Cancer Immunity (15 January 2013) Vol. 13, p. 1

Cancer Immunity
Article

Intraepithelial T cells and tumor-associated macrophages in ovarian cancer patients

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The aims of this study were to evaluate the prognostic significance of tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) in patients with familial ovarian cancer. Clinical and pathological information were retrieved from the Gilda Radner Familial Ovarian Cancer Registry (GRFOCR) in Buffalo, NY. Immunohistochemistry was performed on paraffin-embedded tissue specimens of GRFOCR participants using specific antibodies for CD3+, CD8+, CD25+, FOXP3+, CD68+, and CD163+. The correlation between the frequencies of TILs and TAMs and clinic-pathologic parameters were determined. Overall survival was determined using univariate and multivariate Cox proportional hazards models. High tumor grade correlated with higher frequencies of CD3+ (p = 0.019), CD68+ (p = 0.025), CD163+ (p = 0.018), and T_{reg} (CD25+ FOXP3+) (p = 0.024) cells. Higher stage correlated with higher frequencies of CD163+ cells (p = 0.012). There were correlations between the frequencies of CD68+ and CD3+ (p = 0.029), between T_{reg} and each of CD3+ (p = 0.002), CD8+ (p = 0.018), and CD68+ (p = 0.028) cells. In univariate analysis, age and T_{reg} significantly predicted patient survival. In multivariate survival analysis, T_{reg} frequency was the only significant predictor of prognosis in patients with familial ovarian cancer [HR = 0.92; 95% CI 0.87 - 0.98; p = 0.012]. We concluded that interaction between TILs and TAMs in familial EOC also exists, and tumors with high T_{reg} frequencies have a more favorable outcome. Thus, therapeutic strategies to modulate tumor T_{reg} infiltration could be beneficial for patients with familial ovarian cancer.

Keywords: familial ovarian cancer, CD count, patient outcome

Introduction

Epithelial ovarian carcinoma (EOC) is the fourth leading cause of cancer mortality among women in western countries (1). The incidence of newly diagnosed EOC in the US is estimated to be 22,430 cases per year, with 15,280 deaths (1). The strongest risk factor for ovarian cancer is the presence of an inherited mutation in one of the two ovarian cancer susceptibility genes, BRCA1 or BRCA2. It is estimated that more than 10% of women in North America with invasive ovarian cancer carry a BRCA1 or BRCA2 mutation (2, 3). The majority of these mutation carriers come from high-risk families where several members are affected by breast and/or ovarian cancer. It is estimated that 30% of families with a strong history of breast and ovarian cancers carry one of these mutations (4), but only one in 300 to 500 people in the general population carry the genes (5). While it is clear that alterations in BRCA1 and BRCA2 genes are important for susceptibility to ovarian cancer, it is also well-known that the immune system has the ability to protect the host from cancer and "sculpt" tumor immunogenicity (6, 7). This interaction between the immune system and cancer is complex, consisting of a very delicate balance between immune activation and immune suppression mechanisms (8).

Immune surveillance plays an important role in tumor outcome in patients diagnosed with EOC (9-17). In the study by Sato *et al.*, intraepithelial CD8+ tumor-infiltrating lymphocytes (TILs) and high CD8+/regulatory T cell (T_{reg}) ratio were shown to be associated with favorable prognosis in ovarian cancer (17). The favorable prognosis of intraepithelial CD8+ cells has been confirmed by other studies (11, 12). In contrast to TILs, tumor-associated macrophages (TAMs) have been reported to be associated with poor outcome in EOC (14), suggesting that they might play an immune suppressive role. Thus, TILs and TAMs could interact to modulate and shape the overall immune environment in EOC.

In an effort to define the role of TILs and TAMs in familial EOC using the Gilda Radner Familial Ovarian Cancer Registry (GRFOCR), the goals of our study were (i) to evaluate the presence of TILs (CD3+, CD8+, T_{reg}) and TAMs (CD68+, CD163+) in patients with familial EOC; (ii) to determine the interaction between TILs and TAMs; and (iii) to define the role of TILs and TAMs in the outcome of patients with familial EOC.

