ILCs, the Frontliners of Immune Defense in Mouse Model of Colorectal Cancer

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Research Article

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Abstract

Background and Objective: Innate lymphoid cells (ILCs) have been shown to play important roles in tumor immunity. We studied the frequency of three subsets of circulating ILCs in mouse models of colorectal cancer (CRC).

Methods: Two mouse models of CRC were developed; including a chemically-induced model, via administration of azoxymethane/dextran sulfate sodium (AOM/DSS), and an orthotopic mouse model, using the CT-26 cell line. Based on histopathological examinations, mice were divided into 3 groups of dysplasia group (consists of chemically-induced and orthotopic induced), chemically-induced reparative change group, normal. A sham group was also considered in which mice were screened for stresses that originated from interventions and injections. Flow cytometry analysis was performed to evaluate the frequencies of ILC1, ILC2, and ILC3 in the peripheral blood of all studied mice.

Results: The frequency of ILC1 was significantly higher in the chemically-induced reparative change group compared to the sham and dysplasia groups. ILC2s showed higher frequencies in the dysplasia groups than the sham and chemically-induced reparative change groups. In addition, altered composition of ILCs was observed in peripheral blood of dysplastic mice skewing toward ILC3s in the dysplasia groups compared to sham and chemically-induced reparative change groups.

Conclusions: A higher frequency of ILC1 in the reparative change group suggests a potentially anti-tumorigenic role. Higher ILC2s might be in favor of differentiation from the reparative change stage to the dysplasia. In addition, it seems likely that ILC3s are participating in the primary stages of CRC development.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of death [1]. Clinical diagnosis of CRC has remained challenging due to the asymptomatic progression of CRC until advanced stages. Therefore, a comprehensive understanding of anti-tumor immune responses could be useful for the improvement of diagnostic and therapeutic methods [2]. The innate immune system can influence carcinogenesis via spontaneous priming, recruitment, activation, and clonal expansion of adaptive immune cells, including B- and T-cells. On the other hand, uncontrolled long-lasting activation of the innate immune system might lead to chronic inflammation which could, in turn, facilitate tumorigenesis [3] [4].

The exact role of innate immunity in defense against tumors is poorly understood. Innate lymphoid cells (ILCs) are innate counterparts of T-helper cells which lack re-arranged antigen-specific receptors but produce effector cytokines, like IFN-g, IL-4. These cytokines, in turn, increase the production of the activator cytokines by dendritic cells and stromal cells, which finally acti¬vate ILCs and T-cells [5]. ILCs play an important role in the balance of commensals containment, lymphoid organogenesis, protection of epithelial barriers against infections and inflammation [6] [7]. The ILC family is characterized by a

classic lymphoid morphology and the lacking of rearranged antigen-specific receptors. ILCs are divided into three main groups, based on phenotypic and functional characteristics. In human, group 1 ILCs (ILC1) include CD127low ILC1s, that express CD103, CD56, CD94, and NKp44 and are responsive to IL-12 and IL-15, and CD127high ILC1s which are mainly located in the lamina propria; lack CD56, CD94, and NKp44 expression; and respond to IL-12 and IL-18 by producing IFN-γ. In mice, a population of CD160-expressing ILCs have been defined to be equivalent of CD127low ILC1s. Group 2 ILCs (ILC2s) in human produce type 2 cytokines, mainly IL-5 and IL-13. They are dependent to the transcription factor GATA3 for their development and function. In mice, ILC2s are dependent on GATA3, Notch, TCF-1, and RORa for their development and function (Hazenberg, 2014).

In human prostate and breast tumors, ILC2s produce type 2 cytokines and stimulate MDSCs, thereby mediating a detrimental tolerogenic pathway [8]. Group 3 ILCs (ILC3s) are characterized in two groups based on the expression of natural cytotoxicity receptor NCR1 (NKp46 in mice and NKp44 and NKp46 in humans) or NCR2 [6, 9]. ILC3s respond to IL-6, IL-23, and IL-1β by secreting IL-17 and IL-22. The presence of RORγt + ILC3s within the human breast tumor [10] has been shown to be associated with a higher risk of metastasis. During intestinal inflammation, ILC3s produce IL-22 to maintain epithelial barrier homeostasis. In mouse models, IL-22–producing ILCs could induce and maintain CRC (Hazenberg, 2014).

