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14. ABSTRACT

Colorectal cancer (CRC) is the most common malignancy with high mortality worldwide and is also the 2nd most common cause of cancer deaths in the United States despite important advances in detection, surgery and chemotherapy. An imbalance of commensal bacteria and their gene products underlie mucosal and, in particular, gastrointestinal (GI) inflammation and the predisposition to cancer. Probiotic lactobacilli have received much attention as examples of beneficial microbiota. A prominent member of microbiota is *Lactobacillus acidophilus*, displaying a unique *s*urface *l*ayer *p*rotein (Slp) complex, including SlpA, B and X, all of which differentially activate intestinal innate cells. We previously demonstrated that deletion of specific cell surface molecule(s) of *Lactobacillus acidophilus* significantly modify its protective properties of this bacterium, when delivered orally, against experimental inflammatory bowel disease. Here we clearly show that deletion of lipoteichoic acid (LTA), a TLR2 ligand, normalizes innate and adaptive pathogenic immune responses and critically induces regression of established colonic polyps. Our preliminary data reveals the pro-inflammatory role of LTA and the ability of LTA-deficient *L. acidophilus* to suppress overt inflammation and protect against colon cancer in a novel mouse model. The objective of this proposal thus is to identify critical gene-product of LTA-deficient *L. acidophilus* (called NCK2025) that induce optimal immune regulation in innate immune cells (i.e, DCs), which in turn dampen proinflammatory immune responses, elicited by infiltrated pathogenic T cells in polyp milieu. Our hypothesis is that SlpA of LTA-deficient NCK2025 is the main gene product, which induces regulatory signals to dampen detrimental inflammation. The specific aims are: (**1**) to elucidate the regulatory effects of *L. acidophilus*-Slps on the induced intestinal inflammation. (2) to demonstrate the regulatory effects of *L. acidophilus* SlpA in ablating cancer inflammation of colonic polyposis. Deeply understanding the molecular mechanisms underlying intestinal immune regulation/stimulation in colon cancer is effective when cellular interactions with bacterial products, and critical molecules are identified that culminate in inflammation or anti-inflammatory responses.

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Final Progress report

To study the differential expression of *L. acidophilus*-SlpA and the role(s) of this molecule to either control or elicit inflammatory signals in murine colon cancer we formulated two specific aims.
(1) To elucidate the regulatory effects of *L. acidophilus*-SlpA on induced intestinal inflammation and (**2**) to demonstrate the regulatory effects of *L. acidophilus* SlpA in decreasing cancer-promoting inflammation in colonic polyposis.

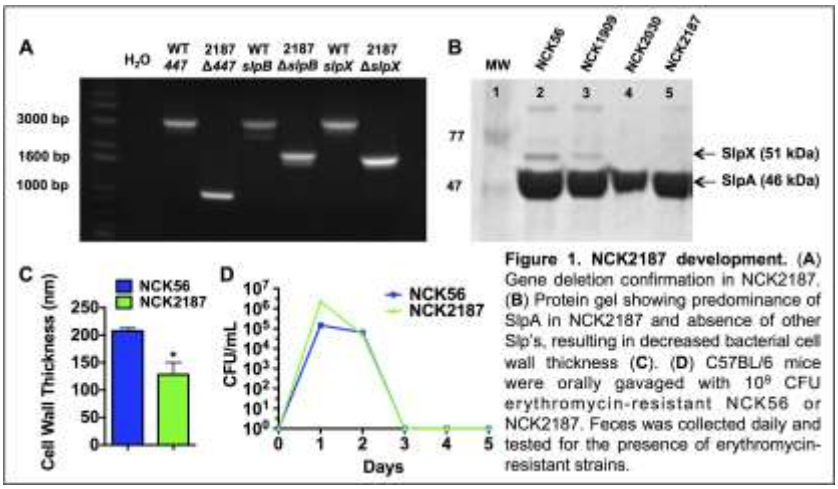

Figure 1. NCK2187 development. (**A**) Gene deletion confirmation in NCK2187. (**B**) Protein gel showing predominance of SlpA in NCK2187 and absence of other Slp's, resulting in decreased bacterial cell wall thickness (**C**). (**D**) C57BL/6 mice were orally gavaged with 10^9 CFU erythromycin-resistant NCK56 or NCK2187. Feces was collected daily and tested for the presence of erythromycin-resistant strains.

