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Granulysin Expression in Lymphocytes that Populate the Peripheral Blood and the Myocardium after an Acute Coronary Event

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Abstract

We aimed to analyse granulysin (GNLY)-mediated cytotoxicity in the peripheral blood of patients with non-ST-segment elevation myocardial infarction (NSTEMI) treated with anti-ischaemic drug therapy. Thirty-nine NSTEMI patients with a median age of 70 years and 28 age-matched healthy subjects were enrolled in this study. On day 7 after MI, the number of $GNLY^+$ lymphocytes in the peripheral blood increased approximately six-fold of that in the healthy subjects, measured by flow cytometry. On day 14, the number of $GNLY^+$ cells significantly decreased in T, NKT, and both $CD56^{+dim}$ and $CD56^{+bright}$ NK subsets. $GNLY^+$ $CD3^+$ and $GNLY^+$ $CD56^+$ cells infiltrated central zone of myocardial infarction (MI). In persons who died in the first week after MI, $GNLY^+$ cells were found within accumulation of apoptotic leucocytes and reached the apoptotic cardiomyocytes in border MI zones probably due to the influence of interleukin-15 in peri-necrotic cardiomyocytes, as it is was shown by immunohistology. By day 28, the percentage of $GNLY^+$ lymphocytes in peripheral blood returned to the levels similar to that of the healthy subjects. Anti-GNLY mAb decreased apoptosis of K562 targets using peripheral blood NK cells from days 7 and 28 after MI, while in assays using cells from days 1 and 21, both anti-GNLY and anti-perforin mAbs were required to significantly decrease apoptosis. Using NK cells from day 14, K562 apoptosis was nearly absent. In conclusion, it seems that $GNLY^+$ lymphocytes, probably attracted by IL-15, not only participate partially in myocardial cell apoptosis, but also hasten resolution of cardiac leucocyte infiltration in patients with NSTEMI.

Introduction

Plaque rupture, mediated by infiltrated immune effectors and superimposed thrombosis in the coronary artery, disrupts the blood supply to the myocardial tissue causing ischaemic myocardial inflammation and cardiomyocyte necrosis [1]. Additionally, apoptotic cardiomyocytes appear at the site of infarction and remote infarction regions [2, 3]. Both apoptosis and necrosis indicate the involvement of accumulated leucocytes and strong cell-mediated immune response in the course of ischaemic inflammation. Interleukin (IL)-1, CXCL8, CCL2, CCL3 and CCL4 are all up-regulated in infracted myocardium, and they facilitate leucocyte recruitment including neutrophils and/or mononuclear cells [4–6]. The recruited neutrophils have potent cytotoxic effects through the release of proteolytic enzymes and enhance the degree of myocardial damage [5, 7]. The accumulation of monocytes denotes the later phase of myocardial infarction (MI; 3–5 months) when the final removal of necrotic cardiomyocytes and apoptotic neutrophils is required [8]. Lymphocyte infiltration is attributed to MI in patients who die suddenly, shortly (4 weeks) or even late (4 months) after coronary thrombosis [2]. In particular, activated $CD3^+$ lymphocytes were found in peri-infarction and in remote infarction regions [2]. This confirms the local inflammatory status, as well as clones of $CD4^+$ $CD28^-$ T cells [9] with cytotoxic activity, resembling that of the NK cells [10] was found in the peripheral blood and plaque of patients with acute coronary

syndrome. Interleukin-15 is an effective chemoattractant for resting and activated NK cells [11]. It augments the binding of NK cells to endothelial cells [11] and controls the cytokine production and cytotoxic potential of NK cells [12], including regulating mRNA expression of perforin and Fas ligand [13] and granulysin (GNLY) [14]. In acute MI, lymphocytes are very effective for triggering the apoptotic death of vascular smooth muscle cells [15] because of the action of perforin protein [16] in concert with pro-apoptotic mediators [17]. This is a critical mechanism for the elimination of one's own injured cells, which directs the targets to an apoptotic rather than necrotic cell death [18].

Granulysin is a member of the family of saposin-like lipid binding proteins [19] with pro-apoptotic features that is expressed in activated T, NK [19] and NKT [20] cells. Mature GNLY (9 kDa) uses multiple mechanisms for target cell killing [19]. It shares the exocytose pathway with perforin [18]. Rapid influx of GNLY into cells through perforin pores causes the release of mitochondrial pro-apoptotic mediators, including apoptosis-inducing factors and cytochrome C, which are able to induce DNA fragmentation in both a caspase-independent and a caspase-dependent manner [21]. GNLY-mediated ceramide generation in the target cell membrane is a slow mechanism that induces chromatin breakdown [22], likely without involving perforin activation [17, 21]. GNLY localizes lysosomal cathepsin B in the cytoplasm of malignant cells, which causes cytochrome c and apoptosis-activating factor release from the mitochondria [21, 23]. The multiple pathways used by GNLY to enter target cells are indicative of its broad cytotoxic activity. Serum GNLY levels reflect the status of cell-mediated immunity in patients with viral and specific infections and cancers, organ transplanted patients and pregnant women with preeclampsia [19]. GNLY was found to cause apoptosis in polymyositis [24], and therefore, it could be worthwhile to investigate GNLY-expressing lymphocytes and their involvement in the pathogenesis of myocardial inflammatory processes such as coronary artery disease within the development of MI, as a leading manifestation of atherosclerosis [25].

The aim of this study was to analyse GNLY protein expression, changes in lymphocyte subpopulations and long-term (18-h) GNLY-mediated NK cytotoxicity against K562 cells *in vitro* in peripheral blood samples from patients with non-ST-segment elevation myocardial infarction (NSTEMI) during the first month after an acute coronary event. The presence and nesting of GNLY-expressing lymphocytes regarding apoptotic cardiomyocytes were investigated. The expression of major histocompatibility complex (MHC) class I molecules and interleukin-15 in the myocardial tissue of persons who died after MI was also analysed. The major results suggested that the prolonged inflammatory reaction that occurs during the development of NSTEMI treated with anti-ischaemic drugs is sustained with GNLY.

Materials and methods

Clinical and laboratory characteristic of patients enrolled in the study. The study included 39 patients with NSTEMI treated conservatively with a median age of 70 years (60/75, 25th/75th percentiles). The group consisted of 20 men and nine women. The control group consisted of 28 sex- and age-matched healthy subjects who underwent clinical examination and routine laboratory analyses at the same hospital as a part of the preventive medical programme conducted by the local authorities. All patients underwent regular physical training for 30 min twice daily at 60–75% of maximum heart rate of VO_2 at the ergospirometry test. All patients with NSTEMI received a beta-blocking agent, an ACE inhibitor, a statin and acetylsalicylic acid. 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, significant valvular deficiency, congestive heart failure, significant peripheral vascular disease, uncontrolled metabolic disease, uncontrolled hypertension (systolic blood pressure >180 mmHg or diastolic >100 mmHg), infectious and autoimmune disease, injury of organs and blood transfusions. This was determined by anamnesis, hospital documentation of the patients and routine laboratory examination during the rehabilitation period. The Ethics Committee of the Clinical Hospital Thalassotherapia Opatija, Opatija, Croatia, and the medical faculty at the University of Rijeka, Rijeka, Croatia, 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.

