OPINION

by Assoc. Prof. Georgi Stoyanov Nikolov, MD, PhD
National Center for Infectious and Parasitic Diseases, Sofia,
Department of Immunology,

Subject: awarding the Doctor of Philosophy (PhD) degree

at Department of Immunology, National Center of Infectious and Parasitsc Diseases

Field of higher education: 7. Health and Sport

Professional Direction: 7.1. Medicine

Scientific specialty: Immunopatology and allergology

PhD student: **Milena Alexova, MD**

Topic: **Post-exposure and post-vaccination T-cell immune response against SARS-COV-2**

General Presentation of the Procedure:

According to the Order № 189/14.06.2024 of Prof. Iva Hristova, MD, DSc, Director of NCIPD, Sofia, and to the Protocol № 02/13.06.2024 of the Scientific Council of the NCIPD I have been elected as internal member of the Scientific Jury for awarding the Doctor of Philosophy (PhD) degree of PhD student Milena Alexova, MD. Based on the decision taken during the first meeting of the Scientific jury I am assigned to prepare an opinion on the procedure for acquiring the educational and scientific degree Doctor of Philosophy.

The set of materials, provided to me on paper/electronical media, fully complies with the requirements of the Law For The Development Of Academic Staff In The Republic Of Bulgaria, as well as the Rules On The Conditions And Procedure For Acquiring Science Degrees and Holding Academic Positions in NCIPD, Sofia.

Dr. Milena Alexova graduated in 2018 from St. Cyril and Methodius University, Skopje, North Macedonia, with a master's degree in medicine.

During the period February 2018 - July 2018, she was trained under the Erasmus+ program at the Medical University, Plovdiv.

In the period 2019 - 2020, she is a general practitioner, the city of Skopje.

From 2021 until now, she is a full-time doctoral student in the Immunopathology and Allergology program in the "Immunology" department of the National Center for Infectious and Parasitic Diseases, Sofia.

Subject relevance

Coronaviruses pose a constant threat to human health and the economy. The rapid spread of SARS-CoV-2 since 2020 has so far affected more than 774 million people and caused more than 7 million deaths.

The immunological response to the SARS-CoV-2 virus depends mainly on the formation, activation and action of virus-specific T and B lymphocytes. The development of immunological memory is essential to control infection, as it creates long-lasting protection and ensures a more rapid immune response upon re-exposure, thereby preventing reinfection and/or disease. Knowledge of these mechanisms is a prerequisite for the development and production of new, effective vaccines to train the immune system to recognize and respond to SARS-CoV-2 and to create a sufficiently durable immune memory. Studies of the immune response against the virus and the factors that determine the qualities of immune memory are essential not only on an individual level. They are critical in developing a comprehensive strategy to combat the evolving virus and put the pandemy under control.

Bearing all this in mind, I believe that the topic of Dr. Alexova's doctorate is relevant and has a specific practical orientation. The PhD is a detailed description of her experience in studying the post-exposure and post-vaccination T-cell immune response against SARS-COV-2.

General characteristics and structure of the dissertation

The dissertation is properly structured and written in a scientific style. It is printed on 187 standard pages and illustrated with 58 figures, 7 tables. It includes all mandatory elements such as: content, abbreviations used, introduction, literature review, aim and objectives, materials and methods, results and discussion, conclusion. Nine conclusions have been formulated and 6 theoretical and 4 applied contributions were identified. The bibliographic reference includes a total of 469 sources in English published in the last 10 years.

Evaluation of the structural parts of the dissertation

The literature review has a volume of 37 pages. It comprehensively presents current data on the characterization, evolution and pathogenesis of SARS-CoV-2 infection. The post-exposure immune response against the virus has been described in detail. Current methods for characterizing the T-cell immune response are analyzed and the mechanisms of the immune response and the emergence of T-cell memory after immunization are discussed.

