LETTER TO THE EDITOR

Immunoregulatory role of circulating endothelial vWF positive cells in patients after acute myocardial infarction

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To the Editor,

The frequency of circulating endothelial cells which express pan-endothelial marker von Willebrand factor (vWF) increases with both endothelial dysfunction and acute myocardial infarction (AMI) (1). Increases in circulating vWF^+ cells correlate with poor clinical outcomes and death in AMI, suggesting that they participate in the pathogenesis of AMI (1, 2). We previously hypothesized that increased vWF^+ cells in the circulation of patients with AMI could act as antigen presenting cells, leading to a direct interaction with NK and T cells and resulting in cytokine secretion, thus enhancing systemic inflammatory reaction and causing additional endothelial damage by cytotoxic potential of lymphocytes (2). This is mostly seen in patients with non-ST-segment elevation myocardial infarction (NSTEMI) who are treated conservatively, as prolonged myocardial ischemia could sustain a local and systemic inflammatory reaction (3, 4). In patients with ST-segment elevation myocardial infarction (STEMI) treated with primary percutaneous coronary intervention (PCI) of the single arterial stenosis responsible for infarct, the inflammation is quenched, whereas it fluctuates for a month in patients with NSTEMI without PCI (2). Under inflammatory conditions, vWF^+ endothelial cells could take on the proinflammatory antigen-presenting phenotype, resembling $CD14^+$ monocytes (2). In patients with AMI, the injured endothelium expresses and secretes interleukin (IL)-15, which is important for T and natural killer (NK) cell proliferation, chemotaxis and binding to endothelial cells, perforin (P) expression, and cytotoxicity (5). P is a cytotoxic mediator constitutively stored in NK cells and induced in T cells, and is released from

Key words: acute myocardial infarction; NK cells; perforin; T cells; von Willebrand factor positive cells

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granules after stimulation to kill targets (6). It is a critical mechanism for the elimination of injured cells (6). However, the dynamics of vWF^+ cells in the circulation and their possible immune functions after AMI are not completely understood. This study investigated the dynamic characteristics and possible interaction of circulating vWF^+ cells with T and/or NK cells in comparison to $CD14^+$ cells in patients with AMI in terms of perforin expression *in vitro*.

MATERIALS AND METHODS

Patients

Patients with AMI who were admitted to the Clinical Hospital Center Rijeka, Croatia, and Special Hospital Thalassotherapia-Opatija, Opatija, Croatia, were enrolled in a prospective study from October 2013 to May 2016. They were treated according to the then current guidelines of the European Cardiac Society (3, 4). All patients with AMI received anti-ischemic therapy (beta-blocker, an angiotensin-converting enzyme inhibitor, statin, acetylsalicylic acid, and clopidogrel). Given the type of AMI and the administration of percutaneous coronary intervention (PCI), patients with AMI were divided into three groups: a) patients with STEMI treated with primary PCI and implantation of one stent in the culprit lesion; b) patients with NSTEMI treated with PCI and implantation of one or two stents in the culprit lesions within the first 24 h; and c) patients with NSTEMI treated conservatively without PCI. Venous peripheral blood (10 mL) samples were taken from all patients with AMI on day one, before PCI (if applied), and on days 7 and 28 during early inpatient cardiac rehabilitation. Blood samples were taken once from healthy subjects who underwent a clinical examination and routine laboratory analyses as part of a preventive medical program. Clinical and laboratory characteristics of patients with AMI and healthy controls are shown in Table I. The exclusion criteria for healthy subjects and patients included generative age in women; chronological age above 80 years for all subjects; unstable angina pectoris; uncontrolled arrhythmia (heart rate >130/min); significant valvular deficiency (Gradus III or IV); congestive heart failure (concentration of N-terminal pro-brain natriuretic protein >150 pg/mL); significant peripheral vascular disease; uncontrolled endocrine disease (plasma glucose >11mmol/L or concentration of thyrotropin-*releasing hormone* >4.20 mU/L); uncontrolled hypertension (systolic blood pressure >180 mmHg or diastolic >100 mmHg); infectious and autoimmune disease; injury to organs and blood transfusions. The Ethics Committees of two hospitals and the Faculty of Medicine, University of Rijeka approved the study according to the "Ethical principles for medical research involving human subjects" in the Declaration of Helsinki outlined by the World Medical Association. All subjects provided written consent for participation in the study.

Routine laboratory analyses

Hematological analysis was performed using an automatic analyzer (Hematology analyzer XS-1000i, Sysmex, Kobe, Japan). Biochemical analysis was determined by a photometric method using Dimension Xpand, (Siemens Healthcare Diagnostics, Newark, DE, USA). Cardiac troponin I (cTn I) was analyzed by the one-step enzyme immunoassay based on the "sandwich" principle with two cTn I-specific monoclonal antibodies in the serum samples using analyzer Dimension RXL (Siemens Healthcare, Diagnostics, New York, DE, USA).

Separation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were extracted from 10 ml blood sample by gradient density centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway), and centrifuged at 600 × g for 20 min (option without brake). The PBMCs were collected with a Pasteur pipette (Hirschmann Laborgerate GmbH, Eberstadt, Germany) from the interface of the Lymphoprep, resuspended in 10 ml Roswell Park Memorial (RPMI) 1640 media (GIBCO, Gaithersburg, MD, USA) and washed (10 min, 350 x g). The cell-pellet was resuspended in 1 ml of RPMI tissue culture medium [RPMI 1640 supplemented with L-glutamate, (2 mM), penicillin 5 U/L), streptomycin sulfate, (0.05 g/L), 10% fetal calf serum (FCS); all from, Kemika, Zagreb, Croatia] and the cells counted in a Neubauer chamber with Trypan blue stain (Serva, Heidelberg, Germany) using a light microscope (Carl Zeiss, Jena, Germany). Cell viability was always >95%.

