Research Paper

The Ratio of Regulatory (FOXP3+) to Total (CD3+) T Cells Determined by Epigenetic Cell Counting and Cardiovascular Disease Risk: A Prospective Case-cohort Study in Non-diabetics

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ABSTRACT

Background: Experimental and clinical evidence indicate that inflammatory processes in atherogenesis and the development of cardiovascular complications are promoted by a loss of regulatory T cell (Treg)-mediated immunological tolerance to plaque antigens. Yet, the association between alterations of systemic Treg frequency and cardiovascular disease incidence remains uncertain.
Methods: A nested case-cohort study was conducted within the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heidelberg, comprising a random subcohort (n = 778) and primary cases of myocardial infarction (MI, n = 276) and ischemic stroke (n = 151). Pre-diagnostic FOXP3 + Treg and total CD3 + T-lymphocyte (tTL) frequencies in blood were measured by epigenetic-based, quantitative real-time PCR-assisted cell counting.
Results: Multivariate, Prentice-weighted Cox regression analyses revealed that lower Treg/tTL ratios were not associated with the risk of either MI (lowest vs. highest sex-specific quartile; hazard ratio: 0.72, 95% confidence interval: 0.46 to 1.13; P_{trend} = 0.51) or stroke (HR: 0.90, 95% CI: 0.51 to 1.60; P_{trend} = 0.78). There were no correlations of Treg/tTL ratios with C-reactive protein, HbA1c, and various lipid parameters.
Conclusions: Among middle-aged adults from the general population, imbalances in the relative frequency of Tregs within the total T cell compartment do not confer an increased risk of MI or stroke.

1. Introduction

Progression and rupture of atherosclerotic plaques in coronary and carotid arteries are important pathogenic factors in the development of myocardial and cerebral infarctions (Falk et al., 2013; Qureshi and Caplan, 2014). Inflammatory processes in the arterial intima play a key role in all stages of atherogenesis (Hansson, 2005; Hansson and Hermansson, 2011) and are strongly modulated by functionally distinct T cell subsets (Ammirati et al., 2015). Accumulating evidence suggests that such inflammation may partly develop owing to reduced immune tolerance, which leads to autoimmune-like responses targeted against plaque antigens (Nilsson and Hansson, 2008; Matsuura et al., 2014). The best documented epitopes capable of activating autoreactive T cells or the generation of autoantibodies are derived from oxidized low-density lipoproteins (oxLDL) (Nilsson et al., 2009) and heat shock proteins (Almanzar et al., 2012; Wick et al., 2014), but also loss of tolerance against unmodified self-proteins, such as apolipoprotein B100, has been found (Nilsson et al., 2012). In healthy persons, escape from peripheral self-tolerance and perturbed immune homeostasis are limited by regulatory T cells (Tregs), which implicates dysregulation of Tregs in atherosclerotic disease (Sakaguchi et al., 2010).

There is evidence from animal models that Tregs exhibit atheroprotective properties by suppression of autoreactive T cell responses or by secretion of anti-inflammatory cytokines (Pastrana et al., 2012). The potential importance of Tregs to cardiovascular disorders in humans was supported by histological studies showing reduced Treg frequencies or their functional defects in human atherosclerotic lesions compared to stable plaques and normal vessel tissue fragments (de Boer et al., 2007; Dietel et al., 2013). Therefore, therapeutic strategies aimed at treating or preventing cardiovascular disease (CVD) by enhancement of physiological immunosuppression have attracted growing interest in recent years, as reviewed in (Meng et al., 2016). However, data are mainly pre-clinical and promising anti-atherogenic effects through vaccination against LDL-derived antigens cannot be solely ascribed to a

