GDAŃSKI UNIWERSYTET MEDYCZNY
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KATEDRA I ZAKŁAD IMMUNOLOGII MEDYCZNEJ

ROZPRAWA DOKTORSKA

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Rola zmian epigenetycznych w regulacji komórek układu odpornościowego – implikacje w terapii cukrzycy typu 1

The role of epigenetic changes in the regulation of immune cells – implications for type 1 diabetes therapy

PROMOTOR:
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Pragnę serdecznie podziękować wszystkim, bez których przedłożona praca nie mogłaby powstać, a w szczególności:

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SPIS TREŚCI

1. WYKAZ PRAC NAUKOWYCH

W skład mojej rozprawy doktorskiej wchodzą prace opublikowane (Publikacje 1-3) oraz dane nieopublikowane, które są szczegółowo opisane w dalszej części przedłożonej rozprawy doktorskiej.

Opublikowane prace naukowe zawarte w rozprawie doktorskiej	
Publikacja 1	**DOI: 10.1016/j.jcyt.2020.07.001** Iwaszkiewicz-Grzes D*, Gliwinski M*, Eugster A, **Piotrowska M**, Dahl A, Marek-Trzonkowska N, Trzonkowski P. Antigen-reactive regulatory T cells can be expanded in vitro with monocytes and anti-CD28 and anti-CD154 antibodies. Cytotherapy. 2020 Nov;22(11):629-641. **Charakt. Merytoryczna:** praca oryginalna **Impact Factor:** 5.414 **Punktacja MEiN:** 100
Publikacja 2	**DOI: 10.3389/fimmu.2021.642678** Iwaszkiewicz-Grzes D,* **Piotrowska M*,** Gliwinski M, Urban-Wójciuk Z, Trzonkowski P. Antigenic Challenge Influences Epigenetic Changes in Antigen-Specific T Regulatory Cells. Front Immunol. 2021 Mar 23;12:642678. **Charakterystyka merytoryczna:** praca oryginalna **Impact Factor:** 8.787 **Punktacja MEiN:** 140
Publikacja 3	**DOI: 10.3390/ijms22137144** **Piotrowska M,** Gliwiński M, Trzonkowski P, Iwaszkiewicz-Grzes D. Regulatory T Cells-Related Genes Are under DNA Methylation Influence. Int J Mol Sci. 2021 Jul 1;22(13):7144. **Charakterystyka merytoryczna:** praca przeglądowa **Impact Factor:** 6.208 **Punktacja MEiN:** 140

*Równy udział w powstaniu pracy

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Inne prace naukowe	
1	**DOI: 10.5114/wo.2019.84110** Gliwiński M*, **Piotrowska M*,** Iwaszkiewicz-Grześ D, Urban-Wójciuk Z, Trzonkowski P. Therapy with $CD4^+CD25^+$ T regulatory cells - should we be afraid of cancer? Contemp Oncol (Pozn). 2019;23(1):1-6. **Charakterystyka merytoryczna:** praca przeglądowa **Punktacja MEiN:** 70

2	**DOI: 10.20452/pamw.16091** Tylicki L*, **Piotrowska M,** Biedunkiewicz B*, Zieliński M, Dąbrowska M, Tylicki P, Polewska K, Trzonkowski P, Lichodziejewska-Niemierko M, Dębska-Ślizień A. Humoral response to COVID-19 vaccination in patients treated with peritoneal dialysis: the COViNEPH Project. Pol Arch Intern Med. 2021 Oct 27;131(10):16091. **Charakterystyka merytoryczna:** praca oryginalna **Impact Factor:** 5.218 **Punktacja MEiN:** 140
3	**DOI: 10.3390/vaccines9101165** Dębska-Ślizień A, Ślizień Z, Muchlado M, Kubanek A, **Piotrowska M**, Dąbrowska M, Tarasewicz A, Chamienia A, Biedunkiewicz B, Renke M, Tylicki L. Predictors of Humoral Response to mRNA COVID19 Vaccines in Kidney Transplant Recipients: A Longitudinal Study-The COViNEPH Project. Vaccines (Basel). 2021 Oct 12;9(10):1165. **Charakterystyka merytoryczna:** praca oryginalna **Impact Factor:** 4.961 **Punktacja MEiN:** 140
4	**DOI: 10.20452/pamw.16142** Dębska-Ślizień A, Muchlado M, Ślizień Z, Kubanek A, **Piotrowska M**, Dąbrowska M, Bzoma B, Konopa J, Renke M, Biedunkiewicz B, Tylicki L. Significant humoral response to mRNA COVID-19 vaccine in kidney transplant recipients with prior exposure to SARS-CoV-2: the COViNEPH Project. Pol Arch Intern Med. 2022 Jan 28;132(1):16142. **Charakterystyka merytoryczna:** praca oryginalna **Impact Factor:** 5.218 **Punktacja MEiN:** 140
5	**DOI: 10.3389/fimmu.2022.832924** **Piotrowska M*,** Zieliński M*, Tylicki L*, Biedunkiewicz B, Kubanek A, Ślizień Z, Polewska K, Tylicki P, Muchlado M, Sakowska J, Renke M, Sudoł A, Dąbrowska M, Lichodziejewska-Niemierko M, Smiatacz T, Dębska-Ślizień A, Trzonkowski P. Local and Systemic Immunity Are Impaired in End-Stage-Renal-Disease Patients Treated With Hemodialysis, Peritoneal Dialysis and Kidney Transplant Recipients Immunized With BNT162b2 Pfizer-BioNTech SARS-CoV-2 Vaccine. Front Immunol. 2022 Jul 22;13:832924. **Charakterystyka merytoryczna:** praca oryginalna **Impact Factor:** 7.300 **Punktacja MEiN:** 140

*Równy udział w powstaniu pracy

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2. INNE OSIĄGNIĘCIA NAUKOWE

KONFERENCJE NAUKOWE

wystąpienia ustne

- Young European Federation of Immunological Sciences 1st Symposium, 11.2022, Germany
  Piotrowska Magdalena, Iwaszkiewicz-Grześ Dorota, Gliwiński Mateusz, Trzonkowski Piotr
  „Foxp3-related molecular and functional changes of antigen-specific regulatory T cells during cell culture"

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  Piotrowska Magdalena, Zieliński Maciej, Tylicki Leszek, Biedunkiewicz Bogdan, Kubanek Alicja, Ślizień Zuzanna, Polewska Karolina, Tylicki Piotr, Muchlado Marta, Renke Marcin, Sudoł Adam, Dąbrowska Małgorzata, Dębska-Ślizień Alicja, Trzonkowski Piotr
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  Piotrowska Magdalena, Zieliński Maciej, Tylicki Leszek, Biedunkiewicz Bogdan, Kubanek Alicja, Renke Marcin, Dębska-Ślizień Alicja, Trzonkowski Piotr
  "Post-vaccine immunity to COVID-19 vaccine in end-stage renal disease patients and kidney transplant recipients "

- XVII Congress of the Polish Society of Experimental and Clinical Immunology, 05.2021, Poland
  Piotrowska Magdalena, Iwaszkiewicz-Grześ Dorota, Gliwiński Mateusz, Trzonkowski Piotr
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  "Histone H3 modifications of regulatory T cells under expansion"

sesje plakatowe

- 19th Immunology of Diabetes Society (IDS) Congress, 05.2023, France
  Piotrowska Magdalena, Iwaszkiewicz-Grześ Dorota, Gliwiński Mateusz, Trzonkowski Piotr
  "Adoptive transfer of regulatory T cells shapes molecular features of CD4+ cells in T1D patients"

NAGRODY I WYRÓŻNIENIA

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Piotrowska M*, Gliwinski M, Trzonkowski P, Iwaszkiewicz-Grzes D. Regulatory T Cells-Related Genes Are under DNA Methylation Influence. Int J Mol Sci. 2021.

Iwaszkiewicz-Grzes D*, **Piotrowska M*,** Gliwinski M, Urban-Wójciuk Z, Trzonkowski P. Antigenic Challenge Influences Epigenetic Changes in Antigen-Specific T Regulatory Cells. Front Immunol. 2021. (Publikacja 2)

2020

Stypendium Santander Universidades dla doktorantów na czynny udział w konferencji naukowej

2020

Wyróżnienie Polskiej Akademii Nauk za publikację:

Iwaszkiewicz-Grześ D*., Gliwiński M., Eugster A., **Piotrowska M.,** Dahl A., Martek-Trzonkowska N., Trzonkowski P., Antigen-reactive regulatory T cells can be expanded in vitro with monocytes and anti-CD28 and anti-CD154 antibodies, Cytotherapy, 2020.

2019

Pierwsze miejsce za prezentację ustną na III Międzynarodowej Konferencji Studenckich Kół Naukowych za pracę: "Histone H3 modifications of regulatory T cells under expansion".

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Primus Inter Pares – Nagroda Senatu Gdańskiego Uniwersytetu Medycznego za wysokie wyniki w nauce i zaangażowanie w społeczność akademicką.

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3. WYKAZ UŻYWANYCH SKRÓTÓW

Ag-spec	–	antygenowo-specyficzne (ang. *antigen-specific*)
APC	–	komórki prezentujące antygen (ang. *antigen presenting cells*)
B7.1/CD80	–	ligandy receptora CD28 (ang. *ligands for the CD28 protein*)
B7.2/CD86	–	ligandy receptora CD28 (ang. *ligands for the CD28 protein*)
BS-seq	–	sekwencjonowanie DNA po reakcji z wodorosiarczanem (ang. *bisulfite sequencing*)
cAMP	–	cykliczny adenozyno-3',5'-monofosforan (ang. *cyclic adenosine monophosphate*)
CAR	–	chimeryczny receptor antygenowy (ang. *chimeric antigen receptor*)
CD	–	antygen różnicowania komórkowego (ang. *cluster of differentiation*)
CNS	–	konserwatywne niekodujące elementy DNA (ang. *conserved non-coding DNA sequences*)
CTLA-4/CD152	–	białko kodowane przez gen CTLA4 (ang. *cytotoxic t cell antigen 4*)
DCs	–	komórki dendrytyczne (ang. *dendritic cells*)
DDI	–	dobowa dawka insuliny (ang. *daily dose of insulin*)
DNMTs	–	metylotransferazy DNA (ang. *DNA methyltransferases*)
DRs	–	regiony demetylowane (ang. *demethylated regions*)
Foxp3	–	czynnik transkrypcyjny (ang. *forkhead box P3*)
GAD65	–	dekarboksylaza kwasu glutaminowego o wielkości 65 kDa (ang. *glutamic acid decarboxylase of 65 kDA*)
GAPDH	–	gen dehydrogenazy aldehydu-3-fosfoglicerynowego (ang. *glyceraldehyde-3-phosphate dehydrogenase*)
HAT	–	acetylotransferaza histonowa (ang. *histone acetyltransferases*)
HD	–	zdrowy dawca krwi (ang. *healthy donor*)
HDAC	–	deacetylazy histonowe (ang. *histone deacetylases*)
HLA	–	ludzkie antygeny leukocytarne (ang. *human leukocyte antigens*)
HMT	–	metylotransferazy histonów (ang. *histone methyltransferases*)
IDO	–	2,3-dioksygenaza indolaminy (ang. *indoleamine 2,3-dioxygenase*)
IFN- γ	–	interferon gamma (ang. *interferon gamma*)

IKZF2 – czynnik transkrypcyjny Helios (ang. *transcription factor Helios*)

IKZF4 – czynnik transkrypcyjny Eos (ang. *transcription factor Eos*)

IL – interleukina (ang. *interleukin*)

IL2RA – receptor alfa interleukiny drugiej (ang. *interleukin 2 receptor subunit alpha*)

IPEX – sprzężony z chromosomem X zespół dysregulacji immunologicznej, poliendokrynopatii i enteropatii (ang. *immunodysregulation polyendocrinopathy enteropathy X-linked syndrome*)

iTreg – Tregs indukowane in vitro (ang. *induced Tregs*)

K – lizyna (ang. *lysine*)

LADA – późno ujawniająca się cukrzyca typu 1 u dorosłych (ang. *latent autoimmune diabetes of adults*)

MFI – średnia intensywność fluorescencji (ang. *mean fluorescence intensity*)

MHC – główny układ zgodności tkankowej (ang. *major histocompatibility complex*)

miRNA – mikroRNA (ang. *micro RNA*)

MMTT – test sekrecji endogennej insuliny (ang. *mixed-meal tolerance test*)

ncRNA – niekodujący RNA (ang. *non-coding RNA*)

NOD – myszy zapadające na cukrzycę pomimo braku otyłości (ang. *non-obese diabetic mice*)

PBMC – jednojądrzaste komórki krwi obwodowej (ang. *peripheral blood mononuclear cells*)

PBS – buforowany roztwór soli fizjologicznej (ang. *phoshate buffered saline*)

PCA – analiza głównych składowych (ang. *principal compontent analysis*)

PTMs – potranslacyjne modyfikacje (ang. *postranslational modifications*)

pTregs – Tregs indukowane na obwodzie (ang. *peripherally induced Tregs*)

qMSP – metylospecyficzna reakcja ilościowej polimerazy w czasie rzeczywistym (ang. *quantitative methylation-specific polymerase chain reaction*)

qPCR – reakcja łańcuchowej polimerazy w czasie rzeczywistym (ang. *quantitative polymerase chain reaction*)

R – arginina (ang. *arginine*)

RISC – kompleks biorący udział w wyciszaniu genów (ang. *RNA-induced silencing complex*)

RT-qPCR – reakcja łańcuchowa polimerazy z odwrotną transkrypcją (ang. *quantitative reverse transcription polymerase chain reaction)*

T1D – cukrzyca typu 1 (ang. *diabetes mellitus type 1*)

TCR – receptor limfocytów T (ang. *T cell receptor*)

Teffs – limfocyty T efektorowe (ang. *effector T cells*)

TET – rodzina enzymów z rodziny TET (ang. *ten-eleven translocation (TET) methylcytosine dioxygenases*)

TF – czynnik transkrypcyjny (ang. *transcription factor*)

TGF-β – transformujący czynnik wzrostu β (ang. *transforming growth factor 1*)

Th – limfocyty T pomocnicze (ang. *helper T cells*)

TNFRSF18 – czynnik GITR (ang. *glucocorticoid-induced tumor necrosis factor receptor - related protein*)

Tregs – limfocyty T regulatorowe (ang. *regulatory T cells*)

TSDR – demetylowany region swoisty dla Tregs (ang. *treg-specific demethylated region*)

tTregs – Tregs powstające w grasicy (ang. *thymus-derived Tregs*)

4. STRESZCZENIE

Cukrzyca typu 1 (T1D; ang. *diabetes mellitus type 1*) jest chorobą objawiającą się defektem wydzielania insuliny na skutek autodestrukcji komórek β trzustki, spowodowanej naciekiem komórkami układu immunologicznego (*insulitis*). W ostatnich latach znaczącą rolę w patogenezie tej choroby przypisuje się modyfikacjom epigenetycznym, które odpowiedzialne są za zmiany ekspresji genów. Komórkami, które w sposób aktywny uczestniczą w hamowaniu prozapalnych komórek efektorowych, są limfocyty T regulatorowe (Tregs). Wykazano, iż u pacjentów z T1D poziom Tregs i/lub ich funkcjonalność są obniżone, dlatego dostępne terapie polegają na poprawie tych parametrów.

Celem niniejszej rozprawy doktorskiej było wygenerowanie komórek antygenowo-specyficznych (Ag-spec), o wysokim potencjale wykorzystania w terapii T1D oraz porównanie ich profilu epigenetycznego z obecnie stosowanym preparatem poliklonalnym. Komórki te uzyskano poprzez stymulację autologicznymi monocytami prezentującymi antygeny charakterystyczne dla T1D (insulina bądź fragment 9-23 łańcucha β insuliny), zaś ich aktywność potwierdzono w badaniach *in vitro*. Następnie Ag-spec Tregs zbadano pod kątem molekularnym określając profil ekspresji genów, modyfikacji histonu H3 oraz poziom metylacji DNA. Ponadto, doświadczenia wykonano na populacji komórek T efektorowych (Teffs) o przeciwnej funkcji biologicznej względem Tregs. Finalnie wytypowano preferencyjny antygen, który umożliwiał otrzymanie Tregs o najwyższej stabilności i określono różnice molekularne pomiędzy subpopulacjami komórek.

W ramach rozprawy doktorskiej, wykonano analizę profilu epigenetycznego i panelu ekspresji genów komórek pochodzących od pacjentów ze świeżo-zdiagnozowaną T1D. Uzyskane dane pokazały, iż w trakcie ekspansji, komórki CD4+ pochodzące od pacjentów z T1D są mniej stabilne, szczególnie w obrębie parametrów dotyczących cząsteczek Foxp3 i CTLA-4.

Biorąc pod uwagę potrzebę rozwoju obecnych terapii wykorzystujących właściwości supresorowe Tregs oraz konieczność prowadzenia długotrwałej ekspansji w celu zwiększenia ilości komórek w końcowym preparacie, doświadczenia, które wykonaliśmy mają realny wpływ na rozwój i udoskonalenie protokołów wykorzystujących te komórki w przebiegu leczenia cukrzycy typu 1.

5. ABSTRACT

Diabetes mellitus type 1 (T1D) is a disease characterized by defects in insulin secretion due to the autodestruction of pancreatic β cells caused by infiltration with immune cells (insulitis). In recent years, a significant role in the pathogenesis of this disease has been attributed to epigenetic modifications, which are responsible for changes in gene expression. The cells actively involved in inhibiting pro-inflammatory effector T cells (Teffs) in the course of T1D are regulatory T cells (Tregs). It has been shown that in patients with T1D the level of regulatory T lymphocytes and/or their functionality is reduced, therefore therapies involving the multiplication and improvement of the functionality of these cells are used.

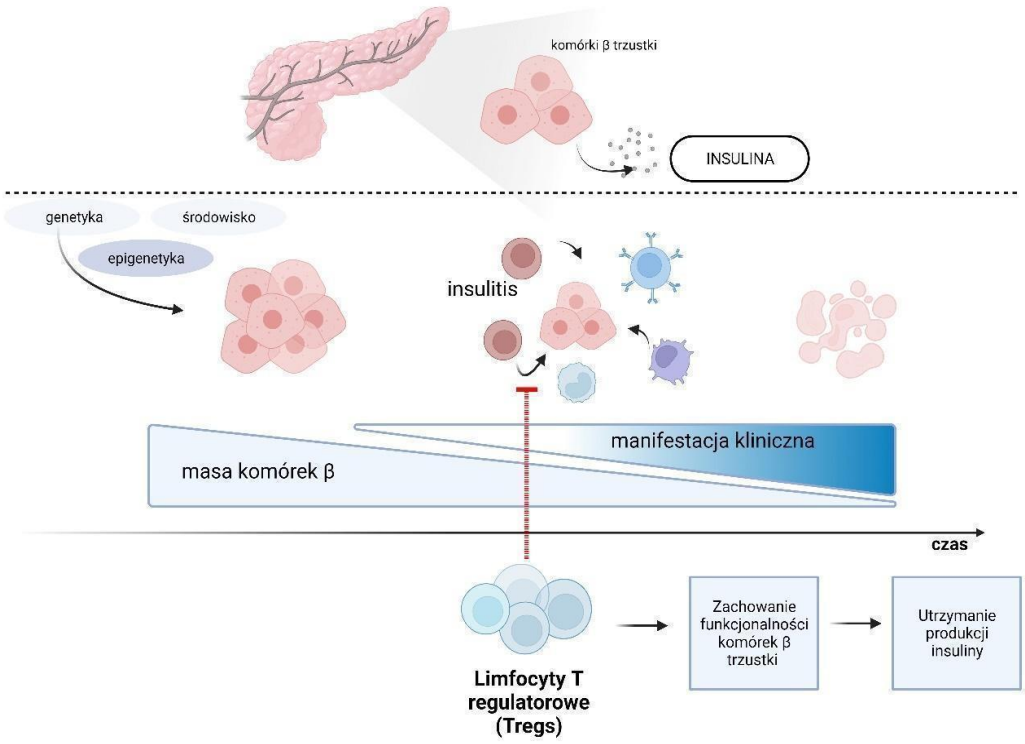
The aim of this doctoral dissertation was to generate antigen-specific (Ag-spec) cells with a high potential for use in T1D therapy and to compare their epigenetic profile with the currently used polyclonal cells. These cells were obtained by stimulation with autologous monocytes presenting antigens characteristic of T1D (insulin or fragment 9-23 of the insulin β chain), and their activity was confirmed in *in vitro* tests. The Ag-spec Tregs were then examined for molecular purposes. For this, the gene expression profile, histone H3 modifications and DNA methylation level were determined. Additionally, experiments were performed on a population of effector T cells (Teffs) with the opposite biological function to Tregs. Finally, a preferential antigen was selected, which allowed obtaining Tregs with the highest stability. Moreover, molecular differences between CD4+ cell subpopulations were determined.

In addition, as part of the doctoral dissertation, an epigenetic profile and a gene expression panel of cells obtained from newly diagnosed T1D patients were determined. We gained data that indicate that during expansion of cells from T1D patients, CD4+ are less stable, particularly in parameters related to Foxp3 and CTLA-4 molecules.

Considering the need to develop current therapies using the suppressor properties of Tregs and the need for long-term expansion to increase the number of cells in the final product, our experiments have a real impact on the development and improvement of protocols using Treg cells in the treatment of type 1 diabetes.

6. WPROWADZENIE

Cukrzyca typu 1 (T1D; ang. *diabetes mellitus type 1*) jest autoimmunologiczną chorobą narządowo-swoistą, w wyniku której dochodzi do niszczenia komórek β trzustki. Jest ona jedną z najczęstszych chorób przewlekłych wieku dziecięcego, a dane statystyczne wskazują na ciągły wzrost częstości zachorowania wśród pacjentów pediatrycznych. Za proces zapalny w obrębie narządu odpowiadają auto-reaktywne komórki układu odpornościowego, które, w tzw. procesie *insulitis*, naciekają wyspy trzustkowe, prowadząc do stopniowej destrukcji narządu, początkowo przebiegającej bezobjawowo. W trakcie manifestacji klinicznej jedynie 10-30% komórek β trzustki utrzymuje swoją aktywność. W związku z tym, współczesne strategie terapeutyczne koncentrują się na zachowaniu i wydłużeniu żywotności tych kluczowych komórek, co znajduje potwierdzenie w literaturze naukowej (Ryc. 1) [1–3].



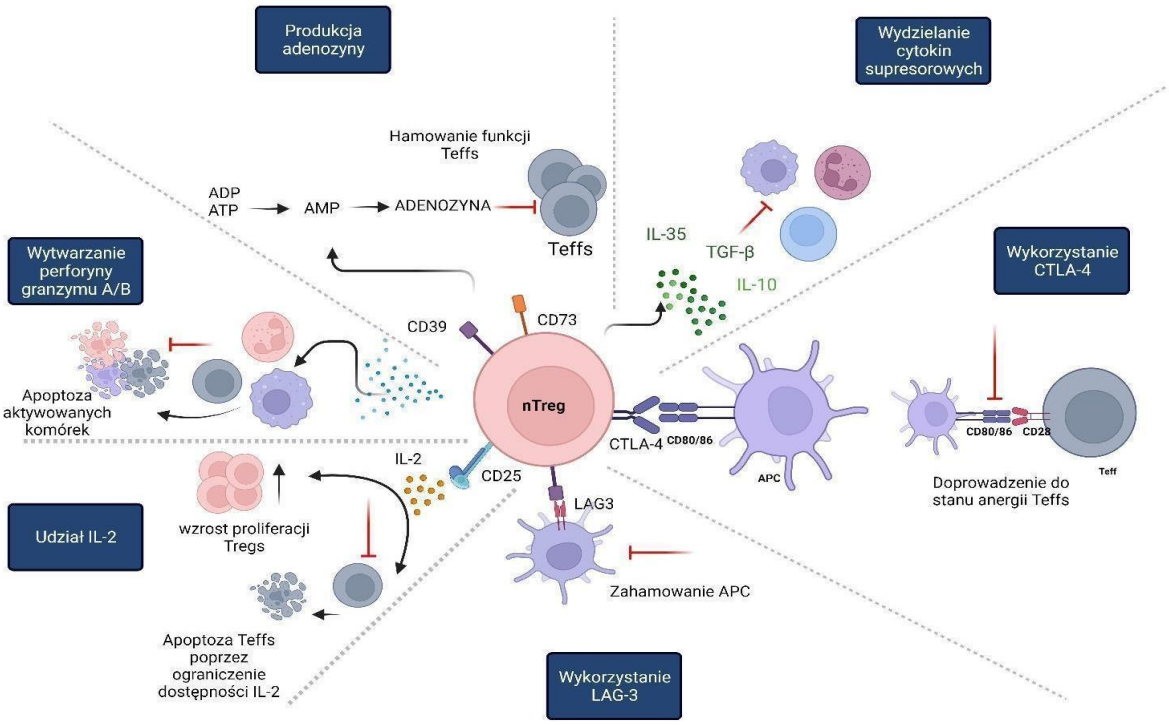
Ryc. 1 Patogeneza cukrzycy typu 1. Schemat infiltracji i niszczenia komórek β trzustki na przestrzeni czasu oraz korzyści terapii limfocytami T regulatorowymi [4].

LIMFOCYTY T REGULATOROWE

Jednym z wykorzystywanych mechanizmów, który znajduje zastosowanie w leczeniu chorób autoimmunologicznych, w tym T1D, są właściwości immunosupresyjne limfocytów T regulatorowych (Tregs). Jest to niewielka populacja, stanowiąca około 5-10% komórek CD4+, która może powstawać w grasicy (tTregs, ang. *thymus-derived Tregs*), bądź być

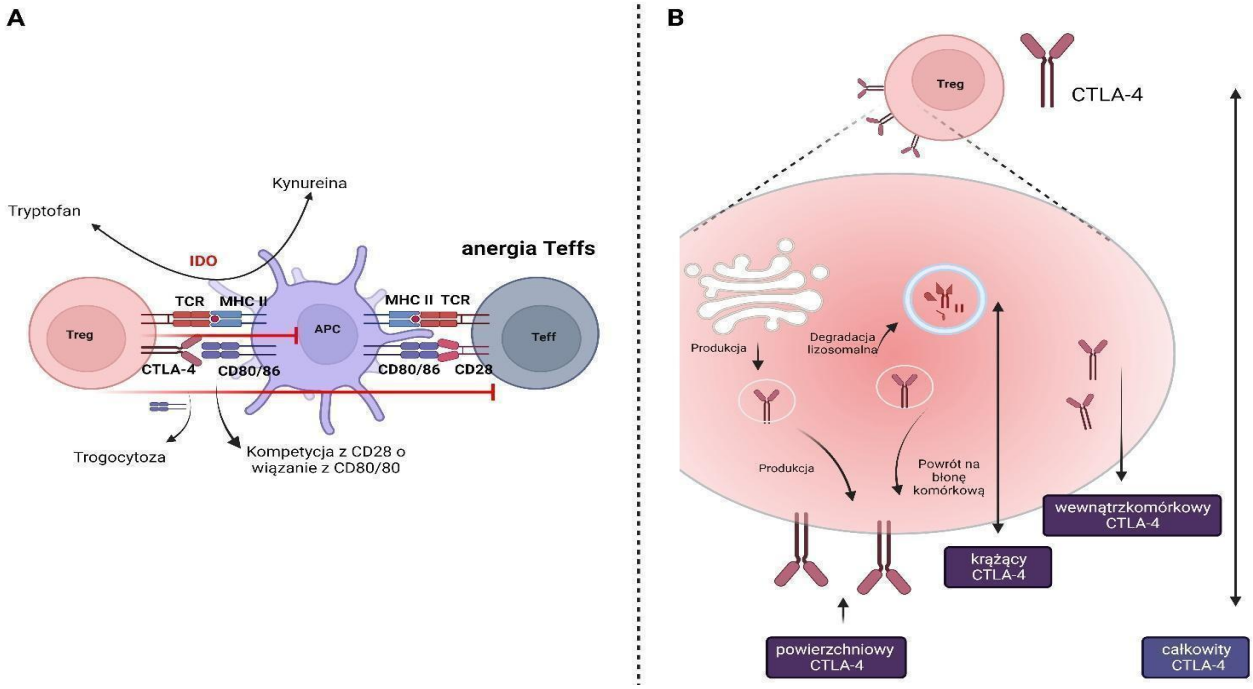
indukowana na obwodzie (pTregs, ang. *peripherally induced Tregs*). Mogą być również indukowane *in vitro* (iTregs) dzięki obecności w środowisku cytokin, np. TGF-β i IL-2. Kluczowym białkiem wewnątrzkomórkowym w procesie ich rozwoju jest Foxp3 (ang. *Forkhead box 3*), który odpowiada za utrzymanie fenotypu. Dzieje się tak m.in. poprzez łączenie z promotorem dla IL-2 i IFN-γ, co w rezultacie prowadzi do blokowania transkrypcji genów prozapalnych. Mutacje w jego obrębie prowadzą do rozwoju chorób autoimmunologicznych, czego przykładem jest sprzężony z chromosomem X zespół deregulacji immunologicznej, poliendokrynopatii i enteropatii, tzw. IPEX (ang. *Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked*) [5,6]. Niemniej jednak, lista cząsteczek odpowiedzialnych za prawidłową funkcjonalność Tregs stale rośnie. Ze względu na swój bezpośredni wpływ na utrzymanie stabilności i funkcjonalności limfocytów T regulatorowych, kluczowa wydaje się obecność CD152 (CTLA-4), IKZF2 (Helios), IKZF4 (Eos) oraz TNFRSF18 (GITR) [7,8].

W układzie odpornościowym rolą limfocytów Tregs jest utrzymywanie stanu homeostazy, poprzez aktywne hamowanie proliferacji komórek T efektorowych (Teffs) i wydzielanych przez nie cytokin. Jest to możliwe w wyniku działania kilku mechanizmów (Ryc. 2) m. in.: wykorzystanie CTLA-4, sekrecja antyzapalnych cytokin (IL-10, TGF-β, IL-35), udział LAG3, produkcja adenozyny przy pomocy CD39 i CD73 oraz sekwestracja IL-2 [9].



Ryc. 2 Wybrane mechanizmy supresji z udziałem limfocytów T regulatorowych [10].

Warto również podkreślić rolę cząsteczki CTLA-4 (CD152) (Ryc. 3), gdyż podobnie do Foxp3, mutacje w jej obrębie doprowadzają do osłabienia działania komórek Tregs, a z drugiej strony jej stabilna ekspresja zapobiega zaburzeniom wynikającym z zachwiania tolerancji immunologicznej [11]. Unikalną cechą CTLA-4 jest jej dystrybucja w obrębie różnych subpopulacji komórkowych. Pojawienie się jej na błonie komórek CD4+/CD8+ wynika z aktywacji poprzez stymulację kompleksu TCR/CD3, a największy poziom na błonie obserwuje się po 24-48h. W zależności od siły interakcji pomiędzy receptorem TCR, a antygenem prezentowanym w kontekście MHC, determinowana jest ilość CD152 na powierzchni i kumulacja tych cząsteczek w obrębie synapsy immunologicznej.



Ryc. 3 Mechanizmy związane z supresją zależną od CTLA-4 (A) oraz dystrybucja CTLA-4 w Tregs (B) [12–14].

W konsekwencji, w większości subpopulacji komórkowych, CD152 przechowywane jest w strukturach wewnątrzkomórkowych, ulegając szybkiemu procesowi endocytozy z błony komórkowej w trakcie aktywacji komórki [15–17]. Jedyną populacją, która posiada zdolność stałej ekspresji CTLA-4, są limfocyty Tregs, zaś ich właściwości immunosupresyjne, są wynikiem kilku mechanizmów:

I. CD152, będąc homologiem CD28, posiadającym wyższe powinowactwo do receptorów CD80/CD86, pośrednio wpływa na obniżenie sygnałów aktywujących limfocyty [18];

II. bezpośrednio hamuje sygnały biegnące z kompleksu TCR/CD3 oraz CD28, poprzez oddziaływanie z białkami z rodziny fosfataz [19,20];

III. cząsteczki te posiadają zdolność do aktywnego usuwania receptorów CD80/CD86 z powierzchni komórek prezentujących antygeny (APC; *ang. Antigen presenting cells*), w procesie tzw. trogocytozy, w wyniku czego upośledzona zostaje ich funkcja prezentacji [12,13];

IV. interakcje CTLA-4 z APC, doprowadzają do produkcji dioksydazy 2,3-indolowej (IDO, ang. *Indoleamine 2,3-dioxygenase*), enzymu, który katalizuje reakcję przekształcenia tryptofanu do kynureniny, co prowadzi do wzbudzenia stanu anergii w limfocytach T i następnie ich apoptozy [12–14].

ZMIANY EPIGENETYCZNE LIMFOCYTÓW T REGULATOROWYCH

W ostatnich latach, znaczącą rolę w patogenezie chorób autoimmunologicznych, w tym T1D, przypisuje się modyfikacjom epigenetycznym. Termin epigenetyka określa zmiany w ekspresji genów i funkcjonalności komórek, które nie powstają na skutek zmian w sekwencjach nukleotydowych. Do podstawowych mechanizmów epigenetycznych należą: metylacja DNA, potranslacyjne modyfikacje histonów oraz udział niekodującego RNA (ncRNA), które mogą skutkować zarówno supresją, jak i aktywacją transkrypcji [21,22].

Pierwszy mechanizm, metylacja DNA, zachodzi głównie w regionach bogatych w pary guanina-cytozyna (wyspy CpG) i skutkuje represją transkrypcji. Polega ona na dodaniu grupy metylowej do węgla C5 cytozyny, poprzez działanie enzymów z grupy metylotransferaz (DNMTs). Enzymy te odpowiadają za metylację *de novo* (DNMT3a, DNMT3b) lub odtwarzają wzór metylacji w trakcie procesu replikacji DNA podczas podziału komórkowego (DNMT1). Z kolei demetylacja, inicjowana jest przez białka z rodziny TET (Tet 1-3), które powodują relaksację chromatyny i wznowienie ekspresji genów [23,24].

