Original Paper

The Assessment of T-Lymphocytes In Gastric Adenocarcinomas

ROXANA-ANDREEA PISICĂ-ILINCA[1], LILIANA STANCA[2], MARIAN VALENTIN ZORILĂ[2], ALEX EMILIAN STEPAN[3]

[1]PhD Student, Department of Pathology, University of Medicine and Pharmacy of Craiova, Romania
[2]Department of Forensic Medicine, University of Medicine and Pharmacy of Craiova, Romania
[3]Department of Pathology, University of Medicine and Pharmacy of Craiova, Romania

ABSTRACT: Gastric adenocarcinomas (GAC) are common cancers in pathology, which pose real problems in practice due to heterogeneity, response to treatment and prognosis. New therapeutic approaches in GAC are aimed at activating the immune system by unblocking or modulating it. In this study, we analyzed the density and distribution of intra- and peritumoral CD4 and CD8 lymphocytes, by immunohistochemistry, in relation to the main prognostic parameters of GAC. CD4 and CD8 lymphocytes were superior in the tumor compartments in the case of discohesive and mixed GAC. They also predominated in discohesive GAC with signet ring cells at the intratumoral level and in those without other specifications at the peritumoral level. CD4 and CD8 were superior in high-grade GAC, in stages II/III and with lymphovascular invasion, more frequently peritumoral. The study indicated differences in the distribution of CD4 and CD8 T lymphocytes that may be useful for improving patient stratification for personalized targeted therapy of GAC.

KEYWORDS: *Gastric adenocarcinoma, lymphocytes, CD4, CD8.*

Introduction

In the current oncological context, gastric adenocarcinoma (GAC) remains a major health challenge, being placed in the top five malignant tumors in terms of global incidence.

Although in recent decades early diagnosis and therapy protocols are superior, tumor heterogeneity and late detection remain major factors that maintain the constant 25-30% survival rate at 5 years [1-3].

This reality in terms of epidemiological data is supported by the biological aggressiveness of GAC, which requires a therapy not only specific to gastric cancers but also personalized, adapted to the immune and genetic status of patients [3,4].

Thus, in the last decade, molecular mechanisms of interference with growth factor receptors (Her-2neu), cell permeability regulators (claudin 18), blocking immune checkpoints that stimulate the immune system, as well as ways to capitalize on the host's defensive potential, such as tumor-infiltrating lymphocyte transfer therapies or the creation of specific oncological sera have been targeted [5-7].

Within the cell populations involved in immunity, αβ T cells (CD4, CD8 and nonCD4/CD8) play a central adaptive role and their presence is associated with numerous benefits related to treatment response and survival [7].

The initial antitumor surveillance action exerted by CD4 T lymphocytes is subsequently complemented by CD8 T lymphocytes that limit tumor growth [7-9].

However, a process of cancer immunoediting occurs, through which cancer cells will select immunophenotypes with low immunogenicity that allow them to survive and later they may develop other mechanisms by which active and effective lymphocytes are exhausted (formation of PD1-PD-L1 complexes, tumor microenvironment interaction) [7,10].

However, the role of the GAC inflammatory infiltrate is dual and complex, in the sense that immune elements are involved not only in combating malignant cells, but can also facilitate their progression by releasing lymphokines, monokines, growth factors and enzymes that stimulate protumor mechanisms [11,12].

Currently, the literature provides contradictory data regarding the prognostic value of inflammatory density and distribution in different tumor compartments [13,14].

While the presence of regulatory T lymphocytes or dendritic cells is sometimes associated with a superior therapeutic response, other studies suggest that certain follicular distributions of macrophages and the presence of neutrophils could indicate increased aggressiveness [12].

This ambivalence emphasizes the need to investigate immune cell populations in GAC, which can transform the tumor

10.12865/CHSJ.52.02.08

microenvironment into an indicator for disease progression and optimization of therapeutic strategies.

Objective

In this study, we followed the immunoexpression of CD4 and CD8 T lymphocytes in gastric adenocarcinoma compartments in relation to the main histopathological prognostic parameters of the lesions, which may have a positive impact on the efficacy of antitumor therapy.

Methods

The study analyzed 54 gastric adenocarcinomas (GAC), which were operated and diagnosed in the Surgical and Pathological Departments Emergency County Clinical Hospital of Craiova, over a five-year period (2019-2023).

