Journal of Cell Identity

Rare subgroup of T Lymphocytes in Oral and Maxillofacial Cancer Patients

Mihnea Ioan Nicolescu[1,2], Octavian Marius Dincă [3,*], Lucian Toma Ciocan[4]

1 Division of Histology, Faculty of Dentistry, „Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania; mihnea.nicolescu@umfcd.ro
2 Laboratory of Radiobiology, „Victor Babeș" National Institute of Pathology, 050096 Bucharest, Romania
3 Division of Oral and Maxillo-Facial Surgery, Faculty of Dentistry, „Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania, octavian.dinca@umfcd.ro
4 Division of Prosthetics Technology, Faculty of Dentistry, „Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania; lucian.ciocan@umfcd.ro

Abstract

Malignant tumors of head and neck region represent a heterogenous group of pathologies. Immune phenotype of each tumor might represent valuable information for a putative personalized treatment in the future. We decided to revisit very interesting previous data from a study we conducted in 2003 where we encountered a rare population of CD4+CD8+ double-positive T cells in lymph nodes surgically removed in patients with head and neck malignancies. The study included 27 patients (22 males and 5 females) who underwent surgical procedures that associated lymphadenectomy during March-July 2003 in the Maxillo-Facial Surgery Clinic of „Dan Theodorescu" University Dental Hospital in Bucharest. We encountered a high percentage of CD4+CD8+ T lymphocytes in peripheral blood of non-Hodgkin Lymphoma and poorly differentiated carcinoma, as well as in lymph nodes of both Hodgkin and non-Hodgkin lymphomas. We would like to emphasize the importance of immune phenotyping, when possible, to discriminate between tumor subtypes, evidence that establishing a true identity of the cells involved might be useful for future studies and/or personalized therapeutic strategies.

Keywords:

T cells; oral cancer; flow cytometry

DOI:

https://www.doi.org/xxxxxxxxxxjocixxxxxxxxx

* Corresponding author:

Correspondence: octavian.dinca@umfcd.ro

Introduction

Malignant tumors of head and neck region represent a heterogenous group of various pathologies. Immune phenotype of each tumor might represent valuable in-formation for a putative personalized treatment in the future. Many of these cancers develop immune defects, and cells might evade immune surveillance due to accumulation of genetic mutations and tumor heterogeneity (Economopoulou et al., 2016). The immune system is a dual player, with roles in both pro- and anti- tumor activities depending on immune cell subsets proportion and disease context (Bohner et al., 2019). Mature T cells are generally considered to express either the CD4 or CD8 coreceptor, in addition to their TCR, and consequently, the T cell pool is commonly divided into two subsets, based on expression of either CD4 or CD8 (Overgaard et al., 2015).

Materials and methods

We have conducted a retrospective study on n=27 patients, who underwent surgical procedures that included lymphadenectomy during March-July 2003 in the Maxillo-Facial Surgery Clinic of „Prof. Dr. Dan Theodorescu" University Dental Hospital, Bucharest, Romania. The criteria for inclusion in the study were: histopathological confirmed malignant tumor, cervical adenopathy clinically present at admittance, tumor that allowed surgical removal. All patients expressed their informed consent. The protocol was part of a dissertation thesis in 2003, approved by request D10365/2003 by Faculty of Dentistry, „Carol Davila" University of Medicine and Pharmacy, Bucharest.

Peripheral blood was collected at that time in EDTA-K vacutainers and on 0.1 ml was added 0.02 ml antibody, followed by 1 min vortexing. Different-sized lymph nodes that were harvested during surgery were processed after classical cell separation (mechanical break-down followed by centrifugation and resuspension). The following BT Biosciences antibodies were used: anti-CD3/CD19, anti-CD4/CD8, anti-CD19/CD16+CD56, anti CD3/HLADR, anti-C25. The probes were incubated for 30 minutes at room temperature and dark. After incubation, each probe was added 2 ml of erythrocyte-lysis buffer. Incubation continued until evidence of erythrocytes lysis. Samples were washed two times in 2 ml PBS with 1% BSA. In the end, they were processed in 0.5 ml PBS with 1% para-formaldehyde. The reading and analyses of samples was done using a FACSCalibur Flow Cytometer (Becton-Dickinson) using CellQuest software. For in vitro blast transformation of lymphocytes, we used PHA protocol, as follows: venous blood harvested in IC65 vessels supplemented with Heparin 100 UI/ml. Lymphocytes were cultured with or without plasma (for the latter – the required blood quantity was supplemented with 10 parts IC65 medium centrifuged at 200g for 10 minutes and the sediment resuspended in a culture medium volume equal with the harvested blood). For culture, 5 parts blood ± plasma was mixed with 95 parts RPMI 1640 cell culture medium buffered with $NaHCO_3$ pH 7.2, enriched with 2mmol glutamine and 100 UI/ml penicillin. Each sample was worked in triplicate, each culture tube had 0.5 ml after the following protocol: (1) unstimulated, without plasma, but supplemented with 10% BFS, (2) stimulated with PHA, without plasma, but supplemented with 10% BFS, (3) unstimulated, with plasma, (4) stimulated, with PHA and plasma, (5) stimulated, with PHA and plasma, and medium conditioned with IL1, (6) stimulated, with PHA and plasma, and thymic extract. In the next stage, the specimens were incubated for 72 hours at 37oC with a humid atmosphere and 5% CO_2. With 24 hours before the end of culture time, 0.1 µCi 3H-Td is added per culture tube, to mark the lymphocytes which underwent blast transformation. At the end of incubation, to measure the isotope quantity that was incorporated, the culture specimens were filtered through a 0.8 µm Millipore membrane and washed with PBS and proteins fixed with 5% trichloroacetic acid solution. The rings fixed on plates were maintained for 24 hours at the thermostat for drying, after which each ring was introduced in a scintillation tube in which 10 ml scintillation fluid was added for beta emissions. The radioactivity was read at a Beckman machine, which gave information on the number of impulses emitted by the radioactive isotope incorporated in the newly synthetized DNA and the values were in number of impulses/minute or as stimulation indexes according to the formula: SI = (number of impulses/minute in stimulated in vitro cultures) / (number of impulses/minute in unstimulated *in vitro* cultures).