Briefly, the GRFOCR is a self-referred registry of families with two or more ovarian cancer cases in blood relatives. It was established in 1981 at the Roswell Park Cancer Institute (RPCI) by Dr. M. Steven Piver to study the incidence of familial ovarian cancer (18-21). The primary function of the registry is to receive family cancer information voluntarily contributed throughout the US by ovarian cancer patients, referring and concerned physicians, concerned women, and patients of the RPCI Gynecologic Oncology Department. The objectives of the registry include (i) obtaining detailed family histories from individuals who are apparently from families with two or more cases of ovarian cancer, or a syndrome possibly related to ovarian cancer; (ii) documenting through medical records and through pathologist review of tissues the occurrence of cancer; (iii) collecting, processing, and storing biological samples, when possible, from registry participants; and (iv) making the information and biological samples available for research under Institutional Review Board (IRB)-approved research protocols. Registry participants are recruited through probands or index persons, and meet at least one of the following criteria: (i) family

Table 1

Clinical and histopathologic characteristics of 73 patients.

No. of evaluable patients		73
Age, years		
	Median	52
	Range	26-80
	Median overall survival,* months	58.81
Histologic diagnosis		
	Serous adenocarcinoma	44 (60)
	Endometrioid adenocarcinoma	21 (29)
	Clear cell carcinoma	3 (4)
	Mucinous adenocarcinoma	2 (3)
	Transitional cell carcinoma	2 (3)
	Undifferentiated carcinoma	1 (1)
Grade		
	1	5 (7)
	2	13 (18)
	3	55 (75)
Stage		
	I	16 (22)
	II	9 (12)
	III	45 (62)
	IV	3 (4)
Status		
	Death	30 (41)
	Alive	43 (59)

Data in parentheses are percentages

history of two or more cases of ovarian cancer; (ii) family history of one case of ovarian cancer and two cases of cancer at any other site; (iii) family history of at least one female with two or more primary tumors with one of the primaries being ovarian cancer; and (iv) family history of two or more cases of cancer with at least one case being ovarian cancer, and the other cancer considered to be of early onset (≤ 45 years old).

Results

Study population and tumor characteristics

Seventy-three patients with adequate tissue material were available for analysis of TILs and TAMs. Patient population and tumor characteristics are presented in Table 1. The age ranged from 26-80 years with a median age of 52. As expected, the majority of patients had FIGO Stage III disease (62%), tumor grade 3 (75%), and serous histology (60%). After a median duration of follow-up of 24.91 months, 30 patients were dead of disease and 43 were alive. The median overall survival (OS) was 58.81 months. Eleven of the 73 patients had BRCA mutation data (five had BRCA1 mutation and six had BRCA2 mutation) and the remaining 62 patients had undefined BRCA mutation status. Therefore, due to the small number of patients with information on BRCA mutation status, we did not further explore any potential association with TILs or TAMs.

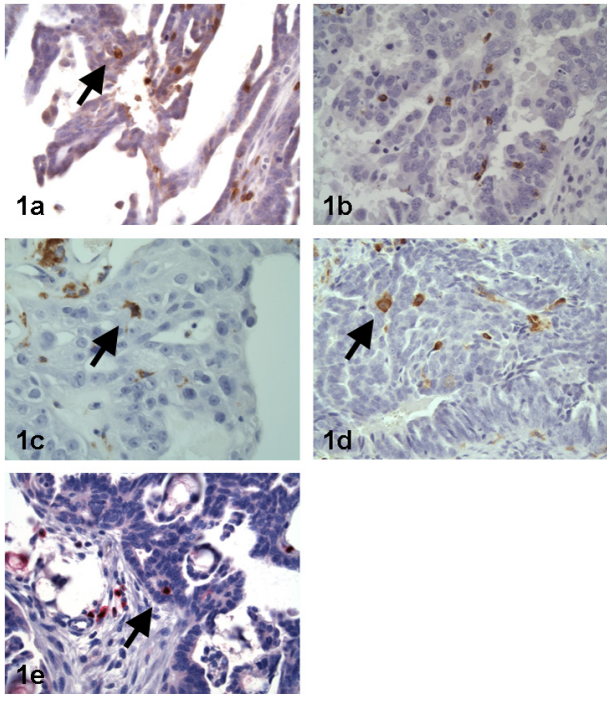
TILs and TAMs in familial ovarian cancer

