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TAMs PD-L1(+) in the reprogramming of germ cell tumors of the testis

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ABSTRACT

Background: In recent years, several studies focused on the process of reprogramming of seminoma (S) cells, which regulates the transition from pure S (P-S) to S component (S-C) of mixed germ cell tumors of the testis (GCTT) and finally to embryonal carcinoma (EC) and other nonseminomatous GCTT (NS-GCTT). The accepted pathogenetic model is driven and regulated by cells (macrophages, B- and T-lymphocytes) and molecules of the tumor microenvironment (TME). Herein, we tested a series of GCTT with double staining (DS) for CD68-PD-L1 to

Abbreviations: GCTT, germ cell tumors of the testis; NS-GCTT, non-seminomatous germ cell tumors of the testis; S, seminoma; P-S, pure seminoma; S-C, seminoma component of mixed GCTT; EC, embryonal carcinoma; YSTpt, yolk sac tumor postpubertal-type; Tpt, teratoma postpubertal-type; CHC, choriocarcinoma; OCT3/4, octamer-binding transcription factor 3/4; PRAME, preferentially expressed antigen of melanoma; SOX2, sex determining region Y-box 2; SOX17, sex determining region Y-box 17; FOXA2, forkhead box protein A2; TME, tumor microenvironment; TAMs, tumor-associated macrophages; PD-L1, programmed death-ligand 1; [TAMs PD-L1+], tumor-associated macrophages with expression of programmed death-ligand 1; DS, double stain; CD68, cluster of differentiation 68; WHO, World Health Organization; AJCC, American Joint Committee on Cancer; H&E, hematoxylin and eosin; OCT4, octamer-binding transcription factor 4; SALL4, sal-like protein 4; CD117/c-kit, cluster of differentiation 117/tyrosine-protein kinase kit; CD30, cluster of differentiation 30; h-GC, human choriogonadotropin; AFP, alpha-fetoprotein; CK AE1/AE3, cytokeratins cocktail; Glypican-3, protein encoded in humans by the *GPC3* gene; GATA3, transcription factor encoded in humans by the *GATA3* gene; DAB-brown color, 3,30-diaminobenzidine; AEC-red color, 3-Amino-9-ethylcarbazole; mv, median value; r, range; miRNA, microRNA; lncRNA, long non-coding RNA; CD3, cluster of differentiation 3; CD4, cluster of differentiation 4; FoxP3, forkhead box protein P3.

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evaluate tumor-associated macrophages (TAMs) expressing programmed death-ligand 1 (PD-L1) [TAMs PD-L1 (+)] and clarify if these cells may be involved in establishing the fate of GCTT.
Methods: We collected 45 GCTT (comprising a total of 62 different components of GCTT). TAMs PD-L1(+) were evaluated with three different scoring systems [*TAMs PD-L1(+)/mm^2*, *TAMs PD-L1(+)/mm^2 H-score*, *TAMs PD-L1 (+) %*], and compared using pertinent statistic tests (*Student's t-test* and *Mann-Whitney U test*).
Results: We found that TAMs PD-L1(+) values were higher in S rather than EC ($p = 0.001$, $p = 0.015$, $p = 0.022$) and NS-GCTT ($p < 0.001$). P-S showed statistically significant differences in TAMs PD-L1(+) values compared to S-C ($p < 0.001$, $p = 0.006$, $p = 0.015$), but there were no differences between S-C and EC ($p = 0.107$, $p = 0.408$, $p = 0.800$). Finally, we found statistically significant differences also in TAMs PD-L1(+) values between EC and other NS-GCTT ($p < 0.001$).
Conclusions: TAMs PD-L1(+) levels gradually decrease during the reprogramming of S cells {P-S [(high values of TAMs PD-L1(+)] → S-C and EC [(intermediate values of TAMs PD-L1(+)] → other NS-GCTT [(low values of TAMs PD-L1(+)], supporting a complex pathogenetic model where the interactions between tumor cells and TME components [and specifically TAMs PD-L1(+)] play a key role in determining the fate of GCTT.

