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Article

ARTICLE

Treg-Mediated Immune Tolerance and the Risk of Solid Cancers: Findings From EPIC-Heidelberg

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Abstract

Background: Laboratory-based, mechanistic, and prognosis studies suggest that a shift from antitumor immunity towards tumor-immune tolerance plays a major role in carcinogenesis. However, prospective epidemiological studies on the consequences of differing immune tolerance levels prior to clinical manifestation are missing.

Methods: A case-cohort study embedded in EPIC-Heidelberg was conducted comprising incident cases of breast (n = 399), colorectal (n = 185), lung (n = 149), and prostate (n = 378) cancer, which occurred during 6.6 years of follow-up, and a subcohort (n = 807). Foxp3+ regulatory T-lymphocytes and CD3+ T-lymphocytes were measured by quantitative polymerase chain reaction–based DNA methylation analysis in prediagnostic leukocyte samples. Hazard ratios (HRs) for associations of cancer risk with the ratio of both parameters, the "cellular ratio of immune tolerance" (ImmunoCRIT), were estimated using Cox regression models. All statistical tests were two-sided.

Results: ImmunoCRIT values were positively associated with the risk of lung (highest vs lowest tertile, HR = 1.98, 95% confidence interval = 1.06 to 3.69, P_{trend} = .0263) and colorectal cancer (HR = 1.59, 95% CI = 0.99 to 2.54, P_{trend} = .0069) after multivariable adjustment, but not with prostate cancer risk. Regarding breast cancer significant heterogeneity by estrogen receptor (ER) status was observed ($P_{heterogeneity}$ = .02), and the ImmunoCRIT was associated with the risk of ER-negative breast cancer (HR = 3.34, 95% CI = 1.52 to 7.35, $P_{trend} \leq .001$), but not ER-positive breast cancer.

Conclusion: The present study indicates that increased peripheral immune tolerance may be an independent risk factor for lung, colorectal, and ER-negative breast cancer, whereas its role on the development of prostate and ER-positive breast tumors remains uncertain.

The ability of tumor cells to evade immune surveillance by suppression of the immune system is a hallmark of carcinogenesis (1,2). Evidence for the existence of immunological defense mechanisms against cancer in humans is based on the observation that cancer progression and prognosis are related to immune status, including the numbers and function of various immune cells infiltrating into a tumor (3).

In healthy subjects, adaptive immune responses are controlled by a balance between total CD3+ T-lymphocytes (tTLs), which mainly consist of effector cells driving the elimination of abnormal cells, and suppressor cells—in particular Foxp3+ regulatory T-lymphocytes (Tregs)—which modulate the aggressiveness of the cellular immune response (4,5). An increased ratio between Tregs and tTLs has been postulated to facilitate cancer development (6), and intratumoral accumulation of Tregs is frequently associated with greater tumor aggressiveness in patients affected by various cancer types (7–10). The notion that the balance between Tregs and tTLs determines immunity against tumors is further

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supported by clinical studies on Interleukin 2 (IL-2) therapies. Both Tregs and activated effector T-lymphocytes express high levels of Interleukin-2 receptor α chain (IL-2Rα). Hence, IL-2 administration—as used for salvage therapy for patients with refractory malignant melanoma (11) and renal cell carcinoma (12)—leads to expansion of both tTLs and Tregs (13). This parallel expansion of antitumor effector cells and their suppressors may explain the limited clinical response to IL-2 treatment observed in the majority of such patients (14). Taken together, these findings suggest that Treg accumulation in tissues could be a dominant mechanism by which malignant cells evade elimination through tumor-specific effector T lymphocyte responses and by which immune tolerance to malignant cells develops.

In addition to IL-2 receptor expression, Tregs also partly share expression of their most specific gene, the transcription factor Foxp3, with activated T cells (15,16). This latter cell type is involved in inflammatory responses. Thus, detection of high IL2R and/or Foxp3 expression in tumor tissue may indicate increased inflammation rather than immune tolerance. Given this ambiguity, it is still being debated whether tumors do indeed exhibit more tolerant microenvironments because of Treg accumulation. If indeed they do, it also remains unclear whether this immune tolerance precedes or follows tumor development, or both (17). Uncertainty also exists as to whether such immune tolerance is mainly a localized phenomenon or has a more systemic character, although increased numbers of Tregs have been reported in peripheral blood of patients with several types of cancer, including pancreas, breast, hepatocellular, prostate, and lung carcinomas (18–24).

