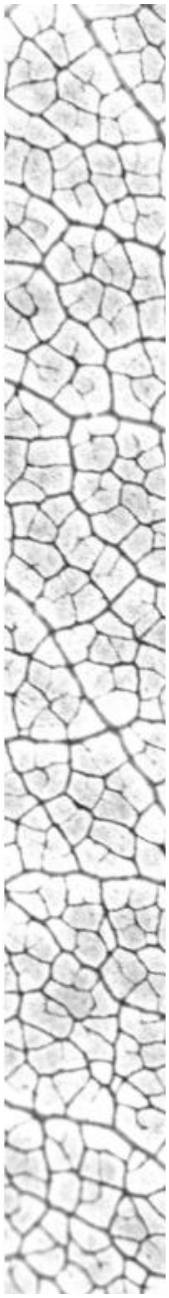

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Early IL-5 transgenesis of colitis-associated colorectal cancer (CA-CRC) mouse model exacerbate the disease severity

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Abstract

Aims: To explore the role of IL-5 in eosinophils induction and cytokine modulation on the development of colitis-associated colorectal cancer mouse model (CA-CRC).

Methods and Material: A plasmid carrying the mouse IL-5 gene (IL-5 transgenesis) was injected to the mice with concurrently induction of CA-CRC mouse model, Then (CA-CRC) development, cytokine gene expression, cell dynamics in tumor vicinity and peritoneal cavity were analysed.

Results: A protumorigenic role of IL-5 was shown on early stages of CRC development as mice experienced severe physical symptoms including: rectal bleeding, diarrhea and significant reduction in body weight gain. Late findings of IL-5 transgenesis revealed higher tumor rates (89.0% vs 50%), significantly higher tumor count (15.1±7.2 vs 7.3±10.6 per colon), higher average tumor size (9.1±5.2 vs 4.4±6.5 mm), higher invasiveness index and shorter colon length. Microscopic examination of tumors revealed the presence of higher plasma cell count but lower eosinophils in AOM-DSS-pIL-5 treated group when compared to AOM-DSS group. Cytokine gene expression pointed out to the downregulation of IL-10 and TGF-β and the overexpression of IFN-γ in both tumor bearing groups compared to pIL-5 group. IL-5 treatment of AOM-DSS mice resulted in insignificant reduction in IL-5 gene expression in colon tissue that was associated with lower eosinophil count in the peritoneal cavity wash.

Conclusions: Immunomodulatory effect of early IL-5 expression in the CA-CRC and its role in the early migration of eosinophils to colon epithelia during colitis development raised the colitis severity and induced higher polyp rate formation and consequently higher tumor load.

Keywords: colitis-associated colorectal cancer, Eosinophils, IL-5, transgenesis

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INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent types of cancer and has become the third in ranking in men and women worldwide (Ferlay et al., 2019). In Jordan, CRC was ranked the second among all cancers in both genders and accounted for 11.3% of all cancers (Cancer Incidence in Jordan, 2012). Ulcerative colitis (UC) is an idiopathic colorectal inflammatory disorder that has a high risk of developing into CRC if not effectively treated, at which point it cannot be resolved (Zhou et al., 2019). Responses of the immune system against colorectal tumors can be identified in early stages of cancers, showing the capability of immune cells to recognize tumors (Di Caro et al., 2014). In the context of colorectal tumors, macrophages, dendritic cells, neutrophils and lymphocytes, such as T cells have all been studied. Tumor progression or rejection has been shown to be controlled by different populations of these cells, which eventually affects the overall outcome for the patient (Fridman WH, 2012).

Cytokines are small proteins secreted by different types of cells and have a specific effect on the interactions and communications between cells (Zhang & An, 2007). Cytokines can have suppressive or enhancing effects on cellular proliferation, differentiation, activation, and motility (Cohen & Cohen, 1996). Moreover, cytokines stimulate the host immune system to produce anti-tumor specific response (Tartour & Fridman, 1998). Interleukin-5 is a cytokine produced by many cell types that function in the maturation and release of eosinophils in the bone marrow (Greenfeder et al., 2001). Eosinophils are circulating granulocytes that contribute to host defense against parasites and

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promotion of allergic reactions (Wen & Rothenberg, 2016). Infiltration of eosinophils in tumor tissue is considered an independent prognostic factor. Recruitment of eosinophils to tumor sites often lead to the release of the granule proteins of eosinophils and tumor-associated cytokines upon activation, thus damaging and killing tumor cells (Gotlib, 2015). Accounting for their functional differences in tumorigenesis, eosinophils are correlated to a favorable prognosis in colorectal, breast and prostate cancers (Sakkal et al, 2016). However, eosinophils infiltrate multiple tumors where they can display either pro- or antitumorigenic functions, leading to controversy about their role (Rothenberg & Hogan, 2006; Ikutani et al., 2012; Reichman et al., 2019). The objective of this study was to explore the role of IL-5 and eosinophils on the development of CRC using the AOM/DSS cancer mouse model.

