ABSTRACT

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Development of approaches for stimulation of T-regulatory cells for immunotherapy of vitiligo

General description of the research. This Ph.D. dissertation is dedicated to studying the phenotypical characteristics of peripheral blood T regulatory cells (Tregs) and develop the approaches to stimulate and generate antigen-specific Tregs with chimeric antigen receptor (CAR) as a cell-based immunotherapy of vitiligo, and the effect of antibiotics to control depigmentation in a mouse model.

Significance of the research. Vitiligo is an incurable and not fully understood autoimmune skin disease affecting 0.5-1% of the world population [1]. According to the official statistics of the RSE on REJ "Kazakh Scientific Center of Dermatology and Infectious Diseases" of the Ministry of Health of the Republic of Kazakhstan for 2018, the prevalence of vitiligo is 9.6 per hundred thousand people [2]. To date, there are no existing targeted treatments for vitiligo due to the limited funding for research on the disease, since vitiligo is not a fatal or a life-threatening disease. Commonly prescribed treatments include topically applied corticosteroids, UVB phototherapy, and for rare cases surgical melanocyte transplantation, and complete depigmentation in the case if the patient has more than 70%-80% vitiligo lesions. These approaches to treating vitiligo are not effective and have serious side effects, and melanocyte transplantation is an extreme surgical technique [3-6]. In terms of quality of life, vitiligo negatively affects the quality of life and well-being of patients. Stigmatization varies in different cultures, and many patients experience psychological stress, low self-esteem, and depression, which can lead to suicide attempts [7].

The etiology of vitiligo is complex and not fully understood yet, but this condition is considered to be a result of the interplay among multiple factors, including stress, genetic predisposition, environmental triggers, and a melanocyte-specific autoimmunity. According to recent studies, disruption of immune tolerance is considered as a main cause of the development of a disease in which cytotoxic T cells attack the patient's own melanocytes. Most autoimmune diseases have a similar etiology associated with impaired regulation of the immune response, including deficiency or impaired activity of T-regulatory cells (Tregs) [8-10]. When recognizing self or non-self-antigens disrupted, the immune system non-specifically destroys cells and tissues of the body and as a result causes autoimmune diseases. The role of Tregs in this case is to actively suppress activation of the immune system and prevent pathological self-reactivity. With vitiligo,

there is a systemic decrease in the proportion of $CD39^+$ and $CD44^+$ Tregs which defines phenotypical characteristics and functional activity**,** as well as a decrease in the migration of Tregs to the lesions of vitiligo, which correlates with the area of depigmentation. It was also previously shown that the adaptive transfer of Tregs to mice with vitiligo led to a temporary stop of depigmentation. Thus, it has been suggested that the use of antigen-specific Tregs as a cell immunotherapy of vitiligo can be highly effective, restoring local immune tolerance and preventing undesired autoimmune reactions.

To restore the immunosuppressive activity of Tregs and increase their homing at the area of autoimmune inflammation, and thus, it was proposed to obtain Tregs with a chimeric antigenic receptor (CAR) specific for antigens of cells undergoing autoimmune attack. Currently, CAR T cells based therapies are widely used to treat various types of cancer. This approach is based on the administration of autologous CAR-modified T cells to patients that recognize specific cancer cell antigens. Some CAR-T cell cancer therapies have been shown to be highly effective in treating patients compared to traditional therapies and some have been approved for use in the clinic. It has been suggested that using this approach for Tregs can be used to treat autoimmune diseases. Thus, we developed a method for producing CAR Tregs specific for ganglioside D3 antigen (GD3) expressed by melanocytes, and investigated the effectiveness of their application to increase their migration to vitiligo lesions and to provide local immune tolerance to melanocytes.

Another promising area of therapy for vitiligo and for other autoimmune diseases is skewing of the microbiome using antibiotics. The importance of the microbiome is currently gaining an increased attention of researchers in connection with its influence on the development of diseases. It was previously reported that the systemic and local effect of antibiotics on microbial diversity affects the progression of diseases in several diseases including acne, psoriasis and atopic dermatitis. While microbial diversity support immune homeostasis, some species may induce pathogenic responses via producing Ro60-producing commensal bacteria in lupus. Another example includes increased abundance of *B. adolescentis* correlates with human autoimmune arthritis. Some microbial peptides might trigger CD8 T cell response; however, these responses are recently not well understood. Thus, a better understanding of bacteria impacting T-cell activation may reveal the influence of gut health to the vitiligo development. To study this, antibiotics were used on vitiligo-prone FH-A2D mouse model to assess the changes in the microbiome and the effect on Treg accumulation in the skin.

