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Perforin-Mediated Cytotoxicity in non-ST Elevation Myocardial Infarction

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Abstract

The aim of this investigation was to examine the role of perforin (P)-mediated cytotoxicity in the dynamics of tissue damage in patients with non-ST-segment elevation myocardial infarction (NSTEMI) treated with anti-ischaemic drugs. We enrolled 48 patients with NSTEMI in this study [age, 71.5 years; 61.5/76 (median, 25th/75th percentiles)]. The percentage of total peripheral blood P⁺ lymphocytes was elevated owing to the increased frequency of P⁺ cells within natural killer (NK) subsets, T and NKT cells in patients on day 1 after NSTEMI when compared with healthy controls. Positive correlations were found between cardiac troponin I plasma concentrations and the frequency of P⁺ cells, P⁺ T cells, P⁺ NK cells and their CD56^{dim} and CD56^{bright} subsets during the first week after the NSTEMI. The expression of P in NK cells was accompanied by P-mediated cytotoxicity against K-562 targets at all days examined, except day 21, when an anti-perforin monoclonal antibody did not completely abolish the killing. The percentage of P⁺ T cells, P⁺ NKT cells and P⁺ NK subsets was the highest on the day 1 after NSTEMI and decreased in the post-infarction period. CD56⁺ lymphocytes were found in damaged myocardium, suggesting their tissue recruitment. In conclusion, patients with NSTEMI have a strong and prolonged P-mediated systemic inflammatory reaction, which may sustain autoaggressive reactions towards myocardial tissue during the development of myocardial infarction.

Introduction

Non-ST elevation myocardial infarction (NSTEMI) is manifested as acute coronary syndrome on the basis of the significant stenosis of one or more coronary artery branches by arteriosclerosis [1]. NSTEMI predominantly appears in individuals of advanced age, mostly women [1]. Patients with NSTEMI have significantly higher mortality rates after discharge than during hospitalization owing to target lesion re-occlusion and new myocardial infarction (MI) in the long term [2]. In patients with MI, a strong inflammatory process, initiated by the damaged endothelium [3], plays an important role in the pathogenesis of coronary artery disease [3–5]. The endothelium activates leucocytes in the blood stream and recruits them to the vessel wall [6] by a wide range of cytokines and chemokines that are upregulated on the endothelial surface or secreted in the blood stream [7–9]. Immune effectors mutually interact within tissue-specific conditions and can significantly damage the vascular endothelium

and smooth muscle cells, as was shown *in vitro* [10]. Particularly, CD4⁺CD28[−] T cells [11], which are able to perform lymphokine-activated killing after interleukin (IL)-12 cytokine stimulation [12], were found in the peripheral blood and in the plaques of patients with acute coronary syndrome. These cells are very effective at triggering the apoptotic death of vascular smooth muscle cells in acute MI [10], probably due to the expression of perforin (P) [13].

P is a primary inflammatory and cytotoxic mediator [14, 15]. It is potentially dangerous and is therefore stored in cytoplasm granules of immune effector T, natural killer T (NKT) and NK cells [16]. After stimulation, T and NK cells can kill target cells predominantly through the granule-mediated death pathway [15]. P induces quick target cell necrosis by pore formation, but it is also capable of inducing apoptosis in target cells at sublytic concentrations or by pore formation in concert with pro-apoptotic granule components, including granzymes, granzulin and the Fas ligand [14, 16].

P is an indispensable mediator of the immune inflammatory reaction. It provides protection against tumours, participates in the pathophysiology of allograft tissue rejection and autoimmune diseases [15] and controls trophoblast invasion during pregnancy [17]. The most important biological function of P is the elimination of cells infected with intracellular pathogens [15]. In a murine model, P⁺ cells were detected in myocardial tissue during the acute and chronic phases of *Trypanosoma cruzi* infection where it contributed to chronic cardiomyopathy [18]. An anti-P-neutralizing antibody reduced myocardial apoptosis during viral myocarditis in mice [19]. The possible contribution of P to the development of cardiac ischaemia injury and chronic heart failure, as a consequence of strong cell-mediated immune response in patients with acute MI [20], is still largely unknown.

Cardiac troponin I (cTn I) is sensitive dynamic biochemical marker of myocardial necrosis and represents cornerstone for the assessment and management of acute coronary syndrome [21]. It is not diagnostically specific for any single myocardial disease process [22]; however, cardiac inflammation and necrosis are associated with increased plasma level of cTn I. Therefore, cTn I is a useful parameter for identifying patients at risk of clinical deterioration [21, 22].

The aim of this study was to analyse the potential role of P-mediated cytotoxicity in the evolution of MI in patients with NSTEMI. We investigated the expression and frequency of P protein in peripheral blood lymphocyte (PBL) subpopulations, P-mediated cytotoxicity of peripheral blood NK cells and the correlation between the expression of P and the levels of cTn I in peripheral blood. The results suggest the involvement of the P-expressing lymphocytes in the pathogenesis of MI. These cells might be involved in the damage of vascular endothelium during development of MI and might be recruited to the myocardium where they could participate in the ischaemic tissue injury.

Materials and methods

Patients. We enrolled 48 patients with NSTEMI who underwent drug treatment with an average age of 71.5 years and an age range of 61.5/76 (median, 25th/75th percentiles). An early medical rehabilitation programme was undertaken by all patients at the Clinical Hospital 'Thalassotherapia Opatija', Opatija, Croatia. Venous peripheral blood samples (20 ml) were taken from the patients on days 1, 7, 14, 21 and 28 after the acute coronary event. The control group consisted of 35 sex- and age-matched healthy subjects who first underwent a clinical examination and routine laboratory analyses at the same hospital as the patients. The examination of healthy controls was a part of the preventive medical programme conducted by the local authorities. The

exclusion criteria for healthy controls and patients with NSTEMI were as follows: generative age in women, chronological age above 80 years for all subjects, unstable angina pectoris, uncontrolled arrhythmia, significant valvular deficiency, congestive heart failure, significant peripheral vascular diseases, uncontrolled metabolic diseases, uncontrolled hypertension (systolic blood pressure > 180 mmHg or diastolic > 100 mmHg), infectious and autoimmune diseases, organ injury and receipt of blood transfusions. The Ethics Committee of the Medical Faculty, University of Rijeka, approved this study according to the 'Ethical principles for medical research involving human subjects' declaration of Helsinki outlined by the World Medical Association. All examinees gave written consent for participation in this study.

