

RESEARCH ARTICLE

Editorial Process: Submission:11/01/2024 Acceptance:09/01/2025 Published:09/12/2025

Effect of Alpha1 Antitrypsin on *PD-1/PD-L1* and *EGFR* in Colitis-Associated Colorectal Cancer BALB/c Mouse Model

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Abstract

Objective: This study aimed to investigate whether Human alpha-1 antitrypsin affects Programmed cell death protein 1 (*PD-1*) and its ligand (*PD-L1*) as well as epidermal growth factor receptor (*EGFR*) expression using a colon cancer tissues in chemically induced colorectal cancer (CRC) model. Previous study indicated a protective role of AAT administration on the development of cancer in CRC mouse model. **Methodology:** The expression of *PD1/PDL1* and *EGFR* were analyzed in colon cancer tissue collected from (AOM/DSS) CRC mice using both real time gene expression (qPCR) and immunohistochemistry(IHC). **Results:** Study results showed that AAT treatment reduced *PD-L1* protein expression by 63.5% (mean H score: 7.8 ± 1.3 to 2.85 ± 0.85 , $p=0.0004$) and *EGFR* protein expression by 48.9% (mean H score: 1.78 ± 0.76 to 0.91 ± 0.60 , $p=0.04$) in the tissue of AOM/DSS-treated mice. In addition, AAT treatment controlled *PD1* gene expression and repressed *EGFR* gene expression by 1.4-fold compared to DSS samples. Immunohistochemical analysis revealed various degrees of *PD1* protein expression, and CD4 and CD8 positive lymphocytes were found in the tumor microenvironment. **Conclusion:** These findings suggest that AAT treatment has immunomodulatory properties and may represent a promising therapeutic strategy for colon cancer.

Keywords: Cancer Immunotherapy- Immune checkpoints- AOM/DSS- Immunohistochemistry- RT-PCR

Asian Pac J Cancer Prev, 26 (9), 3229-3237

Introduction

Programmed death-1 (*PD-1*) is a co-receptor that is expressed predominantly by T cells, B cells, dendritic cells (DCs), natural killer T (NKT) cells, monocytes and activated macrophages [1]. In physiological circumstances, it works as molecule that limits immune response and therefore prevents excessive tissue damage and protects from autoimmunity by binding to its ligand *PD-L1* [2, 3].

PD-L1 is often expressed by various tumor cells and antigen presenting cells in various malignancies [4, 5]. The expression of *PD-L1* on normal cells is rare, thus making it a unique immunoinhibitory molecule [1] and a good target for cancer immunotherapy.

One of the established hallmarks of cancer is the evasion of anti-tumor responses in the tumor microenvironment. Moreover, CD8 T cells that have been exhausted express inhibitory immune checkpoints (IC), such as *PD-1* and *CTLA-4*. T cells undergo apoptosis when they become exhausted and lose their effector functions [6]. However, current anti-tumor immunity-based therapies target the programmed cell death protein-1 (*PD-1*)/*PD-L1* signaling pathway.

Various studies have linked high expression of *PD-L1* with a bad prognosis, including ovarian cancer and melanoma. On the contrary, *PD-1* and *PD-L1* blockade therapy has been shown to reduce tumor growth in ovarian cancer [7]. Yassin and coworkers [8] investigated the expression of *PD-1* and its ligands, *PD-L1*, in colitis-associated colorectal cancer (CRC) using an Azoxymethane (AOM)/Dextran Sodium Sulfate (DSS) mouse model. They found a significant increase in *PD-1* expression on mucosal T-cell subsets of the colon and the ileum, which correlated with disease progression and T-cell exhaustion. In human studies, after anti-*PD-1* antibody administration at the onset of tumor development to potentially inhibit or prevent tumor progression, there was a reduction in the variation in tumor numbers in individuals with cancer, but it did not affect the tumor burden.

Human alpha-1 antitrypsin (AAT), also known as alpha1 proteinase inhibitor and SERPINA1 (Serine Protease Inhibitor, group A, member 1), is one of the classical acute phase response glycoproteins that circulates in the blood and tissues and inhibits neutrophil elastase as well as other serine proteases [9]. Studies reported that AATD can favor the development of different cancers such

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as lung cancer [10, 11], urinary bladder cancer [12], and malignant lymphomas [13]. Even though AAT has been identified as a potential tumor marker, little is known about its role in cancer biology. Additionally, multiple clinical trials have shown that people with AATD have a higher risk of developing hepatocellular carcinomas [9].

Epidermal growth factor receptor (*EGFR*), along with three other related proteins (the ERBB family), plays a critical role in both normal physiological and cancerous conditions [14]. *EGFR* plays a significant role in the progression and development of colorectal cancer (CRC). Overexpression of *EGFR* is detected in approximately 60–80% of CRC and often correlated with a poor prognosis and reduced survival rates. EGF binding to *EGFR* initiates a cascade of downstream signaling cascades, that promote cell growth, angiogenesis, and metastasis [15].