TILs and TAMs in the intraepithelial compartments were counted, as illustrated in Figure 1. The mean cell counts were 55.60±56.13; 62.29±69.77; 27.60±21.46; 30.00±22.95; and 9.56±9.32 for CD3+; CD8+; CD68+; CD163+; and T_{reg} cells, respectively (Figure 2, Supplementary Table 1). Next, we tested the association between the markers of immune cell subsets. As expected, and shown in Table 2, the frequency of CD3+ cells was positively correlated with CD8+ ($p < 0.001$), CD68+ ($p = 0.029$), and T_{reg} ($p = 0.002$) cells. The frequency of CD8+ T cells was also positively correlated with the frequencies of T_{reg} ($p = 0.018$). Furthermore, CD68+ was positively correlated with CD163+ ($p < 0.001$) and T_{reg} ($p = 0.028$). Using the two sample *t*-tests analysis as shown in Table 3, we found that FIGO stage was negatively associated with CD8+ T cell frequency ($p = 0.042$) and positively associated with CD163+ cells ($p = 0.012$). On the other hand, tumor grade was positively associated with CD3+ ($p = 0.019$), CD68+ ($p = 0.025$), CD163+ ($p = 0.018$), and T_{reg} ($p = 0.024$) cells.

Prognostic significance of TILs and TAMs

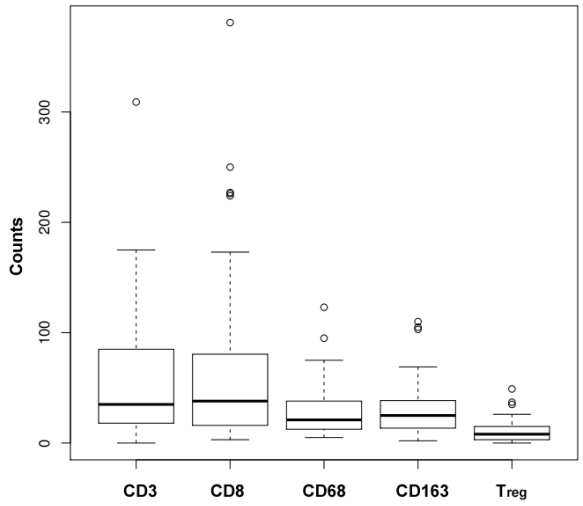
With regard to patient outcome, 30 patients were dead of disease. However, information on definite date of death was missing from six of these patients and they were excluded from further analysis. Thus, we ended with 67/73 with adequate follow-up information. In univariate analysis (Table 4), advanced age was found to be significantly associated with poorer overall survival ($p = 0.002$) among the clinicopathological variables. Using TIL and TAM counts, T_{reg} was the only parameter to be significantly associated with overall survival in univariate analysis ($p = 0.033$, Supplementary Figure 1). The hazard ratio of T_{reg} 0.94, indicated that it was associated with favorable prognosis. In multivariate survival analysis adjusting all clinicopathological covariates (Table 5), the only parameter that appeared to be a significant predictor of poor survival was T_{reg} ($p = 0.012$). The hazard ratio of T_{reg} 0.92 (95% CI = [0.87, 0.98]), suggested that it was associated with improved survival.

Figure 1



Representative illustrations of intraepithelial infiltration by lymphocytes and macrophages in epithelial ovarian cancer. Immunohistochemistry performed for (A) CD3+; (B) CD8+; (C) CD68+; (D) CD163+; (E) T_{reg} (40X magnification).

Figure 2



Box plot for TIL or TAM counts on 73 patients.

Table 2

Correlation between the counts of TILs or TAMs of 67 cases.

		CD3	CD8	CD68	CD163	T_{reg}
	Correlation coefficient		0.560	0.270	0.190	0.370
CD3	*p* value*		**< 0.001**	**0.029**	0.124	**0.002**
	Correlation coefficient			0.160	0.010	0.290
CD8	*p* value			0.205	0.943	**0.018**
	Correlation coefficient				0.610	0.270
CD68	*p* value				**< 0.001**	**0.028**
	Correlation coefficient					0.210
CD163	*p* value					0.091

* *p* values less than 0.05 are marked in bold

Table 3

Association between TIL or TAM counts and FIGO stage and tumor grade.