Results

The sex ratio of the group was 4.4 (22 male patients and 5 female). The average male age was 55 years, and the female average age was 65, resulting in a mean overall age of 57 years. We specifically chose a variety of histopathological confirmed malignancies to compare the percentage of different subsets of lymphocytes, to serve as a preliminary result for future more targeted studies. All the histopathological diagnostics are listed in **Table 1**. We encountered 20 carcinomas (74.07%) and 7 lymphomas (25.93%). Out of these, we decided to compare one out of each representative subgroup, hence we further analyzed four cases: one case of Hodgkin lymphoma, one non-Hodgkin lymphoma, one well differentiated keratinized spinous carcinoma as well as a poorly differentiated carcinoma (cases #1,#3,#9 and #26 from **Table 1**).

In cases of non-Hodgkin lymphoma, the immunophenotyping of peripheral blood showed a slight downshift of T (51%) and B (2%) lymphocytes, compared to normal reported values of 59-85%, and respectively 6-23%. The CD4 T lymphocytes also scored rather low: 24% compared to previously reported values of 29-57%, while CD8 T cells were significantly higher: 41% compared to 11-38% normal values. The low number of T lymphocytes, together with a subunit CD4:CD8 ratio of 0.6 is eloquent for immune suppression. NK cells in peripheral blood were also high: 39%, compared to 6-31% normal values. In these cases, we noticed an increased percentage of B lymphocytes, of about 48%. The lymph node CD4:CD8 T cell ratio was 0.83. Activated lymphocytes (CD3 HLADR) were 23% (while 2% in peripheral blood). Lymph node NK cells were 2%.

In confirmed Hodgkin lymphoma, CD3+ cells were 77% in peripheral blood, and 67% in lymph nodes. B lymphocytes were 4% in peripheral blood and 16% in lymph nodes. As concerns the T cells subsets, the following percentages were recorded: CD4 T cells were 39% and 62%, while CD8 T cells were 40% and 16% in peripheral blood and lymph nodes, respectively. That brought the CD4:CD8 ratio to 1, respectively 3.9. Activated CD3 HLADR lymphocytes were 4% in blood and 15% in lymph nodes. NK cells percentage was 10% in peripheral blood and 2% in lymph nodes.

Table 1
A synopsis of the malignancies histopathological characteristics for cases in the study group

*** Marked cases were further analyzed using flow cytometry.**

Case ID	Gender	Age	Histopathology
1*	M	56	Hodgkin Lymphoma
2	F	72	Unspecified type Lymphoma
3*	M	73	Non-Hodgkin Lymphoma
4	M	68	Non-Hodgkin Lymphoma
5	M	66	Non-Hodgkin Lymphoma
6	F	65	Non-Hodgkin Lymphoma
7	F	71	Non-Hodgkin Lymphoma
8	M	43	Adenosquamous Carcinoma
9*	M	63	Keratinized Spinous Carcinoma
10	M	53	Keratinized Spinous Carcinoma
11	M	49	Keratinized Spinous Carcinoma
12	M	51	Keratinized Spinous Carcinoma
13	M	57	Keratinized Spinous Carcinoma
14	M	43	Keratinized Spinous Carcinoma
15	M	51	Keratinized Spinous Carcinoma
16	M	54	Keratinized Spinous Carcinoma
17	M	57	Keratinized Spinous Carcinoma
18	M	80	Keratinized Spinous Carcinoma
19	M	70	Keratinized Spinous Carcinoma
20	F	53	Keratinized Spinous Carcinoma
21	M	65	Keratinized Spinous Carcinoma
22	M	52	Poorly Differentiated Carcinoma
23	M	49	Poorly Differentiated Carcinoma
24	M	52	Poorly Differentiated Carcinoma
25	M	17	Poorly Differentiated Carcinoma
26*	F	66	Poorly Differentiated Carcinoma
27	M	56	Unkeratinized Carcinoma

Table 2.
Blast transformation of lymphocytes.