Although the role of ILCs in colorectal inflammation and cancer has been mentioned in some studies, little is known about ILC frequency and composition in mouse peripheral blood during colorectal cancer progression. In this experimental study, two models of mouse models of CRC were used, a chemically-induced model to investigate the mechanisms underlying inflammation, and an orthotopic induced model, using the CT-26, a mouse adenocarcinoma cell line, to evaluate ILCs frequency in peripheral blood of mouse during CRC progression and to illustrate whether ILCs are involved in mouse CRC development.

Materials And Methods

Mice

48 male BALB/c mice aged 6-8 weeks, weight range between 18 to 25 grams, were used in this study. Mice were maintained at Laboratory Animal Care Institute in Mazandaran University of Medical Sciences, according to the Animal Care Guidelines. All animals were housed in plastic cages (5 mice/cage) with free access to drinking water and basal diet pellet under controlled conditions of humidity, light/dark cycle, and temperature (23±2°C). They were assigned into 3 experimental groups as chemically-induced (n=18), orthotopic injected (n=12), and the sham group (n=18).

Chemically-induced Mouse Model of CRC

Azoxymethane (AOM), as a genotoxic carcinogen, was purchased from Santa Cruz Chemical (Dallas, TX, US) and dextran sulfate sodium (DSS), as a non-genotoxic carcinogen, was purchased from Sigma Aldrich (Aurora, OH, US). Eighteen mice were treated with an intraperitoneal administration (15 mg/kg body weight) of AOM followed by 7 days of recovery. On day 7 after AOM injection, mice were orally

administered to DSS (2.5% in drinking water) for a week. On day-21, mice were injected again with a single dose of AOM (7.5 mg/kg) and DSS (2.5% in drinking water) during a week (from day-28 to day-35). The sham group was injected with a single dose of normal saline (15 mg/kg) on the days 1 and another dose of PBS (7.5 mg/kg) on day 21; then received 2.5% normal saline in drinking water for further 7 days (on the days 7 to 14 and 28 to 35 after beginning) then no further intervention was performed up to the 80th day.

Orthotopic Mouse Model of CRC

Fourteen mice were injected with CT-26, a mouse colon carcinoma cell line; A number of 2×106 cells were suspended in 1 ml PBS. Mice were anesthetized, shaved, and prepped with povidine iodine. Laparotomy was performed to expose the cecum and then 50 µL of the cell suspension (1×105 cells per mouse) was injected into the cecum (n=14). Finally, the cecum was returned to the abdominal cavity, and the incision was sutured. Two mice out of fourteen died during the surgical process, then 12 remaining mice who underwent the recovery process without receiving antibiotic treatment continued to bear growing tumors until they were proceeding sample collection and sacrificed for pathological diagnosis on days 25 and 40 after cell line injection. Tumor burden was detected in 8 mice (66% evidence rate) of this group; 4 mice were tumor-free, thus excluded from the study. (Figure 1)

Antibodies and preparations

The following anti-mouse Lineage Cocktail antibodies: FITC anti-mCD3/ FITC/ anti-mGr-1/ FITC anti-mCD11b/ FITC anti-mCD45R (B220)/ FITC antimTer-119. In addition, APC anti-mouse IL-33Rα (st2), PE/Cy7 anti-mouse CD45, PE anti-mouse CD117 (c-Kit) were used (all purchased from Biolegend). Corresponding isotype control antibodies were used as controls.

Considering the utility of a 4-color staining panel in the following experiment, prior to evaluation of ILCs in our collected samples, FM2 control and one 4-color control negative panel sample tubes were used to obtain a valid compensation matrix and determine appropriate quadrant marker placement for the panel, also PMT voltages adjusted and optimized for evaluation of target cells.

Sample collection

Twelve mice were selected for each time of sampling on days 80, 105, and 120 (6 mice as the sham group and 6 for the chemically-induced group). In line with Chemically-induced Mice, all orthotopically injected mice were anesthetized with intraperitoneal administration of xylazine (16 mg/kg) and ketamine (120 mg/kg) on days 25 and 40 after cell line injection. Whole blood was collected with cardiac puncture method and blood samples were transferred to ethylene dimetilenotetracetic acid (EDTA) 10% containing tubes and mixed well; then100 µl of blood were aliquoted into 2 ml microtubes and labeled with appropriate amounts of antibodies for 40 min in the dark at 4◦C. Then samples underwent RBC lysis with cold ammonium chloride lysing solution; cells were then washed and suspended in FACS buffer (1X PBS,

