Differences in PD-L1–Expressing Macrophages and Immune Microenvironment in Testicular Germ Cell Tumors

Sam Sadigh, MD,[1] *Sahar J. Farahani, MD,*[1] *Abhishek Shah, MD,*[1] *David Vaughn, MD,*[2] *and Priti Lal, MD*[1]

From the Departments of [1]Anatomic Pathology and [2]Medical Oncology, Hospital of the University of Pennsylvania, Philadelphia.

Key Words: PD-L1; Testicular germ cell tumors; Immune microenvironment

Am J Clin Pathol 2019;XX:1–9

DOI: 10.1093/AJCP/AQZ184

ABSTRACT

Objectives: *To characterize the tumor microenvironment of testicular germ cell tumors (GCTs) using immunohistochemical markers.*

Methods: *Seventy-seven orchiectomies, including 36 nonmetastatic (NM) seminomas, 15 metastatic (M) seminomas, 13 nonmetastatic nonseminomatous germ cell tumors (NSGCTs), and 13 metastatic NSGCTs, were studied with PD-1, PD-L1, FOXP3, CD68, CD163, and mismatch repair (MMR) immunohistochemistry. FOXP3+ and PD-1+ tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) expressing CD68 and CD163 were enumerated. PDL-1 expression was evaluated on tumor cells and macrophages.*

Results: *GCTs primarily express PD-L1 on TAMs, except choriocarcinoma, where true tumor cell positivity was noted. Seminomas reveal increased intratumoral PD-L1+ TAMs compared with NSGCTs (*$P < .05$*). Activated TILs are increased in NM-seminomas compared with M-seminomas (*$P < .05$*). All GCTs retained MMR expression.*

Conclusions: *Robust PD-L1+ TAMs are significantly expanded in seminomas compared with NSGCTs. Among all GCTs, only choriocarcinoma cells reveal true positivity for PD-L1. These findings expand the realm of potentially targeted treatments for GCTs.*

Testicular germ cell tumors (GCTs) are a heterogeneous group of tumors that compose only approximately 1% of all human neoplasms but are the most common solid malignancy in young men aged 20 to 35 years. GCTs generally have a high sensitivity to chemotherapy. The cornerstone of treatment for metastatic GCTs has remained cisplatin-based combination therapy followed by surgical resection of the residual tumor.[1] Approximately 75% of patients with disseminated disease respond favorably to initial treatment. Of the 25% of nonresponders, approximately 50% have a complete response to second-line salvage or high-dose chemotherapy. Given the high rate of survival (5-year survival >95%), GCTs are deemed among the most curable solid malignancies.[1] However, despite this high success, chemotherapy is not a universal cure for all patients, especially those with metastatic disease. About 40% to 80% of patients with relapse after initial chemotherapy will fail salvage treatment, and ultimately approximately 15% of patients will be cisplatin resistant, with incurable relapsed disease, and their prognosis remains dismal.[2,3] Better identifying the aggressive subset of GCTs is critical for prognostication and treatment stratification.[4]

Recent strides in our understanding of the role of tumor microenvironment in cancer progression have led to the rapid development of immunotherapy. One of the targets of immunotherapy is the signaling pathway of programmed death ligand 1 (PD-L1) binding to programmed cell death 1 (PD-1) receptor on the surface of tumor-infiltrating lymphocytes (TILs). In addition, tumor associated macrophages (TAMs) are thought to be involved in immune suppression and tumor aggressiveness and are being explored as potential candidates for immunotherapy. Despite remarkable advancements in the use of immunotherapy for

Table 1
Summary of Clinicopathologic Features[a]

Characteristic	Total No. of Cases	Age, Mean (Range), y	Tumor Size, Mean (Range), cm	Pathologic Stage TNM (AJCC 8th), No. (%)			Clinical Follow-up, Mean, y
				T1	T2	T3	
All germ cell tumors	77	35 (18-69)	4.0 (0.8-20.0)	47 (61)	25 (33)	5 (6)	5.3
All seminomas	51	37 (21-69)	4.0 (0.8-14.5)	33 (65)	16 (31)	2 (4)	5.3
NM-seminoma	36	37 (21-69)	3.9 (0.8-12.0)	22 (61)	13 (36)	1 (3)	5.1
M-seminoma	15	38 (26-54)	4.6 (1.4-14.5)	11 (73)	3 (20)	1 (7)	6.4
All NSGCTs	26	30 (18-51)	4.1 (1.1-20.0)	14 (54)	9 (35)	3 (11)	5.4
NM-NSGCT	13	32 (18-51)	3.5 (1.2-6.0)	8 (62)	3 (23)	2 (15)	7.2
M-NSGCT	13	29 (21-39)	4.6 (1.1-20.0)	6 (46)	6 (46)	1 (8)	3.4

AJCC, American Joint Committee on Cancer; M, metastatic; NM, nonmetastatic; NSGCT, nonseminomatous germ cell tumor.
[a]If multiple tumor foci, largest focus is noted. Tumor size for three cases unavailable. Clinical follow-up for three cases unavailable.

