REVIEW

of a PhD thesis

by Assoc. Prof. Petya Asenova Dimitrova, Laboratory of Experimental Immunotherapy, Department of Immunology, Institute of Microbiology "Stephan Angelov"

a member of the Scientific Jury selected with Order No. I-15/23.01.2025 by the Director of the Institute of Microbiology, BAS and elected as a reviewer by the Scientific Jury with Protocol No. 1/ 30.01.2024

Dissertation with author: **Nikola Ralchev Ralchev**, PhD student in full-time education at the Laboratory of Experimental Immunology of the Department of Immunology, Institute of Microbiology
Topic: Suppression of antigen-specific B lymphocytes by protein engineering molecules in hypersensitivity reactions
Scientific supervisor: Prof. Dr. Andrey Tchorbanov
Research Field: 4.3. Biological Sciences (Immunology)

1. General description of the submitted materials

The author of the dissertation, Nikola Ralchev, is a full-time PhD student at the Laboratory of Experimental Immunology of the Department of Immunology, Institute of Microbiology, Bulgarian Academy of Sciences, with a scientific supervisor, Prof. Andrey Tchorbanov. Nikola Ralchev has submitted the relevant documents in hard copy and in electronic form, according to the requirements of the Regulations for the Development of the Academic Staff of IMikB-BAS, namely: an application for admission to the defense, a copy of a completed master's degree (specialty Cell Biology and Biotechnology, Biological Faculty, Sofia University), an order for enrollment (I-91/31.07.2020), an order for extension of one year (I-91/31.07.2023), an order for dismissal with the right to defense, effective from 31.07.2024. (I-105/30.07.2024), exam reports (English (Protocol 63/12.02.2021), first (Protocol 28.01.2021) and second (Protocol 20.06.2022) minimum in the specialty) and courses in the specialty (Photoshop computer course (23.04.2021), course "Introduction to Cell and Tissue Engineering", course "Theoretical and Practical Foundations of Classical and Modern Histological Methods"), reference for credits obtained according to the requirements of IMikB-BAS (total 502 credits out of a minimum of 250 credits), pre-defense protocol (№2/20.01.2025) and preliminary review of the dissertation, PhD thesis and abstract in Bulgarian, list of publications (2 articles, one in the Scandinavian Journal of Immunology (Q2) and second in the International Journal of Biological Sciences (Q1)), Declaration of Originality and CV.

2. Significance of the topic

Allergic reactions are rapid type I hypersensitivity reactions to antigens, named allergens. Like all other hypersensitivity reactions, they can locally damage tissues and systemically can cause anaphylaxis with anaphylactic shock within 4-8 hours. The severity of allergic reactions depends on the nature, dose and route of allergen penetration. Allergens are small stable proteins or glycoproteins, mostly water-soluble and with protease activity in order to penetrate epithelial and skin barriers. Allergens penetrating through the skin or respiratory tract and eyes are aeroallergens, while those that penetrate through the gastrointestinal tract are food allergens. Aeroallergens are found indoors – house dust mite allergens (HDM) – particles associated with fecal residues of mites, cockroaches, molds, pet fur, and distributed

outdoors (mold allergens, pollen and other particles), part of the air and water particular matters in pollution. Due to continuous encounter with allergens, frequent allergic reactions lead to systemic disruption of the immune response and allergic diseases, such as allergic rhinitis (AR), allergic asthma (AAS), atopic dermatitis (AD), food allergy (FA) and eczema.

Recently, the prevalence of allergic diseases has increased significantly, affecting approximately 10–30% of the world's population. The incidence of allergy is higher in developed than in developing countries. Allergic diseases increasingly affect children. The socio-economic burden of allergies is also significant, associated with frequent exacerbations of symptoms and ongoing costs of treatment. For example, the estimated annual costs of atopic dermatitis in the United States are approximately 5.297 billion US dollars, and for allergic rhinitis - over 2 billion. Allergic diseases are associated with the elevated occurrence of the so-called atopic march - this is a phenomenon in which about a third of patients - adolescents and adults with atopic dermatitis - also develop other allergic respiratory symptoms or asthma. Allergic diseases are heterogeneous, with a complex pathology, dependent on many factors, including the environment during embryonic development (maternal-fetal environment), genetic and epigenetic factors, and the immune status of the organism. Climate change is also a risk factor associated with an increased risk of developing allergic reactions - for example, changes in temperature allow mold growth or more particular matter in the air, carried over long distances during strong winds and hurricanes, increasing the frequency of allergic reactions, especially in genetically predisposed individuals.

