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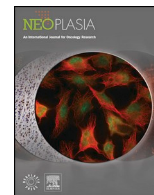

Targeting IL-23 for the interception of obesity-associated colorectal cancer

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ABSTRACT

Inflammation and obesity are two major factors that promote Colorectal cancer (CRC). Our recent data suggests that interleukin (IL)-23, is significantly elevated in CRC tumors and correlates with patient obesity, tumor grade and survival. Thus, we hypothesize that obesity and CRC may be linked via inflammation and IL-23 may be a potential target for intervention in high-risk patients. TCGA dataset and patient sera were evaluated for IL-23A levels. IL-23A [IL-23 p19$^{-/-}$] knockout (KO) mice were crossed to Apc$^{min/+}$ mice and progeny were fed low-fat or high-fat diets. At termination intestines were evaluated for tumorigenesis. Tumors, serum, and fecal contents were analyzed for protein biomarkers, cytokines, and microbiome profile respectively. IL-23A levels are elevated in the sera of patients with obesity and colon tumors. Genetic ablation of IL-23A significantly suppressed colonic tumor multiplicity (76–96 %) and incidence (72–95 %) in male and female mice. Similarly, small-intestinal tumor multiplicity and size were also significantly reduced in IL-23A KO mice. IL-23A knockdown in Apc$^{min/+}$ mice fed high-fat diet, also resulted in significant suppression of colonic (50–58 %) and SI (41–48 %) tumor multiplicity. Cytokine profiling showed reduction in several circulating pro-inflammatory cytokines including loss of IL-23A. Biomarker analysis suggested reduced tumor cell proliferation and immune modulation with an increase in tumor-infiltrating CD4+ and CD8+ T-lymphocytes in the IL-23A KO mice compared to controls. Fecal microbiome analysis revealed potentially beneficial changes in the bacterial population profile. In summary, our data indicates a tumor promoting role for IL-23 in CRC including diet-induced obesity. With several IL-23 targeted therapies in clinical trials, there is a great potential for targeting this cytokine for CRC prevention and therapy.

Introduction

Colorectal cancer (CRC) is the 3rd most common cancer in the United States (US) with an estimated 153,020 new cases and 52,550 deaths in 2023 [1]. Inflammation, a key hallmark of cancer, and obesity are major risk factors for CRC. Studies show that Western-style diet-induced obesity may promote CRC by modulating gut inflammatory mediators. The intestinal tract is constantly exposed to multiple agents, both infectious and noninfectious, that stimulate immune and epithelial cells in the gut lining, and the resulting inflammation creates a favorable environment for cancer initiation, tumor cell survival, and proliferation [2].

The prevalence of obesity in the US continues to increase, recent data indicates that over 40 % of the adult population is obese [3]. Obesity is associated with multiple serious health risks, for example, cancer patients with obesity present with chemoresistance and have a poor

Abbreviations: APC, adenomatous polyposis coli; BMI, body mass index; COX-2, cyclooxygenase-2; CT, colon tumor; CRC, colorectal cancer; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon-γ; IHC, immunohistochemistry; IL-23, interleukin-23; KO, knock out; 5-LOX, 5-lipoxygenase; LBP, lipopolysaccharide-binding protein; mPGES-1, microsomal prostaglandin E synthase; PCA, principal component analysis; PEDF, pigment epithelium-derived factor; SIP, small intestinal polyps; TCGA, the cancer genome atlas; TNF-α, tumor necrosis factor-α.

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prognosis [4]. We previously reported a significant increase in interleukin-23 (IL-23) levels in plasma from human subjects with obesity (BMI> 30 kg/m^2) compared to non-obese individuals (15 individuals/arm; $p < 0.0001$). Furthermore, IL-23A over expression was observed in colonic tumors from humans and rodent models, and this finding is supported by TCGA gene expression data in which IL-23A levels highly correlate with tumor grade, patient weight, and disease-free survival data [2]. These findings suggested that IL-23-mediated pro-inflammatory signaling as a possible important link between obesity and colon tumorigenesis. The present study was designed to understand the role of IL-23 in colorectal carcinogenesis under normal and high-fat diet induced obesity conditions by using an *in vivo* genetic knockout (KO) model.

Many previous studies have assessed the contribution of inflammation promoting enzymes such as cyclooxygenase (COX)-2, 5-lipoxygenase (LOX), and microsomal prostaglandin E synthase (mPGES)-1 to CRC development. However, the role of cytokines has predominantly been studied in inflammatory bowel disease (IBD), ulcerative colitis, or Crohn's disease, [5–8] and the role of inflammation-modulating cytokines in CRC remains understudied. In this context, there is increasing research interest in interleukins based on their potential role in CRC pathogenesis as well as promising results of clinical trials [9–11]. IL-23 is a dendritic cells and macrophage derived cytokine shown to promote skin carcinogenesis [12] and renal cell carcinoma [13]. IL-23p19 is the specific subunit of IL-23 that functions in immune-modulation by positively regulating TH17 and other IL-17-producing cells [14].

Based on this evidence, we hypothesized that disrupting IL-23 by genetic or pharmacological intervention in colon cancer may result in intercepting tumor development. Therefore, to understand the effect of intercepting IL-23 on CRC development, with special emphasis on high-risk groups such as individuals with obesity or genetic predisposition, we performed the following preclinical in vivo experiments. We ablated IL-23A in Apc$^{min/+}$ mice and studied tumorigenesis under normal and high-fat diet conditions. Irrespective of dietary conditions, we observed a significant decrease in intestinal tumors in IL-23A KO mice with a decrease in circulating inflammatory cytokines and increased tumor infiltrating lymphocytes. Further mechanistic studies of the impact of IL-23 on the tumor-promoting microenvironment are needed to extend our knowledge of its biological functions. However, our study highlights opportunities for targeting IL-23 using newly discovered small molecule inhibitors, either alone or in combination with other cancer preventing agents.

Materials & methods

Materials

Apc$^{min/+}$ mice (Stock# 002020) were purchased from the Jackson Laboratory and IL-23A knockout (IL-23 p19$^{-/-}$) mice (stock# 011725) were obtained from MMRRC, UC Davis. High-fat diet (Cat# D12492) was purchased from Research Diets, Inc. Antibodies were purchased from Cell Signaling Technology and Biolegend. The Proteome Profiler Mouse Cytokines Array Kit was from R&D Systems.