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Treg increase (Nilsson et al., 2015). Moreover, lower blood levels of Tregs have been observed in patients with acute coronary syndromes (ACS) compared to patients with stable angina in several studies (Han et al., 2007; Cheng et al., 2008; Mor et al., 2006), while others reported a Treg increase in patients with ST-elevation acute myocardial infarction (MI) and a Treg decrease in non-ST elevation ACS patients (Ammirati et al., 2010). Irrespective of these findings, there is a lack of data on pre-diagnostic Treg frequencies in blood and CVD risk from prospective studies. In the only study so far addressing the potential role of circulating Treg levels in the etiology of human CVD, blood levels of CD4 + FoxP3 + T cells were associated with a higher incidence of MI (Wigren et al., 2012). Yet, this study did not reveal an association of Tregs with stroke incidence and comprised a small, elderly population with a very high prevalence of hypertension. Thus, it remains uncertain as to whether alterations in the circulating Treg compartment represent a consequence of disease manifestation or may be a pre-disposing risk factor reflecting a global immune imbalance (Caligiuri and Nicoletti, 2010).

One core obstacle of epidemiological studies on Tregs and CVD risk is that a flow cytometry-based measurement of immune cells generally requires fresh whole blood samples. In addition, Tregs currently lack specific cell surface markers in humans. Originally, Tregs were identified and characterized by their expression of CD4, forkhead box protein P3 (FOXP3) and CD25. More recently, stable expression of FOXP3 in Tregs was found to be largely controlled by a highly conserved CpG-enriched element in the FOXP3 gene, the Treg-specific demethylated region (TSDR) in mice (Floess et al., 2007) and confirmed in humans. Since activated human conventional CD4 + T cells can also express CD25 and FOXP3 (Wang et al., 2007) whereas TSDR demethylation is not shared by activated T cells (Baron et al., 2007), the Treg-specific epigenetic status provides the most accurate identification and quantification of Tregs (Morikawa and Sakaguchi, 2014). Indeed, Tregs constitute a stable cell lineage, whose state is ensured by DNA demethylation of the Foxp3 locus irrespectively of ongoing Foxp3 expression (Miyao et al., 2012). Using such an epigenetic approach, it has been recently confirmed that reduced Treg frequencies in the circulation, defined by enhanced TSDR methylation, are associated with disease severity in male patients with ACS compared with normal coronary controls (Jia et al., 2013; Lü et al., 2013). In a similar manner, we have also established the epigenetic pattern of the CD3 locus as quantitative measurement system for total CD3 + T-lymphocytes (tTLs) (Sehouli et al., 2011) and showed that the ratio of Tregs to tTLs constitutes a clinically relevant parameter of immune tolerance (Turbachova et al., 2013).

In the present study, we aimed at evaluating the relationship between the Treg/tTL ratio in blood of initially healthy individuals and the risk of MI and stroke in a case-cohort study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heidelberg cohort. Cell-type specific epigenetic assays based on DNA demethylation analysis were applied for immune cell quantification in archived pre-diagnostic leukocyte samples of incident CVD cases and controls. Subsequently, we investigated if low Treg/tTL ratios are associated with an increased CVD risk.

2. Materials and Methods

2.1. 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 aged 35 to 65 years that 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 conducted in compliance with standards indicated by the Declaration of Helsinki. All participants gave written informed consent and the study protocol was approved by the ethics committee of the Medical School of the University of Heidelberg (Riboli et al., 2002; Boeing et al., 1999). Incident cases of MI and stroke were ascertained through follow-up questionnaires at regular intervals of about three years, and all reported cases were validated by study physicians against medical records. The following cardiovascular events of interest were coded according to the International Classification of Diseases, 10th version, 2015 (ICD-10): myocardial infarction (I21); ischemic stroke (I63). Further details on follow-up procedures of EPIC-Heidelberg have been described elsewhere (Bergmann et al., 1999).

