Are Circulating Immune Cells a Determinant of Pancreatic Cancer Risk? A Prospective Study Using Epigenetic Cell Count Measures

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ABSTRACT

Background: Evidence is accumulating that immune cells play a prominent role in pancreatic cancer etiology but prospective investigations are missing.

Methods: We conducted a nested case–control study within the European Prospective Investigation into Cancer and Nutrition (EPIC) study with 502 pairs of incident pancreatic cancer cases and matched controls. Relative counts of circulating immune cells (neutrophils and lymphocyte sublineages: total $CD3^+$, $CD8^+$, $CD4^+$, and $FOXP3^+$ regulatory T cells (Tregs) relative to nucleated cells, (white blood cells) were measured by qRT-PCR. ORs with 95% confidence intervals were estimated using logistic regressions, modeling relative counts of immune cells on a continuous scale.

Results: Neither relative counts of immune cell types taken individually, nor mutually adjusted for each other were associated with pancreatic cancer risks. However, in subgroup analyses by strata of lag-time, higher relative counts of Tregs and lower relative counts of $CD8^+$ were significantly associated with an increased pancreatic cancer risks in participants diagnosed within the first 5 years of follow-up.

Conclusions: These results might reflect reverse causation, due to higher relative counts of Tregs and lower counts of $CD8^+$ cells among individuals with more advanced stages of latent pancreatic cancer, who are closer to the point of developing clinical manifest disease.

Impact: We have shown, for the first time, that increased relative counts of regulatory T cells and lower relative counts of $CD8^+$, cytotoxic T cells may be associated with pancreatic cancer risk or relatively late-stage tumor development.

See related commentary by Michaud and Kelsey, p. 2176

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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Introduction

Animal experiments and clinical studies have shown that innate and adaptive immune responses (immuno-surveillance) play a key role in pancreatic cancer development (1–3). The formation of pancreatic precursor lesions and the further development into an invasive tumor is accompanied by progressive infiltration of various types of immune cells (4). In patients with pancreatic cancer, the total or relative counts of various types of immune cells in tumor tissue (5, 6), but also in peripheral blood (7, 8), have been found to correlate with clinical outcomes. In general, higher total or relative counts of $CD8^+$ cytotoxic T cells, $CD4^+$ T-helper 1 cells, and natural killer cells have been associated with more favorable patient outcomes, whereas higher counts of $CD4^+$ T-helper 2 cells and of $FOXP3^+$ regulatory T cells have been associated with greater immunosuppression, accelerated cancer development and worse prognosis (9). In addition, neutrophil counts have often been found to be higher among patients with cancer, including patients with pancreatic cancer (10), as compared with cancer-free control subjects, and elevated pretreatment ratios of circulating neutrophil-to-lymphocyte (NLR) have been associated with lower overall patient survival (11, 12). However, although substantial evidence now documents the prognostic significance of immune defense among patients, so far no studies have examined whether precisely quantified immune cell homeostasis in initially healthy individuals also determines future pancreatic cancer risk. The major reason for this latter deficit is that blood samples stored in large-scale population cohort studies usually do not contain intact blood cells, prohibiting flow cytometry analyses of immune cell composition.

Recently, we have developed and validated methods that allow the determination of immune cell composition relative to total nucleated cells by qPCR of epigenetic markers specific for different immune cell lineages (13). This type of assay can be applied to DNA extracted from frozen, nonintact white blood cells as well as other tissues. By applying this method to stored buffy coat samples of the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heidelberg cohort, we found that higher counts of $FOXP3^+$ (regulatory) T-cell relative to total nucleated cells were associated with higher risks of cancers of the lung, colorectum and breast, whereas lower relative $CD8^+$ (cytotoxic) T cells counts were inversely associated with lung and breast cancer risks (14). In contrast, this first study showed no associations of cancer risk with relative counts of monocytes, B cells, or natural killer cells, and only marginally suggestive associations (nonsignificant) for neutrophils.

To explore whether similar prospective relationships exist between counts of circulating immune cells relative to total nucleated cells and risk of pancreatic cancer, overall or depending on the prospective lag-time between blood sampling (time of immune cell counts) and cancer diagnosis, we performed a case–control study nested within the Europe-wide EPIC cohort (15), with a focus on estimated relative counts for neutrophils and lymphocyte T-cell sublineages.

