Psoriasis is considered to be a cytokine-driven immune-mediated disease, although the cell cytotoxicity mechanisms involved remain unrecognized. Herein, we analyzed granulysin expression in different lymphocyte subsets of peripheral blood of 40 psoriatic patients (20 with severe and 20 with mild psoriasis) and seven sample of psoriatic skin. The simultaneous detection of intracellular granulysin and cell surface antigens was performed using flow cytometry in peripheral blood and immunohistochemistry in skin lesions. The frequency of granulysin+ cells, mean fluorescence intensity for granulysin, and the frequency of CD8+ T lymphocytes, NK cells, and NKT cells expressing granulysin molecules in peripheral blood were significantly higher in patients with severe psoriasis compared to mild disease and healthy individuals. These were also correlated with disease severity. Furthermore, granulysin+ cells, CD8+granulysin+ T lymphocytes, and CD56+granulysin+ NK cells were present in a higher frequency in the epidermal basal cell layer and in the dermal infiltrate of lesional skin as compared to non-lesional and healthy skin. In conclusion, granulysin+ cytotoxic cells are upregulated in blood and lesions of patients with psoriasis suggesting the involvement of granulysin mediated cytotoxicity in psoriasis pathogenesis.

Key words: cytotoxicity; granulysin; psoriasis; T lymphocytes.

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Psoriasis is a chronic inflammatory skin disease that affects around 2% of the population (1). Although the disease may present with different clinical features, the most common presentation is the vulgar form, characterized by erythematous plaques covering the elbows, knees, umbilical area, or in more severe cases, even larger body surfaces (2). Although the exact mechanism of skin lesion development remains unclear, psoriasis is generally perceived as an inflammatory cell-mediated skin disease (3). The most common inflammatory cells in the peripheral blood, skin, and joints are T lymphocytes, but the significance of dendritic cells (DCs), macrophages, natural killer (NK) cells, and natural killer T (NKT) cells has also been confirmed (4).

Two main cytotoxic lymphocyte subsets, CD8+ T cells and NK cells, are capable of inducing target cell death by releasing cytotoxic armamentarium, such as perforin, granzyme B, and granulysin (GNLY) (5). The upregulation of perforin and granzyme B in the peripheral blood and lesional skin of psoriasis patients has been previously demonstrated (6–8). The presence of GNLY was only recently detected in psoriatic lesions. However, its specific involvement and role in psoriasis pathogenesis is still unclear (9, 10).

GNLY is a cytotoxic molecule with lytic properties expressed in activated T, NK, and NKT cells (11, 12). The cytotoxic effects of GNLY on tumor cells and cells infected by a variety of microorganisms have been confirmed (11, 12). However, GNLY also serves as a chemoattractant for T lymphocytes, monocytes, and NK cells, in addition to attracting and activating DCs (11).

In our previous studies, we have demonstrated upregulation of perforin molecule in peripheral blood of severe psoriasis mainly in the cytotoxic cell subtypes such as CD4+ T lymphocytes, CD8+ T lymphocytes and NK cells (8, 13). We have also found an increase of perforin positive cells in epidermis of psoriasis lesions (7). We suggested an involvement of perforin-mediated cytotoxicity in psoriasis pathogenesis.

Herein, we hypothesized that GNLY expression profiles would play an important role in disease pathogenesis. Consequently, we examined the dynamics of systemic GNLY expression in peripheral blood lymphocytes...
and cytotoxic subtypes of psoriatic patients. We also examined local changes in psoriatic skin lesions and determined the correlation between GNLY levels and disease severity.

**METHODS**

**Study design and participants**

We conducted a cross-sectional analytic study comprising a total of 40 patients, recruited from the Dermatovenerology Department of Clinical Hospital Center Rijeka, all with clinically defined and histopathologically confirmed vulgar psoriasis. The following individuals were excluded from the study: children, persons older than 70 years, those who have psoriatic arthritis (PsA) or any systemic, immunological, or malignant disease, and those who received either systemic psoriasis treatment during the four months before testing or local therapy during the two weeks before the trial. After collecting historical data from the patients and performing an overall physical and dermatological examination, the disease severity was determined according to the psoriasis area and severity index (PASI), where PASI >10 defines severe and PASI <10 defines mild disease (14). The age of patients ranged from 18 to 70 (mean age 49), and the PASI ranged between 1.2 and 37.4 (mean PASI 10.7).