The tumors were radically operated on, and the gastrectomy specimens were fixed in 10% neutral buffered formalin, subsequently processed by paraffin embedding and staining with Hematoxylin-Eosin. The macroscopic processing and microscopic classifictaion of GAC was performed taking into account the latest indications revised by the World Health Organization working group for malignant gastric tumors [15].

To constitute the group of cases for investigation in this study, to ensure homogeneity and exclude changes in the local tissue lymphocyte population, only cases with primitive GAC were included, which did not present immunomodulatory treatments, including oncological or chronic or acute diseases that would alter the local immune status in the last year before surgery.

Selected GACs were analyzed histopathologically, establishing the distribution in relation to tumor type, tumor grade and stage and the presence or absence of lymphovascular invasion. For immunohistochemical analysis (IHC), the semiquantitative intratumoral and peritumoral compartmental expression and CD4 and CD8 lymphocytes in relation to the listed histological parameters were analyzed. The intratumoral compartment was designated as consisting of tumor areas adjacent to other tumor areas on at least one 200x microscopic field (Nikon Eclipse Ei-R microscope), and the peritumoral compartment was formed by the areas that are marginal to the tumor on at most one 200x microscopic field.

As such, serial sections of 3-4µm were made from the paraffin blocks, which were pretreated for incubation with primary and secondary antibodies. The process consisted of: deparaffinization in successive xylene baths, rehydration with alcoholic solutions of decreasing concentration, antigen retrieval in Tris-EDTA (ethylenediamine tetra-acetic acid) solution pH 9 by boiling in the microwave for 20 minutes, blocking of endogenous peroxidase with H2O2 (hydrogen peroxide) at a concentration of 3% in PBS (phosphate-buffered saline), non-specific blocking with BSA (bovine serum albumin) at a concentration of 2% in PBS.

Subsequently, the sections were incubated with primary antibodies overnight at 4ºC, which were represented by monoclonal mouse antihuman CD4 (1/100 dilution, clone 4B12, Dako, Agilent) and antihuman CD8 (1/50 dilution, clone 1A5, Dako, Agilent).

The secondary antibody was a component of the EnVision™ FLEX+ System (Dako, Agilent), and the reactions were visualized with brown chromogen represented by 3,3′-diaminobenzidine (DAB) tetrahydrochloride (Dako, Agilent). After antigen retrieval, antibody incubation and visualization, successive washes with PBS were performed.

Counterstaining with Hematoxylin was followed by a dehydration and permanent mounting protocol. The reactions were validated by using external positive controls represented by tonsils.

For IHC quantification, the positivity index (PI) was established, represented by the number of labeled cells per 200x microscopic field (MF), with the average established for five MF with maximum reactions for each case, at the level of the intratumoral and peritumoral compartments. For counting the labeled inflammatory elements, normal submucosal follicular structures were avoided, as well as areas with necrosis or associated acute inflammation. The counting was performed on microscopes equipped with image acquisition cameras and KoPa Pro software (Eclipse Ei-R), by two pathologists who, in case of discrepancy, repeated the evaluation until consensus was reached.

The statistical investigation used means, standard deviations, medians and comparative tests (Student's test, one-way analysis of variance-ANOVA test, Pearson test) within SPSS12 (Statistical Package for the Social Sciences).

In this study, in addition to the effective mean and standard deviation of PI used for statistics, the median was also used for descriptive analysis, due to the relatively large variations in the number of marked elements between and within groups. In the case of comparison tests, p values <0.05 and <0.1 were considered significant or at the limit of statistical significance.

Results

In this study, most GACs were identified in men (68.5%), with a wide age range of 38-81 years, most between 60-79 years (74.1%), with a mean of 66.3±9.8 years, and maximum tumor dimensions most often up to 10cm (89.1%). Tubular GAC (TGAC) was present in 44.4%, followed by mixed GAC (MX-GAC) with 16.7%, discohesive signet ring cell GAC (PC-SRC GAC) with 14.8%, mucinous GAC (MC-GAC) with 13% and discohesive GAC not otherwise specified (PC-NOS GAC) with 11.1% of cases.

High-grade GAC (55.6%), stage III (50%) and cases with lymphovascular invasion (64.8%) predominated.