Table 2
Summary of Antibodies Used for Immunohistochemistry

Antibody	Clone	Vendor	Catalog No.	Dilution	Pretreatment Solution/Incubation Time
PD-L1	E1J2J	Cell Signaling	15165BF	1:2,000	ER2/20 min
PD-1	NAT105	Abcam	Ab52587	1:40	ER1/20 min
CD68	KP1	Dako	IR60961	Prediluted	ER1/20 min
CD163	10D6	Leica	CD163-L-CE	1:50	ER1/20 min
FOXP3	206D	Biolegend	320102	1:100	ER2/30 min
OCT3/4	N1NK	Leica	PA0193	Prediluted	ER2/20 min
MLH1	G168-15	Biocare	CM220C	1:50	ER2/20 min
MSH2	FE11	Biocare	CM219C	1:50	ER2/20 min
MSH6	BC/44	Biocare	CM265BK	1:50	ER2/20 min
PMS2	EPR3947	Cell Marque	288R-18-ASR	Prediluted	ER2/30 min

ER1, Epitope Retrieval 1; ER2, Epitope Retrieval 2.

melanoma, lung cancer, kidney cancer, or others, GCTs have been bereft of these benefits due to our limited understanding of their immune milieu. Immunotherapies could potentially be added to our armamentarium for treating GCTs to further enhance the success of chemotherapies.

In this retrospective study, we characterized the tumor microenvironment of metastatic vs nonmetastatic testicular GCT. We studied the signaling pathway of PD-1 on the surface of TILs and PD-L1 on the surface of tumor cells and TAMs, as well as FOXP3 expression reflecting activated TILs. In addition, we investigated the mismatch repair (MMR) status of seminoma and nonseminomatous GCTs (NSGCTs) via immunohistochemistry (IHC).

Materials and Methods

The archives of the University of Pennsylvania were searched for all orchiectomy specimens between 1994 and 2018 under an institutional review board. A total of 77 in-house orchiectomies were identified with adequate and available tissue. These included 51 seminomas, of which 36 revealed no pathologic or radiologic evidence of lymph node or retroperitoneal involvement (nonmetastatic [NM] seminoma), and 15 involved either lymph nodes at the time of primary resection or upon follow-up (metastatic [M] seminoma). Among the NSGCTs, a total of 26 in-house cases were identified (13 NM-NSGCTs and 13 M-NSGCTs). The mean age of the cohort was 35 (range, 18-69) years with an average follow-up of 5.3 years Table 1. All cases were reviewed by P.L. and S.S. Slides most representative of the tumor were selected.

Staining for PD-1, PD-L1, FOXP3, CD68, CD163, and MMR was performed with adequate positive and negative controls on formalin-fixed, paraffin-embedded tissue using a Leica Bond-III instrument with the Bond Polymer Refine Detection System (Leica Biosystems DS9800). Heat-induced epitope retrieval was required as follows: Epitope Retrieval 1 solution (cat. AR9961; Leica Biosystems) and Epitope Retrieval 2 solution (cat. AR9640; Leica Biosystems). A proof-of-concept dual immunohistochemical stain for OCT3/4 and PD-L1 was developed and applied to a subset of cases. Costains were performed sequentially on a Leica Bond-III instrument using the Bond Polymer Refine Detection System and the Bond Polymer Refine Red Detection System. Details of antibodies and immunohistochemical stain conditions are illustrated in Table 2.

DOI: 10.1093/AJCP/AQZ184

Table 3
Tumor Immune Microenvironment Immunohistochemistry[a]

Characteristic	Total No. of Cases	PD-1, Mean (SD), %	PD-L1 H-score, Mean (SD)	FOXP3, Mean (SD), %	CD68, Mean (SD), %	CD163, Mean (SD), %	CD163/CD68 Ratio, Mean (SD)
All seminomas	51	12.26 (14.19)	36.78 (45.95)[b]	14.04 (10.46)[b]	18.92 (17.18)	34.31 (20.10)	2.30 (1.43)
NM-seminoma	36	13.06 (15.55)	33.61 (35.41)	15.5 (10.47)	20.83 (17.87)	38.06 (21.32)[c]	2.23 (1.49)
M-seminoma	15	10.33 (10.43)	44.4 (65.72)	10.53 (9.89)	14.33 (14.98)	25.33 (13.56)[c]	2.46 (1.31)
All NSGCTs	26	8.08 (10.59)	12.31 (22.10)[b]	5.38 (6.43)[b]	14.16 (12.76)	25.42 (17.68)	2.13 (0.93)
NM-NSGCT	13	6.92 (9.90)	6.92 (16.53)	4.46 (4.33)	11.77 (12.44)	21.23 (15.51)	2.08 (0.64)
M-NSGCT	13	9.23 (11.52)	17.69 (26.11)	6.31 (8.10)	16.54 (13.13)	29.62 (19.31)	2.16 (1.14)

M, metastatic; NM, nonmetastatic; NSGCT, nonseminomatous germ cell tumor.
[a]PD-L1 is noted as H-score overall. PD-1, FOXP3, CD68, and CD163 are expressed as percentage of tumor area and density overall.
[b]PD-L1 and FOXP3 difference between seminoma and NSGCT, $P < .001$.
[c]CD163 mean percent difference between NM-seminoma and M-seminoma, $P = .04$.

All stains were evaluated by S.S., and a subset of all immunohistochemical stains was evaluated by both pathologists (P.L. and S.S.). TILs positive for FOXP3 and PD-1 were assigned a semiquantitative score based on percentage of tumor area involved; TAMs positive for CD68 and CD163 were identified and enumerated semiquantitatively based on percentage of tumor area involved. Membranous PDL-1 expression was evaluated on both tumor cells and macrophages, with semiquantitative scoring of staining intensity (0, 1+, 2+, 3+) and total percentage of cells positive (macrophages and potential tumor cells combined and divided by total cells). An H-score was subsequently determined by multiplying intensity and percentage.