IL-23A levels in human sera and colorectal tumors

Serum samples and BMI information of human subjects were provided by Dr. Sanghera (IRB Protocol #2911) and Dr. Morris (IRB Protocol #7565) at OUHSC. These samples were used to determine IL-23A levels using human IL-23A Elisa kit (D2300B, R&D systems) following manufacturer's instructions. Online TCGA data analysis tool UALCAN was used to extract IL-23 expression data in human colon and rectal adenocarcinomas (Figs. 1 and Suppl. Fig. 1) [15].

Breeding and genotyping mice

Male Apc$^{min/+}$ mice (Stock# 002020, Jax) and female IL-23A KO mice (stock# 011725, MMRRC) were crossbred to generate hybrid mice that were backcrossed to IL-23A KO mice to generate Apc$^{min/+}$ mice with IL-23A heterozygous (IL-23A$^{+/-}$;Apc$^{min/+}$) or IL-23A KO (IL-23A$^{-/-}$; Apc$^{min/+}$) status. Pups were genotyped as described earlier [16]. Briefly, genomic DNA was isolated from tail biopsy and PCR was performed using gene specific primers, specifically IL-23-Mut-Neo3a: 5′-GCAG CGCATCGCCTTCTATC-3′;

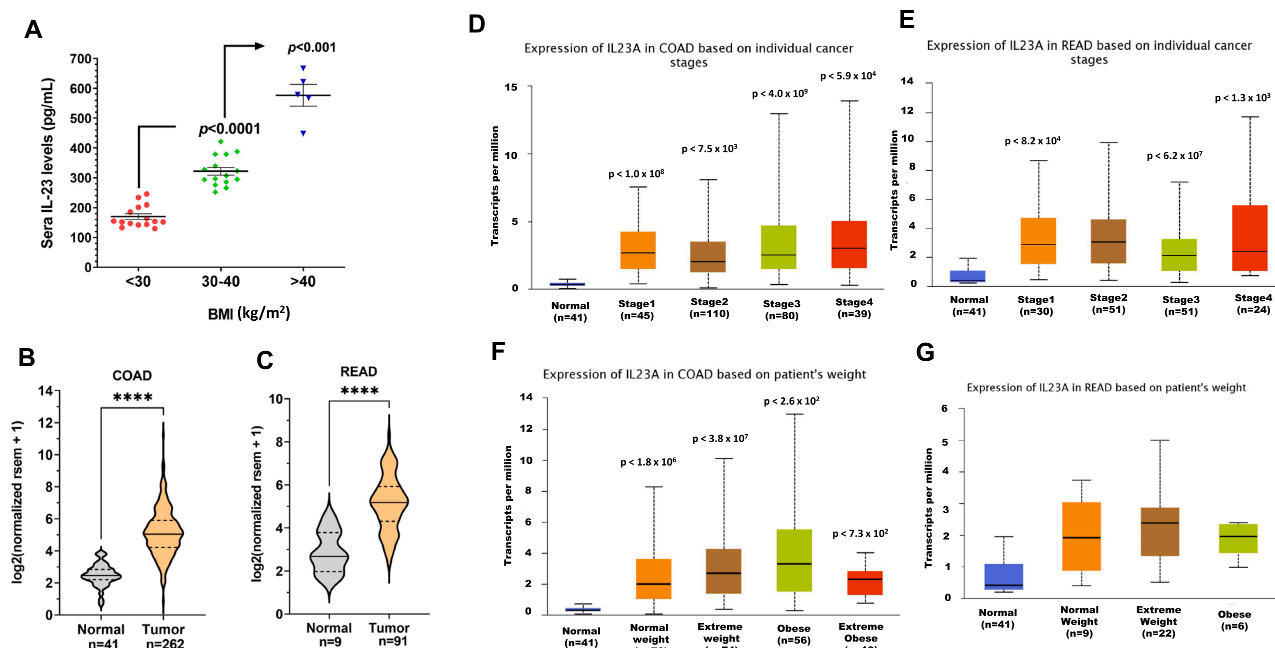

Fig. 1. IL-23 expression is elevated in the serum of individuals with obesity and in colorectal tumors. (A) Sera levels of IL-23 in relation to the BMI of human subjects ($n = 35$) analyzed by ELISA methods. Significantly different from BMI< 30 kg/m^2 group by two-tail Student's *t-test* with Welch's Correction. (B-C) Expression of IL-23A in colon (COAD) and rectal (READ) adenocarcinomas in comparison to corresponding normal tissue. (D-E) Correlation of IL-23A expression in COAD and READ against tumor grade. (F-G) IL-23A gene expression in colorectal cancer patients according to the subject weight.

IL-23-WT-0769-1:5′-TGCAGATCACAGAGCCAGC-3′ & IL-23-Com-0769-3:5′-CTTCCAACCCTCCAGATCC-3′. PCR products were amplified using 55°C annealing temperature and resolved on 1.5 % gels. Amplicon sizes of 305 bp and 250 bp indicated IL-23A wildtype and IL-23A KO alleles respectively. Genotyping for the Apc gene was similarly performed using specific primers and PCR conditions described earlier (Fig. 2) [16].

Bioassay

To determine the effect of IL-23A on intestinal tumorigenesis, six-week-old $Apc^{min/+}$ mice (N ≥ 15/gender) were randomized into groups by IL-23A genotype i.e., Wild type (+/+), heterozygous (+/-), and KO (-/-) then maintained under standard conditions. These mice had unrestricted access to regular chow diet (Lab diet #5053; 4.5 % fat) and water. For diet induced obesity model, another set of mice were fed high fat diet (Research Diets # D12492; 30 % fat or 60 Kcal %fat). At 20 weeks of age, all mice were euthanized by CO_2 asphyxiation and tissues were harvested by individuals blinded to group information. Small and large intestines were longitudinally dissected, freed of fecal matter, and evaluated for tumors. Kidneys, liver, and spleen were weighed. Tumor data was analyzed and differences in incidence, multiplicity, size, and location between the three IL-23A genotypes was compared as described earlier [17].