The relationship between AAT levels and CRC remains controversial. Moreover, high serum AAT concentrations or AAT deficiency are both linked to CRC development according to many studies [9, 16]. A study by our research group demonstrated a beneficial effect for AAT administration on colon cancer development in AOM/DSS mouse model [17]. Lower numbers of large colon tumors, longer colon and less polyps were indicated in AAT treated mice. Thus, the impact of AAT treatment on the expression levels of *PD-1*, *PD-L1* and *EGFR* in tumors was investigated in this study.

Materials and Methods

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 separately in plastic cages and received a normal diet and water ad libitum, with a light/dark cycle of 12:12 h. Housing, anesthesia, and postoperative care concurred with the guidelines established by an Institutional Animal Ethics Committee approval (ACU-2021/11) Yarmouk University.

The AOM/DSS model was based on a single intraperitoneal injection of (10 mg/kg body weight) AOM (ChemCruze, USA), followed by three cycles of 2.5% of DSS (TdB Consultancy, Sweden) in drinking water over a period of ten weeks. The mice were randomly assigned to four groups (n = 10 mice per group): group 1 received water as a control; group 2 received AOM/DSS alone; group 3 received AOM/DSS with an intraperitoneal injection of human AAT protein (Grifols, Germany); and group 4 received AAT alone. AAT (4 mg) was administered into mice at weeks 15, 16, and 17.

After 17 weeks, colons were collected for RNA extraction while keeping them at -80° C until the day of extraction. For histopathological examination, tissues were fixed in 10% formalin and processed into paraffin blocks in the following days.

Immunohistochemistry

Colon tissue sections of 5 µm were prepared using an electronic rotary microtome (Thermo Scientific, USA). The tissue sections were dewaxed with xylene, rehydrated by successive immersing in decreasing concentrations of Ethanol, and finally washed with DW. The sections were

boiled in 10mM sodium citrate (pH 6) using a microwave for antigen retrieval. The slides were rinsed in PBS before being blocked with 3% BSA in PBS, and then incubated overnight at 4°C with rabbit anti-*PD-1* or anti-*PD-L1* antibodies diluted 1:250, and anti-*EGFR* 1:100, both from GenoChem World (Spain). Anti-Rabbit IgG HRP conjugated (Abcam, UK) was used as detection antibody and followed by washing and substrate addition (DAB, Abcam, UK)). Slides were then dehydrated and mounted using D.B.X (Alpha Chemika, India).

Immunofluorescence

For immunofluorescent staining of CD4 and CD8 positive cells in colon tissue, the above protocol was used except that FITC-conjugated Rabbit anti-CD4 and CD8 (ThermoFisher, MA, USA) were used. After incubation with a 1:250 dilution for 1 hr at RT, the slides were washed three times and then incubated with DAPI for 10 min (Merck, Germany).

Determination of Relative Gene Expression of Cytokines by Real Time-PCR

RT-PCR was performed using a TB Green® Premix Ex Taq™ II FAST qPCR (Takara, Japan), according to the manufacturer's instructions. Primers sequences were: for the expression levels of *PD-L1* (F: 5'- AGTATGGCAGCAACGTCACG -3'; R: 5'- TCCTTTTCCCAGTACACCACTA -3', *PD-1* (F: 5'- ACAGTGTCTCAGAGGGGAGCAAA -3'; R: 5'- TATGATCTGGAAGCGGGGCAT -3'), *EGFR*-FW CTGCCAAAAGTTCCAAGATGAGG; RV: GGGGCACTTCTTCACA, GAPDH (F: 5'-GGCATGGACTGTGGTCATGA-3'; R: 5'-TTCACCACCATGGAGAAGGC-3'). Relative gene expression was calculated using the following comparative Ct ($2^{-\Delta\Delta Ct}$) analysis method [18]:

$\Delta Ct = \text{AVG. Ct (gene of interest)} - \text{AVG. Ct (housekeeping gene)}$

$\Delta\Delta Ct = \Delta Ct (\text{treated sample}) - \Delta Ct (\text{control sample})$

Relative quantification of gene expression = $2^{-\Delta\Delta Ct}$

The PCR product was run on 1.5% agarose gel on 120V for 45 min for amplicon validation purposes.

Results

Real-time PCR analysis of genes in colon samples *PD-1* gene expression

The extracted RNA from colon was used for RT-PCR, and their expressions were normalized to the housekeeping gene GAPDH for each sample (Figure 1A). The resulting values were then compared to those of the control group. Additionally, gel electrophoresis was conducted for all RT-PCR samples to verify the presence of a single amplicon, as shown in (Figure 1 B&C. The treatment with AAT resulted in lower expression of *PD-1* mRNA (Fig.2A). However, treatment of mice with AAT intraperitoneally from weeks 14 to 17 after AOM/DSS treatment resulted in elevated levels of *PD-1* gene expression, but did not reach the control baseline level.