Modyfikacje potranslacyjne histonów (PTMs, ang. *Posttranslational modifications*) H2A, H2B, H3 i H4, takie jak np.: metylacja, acetylacja, fosforylacja, monoubikwitynacja oraz sumoilacja, nazywane są „kodem histonowym". Regulują one stan pomiędzy aktywną transkrypcyjnie euchromatyną, a nieaktywną heterochromatyną. Zmiana upakowania i dostępności materiału transkrypcyjnego uwarunkowana jest działaniem enzymów, które katalizują usuwanie lub dodawanie grup do N-końcowych części histonu, bogatych w reszty lizyny (K), argininy (R) lub do rdzenia globulinowego [25]. Większość modyfikacji jest odwracalna i może być katalizowana dwukierunkowo poprzez przeciwstawnie działające grupy

enzymów. Dodawanie grupy acetylowej, poprzez acetylotransferazy skutkuje aktywacją transkrypcji, a proces związany z działaniem deacetylaz histonowych prowadzi do jej represji. Z kolei metylacja i demetylacja histonów, w zależności od miejsca, ilości dodanych grup oraz rodzaju komórki, może skutkować aktywacją, bądź represją transkrypcji. Proces fosforylacji histonów katalizowany jest zaś przez enzymy z grupy kinaz, co wiąże się z aktywacją transkrypcji, natomiast fosfatazy prowadzą do odłączania ujemnie naładowanych grup fosforanowych. Inne modyfikacje, takie jak monoubikwitynacja i sumoilacja, warunkują odpowiednio aktywację lub inhibicję transkrypcji [26,27].

Udział niekodującego RNA wiąże się z wyciszaniem procesu transkrypcji poprzez komplementarne łączenie się miRNA (mikroRNA), krótkiej jednoniciowej cząsteczki RNA, wchodzącej w skład kompleksu RISC (ang. *RNA-induced silencing complex*), indukującego degradację mRNA i ostatecznie doprowadza do obniżenia ilości powstającego białka [28].

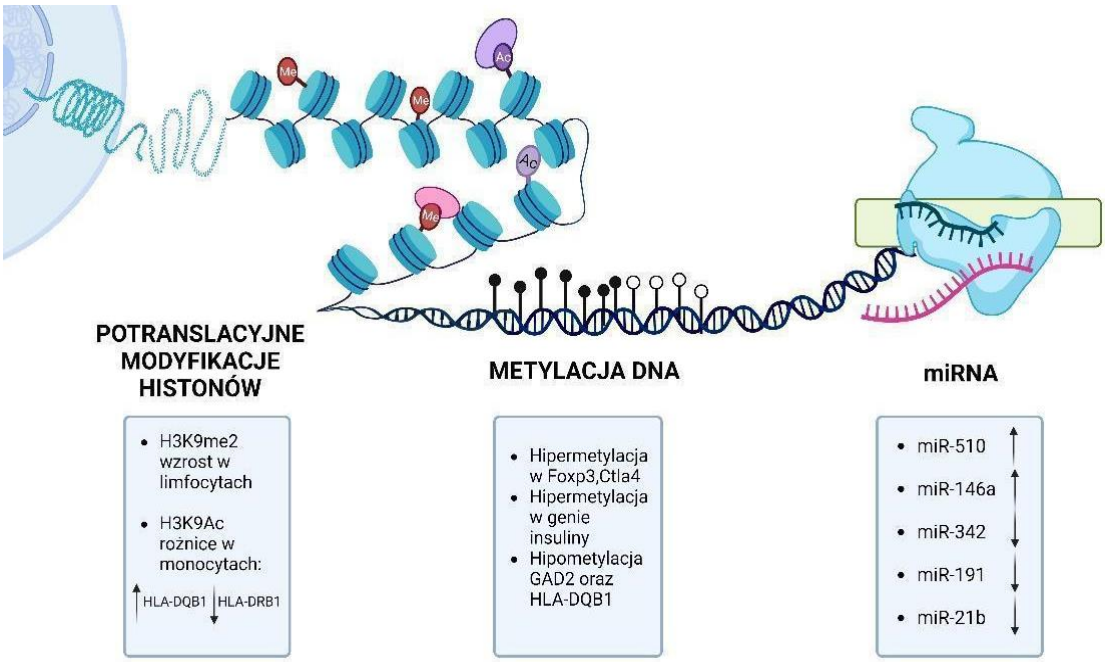
Należy wspomnieć, iż komórki CD4+, pochodzące od osób z T1D, posiadają odmienny wzór epigenetyczny, niż ten obserwowany u osób zdrowych (Ryc. 4). Przykładem jest metylacja promotora w genie *FOXP3*, represyjna modyfikacja H3K9me2 w promotorze genu *CTLA4*, a także wzrost ekspresji miRNA-510, spadek miRNA-342 oraz miRNA-191 [21,29].

Co ważne, wspomniane powyżej geny, będące głównymi elementami regulującymi Tregs, są pod ścisłą kontrolą, a odpowiedni ich status epigenetyczny determinuje fenotyp i stabilność limfocytów [30]. Dodatkowo, profil modyfikacji histonów oraz metylacji DNA, pozwala na odróżnienie Tregs od konwencjonalnych limfocytów T efektorowych, które w trakcie hodowli komórkowej mogą przejściowo produkować sztandarowy marker limfocytów Tregs – Foxp3. Również regiony DNA hipometylowane specyficzne dla Tregs, tzw. Treg-DRs (ang. *Treg-specific DNA demethylated regions*), są określone poprzez wysoką zawartość aktywujących modyfikacji histonów, takich jak: H3K27ac, H3K4me3 oraz H3K1me1, czego nie obserwuje się w limfocytach konwencjonalnych [31,32].

Literatura wskazuje, iż najistotniejsze jest oznaczanie modyfikacji epigenetycznych zachodzących w regionie genu *FOXP3*, gdyż odchylenia w jego obrębie mają znaczący wpływ na jakość preparatu komórkowego [33–35]. Kluczową analizą jest określenie metylacji regionu TSDR (ang. *Treg-specific demethylated region*), będącego częścią konserwatywnych sekwencji niekodujących DNA – CNS (ang. *Conserved non-coding DNA sequence*), zlokalizowanych w locus genu *FOXP3*. TSDR należy utożsamiać z CNS2, znajdującym się w intronie 1, który odpowiada za utrzymanie stabilnej ekspresji czynnika Foxp3, dzięki utrwalonej pamięci epigenetycznej, uwarunkowanej wysokim poziomem demetylacji DNA.

Niemal całkowitą demetylację tego regionu obserwuje się w Tregs generowanych w grasicy, natomiast komórki indukowane *in vitro* oraz limfocyty Teffs posiadają odwrotny profil [32,36].

Należy podkreślić, iż limfocyty T regulatorowe, w trakcie silnej stymulacji receptora TCR oraz w obecności prozapalnego środowiska cytokinowego, mogą tracić swoje właściwości supresorowe oraz różnicować się w komórki Teffs w tzw. procesie plastyczności. Niemniej jednak, ten kontrowersyjny proces nie jest do końca poznany i głównie przypisywany jest Tregs indukowanym *in vitro* oraz niewielkiej subpopulacji komórek Foxp3+, posiadającej niestabilny wzorzec epigenetyczny [35,37].

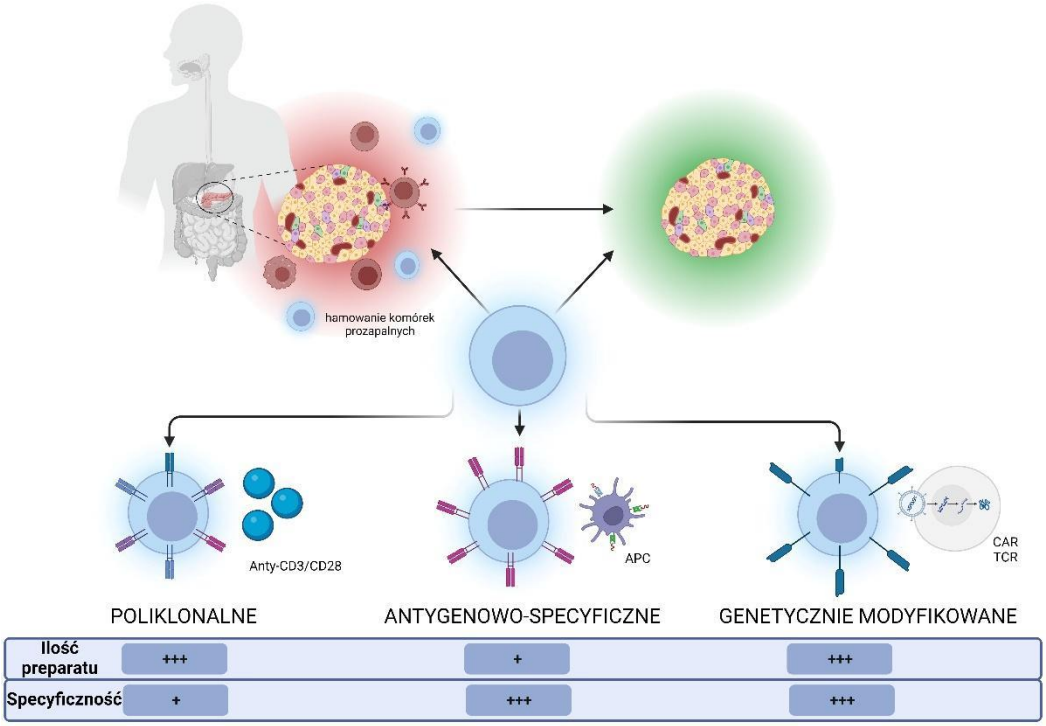


Ryc. 4 Przykładowe mechanizmy epigenetyczne, zachodzące w limfocytach T regulatorowych u osób z T1D [38,39].

ANTYGENOWO-SPECYFICZNE LIMFOCYTY T REGULATOROWE

Limfocyty T regulatorowe są idealnym kandydatem do wykorzystania w leczeniu chorób autoimmunologicznych, w tym T1D. Niemniej jednak, obecnie wykorzystywane komórki mają charakter poliklonalny, czyli są specyficzne względem wielu antygenów, co obniża ich potencjał terapeutyczny. W związku z tym, prowadzone są badania nad stworzeniem komórek dedykowanych dla danej jednostki chorobowej, tzw. antygenowo-specyficznych (Ag-spec). Teoria działania komórek Ag-spec opiera się na ich wysokim powinowactwie do antygenu charakterystycznego dla konkretnej choroby autoimmunologicznej. Uważa się, iż komórki te migrują do miejsca procesu zapalnego i w bardziej efektywny i specyficzny

sposób, przywracają homeostazę w układzie odpornościowym [40]. Dostępnych jest kilka strategii umożliwiających generowanie Ag-spec Tregs (Ryc. 5). Jedną z nich jest zastosowanie metod inżynierii genetycznej w celu produkcji chimerycznych receptorów antygenowych – CAR (ang. *Chimeric antigen receptor*) lub zastosowanie syntetycznych receptorów TCR. Komórki posiadające ekspresję CAR, rozpoznają antygeny niezależnie od kompleksu MHC, natomiast zastosowanie receptorów TCR wiąże się z prezentacją białka w kontekście MHC [41–43].



Ryc.5 Potencjał wykorzystania komórek T regulatorowych w leczeniu T1D [44].

Badania przeprowadzone na modelach mysich wskazują, iż generowane CAR-Tregs, specyficzne względem dekarboksylazy kwasu glutaminowego, izoformy 65 kD (GAD65), czy fragmentowi 10-23 łańcucha β insuliny, indukują tolerancję w obrębie trzustki, hamując proliferację limfocytów autoreaktywnych znacznie efektywniej niż komórki naiwne. Co ważne, w badaniach tych, CAR-Tregs zapobiegały powstawaniu cukrzycy typu 1 w modelu wykorzystującym myszy NOD (ang. *Non-obese diabetic*) [45,46].

Przykładem innej metody generowania komórek antygenowo-specyficznych jest stymulacja komórkami prezentującymi konkretny antygen (APC). Wyniki badań na humanizowanych modelach mysich wskazują, że ekspozycja na alloantygeny prezentowane przez DCs lub komórki B, umożliwia proliferację komórek o wysokiej zdolności do migracji i akumulacji w przeszczepionym narządzie.

Ze względu na niewielką liczbę jaką stanowią Tregs w populacji CD4+, ilość komórek specyficznych względem konkretnego antygenu jest jeszcze mniejsza, a otrzymanie odpowiedniej ilości funkcjonalnego preparatu wiąże się z wieloma problemami technicznymi. W związku z tym, istnieje ciągła potrzeba optymalizacji protokołów umożliwiających otrzymanie pożądanej dawki terapeutycznej, przy zachowaniu ich właściwości [47,48].

PODSUMOWANIE

Wzrost zainteresowania wykorzystaniem komórek antygenowo-specyficznych do leczenia chorób autoimmunologicznych stwarza potrzebę opracowania odpowiednich protokołów, które z jednej strony umożliwiają generowanie optymalnej ilości preparatu, a jednocześnie umożliwiają otrzymywanie komórek o wysokiej funkcjonalności i stabilności fenotypowej.

Biorąc pod uwagę, iż mechanizmy epigenetyczne są zaangażowane w utrzymywanie właściwości Tregs, określenie markerów molekularnych może być kluczowym elementem zwiększającym efektywność terapeutyczną stosowanych preparatów.

Ponadto, w odróżnieniu od mutacji genetycznych, zmiany epigenetyczne mają charakter odwracalny, co stwarza ogromny potencjał do tworzenia nowych terapii chorób autoimmunologicznych, ale także otwiera drogę do zapobiegania powstawaniu chorób z autoagresji.

7. CELE BADAŃ

CELE GŁÓWNE:

Celem pracy było określenie profilu epigenetycznego komórek antygenowo-specyficznych względem antygenów charakterystycznych dla cukrzycy typu 1 (insulina lub fragment 9-23 łańcucha β insuliny) i porównanie ich z obecnie stosowanym preparatem komórek poliklonalnych, jak również ustalenie wzorca molekularnego komórek CD4+ u osób z cukrzycą typu 1, a w szczególności określenie stabilności cząsteczek zaangażowanych w funkcjonalność komórek T regulatorowych w trakcie ekspansji komórkowej.

CELE SZCZEGÓŁOWE:

1. Wygenerowanie antygenowo-specyficznych komórek T regulatorowych do wykorzystania w terapii cukrzycy typu 1.

2. Określenie profilu metylacji DNA oraz zmian w modyfikacjach histonu H3 w wygenerowanym produkcie antygenowo-specyficznych limfocytów T regulatorowych.

3. Wytypowanie preferencyjnego antygenu umożliwiającego otrzymanie komórek antygenowo-specyficznych o stabilnym profilu molekularnym.

4. Porównanie profilu ekspresji wybranych genów związanych z utrzymaniem funkcjonalności limfocytów T regulatorowych pomiędzy osobami ze świeżo-zdiagnozowaną cukrzycą typu 1, a osobami zdrowymi.

5. Określenie wpływu modyfikacji epigenetycznych na zmiany w profilu ekspresji genów u osób ze świeżo-zdiagnozowaną cukrzycą typu 1.

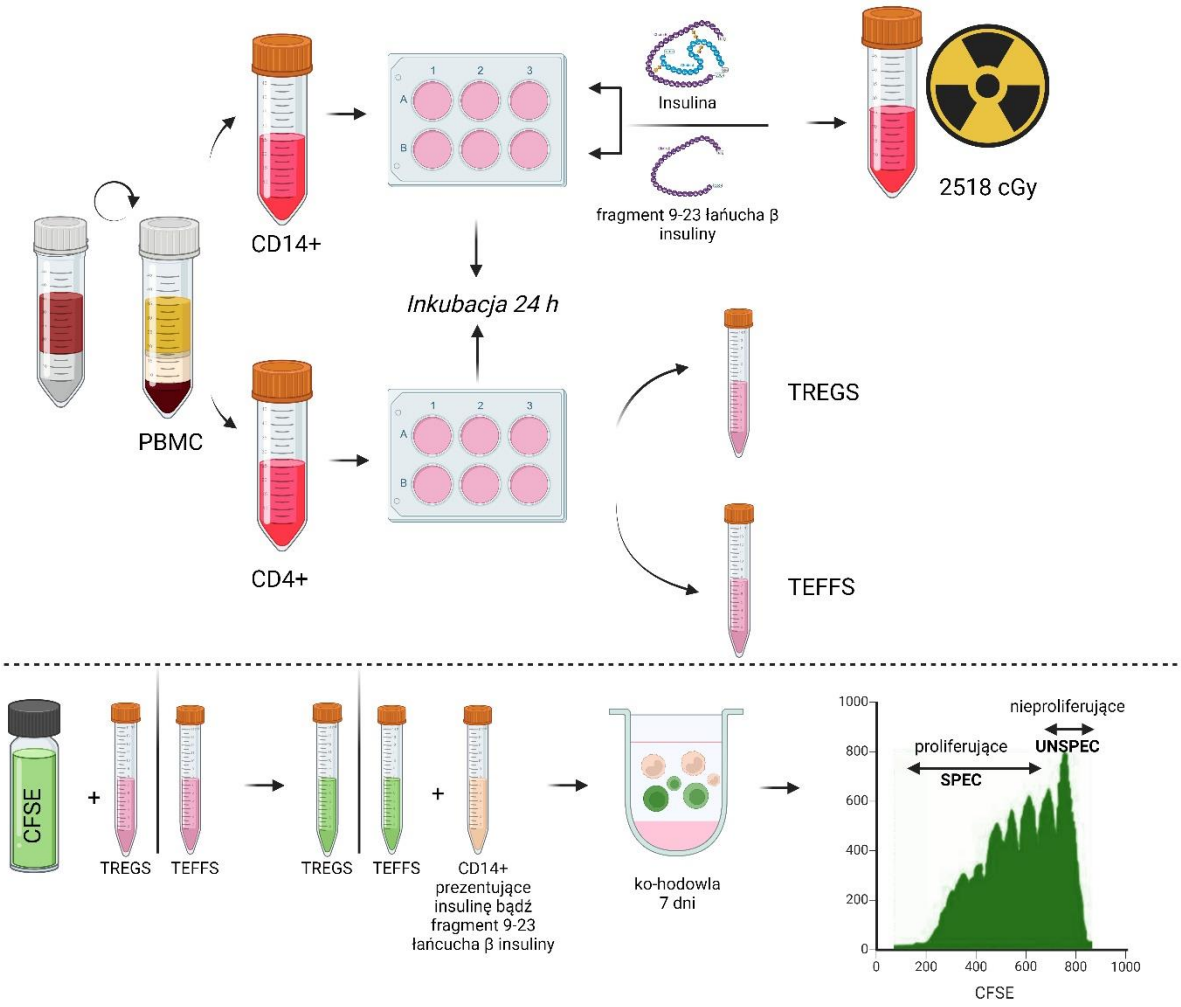
8. MATERIAŁY I METODY

8.1 Materiały i metody ujęte w publikacjach

W publikacji 1, „*Antigen-reactive regulatory T cells can be expanded in vitro with monocytes and anti-CD28 and anti-CD154 antibodies" (doi: 10.1016/j.jcyt.2020.07.001)* opisano metodę otrzymywania antygenowo-specyficznych limfocytów T regulatorowych i określono właściwości supresorowe otrzymanych komórek.

8.1.1 Opracowanie protokołu otrzymywania komórek antygenowo-specyficznych

W tym celu wykorzystano kożuszki leukocytarne zdrowych dawców krwi pozyskanych z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa w Gdańsku zgodnie ze schematem przedstawionym na Ryc. 6.



Ryc. 6 Schemat otrzymywania antygenowo-specyficznych limfocytów T regulatorowych i T efektorowych [opracowanie własne].

Poprzez zastosowanie wirowania w gradiencie gęstości izolowano jednojądrzaste komórki krwi obwodowej (PBMC), z których następnie przy pomocy izolacji immunomagnetycznej, otrzymywano komórki CD4+ oraz CD14+ (monocyty). Limfocyty CD4+ poddawano procesowi sortowania (BD FACSAria IIu) na dwie populacje: Tregs ($CD3+CD4+CD25^{high}CD127-$) oraz Teffs ($CD3+CD4+CD25^{low}CD127+$), a następnie barwiono za pomocą barwnika fluorescencyjnego CFSE (ang. *Carboxtfluorescein diacetate succinimidyl ester*). Autologiczne monocyty stymulowano wybranymi antygenami charakterystycznymi dla cukrzycy typu 1: insulina lub fragment 9-23 łańcucha β insuliny przez 24h i poddawano procesowi napromieniowywania jonizującego (2518 cGy).

W kolejnym etapie zakładano ko-hodowle komórkowe zawierające monocyty prezentujące antygeny i Tregs lub Teffs (monocyty:komórki = 1:1, 37°C, do 7 dni), które suplementowano, bądź nie przeciwciałami anty-CD154 i anty-CD28. Następnie, na podstawie rozcieńczenia barwnika proliferacyjnego CFSE, komórki sortowano na: proliferujące, które odpowiedziały na zaprezentowany antygen – oznaczane w dalszej części badania jako komórki antygenowo-specyficzne (SPEC) oraz nieproliferujące, których poziom barwnika proliferacyjnego odpowiadał poziomowi komórek niestymulowanych – określone jako antygenowo-niespecyficzne (UNSPEC). Kontrolę stanowiły komórki poliklonalne (otrzymywane w wyniku stymulacji kulkami magnetycznymi pokrytymi przeciwciałami anty-CD3 i anty-CD28 w stosunku 1:1). Wygenerowane komórki Tregs poddano testom mającym na celu określenie: fenotypu, funkcjonalności i porównano je z obecnie stosowanymi w terapiach chorób autoimmunologicznych – komórkami poliklonalnymi. Szczegółowe informacje o metodyce zawarte są w publikacji, natomiast poniżej przedstawiono ogólny profil doświadczeń.

8.1.2 Fenotyp Ag-spec

W 7 dniu hodowli cześć komórek pobrano i przeprowadzono barwienie cytometryczne przy użyciu przeciwciał znakowanych fluorescencyjnie skierowanych przeciwko: CD4,CD25, CD127, CD62L, Helios i Foxp3.

8.1.3 Testy funkcjonalne

W 12 dniu hodowli komórkowej przeprowadzono testy funkcjonalne, polegające na ocenie właściwości supresorowych wygenerowanych produktów Ag-spec. W tym celu wykonano test hamowania proliferacji i wydzielania IFN-γ przez autologiczne komórki T efektorowe. Komórki responderowe (Teffs poliklonalne lub Ag-spec Teffs) pozostawiano

bez stymulacji przez 48 h, barwiono przy pomocy barwnika proliferacyjnego VPD450 (*Violet Proliferation Dye 450)*, a następnie zakładano ko-hodowle komórkowe z Tregs w następujących proporcjach (Teffs:Tregs): 1:1, 1:$\frac{1}{2}$, 1: $\frac{1}{4}$ oraz 1: $\frac{1}{8}$ i stymulowano za pomocą monocytów prezentujących antygeny charakterystyczne dla T1D, bądź kulek magnetycznych opłaszczonych przeciwciałami anty-CD3 i anty-CD28. Jako kontrolę negatywną użyto niestymulowanych Teffs. Ko-hodowle komórkowe były utrzymywane do 6 dni. Odczytu profileracji komórek responderowych dokonywano przy użyciu cytometru przepływowego (LSRFortessa, BD), natomiast do pomiaru wydzielonego IFN-γ wykorzystano metodę ELISA (BD OptEIA™ Human IFN-γ ELISA Kit II) oraz ELISpot (Mabtech). Ponadto przeanalizowano repertuar TCR wygenerowanego produktu Ag-spec zgodnie ze schematem doświadczeń przedstawionym przez Eugster i in. [49].

W publikacji 2, *"Antigenic Challenge Influences Epigenetic Changes in Antigen-Specific T Regulatory Cells" (10.3389/fimmu.2021.642678),* ponownie wykorzystano materiał pochodzący od zdrowych dawców krwi z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa w Gdańsku. Następnie generowano antygenowo-niespecyficzne i poliklonalne komórki zgodnie z procedurą przedstawioną w publikacji oraz opisaną w paragrafie 8.1.1. Komórki poddano badaniom molekularnym w celu określenia profilu ekspresji genów, całkowitej metylacji DNA genomowego, metylacji w regionie TSDR oraz wykonano analizę 21 modyfikacji aktywujących i hamujących w histonie H3. Poniżej przedstawiono skrótowy opis doświadczeń wykonanych w ramach tej publikacji.

8.1.4 Izolacja RNA i DNA

Całkowity RNA i DNA izolowano przy użyciu zestawu AllPrep® DNA/RNA Mini Kit (Qiagen) zgodnie z instrukcją producenta. Czystość i stężenie RNA oraz DNA określono na podstawie wartości OD przy długości fali 260 i 280 nm przy użyciu spektrofotometru mikropłytkowego Epoch (BioTek). Otrzymany RNA przechowywano do momentu użycia w temperaturze -80°C, natomiast DNA w temperaturze - 20°C.

8.1.5 RT-qPCR

500 ng RNA poddawano procesowi odwrotnej transkrypcji za pomocą zestawu High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) w standardowych warunkach:

etap 1	25°C	10 min
etap 2	37°C	120 min
etap 3	85°C	5 min
etap 4	4°C	∞

W celu oceny ekspresji genów, cDNA analizowano metodą ilościowej reakcji PCR w czasie rzeczywistym (qPCR) z wykorzystaniem FastStart Essential DNA Probes Master (Roche), zgodnie z protokołem dostarczonym przez producenta. Sekwencje starterów i numery sond (Universal ProbeLibrary Set, Human with Probes; Roche) podano w Tabeli 1. Reakcję qPCR przeprowadzano w objętości 20 µl przy użyciu termocyklera LightCycler®96 (Roche). Zastosowano warunki reakcji:

wstępna inkubacja	95°C	600 s	1 cykl
denaturacja	95°C	10 s	45 cykli
przyłączanie	60°C	30 s	1 cykl

Kontrolę stanowił gen *GAPDH*. Względną ekspresję genów obliczono metodą Livak'a (2-ΔΔCt) [50].

Tabela 1. Sekwencje starterów i numery sond do reakcji Q-PCR.

Gen	Symbol sekwencji referencyjnej	Starter	Sekwencja startera (5'-3')	Sonda (numer) #
GAPDH	NM_001289745.1	Fw	CCCCGGTTTCTATAAATTGAGC	75
		Rv	GGCTGACTGTCGAACAGGA	
FOXP3	NM_014009.3	Fw	ACACTGCCCCTAGTCATGGT	25
		Rv	GAGCTGGTGCATGAAATGTG	
CTLA4	NM_005214.4	Fw	TGGGTCCCAGGGAAGTTT	25
		Rv	TGACCTTGTGTTCTACCTGGTG	
IKZF2	NM_016260.2	Fw	CATCACATTGCTTTGCCCTA	61
		Rv	TCATCACTGTCAGAGAGAGGCTA	
IKZF4	NM_022465.3	Fw	TCAGGCATTTGTTGTGCAGT	3
		Rv	AGGGAAAGGCAGATGCTGT	
TNFRSF18	NM_004195.5	Fw	ACCTGGGTCGGGATTCTC	10
		Rv	CACAGCCAGTTGGACACG	
RUNX1	NM_001754.4	Fw	CCAAAGAGTGTGGAATTTTGGT	55
		Rv	AAACAGGGCGAGTTGCAT	
HMOX1	NM_002133.2	Fw	CCCTTCAGCATCCTCAGTTC	84
		Rv	GACAGCTGCCACATTAGGG	
TET2	NM_001127208.2	Fw	AAAGATGAAGGTCCTTTTTATACCC	68
		Rv	ACCCTTCTGTCCAAACCTTTC	

Fw – przedni *(ang. Forward)*, Rv – wsteczny *(ang. Reverse)*

8.1.6 Metylospecyficzna ilościowa reakcja PCR

W pierwszym etapie, 500 ng DNA poddano konwersji z wodorosiarczynem przy użyciu zestawu EpiTect® Bisulfite Kit (Qiagen), który pozwala na konwersję niemetylowanych cytozyn do uracylu. Reakcję konwersji przeprowadzano w termocyklerze (Arktik Thermal Cycler, Thermo Scientific) według schematu:

95°C	60° C	95°C	60°C	95°C	60°C
5 min	25 min	5 min	85 min	5 min	175 min

Następnie, przekonwertowane DNA, oczyszczono na kolumnach wirówkowych EpiTect (dostarczonych przez producenta) i oceniono poziom metylacji za pomocą ilościowej metylospecyficznej reakcji PCR (qMSP) przy użyciu hot start TaKaRa Taq DNA polymerase (TaKaRa Bio). Pary starterów dla regionu TSDR zostały zaprojektowane w programie MethPrimer 2.0 przez Zafari i in. [51] i są wymienione w Tabeli 2. qMSP przeprowadzono w końcowej objętości reakcji wynoszącej 20 µl, obejmującej 5 pmol starterów odpowiednich dla sekwencji zmetylowanej lub niezmetylowanej oraz 50-100 ng przekonwertowanego DNA. Warunki reakcji qMSP przedstawiono poniżej:

preinkubacja	95°C	30 s	1 cykl
2-stopniowa amplifikacja	95°C	5 s	40 cykli
	60°C	30 s	
określenie temperatury topnienia	95°C	5 s	1 cykl
	60°C	60 s	
	95°C	1 s	
chłodzenie	50°C	30 s	1 cykl

Poziom demetylacji obliczono ze wzoru [51]:

$$demetylacja\ [\%] = \frac{100}{1 + 2^{(Ct^{TG} - Ct^{CG})}}$$

w którym:

- Ct^{TG} oznacza próg cyklu w reakcji ze starterami dla sekwencji niezmetylowanych
- Ct^{CG} próg cyklu osiągnięty w reakcji ze starterami dla sekwencji zmetylowanych

Tabela 2. Sekwencje starterów do określenia poziomu metylacji DNA.

		qMSP		
Gen region	**Pozycja od ATG (+1)**	**Starter**		**Sekwencja startera (5'-3')**
***FOXP3* TSDR**	-2282 to -2376	Fw	ME	GATAGGGTAGTTAGTTTTCGGAAC
		Rv		CCGCCATTAACGTCATAACG
		Fw	UN	GGATAGGGTAGTTAGTTTTTGGAATG
		Rv		CCACCATTAACATCATAACAACCA

ME – startery dla sekwencji zmetylowanych (ang. Methylated), UN – startery dla sekwencji niezmetylowanych (ang. Unmethylated)
Fw – przedni *(ang. Forward)*, Rv – wsteczny *(ang. Reverse)*

8.1.7 Ocena całkowitej metylacji genomowego DNA

Globalny poziom metylacji DNA oceniano przy użyciu zestawu Methylated DNA Quantification Kit (Colorimetric) (Abcam), zgodnie z instrukcją producenta. Do każdej reakcji używano 100 ng/µl DNA. Absorbancję mierzono przy 450 nm, korzystając ze spektrofotometru

Epoch (BioTek) oraz oprogramowania Gene5, a następnie wykorzystywano ją do obliczenia procentu metylacji genomowego DNA poprzez wygenerowanie krzywej standardowej.

8.1.8 Izolacja białek histonowych i analiza profilu modyfikacji histonu H3

Białka histonowe izolowano za pomocą zestawu Histone Extraction Kit (Abcam) zgodnie z instrukcją producenta, natomiast analizę profilu 21 modyfikacji histonu H3 wykonano z wykorzystaniem zestawu ELISA – Histone H3 Modification Multiplex Assay Kit (Colorimetric, Abcam). Do każdej reakcji użyto 150 ng białek histonowych i podążano zgodnie z procedurą dostarczoną przez producenta. Absorbancję mierzono za pomocą spektrofotometru Epoch (BioTek) i wykorzystano ją do obliczenia procentu poszczególnych modyfikacji w histonie H3. W tabeli 3 przedstawiono wszystkie modyfikacje zbadane w przedstawionym doświadczeniu, wraz z określeniem ich wpływu na proces ekspresji genu.

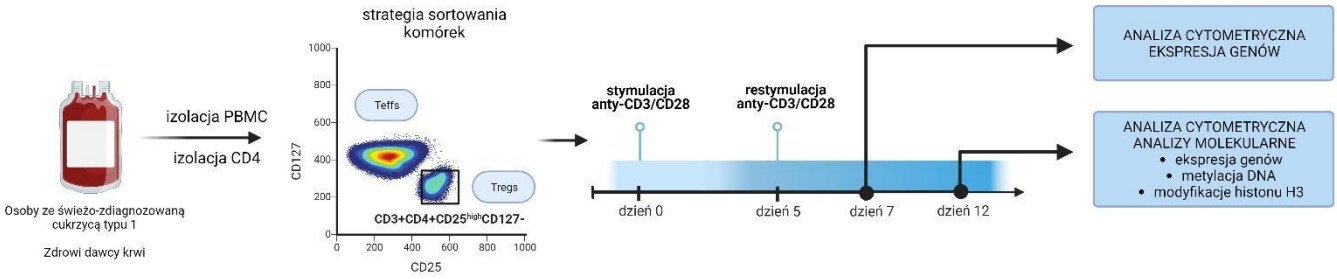
Tabela 3. Modyfikacje histonu H3.

Metylacja	**acetylacja**	**fosforylacja**
H3K4me1↑	H3K9ac↑	H3ser10ph↑
H3K4me2↑	H3K14ac↑	H3ser28ph↑
H2K4me3↑	H3K18ac↑	
H3K9me1↑	H3K56ac↑	
H3K9me2↓		
H3K9me3↓		
H3K27me1↑		
H3K27me2↓		
H3K27me3↓		
H3K36me1↑		
H3K36me2↑		
H3K36me3↑		
H3K79me1↑		
H3K79me2↑		
H3K79me3↑		

↑ modyfikacje aktywujące
↓ modyfikacje hamujące

8.2 Materiały i metody nieujęte w publikacjach

Część wyników wchodzących w skład rozprawy doktorskiej nie została dotychczas opublikowana i zawiera analizy przeprowadzone na materiale pochodzącym od osób ze świeżo-zdiagnozowaną cukrzycą typu 1. Analizowano poziom ekspresji genów określających profil i funkcjonalność komórek Tregs oraz genów zaangażowanych w regulację procesu metylacji DNA. Wykonano również profilowanie całkowitej metylacji DNA oraz określono modyfikację aktywującą (H3K4me3) oraz hamującą (H3K27me3) w histonie H3. Ponadto zbadano wzorzec metylacji DNA w genach *FOXP3* i *CTLA4* w regionach promotorowych i warunkujących ich stabilną ekspresję, tj. TSDR dla *FOXP3* i ekson 2 dla *CTLA4*, a także zbadano te cząsteczki na poziomie białka. Z materiału pochodzącego od pacjentów z T1D i od zdrowych dawców izolowano komórki Tregs i Teffs, a następnie uzyskane limfocyty poddawane były ekspansji przy użyciu kulek magnetycznych pokrytych przeciwciałami anty-CD3/CD28. Hodowle prowadzono przez 12 dni, a komórki w tym czasie były poddawane analizom zgodnie ze schematem przedstawionym na Ryc. 7.



