

Targeting aberrant colon cancer-specific DNA methylation with lipoteichoic acid-deficient *Lactobacillus acidophilus*

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Pathogenic autoinflammatory responses triggered by dysregulated microbial interactions may lead to intestinal disorders and malignancies. Previously, we demonstrated that a lipoteichoic acid (LTA)-deficient *Lactobacillus acidophilus* strain, NCK2025, ameliorated inflammation-induced colitis, significantly reduced the number of polyps in a colonic polyposis cancer model and restored physiological homeostasis in both cases. Nonetheless, the regulatory signals delivered by NCK2025 to reprogram the gastrointestinal microenvironment, and thus resist colonic cancer progression, remain unknown. Accumulating evidence suggest that epigenetic changes, in the presence and absence of pathogenic inflammation, can result in colorectal cancer (CRC). To test possible epigenetic modifications induced by NCK2025, the expression of epigenetically regulated, CRC-associated genes was measured with and without bacterial treatment. In vivo and in vitro, NCK2025 enhanced the expression of tumor suppressor genes that may regulate CRC development. Therefore, differential epigenetic regulation of CRC-related genes by NCK2025 represents a potential therapy against colitis-associated and sporadic CRC.

Introduction

Maintenance of mucosal barrier integrity and low responsiveness to the large numbers of commensal microbes that inhabit the gastrointestinal tract are crucial for intestinal health. The intestinal immune

system is able to accurately and efficiently discriminate between invading pathogens and commensal bacteria, thereby attacking only those that are detrimental to the host while deterring an inflammatory reaction in their absence.^{1,2} However, a breach of commensal-specific tolerance can lead to uncontrolled inflammation in the gut and subsequent inflammatory disorders, such as inflammatory bowel diseases (IBD). Chronic inflammation, characterized by activated immune cells, generation of reactive oxygen species (ROS) and DNA damage, predisposes individuals to developing cancer.³ Consequently, IBD patients have been found to be at increased risk for the development of a subtype of colorectal cancer (CRC) termed colitis-associated cancer (CAC).⁴ In CAC, genetic instability resulting from proinflammatory insults promotes epithelial cell transformation and colon cancer progression through the formation of polyps, adenomas and carcinomas. On the other hand, sporadic CRC is thought to arise independent of pre-existing inflammation and the majority of cases are attributed to environmental toxins and irritants, as well as the composition of the microbiome.^{5,6} Nonetheless, inflammatory anti-tumor responses in sporadic CRC can further induce DNA alterations and tumor-promoting mutations.⁷

In our recent publication,⁸ successful treatment of colonic polyposis was achieved via oral treatment with a genetically modified *Lactobacillus acidophilus* NCFM strain, NCK2025, that reduced localized inflammation and enhanced regulatory responses. The mouse model of colonic polyposis used for our studies

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(APC^{lox468} × TS4-Cre) possesses conditional abnormalities in the *adenomatous coli* (*APC*) gene and demonstrates extensive polyposis throughout the distal ileum and colon.⁸ *APC* mutations account for the onset of familial adenomatous polyposis, a hereditary form of CRC, as well as for the majority of sporadic colorectal tumors.⁹ Inactivating mutations of the APC protein disrupt the formation of the Wnt signaling pathway destruction complex and subsequent degradation of β-catenin, a transcriptional coactivator of target genes that contribute to oncogenesis.⁹ Thus, by decreasing cancer progression in APC^{lox468} × TS4-Cre mice through the anti-inflammatory properties of NCK2025, our data provided further evidence for the deleterious role of inflammation in CRC, including subtypes with genetic predisposition. Here, we explore additional regulatory mechanisms orchestrated by lipoteichoic acid (LTA)-deficient *L. acidophilus* that may help counterbalance mucosal epithelial cell damage and genetic instability caused by pathogenic inflammation.

Aberrant Epigenetic Modifications, Inflammation and Colon Cancer

The view that the accumulation of genetic changes results in cancer has evolved to include the unfavorable role of epigenetic modifications of pre-neoplastic cells.^{10,11} Epigenetics, as opposed to mutations in the DNA sequence, refers to heritable changes in gene expression levels due to structural alterations that influence a gene's ability to be activated or silenced. Epigenetic mechanisms that have been proposed to contribute to cancer include DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs.¹⁰ Together, genetic mutations and epigenetic alterations are thought to promote carcinogenesis through the inactivation of tumor suppressor genes and the activation of oncogenes. The most extensively studied dysregulated epigenetic mechanism in cancer is DNA methylation. In fact, many more methylated genes than genetic mutations have been found in primary CRC tumor samples when compared with normal colon samples.¹² DNA methylation plays an essential role in

development¹³ and involves the addition of a methyl group to the 5' position of cytosines. In cancer, CG rich sequences in the promoter regions of tumor suppressor gene become targets for aberrant DNA methylation. Uncontrolled hypermethylation can lead to transcriptional silencing by (1) directly interfering with the binding of transcription factors or by (2) the recruitment of methyl-CpG binding domain (MBD) proteins. DNA-bound MBD proteins provide docking sites for histone deacetylases and other chromatin remodeling complexes resulting in a chromatin structure that is repressive and inaccessible.