CD4 and CD8 reactions were present in all cases at the level of the membrane and apical cytoplasm of lymphocytes. Considering the entire GAC group analyzed, the mean PI values were higher in the peritumoral compartment compared to the intratumoral compartment, both for CD4 (22.6±19.2 versus 20.1±19.3) and for CD8 (59±47.9 versus 48.9±46.4). The medians of CD4 at the intra- and peritumoral level were 15, and in the case of CD8 30 and 45, so that the differences in the distribution of T lymphocytes were statistically insignificant for both CD4 (p=0.509, Student's t-test) and CD8 (p=0.268, Student's t-test).

CD4 immunoexpression showed differences in relation to the type of GAC. At the intratumoral level, the highest PI values were found in PC-SRC GAC, with a median value of 57.5, followed by PC-NOS GAC, MX-GAC, TGAC and MC-GAC, with median values of 27.5, 20, 10 and 4 (Figure 1A-C).

At the peritumoral level, the highest PI values were present in PC-NOS GAC and MX-GAC, with median values of 47.5 and 40, followed by TGAC, PC-SRC GAC and MC-GAC, with values of 15, 12.5 and 5 (Table 1, Figure 1D-F).

The differences in PI CD4 regarding the GAC type were statistically significant both at the intratumoral level (p<0.001, ANOVA test) and peritumoral level (p<0.001, ANOVA test) (Table 1, Figure1G-H).

Except for PC-NOS GAC, in all other tumor types PI CD4 was significantly higher (TGAC, MX-GAC) or at the borderline of significance (MC-GAC) at the peritumoral level, or at the intratumoral level (PC-SRC GAC) (Table 1).

Table 1. Distribution of CD4 T lymphocytes according to the analyzed parameters.

Parameters/ p value		CD4 IT (PI/ median)	CD4 PT (PI/ median)	p value (Student's t-test)
Histopathological types	TGAC	10.4±4.4/10	15.6±6.9/15	0.003
	PC-NOS GAC	28.3±13.6/27.5	43.3±15.3/47.5	0.104
	PC-SRC GAC	53.1±21.3/57.5	13.1±7/12.5	<0.001
	MX-GAC	24.4±14.4/20	48.8±23/40	0.017
	MC-GAC	3.2±1.6/4	6±3.4/5	0.091
	p value (Anova test)	<0.001	<0.001	
Tumor grades	Low grade	13.8±12/10	22.7±22.7/15	0.097
	High grade	25.2±22.5/15	22.5±16.2/17.5	0.596
	p value (Student's test)	0.03	0.956	
Tumor stages	I	11±6.5/10	17±10.3/15	0.310
	II	20.5±19.8/12.5	27.1±26.3/17.5	0.429
	III	22.8±20.3/15	23.9±16.2/20	0.825
	IV	14.5±20.5/7	9.1±6.9/6.5	0.568
	p value (Anova test)	0.550	0.223	
Lymphovascular invasion	Present	21.3±20/15	22.4±18/15	0.797
	Absent	18±18.3/10	22.8±21.6/15	0.457
	p value (Student's test)	0.552	0.941	

IT: intratumoral; PT: peritumoral; PI: positivity index; TGAC: tubular gastric adenocarcinoma; PC-NOS GAC: poorly cohesive- no other specifications gastric adenocarcinoma; PC-SRC GAC: poorly cohesive- signet ring cells gastric adenocarcinoma; MX-GAC: mixed gastric adenocarcinoma; MC-GAC: mucinous gastric adenocarcinoma.