Patient management. During the study, patients underwent an early cardiac rehabilitation programme. Standard laboratory examinations and a cycle ergospirometry test were administered to all patients. There were no differences in medication between the groups; all patients were taking a beta-blocking agent, ACE inhibitor, statin, acetylsalicylic acid and clopidogrel. During the programme, all patients were enrolled in regular physical training for 30 min, twice daily, at 60–75% of heart rate of VO₂ maximum during ergospirometry testing.

Evaluation of cardiac troponin I. cTn I was measured by the one-step enzyme immunoassay based on the 'sandwich' principle with two cTn I-specific monoclonal antibodies in the serum samples using analyser Dimension RXL (Siemens Healthcare, Diagnostics, New York, DE, USA).

Isolation of peripheral blood mononuclear cells. Blood samples were overlaid onto Lymphoprep (Nycomed Pharma, Oslo, Norway) for gradient density centrifugation (600 g, 20 min). Peripheral blood mononuclear cells (PBMC) were collected from the interface, washed and re-suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Auckland, New Zealand). For the cytotoxicity assay, the adherent cell population was eliminated by allowing them to adhere (45 min, 37 °C, 5% CO₂) to the bottom of a Petri dish (100 × 20 mm; TPP, Trasadingen, Switzerland), and non-adherent lymphocytes were collected.

Immunocytochemistry/Immunohistology. Freshly isolated PBMC (8.0×10^5 cells/ml) were centrifuged onto a glass microscope slide (100 ml, 170 g for 5 min). Cytospins were dried at room temperature (1 h) and fixed in cold acetone (Kemika, Zagreb, Croatia) for 10 min. Indirect immunoperoxidase staining was performed using the EnVision TM G/2 Doublestain System (Dako, Glostrup, Denmark) following the manufacturer's instructions. Briefly, after washing cytopins in Tris-buffered saline (TBS, 0.05 M Tris containing 0.3 M NaCl, pH 7.4) (Kemika, Zagreb, Croatia), endogenous peroxidase activity was blocked with 3% H₂O₂ (Kemika) and

non-specific binding was blocked with 0.1% bovine serum albumin (Sigma-Aldrich Chemie, Steinheim, Germany). Cytospins were incubated for 1 h with primary mouse IgG2b anti-human P (clone Gδ9; 1 µg in 100 µl in TBS) or mouse IgG2b (clone 27-35, undiluted) antibodies (both from the Department of Physiology and Immunology, School of Medicine, University of Rijeka, Croatia). Paraffin-embedded sections were labelled for CD56 using the same principle after the deparaffinization and rehydration of sections with Tissue Clear (3 × 5 min; Sakura Finetek Europe, Zoeterwoude, Netherlands) using decreasing concentrations of ethanol (100%, 96% and 75%; Kemika) and TBS. CD56 molecule retrieval was performed in 10 mM sodium citrate (pH 6.0). Primary mouse anti-CD56 mAb (clone MOC-1, diluted 1:100 in TBS) and isotype-matched mouse IgG1 (undiluted; both from Dako) were added for 1 h at room temperature. The samples were then incubated for 20 min with horseradish peroxidase-labelled polymer (included in the kit). The immunocyto/histology reaction was completed with incubation in the 3,3-diaminobenzidine substrate chromogen. The nuclei were stained with haematoxylin solution Gill No. 3 (Sigma), and the smears were mounted using Aquatex (MerckKGa, Darmstadt, Germany). Slides were analysed with Olympus B × 51 microscope using Olympus DP71 camera, both from Tokyo, Japan. Images were processed by Cell[^]F imaging software (Tokyo, Japan) and ADOBE PHOTOSHOP, version 7.0.1 CE (Adobe Systems Incorporated, San Jose, CA, USA).

Simultaneous detection of surface and intracellular antigens. Surface and intracellular antigens were simultaneously detected according to the previously described method [23]. Briefly, cells (3.0×10^5 cells/sample) were fixed and permeabilized using the Cytotfix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). Anti-P mAb or isotype-matched IgG2b were added to the cells ($3 \mu\text{g}/1.0 \times 10^6$ cells). After washing in fluorescence-activated cell sorter (FACS) buffer (140 mM NaCl, 1.9 mM KH_2PO_4 , 16.5 mM Na_2HPO_4 , 3.75 mM KCl (all from Kemika), 0.96 mM $\text{Na}_2\text{-EDTA}$ (Fluka, Buchs, Switzerland), 1.5 mM NaN_3 (Difco, Detroit, MI, USA)), secondary goat anti-mouse (GAM) polyclonal antibodies (IgG1, IgG2a, IgG2b and IgG3) conjugated with fluorescein isothiocyanate (FITC) were added. The cell membranes were restored in FACS buffer, and the cells were labelled with mouse anti-CD3 mAb (UCHT-1) conjugated with phycoerythrin-CyChrome 5 (PE-Cy 5) and anti-CD56 mAb (B159) conjugated with phycoerythrin (PE). APC- and PE-conjugated mouse IgG1 antibodies were used as controls.

