REGULATORY T CELLS AND T HELPER 17 CELLS EXPRESSING CD39 AND CD73 ECTONUCLEOTIDASE IN CHILDREN WITH SEVERE INJURY

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Frequent resulting disability and case mortality support the urgency of investigation of the immune response mechanisms triggered by severe injury (SI) in children. This study aimed to determine the informative immunological criteria of traumatic injury severity and prognosis in children (n = 43) based on the assessment of expression of CD39 and CD73 ectonucleotidase in populations of regulatory T cells (Treg, $CD4^+CD127^{low}CD25^{high}$) and T-helper 17 cells (Th17, $CD4^+CD161^+CD3^±$) in SI cases grouped by the outcome (favorable (SIfav, n = 24), unfavorable (SIunfav, n = 17) and lethal (n = 2)). With the help of flow cytometry, we identified a pronounced decrease in the absolute number of T_{reg} and Th17, as well as T_{reg} and Th17 expressing CD39 and CD73, in the early post-traumatic period. In the SIfav and SIunfav groups the relative number of T_{reg} and Th17 cells expressing CD39 differed significantly (p <0.05); it was substantially higher form the first to the third day post injury in the SIunfav group. The level of Treg CD39 (44.4%) is a premise for an unfavorable outcome in children surviving an SI. In fatality cases, we registered extremely low ectonucleotidase expression rates: $CD39^+T_{reg}$ — 9.52% (9.52–13.75) and $CD39^+$Th17 — 0.92% (0.74–1.1). In the SIunfav group, the intensity of fluorescence (FL) of CD39 on T_{reg} cells in the early post-traumatic period was higher than seen in the SIfav group. The threshold value for the average fluorescence intensity (FL) of CD39 on T_{reg} was 8.25 c.u. In fatality cases, the T_{reg} CD39 FL values were extremely low: 3.95 c.u. (3.7–4.67). The results of the study indicate that in children, the expression of CD39 and CD73 in T_{reg} and Th17 populations is significantly associated with the severity of injury and outcome of the traumatic disease.

Keywords: children, severe injury, T_{reg}, Th17, CD39, CD73, immune suppression

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Compliance with the ethical standards: the study was approved by the Ethics Committee of the Institute of Urgent Children Surgery and Traumatology of the Department of Health of Moscow (Minutes #2 of May 26, 2020). Parents of all participants of the study have signed the informed consent form in accordance with the principles of the Declaration of Helsinki.

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РЕГУЛЯТОРНЫЕ Т-КЛЕТКИ И Т-ХЕЛПЕРЫ 17-ГО ТИПА С ЭКСПРЕССИЕЙ ЭКТОНУКЛЕОТИДАЗ CD39 И CD73 ПРИ ТЯЖЕЛОЙ МЕХАНИЧЕСКОЙ ТРАВМЕ У ДЕТЕЙ

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Исследование механизмов развития иммунного ответа при тяжелой механической травме (ТМТ) у детей — актуальная и социально значимая задача по причине высокой инвалидизации и летальности. Целью работы было определение информативных иммунологических критериев тяжести и прогноза исхода травматической болезни у детей (n = 43) на основе оценки экспрессии эктонуклеотидаз CD39 и CD73 в популяциях регуляторных Т-клеток (T_{reg}, $CD4^+CD127^{low}CD25^{high}$) и Т-хелперов 17-го типа (Th17, $CD4^+CD161^+CD3^+$) при ТМТ в группах с благоприятным (ТМТбл, n = 24), неблагоприятным (ТМТнебл, n = 17) и летальным исходом (n = 2). С помощью метода проточной цитофлуориметрии было выявлено выраженное снижение абсолютного количества T_{reg} и Th17, а также T_{reg} и Th17, экспрессирующих CD39 и CD73, в раннем посттравматическом периоде ТМТ. В группах ТМТбл и ТМТнебл относительное число T_{reg} и Th17, экспрессирующих CD39, значимо различалось (p <0,05) и было существенно повышено с первых по третьи сутки после травмы для ТМТнебл. Уровень T_{reg} CD39 (44,4 %) является предпосылкой неблагоприятного исхода у выживших детей при ТМТ. Для больных с летальным исходом были получены крайне низкие показатели экпрессии эктонуклеотидаз: $CD39^+T_{reg}$ — 9,52% (9,52–13,75) и $CD39^+$Th17 — 0,92% (0,74–1,1). Для ТМТнебл интенсивность флюоресценции (FL) CD39 на T_{reg} в раннем посттравматическом периоде была повышена в сравнении с ТМТбл. Для средней интенсивности флуоресценции (FL) CD39 на T_{reg} пороговое значение составило 8,25 у.е. Для пациентов с летальным исходом значения FL CD39 на T_{reg} выявлены крайне низкие: 3,95 у.е. (3,7–4,67). Полученные результаты показывают, что экспрессия CD39 и CD73 в популяциях T_{reg} и Th17 в значительной степени связана с тяжестью и исходом травматической болезни у детей.