For the present analyses, an unstratified case-cohort study design was used. After exclusion of prevalent CVD cases among subcohort members (n = 16), the study population comprised primary cases of myocardial infarction (MI, n = 346) and ischemic stroke (n = 187) that occurred between baseline examination and December 31, 2007 as well as a random subcohort of 835 individuals. This subcohort was initially drawn for the EPIC-InterAct case-cohort study on diabetes, and thus did only include participants without a history of diabetes (Langenberg et al., 2011). Consequently, incident cases of MI (n = 47) and stroke (n = 21), who had reported to have diabetes at the baseline of the EPIC-Heidelberg study were excluded from the present analyses as well. Given the possibility of tumor-mediated changes in peripheral immune cell type fractions, we further excluded participants with prevalent cancer (MI: n = 14; Stroke: n = 13, Non-cases: n = 38). Study participants were also excluded when quality control in quantitative PCR (qPCR) analysis failed (MI: n = 5, Stroke: n = 1, Non-cases: n = 6) or there were missing covariate data (MI: n = 4, Stroke: n = 1, Non-cases: n = 13). Thus, statistical analyses were performed based on data of 276 cases of MI, 151 cases of stroke, and 778 subcohort members. The randomly selected subcohort eventually included twenty-two incident CVD cases (MI: n = 11, Stroke: n = 11). Details on the final sample are illustrated in Supplementary Fig. 1.

2.2. Laboratory Methods

At baseline recruitment into the EPIC-Heidelberg cohort, 30 ml of blood was taken from all cohort participants using anticoagulant (citrate) containing monovettes at room temperature and centrifuged for 20 min at 1500 ×g. Buffy coats were separated from the interphase and aliquoted into 500 μl portions which were stored in liquid nitrogen in the EPIC-Heidelberg biobank. Genomic DNA was isolated through proprietary extraction methods by Laboratory of the Government Chemist Limited (LGC Limited, Hoddesdon, UK). DNA solutions for each subject were stored at −80 °C until DNA analyses took place. DNA concentration and quality was measured using Quant-iT PicoGreen dsDNA Assay (Life Technologies, Darmstadt, Germany).

Epigenetic-based quantification of Tregs and tTLs was carried out by Epiontis GmbH (Berlin, Germany), as previously reported (Barth et al., 2015). Briefly, using bisulfite converted DNA as substrate, qPCR-Assays were performed for the selected cell type-specific demethylated loci - the Treg-specific demethylated region (TSDR) in FOXP3 gene and the CD3g/d intergenic region - and for a locus known to be demethylated in all cell types (GAPDH) (Sehouli et al., 2011). The latter was used to allow for determination of total leukocytes. For the present analyses, data of epigenetic-based cell counts are presented as the percentage share of TSDR demethylation divided by CD3 locus demethylation within peripheral blood leukocyte DNA samples by multiplying that ratio by one hundred. The Treg/tTL ratio was then used as a marker of peripheral immune tolerance.

In addition, routine clinical biochemistry markers were analyzed at the SHL Laboratories (Etten-Leur, Netherlands). Straws were opened using a heated wire cutting system followed by aliquoting directly into measurement tubes. Serum and erythrocyte samples were then delivered to the SHL group on dry ice. Serum concentrations of lipid parameters and HbA1c from erythrocytes were determined using the Roche Cobas 6000 analytical system for clinical chemistry according to the manufacturer's protocols.

2.3. Statistical Analyses

Baseline characteristics of individuals with and without incident cardiovascular disease are presented as means ± standard deviations or proportions. Differences between means of continuous variables were assessed by t-tests, and differences in proportions between cases and non-cases were assessed by chi-squared tests. Measurements of T cell subsets are displayed as medians with minimum and maximum values. Spearman's rank correlation coefficients adjusted for age, smoking status (never, former, current) and stratified by sex were used to assess the strengths of associations between waist circumference, BMI, glycated hemoglobin (HbA1c), various lipoprotein–lipid parameters and epigenetic cell counts. Treg/tTL ratios were categorized into quartiles according to sex-specific values in the subcohort and individuals in the highest quartile were considered as the reference group. Prentice-weighted Cox proportional hazards regression models with age at baseline as the underlying time-scale were used to assess the risk for incidence of a primary cardiovascular event between Treg quartiles (Prentice, 1986). All observations in the subcohort were left-truncated at age at baseline and censored at end of follow-up, death or loss to follow-up, whichever came first. Following the Prentice-weighting-scheme, risk sets at a particular event time are composed of all members of the subcohort at risk and cases at that event time, who were only included into the risk set shortly before their event. To adjust for age differences in cases at study entry and other potential age-cohort effects, all analyses were stratified by integer values (in years) of age at baseline. The extended correlation test based on Schoenfeld residuals (Xue et al., 2013) did not indicate any violations to the proportional hazards assumption.

