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Increased percentage of CD8⁺CD28⁻ suppressor lymphocytes in peripheral blood and skin infiltrates correlates with advanced disease in patients with cutaneous T-cell lymphomas

Zwiększony odsetek limfocytów supresorowych CD8⁺CD28⁻ we krwi obwodowej i naciekach skórnych koreluje ze stadium zaawansowania choroby u pacjentów z chłoniakami T-komórkowymi skóry (CTCL)

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

Donata Urbaniak-Kujda^{1,2,AEF}, Katarzyna Kapelko-Słowik^{1AB}, Dariusz Wołowiec^{1DE}, Jarosław Dybko^{1GD}, Agnieszka Hałoń^{3B}, Bożena Jaźwiec^{1B}, Joanna Maj^{4B}, Alina Jankowska-Konsur^{4B}, Kazimierz Kuliczkowski^{1ABG}

¹ Katedra i Klinika Hematologii Nowotworów Krwi i Transplantacji Szpiku Akademii Medycznej we Wrocławiu

² Wyższa Szkoła Fizjoterapii we Wrocławiu

³ Katedra i Zakład Anatomii Patologicznej Akademii Medycznej we Wrocławiu

⁴ Katedra i Klinika Dermatologii Akademii Medycznej we Wrocławiu

Summary

Introduction:

T cells with the CD8⁺CD28⁻ phenotype are CD8⁺ lymphocytes with regulatory function. Their increased numbers were observed in infections, autoimmune and neoplastic diseases, and in elderly healthy individuals. CD8⁺CD28⁻ lymphocyte levels in patients with cutaneous T-cell lymphoma (CTCL) has not yet been described. The aim of the study was to determine their levels in these patients' peripheral blood and cutaneous infiltrates and their relation to the clinical stage of disease.

Material/Methods:

Forty-one untreated patients, 26 males and 15 females, with CTCL were enrolled in the study. CD8⁺CD28⁻ lymphocyte levels were determined by flow cytometry in peripheral blood and by immunochemistry in skin infiltrates.

Results:

The percentage of CD8⁺CD28⁻ lymphocytes in the peripheral blood of the patients was significantly higher than in the controls. Patients with advanced disease displayed a higher percentage of CD8⁺CD28⁻ lymphocytes in the peripheral blood and skin than did the individuals with early stages of the disease. Moreover, positive correlations between CD8⁺CD28⁻ lymphocyte level in peripheral blood and age, clinical stage, and the levels in the skin infiltrates was revealed. Additionally, the percentage of CD8⁺CD28⁻ T cells in the skin infiltrates correlated positively with age and clinical stage of the disease.

Conclusions:

These data suggest that CD8⁺CD28⁻ lymphocytes play an important role in the development of immunotolerance in the progression of cutaneous T-cell lymphoma.

Key words:

CD8⁺CD28⁻ lymphocytes • cutaneous T-cell lymphoma

Streszczenie

Wstęp: Limfocyty CD8⁺CD28⁻ są podtypem limfocytów CD8⁺, które pełnią funkcje regulatorowe. Zwiększona proporcja limfocytów CD8⁺CD28⁻ została udokumentowana w chorobach infekcyjnych, autoimmunologicznych i nowotworowych oraz u ludzi starszych. Nie ma natomiast danych na temat ich liczby w pierwotnych T-komórkowych chłoniakach skóry (CTCL – cutaneous T cell lymphoma). Celem naszych badań była ocena odsetka limfocytów CD8⁺ CD28⁻ we krwi obwodowej i naciekach skórnych u nieleczonych chorych z CTCL oraz zbadanie czy ich liczba jest związana z zaawansowaniem procesu nowotworowego.

Materiał/Metody: Badaniami objęto 41 nieleczonych chorych z rozpoznaniem CTCL: 26 mężczyzn, 15 kobiet. Odsetek komórek CD8⁺CD28⁻ we krwi obwodowej oznaczono przy użyciu cytofluorymetrii przepływowej, natomiast w skórze metodą immunohistochemiczną.