The group of severe psoriasis patients consisted of 13 males and 7 females, with age ranging from 25 to 70 (mean age 49), and a PASI between 10 and 37.4 (mean PASI 22.91). Patients with severe psoriasis had body mass index (BMI) ranging from 25.3 to 28.9 (mean BMI 27.8) and 10 of them have comorbidities such as cardiovascular (4 cases of arterial hypertension), diabetes mellitus (1 case) and depression (2 cases) (Table I). Patients with mild psoriasis comprised a group of 7 males and 13 females, whose age ranged from 18 to 70 (mean age 49), and with PASI between 1.2 and 9.4 (mean PASI 4.97). They had BMI between 22.6 and 28.1 (mean BMI 25.4) and have cardiovascular comorbidities (4 cases of arterial hypertension) and diabetes mellitus (1 case) (Table I).

The control group consisted of 20 healthy volunteers, 8 males and 12 females, age between 23 and 68 (mean age 45) and two of them had cardiovascular comorbidities, namely arterial hypertension (Table I).

The study was approved by the Ethics Committee of the Medical Faculty and Clinical Hospital Center Rijeka and conducted according to the World Medical Association guidelines, outlined in the newest declaration of Helsinki. All patients enrolled in the study provided informed consent.

**Lymphocyte preparation**

Peripheral venous blood samples were collected in a heparinized vacutainer (Becton-Dickinson, Franklin Lakes, NY, USA) from all participants, then overlaid onto a lymphoprep (Nycoderm Pharma, Oslo, Norway), and centrifuged (600 × g, 20 min). Peripheral blood mononuclear cells (PBMCs) accumulating at the interface were collected, washed twice (400 × g, 10 min) in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Auckland, New Zealand), and then resuspended in tissue culture medium (RPMI 1640 supplemented with l-glutamate (2 mM), penicillin (1.0 × 10⁵ U∙l–¹), streptomycin sulfate (0.05 g l–¹), and 10% fetal calf serum (all from GIBCO, Gaithersburg, MD, USA). Cell viability exceeded 90%, and was checked by propidium iodide (0.5 µg∙ml –1/1.0 × 10⁶ cells; Sigma-Aldrich Chemie, Steinheim, Germany) and analyzed by a FACSCalibur flow cytometer using CellQuestPro software (Becton-Dickinson, San Jose, CA, USA).

**Table I. The epidemiological data of patients and healthy individuals**

<table>
<thead>
<tr>
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<th>Healthy individuals (n = 20)</th>
<th>Mild psoriasis (n = 20)</th>
<th>Severe psoriasis (n = 20)</th>
</tr>
</thead>
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<tr>
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<td>23–68 (45)</td>
<td>18–70 (49)</td>
<td>25–70 (49)</td>
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<tr>
<td>Sex, M/F, n</td>
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<td>7/13</td>
<td>13/7</td>
</tr>
<tr>
<td>PASI, range (mean)</td>
<td>–</td>
<td>1.2–9.4 (4.97)</td>
<td>10–37.4 (22.91)</td>
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<tr>
<td>Smoking, M/F, n</td>
<td>7/3</td>
<td>6/4</td>
<td>9/3</td>
</tr>
<tr>
<td>Body mass index, range (mean)</td>
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<td>22.6–28.1 (25.4)</td>
<td>25.3–28.9 (27.8)</td>
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<tr>
<td></td>
<td>Diabetes mellitus 0</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>Depression 0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

**Simultaneous detection of cell surface and intracellular antigens by flow cytometry**

The simultaneous detection of surface and intracellular antigens was performed as described here. PBMCs (3.0 × 10⁶ cells) were fixed, washed, and permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BBD Biosciences, Erembodegen, Belgium). Mouse anti-GNLY monoclonal antibody (mAb; clone RC8) or isotype-matched mouse IgG1 (clone MOPC-1) was added (1 µg/1.0 × 10⁶ cells). After washing, fluorescein isothiocyanate (FITC)-conjugated secondary goat anti-mouse polyclonal antibodies (IgG1, IgG2, IgG2h, and IgG3) were added to the cells. Then, the cells were labeled with mouse anti-CD3 mAb (clone UCHT-1) conjugated with phycoerythrin cytochrome 5 (PE-Cy5), mouse anti-CD4 mAb (clone RPA-T4) conjugated with phycoerythrin (PE), mouse anti-CD8 mAb (clone RPA-T8) conjugated with phycoerythrin (PE) and mouse anti-CD56 mAb (clone NCAM 16.2) conjugated with fluorescein isothiocyanate (FITC). FITC, Cy-PE5 and PE-conjugated mouse IgG1 antibodies were used as controls. All antibodies were provided by BD Biosciences (Erembodegen, Belgium) and used at a concentration of 20 µl/1.0 × 10⁶ cells for 30 min at 4°C. Cells were analyzed by FACSCalibur (Becton-Dickinson) using CellQuestPro software (Becton-Dickinson). GNLY expression was analyzed in the total lymphocyte population and in the CD3⁴ CD4⁴, CD3⁸ CD8⁴, CD3 CD56, and CD3 CD56 subpopulations. The mean fluorescence intensity (MFI) was measured to determine the mean number of molecules per cell. The results were calculated as the difference in the percentage of GNLY⁺ cells or GNLY MI between samples labeled with the anti-GNLY mAb and isotype-matched controls.

