Granulysin expression and granulysin-mediated apoptosis in the peripheral blood of osteoarthritis patients

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Abstract. Osteoarthritis (OA) is a chronic joint disease caused by mechanical damage and metabolic factors that support the development of low-grade inflammation. Increased levels of T helper 1 pro-inflammatory cytokines in the serum of OA patients may support granulysin (GNLY) mediated cytotoxicity, which in-turn may contribute to the pathogenesis of OA. In the present study, GNLY expression and cytotoxic/apoptotic mechanisms mediated by GNLY in the peripheral blood of OA patients were assessed. A total of 40 non-obese women (median age of 64 years old) with knee OA, and 40 controls (median age 62 years old) were enrolled in the study. GNLY, IFN-γ and IL-4 expression levels were investigated in peripheral blood lymphocytes (PBLs) using flow cytometry, immunocytochemistry and/or confocal microscopy. Natural killer (NK) GNLY-mediated apoptosis through NK effectors against K-562 targets was analyzed using the PKH-26 18-h cytotoxicity assay. Serum GNLY levels were assessed using ELISA. The percentage of GNLY+PBLs was higher in the OA patients than that in the controls due to the increase in the proportions of GNLY+ cells in the natural killer (NK), T and natural killer T (NKT) subsets. GNLY localization inside exocytotic lysosomal-associated membrane protein-1+ granules was ~40% in both groups. However, the intensity of GNLY labeling in PBLs was higher in OA patients than in the controls, and it was supported by the increased expression of IFN-γ relative to IL-4 in NK and T cells from OA patients. The serum GNLY concentration was <0.3 ng/ml in both groups. RC8 anti-GNLY mAb by itself was unable to significantly alter early apoptosis, whereas RC8 anti-GNLY mAb combined with anti-perforin mAb significantly reduced NK-mediated early apoptosis of K-562 targets in the OA patients, whilst not exerting a notable effect in the controls. Anti-perforin mAb by itself did not affect apoptosis significantly. These results suggest that in women with knee OA, GNLY expression in the PBL subsets and GNLY-mediated early apoptosis of K-562 targets are increased compared with the controls and accompanied by intracellular dominance of IFN-γ over IL-4 in NK cells.

Introduction

In osteoarthritis (OA), biomechanical forces constantly damage avascular chondrocytes and change their phenotype with intensive production of pro-inflammatory cytokines (1). Increased concentrations of proinflammatory mediators IL-1β, IL-18, IL-15, IL-21, IL-27, IFN-γ, TNF-α, and tolerogenic IL-10 were found in the synovial fluid and/or peripheral blood of OA patients compared to that in controls (1,2). This is consistent with the hypothesis that locally produced inflammatory substances, supported by synovial dendritic cells and polarized macrophages, leak from OA joints into the circulation and induce a systemic immune response mediated by activated lymphocytes during periods of painful OA (3). IL-15 (4), IL-21 (5) and IL-27 (6) act via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, and are crucial factors in the natural killer (NK) cell-associated innate immune response (7). IL-1β and TNF-α act alongside NF-κB (8), which exhibits upregulated expression levels in OA joints, and promotes cellular interaction, proliferation and cytotoxicity (1).

Granulysin (GNLY), a constitutive cytotoxic mediator in NK cells, is upregulated in T cells in a pro-inflammatory

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environment (9,10). After direct stimulation of effector cells in close contact with targets, the cytotoxic GNLY form (9 kDa) is released into the immunological synapse, where it enters the target cells through the perforin pores (10), and kills human cells by inducing apoptosis and DNA fragmentation (9,11). The 15 kDa form of GNLY is released from activated cells spontaneously without direct effector-target cell contact and shows regulatory and chemotactic properties in the dendritic cells (12), but is not cytotoxic (13). To the best of our knowledge, no data exist regarding the expression and cytotoxic/apoptotic mechanisms mediated by GNLY in the peripheral blood of the OA patients, given the mildly pro-inflammatory environment (14-16). We analyzed GNLY, IFN-γ and IL-4 expression in peripheral blood lymphocytes (PBLs), GNLY-mediated cytotoxicity of NK cells at the effector K-562 target cell, and GNLY serum concentration in the OA patients.

Materials and methods

Patients. A total of 40 women with knee OA and 40 control individuals without knee OA were enrolled in a prospective study in the Thallassotherapia-Opatija Hospital, Opatija, Croatia, between the 1st of July 2018 and the 15th of December 2020. The study was approved by the Ethical Committee of the Hospital 'Thallassotherapia-Opatija' (approval no. 01-000-00-450/2018) and all experimental procedures were performed according to the 'Ethical Principles for Medical Research Involving Human Subjects' outlined by the World Medical Association Declaration of Helsinki (17). The median age and age range of the OA group and controls were 64 years old (43-75) and 62 years old (43-73), respectively.

Examinees were evaluated using the 1986 American College of Rheumatology clinical classification criteria (18). Exclusion criteria for both groups were: Infectious and autoimmune diseases, bone marrow and/or lymphatic system disorders, immune deficiency, women of reproductive age, uncontrolled blood glucose level (plasma glucose >11 mmol/l), uncontrolled hypertension (systolic >180 mmHg or diastolic >100 mmHg), chronic liver and/or renal failure, high risk of heart failure as per the New York Heart Association classification (19) III and IV, and injury to organs, blood transfusions, and/or a malignant disease within 5 years. Pain intensity was assessed using the visual analog scale from 0 to 100 mm, as judged by the patients. Radiological staging of OA in the knee was assessed using the Kellgren-Lawrence Scale (20). The clinical and laboratory characteristics of OA patients and controls are shown in Table I. Out of the 40 OA patients, hypercholesterolemia was present in all patients (100%), and glucose intolerance in 11 patients (27.5%); they were controlled with a strict diet (without drug therapy). All controls without knee OA had hypercholesterolemia and glucose intolerance was present in 10 (25%) out of the 40 women, which were regulated only with diet. Arterial hypertension was present in 33 OA patients (82.5%) and 32 controls (80%); it was controlled with β-blockers and/or angiotensin-converting enzyme inhibitors.