Statistical analyses included the Shapiro-Wilk test used to assess the data distribution normality in continuous variables. In the case of nonnormally distributed data, nonparametric tests were used. Mann-Whitney and χ^2 tests were used for continuous and categorical variables, respectively. Logistic regression was performed to determine the association between macrophages, tumor type, and recurrence. When a significant association was present, odds ratio (OR) was calculated to estimate the magnitude of the correlation. The level of statistical significance was considered $P < .05$. All the statistical analyses were performed in Stata software (Stata/SE 13.1; StataCorp).

Results

A total of 77 testicular GCTs were identified in house. Of these, 51 were seminomas and 26 were NSGCTs. All staging data were retrospectively reviewed, and the tumors were staged as per American Joint Committee on Cancer eighth edition. The pathologic stage distribution of the NM-seminoma cohort consisted of 22 pT1s, 13 pT2s, and one pT3. A total of 15 M-seminoma cases had either pathologic evidence of lymph node involvement (n = 8) or radiologic evidence of retroperitoneal mass with clinical suspicion of involvement by GCT (n = 7). Of the patients with NM-seminoma, 23 were followed with active surveillance, five received postorchiectomy adjuvant cisplatin-based chemotherapy, seven patients received postorchiectomy radiation therapy, and one patient was lost to follow-up. All patients with metastatic disease were treated with postorchiectomy chemotherapy and/or radiation. Among the NSGCTs (n = 26), the stage distribution of NM-NSGCTs (n = 13) was eight pT1s, three pT2s, and two pT3s.

Seminoma and NSGCT H&E Evaluation

On H&E evaluation, seminomas primarily revealed lymphocytic infiltrate with various patterns of involvement, including (1) diffuse sparse lymphocytes scattered throughout the tumor, (2) septal infiltrate with or without diffuse intratumoral involvement, (3) large clusters of lymphocytes rimming the tumor without germinal center formation, and (4) large clusters of lymphocytes with germinal center formation. In NSGCTs, the patterns of lymphocytic infiltrate were not as distinct as in seminomas and overall less pronounced. When present in NSGCTs, lymphocytic infiltrates were scattered, without pronounced clustering, follicle formation, or tumor rimming.

Lymphohistiocytic Component of GCT Microenvironment

PD-1- and FOXP3-Positive Lymphocytes

PD-1 and FOXP3 positivity on TILs was reviewed. The percentage of tumor involved by PD-1–expressing lymphocytes and activated FOXP3-positive lymphocytes was calculated individually. There was no statistically significant difference in the PD-1–expressing lymphocytes between seminomas (mean [SD], 12.26% [14.19%]) and NSGCTs (8.08% [10.59%]) Table 3.

DOI: 10.1093/AJCP/AQZ184

There were significantly more activated FOXP3-expressing TILs in seminomas compared with NSGCTs, with a mean (SD) of 14.04% (10.46%) in seminomas and 5.38% (6.43%) in NSGCTs (Table 3; $P < .001$). When considering FOXP3 expression as a binary variable (expression present or absent), the χ^2 test showed that FOXP3-expressing activated T cells were significantly more abundant in NM-seminomas compared with M-seminomas ($P < .05$).

Tumor-Associated Macrophages and Their Subtypes

On H&E evaluation, only rare macrophages were readily identifiable, but on staining with CD68 and CD163, abundant histiocytic infiltrate was highlighted **Image 1**. The histiocytic component was intimately admixed with the lymphocytic infiltrate and closely approximated/interdigitated with tumor cells. TAMs positive for CD68 and CD163 were enumerated semiquantitatively based on percentage of tumor area involved. There was no significant difference in CD68+ or CD163+ TAMs between seminomas (mean [SD], 18.92 [17.18] and 34.31 [20.10]) and NSGCTs (14.16 [12.76] and 25.42 [17.68]). However, within the seminoma group, NM-seminomas showed a significantly higher presence of CD163+ TAMs compared with M-seminomas (Table 3; $P = .04$).

PD-L1 Expression on TAMs and Tumor Cells

Although it initially appeared that some tumor cells might variably express PD-L1, detailed evaluation of the immunohistochemical stains revealed that PD-L1 was strongly positive on macrophages, and the apparent positivity on tumor cells was primarily due to PD-L1–positive macrophages closely rimming them. True tumor cell positivity was rare, with few dim (1+, noncircumferential) positive tumor cells in a few cases (n = 3) accounting for less than 1% of tumor cells in those cases. This finding was further confirmed with a dual stain for OCT3/4 and PD-L1 in a subset of seminomas that showed PD-L1 staining in macrophages but not in tumor cells **Image 2**. With the exception of choriocarcinoma, none of the other GCT subtypes revealed strong membranous (2+ or 3+) or circumferential staining.

A combined positive score of PD-L1 on tumor cells and macrophages together revealed a significantly higher expression in seminomas than in NSGCTs ($P < .001$; Table 3). Moreover, logistic regression showed higher density of macrophages associated with seminoma (OR, 11.9; 95% confidence interval, 1.3-108.6; $P = .03$). There was, however, no statistically significant association with recurrence, TNM stage, or lymphovascular invasion.

There were three main phenotypic categories of cases with regard to the patterns of PD-L1 expression on macrophages.

Group 1 displayed very few PD-L1+ macrophages, and when present, these macrophages were scattered within the septae or fibrous stroma. In this group, the macrophages did not form large aggregates or intersperse within the tumor cells. The second group revealed numerous PD-L1+ macrophages within the septae and stroma and in areas formed aggregates, but they did not extensively intercalate between the tumor cells.

