Non-classical and intermediate monocytes in patients following venous thromboembolism: Links with inflammation

Ewa Wypasek[1,2,A,C,D], Agnieszka Padjas[2,B–D], Magdalena Szymańska[3,B,C], Krzysztof Plens[4,C], Maciej Siedlar[5,E,F], Anetta Undas[1,A,E,F]

[1] Institute of Cardiology, Jagiellonian University Medical College, Kraków, Poland
[2] Department of Medicine, Jagiellonian University Medical College, Kraków, Poland
[3] John Paul II Hospital, Kraków, Poland
[4] Data Analysis Center, Cardiovascular Research Institute, Kraków, Poland
[5] Department of Clinical Immunology, Chair of Clinical Immunology and Transplantation, Institute of Pediatrics, Jagiellonian University Medical College, Kraków, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2019;28(1):51–58

Address for correspondence
Ewa Wypasek
E-mail: ewa.wypasek@wp.pl

Funding sources
The project was supported by a grant from the National Science Centre (UMO-2013/09/B/NZ5/00254, to A.U.)

Conflict of interest
None declared

Received on April 11, 2017
Reviewed on June 1, 2017
Accepted on August 3, 2017

Published online on August 8, 2018

Cite as
Wypasek E, Padjas A, Szymańska M, Plens K, Siedlar M, Undas A. Non-classical and intermediate monocytes in patients following venous thromboembolism: Links with inflammation. *Adv Clin Exp Med*. 2019;28(1):51–58. doi:10.17219/acem/76262

DOI
10.17219/acem/76262

Abstract

Background. Monocyte subsets are involved in atherosclerotic vascular disease and its thromboembolic complications. Moreover, the role of monocytes has been suggested in the pathogenesis of venous thromboembolism (VTE).

Objectives. We hypothesized that pro-inflammatory non-classical and intermediate monocytes are increased in the first months following VTE.

Material and methods. We enrolled 70 patients aged 18–65 years (mean age 41.6 ±11.6) with the first-ever provoked (n = 32; 45.7%) or unprovoked (n = 38; 54.28%) VTE episode, and 46 healthy controls. The exclusion criteria were: acute infection, cancer, autoimmune disorders, previous myocardial infarction (MI), or stroke. Monocyte subsets were assessed 12 (8.5–21.5) months after VTE using flow cytometry and were defined as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$).

Results. Patients with VTE had higher intermediate and non-classical monocyte counts compared to the control group (16.8 ±9.3 vs 10.4 ±4.0 cells/μL, and 64.1 ±25.2 vs 44.1 ±19.2 cells/μL, respectively, both $p < 0.001$). Increased non-classical monocyte counts were observed in patients who experienced a VTE incident within 12 months prior to enrollment (71.5 ±27.4 vs 56.03 ±20.6 cells/μL; $p = 0.01$) and those with unprovoked VTE (70.2 ±4.1 vs 58.8 ±4.3 cells/μL; $p = 0.06$). There were no differences in monocyte subsets related to the current anticoagulation.

Conclusions. Our data has shown for the first time that VTE is associated with an increased number of non-classical and intermediate monocytes, which may indicate the involvement of monocyte-related mechanisms in the pathophysiology of this disease.

Key words: inflammation, venous thromboembolism, non-classical monocytes, intermediate monocytes

Introduction

Venous thromboembolism (VTE), including deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common disease associated with significant mortality and substantial healthcare costs. Venous thromboembolism occurs in approx. 1 to 2 per 1,000 person per year, and the overall VTE incidence is similar to that of strokes.[1] Idiopathic VTE events represent 25–40%. The morbidity rises dramatically after about 45 years of age and is slightly higher for men than for older women.[1–3] Venous thromboembolism is the 3rd most common cause of cardiovascular death worldwide, just after myocardial infarction (MI) and stroke.[4]

There is evidence that inflammation and thrombosis are closely linked, but the nature of this relationship is poorly understood. Increased level of C-reactive protein (CRP), a major marker of inflammation, has been shown to be associated with VTE in the general population as well as DVT, in particular in those with post-thrombotic syndrome that occurs in 20–50% of the patients within the first 2 years since the event.[5–9] In addition, acute infections predispose to DVT, which also supports the role of inflammation in thrombosis.[10,11] Levels of CRP and IL-6 at the time of the DVT diagnosis were associated with thrombotic disease burden, as measured by DVT extent and severity of DVT symptoms and signs.[12]

Identification of internal prothrombotic functions of cells of the innate immune system, which acts in blood vessels, resulted in an intriguing concept of immunothrombosis.[13–16] Involvement of the immune system in the thrombosis process represents a physiological mechanism, an independent line of host defense against microorganisms that mediates in the identification of and protection against pathogens by promoting microthrombi in the vessels.[7–9] Immunothrombosis is triggered and maintained by the local accumulation of innate immune cells, mainly monocytes and neutrophils. Dysregulation of immunothrombotic reactions can, therefore, contribute to thrombotic disorders, including DVT, in individuals free of infections.[7] During development of DVT, activated endothelial cells adopt a proinflammatory phenotype, which initiates the recruitment of innate immune cells, particularly monocytes and neutrophils. Active participation of cells of the innate immune system in the formation of thrombi is a specific attribute of thrombosis, as indicated by studies on mouse models of DVT.[14] It has been demonstrated in animals that the DVT begins as a sterile inflammation characterized by a massive recruitment of neutrophils and monocytes. The role of monocytes in VTE observed in human subjects is, however, unclear.