10.12865/CHSJ.52.02.08

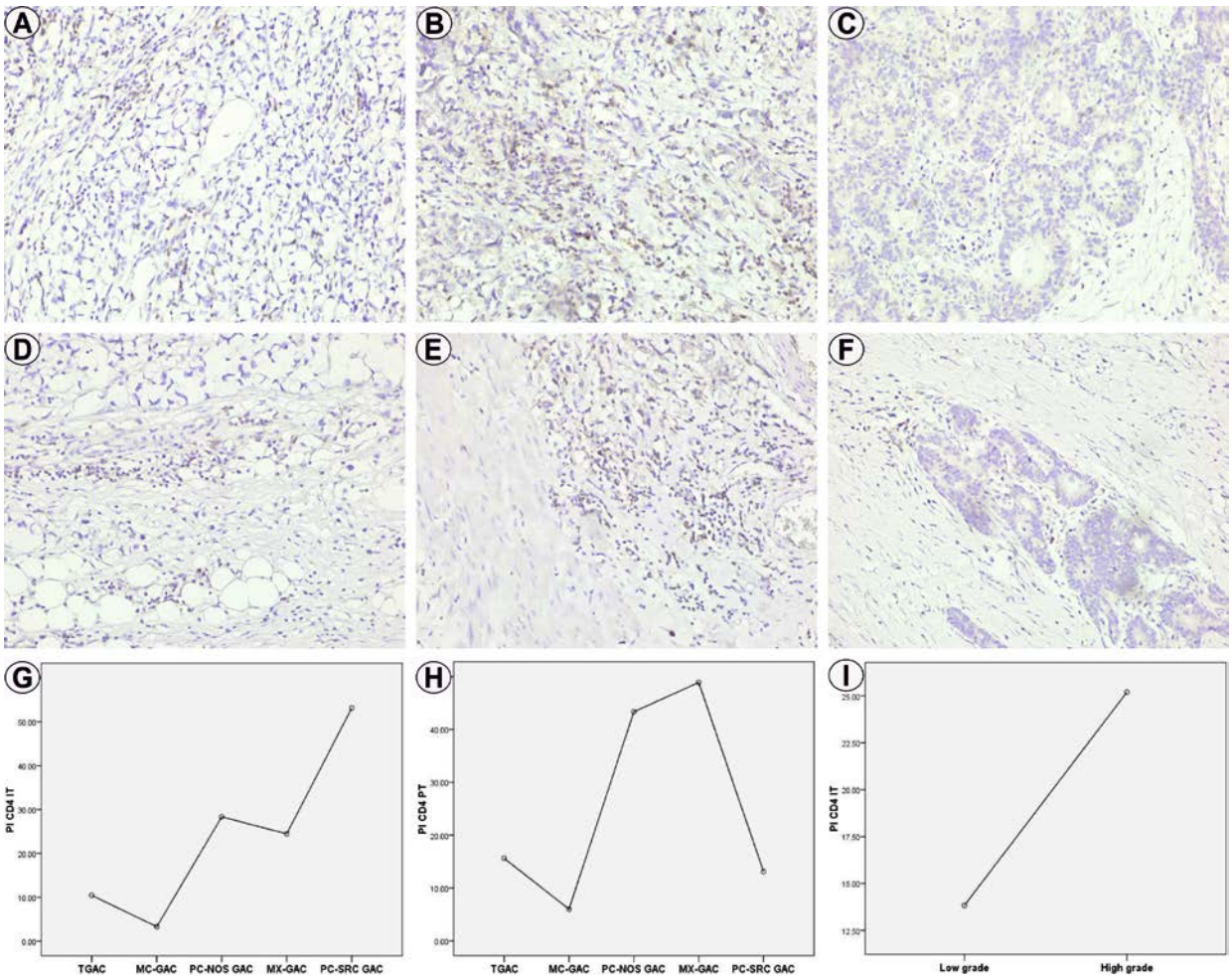

Figure 1. Gastric adenocarcinoma (GAC), CD4 immunostaining, x200.
A. PC-SRC GAC, intratumoral; B. PC-NOS GAC, intratumoral; C. TGAC, intratumoral;
D. PC-SRC GAC, peritumoral; E. PC-NOS GAC, peritumoral; F. TGAC, peritumoral;
G. CD4 positivity index (PI) depending on tumor type, intratumoral; H. CD4 positivity index (PI) depending on tumor type, peritumoral; I. CD4 positivity index (PI) depending on tumor grade, intratumoral.

Regarding the degree of differentiation, the number of labeled T lymphocytes was higher in both the intratumoral and peritumoral compartments in high-grade GAC, with median values of 15/17.5 in the two compartments for high-grade GAC, compared to 10/15 in low-grade tumors, with significant differences only at the intratumoral level (p=0.03, ANOVA test) (Table 1, Figure 1I).

The differences were at the limit of statistical significance in favor of the peritumoral compartment only in the case of low-grade GAC (Table 1).