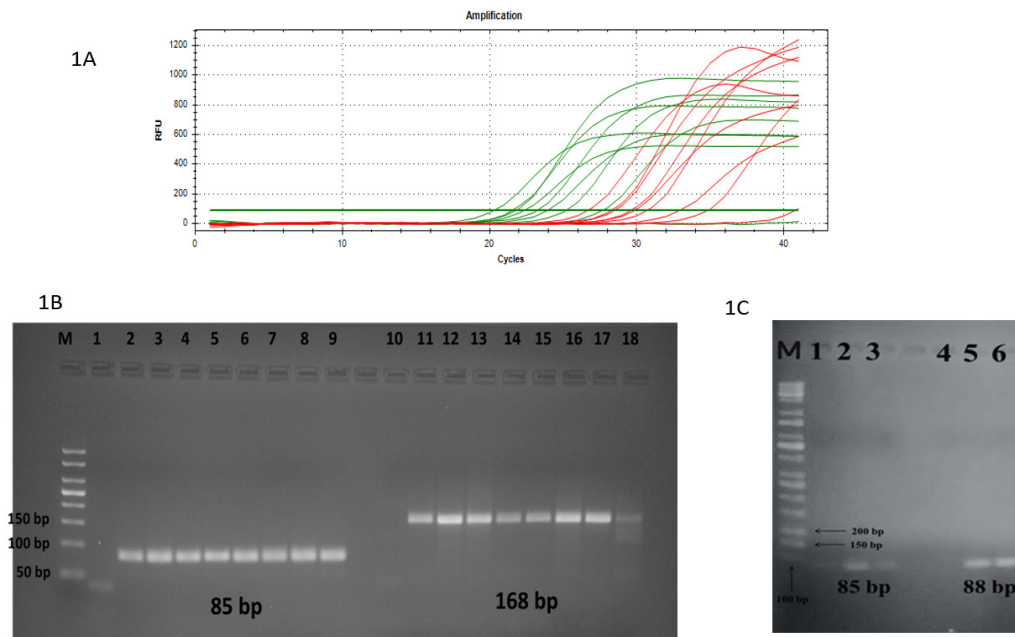


Figure 1. A. RT-PCR charts display Ct value and relative fluorescent unit (RFU) for *GAPDH* gene (green curves) and *PD-1* gene (Red curves). B. Agarose gel electrophoresis of RT-PCR product for colon samples (*GAPDH*, lanes 2-9) and *PD-1* (lanes 11-18). Lanes 1 and 10 are NTC for relevant genes. M: 50 bp ladder). Samples were run by electrophoresis for 00:50 mins, and then visualized under the UV-light C Agarose gel electrophoresis of RT-PCR product for colon samples (*GAPDH*, lanes 2 and 3) and *PD-L1* (lanes 5 and 6). Lanes 1 and 4 are NTC for relevant genes. M: 50 bp ladder). Samples were run by electrophoresis for 00:50 mins, and then visualized under the UV-light

PD-L1 gene expression

As for PD1-L gene expression, AAT treatment reduced the levels of expression measured in the control group (Figure 2B). However, a significant elevation of *PD-L1* was revealed in animals injected with AAT after AOM/DSS treatment. *PD-L1* expression in group treated with AOM/DSS was comparable to control group.

EGFR gene expression

EGFR gene expression was reduced by 1.4-fold in DSS/AAT colon samples compared to DSS samples (which showed a 1.83-fold increase), although this reduction was not statistically significant based on the control group baseline (1-fold expression). (Figure 2C).

Tissue Expression of PD-1

Colon cancer tissues were stained weakly for *PD-1* in about 5% of the lymphocytes in the peritumoral region. The staining intensity and percentage of stained cells were almost similar in the control group, the AAT-treated group, AOM/DSS, and the AAT plus AOM/DSS groups (Figure 3).

AAT treatment reduced the expression of PD-L1 in colon cancer tissues of AOM/DSS mice

PD-L1 protein expression was strongly to moderately stained in the DSS group (mean H score= 7.8 ± 1.3 , which is a histological score or semi-quantification method used in IHC to measure the intensity and distribution of staining in tissue sample. However, DSS/AAT group showed faint to moderate stain (mean H score= 2.85 ± 0.85). Thus, AAT treatment was found to significantly decrease the

expression of PDL-1 (p value= 0.0004) (Figure 4).

