JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2017, 68, 6, 897-906
www.jpp.krakow.pl

K.M. OKONIEWSKA[1,5], A.E. KEDZIERSKA[2], J. OKONIEWSKI[1], A. SLAWEK[2],
K. GRZYMAJLO[3], A. CHELMONSKA-SOYTA[2,3], T. GRABOWSKI[4]

NEW TREGITOPES INDUCING ADAPTIVE REGULATORY T CELLS IN MICE

[1]P.F.O. Vetos-Farma sp. z o.o., Bielawa, Poland; [2]Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland; [3]Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland; [4]Polpharma Biologics, Gdansk, Poland; [5]Department of Clinical Pharmacology, Wroclaw Medical University, Wroclaw, Poland

Epitopes of regulatory T cells (tregitopes) represent linear sequences of amino acids that induce $CD4^+CD25^+Foxp3^+$ T lymphocytes expansion both *in vitro* and *in vivo*. The tregitopes' effectiveness was confirmed in autoimmune disease mouse models and in murine transplant models. Therefore, tregitopes together with regulatory T cells (Tregs) could play a major role in maintaining immune tolerance. The purpose of the presented study was a selection of potential tregitopes and assessment of their impact on Tregs expansion. Eight peptides were selected based on the previously published *in silico* model and their immunotolerogenic functions. To verify, if selected peptides are potential TCR ligands, the affinity of selected peptides to overrepresented in patients with autoimmune diseases, HLA-DRB1*04:01 allele, was measured by surface plasmon resonance. In order to evaluate the impact of potential tregitopes on the induction of Tregs in *in vitro* conditions, $C57BL6Foxp3^{GFP}$ mouse antigen presenting cells were co-cultured with naïve syngeneic T cells under stimulation of selected peptides. $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^+Foxp3^+IL\text{-}10^+$ cells frequency was analyzed using flow cytometry. Based on Tregs induction, two tregitopes derived from yeast and adenovirus protein were identified. In summary, the performed studies allowed an identification of novel putative tregitopes, which application potential includes their use as immunomodulators in mice.

Key words: *tregitope, Saccharomyces, adenovirus, autoimmunity, regulatory T cells,* major histocompatibility complex class II

INTRODUCTION

Regulatory T cells (Tregs) play a major role in maintaining the tolerance to self-antigens, controlling immunopathogenic reactions to infections, establishing the immune privilege during pregnancy and promoting the transplant tolerance (1-4). Each Treg expresses the transcription factor Foxp3 (forkhead box P3), which is a master regulator of the pathway in the development and function of Tregs (5). In Tregs, Foxp3 may be expressed *ab initio* during T lymphocytes' maturation in the thymus (natural Tregs) or induced in the periphery in the presence of specialized antigen presenting cells (APC) and cytokines such as interleukin-2 (IL-2) and transforming growth factor-β (TGF-β) (induced or adaptive Tregs) (6). Natural Tregs are mainly responsible for the maintenance of general homeostasis whereas adaptive Tregs (aTregs) are generated for the control of local inflammatory response (7).

Tregitopes are Tregs' epitopes that are responsible for the suppression of immune response's effector phase towards own antigens. De Groot *et al.* published first reports on tregitopes and indicated that two sequences present in the immunoglobulin G (IgG) fraction were able to induce the expansion of Tregs (8). Tregitopes as IgG-derived Tregs epitopes can be recognized by the major histocompatibility complex class II (MHC II) after immunoglobulin internalization and processing by APC. The sequences comprising a few amino acids that remain after digestive intracellular processing could be bound within the MHC-binding groove and presented to Tregs, resulting in their activation and proliferation (9, 10). So far the effects of tregitopes were confirmed in a number of mouse models such as: reduction of the incidence of diabetes in NOD mice (11), preventing the skin allograft rejection (12) and suppression of experimental autoimmune encephalomyelitis (EAE) in animal model for multiple sclerosis (MS) by deactivation of Th17 cells (13). Moreover, tregitopes effectively induce Tregs in human peripheral blood mononuclear cells that have been derived from patients allergic to either house dust mite *Dermatophagoides pteronyssinus* or to the major birch tree allergen (8). Putative tregitopes' sequences were also identified in other self-proteins like albumin, fibrinogen and osteocalcin (8, 14). It is also known that bacteria, viruses, fungi and parasites contain tolerogenic proteins (15, 19), that may be the source of putative tregitopes' sequences. It was stated that *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Streptococcus thermophilus*, *Bifidobacterium longum*, *Bifidobacterium lactis* and *Escherichia coli* have influence on the level of cytokines (TGF-β, IL-17) involved in a regulation of Foxp3 expression and induction of Tregs (16). Moreover, it was noted that *Borrelia burgdorferi* and *Vibrio cholerae* promote the tolerogenicity of dendritic cells and induce Tregs differentiation (20, 21). Even though the aforementioned bacteria modulate a function of the host immune system, the possible involvement of their tolerogenic compounds has not been fully elucidated.

The presence of tregitopes in drug molecules may demonstrate beneficial as well as detrimental effects on their therapeutic potential (11, 22, 23). For instance, applying the tregitopes that suppress drug immunogenicity in the course IgG therapy, could increase drug safety (24). On the other hand, the removal of tregitopes' sequences from the vaccine proteins reduces their tolerogenic activity and enhances the efficacy of vaccination (25).

Tregitopes possess significant regulatory function in the course of autoimmune diseases verified in the human *in vitro* and mice in *vivo studies* (1-7). They are alternatives to typical immunoglobulin (IVIG) therapy, which is associated with a number of side effects, such as headaches, tachycardia, diarrhea, toxic epidermal necrolysis, meningitis and even acute renal failure (26). Therefore, it is highly probable that tregitopes have the potential to be utilized in the future in the treatment of autoimmune diseases (24).

Based on these observations, the search for new structures with a function similar to currently known tregitopes is in progress. The purpose of the presented study was the selection of new potential tregitopes and their impact on Tregs expansion. The examined peptides were identified and selected from human, rat and microbial proteins. Here, we verified whether our selected peptides derived from non-self proteins induced differentiation and expansion of mouse Tregs to an extent comparable to previously described tregitopes identified by de Groot *et al.* (8).

MATERIALS AND METHODS

Peptides selection

On the grounds of the earlier created *in silico* mathematical model of dependency between chemical structure of tregitopes and binding strength with MHC class I, published in own studies (27), a novel similar *in silico* model in the context of DRB1*04:01 was formulated. The parameters that were taken into consideration in this model, were as follows: minimal value of binding strength with MHC II, isoelectric point, hydrophobicity index, aliphatic index, number of negatively and positively charged residues, hydrogen bond donors and topological polar surface area. Aforementioned parameters were calculated for all selected peptides. The creation of the model was based on the *in silico* analysis of sixteen amino acid sequences of immunoglobulin G with tolerogenic function similar to tregitopes. The linear validated correlation between binding strength with MHC II and physicochemical properties has been suggested instead of present epitope cluster classification (unpublished data). Based on the mathematical model, seven peptides obtained from the proteins demonstrating the impact on the immune system were chosen for the *in vitro* studies. Additionally, one protein without verified impact on immune response that met the model criteria - Nwd2 protein from *Rattus norvegicus* was included into the study (protein code: A0A0G2JZT1, peptide sequence: NHHNMLLSLST SGVL, peptide abbreviation: NHH). Unfortunately, the Authors are not able to publish another part of the studies concerning the *in silico* model of the relationship between regulatory T cell epitope structure and binding to the major histocompatibility complex class II as it is undergoing the patent procedure (patent application number: PCT/PL2017/0050054).