Sex-adjusted and multivariable-adjusted hazard ratios (HRs) along with their 95% confidence intervals (CIs) were estimated. Identification of potential confounders was based on a priori literature search and all those factors were selected that changed the risk estimates by >10%, or were associated with the exposure. Thus, the following potential confounders were additionally included in a uniform multivariable-adjusted model: body mass index (BMI, kg/m^2), physical activity (inactive, moderately inactive, moderately active and active), smoking status (never smokers, former smokers quitting ≥10 years, former smokers quitting <10 years, current smokers < 15 cigarettes per day, current smokers ≥ 15 cigarettes per day), baseline alcohol intake (g/day), use of calcium supplements (yes or no), energy-adjusted dietary intakes of red and processed meat (g/day), and prevalent hypertension or hyperlipidemia at recruitment (yes or no). Potential confounding was also evaluated, but not found, for the following variables: highest educational level, lifetime alcohol intake, regular use of antihypertensive or lipid-lowering drugs, and use of non-steroidal anti-inflammatory drugs.

The ratio of Tregs to tTLs followed an approximately log-normal distribution. Tests for linear trend were carried out based on continuous Treg values on the log2 scale, thus calculating the HR associated with a halving of the Treg/tTL ratio. Sensitivity analyses were conducted excluding cases that were diagnosed within the first 2 years of follow-up. Finally, 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. All statistical tests were 2-sided and P-values below 0.05 were considered to indicate statistical significance. All statistical analyses were performed with SAS 9.3 (SAS Institute, Cary, NC).

3. Results

3.1. Participant Characteristics

Median follow-up time for the study population was 7.2 years ± 2.7. The mean age at diagnosis was 60.7 ± 6.9 years for individuals with incident MI and 60.6 ± 7.8 years among those with incident stroke. The vast majority of cases of MI (N = 218 out of 276) and stroke (N =

142 out of 151) were non-fatal within 28 days of admission. Socio-demographic and life-style characteristics for all study participants are shown in Table 1. Briefly, both endpoints were more common in men (MI: 79%; Stroke: 64%) than women. As compared to the subcohort, individuals who had an incident cardiovascular event were older and had a higher prevalence of established cardiovascular risk factors, including heavy smoking, adiposity, hypertension, and hyperlipidemia. There was no significant difference in the frequency of lipid-lowering medication, anti-hypertensive drugs, or calcium supplement use between cases and subcohort members.

In the subcohort, there were fewer individuals in the lowest compared to the highest quartile of Treg/tTL ratios who have reported to be current smokers, used anti-hypertensive drugs or NSAIDS (Supplementary Table 1). Other potential confounders such as age, education levels, BMI, waist circumference and physical activity were equally distributed across quartiles of Treg/tTL ratios. Finally, high-sensitive C-reactive protein, HbA1c, and lipid parameters did not correlate with the Treg/tTL ratio in the subcohort (Supplementary Table 2).

3.2. Association Between Treg/tTL Ratios and CVD Risk

The distribution of T cell subpopulations across cases of MI, stroke and non-cases is visualized by jittered boxplots in Supplementary Fig. 2. Prospective associations between Treg/tTL ratios in blood and the risk of MI and stroke are shown in Table 2. In Cox models stratified by age and adjusted for sex, lower peripheral Treg/tTL ratios were associated with a reduced risk of MI (lowest vs. highest quartile; HR = 0.62, 95% CI = 0.41 to 0.95; P$_{trend}$ = 0.04). However, this association was

Table 1

Baseline characteristics and laboratory parameters of the study population: EPIC Heidelberg case-cohort study.

