

IMMUNE RESPONSES IN CHILDREN INFECTED
WITH STREPTOCOCCUS A DURING THE SPRING 2023
EPIDEMIC IN BULGARIA

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Abstract

Streptococcus pyogenes, Group A Streptococcus (GAS), is the most common cause of bacterial tonsillitis, scarlet fever, impetigo, etc. in children and adults. Innate and adaptive host immune responses are fundamental for human defense against GAS, but are also important to the clinical manifestations of the infection.

Our aim was to study the humoral and cellular immune responses in children infected with GAS and diagnosed with tonsillitis in the Clinic of Pediatrics in University Hospital "Lozenets".

The study included 22 infected children and 20 healthy controls. The following immune parameters were investigated: i) Humoral immune response – total serum IgG, IgM, IgA; C3, C4 – complement components, as well as the serum cytokines IFN γ , IL-6, IL-1 β , TNF α , IL-17A, IL-10. ii) Cellular immune subsets were tested by immunophenotyping of total T cells, B cells, NK cells, T helper and T cytotoxic subsets.

In this cross-sectional study we found a significant increase in the complement components C3 and C4, increased production of serum IFN- γ , IL-6 and IL-10 and decreased levels of IL-17A in infected children with tonsillitis, compared to healthy controls. The cellular immune parameters in infected children

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showed lymphopenia, reduced total T lymphocytes and T-helper subpopulation, compared to healthy controls.

We assumed that these imbalanced immune responses, found in infected children, are connected with GAS infection and deserved more detailed investigation.

Key words: *Streptococcus pyogenes*, GAS, humoral immunity, cytokines, cellular immunity, tonsillitis

Introduction. Group A beta-hemolytic streptococcus (group A *S. pyogenes*, GAS) is a bacterial pathogen of great clinical importance due to the wide spectrum of diseases it causes [1]. They are characterized by different clinical presentation and severity of manifestation, from asymptomatic carrier to severe and life-threatening invasive infections. *S. pyogenes* is a pathogen that is very well adapted to the human population and the infections which it causes are of interest because of its widespread distribution and epidemic potential, with peaks in incidence mainly during the autumn-winter season in the Northern Hemisphere and in children attending out-of-home daycare centres [1]. These diseases are treated effectively with antibiotic therapy, but ineffective treatment can lead, although rarely, to late non-infectious complications such as rheumatic fever and post-streptococcal glomerulonephritis.

In the 2022/2023 winter/spring season, there was an exponential growth in the number of infections caused by group A beta-hemolytic streptococcus worldwide. Some countries (UK, USA, Netherlands, France, Sweden) reported an increase in severe invasive infections, including an increased number of deaths [2,3]. Children under 10 years of age were at higher risk. In Bulgaria there was also an increase in cases, mainly among children attending kindergarten and primary school [2].

It has been shown that *S. pyogenes* has a plethora of virulence factors (M-proteins, hyaluronic acid capsule, superantigens, etc.) that could induce a strong early immune response of the host innate and adaptive immunity [1,4,5]. Various cells can secrete a range of soluble inflammatory mediators, such as antimicrobial peptides, chemokines and cytokines, which are involved in innate and adaptive immune responses against GAS. An important step in the initial defense against pathogen is phagocytosis optimized by the opsonization with immunoglobulins and various complement components [5]. Mucosal-associated invariant T cells (MAIT) and $\gamma\delta$ TCR+V δ 2+ T cells play an essential role in the mucosal response against GAS infection via the secretion of significant amounts of IFN- γ , TNF α , and IL-2. These cytokines prepare and organize the subsequent specific anti-GAS immune response [5,6]. In acquired immunity an important role play Th-1, Th-17 sub cells and the cytokines produced by them IL-6, TNF α , IFN- γ , etc. [5].

The aim of the present study was a prospective characterization of immune responses in children, infected with *Streptococcus pyogenes*, diagnosed with tonsillitis during the streptococcal epidemic 2022/2023 in Bulgaria, in the Clinic of Pediatrics in University Hospital "Lozenets".

Materials and methods. The study included 42 children who were examined and diagnosed in the Medical Center or Clinic of Pediatrics in University Hospital “Lozenets” in the period February - April 2023. Based on the clinical examinations and tests, the children were divided into three groups as follows:

GAS patients group. Twenty-two children with proven streptococcal infections were included, mean age 7 ± 1.84 years; ten were girls and twelve boys. All patients were infected with *Streptococcus pyogenes*, proved by positive Strep A rapid test. Twelve patients had streptococcal tonsillitis, four were diagnosed with scarlet fever, five had recurrent streptococcal infections, and one had fever and developed a rash.

