Circulating Immune Cell Composition and Cancer Risk: A Prospective Study Using Epigenetic Cell Count Measures

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ABSTRACT

Although ample evidence indicates that immune cell homeostasis is an important prognostic outcome determinant in patients with cancer, few studies have examined whether it also determines cancer risk among initially healthy individuals. We performed a case–cohort study including incident cases of breast ($n = 207$), colorectal ($n = 111$), lung ($n = 70$), and prostate ($n = 201$) cancer as well as a subcohort ($n = 465$) within the European Prospective Investigation into Cancer and Nutrition-Heidelberg cohort. Relative counts of neutrophils, monocytes, and lymphocyte sublineages were measured by qRT-PCR. HRs and 95% confidence intervals were used to measure the associations between relative counts of immune cell and cancer risks. When relative counts of immune cell types were taken individually, a significant positive association was observed between relative counts of FOXP3$^+$ regulatory T cells (Tregs) and lung cancer risk, and significant inverse associations were observed between relative CD8$^+$ counts and risks of lung and breast cancer (overall and ER+ subtype). Multivariable models with mutual adjustments across immune markers showed further significant positive associations between higher relative FOXP3$^+$ T-cell counts and increased risks of colorectal and breast cancer (overall and ER− subtype). No associations were found between immune cell composition and prostate cancer risk. These results affirm the relevance of elevated FOXP3$^+$ Tregs and lower levels of cytotoxic (CD8$^+$) T cells as risk factors for tumor development.

Significance: This epidemiologic study supports a role for both regulatory and cytotoxic T cells in determining cancer risk among healthy individuals.

See related commentary by Song and Tworoger, p. 1801

Introduction

The immune system plays a key role in protecting against cancer. Studies in animal models and in patients with cancer have provided ample evidence that the immune system is able to recognize and eliminate tumor cells through innate and adaptive immune response (immunosurveillance; refs. 1, 2). Although antigen-specific reactions of the adaptive immune system govern actual antitumor response, which may or may not be effective, it has also been reliably observed that the basic counts of different immune cell populations infiltrating tumor tissue or in peripheral blood correlate with clinical outcomes among patients with cancer (1, 3–5). In general, higher counts of CD8$^+$ cytotoxic T cells, CD4$^+$ T-helper 1 cells, natural killer cells, M1 macrophages, and DC1 dendritic cells have been positively associated with favorable antitumor immune responses, whereas CD4$^+$ T-helper 2 cells, M2 macrophages, DC2 dendritic cells, myeloid-derived suppressor cells, and higher ratios of FOXP3$^+$ regulatory T cells have immunosuppressive functions and have been associated with accelerated cancer development and worse prognosis (3–5). However, although there is now abundant evidence that cancer patients' immune defense codetermines tumor progression and clinical prognosis, so far only few human studies have investigated whether immune cell homeostasis also determines cancer risk among initially healthy individuals. The reason for this is that blood samples stored in large-scale population cohort studies usually do not contain intact blood cells, precluding flow cytometry analyses of immune cell counts and composition.

We developed and validated a series of epigenetic assays for the quantification of various leukocyte subpopulations in blood. These assays can be employed and quantitated in a variety of substrates, including DNA extracted from nonintact leukocytes (6). Applying these assays to DNA extracted from stored buffy coat samples of the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heidelberg cohort, we reported a relationship between a higher ratio of Foxp3$^+$ to total CD3$^+$ T-lymphocytes ("ImmunoCRIT" immune tolerance ratio) and increased risk of developing lung, colorectal, and ER− breast cancers (7). The hypothesis addressed in the framework of the EPIC-Heidelberg cohort was that prediagnostic distributions of adaptive immune cells would correlate with the susceptibility to develop manifest tumor diseases. Here, we present extended findings, relating risks of breast, colon, lung, and prostate cancers to a more comprehensive set of quantitative epigenetic markers for total (CD3$^+$), cytotoxic (CD8$^+$), and regulatory (FOXP3$^+$) and non-regulatory (FOXP3−) helper T-lymphocytes, as well as neutrophils, monocytes, natural killer cells, and B-lymphocytes.

Patients and Methods

Study population

EPIC-Heidelberg is an epidemiologic study cohort of 13,611 female and 11,929 male participants ages 35 to 65 years recruited between 1994 and 1998 from the general population of Heidelberg (Germany)

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and surroundings (8), which is part of the larger European EPIC study network (9). At baseline recruitment, all study participants provided extensive data on lifestyle, reproductive factors, and dietary habits via questionnaire (9–11), anthropometric measurements, and a blood sample that was separated into serum, plasma, red blood cells, and a buffy coat fraction. Aliquoted samples of blood fractions were stored under liquid nitrogen (−196°C). The study was approved by the ethics committee of the Heidelberg University hospital and all participants gave written informed consent (8, 9).

Design of case–cohort study

This study was designed as a case–cohort study (12) embedded within the EPIC-Heidelberg cohort. Incident cancer cases were self-reported by follow-up questionnaires and validated by study physicians on the basis of medical records or identified through regional cancer registries (13). For the present analyses, incident cases of invasive breast (ICD-10: C50 $n = 207$), colorectal (ICD-10: C18-C20 $n = 111$), lung (ICD-10: C34 $n = 70$), and prostate cancer (ICD-10: C61 $n = 201$) occurring up to December 31, 2012, were included. The subcohort population ($n = 465$) was selected randomly from all EPIC-Heidelberg study participants; this random sample included 21 incident cancer case participants (breast: $n = 3$; colon: $n = 2$; lung: $n = 2$; prostate: $n = 14$). Overall, this case–cohort study included 1,033 participants.

