

# Abating colon cancer polyposis by *Lactobacillus acidophilus* deficient in lipoteichoic acid

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An imbalance of commensal bacteria and their gene products underlies mucosal and, in particular, gastrointestinal inflammation and a predisposition to cancer. *Lactobacillus* species have received considerable attention as examples of beneficial microbiota. We have reported previously that deletion of the phosphoglycerol transferase gene that is responsible for lipoteichoic acid (LTA) biosynthesis in *Lactobacillus acidophilus* (NCK2025) rendered this bacterium able to significantly protect mice against induced colitis when delivered orally. Here we report that oral treatment with LTA-deficient NCK2025 normalizes innate and adaptive pathogenic immune responses and causes regression of established colonic polyps. This study reveals the proinflammatory role of LTA and the ability of LTA-deficient *L. acidophilus* to regulate inflammation and protect against colonic polyposis in a unique mouse model.

dendritic cells | regulatory T cells | anti-inflammatory

Identifying bacterial gene products that enhance protective versus pathogenic inflammation in the gut is critical to rebalance homeostasis in gastrointestinal (GI) chronic inflammatory diseases and malignancies. Commensal *Lactobacillus* species (i.e., *Lactobacillus acidophilus*) are normal inhabitants of the natural microbiota in the human GI tract (1, 2). *L. acidophilus* stimulates innate cells to produce inflammatory and regulatory cytokines through interaction of their surface layer proteins and other cell surface components (3–7). To investigate the potential role of lipoteichoic acid (LTA) of *L. acidophilus* in the induction of inflammatory signals, we deleted the phosphoglycerol transferase gene (*LBA0447*) that synthesizes LTA. LTA is a zwitterionic glycolipid found in the cell wall of several Gram-positive bacterial strains, including *L. acidophilus*, which stimulates dendritic cells (DCs) through Toll-like receptor 2, resulting in cytokine release (8, 9). Disruption of LTA synthesis generated a *L. acidophilus* derivative (NCK2025) that mitigates colitis in mice (10). Based on these observations and those of others (11, 12), it was proposed that LTA induces inflammation and that its absence significantly attenuates overt intestinal inflammation.

Inflammation has a tumor-promoting role in mice with polyposis and in human colon cancer (13–17). T regulatory cells (Tregs) critically regulate inflammation and play a protective role in polyposis (15, 18) and colon cancer (19–21). However, chronic interaction of Tregs with proinflammatory cells and their cytokines can reverse the anti-inflammatory properties of these cells and render them proinflammatory (15, 16, 22, 23). Consensus suggests that interactions between lymphocytes and myeloid cells regulate pro- vs. antitumor immunity (17, 24), and we hypothesized that the gut microbiota plays an essential role in control of this balance. We used *L. acidophilus* NCK2025, deficient in LTA, to investigate the moderation of pathogenic inflammation within the tumor microenvironment in a unique mouse model of colonic polyposis.