In relation to tumor stage, the highest intra- and peritumoral PI CD4 values were in stage II with medians of 12.5 and 17.5 and stage III with medians of 15 and 20, without significant differences with the other tumor stages (p>0.1, ANOVA test) and in relation to tumor compartments (p>0.1, Student's t-test) (Table 1).

Regarding lymphovascular invasion, a slight predominance of CD4 T lymphocytes was observed in the intratumoral compartment in the case of vascular-invasive tumors, with median values of 15, compared to 10, in the case of tumors that did not present this type of invasion (p>0.1, ANOVA test) (Table 1).

Peritumorally, no differences were reported according to this parameter, the median values being equal to 15, regardless of the presence or absence of invasion (p>0.1, ANOVA test) (Table 1).

There were no significant differences in PI CD4 in the tumor compartments according to lymphovascular invasion (p>0.1, Student’s t-test) (Table 1).

Table 2. Distribution of CD8 T lymphocytes according to the analyzed parameters.

Parameters/ p value		CD8 IT (PI/ median)	CD8 PT (PI/ median)	p value (Student's t-test)
Histopathological types	TGAC	32.5±18.9/27.5	42.7±25.1/35	0.119
	PC-NOS GAC	113.3±31.4/105	146.6±71.1/140	0.329
	PC-SRC GAC	117.5±34.1/100	63.1±35.7/60	0.007
	MX-GAC	25±22.7/15	68.8±34.4/50	0.006
	MC-GAC	2.7±1.8/2	22.8±14.1/20	0.009
	p value (Anova test)	<0.001	<0.001	
Tumor grades	Low grade	24.2±10.6/25	36.2±12.5/35	<0.001
	High grade	68.7±54.3/75	77.3±57.5/60	0.554
	p value (Student's test)	<0.001	0.001	
Tumor stages	I	20.8±11.7/25	30±13.6/30	0.288
	II	39.6±33.5/30	52.1±31.7/47.5	0.288
	III	62.7±54.2/40	72.2±59.7/50	0.542
	IV	35.3±42.5/17.5	42.5±20.4/35	0.720
	p value (Anova test)	0.147	0.179	
Lymphovascular invasion	Present	51.6±45/30	61.2±43.9/50	0.370
	Absent	43.9±49.9/25	55±55.7/35	0.523
	p value (Student's test)	0.564	0.650	

IT: intratumoral; PT: peritumoral; PI: positivity index; TGAC: tubular gastric adenocarcinoma; PC-NOS GAC: poorly cohesive-no other specifications gastric adenocarcinoma; PC-SRC GAC: poorly cohesive-signet ring cells gastric adenocarcinoma; MX-GAC: mixed gastric adenocarcinoma; MC-GAC: mucinous gastric adenocarcinom