Aim 1. **To elucidate the regulatory effects of *L. acidophilus*-Slps on the induced intestinal inflammation.** The rationale for this proposal is that the developmental stages of colon cancer include polyps, adenomas, and carcinomas whereupon undesired inflammation can promote the tumor growth in mice with polyposis and in human colon cancer. Accordingly, such induced pathogenic inflammation when controlled by regulatory mechanisms, including T regulatory cells (Tregs)[1-3] gut homeostasis can be established in order to initiate protective immune responses against induced polyps. To test this notion, we sought to employ *L. acidophilus* strain that lacks lipoteichoic acid (LTA), surface layer B and X but solely expressed SlpA. Rationale: Recently, we showed that transient colonization of the colon with NCK2025 lacking LTA significantly mitigated chemical and T cell-mediated colitis.[2,4-6] Analyzed mechanisms suggested that the induction of regulatory IL-10^+DCs and functional Tregs, activation of pErk1/2, and the downregulation of critical downstream signals (Akt1, p38)[7] are key elements involved in the amelioration of murine colitis.[2,4,7-9] Additionally, NCK2025 significantly abated inflammation-promoting polyposis[6] in our novel *Apc*^{lox468} x TS4-cre mouse model, where protection correlated with the regulation of innate and T cell inflammation.[6] Thus, we concluded that the controlled

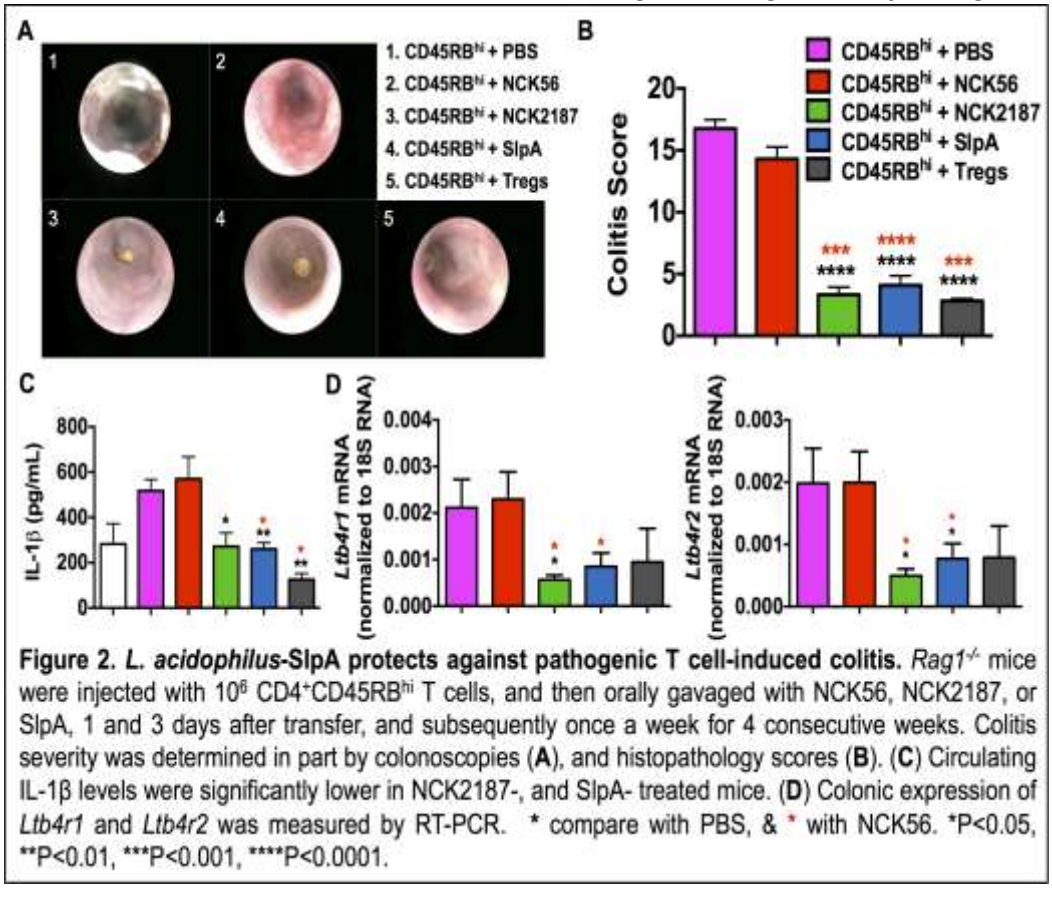

Figure 2. *L. acidophilus*-SlpA protects against pathogenic T cell-induced colitis. *Rag1*$^{-/-}$ mice were injected with 10^6 $CD4^+CD45RB^{hi}$ T cells, and then orally gavaged with NCK56, NCK2187, or SlpA, 1 and 3 days after transfer, and subsequently once a week for 4 consecutive weeks. Colitis severity was determined in part by colonoscopies (**A**), and histopathology scores (**B**). (**C**) Circulating IL-1β levels were significantly lower in NCK2187-, and SlpA- treated mice. (**D**) Colonic expression of *Ltb4r1* and *Ltb4r2* was measured by RT-PCR. * compare with PBS, & * with NCK56. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