MATERIALS AND METHODS

Plasmid Isolation, Injection and Validation

E. coli (Strain GT110) containing plasmids (pORF-mIL-5 from InvivoGen, USA) was cultured in LB media with continuous shaking. Plasmid was extracted from bacterial pellet using E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-Tek, USA) according to the manufacturer's instructions, and concentration was measured spectrophotometrically using NanoDrop Plate (Thermo Scientific™ Multiskan™ GO, Finland). A concentration of 100 µg of purified plasmid containing IL-5 was injected into mouse hind legs intramuscularly. IL-5 gene expression in mouse muscles as well as blood IL-5 concentration were measured over a two months period to evaluate the effectiveness of IL-5 transgenesis.

Animal experiments

Male BALB/C mice aged 8 weeks and weighing 27 g were provided by the animal facility, Yarmouk University, Irbid, Jordan. The animal groups were housed individually in plastic cages and allowed access to normal diet and water ad libitum, with a light/dark cycle of 12:12 h Food and water consumption was comparable between treated and control groups. Measures were taken to avoid all unnecessary distress to the animals. Housing, anesthesia, and postoperative care concurred with the guidelines established by an Institutional Animal Ethics Committee Approval (No. 16/3/3/310 Jordan University of Science and Technology).

Treatment Protocol and Data Collection

The Azoxymethane (AOM) Dextran sodium sulfate (DSS) model (Parang, Barrett, & Williams, 2016) was based on a single intraperitoneal injection of (10 mg/kg body weight) AOM (ChemCruze, USA) and three cycles of (2.5%) inflammatory agent DSS (TdB Consultancy, Sweden) in drinking water, over a period of ten weeks. Forty mice were randomly divided into four groups (n=12 mice per group); group one was the control group, which received water, the second group received AOM/DSS only, the third group received AOM/DSS and was injected intramuscularly with the purified plasmid containing IL-5, and the fourth group received the purified plasmid containing IL-5 only. One hundred micrograms of purified pORF-IL-5 was injected intramuscularly into the hind limbs of mice at week 1 and week 5.

Animals were individually weighed on weekly basis and signs of bloody stool and diarrhea were recorded when noticed. On the day of experiment termination (Week 17), colon was recovered from each mouse and inspected for the presence of abnormalities, including masses and deformation. Colon length and weight were measured then collected for RNA extraction and gross analysis.

For histological examination mice colon tissues were fixed in 10% formalin for 24 hours, and then tissues were cassetted before processing. Tissue processing was performed using Spin Tissue Processor (Thermo Scientific, USA). Samples were serially dehydrated in increasing ethanol baths (70%, 80%, 96%, and 100%) for approximately one and a half hour each. Subsequently, tissue samples were placed in 100% xylene for three hours to allow for tissue clearing. Samples were then incubated in a paraffin wax for four hours. After that, samples were placed into a mold maintaining their original orientation, embedded in 100% paraffin wax and then left to cool at room temperature overnight. Paraffin blocks were cut into thin sections (5 µm) using Electronic Rotary Microtome (Thermo Scientific, USA). Sections were stained with hematoxylin and eosin (H&E) and slides were examined and evaluated microscopically.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from colon tissue using TRIzol reagent (Life Technologies, Carlsbad, Ca, USA) according to the manufacturer instructions. The concentration of RNA was measured using NanoDrop (Thermo Fisher Scientific Multiskan GO, Finland). The isolated RNA was reverse transcribed to cDNA using a Reverse Transcription kit (Applied biosystem, Lithuania) according to procedure supplied by the manufacturer.