Laboratory assays

Relative counts of neutrophils, monocytes, and lymphocyte subpopulations were measured by quantitative epigenetic real-time PCR at Epiontis GmbH (14). DNA was extracted at the German Cancer Research Center (DKFZ, Heidelberg, Germany) from frozen pellets of nonintact, nucleated blood cells (buffy coats). DNA quality was assessed using Quant-iT PicoGreen dsDNA Assay (Life Technologies) and OD 260/280 ratio between 1.7 and 2.0 was considered acceptable. DNA samples from cancer cases and subcohort members were randomly dispersed over analytical batches and sent to EPIONTIS, where laboratory personnel was blinded with regard to the case or noncase status of the samples received. The general procedure for the assays performed at Epiontis is given below; more technical details are given in the Supplementary Data and Methods.

Genomic DNA was treated with ammonium bisulphite, converting unmethylated cytosine to uracil while leaving methylated cytosine unchanged, and relative quantities of different lymphocytes were determined through assays for epigenetic (unmethylated) CpG sites that were shown to be stably associated with specific immune cell type lineages, as described in full detail by Baron and colleagues (2018). Absence of CpG methylation in those gene loci (amplicon regions) were used for the epigenetic cell counting. These loci were *CD3G/CD3D, CD8B, CD4*, and *FOXP3* for quantification of total $CD3^+$ T cells and $CD3CD8^+$ cytotoxic T cells, $CD3^+/CD4^+$ T helper cells and regulatory T cells (Tregs), respectively. On the basis of these data, their according relative proportions were determined for $CD3^+/CD8^+$, $CD3^+/CD4^+$, and $CD3^+/CD4^+/FOXP3+$ cells. Likewise, specific loci in the *PARK2, LRP5, LCN2*, and *MVD* genes were used for quantification of monocytes, B cells, neutrophils, and natural killer (NK) cells. qPCR data for each locus was quantified and expressed as a fraction of total leukocytes measured by real-time qPCR markers for *GAPDH* locus. For quantification, *in silico* bisulfite-converted loci for *GAPDH* (total number of leukocytes) and the various cell-type–specific loci were cloned into vector pUC57 (GenScript USA Inc.), precisely quantified, linearized prior PCR reactions, and used as quantification standard. Further details on DNA preparation, conversion, and oligonucleotide sequences and methods used for epigenetic qPCR have been described in full detail previously (6).

All measurements were subjected to rigorous quality controls. Control DNA from pooled blood and a plasmid-based bisulfite conversion control (containing all native, nonmethylated loci) were carried along with in repetitions with each batch measurement of samples. As these samples were measured in duplicates for each batch, intrabatch and interbatch changes of variation (CV) were determined. They ranged between 4.44% (CD3) and 7.67% (Foxp3) for the intrabatch of the reference blood sample and between 2.50% (Monocytes) and 5.33 (CD3) for intrabatch bisulfite conversion control. Interbatch CVs ranged between 7.69% (CD3) and 11.5% (B cells) for the reference blood sample and 6.37% (CD8) and 8.09% (monocytes) for bisulfite conversion control plasmid. Full data are provided in the Supplementary Data and Methods (Section M2).

The various cell-specific assays have each been validated in independent studies by comparison against relative cell counts by flow cytometry [see Baron and colleagues (6), plus further results in Supplementary Data and Methods], showing correlations (Spearman, Pearson) between 0.71 and 0.94 for epigenetic measurements versus cytometry-based relative counts for blood samples collected from in 25 healthy adult blood donors.

Reproducibility study over time

In a random subsample of EPIC-Heidelberg participants, a reproducibility study was carried out to examine the stability of individuals' relative immune cell counts over time. The method used has previously been described for immunoCRIT (7). In brief, relative counts of immune markers were measured in a random subsample of EPIC-Heidelberg participants who had provided blood samples at three different time points: baseline (T_0), 14 years (T_1), and 15 years (T_2) of follow-up. Intraindividual stability over time were evaluated by partial Spearman correlations over 1 year ($T_1 - T_2$), and over 15 years [T_0 – average (T_1, T_2)] for respectively, a total of 79 and 71 substudy participants with complete and normalized assays for all cell types.

Statistical analyses

As the individual cell lineages measured represent all major nucleated cell types in the circulation, and each were expressed as a relative percentage of total nucleated cells, their percentages should add up to about 100%, and on average this was indeed the case for data on a population level. For single study participants, however, due to random measurement errors the sum of individual cell types added up to values fluctuating around the total sum of 100%. Therefore, we applied a further normalization step to set the sum of all major cell-lineages (neutrophils, B cells, monocytes, NK cells, T cells) to exactly 100% for each single study participant. Likewise, the percentages for the $CD4^+$ and $CD8^+$ T-cell subfractions were recalibrated so as to add up to the fraction of total, renormalized ($CD3^+$) T cells, and the percentages of $FOXP3^+$ and $FOXP3^-$ T helper cells were calculated as fractions of the recalibrated percentage of total $CD4^+$ cells (see also **Fig. 1**, and additional description in Supplementary Data and Methods).