inflammation resulting from the cross-talk between NCK2025, and in particular its surface protein **SlpA**, with intestinal cells, could potentially modulate the expression of epigenetically regulated, colorectal cancer (CRC)-associated genes[10] and restore gut homeostasis to ablate colonic polyps.[6,11,12] These important data were compelling evidence in defining the SlpA of this NCK2025 strain as playing the critical role in the protection against murine colitis and polyposis. *Thus, we hypothesized that the bacterial SlpA interacts with colonic innate cells (e.g., DCs) via SIGNR3 to regulate detrimental signals in innate cells, which in turn, reshape the functional balance of T and B cell[13] homeostasis, resulting in significant mitigation of induced colitis.* The rationale for this hypothesis was that three *L. acidophilus surface layer proteins* (e.g., SlpA, SlpB and SlpX) interact with PRRs[14,15] to deliver signals in intestinal innate cells, however information about the functions of these Slps, and in particular **SlpA**, are relatively limited.[16,17,18] Therefore, to specifically demonstrate the effects of SlpA and its binding to its receptor on intestinal innate cells and the consequences thereafter, the *upp* counterselective knockout strategy was used to generate in-frame deletions in the *SlpB* or *SlpX* genes of NCK2025 lacking LTA,[19] resulting in the generation of **NCK2187 strain**, which expresses only SlpA (**Fig. 1A-C**). Creating this novel bacterial strain was critical to the definitive demonstration of the role of SlpA in controlling pathogenic inflammation, which results in significant mitigation of colitis. To demonstrate that the newly generated NCK2187 transiently colonizes the gut, both the erythromycin-resistant NCK56 wild-type (wt) strain and the **NCK2187** strain were then determined in orally treated C57BL/6 mice (administered once with 10^9 CFU/mouse). Data demonstrate that mice cleared both NCK56 and NCK2187 after 3 days, indicating that deletion of LTA, SlpB, and SlpX in the **NCK2187** strain did not impair its transient colonization when compared to its wt NCK56 (**Fig. 1D**). Furthermore, NCK2187 and SlpA not only significantly mitigated induced colitis in *Rag1*$^{-/-}$ mice that received proinflammatory $CD4^+CD45RB^{hi}$ T cell transfers (**Fig. 2A-B**), but also reduced IL-1β (**Fig. 2C**), and the high affinity receptor for LTB_4 and BLT1[20,21] (*Ltb4r1, Ltb4r2*) (**Fig. 2D**) all of which are significantly involved in the induction of pathogenic inflammation.

Additionally, the barrier function and tight junction proteins (e.g., occludins) were protected (**Fig. 3A-B**), and the composition of microbial symbiosis was maintained (**Fig. 3C-D**) in NCK2187- or SlpA-treated *Rag1*$^{-/-}$ mice**.** In contrast, T cell induced inflammation in PBS- or wt NCK56-treated *Rag1*$^{-/-}$ mice significantly affected the intestinal barrier, tight junction proteins, and gut microbiota (**Fig. 3A-D**).