Immunohistochemistry (IHC)

Formalin fixed paraffin embedded (FFPE) intestinal tumors were sectioned and processed for IHC analysis using standard protocols followed in our laboratory [17]. Briefly, FFPE tumor tissue sections (5 μm) from wild type and KO mice were prepared, deparaffinized and rehydrated using Xylene and graded ethanol solutions respectively. After heat-induced antigen retrieval (in 0.01 M citrate buffer; pH 6.0), endogenous peroxidase quenching (3 % H_2O_2), and blocking steps, tissue sections were incubated with appropriately diluted primary antibody, anti-Ki-67 (cs12202; cell signaling, 1:100) anti-Cyclin D1 (cs55506; cell signaling, 1:50), anti-CD4 (cs25229; cell signaling,1:100), anti-CD8a (201701; Biolegend, 1:100), overnight. Sections were then washed and incubated with HRP-tagged secondary antibodies. Slides were developed using chromogen diaminobenzidine and counterstained with hematoxylin. Multiple representative images were captured with a camera attached to a bright field microscope (Olympus AX71). Positive cells (brown stain) were enumerated and compared between groups.

Cytokine array

Serum was used to analyze cytokine levels using the Proteome Profiler Mouse Cytokine Array (ARY006; R&D systems) following manufacturer's instructions. The mouse Cytokine Array simultaneously analyzes a panel of 40 cytokines and chemokines in duplicate. The cytokines and chemokines represented in this array include: Interleukin (IL)-1A, IL-1B, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, IL-23A, CXCL-1, CXCL-2, CXCL-10, CXCL-11, CCL-2, CCL-4, CCL-5, Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and granulocyte-macrophage colony stimulating factor (GM-CSF). Results are expressed as pg/ml of plasma.

Fecal sample collection and microbiome analysis

At termination, mouse colons were dissected longitudinally, and fecal contents were carefully collected in sterile freezing vials, snap frozen in liquid nitrogen and stored at -80°C until analysis. Fecal pellets were transferred to extraction tubes with buffer, supplied in kits provided by TransnetYX, and shipped for analysis. Microbiome profiling of mouse fecal samples was performed using shallow shotgun, next-generation sequencing at TransnetYX, Inc. (Cordova, TN). For each group, 3 biological replicates were analyzed. Data was analyzed on their OneCodex platform.

Statistical analysis

All values are presented as Mean ± *SEM*. The significance of the differences in organ weights and tumor multiplicity between the groups was analyzed by Student's *t*-test with Welch correction. Tumor incidence was compared using Fisher's Exact test. The data were analyzed using GraphPad Prism 9.4 software (Dotmatics).

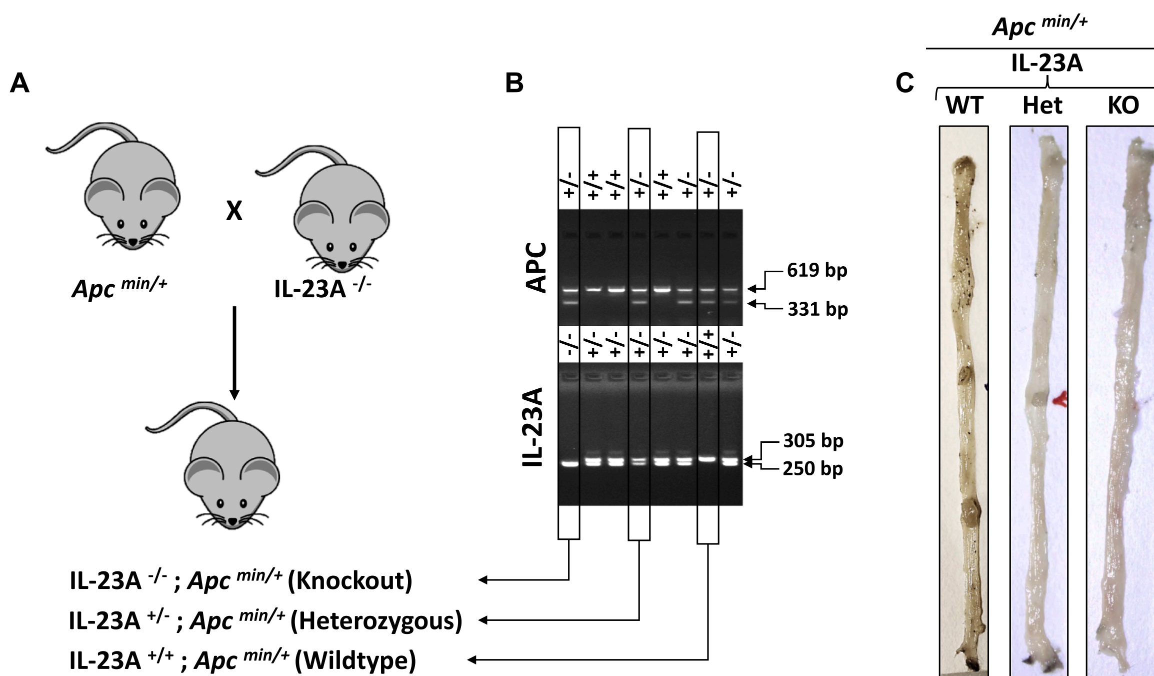

Fig. 2. *Breeding and generation of $Apc^{min/+}$ mouse with various IL-23A genotypes.* (A) $IL\text{-}23A^{-/-}$ mice were crossed to $Apc^{min/+}$ mice to generate male and female mice with $IL\text{-}23A^{+/+}$; $Apc^{min/+}$ (IL-23A wild type), $IL\text{-}23A^{+/-}$; $Apc^{min/+}$ (IL-23A heterozygote), and $IL\text{-}23A^{-/-}$; $Apc^{min/+}$ (IL-23A knockout) genotypes. (B) Representative agarose gel showing PCR amplification products following genotyping. (C) Representative images of the colons from $Apc^{min/+}$ mice with various IL-23A genotypes.

Results

IL-23 levels correlate with obesity and colon tumor grade in humans

Serum for human subjects (n=35) was analyzed to determine circulating levels and compared with BMI. There was clear correlation between cytokine levels and obesity. Significant increase in IL-23 were observed in subject with BMI >30 kg/m^2 and >40 kg/m^2 when compared to those <30 BMI kg/m^2 (Fig 1A). Analysis of the gene expression data from TCGA also indicated an increase in IL-23A mRNA expression in several cancers (Supplementary fig 1A). Importantly, colon and rectal adenocarcinoma tumors showed significant increase in IL-23A expression when compared to normal tissue (Fig. 1B and C). Further, this expression was found to be correlating with tumor stage and weight of the patients (Fig. 1D–G).