Ключевые слова: дети, тяжелая травма, регуляторные Т-лимфоциты, Т-хелперы 17-го типа, CD39, CD73, иммуносупрессия

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Severe mechanical injury (SI) is one of the main reasons behind children's disability and mortality [1, 2]. SI triggers decompensation of the body's life support systems as a result of combined effect of such damage factors as traumatic mechanical damage, blood loss and hypoxia. Mechanical damage is the initiating factor: it triggers the release of damage-associated molecular patterns (DAMPs), which, in turn, can disrupt the cellular immune response to exogenous antigens and pathogen-associated molecular patterns (PAMPs), thus promoting dysfunction of the immune system. Extracellular ATP (eATP) is one of the endogenous tissue DAMPs that trigger and regulate the immune response to damage [3]. In trauma cases, the level of eATP grows persistently in the injury [4, 5]. This compound is one of the main components of the purinergic system; being a strong pro-inflammatory signal, eATP plays an important part in the T cell functioning regulation. As a powerful damage-associated molecular pattern, eATP basically initiates inflammatory response through purinergic P2R receptors. At the same time, the end product of eATP degradation, extracellular adenosine, being an immunosuppressor, plays an important part in limiting that response. Extracellular adenosine functions through the A2A adenosine receptors, blocks the T cell receptor (TCR) signal by inhibiting phosphorylation of the zeta-associated protein 70 (ZAP-70) and activates the activating protein 1 (AP-1), thus decreasing the production of IL2, expression of CD25 and inhibiting the T cell proliferation. The levels of eATP and extracellular adenosine, as well as their biological effects, are strictly regulated by the catalytic effects of CD39 (E-NTPDasa1) and CD73 (Ecto5'NTasa), ectoenzymes expressed on the plasma membrane of immune cells. CD39 metabolizes ATP to ADP, pyrophosphate and AMP. CD73 ectonucleotidase degrades AMP into adenosine and phosphate. Thus, CD39 and CD73 exonucleotidase secure a balance between pro-inflammatory action of ATP and anti-inflammatory action of adenosine in the focus of inflammation [6–9]. In case of a severe trauma, there is usually a period of prominent immunosuppression the pathogenesis of which is largely shaped by the decreasing level of T-lymphocytes. Absolute and relative number of T helper subpopulations is a significant marker in determining the severity of the pathological process and predicting its outcome [10–13]. Establishing the levels of expression of CD39 and CD73 exonucleotidase on various populations of circulating lymphocytes is of great clinical importance in the context of diagnosing and predicting the outcome of a wide range of diseases [14]. Therefore, the purpose of this study was to identify informative immunological criteria for the traumatic disease severity and outcome prognosis as applicable to children. The identification relies on the assessment of absolute and relative number of T lymphocyte subpopulations and the level of expression of CD39 and CD73 ectonucleotidase in T_{reg} and Th17 populations in severe mechanical trauma cases.

METHODS

The study involved 43 patients (28 boys (65.1%), 15 girls (34.9%); 116 observation sessions) with SI, treated at the Department of Anesthesiology and Resuscitation of the Research Institute of Emergency Pediatric Surgery and Traumatology, Moscow, in 2020–2021. We used the laboratory of the National Medical Research Center for Children's Health for laboratory studies, which were prescribed 1 to 5 times, depending on the length of stay of a given child at the Department of Anesthesiology and Resuscitation (DAR). The mean age of the children was 13.0 (6.0–15.0) years (Me (Q_{25}–Q_{75})). The time options for laboratory studies were the first, third, fifth, seventh and 14th days from the day of injury.

The control group was comprised of 41 apparently healthy children; all of them underwent medical examination at the National Medical Research Center for Children's Health. The children were comparable in age and sex: age — 12.41 (7.4–16.2) years; 26 boys (63.4%), 15 girls (36.6%).

Assessing the injury, we relied on the Injury Severity Score (ISS), the Glasgow Coma Scale (GCS) and its modification for patients under 2 years old, the pediatric GCS (pGCS) [15].

The outcome of an SI was assessed with the help of the Glasgow Outcome Scale (GOS) and the Severe Injury Outcomes Scale (OISS) [16], which suggest the following categories: category 1 — full recovery (the patient can live and be as active as before the injury); category 2 — good recovery (there are consequences that do not limit the level of social adaptation, but the patient cannot return to the pre-injury level of functional activity and needs further treatment or rehabilitation); category 3 — moderate disability (there are consequences that disallow return to the pre-injury functional level, but the patient retains independent living skills); category 4 — severe disability (the patient needs assistance of others and cannot live independently); category 5 — death. These scales were applied to assess the condition of the patient at discharge from the hospital.

The patients were included in the study if they had an SI (ISS ≥ 16) and were treated in the ICU. Concomitant acute inflammatory and chronic diseases were a reason for exclusion.