Wyniki: Odsetek limfocytów CD8⁺CD28⁻ we krwi obwodowej chorych był statystycznie istotnie wyższy niż w grupie kontrolnej. Ponadto u pacjentów z zaawansowaną postacią chłoniaka odsetek CD8⁺CD28⁻ we krwi obwodowej i skórze był statystycznie wyższy w porównaniu z pacjentami we wczesnym stadium choroby. Wykazaliśmy także znamienne dodatnią korelację pomiędzy odsetkiem komórek CD8⁺CD28⁻ we krwi obwodowej, a wiekiem, stadium zaawansowania choroby oraz odsetkiem komórek CD8⁺CD28⁻ w skórze. Ponadto odsetek komórek CD8⁺CD28⁻ w skórze osób chorych korelował znamienne dodatnio z wiekiem oraz ze stadium zaawansowania choroby.

Wnioski: Wyniki przeprowadzonych przez nas badań sugerują, że w progresji chłoniaków T-komórkowych skóry istotną rolę odgrywa immunotolerancja guza, w rozwoju której uczestniczą limfocyty CD8⁺CD28⁻.

Słowa kluczowe: limfocyty CD8⁺ CD28⁻ • chłoniaki T komórkowe skóry

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Author's address: dr n. med. Donata Urbaniak-Kujda, Katedra i Klinika Hematologii Nowotworów Krwi i Transplantacji Szpiku AM, ul. Pasteura 4, 50-367 Wrocław; e-mail: durbaniak@post.pl

INTRODUCTION

Cutaneous T-cell lymphomas (CTCL) are classified as non-Hodgkin's lymphomas with clonal CD4 cell proliferation. They account for 1% of all non-Hodgkin's lymphomas. The most common CTCL type is mycosis fungoides or Sezary syndrome (MF/SS). The main prognostic factors in MF/SS include stage of the disease, extracutaneous manifestation, and patient age [5]. The immunological competence of CTCL patients as well as the mechanisms leading to disease progression are still widely analyzed. A proper immune response requires a balance between pathogen-dependent functions and immunotolerance mechanisms. Most protective functions are regulated by CD4⁺ and CD8⁺ T lymphocytes. CD8⁺ T cells play an important role as both effector and regulatory cells in most autoimmune diseases and in organ transplants. Their function manifests in suppressor cytokine production, i.e. interleukin 10 (IL-10) and tumor growth factor beta (TGF-β), as well as the inactivation of dendritic cells. The CD8⁺CD28⁻ T-cell subtype derived from T lymphocytes acts in a dual manner: antigen-dependent through antigen-presenting cells (APCs) and antigen-independent through cytokine secretion. APCs

co-cultured with CD8⁺CD28⁻ T cells become less efficient in inducing a T cell-dependent immune response. Such interaction prevents the upregulation of costimulatory molecules by APCs, hence decreasing the delivery of these signals to CD4⁺ T cells [9]. This results in a reduction in or a complete lack of activation of CD4⁺ Th cells and the development of an anergic state [11]. Increased proportions of CD28⁻ cells were reported in cases of infectious diseases, HIV infection, organ transplantation, and neoplasms (head and neck tumors) [4,11,12]. There are no data regarding the role of CD8⁺CD28⁻ T cells in the course of primary cutaneous T-cell lymphoma. Therefore the aim of this study was to assess CD8⁺CD28⁻ lymphocytes in the peripheral blood and skin infiltrates of patients with cutaneous T-cell lymphoma and to determine whether their count is associated with the clinical stage of the neoplastic disease.

METHODS

Patients

The analysis was conducted on 41 not previously treated patients, 26 males and 15 females aged 33–85 (median: 57



years), diagnosed with primary cutaneous CD4⁺ T cell lymphoma, i.e. mycosis fungoides or Sezary syndrome. Mycosis fungoides was diagnosed in 6 patients in stage T2N0M0, 18 in T3N1M0, 8 in T4N2M1, and 9 in T4N3M1B1 who fulfilled the Sezary syndrome criteria according to TNMB and the WHO-EORTC classification [14,15]. The control group consisted of 29 healthy individuals matched by age and sex to the patient group.