Studies aiming to understand the contribution of proinflammatory mediators to changes in the epigenetic landscape are still in their early stages; nonetheless, recent research has shed some light on this association.¹⁴ In the context of IBD, the DNA methylation status of the colonic mucosa positively correlated with the extent of inflammation of the colon sections analyzed (active distal vs. quiescent proximal).¹⁵ Moreover, interleukin-6 (IL-6), a key tumor-promoting proinflammatory cytokine linked with CAC,^{16,17} has been shown to increase and stabilize the protein levels of DNA methyltransferase-1 (DNMT-1), a CpG island methylator, which then led to the methylation of gene promoter regions and targeted-gene silencing.¹⁸ Of interest, our work indicated that clinical improvement of APC^{lox468} × TS4-Cre mice after NCK2025 treatment correlated with lower levels of serum IL-6.⁸ Another study found that in vitro treatment of an intestinal epithelial cell line with interferon-γ (IFNγ) resulted in enhanced DNA methylation corresponding with increases in the expression levels of DNMT-1.¹⁹ On the other hand, proinflammatory cytokines may promote elevated levels of methyltransferases to prevent the transcription of damaging gene products. For instance, in a mouse model of chemically induced colitis, in vivo inhibition of inflammation-induced DNA methylation of the colonic mucosa exacerbated disease.¹⁹ Consequently, in some cases, inflammation-mediated DNA hypermethylation may arise as a protective mechanism that could then become protumorigenic and detrimental to the host if inflammation persists.

Genetic Modifications Induced by NCK2025

Taken together, these observations prompted us to investigate whether our *L. acidophilus* NCFM strain lacking the gene responsible for the biosynthesis of LTA²⁰ could not only change the proinflammatory milieu in CRC but also prevent aberrant DNA methylation of the mucosal epithelium. Before measuring cytosine methylation levels in the disease state, we simply tested the expression levels of genes commonly methylated in CRC^{11,21,22} after treatment with different *L. acidophilus* NCFM strains. In some experiments, healthy C57B/L6 mice were orally treated with the wild-type strain (NCK56), the protective LTA-deficient strain (NCK2025), or PBS, and gene expression analyses of the distal colon took place 24 h later (Fig. 1A). Additionally, we incubated the human colon carcinoma cell line, HT-29 (kindly provided by Dr Yi Qiu, University of Florida), with NCK56 or NCK2025 for 48 h and again measured gene expression differences compared with untreated controls in vitro (Fig. 1B). Our in vivo and in vitro observations indicate that NCK56 was able to induce the expression of some of the genes tested; however, our LTA-deficient protective strain, NCK2025, consistently promoted higher transcriptional levels of genes that have been found to be silenced through DNA methylation in colonic cancer (Fig. 1).

Control of the immune response in the gut by nonpathogenic and pathogenic microorganisms begins with detection of the bacteria through the interaction of pattern recognition receptors (PRR) expressed on resident innate immune cells and microbe-associated molecular patterns (MAMPS) found on the cell surface of the bacteria. Similarly, intestinal epithelial cells not only protect the host by providing a physical barrier against invading microbes, but can also be intimately involved in the maintenance of gut homeostasis by sensing microorganisms through their own PRR and regulating subsequent innate responses.²³ It is not surprising then that microbiota-mucosal interactions could also alter the epigenetic status of intestinal epithelial cells. The two *L. acidophilus* NCFM strains tested