Allergic reactions begin with an immune response to the allergen, which activates the innate immune cascade in epithelial cells and local dendritic cells, presenting the antigen to naive helper CD4+ T lymphocytes. The latter can polarize to follicular T cells, which promote the differentiation of B cells into plasma cells and the isotype switching for production and secretion of IgE immunoglobulins. The decision of B cells to produce IgE is reinforced by another population of helper cells, which polarize in the Th2 direction. The process is under the control of cytokines, such as IL-3, IL-4, IL-5 and IL-13. The synthesized IgE binds to high-affinity receptors, densely expressed on mast cells in tissues, and basophils circulating in the blood. Upon re-entry of the allergen, surface-bound IgE recognizes it, cross-links it and activates mast cells and basophils to degranulate and release inflammatory mediators. Histamine, prostaglandins, leukotrienes are secreted, increasing the vascular permeability and smooth muscle contraction. Secreted chemokines and cytokines induce subsequent inflammatory reactions (late inflammatory response) with the recruitment of eosinophils, neutrophils, macrophages and further promotion of Th17 immune response, which leads to the symptoms recurrence. This complex dynamics of the immune response indicates that the treatment of allergic diseases should combine different approaches or should target cells that are relevant to the initial stages of the allergic reaction. For example, hypothetically the mechanisms that regulate the initial production of IgE by B lymphocytes, might be better than a suppression of the effector mechanism for the mast cells/basophils degranulation. Such approach underlies the significance of the PhD thesis to tack how the inhibition of allergic-primed B lymphocytes as a source of IgE antibodies can overcome the chronic inflammation induced by repetitive exposure to the house dust allergy.

3. Relevance of the goals and tasks

Nikola Ralchev's dissertation has one aim - to selectively suppress the production of allergen-specific antibodies to house dust by using chimeric molecules containing a monoclonal antibody against an inhibitory receptor (human CR1 or mouse FcγRIIb), conjugated to an epitope-bearing peptide p52-71 of the mite *Dermatophagoides pteronyssinus*. There are 4 formulated tasks – i) construction and

characterization of the mouse Dp52-71 chimera, ii) establishing the experimental models of house dust allergy and iii) application of the chimeric molecules in the relevant experimental models and iv) study of their therapeutic potential.

The PhD thesis exploits 2 experimental models – i) humanized model where the transfer of human PBMC from allergic patients was made into the recipient Rag2-γc- mice - a model of the induction of an allergic immune response, and ii) a mouse model of consecutive treatments with a house dust mite preparation and the induction of chronic allergic inflammation in BALB/c mice to house dust mite.

Experimental allergy models are among the most complex models due to the heterogeneity at the cellular and molecular levels of the allergic response and the specific nature of the allergen. Therefore, it should be noted that their implementation is particularly difficult and shows that the doctoral student had to deal with high-risk implementation tasks.

4. Shaping the problem in the introduction section

Nikola Ralchev has demonstrated excellent knowledge of the recent research and studies of other groups in the field as the literature review is written competently and concisely. Total of nine figures are included in the introduction section, and illustrate the mechanisms associated with the initial encounter with the allergen, early and late allergic reactions, demonstrating the relationships at the cellular and molecular level. There are also figures that show the hypothesis for the new approach of therapy with hybrid molecules. The review contains two informative tables, one with a description of the genetic loci associated with allergic diseases and possible mechanisms of action, and the second for types of house dust allergens. The introduction section shows that Nikola Ralchev is well-informed for the various therapeutic approaches – conventional and novel treatments of allergic diseases, with a particularly good impression being made by the description of the hypothesis that led to the formulation of the goals and objectives of the dissertation. The review cited 4 publications from 2024, 20 publications from 2023, 4 and 51 publications in the period 2020-2022, which is 36% of the totally cited articles. Most of the citations – over 80% are in the last 4 years related to the new concepts and hypotheses for the development of allergies and new therapeutic approaches, indicating that the doctoral student has followed the latest developments in the field and, based on this, has built a good foundation for planning subsequent experiments.

5. Research methodology

Nikola Ralchev uses a diverse methodology. The methods are divided into 2 subsections, those used in an experimental model of transfer of cells from allergic patients into Rag2-γc- mice and those related to the experimental work on induction of allergy to house dust in BALB/c mice. The experimental animals used are precisely described and there are treatment regimens in both experimental models, which give a very accurate idea of the method of induction of an allergic response and inflammation. In the humanized model, there is an information of the methodology of allergic patients selection and, accordingly, the transfer strategy of cells into mice. The production of anti-Dpt IgE, IgA, IgG and total IgE antibodies in fluid after bronchoalveolar lavage (BALF) was studied. Cell phenotyping was also performed as well as a technique for detection of the enzymatic activity of mast cells as an indirect marker for mast cell activation in the lung. The pathological changes in the lung were assessed too. To characterize the allergic response in the second model, quantitative determination of anti-HDM IgG, IgG1, IgM and IgA in serum and BALF, analysis of total IgE antibodies and secreted cytokines IL-4, IL-5, IL-9 and IL-13, cell phenotyping of cells in the lavage and assessment of pathological changes in the lung were performed. In

addition, the protein-engineered chimeric molecules against inhibitory CD32 expressed on mouse cells were constructed with detailed description of the methods for chimera conjugation and techniques used to demonstrate the chimera binding to CD32 and chimera recognition by IgG1 antibodies in serum.

