REVIEW

by Prof. Krasimira Todorova-Hayrabedyan, PhD, DSc
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of a dissertation for awarding the educational and scientific degree of 'Philosophy Doctor',

author: *Nikola Ralchev Ralchev*

on the topic: "*Suppression of antigen specific B lymphocytes by protein engineered molecules in hypersensitivity reactions*",

Professional field 4.3. "Biological Sciences", scientific specialty "Immunology",

with supervisor: *Prof. Andrey Chorbanov, PhD*

Department of Immunology, Laboratory of Experimental Immunology, The "Stephan Angeloff" Institute of Microbiology, Bulgarian Academy of Sciences

1. General Presentation of the Procedure and PhD Student

The presented set of materials on electronic media **is** in accordance with the Law on the Protection of Scientific and Mathematical Works, the Regulations of the Bulgarian Academy of Sciences and the Institute for the Development of the Academic Staff and includes the following documents: Application to the Director of The Stephan Angeloff Institute of Microbiology for admission to the defense of a dissertation; curriculum vitae in European format; copy of a diploma for higher education; orders for enrolment in doctoral studies, for extension of training; for expulsion with the right to defense; Protocols of minimum candidate; minutes of the Seminar for preliminary discussion of the dissertation and the decisions taken for the disclosure of the procedure and for the composition of the scientific jury; dissertation; abstract in Bulgarian and English; a list of scientific publications on the topic of the dissertation; copies of scientific publications; list of participation in scientific forums; a declaration of originality and authenticity of the attached documents; other documents related to the course of the procedure. The documents are neatly and hierarchically arranged in an electronic file. The information presented is detailed. The PhD student has attached 2 publications.

2. Brief biographical data about the PhD student

Nikola Ralchev Ralchev was born on November 1, 1995 and graduated with a Bachelor's degree in Molecular Biology and a Master's degree in Cell Biology and Pathology from Sofia University "St. Kliment Ohridski". Over the years, he has gained significant scientific experience, starting as an intern at the Laboratory of Experimental Immunology at the Stephan Angeloff Institute of Microbiology, where he participated in the development of therapy and animal models of house dust

allergy. Later, as an intern at the Cancer Research Center of Lyon in France, he became involved in research on the non apoptotic functions of proteins of the Bcl 2 family, contributing to the understanding of the molecular mechanisms associated with cell death and tumor progression. Since 2021, she has been working as an assistant at the Laboratory of Experimental Immunology at the Bulgarian Academy of Sciences, where she continues her research in the field of immunotherapy In his scientific work, Nikola Ralchev applies a wide range of modern methods in the field of animal models, immunology, molecular biology and bioinformatics, in which he demonstrates in-depth knowledge. Its ability to combine these techniques allows for detailed studies on the immunoregulation and molecular mechanisms of autoimmune and allergic diseases.

The results of his work have been published in renowned international scientific journals, such as the International Journal of Molecular Sciences, Molecular Nutrition & Food Research and Marine Drugs He is a co-author of more than 15 scientific publications, most of which have a high impact factor, and his works have been cited dozens of times in international scientific literature In addition, he actively presented his results at prestigious scientific conferences, including the European Congress of Immunology and the International Congress of Immunology, in many of which he participated with oral presentations.

Recognition for his scientific work is also expressed in a number of awards. In 2023, he was awarded the "*Ivan Evstratiev Geshov Award for the Youngest Scientist"* for outstanding achievements in biomedicine. The same year he received the award for the best work of a young Bulgarian microbiologist from *the Stefan Angelov Foundation* He has also won awards for presenting scientific reports at international and national forums, which further emphasizes the importance of his research for the development of modern immunology and molecular biology.

Nikola Ralchev participates in various scientific projects aimed at immunotherapy strategies for autoimmune diseases, allergies and oncological conditions. She works in a multidisciplinary environment where she combines fundamental and applied research to develop innovative methods for modulating the immune response. With his knowledge, experience and dedication to science, he has contributed significantly to expanding the understanding of immunoregulation and potential therapeutic approaches in various diseases

3. Relevance of the topics and appropriateness of the set goals and objectives

The problem developed in the dissertation is extremely relevant both from a scientific and scientific applied point of view. Allergic diseases, and especially IgE induced reactions, continue to be serious medical challenges that affect a significant percentage of the population. These diseases not only affect the quality of life of patients but also pose serious social and economic problems. The paper focuses on innovative methods for inhibiting allergen specific B cells through chimeric molecules, which provides a new scientific direction for the treatment of allergies by overcoming the limitations of traditional methods. The relevance of the tasks and approaches in the dissertation is expressed in the solution of existing problems such as insufficient specificity and durability of

traditional therapies, and in the progress in the development of targeted therapeutics that combine immunological innovations with applied commitments to patients.