In addition, triple surface antigen detection of PBMC using anti-CD3 PE-Cy5, anti-CD56 PE and anti-NKG2D plus FITC-conjugated GAM polyclonal antibodies was performed with respect to isotype-matched

controls. All the antibodies were provided by BD Biosciences, Erembodegen, Belgium, and used at $20 \mu\text{l}/1.0 \times 10^6$ cells for 30 min at 4 °C, unless otherwise indicated. Cells were analysed by FACSCalibur (Becton Dickinson) using CellQuestPro software (Becton Dickinson). P expression was analysed in the whole lymphocyte population, $\text{CD}3^-\text{CD}56^+$ NK cells, $\text{CD}3^+\text{CD}56^-$ T cells and $\text{CD}3^+\text{CD}56^+$ NKT cells. To determinate NK cell subsets ($\text{CD}56^{\text{dim}}$ and $\text{CD}56^{\text{bright}}$), the mean fluorescence intensity (MFI) was used to determine the expression of CD56. MFI is a measure of the average number of a particular molecule per single cell. The results are calculated as the difference between the percentage of P⁺ cells or the MFI for P obtained in samples labelled with the anti-P mAb minus the percentage or MFI obtained in the isotype-matched control.

Cytotoxicity assay. NK cell-mediated cytotoxicity was analysed against the NK-sensitive human erythroleukemia K-562 cell line, which does not express major histocompatibility complex (MHC) molecules (provided by Professor E.R. Podack, Department of Immunology and Microbiology, School of Medicine, University of Miami, FL, USA), as previously described [17]. Target K-562 cells were labelled with the PKH-26 lipophylic dye according to the manufacturer's instructions (PKH-26 Red Fluorescent Cell Linker Kit; Sigma Biosciences, St. Louis, MO, USA). The cells were then combined with PBL at effector/target cell ratios of 6:1, 12.5:1, 25:1 and 50:1. Samples of PBL or K-562 cells cultured only in the medium served as the controls. Samples were incubated for 4 h at 37 °C in a humidified atmosphere with 5% CO_2 . Before isolating the cells by FACSCalibur, propidium iodide (PI; Sigma-Aldrich Chemie, Munich, Germany) was added at a final concentration of 5 µg/ml. Some PBL samples were pretreated with 10 µg of anti-P mAb. Killed cells were detected and enumerated as double-labelled PKH-26 (orange, fluorescence FL2 and PI red, FL3)-positive cells. The results are expressed as the difference in the percentage of killed K562 cells at a particular effector/target cell ratio minus the percentage of killed K562 cells cultured only in the medium.

Statistical analysis. Statistical analysis was performed with the STATISTICA 8.0 data analysis software system (StatSoft, Inc., Tulsa, OK, USA). The difference between groups was calculated with the Kruskal–Wallis non-parametric test, and the difference was considered significant at $P < 0.05$. The Mann–Whitney *U*-test was used to establish among which groups the difference existed, with the level of significance adjusted to the number of mutual comparisons. Data are presented as median values and as 25%/75% (25th percentile/75th percentiles). The Spearman rank correlation coefficient was performed to correlate P-expressing cells with cTn I concentrations, and the results are expressed as *r* and *P* values.

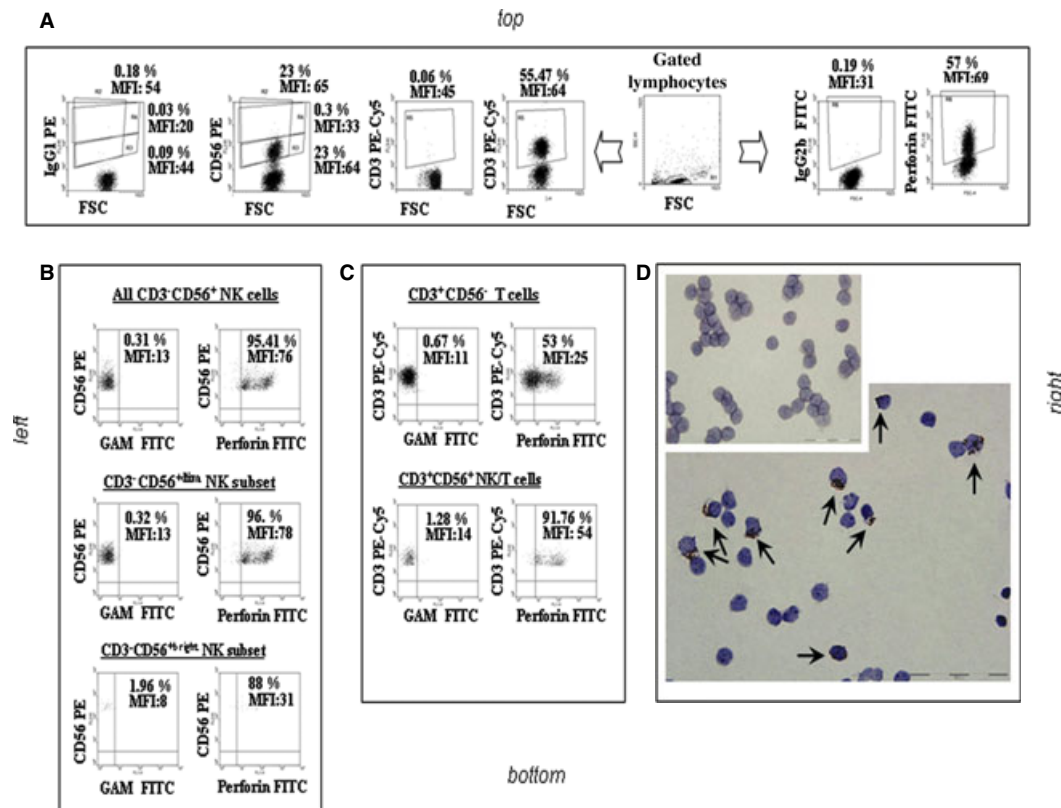


Figure 1 A sample of flow cytometer analysis of perforin (P) expression in peripheral blood lymphocytes (PBLs) in patient after acute coronary event on day 1. Dot plots show PBLs comprised within gate R1 and their gating owing to surface antigens expression as follows: CD56⁺ events – R2, CD56^{dim} events – R3, CD56^{bright} events – R4, CD3⁺ events – R5 and P⁺ events – R6 in respect to their isotype-matched controls (A). Dot plots illustrate P expression in all NK cells and their subsets (B), in T and NKT cells (C) corresponding to isotype-matched controls. Numbers show percentages and mean fluorescent intensity for the marker of interest. Perforin protein is visualized in the same sample by immunocytochemistry as 3, 3'-diaminobenzidine-labelled granules indicated by the arrows (D). Magnification is obtained with objective lens Olympus UPlan Apo, 100 \times /1.35 and Olympus immersion oil for microscopy ordinary use, nd 1.516 (both from Tokyo, Japan). Twelve experiments were performed.