50μM EDTA, 0.2% BSA) for 2 times then suspended in fixation buffer (PBS+2% Paraformaldehyde) until samples evaluation on a BD FACS CALIBUR flow cytometer. (Figure 2)

Histopathological Examinations

Chemically-induced group of mice were sacrificed for macroscopic evaluation of the colon during 3 series of sample collection on days 80, 105, and 120; also orthotopic induced group of mice were sacrificed 25 and 40 days after cell line injection (6 mice per time). Colon and cecum were dissected and fixed in 10% buffered formalin for at least 24 h and prepared on paraffin-embedded sections after hematoxylin and eosin (H&E) staining to proceed histopathological examinations.

Statistical Analysis

Numerical data were analyzed and defined as the mean ± SD (Standard Error Deviation). Kolmogorov-Smirnov normality test, one-way ANOVA tests were used for the calculation of statistical significance, evaluated with GraphPad Prism v 8.1. (P-values less than 0.05 were considered as significant with $*p < 0.05$, $**p < 0.01$, and $***p < 0.0001$)

Results

Mice colonic tissues were examined by a pathologist to detect mucosal ulcerations, dysplasia, and tumors. Samples were assigned mice into 4 groups, based on their pathological status, including the sham group (n = 18), chemically-induced reparative change (n = 10) and dysplasia (n = 8) groups, as well as orthotopic dysplasia group (n = 8); As expected, mice in the sham group showed histopathologically normal appearance with no inflammation; mild infiltrates of inflammatory cells into the lamina properia mucosa with obvious inflammation, often including crypt abscess formation was described as chemically-induced reparative change (n = 10); and dysplasia group which involved colonic mucosa dysplasia diagnosed mice according to all characteristics of dysplasia, such as hyperchromatic nuclei, increased nuclei, mitotic cells consist of either low or high grades (n = 16). (Fig. 3)

ILCs frequencies

To investigate whether conversions of peripheral blood ILCs are involved in CRC development process, we compared the frequency of total ILCs, ILC1s, ILC2s, and ILC3s among all study groups. The frequency of total ILCs in the sham group was slightly higher in comparison to dysplasia and chemically-induced reparative change groups, but it was not statistically significant (Fig. 4).

We observed that ILC1s reflect the most abundant subset, followed by ILC3s and ILC2s (the least characterized subset among mouse peripheral blood ILCs) among all experimental groups, furthermore significantly lower percentage of ILC1 was detected in both of chemically-induced dysplasia group(p.value = 0.0078) and orthotopic induced dysplasia group(p = 0.0092) compared to sham, also it was shown that chemically-induced reparative change group exhibited more enrichment of ILC1 versus SHAM group (p.value = 0.018). Also, the frequency of group 2 ILCs in the orthotopic induced dysplasia

group (p.value = 0.0125) and chemically-induced dysplasia group(p.value = 0.0114) was higher than SHAM, Interestingly we did not observe such a difference in peripheral blood of SHAM and chemically-induced reparative change group mice. The results also showed an ascending ratio of the ILC3 population in the orthotopic induced dysplasia group(p.value = 0.00260) was significantly increased compared to the SHAM group. Similarly, such a change was observed in the chemically-induced dysplasia group (p.value = 0. 0.0011). (Fig. 5)

Discussion

ILCs change in terms of frequency and subset composition has already been explored in this study by taking benefit of two established mouse models of CRC, consists of mouse adenocarcinoma cell line injected model and chemical components (AOM/DSS) treated a well-known inflammation-related CAC, aim to help clarify the ILCs change during CAC progression [11] We found that CRC development acts on the ILC compartment in peripheral blood, it has been shown by our findings that the reparative change group exhibited an elevated number of ILC1s. similar to our findings, higher frequency of ILC1 have been documented in bronchial samples and peripheral blood from patients with chronic obstructive pulmonary disease (COPD), CD (Crohn's disease), synovial fluid, and tissue samples from patients with inflammatory arthritis and those with RA in peripheral blood samples of patients with acute-phase anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV).[12–16].

As it has been reported by our observation, total ILC repertoire is slightly decreased in the peripheral blood of dysplasia and reparative change groups compared with SHAM mice, also it has been shown in this study that ILC1s drastically decrease in the dysplasia group during the progression of CRC, in line with our findings, decreased numbers of ILC1, in patients with acute myeloid leukemia malignancy is documented previously[17].