4. Knowledge of the problem

The PhD student demonstrates excellent awareness of the state of the problem, and in the dissertation presents a comprehensive literature review, which covers both the theoretical foundations of hypersensitivity and the pathophysiology of allergies, as well as modern therapeutic approaches in the field of immunotherapy. He creatively works with the literature material, critically linking existing scientific evidence with the development of an innovative method for selectively suppressing allergen specific B cells through protein engineered molecules, which highlights both the scientific validity and the practical applicability of the study. The literature used varies by year of publication from the early 2000s to the most recent publications, with approximately 60% of the cited material being published in the last 5 years and covering topics related to the pathophysiology of allergies, innovative therapeutic strategies, immunological models and protein engineering.

5. Methodology of study

The methodology of the study is extremely innovative, conceptualizing around the development of chimeric bi specific chimeric molecules recognizing an allergen on the surface of auto reactive B cells and the simultaneous binding and activation of inhibitory receptors in these same cells, leading to suppression or deletion of this auto-reactive branch. This concept was introduced by the scientific supervisor of the PhD student, Prof. Andrey Chorbanov and his team, and has already been successfully proven to act in DNA-specific auto-reactive B-cell clones. Moreover, over the years, a number of successful efforts have been made to move from the application of these chimeric molecules directly to primary human auto reactive cells, to the development of experimental animal and humanized models, in order to recreate the entire complex process of auto reactivity in human pathology, and identify targeted processes to influence it.

The dissertation used two different mouse models to investigate house dust allergy (HDM) and the effectiveness of chimeric molecules as a potential therapy: 1. A humanized mouse model ($Rag2^{-/-}\gamma c^{-/-}$ mice) in which peripheral mononuclear cells (PBMCs) were transferred from sensitized (allergic) patients to a house dust mite (*Dermatophagoides pteronyssinus*, the causative agent of HDM allergy). These mice do not have adaptive immunity and are "tolerant" of xenotransplantation, which allows the survival and functioning of human cells in them. Thus, mice develop an immune response similar to that of humans to allergens from house dust, because human immune cells create a specific human microenvironment in the recipient's immune organs and can interact with each other Subsequently, the pattern is provoked by the allergen (e.g., intranasal administration of *D. Pteronyssinus extract*). Human T and B cells recognize the intranasal allergen (through their human receptors) and reproduce a Th2-mediated allergic response, analogous to that in humans, with the production of allergen-specific IgE antibodies and inflammatory changes in the respiratory tract. These IgE antibodies bind to mouse mast cells, because. Their FcεRI receptor recognizes human IgE and retains it on their surface. Thus, repeated exposure to the same allergen *in vivo* leads to its crosslinking with human IgE on mouse mast cells and causes type I hypersensitivity – mast cell

mediated effector phase with mast cell degranulation and an allergic reaction (bronchoconstriction, vascular permeability, etc.), resembling an asthma attack in humans.

Main advantages of the $Rag2^{-/-}\gamma c^{-/-}$ model: (1) It allows the study of human immune responses in a living organism, overcoming the limitations of *in vitro* experiments. Animal models with human cells provide circulation, a three-dimensional tissue environment and multi-organ interactions, which are currently impossible *in vitro*. (2) The $Rag2^{-/-}\gamma c^{-/-}$ model for allergy mimics a real allergic reaction – human cells communicate with each other (human antigen-presenting cells present the antigen to human T-cells, which help human B-cells switch to IgE, etc.) and produce (IgE) that can activate receptors of the host organism. (3) The model is relatively fast and flexible – by transfusion of sensitized donor cells, the mouse is already allergic, and reactions are induced in days to weeks, instead of waiting for sensitization of a naïve immune system. (4) The model allows for the testing of therapeutic agents (such as the antibody under consideration) in preclinical terms because it allows for the evaluation of their effect on human cells *in vivo*, including pharmacokinetics, distribution and potential side effects on non-target human cells.

