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Immune intratumoral microenvironment differences between sexes in colorectal tumors

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Abstract

Sex-based differences in colorectal cancer (CRC) incidence and progression are well-established, yet their underlying immunological mechanisms remain insufficiently understood. In this study, we characterized and compared systemic and tumor-associated immune responses in male and female mice using the azoxymethane/dextran sodium sulfate (AOM/DSS) model of CRC. At a macroscopic level, males develop greater numbers and larger tumors. In line with this, the histopathological analysis shows that neoplastic tissue is more abundant in males. In contrast, flow cytometric analysis revealed that male mice exhibited increased intratumoral γδ T cells and tumor-associated macrophages, accompanied by increased MCP-1 compared with females. In contrast, females demonstrated a stronger cellular response with a higher infiltrate of cytotoxic lymphocytes, B cells, and plasmatic cells.

In concordance, systemically, males exhibit a higher percentage of T γ δ lymphocytes in the spleen and an increase in MCP-1 in serum, while females have a higher level of CD8+ in the mesenteric ganglia. In summary, these results show a stronger cellular response by females in nearby tissues and in the tumor. Also, suggest that males have a worse prognosis due to the infiltration of macrophages and γδ T cells, and the pro-tumoral action of MCP-1.

Keywords: Sexual dimorphism, colorectal tumors, male, female, immune response.

Introduction

Sexual dimorphism refers to the biological differences between males and females of the same species, which are reflected not only in morphology and physiology but also in the function and regulation of the immune system.

Epidemiological and experimental evidence consistently show that males are more susceptible than females to developing colorectal cancer (CRC). Across populations, men exhibit higher CRC incidence and lower survival rates, indicating that biological sex is a significant risk factor [1]. This disparity cannot be fully explained by lifestyle or environmental factors alone but also reflects intrinsic differences, such as hormonal profiles and genetic factors. For example, male rodents treated with azoxymethane (AOM) and dextran sodium sulfate (DSS) develop more and larger tumors than females[2]. In humans, men are more prone to left-sided colon tumors and have lower survival rates than women, particularly premenopausal women, who benefit from the protective effects of estrogens. These findings underscore the importance of considering sex as a biological variable in CRC research and clinical practice[2,3].

Although we previously reported that tumor number and size are larger in males than in females, these macroscopic parameters do not allow us to determine the degree of histological damage or the specific lesion type. There are three main types of lesions. The most common is the tubular type (≈80%), which generally carries a low malignancy grade. The villous (≈10%) and serrated (≈10%) types, in contrast, exhibit a higher grade of malignancy. All three lesion types may present either low-grade or high-grade dysplasia,

defined by features such as mitotic figures, crypt elongation, hyperchromatic elongation, necrosis, irregular branching, and, in more advanced stages, invasion of the muscularis mucosae (adenocarcinoma). Identifying the lesion type and grade is essential for proposing an appropriate treatment [4].

At the cellular and molecular level, sex-based disparities in immune responses are well documented, with females generally exhibiting stronger innate and adaptive immune activity than males. Women typically produce higher levels of antibodies, mount more robust inflammatory responses, and display greater vaccine efficacy. These advantages confer lower susceptibility to certain infections but also increase the risk of autoimmune diseases. In contrast, males tend to have weaker immune responses, which are associated with greater vulnerability to infectious diseases and reduced vaccine efficiency. These disparities are influenced by hormonal differences such as the immunoenhancing effects of estrogen in females and the immunosuppressive actions of androgens in males, as well as genetic factors, including the expression of immune-related genes on the X chromosome[5].

Experimentally, male animals exhibit heightened early inflammatory responses, characterized by elevated expression of pro-inflammatory mediators including IL-6, COX-2, TNF-α, and NF-κB-related genes, during tumor initiation. This exaggerated inflammatory state may promote tumorigenesis more aggressively in males. In contrast, females display a more controlled inflammatory profile, with higher basal expression of antioxidant and anti-inflammatory molecules such as NQO1, SOD1, and HO-1, which are downstream targets of NRF2 [2,3,6,7].

Despite increasing recognition of sex-based differences in CRC, current knowledge remains largely focused on systemic hormone levels and the expression of soluble inflammatory mediators such as IL-6, TNF-α, COX-2, and IL-1β. A comprehensive characterization of the histopathological analysis and its immune microenvironment and how these parameters differ between sexes remains limited. Moreover, it is still unclear whether systemic immune differences are reflected or disrupted locally during CRC progression.

Based on this background, the aim of this study is to characterize and compare the immune response within the tumor microenvironment and at the systemic level. Also, we perform a histopathological analysis of male and female mice induced with colorectal tumors.

Methodology

Animals and ethical statements

All animal experimental procedures were performed at the Unidad de Modelos Biológicos (UMB), Instituto de Investigaciones Biomédicas (IIB), Universidad Nacional Autónoma de México (UNAM). Animal care and experimental protocols were approved by the Institutional Care and Animal Use Committee (CICUAL), approval ID 6298, adhering to Mexican regulation (NOM-062-ZOO-1999), and in accordance with the recommendations from the National Institute of Health (NIH) of the United States of America (Guide for the Care and Use of Laboratory Animals).

Mice of the syngeneic strain Balb/cAnN were purchased from Modelos Animales y Servicios (MAS) from Harlan, at Facultad de Química, UNAM, México. The animals were housed at the UMB under controlled temperature (22± 2 °C) and 12:12 hours of light-dark cycles. Animals were fed with Purine lab diet provided ad libitum.

Colon tumor induction

Colorectal tumors were induced following the previously described AOM/DSS model reported by Rodríguez-Santiago [2]. Briefly, male and female mice received an intraperitoneal injection of azoxymethane (AOM, Sigma-Aldrich, St. Louis, MO, USA) at 12.5 mg/kg body weight. Five days later, mice were given 2% dextran sodium sulfate (DSS; Alfa Aesar, Ward Hill, MA, USA) in drinking water *ad libitum* for seven consecutive days, followed by two weeks of recovery with regular water. This cycle was repeated three times.

Tissue collection and processing

Seventy days after the start of AOM-DSS treatment, mice were humanely euthanized by inhalation of a mixture of air and sevoflurane (5%), followed by cervical dislocation. Blood samples were collected by cardiac puncture to obtain the serum. Subsequently, the colon, spleen, and mesenteric lymph nodes were excised, and tumors were dissected for flow cytometry analysis.

Flow cytometry

Tumor tissue, mesenteric lymph nodes, and spleens were excised and mechanically dissociated through a 75 µm nylon mesh using cold PBS. After washing, cell suspensions were resuspended in FACS buffer (PBS containing 2% fetal bovine serum and 0.02% sodium azide).

The samples were further digested in RPMI 1640 medium containing DNase I (10 U/mL; Roche, Mannheim, Germany), cOmplete™ Protease Inhibitor Cocktail (ROCHE, Merck KGaA,

Darmstadt, Alemania), and type IV collagenase (0.5 mg/mL; Sigma, St. Louis, MO, USA) for 20 min at 37 °C. The reaction was stopped by adding 50 µL FBS, followed by mechanical disruption in a 75 µm nylon mesh. After the PBS wash, the cells were resuspended in FACS buffer.