Isolation of peripheral blood mononuclear cells. Venous peripheral blood samples (20 ml) were obtained from healthy subjects and patients with NSTEMI on days 1, 7, 14, 21 and 28 after an acute coronary event. Peripheral blood mononuclear cells were isolated using Lymphoprep (Nycomed Pharma, Oslo, Norway), subjected to gradient density centrifugation (600 ***g***, 20 min) and re-suspended in Roswell Park Memorial Institute 1640 medium (Invitrogen, Auckland, New Zealand). For cytotoxicity assays, monocytes and B cells were eliminated by allowing them to adhere to the bottom of a Petri dish (100 × 20 mm; TPP, Trasadingen, Switzerland) for 45 min at 37 °C in 5% CO_2, and non-adherent lymphocytes were collected.

Surface and intracellular antigen detection. The simultaneous detection of surface and intracellular antigens was performed in fixed and permeabilized peripheral blood mononuclear cells (3×10^5/sample) according to the method described previously [26]. All antibodies were

provided by BD Biosciences (Erembodegen, Belgium), and 20 $\mu l/10^6$ cells were used and incubated at 4 °C for 30 min unless otherwise specified.

Mouse anti-GNLY monoclonal antibody (mAb) (RC8, 0.35 μg/sample; MBL International, Woburn, MA, USA) or isotype-matched IgG1 (MOPC-21) was added to the cells. After washing, fluorescein isothiocyanate (FITC)-conjugated secondary goat anti-mouse polyclonal antibodies (IgG1, IgG2a, IgG2b and IgG3) were added to the permeabilized cells (2 μg/sample). Cell membrane integrity was restored by incubation in phosphate-buffered saline (PBS; 33.9 mM $NaHPO_4 \times 12H_2O$, 136.8 mM NaCl, 3 mM KH_2PO_4 in distilled water; Kemika, Zagreb, Croatia) for 5 min. Then, the cells were labelled with mouse anti-CD3 mAb (UCHT-1) conjugated with phycoerythrin cytochrome 5 (PE-Cy5) and anti-CD56 mAb (B159) conjugated with phycoerythrin (PE). Mouse IgG1 antibodies conjugated with PE and PE-Cy5 were used as the controls. K562 cells were indirectly labelled with a mouse IgG1 mAb (W6/32), which recognizes all MHC class I molecules (undiluted supernatant, 100 $\mu g/10^5$ cells; Department of Physiology and Immunology, Medical Faculty, University of Rijeka, Croatia) and was calculated with respect to the IgG1 isotype-matched control. Cells were analysed using a FACSCalibur™ (Becton Dickinson, St Hose, CA, USA) with CellQuestPro software (Becton Dickinson). GNLY protein expression was analysed in the entire lymphocyte population, $CD3^-$ $CD56^+$ NK cells, $CD3^+$ $CD56^-$ T cells, and $CD3^+$ $CD56^+$ NKT cells. To determine the $CD56^{+dim}$ and $CD56^{+bright}$ NK cell subsets, the mean fluorescence intensity (MFI) of CD56 molecule expression was used. Generally, MFI indicates the average number of a particular molecule per cell. The results were calculated as the difference between the percentages of $GNLY^+$ cells, or MFI of GNLY observed in the sample labelled with anti-GNLY mAb minus the percentage or MFI observed in the isotype-matched control.

Immunocytochemistry and histology. Peripheral blood mononuclear cell samples (cytospins) from MI patients and paraffin-embedded myocardial tissue sections (3 μm) from persons who died in the first week or the fifth week after acute MI were stained for GNLY, CD3, CD56 and interleukin-15 using the EnVision™ G|2 Doublestain System (DAKO Corporation, Carpenteria, CA, USA) following the manufacturer's protocol for indirect immunoperoxidase and/or alkaline phosphatase staining. Cytospins from healthy examinees and tissue sections from persons who died from non-myocardial causes were used as controls. Cytospins were fixed in cold acetone, rehydrated in Tris-buffered saline (TBS; 0.05 M Tris, 0.3 M NaCl; Kemika) and 0.1% Tween 20 (Sigma-Aldrich Chemie, München, Germany), pH 7.4. Paraffin-embedded sections were deparaffinized in Tissue Clear (Sakura Finetek Europe, Zoeterwoude, the Netherlands) three times for 5 min each and rehydrated in decreasing concentrations of ethanol (100%, 96% and 75%; Kemika) and TBS prior to staining. Antigens were retrieved using 10 mM sodium citrate, pH 6.0, and the sections were washed in TBS. After blocking endogenous peroxidase and non-specific binding using the component included in the kit, primary mouse anti-CD56 mAb (clone MOC-1, 1 : 100 dilution), rabbit polyclonal anti-CD3 Ab (undiluted), isotype-matched mouse IgG1 (undiluted) or rabbit polyclonal antibody (undiluted) (all from DAKO) were incubated with the sections for 1 h at room temperature, followed by incubation with labelled polymer horseradish peroxidase for 20 min. The reactions were completed with a 4-min incubation in 3,3-diaminobenzidine substrate-chromogen. After washing and double stain blocking (included in the kit), anti-GNLY mAb (1:300 dilution), rabbit polyclonal anti-apoptotic protease activating factor (APAF)-1 (dilution 1:50; Sigma-Aldrich, St Luis, MO, USA), rabbit polyclonal antibodies (undiluted) or mouse IgG1 (undiluted) were applied for 1 h at room temperature. This was followed by incubation with labelled polymer alkaline phosphatase (included in the kit) and a 10-min incubation with Permanent red substrate-chromogen. In some samples, APAF-1 and GNLY were labelled using peroxidase and alkaline phosphate staining, respectively. Interleukin-15 and MHC class I molecules were single labelled using the mouse IgG1 anti-IL-15 mAb (clone 34593; 1:100 dilution; R&D Systems, Minneapolis, MN, USA) or mouse IgG1 anti-MHC class I mAb (clone W6/32 Department of Physiology and Immunology, Medical Faculty, University of Rijeka, Croatia) and alkaline phosphate staining. Nuclei were stained with Shandon haematoxylin solution (Termo Scientific, Soeberg, Denmark), and the specimens were mounted using Acquatex (MerckKGa, Darmstadt, Germany). Cytospins were single labelled for GNLY using the same kit and the above-described protocol. Slides were analysed with an Olympus B × 51 microscope using an Olympus DP71 camera (Olympus, Tokyo, Japan). Images were processed using Cell^F imaging software or Cell^A imaging software, version 3.0 (both from Olympus, Tokyo, Japan) and Adobe Photoshop, version 7.0.1 CE (Adobe Systems Incorporated, San Jose, CA, USA).

