Al-Farabi Kazakh National University

UDC 57.053:612.017:616.5-003.829 Presented as manuscript

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

6D060700-Biology

Dissertation for Doctor of Philosophy (Ph.D.) degree

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Almaty, 2020

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NORMATIVE REFERENCES

This dissertation was written according to following standardized references:

MEMCT 7.32-2001 – Provisions on scientific research. Rules for structure and preparation.

MEMCT 7.1-2003 – Bibliographic record. Bibliographic description. General requirements and rules for structure.

DEFINITIONS

In this dissertation, the following terms are used with the corresponding definitions:

1. Regulatory T cells are subsets of lymphocytes, which are responsible for modulation of immune responses and provide tolerance to self-antigens.

2. Chimeric antigen receptor (CAR) is an engineered chimeric receptor, which is derived from the part of T cell that is responsible for activation, and from variable region of a monoclonal antibody particularly designed for a specific antigen.

3. Retroviral transduction – is a method to introduce external DNA via using retroviruses into cell of interest.

NOTATIONS AND ABBREVIATIONS

GD3	– ganglioside D3
CAR	– chimeric antigen receptor
UV	– ultraviolet
DLQI	– dermatology life quality index
ROS	– reactive oxygen species
HSP70	– heat shock protein 70
DCs	– dendritic cells
LN	– lymph nodes
MHC	– major histocompatibility complex
HLA	– human leucocyte antigen
GWAS	– genome-wide association
CTLA-4	– cytotoxic T-lymphocyte-associated protein 4
IL-2	– interleukin 2
FOXP3	– Forkhead box P3
GITR	– glucocorticoid-induced tumor necrosis factor
LAP	– latency-associated peptide
GARP	– glycoprotein A repetitions predominant
DNA	– deoxyribonucleic acid
RNA	– ribonucleic acid
FBS	– fetal bovine serum
FITC	– fluorescein isothiocyanate
PBS	– phosphate-buffered saline
RPMI 1640	– Roswell Park Memorial Institute medium 1640
PE	– phycoerythrin
DAPI	– 4′,6-diamidino-2-phenylindole
MBEH	– monobenzyl ether of hydroquinone
TCR	– T cell receptor
TGF-B	– transforming growth factor B
IL-5	– interleukin 5
IL-6	– interleukin 6
IL-12	– interleukin 12
IL-15	– interleukin 15
HSCT	– hematopoietic stem cell transplantation
TNP	– 2,4,6-trinitrophenyl
scFv	– single-chain fragment variable
ITAM	– immunoreceptor tyrosine-based activation motif
PBMCs	– peripheral blood mononuclear cells
APC	– antigen presenting cells
GMP	– good manufacturing practices
GDP	– good distribution practices
ATMP	– advanced therapy medicinal product
CTMP	– gene therapy medicinal product
GMO	– genetically modified organism

ELISA	– enzyme-linked immunosorbent assay
TIL	– tumor infiltrating lymphocytes
HBSS	– Hank’s balanced salt solution

INTRODUCTION

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.

1 LITERATURE REVIEW

1.1 Vitiligo

Vitiligo is an autoimmune skin disorder characterized by the loss of functional melanocytes and by the appearance of white pale lesions on the skin [8, p. 227]. The prevalence of vitiligo lies between 0.5-1% worldwide, and the exact etiology is not characterized yet [1, p. 1206]. To date, vitiligo is classified mainly into two types: non-segmental and segmental vitiligo. The most widespread type is non-segmental, which is distributed symmetrically or in an acrofacial pattern, and characterized by unpredictable spread over time. Segmental vitiligo is the second most common type, and lesions show unilateral patterns also affecting follicular melanocyte reservoir that results in hair whitening [11].

1.1.1 Signs, symptoms and diagnosis

The visible signs of vitiligo are depigmented white milky lesions on the skin (Figure 1) [12, p. 39]. Initially, these pale lesions appear in small sizes, and then spread around the skin and tend to expand through changing its shapes. The rate of progression of the disease varies among patients, in some the depigmentation rapidly spreads while in others the process is steady. The loss of pigmentation is most prominent and noticeable on the face, hands, and feet which have a tendency to be exposed to the sun. In addition, patients with vitiligo often struggle with early graying of the hair [13]. However, individuals with vitiligo do not report with a pain or life-threatening symptoms. At this time, vitiligo lies under the “quality of life” category, and patients are usually stigmatized within the society and reports with frequent depression and low self-esteem [14].

Vitiligo can be diagnosed through the use of several methods. First, the physician will do an eye exam. Then, an ultraviolet (UV) can be applied in an early stage of vitiligo to determine and monitor the progression of the disease. When vitiligo skin is checked with UV, it appears as a white milky spots while healthy skin does not show similar skin color change. Final confirmation of vitiligo diagnosis is performing biopsy [15]. A dermoscopy can be performed to reveal the presence of starburst appearance, comet tail appearance, leukotrichia, telangiectasia and identify perifollicular or perilesional changes and altered pigmentary network [16].

1.1.2 Quality of life disease

Vitiligo is identified as a quality of life disease and has a major impact on the well-being of patients. The stigmatization can vary among the different cultures, and many patients experience psychological stress, low-esteem, and depression, which may lead to suicide attempts in extreme cases [17]. In some cultures, vitiligo can be misidentified as leprosy, which is a severe infectious disease that can spread between people. For instance, the first prime minister of India, Pt Jawaharal Nehru declared vitiligo as one of the major diseases in India after leprosy and malaria. According to Indian religious manuscripts, the person who committed “Guru Droh” (sin) in his previous life will suffer from vitiligo in his current life. Therefore, vitiligo impacts the quality of life of children, men, and particularly young women who have a little chance

of getting married in Indian society [18]. Moreover, vitiligo affects more dark-skinned individuals due to the strong contrast in colors. Due to the central role of skin in many aspects of life, and any pathological defect or depigmentation may cause antagonism, avoidance, and whispering comments [19].

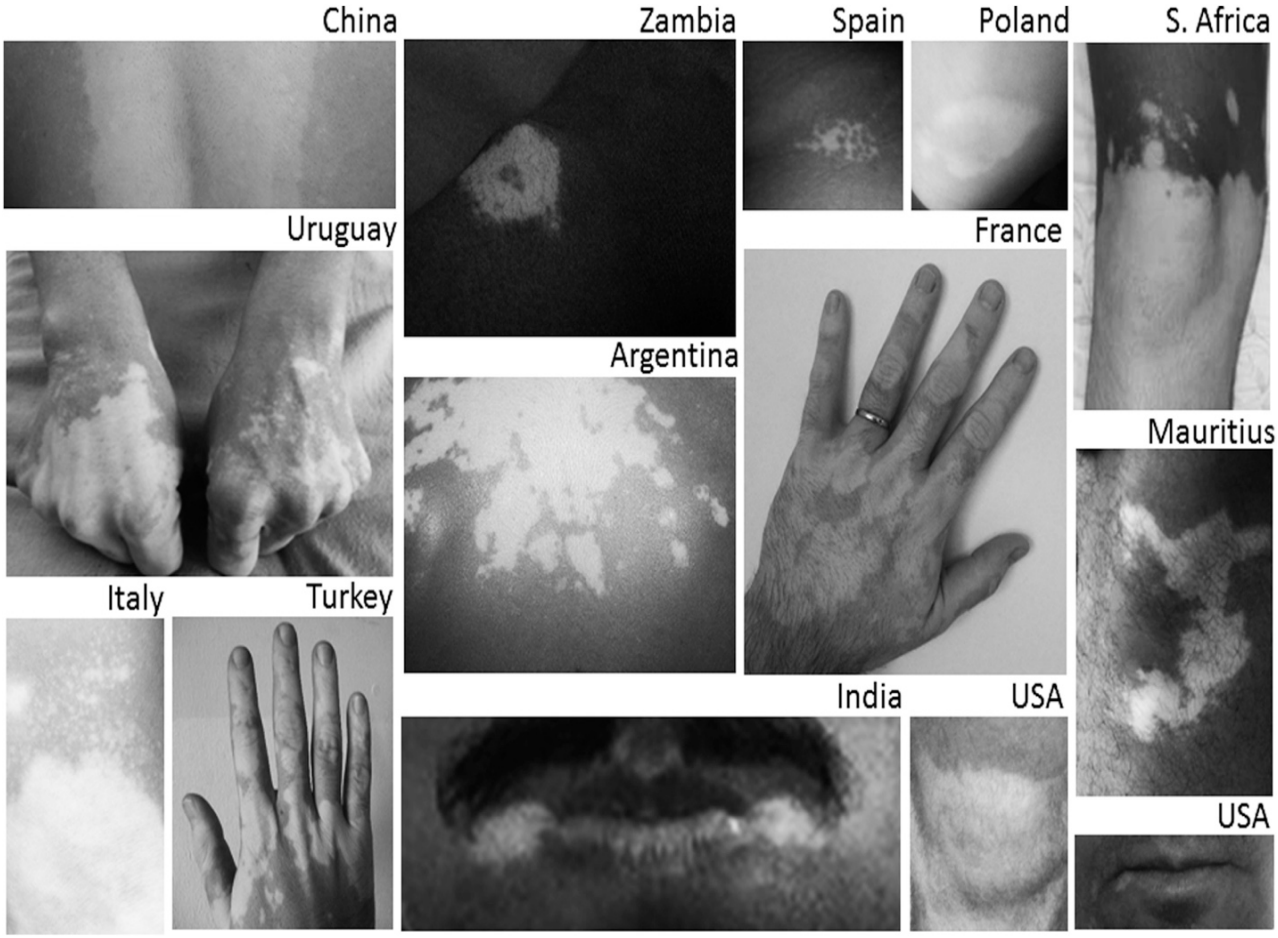


Figure 1 – Representation of vitiligo from around the world [12, p. 39]

Children and teenagers with vitiligo can be discriminated among their peers, and lesions in visible parts of the body such a face, hands or legs may accelerate the embarrassment and they usually avoid sport activities at the school [19, 20]. Humiliating comments from peers may devastate their moods, and eventually, children with vitiligo usually grow in isolation and embarrassment. Currently available treatments such as PUVA appointments and their complications cost a fortune for the parent's budget, and these children loss vital days from the school. When vitiligo patients meet or start a new sexual relationship, they experienced rude remarks from their partners which made them feel anxiety and embarrassment [21].

Dermatology life quality index (DLQI) is a questionnaire dedicated to patients with skin diseases. Vitiligo lesions situated in the face, arms and legs correlate with a lower DLQI. In most cases, stigmatization was the main factor in patient's quality of life that varies in different cultures with a DLQI f 6 in Malaysia and a DLQI of 1 in Italy [22]. These above mentioned psychological stress, stigma and, low self-esteem

can managed by self-help cognitive behavioral therapy to alleviate stress, which is a precipitating factor for further vitiligo development [23]. To promote awareness of vitiligo to the public, and more funding for research will provide hope and facilitate the improvement of quality of life for the patients.

1.1.3 Treatment methods currently available for vitiligo

Effective treatment methods for vitiligo are still under development, and available therapies cannot fully reverse depigmentation. However, standard treatment options are being prescribed to patients with vitiligo worldwide [24]. These options include glucocorticoids and calcineurin inhibitors, which are prescribed as first-line treatments for vitiligo. Phototherapy is commonly prescribed as a second-line therapy for vitiligo. The treatment procedure includes exposing the vitiligo skin to light from the UV-B lamp at 2-3 times a week. This procedure can be performed by the patient at home or in a clinic. Many studies revealed that a combination of UV-B phototherapy with topical steroids moderately improves repigmentation. For example, combined UV with Psoralen increases the sensitivity of the skin to high exposure of light, which promotes melanocytes generation. Posing a high risk of melanoma or sunburns, UV phototherapy is only should be considered when first line standard treatments are not effective [25].

In mild cases, skin camouflage can hide white patches through the use of cosmetics. In case vitiligo patient is pale-skinned, the patches are less visible if the patient avoids tanning. If the vitiligo is widely spread on the skin and covers more parts of the body, the option to full de-pigment may be considered to obtain an even color. Topical drugs such as monobenzone, mequinol, and hydroquinone can permanently remove the skin pigment [13, p. 85].

A study was published using transplantation of melanocytes to vitiligo lesions. This study demonstrated a successful re-pigmentation of vitiligo patches. This procedure involved taking a thin layer of pigmented skin from the gluteal region and separation of melanocytes. These obtained melanocytes subsequently expanded through the use of cell culture. The affected vitiligo skin was denuded with dermabrader and melanocytes skin-graft was applied to those areas. As a result, approximately 70%-85% of patients were experienced with a nearly complete repigmentation which differed among individuals in terms of longevity. In addition, a few transplantation techniques were developed at this point using melanocyte precursors derived from hair follicles. These procedures often are applied for those patients with segmental and stable vitiligo [26]. However, this melanocyte transplantation technique is difficult and expensive surgical procedure with risky consequences.

Recently a drug Afamelanotide is in Phase II clinical trials for vitiligo indication and other derma related diseases. Moreover, a rheumatoid arthritis drug Tofacitinib also has been tested for vitiligo treatment, but both this drugs are not effective in fully treating vitiligo [27].

1.1.4 Mechanism of vitiligo pathogenesis

Decades of research revealed possible mechanisms of melanocyte loss and

vitiligo development, though much remains to be studied. Many hypotheses have been suggested as potential triggers that might be the cause of vitiligo. These triggers could be genetic susceptibility, environmental factors, psychological stress or autoimmunity. Currently leading theories for the cause of vitiligo could be impaired redox status or autoimmunity. According to impaired redox theory, melanocytes die from increased levels of intracellular oxidative stress during melanogenesis. Increased hydrogen peroxide production is considered a cause of low catalase activity, and it has been observed in lesional skin and cultured melanocytes. Moreover, vitiliginous melanocytes have impaired calcium uptake, which is important for maintaining antioxidant activity. As a result, treatment options such as pseudocatalase have been developed to inhibit hydrogen peroxide or reactive oxygen species. However, treatment efficacy with pseudocatalase was minimal where the majority of studies showed no therapeutic effects. This increased sensitivity might be due to genetic or cell abnormalities [28]. A genome-wide association study was performed and revealed a list of loci linked to vitiligo shown in Table 1 [29, p. 312].

Table 1 – Vitiligo susceptibility loci derived from genome-wide association (GWAS) and linkage studies [29, p. 312]

Locus	Candidate gene	Protein	Function
1p13.2	PTPN22	LYP protein tyrosine phosphatase	Regulates T-cell signaling
1p31.3	FOXD3	Forkhead box D3	Transcriptional regulator of neural crest; melanoblast differentiation
1p36.23	RERE	Atrophin-1-like protein isoform b	Lymphoid transcriptional co-repressor; apoptotic regulator
2q24.2	IFIH1	Interferon-induced RNA helicase	Regulates innate antiviral immune responses
2q33.2	CTLA4	Cytotoxic T-lymphocyte-associated-4	Inhibits T cells via interaction with CD80 and CD86

Continued from Table 1

3p13	FOXP1	Forkhead box P1	Transcriptional regulator of B-cell, T-cell, monocyte development
3q13.33	CD80	B-cell activation antigen B7-1	T-cell priming by B cells, T cells, dendritic cells; interacts with CTLA-4
3q28	LPP	LIM domain containing preferred translocation	Transcriptional co-activator?
4p16.1	CLNK	Mast cell immunoreceptor signal transducer	Positive regulator of immunoreceptor signaling
5q22.1	TSLP	Thymic stromal lymphopoietin protein	Cytokine regulator of skin dendritic (Langerhans) cell maturation
6p22.1	HLA-A	Leucocyte antigen A α-chain	Presents peptide antigens
6p22.1	HLA-B-C	Leukocyte antigen B or C α-chain	Presents peptide antigens
6p21.32	HLA-DRB1-DQA1	Major histocompatibility complex class II region	Presents peptide antigens
6q15	BACH2	BTB and CNC homology 1, basic leucine zipper	B-cell transcriptional repressor
6q27	CCR6	Chemokine (C-C motif) receptor 6	Regulates differentiation and function of B cells, T cells, dendritic cells
6q27	SMOC2	SPARC-related modular calcium-binding protein	Regulate cell–extracellular matrix interactions

Continued from Table 1

8q24.22	**TG/SLA**	**Thyroglobulin, Src-like adaptor isoform c**	**Regulates antigen receptor signaling in T cells, B cells, dendritic cells**
10p15.1	IL2RA	Interleukin 2 receptor α-chain	Regulates interleukin 2-mediated activation of T-cells, regulatory T-cells
10q22.1	Gene desert	-	-
10q25.3	CASP7	Caspase 7	Apoptotic executioner protein
11p13	CD44	CD44 antigen	T-cell regulator
11q14.3	TYR	Tyrosinase	Melanin biosynthetic enzyme
11q21	Gene desert	None	TYR regulation?
11q23.3	Gene desert	-	-
12q13.2	IKZF4	Ikaros zinc finger protein, subfamily 1A, 4	T-cell transcriptional regulator
12q24.12	SH2B3	LNK adaptor	B-cell, T-cell developmental regulator
14q12	GZMB	Granzyme B	Mediates CTL-induced target cell apoptosis, helper T-cell apoptosis
15q12-13.1	OCA2	Oculocutaneous albinism II	Melanosomal membrane transporter/pump
16q24.3	MC1R	Melanocortin-1 receptor	Regulates melanogenesis
17p13.2	NLRP1	NLR family, pyrin domain containing 1	Regulates IL-1β innate immune response via NLRP1 inflammasome

Continued from Table 1

19p13.3	TICAM1	Toll-like receptor adaptor molecule 1	Mediates innate antiviral immune responses
21q22.3	UBASH3A	Ubiquitin associated and SH3 domain containing	Regulates T-cell signaling, apoptosis
22q12.1	XBP1 c	X-box binding protein 1	Transcriptional regulator of MHC class II expression, plasma cells
22q12.3	C1QTNF6	C1q and tumor necrosis factor related protein 6	Innate immune response to light-induced apoptosis?
22q13.2	TOB2	Transducer of ERBB2, 2	Inhibitor of cell cycle progression; involved in T-cell tolerance
Xp11.23	FOXP3	Forkhead box P3	Transcriptional regulator of regulatory T-cell function and development

It is widely accepted that vitiligo might be an autoimmune disease where the immune system is responsible for the condition. Support for this theory is accompanied by abundance of cytotoxic T cells and macrophages in the skin of patients with active stage of the diseases. The first evidence of autoimmunity was the presence of circulating antibodies generated to melanocytes in vitiligo patients, which was not present in healthy people. It is suggested that antibody production is a result of vitiligo pathogenesis rather than a direct cause. Moreover, the melanosomal transmembrane protein MelanA, a melanocyte antigen recognized by T cells, induces cytotoxic T cells but not humoral responses. Therefore, autoimmune melanocyte destruction is mediated by cytotoxic T cells, but not by generated antibodies [29, p. 314]. The autoimmune response against melanocytes was described in Figure 2. According to this study, stress conditions and reactive oxygen species (ROS) cause melanocytes to secrete heat shock protein 70 (HSP70). In part, chaperon acting HSP70 present melanocyte-specific antigens to Dendritic cells (DCs) that subsequently recruit antigen-specific CD4 and CD8 T cells in draining lymph nodes (LN). These activated CD8 T cells, in particular, are responsible for mediating the melanocyte death via the granzyme/perforin pathway illustrated in Figure 2 [30, p. 568]. Vitiligo is has a comorbidity with other autoimmune

diseases such as psoriasis, alopecia, systemic lupus erythematosus, and scleroderma [24, p. 74].

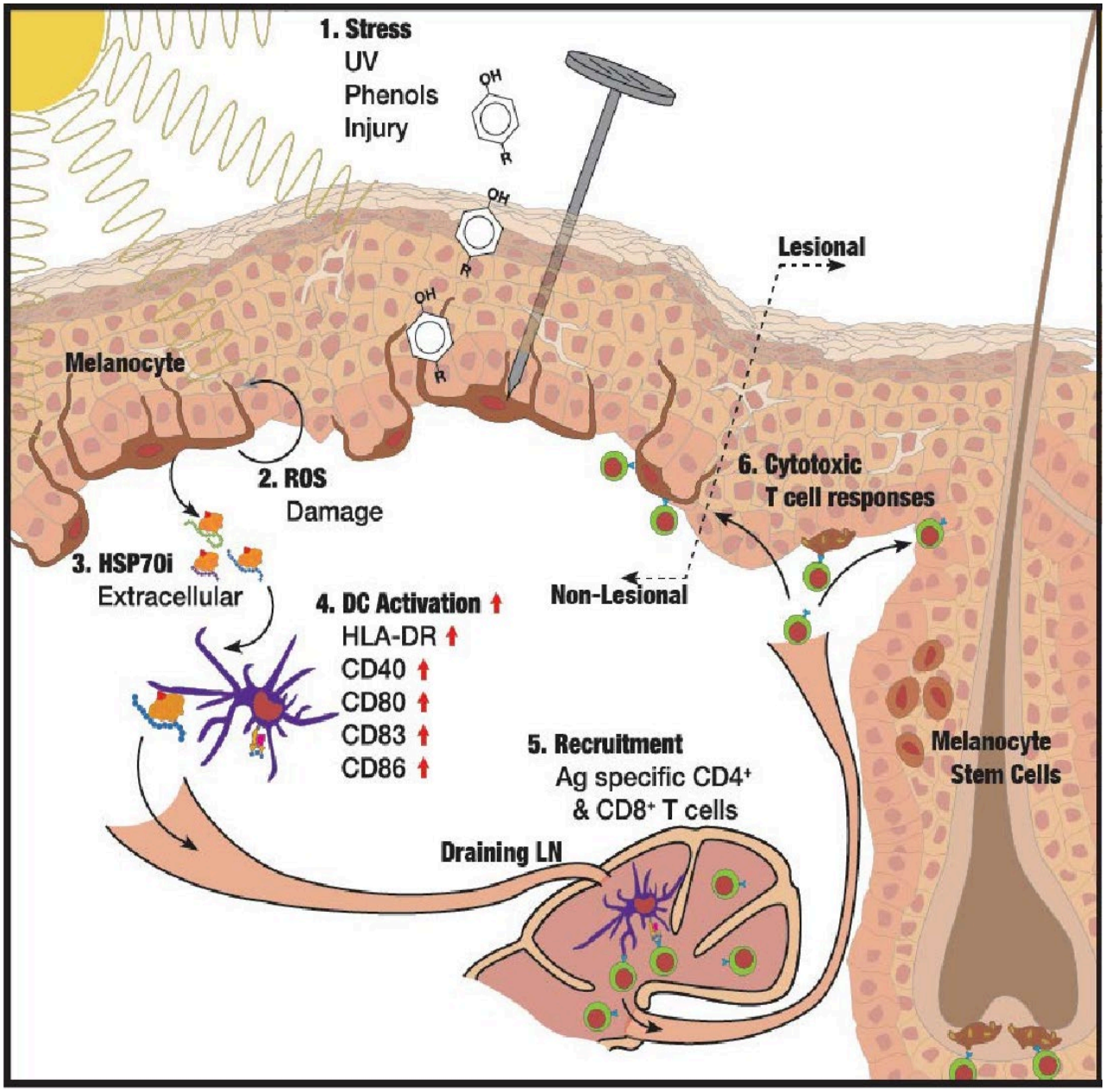


Figure 2 – Autoimmune melanocyte destruction in response to stress factors. Under the stress condition (1), melanocytes lead to ROS generation (2) and secretion of HSP70 (3). These HSP70, in part, chaperon melanocyte-specific antigens and present to DCs, which subsequently recruit antigen-specific CD4 and CD8 T cells in draining LN. These activated CD8 T cells, in particular, are responsible for mediating the melanocyte death (6) via granzyme/perforin pathway [30, p. 568]

1.2 The potential role of microbiota in the development of vitiligo and Treg involvement

Vitiligo progression has been associated with environmental triggers. Autoimmune etiology was recently implicated with intake of antibiotics in some patients, and *H. pylori* were implied in vitiligo [31]. This raised concerns whether

disbalance in microbiome might trigger the vitiligo development. Previous studies reinforce the hypothesis of the role of bacterial diversity in some skin conditions. For example, *P. acnes* is directly associated with acne development [32], and *S. aureus* to psoriasis [33] and to atopic dermatitis [34]. A skin damage provides an opportunity for microbes to enter the organism, and thus, evoking innate, humoral, or T cell-mediated immune responses [35]. In vitiligo skin, the disbalance in microbiome suggests the impact of an intact skin in this condition [36]. Similarly, in a number of autoimmune conditions, gut dysbiosis has a great influence on a systemic immunity, which subsequently affects the disease progression [37]. Although diversity in microbiome promotes the immune homoeostasis, some individual bacterial species can trigger pathogenic responses [38]. As an example, in lupus, a Ro60-producing commensal bacterium is associated with a disease progression [39].

Gut microbiome has an impact on melanoma progression by eliciting immune responses against melanocytes. While enhancing immune response towards melanocytes is essential, dampening the overreacting immune reaction is the objective in vitiligo. Sivan et al reported that *Bifidobacterium* promoted anti-tumor immunity in mice [40]. Thus, some individual bacterial strains can negatively trigger vitiligo, but enhance the immune responses towards melanoma.

In microbiome, the association of some bacterial colonies has to be furtherly investigated. Immunity as a humoral response might be initiated after bacterial infection, and as a result, antibodies will be generated towards bacteria. Some reported that antibody responses to particular bacterial strains have an impact on development of autoimmune responses through molecular mimicry to host antigens due cross-species homology [41]. For example, neomycin-induced segmented filamentous bacteria (SFB) had contributed to arthritic ankle thickening in mice studies [42]. Similar study on autoimmune arthritis demonstrated that increased presence of *B. adolescentis* within small intestine lamina propria has an implication with the disease progression [43]. Thus, a better understanding of gut microbiota diversity and its impact on T cell redistribution including Tregs might reveal whether gut health impacts vitiligo development.

1.3 The role of regulatory T cells in vitiligo

Tregs are a subset of T cells responsible for maintaining immune tolerance to foreign and to self-antigens. Tregs can prevent the development of autoimmune disorders [44]. Tregs are generated from immature CD4 single positive cells receiving TCR signals of intermediate strength to avoid deletion and are directed to differentiate into thymus Tregs (tTregs) [45]. In addition to developing into tTreg, when in the presence TGF-β and IL-2, naïve $CD4^+$ T cells can also differentiate into induced Tregs (iTregs) in the periphery. Some of identified receptors of iTregs further include cytotoxic T lymphocyte-associated antigen (CTLA-4), IL-2 receptor CD25, Forkhead box P3 transcription factor (FOXP3), glucocorticoid-induced tumor necrosis factor (GITR), and neuropilin-1. However, FOXP3 is considered the main and defining marker for Tregs [46]. Among iTregs, type 1 T regulatory (Tr1) cells are the most extensively studied type. Tr1 cells produce high levels of IL-10 and TGF-β, which

played a key role in suppressing antigen-specific T cell responses. Tr1 cells are generated in tolerogenic milieu containing high levels of IL-10 [47].

iTregs provide peripheral tolerance, regulate cytotoxic T cell responses in autoimmune conditions and prevent organ rejection. Generation of *ex vivo* expanded antigen-specific iTregs has potentially promoted their clinical application in autoimmune disorders and Graft vs. Host Disease (GvHD). Tregs in circulation are heterogeneous, and to their instabilities to acquire effector type features is important to prevent and maintain Treg phenotype. Proinflammatory conditions might avert $FOXP3^+$ Tregs into Th17, therefore impairing immune tolerance and contributing to the progression of autoimmune responses [48, 49]. Thus, FOXP3 stability is critical to maintain to prevent the conversion to effector type [50]. The suppression mechanisms of Tregs can be direct, by cell-to-cell contact, or indirect, through a bystander effect. Examples of bystander mechanisms can be via secreting immunosuppressive cytokines such IL-10, TGFB and IL35 [51], and also Tregs can uptake IL-2 and starve effector cell in close proximity [52]. A local deficiency of Tregs in peripheral tissues or impaired Tregs can be the cause for the development of autoimmune conditions including rheumatoid arthritis, alopecia areata, multiple necrosis, and vitiligo [12, p. 38]. The paucity of Tregs were observed in lesional skin of vitiligo patients, thus restoring of Tregs to the skin might halt depigmentation in vitiligo [53, 54].

1.3.1 Approaches to enhance Treg activity

Therapeutics to enhance Tregs function offers a potential tool for the treatment of autoimmune diseases, and can serve to replace the chronic use of immunosuppressants that can be associated with undesirable side effects. Clinical trials have been initiated using polyclonal Tregs for autoimmune skin diseases; a phase I non-randomized, open-label clinical study was conducted for active cutaneous lupus. Patients received a single dose of 10^8 autologous, polyclonal Tregs (NCT02428309), but the study terminated due to participant recruitment feasibility. In an ongoing Treg-based, phase I clinical trial for pemphigus vulgaris and pemphigus foliaceus, patients have been administered a single dose of $1\text{-}2.5\text{x}10^8$ autologous Tregs, and results of this study are pending (NCT03239470). Numerous preclinical studies addressing the treatment of autoimmune skin disorders hail the benefits of using antigen-specific Tregs expressing either a transgenic T cell receptor (TCR) or a chimeric antigen receptor (CAR), and await translation to clinical trial using genetically modified Tregs [55-60].

As an alternative to autologous Treg transfer, therapeutic agents can be used to support Treg function. Drug-based treatment is usually considered more cost effective and may come at a lower risk for adverse events compared to adoptive transfer of live cells. A limited set of clinical trials of this kind is being conducted. This includes a large-scale, ongoing clinical trial to test low-dose IL-2 for the treatment of multiple autoimmune disorders, including skin conditions such as psoriasis, systemic sclerosis and Systemic Lupus Erythematosus (SLE) (NCT01988506) [61, 62]. In animal studies, rapamycin promoted Treg development *via* protein kinase B with subsequent mTOR inhibition [63], and provided benefit by reducing the effector T cell: Treg ratio. However, in clinical trials of rapamycin no significant benefit has been reported to date [64-67].

Supporting Treg skin homing can also be considered for the treatment of autoimmune skin diseases. By cutaneous CCL22 overexpression in a mouse model of vitiligo, Treg numbers were restored and continued treatment was able to suppress vitiligo development [68]. Delivery of CCL22 might be achieved by local needle-free jet injection to attract Treg to the injection site [69]. The same approach can support anti-tumor responses to melanoma, by redirecting Tregs away from the tumor and towards the skin [70].

Supporting microbial diversity or supplying particular microbes can also serve as preventive or treatment measures to drive Treg activity and alleviating autoimmune responses of the skin. This approach is gaining increased attention [71]. In one study, oral administration of *Bifidobacterium infantis* was associated with increased IL-10 secretion and FOXP3 expression in patient blood [72], and in an increase in Tregs in a mouse model of *S. typhimurium* disease [73]. It was found that neomycin treatment can alter the microbiome of vitiligo-prone mice to significantly delay depigmentation and promote the infiltration of Tregs to the skin [74].

1.3.2 The hypothesis of including antigen specificity in Tregs

In recent early-phase clinical trials, polyclonal Tregs have been used to prevent autoimmunity after allogeneic hematopoietic stem cell transplantation (HSCT) and in patients with type-1 diabetes, which demonstrated safety and efficacy [75]. However, the use of polyclonal Tregs for autoimmune diseases might raise some questions. In animal studies, up to 100 times greater numbers of polyclonal Tregs are necessary to achieve the same efficacy as antigen-specific Tregs [76-85]. The risk of developing non-specific immunosuppression is yet another consideration when using polyclonal Tregs [86]. Thus, using antigen-specific Tregs might overcome the two primary challenges with polyclonal Treg therapy: production of potent Tregs with relevant specificity; and importantly, minimizing the risk of non-specific immunosuppression.

1.4 Chimeric antigen receptor (CAR) based cell therapy

Chimeric antigen receptor (CAR) is an artificially engineered chimeric receptor, which combines both antigen-binding and T-cell activating function into a single protein. The first time the term “CAR” was mentioned in 1989 by Gross *et al.* who published an article where they reported the introduction of genetic code for an antibody into cytotoxic T cells. These modified T cells acquired the ability to recognize an antigen called hapten, 2,4,6-trinitrophenyl (TNP), and in vitro studies showed that antigen-specific T cell activity included non-MHC-restricted cytotoxicity and IL-2 production [87].

Structural anatomy of CAR includes four regions: an antigen recognition domain, a hinge region, a trans-membrane domain and an intracellular T-cell signaling domain.

The antigen recognition domain is an extracellular, antigen-binding portion of the chimeric protein responsible to bind to surface antigen expressed on target cells. The antigen recognition part of CAR is derived from the light and heavy chain portions of a monoclonal antibody linked together as a single-chain variable fragment (scFv). This scFv portion imparts to the T cells the ability to recognize and bind the antigen of interest expressed on the cell to which an antibody can be generated [88]. This antigen

recognition domain provides two advantages: potential target antigens can be any proteins, glycoproteins and glycolipids; recognition is not MHC-restricted and no need for antigen processing and presentation. However, one of the drawbacks of using this CAR approach is that it can be only applied for extracellular antigens and solely intracellular antigens cannot be targeted [89]. In addition for CAR antigen recognition domains, there are other non-antibody based molecules including cytokines, innate immune receptors, TNF receptors, growth factors, and structural proteins can also be used as a target antigen [90].

Hinge region is a structural domain situated between the antigen recognition domain and cell membrane. This region enhances the flexibility of the scFv receptor, and reducing the dimensional constraints that promotes synapse formation between the CAR and its target antigen. This hinge region is derived from membrane proximal regions of immunoglobulin G (IgG), CD8 and CD28 [91].

Transmembrane domain is a portion of CAR, which is based on hydrophobic alpha helix that spans the cell membrane. The main function of this transmembrane domain is to anchor the extracellular part of the CAR to the plasma membrane, and to connect the hinge and antigen recognition domain to the intracellular signaling region [90]. The importance of this domain is crucial for the CAR stability, and CD28 transmembrane domain is currently considered as a highly expressed and stable receptor, whereas using CD3-zeta transmembrane domain is not recommended due to the possibility of artificial TCR to be incorporated into native TCR [92].

Intracellular T-cell signaling domain is a portion of CAR located inside the cell. The main role of this signaling domain is to receive a signal from extracellular portion of CAR and transmit to internal cytoplasmic end that initiate cascade of T cell activation [90; 93, p. 37]. This T cell activation is initiated from the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs), which is part of CD3-zeta's cytoplasmic domain. In order to mimic this activation, cytoplasmic domain of the CD3-zeta is commonly used to construct the main CAR endodomain component, and other ITAM-containing domains are considered and found to be not effective [94]. In addition to this CD3 signaling, T cell requires co-stimulatory proteins to retain the prolonged activation. Thus, chimeric domains of co-stimulatory are also added to CAR construct, and currently a variety of co-stimulatory proteins such as CD28, CD27, CD134 (OX40), and CD137 (4-1BB) are being successfully evaluated [90, p. 45; 91, p. 33].

1.4.1 The generations of currently available CARs

CARs are evolved through addition of co-stimulatory proteins and factors including activating cytokines, and ligands to enhance the activity T cells. These engineered CARs are referred as first, second, third, or fourth generations based on their composition.

First generation CARs include an extracellular antigen recognition domain, a hinge region, a transmembrane domain, and intracellular signaling domains. This composition is engineered against to the target antigen with extracellular antigen recognition scFv fragment. Other parts of CAR are responsible to transmit the signals to activate T cells.

Second generation CARs are composed through addition of a co-stimulatory domain such as CD28 or 4-1BB. This addition promotes and enhances the activity of T cells including proliferation, cytokine secretion, protection from apoptosis and prolonged persistence *in vivo*.

Third generation CARs are engineered with multiple co-stimulatory domains, like CD28-41BB or CD28-OX40, to enhance T cell activity. Several preclinical studies report augmented effector activity from T cells transduced with the third generation CARs, which demonstrate improved persistence in animal models compared to the second-generation CARs [95].

Fourth generation CARs is an armored with additional properties that augment T cell proliferation, persistence, improved activity, and also includes cytokines like IL-2, IL-5, IL-12 and co-stimulatory ligands shown in Figure 3 [96; 97, p. 118].

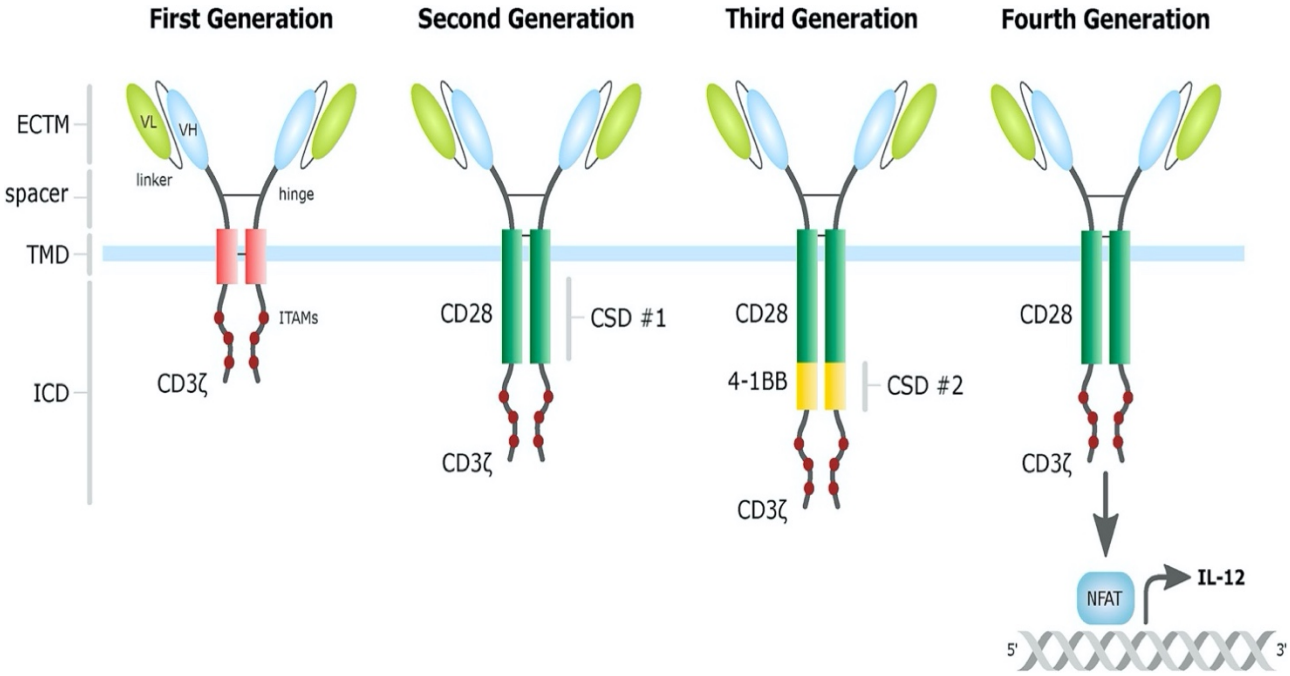


Figure 3 – The evolution of CARs: from basic to armored versions [97, p. 118]

1.4.2 The description of CAR T cell therapy

Adoptive transfer of CAR T cells begins with the isolation of peripheral blood mononuclear cells (PBMCs) through using the leukapheresis or phlebotomy from patients. These obtained cells further are expanded and stimulated to become in state to receive genetic material encoding the CAR of interest. The transduction of the CAR is performed using viral or non-viral methods, which includes retroviruses or lentiviruses. Retroviral vectors integrate only into the genome of the dividing cells, whereas lentiviral vectors can also integrate into non-dividing cells and less susceptible to silencing by host restriction factors, and can deliver larger DNA sequences [98]. After cells reach a sufficient transduction efficiency, they are stimulated for amplification *ex vivo* (Figure 4) [99, p. 1]. The stage of T cell expansion includes the

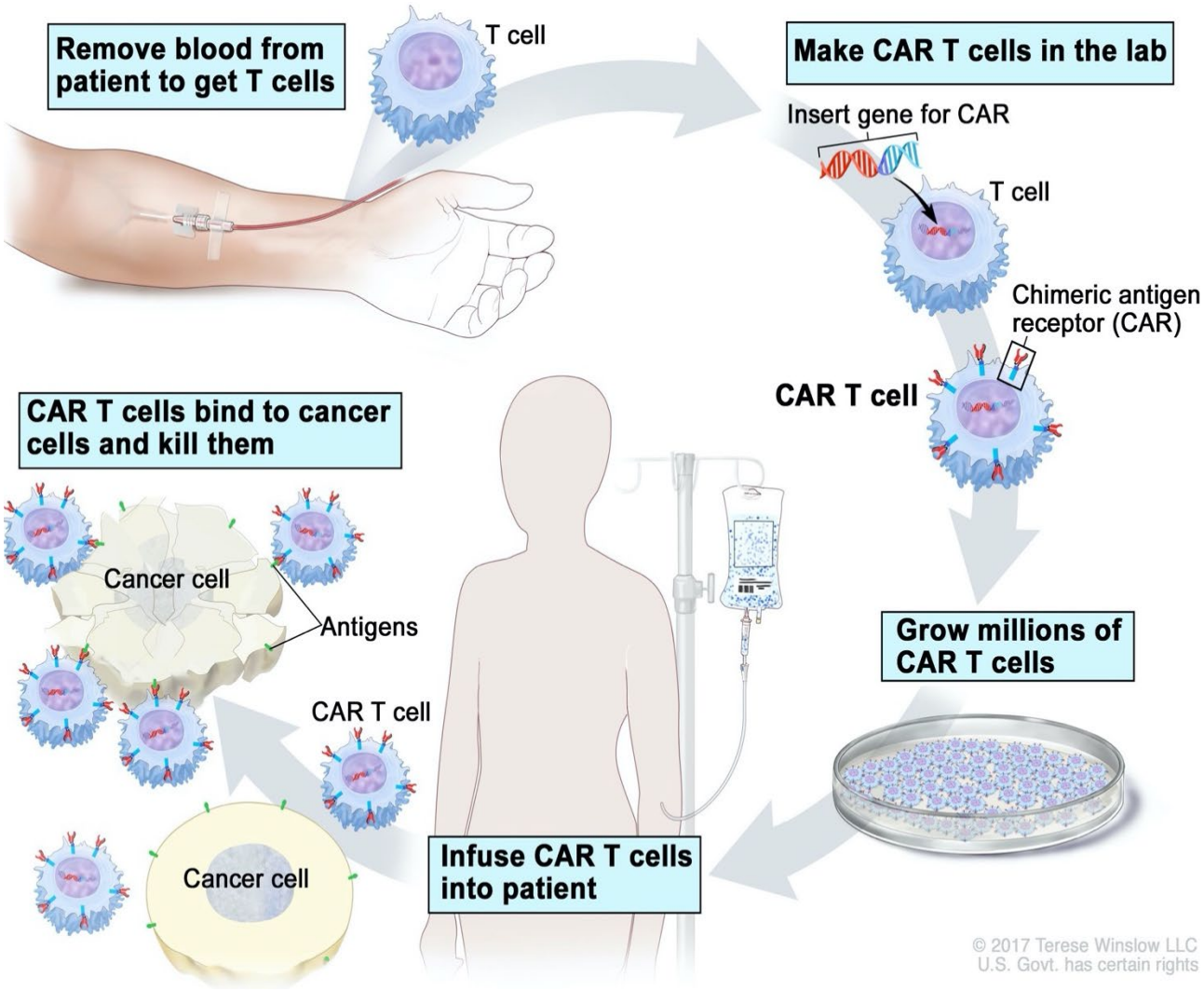


Figure 4 – CAR T-cell therapy diagram. Lymphocytes from patients are collected from peripheral blood using leukapheresis. These derived cells are transduced with CAR of interest, and expanded *in vitro*. Then, millions of the CAR T cells grown in the laboratory are administered back to patients. In the ideal case, CAR T cells migrate and bind to its antigen, and become activated: initiate apoptosis in case of the cancer; suppress immune response in case of autoimmune diseases [99, p. 1]

combined stimulation using anti-CD3 antibodies with or without co-stimulatory anti-CD28, and or cytokines like interleukin-2, -7, -12, and/or -15. Artificial antigen presenting cells (APCs) can also be used for stimulation, and the examples include irradiated K562 tumor cells or EBV-transformed cells. These T cell stimulation methods may differ among the protocols and pre-clinical expertise of the center, which develops an adoptive cell therapy. Available current approaches with different settings include expansion of T cells from several hundred to several thousand-fold in culture with a period ranging from 10 days to 6 weeks [98], p159]. However, using APCs can be very costly and with a longer time in culture to generate sufficient number of T cells for adoptive transfer. The important part is that the functionality of these generated T cells, and their ability of further proliferation and persistence *in vivo* after infusion.

To date, there are two possible ways to prepare the modified T cells with CAR. The first way is to shorten the expansion time period and addition of cytokines, which prevents T cells from exhaustion and excess differentiation. Another way is to choose the defined and sorted population of T cells such as central memory $CD8^+$ T cells, which provides uniform cellular composition, and this approach is being tested in a clinical trial NCT01865617.

Upon adoptive transfer of CAR T cells, they have to traffic to their cognate antigen, get activated, and proliferate. In case of cancer, these CAR T cells must locate tumor and initiate the apoptosis of target cells. Homing molecular appears to be play a crucial role to direct these CAR T cells to demonstrate the high therapeutic effect.

The monitoring of the patients after the CAR T infusion is essential to report any safety issues including concern about insertional mutagenesis, or other unknown complications. To exclude possible complications, non-viral approaches should be considered as these methods are safer and less regulated by authorities. These approaches include transposon/transposase systems (Sleeping Beauty), which can deliver the larger DNA sequences, or gene transfer using RNA electroporation.

Thus, potential CAR T cell therapies have to meet following criteria: (1) sufficient initial number of T cells must be collected; (2) CAR encoded construct has to be transduced efficiently; (3) expansion of cell must be performed with less differentiation and without an exhaustion; (4) upon infusion, CAR T cells has be functional and reach their target; (5) *in vivo* persistence and proliferation; (6) with less toxicity and ideally include suicide gene, which allows to terminate these CAR T cells upon non-desirable outcomes [98, p. 163].

1.4.3 The manufacture and regulatory aspects of CAR T cells

CAR T cells lie under the cell therapy, gene therapy and immunotherapy categories. In European regulations, CAR T cell therapy is classified in the advanced therapy medicinal product (ATMP) and gene therapy medicinal product (GTMP) category. The lack of well-defined regulations for CAR T cell manufacturing and applications are the main obstacles that still require further investigations and development. These drawbacks include lack of knowledge and data from using CAR T cells, and its further ramifications [95, p. 1192].

The manufacture of CAR T cells is a critical part requiring well-defined regulations and the facility with good manufacturing standards (GMPs) associated with hospitals for an easy access to patients. These facilities also require high capacities in generating vector stocks, and provide consistency in each batch with safe CAR T cells with high quality (Table 2) [95, p. 1192].