Materials and methods

The material was a skin lesion biopsy for immunohistochemical and histopathological analysis and EDTA-drawn peripheral blood collected at diagnosis. The immunohistochemical and histopathological analysis was performed at the Department of Pathological Anatomy, Wrocław Medical University, and the cytometric analysis was realized in the lab of the Department of Hematology, Blood Neoplasms, and Bone Marrow Transplantation. Immunohistochemistry

Paraffin-embedded tissue derived from the cutaneous T-cell lymphoma skin biopsies was used for the immunohistochemical analyses. From each block, a slide for simultaneous assessment of CD8 and CD28 antigen expression and a control slide for quality checking and reaction validation were prepared. The primary antibodies used for these reactions were anti-CD28 (BD Biosciences, Germany) and anti-CD8 (Abcam, UK).

The tissue sections were sequentially incubated for 30 minutes at room temperature with 1: 100 dilutions of anti-CD28 and anti-CD8 antibody. The immune complex was then visualized with an EnVision+ HRP, Mouse (DAKO) detection system. The immunocytochemical reaction for the first antibody was developed with 3,3-diaminobenzidine (DAB) and for the second with 3-amino-9-ethylcarbazole (AEC). These two chromogens were applied to obtain differing color reactions: dark-brown (DAB) and intense red (AEC). The expression of CD8 and CD28 antigen was determined using an Olympus light microscope (magnification: 100x) with computer image analysis (AnalySIS DOCU software).

The slides were analyzed by assessing 1000 cells and determining the percentage of antigen-positive cells. The aim of the analysis was to establish the percentage of CD8⁺ cells displaying a CD28-positive reaction. All of the preparations were analyzed twice and mean values were calculated.

Cytometric analysis

The expression of CD28 antigen on the CD8⁺ T lymphocytes was tested on EDTA whole peripheral blood samples. Blood was incubated with the murine monoclonal antibodies anti-CD8-FITC, anti-CD28-PE, and anti-CD3-Cy5 (Becton Dickinson). Erythrocytes were lysed, the cells were washed in PBS by centrifugation, and analyzed on a PAS flow cytometer (Partec, Germany) equipped with FloMax2,4B software. Fifty-thousand cells were analyzed each time. The lymphocytes were gated according to CD45 and CD14 staining (leukogate). The percentage of CD28⁺ cells was expressed as the fraction of double-positive CD3⁺CD8⁺ lymphocytes.

Due to small sample size, the Mann-Whitney *U* test for independent samples and Spearman's rank test were ap-

plied for statistical analysis. A *p* value of 0.05 was considered statistically significant. Stat Soft Statistica software was used to perform the analysis.

RESULTS

The median percentage of lymphocytes in the peripheral blood of all the patients was 28.7% (range: 10.7–86.9%) and of CD3⁺CD8⁺ lymphocytes 20.8% (1.7–48.9%). In the control group the median lymphocyte percentage was 31% (range: 11.3–54.56%) and of CD3⁺CD8⁺ lymphocytes 19.37% (range: 4.41–38.98%).

The median percentage of CD8⁺CD28⁻ cells in the patients' peripheral blood was 49.5% (range: 5.2–92%) and was significantly higher than in the control group (median: 35.51%, range: 6.2–60.10%, *p*=0.001). In the patients with a more advanced disease stage (17 individuals), the percentage range of CD8⁺CD28⁻ lymphocytes was 20–92% and was significantly higher than in the 24 patients with an earlier disease stage, in whom it ranged from 5.2 to 63.5% (*p*=0.00007). The percentage of CD8⁺CD28⁻ lymphocytes in the skin biopsies of the patients with advanced lymphoma was significantly higher than in the patients with less advanced disease (median: 99.5%, range: 76.1–99% and median: 77.4%, range: 17.6–99.5%, respectively, *p*=0.000001).

The whole patient population displayed statistically significant positive correlation between the percentage of CD8⁺CD28⁻ cells in the peripheral blood and age (*R*=0.33, *p*=0.04), disease stage (*R*=0.67, *p*=0.00002), and the percentage of CD8⁺CD28⁻ cells in the skin (*R*=0.72, *p*=0.000002). Moreover, the percentage of CD8⁺CD28⁻ cells in the patients' skin indicated significant positive correlation with age (*R*=0.33, *p*=0.03) and disease stage (*R*=0.88, *p*=0.000001).