Cytotoxicity assay. NK cell-mediated cytotoxicity was analysed against the NK-sensitive human erythroleukaemia cell line K562 (provided by Prof. E. R. Podack, Department of Immunology and Microbiology, School of Medicine, University of Miami, Florida, USA) using the method described previously [27]. K562 target cells were labelled with PKH26 lipophilic dye following the manufacturer's instructions (PKH26 Red Fluorescent Cell Linker Kit; Sigma Biosciences, St. Louis, MO, USA) prior to set up with peripheral blood lymphocytes (PBL) at effector to target cell ratios of 6:1, 12.5:1, 25:1 and 50:1. Samples of PBL and K562 cells cultured in the medium alone served as controls. The samples were incubated for 18 h at

37 °C in a humidified atmosphere containing 5% CO_2. After incubation, samples were labelled with FITC-conjugated annexin V (BD Pharmingen, San Diego, CA, USA) following the manufacturer's instructions (5 $\mu g/10^5$ cells, for 15 min at room temperature in the dark). Propidium iodide (PI, Sigma-Aldrich Chemie) at a final concentration of 5 μg/mL was added before analysis using FACSCalibur™. Some PBL samples were pretreated with anti-perforin δG9 mAb (10 $\mu g/10^5$ PBL, provided by Prof. E.R. Podack), anti-GNLY RC8 mAb (10 $\mu g/10^5$ PBL) or both anti-perforin mAb and anti-GNLY mAb at the indicated concentrations. Cells killed by apoptosis were detected as PI-labelled cells (red, fluorescence FL3) that were FITC-annexin V positive (green, fluorescence FL1) within the population of PKH26-labelled K562 cells (orange, fluorescence FL2). The results are expressed as the difference in the percentage of apoptotic K562 cells at a particular effector to target cell ratio minus the percentage of apoptotic K562 cells cultured in the medium alone.

Statistical analysis. Statistical analyses were performed using Statistica 8.0 data analysis software (StatSoft, Inc., Tulsa, OK, USA). The difference between groups was calculated by the Kruskal–Wallis non-parametric test, and a P value of <0.05 was considered statistically significant. The Mann–Whitney U test was used to determine the difference among groups with the level of significance adjusted to the number of mutual comparisons.

Results

GNLY expression in peripheral blood lymphocytes

Flow cytometry analysis of GNLY expression within gated peripheral blood lymphocytes shows that 4.7% of lymphocytes in healthy person express GNLY with a MFI of 7 (Fig. 1A). The histogram indicates fluctuation in the percentage and MFI of GNLY with respect to isotype-matched controls in patients with NSTEMI (Fig. 1B) on days 1, 7, 14, 21 and 28 after the acute coronary event that matched the summary data shown in the charts (Fig. 1C). The percentage of GNLY-positive lymphocytes was significantly higher (median,

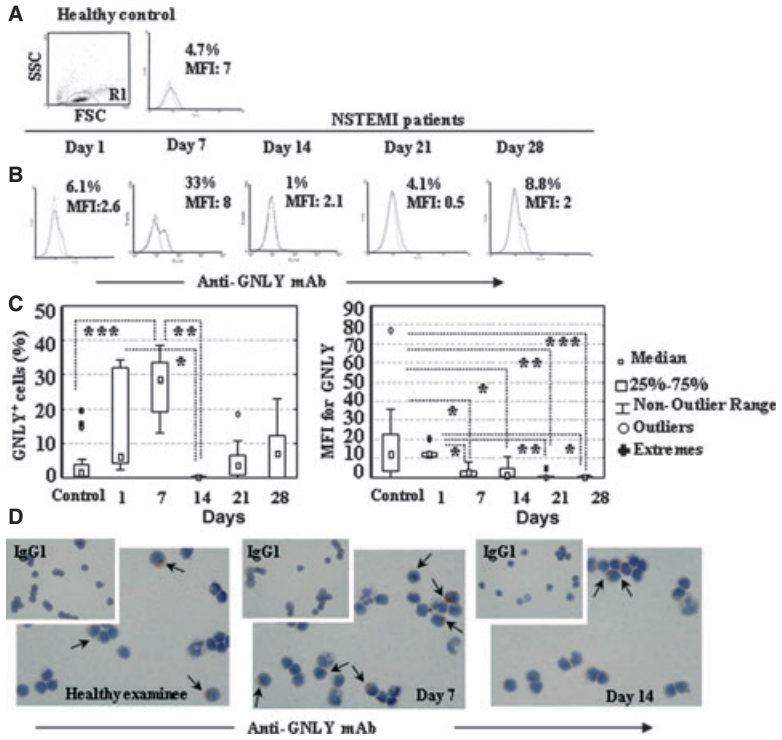

Figure 1 Analysis of granulysin (GNLY) expression in peripheral blood lymphocytes of patients with non-ST-segment elevation myocardial infarction (NSTEMI). A sample flow cytometry analysis of GNLY expression in peripheral blood lymphocytes gated in region 1 (R1) in accordance with forward scatter (FSC) and side scatter (SSC) parameters (dot plot, A). Numbers in the histograms indicate the percentage and mean fluorescence intensity (MFI) of $GNLY^+$ cells (solid histogram curves) calculated with respect to isotype-matched controls (dashed histogram curves) in the healthy examinees (A), and patients with NSTEMI (B). Median (25th, 75th percentiles) of the frequency and MFI of GNLY (C) at the indicated time points after acute coronary event were compared among themselves and with healthy examinees. Twenty-eight experiments were performed. Levels of statistical significance: $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$. GNLY is visualized as red cytoplasmic granules (indicated by the arrows) using alkaline phosphatase, anti-GNLY mAb (RF8) and Permanent red substrate (D). The controls, shown in the upper part of the photos, are isotype-matched. Shown is representative experiment of the 3 performed. Magnification is obtained with objective lens Olympus UPlan Apo, 100 × /1.35 and Olympus immersion oil for microscopy use, nd 1.516 (both from Tokyo, Japan).

28.67) on day 7 after the acute coronary event compared with healthy examinees (median, 2.6) or with values on day 14 (median 0.28). On day 1, GNLY was slightly increased compared to healthy examinees, but it was significantly higher when compared to that of patients with NSTEMI on day 14 (Fig. 1C). MFI of GNLY in lymphocytes decreased significantly from day 7 to day 28 compared to healthy examinees or to day 1 (Fig. 1C).

Using immunocytochemistry, GNLY protein was visualized as red-labelled granules beneath the cell membrane of lymphocytes in healthy examines and patients with NSTEMI. The highest expression of GNLY was on day 7, and the lowest expression of GNLY was

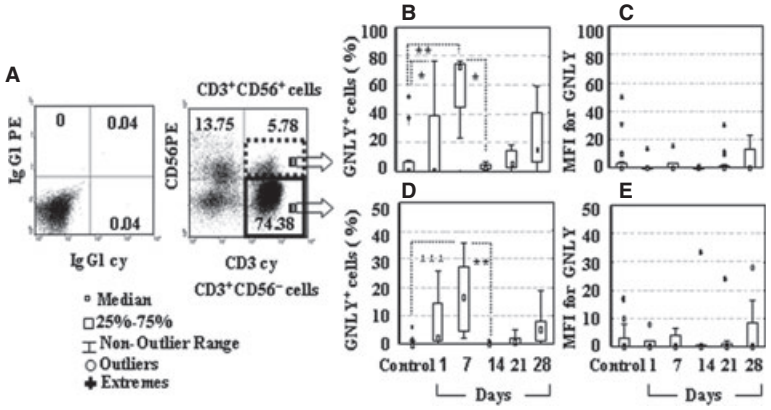

Figure 2 Granulysin (GNLY) expression in peripheral blood T cells and NKT cells from patients with non-ST-segment elevation myocardial infarction (NSTEMI). Dot plots (A) illustrate the selection of $CD3^+$ $CD56^-$ T cells (solid line rectangle) and $CD3^+$ $CD56^+$ NKT cells (dashed line rectangle) for GNLY expression analysis with respect to isotype-matched controls by flow cytometry. Charts show the percentage and mean fluorescence intensity (MFI) of GNLY within NKT cells (B and C) and T cells (D and E) from the peripheral blood of healthy controls and patients with NSTEMI at the indicated time points after acute coronary event. In each group, 12–15 experiments were performed. Levels of statistical significance: $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$.