AAT treatment reduced the expression of EGFR in colon cancer tissues of AOM/DSS mice

EGFR was strongly to moderately stained in the DSS group (mean H score= 1.78 ± 0.76); whereas the DSS/AAT group had faint to moderate stain (mean H score= 0.91 ± 0.60). By comparing the untreated colitis-induced group (DSS) with the AAT-treated group (DSS/AAT), the AAT treatment was found to significantly decrease the expression of *EGFR* (p value= 0.04)(Figure 5A&B).

Localization of CD4 and CD8 in colon tumors

Analysis of the localization of CD4 and CD8 positive lymphocytes in the tumor microenvironment was assessed using FITC stained antibodies (Fig. 6). Both CD4 and CD8 cells were encountered in the tumor tissue.

Discussion

This is the first study to elucidate the relationship between AAT administration and the expression levels of (*PD-1*)/*PD-L1* and *EGFR* in colon cancer animal model. AAT treatment in CRC mouse model resulted in a significant reduction of colon cancer development as indicated by the restoration in colon length, lower number of polyps and colon tumors [17].

It has been indicated that AAT deficiency favors the development of different cancers such as lung cancer [10, 11], urinary bladder cancer [12], and malignant lymphomas [13]. Thus our hypothesis was that the administration of AAT to AOM/DSS CRC may reduce cancer development

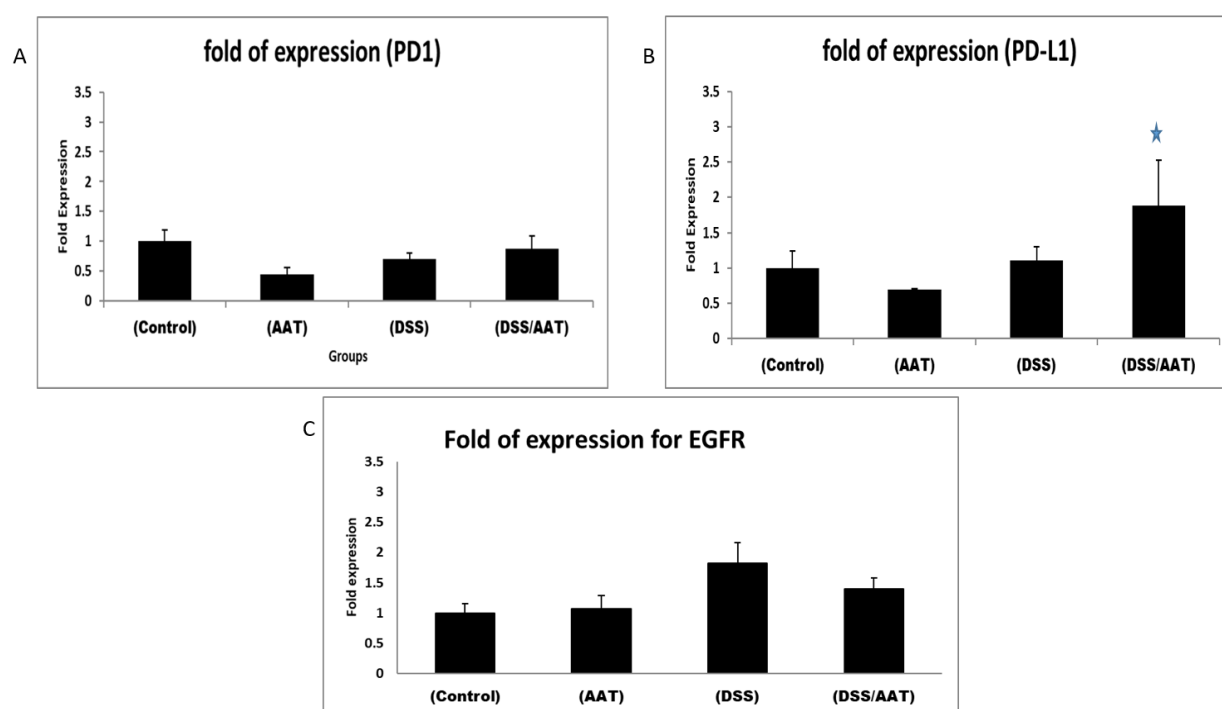


Figure 2. A. fold expression of *PD-1* gene in colon tissue collected from AOM/DSS colorectal cancer mouse groups. B. Fold expression of *PD-L1* gene in colon tissue C Fold gene expression of *EGFR* in colon samples collected from AAT treated and control AOM/DSS mouse model. Data shows mean $\pm$ SD. Fold gene expression of all used samples was calculated using the $(-\Delta\Delta Ct)$ equation. Mann-Whitney t-test was performed on the values using graph prism to assess the data significance at $p \leq 0.05$. *GAPDH* was used as a housekeeping gene to normalize gene expression levels.

by decreasing *PD-L1* and *PD-1* expression, thereby enhancing cancer immune surveillance and protection.