The design of the panels was as follows: for T lymphocyte: FITC-conjugated anti-CD3ε (Cat# 100306, Biolegend) 1:300, PE-conjugated antiCD4 (Cat# 100408, Biolegend) 1:300, Pe Cy5-conjugated anti-CD8 (Cat# 100719, Biolegend) 1:200, AlexaFluor 647-conjugated antiFoxp3 (Cat# 320014, Biolegend), PE/Dazzle 594-conjugated antiCD25 (Cat# 101920, Biolegend) 1:200 and for γδ T cells PE-conjugated TCR γδ. For NK: PE-conjugated anti-NKP46+ (Cat# 137604, Biolegend), For macrophage APC-conjugated anti-F4/80 (Cat# 123116, Clone 29A1.4, RRID:AB_2235755), and for mast cells APC-conjugated anti-CD117+. For B lymphocyte: PERCP-Cy5-conjugated anti-CD19 (Cat# 118108, Biolegend), 1:200, plasmatic cells: APC/Cy7 anti-mouse CD138 (Cat# 142529, Biolegend).

A minimum of 10^4 events per sample were acquired using an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Histopathological processing

The distal colon and rectum were harvested the distal colon and rectum after euthanizing male and female BALB/c mice. The samples were fixed by immersion in 4% paraformaldehyde. They were then dehydrated in increasing grades of ethanol and cleared in xylene. Next, the samples were paraffin embedded. The blocks were cut into 5µm-thick serial sections, which were stained with hematoxylin and eosin. For the analysis, we used a brightfield BX50 Olympus microscope with a 4x objective. We also used a digital camera and Infinity Analyze software, v6.3.0, to image

the histopathological lesions. We measured the outer perimeter and areas of each histopathological section. Then, we counted the colonic changes, such as high-grade dysplasia, adenomas, and adenocarcinomas. These measurements included both villous and serrated polyps on each tissue section. The review was done blindly. The data for the counted adenomas were unblinded.

Cytokine measurement

Nine cytokines, IL-1ββ, IL-4, IL-5, IL-6, IL-10, TNF-α, IP-10, MCP-1, were measured in serum and tumor using the MILLIPLEX mouse Cytokine/Chemokine Magnetic Bead Panel (MCYTOMAG-70K) (MERCK, Darmstadt, Germany).

Statistical analysis

Data are presented as mean ± ~~+/-~~ standard deviation (SD) from two independent experiments. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA followed by Tukey's *post hoc* test was applied to compare multiple groups. Differences were considered statistically significant at $p < 0.05$.

Results

Macroscopic tumor quantification

After excision, the tumors were photographed and later counted; males develop greater tumors in number and size **(Figure 1)**.

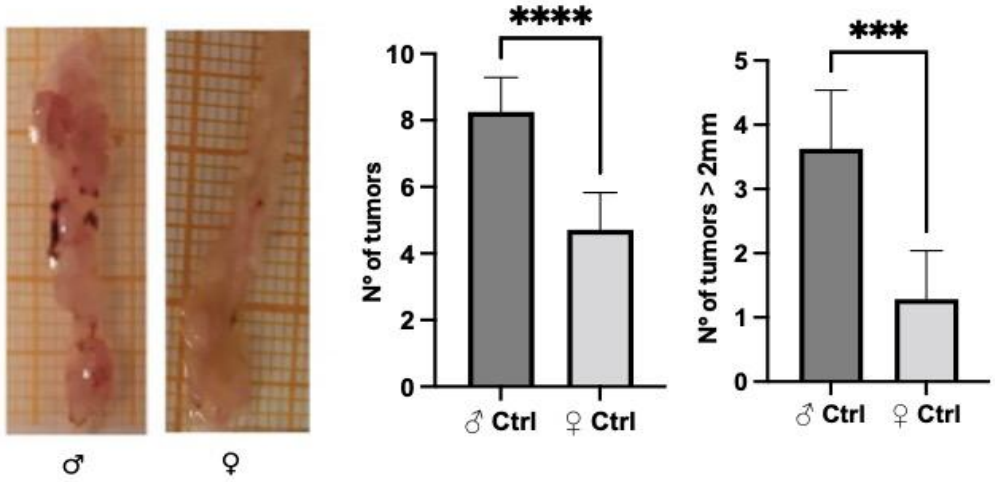
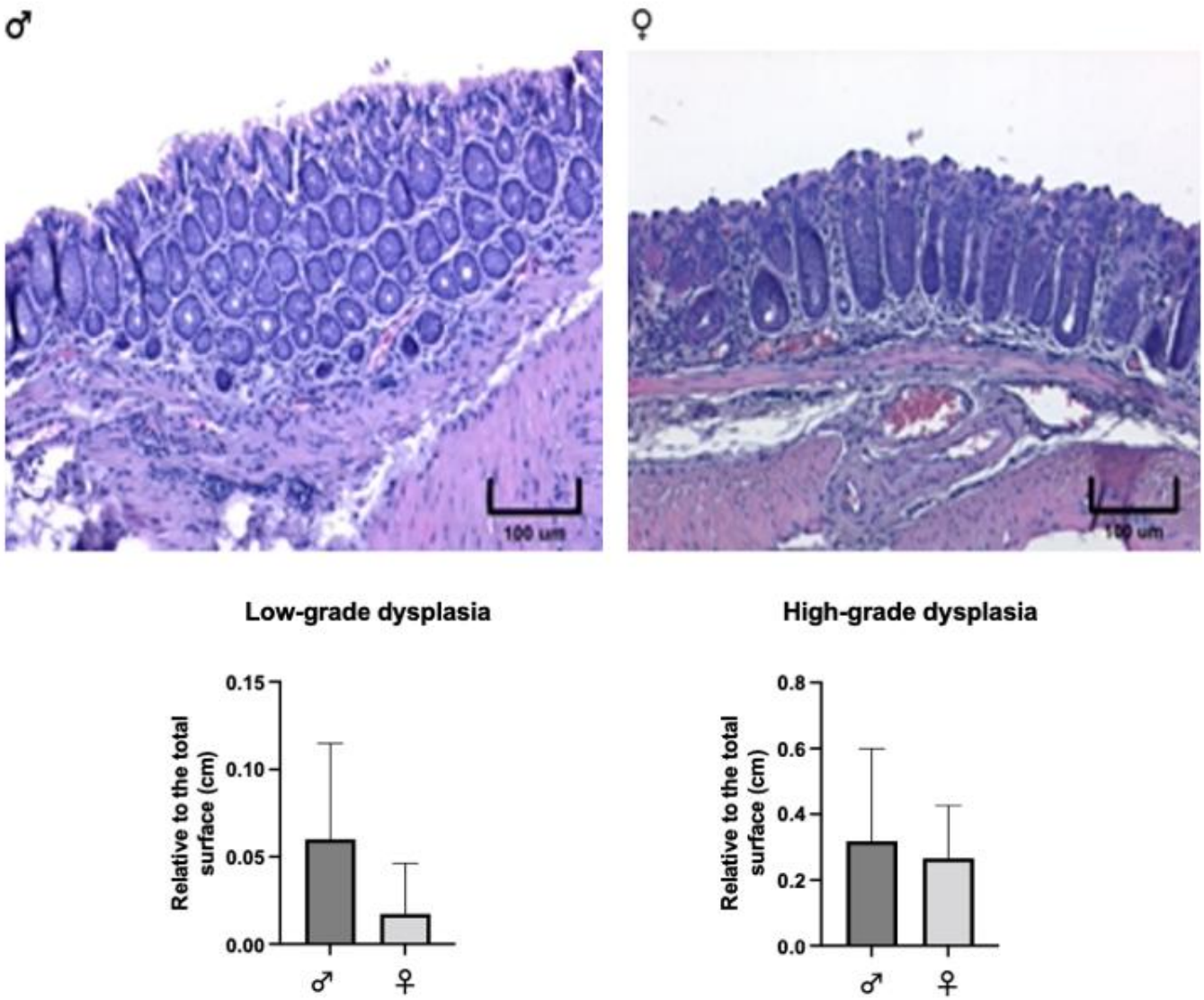


Figure 1. Macroscopic observation and quantification of tumor induction in male and female. $P < 0.05$ was considered statistically significant.

