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Research Article

Zoology

Tumor burden in mice with Ehrlich ascites induces lymphopenia in the peripheral blood leukocytes

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KEY WORDS

EAC, CD4 T-cells, CD8 T-cells, Tumor

ABSTRACT

The purpose of this study is to investigate how EAC affects the numbers of CD4 T-cells, CD8 T-cells, and activated T-cells in the spleen and PBL. Ehrlich's tumor has historically been used in experimental oncology to evaluate the anti-tumor efficacy of various drugs. However, knowledge of the immunological pathways involved in Ehrlich carcinogenesis is still limited. The study aimed to examine the impact of tumor (EAC) on the total number of T lymphocytes (CD4 / CD8) and activated T cell ($CD4^+CD69^+$ / $CD8^+CD69^+$) populations in the spleen and peripheral blood (PBL). Twelve female albino mice were allocated into two groups: the first was the control (naïve group), while the second was injected interperetonial with 2.5×10^6 EAC. After 14 days of inoculation, blood samples were collected and spleen cells were harvested. Cells were stained with the mAbs and analyzed using Flow Cytometry. In the EAC group, the relative and absolute numbers of CD4 T lymphocytes and CD4+CD69+ T lymphocytes increased compared to the naïve group in the spleen. Additionally, The EAC group showed an increase in the absolute number of CD8 T lymphocytes and $CD8^+CD69^+$ T lymphocytes compared to the naïve group in the spleen. However, the relative and the absolute number of CD4, CD8 T lymphocytes, and $CD8^+CD69^+$ T lymphocytes decreased in the EAC group compared to naïve cells in the PBL. These results could be utilized to enhance cancer treatments, similar to those for Ehrlich's tumor.

Introduction

The presence of a tumor significantly alters the entire tumor microenvironment (TME), impacting various cellular and molecular components **(Anderson and Simon, 2020)**. Tumors frequently enlist diverse immune cells (macrophages, T lymphocytes, and suppressor cells derived from myeloid cells (MDSCs)). Tumors can affect systemic immunity, changing peripheral immune cell populations and function **(Hiam-Galvez et al., 2021; Ruffell and Coussens, 2015)**. These alterations create an immune-suppressive environment that facilitates tumor growth and metastasis **(Wang et al., 2023),** highlighting the importance of studying EAC to understand cancer immunology and develop therapeutic strategies.

Ehrlich ascites carcinoma (EAC) is a commonly used model for studying cancer and its effects on the immunological system **(Radulski et al., 2023; Saleh et al., 2022)**. Ehrlich's tumor is significant as transplantable cancer originating from malignant epithelial cells, is specific to certain species, and resembles breast adenocarcinoma found in female mice **(Calixto-Campos et al., 2013; Radulski et al., 2023)**. This tumor develops as ascitic (EAC) in different mouse strains when administered into the peritoneal cavity and as a solid tumor when administered subcutaneously. It exhibits rapid growth and comprises extremely anaplastic and pleomorphic cells, distinguished by a high nucleus-to-cytoplasm ratio **(Abu-Zeid et al., 2024; Frajacomo et al., 2016).** When mice are implanted with EAC, significant alterations happen in their immune cell populations. Since the EAC model closely resembles pathological and behavioral processes in humans, it is commonly used in cancer research to gain insights into tumorigenesis, immune responses, and physiological processes, as well as to assess new diagnostic techniques and the effects of therapeutic drugs **(Feitosa et al., 2021).** Tumors induce an immunological response in individuals, and while considerable data supports anti-tumor immune reactivity in humans, immunity to cancers is more frequently demonstrated through experimental animal investigations **(Mukherjee et al., 2022).**

Prolonged exposure to tumor antigens can result in T cell exhaustion, marked by reduced T cell efficacy and increased expression of inhibitory receptors (such as PD-1) **(Pauken and Wherry, 2015)**. This can minimize T cells' quantity and efficacy **(Takeuchi and Nishikawa, 2016)**. Some tumors can induce apoptosis in T cells that recognize tumor

antigens, effectively reducing their numbers **(Lu and Finn, 2008)**. Additionally, tumors can reduce the expression of MHC class I molecules, complicating the ability of CD8 T cells to recognize and eradicate them **(Cornel et al., 2020)**. The combined impact of tumor-induced alterations in CD4 and CD8 T cells leads to a weakened immune response, facilitating tumor growth and metastasis **(Mittal et al., 2014).** This study aims to examine the impact of EAC on the counts of CD4, CD8 T lymphocytes and activated T-cells in the spleen and PBL.

Materials and methods

Mice:

Female Swiss Albino (CD-1) mice, aged 10 to 12 weeks and weighed between 22-25 grams at the start of the experiments, were bought from the Pharmacology and Experimental Oncology Unit of the National Cancer Institute, Cairo University, Egypt. The mice were housed in a particular pathogen-free environment at the Faculty of Science, Tanta University, under the ethical criteria (Rec-Sci-Tu0023) established by the local Institutional Animal Care and Use Committee. They were partitioned into two groups of six mice each.