Monocytes represent a heterogeneous cell population in both phenotype and function. Based on the expression of CD14 and CD16, 3 monocyte subsets can be differentiated: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$). CD16 antigen is identified as FcyRIIIA and is involved in innate immunity, while CD14 is a coreceptor of toll-like receptor 4 that binds lipopolysaccharide (LPS). The correct count of the number of individual subsets of monocytes requires staining and appropriate gating strategy, which includes a 3rd pan-monocyte marker, i.e., HLA-DR or CD86. $CD16^{+}$ monocytes are the main producers of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, which indicates that intermediate or non-classical monocytes or both jointly produce the largest quantities of the proinflammatory cytokines.[17–20]

Recently, Mukherjee et al. have shown that non-classical $CD14^{+}CD16^{++}$ subtype of monocytes displays inflammatory characteristics and properties for antigen presentation.[21] In turn, intermediate $CD14^{++}CD16^{+}$ appear to be transitional monocytes that display both phagocytic and inflammatory functions, whereas classical monocytes $CD14^{++}CD16^{-}$ are phagocytic with low inflammatory attributes.[21]

Growing evidence suggests that proinflammatory subsets of monocytes are involved in atherosclerosis and its thromboembolic complications. Higher numbers of proinflammatory intermediate or non-classical monocytes have been shown in patients with a stable and unstable coronary artery disease (CAD).[22,23] In patients with stable CAD, cardiovascular events can be predicted by elevated counts of intermediate monocytes.[24] Intermediate monocytes have also been shown to be positively correlated with peak cardiac troponin and inflammatory markers in patients with acute ST segment elevation MI that is caused in the vast majority of cases by a thrombus occluding a coronary artery.[25] In unstable angina patients counts of intermediate monocytes, intermediate monocyte-platelet aggregates and total monocyte-platelet aggregates are increased, and are independent of traditional risk factors.[26] Hypercholesterolemia is also associated with an elevated number of non-classical monocytes, while HDL-cholesterol showed a negative association.[27] Elevated counts of intermediate monocytes have also been demonstrated in patients during the first days after an ischemic stroke.[28]

Despite the fact that monocyte subpopulations have been assessed in various diseases and experimental studies have shown a causative role of monocytes in the pathogenesis of VTE, to our knowledge there have been no published studies assessing various subsets of circulating monocytes in patients following VTE.[29,30]

Methods

Patients

We investigated 70 consecutive Caucasian patients, aged 18–65 years, with a history of the first-ever provoked or unprovoked DVT alone or in combination with PE, referred to an outpatient clinic between October 2012

and June 2015. The diagnosis of DVT was established by a positive finding on color duplex sonography (visualization of an intraluminal thrombus in calf, popliteal, femoral, or iliac veins). The diagnosis of PE was based on the presence of typical symptoms and positive results of computed tomography pulmonary angiography (CT). Patients with signs of acute infection, known cancer, chronic inflammatory disease, or autoimmune disorders (including antiphospholipid syndrome), previous MI or stroke, serum creatinine ≥120 μM, liver injury, pregnancy were ineligible. All patients were treated with unfractionated or low-molecular-weight heparin, and then vitamin K antagonists (VKA) were continued for at least 3 months in patients with VTE triggered by transient risk factors and for 6 months or longer on the discretion of the treating physicians in patients with unprovoked VTE. A VTE episode was defined as unprovoked (idiopathic) if the patient had no history of cancer, surgery requiring general anesthesia, major trauma, a plaster cast or hospitalization within the last month, or pregnancy or delivery within the last 3 months. A proximal DVT was defined as thrombosis in the popliteal veins, including the trifurcation, the femoral and iliac veins.

Forty-six consecutive healthy volunteers served as the control group. The exclusion criteria were: personal and/or family history of cardiovascular diseases including VTE, MI, CAD, heart failure, stroke, and any of chronic diseases except for arterial hypertension, as well as age over 65 years. All subjects denied taking any medication on a long-term basis and within the previous month.

Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Obesity was defined as a BMI of 30 kg/m^2 or higher. Diabetes mellitus was defined as the previous diagnosis of diabetes, or at least 2 random fasting glucose levels of >7 mmol/L. Arterial hypertension was diagnosed based systolic or diastolic pressure ≥140 mm Hg or ≥90 mm Hg, respectively, on at least 2 different occasions or the use of antihypertensive treatment. Hypercholesterolaemia was diagnosed based on low-density lipoprotein cholesterol (LDL-C) level >3.0 mmol/L or previously diagnosed hypercholesterolaemia. The diagnosis of MI was based on the 2012 American Heart Association, European Society of Cardiology, American College of Cardiology Foundation, and World Heart Federation (ESC/ACCF/AHA/WHF) guidelines. Ischemic stroke was diagnosed according to the World Health Organization criteria. Smoking was defined as the use of at least 1 cigarette per day.

The Ethical Committee by Regional Medical Council in Kraków (No. 135/KBL/OIL/2013) approved the study and all the participants provided their written informed consent.

Laboratory investigations

Fasting blood samples were drawn between 8 a.m. and 12 a.m. from an antecubital vein with minimal stasis. Serum triglycerides, total cholesterol, LDL-C, high-density lipoprotein cholesterol (HDL-C), creatinine and glucose, total protein and albumin, and complete blood count were assayed using a biochemical analyser Cobas 6000™ (Roche Diagnostics GmbH, Mannheim, Germany). Fibrinogen was determined using the Clauss method. A high-sensitivity CRP (hs-CRP) was determined using immunoturbidimetry (Roche Diagnostics GmbH).

