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CD4+CD39 T CELLS IN THE PERIPHERAL BLOOD AND SPLEEN OF PATIENTS WITH IMMUNE THROMBOCYTOPENIA

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CD4+CD39+ T უჯრედები პერიფერიულ სისხლსა და ელენთაში იმუნური თრომბოციტოპენიის მქონე პაციენტებში

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რეზიუმე

პირველადი იმუნური თრომბოციტოპენია (ითპ) ხასიათდება თრომბოციტების რაოდენობის შემცირებით და სისხლდენის მაღალი რისკით. ითპ-ის პათოგენეზი ჯერ კიდევ შესწავლის საგანია, თუმცა ლიმფოციტების მიერ აუტოანტისხეულების წარმოქმნას T-ჰელპერული უჯრედები მართავენ. ითპ პათოგენეზში აქტიურად შეისწავლება მარეგულირებელი T-უჯრედების როლიც. უკანასკნელი კვლევებით, ითპ პაციენტების პერიფერიულ სისხლში ნანახია (Treg) უჯრედების კლება, თუმცა ასევე აღსანიშნავია პუბლიკაციები სადაც (Treg) უჯრედები არ განსხვავდება საკონტროლო ჯგუფისგან. (Treg) უჯრედებზე ექსპრესირებული CD39 მნიშვნელოვნად განსაზღვრავს (Treg) უჯრედების იმუნოსუპრესორულ პოტენციალს. აღნიშნულ კვლევაში ჩვენს მიზანს წარმოადგენდა შეგვესწავლა CD4+CD39+ T-ლიმფოციტები იმ ითპ პაციენტთა სისხლსა და ელენთაში, რომლთაც პირველი რიგის მკურნალობაზე პასუხი ვერ მიიღეს და ჩაუტარდათ სპლენექტომია, როგორც მეორე რიგის თერაპია. სპლენექტომირებული პაციენტები რომელთაც არ ჰქონდათ ითპ წარმოდგენილი იყვნენ საკონტროლო ჯგუფში. ჩვენი შედეგების მიხედვით CD4+CD39+ T-უჯრედების პროცენტული მაჩვენებელი სარწმუნოდ დაბალია ითპ პაციენტების ელენთაში საკონტროლო ჯგუფთან შედარებით. აღსანიშნავია რომ ეს სხვაობა არ აღინიშნება სისხლში. ასევე აღსანიშნავია, რომ CD4+CD39+ T-უჯრედების სიხშირე უფრო სტაბილურია ითპ პაციენტების ელენთაში, ვიდრე სისხლში. ჩვენი კვლევა მიუთითებს CD39+-ის, როგორც ითპ-ის პოტენციური ბიომარკერის მნიშვნელობაზე და ადასტურებს ელენთის ქსოვილში იმუნური მარკერების კლინიკურ და სამეცნიერო ღირებულებას აღნიშნულ პათოლოგიაში.

Introduction

Immune thrombocytopenia (ITP, also called idiopathic thrombocytopenic purpura) is an acquired thrombocytopenia characterized by a reduced platelet lifespan (<100 G/L) due to antibody mediated destruction [1,2]. The review of published reports determined an annual ITP incidence of approximately 2,6-6,6 per 100,000 in adults [3-5]. The pathogenesis of ITP is incompletely understood. The principal mechanism is thought to involve specific autoantibodies produced by the patients B cells (IgG) most often directed against platelet membrane glycoproteins such as GPIIb/IIIa. The involvement of helper T cells in ITP pathogenesis is crucial [6,7]. The T follicular helper cells (TFH) support B cell differentiation and antiplatelet production [8,9]. However, other mechanisms are important, including cytotoxic T cells, [10-12] as well as humoral and cellular autoimmunity directed at megakaryocytes, causing impaired platelet production [2,13-16].

Recent evidence proves the significance of Regulatory T cells (T cell subset with a CD4+CD25-high+Foxp3 phenotype) in ITP patients. The low frequency of circulating Tregs has been observed in most of studies, [16-18] however similar levels with controls have also been reported [19]. Audia et al demonstrated that the percentage of circulating regulatory T cells (Tregs) was similar to that in controls,

however splenic Tregs were reduced in ITP patients. Interestingly, the ratio of proinflammatory Th1 cells to suppressive Tregs was increased in the spleens of patients who failed RTX therapy [20].