Real Time PCR

Gene expression of cytokines was quantified using Quantifast SYBR green qPCR kit according to the manufacturer instructions (Qiagen, USA). The qPCR reaction was started by adding 10 µl 2X Quantifast mix, 1 µl (500 ng/µl) cDNA, 0.8 µl forward, 0.8 µl reverse primers and 7.4 µl nuclease free water to PCR tubes with a final volume of 20 µl. All cytokine expression levels were normalized to GAPDH gene. The primer sequences for different genes under study were as follows: pORF-mIL-5*

FW:AGAGACCTTGACACAGCTGT,RV:CTCTCCT CGCCACACTTCTC, INF-γ (Al-Omari et al, 2019) FW: TTCTTCAGCAACAGCAAGGC,RV:TCAGCAGCGACT CCTTTTCC, IL-10 (Al-Omari et al., 2019) FW: ATAACTGCACCCACTTCCCA,RV:GGGCATCACTTC TACCAGG, TGF-β1 (Al-Omari et al., 2019) FW: CCTGCAAGACCATCGACATG,RV:TGTTGTACAAAG CGAGCACC, GAPDH* (Housekeeping gene) FW:TGCAGTGCCAGGTGAAAATC,RV:ATCACGTCCT CCATCATCCC.* Custom designed primers.

The PCR cycling conditions were 95°C initial denaturation for 30 seconds, followed by 40 cycles of 95°C denaturation for 10 seconds, annealing at 60°C for 20 seconds and template extension for 20 seconds at 72°C. The relative expressions of cytokine genes were calculated using comparative Ct (2-ΔΔCt) analysis methods and assayed by Roter-Gene Q-QIAGEN (Germany), as in the equations below.

Relative expression= 2-ΔΔCt

ΔΔCt = ΔCt (treated sample) –ΔCt (control sample)

ΔCt= AVG. Ct (gene of interest) –AVG. Ct (housekeeping gene).

Flow Cytometry

On the day of experiment termination, mice were euthanized and peritoneal cells were collected in cold PBS with 1% BSA according to a standard protocol (Al-Qaoud et al 2015). Approximately 2×106 single-cell suspensions of mouse peritoneal cells were incubated on ice for 30 minutes with 1 ml of blocking buffer (0.5% BSA and 2% normal fetal bovine serum in 1X PBS). Next, samples were centrifuged and pellet resuspended with 125 µL FACS buffer (0.5% BSA and 0.05% Sodium Azide (NaN3) in 1X PBS) containing diluted primary antibody as per manufacturer's recommendations, PE-conjugated anti-Siglec-F and FITC-conjugated CD11c (Invitrogen, USA) and incubated for 30 minutes on ice in the dark. Then, samples were rinsed 3 times in FACS buffer by centrifugation for 5 minutes with minimum light exposure. Cells were fixed with 1 ml of fixation buffer (2% paraformaldehyde in 1X PBS) and analyzed using BD FACSCanto™ II (BD Biosciences, USA).

Statistical Analysis

Data was statistically analyzed using the Statistical Package for the Social Sciences (SPSS) software. Experimental groups were compared with the negative and positive control groups using Kruskal-Wallis followed by Mann-Whitney U test. The mean and standard errors were calculated with a significant probability level of p≤ 0.05.

RESULTS

Testing of IL-5 Expression in Plasmid Injected Mice

A preliminary experiment was set to evaluate the expression level of IL-5 in mice sera after the intramuscular injection of IL-5 containing plasmid (pORF-mIL-5). Muscle pieces were excised from the site of mouse injection and IL-5 mRNA levels were evaluated by RT-PCR (**Fig. 1**). The serum IL-5 level of normal mice was measured using ELISA method at different time-points (**Fig. 1**). Expression levels started to increase after two days and remained high at 30 days. However, declining levels were seen between 60 and 90 days. Based on these data we decided to repeat the injection of mice with the optimized dose of pIL-5 every month.

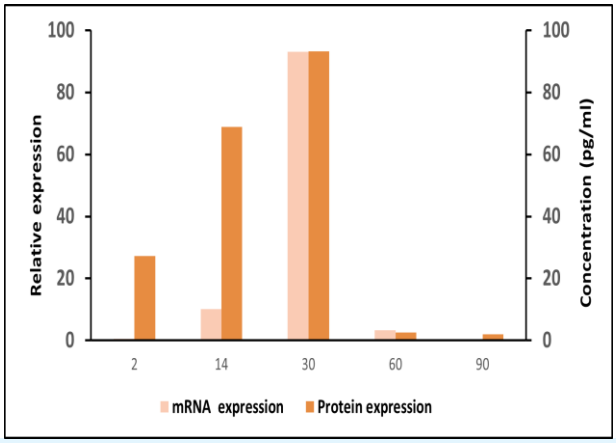

Fig. 1. IL-5 gene expression levels of normal BALB/c mice. Expression of mRNA was measured by real-time PCR from muscle and protein concentration was measured by ELISA from serum at different time-points