A complete blood count was determined using the hematological analyzer Sysmex XT2000i (Sysmex Corporation, Kobe, Japan). Anti-nuclear antibodies (ANA) were tested using indirect fluorescent assay (IFA) in sera diluted at 1:160 (Euroimmun, Lübeck, Germany).

Flow cytometry

The number of monocytes was assessed in blood samples an average of 12 months after the incident of VTE. Whole blood samples were drawn into EDTA-K3 collection tubes and were prepared for flow cytometry within 30 min. Briefly, 50 μL of whole blood was incubated with antibody mix containing 10 μL of FITC-labeled anti-human CD14 (B36297, Beckman Coulter, Brea, USA), 10 μL PE-labeled anti-human CD16 (332779, BD Biosciences, San Jose, USA), 10 uL PerCP-labelled anti-human HLA-DR (347402, BD Biosciences), 5 μL APC-labeled anti-human CD45 (340910, BD Biosciences) in BD Trucount tubes (all from BD Bioscience) for 30 min in room temperature in the dark. The isotype control was run in parallel. Lysis of erythrocytes was performed using 450 μL of BD FACS Lysing Solution (BD Bioscience) for 5 min. Determination of monocytes subsets were performed on FACSCanto II flow cytometry (BD Bioscience) and analyzed by FACSDiva software v. 7.0 (BD Biosciences). The following calculation has been performed: [(number of events in quadrant containing cell population)/(number of events in absolute-count bead region)] × [(number of beads per test defined by manufacturer)/(test volume)], to obtain the number of monocytes per microliter.

Monocyte subpopulation identification

The absolute number of monocyte subpopulations was determined as described previously.[31] Briefly, based on CD45-positive and SSC characteristics, monocytes were gated together with adjacent lymphocytes, including NK cells (Fig. 1A). Then, to exclude CD14-negative and HLA-DR-negative NK cells (gate "d"), a gate "c" was defined including CD14-positive and HLA-DR-positive events (Fig. 1B). All events from gate "c" were then divided based on CD14 and CD16 expression into: classical monocytes CD14^{++}CD16^{-} (gate "e") expressing high levels of CD14 but no CD16; intermediate monocytes CD14^{++}CD16^{+} (gate "f") expressing high levels of CD14 and low CD16; and non-classical monocytes CD14^{+}CD16^{++} (gate "g") expressing low CD14 but high CD16 (Fig 1C).

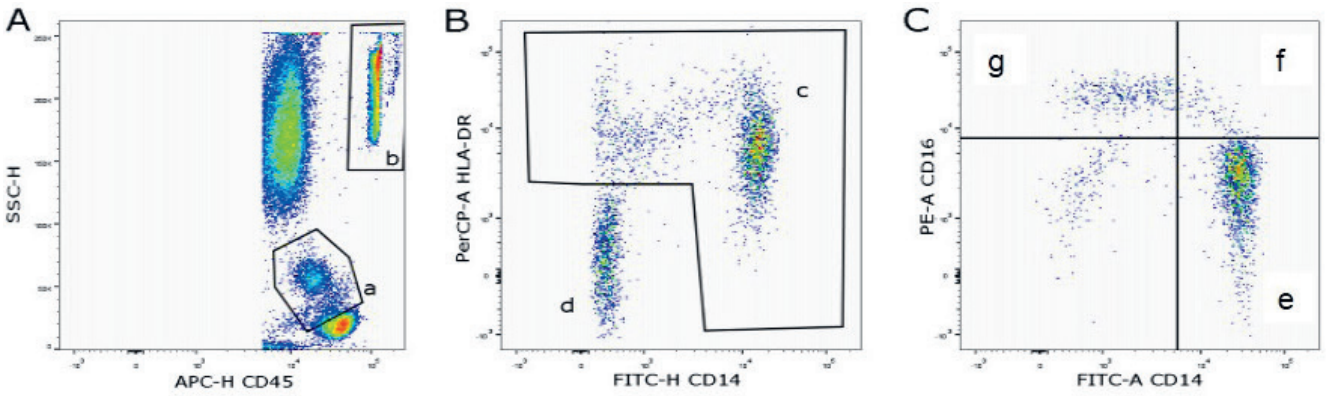

Fig. 1. Gating strategy of monocytes subsets analysis by flow cytometry. Monocytes were gated as (A) CD45-positive cells vs side scatter (SSC) plot: ("a") monocytes, ("b") counting beads for determination of the absolute cell count. (B) CD14-positive cells from gate "a" were then gated ("c") to exclude CD14-HLA-DR-negative NK cells ("d") and finally analyzed for CD14 and CD16 expression (C) as: classical monocytes ($CD14^{++}CD16^{-}$, "e"), intermediate monocytes ($CD14^{++}CD16^{+}$, "f") and non-classical monocytes ($CD14^{+}CD16^{++}$, "g")

Statistical analysis

Assuming a standard deviation (SD) for non-classical monocytes of 25/μL, the study would require a sample size of 44 for each group to demonstrate 2-sided equality and to achieve a power of 0.8 and a level of significance of 0.05, for detecting a difference in means of this monocyte subset between the VTE and the control group of 15/μL.[32]

Categorical variables are presented as numbers and percentages. Continuous variables are expressed as mean ± SD or median and interquartile range (IQR). Normality was assessed by the Shapiro-Wilk test. Equality of variances was assessed using Levene's test. Differences between groups were compared using the Student's or the Welch's t-test depending on the equality of variances for normally distributed variables. The Mann-Whitney U test was used for non-normally distributed continuous variables. Categorical variables were compared by Fisher's exact test. The Pearson's correlation coefficient was computed to measure the linear association between 2 variables. The Spearman's rank correlation coefficient was calculated to measure the monotonic trend between 2 variables. Multivariate logistic regression models were used to adjust the results to age and BMI. Two-sided p-values < 0.05 were considered statistically significant. All calculations were done with JMP v. 9.0.0 (SAS Institute Inc., Cary, USA).