Prentice-weighted Cox proportional hazards regression models (15) were used to estimate HRs and 95% confidence intervals (CI) for each immune cell percentage. The models used age as the underlying timescale. All observations in the subcohort were left-truncated at age at recruitment and right-censored at end of follow-up, death, or loss to follow-up, or the occurrence of any cancer (including cancers other than those of the lung, breast, prostate, or colorectum), whichever came first.

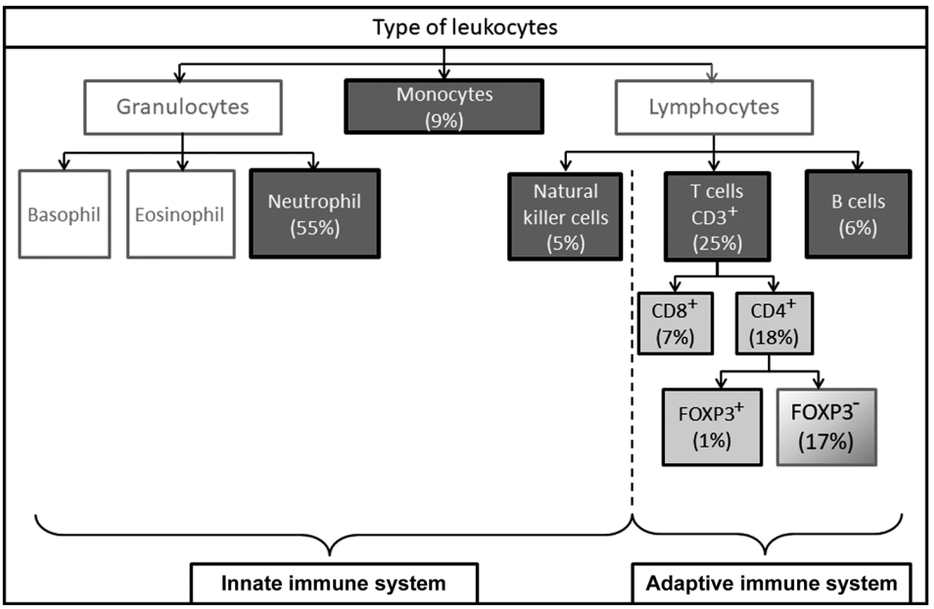

Figure 1.
Leukocyte decomposition scheme. Gray filling (light and dark) represents relative immune cell counts measured for the study. Relative counts (percentages) of cell types marked in dark gray were renormalized so as to add up to a total of 100%. Fractions of $CD4^+$ and $CD8^+$ cells were recalibrated so as to add up to the renormalized fraction of total ($CD3^+$) T cells; fractions of $FOXP3^+$ and $FOXP3^-$ cells were recalibrated so as to add up to fraction of renormalized $CD4^+$ cells. White filling represents immune cells (basic lineages) not measured in the present study. Gray gradient represents the fraction of $CD4^+/FOXP3^-$ cells, calculated as the difference between total $CD4^+$ and $FOXP3^+$ cells. Percentage in brackets represents the average proportion of immune marker onto total leukocytes after normalization.

Risk associations were examined for cell types individually, as well as with mutual adjustments for the other major cell types. With mutual adjustments across different cell types, a series of models were fitted in which first the variable for total T cells ($CD3^+$) was broken down into its $CD8^+$ and $CD4^+$ subcomponents, and then further $CD4^+$ was broken down into its $FOXP3^+$ and $FOXP3^-$ (complementary) subcomponents. In this stepwise decomposition approach, log-likelihood ratio tests were used to examine improvements in overall model fit. As the variables for subcomponents always add up precisely to those for the total of higher-order T-cell lineages (i.e., $CD4^+$ plus $CD8^+$ equals total $CD3^+$, and $FOXP3^+$ plus $FOXP3^-$ equals $CD4^+$), models within this two-step decomposition hierarchy can be considered nested, and stepwise improvements in model fit indicate whether, or not, subcomponent lineages have identical associations with cancer risk as compared with their higher-order sum. This approach of fitting a hierarchical series of nested models has also been used, for example, to examine the association of disease risk with nutrient composition of diet, decomposing total energy (calorie) intake into calories from different nutrient sources (16), or with alcohol consumption, overall or from different types of beverage (17).

All models were estimated first with minimal adjustments for age at blood donation and, in colorectal cancer and lung cancer cases, for sex. To examine further potential confounding variables, models also tested with additional adjustments for covariates showing significant cross-sectional associations with immune cell composition, as identified by Dirichlet regression models (18). Thus, likelihood ratio tests were computed comparing models with and without the following covariates: age (years), physical activity (active/inactive), level of education (having or not having a university degree), body mass index (BMI, kg/m^2), height (cm), alcohol consumption (g/day), processed meat consumption (g/day), fiber consumption (g/day), smoking status (never, former, current), as well as full term pregnancy (yes/no), pill user (ever/never), postmenopausal hormone use (yes/no), and menopausal status (pre-/postmenopausal, perimenopausal were grouped within premenopausal women). For breast cancer, likelihood ratio tests were used to examine statistical significance of heterogeneity in risk associations by subgroups defined by estrogen receptor (ER) status (19).

As multiple cell types (either individually or with mutual adjustment) were tested for their associations with risk of four different types of cancer, we decided to judge the significance of our findings based on *P*-values from permutation tests (20). This resampling approach was chosen to account for the interdependence between the relative counts for different cell types, which may lead to statistical dependence between tests for each of the various cell lineages examined. For all Prentice-weighted Cox proportional hazards regression models, we calculated empirical *P* values for all cell types or cell composition profiles (in the mutually adjusted models) based on 1,000 permutations of the independent variable(s) of interest. For the mutually adjusted models, individuals' entire cell composition profiles were permuted and not the individual cell-type percentages.