**Skin biopsies**

Skin specimens, each 5 mm in diameter, were taken from 7 participants under local anesthesia. Two biopsies were obtained from every patient; one from the inside border of psoriatic plaques, and the other from uninvolved skin, at least 3 cm from the affected skin. Skin samples from healthy controls were obtained from the peripheries of surgical excisions of benign tumors (fibromas). Tissue samples were then fixed in buffered formalin, embedded in paraffin, and processed for histopathological diagnosis confirmation. The rest of the tissues were used for immunohistochemical investigation.

**Immunohistochemistry**

Paraffin-embedded tissue was cut into 3 µm sections, deparaffinized and rehydrated through graded alcohols. Antigen retrieval was performed by microwave treatment in Tris/EDTA buffer with pH 9 for 15 min, followed by cooling for 20 min at 22°C (room temperature; RT). Slides were blocked with 5% BSA before incubation.
with mouse anti-GNLY monoclonal antibody (Leica Biosystems, Novocastra, UK) or antibody diluent (DAKO, Carpinteria, CA, USA) for 1 h at RT. The dilution ratio was 1:20. Specific binding detection was provided by EnVision/DAB+ System (DAKO, Glostrup, Denmark). Tris-buffered saline (TBS) was used to wash slides between individual steps. A diaminobenzidine (DAB) was distilled on the slides and left for 10 min. The slides were then washed and incubated for 30 min with mouse anti-CD8 monoclonal antibody (clone C8/144B) (diluted 1:100) or mouse anti-CD56 monoclonal antibody (clone MRQ-42) (diluted 1:1000) (both DAKO, Glostrup, Denmark) for 30 min at RT. For the negative control, an irrelevant mouse monoclonal IgG antibody was used. After washing, biotinylated secondary goat anti-mouse antibodies were added for 30 min, followed by streptavidin with alkaline phosphatase for 30 min (DAKO Real Detection System Alkaline Phosphatase/RED Rabbit Mouse) at RT. Tris-buffered saline (TBS) was used to wash slides between individual steps. Sections were counterstained with hematoxylin. Positive cells were counted under 400× magnification in 5 fields. The number of positive cells per 5 fields was analyzed by two independent investigators in a blinded fashion. The results are shown as a percentage of double positively stained cells among all CD8+ T lymphocytes and NK cells, respectively (DAB stained cells).

Statistical analysis

Statistical analysis was performed with the Statistica 13.2 data analysis software system (StatSoft, Inc., Tulsa, OK, USA). Differences in the values of quantitative variables between two groups were assessed with the Mann-Whitney U-test, while the differences between 3 groups were evaluated with the Kruskal-Wallis test. This was followed by post-hoc analysis for detection of differences between individual groups. Statistical significance was set at \( p < 0.05 \). Data are presented as median values and as 25/75% values (25th percentile/75th percentile). For the purpose of investigating the correlation of continuous numerical variables, the correlation was used. Only statistically significant coefficients of correlation (with criterion \( p < 0.05 \)) were interpreted.

RESULTS

Granulysin is highly expressed in cytotoxic cell populations of peripheral blood in psoriatic patients compared to healthy individuals

The frequency of GNLY+ cells in peripheral blood (Fig. 1) was significantly higher in patients with severe disease than in patients with mild disease, and was higher in both disease groups than in healthy individuals (Fig. 1b). Similar results were also obtained for the MFIs (Fig. 1c), suggesting an abundance of GNLY in cytotoxic cells of psoriatic patients. Significant positive correlation between GNLY expression and disease severity (represented by PASI) was confirmed by chi-square testing (Fig. 1d) (see also Tables SI and SII1).