At the first stage, we analyzed the results from the control group and the SI group. At the second stage, we analyzed the two groups formed with the help of GOS and OISS, the favorable outcome group (SIfav, n = 24) and the unfavorable outcome group (SIunfav, n = 17) (Table 1). The distribution into these groups was based on the scores: patients were allocated to the SIfav group if they scored 4–5 points on the GOS scale and 1–2 points on the OISS scale, and to the SIunfav group if they scored 2–3 points on the GOS scale and 3-4 points on the OISS scale. A group of fatal cases (SIdeath, n = 2) was described separately (Table 1).

We assessed the quantity of Th17 lymphocytes (Th17 — $CD3^+CD4^+CD161^+$), regulatory T lymphocytes (T_{reg} — $CD4^+CD127^{low}CD25^{high}$) in the patients and established the level of expression of purinergic signaling receptors on T_{reg} ($CD39^+T_{reg}$ — $CD4^+CD127^{low}CD25^{high}CD39^+$ and $CD73^+T_{reg}$ — $CD4^+CD127^{low}CD25^{high}CD73^+$) and Th17 lymphocytes ($CD39^+$Th17 — $CD3^+CD4^+CD161^+CD39^+$ and $CD39^+$Th17 — $CD3^+CD4^+CD161^+CD73^+$). Two-platform technology enabled assessment of the quantitative indicators of the subpopulation composition of peripheral blood T lymphocytes. The absolute number of lymphocytes was calculated with the help of a Sysmex XT-2000i hematology analyzer (Sysmex Corporation; Japan). The preparation of samples for cytofluorimetric analysis included incubation of 100 µl of whole blood with 10 µl of monoclonal antibodies tagged with fluorochromes for 20 min in a dark place. The erythrocytes were lysed with BD FACS™ Lysing Solution (BD Biosciences; USA); the duration of incubation therewith in the dark at room temperature did not exceed 10–12 minutes. The resulting samples were analyzed in a Novocyte flow cytometer (ACEA Biosciences; USA). The surface markers used to determine lymphocyte subpopulations were as follows: CD45, IgG1, IgG2a, CD3, CD4, CD25, CD127, CD161, CD39, CD73 (Beckman Coulter, USA; BD Biosciences, USA; SONY corp., Japan).

We used MS Excel 2016 (Microsoft corp.; USA), Statistica 10 (StatSoft, Inc.; USA), and IBM SPSS Statistics 25 (IBM corp.; USA)

Table 1. Clinical characteristics of patients

Factor		SI outcome		
		SIfav	SIunfav	SIdeath
n		24	17	2
Sex, %	girls	9 (37.5)	6 (35.3)	–
	boys	15 (62.5)	11 (64.7)	2 (100.0)
Age (Me [IQR]), years		12.5 [6.0–15.0]	13.0 [8.0–14.0]	7.5 [4.7–10.2]
Days in DAR (Me [IQR])		9.00 [7.00–13.25]	16.00 [10.00–25.00]	6.00 [6.00–6.00]
Total days in hospital (Me [IQR])		23.00 [16.00–29.25]	53.00 [23.00–58.00]	6.00 [6.00–6.00]
ISS (Me [IQR])		26 (19–29)	27 (26–34)	25 и 35
TBI, %		21 (87.5)	16 (94.1)	100
GCS (Me [IQR]), points		12 (8–12)	7 (4–13)	7 и 3
Coma, %		5 (20.8)	8 (47.0)	2 (100)
Concomitant injury, %		21 (87.5)	16 (94.1)	2 (100)
Multiple trauma, %		11 (45.8)	7 (41.1)	1 (50)
Blood loss, %		16 (66.6)	13 (76.4)	1 (50)
Unstable hemodynamics, %		8 (33.3)	12 (70.5)	2 (100)
Respiratory support (ALV), %		16 (66.6)	16 (94.1)	2 (100)
Multiple organ failure, %		1 (4.1)	2 (11.7)	2 (100)

to process the data obtained. The results are presented as a median (Me) and quartiles (Q_{25}–Q_{75}). Mann–Whitney *U*-test with Bonferroni correction enabled comparison of differences in the attributes. Spearman's rank correlation coefficient (R) was used to assess relations between the attributes. The significance of quantitative indicators was assessed and threshold values (cut-off points) chosen with the help of the receiver operating characteristic curve (ROC curve). The threshold values were determined factoring in the maximum sensitivity and specificity requirements. The conclusions were considered significant at $p < 0.05$ (*).

RESULTS

The analysis of data from the control and SI groups revealed a pronounced decline in the absolute number of T_{reg} and Th17 in the early post-traumatic period. The values of these indicators in SI patients were significantly different from the respective values registered in the control group (Table 2, 3). Third to fifth day post injury, the T_{reg}/Th17 ratio was decreased in the SI group compared to the control group, which is the result of gradual growth of the level of Th17 from the third day on (Tables 2, 3).