The model has some limitations at the same time: (1) Short-term and resistant graft: PBMCs from an adult donor do not persist for long in the mouse; (2) Potential for a reaction against the mouse organism (GvHD); (3) Lack of a complete human environment: Sometimes human cells do not receive the necessary signals, or vice versa – mouse cells do not respond to human signaling molecules. The Th2 secreted by humans may not interact optimally with the receptors of mouse eosinophils or epithelial cells, which can alter the pattern of inflammation (4) Limited innate immunity: In PBMC transfer, human neutrophils, eosinophils and basophils are absent (they are not transferred to the PBMC fraction), therefore the pattern of inflammation could be limited to some extent relative to real human ones. In this model, human CR1 is expressed mainly on the introduced human B-lymphocytes, which is why cross-species interactions with the mouse complement system are limited due to the species specificity of the latter. Human CR1 may not bind mouse C3b effectively, meaning that human B cells in the model are probably not regulated by complement as they would in *vivo* in humans.

Although humanized mouse models by hematopoietic stem cell transplantation (HSC) give a longer term recovery of the entire immune system (including the development of B and T cells inside the mouse), they are more complex and slower, while the presented model has the advantage of speed and relatively less complexity, respectively higher reproducibility.

The main idea behind the use of chimeric molecules is the selective elimination of allergen specific B cells that produce IgE antibodies against HDM. Chimeric molecules consist of: A monoclonal antibody specific to an inhibitory receptor on B cells (human CR1 or mouse FcγRIIb). Allergen epitope (Dp52 71) of *Dermatophagoides pteronyssinus* (Der p 1), which recognizes and connects Der p 1 specific B cells Binding of the chimeric molecule to allergen specific B cells via the BCR (B-cell receptor) that recognizes the Der p 1 epitope also leads to simultaneous activation of the inhibitory receptor (CR1 or FcγRIIb), resulting in a signaling cascade that suppresses the activity and survival of the B cell. As a result, these cells die or stop producing IgE, reducing the allergic response.

To determine whether chimeras reduce the allergen specific immune response and pulmonary inflammation, the following indicators were monitored: levels of anti Der p 1 IgE antibodies in BALF

(bronchoalveolar lavage), mast cell degranulation (β-hexosaminidase activity), phenotyping of pulmonary infiltrates by FACS, histological analysis of the lungs.

Results of the humanized Rag2 γc model: significantly reduced the levels of anti Der p 1 IgE in BALF compared to the control group, reduced degranulation of mast cells and lower infiltration of inflammatory cells (especially lymphocytes) in the lungs, with a reduction in histological findings of inflammation.

Questions: Due to the use of a monoclonal antibody to generate the chimeric bi-specific antibody, I have questions about its anti-CR1 specificity. Are there structural allotypes of CR1, out of the available four associated with their varying number of repeat domains ("long homologous repeats"), that the antibody could miss? would you create other variants of the antibody in the future or is this not necessary (if the CR1 domain connecting C3b/C4b is recognized)?

Is it possible that there are known single nucleotide polymorphisms that could lead to lower recognition? For example, single polymorphisms in exon 29, whose affected products are localized extracellularly and define blood-group antigens of the Knops system? Do you have such phenomena in mind when developing the next generation of chimeric molecules?

2. Chronic mouse allergy model (BALB/c mice): It uses a BALB/c mouse model in which mice are sensitized and repeatedly induced with HDM allergens to create chronic allergic inflammation. Through this model, the long term effects of the therapy and its impact on the systemic and local immune response are tracked Mice were treated with Dp52 71 chimeric molecules and the levels of anti HDM IgE, IgG1, IgA, IgM in serum and BALF, the cytokine profile (IL-4, IL 5, IL 9, IL 13) in BALF were measured.

In this model, serum levels of IgE and IgG1 to HDM and levels of inflammatory cytokines in BALF were significantly reduced in chimeric molecule-treated mice, indicating reduced Th2-mediated immune activation. Reduced infiltration of eosinophils, significantly less inflammation in the lungs is observed.

It can be concluded that chimeric molecules effectively eliminate allergen-specific B cells and reduce the IgE dependent immune response in both models, reducing the inflammatory process in the lungs

In conclusion, although the data from the humanized and BALB/c models cannot be directly related due to their different cellular and immune environments, they together provide a comprehensive picture of the therapeutic potential of the chimeric molecule. The first model confirms the effectiveness of the therapy on human immune cells, while the second model – albeit with mouse components – allows for an upgrade through a longer and more complex analysis of the changes that occur during the allergic reaction. This synergistic approach is essential for assessing the potential clinical applicability of the new therapy.