1. Introduction

In recent years, numerous studies have focused on the biological process that drives the transition from seminoma (S) to non-seminomatous germ cell tumors of the testis (NS-GCTT), the "so-called" reprogramming of S cells [1–21]. Different transcription factors (PRAME, OCT3/4, SOX2, SOX17, FOXA2, etc.) have been identified as selectively involved in the maintenance of the stem cell phenotype [primordial germ cell-type in S and embryonal cell-type in embryonal carcinoma (EC)] and the divergent differentiation programs observed in the NS-GCTT [1–21]. Defining the intricate network of interactions among all these factors that drive the transition from pure S (P-S) to S component (S-C) of mixed GCTT and finally to other NS-GCTT (with no S component) is essential to clarify the biology of GCTT and develop novel therapies for these tumors [1–21]. We recently found that P-S, S-C, EC, and other NS-GCTT show different patterns of expression of PRAME and SOX2, suggesting that the immunohistochemical analysis of the different evolutionary steps of the reprogramming of S cells (PS → S-C → EC → other NS-GCT) may provide useful clues about this intriguing process [16,17]. Additionally, several authors have found that the tumor microenvironment (TME) plays a key role in the reprogramming of S cells [18–21]. Specifically, the different cells of the TME and the molecules expressed by them (membrane receptors, cytokines, chemokines, etc.) constitute a complex and heterogeneous niche that interacts with the tumor cells regulating the expression of the transcription factors involved in this process [18–21]. Since numerous studies have shown that tumor-associated macrophages (TAMs) and specifically those expressing programmed death-ligand 1 (PD-L1) have a pivotal role in the biology of several tumors including germ cell tumors of the testis (GCTT), we hypothesized that TAMs expressing PD-L1 [TAMs PD-L1(+)] could also be involved in the reprogramming of S cells [21–29]. To test our hypothesis, we interrogated a retrospective case series of GCTT from a single institution adopting a double stain (DS) for CD68/PD-L1 to score TAMs PD-L1(+) in the sequential evolutionary steps observed in the reprogramming of S cells and assess whether these cells are involved in establishing the fate of GCTT.

2. Materials and methods

We retrospectively collected GCTT diagnosed between January 1st 2019 and April 1st 2022 at our Institution (Pathology Unit, Maggiore Hospital-AUSL Bologna). Clinical parameters (age and tumor dimension) were retrieved from the digital records of the Urology Department, Maggiore Hospital-AUSL Bologna. All hematoxylin and eosin (H&E) stained slides were reviewed to confirm the diagnosis and select a representative block for immunohistochemistry. One 3-μm section cut from each paraffin-embedded tissue block was stained with DS for CD68/PD-L1 (BenchMark ULTRA automated immunostainer; Ventana Medical Systems-Roche Diagnostics, Switzerland), as previously described [30]. Slides stained for CD68/PD-L1 were read by two uropathologists with specific training on PD-L1 evaluation (M.F. and C. R.: routine evaluation of PD-L1 cases, evaluation of PD-L1 cases for research purposes, national and international meetings on this topic, inter- and intra-departmental courses on PD-L1 assessment for the evaluation of inter- and intra-observer agreement) on a multi-head microscope. TAMs [CD68(+)] were considered PD-L1(+) in presence of membrane staining, while cytoplasmic staining was considered positive only if there was concomitant membrane staining [30]. Since no specific cut-offs or methodologies are validated for scoring TAMs PD-L1(+), we adopted three different scoring systems, as previously described [30–36]:

1) CD68/PD-L1 double-positive cells [TAMs PD-L1(+)] counted in hotspot areas adding up to 1 mm^2; the total number of counted cells is reported as "*TAMs PD-L1(+)/mm^2*".

2) *TAMs PD-L1(+)/mm2* combined with the intensity of PD-L1 staining (0, 1+, 2+, 3+) to calculate the "H-score"; the resulting value (0−300) is reported as "*TAMs PD-L1(+)/mm^2 H-score*".

3) TAMs PD-L1(+) and CD68 single-positive cells (TAMs) assessed on the entire selected slide, and adopting the formula TAMs PD-L1 (+)/TAMs x 100; the resulting value is reported as "*TAMs PD-L1(+) %*".