Analysis of real-time interaction of tregitopes with MHC II by surface plasmon resonance

The binding of peptides to MHC II DRB1*04:01 (TCMetrix, Switzerland) immobilized on NTA (GE Healthcare) sensor chips, was analyzed by surface plasmon resonance (SPR) using BIAcore

Table 1. Characteristics of examined peptides selected for the *in vitro* analysis.

Protein code	Protein name	Peptide sequence	Peptide abbreviation	Impact on the immune system	References
P01857	region C of human IgG, chain 1	EEQYNSTYRVVSVLTV LHQDW	EEQ	positive control - verified impact on immune response modulation	(11)
P03244	E1B 55 kDa protein of human adenovirus C, serotype 2 (HAdV-2)	FSGTVFLANTNLILH	FSG	adenoviral vectors as means of circumventing of innate immune responses induction	(30)
P52960	peroxisome proliferation transcriptional regulator from *S.cerevisiae*	WLSIISMATLESSLK	WLS	regulation of host immune response as a mechanism of probiotics action	(31)
C7DQA2	external surface of protein A from *B.bavariensis*	LKDFALEGTLAADKT	LKD	presence of Treg during the *B. burgdorferi sensu lato* infection	(20)
Q93009	human hydrolase 7 C of the end of ubiquitin	DELLECLSPATSRTF	DEL	increase of Treg suppressive capacity by stabilizing transcription factor Foxp3	(28)
Q5H9R7	subunit 3 of human phosphatase 6 of proteins serine/threonine	LMKLYSFLLNDSPLN	LMK	potential important role in maintaining immune self-tolerance	(32)
Q9UQC1	human heat shock protein 72	LNVLRIINEPTAAAI	LNV	indirect or direct stimulation of Tregs	(33)
T2BRB8	subunit of cholera toxin A from *V.cholerae*	SLRSAHLVGQTILSG	SLR	promotion of dendritic cells tolerogenicity and Treg differentiation	(21)
Edratide	synthetized peptide based on the complementarity-determining region 1 of a human anti-DNA mAb	GYYWSWIRQPPGKGEE WIG	GYY	lack of impact on immune response and efficacy of the drug	(34)

IgG, immunoglobulin G; Treg, regulatory T cells; Foxp3, transcription factor of regulatory T cells; mAb, monoclonal antibody; kDa, kilodalton; single-letter amino acid symbols: A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid, G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan, Y, tyrosine; V, valine.

T200 (GE Healthcare). All binding experiments were carried out at 25ºC with a 30 µL/min flow rate. To determine peptides' affinity to the immobilized protein, 150 µL of five different concentrations (0, 50, 100, 200, 400 nM) of peptides' solutions (*Table 1*), as well as a sample buffer blank, were passed over the ligand-immobilized chip surface (association phase) for 240 seconds, followed by 720 seconds of dissociation with the running buffer. The same samples were passed over a control chip surface without immobilized ligand. Three replicates of each analyte concentration were injected. The resulting sensograms were obtained first by subtracting the buffer blank from curves recorded for the interactions of peptides with immobilized protein. Then the curves recorded when peptides were passed over the blank surface were subtracted from such sensograms. The equilibrium constants were determined using BIAevaluation software. For global fitting a 1:1 Langmuir binding model with an included mass transport step was applied based on criteria provided by the BIAevaluation handbook.

As a result of SPR analysis, kinetic parameters of peptides' affinity to MHC II were determined. Among these kinetic parameters were: association rate (k_a) and dissociation rate (k_d), that enabled calculation of binding affinity constant $K_D = k_d / k^a$.

In vitro studies

The homology analysis, mentioned in the patent of Epivax by de Groot *et al.* (14), has shown a tregitopes' conservation across non-human species such as mouse, rat, cat, camel, cow and non-human primates. On this basis, we investigated the impact of selected peptides on mouse cells (13).

Peptides

In vitro T lymphocytes stimulation was performed with the use of all designed peptides (*Table 1*). The purity of peptides was in the range of 90.07% to 95.24% (*Table 2*). A positive control was tregitope 289 (EEQ) with confirmed regulatory function (14) and a negative control was a cell culture stimulated without any peptides. To verify the impact of utilized peptides' solvents on Tregs proliferation, the cells were also cultured with the same combination and concentration of added solvents.

On the basis of the molecular charge differences, three groups of peptides were identified (with negative total charge, neutral total charge, positive total charge). Each of peptides was dissolved according to the manufacturer's recommendations (GeneCust Europe Laboratoire de Biotechnologie du Luxembourg S.A.) (*Table 2*).

Animals

Thirty adult (6 – 8 week-old) female of C57BL6 Foxp3^{GFP} strain were used in an experiment. These transgenic mice expressed green fluorescent protein (GFP) under control of the mouse Foxp3 promoter. It was shown that intensity of the GFP fluorescence was proportional to the Foxp3 transcript level. Thus, it allowed the identification of Tregs by the level of expression of the GFP.

All of the mice were housed in a dark-light cycle (12:12) under optimal (temperature: 20 – 24ºC; humidity: 50 ± 10%) and specific pathogen free conditions in the Laboratory Animal Facilities of the Hirszfeld Institute of Immunology and Experimental Therapy, of the Polish Academy of Sciences in Wroclaw. During the experiments the animals received standard autoclaved food for rodents (Ssniff, V1534-300) and water *ad libitum*. The study was carried out in strict accordance with the most recent legal regulations and did not require ethical approval (35, 36). Each peptide was repeated fifteen times utilizing 30 mice. Because of the insufficient number of cells, it was not possible to utilize only 15 mice to verify all selected peptides.

Isolation of splenocytes and magnetic cell sorting of antigen presenting cells and CD4$^+$CD25$^-$

Animals were anesthetized by using an automatic delivery system that provides a mixture of isoflurane and oxygen and euthanized by cervical dislocation and then a spleen was dissected. A single cell suspension of splenocytes was obtained as previously described (37, 38). In brief, isolated spleens were squeezed through a 40 µm cell strainer (BD) to the 0.84% ammonium chloride solution and washed twice (4ºC, 300 × g, 10 min) in Sorting Buffer (PBS buffer supplemented with 2 mM

Table 2. The characteristics of examined peptides with reference to the total molecular charge, used solvents and peptides' purity.

Molecular charge	Peptide sequences	Solvents	Purity
Negative charge	EEQYNSTYRVVSVLTVLHQDW	Distilled water	90.03%
	LKDFALEGTLAADKT	Distilled water	91.66%
	DELLECLSPATSRTF	Distilled water	91.31%
Neutral charge	GYYWSWIRQPPGKGEEWIG	Acetonitrile	90.07%
	WLSIISMATLESSLK	Acetonitrile, DMSO	90.08%
	LMKLYSFLLNDSPLN	Acetonitrile, DMSO, urea	93.43%
	LNVLRIINEPTAAAI	Acetonitrile	93.86%
Positive charge	SLRSAHLVGQTILSG	Distilled water	92.08%
	FSGTVFLANTNLILH	Distilled water, acetic acid, TFA	95.24%
	NHHNMLLSLSTSGVL	Distilled water	91.88%

Single-letter amino acid symbols: A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid.

EDTA and 2% fetal bovine serum) (Biowest). Next, following cell subpopulations were sorted sequentially with the use of the positive selection method according to the manufacturer's recommendations (Stemcell Technologies): $CD11c^+$ cells, $CD11b^+$ cells and $CD4^+CD25^-$ cells. Due to the requirement of higher purity of the sorted subpopulations, no more than 4×10^6 splenocytes were separated. Purity of isolated cells was confirmed by flow cytometry analysis by staining with: anti-mouse CD4 PE antibody (eBioscience, clone RM4-5), anti-mouse CD25 PE-Cy7 antibody (eBioscience, clone PC61.5), anti-mouse CD11c FITC antibody (eBioscience, clone N418) and anti-mouse CD11b antibody APC (eBioscience, clone M1-70). The average purity of all sorted cells was 85% or higher.