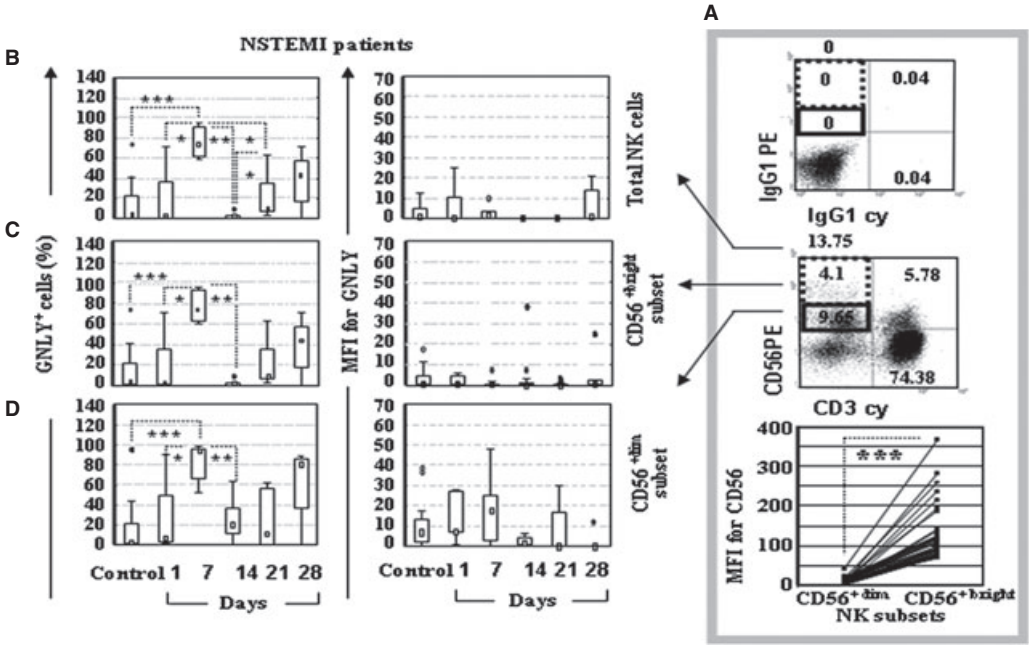

Figure 3 Granulysin (GNLY) expression in peripheral blood NK cells and their subsets. Dot plots (A) illustrate the selection of $CD3^-$ $CD56^+$ NK cell subsets: $CD56^{+dim}$ subset (solid line rectangle) with respect to isotype-matched controls by flow cytometry. Mean fluorescence intensity (MFI) of the CD56 molecule between $CD56^{+dim}$ and $CD56^{+bright}$ subsets is shown in chart A. Frequency of $GNLY^+$ cells and MFI of GNLY within total NK cells (row B), $CD56^{+bright}$ (row C) and $CD56^{+dim}$ (row D) NK subsets in the peripheral blood of patients with non-ST-segment elevation myocardial infarction at the indicated time points after acute coronary events were compared among themselves and with the control. In each group, 12–15 experiments were performed. Levels of statistical significance: $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$.

on day 14 (Fig. 1D). Labelling with irrelevant isotype-matched mouse immunoglobulin G1 (IgG1) was negative (upper left microphotographs in Fig. 1D).

In the dot plots of PBL from healthy examinees shown in Fig. 2A, $CD3^+$ $CD56^-$ T cells are located within the solid line rectangle and $CD3^+$ $CD56^+$ NKT cells are presented within the dashed line rectangle with respect to isotype-matched control. In patients with NSTEMI, the frequency of GNLY-positive NKT cells (Fig. 2B) and T cells (Fig. 2D) was increased on day 7 compared to the percentage observed in healthy examinees and in patients with NSTEMI on day 14 after an acute coronary event. On day 1, the percentage of $GNLY^+$NKT cells was higher than in healthy examinees (Fig. 2B). The MFI of GNLY essentially did not change in NKT (Fig. 2C) and T cells (Fig. 2E) during the investigation period.

The dot plots in Fig. 3A show a sample flow cytometry with the gates set up for the analysis of GNLY expression in total NK cells and their subsets. Total $CD3^-$ $CD56^+$ events (cells) were divided into $CD56^{+dim}$ (solid line rectangle) and $CD56^{+bright}$ (dashed line rectangle) NK subsets. In the sample of 13.75% of total NK cells, 4.1% of $CD56^{+bright}$ NK cells and 9.65% of $CD56^{+dim}$ NK cells were found to express GNLY compared to the isotype-matched control (0%). The chart in Fig. 3A shows significantly lower expression of the CD56 molecule in the $CD3^-$ $CD56^{+dim}$ subset compared to the $CD3^-$ $CD56^{+bright}$ subset ($P < 0.0001$), as it is determined by MFI. In patients with NSTEMI, the frequency of GNLY-positive total NK cells was elevated on day 7 after an acute coronary event compared to healthy examinees and to patients with NSTEMI on days 1, 14 and 21 (Fig. 4B). The lowest frequency of GNLY-positive cells was found on day 14 after an acute coronary event, which is significantly lower than on days 7 and 21, although it did not differ from day 28 or from the healthy controls (Fig. 4B). In both NK subsets, the percentage of cells expressing GNLY was higher on day 7 compared to on days 1 and 14 after MI and to healthy controls (Fig. 4C,D). In general, the MFI of GNLY basically did not change in NK cells (Fig. 3).