## Results

**Ablation of Polyps by LTA-Deficient *L. acidophilus* in TS4Cre × APC<sup>lox468</sup> Mice.** To target a key carcinogenic event in the colon, homologous recombination was used to introduce loxP sites on either side of exons 11 and 12 of the mouse adenomatous polyposis coli (APC<sup>lox468</sup>) gene (Fig. 1 *A* and *B*). These mice (APC<sup>lox468</sup>) were crossed to TS4-Cre transgenic mice (25) that express Cre specifically in the colon and distal ileum. Mice double heterozygous for conditional APC<sup>lox468</sup> and TS4-Cre were generated and aged. As early as 4 to 5 mo of age, extensive polyposis was detected throughout the colon and distal ileum, but not in the proximal ileum, jejunum, or duodenum (Fig. 1 *C* and *D*). To analyze the immunomodulatory properties of NCK2025, TS4Cre × APC<sup>lox468</sup> mice 5 mo of age were treated daily with oral doses of NCK2025 (5 × 10<sup>8</sup> cfu/mouse), or were fed water as a control for 4 wk. To specifically address the role of LTA, a third group of mice was treated in a similar manner with the parental LTA<sup>+</sup> strain, *L. acidophilus* NCK56. After 4 wk of treatment, all mice were euthanized and analyzed. There was little to no change in polyposis in NCK56-treated mice compared with control PBS solution-treated mice. In contrast, NCK2025-treated mice had a reduced number of polyps in the distal ileum and significantly decreased polyps in the colon (Fig. 2 *A* and *B*). Mitotic and apoptotic, but not intranuclear, β-catenin activities (data not shown) were significantly reduced in polyps of NCK2025-treated mice compared with PBS solution- and NCK56-treated mice (Fig. 2 *C* and *D*). Mice treated with NCK56 had higher intrapolymp mitotic and apoptotic activities compared with untreated or NCK2025-treated mice (Fig. 2 *C* and *D*). These observations demonstrate the therapeutic properties of oral NCK2025 treatment in mice with preestablished colonic polyposis.

**Immune Regulation by LTA-Deficient *L. acidophilus*.** To provide evidence for dampened inflammation in NCK2025-treated mice, immunofluorescent staining was performed throughout the colon. Previously, the expansion and activation of mast cells in adenomatous polyps was reported, and the evidence supported their tumor-promoting role (13, 17, 26). Therefore, we quantified intrapolymp mast cell densities in TS4Cre × APC<sup>lox468</sup> NCK2025-treated mice compared with mice fed the parental NCK56. Significant decreases in the intrapolymp mast cell counts in NCK2025-treated mice were observed; however, no differences

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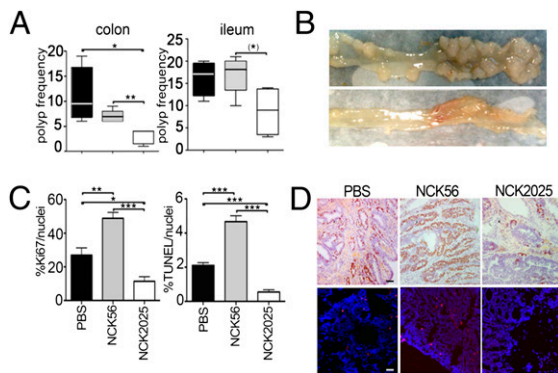
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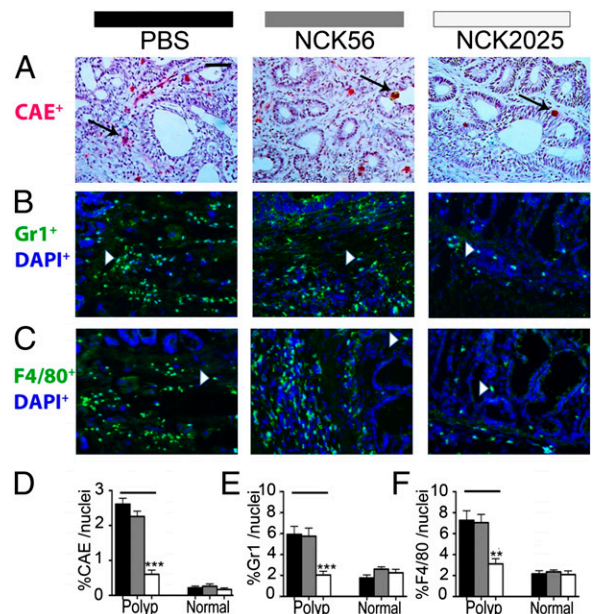