Peripheral venous blood (10 ml) was drawn from the patients and controls according to standard procedures; routine laboratory tests (erythrocytes, hemoglobin, leukocytes, thrombocytes, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transferase, creatinine, urate and high sensitivity C-reactive protein) were performed using the Hematology analyzer XS-1000i (Sysmex, Kobe, Japan) and Biochemical analyzer Dimension Xpand (Siemens Healthcare Diagnostics, Newark, DE, USA).

Isolation of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated by gradient density centrifugation (Lymphoprep solution, Nycomed Pharma; 600 x g, 20 min at 4˚C) (21). PBMCs were collected and washed (400 x g, 10 min at 4˚C) in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.), re-suspended in tissue culture medium [RPMI-1640 supplemented with L-glutamate (2 mM), penicillin ($1x10^5$ IU/l), streptomycin sulphate (0.05 g/l), and 10% FBS; Gibco; Thermo Fisher Scientific, Inc.], and counted. The cell viability exceeded 98% as measured using propidium iodide (PI; 0.5 µg/ml/$1x10^6$ cells; 5 min at 22-25˚C; MerckMillipore) and a FACSCalibur flow cytometer with CellQuest Pro software version 6.0 (both from Becton Dickinson).

Antigen detection by flow cytometry. The simultaneous detection of surface and intracellular antigens was performed as described previously (21,22). Primary antibodies, mouse IgG1 anti-human GNLY (cat. no. D-185-3; RC8 clone; MBL International) or isotype-matched IgG1 (cat. no. bd554121; MOPC-21 clone; BD Pharmingen) were added to cell samples (1 µg/10^6 cells, 30 min, 4˚C), which were previously washed (400 x g, 10 min at 4˚C) in 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 Na_2-EDTA (Fluka), 1.5 mm NaN_3 (Difco), fixed (4% paraformaldehyde, 10 min at 22-25˚C), permeabilized in saponin buffer (0.1% saponin, Sigma, Poole, Dorset, USA), 2% fetal calf serum in PBS ($NaHPO_4x12H_2O$ 33.9 mM, NaCl 136.8 mM, KH_2PO_4 3 mM of distilled water; all from Kemika), 0.05% BSA (MerckMillipore), and incubated with 10% heat-inactivated fetal calf serum for 20 min at 22-25˚C. The cells were washed twice in saponin buffer (400 x g, 10 min at 4˚C) and secondary goat anti-mouse polyclonal antibodies conjugated with fluorescein isothiocyanate (FITC; cat. no. bd554001; BD Pharmingen, 1 µg/$1x10^6$ cells) were added for 30 min at 4˚C. After two washes in saponin buffer, the cell membranes were restored using 1 ml FACS buffer (5 min, at 22-25˚C). Cell surface labeling was performed using a combination of phycoerythrin (PE)-conjugated anti-CD3 (cat. no. bd555332, UCHT-1 clone; BD Pharmingen; 20 µl/$1x10^6$ cells) and PE-CyChrome5 (PE-Cy5)-conjugated anti-CD56 (cat. no. bd569104, B159 clone; BD Pharmingen; 20 µl/$1x10^6$ cells) mAbs (30 min, 4˚C). Samples of PBMCs ($1x10^6$/ml) were incubated with phorbol-myristate-acetate (10 ng/ml), ionomycin (1 µM), and monensin (3 µM) (all from MerckMillipore) for 5 h at 37˚C in a humidified incubator supplied with 5% CO_2 and intracellularly labeled for IFN-γ or IL-4 in lymphocyte subsets using PE-Cy5-conjugated anti-CD56 (20 µl/$1x10^6$ cells), FITC-conjugated anti-CD3 (cat. no. bd561808, UCHT1 clone, 20 µl/$1x10^6$ cells); and PE-conjugated IFN-γ (cat. no. bd554701, B27 clone, 0.25 µg/$1x10^6$ cells) or PE-conjugated IL-4 (cat. no. bd551774, 8D4-8 clone; 20 µl/$1x10^6$ cells) (all from BD Pharmingen). Mouse IgG1 (MOPC-21 clone) conjugated with FITC (cat. no. bd556649), PE-Cy5 (cat. no. bd555750), or PE (cat.

Table I. Demographic, clinical and laboratory characteristics of the OA patients and controls.

Demographic characteristics	OA patients	Controls
Age, years (age range)	64 (43-75)	62 (43-73)
Clinical characteristics[a]		
Body mass index, kg/m^2	25 (23-29)	26 (22-27)
Duration of diagnosis, years	6 (3.5-12)	-
Kellgren-Lawrence Scale, grade	2 (1-3)	-
Medial knee compartment involvement, n=40	40	-
Medial and lateral knee compartment involvement, n=40	14	-
Morning stiffness, min	18 (10-20)	-
Visual analog scale for pain, 1-100 mm	40 (20-50)	-
Systolic blood pressure, mmHg	145 (130-145)	140 (135-145)
Diastolic blood pressure, mmHg	85 (80-105)	88 (85-92)
Laboratory characteristics[a]		
High sensitivity C-reactive protein, mg/l	2.1 (1.8-3)	2.0 (1.5-2.9)
Erythrocytes, x10^{12}/l	4.6 (4.5-4.8)	4.6 (4.4-4.8)
Hemoglobin, g/l	133 (132-147)	135 (131-144)
Leukocytes, x10^9/l	6.4 (6.2-6.6)	6.9 (5.2-7.1)
Thrombocytes, x10^9/l	264 (223-283)	280 (222-325)
Alanine aminotransferase, IU/l	35 (28-38)	26 (17-30)
Aspartate aminotransferase, IU/l	20 (20-22)	18 (15-22)
γ-glutamyl transferase, IU/l	21(16-27)	17 (16-22)
Urate, mmol/l	285 (232-308)	233 (204-271)
Creatinine, mmol/l	86 (78.5-91.5)	75 (68-82)
Glucose, mmol/l	5.5 (5.1-6.4)	6.2 (5.1-6.4)
Low density lipoprotein-cholesterol, mmol/l	3.9 (3.6-4.9)	4.01 (3.1-5.2)
Granulysin, ng/ml	<0.3	<0.3

[a]Data are presented as the median (25-75th percentile).

no. bd556650) (all 20 μl/1x10^6 cells; all from BD Pharmingen) were used as isotype controls All the incubations were performed at 4˚C for 30 min. Samples were resuspended in 2% paraformaldehyde, and processed immediately using flow cytometry, and analyzed using WinMDI 2.9 (The *Scripps Research Institute*).