Materials and Methods

Study population

The current investigation was based on a case–control study nested within the European EPIC cohort—a multicenter prospective study in Europe described in detail previously (15). Briefly, 519,978 healthy men and women, mostly aged between 35 and 70 years, were recruited by 23 collaborating centers in 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom) between 1992 and 2000. At baseline, participants filled in comprehensive questionnaires on lifestyle, nutrition, and diseases and their height, weight, and body circumferences (waist and hip) were measured. In addition, study participants provided blood samples, which were processed into serum, plasma, and red blood cells, as well as a buffy coat containing all nucleated cells. Processed blood cell fractions were frozen and stored centrally at the International Agency for Research on Cancer (IARC), as well as locally in each recruitment center. The EPIC centers in Malmö (Sweden) and Greece did not contribute to this study.

Incident cancer cases were identified by population cancer registries (Denmark, Italy, the Netherlands, Spain, Sweden, and the United Kingdom) or by a combination of methods including linkage with cancer and pathology registries and systemic verification of active follow-up (France, Germany). In all EPIC centers, data on vital status were collected through population registries, in combination with national health insurance data (France).

Nested case–control design

The present project is based on incident pancreatic cancer cases diagnosed between 1993 and 2008.

Pancreatic cancer incidence data were coded according to ICD-10 and included all invasive exocrine pancreatic cancers that were coded as C25 (25.0–25.3, 25.7–25.9). Exclusion criteria were the occurrence of other malignant tumors preceding the diagnosis of pancreatic cancer, except for nonmelanoma skin cancer, and nonavailability of blood specimens. Seventy-three percent were adenocarcinomas whereas the other 27% were not otherwise specified. Of these 145 unspecific cases, 11% were of advanced and 74% of unknown stage. Microscopically confirmed were 391 cases (78%), whereas the diagnosis of the remaining 22% was based on clinical observations, imaging, or autopsy.

For each case subject, one control subject was selected using an incidence density sampling protocol. Case and control subjects were matched on study recruitment center, sex, age at blood collection ($\pm$6 months), date of blood collection ($\pm$2 month), time of blood collection ($\pm$2 hours), length of follow-up, and time between blood sampling and time of last consumption of any foods or drinks (<3, 3–6, ≥6 hours). We included initially 530 incident pancreatic cancer cases and 533 controls, building 502 matched case–control pairs and 59 single subjects (31 controls, 28 cases) with no matching partner due to depleted blood samples in the biorepositories.

Laboratory methods: quantification of immune cells

Relative counts of neutrophils and lymphocyte subpopulations were measured by quantitative epigenetic real-time PCR at EPIONTIS GmbH (13).

DNA was extracted at the IARC, from frozen pellets of nucleated blood cells (buffy coats) using the Autopure LS DNA preparation platform (Autogen; ref. 16). Relative counts of immune cells in genomic DNA extracted from buffy coats were assessed by qRT-PCR assisted cell counting (qPACC). Descriptions of the laboratory methods are given on the Epiontis web site (http://www.epiontis.com) have been published previously (13, 14, 17–19), and are detailed in the Supplementary Methods and Materials M1 to M4. In brief, for each subject, 1.5 μg of genomic DNA bisulfite treated prior to qPCR analysis. Relative counts of neutrophils, and lymphocyte sublineages (Tregs, $CD4^+$, and $CD8^+$ T cells) were assessed by qPACC. The assays are based on the measurement of genetic loci that have been shown to be specifically unmethylated in the target cell type, whereas methylated in all other nucleated cells of the blood. Specific gene loci (amplicon regions) used for the epigenetic cell count quantification include

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CD3G/CD3D (all $CD3^+$ T cells), CD8B ($CD3^+/CD8^+$ T cells), CD4 ($CD4^+$ T cells), FOXP3 ($CD3^+/CD4^+/FOXP3^+$ Tregs), and LCN2 ($CD15^+$ neutrophils). The qPCR signals for each of the immune cell types were normalized relative to the number of nucleated (white blood) cells by using a constitutively unmethylated locus in the GAPDH gene. Each of the assays has been extensively validated in various studies by comparison against absolute cell counts by flow cytometry and show very high correlations (0.85 and higher), both for absolute cells counts (per mm^3 of whole blood) and for relative counts as a proportion of total nucleated cells (in whole blood; refs. 13, 20).

Statistical analyses

Each individual cell lineage measured was expressed as a relative percentage of total nucleated cells in the circulation. In addition, for each study participant, the percentages for the $CD4^+$ and $CD8^+$ T-cell subfractions were further normalized (recalibrated) so as to add up to the fraction of total $CD3^+$ 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 Supplementary Fig. S1, additional description in Supplementary Methods and Materials M1–M4 and in ref. 14). The latter, hierarchical recalibration of T-cell subfractions allows an evaluation of improvement in model fit upon stepwise decomposition of the total $CD3^+$ T cells into $CD4^+$ and $CD8^+$ sublineages, and of $CD4^+$ into $FOXP3^+$ and $FOXP3^-$ sublineages, as discussed below.