**Fig. 2.** Status of polyps in TS4Cre  $\times$  cAPC<sup>lox468</sup> mice ( $n = 6$ ) treated with PBS solution, NCK56, or NCK2025. (A) Polyps were counted in the ileum and colon, and results are presented in box-and-whisker plots. (B) Photographs of representative colons of mice treated with PBS solution (Upper) or NCK2025 (Lower). (C) Quantification of Ki-67<sup>+</sup> and TUNEL<sup>+</sup> cells in polyps of mice treated with PBS solution, NCK56, or NCK2025. (D) Staining of colonic polyp for Ki-67 (Upper) and TUNEL (Lower) of 6- $\mu$ m sections from paraffin-embedded tissue of mice treated with PBS solution, NCK56, or NCK2025. (Scale bar: 50  $\mu$ m.) Images were acquired by using Tissue Gnostics and analyzed with ImageJ. The data were statistically analyzed by unpaired  $t$  test ( $***P < 0.0001$ ,  $**P < 0.004$ , and  $*P < 0.0176$ ). Ki-67 (iHistochem) and TUNEL (Millipore) staining was performed per manufacturers' instructions. Data are representative of three independent experiments.

Together, these findings demonstrate that administration of NCK2025 to mice with established polyps down-regulates inflammation and resets both local and systemic immunity, whereas the parental *L. acidophilus* NCK56 elicits an intermediate response.

**Sustained Phenotype of Foxp3<sup>+</sup> Regulatory T Cells by LTA-Deficient *L. acidophilus*.** Tregs play a dual role in cancer, increasing in total number and suppressing protective T-cell immunity as well as protecting against cancer through suppression of inflammation. In cancer and chronic inflammation, Tregs can lose their anti-inflammatory properties. This results via expression of ROR $\gamma$ t and loss of Foxp3, leading to conversion to TH17 cells (32–35). Recently, we reported that, in polyposis and colon cancer, Tregs can gain proinflammatory TH17-like properties while maintaining their ability to suppress T cells (15, 16, 36, 37). This subset of Tregs critically contributes to escalation of cancer-associated inflammation and promotes tumor growth (15, 16, 22). In consideration of the reduced inflammation in mice treated with NCK2025, we assessed the frequencies of bona fide Foxp3<sup>+</sup> Tregs defined as Foxp3<sup>+</sup>ROR $\gamma$ t<sup>-</sup> stained cells vs. Tregs with TH17 characteristics, defined as Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> cells. The overall densities of Foxp3<sup>+</sup> cells decreased in polyps of mice treated with NCK2025, but not in mice treated with the parental NCK56 (Fig. 6 B and D). Interestingly, double staining for Foxp3 and ROR $\gamma$ t, revealed a critical change in the quality of polyp-infiltrating Tregs with differential changes in densities of bona fide vs. proinflammatory Tregs. Thus, polyps of NCK2025-treated mice showed significant increases in densities of bona fide Foxp3<sup>+</sup> Tregs (Fig. 6 B and E), but decreases in densities of Foxp3<sup>+</sup>ROR $\gamma$ t<sup>+</sup> proinflammatory Tregs (Fig. 6 B and F); mice treated with NCK56 showed intermediate responses (Fig. 6 B, E, and F). Levels of ROR $\gamma$ t-expressing non-Tregs did not change with NCK2025, whereas proinflammatory CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> T cells were reduced (Fig. 6 B and G). These observations illustrate a shift in balance from pro- to anti-inflammatory Tregs in the polyp microenvironment of NCK2025-treated mice, with a corresponding reduction in inflammatory helper T cells. These findings highlight the importance of analyzing Treg subsets