More recently, a Treg-specific demethylated region (TSDR) has been identified in mice (25) and confirmed in humans. Along with this, it was shown that TSDR demethylation is not shared by activated T cells (26) and is therefore regarded as the currently most specific Treg marker (27,28). Indeed, it has been shown that Tregs constitute a stable cell lineage, whose state is ensured by DNA demethylation of the Foxp3 locus irrespective of ongoing Foxp3 expression (29). Based on these findings, quantitative polymerase chain reaction (qPCR) approaches were developed to determine the relative amount of Tregs (30) and, based on an equivalent assay for the CD3g/d intergenic region, the total CD3+ T lymphocytes (tTLs) (31). The ratio of Tregs to tTLs was shown to be increased in tumor biopsies and dubbed Cellular Ratio of Immune Tolerance ("ImmunoCRIT") (32). This is because the Treg/tTL ratio is thought to be an important determinant of immune tolerance. For the current study, it is important to note that the technology on which epigenetic qPCRs are based allows the assessment of the Treg/tTL ratio in long-term stored blood samples.

While data from studies of patients with clinically manifest cancers support the concept that Treg-mediated tolerance may represent an important barrier against the induction of antitumor immunity (33), epidemiological studies on its role in earlier stages of cancer development are missing. Here, we report the findings from a case-cohort study within the EPIC-Heidelberg cohort, including incident cases of the four most frequent cancer types (breast, colorectal, lung, and prostate cancer). Our prior hypothesis was that an elevated ratio of Tregs-to-tTL in the blood of initially healthy subjects might be associated with an increased cancer risk.

Methods

Study Population

The European Prospective Investigation into Cancer and Nutrition (EPIC)–Heidelberg study was initiated as part of the Europe-wide EPIC project and includes 11 929 male and 13 611 female participants age 35 to 65 years, who were recruited from the local general population. Baseline examinations were carried out from 1994 through 1998 and included blood sampling, anthropometric measurements, and self-administered questionnaires on diet, lifestyle, and reproductive health. The study was approved by the ethics committee of the Medical School of the University of Heidelberg, and all participants gave written informed consent (34,35). Incident cancer cases were ascertained by follow-up questionnaires and by record linkage, and all cases were verified by study physicians based on medical records. Further details on follow-up procedures of EPIC-Heidelberg have been described elsewhere (36).

More recently, after a median follow-up time of 14 years, a random subsample of EPIC study participants was reinvited for a validation substudy. As part of this substudy, two further blood samples were collected with an average interval of one year, which were used for analyses of the reproducibility of blood-based biomarker measurements over time, including the Treg/tTL ratio measured for the present study. Further details on the setup and analysis of this substudy are provided in the Supplementary Methods (available online) and Supplementary Table 1 (available online).

A case-cohort study embedded in EPIC-Heidelberg was set up for the present project.

After exclusion of prevalent cancer cases, the study population comprised 150 case patients with lung cancer (International Classification of Diseases [ICD-10: C34]), 194 case patients with colorectal cancer (ICD-10: C18-20), 410 case patients with breast cancer (ICD-10: C50), and 394 case patients with prostate cancer (ICD-10: C61) that occurred between baseline examination and December 31, 2006, as well as a random subcohort of 813 subjects who had initially been drawn for the EPIC-InterAct case-cohort study (37). For the primary analysis, study subjects were excluded when there were missing covariate data (case patients: n = 2 colorectal, n = 1 prostate) or quality control in qPCR analysis failed (case patients: n = 1 lung, n = 7 colorectal, n = 11 breast, n = 15 prostate; noncases: n = 6). Thus, statistical analyses were performed on 149, 185, 399 and 378 case patients with lung, colorectal, breast, and prostate cancer, respectively, and 807 subcohort members. The randomly selected subcohort included 22 incident cancer case patients (lung: n = 4, breast: n = 2, prostate: n = 16).