The requirements for GMP-compliant and manufacture for CAR T cells might delay the timely translation to the patients. First issue includes the source of the starting material, which is highly variable in quality from patient to patient. Transduction efficiency is another concern, which directly impacts the safety and efficacy of CAR T cell therapy. In addition to transduction, the level of CAR expression and the copy numbers per cell has to be evaluated and addressed. However, manufacturing under the GMP standards fortify the safety profile of CAR T cells with harmonized guidance that applies for all the facilities and hospitals providing the cell therapy. Another

concern is due the nature of CAR T cells as they are classified as a genetically modified organism (GMO), which raises an additional burden in terms of regulations.

A clinical design of CAR T cells are encouraged to initiated with scientific advice and recommendation from competent regulatory agencies. This early actions will prevent the hurdles raised in later stages of clinical development and review on marketing authorization in the future [98, p. 164].

Table 2 – Hurdles and possible solutions for the clinical translations [95, p. 1192]

Hurdles	**Possible solutions**
Infrastructure for efficient translation missing	Support for establishing clinical centers that combine basic research, GMP production, and clinical research
CAR T cells are genetically modified organisms (GMOs) in certain EU member states and therefore require a release certificate prior to clinical evaluation	Facilitate process by putting together a universal documentation on the GMO characteristic of CAR T cells, which will then be applicable to any CAR T cell product
Different requirements among EU member states	Harmonize requirements between member states. To improve the current situation, the Voluntary Harmonization Procedure (VHP) was established (regulation 536/2014 EC)
Lack of disseminated knowledge/specific guidance	Set up databases for ATMP clinical trials and products as well as technology transfer networks
	Preparation of CAR T cell-specific guidelines
	Early contact with national competent authorities or EMA

Continue of Table 1

GMP compliance (high burden of documentation already in early phase of application even for clinical trials driven by academia)	**A GMP-specific guideline for ATMPs including provisions for early clinical trial material is currently under development by the Commission in consultation with EMA**
Product chain identity	Develop a general identifier encoding for all relevant information for the hospital and manufacturer to circumvent patient–product mismatches
Toxicities in clinical trials	Better animal models to predict the potential toxicities of CARs

The transportation of CAR T cells from manufacturing sites to hospitals must be highly regulated under good distribution practice (GDP) guidance with an indication of the delivery to a different continent. The tracking of the CAR T cells and preventing the patient–product mismatch is a critical part of the therapy. To address this concern, a harmonized tracking process is currently being addressed by the "CARAT (Chimeric Antigen Receptors for Advanced Therapies) consortium funded by HORIZON 2020 (http://www.carat-horizon2020.eu/).

Clinical development of CAR T cells are not currently conducted phase III trials for any of these CAR T cell products dedicated for cancer therapy. To date, only a conditional marketing authorization has been granted based on single-arm phase II studies for cancer therapies if they are defined under the "orphan disease category" or life-threatening conditions after assessment of convincing efficacy with a positive benefit–risk balance. Upon conditional approval, the CAR T cell provider have to update the data from ongoing clinical trials, and the benefit–risk ratio of the product to evaluate the long-term outcome from conducted clinical trials.

1.4.5 TCRs and CARs – a functional comparison

Antigen-specific Tregs can be generated using traditional methods such as expansion with APCs and specific antigens, but few antigen-specific Tregs will be among numerous polyclonal Tregs, which makes this method inefficient. Engineered Tregs with specific T-cell receptors (TCRs) can be generated using viral constructs with high efficiency, but these TCR-Tregs are limited by MHC-restriction, not allowing all patients to benefit from the same therapy [100]. Tregs transduced with genes encoding a chimeric antigen receptor will overcome this MHC-dependency. This is because chimeric antigen receptors recognize their targets based on interaction with an antibody variable domain, which can respond to epitopes presented on the cell surface. Instead of employing a T cell receptor (lower affinity), these constructs are

instead made of a single-chain variable fragment, scFv – the binding portion of a monoclonal antibody, followed by an extracellular hinge, a transmembrane region, and intracellular signaling domains. Resulting CAR-Tregs can be obtained by fusing the variable domains of an antibody to the signaling portion of a TCR, enforced with domains from costimulatory molecules to further enhance T cell function. In CAR-T cell therapy, optimizing and selecting the correct CAR affinity and intracellular signaling domains is crucial for the resulting therapeutic activity and cellular persistence of the regulatory T cells. Since CARs are constructed using antibody variable regions, they hold higher affinity to their cognate antigen compared to TCRs. It was previously shown that high-affinity CAR stimulation in CD8 T cells can result in reduced activity and loss of specificity of host T cells [56, p. 1232]. In contrast, studies of thymic development showed distinct requirements for TCR affinity in Tregs, which are typically higher in comparison to those leading to selection of conventional T cells [101]. Therefore, the optimal affinity range for the domains responsible for specificity of the CARs, and the effect of high affinities for the function of resulting Tregs still needs to be studied.

1.4.6 Currently available TCRs and CARs of potential use for autoimmune skin diseases

Current cancer immunotherapies involve using TCRs and CARs, and outcomes from these treatments have reached clinical trials that could hold promise in the treatment of autoimmune diseases**.** *Ex vivo*-expanded autologous polyclonal CD4+CD25^{high} CD127^{-} regulatory T cells have been in clinical trials for Type 1 Diabetes and GvHD [102, 103]. The successful use of CARs for the generation of alloantigen-specific Tregs has shown promises in preclinical studies in transplantation. MacDonald K. *et al.* generated Tregs expressing HLA-A2–specific CAR (A2-CAR), which maintained stable phenotype, superior suppressive activity prior to, during, and post A2-CAR–present stimulation *in vitro* and prevented xenogeneic GvHD caused by HLA-A2^{+} T cells in mice model [58, p. 1418] .

For autoimmune skin conditions, a chimeric autoantibody receptor (CAAR) specific for pathogenic antibody-producing B cells has been developed for the treatment of PV. This CAAR was generated by fusing an autoantigen (Dsg3) to a CD137/CD3z signaling domain. Preclinical studies showed that these Dsg3-CAAR T cells were reactive to human anti-Dsg3 BCR-expressing B cells by preventing blistering, and demonstrated no major off-target toxicity.

Thus, CAAR T-cell therapy might be potential clinical route for deleterious PV by eliminating self-reactive B cells and opening an avenue using CAR Tregs for other autoimmune skin conditions [104, 105].

1.4.7 Opportunities for antigen-specificity in vitiligo

The primary dermatologic application of CAR T cells are currently dedicated to treating tumors, including melanoma. In particular, engineered CAR T cells targeting gp100 hold a promise as this antigen is expressed in more than 90% of melanomas [106]. Melanocytes express several currently known melanosomal proteins such as gp100, MART-1, Tyrosinase, TRP-1 and TRP-2. GD3 meanwhile, is a surface

glycoprotein that can be targeted by CAR T cells [107]. Similarly, the GD3 antigen can serve as a target for CAR Tregs. GD3 is an antigen, expressed by neuroendocrine cells including melanoma cells, and melanocytes. GD3 was markedly overexpressed in perilesional skin from vitiligo patients. The concept is then, that GD3 can serve as a target for antigen-specific Tregs to suppress autoreactive T cells in vitiligo without eliciting non-specific immunosuppression [108, 109]. Especially with the local deficiency of peripheral Tregs in vitiligo in mind, adoptive transfer of antigen-specific Tregs responsive to perilesional epithelial cells might prevent general immunosuppression and bring superior efficiency of the potential therapy [110].

Ex vivo generation of human CD4+CD25^{high}CD127$^{low/-}$ can be a challenge due to plasticity of Tregs and lack of proliferation, and sufficient numbers for clinical application only can be generated *in vitro*. Moreover, lineage stability of such peripheral human Tregs and prevention of antigen-specific Tregs from turning into effector T cells can be a challenge. The risks of such Tregs might be mitigated by including suicide genes and maintaining FOXP3 expression [111].

Applicability of antigen-specific Tregs can be limited if the autoimmune skin diseases are not life threatening and cytokine release syndrome and non-specific immunosuppression may raise safety concerns. Another challenge is the cost of adoptive cell transfer for an individual patient which can be addressed via creating universal Tregs by gene editing [112]. Several candidates for a potential target antigens are known, and this provides a possibility to generate antigen-specific Tregs for vitiligo [113]. However, infusion of Tregs periodically might pose a hurdle in terms of the cost and risks with each administration, but local application of chemokines can promote Treg accumulation in the skin to modulate disease progression in vitiligo. Based on provided studies above, antigen-specific Tregs can potentially provide a promising novel approach to treat vitiligo.

1.5 Literature review summary

Vitiligo is a cutaneous autoimmune disease resulting in melanocytes death that leads to white lesions on the skin [24; 114, p. 12]. The prevalence of vitiligo comprises 0.5-1% worldwide, and patients experience psychological issues and social stigma [115-117]. The available treatment options provide limited efficacy, and finding effective therapies is still relevant to treat this quality of life skin disease [118]. Exact etiology of vitiligo is currently unknown, and several factors have been associated with the pathogenesis of vitiligo. One mechanism is via oxidative stress in melanocytes, which in response secrete HSP70i [119]. This stress chaperone, in turn, can transport melanosomal antigens to APCs that directly activates immune cells [120, 121]. As a result, these APCs recruit cytotoxic T cells to mediate melanocyte-specific destruction in vitiligo [122].

The impaired immune tolerance to self-antigens also involves infiltration of skin resident Treg activity [123]. Tregs provide immune tolerance in periphery towards self-reactive, cytotoxic T cells, and maintain immune homeostasis [124]. The deficiency and impaired functions of Tregs have been reported in several autoimmune disorders [125]. In vitiligo, Tregs were greatly reduced in lesional, non-lesional, and perilesional

part of the skin. This suggests that reduced numbers of Tregs affects to provide limited immune tolerance in periphery [68, p. 478].

To date, the infusion of polyclonal Tregs has been applied in clinical trials for several autoimmune disorders, wherein autologous cells from patients were expanded *ex vivo* and re-administered to patients, with limited success [126]. In a vitiligo mouse model, the adoptive transfer of polyclonal Tregs provided a short-term benefits via favoring Treg to effector T cell ratio in this setting [127]. However, administration of polyclonal Tregs might result in systemic immunosuppression through dampening of immune responses to infection or malignancies, which can be a serious clinical consideration [128]. Furthermore, clinical application of Tregs require high numbers of Tregs generated *ex vivo,* which can be challenging. The limitation with generic immunosuppression can be overcome via adding antigen specificity to Tregs. In transplant biology, recently, antigen-specific Tregs are demonstrating promising results to provide local immunosuppression, where the disease is progressing by targeting HLA discrepancies between donor and recipient [129]. Thus, antigen-specific Tregs might offer a potential significant advantages for autoimmune diseases, including vitiligo.

Several approached cane be used to generate antigen-specific Tregs, such as incorporation of TCRs, or using CARs. The limitation of using TCRs is the MHC-restriction, which do not allow for all patients to benefit from the treatment [130]. In this study, antigen-specific Tregs were generated using transduction approach to insert a CAR construct, which overcomes MHC-restriction. Selection of CAR with high affinity to the cognate antigen with intracellular signaling domains is crucial in CAR-T cell therapy. Since CARs are constructed from incorporating the variable part of the monoclonal antibody, they hold higher towards their cognate antigen when compared to TCRs [56, p. 1678]. The identification of the surface antigens is important, and their relatively abundant expression on target cells or affected tissues provide CAR Tregs to suppress the autoimmunity. In vitiligo, the expression of surface antigen on the epithelial skin cells can be served as a target antigen for antigen-specific CAR-Tregs. When these antigen-specific Tregs are infused, they might selectively suppress melanocyte-specific, cytotoxic T cells in vitiligo [131]. These provided results can be compared with reported studies on using a tyrosinase-reactive TCR. This tyrosinase-reactive TCR was tested in an immunodeficient melanoma model, and this was the first time to show the activity infused antigen specific Treg [132]. It has been reported that both tyrosinase and HLA-A2 encoding-genes have an association with vitiligo development, rendering relevance to tyrosinase-reactive T cells, and the association with HLA-A*0201 restriction [133]. When the h3TA2 mouse model of vitiligo generated, the same combination of TCR and MHC transgenes was used, and CAR transgenic Tregs described in this dissertation were tested on this mouse model [55]. GD3 is an antigen expressed on stressed melanocytes and epithelial skin cells, and holds relevance as a target antigen for vitiligo [108, p. 627]. This GD3 antigen has been targeted for melanoma to prepare cell based immunotherapy due to its abundant expression on cell membrane and easy access to antibodies [134, 135]. Another contribution of GD3 includes melanogenesis, cell growth, and cell dendricity [136, 137].

Adoptive transfer of antigen-specific Tregs potentially bring an exciting opportunity to control depigmentation, and to complement up-and-coming therapeutics such as modified inducible HSP70 to tolerize DCs and Janus kinase inhibitors to mitigate T cell activation [69; 138, p. 18].

2 MATERIALS AND METHODS

2.1 Research materials

2.1.1 Study subjects

Peripheral blood samples were derived from healthy donors (average age 34.5 ± 9.6 years (26-51), n = 8; women: average age 42.3 ± 12.5 years, n = 3; men: average age 29.8 ± 3.0 years, n = 5), without autoimmune diseases or obvious signs of other diseases, and people with active and stable forms of vitiligo (average age - 25.0 ± 7.3 years (20- 31), n = 6; women: average age 21.0 ± 1.4 years, n = 2; men: average age 27.8 ± 2.5 years, n = 4), characteristics of patients with vitiligo donors are presented in Table 3. The study was approved by the local ethics committee of the Aitkhozhin Institute of Molecular Biology and Biochemistry. All donors provided informed consent, and the study was conducted in accordance with the ethical principles of the Helsinki Declaration.

Table 3 - Characteristics of vitiligo patients participating in the study

No.	Gender	Age	Duration of disease	Current status of disease progression	Clinical description
1	Male	26	17 years	Stable (8 year)	Localized form (~4% of total skin area are lesional)
2	Male	26	4 years	Stable (5 years)	Localized form (~5% of total skin area are lesional)
3	Female	22	15 years	Active	Generalized form (~30-40% of total skin area) is lesional
4	Male	31	1.5 month	Active	Localized form (~1% of total skin area are lesional)
5	Female	20	6 years	Active	Localized form (~2% of total skin area are lesional)
6	Male	18	~10 years	Stable	Generalized form (~30-40% of total skin area) is lesional

2.1.2 Mice

FH-A2D transgenic mice are used as recipients for study of an impact of antibiotic treatment in microbiome change and redistribution of Treg cells *in vivo*. This transgenic mouse is generated by incorporating TCR reactive to tyrosinase, specifically to the region of 369 to 377 in the amino acid sequence, which is derived from AAD^{+} albino mice.

FoxP3 eGFP – (B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J, B6-Foxp3^{EGFP}) transgenic mice, is used as a source for FoxP3 expressing CD4$^+$ T cells with co-expression of eGFP, are purchased from the Jackson Laboratories, Maine, USA. These mice are homozygous, viable and fertile with regular T cell and B cell development as of C57BL/6. Regulatory T cell specific, transcription factor FoxP3 is designed to co-express with eGFP under the endogenous promoter control. The expression of eGFP is directly and accurately correlates with FoxP3 expression with more than 97% FoxP3 positive T cells, and mRNA expression of FoxP3 strictly segregates with eGFP T cells. This strain targets the GFP cassette downstream of the stop codon of endogenous FoxP3, and no defects found in FoxP3 or regulatory function of these cells. One particular note is that in female mice, the number of CD4$^+$ eGFP $^+$ T cells is approximately half of found in hemizygote males, due to X-inactivation in females. These CD4$^+$ eGFP $^+$ T cells demonstrate regular Treg activity through suppressing effector T cell proliferation in the presence of anti-CD3/CD28 activator beads. In addition, some eGFP were expressed on a small population of CD8$^+$ T cells. These transgenic mice can be useful in several immunological studies including suppression assays, primary immune response, antigen independence, autoimmunity research and *in vivo* cell tracing in mice.

h3T – transgenic mice with human tyrosinase reactive epitope (TIL1383I TCR) were developed using C57BL/6 as a background mice described in Mehrotra *et al* [55]. This mouse model was created using genomic DNA isolated from TIL1383I, an HLA-A2$^+$ human tyrosinase$_{368\text{-}376}$(YMDGTMSQV) specific CD4$^+$ T cell line. The steps of development include cloning of fragments containing the genomic V-J and V-D-J regions of the TCR α- and β-chains, sequencing, and sub-cloning these fragments to TCR cassette described in the earlier study [139]. This TCR cassette then co-injected to fertilized C57BL/6 embryos yielding transgenic founder lines (transgenic core facility at the Medical University of South Carolina). Mice were bred and maintained at Center for comparative medicine (CCM) under the approved animal study protocols of the Institutional Animal Care and Use Committee at Northwestern University, Feinberg School of Medicine.

h3TA2 – vitiligo-prone mice were obtained by crossing h3T mice with HLA-A2 mice (from Jackson laboratories, ME, USA) as T cells with TIL1383I TCR reactive only to tyrosinase from HLA-A2 mice. Due to this properties of this mouse model, TIL1383I TCR carrying T cells attacks melanocytes of h3TA2 heterozygous mice and then they spontaneously develop depigmentation over the time. The depigmentation is observed starting in five weeks, however, the melanocyte destruction is form two weeks onwards and no evidence of remaining melanocytes after active depigmentation (60 – 70 % by 30 weeks of age in h3T-A2 mice).

h3T-FoxP3 eGFP – heterozygous mice were obtained from crossing h3T male with FoxP3 eGFP female mice. The F1 offspring were used as donor mice to isolate h3T-FoxP3 eGFP Tregs to check the suppressive activity of antigen (tyrosinase) specific Tregs *in vivo* using h3TA2 mice as recipient mice.

2.1.3 Tissue procurement, cell culture and reagents

"Human perilesional skin samples were obtained along with informed consent from patients with vitiligo attending the Dermatology clinic at Loyola University Medical Center in Maywood, IL. Studies were approved by the Institutional Review Board in adherence to principles described in the Declaration of Helsinki. Mouse derived naive $CD4^+$ T cells and $CD4^+FoxP3^+$ Tregs were cultured in RPMI media supplemented with 10% FBS, 1X Non-essential amino acids (Corning, USA), 50U/ml Penicillin-Streptomycin (Thermo Fisher Scientific, USA), 1mM Sodium Pyruvate (Gibco, USA), 10mM HEPES (Gibco, USA), and 50 μM β-Mercaptoethanol (Sigma, USA). Human melanocytes were cultured in Human Melanocyte Growth Supplement-2, PMA-free (HMGS-2) (Thermo Fisher Scientific, USA) added to Medium 254 (Thermo Fisher Scientific, USA) with 10mM L-Glutamine (Thermo Fisher Scientific, USA) and 1x antibiotic-antimycotic (Thermo Fisher Scientific, USA). Rabbit anti-GD3 CAR sera and stable GD3 CAR virus producing cells were kindly provided by Dr. Richard P. Junghans (Tufts Medical Center). Stable GD3 CAR virus producing cells were maintained in the above described T cell medium for virus production" [140, p. 2].

2.2 Research methods

2.2.1 Isolation of naive $CD4^+$ T cells and polarization to $CD4^+$ $FoxP3^+$Tregs *in vitro*

"Naïve mouse $CD4^+$ T cells were isolated from spleens of 8-10 week-old B6.Cg-*Foxp3*^{tm2Tch}/J ('FoxP3 eGFP') reporter mice (Jackson Laboratories) using EasySep Mouse naive CD4+ T cell isolation kit (StemCell Technologies) following the manufacturer's protocol. These mice co-express eGFP, which is restricted to the T cell lineage, primarily to the $CD4^+$ T cell population. Naïve $CD4^+$ T cells were polarized to $CD4^+$ $FoxP3^+$ using 30 ng/ml human TGF-β (eBioscience) in the presence of Dynabeads™ Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific) with a 1:1 bead to cell ratio and 300 IU/ml rhIL-2 (NIH, Bethesda, MD) for 5 days. Human TGF-β was used to polarize murine Tregs as mouse and human TGF-β share 99% sequence homology with high cross-species reactivity [141, 142]. Human IL-2 was used as human and mouse IL-2 share 57% of homology, and human IL-2 efficiently stimulates mouse IL-2 receptor, whereas mouse IL-2 do not elicit efficient binding to human IL-2 receptors [143, 144]" [140, p. 3].

2.2.2 Generation of GD3 CAR transduced mouse Tregs

"Twenty-four well non-tissue culture plates were coated with 10 μg/ml retronectin (Takara Bio USA Inc.). MFG retroviral vector based second generation CAR construct (sFv-CD28/TCRζ) reactive GD3 was generated [145]. Conditioned medium supernatant from Phoenix E retroviral producer cells [146] consisting of GD3 CAR-encoding virus (80% confluent) [145, 147] was transferred to retronectin coated plates and centrifuged at 2,000xg. Supernatant was carefully removed and activated $CD4^+$ $FoxP3^+$ Tregs were transferred to the retronectin coated plates with additional viral supernatant, 5 μg/ml protamine sulfate (Sigma Aldrich) and 300 IU/mL rhIL2. Plates were centrifuged at 1,000xg, before incubation with complete T cell culture medium and mouse T-Activator CD3/CD28 beads and rhIL-2 as above. The transduction was

then repeated to increase the transduction efficiency. Transduced Tregs were reactivated with CD3/CD28 beads, 30 ng/ml human TGF-β and rhIL-2 for 2 days before flow analysis" [140, p. 3].

2.2.3 *In vitro* co-culture experiments

"Human HLA-$A2^+$ and HLA-$A2^-$ melanocytes were plated with tyrosinase reactive h3T effector T cells [55] and either untransduced or GD3 CAR-transduced suppressor Tregs at 10:1:1 effector to target to suppressor ratio for 36 hours. Co-cultures were seeded in triplicates and incubated using IncuCyte® Caspase-3/7 Red Apoptosis Assay Reagent (Sartorius). Images were taken every three hours, in triplicate, using the IncuCyte live-cell analysis system (Sartorius). Supernatants were saved for mouse IFN-γ (R&D systems, Minnesota, MN) and IL-10 ELISA assay (Mabtech AB, Stockholm, Sweden) performed according to the manufacturer's protocols. Cytotoxicity was examined by quantifying live cells relative to control wells using Adobe Photoshop (Adobe Systems, San Jose, CA)" [140, p. 3].

2.2.4 Antibiotic administration

Transgenic FH-A2D recipient mice with T cells expressing a TCR reactive to the tyrosinase 369-377 epitope were maintained under protocols approved by Northwestern University's Institutional Animal Care and Use Committee (IACUC) following guidelines for the care and use of laboratory animals as outlined by the US National Research Council. Recipient FH-A2D mice were administered different antibiotics per group after pregnancy, and after litters were delivered, they were received ampicillin (n=10, 5♂, 5♀), or neomycin (n=8, 5♂, 3♀), and one group of mice were left untreated (n=10, 6♂, 4♀). The administration of antibiotics were at 1g/L concentration for ampicillin sodium salt (Sigma-Aldrich, St. Louis, USA) or 1 g/L of neomycin sulfate (Fisher BioReagents, Pittsburgh, USA). For the vehicle, 2.5 g/L of Equal® Sweetener was dissolved in water. The treatment continued during the lactation period and after the pups was weaned. Skin biopsies, spleen, ileum, lymph nodes were maintained in OCT, and serum were stored for cytokine analysis [74, p. 684].

2.2.5 Study design for adoptive transfer of Tregs in h3TA2 mice

"The purpose of this study was to investigate the efficacy of antigen-specific Tregs to halt progressing depigmentation in a mouse model of human vitiligo. Tregs were polarized and amplified *in vitro* before generating antigen-specific Tregs using a CAR construct to generate GD3 CAR Tregs with high transduction efficiencies. To define *in vitro* effects, we co-cultured human HLA-$A2^+$ melanocytes along with tyrosinase reactive effector T cells in the presence of untransduced Tregs and GD3 CAR Tregs. For *in vivo* efficacy, we adoptively transferred untransduced Tregs, GD3 CAR Tregs and HBSS vehicle into a humanized mouse model prone to develop vitiligo, subjecting the animals to depigmentation analysis, while evaluating the local and distant effects of CAR Tregs by immunohistology and multiplex cytokine analysis. Human skin from vitiligo patients were stained for GD3 expression on lesional, perilesional and non-lesional skin.

Transgenic h3T-A2 recipient mice with T cells expressing a TCR reactive to the human tyrosinase 368-376 (YMDTMSQV) epitope [127] were maintained under protocols approved by Northwestern University's Institutional Animal Care and Use Committee (IACUC) following guidelines for the care and use of laboratory animals as outlined by the US National Research Council. Mice were retro-orbitally administered 2 x 10^5 untransduced Tregs/per animal (n=11; 6♂, 5♀) or 2 x 10^5 GD3 CAR Tregs/per animal (n=11; 6♂, 5♀), or treated with vehicle (HBSS) alone (n=12; 6♂, 6♀) four times, every two weeks, starting at 5 weeks of age. All groups received recombinant human IL-2 (3,000 IU) 3 times a week throughout the entire experiment. Animals were maintained for 15 weeks and humanely euthanized. Skin biopsies, spleen, brain, ileum, lymph nodes were maintained in OCT, and serum were stored for cytokine analysis.

Sample sizes for *in vivo* experiments were determined based on statistical power calculations from previous studies and past experience with the h3TA2 mouse model of vitiligo. For *in vivo* studies, mice were randomly assigned to groups with regard to gender, and investigators were blinded to the experimental conditions and to the further analysis. All the experimental samples and animals were included in the analysis, with no exclusion of outliers. Sample sizes, replicates, and statistical methods are indicated in the results and in the figure legends" [140, p. 2].

2.2.6 Bacterial DNA isolation and amplicon sequencing of 16S ribosomal RNA

For microbiome analysis, samples from feces and skin from antibiotic treated mouse were collected and stored at -80°C. According to the protocols from manufacturers, DNA was isolated from feces using QIAmp DNA stool mini kits (Qiagen, Hilden, Germany), and from abdominal skin using DNAeasy Blood and Tissue Kits (Qiagen, USA). (Eu) bacterial primers were used to evaluate and estimate bacterial diversity in DNA from feces as described by Barman et al., 2008. by qRT-PCR (Applied Biosystems, USA). For a reference, *R. productus* was used as necessary for __Ct analysis. 16S rRNA sequencing was performed using DNA samples from both skin and feces at University of Illinois at Chicago campus using Research Informatics Core (RIC). All the necessary steps including basic processing of samples, annotation and analysis of amplicon sequences was incorporated using primer pair consisting of 515 forward: ACACTGACGACATGGTTCTACAGTGYCAGCMGCCGCGGTAA and using for the sequencing 806 reverse primer: TACGGTAGCAGAGACTTGGTCTGGACTACNVGGGTWTCTAAT. Sequence data from readouts were analyzed using BaseSpace online software (Illumina, San USA) [74, p. 685].

2.2.7 Immunoblotting

Colon samples from experimental mice were combined with Laemmli sample buffer (Bio-Rad Laboratories, USA) and 2-Mercaptoethanol (Sigma Aldrich, USA) for further denaturation. Then, the samples were loaded onto a 4-20% Mini-PROTEAN TGX gel (Bio-Rad), and for further immunostaining they were transferred to a polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich, USA). The expression of HSP70i and 4-Hydroxynonenal were assessed using immunoblotting. Following

anti-HSP70i [C92F3A-5] (Enzo, Germany), anti-4 Hydroxynonenal (4-HNE) (Abcam, USA), and anti-actin [C4/actin] (BD Biosciences, USA) were used to immunostaining in membranes. For the secondary antibodies, following ones have been used: horseradish peroxidase (HRP) labeled reactive to mouse IgG1 (Southern Biotech, USA) for detection of HSP70i and for actin, and reactive to rabbit IgG (Agilent Technologies, USA) for 4-HNE. These immunostainings were detected using enhanced chemiluminescence substrate (Thermo Fisher Scientific, USA) and film (Denville Scientific, Thomas Scientific), USA). The obtained results from scanned images of the film (Epson, Japan) were analyzed using ImageJ, and the calculations are made taking the actin as relative levels [74, p. 685].

2.2.8 Immunohistology

Tissue samples from antibiotic received experimental mice including ileum, colon and abdominal skin samples were frozen using Optimal Cutting Temperature Compound (Sakura Finetek, USA). Sectioning at 8μm was performed for all tissues using cryostat (Leica, Germany). For immunohistology stainings, CD3ε was pre-blocked with Biotin Block (ScyTek Laboratories), and then SuperBlock was added to prevent unspecific bindings. Biotinylated rat-raised antibody 145-2C11 reactive to mouse CD3ε (Becton Dickinson Biosciences, USA) was used to detect mouse CD3ε. Ileum tissue sections were stained with an antibody, clone RM4-5 reactive to mouse CD4 (BD Biosciences, USA) or with a clone 53-6.7 to mouse CD8a (BD Biosciences, USA). For skin tissue staining, antibody with a clone D-18 reactive to mouse TRP-2 (Santa Cruz Biotechnology) used to detect TRP-2 in the mouse skin. To detect biotinylated CD3ε, HRP-labeled streptavidin (Southern Biotech, USA) was used, and for CD4 and CD8a – HRP-labeled goat antirat secondary antibody (SouthernBiotech) used for ileum tissues. In the skin, TRP-2 was detected using HRP-labeled swine anti-goat secondary antibody (SouthernBiotech). HRP-labelled streptavidin incubated were developed with aminoethylcarbazole (AEC) substrate (Abcam, UK) and counterstained with Harris hematoxylin (Sigma-Aldrich, USA). Immunofluorescence staining was performed to detect CD4 and CD8a in the mouse skin, and antibody labeled with Alexa Fluor 488 (AF488) clone GK1.5 reactive to mouse CD4 (BioLegend, USA) or biotinylated clone 53-6.7 to mouse CD8a (BioLegend, USA) was used to incubate on mouse skin tissues. Biotinylated anti-CD8a was followed with PE-labeled streptavidin (SouthernBiotech). Nuclear detection was obtained via using 4’,6-diamidino-2-phenylindole (DAPI) (BD Biosciences, USA) staining. Transcription factor FoxP3 staining was performed using AF488-labeled with clone MF-14 reactive to FoxP3 (BioLegend, USA) and PE-labeled anti-CD3ε (BioLegend, USA) was used in staining, and for nuclear staining, DAPI was used. TUNEL tissue sections were fixed using 1% PFA in PBS and permeabilized in 2:1 ethanol:acetic acid prior to TdT enzyme and anti-digoxigenin conjugate incubation. AEC substrate used to develop TUNEL staining, Mayer’s hematoxylin (Thermo Fisher Scientific, USA) was used to counterstain the tissues. Imaging of stained sections were performed using a Revolve microscope (Echo Laboratories, USA) or TissueFAXS PLUS imaging system (TissueGnostics). Adobe Photoshop software was used to quantify the positively stained cells [74, p. 685].

“Mouse skin from adoptively CAR Treg transferred and human skin samples were frozen using Optimal Cutting Temperature Compound (Sakura Finetek) on dry ice. Eight µm cryosections were cut (Leica). For FoxP3/CD3 staining, sections were paraformaldehyde-fixed and permeabilized using True-Nuclear Transcription factor buffer (BioLegend). Sections were treated with SuperBlock (ScyTek Laboratories, Logan, UT). PE-labeled antibody 145-2C11 to mouse CD3ε (Biolegend) and AF488-labeled antibody MF-14 to mouse FoxP3 (BioLegend) were used for double staining procedures, followed by DAPI (BD Biosciences) nuclear staining. For other tissue stainings, mouse and human skin sections were fixed in cold acetone. Mouse skin sections were blocked with SuperBlock and then incubated with either antibody H-90 to TRP-1 (Santa Cruz Biotechnology, Dallas, TX) followed by Alexa Fluor 555 labelled donkey anti-rabbit antibody (abcam), or PE-labeled MB3.6 to GD3 (Santa Cruz Biotechnology), or PE-labeled antibody YGITR 765 to GITR (Biolegend), or AF488-labeled antibody B56 to Ki67 (BD Biosciences), all followed by DAPI nuclear staining. Human skin sections were blocked with 10% normal human serum (Gemini Bio Products, West Sacramento, CA) and then incubated with Ta99 to TRP-1 (BioLegend) or R24 to GD3 (Abcam, Cambridge, UK). Both were detected by an HRP-conjugated goat anti-mouse IgG antibody (Agilent Dako, Santa Clara, CA). These stainings were developed using AEC substrate (Abcam) and nuclei were subsequently detected by incubation in Mayer’s hematoxylin (Sigma-Aldrich) and blued in Scott’s tap water (Sigma-Aldrich). Slides were imaged on a Revolve microscope (Echo Laboratories). Cells were quantified using Adobe Photoshop software” [140, p.3].

2.2.9 *In vitro* splenocyte stimulation

Antibiotic treated mice splenocytes were cryopreserved after tissue collection. Prior to stimulation, splenocytes were thawed into media containing RMPI 1640 with 10% FBS, 1% antibiotics, 50µM 2-mercaptoethanol and 20µg/ml DNAseI (Thermo Fisher Scientific, USA). For stimulation, one million splenocytes were added to wells specified as 5, 24 or 48 hours. Each conditioned with without 30µg/ml murine tyrosinase (369-377) (GenScript, USA). For activation, leukocyte activation cocktail (BD Biosciences; 5 hours only) or 3:1 Dynabeads Mouse TActivator CD3/CD28 (Thermo Fisher Scientific) was used maintain splenocytes for for 24 and 48 hours. Brefeldin A was added to retain cytokines within the cells 5 hours prior analysis. Supernatants from each condition were in further used in ELISAs. Cells were stained for further analysis using flow cytometry [74, p. 685].

2.2.10 Flow cytometry

Human peripheral blood mononuclear cells were isolated from whole blood by centrifugation on Histopaque-1,077 (Sigma-Aldrich, USA) for 20 min at 3000g, 20 °C and washed with RPMI-1640 medium (Sigma-Aldrich, USA). Further, the phenotype and expression of suppressor molecules was evaluated by flow cytometry, analyzing Treg cells in a $CD4^{+}CD25^{+}$ gate. For this, cells were labeled with fluorescently-labeled antibodies to CD4-PE, CD39-APC, CTLA4-APC, IL10-FITC, GITR-APC, CD73-APC, LAP (TGFβ1) -APC, TGFβ1-PE, FoxP3-APC (Miltenyi Biotech, USA), CD4-

FITC, CD25-PerCP-Cy5.5, FoxP3-PE, CD44-FITC (BD Biosciences, USA), IL35-APC (R&D Systems, USA). First, surface markers were labeled according to the protocols provided by manufacturers, then the cells were fixed, permeabilized and washed using the FOXP3 Staining Buffer Set (Miltenyi Biotec, USA), then intracellular staining was performed and analyzed using the FACSCalibur cytofluorimeter and BD CellQuest Pro Software (BD Biosciences, USA) [74, p.685].

Flow cytometry analysis also was performed for *in vitro* stimulated splenocytes. Prior surface staining, splenocytes were blocked with mouse Fc Block (Biolegend, USA) and LIVE/DEAD Fixable Near IR Dead Cell dye (Thermo Fisher Scientific, USA) to eliminate dead cells from analysis. Antibodies for surface staining are following: anti-CD45 BB515 (30-F11), CD3 BUV395 (145-2C11), CD4 BUV737 (GK1.5; all BD Biosciences, USA), CD8 BV786 (53-6.7; Biolegend, USA). BD Cytofix/Cytoperm kit (BD Biosciences) was used to fix and permeabilize cells prior to stain intracellular cytokines such as anti-IFN-γ APC (XMG1.2) and anti-IL-17A PE (TC11-18H10.1; both Biolegend, USA) antibodies. Flow cytometry analysis was performed on a BD FACSymphony flow cytometer, and results were analyzed using FlowJo v10.3.0 (FlowJo LLC, USA) [74, p. 685].

To assess the transduction efficiency on GD3-specific CAR Tregs, flow cytometry was performed on transduced Tregs. "Prior to surface staining, cell from CAR transduction were incubated with mouse Fc Block (BioLegend) and LIVE/DEAD Fixable Near-IR Dead Cell dye (Thermo Fisher Scientific) according to the manufacturer's instructions. Surface staining of directly labeled antibodies included BUV395-labeled anti-mouse CD3 clone 145-2C11 (BD Biosciences), and BV421-labeled anti-mouse CD4 clone GK1.5 (BioLegend). The eGFP marker expressed under the FoxP3 promoter in Treg reporter mice, as well as BB700- labeled rat anti-mouse CD25 clone PC61 (BD Biosciences) were used to identify Tregs. Unlabeled anti-GD3 CAR rabbit sera detected by anti-rabbit APC (Invitrogen) antibodies were used to evaluate CAR expression by transgenic Tregs. Stained cells were analyzed using a BD FACSymphony flow cytometer and FlowJo v10.3.0 software (FlowJo LLC, OR, USA)" [140, p. 4].

2.2.11 ELISA cytokine assay

IFN-γ and IL-17 was evaluated using Quantikine ELISA kits (R&D Systems, USA) for supernatants from 24 and 48hr splenocyte stimulations. ELISA plate were recorded using a Synergy HT reader (Biotek, USA) equipped with Gen5 v1.08 (Biotek, USA), and generated results were analyzed using Prism v7.03 (GraphPad Software, USA) [74, p. 685].

"Included in cytokine analysis were supernatants from *in vitro* suppression assays (IncuCyte experiments), collected 36 hours post-co-culture, and serum samples from HBSS vehicle (n=11), untransduced (n=10) and GD3 CAR Tregs (n=9) treated mouse groups. Detection of murine IFN-γ, TNF-α, IL-4 and IL-10 was performed by using a V-Plex Proinflammatory Panel 1 Mouse kit (Meso Scale Diagnostics, LLC) according to manufacturer's instructions. Data were acquired on a Synergy HT reader (Biotek) equipped with Gen5 v1.08 (Biotek) and analyzed using Prism version 8.3.0 (GraphPad Software)" [140, p. 4].

2.2.12 Cytokine array analysis

Cytokine array was performed on previously cryopreserved colon samples. Prior to analysis, colon was homogenized by BeadBug Microtube Homogenizer and 1.5 mm Triple-Pure High Impact Zirconium pre-filled tubes (Benchmark Scientific, USA) in the presence of T-PER tissue protein extraction reagent (ThermoFisher Scientific) and protease inhibitor cocktail (ThermoFisher Scientific, USA). Pierce BCA Protein Assay Kit was used to for colon protein quantification and Proteome Profiler Mouse Cytokine Array Kits (R & D Systems, Minneapolis, MN) was utilized for protein analysis following the manufacturer's protocol. Serum from antibiotic treated mouse were pooled (N=4) using 25 µl of serum from each mouse. Acquired membranes were imaged on a LAS-3000 luminescent imager (Fujifilm, Japan). Obtained images were further analyzed using Image J software [74, p. 685].

2.2.13 Depigmentation analysis

"From 5 weeks to 15 weeks of age, mice were scanned weekly on a flatbed scanner (Hewlett-Packard, Palo Alto, CA) under isoflurane anesthesia. Using Adobe Photoshop software (Adobe Systems) ventral and dorsal luminosity was measured to calculate depigmentation, as previously described [148]. Depigmentation was graphed over time, and statistical significance was determined by the time-adjusted AUC. Representing change in depigmentation from treatment initiation was calculated using the trapezoidal rule. No imputation was done for missing data, and the AUC for each mouse was divided by the total number of weeks of available data minus 1. The WRS test was used to compare the time-adjusted AUC among groups" [140, p. 4].

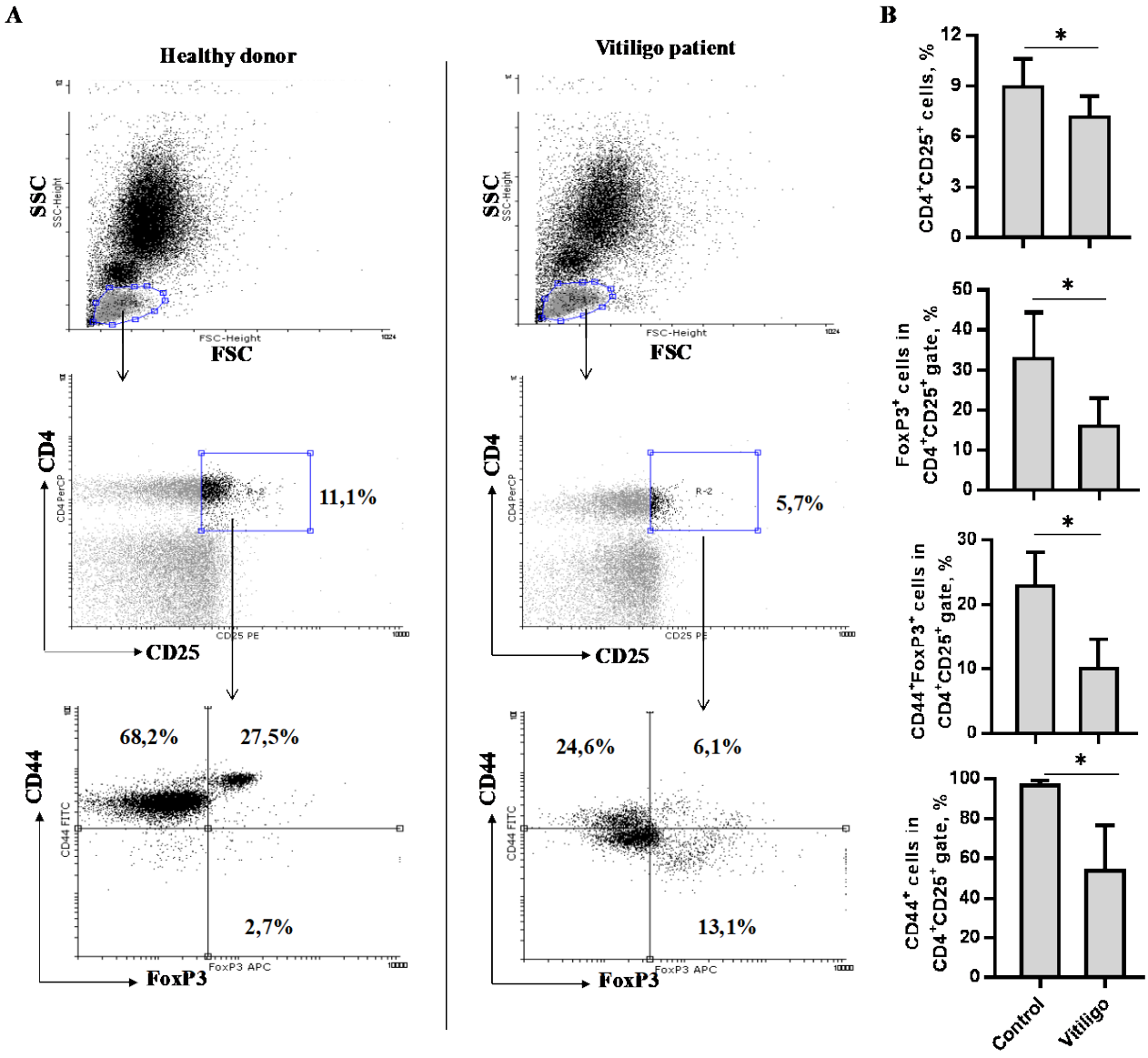
2.2.14 Statistical analysis

"Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad) and R-software. Data are presented as bars and dot plots with mean values ± standard deviation. The statistical significance was determined by the criterion of Student (T test) for phenotypical characteristics of Tregs in vitiligo patients. The data were evaluated by one-way ANOVA analysis of variance accounting for different variances across the treatment groups, with post-hoc Tukey-Kramer comparisons. To determine statistical significance for immunosuppression *in vitro*, two-way ANOVAs were used with aligned rank transformation followed by multiple pairwise comparisons testing using Tukey approach. For depigmentation, the time-adjusted AUC, representing change in depigmentation from treatment initiation, was calculated using the trapezoidal rule. No imputation was done for missing data, and the AUC for each mouse was divided by the total number of weeks of available data minus 1. The Wilcoxon rank sum (WRS) test was used to compare the time-adjusted AUC among groups. Statistical significance is represented as *$p<0.05$, ** $p<0.01$, *** $p<0.001$ or **** $p<0.0001$" [140, p. 4].

3. RESULTS AND DISCUSSION

3.1 Phenotypical characterization of Tregs in patients with vitiligo

To date, there are disagreements in the literature regarding changes in the proportion of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells in peripheral blood in vitiligo patients [113]. We studied these populations in a group of healthy donors and vitiligo patients. In the course of the study, it was found that in the group of vitiligo patients, the proportion of circulating $CD4^+CD25^+$ Treg cells and FoxP3 expressing $CD4^+CD25^+$ Treg cells was significantly reduced compared with the group of healthy donors (Figure 5).



Representative (A) and generalized data are presented in the form M±SD (B), the significance of differences between the groups is presented as * $p<0.05$ (by Student's criterion).

Figure 5 – Proportion of $CD4^+CD25^+$ Treg cells expressing $FoxP3^+$, $CD44^+FoxP3^+$ and $CD44^+$ in peripheral blood of healthy donors (Control) and vitiligo patients (Vitiligo)

When comparing the proportion of peripheral blood $CD4^+CD25^+$ Treg cells between the control group and a group of vitiligo patients with a progressive form of the disease, it was also found a significant decrease in the number of these cells (9.0±1.6 (n=8) 6.4±0.04 (n=4), p=0.003, respectively). The proportion of peripheral blood $CD4^+CD25^+$ Treg cells did not differ between the groups of healthy donors and patients with stable form of vitiligo and between the groups of patients with active and stable forms of vitiligo (9.0±1.6 (n=8), 8.2±1.3 (n=3), p=0.4; 6.4±0.04 (n=4), 8.2±1.3 (n=3), p=0.1, respectively). Meanwhile, when comparing to healthy donors, the proportion of FoxP3-expressing $CD4^+CD25^+$ Treg cells was down-regulated in both patients groups with active and stable forms of vitiligo (33.0±11.4 (n=8), 16.7±8.7 (n=4), p=0.02; 33.0±11.4 (n=8), 15.1±5.3 (n=3), p=0.008, respectively). The level of expression of FoxP3 by Treg cells also did not differ significantly between the patients' groups (p=0.8).

Our results are in agreement with previously published data, according to which the proportion of $CD4^+$ $CD25^+$ Treg cells expressing FoxP3 is reduced in the peripheral blood of patients with progressive vitiligo compared with healthy people and patients with an inactive form of the disease [149]. Thus, obtained data support the findings showed that the number of circulating $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ Treg cells correlates with the progression of vitiligo and confirm the role of Treg cell number reduction in the insufficient regulation and suppression of autoimmune response leading to vitiligo progression.