Ehrlich Ascites Carcinoma (EAC)

EAC was bought from the National Cancer Institute in Cairo, Egypt, and was preserved in its ascitic state by several passages in Swiss mice using bi-weekly intraperitoneal transplantations of 2.5×10^5 tumor cells per mouse. Ascitic fluid was extracted using a syringe and tumor cells were enumerated utilizing a Neubauer hemocytometer. Viable cells were evaluated via the Trypan Blue dye exclusion test.

Reagents and antibodies

Chemicals and reagents

Cells were stained with mAbs: (anti-CD8 APC, anti-CD4 PE, and anti-CD69: PEcy7 purchased from (BD Pharmingen, USA). The FACS lysing solution was obtained from BD Biosciences, in San Jose, CA, USA. ACK buffer, which contains NH_4Cl (8.29 g/l), $KHCO_3$ (1.00 g/l), and disodium EDTA.$2H_2O$ (0.0372 g/l), was used to lyse red blood cells (RBCs) at a pH of 7 in both blood and spleen samples.

Cell Viability

Splenocytes were homogenized using a sterile syringe or plunger to gently push the tissue through a 70 µm cell strainer over 50 mL conical tubes, filtering out debris and aggregates. The cell strainer was washed with 8 mL of PBS, after that, red blood cells (RBCs) red blood cells (RBCs) were lysed using ACK buffer for 5-minute incubation. The cells were washed twice and then evaluated using an exclusion assay with 0.2% trypan blue solution, added for 1 minute before counting the cells.

Complete Blood Count (CBC)

All mice were bled and sacrificed after 2 weeks and prepared for different assays. Total white blood cells (WBCs) were counted in both peripheral blood using an automated complete blood count (CBC) instrument (ABX Micros 60 hematology analyzer, Horiba Medical).

Detection of T lymphocytes and activated T cells by Flow Cytometry

Fresh single-cell suspensions of leukocytes were prepared from blood and spleen samples. Peripheral blood was collected by bleeding each mouse from the retro-orbital plexus. Approximately 1 × 10^6 cells were stained with anti-CD4, anti-CD8, and anti-CD96 antibodies and incubated on ice for 30 minutes. All samples were re-suspended by PBS and vortexed gently then analyzed by cytometer BD FACS Canto II flow cytometer equipment. After samples acquisition row data could be exported as FSC files. Data were analyzed using (Flow logic, Miltenyi Biotec, USA). Total leucocytes were gated, CD4 and CD8 were gated from total leucocytes then CD96 gated from CD4 and CD8 numbers.

Statistical analysis

Numerical data from the experiments were presented as mean ± SE, and statistical differences between the experimental and control groups were evaluated using Student's t-test. GraphPad Prism (GraphPad Software, Inc., San Diego, CA) was employed to analyze the P values, with P values of $\leq$ 0.05 deemed statistically significant.

Results

Effect of EAC on the number of splenocytes and total WBCs in the PBL

To investigate the effect of EAC on the number of splenocytes in the spleen and total WBCs in the PBL, we found the number of splenocytes showed a decrease in the EAC group as compared to the naïve group. However, Total W.B.Cs induced an increase by 2-fold in the EAC group as compared to the naïve group as shown in Fig. (1).

Effect of EAC on the number of the types of Leukocytes in the PBL

The absolute number of lymphocytes induced increases by 2.9-fold after 2 weeks of I.p injection of the EAC group as compared to the naïve group. However, there were no changes in the absolute number of monocytes and neutrophils after 2 weeks of injection of the EAC group as compared to the naïve group as shown in Fig. (2).