Thus, this study aimed at studying the phenotypic characteristics of peripheral blood Tregs in vitiligo patients and developing a method for producing ganglioside D3 (GD3)-specific CAR-Tregs (GD3-specific CAR-Tregs) and studying its effectiveness for treating vitiligo *in vitro* and *in vivo,* as well as a study of the effect of the use of antibiotics that alter microbiomes, which redistributes T cells and accumulate Tregs in vitiligo, is novel and relevant for the development of new approaches to immunotherapy of vitiligo, and other serious autoimmune diseases.

The purpose of the research. The purpose of the research is to study the phenotypical characteristics of peripheral blood Tregs of patients with vitiligo and to develop approaches for stimulating and generating antigen-specific Tregs based on CAR, and assessing them *in vitro* and *in vivo* for a potential immunotherapy of vitiligo, as well as evaluating the effect of antibiotics to control depigmentation in a mouse model.

The main tasks of the research to accomplish purpose are as following:

1. To study the phenotypical characteristics of Tregs from peripheral blood of patients with vitiligo.
2. To develop approaches for maintaining immunosuppressive Treg phenotype *ex vivo*.
3. To develop a method for generating antigen-specific Tregs for a cell-based immunotherapy for vitiligo.
4. To study the immunosuppressive activity of GD3-specific CAR-Tregs *in vitro*.
5. To study the effectiveness of GD3-specific CAR-Tregs *in vivo* using vitiligo-prone mouse model.
6. To study the effect of antibiotics for microbial diversity to control depigmentation in vitiligo.

The research objects and materials. Regulatory T cells of the peripheral blood of vitiligo patients, $CD3^+$ T cells, naïve $CD4^+$ cells, $CD4^+$ $CD25^+$ Mouse Tregs, human HLA-A2-positive and -negative melanocytes, TCR-transgenic mice (h3TA2), FH-A2D transgenic mice.

Research Methods. Cell culture, transfection, viral transduction, flow cytometry, immunohistochemistry, immunofluorescence staining, fluorescence microscopy, enzyme-linked immunosorbent assay (ELISA), fluorescence imaging of live cells, caspase-3/7 mediated apoptosis assay, adoptive cell transfer, flat-bed mouse scanning, Adobe Photoshop image analysis, and GraphPad Prism and R statistical analysis tools.

The scientific novelty of the research. During the study, it was first discovered that the proportion of $CD39^+$ and $CD44^+$ Tregs in peripheral blood was significantly reduced in vitiligo. It was also found that in patients with vitiligo who are in remission, the proportion of Tregs with the phenotype $CD39^+$ and $CD44^+$ $FoxP3^+$ is also reduced in comparison with the control. The obtained data indicate the dysfunction of Treg cells in vitiligo may indicate a decrease in their immunosuppressive properties and ability to migrate efficiently to the lesion site of depigmentation, which may lead to uncontrolled activity of melanocyte-specific T cells and disease progression. The results contribute to an understanding of the mechanisms of impaired immune regulation in vitiligo and can serve as the basis for the development of new approaches to vitiligo treatment based on increased suppressor activity of Tregs and their recruitment into affected vitiligo skin areas.

Overexpression of GD3 was first detected in the epithelial cells and melanocytes, in the vitiligo-affected areas of the skin of humans and mice. Increased expression of the surface antigen GD3 has been identified as a target antigen for vitiligo.

This dissertation also describes a method for generating antigen-specific Tregs that carry CAR to the antigen expressed by melanocytes, and investigated the effectiveness of the use of GD3-specific CAR-Tregs for restoration of immune tolerance *in vitro* and *in vivo*. In particular, a viral transduction method has been developed that provides Treg with high expression of CAR – specific to GD3. Also, a new approach was developed and optimized for polarizing naive $CD4^+$ T cells into $CD4^+$ $FoxP3^+$ Tregs and expanding the Treg pool *in vitro* while maintaining their phenotype. For the first time, the GD3-encoded CAR construct was used to generate highly transduced antigen-specific Tregs *ex vivo* using an optimized transduction protocol using retroviruses. *In vitro* studies have shown that the obtained GD3-specific CAR Tregs have antigenic specificity and a high level of production of the immunosuppressive cytokine IL-10. *In vitro* melanocyte viability assay was also conducted for the first time using a new live cell imaging system to evaluate the immunosuppressive activity of GD3-specific CAR Tregs. Moreover, the introduction of GD3-specific CAR Tregs into the co-culture of human melanocytes and melanocyte-reactive cytotoxic T cells led to an increase in the level of viable melanocytes. It was also found that GD3-specific CAR Tregs have greater immunosuppressive activity against melanocyte-reactive cytotoxic T cells compared to untransduced Tregs.