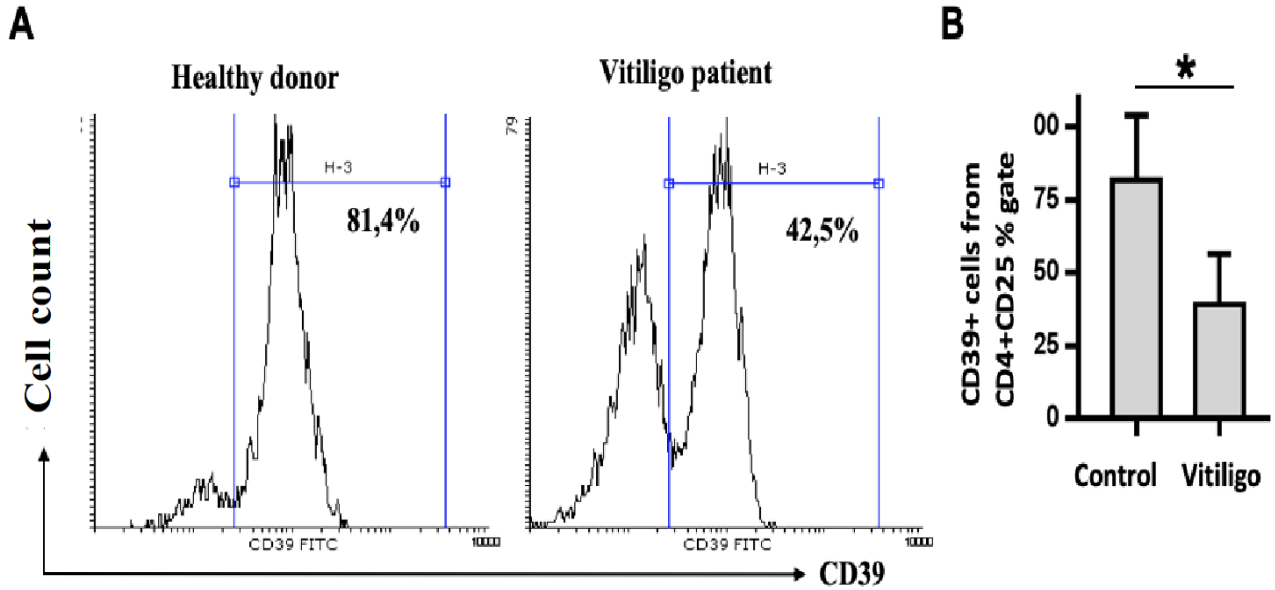
It is known that the proportion of $CD4^+CD25^+$ Treg cells that infiltrate foci of vitiligo depigmentation is significantly reduced, which is considered the main reason for the lack of regulation of autoimmune response against melanocytes. However, in previous studies, attempts to identify the causes of a decrease in the migration of Treg cells to the affected areas of the dermis were unsuccessful. In particular, no abnormalities were found in the expression of adhesion and homing receptors for CCR4, CCR5, CCR8, and CLA by peripheral blood Treg cells in vitiligo [20]. However, the expression of one of the central receptors for attracting Treg cells from the bloodstream to the site of inflammation — CD44 has not been previously studied in vitiligo. Thus, the next step in our study was to study the expression of CD44 by $CD4^+CD25^+$ Treg cells in the peripheral blood of patients with active and stable forms of vitiligo.

In the course of the study, it was found that the proportion of circulating $CD4^+CD25^+CD44^+$ Treg cells was significantly reduced in the group of vitiligo patients compared with healthy volunteers. Moreover, the percentage of $CD44^+FoxP3^+$ Treg cells analyzed in the $CD4^+CD25^+$ gate was also significantly reduced in the vitiligo group relative to the control group (Fig. 5). Similar data were obtained regarding the proportion of $CD44^+$ and $CD44^+FoxP3^+$ Treg cells when comparing the control group with a group of patients with an active form of vitiligo (96.9±2.3 (n=8), 46.3±26.3 (n=4), p=0.03; 22.3±8.0 (n=8), 7.8±4.6 (n=4), p=0.001, respectively). The proportion of $CD44^+FoxP3^+$ cells analyzed in the $CD4^+CD25^+$ gate was also significantly higher in the peripheral blood of healthy donors compared with donors with a stable form of vitiligo (22.3±8.0 (n=8), 14.3±4.1 (n=3), p=0.002, respectively). There were no significant differences in the level of expression of $CD44^+$ by

$CD4^+CD25^+$ cells between a group of healthy donors and patients with a stable form of vitiligo, as well as expression of CD44 by $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ cells between the groups of patients with active and stable forms of vitiligo.

Thus, expression of the CD44 homing receptor by peripheral blood Treg cells in vitiligo is reduced. We can hypothesize that this dysfunction of Treg cells can lead to a decrease in the homing of Treg cells in the dermis of vitiligo foci. Moreover, earlier studies in mice showed that the expression of CD44 positively correlates with the expression of FoxP3, IL-10 production, increased proliferation and suppressor activity of Treg cells with respect to T cell proliferation, . The ability to bind the intercellular matrix component, hyaluronan, by active isoforms of CD44 discriminates Treg cells with an increased suppressor potential and degree of activation. It was also previously shown that Treg cells of mice with the absence of a gene encoding CD44 have a reduced ability to suppress T-cell immunity. We can hypothesize that a decrease in the proportion of Treg cells expressing CD44 during the development of vitiligo indicates a decrease in the pool of Treg cells capable of effectively inhibiting the activity of melanocyte-specific $CD8^+$ T cells in patients.

Next, the expression level of functional markers by Treg cells was compared in peripheral blood of vitiligo patients and healthy donors. No significant differences in the expression of CTLA-4, IL-10, GITR, CD73, LAP, TGFβ and IL-35 among neither $CD4^+CD25^+$ nor $CD4^+CD25^+FoxP3^+$ Treg cells was observed between the studied groups (data not shown). However, a significant decrease in the expression of CD39 by $CD4^+CD25^+$ Treg cells was found in the vitiligo group, compared to healthy donors (Figure 6).



Representative (A) and generalized data are presented in the form M ± SD (B), the significance of differences between the groups is presented as * $p<0.05$ (by Student's criterion)

Figure 6 – Percentage of $CD4^+CD25^+$ Treg cells expressing CD39 in the peripheral blood of healthy donors (Control) and vitiligo patients (Vitiligo)

Interestingly, the proportion of $CD4^+CD25^+$ Treg cells expressing CD39 was significantly higher in the group of healthy donors when compared with the group of patients suffering from the active form of vitiligo, but not stable vitiligo (86.9±8.3 (n=8), 23.9±22.0 (n=4), p=0.03; 48.6±30.1 (n=3), p=0.1, respectively). Also, in comparison with control group, a significant decrease in the proportion of $CD39^+FoxP3^+$ Treg cells ($CD4^+CD25^+$ gate) was found in the vitiligo group including all the patients, group of active and stable form of vitiligo (27.2±10.0 (n=8), 13.5±6.5 (n=7), p=0.002; 11.2±3.5 (n=4), p=0.003; 12.3±5.4 (n=3), p=0.01, respectively). There were no differences in the proportion of $CD39^+$ or $CD39^+FoxP3^+$ cells in $CD4^+CD25^+$ Treg cells gate between the groups of patients with an active and stable form of vitiligo.

The expression of CD39 is associated with the suppressor activity of Treg cells and the catalytic inactivation of surface ATP by the CD39 molecule is one of the key immunosuppressive mechanisms of Treg cells. CD39 hydrolyzes both adenosine triphosphate and adenosine diphosphate to monophosphate. The binding of adenosine to its A2A receptor, which is present on the membrane of effector T cells and dendritic cells, leads to an increase in intracellular cyclic adenosine monophosphate and suppression of the function of these cells. There is experimental evidence that suppression of the proliferation of T cells of mice knocked out by the A2A gene is much lower than in wild mice. Transduction of the *foxp3* gene into murine $CD25^-$T cells induces their expression of CD39, and pre-incubation of Treg cells in an ATP-containing medium reduces ATP-dependent DC maturation upon further co-incubation. In addition to the direct suppressor effect, the removal of ATP from the cytoplasmic membrane of cells by the CD39 molecule allows Treg cells to penetrate into the area of inflammation and suppress ATP-induced inflammatory reactions.

Since obtained data indicate a decrease in the expression of the CD39 suppressor molecule by Treg cells possessing $CD4^+CD25^+FoxP3^+$ phenotype in case of progressive vitiligo and in disease remission, it might suggest the contribution of Treg cell dysfunction to the pathogenesis of vitiligo. Decreased homing and expression of CD39 by Treg cells can lead to an uncontrolled increase in the number and activation of melanocyte-specific cytotoxic T cells and their autoimmune cytotoxic effect in vitiligo foci.

Thus, the proportion of the total population of Treg cells with $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ phenotype was observed, as well as the proportion of $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ Treg cells expressing the immunosuppressive molecule CD39 and the cell adhesion and migration receptor CD44, are reduced in the peripheral blood of patients with progressive and stable vitiligo form of the disease. The obtained data indicate the dysfunction of Treg cells in vitiligo and may indicate a decrease in their immunosuppressive properties and ability to migrate efficiently to the foci of depigmentation, which may lead to uncontrolled activity of melanocyte-specific cytotoxic 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 the treatment of vitiligo based on increasing the suppressor activity of Treg cells and their recruitment into affected vitiligo skin areas.

3.2 Microbiota change after antibiotic treatment (neomycin) impacts peripheral Treg infiltration in the skin, and drive (ampicillin) vitiligo development in mice

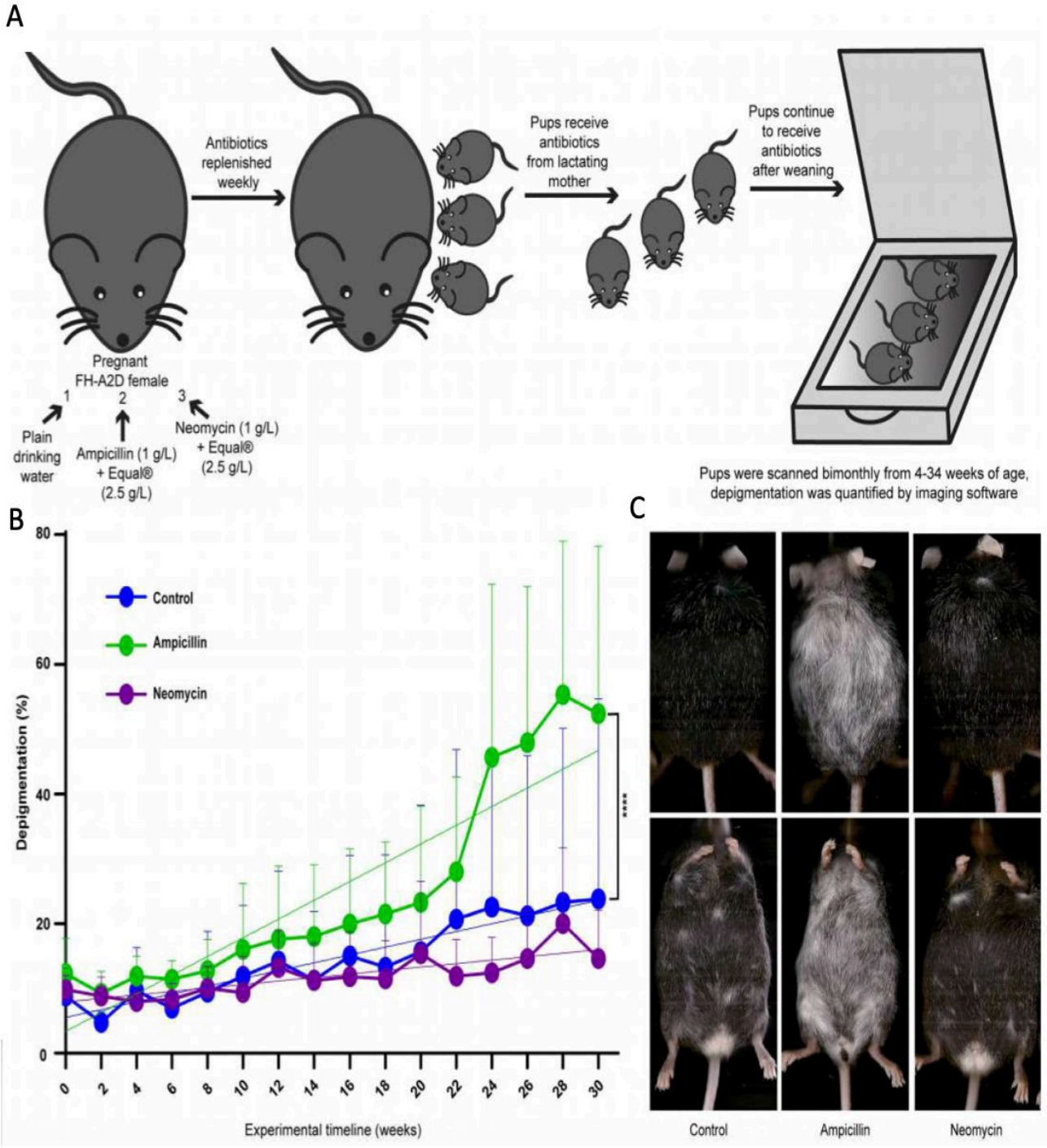
3.2.1 Antibiotic application impacts vitiligo development in mouse model

Several studies previously reported that microbiota diversity was associated with the development of autoimmune diseases [37, p. 78]. Chang et al. reported that *Staphylococcus aureus* contributes to psoriasis development, and similar study by Seddon and Hughes et al. also linked atopic dermatitis with this cutaneous bacteria [33, p. 154; 34, p. 1234]. The impact of antibiotic application, and change in microbiome affected depigmentation in vitiligo prone transgenic mice. In case for vitiligo patients, studies demonstrated that disbalance in bacterial diversity and scarce of *Corynebacteriaceae*, and abundance of *Gammaproteobacteria* and *Flavobacteria* are observed, which can be associated with depigmentation [36, p. 18761]. Moreover, vitiligo patients prior to diagnosis, reported antibiotic treatment in the etiologic factors, and study showed that *Helicobacter pylori* was associated in vitiligo [31], p. 1513]. Thus, it was attempted to understand the impact of antibiotics on microbiome, and the vitiligo progression in animal model. From a list of currently prescribed antibiotics, ampicillin and neomycin were chosed in our experiment. The main reason of applying these antibiotics is due to their "broad spectrum" for multiple gram-positive bacteria (ampicillin) and high efficiency against gram-negative bacteria (neomycin).

To evaluate the impact of the antibiotics, the recipient vitiligo-prone FH-A2D mice received ampicillin and neomycin with drinking water during neonatal period, and followed by 35 weeks after birth. The depigmentation measures were taken every two weeks, and the analyzed images of depigmentation were shown in Figure 7A. The results from ampicillin treated group demonstrated the acceleration in depigmentation (p<0.0001) while neomycin treated group protected and delayed the vitiligo development (p=0.07) (Figure 7B). Representative examples from mice treated with ampicillin group shows a total depigmentation presented in Figure 7C. Depigmentation rates were differed among groups. Mice from untreated group (n=10) depigmented (0,6±0,16%), whereas neomycin (n=8) received group (0,27±0,06%) and ampicillin group (n=10) depigmented (1.45±0.14%) per week. Interestingly, 2.4 fold increases in depigmentation was observed in ampicillin received mice [74, p. 677]

Microbiome alteration after antibiotic application influenced the progression of vitiligo in mouse model. A few studies report similar results regarding antibody mediated autoimmune response against microbes through humoral activity, while cytotoxic T cells are not capable to target bacteria [150]. Thus, a mechanism of enhanced autoimmune response of T cells against self-antigens is not known yet. However, recently, the impact of microbiome towards immune system and disease development is gaining more attention [151], and to evaluate the influence of bacterial diversity, antibiotics can be used as an useful investigative tool [152]. When compared, human and mice microbiome showed similarity which justified the use of mouse as an animal model [153], and the ileum gains a significant role as bacterial adherence and T cell activation occur in this part of the gut [154]. An important question is that how gut microbiome influence immune activity in a distant organs such a skin [155].

Alternatively, the impact of skin resident commensal bacteria could trigger the immune response to non-classical MHC-I-restricted T cells [74, p. 678; 156].



(A) Experimental design of antibiotic treatment and bimonthly scanning. (B) Images were taken by flat-bed scanning, and measured biweekly. The significance of differences between the groups is presented as **** $p<0.0001$ (by Student's criterion). (C) Images from end-point of the experiment

Figure 7 – The impact of antibiotics to vitiligo development in mice [74, p. 678]

Tyrosinase related protein (TRP-2), a surface marker for melanocytes were stained using immunohistochemistry to identify survived melanocytes in the skin of

FH-A2D mice. A post-confirmatory staining of skin tissues demonstrated the loss of melanocytes almost in all three groups at the end of the *in vivo* study. However, a faint expression of TRP-2 was still presented in skin of the control group, while pigmented and melanized hairs were observed in neomycin received and control groups shown in Figure 8. Therefore, it was suggested that microbiome might impact the overall autoimmunity to melanocytes and promote depigmentation.

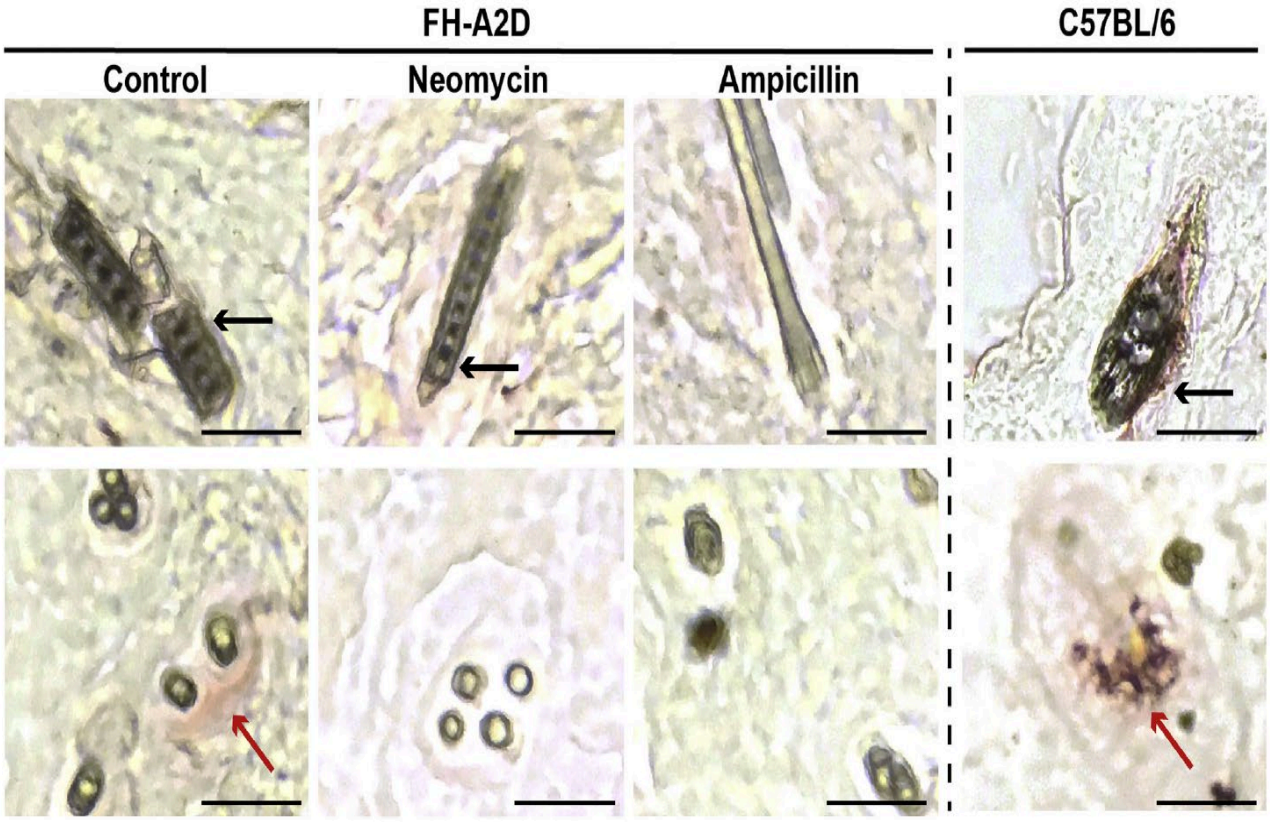


Figure 8 – Melanocyte (red) and the absence of melanized (black) hairs in the skin of FH-A2D mice [74, p. 687.e2]

It was previously reported that ampicillin can induce the creation and accumulation of reactive oxygen species (ROS) in both bacterial and human cells. This ROS formation might influence gut permeability [157]. Mitra *et al*. reported that ROS formation has been related to vitiligo where high cytokine release correlated with elevated ROS and decreased antioxidants [158]. Since an association of increased ROS accumulation after long term antibiotic (ampicillin) administration was reported, and melanocyte stress was associated with ROS formation, and then it was attempted to evaluate the oxidative stress in FH-A2D transgenic mice. Expression of metabolic marker of ROS, hydroxy-2-nonenal (4-HNE) and inducible heat shock protein 70 (HSP70) were compared in colon homogenates of ampicillin treated group (Figure 9). An intense depigmentation could not been ascribed to increased level of intestinal oxidative stress, then it was pursued to analyze the microbial dysbiosis as a potential cause for the disease development via determining the composition of microbes residing in skin and gut.

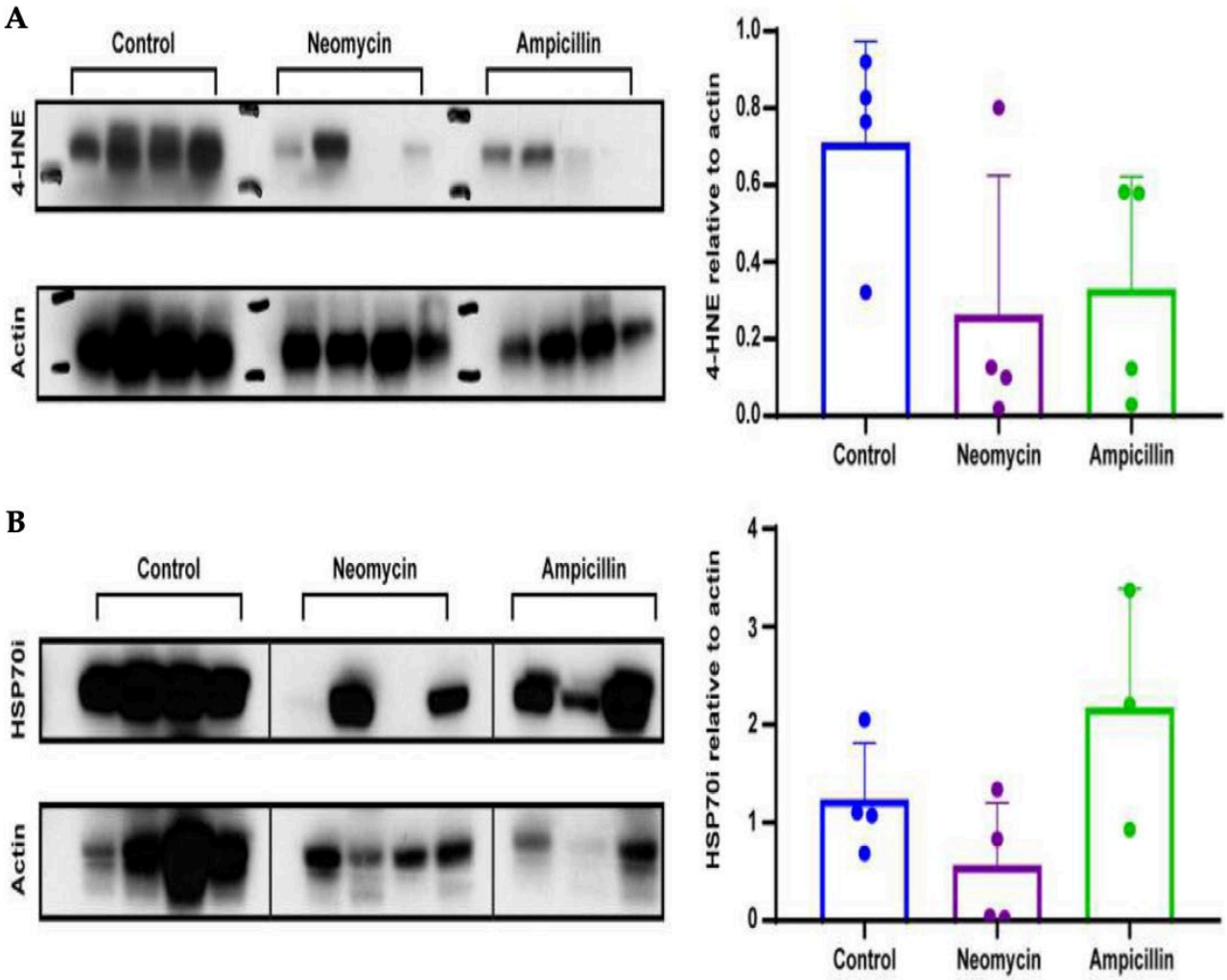


Figure 9 – Depigmentation in antibiotics treated FH-A2D mice was not ascribed with an elevated oxidative stress. The expression levels of (A) 4-HNE and (B) HSP70i were not significantly different among treatment group [74, p. 687.e3]

3.2.2 Systemic application of antibiotics predominantly limits microbiome in the gut but not affect the skin

A study by Byrd et al. revealed an association between gut microbiota and autoimmunity in skin diseases, psoriasis [159]. It is already known that antibiotics reduce the gut bacteria abundance while increasing the shedding derived from bacterial components [160]. In the next step of the study, it was investigated whether the microbial diversity was impacted in gut or in the distant skin tissues. For this, DNA was isolated from collected skin microbiome and excrements, and then 16S rRNA ribosomal genes used in polymerase chain reactions (PCR) to amplify degenerate primers to differentiate the species. The generated amplicons were sequenced for 16S r RNA to identify the bacterial abundance in skin and gut microbiome.

The relative percentage of microbial composition of skin derived from 16S rRNA ribosomal gene sequence analysis is shown in Figure 10. The skin samples are presented from control group (n=3), ampicillin received (n=3), and neomycin (n=5) treated groups.

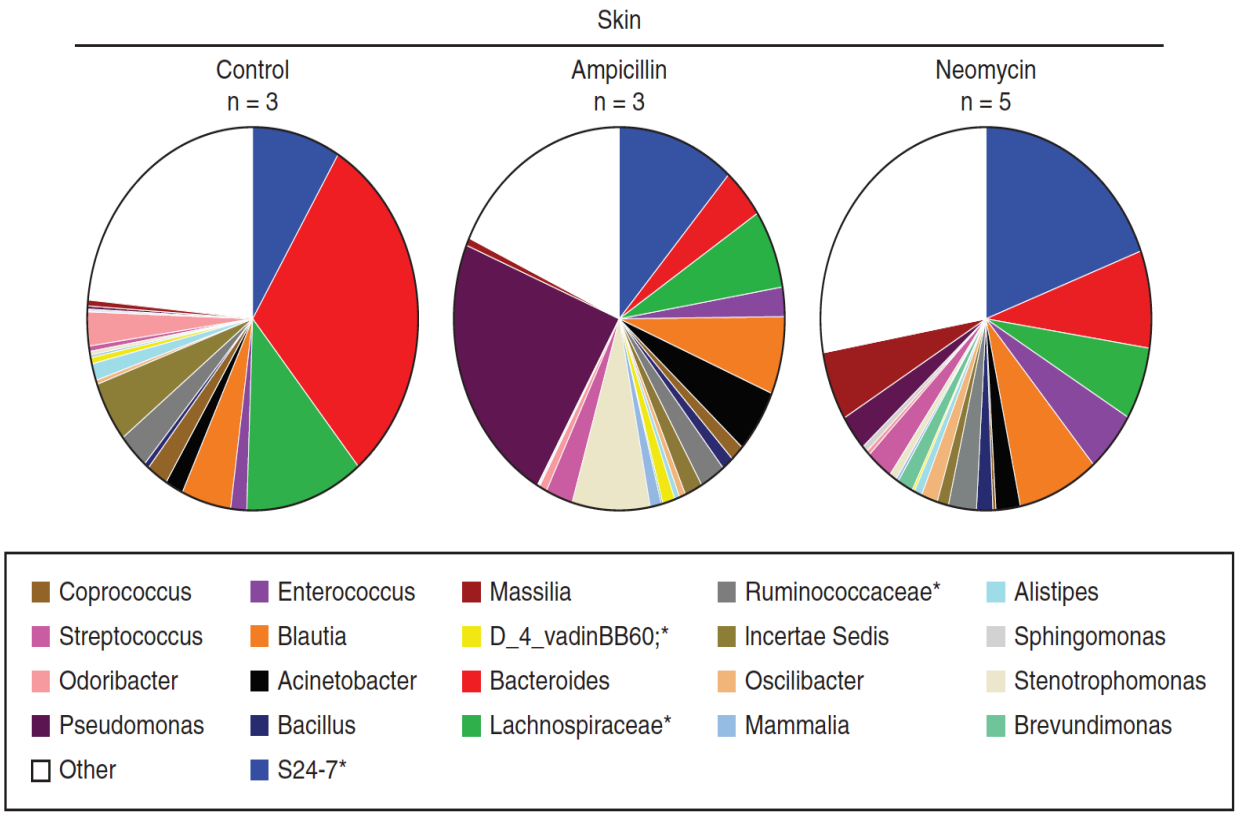


Figure 10 – The relative abundance of skin microbiome [74, p. 679]

Obtained results showed no significant, minimal differences among the skin resident bacteria among groups in the experiment, except the decrease of *Incertae Sedis* species (p=0.004) residing the skin of neomycin received group. This significant difference has not affected the depigmentation (Figure 11) indicating the absence of impact from this particular bacteria (Figure 12).

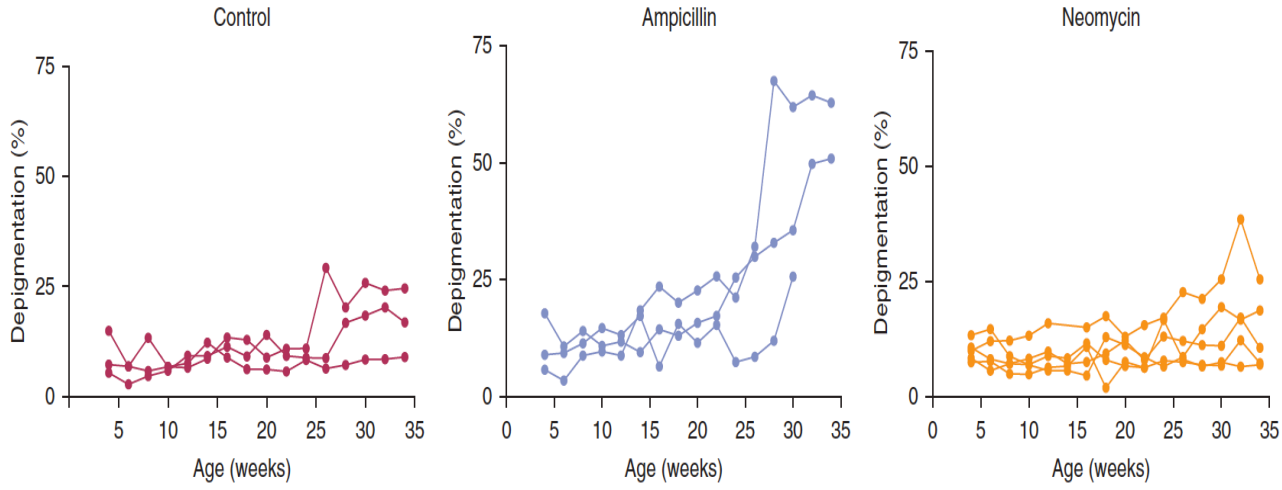


Figure 11 – Depigmentation graphs derived from scan images from mice from which skin tissues were used for 16S rRNA ribosomal sequence analysis [74, p. 687.e3]

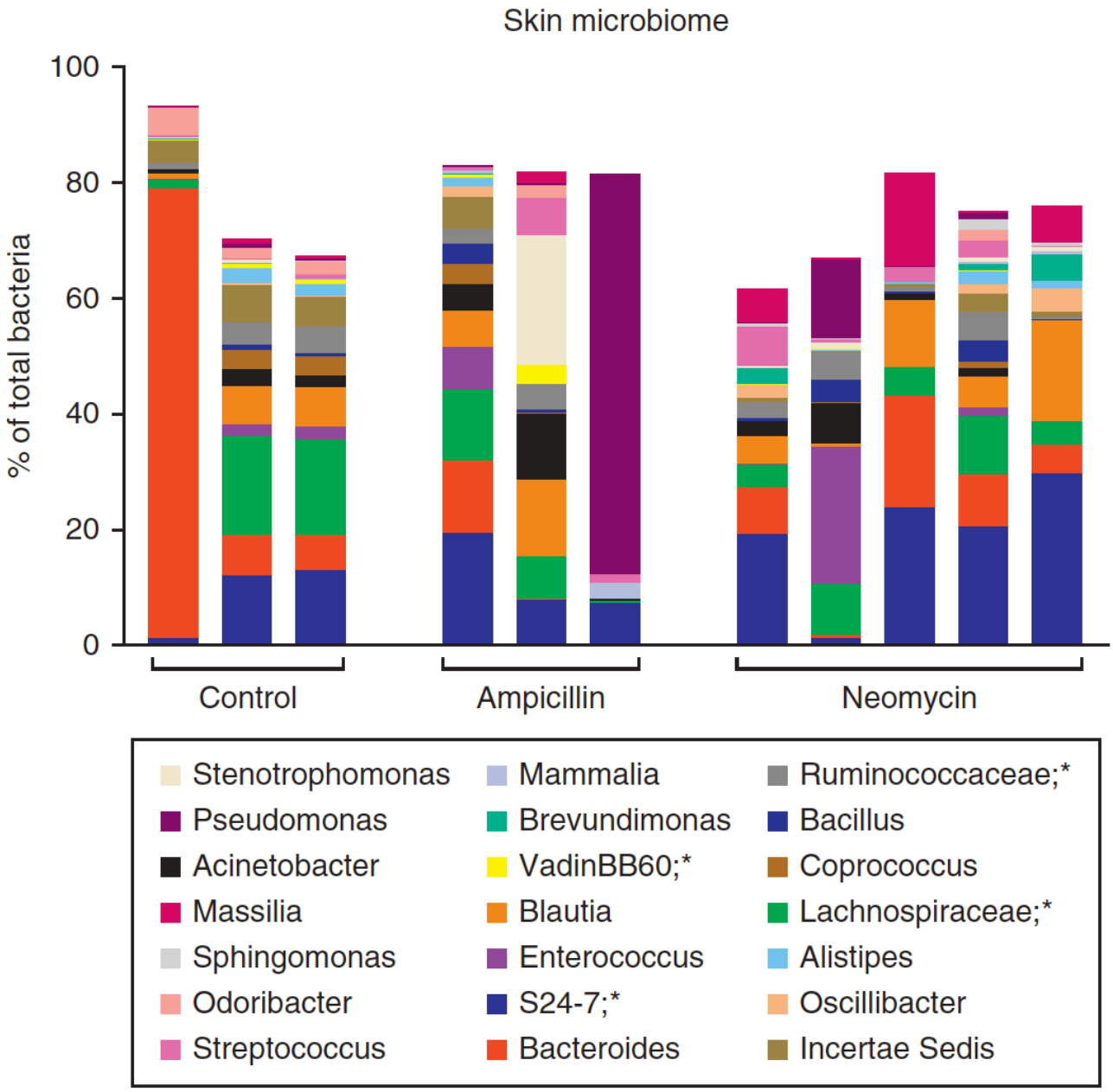


Figure 12 – Percentual variability of skin microbiome [74, p. 687.e4]

From abovementioned skin microbiome results, it can be concluded that antibiotics from drinking water had a minimal impact on the skin microbiome composition. Thus, this observation suggests that enhanced autoimmune response in the skin were due to the change of distant microbes, and their overall effect to the organism. Thus, the assessment of gut microbiome composition in correlation with depigmentation values was performed.

Next, the gut microbiome diversity was evaluated in mice treated with neomycin (n=8) and ampicillin (n=5) along with control (n=9) animals. First, total mass of bacterial DNA was assessed to see the capacity of different antibiotics to reduce the abundance of total bacteria in the gut, which is displayed in Figure 13.

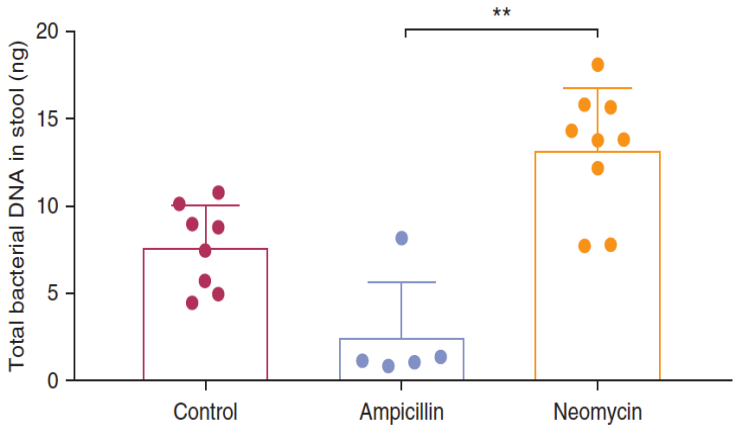


Figure 13 – Relative mass of total microbial DNA in the stool [74, p. 679]

The results from evaluating the total mass of microbial DNA in the gut showed that a significant decrease in DNA mass in ampicillin received mice group, whereas the DNA mass was increased in neomycin received group comparing to control animals. Excrements from neomycin received mice contained significantly more DNA than from ampicillin treated group (p=0.0002), suggesting that ampicillin greatly reduces the overall population of gut microbiome.

Neomycin- and ampicillin-treatment both affected gut microbiome decreasing the relative abundance of *Alkaliphilus* (p<0.0001), *Dysgonomonas* (p<0.0001), *Odoribacter* (p=0.0009), *Oscillospira* (p=0.006), *Pedobacter* (p<0.0001 ampicillin, and p=0.001 neomycin) and *Ruminococcus* (p=0.003) and *Parabacteroides* (p<0.0001 ampicillin (Figure 14).

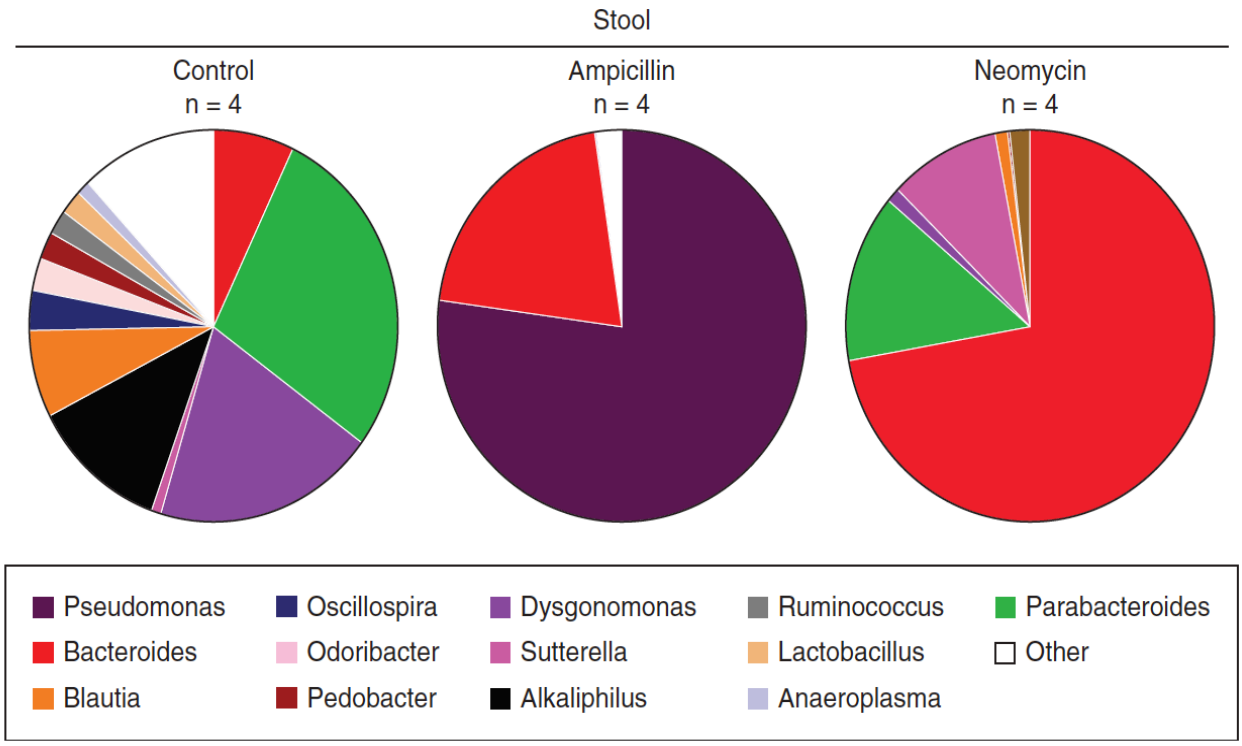


Figure 14 – The relative diversity of gut microbiome [74, p. 679]

Interestingly, the significant differences were noted in strains of *Sutterella* genus resulting in decrease after ampicillin-treatment and increase in the neomycin-received group (both p=0.0003). Ampicillin promoted the dominant inhabitation of *Pseudomonas* in gut microbiota, while neomycin affected the *Bacteroides* dominance (p<0.0001). In comparison to skin, the diversity of gut microbiome were consistent among groups (Figure 15).

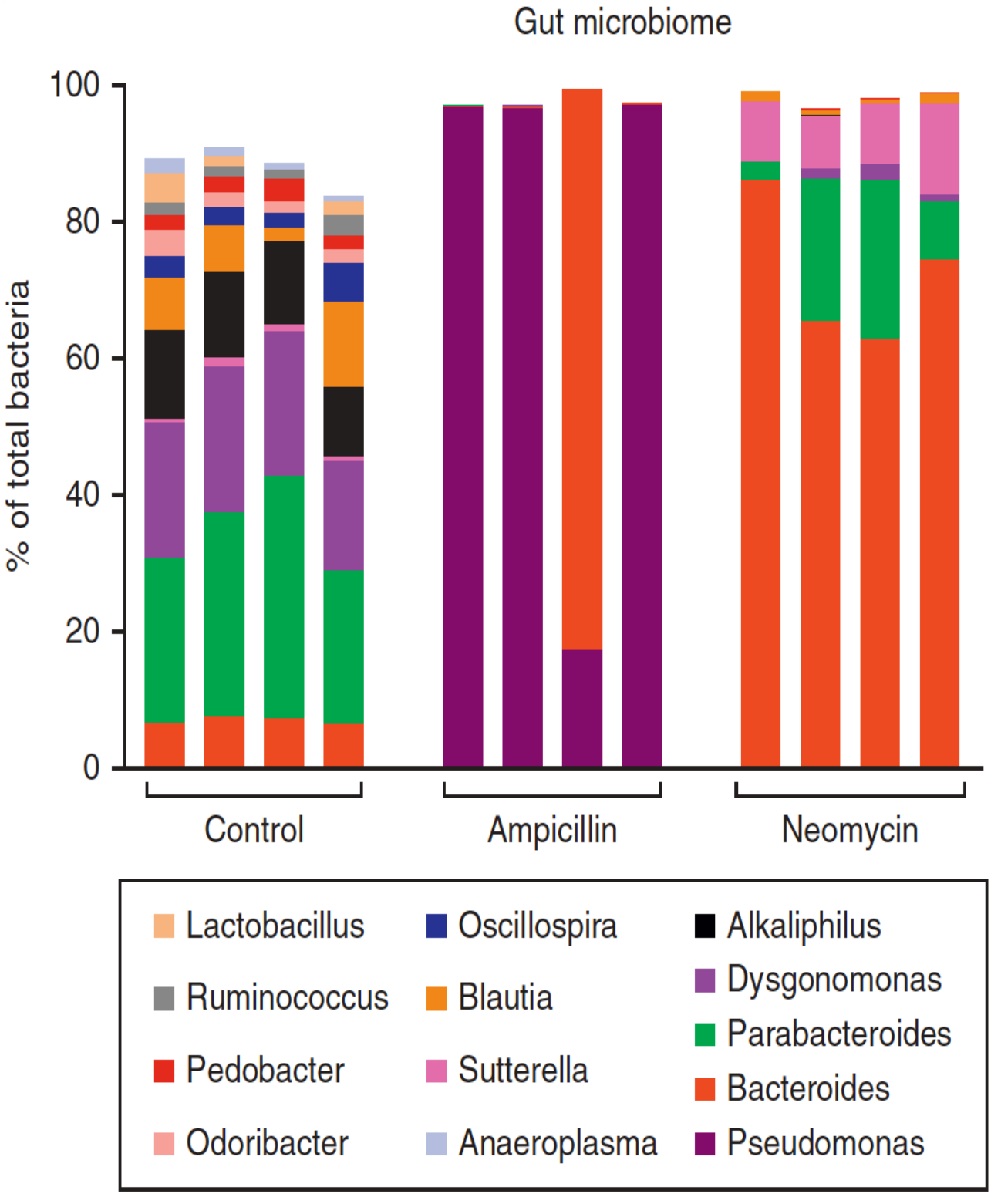


Figure 15 – Percentual variability of gut microbiome [74, p. 687.e4]

Importantly, species of *Pseudomonas aeruginosa* and *Pseudomonas tropicalis* *Pseudomonas* are predominantly observed in ampicillin, while *Bacteroides vulgatus* in neomycin treated groups (Figure 16).

