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Tregitopes as a novel immunoregulators of pregnancy tolerance in mouse abortion prone model

Tregitopy – nowe cząsteczki immunoregulatorowe w mysim modelu ciąży zagrożonej poronieniem

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The doctoral thesis was conducted in the Laboratory of Reproductive Immunology of the Department of Experimental Therapy

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2. Streszczenie

Streszczenie

Niezbędnym warunkiem rozwoju ciąży jest ustalenie prawidłowych interakcji między matką a płodem. Obecność w organizmie matczynym alogenicznego płodu wyzwala stan tolerancji immunologicznej, który umożliwia implantację, a następnie rozwój ciąży. Zaburzenia ciąży takie jak poronienia i stan przedrzucawkowy powiązane są z zakłóconymi mechanizmami tolerancji immunologicznej. Wśród poznanych typów komórek przyczyniających się do tolerancji antygenów płodu, główną rolę przypisuje się limfocytom T regulatorowym (Tregs). Dowiedziono, że zarówno u ludzi jak i u myszy podczas prawidłowej ciąży dochodzi do wzrostu liczby komórek regulatorowych, natomiast spadek ich liczby oraz aktywności jest obserwowany w przypadkach spontanicznych poronień. Efekt resorpcji płodów i poronień w typowym modelu poronnym myszy można odwrócić poprzez wczesny transfer adoptywny Tregs. Wykazano, że krótkie peptydy znajdujące się w ludzkich i mysich immunoglobulinach (IgG) mogą indukować ekspansję Tregs. Te naturalne, silnie konserwatywne epitopy Tregs, zwane tregitopami, mają wysokie powinowactwo do wiązania z ludzkimi antygenami głównego kompleksu zgodności tkankowej klasy II (MHCII).

Stąd też, celami rozprawy doktorskiej było: **1)** zidentyfikowanie nowych tregitopów nie pochodzących z IgG, które mogą wiązać się z MHCII z wysokim powinowactwem i promować ekspansję Tregs *in vitro;* **2)** zbadanie czy znane mysie tregitopy 167 (T167) i 289 (T289) oraz zaprojektowane *in silico* nowe peptydy (SGS i LKD) uczestniczą w rozwoju alotolerancji w przebiegu ciąży u myszy.

W pierwszej pracy, mysie naiwne limfocyty T pochodzące z myszy szczepu C57BL6Foxp3^{GFP} hodowano z komórkami prezentującymi antygen i stymulowano znanymi tregitopami i zaprojektowanymi *in silico* peptydami o potencjalnej aktywności stymulującej rozwój limfocytów Treg. Następnie, metodą cytometrii przepływowej analizowano odsetek komórek o fenotypie CD4^{+}CD25^{+}Foxp3^{+} i CD4^{+}CD25^{+}Foxp3^{+}IL-10^{+}.

W drugim i trzecim artykule, samicom myszy CBA/J skojarzonym z samcami myszy DBA/2J, (model ciąży zagrożonej poronieniem) tuż po kryciu, podawano dootrzewnowo, oddzielnie 100 µg każdego z tregitopów tj.: T167, T289 i SGS oraz peptydu LKD lub 150 µl soli fizjologicznej buforowanej fosforanem jako kontroli. W czternastym dniu ciąży oceniano liczbę zresorbowanych i żywych zarodków. W trzecim i czternastym dniu ciąży zmierzono poziom cytokin za pomocą testu immunoenzymatycznego. Dodatkowo, za pomocą cytometrii

przepływowej zbadano frekwencje limfocytów T i B regulatorowych oraz ekspresję cząsteczek kostymulujących (CD80, CD86, CD40) oraz głównego układu zgodności tkankowej klasy II na komórkach prezentujących antygen.

W pierwszej pracy, wykazano, że część zaprojektowanych *de novo* peptydów wykazywała właściwości tregitopów i indukowała ekspansję śledzionowych limfocytów Tregs. W kolejnych dwóch pracach wykazano, że podanie mysiego tregitopu 167 i 289 oraz nowo zaprojektowanego tregitopu SGS spowodowało statystycznie istotne zmniejszenie liczby zresorbowanych płodów w porównaniu z grupą kontrolną. Jednocześnie, w przedimplantacyjnym okresie ciąży zaobserwowano zwiększenie puli śledzionowych Tregs. Zademonstrowano również, że nowo zaprojektowany tregitop SGS i mysie tregitopy 167 i 289 obniżyły odpowiednio ekspresję cząsteczki kostymulującej CD80 lub CD86 na komórkach prezentujących antygen. Dodatkowo, w przedimplantacyjnym okresie ciąży tregitop SGS podwyższał poziom interleukiny 2 i interleukiny 10 w surowicy badanych myszy.

Przeprowadzone badania *in vitro* pozwoliły na identyfikację nowych peptydów o właściwościach tregitopów. Ponadto wykazano, że znane tregitopy 167 i 289 oraz nowy peptyd SGS mają korzystny wpływ na przebieg ciąży poprzez ograniczenie śmiertelności płodów. Skuteczność w przeciwdziałaniu poronieniom stosowanych tregitopów może wynikać ze zwiększenia puli Tregs oraz zmiany odpowiedzi kostymulacyjnej komórek prezentujących antygen po ich podaży. W przyszłości, właściwości immunomodulujące tregitopów mogą być wykorzystane w potencjalnych strategiach terapeutycznych w przypadku niepowodzeń ciąży wywołanych dysregulacją mechanizmów tolerancji immunologicznej.

3. Abstract

Abstract

An essential condition for the development of a healthy pregnancy is the establishment of correct interactions between the mother and the foetus. The presence of the allogeneic foetus induces state of the immune tolerance that allows embryo to implant and later the foetus to develop. The loss of immune tolerance to foetal antigens may result in reproductive failure. The disturbed mechanisms of immune tolerance may cause pregnancy disorders such as miscarriage and pre-eclampsia. The regulatory T lymphocytes (Tregs) are critical for establishing immune tolerance to the foetal antigens. It has been proved that, both in humans and mice, there is an increase of Tregs during normal pregnancy and decrease in their number and activity in spontaneous abortion cases. Moreover, the adoptive transfer of Tregs mitigates pregnancy loss in abortion-prone mice. It has been shown that short peptides found in human and mouse immunoglobulins (IgGs) may induce Tregs expansion. These natural Treg epitopes, named tregitopes are highly conserved and have high binding affinity to human class II major histocompatibility complexes.

The doctoral thesis aimed to 1) identify novel non-IgG source tregitopes that can bind to MHC II with high affinity and promote expansion of Tregs *in vitro*; 2) investigate the role of designed SGS and LKD peptides and mouse tregitope 167 and 289 in alleviating immune imbalance and preventing pregnancy failure in an abortion-prone mouse model.

In the first paper, C57BL6Foxp3^{GFP} mouse naïve T cells were co-cultured with antigen presenting cells (APCs) under stimulation of selected peptides and known tregitopes. Then, the frequency of $CD4^{+}CD25^{+}Foxp3^{+}$ and $CD4^{+}CD25^{+}Foxp3^{+}IL\text{-}10^{+}$ cells were analyzed using flow cytometry. In the second and third paper, CBA/J female mice mated with DBA/2J male mice were intraperitoneal injected with 100 µg tregitope 289 or 100 µg tregitope 167 or 100 µg SGS tregitope or 100 µg LKD peptide or 150 µL phosphate-buffered saline (PBS; vehicle) on day 0 post-coitum. On the fourteenth day of pregnancy the number or resorbed and viable embryos were evaluated. On the third day and fourteenth day of pregnancy, the T-helper cell 1/T-helper cell 2-related cytokine levels were measured using enzyme-linked immunosorbent assay. Additionally, the proportions of T and regulatory B lymphocytes, and the expression of costimulatory molecules (CD80, CD86, CD40) and major histocompatibility class II molecules on APCs were examined using flow cytometry.

3. Abstract

In the first paper, it was shown that some of the *de novo* designed peptides exhibited tregitope properties and stimulated the development of Treg lymphocytes in the splenic lymphocyte population. In the second and third paper, it was demonstrated that the administration of mouse tregitope 167, 289 and newly designed SGS tregitope resulted in a statistically significant reduction in the foetal death rate compared with the control group. Flow cytometry analysis demonstrated that administration of either tregitope significantly increased the splenic pool of Tregs at the preimplantation stage of pregnancy. We also demonstrated that newly designed SGS tregitope and mouse 167 and 289 tregitopes downregulated the expression of the CD80 or CD86 costimulatory molecule of antigen-presenting cells, respectively. Additionally, SGS tregitope upregulated the serum levels of interleukin 2 and interleukin 10 at the preimplantation stage of pregnancy.

The performed *in vitro* studies identified two novel tregitopes. Moreover, the *ex vivo* studies demonstrated that treatment with known 167 and 289 tregitopes and novel SGS tregitope significantly increased the frequency of Tregs, enhanced the production of IL-10 by Tregs and changed the costimulatory phenotype of APCs, contributing to improved pregnancy outcome. Moreover, we confirmed that known tregitopes 167 and 289 and novel SGS tregitope have a beneficial effect on pregnancy outcome by limiting the foetal death rate. Tregitope-mediated immunomodulation can be a potential therapeutic strategy for immune dysregulation-induced pregnancy failure.

4. The list of publications included in the dissertation

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1. Krystyna Marta Okoniewska¶*, **Anna Ewa Kedzierska¶**, Jakub Okoniewski, Anna Slawek, Krzysztof Grzymajlo, Anna Chelmonska-Soyta, Tomasz Grabowski, New Tregitopes Inducing Adaptive Regulatory T Cells, Journal of Physiology and Pharmacology, 2017, 68(6), 897-906.

2. **Anna Ewa Kedzierska***, Daria Lorek, Anna Slawek, Anna Chelmonska-Soyta, Tregitopes regulate the tolerogenic immune response and decrease the foetal death rate in abortion-prone mouse matings, Scientific Reports, 2020, 10(1):10531.

3. **Kedzierska Anna Ewa***, Lorek Daria, Slawek Anna, Grabowski Tomasz, Chelmonska-Soyta Anna. CD91 Derived Treg Epitope Modulates Regulatory T Lymphocyte Response, Regulates Expression of Costimulatory Molecules on Antigen-Presenting Cells, and Rescues Pregnancy in Mouse Pregnancy Loss Model. International Journal of Molecular Sciences. 2021; 22(14):7296.

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Contributor	Contribution [%]	Description of main tasks
Anna Ewa Kedzierska¶	35 %	Conceptualization; methodology - establishing the cells sorting protocol, cytokine concentrations, culture conditions for tregitopes and antigen-presenting cells co-culture, developing a flow cytometry methodology – antibodies selection, cell analyzer settings; reading and analyzing data, statistical analysis; preparing figures, writing—original draft preparation
Krystyna Marta Okoniewska¶*	35%	investigation - cells sorting, setting up the co-culture of antigen-presenting cells and T cells with tregitopes; samples staining, raw data analysis; preparing tables; writing—original draft preparation; corespondence
Jakub Okoniewski	5 %	writing—review and editing, raw data analysis
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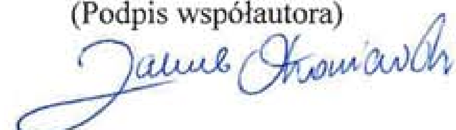

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Anna Ewa Kedzierska*, Daria Lorek, Anna Slawek, Anna Chelmonska-Soyta, Tregitopes regulate the tolerogenic immune response and decrease the foetal death rate in abortion-prone mouse matings, Scientific Reports, 2020, 10(1):10531. *correspondence

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Contributor	Contribution [%]	Description of main tasks
Anna Ewa Kędzierska	70 %	Conceptualization; funding acquisition; methodology: selection of antibodies, determination of peptide doses, cell culture conditions and, staining protocol, cell analyzer settings; animals: cytological swabs, mating, injection of peptides, material collection; investigation: assessment of the mice reproductive cycle phase, embryo flushings, cell isolation, cell culture, samples staining, sample reading, determination of serum cytokines; raw data analysis; statistical analysis; preparation of figures and tables; preparation of the manuscript; correspondence
Daria Lorek	20 %	Animals: cytological swabs and mating; investigation: sample staining; writing: review and editing
Anna Sławek	5 %	Determining the flow cytometry protocol; writing: review and editing
Anna Chełmońska-Soyta	5 %	Conceptualization; writing: review and editing

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20.07.2021

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I hereby declare that my contribution to the following manuscript:

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NEW TREGITOPES INDUCING ADAPTIVE REGULATORY T CELLS IN MICE

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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-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

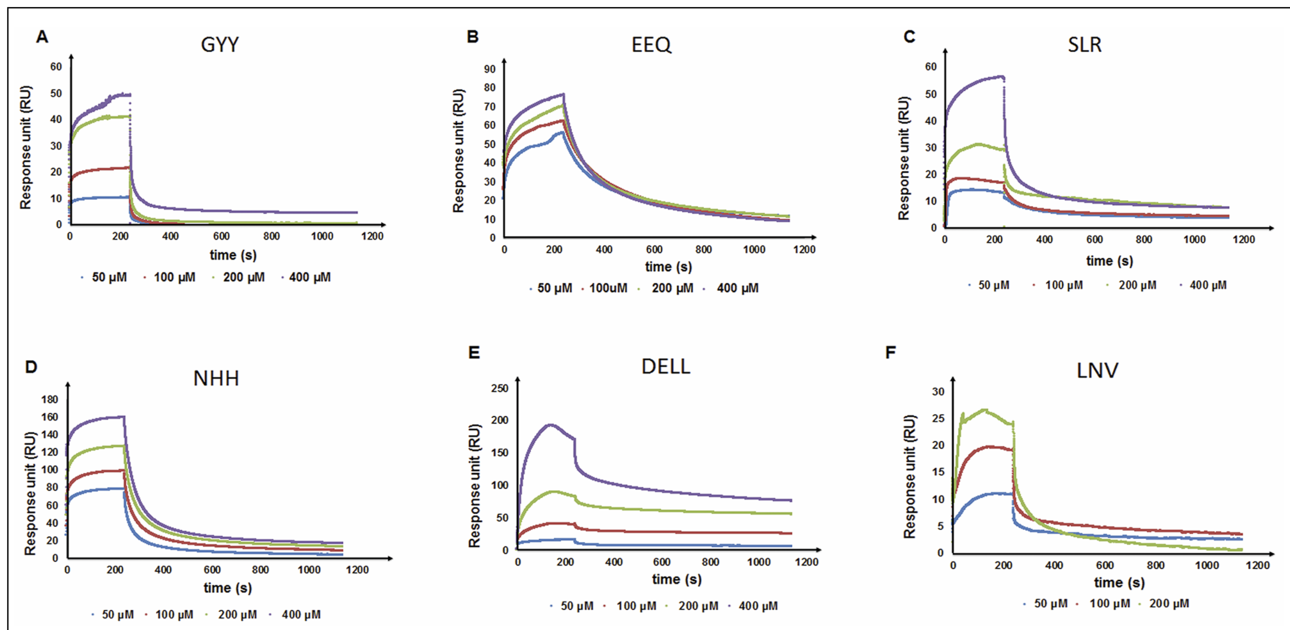

Fig. 1. The binding of selected peptides to MHC II DRB1*04:01 allele analyzed by surface plasmon resonance. Interaction of (A) GYY peptide (Edratide) and (B) EEQ peptide (positive control, tregitope 289) (C) SLR, (D) NHH, (E) DELL (F) LNV with immobilized MHC II with the use of SPR. Lines represent different 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

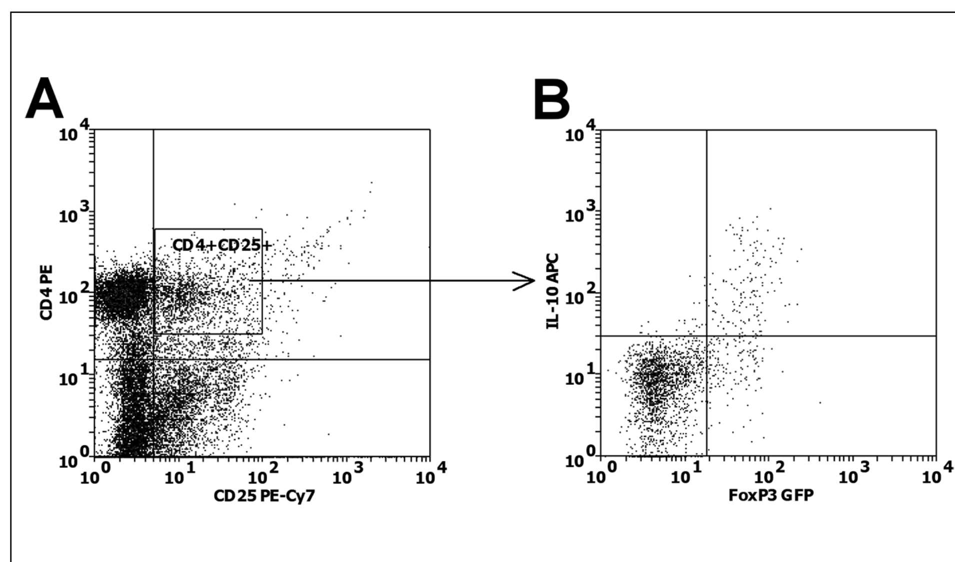

Fig. 2. Representative dot plots of flow cytometric analysis of mouse splenocytes under stimulation of selected FSG peptide. Naïve T cells were co-cultured with mouse antigen presenting cells and stimulated with one of the selected peptide (FSG peptide). Cells were tri-colour stained for the presence 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).