Clinical Assessment of Colitis and Tumor Progression

The weight of mice in each group was recorded in the beginning of every week and at the end of the experiment. The average weight of AOM-DSS and AOM-DSS-pIL-5 treated groups significantly decreased following each round of DSS treatment. Thus, at the end of the study in termination (week 17), the average weight of AOM-DSS-pIL-5 group was significantly lower than that of the other groups. The percentage of weight gain showed that AOM-DSS-pIL-5 group had the lowest weight gain in comparison to AOM-DSS treated group (**Table 1**). Weight gain in the control and pIL-5 groups was steadily higher compared to the other groups.

Rectal bleeding, diarrhea, and fecal occult blood (FOB) were regularly monitored during the experiment period. The fecal occult blood was positive in all mice in AOM-DSS treated groups after the first cycle of DSS. The treatment in group AOM-DSS resulted in the death of 5 mice after the second cycle (data not shown) which reduced the group size.

Macroscopic Examination of Mice Colons

The total colonic length of the AOM-DSS-pIL5 group was significantly shorter than that of the AOM-DSS group. Also, the gross inspection of dissected colons (**Figs. 2** and **3**) revealed the development of tumors in half of the AOM-DS were more abundant in the AOM-DSS-pIL-5 group (Figure 4 and 6). Mice (3 out of 6), whereas tumors were observed in most mice of the

Table 1. Average weight of mice and weight gain week 1,5 and 17. Data are presented as the mean ± SD. Kruskal-Wallis test followed by Mann-Whitney U-test were used for statistical analysis. Results were significant at p-value ≤ 0.05

	Week 0	Week 5	Week 17
AOM/DSS (mean weight (g))± SD	27.8±2.8	31.2±2.0	31.14 ±2.3
% weight gain	0%	11.60%	7.47%
AOM/DSS+pIL-5 (mean weight (g))± SD	27.0±1.9	28.7±1.5	26.75 ±4.4*/**
% weight gain	0%	5.53%	-3.6%
pIL-5(mean weight (g))± SD	27.3±1.4	28.7±1.2	31.1±1.7
% weight gain	0%	5.36%	14.2
Control (mean weight (g))± SD	27.5±2.3	29.1±1.1	30.3±0.7
% weight gain	0%	6.33%	13.17%

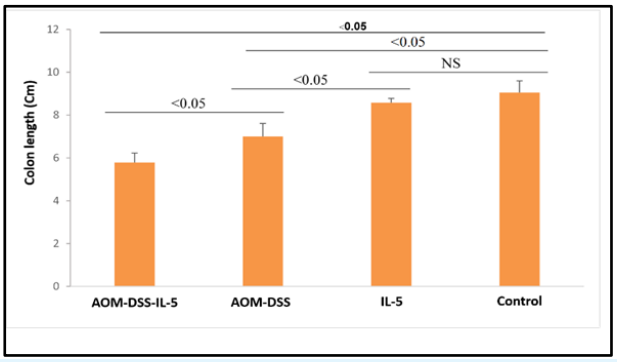

Fig. 2. The average length of mice colon in each group at the end of experiment. Data. are presented as the mean ± SD. Kruskal-Wallis test followed by Mann-Whitney U-test were used for statistical analysis. Results were significant at p-value ≤ 0.05. NS: not significant

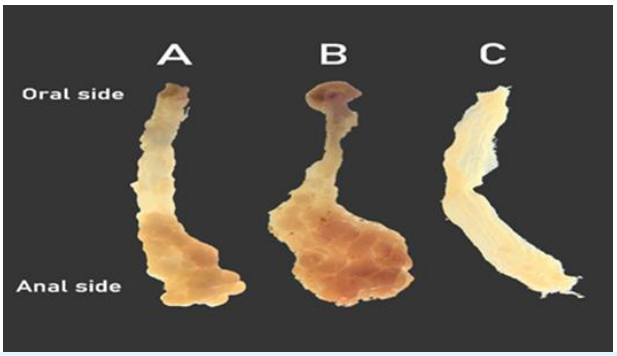

Fig. 3. Macroscopic view of the location of oral and anal side of mouse colon. (A) AOM-DSS group mouse colon, (B) AOM-DSS-pIL-5 group mouse colon, (C) Normal mouse colon. AOM-DSS; Dextran Sodium Sulphate-Azoxymethane treated group, AOM-DSS-pIL-5; Dextran Sodium Sulphate-Azoxymethane with pORF-mIL-5 treated group, pIL-5; pORF-mIL-5 treated group

AOM-DSS-pIL-5 group (8 out of 9). No tumors were found in both control groups.