The third group revealed extensive presence of PD-L1+ macrophages around and in between the tumor cells. The percentages of cases from each of the three subgroups described above are summarized in **Table 4**.

On comparison, there were significantly more seminoma cases with extensive intratumoral PD-L1+ macrophages compared with NSGCTs ($P < .05$). The teratoma component of NSGCTs showed nonspecific CD163 staining in fibroblasts and stromal elements without significant PD-L1+ TAMs. Representative images of the three main patterns of PD-L1+ TAMs are summarized in Image 1.

PD-L1 Expression in Choriocarcinoma

Choriocarcinomas were the only subset of GCTs that definitively expressed PD-L1 in tumor cells, including cytotrophoblasts as well as syncytiotrophoblasts. These positive cases showed strong membranous PD-L1 expression in the tumor cells, whereas there seemed to be a paucity of macrophages in these areas.

Germ Cell Tumors Have Retained MMR IHC

All cases of seminomas and NSGCTs (including embryonal carcinoma, yolk sac tumor, teratoma, and choriocarcinoma) had retained MMR IHC. There was strong nuclear expression of MLH1, PMS2, MSH2, and MSH6 in tumor cells as well as background nuclei for every case. Representative images of MMR protein IHC in seminomas and NSGCTs are depicted in **Image 3**.

Discussion

The observation of immune reaction induced by GCTs as evidenced by lymphocytic and granulomatous intratumoral infiltrates dates back to 1964, when it was published in *Lancet*.[5] The rich infiltration of testicular GCTs, especially seminomas, suggests an involvement of immune system in their biology. The immune cell characterization of seminomas has shown the presence of TILs, including CD3+ and T memory cells, while B cells and plasma cells were noted to be present less frequently.[6] Interestingly, seminoma in situ was found to

DOI: 10.1093/AJCP/AQZ184

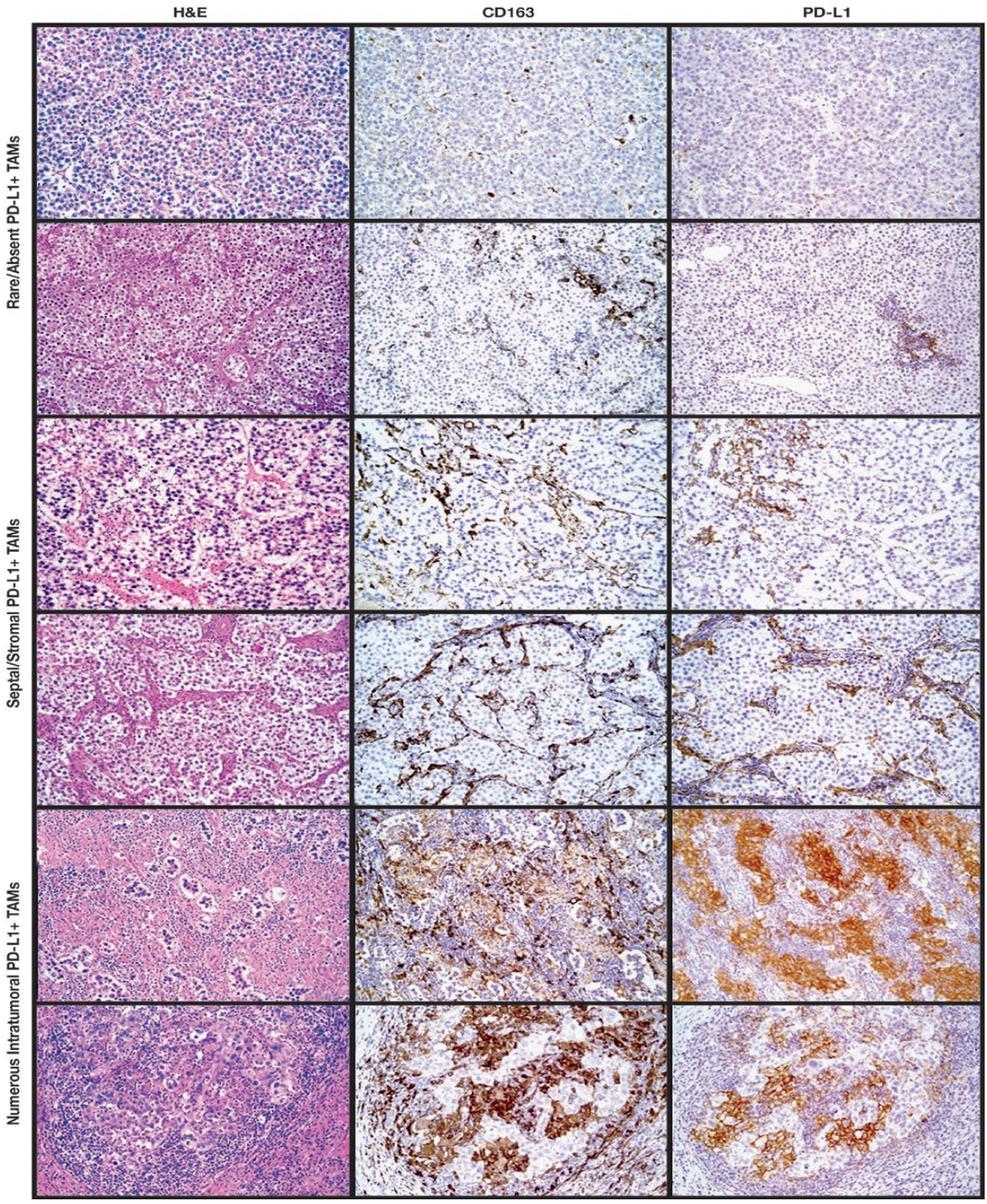

Image 1 Patterns of programmed death ligand 1 (PD-L1) expression in CD163+ macrophages in representative seminoma cases (panels include two representative cases for each category). Rare/absent PD-L1+ tumor-associated macrophages (TAMs), septal/stromal PD-L1+ TAMs, and numerous intratumoral PD-L1+ TAMs are shown. (H&E, ×20; CD163, ×20; PD-L1, ×20)