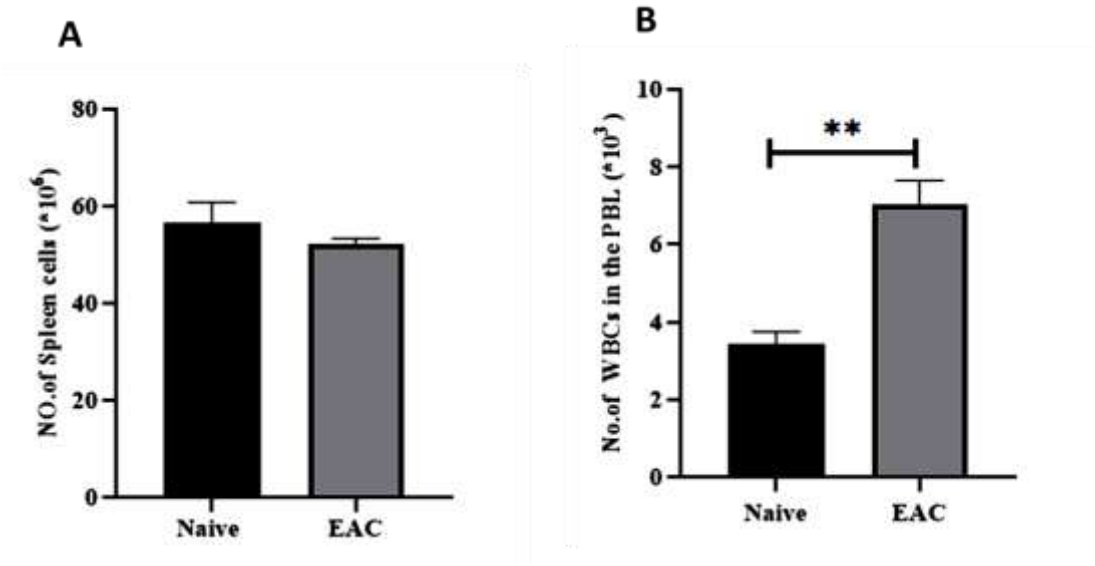

Fig. (1): The total number of splenocytes (A) and leucocytes (B) in the spleen and PBL: 2.5×10^{6} of EAC cells were injected IP at day 0, and then cells were harvested after two weeks of EAC injection in the studied groups. Data represent the mean ± SE (n = 3). * Significant at $p\leq 0.05$.

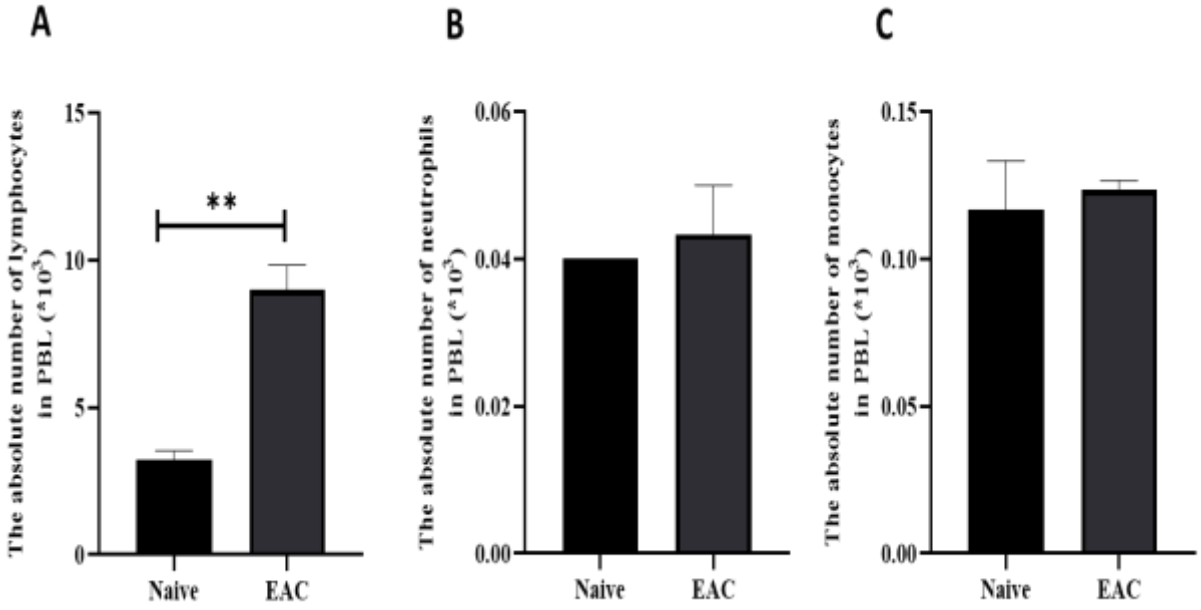

Fig. (2): The absolute numbers of Differential W.B.Cs of the studied groups in PBL. A) The absolute number of lymphocytes in naïve and EAC groups. B) The absolute number of neutrophils in naïve and EAC groups. C) The absolute number of monocytes in naïve and EAC groups: 2.5×10^{6} of EAC cells were injected IP at day 0 and then cells were harvested after two weeks of EAC injection in the studied groups. Data represent the mean ± SE (n = 3). * Significant at $p\leq 0.05$.

Hematological indices alter in the EAC group

The number of RBCs and platelets induced decreases in the EAC group as compared to the naïve group. However, there was no change in the concentration of hemoglobin as shown in Table (1).

Table (1): Hematological indices alter in EAC group. 2.5×106of EAC cells were injected IP at day 0, and then all mice were bled after two weeks of EAC injection in the studied groups. Data represent the mean ± SE (n = 3). * Significant at p≤ 0.05.

Groups	RBC (10^6/µl)	HGB (g/dl)	PLT (10^3/µl)
Naïve	8.7±0.1	13.6 ± 0.2	741.0 ± 23.7
EAC	6.5 ± 0.3	12.1 ± 0.5	395.7±22.4
P value	0.01*	0.12	0.001**

Effect of EAC on the number of CD4 and $CD4^+CD69^+$ T-cells in the spleen

The relative and absolute numbers of CD4 induced increases in the EAC group by1.5- 2- fold, respectively as compared to the naïve group. As well as, the absolute number of $CD4^+CD69^+$ showed increases by 2.5-fold in the spleen of the tumor group (EAC) compared to the naïve group. However, there is no significant change in the relative number of $CD4^+CD69^+$ in the spleen of the EAC group as shown in Fig. (3).