Results

Perforin expression in peripheral blood lymphocytes

A sample of flow cytometry analysis of P expression in gated lymphocyte subpopulations of PBL on day 1 after an acute coronary event in patients with NSTEMI is shown in Fig. 1. Lymphocytes are gated by size (forward scatter or FSC) and complexity of cellular structure (side scatter or SSC) in gate region (R) 1 and then further gated (Fig. 1A). Using the combination of gates R1 and R2, R1 and R3, or R1 and R4, with the exclusion of CD3⁺ events, CD3⁺CD56⁺ NK cells, the CD3⁺CD56^{dim} subset or the CD3⁺CD56^{bright} subset was detected (Fig. 1B). The frequency of P-expressing cells in each NK cell population was ~90% (Fig. 1B). Perforin expression in T cells gated within R1 and R5, but not in the R2 gate, was 53% (Fig. 1C). The frequency of P-expressing NKT cells, belonging to the R1 and R2 and R5 gates, was 91.76% (Fig. 1C). Immuno-

cytochemistry revealed the storage of P protein within large and small intracytoplasmic granules in PBMC from patients on day 1 after an acute coronary event (Fig. 1D, indicated by the arrows).

In patients with NSTEMI, there were a significantly higher percentage of P⁺ lymphocytes (Fig. 2A) on days 1 and 7 after the acute coronary event than in healthy controls and samples from day 28, when the percentage of P⁺ cells was at the same level as controls. The MFI for P (Fig. 2A) was significantly higher on day 7 after the MI when compared with healthy controls or with values found on days 21 and 28 (Fig. 2A).

The frequency of P⁺ NK cells was significantly elevated on days 1, 7 and 21 after the acute coronary event (Fig. 2B). Very similar patterns of P fluctuations were found in the percentages of the P⁺CD56^{dim} and P⁺CD56^{bright} NK subsets (Fig. 2C or 2D, respectively). The percentage of P⁺ T cells was increased on day 1 and decreased significantly thereafter (Fig. 2E). A similar pattern was observed in P⁺ NKT cells (Fig. 2F).

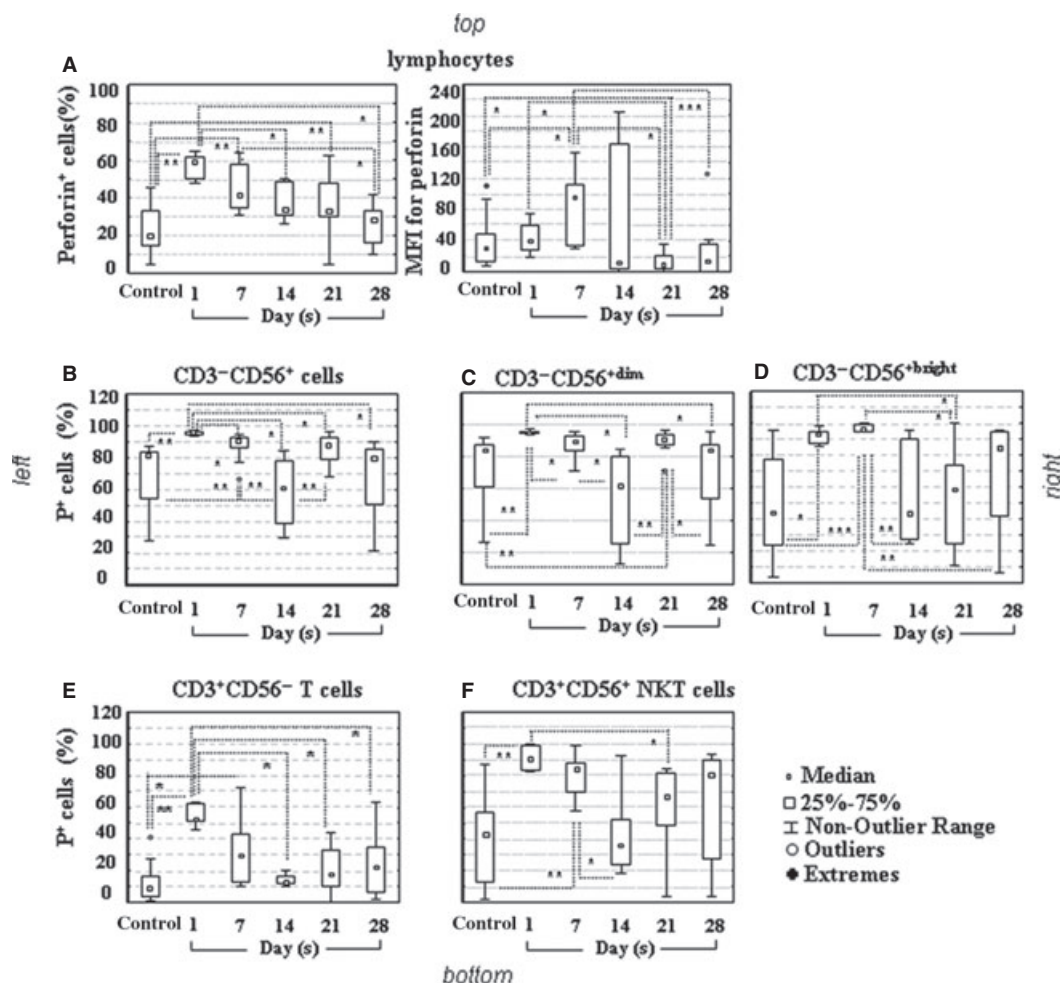


Figure 2 The dynamics of perforin (P) expression and mean fluorescence intensity for P in the peripheral blood lymphocytes in non-ST-segment elevation myocardial infarction. The percentages of all P⁺ lymphocytes (A), the frequencies of P⁺ NK cells (B), CD56^{dim} (C) and CD56^{bright} (D) NK subsets, CD3⁺CD56⁻ T cells (E) and CD3⁺CD56⁺ NKT cells (F) are shown at indicated time points after the onset of acute coronary event. Twelve experiments were performed. Levels of statistical significance: * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.