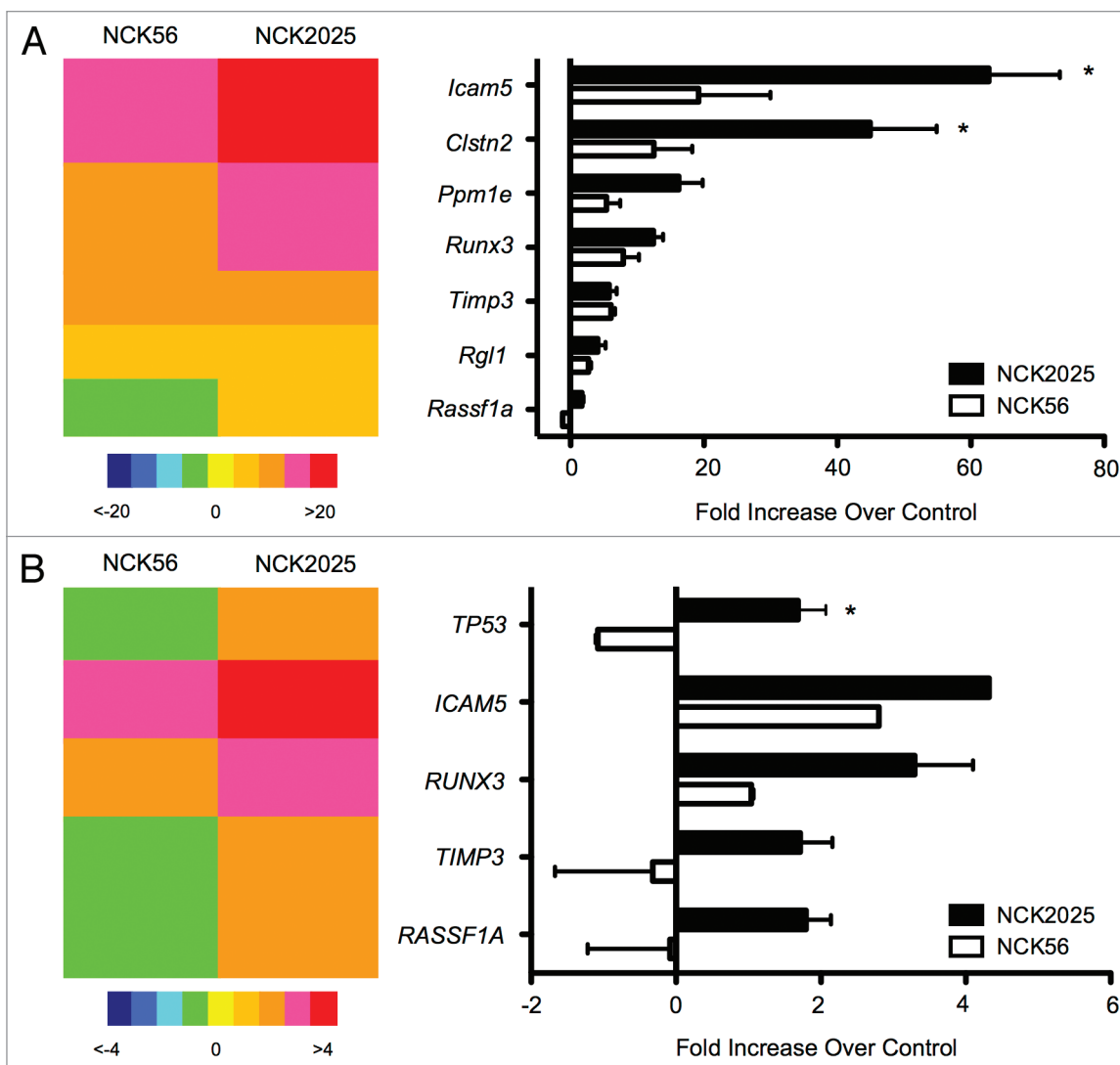


Figure 1. LTA-deficient *L. acidophilus* induces the expression of genes commonly silenced through DNA methylation in CRC. **(A)** Healthy C57B/L6 mice were treated orally with wild-type *L. acidophilus* NCFM (NCK56), protective LTA-deficient *L. acidophilus* NCFM (NCK2025), or PBS, and colonic tissue samples were collected 24 h after treatment. Expression levels of epigenetically regulated, CRC-associated genes were measured by Real-time PCR and normalized to PBS-treated levels. **(B)** HT-29 colon carcinoma cells were treated for 48 h and analyzed as above. * denotes statistical significance $p < 0.05$.

in our studies differ in their expression of LTA on their cells surface. Therefore, our data suggest that the gene expression changes observed are due to other *L. acidophilus* NCFM cell surface components. LTA has been postulated to induce pro-inflammatory immune responses in the gut.^{8,20,24} Conversely, the absence of the surface layer protein A (SlpA) upregulates the production of proinflammatory cytokines,²⁵ indicating an anti-inflammatory effect of this protein. However, contributions of SlpA to epigenetic modifications and auto-inflammation in the host have not been explored. In this respect, the

current focus of our laboratory centers on the role of Slps of *L. acidophilus*, including SlpA, in intestinal immune modulation.