DOI: 10.1093/AJCP/AQZ184

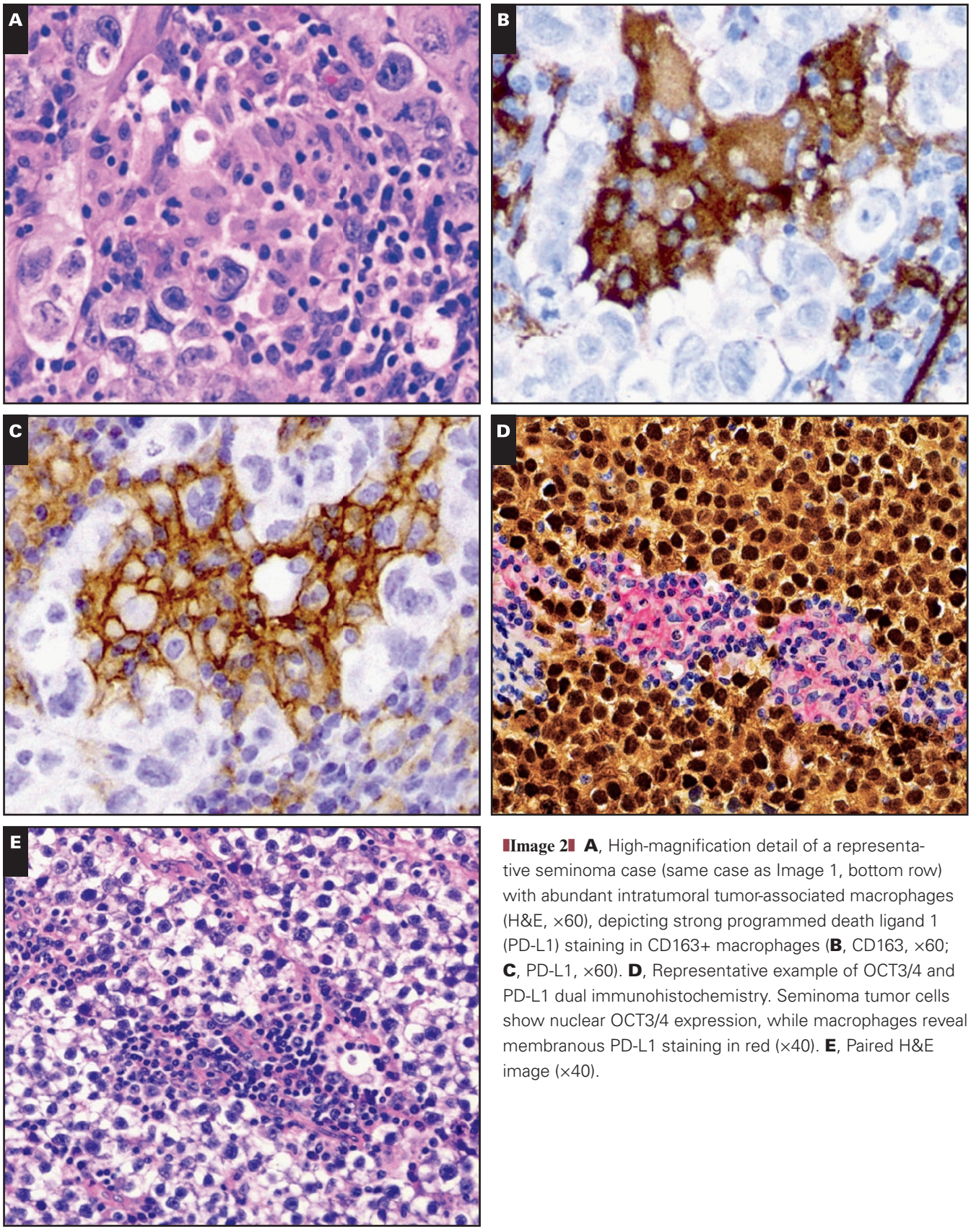

Image 2 **A**, High-magnification detail of a representative seminoma case (same case as Image 1, bottom row) with abundant intratumoral tumor-associated macrophages (H&E, ×60), depicting strong programmed death ligand 1 (PD-L1) staining in CD163+ macrophages (**B**, CD163, ×60; **C**, PD-L1, ×60). **D**, Representative example of OCT3/4 and PD-L1 dual immunohistochemistry. Seminoma tumor cells show nuclear OCT3/4 expression, while macrophages reveal membranous PD-L1 staining in red (×40). **E**, Paired H&E image (×40).