Immunocytochemistry. Cytospins were prepared from freshly isolated PBMCs suspended in PBS/0.05% BSA to reach a final concentration of 8x10^5 cells/ml. Subsequently, 100 μl of the suspension was centrifuged (Cytospin centrifuge; Shandon Inc.; 210 x g, 5 min at 4˚C) onto a glass microscope slide (Marienfeld microscope; Paul Marienfeld GmbH & Co.). The cytospin samples were air-dried (1 h) and subsequently fixed in pure acetone (5 min at 22-25˚C; Kemika). Labeling of GNLY was performed using the immunoperoxidase staining method with an LSAB™-HRP kit (Dako; Agilent Technologies, Inc.). Briefly, sections preincubated (20 min) with LSAB™-HRP kit blocking solution were incubated (30 min) with anti-human GNLY mAb (RC8 clone) or mouse IgG1 (R312-Mouse IgG1 clone, Sigma Aldrich) diluted 1:100 in PBS/0.05% BSA. Incubation (10 min) with biotinylated anti-mouse secondary antibodies was followed by incubation with streptavidin for 10 min (both from LSAB™-HRP kit). The reaction was developed using aminoethyl carbazole (MerckMillipore); nuclei were counterstained with hematoxylin (Gill III; MerckMillipore). All incubations were performed at 22-25˚C; samples were washed twice in PBS for 5 min between labeling steps. Subsequently, slides were mounted with Entellan (MerckMillipore) and imaged using an Olympus BX51 microscope with an Olympus DP71 camera and CellA software, v3.0 (magnification, x1,000) (Olympus Corporation).

Confocal microscopy. Confocal microscopy was performed as described by Donaldson (19). Samples were fixed with 4% paraformaldehyde in PBS (10 min at 22-25˚C), permeabilized with Triton X-100 (Rohm and Haas Corporate Headquarters, 7 min), and incubated in 20% AB serum (Developed in-house) in PBS (45 min at 22-25˚C). The samples were then incubated with a combination of anti-human GNLY mAb (dilution 1:100, D185-3 clone) and rat IgG2a anti-lysosomal-associated membrane protein (LAMP)-1 mAb (dilution 1:100, 1D4B clone, BD Pharmingen) in PBS/1% BSA at 4˚C overnight. After washing, cells were labeled with secondary antibodies, Alexa Fluor 594-conjugated donkey anti-mouse (IgG H+L polyclonal; cat. no. A32744; Invitrogen; Thermo Fisher Scientific, Inc., dilution 1:500) and Alexa Fluor 488 goat anti-rat (IgG H+L polyclonal; cat. no. A11006; Invitrogen; Thermo Fisher

Scientific, Inc., dilution 1:300) in PBS for 1 h at 22-25˚C, washed, and mounted with Mowiol (MerckMillipore). All washing steps were performed in PBS (three washes, 3 min each). Images were taken using a confocal imaging system (magnification x600; Olympus Fluoview FV3000) with CellA software. The cells were randomly chosen and demonstrated a well-resolved pattern.

Isolation of NK cells. Freshly isolated PBLs were obtained from the non-adherent fraction of PBMCs after 45 min incubation in a tissue culture Petri dish (60x15 mm; Techno Plastic Products). NK cells were purified by negative magnetic cell separation using the NK Cell Isolation Kit (cat. no. 130-092-657, Miltenyi Biotec, GmbH). The NK Cell Biotin-Antibody Cocktail (10 μl) and 40 μl ice-cold filtered FACS buffer were added to 1×10^7 pelleted PBLs, mixed thoroughly, and incubated for 10 min at 4˚C. Subsequently, a mixture of 30 μl FACS buffer and 20 μl NK Cell MicroBead Cocktail was added per 1×10^7 total cells and refrigerated for an additional 15 min (4˚C). The suspension was washed (300 x g, 10 min at 4˚C) and the supernatant was aspirated and replaced with 500 μl FACS buffer. The cell suspension was loaded onto a prewashed medium-sized column and placed in the magnetic field of the VarioMACS separator (both from Miltenyi Biotec, GmbH). Unlabeled cells that passed through the column were collected as NK cells. The purity and viability of the NK cells were >95%, as estimated using flow cytometry.

K cytotoxicity assay. NK cell cytotoxicity was estimated by assessing early apoptosis of K-562 cells (23). Human erythroleukemia K-562 cells (Department of Physiology and Immunology, Faculty of Medicine, University of Rijeka, Croatia) were labeled with PKH26 (5 min at 22-25˚C) red lipophilic dye (PKH 26 Red Fluorescent Cell Linker Kit; MerckMillipore). The samples of effector NK and target K-562 cells, at different ratios in a total volume of 200 μl, were incubated in tissue culture medium for 18 h at 37˚C with 5% CO_2, and labeled with FITC-conjugated Annexin V (BD Pharmingen) (5 μg/10^5 cells, for 15 min at 22-25˚C in the dark). PI, at a final concentration of 5 μg/ml, was added to the samples 15-20 min before performing FACS using the FACSCalibur. NK cell samples were untreated, pre-treated with mouse IgG2b anti-human perforin (cat. no. NBP1-45774, δG9 clone; 1 μg/1×10^5 NK cells; Novus Biologicals), anti-human GNLY (cat. no. D185-3, RC8 clone; MBL International, 1 μg/1×10^5 NK cells) or a combination of anti-perforin and anti-GNLY mAbs for 30 min at 4˚C The subset of PKH26 labeled K-562 cells (stained red), detected as PI-negative and FITC-Annexin V-positive, were considered early apoptotic cells (23). The results are expressed as the difference in the percentage of early apoptotic K-562 cells in pretreated samples at a particular effector to target cell ratio, minus the percentage of apoptotic K-562 cells cultured in the medium only.