Multiple imputation was used to impute relative counts of immune cells missing at random in complete matched case sets, that is, neutrophils (proportion of missing 11.1%), $CD3^+$ (1.7%), $CD8^+$ (1.8%), $CD4^+$ (2.7%), $FOXP3^+$ (5.5%), adjusted for smoking status (ever vs. never), body mass index (BMI, kg/m^2), diabetes (yes, no, unknown), and case/control status. This allowed us to perform analyses in 502 matched case–control sets instead of 416 sets with originally complete immune cell data. The Markov Chain Monte Carlo method for arbitrary missing patterns was used to generate 25 imputed datasets and results were combined to generate valid statistical inferences (21). Comparable results were obtained between imputed and nonimputed datasets, albeit number of case sets were different in the two analyses and risk estimates not significant (Supplementary Table S2).

To examine cross-sectional relationships between relative counts of immune cells and selected baseline risk covariates in controls, partial correlations were calculated adjusted for age at blood draw and sex, for each of the 25 imputed dataset. Resulting correlation coefficients were combined using Fisher z transformation as explained in the SAS documentation PROC MIANALYZE. In addition, a generalized linear model (GLM) was performed to estimate the association between immune markers and diabetes risk in controls.

Conditional logistic regression models were used to examine associations of relative immune cell counts with pancreatic cancer risk, modeling immune cells as continuous variables. Statistical analyses focused in parallel on: (i) the association of pancreatic cancer risk with relative neutrophil counts; and (ii) the association of pancreatic cancer risk with relative blood counts of total ($CD3^+$) T cells and T-cell subfractions ($CD4^+$ vs. $CD8^+$, and within the $CD4^+$ fraction $FOXP3^+$ vs. other CD4 helper cells). Risk associations were examined for neutrophils and overall $CD3^+$ T cells (Model 1), as well as in a series of nested models. Stepwise decomposition models were fitted within the recalibrated T cells, breaking down total T-lymphocytes ($CD3^+$) into its $CD8^+$ (cytotoxic) and $CD4^+$ (helper cell) subfractions (Model 2), and $CD4^+$ into its $FOXP3^+$ (regulatory) and $FOXP3^-$ (nonregulatory) subfractions (Model 3; ref. 14). 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. For example, if the model including separate variables for $CD4^+$ and $CD8^+$ cells shows better fit than a model including a variable only for $CD3^+$ cells (the sum of $CD4^+$ and $CD8^+$) this implies that pancreatic risk has significantly different associations with $CD4^+$ and with $CD8^+$, and that these two risk associations are not summarized well by association of pancreatic cancer risk with the single variable of total $CD3^+$ cells.

Analyses were performed for the full, overall case–control sets as well as by strata of lag-time (i.e., strata of 5-years follow-up) between blood draw and cancer diagnosis (<5, 5–10, and ≥10 years). Heterogeneity of the associations of pancreatic cancer risk with immune cell counts by strata of prospective follow-up time was assessed as statistical interaction effect, using likelihood ratio tests. Additional adjustments for potential confounders including smoking status (never, former, current), BMI (kg/m^2), self-reported preexisting diabetes at baseline (yes/no), and alcohol lifetime intake (g/day) were examined separately in each model. Although some potential confounders were associated with pancreatic cancer risk (BMI, smoking, diabetes), none were related to immune cell counts and none of the potential confounders changed risk estimates by more than 10% and, therefore, we discarded them from our main models. ORs adjusted for lifestyle factors are very similar and shown in Supplementary Table S3. Sensitivity analyses were performed in microscopically confirmed cases only ($n = 391$, 78% of cases) and by modeling lag-time in shorter intervals close to baseline (i.e., ≤2 years and 2–5 years).

All statistical analyses and imputations were conducted using the Statistical Analysis System (SAS) software package, version 9.4 (SAS Institute Inc.).

Ethics approval and consent to participate

This research project has been performed in accordance with the Declaration of Helsinki and has been approved by the Ethical Committee at IARC (IEC 14-01) and the Medical Faculty Heidelberg (S-103/2015).

Data availability

The EPIC project was launched in the 1990s. Unlike in new studies that we run today, public access to data from the EPIC population was not part of the study protocol at that time. Thus, the data protection statement and informed consent of the EPIC participants do not cover the provision of data in public repositories. Nevertheless, we are open to providing our dataset upon request for (i) statistical validation by reviewers and (ii) pooling projects under clearly defined and secure conditions and based on valid data transfer agreements.