Association		*p* value*	Mean_G1	Mean_G2
CD3	Stage^	0.212	65.35	53.45
	Grade^	**0.019**	37.35	64.40
CD8	Stage	**0.042**	88.13	50.32
	Grade	0.356	56.71	65.54
CD68	Stage	0.253	25.78	29.09
	Grade	**0.025**	19.88	30.70
CD163	Stage	**0.012**	22.00	33.02
	Grade	**0.018**	18.00	33.06
T_{reg}	Stage	0.477	10.09	9.93
	Grade	**0.024**	6.47	11.18
	Grade	**0.024**	6.47	11.18

^ The stage was regrouped as G1=I/II, and G2 as III/IV
^ The grade was regrouped as group 1 as G1 and G2, and group 2 as G3
* *p* values less than 0.05 are marked in bold

Discussion

There is a higher risk of ovarian cancer in individuals with a family history of one or more affected first degree relatives with ovarian cancer. While the lifetime risk of EOC in the general population is approximately 1 in 100, this risk increases to 1 in 40 if a first degree relative is affected by ovarian cancer (25). Indeed, an unaffected woman with 2-3 relatives with ovarian cancer had a 4.6 times increased risk compared to the general population risk, i.e., a 7.2% lifetime probability of ovarian cancer (25). Since it is also now clear that the immune system protects against development of cancer (7), our study addressed, for the first time, a potential link between anti-cancer immunity and progression of familial ovarian cancer.

TILs and TAMs are considered major components of the cellular immune response against cancer. While numerous studies have shown infiltration of ovarian cancer by CD8+ TILs to be positively associated with survival (9, 17, 26), multiple immune suppressive mechanisms have also been shown to counteract the beneficial effects of CD8+ T cells. These mechanisms include T_{reg} that mediate homeostatic peripheral tolerance by suppressing autoreactive T cells (27, 28); co-inhibitory molecules such as PD-1 and LAG-3 (8); tolerance-inducing plasmacytoid dendritic cells (pDCs) (18); B7-H4+ macrophages (15, 16, 29); immune suppressive cytokines such as TGF-β (30); and myeloid-derived suppressor cells (MDSCs) (31). The majority of these mechanisms are orchestrated by ovarian cancer cells in order to create a microenvironment that favors escape from immune elimination (32).

In familial EOC, we showed considerable amounts of TILs and TAMs in the intraepithelial compartment, indicating that the immune response is still triggered in this population of ovarian cancer patients. We also showed evidence of a significant relationship among TIL subsets, and between TILs and TAMs. The relationship seen between T_{reg} and CD68+ would suggest an interaction between macrophages and T_{reg} in order to contribute to the profound suppressive environment in ovarian cancer. In support of this observation is a previous report by Kryczek *et al.* which demonstrated interaction between macrophages and T_{regs} in sporadic EOC (16). Furthermore, we found that as grade of ovarian tumors increased, the number of intraepithelial tumor-infiltrating CD3+, CD68+, CD163+, and T_{reg} cells also increased. In line with this observation, we found that as tumors became more advanced, there was higher tumor infiltration by CD163+ cells.

T_{reg}, as documented by CD25+ FOXP3+ cells in our study, are a specific population of T cells with immunosuppressive properties. T_{reg} normally function as suppressors of inappropriate immune responses, preventing any unwanted autoimmune reactions (33, 34). Previous studies demonstrated an increase of T_{reg} counts in late-stage and in high-grade EOC (10, 11). Also, infiltration of tumor cells by T_{reg} seemed to suppress tumor-specific T cell immune response, leading to tumor cell growth. Infiltration of tumor cells by T_{reg} was shown to be associated with poor survival in patients with EOC and in other solid tumors such as hepatocellular carcinoma (10, 17, 35). However, this finding was not confirmed by others (13, 34). The difference of results among studies could be mainly due to the methods used in the analysis of T cell subsets. Sato *et al.* found that high CD8+/T_{reg} count ratio to be the strongest predictive factor for prolonged survival, with a nearly 70% reduction of mortality rate in patients with EOC. In our study on familial EOC, we found that among all immune cell populations and their ratios, T_{reg} was the only independent prognostic factor in predicting favorable outcome in familial EOC. This finding is the first in the literature in familial EOC and it is consistent with the reports by others, where similar results were described in patients with sporadic EOC (13, 14).

Lastly, there are several limitations of the present study—the GRFOCR is a self-referred registry; the documentation of residual disease after optimal debulking (one of the prognostic factors in EOC) was not assessed in this study. The differences in clinical characteristics, together with the relatively small sample

size used in this study, might contribute to the difference between prior studies of CD8+ T cells and our study. Also, disease-free survival could not be documented as the registry only knows whether a registry member is alive or dead. However, despite these limitations, our study is the first to shed some light on the role of the immune system in familial ovarian cancer.