Two control groups of ten children each were included in the study.

Control group 1 (used for comparison of humoral and cellular immunity) consisted of ten children: eight boys and two girls, mean age 5.9 ± 2.7 years.

Control group 2 consisted of ten children: six boys and four girls, mean age 10.9 ± 3.04 years, was used for comparison of cytokine parameters between patients and healthy individuals.

Methods and parameters investigated. All children included in the study were tested with rapid OSOM Strep A Test (SEKISUI Diagnostics, USA). All patients were positive for Strep A test and the controls were negative.

Immunoglobulins and complement components. Total immunoglobulins IgG, IgM, IgA, complement components C3 and C4 were measured by Chemiluminescent Microparticle Immuno Assay on Alinity automated analyzer (Abbott, USA).

Cytokine quantification by ELISA. Serum cytokines IFN γ , IL-6, IL-1 β , TNF α , IL-17A and IL-10 were measured by the respective commercially available ELISA quantification kit (Diacclone SAS, France), according to the manufacturer's instructions.

Flowcytometry analysis. Peripheral venous blood samples (EDTA anticoagulated) were collected from all participants. The absolute counts and percentages (%) of CD3+ T cells, CD4+ T cells, CD8+ T cells, B cells, and NK cells were determined using 6-colour TBNK (BD Biosciences) kit, according to the manufacturer's instructions. Briefly, 50 μ L of whole blood was stained with 10 μ L of a 6-colour TBNK antibody cocktail for 20 min. After adding 450 μ L of lysis solution (BD Biosciences) and after 10 min of incubation, the samples were analysed using FACS Canto II flow cytometer (BD). All data were analysed using BD FACS Canto v3.0 software.

Statistical methods. Kolmogorov–Smirnov, Shapiro–Wilk test, Student's *t* test and Mann–Whitney U test for data analysis, were used. We used statistical

software IBM SPSS Statistics 25 and GraphPad Prism. Differences at $p < 0.05$ were considered statistically significant.

Results. Distribution of the obtained raw data. We found that serum IgG, IgM, IgA and C3, C4 complement fractions as well as lymphocyte subpopulations data had a normal Gaussian distribution, which justified the use of the Student's t test. The distribution of the cytokine data showed non-normal distribution; therefore, we used non-parametric Mann–Whitney U test.

Study of humoral immunity. The following parameters were examined in the sera of the participants. Total IgG, IgM, IgA and complement components C3, C4; as well as serum cytokine levels. The obtained results for total immunoglobulins and complement components are shown in Fig. 1.