Results

Descriptive analyses

At baseline, subcohort participants were younger than those who developed cancer (50.8 years vs. 51.4 years for breast, 55.8 years for colorectal, 55.0 years for lung, and 57.7 years for prostate cancer; **Table 1**). The average follow-up time for the subcohort participants was 13.4 years (range: 0.3–16.5) against 6.7 years (range: 0.08–15.4) for cancer cases, up to their diagnosis. Subcohort participants were slightly overweight (BMI = 25.9 kg/m^2) at baseline, and approximately half of women and two-thirds of men were self-reported ever smokers, including 20% and 25% of current smokers, respectively. Higher proportions of ever- and current smokers were reported by those who developed lung cancer (women: 74%, men: 98%) or colorectal cancer (women: 66%, men: 70%).

The quantitatively most abundant immune cell type was neutrophils (in the subcohort: mean = 54.5%), followed by $CD3^+$ (25.4%) and $CD4^+$ (18.0%) T cells (**Fig. 2**). On average, monocytes, B cells, and $CD8^+$ T cells each represented less than 10% of cells present and lowest percentages were for natural killer (4.8%) and $FOXP3^+$ cells (1.4%). These data are in line with data reported in the literature and detected with flow cytometry for all cell types. Adjusting for age and sex, relative counts of neutrophils—the most abundant cell type, with greatest absolute variability across

Table 1. Characteristics of the study population (n = 1,033).

	Incident cancer cases				Subcohort		
	Breast	**Colorectal**	**Lung**	**Prostate**	**Men**	**Women**	**Total**
N	207	111	70	201	210	255	465
Age at blood draw (years)	51.4 (8.1)	55.8 (6.4)	55.0 (7.5)	57.7 (5.3)	52.2 (6.9)	49.6 (8.5)	50.8 (7.9)
Age at diagnosis (years)	57.8 (7.8)	62.2 (6.7)	61.8 (7.2)	64.9 (5.2)			
Time between blood draw and diagnosis (years)	6.4 (3.5)	6.4 (3.4)	6.9 (3.3)	7.2 (3.3)			
Body mass index (kg/m2)	25.5 (4.9)	27.4 (3.7)	27.4 (4.5)	27.2 (3.2)	26.7 (3.7)	25.2 (4.5)	25.9 (4.2)
Height (cm)	164.8 (5.7)	172.2 (8.1)	169.5 (8.8)	175.0 (6.8)	176.2 (6.2)	163.7 (6.2)	169.3 (8.8)
Physically active[a]	108 (52.2%)	55 (49.5%)	29 (41.4%)	102 (50.7%)	116 (55.2%)	135 (52.9%)	251 (54.0%)
University degree	55 (26.6%)	33 (29.7%)	10 (14.3%)	69 (34.3%)	92 (43.8%)	62 (24.3%)	154 (33.1%)
Alcohol consumption at baseline (g/day)	11.7 (12.5)	30.5 (46.0)	20.7 (22.9)	26.0 (21.8)	25.3 (23.1)	10.5 (13.1)	17.2 (19.7)
Former smokers	58 (28.0%)	47 (42.3%)	17 (24.3%)	87 (43.3%)	90 (42.9%)	71 (27.8%)	161 (34.6%)
Current smokers	37 (17.9%)	30 (27.0%)	46 (65.7%)	33 (16.4%)	52 (24.8%)	51 (20.0%)	103 (22.2%)
Processed meat consumption (g/day)	42.6 (30.4)	55.9 (34.8)	66.2 (49.3)	57.2 (34.8)	60.5 (45.9)	41.6 (28.2)	50.1 (38.3)
Fiber consumption (g/day)	19.1 (6.6)	19.6 (6.5)	19.2 (7.0)	21.2 (6.0)	21.4 (6.8)	19.1 (6.6)	20.1 (6.8)
Women	207 (100.0%)	38 (34.2%)	23 (32.9%)			255 (100%)	255 (54.8%)
Postmenopausal women	107 (51.7%)	21 (55.3%)	15 (65.2%)			100 (39.2%)	100 (39.2%)
Hormone therapy user[b]	70 (65.4%)	11 (52.4%)	6 (40.0%)			46 (46.5%)	46 (46.5%)
Full term pregnancy	164 (79.2%)	32 (84.2%)	20 (87.0%)			204 (80.3%)	204 (80.3%)
Pill ever user	165 (79.7%)	29 (76.3%)	14 (60.9%)			205 (80.7%)	205 (80.7%)
Relative immune cell counts							
Neutrophils	56.0 (10.5)	55.1 (10.7)	56.2 (9.0)	55.1 (9.3)	54.2 (10.9)	54.8 (10.7)	54.5 (10.8)
Monocytes	8.7 (2.7)	9.4 (3.2)	9.0 (3.2)	9.9 (3.2)	9.8 (2.9)	8.9 (2.9)	9.3 (2.9)
Natural killer	4.5 (1.8)	5.2 (2.5)	4.5 (2.1)	5.5 (2.5)	5.1 (2.4)	4.5 (1.9)	4.8 (2.2)
B-lymphocytes	5.8 (2.6)	5.9 (4.1)	6.3 (2.9)	5.6 (2.6)	6.1 (2.8)	6.0 (2.4)	6.0 (2.6)
Cd3	24.9 (7.7)	24.4 (7.4)	24.0 (6.1)	23.8 (6.3)	24.9 (7.6)	25.8 (7.8)	25.4 (7.7)
Cd8	6.7 (2.5)	6.9 (2.9)	6.0 (2.4)	6.9 (3.3)	7.4 (4.0)	7.4 (3.1)	7.4 (3.5)
Cd4	18.2 (6.0)	17.5 (5.6)	17.9 (4.9)	17.0 (4.7)	17.5 (5.5)	18.4 (5.8)	18.0 (5.7)
$FOXP3^+$	1.5 (0.6)	1.4 (0.6)	1.5 (0.6)	1.2 (0.5)	1.3 (0.6)	1.5 (0.6)	1.4 (0.6)
$FOXP3^-$	16.7 (5.5)	16.1 (5.2)	16.4 (4.5)	15.7 (4.3)	16.2 (5.1)	16.9 (5.4)	16.6 (5.3)