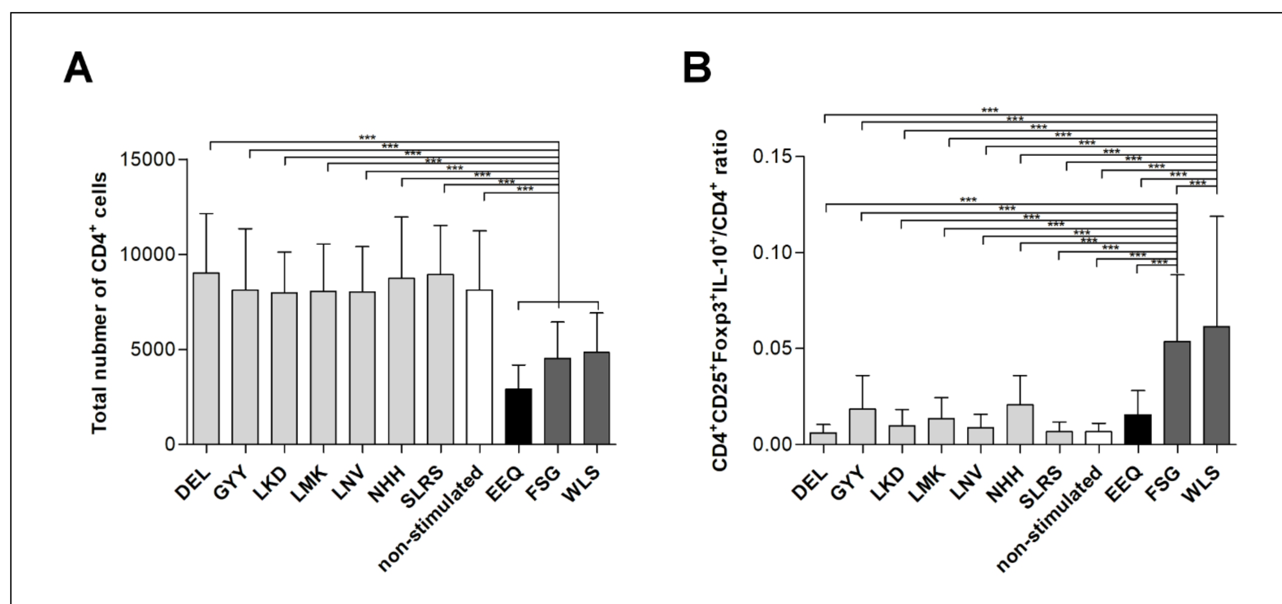

Fig. 3. The total number of $CD4^+$ and $CD4^+CD25^+Foxp3^+IL\text{-}10^+/CD4^+$ ratio after stimulation of APCs/$CD4^+CD25^-$ co-culture with selected peptides. Number of (A) $CD4^+$ cells, (B) $CD4^+CD25^+Foxp3^+IL\text{-}10^+/CD4^+$ ratio after co-culture of naïve T cells with mouse antigen presenting 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, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

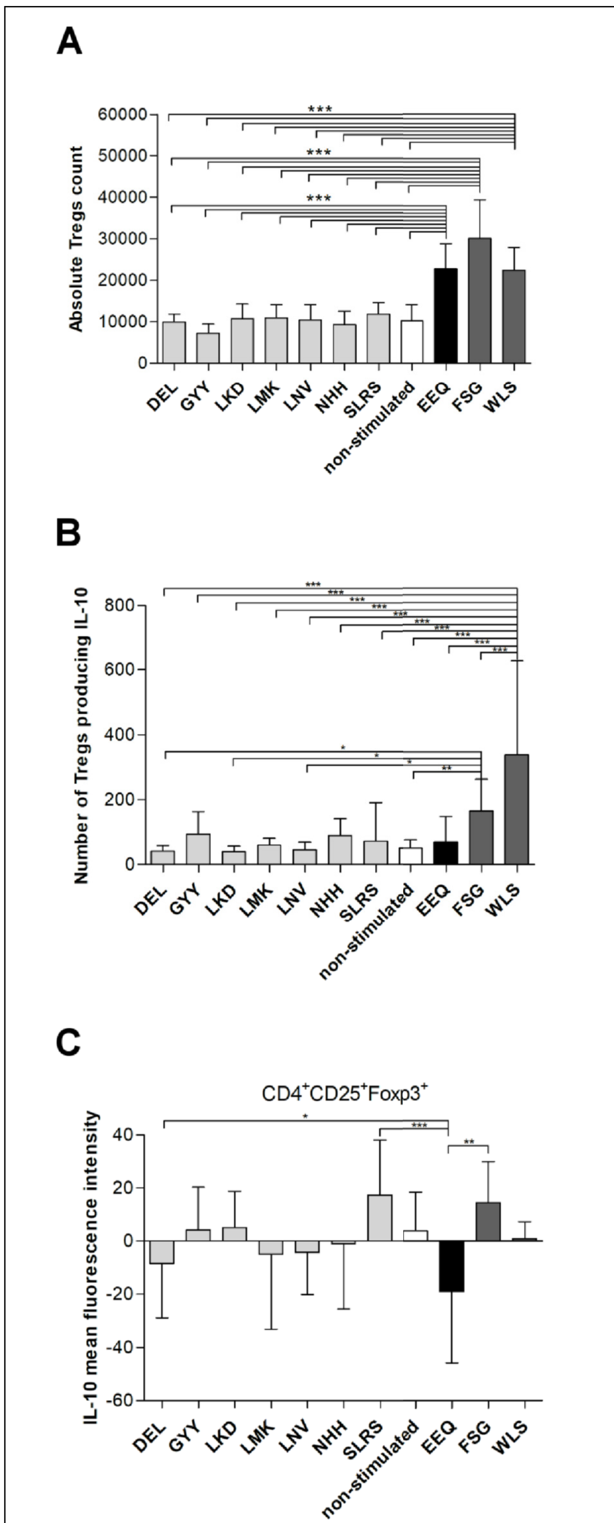

Fig. 4. The absolute Tregs and Tregs producing IL-10 count after stimulation of APCs/CD4$^+$CD25$^-$ co-culture with selected peptides and mean fluorescence intensity (MFI) of IL-10 in CD4$^+$CD25$^+$Foxp3$^+$ cells. Number of (A) CD4$^+$CD25$^+$Foxp3$^+$ cells, (B) CD4$^+$CD25$^+$Foxp3$^+$IL-10$^+$ 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-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-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-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.

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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.

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Tregitopes regulate the tolerogenic immune response and decrease the foetal death rate in abortion-prone mouse matings

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The imbalance in immune tolerance may cause the variety of reproductive failures. An intravenous immunoglobulin infusion (IVIg) therapy is used to improve the live birth rate in women suffering from recurrent pregnancy loss, recurrent spontaneous abortions and recurrent implantation failures. However, the results of IVIg studies are still inconclusive as IVIg infusion in women suffering from pregnancy loss is sometimes ineffective. One of the mechanisms of action of this treatment is inhibition of B cells differentiation and expansion of Tregs and secretion of interleukin 10. It was proposed that immunomodulatory effects of IVIg may be attributed to tregitopes - self-IgG-derived epitopes present in the structure of immunoglobulins. Similarly to IVIg, tregitopes cause the expansion of Tregs and secretion of antigen-specific effector cytokine response. Here, we studied whether the administration of mouse tregitope 167 and/or 289 can prevent abortions in mouse abortion-prone mouse matings. We revealed that tregitopes reduce the foetal death rate. This may be driven by observed higher pool of peripheral Tregs, increased production of IL-10 by Tregs and Bregs and/or maintaining the tolerogenic phenotype of antigen-presenting cells. We believe that our findings may indicate a potential alternative to IVIg for therapeutic intervention in case of pregnancy failures.

The presence of an antigenically foreign foetus induces a state of immune tolerance in the mother organism that is crucial to embryo implantation and foetus development. Imbalances in immune tolerance may cause a variety of reproductive failures such as preeclampsia or spontaneous and recurrent miscarriage. It is estimated that the problem of miscarriage affects one in four recognized pregnancies, with 85% of them being lost in the first trimester[1,2]. Despite continuous advances in reproductive medicine, the problem of recurrent miscarriage is still unsolved, and it is believed that more than half of such pregnancies do not have a clearly defined aetiology[3]. Therapies that have been proposed for the treatment of autoimmune-mediated pregnancy/reproductive failure[4] include intravenous infusion of immunoglobulin (IVIg), which contains a broad range of antibodies derived from the pooled plasma of healthy donors. In past years, IVIg has been used to improve the live birth rate in women suffering from recurrent pregnancy loss (RPL)[5–7], recurrent spontaneous abortion (RSA)[8–10] and recurrent implantation failure (RIF)[11–14]. The major mechanism of action of IVIg is related to natural killer cell inhibition, modulation of the functions of antigen-presenting cells (APCs), neutralization of cytokines and autoantibodies, inhibition of B cell differentiation and expansion of regulatory T lymphocytes (Tregs)[15–20]. A hypothesis involving Tregs expansion and secretion of tolerogenic interleukin 10 (IL-10) after IVIg treatment has been described by de Groot and co-workers[21]. They proposed that nonspecific antibodies present in IVIg might be internalized and processed by APCs and that self-immunoglobulin-derived epitopes are then presented to Tregs via the major histocompatibility complex II (MHCII). Formation of the TCR (T-cell receptor) epitope-MHCII complex leads to a decrease in the production of costimulatory molecules (CD80, CD86) by APCs and activation and proliferation of Tregs. Therefore, these natural Treg epitopes, with high-affinity binding to human class II Major Histocompatibility Complexes (HLA class-II) that are responsible for the suppression of immune response's effector phase towards own antigens were called tregitopes (T regulatory cell epitopes)[21]. Many recent studies have shown that T cells

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exhibit a typical $CD4^+CD25^+FOXP3^+$ regulatory cell phenotype in response to mouse and human tregitopes. *In vitro* co-incubation of T cells with immunogenic peptides inhibits the effector T cell response and the initiation of an antigen-specific effector cytokine response[22]. The sequences of natural Treg epitopes are highly conserved and are found in the light and heavy chains of human and mouse immunoglobulins (IgG). Two of five identified tregitopes, tregitope 167 (located in the first constant domain C_H1) and tregitope 289 (located in the second constant domain C_H2), bind to HLA class-II with the highest affinity as calculated by EpiMatrix scores[21]. Tregitopes have already been shown to regulate the immune response by increasing the expansion of Tregs in several autoimmune diseases, e.g., mouse models of diabetes[23], experimental autoimmune encephalomyelitis (EAE)[24] and cockroach allergy[25]. In mammalian pregnancy, Tregs are essential for the development of tolerance to foetal antigens. In both humans and mice, the levels and the activity of Tregs increase during normal pregnancy compared to non-pregnant controls and decrease in cases of spontaneous abortion when compared to normal pregnancy but not to non-pregnant subjects[26–31]. It was also recently shown that regulatory B lymphocytes (Bregs) may contribute to pregnancy maintenance based on the fact that, their number increases during normal pregnancy when compared to non-pregnant subjects and decreases in abortion-prone mice after mating and in spontaneous abortions cases in comparison to healthy pregnant women[32–35]. In CBA/JxDBA/2J mice, the most widely studied animal model of pregnancy failure caused by immune imbalance, the occurrence of abortion can be reduced by the adoptive transfer of regulatory B and/or T cells[36,37] and, most importantly, by IVIg administration[38,39]. In this mating, the high abortion rate may be provoked by an adverse reaction against paternal antigens present in the semen of DBA/2 J males[40]. This semen induce unfavourable immune response disrupting pregnancy tolerance what may lead to increased abortion rate in mated CBA/J females. As previously mentioned, it was already shown that tregitopes are able to induce expansion of Tregs and effectively suppress adverse immune response caused by auto- and alloantigens. Moreover, simultaneous co-administration of autoantigen and tregitopes to non-obese diabetic mice induced antigen-specific adaptive tolerance more effectively than tregitopes alone[41]. Therefore, in our study we propose that semen antigens together with early (within eight hours after mating) administration of tregitopes may suppress effector immune response against delivered antigens. Thus, the aim of this study was to investigate whether the early administration of two selected IgG-derived epitopes, mouse tregitopes 167 and 289 can cause the expansion of regulatory lymphocytes and prevent abortion in a mouse abortion-prone model.

Results

Tregitopes decrease the abortion rate. To determine whether tregitope administration is beneficial to pregnancy maintenance in abortion-prone mice, we calculated the foetal death rate at the 14th day of pregnancy according to the formula described in the Methods section. Administration of tregitope 167 (T167) or tregitope 289 (T289) resulted in a significant decrease ($p=0.0009$ and $p=0.0059$, respectively) in the foetal death rate compared to the foetal death rate in female mice that received only PBS (14.29% and 16.35%, respectively, vs 36.20%) (Fig. 1a). The injection of tregitopes did not change the number of viable embryos (Fig. 1b), however, administration of either of the tested tregitopes led to a significant reduction in the number of resorbed embryos ($p=0.0001$ for T167 and $p=0.0112$ for T289) and total implantation sites ($p=0.0004$ for T167 and $p=0.0082$ for T289) compared to the control (Fig. 1c,d respectively).

Tregitope 289 enhances the TGFβ1 level. To determine whether tregitope treatment restores the Th1/Th2 balance during pregnancy in abortion-prone mice, we examined the levels of IL-2, IL-4, IL-10, IFNγ and TGFβ1 cytokines in blood sera. The absorbances obtained for IL-2, IL-4, IL-10 and IFNγ on 3rd and 14th day of pregnancy were below the level of detection provided by the kit (data not shown). However, on the 3rd day of pregnancy, mice treated with tregitope 289 had higher serum concentrations of TGFβ1 (196.6 ± 21.85) than control mice (149.8 ± 22.25) (Fig. 1d; $p=0.0003$). At the 14th day of pregnancy, we did not observe any differences in the TGFβ1 levels in the investigated groups of mice.

Tregitopes administration increased Tregs frequency and IL-10 production. To determine whether tregitopes administration to abortion-prone mice after mating could stimulate Tregs expansion, the percentage of $CD4^+CD25^+FOXP3^+$ and $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ cells in the spleens and uterine-draining lymph nodes (Fig. 3) of the animals were analysed based on the gating strategy shown in Fig. 2.

We found that at the 3rd day of pregnancy, both tregitopes induced expansion of $CD4^+CD25^+FOXP3^+$ cell populations in the spleens of the treated animals (T167 $p=0.0245$; T289 $p=0.0399$) (Fig. 3a) but not in the uterine-draining lymph nodes (Fig. 3b). The frequency of $CD4^+CD25^+FOXP3^+$ cells in the spleen was almost two-fold higher in the treated animals (0.4212 ± 0.2498 for T167 and 0.3764 ± 0.1113 for T289) than in the vehicle group (0.2139 ± 0.1120). However, only T167 induced a higher percentage of $CD4^+CD25^+FOXP3^+$ cells in the $CD4^+$ splenocyte population (Fig. 3c; $p=0.041$). We did not observe any alterations in the frequencies of $CD4^+CD25^+FOXP3^+$ cells among $CD4^+$ lymph node cells (Fig. 3d). Analysis of $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ cells on the 3rd day of pregnancy revealed that only T167 administration significantly increased the frequency of Tregs producing IL-10 in splenocytes (Fig. 3e; $p=0.0249$) and in splenic $CD4^+$ cells (Fig. 3g; $p=0.041$), but no differences in uterine-draining lymph nodes were observed between tregitopes and controls (Fig. 3f,h). However, in uterine-draining lymph nodes, lower levels of IL-10 MFI ($p=0.0055$) were found in the examined cells in mice treated with tregitope 167 in comparison to the control group (Fig. 3j).

No differences were observed in the frequency of $CD4^+CD25^+FOXP3^+$ cells (Tregs) on the 14th day of pregnancy in any of the examined tissues (Fig. 3a,b). Nevertheless, T289 administration decreased the percentage of $CD4^+CD25^+FOXP3^+$ cells in the $CD4^+$ splenocyte population (Fig. 3c; $p=0.0003$). Analysis of $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ cells on the 14th day of pregnancy revealed that individual administration of T167 ($p=0.004$) and T289 ($p=0.0008$) increased the frequency of these cells among splenocytes (Fig. 3e; $p=0.004$ or $p=0.0008$, respectively) and $CD4^+$ cells (Fig. 3g; $p=0.008$ or $p=0.0047$, respectively). At the same time,

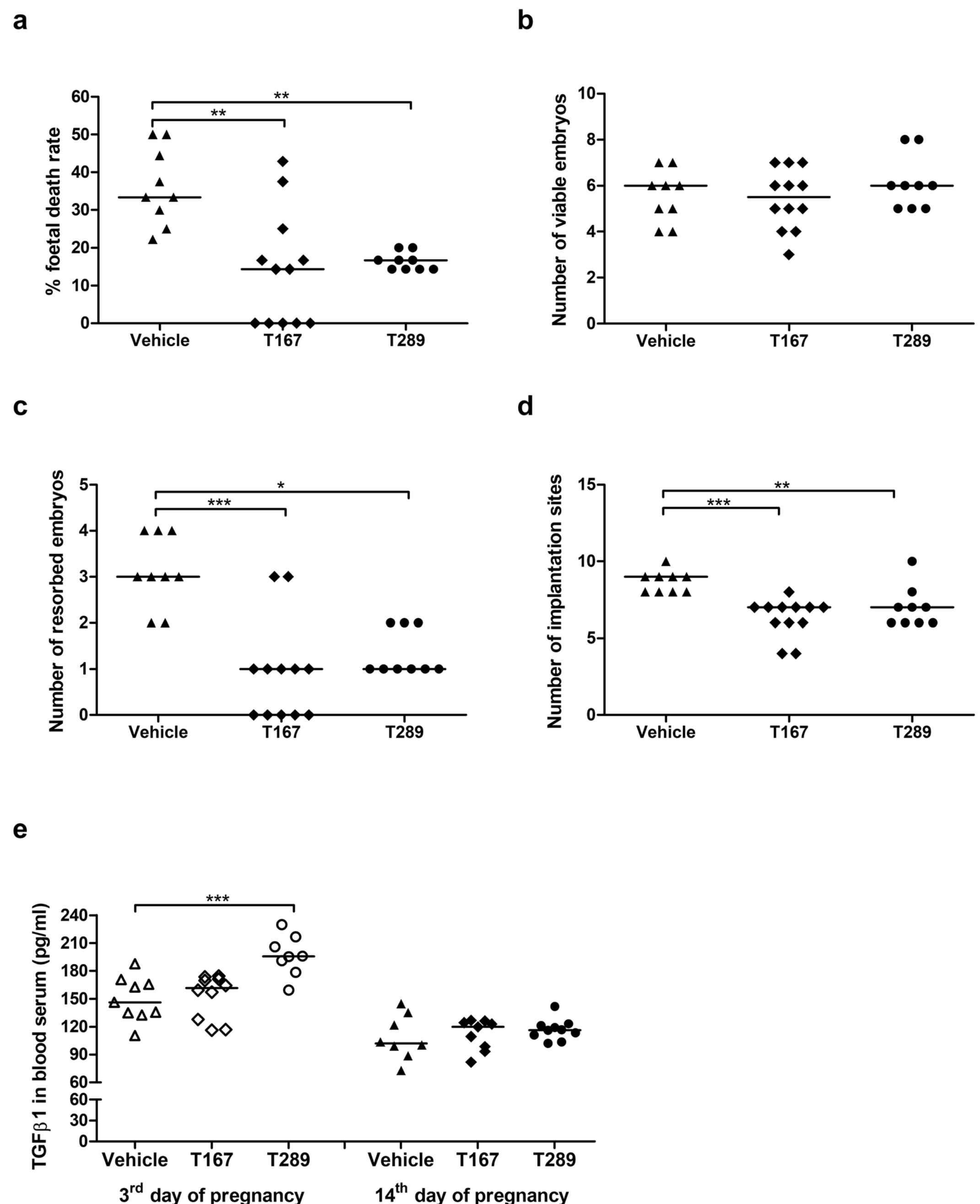

Figure 1. Effect of tregitope treatment on foetal death rates, numbers of viable and resorbed embryos, total implantation sites and cytokine levels in a murine abortion-prone pregnancy model. **(a)** Effect of tregitope injection on foetal death rate and numbers of **(b)** viable, **(c)** resorbed embryos and **(d)** total implantation sites evaluated at the 14th day of pregnancy. **(e)** Effect of tregitope injection on TGFβ1 concentrations in serum on the 3rd and 14th days of pregnancy. The data were analysed by the Kruskal-Wallis test (non-normal distribution) with Dunn's multiple comparison post-hoc test ($P < 0.05$) and are presented as individual values with median (at 14dpc $n = 12$ for T167, $n = 9$ for T289, $n = 9$ for Vehicle; at 3dpc $n = 11$ for T167, $n = 11$ for T289, $n = 9$ for Vehicle); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

administration of tregitopes also significantly enhanced the level of IL-10 (the MFI value) in splenic Tregs (T167 $p = 0.0422$; T289 $p = 0.0155$) in comparison to the control (Fig. 3i). We also found that T167 augmented not only the frequency of IL-10-producing Treg cells (Fig. 3f; $p = 0.0264$) but also the level of IL-10 in uterine-draining lymph nodes on the 14th day of pregnancy (Fig. 3j; $p = 0074$) in comparison to the vehicle group.