Table 2. Subpopulations of $CD4^+$-lymphocytes, T_{reg} and Th17 expressing CD39 and CD73, and ectonucleotidase fluorescence intensity on T_{reg} and Th17 in SI and control groups, regardless of the traumatic disease outcome

Indicators	Control group	SI (days elapsed since injury)				
		1st day	3rd day	5th day	7th day	14th day
	n = 41	*n* = 18	*n* = 33	*n* = 16	*n* = 21	*n* = 24
T_{reg}, abs	72.2 [57.3–86.2]	34.9 [22–48]*	38.3 [24.2–54.4]*	36.5 [24–67.2] *	36.5 [24–67.2]*	61 [49.1–78.9]
Th17, abs	144.6 [97.7–150.6]	78.1 [54.7–97.2]*	87.2 [64.4–136.3]*	93.2 [75.3–145.9]	93.2 [75.3–145.9]	163.3 [118.4–232.9]
T_{reg}/Th17	0.6 [0.5–0.8]	0.4 [0.3–0.7]	0.4 [0.3–0.5]*	0.4 [0.3–0.5]*	0.4 [0.3–0.5]	0.4 [0.3–0.5]*
$CD39^+$, % T_{reg}	35.2 [29.1–39.4]	27.6 [17.3–43.1]	33.3 [15.4–53.2]*	36.4 [15.8–49.6]	36.4 [15.8–49.6]	43.4 [28–52]
$CD39^+$, abs T_{regs}	27 [18.3–31.7]	9.3 [5.9–13.1*	10 [7–14.2]*	12.4 [6.7–18.8]*	12.4 [6.7–18.8] *	23.2 [10.9–38.7]
$CD39^+$, % Th17	9.6 [8.6–12.1]	9.8 [6.5–12.4]	7.7 [3.4–10.6]*	6.8 [5.3–10.7]*	6.8 [5.3–10.7]	7.3 [4–8.9] *
$CD39^+$, abs Th17	12.5 [10.9–14.7]	7.9 [3.5–9.2]*	6.0 [2.2–9.6]*	7.1 [4–10.5]*	7.1 [4–10.5]	11.3 [4.3–18.5]
$CD73^+$, % T_{reg}	8.9 [7.3–11.1]	6.5 [4.1–13.1]	6.9 [4.9–11.8]	11.2 [5.1–22.3]	11.2 [5.1–22.3]	6.7 [4.6–16.9]
$CD73^+$, abs T_{regs}	8 [3–10]	2.7 [1.3–3.3]*	2.2 [1.6–4.7]*	5.2 [2.7–6.5]	5.2 [2.7–6.5]	4.3 [2.5–8]
$CD73^+$, % Th17	10.2 [7.3–14.4]	8.1 [6.1–13.7]	10.8 [7.4–19]	13.8 [10.6–16.5]	13.8 [10.6–16.5]	15* [9.2–19.8]
$CD73^+$, abs Th17	13.6 [8.4–17]	6.5 [3.3–9.2]*	10.3 [4.3–22.4]	14.7 [11.9–28.2]	14.7 [11.9–28.2]	26.7 [12.3–34.9]*
CD39/CD73 T_{reg}	3.4 [2.6–5.1]	3.9 [1.9–6.9]	4.5 [1.9–7.8]	2.8 [1.6–5.4]	2.8 [1.6–5.4]	4.6 [2.5–9.1]
CD39/CD73 Th17	1.1 [0.7–1.7]	1.4 [0.5–2.2]	0.7 [0.2–1.3]	0.5 [0.2–1.3]	0.5 [0.2–1.3]	0.5 [0.1–0.7]*
FL CD39 T_{reg}	7.9 [7–9.2]	8 [6.7–13]	8.4 [6.2–11.3]	8.1 [5.6–10]	8.1 [5.6–10]	9.4 [7.1–12]
FL CD39 Th17	7.2 [5.8–8.9]	6.6 [5.4–7.7]	7.8 [6.2–9.3]	7.2 [6.6–9.1]	7.2 [6.6–9.1]	7.8 [6.8–8.8]
FL CD73 T_{reg}	3.3 [2.7–3.7]	3.2 [2.7–4.1]	4.2 [3.2–6.2]*	3.3 [2.8–4.7]	3.3 [2.8–4.7]	4.4 [3.6–5.7]*
FL CD73 Th17	3.6 [3.3–4.7]	4 [3.2–6.1]	4.6 [3.2–5.7]	4.1 [3.9–6.8]	4.1 [3.9–6.8]	4 [3.3–6.4]

Note: Me [Q_{25}–Q_{75}%]; * — $p < 0.05$, Mann–Whitney *U*-test with Bonferroni correction, compared groups (healthy children, SI).