	MI cases (N = 276)	Stroke cases (N = 151)	Subcohort (N = 778)
Male sex (N, %)	219 (79)*	96 (64)*	355 (46)
Age at blood draw (years)	55.6 ± 6.3*	55.6 ± 7.1*	50.6 ± 8.0
Age at diagnosis (years)	60.7 ± 6.9	60.6 ± 7.8	–
University degree (N, %)	66 (24)*	39 (26)	244 (31)
Physically inactive (N, %)	42 (15)	25 (17)	89 (11)
BMI (kg/m^2)	27.9 ± 4.0*	27.3 ± 3.9*	25.7 ± 4.2
Lifetime alcohol intake (g/day)	24.9 ± 30.5*	22.2 ± 29.3*	16.8 ± 24.4
Red and processed meat intake ≥ 120 g/day (N, %)[a]	96 (35)*	43 (28)	170 (22)
Smoking status (N, %)			
Never smokers	85 (31)	48 (32)	338 (43)
Former smokers ≥ 10 years	55 (20)	37 (25)	175 (22)
Former smokers < 10 years	23 (8)	13 (9)	84 (11)
Current smokers < 15 cig/day	28 (10)	13 (9)	79 (10)
Current smokers ≥ 15 cig/day	85 (31)*	40 (26)*	102 (13)
Hyperlipidemia (N, %)[b]	145 (53)*	76 (50)*	263 (34)
Use of lipid-lowering drugs (N, %)[c]	42 (29)	19 (25)	66 (25)
Hypertension (N,%)[b]	126 (46)*	71 (47)*	209 (27)
Use of anti-hypertensive drugs (N, %)[c]	92 (73)	51 (72)	134 (64)
Use of NSAIDs (N, %)[b]	33 (12)	17 (11)	68 (9)
Use of calcium supplements (N, %)[b]	13 (5)*	4 (3)	16 (2)
Laboratory measurements of T cell subsets (median, range)			
% CD3 + tTL of leukocytes	19.7 (3.9–48.7)	19.9 (8.6–41.5)	20.0 (4.0–61.3)
% Foxp3 + Treg cells of leukocytes	1.0 (0.3–3.1)	1.0 (0.3–2.4)	1.0 (0.2–4.9)
Treg/tTL ratio[d]	5.2 (1.7–14.0)	5.1 (1.8–12.0)	5.1 (1.5–15.5)

BMI: body mass index. NSAIDs: non-steroidal anti-inflammatory drugs. tTL: total CD3 + T-lymphocytes.
Values are means ± standard deviation or percentages unless otherwise stated.
Data are missing for lipid-lowering (n = 2) medication.
[a] Adjusted for total energy intake using the residual method.
[b] Prevalent.
[c] Among individuals with prevalent hypertension or hyperlipidemia, respectively.
[d] Ratio multiplied by 100.
* P < 0.05 for case vs. subcohort. P-value for difference was calculated using the chi-squared test for categorical variables and the t-test for continuous variables.

markedly attenuated and no longer statistically significant when multiple cardiovascular risk factors were included in Cox regression models (HR = 0.72, 95% CI = 0.46 to 1.13, P_{trend} = 0.51). No significant associations were observed for ischemic stroke, with a multivariable-adjusted hazard ratio between extreme quartiles of 0.90 (95% CI = 0.51 to 1.60; P_{trend} = 0.78).