Changes in NK cell-mediated cytotoxicity and NKG2D expression

In healthy controls, NK cells from freshly isolated PBL spontaneously killed 10–30% of the NK-sensitive K-562 cell targets in the 4-h cytotoxicity assay, depending on the effector/target cell ratio (Fig. 3A). On day 21, after the acute coronary event, the cytotoxicity level was the highest and significantly higher when compared with days 7 ($P = 0.0001$) and 28 ($P = 0.0001$) or to healthy controls ($P = 0.001$) at an effector/target ratio of 50:1 (Fig. 3A). The anti-P mAb completely abrogated the killing of the K562 targets by NK cells from healthy controls or patients with NSTEMI at all time points investigated, except for day 21 (Fig. 3A). The expression of NKG2D on the surface of peripheral blood NK cells in patients with NSTEMI was always high and above the levels observed for the isotype-matched control (Fig. 3B). NKG2D expression did

not differ significantly from the levels observed for NK cells from healthy controls, which was 90.31, 84.08/95.45 (median, 25th/75th percentiles) (Fig. 3B).

Correlation between cardiac troponin I and the frequency of perforin-expressing lymphocytes in peripheral blood

In patients with NSTEMI, the plasma concentration of cTn I was significantly higher than in healthy controls (Fig. 4A). The concentration of cTn I on day 1 after coronary artery thrombosis was significantly higher from those at the admission (time point '0') and on day 3 (Fig. 4A).

The frequency of T cells significantly decreased on days 7 and 14 and was restored on day 28 after the acute coronary event when compared with healthy controls (Fig. 4B). NKT cells were significantly increased on days 1 and 14 when compared with healthy controls and with

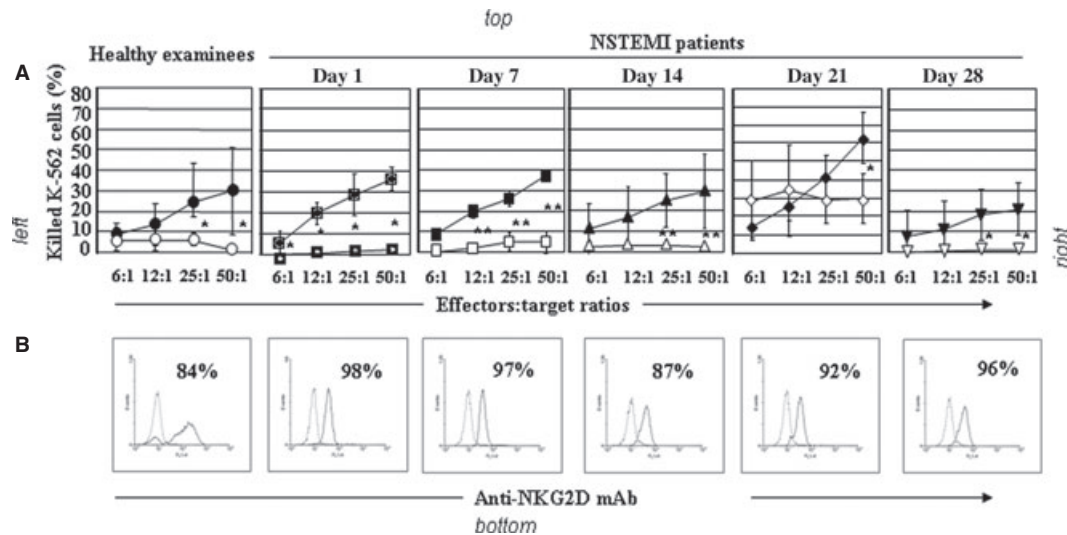


Figure 3 The short-term (4 h) cytotoxicity of peripheral blood NK cells and their NKG2D receptor expression. The cytotoxicity of peripheral blood NK cells from healthy controls (●) and patients with non-ST-segment elevation myocardial infarction (NSTEMI) against K-562 cell line are measured on days 1 (□), 7 (■), 14 (▲), 21 (◆) and 28 (▼) after acute coronary event at indicated effector/target ratios (A). Some samples were treated with anti-P Gd9 mAb on days 1 (□), 7 (■), 14 (▲), 21 (◆) and 28 (▼) or healthy control (○) (A). Solid histogram curves show labelling of peripheral blood NK cells with anti-NKG2D mAb in comparison with the isotype-matched (IgG2a) control (dashed curves, B). Five to eight experiments were performed in each group. Levels of statistical significance: * $P < 0.01$, ** $P < 0.001$.