Tregitopes administration enhanced IL-10 production by Bregs cells. To verify whether tregitopes administration stimulates the expansion of B regulatory lymphocyte populations in abortion-prone mice, we examined the percentage of $CD19^{+}CD5^{+}CD1d^{hi}IL\text{-}10^{+}$ cells (Bregs) in the spleens and uterine-draining lymph

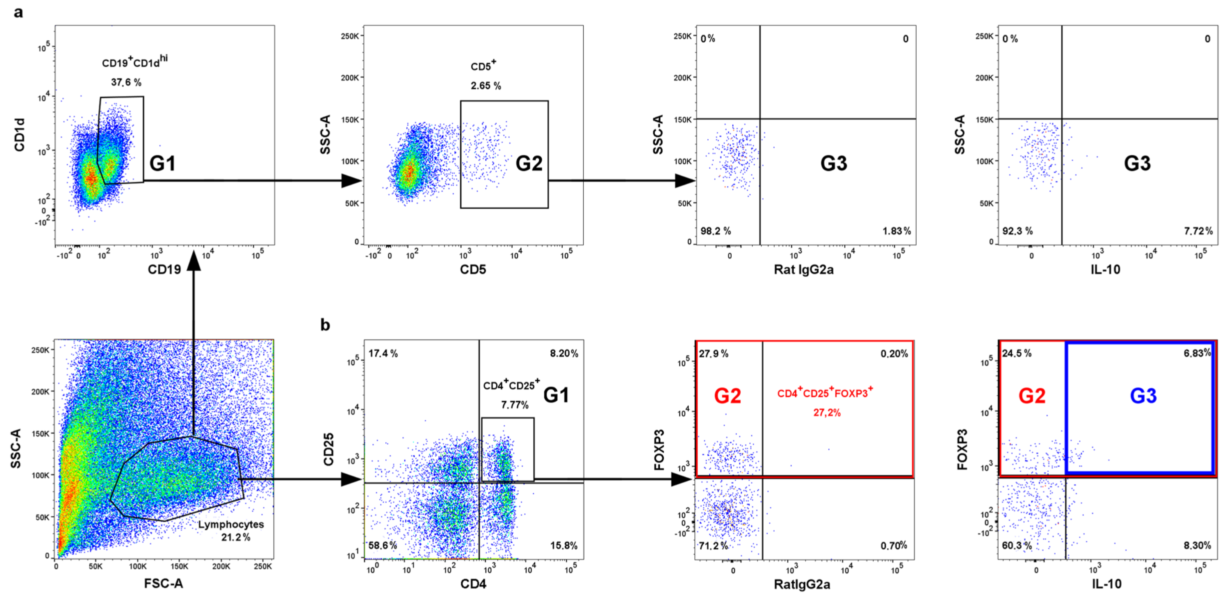

Figure 2. Representative dot plots for the process of regulatory T and B lymphocytes gating in spleen sample. **(a)** Gating strategy for $CD19^+CD1d^+CD5^+IL\text{-}10^+$ lymphocytes: **G1** represents $CD19^+CD1d^+$ cells, **G2** represents $CD19^+CD1d^+CD5^+$ cells, and **G3** represents $CD19^+CD1d^+CD5^+IL\text{-}10^+$ cells. **(b)** Gating strategy for $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ lymphocytes: **G1** represents $CD4^+CD25^+$ cells, **G2** represents overall $CD4^+CD25^+FOXP3^+$ cells, and **G3** represents $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ cells.

nodes of the treated mice (Fig. 4) based on the gating strategy shown in Fig. 2. No differences in the frequency of Bregs in the spleens (Fig. 4a) or uterine-draining lymph nodes (Fig. 4b) of the treated animals were observed at the 3rd or 14th days of pregnancy in comparison to mice that received PBS ($p > 0.05$). However, at the 14th day of pregnancy, $CD19^+CD5^+CD1d^{hi}$ splenocytes from mice treated with T167 or T289 were found to produce higher levels of IL-10 (Fig. 4c) than $CD19^+CD5^+CD1d^{hi}$ splenocytes from control mice (T167 $p = 0.0385$; T289 $p = 0.0462$). Moreover, T167 significantly enhanced the level of IL-10 in $CD19^+CD5^+CD1d^{hi}$ cells in uterine-draining lymph nodes (Fig. 4d; $p = 0.0004$).

Tregitopes administration modulates the costimulatory phenotype of APCs. Cousens *et al.* proposed that tregitopes are able to modify the costimulatory phenotype of antigen-presenting cells[22]. To test that hypothesis in our model, we selected dendritic cells ($CD11c^+$, DCs), B lymphocytes ($CD19^+$) and B cells with expression of CD11c ($CD19^+CD11c^+$) as populations of antigen-presenting cells and examined the surface expression of CD40, CD80, CD86 and MHC class II by measuring the median fluorescence intensity (MFI) of the cells (Fig. 5, Supplementary Table S1) based on the gating strategy shown in Fig. 6. The proportions of antigen-presenting cells with expression of the aforementioned costimulatory molecules are shown in Supplementary Fig. S1.

We found that at the 3rd day of pregnancy, $CD11c^+$ splenocytes from mice treated with tregitopes 167 and 289 showed decreased expression of CD86 ($p = 0.0004$ and $p = 0.0001$, respectively) compared with $CD11c^+$ splenocytes from control mice (Fig. 5a). At the same time, T167 treatment increased MHC class II expression ($p = 0.0126$), and T289 decreased CD80 expression ($p = 0.0178$) on splenic $CD11c^+$ cells (Fig. 5a). In mice treated with T167, there were no differences in the expression of costimulatory molecules or MHC class II on $CD19^+$ (Fig. 5c) and $CD19^+CD11c^+$ (Fig. 5e) splenocytes. In contrast, T289 enhanced the expression of CD40 on $CD19^+$ (Fig. 5c; $p = 0.0013$) and $CD19^+CD11c^+$ splenocytes (Fig. 5e; $p = 0.0148$). In uterine-draining lymph nodes, we observed no differences in the expression of selected molecules on $CD19^+$, $CD11c^+$ and $CD19^+CD11c^+$ cells in mice treated with tregitope 167 and controls (Fig. 5b,d,f respectively). However, tregitope 289 increased the expression of MHC class II and CD40 on $CD11c^+$ (Fig. 5b; $p = 0.017$ and $p = 0.0195$, respectively) and $CD19^+CD11c^+$ cells (Fig. 5f; $p = 0.0022$ and $p = 0.0211$, respectively) in uterine-draining lymph nodes.

At the 14th day of pregnancy, mice treated with tregitope 167 showed decreased expression of CD86 ($p = 0.014$ on $CD11c^+$ cells and $p = 0.0085$ on $CD19^+$ cells) and enhanced expression of MHC class II ($p = 0.0432$ on $CD11c^+$ cells and $p = 0.0085$ on $CD19^+$ cells) and CD40 ($p = 0.0203$ on $CD11c^+$ cells and $p = 0.0316$ on $CD19^+$ cells) on splenic $CD11c^+$ (Fig. 5a) and $CD19^+$ cells (Fig. 5c). T167 also inhibited the expression of CD86 ($p = 0.0141$) and enhanced CD40 ($p = 0.0134$) expression on splenic $CD19^+CD11c^+$ cells in comparison with mice treated with vehicle (Fig. 5e). At the same time, tregitope 289 decreased the expression of CD80 ($p = 0.013$) and CD86 ($p = 0.037$) on splenic $CD11c^+$ cells compared to controls (Fig. 5a). In addition, we observed inhibition of CD86 ($p = 0.0483$ on $CD19^+$ cells and $p = 0.0028$ on $CD19^+CD11c^+$ cells) and enhancement of CD40 ($p < 0.0001$) expression on splenic $CD19^+$ (Fig. 5c) and $CD19^+CD11c^+$ (Fig. 5e) cells as well as enhancement of MHC class II expression on $CD19^+$ cells (Fig. 5c; $p = 0.007$). In uterine-draining lymph nodes, there was no difference in the

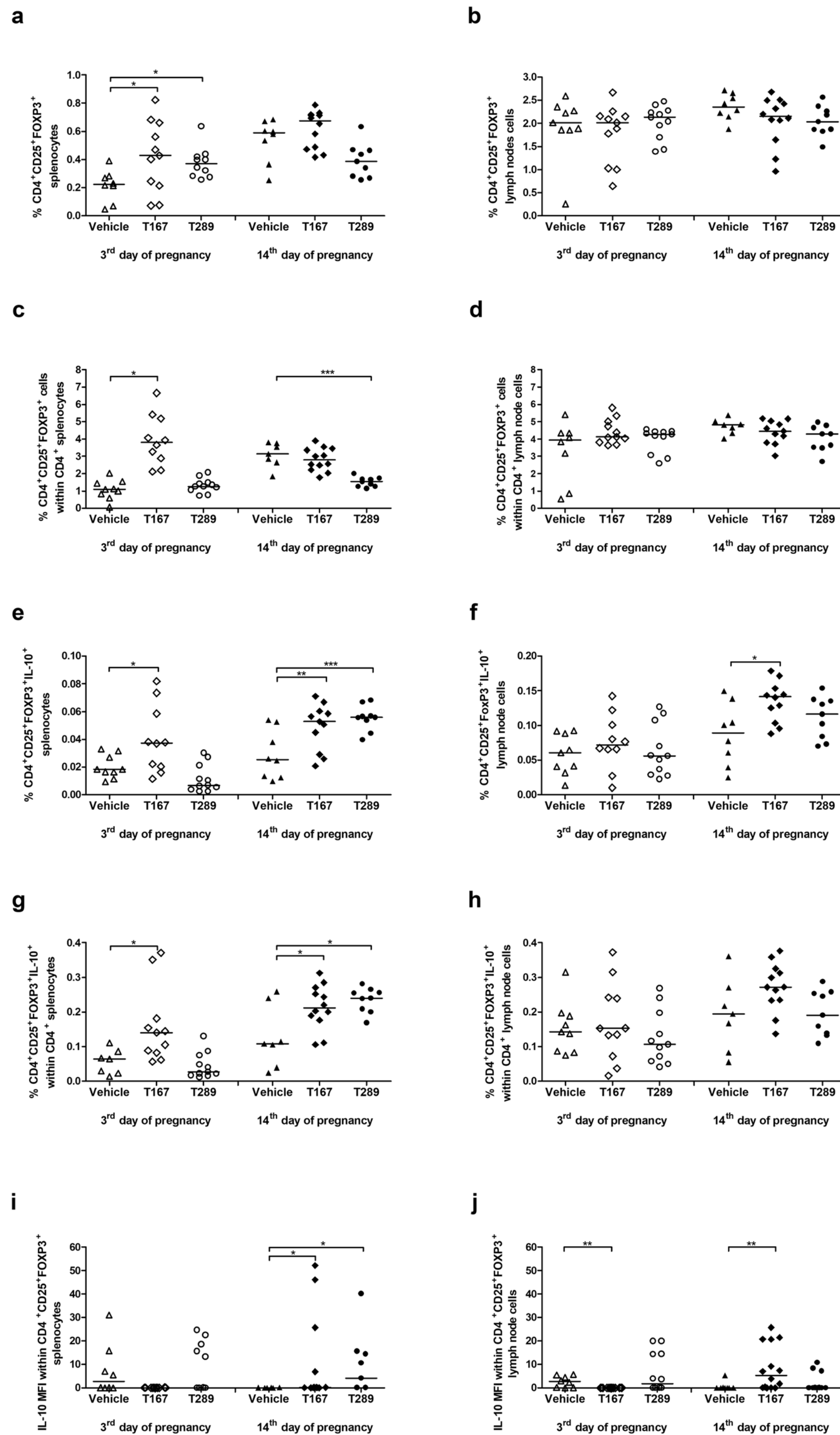

Figure 3. Effect of tregitope treatment on regulatory T lymphocytes in a murine abortion-prone pregnancy model. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A and monensin and the frequencies of CD4+CD25+Foxp3+ **(a)** splenocytes and **(b)** uterine-draining lymph node cells within CD4+ **(c)** splenocytes and **(d)** lymph node cells and the frequencies of CD4+CD25+FOXP3+ IL-10+ splenocytes **(e)** and **(f)** lymph node cells within CD4+ **(g)** splenocytes and **(h)** lymph nodes and the median fluorescence intensity (MFI) of IL-10 within CD4+CD25+FOXP3+ **(i)** splenocytes and **(j)** uterine-draining lymph node cells were measured. The data were analysed by one-way ANOVA (normal distribution) or the Kruskal-Wallis test (non-normal distribution) with Dunn's multiple comparison post hoc test ($P < 0.05$) and are presented as individual values with median (at 14dpc n = 12 for T167, n = 9 for T289, n = 9 for Vehicle; at 3dpc n = 11 for T167, n = 11 for T289, n = 9 for Vehicle). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

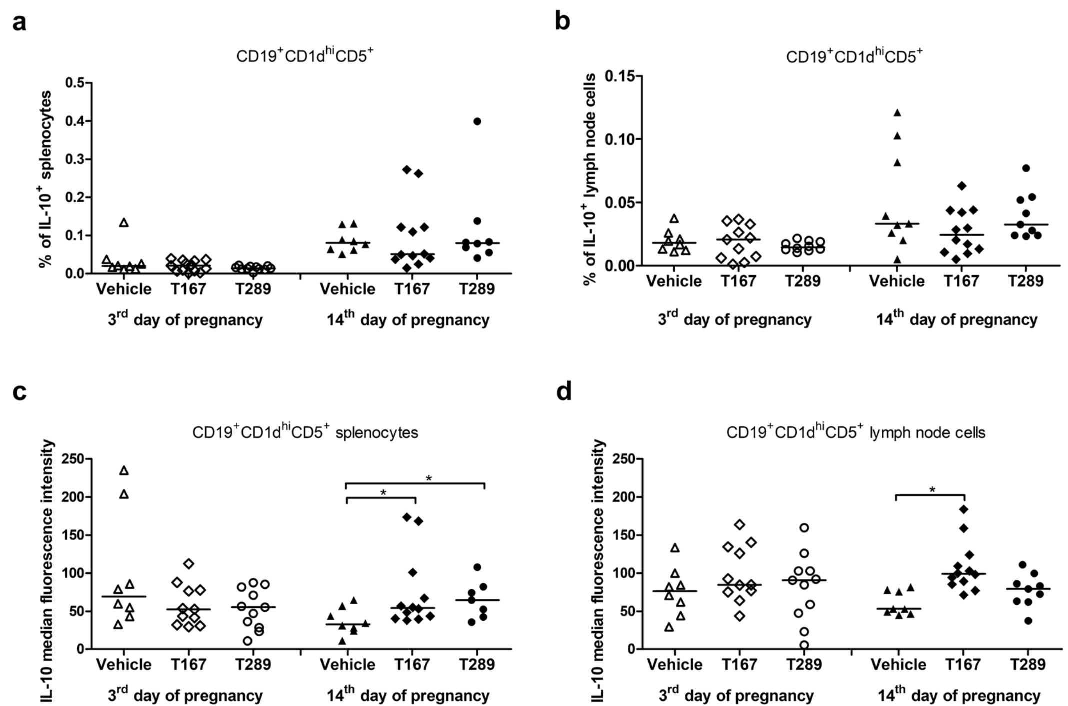

Figure 4. Effect of tregitope treatment on regulatory B lymphocytes in a murine abortion-prone pregnancy model. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A and monensin and the frequencies of $CD19^+CD1d^+CD5^+IL\text{-}10^+$ (**a**) splenocytes and (**b**) uterine-draining lymph node cells, and the production of IL-10 by $CD19^+CD1d^+CD5^+$ (**c**) splenocytes and (**d**) lymph node cells were analysed by intracellular staining. The data were analysed by one-way ANOVA (normal distribution) or the Kruskal-Wallis test (non-normal distribution) with Dunn's multiple comparison post hoc test ($P < 0.05$) and are presented as individual values with median (at 14dpc n = 12 for T167, n = 9 for T289, n = 9 for Vehicle; at 3dpc n = 11 for T167, n = 11 for T289, n = 9 for Vehicle). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

expression of costimulatory molecules on selected APCs after T167 administration (Fig. 5b,d,f). In contrast, T289 administration increased the expression of MHC class II ($p = 0.0433$) and CD40 ($p < 0.0001$) on B cells (Fig. 5d) and enhanced the expression of CD40 ($p < 0.0001$) on $CD19^+CD11c^+$ cells (Fig. 5f) in uterine-draining lymph nodes.