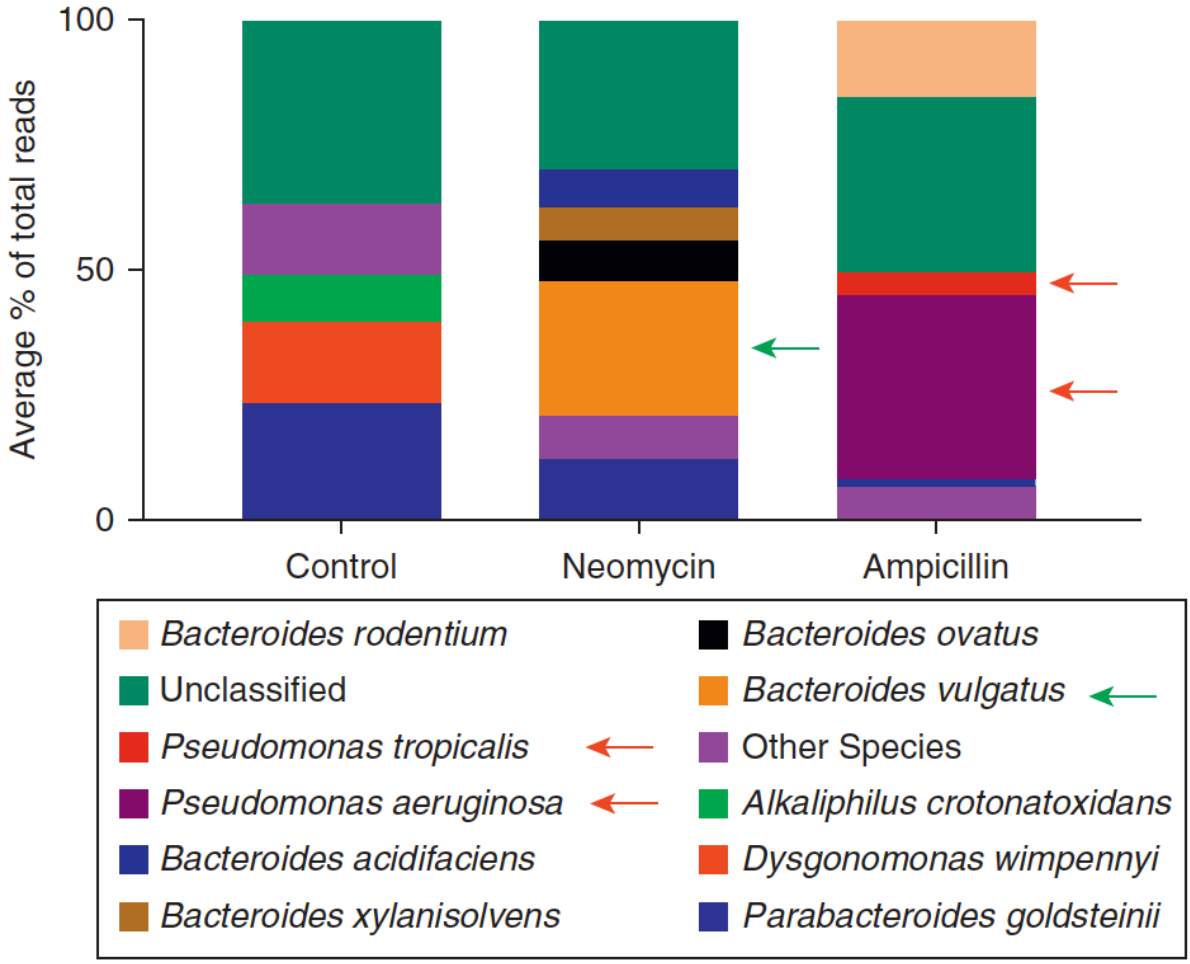


Figure 16 – Abundance of *Pseudomonas aeruginosa* and *Pseudomonas tropicalis* in ampicillin received group (red arrows), and *Bacteroides vulgatus* in neomycin treated groups (green arrows) [74, p. 687.e4]

3.2.3 Reduction in diversity of gut microbiome affect T cell distribution in vitiligo prone FH-A2D mice

The different sets of antibiotics can impact to the development of autoimmunity in various diseases. It was reported that neomycin can induce B cell response to autoimmune arthritis [161], while ampicillin promote cytotoxic T cell-mediated autoimmunity in vitiligo [8, p. 229]. These studies suggest that a particular antibiotic favors an individual subsets of immune cells, in particular lymphocytes. To understand the impact of ampicillin and neomycin, immunohistochemistry and immunofluorescence staining were performed to locate and quantify T cell distribution in the skin. The results displayed in the Figure 17 (A-C) shows ampicillin-mediated T cell decrease in the skin samples by 68% compared to both neomycin ($p=0.047$) and control group ($p=0.014$) quantified in the graph (Figure 17 G).

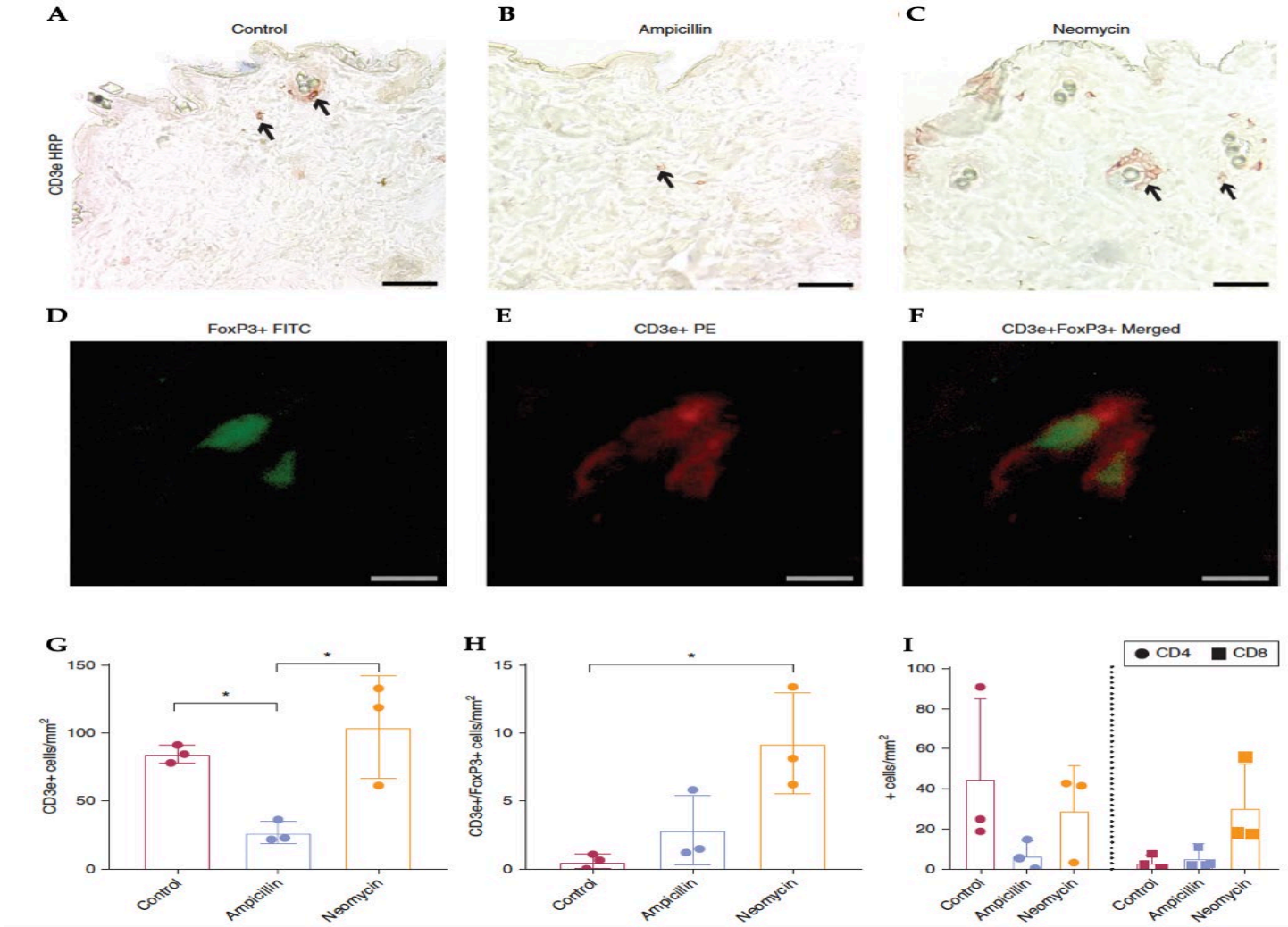


Figure 17 – Ampicillin mediates T-cell depletion from the skin [74, p. 68]

The Figure 18 shows the remaining population of T cells, which are infrequently distributed around not-melanized part of the hair follicles. Interestingly, the number of Tregs were significantly increased with 16-fold in neomycin-treated group skin (p=0.02) compared to control group suggesting the immune control that might prevent vitiligo (Figure 17 D-F). This Treg presence might explain the pigmentation in the hair of neomycin received mice group.

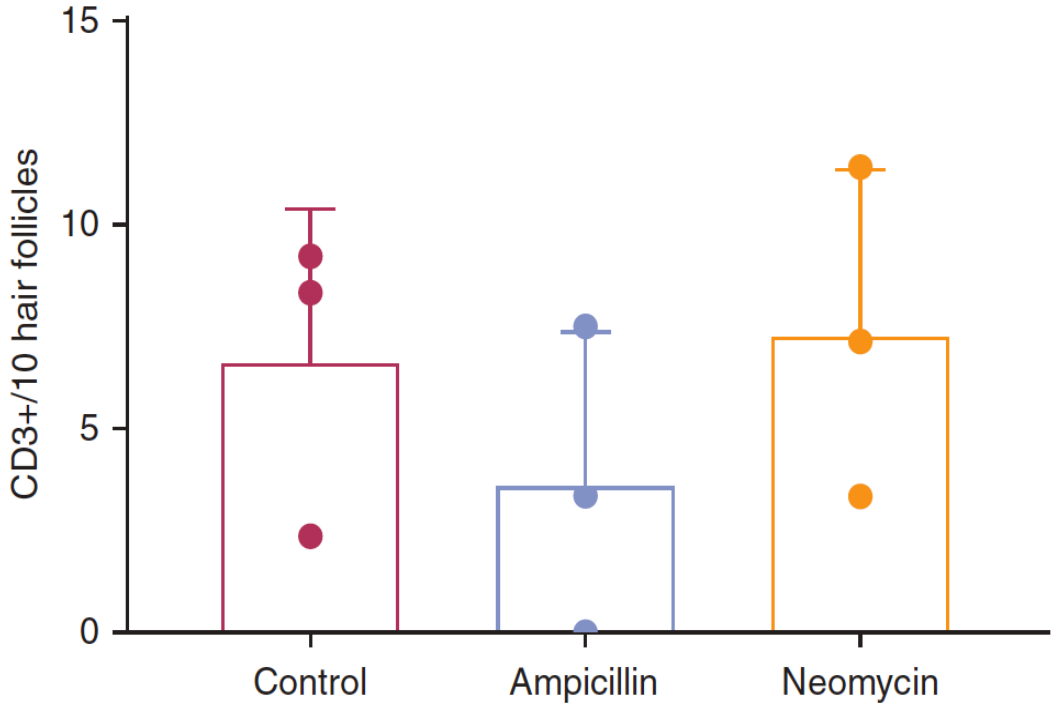


Figure 18 – Total T cell quantification in the hair follicles from mice skin included in the experiment [74, p. 687.e4]

The gut T cells were assessed through staining for microbial diversity using ileum sections, and the results demonstrated the quantifiable differences in numbers of T cells among groups (Figure 19). The quantification of CD4 T cells showed 51% reduction in neomycin (n=3) received group (P=0.04) and 83% decrease in ampicillin (n=3) received group (p=0.004) shown in Figure 20A. The distribution of CD4 T cells in control group are peripherally located within villi, whereas in ampicillin and especially neomycin group the CD4 T cells are situated in central in villi shown in Figure 20 (B-D).

The reduction in cytotoxic T cells infiltration are shown in Figure 20E, where 65% is mediated by neomycin (p=0.001), and 92% by ampicillin treatment (p=0.0002). Neomycin treatment impacted the distribution of CD4 T cells via making fewer contact with gut contents (Figure 20 F-H). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining confirmed that ampicillin did not affect the increase in apoptosis gut (Figure 21). Thus, the treatment including antibiotics affect the distribution and excess number of T cells in the gut. This suggests that microbial viability and mobility in the gut is influenced by antibiotics, which in response possibly relocate the sites of antigen expression that might trigger autoimmunity in vitiligo.

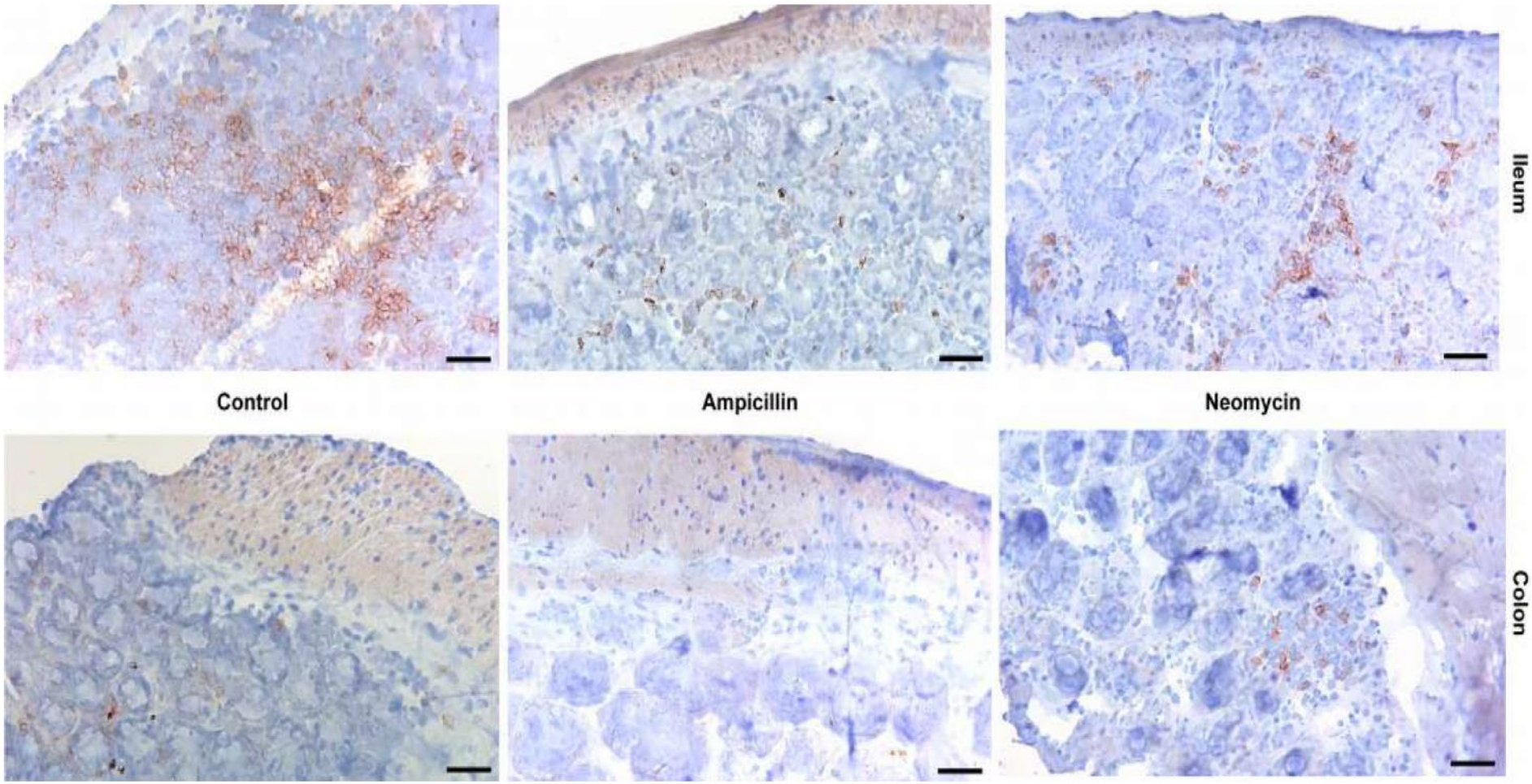


Figure 19 – T cell distribution in ileum from antibiotic treated mice [74, p. 687.e5]

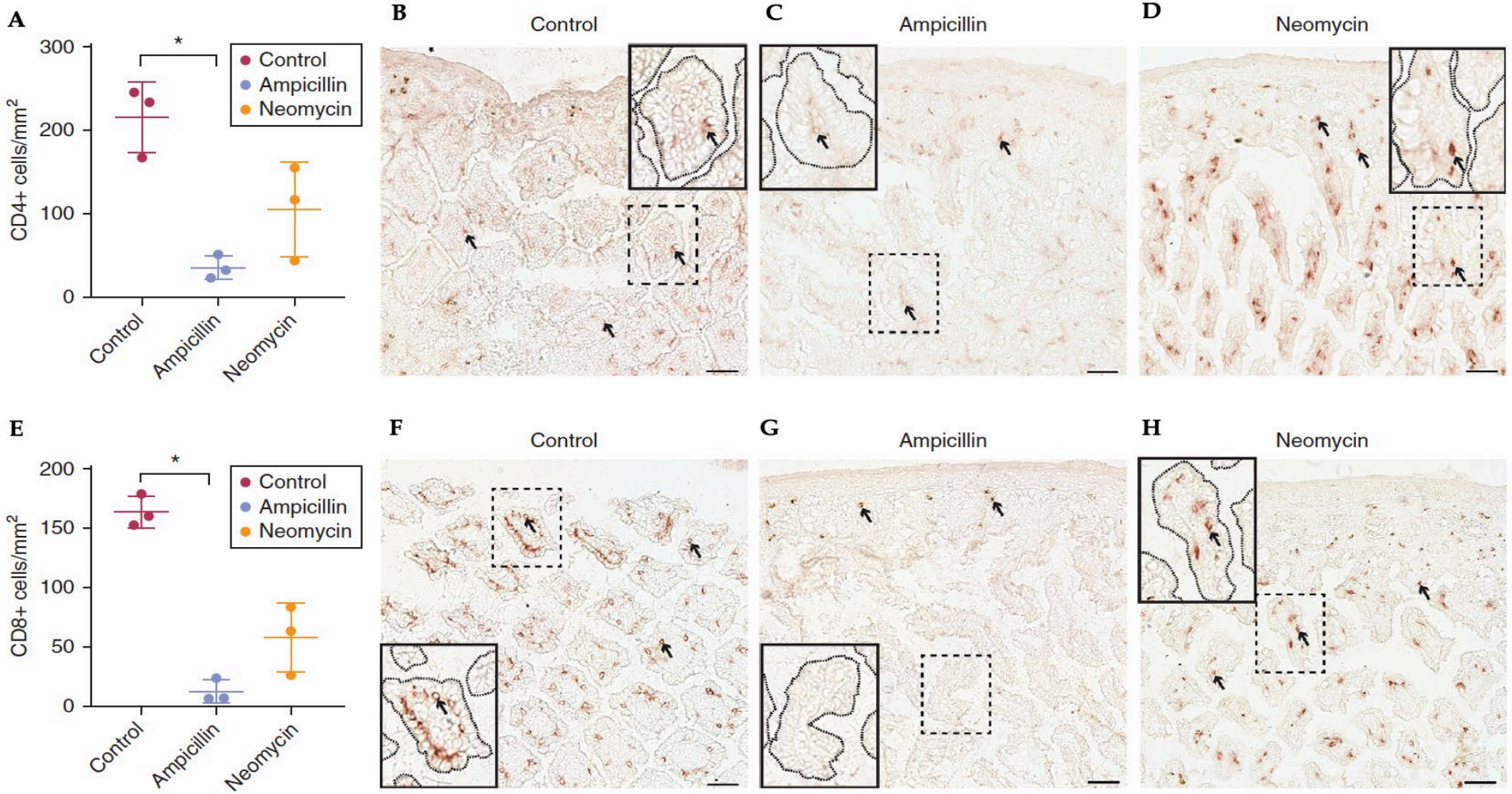


Figure 20 – T cell distribution in the gut from antibiotic treated mice redistribution [74, p. 681]

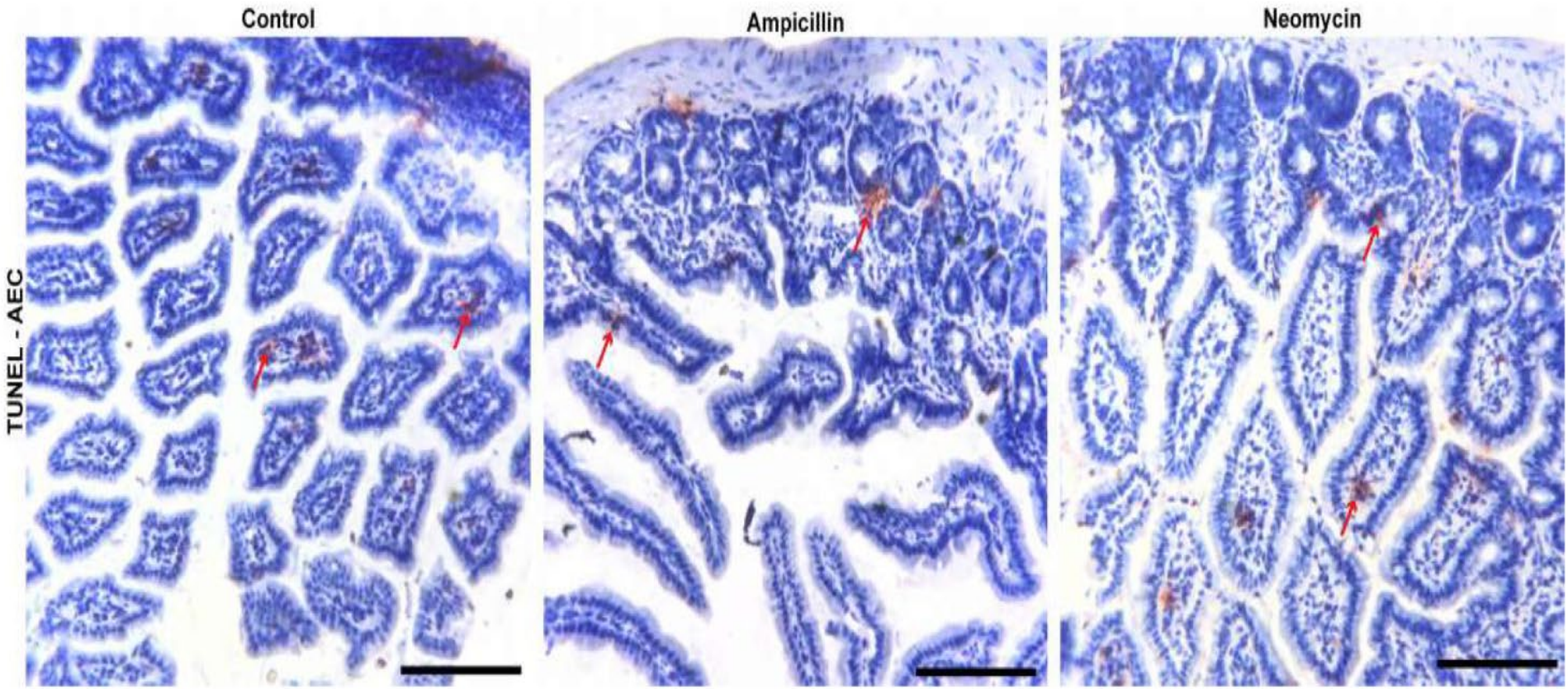


Figure 21 – Differences from T cell abundance in TUNEL samples [74, p. 687.e5]

3.2.4 Long-term ampicillin treatment impacts altered cytokine secretion

As previously mentioned, antibiotic intake impact vitiligo development, which might suggest that bacterial mediated signal to the distant tissues from gut chemokines such as CXCL10 promote cutaneous T cell migration and recruitment [162], affect melanocyte viability [120, p. 2178], and thus, influence depigmentation in vitiligo.

The results from ampicillin received mice showed an excessive expansion of digestive system shown in Figure 22A, which suggests the induction of abnormality in gut permeability. To evaluate the prolonged effect of antibiotics, the level of cytokine profiles were assessed in the serum of mice included in the experiment. Homogenates from gut were tested using microarray analysis for cytokines shown in Figure 22B.

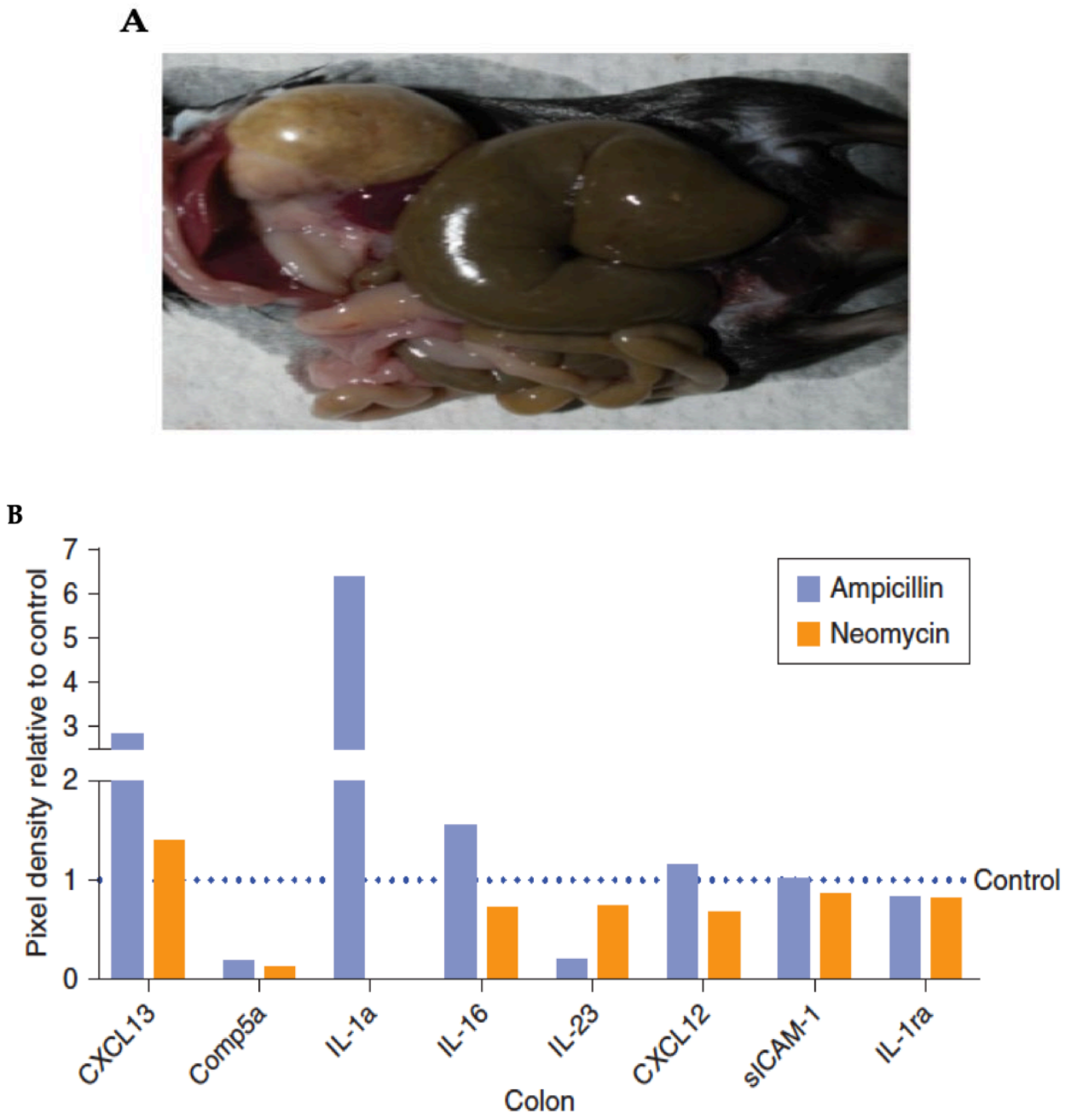


Figure 22 – Antibiotic intake impact vitiligo development via tissue damage and cytokine release in the gut [74, p. 682]

According to the results, the affect after antibiotic treatment, complement 5A were decreased by 82% for ampicillin received group and 88% for neomycin treated mice. In contrast, ampicillin only reduced IL-23 level, while selectively increasing CXCL13 by 2.8 fold. The significant difference was observed in IL- 1α, where the levels greatly increased by 6.3 fold in ampicillin received group, while this cytokine was completely absent in neomycin received mice. This numbers suggest the different response to the ampicillin and neomycin. To access the effect of long-term antibiotic treatment, pooled serum cytokines were measured to evaluate changes in systemic immune response (Figure 23).

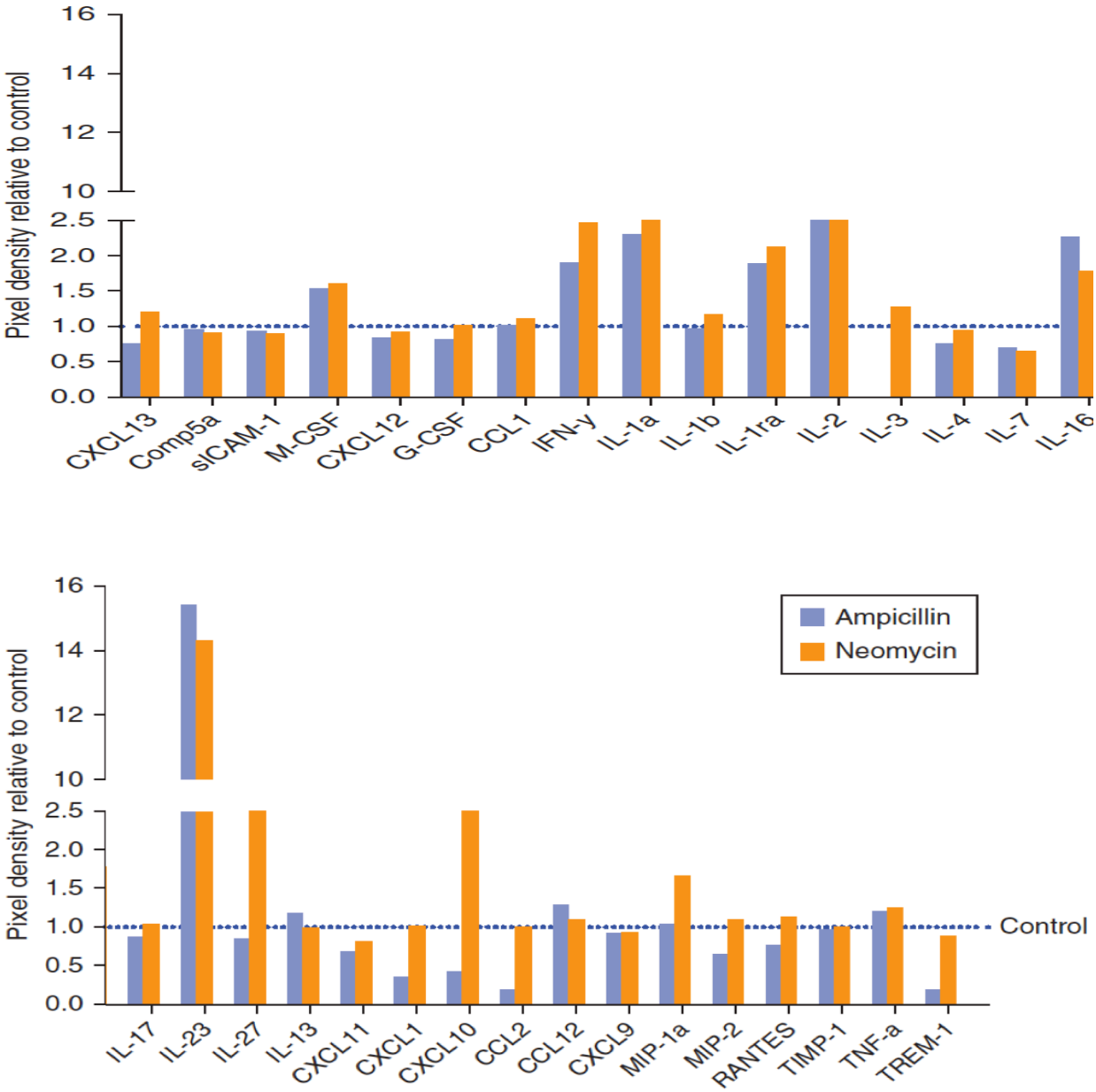


Figure 23 – Long-term effect of antibiotics to the cytokine profiles in serum [74, p. 682]

Overall, ampicillin and neomycin treatment had an association in over secretion of M-CSF, IFN-γ, IL-1α, IL-1ra, IL-16, and IL-2 with an increase by <2.5 fold, and a greatly increase of IL-23 by ~15-fold was also been observed. These results suggest

that antibiotic treatment enhance inflammatory cytokine release. Thus, the increased depigmentation can be explained by ampicillin driven cytokine release, which include abolished IL-3 production that might affect plasmacytoid dendritic cells (pDCs) activation. In addition, several molecules are reduced including neuroprotective CXCL1 (65%), Th2 cytokine CCL-2 (80%), innate pro-inflammatory TREM-1 (75%), and vitiligo biomarker CXCL-10 in ampicillin treated group. In contrast, CXCL-10 was increased by 2.5-fold when neomycin was used in the mice group. Therefore, the enhanced depigmentation can be associated with high inflammatory environment and with a decrease of type 2 cytokines.

As CXCL-10 levels were upregulated in serum of mice treated with neomycin while downregulated in ampicillin received mice. Despite the absence of correlation of CXCL-10 levels with depigmentation, this cytokine might relate with rerouting the persisting autoimmune response to other tissues than the skin.

3.2.5 Antibiotic treatment affects T cell distribution and functionality in vitiligo prone FH-A2D mice

As the functions of T cells has been linked to depigmentation in vitiligo, and altered cytokine profiles was noted, then next, the impact of antibiotics was assessed to the systemic T cell functionality. T cells and their functionality was evaluated using multiparameter FACS analysis shown in Figure 24. Splenocytes directly was analyzed, and the results showed in some samples representing up to 90% of $CD45^{+}$ positive T cells (Figure 24A).

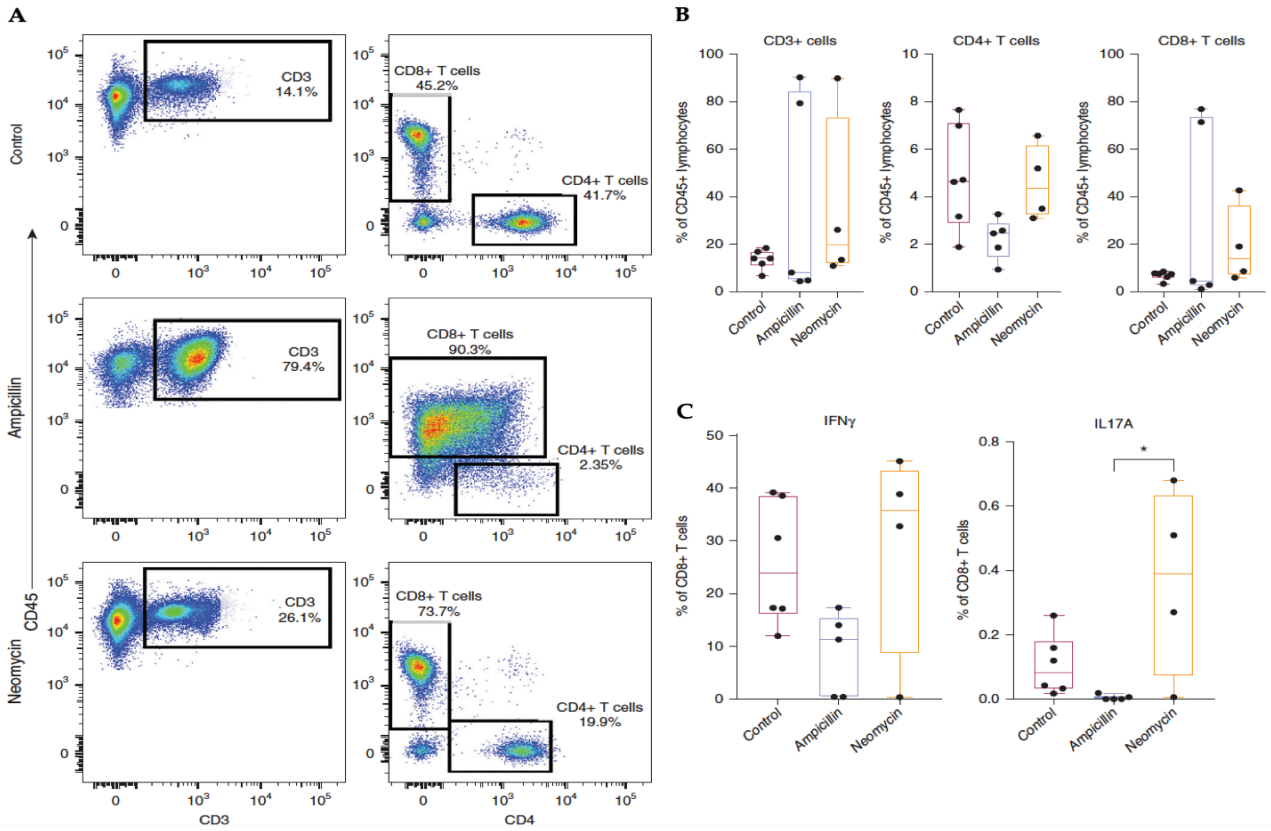


Figure 24 – T cell distribution was skewed by antibiotic treatment in experimental mice [74, p. 683]

This can explained by the chronic activation in those mice treated with ampicillin with elevated $CD8^+$ T cell numbers which co-expressed with $CD4^+$ [163]. In contrast, the percentage of $CD4^+$ is fewer in ampicillin treated group compared to other groups (Figure 24B).

In vitro nonspecific stimulation was performed to evaluate the activity of T cells. Results showed that ampicillin treated $CD8^+$ T cells were become refractory via producing 65% less IFN-γ-producing cells, which also accompanied by abolished of IL-17A-producing cells compared to neomycin received mice group (p=0.014) displayed in Figure 25. When anti-CD3 and anti-CD28 activator beads or murine tyrosinase 269-276 as an antigen were used for T cell stimulation, similar results were obtained. When assessing IFN-γ production among groups, neomycin elevated IFN γ levels, while minimal IFN-γ was observed in ampicillin treated group (p=0.004) and some expression in control mice (p=0.04), which was illustrated in Figure 25 (B,C). Similarly, IL-17 expression was enhance in neomycin group compared to ampicillin received group (p=0.004) shown in Figure 25C. For both IFN-γ and IL-17, the differences were smaller after 24 hours incubation. Thus, obtained results after ampicillin treatment indicate that impaired and exhausted T cells with elevated cytokine release correspond with enhanced depigmentation in FH-A2D experimental mice. The gating strategy for quality control of for FACS analysis is shown in Figure 26.

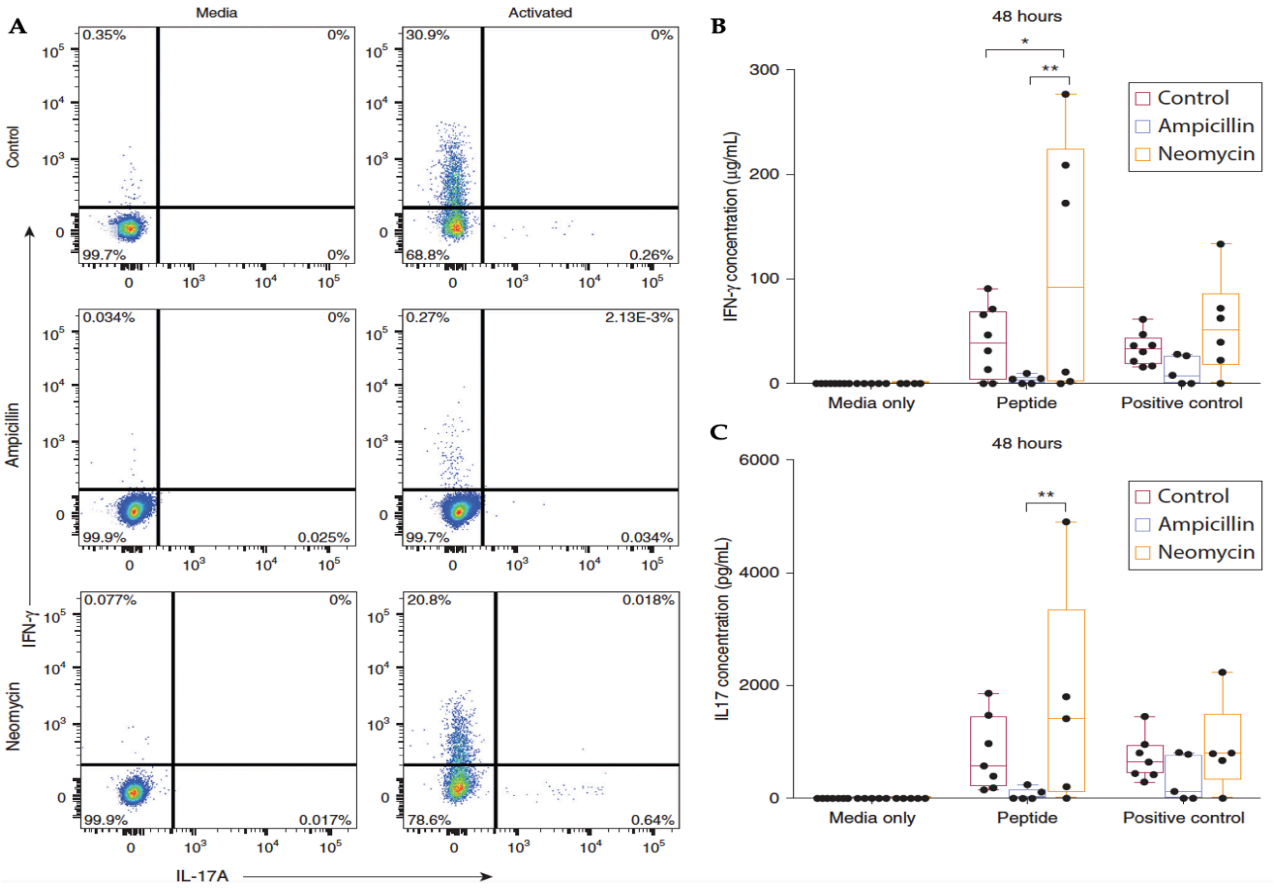


Figure 25 – T cell functionality was skewed by antibiotic treatment in experimental mice [74, p. 684]

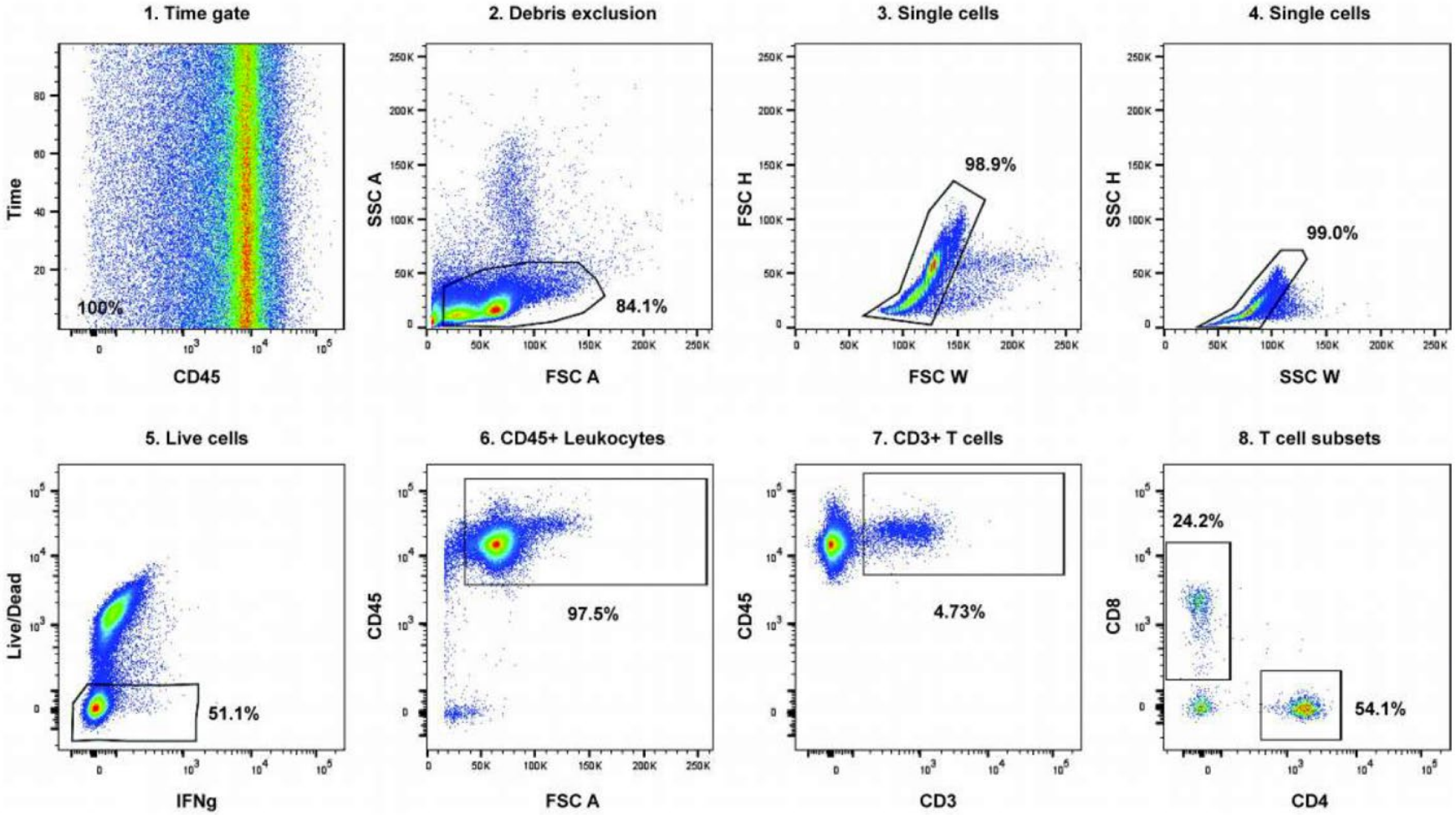


Figure 26 – Flow analysis of $CD4^+$ and $CD8^+$ T cell population from the splenocytes of antibiotic treated mice [74, p. 687.e6]

From these results described above, it can be proposed that ampicillin treatment can promote the growth of proinflammatory gut species and/or produce antigens, which can be delivered by antigen-presenting dendritic cells (DCs) to activate cytotoxic T cells towards melanocytes. Besides, the cytokine assay results from gut homogenates revealed the patterns of inflammatory cytokines that possibly help to activate antigen-presenting DCs and T cells to induce autoimmune activity [164].

Similar studies reported that antigen epitopes derived from bacterial shedding possibly identified as self-antigens due to the antigen homology homogenates possible [165]. In the development of lupus, antigens from (Ro60) commensal bacteria might elicit autoimmune and pathogenic response [39]. Importantly, vitiligo is only developed in those mice that carry tyrosinase-reactive transgenic T cell with haplotype of HLA A2D, suggesting that self-reactive T cells response is driven peptide HLA complexes. Meantime, ampicillin treated mice T cells become unresponsive, after melanocyte elimination, suggesting that these melanocyte-reactive T cells either become exhausted or underwent apoptosis after melanocyte elimination [166].