Table 3. Adjusted level of reliability of the analyzed parameters (with Bonferroni correction), control and SI groups, regardless of the traumatic disease outcome

Parameter	Mann–Whitney *U*-test (control group/SI)				
Days since injury	1	3	5	7	14
# of observation sessions	18	33	16	21	24
T_{reg}, abs	0.0000*	0.0000*	0.006*	0.03 *	0.605
Th17, abs	0.0000*	0.003*	0.215	1.407	0.232
T_{reg}/Th17	0.1035	0.005*	0.015*	0.3665	0.011*
$CD39^+$, % T_{reg}	0.509	4.1445	3.206	4.2395	0.3365
$CD39^+$, abs T_{reg}	0.0000*	0.0000*	0.002*	0.017*	1.9045
$CD39^+$, % Th17	4.13	0.0335*	0.0125*	1.9935	0.0015*
$CD39^+$, abs Th17	0.0005*	0.0000*	0.0165*	0.2125	1.091
$CD73^+$, % T_{reg}	2.2375	1.0035	2.6035	1.836	3.6035
$CD73^+$, abs T_{reg}	0.0015*	0.0000*	0.206	0.98	0.926
$CD73^+$, % Th17	1.6255	1.418	0.758	2.152	0.063
$CD73^+$, abs Th17	0.006*	2.032	1.1885	4.7335	0.0065*
CD39/CD73 T_{reg}	4.3845	4.1865	1.2375	1.4005	1.9915
CD39/CD73 Th17	4.462	0.1255	0.0885	1.2715	0.007*
FL CD39 T_{reg}	4.8375	4.8275	3.333	3.079	1.4725
FL CD39 Th17	1.936	1.105	2.7475	2.547	2.438
FL CD73 T_{reg}	2.5445	0.0245 *	3.462	0.1695	0.0005*
FL CD73 Th17	1.584	0.5755	0.223	2.6665	0.993

Note: Me [Q_{25}–Q_{75}%]; * — $p < 0,05$, Mann–Whitney *U*-test, compared groups (control group, SI).

The dynamics of the absolute number of Treg and Th17 cells expressing CD39 and CD79 was similar to the dynamics of small subpopulations of $CD4^+$ lymphocytes during the acute post-traumatic period, but the changes were more pronounced in case of T_{reg} cells (Tables 2, 3). The relative amount of $CD39^+T_{reg}$ in children with SI varied from 6.3 to 76.6% and significantly exceeded the value of $CD39^+$Th17 (range of variability: 0.3–24.1%) (Table 2). As for CD73, the relative number of this marker was significantly higher on Th17 (range of variability: 2.6–99.9%) than on T_{reg} (range of variability: 0.5–55.2%). We uncovered no significant differences with the control group. However, at some observation sessions the registered values significantly exceeded the maximum levels seen in the control group (Table 2).

The analysis of ectonucleotidase mean fluorescence intensity (FL) on T_{reg} and Th17 revealed differences for CD73 on T_{reg}. Compared to the control group, the FL values for CD73 three days after the injury were increased (Tables 2, 3).

Correlation analysis revealed a relationship between the percentage of T_{reg} and Th17 expressing CD39 and CD73 and the intensity of marker fluorescence. In case of Th17, the percentage of enzyme-expressing cells increases slightly

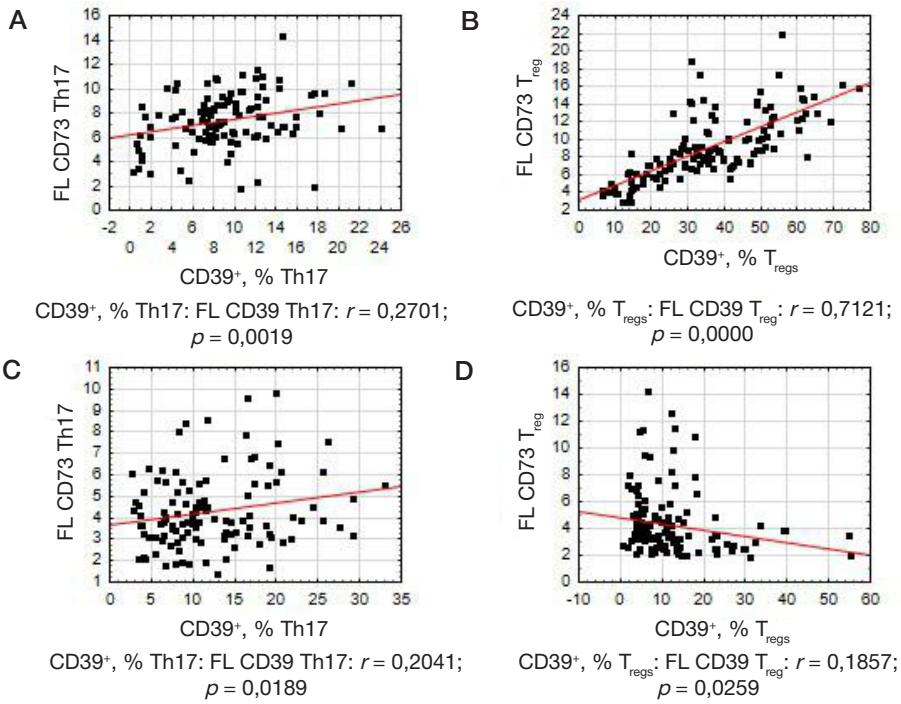

Fig. 1. Dependence of the percentage of T_{reg} and Th17 cells expressing CD39 and CD73 enzymes on the intensity of fluorescence (FL) of CD39 and CD73. **A**. FL CD39 Th17: $CD39^+$, Th17%. **B**. FL CD39 T_{reg}: $CD39^+$, T_{reg}%. **C**. FL CD73 Th17: $CD73^+$, Th17%. **D**. FL CD73 T_{reg}: $CD73^+$, %T_{reg}