General observations in IL-23A knockout mice

IL-23A KO mice were viable, fertile and their appearance was similar to IL-23A wildtype mice. When crossed with Apc$^{min/+}$ mice, the pups exhibited mendelian inheritance (Fig. 2A). Hybrid mice grew normally with gradual body weight gain. Generally, Apc$^{min/+}$ mice develop cachexia as they age due to increasing tumor burden [18]. Interestingly, IL-23A heterozygous and IL-23A KO mice had greater weight gain with significantly higher terminal bodyweight compared to IL-23A$^{+/+}$ Apc$^{min/+}$ mice. Splenomegaly is another common feature in Apc$^{min/+}$ mice,[19] and this was also significantly decreased in IL-23A KO Apc$^{min/+}$ mice (Suppl. Figs 2 & 3). This data indicates overall improved health with IL-23 knock down.

Knocking down IL-23A significantly suppressed colonic tumors in CRC mouse model

Colonic tumor (CT) multiplicity data indicated that genetic ablation of IL-23A led to significant suppression of large intestinal tumors of Apc$^{min/+}$ mice in both genders (Figs 2C & 3). Male and female Apc$^{min/+}$ mice with a wildtype IL-23A genotype had mean CT multiplicities of 1.35 ± 0.16 and 0.61 ± 0.13, respectively. Among males, IL-23A$^{+/-}$; Apc$^{min/+}$ mice exhibited 76 % lower CT multiplicity (0.31 ± 0.15; $p <$ 0.0001) while the IL-23A$^{-/-}$; Apc$^{min/+}$ mice exhibited 96 % inhibition (0.05 ± 0.05; $p < 0.0001$) in comparison to IL-23A$^{+/+}$; Apc$^{min/+}$ mice (Fig. 3A). In the female mice, both the IL-23A$^{+/-}$; Apc$^{min/+}$ and IL-23A$^{-/-}$; Apc$^{min/+}$ groups had near 90 % fewer CT (0.06 ± 0.09; $p < 0.001$) when compared to IL-23A$^{+/+}$; Apc$^{min/+}$ mice (Fig. 3B).

CT incidence was also assessed in the mice with various IL-23 genotype groups. While 91 % of IL-23A$^{+/+}$; Apc$^{min/+}$ male mice had CT, the incidence was reduced by 72 % ($p < 0.0001$) in IL-23A$^{+/-}$; Apc$^{min/+}$ and by 95 % ($p < 0.0001$) in the IL-23A$^{-/-}$; Apc$^{min/+}$ mice (Fig. 3C). Similarly, in IL-23A$^{+/+}$; Apc$^{min/+}$ female mice, CT incidence was 52 %, and the CT incidence was over 88 % lower ($p < 0.005$) in both IL-23A$^{+/-}$; Apc$^{min/+}$ and IL-23A$^{-/-}$; Apc$^{min/+}$ group mice (Fig. 3D). Thus, a gene-dose dependent decrease in colonic tumor number was observed in both male and female Apc$^{min/+}$ mice indicating that targeting IL-23 may intercept CT formation.

Small intestinal polyps (SIP) are strongly suppressed with IL-23A knockdown in Apc$^{min/+}$ mice

A profound suppressive effect of IL-23A knockdown on SIP multiplicity was also evident (Fig. 3E–J). Total SIP in IL-23A$^{+/+}$; Apc$^{min/+}$ male mice were 27.48 ± 2.38, in IL-23A$^{+/-}$; Apc$^{min/+}$ and IL-23A$^{-/-}$; Apc$^{min/+}$ mice SIP were reduced by 54 % (12.56 ± 1.64; $p < 0.0001$) and 58 % (11.45 ± 1.03; $p < 0.0001$) respectively (Fig. 3E). In female mice we saw 55 % (13.88 ± 1.05; $p < 0.0001$) and 59 % (12.73 ± 2.03; $p <$ 0.0001) fewer SIP respectively in the IL-23A$^{+/-}$; Apc$^{min/+}$ and IL-23A$^{-/-}$; Apc$^{min/+}$ mice when compared to IL-23A$^{+/+}$; Apc$^{min/+}$ mice (30.90 ± 2.54) (Fig. 3F).

The suppressive effect of IL-23A knockdown was so profound that it was also reflected in reductions of SIP of all size and in all locations in both male and female mice. A significant reduction in small, medium, and large SIPs was observed with IL-23A knockdown in both male and female mice (Fig. 3G and H). Most importantly, large polyps were significantly inhibited in both genders (> 95 % inhibition; $p < 0.0001$). SIP multiplicity, based on its location in the small intestine, was also evaluated and we found that SIPs were significantly inhibited along the entire small intestine (duodenum, jejunum, and ileum) in both IL-23A$^{+/-}$; Apc$^{min/+}$ and IL-23A$^{-/-}$; Apc$^{min/+}$ mice ($p < 0.001$) (Fig. 3I and J).

Intestinal tumorigenesis was also suppressed in IL-23A$^{-/-}$; Apc$^{min/+}$ mice fed a high-fat diet

To determine if IL-23A knockdown has a similar tumor suppressive effect on high-fat diet -induced obesity-associated CRC, we fed high-fat diets to IL-23A$^{+/+}$; Apc$^{min/+}$ and IL-23A$^{-/-}$; Apc$^{min/+}$ *mice*. Upon termination, CT multiplicity was compared; similar to the above findings, IL-23A knockdown had a strong inhibitory effect on high-fat diet driven CT and SIP (Fig 4). IL-23A$^{-/-}$; Apc$^{min/+}$ male mice had 50 % less CT (0.81 ± 0.25 vs 1.61 ± 0.27; $p < 0.05$) while female mice had 58 % fewer CT (0.40 ± 0.16 vs 0.94 ± 0.21; $p = 0.05$) compared to the IL-23A$^{+/+}$; Apc$^{min/+}$ control mice (Fig. 4A and B). Similarly, CT incidence was suppressed by 36% and 48% in male and female IL-23A$^{-/-}$; Apc$^{min/+}$ mice respectively compared to control mice (Figs 4C and 4D). SIP multiplicity was also inhibited by 48 % (15.00 ± 1.13 vs 28.54 ± 1.11; p < 0.0001) and by 41 % (14.83 ± 1.35 vs 25.18 ± 1.71; $p < 0.0005$) in male and female IL-23A$^{-/-}$; Apc$^{min/+}$ mice respectively compared to IL-23A$^{+/+}$; Apc$^{min/+}$ (Fig. 4E and F). Both small and large SIP were significantly inhibited in both male and female IL-23A$^{-/-}$; Apc$^{min/+}$ *mice* (Fig. 4G and H) compared to respective control mice. Distribution analysis suggested significant inhibition of SIP in the jejunum which was the location of most of the SIP (Fig. 4I and J).