NK cell-mediated apoptosis of K562 cells

In healthy examinees, NK cells from freshly isolated PBL spontaneously induced apoptosis of NK-sensitive K562

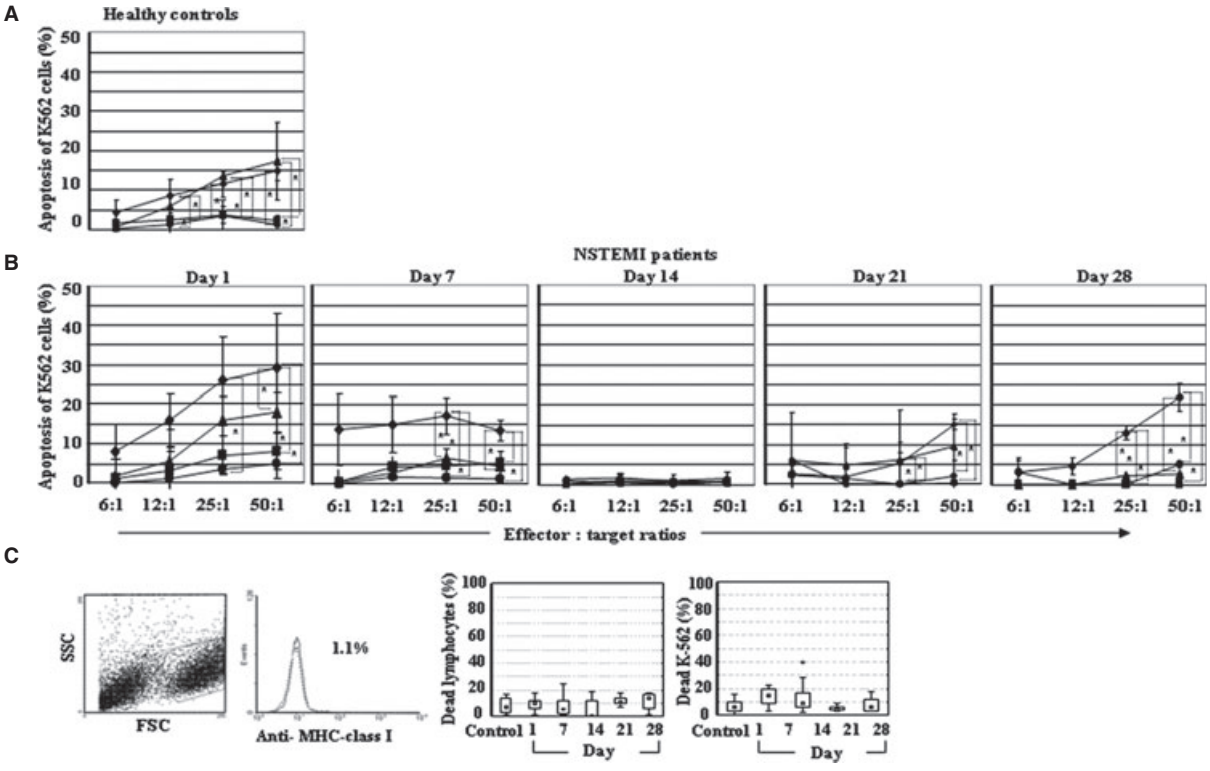

Figure 4 Long-term (18-h) granulysin (GNLY)-mediated apoptosis. Apoptosis against K562 cells mediated by peripheral blood NK cells from healthy examinees (A) and patients with non-ST-segment elevation myocardial infarction (NSTEMI) was measured at the indicated time points after an acute coronary event (row B). Cells were cultured in medium alone (♦) or pretreated with δG9 anti-perforin mAb (■), RC8 anti-GNLY mAb (▲) or both antibodies (●) at 10 $\mu g/10^5$ effector cells. In the charts, the data are expressed as the percentage of apoptotic propidium iodide- and annexin V-positive K562 cells and the results are shown as the mean (SD) of 8–12 experiments performed in each group. Level of statistical significance: $^*P < 0.01$. Major histocompatibility complex (MHC) class I expression gated on target K562 cells with respect to the isotype-matched control, and apoptosis (PI^+ and annexin V^+) of lymphocytes and K562 targets cultured for 18 h in medium alone are shown in part C.

target cells in a 18-h cytotoxicity assay from 5 to 15% depending on the effector to target cell ratio, ranging from 6:1 to 50:1 (Fig. 4A). Anti-perforin mAb almost completely abrogated apoptosis at effector to target ratios from 12:1 to 50:1, as did the combination of anti-perforin and anti-GNLY mAbs, whereas anti-GNLY mAb alone was ineffective at abolishing apoptosis (Fig. 4A). On days 7 and 28 after an acute coronary event, the apoptosis of K562 cells was significantly inhibited by the addition of anti-perforin mAb, anti-GNLY mAb, and the combination of anti-perforin and anti-GNLY mAbs at effector to target cell ratios of 50:1 and 25:1 (Fig. 4B). On day 14, apoptosis was generally negligible (Fig. 4B). On day 21, anti-perforin mAb and a combination of anti-perforin and anti-GNLY mAbs significantly decreased K562 apoptosis at ratios of 50:1 and 25:1, whereas anti-GNLY mAb by itself was ineffective (Fig. 4B). A negligible percentage of gated K562 cells expressed MHC class I molecules (1.2%) on the surface compared to the isotype-matched control, as was shown in the representative sample (Fig. 4C). In all experiments, the apoptosis of K562 cells and lymphocytes cultured in medium alone was comparable and was <15% (Fig. 4C).

Assessment of GNLY, CD3, CD56, APAF-1, MHC class I and interleukin-15 antigens in myocardial tissue sections

In leucocyte infiltrations, $CD3^+$ and $CD56^+$ cells were found rarely, but they were present (Fig. 5A). The double labelling of paraffin-embedded myocardial tissue sections from patients who died in the first week after an acute coronary event confirmed the presence of GNLY in cells with a $CD3^+$ and $CD56^+$ phenotype, compared to the isotype-matched control (Fig. 5A). $CD3^+$ cells expressing GNLY were found more often than GNLY-expressing $CD56^+$ cells (Fig. 5A). In patients who died late after an acute coronary event, a thinning and loss of myofibrils were observed (Fig. 5B), whereas neither lymphocyte infiltration nor GNLY-positive cells were found (Fig. 5B,C). Remaining myofibrils were without centrally positioned nucleuses, contrary to maintained nucleases in cardiomyocytes of patients who died from non-myocardial causes (Fig. 5C). Both $CD3^+$ and $CD56^+$ cells are found in the vicinity of weakly APAF-1^+ myocardial filaments with a reduced number of nucleuses (Fig. 6). Moreover, GNLY-positive cells were found close to weakly APAF-1^+ cells placed in the border zones of the infarct foci in tissue sections of persons who died in the first week after myocardial infarction (Fig. 7A). Additionally, $GNLY^+$ cells were found in the accumulations of apoptotic leucocytes, infiltrating myocardium, early after the acute coronary event (Fig. 7A). In sections of persons who died in the fifth week after the MI, rare $GNLY^+$ cells were seen only in the vessels, although APAF-1^+ filaments were detected all over the myocardium (Fig. 7B). Myocardium of person who died from non-myocardial causes did not contain APAF-1^+ cells (Fig. 7C). IL-15 protein expression was observed in the patients who died in the first week after the acute coronary event within viable cardiomyocytes encircling the necrotic region (Fig. 8A). At the site of the necrosis, consisted of damaged myofibrils without nucleuses, oedema and leucocyte infiltration,