The review concludes with a brief summary of the outstanding questions regarding SARS-CoV-2 immunological memory, which clearly presents the objective of the present dissertation, namely: "to investigate the emergence, persistence and functional characteristics of SARS-CoV-2-specific T cells , induced after infection, immunization or hybrid exposure".

For the fulfillment of the set goal, Dr. Alexova has focused her efforts on solving 7 tasks that structure a grounded approach to the study.

The setting of the study, the stages of the study and the research methods used are well described. The chosen design allows successful implementation of the specific tasks and ensures the achievement of the main goal of the dissertation work.

The study included:

A. 562 patients with proven SARS-CoV-2 infection.

- in the acute phase of COVID-19 during hospitalization and/or < 20 days after PCR (+) result – 243.
- up to 3 months after PCR (+) result - 105
- from 3 to 9 months after PCR (+) result – 119
- from 9 to 12 months after PCR (+) result - 26
- more than 12 months after PCR (+) result – 69

They were followed over time as follows: 124 patients - twice, 41 - three times and 12 - four times (on average, respectively, 93, 254 and 378 days after PCR+ result).

B. Control groups:

- 120 persons without data on past infection, vaccinated against SARS-CoV-2.
- 38 clinically healthy persons, without data on past infection, unvaccinated.

Isolated lymphocytes from 279 donors and serum/plasma from 449 donors were stored.

A database of clinical-epidemiological data was created for the examined patients, containing the following information: date and result of SARS-CoV-2 PCR and/or rapid test; symptoms and complications, severity of COVID-19, according to WHO criteria, co-morbidities and syndromes, vaccination status (administered doses and type of vaccine).

A variety of modern clinical, laboratory, instrumental immunological methods have been appropriately used, which contribute to solving the development tasks.

The survey data has been processed statistically with appropriate methods of analysis and illustrated with a sufficient number of figures and tables.

The results of each research task are presented in a comprehensive volume and are suitably illustrated, making their perception easy and convincing.

The conducted study found that absolute lymphocyte count (LyAC) is a major criterion

for determining the clinical severity of SARS-CoV-2 virus infection.

Depending on the number of lymphocytes, patients were classified into three subgroups: Group A - with LyAC within the reference values for healthy controls (>1100), Group B - with first degree lymphopenia (LyAC from 1100 to 800 cells/μl) , and Group C - with 2nd and greater degree of lymphopenia (< 800 cells/μl).

A significantly higher proportion of Th1 lymphocytes in group A compared to B and C and an increase in the proportion of activated (CD38+DR+) CD8 T-cells were found. Decreased levels of helper and suppressor T cells, especially in severe cases, are a sign of an impaired immune response. An increase in naïve helper T cells and a decrease in memory helper T cells were also observed, suggesting a compromised immune defense against SARS-CoV-2. At the same time, an increase in the T-regulatory subpopulation with non-specific inhibitory activity (CD25hiCD127-CD4+) was found in patients with lymphopenia. These cells play a key role in the immunopathological changes in severe forms of COVID-19 by suppressing the effector functions of T-lymphocytes without limiting pathological immune inflammation. The CD39+Treg subpopulation distinguishes patients with moderate and severe COVID-19 and can be used as a predictive marker.

Severe infection is associated with a specific cytokine imbalance: a significantly reduced IFN-γ/IL-10 ratio and an increase in the IL-6/IL-10 ratio. This affects the effector function of T-lymphocytes. Immune inflammation is not controlled, and the induction of long-term immune memory is compromised. The combined use of IFNg/IL-10 and IL-6/Il-10 ratios is suitable for monitoring the clinical course of COVID-19.

After the 9th month of infection, circulating virus-specific IFNγ+ T cells decrease significantly and the application of a modified stimulation or labeling protocol (activation-induced molecules, AIMs) is a suitable method for the detection of SARS-CoV-2-specific memory (including stem memory) cells in the late stages of infection.