ELISA. Serum GNLY concentrations were measured using the LEGEND MAX™ Human Granulysin ELISA Kit (cat. no. 438007; BioLegend, Inc.). The sera of OA patients and controls were prepared from peripheral venous blood samples (3 ml/sample), which were taken by venipuncture in plastic Serum Separator Tubes, 3.5 ml (Greiner Bio-One GmbH), allowed to clot for 20 min at 22-25˚C, and centrifuged (1,000 x g, 10 min, at 4˚C). The supernatant (serum) was collected in Cryo.S, 2 ml, round bottom, screw-cap vial (Greiner Bio-One GmbH) and stored at -80˚C until required for ELISA. Assays were performed following the manufacturer's instructions, and the absorbance was measured using an MRX Revelation microplate reader (Dynex Technologies Inc.).

Statistical analysis. Data are presented as the median (25-75th percentile). The difference between two groups was assessed using the non-parametric Mann-Whitney U test (TIBCO Statistica, version 13.4.0.14). Differences between multiple groups were calculated using a Kruskal-Wallis non-parametric test and a post hoc Dunn's test using MedCalc Statistical version 20.011 (MedCalc Software Ltd.). P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical and laboratory characteristics of the patients. Table I compares the clinical and laboratory characteristics of the group of postmenopausal women with primary OA and controls. The median OA patients' BMI (25 and 75th percentiles) of 25 kg/m² (23-29 kg/m²) was comparable to that of the control group (26; 22-27 kg/m²), as were the values for systolic and diastolic blood pressure and glucose and LDL-cholesterol concentrations. Routine hematological parameters (erythrocytes, hemoglobin, leukocytes, thrombocytes), liver parameters (alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transferase), creatinine, urate and high sensitivity C-reactive protein levels did not differ between the groups. The patients were diagnosed with primary OA of the knee 6 (3.5-12) years ago. At the time of participation in the study, OA patients suffered from a pain intensity of 40/100 (20/100-50/100) mm and morning stiffness lasting 18 (10-20) min. Radiological staging of the disease in the affected joint was 2 (1-3) according to the Kellgren-Lawrence Scale. The medial knee compartment was affected in all 40 patients with OA, whereas both compartments (medial and lateral) were affected in 14/40 OA patients (35%).