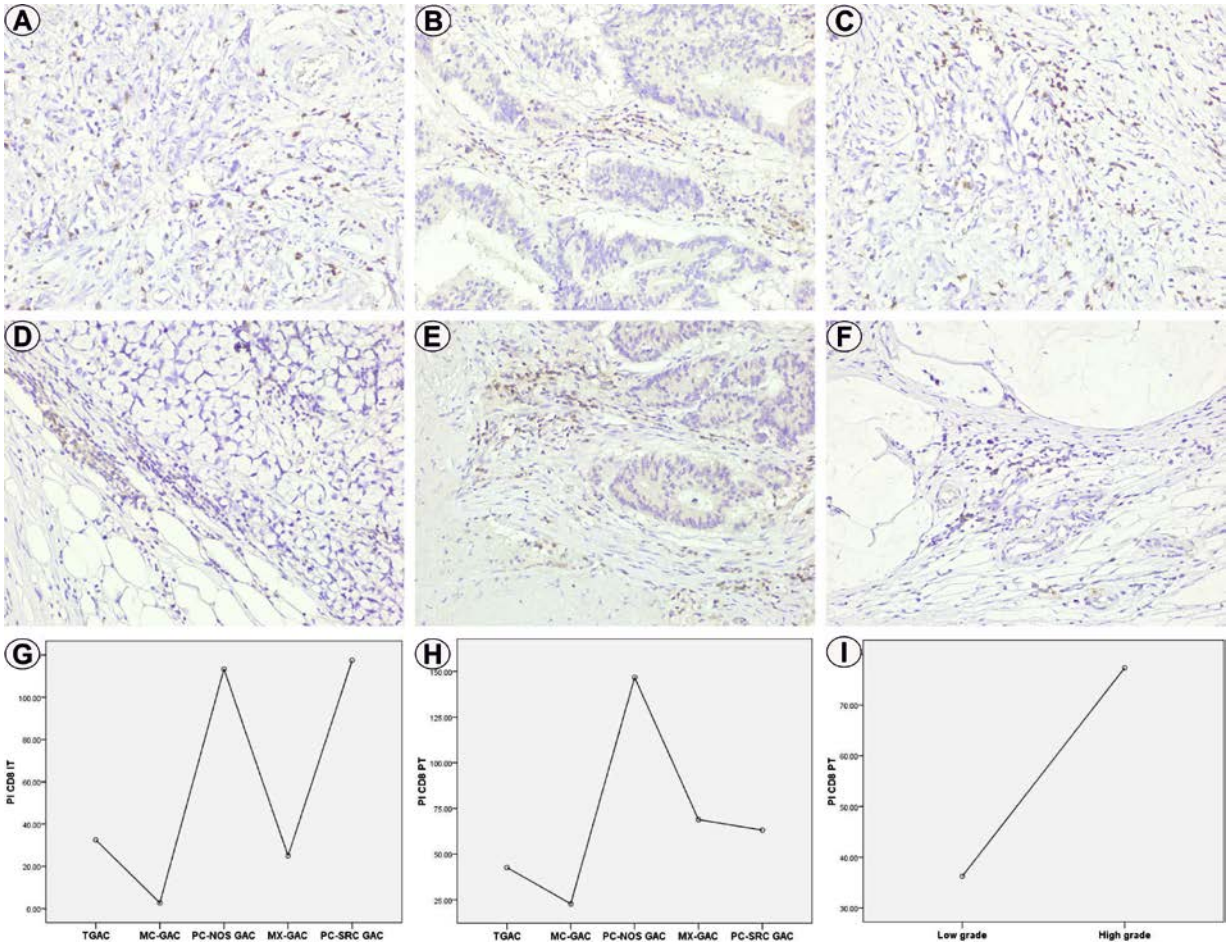

Figure 2. Gastric adenocarcinoma (GAC), CD8 immunostaining, x200.
A. PC-NOS GAC, intratumoral; B. TGAC, intratumoral; C. PC-NOS GAC, peritumoral; D. PC-SRC GAC, peritumoral; E. TGAC, peritumoral; F. MC-GAC, peritumoral; G. CD8 positivity index (PI) depending on tumor type, intratumoral; H. CD8 positivity index (PI) depending on tumor type, peritumoral; I. CD8 positivity index (PI) depending on tumor grade, peritumoral.

10.12865/CHSJ.52.02.08

For CD8, higher PI values were observed in the intratumoral compartment in the case of discohesive GAC, with median values for PC-NOS GAC and PC-SRC GAC of 105 and 100, compared to values of 17.5 in TGAC, 15 in MX-GAC and 2 in MC-GAC (Table 2, Figure 2A-B).

The same distribution was also reported in the peritumoral compartment, where a superiority was observed in the case of PC-NOS GAC, with a median value of 140, compared to PC-SRC GAC with a median of 60, followed by MX-GAC TGAC and MC-GAC with medians of 50, 35 and 20 (Table 2, Figure C-F).

PI CD8 differences regarding GAC type were statistically significant both at intratumoral ($p<0.001$, ANOVA test) and peritumoral ($p<0.001$, ANOVA test) levels (Table 2, Figure 2G-H).

Except for PC-SRC GAC, where CD8 T lymphocytes predominated in the intratumoral compartment, in all other types a peritumoral superiority was observed, with statistically significant values in the case of PC-SRC GAC, MX-GAC and MC-GAC (Table 2).

Regarding tumor grade, a predominance of CD8 expression was observed in high-grade GAC in both compartments, with median values of 75 and 60 intratumoral/peritumoral, compared to values of 25 and 35 in low-grade tumors (Table 2, Figure 2I).

The differences were significant between high- and low-grade GAC both intratumoral ($p<0.001$, ANOVA test) and peritumoral ($p=0.001$, ANOVA test) (Table 2).

PI CD8 was superior peritumorally in both high- and low-grade GAC, but statistically significant only in the case of low-grade (Table 2).