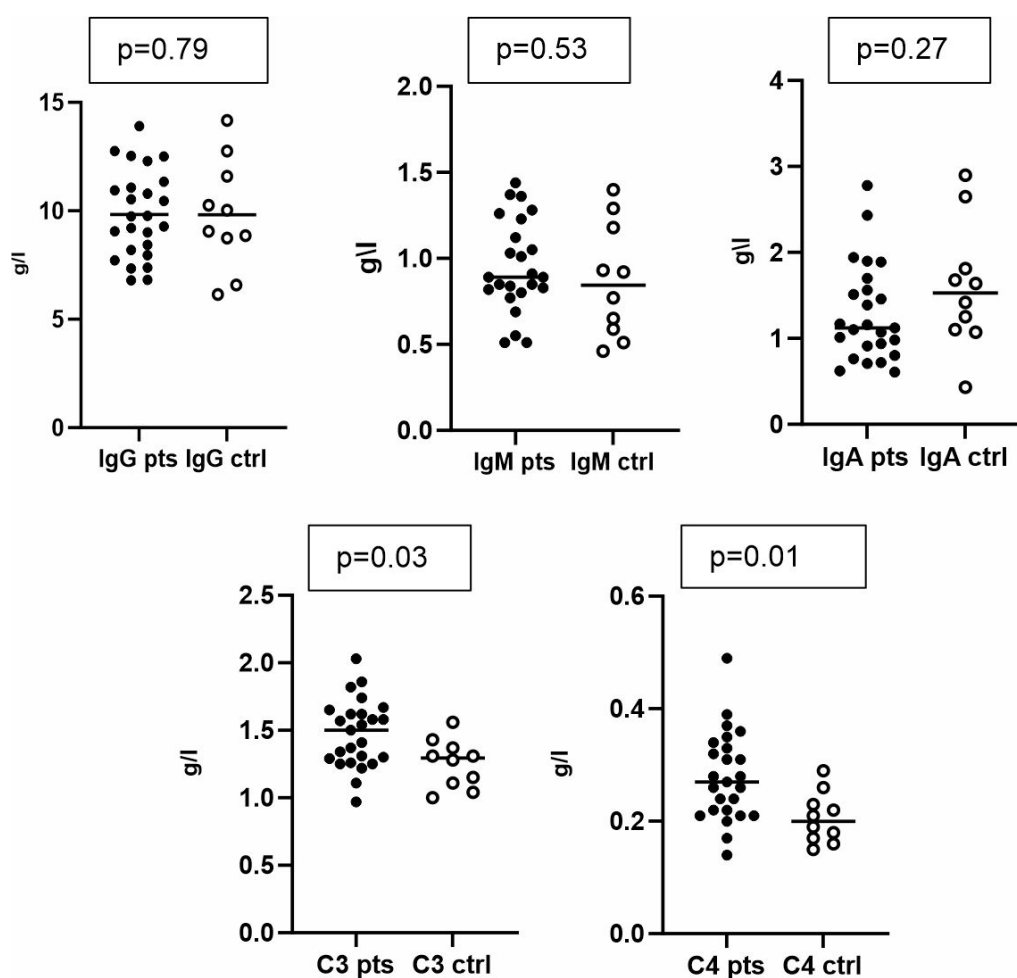


Fig. 1. Serum levels of IgG, IgM, IgA and C3, C4 complement fractions in children with Streptococcal infection and healthy controls. Legend: pts – patient group; ctrl – control group 1

Among the total immunoglobulin levels of IgG, IgM, IgA, there were no significant differences between infected and healthy control children. C3 and C4 complement components in serum were significantly increased in the patient group compared to healthy control ($p = 0.03$ and $p = 0.01$, respectively). The increase of C3 and C4 complement is a common phenomenon in active bacterial infections, as we observed in our patient group.

The presence and concentration of cytokines in serum are associated with activation of different cell populations, both from the innate immunity (MAIT and $\gamma\delta$ T cells) and from T lymphocytes associated with the adaptive immune responses. The results obtained from the cytokine analysis in GAS infected children are presented in Fig. 2.

The concentration of IL-6 in patients was more than three times higher ($p < 0.001$) than that of healthy children. The concentration of IL-10 in the serum of children with Strep A infection was over 4-fold higher than in the control group ($p = 0.003$). There was also a statistically significant increase in IFN γ levels in the patients compared to the control group ($p = 0.02$). The serum level of IL-17A