Flow cytometry

Labeling of cell markers was performed in isolated PBMCs (2×10^5/sample) from patients with AMI on days 1, 7, and 28, and healthy subjects. Antibodies were obtained

Table I. *Demographic, clinical and laboratory characteristics of patients with acute myocardial infarction*

	Value / number of patients (N)			
	Healthy Control (N28)	**STEMI with PCI (N16)**	**NSTEMI without PCI (N15)**	**NSTEMI with PCI (N17)**
Clinical characteristics				
Male / Female	13/15	12/4	6/9	7/10
Fatal outcome	0/0	0/0	0/2	1/0
Diabetes	0	7	3	3
Arterial hypertension	0	16	15	17
Hyperlipoproteinemia	0	16	8	15
Oral hypoglycemic drugs	0	3	2	3
Insulin therapy	0	1	1	0
Anti-ishemic therapy	0	16	15	17
Coronary culprit lesion (PCI)		LAD (8×), D1 (1×), RCA(6×), OM1(1×)		LAD(11×), D1(1×), D2(1×), RCA(5×), LCX(2×), OM1(1×)
	Value / median (percentile 25th, percentile 75th)			
Age Male (years)	60 (58, 64)	62 (54,71)	62 (54, 70)	60 (55, 69)
Age Female (years)	68 (60, 70)	67 (59, 75)	70 (62, 76)	71 (64, 70)
Body mass index (kg/m2)	25 (22, 27) [a,b] *p = 0.002*	29 (26, 31)	28 (25, 31)	28 (26, 32)
Systolic pressure (mm Hg)	126 (119,130) [a,b] *p = 0.002*	130 (115, 140)	130 (120, 148)	130 (120, 140)
Diastolic pressure (mm Hg)	78 (70, 80) [a, b, c] *p = 0.002*	70 (65, 80)	70 (65, 80)	70 (65, 75)
Laboratory characteristics				
hs CRP (mg/L)	2.85 (1.5, 4.1) [a]*p = 0.0001,* [b,c]*p = 0.001*	13.3 (6.8, 31.1)	15,8 (10.7, 18.9)	11.8 (5.4, 20.4)
CTn I (ng/L)	0.02 (0.01, 0.03) [a,b,c] *p = 0.0006*	33.2 (13, 74.2)	6.3 (2.7, 16.6)	4.1 (1.3, 10)
NT pro-BNP	14 (10.7, 20) [a,b,c] *p = 0.0004*	67 (48, 123)	66 (40, 87)	58 (35, 81)
Erythrocytes ($\times 10^{12}$/L)	4.8 (4.5, 5)	4.4 (4.2, 4.6)	4.2 (4, 4.4)	4.15 (4, 4.6)
Leukocytes ($\times 10^9$ /L)	6.2 (5.3, 7.1) [a] *p = 0.002*	11.8 (10, 13.7)	7.65 (6.5, 8.7)	7.3 (5.4, 8.1)
Thrombocytes ($\times 10^9$ /L)	278 (249, 310) [a] *p =0.002*	188 (135, 197)	302 (173, 305)	228 (172, 256)
AST (IU/L)	18 (14, 19) [a] *p = 0.0000*	77 (40, 137)	19 (16, 25)	15 (13, 53)
ALT (IU/L)	20 (17, 23) [a] *p = 0.0001*	49.4 (35, 60)	19 (14, 21)	28 (18, 83)
Urate (μmol/L)	314 (264, 384) [a] *p =0.001*	428 (406, 454)	345 (286, 414)	314 (264, 344)
Creatinine (μmol/L)	87 (85, 92)	86 (63.5, 121)	90 (82, 110)	82.5 (76, 118)
Glucose (mmol/L)	5.6 (5.3, 5.9)	8 (6.7, 8.9)	8.7 (7.7, 9.1)	5.5 (5, 7.7)
LDL cholesterol (mmol/L)	3,2 (1.9, 3.8)	3.9 (3.4, 4.1)	2.2 (1.7, 2.9)	2.2 (1.9, 2.7)
HDL cholesterol (mmol/L)	1.8 (1.6-2.3) [a] *p = 0.0006*	1.3 (1-1.4)	0.8 (0.7-1.4)	1 (0.8, 1.3)
Triglicerides (mmol/L)	0.9 (0.6, 1.1) [a] *p= 0.0001*	2 (1.9, 3.1)	0.9 (0.9-1.2)	1.1 (1, 1.3)