Antigen presenting cells / $CD4^+CD25^-$ co-cultures

APCs: $CD11c^+$, $CD11b^+$ were pooled in 1:1 proportion. $CD4^+CD25^-$ lymphocytes and APCs were then suspended in culture medium: RPMI 1640 (Sigma-Aldrich), 10% fetal bovine serum (Gibco), L-glutamine-penicillin-streptomycin (Sigma-Aldrich, stock 1:100), 50 µM β-mercaptoethanol (Sigma-Aldrich), sodium pyruvate (Gibco, stock 1:100), HEPES (Gibco, stock 1:100), MEM non-essential amino acids solution (Gibco, stock 1:100). Then, APCs were placed in a 96-well round-bottomed culture plate in an amount of 5×10^4 cells/well. Then, the cells were treated with mitomycin C (Cayman Chemical) for 30 min at 37ºC. After triple washing in RPMI 1640 medium, the cells were resuspended in medium supplemented with IL-2 (8 ng/mL), TGF-β (4 ng/mL) and 1×10^5 $CD4^+CD25^-$ cells/well. Next, examined peptides in a concentration of 100 µg/mL were added to each well (39). The cells were incubated for 6 days, in 5% CO_2 and at 37ºC. Every second day, 50% of the medium was changed and refilled with fresh medium supplemented with IL-2 and TGF-β.

Flow cytometry staining and analysis

For intracellular staining of IL-10, the last 6 hours of co-culture, cells were stimulated with brefeldin A: 10 µg/mL (Cayman Chemical), ionomycin: 1 µg/mL (Cayman Chemical), phorbol 12-myristate 13-acetate: 0.1 µg/mL (Cayman Chemical) and monensin A: 2 µM (eBioscience). After incubation, cells were washed twice and the intracellular staining was performed according to the manufacturer's instructions (Foxp3/Transcription Factor Staining Buffer Set, eBioscience). Briefly, cells were stained with anti-mouse CD4-PE antibody (eBioscience, clone RM4-5) and anti-mouse CD25-Pe-Cy7 antibody (eBioscience, clone PC61.5) for 30 min at 4ºC in the dark and washed with Staining Buffer (PBS buffer, 0.5 mM EDTA, 1% fetal bovine serum and 0.002% NaN3). Then, cells were fixed for 14 h at 4ºC in the dark, washed twice with Fixation/Permeabilization Buffer (eBioscience) and stained for 1 hour at 4ºC in the dark with anti-mouse IL-10 APC antibody and the appropriate isotype control (RatIgG2bκ clone JES5-16E3, eBioscience) in the same concentration as the specific antibody (1 hour, 4ºC, in the dark). Afterwards, cells were washed twice with Permeabilization Buffer and analyzed on FACSCalibur II (BD Pharmingen). All analyses were carried out with the use of Weasel 3.0.2 software (Walter and Eliza Hall Institute, Parkville, Australia).

Absolute regulatory T cells count

Absolute Tregs count (a number of cells in the sample withdrawn from one well of culture plate) was determined with the use of the 'dual platform' method (40). It consisted of three steps. Firstly, the number of lymphocytes in the selected wells was counted with the use of Bürker's chamber. Secondly, that sample was analyzed by flow cytometer and the percentage of Tregs in the whole lymphocyte population was counted. Finally, these percentage values were calculated with reference to absolute lymphocyte count. In that way, absolute $CD4^+CD25^+Foxp3^+$ (Tregs) count and absolute Tregs producing IL-10 count were calculated. Apart from that, the total number of $CD4^+$ and $CD4^+CD25^+Foxp3^+IL\text{-}10^+/CD4^+$ ratio was calculated based on the gating procedure (*Fig. 2*). The relative level of IL-10 in gated $CD4^+CD25^+Foxp3^+$ cells was shown as the specific mean fluorescence intensity (MFI) based on the difference between the

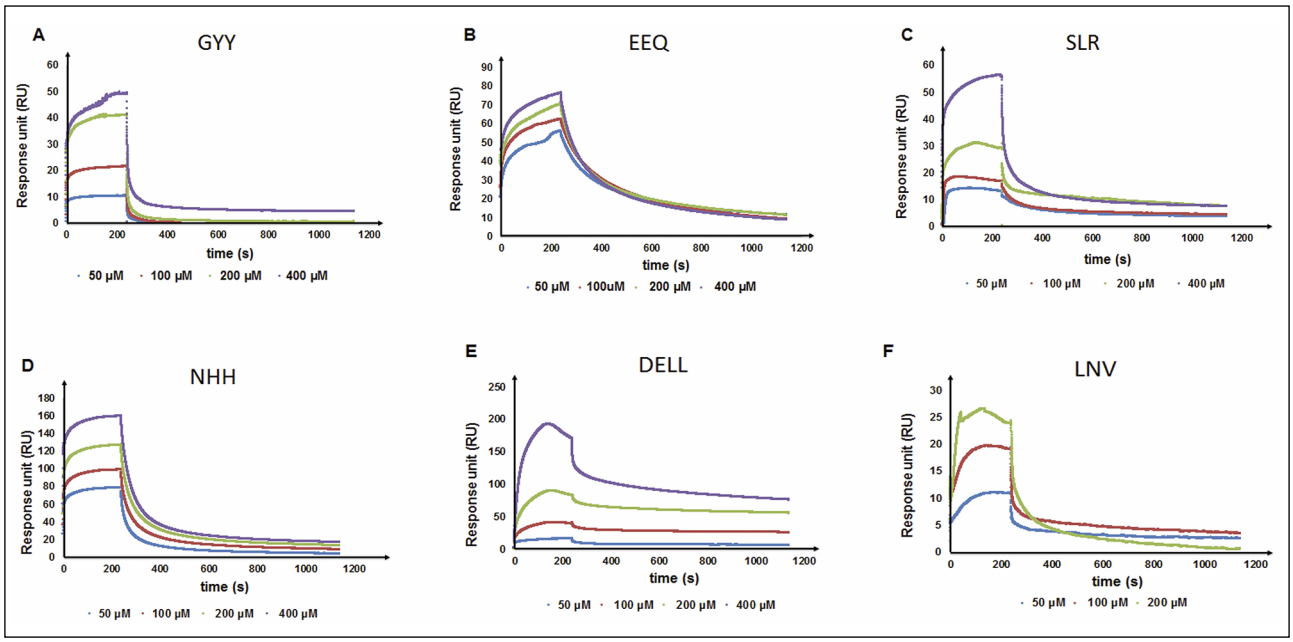

Fig. 1. The binding of selected peptides to MHC II DRB1*04:01 allele analyzed by surface plasmon resonance. Interaction of (A) GYY peptide (Edratide) and (B) EEQ peptide (positive control, tregitope 289) (C) SLR, (D) NHH, (E) DELL (F) LNV with immobilized MHC II with the use of SPR. Lines represent different analyte concentration in HBS buffer. Binding data collected at flow rate 30 µL/min and represented as sensograms in relative units (RU).

MFI of the specific stained cells and the isotype-matched control stained cells.

Statistical analysis

All statistical calculations were performed in GraphPad 6 (GraphPad Software, Inc., La Jolla, CA, USA). The surface plasmon resonance was conducted in at least three independent runs. The statistical analysis was performed using Student's t-test. For a comparison of Tregs induction and their IL-10 production under the influence of selected peptides Kruskal-Wallis test and post hoc Dunn's multiple comparison test were used. A P-values less than 0.05 were considered as statistically significant.