In summary, our study supported a role for the immune system in the progression of familial ovarian cancer. Since the frequency of intratumoral T_{reg} was the most significant predictor of outcome in this population of patients, future studies are warranted to examine the relationship between the known ovarian cancer predisposition genes and genes that regulate T_{reg} differentiation and expansion.

Table 4
Univariate Cox proportional hazard analysis of 67 patients.

		Cox Survival	
Variable	Hazard Ratio	95% C.I.	*p* value*
Age, years	1.06	1.02-1.09	**0.002**
Histology (serous. vs. others)	0.79	0.35-1.78	0.565
Grade (3 vs. 1,2)	1.60	0.55-4.66	0.393
Stage (III/IV vs. I/II)	1.85	0.73-4.64	0.193
CD3	0.99	0.99-1.01	0.459
CD8	1.00	0.99-1.01	0.120
CD68	1.00	0.98-1.02	0.706
CD163	1.01	0.99-1.02	0.835
T_{reg}	0.94	0.89-1.00	**0.033**
CD8/CD3^	0.74	0.47-1.17	0.198
T_{reg}/CD3^	0.57	0.19-1.69	0.311
T_{reg}/CD8	0.54	0.12-2.40	0.421
CD163/CD68	1.14	0.85-1.51	0.390

^ One extra case was deleted in the analysis of CD8/CD3 and T_{reg}/CD3 due to the zero value in CD3 counts
* *p* values less than 0.05 are marked in bold

Abbreviations

TIL, tumor-infiltrating lymphocyte; TAM, tumor-associated macrophage; GRFOCR, Gilda Radner Familial Ovarian Cancer Registry; CD, cluster of differentiation; T_{reg}, regulatory T cell; EOC, epithelial ovarian cancer

Acknowledgements

We would like to thank Mrs. Cathy Fahey for her help and assistance in providing the data and specimens. Also, we thank Mrs. Mary Vaughn for her expertise in immunohistochemistry. This work was funded by the Gilda Radner Familial Ovarian Cancer Registry, Buffalo, NY.

References

1. Siegel R, Naishadham D, Jemal A. Cancer Statistics, 2012. *CA Cancer J Clin* 2012; **62:** 10-29. (PMID: 22237781)

2. Berchuck A, Heron KA, Carney ME, Lancaster JM, Fraser EG, Vinson VL, Deffenbaugh AM, Miron A, Marks JR, Futreal PA, Frank TS. Frequency of germline and somatic BRCA1 mutations in ovarian cancer. *Clin Cancer Res* **1998: 4:** 2433-2437. (PMID: 9796975)

3. Risch HA, McLaughlin JR, Cole DE, Rosen B, Bradley L, Kwan E, Jack E, Vesprini DJ, Kuperstein G, Abrahamson JL, Fan I, Wong B, Narod SA. Prevalence and penetrance of germline BRCA1 and BRCA2 mutations in a population series of 649 women with ovarian cancer. *Am J Hum Genet* 2001; **68:** 700-710. (PMID: 11179017)

4. James PA, Doherty R, Harris M, Mukesh BN, Milner A, Young MA, Scott C. Optimal selection of individuals for BRCA mutation testing: a comparison of available methods. *J Clin Oncol* 2006; **24:** 707-715. (PMID: 16446345)

5. Nelson HD, Huffman LH, Fu R, Harris EL. Genetic risk assessment and BRCA mutation testing for breast and ovarian cancer susceptibility: systematic evidence review for the U.S. Preventive Services Task Force. *Ann Intern Med* 2005; **143:** 362-379. (PMID: 16144895)

6. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol* 2004; **22:** 329-360. (PMID: 15032581)

7. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 2004; **21:** 137-148. (PMID: 15308095)

8. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, Beck A, Miller A, Tsuji T, Eppolito C, Qian F, Lele S, Shrikant P, Old LJ, Odunsi K. Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. *Proc Natl Acad Sci U S A* 2010; **107:** 7875-7880. (PMID: 20385810)

9. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 2003; **348:** 203-213. (PMID: 12529460)