GNLY expression. Fig. 1 illustrates GNLY expression in PBLs. All PBLs were observed to be within the lymphocyte gate region (R)1 in the forward scatter/side scatter dot plot in the OA patients (Fig. 1A) and controls (Fig. 1B). The dot plot of OA patients revealed 8.9% of GNLY+ cells gated in R2 compared to the IgG1 isotype control (0.2%), visualized in the histogram (Fig. 1A); 3.2% GNLY+ cells were found in the controls when compared with the isotype control (0.9%) (Fig. 1B). Immunoperoxidase staining was used to detect higher levels of GNLY in the OA patients compared with the controls, observed as red-labeled granules (Fig. 1C). Confocal microscopy provided better insight into the colocalization of GNLY (red fluorescence) and LAMP-1, a marker of exocytosis (green fluorescence) in OA patients (Fig. 1D) and controls (Fig. 1E). The colocalization of GNLY and LAMP-1 exhibited yellow fluorescence, resulting from the overlapping of green and red fluorescence in the cytotoxic effector cells of controls, particularly in OA patients, where it accumulated on one half
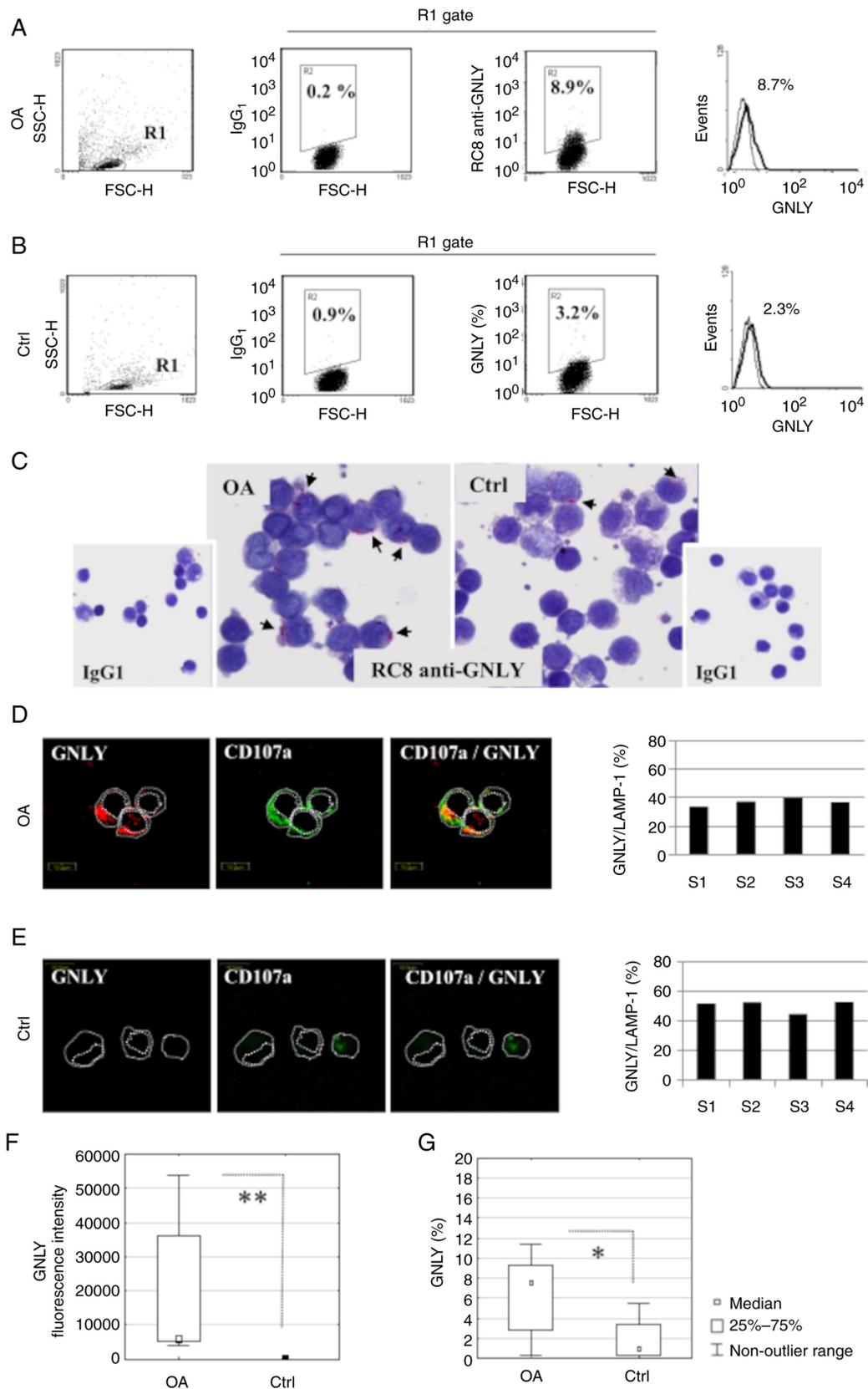
Figure 1. GNLY expression in the peripheral blood lymphocytes of OA patients and controls. Representative examples of dot plots and histograms showing the results from flow cytometry analyses of the R1 lymphocyte gate for R2 GNLY+ cells in the (A) OA patients and (B) controls in comparison to that of the isotype control (n=18). (C) Images show immunohistochemically labeled GNLY in lymphocytes (red color, indicated with arrows) compared with the isotype control. One of three independent samples is shown. Magnification, x1,000. Confocal laser microscopy of GNLY (red fluorescence) and LAMP-1 (green fluorescence) in lymphocytes from (D) OA patients and (E) controls. The colocalization of GNLY and LAMP-1 exhibited yellow fluorescence. The original magnification of the images are 900 x 8. Scale bar, 10 μm. The percentage of GNLY and LAMP-1 colocalization is shown for each of the four independent experiments/samples. (F) The intensities of GNLY fluorescence measured using confocal microscopy in 12 cells from OA patients and controls. (G) Percentage of GNLY+ lymphocytes in OA patients and controls measured using flow cytometry (n=18). *P=0.003, **P=0.000001 (Mann-Whitney U test). GNLY, granulysin; OA, osteoarthritis; R, region; LAMP-1, lysosomal-associated membrane protein-1; FSC-H, forward scatter-height; SSC-H, side scatter-height.
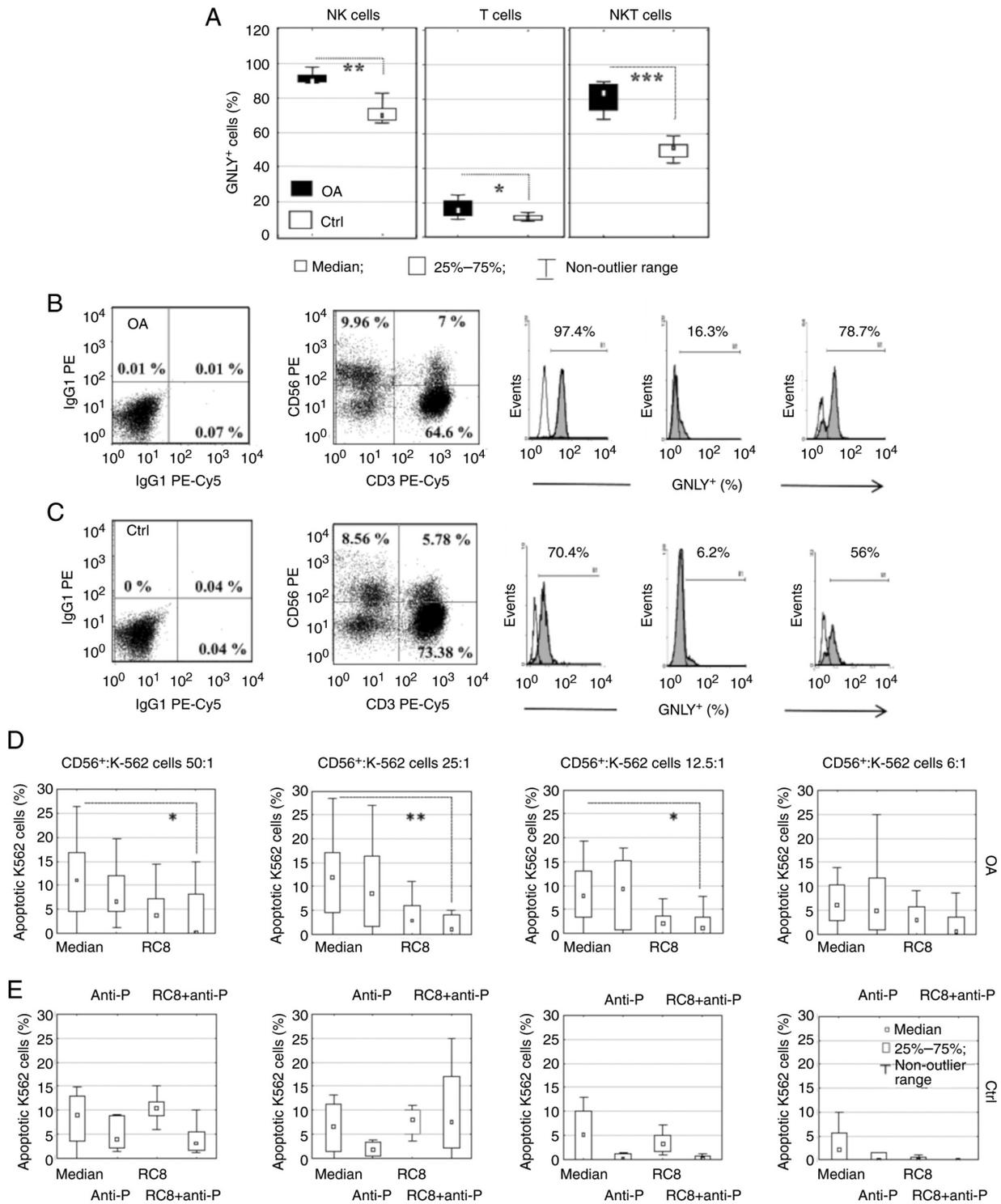
Figure 2. Proportion of GNLY+ cells in the peripheral blood subpopulations and analysis of GNLY mediated apoptosis in NK cells. (A) Frequency of GNLY in CD3⁻CD56⁺ NK cells, CD3⁺CD56⁻ T cells and CD3⁺CD56⁺ NKT cells between OA patients and controls. *P=0.03, **P=0.01, ***P=0.003 (Mann-Whitney U test) in 18 experiments. Quadrants in dot plots illustrate the analysis of lymphocyte subpopulations from (B) OA patients and (C) controls pertaining to CD3/CD56 labeling or isotype control, respectively. GNLY⁺ cells within lymphocyte subpopulations are shown as histograms for the (B) OA patients and (C) controls. Bars in the charts depict the median values of early apoptosis of K-562 cells cultured with NK cells from (D) OA patients or (E) control at the indicated ratios in the medium alone, with an anti-perforin mAb, with an anti-GNLY RC8 mAb, or with a combination of anti-perforin and anti-GNLY RC8 mAbs (n=5-6 per group). *P=0.01, **P=0.006 (Kruskal-Wallis and post-hoc Dunn's test). GNLY, granulysin; OA, osteoarthritis; anti-P, anti-perforin; PE, phycoerythrin.