Discussion

It is well known that improper immune tolerance towards foetal antigens can result in miscarriage. IVIg infusion is one of the therapies currently used to increase the live birth rate in women suffering from pregnancy complications/failures. However, the results of IVIg studies are still inconclusive. Despite promising results in some studies, meta-analysis and systematic reviews of clinical trials of IVIg have failed to prove its effectiveness in pregnancy outcome[42–46]. In general, IVIg treatment is relatively safe; however, its high cost and limited supply makes it desirable to develop a new therapy that can be used to improve immune tolerance in pregnancy. The possibility of such a therapy arose in 2008 when the complete amino acid sequence of IgG was determined and natural Treg epitopes, which display high-affinity binding to human class II major histocompatibility complexes (HLA-DR), were discovered[21]. These natural Treg epitopes, called tregitopes, are conserved in all IgG allotypes; they have the ability to bind to various HLAs with high affinity and, like IVIg therapy, are capable of causing Treg expansion.

To investigate the involvement of tregitopes in pregnancy maintenance, we used a commonly studied murine model of immune-mediated pregnancy failure (CBA/J female mice mated with DBA/2 J males) that is believed to share some similarities with human pregnancy loss[47–49]. Similarly to human pregnancies, in this model the key role in suppressing the response towards foreign foetal antigens is played by regulatory T cells that express the Foxp3 transcription factor. In women who suffer from spontaneous miscarriages and preeclampsia, there is a decrease in the number and activity of Treg[28,50,51]. In a murine abortion-prone model, reduced frequencies of Treg cells were observed both at the periphery and locally in the uterus during the pre- and post-implantation period of pregnancy compared with females presenting a normal pregnancy[32,52].

Using the aforementioned model, we analysed pregnancy outcome at 14 dpc after administration of tregitopes by assessing the numbers of healthy and resorbed foetuses. Our findings showed that administration of either of the tested tregitopes led to a significant reduction in the foetal death rate compared to the control. The unchanged number of viable embryos and decreased number of total implantation sites between groups of mice, may suggest that tregitope's treatment rather than rescuing the pregnancy, may ensure that only good quality embryos are implanted. Implantation of those "healthy" embryos may be beneficial for mother's organism to avoid an unnecessary energy investment in developing pregnancy that is doomed to fail[53]. One possible explanation for

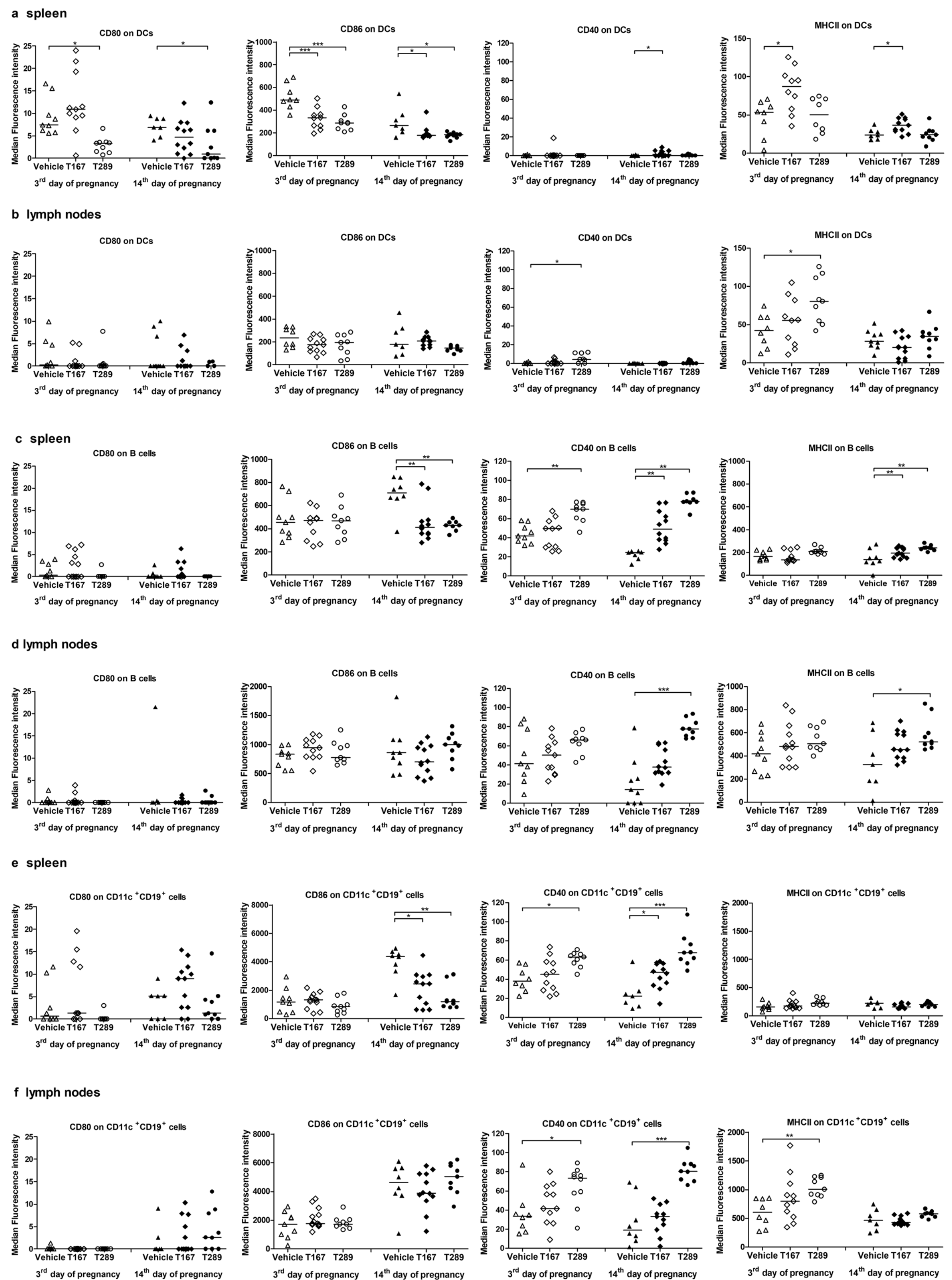

Figure 5. Effect of tregitope treatment on the expression of costimulatory molecules on antigen-presenting cells in abortion-prone mice. The data shown the specific fluorescence intensity of CD40, CD80, CD86 and MHC class II proteins on the surfaces of $CD11c^{+}$ **(a)** splenocytes and **(b)** uterine-draining lymph node cells, $CD19^{+}$ **(c)** splenocytes and **(d)** uterine-draining lymph node cells and $CD11c^{+}$ $CD19^{+}$ **(e)** splenocytes and **(f)** uterine-draining lymph node cells at the 3rd and 14th days of pregnancy. The data were analysed by one-way ANOVA (normal distribution) or the Kruskal-Wallis test (non-normal distribution) with Dunn's multiple comparison post hoc test ($P < 0.05$) and are presented as individual values with median (at 14dpc $n = 12$ for T167, $n = 9$ for T289, $n = 9$ for Vehicle; at 3dpc $n = 11$ for T167, $n = 11$ for T289, $n = 9$ for Vehicle). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

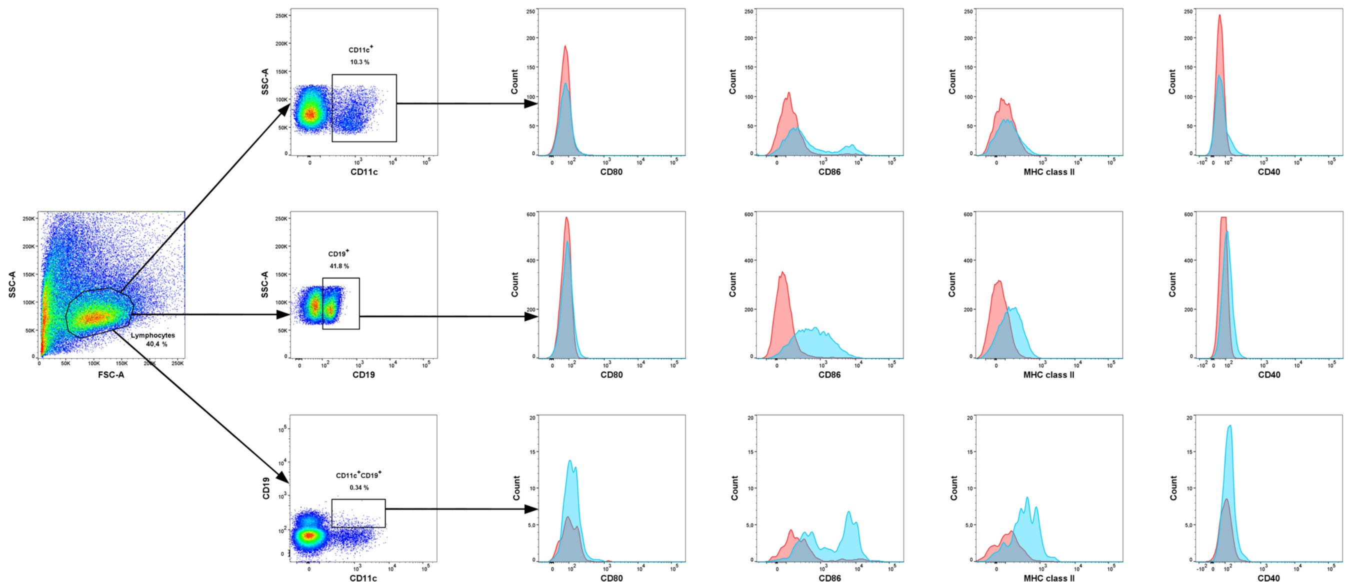

Figure 6. Representative overlay histograms comparing the expression of CD40, CD80, CD86 and MHC class II molecules (blue histograms) with that in the respective isotype-matched controls (red histograms) in CD11c^{+} cells, CD19^{+} cells, and CD19^{+}CD11c^{+} cells derived from spleen of abortion-prone mice.

the effect of tregitopes could be induction of tolerance through increased frequencies of Tregs. We observed that after administration of tregitopes, the peripheral splenic Tregs pool was already higher in early pregnancy (3 dpc), that is, before implantation even occurs, in comparison to the vehicle group. However, only T167 increased the frequency of Tregs producing IL-10 compared to controls. The higher proportion of splenic Tregs expressing IL-10 was maintained in the subsequent stage of pregnancy (14 dpc, mid-pregnancy) after injection of either tregitope in comparison to mice that received vehicle. On the other hand, in paraortal uterine-draining lymph nodes (PALN), only T167 significantly induced Tregs producing IL-10 and also enhanced the production (MFI) of IL-10 compared to control mice. The limited changes in the Tregs proportions in paraortal uterine-draining lymph nodes suggest that tregitopes treatment induce systemic changes in Tregs population, but the specific impact on the IL-10^{+} population in PALN. According to Langenhorst *et al*. Tregs (FOXP3^{+}) lymphocytes producing IL-10 are fully activated, terminally differentiated cells with a limited life-span[54]. Therefore, observed by us the presence of this rare phenotype in uterine-draining lymph nodes indicates that the intraperitoneal administration of tregitopes can induce not only peripherally but also locally effective immunosuppression. Thus, it is tempting to speculate that T167 is more effective than T289 in the induction of Tregs. Our data support other studies that have utilized tregitopes 167 and 289 to treat diseases with immune aetiology. In those studies, increased Tregs frequency was also observed after the administration of tregitopes[21,23,24]. Here, we demonstrate that most probably these Tregs are functional and activated as they produce more IL-10 than is found in control mice. The production of IL-10 is one of the main attributes of tolerance induction by Tregs. The importance of the anti-inflammatory cytokine IL-10 in normal pregnancy development has been confirmed in humans as well as in murine studies[55,56]. It was shown that the administration of exogenous IL-10 caused a decrease in pregnancy loss in a murine abortion-prone model[57]; this is one of the mechanisms that may be responsible for the observed decrease in the foetal death rate.

In this paper, we were also interested in whether tregitopes affect another type of regulatory cells, regulatory B lymphocytes (Bregs). The role of B cells in pregnancy has been studied extensively[17,58–60]. The regulatory and suppressive functions of B cells in pregnancy have been mainly attributed to the production of IL-10 and interaction with T cells to inhibit the immune response. In mice, IL-10-producing B cells (B10 cells) were found mainly among CD19^{+}CD1d^{+}CD5^{+} cells; however, other subsets of B cells may also have suppressive/regulatory functions. In an abortion-prone model, a diminished percentage of splenic CD19^{+}IL-10^{+} was observed compared to mice presenting normal pregnancies. Transfer of B10 cells into these mice prevented foetal resorption and led to Tregs expansion[36]. It is believed that the main function of Bregs is control of Tregs function via direct cell-to-cell contact that is mediated by costimulatory molecules and/or indirectly through the cytokine milieu, e.g., the secretion of soluble factors such as IL-10 or TGFβ. Here, we observed that both tregitopes augmented IL-10 expression in CD19^{+}CD1d^{+}CD5^{+} cells (calculated as MFI) at mid-pregnancy. The results presented here suggest that tregitopes may modulate the cross-talk between Bregs and Tregs and that such cross-talk is important in rescuing foetuses from immune rejection. The tregitopes-induced elevated expression of IL-10 in both Tregs and Bregs suggests that indirect contact between these cells may be involved. However, their interplay is probably more complex, as we also found changes in the expression of costimulatory molecules on B cells after the administration of tregitopes.

At present, it is undebatable that in addition to producing autoantibodies and pro- and anti-inflammatory cytokines, B cells, along with dendritic cells (DCs), are professional antigen- presenting cells that are able to

present antigens to T cells in the context of MHC class II molecules. The antigen presentation, together with high expression of MHCII, costimulatory molecules (CD80, CD86) and secretion of cytokines, leads to T cell proliferation, cytokine production and the development of effector functions. Antigen presentation by APCs in the absence of a costimulatory signal may cause T cells to differentiate into Tregs. Here, we found that at the preimplantation stage of pregnancy both tregitopes caused decreased expression of CD86 and CD80 on $CD11c^+$ cells. It is well known that low expression of maturation markers as well as costimulatory molecules is a determinant of the immature state of DCs (iDCs) that enables the establishment of tolerance toward self-antigens[61,62]. Therefore, we hypothesize that these tregitopes may trigger an immature status of DCs and that these DCs in turn effectively induce Treg expansion upon antigen presentation. The iDCs phenotype is maintained even at mid-pregnancy, as we observed decreased expression of CD86 on $CD11c^+$ cells and other APCs ($CD19^+$). On the other hand, we observed the enhancement of CD40 and MHC class II molecules on splenic and lymph node APCs during the course of pregnancy after the administration of tregitopes. This may suggest that DCs became activated rather than being immature and indicate the process of activation of T cells. However, it was shown that increased expression of MHC II molecule on DCs[63,64] enable T cell activation that may contribute to Tregs development as its enhancement is required for Treg homeostasis[65]. Furthermore, the immature/mature form of DCs depend not only on costimulatory molecules but also on timing, dose and signal strength of many factors that interact with DCs like the inflammatory cytokines or chemokines[66]. Therefore, we believe that the observed increased production of IL-10 by Bregs and Tregs may lead to DCs shift towards iDCs because it was previously shown that IL-10 produced by B10 cells is responsible for keeping DCs in an immature state and that it inhibits their ability to present antigens to T cells[67]. However, further studies, including detailed DCs phenotyping, needs to be performed to clarify the DCs state after tregitopes administration. Like IL-10, TGFβ is one of the cytokines that is crucial in controlling the phenotype of DCs and the differentiation of Tregs[68–70]. We found that the concentration of plasma TGFβ1 during early pregnancy is higher in mice treated with T289. We believe that TGFβ may be another factor that is involved in the induction and maintenance of Tregs and that it may be beneficial for pregnancy maintenance after administration of tregitopes.

The downregulation of CD80 and CD86 observed in this study may partially explain the findings of Jin *et al.* and Zhou *et al.* that systemic blockade of CD80 and CD86 or of CD86 alone at implantation inhibits maternal rejection of allogenic foetuses in CBA/JxDBA/2 J matings[71,72]. We hypothesize that the alteration in the expression of CD86 and CD80 on APCs, including splenic B cells, that is observed after the administration of tregitopes may be crucial for proper embryo implantation and subsequent embryonic development through activation of immune tolerance toward foetal antigens. Our results confirmed data obtained in previous studies that also demonstrated downregulation of CD80 and CD86 costimulatory molecules on APCs after exposure to tregitopes[22]. All of these findings support the hypothesis proposed by Cousens *et al.* that tregitopes may generate immune tolerance to self-antigens and probably to alloantigens through their presentation by APCs.

Conclusions

In summary, our study demonstrated that treatment with tregitopes significantly increased the frequency of Tregs, enhanced the production of IL-10 by Tregs and Bregs and changed the costimulatory responses of APCs, contributing to the reduction of the foetal death rate. Our findings for the first time indicate, that tregitopes may be a potential tool for therapeutic intervention in cases of immune-mediated pregnancy failures. However, further studies should be performed to confirm the ability of tregitopes to modulate the immune response in humans, especially in the context of IVIg therapies.