*ALT: alanine transaminase; AST: aspartate transaminase; D1: the first diagonal artery; D2: the second diagonal artery; ESR: erythrocyte sedimentation rate; HDL: high density lipoprotein; hsCRP: high sensitive C: reactive protein; CTn I: cardiac troponin I; LAD: left anterior descending artery; LCX: the left circumflex artery; LDL: low density lipoprotein; NT proBNP: N-terminal pro brain natriuretic peptide; OM1: the first obtuse marginal artery; RCA: right coronary artery. *a (control vs STEMI with PCI); b (control vs NSTEMI with PCI); c (control vs NSTEMI without PCI).*

from BD Biosciences (San Jose, USA), and used at 20 μL/10^6 cells for 30 min at 4°C, unless otherwise indicated. All washes (2 × 5 min at 400 × g) were performed in fluorescence-activated cell sorter (FACS) buffer [NaCl (140 mM), KH_2PO_4 (1.9 mM), Na_2HPO_4 (16.5 mM), KCl (3.75 mM) (all from Kemika), Na_2EDTA (0.96 mM, Fluka, Buchs, Switzerland), NaN_3 (1.5 mM, Difco, Detroit, Michigan, USA)], except in intracellular antigen labeling, when saponin buffer [0.1% saponin (Sigma, Poole, Dorset, USA), 2% fetal calf serum in phosphate-buffered saline (PBS: $NaHPO_4 \times 12H_2O$ 33.9 mM, NaCl 136.8 mM, KH2PO4 3 mM of distilled water, all from Kemika) 0.05% bovine serum albumin (Sigma, Poole, Dorset, USA)] was used. Samples were initially incubated with 10% heat-inactivated fetal calf serum (Gibco) for 20 min at room temperature (RT) to block Fc receptor binding and washed. The pellet was resuspended, and the cells indirectly labeled with vWF monoclonal antibody (mAb) (mouse IgG1, 3E2D10, Abcam, Cambridge, UK, dilution 1:100) or CD14 mAb (mouse IgG2a, M5E2, dilution 1:100) followed by incubation with secondary goat anti-mouse (GAM) polyclonal antibodies conjugated with fluorescein isothiocyanate (FITC). In some samples, HLA DR, CD80, or CD86 were labeled by the addition of phycoerythrin (PE)-conjugated anti-HLA-DR mAb (mouse IgG2a, G46-6), anti-CD80 mAb (mouse IgG1, L307.4), or anti-CD86 mAb (mouse IgG1, FUN-1), respectively. During magnetic separation, cell fractions were single labeled using unconjugated antibodies directed toward vWF, CD14 or CD1a, (mouse IgG1, OKT-6, Department of Immunology and Inflammation, Clinical Institute Humanitas, Rozzano, Italy, dilution 1:100), PE-conjugated CD56 mAb (mouse IgG1, B159) and FITC conjugated CD3 (mouse IgG1, UCHT1). Detection of IL-15 was performed in permeabilized and pre-labeled vWF^+ cells, according to the principle of the method described earlier (7). Briefly, freshly isolated PBMCs (10⁶/mL) were incubated with phorbol-myristate-acetate (PMA, 10 ng/mL), ionomycin (1 μM), and monensin (3 μM) (all from Sigma-Aldrich Chemie, Steinheim, Germany) for 5 h at 37°C, in a humidified atmosphere containing 5% CO_2. Then, non-specific binding was blocked, and the cells were labeled using anti-vWF mAb and GAM-FITC. Next, the cells were fixed in 4% paraformaldehyde (Kemika, Zagreb, Croatia), pH 7.4 for 10 min at RT, washed, and permeabilized in 100 uL saponin buffer for 20 min at RT. PE-conjugated IL-15 mAb (mouse IgG1, 34559, R&D Systems, Minneapolis, USA, 20 μL/10^6 cells) was added into cell samples. After two washes in saponin buffer, the cells were resuspended in 1 mL of FACS buffer to restore membrane integrity, washed in FACS buffer, and resuspended in 200 μL of 2% paraformaldehyde. P was detected in CD3/CD56 labeled cells after coculture with purified vWF^+ cells, $CD14^+$ or $CD14^-$ cells at cell ratios 2.5 and 5:1, or after culture in medium only for 18 h at 37°C in a humidified atmosphere with 5% CO_2. P was labeled intracellularly using unconjugated anti-P mAb (mouse IgG2b, δG9, Department of Physiology and Immunology, Medical Faculty University of Rijeka, Rijeka Croatia, 3μg/10^6 cells) and GAM FITC in fixed and permeabilized cells. Then surface CD3 and CD56 was labeled directly using FITC-conjugated CD3 mAb (mouse IgG1, UCHT-1) and PE-conjugated CD56 mAb (mouse IgG1, B159). We used unconjugated mouse IgG1 (MOPC-31C, dilution 1:100), FITC or PE-conjugated mouse IgG1 (MOPC-21), unconjugated or PE-conjugated mouse IgG2a (clone 155-178), and unconjugated mouse IgG2b (clone 27-35) as the control, according to the isotype of specific mAb used. All labeled cell samples were acquired immediately by FACS Calibur flow cytometry and CellQuestPro Software (Becton Dickinson, New Jersey, USA). The viability of vWF^+ and $CD14^+$ cells was assessed with propidium iodide (PI; 0.5 μg/mL/10^6 cells, Sigma-Aldrich Chemie, Daisenhofen, Germany) and flow cytometry.