While some studies had different opinions which claim peripheral blood ILC1s are increased at metastatic CRC patients[18], These differences in results and observations can be attributed to the different manner of ILCs during several stages and conditions of cancer.

ILC1s are characterized by secretion of the pro-inflammatory cytokines interferon (IFN)-γ and tumor necrosis factor, It seems likely that ILC1s are able to promote anti-tumor immunity in primary stages of cancer, though their protective role might be hindered during cancer progression. On other hand, ILC1s sharp increase and overactivity may favor(s) Long-standing intestinal inflammation, which considered as a protumorigenic factor, which plays an efficacious role in the malignant transformation/ tumor formation process.

Notably, our findings represent a significant upregulation of group 2 innate lymphoid cells in dysplastic mice compared with sham and reparative change groups, which suggests a protumorigenic role for ILC2, despite their small number population, several studies have been revealed their importance in the context of cancer- immune defense, ILC2s are able to promote cancer progression and metastasis by producing suppressor cytokines like IL-33 lead(s) to the accumulation of myeloid-derived suppressor cells and

alternatively activated M2 macrophages were described as a key factor driving immune-evasion and metastases in 4T1 model of breast cancer [19, 20] Furthermore, in patients with gastric cancer, a higher frequency of ILC2 was observed in peripheral blood and was thought to lead to an immunosuppressive microenvironment. In line with our finding, it was reported by other studies that ILC2-Cs and ILC-reg exhibit an ascending trend in the late stage of CRC and dramatically promote tumor development [20]. Also, we observed a significantly higher frequency of ILC3s in the dysplasia group which was accompanied by CRC development, recent studies declared that ILC3s have also been involved in initiation and perpetuation, metastatic spread Of murine 4T1 breast carcinoma cells in Rag1 -/- mice. Interestingly, increased numbers of RORγt + ILC3s have been found to correlate with an increased incidence of metastasis in human breast carcinoma.[21].

Evidence from animal models suggests that IL23 responsive ILC3s are thought to be the source of IL-17A and IL-22 and are found in higher frequencies in this model. ILCs and especially ILC3 may contribute to intestinal Carcinogenesis by producing IL22 and play a crucial role in colitis-associated cancer model, Thus, CRC did not develop in mice that were depleted of IL-22–producing ILC3, and treatment with IL-22–blocking agents did protect against the development of CRC in these mice [22]. recent studies suggest that elevated IL-17 and IL-22 expression which in turn caused exacerbated epithelial cell proliferation and promoting several human tumors, such as gastric, prostate, breast, hepatocellular carcinoma.[23]. which are pivotal for the adenoma formation In CRC, can be considered as triggers for angiogenesis via vascular endothelial growth factor (VEGF) secretion by tumor cells and is associated with poor prognosis [24]. Former studies in the context of innate lymphoid cells confirm accumulation and alteration of ILCs composition in several human/animal model cancer, an illustration about the regulation of immune cells in peripheral blood is necessary for better understanding of a complex immune cells network in cancer as a dynamic process that involves cell trafficking through the peripheral blood. [24, 25]. Collectively, these results suggest an association of altered ILCs proportions with the occurrence of CRC/CAC in a mouse model, revealed an important role of considering the ILC compartment in Understanding of immune regulation toward cancer, aim to better define immune scores and eventually to the development of future therapeutic strategies.

Declarations

Author Contributions

All authors contributed to the study. Abolghasem Ajami, Mohsen Tehrani, and Hossein Asgarian-Omran designed and conducted the research. Mohsen Rashidi managed the induction of cancer model in mice. Laleh vahedi larijani assessed the pathological scores of samples. Mohsen Keykhosravi and Seyed Mohammad Javadzadeh carried out the assays, contributed to data collection and analysis, and prepared the manuscript.