Concluding Remarks

Although the importance of gut microbiota in mucosal immune maturation is appreciated,²⁶ the early results presented here indicate a potential link between commensal and pathogenic bacteria in the colon and the epigenetic status of the intestinal epithelium, which may consequently result in protection from or susceptibility to inflammatory diseases and CRC (Fig. 2). Indeed,

this area of research is presently gaining significant interest.^{27,28} As our previous work demonstrates that NCK2025 protects against precancerous colonic polyps in an animal model of the disease,⁸ future work will focus on the molecular mechanisms responsible for protection beyond changes in the immune response. Namely, we will explore the DNA methylation landscape of diseased mice with and without oral treatment with our bacterial strain of interest. Our current hypothesis is that NCK2025 contributes to protection from CRC by normalizing pathogenic inflammation in the gut, which in turn prevents

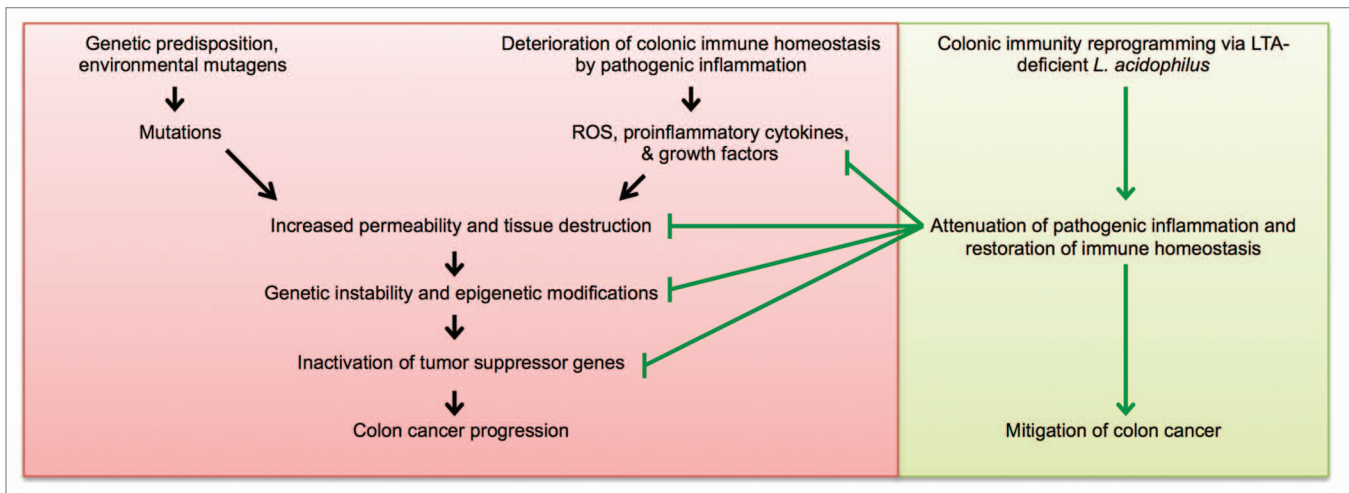


Figure 2. Schematic summary of the potential role of LTA-deficient *L. acidophilus* in CRC mitigation. In sporadic CRC and CAC, tissue destruction and increased permeability lead to genetic instability and aberrant epigenetic alterations, thereby activating a deleterious cascade that culminates in colon cancer progression. Our previous studies demonstrate that NCK2025 dampens pathogenic inflammation in the gut and restores immunological homeostasis. As a result, proinflammatory mediators no longer promote oncogenesis. Data presented here suggest that in addition to its immunomodulatory role, NCK2025 may also be involved in the prevention of epigenetic modifications that are conducive to CRC development. Consequently, LTA-deficient *L. acidophilus* may not only be helpful in the treatment of CAC, but also in the prevention of sporadic CRC.

further tissue damage, genetic instability and propensity to carcinogenesis (Fig. 2). Alternatively, NCK2025 may directly influence the mucosal epithelium to avoid aberrant epigenetic modifications that may inactivate tumor suppressor genes. We have generated a model of invasive colon cancer that, in addition to the *APC* abnormality, also harbors a conditional deletion of the *phosphatase and tensin homolog deleted on chromosome 10 (PTEN)* gene²⁹ ($APC^{\text{lox468}} \times PTEN^{\text{lox}} \times TS4\text{-Cre}$). This animal model provides an additional tool to dissect the molecular events taking place during all stages of colon cancer progression and/or prevention with NCK2025 therapy. Further understanding of the immunomodulatory effects of NCK2025 and the molecular consequences of its treatment in the host will help move this probiotic agent one step closer to clinical application.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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