In contrast to our expectations, quantification using RT-PCR revealed the low expression of both *PD-1* and *PD-L1* in colon tissues. This may be due to the fact that

we measured the gene expression in early stages of cancer development [17]. The 16-week period did not result in lymph node involvement and metastasis. Most studies indicated the correlation between the cancer stage and the expression of *PD-1*. Shan Tow and coworkers described

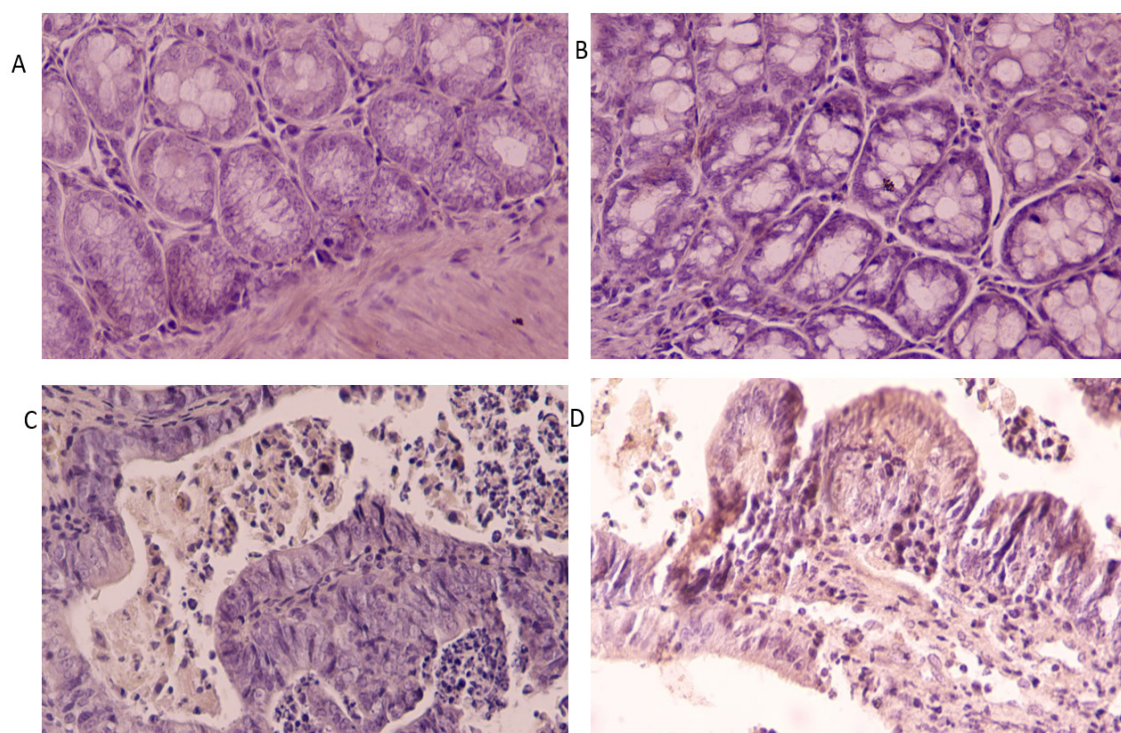


Figure 3. Immunohistochemistry Staining of *PD-1* in Colon Tissue Samples of Mice Treated with Control (A), AAT alone (B), AOM/DSS (C) AOM/DSS+AAT (D). 400X magnification.

