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The association of peripheral blood regulatory T-cell concentrations with epithelial ovarian cancer: A brief report

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Abstract

Objective—There is a mounting body of evidence demonstrating higher percentages of regulatory T (Treg) cells in the peripheral blood of cancer patients in comparison to healthy controls, but there is a paucity of epidemiological literature characterizing circulating Treg cells among epithelial ovarian cancer (EOC) patients. To investigate the role of peripheral Treg cells in ovarian neoplasms, we conducted a case-control study to characterize circulating concentrations of Treg cells among EOC patients, women with benign ovarian conditions, and healthy controls without a history of cancer.

Materials and Methods—Participants were identified for inclusion due to their participation in the Data Bank and BioRepository program at Roswell Park Cancer Institute in Buffalo, NY. Patients included 71 women with a primary diagnosis of EOC and 195 women with a diagnosis of benign ovarian conditions. Controls included 101 age- and race-matched women without a history of cancer. Non-fasting, pre-treatment peripheral blood levels of CD3+CD4+CD25+FOXP3+ Treg cells were measured using flow cytometric analyses and expressed as a percentage of total CD3+ cells and as a percentage of total CD3+CD4+ cells.

Results—Compared to healthy controls and women with benign ovarian conditions, EOC patients had significantly higher frequency of Treg cells ($p<0.04$). In multivariable logistic regression analyses utilizing Treg frequency expressed as a percentage of CD3+ cells, we observed a significant positive association between Treg cell percentage and EOC risk, with each one percent increase associated with a 37% increased risk of EOC (OR=1.37, 95% CI: 1.04-1.80). We observed a similar trend when Treg frequency was expressed as a percentage of CD3+CD4+ cells (OR=1.22, 95% CI: 0.99-1.49).

Conclusions—The current study provides support that peripheral Treg cell frequency is elevated in EOC patients in comparison to women with benign ovarian conditions and healthy controls.

Keywords

T-Regulatory Cells; Treg cells; epithelial ovarian cancer

Introduction

In the United States, invasive epithelial ovarian cancer (EOC) is the deadliest gynecological cancer, ranking fifth among all cancer deaths among U.S. women [1]. While some well-established risk factors for EOC have been previously identified, much of the etiology of this disease remains unknown. Therefore, further patient-oriented research to elucidate novel risk factors, biomarkers, and/or therapeutic regimens is warranted. To this end, host tumor immunosuppressive factors such as regulatory T (Treg) cells have become an important area of inquiry into identifying underlying biological mechanisms associated with the development and progression of EOC [2]. While Treg cells are most commonly considered as inhibitors of CD4+ and CD8+ T lymphocytes [3], evidence also indicates that they suppress natural killer cells and professional antigen presenting cells, such as dendritic cells [4, 5].

Furthermore, while it is well accepted that Treg cells in ovarian tumors are directly associated with both poor prognosis and unfavorable disease characteristics [6-9], the clinical usefulness of assessing peripheral concentrations of Treg cells has not been well established, and these associations have not been investigated in well-designed case-control analyses. Thus, we sought to characterize the peripheral blood concentrations of immunosuppressive Treg cells in three populations of women: patients diagnosed with EOC, patients diagnosed with benign ovarian conditions, and healthy controls without a history of cancer.

Materials and Methods

We conducted a case-control study including incident EOC patients, benign ovarian tumor patients, and healthy controls recruited from Roswell Park Cancer Institute (RPCI), Buffalo, NY. All participants were identified for inclusion by their consent to participate in the Data Bank and Bio Repository (DBBR) program, a shared core research resource at RPCI [10]. The DBBR program is designed to collect biospecimens and extensive clinical and epidemiological data from patients treated at RPCI.

Study Population

Participants were enrolled from October 2009 through February 2015. During this time, the gynecological clinic at RPCI yielded an 81% participation rate in the DBBR. A total of 71 pathologically confirmed EOC patients, 101 age- and race-matched controls, and 195 benign ovarian tumor patients participated in the current investigation. EOC and benign patients included women aged 18 years and older with newly diagnosed neoplasms who were seeking treatment at RPCI. Controls included friends, family members, and other visitors at RPCI who consented to participate in the DBBR and who self-reported as having no history of cancer upon enrollment.

Data Collection

All participants provided face-to-face informed consent to donate at least one blood sample and to complete a self-administered in-depth epidemiologic questionnaire. At the time of data analysis (December 2015), 287 (78%) of blood sample donors had returned their epidemiologic questionnaires to the DBBR for data processing. Women with missing data on variables of interest in this study, such as age, parity, or use of oral contraceptives, were excluded from analyses. The study protocol was approved by the RPCI Institutional Review Board.