Effect of EAC on the number of CD8 and $CD8^+CD69^+$ T-cells in the spleen

There was no change in the relative number of CD8 in the spleen in the EAC group as compared to the naïve group. However, the absolute number of CD8 and $CD8^+CD69^+$ induced increases in the spleen of tumor group (EAC) by 1.5-2-folds, respectively compared to the naïve group as shown in Fig. (4).

Effect of EAC on the number of CD4 and $CD4^+CD69^+$ T-cells in the PBL

The relative numbers of CD4 induced a decrease in the EAC group by 2.5fold compared to the naïve group as shown in Figure 5. As well as, the absolute number of CD4 and the relative and absolute numbers of $CD4^+CD69^+$ showed a no-significant decrease in the EAC group as compared to the naïve group as shown in Fig. (5).

Effect of EAC on the number of CD8 and $CD8^+CD69^+$ T-cells in the PBL

The relative numbers of CD8 induced a decrease in the EAC group by 4-fold as compared to the naïve group. As well as,

the absolute number of CD8 and $CD8^{+}CD69^{+}$ induced reduction in the PBL of the EAC group compared to the naïve group. However, the relative number of $CD4^{+}CD69^{+}$ induced increases in the PBL of the EAC group as shown in Fig.(6).

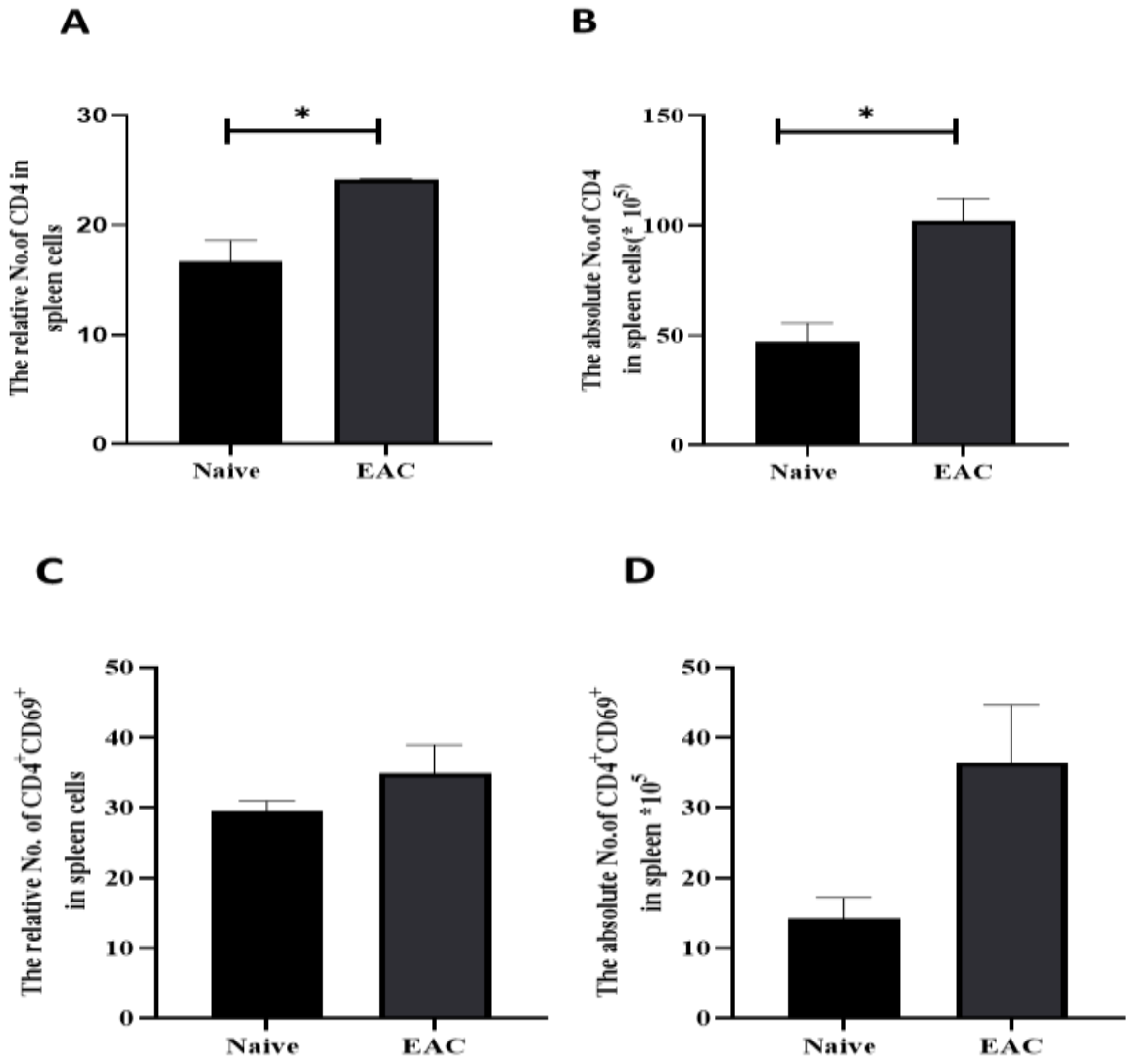