10. Leffers N, Gooden MJM, de Jong RA, Hoogeboom BN, ten Hoor KA, Hollema H, Boezen HM, van der Zee AG, Daemen T, Nijman HW. Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer. *Cancer Immunol Immunother* 2009; ; **58:** 449-459. (PMID: 18791714)

11. Stumpf M, Hasenburg A, Reiner MO, Jütting U, Wang C, Shen Y, Orlowska-Volk M, Fisch P, Wang Z, Gitsch G, Werner M, Lassmann S. Intraepithelial CD8-positive T lymphocytes predict survival for pateints with serous stage III ovarian carcinomas: relevance of clonal selection of T lymphocytes. *Br J Cancer* 2009; **101:** 1513-1521. (PMID: 19861998)

12. Adams SF, Levine DA, Cadungog MG, Hammond R, Facciabene A, Olvera N, Rubin SC, Boyd J, Gimotty PA, Coukos G. Intraepithelial T cells and tumor proliferation: impact on the benefit from surgical cytoreduction in advanced serous ovarian cancer. *Cancer* 2009; **115:** 2891-2902. (PMID: 19472394)

13. Milne K, Köbel M, Kalloger SE, Barnes RO, Gao D, Gilks CB, Watson PH, Nelson BH. Systemic analysis of immune infiltrates in high-grade serous ovarian cancer reveals CD20, FoxP3 and TIA-1 as positive prognostic factors. *PLoS One* 2009; **4:** e6412. (PMID: 19641607)

14. Shah CA, Allison KH, Garcia RL, Gray HJ, Goff BA, Swisher EM. Intratumoral T cells, tumor-associated macrophages, and regulatory T cells: association with *p53* mutations, circulating tumor DNA and survival in women with ovarian cancer. *Gynecol Oncol* 2008; **109:** 215-219. (PMID: 18314181)

15. Kryczek I, Zou L, Rodriguez P, Zhu G, Wei S, Mottram P, Brumlik M, Cheng P, Curiel T, Myers L, Lackner A, Alvarez X, Ochoa A, Chen L, Zou W. B7-H4 expression identifies a novel suppressive

macrophage population in human ovarian carcinoma. *J Exp Med* 2006; **203:** 871-881. (PMID: 16606666)

16. Kryczek I, Wei S, Zhu G, Myers L, Mottram P, Cheng P, Chen L, Coukos G, Zou W. Relationship between B7-H4, regulatory T cells, and patient outcome in human ovarian carcinoma. *Cancer Res* 2007; **67:** 8900-8905. (PMID: 17875732)

17. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C, Kepner J, Odunsi T, Ritter G, Lele S, Chen YT, Ohtani H, Old LJ, Odunsi K. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A* 2005; **102:** 18538-18543. (PMID: 16344461)

18. Piver MS, Mettlin CJ, Tsukada Y, Nasca P, Greenwald P, McPhee ME. Familial Ovarian Cancer Registry. *Obstet Gynecol* 1984; **64:** 195-199. (PMID: 6738953)

19. Piver MS, Goldberg JM, Tsukada Y, Mettlin CJ, Jishi MF, Natarajan N. Characteristics of familial ovarian cancer: a report of the first 1,000 families in the Gilda Radner Familial Ovarian Cancer Registry. *Eur J Gynaecol Oncol* 1996; **17:** 169-176. (PMID: 8780913)

20. Piver MS, Tsukada Y, Werness BA, DiCioccio RA, Whittemore AS, Ponder BA. Comparative study of ovarian cancer histopathology by registry pathologists and referral pathologists: a study by the Gilda Radner Familial Ovarian Cancer Registry. *Gynecol Oncol* 2000; **78:** 166-170. (PMID: 10926797)

21. Piver MS. Hereditary ovarian cancer. Lessons from the first twenty years of the Gilda Radner Familial Ovarian Cancer Registry. *Gynecol Oncol* 2002; **85:** 9-17. (PMID: 11925114)

22. Piver MS, Rose PG, Freedman MF. Change in International Federation of Gynecology and Obstetrics staging. *Am J Obstet Gynecol* 1988; **158:** 678-679. (PMID: 3348332)

23. The International Agency for Research on Cancer. *World Health Organization classification of tumours. Pathology and genetics of tumours of the breast and female genital organs.* Tavassoli FA, Devilee P. (Eds.) Lyon: IARC Press-WHO; 2003; 114-115.