The ectoenzyme CD39 within CD4+CD25+ cells highly suggest the immunosuppressive function of Tregs and might be considered as a biomarker in autoimmune disease [21]. In the recent study where patients with newly diagnosed primary Immune thrombocytopenia (ITP) were enrolled, the expression of CD39 in CD4+CD25+ Treg cells was initially decreased compared to normal controls. After high dose of dexamethasone therapy, the response group showed elevated CD39 expression within Treg cells, while non-responder group did not show any difference in compression to that before treatment [22]. In these studies, the readouts of CD39 in ITP patients are associated with the first line treatment.

Splenectomy is a second line therapy of ITP [23]. This therapeutic approach presents a very rare clinical opportunity to study splenic cells in patients as spleen is the primary site of the autoimmune response during ITP.

We aimed to study frequency of CD4+CD39+ T lymphocytes in the spleen and blood of patients with ITP and in control group that might further reveal new targets for therapeutic intervention.

Materials and Method

Patients

This study was carried out in accordance with the principles of the 1975 Declaration of Helsinki and its later amendments or comparable ethical standards, and was approved by Tbilisi State Medical University Biomedical Research Ethics Committee. Formal consent was not required for this retrospective study, while all data were kept confidential.

The main inclusion criterion was thrombocytopenia (platelet count<100×109/L). Familial, viral, or drug-induced etiologies were excluded. Patients suffering from other autoimmune diseases (eg, systemic lupus erythematosus and antiphospholipid syndrome) were also excluded. Most of the patients were treated with steroids and, if necessary, with IVIg as first-line therapies. All of these patients were refractory to first line treatment and had splenectomy as a second line therapy.

Blood and spleen samples were obtained from ITP and non ITP patients as controls. None of control group individuals had other hematologic disease, cancer, acute or chronic infections, liver or kidney disease. Blood was collected (from ITP patients and Controls) 1 hour before splenectomy.

Spleen preparation

The spleen samples were obtained from patients during scheduled surgery within the hour of splenectomy. Sterile spleen tissues were mechanically disrupted using a syringe plunger. After the cells were dissociated, they were filtered through a 100-μm nylon strainer, BD Bioscience. Cell suspension was incubated for 10 minutes in a hemolytic solution (150mM ammonium chloride, 10mM potassium bicarbonate, and 0.1mM EDTA) at room temperature to remove RBCs. Cells were then washed in medium (RPMI with 10% FBS) and filtered again. Samples were then divided for Flow cytometry (FCM) analysis; remaining cells were prepared for cryopreservation.

Blood samples

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll gradient centrifugation and prepared for analysis as described in spleen preparation.

Flow cytometry (FCM)

CD4-APC/CD39-PE staining was performed following the manufacturer's instructions (eBioscience). Data were acquired on FacsCalibur flow cytometer and analyzed using FlowJo® v10 software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Two-tailed unpaired t-test was used to compare patients and controls.

Results and discussion

ITP patients and Control individuals included in our study were characterized according to gender and age. No differences were found between the groups (Table 1).

Table 1 Characteristics of splenectomized ITP patients

Patient	Sex	Age	Previous treatments
1	M	20	Steroids
2	F	24	Steroids;Thrombopoietin agonist (TPO-RA).
3	F	47	Steroids
4	M	60	Steroids
5	F	67	Steroids
6	M	76	Steroids
7	M	25	Steroids
8	F	66	Steroids
9	F	74	Steroids

Recent immunotherapeutic approaches aim to trigger the functional state of immunosuppressive cells in autoimmune pathologies. Controversial findings are available regarding immunosuppressive cells frequencies are functions in ITP patients.

In our study the frequencies of CD4+CD39+ T cells were comparable in ITP patient's blood and spleen (Figure 1). There was no difference in the blood levels of these cells between controls and ITP patients (Figure 2). The expression of ectoenzyme CD39 was diminished in CD4+ T cells in spleen of the patients with immune thrombocytopenia compared to controls (15.80%±2.205% to 37.15% ± 6.482%, P=0.0325) (Figure 3).