Table 4. Relative amount of T_{reg} and Th17 cells expressing CD39 and CD73 enzymes, first through third days, children with SI

Indicator	SIunfav	SIfav	SIdeath	Statistical significance level *p*, SIunfav and SIfav
# of observation sessions	19	28	3	
T_{reg}, % CD4	9.24 [8.12–10.84]	8.9 [8.48–11.4]	9.9 [8.84–10.5]	0.968
CD39, % T_{reg}	52.33 [43.7–62.2]*	21.7 [14.9–25.2]	9.52 [9.52–13.75]	0.000026
CD73, % T_{reg}	6.24 [3.2–8.8]	6.54 [4.0–9.2]	4.9 [3.53–7.2]	0.84
Th17, % CD4	30.76 [25.2–35.2]*	15.5 [12.2–17.8]	19.5 [17.91–28.5]	0.0008
CD39, % Th17	14.55 [8.9–19.1]*	6.72 [3.14–9.0]	0.92 [0.74–1.1]	0.012
CD73, % Th17	12.38 [7.7–19.21]	10.38 [4.15–15.77]	5.7 [4.7–6.7]	0.599

Note: Me [Q_{25}–Q_{75}%]; Mann–Whitney *U*-test, compared groups: SIunfav, SIfav.

as the intensity of fluorescence of CD39 (r = 0.27; p = 0.002) and CD73 (r = 0.20; p = 0.018) grows (Fig. 1A, B). In case of Treg, as the fluorescence intensity grows, the share of enzyme-expressing cells increases for CD39 (r = 0.71; p < 0.001) and decreases slightly for CD73 (r = –0.18; p < 0.05; Fig. 1). The strongest direct dependence was found for $CD39^+T_{reg}$ (Fig. 1B).

A comparative analysis of the post-traumatic period data from SIfav and SIunfav groups has shown a significant increase in the relative amount of Th17 that occurred first through third days in the SIunfav group. At the same time, there were no differences between groups in terms of the number of T_{reg} cells (Table 4). The levels of expression of CD39 on T_{reg} and Th17 lymphocytes differed significantly in the SIfav and SIunfav groups: patients from the latter group had it considerably higher (Table 4, Fig. 2). We did not do the comparative analysis for the STMdeath group (n = 2) because of the small number of observation sessions (three), but it can be noted that in case of such patients, with the relative amounts of T_{reg} and Th17 being comparable, the registered expression of ectonucleotidase on T_{reg} and Th17 was extremely low (Table 4).

The following clinical cases show the dynamics of CD39 expression on T_{reg} and Th17 in patients with unfavorable (Case #1, Fig. 3) and favorable (Case #2, Fig. 3) injury outcome.

The analysis of fluorescence of ectonucleotidase on T_{reg} and Th17 in children from the SIfav and SIunfav groups revealed that the respective parameter differed significantly between the groups in case of CD39 on T_{reg}. In the SIunfav group we registered a slight increase in the fluorescence of CD39 on T_{reg} days 1 through 7 post-injury (Table 5). As for the SIdeath group, the fluorescence values there were as follows: FL CD39 T_{reg} — 3.95 (3.7–4.67), FL CD73 T_{reg} — 4 (2.55–4.55), FL CD39 Th17 — 6 .77 (5–8.55), FL CD73 Th17 3.52 (3.1–3.95). Compared to SIfav and SIunfav, the FL CD39 T_{reg} values there were extremely low (Table 5).

We built a ROC curve (both SIfav and SIunfav groups) for the indicators that proved to be of high prognostic significance in traumatic disease cases in children. A good quality division model was generated for $CD39^+$ T_{reg} % (AUC = 0.741) and FL CD39 T_{reg} (AUC = 0.721). The resulting cut-off for $CD39^+T_{reg}$ was 44.4% (sensitivity — 66.6, specificity — 84.7) and FL CD39 Treg — 8.25 c.u. (sensitivity — 87.5, specificity — 62.5).