Tumor type (gender, age, case ID)	Lymphocytes	Unstimulated	Stimulated 1% PHA	Stimulation index (SI)
Non-Hodgkinlymphoma (male, 73yrs, case#3)	Submandibular lymph node	3958	6221	1.57
	Jugulodigastric lymph node	4706	44656	9.48
	Washed blood + 10% BFS	253	13881	54.86
	Blood with plasma	401	16441	41
Hodgkin lymphoma (male, 56yrs, case#1)	Submandibular lymph node	3230	10895	3.37
	Washed blood + 10% BFS	208	3506	16.85
	Blood with plasma	408	5164	12.65
Keratinized spinous carcinoma (male, 63yrs, case#9)	Jugulodigastric lymph node	565	4153	7.35
	Washed blood + 10% BFS	157	6755	43.02
	Blood with plasma	169	1999	11.82
Poorly differentiated carcinoma (female, 66yrs, case#26)	Washed blood + 10% BFS	232	3960	17.06
	Blood with plasma	200	2969	14.84

In **Table 2** we present a comparison of the results of lymphocytes blast transformation in four patients with different histopathological diagnosis, in which Stimulation Index (SI) is defined as (number of impulses/minute in stimulated in vitro cultures) / (number of impulses/minute in unstimulated in vitro cultures).

Flow-cytometry results of peripheral blood and selected lymph nodes show CD4+CD8+ double positive T cells as well as activated T cells in non-Hodgkin lymphoma (**Figure 1**), Hodgkin lymphoma (**Figure 2**), keratinized spinous carcinoma (**Figure 3**) and poorly differentiated carcinoma (**Figure 4**).

Discussion

Firstly, we would like to underline the high SI value in non-Hodgkin lymphoma (54.86) compared with only 16.85 in Hodgkin lymphoma (Table 2). Although a wide interval of „normal" values have been reported in healthy individuals (Alsalamah et al.,2019), we have presented this result in order to point out the lack of any default correlation with the presence of a higher percentage of CD4+CD8+ T lymphocytes.

There have been reports on the presence of double positive CD4+CD8+ T lymphocytes in a variety of species and tissues (Overgaard et al., 2017). Expression of both these receptors by T cells is encountered during T lymphocytes maturation in the thymus. After the negative selection process, T cells commit to either the T helper route (CD4+) or to cytotoxic lineage (CD8+). Some authors advanced the hypothesis of upregulation of one of the coreceptors by mature T lymphocytes (Overgaard et al., 2015). This can be achieved by either expressing CD8 on CD4+ cells (Zuckermann et al., 1999) or the other way around, by expressing CD4 on CD8+ cells (Sullivan et al., 2001).

Figure 1.
Flow cytometry. Non-Hodgkin lymphoma (case ID #3). (A) Peripheral blood; (B) and (C) Lymph nodes. Left panels: CD4:CD8 cells; Right panels: activated lymphocytes (CD3:HLADR).