Because of the more rapid decline in RBD-IgG and IgA titers, there is a lack of correlation between virus-specific humoral and T-cell responses, and the significantly higher values of HKU-1 IgG antibodies in asymptomatic persons, in combination with well-expressed SARS-A CoV-2 specific CD8 T-response and absent SARS-CoV-2 antibodies is evidence of cross-protective immunity following prior corona virus infections.

Compared to infection, immunization and hybrid exposure (immunization after recovery) provided a significantly stronger RBD-specific antibody response associated with the formation of a larger CD4 SCM pool and more intense CD4 S1-responses.

After the 9th month and up to 24 months after infection, AIM+ T-cells recognizing

SARS-CoV-2 peptides were detected in all studied donors. Virus-specific CD4 T-lymphocytes have a predominantly naïve and central-memory phenotype, declining more rapidly over time; virus-specific CD8 T cells are predominantly effector and terminal effector phenotypes and are maintained at a lower but constant level.

More than 12 months after infection, memory CD4 and CD8 T-cells are detected that recognize variants that arose later and significantly diverged from the initial variant. Hybrid exposure stimulates and maintains the stem-memory pool. CD8+ TSCMs are capable of rapid reverse differentiation into effector cells, thereby providing immediate protection regardless of the variant that induced them. Therefore, virus-specific TSCMs are a robust marker of protection in the setting of circulating and evolving SARS-CoV-2.

The obtained original results of the study have been competently discussed, a thorough analysis has been made and relevant conclusions have been formulated

The dissertation work is designed precisely and I have no significant remarks on the methodology, the presentation of the results and their analysis.

Assessment of contributions, publications and personal contribution of the PhD student

I accept the formulated contributions and believe that they objectively reflect the real results of the conducted studies. I value the following contributions as more significant:

- The first prospective study of T-cell immune memory to SARS-CoV-2 was carried out on statistically reliable groups of the Bulgarian population and it was shown that the early increase of induced T-regulatory cells (Treg/CD39+) is associated with a more severe course of SARS-CoV-2 infection.
- A hypothesis was formulated for a cross-protective effect of infections with the seasonal HCoV HCU-1, as a result of stimulation of memory CD8 T-cells recognizing structurally similar peptide epitopes from SARS-CoV-2.
- SARS-CoV-2-specific CD8+ stem-like memory cells (TSCM) have been demonstrated to be an accessible and relatively independent marker of viral evolution for long-term protection against COVID-19.
- The cross-reactivity of virus-specific memory T lymphocytes with evolved variants of SARS-CoV-2 (XBB, BA) depends significantly on the severity of infection, subsequent immunization and the presence of RBD-specific IgA.
- A modification of the IFN gamma-based ELIspot test was introduced, which allows detection of SARS-CoV-2 specific T-lymphocytes more than 12 months after exposure.

A list of 4 publications and 17 participations in scientific forums, related to the dissertation work is presented in the abstract, as well as participation in a research projects.

In terms of number and quality, the scientific works exceed the minimum requirements for the PhD dergree, according to the quantitative criteria regarding the publication activity laid down in the regulations of NCIPD, Sofia.

Abstract

The abstract is written on 75 pages and is prepared according to the requirements, accurately reflecting the dissertation research, results and conclusions.

CONCLUSION

In conclusion, the doctoral thesis of Milena Alexova, MD meets the regulatory requirements and is the result of the doctoral student's own development.

It concerns a significant problem of modern medicine related to the study of the post-exposure and post-vaccination T-cell immune response against the SARS-COV-2 virus. The specific tasks that ensure the achievement of the main objective of the dissertation have been successfully completed and the results obtained lead to the formulation of conclusions and contributions that I accept.

All this gives me grounds to give a positive assessment of the dissertation paper and I propose to the members of the esteemed Scientific jury to award to Milena Alexova, MD Doctor of Philosophy degree in the doctoral program of Immunopathology and allergology.

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02. 08. 2024

Assoc. Prof. Georgi Nikolov, MD,PhD