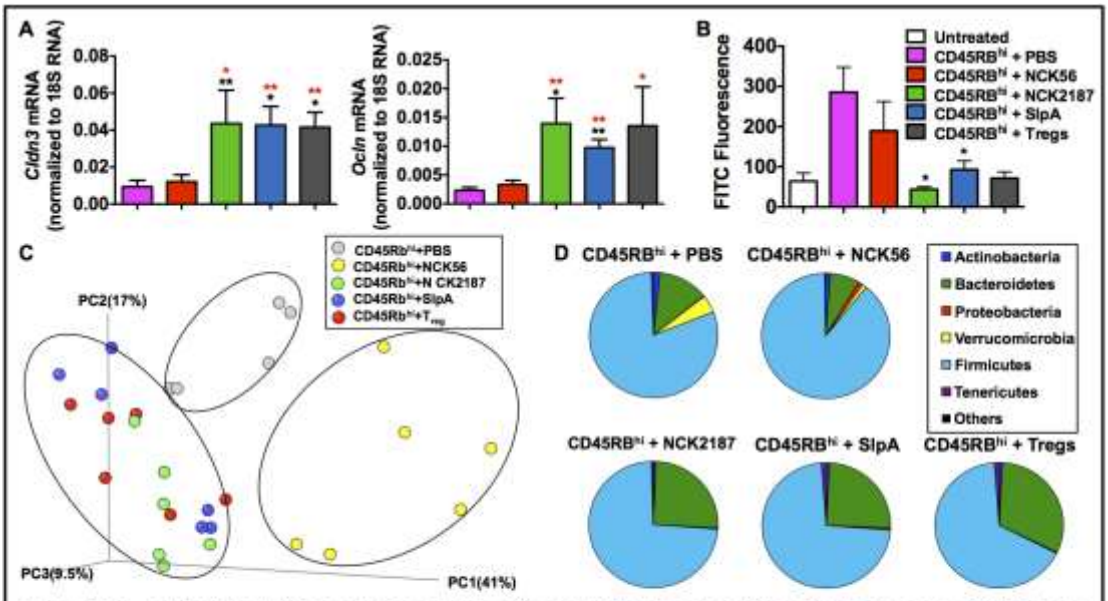

Figure 3. *L. acidophilus*-SlpA protects against pathogenic T cell-induced intestinal damage and dysbiosis. *Rag1*$^{-/-}$ mice were injected with 10^6 $CD4^+CD45RB^{hi}$ T cells, and then orally gavaged with NCK56, NCK2187, or SlpA, 1 and 3 days after transfer, and subsequently once a week for 4 consecutive weeks. (**A**) Colonic expression of tight junction-related genes was measured by RT-PCR. (**B**) Injected *Rag1*$^{-/-}$ mice were orally gavaged with FITC-Dextran, and FITC fluorescence in the serum measured as a measure of intestinal permeability. (**C**) UniFrac analyses were used to calculate distances between samples and three dimensional scatterplots were generated by using principal coordinate analyses (PCoA). Protected mice that received Tregs clustered with NCK2187 & SlpA-treated *Rag1*$^{-/-}$ mice. (**D**) Changes in the relative abundance of different phyla were also apparent in diseased mice. * compare with PBS, & * with NCK56. *P<0.05, **P<0.01.

To address the direct effects of the monoassociation of NCK2187 versus wt NCK56 on intestinal responses, germ free (GF) mice were then employed. Monoassociation of NCK2187 regulated IL-1β, and IL-6 in DCs of GF C57BL/6 (**Fig. 4A**). Importantly, NCK2187 not only induced the formation of colonic Tregs (**Fig. 4B**), but also IL-10^{+} IgA^{+} B cells (**Fig. 4C**) in GF mice when compared with PBS-, or NCK56-treated mice, suggesting that SlpA plays a critical role in the immune regulation that contributes to the mitigation of colitis.

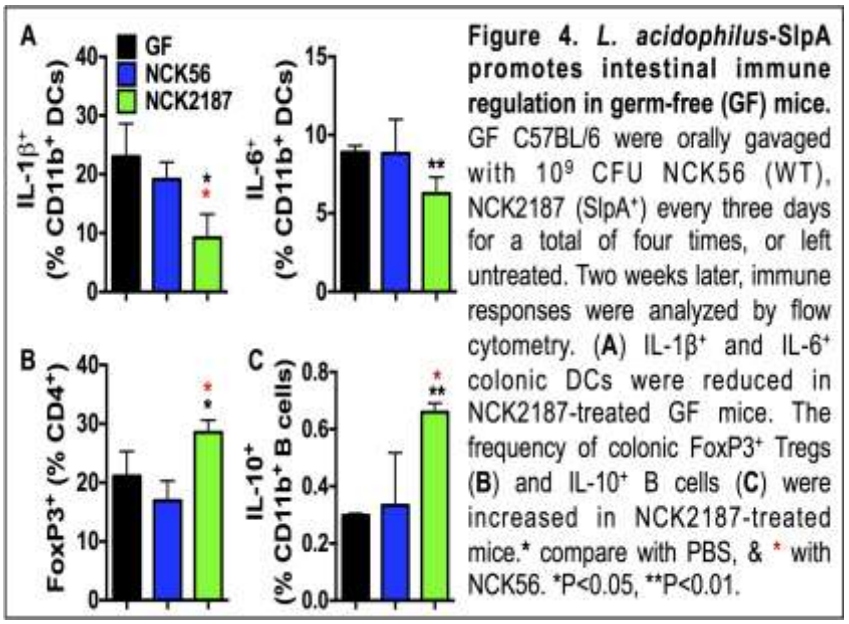

Figure 4. *L. acidophilus*-SlpA promotes intestinal immune regulation in germ-free (GF) mice. GF C57BL/6 were orally gavaged with 10^9 CFU NCK56 (WT), NCK2187 (SlpA^{+}) every three days for a total of four times, or left untreated. Two weeks later, immune responses were analyzed by flow cytometry. (**A**) IL-1β^{+} and IL-6^{+} colonic DCs were reduced in NCK2187-treated GF mice. The frequency of colonic FoxP3^{+} Tregs (**B**) and IL-10^{+} B cells (**C**) were increased in NCK2187-treated mice.* compare with PBS, & * with NCK56. *P<0.05, **P<0.01.

Next, by utilizing a gene screening approach for all known murine **SIGNR1-8**, the SIGNR1 and SIGNR3 genes were determined to be differentially activated in the colonic cells of the mice orally treated with NCK2187 (**Fig. 5A**), prompting us to evaluate the binding of SlpA to SIGNR3 and SIGNR1 that potentially regulates critical signals in colonic cells. Therefore, the corresponding extracellular domains of SIGNR1 and SIGNR3 were fused to the *Fc* part of human IgG$_1$ (*Signr1-hFc*, *Signr3-hFc*) and then transiently expressed in Chinese hamster ovary (CHO)-S cells (not shown). Our data demonstrated that while expressed SIGNR3-hFC, but not SIGNR1-hFC, bound to purified SlpA coated on charged beads, DCAR-hFc (control irrelevant protein), hFc, or the secondary rat anti-human Fc alone did not, suggesting SlpA-binding specificity to SIGNR3 but not SIGNR1 (**Fig. 5B**). Moreover, given that SIGNR3 was found to be the mouse ortholog of DC-SIGN, most closely resembling its human homolog in terms of ligand binding,[22] *in vitro* binding assays confirmed that purified SlpA binds to DC-SIGN-Fc (**Fig. 5C**), as well as to CHO-S expressing DC-SIGN (**Fig. 5D**). This very critical observation led us to focus on SIGNR3-properties in the intestinal immune regulation upon its interaction with SlpA.