Laboratory Methods

Details on storage and processing of blood samples at recruitment in the EPIC-Heidelberg cohort are described in the Supplementary Methods (available online).

For epigenetic analysis, genomic DNA was chemically modified by sodium bisulphite. In this reaction, unmethylated cytosine is converted to uracil, while methylated cytosine remains unchanged. From the genomic DNA of each blood sample, about 1.6 µg DNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen Hilden, Germany) following the manufacturer's protocol. DNA fragments corresponding to unmethylated, bisulfite-converted DNA at the Foxp3, CD3, and GAPDH loci were cloned into vector pUC57 (GenScript USA Inc., Piscataway, NJ). The resulting 3787 bp plasmid was linearized and used for the qPCR reactions detailed below in the following serially diluted final concentrations of 12.97, 2.59, 0.52, 0.1, 0.02, and 0.01 pg/mL, yielding 15625, 3135, 625, 125, 25, and 15 plasmid copies. Epigenetic qPCR reactions contained 7.5 pmol forward and reverse primers, 1.25 pmol hydrolysis probe, 1x Roche LightCycler 480 Probes Master, and

approximately 70 ng bisulfite-converted DNA or the above final concentrations of plasmid for standard curve design. Each reaction was performed in a final volume of 5 µl. Tregs, tTLs, and all leukocytes (GAPDH) for all blood samples were analyzed in triplicate using a LightCycler 480 System (Roche, Basel, Switzerland). Cycling conditions were: 1 time 95° C preheating for 10 minutes and 50 cycles of 95°C for 15 seconds followed by 1 minute at 61°C. Template copy numbers were estimated from standard curve by linear regression on crossing point (CP) using second derivative maximum method as defined by Roche Light Cycler 480 Software. The proportion of a specific cell type was determined as follows: Using bisulfite-converted DNA as substrate, qPCRs were designed and performed for the selected cell type–specific demethylated loci (Foxp3 and CD3) and for a locus known to be demethylated in all cell types (GAPDH) (31). Then, the ratio of Foxp3 and CD3 values was determined and is referred to as ImmunoCRIT.

Statistical Analyses

Selected baseline characteristics of case patients and subcohort members are presented as means ± standard deviations or proportions (Table 1). ImmunoCRIT values were displayed as means, medians, and extreme values. For analyses on cancer risk, ImmunoCRIT measurements were categorized into tertiles using cutpoints based upon the distribution in the subcohort and subjects in the lowest tertile were considered the reference group. We used Prentice-weighted Cox proportional hazards regression models with age as the underlying time-scale (38). All observations in the subcohort were left-truncated at age at recruitment and censored at end of follow-up, death, or loss to follow-up, whichever came first. Following the Prentice-weighting scheme, case patients were only included shortly before their event. To adjust for age differences in case patients at study entry and other potential age-cohort effects, all analyses were stratified by integer values (in years) of age at recruitment. The extended correlation test based on Schoenfeld residuals (39) did not indicate any violations to the proportional hazards assumption. Sex-adjusted (if appropriate) and multivariable-adjusted hazard ratios (HRs), along with their 95% confidence intervals (CIs) for the association between ImmunoCRIT and cancer risk, were estimated. The multivariable-adjusted models included all those potential confounding variables that changed the risk estimates by more than 10% or were clearly associated with the exposure; these are indicated in the footnotes to Table 2. The ImmunoCRIT values followed an approximately log-normal distribution, and tests for linear trend were carried out based on continuous values of the ImmunoCRIT on the log2 scale, thus calculating the HR associated with a doubling of the ImmunoCRIT.