of the cell, forming a signet ring shape. The individual values for each of the four independent experiments per group (bar samples S1-S4) are shown in the charts (Fig. 1D and E). The median percentage (25-75th percentiles) of GNLY localized

inside LAMP-1⁺ granules was 37.2% (35.4-38.5%) in the OA patients and did not significantly differ compared to the controls [51% (48-51.4%)] (Mann-Whitney U test). However, the intensity of GNLY labeling was significantly higher in
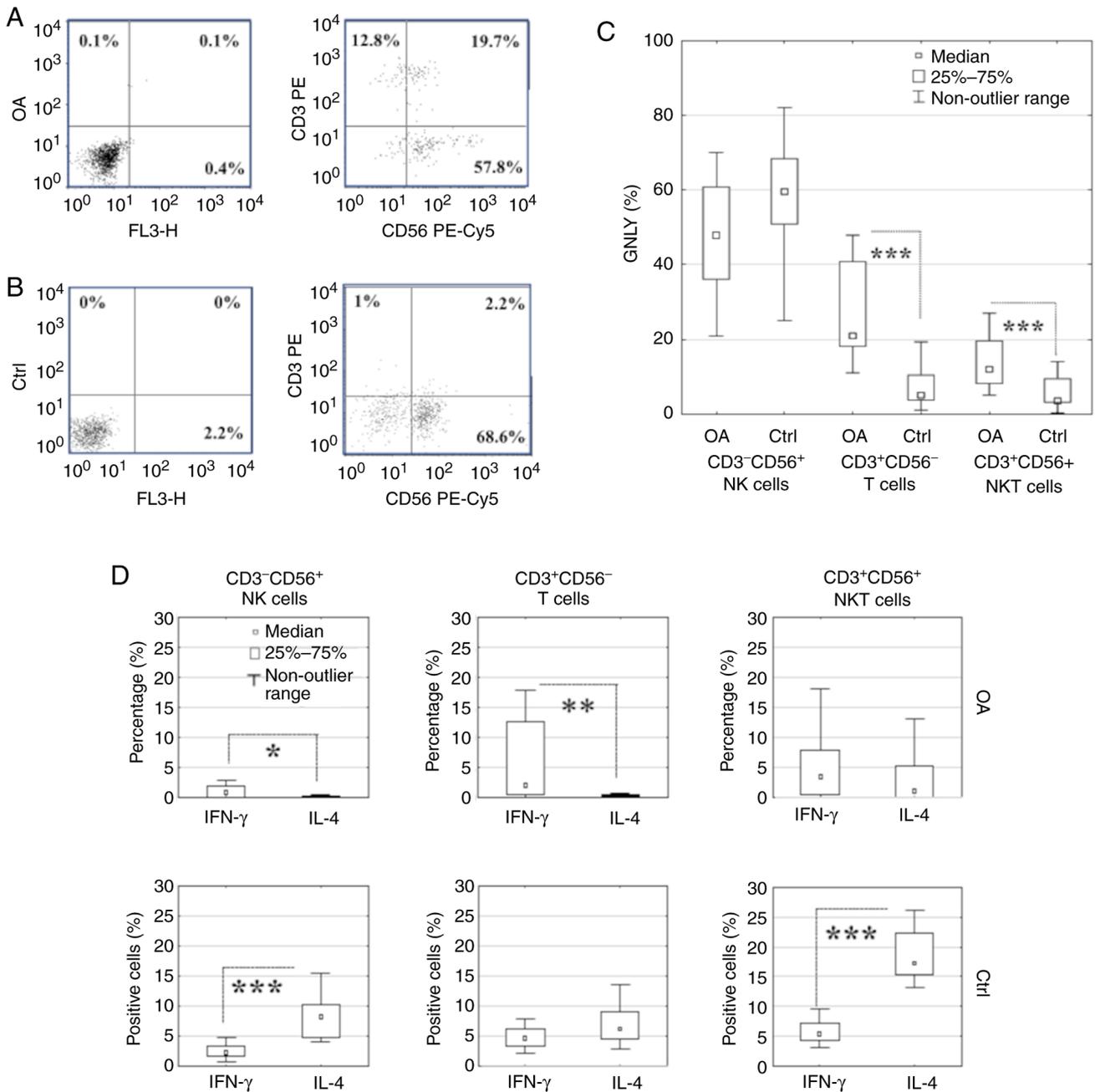
Figure 3. Distribution of GNLY and cytokine expression in the peripheral blood lymphocyte subpopulation. Dot plots show the distribution of GNLY gated cells in lymphocyte subpopulations pertaining to CD3/CD56 labeling or isotype control, respectively in (A) OA patients and (B) controls (n=6). ***P=0.004 (Kruskal-Wallis and post-hoc Dunn's test) (C). Comparison of intracellular IFN-γ and IL-4 expression in CD3‾CD56⁺ NK, CD3⁺CD56‾ T, and CD3⁺CD56⁺ NKT cells in the (D) OA patients and (E) controls (n=10-12). *P=0.03, **P=0.01, ***P=0.004 (Mann-Whitney U test). GNLY, granulysin; OA, osteoarthritis; PE, phycoerythrin; FL3, fluorescence channel 3.