DISCUSSION

This study shows that a severe mechanical trauma in children unbalances the T_{reg}/Th17 ratio in the early post-injury period, the imbalance translating into a slight shift towards Th17 while the absolute number of T_{reg} and Th17 cells decreases noticeably. These findings are consistent with the data published by other researchers [11–13, 17]. Among Treg and Th17, the

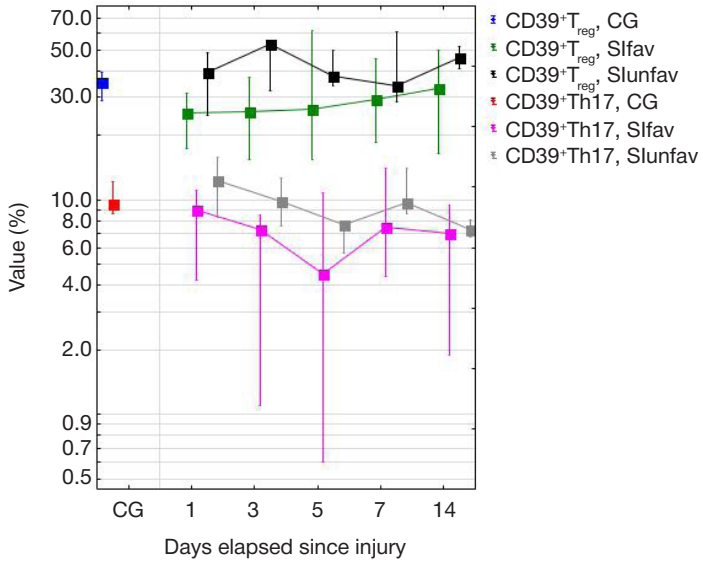

Fig. 2. Relative amount of Th17 and T_{reg} cells expressing CD39, SIfav and SIunfav and control groups. Me [Q_{25}–Q_{75}%]; compared groups: SIunfav, SIfav, control group (CG)

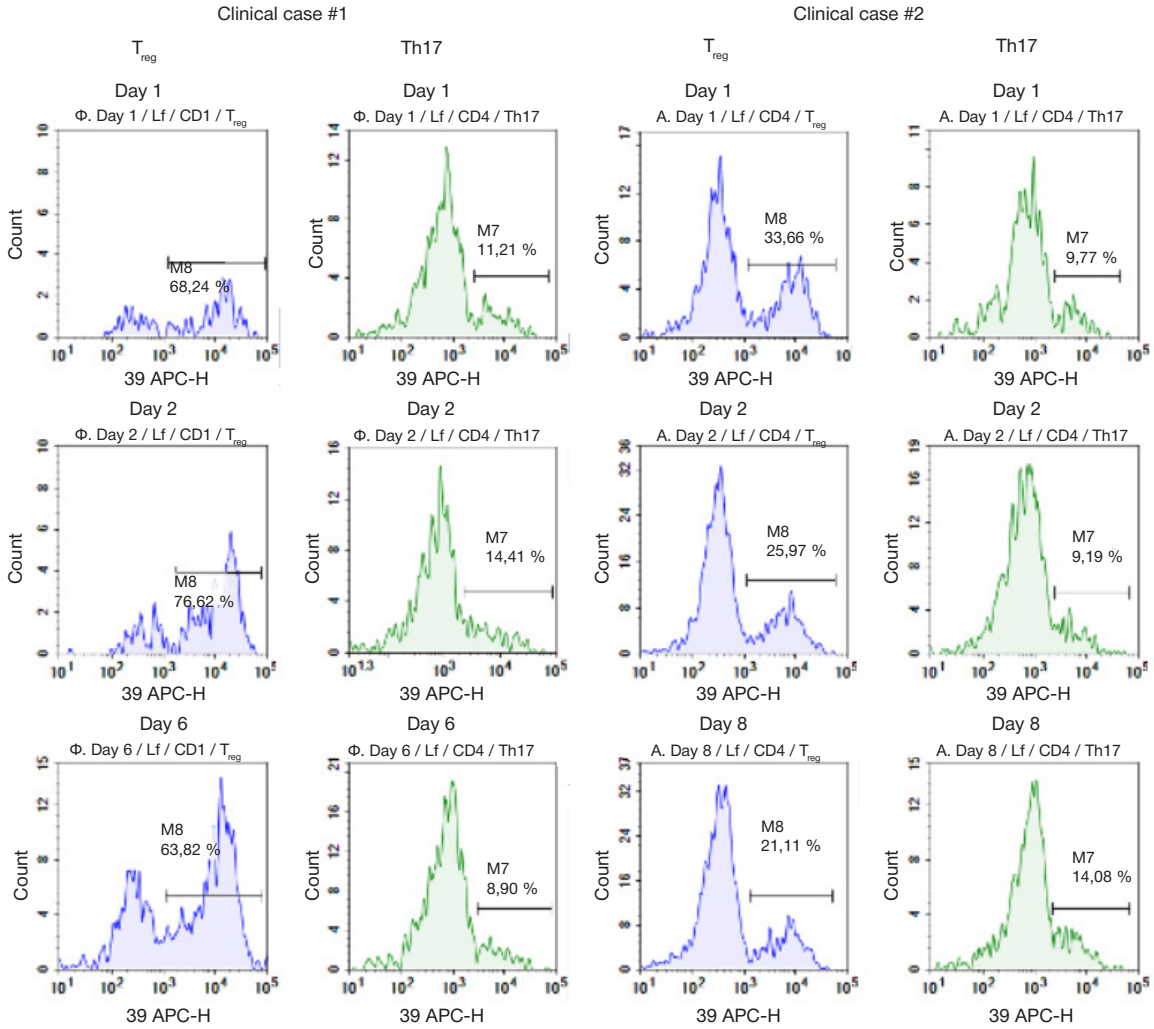

Fig. 3. Dynamics of the relative amount of Th17 and T_{reg} expressing CD39, critical period, severe injury, children with unfavorable (Case #1) and favorable (Case #2) outcome

absolute number of cells expressing CD39 and CD73 also proportionally decreases in the critical period of traumatic disease.