Enrichment of peripheral blood cell-subpopulations

PBMC cells were cultured overnight (18 h) in tissue culture Petri dishes 100 × 20 mm (TPP, Trasadingen, Switzerland) at 37°C, in a humidified 5% CO_2 incubator to obtain non-adherent and adherent cells (fractions). The non-adherent cells were lymphocytes, which consisted of T and NK cells. They were used for the positive separation of $CD56^+$ cells. For this purpose, non-adherent cells were collected and filtered through 100 μm nylon mesh to remove possible clumps. Subsequently, the cells were resuspended in 80 μL of ice-cold FACS buffer and 20 uL of microbeads-conjugated anti-CD56 mAb (mouse IgG1, Miltenyi Biotec, Bergisch Gladbach, Germany, 20 μL/10^7 cells). Following a 15-min incubation at 4°C, the cells were centrifuged at 400 × g for 10 min, resuspended in 500 μL of FACS buffer, applied onto the large column, and placed in the magnetic field of Vario-MACS separator

(both from Miltenyi Biotec). After the cells ran through, the column was washed with 3 mL of FACS buffer four times, then removed from the magnetic field. The $CD56^+$ cells were flushed out using the same medium, whereas CD56 negative cells were collected separately and were used as a source of enriched and functionally untouched $CD3^+$ cells, however with the possible impurity of $CD56^+$ cells. The adherent fraction contained cells with adhesive properties ($CD14^+$ monocytes, $CD1a^+$ dendritic cells, and vWF^+ endothelial cells), which were collected by a pipette and filtered through 100 μm nylon mesh, then pelleted, and used for the separation of vWF^+ cells. The adherent cells were incubated with anti-vWF mAb (1 μg per 10^6 of total cells) for 30 min in the presence of FACS buffer (60 μL) and blocking reagent (20 μL, Miltenyi Biotec) to reduce non-specific antibody binding. Then, the cells were washed and resuspended in 60 μL of ice-cold FACS buffer. Secondary polyclonal goat anti-mouse IgG MicroBeads (Miltenyi Biotec, 20 μL/10^6 cells) were added, and cells were incubated for 15 min, at 4°C. The following steps of vWF^+ cell isolation were identical to the isolation procedure of $CD56^+$ cells. $CD14^+$ cells were purified from vWF^- fraction using unconjugated anti-CD14 mAb (mouse IgG2a, M5E2, BD Bioscience, dilution 1:100/10^6 cells). Enriched vWF^+, $CD14^+$ and $CD14^-$ fractions were tested for the presence of $CD14^+$ monocytes and $CD1a^+$ dendritic cells with strong antigen-presenting properties.

Statistical analyses

Statistical analyses were performed using the data analysis software system TIBCO Statistica, Version 13.4.0.14 (Palo Alto, CA, USA). The Kruskal-Wallis non-parametric test was used to calculate the differences between groups. A P-value of <0.05 for the differences between the groups was determined to be significant, using the non-parametric Kruskal-Wallis test. The Mann-Whitney U-test evaluated differences between two groups of interest with significance levels adjusted to several mutual comparisons.

RESULTS

Dynamic changes of vWF^+ and $CD14^+$ cells in patients with AMI

In all patients with AMI, the percentage of vWF^+ cells was higher on days 1, 7, and 28 after AMI when compared to the healthy controls, which was 1.64% (1.3%; 2.2%), [median (25th; 75th percentiles)] (Fig. 1 A-C). In STEMI patients treated with primary PCI, the percentage of vWF^+ cells was the highest on day 7 (approximately 5%) and statistically significantly differed from that on days 1 and 28 after acute coronary event (Fig. 1A). In NSTEMI patients without PCI, vWF^+ cells were approximately 15% on day 1 and around 12% on day 28 (Fig. 1B). Both values were statistically significantly higher when compared to day 7 (4%, Fig. 1B). In NSTEMI patients treated with primary PCI, the percentage of vWF^+ cells was 15% on day 1 before the intervention, and then 18% and 12% on days 7 and 28, respectively (Fig. 1C). In these patients, vWF^+ cells were highest on day 7, and it was significantly higher than on day 1 and day 28 (Fig. 1C).

In STEMI patients treated with primary PCI, the frequency of $CD14^+$ cells increased significantly on day 1 after AMI, whereas the highest percentage of $CD14^+$ cells was found on day 7 in these patients (Fig. 1D) and NSTEMI patients without PCI (Fig. 1E). The percentage of $CD14^+$ cells on day 7 were higher than that of day 28 and of healthy controls in both groups (Fig. 1D and 1E). Additionally, in NSTEMI patients without PCI, the percentage of $CD14^+$ cells on day 7 was higher than that of day 1 as the proportion of $CD14^+$ cells on day 1 remained almost unchanged compared to that of the controls (Fig. 1E).

Phenotype of vWF^+ cells and $CD14^+$ cells in patients with AMI

Dynamic changes in HLA-DR, CD80, CD86, and IL-15 expression (Fig. 1 F-I) and viability of vWF^+ cells (Fig. 1J) were observed during the month after AMI. The frequency of HLA-DR expressing vWF^+ cells statistically increased on days 1 and 7 compared to the controls and day 28 (Fig. 1F), whereas CD80 expression decreased (Fig. 1G). CD86 expression remained statistically unchanged on days 1 and 7 and significantly decreased on day 28 (Fig. 1H). IL-15 expression in vWF^+ cells dropped on day 7 after AMI, and was statistically marginally lower compared to day 28 ($P = 0.029$, Fig. I). A significantly higher percentage was found of killed vWF^+ cells in the circulation on

day 1 and 28 after AMI than in the control group. At the same time, they dropped on day 7 and became marginally significant toward day 1 (P = 0.02) and day 28 (P = 0.05, Fig. J). Further, we compared the phenotype of vWF$^+$ and CD14$^+$ cells in NSTEMI patients on day 1 after AMI before PCI, as their number was the highest at that time-point. The percentage of HLA-DR$^+$ cells did not differ between vWF$^+$ cells and CD14$^+$ cells (Fig. 1K). The frequency of vWF$^+$ cells expressing CD80 was negligible (Fig. 1L), whereas CD86 was expressed in 40% -75% of vWF$^+$ cells (Fig. 1M). Both were statistically significantly lower when compared to CD14$^+$ cells. IL-15 was significantly lower in vWF$^+$ cells than in CD14$^+$ cells (Fig. 1N). Percentages of killed CD14$^+$ cells did not significantly differ one month after AMI (Fig. 1O).