Sensitivity analyses by excluding cases that occurred within the first 2 years since blood draw (MI: n = 46, Stroke: n = 27) yielded results that were consistent with those of our main analyses; the fully adjusted HR (95% CI) of MI and stroke, respectively, was 0.68 (95% CI = 0.42 to 1.09, P_{trend} = 0.41) and 0.91 (95% CI = 0.49 to 1.68, P_{trend} = 0.71) comparing extreme quartiles of the Treg/tTL ratio. Interactions between red meat intake on a continuous scale and Treg/tTL ratios were observed with respect to both MI and stroke risk (MI: $P_{interaction}$ = 0.02; Stroke: $P_{interaction}$ = 0.01). Additionally, there was a significant trend for interaction between smoking - categorized as never, former, current smokers - and Tregs in Cox regression analyses on MI ($P_{interaction}$ = 0.045), but not stroke. Multivariable-adjusted subgroup analyses that were stratified according to strata of sex-specific red meat intake tertiles showed no significant associations with MI or stroke risk across the risk strata (data not shown). In contrast, a statistically significant inverse trend in MI risk across quartiles of Treg/tTL ratios was found among former smokers in multivariable-adjusted models (HR_{log2} = 0.56, 95% CI = 0.31 to 0.99, P_{trend} = 0.047), but not current or never smokers (Supplementary Table 3).

4. Discussion

In the present population-based study, we assessed the relationship between pre-diagnostic Treg to tTL ratios and future cardiovascular events using a highly specific epigenetic assay for the quantification of T cell subsets. After a median follow-up time of about 7 years, lower Treg/tTL ratios were not associated with either an increased incidence of myocardial infarction or ischemic stroke in multivariable models accounting for traditional risk factors of atherosclerotic CVD. These findings do not support a potential role of altered Treg-mediated immune tolerance as a risk factor for human CVD.

Previous studies in humans were centered almost exclusively on patients with established cardiovascular disease and therefore it remained uncertain whether reduced circulating levels of Tregs are an important etiological factor, or rather reflect facilitated Treg trafficking to ischemic tissues as part of regulatory mechanisms involved in resolution of post-infarction inflammatory response (Frangogiannis, 2012). In accordance with a protective role of Tregs in CVD development, the Malmö Diet and Cancer Study cardiovascular cohort (MDCS-CV) study showed that low circulating Treg levels, defined as CD4 + FoxP3 + T cells, were

associated with an increased risk for acute myocardial infarction (hazard ratio 1.9 for the lowest Treg tertile) during a 15-year follow-up. On the contrary, no association of Treg frequencies with overall stroke risk was found (Wigren et al., 2012). Our results for ischemic stroke are in agreement with these findings by Wigren and colleagues, whereas absence of an association between low Treg/tTL ratios and MI risk contrasts with their results. However, the present study differs from the MDCS-CV study in several respects. We used an epigenetic approach for immune cell quantification as compared with a cytokine release assay combined with flow cytometry in the previous study. This difference may be relevant because Tregs form only a minor fraction of lymphocytes in blood and epigenetic signatures involving the FOXP3 TSDR demethylation status are currently considered to be the most specific marker of stable Tregs (Floess et al., 2007; Polansky et al., 2008; Baron et al., 2007). An earlier comparison between quantification of Treg/tTL ratios in cord and adult blood samples using epigenetic qPCR and flow cytometry assays showed reasonable method agreement, even though it has to be noted that differences to some degree between the two methods can be expected as the epigenetic tool allows a more specific detection of functionally stable FOXP3 + Tregs compared to surface marker-based flow cytometry (Nettenstrom et al., 2013). Besides the different assays applied in the present study and the study by Wigren et al., deviating denominators used to determine relative Treg frequencies (CD3 + T cells in EPIC-Heidelberg vs. CD4 + T cells in the MDCS-CV study) must be considered when making a direct comparison of results from the two studies. In fact, there may be differences in the directions of associations between distinct T cell subpopulations, such as CD4 + and CD8 + T cells, with inflammatory and autoimmune-mediated pathogenesis of atherosclerotic CVD (Ammirati et al., 2015), and CD3 + CD8 + T cells were positively associated with MI risk in the MDCS-CV study (Kolbus et al., 2013). Moreover, EPIC-Heidelberg cohort participants included here were free of diabetes (0% vs. 21% and 11% among cases and controls from the MDCS-CV), younger (mean baseline age 55 years vs. 65 years), and incident MI cases had less frequently reported hypertension at baseline (46% vs. 87%) compared to the MDCS-CV study. In contrast, MI cases occurred more frequently in men as compared with those cases identified in the MDCS-CV (79% vs. 53%). Of note, male gender, increasing age as well as other established CVD risk factors such as diabetes and arterial hypertension have all been proposed to be inversely associated with absolute Treg numbers (Kornete et al., 2013; Idris-Khodja et al., 2014). Inconsistency of results between both studies may thus be due to heterogeneity concerning the cardiovascular risk profile, and could imply that the role of circulating Tregs in CVD development depends on the extent or type of pre-existing arterial remodeling. Further clarification of this issue will require verification in other cohorts including atherosclerosis imaging approaches.