For the analyses on breast cancer risk, heterogeneity by estrogen receptor (ER) status was assessed by Cochran's Q-test. Multiplicative statistical interactions with risk factors were tested for by including cross-product terms along with the main effect terms into the multivariable adjusted models. Sensitivity analyses were conducted excluding case patients who were diagnosed within the first two years of follow-up. To examine mid- and long-term partial correlations between ImmunoCRIT values assessed at baseline, after 14 years and after 15 years, Spearman correlation coefficients, adjusted for age at recruitment and sex,

Table 1. Characteristics and laboratory results of the study population*

	Incident cancer cases				Subcohort		
Characteristic	Lung	Colorectum	Breast	Prostate	Total	Women	Men
N	149	185	399	378	807	436	371
Socio-demographic factors							
Women, %	30.2	35.1	100.0	-	54.0	100.0	-
Age at recruitment, y	55.1±7.4	56.1±6.3	51.6±7.8	57.7±5.4	50.7±8.0	49.1±8.4	52.5±7.0
Education level†, %							
Primary school	45.6	33.5	26.6	34.9	25.2	25.2	25.1
Secondary school	38.9	36.8	47.2	31.2	43.4	51.6	33.7
University	15.4	29.7	26.1	33.9	31.5	23.2	41.2
Case patient characteristics							
Age at diagnosis, y	61.8±7.5	62.4±6.8	57.8±7.8	64.7±5.4	-	-	-
Stage at diagnosis, %							
Local	18.8	38.9	60.9	70.6	-	-	-
Regional	26.2	41.1	34.1	24.1	-	-	-
Distant	45.0	19.5	2.0	4.0	-	-	-
Unknown	10.1	0.5	3.0	1.3	-	-	-
Lifestyle factors, %							
Abdominal adiposity‡	32.9	40.5	25.8	25.7	23.2	22.7	23.7
Physically Inactive§	51.7	49.7	47.9	46.6	44.4	45.4	43.1
Ever smokers†	91.3	67.0	44.2	58.2	57.2	49.7	66.1
Heavy drinkers‖	38.9	45.9	33.6	43.5	34.6	31.0	38.8
Laboratory measurement, ImmunoCRIT, %							
Mean	5.9	5.5	6.0	5.0	5.3	5.7	4.9
Median (range)	5.9 (2.1–11.3)	5.3 (2.6–11.5)	5.7 (2.3–18.0)	4.8 (1.2–11.0)	5.1 (1.4–15.5)	5.5 (2.4–11.9)	4.7 (1.4–15.5)

* Values are means ± standard deviations or proportions, unless otherwise stated.
† Education data missing for one breast cancer case. Smoking data missing for one colorectal as well as one breast cancer case patient and three subcohort members.
‡ Defined by waist circumference ≥ 102 cm for men and ≥ 88 cm for women according to World Health Organization cutoffs.
§ Summary variable for inactive and moderately inactive.
‖ Defined by alcohol intake at baseline >24 g/d for men and >12 g/d for women.

Table 2. Sex-adjusted (if appropriate) and multivariable-adjusted HRs for the association between ImmunoCRIT and solid cancers*