RESULTS

Surface plasmon resonance

The affinity of peptides to MHC II complex was analyzed by SPR (*Fig. 1*). Mean KD of positive control reached 8.11×10^{-8} and was linked to stronger interparticle interactions. The binding affinity of the Edratide (GYY) to MHC II was below the detection level of the instrument, which means that KD was lower than 10^{-6}. Statistical significance between GYY and positive control was demonstrated at the level of $P < 0.001$. Calculated K_D values for peptides NHH, DEL and SLR were as follows: 5.41×10^{-9}, 2.03×10^{-9}, 2.31×10^{-8}, respectively. The determination of K_D values for LKD, LNV and LMK were

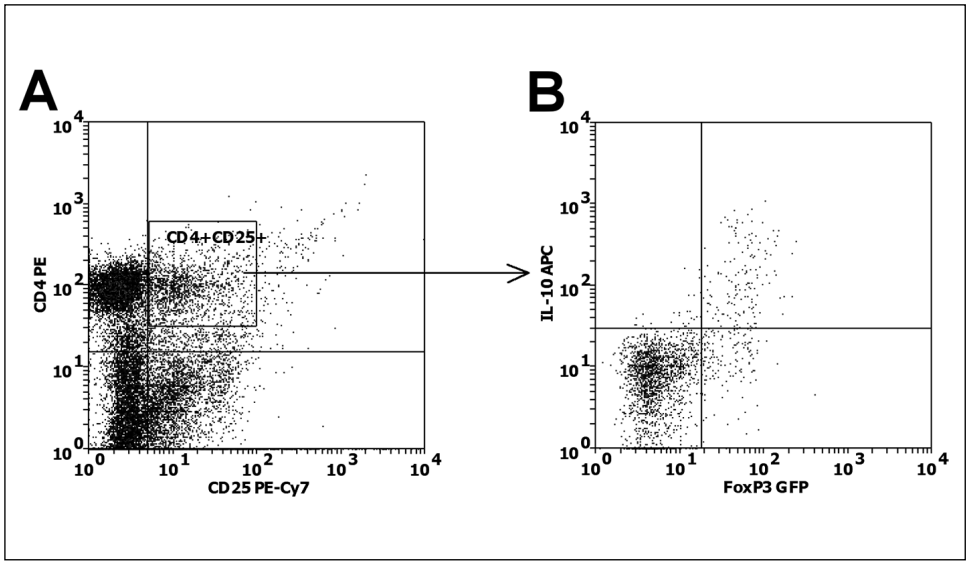

Fig. 2. Representative dot plots of flow cytometric analysis of mouse splenocytes under stimulation of selected FSG peptide. Naïve T cells were co-cultured with mouse antigen presenting cells and stimulated with one of the selected peptide (FSG peptide). Cells were tri-colour stained for the presence of surface (CD4 and CD25) and intracellular IL-10 antigens. $CD4^+CD25^+$ cells are located in the rectangular gate (A). The distribution of subpopulation of gated $CD4^+CD25^+$ cells stained for IL-10 antigen (X axis) and for Foxp3 expression (Y axis) is presented on the second dot-plot (B).

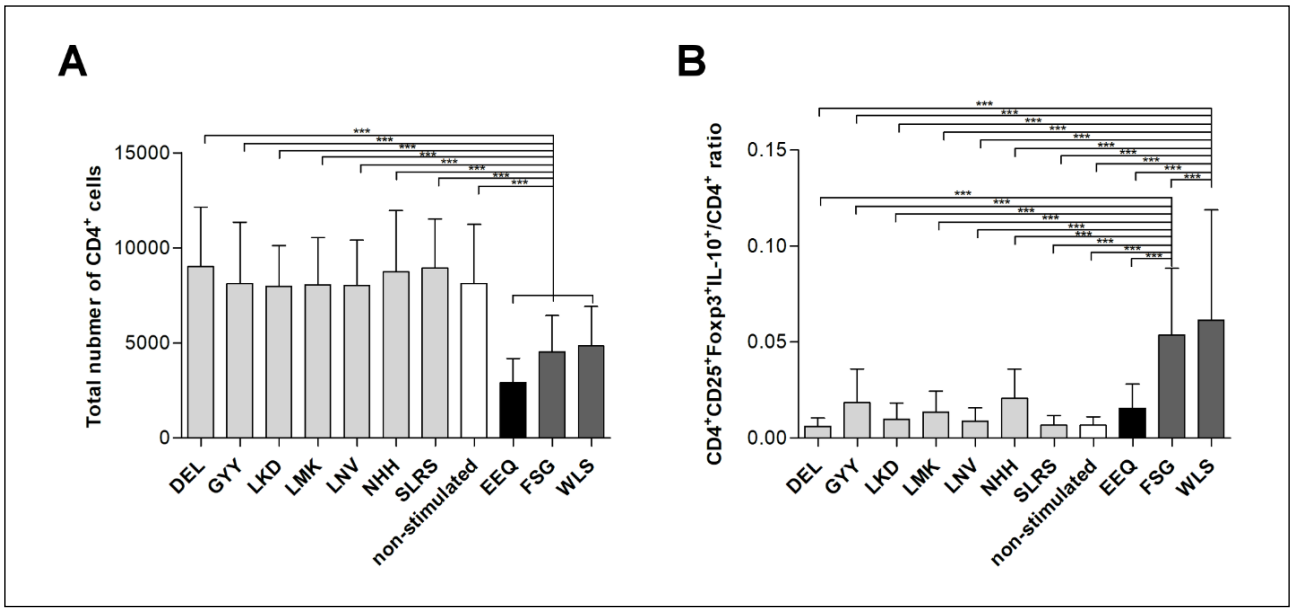

Fig. 3. The total number of $CD4^+$ and $CD4^+CD25^+Foxp3^+IL\text{-}10^+/CD4^+$ ratio after stimulation of APCs/$CD4^+CD25^-$ co-culture with selected peptides. Number of (A) $CD4^+$ cells, (B) $CD4^+CD25^+Foxp3^+IL\text{-}10^+/CD4^+$ ratio after co-culture of naïve T cells with mouse antigen presenting cells and stimulated with selected peptides. Results are expressed as the mean number of $CD4^+$ cells and $CD4^+CD25^+Foxp3^+IL\text{-}10^+$ from one well (± S.D.) calculated using the dual platform method (n = 15 per peptide, Kruskal-Wallis with the Dunn's multiple comparison post hoc test, *P < 0.05, **P < 0.01, ***P < 0.001).