Methods

Animals. CBA/J female and DBA/2 J male mice (Charles River Laboratories, Sulzfeld, Germany), were housed under specific pathogen-free (SPF) conditions under/in a dark-light cycle of 12 h:12 h. The reproductive cycle of 6-8-week-old female CBA/J mice was monitored by Cytocolor staining (Merck Millipore, USA) of vaginal smears. Females at the proestrus phase were placed with stud DBA/2 J males at 19:00 and copulatory plug were checked at 7:00-8:00. The morning the plug appears was defined as day 0 *post coitum* (dpc). Two periods of pregnancy were investigated: 3rd day of pregnancy (preimplantation pregnancy) and 14th day of pregnancy. Three groups of mice for each period of pregnancy were examined: a group of pregnant mice after administration of tregitope 167, a group of pregnant mice after administration tregitope 289 or a group of pregnant mice after administration PBS buffer. Immediately after detection of a copulatory plug, female mice were intraperitoneally injected with 100 µg (dissolved in 150 µl PBS) of mouse tregitope 167 (T167) with a sequence corresponding to the Fc fragment of IgG (PAVLQSDLYTLSSSVTVPSS, purity >90%, GeneCust, Luxembourg), 100 µg of mouse tregitope 289 (T289) with a sequence corresponding to Fc fragment of IgG (EEQFNSTFRSVSELPIMHQ, purity >90%, GeneCust, Luxembourg) or phosphate-buffered saline (vehicle) as a control. The tregitopes concentrations were selected based on our previous observations[73] and other studies that used tregitopes to treat autoimmune diseases in mouse models[23–25]. Pregnancy was confirmed *post mortem* by the presence of embryos in the uterine horns after flushing at 3 dpc or by observation of the number of foetuses and implantation sites at 14 dpc. At the 3rd and 14th days of pregnancy, the pregnant mice were anaesthetized, blood samples were collected, and the animals were euthanized by cervical dislocation. The spleens, the paraortal uterine-draining lymph nodes (PALN) and the uteri were dissected for further analyses. All efforts were made to minimize the animals' suffering. The animal experiments were approved by the Local Ethics Committee for Experiments on Animals at the Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw (No. 53/2015). All methods used in this study were performed in accordance with the relevant guidelines and regulations.

Tissue processing. The animals' spleens and lymph nodes were processed according to a standard protocol as described previously[73,74]. In brief, spleens and uterine-draining lymph nodes were squeezed through a 40-μm cell strainer (Falcon) into 0.84% ammonium chloride solution and sorting buffer (PBS buffer supplemented with 2 mM EDTA and 2% foetal bovine serum (Biowest, France)), respectively, and washed twice (4 °C, 300 × g, 10 minutes) in sorting buffer. The isolated cells were used in flow cytometry analysis.

Uteri from 14 dpc pregnant females were used to determine the foetal death rate. The rate was calculated as the number of resorbed embryos divided by the total number of embryos (resorbed plus viable embryos) multiplied by one hundred. The abortion sites were identified by their small size accompanied by a necrotic, haemorrhagic appearance compared with normal embryos and placentas[32].

Flow cytometry. Splenic and lymph node cells (1×10^6 cells) were stimulated with 0.1 μg/ml phorbol 12-myristate 13-acetate (Cayman Chemical, USA), 1 μg/ml ionomycin (Cayman Chemical, USA), 10 μg/ml brefeldin A (eBioscience, USA) and 2 μM monensin (eBioscience, USA) in RPMI-1640 medium supplemented with 10% FBS and 1x penicillin/streptomycin (from 100×) (Merck Millipore, USA) in a tissue culture incubator at 37 °C and 5% CO_2 for 6 hours. After incubation, the cells were stained as previously described[75]. In brief, the cells were stained with anti-mouse CD4 Alexa Fluor 700 (eBioscience, USA, clone: Gk1.5), CD25 APC-Cy7 (BD Biosciences, clone: PC61), CD19 FITC (eBioscience, USA, clone: eBio1D3), CD1d PE (eBioscience, USA, clone: 1B1), CD5 Pacific Blue (eBioscience, USA, clone: 53-7.3), CD11c APC-Cy7(eBioscience, USA, clone: N418), CD80 Pacific Blue (eBioscience, USA, clone: 16-10A1), CD86 PE-Cy7 (eBioscience, USA, clone: GL1), CD40 PE (eBioscience, USA, clone: 1C10) and MHC Class II (IA/IE) Alexa Fluor 700 (eBioscience, USA, clone: M5/114.15.2) antibodies or appropriate isotype controls at 4 °C for 30 minutes in the dark. The cells were then washed twice with staining buffer, and intracellular staining was performed according to the instructions supplied with the FOXP3/Transcription Factor Staining Buffer Set (eBioscience, USA). In brief, the cells were fixed for 14 h at 4 °C in the dark, washed twice with permeabilization buffer (eBioscience, USA) and stained for 15 minutes at 4 °C with anti-CD16/CD32 antibodies to block nonspecific binding. The cells were then stained for 1 h at 4 °C in the dark with anti-mouse FOXP3 PE-Cy7(eBioscience, USA, clone: FjK-16s) and anti-IL-10 APC (eBioscience, USA, clone: JES5-16E3) antibodies or the appropriate isotype control at the same concentration as the specific antibody. The cells were washed twice with permeabilization buffer, and cellular fluorescence was immediately measured on an LSRFortessa cell analyzer (Becton Dickinson, USA). The relative levels of CD40, CD80, CD86, MHC-class II and IL-10 antigens are shown as the specific median fluorescence intensity (MFI) based on the difference between the median fluorescence intensity of the specifically stained cells and the isotype-matched control cells gated for the populations of interest. The MFI of CD40, CD80, CD86, MHC-class II molecules were measured/calculated in total: B cells, DCs ($CD11c^+$ cells) and $CD11c^+CD19^+$ cells. The MFI of IL-10 were measured/calculated in $CD4^+CD25^+FOXP3^+$cells and $CD19^+CD1d^+CD5^+$ cells. All analyses were conducted using Weasel 3.0.2 software (Walter and Eliza Hall Institute, Parkville, Australia).

Cytokines ELISA. Blood samples were collected from all examined mice and centrifuged at 10,000 × g at 4 °C for 10 minutes. The obtained sera were stored at −80 °C. The concentrations of TGFβ1, IFN-γ, IL-2, IL-4 and IL-10 in the sera were measured using the murine Ready-SET-Go! kit (eBioscience, USA) according to the manufacturer's instructions. Briefly, 96-well ELISA plates were coated with specific antibodies overnight at 4 °C and then blocked for 1 hour at room temperature (RT). Sera diluted 1:2 (IFN-γ, IL-2, IL-4 and IL-10) or 1:5 (TGFβ1) and standard concentrations of cytokines (100 μl/well) were then added to the wells, and the plates were incubated overnight at 4 °C. The wells were then washed three times and incubated with biotinylated specific antibodies for 60 minutes at RT. After further triple washing, horseradish peroxidase-conjugated streptavidin was added, and the plates were incubated for 30 minutes at RT. The washed plates were incubated for 10 minutes in the dark at RT with TMB substrate, and the reaction was stopped with 50 μl of 1 M H_2SO_4. The absorbance (A_{450}) was measured on a Wallac 1420 Victor2 Microplate Reader (PerkinElmer, USA) within 15 minutes of the endpoint of the protocol.

Statistical analysis. Statistical calculations were performed in GraphPad Prism 7 (GraphPad Software, USA). Data distribution was assessed using the Shapiro-Wilk normality test. Homoscedasticity was tested with Brown-Forsythe test. One-way ANOVA (parametric) or the Kruskal-Wallis test (nonparametric) with Dunn's multiple comparison post hoc test were performed according to the data distribution. A p value <0.05 was considered statistically significant.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

A.E.K.: conceptualization, methodology, investigation, resources, writing original draft, visualization, funding acquisition; D.L.: investigation, writing-review and editing: A.S.: investigation, writing-review and editing; A.C.S.: conceptualization, writing-review and editing.

Competing interests

The authors declare no competing interests.

Additional information

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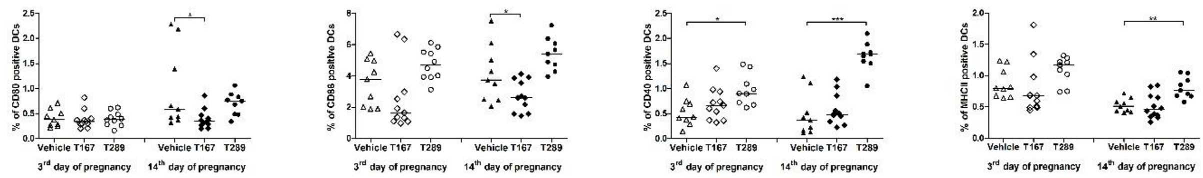
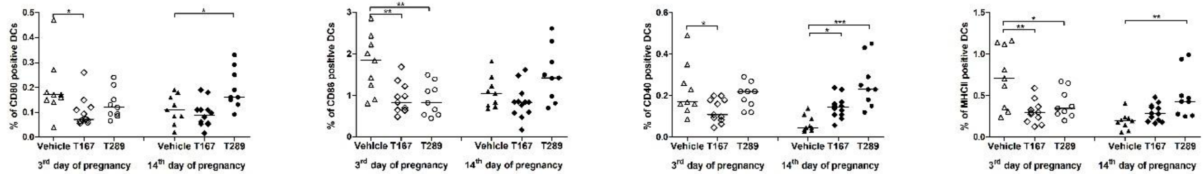
Tregitopes regulate the tolerogenic immune response and decrease the foetal death rate in abortion-prone mouse matings

Anna Ewa Kedzierska, Daria Lorek, Anna Slawek, Anna Chelmonska-Soyta

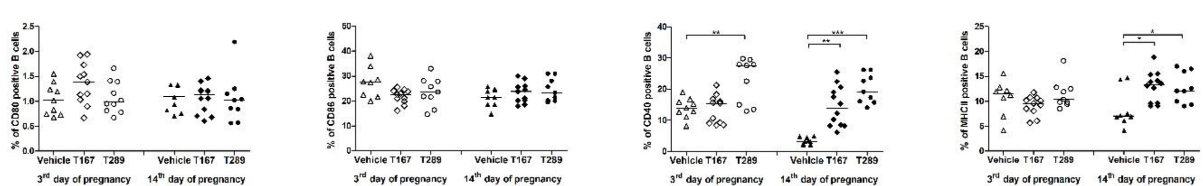
a spleen



b lymph nodes



c spleen



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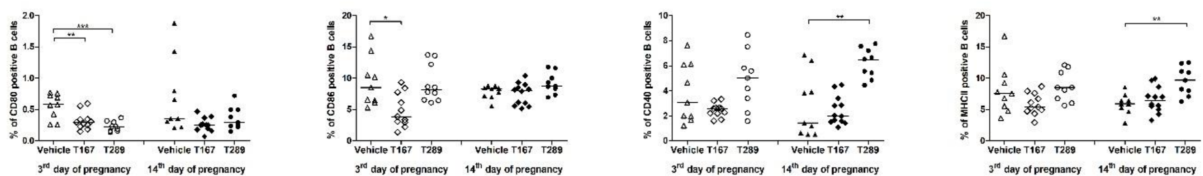
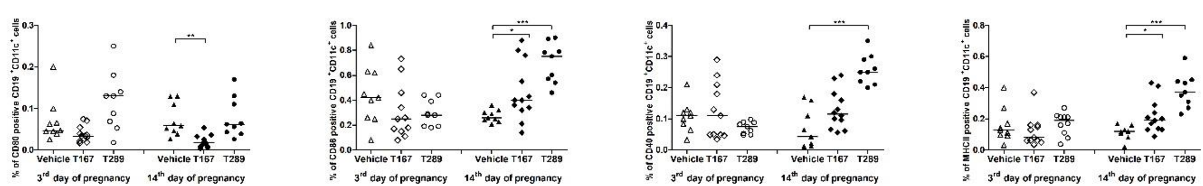
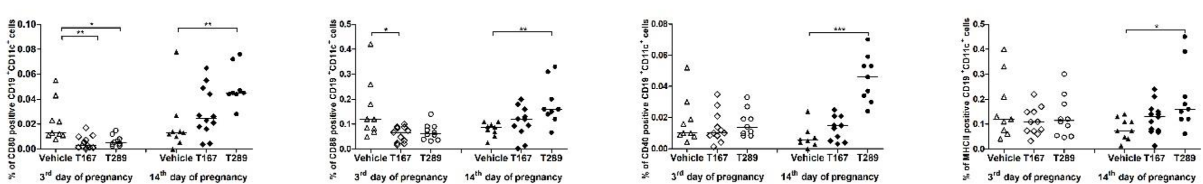


e spleen



f lymph nodes



Supplementary Figure S1. Effect of tregitope treatment on the antigen-presenting cells with expression of costimulatory molecules in abortion-prone mice. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A and monensin and the frequencies of CD40, CD80, CD86 and MHC class II proteins on the surfaces of CD11c^{+} **(a)** splenocytes and **(b)** uterine-draining lymph

node cells, $CD19^+$ **(c)** splenocytes and **(d)** uterine-draining lymph node cells and $CD11c^+$ $CD19^+$ **(e)** splenocytes and **(f)** uterine-draining lymph node cells at the 3rd and 14th days of pregnancy. The data were analysed by one-way ANOVA (normal distribution) or the Kruskal-Wallis test (non-normal distribution) with Dunn's multiple comparison post hoc test ($P < 0.05$) and are presented as individual values with median (at 14dpc n=12 for T167, n=9 for T289, n=9 for Vehicle; at 3dpc n=11 for T167, n=11 for T289, n=9 for Vehicle). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Tregitopes regulate the tolerogenic immune response and decrease the foetal death rate in abortion-prone mouse matings

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	CD11c$^+$				CD19$^+$				CD19$^+$CD11c$^+$			
	CD80	**CD86**	**MHCII**	**CD40**	**CD80**	**CD86**	**MHCII**	**CD40**	**CD80**	**CD86**	**MHCII**	**CD40**
TREGITOPE 167												
3 dpc spleen		*** ↓	* ↑									
3 dpc LN												
14 dpc spleen		* ↓	* ↑			** ↓	** ↑	** ↑		* ↓		* ↑
14 dpc LN												
TREGITOPE 289												
3 dpc spleen	* ↓	*** ↓						** ↑				* ↑
3 dpc LN			* ↑	* ↑							** ↑	* ↑
14 dpc spleen	* ↓	* ↓				* ↓	** ↑	*** ↑		** ↓		*** ↑
14 dpc LN							** ↑	*** ↑				*** ↑

Supplementary Table S1. Summary of expression of costimulatory molecules on antigen-presenting cells in abortion-prone mice. The data show the changes in the specific fluorescence intensity due to CD40, CD80, CD86 and MHC class II proteins on the surfaces of splenic and uterine-draining lymph node cells at the 3rd and 14th days of pregnancy compared to cells obtained from control mice. The data were analysed by one-way ANOVA (normal distribution) or the Kruskal-Wallis test (non-normal distribution) with Dunn's multiple comparison post hoc test ($P < 0.05$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Legend: LN: lymph nodes, ↑: increased MFI compared to control mice, ↓: decreased MFI compared to control mice

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CD91 Derived Treg Epitope Modulates Regulatory T Lymphocyte Response, Regulates Expression of Costimulatory Molecules on Antigen-Presenting Cells, and Rescues Pregnancy in Mouse Pregnancy Loss Model

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Abstract: The loss of immune tolerance to fetal antigens may result in reproductive failure. The downregulated number and activity of T regulatory lymphocytes, which are critical for the establishment of immune tolerance to fetal antigens, during pregnancy may lead to miscarriage. The adoptive transfer of Tregs prevents fetal loss in abortion-prone mice. Recently, we demonstrated that the administration of tregitopes, which are short peptides found in human and mouse immunoglobulins (IgGs), decreased the incidence of abortions in female CBA/J mice mated with DBA/2J mice. Here, two non-IgG source peptides (SGS and LKD) that can potentially bind to the major histocompatibility complex II (MHC II) with high affinity and induce Treg expansion were designed in silico. The immune dysregulation-induced pregnancy failure mouse model was used to evaluate the effect of SGS and LKD on immune response and pregnancy outcome. The fetal death rate in the SGS-treated group was lower than that in the phosphate-buffered saline-treated group. SGS and LKD upregulated the splenic pool of Tregs and modulated the T-helper cell (Th1)/Th2-related cytokine response at the preimplantation stage. Additionally, SGS and LKD downregulated the expression of CD80 and MHC class II molecules in splenic $CD11c^{+}$ antigen-presenting cells. Thus, SGS treatment can result in beneficial pregnancy outcomes. Additionally, SGS peptide-mediated immunomodulation can be a potential therapeutic strategy for immune dysregulation-induced pregnancy failure.

Keywords: tregs; bregs; pregnancy; epitope; CD80; CD86; antigen-presenting cells; miscarriage; MHCII; CD91

1. Introduction

The cross-talk between the mother and the conceptus is one of the fundamental prerequisites for the development of a healthy pregnancy. The recognition of foreign paternal antigens in the semen as well as in the embryo promotes immune tolerance to fetal antigens and enables embryo implantation and fetal development. The mechanisms underlying natural tolerance during pregnancy have not been elucidated. However, dysregulated immune tolerance during pregnancy is reported to promote various reproductive complications such as preeclampsia or spontaneous and recurrent miscarriage. Miscarriages affect one in four diagnosed pregnancies, and about half of them do not have determined etiology. It is believed that women with recurrent miscarriages have impaired tolerance to autoantigens and fetal antigens [1]. It is well known that regulatory T lymphocytes (Tregs) and regulatory B lymphocytes (Bregs) contribute to the development of tolerance to fetal antigens [2,3]. In both humans and mice, the proportion of Tregs in pregnant individuals

is higher than that of those in non-pregnant controls. However, the proportion of Tregs in spontaneous abortion cases is lower than that of healthy pregnancy cases but higher than that of non-pregnant cases [4–10]. Similarly, compared to non-pregnant subjects, the proportion of Bregs is higher in pregnancy cases. Additionally, the proportion of Bregs in spontaneous abortion cases is lower than it is in healthy pregnancy cases [11–13]. The abortion-prone model (CBA/J female mice mated with DBA/2J males), which is a widely studied murine model of immune-mediated pregnancy failure, is characterized by a high rate of fetal resorption and mortality [14]. Previous studies have demonstrated that the adoptive transfer of regulatory B and/or T cells can reverse the high abortion rate in abortion-prone mice if the cells are administered immediately after fertilization [12,15]. Recently, we demonstrated that the early administration of T regulatory cell epitopes (tregitopes), which are short peptides found in the light and heavy chains of human and mouse immunoglobulins (IgGs), decreased the incidence of abortion in abortion-prone mice [16]. These tregitopes may bind to the major histocompatibility complex (MHC II)-binding groove, and this complex is presented to Tregs, which results in the activation and proliferation of Tregs. IgG-derived tregitopes, identified by de Groot and coworkers, are formed after IgG is internalized and processed by the antigen-presenting cells (APCs) [17]. However, the sequences of putative tregitopes have also been reported in other self-proteins, such as albumin, fibrinogen, and osteocalcin [17,18]. Moreover, viruses, fungi, and bacteria are reported to express tolerogenic proteins [19–22], which may be the source of putative tregitope sequences. Therefore, this study aimed to identify novel non-IgG source peptides that can bind to MHC II with high affinity based on an in silico model and to investigate whether their early administration promotes the expansion of regulatory lymphocytes and prevent abortion in a mouse abortion-prone model. The findings of this study suggested that the early administration of CD91 derived Treg epitope (SGS peptide) induces the expansion of Tregs and may prevent abortions in an abortion-prone mouse model. The identified SGS peptide meets the criteria of a tregitope, as its sequence is present in prevalent human proteins, conservative across many species, and can bind to the human leukocyte antigen-DR isotype (HLA-DR) [23].