Comparative evaluation of H&E and pertinent immunohistochemistry (SALL4, OCT4, CD117, CD30, Glypican-3, h-CG, GATA3, AFP, and CK AE1/AE3) was used to assess TAMs and TAMs PD-L1(+) in the different components of mixed GCTT. Immunohistochemical protocols, antibody clones, and other technical data are summarized in Supplementary Material 1-Table S1. All cases had been diagnosed and staged according to the 5th edition (2022) of the WHO classification of urinary and male genital tumors and the 8th edition of the AJCC Cancer Staging Manual [37,38]. All clinical-pathological investigations were conducted according to the principles of the Declaration of Helsinki, and the study has been approved by the Review Board of the Area Vasta Emilia Centro-AVEC (protocol n.463–2022-AUSLBO-22092-ANAPAT TESTIS 03).

2.1. Statistical analyses

This is an observational retrospective cohort study. A sample size calculation was not performed because all eligible patients in the Pathology Unit, Maggiore Hospital-AUSL Bologna were included. TAMs PD-L1(+) values measured with the three different scoring systems described above were compared between different groups (S and NS-GCTT, S and EC, P-S and S-C, S-C, and EC) using *Student's t-test* (normal distribution) or *Mann-Whitney U test* (non-normal distribution). Skewness and Kurtosis tests were used to establish the normal or non-normal distribution of TAMs PD-L1(+) results in the analyzed groups (normal distribution was assumed when scores at both tests were between −1 and 1). Statistical analyses were performed using the IBM SPSS software, with a p-value < 0.05 (two-sided) indicating statistical significance.

3. Results

3.1. Study population

Forty-five GCTT were included in the study, with a total of 62 different components of GCTT collected: 36/62 (58 %) seminoma (S), 11/62 (17.7 %) embryonal carcinoma (EC), 3/62 (4.8 %) yolk sac tumor postpubertal-type (YSTpt), 10/62 (16.1 %) teratoma postpubertal-type (Tpt), and 2/62 (3.2 %) choriocarcinoma (CHC). The mean value (mv) of age at diagnosis was 39.8 years [range (r): 23–64 years] and the mv of dimension was 4.6 cm (r: 0.9–10 cm). The pathologic stage was pT1 in 43/62 (69.4 %) patients and pT2 in 19/62 (30.6 %) patients; in the subgroup of pure S (P-S), the pathologic stage was pT1a in 7/30 (23.3 %), pT1b in 9/30 (30 %), and pT2 in 14/30 (46.7 %) patients.

3.2. TAMs PD-L1(+) results [TAMs PD-L1(+)/mm^2, TAMs PD-L1 (+)/mm^2 H-score, TAMs PD-L1(+) %]

We found that TAMs PD-L1(+) measured by all three scoring systems were significantly more numerous in S than in NS-GCTT ($p < 0.001$ for the three scoring systems) and EC ($p = 0.001$, $p = 0.015$, and $p = 0.022$). P-S showed significantly higher TAMs PD-L1(+) values compared to S-C ($p < 0.001$, $p = 0.006$, and $p = 0.015$). However, TAMs PD-L1(+) values of S-C did not significantly differ from those of EC ($p = 0.107$, $p = 0.408$, and $p = 0.800$). Finally, we found statistically significant differences in TAMs PD-L1(+) values between EC and the other NS-GCTT ($p < 0.001$ for the three scoring systems). Clinico-pathologic features and TAMs PD-L1(+) values are summarized in Table 1. A summary of the results [*TAMs PD-L1(+)/mm^2, TAMs PD-L1(+)/mm^2 H-score, TAMs PD-L1(+) %*] in the different analyzed groups is shown in Table 2. Illustrative examples of P-S, S-C, EC, and NS-GCTT (H&E and CD68/PD-L1) are shown in Fig. 1.

4. Discussion

The development of GCTT is regulated by a complex network that involves numerous genes, transcription factors, hormones, cytokines, and other different molecules (miRNA, lncRNA, etc.) [1–21]. In recent years, numerous approaches have been used in an attempt to better understand the biological processes that drive the transition from S to NS-GCTT (i.e., the "so-called" reprogramming of S cells) [1–21]. It is now recognized that the maintenance of a stem-cell phenotype in S and

Table 1
Clinico-pathologic features, diagnosis (GCTT histotypes) and TAMs PD-L1(+) values. For the criteria adopted in the three scoring systems (*TAMs PD-L1(+)/mm^2, TAMs PD-L1(+)/mm^2 H-score, TAMs PD-L1(+) %*) see *Materials and methods*.