Results

Baseline characteristics of the nested case–control participants are displayed in **Table 1**. Pancreatic cancer cases were on average 64 years old at diagnosis (range: 37–87) with a median follow-up of 8 years (range: 0–16) after baseline blood sampling. More than half of case and control subjects were overweight at recruitment (62% of cases and 55% of controls). Compared with control subjects, cases more often reported current smoking (32% vs. 23%) and preexisting diabetes

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Table 1. Baseline characteristics of the study subjects in the nested case–control study within EPIC [median (min–max) or *n* (%)].

Variable	Cases ($n = 502$)	Controls ($n = 502$)
Women	248 (49)	248 (49)
Age at recruitment (years)	57 (30–76)	57 (30–76)
Age at diagnosis (years)	64 (37–87)	-
Follow-up (years)	8 (0–16)	
BMI (kg/m^2)		
<25	193 (38)	224 (45)
≥25	309 (62)	278 (55)
Alcohol intake at recruitment (g/d)[a]		
Men	15 (0–138)	14 (0–147)
Women	3 (0–81)	3 (0–59)
Smoking status[a]		
Never	187 (37)	222 (44)
Former	148 (30)	164 (33)
Current	162 (32)	114 (23)
Pack-years	5.0 (0–86)	0.4 (0–81)
Diabetes status[a]		
Self-reported diabetes at recruitment	29 (6)	19 (4)
No diabetes	418 (83)	434 (87)
Counts of immune markers relative to total nucleated cells (%)[a,b]		
Neutrophils	55 (3–107)	54 (4–97)
CD3$^+$	22 (4–60)	22 (6–54)
CD8$^+$	7 (1–28)	7 (0–27)
CD4$^+$	15 (2–40)	15 (1–42)
FOXP3$^+$	1 (0–4)	1 (0–4)
FOXP3$^-$	14 (1–37)	14 (0–39)

[a]Missing (*n* case/*n* control): alcohol intake at recruitment (2/3), smoking status (5/2), pack year (72/55), diabetes (55/49), neutrophils (62/49), CD3$^+$ (7/10), CD8$^+$ (9/9), CD4$^+$ (15/12), FOXP3$^+$ (26/29), FOXP3$^-$ is the difference between CD4 and FOXP3$^+$.

[b]Immune marker averages calculated after multiple imputation and recalibration. Neutrophils were not recalibrated; therefore individual percentage can exceed 100.

(6% vs. 4%) at the time of recruitment. Cases and controls showed no differences in alcohol intake (g/day) at recruitment in either men (15% of cases and 14% of controls) or women (3% in cases and controls).

Among the control subjects, on average the neutrophils constituted 54% (range: 4–97) of all nucleated cells, whereas the CD3$^+$ cells represented about 22% (6–54). Within the CD3$^+$ T-cell compartment, 15% and 7% of the total nucleated cells were CD4$^+$ and CD8$^+$ cells, respectively, and within the CD4$^+$ compartment 1% of total nucleated cells were FOXP3$^+$. Percentages were similar among the cases (**Table 1**). Adjusting for age at blood draw and sex, the relative counts of total CD3$^+$ T-lymphocytes and all T-cell subfractions (CD8$^+$, CD4$^+$, FOXP3$^+$) were inversely correlated with neutrophils ($r \leq -0.40$) in controls. Within the T-cell lineage, there was a moderate correlation ($r = 0.51$) between the relative counts of CD8$^+$ T and CD4$^+$ T cells, and a positive correlation ($r = 0.65$) was also observed between relative counts of FOXP3$^+$ and total CD4$^+$ cells. All cell types showed only weak and nonsignificant correlations ($r < 0.16$) with BMI, waist circumference, smoking (pack years), time from quitting smoking or alcohol consumption (**Fig. 1**). In addition to using GLM, the average of relative counts of immune cells were not statistically different between diabetes status (e.g., neutrophils $P = 0.21$, CD3$^+$ $P = 0.83$, CD8$^+$ $P = 0.78$, CD4$^+$ $P = 0.90$, FOXP3$^+$ $P = 0.89$).