2. Results

2.1. Peptides Selection

Here, two peptides, the sequences of which were identified in the proteins involved in the immune system were chosen (Table 1). The sequence of the first synthetic peptide was LKDFALEGTLAADKT (LKD). The calculated half-maximal inhibitory concentration value (IC_{50}) of LKD against the MHC II molecule was 108 nM. The LKD peptide was chosen as a control peptide because we have previously shown that it did not induce Tregs expansion in vitro [24] and could not be treated as a tregitope. The sequence of the second peptide was SGSVVLRNSTTLVMH (SGS). The IC_{50} of SGS against MHC II was 146 nM. The peptides were commercially synthesized, and the sequence purity was above 90% (GeneCust, Luxembourg).

Table 1. Characteristics of designed peptides.

Protein Code	Protein Name	Peptide Sequence and Abbreviation	Impact on Immune System
Q91ZX7.1 in *Mus musculus* Q07954.2 in *Homo sapiens*	prolow-density lipoprotein receptor-related protein 1 isoform X1 (LRP1, CD91)	SGSVVLRNSTTLVMH (SGS)	Scavenger receptor that regulates adaptive immunity and inflammation; the interaction with a variety of ligands and adaptor molecules involved in cell signaling, phagocytosis, and endocytosis [25]
Q09089.1	Outer surface protein A from *Borrelia burgdorferi* (OspA)	LKDFALEGTLAADKT (LKD)	T cells proliferation and secretion of interferon-gamma [26]

2.2. SGS Peptide Decreases the Abortion Rate

The protective role of SGS and LKD during pregnancy in abortion-prone mice was examined by analyzing the number of non-resorbed and resorbed embryos. The fetal death rate at 14 dpc was calculated according to the formula described in the methods section.

The fetal death rate (15.03%) and the number of resorbed embryos in the SGS-treated group were significantly lower than those in the PBS-treated group (36.04%) (Figure 1b,c) ($p < 0.05$). The number of viable embryos in the SGS-treated and LKD-treated groups was not significantly ($p > 0.05$) different from that of the PBS-treated group (Figure 1d). However, the total number of implantation sites in the LKD-treated and SGS-treated group was significantly ($p < 0.05$) higher than that of the PBS-treated group (Figure 1e).

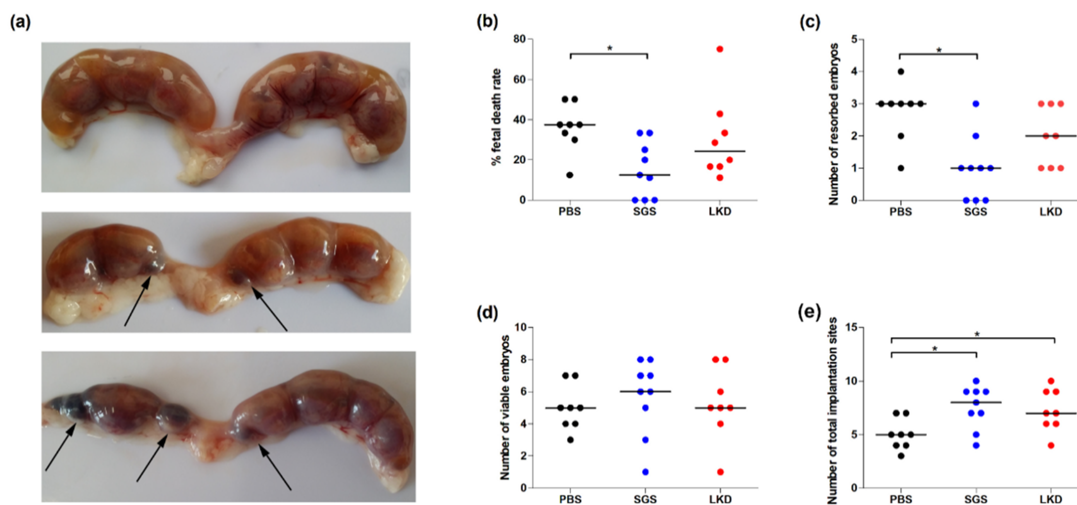

Figure 1. (**a**) Representative pictures of the uteri showing resorbing (arrows) and viable implantation sites and the effect of SGS and LKD peptides on the (**b**) fetal death rate, (**c**) number of resorbed embryos, (**d**) number of viable embryos, and (**e**) total implantation sites at 14 dpc in abortion-prone mice. The data were analyzed using one-way analysis of variance (normally distributed data), followed by Bonferroni's multiple comparison post hoc test or Kruskal–Wallis test (non-normal distributed data), followed by Dunn's multiple comparison post hoc test ($p < 0.05$). Data are presented as individual values with the median. * $p < 0.05$.

2.3. Designed Peptides Enhance Serum Interleukin 10

The effect of SGS and LKD on the T-helper cell (Th1)/T-helper cell (Th2) balance during pregnancy was examined in abortion-prone mice by measuring the serum levels of interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 10 (IL-10), transforming growth factor-beta 1 (TGFβ1), and interferon-gamma (IFNγ). At 3 dpc, the serum level of IL-2 was upregulated in the SGS-treated group (Figure 2a). However, the IL-2 serum levels were not significantly ($p > 0.05$) different between the experimental groups at 14 dpc (Figure 2a).

The serum levels of IL-4 on the third and fourteenth days of pregnancy were not significantly different ($p > 0.05$) between the experimental groups (Figure 2b). Compared to those in the PBS-treated group, the IL-10 serum levels on the third day of pregnancy were significantly upregulated in the SGS-treated and LKD-treated groups ($p < 0.05$) (Figure 2c). At 14 dpc, the serum levels of IL-10 were not significantly different between the experimental groups. On the third day of pregnancy, the TGFβ1 serum levels were not significantly different between the experimental groups (Figure 2d). However, the TGFβ1 serum levels in the SGS-treated and LKD-treated groups were significantly lower than those in the PBS-treated group at 14 dpc (Figure 2d; $p < 0.05$). The absorbance values of IFNγ on the third and fourteenth day of pregnancy were below the detection level of the kit (data not shown).

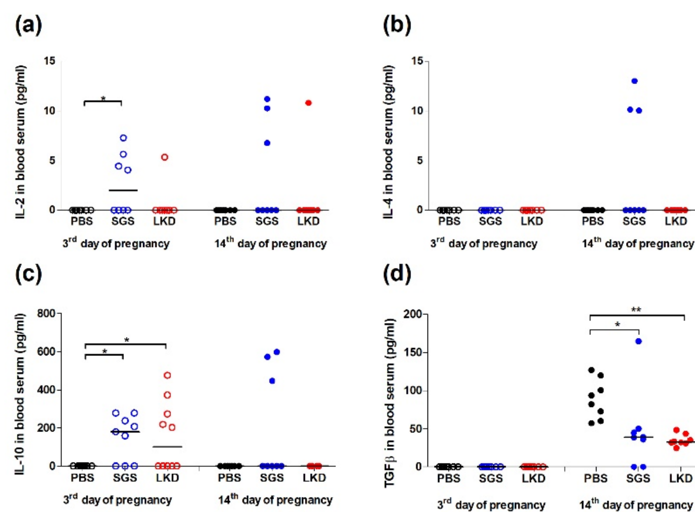

Figure 2. Effect of SGS and LKD on the levels of (**a**) IL-2, (**b**) IL-4, (**c**) IL-10, and (**d**) TGFβ1 at 3 dpc and 14 dpc in abortion-prone mice. Data were analyzed using one-way analysis of variance (for normally distributed data), followed by Bonferroni's multiple comparison post hoc test or the Kruskal–Wallis test (for non-normally distributed data), followed by Dunn's multiple comparison post hoc test ($p < 0.05$). Data are presented as individual values with the median. * $p < 0.05$ and ** $p < 0.01$.

2.4. SGS and LKD Peptides Upregulate Treg Frequency

Next, the effect of SGS and LKD on Treg expansion was examined in abortion-prone mice. The number of $CD4^+CD25^+FOXP3^+$ lymphocytes in the spleen and para-aortic uterine-draining lymph nodes (PALNs) (Figure 3) of the pregnant mice were analyzed. The gating strategy for analyzing Tregs is shown in Figure 4.

At 3 dpc, the frequencies of splenic $CD4^+CD25^+FOXP3^+$ cells in the SGS-treated and LKD-treated groups were almost two times higher ($p < 0.01$) than those in the PBS-treated group (Figure 3a). The frequencies of $CD4^+CD25^+FOXP3^+$ cells in the PALN were similar among the experimental groups (Figure 3b). The proportion of $CD4^+CD25^+FOXP3^+$ cells was upregulated in the $CD4^+$ splenocyte population of the SGS-treated and LKD-treated groups (Figure 3c). The frequency of Tregs among the $CD4^+$ PALNs was similar among the experimental groups (Figure 3d). Furthermore, the levels of IL-10 (calculated as MFI) in the splenic and lymph node Tregs were similar between the SGS-treated, LKD-treated, and PBS-treated groups (Figure 3e,f).

The administration of SGS and LKD did not affect the frequency of Tregs in the spleen and PALN at 14 dpc (Figure 3a,b). Moreover, the proportion of $CD4^+CD25^+FOXP3^+$ cells within $CD4^+$ cells in the spleen and PALN of the SGS-treated and LKD-treated groups was similar between the experimental groups (Figure 3c,d). Additionally, the IL-10 levels in the splenic and lymph node Tregs ($p > 0.05$) were similar among the experimental groups (Figure 3e,f).

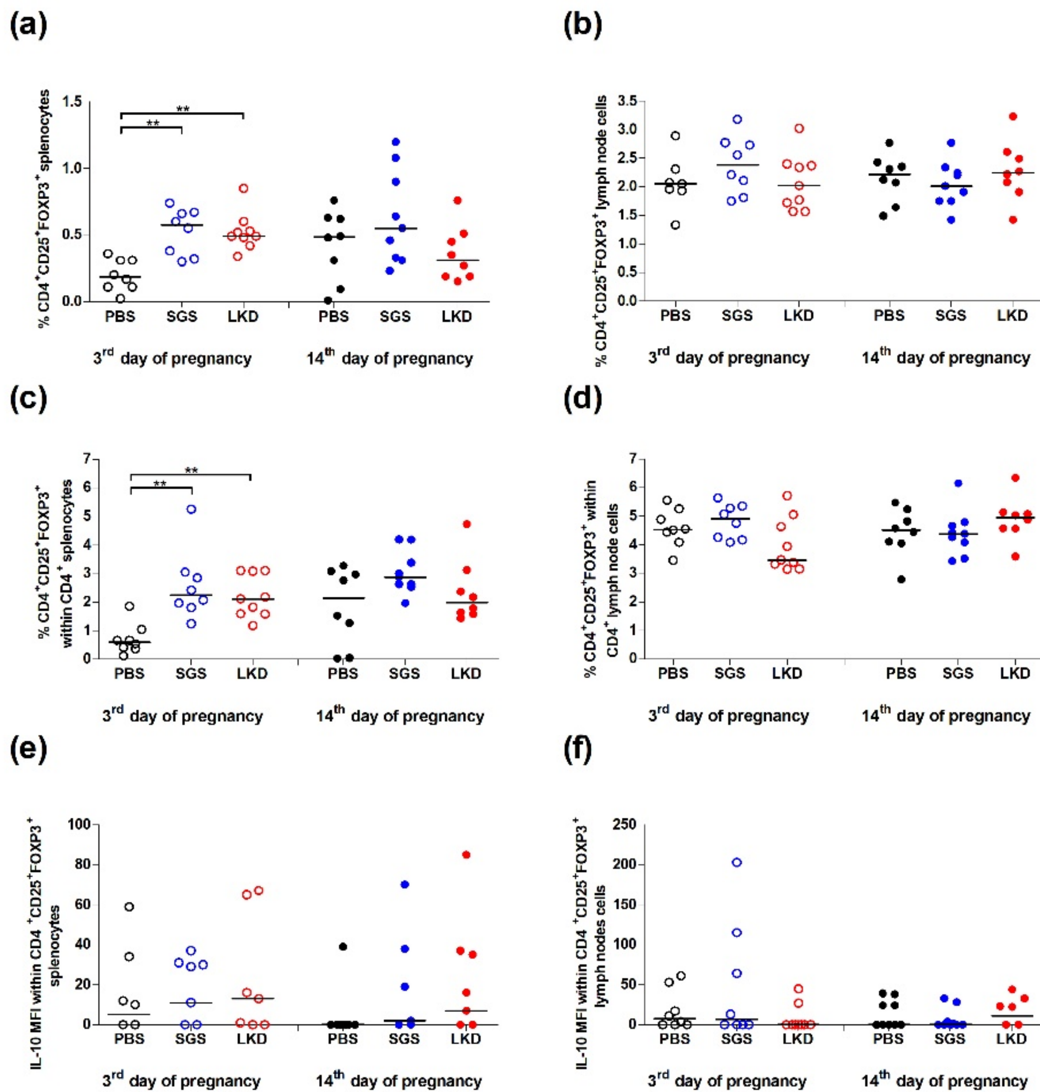

Figure 3. Frequencies of CD4+CD25+FOXP3+ (**a**) splenocytes and (**b**) uterine-draining lymph node cells among the (**c**) CD4+ splenocytes and (**d**) CD4+ lymph node cells, respectively, of the SGS-treated and LKD-treated groups at 3 dpc and 14 dpc in abortion-prone mice. The median fluorescence intensity (MFI) of IL-10 in the CD4+CD25+FOXP3+ (**e**) splenocytes and (**f**) uterine-draining lymph node cells of the SGS-treated and LKD-treated groups at 3 dpc and 14 dpc in abortion-prone mice. Data were analyzed using one-way analysis of variance (for normally distributed data), followed by Bonferroni's multiple comparison post hoc test or the Kruskal–Wallis test (for non-normally distributed data), followed by Dunn's multiple comparison post hoc test ($p < 0.05$). Data are presented as individual values with the median. ** $p < 0.01$.

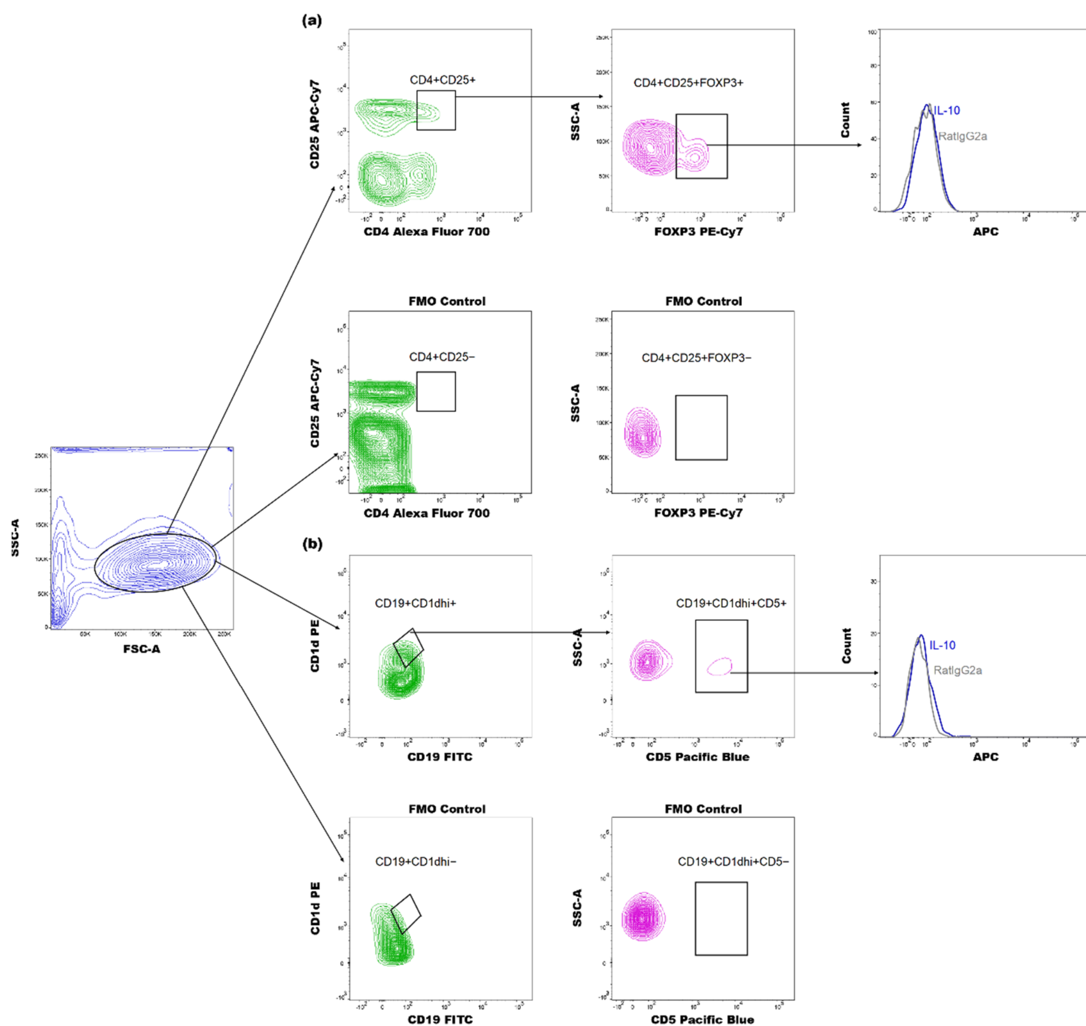

Figure 4. Representative dot plots for gating regulatory T (Tregs) and B lymphocytes (Bregs) and representative histograms of the expression of IL-10 (blue histograms) overlayed with those in the respective isotype-matched controls (red histograms) derived from the spleens of abortion-prone mice. (**a**) Gating strategy for CD4$^+$CD25$^+$FOXP3$^+$ lymphocytes and IL-10 expression in these cells. (**b**) CD19$^+$CD1d^{high+}CD5$^+$ lymphocytes and expression of IL-10 in these cells.