Fig. (3): The relative and absolute numbers of total CD4 and activated T cells CD4+CD69+ in the studied groups in the spleen**:** A) The relative number of CD4 in naïve and EAC groups in spleen. B) The absolute number of CD4in naïve and EAC groups in spleen. C) The relative number of CD4+CD69+ in naïve and EAC groups in PBL. D) The absolute number of CD4+CD69+ in naïve and EAC groups in PBL. 2.5×10^{6} of EAC cells were injected IP at day 0, then cells were harvested after two weeks of EAC injection in the studied groups , stained, and acquired by flow Cytometry Data represent the mean ± SE (n = 3). * Significant at $p\leq 0.05$.

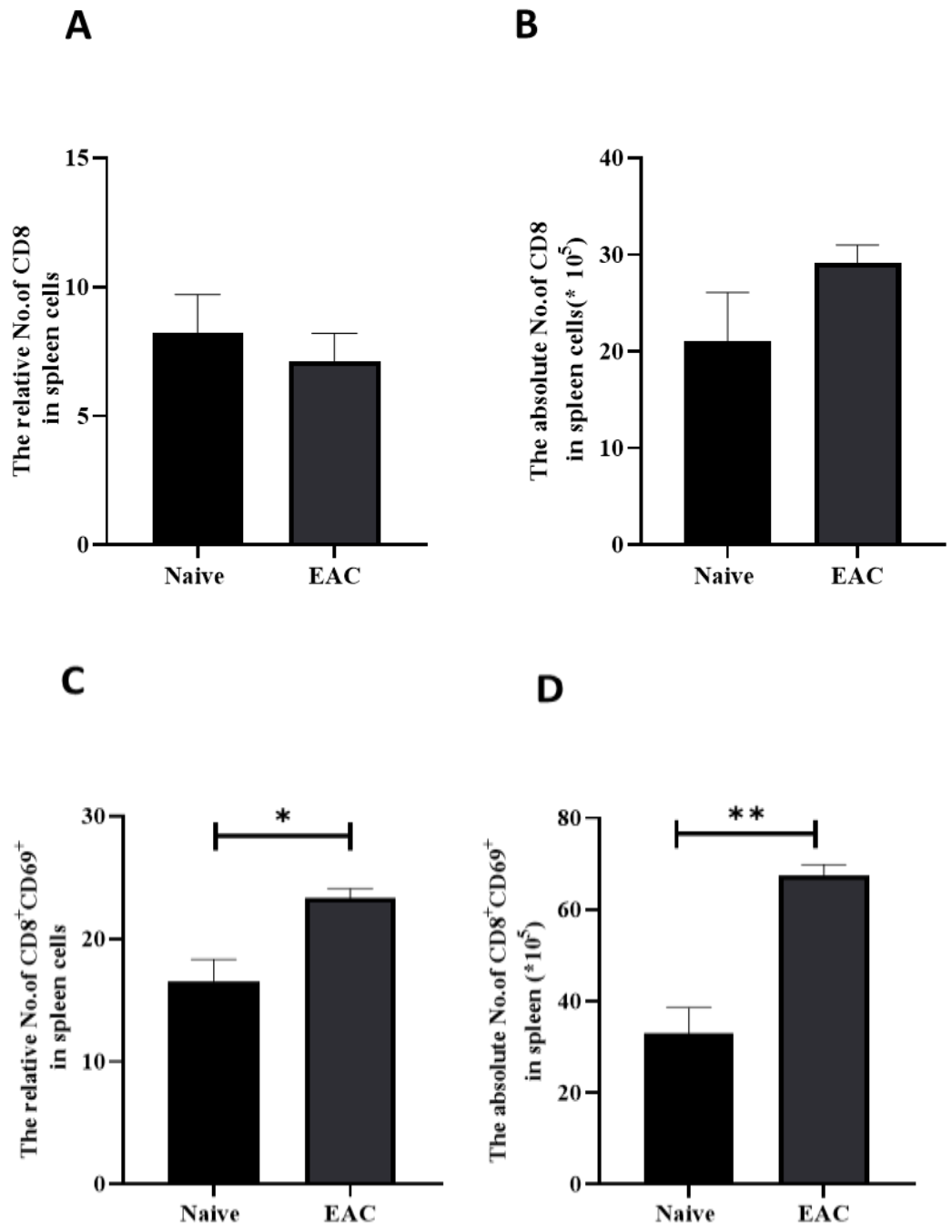

Fig. (4): The relative and absolute numbers of total CD8 and activated T cells CD8+CD69+ in the studied groups in the spleen and PBL: A) The relative number of CD8 in naïve and EAC groups in spleen. B) The absolute number of CD8 in naïve and EAC groups in spleen. C) The relative number of CD8+CD69+ in naïve and EAC groups in PBL. D) The absolute number of CD8+CD69+ in naïve and EAC groups in PBL. 2.5×10^{6} of EAC cells were injected IP at day 0, then cells were harvested after two weeks of EAC injection in the studied groups , stained, and acquired by flow Cytometry Data represent the mean ± SE (n = 3). * Significant at $p\leq 0.05$.