Enrichment of vWF$^+$ cells, CD14$^+$ cells and CD3$^+$ lymphocytes

We showed the placement of vWF$^+$ events within an oval fence (set up on a "back gating" principle) in dot plots specified by size or FSC (X-axis) and

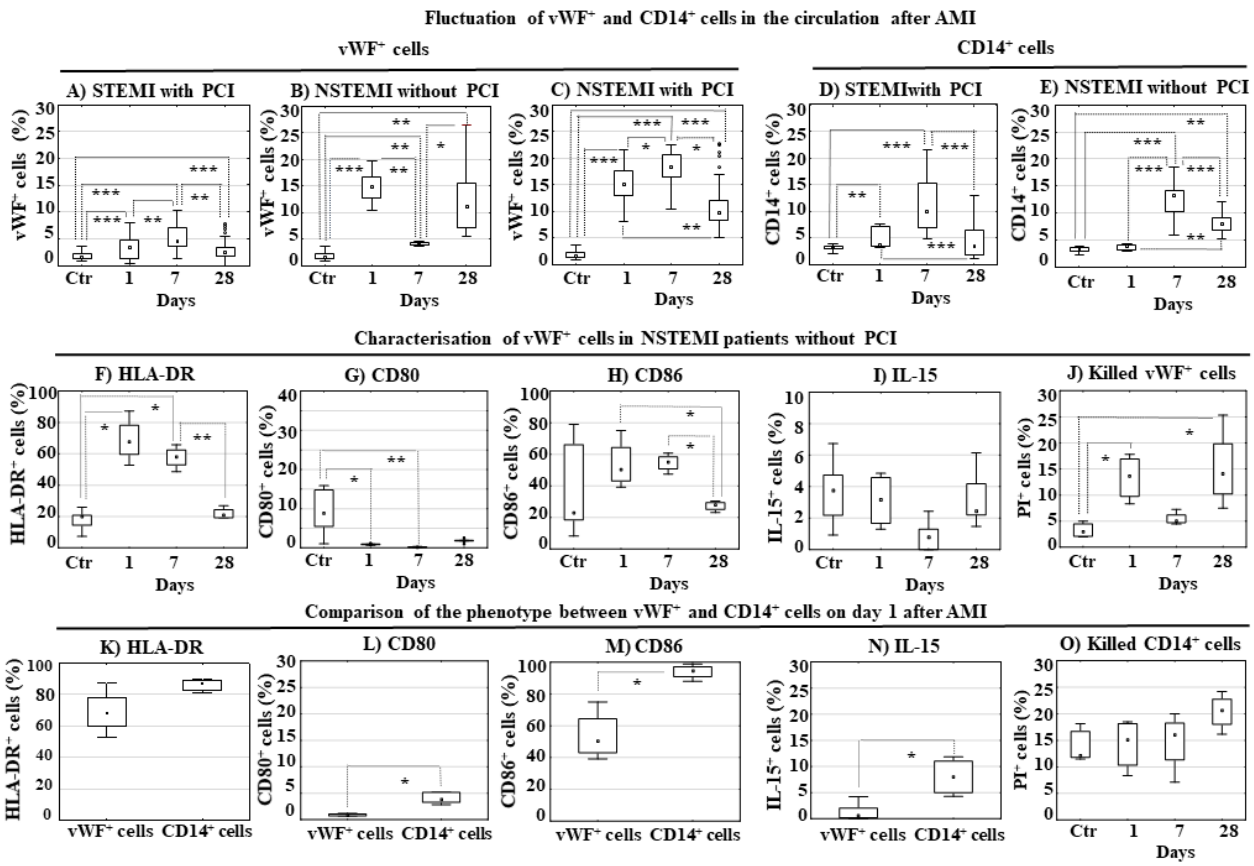

Fig. 1. *Fluctuation of vWF$^+$ and CD14$^+$ cells in the circulation after acute myocardial infarction (AMI). The proportion of circulating vWF$^+$ cells (**A, B, C**) and CD14$^+$ cells (**D, E**) in patients with ST-elevation myocardial infarction (STEMI) and primary percutaneous coronary intervention (PCI) (A, D), patients with non - ST- elevation myocardial infarction (NSTEMI) without PCI (B, E) and patients with NSTEMI and PCI (C) on day 1, day 7 and day 28 are compared with the controls (Ctr). Graphs show 8-32 experiments per group in the upper row, and the levels of statistical significance are *P<0.012, **P<0.005, ***P<0.0001. Dynamic changes in HLA-DR (**F**), CD80 (**G**), CD86 (**H**) and IL-15 (**I**) expression, as well as killed vWF$^+$ cells (**J**) in NSTEMI patients without PCI (middle row). Comparison of HLA-DR (**K**), CD80 (**L**), CD86 (**M**) and interleukin (IL)-15 (**N**) between vWF$^+$ and CD14$^+$ cells in NSTEMI patients without PCI on day 1 and fluctuation of killed CD14$^+$ cells (**O**) in NSTEMI patients without PCI (lower row). Graphs show 4-6 experiments per group in the middle and lower row, and the levels of statistical significance are *P=0.04; **P=0.03, ***P=0.01. Bars represent median (▫), 25% - 75% (□), non-outlier range (I).*