All authors read and approved the final manuscript

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethics approval

This study was approved by ethical committee of Mazandaran university of medical science, sari, iran [IR.MAZUMS.IMAMHOSPITAL.REC.1398.5177]

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Figures

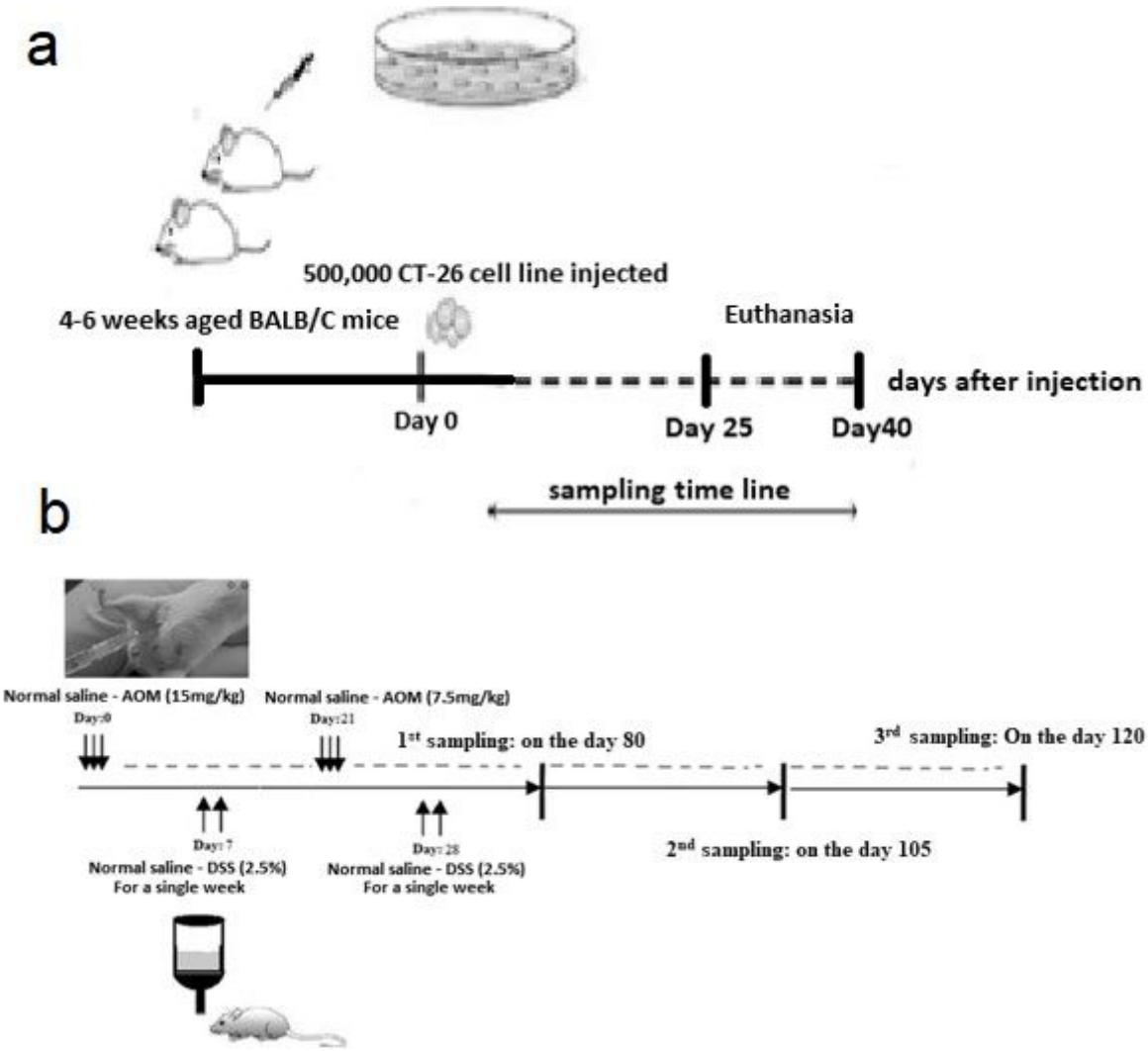

Figure 1

Depicted scheme showing development of CT-26 adenocarcinoma cell line induced mouse model of CRC (a). Treatment scheme of AOM/DSS in chemically-induced mouse CRC model (b)