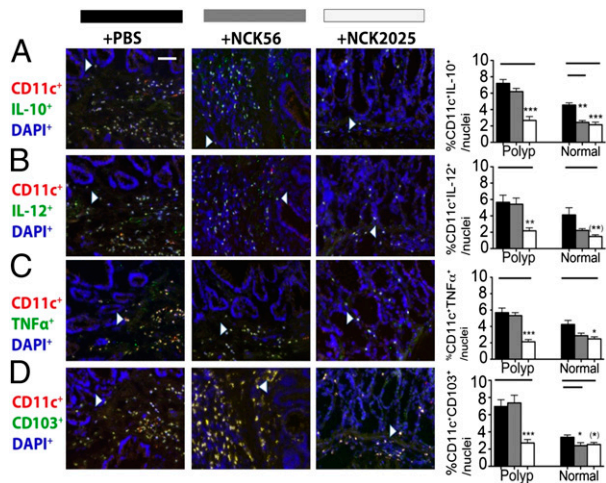
**Fig. 3.** Attenuation of intrapolyp inflammation in TS4Cre  $\times$  cAPC<sup>lox468</sup> by NCK2025 treatment. (A) CAE-positive mast cells, (B) Gr1<sup>+</sup> granulocytes, (C) F4/80<sup>+</sup> macrophages, (D) frequencies of intrapolyp CAE<sup>+</sup> cells ( $***P < 0.0001$ ), (E) frequencies of intrapolyp Gr1<sup>+</sup> cells ( $***P < 0.0005$ ), and (F) frequencies of intrapolyp F4/80<sup>+</sup> cells ( $**P < 0.006$ ). (Scale bar: 50  $\mu$ m.) Black arrows show mast cells, and white arrowheads show F4/80<sup>+</sup> or Gr1<sup>+</sup> cells for PBS solution-, NCK56-, and NCK2025-treated mice ( $n = 5$  per group). Error bars show SEM. Data were analyzed by two-tailed Student  $t$  test, type 2, compared with untreated mice. Sections (5  $\mu$ m) of frozen colon tissues were stained with CAE-specific antibodies as described previously (13). For immunostaining, primary antibodies used were rat anti-mouse F4/80 (Abcam), biotin anti-mouse Gr1, and hamster anti-mouse CD11c (BD Biosciences); secondary antibodies (DAPI; Invitrogen) were anti-hamster Alexa Fluor 594 and anti-rat Alexa Fluor 488. Tissues were then reacted with streptavidin 488 and DAPI to reveal nuclei. Images were acquired by using a TissueGnostics Tissue/Cell High Throughput Imaging and Analysis System and analyzed by using ImageJ software. Data are representative of three independent experiments.

separately, and implicate NCK2025 in the improvement of Treg protective function in mice with polyposis. Treatment with NCK2025 reduced the frequencies of IFN- $\gamma$ -producing CD4<sup>+</sup> T-cells, in line with the attenuation of inflammation in these mice (Fig. 6 C and H).

## Discussion

Colorectal cancer is the second most common cause of cancer deaths in the United States and other developed countries, despite important advances in detection, surgery, and chemotherapy (38). In the past few decades, the link between induced pathogenic inflammation and colonic tumorigenesis has been well established, with significant supporting evidence from immunogenetic, pharmacological, and epidemiological studies. Inflammation and its establishment are known to be complex processes in response to changes in the homeostatic condition. It has been postulated that, although inflammation is beneficial for the host, it can often progress beyond manageable levels with deleterious results. Thus, with the initiation of inflammatory responses, simultaneous commencement of anti-inflammatory signals is triggered to reestablish a balanced homeostasis.

Insufficient levels of these anti-inflammatory or regulatory signals can lead to chronic pathogenic inflammation that is believed to promote carcinogenesis, including colon cancer. To address whether the LTA-deficient *L. acidophilus* strain NCK2025 could regulate induced detrimental inflammation in