The results are displayed in Figures 1–4.

DISCUSSION

Immune system homeostasis is maintained through the normal function of regulatory lymphocytes. Among them, the T-suppressor cell CD8⁺CD28⁻ subtype is characterized by acting in an antigen-dependent or -independent manner. According to Colovai et al., CD8⁺CD28⁻ lymphocytes recognize MHC class I antigen on APCs and suppress CD4 lymphocyte proliferation by a cell-to-cell contact mechanism [2]. Another mechanism, suggested by Filaci et al. [4], proposes that CD8⁺CD28⁻ lymphocyte-mediated suppression is associated with the secretion of the suppressing cytokines IL-10 and TGF-β [4]. Elevated CD8⁺CD28⁻ lymphocyte counts were observed in autoimmune disorders, head and neck tumors, infectious diseases, and in elderly people [1,7,11,13].

In head and neck cancer, elevated CD8⁺CD28⁻ lymphocyte count correlated with active disease and patient age [11]. Disruption of the CD8⁺CD28⁻ cell production and function was also observed in autoimmune diseases, for example systemic lupus erythematosus, scleroderma, and multiple sclerosis [4]. Tulunay et al. found a decrease in circulating CD8⁺CD28⁻ lymphocytes in lupus erythematosus patients which was associated with diminished suppression resul-

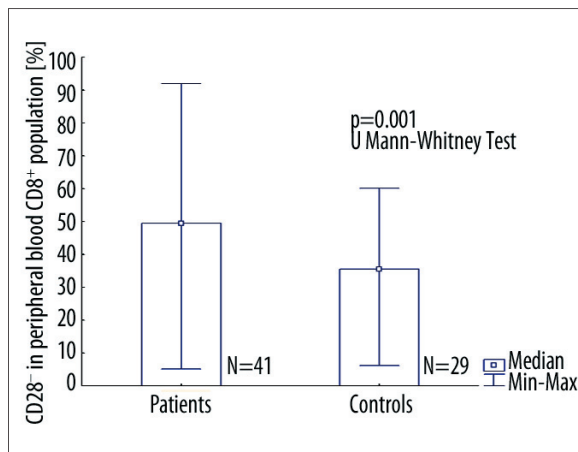


Figure 1. The percentages of CD28- cells in the peripheral blood CD8+ population in CTCL patients and healthy controls

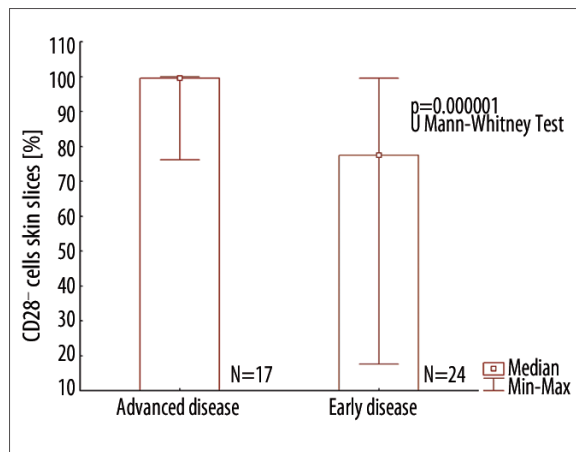


Fig.3. The percentages of CD28- cells in skin infiltrates sections of patients with advanced and early stages of CTCL

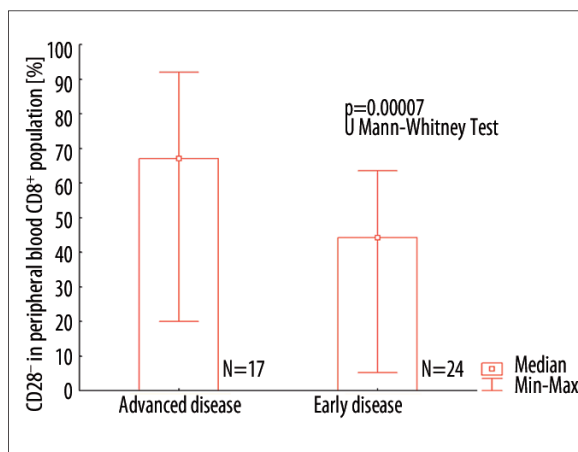


Fig. 2. The percentages of CD28- cells in the peripheral blood CD8+ population in CTCL patients with advanced and early stages of CTCL