Note: Values are *n* (proportions) for categorical variables or means (SD) adjusted for age and sex (if applicable) in generalized linear model for continuous variables. Missing values: one hormone therapy user, one full-term pregnancy, one pill ever user.
[a]According to the Cambridge Physical Activity Index.
[b]In postmenopausal women.

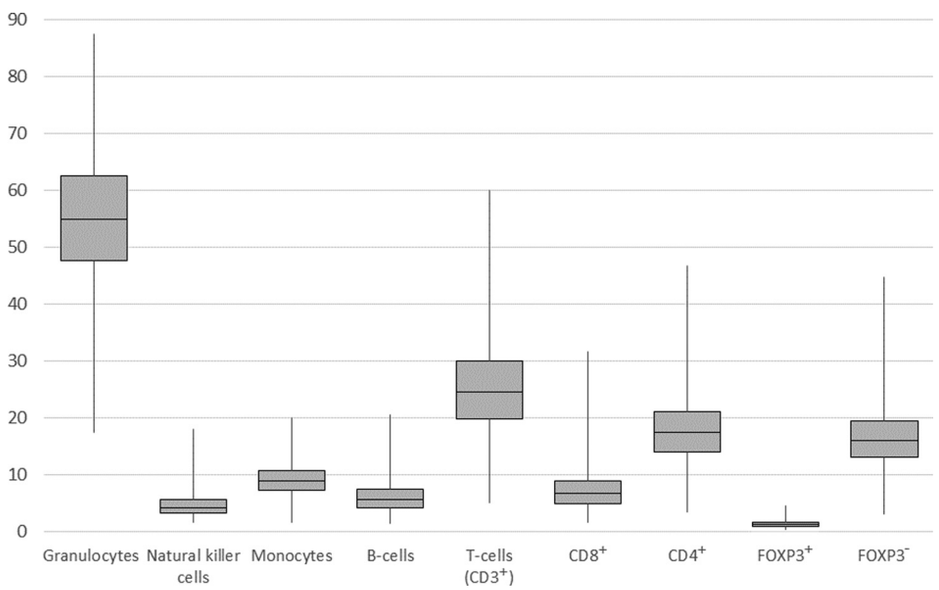

Figure 2.
Relative counts of circulating immune cells in the subcohort (n = 465). Box plots show summary statistics of each immune cell type; the extreme values indicate the range, the boundary of the box closest to zero represents the lower (Q1) quartile, the farthest from zero represents the upper (Q3) quartile, and the line within the box indicates the median.

individuals—showed strong inverse correlations with the proportions of CD3+ (partial Spearman's correlation: $\rho = -0.90$) and $CD4^+$ ($\rho = -0.82$) T cells, and more moderate inverse correlations with the other immune cells ($-0.46 \le \rho \le -0.68$; Supplementary Table S1). Within the T-cell lineage, strong positive correlations were found between relative counts of $CD3^+$ T and $CD8^+$ T cells ($\rho = 0.77$), and between $CD3^+$ T and $CD4^+$ T cells ($\rho = 0.90$), whereas relative counts of $CD8^+$ T and $CD4^+$ T cells were more moderately correlated ($\rho = 0.47$). A positive correlation was observed between relative counts of $FOXP3^+$ and $CD4^+$ T cells ($\rho = 0.75$). Weaker positive correlations ($\rho \le 0.44$) were also observed between relative proportions of monocytes, natural killer cells, and B-lymphocytes.

In the subcohort, Dirichlet regression identified significant cross-sectional associations of circulating immune cell composition with smoking status, alcohol and processed meat consumption in men and women combined, full-term pregnancy, postmenopausal hormone use, level of education, and processed meat consumption in women only, and smoking status and alcohol consumption in men only (Supplementary Table S2; Supplementary Fig. S1). No further associations were found between immune cell composition and physical activity, BMI, height, menopausal status, and past or current exogenous hormone use.

Stability of relative immune cell counts over time

Both over 1 year (T_1 – T_2), and over a 14- to 15-year interval, individuals' relative cell count values showed good reproducibility with age- and sex-adjusted partial Spearman correlations ranging from $r = 0.46$ (monocytes) to $r = 0.68$ ($CD8^+$ T-lymphocytes) 1 year apart, and from $r = 0.48$ (monocytes and $CD4^+$ T-lymphocytes) to $r = 0.67$ ($CD8^+$ T-lymphocytes) 14 to 15 years apart, and with correlations 14 to 15 years apart of 0.83 and 0.51, respectively, for the $CD4^+/CD8^+$ and $FOXP3^+/CD4^+$ ratios (Supplementary Data and Methods; Supplementary Table S3).