Results

Study participants

The characteristics of VTE patients and healthy controls are presented in Table 1.

Venous thromboembolism patients were slightly older and more overweight. The age range for the control group was 20–58 years and 18–64 years for the VTE patients. Twenty-three (32.8%) of VTE subjects had isolated DVT and 24 (34.3%) subjects had symptomatic PE with concomitant DVT. Proximal DVT occurred in 56 (80.0%) subjects. More than 80% of VTE patients were treated with oral anticoagulants (Table 1). One patient with VTE (1.4%) had a history of previous MI and another one (1.4%) experienced ischemic stroke in the past.

Most laboratory investigations were similar in both groups. Fibrinogen was higher in VTE patients, while CRP concentrations were similar (Table 1). However, the proportion of VTE patients with CRP >3 mg/L was 2-fold larger compared to that found in healthy volunteers (Table 1). The difference remained significant after adjustment for age and BMI. In VTE patients, glucose was slightly higher; while creatinine and albumin concentrations were lower (Table 1). Only the relative frequency, but not the absolute count of monocytes and lymphocytes, was lower in VTE patients, which seems to be due to the increase in neutrophils without changes in the absolute blood level of monocytes and lymphocytes (Table 1).

Positive ANA was detected more commonly among VTE patients compared to the controls (Table 1).

Monocyte characteristics

Patients with VTE had higher intermediate $CD14^{++}CD16^{+}$ and non-classical $CD14^{+}CD16^{++}$ monocyte counts compared to the control group (Table 1). These differences remained significant after adjustment for age and BMI. There were no intergroup differences in classical monocyte $CD14^{++}CD16^{-}$ counts (Table 1).

The absolute number of all 3 monocyte subsets in VTE patients was not associated with comorbidities, type of VTE or the medications used. Among the VTE patients, the non-classical $CD14^{+}CD16^{++}$ monocytes were positively associated with age ($r = 0.33$; $p = 0.008$), weight ($r = 0.33$; $p = 0.005$) and BMI ($r = 0.31$; $p = 0.009$), while in the control group the only significant association was found for this monocyte subpopulation and age

Table 1. Characteristics of the study participants

Parameters	Control (n = 46)	VTE (n = 70)	p-value
Age [years]	36.98 ±10.09	41.61 ±11.55	0.03
Male sex, n [%]	22 (47.83)	23 (32.86)	0.12
Body mass index [kg/m^2]	24.19 ±3.78	26.82 ±5.25	0.004
Obesity, n [%]	3 (6.52)	15 (21.43)	0.04
Clinical characteristics, n [%]			
Current smoking	6 (13.04)	14 (20.00)	0.25
Arterial hypertension	0	17 (24)	–
Diabetes mellitus	0	5 (7.14)	–
Hypercholesterolemia	20 (43)	36 (51.42)	0.31
Unprovoked VTE	0	38 (54.28)	–
DVT alone	0	23 (32.85)	–
PE + DVT	0	24 (34.28)	–
Proximal DVT	0	56 (80.00)	–
Family history of VTE	0	25 (35.71)	–
Time from the last VTE event [months]	n.a.	12.0 (8.5–21.5)	–
Laboratory parameters			
Glucose [mmol/L]	4.98 ±0.55	5.38 ±0.66	0.002
Creatinine [µmol/L]	77.17 ±13.01	71.27 ±14.16	0.01
Total cholesterol [mmol/L]	4.88 ±0.86	5.13 ±0.95	0.11
HDL cholesterol [mmol/L]	1.64 ±0.32	1.60 ±0.41	0.31
LDL cholesterol [mmol/L]	3.08 ±0.83	3.31 ±0.89	0.10
Triglycerides [mmol/L]	1.13 ±0.68	1.24 ±0.79	0.62
Total protein [g/L]	74.84 ±4.41	74.49 ±4.34	0.83
Albumin [g/L]	40.80 ±2.63	39.40 ±2.80	0.007
Albumin/globulin ratio	1.21 ±0.16	1.14 ±0.17	0.01
ANA, n [%]	9 (19.57)	24 (42.11)	0.004
Blood cell variables			
Red blood cells [10^6/µL]	4.83 ±0.46	4.76 ±0.38	0.36
Hemoglobin [g/dL]	13.70 ±1.43	13.81 ±1.33	0.66
Hematocrit [%]	40.76 ±3.59	41.21 ±3.30	0.48
Platelets [10^3/µL]	237 ±54	255 ±59	0.16
Leukocytes [10^3/µL]	5.81 ±1.29	6.53 ±1.91	0.045
Neutrophils [10^3/µL]	3.20 ±0.94	4.05 ±1.65	0.004
Lymphocytes [10^3/µL]	1.94 ±0.49	1.79 ±0.54	0.08
Monocytes [10^3/µL]	0.55 ±0.26	0.60 ±0.77	0.41
Eosinophils [10^3/µL]	0.15 ±0.09	0.14 ±0.09	0.62
Basophils [10^3/µL]	0.03 ±0.02	0.03 ±0.01	0.40
Neutrophils [%]	54.34 ±7.12	60.80 ±9.49	<0.001
Lymphocytes [%]	33.68 ±6.35	28.63 ±8.50	<0.001
Monocytes [%]	8.82 ±1.54	7.78 ±1.96	<0.001
Eosinophils [%]	2.53 ±1.56	2.22 ±1.34	0.36
Basophils [%]	0.43 ±0.28	0.43 ±0.24	0.70
Inflammatory markers			
Fibrinogen [g/L]	2.66 ±0.57	3.24 ±0.90	<0.001
hsCRP [mg/L]	1.11 (0.70–1.98)	1.23 (0.69–2.59)	0.27
hsCRP >3 mg/L, n [%]	5 (10.87)	17 (24.29)	0.02
Monocyte subsets [10^3/µL]			
Classical	370.48 ±113.04	363.90 ±143.71	0.43
Intermediate	10.43 ±4.01	16.79 ±9.29	<0.001
Non-classical	44.09 ±19.21	64.09 ±25.21	<0.001
Non-classical/classical monocytes ratio	0.14 (0.11–0.18)	0.18 (0.13–0.23)	0.003
Treatment			
Vitamin K antagonists	n.a.	30 (42.86)	–
Rivaroxaban	n.a.	24 (34.28)	–
No anticoagulation	n.a.	10 (14.29)	–