Ryc. 7 Schemat pozyskania materiału do badania i jego dalsze wykorzystanie [opracowanie własne].

Badanie zostało zaakceptowane i zatwierdzone przez komisję bioetyczną przy Gdańskim Uniwersytecie Medycznym (NKBBN/376-410/2021), a szczegółowy opis metod przedstawiono w dalszej części tego rozdziału.

8.2.1 Charakterystyka grupy badanej

Do badania włączono pacjentów ze świeżo-zdiagnozowaną cukrzycą typu 1, spełniających kryteria do otrzymania preparatu zawierającego poliklonalne komórki T regulatorowe (Tabela 4). Charakterystyka kliniczna grupy została podana w Tabeli 5. Do grupy kontrolnej zakwalifikowano zdrowych dawców krwi z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa w Gdańsku, w wieku powyżej 20 lat, co umożliwiło zredukowanie

prawdopodobieństwa wystąpienia T1D w przyszłości [52,53] (n=10; wiek$_{(średnia±SD)}$=26.9; płeć$_{(\%mężczyzn)}$=50%).

Tabela 4. Kryteria włączenia i wyłączenia uczestnika badania [54].

Kryteria włączenia	Kryteria wyłączenia
● Wiek uczestnika: 8-16 lat. ● BMI: 25-75 percentyl (wg projektu OLAF). ● Peptyd C w osoczu na czczo > 0,7 ng/ml, a w teście stymulacyjnym wzrost ≥100%. ● Obecność co najmniej jednego autoprzeciwciała przeciw wyspom trzustkowym (ICA, IAA, GAD): wysokie miano IAA lub GAD (≥4-krotność normy) lub niskie miano (2-4-krotność normy) co najmniej dwóch z nich przeciwciała. ● Możliwość wyrażenia pisemnej świadomej zgody przez rodziców (i pacjentów w wieku >16 lat). ● Zaangażowanie pacjentów i rodziców w intensywne leczenie cukrzycy definiowane jako samokontrola stężenia glukozy nie rzadziej niż 3 razy dziennie oraz podawanie insuliny. ● Odpowiedni dostęp żylny do pobrania krwi.	● Brak zgody na udział w badaniu i brak podpisanej świadomej zgody. ● Inne niż autoimmunologiczna cukrzyca typu 1. ● Wiek <8 i >16 lat. ● Niedobór IgA lub inna wada genetyczna. ● BMI <25 lub >75 percentyla dla danego wieku. ● Nadwrażliwość na rytuksymab, przeciwciało anty-CD20 lub inny składnik preparatu. ● Obecność lub historia aktywnej infekcji, w tym wirusowego zapalenia wątroby typu B, zapalenia wątroby typu C, HIV, gruźlicy lub kiły. Osoby z laboratoryjnymi dowodami aktywnej infekcji zostały wykluczone nawet w przypadku braku klinicznych dowodów aktywnej infekcji. ● Obecność aktywnego zakażenia wirusem EBV (dodatnia IgM). ● Obecność lub historia aktywnej ogólnoustrojowej infekcji grzybiczej. ● Jakakolwiek historia nowotworu złośliwego. ● Niedokrwistość, limfopenia, neutropenia lub małopłytkowość poniżej dolnych granic zakresu referencyjnego w ciągu 6 tygodni przed badaniem. ● Stany nadkrzepliwości. ● Leczenie wymagające przewlekłego stosowania leków innych niż insulina dłużej niż 3 miesiące. ● Leczenie jakimkolwiek lekiem przeciwcukrzycowym innym niż insulina w ciągu 4 tygodni od włączenia do badania. ● Retinopatia cukrzycowa. ● Nadciśnienie tętnicze. ● Obecność lub historia makroalbuminurii. ● W przypadku kobiet w wieku powyżej 15 lat: pozytywny wynik testu ciążowego lub niechęć do stosowania skutecznych środków antykoncepcyjnych przez czas trwania badania i, w stosownych przypadkach, 4 miesiące po jego zakończeniu. ● W przypadku mężczyzn: zamiar prokreacji w trakcie trwania badania lub w stosownych przypadkach w ciągu 4 miesięcy po jego przerwaniu. ● Nadmierny niepokój pacjenta lub rodziców związany z zabiegami. ● Każdy stan chorobowy, który w opinii badacza będzie zakłócał bezpieczne uczestnictwo w badaniu. ● Dla rodziców i dzieci w wieku powyżej 15 lat: znane nadużywanie aktywnego alkoholu lub substancji psychoaktywnych.

ICA – przeciwciała przeciwwyspowe, IAA – przeciwciała przeciwko insulinie, GAD – przeciwciała przeciwko dekarboksylazie kwasu glutaminowego

Tabela 5. Charakterystyka grupy badanej.

Pacjenci ze świeżo-zdiagnozowaną cukrzycą typu 1 (n=22)

Parametr	Średnia (SD)
$BMI_{(kg/m^2)}$	18.30 (2.307)
$Wiek_{(lata)}$	12.05 (2.439)
$Płeć_{(\%mężczyzn)}$	68
$HbA1c_{(\%)}$	6.186 (0.887)
$DDI_{(unit/kg)}$	0.113 (0.148)
$C\text{-}peptyd_{(ng/dl)}$	0.899 (0.343)

DDI – dobowa dawka insuliny

8.2.2 Izolacja limfocytów Tregs i Teffs

Jednojądrzaste komórki krwi obwodowej (PBMC) otrzymywano poprzez wirowanie w gradiencie gęstości. W tym celu krew od pacjentów z T1D lub od zdrowych dawców krwi rozcieńczano w stosunku 1:1 z PBS (ang. *Phosphate buffered saline*), nawarstwiano na Ficoll-Paque Plus i wirowano przez 20 min (800 x g). Z uzyskanych frakcji:

górna – składająca się z osocza,

środkowa – zawierająca PBMC,

dolna – zawierająca głównie erytrocyty,

zbierano PBMC, trzykrotnie przepłukiwano buforem PBS (5 min, 350 x g) i na podstawie selekcji negatywnej, izolowano immunomagnetycznie komórki CD4+ (EasySep™ Human CD4+ *T Cell Isolation*; StemCell Technologies) zgodnie z instrukcją producenta. Następnie, komórki barwiono przeciwciałami: anty-CD3 (PacificBlue), anty-CD4 (APC), anty-CD25 (PE), anty-CD127 (FITC) i sortowano na dwie populacje: Tregs ($CD3+CD4+CD25^{high}CD127$) oraz Teffs ($CD3+CD4+CD25^{low}CD127+$) z wykorzystaniem sortera FACSAria IIu (BD Biosciences). Wyizolowane komórki przenoszono na płytki 96-dołkowe, zawieszano w kompletnym medium (X-VIVO 20, 10% inaktywowanej ludzkiej surowicy AB, IL-2 10000 U/ml, penicylina 100 U/ml i streptomycyna 100 mg/ml) i stymulowano przy pomocy kulek magnetycznych pokrytych przeciwciałami anty-CD3/anty-CD28 w stosunku 1:1 (Miltenyi Biotech). Komórki inkubowano przez 12 dni, a ich analizę oraz archiwizację przeprowadzano w 7 i 12 dniu hodowli. W trakcie hodowli komórkowej stopniowo zmniejszano poziom stosowanej IL-2. Dodatkowo, 5 dnia komórki zostały ponownie stymulowane przy pomocy anty-CD3/anty-CD28, a temperatura hodowli obniżona z 37°C na 33°C.

8.2.3 Izolacja RNA i analiza ekspresji genów

Z uzyskanych komórek izolowano RNA zgodnie z opisem przedstawionym w paragrafie 8.1.4, natomiast analizę profilu ekspresji genów przeprowadzono zgodnie z warunkami przedstawionymi w 8.1.5. Sekwencje starterów i numery sond podano w Tabeli 6.

Tabela 6. Sekwencje starterów i numery sond do reakcji Q-PCR.

Gen	Symbol sekwencji referencyjnej	Starter	Sekwencja startera (5'-3')	Sonda (numer) #
GAPDH	NM_001289745.1	Fw	CCCCGGTTTCTATAAATTGAGC	75
		Rv	GGCTGACTGTCGAACAGGA	
FOXP3	NM_014009.3	Fw	ACACTGCCCCTAGTCATGGT	25
		Rv	GAGCTGGTGCATGAAATGTG	
CTLA4	NM_005214.4	Fw	TGGGTCCCAGGGAAGTTT	25
		Rv	TGACCTTGTGTTCTACCTGGTG	
IKZF2	NM_016260.2	Fw	CATCACATTGCTTTGCCCTA	61
		Rv	TCATCACTGTCAGAGAGAGGCTA	
IKZF4	NM_022465.3	Fw	TCAGGCATTTGTTGTGCAGT	3
		Rv	AGGGAAAGGCAGATGCTGT	
TNFRSF18	NM_004195.5	Fw	ACCTGGGTCGGGATTCTC	10
		Rv	CACAGCCAGTTGGACACG	
RUNX1	NM_001754.4	Fw	CCAAAGAGTGTGGAATTTTGGT	55
		Rv	AAACAGGGCGAGTTGCAT	
CREB1	NM_0012874891.1	Fw	GGAGCTTGTACCACCGGTAA	50
		Rv	GCATCTCCACTCTGCTGGTT	
HMOX1	NM_002133.2	Fw	CCCTTCAGCATCCTCAGTTC	84
		Rv	GACAGCTGCCACATTAGGG	
STAT5A	NM_001288718.2	Fw	GCTCCCTATAACATGTACCCACA	58
		Rv	TGGTCTCATCCAGGTCGAA	
STAT5B	NM_01448.3	Fw	TGAAGGCCACCATCATCAG	40
		Rv	TGTTCAAGATCTCGCCACTG	
NFATC1	NM_0061162.4	Fw	TGGACCAGTTGTACCTGGATG	67
		Rv	GTGCTCCAATGTGGCAACTA	
NFATC2	NM_012340.4	Fw	TATTACCTGCGGGGGTGAC	68
		Rv	CCAGCTAAGGTGTGTGTCTATCA	
TET2	NM_001127208.2	Fw	AAAGATGAAGGTCCTTTTTATACCC	68
		Rv	ACCCTTCTGTCCAAACCTTTC	
TET3	NM_001287491.1	Fw	CCATTGCAAAGTGGGTGA	65
		Rv	CGCACCAGGCAGAGTAGC	
DNMT3A	NM_022552.4	Fw	ACTACATCAGCAAGCGCAAG	75
		Rv	CACAGCATTCATTCCTGCAA	
DNMT1	NM_001379.3	Fw	GGCTAAGCGTTCAAGAGACC	30
		Rv	TGTAATCCTGGGGCTAGGTG	
LRBA	NM_001199282.2	ASSAY ID: HS01032231_M1 (CATALOG # 4331182)		

Fw – przedni *(ang. Forward)*, Rv – wsteczny *(ang. Reverse)*

8.2.5 Izolacja DNA

DNA z uzyskanych komórek izolowano i przechowywano zgodnie z informacjami podanymi w rozdziale 8.1.4.

8.2.6 Ocena całkowitej metylacji genomowego DNA

Globalny poziom metylacji DNA (100 ng/reakcja) oceniano przy użyciu zestawu MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Epigentek) zgodnie z instrukcją producenta. Absorbancję mierzono przy 450 nm, korzystając ze spektrofotometru Epoch (BioTek) oraz oprogramowania Gene5. Całkowitą metylację (% 5-mC) obliczono zgodnie ze wzorem podanym przez producenta.

8.2.7 Ocena metylacji DNA w regionach kluczowych dla genów FOXP3 i CTLA4

Doświadczenia przeprowadzono zgodnie z opisem przedstawionym w sekcji 8.1.6, zawierającym warunki konwersji z wodorosiarczynem, oczyszczania produktu i reakcji qMSP. Pary starterów odpowiednio dla sekwencji zmetylowanych i niezmetylowanych w regionach promotorowych i specyficznych dla Tregs w genie *FOXP3* i *CTLA4* zostały zaprojektowane w programie MethPrimer 2.0 i są wymienione w Tabeli 7 – qMSP. Do obliczenia poziomu metylacji DNA wykorzystano wzór podany w rozdziale 8.1.6. W przypadku analizy materiału od kobiet równanie to skorygowano współczynnikiem 2 (jeden z dwóch alleli TSDR jest metylowany w wyniku inaktywacji chromosomu X [51].

Tabela 7. Sekwencje starterów do określenia poziomu metylacji DNA.

qMSP				
Gen region	**Pozycja od ATG (+1)**	**Starter**		**Sekwencja startera (5'-3')**
***FOXP3* TSDR**	-2282 to -2376	Fw	ME	GATAGGGTAGTTAGTTTTCGGAAC
		Rv		CCGCCATTAACGTCATAACG
		Fw	UN	GGATAGGGTAGTTAGTTTTTGGAATG
		Rv		CCACCATTAACATCATAACAACCA
***FOXP3* PROMOTOR**	-6211 to -6273	Fw	ME	AAAATTTAAAATTTTAAAATTTTCGT
		Rv		CACGCTATACGATATAAAAACCG
		Fw	UN	AAAATTTAAAATTTTAAAATTTTTGT
		Rv		CACACTACAATATAAAAACCACA
***CTLA4* EKSON 2**	+2686 to +2792	Fw	ME	GGTATTGGTTAGTAGTCGAGG
		Rv		ATTCCCCATCATATAAATTACC
		Fw	UN	GGTATTGGTTAGTAGTTGAGG
		Rv		ATTCCCCATCATATAAATTACC
***CTLA4* PROMOTOR**	-2582 to -2721	Fw	ME	GAGATTAGTTTGGTTAATATGGCGA
		Rv		CCAAATTAAAATACAATAACGCGAT
		Fw	UN	GAGATTAGTTTGGTTAATATGGTGA
		Rv		CCCAAATTAAAATACAATAACACAAT
BS-Seq				
***CTLA4* EKSON 2**	+2686 to +2758	Fw	NA	GTTTGTTGTGGTATTGGTTAGTAGT
		Rv		TCATCTAAAAAAATCAACTCATTCC

ME – startery dla sekwencji zmetylowanych (ang. Methylated), UN – startery dla sekwencji niezmetylowanych (ang. Unmethylated)
Fw – przedni *(ang. Forward)*, Rv – wsteczny *(ang. Reverse)*, NA – nie dotyczy (ang. not applicable)

8.2.8 Sekwencjonowanie po reakcji z wodorosiarczanem

W celu zidentyfikowania wzoru metylacji w poszczególnych cytozynach przeprowadzono sekwencjonowanie DNA po reakcji z wodorosiarczanem (BS-seq) w eksonie 2 w genie *CTLA4*. W tym celu przeprowadzono konwersję, oczyszczanie DNA i reakcję PCR zgodnie z parametrami przedstawionymi w pkt. 8.1.6 (sekwencje starterów do reakcji PCR przedstawiono w Tabeli 7 – BS-Seq). Następnie otrzymane produkty PCR zostały zsekwencjonowane przez firmę zewnętrzną Genomed. Procent metylacji każdego regionu CpG obliczono mierząc stosunek pomiędzy wysokościami pików dla cytozyny (C) i tyminy (T). Równanie % metylacji cytozyny (mC) [55]:

$$\%mC = \frac{C}{C+T} * 100$$

8.2.9 Izolacja białek histonowych i określenie profilu H3

Białka histonowe izolowano przy użyciu zestawu EpiQuik™ Total Histone Extraction Kit (Epigentek) zgodnie z instrukcją producenta. Ocenę stężenia i czystości wyizolowanych białek histonowych określono na podstawie wartości OD (BSA użyto jako standard) za pomocą spektrofotometru Epoch (BioTek).

8.2.10 Analiza profilu białek histonowych H3

Wykorzystując metodę ELISA ocenione zostały dwie modyfikacje H3: permisywna - H3K3me3 i represyjna - H3K27me3, z wykorzystaniem zestawu EpiQuik™ Global Tri-Mehyl Histone H3-K4/H3-K27 Quantification Kit (Colorimetric, Epigentek). Zgodnie z wytycznymi producenta, na podstawie odczytanej absorbancji dokonano obliczeń ilości trimetylacji lizyny w pozycjach 27 i 4, wyrażając wyniki w jednostkach ng/µg białka.

8.2.11 Badanie cytometryczne poziomu białka Foxp3 i CTLA-4

Poziom białka Foxp3 i CTLA-4 określony został poprzez barwienie cytometryczne na urządzeniu LSRFortessa (BD Biosciences) z wykorzystaniem fluorescencyjnie znakowanych przeciwciał.

Barwienie zewnątrzkomórkowe

Frakcję CTLA-4 obecną na powierzchni komórki (surface CTLA-4, ang. S*urface* - *powierzchniowy*) oznaczono przy użyciu przeciwciała anty-CD152 (CF594, BD Biosciences). Barwienie przeciwciałami prowadzono w temperaturze 4°C przez 30 min. Następnie po płukaniu za pomocą PBS wykonywano odczyt.

Barwienie wewnątrzkomórkowe

W przypadku barwienia czynnika transakcyjnego Foxp3 i poziomu całkowitego CTLA-4 obecnego w komórce (total CTLA-4, ang. *Total* - *całkowity*), zastosowano procedurę polegającąna permeabilizacji błony komórkowej używając zestawu eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (eBioscences), która wykonywana była w 4°C przez 30 min. Po wypłukaniu odczynnika do permeabilizacji, komórki barwiono za pomocą przeciwciał anty-CD152 (CF594, BD Biosciences) oraz anty-Foxp3 (APC, Life Technologies) przez 30 min w 4°C. Następnie po płukaniu za pomocą PBS odczyt wykonywano przy użyciu cytometru LSRFortessa (BD Biosciences).

8.2.12 Analiza statystyczna

Wszystkie analizy statystyczne przeprowadzono z wykorzystaniem oprogramowania GraphPad Prims 10. Wyniki statystycznie istotne zostały oznaczone za pomocą * ($p<0.05$), ** ($p<0.01$), lub ***($p<0.001$). Normalność rozkładu danych sprawdzono przy użyciu testu Skapiro-Wilka. Dane o normalnym rozkładzie porównywano dzięki testowi t-Studenta, natomiast dane o charakterze nieparametrycznym analizowano z wykorzystaniem testu U-Manna-Whitney'a lub za pomocą rangowanych znaków (Test Wilcoxona). W celu określenia związku między dwoma zmiennymi wyznaczono współczynnik korelacji r-Pearsona.

9. WYNIKI I WNIOSKI

W skład mojej rozprawy doktorskiej wchodzą zarówno wyniki opublikowane, jak i nieopublikowane, tworząc kompleksowy zbiór badań zawierający profil fukncjonalny i molekularny produktu antygenowo-specyficznych komórek Tregs jako potencjalnego preparatu w leczeniu chorób autoimmunologicznych, na przykładzie cukrzycy typu 1 (Publikacja 1 oraz 2), jak również określający zmiany molekularne zachodzące w trakcie ekspansji komórek CD4+ pochodzących od osób z cukrzycą typu 1 w odniesieniu do osób zdrowych (badanie nieopublikowane). Rozprawa zawiera również przegląd literaturowy w powyższej tematyce (Publikacja 3).

9.1 PRACE OPUBLIKOWANE

9.1.1 PUBLIKACJA 1

Iwaszkiewicz-Grzes D*, Gliwinski M*, Eugster A, **Piotrowska M**, Dahl A, Marek-Trzonkowska N, Trzonkowski P. Antigen-reactive regulatory T cells can be expanded in vitro with monocytes and anti-CD28 and anti-CD154 antibodies. Cytotherapy. 2020 Nov;22(11):629-641.

Głównym celem eksperymentów zawartych w tej publikacji było opracowanie protokołu otrzymywania preparatu antygenowo-specyficznych komórek T regulatorowych do zastosowania u pacjentów z cukrzycą typu 1. Wygenerowane zostały dwa produkty: Tregs specyficzne względem insuliny albo względem peptydu 9-23 łańcucha β insuliny. Tak przygotowane preparaty komórkowe, poddawane były ekspansji, a następnie testom funkcjonalnym, w celu porównania ich z obecnie stosowanymi w terapiach chorób autoimmunologicznych komórkami poliklonalnymi. W trakcie opracowywania protokołu potwierdziliśmy, iż stosowanie przeciwciał: anty-CD28 i anty-CD154 umożliwiało lepszą proliferację komórek antygenowo-specyficznych, a także utrzymanie wysokiego poziomu czynnika transkrypcyjnego Foxp3.

Co ważne, wykazaliśmy, iż stymulacja Tregs za pomocą monocytów prezentujących antygeny umożliwia proliferację komórek reaktywnych względem tego antygenu i uzyskanie optymalnej ilości Tregs do terapii komórkowych. Pomimo tego, że produkt zawierał wiele klonów komórkowych, repertuar receptorów TCR preparatu zawierającego komórki antygenowo-specyficzne znacząco różni się od preparatu komórek poliklonalnych. Ponadto

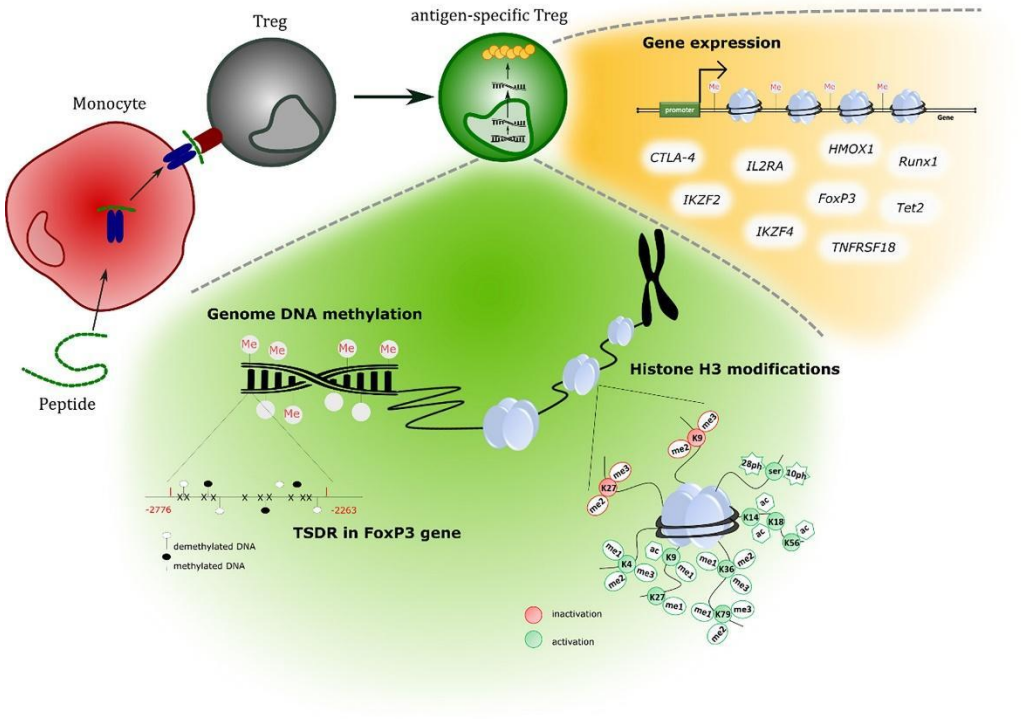
zaobserwowaliśmy, iż rodzaj zaprezentowanego antygenu ma wpływ na siłę proliferacji Tregs. Uszczegółowiając, prezentacja peptydu 9-23 łańcucha β insuliny warunkowała uzyskanie większej ilości reaktywnych Tregs, które znacznie lepiej radziły sobie w testach funkcjonalnych polegających na hamowaniu proliferacji i wydzielania INF-γ poprzez autologiczne komórki T efektorowe w porównaniu do komórek poliklonalnych.

Potencjał naszej terapii, nie ogranicza się jedynie do leczenia T1D. Dzięki zastosowaniu monocytów prezentujących antygeny, specyficzne względem innych chorób autoimmunologicznych, możliwe będzie poszerzenie wachlarza terapii celowanych z wykorzystaniem Tregs.

9.1.2 PUBLIKACJA 2

Iwaszkiewicz-Grzes D,* **Piotrowska M*,** Gliwinski M, Urban-Wójciuk Z, Trzonkowski P. Antigenic Challenge Influences Epigenetic Changes in Antigen-Specific T Regulatory Cells. Front Immunol. 2021 Mar 23;12:642678.

W związku z niepodważalną rolą mechanizmów epigenetycznych zaangażowanych w utrzymanie prawidłowej funkcji i fenotypu Tregs, w powyższym artykule określono profil epigenetyczny antygenowo-specyficznych Tregs, otrzymanych dzięki zastosowaniu pionierskiej metody wykorzystującej stymulację monocytami prezentującymi antygeny charakterystyczne dla cukrzycy typu 1. W pracy porównano ich profil molekularny w odniesieniu do obecnie stosowanych w terapiach komórek poliklonalnych. Ponadto doświadczenia przeprowadzono na populacji komórek T efektorowych, o przeciwnym działaniu do Tregs, co umożliwiło rozszerzenie wiedzy z zakresu różnic biologicznych tych populacji. W badaniu wykonano analizy metylacji DNA globalnie oraz w specyficznym regionie TSDR w genie *FOXP3*, a także przeanalizowano 21 modyfikacji histonu H3 oraz poziom ekspresji genów kluczowych dla Tregs (*CTLA4, IL2RA, IKZF2, IKZF4, FOXP3, TNFRSF18, RUNX1, HMOX1, TET2*) (Ryc. 8).



Ryc. 8. Zakres badań molekularnych przeprowadzonych na komórkach Ag-spec.

Uzyskane wyniki pozwoliły potwierdzić, iż w zależności od prezentowanego antygenu, profil epigenetyczny uzyskiwanych komórek ulega zmianom. Odkryliśmy charakterystyczny wzór, związany z rodzajem stymulacji, w poszczególnych parach Treg/Teff. Mianowicie, niski poziom modyfikacji histonu H3, w jednym podzbiorze z pary, zawsze wiązał się z wysokim poziomem modyfikacji w drugiej subpopulacji. A zatem ze względu na fakt, że ogólny profil epigenetyczny komórek T efektorowych zmienił się po procesie stymulacji z monocytami prezentującymi antygeny, podkreśliliśmy szczególną potrzebę kontrolowania jakości metod umożliwiających otrzymywanie Ag-spec Tregs do terapii komórkowych, tak aby uzyskany preparat był jak najbardziej bezpieczny dla pacjenta.

Co ważne, wszystkie wygenerowane Tregs posiadały znamiennie wyższy profil ekspresji genów zaangażowanych w ich funkcje w porównaniu do Teffs, za wyjątkiem *IKZF4 i TNFRSF18*, których wysoką lub umiarkowaną ekspresję obserwowano w Teffs specyficznych względem peptydu 9-23 łańcucha β insuliny oraz Teffs specyficznych względem insuliny. Ponadto uzyskane Tregs posiadały wysoki poziom demetylacji w TSDR (powyżej 75%), natomiast Teffs były praktycznie zmetylowane w tym regionie.

W przypadku analizy różnych kondycji Tregs, wyraźne różnice w profilu molekularnym były szczególnie widoczne w parze komórek specyficznych względem insuliny ($SPEC_{INS}$) i peptydu 9-23 łańcucha β insuliny ($SPEC_{B:9-23}$). Komórki Tregs $SPEC_{B:9-23}$ posiadały znacznie lepsze parametry pozwalające na utrzymanie stabilnego fenotypu oraz właściwości supresorowych podczas hodowli komórkowej. Było to związane z niższym poziomem metylacji całkowitego DNA oraz z wyższą demetylacją regionu TSDR (powyżej 90% w Tregs $SPEC_{B:9-23}$ do około 80% w Tregs $SPEC_{INS}$). Ponadto Tregs $SPEC_{B:9-23}$ posiadały wyższy poziom ekspresji genów kluczowych dla ich funkcjonalności w porównaniu do komórek specyficznych względem insuliny. Analizując PTMs w histonie H3, zauważyliśmy, że Tregs $SPEC_{B:9-23}$ charakteryzowały się wyższym poziomem wszystkich modyfikacji, z wyłączeniem H3K18ac, H3K9me1, H3K9me3 i H3K36me2.

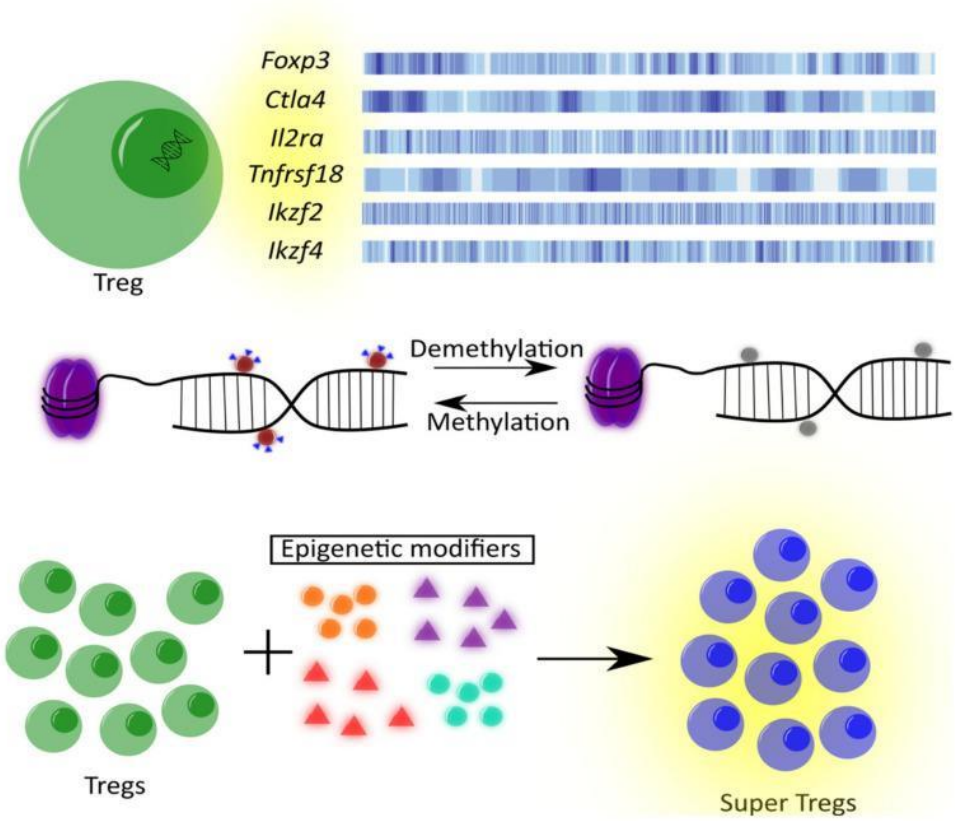
Podsumowując, wygenerowane przez as Ag-spec Tregs posiadały wszystkie cechy umożliwiające utrzymanie właściwości supresorowych, co potwierdza skuteczność opracowanego przez nas protokołu. Uważamy, iż stymulacja Tregs poprzez monocyty prezentujące fragment 9-23 łańcucha β insuliny wywiera korzystniejsze zmiany, poprzez zachowanie tzw. pamięci epigenetycznej.

9.1.2 PUBLIKACJA 3

Piotrowska M, Gliwiński M, Trzonkowski P, Iwaszkiewicz-Grzes D. Regulatory T Cells-Related Genes Are under DNA Methylation Influence. Int J Mol Sci. 2021 Jul 1;22(13):7144.

W publikacji tej przedstawiono aktualny stan wiedzy na temat wpływu metylacji DNA na funkcjonalność genów kluczowych dla limfocytów T regulatorowych (*FOXP3*, *CTLA4*, *IL2RA*, *TNFRSF18*, *IKZF2*, *IKZF4*). Określiliśmy wzór metylacji, który warunkuje utrzymanie stabilnego fenotypu komórek T regulatorowych w trakcie trwania hodowli komórkowej oraz przedstawiliśmy skutki ewentualnych zaburzeń na funkcjonalność konkretnych genów.

Ponadto, podkreśliliśmy relację zmian w profilu metylacji powyższych genów z poszczególnymi jednostkami chorób autoimmunologicznych. Ważnym elementem przeglądu było podkreślenie odwracalnego charakteru zmian epigenetycznych i usystematyzowanie obecnie dostępnych preparatów regulujących poziom metylacji DNA, które mogłyby zwiększyć potencjał terapii personalizowanych z wykorzystaniem Tregs (Ryc.9).



Ryc. 9 Geny omówione w publikacji i określenie wpływu zmian w profilu ich metylacji DNA na funkcje Tregs.

9.2 WYNIKI NIEPUBLIKOWANE

Wykorzystanie limfocytów T regulatorowych w terapiach komórkowych jest wciąż w fazie znacznego rozwoju. Ich właściwości supresorowe znalazły szerokie zastosowanie w wielu badaniach klinicznych, m.in. w transplantologii [56,57], jak również w leczeniu chorób autoimmunologicznych [58]. Udowodniono, że administracja Tregs jest bezpieczną procedurą, a terapia jest dobrze tolerowana i nie powoduje poważnych skutków ubocznych [57]. Badania potwierdziły jej skuteczność w zachowaniu funkcji komórek β trzustki, na co wskazuje wyższy poziom C-peptydu i mniejsze zapotrzebowanie na insulinę [59,60]. Niemniej jednak, pojedyncza dawka okazała się nie wystarczająca i pacjenci ze świeżo-zdiagnozowaną T1D ostatecznie rozwijają pełno objawową chorobę. Jednakże, osoby otrzymujące dwie dawki preparatu Tregs w skojarzeniu z rytuksymabem (przeciwciało anty-CD20), uzyskują lepsze wyniki kliniczne, o czym świadczy poprawa kontroli glikemii i lepsze wyniki testu sekrecji endogennej insuliny (MMTT, ang. *Mixed-meal tolerance test*) [54,61].

Należy podkreślić fakt, że przygotowywanie komórek do terapii związane jest długotrwałą ekspansją *in vitro,* w celu uzyskania odpowiedniej ich ilości. W związku z tym, wymagane jest stosowanie standaryzowanych protokołów, które umożliwiają zachowanie funkcji i fenotypu, aby ograniczyć ryzyko plastyczności Tregs w kierunku komórek o fenotypie Th17 – co może doprowadzić do zmniejszenia skuteczności terapii lub nawet zaostrzenia choroby podstawowej [62,63].