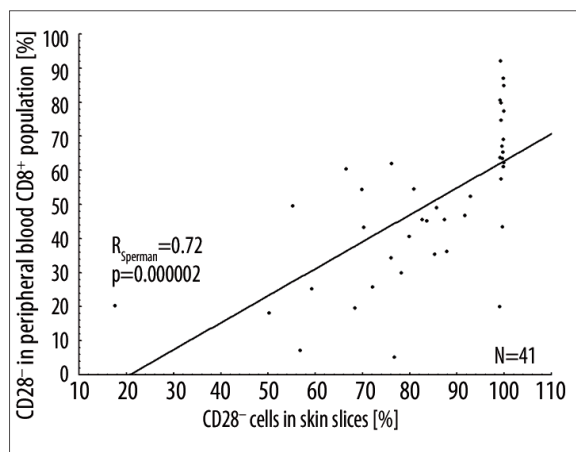


Fig.4. The correlation between the percentage of CD28- cells in infiltrated skin sections and of CD28- cells of the peripheral blood CD8+ population in CTCL patients

ting in autoreactive B-cell and antibody production and overproduction [12]. The immunopathogenesis of cutaneous T-cell lymphoma as well as the patient's immunological state are still widely analyzed in clinical studies.

The impaired T-cell suppressor function in CTCL may be triggered by increased cytokine production (mainly IL-4 and IL-10) in the neoplastic clone [5]. Moreover, constant secretion of these cytokines is believed to cause disruption of macrophage and CD4+ lymphocyte function, leading to disruption of the recognition and destruction of cancer cells [3].

The presence of CD8+CD28- lymphocytes in neoplastic diseases is relatively poorly described in the literature. Research has focused to date on detecting these cells in peripheral blood and solid tumors. There are no data regarding the assessment of CD8+CD28- cells circulating in the blood or present in skin infiltrates of CTCL patients. Our study included CTCL CD4+ treatment-naïve patients in whom we measured peripheral blood and skin-derived CD8+CD28- lymphocytes. We demonstrated a significantly higher percentage of CD8+CD28- cells in the patients' peripheral blood than in the control group and higher counts

in advanced disease stage than in early stage. Additionally, the statistical analysis revealed that the CD8+CD28- cell count in peripheral blood increases with age and disease stage and correlates with the CD8+CD28- cell count in skin infiltrates. Moreover, the percentage of CD8+CD28- lymphocytes in skin infiltrates increases with age and disease stage.

Our results are consistent with those obtained by Tsukishiro et al. for head and neck cancer, both regarding the circulating CD8+CD28- cell count and the positive correlation with patient age. These authors observed decreased CD8+CD28- cell counts after tumor removal [11]. The presence of these lymphocytes in CTCL skin infiltrates observed in our study is also consistent with the results obtained by Filaci et al. and their hypothesis concerning a suppressor role of CD8+CD28- cells, which induces and maintains immunotolerance to neoplastic antigens in the tissue [4]. Additionally, we found positive correlation of CD8+CD28- cell percentage with disease stage and patient age.

We wish to stress the observation that the correlation coefficient between CD8+CD28- lymphocyte percentage, both in peripheral blood and skin infiltrates, and disease



stage was significantly higher than that between this percentage and patient age ($R=0.67$, $R=0.88$, and $R=0.33$, respectively). This indicates that the connection of this lymphocyte subtype with disease stage is stronger than with patient age.

The results obtained in this study seem to confirm the important role of CD8⁺CD28⁻ cells in cutaneous T-cell lymphoma progression, in which the main causative factor is immunotolerance to cancer antigens. Therefore, suppressing CD8⁺CD28⁻ cell function is one of the therapeutic perspectives in cutaneous T-cell lymphoma management.

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