Tumor cell proliferation was suppressed while T-lymphocyte infiltration increased in IL-23A KO mice

IHC analysis of FFPE tumor tissue sections was performed to determine level of cell proliferation markers. The data showed a decrease in Ki-67 and cyclin D1 in tumors from IL-23A$^{-/-}$; Apc$^{min/+}$ mice compared to IL-23A$^{+/+}$; Apc$^{min/+}$ (Fig. 5A and B). CD4 and CD8 staining was performed to understand the effect on T-lymphocyte cell infiltration. We observed increased CD4+ and CD8+ T-lymphocytes in the villi and tumors from IL-23A$^{-/-}$; Apc$^{min/+}$ mice compared to controls (Fig. 5C and D). These data suggest that IL-23 contributes to immune evasion of the tumor cell and promotes their survival. Therefore, intercepting IL-23 may enhance immune cell infiltration and tumor regression by immune modulation.

Proinflammatory cytokines are decreased in the IL-23A KO mice

Plasma prepared from whole blood collected upon termination was analyzed to determine the effect of IL-23A KO on circulating levels of various cytokines and chemokines. Plasma from IL-23A$^{-/-}$; Apc$^{min/+}$ showed significant reductions in proinflammatory cytokine and chemokines levels compared to IL-23A$^{+/+}$; Apc$^{min/+}$ mice. While IL-23 and IL17 were not detected, other cytokines including IL-1β, IL-10, IL-13, CCL-2, CCL-3, CCL-5, TNFα, and IFNγ were greatly reduced. There was an increase in CXCL1 with no change in CXCL12 or C5/C5a (Fig. 5E and F)

High fat diet-induced microbial dysbiosis

Fecal samples from IL-23A$^{+/+}$; Apc$^{min/+}$ *and* IL-23A$^{-/-}$; Apc$^{min/+}$ mice fed either the regular or high fat diet were analyzed to generate gut