Cancer type	Tertiles†			HR (95% CI) $_{log2}$	P_{trend}‡
	1 (Ref.)	2	3		
Lung cancer					
Tertile median (range)	3.9 (1.4–4.6)	5.1 (4.6–5.8)	6.7 (5.8–15.5)		
No. of case patients/subcohort§	35 / 265	36 / 265	78 / 264		
Sex-adjusted	1.00	1.43 (0.82 to 2.51)	3.45 (1.97 to 6.04)	3.44 (2.11 to 5.62)	<.0001
MV-adjusted*	1.00	0.99 (0.52 to 1.89)	1.98 (1.06 to 3.69)	1.95 (1.08 to 3.52)	.0263
Colorectal cancer					
Tertile median (range)	3.9 (1.4–4.6)	5.1 (4.6–5.8)	6.6 (5.8–15.5)		
No. of case patients/subcohort§	59 / 265	55 / 264	71 / 264		
Sex-adjusted	1.00	1.31 (0.85 to 2.02)	1.70 (1.10 to 2.64)	1.81 (1.22 to 2.70)	.0035
MV-adjusted*	1.00	1.32 (0.83 to 2.11)	1.59 (0.99 to 2.54)	1.81 (1.18 to 2.77)	.0069
Breast cancer					
Tertile median (range)	4.3 (2.4–4.9)	5.5 (5.0–6.2)	7.1 (6.2–11.9)		
No. of case patients/subcohort§	117 / 145	129 / 145	153 / 145		
Crude	1.00	1.10 (0.76 to 1.59)	1.23 (0.86 to 1.75)	1.47 (0.99 to 2.19)	.0567
MV-adjusted*	1.00	1.03 (0.69 to 1.53)	1.11 (0.77 to 1.61)	1.34 (0.90 to 2.01)	.1624
ER-positive breast cancer‖					
No. of case patients/subcohort§	94 / 144	98 / 146	115 / 144		
Crude	1.00	1.00 (0.67 to 1.50)	1.10 (0.75 to 1.62)	1.30 (0.84 to 2.02)	.2421
MV-adjusted*	1.00	0.91 (0.59 to 1.41)	0.99 (0.67 to 1.46)	1.18 (0.75 to 1.85)	.4810
ER-negative breast cancer‖					
No. of case patients/subcohort§	14 / 144	23 / 145	34 / 144		
Crude	1.00	1.99 (0.89 to 4.44)	3.09 (1.46 to 6.55)	3.44 (1.76 to 6.72)	<.001
MV-adjusted*	1.00	2.28 (1.05 to 4.93)	3.34 (1.52 to 7.35)	3.73 (1.80 to 7.73)	<.001
Prostate cancer					
Tertile median (range)	3.4 (1.4–4.2)	4.7 (4.2–5.3)	6.1 (5.3–15.5)		
No. of case patients/subcohort§	124 / 122	102 / 123	152 / 122		
Crude	1.00	0.92 (0.60 to 1.40)	1.11 (0.75 to 1.66)	1.01 (0.71 to 1.45)	.9487
MV-adjusted*	1.00	0.90 (0.58 to 1.39)	1.39 (0.91 to 2.13)	1.17 (0.80 to 1.70)	.4505

* Prentice-weighted Cox regression models were used to evaluate the association between ImmunoCRIT (=cellular ratio of immune tolerance) and cancer risk. Multivariable models were adjusted for the following factors: lung cancer: smoking status (never smokers, former smokers ≥10 years, former smokers <10 years, smokers <15 cigarettes/d, smokers ≥15 cigarettes/d), smoking duration (y), NSAID use (yes/no), history of myocardial infarction or stroke (yes/no), red meat consumption (g/d), and height (cm); colorectal cancer: waist circumference (cm), alcohol intake (g/d), processed meat consumption (g/d), hyperlipidemia (yes/no), height (cm), smoking status (see above), and smoking duration (y); breast cancer: exogenous hormone use (OC/HRT; yes/no), height (cm), menopausal status (pre-, peri-, postmenopausal including surgical hysterectomy), and NSAID use (yes/no); estrogen receptor (ER)–positive: see overall breast cancer model; ER-negative: menopausal status (see above), exogenous hormone use (yes/no), and time between menarche and first birth (y); prostate cancer: smoking status (see above), smoking duration (y), calcium intake (mg/d), and wholegrain intake (g/d). All statistical tests were two-sided. CI = confidence interval; HR = hazard ratio; MV = multivariable; Ref = referent category.
† Tertile cutpoints were based on the distribution in the subcohort.
‡ Tests for linear trend were carried out based on the continuous values of ImmunoCRIT on the log2 scale, included along with the main effect terms into the multivariable adjusted models.
§ Exclusions because of missing covariates for case patients/subcohort: lung cancer (0/13), colorectal cancer (2/14), overall breast cancer (0/1), prostate cancer (1/4).
‖ Receptor status could not be determined for n = 21 breast cancers. Thus, analyses were performed for n = 307 ER-positive breast cancers and n = 71 ER-negative breast cancers.

were calculated within a reproducibility study of 100 subjects. All statistical tests were two-sided, and *P* values below .05 were considered statistically significant. All analyses were performed using SAS 9.3 (SAS Institute, Cary, NC).