Moreover, the average number of tumors masses in AOM-DSS-pIL-5 group was higher than in AOM-DSS group, which was also significantly higher in tumor average size (9.1±5.2 vs 4.4±6.5 cm) and invasiveness rate (88.8% vs 50%).

Microscopic Examination of Tumor Masses

Microscopic examination of the Hematoxylene Eosin stained sections of tumor masses revealed the presence of significantly higher eosinophils count in the AOM-DSS group compared to AOM-DSS-pIL-5 (**Figs. 4** and **5**),

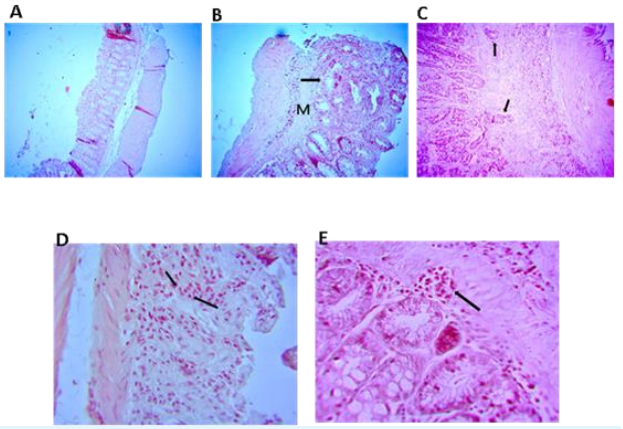

Fig. 4. Representative histopathological analysis in the distal colon with H-E staining, A. normal colonic wall in control group B. a small dysplastic focus (arrowhead) with no invasion to the muscularis mucosa (M) or the lamina propria in AOM-DSS group C. A large tumor with invasion into the lamina propria (arrowhead) in AOM-DSS-pIL-5 group D. eosinophils (arrowhead) among other inflammatory cells in the background in AOM-DSS group E. A collection of plasma cells in AOM-DSS-pIL-5 group magnification objective is 10x

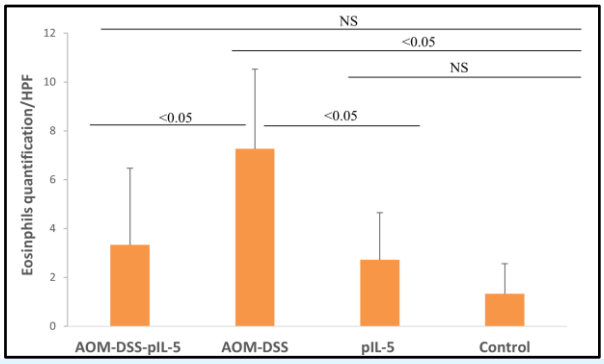

Fig. 5. Eosinophils quantification/HPF in sections prepared from the colon of mice groups stained with hematoxylin and eosin stain. The average of 10 observed fields is represented in this figure. Kruskal-Wallis test followed by Mann-Whitney U-test were used for statistical analysis. Results were significant at p-value ≤ 0.05. NS: not significant

while plasma cells were more abundant in the AOM-DSS-pIL-5 group (**Figs. 4** and **6**).

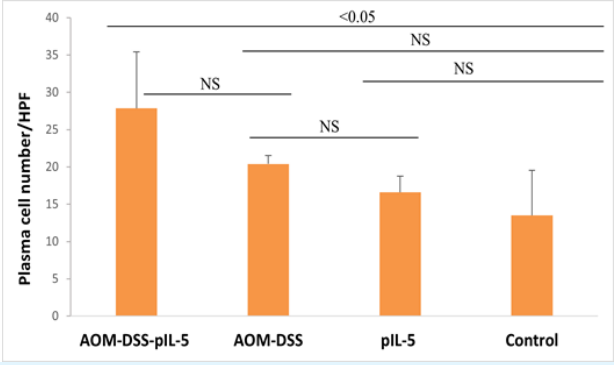

Fig. 6. Plasma cell quantification/HPF in sections prepared from the colon of mice groups stained with hematoxylin and eosin stain. The average of 10 observed fields is represented in this figure. Data are presented as the mean ± SD. Kruskal-Wallis test followed by Mann-Whitney U-test were used for statistical analysis. Results were significant at p-value ≤ 0.05. NS: not significant