Table 2

Hazard ratios (95% confidence intervals) for incident cardiovascular events by sex-specific quartiles of the Treg/tTL ratio.

Outcome	Quartiles[c]				HR (95% CI)$_{log2}$	P_{trend}[d]
	1 (high)	2	3	4 (low)		
	6.4/7.4	5.1/6.0	4.3/5.1	3.2/4.1		
Myocardial infarction						
N cases/subcohort	97/194	63/195	61/195	55/194		
Sex-adjusted[a]	1.00	0.71 (0.47, 1.08)	0.71 (0.47, 1.08)	0.62 (0.41, 0.95)	0.70 (0.50, 0.98)	0.04
MV-adjusted[b]	1.00	0.58 (0.37, 0.93)	0.86 (0.55, 1.33)	0.72 (0.46, 1.13)	0.88 (0.60, 1.29)	0.51
Ischemic stroke						
N cases/subcohort	47/194	30/195	41/195	33/194		
Sex-adjusted[a]	1.00	0.76 (0.45, 1.26)	0.95 (0.59, 1.54)	0.77 (0.46, 1.29)	0.78 (0.52, 1.17)	0.23
MV-adjusted[b]	1.00	0.66 (0.37, 1.19)	1.14 (0.68, 1.89)	0.90 (0.51, 1.60)	0.94 (0.59, 1.48)	0.78

[a] Derived from Prentice-weighted Cox proportional-hazards regression with age as underlying time variable, stratified by age at baseline (in 1-year categories), and adjusted for sex.

[b] The hazard ratio in multivariable (MV) models was additionally adjusted for the following traditional risk factors: presence or absence of hypertension or hyperlipidemia, smoking status, body mass index, physical activity, baseline alcohol intake, energy-adjusted dietary intakes of red and processed meat, use of calcium supplements.

[c] Quartile cut-points, displayed as medians in men/women, were based on the distribution in the female and male subcohort.

[d] Tests for linear trend were carried out based on the continuous values of Treg/tTL ratios on the log2 scale, which were included along with the main effect terms into the MV-adjusted models.

It could be argued that the lack of association between pre-diagnostic Treg/tTL ratios and CVD risk in the present study is due to the fact that plaques represent a different immunological compartment than blood (Grivel et al., 2011), and it remains to be determined whether Treg levels in blood adequately reflect sustained immune responses in the arterial wall. Actually, a correlation of circulating Tregs with the carotid intima-media thickness, a proxy of subclinical atherosclerosis, could not be found in both healthy populations (Wigren et al., 2012; Ammirati et al., 2010) and ACS patients (Ammirati et al., 2010). Nevertheless, it is increasingly acknowledged that antigen-specific atherogenic T cell responses are initiated in secondary lymphoid organs by presentation of plaque antigens (Ammirati et al., 2015) and a systemic nature of immune reactions related to atherosclerosis is further supported by the fact that both local and systemic inflammation affect early steps of atherogenesis and CVD (Hansson, 2005). In a previous study, we have also shown that higher pre-diagnostic Treg/tTL ratios in blood are associated with the risk of lung, colorectal and ER-Negative breast cancer (Barth et al., 2015). These observations support the relevance of Tregs in the periphery to local immune homeostasis and tolerance.