OA patients than in controls (P=0.000001), as measured by flow cytometry (Fig. 1F). The frequency of GNLY⁺ PBLs, assessed by flow cytometry, was also higher [7.5% (2.8-9%)] in the OA patients compared with the controls [1.7% (0.3-3.9%)] (Fig. 1G). In the OA patients and controls, the GNLY serum concentrations, determined by ELISA were <0.3 ng/ml (Table I).

Cytotoxic potential of GNLY⁺ effector cells. The proportion of GNLY-expressing CD3‾CD56⁺ NK cells, CD3⁺CD56‾ T cells and CD3⁺CD56⁺ NKT subsets were significantly increased in the OA patients compared with the controls (P=0.01, P=0.03

and P=0.003, respectively; Fig. 2A). Dot plots illustrate the analysis of PBL subsets from OA patients (Fig. 2B) and controls (Fig. 2C) and show the frequency of CD3‾CD56⁺ NK (10 vs. 8.6%), CD3⁺CD56⁺ NKT (7 vs. 5.8%), and CD3⁺CD56‾ T cells (64.6 vs. 73.4%), with respect to isotype control. These values correspond to the median (25-75th percentiles) of the percentage of NK cells [8.3% (6.8-10%)], T cells [73.2% (70.4-76.5%)] and NKT cells [2.9% (2.1-3.9%)] in OA patients, and these did not significantly differ from the values of NK cells [10.3% (7.2-14.5%)], T cells [70.6% (67.2-75%)], and NKT cells [3.5% (2.3-5.4%)] in the controls. Histograms included in Fig. 2B and C illustrate the frequency

of GNLY-expressing PBL subsets in the OA patients and controls, respectively. The histograms were calculated based on the difference between the percentages of the GNLY$^+$ cells minus the percentage obtained in the isotype-matched control.

Early apoptosis did not differ significantly between the OA patients and controls when obtained cells were cultured in medium only [Fig. 2D and E, Mann-Whitney U test for each, effector: target ratio (E:T)]. However, the analysis of molecular mechanisms involved in the NK cell-mediated killing of K-562 targets revealed GNLY-mediated early apoptosis only in the OA patients (Fig. 2D). The combination of anti-GNLY and anti-perforin mAbs almost completely abolished early apoptosis in cells, at E:T cell ratios of 50:1 (P=0.01), 25:1 (P=0.006) and 12.5:1 (P=0.01) (Fig. 2D), but without an additional effect compared to the cells treated with anti-GNLY antibody alone. In controls, the addition of anti-GNLY antibody or the combination of anti-GNLY antibodies with an anti-perforin mAb did not significantly alter the proportion of early apoptotic cells (Fig. 2E). Anti-perforin mAb alone did not affect the early apoptosis of K-562 cells in the OA patients (Fig. 2D) or controls (Fig. 2E).

Distribution of GNLY and intracellular cytokine expression in PBL subsets. The distribution of GNLY was analyzed in gated GNLY$^+$ cells observed in the fluorescence 2/fluorescence 3 dot plots showing CD3/CD56 labeling in OA patients (Fig. 3A) and controls (Fig. 3B) in comparison to their isotype controls. Considering the samples from OA patients vs. that from controls, the GNLY distribution percentages were 57.8 vs. 68.6% in CD3$^-$CD56$^+$ NK cells, 12.8 vs. 1% in CD3$^+$CD56$^-$ T cells, 19.7 vs. 2.2% in CD3$^+$CD56$^+$ NKT cells, and negligible in the isotype controls (Fig. 3A and B). The T and NKT cells of OA patients had significantly higher GNLY distribution than that in the controls (P=0.004; Fig. 3C).

Analysis of intracellular IFN-γ and IL-4 expression levels revealed differences in the balance between the OA patients (Fig. 3D) and controls (Fig. 3E). IFN-γ dominated over IL-4 in NK and T cells of OA patients (P=0.03 and P=0.01, respectively), while they were codominant in NKT cells (Fig. 3D). In the controls, IL-4 levels were higher than IFN-γ levels in NK and NKT cells (P=0.004), and it was only marginally increased in T cells (P=0.07; Fig. 3E).