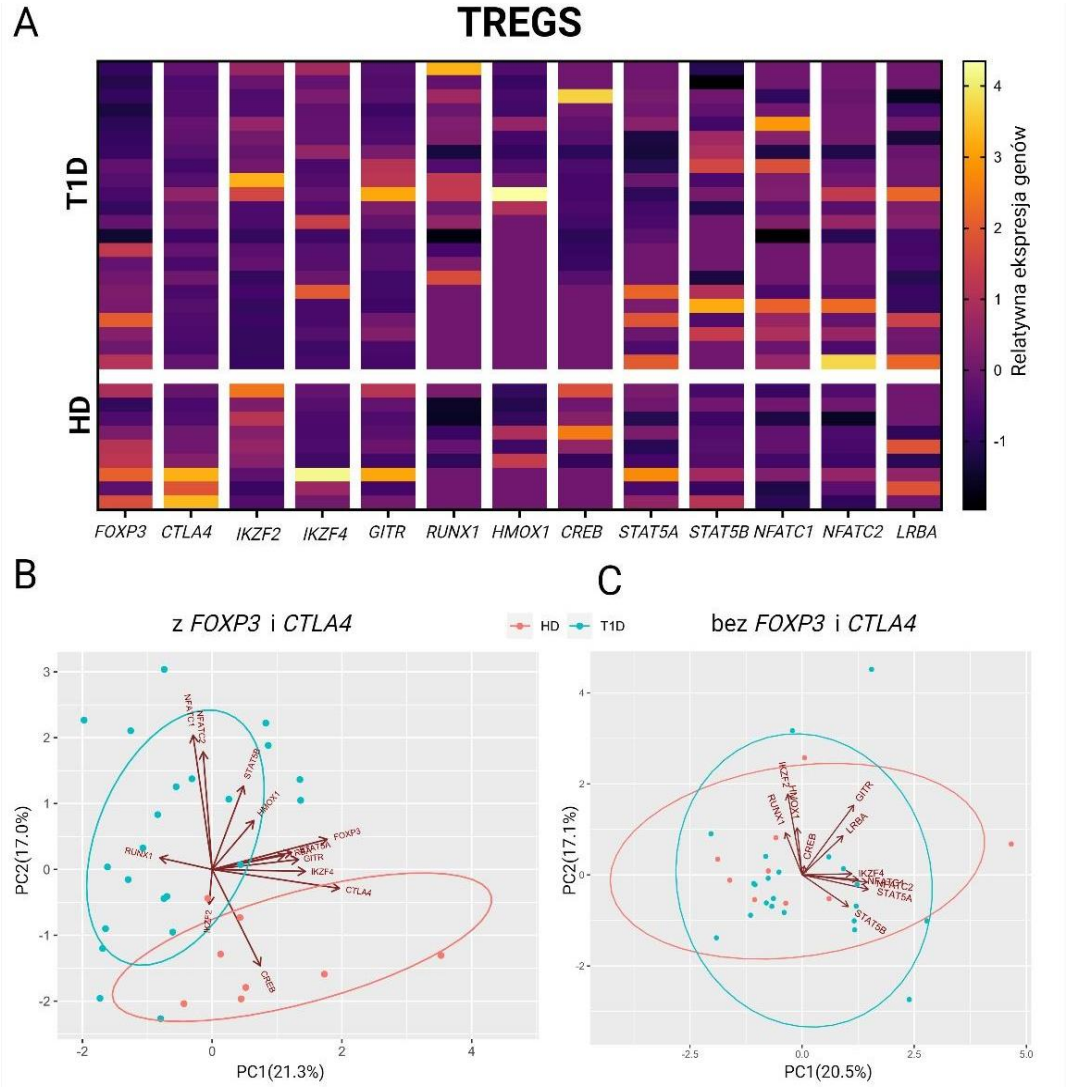
W badaniach przeprowadzonych na materiale pochodzącym od pacjentów z T1D, mających na celu określenie wpływu stymulacji *in vitro* limfocytów Tregs kulkami magnetycznymi opłaszczonymi przeciwciałami anty-CD3/CD28 wykazano, że ekspansja w obecności wysokiej dawki IL-2 wzmacnia ich funkcje supresorowe poprzez korygowanie odpowiedzi pSTAT5 oraz zwiększenie ekspresji CD25, LAP i CTLA-4 [57,64]. Należy natomiast pamiętać, iż nadmierna stymulacja TCR może prowadzić do rozregulowania w wyniku m.in. hipermetylacji regionów specyficznych dla Treg (Treg-DR), a także wiązać się z tzw. wyczerpaniem komórkowym [65,66].

Wobec powyższego, głównym celem przedstawionych poniżej badań było określenie stabilności Tregs pochodzących od pacjentów ze świeżo-zdiagnozowaną cukrzycą typu 1 w trakcie trwania ekspansji komórkowej i wykazanie ewentualnych różnic w ich profilach molekularnym w porównaniu do materiału pochodzącego od osób bez chorób autoimmunologicznych. Ponadto Tregs porównano z kontr-populacją CD4+ jaką są konwencjonalne komórki T efektorowe. Wszystkie limfocyty hodowano nie dłużej niż 12

dni, a główne analizy przeprowadzono w 7 i 12 dniu od stymulacji poliklonalnej (anty-CD3/anty-CD28).

9.2.1 Profil ekspresji genów w Tregs i Teffs.

W pierwszym etapie wykonaliśmy analizę ekspresji genów, wśród których uwzględniliśmy geny kluczowe, charakteryzujące stabilną populację Tregs (*FOXP3, CTLA4, IKZF2, IKZF4, TNFRSF18*) oraz geny pomocnicze, warunkujące ich prawidłowe funkcjonowanie (*CREB, RUNX1, HMOX1, LRBA, STAT5A, STAT5B, NFATC1, NFATC2*) (Ryc. 10A).

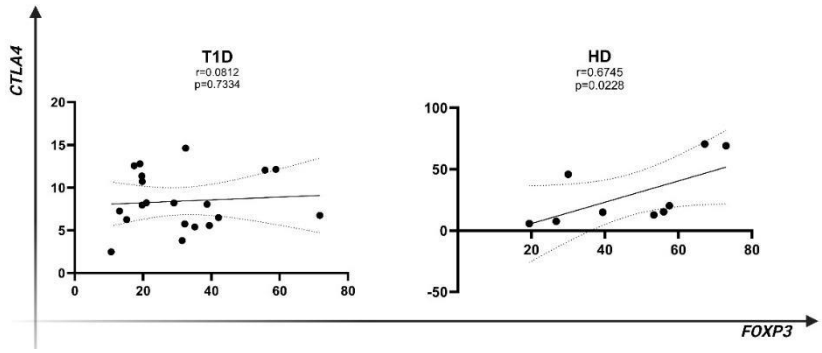


Ryc. 10 Profil ekspresji genów w limfocytach Tregs od pacjentów ze świeżo-zdiagnozowaną T1D, w porównaniu do zdrowych dawców (HD). A – Mapa ciepła przedstawiająca relatywną ekspresję genów (kolumna odpowiada konkretnemu genowi, natomiast wiersz zawiera dane pochodzące z analizy od jednego pacjenta). Surowe dane poddano transformacji logarytmicznej. **B –** Biplot przedstawiający analizę głównych składowych (PCA) wszystkich genów, zawierający pierwszą (PC1) i drugą składową (PC2). **C –** Analiza PCA wszystkich genów z pominięciem *FOXP3* i *CTLA4*. Odległość euklidesową pomiędzy populacjami Tregs wywodzącymi się z T1D oraz grupy zdrowych dawców oznaczono jako elipsę. Dane do analizy PCA poddano procesowi imputacji wielowymiarowej za pomocą równań łańcuchowych (MICE, ang. *Multivariate imputation by chained equations*) metodą predictive mean matching (PMM). T1D – pacjenci ze świeżo-zdiagnozowaną cukrzycą typu 1, HD – zdrowi dawcy (ang. *Healthy donors*).

Ocena genów charakterystycznych dla populacji Tregs wskazała, że największe różnice pomiędzy osobami z T1D, a zdrowymi dawcami (HD), występowały w *CTLA4* i *FOXP3*. Jednocześnie, osoby ze świeżo-zdiagnozowaną chorobą posiadały niższy poziom mRNA dla *FOXP3* i *CTLA4*. Ponadto zaobserwowano obniżenie transkryptu *IKZF2*, a niektóre geny, takie jak *RUNX1, NFATC1* i *NFATC2,* wykazywały wyższą ekspresję, w porównaniu do grupy kontrolnej (Ryc. 10A).

W dalszym kroku przeprowadzono analizę głównych składowych – PCA (ang. *Principal component analysis*). Wykazano, że Tregs pochodzące od pacjentów z T1D skupiały się w większej odległości od komórek wyizolowanych z grupy kontrolnej (Ryc. 10B). Analiza pokazała, iż na główną składową pierwszą (PC1) największy wpływ miała ekspresja *FOXP3*, *CTLA4* i *STAT5A*. Biorąc pod uwagę, że w grupie osób z T1D ekspresja *CTLA4* i *FOXP3* była niższa, w dalszym kroku wykonano analizę PCA z pominięciem tych dwóch genów i zauważono, że badane grupy pokrywały się ze sobą w znacznym stopniu (Ryc. 10C). Wobec powyższego wykazaliśmy, iż *FOXP3* i *CTLA4* determinują różnic w Tregs pomiędzy badanymi populacjami.

Co ciekawe, na podstawie współczynnika korelacji Pearsona, wykazaliśmy, że geny te korelowały dodatnio w grupie zdrowej, podczas gdy u pacjentów z T1D zależności tej nie zaobserwowaliśmy (Ryc. 11).

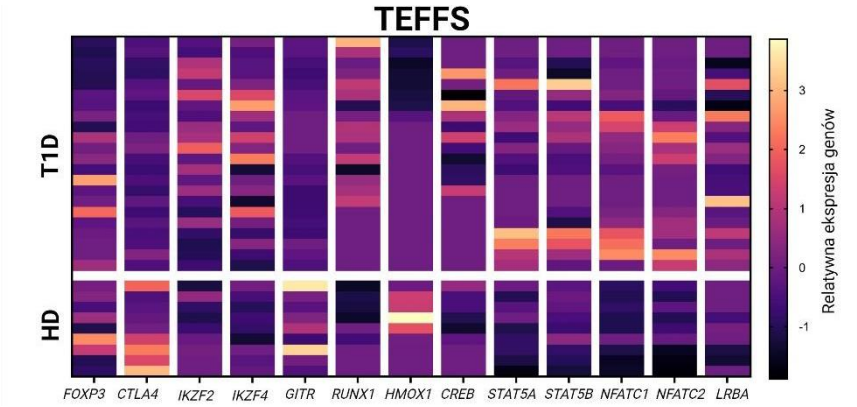


Ryc.11 Korelacja pomiędzy relatywną ekspresją genów *CTLA4* i *FOXP3* w Tregs pozyskanych od pacjentów z T1D i od zdrowych dawców krwi (HD).

W literaturze istnieje wiele rozbieżności na temat profilu ekspresji tych cząsteczek u pacjentów z T1D. W niektórych badaniach obserwowane były zmiany, natomiast w innych nie wykazano żadnych odchyleń [36]. Dzieje się tak głównie dlatego, że profil komórek niestymulowanych różni się od tego, jaki występuje po aktywacji anty-CD3/anty-CD28. Dlatego też, początkowo niewidoczne zmiany, można stopniowo zaobserwować w trakcie ekspansji komórkowej. Istnieje jednak szansa, że pierwotne zmiany zostaną zamaskowane poprzez powyższą stymulację. Jednym z przykładów jest zmniejszona ekspresja czynnika

transkrypcyjnego Helios w limfocytach Tregs poddanych ekspansji. Co ciekawe, pomimo niższej zawartości tego czynnika w preparacie, uzyskane komórki zachowywały swoją funkcjonalność [67]. Innym przykładem nieścisłości danych jest to, że nie wszystkie analizy genów przeprowadzane są na wyselekcjonowanej populacji Tregs. Niektóre badania opierają się na PBMC, inne na wzbogaconej populacji Tregs. Niemniej jednak, zdecydowana większość danych pokazuje, że u pacjentów z T1D dochodzi do obniżenia ekspresji powyższych genów [67–69]. W związku z tym, iż zaobserwowano obniżenie się poziomu ekspresji genów kluczowych dla Tregs w grupie pacjentów z T1D, postanowiliśmy sprawdzić, czy komórki te posiadają odmienny profil molekularny w stosunku do Teffs, które pełnią przeciwną funkcję biologiczną.

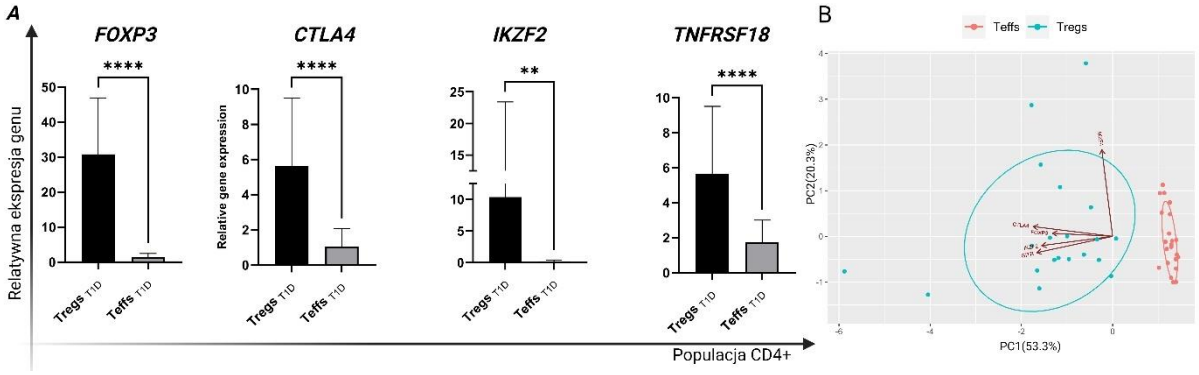
W pierwszym etapie Teffs poddano analizie ekspresji genów, które były analizowane początkowo w populacji Tregs i wykazaliśmy, że w przypadku Teffs heterogeniczność między badanymi grupami wynikała z obniżenia poziomu *CTLA4*, *TNFRSF18* i *HMOX1* oraz zwiększenia poziomu *RUNX1*, *STAT5A* i *NFATC1/C2* w grupie T1D (Ryc. 12).



Ryc. 12 Profil ekspresji genów w limfocytach Teffs od pacjentów ze świeżo-zdiagnozowaną T1D, w porównaniu do zdrowych dawców (HD). Mapa ciepła przedstawia relatywną ekspresję genów (kolumna odpowiada konkretnemu genowi, natomiast wiersz zawiera dane pochodzące z analizy jednego pacjenta). Surowe dane poddano transformacji logarytmicznej. T1D – pacjenci ze świeżo-zdiagnozowaną cukrzycą typu 1, HD – zdrowi dawcy (ang. *Healthy donors*).

A zatem zarówno w Tregs, jak i Teffs pochodzących od pacjentów z T1D zaobserwowano obniżenie poziomu *CTLA4* oraz jednoczesny wzrost *RUNX1*, *NFATC1/C2*.

W kolejnym kroku porównano ekspresję genów warunkujących właściwości supresorowe Tregs i wykazano, iż Tregs pochodzące od pacjentów z T1D posiadają znamiennie wyższy poziom mRNA tych genów w porównaniu do Teffs (Ryc. 13A), co zobrazowane jest w analizie PCA, w której obserwowaliśmy wyraźne odseparowanie grup (Ryc. 13B).

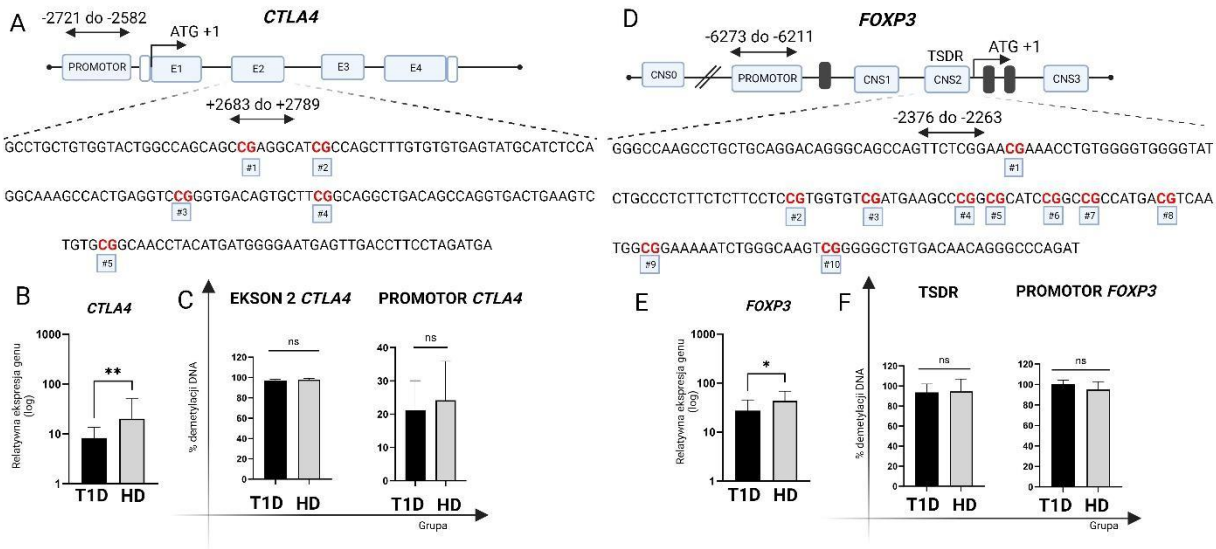


Ryc. 13 Profil ekspresji genów w limfocytach Tregs i Teffs, pochodzących od pacjentów z T1D, poddanych poliklonlnej stymulacji. A – Analiza genów charakterystycznych dla stabilnej populacji limfocytów Tregs (*FOXP3, CTLA4, IKZF2, TNFRSF18*). Wyniki przedstawiono jako medianę ± SD. Istotność statystyczną oznaczono jako * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$). **B** – Analiza głównych składowych (PCA), zawierająca ocenę genów charakterystycznych dla Tregs (*FOXP3, CTLA4, IKZF2, TNFRSF18*) przedstawiająca pierwszą (PC1) i drugą składową (PC2). Odległość euklidesową pomiędzy populacjami Tregs i Teffs wywodzącymi się z grupy T1D oznaczono jako elipsę.

9.2.2 Metylacja DNA w genach FOXP3 i CTLA4

W dalszych badaniach sprawdziliśmy, czy obniżona ekspresja *FOXP3* i *CTLA4* u pacjentów z T1D jest związana ze zmianą wzorca metylacji DNA. Przeanalizowano fragmenty promotorów powyższych genów oraz regiony, które są wysoce demetylowane w przypadku populacji Tregs, tzw. Treg-DRs. Figura 14A przedstawia schematyczne fragmenty eksonu 2 genu *CTLA4*, z zaznaczeniem dinukleotydów CG w obrębie wysp CpG, które analizowano metodą qMSP. W dostępnej literaturze przedstawione są dane na temat niższej ekspresji *CTLA4* u pacjentów z chorobami autoimmunologicznymi, które związane są ze zmianami w profilu metylacji DNA. Jednym z przykładów jest zwiększenie metylacji promotora *CTLA4* u pacjentów z reumatoidalnym zapaleniem stawów, które w konsekwencji doprowadza to obniżenia poziomu CTLA-4 i negatywnie wpływa na supresorowe funkcje limfocytów T regulatorowych [14,70]. W naszych doświadczeniach zaobserwowaliśmy niższy, chociaż nieistotnie statystyczny, poziom demetylacji w genie *CTLA4* w grupie T1D, szczególnie w regionie promotorowym (Ryc. 14C).

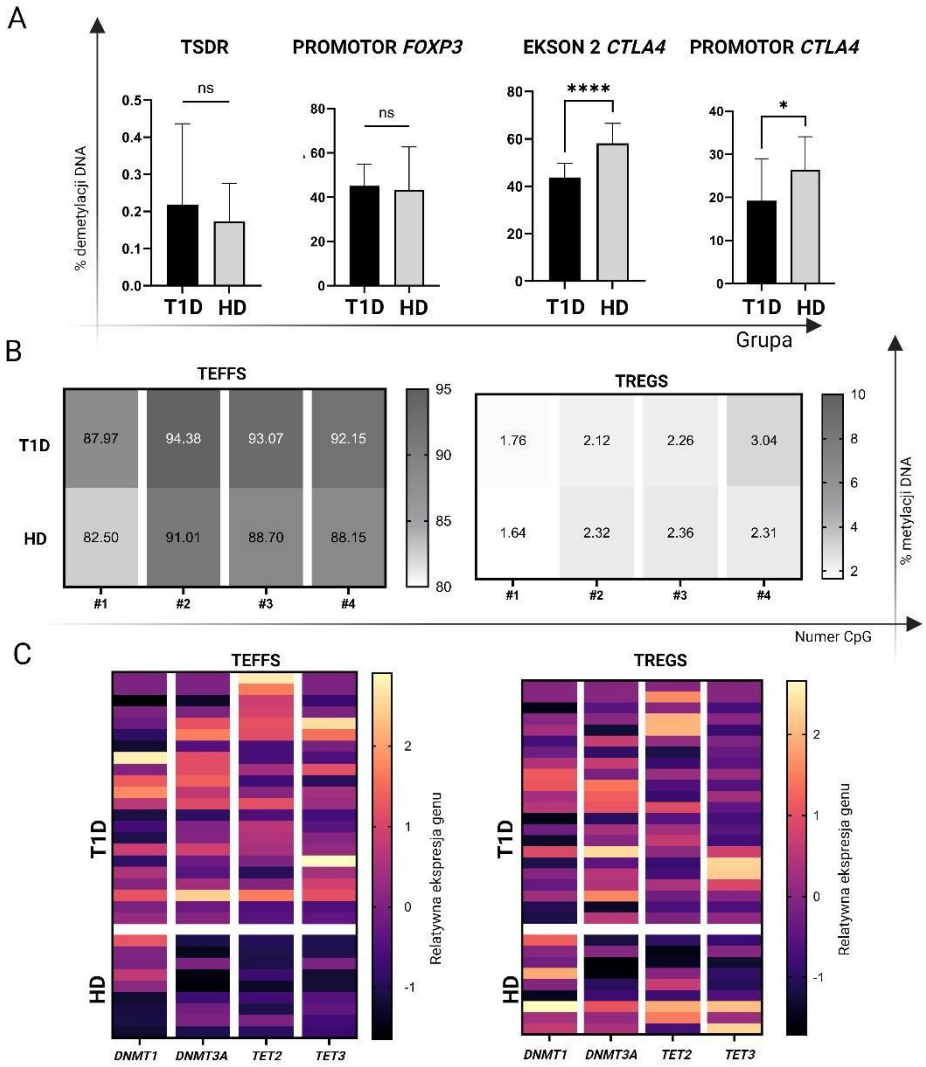
Odpowiedni schemat doświadczeń wykonano w genie *FOXP3*. Figura 14D przedstawia poszczególne motywy CpG w obrębie regionu TSDR w genie *FOXP3*. W naszym badaniu nie stwierdziliśmy istotnych statystycznie zmian w profilu metylacji *FOXP3* pomiędzy T1D, a grupą kontrolną, w związku czym nie mogliśmy powiązać zmniejszonej ekspresji z wyższą metylacją DNA (Ryc. 14 E i F).



Ryc.14 Ekspresja genów *FOXP3* i *CTLA4*, a metylacja DNA w regionach promotorowych i Tregs-DRs (ang. *Specific demethylated regions*). A – Schematyczne przedstawienie genu *CTLA4* zawierające szczegółową sekwencję nukleotydów w eksonie 2, z podkreśleniem konkretnych CpG, które analizowane były dzięki qMSP. Dwukierunkowe strzałki określają fragmenty promotora i eksonu 2 w odniesieniu do miejsca startu translacji (ATG), w których określano poziom demetylacji. **B –** Relatywna ekspresja *CTLA4* w Tregs pozyskanych od osób z T1D i HD. **C –** % demetylacji regionu promotora i eksonu 2 w genie *CTLA4*. **D –** Schematyczne przedstawienie genu *FOXP3* zawierające szczegółową sekwencję nukleotydów w regionie TSDR, z podkreśleniem konkretnych CpG, które analizowane były dzięki qMSP. Dwukierunkowe strzałki określają fragmenty promotora i TSDR w odniesieniu do miejsca startu translacji (ATG), w których określano poziom demetylacji. **E –** Relatywna ekspresja *FOXP3* w Tregs pozyskanych od osób z T1D i od zdrowych dawców krwi (HD). **F –** % demetylacji regionu promotora i TSDR w genie *FOXP3*. Wyniki przedstawione są jako mediana ± SD. Istotność statystyczną oznaczono jako * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), ns – nieistotne statystycznie.

W kontekście naszych wyników, Anne M. Pesenckler i in. wykazali, że u świeżo-zdiagnozowanych pacjentów z T1D obserwuje się podobną demetylację w regionie TSDR w porównaniu do grupy kontrolnej. Jednocześnie stwierdzono u nich obniżony poziom mRNA *FOXP3* [69]. Tymczasem, w badaniu przeprowadzonym przez Yijun Li i in., u pacjentów z późno ujawniającą się cukrzycą typu 1 (LADA, ang. *Latent autoimmune diabetes in adults*) zaobserwowano obniżony poziom *FOXP3*, który był powiązany z wyższą metylacją promotora *FOXP3* [71].

Profil metylacji DNA został określony również w komórkach Teffs. Zgodnie z oczekiwaniami, nie wykazano różnic w przypadku metylacji DNA w regionie *FOXP3*. Z kolei obniżony poziom mRNA *CTLA4* wiązał się ze wzrostem metylacji DNA w promotorze *CTLA4* (Ryc. 15A), co jest zgodne z innymi badaniami, w których wykazano wzrost metylacji tego regionu u pacjentów z T1D [72]. Dodatkowo, w Teffs zaobserwowaliśmy hipermetylację eksonu 2 w genie *CTLA4*. Dlatego też, przeprowadzona została również szczegółowa analiza profilu metylacji DNA w eksonie 2 z wykorzystaniem sekwencjonowania DNA po reakcji z wodorosiarczynem (BS-seq, ang. *Bisulfite sequencing*) (Ryc. 15B).



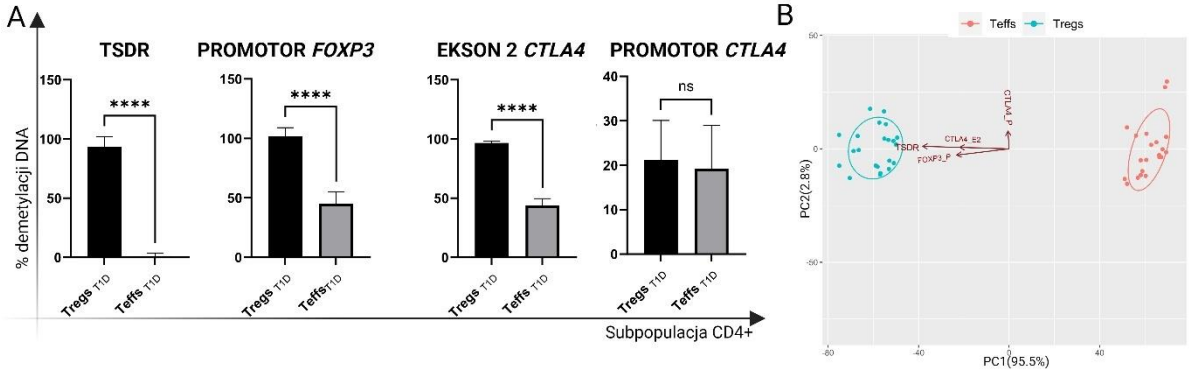
Ryc.15 Teffs pozyskane od pacjentów z T1D posiadają odmienny profil metylacji w porównaniu do Teffs od zdrowych dawców. A – % demetylacji regionu TSDR w genie *FOXP3* i eksonu 2 w genie *CTLA4* oraz analiza demetylacji fragmentów promotorów *CTLA4* i *FOXP3* w Teffs. **B** – Wyniki BS-seq przedstawiające % metylacji konkretnych CpG (określonych zgodnie z numeracją przedstawioną na Rycinie 14A jako # - numer CpG) w eksonie 2 w genie *CTLA4*. Dane pokazane są dla populacji Tregs i Teffs pochodzących od pacjentów z T1D i zdrowych dawców (HD). **C** – Mapa ciepła przedstawiająca relatywną ekspresję genów związanych z regulacją poziomu metylacji DNA w Tregs i Teffs badanych grup (kolumna odpowiada genowi, natomiast wiersz zawiera dane pochodzące od z analizy jednego pacjenta). Surowe dane poddano procesowi transformacji logarytmicznej. Wyniki przedstawione są jako mediana ± SD. Istotność statystyczną oznaczono jako * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), ns – nieistotne statystycznie.

Podczas analizy Teffs, zaobserwowaliśmy, że wszystkie cztery regiony CpG posiadały znamiennie wyższy poziom metylacji u osób ze świeżo-zdiagnozowaną T1D. Natomiast w przypadku komórek Tregs pacjenci z T1D mieli nieco wyższy, chociaż statystycznie istotny poziom metylacji w regionie oznaczonym jako #4 CpG (+2755 od ATG) (Ryc. 15B).

W związku z powyższym, postanowiliśmy określić poziom metylotransferaz DNA i enzymów z rodziny Tet, zaangażowanych w regulację metylacji cytozyny w dinukleotydach CpG. Analiza ekspresji pokazała, że największe różnice pomiędzy badanymi grupami wystąpiły w komórkach Teffs, które charakteryzowały się wyższą ekspresją *DNMT3A*, *TET2*

oraz *TET3* w stosunku do grupy zdrowej (Ryc. 15C). W przypadku Tregs, nie wykazaliśmy statystycznie istotnych różnic pomiędzy osobami z grupy T1D i HD.

Finalnie, porównaliśmy wzór metylacji pomiędzy limfocytami Tregs i Teffs pochodzącymi od pacjentów z T1D. Wykazaliśmy, iż populacje tych komórek różnią się znamiennie pod kątem demetylacji w Treg-DRs (TSDR i ekson 2 w genie *CTLA4*), a Tregs charakteryzują się prawie całkowitą demetylacją w tych regionach (Ryc. 16A).



Ryc. 16 Porównanie profilu metylacji DNA Tregs i Teffs pozyskanych od pacjentów z T1D. **A** – % demetylacji regionów związanych z *FOXP3* (TSDR i promotor) oraz *CTLA4* (ekson 2 i promotor) w Tregs i Teffs osób z T1D. **B** – Analiza głównych składowych (PCA), zawierająca ocenę profilu demetylacji Tregs i Teffs przedstawiająca pierwszą (PC1) i drugą składową (PC2). Odległość euklidesową pomiędzy populacjami Tregs i Teffs wywodzącymi się z grupy T1D oznaczono jako elipsę. Wyniki przedstawione są jako mediana ± SD. Istotność statystyczną oznaczono jako * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), ns – nieistotne statystycznie.

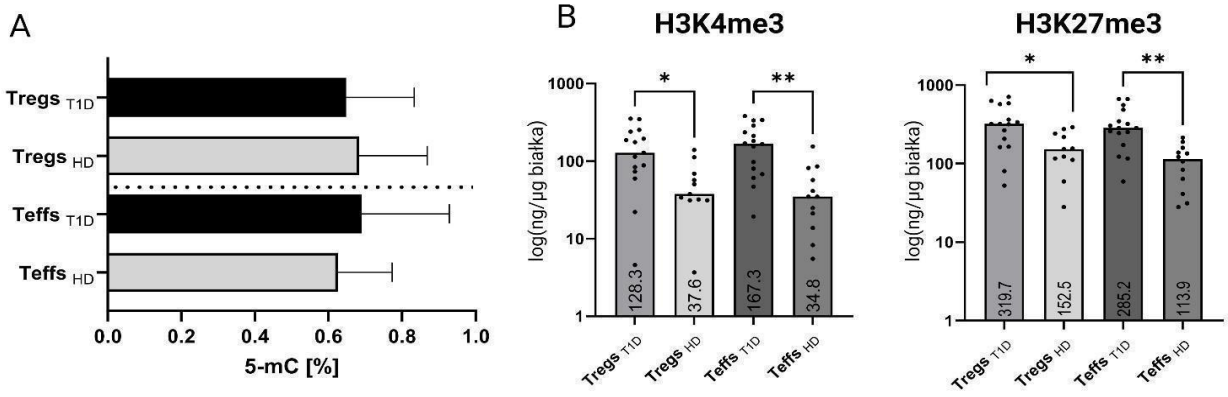
Jest to szczególnie ważne, gdyż udowodniono, że Tregs hodowane w warunkach nadmiernej stymulacji mogą tracić ekspresję markerów liniowych z powodu wzrostu metylacji w kluczowych regionach, co może negatywnie wpłynąć na skuteczność terapii [66]. Powyższe obserwacje potwierdzają również skuteczność stosowanego przez nas protokołu do izolacji Tregs, który umożliwia otrzymanie preparatu o czystości ≥98%. Jest to istotne, ponieważ badania pokazują, że niewystarczająca jakość sortowania Tregs może mieć wpływ na stabilność i późniejszą jakość preparatu komórkowego [73,74]. W przypadku demetylacji promotora genu *FOXP3*, również obserwowaliśmy znaczną demetylację w komórkach Tregs, natomiast poziom metylacji promotora *CTLA4* nie różnił się pomiędzy badanymi populacjami, co znajduje też potwierdzenie w dostępnej literaturze [70]. Przeprowadzona analiza PCA, potwierdziła całkowity rozdział Tregs od Teffs na podstawie profilu metylacji DNA (Ryc. 16 B).

9.2.3 Całkowita metylacja DNA i modyfikacje histonu H3

Zmiany epigenetyczne zostały również określone na poziomie globalnym. Oceniono całkowitą zawartość 5-metylocytozyny oraz poziom trimetylacji histonu H3 w lizynie 4-tej

(H3K4me3) i lizynie 27-mej (H3K27me3). Nie znaleźliśmy istotnych różnic w globalnej metylacji DNA (Ryc. 17A). Analizując modyfikacje histonu związane z represją (H3K27me3) i aktywacją (H3K4me3) transkrypcji, ujawniliśmy, że ich poziomy w Tregs i Teffs od pacjentów z T1D były znacznie wyższe w porównaniu do grupy kontrolnej (Ryc. 17B).