Case number	Age (years)	Dimension (cm)	pT	Diagnosis	*TAMs PD-L1 (+)/mm^2*	*TAMs PD-L1 (+)/mm^2 H-score*	*TAMs PD-L1 (+) %*
1	31	3.1	pT1b	S	362	110	60
2	31	3.5	pT1b	S	140	220	90
3	45	5.2	pT2	S	220	70	40
4	59	7.2	pT2	S	252	190	80
5	39	3.1	pT1b	S	130	110	80
6	47	2.3	pT1a	S	240	185	95
7	39	5	pT1b	S	58	70	40
8	36	7	pT1	S-EC	80–65	80–55	60–45
9	50	3.2	pT1b	S	71	80	40
10	23	5.5	pT2	S	266	170	90
11	64	2	pT1a	S	233	140	90
12	46	5.5	pT1b	S	108	60	30
13	53	8	pT2	S	211	120	60
14	26	1.5	pT2	EC	31	9	5
15	53	1.4	pT1a	S	382	170	90
16	44	3.1	pT1	S-EC-Tpt-YSTpt	5–35–0–3	5–80–0–3	5–80–0–3
17	29	1.8	pT2	EC	22	5	5
18	38	3	pT2	S	90	100	60
19	60	6	pT2	S	164	160	70
20	39	7.5	pT1	Tpt	7	4	2
21	35	7	pT1	YSTpt	3	1	1
22	40	3.5	pT1	S-Tpt	22–4	12–2	10–2
23	27	5.9	pT2	S-EC-Tpt-YSTpt	18–17–0–7	10–5–0–2	10–5–0–2
24	23	3	pT1	S-Tpt	17–0	80–0	70–0
25	28	8	pT1	EC-Tpt	90–8	90–1	50–1
26	33	1.5	pT1a	S	158	170	80
27	29	7	pT2	S	376	170	70
28	45	4.7	pT1	EC-Tpt	130–30	55–5	20–5
29	38	7	pT2	S	106	80	40
30	37	6	pT2	S	230	155	65
31	62	4.8	pT2	S	62	30	20
32	53	4.5	pT1b	S	20	3	3
33	41	4.2	pT1b	S	187	235	85
34	45	7	pT2	S	93	35	30
35	32	1.5	pT1a	S	255	245	95
36	46	0.9	pT1a	S	336	180	90
37	27	5	pT2	S	363	200	90
38	33	5	pT2	S	423	205	95
39	27	10	pT2	EC-Tpt	90–0	50–0	30–0
40	38	2	pT1a	S	210	155	95
41	40	4.5	pT1	EC-CHC-Tpt	101–0–73	140–0–20	70–0–15
42	27	1.9	pT1b	S	25	5	5
43	52	2.8	pT2	S-EC	96–35	50–15	25–15
44	27	3.8	pT1	EC-CHC-Tpt	223–0–0	120–0–0	60–0–0
45	31	3.6	pT2	S	305	195	85

germ cell tumors of the testis (GCTT); yolk sac tumor postpubertal-type (YSTpt); seminoma (S); embryonal carcinoma (EC); teratoma postpubertal-type (Tpt); choriocarcinoma (CHC); tumor-associated macrophages (TAMs); programmed death-ligand 1 (PD-L1);

Table 2
Summary of the results [*TAMs PD-L1(+)/mm²*, *TAMs PD-L1(+)/mm² H-score*, *TAMs PD-L1(+) %*] in the different analyzed groups. For the criteria adopted in the three scoring systems [*TAMs PD-L1(+)/mm²*, *TAMs PD-L1(+)/mm² H-score*, *TAMs PD-L1(+) %*] see *Materials and methods*.