24. Silverberg SG. Histopathologic grading of ovarian carcinoma: a review and proposal. *Int J Gynecol Pathol* 2000; **19:** 7-15. (PMID: 10638449)

25. Kerlikowske K, Brown JS, Grady DG. Should women with familial ovarian cancer undergo prophylactic oophorectomy? *Obstet Gynecol* 1992; **80:** 700-707. (PMID: 1407898)

26. Tomsová M, Melichar B, Sedláková I, Steiner I. Prognostic significance of CD3+ tumor-infiltrating lymphocytes in ovarian carcinoma. *Gynecol Oncol* 2008; **108:** 415-420. (PMID: 18037158)

27. Salvi S, Segalla F, Rao S, Arienti F, Sartori M, Bratina G, Caronni E, Anichini A, Clemente C, Parmiani G, Sensi M. Overexpression of the T-cell receptor beta-chain variable region TCRBV14 in HLA-A2-matched primary human melanomas. *Cancer Res* 1995; **55:** 3374-3379. (PMID: 7614474)

28. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; **10:** 942-949. (PMID: 15322536)

29. Chen W, Liang X, Peterson AJ, Munn DH, Blazar BR. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. *J Immunol* 2008; **181:** 5396-5404. (PMID: 18832696)

30. Wahl SM, Swisher J, McCartney-Francis N, Chen W. TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. *J Leukoc Biol* 2004; **76:** 15-24. (PMID: 14966194)

31. Serafini P, De Santo C, Marigo I, Cingarlini S, Dolcetti L, Gallina G, Zanovello P, Bronte V. Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol Immunother* 2004; **53:** 64-72. (PMID: 14593498)

32. Yigit R, Massuger LF, Figdor CG, Torensma R. Ovarian cancer creates a suppressive microenvironment to escape immune elimination. *Gynecol Oncol* 2010; **117:** 366-372. (PMID: 20144842)

33. Dietl J, Engel JB, Wischhusen J. The role of regulatory T cells in ovarian cancer. *Int J Gynecol Cancer* 2007; **17:** 764-770. (PMID: 17309663)

34. Barnett B, Kryczek I, Cheng P, Zou W, Curiel TJ. Regulatory T cells in ovarian cancer: biology and therapeutic potential. *Am J Reprod Immunol* 2005; **54:** 369-377. (PMID: 16305662)

35. Sasaki A, Tanaka F, Mimori K, Inoue H, Kai S, Shibata K, Ohta M, Kitano S, Mori M. Prognostic value of tumor-infiltrating FOXP3+ regulatory cells in patients with hepatocellular carcinoma. *Eur J Surg Oncol* 2009; **34:** 173-179. (PMID: 17928188)

36. *The R Project.* Accessed from: http://www.r-project.org/

Materials and methods

Patient population

Study subjects for this work derived from the Gilda Radner Familial Ovarian Cancer Registry (formerly referred to as the Familial Ovarian Cancer Registry) under a protocol that has been reviewed and approved by the Roswell Park Cancer Institute IRB.

Patients were included in our study only when a staging procedure was performed at the time of the original surgery, or within the following months using the FIGO (International Federation of Gynecology and Obstetrics) staging criteria (22). All patients received standard care for EOC consisting of cytoreductive surgery, followed by platinum-based chemotherapy. At follow-up, patients had two outcomes: alive (A) or dead of disease (DOD). Information on the status of the residual disease or time to disease relapse was not available.

Patient samples

Hematoxylin and eosin (H&E) slides were available for histologic review. The tumor subtypes and grade were re-reviewed for confirmation by one experienced pathologist (PM-F). Histologic subtypes were based on World Health Organization (WHO) classification of tumors (23). The histologic grade was determined according to criteria used by

the Silverberg's grading system (24).