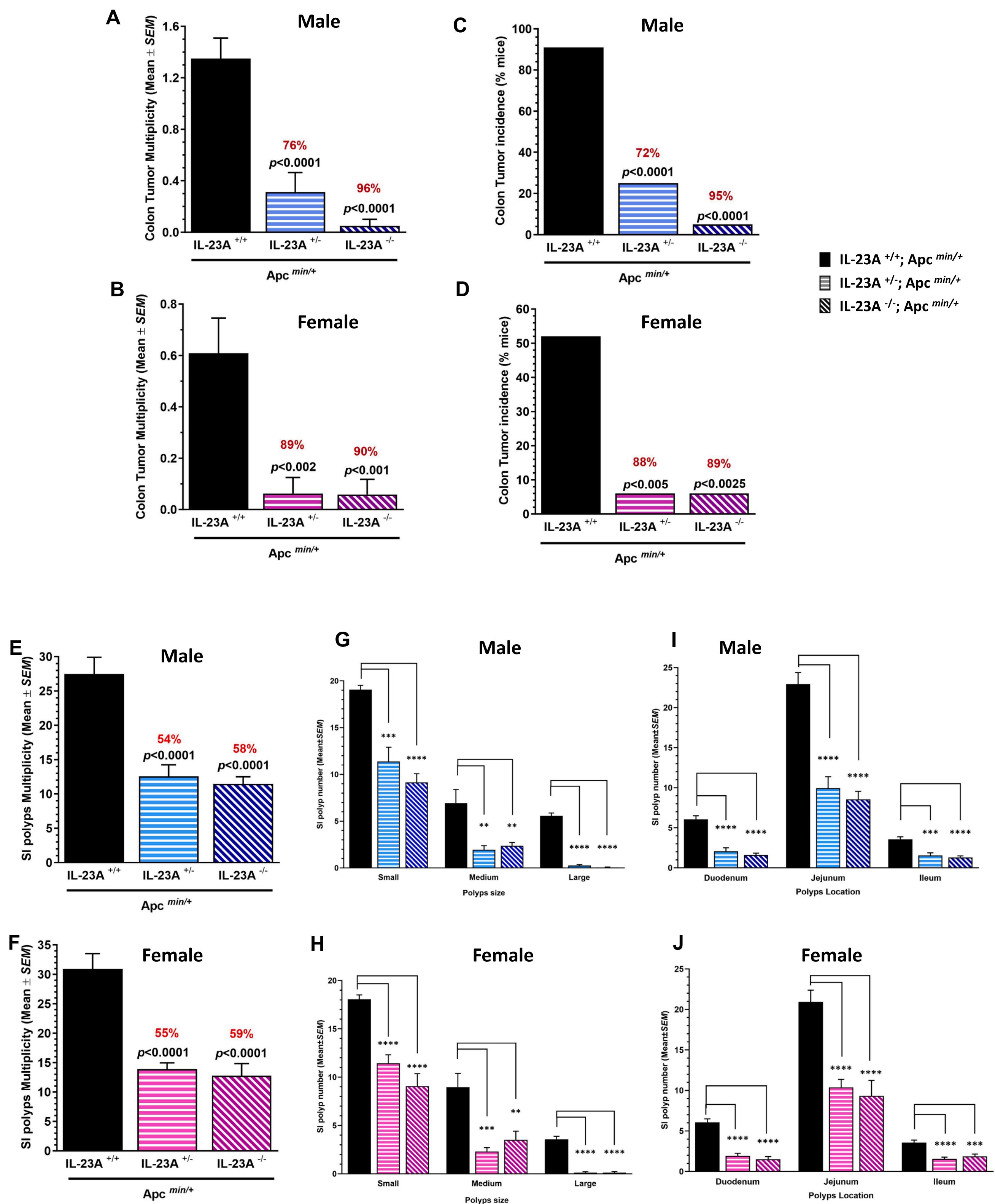

Fig. 3. *IL-23 knockdown led to suppression of colonic and small intestinal tumors in Apc$^{min/+}$ mouse.* IL-23A$^{-/-}$ mice were crossed to Apc$^{min/+}$ mice to generate male and female Apc$^{min/+}$ mice with IL-23A$^{+/+}$; Apc$^{min/+}$ (IL-23A wild type), IL-23A$^{+/-}$; Apc$^{min/+}$ (IL-23A heterozygote), and IL-23A$^{-/-}$; Apc$^{min/+}$ (IL-23A knockout) genotypes. Starting at 6-weeks of age mice were fed regular chow diet. Mice were grouped by genotype and gender (n ≥ 15 per group). At 20-weeks of age, mice were euthanized, and colons were analyzed for tumors. Colonic tumor multiplicity and incidence were significantly suppressed in both male (A, C) and female (B, D) mice of the genotypes IL-23A heterozygous (IL-23A$^{+/-}$; Apc$^{min/+}$) and IL-23A knockout (IL-23A$^{-/-}$; Apc$^{min/+}$). Small intestinal polyp multiplicity was suppressed in both male (E) and female (F) mice either IL-23A heterozygous or lacking IL-23A. Analysis of small intestinal polyps data by all polyp size (G-H) and distribution across small intestine (I-J) was also significantly altered in both male and female mice either IL-23A heterozygous or lacking IL-23A. Significance calculated using students t-test, *=$p < 0.05$; **=$p < 0.01$; ***=$p < 0.001$; ****=$p < 0.0001$.

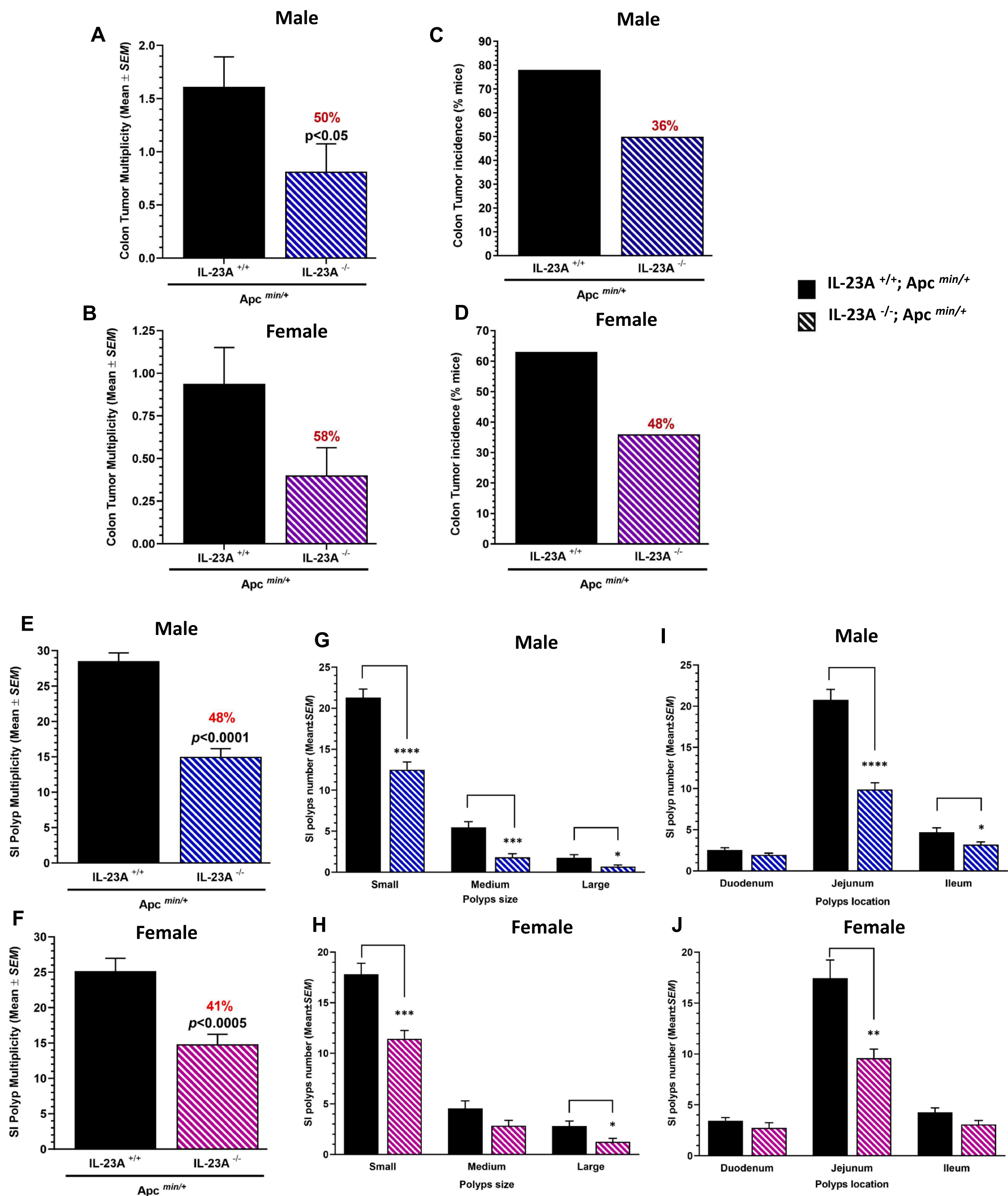

Fig. 4. *Large and small intestinal tumors were inhibited in high-fat diet fed Apc$^{min/+}$ mice with IL-23 knockdown.* IL-23A$^{-/-}$ mice were crossed to Apc$^{min/+}$ mice to generate male and female Apc$^{min/+}$ mice with IL-23A$^{+/+}$; Apc$^{min/+}$ (IL-23A wild type), and IL-23A$^{-/-}$; Apc$^{min/+}$ (IL-23A knockout) genotypes. Starting at 6-weeks of age mice were fed a high-fat diet. At 20-weeks of age, mice were euthanized, and colons were analyzed for tumors. IL-23 knockdown resulted in reduced tumor multiplicity in high-fat diet fed male (A) and female (B) mice. Colonic tumor incidence was also significantly reduced in (C-D) IL-23A KO mice fed a high-fat diet. IL-23 knockdown resulted in significant reduction of small intestinal polyps (E-F). Also, there were significant differences in small intestinal polyp numbers when categorized by various sizes (G-H) and by distribution (I-J) in IL-23A KO mice fed a high-fat diet compared to controls. Significance calculated using students t-test, *=$p < 0.05$; **=$p < 0.01$; ***=$p < 0.001$; ****=$p < 0.0001$.