In relation to tumor stage, both in the intratumoral and peritumoral compartments, an increase in the number of labeled lymphocytes was observed with advancing stage, reaching a maximum value in stage III with medians of 40 and 50, while in stage IV the medians reached values of 17.5 and 35 ($p>0.1$, ANOVA test) (Table 2).

Also, regardless of stage, the PI and median values were higher in the peritumoral area, but without statistical significance ($p>0.1$, Student's t-test) (Table 2).

In vascular-invasive tumors, CD8+T lymphocytes were present in higher quantities both intratumorally and peritumorally, with median values of 30 and 50, compared to 25 and 35 in tumors without lymphovascular invasion ($p>0.1$, ANOVA test) (Table 2).

Their number was also higher in the peritumoral compartment, regardless of the presence/absence of this type of invasion ($p>0.1$, Student's t-test) (Table 2).

Analysis of the CD4/CD8 ratio in this study indicated the predominance of CD8 in both tumor compartments of GAC.

Analysis of the PI CD4 and CD8 values indicated a significant positive linear correlation both at the intratumoral level ($p<0.001$, Pearson test) and at the peritumoral level ($p=0.035$, Pearson test).

Discussion

While CD8 lymphocytes control tumor growth through the direct effect of cytotoxicity, CD4 lymphocytes (Th1 and Th2 helper) have complex roles, are cytokine-dependent and cytokine-secreting directly or inducing and are involved in long-term immune defense [7,16].

However, the role of CD4 T lymphocytes is much more complex, being able to differentiate towards cell lineages such as TH17 with antitumor effects in some cancers (head and neck, ovarian, esophageal) or protumor in others (breast), most likely depending on the type of cancer and the immune status of the host [7,17,18].

CD4 is a glycoprotein belonging to the immunoglobulin family, expressed on the surface of T helper cells [19].

CD4 T cells have an essential role in the coordination of adaptive immunity, through the ability to differentiate into various functional subtypes, in response to received signals, each subtype having a different role, determining the production of specific cytokines and transcription factors [20].

The data in the literature related to the distribution and density of CD4 T lymphocytes in carcinomas in general and in gastric carcinomas are heterogeneous, and compartmental investigation is rarely addressed [21].

In our study, CD4 responses showed differences in histological parameters and tumor compartments. Thus, PI CD4 was higher in PC-SRC GAC at intratumoral level and in PC-NOS GAC and MX-GAC at intratumoral level, the three types having generally the highest values. High-grade GAC, in stages II/III and with lymphovascular invasion generally showed superior responses, and CD4 predominance was mainly peritumoral, with

exceptions, respectively in PC-SRC GAC and high-grade GAC.

CD4 T cells are an important component of the tumor microenvironment. Recent studies have shown that CD4 T cells may elicit an antitumor immune response superior to CD8 T cells [22,23], and even independently of them [23,24].

One study showed that, in colorectal cancer, activated CD4 T lymphocytes were found in significantly higher density intratumorally, compared to normal tissues [25].

They also predominated in stages T1-T2, compared to T3-4 [25].

In lung adenocarcinomas, they have been associated with hypometabolic activity and a favorable prognosis [26].

In gastric adenocarcinomas, their role is controversial, with studies supporting their association with more advanced stages [27] and tumor proliferation [28], while other studies show a correlation with a favorable prognosis [29,30].

A study has highlighted that tumor infiltration with CD4 T cells, respectively a low serum level of CD4 lymphocytes, may be a negative prognostic marker for patients with gastric adenocarcinomas [You Q, 2021].

CD8 is a cell surface glycoprotein, a member of the immunoglobulin family, expressed on normal and malignant T cells [31].

Cytotoxic CD8 T lymphocytes recognize MHC (Major Histocompatibility Complex) class I peptides expressed on the surface of tumor cells and, through various mechanisms (cytokines - interferon-γ, tumor necrosis factor and enzymes - perforin, granzyme B), inhibit tumor progression [32].

Studies conducted so far in carcinomas with different locations have led to heterogeneous conclusions regarding their distribution, degree of activation and prognostic significance.

In our study, PI CD8 were superior in PC-GAC at the intratumoral level and PC-GAC and MX-GAC at the peritumoral level; with the exception of PC-SRC GAC, the elements predominated peritumorally, being superior in high-grade GAC, in stage II/III and with lymphovascular invasion.