Specimen Collection—Non-fasting peripheral blood (30 ml) was drawn at the phlebotomy service at RPCI, and 10 ml of whole blood was immediately transported via pneumatic tube to the Flow and Image Cytometry resource at RPCI. The remaining blood sample (including serum, red blood cells, plasma, buffy coats, and whole blood) was logged by a unique barcode identification number and banked in 0.5 ml straws.

Treg Cell Assessment—We used CD3+CD4+CD25+FOXP3+ as the principal definition of Treg cells and further identified two Treg cell frequency variables, our exposures of interest, as the percentage of CD3+ T cells and the percentage of CD3+CD4+ T cells. To measure Tregs, cells were thawed, washed and checked for viability using Fixable Live Dead Violet (Thermo Fisher, Waltham, MA). The cells were stained with FOXP3 Ax488 (clone 206D, BioLegend, San Diego, CA), CD127 PE (clone R34.34, Beckman Coulter, Miami, FL), CD4 PerCP (clone OKT-4, BioLegend), CD3 PECy7 (clone SK7 8-11, BD Bioscience San Jose, CA), and CD25 APC (clone 2A3, BD Bioscience), as previously described [11]. For each sample, a fluorescence minus one (FMO) control, substituting an isotype control (IgG1K AX488, BioLegend) for FOXP3, was used to establish the boundaries for FOXP3 negativity.

Cytofluorometric analysis was performed using a FACSCanto II (BD BioSciences) flow cytometer equipped with air cooled 405, 488, and 633 nm lasers. Forward scatter, side scatter and five fluorescent parameters were collected with a threshold set on forward scatter to eliminate debris from list mode data. Lymphocytes (5×10^5) were stained with surface marker first, then processed in fixation/permeabilization (Fix/Perm) Buffer (eBioscience, San Diego, CA). After staining with FOXP3 or isotype control, the cells were fixed in 2% formaldehyde, acquired on the Canto II and analyzed by WinList. Cells were gated for CD3, CD4, CD25, and FOXP3 markers.

Data Analysis

Chi-square test for independence and one-way ANOVA were utilized to examine differences in participant characteristics and Treg cell frequency by patient status. Unconditional age-adjusted logistic regression models were utilized to estimate odds ratios (OR) and 95% confidence intervals (CI) representing the associations of well-established risk factors with EOC status. Next, we estimated multivariable logistic regression models representing the association between Treg cell frequency and EOC status.

In multivariable analyses we modeled Treg frequency as continuous and categorical variables, with the distribution among controls as a guide for tertile cutoff points. We pre-specified well-established risk factors of EOC as important adjustment variables and we also adjusted for body mass index (BMI) and physical activity because of their probable association with EOC risk and Treg cell populations in healthy women [12]. Additional potential confounders were identified via the ten percent change-in-estimate guideline [13]. Based upon these criteria, multivariable logistic regression models were adjusted for age (continuous), parity (number of live births), oral contraceptive use (ever/never), a family history of breast or ovarian cancer (yes/no), breastfeeding (number of children breastfed), menopausal status (pre/post), BMI (continuous), and participation in regular recreational physical activity (yes/no). The alpha level for statistical significance was set *a priori* at less than 0.05.

Results

Descriptive characteristics of the study population are depicted in Table 1. Patients with benign conditions were younger and were more likely to be pre-menopausal than EOC cases and controls. Further, EOC cases were less likely to have ever used oral contraceptives and mean frequency of Treg cells, expressed as a percentage of CD3+ T cells, was significantly higher in EOC cases (3.69 $\pm$ 2.13%) than controls (2.78 $\pm$ 1.61%) and patients with benign conditions (3.09 $\pm$ 1.76, ANOVA p = 0.006) (Figure 1a). We observed the same trend when Treg frequency was expressed as a percentage of CD3+CD4+ T cells (Figure 1b).

Utilizing Treg cell frequency as a percentage of CD3+ cells, we observed a significant positive trend in the association between increasing tertiles of Treg cells and EOC status, with nearly a threefold significant increased odds of EOC among those women with the highest Treg frequencies: (second tertile OR=1.86, 95% CI: 0.80-4.34; third tertile OR=2.83, 95% CI: 1.25-6.41; p for trend=0.012) (Table 2). However, in multivariable models, the

observed association for the third tertile of Treg cells was attenuated (OR =1.72, 95% CI: 0.58-5.14). When logistic regression models were estimated with Treg cell frequency as a continuous variable, we observed a significant association between Treg cell frequency and EOC status after adjusting for all confounders. Specifically, each one percent increase in Treg cell frequency was associated with 37% higher odds of EOC (OR=1.37, 95% CI: 1.04-1.80) (Table 2). When Treg frequency was expressed as a percentage of CD3+CD4+ T cells, there was no association between tertiles of Treg cell frequencies with EOC, but we observed a borderline significant association when Treg cell frequency was modeled as a continuous variable (OR=1.22, 95% CI: 0.99-1.49) (Table 2).