When studying the effectiveness of using GD3-specific CAR Tregs *in vivo*, it was found that the adoptive transfer of the obtained Tregs to transgenic mice from vitiligo provides a more effective restoration of immune tolerance in the lesions and a decrease in the area of depigmentation. Compared to untransduced Tregs, GD3-specific CAR Tregs have better melanocyte homing ability and greater immunosuppressive activity in relation to proliferation of melanocyte-specific cytotoxic T cells.

Microbial diversity was first investigated after the administration of antibiotics such as ampicillin and neomycin, and its effect on the development of vitiligo in an experimental mouse model (FH-A2D mice). It was found that ampicillin leads to increased depigmentation, while neomycin inhibits the development of the disease by indirectly affecting skin Treg infiltration.

The data obtained reveal the mechanisms of development of vitiligo and can be used to create approaches to cell immunotherapy for vitiligo, based on the use of antigen-specific Tregs.

Theoretical and practical significance of the research. The dissertation contributes to the fundamental aspects of autoimmunity. Studying the phenotypical characteristics of Tregs provides the understanding of the role of different subsets of Tregs that mediate different autoimmune disorders. Studying of the immunosuppressive ability of GD3-specific CAR Tregs enlightens the field of bystander effect of Tregs *in vivo*, and, helps to get one step closer to reveal the full mechanism immunosuppression defects in autoimmune diseases.

The practical significance of the obtained results lies in the development of new approaches to the treatment of autoimmune diseases using transgenic CAR based Tregs with antigen specificity. The data obtained suggest that GD3-specific CAR Tregs can

efficiently recognize the antigen and provide local immune tolerance towards melanocytes *in vivo.* The data obtained in the course of the dissertation indicate the effectiveness of adoptive transfer of GD3-specific CAR Tregs for the treatment of vitiligo, which indicates that this method is promising for further clinical trials. Moreover, the data obtained and the developed methods can serve as the basis for the development of immunotherapy of other autoimmune diseases, such as pemphigus vulgaris, pemphigus bullous, psoriasis, multiple sclerosis, celiac disease, Crohn's disease, lupus, etc.

Another practical significance of the obtained results is to identify the indirect effect of neomycin on the increase in infiltration of skin areas by Tregs and the halt in depigmentation in the experimental model of vitiligo (FH-A2D mice). The data obtained can also be used to develop approaches for the treatment of vitiligo.

In the course of the study, a detailed transduction protocol was also prepared and optimized, which provides Tregs with high expression of antigen-specific CAR. Experimental protocols have been developed to study the level of immunosuppression *in vitro* using human melanocytes expressing GD3 as target cells, cytotoxic T cells specific for h3T, as effector cells, as well as GD3-specific CAR Tregs or untransduced Tregs, expressing eGFP linked to FoxP3 as suppressor cells. Also, to analyze the level of viability of melanocytes, the approach was optimized using the new IncuCyte live cell imaging system, which allows it to take several images over time and using caspase-3/7 Red reagent, which detects cells undergoing apoptosis mediated by caspase-3/7. The developed experimental methods can be used in research in the field of molecular and cellular immunology.

Thus, the thesis has theoretical and practical significance.

The main provisions for the defense:

1. The proportion of $CD39^+$ and $CD44^+$ Tregs of peripheral blood is significantly reduced in vitiligo.
2. The expression of ganglioside D3 is increased by human and mouse epithelial cells and melanocytes in vitiligo.
3. A protocol has been developed for expanding the pool of Tregs that preserve the immunosuppressive phenotype *in vitro*.
4. Tregs can be transduced with high efficiency using the CAR construct encoding GD3 by retroviral transduction.
5. GD3-specific CAR Tregs are highly specific for GD3 antigen and have enhanced immunosuppressive activity *in vitro*.
6. Adoptive transfer of GD3-specific CAR Tregs halts depigmentation in vitiligo-prone transgenic h3TA2 mice
7. GD3-specific CAR Tregs efficiently recognize antigen on affected epithelial cells and melanocytes, and provide local immune tolerance towards melanocytes in mice.
8. Neomycin causes an increase in Treg infiltration on skin samples by indirectly affecting microbial diversity and helps control depigmentation in vitiligo in mice.