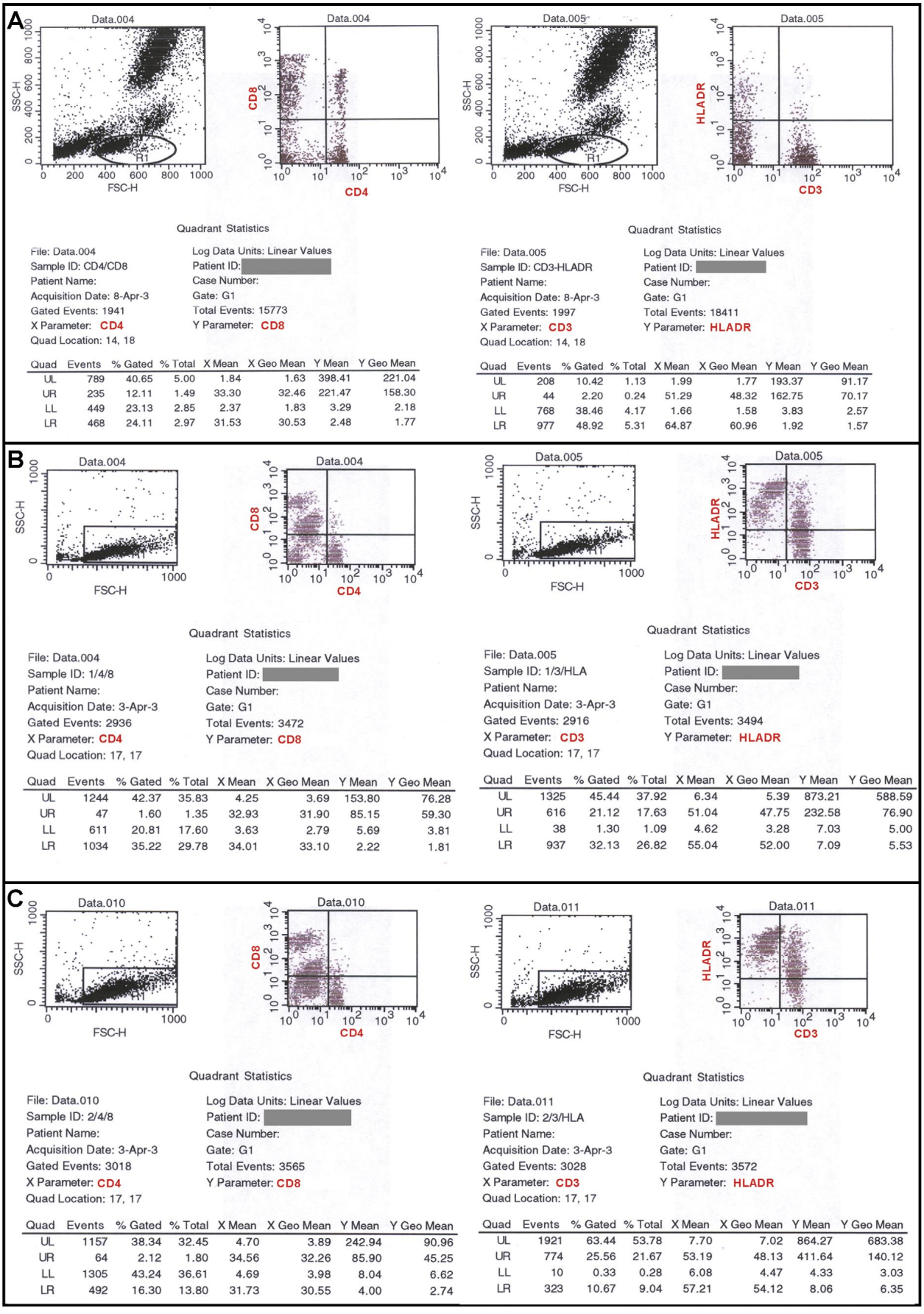
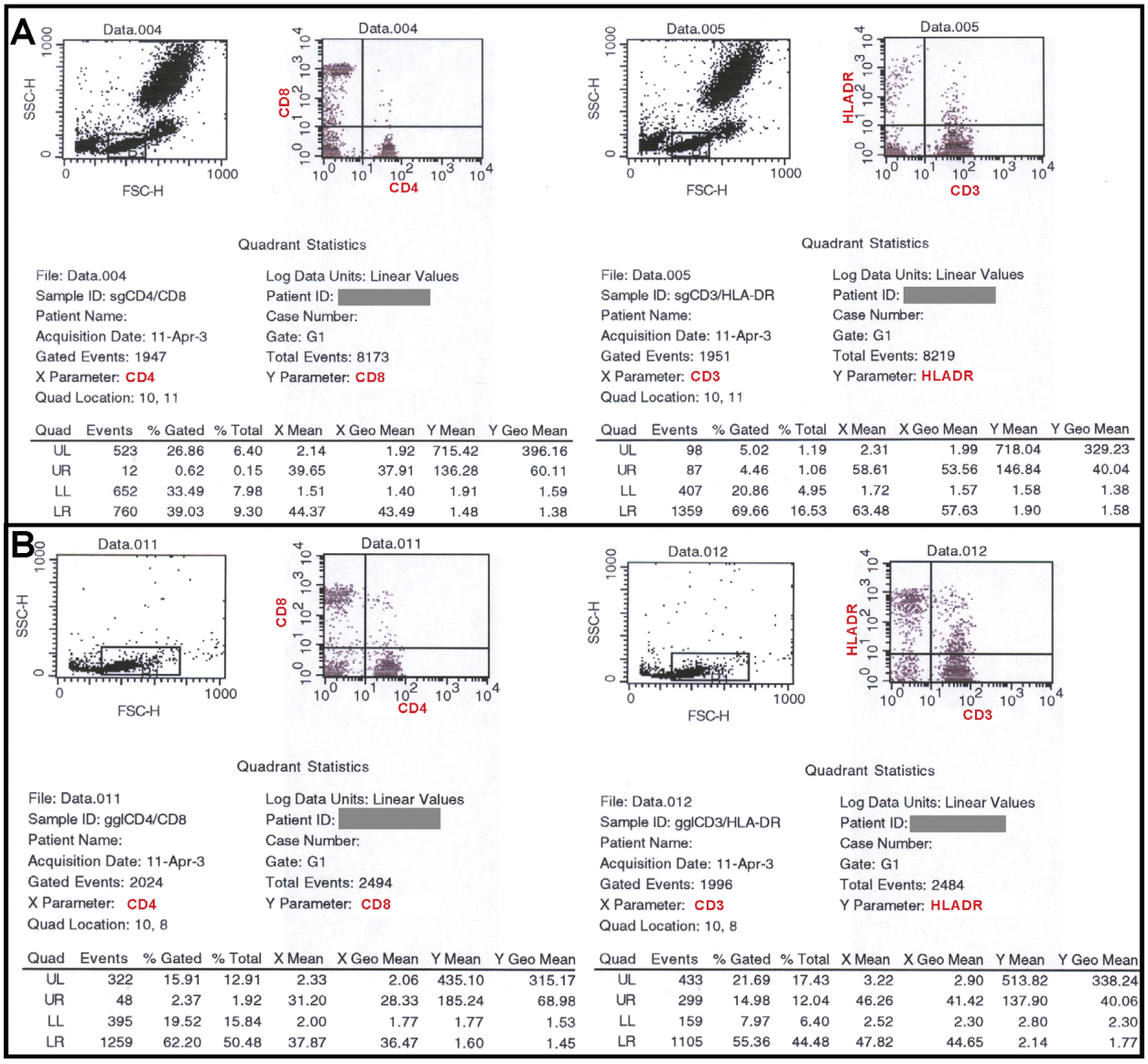