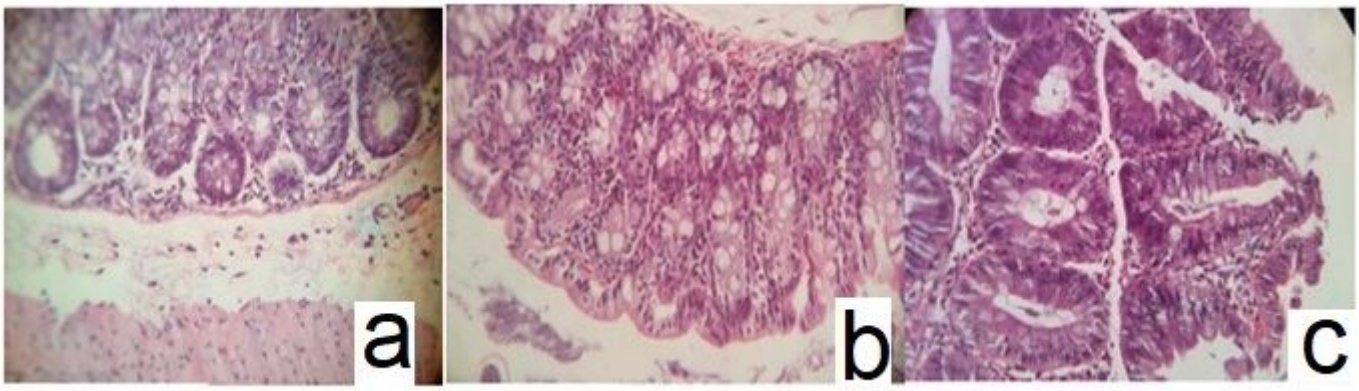

Figure 2

Histological microscopic examination (magnification, ×400) in the Haematoxylin-eosin-stained cross sections of mice colonic tissues, showing normal appearance of sham (A) reparative change have been depicted with hemorrhagic and inflamed scheme (B) represents microscopic observation of dysplastic alteration in mouse colon. (C)

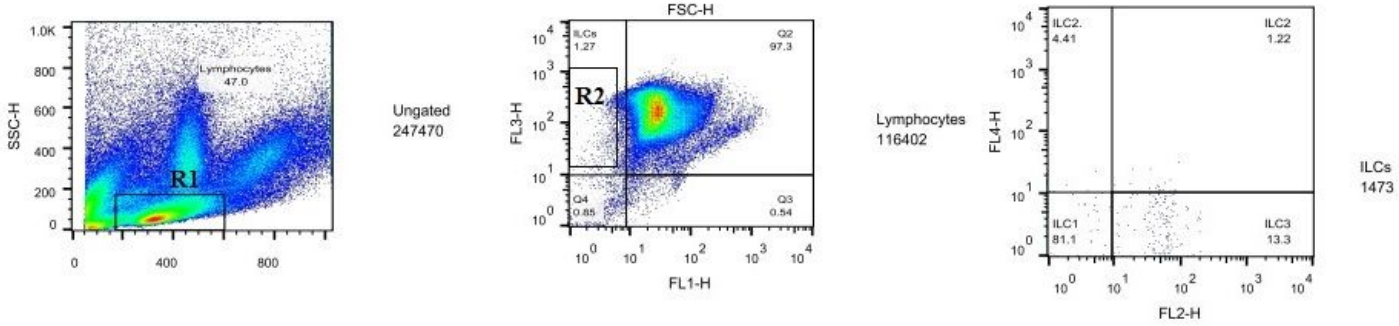

Figure 3

Samples were analysed using flow cytometry; lymphocyte population were gated in agreement with the conventional identification, Lin - CD45 + events showing total ILCs; aim to analyse the ILC subpopulations, daughter dot plot was Derived from Lin- CD45+ and Events were further gated into ILC1 (Lin- CD45+ CD117- ST-2-), ILC2 (Lin- CD45+ CD117+/- ST-2+), and ILC3 (Lin- CD45+ CD117+ ST-2-)