Results

Descriptive Statistics

As compared with the subcohort, subjects who developed cancer were older and characterized by a higher prevalence of unfavorable lifestyle behaviors, as shown in Table 1. The mean lag time from blood donation to time of diagnosis was 6.3, 6.4, 6.7, and 7.0, respectively, for the case patients with breast, colorectal, lung, and prostate cancer. The proportions of women among lung cancer case patients (30.2%) and colorectal cancer case patients (35.1%) were smaller than in the subcohort (54.0%). In participants of the subcohort, geometric means of ImmunoCRIT were statistically significantly higher in women and ever smokers, with some indication for an increase by both cumulative lifetime smoking history and current smoking status at the time of blood sampling (Supplementary Table 2, available online). There were no differences in ImmunoCRIT levels across strata of age, waist circumference, alcohol intake, and current NSAID use. Among women, ImmunoCRIT values did not differ statistically by menopausal status, exogenous hormone use, and pregnancy-related factors (eg, parity) (Supplementary Table 3, available online).

Stability of the ImmunoCRIT over time

In a random sample of 100 participants from the EPIC-Heidelberg substudy, a reproducibility study was carried out to evaluate the correlation between ImmunoCRIT values at baseline (T0) and two time points of the substudy, after 14 (T1) and 15 (T2) years of follow-up, as illustrated in Supplementary Figure 1 (available

online). Upon internal quality control, the following numbers of measurements were available at each time point: n = 92 (T0-T1 and T0-T2), n = 89 (T1-T2). Over one year (T1-T2), intra-individual values showed good reproducibility with a Spearman coefficient of correlation of 0.73 after adjustment for sex and age. Long-term correlations were moderately high after 14 years (T0-T1; r = 0.56) and 15 years (T0-T2; r = 0.53).

ImmunoCRIT Values and Cancer Risk

The distribution of ImmunoCRIT measurements among case patients and participants in the subcohort is visualized with boxplots in Figure 1. Median ImmunoCRIT values were 5.7% in breast, 5.3% in colorectal, 5.9% in lung, and 4.8% in prostate cancer case patients, whereas the subcohort showed median values of 5.1% in total, as well as 5.5% and 4.7% among female and male subjects, respectively.

Associations between the ImmunoCRIT and risks of lung, colorectal, breast (overall and by estrogen receptor status), and prostate cancer are presented in Table 2. After multivariable adjustment, Cox regression analyses showed statistically significant positive associations between ImmunoCRIT values and lung cancer risk (highest vs lowest tertile; HR = 1.98, 95% CI = 1.06 to 3.69, P_{trend} = .0263) as well as colorectal cancer risk (HR = 1.59, 95% CI = 0.99 to 2.54, P_{trend} = .0069). For colorectal cancer, associations in the crude and multivariable-adjusted model were of similar magnitude, whereas associations between the ImmunoCRIT and lung cancer risk were attenuated after adjustments, particularly when smoking was accounted for. There were no associations of the ImmunoCRIT with overall breast and prostate cancer risk in multivariable models. Sensitivity analyses, excluding cases that occurred within the first two years of follow-up, showed no major change in any of the risk estimates, as presented in Supplementary Table 4 (available online).

There were no statistically significant interactions between the ImmunoCRIT and any of the adjustment factors. However, significant heterogeneity in the associations between ImmunoCRIT and breast cancer risk by ER status ($P_{heterogeneity}$ = .02) was observed. Subgroup analyses by ER status revealed a positive association between the ImmunoCRIT and the risk of ER-negative breast cancer (HR = 3.34, 95% CI = 1.52 to 7.35, P_{trend} ≤ .001) (Table 2) but no statistical association with the risk of ER-positive breast cancer. A statistically significant direct association of the ImmunoCRIT with breast cancer risk was found among women diagnosed younger than age 50 years (highest vs lowest tertile; HR = 2.26, 95% CI = 1.12 to 4.56, P_{trend} = .0388) (see Supplementary Table 5, available online) but not women older than 50 years.

Kaplan-Meier curves analyzing the event-free survival for each of the cancer types by tertiles of ImmunoCRIT—as shown in Supplementary Figure 2 **(available online)**—support the age-adjusted ratios observed in Cox regression analyses by indicating a shift towards earlier diagnosis dependent on ImmunoCRIT value.