Regarding associations with pancreatic cancer risk, conditional logistic regression showed no significant associations per percent unit increase of neutrophils or total CD3$^+$ T-lymphocytes with pancreatic cancer, irrespectively of the lag time between blood donation and cancer diagnosis (**Table 2**). Likewise, in the full case-control study set there were no significant improvements in the overall fit for multivariable models when the counts for overall CD3$^+$ T cells (model M1) were broken down into the constituent counts for cytotoxic (CD8$^+$) T-lymphocytes and CD4$^+$ (helper) T cells (model M2), or when counts of the CD4$^+$ compartment were further broken down into regulatory (FOXP3$^+$) and nonregulatory (FOXP3$^-$) T-lymphocytes (model M3). In analyses by strata of lag-time, however, the hierarchical decomposition into T-cell constituents resulted in borderline significant improvement of model fit when focusing on pancreatic cancer cases whose blood samples were collected less than 5 years prior to cancer diagnosis ($P_{M2M3} = 0.05$ or $P_{M1M3} = 0.03$). Within this 0- to 5-year lag-time interval, the decomposed, mutually adjusted model showed higher pancreatic cancer risk for higher relative counts of regulatory (FOXP3$^+$) T-lymphocytes [for a percent unit: OR = 1.80; 95% confidence interval (CI), 1.01–3.20], and for lower counts of cytotoxic CD8$^+$ lymphocytes (OR = 0.92; 95% CI, 0.85–1.00; **Table 2**). For lag-times between blood donation and cancer diagnosis longer than 5 years, these associations and improvements in overall model fit were not observed. Above observed risks were stronger in analyses restricted to microscopically confirmed cases, that is, OR = 2.22 (95% CI, 1.12–4.40) for higher FOXP3 and OR = 0.92 (95% CI, 0.83–1.02) for higher CD8$^+$ within the first 5 years of follow-up ($P_{M2M3} = 0.01$ or $P_{M1M3} = 0.02$; not tabled). Further detailed lag-time analyses showed step-wise higher nonsignificant associations for FOXP3 the shorter the follow-up, that is, the closer to baseline (≤2 years OR = 2.15; 95% CI, 0.55–8.40 with $n_{cases} = 52$, 2–5 years OR = 1.74; 95% CI, 0.92–3.31 with $n_{cases} = 95$, and 5–10 years OR = 1.06; 95% CI, 0.69–1.63; data not in tables). Significant Likelihood ratio tests were observed for the interaction between length of follow-up and relative immune cell counts of FOXP3$^+$ across follow-up categories (<5 years vs. 5–10 years, $P = 0.04$).

No significant associations were observed in analyses stratifies by smoking status (ever/never; not tabled). Further stratification by follow-up, however, did show higher pancreatic cancer risk with higher FOXP3$^+$ in ever smokers (for a percent unit: OR = 1.87; 95% CI, 1.05–3.33) with follow-up <5 years, although not significant in fully adjusted models.

Additional adjustments for relative counts of neutrophils did not materially alter the fit of any of the above models or alter any of the estimated associations (logistic regression coefficients) between relative cell counts and pancreatic cancer risk.

Discussion

Using a nested case–control design within the European EPIC cohort, we examined the prospective relationship of relative immune cell counts in blood of initially healthy individuals with pancreatic cancer risk, focusing on neutrophils and total (CD3$^+$), cytotoxic (CD8$^+$), and regulatory (FOXP3$^+$) and nonregulatory (FOXP3$^-$) CD4$^+$ (helper) T-lymphocytes. To determine the relative quantities of these cell types, we used quantitative real-time PCR assays of DNA methylation markers, which provide highly accurate measurements as validated by comparison to absolute to relative cell counts by flow cytometry (13).

The adaptive immune response among healthy individuals depends on the balance between cytotoxic CD8+ effector T cells, which drive

