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

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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.²⁻³

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.⁵⁻⁶

In addition, acute infections predispose to DVT, which also supports the role of inflammation in thrombosis.⁷⁻¹¹

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 immunothrombolic 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 FcγRIIIA 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.²¹ 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 (visualiza-
tion of an intraluminal thrombus in calf, popliteal, femoral,
or iliac veins). The diagnosis of PE was based on the pres-
ence of typical symptoms and positive results of computed
tomography pulmonary angiography (CT). Patients with
signs of acute infection, known cancer, chronic inflam-
matory disease, or autoimmune disorders (including an-
tiphospholipid syndrome), previous MI or stroke, serum creatinine ≥120 μM, liver injury, pregnancy were ineligible.
All patients were treated with unfractionated or low-mo-
lecular-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 pa-
tients with unprovoked VTE. A VTE episode was defined as
unprovoked (idiopathic) if the patient had no history of
cancer, surgery requiring general anesthesia, major trau-
a, a plaster cast or hospitalization within the last month,
or pregnancy or delivery within the last 3 months. A prox-
imal 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 ki-
lograms divided by the square of height in meters. Obes-
ity was defined as a BMI of 30 kg/m² 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 dia-
stolic pressure ≥140 mm Hg or ≥90 mm Hg, respectively,
on at least 2 different occasions or the use of antihyper-
tensive treatment. Hypercholesterolaemia was diagnosed
based on low-density lipoprotein cholesterol (LDL-C) level
>3.0 mmol/L or previously diagnosed hypercholesterola-
mia. The diagnosis of MI was based on the 2012 American
Heart Association, European Society of Cardiology, Amer-
ican College of Cardiology Foundation, and World Heart
Federation (ESC/ACCF/AHA/WHF) guidelines. Ischemic
stroke was diagnosed according to the World Health Or-
ganization 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. Se-
rum 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 immunoturbidim-
etry (Roche Diagnostics GmbH).

A complete blood count was determined using the he-
matological analyzer Sysmex XT2000i (Sysmex Corpora-
tion, 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 sam-
plers 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 Biosci-
ences, San Jose, USA), 10 μL APC-labeled anti-human
HLA-DR (347402, BD Biosciences), 5 μL APC-labeled
anti-human CD45 (340910, BD Biosciences) in BD Tru-
count 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 per-
formed on FACS Canto II flow cytometry (BD Bioscience)
and analyzed by FACS Diva software v. 7.0 (BD Biosci-
ces). The following calculation has been performed:
[(number of events in quadrant containing cell popula-
tion)/(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. 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).
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.

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.
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.
arterial walls enhancing the inflammatory and immune processes characteristic of atherosclerosis.\textsuperscript{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 idiopathic VTE were more likely to have carotid artery plaque (47%) than patients with provoked VTE (27%) or age- and sex-matched controls (32%).\textsuperscript{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.\textsuperscript{36,37} Also, symptomatic cardiovascular events may precede the incident of VTE.\textsuperscript{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-\(\alpha\) and IL-1\(\beta\), 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-\(\alpha\) and the metalloprotease ADAM17 gene, which are involved in the processing of TNF-\(\alpha\) from the cell surface.\textsuperscript{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.\textsuperscript{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.\textsuperscript{39}

It should be also underlined that TNF-\(\alpha\) may exert procoagulant activity and its expression by non-classical monocytes may lead to enhanced thrombin formation. It has been shown that TNF-\(\alpha\) may downregulate thrombomodulin, a cofactor in protein C activation.\textsuperscript{40} Moreover, a study performed in healthy volunteers showed that TNF-\(\alpha\) is able to induce a rapid inhibition of fibrinolysis mediated by a delayed increase in plasminogen activator inhibitor-1.\textsuperscript{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.\textsuperscript{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. 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. Moreover, we found that non-classical monocytes correlated positively with BMI and glucose, which is in line with the previous findings. 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. 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 monocyte numbers. 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.

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. 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. 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.

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