W dostępnej literaturze pojawiają się informacje na temat zmian w profilu modyfikacji histonu H3 u osób z T1D. A mianowicie regiony genów regulujących Tregs miały inny wzór modyfikacji histonów w porównaniu z osobami wolnymi od chorób autoimmunologicznych. Przykładem jest wzrost represyjnej modyfikacji H3K9me2 w promotorze *CTLA4* u osób z T1D [75,76]. A zatem w przypadku naszego badania obniżony poziom mRNA *FOXP3* i *CTLA4* w Tregs może być wynikiem zmian w profilu modyfikacji histonu H3, niemniej jednak potrzebne są dalsze analizy w celu potwierdzenia tej hipotezy.



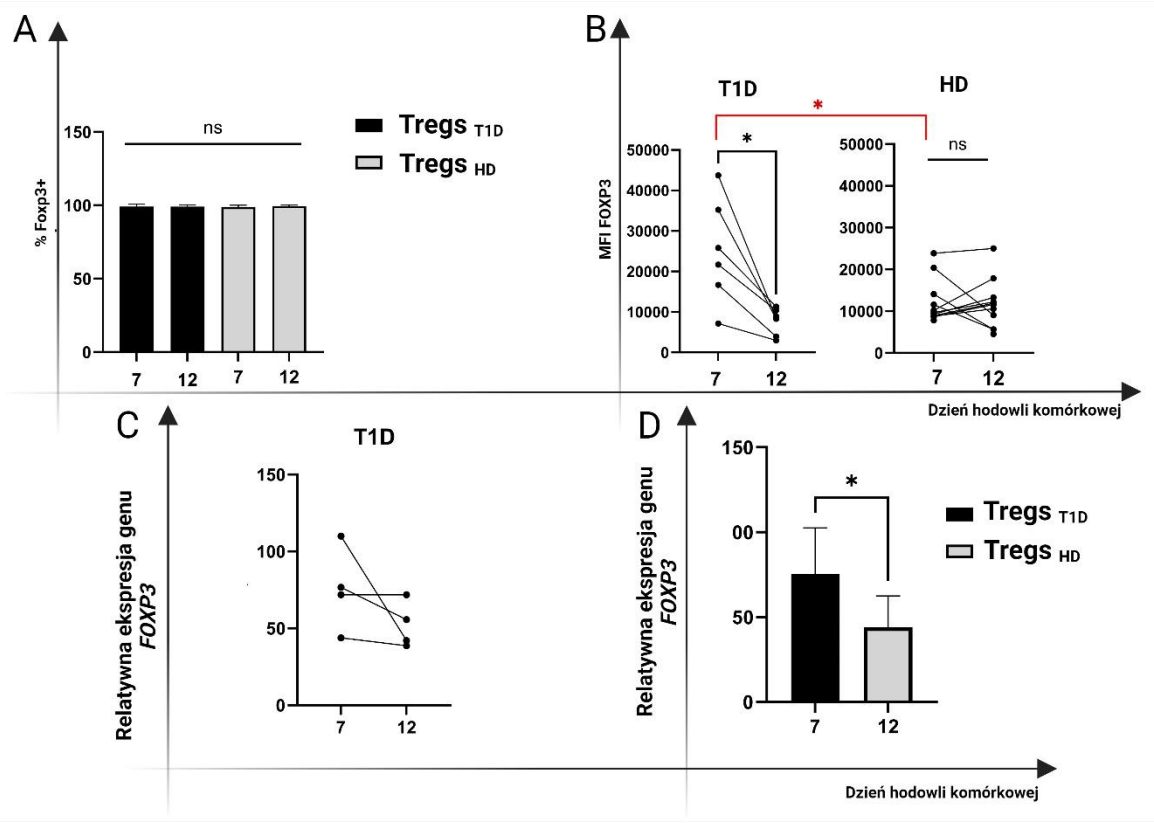
Ryc. 17 Poziom (%) całkowitej metylacji DNA w Tregs i Teffs (A) oraz ocena ilości (ng/µg białka) modyfikacji histonu H3: H3K4me3 (aktywująca) oraz H3K27me3 (hamująca) (B) w komórkach Tregs i Teffs w badanych grupach. Wyniki przedstawione są jako mediana ± SD. Istotność statystyczną oznaczono jako * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), ns – dane nieistotne statystycznie. T1D – pacjenci ze świeżo-zdiagnozowaną cukrzycą typu 1, HD – zdrowi dawcy (ang. *Healthy donors*).

9.2.4 Foxp3 i CTLA-4 – poziom białka

Biorąc pod uwagę wykazane różnice na poziomie molekularnym w komórkach pochodzących od pacjentów z T1D, sprawdziliśmy, czy mają one wpływ na regulację poziomu białka Foxp3 i CTLA-4. Dodatkowo postanowiliśmy określić dynamikę ewentualnych zmian w trakcie trwania ekspansji komórkowej. Dlatego też pobraliśmy materiał do badań w siódmym i dwunastym dniu hodowli komórkowej.

Pomimo, iż ogólny odsetek komórek Foxp3+ nie różnił się pomiędzy grupami, średnia intensywność fluorescencji (MFI, ang. *Mean fluorescence intensity*) znacznie obniżyła się w trakcie hodowli Tregs od pacjentów z T1D (Ryc. 18 A i B). Jest to zgodne z innymi badaniami, w których różnice pomiędzy pacjentami z T1D, a grupą kontrolną były najbardziej

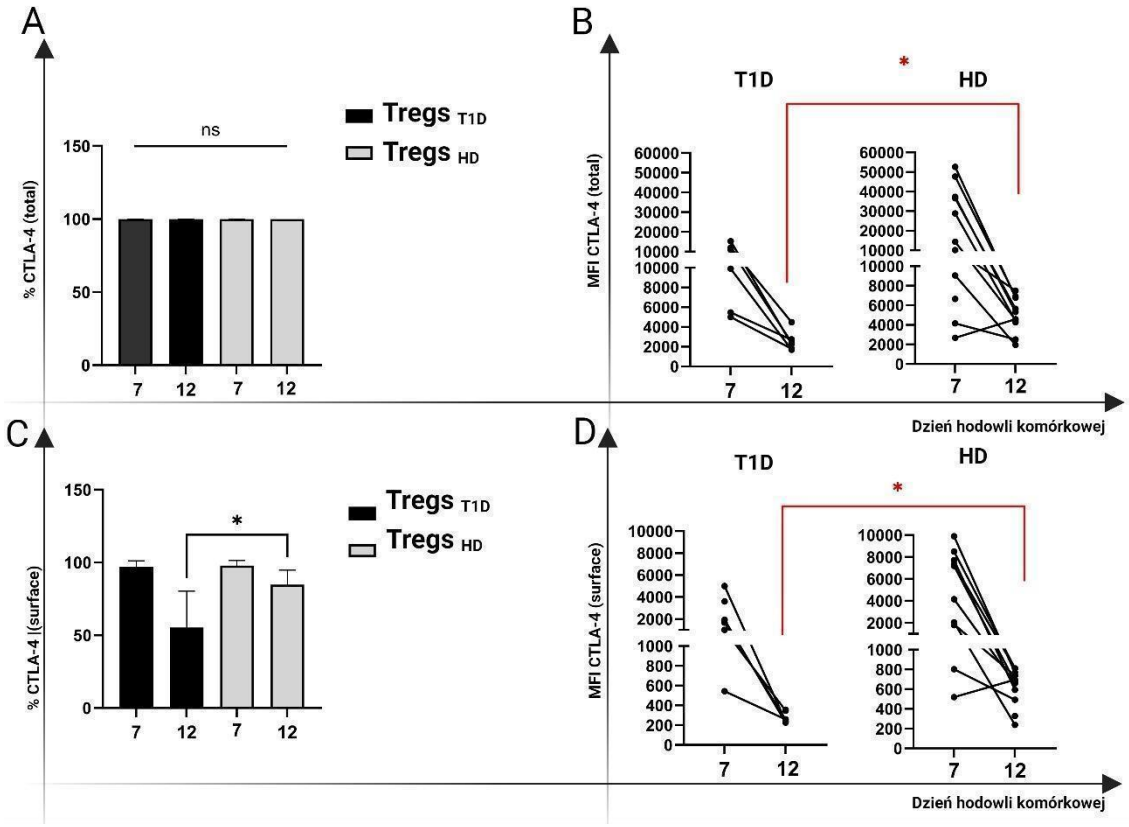
widoczne w analizie MFI [77,78]. Interesujący może być fakt, że siódmego dnia, Tregs z grupy T1D posiadały wyższy poziom Foxp3 (MFI) w porównaniu do osób bez obciążeń autoimmunologicznych. Obserwacja ta skłoniła nas do porównania ilości mRNA *FOXP3* w siódmym i dwunastym dniu hodowli komórkowej, co pokazało znaczy wzrost ekspresji *FOXP3* w siódmym dniu hodowli, która była 1.5 razy wyższa od poziomu obserwowanego w grupie zdrowej dnia dwunastego (Ryc. 18 C i D). Jednakże, należy podkreślić, że w trakcie trwania hodowli ekspresja Foxp3 znacznie spadła w grupie pacjentów z T1D.



Ryc. 18 Poziom Foxp3 w limfocytach T regulatorowych 7-go i 12-go dnia hodowli komórkowej. **A** – % Foxp3+ w Tregs pozyskanych od pacjentów z T1D i od zdrowych dawców (HD) **B** – Porównanie wartości MFI dla Foxp3. **C –** Relatywna ekspresja genu Foxp3 w Tregs pobranych od osób z T1D w trakcie trwania hodowli komórkowej. **D –** Porównanie relatywnej ekspresji genu *FOXP3* w Tregs pomiędzy pacjentami z T1D i zdrowymi dawcami krwi (HD). Wyniki przedstawione są jako mediana ± SD. Istotność statystyczną oznaczono jako * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), ns –nieistotne statystycznie.

CTLA-4 jest cząsteczką, której powierzchniowa ekspresja ogranicza się do komórek aktywowanych, a większość CD152 znajduje się w strukturach wewnątrzkomórkowych. Wyjątek stanowią limfocyty Tregs, które posiadają konstytutywną ekspresję tego antygenu na powierzchni błony komórkowej. Niemniej jednak, transport CTLA-4 w komórkach jest procesem dynamicznym, podczas którego CTLA-4 podlega procesom internalizacji,

lizosomalnej sekrecji albo degradacji [79,80]. Wobec tego, zmierzyliśmy poziom CTLA-4 na powierzchni limfocytów T oraz określiliśmy całkowitą ilość CD152 w komórce.



Ryc. 19 Poziom CTLA-4 w limfocytach T regulatorowych 7-go i 12-go dnia hodowli komórkowej. A i B – analiza całej puli CTLA-4 w Tregs (określona na wykresie jako total – ang. *Total*-całkowity). Wyniki zostały przedstawione jako % CTLA-4 pozytywnych komórek (**A**) oraz MFI CTLA-4 (**B**). **C i D** – Ocena ekspresji CTLA-4 wyłącznie na powierzchni błony komórkowej (oznaczone na wykresach jako *surface* – ang. *Surface*-powierzchnia). Dane zostały przedstawione jako % CTLA-4 pozytywnych komórek (**C**) oraz MFI CTLA-4 (**D**). Wyniki przedstawione są jako mediana ± SD. Istotność statystyczną oznaczono jako * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), ns –nieistotne statystycznie. T1D – pacjenci ze świeżo-zdiagnozowaną cukrzycą typu 1, HD – zdrowi dawcy (ang. *Healthy donors*).

Analizując poziom całkowitego CTLA-4, nie stwierdziliśmy różnic w ilości komórek pozytywnych względem CD152, jednakże, porównując MFI zaobserwowaliśmy zmniejszoną ekspresję cząsteczki u pacjentów z T1D (Ryc. 19 A). Niższy poziom MFI dla CTLA-4 był obserwowany już siódmego dnia hodowli, jednak różnice te nie były istotne statystycznie. Niemniej jednak, analizy przeprowadzone dnia dwunastego hodowli pokazały znaczne obniżenie MFI dla CTLA-4 w grupie pacjentów z T1D (Ryc. 19 B). Co ciekawe, w przypadku analizy CTLA-4 wyłącznie na powierzchni komórki, zarówno odsetek komórek pozytywnych, jak i MFI, były znacznie niższe u pacjentów z T1D. Jednocześnie, Tregs z grupy T1D miały tendencję do zmniejszonej ekspresji CTLA-4 na powierzchni komórki w trakcie hodowli

komórkowej, podczas gdy Tregs od zdrowych dawców wykazywały bardziej stabilną ekspresję (Figura 19 C i D).

Zgodnie z oczekiwaniami, ekspresja i dystrybucja CTLA-4, były znacząco niższe w Teffs w porównaniu z komórkami Tregs (Tabela 8).

Tabela 8. Analiza cytometryczna CTLA-4 całkowitego (ang. *Total*) oraz na powierzchni (ang. *Surface*) w Tregs i Teffs od pacjentów z T1D.

	% CTLA-4 (surface)	MFI CTLA-4 (surface)	% CTLA-4^{hi} (total)	MFI CTLA-4 (total)	Dzień hodowli
TREGS	98.59	1801	99.50	10463	7
TEFFS	27.59	177	21.41	778	
TREGS	58.08	258	86.83	2423	12
TEFFS	6.38	69	9.02	460	

W nawiązaniu do uzyskanych przez nas wyników, inne grupy badawcze wykazały niższą powierzchniową ekspresję CD152 u pacjentów ze stwardnieniem rozsianym, miastenią i reumatoidalnym zapaleniem stawów [70,81,82]. A zatem wykrywanie frakcji powierzchniowej może być bardziej informatywne, aniżeli analiza całkowitego CTLA-4, w przypadku pacjentów z zaburzeniami autoimmunologicznymi.

Pomimo wykazanych różnic na poziomie molekularnym w Teffs pozyskanych od pacjentów z T1D, analizy cytometryczne nie pokazały różnic na poziomie białka, co wskazuje na obecność innych mechanizmów zaangażowanych w ekspresję CTLA-4, które muszą być zbadane w przyszłości.

10. PODSUMOWANIE

Badania zrealizowane w ramach niniejszej rozprawy doktorskiej przyczyniły się do określenia funkcjonalności i profilu molekularnego innowacyjnego preparatu antygenowo-specyficznych komórek Tregs. Dzięki przeprowadzonym eksperymentom potwierdziliśmy stabilność fenotypową preparatu Tregs, a także wykazaliśmy, że otrzymane Ag-spec Tregs mają właściwości supresorowe i preferencyjnie hamują autoreaktywne klony komórek T efektorowych. Co istotne, zauważyliśmy, iż rodzaj prezentowanego antygenu ma wpływ na jakość uzyskiwanego preparatu komórek Ag-spec Tregs, a w konsekwencji na skuteczność terapii z wykorzystaniem tych komórek.

Co ważne, wykazaliśmy, że profil molekularny zarówno Tregs, jak i Teffs podlega zmianom uwarunkowanym stymulacją monocytami prezentującymi antygeny typowe dla T1D. Wskazuje to na istotność procesu sortowania subpopulacji CD4+, który powinien umożliwiać otrzymanie Tregs o jak najwyższym poziomie czystości.

Wyniki pokazały, że powyższa stymulacja generuje różnice w profilu metylacji DNA oraz w poziomie modyfikacji histonu H3, co z kolei może mieć wpływ na ekspresję genów zaangażowanych w regulację Tregs. Co ważne, wybór antygenu do stymulacji Tregs ma duże znaczenie, ponieważ wpływa on na kierunek zachodzących zmian. Dzięki tym obserwacjom wytypowaliśmy preferencyjny antygen do zastosowania w protokole generowania Ag-spec Tregs do terapii T1D, jakim był peptyd 9-23 łańcucha β insuliny. W odróżnieniu od insuliny, peptyd 9-23 łańcucha β insuliny umożliwiał otrzymanie Tregs o stabilnym profilu epigenetycznym, charakterystycznym dla limfocytów Tregs oraz warunkował zachowanie wysokiej ekspresji genów regulujących ich funkcje supresorowe.

W związku z powyższym uważamy, że badanie statusu epigenetycznego może usprawnić proces poszukiwania peptydów o najwyższym potencjale terapeutycznym do zastosowania w leczeniu chorób autoimmunologicznych.

Dzięki analizie profilu epigenetycznego i panelu ekspresji genów komórek pochodzących od pacjentów ze świeżo-zdiagnozowaną cukrzycą typu 1 uzyskaliśmy dane, które wskazują, iż w trakcie hodowli komórkowej, limfocyty pochodzące od pacjentów z T1D są mniej stabilne, szczególnie w obrębie parametrów dotyczących cząsteczek Foxp3 i CTLA-4, które z kolei mogą być następstwem rozbieżności jakie występowały we wzorze metylacji DNA i w profilu modyfikacji histonu H3 u osób z T1D. Niemniej jednak, należy pokreślić, że profil Tregs pochodzących od pacjentów z T1D znamiennie różnił się od wzorca jaki obserwowany był w Teffs. Podkreśla to znamiennie wyższa ekspresja genów kluczowych dla Tregs (*FOXP3,*

CTLA4, IKZF2, TNFRSF18), a także różnice w profilu metylacji DNA pomiędzy tymi subpopulacjami komórkowymi. A zatem określone przez nas zmiany nie powodują nabycia przez Tregs cech charakterystycznych dla kontr-populacji, jaką są Teffs.

Analizy na poziomie białka pokazały, że w przypadku Foxp3, Tregs z grupy T1D, w pierwszych dniach po ekspansji produkują znaczne ilości czynnika transkrypcyjnego, nawet na wyższym poziomie aniżeli ten obserwowany u osób zdrowych. Co istotne, w trakcie trwania hodowli stopień ekspresji *FOXP3* obniża się, co potwierdza jego niestabilną produkcję przez komórki pochodzące od pacjentów z T1D. Dzięki analizie CTLA-4, przeprowadzonej osobno dla frakcji zewnątrzkomórkowej i całkowitej, wskazaliśmy, że w limfocytach T regulatorowych od pacjentów z T1D, w trakcie poliklonalnej stymulacji dochodzi do obniżenia ekspresji cząsteczki CTLA-4, co szczególnie było widoczne w przypadku puli CTLA-4 transportowanego na zewnątrz błony komórkowej. Wobec tego, badanie frakcji zewnątrzkomórkowej może nieść ze sobą więcej informacji w przypadku oceny układu odpornościowego u osób z chorobami autoimmunologicznymi.

Ze względu na stały wzrost częstości występowania oraz heterogeniczność chorób autoimmunologicznych, istnieje potrzeba opracowywania innowacyjnych metod terapeutycznych. Niezaprzeczalna rola mechanizmów epigenetycznych w utrzymanie stabilnej funkcji komórek Tregs i ich odwracalny charakter stanowią solidną podstawę do udoskonalania współczesnych terapii komórkowych.

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FULL-LENGTH ARTICLE

Basic Research

Antigen-reactive regulatory T cells can be expanded *in vitro* with monocytes and anti-CD28 and anti-CD154 antibodies

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ABSTRACT

Background: In recent years, therapies with $CD4^+CD25^{high}FoxP3^+$ regulatory T cells (Tregs) have been successfully tested in many clinical trials. The important issue regarding the use of this treatment in autoimmune conditions remains the specificity toward particular antigen, as because of epitope spread, there are usually multiple causative autoantigens to be regulated in such conditions. Methods: Here we show a method of generation of Tregs enriched with antigen-reactive clones that potentially covers the majority of such autoantigens. In our research, Tregs were expanded with anti-CD28 and anti-CD154 antibodies and autologous monocytes and loaded with a model peptide, such as whole insulin or insulin β chain peptide 9–23. The cells were then sorted into cells recognizing the presented antigen. The reactivity was verified with functional assays in which Tregs suppressed proliferation or interferon gamma production of autologous effector T cells (polyclonal and antigen-specific) used as responders challenged with the model peptide. Finally, we analyzed clonotype distribution and *TRAV* gene usage in the specific Tregs. Results: Altogether, the applied technique had a good yield and allowed us to obtain a Treg product enriched with a specific subset, as confirmed in the functional tests. The product consisted of many clones; nevertheless, the content of these clones was different from that found in polyclonal or unspecific Tregs. Conclusions: The presented technique might be used to generate populations of Tregs enriched with cells reactive to any given peptide, which can be used as a cellular therapy medicinal product in antigen-targeted therapies.

Introduction

Regulatory T cells (Tregs) constitute only about 1% of all peripheral blood lymphocytes, but they are instrumental in maintaining tolerance to self-tissues [1–3]. Lack of Tregs leads to numerous autoimmune diseases and allergies, as seen in immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome [4]. Thanks to the capability to suppress the immune system in a targeted manner, Treg cells can be called "intelligent steroids" [5]. Clinical trials indicate that therapy with Treg cells is safe and does not impair the immune response against foreign and dangerous antigens, such as viruses, bacteria and cancer cells [6–8].

Tregs are able to efficiently suppress the proliferation and differentiation of effector T (Teffs) cells *in vivo* as well as the effector functions of mature T, B and natural killer cells; natural killer T cells; macrophages; and dendritic cells [9]. Tregs utilize several mechanisms to execute this surveillance. The secretion of inhibitory cytokines transforming growth factor β, IL-10 and IL-35 seems to be an important contributor to their regulatory function [10,11]. There is also a metabolic disruption dependent on high-affinity IL-2 receptor alpha (CD25) in which Tregs uptake all available IL-2 and cause cytokine-deprivation apoptosis of other T cells. Tregs can also actively kill

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other cells [12]. Finally, they modulate dendritic cells. For example, LAG3 major histocompatibility complex class II coupling inhibits dendritic cell maturation, and CTLA-4 and CD80/CD86 coupling induces the suppressive enzyme indoleamine-2,3-dioxygenase [10,13,14].

Recently, Tregs have become used as a cellular drug. Currently, about 40 clinical trials have been conducted with these cells around the world. The major advantage of these cells is intelligent immunosuppression, which inhibits unwanted immune reactions without impairment of the physiological immune response [15]. There are different clinical targets of this therapy, such as therapy/prevention of graft-versus-host disease (GVHD) [16], autoimmunity and induction of tolerance in allotransplantation [16,17]. Our research group has been conducting research on the biology and clinical use of Tregs for over 20 years, mostly in autoimmune conditions like type 1 diabetes and multiple sclerosis [6,15,18,19].

Tregs for use in therapy are usually obtained from peripheral blood, apheresis of patients or umbilical cord blood from newborns. The best method of isolation is fluorescence-activated cell sorting (FACS), which results in a very pure (97–100%) population for further expansion. Typically, the following phenotypes of lymphocytes are sorted: $CD3^+CD4^+CD25^{high}$ or $CD3^+CD4^+CD25^{high}CD127^-$ or $CD3^+CD4^+CD25^{high}CD127^{low}$ [1,6,7,15,18]. There are many other phenotypes proposed in the literature, such as the relatively highly suppressive $CD45RA^+$ Tregs, but this is not always practical for clinical purposes, where high yield is required [19,20]. To obtain a sufficient amount of polyclonal Tregs for administration to a patient, sorted Tregs are expanded in the presence of IL-2 and anti-CD3 and anti-CD28 antibodies for 10–14 days [1,18]. The effective expansion must be carried out under conditions maintaining the full phenotype, including the main marker of Tregs, which is the expression of *FoxP3*-transcriptional factor [5,20]. In addition, the expansion of Tregs for clinical applications must be carried out in accordance with the standards of Good Manufacturing Practice, as Tregs are classified as medicinal products; for example, in Europe they are called advanced therapy medicinal products [21,22] Also, scientific consortia have defined some rules for cellular medicinal products like Tregs [23].

The bulk of Tregs isolated and expanded as described earlier are polyspecific/polyclonal (specific against many different peptide antigens), and thus their effectiveness at suppressing in a tissue-specific manner after administration is limited. The fact that Tregs can circulate and migrate to inflammatory sites to exert suppressor activities, as well as their ability to convert other cells to the regulatory phenotype via infectious tolerance, speaks to the high efficiency of polyspecific products [24]. Nevertheless, the efficacy of such a product can be increased by selecting antigen-specific Tregs that potentially migrate to the sites of antigen expression, where they selectively inhibit the activity of pathological effector cells with similar specificity [25]. Thus, applying antigen-specific Treg therapy in autoimmune diseases could possibly halt the destruction of the affected structures, such as insulin-producing pancreatic islets in type 1 diabetes or myelin sheaths in multiple sclerosis. At the same time, this would limit potential systemic side effects of Tregs, which, instead of traveling through the entire lymphatic system, would be targeted only to the sites expressing the specific antigen they are sensitized to [25]. However, this approach should be further clarified in autoimmune conditions in which—because of epitope spread—a variety of autoantigens ignite responses in multiple T-cell clones. For this reason, a proper cellular therapeutic should contain several clones with a specificity toward all or at least the majority of autoantigens that drive the disease [15].

Here we describe a method that allows the *in vitro* preparation of antigen-reactive Tregs (Tregs SPEC) for clinical use in the treatment of autoimmune diseases. We decided to isolate Tregs that recognize antigens important in type 1 diabetes—whole insulin or insulin β chain peptide 9–23—but the same method can potentially be used in the manufacturing of Tregs with specificity to any other autoantigen and applied in the treatment of conditions such as multiple sclerosis or rheumatoid arthritis. We tested the suppressor capabilities of the manufactured specific Tregs in functional tests measuring proliferation or interferon gamma (IFNγ) secretion of autologous responders. In addition, we sequenced the T-cell receptor α (TCRα) chains in the obtained subsets (Tregs: polyclonal - index POLY, antigen-reactive -index SPEC, antigen-unrective - index UNSPEC; Teffs: polyclonal - index POLY, antigen-reactive -index SPEC, antigen-unrective - index UNSPEC) to determine the TCR repertoire. In our opinion, the development of a safe, simple and economically viable method to multiply Tregs with chosen antigenic reactivity and high suppressor potential is an interesting avenue in the search toward clinical trial success using Tregs as a therapeutic tool.

Methods

An overview of the experimental procedure is shown in Figure 1.

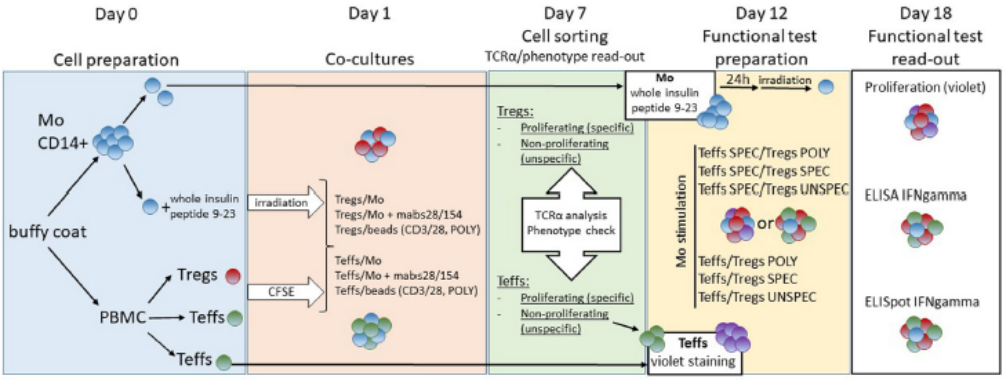

Figure 1. Experimental overview. Monocytes and lymphocytes are isolated from buffy coats. Tregs (red) and Teffs (green) are isolated from PBMCs. All Tregs and some Teffs are stained with CFSE and stimulated with irradiated autologous monocytes previously loaded with antigen (whole insulin or insulin β chain peptide 9–23). Polyclonal Tregs and Teffs (index POLY) are generated by stimulation with anti-CD3/anti-CD28 beads. After 6 days of co-culture, the cells are separated into antigen-specific/reactive (index SPEC) and unspecific/unreactive cells (index UNSPEC) according to diluted fluorescence of CFSE using the protocol shown in supplementary Figures 1, 2C,D,E. The obtained Tregs POLY, Tregs SPEC and Tregs UNSPEC cells are then used in functional tests. Unstimulated (without anti-CD3/anti-CD28 beads) Teffs as polyclonal (violet) cells and antigen-reactive Teff (reactive to whole insulin or insulin β chain peptide 9–23 [Teffs SPEC], violet) cells stained with violet were used. The results of these tests are shown in Figures 2–6. TCRα analysis includes NGS (Figures 4–6; also see supplementary Figures 3, 5–7) and clonality study using flow cytometry (see supplementary Figure 4). To study the effects of Tregs on Teff proliferation, we used Teffs stained with violet. For ELISA and ELISpot, we used unstained cells. ELISA, enzyme-linked immunosorbent assay; mabs, monoclonal antibodies; Mo, monocytes; NGS, next-generation sequencing; PBMC, peripheral blood mononuclear cell. (Color version of figure is available online).

Blood donors

Buffy coats, with unknown HLA, were obtained from healthy volunteers from the Regional Centre for Blood Donation and Treatment in Gdańsk. The study has been approved by the Institutional Review Board of the Medical University of Gdańsk (approval no NKBBN/32/2015).

Insulin β chain peptide 9–23 and whole insulin

Insulin β chain peptide 9–23 was synthesized at Lipopharm (Gdansk, Poland) with purity >90% using the high-performance liquid chromatography method. Peptide was dissolved in deionized, autoclaved water for a final concentration of 0.5 μg/μL and stored at −70°C for no longer than 3 months. The samples of insulin used in tests were commercially available (Actrapid Penfill; Novo Nordisk A/S).

Cell isolation and sorting

Tregs and Teffs (Day 0)

Peripheral blood mononuclear cells were isolated from buffy coats obtained from healthy volunteers by Ficoll-Hypaque gradient centrifugation and were used fresh. Tregs and autologous Teffs were freshly isolated according to our previously described protocol [1,18,26]. Briefly, $CD4^+$ T cells were separated by negative selection using an EasySep human CD4+ T-cell enrichment kit (StemCells Technologies, Canada) according to the manufacturer's instructions. Subsequently, $CD4^+$ T cells were stained with monoclonal antibodies specific for the following antigens: CD3, CD4, CD25 and CD127. Cells were sorted with a FACSAria IIu sorter (BD Biosciences, USA) into Treg phenotype $CD3^+CD4^+CD25^{high}CD127^{-/}lin^-doublet^-$ and Teff $CD3^+CD4^+CD25^-CD127^+lin^-doublet^-$. Isolated Tregs and Teffs were cultured on separate plates and incubated at 37°C in X-VIVO 20 (Lonza, Belgium) culture medium, fulfilling Good Manufacturing Practice standards. The medium was supplemented with heat-inactivated human AB serum (10%), IL-2 100 U/mL (Proleukin; Novartis, USA), penicillin 100 U/mL and streptomycin 100 mg/mL for 24 h.

Monocytes (Day 0)

Autologous $CD14^+$ cells were isolated by positive selection using an EasySep human CD14 positive selection kit II (StemCells Technologies, Canada) according to the manufacturer's instructions, with purity >95%. Isolated monocytes were cultured (10^6 cells/well) in X-VIVO 20 (Lonza, Belgium) culture medium and incubated at 37°C. Previously prepared insulin β chain peptide 9–23 solution (25 μg/well/mL) or whole insulin (100 μL/well/mL) was added for 24 h of incubation. Two conditions were prepared: monocytes stimulated with insulin β chain peptide 9–23 or whole insulin.

Dye labeling and cell expansion

Monocytes (Day 1)

After 24 h of incubation, monocytes from all conditions were collected and γ irradiated at at least 2518 cGy for 10 min, counted and resuspended in fresh medium (X-VIVO 20; Lonza, Belgium) at a final concentration of 1×10^6 cells/mL. Irradiated cells were used as stimulators and co-cultured with autologous lymphocytes.

Tregs and Teff cells (Day 1)

After 24 h of incubation, Tregs and part of the Teffs were washed and resuspended in phosphate-buffered saline (PBS) at a concentration of 1×10^6 cells/mL and stained with carboxyfluorescein succinimidyl ester (CFSE) using a CellTrace CFSE cell proliferation kit (Life Technologies, USA), with a final CFSE concentration between 1 and 5 μM [27]. The cells were incubated at 37°C for 20 min in the dark and washed several times with PBS and then culture medium X-VIVO 20 (Lonza, Belgium), 10% heat-inactivated human serum, IL-2 100 U/mL, penicillin and streptomycin.

Polyclonal stimulation (Day 1)

After the dye labeling, part of the Tregs and Teffs were suspended in fresh medium (X-VIVO 20; Lonza, Belgium) containing 10% heat-inactivated human serum (National Blood Bank, Gdańsk, Poland), IL-2 100 U/mL, penicillin and streptomycin (Sigma Aldrich, Poland); seeded in 96-well plates (1×10^5 cells/well); and either stimulated with magnetic beads coated with anti-CD3 and anti-CD28 antibodies using a Treg expansion kit (Miltenyi Biotech, Germany) in a 1:1 ratio (beads to cells) or not (control) and cultured for 6 days. Two types of polyclonal cells were prepared: Treg POLY and Teff POLY.

Antigen stimulation (Day 1)

After the dye labeling, part of the Tregs and Teffs were suspended in fresh medium (X-VIVO 20; Lonza) containing 10% heat-inactivated human serum (National Blood Bank, Gdańsk, Poland), IL-2 100 U/mL, penicillin and streptomycin (Sigma Aldrich, Poland); seeded in 96-well plates (1×10^5 cells/well); and stimulated with autologous monocytes loaded with antigen insulin β chain peptide 9–23 or whole insulin in a 1:1 ratio (monocytes to Tregs/Teffs). Sterile anti-CD154 (purified NA/LE mouse anti-human CD154; BD Biosciences, USA) and anti-CD28 (purified NA/LE mouse anti-human CD28; BD Biosciences, USA) at a final concentration of 5 μg/mL/well were added to the co-culture. The co-culture was incubated at 37°C in 5% CO_2 in culture medium (X-VIVO 20; Lonza, Belgium) containing 10% heat-inactivated human AB serum, IL-2 100 U/mL, penicillin and streptomycin. In parallel, cells stimulated with antigen-loaded monocytes, but without anti-CD28 and anti-CD154, were prepared. Unstimulated, non-proliferating cells (without monocytes) were used as negative controls. As a positive control, we used polyclonal cells. Cells in all conditions were cultured for 5–7 days. Part of the Teffs were left without stimulation and used as responders at day 13 during functional tests.