Relative cell counts and cancer risk

Considering relative counts for single cell types, adjusting only for age and sex, proportional hazards models showed significant negative associations for relative $CD8^+$ counts with risks of lung cancer and breast cancer, overall and ER+ subtype (**Table 2**) and significant positive associations for relative $FOXP3^+$ counts with risks of lung cancer. Mutually adjusting across immune cell components, models showed significant improvements in the overall fit when the counts for overall $CD3^+$ T cells were broken down stepwise into the constituent counts for cytotoxic ($CD8^+$) and helper ($CD4^+$) T-lymphocytes (cancers of the lung and breast including ER+ subtype), and then further into regulatory ($FOXP3^+$) and nonregulatory ($FOXP3^-$) T-lymphocytes [cancers of lung, breast (overall, ER+, and ER− subtypes) and colorectum]. In the fully decomposed models, based on permutation testing higher relative counts of the cytotoxic $CD8^+$ cells were found to be associated with significantly reduced risks of cancers of the lung and breast (overall, as well as ER+ subtype), whereas higher relative counts of regulatory ($FOXP3^+$) T cells were associated with increased risks of lung, colorectal, and breast cancers (overall and ER− subtype; **Table 2**). Heterogeneity in risk associations by breast cancer ER subtypes were not significant. Relative counts of monocytes, natural killer cells, and B cells showed no association with any of the cancer outcomes. In contrast to all other cancer entities, no associations were observed for relative counts of immune cells with risk of prostate cancer.

Adjusting additionally for factors that showed cross-sectional relationships to immune composition by Dirichlet regression did not fundamentally change any of the HR estimates (Supplementary Table S4), although the association between $FOXP3^+$ and lung cancer weakened and was no longer statistically significant. Furthermore, sensitivity analyses excluding subjects with follow-up shorter than two years showed similar results to the main analyses (Supplementary Table S5), although associations between relative counts of $FOXP3^+$ and breast cancer [overall (HR = 1.51; 95% CI = 0.96–2.37), and ER− subtype (HR = 2.09; 95% CI = 0.93–4.70)] were no longer statistically significant.

Discussion

Using DNA methylation markers for the specific quantification of major immune cell lineages in stored blood DNA (buffy coat) samples, we examined the relationship of relative immune cell counts in blood of initially healthy individuals with subsequent cancer risk. Statistical modeling of the associations of relative cell counts, with stepwise decomposition of total ($CD3^+$) T cells into cytotoxic ($CD8^+$), regulatory ($FOXP3^+$), and nonregulatory ($FOXP3^-$) helper cells, and adjusting for the overall proportions of other major cell lineages, showed an increased risk of cancer (lung, breast) among individuals with lower proportions of $CD8^+$ T cells within the overall T-cell compartment and, for cancers of the lung, breast, and colorectum, with higher proportions of $FOXP3^+$ regulatory T cells among the total $CD4^+$ helper T cells in the circulation. Counts of $CD8^+$ or $FOXP3^+$ T cells showed no associations with risk of prostate cancer. Other than T cells, none of the immune cell types considered showed any association with cancer risk.

We previously reported a significant positive association between the ratio of $FOXP3^+$ to total $CD3^+$ T-lymphocytes— "Immuno-CRIT" immuno-tolerance ratio—and risk of lung, colorectal, and ER− breast cancers (7). Although the associations of cancer risk with this ratio remain fully present and statistically significant within the sub-set of study participants used for the present study, our present analyses, using a more generalized modeling framework to examine the association of disease risks with the relative counts of multiple cell types and with stepwise decomposition within cell lineages, shows that within the overall T-cell lineage, cancer risk is associated with relative numbers of T cells on two levels, namely: (i) the relative counts of $CD8^+$ versus $CD4^+$ cells, and independently; and (ii) within the $CD4^+$ cells, the relative counts of regulatory ($FOXP3^+$) and versus other (nonregulatory) $CD4^+$ cells. Overall, our data indicate increased cancer risks among individuals who have lower $CD8^+$ counts in the overall T-cell compartment, or who within the $CD4^+$ T helper cell department have a higher proportion of $FOXP3^+$ regulatory T cells. In healthy subjects, the balance between cytotoxic effector T cells, which drive the elimination of abnormal cells, and $FOXP3^+$ regulatory T lymphocytes (Tregs), which modulate the aggressiveness of the cellular immune response, controls adaptive immune response (21, 22). Therefore, substantial variance of immune cell count ratio in healthy immune system may provide clues about the likelihood to develop cancer. In line with our results, higher intratumoral accumulation of Tregs and lower accumulation of $CD8^+$ effector cells both have been frequently associated with greater tumor aggressiveness in patients affected by various cancer types, and both factors have been postulated to facilitate cancer development (1–5, 23).

Neutrophils, the most abundant type of leukocyte in human circulation, increasingly are also being recognized as part of the immune reaction to cancer. Patients with cancer, especially those with advanced-stage disease, frequently have increased neutrophil counts in

Table 2. HRs for the association between circulating immune cell composition (relative counts) and cancer risk ($n = 1{,}033$).