Another potentially important result of this study is that CVD risk across quartiles of Treg to tTL ratios was attenuated on adjustment for smoking, which is in line with previous data demonstrating that both acute and cumulative smoking exposure positively correlates with the Treg/tTL ratio (Wiencke et al., 2012). Although largely unknown, a smoking-associated Treg increase in blood may either reflect a more general state of suppressed immune and inflammatory responses (Stampfli and Anderson, 2009; Shiels et al., 2014) or relates to facilitated mobilization and migration of Tregs to tissue sites affected by smoke exposure to dampen local inflammation (Ritter et al., 2005). In fact, our subgroup analyses by smoking status did point to some heterogeneity in the associations between Treg frequencies and MI but not stroke risk. With respect to MI, a significant inverse association with Treg frequencies was only observed in former smokers, while there were no significant associations in current and never smokers. Admittedly, our subgroup samples were rather small and possible smoking-related influences on the relationship between peripheral Treg variability and MI risk may require further study in a larger sample.

The strengths of our study include its prospective design, the large sample size for our main analyses, and the use of epigenetic assays, which enable the quantification of immune cells in buffy coat samples after long-term storage. There are also some limitations to this study that have to be considered. First, the generalizability of the association between cellular immune markers and CVD development is limited to some extent because the EPIC-Heidelberg cohort represents a population with a higher socio-economic status and more favorable lifestyle factor profile compared to populations from other regions in Germany (Boeing et al., 1999). This overrepresentation of health-conscious individuals could have become even more pronounced by restriction of our investigation to non-diabetics. With regard to the quantification of overall Tregs, it must be noted that we could not address the issue of heterogeneity within the Treg compartment in our study and that different T cell subsets may have distinct properties in CVD development. Our study was set up to analyze Treg-mediated immune tolerance as a global phenomenon, but studies on more specific functions of immune cell subsets are clearly needed. Another general limitation of long-term observational studies is that exposures are measured only at a single point in time. However, in a reproducibility study of 100 healthy EPIC-Heidelberg participants with repeated measurements, we have previously shown a moderate-high correlation between individual's peripheral Treg/tTL ratio over time periods of one year ($r = 0.73$) and up to fifteen years ($r = 0.53$) (Barth et al., 2015). Thus, a single quantification may provide a good proxy for long-term FOXP3 + Treg values and supports the notion that relative numbers of these cells in the periphery are kept in a homeostatic state throughout life (Liston and Gray, 2014; Fessler et al., 2013).

In conclusion, we observed no association between pre-diagnostic Treg to tTL ratios in peripheral blood and future cardiovascular events in a cohort of non-diabetic, middle-aged individuals. Unlike previous findings from experimental studies and a smaller epidemiological study in elderly individuals, our results do not indicate that lower Treg frequencies in peripheral blood, as assessed relative to total T cells, are a risk factor of CVD.

Funding Sources

Conflicts of Interest

Sven Olek is an employee and owner of Epiontis, Berlin, a company that develops epigenetic assays. Janika Schulze and Katharina Gellhaus are employees of Epiontis, Berlin. Sven Olek holds patents on assays for the quantification of Foxp3 + and CD3 + T cells. The other authors declare that they have no competing financial interest.

Author Contributions

RK and SO conceived and designed the study. SO coordinated the laboratory analyses.

TJ prepared the samples. JS performed the laboratory analyses with support from KG. SB analyzed the data. TK and VK verified the statistical analyses. SB wrote and TK revised the manuscript. All authors critically revised and approved the final version of the manuscript.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.07.035.

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