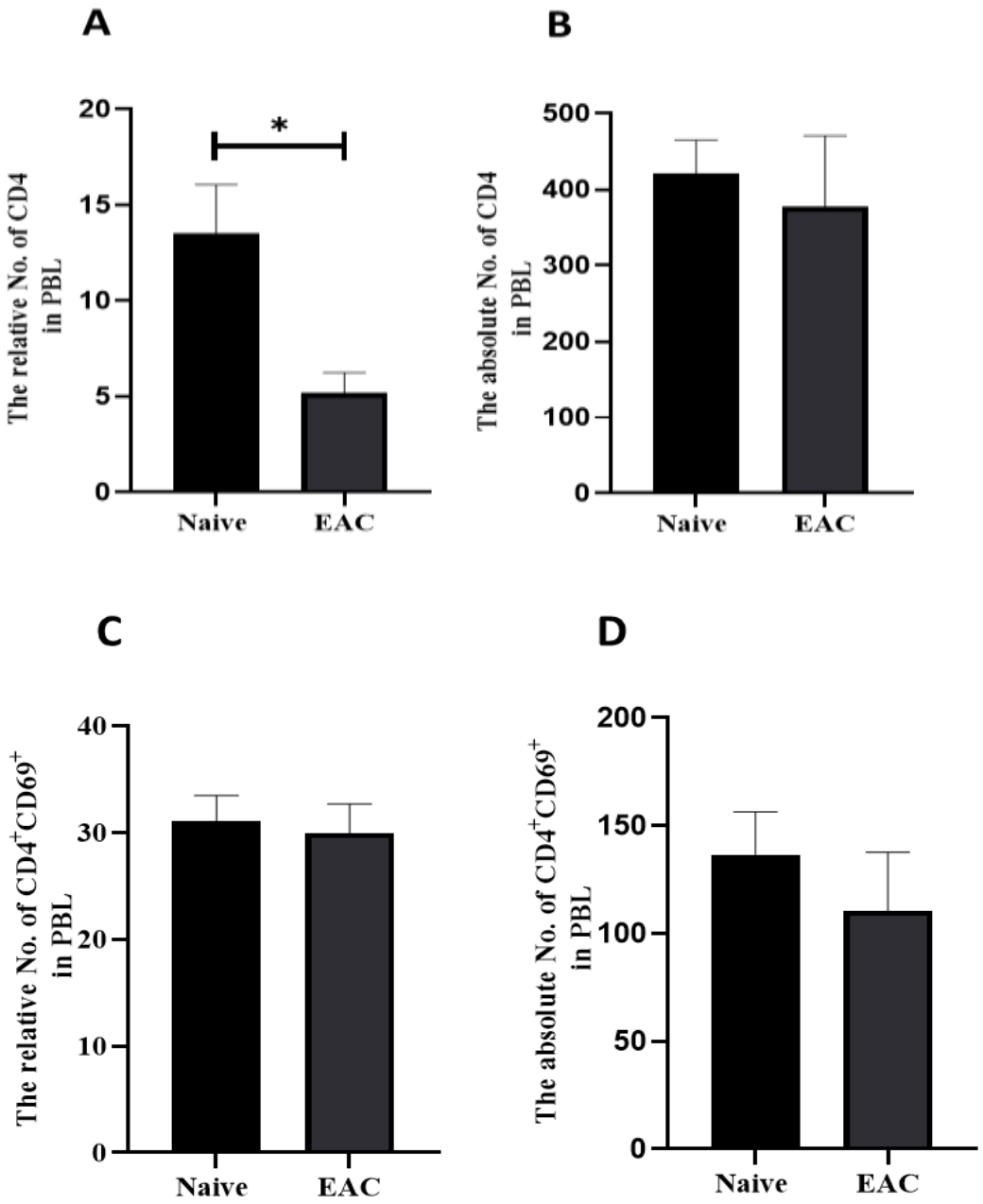

Fig. (5): The relative and absolute numbers of total CD4 and activated T cells CD4+CD69+ in the studied groups in the spleen and PBL: A) The relative number of CD4 in naïve and EAC groups in PBL. B) The absolute number of CD4 in naïve and EAC groups in PBL. C) The relative number of CD4+CD69+ in naïve and EAC groups in PBL. D) The absolute number of CD4+CD69+ in naïve and EAC groups in PBL. 2.5×10^{6} of EAC cells were injected IP at day 0 and then cells were harvested after two weeks of EAC injection in the studied groups, stained, and acquired by flow Cytometry. Data represent the mean ± SE (n = 3). * Significant at $p\leq 0.05$.