Lui *et al.* reported that gut microbiome promote and support the tolerogenic differentiation of T cells [167]. T cell redistribution occurred in the ileum of mice treated with neomycin, and increased Treg infiltration was observed in pre-vitiliginous skin suggesting Treg differentiation might be favored if neomycin used in the treatment. In addition, increased infiltration of Tregs accompanies by reduced abundance of skin resident *Incertae Sedis*. In the gut, mucosal-associated invariant T cells (MAIT cells) could be responsible explaining the connection between gut dysbiosis and autoimmunity, because these MAIT cells can react to bacterial metabolites while mounting both antimicrobial and effector T cell functions. This observations could suggest the development of several autoimmune conditions including multiple sclerosis [168].

In the course of study, the abundance of *Pseudomonas aeruginosa* was observed, and correlated with accelerated depigmentation in mice. Similar studies on ulcerative colitis showed the increased presence of *Pseudomonas aeruginosa* [169], suggesting that abundance of this strain might contribute to gut permeability after ampicillin treatment [170].

Overall, in this study, it was observed that *Bacteroides* species were associated with delayed autoimmunity in the mouse model after treating with neomycin treatment. It was previously reported that *Bacteroides* gut colonization can help prevent the development of inflammatory bowel disease [171]. The important dilemma exists relating that a diverse healthy gut microbiome can promote anti-tumor responses including melanoma. This is also true for vitiligo, and thus, suggests that an individual species might enhance the vitiligo development, while eliciting the activation of anti-melanoma T cells.

In summary, depigmentation in vitiligo is the result of melanocyte-reactive T cell mediated autoimmunity towards melanocytes that have been activated against self-antigens via some triggering factors. It was hypothesized that gut dysbiosis is one such trigger, and in this study, some evidence were provided that altered gut microbiota can drive or delay depigmentation in mice depending upon which species are present [74] p. 678].

3.3 Antigen-specific Tregs enhance immunosuppression in vitiligo prone mouse model

3.3.1 Proof of principle evaluation of suppressive capacity of TCR transgenic Tregs in h3TA2 mouse model

Previous studies demonstrated that adoptive transfer of polyclonal Tregs immunosuppress autoreactive cytotoxic T cells and prevent autoimmunity in vitiligo mouse model. However, these polyclonal Tregs might invoke non-specific immunosuppression, and using antigen-specific Tregs can improve suppressive ability through targeted delivery of cells. The assessment of antigen-specific immunosuppression requires either TCR based or CAR based Tregs. First, it was attempted to evaluate TCR transgenic Tregs for their capacity to suppress ongoing autoimmunity towards melanocytes in the vitiligo prone, h3TA2 mouse model that spontaneously depigment over time. This concept was derived from the study by Bluestone group, in which, TCR transgenic Tregs with artificially inserted epitope, reactive to human tyrosinase were tested in a tumor model [132, p. e11726]. The results from this study showed that these tyrosinase reactive TCR transgenic Tregs (h3T-Tregs) facilitated the tumor growth through the immune suppression in the mice with injected tumors. With this in mind, we attempted to test these h3T-Tregs in preventing the autoimmune depigmentation in vitiligo mouse model.

h3T-Tregs can be readily isolated from transgenic mouse model specifically designed to carry tyrosinase reactive TCR. However, tracing Tregs *in vivo* is important to monitor their activity, and to obtain this feature, FoxP3 eGFP reporter mouse was used as their FoxP3 expression directly correlates with eGFP expression. Thus, h3T mice was crossed with Foxp3 eGFP (C57BL/6 background) mice to obtain tracible antigen-specific h3T-Tregs for adoptive-transfer to h3TA2 recipient mice. As a candidate mice to generate *in-vivo* traceable Tregs, h3T homozygous ♂ (25 weeks old) crossed with FoxP3 homozygous ♀ (7 weeks old) mouse. Their offspring carried one copy of h3T and one copy of FoxP3 linked with eGFP reporter, and these heterozygous (F1) mice were served as donors for traceable h3T-Tregs isolation. The hypothesis behind the reason to obtain this h3T positive with FoxP3 eGFP was that this genotype has number of Tregs as in C57BL/6 background. The donor mice with desired genotype were serotyped using cheek bleed for flow cytometry, and the results are shown as in Figure 27 and Figure 28. According to the results, four different of genotype sets were among the F1 offspring: h3T positive, FoxP3 eGFP positive, HLA-A2 negative (desired genotype); h3T negative, FoxP3 eGFP positive, HLA-A2 negative; h3T negative, FoxP3 eGFP positive, HLA-A2 positive; h3T negative, FoxP3 eGFP positive, HLA-A2 negative; and h3T positive, FoxP3 eGFP positive, HLA-A2 positive. Other unused mice different genotypes were either used for breeding to generate mice for different experiments or humanely euthanized according to IACUC regulations.

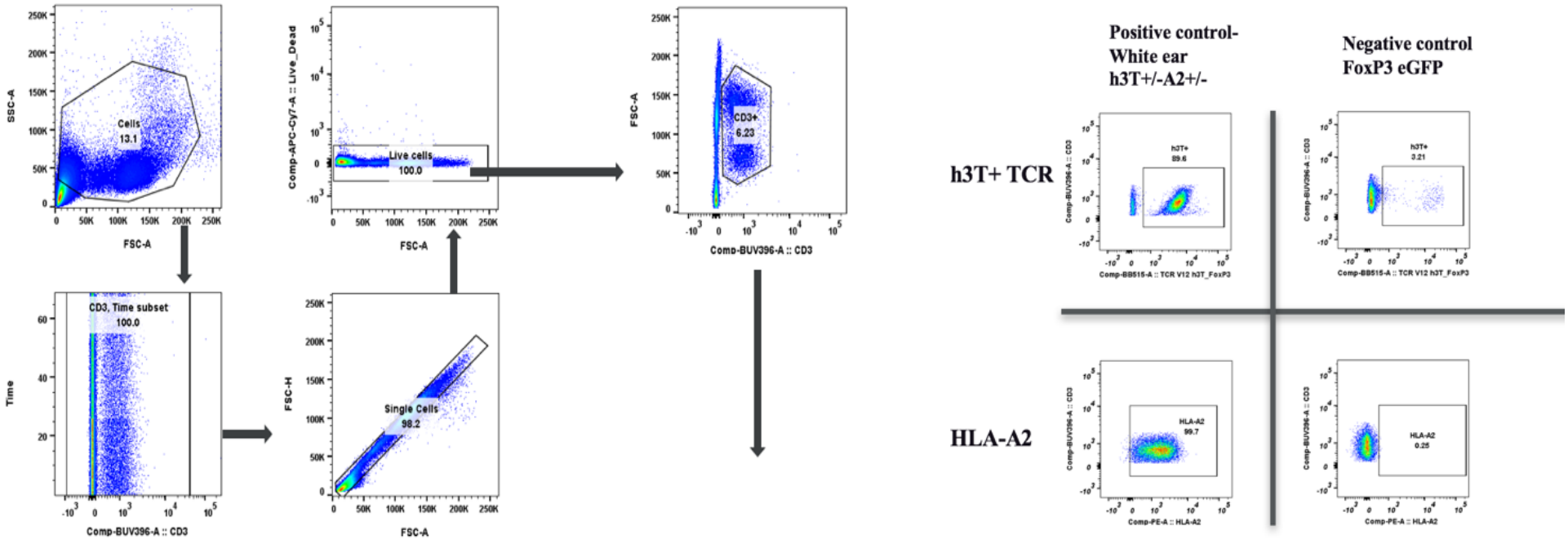


Figure 27 – Gating strategy for CD3^{+} T cells derived from mouse blood from cheek bleeds and with h3T TCR and FoxP3 eGFP without HLA-A2 haplotype are shown in the flow images

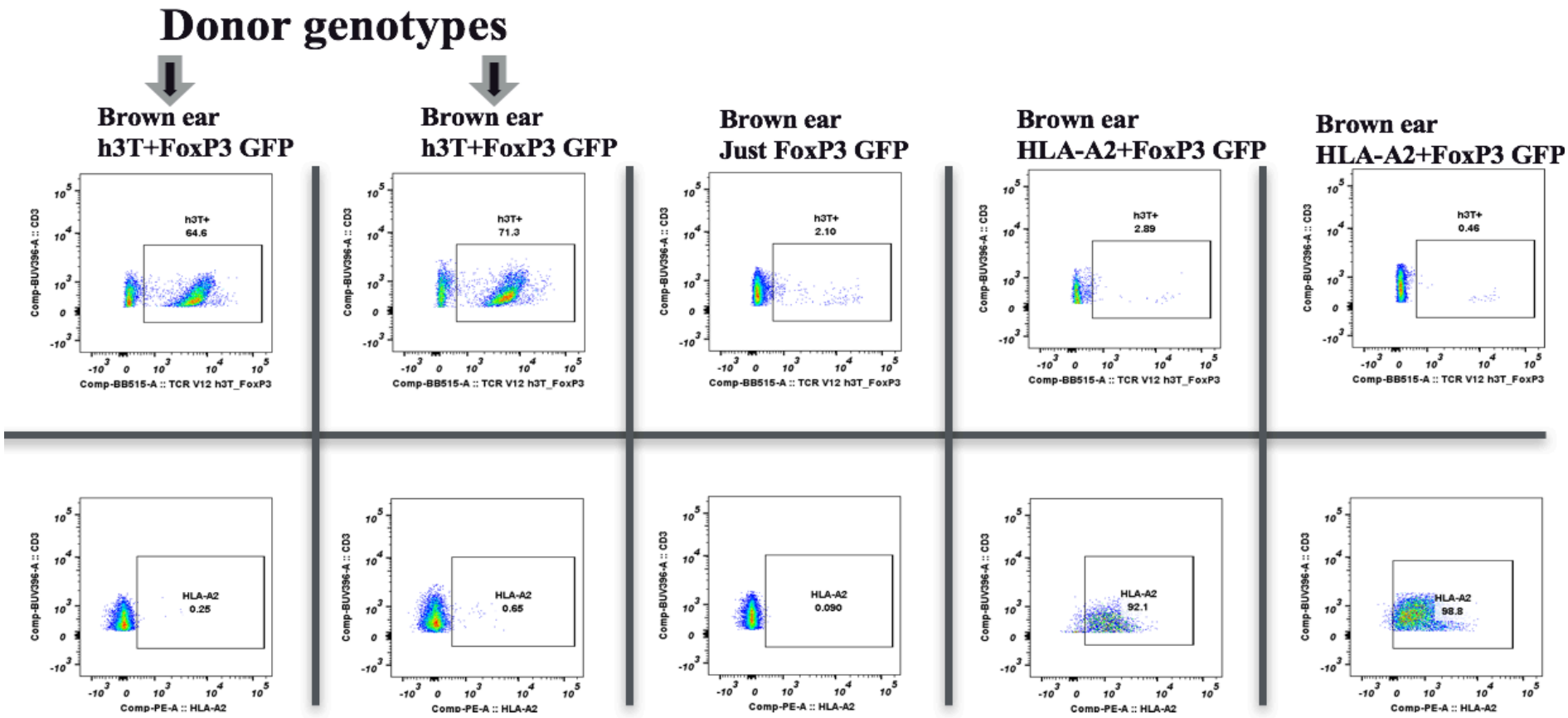


Figure 28 – Serotyping mice for identification of donors with desired genotypes which have to have brown ears and tails. There are four possible genotypes: h3T positive FoxP3 eGFP positive HLA-A2 negative (desired genotype); h3T negative FoxP3 eGFP positive HLA-A2 negative; h3T negative FoxP3 eGFP positive HLA-A2 positive; h3T negative FoxP3 eGFP positive HLA-A2 negative; h3T positive FoxP3 eGFP positive HLA-A2 positive

Donor mice with tracible h3T-Tregs with FoxP3 eGFP marker were maintained till the age of 8 weeks before they were sacrificed to obtain sufficient number of h3T-Tregs for adoptive transfer. Along with these donor mice for antigen-specific h3T-Tregs, FoxP3 eGFP only mice were used to isolate polyclonal Tregs as a control to access the efficiency of antigen-specific Tregs over polyclonal Tregs.

The experimental outline for *in vivo* evaluation of h3T-FoxP3 Tregs are shown in Figure 29. The recipient h3TA2 mice were administered antigen-specific h3T-FoxP3 Tregs (n=5), polyclonal FoxP3 eGFP Tregs (n=5), and untreated (n=5) starting at 9-week age biweekly two rounds of retro-orbital injection. This study was performed after our group with collaborators reported the results where a single injection of polyclonal Tregs at age of 3-week delayed the depigmentation in h3TA2 mice [127, p. 1288] . However, vitiligo was only halted till week-9 due to the aggressive nature of depigmentation in this mouse model. This can be explained by the efficacy of polyclonal Tregs to delay the depigmentation up to the point before the disease begins its aggressive stage between weeks 9 till the week 15. Therefore, the experiment was outlined starting at week-9 with in mind of enhanced protective capacity of antigen-specific Tregs from autoimmunity in this vitiligo prone mouse model. In the course of experiment, h3TA2 mice received 2x10^5 of either antigen-specific h3T-Tregs or polyclonal Tregs for two-rounds. Following the treatment, IL-2 injections (3000 IU) were accompanied intraperitoneally to promote Treg activity and persistence *in vivo*.

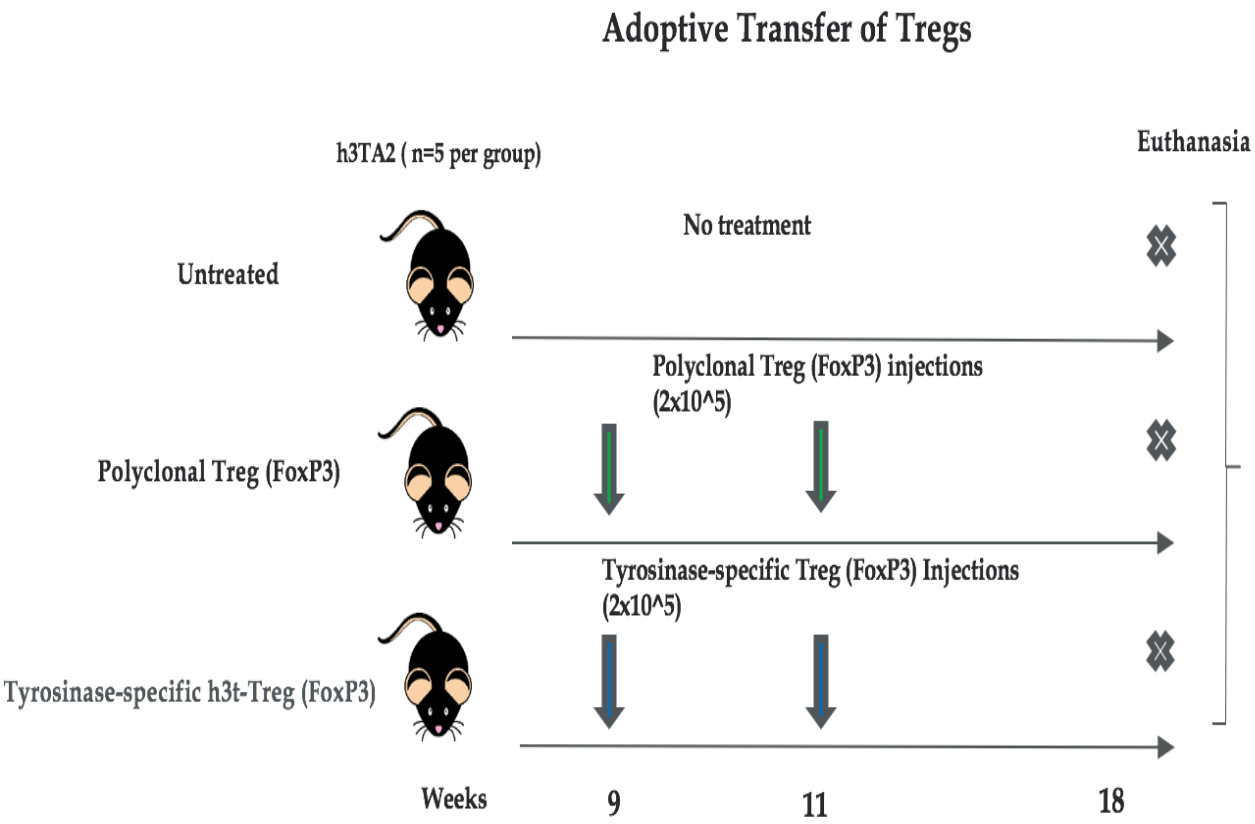


Figure 29 – Experimental outline of adoptive transfer of antigen-specific h3T-Treg transfer to vitiligo prone h3TA2 mouse model. Recipient h3TA mice were administered retro-orbitally h3T-Tregs (n=5), polyclonal Tregs (n=5) and untreated (n=5) starting at age week-9 biweekly till week 11. Mice received two-rounds of retro-orbital injections of 2x10^6 of either h3T-Tregs or polyclonal Tregs

The recipient mice were scanned weekly for depigmentation on a dorsal and ventral side using flat-bed scanner under isoflurane anesthesia. The obtained images were analyzed, and results were shown for the dorsal side of the mouse in Figure 30. The fold change in depigmentation over time has been calculated and depicted in the graph. Due to the slower depigmentation rate of dorsal side, no significant increase was observed from week-9 till week-12. Following this period, depigmentation incrementally from week-13 till week-17, and escalated in the last weeks of the experiment.

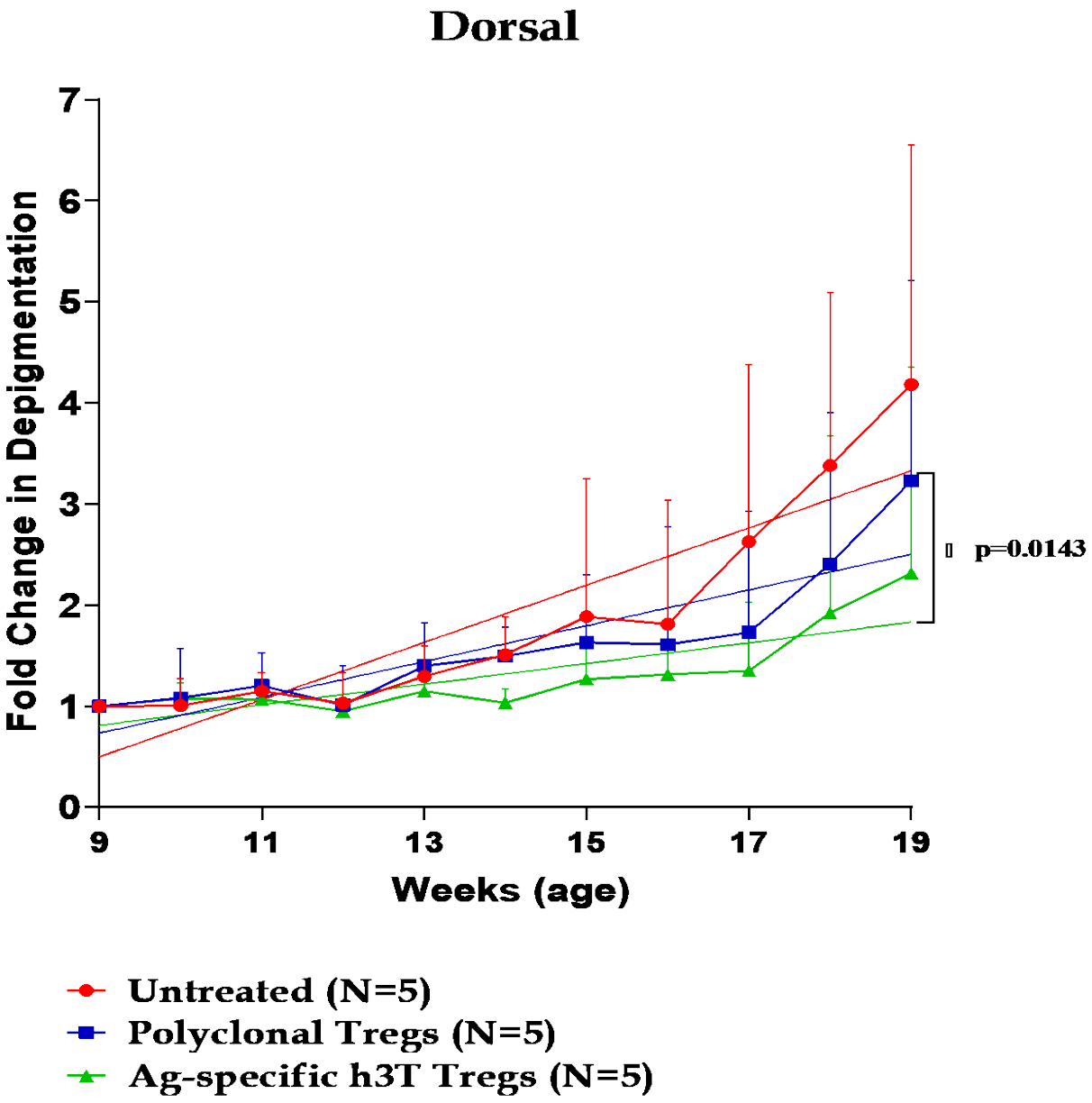


Figure 30 – Dorsal depigmentation measurements for after adoptive transfer of h3T-Tregs and polyclonal Tregs along with untreated control mice

No significant delay in depigmentation was observed between polyclonal Tregs and untreated groups, whereas antigen-specific h3T-Tregs significantly halt depigmentation compered to untreated group (p=0.0143).

In contrast to dorsal side, ventral depigmentation was not provided significance among groups. Similar trend was noticed between h3T-Treg and polyclonal Treg treated group with a delay in depigmentation in the first four weeks of the treatment compared to the control group (Figure 31).

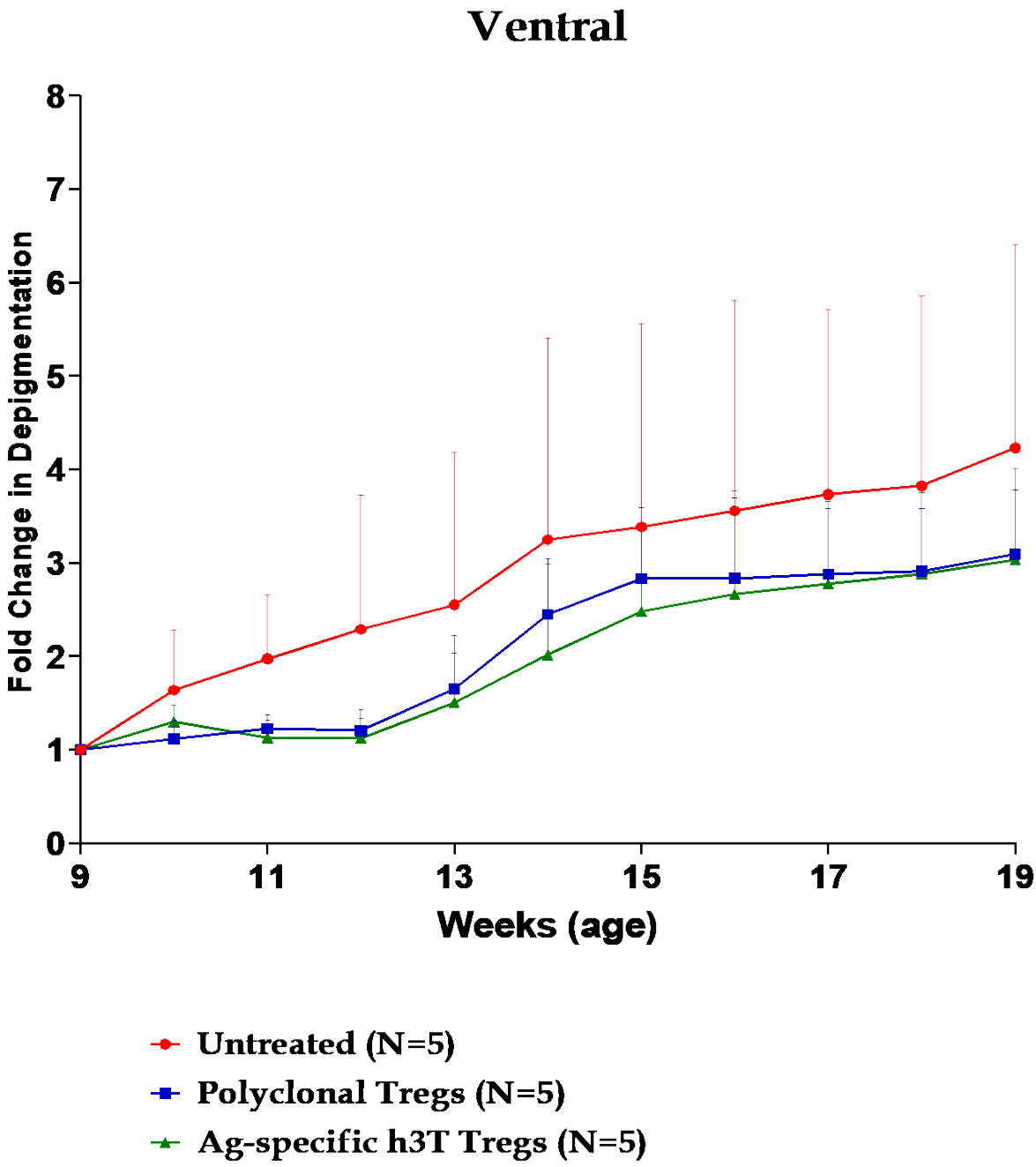


Figure 31 – Ventral depigmentation measurements for after adoptive transfer of h3T-Tregs and polyclonal Tregs along with untreated control mice

The representative scan images for antigen-specific h3T-Treg and polyclonal Tregs along with untreated groups are shown in the Figure 32 for dorsal side and Figure 33 for ventral side, respectively. Four sets of time period scan images were represented from week 9, 12, 15, and 18.

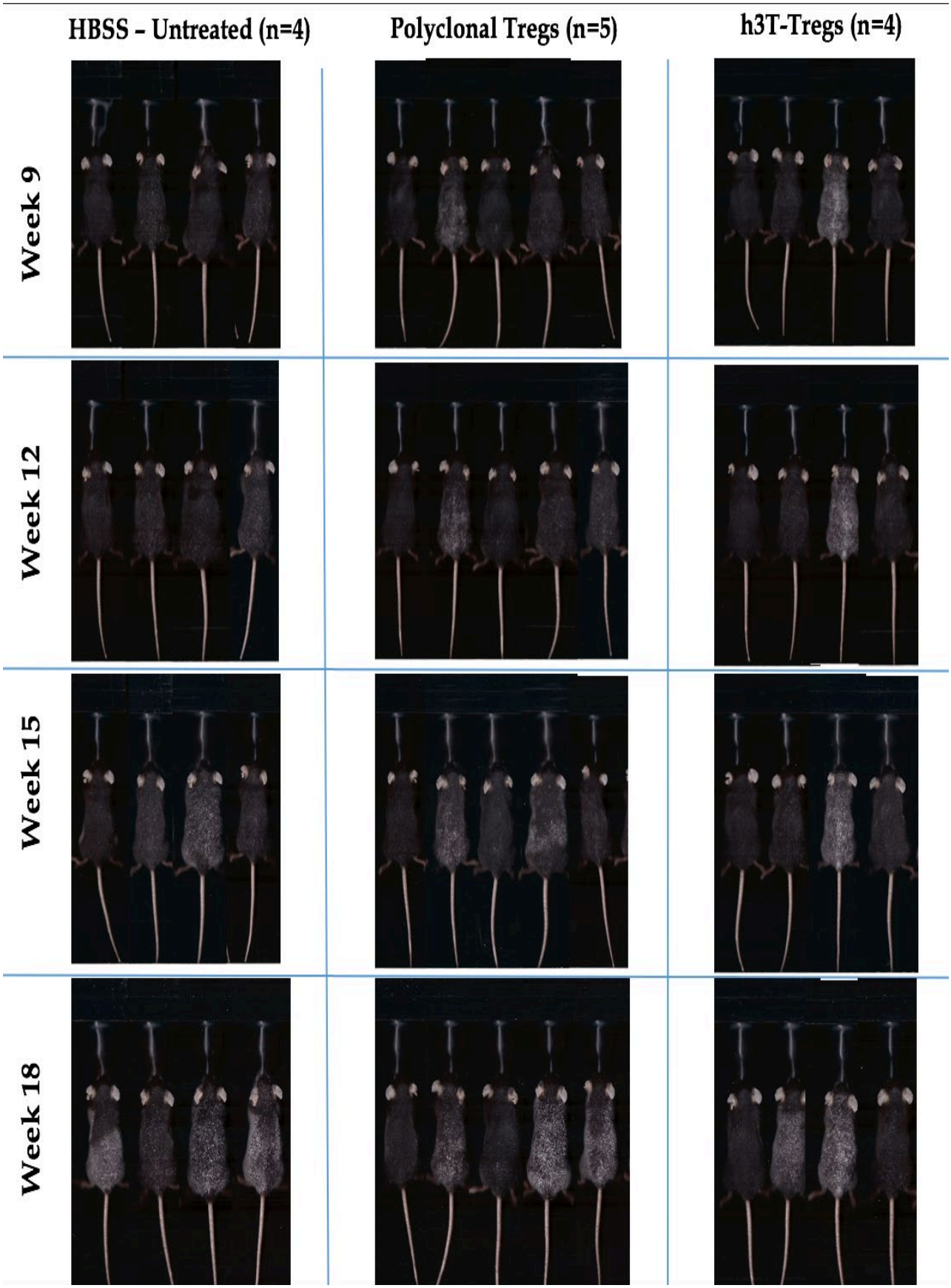


Figure 32 – Images from dorsal part of the mice scanned using from a flat-bed scanning

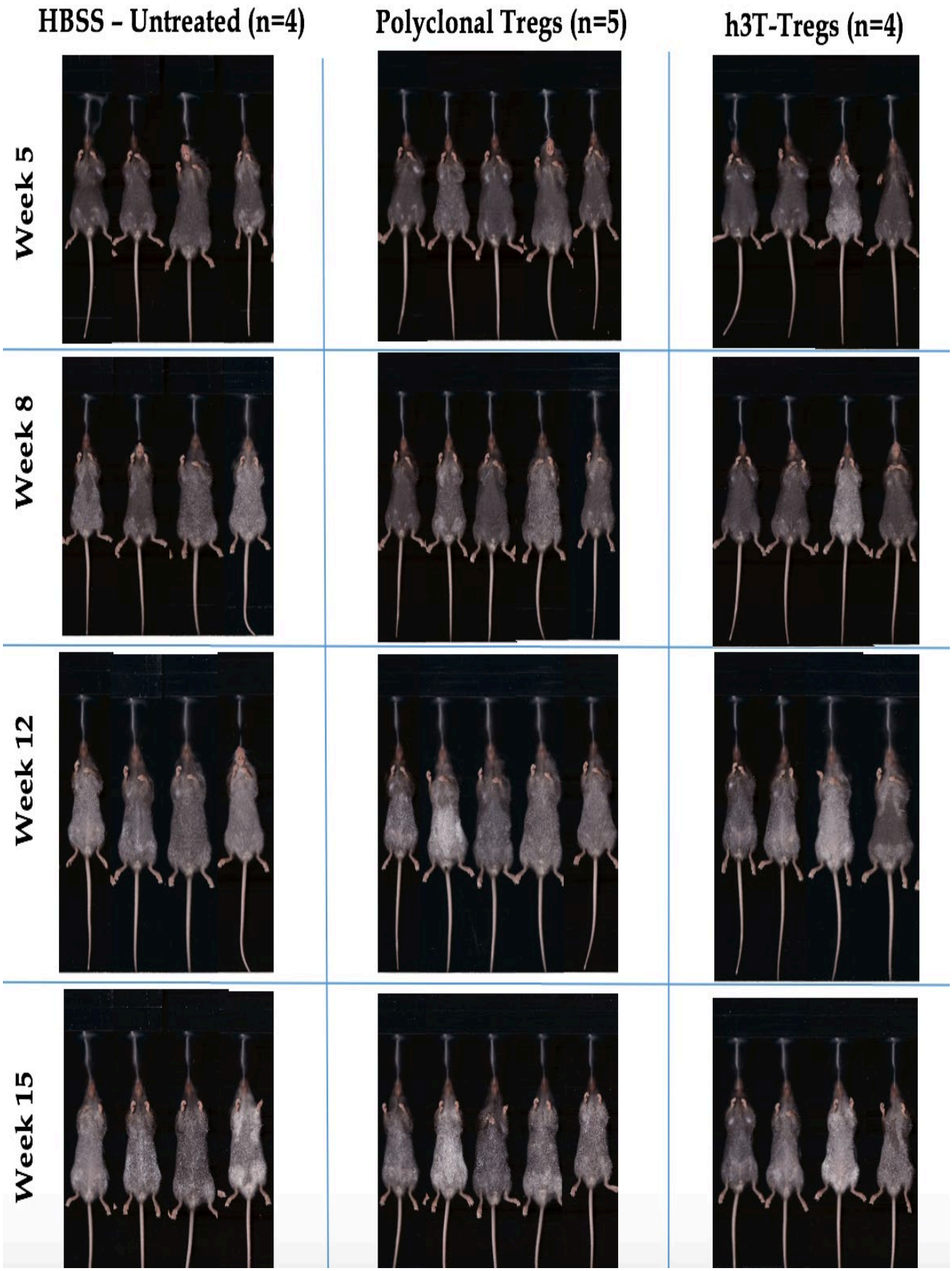


Figure 33 – Images from ventral part of the mice scanned using from a flat-bed scanning

Although engineered TCR transgenic Tregs can be generated using viral constructs with high efficiency, these TCR-Tregs are limited by MHC-restriction, not allowing all patients to benefit from the same therapy [100, p. 346]. Tregs transduced with genes encoding CAR will overcome this MHC-dependency. Instead of employing a (lower affinity) TCR, these CAR constructs are instead made of a single-chain variable fragment, scFv - the binding portion of a monoclonal antibody, followed by an extracellular hinge, a transmembrane region, and intracellular signaling domains. Resulting CAR-Tregs can be obtained by fusing the variable domains of an antibody to the signaling portion of a TCR, enforced with domains from costimulatory molecules to further enhance T cell function. In CAR-T cell therapy, optimizing and selecting the correct CAR affinity and intracellular signaling domains is crucial for the resulting therapeutic activity and cellular persistence of the regulatory T cells. Since CARs are constructed using antibody variable regions, they hold higher affinity to their cognate antigen compared to TCRs. Therefore, using CAR based Tregs over TCR transgenic Tregs are favored due to the MHC-independency and a higher affinity to a cognate antigen. After the evaluation of advantages and drawbacks of both TCR- and CAR-based methods of generating antigen-specific Tregs, CAR-based Tregs were chosen due to abovementioned properties of this technology.

3.3.2 Evaluation of suppressive capacity of antigen-specific CAR Tregs in h3TA2 mouse model

The generation of antigen-specific CAR Tregs for vitiligo firstly required to identify a target antigen of this skin condition. After the identification of the target antigen, the sufficient number of Tregs for adoptive transfer has to be generated and expanded *ex-vivo* due to the low percentage of Tregs among total T cell population. Subsequently, antigen encoding CAR construct is necessary to generate virus for transduction of Tregs to obtain antigen-specific Tregs. These transduced antigen-specific CAR Tregs, then, can be tested *in vitro* and *in vivo* for immunosuppressive capacity to prevent autoimmunity towards melanocytes. The descriptive details and results of each steps of antigen-specific CAR Treg preparation and their suppressive activity are described in this part of the dissertation.

3.3.2.1 Ganglioside D3 (GD3) is a target antigen, expressed on perilesional epithelial cells including stressed melanocytes in vitiligo, both in human/mouse skin

A surface expression of GD3 was first reported as melanoma associated antigen, and this molecule was also expressed in human melanocytes. Otake *et al.* with colleagues reported that increased GD3 expression is associated with enhanced melanocyte migration [172, 173]. In vitiligo, the overexpression of O-acetylated GD3 is reported in the skin with disease progression [108; 174, p. 742]. This provide a prompt to assess the GD3 expression in perilesional skin of the vitiligo skin. In Figure 34A, the marked expression of GD3 was detected in human skin derived from perilesional skin of vitiligo. No presence of melanocytes were observed from the border of the skin biopsy section, shown in Figure 34B. Epidermal GD3 expression was not detected in the skin from healthy control skin (Figure 34C), while melanocytes are readily observed in healthy skin (Figure 34D). Similarly, when mouse skin of

h3TA2 mice was evaluated, GD3 expression was also detected (Figure 34E). This detection of GD3 in skin, and particularly in melanocytes prompted us to target this antigen with a hypothesis to test using the concept of CAR T cell therapy. In the case of autoimmune conditions, Tregs are known to suppress autoimmune response and assessment of antigen-specific Tregs to augment prolonged effect of the treatment for vitiligo [140, p. 5].

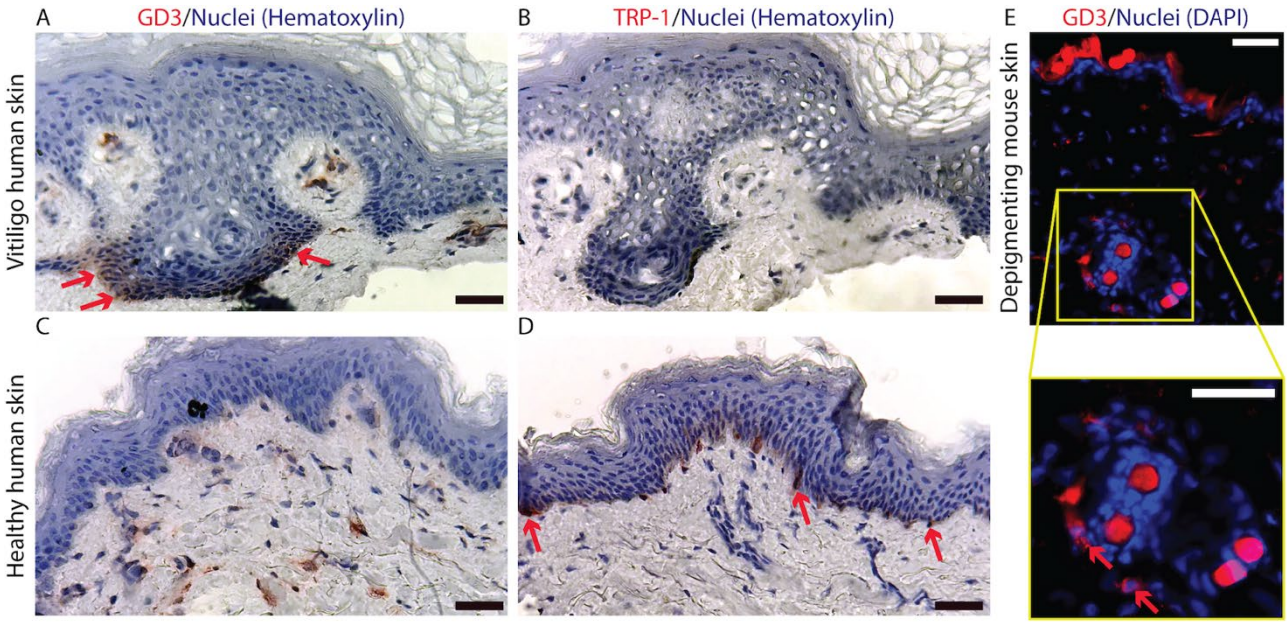


Figure 34 – Ganglioside D3 is expressed both in human and mouse skin with progressing depigmentation. (A) GD3 expression and (B) an absence of melanocytes, as illustrated by no detection of TRP-1+ staining, were observed in depigmenting human vitiligo skin. (C) Limited GD3 expression and (D) abundant TRP-1 expression are presented in healthy control human skin. (E) In vitiligo-prone mouse skin, GD3 expressing cells were detected in hair follicles and in proximity to the epidermis. Representative image from of GD3 expressing cells is depicted in red with nuclei in blue (Scale bar = 50μm) [140, p. 5]

3.3.2.2 Naïve $CD4^+$ polarized in the presence of TGF-β to obtain high numbers of $CD4^+FoxP3^+$ Tregs

Adoptive cell transfer requires a high number of Tregs with a stable phenotype. The normal percentage of Tregs varies between 2-5% of total T cells in peripheral blood, and this is not sufficient for a single adoptive cell transfer. In addition to this hurdle, the plasticity of Tregs is another major concern to maintain in *ex vivo* culture. Previous studies reported the possibility to polarize naïve $CD4^+$ T cells into $CD4^+FoxP3^+$ Tregs to obtain a sufficient initial numbers with more stable phenotype in the presence of TGF-β. It is essential to maintain the stable phenotype of Tregs as they might convert to effector type, which is highly not desired. To maintain the phenotype in check, these polarized has to be characterized at every phase of cell preparation. The intermediate polarization has been shown in Figure 35, which is evaluated using flow cytometry [140, p. 5].

Naïve $CD4^+$ T cells have been chosen as they highly proliferate and less differentiated. In the study, these $CD4^+$ T cells were partially polarized to highly $FoxP3^+$ expressing Tregs with 51.6% efficiency in 5 days in culture. Further, the presence of TGF-β is maintained to keep the Treg phenotype and increase the percentage of highly expressing $FoxP3^+$ Tregs. Only small number of Treg phenotype is sufficient to show the suppressive activity, which is due to the cell-to-cell contact or bystander effect.

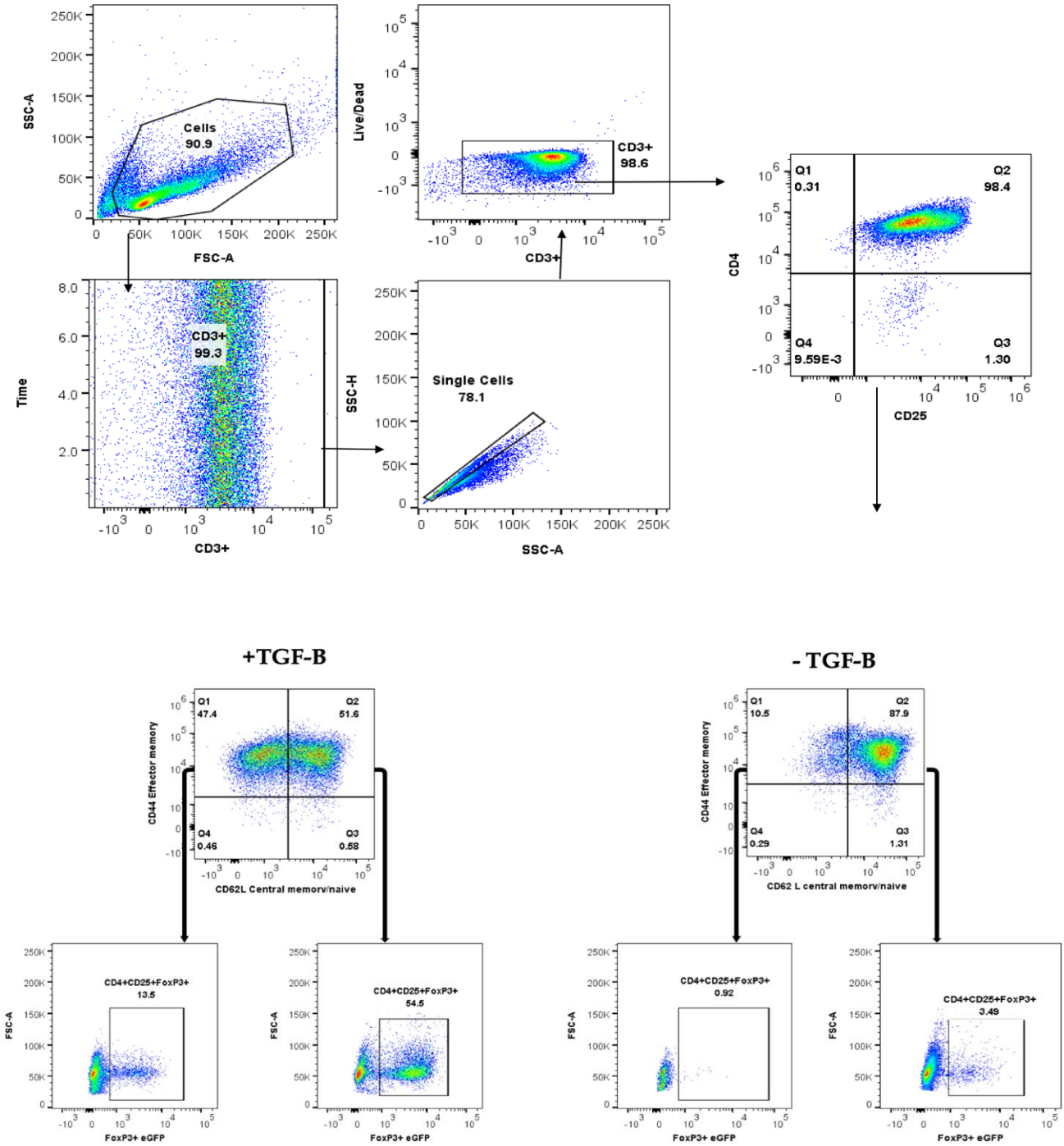


Figure 35 – Polarization of naïve $CD4^+$ T cells into $CD4^+FoxP3^+$ Tregs in the presence of TGF-β

3.3.2.3 High viral transduction of Tregs with GD3-encoded CAR construct

To generate antigen-specific Tregs, $FoxP3^+CD4^+$ T cells were polarized from $CD4^+$ T cells, and followed by transduction with GD3-reactive CAR construct (Figure 36). In the study, approximately $1.5x10^6$ naïve $CD4^+$ T cells were isolated from $3x10^8$ splenocytes, cultured in presence of TGF-β, and successfully polarized and expanded to approximately $1.6x10^7$ Tregs per spleen of the donor mouse. Next, TGF-β-polarized $FoxP3^+CD4^+$ T cells were transduced using retroviruses, and GD3 CAR expression was assessed using flow cytometry shown in Figure 37. The flow cytometry results are shown in in Figure 37A, with the transduction efficiency of 86.7% from total $CD4^+$ T cells to express GD3 CAR (Figure 37B). After further amplification, 64±3.5% among the transduced cells were $FoxP3^+$ expressing Tregs (Figure 37C). From the initial transduced $4x10^6$ $FoxP3^+$ Tregs, $2.1x10^7$ were GD3 CAR-expressing, $FoxP3^+$ Tregs. The CAR transduced with high efficiency of transduction were further planned to be tested *in vitro* to test their immunosuppressive capacity. FoxP3 expression directly correlates with suppression capacity and phenotype stability of Tregs [140, p. 6].