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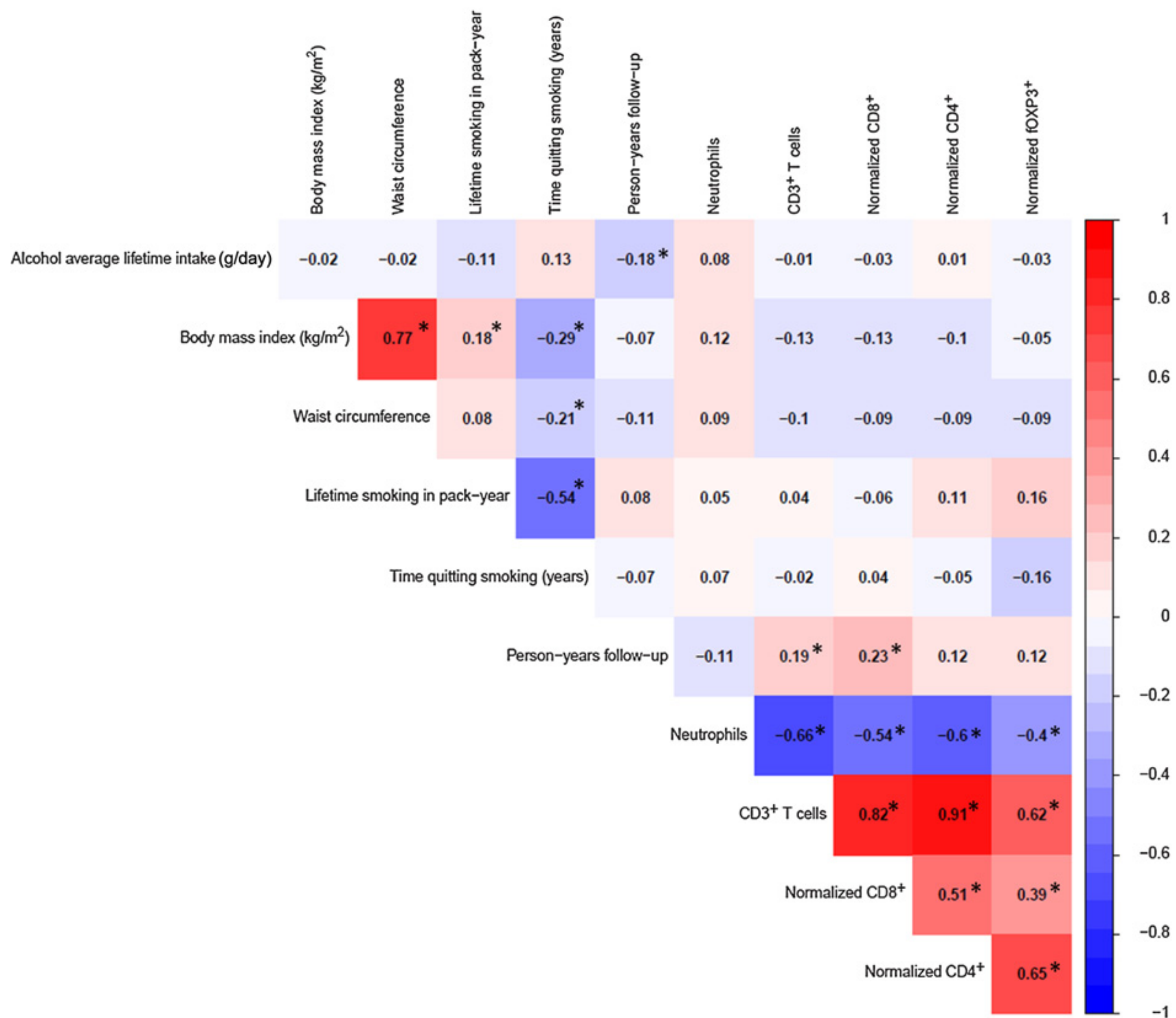

Figure 1.

Heatmap representing correlations between immune markers and selected baseline factors in 502 controls, adjusted for age and sex, and combined using Fisher transformation. *, Denotes significance $P < 0.05$. Missing (*n* case/*n* control): alcohol average lifetime intake (87/82), waist circumference (56/56), Lifetime smoking in pack-year (72/55), time quitting smoking in former smokers (16/15), neutrophils (62/49), T-lymphocytes after normalization.

the elimination of abnormal cells, and FOXP3$^+$ regulatory T lymphocytes (Tregs), which modulate the aggressiveness of the cellular immune response (22, 23). In patients with pancreatic cancer, studies have found greater tumor aggressiveness and worse survival when tumor tissue (especially the tumor center) showed higher FOXP3$^+$ T-cell infiltration, whereas high infiltration of particularly CD8$^+$ T cells generally corresponded with better outcomes (24, 25). Similarly, studies have shown worse oncologic outcomes, for example, cancer-specific survival including pancreatic cancer (9), among patients who present with higher Tregs and lower CD8$^+$ T-lymphocyte counts in blood (26–28). It thus appears plausible that, also among initially healthy individuals, lower relative blood cell counts of CD8$^+$ effector T-lymphocytes and higher counts of Tregs within the CD4$^+$ T helper cell compartment may be related to higher risk of future cancer development.