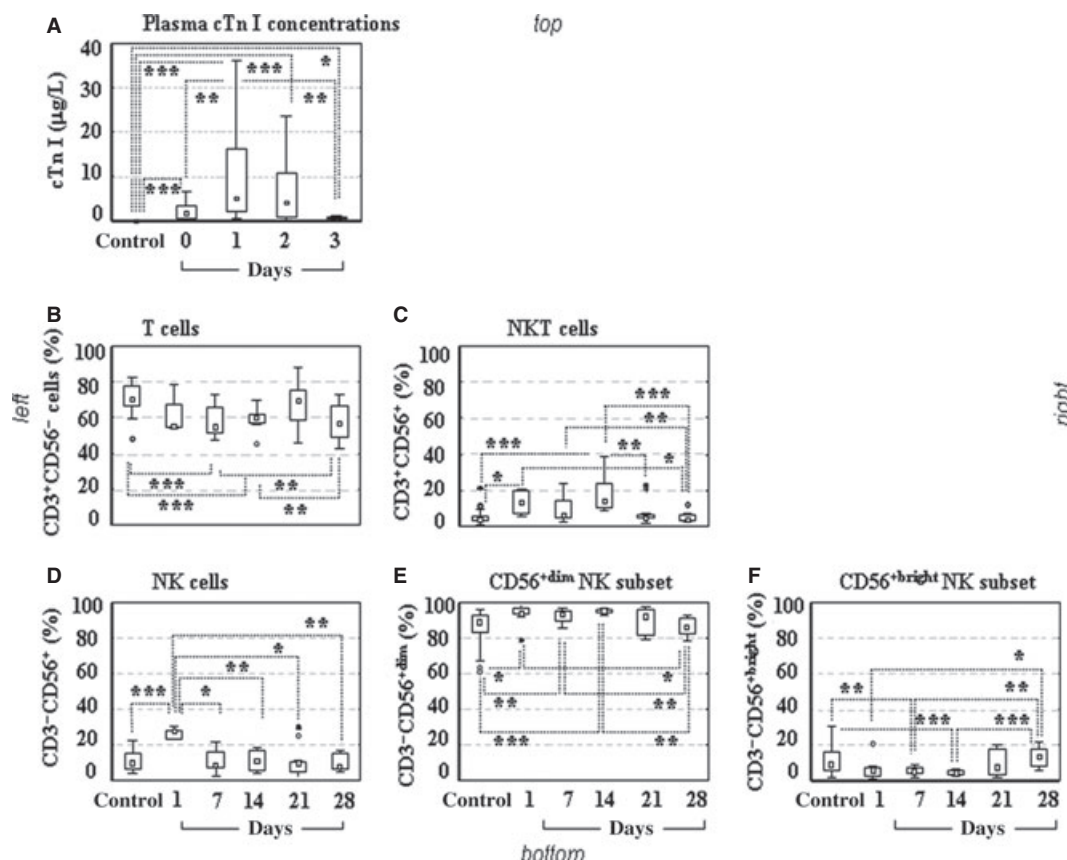


Figure 4 The dynamics of cardiac troponin I (cTn I) concentrations and the frequencies of lymphocyte subpopulations in peripheral blood of patient with non-ST-segment elevation myocardial infarction (NSTEMI). Plasma concentrations of cTn I (A) and frequency of lymphocyte subpopulations including T cells (B), NKT cells (C), NK cells (D), CD56^{dim} NK subset (E) and CD56^{bright} NK subset (F) at indicated time points after the onset of acute coronary event. Twelve experiments were performed. Levels of statistical significance: * $P < 0.01$, ** $P = 0.001$, *** $P = 0.0001$.

those on day 28, while their frequency returned to the same levels as control on day 21 (Fig. 4C). On day 1, the percentage of NK cells was higher than at the other time points (Fig. 4D). The proportion of $CD56^{+dim}$ NK subset significantly increased (Fig. 4E) and the proportion of $CD56^{+bright}$ NK subset significantly decreased (Fig. 4F) on days 1, 7 and 14 after the acute coronary event when compared with healthy controls. Significant positive correlations were found between cTn I concentrations and the frequency of P^{+} cells in all PBL and in $CD3^{+}CD56^{-}$ T cells on day 1, in $CD3^{-}CD56^{+}$ NK cells and their $CD56^{+dim}$ and $CD56^{+bright}$ subsets on day 4 and in the $CD56^{+bright}$ NK subset on day 7 (Fig. 5).

Presence of NK cells in the myocardium

$CD56^{+}$ cells (Fig. 6A, indicated by the arrows) were found within the lymphocyte infiltrate of damaged myocardial fibres in the paraffin-embedded myocardial tissue sections from patients who died in the 1st week after the cardiovascular event when compared with the isotype-matched mouse IgG1 control (Fig. 6B). There was no lymphocyte infiltration with $CD56^{+}$ cells in the paraffin tissue sections from patients who died in the 5th week after the acute coronary event (Fig. 6C) or from non-myocardial reasons (Fig. 6E) when compared with isotype-matched controls (Fig. 6D or 6F, respectively).

Discussion

On the basis of the ischaemic heart disease is the atherosclerotic process, which begins as an endothelial dysfunction very early during lifetime and represents chronic, progressive, pro-inflammatory and potentially dangerous process [3–5]. The intensity of this inflammatory reaction reaches the peak rapidly after an acute coronary event, simultaneously with the myocardial necrosis [24]. The leaking of various cytokines from the damaged and/or necrotic cells [25–27] activates innate and adaptive cell-mediated immune response including cytotoxic mediators' expression leading to potentially harmful immune reactions directed towards the endothelium [28] and smooth muscle cells of cardiovascular system [10]. It has been shown that activated T cells enter into the plaque and destabilize the lesion [29]. Coronary artery plaque rupture, mediated by T cells, leads to local thrombosis, myocardial ischaemia and the infarction of the irrigation area. Myocardial ischaemia is the basis of strong local and systemic inflammatory response. Activated peripheral blood T cells accumulate in the myocardium after an acute coronary event and damage the peri-ischaemic myocardial tissue [20]. NKT cells, expressing CD3 and CD56 surface molecules, contribute to cardiovascular impairment owing to their activation after the recognition of glycolipid antigens [10].