Figure 2.
Flow cytometry. Hodgkin lymphoma (case ID#1). (A) Peripheral blood; (B) Lymph node. Left panels: CD4:CD8 cells; Right panels: activated lymphocytes (CD3:HLADR).

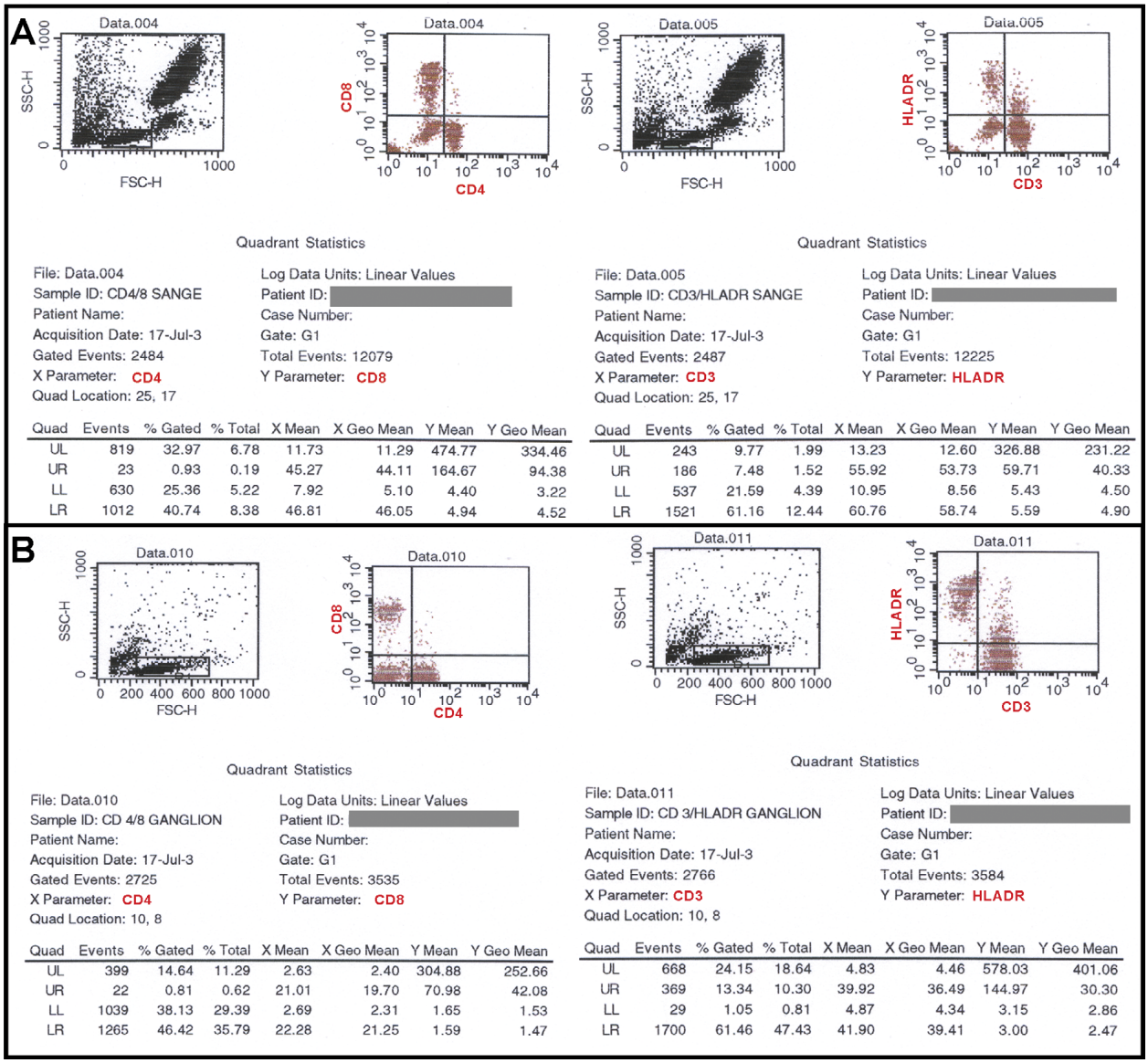


Other authors consider the presence of these double positive CD4+CD8+ T lymphocytes as an indication of a reactive subset that might prove useful in diagnosing cases with scarce or limited tissue availability (Rahemtullah et al., 2006). CD4+CD8+ T cells might exist as a small population in the peripheral blood of healthy people. Nevertheless, they have been reported to be increased in patients with past or present viral infections, in whom these cells may exhibit antiviral functions (Nascimbeni et al., 2004).

We report here a significant percentage of CD4+CD8+ double positive T lymphocytes in the peripheral blood of non-Hodgkin Lymphoma (12,11% in Figure 1, A) and poorly differentiated carcinoma (7,33% in Figure 4), compared to values under 1% in Hodgkin Lymphoma (0,62% in Figure 2, A) and keratinized spinous carcinoma (0,93% in Figure 3, A). They might represent a cell population with regulatory functions, as previously suggested (Das et