Contrary to our expectations, our present study shows no long-term associations of pancreatic cancer risk with the relative quantities of CD8$^+$, or FOXP3$^+$ and FOXP3$^-$ (CD4$^+$) T-cell components, when we used statistical models with stepwise decomposition of total (CD3$^+$) T cells into cytotoxic (CD8$^+$), regulatory (FOXP3$^+$), and nonregulatory (FOXP3$^+$) helper cells. These overall null findings stand in contrast to our earlier results from the EPIC-Heidelberg cohort, where over prospective follow-up times up to 15 years (median 6.7 years) this step-wise modeling approach revealed a higher long-term risk of cancers (lung, breast) among initially healthy individuals who had lower proportions of CD8$^+$ T cells within the overall T-cell compartment, or who had higher proportions of FOXP3$^+$ regulatory T cells among the total circulating CD4$^+$ helper T cells (cancers of the lung, breast, and colorectum; ref. 14). However, in subgroup analyses, considering blood samples collected no more than 5 years prior to cancer diagnosis, our present findings did suggest an association of pancreatic cancer risk with a T-lymphocyte signature similar to that identified in our previous EPIC-Heidelberg study, namely higher pancreatic cancer risk among those who had higher relative counts

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Table 2. ORs for the association between circulating immune cell composition (relative counts) and pancreatic cancer risk.

	Models[a]	Neutrophils	T cells					Model fit improvement
			Total CD3$^+$	CD8$^+$	CD4$^+$	FOXP3$^+$	FOXP3$^-$	*P* value[b]
All (502 cases/502 controls)								
Cells modeled individually	M1	1.01 (1.00,1.02)	0.99 (0.97,1.01)					
Cells modeled with mutual adjustments across	M2			0.99 (0.95,1.03)	0.99 (0.96,1.02)			$P_{M1M2} = 0.89$
T-lymphocyte cells	M3			0.99 (0.95,1.03)		1.18 (0.89,1.57)	0.98 (0.94,1.01)	$P_{M2M3} = 0.22$
								$P_{M1M3} = 0.45$
Lag time < 5 years (147 cases/147 controls)								
Cells modeled individually	M1	1.01 (0.99,1.03)	0.99 (0.96,1.03)					
Cells modeled with mutual adjustments across	M2			0.93 (0.86,1.01)	1.03 (0.98,1.08)			$P_{M1M2} = 0.08$
T-lymphocyte cells	M3			0.93 (0.85,1.00)		1.80 (1.01,3.23)	0.99 (0.94,1.06)	$P_{M2M3} = 0.05$
								$P_{M1M3} = 0.03$
Lag time 5 to 10 years (221 cases/221 controls)								
Cells modeled individually	M1	1.00 (0.99,1.02)	0.99 (0.96,1.02)					
Cells modeled with mutual adjustments across	M2			1.04 (0.98,1.10)	0.96 (0.92,1.00)			$P_{M1M2} = 0.06$
T-lymphocyte cells	M3			1.04 (0.98,1.10)		1.08 (0.70,1.66)	0.95 (0.90,1.00)	$P_{M2M3} = 0.58$
								$P_{M1M3} = 0.14$
Lag time 10 years or more (134 cases/134 controls)								
Cells modeled individually	M1	1.00 (0.98,1.03)	0.98 (0.95,1.02)					
Cells modeled with mutual adjustments across	M2			0.97 (0.90,1.04)	1.00 (0.93,1.07)			$P_{M1M2} = 0.60$
T-lymphocyte cells	M3			0.97 (0.90,1.05)		0.92 (0.53,1.62)	1.00 (0.93,1.08)	$P_{M2M3} = 0.77$
								$P_{M1M3} = 0.81$

Note: $n = 502$ controls matched to 502 cases on study recruitment centre, sex, age at blood collection, date and time of blood collection, length of follow-up, and fasting status.
[a]M1: model including CD3$^+$ only, M2: model including CD8$^+$ and CD4$^+$, M3: model including CD8$^+$, FOXP3$^+$, FOXP3$^-$.
[b]Improvement in fit between M1, M2, and M3.
Conditional logistic regression, modelling immune cells continuously as percent counts. Foxp3$^+$ with unit 0.1.
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.

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for FOXP3$^+$ regulatory T cells among the total CD4$^+$ helper T cells, and lower proportions of cytotoxic (CD8$^+$) T-lymphocytes. For pancreatic cancer patients whose blood samples had been collected more than 5 years prior to diagnosis, and their matched control subjects, this association pattern was not observed.

The most plausible explanation for seeing associations between relative immune cell counts and pancreatic cancer risk only in the first five years of follow-up is reverse causation, the associations reflecting progressive increases in circulating Tregs, and decreases in CD8$^+$ cells, as future pancreatic cancer patients gradually develop more advanced-stage and eventually symptomatic tumors. The well-described model of pancreatic cancer development via precursor lesions (29) and massive infiltration and later shedding of immune cells to the circulation (3) may also support this latter interpretation. Unfortunately, we could not examine whether the associations of immune cell composition with pancreatic cancer within the first 5 years of prospective follow-up varied according to stage at diagnosis, due to incomplete information on tumor stage. Most pancreatic cancers, however, are diagnosed in advanced stage (30).