2.5. LKD Upregulates Breg Frequency

The effect of SGS and LKD on the expansion of Bregs was examined in abortion-prone mice. The proportion of CD19$^+$CD5$^+$CD1d^{hi} cells (Bregs) in the spleen and PALN was examined (Figure 5) based on the gating strategy shown in Figure 4b.

The frequency of Bregs in the spleens and among B cell populations (Figure 5a,c, respectively) was similar among the experimental groups on the third day of pregnancy ($p > 0.05$). However, at 3 dpc, the proportion of Bregs ($p < 0.01$) in the PALN ($p < 0.01$) in the LKD-treated group was higher than that of the PBS-treated group (Figure 5b). The frequency of Bregs among B cell populations in the PALN in the SGS-treated and LKD-treated groups was not significantly different from that of the PBS-treated group ($p > 0.05$) (Figure 5d). The IL-10 levels in the Bregs cells were not markedly different in the spleen and PALN at 3 dpc (Figure 5e,f).

On the fourteenth day of pregnancy, the frequency of Bregs in the spleen ($p < 0.01$) and among splenic B cell populations ($p < 0.001$) in the LKD-treated group was higher than that of the PBS-treated group (Figure 5a,c). However, at 14 dpc, the proportion of Bregs in the PALN in the SGS-treated and LKD-treated groups was not significantly different from that of the PBS-treated group ($p > 0.05$) (Figure 5b). However, the frequency of Bregs among B cell populations in the PALN in the LKD-treated groups was not significantly

different from that in the PBS-treated group ($p > 0.05$) (Figure 5d). Additionally, the level of IL-10 in the splenic and lymph node Tregs in the SGS-treated and LKD-treated groups was similar to that of the PBS-treated group (Figure 5e,f).

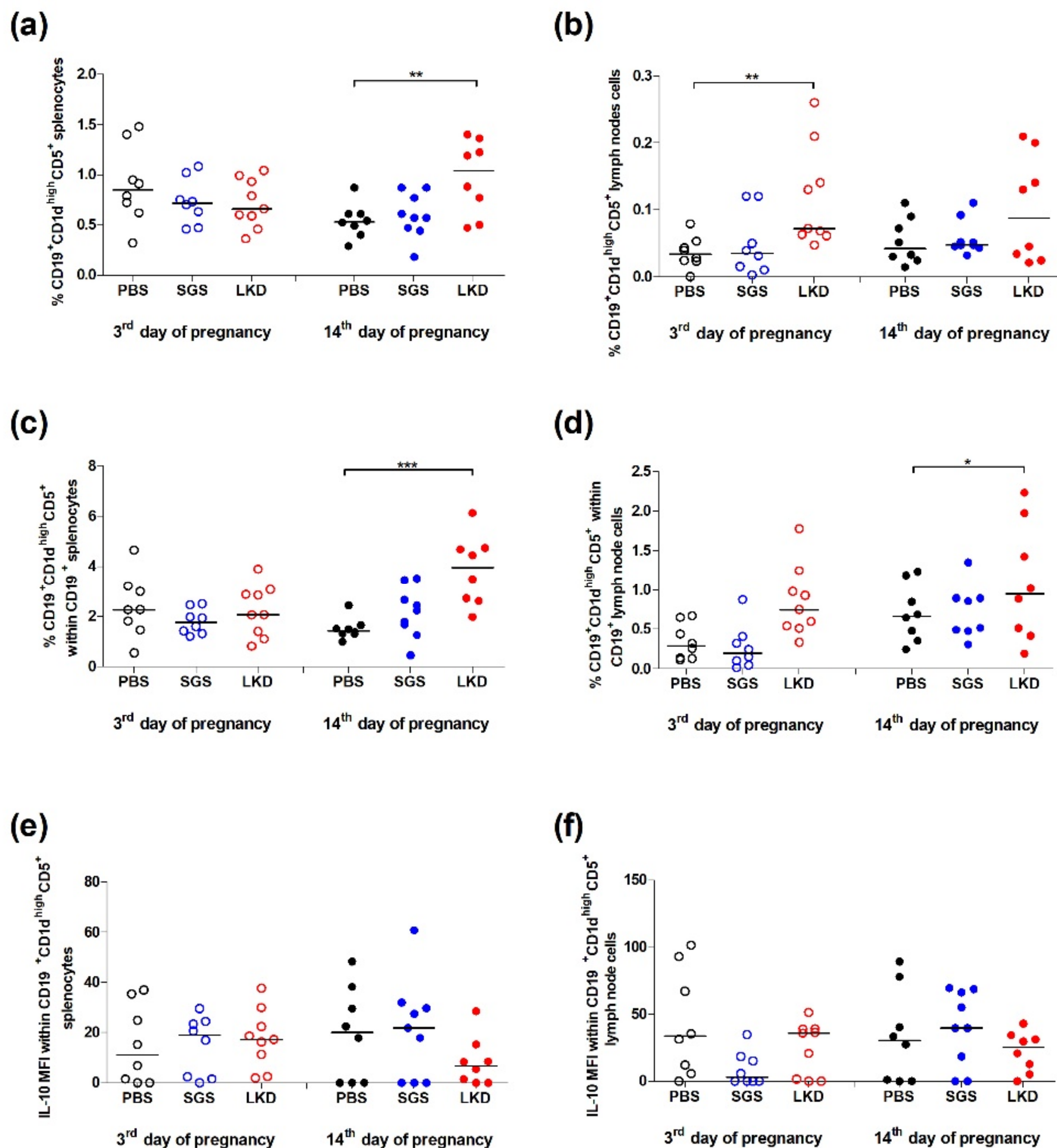

Figure 5. Effect of SGS and LKD on the proportions of $CD19^+CD1d^+CD5^+$ (**a**) splenocytes and (**b**) uterine-draining lymph node cells in the (**c**) $CD19^+$ splenocytes and (**d**) $CD19^+$ uterine-draining lymph nodes, respectively, and the production of IL-10 by the $CD19^+CD1d^+CD5^+$ (**e**) splenocytes and (**f**) lymph node cells at 3 dpc and 14 dpc in abortion-prone mice. Data were analyzed using one-way analysis of variance (for normally distributed data), followed by Bonferroni's multiple comparison post hoc test or the Kruskal–Wallis test (for non-normal distributed data), followed by Dunn's multiple comparison post hoc test ($p < 0.05$). Data are presented as individual values with the median. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

2.6. SGS and LKD Peptides Modulate the Costimulatory Phenotype of APCs

Next, the effect of SGS and LKD on the costimulatory phenotype of APCs was examined. The expression of CD40, CD80, CD86, and MHC class II molecules on the surface of DCs ($CD11c^+$) and B lymphocytes ($CD19^+$) was analyzed based on the gating strategy shown in Figure 6.

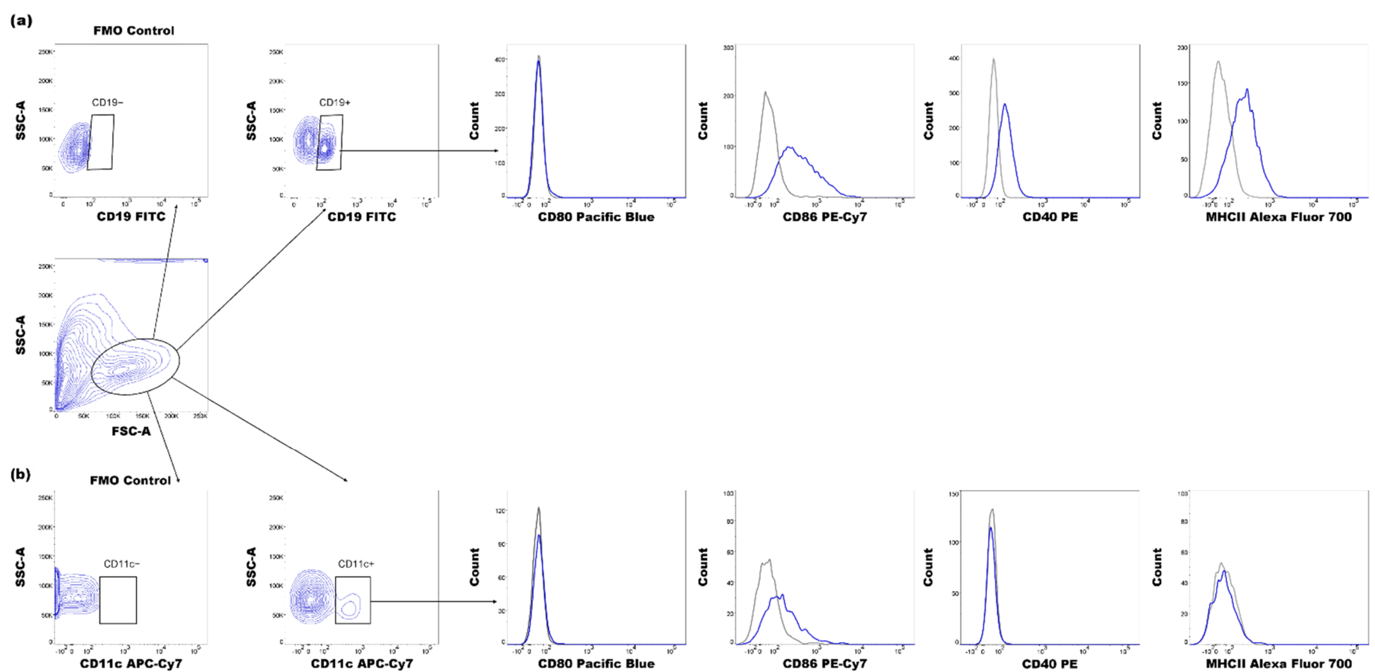

Figure 6. Representative histograms of the expression of CD40, CD80, CD86, and major histocompatibility class II molecules (blue histograms) overlayed with those in the respective isotype-matched controls (gray histograms) in (**a**) CD19$^+$ cells and (**b**) CD11c$^+$ cells derived from the spleens of abortion-prone mice.

On the third day of pregnancy, the expression levels of CD80 on the splenic CD11c$^+$ cells in the SGS-treated and LKD-treated groups ($p < 0.01$, LKD peptide; $p < 0.001$ for SGS peptide) were significantly downregulated when compared with those in the PBS-treated group (Figure 7a). However, the expression levels of CD80 on the CD11c$^+$ splenic cells in the PALN were similar between the SGS-treated, LKD-treated, and PBS-treated groups (Figure 7b). The expression levels of CD86 and CD40 on the DCs ($p > 0.05$) in the spleen and PALN were not significantly different between the experimental groups (Figure 7a,b). Meanwhile, the expression levels of MHC II molecules on the DCs in the spleen and PALN of the SGS-treated and LKD-treated groups were downregulated when compared to those in the spleen and PALN of the PBS-treated group (Figure 7a,b). SGS and LKD did not affect the expression of CD80, CD40, and MHC II on the B cells in the PALN and spleen (Figure 7c,d). However, treatment with SGS inhibited CD86 expression on B cells in the spleen ($p < 0.001$) but not on those in the PALN (Figure 7c,d).

On the fourteenth day of pregnancy, SGS and LKD did not upregulate the expression of CD80, CD86, and CD40 molecules on DCs or B cells in the spleen and PALN ($p > 0.05$). The expression of MHC II molecules on the splenic B cells, but not on splenic DCs, was downregulated in the LKD-treated group when compared to that of the PBS-treated group. However, the expression of MHC II molecules in the PALN was similar between the SGS-treated, LKD-treated, and PBS-treated groups (Figure 7c,d).

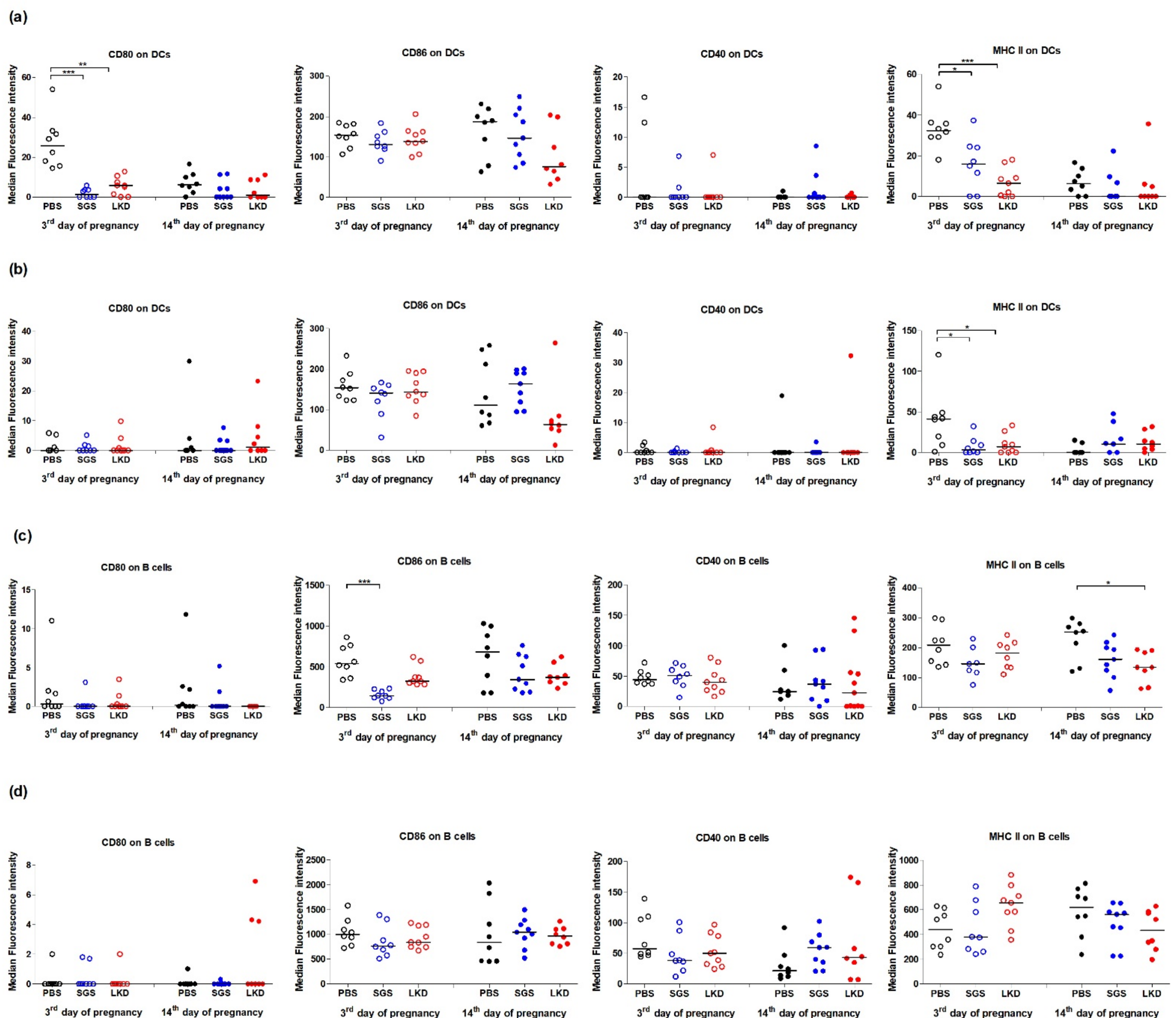

Figure 7. Effect of SGS and LKD on the expression of CD40, CD80, CD86, and major histocompatibility class II proteins on the surface of (**a**) CD11c$^+$ splenocytes and (**b**) CD11c$^+$ uterine-draining lymph node cells, (**c**) CD19$^+$ splenocytes, and (**d**) CD19$^+$ uterine-draining lymph node cells at 3 dpc and 14 dpc in abortion-prone mice. Data were analyzed using one-way analysis of variance (for normally distributed data), followed by Bonferroni's multiple comparison post hoc test or the Kruskal–Wallis test (for non-normally distributed data), followed by Dunn's multiple comparison post hoc test ($p < 0.05$). Data are presented as individual values with the median. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Discussion

Dysregulated immune tolerance to fetal antigens may cause various reproductive complications. The decreased number of Tregs, which are critical for the establishment of immune tolerance to fetal antigens, during pregnancy may result in miscarriage. In this study, the mouse model of immune imbalance-mediated pregnancy failure was used to study the effect of newly designed peptides on pregnancy outcomes.

Tregitopes, which are short amino acid sequences found in the light and heavy chains of human and mouse IgG, can bind to MHC II molecules with high affinity. Additionally, tregitopes presented with MHC II promote the expansion of Tregs and suppress the effector T cell responses by enhancing the production of cytokines [17]. Based on a previously reported in silico model [24], two potential non-IgG source Treg epitopes that can induce

the expansion of Tregs were identified in this study. The main assumption of the in silico analysis in this study was that the designed tregitopes can bind to MHC II molecules, which is an essential attribute for presenting tregitopes. However, binding with a high affinity to MHC II does not always result in the induction of Tregs. To ensure that the selected tregitopes exhibit immunomodulatory features, other factors, such as hydrophobicity index, the number of negatively and positively charged residues, and immune function, were incorporated in the analysis [24].

In this study, the number of resorbed and viable fetuses was examined to analyze the effect of SGS and LKD on pregnancy outcomes. The fetal death rate and the number of resorbed embryos in the SGS-treated group were significantly lower than those of the PBS-treated group. Additionally, treatment with SGS increased the number of implantation sites, which suggested that SGS can potentially rescue pregnancy in abortion-prone mice. Previously, we had demonstrated that known tregitopes decreased the occurrence of abortion in abortion-prone mice [16]. SGS may rescue pregnancy in abortion-prone mice by promoting the expansion of Tregs and regulating the levels of serum cytokines.