The analysis of cells expressing CD39 and CD73 ectonucleotidase in $CD4^+$-lymphocyte populations in children with SI revealed that the highest expression of CD39 in the Treg population is up to 76.6%, that of CD73 in Th17 — up to 99.9%. In apparently healthy children, by contrast, the CD39 expression in the Treg population ranged from 19 to 49%, and that of CD73 by Th17 — from 7 to 35% [18].

We have established that depending on the traumatic disease outcome, the expression of ectonucleotidase in children going through the early post-injury period may be different. Compared to the patients for whom the outcome was favorable, children from the Slunfav group had the percentage of CD39 on T_{reg} and Th17 increasing and the intensity of CD39 fluorescence on T_{reg} growing on days first through seventh post-injury. A possible reason therefor is the role played by ectonucleotidases, especially CD39, in enhancing the hydrolysis of eATP and accumulation of extracellular adenosine

Table 5. Fluorescence parameters (FL) of CD39 and CD73 purinergic signaling on T_{reg} and Th17, critical period, SI, children

Indicators	SIfav, days since injury					Slunfav, days since injury				
	1	3	5	7	14	1	3	5	7	14
	n = 10	*n* = 18	*n* = 6	*n* = 14	*n* = 12	*n* = 6	*n* = 13	*n* = 8	*n* = 7	*n* = 12
FL CD39 T_{reg}	7.7 [5.1–9.3]*	7.3 [6.2–9] *	9.7 [5.6–14.8]	7.7 [5.6–9.9] *	7.5 [6.3–11.8]	10.7 [8.5–14]	10.8 [8.8–12.4]	8.5 [7.9–8.8]	12.9 [9.4–14.1]	10.3 [8.3–12]
FL CD39 Th17	6.5 [5.4–7]	7.3 [5.7–8.4]	9.9 [6.1–10.6]	7.9 [6.4–8.2]	7.3 [6.1–8.8]	7.3 [6–8.7]	8.7 [7.1–9.8]	7 [6.8–7.9]	9.3 [5.3–10.3]	8.2 [7.1–9]
FL CD73 T_{reg}	3.2 [2.7–5.9]	3.8 [3–4.8]	3.2 [3.1–4.9]	3.9 [2.4–4.8]	4.6 [2.8–6]	3.2 [2.6–3.7]	5.1 [3.7–8.7]	3.4 [2.8–5.5]	4.7 [4.3–7.1]	4.4 [3.8–5.6]
FL CD73 Th17	3.7 [3.1–6.1]	4.2 [3.7–4.8]	4.1 [3.9–5.1]	3.9 [3.1–5.7]	4.3 [3.3–7.4]	4.3 [4–6.9]	5.1 [3.3–8.4]	4.5 [3.9–7.9]	4.7 [3.3–5.9]	3.9 [3.2–5.3]

Note: Me [Q_{25}–Q_{75}%]; *p* is the adjusted significance level (Bonferroni correction applied); * — $p < 0.05$ significance level, Mann–Whitney *U*-test, compared groups (SIfav and Slunfav on the first, third, fifth, seventh, and 14th days post injury).

in the injury locus, which triggers a cascade of reactions through the A2R system of adenosine receptors, this cascade ultimately leading to suppression of the immune response to prevent massive tissue damage [14]. The direct correlation between the level of CD39 fluorescence and the percentage of $CD39^{+}T_{reg}$ that we discovered indicates that the proportion of Treg abundantly expressing CD39 ectonucleotidase increases in response to injury. Previous studies that involved healthy adult donors have shown that cells with a large amount of CD39 on T_{reg} hydrolyze ATP more efficiently [8]. As for the CD73 ectonucleotidase, we established no correlation between its percentage and fluorescence intensity, probably due to the fact that CD73 is found both on the cell membrane and in soluble form [19]. In the deceased patients, the identified values of ectonucleotidase expression were extremely low, which may signal of development of decompensation of the immune system functions when the injuries are extremely severe.

CONCLUSIONS

The results of the study indicate that in children, the expression of CD39 and CD73 in T_{reg} and Th17 populations is significantly associated with the severity of injury and may be used to predict outcome of the traumatic disease. A deeper understanding of the role of purinergic signaling in the pathogenesis of traumatic disease suggests therapeutic potential of biopreparations based on the soluble forms of ectonucleotidase that may be designed to manipulate the immune system in such critical conditions as severe traumatic injury [20].

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