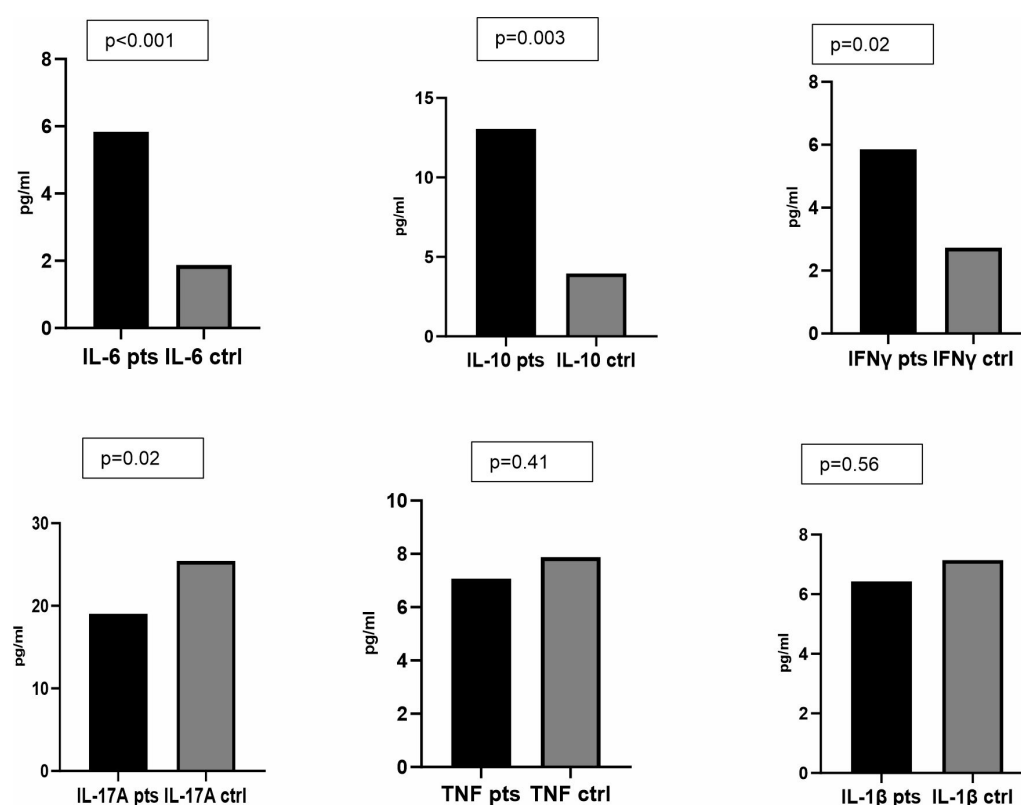


Fig. 2. Comparison of median values of serum cytokines between children infected with Strep A (black columns) and control group 2 (gray columns)

T a b l e 1

Cell subpopulations and populations in children with Streptococcus infection compared to healthy individuals, percentage and absolute counts in patients

Lymphocytes subsets	Control group 1, $N = 10; \bar{x} \pm SD$	Patients $N = 22; \bar{x} \pm SD$	p
WBC $\times 10^9$	7.8 ± 2.6	11.9 ± 6.4	0.063
% Lymphocytes	47.7 ± 11.7	28.6 ± 16.56	0.003
%* CD3 ⁺	71 ± 4.7	65 ± 6.4	0.017
abs. count CD3 ⁺	2550 ± 809	1793 ± 713	0.012
% CD4 ⁺	42.3 ± 6.2	33 ± 7.3	0.004
abs. count CD4 ⁺	1538 ± 595	924 ± 407	0.002
% CD8 ⁺	22 ± 3.6	22 ± 5.2	0.60
abs. count CD8 ⁺	780 ± 267	648 ± 335	0.28
CD4:CD8 ratio	1.9 ± 0.55	1.58 ± 0.62	0.089
% CD19 ⁺	16.3 ± 2.22	18.2 ± 5	0.23
abs. count CD19 ⁺	582 ± 216	461 ± 245	0.34
%CD3 ⁻ CD16 ⁺ 56 ⁺	11.5 ± 5.05	15 ± 5.1	0.078
abs. count CD3-CD16 ⁺ 56 ⁺	433 ± 293	394 ± 221	0.77

Legend: WBC – white blood cells; CD3+ – total T-lymphocytes; abs. count. – absolute count, CD4⁺ – T helper cells; CD8⁺ – T cytotoxic cells; CD19⁺ – B cells; CD3⁻ CD16⁺ 56⁺ – NK cells; * – % from lymphocytes

in the patient group was statistically significantly lower, compared to the control group ($p = 0.02$). For cytokine levels of TNF α and IL-1 β , we found no significant differences between the infected children and healthy controls.

Lymphocyte subpopulations in children infected with Strep A. The results of flow cytometric immunophenotyping of peripheral blood lymphocytes are presented in Table 1. Both % distribution and absolute counts of lymphocyte populations and T lymphocyte subpopulations were analyzed. The distribution of the primary data was normal, Gaussian, and therefore analysis was performed using the parametric Student's t test.

Our results showed significantly decreased lymphocytes – lymphopenia in the peripheral blood of infected children compared to the group of controls ($p = 0.003$). The total T lymphocyte (CD3+) and T helper lymphocyte (CD4+) populations in the group of patients were also significantly decreased compared to the group. Although not statistically significant, there was an increase in the percentage of NK cells of the patients. We observed no significant differences of CD8+ and B cells between the patients group and controls.

Discussion. In this first Bulgarian, prospective study of the immune status of children infected with Strep A, who developed tonsillitis during the winter/spring epidemic of 2023, we found differences in the immune status between GAS infected children and controls. We studied the humoral immune response and found out that there was no significant difference of total IgG, IgA and IgM

levels between patients and controls. However, the C3 and C4 complement factors were increased in the patient group. This may influence the opsonization of the streptococcal microorganism with subsequent phagocytic destruction. Like all gram-positive bacteria, GAS is resistant to humoral lysis by the host membrane-attack complex (MAC) [7, 8].