Data is shown as mean ± standard deviation (SD) or a median (interquartile range (IQR)) or number (percentage). VTE – venous thromboembolism; DVT – deep vein thrombosis; PE – pulmonary embolism; HDL – high-density lipoprotein; LDL – low-density lipoprotein; ANA – anti-nuclear antibodies, hsCRP – high sensitivity C-reactive protein; n.a. – not applicable.

(r = 0.39; p = 0.01). Additionally, the non-classical CD14$^+$CD16^{++} monocyte counts showed positive correlations with glucose (r = 0.26; p = 0.03) and albumin to globulin ratio (r = 0.26; p = 0.03).

Classical CD14^{++}CD16$^-$ and intermediate CD14^{++}CD16$^+$ monocyte counts were positively associated with leukocyte counts (r = 0.67 and r = 0.51, respectively, p < 0.0001 for both). Classical CD14^{++}CD16$^-$ and non-classical CD14$^+$CD16^{++} monocyte counts showed positive correlations with hsCRP (r = 0.25 and r = 0.27, respectively, p = 0.03 for both). However, after removing of 3 outliners, only the trend towards correlation between non-classical monocytes and CRP was found (r = 0.2; p = 0.08).

Only intermediate CD14^{++}CD16$^+$ monocyte counts were positively associated with FVIII activity (r = 0.5; p = 0.03). There was no correlation of these counts with D-dimer and ANA antibody. Interestingly, the patients who had a VTE incident within 12 months prior to enrollment (9 (6–10) months) were characterized by an increased number of non-classical CD14$^+$CD16^{++} monocytes as compared to those who experienced a VTE event at an earlier, more distant time point (21 (15–29) months) (71.5 ±27.4 vs 56.03 ±20.6 cells/µL, p = 0.01; Fig. 2).

As shown in Fig. 3, non-classical CD14$^+$CD16^{++} monocyte counts tended to be higher in patients following unprovoked VTE as compared to individuals with provoked VTE (70.2 ±4.1 vs 58.8 ±4.3 cells/µL; p = 0.06).

Discussion

This study shows that a history of VTE, regardless of the type of thrombotic event and anticoagulant treatment, is associated with increased counts of non-classical CD14$^+$CD16^{++} and intermediate CD14^{++}CD16$^+$ monocytes.

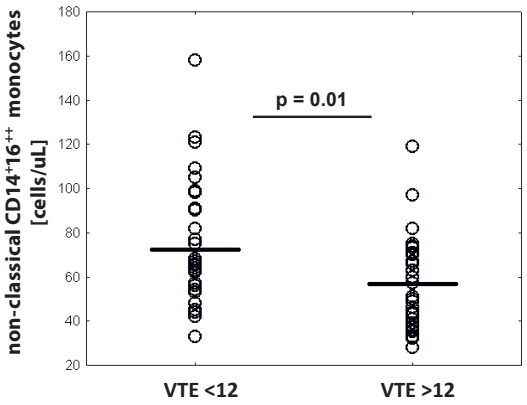

Fig. 2. The absolute number of non-classical CD14^{+}CD16^{++} monocytes in venous thromboembolic patients (VTE) who experienced a last VTE event <12 and >12 months

This effect was not associated with unprovoked or provoked VTE.

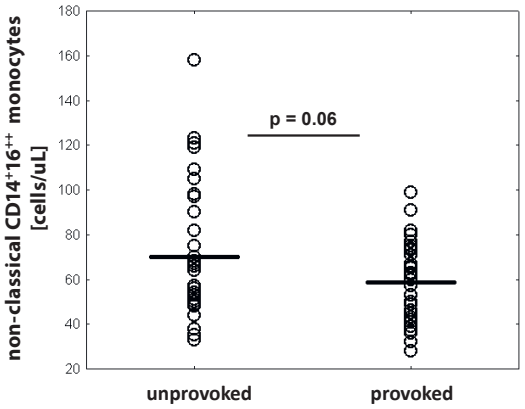

Fig. 3. Non-classical CD14^{+}CD16^{++} monocyte counts in patients with unprovoked and provoked venous thromboembolism (VTE)

This phenomenon is more pronounced in our patients aged 65 years or less assessed in the first 12 months from the VTE event, and it is in partly driven by inflammation, reflected by positive correlations with elevated hsCRP.