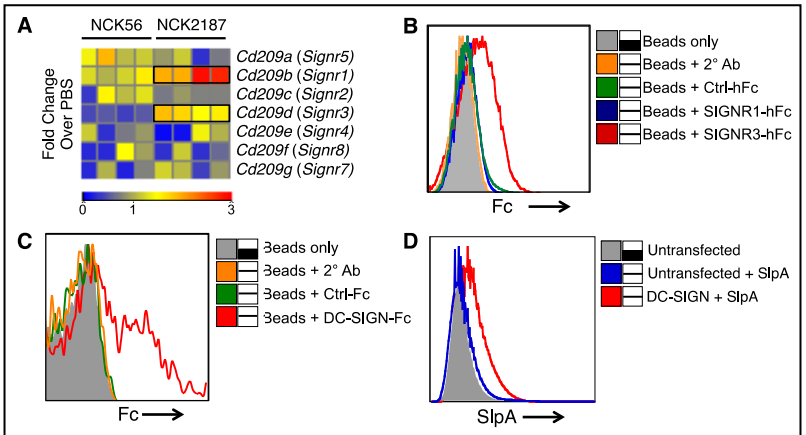

Figure 5. *L. acidophilus*-SlpA binds to mouse SIGNR3 and DC-SIGN. (A) C57BL/6 mice were orally gavaged with 10^9 CFU NCK56 (WT) or NCK2187 (SLP+) and the colonic gene expression of murine C-type lectin receptors were measured by qRT-PCR. (B) Binding of SlpA to murine lectin-Fc proteins were analyzed by flow cytometry. (C) Binding of SlpA to human DC-SIGN were analyzed by flow cytometry. (D) CHO-S cells were transfected with DC-SIGN expressing plasmid and binding of labeled SlpA-coated beads were assessed for the their binding.

Subsequently, *Signr3*$^{-/-}$ mice were generated in our laboratory to fortify the hypothesis that **SlpA** regulates colonic immunity via **SIGNR3** signaling to resist pathogenic inflammation in dextran sulfate sodium (DSS)-induced colitis.[2] In this colitis model, while NCK2187 or its purified SlpA protected wt *Signr3*$^{+/+}$ mice from severe induced colitis, such protection was not observed in *Signr3*$^{-/-}$ mice (**Fig. 6A-B**), indicating that this SIGNR3 is highly involved in immune regulation via its interaction with SlpA. Analyses of the microbiota of wt mice treated with NCK2187 or its SlpA compared to NCK56 clearly illustrated a significant rebalance of the composition of the natural mouse microbiota, which was not evident in *Signr3*$^{-/-}$ mice (**Fig. 6C-D**). Importantly, both NCK2187 and its purified SlpA also significantly mitigated already established DSS-induced colitis. Such a trend,

however, was not observed in *Signr3*$^{-/-}$ mice (not shown). Below, we will further elucidate SlpA:SIGNR3 mechanisms to ascertain their impact on intestinal immune responses, and barrier functions. To further investigate the regulatory role of *L. acidophilus*-SlpA we then selected *Citrobacter rodentium* that results in a breach of the intestinal epithelial barrier, potentially orchestrated by unrestrained pro-inflammatory immune responses.[23] Obtained data demonstrate that treatment of mice with NCK2187 and its purified SlpA significantly accelerated pathogen clearance (**Fig. 7A**), resulting in reduced size of the draining lymph nodes (**Fig. 7B**), and decreased colonic IL-1β expression (**Fig. 7C**). Conversely, this trend was not observed in *C. rodentium*-infected mice that were treated with NCK56 or PBS, suggesting that *L. acidophilus*-SlpA regulates induced proinflammation (e.g., IL-1β) resulting in less colonic damage and bloody diarrhea (**Fig. 7D-E**). Furthermore, histologic analyses of colonic mucosal damage (e.g., abnormal crypts) and induced transmural inflammation with *C. rodentium* alone or *C. rodentium* plus NCK56 revealed increased lymphoplasmacytic with lesser but mildly increased neutrophilic infiltrates within the lamina propria and colonic submucosae, which were decreased in NCK2187 and its purified SlpA-treated groups (**Fig. 7D**). Thus, these data indicate that the magnitude of innate cell activation must be precisely regulated so as to minimize the pathologic conditions characterized by hyperimmune responses that result in colitis.[24] Such regulation is also critical in limiting the later stages of pathogen-specific immunity and pathogen-induced inflammation as a means of <u>preventing inflammation associated tissue damage.</u>[25]