	TAMs PD-L1 (+)/mm²		*TAMs PD-L1 (+)/mm² H-score*		*TAMs PD-L1 (+) %*	
S (n = 36)	mv: 175.5, r: 5–423	$p <$ *0.001*[a]	mv: 118.2, r: 3–245	$p <$ *0.001*[b]	mv: 59.5 % r: 3–95	$p <$ *0.001*[b]
NS-GCTT (n = 26)[1]	mv: 37.5, r: 0–223		mv: 25.5, r: 0–140		mv: 16, r: 0–80	
S (n = 36)	mv: 175.5, r: 5–423	$p =$ *0.001*[a]	mv: 175.5, r: 5–423	$p =$ *0.015*[b]	mv: 175.5, r: 5–423	$p =$ *0.022*[b]
EC (n = 11)	mv: 76.3, r: 17–223		mv: 56.7, r: 5–140		mv: 35, r: 5–80	
P-S (n = 30)	mv: 202.5, r: 20–423	$p <$ *0.001*[a]	mv: 133.9, r: 3–245	$p =$ *0.006*[b]	mv: 65.4, r: 3–95	$p =$ *0.015*[b]
S-C (n = 6)	mv: 39.7, r: 5–96		mv: 39.5, r: 5–80		mv: 30, r: 5–70	
S-C (n = 6)	mv: 39.7, r: 5–96	$p =$ *0.107*[b]	mv: 39.5, r: 5–80	$p =$ *0.408*[a]	mv: 30, r: 5–70	$p =$ *0.800*[b]
EC (n = 11)	mv: 76.3, r: 17–223		mv: 56.7, r: 5–140		mv: 56.7, r: 5–140	
EC (n = 11)	mv: 76.3, r: 17–223	$p <$ *0.001*[b]	mv: 56.7, r: 5–140	$p <$ *0.001*[b]	mv: 56.7, r: 5–140	$p <$ *0.001*[b]
Other NS-GCTT (n = 15)[c]	mv: 9, r: 0–73		mv: 2.5, r: 0–20		mv: 2.1, r: 0–15	

germ cell tumors of the testis (GCTT); non-seminomatous germ cell tumors of the testis (NS-GCTT); seminoma (S); pure seminoma (P-S); seminoma component of mixed GCTT (S-C); embryonal carcinoma (EC); tumor-associated macrophages (TAMs); programmed death-ligand 1 (PD-L1); median value (mv); range (r);

[a] Student t-test (normal distribution);

[b] Mann-Whitney U-test (non-normal distribution);

[c] In case of mixed tumors, H-score of each component of GCTT has been separately assigned and evaluated.