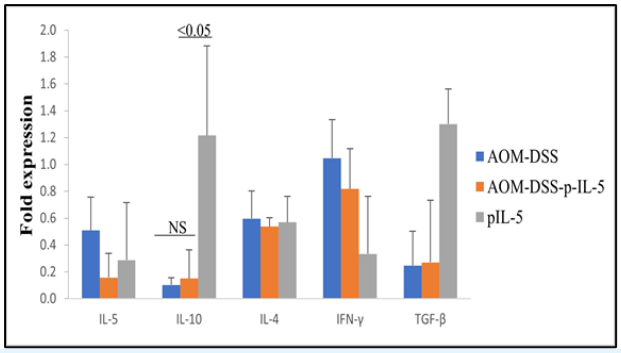

Fig. 7. The fold expression level of IL-5, IL-10, IL-4, IFN-γ, TGF-β and IL-4 in mouse colon tissues. Values are given as mean ± SD. Kruskal-Wallis test followed by Mann-Whitney U-test were used for statistical analysis. Results were significant at p-value ≤ 0.05. NS: not significant

Cytokines Gene Expression of Colon Tissue by Real-time PCR

The total mRNA of colonic tumors was harvested to evaluate the expression of selected cytokines gene at week 17. The cytokine mRNA expression was normalized to a value relative to GAPDH mRNA expression and was presented as relative to the values for the control mice group. Gene expression of IFN-γ was higher in AOM-DSS treated groups compared to control groups, whereas the gene expression of IL-10 and TGF-β in both groups was lower than control groups. Regarding IL-5 gene expression, AOM-DSS treated group expressed higher level compared to AOM-DSS-IL-5 group. However, no significant difference in the expression of IL-4 gene was observed between the groups IL-4 gene expression was almost the same in all groups (**Fig. 7**)

Quantification of Eosinophils in Peritoneal Cavity

To further evaluate the changes in eosinophils in tumor surrounding environment, peritoneal cell

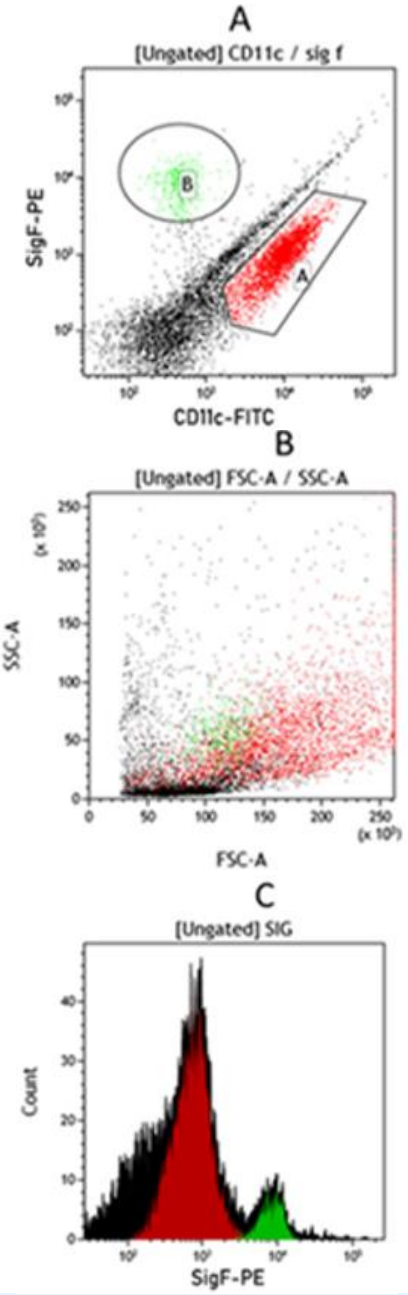

Fig. 8. Macroscopic view of the location of oral and anal side of mouse colon. (A) AOM-DSS group mouse colon, (B) AOM-DSS-pIL-5 group mouse colon, (C) Normal mouse colon. AOM-DSS; Dextran Sodium Sulphate-Azoxymethane treated group, AOM-DSS-pIL-5; Dextran Sodium Sulphate-Azoxymethane with pORF-mIL-5 treated group, pIL-5; pORF-mIL-5 treated group

populations were examined using specific Siglec-F monoclonal antibodies in combination with anti CD11c antibodies (**Fig. 7a**). Siglec-F+/CD11c− population was the lowest in AOM-DSS-IL-5 treated mice (0.57% ± 0.44%) and was significantly different from IL-5 treated mice (2.7% ± 1.2%) (**Fig. 8**).