The pattern of monocytes counts in peripheral venous blood observed following VTE is similar to that found in patients with atherosclerotic vascular disease.[25,26] It may be speculated that the non-classical and intermediate monocytes are involved in the similar processes in patients with VTE and those with atherosclerosis. The recent data has suggested that VTE and atherothrombosis share similar pathophysiological pathways like low-grade systemic inflammation and hypercoagulability state.[33] The mechanisms typical of atherosclerosis like platelet activation and neutrophil recruitment with the formation of neutrophil extracellular traps are also involved in the pathogenesis of DVT.[34] The same coagulation proteins which play a crucial role in the pathophysiology of VTE are also expressed in the atherosclerotic arterial walls enhancing the inflammatory and immune processes characteristic of atherosclerosis.[33] Additionally, the clinical association of VTE and atherothrombosis has been shown for the first time by Prandoni et al., who demonstrated that patients with idiopatic VTE were more likely to have carotid artery plaque (47%) than patients with provoked VTE (27%) or age- and sex-matched controls (32%).[35] Further studies have reported an increased risk of acute MI and stroke among patients with a prior history of VTE than among those without such a history.[36,37] Also, symptomatic cardiovascular events may precede the incident of VTE.[33] Our findings suggest that monocytes might contribute to the links between atherosclerosis and VTE.

Molecular mechanisms underlying the current findings study are likely complex. Increased numbers of non-classical monocytes, which are primary producers of TNF-α and IL-1β, may regulate the immune response by enhancing cells proliferation, migration and receptor expression in VTE patients. Accordingly, it has been shown that the gene expression profile exhibited by non-classical monocytes showed the highest expression of *TNF-α* and the metalloprotease *ADAM17* gene, which are involved in the processing of TNF-α from the cell surface.[38] Moreover, an in vitro study demonstrated that only non-classical CD14^{+}CD16^{++} monocytes are able to produce high levels of IL-6, CCL2 chemokine and matrix metalloproteinase-9.[39] Thus, their increased number and accumulation onto endothelium may result in the recruitment of monocytes and T cell subsets at sites of inflammation in response to CCL2 and IL-6-induced cell activation and/or differentiation, and MMP-9-mediated vascular and tissue injury.[39]

It should be also underlined that TNF-α may exert procoagulant activity and its expression by non-classical monocytes may lead to enhanced thrombin formation. It has been shown that TNF-α may downregulate thrombomodulin, a cofactor in protein C activation.[40] Moreover, a study performed in healthy volunteers showed that TNF-α is able to induce a rapid inhibition of fibrinolysis mediated by a delayed increase in plasminogen activator inhibitor-1.[41] It may be speculated that in VTE patients non-classical monocytes may play a role in immunothrombosis. In the mouse model of flow restriction-induced DVT, it has been shown that the rapid accumulation of neutrophils and monocytes is observed within a forming thrombus and innate immune cells initiate local fibrin formation predominantly through the delivery of TF.[17] Further studies are needed to elucidate monocyte-derived mediators involved in VTE and the consequences of elevated non-classical monocytes.

In the current study we also found the associations between non-classical monocytes and age. It has been shown earlier that aging is associated with significant changes in monocyte subsets, which may have implications for the development of age-related diseases.

In a cross-sectional study involving 91 healthy individuals, age was associated with an increased proportion of intermediate and non-classical monocytes.[42] Most recently, it has been shown by Puchta et al. that intermediate human and mice monocytes produced more of the inflammatory cytokines IL-6 and TNF-α with age, both in the steady state and when stimulated with bacterial products.[43]

Moreover, we found that non-classical monocytes correlated positively with BMI and glucose, which is in line with the previous findings.[44,45] It has been shown that the proportion of intermediate and non-classical monocyte positively correlated with BMI and fasting glycemia in obese and type 2 diabetic patients.[44] Moreover, it has been shown that CD16 positive monocyte subsets were reduced by drastic fat mass loss. This feature suggests that increased glycemia could be a parameter regulating intermediate and non-classical monocytes numbers.[44] Furthermore, the I LIKE HOMe study reveals a significant association between counts of non-classical monocytes but not of total monocytes or classical monocytes, and both obesity as well as subclinical atherosclerosis in a large cohort in low-risk individuals.[45]

Of note, we observed that the elevated number of non-classical monocytes is decreased after 12 months of the VTE event, when the inflammatory process is resolved. This suggests the involvement of this subset in the acute thrombosis and the subsequent thrombus resolution.

We also showed a positive correlation of non-classical monocytes with elevated hsCRP level.

Data on the relationship between monocyte subsets and inflammatory markers yielded inconsistent findings. In patients with unstable angina, CD16-positive monocytes were associated with hsCRP levels, but no such association was found in stable angina.[22,23] Similar to our observations, an association of CRP levels and non-classical monocyte counts has been found in patients with rheumatoid arthritis and type 1 diabetes mellitus.[46,47] It should also be noted that specific single nucleotide polymorphisms in the *CRP* gene and other inflammatory and coagulation biomarkers are strongly associated with their plasma concentrations and may regulate the inflammatory processes.[48]

The present findings increase our knowledge on the role of immune responses in the pathophysiology of VTE by providing a new, monocyte-associated aspect of immunothrombosis, which suggests the involvement of non-classical and intermediate monocytes in the early phase following an acute VTE episode.