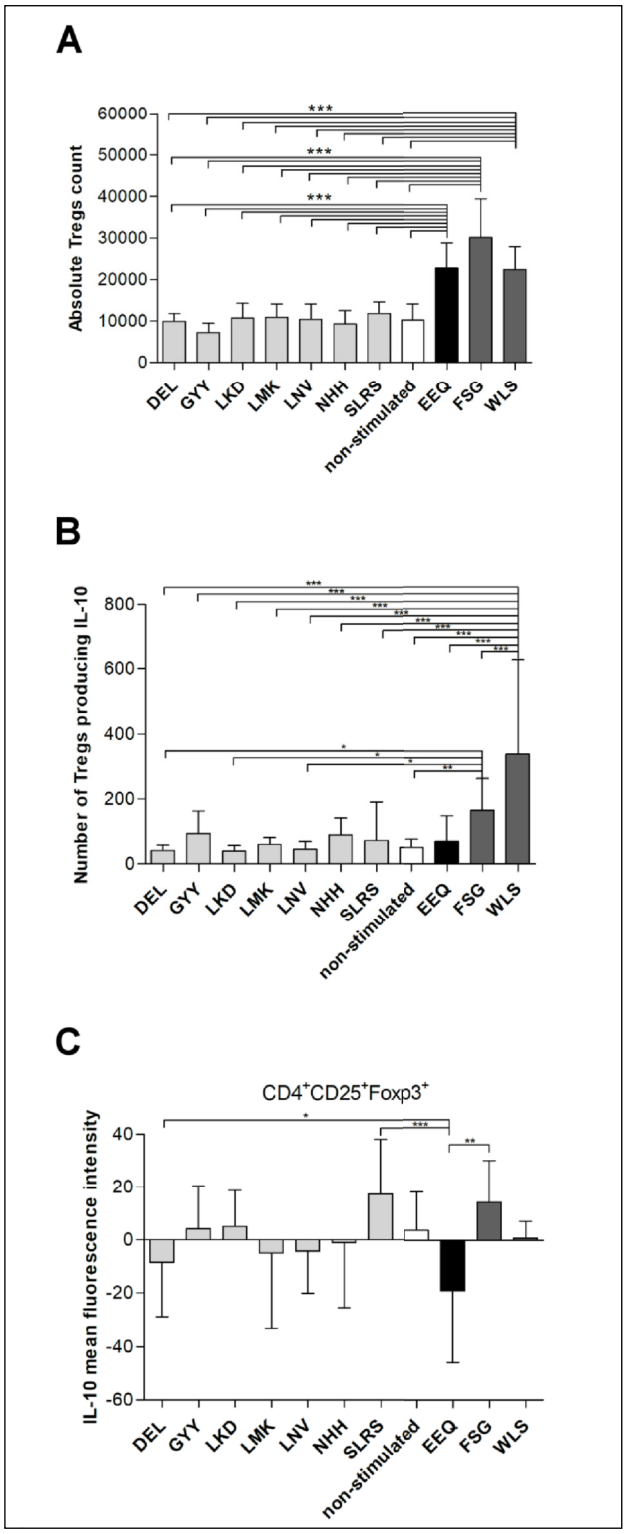

Fig. 4. The absolute Tregs and Tregs producing IL-10 count after stimulation of APCs/$CD4^+CD25^-$ co-culture with selected peptides and mean fluorescence intensity (MFI) of IL-10 in $CD4^+CD25^+Foxp3^+$ cells. Number of (A) $CD4^+CD25^+Foxp3^+$ cells, (B) $CD4^+CD25^+Foxp3^+IL\text{-}10^+$ cells and (C) the relative level of IL-10 in $CD4^+CD25^+Foxp3^+$ cells (MFI) after co-culture of naïve T cells with mouse antigen presenting cells and stimulated with selected peptides. Results are expressed as the mean number of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^+Foxp3^+IL\text{-}10^+$ cells from one well (± S.D.) calculated using the dual platform method (n = 15 per peptide, Kruskal-Wallis with the Dunn's multiple comparison post hoc test, *P < 0.05, **P < 0.01, ***P < 0.001).

below the detection level of BIAcore T200. Moreover, the KD values for WLS and FSG could not have been calculated.

Regulatory T cells expansion and interleukin-10 production

To verify the immunosuppressive function of selected peptides and whether the peptides could stimulate the expansion of aTregs, absolute number of Tregs and total number of $CD4^+$ cells were calculated based on the gating procedure (*Fig. 2*). Investigation of the total number of $CD4^+$ cells revealed that addition of GYY, NHH, LKD, LMK, LNV, DEL and SLR peptides did not change the number of $CD4^+$ cells and maintained at the same level as negative control (*Fig. 3A*) Whereas, three other peptides: EEQ, FSG and WLS significantly decreased the number of $CD4^+$ cells (2901.29 ± 1277, 4532.82 ± 1915, 4849 ± 2076, respectively) comparing to non-stimulated cells (8135 ± 3094) ($P < 0.0001$). At the same time aforementioned peptides significantly increased the absolute number of $CD4^+CD25^+Foxp3^+$ (Tregs) in comparison to negative control as well as to remaining peptides (*Fig. 4A*) ($P < 0.0001$). However, among those peptides, only WLS peptide significantly induced the number of Tregs producing IL-10 at the level of 338.4 ± 289.7 in comparison to all examined peptides and negative control (*Fig. 4B*). Similarly, the FSG peptide also significantly increased the number of cells producing IL-10 (165.2 ± 98.74) (*Fig. 4B*) but only in comparison to negative control (50.91 ± 24.72) and to LNV (44.76 ± 24.28), LKD (39.78 ± 17.46) and DEL (41.32 ± 17.88) peptides ($P < 0.01$). Interestingly, the positive control did not show any differences in the number of Tregs producing IL-10 comparing to negative control (*Fig. 4B*) ($P > 0.05$). Although FSG, WLS and EEQ peptides increased the number of Tregs, they did not significantly increase the level of IL-10 (MFI value) in comparison to negative control (*Fig. 4C*). However, we observed higher MFI values after addition of DEL, SLR, FSG peptides in comparison to positive control, that were equal to –8.360 ± 20.41, 17.26 ± 20.67, 14.48 ± 15.36, respectively (*Fig. 4C*).

The calculated $CD4^+CD25^+Foxp3^+IL\text{-}10^+/CD4^+$ ratio for EEQ, FSG, WLS peptides was significantly higher than for the rest of the peptides and 3.67; 4.67; 8.88 times higher than for non-stimulated cells, respectively ($P < 0.0001$) (*Fig. 3B*). Interestingly, with regard to FSG and WLS peptides, the $CD4^+CD25^+Foxp3^+IL\text{-}10^+/CD4^+$ ratio was 1.27 and 2.27 times higher than positive control, respectively ($P < 0.0001$) (*Fig. 3B*).

The verification of the effects of the utilized solvents in concentrations and combinations added in the experiment did not show any impact on the number of regulatory T cells and Tregs inducing IL-10 production (data not shown).

DISCUSSION

The tregitopes definition concerns the amino acid sequences presented *via* MHC II molecules that are able to convert naïve T lymphocytes to aTregs and finally suppress the effector T cell responses by up-regulating the production of associated cytokines (8). Recent scientific contributions on proposed mechanisms of induction and expansion of Treg cells by intravenous immunoglobulin (IVIG) have revealed multiple pathways dependent on cyclooxygenase 2, DC immunoreceptor, interleukin 33, among which tregitopes, could have the crucial meaning (41). From 2008, when first reports on tregitopes were presented, no putative tregitopes' sequences have been discovered so far. Therefore, we selected potential tregitopes and examined their impact on Tregs expansion in mice. The induction of Tregs leading to an immune response suppression could prevent autoimmune diseases and allergies (42-44). Thus,

seeking for new biological drugs that could enable an effective therapy is demanded and still in progress.

One of the assumptions of the in silico studies was that the peptides' binding to MHC II is one of the important property of potential tregitopes. It is known that the affinity to MHC II is a necessary feature for presenting tregitopes. However, it does not mean that any peptide presented by MHC II can cause the Tregs induction.

The presented studies proceeded through several stages. The first one consisted of *in silico* analysis of potential tregitopes whereas the second stage was the analysis of the peptides' binding strength to HLA-DRB1*04:01 allele by SPR as a verification of probability of the utilized tregitopes' presentation by APC in humans.

Then, the analysis of the selected peptides was performed in the *in vitro* mouse model experiments. The research carried out by de Groot *et al.* (8, 14) showed that tregitopes' structures derived from IgG are conserved among species and are potent to induce Tregs in different species.