					T cells					Model fit improvement *P* value[a]
	Neutrophils	NK cells	Monocytes	B cells	Total $CD3^+$	$CD8^+$	$CD4^+$	$FOXP3^+$	$FOXP3^-$	
Lung cancer (No. of case patients = 70 / subcohort = 465)										
Age and sex adjusted										
Cells modeled individually	1.02 (1.00,1.04)	0.89 (0.77,1.02)	0.93 (0.84,1.04)	1.07 (0.97,1.19)	0.98 (0.95,1.01)	**0.88*** (0.81,0.96)**	1.00 (0.96,1.04)	**1.44* (1.05,1.98)**	0.99 (0.95,1.04)	
Cells modeled with mutual adjustments		0.91 (0.79,1.06)	0.95 (0.84,1.08)	1.12 (1.00,1.26)	0.97 (0.94,1.01)					
		0.93 (0.80,1.08)	0.94 (0.82,1.07)	1.10 (0.97,1.25)		**0.87* (0.78,0.96)**	1.02 (0.96,1.08)			0.008
		0.93 (0.81,1.08)	0.94 (0.82,1.07)	1.09 (0.95,1.25)		**0.86* (0.78,0.96)**		**1.85** (1.16,2.95)**	0.96 (0.89,1.04)	0.003
Colorectal cancer (No. of case patients = 111 / subcohort = 465)										
Age and sex adjusted										
Cells modeled individually	1.01 (0.99,1.03)	1.03 (0.93,1.13)	0.99 (0.91,1.07)	1.02 (0.90,1.15)	0.98 (0.96,1.01)	0.97 (0.92,1.02)	0.99 (0.95,1.02)	1.20 (0.87,1.64)	0.98 (0.94,1.02)	
Cells modeled with mutual adjustments		1.04 (0.94,1.16)	0.98 (0.90,1.08)	1.04 (0.92,1.18)	0.98 (0.95,1.01)					
		1.05 (0.94,1.16)	0.98 (0.90,1.08)	1.04 (0.91,1.19)		0.97 (0.92,1.02)	0.98 (0.94,1.03)			0.70
		1.04 (0.94,1.15)	0.98 (0.90,1.08)	1.03 (0.90,1.18)		0.97 (0.91,1.02)		**1.59* (1.04,2.42)**	0.94 (0.89,1.01)	0.01
Breast cancer, all (no. of case patients = 207 / subcohort = 255)										
Age adjusted										
Cells modeled individually	1.01 (0.99,1.03)	1.00 (0.91,1.10)	0.98 (0.92,1.05)	0.97 (0.89,1.05)	0.99 (0.96,1.01)	**0.93* (0.87,0.99)**	0.99 (0.96,1.03)	1.12 (0.82,1.54)	0.99 (0.96,1.03)	
Cells modeled with mutual adjustments		1.02 (0.92,1.13)	0.98 (0.92,1.05)	0.99 (0.90,1.08)	0.99 (0.96,1.01)					
		1.04 (0.93,1.15)	0.99 (0.92,1.06)	0.97 (0.89,1.07)		**0.91* (0.85,0.98)**	1.02 (0.98,1.06)			0.003
		1.04 (0.93,1.16)	0.98 (0.91,1.05)	0.97 (0.89,1.07)		**0.89** (0.83,0.97)**		**1.57* (1.02,2.44)**	0.99 (0.94,1.04)	0.006
Breast cancer, ER+ (no. of case patients = 159 / subcohort = 255)										
Age adjusted										
Cells modeled individually	1.01 (0.99,1.03)	1.00 (0.90,1.12)	0.99 (0.92,1.06)	0.98 (0.89,1.07)	0.99 (0.96,1.02)	**0.93* (0.87,0.99)**	1.00 (0.97,1.04)	1.13 (0.80,1.61)	1.00 (0.96,1.04)	
Cells modeled with mutual adjustments		1.02 (0.91,1.14)	0.99 (0.92,1.07)	0.99 (0.90,1.09)	0.99 (0.96,1.02)					
		1.04 (0.92,1.17)	0.99 (0.92,1.08)	0.98 (0.88,1.08)		**0.90** (0.83,0.98)**	1.03 (0.99,1.08)			0.002
		1.04 (0.92,1.17)	0.99 (0.91,1.07)	0.97 (0.88,1.08)		**0.88** (0.81,0.96)**		1.49 (0.93,2.38)	1.01 (0.95,1.06)	0.04
Breast cancer, ER− (no. of case patients = 36 / subcohort = 255)										
Age adjusted										
Cells modeled individually	1.02 (0.99,1.05)	1.03 (0.88,1.20)	0.99 (0.90,1.10)	0.93 (0.79,1.08)	0.97 (0.93,1.00)	0.91 (0.82,1.01)	0.96 (0.91,1.02)	1.13 (0.70,1.82)	0.96 (0.90,1.01)	
Cells modeled with mutual adjustments		1.07 (0.90,1.27)	1.00 (0.90,1.12)	0.96 (0.82,1.13)	0.97 (0.93,1.01)					
		1.08 (0.91,1.28)	1.00 (0.90,1.12)	0.95 (0.81,1.12)		0.91 (0.80,1.03)	1.00 (0.94,1.06)			0.34
		1.09 (0.92,1.28)	0.99 (0.88,1.11)	0.95 (0.80,1.14)		0.87 (0.76,1.01)		**2.26* (1.11,4.58)**	0.94 (0.86,1.02)	0.03
Prostate cancer (no. of case patients = 201 / subcohort = 210)										
Age adjusted										
Cells modeled individually	1.01 (0.99,1.03)	1.03 (0.95,1.11)	0.99 (0.92,1.07)	1.02 (0.93,1.12)	0.98 (0.95,1.01)	0.97 (0.92,1.02)	0.98 (0.94,1.02)	0.89 (0.62,1.28)	0.98 (0.94,1.02)	
Cells modeled with mutual adjustments		1.04 (0.95,1.13)	1.00 (0.92,1.09)	1.05 (0.94,1.16)	0.97 (0.94,1.00)					
		1.04 (0.95,1.13)	1.00 (0.92,1.09)	1.05 (0.94,1.17)		0.97 (0.92,1.02)	0.97 (0.93,1.02)			0.90
		1.04 (0.95,1.13)	1.00 (0.92,1.09)	1.05 (0.94,1.17)		0.97 (0.92,1.02)		1.01 (0.59,1.73)	0.97 (0.91,1.03)	0.81