The levels of research organization. The research described in this dissertation performed on molecular, cellular, tissue, organ, and organism level.

Relationship of the research with the scientific project. This Ph.D. research on developing approaches for vitiligo immunotherapy using antigen-specific Tregs was supported by the National Institute of Health grant RO1 AR057643 to Dr. I. Caroline Le Poole. The majority of the research has been performed at Dr. Le Poole's laboratory, which specializes in immunology and dermatology. This research also was supported by Dr. Le Poole's initiative, which had provided a Ph.D. level stipend, and she also advised this research throughout a Ph.D. time period. In Kazakhstan, a local scientific advisor, Ostapchuk Yekaterina. O. supported this Ph.D. research via advising from the first year of the research and providing advice at every point. The study of phenotypical characteristics of Tregs of vitiligo patients was supported by grant AP05131691 "Molecular mechanisms of the influence of T-regulatory cells on the activity of tumor cells" provided by Committee of Science, Ministry of Education and Science of the Republic of Kazakhstan, to Ostapchuk Yekaterina O., and was performed at the Aitkhozhin Institute of Molecular Biology and Biochemistry.

The contribution of the author for the results described in this dissertation. All the main results described here are performed and collected by the author. In addition, main research results, analyses, tables, data, and figures are generated by the author, and all the new observations and conclusions are made from the results derived from Ph.D. candidate's work and research.

Research approbation. The main results and observations are presented and discussed at international conferences and symposiums:

– at international scientific conference, Oral Presentation: Awardee of Society for Investigative Dermatology Eugene M. Farber Travel Awards for Young Investigators " The Joint Montagna Symposium on the Biology of Skin/Pan American Society for Pigment Cell Research Annual Meeting "Melanoma to Vitiligo: The Melanocyte in Biology and Medicine" (2018, Salishan Resort, Oregon);

– at international scientific summit "2nd Antigen-Specific Immune Tolerance Drug Development Summit 2019" (2019, Boston, USA);

– at scientific conference "Northwestern Research Day 2019" (2019, Chicago, USA);

– at International scientific conference for students and young scientists "Farabi Alemi" (2018, Almaty, Kazakhstan);

– at international scientific conference "Society for Investigative Dermatology (SID) Annual Meeting" (2019, Chicago, USA);

– at international scientific conference "The Society for Immunotherapy of Cancer (SITC), 34th Annual Meeting & Pre-Conference Programs, Nov. 6–10, (National Harbor, 2019, Chicago, USA);

– at the International Scientific Conference of Young Scientists "Fundamental Research and Innovation in Molecular Biology, Biotechnology, Biochemistry" on the occasion of the 80th birthday of Academician Murat Abenovich Aitkhozhin November

28-29 (2019, Almaty, Kazakhstan);

– at international scientific conference “PanAmerican Society for Pigment Cell Research (PASPCR), Oct 02 - 04, 2019 at The Jackson Laboratory, Bar Harbor, Maine, USA”.

Publications. The majority of this dissertation content was published in 13 scientific works, including 1 research article with impact factor (IF)=6.29, and 3 abstracts published in journals with impact factor (IF=8.728; IF=6.29; IF=4.172) according to *SCOPUS* database, 4 articles in scientific journals recommended by Education and Science Monitoring Committee of the Ministry of Education and Science of the Republic of Kazakhstan (CCESF MES RK), and 5 abstracts in the materials of international conferences, symposiums, and summits. The provisional US Patent (serial No. 62/915,945 in USA) was filed on 10/16/2019 with the title of “Materials and Methods for Treating Vitiligo”, followed with United States and PCT international patent (Utility & PCT, serial No. 17/072,939 & PCT/US2020/056104) filed as “MATERIALS AND METHODS FOR TREATING VITILIGO” on 10/16/2020 (internal reference: NU2019-172-02 &-03).

Dissertation structure. This dissertation is written in 122 pages, and contains notations and abbreviations, introduction, literature review, materials and methods, results and discussions, conclusions, references and appendices from 215 sources where 207 are in English, contains 3 tables, and 50 figures.