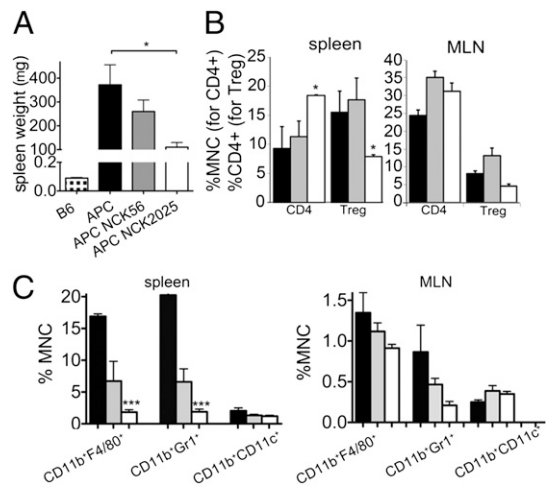


**Fig. 4.** Representation and quantification of infiltration in the polyps and normal adjacent tissues by DCs expressing immune-suppressive and proinflammatory cytokines. (A) CD11c<sup>+</sup>IL-10<sup>+</sup> cells ( $***P < 0.0001$  and  $**P < 0.001$ ), (B) CD11c<sup>+</sup>IL-12<sup>+</sup> cells ( $**P < 0.006$  and  $**P < 0.004$ ), (C) CD11c<sup>+</sup>TNF $\alpha$ <sup>+</sup> cells ( $***P < 0.0005$  and  $*P < 0.01$ ), and (D) CD11c<sup>+</sup>CD103<sup>+</sup> cells ( $***P < 0.0009$  and  $*P < 0.05$ ). Significant increase in intrapolyp compared with extrapolyp CD103<sup>+</sup> DCs ( $**P < 0.0014$ ). (Scale bar: 50  $\mu$ m). White arrowheads show double-positive cells. Data were analyzed by two-tailed Student *t* test, type 2, compared with untreated mice. Immunostaining, acquisition and analyses were performed as described for Fig. 2. Antibodies were hamster anti-mouse CD11c (BD Biosciences); rat anti-mouse IL-10, IL-12, IFN- $\gamma$ , and TNF- $\alpha$  (BioLegend); anti-hamster Alexa Fluor 594; and anti-rat Alexa Fluor 488. Black bars indicate PBS solution treatment; gray bars, NCK56; and white bars, NCK2025. Data are representative of three independent experiments.

polyposis, as has previously been demonstrated in a murine colitis model, we generated a polyposis model whereby polyps could be developed in the colons of these mice.

The data reported here demonstrate that pathologic inflammation in mice with precancerous colonic polyps can be reset to a protective mode by oral treatment with a probiotic microbial strain with anti-inflammatory properties, *L. acidophilus* NCK2025. Healthy intestinal immunity is positioned in a state of equilibrium that permits accurate and rapid protective responses against pathogens while curtailing pathogenic induced inflammatory processes. This balance is achieved through microbiota-driven inflammation (39) and IL-10-dependent suppression of inflammation by Tregs (40–43). Proinflammatory and anti-inflammatory CD4<sup>+</sup> T cells are differentiated and activated via their interactions with innate immune cells, including DCs. Regulatory or proinflammatory DCs are recognized by their expression of cytokines that induce generation of extrathymic Tregs and by proinflammatory cytokines that commit T cells to TH1 or TH17 lineages (44, 45). Gut-derived DCs play a significant role in imprinting gut homing properties to these T cells in a CD103/retinoic acid-dependent manner (28–31). In this study, the absence of LTA in the *L. acidophilus* strain NCK2025 results in immunomodulatory changes that alter inflammation and immunity that may oppose further progression of established colonic polyps.

Cancer inflammation is regulated by Tregs, which, in polyposis and cancer, tend to revert to a proinflammatory phenotype (15, 16). An interesting outcome of oral treatment of mice with NCK2025 was the change in polyp-infiltrating Treg subsets. Although densities of proinflammatory cells in the gut as well as in the spleen were down-regulated, relative densities of effector Foxp3<sup>+</sup>ROR $\gamma$ <sup>+</sup> Tregs increased, whereas Foxp3<sup>+</sup>ROR $\gamma$ <sup>+</sup> proinflammatory Tregs decreased. These changes appeared to reset healthy immunity in the NCK2025-treated mice, as evidenced