Neutrophils were originally considered to have pro-inflammatory functions as part of the innate immune responses and as effectors of acute inflammation, but are increasingly being recognized to also exert a broader array of specialized functions in adaptive immune reactions and chronic inflammatory responses to cancer and other diseases (31, 32). In blood, patients with various types of solid tumors, including pancreatic cancer (10, 33, 34), often show increased neutrophil counts compared with cancer-free control subjects, and higher ratios of circulating NLR have been associated with more advanced disease stage and poorer cancer-specific survival rates (35, 36). Again, however, contrary to our initial expectations our present data show no significant association of prediagnosis relative counts of peripheral neutrophils with risk of developing pancreatic cancer. It is worth noting that, in our previous study in the EPIC-Heidelberg cohort, we also did not identify any prospective association of relative neutrophil counts with risks of cancers of the lung, breast, colorectum, or prostate (14).

To our knowledge, our analyses are the first to relate pancreatic cancer risk to relative measures of T-lymphocyte composition using a biologically validated set of cell lineage-specific epigenetic markers, in individuals initially free of known cancer. Cell proportions estimated from epigenetic cell lineage markers—derived from methylation arrays—are nowadays more frequently used. Although this chip array technology is functionally not associated and the role and association of individual CpGs is mostly unknown, these approaches do confirm our currently used approach. Michaud and colleagues recently published on DNA methylation derived immune cell profiles and pancreatic cancer risk but did not observe any association with CD3$^+$, CD4$^+$, and neutrophils, among others, irrespective of lag-time (37). Their method differs from ours, and used deconvolution algorithms to estimate relative immune cell counts from a broader series of epigenome-wide methylation markers, whereas our more precise and more powerful method uses not only cell type associated but also comethylated regions that were biologically valid and extensively validated against flow cytometry (13).

A limitation of our study is that the epigenetic assays for buffy coat samples allowed quantification only of relative immune cell counts, relative either to total nucleated cells or to a higher-order cell lineage for T-cell compartments, but not of absolute cell counts relative to blood volume. Nonetheless, relative counts by our epigenetic assays show excellent correlations ($r \geq 0.85$) with counts obtained by flow cytometry (13). Another limitation is that only a single, baseline blood sample is available for the EPIC cohort participants. In our previous study in the EPIC-Heidelberg cohort, however, we found that there was a relatively high intra-individual stability of relative immune cell counts between repeat blood samples collected over time for a sub-set of study participants, with age- and sex-adjusted partial Spearman correlations over a 15-year time interval of about 0.50 for neutrophils, total CD3$^+$, total CD4$^+$ and FOXP3$^+$ cells, and of 0.67 for CD8$^+$ cells (14).

In summary, this study in initially cancer-free individuals showed no long-term relationship between blood counts of neutrophils or T-lymphocyte sublineages with later risk of developing pancreatic cancer. However, within prospective follow-up times of less than 5 years between blood sampling and cancer diagnosis, our data suggest a possible higher pancreatic cancer risk in relation to circulating immune cell signature characterized by higher Treg-mediated immune tolerance, and lower CD8$^+$ mediated cytotoxicity—a signature that we previously found to be associated with higher risks of cancers of the lung, breast, and colorectum. These time-restricted associations may reflect reverse causation—that is, alterations in circulating immune cell composition induced that result from progressive pancreatic cancer development.

To our knowledge, these analyses are the first to relate pancreatic cancer risk to specific and well-validated epigenetic markers as quantitative measures of circulating T-lymphocyte composition and neutrophil counts in individuals initially free of known cancer. Further studies will be needed to confirm our observations and to assess whether, at all, increased Treg-mediated immune tolerance or reduced CD8$^+$ mediated cytotoxicity have any impact on pancreatic cancer risk, and if so, whether this impact is mostly on relatively late-stage tumor development, that is, within relatively short lag-times between blood donation and cancer diagnosis. If further epidemiologic studies confirm findings and show a consistent pattern of associations between circulating immune cell composition and cancer risk, we hope this might open up novel avenues for cancer prevention, focusing on ways to optimize individuals' general immune defense.

Authors' Disclosures

M.-C. Boutron-Ruault reports personal fees from MAYOLI-SPINDLER and GILEAD outside the submitted work. S. Olek reports a patent for US2012107810A1 issued, a patent for WO2010069499A2 issued, a patent for WO2013014122A1 issued, and a patent for WO2011135088A1 issued. No disclosures were reported by the other authors.

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