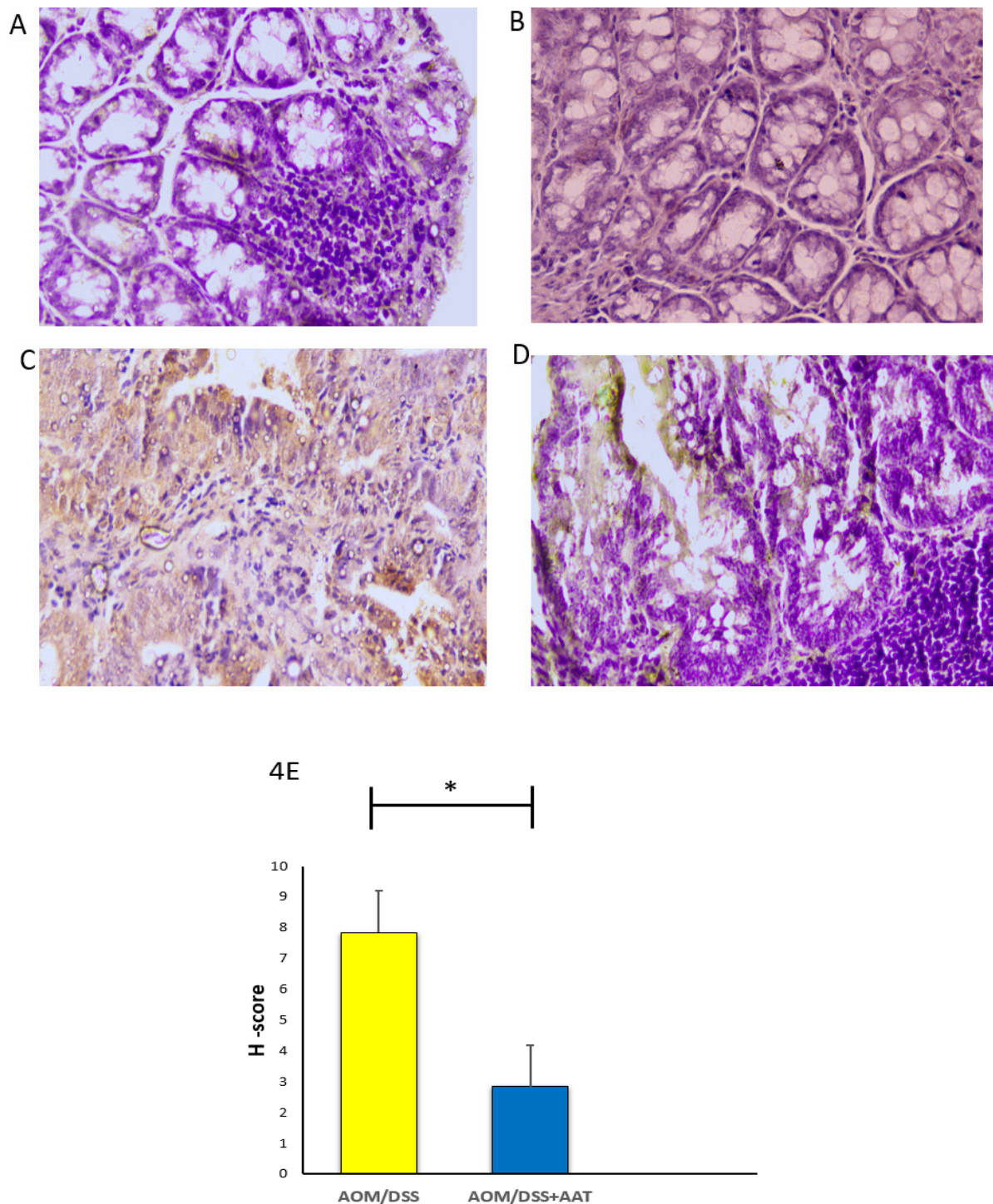


Figure 4. A. Immunohistochemistry staining of *PD-L1* in colon tissue samples of mice treated with AAT alone (A), No treatment control (B), AOM/DSS (C), AOM/DSS+AAT (D). 400X magnification. E The mean $\pm$ SD for H-score of *PD-L1* expression in colon tissues. All samples of DSS group and DSS/AAT group were used in calculating the protein expression and the values were displayed using a graph prism to assess their significance, with * indicating significance and $p \leq 0.05$, ($P=0.0004$)

the positive correlation of *PD-L1* expression with TNM stage, LN involvement and distant metastasis [19].

The low expression levels of *PD-I* in colon tumors in this study is not in agreement with other studies that utilized the same mouse model [8]. Researchers found a significant increase in *PD-I* expression on mucosal T-cell subsets of the colon and the ileum, which correlated with

disease progression and T-cell exhaustion. In contrast to *PD-I*, the elevation of *PD-L1* in DSS treated mice shown in this is consistent with findings by Sumiyoshi and colleagues, who reported significantly higher levels of *PD-L1* in AOM/DSS-treated mice compared to normal controls in colorectal cancer [20]. They presented an association with expression of the suppressive molecules