EC (primordial germ cell-type in S and embryonal cell-type in EC) and the induction of differentiation programs in the other NS-GCTT requires a complex network of transcription factors (OCT3/4, PRAME, SOX2, SOX17, FOXA2, etc.) acting in a coordinated fashion [1–21]. However, the mechanisms that regulate the expression of these molecules, thus determining the ultimate fate of GCTT, remain incompletely understood [1–21]. Previous studies have shown that specific TME cells (T-lymphocytes and macrophages) regulate the expression of pluripotency markers in cocultures with TCam-2 cells [3,11,13,18–21]. In addition, other authors found that individual GCTT cell lines differentiate to different GCTT components depending on the TME they interact with [1,3,11,15,18–21]. These data suggest that the TME may represent a major determinant of the fate of GCTT by regulating the molecular networks involved in reprogramming [1,3,11,13,15,18–21]. According to this theory, TME cells (B- and T-lymphocytes, macrophages M1 and M2, etc.) and molecules (membrane receptors, cytokines, chemokines, etc.) collaborate to create a niche that cross-talks with GCTT cells, regulating the intracellular expression of the molecules involved in the reprogramming [18–21]. This theory may clarify the genesis of GCTT and explain why these tumors often change morphology/histotype in metastatic sites (especially in lymph nodes, where tumor cells cross-talk with a completely different TME) and/or after chemotherapy [18–21]. It could justify the relationship between the degree of infiltration by specific immune cells populations (lower levels of CD20 and CD3 lymphocytes) and GCTT aggressiveness (higher pT stage and rete testis invasion), the increased incidence of GCTT in patients with immunosuppression, and also the phenomenon of completely regressed GCTT [37,39]. Moreover, this theory may have potential therapeutic implications [18–21]. More specifically, modifying the TME with specific chemo- and immuno-therapy may control the reprogramming and prevent the shift of GCTT toward aggressive and/or chemo-resistant histotypes [18–21]. Although several studies have investigated the role of TME in GCTT, no previous studies have focused on the role of TME in the reprogramming of S cells [1,3,11,13,15,18–21,27–29]. In the present study, we adopted a DS for CD68/PD-L1 to evaluate and score TAMs PD-L1(+) in the sequential evolutionary steps of the reprogramming (P-S → S-C → EC → other NS-GCTT). TAMs PD-L1(+) are involved in a wide range of biological processes (development, acquisition of infiltrative and/or aggressive behavior, metastatic phenotype, acquisition of chemo-resistance, etc.) in GCTT and several other tumor types, but no previous studies have analyzed its potential involvement in the reprogramming of S cells [22–29]. We found statistically significant differences in TAMs PD-L1(+) levels between P-S, S-C, EC, and other NS-GCTT adopting three different score systems for TAMs PD-L1(+); specifically, we found that the TAMs PD-L1(+) gradually decrease during the transition from P-S to other NS-GCTT (P-S > S-C and EC > other NS-GCTT). This suggests that the preservation of stem-cell phenotypes (P-S, S-C, and EC) and the induction of divergent differentiation programs (other NS-GCTT) are influenced by the interplay between TAMs PD-L1(+) and GCTT cells (Table 2). Based on our results and according to the theory recently proposed by *Nettersheim* et al., we presented a model to explain how TAMs PD-L1(+) could influence the fate of GCTT (Fig. 2) [2,3,6,11,17,20]. In P-S (Phase 1-Fig. 2), high TAMs PD-L1(+) levels help to preserve the stem-cell phenotype by interacting with S cells and inducing high expression levels of PRAME and SOX17 levels, the interaction between SOX17 and OCT3/4, and binding of SOX17-OCT3/4 complex to specific motifs involved in the preservation of the stem-cell signature of primordial germ cell-type [2,3,6,11,17,20]. During the reprogramming of P-S in S-C and EC (Phase 2-Fig. 2), the decreased TAMs PD-L1(+) levels are required to allow a shift towards an embryonic-type stem-cell signature and the acquisition of primordial attributes required for reprogramming to NS-GCTT [2,3,6,11,17,20]. Since the levels of TAMs PD-L1(+) do not modify passing from S-C to EC, other TME actors are probably required to induce the changes that mediate this step (S-C: synchronous decrease of PRAME and SOX17 levels, increase of SOX2 levels, replacement of SOX17 by SOX2, binding of SOX2-OCT3/4 complex to specific motifs that shift towards an embryonic-type stem-cell signature; EC: drastic decrease of PRAME and further increase in SOX2 levels, which simultaneously preserve the embryonic-type stem-cell signature and predispose the GCTT cells to the divergent differentiation programs occurring in the other NS-GCTT) [2,3,6,11,17,20]. *Miyai K* et al. identified genetic evidence of progression from S to EC and showed that P-S and S-C demonstrate different pattern of gene expression (154 differentially regulated genes) [9]. Subsequently, *Medvedev KE* et al. revealed that P-S could be classified into two subtypes (1 and 2), with significative differences in pluripotency stage, DNA repair mechanisms, loss of heterozygosity, lncRNA expression, and cisplatin resistance. P-S subtype 1 is characterized by a higher pluripotency state, while P-S subtype 2 shows initial attributes of reprogramming and may be the putative precursor of S-C, EC, and other NS-GCTT [39]. According to these findings and in line with our results, the genetic/epigenetic/histological changes occurring during the transition from P-S to S-C and E-C could begin in a subset of P-S (i.e., P-S subtype 2 may evolve into S-C, EC, and other NS-GCTT) and be regulated by variations in TME composition and TAMs PD-L1(+) levels [39]. In the other NS-GCTT (Phase 3-Fig. 2), the drastic decrease of TAMs PD-L1(+) levels seems to be associated with loss of the stem-cell phenotype, allowing GCTT to activate divergent differentiation programs observed in YSTpt, Tpt, and CHC [2,3,6,11,17,20]. In line with