Discussion

In the present study, it was shown that the frequency of GNLY$^+$ PBLs was higher in a group of primary OA patients compared with the control group using an anti-GNLY mAb of RC8 clone, which binds to the common core epitope of both the 9 and 15 kDa isoforms (24). Due to a high percentage of GNLY+ cells (~30%) in the peripheral blood of OA patients and a wide scattering of the results in our pilot study (3), the OA group and controls in this investigation were defined more precisely according to the exclusion criteria mentioned above. Increased expression of GNLY in NK, T, and NKT cells in the OA patients is consistent with the slightly dominant systemic, low grade pro-inflammatory immune response in OA (1,16), and the knowledge that GNLY increases inflammation (25,26). Here, the dominance of Th1 IFN-γ over Th2 IL-4 in the NK and T cells he peripheral blood of OA patients

was demonstrated, whereas in controls IL-4 dominated over IFN-γ in the NK and NKT cell subsets. Correspondingly, Th1 and cytotoxic T cells producing IFN-γ were found in the synovial fluid and joint tissues of OA patients (2). IFN-γ mRNA and protein expression levels in T and NK cell types depends on the JAK/STAT signaling pathway, which is known to enhance the immune response (7,15). This corresponds to an increased intensity of GNLY labeling in PBLs of OA patients, compared with the negligible fluorescent signals of GNLY in controls. It is known that mild expression of GNLY in T cells and constitutive expression of GNLY in innate NK cell-effectors increases following activation by antigen or NK-cell receptor ligands (10). Immunocytochemistry illustrated a greater proportion of GNLY+ PBLs in the OA patients compared with the controls. Moreover, it was shown here for the first time that the NK cells of OA patients employed GNLY-mediated apoptosis to kill K-562 cells *in vitro*, in contrast to the controls, whose NK cells exhibited low levels of GNLY and thus could not employ GNLY-mediated cytotoxicity. GNLY acts in concert with perforin in the realization of K-562 cell apoptosis, as the anti-GNLY mAb of RC8 clone significantly decreased early apoptosis in the OA patients only. The perforin pores enable the entry of GNLY into cells, but perforin does not perform apoptosis by itself (10,27). The apoptotic process is initiated by intracellular Ca^{2+} increase, membrane damage, mitochondrial reactive oxygen species generation, release of apoptosis-inducing factor, cytochrome C and activation of caspase 3, which disrupt the normal physiology of targets (10,26). Target cells are killed through induction of apoptosis, but also by necrosis 4 h after the rapid entry of GNLY through perforin pores (26,27), which meant a proportion of cells were not included in the Annexin$^+$PI$^-$ subset (gate) used for the detection of early apoptotic targets by flow cytometry at the end of an 18 h cytotoxicity test (23).

Killing of K-562 targets, which are devoid of HLA class I antigens, represents a model for *in vitro* analysis of NK cell-mediated self-aggression, as damaged tissue cells down-regulate HLA class I antigens and become more susceptible to NK cell cytotoxicity (28). This implies that activated NK cells in the circulation may harm endothelial cells during atherogenesis, independently or in concert with classical metabolic risk factors and support endothelial dysfunction underlying cardiovascular diseases (29). It was previously shown that >80% of OA patients suffer from arterial hypertension and ~30% are diabetic (30), as reflected in the OA patients group in this investigation, signifying metabolically triggered systemic inflammation (31,32). All patients with OA enrolled in this study, as well as the controls, had hypercholesterolemia, as a component of the metabolic syndrome. An intake of particular kinds of fats could affect knee OA progression independent of weight and induce metabolically triggered low grade, systemic inflammation (31,33). We enrolled the non-obese patients with primary OA, as patients with disease of unknown cause. However, the contribution of biochemical and metabolic factors, together with the factors that can be modified by age, heritage and behavior cannot be completely excluded (34). Additionally, knee OA affects men and women differently, with women exhibiting increased disease frequency, severity and disability (35). To avoid sex-based differences and the possible role of estrogen in granulysin expression (36), only

postmenopausal women were recruited in the OA group and controls in this study. To visualize GNLY and LAMP-1, a marker of lysosome-related secretory organelles (37), confocal microscopy was used. Confocal microscopy confirmed more frequent visualization of GNLY in PBLs from the OA group compared with the controls, although the colocalization of GNLY and LAMP-1 was ~40% in both groups. It indicates the accumulation of cytotoxic 9 kDa GNLY in dense, exocytic granules, whereas 15 kDa GNLY is not associated with LAMP-1⁺ organelles and does not overlap with 9 kDa GNLY (9). The 15 kDa form of GNLY is released by activated cells in a protein kinase C-dependent manner (9) and lacks cytotoxic activity in eukaryotic cells (13). It represents the soluble serum GNLY with regulatory functions (24), which was negligible in the OA patients and controls.

Based on the results shown herein, it was concluded that in women with knee OA, GNLY expression in the PBL subsets and GNLY-mediated apoptosis of K-562 targets were higher than that in the controls, alongside lymphocyte pro-inflammatory polarization. However, it would be optimal to broaden the study with a larger number of patients and different OA phenotypes in both sexes to reach a more definitive conclusion and/or determine differences between the sexes.

Acknowledgements

Availability of data and materials

The data sets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, acquisition of data and analysis were performed by VD, GL, BĆC, LK, MA, MR, TK and DL. The first draft of the manuscript was written by GL and DR. All authors revised the manuscript. All authors have read and approved the final manuscript (except DR who has unfortunately passed away). VD, GL and BĆC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All participants provided written informed consent. The study was approved by the Ethical Committees of Thalassotherapia-Opatija Hospital and the Faculty of Medicine, University of Rijeka, Rijeka, Croatia (approval no. 01-000-00-450/2018). All experimental procedures were performed according to the ethical principles for medical research involving human subjects outlined in the World Medical Association Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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