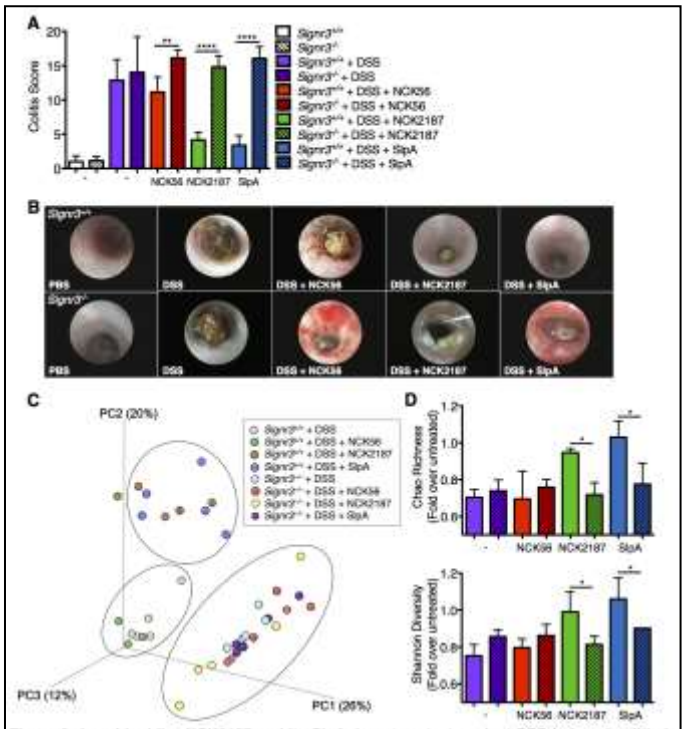

Figure 6. *L. acidophilus* NCK2187 and its SlpA do not protect against DSS-induced colitis in *Signr3*$^{-/-}$ mice. WT B6 or B6 *Signr3*$^{-/-}$ (KO) mice were orally gavaged with NCK56, NCK2187 or SlpA on days -3 and -1, and 3% DSS was given in the drinking water. Mice were gavaged with bacteria or purified SlpA every other day for an additional 3 times and monitored for disease progression. **A.** Colitis scores based on histopathology were used as a measure of disease. n = 5 mice/group. **B.** Colonoscopies were performed in the different groups with a Multi-Purpose Rigid™ Telescope attached to a TELE PACK X on day 10. **C.** 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. **D.** 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. n = 4-6 mice/group. Data are shown as mean ± SEM, and are representative of three different experiments. *P<0.05, **P<0.01, ****P<0.0001.

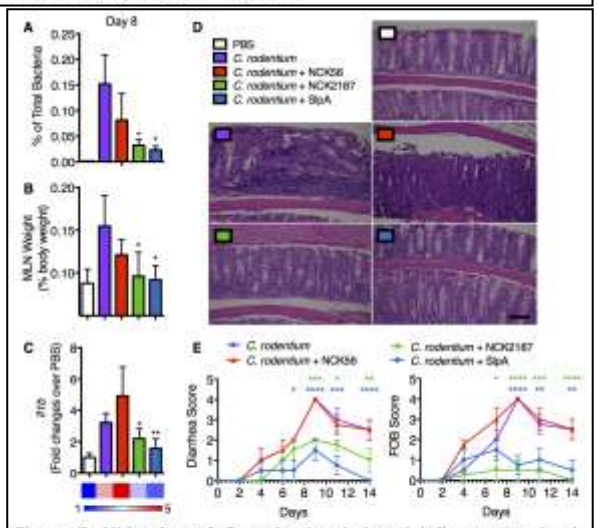

Figure 7. Mitigation of *C. rodentium*-induced inflammation by *L. acidophilus* and its SlpA. B6 mice were orally gavaged with NCK56 (red), NCK2187 (green), or SlpA (blue), 3 and 1 days prior to infection with *C. rodentium*, and every other day thereafter for 2 weeks, or left untreated (magenta). A group of mice remained uninfected and was fed PBS as control (white). **A.** *C. rodentium*-specific primers and 16S rDNA universal primers were used to test the percentage of *C. rodentium* relative to total bacteria. After 8 days post-infection of *C. rodentium*, accelerated clearance of *C. rodentium* was observed in the groups orally gavaged with NCK2187 and SlpA, but not in the NCK56 group. **B.** Relative weight of the mesenteric lymph nodes (MLNs) of uninfected and infected mice at day 14. MLNs isolated from NCK2187- and SlpA-treated animals were of similar size to uninfected mice. **C.** Colonic expression of *Il1b* was determined by RT-PCR relative to uninfected mice at day 14. **D.** Histopathology of colons isolated 14 days post-infection demonstrated increased inflammation in *C. rodentium* only and *C. rodentium* + NCK56 groups. **E.** Colitis severity was also determined in part by diarrhea scores and FOB. n = 5 mice/group. Data represent two individual experiments and are shown as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001, compared to the *C. rodentium* only group.

The rationale of characterization this *L. acidophilus* strain, NCK2187, was then to fully understand the regulatory/stimulatory role of SlpA in the regulation of pathogenic inflammation induced in polyposis model as demonstrated above in various infections and inflammatory induced colitis murine models.