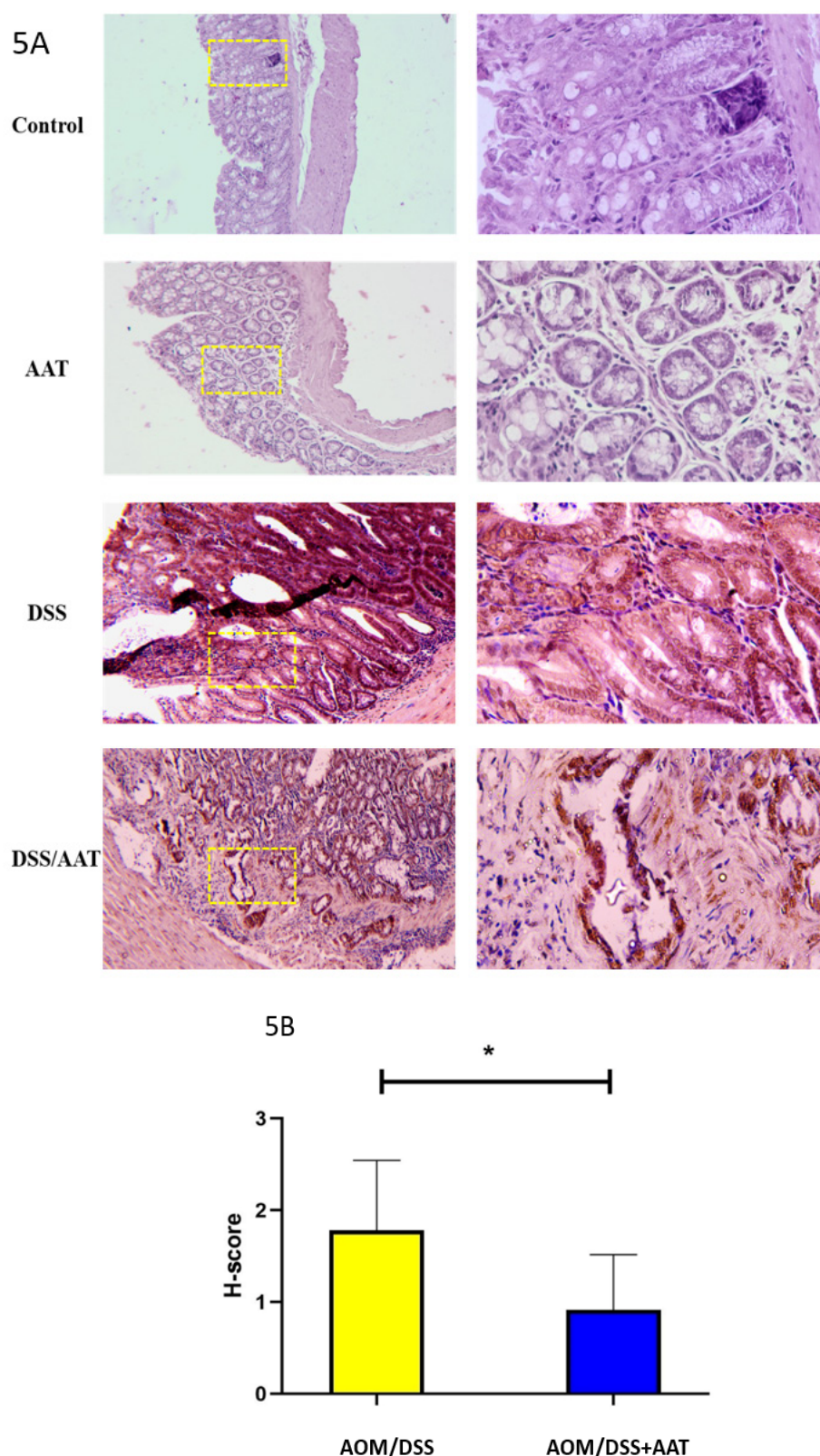


Figure 5. A. Immunohistochemical staining of EGFR protein expression in colon sections in all mouse groups (DSS, DSS/AAT, AAT and control) at 100 X magnifications to the left and 400X to the right. The yellow squares indicate the tissue field magnified at 100X. B. The mean $\pm$ SD for H-score of EGFR protein expression in colon tissues. All samples of DSS group and DSS/AAT group were used in calculating the protein expression and the values were displayed using a graph prism to assess their significance, with * indicating significance and $p \leq 0.05$, ($P=0.04$)

ARG1, INOS, ROS, and *PD-L1* in DSCs induced by CRC tumors [21]. The presence of cells in colon tissue of normal mice stained for *PD-L1* is in agreement with

others observations that showed the expression of *PD-L1* in 6.3% of normal colon tissue [19].

The low expression of *PD-L1* in tissue from AOM/DSS

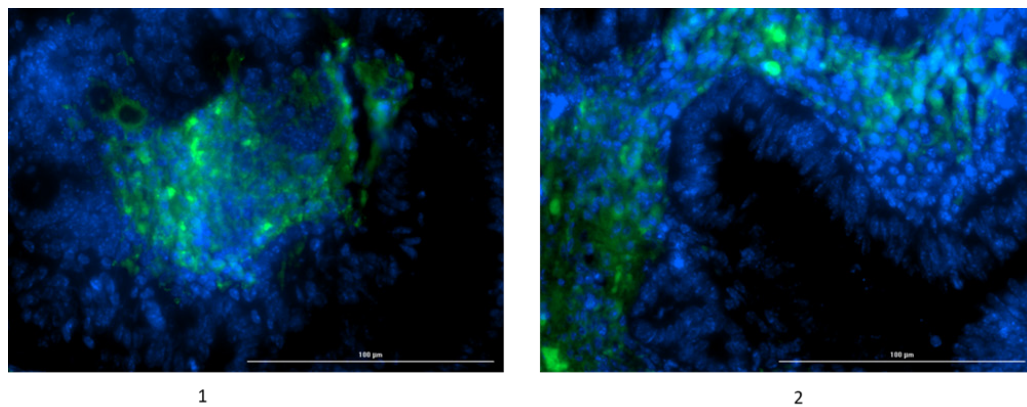


Figure 6. Immunofluorescence Staining of Colon Tumors from AOM/DSS-AAT Treated Mouse. Tissue was incubated with anti-mouse CD4 FITC conjugated (1) and anti-mouse CD8 FITC conjugated. Green fluorescent color is merged with DAPI stained cells (blue). 400X magnification.