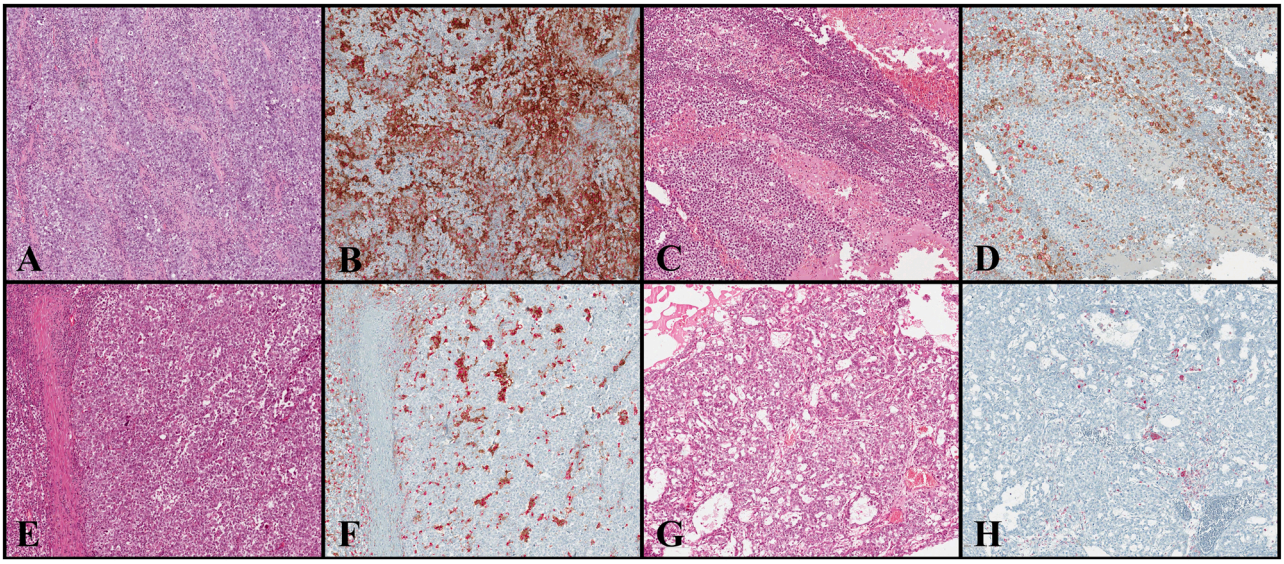

Fig. 1. Illustrative examples of P-S, S-C, EC, and NS-GCTT (H&E and CD68/PD-L1). non-seminomatous germ cell tumors of the testis (NS-GCTT); seminoma (S); pure seminoma (P-S); seminoma component of mixed GCTT (S-C); embryonal carcinoma (EC); yolk sac tumor postpubertal-type (YSTpt); tumor-associated macrophages with expression of programmed death-ligand 1 [TAMs PD-L1(+)]; hematoxylin and eosin (H&E); cluster of differentiation 68 (CD68); programmed death-ligand 1 (PD-L1); 3,30-diaminobenzidine (DAB)-brown color; 3-Amino-9-ethylcarbazole (AEC)-red color; **A-B: P-S (A: H&E, original magnification 100x; B: CD68/PD-L1, original magnification 100x). C-D: S-C (C: H&E, original magnification 100x; D: CD68/PD-L1, original magnification 100x). E-F: EC (E: H&E, original magnification 100x; F: CD68/PD-L1, original magnification 100x). G-H: YSTpt (G: H&E, original magnification 100x; H: CD68/PD-L1, original magnification 100x).** Note as TAMs PD-L1(+) levels gradually decrease during the reprogramming of S cells (P-S→S-C and EC → other NS-GCTT). P-S: high values of TAMs PD-L1(+), S-C and EC: intermediate values of TAMs PD-L1(+), other NS-GCTT: low values of TAMs PD-L1(+). *The chromogen (labelling) for CD68 is AEC; the chromogen (labelling) for PD-L1 is DAB.*