Histopathological analysis of tumors

There is no difference between low and high grade of dysplasia **(Figure 2)**.



207

208 **Figure 2.** Histopathological analysis of dysplasia of low and high grade.

209 Nevertheless, the analysis of the subtype of dysplasia show that males develop a greater
210 number of villous lesions than females **(Figure 3)**. These lesions carry the highest risk of
211 developing adenocarcinomas.
212

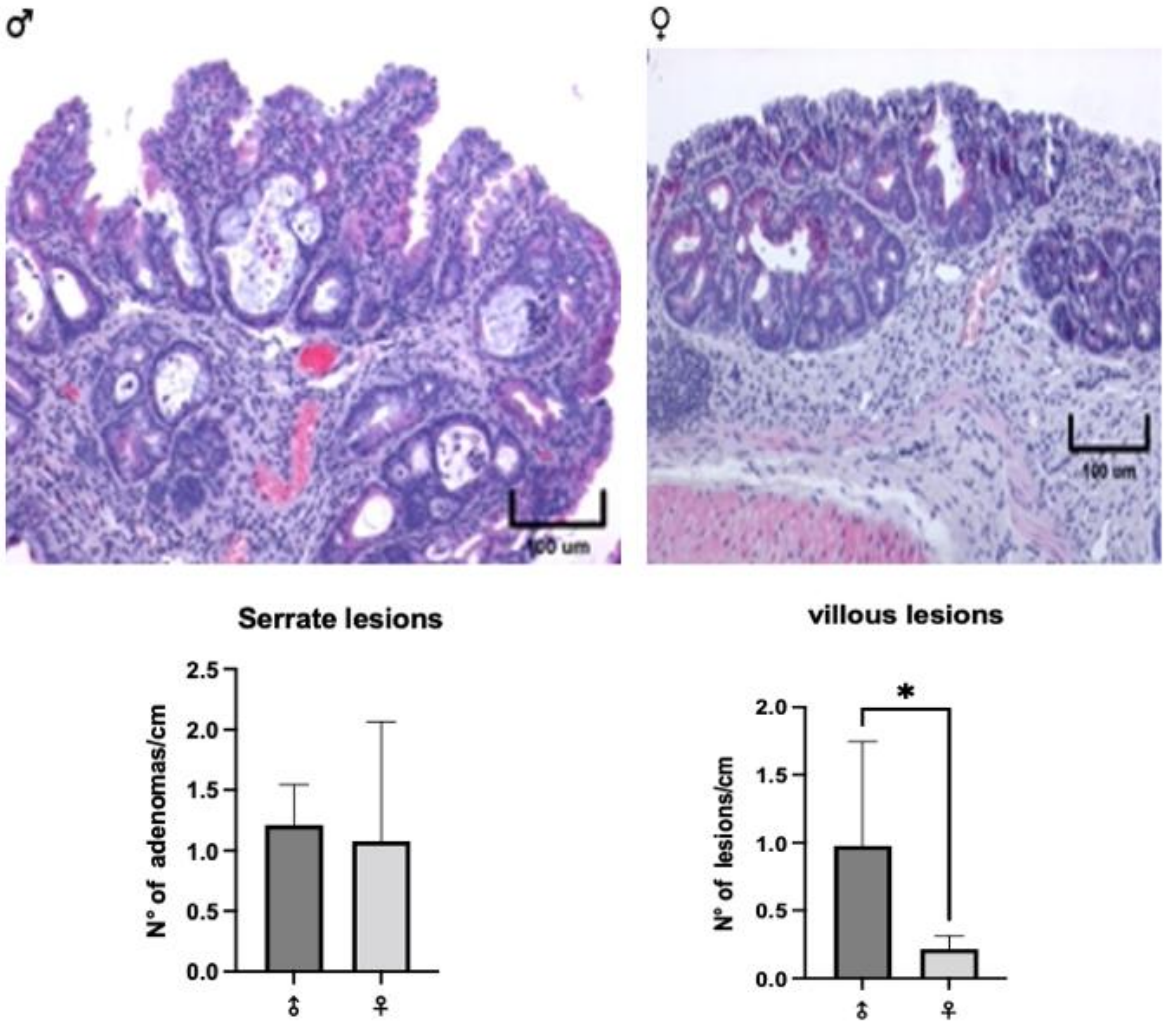


Figure 3. Histopathological analysis of type of lesions. $P < 0.05$ was considered statistically significant.

In line, we observed a drastically higher formation of adenocarcinomas in males than in females **(Figure 4)**.