as well as AOM/DSS-AAT treated mice indicates that the tumor microenvironment is not rich in immune cells that express *PD-1*. This molecule is expressed predominantly by T cells, B cells, dendritic cells (DCs), natural killer T (NKT) cells, monocytes and activated macrophages [1]. In an attempt to elucidate the immune cell distribution in the tissues, immunofluorescence conjugated anti CD4, CD8 and CD25 were utilized. A low cell count was observed in the tissue of mouse treated with AAT which needs further investigation and comparison to untreated groups.

To understand the association between AAT and (*PD-1*)/*PD-L1* expression, the study of chronic obstructive pulmonary disease (COPD) which is linked to a deficiency of alpha-1 antitrypsin (A1AT), was found to be associated with overactive innate immune responses, an elevation in the numbers of T regulatory cells (Tregs), and myeloid-derived suppressive cells (MDSCs). This type of cell inhibits the host's response to pathogens, making the host vulnerable to recurrences of infections. Moreover, researchers showed a reduction in the expression of *PD-L1* in alveolar macrophages, Th1 polarization, and stimulation of CD8⁺ T cells to IFN-secretion, which facilitate cell-mediated tissue destruction in patients with emphysema [22]. Additionally, the tumor cells can also interact with their microenvironment, thereby establishing an immunosuppressive milieu that contains inhibitory molecules and suppressive mediators, such as Tregs and MDSCs, which enhance the immune system's suppression of tumor-specific T cells. This may explain the reduction of both *PD-1* and *PD-L1* in AAT injected mice compared to their littermate control in our study.

The significant reduction of *EGFR* expression observed in the DSS/AAT group compared to the DSS group shows that AAT treatment can regulate the *EGFR* signal pathway, and therefore mitigate its pro-inflammatory and tissue-injurious effects in colitis. This is in accordance with previous work that has pointed to *EGFR*'s involvement in inflammatory bowel disease (IBD) pathogenesis [23, 24]. Decreased expression of *EGFR* after treatment with AAT may indicate therapeutic effect since *EGFR* inhibition has been shown to reduce inflammation and improve healing in experimental colitis [25, 26]. These findings confirm the hypothesis that the anti-inflammatory effect of AAT can

in part be mediated through its effect on *EGFR* signaling. Further research would uncover the mechanisms of this effect and delineate the therapeutic potential of AAT in targeting *EGFR* in IBD. Research has shown that *EGFR* signaling can regulate *PD-L1* expression in cancer cells. Activation of *EGFR* can induce *PD-L1* expression through various signaling pathways, including the PI3K/AKT pathway [27]. This interplay between *EGFR* and *PD-L1* suggests that targeting both pathways may be an effective therapeutic strategy.

Whether AAT can be used as immunotherapy for cancer either alone or in conjunction with immune checkpoints inhibitors (ICI) is still to be evaluated. ICIs approved by the FDA include three *PD-1* inhibitors (pembrolizumab, nivolumab, cemiplimab, and dostarlimab), and three *PD-L1* inhibitors (avelumab, durvalumab, and atezolizumab) [28-30]. As part of the cancer therapeutic approach, the PD1/PD1-L blockade has been reported to be effective in treating melanoma, head, and neck cancer, metastatic urothelial cancer, and colorectal cancer [31]. Thus the current study recommends the use of combinational therapy for CRC mouse model to elucidate a possible synergistic activity that may also decrease the side effects of antibody therapeutics. Furthermore, studies on a combinational therapy with AAT and ICIs are recommended.

Author Contribution Statement

All authors contributed to the study conception and design. M.A, A.O, T.A & R.M performed data collection and analysis. N.A read the Immunohistochemistry slides. K.A & M.A drafted the manuscript. All authors read and approved the final manuscript

Acknowledgements

Funding statement

This work was supported by the Deanship of Scientific Research and Graduate Studies at Yarmouk University (Grant No. 2023/104)

Availability of data

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

Ethical Consideration

This study was conducted in accordance with the guidelines established by the Institutional Animal Ethics Committee (IAEC) at Yarmouk University (approval number ACU-2021/11). All animal procedures were performed in compliance with relevant regulations and standards

Conflict of interest

We declare no conflicts of interest

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