To target a key carcinogenic event in the colon, we have used homologous recombination to introduce loxP sites on either side of exons 11 and 12 of the mouse adenomatous polyposis coli (APC^{lox468}) gene, such that Cre-mediated recombination would generate a truncated APC gene product extending only as far as exon 468. These mice (APC^{lox468}) were crossed to TS4-Cre transgenic mice that express Cre specifically in the colon and distal ileum. Mice double heterozygous for conditional APC^{lox468} and TS4-Cre were generated and aged where as early as 4 months of age, extensive polyposis is detected throughout the colon and distal ileum, but not in the proximal ileum, jejunum or

duodenum (**Fig. 8**). Afterwards, we tested the hypothesis that *L. acidophilus* surface layer protein A (SlpA) interacts with colonic DCs via a c-type lectin receptor (CLR), such as dendritic cell specific ICAM-3-grabbing nonintegrin (SIGNR3), to regulate detrimental signals in innate cells resulting in significant mitigation of pathogenic inflammation that potentially results in the growth of polyposis (**Fig. 8**).

Aim 2. To demonstrate the regulatory effects of *L. acidophilus* SlpA in decreasing cancer promoting inflammation of colonic polyposis in TS4Cre-APC^{lox468} **transgenic mice.**

To analyze the immunomodulatory properties of NCK2187 versus its control strains, TS4Cre-APC^{lox468} mice were treated for four weeks and then euthanized and analyzed. We looked for a difference in the number of polyps in the small intestine and colon. We also investigated the differential frequencies of immune cells and differential cytokine expression throughout the intestines and the draining lymph nodes. Previously we reported that inflammation associated with polyposis becomes systemic in mouse models of hereditary polyposis, as evidenced by splenomegaly and elevated levels of pro-inflammatory cytokines in the sera.[1] Once again data demonstrated the promising trend as previously demonstrated.[3] After this significant progress demonstrating that NCK2187 and its SlpA play a critical role in tuning down the inflammation we then established *PTEN^{lox}APC^{lox468}TS4-Cre* mouse strain to meet further the milestones outlined in Aim 2.

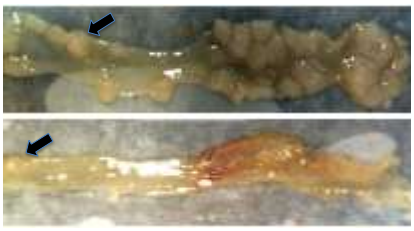

Fig. 8. NCK2025 *L. acidophilus* protects against colon cancer. Colons from polyposis mice, untreated (top) or orally treated with 5x10^{8} NCK2025 everyday for 4 weeks. Arrows point to polyps.

***PTEN^{lox}APC^{lox468}TS4-Cre* mouse model.** The transition from benign to invasive cancer involves secondary genetic mutations that can potentially render the neoplasm less dependent on the immediate tumor microenvironment. To test the protective or otherwise harmful potential of *L. acidophilus* strains and their gene products (SlpA), we have developed a mouse model of invasive cancer that is based on the superimposition of a secondary genetic event on the tumor suppressor APC genetic defect. Large scale studies have revealed loss of heterozygosity at the *p*hosphatase and *ten*sin homolog deleted on chromosome 10 (PTEN) tumor suppressor gene locus in up to 50% of primary tumors tested, making it one of the earliest and most commonly mutated genes in human cancer. By crossing TS4-Cre mice to mice genetically modified to harbor Cre-dependent conditional PTEN and APC alleles, we generated homozygous compound mutant mice that inactivate both APC and PTEN in colonic epithelial cells (**Fig. 9**). The resulting *PTEN^{lox} APC^{lox468}TS4-Cre* mice develop invasive colon cancer starting from 5 months of age. To test the response to *L. acidophilus* strains and purified SlpA in this murine colon cancer model, we carried out the same series of experiments in the *PTEN^{lox}APC^{lox468}TS4-Cre* mice already described in **Fig. 8**. Briefly, *PTEN^{lox}APC^{lox468}TS4-Cre* mice were constructed and crossed to obtain the double mutations in APC and PTEN genes in these mice. Subsequently, these mice were aged (3-4 months) to conduct studies dealing with local and systemic immune regulation by NCK2187 or its purified SlpA to reduce polyps in these mice. By testing the anti-carcinogenic effects of NCK2187 on our TS4-Cre-*APC^{lox468} PTEN^{lox}* mouse