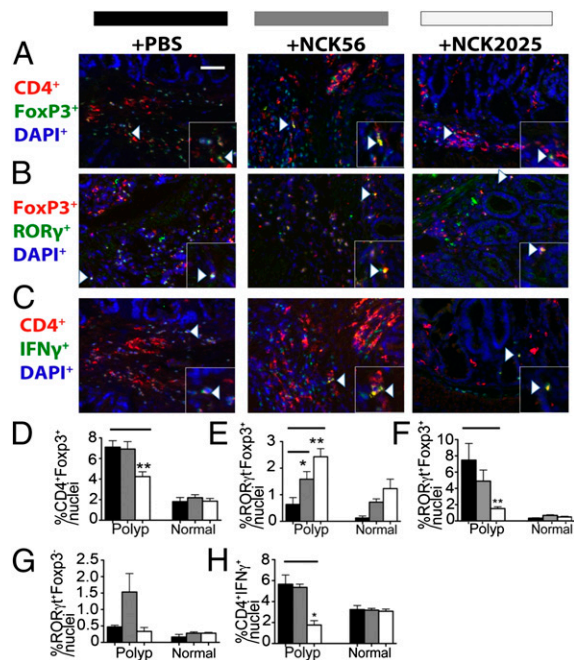
**Fig. 5.** Attenuation of systemic inflammation in TS4Cre  $\times$  cAPC<sup>lox468</sup> mice by NCK2025 treatment. (A) Splenic weights in TS4Cre  $\times$  cAPC<sup>lox468</sup> mice ( $n = 5$  per group) that were untreated or treated with *L. acidophilus* strains ( $*P < 0.026$ ). (B) Frequencies of CD4<sup>+</sup>Foxp3<sup>-</sup> effector T cells and CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in the spleen and MLN of untreated and treated TS4Cre  $\times$  cAPC<sup>lox468</sup> mice; splenic CD4<sup>+</sup> T cells ( $*P < 0.065$ ) and splenic Tregs ( $P < 0.026$ ). (C) Frequencies of CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages, CD11b<sup>+</sup>Gr1<sup>+</sup> granulocytes, and CD11b<sup>+</sup>CD11c<sup>+</sup> DCs among spleen-derived leukocytes of untreated or treated mice; splenic CD11b<sup>+</sup>F4/80<sup>+</sup> cells ( $***P < 0.0001$ ) and splenic CD11b<sup>+</sup>Gr1<sup>+</sup> cells ( $***P < 0.0004$ ). (C) Same frequencies among MLN-derived leukocytes. Histograms show compiled results with SEM; trends shown were not statistically significant. Student *t* test was performed with two tails, type 2 between untreated and treated groups. Single cell suspensions were filtered (40  $\mu$ m), and red blood cells were lysed by using Ack Lysing Buffer (BioWhittaker). Cells were washed and then incubated with Fc block (BD Bioscience). Dead cells were excluded (LIVE/DEAD Violet Dead cell Stain kit; Invitrogen). Antibodies used were CD11c FITC (HL3), GR1 APC (RB6-8C5), CD4 Percp (RM4-5), CD25 biotin (7D4), and streptavidin FITC (BD Pharmingen); and F4/80 Percp (BM8) and CD11b PE-Cy7 (M1/70; Biolegend). Data were acquired with a FACScanto II device (BD) and analyzed by using FlowJo software (Tree Star). Black bars indicate untreated mice, gray bars NCK56-treated mice, and white bars NCK2025-treated mice. Data are representative of two independent experiments.

by a reduced number of polyps in the distal ileum and significantly decreased polyps in the colon.

Beyond emphasizing the important role of the gut microbiota in mucosal immune regulation and in cancer initiation and progression, this work demonstrates the reversibility of preneoplasia. Genetically modified *L. acidophilus* strains may provide new opportunities for regulating intestinal immunity to protect against pathogenic inflammation and the susceptibility to colon cancer. Additional studies are required to further characterize bacterial gene products which induce regulatory signals in innate immune cells (e.g., DCs, macrophages) that, in turn, affect intestinal homeostasis and induced inflammation, all of which play critical roles in colon cancer.