6. Characteristics and evaluation of the dissertation

The dissertation, spanning 106 pages and containing 2 tables and 29 figures, is distinguished by a comprehensive and systematic approach to the study of hypersensitivity and allergies. The paper begins with an extensive literature review that lays the theoretical basis for the formulation of a new

scientific hypothesis – selective suppression of allergen-specific B-cells by protein-engineered molecules. The theoretical part (about 30 pages) presents modern theories and approaches to the treatment of allergies, including hypotheses for therapy that targets specific B-cells and broadly grounded scientific definitions. Chapter analysis integrates modern concepts in immunology with a detailed description of two experimental mouse models: the humanized Rag2 γc model, in which PBMCs from allergic patients are introduced into immunodeficiency mice, and the classic BALB/c model, in which chronic allergic inflammation is simulated through repeated sensitization. The methods used (FACS, ELISA, histopathological analysis) provide high reliability of the data and prove that the innovative approach leads to a significant reduction in IgE production and inflammatory response. The discussion is analytically written, with a critical and objective presentation of hypotheses, findings, conclusions. The contributions are valuable, presented briefly and without speculation, which gives exceptional weight to the work

7. Contributions and relevance of the development to science and practice

The scientific contributions of Nikola Ralchev's dissertation are significant both in theoretical and applied aspects. The main scientific contribution of fundamental importance is the demonstration of the overexpression of the FcγRIIb receptor on B lymphocytes, including IgE positive B cells, in the context of house dust allergy, which presents a new understanding of the involvement of FcγRIIb in allergic reactions This discovery offers a new perspective in the pathogenesis of allergy and may have the potential for future research in immunological-medical science related to the specific regulation of IgE-producing B cells. An additional scientific contribution is the established association between anti-HDM IgG1 antibody levels with specific indicators of allergic inflammatory activity, which may reveal the role of IgG1 antibodies in the pathogenesis of HDM allergy and support extended research into this interaction. From a scientific and applied point of view, the dissertation presents a real innovation with the creation of two experimental mouse models for house dust allergy – humanized and chronic, which provide a solid scientific basis for further research into the mechanisms of the disease and trials of new therapeutic approaches The protein engineered chimeric technology used to selectively eliminate allergen-specific B cells demonstrates significant therapeutic potential. Not only does this technology offer new options for specific allergy therapy, but it also lays the foundations for the development of therapeutics that can be adapted to individual patient characteristics and their respective immunological profiles. These scientific and applied scientific achievements prove that the methodology based on chimeric molecules and specific mouse models has great potential for future clinical research and implementation in the practice of treating allergic diseases, providing an important tool for expanding modern therapeutic approaches

8. Evaluation of the publications on the dissertation and personal participation of the PhD student

Nikola Ralchev has made a significant personal contribution to the dissertation research, which is confirmed by his active participation in the published scientific results. He is the first author in both publications that have IF, and the highest quartiles Q1 Q2 He is a co-author in a total of 15 scientific publications, where in 5 of which he is the first author, which many times exceeds the output of a PhD student. This clearly shows that his contribution is not limited to technical or experimental work,

but covers the entire process of scientific research, from the planning and execution of experiments to the analysis of results and the design of publications. The fact that he is the first author in a significant number of these publications highlights his leading role in the development of the concept, research design, and interpretation of the data. His leading participation in the research, as well as his significant scientific achievements, give reason to conclude that the formulated contributions and the results obtained in the dissertation are predominantly his personal merit.

9. Abstract

The abstract is extremely well structured and meets the requirements of the relevant regulations, clearly formulating the goals, methodology and main achievements of the dissertation. It adequately reflects the key results associated with the innovative therapeutic approach for selective suppression of allergen specific B cells, as well as the contribution of the humanized and BALB/c mouse models used to establish the effect on IgE production and inflammatory response. This confirms that the abstract presents the essential scientific and scientific-applied results of the dissertation in a clear and justified way.

CONCLUSION

The dissertation shows that the PhD student Nikola Ralchev Ralchev has in-depth theoretical knowledge and professional skills in the scientific specialty "Immunology", demonstrating qualities and skills for independent scientific research. In view of the above, I confidently give my positive assessment of the research carried out, presented by the above-reviewed dissertation, abstract, results achieved and contributions, and I propose to the honorable scientific jury to award the educational and scientific degree "Philosophy Doctor" to Nikola Ralchev Ralchev in the doctoral program in "Immunology" at the Department of Immunology, Laboratory of Experimental Immunology, The Stephan Angeloff Institute of Microbiology.

24.2.25

Sofia

Reviewer:

Prof. K. Todorova, PhD, DSc