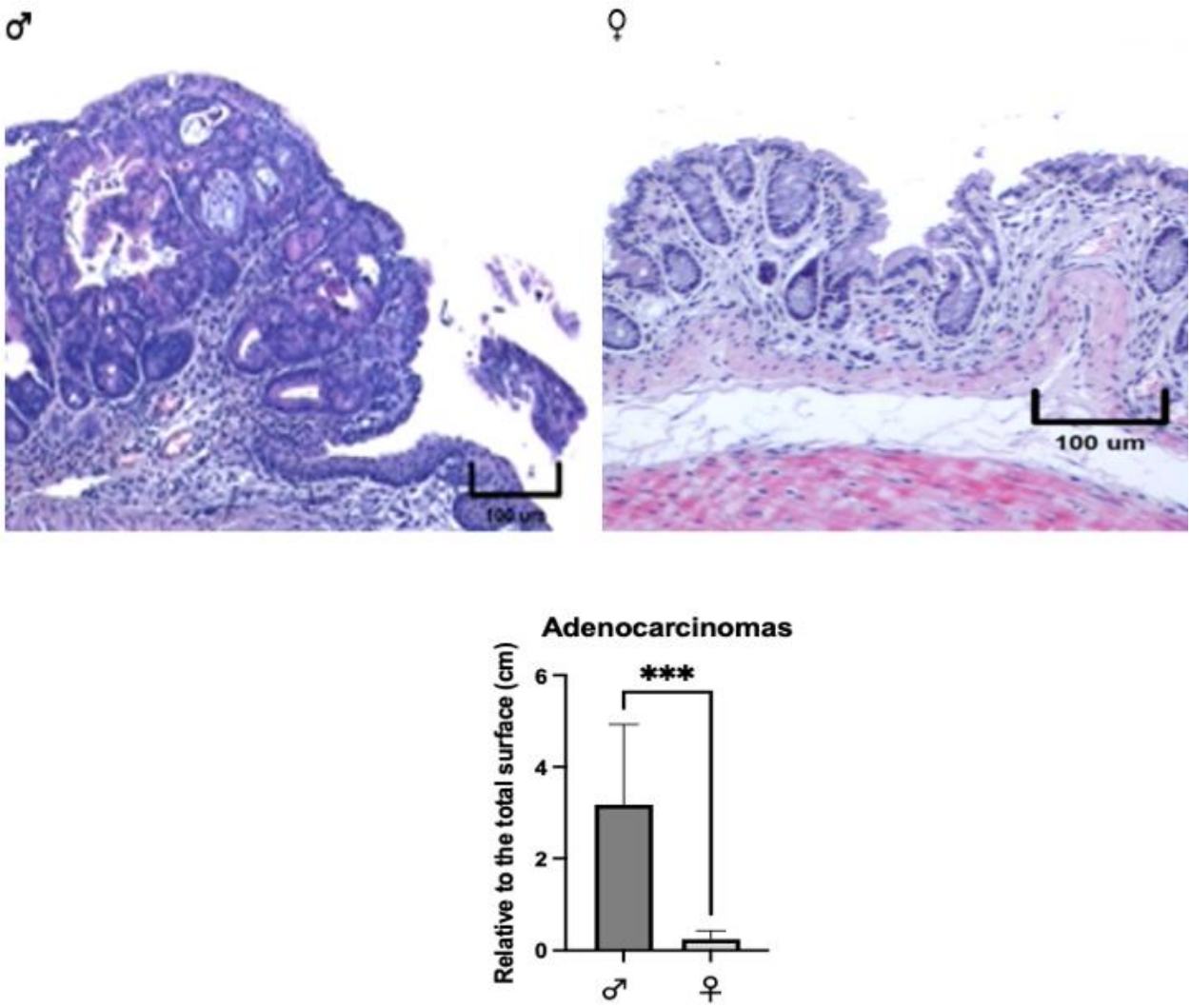


Figure 4. Histopathological analysis of adenocarcinoma proportion. $P < 0.05$ was considered statistically significant.

Strategy selection of immunological subpopulations

The gating strategy used to identify innate and adaptive immune subpopulations is shown in Figure 3. Representative plots illustrate the sequential selection of viable lymphocytes, followed by discrimination of macrophages, mast cells, neutrophils, NK cells, B lymphocytes, T cell subsets ($CD4^+$, $CD8^+$, γ δ T cells), and plasmatic cells **(Figure 5)**.

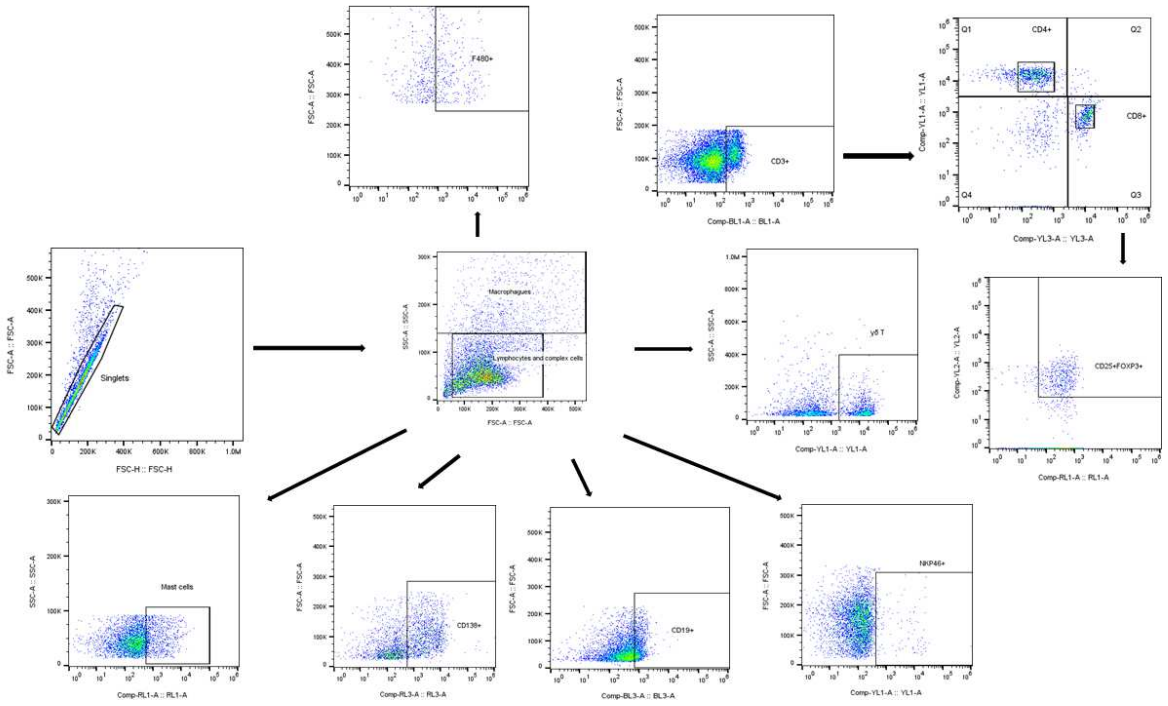


Figure 5. Gating strategy for the identification of innate and adaptive immune cells. populations by flow cytometry. $P < 0.05$ was considered statistically significant.

Tumor microenvironment

Innate* immune *response

We quantify the percentage of cells in the tumor. Males had a higher level of macrophages within the colon compared with females; in concordance, they had a higher concentration of CCL-2 (See soluble factors section). These suggest that these cells have a pro-tumoral phenotype, since CCL-2 favors the polarization of macrophages toward a M2 phenotype. In addition, this group had an augmented level of γ δ T cells. These suggest that the susceptibility to the growth of neoplastic lesions in males is mediated by cells and soluble factors associated with the innate immune system. Interestingly, there were no changes in the remaining cytokines analyzed **(Figure 6)**.

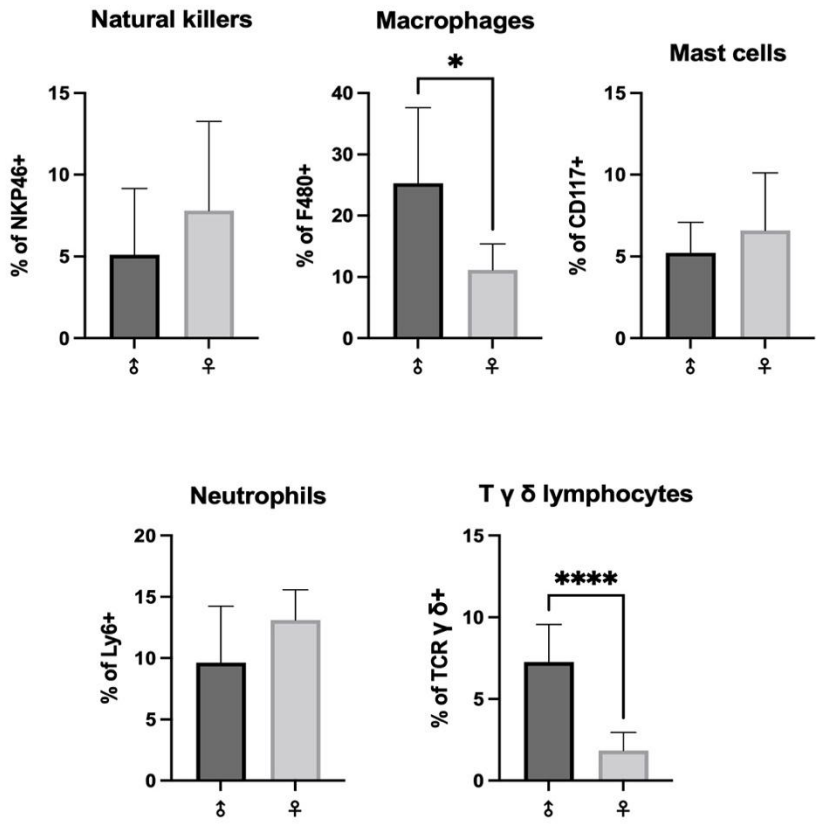


Figure 6. Innate immune profiles within the colorectal tumor microenvironment. $P < 0.05$ was considered statistically significant.