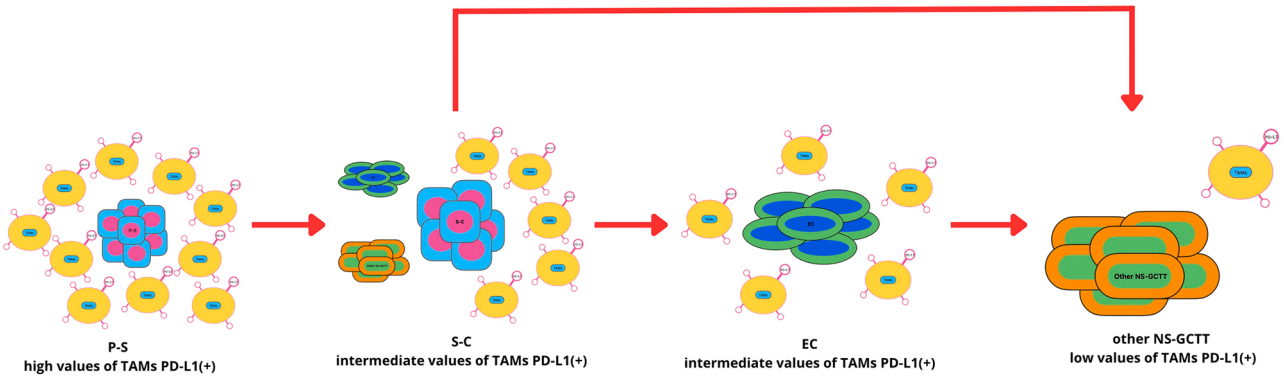

Fig. 2. Schematic model of TAMs PD-L1(+) levels in the reprogramming of S cells. seminoma (S); pure seminoma (P-S); seminoma component of mixed GCTT (S-C); embryonal carcinoma (EC); non-seminomatous germ cell tumors of the testis (NS-GCTT); tumor-associated macrophages with expression of programmed death-ligand 1 [TAMs PD-L1(+)]; cluster of differentiation 68 (CD68); programmed death-ligand 1 (PD-L1); From left to right the three phases of the reprogramming of S cells (Phase 1: P-S, Phase 2: S-C and EC, Phase 3: other NS-GCTT) with red arrows indicating the possible evolution pathways. In P-S (Phase 1): high levels of TAMs PD-L1(+); In S-C and EC (Phase 2): intermediate levels of TAMs PD-L1(+); In other NS-GCTT (Phase 3): low levels of TAMs PD-L1(+);.

our findings, *Sadigh S* et al. found that TAMs PD-L1(+) levels are significantly higher in S compared to NS-GCTT [29]. However, the authors did not analyze the differential TAMs PD-L1(+) levels in P-S, S-C, EC, and other NS-GCTT, precluding an evaluation of the role of these cells in the sequential steps of the reprogramming of S cells [29]. Other authors have found significative variations in T-lymphocytes (with and without PD-L1 expression) but not in macrophages levels between S and NS-GCTT, thus assuming a pivotal role for T-lymphocytes and a minor role for macrophages in the biology of these tumors [21,40–43]. Nonetheless, the model used in these studies (in vitro model with TCam-2 cell and immune cells purified from human peripheral blood) does not properly mimic the normal in vivo physiology [21,40–43]. Also, discrepancies between the studies could be influenced by differences in the studies' design, including: a) standard immunohistochemistry, bright-field multiplex immunohistochemistry, and gene expression profile analysis; b) different scoring systems for the evaluation of immune cells; c) analysis of specific T-lymphocytes classes (CD3, CD4, FoxP3, etc.) but not of specific macrophages classes [PD-L1(+) or not, M1 or M2, etc.] [21,40–43]. In addition, although the recently developed bright-field multiplex immunohistochemistry allows investigating multiple markers on the same slide and helps to analyze the TME of different tumors in detail, the comprehensive TME characterization on human neoplastic tissue is extremely difficult (and probably quite impossible) [21,44–46].

In conclusion, we found that TAMs PD-L1(+) levels gradually decrease during the reprogramming of S cells (P-S →S-C and EC → other NS-GCTT), suggesting that these cells play a major role in the preservation of stem-cell phenotypes [(P-S: high values of TAMs PD-L1(+), S-C and EC: intermediate values of TAMs PD-L1(+)] and the divergent differentiation programs that determine the fate of GCTT [other NS-GCTT:

low values of TAMs PD-L1(+)]. Additional studies are needed to further elucidate the interactions between TAMs PD-L1(+), other components of the TME (B- and T-lymphocytes), and different tumor cells, as well as to assess the molecular processes regulated by these interactions.

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CRediT authorship contribution statement

Sofia Melotti, Costantino Ricci: Conceptualization, Data curation, Investigation, Visualization, Roles/Writing - original draft; Writing - review & editing; **Francesca, Ambrosi, Tania Franceschini, Francesca Giunchi**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology; **Giorgia Di Filippo, Eugenia Franchini:** Data curation, Formal analysis, Investigation, Methodology; **Francesco Massari, Veronica Mollica, Valentina Tateo, Federico Mineo Bianchi:** Conceptualization, Data curation, Methodology; **Maurizio Colecchia, Andres Martin Acosta, Joao Lobo, Michelangelo Fiorentino**: Conceptualization, Data curation, Investigation, Visualization, Roles/Writing - original draft; Writing - review & editing.

Declaration of Competing Interest

All the authors present in his article declare no conflict of interests.

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Author's statement

All Authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the author's original work.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2023.154540.

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