In colorectal and pancreatic carcinomas, increased CD8 T cell counts are a favorable prognostic factor, being associated with a better survival rate [33,35].

In gastric carcinomas, increased CD8 T cell counts appear to be negatively correlated with lymphovascular invasion and lymph node metastasis, with patients presenting in some studies a significantly better recurrence-free survival rate [36].

The lack of significant correlation between CD8 T cells and tumor size, tumor differentiation, tumor depth, or TNM stage is also highlighted [37].

Other authors have reported a correlation between high CD8 T cell density and tumor stages I-II, compared to more advanced stages, and in tumors with a high degree of differentiation, but without association with other parameters [38].

One study reported a significant association of increased CD8 T cell counts with small tumor size (<5cm) [39].

Another study in the literature confirms the lack of association of CD8 lymphocytes with the depth of tumor invasion or the presence of lymphatic invasion, but highlights a significant association of their high density with a lower frequency of lymph node metastases compared to groups with low density [12].

On the other hand, one study reported an association of high density of intratumoral/stromal CD8 T cells with a lower survival rate, compared to that of patients with low densities [40].

These data are confirmed by a study that highlighted the association of the tumor microenvironment rich in inactive CD8 T cells with an unfavorable prognosis, compared to the group of tumors that presented an average density of this cell type [41].

Also, a low density of CD8 T lymphocytes is associated with a reduced survival rate, the cause being, most likely, diminished antitumor activity due to the absence of tumor antigens [41].

Although previous studies have shown a predominance of CD8+ T cells compared to CD4+ T cells in patients with neuroblastoma [42], in gastric cancer it seems that CD4 lymphocytes are more frequent at the tumor level, resulting in an increased CD4/CD8 ratio, an aspect that may be relevant in immunotherapy [43].

However, the density of T lymphocytes is dependent on the tumor type and immune status, and in our study, CD8 lymphocytes predominated in both tumor compartments compared to CD4 lymphocytes, with which they had a positive linear correlation.

In this context, it should be mentioned that CD4/CD8 double-positive T lymphocyte populations with an uncertain role but

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susceptible to be cytotoxic for malignant tumors have also been described [44], which constituted a limitation of this study in terms of separate quantification.

Overall, it can be considered necessary and beneficial for the perspective of improving the prognosis of the oncological patient in general, to establish in future studies the complex integrated role of lymphocytes, macrophages and leukocytes in GAC.

Conclusions

CD4 and CD8 T lymphocytes predominated in discohesive and mixed gastric carcinomas, in high-grade and stage II/II tumors, with lymphovascular invasion, with differences between the two tumor compartments.

CD8 lymphocytes predominated and varied in the same direction as CD4 lymphocytes.

The results may suggest the existence of a complex immune response in GAC involved in tumor structural and functional modeling, but which must be interpreted in the context of the degree of lymphocyte activation and dual lymphocyte phenotypes, which may provide a personalized perspective of specific therapy.

Acknowledgement

None to declare.

Author Contributions

Conceptualization, A.E.S. and R.A.P-I.; Methodology, R.A.P-I., L.S. and M.V.Z; Investigation, R.A.P-I.; Data analysis, A.E.S, R.A.P-I. and L.S.; Manuscript writing and initial draft preparation, R.A.P-I.; Manuscript review and editing R.A.P-I., L.S. and M.V.Z.; Supervision, A.E.S.

All authors read and approved the final manuscript.

Funding

This research received no specific funding.

Conflicts of interest

The authors declare no competing interests.

Institutional Review Board

The study was conducted according to the guidelines of the Declaration of Helsinki; the study and the protocols utilized therein were approved by the Ethics Committee of University of Medicine and Pharmacy of Craiova (11/16.03.2020).

Consent Statement

Not applicable for a retrospective descriptive study.

Data availability

All data presented in the manuscript are available from the authors upon request.

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Corresponding Authors: Liliana Stanca, Department of Forensic Medicine, University of Medicine and Pharmacy of Craiova, Romania, e-mail: liliana.stanca@umfcv.ro;

Marian Valentin Zorilă, Department of Forensic Medicine, University of Medicine and Pharmacy of Craiova, Romania, e-mail: valentin.zorilă@umfcv.ro