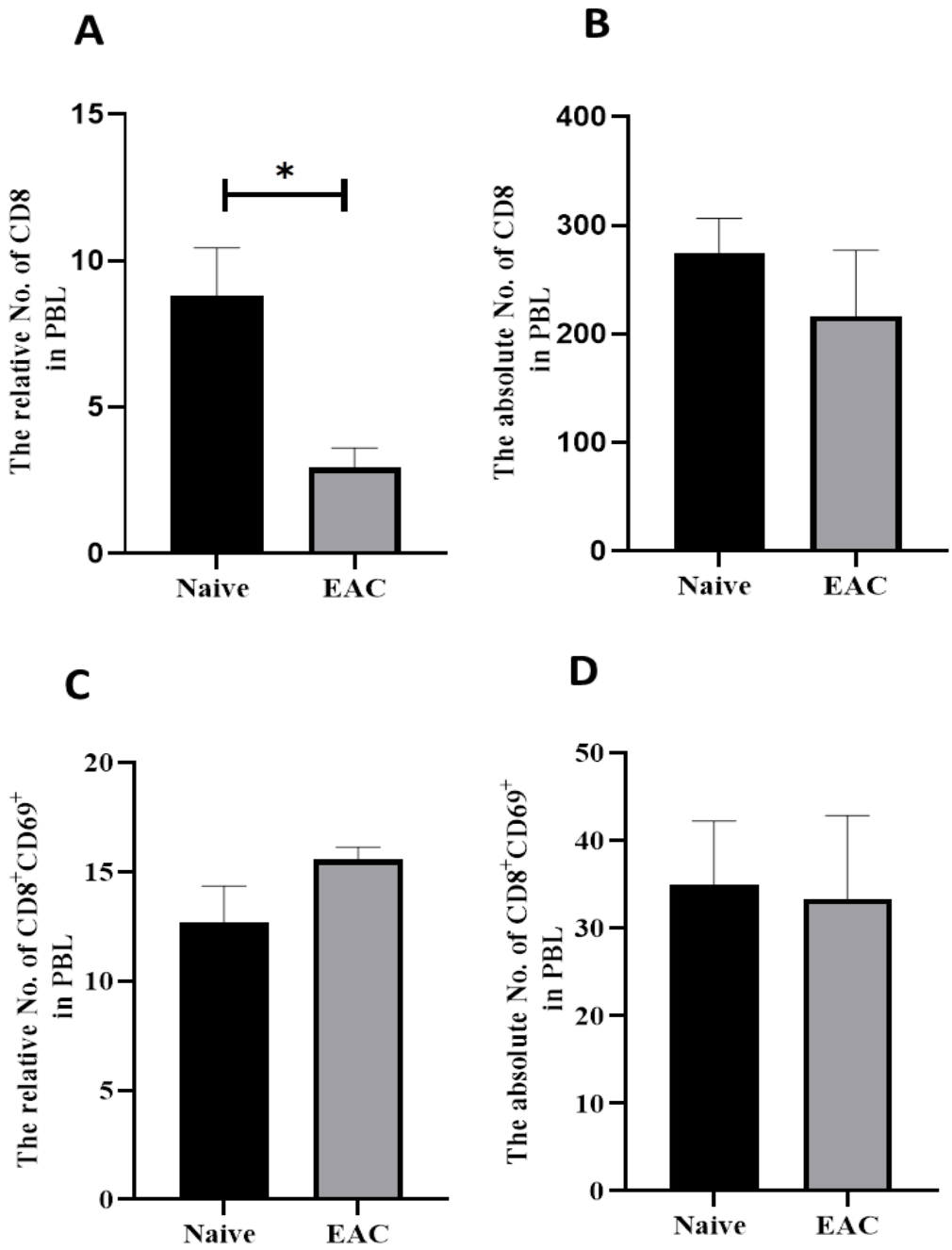

Fig. (6): The relative and absolute numbers of total CD8 and activated T cells CD8+CD69+ in the studied groups in the spleen and PBL: A) The relative number of CD8 in naïve and EAC groups in PBL. B) The absolute number of CD8 in naïve and EAC groups in PBL. C) The relative number of CD8+CD69+ in naïve and EAC groups in PBL. D) The absolute number of CD8+CD69+ in naïve and EAC groups in PBL. 2.5×10^{6} of EAC cells were injected IP at day 0 and then cells were harvested after two weeks of EAC injection in the studied groups, stained, and acquired by flow Cytometry. Data represent the mean ± SE (n = 3). * Significant at $p\leq 0.05$.