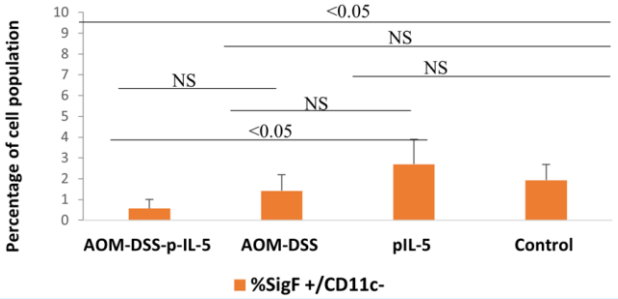

Fig. 8 (continued). Macroscopic view of the location of oral and anal side of mouse colon. (A) AOM-DSS group mouse colon, (B) AOM-DSS-pIL-5 group mouse colon, (C) Normal mouse colon. AOM-DSS; Dextran Sodium Sulphate-Azoxymethane treated group, AOM-DSS-pIL-5; Dextran Sodium Sulphate-Azoxymethane with pORF-mIL-5 treated group, pIL-5; pORF-mIL-5 treated group

DISCUSSION

This study aimed to investigate the effect of IL-5 transgenesis on the development of CRC in a chemically induced mouse model and to explore the possibility of using IL-5 in immunotherapy in CRC cases. Our results showed a protumorigenic role of IL-5 on early stages of CRC development as mice developed physical symptoms including rectal bleeding, diarrhea and reduced weight gain. The experiment was terminated intentionally after 17 weeks to give enough time for prominent tumor development and better tumor evaluation (Lee et al., 2016). However, as the IL-5 plasmid was injected at weeks 1 and 5, our results may point to early effect of IL-5 transgenesis, i.e. effect on colitis development and early polyp stages. In association with a higher load of chemically-induced tumors, IL-5 injected mice, exhibited lower eosinophil count in tissues and peritoneal cavity and lower IL-5 gene expression, but higher plasma cell count in tissues compared to non-injected AOM-DSS mice. However, AOM-DSS-p-IL-5 group expressed lower IL-5, TGF-β and IL-10, but higher IFN-γ, which indicates that IL-5 may enhance or inhibit the action of other cytokines.

AOM-DSS treatment induced shortening of the colon, which is one of the biological markers of severity of colonic inflammation. Similarly, a study by Inoue et al. (Inoue et al., 2007) showed that colorectal length was significantly shortened compared to the normal control group after 120 days from the completion of DSS administration, as the length in the 5% DSS, 2% DSS and normal control groups was 8.2±0.9 cm, 9.1±0.6 cm and 12.1±0.6 cm, respectively. Our study clearly indicated that tumor number and size were markedly higher in IL-5 injected mice, which also showed higher invasive carcinoma and plasma cells, but lower eosinophils count in colon tissue and peritoneal cavity. Consistent data on the role of eosinophils in CRC have been reported by many studies. Cho et al. reported a decrease in the number of eosinophils in the tumor tissue of patients with CRC. Moreover, it is evident that better disease prognosis is associated with an increase in tissue-infiltrating eosinophils in colorectal cancer (Cho et al., 2016). Therefore, decreased tissue eosinophilia may represent an immune evading strategy of colon cancer.

This sum of data on the of positive role of eosinophils in CRC control is consistent with the data obtained from our study (higher tumor load associated with lower eosinophils count), but unfortunately indicated that the treatment regimen we followed did not induce a sustainable high eosinophilia in mice all over the period of experiment as noticed at week 17. As we proved in the preliminary pilot experiment that the expression of IL-5 and eosinophils induction was successful (**Fig. 4**), the low eosinophils and IL-5 expression at late stages may be explained by two possible ways: one is that early colitis developed in mice and this is known to be sustained for about 8 weeks until the polyps start to develop (Tanaka et al., 2003).That coincided with IL-5 peak of expression depicted as higher eosinophilia, worsening inflammation (as indicated by frequent mice death and higher bleeding rate and diarrhea) and as a consequence, induced polyp formation, as supported by Reichman et al (Reichman et al., 2019). But at later stages, the expression of IL-5 ceased and the early stage eosinophils disintegrated without being substituted. The second explanation is the antagonistic role of IFN-γ. The high levels of IFN-γ gene expression in AOM-DSS-pIL-5 and AOM-DSS groups seem to be specific to the tumor microenvironment. Recent evidence showed that IFN-γ also plays an important role in the escape phase, which characterizes the protumorigenic face of IFN-γ and epitomizes the paradoxical nature of IFN-γ signaling pathway in cancer (Zaidi, 2019). Ni et al. have reported that while IFN-γ induces angiostasis, IFN-γ-mediated signaling also dissociates perivascular cells from blood vessels, which contributes to the acceleration of tumor metastasis (Ni et al., 2017).