The frequency of CD3+CD4+GNLY+ cells and CD3+CD8+GNLY+ cells in both groups of psoriasis pa-
Granulysin expression increase in severe psoriasis

Patients was significantly higher than in healthy individuals (Fig. 2a, b). There was also a higher frequency of GNLY in CD3+CD8+ T lymphocytes in severe psoriasis patients compared to those with mild disease (Fig. 2b). Similar results were obtained for CD3−CD56+ NK cells and CD3+CD56+ NKT cells, with higher frequencies of these cells among the PBLs of psoriasis patients (Fig. 2c, d). GNLY expression among NKT cells in severe disease was also higher than in the mild form (Fig. 2d). Positive correlations between both CD3+CD8+GNLY+ T lymphocytes and CD3+CD56+GNLY+ NKT cells and PASI were confirmed by chi-square testing (Fig. 2e, f). There was no correlation between CD56+GNLY+ cells and PASI (not shown).

Fig. 2. Triple-positive (granulysin+ [GNLY] and surface markers) peripheral blood lymphocytes of patients with severe and mild psoriasis and healthy individuals. Percentages of CD3+CD4+GNLY+ cells (a), CD3+CD8+GNLY+ cells (b), CD3+CD56+GNLY+ natural killer (NK) cells (c) and CD3+CD56+GNLY+ NKT cells (d) in peripheral blood lymphocytes of healthy controls (white bar) compared to mild psoriasis patients (gray bar) and severe psoriasis patients (black bar) (n = 20 per group). Levels of significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (e) Correlation between GNLY expression in CD8+ T lymphocytes and disease severity (PASI index) (r = 0.529, p < 0.001). (f) Correlation between GNLY expression in CD3+CD56+ NKT cells and disease severity (PASI index) (r = 0.3106, p < 0.05).

Fig. 3. Immunohistochemical evaluation of granulysin+ (GNLY+) cells in lesional psoriatic skin compared to uninvolved skin and healthy skin. Frequency of GNLY+ cells (median value: 25th/75th percentile) in healthy skin (white bar), nonlesional skin (gray bar), and lesional skin of psoriatic patients (black bar) in 7 experiments in each group, in both skin compartments (a) and separately in the epidermis (b) and dermis (c) (n = 7 per group). Levels of significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Distribution of GNLY+ cells in psoriasis lesional skin [magnification ×200 (d) and ×400 (e)], nonlesional skin (f), and healthy skin (g). GNLY+ cells are indicated with a red arrow.
Granulysin$^+$ cells are highly abundant in psoriatic plaques compared to nonlesional and healthy skin

GNLY$^+$ cells are present with higher frequency in lesional skin compared to nonlesional and healthy skin (Fig. 3a). This upregulation of GNLY$^+$ cells was found in the epidermal and dermal compartments of lesional psoriatic skin (Fig. 3b, c). Distribution analysis showed that in psoriatic plaques, GNLY$^+$ cells were mostly found in dermal infiltrates and in the basal and suprabasal compartments of the epidermis (Fig. 3d, e). Meanwhile, there were scarce positive cells found in nonlesional skin (Fig. 3f). Positive cells were completely absent in healthy skin (Fig. 3g).

**CD8$^+$ T lymphocytes and natural killer cells that express granulysin predominate in psoriatic skin lesions compared to nonlesional and healthy skin**

A significantly higher accumulation of CD8$^+$ GNLY$^+$ cells in psoriatic lesional skin was found compared with nonlesional and healthy skin (Fig. 4a). The majority of CD8$^+$ GNLY$^+$ cells were located in the epidermis of psoriatic plaques (12%), compared to 2% in nonlesional skin and complete absence in the epidermal compartment of healthy skin (Fig. 4b). In the dermis, CD8$^+$ GNLY$^+$ cells were also found with higher frequency in lesional skin than nonlesional and healthy skin (Fig. 4c). The distribution of CD8$^+$ GNLY$^+$ cells in psoriatic plaque, nonlesional skin, and healthy skin showed the prevalence of those cells (indicated by a red arrow) in the epidermis, mainly in the basal and suprabasal epidermal layer of psoriatic lesion (Fig. 4d, e). There were very scarce CD8$^+$ GNLY$^+$ cells in nonlesional skin (Fig. 4f) and complete absence of these cells in healthy skin (Fig. 4g).