Several study limitations should be acknowledged. The number of patients with VTE and healthy controls was limited; however, the study was sufficiently powered. Nevertheless, the subgroup analysis should be interpreted with caution. A well-matched control group is recommended to be used for future study on this topic. Furthermore, we determined each variable at a single time point. Our findings cannot be easily extrapolated to the elderly or patients with severe comorbidities, in particular cancer, who were excluded from our study. Associations reported here do not necessarily mean the cause-effect relationship and should be regarded as the hypothesis-generating investigation, which can, however, have important implications. Moreover, observation at different time intervals after VTE event would introduce additional information on the presence and proportion of each subpopulation of monocytes. Finally, a long-term follow-up study is needed to assess a potential prognostic role of the current findings and investigate whether and when the intermediate and non-classical monocyte subset populations can normalize.

To our knowledge, this is the first study which shows increased counts of non-classical and intermediate monocyte subsets in patients following VTE, suggesting a new role of the immune system in this disease. Monocyte-related immunothrombotic mechanisms of VTE, that are more pronounced within the first months since the event, in particular that of unprovoked nature, provide new insights into the pathophysiology of this common disease.

References

1. Heit JA, Spencer FA, White RH. The epidemiology of venous thromboembolism. *J Thromb Thrombolysis.* 2016;41(1):3–14.
2. Zhu T, Martinez I, Emmerich J. Venous thromboembolism risk factors for recurrence. *Arterioscler Thromb Vasc Biol.* 2009;29(3):298–310.
3. Cushman M. Epidemiology and risk factors for venous thrombosis. *Semin Hematol.* 2007;44(2):62–69.
4. Goldhaber SZ, Bounameaux H. Pulmonary embolism and deep veins thrombosis. *Lancet.* 2012;379(9828):1835–1846.
5. Folsom AR, Lutsey PL, Astor BC, Cushman M. C-reactive protein and venous thromboembolism. A prospective investigation in the ARIC cohort. *Thromb Haemost.* 2009;102(4):615–619.
6. Zacho J, Tybjaerg-Hansen A, Nordestgaard BG. C-reactive protein and risk of venous thromboembolism in the general population. *Arterioscler Thromb Vasc Biol.* 2010;30(8):1672–1678.
7. Roumen-Klappe EM, den Heijer M, van Uum SH, van der Ven-Jongekrijg J, van der Graaf F, Wollersheim H. Inflammatory response in the acute phase of deep vein thrombosis. *J Vasc Surg.* 2002;35(4):701–716.
8. Pesavento R, Villalta S, Prandoni P. The post-thrombotic syndrome. *Intern Emerg Med.* 2010;5(3):185–192.
9. Rabinovich A, Kahn SR. How to predict and diagnose postthrombotic syndrome. *Pol Arch Med Wewn.* 2014;124(7–8):410–416.
10. Bucek RA, Reiter M, Quehenberger P, Minar E. C-reactive protein in the diagnosis of deep vein thrombosis. *Br J Haematol.* 2002;119(2):385–389.
11. Smeeth L, Cook C, Thomas S, Hall AJ, Hubbard R, Vallance P. Risk of deep vein thrombosis and pulmonary embolism after acute infection in a community setting. *Lancet.* 2006;367(9516):1075–1079.
12. Rabinovich A, Cohen JM, Cushman M. BioSOX Investigators. Association between inflammation biomarkers, anatomic extent of deep venous thrombosis, and venous symptoms after deep venous thrombosis. *J Vasc Surg Venous Lymphat Disord.* 2015;3(4):347–353.
13. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. *Nat Rev Immunol.* 2013;13(1):34–45.
14. von Bruhl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med.* 2012;209(4):819–835.
15. Pfeiler S, Massberg S, Engelmann B. Biological basis and pathological relevance of microvascular thrombosis. *Thromb Res.* 2014;133(Suppl 1):S35–37.
16. van der Poll T, Herwald H. The coagulation system and its function in early immune defense. *Thromb Haemost.* 2014;112(4):640–648.
17. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood.* 1989;74(7):2527–2534.