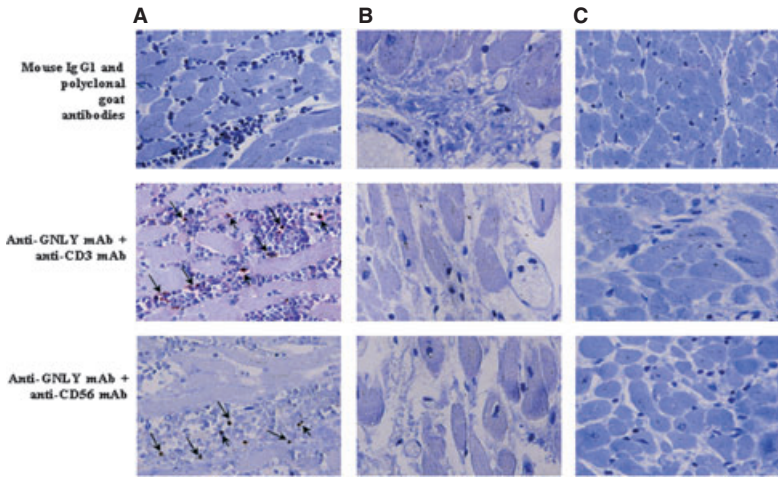

Figure 5 Analysis of granulysin (GNLY)-expressing lymphocytes within infarcted myocardium. Immunohistology of paraffin-embedded myocardial tissue sections from patients who died in the first week (A) or in the fifth week (B) after an acute coronary event and from patients who died from non-cardiac causes (C) using mouse IgG1, polyclonal goat antibodies, mouse RC8 anti-GNLY mAb, mouse anti-CD56 mAb and goat polyclonal anti-CD3 mAb is shown. Double labelling using indirect immunoperoxidase and alkaline phosphatase staining was used. $GNLY^+$ cells appear as red-labelled cells by Permanent red, and $CD3^+$ cells or $CD56^+$ cells appear as brown labelled cells by 3,3-diaminobenzidine (DAB). Co-expression of GNLY (red labelling) and CD56 or CD3 (brown labelling) in the same cell is indicated by the arrows. Four samples were labelled in each group. Magnification is obtained with objective lens Olympus UPlan Apo, 100 × /1.35 and Olympus immersion oil for microscopy use, nd 1.516 (both from Tokyo, Japan).

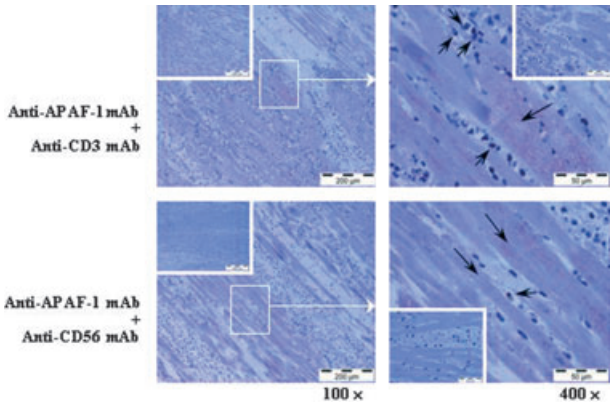

Figure 6 Analysis of co-localization of APAF-1 expressing myocardiocytes and lymphocytes within infarcted myocardium. Immunohistology of paraffin-embedded myocardial tissue sections from patients who died in the first week after an acute coronary event using mouse IgG1, rabbit polyclonal antibodies, rabbit polyclonal anti-APAF-1 mAb, mouse anti-CD56 mAb and rabbit polyclonal anti-CD3 mAb is shown. Double labelling using indirect immunoperoxidase and alkaline phosphatase staining was performed. APAF-1$^+$ cells appear as red-labelled cells by Permanent red (indicated by the long arrows), and CD3$^+$ or CD56$^+$ cells appear as brown labelled cells by 3,3-diaminobenzidine (indicated by the short arrows). Inserts in the corners of microphotographs show isotype-matched controls. Three samples were labelled in each group. Magnifications 100× and 400× are performed with objective lens Olympus UPlan FLN 10 × /0.30 or Olympus UPlan FLN 40 × /0.75 (all from Tokyo, Japan).

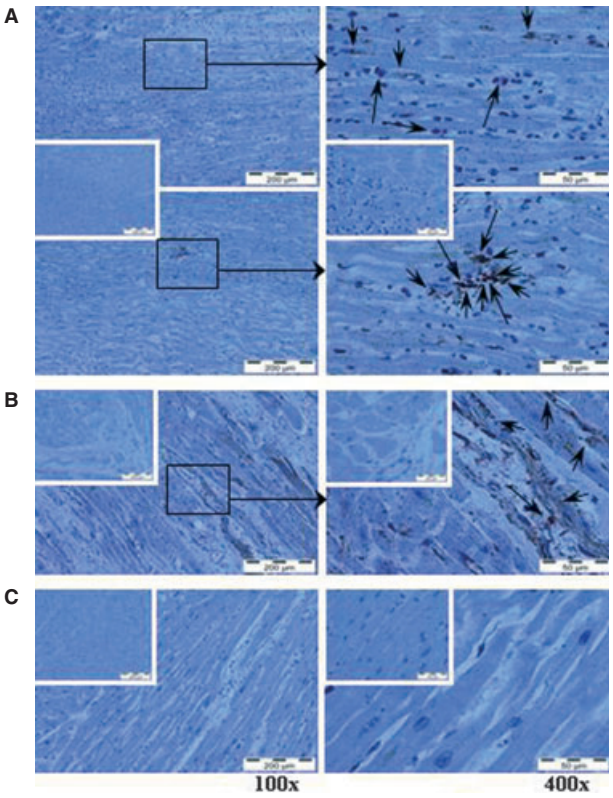

Figure 7 Analysis of co-localization between APAF-1 expressing myocardiocytes and GNLY within infarcted myocardium. Immunohistology of paraffin-embedded myocardial tissue sections from patients who died in the first week (A) or in the fifth week (B) after an acute coronary event and from patients who died from non-cardiac causes (C) using mouse IgG1, polyclonal rabbit antibodies, mouse anti-GNLY mAb, rabbit polyclonal anti-APAF-1 mAb is shown. Double labelling using indirect immunoperoxidase and alkaline phosphatase staining was performed. GNLY$^+$ cells appear as red-labelled cells by Permanent red (indicated by the long arrow), and APAF-1$^+$ cells appear as brown labelled cells by 3,3-diaminobenzidine (indicated by the short arrows). Insert sin the corners of microphotographs show isotype-matched controls. Three samples were labelled in each group. Magnifications 100× and 400× are performed with objective lens Olympus UPlan FLN 10×/0.30 or Olympus UPlan FLN 40×/0.75 (all from Tokyo, Japan).

IL-15 was not found (Fig. 8B). IL-15 completely disappeared from the myocardial tissue sections of persons who died 5 weeks after an acute coronary event (Fig. 8C), and it was not found in myocardial tissue sections from persons who died from non-cardiac causes (Fig. 8D). MHC class I molecules were down-regulated in the centre of the infarct foci, whereas they were present in peri-necrotic region (Fig. 9A,B), as well as in person who died later after myocardial infarction (Fig. 9C) or non-myocardial causes (Fig. 9D).