Sorting of antigen-reactive cells (Day 7)

At day 7, the cells were collected and washed with fresh medium (X-VIVO; Lonza, Belgium). Cells were sorted using a FACSAria IIu sorter (BD Biosciences, USA) from the side scatter-A dot plot versus 488-nm channel for CellTrace CFSE cell proliferation kit (Life Technologies, USA) (see supplementary Figure 1). Cells proliferating, as response to the antigen presented by monocytes, (index SPEC) were identified as those that showed a fluorescence lower than the cells from the negative control (cutoff for the sorting gate assumed for fluorescence intensity below the negative control peak, goal containing no more than 5% peak events, negative control with the lowest fluorescence) and unreactive, non-proliferating cells (index UNSPEC) as those whose fluorescence was comparable to cellular fluorescence from the negative control (sorting gates assumed for fluorescence intensity of the negative control peak, goal containing not less than 80% of events of the negative control peak). Sorted cells were expanded for another 5 days with an anti-CD3/anti-CD28 Treg expansion kit (Miltenyi Biotech, Germany) in a 1:1 ratio (beads to cells), washed free of the beads, left for 48 h in culture medium without stimulation for resting and then subjected to phenotype control and suppression functional tests.

Phenotype check (Day 7)

At day 7th of the expansion, samples of Tregs and Teff cells were labeled with monoclonal antibodies against antigens CD4, CD25, CD127, CD62L, Helios (Life Technologies, USA) and FoxP3 using a FoxP3 staining buffer set (eBioscience, USA) and analyzed by flow

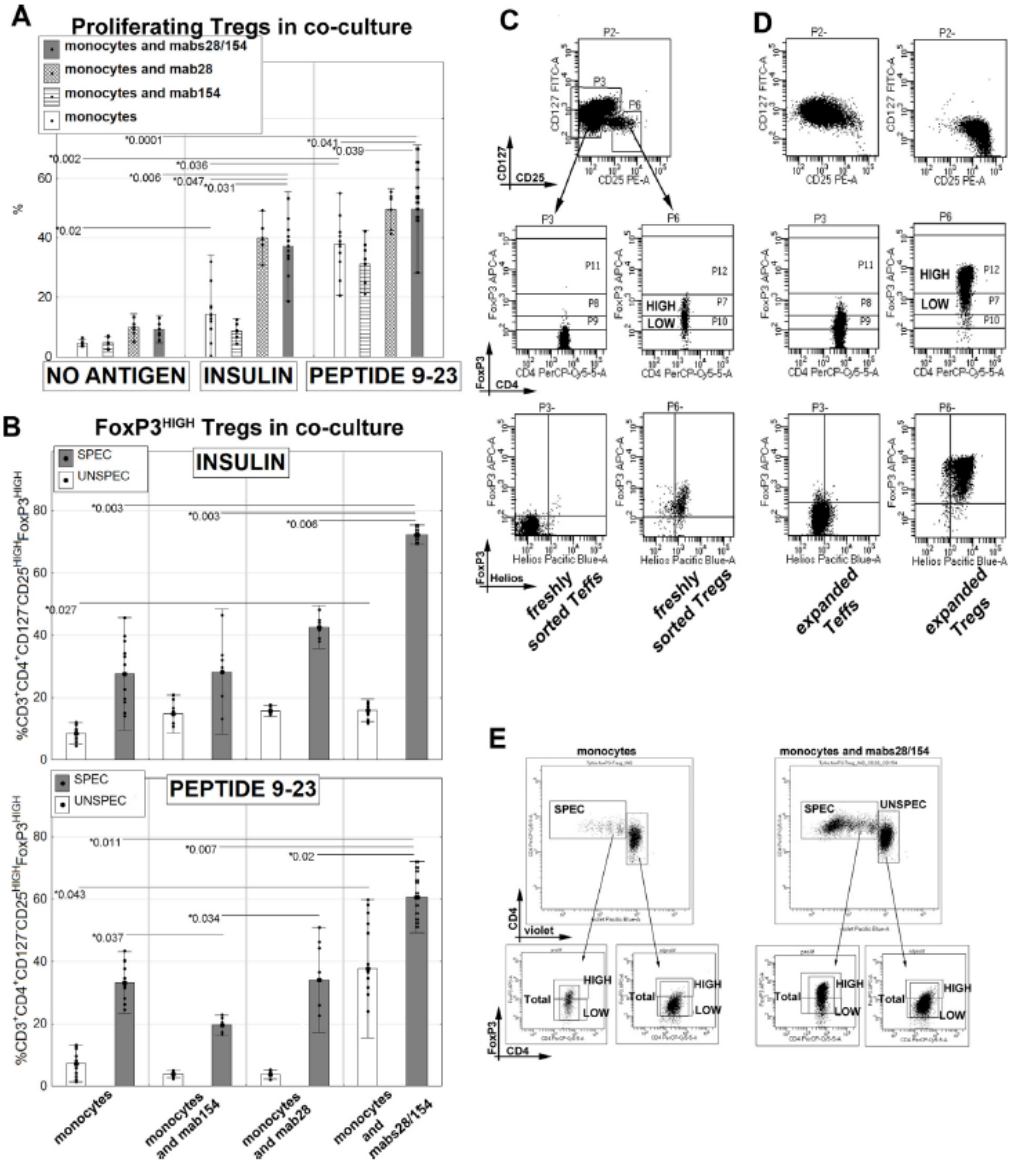

Figure 2. Percentage of Tregs responding to antigens in co-cultures with monocytes. The percentage of proliferating (antigen-reactive, SPEC) Tregs (A) and Tregs expressing a high intensity of FoxP3 expression (FoxP3^{high}) in specific (SPEC, gray bars) and unspecific (UNSPEC, transparent bars) Tregs (B) was assessed in different culture conditions. The results are presented from co-cultures stimulated with monocytes only (▭), monocytes and anti-CD154 antibody (▯), monocytes and anti-CD28 antibody (▯) and monocytes and anti-CD28 and anti-CD154 antibodies (▭). The results from 11 independent experiments (each time with a different donor's material) are presented as mean ± SD, and dots (•) represent individual results. The strategy to assess FoxP3 expression is shown in freshly sorted cells from peripheral blood (C) and those expanded *in vitro* (D). The sorting from peripheral blood (C) was based on the expression of CD25 and CD127 receptors on CD4$^+$ T cells into CD25$^{-/low}$CD127$^+$ Teff cells (P3 gate) and CD25^{high}CD127$^-$ Tregs (P6 gate), with the purity of sorted Tregs ≈97%. The cutoff for positive signal for the evaluation of FoxP3 expression was set based on isotype controls using an FMO approach. The splitting of FoxP3^{high} from FoxP3^{low} Tregs was based on the expression of FoxP3 in Teffs. The intensity of the signal above that seen in Teffs was treated as FoxP3^{high} and the intensity present in Teff cells as FoxP3^{low}. As the expression of FoxP3 in the expansion cultures (D) increases for at least one decade (and could be elevated more than 2 decades), the cutoff for all FoxP3+ Tregs was also set higher, above the FoxP3^{low} signal in Tregs in peripheral blood. This ensured that ex-Tregs and FoxP3$^+$ Teff cells were excluded from the analysis and the purity of Tregs in all cultures throughout the experiments was >90%. The splitting between FoxP3^{high} and FoxP3^{low} expanded Tregs (ex-Tregs) was based on the expression of FoxP3 in the expanded Teff cells. To avoid problems with extremely high intensity signal of FoxP3 in the expansion Treg cultures, the voltage of the photomultiplier for the FoxP3 signal was decreased in the routine acquisition of samples from expansion cultures (E). In addition, to confirm Treg phenotype, Helios expression was co-analyzed with FoxP3 signal (E) (the lowest dot plots). The example of the FACS gating strategy for analyzing proliferation and FoxP3 expression is shown from co-cultures of Tregs stimulated with monocytes loaded with specific peptides (insulin in the example). Gates in the dot plots for CD4 versus CFSE show proliferating/antigen-reactive Tregs (SPEC) (left) and non-proliferating/unreactive Tregs (UNSPEC) (right). Arrows link corresponding dot plots to show the expression of FoxP3. Upper gates represent FoxP3^{high} and lower FoxP3^{low} subsets of the cells. Comparison of the cultures stimulated additionally with monoclonal antibodies anti-CD28 and anti-CD154 as a second signal and cultures without antibodies is shown. For result reliability, the compared cultures come from the same donor. Ex-Tregs, Foxp3– Tregs; FMO, fluorescence minus one; mab, monoclonal antibody; SD, standard deviation.

cytometry (LSRFortessa; BD Biosciences, USA). Gates and cytometer configuration were set as previously described (Figure 2) [5,18].

Functional tests

General procedure for functional tests (Day 12)

At day 12 of the expansion, functional assays were performed. We measured the ability of Tregs to inhibit proliferation and secretion of IFNγ of autologous Teffs (polyclonal and antigen-specific). Prior to commencing the assays, Tregs and Teff cells were washed and left in medium for 48 h without stimulation for resting. Next, autologous Teffs (Teff SPEC or Teffs) were used as responders and mixed in the following proportions with Tregs: 1:1, 1:$^1/_2$, 1:$^1/_4$ and 1:$^1/_8$ (Teffs to Tregs). The co-cultures were suspended in fresh culture medium containing 10% heat-inactivated human AB serum, IL-2 100 U/mL, penicillin and streptomycin. Irradiated autologous monocytes loaded with appropriate antigen (whole insulin or insulin β chain peptide 9–23) were used as stimulants and added in a 1:1 ratio to the Teffs. Teffs only (without Tregs) stimulated with monocytes loaded with antigen or microspheres coated with anti-CD3 and anti-CD28 antibodies were used as a positive control. Teffs without monocyte stimulation (reference to read in cytometer) were used as a negative control. The co-cultures were incubated for 5 days at 37°C in 5% CO_2 in culture medium (X-VIVO 20; Lonza, Belgium) containing 10% heat-inactivated human AB serum, IL-2 100 U/mL, penicillin and streptomycin and then harvested and analyzed.

Proliferation inhibition assay (Day 12)

Prior to functional test, autologous Teff cells used as responders in proliferation inhibition assay were washed with PBS buffer, counted and stained with violet (CellTrace violet cell proliferation kit; Life Technologies, USA) 1 μM at 37°C for 15 min to analyze their proliferation in the presence of unstained autologous Tregs after 6 days of incubation [27]. The readout of the test was obtained using a flow cytometer (LSRFortessa; BD Biosciences, USA). Unstimulated Teff responders cultured without Tregs were used as 100% of undividing cells. Stimulated Teff responders cultured without Tregs were used as 0% of undividing cells.

Enzyme-linked immunosorbent assay IFN-γ production (Day 12)

After 6 days of incubation, as described in the general procedure, supernatants were harvested from the culture, and levels of secreted IFNγ were determined using a BD OptEIA human IFN-γ enzyme-linked immunosorbent assay kit II (BD Biosciences, USA) according to the manufacturer's instructions.

Enzyme-linked immune absorbent spot IFN-γ production (Day 12)

Co-cultures of Teffs (responders) were mixed with Tregs as described in the general procedure and incubated for 48 h on enzyme-linked immune absorbent spot (ELISpot) plates (Mabtech, Sweden). After incubation, cells were removed from the plates by washing, and plates were stained according to the manufacturer's procedure. Readouts were made on an ELISpot plate reader (ImmunoSpot 5; CTL, USA).

Clonal vβ repertoires (Day 7)

The TCR repertoire was analysed using the IOTest beta mark kit (Beckman Coulter, USA), combined with naive/memory phenotype and intracellular FoxP3 staining, according to the manufacturer's protocol. This multiparametric kit allows for about 70% coverage of TCR vβ repertoire of human T lymphocytes by flow cytometry.

Preparation and sequencing for Next-Generation Sequencing of TCRα chains (Day 7)

Libraries for Next-Generation Sequencing of TCRα chains were prepared and sequenced as described by Eugster *et al.* [28]. Briefly, for RNA isolation, cells were thawed and processed using RNeasy mini and micro kits (Qiagen, Valencia, CA, USA). Quantification and quality control were performed with the RNA 6000 pico kit on the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

First-strand complementary DNA (cDNA) was synthesized by rapid amplification of 5' complementary DNA ends. RNA, 1 mM deoxynucleotide triphosphate and 0.125 μM final of the 3' primer binding to the TCRα C region (5'-CACTGTTGCTCTTGAAGTCC-3') were denatured for 5', 65°C. A mix containing 2.5 μM final of the template-switching primer (5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTrGrGrG-3'), 2 mM dithiothreitol, 20 U rRNAsin (Promega, Madison, WI), 50 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 8), 187 mM potassium chloride, 3 mM manganese(II) chloride tetrahydrate and 0.05% Tween 20 was added, for a final volume of 20 μL.

After pre-incubation (2', 42°C), 200 U SuperScript II reverse transcriptase (Invitrogen) was added, and incubation was continued for 90', 42°C and 15', 70°C. The cDNA was purified using a MinElute polymerase chain reaction (PCR) purification kit (Qiagen, Germany). Whole cDNA was amplified over 3 rounds of PCR with PrimeSTAR high-sensitivity DNA polymerase (Takara, Japan), allowing the addition of barcodes and adaptors for Illumina sequencing. Primers were:

(i) 5'-TCGGTGAATAGGCAGACAGA-3' and 5'-GTGACTGGAGTTCAGACGTG-3'
(ii) 5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGCAGGGTCAGGGTTCTGGAT-3' and 5'-CAAGCAGAAGACGGCATACGAGATindexGTGACTGGAGTTCAGAC-3'

(iii) 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACAC-3' and
5'-CAAGCAGAAGACGGCATACGAGATindexGTGACTGGAGTTCAGAC-3'

Amplification was for 15, 12 and 25 cycles with the following conditions: (i) 50 μL total (98°C for 10 min, 56°C for 7 min, 72°C for 1 h 50 min); (ii) 1 μL first PCR for 20 μL total (98°C for 10 min, 49°C for 7 min at 2°C increments per cycle, 72°C for 1 h 50 min); and (iii) 1 μL second PCR (1:100) for 50 μL total (98°C for 10 min, 58°C for 7 min, 72°C for 1 h 50 min).

The final product was purified using AMPure beads. The final TCRα construct obtained from the library preparation contained the nucleotide sequence for the variable region of TCRα (V and J segments) with the entire complementary determining region (CDR3); 150 base pair reads were generated using the Illumina HiSeq 2500. TCR CDR3 region sequence extraction and PCR error correction were carried out as described with MiXCR [29].

The libraries were sequenced with 20 reads/cell, resulting in a mean of 69% usable reads (range: 49–81%). Non-productive TCR sequences were removed. TCRα chains were considered 1 clonotype if they had identical CDR3 amino acid sequences. Downstream analysis was done using the R package Immunarch (ImmunoMind) and KNIME. Cell numbers, total reads and TCR reads obtained are shown in Table 1.

Statistics

The analysis was based on 2-tailed parametric analysis of variance (ANOVA), *t*-tests, Pearson correlation and Kruskal-Wallis test, as indicated by data distribution. $P < 0.05$ was recognized as significant.

Table 1
Sample characteristics.

Sample ID	Antigen	Cell type	Input cells (mio)	TCRα reads (Mio)	Clonotypes
S1	WHOLE INSULIN	Tregs POLY	1	5.38	36076
S1		Tregs SPEC	0.7	9.18	12448
S1		Tregs UNSPEC	1	6.81	139549
S1		Teff POLY	4.5	14.78	48070
S1		Teff SPEC	4	5.07	49172
S2		Tregs POLY	1	14.68	254832
S2		Tregs SPEC	1	6.86	249220
S2		Tregs UNSPEC	1	15.89	184561
S2		Teff POLY	1	14.66	238868
S2		Teff SPEC	1	16.58	27654
S3		Tregs POLY	1	25.26	66161
S3		Tregs SPEC	1	30.3	16051
S3		Tregs UNSPEC	1	10.6	90003
S3		Teff POLY	1	18.11	110327
S3		Teff SPEC	1	19.4	34000
S3		Teff UNSPEC	1	10.92	167092
S1	INSULIN PEPTIDE 9-23	Tregs POLY	1	7.34	6722
S1		Tregs SPEC	1	12.5	96289
S1		Tregs UNSPEC	1	8.03	140883
S1		Teff POLY	1	8.1	237344
S1		Teff SPEC	1	11.12	35229
S1		Teff UNSPEC	1	6.75	65158
S2		Tregs POLY	1	2.38	16198
S2		Tregs SPEC	1	5.09	13991
S2		Tregs UNSPEC	1	6.2	26385
S2		Teff POLY	1	37.04	78272
S2		Teff SPEC	1	35.3	53115
S2		Teff UNSPEC	1	63.44	227414
S3		Tregs POLY	2	11.04	134664
S3		Tregs SPEC	2	10.01	3455
S3		Tregs UNSPEC	1	6.99	17343
S3		Teff POLY	2	8.83	39143
S3		Teff SPEC	2	6	7809
S3		Teff UNSPEC	2	6.39	3776

Software used was FACSDiva 8, FlowJo 10, Prism 7 (GraphPad) and Statistica 11.0.

Results

The generation of antigen-specific Tregs through co-cultures with antigen-presenting monocytes and the use of anti-CD28 and anti-CD154 antibodies

Compared with stimulation with monocytes without antigen, stimulation with specific peptides led to significantly higher proliferative responses (*t*-test difference, no antigen/insulin, $P = 0.0001$ and no antigen/insulin β chain peptide 9–23, $P = 0.006$). To expand isolated Tregs *in vitro*, they were cultivated in the presence of monocytes presenting the peptides of choice and anti-CD28 and anti-CD154 antibodies. The percentage of proliferating antigen-reactive Tregs generated by co-culture with autologous antigen-presenting monocytes was significantly higher when anti-CD28 and anti-CD154 antibodies were added to the co-cultures (*t*-test difference with/without the addition of antibodies, whole insulin, $P = 0.047$ and insulin β chain peptide 9–23, $P = 0.041$) (Figure 2A). Compared with whole insulin, stimulation with monocytes loaded with insulin β chain peptide 9–23 led to a significantly higher percentage of proliferating Tregs (*t*-test, $P = 0.036$) (Figure 2A).

When analyzing the role of the antibodies as a stimulus of proliferation, it appeared that anti-CD28 was mainly responsible. There was no significant difference between the cultures with anti-CD28 antibody and anti-CD28 and anti-CD154 antibodies (*t*-test, $P > 0.05$) and significantly lower proliferative responses between cultures with anti-CD154 antibody and anti-CD28 and anti-CD154 antibodies (*t*-test, anti-CD154/anti-CD28/anti-CD154 antibodies, whole insulin, $P = 0.031$ and insulin β chain peptide 9–23, $P = 0.039$).

FoxP3 expression in generated antigen-specific Tregs

In all cultures of Tregs throughout the entire experiment, the percentage of lymphocytes expressing FoxP3 did not fall below 90%, whereas expression of FoxP3^{high} was between 16% and 79%, depending on the type of cells (see supplementary Figure 2). The highest comparable percentage of Tregs showing high expression of FoxP3 transcription factor (CD3$^+$CD4$^+$CD25^{high}CD127$^-$FoxP3^{high} phenotype) was seen in polyclonal Tregs (mean ± standard deviation, 76.8 ± 2.79) and antigen-reactive Tregs (SPEC) stimulated with peptide-loaded monocytes and CD28 and CD154 antibodies (mean ± standard deviation, insulin, 72.35 ± 3.06 and insulin β chain peptide 9–23, 60.68 ± 11.45) (Figure 2B). The percentage of FoxP3^{high}Tregs stimulated by autologous monocytes presenting whole insulin or insulin β chain peptide 9–23 (SPEC) was significantly higher for antigen-reactive/proliferating populations compared with the corresponding unreactive/non-proliferating Tregs (UNSPEC) (all *t*-tests, $P < 0.05$) (Figure 2B,E).

Stimulation with anti-CD28 and anti-CD154 antibodies additionally increased the percentage of FoxP3^{high} Tregs in both reactive/proliferating Tregs and unreactive/non-proliferating Tregs (*t*-test difference with/without antibodies, Tregs [SPEC], whole insulin, $P = 0.003$, insulin β chain peptide 9–23, $P = 0.011$ and Tregs [UNSPEC], whole insulin, $P = 0.027$, insulin β chain peptide 9–23, $P = 0.043$). Interestingly, neither of the antibodies separately was able to induce the percentage of FoxP3^{high} Tregs noted in the co-cultures stimulated with both anti-CD28 and anti-CD154 antibodies. The percentage of FoxP3^{high} Tregs reactive to the antigen in the co-cultures stimulated with both antibodies was significantly higher than the percentage in other culture conditions (*t*-test difference for insulin, monoclonal antibodies CD28 and CD154 versus monocytes, $P = 0.003$, monoclonal antibodies CD28 and CD154 versus monoclonal antibody CD154, $P =$

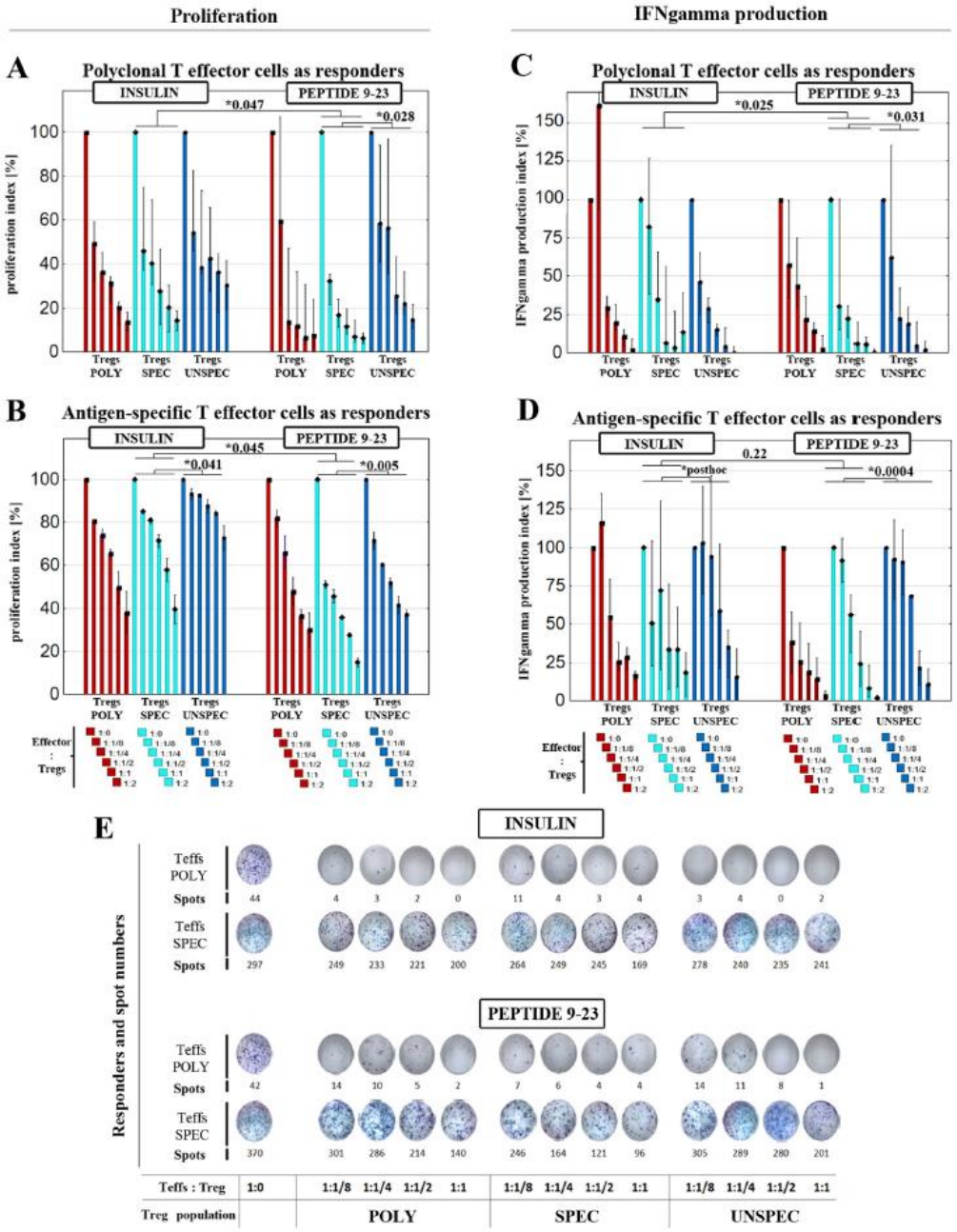

Figure 3. Functional tests, inhibition of Teff lymphocyte proliferation and IFNγ production. POLY, SPEC (toward insulin or insulin β chain peptide 9–23) and UNSPEC Tregs were co-cultured with autologous Teff cells as responders. We used polyclonal (not stimulated with antigen or bead anti-CD3/anti-CD28) (A, C, E) and antigen-reactive (B, D, E) Teff cells in the proportions shown. (A, B) Co-cultures were stimulated with irradiated monocytes pre-loaded with whole insulin or insulin β chain peptide 9–23. (A, B) Suppression capacity of proliferation of responder cells (antigen-reactive or polyclonal) stained with violet by Tregs (POLY, SPEC, UNSPEC). (C, D) Suppression of IFNγ secretion by responder cells (polyclonal or antigen-reactive) caused by Tregs (POLY, SPEC, UNSPEC) using the IFNγ ELISA test. Results are indexed to the cultures with responders only (no Tregs), where proliferation was set to 100%. Three independent experiments were performed for the assessment of suppression of proliferation (A, B) and another 3 experiments for suppression of IFNγ secretion (C, D). Results are shown as mean ± min/max. Significant differences are marked with * and P value. (E) Suppression of IFNγ production by single cell responder caused by Tregs (POLY, SPEC, UNSPEC) using the IFNγ ELISpot test. Results are shown as pictures of cultures and number of spots in particular wells. The ELISpot experiment with all conditions included was performed using the cells of one donor. ELISA, enzyme-linked immunosorbent assay; min/max, minimum/maximum. (Color version of figure is available online).

0.003, monoclonal antibodies CD28 and CD154 versus monoclonal antibody CD28, $P = 0.006$ and t-test difference for insulin β chain peptide 9–23, monoclonal antibodies CD28 and CD154 versus monocytes, $P = 0.011$, monoclonal antibodies CD28 and CD154 versus monoclonal antibody CD154, $P = 0.007$, monoclonal antibodies CD28 and CD154 versus monoclonal antibody CD28, $P = 0.02$). The expression of another marker of Treg quality used in our laboratory, CD62L receptor, was noted on more than 85% of Tregs throughout the entire experiment (data not shown).

Functional tests: proliferation and IFNγ secretion

The efficacy of Tregs specific to whole insulin and insulin β chain peptide 9–23 was evaluated in functional tests in which the suppression of proliferation and interferon secretion was assessed in responder cells. We conducted 2 sets of tests in which autologous Teff cells—polyclonal Teff cells (unstimulated with antigens) and antigen-specific Teff cells (stimulated and proliferation-responsive to the appropriate antigen)—acted as responders. The obtained results suggested suppressive effects exerted by most Treg subpopulations (ANOVA, $P < 0.05$) (Figure 3).

Compared with Tregs reactive toward whole insulin, Tregs reactive to insulin β chain peptide 9–23 had a stronger capacity to supress both polyclonal Teff cells (ANOVA, F = 8.03, $P = 0.047$) (Figure 3A) and reactive Teff cells (ANOVA, F = 20.40, $P = 0.045$) (Figure 3B). In addition, the suppression exerted by Tregs reactive to insulin β chain peptide 9–23 was stronger than the suppression of unreactive Tregs when the responders were polyclonal Teff cells (insulin β chain peptide 9–23 ANOVA, F = 8.21, $P = 0.028$ and whole insulin ANOVA, F = 1.31, $P = 0.33$) (Figure 3A) as well as antigen-reactive Teff lymphocytes (insulin β chain peptide 9–23 ANOVA, F = 186.32, $P = 0.005$ and whole insulin ANOVA, F = 22.47, $P = 0.041$) (Figure 3B).

The secretion of IFNγ by polyclonal Teff cells acting as responders was suppressed more efficiently by Tregs reactive to insulin β chain peptide 9–23 than Tregs reactive to whole insulin (ANOVA, F = 5.78, $P = 0.025$) (Figure 3C). A similar difference was observed when the responders were antigen-reactive Teff cells; however, the difference did not reach statistical significance (ANOVA, F = 1.86, $P = 0.22$) (Figure 3D). Compared with unreactive Tregs, Tregs reactive toward insulin β chain peptide 9–23 suppressed significantly stronger IFNγ secretion when the responders were both polyclonal Teff cells (ANOVA, F = 5.3, $P = 0.031$) and Teffs reactive to insulin β chain peptide 9–23 (ANOVA, F = 111.84, $P = 0.0004$). Stronger inhibition was also observed in cases of Tregs reactive to whole insulin compared with unreactive Tregs when responders were Teff cells reactive to insulin. However, the statistical significance of the effect was only observed in some experiments and post hoc analysis, whereas the overall analysis yielded no significant results (ANOVA, F = 0.31, $P = 0.56$). There were no differences between insulin-reactive and unreactive Tregs to the polyclonal responders (ANOVA, F = 0.0004, $P = 0.94$). All results were confirmed with IFNγ ELISpot tests (Figure 3E).

TCR repertoires of specific and unspecific Tregs

The TCRα repertoires obtained after stimulation of Tregs (or Teffs) with whole insulin or insulin β chain peptide 9–23 were surprisingly large, and repertoire composition, diversity and clonality were very variable between methods and samples. As expected, the reactive TCRα repertoires of all analyzed samples (3 Treg and 3 Teff samples were stimulated with whole insulin and 3 Treg and 3 Teff samples with insulin β chain peptide 9–23) contained fewer clonotypes than the TCRα repertoires of polyclonally stimulated or unstimulated cells (Table 1). This was true even if the starting cell number was higher for the repertoire of unstimulated cells (sample 3, insulin β chain peptide 9–23). The TCRα repertoires of reactive Tregs (and also of reactive Teffs; data not shown) were characterized by a higher proportion of clonal expansions than found in the repertoires of unreactive or polyclonal subsets (Figure 4A,B; also see supplementary Figure 3). Similarly, the diversity of reactive Treg repertoires was lower than that seen in the repertoires of polyclonally or unexpanded Tregs, as shown by the inverse Simpson index [30] and Hill numbers [31] (see supplementary Figure 5). In each experiment, various clones with different *TRAV*and *TRBV*genes were expanded, although the overall *TRAV*and *TRBV* gene usage profiles of the 3 different samples were maintained (Figure 5; also see supplementary Figure 4; data not shown for stimulation with insulin β chain peptide 9–23). The differences in gene usage between the three groups were subtle, except for some prominent expansions (unreactive, *TRAV21* or polyclonal, *TRAV 26-1* in sample 2). Clonotypes highly expanded in the reactive repertoires were only partially found in polyclonal or unreactive repertoires, confirming that the latter repertoires were different from the former (Figure 5; data not shown for stimulation with insulin β chain peptide 9–23).

The cumulative frequency of the top 30 TCR repertoires found among the reactive Tregs (range, 0.4–0.9% in sample 1, 0.13–14% in sample 2 and 0.3–1.5% in sample 3) was lower in polyclonal and unreactive Tregs (Figure 6; also see supplementary Figure 6). The Morisita overlap index [32] showed that the overlap between the polyclonal and unreactive Treg repertoires was higher than the overlap between either the polyclonal or the unreactive and reactive Treg repertoires, confirming that the specifically stimulated Treg repertoires differed substantially from the others in all 3 samples (see supplementary Figure 7; data not shown for stimulation with insulin β chain peptide 9–23). For sample 3, the only one in which Teff repertoires were available for the 3 stimulation conditions, we observed the same. There was relatively little overlap between the specific Treg repertoires and the reactive Teff repertoires. Rather, the overlap between all 3 stimulation conditions as well as between Tregs and Teffs was within the same range, confirming that reactive Treg repertoires differ substantially from reactive Teff repertoires.

Discussion

The subject of this article was an *in vitro* method for the generation of model antigen-reactive Tregs for clinical use. Such cells could be used in the treatment of autoimmune diseases and to inhibit unwanted immune reactions, such as transplant rejection, allergies and GVHD. Now, Tregs used in the clinic are polyclonal, which means that they recognize many different antigens, and therefore their efficacy may be diluted [1,2,6,7,16,18,19,26,33–36]. Here we present a workflow allowing the selection of Tregs reactive to a defined antigen. Such Tregs might travel to tissues expressing particular antigens and suppress autoreactive lymphocytes responsible for the inflammatory response against specific antigens locally. The use of antigen-reactive Tregs will allow for more precise treatment and reduction in Treg dose and will potentially increase the effectiveness of treatment and reduce possible side effects.