Note: Prentice-weighted Cox regression adjusted for age at recruitment and sex (if applicable). Bold HRs are significant. Codes for *P* values from permutation tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. Fractions of $CD4^+$ and $CD8^+$ cells were recalibrated so as to add up to the renormalized fraction of total ($CD3^+$) T cells; fractions of $FOXP3^+$ and $FOXP3^-$ cells were recalibrated so as to add up to fraction of renormalized $CD4^+$ cells.
[a]Improvement in fit between the present model and the one from the line above.

peripheral blood in comparison to cancer-free control subjects, and higher pretreatment ratios of circulating neutrophil-to-lymphocyte (NLR) have been associated with reduced overall and cancer-specific survival in patients with various types of solid tumors, including tumors of the lung, colorectum and breast (24–26). In our data, contrary to our expectations, we observed no significant association of prediagnosis relative counts of peripheral neutrophils with cancer risk. Possibly, an elevated NLR ratio is a characteristic of later stage cancer patients, reflecting tumor-induced inflammatory responses.

To our knowledge, our analyses in the EPIC-Heidelberg cohort are the first to relate comprehensive quantitative measures of circulating immune cell composition in individuals initially free of known cancer to later cancer risk. A limitation of applying epigenetic assays to buffy coat samples is that it allows quantification only of relative immune cell composition, but not of absolute cell counts relative to blood volume. Nonetheless, the average proportions of circulating leukocytes as measured by our epigenetic markers correspond well to reference values based on classical cell counting by flow cytometry (27), and showed also high correlations with measures of relative counts based on flow-cytometry (Supplemental Data and Methods, Section M4; ref. 6). A further limitation of our present study is the still relatively limited set of only main immune cell lineages that were addressed. Epigenetic marker assays of immune cell subsets, for example CD56bright and CD56dim, $CD4^+$ Treg subtypes, $CD8^+$ subtypes (28, 29) or M1 and M2 macrophages (30), which all might have differential impacts on early cancer development, were not available for this study, but are in development (6, 31–33). Although, our study is based on only a single blood sample per person, the findings from our embedded longitudinal reproducibility substudy showed good correlations between individuals' relative immune cell counts measured repeatedly over a longer prospective time interval. Sensitivity analyses excluding cases diagnosed within less than 2 years after blood donation provided no strong evidence of reverse causation bias. Although the association of relative $FOXP3^+$ counts with breast cancer risk (overall and ER− subtype) was no longer statistically significant in these analyses, likely because of small numbers of cancer cases in ER− subtype ($n = 36$ before and $n = 29$ after exclusion), the effect estimates were not meaningfully different.

Despite its limitations, our present study demonstrates the potential power of using DNA methylation markers for the quantification of relative immune cell counts in blood, combined with basic hierarchical decomposition modeling, to prospectively examine relationships of individuals' immune status with cancer risk in the context of existing epidemiologic studies with biobanks of stored leukocyte DNA, so as to gain further insight into the role of immune status as a risk factor for cancer development among initially disease-free individuals. Although limited numbers of incident cancer cases precluded a more precise analysis, our analyses show a clear improvement in model fit at the deepest, compared with the lower level of decomposition, for breast, colorectal, and lung cancer, reaffirming the association between higher relative $FOXP3^+$ T-cell counts and cancer risk, whereas additionally showing increased risks of lung and breast cancer (overall and ER+) at lower $CD8^+$ T-cell counts. Our results are in line with the notion that abnormal cells are eliminated efficiently by cytotoxic T cells, whereas $FOXP3^+$ regulatory T lymphocytes (Tregs), which weaken cellular immune response by impeding the activation of T effector cells, may preserve abnormal cells from elimination (1, 2).

In summary, our findings confirm that in healthy individuals, not only increased Treg-mediated immune tolerance, but also reduced $CD8^+$-mediated cytotoxicity may both promote cancer development and occurrence of cancer later in life. Although our sample numbers were too small to draw definitive and more quantitative conclusions regarding the strength of associations between immune cell composition and cancer risk, the clear trends observed in our analyses motivate research in larger study populations and using additional methylation markers for more extended series of immune cell subtypes. Understanding the role of individuals' general immune profiles as a contributing cause for cancer development may help identify individuals at increased cancer risk who may benefit from targeted prevention strategies, for example, harnessing an individual's immune system against cancer through lifestyle changes (34–37) chemopreventive drugs (37–39) or prophylactic vaccinations (40–42).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: S. Olek
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