al., 2003; Nascimbeni et al., 2004). The presence of this double positive T cells has been acknowledged in non-Hodgkin Lymphoma Rahemtullah et al., 2006) and melanomas (Desfrançois et al., 2010), but is not so common in poorly differentiated carcinomas.

The presence of CD4+CD8+ T cells in intestinal mucosa has been shown to suppress intestinal inflammation in a regulatory fashion via IL-10 route (Das et al., 2003). In urological cancers, the same cells also act using an immu-

Figure 3.
Flow cytometry. Keratinized spinous carcinoma (case ID#9). (A) Peripheral blood; (B) Lymph node. Left panels: CD4:CD8 cells; Right panels: activated lymphocytes (CD3:HLADR).

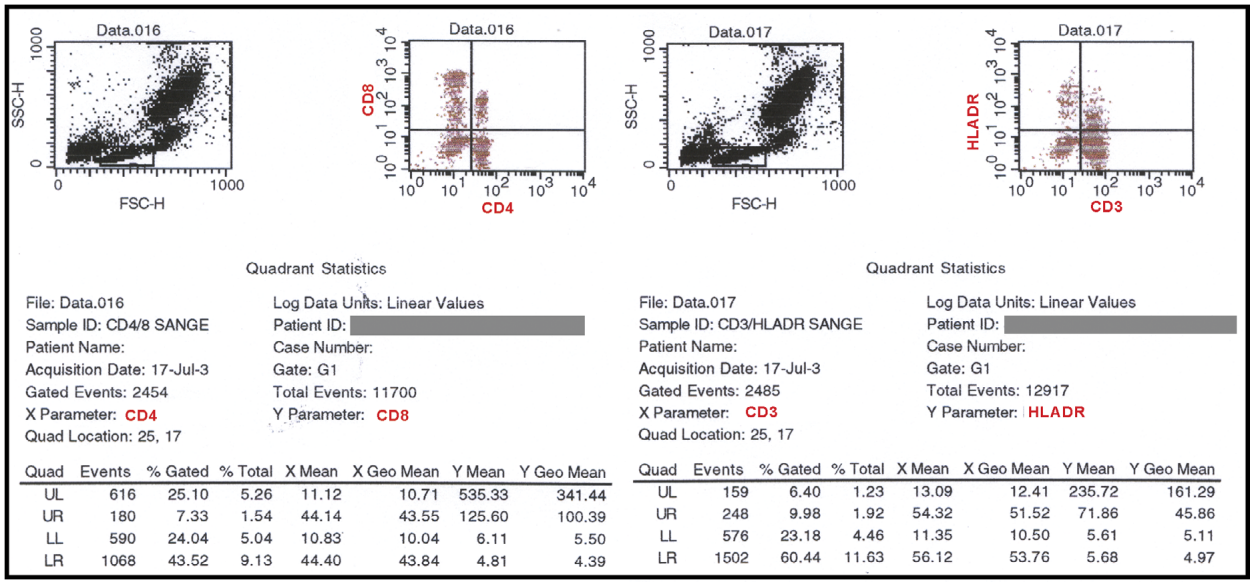


noregulatory mechanism, by restraining Th1 induction and enhancing Th2 production (Bohner et al., 2019). Nevertheless, diverse microenvironments may shift these double positive T lymphocytes behavior, as previously pointed out (Overgaard et al., 2015). The CD4+CD8+ double positive T cells have been considered to have a role as effector cells in active infections or to be reactive to still insufficiently defined underlying stimuli (Chen et al., 2022).