Conclusions

In this epidemiological investigation, we observed significantly higher mean frequencies of Treg cells in newly diagnosed EOC patients than in women with benign ovarian conditions and cancer-free controls. We also observed evidence that Treg cell frequency positively associates with EOC when modeled as a continuous variable. These findings are consistent with a previous study demonstrating that Treg cell percentage was significantly higher in ovarian cancer patients than controls ($5.7 \pm 3.1\%$ versus $2.8 \pm 1.4\%$, $p=0.002$) [14].

The findings of this study should be interpreted with caution. Due to the limited sample size, we were not powered to detect significant associations when Treg cells were parameterized as a categorical variable. Temporality of the association between Treg cells and ovarian cancer cannot be established due to the case-control nature of the study. Further, we did not examine Treg cell levels in tumor specimens and it is unclear whether Treg cell levels in the peripheral blood are strongly correlated with Treg cell tissue concentrations. Lastly, we did not assess CD3+CD8+ T cells, so we were unable to compare CD8 cells among EOC cases and controls. Nevertheless, the current research contains several noteworthy strengths. To our knowledge, this is the first epidemiological study utilizing an age- and race-matched control group to evaluate the association between peripheral Treg cells and EOC. Secondly, because previous research has demonstrated that the density and distribution of Treg cells is affected by treatment [15], we collected blood samples prior to treatment. Third, our use of multiple markers to identify Treg cells, rather than relying on a single marker of FOXP3 expression, enhanced our ability to accurately assess Treg cell levels. Lastly, data on a wide range of epidemiological variables, including demographic, reproductive, and lifestyle factors, were available for consideration as potential confounding variables.

The findings of our study provide additional insight into the association between pre-treatment peripheral blood levels of immunosuppressive Treg cells and EOC status. Further, these results underscore the notion that immunosuppression is an important biological process in the etiology of EOC.

Acknowledgments

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Figure 1. Violin plots of Treg cell frequencies across groups of controls, benign ovarian disease, and epithelial ovarian cancer

These violin plots show the percentage of Treg cells (x axis) across participant groups (y axis). Specifically, the median Treg cell frequencies (white dots), interquartile ranges (thick black bars), and 95% confidence intervals (thin black bars) for Treg cell frequencies in controls, benign conditions and ovarian cancer cases are displayed. 1a. The mean (standard deviation) of Treg cell frequencies, defined as a percentage of CD3+ cells were 3.69 (2.13), 3.09 (1.76), and 2.78 (1.61) for ovarian cancer, benign cases and controls, respectively ($p_{ANOVA}=0.006$). 1b. The mean (standard deviation) of Treg cell frequencies, defined as a percentage of CD3+CD4+ cells were 3.93 (2.06), 4.63 (3.03), and 4.99 (2.89) for ovarian cancer, benign cases and controls, respectively ($p_{ANOVA}=0.035$). Each group is also plotted with a kernel density plot, which shows the probability density function. The thicker the violin, the more probable it is that the participant group would have Treg cell levels at this value.

Table 1Descriptive characteristics of the study population by participant status (N=367)¹

Variable	Healthy Controls (N=101)	Benign Conditions (N=195)	EOC Cases (N=71)	P-value ² all 3 groups
Reference Age	57.2 (10.9)	50.7 (12.8)	58.1 (11.0)	<0.001
Regulatory T Cell Frequency ³ (Percentage of CD3+ Cells)	2.78 (1.61)	3.09 (1.76)	3.69 (2.13)	0.006
Regulatory T Cell Frequency ⁴ (Percentage of CD3+4+ Cells)	3.93 (2.06)	4.63 (3.03)	4.99 (2.89)	0.035
Race				
White	96 (95.0%)	162 (83.1%)	66 (93%)	0.194
Black	5 (5.0%)	17 (8.7%)	2 (2.8%)	
Other	0 (0.0%)	16 (8.2%)	3 (4.2%)	
Education				
Less than High School	2 (2.7%)	10 (24.0%)	2 (4.4%)	0.463
High School	18 (24.0%)	33 (24.0%)	9 (20.0%)	
Greater than High School	55 (73.3%)	83 (65.9%)	34 (75.6%)	
Smoking Status				
Never	36 (48.0%)	64 (49.2%)	23 (48.9%)	0.706
Former	28 (37.3%)	41 (31.5%)	13 (27.7%)	
Current	11 (14.7%)	25 (19.2%)	11 (23.4%)	
BMI Classification (kg/m ²)				
Underweight (<18.5)	1 (1.3%)	2 (1.6%)	1 (2.2%)	0.649
Normal weight (18.5-24.9)	24 (32.0%)	27 (21.3%)	13 (28.9%)	
Overweight (25.0-29.9)	24 (32.0%)	39 (30.7%)	14 (31.1%)	
Obese (≥ 30.0)	26 (34.7%)	59 (46.5%)	17 (37.8%)	
Current Physical Activity				
Inactive	24 (31.6%)	48 (36.1%)	16 (34.0%)	0.803
Active	52 (68.4%)	85 (63.9%)	31 (66.0%)	
Menopause Status				
Pre/Peri	27 (35.5%)	77 (57.5%)	14 (29.8%)	0.003
Post	48 (63.2%)	56 (41.8%)	33 (70.2%)	
Don't Know	1 (1.3%)	1 (0.7%)	0 (0.0%)	
Parity (# of live births)				
0	12 (16.0%)	29 (22.0%)	9 (19.6%)	0.579
1	14 (18.7%)	21 (15.9%)	14 (8.7%)	
2-3	34 (45.3%)	64 (48.5%)	34 (56.5%)	