Furthermore, as important humoral factors we also measured a panel of six cytokines associated with pro-inflammatory (Th-1, Th-17) and protective immune responses. They are formed by various activated cells as the result of an infection. We found significantly higher levels of IL-6 and IFN γ , which characterizes Th-1 immune response. Also we observed an increase of IL-10 in patients compared to controls. IL-10 levels characterize an anti-inflammatory response (protection) in the patients. Unexpectedly, we found decreased levels of IL-17A in GAS-infected children compared to controls. Elevated levels of IL-17A indicate a Th-17 pro-inflammatory response, but in some infections (e.g. with staphylococci) it may also characterize protection [9]. There were no differences in serum levels of TNF α and IL-1 β between the two studied groups.

It has been shown that cells of the innate immune system secrete a number of inflammatory mediators, including cytokines, chemokines, adenosine monophosphate (AMP) and prostaglandins (PG), as part of a highly coordinated immune response against streptococcal infection [1, 5]. Mucosal-attached invariant T cells, a subclass of innate lymphocyte-like T cells, are accepted as a major source of IFN γ and pro-inflammatory cytokines such as TNF α , IL-2 [6]. Another group of lymphocytes such as T helper cells with different phenotypes: Th-1, Th-2, Th-17 and Tregs, also play a very significant role in the pathogenesis of GAS infection [5]. Recently, it has been observed that GAS antigens induce a pro-inflammatory immune response via Th-1 and Th-17 cell pathways, with IFN γ release [10]. It has been suggested that IL-17A-producing T helper cells are particularly important in providing host immune protection against extracellular microorganisms which attack the superficial epithelium, playing a major role in the clearance of GAS-infection in acute pharyngitis [11]. In our cohort of children, we found decreased serum IL-17A levels, which may be associated with more complex regulatory mechanisms of IL-17A induction and production [12]. It has been shown that during GAS systemic infection, an anti-inflammatory cytokine production is probably induced, which is in agreement with our findings of increased IL-10 in patients. Furthermore, other authors found increased T regulatory lymphocytes that lead to suppression of the active immune response against GAS [13]. T regulatory cells can also be induced by superantigens or M-protein, triggering cross-linking of TCR and CD46 on naïve T cells. GAS M-protein and superantigens can both activate Th-17 (activation of the immune response) and to induce and activate Tregs (immunosuppressive/immunomodulatory effect), which in turn to facilitate antigen evasion from the immune system, and furthermore to promote the advancement of the streptococcal infection.

We found markers for pro-inflammatory immune response (increased IL-6 and IFN γ) and also evidence of a suppressive one (decreased CD4+, decreased IL-17A and increased IL-10 levels).

Our results on the major lymphocyte subpopulations revealed lymphopenia in peripheral blood. Total T lymphocytes and T helper subpopulations were also significantly reduced in infected children. This may be related to the reduced pro-inflammatory Th-17 response. The study of cell-mediated immunity by TBNK immunophenotyping did not provide additional information on the type of cell-mediated immune phenomena associated with GAS infection. Such information on the pro-inflammatory Th-1 and Th-17 pathways and protective immunity was obtained from the cytokine assay.

In an elegant study held by ANDERSON et al. [14], the first human in vivo model of GAS infection was established by induction of acute pharyngitis. The study monitored the acute immune responses before antibiotic treatment of the participants. An in-depth dissection of the immune responses that developed over the course of this model was performed. They found: 1) elevation of serum IL-1Ra, IL-6, IFN γ , IP-10, and IL-18; 2) elevation of blood dendritic cells and monocytes; 3) decreased levels of blood B and CD4+ T lymphocytes (Th-1, Th-17, Tregs, TFH); and 4) activation of unconventional $\gamma\delta$ TCR+V δ 2+ T cells and MAIT, which the authors refer to as “immune signature” of GAS infection. The authors recommend more extensive studies in the setting of a natural Strep A infection.

In conclusion, the detailed study of humoral and cellular immunity in children with streptococcal infection showed changes in both humoral immune responses (an increase in C3 and C4 complement components in serum) and in the cellular immune responses of adaptive immunity (lymphopenia, decreased total T lymphocytes, especially in the T helper subpopulation). This was also accompanied by significantly increased production of IFN γ , IL-6 and IL-10 and reduced serum levels of IL-17, compared to healthy controls. Our study can be regarded as a thorough dissection of the immune response against GAS infection with clinical manifestation of tonsillitis in the setting of a naturally evolving epidemic in Bulgaria. Moreover, our findings are in agreement with the “immune signature” found by Anderson et al. [14].

The obtained results are original for the Bulgarian population of children infected with Strep A infection, they contribute valuable information to the world experience and hypotheses on the pathogenesis of streptococcal infection.

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