An interesting trait of CD4+CD8+ double positive T lymphocytes is the exhibit of a higher expression of surface activation markers, such as HLA-DR (Gonzalez-Mancera et al., 2020). When we compared the two types of tumors with a high percentage of CD4+CD8+ T cells, we noticed a difference in the activation markers: in carcinomas the activation percentage was significantly higher (7.48% and 9.98%) compared to under 5% values in lymphomas (4.56% in Hodgkin's and merely 2.2% in non-Hodgkin's). When compared to non-lymphomas, the percentage of CD4+CD8+ double positive T cells was either „normal" – i.e. under 1% (see Figure 3 – left panels) in both peripheral blood and lymph node, or surprisingly above 7% in the peripheral blood of a patient with poorly differentiated carcinoma (Figure 4 – left panel).

Characterization of tumor microenvironment may be regarded as a future checkpoint to assess immunosuppressive molecules. As previously pointed out, by accurate identification

Figure 4.
Flow cytometry of peripheral blood in poorly differentiated carcinoma (case ID #26). Left panel: CD4:CD8 cells; Right panel: activated lymphocytes (CD3:HLADR).



Quadrant Statistics

File: Data.016
Sample ID: CD4/8 SANGE
Patient Name:
Acquisition Date: 17-Jul-3
Gated Events: 2454
X Parameter: CD4
Quad Location: 25, 17

Log Data Units: Linear Values
Patient ID:
Case Number:
Gate: G1
Total Events: 11700
Y Parameter: CD8

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	616	25.10	5.26	11.12	10.71	535.33	341.44
UR	180	7.33	1.54	44.14	43.55	125.60	100.39
LL	590	24.04	5.04	10.83	10.04	6.11	5.50
LR	1068	43.52	9.13	44.40	43.84	4.81	4.39

Quadrant Statistics

File: Data.017
Sample ID: CD3/HLADR SANGE
Patient Name:
Acquisition Date: 17-Jul-3
Gated Events: 2485
X Parameter: CD3
Quad Location: 25, 17

Log Data Units: Linear Values
Patient ID:
Case Number:
Gate: G1
Total Events: 12917
Y Parameter: HLADR

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	159	6.40	1.23	13.09	12.41	235.72	161.29
UR	248	9.98	1.92	54.32	51.52	71.86	45.86
LL	576	23.18	4.46	11.35	10.50	5.61	5.11
LR	1502	60.44	11.63	56.12	53.76	5.68	4.97

one may provide a basis for further manipulating the signaling repertoaire present in a specific pathological condition (Hinescu, 2020). This could prove pivotal to deconvolve mechanisms of immunotherapies in the complex therapeutic landscape. A standardized algorithm could be developed to dictate antitumor immune responses using quantitative pathology, proposed as a stronger prognostic tool that has not been applied yet in head and neck cancers (Galon et al., 2014).

Double positive CD4+CD8+ T lymphocytes in diagnosis of Hodgkin/Non-Hodgkin lymphomas should not be misinterpreted as solely a population derived from a common precursor. In such neoplasms we could be talking about an expansion of a normal cell population with a possible reactive/regulatory function. The prognostic significance of lymphocytes subsets may unravel an (un)favorable outcome: further investigation and the clinical translation of T-cell-tumor infiltrating lymphocytes markers, as well as more extensive homogeneous patients/treatments studies are needed. One valuable addition to flow cytometry could be multiplex fluorescence immunohistochemistry – a promising technique to check T cells subsets.

The prognostic meaning of tumor-infiltrating lymphocytes in head & neck squamous carcinoma has been recently reviewed (Borsetto et al., 2021). One important statement that arises from that review is that timing of immune phenotyping is of pivotal importance in continuing/modifying the therapeutic approach for the best possible patient outcome. That is another reason for taking as much information as possible in establishing an immune panel – i.e. the activation percentage of lymphocytes is equally important as the presence of specific subsets.

Conclusions

There is an increasing incidence of late-stage head and neck cancers (Thompson-Harvey et al., 2020). Hence, we would like to emphasize the importance of immune phenotyping, when possible, to reveal as much as possible of the identity of the involved cells and to discriminate between tumor subtypes and consequently possibly choose a different immune therapy as part of the multimodal treatment of head and neck malignancies (Lo Nigro et al., 2017). We brought evidence that might be useful for future studies and/or personalized therapeutic strategies.