## Materials and Methods

**Generation of TS4Cre  $\times$  APC<sup>lox468</sup> Mice.** A cosmid clone spanning exons 10 to 14 of the mouse APC gene was modified to encode a loxp flanked neoR gene cassette upstream of exon 11 and a third loxp sequence carrying an SphI site immediately downstream of APC exon 12. The entire cassette was then excised and transfected into TC1 (129/SV) embryonic stem cells (46), and NeoR clones were picked. To facilitate selection of transfected ES cells, a neoR cassette flanked by loxp sites was introduced upstream of exon 11. An external and an internal probe were used to detect homologous recombination by DNA Southern blot, as revealed by SphI or EcoRI fragments spanning upstream sequences; 2.3 kb and 6.5 kb for *wt* and recombinant alleles, respectively, or downstream (11 kb and 8.5 kb for *wt* and recombinant alleles, respectively). The neoR cassette proved to interfere with gene expression and was removed by crossing the initial founder mouse to mice expressing Cre universally but inefficiently (synaptonemal-Cre mice) (47). In



**Fig. 6.** Change in quality of polyp-infiltrating Tregs and helper CD4<sup>+</sup> T cells. Representations of polyp-infiltrating (A) CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs, encompassing anti- and proinflammatory subsets; (B) cells expressing RORγt and/or Foxp3, encompassing anti- and proinflammatory Tregs, as well as proinflammatory T cells. (C) CD4<sup>+</sup>IFN-γ<sup>+</sup> proinflammatory T-cells; mice ( $n = 5$  per group) were untreated or treated with PBS solution, NCK56, or NCK2025 as indicated. Quantification in the polyps and normal adjacent tissue of (D) CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs, encompassing anti- and proinflammatory subsets ( $*P < 0.004$ ); (E) RORγt<sup>+</sup>Foxp3<sup>+</sup> anti-inflammatory Tregs ( $**P < 0.002$ ,  $n = 5$ ; and  $*P < 0.04$ ,  $n = 5$ ); (F) RORγt<sup>+</sup>Foxp3<sup>-</sup> proinflammatory Tregs ( $**P < 0.008$ , PBS solution and NCK2025); (G) RORγt<sup>+</sup>Foxp3<sup>-</sup> proinflammatory T cells (trend not statistically significant); and (H) CD4<sup>+</sup>IFN-γ<sup>+</sup> proinflammatory effector T cells ( $*P < 0.016$ ). (Scale bar: 50 μm.) White arrowheads represent F4/80<sup>+</sup> or Gr1<sup>+</sup> cells. Data were analyzed by two-tailed Student *t* test, type 2, compared with untreated mice. Immunostaining, acquisition, and analyses were performed as described for Fig. 3. Antibodies used were mouse anti-mouse CD4 (Abcam), rat anti-mouse IFN-γ (BioLegend), rat anti-mouse Foxp3 (eBiosciences), rabbit anti-mouse RORγt, anti-rat Alexa Fluor 488, anti-mouse Alexa Fluor 594, and anti-rabbit Alexa Fluor 488. Black bars indicate PBS treatment, gray bars NCK56, and white bars NCK2025. Insets in corners of each image show higher magnifications. Data are representative of three independent experiments.

the final founder, only two loxP sites remained flanking exons 11 and 12. Cre-mediated recombination truncated the APC gene at exon 468. The remaining sequences missed all β-catenin binding sites as well as the armadillo repeats, and encoded the most severely truncated APC gene product reported to date. Loss of the third loxP site resulted in a smaller SphI fragment of 1.3 kb. Subsequently, the neoR cassette was removed by crossing to synaptonemal-Cre mice and screening. Mice were backcrossed for more than 20 generations to C57BL/6J. Mice were maintained in microisolator cages under specific pathogen-free, *Helicobacter*-free conditions at the animal care facility. Experiments were performed in an accredited establishment

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according to National Institutes of Health Guidelines for the Guide for Care and Use of Laboratory Animals (NIH-72-23), and the local ethics committee approved animal protocols.