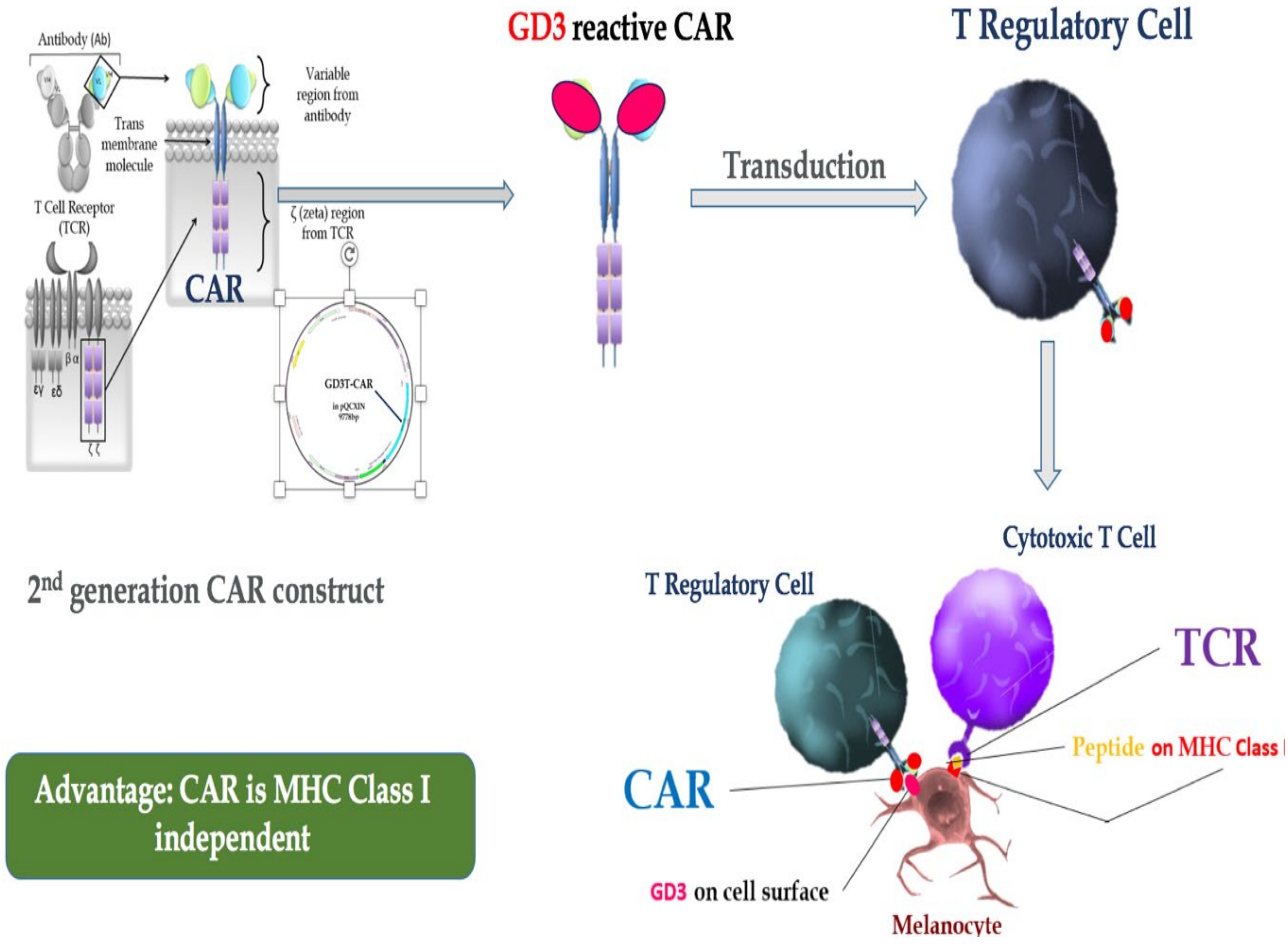


Figure 36 – Illustration of second generation of CAR and transduction with a hypothesis of further antigen recognition and the protection of melanocytes

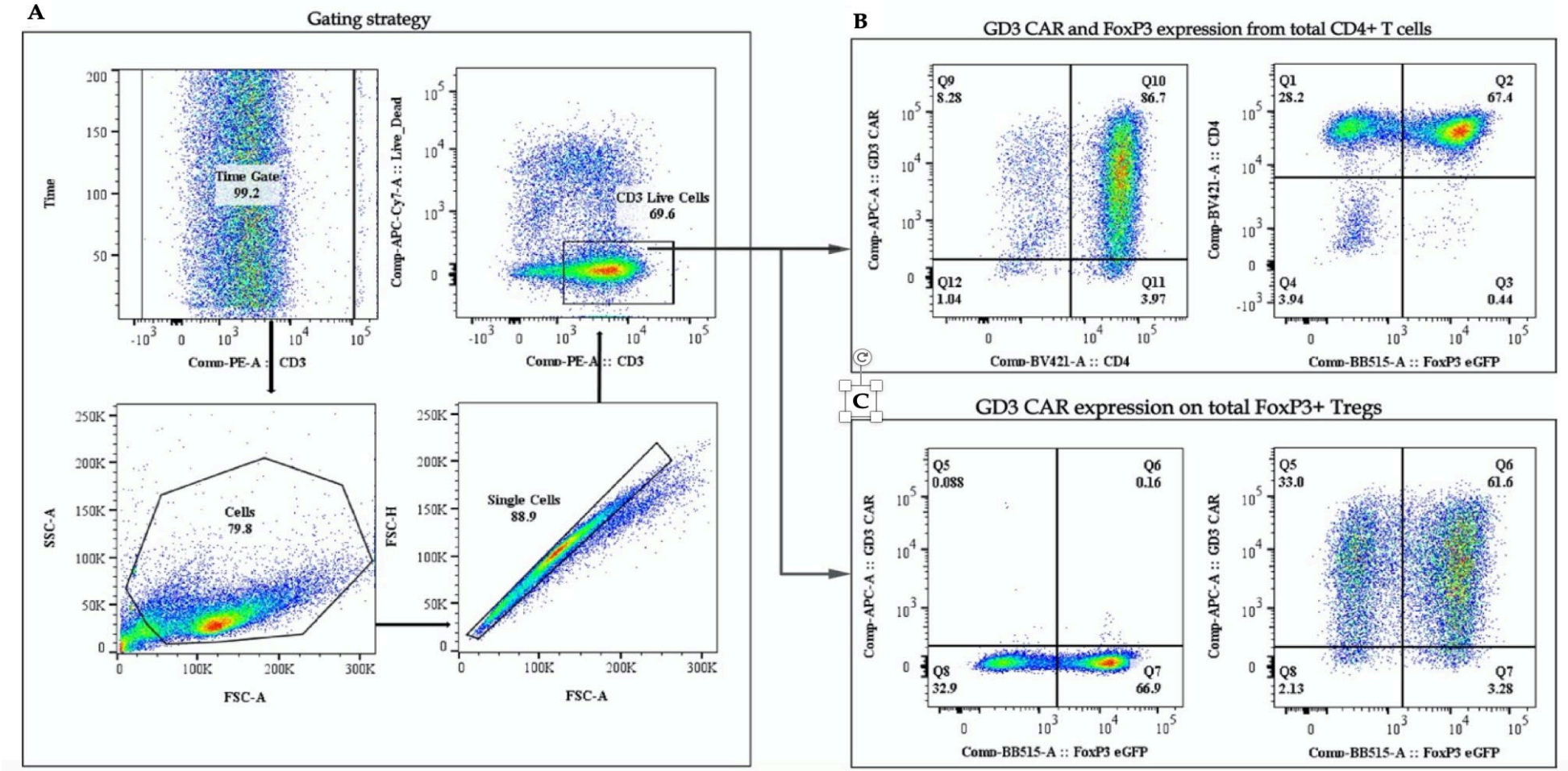


Figure 37 –Tregs were highly transduced with GD3 CAR construct. (A) The gating strategy shows a time gate that followed by sequentially gating on lymphocytes, single cells, and live cells. (B) 86% of total $CD4^+$ T cells were efficiently transduced with GD3 CAR construct, (C) and among them 67% of cell population expresses $FoxP3^+$ [140, p. 6]

To maintain and promote FoxP3 expression *in vitro* conditions, TGF-β was also added during the transduction period. Similar clinical studies provided the expansion protocols of Tregs *ex vivo* with high stable Treg phenotype. An example of Treg expansion was during the first clinical trial for GvHD, where freshly isolated Tregs have been used to achieve large numbers for clinical administration [175-177]. Fraser *et al.* prepared polyclonal Tregs for clinical trials *ex vivo* with a 300-fold expansion in 36 days using anti-CD3/CD28 coated activator beads in the presence of high dose IL-2 [178]. To maintain the higher stability of Tregs, some clinical protocols involve using Rapamycin. Besides, rapamycin blocks AKT-mTOR-SMAD3 signaling that favors FOXP3 upregulation [179], and confers a higher stability and suppression ability for the expanded Tregs. In the presence of rapamycin, Tregs from T1D patients and patients with cirrhosis recover their suppressive capacity during the expansion [180-182]. Levental's group developed a Treg expansion protocol using TGF-β together with rapamycin and IL-2, and these expanded Tregs demonstrated higher suppression compared to the use of rapamycin alone [183]. Putnam *et al.* developed a preclinical protocol to generate antigen-specific Tregs from co-culture with CD40-activated allogeneic B-cells or donor-derived DCs in the presence of IL-2. These antigen-specific Tregs have been shown enhanced suppression for alloimmune responses *in vitro* and *in vivo* compared to the polyclonally expanded Tregs [184]. The abovementioned examples of clinical trials support the need for already approved conditions to better maintain the Treg stability *ex vivo* and their sufficient proliferation.

3.3.2.4 GD3-specific CAR Tregs secrete a higher immunosuppressive cytokine

To test the suppressive activity of generated GD3-specific CAR Tregs, HEK293 transduced to express GD synthase (SIAT8 enzyme) was used as a target cells along with HEK293 cells as a control. The aim of *in vitro* assay was to evaluate whether GD3-specific CAR Tregs recognize its antigen, and to show the difference in production of suppressive cytokines. For a control, untransduced Tregs were used to compare the efficiency of GD3-specific CAR Tregs. The results demonstrated a significance 2.5-fold increase in mouse IL-10 production from GD3-specific CAR Tregs compared to untransduced Tregs ($p<0.05$) over 24 hour incubation shown in Figure 38. This results indicate that GD3-specific CAR Tregs recognized its antigen on target cells, and subsequently become activated and produce substantially higher amount of suppressive cytokine, IL-10, which plays an important role in immunosuppression. The results were obtained from ELISA tests which was performed using the media where co-culture of Tregs with target cells.

This generated results provided an insight that these GD3-specific CAR Tregs are functional and stable, which provided a promising results *in vitro* regarding the antigen-specific immunosuppression of Tregs. These observations prompted us to continue for further evaluation using different assays. HLA-A2 positive melanocytes can present antigens to effector cells (h3T cytotoxic T cells towards melanocytes), and it has been thought that human melanocytes with this haplotype, would be an ideal candidate as target cells to test the activity of GD3-specific CAR Tregs *in vitro.*

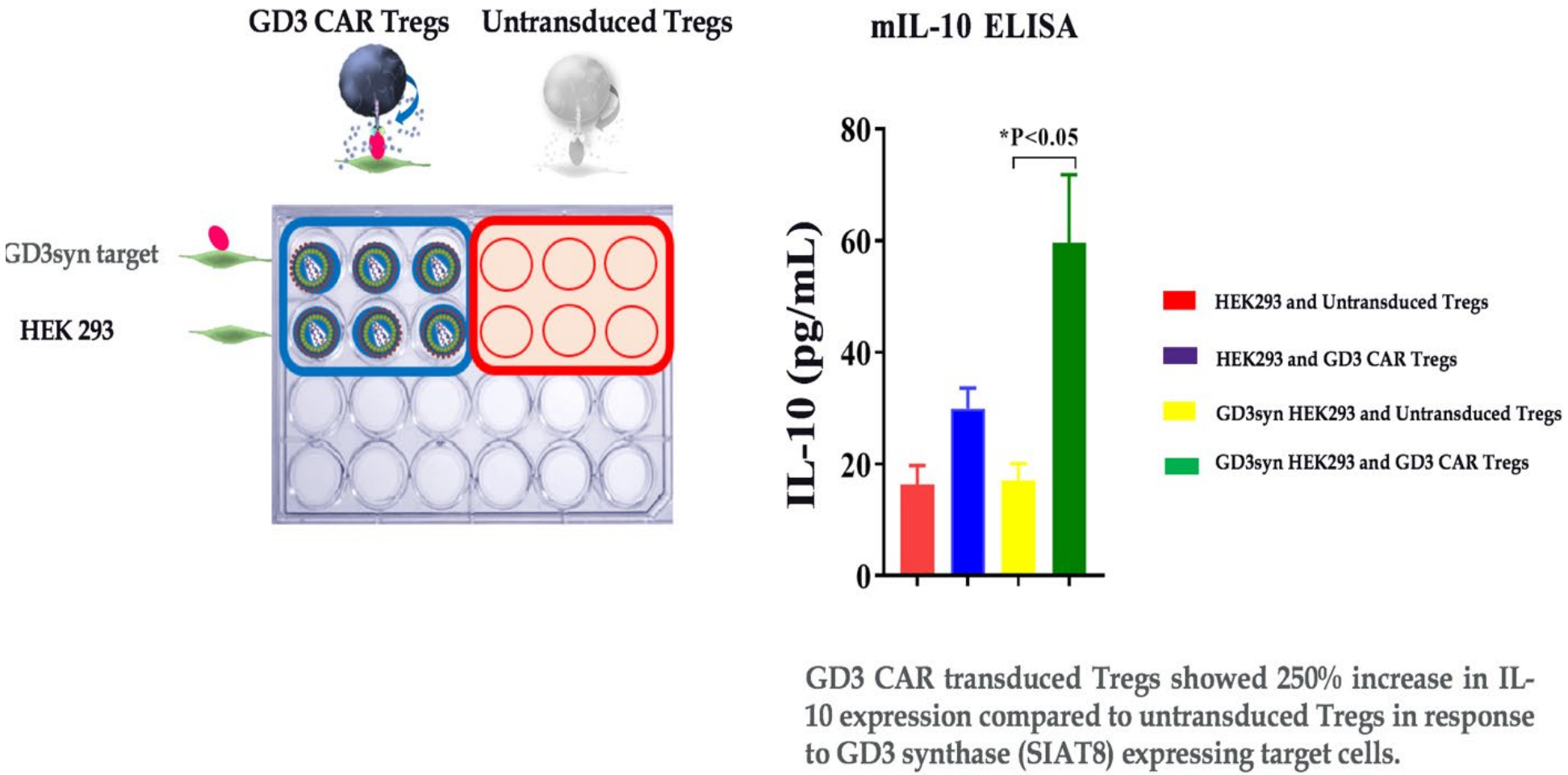


Figure 38 – *In vitro* suppression assay to check the suppressive activity of CAR Tregs and untransduced Tregs in the presence of GD3 synthase (SIAT8) expressing target cells. IL-10 expression was evaluated using ELISA assay

3.3.2.5 GD3-specific CAR Tregs provides enhanced protection towards melanocytes *in vitro*

Cytokine panel relevant to immune activation or immunosuppression was performed to measure the level of IFN-γ, TNF-α, IL-4 and IL-10 from *in vitro* culture of GD3 CAR Tregs or untransduced Tregs with tyrosinase-reactive h3T effector T cells (Teffs) and their HLA-matched targets (1:10:1), measuring concentrations 42 hours after cells were combined in culture in presence of IL-2 (Figure 39). These effector T cells were designed to recognize human HLA-A2-matched melanocytes [55]. The production of IFN-γ showed no difference among groups, suggesting that the presence of Tregs had a little influence on the production of this cytokine at this Treg to Teff ratio (Figure 39A). In combinations with GD3 CAR Tregs, production of TNF-α (Figure 39B, p=0.0005), IL-4 (Figure 39C, p=0.03), and IL-10 (Figure 39D, p=0.0005) was significantly increased, while overall IL-4 production remained consistently low. Importantly, the production of IL-10 regulatory cytokine was observed only in presence of cytotoxic T cells and HLA-matched human melanocytes. Taken together, antigen-specific Tregs provided enhanced immunosuppressive ability in the presence of activated Teffs. Next, melanocyte viability was evaluated in the presence of Teffs and Tregs, and whether this cytokine environment might translate to the better antigen-specific protection [140, p. 7].

Tregs elicit suppressive activity via cytokines, by cell-to-cell contact or through bystander effects [185]. To measure the suppressive activity of both untransduced and GD3 CAR Tregs, co-culture of targets, effector T cells, and Tregs were set up for 36 hrs *in vitro*. In figure 40A, the viability of targeted HLA-$A2^+$ human melanocytes was evaluated in the presence of target cells, effector T cells and Tregs with the ratio of 1:10:1. The target cells alone were increased slightly over time, whereas 82.2% cytotoxicity ($p<0.0001$) was observed when co-cultured with effector T cells after 36 hours. Untransduced Tregs provided 35.8% (p=0.02) protection from cytotoxic T cells over time. A two-way ANOVA was performed with aligned rank transformation using R-software, and pairwise post-hoc multiple comparison testing according to Tukey to determine that in presence of CAR Tregs, cytotoxicity towards melanocytes was 62.0% prevented (p=0.0004). Representative images for each combination of cells including targets alone (Figure 40B), targets and Teff (Figure 40C), and the latter combination in presence of untransduced Tregs (Figure 40D) or CAR Tregs (Figure 40E) at different time points likewise reveal most inhibition of cytotoxicity in a combination that includes GD3 CAR Tregs [140, p. 7].

Thus, results from melanocyte viability assay, it can be observed that both untransduced Tregs and GD3 CAR Tregs provide a significant protection. Importantly, GD3 CAR Tregs provided a significant protection compared to untransduced Tregs *(*p=0.04*)*, eliciting the added benefit of the antigen specificity to enhance immunosuppression. These findings also may suggest that GD3 CAR Tregs remained for longer term in the skin via being activated by GD3 surface antigen on perilesional epithelial cells including melanocytes, and the presence of melanocytes after adoptive transfer of CAR Tregs suggests that antigen-specific suppression of cytotoxic T cells occurred and melanocytes survived. Thus, we next evaluated the therapeutic effects of GD3 CAR Tregs *in vivo* [140, p. 7].

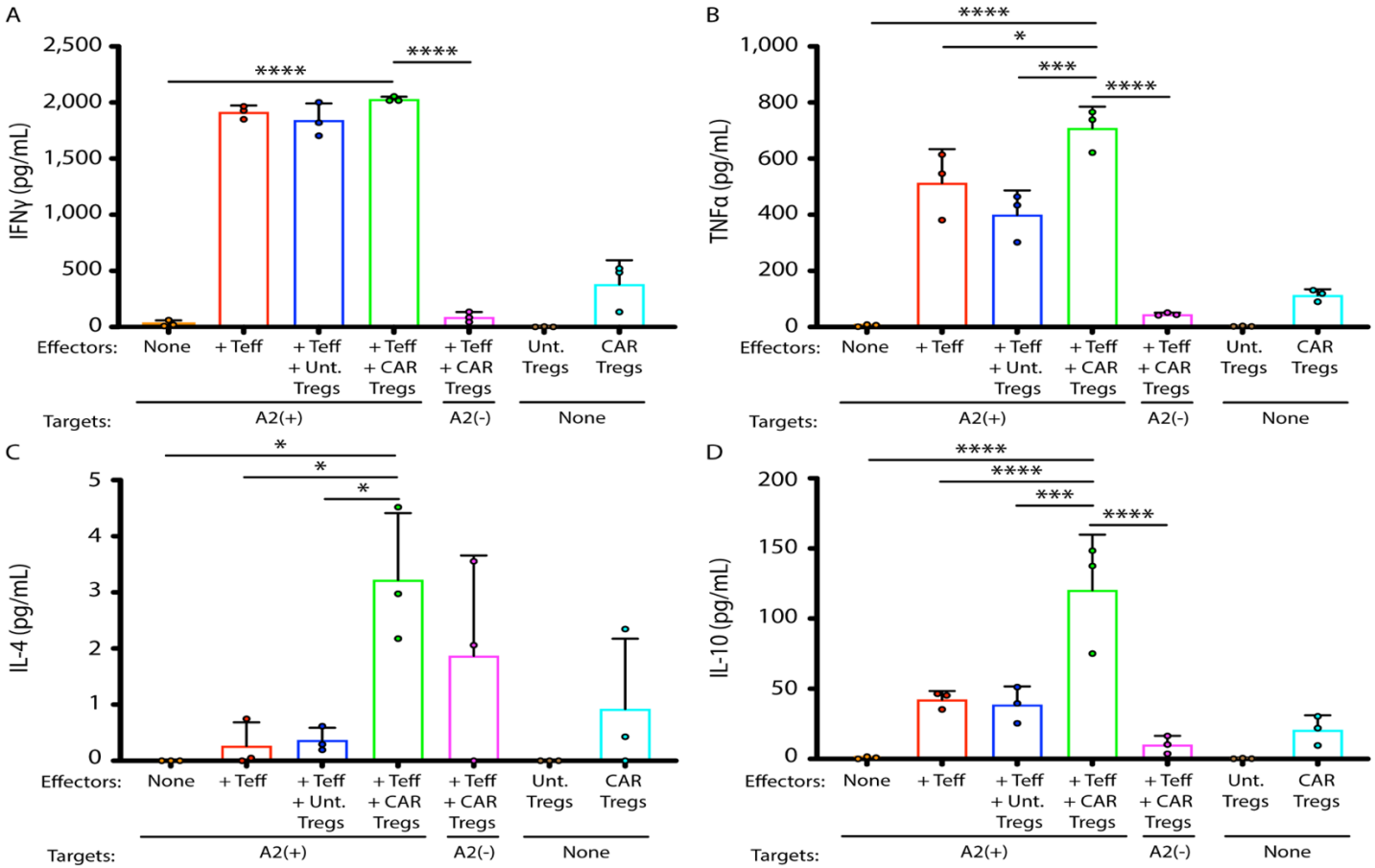


Figure 39 – GD3 CAR Tregs produce immunosuppressive cytokines in presence of activated T cells. Supernatants from cocultures of melanocytes, Teffs, and in presence and absence of untransduced or CAR-transduced Tregs were subjected to cytokine measurement. Cytokine levels are presented for (A) IFN-γ, (B) TNF-α, (C) IL-4, and (D) IL-10. Statistical analysis was performed by a one-way ANOVA test followed by Tukey's post-hoc test for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 [140, p. 8]

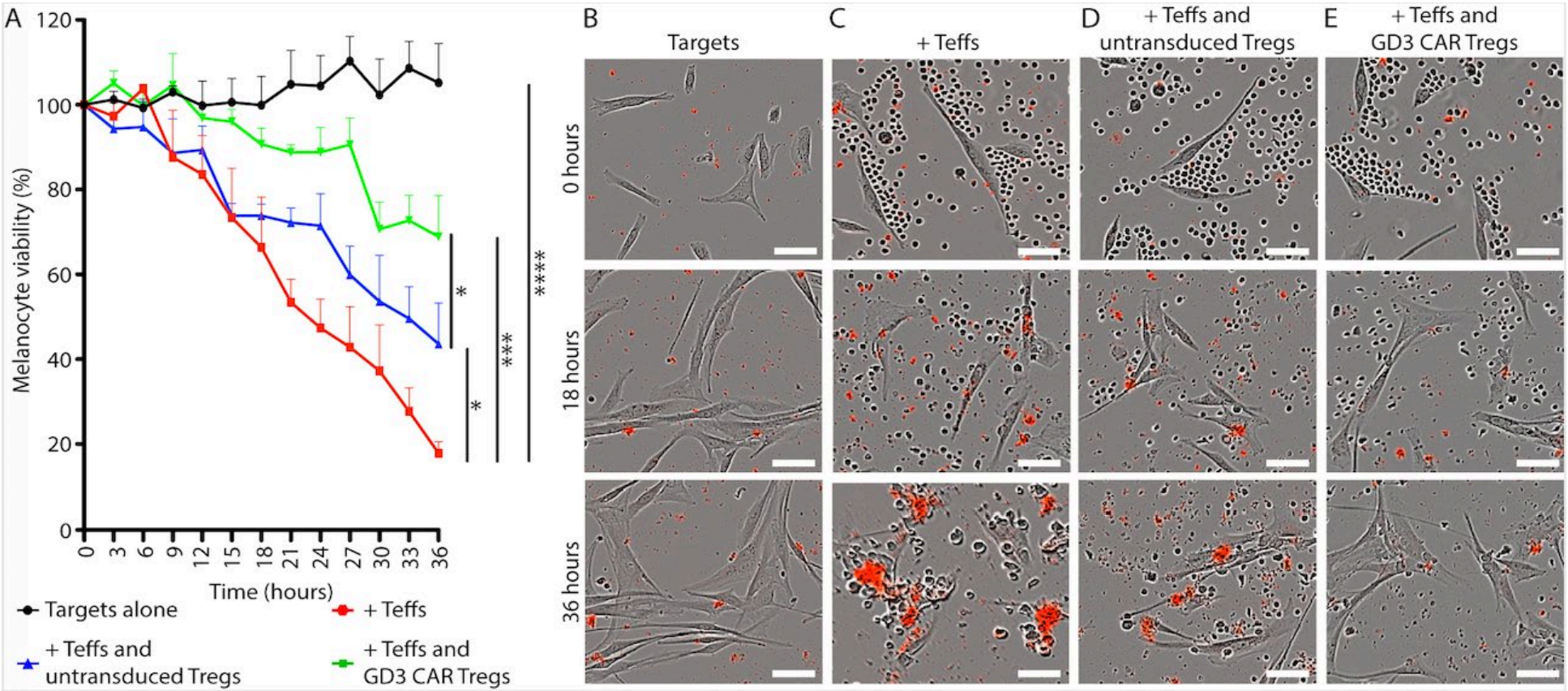


Figure 40 – "GD3 CAR Tregs provide enhanced protection for melanocytes from T cell-mediated cytotoxicity *in vitro*. The immunosuppressive capacity of both GD3 CAR Tregs and untransduced Tregs was evaluated. (A) Viability of HLA-A2+ human melanocytes (targets) in the presence or absence of Teffs and Tregs (1:10:1) is represented over time. Representative images of (B) HLA-A2+ human melanocytes (C) combined with Teffs, and additionally with (D) untransduced Tregs, or (E) GD3 CAR Tregs. Dead cells are marked by a red precipitate formed by caspase activity. Statistical significance was determined by two-way ANOVA with aligned rank transformation followed by Tukey's pairwise multiple comparisons test. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$ (Scale bar = 50μm)" [140, p. 8]

3.3.2.6 GD3-specific CAR Tregs enhance immunosuppression in h3TA2 mice

After the promising results *in vitro,* GD3-specific CAR Tregs are tested *in vivo* in vitiligo prone h3TA2 transgenic mice. This animal model elicits the progressive vitiligo starting at early age with rapid depigmentation over time. It was previously reported that single adoptive transfer of polyclonal Tregs control depigmentation in vitiligo-prone h3TA2 transgenic mice at early age (3 weeks to 9 weeks). Transgenic T cells reactive to human tyrosinase (h3T T cells) aggressively destroy melanocytes in the skin of this mouse model starting from early age. Treating with polyclonal Tregs controlled the depigmentation during 6-week observation period, and h3T T cell number remained unchanged suggesting that the only presence of transferred Tregs prevented the progression of the disease [127, p. 1290].

The suppressive activity of GD3 CAR Tregs was evaluated in vitiligo-prone h3TA2 mouse model. The depigmentation was measured starting from 5 weeks of age. In this animal mode, depigmentation progresses at early age, and display half-maximum of the depigmentation approximately at 23 weeks [186]. The experimental outline for adoptive transfer of untransduced Tregs, GD3 CAR Tregs or vehicle control is shown in Figure 41A. The retro-orbital injection was performed once every two weeks from week 5 to week 11. Images from ventral and dorsal side of mouse from experimental groups of untransduced Tregs, GD3 CAR Tregs, or vehicle control are presented in Figure 41B. To determine significance, the Wilcoxon rank sum (WRS) test was applied to compare the time-adjusted area under the curve (AUC) among groups in the experiment. The depigmentation outcomes for both vehicle and untransduced Treg control groups did not differ during and after the experiment (dorsal $p=0.97$, ventral $p=0.88$). Therefore, the vehicle and untransduced Treg groups were merged, and compared to the GD3 CAR Treg-treated group. In a one-sided t-approximation for the WRS test, the AUC for dorsal depigmentation dropped by 73.0% ($p=0.028$) for CAR Treg treated mice ($n=11$) for the 15-week observation period. When compared, ventral depigmentation progressed more rapidly and was evaluated separately. In this experiment, GD3 CAR Treg received mice ($n=11$) showed a significantly delayed depigmentation over the follow-up period (Figure 41C) resulting in a 60.5% reduction in the AUC ($p=0.006$) (Figure 41D). In Figures 42 and 43, individual scanned images were shown for both dorsal and ventral side of mice from HBSS, Treg and CAR Treg treated groups, and their depigmentation values are presented in Figure 44 [140, p. 8].

The enhanced protection from autoimmune depigmentation might be via expression of target, GD3 antigen in lesional skin, and the presence of activated effector T cells on site. To evaluate this, serum cytokine profile content was measured for IFN-γ, TNF-α, IL-4 and IL-10 from serum samples of mice received vehicle alone ($n=11$), untransduced Tregs ($n=10$), or GD3 CAR Tregs ($n=9$). Results showed that the levels of cytokines were remarkably consistent among the groups at end point shown in Figure 45. These results from cytokine levels in serum samples support the concept, in which, Tregs, including CAR Tregs, may be preferentially activated on site in areas of immune activity. No adverse events were observed in the course of the experiment by any groups. At the termination of the experiment, no abnormalities were found during internal organ examination from any groups. Next, it was probed whether

reduced depigmentation was accompanied by a sustained presence of melanocytes and changes in T cell populations [140, p. 8].

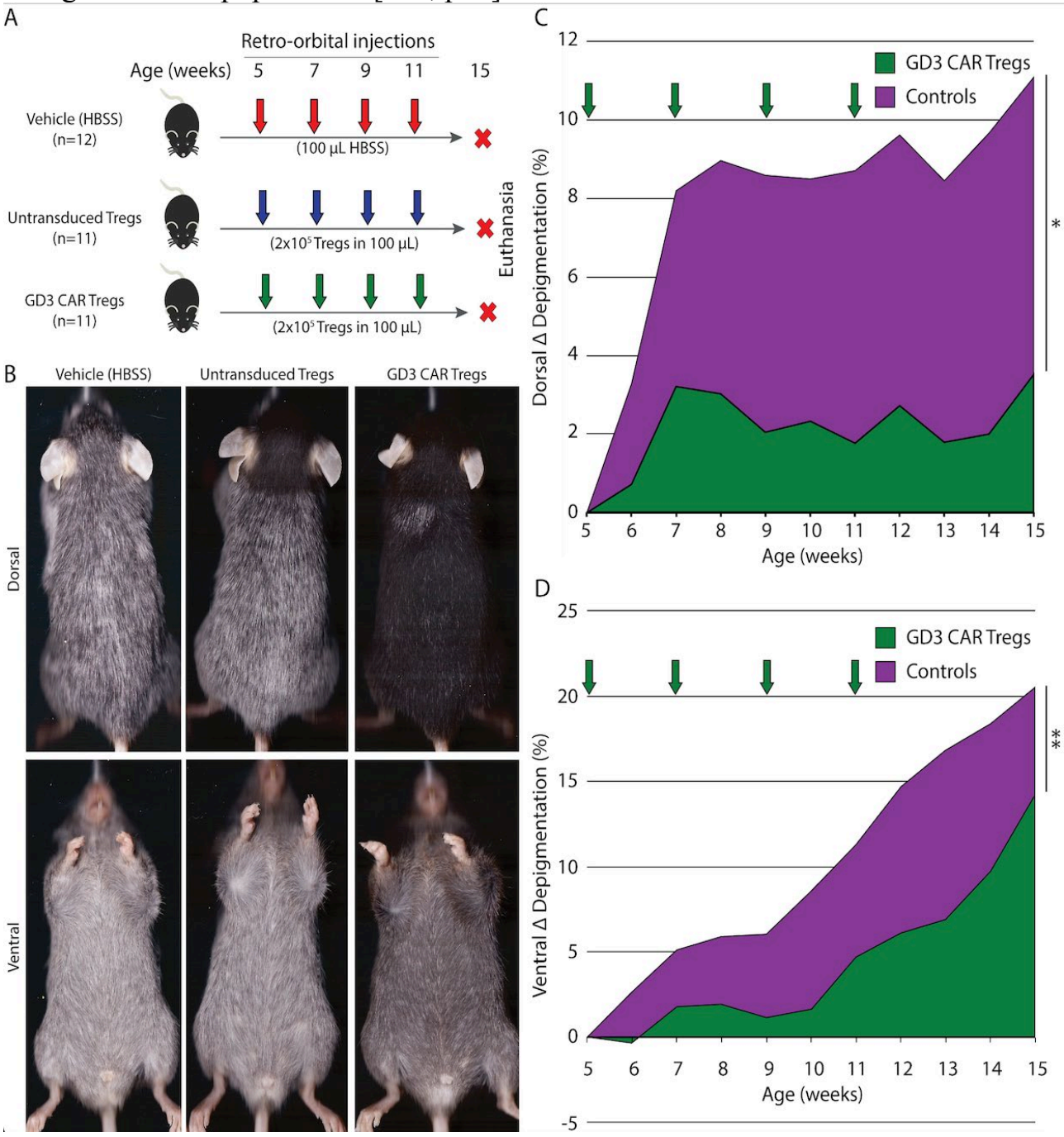


Figure 41 – Antigen-specific GD3 CAR Tregs elicit significant protection from depigmentation in h3TA2 vitiligo mouse model. (A) Adoptive transfer scheme for recipient h3T-A2 mice administered with vehicle alone (n=12), or untransduced Tregs (n=11) or GD3 CAR Tregs (n=11). Adoptive transfer of Tregs initiated at 5 weeks of age and continued biweekly until 11 weeks of age. Mouse skin was scanned for depigmentation weekly from 5-15 weeks of age. (B) Representative scan images from dorsal and ventral side from the HBSS vehicle, untransduced Treg, and GD3 CAR Treg treated mice groups. (C) Depigmentation quantified on dorsal and (D) ventral sides throughout the experiment. The Wilcoxon rank sum (WRS) test was used to compare the time-adjusted AUC among groups. Arrows: treatment times. *$p<0.05$; **$p<0.01$ [140, p. 9]

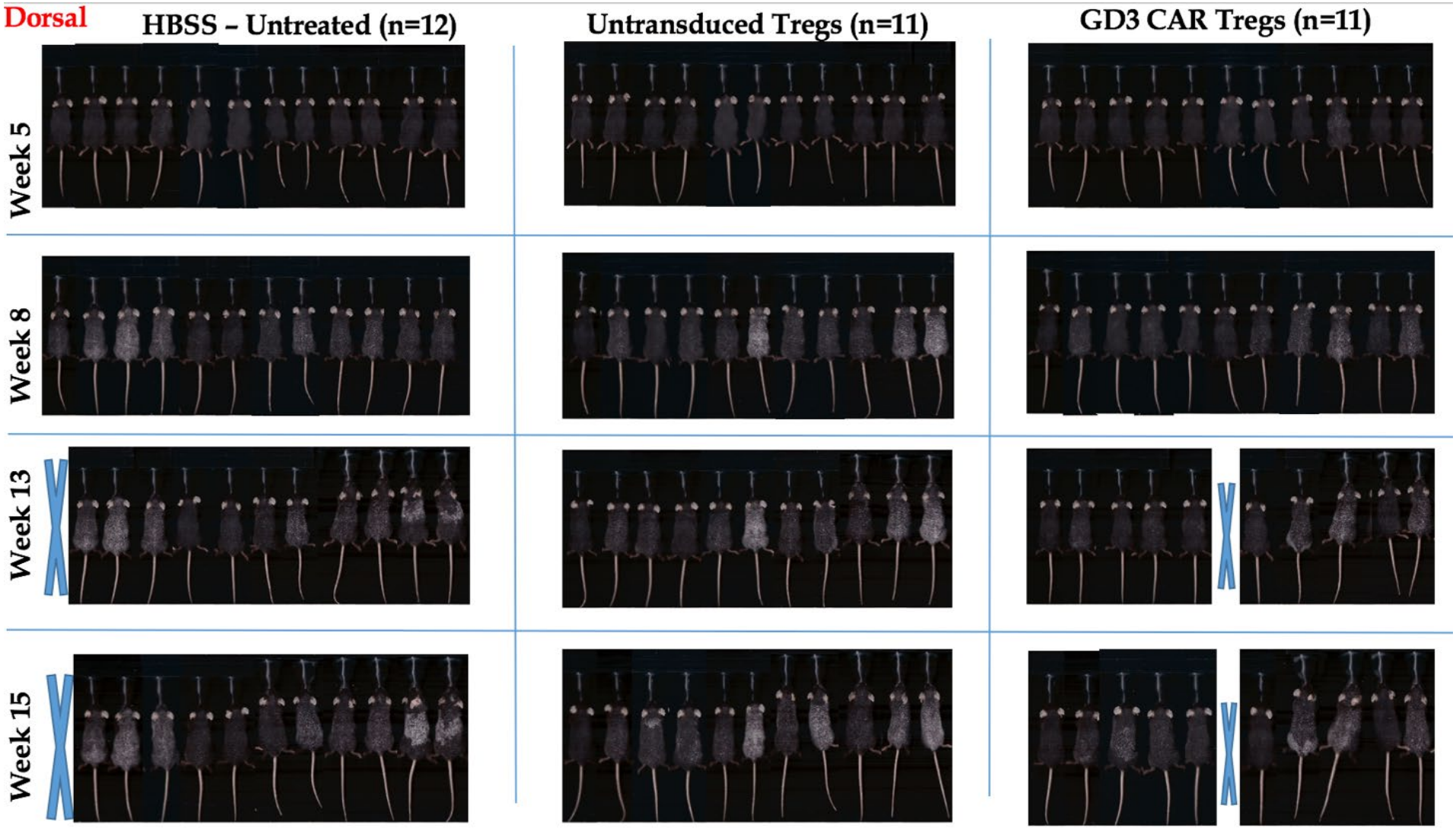


Figure 42 – Adoptive transfer of CAR Tregs control depigmentation in h3TA2 mice (Dorsal)

Figure 43 – Adoptive transfer of CAR Tregs control depigmentation in h3TA2 mice (Ventral)

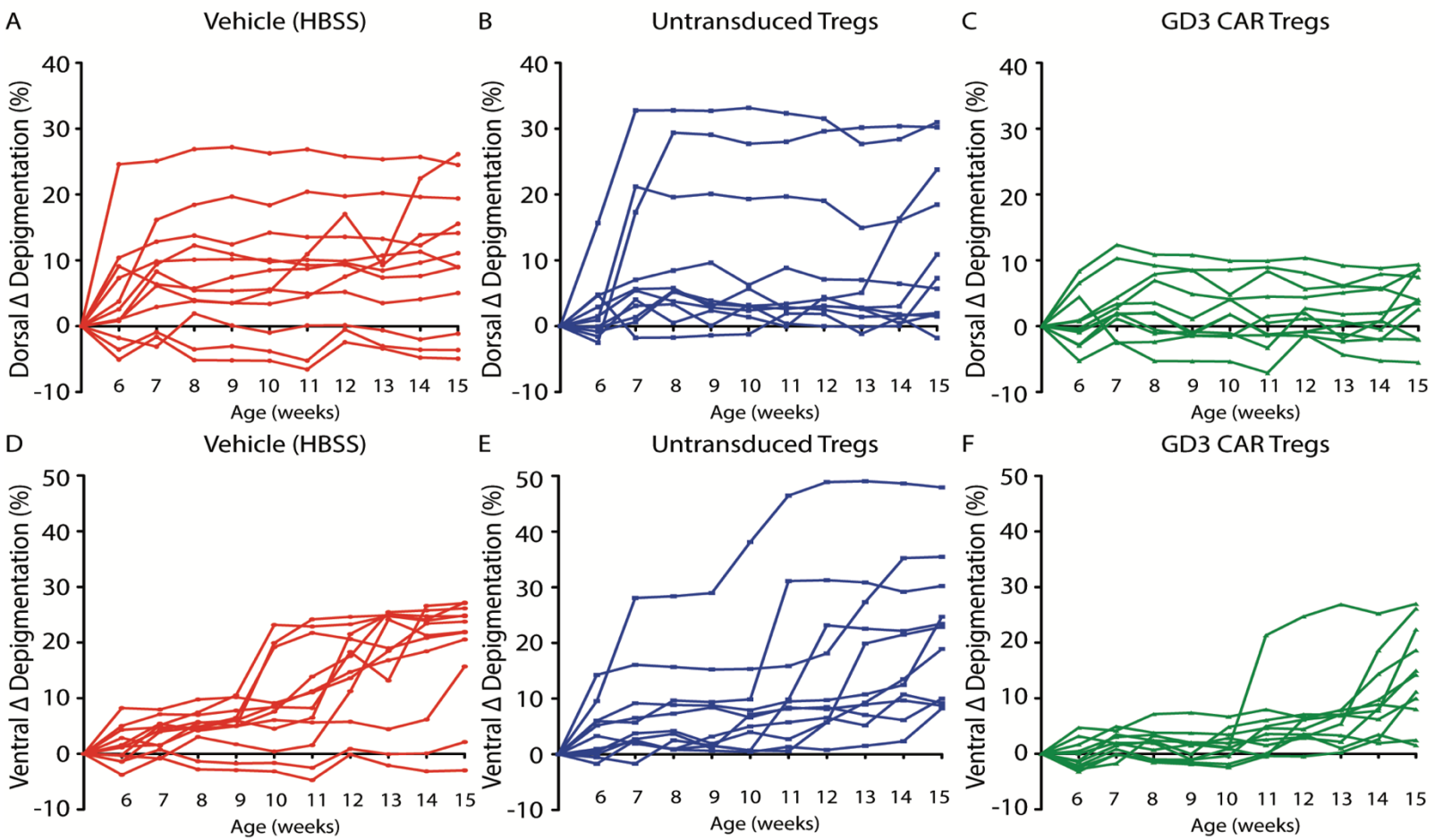


Figure 44 – **“**Individual mouse depigmentation values over time support the treatment effects of CAR Tregs. Dorsal depigmentation, represented as the change from baseline over time, is shown for each individual mouse from the (A) vehicle treated (n=12), (B) untransduced Treg (n=11), and (C) GD3 CAR Treg (n=11) treated groups. Respective ventral depigmentation values for (D) vehicle, (E) untransduced Tregs and (F) CAR Treg are also presented” [140, p. S1]

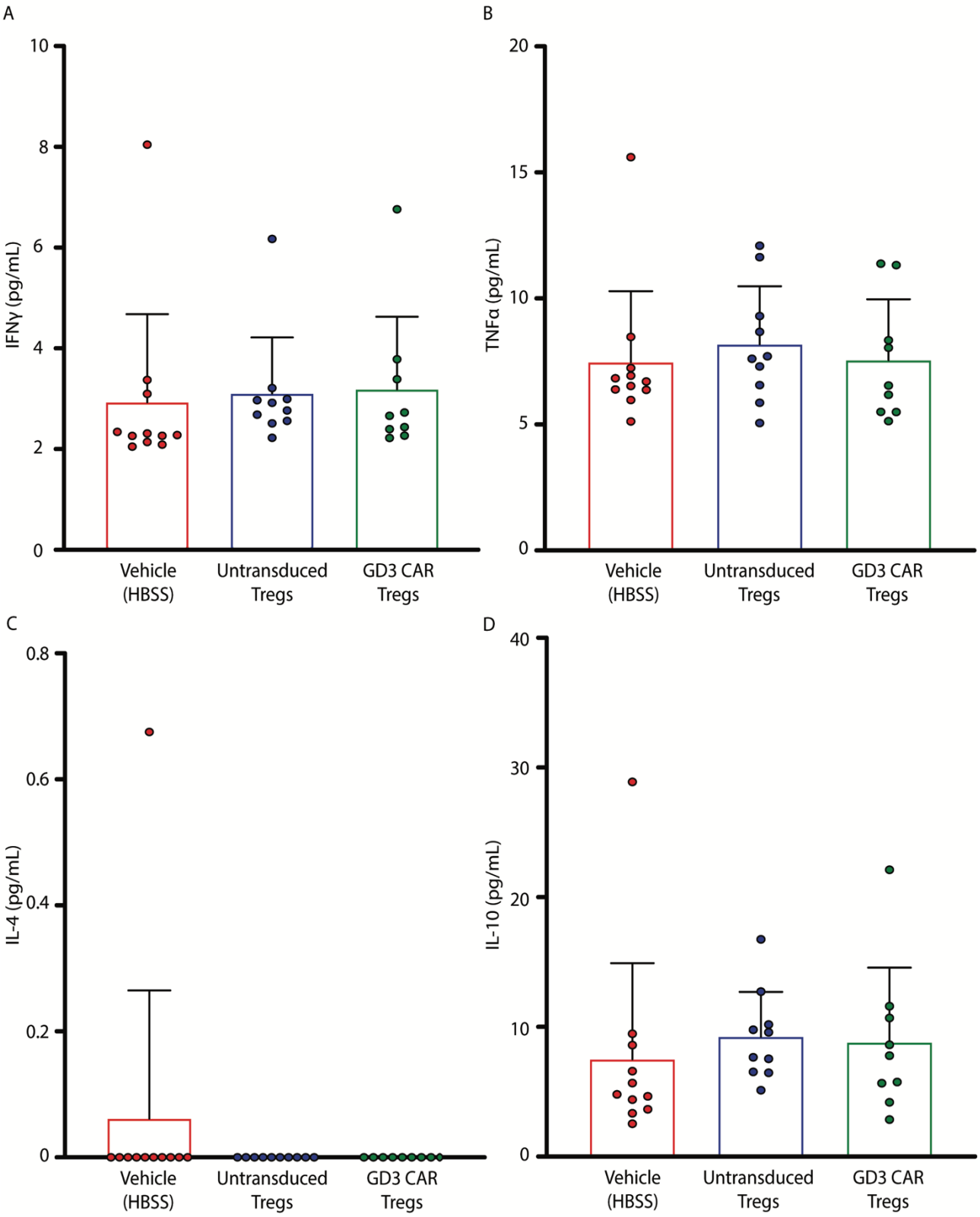


Figure 45 – "No differences in relevant cytokine titers were observed in serum from experimental animals. Detection of (A) IFN-γ, (B) TNF-α, (C) IL-4 and (D) IL-10 in serum harvested at 15 weeks of age from vehicle treated (n=11), untransduced Treg treated (n=10), and GD3 CAR Treg treated (n=9), vitiligo-prone mice. Murine cytokine levels were unchanged between groups with $p < 0.05$ by one-way ANOVA followed by a Tukey post-test to correct for multiple comparisons" [140, p. S2]

3.3.2.7 Increased number of GD3-specific CAR Tregs in the spleen

The confirmatory examination of GD3-specific CAR Tregs/or polyclonal Tregs was performed using immunofluorescence staining in the spleen tissues derived from adoptively transferred recipient mice. The results showed that GD3-specific Tregs persistently remained for in the spleen after injection (Figure 46). This indicates that antigen-specific Tregs might longer circulate in the presence of a cognate antigen compared to polyclonal Tregs.