IL-5 association with colon cancer was evident (Anagnostopoulos et al., 2005; Kato et al., 2010). According to the study by Baier et al. (2005) a significant increase of IL-5 in tumor tissue compared to normal mucosa in colon carcinoma was found, upon which they hypothesized a contribution to local immune escape mechanisms (Baier et al, 2005). IL-5 was originally identified as a B-cell growth and differentiation factor (Kinashi et al., 1986). Its role in plasma cell development and longevity was reported by Brynjolfsson et al. (2018), who showed that long lived plasma cells play an important role against diseases (Brynjolfsson, et al., 2018). Our data regarding the presence of significantly high number of plasma cells in colons of mice injected with pIL-5 may be explained by the early induction of plasma cell development and settlement in the colon

tissue. Thus, the role of these cells in antibody secretion mainly IgA urges further studies.

Our results showed reduction in IL-10 gene expression in AOM-DSS and AOM-DSS-p-IL-5 groups. These findings are supported by Čačev et al. (2008), who revealed a statistically significant decrease in IL-10 mRNA expression in tumor tissue compared with corresponding normal colon tissue (Čačey et al, 2008). Many of the proinflammatory cytokines are abundantly expressed in chronic

inflammatory lesions and the tumor tissues, but it was reported that, unlike these proinflammatory cytokines, IL-10 is not expressed in tumor tissues (Mumm et al., 2011). IL-10 is a pleiotropic cytokine, although it was initially described as an immunosuppressive and anti-inflammatory. Recent findings indicate that the stimulating properties of IL-10 depend on the type of tumor and the disease condition analyzed (Asadullah et al, 2003).

The down-regulation of TGF-β in both AOM-DSS groups is consistent with a study by Matsushita et al., (1999), who showed that TGF-β receptor mRNAs were faintly expressed in eight of 22 colorectal cancers, and that down-regulation was greater for TGF-β RI than for TGF-β RII (Matsushita et al, 1999). As for the meaning of down-regulation of TGF-β RI, Wang et al. reported that low TGF-β RI expression levels in a human colon carcinoma cell can be a limiting factor for TGF-β response and autocrine-negative activity (Wang et al., 1996). The low expression levels of TGF-β in our model could have been initiated by IL-5 treatment in early stages of adenoma formation as both cytokines have antagonistic activity to each other (Kanzaki et al., 2007; Xie et al, 2011), or may have been dysregulated by developed tumor as proposed by other studies (Villalba et al, 2017).

The peritoneum serves as a reservoir for eosinophils and expresses high levels of receptor Siglec-F (Ohnmacht et al, 2007); therefore, it represents the nearest pouch environment surrounding the digestive system. Some studies attempted to dissect the cell population to understand cancer development and metastasis (Castro-Giner & Aceto, 2020). Eosinophils in peritoneal cells (PC) confirmed the data obtained from cancer tissue. AOM-DSS-pIL-5 eosinophils were lower than its counter group (AOM-DSS) (**Fig. 7**). Moreover, flow cytometry results indicated the presence of many cell populations with distinct features that can be used for further study of cell tumor interaction and cytokine interplay in this vicinity.

In conclusion, IL-5-induced eosinophils seem to have a significant protumorigenic effect on tumor development that is distinct from its role in regulation of immune cells, in a way that involves pro-inflammatory and anti-inflammatory cytokine profile changes. We may hypothesize that the expression of IL-5 gene in early stages of colitis induction forced eosinophil migration to the inflamed area, which resulted in higher colitis severity, and consequently favored higher and more severe cell dysplasia. Moreover, the absence of eosinophils in late stages in IL-5 treated group due to short eosinophils half-life or a halt in injected IL-5 gene expression pave the way for development of significantly higher invasive adenoma. Furthermore, the understanding of direct interactions between tumor cells and IL-5 may help in manipulations that could prevent the progression of colorectal cancer.

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