The frequency of Tregs during a healthy pregnancy is reported to be higher than during a non-pregnancy period. Additionally, the frequency of Tregs in abortion cases is lower than that in healthy pregnancy cases [7,8]. Compared to those in mice with healthy pregnancies, the local and peripheral levels of Tregs in the uterus are downregulated during pregnancy in abortion-prone mice [27]. The adoptive transfer of splenic and thymic $CD4^{+}CD25^{+}$ cells from CBA/J mice mated with BALB/c males (healthy pregnancy) into abortion-prone mice inhibited the incidence of abortion [27]. In this study, SGS and LKD promoted peripheral changes in the frequency of Tregs at the preimplantation stage of pregnancy. The SGS-induced or LKD-induced expansion of Tregs is consistent with the results of our previous studies, which reported that the administration of known tregitopes promoted the induction of splenic Tregs [16]. However, the results of our previous study indicated that tregitopes increased the level of IL-10 produced by Tregs, which was not observed in this study. The IL-10 serum levels were upregulated at the preimplantation stage of pregnancy in the SGS-treated and LKD-treated groups. Cytokines are essential factors for the survival of the fetus [28–33]. The abortion-prone mouse model exhibits a dysregulated cytokine balance and downregulated levels of IL-10 and IL-4 in feto-placental units [34]. The administration of exogenous IL-10 prevents fetal loss in abortion-prone mice, whereas the depletion of IL-10 promotes abortion [34]. This indicates that SGS protects against fetal loss through the upregulation of the IL-10 level. Like IL-10, TGFβ is one of the cytokines that is crucial in the modulation of the dendritic cells phenotype and the differentiation of Tregs [35,36]. It is well known that TGFβ may inhibit the proliferation and differentiation of mature Treg. Here, we demonstrated an increased level of IL-10 at 3 dpc and a decreased TGFβ1 level at 14 dpc. The upregulation of IL-10 may be related to the observed expansion of Tregs at 3 dpc. However, the lack of differences in Tregs expansion at 14 dpc suggests that the downregulation of TGFβ1 did not negatively influence immune tolerance. This conclusion may be supported by the fact that, despite decreased TGFβ1 concentration, the SGS-treated mice had a reduced fetal death rate. However, this is very speculative, and additional studies are needed to explain this phenomenon. Additionally, SGS upregulated the IL-2 level at the preimplantation stage of pregnancy. The cytokine microenvironment plays a key role in the differentiation, expansion, and function of Tregs. IL-2 is one of the cytokines essential for the development, peripheral homeostasis, and stability of Tregs [37]. Therefore, the effectiveness of SGS can be also attributed to its ability to upregulate IL-2 levels. The protective role of the SGS peptide on pregnancy outcome may be due to its origin. The sequence of the SGS peptide was identified in an extracellular α-chain with binding sites for ligands of prolow-density lipoprotein receptor-related protein 1 isoform X1 from various species, such as *Mus musculus* and *Homo sapiens*. The prolow-density lipoprotein receptor-related protein 1 (LRP1), also known as the CD91 molecule, is an endocytic receptor that recognizes over thirty different ligands, which play various roles in many biological processes such as cell signaling, the activation of

lysosomal enzymes, lipoprotein metabolism, phagocytosis, and endocytosis [25]. CD91 is expressed by most APCs and participates in the regulation of inflammatory processes and cross-presentation of the heat shock proteins (HSPs)-chaperoned peptide [38]. Through metalloproteinases, a shed form of LRP1 may be released into the extracellular matrix in response to stress and inflammatory mediators and to function as a decoy receptor. LRP1/CD91 shedding results in the release of ligand-binding α-chain from the cell [39]. The released soluble LRP1 (sLRP1) can be detected in the brain, plasma, cerebrospinal fluid, and the peripheral nervous system [39–41]. During the incubation of human plasma sLRP1, purified mouse LRP1 with RA 264.7 macrophage-like cells increased mRNA for IL-10 [42]. The exact mechanism associated with the biological activity and immune functions of LRP1/CD91 remains unresolved. However, we believe that the delivered amount of SGS peptide might induce, as observed in this study, increased concentration of the IL-10 in blood sera, which in turn might prompt Tregs and contribute to improved pregnancy outcomes in abortion-prone mice.

Although the LKD peptide could not rescue pregnancy, it increased the number of $CD19^{+}CD5^{+}CD1d^{high+}$ cells (Bregs) in the lymph nodes (at the preimplantation stage) and spleen at 14 dpc, which was not observed after treatment with the SGS peptide. Moreover, LKD administration increased the serum level of IL-10 but did not upregulate the levels of IL-10 (calculated as MFI) in the splenic and lymph node Bregs. It was already demonstrated that the proportion of Bregs, called B10 cells in abortion-prone mice, is lower than that of mice with healthy pregnancies. Moreover, the adoptive transfer of IL-10-producing B cells mitigated the incidence of abortion in abortion-prone mice [11]. It was also demonstrated that IL-10 secreting B cells (B10), but not B effector cells that do not produce IL-10, are prominent in the maintenance of the immune balance during pregnancy [43]. Furthermore, in our previous work, we have shown that at 14 dpc, abortion-prone mice have an increased proportion of $CD19^{+}CD5^{+}CD1d^{high+}$ cells in the uterine draining lymph nodes when compared to that of mice with healthy pregnancies [44]. What is more, the frequency of $CD19^{+}CD5^{+}$ cells in preeclampsia cases is higher than that of the second and third trimester of healthy pregnancy cases and is correlated with the production of autoantibodies. The autoantibodies generated from the $CD19^{+}CD5^{+}$ cells may reach the placenta and consequently lead to pregnancy failure [45]. Therefore, B cells are reported to exert both beneficial and adverse effects on pregnancy. These contradictory findings can be attributed to the complicated biology of B cells and the differential roles of B cell subpopulations in adaptive and innate immunity [3,46,47]. Thus, we hypothesized that the elevated frequencies of $CD19^{+}CD5^{+}CD1d^{high+}$ cells after LKD treatment may have an adverse effect on pregnancy outcome.

B cells and DCs can present antigens to the T lymphocytes through MHC II molecules. Antigen presentation in the absence of CD80/CD86 costimulatory molecules and cytokines, such as IL-10, induces the differentiation of T cells into Tregs. Previously, we demonstrated that the early administration of known IgG-derived epitopes (mouse tregitopes 289 and 167) downregulated the expression of CD80 and CD86 antigens on DCs and B cells at 3 dpc and 14 dpc [16]. In this study, SGS and LKD downregulated the expression of CD80 and MHC II molecules on splenic DCs. Additionally, SGS and LKD downregulated the expression of MHC II on DCs residing in the lymph nodes at the preimplantation stage of pregnancy. Among the costimulatory molecules on B cells, SGS downregulated the expression of the CD80 antigen and consequently inhibited maternal rejection of fetuses in abortion-prone mice. The downregulated expression of costimulatory molecules on DCs promotes immune tolerance to self-antigens. Similar observations were reported for B cells in a previous study, which demonstrated that the inhibition of CD86 induced antigen-specific peripheral tolerance [48]. Muzzio et al. (2014) demonstrated that the proportions of peritoneal $CD19^{+}CD23^{-}CD5^{+}CD86^{+}$ B-1a B cells in mice with healthy pregnancy was significantly lower than that of animals associated with fetal loss or non-pregnant animals [49]. Previously, we had reported that the mRNA expression of CD86 in the splenic B cells of mice with healthy pregnancy is downregulated when compared to

that in the splenic B cells of mice with disturbed pregnancy [50]. Furthermore, the systemic inhibition of CD80 and CD86 at the implantation period of pregnancy inhibited [51,52] the rejection of fetuses in abortion-prone mice. Therefore, we hypothesized that the SGS-mediated inhibition of costimulatory molecules and MHC II on APCs may exert beneficial effects on fetal development by inducing tolerance to embryo antigens. SGS-mediated downregulation of CD80, CD86, and MHC II on APCs and the expansion of Tregs are consistent with the findings of de Groot et al., who reported that treatment with known tregitopes downregulated the expression of costimulatory molecules [53].

In summary, this study demonstrated that treatment with the novel CD91 derived peptide (SGS) with tregitope activity increased the proportions of Tregs, upregulated the concentrations of IL-2 and IL-10, and downregulated the expression of the costimulatory molecules of APCs, which resulted in improved pregnancy outcomes. The findings of this study indicate that the SGS peptide may mimic the pro-tolerogenic effects of known tregitopes and that SGS is a potential therapeutic for immune dysregulation-induced pregnancy failure. However, further studies are needed to evaluate the ability of SGS to regulate the immune response in humans. Although LKD promoted the expansion of Tregs and Bregs, it did not decrease the incidence of abortions, which may be due to its unverified tregitope activity [24] and therefore its insufficient immunomodulatory effects.

4. Materials and Methods

4.1. Peptides Selection and Design

The tregitopes were designed as described previously [24]. First, tregitopes were generated using an in silico mathematical model for the correlation between the chemical structure of tregitopes and binding strength with MHC II (DRB1*04:01). In this study, the following parameters were calculated for the tregitope sequences: the half-maximal inhibitory concentration value against MHC II, the hydrophobicity index, the number of negatively and positively charged residues, the aliphatic index, the isoelectric point, the hydrogen bond donors, and the topological polar surface area. In silico analysis of 16 amino acid sequences of IgG with tolerogenic function similar to that of tregitopes was performed for mathematical selection. The linear validated correlation between binding strength with MHC II and physicochemical properties has been suggested instead of the present epitope cluster classification (unpublished data).

4.2. Animals

DBA/2J male mice and CBA/J female mice, which were purchased from Charles River Laboratories (Sulzfeld, Germany), were housed under specific pathogen-free conditions with a 12 h dark/light cycle. The vaginal swabs were collected from female CBA/J mice aged 6–8 weeks every morning to determine the phase of the reproductive cycle. Next, the vaginal smears were fixed and stained using the Cytocolor reagent (Merck Millipore, Burlington, Massachusetts, USA), as per the manufacturer's instructions. Female mice at the proestrus phase were mated with DBA/2J males in the evening (at 7 pm) on the same day. Copulation was confirmed the next morning based on the presence of a vaginal plug. Pregnancy was defined as 0 days post coitum (dpc). At 0 dpc, the female mice were intraperitoneally injected with 100 µg (dissolved in 150 µL phosphate-buffered saline (PBS)) SGS peptide (SGS-treated group, n = 8 at 3 dpc and n = 9 at 14 dpc), 100 µg LKD peptide (LKD-treated group, n = 9 at 3 dpc and n = 8 at 14 dpc), or 150 µL PBS (PBS-treated group, n = 8). At 3 and 14 dpc, the blood samples, para-aortic uterine-draining lymph nodes (PALNs), and uteri were collected for further analyses. All efforts were made to minimize animal suffering. The animal experiments were approved by the Local Ethics Committee for Experiments on Animals at the Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw (No. 53/2015).

4.3. Tissue Processing

The spleen and PALN were isolated as described previously [16]. First, the spleen and PALN were passed through a 40-µm cell strainer (Falcon) into lysis solution (0.84% ammonium chloride) and wash buffer (PBS supplemented with 2 mM ethylenediaminetetraacetic acid (EDTA) and 2% fetal bovine serum (FBS; Biowest, France)), respectively. The cells were washed twice (at 4 °C and 300× *g* for 10 min) with wash buffer and stimulated as described in the flow cytometry section.

The uterus was collected from pregnant females at 14 dpc to determine the fetal death rate, which was calculated as follows: [(number of resorbed embryos)/(resorbed embryos + viable embryos)] × 100. The abortion sites were identified (Figure 2a) as described previously [16].

4.4. Flow Cytometry

The cells (1×10^6 cells) were stimulated as previously described [16]. First, the cells were incubated with 1 µg/mL ionomycin (Cayman Chemical, Ann Arbor, MI, USA), 0.1 µg/mL phorbol 12-myristate 13-acetate (Cayman Chemical, Ann Arbor, MI, USA), 2 µM monensin (eBioscience, Waltham, MA, USA), and 10 µg/mL brefeldin A (eBioscience, Waltham, MA, USA) in Rosewell Park Memorial Institute-1640 medium supplemented with 10% FBS and 1X penicillin/streptomycin (from 100X) (Merck Millipore, Burlington, MA, USA) at 37 °C and 5% CO_2 for 6 h. The cells were then stained with allophycocyanin (APC)/Cy7-conjugated anti-mouse CD25 (BD Biosciences; clone: PC61), Alexa Fluor 700-conjugated anti-CD4 (eBioscience, USA; clone: Gk1.5), phycoerythrin (PE)-conjugated anti-CD1d (eBioscience, USA; clone: 1B1), fluorescein isothiocyanate (FITC)-conjugated anti-CD19 (eBioscience, USA; clone: eBio1D3), pacific blue-conjugated anti-CD5 (eBioscience, USA; clone: 53-7.3), FITC-conjugated anti-CD19 (eBioscience, USA; clone: eBio1D3), APC/Cy7-conjugated anti-CD11c (eBioscience, USA; clone: N418), PE/Cy7-conjugated anti-CD86 (eBioscience, USA; clone: GL1), pacific blue-conjugated anti-CD80 (eBioscience, USA; clone: 16-10A1), Alexa Fluor 700-conjugated anti-MHC class II (IA/IE) (eBioscience, USA; clone: M5/114.15.2), PE-conjugated anti-CD40 (eBioscience, USA; clone: 1C10) antibodies, or isotype controls for 30 min at 4 °C in the dark. Next, the cells were washed and fixed with fixation/permeabilization buffer (eBioscience, USA) for 14 h at 4 °C in the dark. The cells were then washed twice with permeabilization buffer (eBioscience, USA) and blocked with anti-CD16/CD32 antibodies for 15 min at 4 °C in the dark. The cells were then stained with APC-conjugated anti-mouse interleukin 10 (IL-10) (eBioscience, USA; clone: JES5-16E3) and PE/Cy7-conjugated anti-Foxp3 antibodies (eBioscience, USA, clone: FjK-16s) or isotype controls for 1 h at 4 °C in the dark. The cells were washed twice with permeabilization buffer (eBioscience, USA), and the fluorescence intensity was measured using an LSRFortessa cell analyzer (Becton Dickinson, Franklin Lakes, NJ, USA). The relative levels of costimulatory molecules (CD40, CD80, and CD86) and MHC class II antigen in the $CD19^+$ cell population (B cells) and $CD11c^+$ cell population (dendritic cells (DCs)) were calculated as follows: median fluorescence intensity (MFI) of the stained cells—MFI of the isotype-matched control cells. The expression levels of costimulatory molecules are presented as specific MFI. Similarly, the levels of IL-10 in the $CD4^+CD25^+FOXP3^+$ cells or $CD19^+CD1d^+CD5^+$ cells were calculated as follows: MFI of cells stained with anti-IL-10 antibody—MFI of cells stained with isotype-matched control. In total, 200,000 events were recorded at a rate of 600–800 events per second. Cytometer Setup and Tracking beads (CS&T Research Beads, Becton Dickinson, USA) were used for automated quality assurance and control of machine performance. The analyses were conducted using FlowJo™ software version 10.6.2 (Becton Dickinson, Franklin Lakes, NJ, USA).

4.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The blood samples collected from the pregnant mice were centrifuged at 10,000× *g* and 4 °C for 10 min, and the serum samples were stored at −80 °C. The concentrations of TGFβ1, IFN-γ, IL-2, IL-4, and IL-10 were evaluated elevated using the murine Ready-

SET-Go! Kit (eBioscience, USA) as previously described [16]. First, the wells of the microplates were incubated with anti-TGFβ1, anti-IFN-γ, anti-IL-2, anti-IL-4, and anti-IL-10 antibodies overnight at 4 °C. Next, the plates were blocked for 1 h at room temperature (RT). The standard concentrations of cytokines or diluted sera (1:5 for TGFβ1 measurements or 1:2 for IFN-γ, IL-2, IL-4, and IL-10 measurements) were added to the wells and incubated overnight at 4 °C. The wells were washed and incubated with biotinylated antibodies for 1 h at RT. The plates were washed thrice and incubated with horseradish peroxidase-conjugated streptavidin for 30 min at RT. Finally, the plates were incubated with 3,3′,5,5′-tetramethylbenzidine substrate for 10 min in the dark at RT. The reaction was terminated with the addition of 50 μL of 1 M H_2SO_4. The absorbance at 450 nm (A_{450}) was measured using a Wallac 1420 Victor2 microplate reader (PerkinElmer, Waltham, Massachusetts, USA) within 15 min of the endpoint of the protocol.

4.6. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). Normal distribution was assessed using the Shapiro–Wilk normality test, while homoscedasticity was examined using the Brown–Forsythe test. The normally distributed data were analyzed using one-way analysis of variance (parametric), followed by Bonferroni's multiple comparison post hoc test. The non-normally distributed data were analyzed using the Kruskal–Wallis test (nonparametric), followed by Dunn's multiple comparison post hoc test. The differences were considered significant at $p < 0.05$.

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Informed Consent Statement: Not applicable.

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7. Conclusions

Conclusions

1. *In vitro* analysis of the designed sequences enabled positive verification of the tolerogenic potential of new tregitopes (Okoniewska et al., 2017).

2. The treatment with two tregitopes with known sequences derived from mouse IgG significantly increased the Tregs pool, increased IL-10 production by Tregs and Bregs, and altered the costimulatory response of antigen-presenting cells, thus contributing to the reduction of the foetal death rate in abortion-prone mice (Kedzierska et al., 2020).

3. The treatment with the novel CD91 derived peptide (SGS) with tregitope activity increased the frequencies of Tregs, increased the concentrations of IL-2 and IL-10, and decreased the expression of the costimulatory molecules of antigen-presenting cells, which resulted in improved pregnancy outcomes in abortion-prone mice (Kedzierska et al., 2021).

4. The identified CD91 derived peptide (SGS) meets the criteria of a tregitope, as its sequence is present in prevalent human proteins, is conservative across many species, and may bind to the MHCII (Kedzierska et al., 2021).