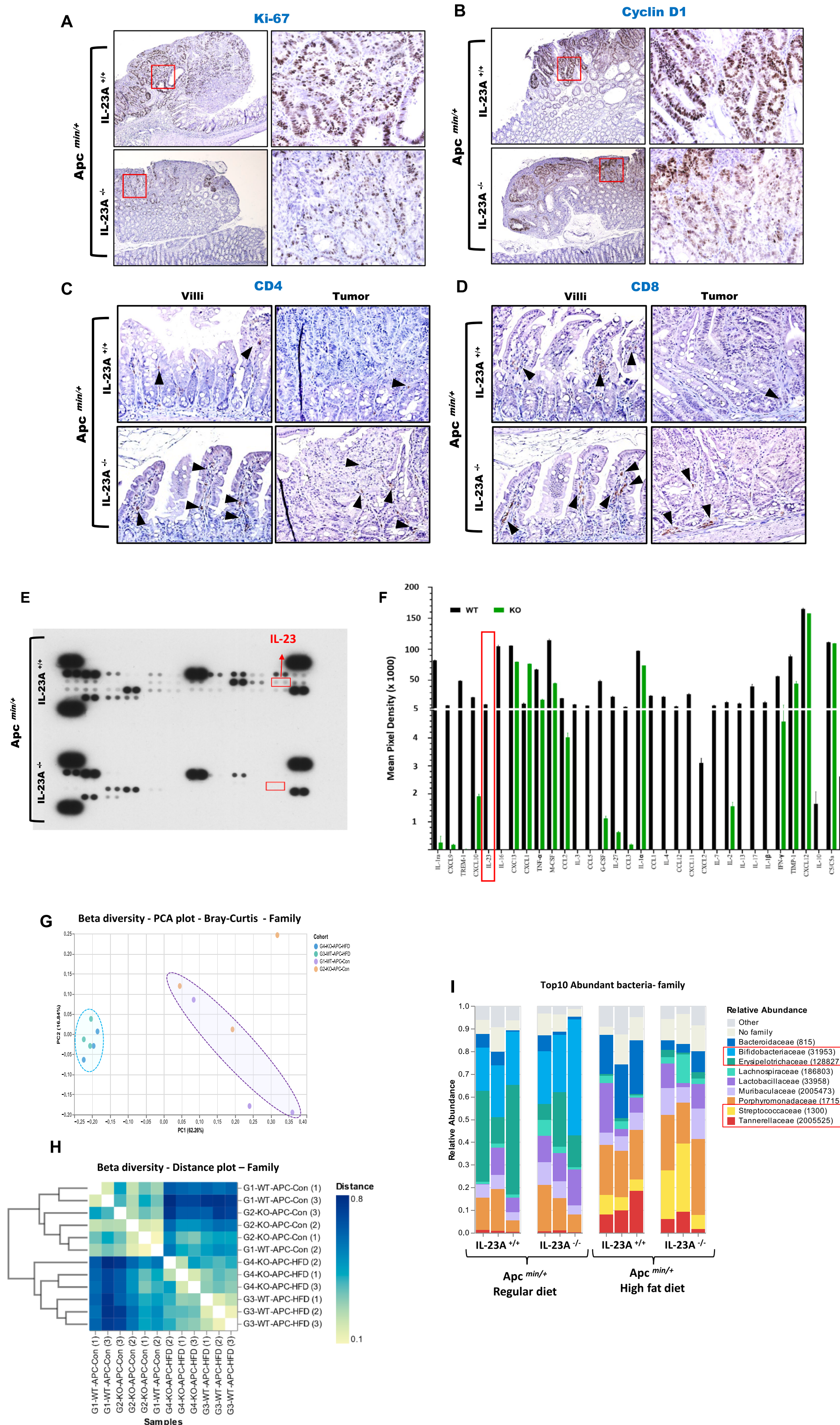

Fig. 5. *Tumor cell proliferation was inhibited alongside changes in T-lymphocytes infiltration, circulating cytokine and chemokine levels.* Immunohistochemistry of formalin fixed tumors showed reduction in Ki-67 (A) and cyclin-D1 (B) positive cells in IL-23A$^{-/-}$; Apc$^{min/+}$ mice tumors compared to controls. IL-23A$^{-/-}$; Apc$^{min/+}$ mice tumors showed increased CD4+ (C) and CD8+ (D) cells when compared to IL-23A$^{+/+}$; Apc$^{min/+}$ mice tumors. Mouse plasma was analyzed using the Proteome Profiler Mouse Cytokine Array. IL-23A$^{-/-}$; Apc$^{min/+}$ mice showed reduction in circulating cytokine and chemokine levels. Image showing the cytokine array (E) and densitometric scanning results for each cytokine (F). (G-I) *High-fat diet fed mice had altered microbiome compared to control diet fed mice.* Fecal samples collected from mice fed the regular diet or the high-fat diet were analyzed to determine microbiome profile using shallow shotgun, next-generation sequencing at TransnetYX, Inc. (Cordova, TN). Data obtained were analyzed on their OneCodex platform. Images show principal component analysis plot for beta diversity (G), distance plot for beta diversity (H), and relative abundance of top 10 bacterial families (I).

microbiome profiles. Principal component analysis (PCA) and beta diversity of the fecal microbiome at the family level showed high similarity in the gut bacteria composition based on type of diet fed rather than the IL-23A genotype (Fig. 5G and H). When the most abundant microbial species were analyzed by mouse IL-23A genotype, *Faecalibaculum rodentium, Parabacteroides distasonis, Akkermansia muciniphila, Bacteroides uniformis,* and *Bacteroides vulgaris* appeared to be less abundant in IL-23A$^{-/-}$; Apc$^{min/+}$ mice compared to IL-23A$^{+/+}$; Apc$^{min/+}$ mice. On the other hand, *Muribaculum intestinale, Lactobacillus murinus,* and *Lactococcus lactis* showed an opposite trend (Suppl. Fig. 4). *Bifidobacteriaceae* and *Erysipelotrichaceae* members were highly abundant in the mice fed regular diet, and present at much lower levels in the high-fat diet fed mice. *Streptococcacea* and *Tannerellaceae* members were abundant in high-fat diet fed mice while their number was very low in regular diet mice (Fig 5I). Notably several bacterial species, such as *Bifidobacterium pseudolongum,* [20,21] *Faecalibaculum rodentium,* [22,23] and *Erysipelotrichaceae bacterium,* [24] which are known to have beneficial effects are lost in high-fat diet-fed mice. *Lactobacillus johnsonii;* [25] *Lactobacillus murinus,* [26] and *Lactobacillus reuteri* were present at similar levels in both regular and high-fat diet fed mice, while *Parabacteroides distasonis,* [27] *Lactococcus lactis,* [28,29] *Bacteroides uniformis,* [30,31] *Muribaculum intestinale,* [32] and *Akkermansia muciniphila* were more abundant in high-fat diet fed mice than in those fed a regular diet (Suppl. Fig 4).