DOI: 10.1093/AJCP/AQZ184

Table 4
Patterns of PD-L1–Expressing Macrophages in Germ Cell Tumors

Characteristic	Total No. of Cases	Rare PD-L1+ Macrophages, No. (%)	Intraseptal/Stromal PD-L1+ Macrophages, No. (%)	Extensive Intratumoral PD-L1 Macrophages, No. (%)
All seminomas	51	15 (29)	22 (43)	14 (28)[a]
NM-seminoma	36	11 (31)	14 (39)	11 (30)
M-seminoma	15	4 (27)	8 (53)	3 (20)
All NSGCTs	26	10 (38)	14 (54)	2 (8)[a]
NM-NSGCT	13	6 (46)	6 (46)	1 (8)
M-NSGCT	13	4 (31)	8 (62)	1 (7)

AJCC, American Joint Committee on Cancer; M, metastatic; NM, nonmetastatic; NSGCT, nonseminomatous germ cell tumor.
[a]Significantly more seminoma cases with extensive intratumoral PD-L1+ macrophages compared with NSGCTs, $P < .05$.

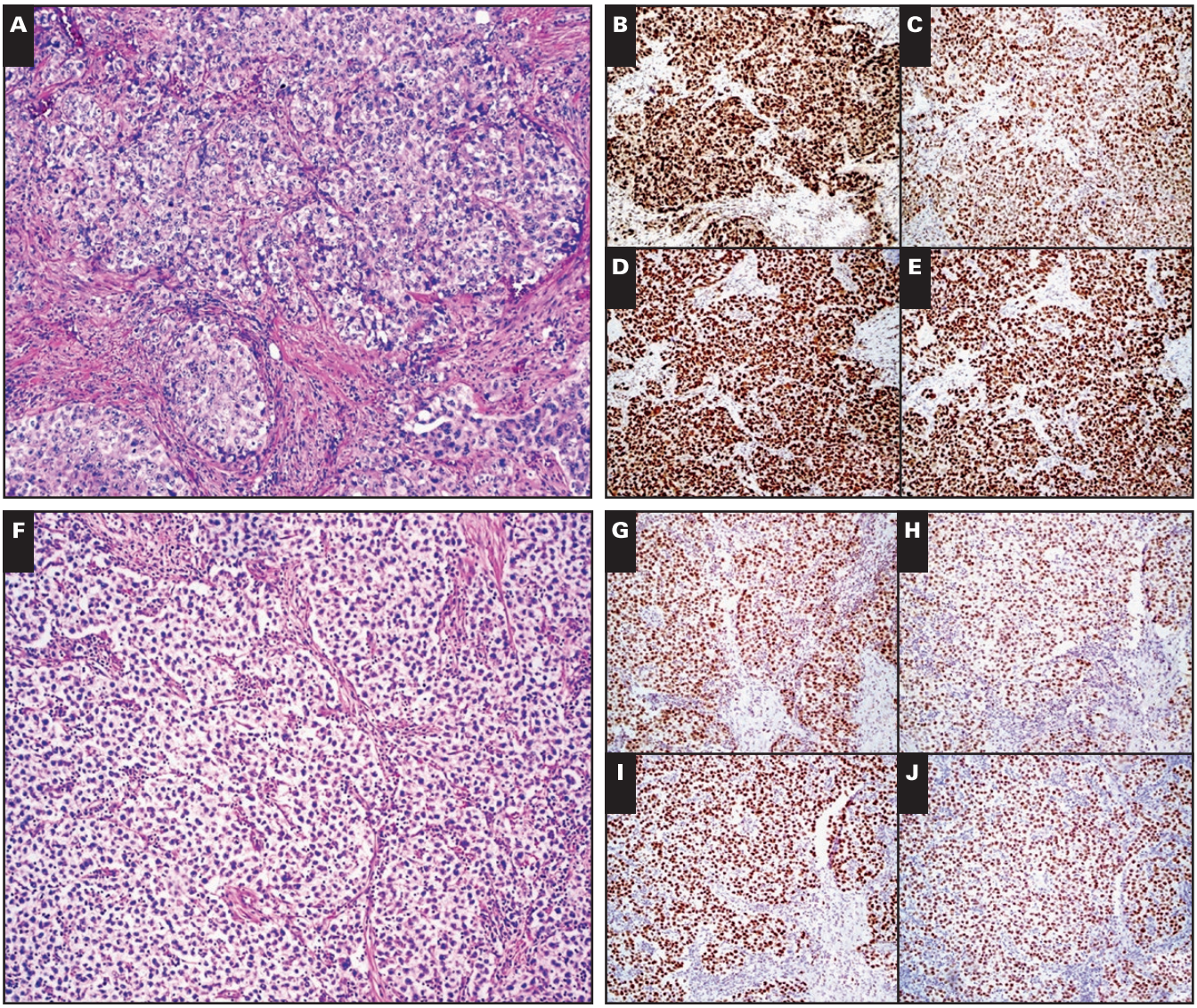

Image 3 Retained mismatch repair immunohistochemistry (IHC) in germ cell tumors. Embryonal carcinoma (**A**) and seminoma (**F**) showing retained expression of MLH1 (**B** and **G**), PMS2 (**C** and **H**), MSH2 (**D** and **I**), and MSH6 (**E** and **J**) in tumor cells. (H&E, ×10; IHC, ×10)

be infiltrated by CD4+ and CD8+ TILs and then followed by B cells, dendritic cells, natural killer cells, and macrophages.[6] This study was also among the first to provide data on the prognostic significance of TILs, where a lower number of TILs were associated with poorer prognosis.