Discussion

The early post-infarction period is characterized with systemic pro-inflammatory condition that activate peripheral blood T and NK cells inducing their cytotoxic potential [9, 15]. Pro-inflammatory IFN-γ and TNF-α cytokines production are found elevated in cultures of lymphocytes from patients with acute MI compared with group of stable angina or healthy subjects, suggesting their contributions to plaque instability and clinical manifestations [28, 29]. Additionally, significant increase in pro-inflammatory markers IL-6, CXCL8 and C-reactive protein ware found in patients with coronary artery disease with subsequent MI when compared to coronary artery disease group without MI [6]. Serum level of pro-inflammatory IL-1β cytokine increased in MI patients within the first few hours after the onset of chest pain, but it could not be found elevated latter in MI patients, despite the significant IL-1β up-regulation in the infracted myocardium [4]. It is likely that the role of IL-1β is in attraction of lymphocytes in the myocardium and it alone or in the combinations with IFN-γ and TNF-α induces cardiomyocyte apoptosis [4]. Interleukin-15 shares pro-inflammatory properties with IL-2, and it attracts [11] and activates the cytotoxicity of CD56$^{+\,dim}$ and CD56$^{+\,bright}$ subsets [12]. It was shown previously to recruit NK cells in an atherosclerotic plaque [11]. Therefore, it is likely that IL-15 plays an important role in the recruitment of activated leucocytes from the blood stream in the infracted myocardial region of persons who died early after an acute coronary event. This hypothesis is supported by the abundant IL-15 expression in the border-necrotic, viable

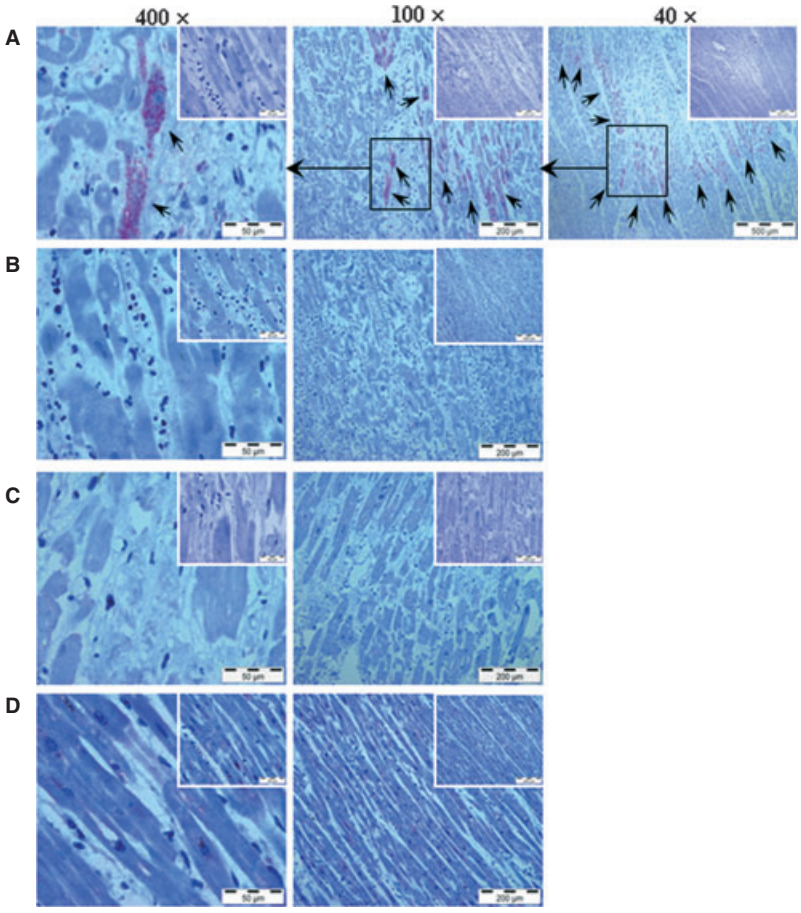

Figure 8 Interleukin-15 expression within infarcted myocardium. Immunohistology of paraffin-embedded myocardial tissue sections at the peri-necrotic region (A) and at the central necrotic region (B) from patients who died in the first week or in the fifth week (C) after an acute myocardial event, and tissue sections from patients who died from non-cardiac causes (D). Indirect alkaline phosphatase staining was performed using anti-interleukin-15 mAb or mouse isotype-matched IgG1. Red coloration by Permanent red indicates specific interleukin-15 labelling (indicated by the arrows). Inserts in the upper right part of each microphotograph show control labelling. Four samples were labelled in each group. Magnifications 4×, 100× and 400× are performed with objective lens Olympus UPlan FLN 4×/0.13, Olympus UPlan FLN 10×/0.30 or Olympus UPlan FLN 40×/0.75 (all from Tokyo, Japan).

myocardiocytes that surround lymphocytes infiltration in the form of necklace. Although the $CD56^{+bright}$ NK cell subset represents mostly cytokine-producing, regulatory NK cells in a steady state condition, they are able to become highly cytotoxic under tissue-specific inflammatory Th1 cytokine stimulation, such as the combination of IL-15 with other cytokines [12]. This was confirmed *in vitro* even with decidual $CD56^{+bright}$ NK cells [27], whose cytotoxicity is normally strongly down-regulated *in situ* by local immune-endocrine interactions during the first trimester of pregnancy. However, there is no clear evidence for the involvement of particular cytotoxic mediator(s) in the apoptosis of myocardial tissue after infarction. Here, we show for the first time the presence of the pro-apoptotic molecule GNLY in the cytoplasm of $CD3^+$ and $CD56^+$ cells, which take part in lymphocyte infiltration in the centre of MI in the patients who died in the first week after coronary artery thrombosis. GNLY can be easily released from the cells upon pro-inflammatory stimulation [19], what is supported with significantly lower MFI for GNLY in peripheral blood lymphocytes of MI patients when compared with healthy control. In turn, the soluble mature form of GNLY could enhance secretion of Th1 chemokines from macrophages and exhibit chemotactic properties for monocytes, mature dendritic cells, NK cells, and $CD4^+$ and $CD8^+$ T cells with a $CD45RO^+$ phenotype, but not naïve $CD45RA^+$ cells, as was shown previously [19], thus contributing to the accumulation of immune effectors in the myocardium after infarction [2]. On the other side, GNLY could hasten resolution rather than worsen cardiac post-infarction inflammation because of the finding of $GNLY^+$ cells within accumulations of apoptotic leucocytes 1 week after the acute coronary event.

K562 killing represents a model for *in vitro* testing of NK cell-mediated self-aggression, because K562 cells do not express MHC class I protein forms, as is known for damaged tissue cells [30]. Significant spontaneous peripheral blood NK cell- and GNLY-mediated apoptosis of K562 cells, which occurs in the first week after the acute coronary event, disappeared on day 14, with a concomitant decrease in the percentage of $GNLY^+$ cells and the $GNLY^+$ $CD56^{+\ bright}$ NK cell subset in the circulation. The decrease in GNLY-mediated apoptosis is most likely a consequence of the recruitment of cytotoxic peripheral blood lymphocytes into damaged myocardial tissue, as it was recently reported for T cells [2] and NK cells in patients with NSTEMI [31]. They could additionally damage myocardial tissue, because MHC class I proteins disappeared in the central infarction sites, whereas their