**Bacterial Strains.** *L. acidophilus* NCK56 (NCFM) and NCK2025 were inoculated at 1% and propagated in de Man, Rogosa, and Sharpe broth (Difco) at 37 °C for 15 h. Subsequently, 1 mL of each culture was transferred to 10 mL of fresh de Man, Rogosa, and Sharpe broth and incubated at 37 °C for 18 h. In preparation for oral treatment, the concentration of each *L. acidophilus* strain was adjusted to  $1 \times 10^9$  cfu/mL based on OD<sub>600</sub> readings that had previously been correlated with cfu numbers (24). Each mouse was orally treated with  $5 \times 10^8$  cfu bacterial strain; therefore, 500 μL of  $1 \times 10^9$  cfu/mL suspension was centrifuged, pelleted, and then resuspended in 100 μL of PBS solution. Each mouse was then treated with 100 μL PBS solution (control group) or  $5 \times 10^8$  cfu of either *L. acidophilus* strain orally in 100 μL sterile PBS solution.

**Immunofluorescence.** Colons of mice treated with NCK56, NCK2025, or PBS solution alone were fillet-opened, rolled, and snap frozen at –80 °C in optimal cutting temperature compound. Sections (5 μm) were cut, fixed in ice-cold methanol (–20 °C) for 15 min, and blocked with 1% BSA. Subsequently, sections were incubated overnight at 4 °C with purified hamster anti-mouse CD11c (BD Biosciences), CD4, FoxP3, RORγ, IFN-γ, TNF-α, IL-12, GR1, F4/80, CD103, and rat anti-mouse IL-10 (BioLegend), followed by two washes with PBS solution and incubation with anti-hamster Alexa Fluor 594 and anti-rat Alexa Fluor 488 (Invitrogen) for 1 h. Sections were then washed two times with PBS solution and incubated for 10 min with DAPI (Invitrogen), washed with PBS solution two times, and mounted with antifade mounting medium as described previously (14). Images were acquired by using a Tissue/Cell High Throughput Imaging and Analysis System (TissueGnostics) and analyzed by using ImageJ software. In some experiments, mice were killed, and colon cross-sectional Swiss rolls were fixed in 10% (vol/vol) formaldehyde and embedded in paraffin. Tissue sections (4 μm) were stained for chloroacetate esterase (CAE) and subsequent H&E.

**Flow Cytometry.** Single-cell suspensions of spleens and MLNs of TS4Cre × cAPC<sup>lox468</sup> mice that were treated with PBS solution, NCK56, or NCK2025 were filtered (40 μm), and red blood cells were lysed by using ACK Lysing Buffer (BioWhittaker). Cells were washed and then incubated with Fc block (BD Bioscience). To exclude dead cells, a LIVE/DEAD Violet Dead cell Stain kit was used (Invitrogen). Subsequently, cells were stained with the following antibodies: CD11c FITC (HL3), GR1 APC (RB6-8C5), CD4 Percp (RM4-5), CD25 biotin (7D4), and streptavidin FITC (BD Pharmingen); and F4/80 Percp (BM8) and CD11b PE-Cy7 (M1/70; Biolegend). Data were acquired with an FACS-Canto II device (BD) and analyzed by using FlowJo software (Tree Star).

**Multiplex ELISA.** Sera of PBS solution-, NCK56-, or NCK2025-treated TS4Cre × cAPC<sup>lox468</sup> mice were collected and analyzed for cytokines. Multiplex ELISA was conducted according to the manufacturer's instructions (Millipore) on filtered (0.22 μm) serum. Subsequently, results were acquired with a Luminex 100 instrument and analyzed by using xPONENT software (Luminex).

**Statistical Analysis of Data.** For paired comparisons, we used a paired Student *t* test run with two tails, type 2 between untreated and treated groups. For unpaired comparisons, we used an unpaired Student *t* test.

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