Adaptive immune response

On the other hand, females had a greater infiltration of adaptive immune cells. They had a higher percentage of cytotoxic lymphocytes, B lymphocytes, and plasmatic cells. These suggest lower development of tumors, and the minor tissue damage is mediated by cellular adaptive immune response. However, it will be interesting to evaluate other molecules such as antibodies against tumor antigens **(Figure 7)**.

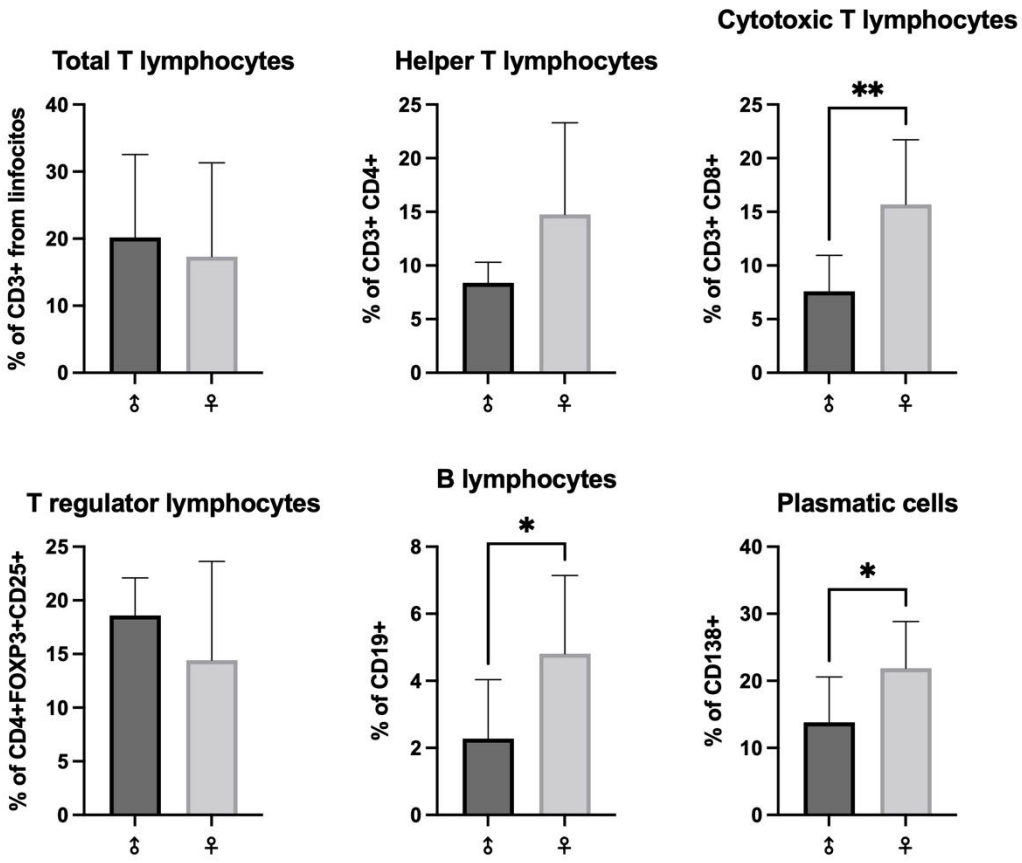


Figure 7. Adaptive immune profiles within the colorectal tumor microenvironment. $P < 0.05$ was considered statistically significant.

Intratumoral soluble factors

As mentioned, we only observed changes in CCL-2, suggesting that—despite differences in immune cell infiltration between males and females—redundant mechanisms for the secretion of these molecules also exist **(Figure 8)**.

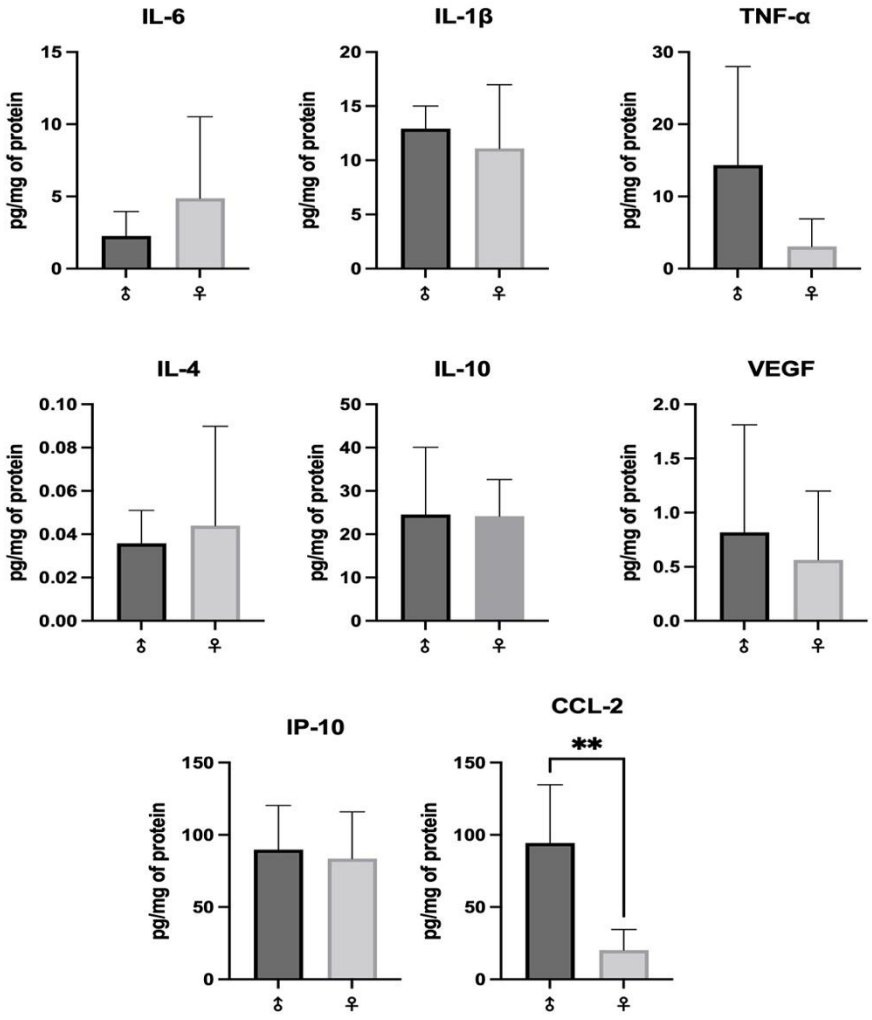


Figure 8. Quantification of soluble factor concentration. $P < 0.05$ was considered statistically significant.