Therefore, we utilized in our mouse model IgG-derived tolerogenic peptides - EEQ and GYY called Eratide that is a synthetized peptide based on the complementarity-determining region 1 (hCDR1) of a human anti-DNA mAb.

Aforementioned *in vitro* analysis was preceded by an analysis of the peptides binding strength to HLA-DRB1*04:01 allele by SPR. The HLA-DRB1*04:01 allele was selected based on its common overrepresentation in patients with severe autoimmune diseases (45). Experimental methods enabled determining KD for only half of examined peptides. The binding of LMK, LNV and LKD peptides to MHC II HLA DRB1*04:01 in tested conditions was below the detection level of BIAcore T200. Due to limited solubility of FSG and WLS peptides and, therefore presence of moderate concentrations of acetonitrile, trifluoroacetic and acetic acid, it was impossible to determine unique dissociation constants. Statistically significant difference in the affinity of GYY and positive control (EEQ) peptides to MHC II ligand indicates that EEQ is MHC II-binding sequence and possesses one of major characteristics for tregitopes. Lack of binding with MHC II for LMK and LNV peptides and negative value of IL-10 MFI proves that none of them could be fully presented by APC and therefore could not induce Tregs expansion.

Although the SPR analysis demonstrated moderate or even high affinity to MHC II ligand for GYY, NHH, SLR and DEL peptides, the *in vitro* studies did not confirm their ability to induce Tregs. This means that not every peptide binding to MHC II should be classified as tregitope. Moreover, the results of *in vitro* investigations have shown that sample with GYY (Edratide) and negative control which was cell culture without any peptides, did not statistically induce the expansion of $CD4^+CD25^+Foxp3^+$ cells with reference to positive control ($P < 0.001$). This fact was demonstrated in other studies, in which Edratide failed the second phase of clinical trials on SLE (34) because of general efficacy absence. Although the earlier presented studies showed very moderate effects of Edratide on downregulation of pro-inflammatory cytokines *in vivo* and *in vitro* in murine SLE models and in human lupus and upregulation of immunosuppressive cytokines with induction of Tregs, our research did not confirm any immunosupresive activity of Edratide. So far, only one group of scientists points to the tolerogenic function of Edratide concerning the $CD4^+CD25^+Foxp3^+$ proliferation. In our model Edratide's mode of action was not proven. However, utilized positive control, demonstrated the impact on Tregs proliferation. At this stage of research it is not possible to indicate the main reason of these phenomena. Currently, it requires further investigations and verification of the model sensitivity.

Similarly to Edratide, the SLR sequence from fusion protein CTA1R9K-hMOG10-60-DD was not classified positively as a tregitope in our studies (46). Both potential tolerogenic sequences were verified negatively in second phase of clinical trials on SLE and primate model of EAE.

On the other hand, two other peptides: FSG and WLS induced the expansion of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^+Foxp3^+IL\text{-}10^+$ cells. Although those peptides increased the number of Tregs producing IL-10, we did not observe increased MFI value in comparison to non-stimulated cells. However, both of them demonstrated positive MFI values in opposite to verified tregitope - EEQ peptide, which showed negative MFI value. Also, DEL, LMK, LNV and NHH peptides showed negative value of MFI. This observation could be caused by a small $CD4^+CD25^+Foxp3^+IL\text{-}10^+$ population in face of a huge $CD4^+CD25^+Foxp3^+IL\text{-}10^-$ population. Notably, negative values could be a consequence of two different operations. Firstly, a subtraction process that instruments do on an event-by-event basis before reporting the data. This process can result in a value less than zero for events that have essentially no fluorescence, because the error in the estimation of the background can exceed the absolute magnitude of the background itself. Secondly, the compensation, which subtracted values from the fluorescence signal based on the fluorescence in other channels and the error in the estimation of the amount to subtract can therefore exceed the absolute magnitude of the value after subtraction. With both of these operations, this could end up with observed in our experiment negative MFI values (47). Although WLS peptide significantly increased the number of IL-10 producing Tregs, the IL-10 MFI was not statistically different among the peptides. It could be caused by significant differences in IL-10 expression among the cells. Similar results were observed in the Su and Rossi studies on immunogenicity of proven tregitopes: 167 and 289 (39). Interestingly, in two selected peptides (WLS and FSG) and positive control, we observed the decreased level of total $CD4^+$ cells number, which could indicates their immunosuppressive impact on the $CD4^+$ cells. In addition, the high $CD4^+CD25^+Foxp3^+IL\text{-}10^+/CD4^+$ ratio in both peptides - WLS and FSG can confirm the fact that active Tregs do not contribute to $CD4^+$ cells proliferation, despite the favourable cytokine conditions (Il-2, TGF-β), and lead to switch on the tolerogenic mechanisms of immune system.

Here we present two novel tregitopes. The conducted study enabled a search and confirmation of Tregs' induction of two so far undiscovered tregitopes. FSG tregitope was identified in P03244 protein that came from human adenovirus C, serotype 2 (HAdV-2) (28, 29). Whereas, the second one, WLS, was identified in P52960 protein - peroxisome proliferation transcriptional regulator from *Saccharomyces cerevisiae* (28, 29).

FSG tregitope was discovered in an adenovirus protein that is pathogenic towards humans. It might be an indirect evidence that pathogens, which possess putative tregitopes sequences, decrease the level of host immune response and enable further organism penetration whilst host immune response impairment. It has been scientifically proven that an influence of viruses on organism immune system is related to a suppression of Tregs' function (48). The adenovirus vector - mediated gene transfer studies on a treatment for cystic fibrosis demonstrated significant decrease of Tregs activity (49). On the grounds of these results, it might be hypothesized that tolerogenic sequences are one of the methods of host virus-directed immune response avoidance.

The second of discovered tregitopes was WLS. A presence of that peptide in *Saccharomyces cerevisiae*, is an indirect evidence of promoting intestinal immune tolerance by occasional digestive commensals (50). Therefore, it might be concluded that the presence of tregitopes in the commensals' proteins could play the

role in one of the homeostasis maintenance mechanisms. Similarly to gut microbiota, *Saccharomyces boulardii*, a strain of *Saccharomyces cerevisiae*, with known probiotic properties demonstrates the beneficial effects on immune system modulation (51). Its ability to engage immune cells and influence cytokine secretion *in vitro* was confirmed in the studies on the rodent colitis models where positive impact on disease outcome was proven (52). Additionally, it was shown that *S. boulardii* reduces pro-inflammatory response (53), promotes intestinal mucosal anti-inflammatory signalling effects by Tregs stimulation (54) and increase of anti-inflammatory cytokine IL-10 expression (55). Thus, presented research indicates a meaningful role of P52960 protein with reference to the immune system balance of the large intestine.

From 16 amino acid sequences that were selected in *in silico* study only 2 of them demonstrated tolerogenic properties in *in vitro* conditions. That indicates the necessity to include new, other potential tolerogenic sequences to precise the *in silico* model, that could be utilized to search for further amino acid sequences. In general, the conclusion of all *in silico* studies concerning chemical structures as well as biological drugs is that every in silico study should be verified in *in vitro* conditions and animal models (11, 27). However, as indicated in our own studies, which showed differential response of mouse cells towards EEQ and GYY peptides in comparison to human cell response, the investigations carried out utilizing mouse model need further verification in humans.