CD56$^+$ GNLY$^+$ NK cells were also upregulated in psoriatic skin lesions (Fig. 5a). In the epidermal compartment of lesional skin, we found 80% double positive cells compared to only 0.5% in nonlesional skin ($p<0.01$). Meanwhile, there were no CD56$^+$ GNLY$^+$ NK cells in healthy skin (Fig. 5b). In the dermis, CD56$^+$ GNLY$^+$ cells were mainly found in dermal infiltrates (around 60%), compared to 20% in nonlesional skin and 15% in healthy skin (Fig. 5c). A distribution of these cells showed that they were mainly present in the basal cell layer of the epidermis and in dermal infiltrates of lesional psoriatic skin (indicated by a red arrow) (Fig. 5d, e). However, they were scarce in nonlesional and healthy skin (Fig. 5f, g).

**DISCUSSION**

Cell-mediated cytotoxicity is the ability of certain cells, mostly CD8$^+$ and NK cells, to induce target cell lysis (15, 16). This mechanism involves granule exocytosis and the release of cytolitic molecules, such as perforin,
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Perforin and granzyme B are found in psoriatic plaques (6, 17). Perforin is located in the epidermal compartment of psoriatic plaque, mainly at the epidermal–dermal junction and to a lesser degree, in dermal infiltrates resembling sites of CD8 infiltration (7, 17). Expression of granzyme B is also increased in psoriasis in both CD4+ and CD8+ T cells (17).

A spongiform degeneration of the basal membrane was seen at the site where dermal perforin+ cells migrate into epidermis suggesting their possible attack on psoriatic keratinocytes (7).

GNLY+ T cells are mainly found in the papillary dermis of psoriatic plaques and their number increases with increasing clinical severity of disease (9, 10). Herein, for the first time, we demonstrated that CD8+ T lymphocytes and NK cells expressing GNLY molecule were several times more frequent in psoriasis skin, especially in epidermis compared to nonlesional and healthy skin.

The involvement of perforin in the systemic immune response in psoriasis has also been demonstrated, and the majority of T lymphocytes that express perforin in peripheral blood are of the CD8 and NK cell phenotype (8, 13, 18). GNLY-mediated cytotoxicity in psoriasis pathogenesis was highlighted by the results presented in this paper. For the first time we showed that GNLY molecule is highly expressed in CD8+ T lymphocytes, NK and NKT cells in peripheral blood and that frequency of those cells correlates with disease severity. We also observed a rise in the MFI in severe psoriasis. The abundance of cytotoxic cells in circulation is a source of new cells migrating to skin lesions, mainly epidermis and to a certain extent dermis, possibly performing cell mediated lysis.

Increases in GNLY expression in tissue and blood have been reported in various clinical entities (19, 20). In psoriatic arthritis, GNLY is detected at higher levels in PBLs, mainly in NK cells (21). In skin lesions, GNLY has so far been discovered in severe cutaneous adverse reactions, such as Stevens-Johnson syndrome and toxic epidermal necrolysis, where it serves as a key mediator for disseminated keratinocyte death (22, 23). The cytotoxic effect of GNLY has also been implicated in the apoptosis of basal keratinocytes in lichen planus lesions (24).

In psoriatic skin lesions the majority of CD8+ T cells in the epidermis show oligoclonality, so they probably respond to a still undetermined antigen that is presented by antigen presenting cells, such as dendritic cells or keratinocytes (25, 26). Dermal Langerhans cell-like DC stimulate CD8+ T cells to a subsequent proliferation directly or indirectly with the help of CD4+ T cells (27). The epidermal CD8+ T lymphocytes are in close association with keratinocytes, and rich with perforin, GNLY, and other cytotoxic molecules (6, 7). Although we have not examined effector function of GNLY+CD8+ T lymphocytes directly on keratinocytes in this study we could hypothesize that virally and bacterially damaged keratinocytes could be a potential target to GNLY/perforin cytotoxic attack. This could lead to withdrawal of damaged keratinocytes that are the possible proliferation...
stimulus and subsequent stoppage of further keratinocyte proliferation. However, the extended experiments on cytotoxic effect of GNLY molecule in keratinocyte cultures derived from psoriasis skin lesions are needed for better insight in the role of GNLY molecule in the beginning, development or even termination of the immunologic events in psoriasis.

In conclusion, the results of this study showed higher frequency of CD8+ T lymphocytes and NK and NKT cells that express GNLY molecule in blood and lesions of severe psoriasis and its correlation with disease severity suggesting that besides the complex cytokine network involved in psoriasis and its effects on immune cells and keratinocytes (28), cytolytic molecules could be strongly involved in disease pathogenesis.

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The authors have no conflicts of interest to declare.

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