A correlation analysis was performed between the individual parameters - cell phenotype and cytokines and factors in order to establish dependencies when introducing the model and assess the likelihood result after the treatment with the chimeric molecules. The statistical methods are adequate and guarantee repeatability of the obtained data.

Certainly, during the experimental work, Nikola has acquired a lot of practical skills that would give him an advantage in his future career development and excellent skills to plan new experimental tasks when implementing scientific projects.

6. Characteristics of the PhD thesis

The dissertation has a structure according to the requirements of the Regulations for the Development of the Academic Staff of the Institute of Microbiology and Immunology, Bulgarian Academy of Sciences. It contains the following chapters:

- Title
- Abbreviations used
- Introduction
- Literature review - 40 pages, with subsections for a theoretical review of allergic reactions, mechanisms, specifically pathology in house dust allergies, therapeutic approaches and allergen-specific therapy, justification of the need for new therapies and hypothesis.
- Goals and objectives - 1 pages, with 4 tasks
- Materials and methods - are divided into 2 subsections and a description of the set of techniques, described on 14 pages and illustrated with 2 figures demonstrating the schedule of the animal treatment
- Results - 28 pages, 17 figures with more than 6 panels.
- Discussion – 9 pages, divided into two, according to the results obtained from the humanized model and the mouse model
- Conclusions – 1 page
- Contributions – 1 page – divided into contributions of a fundamental and scientific-applied nature
- Literature sources – 211 sources.

7. Contributions and significance of the research data

The contributions of the research data are:

- Conceptual contribution

1. For the first time, it has been shown that FcγRIIb is overexpressed on the surface of all lung B lymphocytes, including IgE-positive B cells isolated from mice injected with house dust allergens.

2. For the first time, a model of chronic allergic inflammation in mice after house dust injection has been used, in which an increased level of serum anti-HDM IgG1 antibodies was detected and may be used as a biomarker for the development of an allergen-specific response in the experimental model.

- Significance for vast data application

3. Experimental models of allergic response and inflammation have been developed, which can be used to study the mechanisms of the disease, as well as to study proof-of-principles hypotheses for new therapeutic approaches.

4. The protein-engineered chimeric technology has been demonstrated to have an effect on late immune responses involving eosinophils. This shows a potential application for diseases mediated by eosinophil activation – for example, immune responses to parasites or after respiratory virus infections.

The significance of the data was well-shaped in the discussion section, demonstrating Nikola's skill for critical analysis of his own experimental data.

7. Publications

The doctoral student has 2 publications and fulfills the required credits and requirements of the Institute of Microbiology. Nikola Ralchev has an article in the Scandinavian Journal of Immunology 2022: Targeted suppression of Dpt-specific B cells in humanized Rag2- γc- mouse model of HDM allergy and an article in Int. J. Mol. Sci. 2024: Suppression of Pathological Allergen-Specific B Cells by Protein-Engineered Molecules in a Mouse Model of Chronic House Dust Mite Allergy.

In both publications, Nikola is the first author. The papers are related with topic of the dissertation and meets the requirements for being in refereed publications being with Q2 and Q1 in 2023.

8. Critical remarks and recommendations

My questions to Nikola Ralchev are:

1. What are the advantages and disadvantages of the mouse model of allergic chronic inflammation?

CONCLUSION

Nikola Ralchev's dissertation contains scientific, applied scientific results that represent an original contribution to science and meet the requirements of the Act on the Development of Academic Staff in the Republic of Bulgaria, the Regulations for the Implementation of the Act and the Regulations for the Implementation of the Act in the Bulgarian Academy of Sciences. The presented PhD thesis corresponds to the specific requirements of the Regulations of the Institute of Microbiology.

The dissertation shows that Nikola Ralchev possesses theoretical knowledge and professional skills by demonstrating qualities and skills for independent conduct of scientific research.

Due to the above, I give my positive assessment of the conducted research, and I propose that the Nikola Ralchev is awarded with the educational and scientific degree 'Doctor' in field 4.3. Biological Sciences (Immunology).

24.02.2025

Reviewer: .....

Assoc. Prof. Petya Dimitrova