To obtain such a preparation, we decided to physically separate *in vitro* Tregs reactive to an antigen of choice from polyclonal Tregs by using monocytes loaded with the antigen. $CD3^+CD4^+CD25^{high}CD127^-$ Tregs grown with autologous γ-irradiated monocytes presenting a specific antigen (e.g., whole insulin or insulin β chain peptide 9–23) proliferate only when they are reactive for the antigen presented by the monocytes. The sorting of a pure antigen-reactive Treg population was done using the FACS cell sorter, as this is the most precise method of obtaining a pure population of Treg cells. Sorting of the reactive Treg population was possible thanks to prior staining of the Tregs with a fluorescent dye (here CFSE) diluted in daughter cells upon cell division after activation. Fluorescence intensity decreased by about half with each subsequent cell division. This change in fluorescence, from high fluorescence in non-proliferating cells (non-reactive Treg

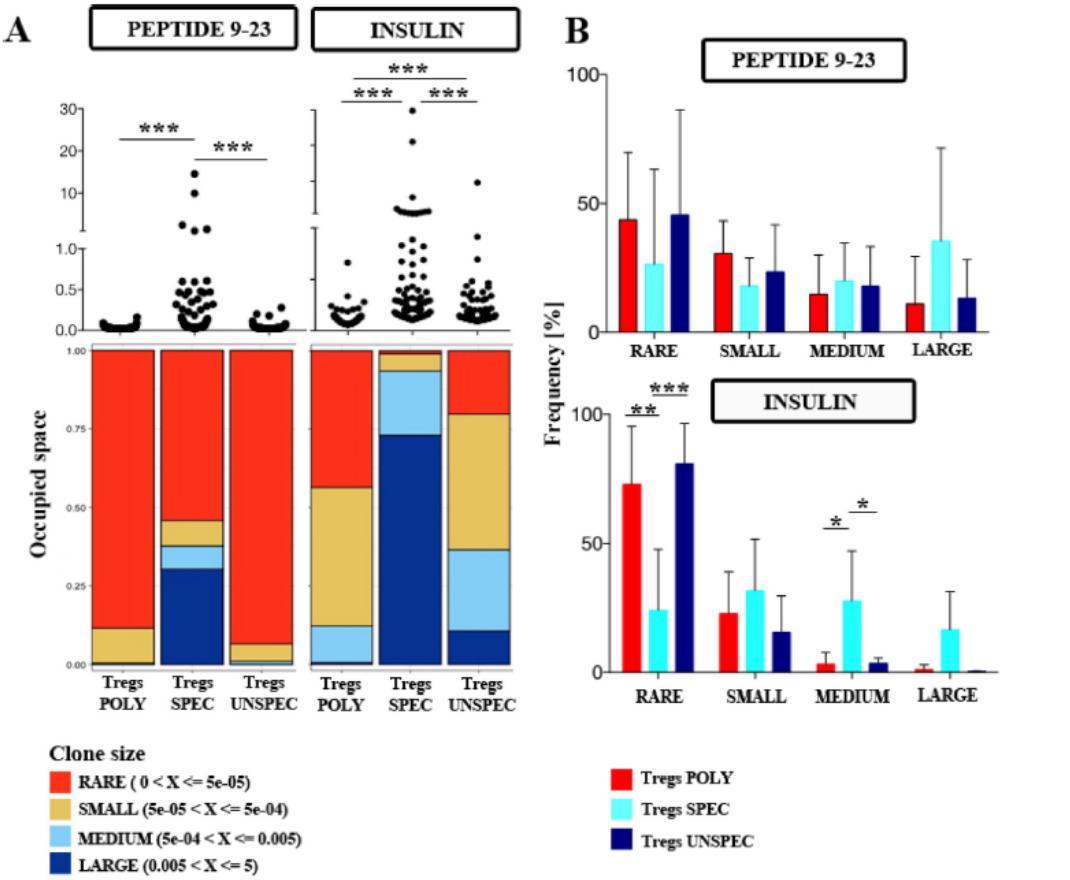

Figure 4. Clonotype distribution in stimulated Tregs repertoires. High-throughput TCRα chain sequencing was used to analyze clonotype frequencies in Tregs (reactive, polyclonal or unreactive). (A) Relative frequencies (%) of the 100 most abundant clonotypes are shown (top). The proportion of space occupied by clonotypes of a certain size range is plotted for POLY, SPEC and UNSPEC Tregs (bottom). Shown are the data from one exemplary sample for each peptide used. (B) The relative frequency of clonotypes of a certain size range is plotted for POLY, SPEC and UNSPEC Tregs from 3 samples. Significance was calculated using the Kruskal-Wallis test. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$. (Color version of figure is available online).

lymphocytes) to low fluorescence in proliferative cells (antigen-reactive Treg lymphocytes), allowed the separation and sorting of cells that could be further grown, expanded and used for treatment or in functional tests to verify their specificity and potency.

The possibility of regulating only immune cells reacting to a specific antigen is important from a therapeutic point of view. Physiologically, the immune system recognizes and destroys foreign and dangerous antigens, whereas it tolerates antigens arising from its own tissues. Nevertheless, in the case of autoimmune diseases such as multiple sclerosis, diabetes mellitus type 1, psoriasis, systemic lupus erythematosus and rheumatoid arthritis, this mechanism is compromised [6,15,37–41]. Effector lymphocytes recognize autoantigens as foreign and begin to destroy self-tissues. This process leads to irreversible changes. Currently, the treatment of autoimmune diseases is most often limited to pharmacological immunosuppression and inhibition of the inflammatory response. Such a therapy turns out to be ineffective over time [42]. Despite initial improvement, disease progression cannot be completely stopped, and interruption in therapy is usually associated with exacerbation of the disease. This treatment is also associated with a deep reduction in immunity [42]. Therefore, patients may develop susceptibility to infections, which, in patients receiving immunosuppressive drugs, have a more serious course than that seen in healthy people. Nonspecific immunosuppression also poses the risk of cancer, which is more common among patients receiving immunosuppressive drugs than in the general population [43,44]. The same applies to solid organ transplant recipients, who are in need of constant administration of strong immunosuppressive drugs to prevent organ rejection, whereas bone marrow transplant recipients require immunosuppression to avoid fatal GVHD [16,45]. The alternative to such excessive immunosuppression in all these cases can be precisely tailored antigen-specific cellular immunosuppression. This should be relatively easy in allogeneic solid organ transplants and bone marrow hematopoietic cell transplants, as alloantigens are very well defined as HLA mismatches, but it could be more difficult in autoimmune conditions, as the causative autoantigens are not clearly defined in many of them, and different autoantigens may be present in different stages of the disease [46].

The usefulness of antigen-specific Treg cells has been described in animal models, and a few years ago the first attempts to obtain such cells in humans were initiated. At the beginning, efforts were made to develop antigen-specific type 1 regulatory cells; later this was extended to natural Treg lymphocytes [46,47]. Recently, attempts have also been made to create extremely specific engineered Tregs with cloned TCR or chimeric antigen receptors specific against a

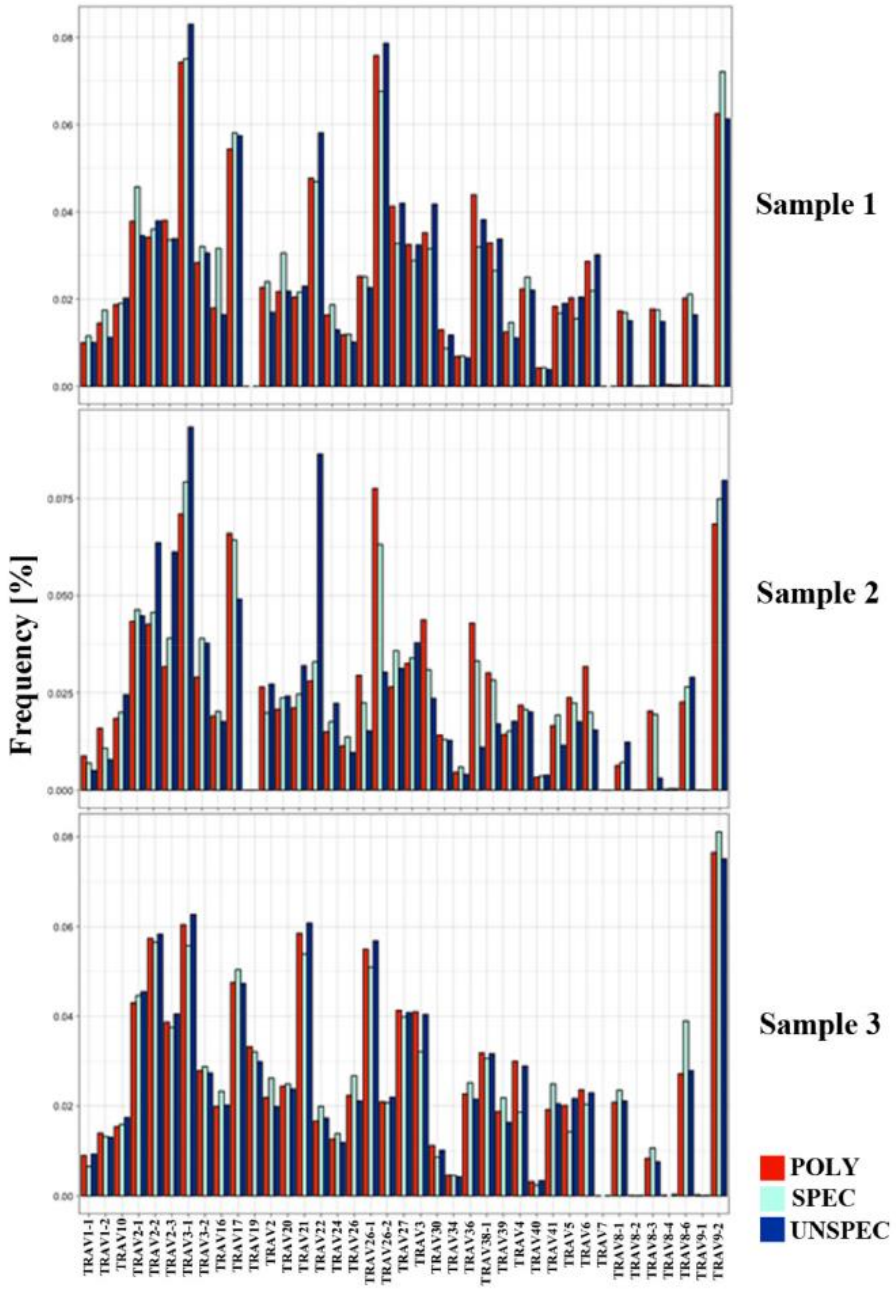

Figure 5. *TRAV* gene usage of stimulated Tregs repertoires. High-throughput TCRα chain sequencing was used to compare *TRAV* gene usage of Tregs in SPEC, POLY and UNSPEC cells. Shown are the frequencies (%) of the individual T-cell receptor alpha variable (*TRAV*) genes for 3 samples tested. (Color version of figure is available online).

particular antigen [46–48]. However, this approach may be too restrictive in the case of autoimmune diseases, as it restrains the response to a single antigen only, whereas a series of autoantigens and their epitopes may be responsible in such conditions. Moreover, these autoantigens change with the progression of disease [49]. When such an epitope spread occurs, a specificity toward several peptide sequences might be more efficient in the process of controlling the autoimmune response.

Our approach resulted in a large number of responding Treg (and Teff) clones. As shown in other immune repertoire studies, this type

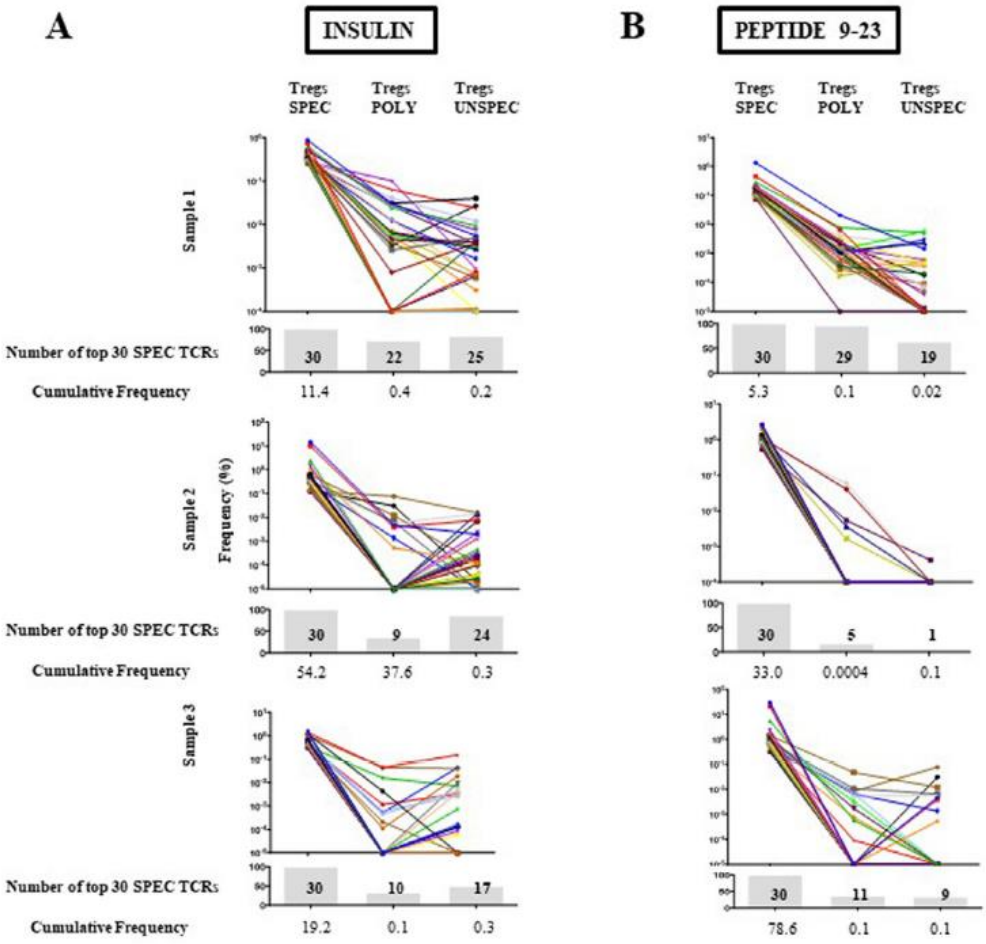

Figure 6. Sharing of the 30 most frequent reactive Tregs clonotypes. Tracking the 30 most frequent clonotypes of whole insulin (A) and insulin β chain peptide 9–23 (B) reactive Tregs repertoires from 3 samples in the repertoires of polyclonally and unspecifically stimulated Tregs obtained by high-throughput TCRα chain sequencing. Every colored line represents a particular clone. The y-axis shows the frequency (%). The bar plots show the counts of the 30 most frequent TCRs of the specific Tregs repertoires found in the other 2 repertoires. Counts are written in the bars. Numbers below the bar plots show the cumulative frequency of these 30 TCRs for each repertoire. (Color version of figure is available online).

of stimulation always activates dozens to even millions of different TCR clones [28,50], resulting in cultures being enriched with several clones of Treg cells responding to one antigen. This might be a biologically relevant finding, but it might also be due to the nature of the assay. One reason is cross-reactivity of TCRs from a number of clones toward the used antigen. Another is that the proliferating Tregs or Teffs include bystander-activated cells that dilute the true responder pool. This may result in a better understanding of the antigen-specific regulation of the immune response, which may be dependent on a group of clones rather than on a single cell clone. Acknowledging this, we should highlight that the response most probably involves both antigen-specific clones and cells activated via bystander mechanisms and cross-reactive cells. No doubt, the cytokine milieu and interactions between cells can also ignite unspecific responses [51]. Hence, we might describe the cells generated in the presented protocol as antigen-specific-enriched or antigen-reactive only. Nevertheless, the stronger suppression exerted by SPEC Tregs compared with UNSPEC Tregs in our study clearly confirms that such an enrichment improves the quality of Tregs when challenged with a particular antigen. An alternative to our approach could be the administration of low doses of IL-2, which has been found to stimulate preferentially specific Tregs [52]. Nevertheless, this approach should be combined with T-cell depletion. Otherwise, the sum of IL-2 administered exogenously and that produced by conventional CD4+ T cells in fully competent subjects is uncontrollable and may easily exceed the threshold when the cytokine activates preferentially T cytotoxic/effector cells and, as a result, accelerates autoimmunity or organ rejection [53,54].

Indeed, the final outcome of the immune response is very much dependent on the antigen that elicits it and the balance between Tregs and Teff cells activated by the challenge. Different peptides may differ in the strength of activation, as suggested by the differences in response to whole insulin and insulin β chain peptide 9–23 in our experiments. Another risk, notably in autoimmune diseases, may be a preferential affinity of the peptides toward Teffs instead of Tregs. As a result, an ignition of antigen-specific responses with such peptides may exaggerate autoimmunity instead of quenching it. As shown in our experiments, antigen-reactive Teff cells are much more

difficult to regulate than polyclonal Teffs when used as responders in functional tests. Our recent studies in patients with type 1 diabetes mellitus have confirmed that the same autoantigens—probably fragments of pro-insulin—activate both Tregs and Teff cells [55]. This additionally highlights the need for careful choice of antigen when antigen-specific therapy is considered, regardless of the specific cells or pure peptides being administered. It also implies that the preparation should be given as early as possible, if both regulatory and effector arms are activated by the same stimuli. This is mainly because Tregs expand slowly and could be quickly overtaken by autoreactive Teffs during the autoimmune process [20].

The proliferation of Tregs is a fragile process during which they easily turn into pro-inflammatory cells. For clinical use, a relatively high number of Treg cells is required. In many cases, Tregs have only low affinity to the antigens, and their stimulation is too weak to elicit any response or proliferation. Hence, the conditions under which a co-culture is carried out are crucial to provide vigorous proliferation and, at the same time, to maintain regulatory and suppressor properties of the cells. In our co-cultures, both conditions were met after the addition of anti-CD28 and anti-CD154 antibodies, which provided the missing second signal to Tregs. Interestingly, the proliferation was mainly dependent on anti-CD28 stimulation, whereas keeping the high percentage of FoxP3^{high}-specific Tregs depended on the synergistic activity of anti-CD28 and anti-CD154. Neither of the antibodies alone was able to induce the high percentage of FoxP3^{high}-specific Tregs seen in the co-cultures stimulated with the two antibodies together. Tregs specific for the presented antigen in the presence of anti-CD28 and anti-CD154 antibodies began to proliferate without losing stability, defined as expression of the FoxP3 factor and preserved activity in functional suppression assays. The inhibition of proliferation and IFNγ production exerted by the generated antigen-specific Treg cells was higher than the activity of the starting and expanded polyclonal Treg populations. The high expression of FoxP3^{high} together with the reactivity toward a specific antigen may explain the superior suppressor properties of antigen-reactive Tregs [15,17].

One million sorted Tregs allowed the expansion of around 200 000 to 400 000 Tregs reactive to the antigen. Depending on the total number of Tregs sorted from the peripheral blood, further expansion of such Tregs should yield tens to even hundreds of millions of Tregs in the final product. This is 1–2 logs less than in the case of a polyclonal product, but the specificity should make the antigen-reactive product much more effective.

Taken together, we see a potential for the use of specific/reactive Tregs produced by stimulation with monocytes loaded with a specific antigen as targeted therapy—for example, in type 1 diabetes. We furthermore believe that our study would be a good starting point for the development of cellular drugs in other autoimmune diseases in which a specific antigen is known and treatment enables the use of Tregs.

Conclusions

Using this method, we are able to manufacture a cellular product enriched with antigen-reactive Tregs. The most important features of this protocol are the use of monocytes loaded with a specific antigen and the use of a combination of anti-CD28 and anti-CD154 antibodies to activate the proliferation of antigen-reactive Tregs.

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Declaration of Competing Interest

DI-G, MG and PT are co-inventors of a patent related to the presented content. DI-G, MG, NMT and PT are members of COST Action BM1305 AFACTT, supported by COST (European Cooperation in Science and Technology), which is part of European Union Framework Programme Horizon 2020.

Author Contributions

Conception and design of the study: DI-G, MG and PT. Acquisition of data: DI-G, MG, MP, NM-T and PT. Analysis and interpretation of data: DI-G, MG, AE, MP, AD, NM-T and PT. Drafting or revising the manuscript: DI-G, MG, AE and PT. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.07.001.

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Antigenic Challenge Influences Epigenetic Changes in Antigen-Specific T Regulatory Cells

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Background: Human regulatory T cells (Tregs) are the fundamental component of the immune system imposing immune tolerance *via* control of effector T cells (Teffs). Ongoing attempts to improve Tregs function have led to the creation of a protocol that produces antigen-specific Tregs, when polyclonal Tregs are stimulated with monocytes loaded with antigens specific for type 1 diabetes. Nevertheless, the efficiency of the suppression exerted by the produced Tregs depended on the antigen with the best results when insulin β chain peptide 9-23 was used. Here, we examined epigenetic modifications, which could influence these functional differences.

Methods: The analysis was pefromed in the sorted specific (SPEC, proliferating) and unspecific (UNSPEC, non-proliferating) subsets of Tregs and Teffs generated by the stimulation with monocytes loaded with either whole insulin (INS) or insulin β chain peptide 9-23 (B:9-23) or polyclonal cells stimulated with anti-CD3/anti-CD28 beads (POLY). A relative expression of crucial Tregs genes was determined by qRT-PCR. The Treg-specific demethylated region (TSDR) in FoxP3 gene methylation levels were assessed by Quantitative Methylation Specific PCR (qMSP). ELISA was used to measure genomic DNA methylation and histone H3 post-translational modifications (PTMs).

Results: Tregs $SPEC_{B:9-23}$ was the only subset expressing all assessed genes necessary for regulatory function with the highest level of expression among all analyzed conditions. The methylation of global DNA as well as TSDR were significantly lower in Tregs $SPEC_{B:9-23}$ than in Tregs $SPEC_{INS}$. When compared to Teffs, Tregs were characterized by a relatively lower level of PTMs but it varied in respective Tregs/Teffs pairs. Importantly, whenever the difference in PTM within Tregs/Teffs pair was significant, it was always low in one subset from the pair and high in the other. It was always low in Tregs $SPEC_{INS}$ and high in Teffs $SPEC_{INS}$, while it was high in Tregs $UNSPEC_{INS}$ and low in Teffs $UNSPEC_{INS}$. There were no differences in Tregs/Teffs $SPEC_{B:9-23}$ pair and the level of modifications was low in Tregs $UNSPEC_{B:9-23}$ and high in Teffs $UNSPEC_{B:9-23}$. The regions of PTMs in which differences were significant overlapped only partially between particular Tregs/Teffs pairs.

Conclusions: Whole insulin and insulin β chain peptide 9-23 affected epigenetic changes in CD4$^+$ T cells differently, when presented by monocytes. The peptide preferably favored specific Tregs, while whole insulin activated both Tregs and Teffs.

Keywords: TSDR, antigen-specific, DNA methylation, histone H3, gene expression, epigenetics

INTRODUCTION

T regulatory cells (Tregs) constitute a subset of CD4$^+$ T lymphocytes which is pivotal in immune tolerance due to their ability to suppress effector cells. There are two main subpopulations of Tregs: natural (nTregs or tTregs) which develop in the thymus during thymopoesis and peripheral (pTregs) which differentiate from naïve CD4$^+$T cells in the periphery during TCR stimulation in the presence of cytokines (e.g. IL-2, TGF-β). Natural T regulatory cells (CD4$^+$CD25^{high}CD127$^-$FoxP3$^+$; Tregs) are mainly predisposed to exert suppressive functions over effectors, which is highlighted by stable genomic architecture in this subset of Tregs. Transcriptional factor FoxP3 (forkhead box P3) is a master regulator of Tregs. Its expression in Tregs is kept stable *via* Treg-specific demethylated region (TSDR) in the promoter of *FoxP3* gene. The sustained expression of FoxP3, possible due to demethylated TSDR, allows the expression of a wide range of other genes encoding such as: *Eos, GITR, CTLA4*, and simultaneously suppresses activation of: IL-2, IL-4 and INF-γ(1–4). Other important function-associated genes in Tregs are: *IL2RA* (CD25), *CTLA4* (CD152), *TNFRSF18* (GITR), *IKZF2* (Helios), *IKZF4* (Eos) and *Tet2* (5–8).

Tregs ability to prevent excessive immune response has been tested in many clinical trials. In human autoimmune diseases or transplantation, a broken tolerance can be restrained by administration of Tregs. In our hands, the therapy with expanded Tregs was successfully administered in type 1 diabetes or graft versus host disease (GvHD) after bone marrow transplantation (9–11). Until now, mainly polyclonal Tregs have been used in clinical therapies due to the problem in technical expansion of antigen-specific cells (12, 13). Only recently, we have developed a technique, which allows for efficient production of bulk quantities of antigen-specific Tregs, which seems to be a promising tool for autoimmune therapies (14). Our method is based on antigen-loaded monocytes which preferentially activate Tregs specific to presented antigen. Because our work is mainly focused on type 1 diabetes, we used either whole insulin or insulin β chain peptide 9-23 as antigens. Surprisingly, we have found that Tregs generated with β chain peptide 9-23 were significantly more suppressive than those generated with the whole insulin.

Looking for the reasons of such a difference, we examined epigenetic features, presented at **Figure 1**, of both: Tregs (CD4$^+$CD25$^{high/+}$CD127$^-$) and T effector cells (CD4$^+$CD25$^{low/-}$CD127$^+$; Teffs) generated with monocytes loaded with either whole insulin or insulin β chain peptide 9-23 sorted as antigen-specific (index SPEC) cells. We have also looked at Tregs and

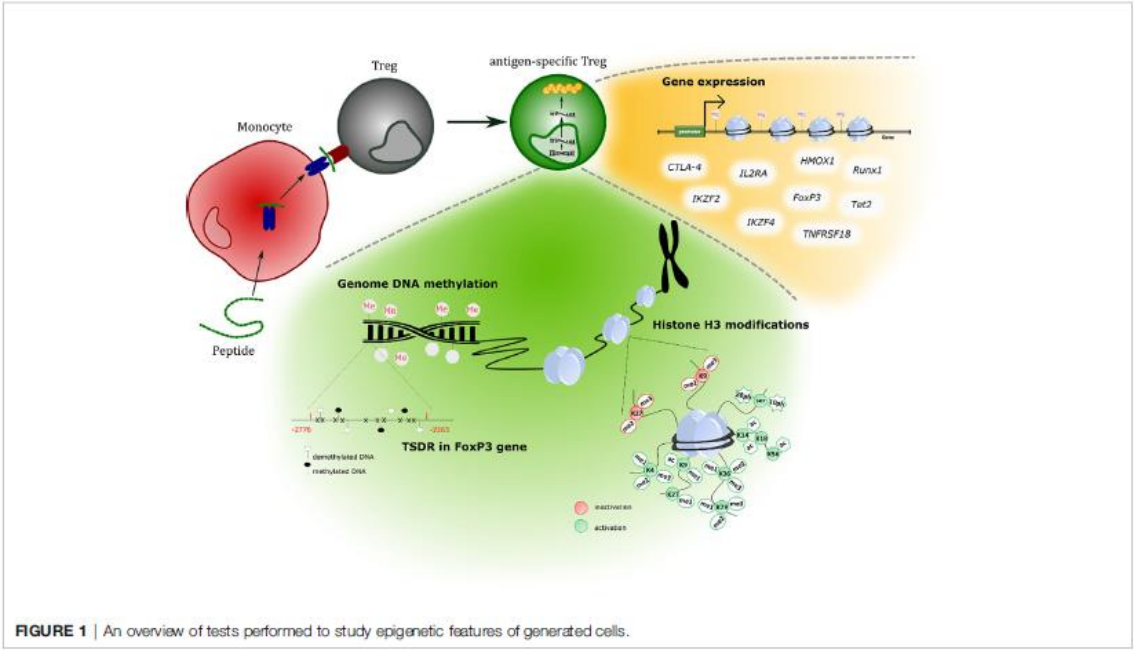

FIGURE 1 | An overview of tests performed to study epigenetic features of generated cells.

Teffs unspecific to the antigens (index UNSPEC) as well as those expanded with anti-CD3/anti-CD28 beads used currently as the polyclonal (index POLY, 1:1 ratio bead:cell) in the treatment of type 1 diabetes. Taking into account already known TSDR-mediated regulation of *FoxP3* gene, we assumed that other epigenetic changes could be also very important in the activity of the manufactured cells and therefore we investigated global genomic DNA methylation, methylation in specific TSDR region of *FoxP3* gene and histone H3 post-translational modifications (PTMs). In addition, we assessed in all subsets the expression of genes crucial in the activity of Tregs, such as: *FoxP3, CTLA-4, IKZF2, IKZF4, IL2RA, TNFRSF18, Tet2, Runx1* and *HMOX1*. Indeed, we found significant differences between the subsets, which could impact the activity of the cells.

MATERIALS AND METHODS

Research Material

Buffy coats, with unknown HLA, were obtained from the Regional Centre for Blood Donation and Treatment in Gdańsk from volunteers donating blood. All tests were conducted on male volunteers aged 18-65.

Cells Preparation

Detailed procedure for cells preparation was described by Iwaszkiewicz-Grzes D. et al. previously (14). General procedure is presented at the workflow in **Figures 2** and **S1**.

Tregs and Teffs were freshly isolated from buffy coats obtained from anonymous healthy volunteer blood donors according to previously described protocol (15). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by gradient centrifugation using Ficoll-Paque Plus. Collected PBMCs were counted and separated into two tubes, first tube for isolation of $CD4^+$ cells using negative immunomagnetic selection method (StemCell EasySep™ Human CD4 Negative Selection Kit, StemCell Technologies, Canada) and second tube for isolation of CD14+ cells using positive immunomagnetic selection method (StemCell EasySep™ Human CD14 Positive Selection Kit, StemCell Technologies, Canada).

$CD4^+$ T cells were transferred into cell culture flask in X-VIVO culture medium with addition of penicillin/streptomycin and remained to the next day under standard conditions (37°C, 5% CO_2, 95% O_2). At the same time, $CD14^+$ monocytes (Mo) were isolated according to the manufacturer's instructions. Next, $CD14^+$ cells were suspended in X-VIVO culture medium (Lonza), spread out into plates and stimulated for 24h with tested antigens: whole insulin (index INS, 350µg/well/ml (16), Actrapid® Penfill®, Novo Nordisk A/S) and insulin β chain peptide (index B:9-23, 25µg/well/ml; Lipopharm; Gdansk, Poland) under standard conditions (37°C, 5% CO_2, 95% O_2).

After 24h $CD4^+$ T cells were stained with monoclonal antibodies (mAbs): CD3, CD4, CD25 and CD127, and sorted with FACS AriaII sorter (BD Biosciences, USA) into Tregs ($CD3^+CD4^+CD25^{high/+}CD127^{-/low}lin^-doublet^-$) and Teffs ($CD3^+CD4^+CD25^{low/-}CD127^+lin^-doublet^-$).

Next, Tregs and Teffs were stained with violet (Cell Trace Violet Cell Proliferation Kit, Life Technologies, 1µM; 15min, 37°C) (17). At the same time, previously prepared monocytes were collected and irradiated.

We prepared the following conditions: Tregs/Mo stimulated with INS or B:9-23, Tregs/beads (ExpAct Treg Beads conjugated to CD28, Anti-Biotin, and CD3-Biotin monoclonal antibodies; MACS®GMP; in 1:1 ratio (bead:cell)), Teffs/Mo stimulated with INS or B:9-23 and Teffs/beads in X-VIVO culture medium with addition of IL2 (100 IU/ml), heat-inactivated human serum (10%) and penicillin/streptomycin. Cultures containing monocytes were additionally stimulated with anti-CD28 and anti-CD154 antibodies at a final concentration of 5µg/ml/well each (BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD154/CD28). After 6 days of expansion cells were collected and sorted based on violet fluorescence (**Figure S1**) (17). Cells responding to the antigen presented by the monocytes were identified as antigen-specific (index SPEC), non-proliferating cells were identified as unspecific (index UNSPEC). During whole procedure cells were stimulated only once with monocytes. Cells stimulated with beads were treated as polyclonal (specific against many antigens, index POLY).

Obtained cells were expanded with beads (no longer than 5 days) in order to obtain enough cells for all tests, at least 1 million cells per condition (Tregs: POLY, SPEC, UNSPEC; Teffs: POLY, SPEC, UNSPEC) for cells stimulated with whole insulin and insulin β chain peptide 9-23 and stored in -70°C maximum 1 month.

RNA Extraction and RT-qPCR

Total RNA was isolated using AllPrep® DNA/RNA Mini Kit (Qiagen, USA) following the manufacturer's instruction. Assessment of RNA concentration and purity was measured *via* spectrophotometer (Epoch, BioTek). Obtained RNA was stored in -70°C until use.

FIGURE 2 | The scheme of obtaining cells and their further use.

500ng total RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) under standard conditions: step 1 - 25°C/10 min; step 2 - 37°C/120 min; step 3 - 85°C/5 min; step 4 - 4°C/∞ min. The expression of target genes, characteristic for Tregs: *FoxP3, IKZF2, IKZF4, IL2RA, TNFRSF18, Tet2, Runx1* and *HMOX1* was detected using FastStart Essential DNA Probes Master (Roche, Switzerland) on LightCycler®96 (Roche, Switzerland) in accordance to the manufacturer's protocols in prepared cell populations (Tregs: POLY, SPEC, UNSPEC; Teffs: POLY, SPEC, UNSPEC). The primer sequences (Sigma-Aldrich) and used probes (Universal ProbeLibrary Set, Human with Probes; Roche, Switzerland) were designed using Assay Design Center Software (Roche) and are listed in **Table 1**. GAPDH was used as the housekeeping gene and the normalized expression ratio of the target genes in prepared cell populations was calculated using the $2^{-\Delta\Delta Ct}$ (Livak method) (18). All reactions were carried out in triplicate from three independent experiments.

Genomic DNA Extraction and Global DNA Methylation

DNA was isolated using AllPrep® DNA/RNA Mini Kit (Qiagen, Germany) following the manufacturer's instruction from following cells: Tregs: POLY, SPEC, UNSPEC; Teffs: POLY, SPEC, UNSPEC for cells stimulated with whole insulin and insulin β chain peptide 9-23. Assessment of DNA concentration and purity was measured *via* spectrophotometer (Epoch, BioTek). Obtained DNA was stored in -20°C until use.

Quantification of genomic DNA methylation was performed using Methylated DNA Quantification Kit (Colorimetric) (Abcam, UK). 100 ng/µl of DNA was used per reaction (well) under manufacturer's instruction. Absorbance was read at 450 nm *via* Epoch (BioTek) spectrophotometer with Gene5 software. Obtained absorbance was used to calculate percentage of genomic DNA methylation in each cell population. The total/global amount of methylated DNA was calculated by generation of a standard curve. Next, the slope (OD/ng) of the standard curve was determined using linear regression and then the analysis of absolute/total quantification of 5-mC in total DNA was determined.

Histone Extraction and Histone H3 Modification

Cells stimulated with beads, whole insulin or insulin β chain peptide 9-23 (Tregs: POLY, SPEC, UNSPEC; Teffs: POLY, SPEC, UNSPEC) were pelleted and histones isolated with Histone Extraction Kit (Abcam, UK) according to the manufacturer's instructions. Two Assay Control Proteins were prepared with the final concentrations of 5ng/µl and 25ng/µl. 150ng of histone extract per well for each modification were used in triplicate. Assessment of histone concentration and quality were measured *via* spectrophotometer Epoch (BioTek). 21 histone H3 modifications, which include the most important and the most well characterized patterns, were measured using Histone H3 Modification Multiplex Assay Kit (Colorimetric, Abcam) and are listed in **Table 2**. Obtained absorbance was used to calculate % of individual histone H3 modifications.

DNA Bisulfite Conversion and Methylation-Specific PCR

Quantitative methylation-specific polymerase chain reaction with methylated (M) and unmethylated (U) primers was used for detection of methylation of the TSDR region in *FoxP3* gene. Briefly, genomic DNA was extracted from maximum 1 million of cells using AllPrep® DNA/RNA Mini Kit (Qiagen, Germany) and submitted to bisulfite conversion using the EpiTect® Bisulfite Kit (Qiagen, Germany) under manufacturer's instruction. First, DNA bisulfite conversion was performed in which unmethylated cytosine

TABLE 1 | Sequences of primers used for real-time PCR.