FITC fluorescence (Y-axis) for adherent cells (Fig. 2A) and non-adherent cells (Fig. 2B). In the dot plot shown, the adherent cells contained 13.2% of vWF^+ cells compared to the control labeling of 0.2% (Fig. 2A), whereas vWF^+ cells accounted for only 0.7% in the non-adherent cells compared with control (0.3%) (Fig. 2B). A higher frequency of vWF^+ cells in adherent [13% (10.1%; 15.2%)] than in non-adherent fraction ($P < 0.05$) derived from six independent experiments determined further separation of vWF^+ cells from the adherent cell fraction. In the sample shown, enrichment of vWF^+ cells was 78.71% after positive magnetic separation (Fig. 2C) and was in accordance with the median of 72.2% (70.5%; 77.9%) in the six experiments performed. The enriched vWF^+ subpopulation did not contain $CD1a^+$ cells, whereas $CD14^+$ cells were represented with 2.9% in the histogram shown (Fig. 2C). $CD14^+$ cells were selected by positive magnetic separation from vWF negative fraction to the enrichment of 82.42% (Fig. 2D). The median enrichment of $CD14^+$ cells was 80% (78%; 81.2%). We did not find $CD14^+$ cells in the $CD14^-$ adherent fraction (Fig. 2D). $CD14^+$ and $CD14^-$ fraction did not contain $CD1a^+$ cells (Fig. 2D), therefore the $CD14^-$ fraction served as a control in T cell coculture experiments.

$CD56^+$ cells were separated from the non-adherent cells to a degree of purification of 97.06% (Fig. 2E), which corresponds to 96.33% (95%; 97.7%) of purity in five experiments performed. Functionally intact $CD3^+$ T cells remained in the non-adherent fraction of PBMCs after removing $CD56^+$ cells by positive magnetic separation (Fig. 2E). They were enriched to 69% in the shown sample and corresponded to

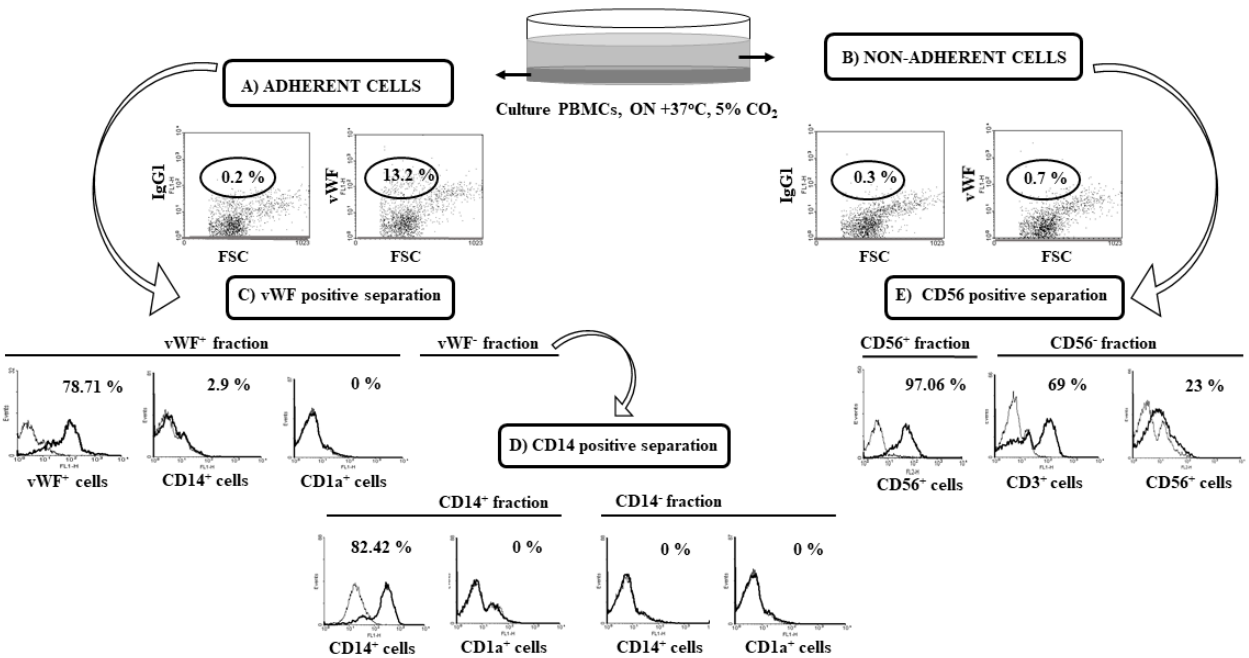

Fig. 2. *Enrichment strategy for subpopulations from peripheral blood mononuclear cells (PBMCs) in NSTEMI patients without PCI treatment on the first day after acute myocardial infarction. The location and proportion of vWF^+ cells concerning isotype-matched controls are shown in sample dot plots in numbers within an oval fence (set up on a "back gating" principle) in adherent cells (**A**) and non-adherent cells (**B**). The thick curve laid above the thin curve in histograms illustrate the enrichment of vWF^+ cells and admixture of $CD14^+$ and $CD1a^+$ cells in adherent vWF^+ cell fraction (**C**); the enrichment of $CD14^+$ cells and the admixture of $CD1a^+$ cells in adherent $CD14^+$ fraction, and the percentage of $CD14^+$, $CD1a^+$ cells in adherent $CD14^-$ fraction (**D**); the purification of $CD56^+$ cells in non-adherent $CD56^+$ fraction, and the enrichment of $CD3^+$ cells and admixture of $CD56^+$ cells in the non-adherent $CD56^-$ cell fraction (**E**). The numbers above the histograms indicate the difference in the percentage of labeled events with specific antibodies (thick histogram curve) and isotype control (thin histogram curve).*