Discussion

To our knowledge, this is the first prospective study among initially healthy subjects to address the relationship between interindividual variations in peripheral immune tolerance and cancer risk. The use of DNA methylation markers enabled the analysis of Treg/tTL ratios (ImmunoCRIT) in long-term stored samples, which provides an index for the level of cell-mediated immune tolerance. We observed that an increased ImmunoCRIT was clearly associated with a higher risk of lung and colorectal cancer. Moreover, there was a statistically significant direct association between elevated ImmunoCRIT values and the risk of ER-negative breast cancer. No statistically significant relationships were found with respect to ER-positive breast and prostate cancer.

Overall, our findings are in line with the notion that Treg-mediated immune tolerance may play an important role throughout cancer development. In fact, the observed statistical associations were still present after excluding subjects

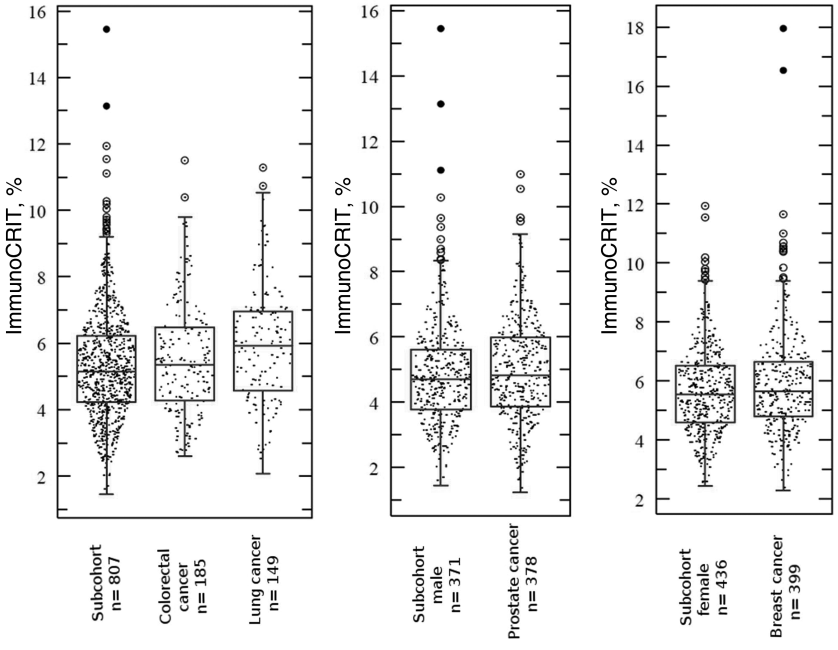

Figure 1. Boxplots showing ImmunoCRIT data by cancer type. **Horizontal lines** are medians, **whiskers** cover the data up to a maximum of 1.5*interquartile range above the third quartile or below the first quartile, and **open and filled circles** represent values beyond this area.

diagnosed with cancer within two years after blood draw, further supporting the idea that increased immune tolerance may facilitate carcinogenesis rather than being a consequence of tumor development. Although previous studies had indicated an association between immune profile and various cancer entities, these studies were not prospective and hence could not assess whether disturbed peripheral balance preceded disease onset or occurred after it (40,41). So far, there is only one other prospective study, in which the association between prediagnostic markers of cellular immune function in relation to cancer risk was examined, although this study focused on cytotoxic activity of mononuclear cells rather than peripheral immune tolerance (42). Imai et al. reported that higher natural cytotoxic activity of peripheral blood lymphocytes was inversely associated with overall cancer risk (42). Both the study of Imai et al. and ours add to the experimental evidence on the importance of immunological host defense mechanisms among healthy humans against cancer development.