DOI: 10.1093/AJCP/AQZ184

T lymphocytes and macrophages were also documented in embryonal carcinoma.[7] A recent study described distinct molecular signatures of immune cells specifically for seminoma showing elevation of expression signatures for B cells, cytotoxic T cells, Th17 cells, and T-regulatory cells. This increase was associated with an increase in specific cytokines and immune checkpoints (CTLA4, LAG3, and PD-L1).[8] Despite these strides in our understanding, the question of the specific role of immune surveillance in GCT development and treatment outcomes of testicular germ cell neoplasms remains open.

Our study explored the immune microenvironment of testicular GCTs. We found a robust presence of activated T cells and PD-L1–expressing macrophages in seminomas. There was similar expression of PD-L1 in M-seminomas and NM-seminomas, but NM-seminoma cases had an increased FOXP3+ activated T-cell reaction. Prior studies have shown mixed findings as to the prognostic role of FOXP3 expression in human neoplasia.[9] Some data indicate FOXP3+ lymphocytic tumor infiltrate and tumor cell FOXP3 expression as being a poor prognostic marker,[10] while others have shown that tumors with a low density of FOXP3+ T cells are indeed at higher risk of progression.[11] Siska and colleagues[12] performed immune profiling using multiplexed fluorescence immunohistochemistry for T-cell subsets and found that seminomas were associated with increased CD3 T-cell infiltration, decreased regulatory T cells, increased PD-L1, and increased PD-1/PD-L1 spatial interaction compared with nonseminomas. In addition, immune characterization using IHC and gene expression profiling identified activated T-cell infiltration correlated with seminoma histology and good prognosis. Our results are in line with this study and build on an intriguing finding that may be suggestive of successful immune evasion in tumors that ultimately develop progression.

Previous studies have reported PD-L1 expression in a large subset of seminoma and NSGCT cases.[12-16] To our knowledge, this is the first study to delineate and separate PD-L1 expression in specific compartments of testicular GCT (ie, in tumor cells vs TILs and TAMs using whole sections). We found that choriocarcinoma was the only GCT type with definitive tumor PD-L1 expression, whereas other subtypes had varying levels of PD-L1 expression on TAMs without true PD-L1 expression on tumor cells themselves. This finding was further confirmed on a subset of seminoma cases with a dual stain for OCT3/4 and PD-L1. There is a growing evidence in the gynecologic pathology literature showing PD-L1 expression in trophoblastic tumors and choriocarcinomas,[17,18] and our findings expand on that within the realm of testicular GCTs. It has been shown that human trophoblastic cells, including placental tissue, express PD-L1,[18] and it has been speculated that this expression may in fact be constitutive in trophoblastic derived cells.

Since prior studies did not separate tumor cells from macrophages when evaluating PD-L1 expression, it is feasible that at least some of the PD-L1 staining captured in these data was in fact on the intratumoral macrophages that were intimately associated and circumferentially surrounding tumor cells. For example, Fankhauser and colleagues[13] performed PD-L1 staining on tissue microarrays but did not use cell-specific staining to distinguish macrophages from tumor cells and did not provide paired H&E images, and thus it is challenging to confidently ascertain from their images which cells are in fact staining with PD-L1. Nonetheless, in their Figure 1B, it appears that septal macrophages are strongly staining with PD-L1 in a pattern that corresponds to the second phenotypic group of PD-L1+ macrophages in the current study. Other studies similarly have used tissue microarrays and lack any detailed distinction between the cell type(s) that show staining with PD-L1.[14,15] Since tissue microarrays only sample a minute segment of the tumor and since the expression of PD-L1 may be patchy, the data from TMAs at best tell only part of the story. Our approach of staining whole sections enabled us to conduct an in-depth study of the relationship of the tumor cells to the inflammatory component, with a special focus on macrophages. There is a growing body of evidence that macrophages play critical roles in various stages of tumor progression and can have both protumoral and antitumoral effects.[19] TAMs can limit the antitumoral activity of conventional chemotherapy and radiotherapy. Based on such findings, TAM-centered approaches to anticancer therapy are under investigation and include inhibition of macrophage recruitment to and/or survival in tumors, functional reeducation of TAMs to an antitumor, "M1-like mode," manipulation of macrophage-mediated extracellular killing, or phagocytosis and intracellular destruction of cancer cells.[20] Our study suggests that GCTs are rich in TAMs in primary seminomas and NSGCTs and may play an important role in subsequent disease recurrence.

We also investigated the status of MMR protein expression and the possibility of MMR deficiency in testicular GCTs and found that all cases of seminomas and NSGCTs in our cohort showed retained MMR by IHC. Although few early studies had reported a subset of testicular GCTs can exhibit microsatellite instability and MMR deficiency,[21,22] more recent studies have found retained MMR via IHC.[23] In keeping with our findings, these results may in part be due to a difference in assessment of MMR loss in earlier studies, where "low staining" or "reduction in signal" may have at the time been interpreted

DOI: 10.1093/AJCP/AQZ184

as loss, while our evaluation required complete loss of nuclear staining in all tumor cells to be considered deficient. Furthermore, it is possible that some cases with microsatellite instability pathways have retained MMR antigenicity while having lost protein function. Our study would not have detected such potential cases. Our overall data may suggest that microsatellite instability does not play a significant role in testicular germ cell neoplasia.

There is a small clinical literature showing mixed results of immune checkpoint blockade for GCT with both a successful case report and a larger clinical trial that did not show significant activity of single-agent immune blockade in refractory nonseminomatous GCTs.[24,25] Another study reported results of PD-1 inhibitor use in a small cohort of patients with GCT and showed promising effects in a subset.[16] Our findings build on the notion that careful analysis of the immune microenvironment, particularly with respect to PD-L1–expressing macrophages, might help stratify patients who may benefit from immunotherapeutics. Future directions include investigating a larger cohort for a stronger powered study and ultimately further expanding our understanding of the role of the immune microenvironment in testicular GCT behavior.