Systemic immune response

We analyzed the same cell populations in the spleen and in the mesenteric ganglia **(Figure 1 of supplementary material)**. Although the tumor is the site where dysregulation is most pronounced, in the spleen, we observe notable differences, with females exhibiting drastically lower levels of B cells (CD19+) compared to males, as well as NK cells. In line, we

found a lower percentage of NK cells in mesenteric ganglia **(Figure 2 of supplementary material)**.

To evaluate the systemic alteration of soluble factors, we analyzed the concentration of these molecules in the serum. We found that males exhibit a higher concentration of MCP-1, which is also increased in tumor tissue. Also, IL-1β showed a higher concentration **(Figure 3 of supplementary material)**.

Discussion

Macroscopic observation and histopathological analysis

Our study focuses on late stages of CRC, in which a mutagenic inducer is administered alongside three cycles of DSS to evaluate the effect of chronic inflammation. At the macroscopic level, males exhibited a higher tumor burden compared to females. However, although this tumor burden is associated with the degree of dysplasia, it does not allow us to determine the lesion type or severity. To address this, we performed hematoxylin-eosin staining and histopathological analysis.

Interestingly, males presented a higher number of villous lesions. These lesions are characterized by originating from the surface epithelium and presenting a larger surface area due to their villous morphology. Human studies have found that these lesions are primarily located in the rectum (55%) and are mostly adenocarcinomas, indicating high severity [8]. In line with this, our study showed that males had a higher proportion of adenocarcinomas compared to females. These lesions have a glandular morphology and account for 98% of colorectal malignancies. The other lesions evaluated were serrated; however, no sex differences were observed in these.

At the molecular level, villous lesions exhibit a high frequency of *KRAS* mutations. This mutation enables the constant activation of the mitogen-activated protein kinase (MAPK/ERK) pathway and the phosphatidylinositol 3'-kinase (PI3K)-Akt pathway. These pathways promote tumor growth and narrow the range of available treatments due to the resistance they confer to anti-EGFR therapies [9].

Our findings confirm that the macroscopic observations in males accurately reflect the histological lesions, and that males develop higher-grade dysplasia and more aggressive lesion types than females. Although this is an animal model, it is consistent with studies in patients, suggesting that the higher incidence observed in men stems from biological sex differences, which may also influence treatment responses.

Intratumoral immune response

In this study, we evaluated the infiltration of immune system cells and the presence of soluble factors within the tumor. Males exhibited a higher percentage of macrophages compared to females and, accordingly, a higher concentration of CCL-2. This chemokine plays a crucial role in macrophage recruitment and is elevated in dysplastic tissue from colorectal cancer (CRC) patients and in animal models that develop spontaneous adenomas[10]. Moreover, CCL-2 regulates other macrophage functions, including polarization toward an M2 phenotype, which is characterized by the secretion of pro-tumor enzymes such as COX-2 [10]. For instance, *Cox-2*−/− mice show an 86% reduction in polyp numbers [11]. Similarly, COX-2 promotes colorectal cancer cell proliferation and invasion while also increasing the production of anti-apoptotic molecules [12]. These findings suggest that macrophages in males adopt an M2-like phenotype that favors tumor growth.

In addition, males had greater infiltration of γδ T lymphocytes. Unexpectedly, many studies report that the infiltration of these cells is associated with a good prognosis, as they can eliminate tumors through the secretion of soluble factors such as IFN-γ [13]. However, this depends on the subtype, since γδ T cells can also secrete IL-17, which promotes angiogenesis, metastasis, and the secretion of immunosuppressive factors [14]. Therefore, characterizing the infiltrating subtype is essential to understanding their role within the tumor.

Conversely, tumors from females exhibited a higher percentage of adaptive immune cells. For example, they showed greater B-cell tumor infiltration. In line with this, B-cell depletion in murine models enhances T-cell capacity to eliminate tumor cells; however, other studies attribute antitumor features to B lymphocytes [15]. This apparent contradiction can be explained by the broad diversity of B-cell subtypes. In our study, we labeled B cells with CD19+, a lineage-restricted coreceptor involved in lowering the threshold needed for B-cell receptor activation [16]. This suggests that females mount a more potent antitumor response due to superior antigen-presenting capacity compared to males. Supporting this idea, females presented a higher percentage of cytotoxic T lymphocytes, which are clearly associated with improved patient prognosis and are capable of directly eliminating tumor cells [17]. Lastly, females displayed greater infiltration of plasma cells. In CRC patients, the presence of these cells correlates with increased survival [18]. Although the mechanisms underlying this apparent protective effect have not been elucidated in CRC, studies in other solid tumors indicate that plasma cells produce immunoglobulins against tumor antigens, enabling T cells and neutrophils to eradicate cancer cells [19].

Systemic immune response

The spleen is a secondary lymphoid organ that serves as a reservoir for both innate and adaptive immune cells. It is also a site where T cells, B cells, and dendritic cells undergo activation. Its role in cancer immunity has recently gained attention [20]. Tumor antigens travel via the bloodstream; upon reaching the spleen, they trigger clonal expansion and activation of T cells destined to recognize neoplastic cells. Additionally, the spleen stores memory T and B cells, which is particularly relevant for invasion, metastasis, and recurrence (or secondary tumor challenge in animal models). Conversely, it can also give rise to myeloid-derived suppressor cells, which promote tumor expansion and hinder immune-mediated elimination. Although the spleen has been investigated in breast cancer and melanoma models, its role in colorectal cancer remains largely unexplored [20].

In our study, we found a higher proportion of CD19+ B cells in females, suggesting that these cells are skewing toward a memory phenotype, thereby enhancing the antitumor response. Other differences included higher percentages of NK cells and neutrophils in males. However, further studies are needed to evaluate the specific roles of these splenic differences and elucidate their significance.

On the other hand, soluble factors in the blood can serve as biomarkers of intratumoral dysregulation and could offer less invasive alternatives to colonoscopy for diagnosing neoplastic lesion development. Nonetheless, comprehensive studies are required to identify markers specifically indicative of this pathology [21]. In line with the alterations observed within the tumor, our work revealed that CCL-2 is present at higher concentrations in males than in females, suggesting that it serves as a circulating biomarker reflective of the

tumor microenvironment. We consider that the proposal of any diagnostic or prognostic marker should consider multiple soluble factors and their combined concentrations.

Translational implications to clinical therapeutic approaches

Our findings highlight the importance of considering sex as an essential variable in both colorectal cancer (CRC) research and treatment administration. Clinically, it has been reported that treatment outcomes differ according to sex; women experience greater drug toxicity, which leads to treatment delays and reduced efficacy [22]. Furthermore, sex-related differences in the immune infiltrate have been identified, such as higher infiltration of cytotoxic and helper T lymphocytes in women [23].

However, the tumor microenvironment is highly complex, and one must consider the cells and molecules that play significant roles in tumor development. Macrophages are a clear example: in our study, males showed higher macrophage infiltration than females, consistent with higher concentrations of the chemokine CCL-2 in both serum and tumor tissue. Targeting tumor-associated macrophages has been proposed as an important therapeutic strategy, given their involvement in dysbiosis, angiogenesis, immunosuppression, invasion, and metastasis. One potential approach is to inhibit CCL-2 activity, as this chemokine has been found elevated in the serum of CRC patients, with higher average levels observed in male patients [24]. In other solid tumors, such as bladder, melanoma, and breast cancer, treatment with a CCL-2 receptor inhibitor has shown synergistic effects when combined with anti-PD-L1 therapy and also reduces lung metastasis [25].