Discussion

In Ehrlich's tumor, numerous investigations show how the tumor utilizes evasion tactics to circumvent the immune system, while other research emphasizes the immune response that can inhibit tumor development, whether in ascetic or solid form **(Feitosa et al., 2021)**. Although numerous research have recorded immune responses to Ehrlich's tumor, a definitive elucidation of how, what, and to what degree the immunological attributes of Ehrlich's tumor can be comparable, predictive, and relevant to comprehending immunopathology in humans remains absent. The most recent review on Ehrlich's tumor was published, focusing on the biochemical and physiological properties of the ascetic variety **(Radulski et al., 2023).**

The tumor microenvironment is a dynamic and heterogeneous ecosystem that controls tumor behavior, immune response, and therapeutic efficacy. Understanding these interactions is essential for developing successful cancer treatments and immunotherapies **(Xiao and Yu, 2021)**. Consequently, this work seeks to examine the effect of the microenvironment of the tumor on T-cell activation within this experimental cancer model.

In PBL, the EAC group showed an increase in the number of Leucocytes, Lymphocytes and neutrophils and monocytes than the naïve group. This is supported by **(Karthigayan et al., 2007)** as neutrophils were increased in the EAC-bearing mice in comparison to the normal animals and also in clinical study they observed that peripheral neutrophils in the blood are increased in cancer patients **(Inzhevatkin and Savchenko, 2017; Radulski et al., 2023).**

There was a decrease in the number of red blood cells and platelets and a non-significant decrease in the amount of hemoglobin. This observation is in line with the recent studies **(Karthigayan et al., 2007)** that reported that In EAC-bearing mice, red blood cells and hemoglobin were significantly reduced in comparison to normal animals.

In spleen, According to the current study, compared to the naïve group, the EAC-bearing mice had fewer leukocytes in the spleen and more CD4 and CD8 T-cells in total. In agreement with our findings, **Mandal et al., (2006)** discovered that during the initial weeks following tumor injection in mice treated with EAC, the populations of CD4 and CD8 T-cells are reduced **(Feitosa et al., 2021)**. During the initial two weeks following tumor inoculation,

the thymus enhances the production of CD4 and CD8 T-cells. This signifies that the thymus is actively replenishing these populations in the spleen and peripheral circulation to mitigate initial depletion, potentially aiding in tumor growth restriction during this interval.

In PB, the EAC group showed an increase in the total number of CD4 and CD8 T lymphocytes in comparison with the naïve group. This observation lines up with the findings of Mandal et al., (2006) that found in EAC-bearing mice, a significant reduction in CD4 and CD8 lymphocytes in peripheral blood was observed, followed by substantial thymic destruction by the 21st-day post-tumor inoculation.

Conclusion

Tumors can profoundly affect T cell behavior, leading to impaired immune responses that allow for tumor growth and progression. Understanding these interactions is crucial for developing effective immunotherapies and enhancing the ability of the immune system to resist cancer.

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نقصان عدد الخلايا الليمفاوية في خلايا الدم البيضاء بالدم الطرفي في الفئران الحامله لورم ايرليش

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تهدف هذه الدراسة لمعرفة تأثير الورم على أعداد الخلايا الليمفاويه التائية والقاتله وكذلك المنشط منها في الطحال والدم .ويعد نموذج الورم إيرليش(Ehrlich) من النماذج المستخدمة في أبحاث الأورام التجريبية لتقييم فعالية الأدوية المضادة للأورام، ومع ذلك، فإن معرفة المسارات المناعية المرتبطة بتكون هذا الورم لا تزال محدودة . وللقيام بهذه الدراسة تم إستخدام اثني عشر فأرة ألبينو أنثوية ووضعهم فى مجموعتين: الأولى كانت مجموعة غير مصابة (سليمة) كمجموعة ضابطه ، بينما تم حقن الثانية داخل التجويف البطني بجرعة (٢.٥×١٠^٦) من خلايا ايرليش (EAC) (مصابه) وبعد مرور ١٤ يومًا من الحقن (إصابتها بالورم) ثم تم جمع عينات الدم واستخراج خلايا الطحال و تم صبغ الخلايا بالأجسام المضادة الأحادية (mAbs) وتحليلها باستخدام تقنية تدفق الخلايا (Flow Cytometry). حيث أظهرت نتائج الدراسة أن المجموعة المصابة أظهرت زيادة في العدد النسبي والمطلق للخلايا اللمفاوية التائية CD4 والخلايا اللمفاوية التائية النشطة +CD4+CD69 في الطحال مقارنة بالمجموعة السليمه الخالية من الورم. كما لوحظ أيضًا زيادة في العدد المطلق للخلايا التائية CD8 وخلايا التائية النشطه +CD8+CD69 في الطحال للمجموعة المصابة بالورم (EAC) مقارنة بالمجموعة السليمة. لوحظ ايضا انخفاض في العدد النسبى والمطلق لخلايا CD4 و CD8 و +CD8+CD69 في الدم لدى المجموعة المصابة بالورم مقارنة بالمجموعة السليمة (الضابطة).

يمكن الاستفادة من هذه النتائج لتحسين علاجات السرطان، وذلك على غرار العلاجات المستخدمة للورم إيرليش (Ehrlich).