Immunohistochemistry

Four μm thick sections were deparaffinized with xylene, and washed with ethanol. Sections were cooled for 20 min and then incubated for 10 min with 3% H_2O_2 to quench endogenous peroxidase activity. Blocking was performed using serum-free protein block, Dakocytomation (Carpenteria, CA) for 30 min. A pretreatment with Tris Buffer saline solution/EDTA, high pH, for 10 min was used for CD3+, CD8+, CD68+ and steamer for CD163+ staining. Afterwards, sections were incubated with antibodies for 30 min at room temperature for CD3+, CD8+, CD68+, and for 60 min for CD163+. The description of the antibodies used are as follows: CD3+ (ready to use; polyclonal; 1:100; Dakocytomation), CD8+ (clone CD8/114B; monoclonal; 1:200; Dakocytomation), CD68+ (clone PG-M1; monoclonal; 1:3000; Dakocytomation), CD163+ (clone 10D6; monoclonal; 1:50; Novacastra, Cambridge, UK), CD25+ (clone 4C9; monoclonal; 1:50; Vector Labs, Burlingame, CA), and anti-FOXP3 (clone 259D; polyclonal; 1:20; BioLegend, San Diego, CA). Diaminobenzidine tetrahydrochloride was then added for development for 10 min, followed by counterstaining with hematoxylin solution. T_{reg} was defined as CD25+ FOXP3+ cells and, for this purpose, a dual staining procedure for CD25+ FOXP3+ was performed as described previously (17). Negative control slides omitting the primary antibody were included in all assays.

Quantification of TILs and TAMs

Evaluation of the IHC slides was performed by two pathologists independently (PM-F and LA). At first, the entire tumor section was evaluated by using 20X objective lens on an Olympus microscope (Olympus, Holly, MI). Ten independent areas with the most abundant intraepithelial T cell infiltrates (CD3+, CD8+, T_{reg}) and intraepithelial tumor-associated macrophages (CD68+, CD163+) were selected. Intraepithelial tumor infiltrates is defined by macrophages and T cells highlighted by immunohistochemistry that are seen in the epithelial tumor cells. Those seen in the stroma were not counted. The count is done at 40X magnification. Each pathologist counted 10 areas and scored their counts. The scores of the two pathologists were compared and when a discrepancy (count difference of more than 10 cells infiltrates) was noticed, the two pathologists sat down at a double head microscope to solve the issue and reach a final score. Disagreement was not very frequent between the first and the second read and occurred roughly around 5% of the cases. The mean number of tumor infiltrates per 10 high power fields was evaluated as continuous variable.

Statistical analysis

Statistical analyses were performed by The R Project (36). The clinical parameters used for modeling are age, stage, grade, and counts of TILs and TAMs. To find the association between the CD counts and FIGO stage and tumor grade, two sample *t*-tests were performed to determine if two groups of cluster of differentiation (CD) counts differ significantly in different categories. Univariate and multivariate Cox proportional hazards models were used to estimate hazard ratio that represents the correlation between relative risk of death among patients and clinical parameters. The *p* value for the relevant parameter is derived from the Wald test. The proportional hazards assumption for a Cox model fit is tested using the cox.zph function in The R Project. Fair proportional assumption holds for each variable ($p > 0.017$) except CD163+. CD163+ is not a significant variable using either Cox proportional hazards model ($p = 0.835$, shown in Table 5) or logistic model ($p = 0.594$, data not shown). Hence, Cox proportional hazards models were used throughout the study. Pearson correlation coefficients are calculated to access the relationship of TIL and TAM counts. A *p* value of < 0.05 is considered statistically significant.

Table 5
Multivariate Cox proportional hazard analysis of 67 patients.

		Cox Survival	
Variable	**Hazard Ratio**	**95% C.I.**	***p* value***
CD3	0.99	0.99-1.01	0.671
CD8	0.99	0.99-1.01	0.296
CD68	0.99	0.98-1.02	0.968
CD163	1.01	0.98-1.02	0.694
T_{reg}	0.92	0.87-0.98	**0.012**
CD8/CD3^	0.71	0.41-1.21	0.208
T_{reg}/CD3^	0.26	0.06-1.08	0.064
T_{reg}/CD8	0.20	0.04-1.07	0.059
CD163/CD68	1.07	0.79-1.46	0.660

Multivariate Cox proportional hazards model included age, histology, stage, and grade as covariates
^ One extra case was deleted in the analysis of CD8/CD3 and T_{reg}/CD3 due to the zero value in CD3 counts
* *p* values less than 0.05 are marked in bold

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Supplemental data

Supplementary Figure 1. High resolution version of Figure 1.

Download from http://www.cancerimmunity.org/130101-suppl-fig-1.pdf (0.64 MB PDF file).

Supplementary Table 1. High resolution version of Table 1.

Download from http://www.cancerimmunity.org/130101-suppl-tab-1.pdf (0.55 MB PDF file).