Together, these findings indicate that understanding sex differences at the histological, cellular, and molecular levels will enable better decision making in both the research and clinical application of CRC treatments.

Scopes and limitations

This study reveals clear sex differences at the macroscopic, histological, and molecular levels. Beyond this descriptive perspective, it proposes several candidate molecules that could be evaluated in tumor biopsies from CRC patients, potentially serving as therapeutic targets for the development of more effective treatments that incorporate sex as an essential variable. Nevertheless, based on our results additional factors would be worth exploring, such as the production of specific antibodies against tumor antigens, given the observed shifts in B-cell and plasma cell populations, as well as the detailed phenotyping of the immune cells assessed.

Furthermore, in a review we previously published, we advocated for approaching CRC from a neuro-immune-endocrine perspective, considering the expression of cytokines, neurotransmitters, and hormones in the study of this disease pathophysiology [3]. Considering the expression of receptors for all these soluble factors would provide a broader view of the complex dysregulation present within the tumor and would aid in proposing more effective combination therapies.

Lastly, it is important to note that we evaluated the tumor microenvironment at an advanced stage of CRC. However, from a prognostic standpoint, it would be of great interest to assess these parameters from the onset of non-malignant lesions or the first stages of ulcerative colitis.

Conclusions

1. Males exhibited a higher tumor burden at the macroscopic level and a greater proportion of adenocarcinomas in the colon.
2. Males had a higher proportion of villous lesions compared with females.
3. Males also showed a higher percentage of intratumoral macrophages and elevated CCL-2 concentrations.
4. In contrast, females displayed greater intratumoral infiltration of cytotoxic lymphocytes, B cells, and plasma cells.
5. At the systemic level, females had lower percentages of B cells in the spleen and reduced NK cell proportions in both the spleen and mesenteric lymph nodes.
6. In serum, males presented higher concentrations of CCL-2 and IL-1β.

Declarations

Funding

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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.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All experimental procedures in the animals were approved by the Institutional Care and Animal Use Committee (Full name: Consejo para el Cuidado y Uso de Animales de Laboratorio, CICUAL), adhering to Mexican regulation (NOM-062-ZOO-1999), in accordance with the recommendations outlined in the National Institutes of Health (NIH) of the United States of America (Guide for the Care and Use of Laboratory Animals). The animal care and experimental practices were conducted at Unidad de Modelos Biológicos (UMB) in the Instituto de Investigaciones Biomédicas (IIB), Universidad Nacional Autónoma de México (UNAM). All experimental procedures on the animals were approved by the CICUAL, ID

Number 6298. The present study adheres to the ARRIVE guidelines. For tumor induction, we utilized the gold standard protocol for inducing colorectal tumors (check materials and methods). Throughout the study, the animals were monitored daily to promptly identify and alleviate signs of pain or distress.

Consent for publication

Not applicable.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Materials availability

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Code availability

Not applicable.

Author contribution

YRS: Design of the study, execution of experiments, processing of biological material, formal analysis, data interpretation, and writing the original draft. CAGC: execution of experiments, processing of biological material, formal analysis, and data interpretation, reviewing the original draft. APT: histological analysis, data interpretation; VHDRA: Design of the study, execution of experiments, and data interpretation. LIT: design of the study and data interpretation. MSRA: execution of experiments, processing of biological material, and data interpretation. CAZL: execution of experiments, processing of biological material and data

interpretation. JMM: design of the study, data interpretation, acquisition of funds for the project, writing the original draft, and writing the final draft.

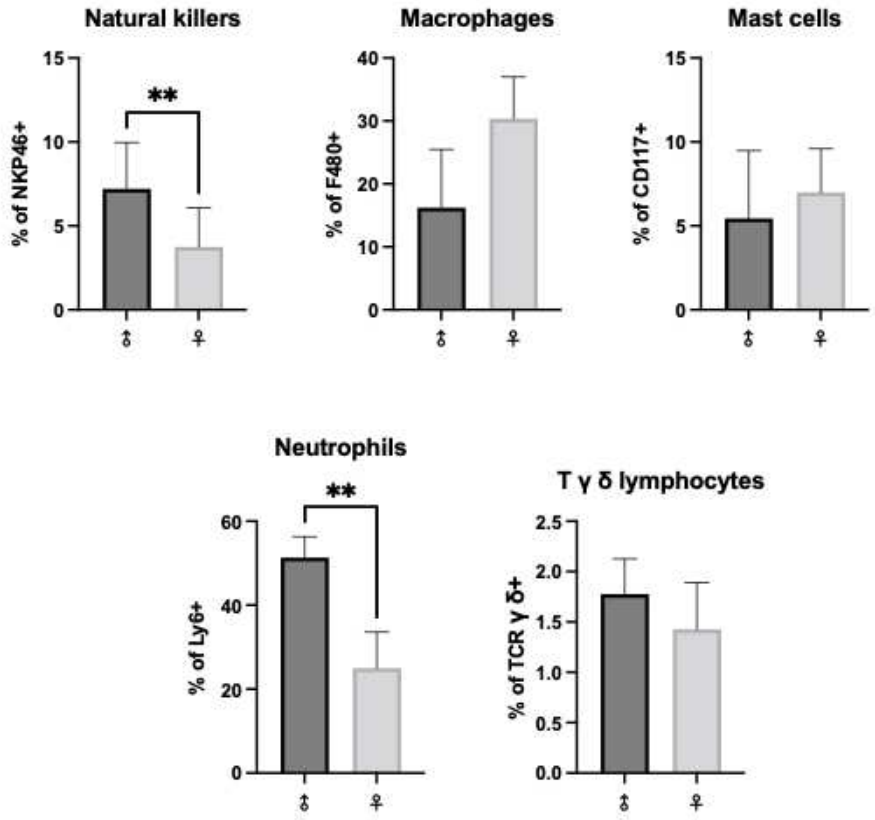
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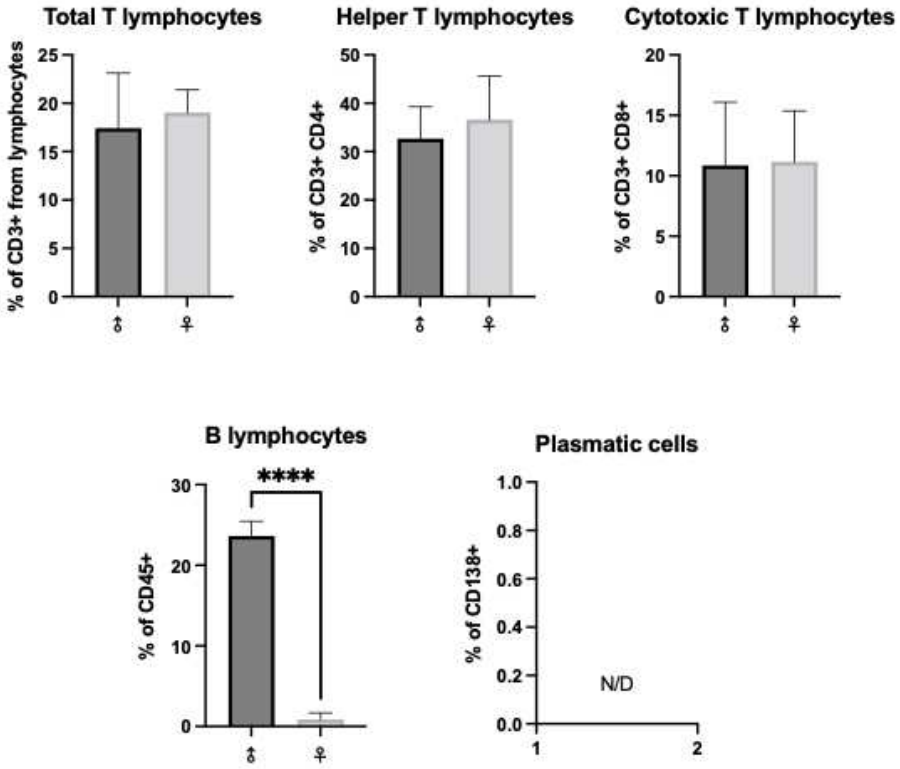
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Supplementary material.