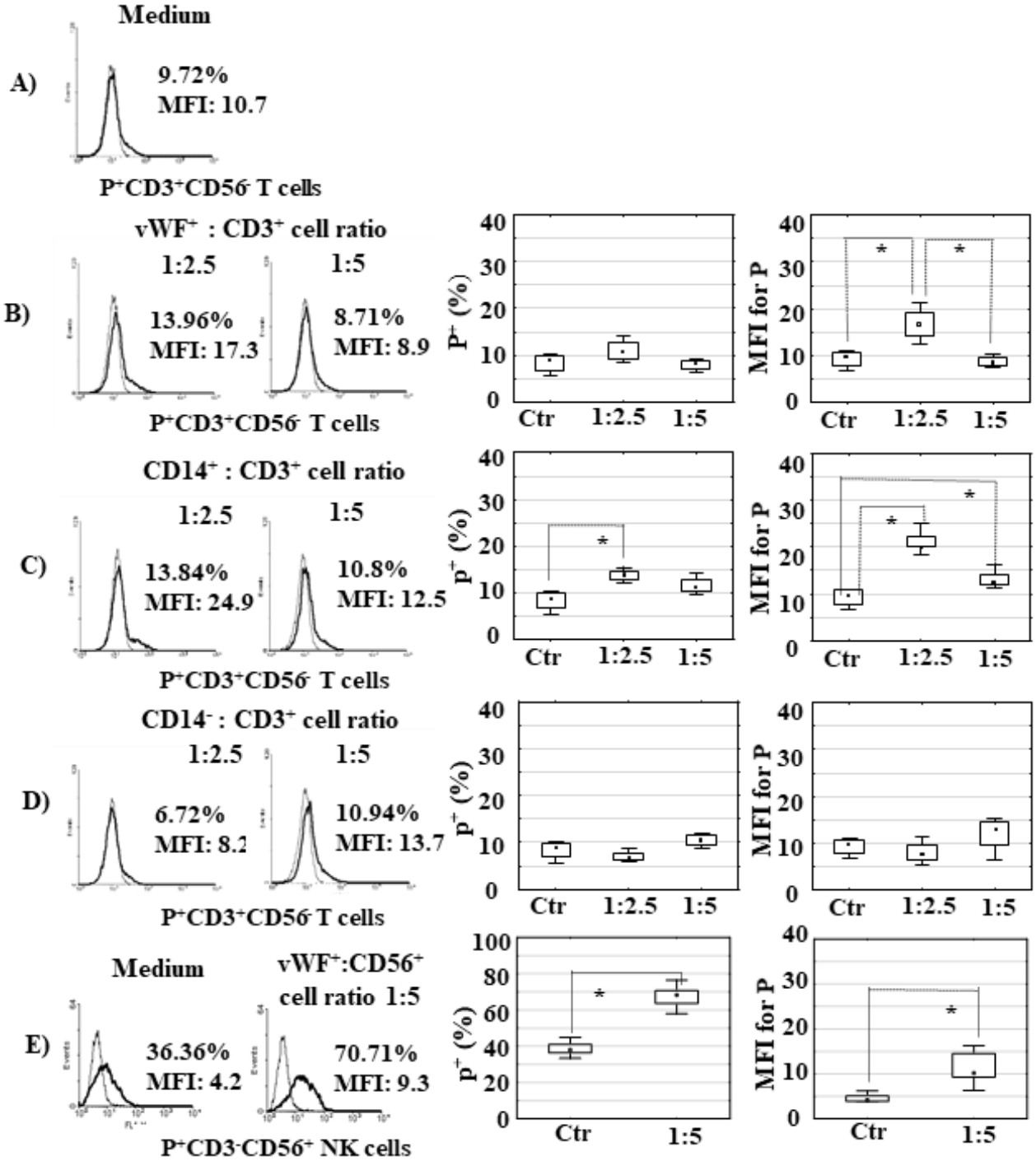

Fig. 3. *Perforin (P) expression in $CD3^+CD56^-$ T cells and $CD3^-CD56^+$ NK cells in NSTEMI patients before PCI on the first day after acute myocardial infarction. Histograms and bars show the percentage of P^+ T cells and mean fluorescence intensity (MFI) for P in T cells cultured in medium only (**A**) or cocultured with vWF^+ cells (**B**), $CD14^+$ cells (**C**), and $CD14^-$ cells (**D**) at ratios 2.5:1 and 5:1 after 18 h. Percentage and MFI for P in NK cells cultured in medium only or cocultured with vWF^+ cells at ratio 5:1 after 18 h is illustrated in histograms and shown in the charts (**E**). The numbers above the histograms indicate the difference in the percentage of P^+ cells (thick histogram curve) and isotype IgG2b controls (thin histogram curve), while bars represent the median (25^{th}; 75^{th} percentiles) of 3-5 experiments performed in each group. The level of statistical significance is *P = 0.019.*

the median of 68% (56%; 69%) in four experiments performed. These cells contained $CD56^+$ cells in 23% in the shown histogram (Fig. 2E).