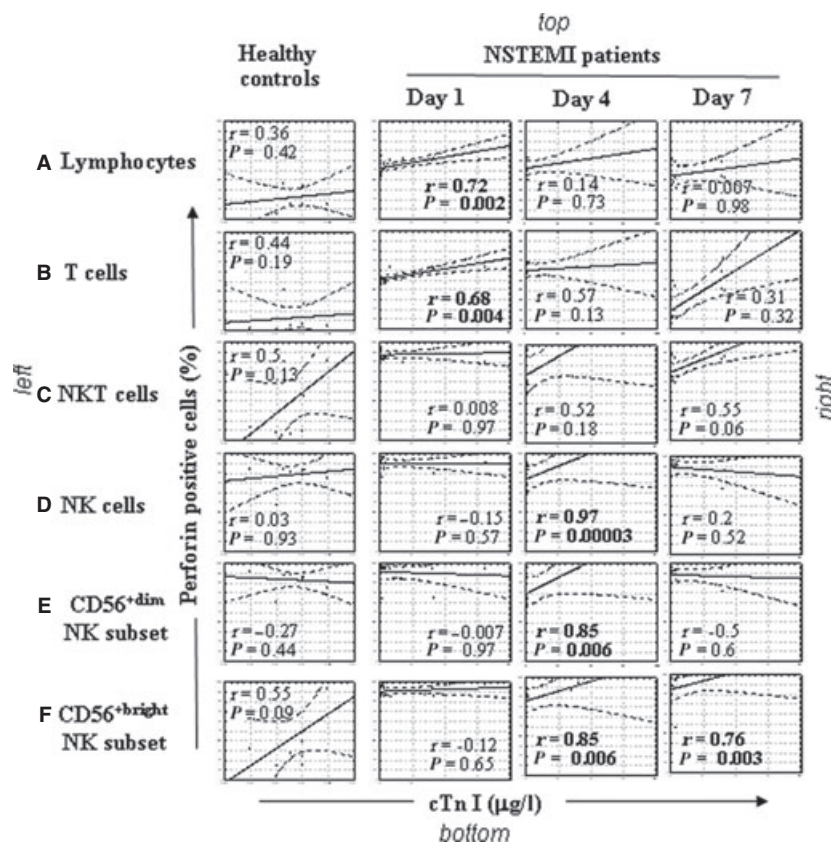


Figure 5 Correlation between cardiac troponin I (cTn I)- and perforin (P)-expressing lymphocytes in peripheral blood of patient with non-ST-segment elevation myocardial infarction (NSTEMI). cTn I was correlated with the percentage of all P^{+} lymphocytes (A), frequency of P-expressing $CD3^{+}CD56^{-}$ T cells (B), P-expressing $CD3^{+}CD56^{+}$ NKT cells (C), P-expressing $CD3^{-}CD56^{+}$ NK cells (D), P-expressing $CD56^{+dim}$ NK subset (E) and P-expressing $CD56^{+bright}$ NK subset (F) in healthy controls and patients with NSTEMI are shown at indicated time points after the onset of the chest pain. Twelve to 15 experiments were performed for patients with NSTEMI in each group and 12 for healthy controls. Correlation coefficient (r) and level of statistical significances (P) are indicated in the charts.

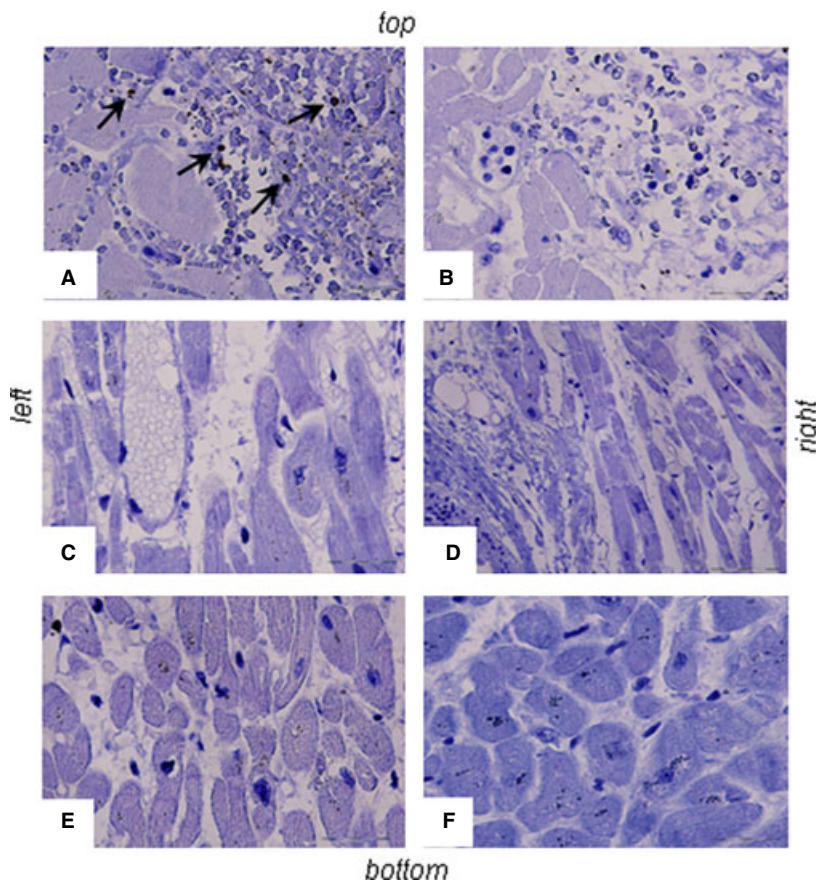


Figure 6 The presence of CD56⁺ cells in myocardial infarction. The immunohistology of paraffin-embedded myocardial tissue sections from patient who died in the first week (A, B) and in the fifth week (C, D) after the acute coronary event, as well as from patient who died from non-cardiac reasons (E, F) using mouse anti-CD56 mAb (A, C and E) or isotype-matched mouse IgG1 (B, D and F) and immunoperoxidase staining method is shown. The CD56⁺ cells appeared as brown-labelled cells by 3,3-diaminobenzidine (DAB) (indicated by the arrows). Magnification is obtained with objective lens Olympus UPlan Apo, 100 \times /1.35 and Olympus immersion oil for microscopy ordinary use, nd 1.516 (both from Tokyo, Japan). Four labelling were performed in each group.