Interestingly, our data show a risk association with ImmunoCRIT levels only for tumors of the colorectal, lung, and ER-negative breast cancers, but not for prostate and ER-positive breast cancers. One tentative explanation for this heterogeneity of association of tumor risks with ImmunoCRIT is that colorectal and lung tissues may be more immunologically exposed than prostatic tissue, for example. Indeed, reports indicate that up to 20% of cells in colorectal and lung tissues are tTLs, a value similar to peripheral T-cell levels (31), and tumors in these tissues show more aggressive hematogenous spreading and formation of distant metastases than tumors in less exposed tissues, such as, for example, brain tissue, which contains only 1% to 7% T-cell infiltrates (43) and presumably prostate (44). We thus speculate that the degree of normal-tissue immune infiltration may determine our observed heterogeneity of associations of ImmunoCRIT with tumor risks at different organ sites. We also realize, however, that this concept may not fully explain all of the heterogeneity observed in our study, eg, between ER-negative and -positive breast cancers, and therefore we suggest that future studies linking cancer risk to the Treg/tTL ratio in blood should include assessments of tissue infiltration by immune cells.

Another finding of the present study that is worth highlighting is the utility of the ImmunoCRIT as a long-term biomarker of immune tolerance. The remarkable one-year and 14-year within-subject reproducibility of the ImmunoCRIT suggests that in epidemiological studies a single measurement may provide a good proxy for long-term values, in line with the theory of a tight homeostatic control of Tregs (45).

Although the ratio of peripheral Treg levels and tTLs is rather stable over time, it is still also worthwhile identifying potential modulators of the ImmunoCRIT. In our study, the only factors that showed statistically significant associations with ImmunoCRIT values were sex and smoking. Heterogeneity of the ImmunoCRIT by sex could be because of the fact that one of the two Foxp3-TSDR alleles, which should be methylated as a result of X inactivation in women, may be not entirely inactivated (46). Because methylation of the CD3D gene is not sex-dependent, this may well imply that clinical reference ranges of ImmunoCRIT values should indeed be sex-specific. Nonetheless, our analyses showed no significant heterogeneity of the association between ImmunoCRIT and cancer risk by sex. While the present study is based on the assumption that an increase of the ratio between Tregs and tTL constitutes an individual and predisposing biological feature for certain cancers, an alternative hypothesis is that alterations of the ImmunoCRIT may be a consequence of chronic inflammation. In this context, immunosuppression could result from long-term production and accumulation of inflammatory factors and be reflected by an increase of the Treg count and ratio. This hypothesis was not addressed in the current work and needs to be assessed in future studies.

Our observation of a positive association between ImmunoCRIT values and smoking confirms previous findings of elevated Treg levels in female smokers (47) and of higher Treg/tTL ratios, within the present study referred to as ImmunoCRIT, among current smokers as compared with past or never smokers (48), and is in line with experimental data pointing to compromised immunity induced by smoking (49,50). In this context, it is of note that statistical adjustment for smoking led to substantial attenuation of the association between ImmunoCRIT values and lung cancer risk. While we acknowledge that smoking assessment is prone to some measurement error and that residual confounding may have influenced our results on lung cancer risk, it is also plausible that the adverse effect of smoking with respect to lung cancer may in part be mediated by immune suppression (51). However, associations between ImmunoCRIT and covariates in our study were merely cross-sectional. Therefore, further research on the possible interaction between ImmunoCRIT and lifestyle factors is needed.

The prospective design of this study and the novel epigenetic assay enabling the quantification of immune cells in buffy coat samples after long-term storage provided a unique opportunity to clarify the relationship between Treg/tTL ratio in peripheral blood and cancer risk. The long-term within-subject reproducibility of ImmunoCRIT values was demonstrated based on repeated blood draws, over medium (1-year) and long-term (14-year) time intervals. However, while our main analyses on breast, prostate, lung, and colorectal cancer were well powered, we acknowledge that our subgroup finding of an association between ImmunoCRIT values and ER-negative breast cancer may require replication in a larger study.

In summary, the present study within the prospective EPIC-Heidelberg cohort indicates that a higher Treg/tTL ratio (ImmunoCRIT) in peripheral blood may facilitate cancer development independently from well-established risk factors, at least with regard to colorectal, lung, and ER-negative breast cancer. Overall, our findings suggest a role of a positively skewed Treg/tTL ratio in suppressing immune surveillance of human carcinomas at selected sites by inducing immune tolerance. Consequently, the reduction of peripheral tolerance might be a promising target for the prevention of cancer.

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Notes

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