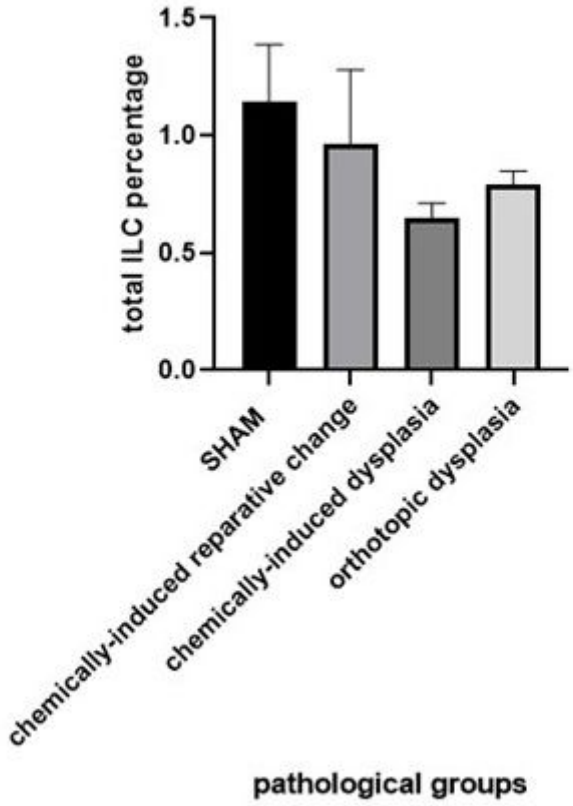

Figure 4

Mean total ILC frequencies in chemically-induced reparative change, chemically-induced dysplasia, orthotopic induced dysplasia, and the sham groups. Bars show mean±SEM, * $p < 0.05$, ** $p < 0.01$, and *** $p<0.001$

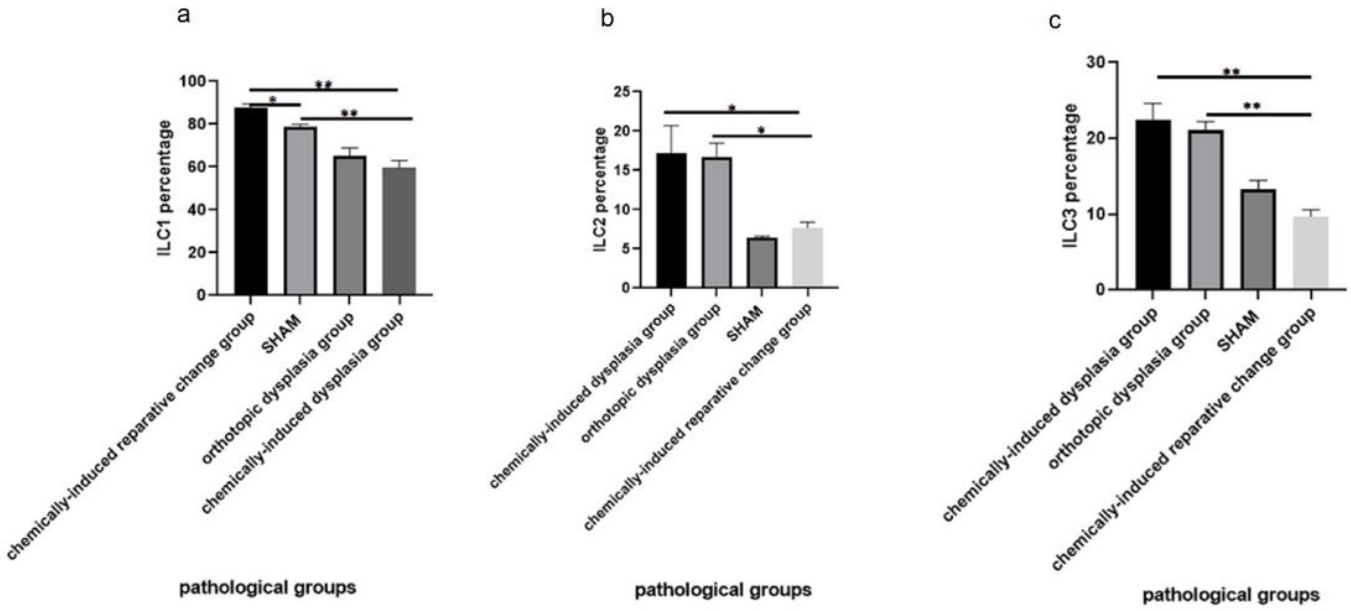

Figure 5

Representative columns of comparison dysplasia (chemically-induced dysplasia group and orthotopic induced dysplasia group), chemically-induced reparative change group, SHAM groups in term of ILC1s frequency(a) ILC2s subpopulation are participated to CRC progression. Representative columns showing ILC2 Abundance among pathological groups(b) ILC3s have promoting trend during CRC development Representative columns of ILC3s percentage in several experimental groups(c). Bars show mean±SEM, * $p < 0.05$, ** $p < 0.005$, *** $p<0.001$, and ****$p < 0.0001$