Discussion

CRC is a preventable disease; however, low colonoscopy rates and lack of effective preventive agents make it a life-threatening disease primarily due to late diagnosis and aggressive metastatic tumors. Chronic inflammation is a well-established risk factor in CRC that contributes to tumor initiation and progression. Various cell types, including immune cells, endothelial cells, and cancer-associated fibroblasts, are present in the tumor microenvironment and interact with tumor cells via secretion of signaling molecules such as cytokines, chemokines, growth factors, and other enzymes. Aberrant expression of such signaling molecules, particularly cytokines, and their complex interactions, determine the pro- or anti-tumorigenic signals within the tumor environment thus shaping tumor progression, therapeutic response, and disease outcomes [33]. Thus, understanding the role of individual cytokines during CRC tumorigenesis will provide insights into mechanistic details and facilitate the development of novel agents for both therapy and prevention [10].

IL-23 is a pro-inflammatory cytokine produced by macrophages and dendritic cells in response to exogenous or endogenous signals. It is made up of two subunits, IL-23A (p19) and IL-12/23B (p40–shared with IL-12). IL-23 affects the differentiation/activation of T-helper 17 cells and production of other cytokines like IL-17A, IL-17F, IL-6, IL-22, and TNF-α. Interestingly, in our study we observed depletion of IL-17 along with IL-23 in the IL-23A knockout mice. The IL-23/IL-17 signaling pathway is well studied in the development of several inflammatory diseases, such as psoriasis, rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis, and Sjogren's syndrome [34–36]. Although the role of IL-23 in IBD, [37] ulcerative colitis, [38] and Crohn's disease is well studied, an understanding of its contribution to cancer continues to evolve [39]. These studies have led to development of multiple agents against IL-23 that target either the p19 subunit (mirikizumab, risankizumab, brazikumab, guselkumab) or the p40 subunit (ustekinumab, briakinumab) which are under clinical investigation [38,40]. While these monoclonal antibodies exhibit efficacy, they also have some side-effects, and some patients have no response or a partial response. Small molecule inhibitors of IL-23 as well as alternative approaches, such as inhibiting intracellular signaling cascades, are also being developed [41].

Transgenic mice lacking production or function of individual interleukin have been invaluable tools to study the role of cytokines in various diseases. For example, use of IL-4 and IL-13 KO mice treated with the colon carcinogen azoxymethane (AOM) established the pro-tumorigenic role of IL-4 and IL-13. In another study, mice over-expressing human IL-8 exhibited increased tumor number suggesting its tumor promoting role. Herein, we used IL-23p19 KO mice crossed to Apc$^{min/+}$ mice, which spontaneously develop both small and large intestinal tumors, to study its role in CRC development. Apc$^{min/+}$ lacking IL-23 production developed significantly fewer tumors in both the small intestine and the colon. These data strongly support the pro-tumorigenic role of IL-23 in CRC, and is consistent with similar observations in the CPC-Apc model where the Apc mutant was driven by Cdx2- cre leading to development of distal colon tumors [42]. Both these studies show that disrupting IL-23 can result in significant inhibition of intestinal tumors. Moreover, IL-23 is also shown to promote other cancers including those of the skin [12] and kidneys.

Obesity is a modifiable risk factor for CRC; recent epidemiological studies indicate that approximately 50 % of US adults are obese and its prevalence is rising [3]. Excess dietary fat intake is clearly associated with elevated CRC risk; a high-fat diet is known to promote CRC through diet induced cytokines and adipokines, and by modulating the gut microbiota [43,44]. In the present study high fat-diet fed IL-23A$^{-/-}$; Apc$^{min/+}$ mice developed significantly fewer tumors than IL-23A$^{+/+}$; Apc$^{min/+}$ mice on the same diet, suggesting that targeting IL-23 may be effective in obesity-associated CRC patients. While our study highlights the role of IL-23 in obesity mediated inflammation and its contribution to CRC, it should be noted that obesity and inflammation are complex conditions involving several circulating signal mediators that can influence the tumor cell in multiple ways [45]. Some of these cytokines have been very well studied, but the contribution of a few others such as pigment epithelium-derived factor (PEDF) [46], lipopolysaccharide-binding protein (LBP) is less explored [47]. Studies indicate that circulating levels of these cytokines correlate with individuals' BMI, metabolic deterioration, and insulin resistance. Further, a correlation between these cytokines and well established proinflammatory cytokines like TNF-α, IL-1β, IL-6 etc. was observed in sera of subjects with obesity [46,47]. Although the role of these circulating cytokines is studied in obesity models, their role in cancer and their potential as target for cancer prevention needs to be investigated.

Splenomegaly is an indicator of underlying inflammatory conditions. In Apc$^{min/+}$ mice spleen size correlates with tumor burden [19]. In our study, IL-23A KO Apc$^{min/+}$ mice had smaller spleens than did IL-23 wild type Apc$^{min/+}$ mice. These data also correlated with the reduced tumor multiplicity and tumor size in the IL-23A KO Apc$^{min/+}$ mice and are similar to data reported in the CPC-APC model [42]. Our findings support the hypothesis that monitoring spleen size can be used to interpret intestinal tumors in Apc mutant mouse models [19].

Although we could not study the role of p40 / IL12, our study shows that IL-23p19 KO mice develop significantly less tumors than do IL-23 wild type mice, and that IL-23 is a promising target against CRC. IL-23 targeted therapies, particularly those targeting p19, may be useful in CRC management/prevention. Our results clearly demonstrate the colon tumor-promoting role of IL-23 and strengthen our hypothesis that this target will be useful for CRC interception in high-risk individuals with obesity.

CRediT authorship contribution statement

Venkateshwar Madka: Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Srikanth Chiliveru:** Formal analysis, Methodology. **Janani Panneerselvam:** Formal analysis, Methodology. **Gopal Pathuri:** Formal analysis, Methodology. **Yuting Zhang:** Formal analysis, Methodology. **Nicole Stratton:** Formal analysis, Methodology. **Nandini Kumar:** Formal analysis, Methodology. **Dharambir K. Sanghera:** Resources, Writing – review & editing. **Chinthalapally V. Rao:** Conceptualization, Data curation, Formal

analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2023.100939.

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