Variable	Healthy Controls (N=101)	Benign Conditions (N=195)	EOC Cases (N=71)	P-value ² all 3 groups
4+	15 (20.0%)	18 (13.6%)	15 (15.2%)	
Tubal Ligation				
No	53 (69.7%)	92 (68.7%)	33 (70.2%)	0.975
Yes	23 (30.3%)	42 (31.3%)	14 (29.8%)	
Contraceptive Use				
No	26 (34.7%)	38 (29.0%)	24 (51.1%)	0.024
Yes	49 (65.3%)	93 (71.0%)	23 (48.9%)	
Breastfeeding				
No	34 (45.3%)	44 (33.3%)	17 (37.0%)	0.604
Yes, 1-2 children	18 (24.0%)	43 (32.6%)	14 (30.4%)	
Yes, 3+ children	11 (14.7%)	15 (11.4%)	6 (13.0%)	
NA	12 (16.0%)	30 (22.7%)	9 (19.6%)	
Family History of Breast or Ovarian Cancer				
Yes	14 (18.4%)	36 (26.9%)	10 (21.3%)	0.355
No	62 (81.6%)	98 (73.1%)	37 (78.7%)	

¹ Numbers may not sum to total due to missing data;

² Chi square test for independence was performed for categorical variables, and One-way ANOVA was performed for reference age and Treg percentage;

³ Treg frequency is defined as the percentage of CD3+CD4+CD25+FOXP3+ cells from CD3+ cells

⁴ Treg frequency is defined as the percentage of CD3+CD4+CD25+FOXP3+ cells from CD3+CD4+ cells

Table 2

The association between Treg cell frequency and epithelial ovarian cancer.

Treg cell frequency		Case N ³	Control N ³	Unadjusted OR & 95% CI	Age-Adjusted OR & 95% CI	Multivariable-Adjusted ⁴ OR & 95% CI	
Treg Cell Frequency as a percentage of CD3+ cells ¹	Treg Cell Frequency (tertiles)	0%-1.71%	33	Referent	Referent	Referent	
		1.72%-3.32%	34	1.86 (0.80-4.34)	1.22 (0.45-3.26)	0.81 (0.27-2.43)	
		=>3.33%	33	2.83 (1.25-6.41)	1.84 (0.68-5.01)	1.72 (0.58-5.14)	
	P for trend			0.012	0.213	0.260	
Treg Cell Frequency as a percentage of CD3+CD4+ cells ²	Treg Cell Frequency (continuous)		71	101	1.35 (1.14-1.59)	1.41 (1.16-1.71)	1.37 (1.04-1.80)
	Treg Cell Frequency (tertiles)	0%-2.69%	17	32	Referent	Referent	Referent
		2.70%-4.81%	19	34	1.05 (0.47-2.37)	0.71 (0.27-1.83)	0.40, 0.13-1.18
		=>4.82%	33	33	1.88 (0.88-4.02)	1.14 (0.44-2.90)	1.01, 0.35-2.92
P for trend			0.87		0.714	0.950	
Treg Cell Frequency (continuous)		71	101	1.20 (1.05-1.37)	1.14 (0.96-1.36)	1.22 (0.99-1.49)	

¹Treg frequency is defined as the percentage of CD3+CD4+CD25+FOXP3+ cells from CD3+ cells

²Treg frequency is defined as the percentage of CD3+CD4+CD25+FOXP3+ cells from CD3+CD4+cells

³Numbers may not sum to total due to missing epidemiological data

⁴Model adjusted for age, parity, birth control use, breastfeeding, menopause status, family history of breast or ovarian cancer, BMI, physical activity