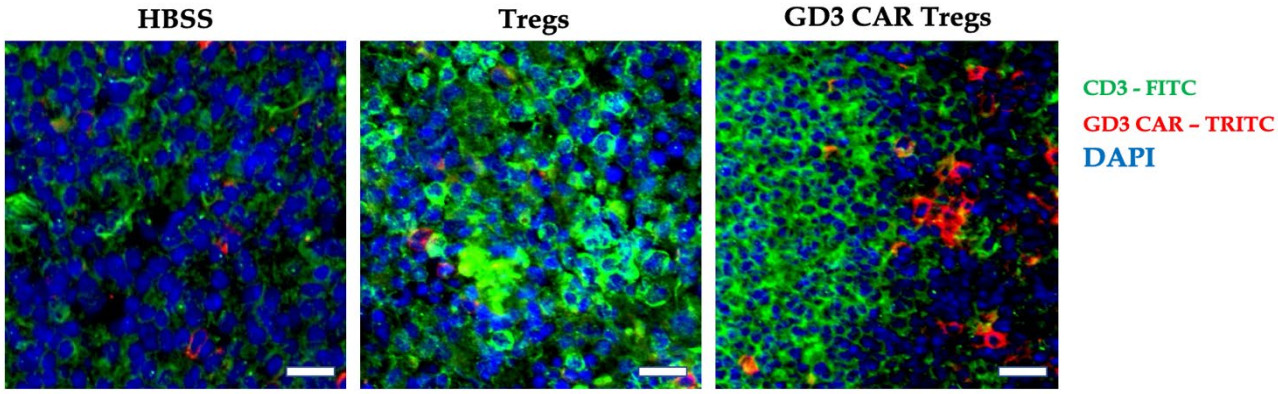


Figure 46 – Immunofluorescence staining of GD3-specific CAR Tregs/or polyclonal Tregs in the spleen tissues of adoptively transferred recipient mice (Scale bar =20µm)

3.3.2.8 Melanocytes are protected in the presence of GD3-specific CAR Tregs

Melanocyte presence was evaluated on dorsal skin of experimental mice. To detect melanocytes, antibodies to TRP-1 was used, and outcome is presented in Figure 47. The quantification of melanocytes is shown Figure 47. Complete loss of melanocytes were observed on skin biopsies from vehicle treated mice (n=3 per group) Only a few remaining melanocytes were detected from the skin of untransduced Treg treated mice (n=3 per group). A greater abundance of melanocytes were observed in GD3 CAR Treated mouse group. One-way ANOVA was performed followed by Tukey's post-hoc test to show the difference, and GD3 CAR Treg received mice skin contained a significantly greater number of melanocytes when compared to mice treated with untransduced Tregs ($p=0.025$), and to vehicle treated controls ($p=0.006$). Representative skin images from TRP-1 staining are shown for vehicle, untransduced Treg, and CAR Treg-treated mice in Figures 47B-D, and overlaid with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) nuclear staining in Figures 47E-G, respectively. Similar results were observed from evaluation of GD3 expression on the skin samples. GD3 expressing cells were quantified, and skin samples of CAR Tregs infused groups revealed significantly more GD3 expressing cells than the vehicle HBSS-treated mice ($p=0.003$) or mice transfused with untransduced Treg ($p=0.003$) (Figure 48). This staining results supports the concept that GD3 expressing cells were undergone less cytotoxicity compared to in vehicle or polyclonal Treg received mice. This confirmatory staining for melanocyte mainly corresponds with *in vivo* data presented in Figure 47, suggesting the enhanced suppressive ability of CAR Tregs. Next, Treg infiltration was evaluated from the skin samples of each treatment groups to explain the melanocyte maintenance [140, p. 9].

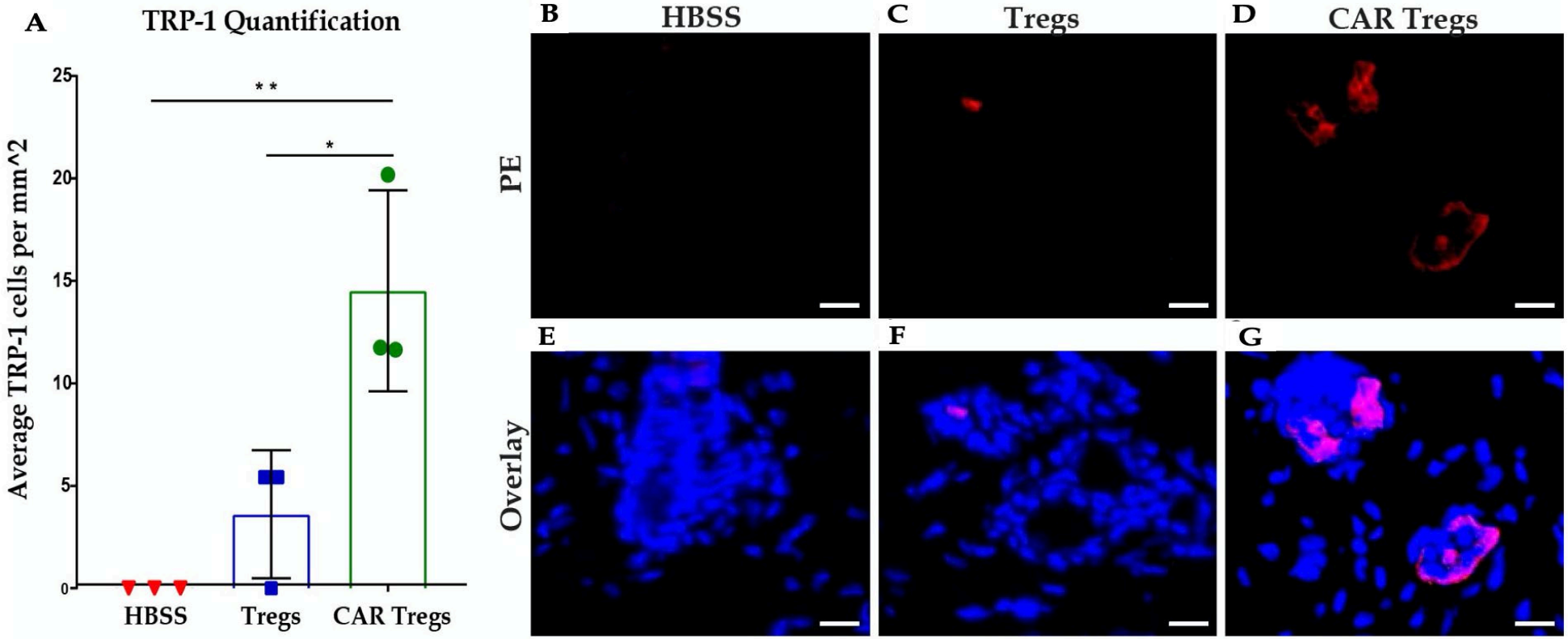


Figure 47 – "GD3 reactive CAR Tregs provides protection towards melanocytes from T-cell mediated cytotoxicity. Mouse skin was stained for melanocytes using antibodies to TRP-1. (A) Quantification of melanocytes, as well as accompanying representative images of TRP-1 staining from (B) HBSS vehicle, (C) untransduced Tregs, and (D) GD3 CAR Tregs treated mice (n=3 per group), with (E-G) the respective overlay including DAPI nuclear staining in blue is shown. Quantification of skin staining ± SD (*n*=3 per group) for (N) T cells and (O) Tregs are shown, respectively. Statistical analysis was performed by non-parametric t tests. *p<0.05, **p<0.001 (Scale bar = 20μm)" [140, p. 9]

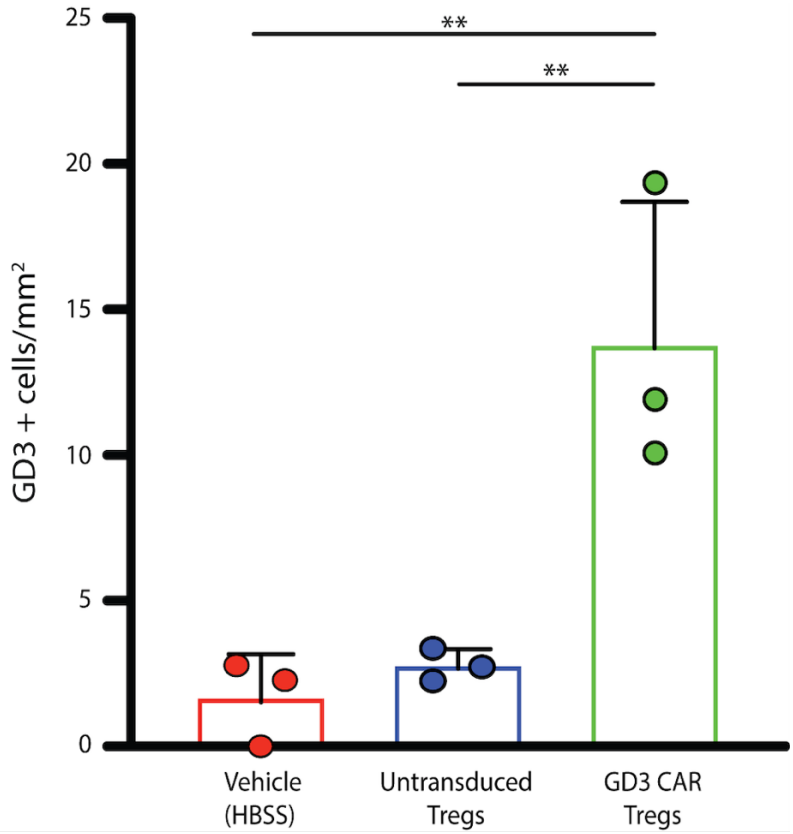


Figure 48 – "Treg transfusion helps maintain GD3 expressing cells in h3T-A2 vitiligo mouse skin. Quantification of GD3 expressing cells from h3T-A2 mouse skin at end point (mean ± SD) is compared across recipients of vehicle treatment, adoptive transfer by untransduced Tregs, or by GD3 CAR Tregs (n=3 per group). Statistical significance was determined by one-way ANOVA followed by a Tukey post-test to correct for multiple comparisons *p< 0.05; **p < 0.01" [140, p. S3]

3.3.2.9 GD3-specific CAR Tregs gravitate towards GD3 expressing cells in the skin

To understand the correlation between melanocyte survival and Treg activity, T infiltration was evaluated in the skin samples from each group. T cells were stained with antibodies to CD3ε$^+$, and Tregs were identified as CD3ε$^+$ FoxP3$^+$. Representative images from mouse skin from each group is presented in Figure 49. Stained Tregs were shown in Figures 49A-C for vehicle (HBSS), untransduced, and GD3 CAR Tregs, respectively, and overlaid with DAPI nuclear staining in Figures 49D-F. The quantification of CD3ε$^+$ cell and CD3ε$^+$/FoxP3$^+$ Treg were performed as mean ± SD (at n=3 per group) for each treatment group. The average number of infiltrating CD3ε$^+$ T cells at end point was 2.3-fold greater (p=0.02) in the control groups as compared to the GD3 CAR Treg received group (Figure 49G), when a one-way ANOVA followed by Tukey's post-hoc test was performed. CD3$^+$FoxP3$^+$ Tregs were only observed in skin samples of GD3 CAR Treg treated, whereas no (remaining) CD3$^+$FoxP3$^+$ Tregs were detected in either control group 10 weeks after adoptive transfer (Figure 49H). GITR-expression was evaluated for Treg numbers at the end point, and an significant increase in Treg numbers was again observed in skin from CAR Treg treated mice

compared to those treated with untransduced Tregs (p=0.0059) or vehicle alone (p=0.0089). However no difference was observed in abundance of proliferating $GITR^{+}Ki67^{+}$ cells across the treatment groups. This suggest that differences in Treg numbers may instead be defined by increased influx or decreased efflux of Tregs from the skin in GD3 CAR Treg treated mice (Figure 50). Nevertheless, the increased numbers of Tregs in GD3 CAR Treg treated group at end point might explain the enhanced suppressive activity by GD3 CAR Tregs and suggests that maintenance of a Treg presence on site is supported by local antigen recognition. In summary, the data show that antigen specificity prolonged the suppressive activity of adoptively transferred Tregs [140, p. 9].

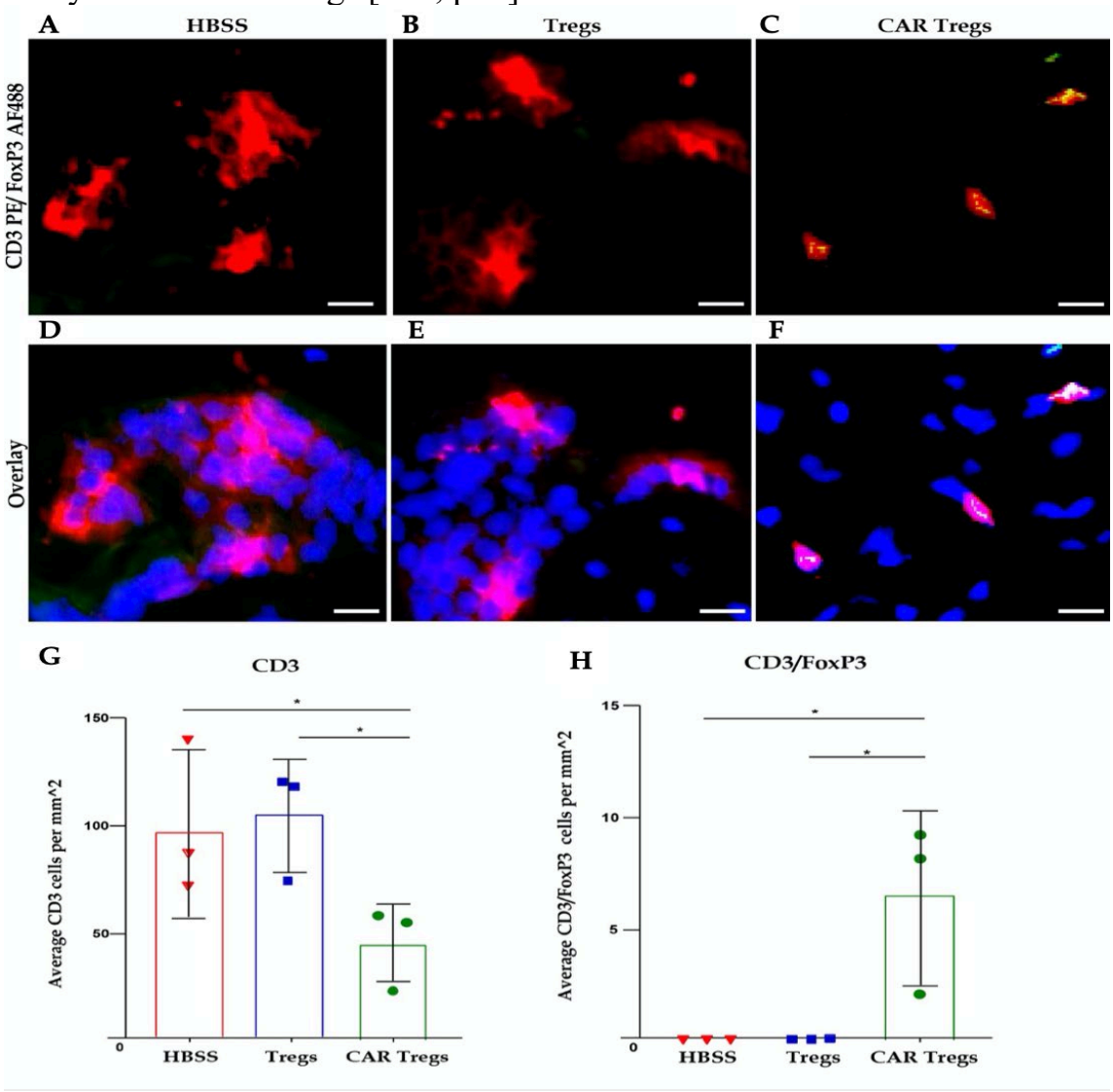


Figure 49 – "Mouse skin tissues were evaluated for T cell infiltration using antibodies to CD3ε and FoxP3, and examples of staining in skin from (A) vehicle control, (B) untransduced Tregs, and (C) GD3 CAR Tregs administered mice are shown. Representative samples were used to quantify $CD3\varepsilon^{+}$ T cells (red), $FoxP3^{+}$ cells (green), and double positive Tregs; (D-F) respective overlays with DAPI (blue) are also shown. Quantification of skin staining ± SD (*n*=3 per group) for (G) T cells and (H) Tregs are shown, respectively. Statistical analysis was performed by non-parametric t tests. *p<0.05, **p<0.001 (Scale bar = 20μm)" [140, p. 9]

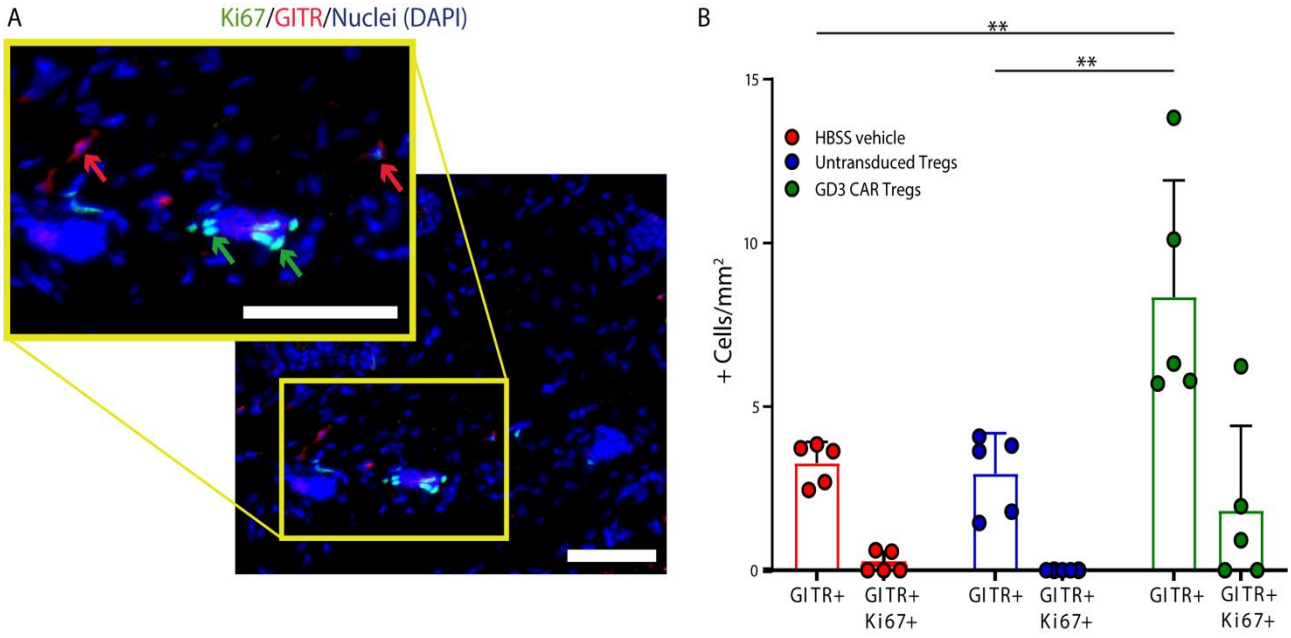


Figure 50 – "Proliferation of GITR$^+$ Tregs is not increased in the skin of CAR Treg recipient mice at 15 weeks. Cells expressing Ki67 (green) and GITR (red) in the skin of vitiligo-prone h3T-A2 mice were quantified at 15 weeks of age (mean ± SD) and compared among recipients of vehicle treatment, of untransduced Tregs, or of GD3 CAR Tregs (n=5 skin samples per group). Statistical significance was determined by one-way ANOVA followed by a Tukey post-test to correct for multiple comparisons. *p< 0.05; **p < 0.01 (Scale bar = 50μm)" [140, p. S4]

3.3.2.10 Summary of GD3-specific CAR Tregs for vitiligo immunotherapy

Here in this study, therapeutic potential of antigen-specific GD3 CAR Tregs was described to provide immune tolerance towards melanocytes in vitiligo. When compared to traditional immunosuppressive steroids, biologics, and antimetabolites, Tregs can elicit complex effect on providing immune tolerance in several autoimmune diseases [126, p. 751] . Many studies have already evaluated polyclonal CD4$^+$CD25$^+$FoxP3$^+$ Tregs to restore immune tolerance in animal models, including vitiligo [127]. Bluestone group has established a robust protocol to purify Tregs with high efficiency (>90%). Further, *ex vivo* amplification protocol has been developed to obtain 3x10^9 Tregs from a peripheral blood of a single donor as described here [187]. A few phase I clinical trials have been established to efficiently isolate and expand Tregs to perform Tregs immunotherapy in various autoimmune diseases [102, 188-190, p. 315]. However, polyclonal Treg infusion is not shown to be efficient yet [140, p. 10].

The studies reported a conflicting results in numbers of Tregs in vitiligo. While several reported no significant differences in circulating Treg numbers [53, 191], some showed a great difference in Treg numbers in peripheral blood of vitiligo patient and healthy donors [149, p. 589; 192]. However, recent studies showed a local deficiency of cutaneous Tregs in vitiligo, which support the loss of the peripheral immune tolerance [12, p. 489; 53, p. 283; 191, p. 134] .

Infusion of islet-specific Tregs showed a greater efficiency compared to

polyclonal Tregs in type 1 diabetes progression [126, p. 162; 193; 194]. Unfortunately, pancreas is nearly complete when patients with type I diabetes are diagnosed, and adoptive transfer of Tregs might not be beneficial for this condition. However, other autoimmune diseases, in which the number of autoimmune destructed target cells can still be renewable, can benefit from the infusion of the antigen-specific Tregs. Many preclinical studies demonstrated a greater efficiency of antigen-specific Tregs in organ transplantation [85, p. 1691; 195-197].

In vitiligo-prone mouse, cytotoxic T effector cells are continuously reacting to melanosomal antigens and destroying melanocytes, which describes the diseases progression. Adoptive transfer of the Tregs control autoimmune depigmentation for the duration of treatment, and when infusion is halted, the disease can progress again. This suggests that adoptive transfer of Tregs are most efficient when administered during the active phase of vitiligo. In contract to continuous depigmentation in mice, human patients experience progressive disease periods with periods of inactivity that provide an opportunity to differentiate and regenerate melanocytes from stem cells. Wen treated with JAK inhibitors, effector T cells can be suppressed if combined with UV light therapy [198]. Intermittent infusion Tregs will be beneficial when active progression occurred in vitiligo, thus cryopreservation of the engineered GD3 CAR Tregs is needed for later use [140, p. 10; 199].

Antigen-specific Tregs by their virtue can provide a greater efficiency with more favorable safety profiles to prevent generic immunosuppression. This claim can be supported by increased IL-10 production in *in vitro* when combined with target cells and effector cells describe in this dissertation. IL-10 production is significantly increased when second generation CARs with a 28ζ costimulatory domain is used to engineer Tregs [200], and similar results were observed in this study shown here. Thus, using antigen-specific Tregs based on TCR and CAR transduction might potent treatment option in vitiligo [8]. It was observed that TNF-α was significantly increased in both in *in vitro* culture and in mouse serum. Nevertheless, it was previously shown that TNF-α knock out was not associated with the development of vitiligo. Moreover, no difference in depigmentation was observed in comparison to wild type h3TA2 mice [127, p.1290; 140, p. 10].

It was investigated whether antigen-specific Tregs hold superior efficacy in autoimmune vitiligo. Having a complex etiology [114, p. 651], vitiligo is presented with melanocyte loss, and prevention of autoimmunity is a need real need for a new treatment strategies. TCR- and CAR-transduced Tregs differs in terms of mechanistic and functional properties. TCR Tregs can be activated with low-antigen expression, while CAR Tregs require high density of antigens [201, 202]. This suggests choosing the right approach of using TCRs or CARs could be decided on the level expression of antigen on target cells or tissue. Main advantages of the CAR Tregs are to not being subjected to HLA restriction, and having a higher affinity for their targets moieties [203]. Therefore, CAR-based antigen-specific Tregs can become a universal application for all patients with vitiligo [140, p. 11].

In the course of this study, a potential target antigen was identified for vitiligo. In vitiligo-prone mice, engineered GD3 CAR Tregs protect melanocytes cytotoxic, melanocyte-specific T cells that expresses a human TCR and matched human MHC.

Thus, these transgenic T cells are capable of responding to human melanocytes. Importantly, target antigen does not required to be expressed by target cells spared in the response themselves [204].

After post-examination of mice, all internal organs were observed with no side effects. This suggests that infusion of the GD3 CAR Tregs were safe, and major concerns such as cytokine release syndrome (CRS) or neurotoxicity were not found in these experimental mice [205]. CRS is more likely to occur when applied for liquid tumors than in solid tumors [206]. To prevent any potential side effects, a caspase-based suicide gene was incorporated construct to inactivate the GD3 CAR Tregs when needed *in vivo* [140, p. 11; 207, 208].

Intravenous administration of antigen-specific Tregs, and the migration of these Tregs to the site of autoimmune inflammation can raise a possible concern in vitiligo [194, p. 154]. Alternative option can be a local application of Tregs in the skin, or the CCR4 Treg homing receptor ligand CCL22 can be introduced to attract systemically applied Tregs [68, p. 1578]. This suits autoimmune skin diseases for using antigen specific Tregs when relevant antigens can be identified. Besides, GD3 might support keratinocyte proliferation as O-acetylated GD3 was overexpressed in skin with psoriatic lesions [209, 210]. These mentioned findings suggest that GD3 CAR Tregs might provide a local immune tolerance in the skin and might be applied in psoriasis as well [140, p. 11].

In clinical trials, recently, only alloantigen-reactive Tregs are being evaluated for organ transplantation purposes [194, p. 155]. In this dissertation, GD3 reactive CAR Tregs generated and expanded in amounts sufficient for adoptive transfer in mice. The same approach can be pursued in patients, and cryopreservation of these Tregs can make it suitable for the future use [211-214]. To overcome the cost and time for the preparation of Tregs for each infusion, off-the-shelf “Universal CAR Tregs” can be developed. From the universal CAR Tregs, all patients can benefit, and require less cost for the therapy [140, p. 11; 215]. In summary, the results provided in this dissertation strongly support the application of antigen-specific CAR Tregs as a cell-based immunotherapy for vitiligo, as they halt depigmentation and provide immune tolerance in vitiligo.

CONCLUSION

Vitiligo is a skin condition characterized by white milky patches due to the loss of melanocytes. The etiology of the disease is still unknown, and effective treatment options are not available at current time. The condition may cause psychological distress, depression, and in some cultures stigmatization can also occur due to misidentification as a contagious disease. Therefore, finding an effective cure for vitiligo remains one of the main tasks in dermatology.

During this Ph.D research, it was observed that the proportion of $CD39^+$ and $CD44^+$ was decreased in peripheral blood of vitiligo patients. Besides, $CD39^+$ and $CD44^+$ $FoxP3^+$ Tregs are reduced in patients with vitiligo remission when compared to healthy controls. These findings suggest that the decrease in number of this Treg subpopulation might be associated with vitiligo development and progression.

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. It was found that ampicillin leads to increased depigmentation, while neomycin inhibits the development of the disease by indirectly affecting skin Treg infiltration.

Stress conditions have been associated with vitiligo development. Thus, identification stress markers were crucial to develop a therapy for diseases. In case of vitiligo, the overexpression of GD3 was firstly identified in the epithelial cells and melanocytes, in both human and mice perilesional skin. Therefore, GD3 was hypothesized as a target antigen for vitiligo. The 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 transduction method has been developed that provides Treg with high expression of CAR – specific for 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 produce 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 human polyclonal 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 polyclonal Tregs, GD3-specific CAR

Tregs have better melanocyte homing ability and greater immunosuppressive activity in relation to proliferation of melanocyte-specific cytotoxic T cells.

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

REFERENCES:

1 Kruger C., Schallreuter K. U. A review of the worldwide prevalence of vitiligo in children/adolescents and adults // Int J Dermatol. – 2012. – Т. 51, № 10. – С. 1206-12.

2 Абишев А.Т. И. Е. Н., Джусупгалиева М.Х., Таубаева А.А. Основные характеристики дерматовенерологической службы рк в 2018 году: коечный фонд, кадровый потенциал, общая заболеваемость населения. Основные медико-экономические характеристики –2018. – URL: http://kncdiz.kz/files/00007050.pdf?sid=dpqj1jqtgrmk82llmqthsd24m4. 11.01.2020.

3 Felsten L. M., Alikhan A., Petronic-Rosic V. Vitiligo: a comprehensive overview Part II: treatment options and approach to treatment // J Am Acad Dermatol. – 2011. – Т. 65, № 3. – С. 493-514.

4 Kim S. M., Lee H. S., Hann S. K. The efficacy of low-dose oral corticosteroids in the treatment of vitiligo patients // Int J Dermatol. – 1999. – Т. 38, № 7. – С. 546-50.

5 Lee D. Y., Kim C. R., Lee J. H., Yang J. M. Recent onset vitiligo treated with systemic corticosteroid and topical tacrolimus: Need for early treatment in vitiligo // J Dermatol. – 2010. – Т. 37, № 12. – С. 1057-9.

6 Rusfianti M., Wirohadidjodjo Y. W. Dermatosurgical techniques for repigmentation of vitiligo // Int J Dermatol. – 2006. – Т. 45, № 4. – С. 411-7.

7 Silverberg J. I., Silverberg N. B. Association between vitiligo extent and distribution and quality-of-life impairment // JAMA Dermatol. – 2013. – Т. 149, № 2. – С. 159-64.

8 Le Poole I. C., Luiten R. M. Autoimmune etiology of generalized vitiligo // Curr Dir Autoimmun. – 2008. – Т. 10. – С. 227-43.

9 Мухатаев Ж.Е. Остапчук Е. О. Снижение доли регуляторных Т-клеток периферической крови при витилиго // Материалы Международной научной конференции Студентов и молодых ученых "Фараби әлемі". – 2018. – С. 162.

10 Мухатаев Ж.Е. Остапчук Е. О. Доля Т-регуляторных клеток, экспрессирующих маркеры CD39 и CD44, снижена в периферической крови больных витилиго // Международная научная конференция молодых ученых «Фундаментальные исследования и инновации в молекулярной биологии, биотехнологии, биохимии» к 80-летию со дня рождения академика Мурата Абеновича Айтхожина. – 2019. – С. 48.

11 Huggins R. H., Schwartz R. A., Janniger C. Vitiligo // Acta Dermatovenerol Alp Pannonica Adriat. – 2005. – Т. 14, № 4. – С. 137-42, 144-5.

12 Le Poole I. C., Mehrotra S. Replenishing Regulatory T Cells to Halt Depigmentation in Vitiligo // J Investig Dermatol Symp Proc. – 2017. – Т. 18, № 2. – С. S38-s45.

13 Daniel B. S., Wittal R. Vitiligo treatment update // Australas J Dermatol. – 2015. – Т. 56, № 2. – С. 85-92.

14 Picardi A., Pasquini P., Cattaruzza M. S., Gaetano P., Melchi C. F., Baliva G., Camaioni D., Tiago A., Abeni D., Biondi M. Stressful life events, social support,

attachment security and alexithymia in vitiligo. A case-control study // Psychother Psychosom. – 2003. – Т. 72, № 3. – С. 150-8.

15 Gawkrodger D. J., Ormerod A. D., Shaw L., Mauri-Sole I., Whitton M. E., Watts M. J., Anstey A. V., Ingham J., Young K. Guideline for the diagnosis and management of vitiligo // Br J Dermatol. – 2008. – Т. 159, № 5. – С. 1051-76.

16 Kumar Jha A., Sonthalia S., Lallas A., Chaudhary R. K. P. Dermoscopy in vitiligo: diagnosis and beyond // International Journal of Dermatology. – 2018. – Т. 57, № 1. – С. 50-54.

17 Cotterill J. A., Cunliffe W. J. Suicide in dermatological patients // Br J Dermatol. – 1997. – Т. 137, № 2. – С. 246-50.

18 Mattoo S. K., Handa S., Kaur I., Gupta N., Malhotra R. Psychiatric morbidity in vitiligo: prevalence and correlates in India // J Eur Acad Dermatol Venereol. – 2002. – Т. 16, № 6. – С. 573-8.

19 Parsad D., Dogra S., Kanwar A. J. Quality of life in patients with vitiligo // Health and Quality of Life Outcomes. – 2003. – Т. 1, № 1. – С. 58.

20 Ingordo V., Cazzaniga S., Gentile C., Iannazzone S. S., Cusano F., Naldi L. Dermatology Life Quality Index score in vitiligo patients: a pilot study among young Italian males // G Ital Dermatol Venereol. – 2012. – Т. 147, № 1. – С. 83-90.

21 Hill-Beuf A., Porter J. D. Children coping with impaired appearance: social and psychologic influences // Gen Hosp Psychiatry. – 1984. – Т. 6, № 4. – С. 294-301.

22 Kruger C., Schallreuter K. U. Cumulative life course impairment in vitiligo // Curr Probl Dermatol. – 2013. – Т. 44. – С. 102-17.

23 Shah R., Hunt J., Webb T. L., Thompson A. R. Starting to develop self-help for social anxiety associated with vitiligo: using clinical significance to measure the potential effectiveness of enhanced psychological self-help // British Journal of Dermatology. – 2014. – Т. 171, № 2. – С. 332-337.

24 Ezzedine K., Eleftheriadou V., Whitton M., van Geel N. Vitiligo // Lancet. – 2015. – Т. 386, № 9988. – С. 74-84.

25 Scherschun L., Kim J. J., Lim H. W. Narrow-band ultraviolet B is a useful and well-tolerated treatment for vitiligo // J Am Acad Dermatol. – 2001. – Т. 44, № 6. – С. 999-1003.

26 Olsson M. J., Juhlin L. Melanocyte transplantation in vitiligo // Lancet. – England, 1992. – С. 981.

27 Fabrikant J., Touloei K., Brown S. M. A review and update on melanocyte stimulating hormone therapy: afamelanotide // J Drugs Dermatol. – 2013. – Т. 12, № 7. – С. 775-9.

28 Gauthier Y., Cario Andre M., Taieb A. A critical appraisal of vitiligo etiologic theories. Is melanocyte loss a melanocytorrhagy? // Pigment Cell Res. – 2003. – Т. 16, № 4. – С. 322-32.

29 Spritz R. A. Modern vitiligo genetics sheds new light on an ancient disease // The Journal of Dermatology. – 2013. – Т. 40, № 5. – С. 310-318.

30 Mosenson J. A., Eby J. M., Hernandez C., Le Poole I. C. A central role for inducible heat-shock protein 70 in autoimmune vitiligo // Exp Dermatol. – 2013. – Т. 22, № 9. – С. 566-9.

31 Magen E., Delgado J. S. Helicobacter pylori and skin autoimmune diseases // World J Gastroenterol. – 2014. – Т. 20, № 6. – С. 1510-6.

32 Liu P. F., Hsieh Y. D., Lin Y. C., Two A., Shu C. W., Huang C. M. Propionibacterium acnes in the pathogenesis and immunotherapy of acne vulgaris // Curr Drug Metab. – 2015. – Т. 16, № 4. – С. 245-54.

33 Chang H. W., Yan D., Singh R., Liu J., Lu X., Ucmak D., Lee K., Afifi L., Fadrosh D., Leech J., Vasquez K. S., Lowe M. M., Rosenblum M. D., Scharschmidt T. C., Lynch S. V., Liao W. Alteration of the cutaneous microbiome in psoriasis and potential role in Th17 polarization // Microbiome. – 2018. – Т. 6, № 1. – С. 154.

34 Seddon O., Hughes H. Staphylococcus aureus and atopic dermatitis: a complex relationship // Br J Dermatol. – 2018. – Т. 178, № 6. – С. 1234.

35 Prescott S. L., Larcombe D. L., Logan A. C., West C., Burks W., Caraballo L., Levin M., Etten E. V., Horwitz P., Kozyrskyj A., Campbell D. E. The skin microbiome: impact of modern environments on skin ecology, barrier integrity, and systemic immune programming // World Allergy Organ J. – 2017. – Т. 10, № 1. – С. 29.

36 Ganju P., Nagpal S., Mohammed M. H., Nishal Kumar P., Pandey R., Natarajan V. T., Mande S. S., Gokhale R. S. Microbial community profiling shows dysbiosis in the lesional skin of Vitiligo subjects // Scientific Reports. – 2016. – Т. 6, № 1. – С. 18761.

37 Honda K., Littman D. R. The microbiota in adaptive immune homeostasis and disease // Nature. – 2016. – Т. 535, № 7610. – С. 75-84.

38 Duvallet C., Gibbons S. M., Gurry T., Irizarry R. A., Alm E. J. Meta-analysis of gut microbiome studies identifies disease-specific and shared responses //.

39 Greiling T. M., Dehner C., Chen X., Hughes K., Iniguez A. J., Boccitto M., Ruiz D. Z., Renfroe S. C., Vieira S. M., Ruff W. E., Sim S., Kriegel C., Glanternik J., Chen X., Girardi M., Degnan P., Costenbader K. H., Goodman A. L., Wolin S. L., Kriegel M. A. Commensal orthologs of the human autoantigen Ro60 as triggers of autoimmunity in lupus // Sci Transl Med. – 2018. – Т. 10, № 434.

40 Sivan A., Corrales L., Hubert N., Williams J. B., Aquino-Michaels K., Earley Z. M., Benyamin F. W., Lei Y. M., Jabri B., Alegre M. L., Chang E. B., Gajewski T. F. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy // Science. – 2015. – Т. 350, № 6264. – С. 1084-9.

41 Proal A. D., Albert P. J., Marshall T. G. The human microbiome and autoimmunity // Curr Opin Rheumatol. – 2013. – Т. 25, № 2. – С. 234-40.

42 Wu H. J., Ivanov, II, Darce J., Hattori K., Shima T., Umesaki Y., Littman D. R., Benoist C., Mathis D. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells // Immunity. – 2010. – Т. 32, № 6. – С. 815-27.

43 Tan T. G., Sefik E., Geva-Zatorsky N., Kua L., Naskar D., Teng F., Pasman L., Ortiz-Lopez A., Jupp R., Wu H.-J. J., Kasper D. L., Benoist C., Mathis D. //.

44 Chen W. Tregs in immunotherapy: opportunities and challenges // Immunotherapy. – 2011. – Т. 3, № 8. – С. 911-4.

45 Famili F., Wiekmeijer A. S., Staal F. J. The development of T cells from stem cells in mice and humans // Future Sci OA. – 2017. – Т. 3, № 3. – С. Fso186.

46 Kretschmer K., Apostolou I., Hawiger D., Khazaie K., Nussenzweig M. C., von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen // Nat Immunol. – 2005. – Т. 6, № 12. – С. 1219-27.

47 Gol-Ara M., Jadidi-Niaragh F., Sadria R., Azizi G., Mirshafiey A. The role of different subsets of regulatory T cells in immunopathogenesis of rheumatoid arthritis // Arthritis. – 2012. – Т. 2012. – С. 805875.

48 Gregori S., Roncarolo M. G. Engineered T Regulatory Type 1 Cells for Clinical Application // Front Immunol. – 2018. – Т. 9. – С. 233.

49 Komatsu N., Okamoto K., Sawa S., Nakashima T., Oh-hora M., Kodama T., Tanaka S., Bluestone J. A., Takayanagi H. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis // Nat Med. – 2014. – Т. 20, № 1. – С. 62-8.

50 Остапчук Е.О. Т. Р. Т., Каргажанова А., Мухатаев Ж.Е., Перфильева Ю.В. Натуральные Т-регуляторные клетки повышают пролиферативную активность и резистентность к действию противоопухолевых препаратов клеток линии эритролейкемии человека к562 // Серия биологическая. – 2019. – Т. 79. – С. 169-177.

51 Vignali D. A., Collison L. W., Workman C. J. How regulatory T cells work // Nat Rev Immunol. – 2008. – Т. 8, № 7. – С. 523-32.

52 Sojka D. K., Huang Y. H., Fowell D. J. Mechanisms of regulatory T-cell suppression - a diverse arsenal for a moving target // Immunology. – 2008. – Т. 124, № 1. – С. 13-22.

53 Klarquist J., Denman C. J., Hernandez C., Wainwright D. A., Strickland F. M., Overbeck A., Mehrotra S., Nishimura M. I., Le Poole I. C. Reduced skin homing by functional Treg in vitiligo // Pigment Cell Melanoma Res. – 2010. – Т. 23, № 2. – С. 276-86.

54 Mukhatayev Z., Ostapchuk Y.O. TRENDS OF RESEARCH IN VITILIGO // Scientific-Practical Journal of Medicine, Vestnik KazNMU. – 2018. – Т. 4, № 1. – С. 37-41.

55 Mehrotra S., Al-Khami A. A., Klarquist J., Husain S., Naga O., Eby J. M., Murali A. K., Lyons G. E., Li M., Spivey N. D., Norell H., Martins da Palma T., Onicescu G., Diaz-Montero C. M., Garrett-Mayer E., Cole D. J., Le Poole I. C., Nishimura M. I. A coreceptor-independent transgenic human TCR mediates anti-tumor and anti-self immunity in mice // J Immunol. – 2012. – Т. 189, № 4. – С. 1627-38.

56 Oren R., Hod-Marco M., Haus-Cohen M., Thomas S., Blat D., Duvshani N., Denkberg G., Elbaz Y., Benchetrit F., Eshhar Z., Stauss H., Reiter Y. Functional comparison of engineered T cells carrying a native TCR versus TCR-like antibody-based chimeric antigen receptors indicates affinity/avidity thresholds // J Immunol. – 2014. – Т. 193, № 11. – С. 5733-43.

57 Tsang J. Y., Tanriver Y., Jiang S., Xue S. A., Ratnasothy K., Chen D., Stauss H. J., Bucy R. P., Lombardi G., Lechler R. Conferring indirect allospecificity on CD4+CD25+ Tregs by TCR gene transfer favors transplantation tolerance in mice // J Clin Invest. – 2008. – Т. 118, № 11. – С. 3619-28.

58 MacDonald K. G., Hoeppli R. E., Huang Q., Gillies J., Luciani D. S., Orban P. C., Broady R., Levings M. K. Alloantigen-specific regulatory T cells generated with

a chimeric antigen receptor // J Clin Invest. – 2016. – T. 126, № 4. – C. 1413-24.

59 Tenspolde M., Zimmermann K., Weber L. C., Hapke M., Lieber M., Dywicki J., Frenzel A., Hust M., Galla M., Buitrago-Molina L. E., Manns M. P., Jaeckel E., Hardtke-Wolenski M. Regulatory T cells engineered with a novel insulin-specific chimeric antigen receptor as a candidate immunotherapy for type 1 diabetes // Journal of Autoimmunity. – 2019. – T. 103. – C. 102289.

60 Yoon J., Schmidt A., Zhang A. H., Konigs C., Kim Y. C., Scott D. W. FVIII-specific human chimeric antigen receptor T-regulatory cells suppress T- and B-cell responses to FVIII // Blood. – 2017. – T. 129, № 2. – C. 238-245.

61 Rosenzwajg M., Lorenzon R., Cacoub P., Pham H. P., Pitoiset F., El Soufi K., C R. I., Bernard C., Aractingi S., Banneville B., Beaugerie L., Berenbaum F., Champey J., Chazouilleres O., Corpechot C., Fautrel B., Mekinian A., Regnier E., Saadoun D., Salem J. E., Sellam J., Seksik P., Daguenel-Nguyen A., Doppler V., Mariau J., Vicaut E., Klatzmann D. Immunological and clinical effects of low-dose interleukin-2 across 11 autoimmune diseases in a single, open clinical trial // Ann Rheum Dis. – 2019. – T. 78, № 2. – C. 209-217.

62 Tahvildari M., Dana R. Low-Dose IL-2 Therapy in Transplantation, Autoimmunity, and Inflammatory Diseases // The Journal of Immunology. – 2019. – T. 203, № 11. – C. 2749.

63 Sauer S., Bruno L., Hertweck A., Finlay D., Leleu M., Spivakov M., Knight Z. A., Cobb B. S., Cantrell D., O'Connor E., Shokat K. M., Fisher A. G., Merkenschlager M. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR // Proc Natl Acad Sci U S A. – 2008. – T. 105, № 22. – C. 7797-802.

64 Schwaiger T., van den Brandt C., Fitzner B., Zaatreh S., Kraatz F., Dummer A., Nizze H., Evert M., Broker B. M., Brunner-Weinzierl M. C., Wartmann T., Salem T., Lerch M. M., Jaster R., Mayerle J. Autoimmune pancreatitis in MRL/Mp mice is a T cell-mediated disease responsive to cyclosporine A and rapamycin treatment // Gut. – 2014. – T. 63, № 3. – C. 494-505.

65 Wong V. W., You F., Januszyk M., Gurtner G. C., Kuang A. A. Transcriptional profiling of rapamycin-treated fibroblasts from hypertrophic and keloid scars // Ann Plast Surg. – 2014. – T. 72, № 6. – C. 711-9.

66 Wei K. C., Lai P. C. Combination of everolimus and tacrolimus: a potentially effective regimen for recalcitrant psoriasis // Dermatol Ther. – 2015. – T. 28, № 1. – C. 25-7.

67 Siklar Z., Karatas D., Dogu F., Hacihamdioglu B., Ikinciogullari A., Berberoglu M. Regulatory T Cells and Vitamin D Status in Children with Chronic Autoimmune Thyroiditis // J Clin Res Pediatr Endocrinol. – 2016. – T. 8, № 3. – C. 276-81.

68 Eby J. M., Kang H. K., Tully S. T., Bindeman W. E., Peiffer D. S., Chatterjee S., Mehrotra S., Le Poole I. C. CCL22 to Activate Treg Migration and Suppress Depigmentation in Vitiligo // J Invest Dermatol. – 2015. – T. 135, № 6. – C. 1574-1580.

69 Henning S. W., Fernandez M. F., Mahon J. P., Duff R., Azarafrooz F., Guevara-Patino J. A., Rademaker A. W., Salzman A. L., Le Poole I. C. HSP70iQ435A-

Encoding DNA Repigments Vitiligo Lesions in Sinclair Swine // J Invest Dermatol. – 2018. – T. 138, № 12. – C. 2531-2539.

70 Klarquist J., Tobin K., Farhangi Oskuei P., Henning S. W., Fernandez M. F., Dellacecca E. R., Navarro F. C., Eby J. M., Chatterjee S., Mehrotra S., Clark J. I., Le Poole I. C. Ccl22 Diverts T Regulatory Cells and Controls the Growth of Melanoma // Cancer research. – 2016. – T. 76, № 21. – C. 6230-6240.