Effect of vWF^+ and $CD14^+$ cells on the maintenance of perforin in cultured T and NK cells

Fig. 3 shows the expression of P in $CD3^+CD56^-$ T cells after an 18- h coculture with vWF^+ cells, $CD14^+$ or $CD14^-$ cells, as well as in $CD3^-CD56^+$ NK cells after the coculture with vWF^+ cells in NSTEMI patients on day 1 after AMI before PCI. In the shown histogram sample, 9.72% of unstimulated T cells expressed P with mean fluorescence intensity, MFI 10.7 after an 18-h culture in medium only (Fig. 3A). It corresponds to a median of 8.96% (6.83%; 9.96%) in three experiments. Histograms in Fig. 3B illustrate P expression in T cells after coculture with vWF^+ cells at a ratio 1:2.5 (13.96%, MFI 17.3) and a ratio 1:5 (8.71%, MFI 8.9). The median percentage of P^+ T cells cocultured with vWF^+ cells did not significantly change, whereas MFI for P significantly increased in the T cells cocultured with vWF^+ cells at the ratio of 2.5:1 when compared to the ratio of 5:1 or T cells cultured in medium only (Fig. 3B).

Fig. 3C shows P regulation by $CD14^+$ cells. Histograms illustrate the percentage and MFI for P in T cells after the coculture with $CD14^+$ cells at ratio 1:2.5 (13.84%, MFI 24.9) and the ratio 1:5 (10.8%, MFI 12.5), when compared to isotype control. Bars proved that $CD14^+$ cells significantly increased the frequency of P^+ T cells at cell ratio 1:2.5 and MFI for P in both tested ratios compared to unstimulated T cells (Fig. 3C). P expression and MFI for P in T cells did not change after coculture with $CD14^-$ cells (Fig. 3D).

In Fig. 3E, histograms illustrate P expression in $CD3^-CD56^+$ NK cells after an18-h coculture with vWF^+ cells at cell ratio 5:1 (70.71%, MFI 9.3), when compared to NK cells cultured in medium only (36.36%, MFI 4.2). These differences in the percentage and the MFI were statistically significant, as shown in the chart (Fig. 3E**).**

DISCUSSION

We found a higher proportion of circulating vWF^+ cells in NSTEMI patients treated conservatively and before PCI implementation than in STEMI patients on day 1 after the AMI, which confirmed the greater inflammatory response in NSTEMI patients, as previously shown (8). The early peak of the circulating vWF^+ cells in NSTEMI patients appeared simultaneously as the largest increase in the frequency of $CD3^-CD56^+$ NK cells, their cytotoxic $CD56^{dim+}$ subset, P^+ NK cells, and P^+ T cells (8), suggesting the early interaction of circulating vWF^+ cells and lymphocytes. HLA-DR and CD86 expression in circulating vWF^+ cells on days 1 and 7 enable their antigen-presenting capacities to T cells, as shown for $CD14^+$ monocytes (9), while monocytes peak later in the circulation, after the NSTEMI and STEMI. In NSTEMI patients, early circulating vWF^+ cells with negligible CD14 expression increased MFI for P in autologous $CD3^+CD56^-$ T cells at 1:2.5 cell ratio in a direct contact after 18 h. Enriched $CD14^+$ cells more efficiently enhanced frequency and MFI for P on the first day after NSTEMI in a dose-dependent manner, due to the higher expression of CD80 and CD86 costimulatory molecules. However, vWF^+ cells enhanced P in $CD3^-CD56^+$ NK cells, possibly due to the sustained production of IL-15, a strong proinflammatory activator (5). Upregulation of cytotoxic mediator P may enable cytotoxic effectors to destroy circulating vWF^+ cells, as cytotoxic effector cells promote rupture of vulnerable atherosclerotic lesions which are covered with vWF^+ endothelial cells, involving NKG2D/MICA/B endothelial cell recognition mechanism (10). It matches the increased frequency of killed vWF^+ cells on day 1 and statistically marginally decreased number of circulating vWF^+ cells on day 7 in NSTEMI patients. A drop in circulating vWF^+ cells may temporarily reduce the systemic cellular immune response, which mirrors the subsequent decrease in NK cells, P^+ NK cells, and their P^+CD56^{dim+} subset on day 14 (8). However, circulating vWF^+ cell regrowth in NSTEMI patients treated conservatively on day 28, concomitantly with the enhanced degree of inflammation, is a characteristic of multivessel chronic coronary artery disease (11). In contrast, the frequency of vWF^+ cells in NSTEMI patients treated with PCI decreased gradually. Their dynamic resembles the dynamic in STEMI patients, where

primary PCI removed the culprit stenosis and the acute ischemic inflammation. However, an explicit increase in the count of circulating vWF^+ cells appeared on day 1, possibly due to PCI (12). It seems that circulatory vWF^+ cells fine-tune T and NK cell functions early after AMI. In order to reach a final conclusion, it is necessary to expand the research and try to remove the weaknesses of this investigation, for example, related to the purification of cellular subpopulations.

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