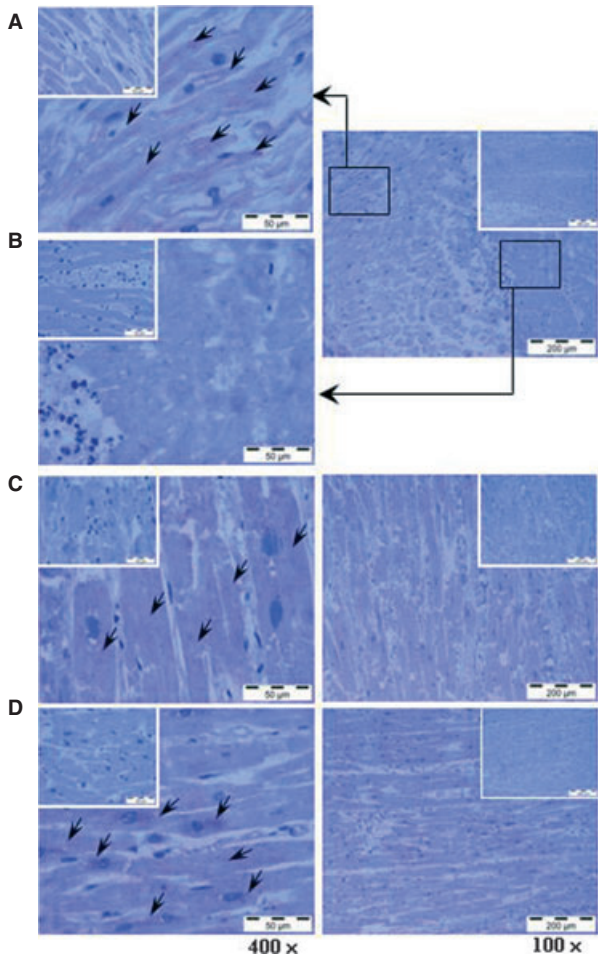

Figure 9 Major histocompatibility complex (MHC) class I molecules expression within infarcted myocardium. Immunohistology of paraffin-embedded myocardial tissue sections at the peri-necrotic region (A) and at the central necrotic region (B) from patients who died in the first week or in the fifth week (C) after an acute coronary event, and tissue sections from patients who died from non-cardiac causes (D). Indirect alkaline phosphatase staining was performed using rabbit anti-MHC class I mAb or rabbit polyclonal antibodies, as isotype control. Red coloration by Permanent red indicates specific MHC class I labelling (indicated by the arrows). Inserts in the upper right or left part of the microphotograph show isotype control labelling. Four samples were labelled in each group. Magnifications 100× and 400× are performed with objective lens Olympus UPlan FLN 10×/0.30 or Olympus UPlan FLN 40×/0.75 (all from Tokyo, Japan).

expression was conserved, but weaker in the surrounding peri-necrotic zones of the MI 1 week after an acute coronary event when compared to myocardial tissue sections of persons who died 5 weeks after an acute coronary event. It suggests susceptibility of peri-infarction zones for NK cell killing mediated by cytotoxic mediators. $GNLY^+$ $CD3^+$ cells and rarely $GNLY^+$ $CD56^+$ cells reach the apoptotic $APAF\text{-}1^+$ cardiomyocytes in the border infiltration zone of persons who died 1 week after the acute coronary event and could participate in the apoptosis of these cells. Accordingly, apoptotic single-stranded DNA–positive cells were found in the border zones and granulation tissue cells in the infarct region by Akasaka *et al.* [7]. But, it is unlikely that $GNLY^+$ cells cause significant cardiomyocytes apoptosis because of their small numbers. In addition, later after the MI, the $APAF\text{-}1^+$ apoptotic myocardial cells are found without close contact with $GNLY^+$ cells, suggesting implementation of GNLY-independent mechanism of cellular loss. A formation of apoptosome after the binding of APAF-1 protein with cytochrome C could induce caspase 9 dimerization and autocatalysis [32]. Indeed, apoptotic markers (caspase 3 and apoptotic bodies) are present in the surviving zone of the heart, remote from the infarct region, as early as day 1 after MI and persist for up to 1 month [3, 33]. Additionally, on day 7 after an acute coronary event, the significant increase in the percentage of peripheral blood $GNLY^+$ NK cells enables GNLY-mediated K-562 apoptosis, as the mechanism attributed to perforin-mediated cytotoxicity [31]. GNLY probably accesses the K562 target cell cytoplasm through perforin pores or by other mechanisms that involve sublytic perforin concentrations in agreement with Lettau *et al.* [18], because an additive effect between GNLY- and perforin-mediated cytotoxicity has not been found. This suggests that they probably use the same mechanism for entering cells. On day 14, in patients with NSTEMI, GNLY expression, as well as perforin expression [31], in all peripheral blood lymphocyte subpopulations was the lowest and it was reflected in negligible NK cell apoptotic activity against K-562 cells. The lower percentage of GNLY-positive NK cells in patients with NSTEMI on day 21 as compared to day 7, correlated well with mostly perforin-mediated NK cell killing as a redundant apoptotic mechanism [27]. At the end of a 1-month rehabilitation period in patients with NSTEMI, we again found significant participation of GNLY in K562 apoptosis as a result of restored GNLY expression in peripheral blood NK cells. It correlates well with the restored perforin protein expression in lymphocytes from patients with NSTEMI at the end of the first month of the rehabilitation period [31]. Our results are supported by the findings of Kuroki *et al.* [34] and Klarlund *et al.* [35], which showed higher short-term NK cell killing of K562 targets in MI patients on days 7 and 28 after coronary artery occlusion compared to the first hospital day, although the total number of NK cells, identified as large granular lymphocytes, was unchanged. Restored granulysin-mediated cytotoxicity at the end of rehabilitation period could be the consequence of gradual decrease in early post-infarction inflammatory condition during the first month after MI, as it is confirmed with statistically significant lower plasma concentration of CXCL-8, TNF-α, fibrinogen and C-reactive protein when compared with day 7 after MI [36].

In conclusion, this study demonstrated the increased frequency of $GNLY^+$ peripheral blood lymphocytes

within the T, NK and NKT cell subpopulations in patients with NSTEMI treated with anti-ischaemic drugs on day 7 after the acute coronary event, which probably preceded the recruitment of $GNLY^+$ cells in the myocardium, under the influence of IL15. Concomitant with the increased GNLY expression in peripheral blood, increased GNLY-mediated cytotoxicity was seen against K562 cells *in vitro*, as a model of self-aggression. Additionally, we showed for the first time the presence of GNLY within $CD3^+$ and $CD56^+$ lymphocytes infiltrating central zone of MI and reaching the apoptotic cells in border MI zones of patients who died shortly after coronary artery thrombosis, suggesting that GNLY-mediated apoptosis at least partly participate in myocardial cell injury, but also hasten resorption of leucocytes infiltration.

Disclosure

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

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Authors contribution

Viktor Persic, Alen Ruzic and Bojan Miletic analysed data and discussed the scientific results; Dijana Travica Samsa and Marijana Rakic performed experimental work and analysed data Damir Raljevic collected and analysed data; Vesna Pehar Pejcinovic collected data and performed clinical follow-up of the patients; Senija Eminovic collected data and carried out immunohistology studies; Luka Zaputovic and Gordana Laskarin provided theoretical background; Alen Ruzic and Gordana Laskarin discussed the scientific results and wrote the manuscript.

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