18. Ziegler-Heitbrock L, Ancuta P, Crowe S , et al. Nomenclature of monocytes and dendritic cells in blood. *Blood.* 2010;116(16):74–80.
19. Ziegler-Heitbrock L. Blood monocytes and their subsets: Established features and open questions. *Front Immunol.* 2015;6:423.
20. Belge KU, Dayyani F, Horelt A, et al. The proinflammatory $CD14^+$ $CD16^+DR^{++}$ monocytes are a major source of TNF. *J Immunol.* 2002; 168(7):3536–3542.
21. Mukherjee R, Kanti Barman P, Kumar Thatoi P, Tripathy R, Kumar Das B, Ravindran B. Non-classical monocytes display inflammatory features: Validation in sepsis and systemic lupus erythematous. *Sci Rep.* 2015;5:13886.
22. Schlitt A, Heine GH, Blankenberg S, et al. $CD14^+CD16^+$ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. *Thromb Haemost.* 2004;92(2):419–424.
23. Imanishi T, Ikejima H, Tsujioka H, et al. Association of monocyte subset counts with coronary fibrous cap thickness in patients with unstable angina pectoris. *Atherosclerosis.* 2010;212(2):628–635.
24. Rogacev KS, Cremers B, Zawada AM , et al. $CD14^{++}CD16^+$ monocytes independently predict cardiovascular events: A cohort study of 951 patients referred for elective coronary angiography. *J Am Coll Cardiol.* 2012;60(16):1512–1520.
25. Tapp LD, Shantsila E, Wrigley BJ, Pamukcu B, Lip GY. The $CD14^{++}CD16^+$ monocyte subset and monocyte-platelet interactions in patients with ST-elevation myocardial infarction. *J Thromb Haemost.* 2012; 10(7):1231–1241.
26. Zeng S, Zhou X, Ge L, et al. Monocyte subsets and monocyte-platelet aggregates in patients with unstable angina. *J Thromb Thrombolysis.* 2014;38(4):439–446.
27. Rothe G, Gabriel H, Kovacs E, et al. Peripheral blood mononuclear phagocyte subpopulations as cellular markers in hypercholesterolemia. *Arterioscler Thromb Vasc Biol.* 1996;16(12):1437–1447.
28. Kaito M, Araya S, Gondo Y, et al. Relevance of distinct monocyte subsets to clinical course of ischemic stroke patients. *PLoS One.* 2013;8(8): e69409.
29. Maldonado-Peña J, Rivera K, Ortega C, Betancourt M, Lugo JE, Camargo E. Can monocytosis act as an independent variable for predicting deep vein thrombosis? *Int J Cardiol.* 2016;219:282–284.
30. Go SI, Kim RB, Song HN, et al. Prognostic significance of the absolute monocyte counts in lung cancer patients with venous thromboembolism. *Tumour Biol.* 2015;36(10):7631–7639.
31. Siedlar M, Strach M, Bukowska-Strakova K, et al. Preparations of intravenous immunoglobulins diminish the number and proinflammatory response of $CD14^+CD16^{++}$ monocytes in common variable immunodeficiency (CVID) patients. *Clin Immunol.* 2011;139(2):122–132.
32. Chow S, Shao J, Wang H. *Sample Size Calculations in Clinical Research*. 2nd ed. New York-Basel: Marcel Dekker Inc.; 2008.
33. Riva N, Donadini MP, Ageno W. Epidemiology and pathophysiology of venous thromboembolism: Similarities with atherothrombosis and the role of inflammation. *Thromb Haemost.* 2015;113(6):1176–1183.
34. Fuchs TA, Brill A, Wagner DD. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. *Arterioscler Thromb Vasc Biol.* 2012; 32(8):1777–1783.
35. Prandoni P, Bilora F, Marchiori A, et al. An association between atherosclerosis and venous thrombosis. *N Engl J Med.* 2003;348(15):1435–1441.
36. Prandoni P, Ghirarduzzi A, Prins MH, et al. Venous thromboembolism and the risk of subsequent symptomatic atherosclerosis. *J Thromb Haemost.* 2006;4(9):1891–1896.
37. Sørensen HT, Horvath-Puho E, Pedersen L, et al. Venous thromboembolism and subsequent hospitalization due to acute arterial cardiovascular events: A 20 year cohort study. *Lancet.* 2007;370(9601): 1773–1779.
38. Gren ST, Rasmussen TB, Janciauskiene S, Håkansson K, Gerwien J, Grip O. A single-cell gene-expression profile reveals inter-cellular heterogeneity within human monocyte subsets. *PLoS One.* 2015; 10(12):e0144351.
39. Ancuta P, Wang J, Gabuzda D. $CD16^+$ monocytes produce IL-6, CCL2, and matrix metalloproteinase-9 upon interaction with CX3CL1-expressing endothelial cells. *J Leukoc Biol.* 2006;80(5):1156–1164.
40. Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J Exp Med.* 1986;163(3):740–745.
41. van der Poll T, Levi M, Büller HR, et al. Fibrinolytic response to tumor necrosis factor in healthy subjects. *J Exp Med.* 1991;174(3):729–732.
42. Hearps AC, Martin GE, Angelovich TA, et al. Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function. *Aging Cell.* 2012;11(5):867–875.
43. Puchta A, Naidoo A, Verschoor CP, et al. TNF drives monocyte dysfunction with age and results in impaired anti-pneumococcal immunity. *PLoS Pathog.* 2016;12(1):e1005368.
44. Poitou C, Dalmas E, Renovato M, et al. $CD14dimCD16^+$ and $CD14^+CD16^+$ monocytes in obesity and during weight loss: Relationships with fat mass and subclinical atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2011;31(10):2322–2330.
45. Rogacev KS, Ulrich C, Blomer L, et al. Monocyte heterogeneity in obesity and subclinical atherosclerosis. *Eur Heart J.* 2010;31(3):369–376.
46. Kawanaka N, Yamamura M, Aita T, et al. $CD14^+$, $CD16^+$ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis Rheum.* 2002;46(10):2578–2586.
47. Myśliwska J, Smardzewski M, Marek-Trzonkowska N, Myśliwiec M, Raczyńska K. Expansion of $CD14^+CD16^+$ monocytes producing TNF-α in complication-free diabetes type 1 juvenile onset patients. *Cytokine.* 2012;60(1):309–317.
48. Montagnana M, Danese E, Lippi G. Genetic risk factors of atherothrombosis. *Pol Arch Med Wewn.* 2014;124(9):474–482.