71 Konieczna P., Akdis C. A., Quigley E. M., Shanahan F., O'Mahony L. Portrait of an immunoregulatory Bifidobacterium // Gut Microbes. – 2012. – T. 3, № 3. – C. 261-6.

72 Konieczna P., Groeger D., Ziegler M., Frei R., Ferstl R., Shanahan F., Quigley E. M., Kiely B., Akdis C. A., O'Mahony L. Bifidobacterium infantis 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells // Gut. – 2012. – T. 61, № 3. – C. 354-66.

73 O'Mahony C., Scully P., O'Mahony D., Murphy S., O'Brien F., Lyons A., Sherlock G., MacSharry J., Kiely B., Shanahan F., O'Mahony L. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation // PLoS Pathog. – 2008. – T. 4, № 8. – C. e1000112.

74 Dellacecca E. R., Cosgrove C., Mukhatayev Z., Akhtar S., Engelhard V. H., Rademaker A. W., Knight K. L., Le Poole I. C. Antibiotics Drive Microbial Imbalance and Vitiligo Development in Mice // J Invest Dermatol. – 2019.10.1016/j.jid.2019.08.435.

75 Trzonkowski P., Bacchetta R., Battaglia M., Berglund D., Bohnenkamp H. R., ten Brinke A., Bushell A., Cools N., Geissler E. K., Gregori S., Marieke van Ham S., Hilkens C., Hutchinson J. A., Lombardi G., Madrigal J. A., Marek-Trzonkowska N., Martinez-Caceres E. M., Roncarolo M. G., Sanchez-Ramon S., Saudemont A., Sawitzki B. Hurdles in therapy with regulatory T cells // Sci Transl Med. – 2015. – T. 7, № 304. – C. 304ps18.

76 Green E. A., Choi Y., Flavell R. A. Pancreatic lymph node-derived CD4(+)CD25(+) Treg cells: highly potent regulators of diabetes that require TRANCE-RANK signals // Immunity. – 2002. – T. 16, № 2. – C. 183-91.

77 Tang Q., Henriksen K. J., Bi M., Finger E. B., Szot G., Ye J., Masteller E. L., McDevitt H., Bonyhadi M., Bluestone J. A. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes // J Exp Med. – 2004. – T. 199, № 11. – C. 1455-65.

78 Tarbell K. V., Yamazaki S., Olson K., Toy P., Steinman R. M. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes // J Exp Med. – 2004. – T. 199, № 11. – C. 1467-77.

79 Masteller E. L., Warner M. R., Tang Q., Tarbell K. V., McDevitt H., Bluestone J. A. Expansion of functional endogenous antigen-specific CD4+CD25+ regulatory T cells from nonobese diabetic mice // J Immunol. – 2005. – T. 175, № 5. – C. 3053-9.

80 Tarbell K. V., Petit L., Zuo X., Toy P., Luo X., Mqadmi A., Yang H., Suthanthiran M., Mojsov S., Steinman R. M. Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD

mice // J Exp Med. – 2007. – T. 204, № 1. – C. 191-201.

81 Stephens L. A., Malpass K. H., Anderton S. M. Curing CNS autoimmune disease with myelin-reactive Foxp3+ Treg // Eur J Immunol. – 2009. – T. 39, № 4. – C. 1108-17.

82 Sanchez-Fueyo A., Sandner S., Habicht A., Mariat C., Kenny J., Degauque N., Zheng X. X., Strom T. B., Turka L. A., Sayegh M. H. Specificity of CD4+CD25+ regulatory T cell function in alloimmunity // J Immunol. – 2006. – T. 176, № 1. – C. 329-34.

83 Nishimura E., Sakihama T., Setoguchi R., Tanaka K., Sakaguchi S. Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+CD25+CD4+ regulatory T cells // Int Immunol. – 2004. – T. 16, № 8. – C. 1189-201.

84 Joffre O., Santolaria T., Calise D., Al Saati T., Hudrisier D., Romagnoli P., van Meerwijk J. P. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes // Nat Med. – 2008. – T. 14, № 1. – C. 88-92.

85 Golshayan D., Jiang S., Tsang J., Garin M. I., Mottet C., Lechler R. I. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance // Blood. – 2007. – T. 109, № 2. – C. 827-35.

86 Brunstein C. G., Blazar B. R., Miller J. S., Cao Q., Hippen K. L., McKenna D. H., Curtsinger J., McGlave P. B., Wagner J. E. Adoptive transfer of umbilical cord blood-derived regulatory T cells and early viral reactivation // Biol Blood Marrow Transplant. – 2013. – T. 19, № 8. – C. 1271-3.

87 Gross G., Waks T., Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity // Proceedings of the National Academy of Sciences. – 1989. – T. 86, № 24. – C. 10024.

88 Krebs S., Chow K. K., Yi Z., Rodriguez-Cruz T., Hegde M., Gerken C., Ahmed N., Gottschalk S. T cells redirected to interleukin-13Ralpha2 with interleukin-13 mutein--chimeric antigen receptors have anti-glioma activity but also recognize interleukin-13Ralpha1 // Cytotherapy. – 2014. – T. 16, № 8. – C. 1121-31.

89 Rapoport A. P., Stadtmauer E. A., Binder-Scholl G. K., Goloubeva O., Vogl D. T., Lacey S. F., Badros A. Z., Garfall A., Weiss B., Finklestein J., Kulikovskaya I., Sinha S. K., Kronsberg S., Gupta M., Bond S., Melchiori L., Brewer J. E., Bennett A. D., Gerry A. B., Pumphrey N. J., Williams D., Tayton-Martin H. K., Ribeiro L., Holdich T., Yanovich S., Hardy N., Yared J., Kerr N., Philip S., Westphal S., Siegel D. L., Levine B. L., Jakobsen B. K., Kalos M., June C. H. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma // Nat Med. – 2015. – T. 21, № 8. – C. 914-921.

90 Chandran S. S., Klebanoff C. A. T cell receptor-based cancer immunotherapy: Emerging efficacy and pathways of resistance // Immunol Rev. – 2019. – T. 290, № 1. – C. 127-147.

91 Hudecek M., Sommermeyer D., Kosasih P. L., Silva-Benedict A., Liu L., Rader C., Jensen M. C., Riddell S. R. The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity // Cancer Immunol

Res. – 2015. – T. 3, № 2. – C. 125-35.

92 Bridgeman J. S., Hawkins R. E., Bagley S., Blaylock M., Holland M., Gilham D. E. The optimal antigen response of chimeric antigen receptors harboring the CD3zeta transmembrane domain is dependent upon incorporation of the receptor into the endogenous TCR/CD3 complex // J Immunol. – 2010. – T. 184, № 12. – C. 6938-49.

93 Zhang C., Liu J., Zhong J. F., Zhang X. Engineering CAR-T cells // Biomark Res. – 2017. – T. 5. – C. 22.

94 Dotti G., Gottschalk S., Savoldo B., Brenner M. K. Design and development of therapies using chimeric antigen receptor-expressing T cells // Immunol Rev. – 2014. – T. 257, № 1. – C. 107-26.

95 Hartmann J., Schüßler-Lenz M., Bondanza A., Buchholz C. J. Clinical development of CAR T cells-challenges and opportunities in translating innovative treatment concepts // EMBO Mol Med. – 2017. – T. 9, № 9. – C. 1183-1197.

96 Kueberuwa G., Kalaitsidou M., Cheadle E., Hawkins R. E., Gilham D. E. CD19 CAR T Cells Expressing IL-12 Eradicate Lymphoma in Fully Lymphoreplete Mice through Induction of Host Immunity // Mol Ther Oncolytics. – 2018. – T. 8. – C. 41-51.

97 Halim L., Ajina A., Maher J. Pre-clinical development of chimeric antigen receptor T-cell immunotherapy: Implications of design for efficacy and safety // Best Practice & Research Clinical Haematology. – 2018. – T. 31, № 2. – C. 117-125.

98 Gill S., Maus M. V., Porter D. L. Chimeric antigen receptor T cell therapy: 25years in the making // Blood Reviews. – 2016. – T. 30, № 3. – C. 157-167.

99 The website of the National Cancer Institute (https://www.cancer.gov). – (дата обращения: 01/02/2020.2020).

100 Boardman D., Maher J., Lechler R., Smyth L., Lombardi G. Antigen-specificity using chimeric antigen receptors: the future of regulatory T-cell therapy? // Biochem Soc Trans. – 2016. – T. 44, № 2. – C. 342-8.

101 Hogquist K. A., Jameson S. C. The self-obsession of T cells: how TCR signaling thresholds affect fate 'decisions' and effector function // Nat Immunol. – 2014. – T. 15, № 9. – C. 815-23.

102 Bluestone J. A., Buckner J. H., Fitch M., Gitelman S. E., Gupta S., Hellerstein M. K., Herold K. C., Lares A., Lee M. R., Li K., Liu W., Long S. A., Masiello L. M., Nguyen V., Putnam A. L., Rieck M., Sayre P. H., Tang Q. Type 1 diabetes immunotherapy using polyclonal regulatory T cells // Sci Transl Med. – 2015. – T. 7, № 315. – C. 315ra189.

103 Marek-Trzonkowska N., Mysliwiec M., Iwaszkiewicz-Grzes D., Gliwinski M., Derkowska I., Zalinska M., Zielinski M., Grabowska M., Zielinska H., Piekarska K., Jazwinska-Curyllo A., Owczuk R., Szadkowska A., Wyka K., Witkowski P., Mlynarski W., Jarosz-Chobot P., Bossowski A., Siebert J., Trzonkowski P. Factors affecting long-term efficacy of T regulatory cell-based therapy in type 1 diabetes // J Transl Med. – 2016. – T. 14, № 1. – C. 332.

104 Ellebrecht C. T., Bhoj V. G., Nace A., Choi E. J., Mao X., Cho M. J., Di Zenzo G., Lanzavecchia A., Seykora J. T., Cotsarelis G., Milone M. C., Payne A. S.

Reengineering chimeric antigen receptor T cells for targeted therapy of autoimmune disease // Science. – 2016. – T. 353, № 6295. – C. 179-84.

105 Ellebrecht C. T., Payne A. S. Setting the target for pemphigus vulgaris therapy // JCI Insight. – 2017. – T. 2, № 5. – C. e92021.

106 Zhang G., Wang L., Cui H., Wang X., Ma J., Han H., He W., Wang W., Zhao Y., Liu C., Sun M., Gao B. Anti-melanoma activity of T cells redirected with a TCR-like chimeric antigen receptor // Sci Rep. – 2014. – T. 4. – C. 3571.

107 Tomas A S. S., Dellacecca E.R., Lanski N, Barse L, Zamora-Pineda J, Akhtar, Picken M, Scholtens D, Dilling D, Junghans R, Le Poole C. Loss of function of the TSC1-TSC2 complex renders tumors eligible for GD3 CART therapy // Journal for ImmunoTherapy of Cancer. – 2019. – C. 106.

108 Le Poole I. C., Stennett L. S., Bonish B. K., Dee L., Robinson J. K., Hernandez C., Hann S. K., Nickoloff B. J. Expansion of vitiligo lesions is associated with reduced epidermal CDw60 expression and increased expression of HLA-DR in perilesional skin // Br J Dermatol. – 2003. – T. 149, № 4. – C. 739-48.

109 Zhussipbek Mukhatayev K. P.-M., Jonathan M. Eby S. M., Richard Daniel, Peiffer R. J., I. Caroline Le Poole. Adoptive transfer of antigen-specific Tregs controls depigmentation in vitiligo-prone mice // PASPCR Annual Meeting 2019. – T. 1 –, 2019. – C. 31.

110 Mukhatayev Z.M. P.-M. K., Eby J.M., Peiffer D., Mehrotra S., Junghans R.P., Le Poole I.C. GD3 CAR-Tregs to mount antigen-specific immunosuppression in vitiligo // Pigment Cell Melanoma Res. – 2018. – T. 32. – C. 184.

111. DuPage M., Bluestone J. A. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease // Nat Rev Immunol. – 2016. – T. 16, № 3. – C. 149-63.

112 Serra P., Santamaria P. Antigen-specific therapeutic approaches for autoimmunity // Nat Biotechnol. – 2019. – T. 37, № 3. – C. 238-251.

113 Dwivedi M., Kemp E. H., Laddha N. C., Mansuri M. S., Weetman A. P., Begum R. Regulatory T cells in vitiligo: Implications for pathogenesis and therapeutics // Autoimmun Rev. – 2015. – T. 14, № 1. – C. 49-56.

114 Kundu R. V., Mhlaba J. M., Rangel S. M., Le Poole I. C. The convergence theory for vitiligo: A reappraisal // Exp Dermatol. – 2019. – T. 28, № 6. – C. 647-655.

115 Henning S. W., Jaishankar D., Barse L. W., Dellacecca E. R., Lancki N., Webb K., Janusek L., Mathews H. L., Price R. N., Jr., Le Poole I. C. The relationship between stress and vitiligo: Evaluating perceived stress and electronic medical record data // PLoS One. – 2020. – T. 15, № 1. – C. e0227909.

116 Grimes P. E., Miller M. M. Vitiligo: Patient stories, self-esteem, and the psychological burden of disease // Int J Womens Dermatol. – 2018. – T. 4, № 1. – C. 32-37.

117 Osinubi O., Grainge M. J., Hong L., Ahmed A., Batchelor J. M., Grindlay D., Thompson A. R., Ratib S. The prevalence of psychological comorbidity in people with vitiligo: a systematic review and meta-analysis // Br J Dermatol. – 2018. – T. 178, № 4. – C. 863-878.

118 Frisoli M. L., Essien K., Harris J. E. Vitiligo: Mechanisms of Pathogenesis and Treatment // Annu Rev Immunol. – 2020.10.1146/annurev-immunol-100919-023531.

119 Mosenson J. A., Flood K., Klarquist J., Eby J. M., Koshoffer A., Boissy R. E., Overbeck A., Tung R. C., Le Poole I. C. Preferential secretion of inducible HSP70 by vitiligo melanocytes under stress // Pigment Cell Melanoma Res. – 2014. – Т. 27, № 2. – С. 209-20.

120 Tulic M. K., Cavazza E., Cheli Y., Jacquel A., Luci C., Cardot-Leccia N., Hadhiri-Bzioueche H., Abbe P., Gesson M., Sormani L., Regazzetti C., Beranger G. E., Lereverend C., Pons C., Khemis A., Ballotti R., Bertolotto C., Rocchi S., Passeron T. Innate lymphocyte-induced CXCR3B-mediated melanocyte apoptosis is a potential initiator of T-cell autoreactivity in vitiligo // Nat Commun. – 2019. – Т. 10, № 1. – С. 2178.

121 Jacquemin C., Rambert J., Guillet S., Thiolat D., Boukhedouni N., Doutre M. S., Darrigade A. S., Ezzedine K., Blanco P., Taieb A., Boniface K., Seneschal J. Heat shock protein 70 potentiates interferon alpha production by plasmacytoid dendritic cells: relevance for cutaneous lupus and vitiligo pathogenesis // Br J Dermatol. – 2017. – Т. 177, № 5. – С. 1367-1375.

122 Le Poole I. C., Wankowicz-Kalinska A., van den Wijngaard R. M., Nickoloff B. J., Das P. K. Autoimmune aspects of depigmentation in vitiligo // J Investig Dermatol Symp Proc. – 2004. – Т. 9, № 1. – С. 68-72.

123 Bhardwaj S., Rani S., Kumaran M. S., Bhatia A., Parsad D. Expression of Th17- and Treg-specific transcription factors in vitiligo patients // Int J Dermatol. – 2020. – Т. 59, № 4. – С. 474-481.

124 Levings M. K., Sangregorio R., Roncarolo M. G. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function // J Exp Med. – 2001. – Т. 193, № 11. – С. 1295-302.

125 Zhang X., Olsen N., Zheng S. G. The progress and prospect of regulatory T cells in autoimmune diseases // J Autoimmun. – 2020.10.1016/j.jaut.2020.102461. – С. 102461.

126 Ferreira L. M. R., Muller Y. D., Bluestone J. A., Tang Q. Next-generation regulatory T cell therapy // Nat Rev Drug Discov. – 2019. – Т. 18, № 10. – С. 749-769.

127 Chatterjee S., Eby J. M., Al-Khami A. A., Soloshchenko M., Kang H. K., Kaur N., Naga O. S., Murali A., Nishimura M. I., Caroline Le Poole I., Mehrotra S. A quantitative increase in regulatory T cells controls development of vitiligo // J Invest Dermatol. – 2014. – Т. 134, № 5. – С. 1285-1294.

128 Serra P., Santamaria P. Nanoparticle-based autoimmune disease therapy // Clin Immunol. – 2015. – Т. 160, № 1. – С. 3-13.

129 Mathew J. M., Voss J. H., McEwen S. T., Konieczna I., Chakraborty A., Huang X., He J., Gallon L., Kornbluth R. S., Leventhal J. R. Generation and Characterization of Alloantigen-Specific Regulatory T Cells For Clinical Transplant Tolerance // Sci Rep. – 2018. – Т. 8, № 1. – С. 1136.

130 Dawson N. A. J., Levings M. K. Antigen-specific regulatory T cells: are

police CARs the answer? // Transl Res. – 2017. – T. 187. – C. 53-58.

131 Abbott R. C., Cross R. S., Jenkins M. R. Finding the Keys to the CAR: Identifying Novel Target Antigens for T Cell Redirection Immunotherapies // Int J Mol Sci. – 2020. – T. 21, № 2.

132 Brusko T. M., Koya R. C., Zhu S., Lee M. R., Putnam A. L., McClymont S. A., Nishimura M. I., Han S., Chang L. J., Atkinson M. A., Ribas A., Bluestone J. A. Human antigen-specific regulatory T cells generated by T cell receptor gene transfer // PLoS One. – 2010. – T. 5, № 7. – C. e11726.

133 Jin Y., Ferrara T., Gowan K., Holcomb C., Rastrou M., Erlich H. A., Fain P. R., Spritz R. A. Next-generation DNA re-sequencing identifies common variants of TYR and HLA-A that modulate the risk of generalized vitiligo via antigen presentation // J Invest Dermatol. – 2012. – T. 132, № 6. – C. 1730-3.

134 Kemminer S. E., Conradt H. S., Nimtz M., Sagi D., Peter-Katalinić J., Diekmann O., Drmić I., Müthing J. Production and molecular characterization of clinical phase i anti-melanoma mouse IgG3 monoclonal antibody R24 // Biotechnol Prog. – 2001. – T. 17, № 5. – C. 809-21.

135 Kniep B., Flegel W. A., Northoff H., Rieber E. P. CDw60 glycolipid antigens of human leukocytes: structural characterization and cellular distribution // Blood. – 1993. – T. 82, № 6. – C. 1776-86.

136 Nakano J., Yasui H., Lloyd K. O., Muto M. Biologic roles of gangliosides G(M3) and G(D3) in the attachment of human melanoma cells to extracellular matrix proteins // J Investig Dermatol Symp Proc. – 1999. – T. 4, № 2. – C. 173-6.

137 Birklé S., Gao L., Zeng G., Yu R. K. Down-regulation of GD3 ganglioside and its O-acetylated derivative by stable transfection with antisense vector against GD3-synthase gene expression in hamster melanoma cells: effects on cellular growth, melanogenesis, and dendricity // J Neurochem. – 2000. – T. 74, № 2. – C. 547-54.

138 Karagaiah P., Valle Y., Sigova J., Zerbinati N., Vojvodic P., Parsad D., Schwartz R. A., Grabbe S., Goldust M., Lotti T. Emerging drugs for the treatment of vitiligo // Expert Opin Emerg Drugs. – 2020. – T. 25, № 1. – C. 7-24.

139 Kouskoff V., Signorelli K., Benoist C., Mathis D. Cassette vectors directing expression of T cell receptor genes in transgenic mice // J Immunol Methods. – 1995. – T. 180, № 2. – C. 273-80.

140 Mukhatayev Z., Dellacecca E. R., Cosgrove C., Shivde R., Jaishankar D., Pontarolo-Maag K., Eby J.E., Ostapchuk Y.O., Cedercreutz K., Issanov A., Mehrotra S., Junghans R.P., Leventhal J.R., Le Poole I.C. Antigen specificity enhances disease control by Tregs in vitiligo // Front. in Immunol. – 2020. – T. 11, №1. – C. 581433.

141 Abnaof K., Mallela N., Walenda G., Meurer S. K., Seré K., Lin Q., Smeets B., Hoffmann K., Wagner W., Zenke M., Weiskirchen R., Fröhlich H. TGF-β stimulation in human and murine cells reveals commonly affected biological processes and pathways at transcription level // BMC Systems Biology. – 2014. – T. 8, № 1. – C. 55.

142 Tsang M. L., Zhou L., Zheng B. L., Wenker J., Fransen G., Humphrey J., Smith J. M., O'Connor-McCourt M., Lucas R., Weatherbee J. A. Characterization of recombinant soluble human transforming growth factor-beta receptor type II (rhTGF-

beta sRII) // Cytokine. – 1995. – T. 7, № 5. – C. 389-97.

143 Arkin M. R., Randal M., DeLano W. L., Hyde J., Luong T. N., Oslob J. D., Raphael D. R., Taylor L., Wang J., McDowell R. S., Wells J. A., Braisted A. C. Binding of small molecules to an adaptive protein–protein interface // Proceedings of the National Academy of Sciences. – 2003. – T. 100, № 4. – C. 1603.

144 Arenas-Ramirez N., Woytschak J., Boyman O. Interleukin-2: Biology, Design and Application // Trends Immunol. – 2015. – T. 36, № 12. – C. 763-777.

145 Lo A. S., Ma Q., Liu D. L., Junghans R. P. Anti-GD3 chimeric sFv-CD28/T-cell receptor zeta designer T cells for treatment of metastatic melanoma and other neuroectodermal tumors // Clin Cancer Res. – 2010. – T. 16, № 10. – C. 2769-80.

146 Lo A. S. Y., Ma Q., Liu D. L., Junghans R. P. Anti-GD3 Chimeric sFv-CD28/T-Cell Receptor ζ Designer T Cells for Treatment of Metastatic Melanoma and Other Neuroectodermal Tumors // Clinical Cancer Research. – 2010. – T. 16, № 10. – C. 2769.

147 Yun C. O., Nolan K. F., Beecham E. J., Reisfeld R. A., Junghans R. P. Targeting of T lymphocytes to melanoma cells through chimeric anti-GD3 immunoglobulin T-cell receptors // Neoplasia. – 2000. – T. 2, № 5. – C. 449-59.

148 Denman C. J., McCracken J., Hariharan V., Klarquist J., Oyarbide-Valencia K., Guevara-Patiño J. A., Caroline Le Poole I. HSP70i Accelerates Depigmentation in a Mouse Model of Autoimmune Vitiligo // Journal of Investigative Dermatology. – 2008. – T. 128, № 8. – C. 2041-2048.

149 Dwivedi M., Laddha N. C., Arora P., Marfatia Y. S., Begum R. Decreased regulatory T-cells and CD4(+) /CD8(+) ratio correlate with disease onset and progression in patients with generalized vitiligo // Pigment Cell Melanoma Res. – 2013. – T. 26, № 4. – C. 586-91.

150 Lopez P., Sanchez B., Margolles A., Suarez A. Intestinal dysbiosis in systemic lupus erythematosus: cause or consequence? // Curr Opin Rheumatol. – 2016. – T. 28, № 5. – C. 515-22.

151 Ash C., Mueller K. Manipulating the Microbiota // Science. – 2016. – T. 352, № 6285. – C. 530-1.

152 Blaser M. J. Antibiotic use and its consequences for the normal microbiome // Science. – 2016. – T. 352, № 6285. – C. 544-5.

153 Krych L., Hansen C. H., Hansen A. K., van den Berg F. W., Nielsen D. S. Quantitatively different, yet qualitatively alike: a meta-analysis of the mouse core gut microbiome with a view towards the human gut microbiome // PLoS One. – 2013. – T. 8, № 5. – C. e62578.

154 Schnupf P., Gaboriau-Routhiau V., Sansonetti P. J., Cerf-Bensussan N. Segmented filamentous bacteria, Th17 inducers and helpers in a hostile world // Curr Opin Microbiol. – 2017. – T. 35. – C. 100-109.

155 Salem I., Ramser A., Isham N., Ghannoum M. A. The Gut Microbiome as a Major Regulator of the Gut-Skin Axis // Front Microbiol. – 2018. – T. 9. – C. 1459.

156 Linehan J. L., Harrison O. J., Han S. J., Byrd A. L., Vujkovic-Cvijin I., Villarino A. V., Sen S. K., Shaik J., Smelkinson M., Tamoutounour S., Collins N., Bouladoux N., Dzutsev A., Rosshart S. P., Arbuckle J. H., Wang C. R., Kristie T. M.,

Rehermann B., Trinchieri G., Brenchley J. M., O'Shea J. J., Belkaid Y. Non-classical Immunity Controls Microbiota Impact on Skin Immunity and Tissue Repair // Cell. – 2018. – T. 172, № 4. – C. 784-796 e18.

157 Kalghatgi S., Spina C. S., Costello J. C., Liesa M., Morones-Ramirez J. R., Slomovic S., Molina A., Shirihai O. S., Collins J. J. Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in Mammalian cells // Sci Transl Med. – 2013. – T. 5, № 192. – C. 192ra85.

158 Mitra S., De Sarkar S., Pradhan A., Pati A. K., Pradhan R., Mondal D., Sen S., Ghosh A., Chatterjee S., Chatterjee M. Levels of oxidative damage and proinflammatory cytokines are enhanced in patients with active vitiligo // Free Radic Res. – 2017. – T. 51, № 11-12. – C. 986-994.

159 Byrd A. L., Belkaid Y., Segre J. A. The human skin microbiome // Nat Rev Microbiol. – 2018. – T. 16, № 3. – C. 143-155.

160 Vazquez-Mendoza P., Elghandour M. M. M., Alaba P. A., Sanchez-Aparicio P., Alonso-Fresan M. U., Barbabosa-Pliego A., Salem A. Z. M. Antimicrobial and bactericidal impacts of Bacillus amyloliquefaciens CECT 5940 on fecal shedding of pathogenic bacteria in dairy calves and adult dogs // Microb Pathog. – 2018. – T. 114. – C. 458-463.

161 Wu H. J., Ivanov I., Darce J., Hattori K., Shima T., Umesaki Y., Littman D. R., Benoist C., Mathis D. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells // Immunity. – 2010. – T. 32, № 6. – C. 815-27.

162 Rashighi M., Agarwal P., Richmond J. M., Harris T. H., Dresser K., Su M. W., Zhou Y., Deng A., Hunter C. A., Luster A. D., Harris J. E. CXCL10 is critical for the progression and maintenance of depigmentation in a mouse model of vitiligo // Sci Transl Med. – 2014. – T. 6, № 223. – C. 223ra23.

163 Nascimbeni M., Shin E. C., Chiriboga L., Kleiner D. E., Rehermann B. Peripheral CD4(+)CD8(+) T cells are differentiated effector memory cells with antiviral functions // Blood. – 2004. – T. 104, № 2. – C. 478-86.

164 Wekerle H. Brain Autoimmunity and Intestinal Microbiota: 100 Trillion Game Changers // Trends Immunol. – 2017. – T. 38, № 7. – C. 483-497.

165 Li B., Selmi C., Tang R., Gershwin M. E., Ma X. The microbiome and autoimmunity: a paradigm from the gut-liver axis // Cell Mol Immunol. – 2018. – T. 15, № 6. – C. 595-609.

166 Ekmekciu I., von Klitzing E., Fiebiger U., Escher U., Neumann C., Bacher P., Scheffold A., Kuhl A. A., Bereswill S., Heimesaat M. M. Immune Responses to Broad-Spectrum Antibiotic Treatment and Fecal Microbiota Transplantation in Mice // Front Immunol. – 2017. – T. 8. – C. 397.

167 Lui J. B., McGinn L. S., Chen Z. Gut microbiota amplifies host-intrinsic conversion from the CD8 T cell lineage to CD4 T cells for induction of mucosal immune tolerance // Gut Microbes. – 2016. – T. 7, № 1. – C. 40-7.

168 Buscarinu M. C., Romano S., Mechelli R., Pizzolato Umeton R., Ferraldeschi M., Fornasiero A., Renie R., Cerasoli B., Morena E., Romano C., Loizzo N. D., Umeton R., Salvetti M., Ristori G. Intestinal Permeability in Relapsing-Remitting

Multiple Sclerosis // Neurotherapeutics. – 2018. – Т. 15, № 1. – С. 68-74.

169 Walujkar S. A., Kumbhare S. V., Marathe N. P., Patangia D. V., Lawate P. S., Bharadwaj R. S., Shouche Y. S. Molecular profiling of mucosal tissue associated microbiota in patients manifesting acute exacerbations and remission stage of ulcerative colitis // World J Microbiol Biotechnol. – 2018. – Т. 34, № 6. – С. 76.

170 Yu P., Martin C. M. Increased gut permeability and bacterial translocation in Pseudomonas pneumonia-induced sepsis // Crit Care Med. – 2000. – Т. 28, № 7. – С. 2573-7.

171 Samborski P., Grzymislawski M. The Role of HSP70 Heat Shock Proteins in the Pathogenesis and Treatment of Inflammatory Bowel Diseases // Adv Clin Exp Med. – 2015. – Т. 24, № 3. – С. 525-30.

172 Takeuchi R., Kambe M., Miyata M., Jeyadevan U., Tajima O., Furukawa K., Furukawa K. TNFα-signal and cAMP-mediated signals oppositely regulate melanoma-associated ganglioside GD3 synthase gene in human melanocytes // Scientific Reports. – 2019. – Т. 9, № 1. – С. 14740.

173 Otake A. H., de Freitas Saito R., Duarte A. P. M., Ramos A. F., Chammas R. GD3 ganglioside-enriched extracellular vesicles stimulate melanocyte migration // Biochim Biophys Acta Mol Cell Biol Lipids. – 2019. – Т. 1864, № 3. – С. 422-432.

174 Wu X., Ragupathi G., Panageas K., Hong F., Livingston P. O. Accelerated tumor growth mediated by sublytic levels of antibody-induced complement activation is associated with activation of the PI3K/AKT survival pathway // Clin Cancer Res. – 2013. – Т. 19, № 17. – С. 4728-39.

175 Martelli M. F., Di Ianni M., Ruggeri L., Falzetti F., Carotti A., Terenzi A., Pierini A., Massei M. S., Amico L., Urbani E., Del Papa B., Zei T., Iacucci Ostini R., Cecchini D., Tognellini R., Reisner Y., Aversa F., Falini B., Velardi A. HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse // Blood. – 2014. – Т. 124, № 4. – С. 638-44.

176 Golovina T. N., Mikheeva T., Brusko T. M., Blazar B. R., Bluestone J. A., Riley J. L. Retinoic acid and rapamycin differentially affect and synergistically promote the ex vivo expansion of natural human T regulatory cells // PLoS One. – 2011. – Т. 6, № 1. – С. e15868.

177 Scottà C., Esposito M., Fazekasova H., Fanelli G., Edozie F. C., Ali N., Xiao F., Peakman M., Afzali B., Sagoo P., Lechler R. I., Lombardi G. Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4(+)CD25(+)FOXP3(+) T regulatory cell subpopulations // Haematologica. – 2013. – Т. 98, № 8. – С. 1291-9.

178 Fraser H., Safinia N., Grageda N., Thirkell S., Lowe K., Fry L. J., Scotta C., Hope A., Fisher C., Hilton R., Game D., Harden P., Bushell A., Wood K., Lechler R. I., Lombardi G. A Rapamycin-Based GMP-Compatible Process for the Isolation and Expansion of Regulatory T Cells for Clinical Trials // Mol Ther Methods Clin Dev. – 2018. – Т. 8. – С. 198-209.

179 Thomson A. W., Turnquist H. R., Raimondi G. Immunoregulatory functions of mTOR inhibition // Nat Rev Immunol. – 2009. – Т. 9, № 5. – С. 324-37.

180 Battaglia M., Stabilini A., Migliavacca B., Horejs-Hoeck J., Kaupper T., Roncarolo M. G. Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients // J Immunol. – 2006. – Т. 177, № 12. – С. 8338-47.

181 Алжанұлы Б. Мухатаев Ж. Е., Ботбаев Д.М, Хансейітова А.К. . Адам жасушаларынан инсулин синтездеуші жасушалар жасау // Вестник, Научно-практический журнал –2019. – Т. 3. – С. 215-218.

182 Алжанулы Б Мухатаев Ж. Е., Ботбаев Д.М., Шарипов К.О. Получение инсулин синтезирующих β-подобных клеток из человеческих клеток для клеточной терапии против сахарного диабета // Международная научная конференция молодых ученых «Фундаментальные исследования и инновации в молекулярной биологии, биотехнологии, биохимии» к 80-летию со дня рождения академика Мурата Абеновича Айтхожина. – 2019. – С. 71.

183 Mathew J. M., J H. V., LeFever A., Konieczna I., Stratton C., He J., Huang X., Gallon L., Skaro A., Ansari M. J., Leventhal J. R. A Phase I Clinical Trial with Ex Vivo Expanded Recipient Regulatory T cells in Living Donor Kidney Transplants // Sci Rep. – 2018. – Т. 8, № 1. – С. 7428.

184 Putnam A. L., Safinia N., Medvec A., Laszkowska M., Wray M., Mintz M. A., Trotta E., Szot G. L., Liu W., Lares A., Lee K., Laing A., Lechler R. I., Riley J. L., Bluestone J. A., Lombardi G., Tang Q. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation // Am J Transplant. – 2013. – Т. 13, № 11. – С. 3010-20.

185 Schmidt A., Oberle N., Krammer P. H. Molecular mechanisms of treg-mediated T cell suppression // Front Immunol. – 2012. – Т. 3. – С. 51.

186 Eby J. M., Kang H. K., Klarquist J., Chatterjee S., Mosenson J. A., Nishimura M. I., Garrett-Mayer E., Longley B. J., Engelhard V. H., Mehrotra S., Le Poole I. C. Immune responses in a mouse model of vitiligo with spontaneous epidermal de- and repigmentation // Pigment Cell Melanoma Res. – 2014. – Т. 27, № 6. – С. 1075-85.

187 Putnam A. L., Brusko T. M., Lee M. R., Liu W., Szot G. L., Ghosh T., Atkinson M. A., Bluestone J. A. Expansion of human regulatory T-cells from patients with type 1 diabetes // Diabetes. – 2009. – Т. 58, № 3. – С. 652-62.

188 Marek-Trzonkowska N., Mysliwiec M., Dobyszuk A., Grabowska M., Techmanska I., Juscinska J., Wujtewicz M. A., Witkowski P., Mlynarski W., Balcerska A., Mysliwska J., Trzonkowski P. Administration of CD4+CD25highCD127-regulatory T cells preserves β-cell function in type 1 diabetes in children // Diabetes Care. – 2012. – Т. 35, № 9. – С. 1817-20.

189 Marek-Trzonkowska N., Myśliwec M., Siebert J., Trzonkowski P. Clinical application of regulatory T cells in type 1 diabetes // Pediatr Diabetes. – 2013. – Т. 14, № 5. – С. 322-32.

190 Marek-Trzonkowska N., Mysliwiec M., Dobyszuk A., Grabowska M., Derkowska I., Juscinska J., Owczuk R., Szadkowska A., Witkowski P., Mlynarski W., Jarosz-Chobot P., Bossowski A., Siebert J., Trzonkowski P. Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic

islets - results of one year follow-up // Clin Immunol. – 2014. – T. 153, № 1. – C. 23-30.

191 Ben Ahmed M., Zaraa I., Rekik R., Elbeldi-Ferchiou A., Kourda N., Belhadj Hmida N., Abdeladhim M., Karoui O., Ben Osman A., Mokni M., Louzir H. Functional defects of peripheral regulatory T lymphocytes in patients with progressive vitiligo // Pigment Cell Melanoma Res. – 2012. – T. 25, № 1. – C. 99-109.

192 Lili Y., Yi W., Ji Y., Yue S., Weimin S., Ming L. Global activation of CD8+ cytotoxic T lymphocytes correlates with an impairment in regulatory T cells in patients with generalized vitiligo // PLoS One. – 2012. – T. 7, № 5. – C. e37513.

193 Raffin C., Vo L. T., Bluestone J. A. T(reg) cell-based therapies: challenges and perspectives // Nat Rev Immunol. – 2020. – T. 20, № 3. – C. 158-172.

194 Bluestone J. A., Tang Q. T(reg) cells-the next frontier of cell therapy // Science. – 2018. – T. 362, № 6411. – C. 154-155.

195 Trenado A., Charlotte F., Fisson S., Yagello M., Klatzmann D., Salomon B. L., Cohen J. L. Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia // J Clin Invest. – 2003. – T. 112, № 11. – C. 1688-96.

196 Sagoo P., Ali N., Garg G., Nestle F. O., Lechler R. I., Lombardi G. Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells // Sci Transl Med. – 2011. – T. 3, № 83. – C. 83ra42.

197 Noyan F., Lee Y. S., Hardtke-Wolenski M., Knoefel A. K., Taubert R., Baron U., Manns M. P., Jaeckel E. Donor-specific regulatory T cells generated on donor B cells are superior to CD4+CD25high cells in controlling alloimmune responses in humanized mice // Transplant Proc. – 2013. – T. 45, № 5. – C. 1832-7.

198 Liu L. Y., Strassner J. P., Refat M. A., Harris J. E., King B. A. Repigmentation in vitiligo using the Janus kinase inhibitor tofacitinib may require concomitant light exposure // J Am Acad Dermatol. – 2017. – T. 77, № 4. – C. 675-682.e1.

199 MacDonald K. N., Ivison S., Hippen K. L., Hoeppli R. E., Hall M., Zheng G., Dijke I. E., Aklabi M. A., Freed D. H., Rebeyka I., Gandhi S., West L. J., Piret J. M., Blazar B. R., Levings M. K. Cryopreservation timing is a critical process parameter in a thymic regulatory T-cell therapy manufacturing protocol // Cytotherapy. – 2019. – T. 21, № 12. – C. 1216-1233.

200 Boroughs A. C., Larson R. C., Choi B. D., Bouffard A. A., Riley L. S., Schiferle E., Kulkarni A. S., Cetrulo C. L., Ting D., Blazar B. R., Demehri S., Maus M. V. Chimeric antigen receptor costimulation domains modulate human regulatory T cell function // JCI Insight. – 2019. – T. 4, № 8.

201 Watanabe K., Terakura S., Martens A. C., van Meerten T., Uchiyama S., Imai M., Sakemura R., Goto T., Hanajiri R., Imahashi N., Shimada K., Tomita A., Kiyoi H., Nishida T., Naoe T., Murata M. Target antigen density governs the efficacy of anti-CD20-CD28-CD3 ζ chimeric antigen receptor-modified effector CD8+ T cells // J Immunol. – 2015. – T. 194, № 3. – C. 911-20.

202 Walker A. J., Majzner R. G., Zhang L., Wanhainen K., Long A. H., Nguyen S. M., Lopomo P., Vigny M., Fry T. J., Orentas R. J., Mackall C. L. Tumor Antigen and Receptor Densities Regulate Efficacy of a Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase // Mol Ther. – 2017. – Т. 25, № 9. – С. 2189-2201.

203 Chmielewski M., Hombach A. A., Abken H. //.

204 Raffin C., Zhou Y., Piccoli L., Lanzavecchia A., Sadelain M., Bluestone J. A. Development of citrullinated-vimentin-specific CAR for targeting Tregs to treat autoimmune rheumatoid arthritis // The Journal of Immunology. – 2016. – Т. 196, № 1 Supplement. – С. 210.19.

205 Hua J., Inomata T., Chen Y., Foulsham W., Stevenson W., Shiang T., Bluestone J. A., Dana R. Pathological conversion of regulatory T cells is associated with loss of allotolerance // Sci Rep. – 2018. – Т. 8, № 1. – С. 7059.

206 Junghans R. P. The challenges of solid tumor for designer CAR-T therapies: a 25-year perspective // Cancer Gene Therapy. – 2017. – Т. 24, № 3. – С. 89-99.

207 Jones B. S., Lamb L. S., Goldman F., Di Stasi A. Improving the safety of cell therapy products by suicide gene transfer // Front Pharmacol. – 2014. – Т. 5. – С. 254.

208 Rossignoli F., Grisendi G., Spano C., Golinelli G., Recchia A., Rovesti G., Orsi G., Veronesi E., Horwitz E. M., Dominici M. Inducible Caspase9-mediated suicide gene for MSC-based cancer gene therapy // Cancer Gene Ther. – 2019. – Т. 26, № 1-2. – С. 11-16.

209 Paller A. S., Arnsmeier S. L., Alvarez-Franco M., Bremer E. G. Ganglioside GM3 inhibits the proliferation of cultured keratinocytes // J Invest Dermatol. – 1993. – Т. 100, № 6. – С. 841-5.

210 Huang B. B., Bonish B. K., Chaturvedi V., Qin J. Z., Nickoloff B. J. Keratinocyte CDw60 expression is modulated by both a Th-1 type cytokine IFN-gamma and Th-2 cytokines IL-4 and IL-13: relevance to psoriasis // J Invest Dermatol. – 2001. – Т. 116, № 2. – С. 305-12.

211 Safinia N., Vaikunthanathan T., Fraser H., Thirkell S., Lowe K., Blackmore L., Whitehouse G., Martinez-Llordella M., Jassem W., Sanchez-Fueyo A., Lechler R. I., Lombardi G. Successful expansion of functional and stable regulatory T cells for immunotherapy in liver transplantation // Oncotarget. – 2016. – Т. 7, № 7. – С. 7563-77.

212 Hippen K. L., Merkel S. C., Schirm D. K., Sieben C. M., Sumstad D., Kadidlo D. M., McKenna D. H., Bromberg J. S., Levine B. L., Riley J. L., June C. H., Scheinberg P., Douek D. C., Miller J. S., Wagner J. E., Blazar B. R. Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity // Sci Transl Med. – 2011. – Т. 3, № 83. – С. 83ra41.

213 Weiner J., Duran-Struuck R., Zitsman J., Buhler L., Sondermeijer H., McMurchy A. N., Levings M. K., Sykes M., Griesemer A. Restimulation After Cryopreservation and Thawing Preserves the Phenotype and Function of Expanded Baboon Regulatory T Cells // Transplant Direct. – 2015. – Т. 1, № 1. – С. 1-7.

214 Guo H., Zhang H., Lu L., Ezzelarab M. B., Thomson A. W. Generation, cryopreservation, function and in vivo persistence of ex vivo expanded cynomolgus monkey regulatory T cells // Cell Immunol. – 2015. – Т. 295, № 1. – С. 19-28.

215 Koristka S., Kegler A., Bergmann R., Arndt C., Feldmann A., Albert S., Cartellieri M., Ehninger A., Ehninger G., Middeke J. M., Bornhäuser M., Schmitz M., Pietzsch J., Akgün K., Ziemssen T., Steinbach J., Bachmann M. P. Engrafting human regulatory T cells with a flexible modular chimeric antigen receptor technology // J Autoimmun. – 2018. – T. 90. – C. 116-131.

APPENDICES

Appendix 1.

Attorney Docket No. 30938/2019-172P
NU Reference No. 2019-172-01

ASSIGNMENT

Application Serial No: 62/915,945 Filing Date: 10/16/2019
Inventors: Isabelle Caroline Le Poole, Zhussipbek Mukhatayev
Title of Patent Application: Materials and Methods for Treating Vitiligo

For good and valuable consideration, the receipt and sufficiency of which are hereby agreed, each of the undersigned hereby assigns to Northwestern University, an Illinois not-for-profit corporation, having a place of business at 633 Clark Street, Evanston, Illinois 60208 and its successors and assigns ("Assignee") the entire right, title and interest in and to the inventions of such undersigned disclosed in the application listed above, and in any and all other applications anywhere in the world which the undersigned may file and/or be named as an inventor of, solely or jointly, on said inventions, in any and all patents which may be obtained on any of said applications and in any and all reissues, reexaminations, supplemental examinations, inter partes reviews, oppositions, post-grant reviews, supplementary protection certificates and/or extensions thereof, including without limitation, the right to bring suit and to claim and retain all damages and/or seek other remedies for the past, present and future infringement of any of the foregoing, the right of priority, including without limitation to claim priority benefit of or to said patent applications, and request the Commissioner for Patents in the United States and similar authorities outside the United States to issue said patents to and in the name of the Assignee.

Each of the undersigned states that the application listed above is or was made or authorized to be made by him or her. Each of the undersigned believes himself or herself to be the original inventor or a joint inventor with another undersigned of a claimed invention in the application listed above. Each of the undersigned acknowledges that any willful false statement made by him or her in this paragraph is punishable under 18 U.S.C. §1001 by fine or imprisonment of not more than five years, or both.

Except in favor of Assignee, each of the undersigned warrants that: (i) he or she is the owner of all its rights, titles and interests herein assigned and has the right to make this unconditional and irrevocable assignment to Assignee without obtaining any approval or permission of a third party; and (ii) there are no outstanding encumbrances, liens, prior assignments, licenses, or other obligations or restrictions on the rights, titles and interests herein assigned.

Upon the request of Assignee and at no expense to the undersigned, each of the undersigned hereby agrees to execute any and all applications on said inventions, including without limitation for the reissue, reexamination, supplementary protection certificate or extension thereof and any oath, declaration or

affidavit relating thereto that said Assignee may deem necessary or expedient, and to cooperate to the best of the ability of the undersigned with and perform any and all affirmative acts requested by Assignee to prepare, file, prosecute, maintain, defend, enforce and vest in Assignee the rights, titles and interests assigned herein, including without limitation, preparing and executing statements and giving and producing evidence in support thereof, whereby said rights, titles and interests will be held and enjoyed by said Assignee to the full end of the term for which said patents may be granted as fully and entirely as the same would have been held and enjoyed by the undersigned if this assignment had not been made.

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Attorney Docket No. 30938/2019-172P
NU Reference No. 2019-172-01

Application Serial No: 62/915,945
Filing Date: 10/16/2019
Inventors: Isabelle Caroline Le Poole, Zhussipbek Mukhatayev
Title of Patent Application: Materials and Methods for Treating Vitiligo

Undersigned (ASSIGNORS) continued:

Inventor Name: Zhussipbek Mukhatayev — 11/24/2019 DATE

State of Commonwealth of Virginia

County of Arlington ss.

On this 24th day of November 2019, before me, a Notary Public in and for the County and State aforesaid, appeared, to me personally known to be the same person whose name is subscribed to the foregoing instrument, and acknowledged that he or she executed said instrument as his or her free and voluntary act and for the uses and purposes therein expressed.

[NOTARY SEAL] KSENIA KASHEVA NOTARY PUBLIC COMMONWEALTH OF VIRGINIA REGISTRATION # 7720135

NOTARY PUBLIC

My Commission Expires: 01-31-2020

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