Little is known about the role of NK cells during an acute coronary event. Szodoray *et al.* [30] claimed an elevated percentage of peripheral blood NK cells in a group of patients with acute coronary syndrome, as well as we did found on day 1. Klurlund *et al.* [31] showed that the number of peripheral blood NK cells, identified simply as large granular lymphocytes, was not reduced in patients with acute ST-segment elevation MI on days 1, 3 and 14 and at 6 weeks after an acute coronary event. The peripheral blood NK cells from these patients were considered to be functionally defective on the basis of K562 cell killing [31, 32], although this observation cannot be completely equated with the NK cytotoxic reactions against autologous cells *in vivo*. The cytotoxicity of NK cells is regulated by the precise, dynamic activation-dependent expression of NK cell receptors that bind to their ligands, which mostly belong to MHC class I or MHC class I-related molecules [33]. The absence of MHC class I molecules on target cells provides NK cells with the signal for killing [33]. A lethal hit for targets could also be provided by NKG2D receptor signalling after ligation with MHC class I-related chain A/B (MICA/B) [34]. We demonstrated that almost all NK cells from patients with NSTEMI express the P and the NKG2D receptor, implying the presence of cytotoxicity directed towards

activated MICA/B⁺ endothelial cells during MI [35]. Particularly, the cytotoxic CD56^{dim} NK subset may participate in vascular tissue damage early at the time of MI owing to the expansion and the stimulation from pro-inflammatory myocardial and endothelial soluble factors. Fractalkine [7], IL-12 [12] and IL-15 [13] are found at increased concentrations in the peripheral blood of patients with coronary syndrome, and these molecules may support NK cell proliferation and upregulate the expression of P and pro-apoptotic molecules at the mRNA and protein levels in NK cells, thereby inducing lymphokine-activated killing, as shown in different pathophysiological processes [17, 36, 37]. Besides the cytotoxic CD56^{dim} NK subset, the CD56^{bright} NK subset, with low cytotoxicity, considerable cytokine production and the capacity to modulate immune reactions [38], becomes effective killers after their activation with pro-inflammatory mediators. IL-2, IL-12, IL-15 and/or IL-18 can stimulate even the normally suppressed, early pregnancy decidual, P-expressing CD56^{bright} NK subset, which become very effective at killing NK-sensitive and NK-resistant targets *in vitro* [39] and *in vivo* during spontaneous, recurrent abortions [36]. IL-15, expressed on endothelial cells [9], as well as in myocardium after ischaemic injury (our unpublished observation), is the most important early proliferation and differentiation

factor for NK cells [39]. IL-15 is also able to chemoattract NK cells and stimulate their adhesion to vascular endothelium [9]. Accordingly, IL-15 could be one of the factors governing NK cell recruitment in the myocardium, which we found in the ischaemic region in the person who died in the first week after an acute coronary event (Fig. 6). The possible role of local NK cells in ischaemic myocardial injury during lifetime is insufficiently investigated, owing to the ethical reasons in the tissue collection.

P plays an important role in atherogenesis [13]; however, its role in the pathogenesis of acute MI is still far for clear. We detected a threefold increase in the frequency of P⁺ lymphocytes within the 1st week after an acute coronary event, owing to the increased expression of P in all lymphocyte subpopulations. This increase indicates the potential significance of P-mediated cytotoxicity in the tissue damage following an acute coronary event. The highest level of NK cell cytotoxicity, as measured by an *in vitro* test of their ability to kill the K-562 NK-sensitive cell line, was obtained on day 21 after the acute coronary event. The addition of an anti-P mAb to the test system completely blocked this cytotoxicity at all days post-MI, except for day 21, suggesting an additional enhancement of the inflammatory reaction when the other cytotoxic/apoptotic molecules are involved in cell death and tissue damage. Candidates for this induction of cell death include granzyme B, which is elevated in the plasma of patients with MI [40], or FasL, which is expressed on the surface of lymphocytes [41], whose actions are redundant to the P-mechanism. The P released from the cytotoxic granules of activated immune effectors could participate in cardiomyocyte death, because it facilitates the access of ions and pro-apoptotic molecules to target cells in a pore-dependent [42] and pore-independent [14] manner.

Plasma cTn I level reflects myocardial necrosis, and it was significantly higher in patients with MI with larger tissue damage [43]. Oxygen supply/demand mismatch, the direct effect of pro-inflammatory cytokines and microvascular thrombus set cardiac inflammation and an increase in cTn I [21]. Therefore, it could be worth to pay attention to the expression of primary, cytotoxic and inflammatory mediator P in lymphocyte subpopulations in terms of increased cTn I in peripheral blood of patients with MI. cTn I shows dynamic fluctuation in the first days after MI and assessed at 72 h from symptoms onset and shows the best correlation with the size of necrotic region [43]. The most suitable period for the examination of P protein expression is also during the first week. We found indeed a positive correlation between cTn I and P-expressing T cells on day 1, whereas level of cTn I positively correlated with P-expressing NK cells and their CD56^{dim} and CD56^{bright} subsets on day 4 and/or day 7.

In conclusion, patients with NSTEMI have a strong and prolonged cell-mediated systemic inflammatory reaction that may sustain autoaggressive reactions towards myocardial tissue during the development of MI by a perforin-mediated mechanism. Because the positive correlation between cTn I plasma levels and P expression in lymphocyte subpopulations exists, perforin could be an indirect biochemical parameter for the estimation of the degree of myocardial ischaemic injury.

Disclosure

The authors declare that they do not have any conflict of interest.

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Author contributions

G. Laskarin and V. Persic contribute equally to this work. G. Laskarin, theoretical background, statistical analysis, discussion of the scientific results, writing of the manuscript; V. Persic, data analysis, discussion of the scientific results, writing of the manuscript; A. Ruzic, discussion of the scientific results and theoretical background; B. Miletic, data collection, discussion of the scientific results; M. Rakic and D. T. Samsa, experimental work, data analysis; D. Raljevic, experimental work, figure editing; V. P. Pejcinovic, data collection, discussion of the scientific results; R. Miskulin, data collection; D. Rukavina, theoretical background, discussion of the scientific results, writing of the manuscript.

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