Tregitopes discovered 10 years ago are a novel direction of researches on the suppressive function of immune system. The earlier proposed $CD4^+CD25^+Foxp3^+$ T lymphocytes *in vitro* expansion induced by tregitopes has also been confirmed in this paper, with reference to two new tregitopes, FSG and WLS. Known tregitopes 167 and 289, as well as FSG and WLS could be therapeutic agents in autoimmune diseases such as: SLE, diabetes mellitus, rheumatoid arthritis and MS. Therefore, there is a need for further studies on the application potential of tregitopes in therapy of aforementioned diseases. One of the directions, where both discovered tregitopes should be used is an optimization of immunogenic biological drugs, for instance mAb. However, a detailed functional analysis of the presented sequences still needs to be verified under *in vitro* and *in vivo* conditions.

The presented manuscript indicates the potential use of tregitopes concerning immunomodulatory mode of action in a range of suppression of immune system through: inhibiting the antigen-specific activity of nearby auto-reactive effectors and/or by changing the phenotype of the effectors to an induced Treg (iTreg) phenotype, by direct suppression of bystander T cells, through expression of certain cytokines and/or by modulation of the antigen-presenting cell (APC) towards a tolerance-inducing phenotype (56).

On the other hand there are two reports undermining the mechanism of action of tregitopes (34). In the Sorde *et al.* paper, authors did not realise that one molecule of IgG contains approximately 10^5 duplicated sequences of potential tregitopes (58). This means that the analysis of the impact of IVIG on the percentage of Tregs could not be an element of comparison for the influence of a single sequence on Tregs percentage. A process that would actually correspond to such a model would be the use of a mixture of an appropriate number of tregitopes. In this case, the comparison concerns completely different doses of single tregitopes, that should be 100 times higher. On this basis, it is difficult to draw a meaningful conclusion and to compare our results with work of Sorde *et al.* (58). In order to be able to compare the effect of synthesized tregitopes as well as IVIG, and their effect on Tregs percentage, it would be necessary to significantly reduce the IVIG concentration or increase the number of synthetic tregitopes.

Initially, it was assumed that IVIG therapy could increase immune tolerance resulting in the discovery of tolerogenic peptides, which in many *in vitro* tests and in *in vivo* models stimulated Tregs expansion. However, transformation of whole proteins leading to peptides' presentation in the context of MHC II and Tregs induction is a multi-step process and probably not all native peptides are able to imitate phenomena of a full antigen presentation process. Hence, the lack of tregitopes' Tregs stimulation in Sorde *et al.* study and the failure of the second clinical phase of Edratide were observed (34, 58). Therefore, in the presented study, we suggest looking for new tregitopes considering a different algorithm for their selection.

Moreover, the aforementioned study may contribute to further investigations on: the use of tregitopes as a tolerogenic sequences for the treatment of allergies, the minimization of antidrug antibodies' response, the elimination of tregitopes from the sequences of proteins present in vaccines to enhance antigenicity and immunogenicity, the search for tregitopes produced by pathogens and commensals to clarify the pathogenesis of autoimmune diseases and their role in maintaining homeostasis. However, despite the presented study confirmed the action of two tregitopes of foreign origin, a full verification of their immunosuppressive function still needs to be performed under *in vitro* and *in vivo* conditions in humans since the Tregs expansion under treitopes' stimulation in mice and humans may vary.

To sum up, these studies broaden the current knowledge of tregitopes' source and application potential. The result of this study is the discovery of two diverse tregitopes isolated from virus and yeast proteins with a verified impact on Tregs induction and their IL-10 production in mice.

Acknowledgements: Special thanks are expressed to Artur Stachowiak and Alexander Lim Geng Wang for correcting grammar and language of the manuscript. Additionally the Authors have no funding to report.

K.M. Okoniewska and A.E. Kedzierska equally contributed to this work.

Conflict of interests: The author (K.M.O.) is a worker of P.F.O. Vetos-Farma Ltd. pharmaceutical company that provides service and access to the patented tregitope structure on a fee-for-service basis. Due to this relationship with P.F.O. Vetos-Farma Ltd., the author acknowledges that there is a potential conflict of interest inherent in the publication of this manuscript, and asserts that she made an effort to reduce or eliminate that conflict where possible. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject of matter or materials discussed in the manuscript apart from those disclosed.

REFERENCES

1. Maizels RM, Smith KA. Regulatory T cells in infection. *Adv Immunol* 2011; 112: 73-136.
2. Shalev I, Selzner N, Shyu W, Grant D, Levy G. Role of regulatory T cells in the promotion of transplant tolerance. *Liver Transpl* 2012; 18: 761-770.
3. Guerin LR, Prins JR, Robertson SA. Regulatory T-cells and immune tolerance in pregnancy: a new target for infertility treatment? *Hum Reprod Update* 2009; 15: 517-535.
4. Racz GZ, Kadar K, Foldes A, *et al.* Immunomodulatory and potential therapeutic role of mesenchymal stem cells in periodontitis. *J Physiol Pharmacol* 2014; 65: 327-339.

5. Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol* 2009; 10: 689-695.
6. Sakaguchi S. Naturally arising Foxp3-expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; 6: 345-352.
7. Yadav M, Stephan S, Bluestone JA. Peripherally induced Tregs - role in immune homeostasis and autoimmunity. *Front Immunol* 2013; 4: 232. doi: 10.3389/fimmu.2013.00232
8. De Groot AS, Moise L, McMurry JA, *et al.* Activation of natural regulatory T cells by IgG Fc-derived peptide 'Tregitopes'. *Blood* 2008; 112: 3303-3311.
9. Bergtold A, Desai DD, Gavhane A, Clynes R. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. *Immunity* 2005; 23: 503-514.
10. Regnault A, Lankar D, Lacabanne V, *et al.* Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* 1999; 189: 371-380.
11. Cousens LP, Najafian N, Mingozzi F, *et al.* In vitro and in vivo studies of IgG-derived Treg epitopes (Tregitopes): a promising new tool for tolerance induction and treatment of autoimmunity. *J Clin Immunol* 2013; 33 (Suppl. 1): S43-S49.
12. Karim M, Kingsley CI, Bushell AR, Sawitzki BS, Wood KJ. Alloantigen-induced $CD25^+CD4^+$ regulatory T cells can develop in vivo from $CD25^-CD4^+$ precursors in a thymus-independent process. *J Immunol* 2004; 172: 923-928.
13. Elyaman W, Khoury SJ, Scott DW, De Groot AS. Potential application of tregitopes as immunomodulating agents in multiple sclerosis. *Neur Res Inter* 2011; 2011: 256460. doi: 10.1155/2011/256460
14. de Groot A, Rivera DS. Epivax, Inc. Regulatory T cell epitopes, compositions and uses thereof. United States patent 20090018067 A1; 2007.
15. Taylor MD, Harris A, Babayan SA, *et al.* CTLA-4 and $CD4^+$ $CD25^+$ regulatory T cells inhibit protective immunity to filarial parasites in vivo. *J Immunol* 2007; 179: 4626-4634.
16. Donkor ON, Ravikumar M, Proudfoot O, *et al.* Cytokine profile and induction of T helper type 17 and regulatory T cells by human peripheral mononuclear cells after microbial exposure. *Clin Exp Immunol* 2012; 167: 282-295.
17. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 2012; 336: 1268-1273.
18. Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 2012; 489: 231-241.
19. Kaewraemruaen C, Sermswan RW, Wongratanacheewin S. Induction of regulatory T cells by Opisthorchis viverrini. *Parasite Immunol* 2016; 38: 688-697.
20. Fryland L. Immune mechanisms in Borrelia burgdorferi sensu lato infection in relation to clinical outcome. Linkoping University Medical Dissertation No 1347, Linkoping University, Sweden, 2012.
21. Maldonado RA, von Andrian UH. How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol* 2010; 108: 111-165.
22. Cousens LP, Mingozzi F, van der Marel S, *et al.* Teaching tolerance: new approaches to enzyme replacement therapy for Pompe disease. *Hum Vaccin Immunother* 2012; 8: 1459-1464.
23. D Seeliger. Development of scoring functions for antibody sequence assessment and optimization. *PLoS One* 2013; 8: e76909. doi: 10.1371/journal.pone.0076909
24. De Groot AS, Cousens L, Mingozzi F, Martin W. Tregitope peptides: the active pharmaceutical ingredient of IVIG? *Clin Dev Immunol* 2013; 2013: 493138. doi: 10.1155/2013/493138
25. Aguirre D. Deletion of antibody encoded tolerogenic signals to improve a dendritic cell targeted vaccine delivery platform system. Master's Thesis. University of Rhode Island, 2014.
26. Orbach H, Katz U, Sherer Y, Shoenfeld Y. Intravenous immunoglobulin: adverse effects and safe administration. *Clin Rev Allergy Immunol* 2005; 29: 173-184.
27. Okoniewska KM, Okoniewski J, Grabowski T. Relationship between tregitopes structure and binding with major histocompatibility complex class I. *Drug Res* (*Stuttg*) 2016; 66: 211-216.
28. http://www.uniprot.org/
29. Apweiler R, Bairoch A, Wu CH. Protein sequence databases. *Curr Opin Chem Biol* 2004; 8: 76-80.
30. Chahal JS, Qi J, Flint SJ. The human adenovirus type 5 E1B 55 kDa protein obstructs inhibition of viral replication by type I interferon in normal human cells. *PLoS Pathog* 2012; 8: e1002853. doi: 10.1371/journal.ppat.1002853
31. Yan F, Polk DB. Probiotics and immune health. C*urr Opin Gastroenterol* 2011; 27: 496-501.
32. Barbi J, Pardoll DM, Pan F. Ubiquitin-dependent regulation of Foxp3 and Treg function. *Immunol Rev* 2015; 266: 27-45.
33. Brenu EW, Staines DR, Tajouri L, Huth T, Ashton KJ, Marshall-Gradisnik SM. Heat shock proteins and regulatory T cells. *Autoimmune Dis* 2013; 2013: 813256. doi: 10.1155/2013/813256
34. Urowitz MB, Isenberg DA, Wallace DJ. Safety and efficacy of hCDR1 (Edratide) in patients with active systemic lupus erythematosus: results of phase II study. *Lupus Sci Med* 2015; 2: e000104. doi: 10.1136/lupus-2015-000104
35. FDA. Guidance for Industry Animal Models - Essential Elements to Address Efficacy Under the Animal Rule. 2009.
36. EC. Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for Scientific Purposes. 2010.
37. Maj T, Slawek A, Chelmonska-Soyta A. CD80 and CD86 costimulatory molecules differentially regulate OT-II $CD4^+$ T lymphocyte proliferation and cytokine response in cocultures with antigen-presenting cells derived from pregnant and pseudopregnant mice. *Mediators Inflamm* 2014; 2014: 769239. doi: 10.1155/2014/769239
38. Chelmonska-Soyta A, Ozgo M, Lepczynski A, *et al.* Proteome of spleen CD4 lymphocytes in mouse preimplantation pregnancy. *J Physiol Pharmacol* 2014; 65: 719-731.
39. Su Y, Rossi R, De Groot AS, Scott DW. Regulatory T cell epitopes (Tregitopes) in IgG induce tolerance in vivo and lack immunogenicity per se. *J Leukoc Biol* 2013; 94: 377-383.
40. Holcar M, Goropevsek A, Ihan A, Avcin T. Age-related differences in percentages of regulatory and effector T lymphocytes and their subsets in healthy individuals and characteristic STAT1/STAT5 signalling response in helper T lymphocytes. *J Immunol Res* 2015; 2015: 352934. doi: 10.1155/2015/352934
41. Maddur MS, Kaveri SV, Bayry J. Circulating normal IgG as stimulator of regulatory T cells: lessons from intravenous immunoglobulin. *Trends Immunol* 2017; 38: 789-792.
42. Dejaco C, Duftner C, Grubeck-Loebenstein B, Schirmer M. Imbalance of regulatory T cells in human autoimmune diseases. *Immunology* 2006; 117: 289-300.
43. Robinson DS, Larche M, Durham SR. Tregs and allergic disease. *J Clin Invest* 2004; 114: 1389-1397.
44. Bak M, Jazwa A, Kasper L, *et al.* Involvement of microRNAs in the inflammatory pathways of pulmonary sarcoidosis. *J Physiol Pharmacol* 2015; 66: 635-642.
45. Flam ST, Gunnarsson R, Garen T, Lie BA, Molberg O. The HLA profiles of mixed connective tissue disease differ distinctly from the profiles of clinically related connective tissue diseases. *Rheumatology* (*Oxford*) 2015; 54: 528-535.

46. Kap YS, van Driel N, Arends R, *et al.* Immune modulation by a tolerogenic myelin oligodendrocyte glycoprotein (MOG)10-60 containing fusion protein in the marmoset experimental autoimmune encephalomyelitis model. *Clin Exper Immunol* 2015; 180: 28-39.
47. http://flowjo.typepad.com/the_daily_dongle/2007/02/negative_fluore.html
48. Boer MC, Joosten SA, Ottenhoff TH. Regulatory T-cells at the interface between human host and pathogens in infectious diseases and vaccination. *Front Immunol* 2015; 6: 217. doi: 10.3389/fimmu.2015.00217
49. Nagai Y, Limberis MP, Zhang H. Modulation of Treg function improves adenovirus vector-mediated gene expression in the airway. *Gene Ther* 2014; 21: 219-224.
50. Rea PK. Reversibility of chronic disease and hypersensitivity: The effects of environmental pollutants on the organ system. *CRC Press* 2014; 15: 321-325.
51. Smith IM, Christensen JE, Arneborg N, Jespersen L. Yeast modulation of human dendritic cell cytokine secretion: An in vitro study. *PLoS One* 2014; 9: e96595. doi: 10.1371/journal.pone.0096595
52. Thomas CM, Versalovic J. Probiotics-host communication: modulation of signaling pathways in the intestine. *Gut Microbes* 2010; 1: 148-163.
53. Kelesidis T, Pothoulakis C. Efficacy and safety of the probiotic Saccharomyces boulardii for the prevention and therapy of gastrointestinal disorders. *Therap Adv Gastroenter* 2012; 5: 111-125.
54. Jahn HU, Ullrich R, Schneider T, *et al.* Immunological and trophical effects of Saccharomyces boulardii on the small intestine in healthy human volunteers. *Digestion* 1996; 57: 95-104.
55. Generoso SV, Viana M, Santos R, *et al.* Saccharomyces cerevisiae strain UFMG 905 protects against bacterial translocation, preserves gut barrier integrity and stimulates the immune system in a murine intestinal obstruction model. *Archiv Microbiol* 2010; 192: 477-484.
56. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008; 9: 239-244.
57. Baecher-Allan C, Hafler DA. Human regulatory T cells and their role in autoimmune disease. *Immunol Rev* 2006; 212: 203-216.
58. Sorde L, Spindeldreher S, Palmer E, Karle A. Tregitopes and impaired antigen presentation: drivers of the immunomodulatory effects of IVIg? *Imm Inflamm Dis* 2017; 5: 400-415.

Received: September 12, 2017
Accepted: December 15, 2017

Author's address: Dr. Krystyna Marta Okoniewska, P.F.O., Vetos-Farma sp. z o.o., 21 Dzierzoniowska Street, 53-260 Bielawa, Poland.
E-mail: krystyna.okoniewska@vetos-farma.com.pl