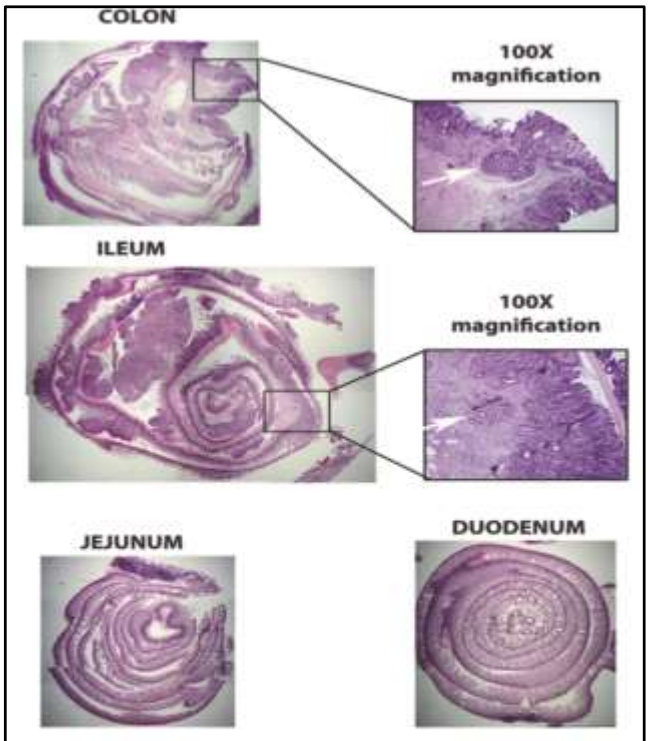

Fig 9 H&E of paraffin embedded "jelly-rolls" of APC^{lox468} x PTEN^{lox} mouse intestine. Note invasive lesions in the colon and distal ileum, (white arrow, 100x magnification).

model we found that this mouse model is extremely difficult to significantly reduce the induced polyps by NCK2187 potentially due to deletion of two very important genes in the same mice (**Fig. 10).** After so many attempts we concluded that much more time and funds are highly required to tune up the system in this novel mouse model. Additionally, we also established a new method for isolation of SlpA using NaCl and developed a novel monoclonal antibody that can detect the uptake of orally gavaged SlpA by gut immune cells.[26] Using this method, experiments are outlined in a proposal that has just been submitted to NIH indicating that SlpA can be potentially used in first clinical trials that will be performed at the University of Florida.

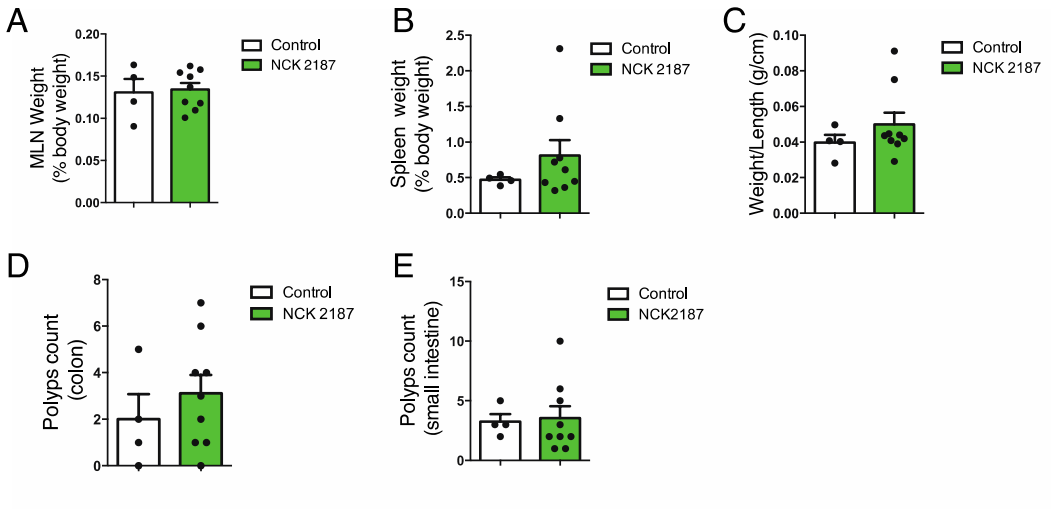

Fig. 10. *PTEN^{lox}APC^{lox468}TS4-Cre* mice were gavaged with NCK 2187 (10^9 CFU/mouse) or with PBS (Control) once a week during nine consecutive weeks. Mice were sacrificed two week later. A. MLN weight (% of body weight), B. Spleen weight (% of body weight), C. Ratio weight/Length (g/cm), D. Polyps counts (colon), E. Polyps counts (small intestine). Experiments were repeated at least 3 times with the same trend.

Conclusion

This proposal took advantage of innovative concepts and tools to rebalance intestinal immunity by novel *L. acidophilus* strain, called NCK2187 and newly purified SlpA, to achieve therapeutic outcomes in colon cancer. The goal of the study was to discover genetically modified *L. acidophilus* strain with cancer preventing and therapeutic properties using unique mouse models that have been established in our laboratory. These studies could have significant impact on our understanding of the communication between beneficial microbes such as modified *L. acidophilus* (NCK2187) and the host immune system involved in inflammatory responses, and would potentially lead to the development of novel probiotic platforms for cancer therapeutic application. We thus believe that such mechanistic studies provide a better understanding of signals delivered by *L. acidophilus*-SlpA to intestinal cells, particularly DCs, which might initiate critical immune responses to mitigate intestinal diseases.

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