Acknowledgements

We would like to thank Dr. Crina Stăvaru and Dr. Dorel Radu for their help at the time of the initial experiments.

Funding

This work was supported by the Romanian Ministry of Research, Innovation and Digitalization under Grant number 31PFE/2021.

Competing interests/conflict of interests

The authors declare no conflict of interest

References

Alsalamah, M. et al. (2019) Establishing reference ranges for lymphocyte proliferation responses to phytohemagglutinin in patients with T cell dysfunction. LymphoSign J. 6:26–30. doi:10.14785/lymphosign-2019-0002

Bohner, P. et al. (2019) Double Positive CD4+CD8+ T Cells Are Enriched in Urological Cancers and Favor T Helper-2 Polarization. Front. Immunol. 10: 622. doi:10.3389/fimmu.2019.00622

Borsetto, D. et al. (2021) Prognostic Significance of CD4+ and CD8+ Tumor-Infiltrating Lymphocytes in Head and Neck Squamous Cell Carcinoma: A Meta-Analysis. Cancers (Basel). 13:781. doi:10.3390/cancers13040781

Chen, Z.W. et al. (2022) Flow Cytometric Detection of the Double-Positive (CD4+CD8+)/PD-1bright T-Cell Subset Is Useful in Diagnosing Nodular Lymphocyte-Predominant Hodgkin Lymphoma. Arch. Pathol. Lab. Med. 146:718–726. doi:10.5858/arpa.2020-0726-OA

Das, G. et al. (2003) An important regulatory role for CD4 + CD8αα T cells in the intestinal epithelial layer in the prevention of inflammatory bowel disease. Proc. Natl. Acad. Sci. 100:5324–5329. doi:10.1073/pnas.0831037100

Desfrançois, J. et al. (2010) Double Positive CD4CD8 αβ T Cells: A New Tumor-Reactive Population in Human Melanomas. PLoS One, 5:e8437. doi:10.1371/journal.pone.0008437

Economopoulou, P. et al. (2016) The emerging role of immunotherapy in head and neck squamous cell carcinoma (HNSCC): anti-tumor immunity and clinical applications. Ann. Transl. Med. 4:173–173. doi:10.21037/atm.2016.03.34

Galon, J. et al. (2014) Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. J. Pathol. 232:199–209. doi:10.1002/path.4287

Gonzalez-Mancera, M.S. et al. (2020) Percentages of CD4+CD8+ Double-positive T Lymphocytes in the Peripheral Blood of Adults from a Blood Bank in Bogotá, Colombia. Turkish J. Haematol. 37:36–41. doi:10.4274/tjh.galenos.2019.2019.0256

Hinescu, M.E. (2020) Why cell identity matters. J. Cell Ident. 1:5-13. doi:10.47570/joci.2020.001

Lo Nigro, C. et al. (2017) Head and neck cancer: improving outcomes with a multidisciplinary approach. Cancer Manag. Res. 9:363–371. doi:10.2147/CMAR.S115761

Nascimbeni, M. et al. (2004) Peripheral CD4+CD8+ T cells are differentiated effector memory cells with antiviral functions. Blood, 104:478–486. doi:10.1182/blood-2003-12-4395

Overgaard, N.H. et al. (2015) CD4 + /CD8 + double-positive T cells: more than just a developmental stage? J. Leukoc. Biol. 97:31–38. doi:10.1189/jlb.1RU0814-382

Overgaard, N.H. et al. (2017) CD4 + CD8β + double positive T cells in skin draining lymph nodes respond to inflammatory signals from the skin. J. Leukoc. Biol. 102:837–844. doi:10.1189/jlb.1AB0217-065R

Rahemtullah, A. et al. (2006) A Double-Positive CD4+CD8+ T-Cell Population Is Commonly Found in Nodular Lymphocyte Predominant Hodgkin Lymphoma. Am. J. Clin. Pathol. 126:805–814. doi:10.1309/Y8KD-32QG-RYFN-1XQX

Sullivan, Y.B. et al. (2001) Upregulation of CD4 on CD8+ T cells: CD4dimCD8bright T cells constitute an activated phenotype of CD8+ T cells. Immunology 103:270–280. doi:10.1046/j.1365-2567.2001.01243.x

Thompson-Harvey, A. et al. (2020) Rising incidence of late-stage head and neck cancer in the United States. Cancer 126:1090–1101. doi:10.1002/cncr.32583

Zuckermann, F.A. (1999) Extrathymic CD4/CD8 double positive T cells. Vet. Immunol. Immunopathol. 72:55–66. doi:10.1016/S0165-2427(99)00118-X