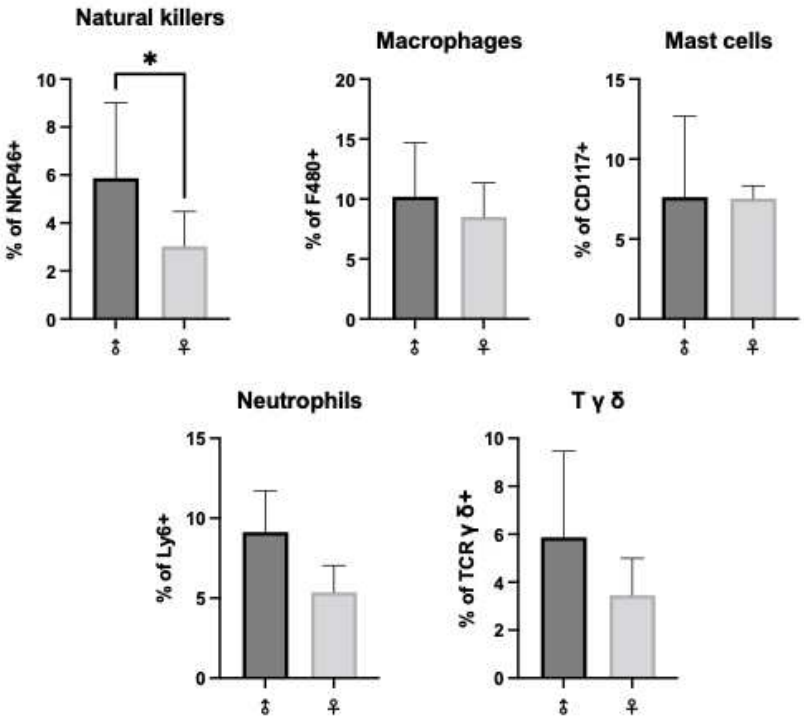
Supplementary figure 1. Innate response of the spleen.



Supplementary figure 2. Adaptive immune response of the spleen.

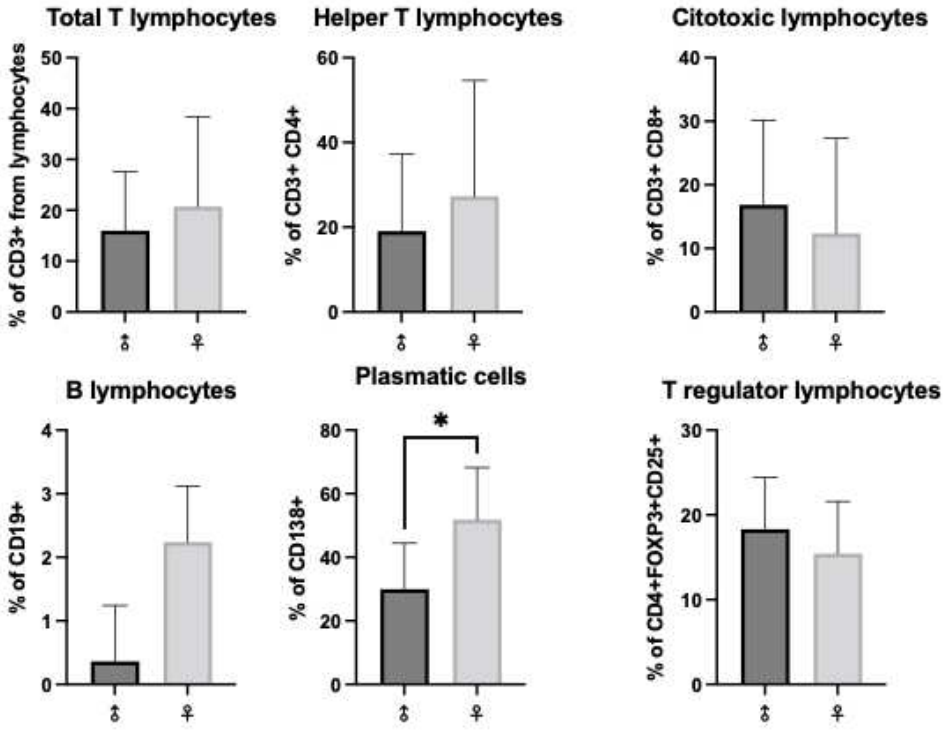


Supplementary figure 3. Innate response of mesenteric ganglia.



606 **Supplementary figure 4. Adaptative immune response of mesentery ganglia.**

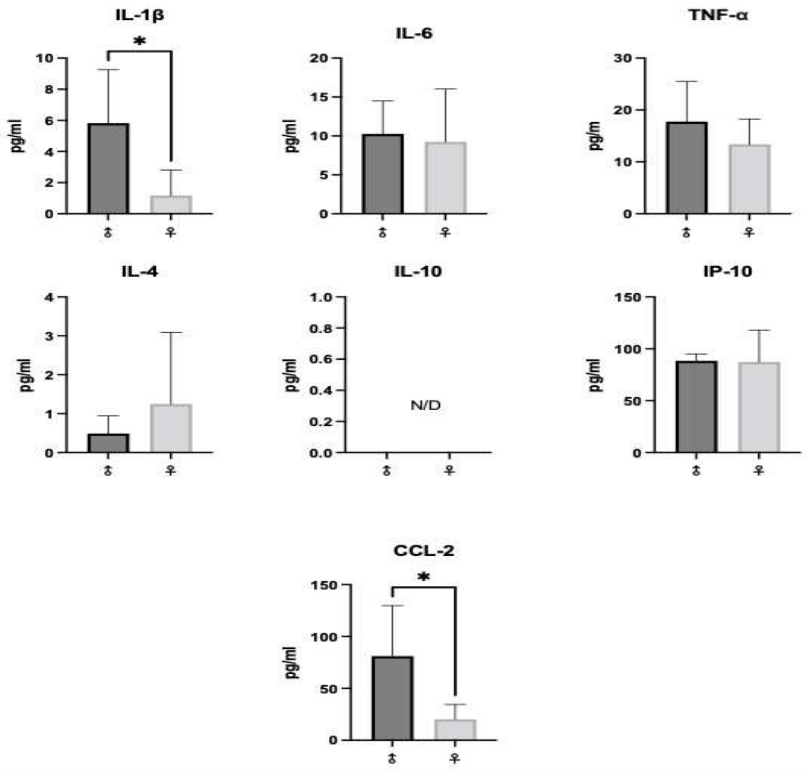
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608 **Supplementary figure 5. Analysis of cytokines in serum.**

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