Figure 1. Spleen vs. blood levels of CD39 in CD4+ T lymphocytes in ITP

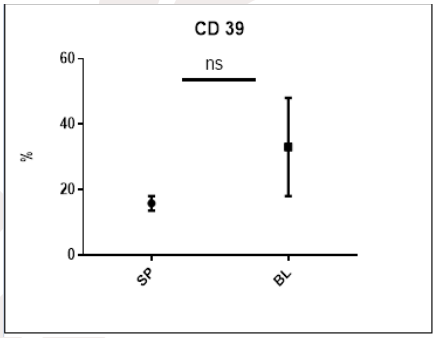


Figure 2. Blood levels of CD39 in CD4+ T lymphocytes in ITP vs. Control

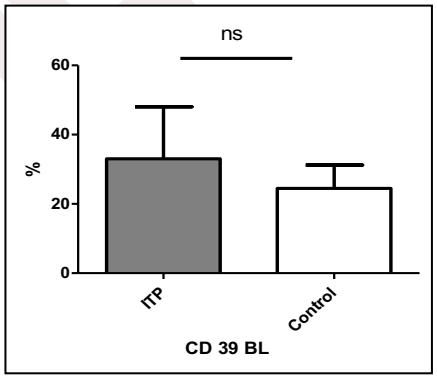
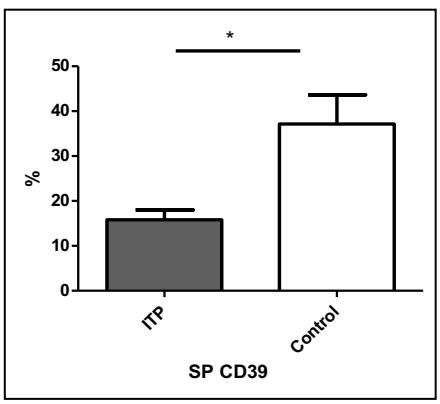


Figure 3. Splenic levels of CD39 in CD4+ T lymphocytes in ITP vs. Control



Pathogenesis of ITP still remains complex and its biomarkers uncovered. These complicate disease management and reflect in the frequent unresponsiveness of patients to the first line treatment. [24-26]. Splenectomy as a second line therapy of ITP removes the major site of phagocytosis of antibody-coated platelets, as well as lymphocytes that resides in the spleen that might be responsible for producing antiplatelet antibodies [23,27,28]. This therapeutic approach presents a very rare clinical opportunity to study splenic cells in patients as spleen is the primary site of the autoimmune response during ITP [20].

Increasing evidence suggest the importance of measuring CD39 expression in T lymphocytes to speculate on their suppressive functional state. CD39 and CD73 are coexpressed on the surface of murine and human Treg cells and generate extracellular adenosine, contributing to Treg immunosuppressive activity [21,29]. A high level of CD39+ within CD4+CD25+Treg cells highly suggests their immunosuppressive function [21]. Lu et al have demonstrated decreased expression of CD39 in circulating CD4+ CD25+ Treg cells of ITP patients compared to normal controls. CD39 expression was elevated within Treg cells after high dose of dexamethasone therapy in the response group, while non-responder group did not show any difference in compression to that before treatment [22]. Another study has demonstrated that methotrexate (MTX) unresponsiveness in rheumatoid arthritis (RA) is associated with low expression of CD39 in peripheral blood CD4+CD25+FoxP3+ Tregs and the decreases suppressive activity of these cells [30,31].

Precedent findings suggest that the expression of CD39 on Tregs can predict the response on treatment, however they all report the results based on blood Tregs in humans. In our study, we show for the first time in ITP patients that CD39 expression is diminished in splenic CD4+ T lymphocytes. Therefore, our findings have important scientific and clinical value for understanding ITP pathogenesis and open new avenues for further investigations in this field.

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CD4+CD39 T CELLS IN THE PERIPHERAL BLOOD AND SPLEEN OF PATIENTS WITH IMMUNE THROMBOCYTOPENIA

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SUMMARY

Primary immune thrombocytopenia (ITP) is characterized with decreased platelet count and increased risk of bleeding. The mechanism of thrombocytopenia in ITP is incompletely understood but thought to involve autoantibodies which are produced by the B cells and are stimulated by helper T cells. Regulatory T cells (Treg) have been seen to be significant in ITP pathogenesis. Recent studies have reported reduction of circulating Treg cells in ITP patients but similar levels with controls have also been observed. The ectoenzyme CD39 is highly expressed on the surface of Treg cells and can suggest its immunosuppressive function.

In this study we aimed to analyze CD4+CD39+ T lymphocytes both in the blood and spleen of patients with ITP who did not respond to the first line treatment and underwent splenectomy as a second line therapy. Non-ITP patients undergoing splenectomy were involved in the control group. Our data demonstrates significant diminution of in splenic but not circulating CD4+CD39+ T cells in ITP patients compared to controls. Of note, the comparison of spleen and peripheral CD4+CD39+T lymphocytes indicates that the frequency of CD39+ Treg cells is more stable in spleen compared to blood in ITP patients. Our data suggests the potential of CD39 as an important biomarker for ITP and underlines the clinical and scientific value of spleen immune analyzes in this pathology.

Keywords: Primary immune thrombocytopenia, CD4+CD39+ T cells, blood, spleen