Corresponding author: Priti Lal, MD; priti.lal@pennmedicine.upenn.edu.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin.* 2018;68:7-30.
2. Motzer RJ, Sheinfeld J, Mazumdar M, et al. Paclitaxel, ifosfamide, and cisplatin second-line therapy for patients with relapsed testicular germ cell cancer. *J Clin Oncol.* 2000;18:2413-2418.
3. Einhorn LH, Williams SD, Chamness A, et al. High-dose chemotherapy and stem-cell rescue for metastatic germ-cell tumors. *N Engl J Med.* 2007;357:340-348.
4. Riggs SB, Burgess EF, Gaston KE, et al. Postchemotherapy surgery for germ cell tumors–what have we learned in 35 years? *Oncologist.* 2014;19:498-506.
5. Marshall AH, Dayan AD. An immune reaction in man against seminomas, dysgerminomas, pinealomas, and the mediastinal tumours of similar histological appearance? *Lancet.* 1964;2:1102-1104.
6. Bols B, Jensen L, Jensen A, et al. Immunopathology of in situ seminoma. *Int J Exp Pathol.* 2000;81:211-217.
7. Torres A, Casanova JF, Nistal M, et al. Quantification of immunocompetent cells in testicular germ cell tumours. *Histopathology.* 1997;30:23-30.
8. Hollern DP, Hoadley K, Vincent B, et al. A genomic characterization of testicular germ cell tumor immune microenvironment. *Cancer Res.* 2017;77:3703. Abstract 3703.
9. Chaudhary B, Elkord E. Regulatory T cells in the tumor microenvironment and cancer progression: role and therapeutic targeting. *Vaccines (Basel).* 2016;4:28.
10. Takenaka M, Seki N, Toh U, et al. FOXP3 expression in tumor cells and tumor-infiltrating lymphocytes is associated with breast cancer prognosis. *Mol Clin Oncol.* 2013;1:625-632.
11. Cavalleri T, Bianchi P, Basso G, et al; Alleanza contro il Cancro (ACC) Colorectal Cancer Working Group. Combined low densities of FoxP3+ and CD3+ tumor-infiltrating lymphocytes identify stage II colorectal cancer at high risk of progression. *Cancer Immunol Res.* 2019;7:751-758.
12. Siska PJ, Johnpulle RAN, Zhou A, et al. Deep exploration of the immune infiltrate and outcome prediction in testicular cancer by quantitative multiplexed immunohistochemistry and gene expression profiling. *Oncoimmunology.* 2017;6:e1305535.
13. Fankhauser CD, Curioni-Fontecedro A, Allmann V, et al. Frequent PD-L1 expression in testicular germ cell tumors. *Br J Cancer.* 2015;113:411-413.
14. Chovanec M, Cierna Z, Miskovska V, et al. Prognostic role of programmed-death ligand 1 (PD-L1) expressing tumor infiltrating lymphocytes in testicular germ cell tumors. *Oncotarget.* 2017;8:21794-21805.
15. Jennewein L, Bartsch G, Gust K, et al. Increased tumor vascularization is associated with the amount of immune competent PD-1 positive cells in testicular germ cell tumors. *Oncol Lett.* 2018;15:9852-9860.
16. Zschäbitz S, Lasitschka F, Hadaschik B, et al. Response to anti-programmed cell death protein-1 antibodies in men treated for platinum refractory germ cell cancer relapsed after high-dose chemotherapy and stem cell transplantation. *Eur J Cancer.* 2017;76:1-7.
17. Lu B, Teng X, Fu G, et al. Analysis of PD-L1 expression in trophoblastic tissues and tumors. *Hum Pathol.* 2019;84:202-212.
18. Veras E, Kurman RJ, Wang TL, et al. PD-L1 expression in human placentas and gestational trophoblastic diseases. *Int J Gynecol Pathol.* 2017;36:146-153.
19. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity.* 2014;41:49-61.
20. Mantovani A, Marchesi F, Malesci A, et al. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol.* 2017;14:399-416.
21. Mayer F, Gillis AJ, Dinjens W, et al. Microsatellite instability of germ cell tumors is associated with resistance to systemic treatment. *Cancer Res.* 2002;62:2758-2760.
22. Velasco A, Riquelme E, Schultz M, et al. Mismatch repair gene expression and genetic instability in testicular germ cell tumor. *Cancer Biol Ther.* 2004;3:977-982.
23. Al-Obaidy KI, Trevino KE, Idrees MT. Clinicopathologic characterization of bilateral testicular germ cell tumors with immunohistochemical evaluation of mismatch repair and *BRAF* (V600E) genes mutations. *Int J Surg Pathol.* 2019;27:619-623.
24. Chi EA, Schweizer MT. Durable response to immune checkpoint blockade in a platinum-refractory patient with nonseminomatous germ cell tumor. *Clin Genitourin Cancer.* 2017;15:e855-e857.
25. Adra N, Einhorn LH, Althouse SK, et al. Phase II trial of pembrolizumab in patients with platinum refractory germ-cell tumors: a Hoosier Cancer Research Network Study GU14-206. *Ann Oncol.* 2018;29:209-214.

DOI: 10.1093/AJCP/AQZ184