Gene Name	RefSeq Accession Number	Primer	Amplicon Size (nt)	%GC	Tm	Primer Sequence (5'-3') Forward	Probe #
GAPDH*	NM_001289745.1	Fw	70	45	60	CCCCGGTTTCTATAAATTGAGC	#75
		Rv		58	59	GGCTGACTGTCGAACAGGA	
FoxP3	NM_014009.3	Fw	102	55	59	ACACTGCCCCTAGTCATGGT	#25
		Rv		50	60	GAGCTGGTGCATGAAATGTG	
CTLA-4	NM_005214.4	Fw	65	56	60	TGGGTCCCAGGGAAGTTT	#25
		Rv		50	60	TGACCTTGTGTTCTACCTGGTG	
IKZF2	NM_016260.2	Fw	64	45	59	CATCACATTGCTTTGCCCTA	#61
		Rv		48	59	TCATCACTGTCAGAGAGAGGCTA	
IKZF4	NM_022465.3	Fw	68	45	60	TCAGGCATTTGTTGTGCAGT	#3
		Rv		53	59	AGGGAAAGGCAGATGCTGT	
IL2RA	NM_000417.2	Fw	73	55	59	CCAACTTCCCAGTTCAGGAG	#45
		Rv		44	59	GGGTAGAGTGTGTGTGTTGTGTATT	
TNFRSF18	NM_004195.2	Fw	92	61	59	ACCTGGGTCGGGATTCTC	#10
		Rv		61	59	CACAGCCAGTTGGACACG	
Tet2	NM_001127208.2	Fw	93	36	59	AAAGATGAAGGTCCTTTTTATACCC	#68
		Rv		48	59	ACCCTTCTGTCCAAACCTTTC	
Runx1	NM_001754.4	Fw	61	41	60	CCAAAGAGTGTGGAATTTTGGT	#55
		Rv		50	59	AAACAGGGCGAGTTGCAT	
HMOX1	NM_002133.2	Fw	61	55	59	CCCTTCAGCATCCTCAGTTC	#84
		Rv		58	59	GACAGCTGCCACATTAGGG	

GAPDH was used as an endogenous control.

TABLE 2 | Histone H3 modifications.

Methylation	Acetylation	Phosphorylation
H3K4me1↑	H3K9ac↑	H3ser10ph↑
H3K4me2↑	H3K14ac↑	H3ser28ph↑
H2K4me3↑	H3K18ac↑	
H3K9me1↑	H3K56ac↑	
H3K9me2↓		
H3K9me3↓		
H3K27me1↑		
H3K27me2↓		
H3K27me3↓		
H3K36me1↑		
H3K36me2↑		
H3K36me3↑		
H3K79me1↑		
H3K79me2↑		
H3K79me3↑		

↑ *activating modification.*
↓ *inactivating modification.*

residues are deaminated to uracil and methylated cytosine (5-mC) residues remain intact. 500 ng of isolated DNA per reaction were used. Bisulfite reaction was performed in the thermocycler with the following parameters: 95°C for 5 min, 60°C for 25 min, 95°C for 5 min, 60°C for 85 min, 95°C for 5 min and 60°C for 175 min. After conversion, DNA was subjected to quantitative methylation-specific PCR procedure using TB Green Premix Ex Taq II (Takara, Japan) on LightCycler®96 (Roche, Switzerland) in accordance with the manufacturer's protocols. EpiTect® PCR Control DNA Set (Qiagen, Germany) was used as positive and negative controls. Unconverted DNA was considered as a negative control. Methylated and unmethylated primers for *FoxP3* gene intron 1 were designed using MethPrimer 2.0 Software by Zafari et al. and are listed in **Table 3** (19, 20). Real-time PCR was performed in a final reaction volume of 20 µl using the Roche Life Science LightCycler® 96 including 5 pmol of each forward and reverse methylated/demethylated primer and 50–100 ng of bisulfite-treated genomic DNA. PCR consisted of an initial denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, followed by 60°C for 30 s, 1 cycle of melting at 95°C for 5 s, followed by 60°C for 1 min, 1 cycle of cooling at 50°C for 30 s. The level of methylation was also verified by electrophoresis on a 2% agarose gel using 100 bp DNA Ladder (Invitrogen, USA) as a marker.

Statistics

The Statistica 13.0 software was used to perform all statistical analysis. Significance was calculated using the *t*- test. Significant results are marked with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

RESULTS

Influence of Antigen Stimulation on Gene Expression

In the current study we selected several genes known to be involved in the function of Tregs and investigated their expression in particular subsets of cells. We compared polyclonal Tregs, which are a cellular medicinal product used in the treatment of type 1 diabetes, with Tregs specific and unspecific against whole insulin (index INS) or insulin β chain peptide 9-23 (index B:9-23). Polyclonal Tregs were treated as a reference for other cells. To obtain fully valuable results, we also performed the same tests for effector T cells: polyclonal, specific and unspecific against presented antigen. The obtained results are presented in **Figure 3**, and the p values of statistical significance in **Table S1**.

Figure 3A shows gene expression in all populations in a form of a heatmap. With the exception of the IKZF4 gene, Tregs showed higher expression of the studied genes than Teffs. Teffs $SPEC_{INS}$ were the only ones among the effectors which showed low but noticeable expression of IKZF4, TNFRSF18 and HMOX1 genes. Among Tregs subsets (**Figure 3B**), Tregs $SPEC_{B:9-23}$ and Tregs POLY were of special interest as they expressed all analyzed genes. Tregs $SPEC_{B:9-23}$ showed the highest levels of expression of RUNX1 and IKZF4 genes but the expression of other genes was moderate. On the other hand, Tregs $SPEC_{INS}$ were the cells with the lowest expression of IKZF2, FoxP3, IKZF4, Tet2 and RUNX1 genes. Tregs $UNSPEC_{INS}$ also showed low expression of the majority of genes, especially IL2RA, HMOX1 and FoxP3, when compared to Tregs $SPEC_{B:9-23}$.

We could observe statistically significant differences between particular subsets in the expression of five genes: FoxP3, IKZF2, IKZF4, IL2RA and TNFRSF18 which are presented separately in **Figure 3C**. Values of statistical significance for gene expression are presented in **Table S1**.

The Influence of Antigen Stimulation on Genomic DNA Methylation

Based on a colorimetric assay for quantification of global DNA methylation by measuring levels of 5-methylocytosine (5-mC) we observed statistically significant differences between cells (**Figure 4**). Tregs $SPEC_{INS}$ and Tregs $UNSPEC_{B:9-23}$ had the highest level of methylation. Tregs $SPEC_{B:9-23}$ were much less affected. Their methylation differed significantly from Tregs $UNSPEC_{B:9-23}$ (t-test; $p < 0.0001$), Tregs $SPEC_{INS}$ (t-test; $p < 0.0001$) and Teffs $SPEC_{B:9-23}$ (t-test; $p < 0.0001$). Tregs

TABLE 3 | Primers for TSDR analysis (19).

Primer set	Primer	Primer Sequence (5'-3') Forward	PCR product size	Tm
demethylated	Fw	GGATAGGGTAGTTAGTTTTTGGAATG	117	62.6
	Rv	CCACCATTAACATCATAACAACCA		64.1
methylated	Fw	GATAGGGTAGTTAGTTTTCGGAAC	116	59.9
	Rv	CGCCATTAACGTCATAACG		64.9

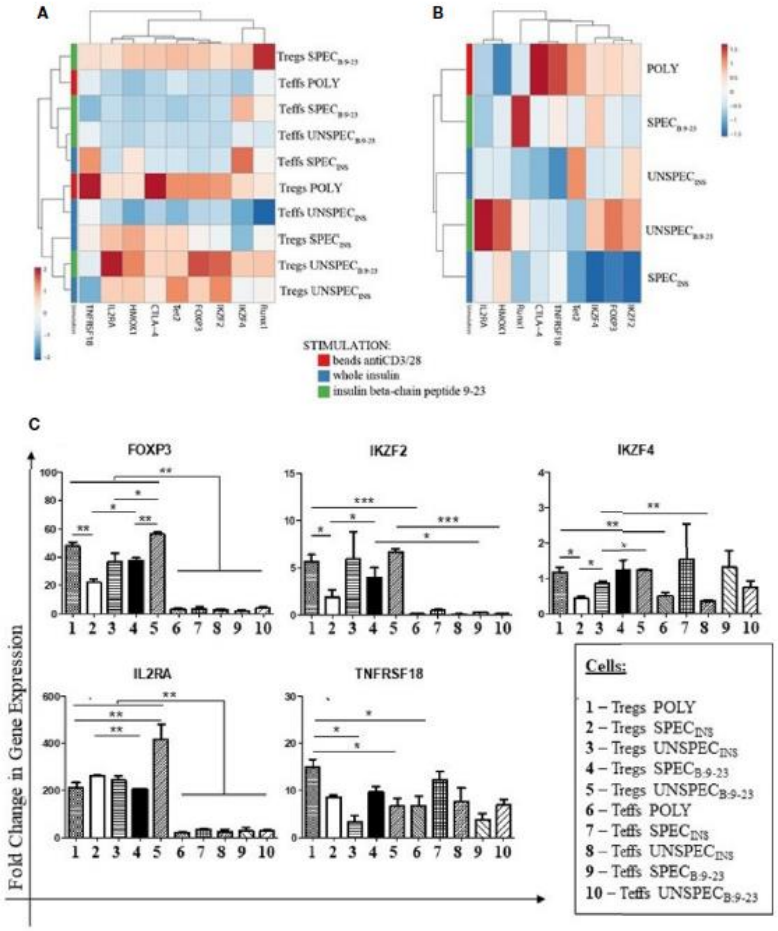

FIGURE 3 | Fold Change in Gene Expression in Tregs and Teffs. **(A):** Clustering and heat maps analysis of gene expression in Tregs: POLY, SPEC, UNSPEC and Teffs: POLY, SPEC, UNSPEC. **(B)** Clustering and heat maps analysis of gene expression only in Tregs. **(C)** Graphs presenting changes in cells populations. Only the expressions in which statistical significance occurred are presented. The cells were stimulated with beads anti-CD3/anti-CD28 (index POLY) or monocytes loaded with whole insulin (index INS) or insulin β chain peptide 9-23 (index B:9-23). The cells responding to antigen have an index SPEC, cells not recognizing the antigen have an index UNSPEC. All stimulations were performed in triplicates (three tests for whole insulin and three tests for insulin β chain peptide 9-23). The results are presented as mean+/- SD. Significance was calculated using the t- test, significant results are marked with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). Heat maps were prepared using ClustVis tool based on correlation distance and average linkage between clusters (21).

$SPEC_{B:9-23}$ were also significantly different from antigen-specific Teffs stimulated with the same antigen (Teffs $SPEC_{B:9-23}$; t-test; $p<0.0001$). We did not observe significant differences between Tregs POLY and other cells.

The Influence of Antigen Stimulation on TSDR Methylation in FoxP3 Gene

We next used quantitative methylation-specific polymerase chain reaction with methylated and unmethylated primers for detection of methylation in TSDR region of FoxP3 gene. TSDR in all Tregs showed a level of demethylation over 75%, which was significantly more demethylated than TSDR of Teffs (t-test; $p<0.0001$) (**Figure 5A**). These results were also confirmed by agarose gel electrophoresis (**Figure 5B**). Tregs $SPEC_{INS}$ showed the lowest level of demethylation (75%) among Tregs subsets. It was significantly less compared to Tregs $UNSPEC_{INS}$ (t-test; $p=0.0020$) and Tregs $SPEC_{B:9-23}$ (t-test; $p=0.0065$). Tregs POLY, whose demethylation was ≈80%, were significantly less demethylated than each of the three subsets of Tregs: Tregs $SPEC_{B:9-23}$ (t-test; $p=0.0229$), Tregs $UNSPEC_{B:9-23}$ (t-test;

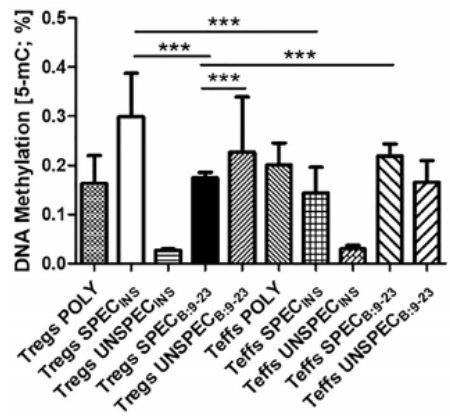

FIGURE 4 | Global DNA methylation in Tregs and Teffs. Comparison of 5-methylocytosine (5-mC) production in Tregs: POLY, SPEC, UNSPEC and Teffs: POLY, SPEC, UNSPEC. During the tests cells were stimulated with beads anti-CD3/ant-CD28 (index POLY) or monocytes loaded with whole insulin (index INS) or insulin β chain peptide 9-23 (index B:9-23). Cells responding to antigen have an index SPEC, cells not recognizing the antigen have an index UNSPEC. All analyzes were performed for six tests (three tests for whole insulin and three tests for insulin β chain peptide 9-23) in triplicate. The results are presented as mean+/- SD. Significance was calculated using the t- test, significant results are marked with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

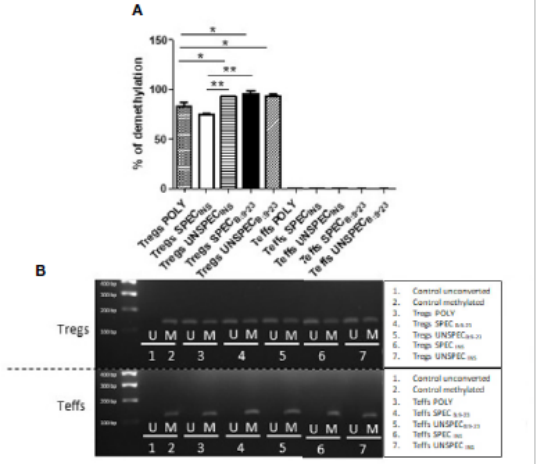

FIGURE 5 | FoxP3 demethylation in TSDR region in Tregs and Teffs. **(A)** Comparison of percentage of demethylation in Treg-specific demethylated region (TSDR) in Tregs: POLY, SPEC, UNSPEC and Teffs: POLY, SPEC, UNSPEC. **(B)** Representative results showing FoxP3 demethylation in all cell cultures was detected by agarose gel electrophoresis [reaction with methylated (M) and unmethylated (U) primer]. During the tests cells were stimulated with anti-CD3/anti-CD28 beads (index POLY) or monocytes loaded with whole insulin (index INS) or insulin β chain peptide 9-23 (index B:9-23). Cells responding to the antigen have an index SPEC, cells not recognizing presented antigen have an index UNSPEC. All analyzes were performed for six tests (three tests for whole insulin and three tests for insulin β chain peptide 9-23) in triplicate. The results are presented as mean+/- SD. Significance was calculated using the t- test, significant results are marked with *($p<0.05$), **($p<0.01$) or ***($p<0.001$).

$p=0.0433$) and Tregs UNSPEC$_{INS}$ (t-test; $p=0.0451$). All these three remaining Treg subsets showed demethylation over 90%.

The Influence of Antigen Stimulation on Histone H3 Modifications

The total concentration of histone H3 protein and individual modifications in ng was calculated and compared at heat-maps (**Figure S2**). Then, based on Total H3, the percentage of different cell modifications was determined (**Figure 6**). We analyzed gene activating modifications (↑) such as methylation: H3K4me(1-3), H3K9me1, H3K27me1, H3K36me(1-3), H3K79me(1-3); acetylation: H3K9ac, H3K14ac, H3K18ac, H3K56ac; phosphorylation: H3ser28P, H3ser10P and gene inactivating modifications (↓) such as methylation: H3K9me2, H3K9me3, H3K27me2, H3K27me3 (**Table 2**). All cultures were performed in triplicates.

The comparison of the precentage of histone H3 modifications in total H3 between Tregs and Teffs (**Figure 6A**) confirmed that the populations of Tregs showed opposite pattern of modifications than Teffs. Teffs, mainly Teffs SPEC$_{B:9-23}$, Teffs SPEC$_{INS}$ and Teffs UNSPEC$_{B:9-23}$, were characterized by a relatively high level of modifications.

While studying the differences between differently stimulated Tregs (**Figure 6B**), Tregs POLY exhibited the highest level of histone modification, when compared to monocyte stimulated cells. When comparing antigen-specific subsets, we could observe that Tregs SPEC$_{INS}$ showed a lower level of modification than Tregs SPEC$_{B:9-23}$, excluding H3K18ac, H3K9me1, H3K9me3 and H3K36me2.

Interestingly, when compared to Teffs, Tregs were characterized by a relatively lower level of PTMs but it varied in respective Tregs/Teffs pairs (**Figure 6C**, **Table 4**). Importantly, whenever the difference in PTMs within Tregs/Teffs pair was significant, it was always low in one subset from the pair and always high in the other. The level of modifications in Tregs SPEC$_{INS}$ was significantly lower than that in Teffs SPEC$_{INS}$ in 7 out of 11 regions, in which any significant differences occurred (H3K4me3, H3K9me2, H3K27me1, H3K79me1, H3K79me3, H3K14ac and, H3ser28P). At the same time, there was no single modification, which level was different between Tregs SPEC$_{B:9-23}$ and Teffs SPEC$_{B:9-23}$ (**Figure 6C**). Tregs UNSPEC$_{INS}$ showed a significantly higher degree of modifications than Teffs UNSPEC$_{INS}$ in 5 out of 11 regions, in which any significant differences occurred (H3K9me2, H3K27me3, H3K79me1, H3K79me3 and, H3K14ac) (**Figure 6C**). The level of modifications in Tregs UNSPEC$_{B:9-23}$ was significantly lower than that in Teffs UNSPEC$_{B:9-23}$ in 6 out of 11 regions, in which any significant differences occurred (H3K27me3, H3K36me1, H3K79me1, H3K9ac, H3K14ac and, H3ser28P). Interstingly, H3K79me1 was the only one modified

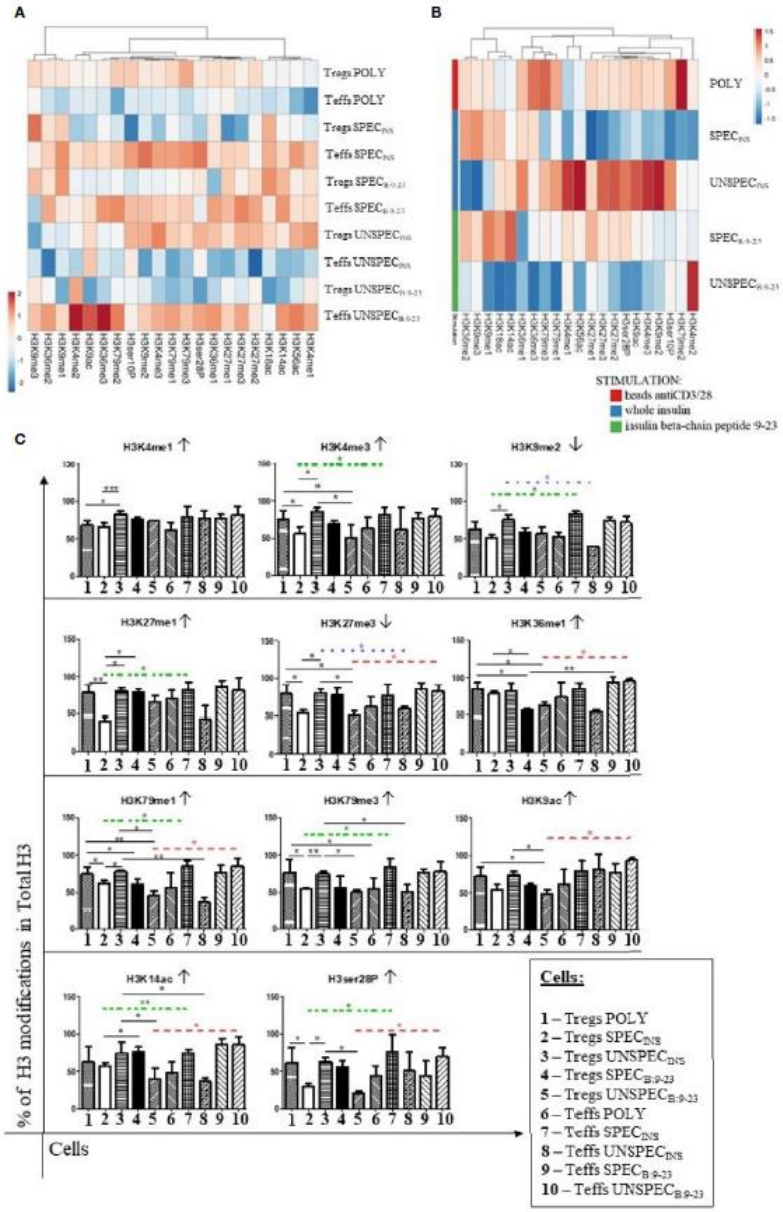

FIGURE 6 | Percentage of histone H3 modification in total H3 in Tregs and Teffs. **(A)** Comparison of percentage of modifications in Tregs: POLY, SPEC, UNSPEC and Teffs: POLY, SPEC, UNSPEC. **(B)** Comparison of modifications only in Tregs. Cells were stimulated with anti-CD3/anti-CD28 beads (index POLY) or monocytes loaded with whole insulin (INS) or insulin β chain peptide 9-23 (B:9-23). **(C)** Graphs presenting changes in cells populations. Only modifications in which statistical significance occurs are presented. Cells responding to the antigen have an index SPEC, cells not recognizing the antigen have an index UNSPEC. All analyzes were performed for six tests (three tests for whole insulin and three tests for insulin β chain peptide 9-23) in triplicate. The results are presented as mean+/- SD. Significance was calculated using the t- test, significant results are marked with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). Heat maps were prepared using ClustVis tool based on correlation distance and average linkage between clusters (21). ↑ - activating modification; ↓ - inactivating modification; ···· difference between specific Tregs and Teffs stimulated with insulin; ···· difference between unspecific Tregs and Teffs stimulated with insulin; ···· difference between unspecific Tregs and Teffs stimulated with peptide 9-23.

TABLE 4 | Selectivity index (SI) for histone modifications.

Histone modification	SI [Teffs/Tregs]				
	POLY	$SPEC_{INS}$	$UNSPEC_{INS}$	$SPEC_{B:9-23}$	$UNSPEC_{B:9-23}$
H3K4me1	0,90	1,22	0,93	1,02	1,11
H3K14ac	0,78	**1,31**	0,49	1,13	**2,16**
H3K56ac	0,81	1,22	0,72	1,05	1,56
H3K4me3	0,84	**1,44**	0,73	1,12	1,59
H3K9me2	0,83	**1,64**	**0,51**	1,28	1,28
H3K27me1	0,89	**2,00**	0,53	1,08	1,24
H3K27me3	0,79	1,43	**0,75**	1,09	**1,62**
H3K36me2	0,78	1,07	0,60	1,06	1,37
H3K79me1	0,75	**1,37**	0,47	1,26	**1,86**
H3K79me3	0,70	**1,56**	0,67	1,35	1,56
H3ser28P	0,72	**2,55**	0,80	0,79	**3,30**
H3K4me2	0,92	1,16	0,82	0,98	1,10
H3K9me1	0,79	1,11	0,82	0,93	1,40
H3K9me3	0,95	0,97	1,07	0,83	1,19
H3K27me2	0,80	0,98	0,61	1,14	1,12
H3K36me1	0,88	1,08	0,67	1,69	**1,53**
H3K36me3	0,93	1,13	1,04	1,19	1,30
H3K79me2	0,75	1,18	0,87	1,11	1,27
H3K9ac	0,84	1,48	1,08	1,29	**1,93**
H3K18ac	0,91	1,02	0,91	0,90	1,36
H3ser10P	0,79	1,51	0,88	1,28	1,14

The selectivity index was calculated according to formula $SI = \frac{Teffs}{Tregs}$, *where:*
Teffs – percentage of histone H3 modification in total H3 in T effector cells.
Tregs – percentage of histone H3 modification in total H3 in T regulatory cells.
green - difference between specific Tregs and Teffs stimulated with insulin;
blue - difference between unspecific Tregs and Teffs stimulated with insulin;
red - difference between unspecific Tregs and Teffs stimulated with peptide 9-23.

region, in which the significant differences were found between all three respective Tregs/Teffs pairs.

Modifications with significant differences are shown in **Figure 6C** and p values in **Table S2**. Modifications without correlations are presented in supplementary materials at **Figure S3**.

DISCUSSION

In this study we aimed to examine the epigenetic background of the difference in the activity between Tregs expanded with monocytes loaded with different peptides, such as whole insulin or insulin β chain peptide 9-23. We have found different pattern of histone PTMs and different level of DNA methylation as well as different expression of genes crucial for Tregs development and suppressive function between the subsets expanded with these different antigens.

Until now, polyclonal T regulatory cells have been used in many clinical trials as a potent medicinal product that downregulates immune response during autoimmune diseases (9). Polyclonal cells, obtained by anti-CD3 and anti-CD28 stimulation, exert positive effect on patients with type 1 diabetes and significantly reduce the inflammatory response (16). One attempt to improve this therapy is to use specific Tregs directed toward disease-causing antigens. Such Tregs should traffic only into the inflamed tissue and suppress autoreactive lymphocytes *in situ* by response against specific antigens. Such an antigen-specific preparation may improve effectiveness of the currently administered treatment with polyclonal Tregs, reduce the required dose and limit adverse effects related to the interaction of Tregs with distant unrelated tissues. In our previous study, we proved that Tregs responding to a particular antigen showed higher potency to suppress Teffs than polyclonal cells. Antigen-specific Tregs retained a higher level of $FoxP3^{high}$ expression and also maintained suppressive phenotype, which makes them more potent to surpass the excessive immune response during autoimmunity (14). Interestingly, Tregs specific to insulin β chain peptide 9-23 were more suppressive than those generated with whole insulin. We found that antigen-specific Teffs could be generated with monocytes loaded with antigens, too. These results are important as they confirmed our *in vivo* data from type 1 diabetes in which we found that the disease-specific antigens can induce both specific Tregs and Teffs and the balance between these two subsets might be associated with the course of the disease (22). Importantly, current report confirms *in vitro* that the whole insulin is a poor stimulator of Tregs and the efficient induction of tolerance should be performed with other peptides, like β chain peptide 9-23.

It is highly interesting which epigenetic changes are exerted by the particular stimuli. It is widely known that the sustained suppressive phenotype of Tregs requires progressive demethylation in Tregs-specific signature genes (23). The majority of the studies focus on *FoxP3* gene and its regulation, because *FoxP3* is a master regulator that provides Tregs function and ensures phenotype maintenance. However, recent data shows that *FoxP3* expression alone is unable to preserve Tregs function without acquisition of Treg-specific epigenome. It is well-established that Tregs deprived of CNS2 or Tregs with high TSDR methylation lack *FoxP3* expression and suppressive function and could even acquire abilities to produce pro-inflammatory cytokines (24, 25). TSDR demethylation within the first intron of *FoxP3* gene locus is specific for Tregs, while in Teffs this region is highly methylated. The analysis of methylation status is reliable and correlates with the generation of stable Tregs (26, 27). The state of demethylation is needed for binding with other transcription factors such as: CREB, NFAT, RUNX to enable *FoxP3* expression (28). The research conducted by Miyao et al. (29) has revealed that TSDR demethylation acts as epigenetic memory that provides linage stability, even in the environment that contributes to *FoxP3* downregulation and thus indicate that stable CNS2 ensures Tregs persistence. In a similar study on effectiveness of antigen-specific Tregs obtained after stimulation with APCs, scientists confirmed that antigen-specific Tregs possessed a comparable average demethylation level (range 70,1-95.2%) to polyclonal cells, while specific and polyclonal Teffs had less than 0,2% of demethylation (30). For these reasons, the measurement of TSDR demethylation was applied as a useful quality control tool in the manufacturing of expanded polyclonal Tregs product (15, 31). In our research, all Tregs showed demethylation over 75% and Teffs were almost 100% methylated, which indicates that during cell culture all Tregs

remained stable. Worth emphasizing are Tregs $SPEC_{B:9-23}$, whose demethylation was significantly higher than Tregs $SPEC_{INS}$ and POLY, which confirms their usefulness as a drug candidate superb to polyclonal Tregs.

The process of genomic DNA methylation is rapid and flexible during T cell activation and differentiation (32). 5'-methylcytosine (5-mC) depletion is a hallmark of active transcription and it is involved in determination of lymphocyte function (33). In our study we examined total percentage of DNA methylation in T lymphocytes and we saw various pattern of methylation in particular subsets. Nevertheless, Tregs $SPEC_{B:9-23}$ were significantly less methylated than Tregs $SPEC_{INS}$. We performed correlation analysis between global DNA methylation and TSDR methylation, and we did not observe any statistical significance. However, we can notice a trend toward increased DNA methylation and diminished TSDR demethylation in Tregs $SPEC_{INS}$, and low global methylation and substantial TSDR demethylation in Tregs $UNSPEC_{INS}$. Moreover, higher % of total DNA methylation does not affect TSDR demethylation in Tregs $UNSPEC_{9-23}$.

Histone PTMs play a major role in chromatin remodeling, due to changes in electrostatic charge of histone protein tails, and creation of docking sites for proteins containing bromodomains or chromodomains that recognize acetylated or methylated lysine, respectively (34). The term "histone code" is used to describe the influence of histone modifications on gene expression and indicates that histone machinery decides which part of gene is transcribed (35). Genome-wide studies have revealed that different regions have distinct histone-modifications patterns, enabling expression of specific class of genes. A large number of studies contributed to understanding the functions of individual histone modifications. And thus, acetylation of lysine residues, is believed to be enriched in highly active promoters and increase transcription. Lysine: 4, 36, 79 mono-, di-, tri-methylation and 9, 27 mono-methylation is associated with active genetic status. Conversely, lysine: 9, 27 di- and tri-methylation is a repressive mark, resulting in gene inactivation. In turn, H3ser10P is responsible for gene activation and, like H3ser28P, for chromosome condensation during mitosis (36, 37). Histone modifications can alter as a result of activation process in CD4+ cells. Lamere SA. et al. (38) have revealed, that upon CD4+ activation, the dynamics of H3K4 methylation in promoter varies, and matches the RNA expression. Many differences in histone H3 methylation have been observed in gene promotors between Tregs and Teffs (39). In our study we decided to measure 21 histone H3 modifications, which consisted of lysine: 4, 9, 27, 36, 79 methylation, serine: 10, 28 phosphorylation and lysine: 9, 14, 18, 56 acetylation. We conclude that the type of stimulation (whole insulin or insulin β chain peptide 9-23) has an impact on PTMs.

The process of histone alteration is dynamic upon environmental conditions and is believed to be an indicator of gene activation status (40). The study of Th1 and Th2 differences in histone modifications in crucial gene signatures confirms the presence of active marks in given cell population with repressive histone marks in opposing cell line (41, 42). Moreover, substantial differences were not found between Tregs and conventional T cells based on H3K4me4 and H3K27me3 modifications (39). In our study we also did not see many differences between polyclonal Tregs and Teffs, except H3K79me3, with a predominance in Tregs (**Figure 6A**). However, upon antigen stimulation we observed changes in histone H3 modifications. In general, permissive H3 modifications (H3K4me1, H3K4me3) are abundant in indispensable regions such as: *FoxP3* promoter and intronic enhancer elements, and are connected with active promoters of up-regulated genes (*IL2RA, CTLA4, TNFRSF18, FOLR4*) (28, 43). All the above allow to maintain stable *FoxP3* expression and cell lineage commitment. In our study, Tregs $SPEC_{INS}$ were the least modified of H3K4me1/3, but high amount of such modifications was seen in all Tregs subsets, with the predominance of Tregs $UNSPEC_{INS}$ (**Figure 6A**). Another important modification regarding Tregs is H3 acetylation by histone acetyltransferases (HATs) CBP and p300. It allows proper development and maintenance of the suppressive function of Tregs. Upon activation HATs mediate acetylation of Tregs-related genes permitting their stable function. Disruption of p300 causes Tregs instability and promotes autoimmunity (44). Our research has revealed, that Tregs $SPEC_{9-23}$ were more enriched in lysine 9,14,18,56 acetylation compared to Tregs $SPEC_{INS}$ (**Figure 6B**). Regarding H3K27 methylation, the EZH2 methyltransferase contributes to cell stability and normal function.

It might be connected with the closed chromatin state in genes that are down-regulated by *FoxP3* (45–47). Moreover, EZH2 disruption leads to Tregs impairment and strengthens the anti-tumor immunity (48), which indicates the prominent role of H3K27me3 in Tregs. Here, we saw that Tregs $SPEC_{9-23}$ and Tregs $UNSPEC_{INS}$ have higher % of H3K27me3 modification than Tregs $UNSPEC_{9-23}$ and Tregs $SPEC_{INS}$ (**Figure 6A**).

Interestingly, we found a characteristic pattern related to the kind of stimulation in particular Treg/Teff pairs. Namely, a low level of PTMs in one subset from the pair was always associated with high level of PTMs in the other. This trend was found in the cells specific to insulin where low level of PTMs in Tregs $SPEC_{INS}$ was associated with high level of PTMs in Teffs $SPEC_{INS}$ and high level of PTMs in Tregs $UNSPEC_{INS}$ was associated with low level of PTMs in Teffs $UNSPEC_{INS}$. According to global DNA methylation there is an interdependence between low methylation level and high abundance of histone modifications in Tregs $UNSPEC_{INS}$ and Tregs $SPEC_{9-23}$, and decreased H3 modifications in Treg $SPEC_{INS}$ and Tregs $UNSPEC_{9-23}$ in relation to higher percentage of DNA methylation. But this trend did not occur in Teffs subset. There was no difference in Tregs/Teffs $SPEC_{B:9-23}$ pair and the low level of modifications in Tregs $UNSPEC_{B:9-23}$ was associated with the high one in Teffs $UNSPEC_{B:9-23}$. The PTMs in which the differences were significant overlapped only partially between particular Tregs/Teffs pairs, which suggests that the stimulation with different peptides differently influenced PTMs. Nevertheless, mainly activating PTMs, such as H3K18ac, H3K9me1 and, H3K36me2 were modified in Tregs $SPEC_{B:9-23}$

and Tregs $SPEC_{INS}$ and additionally H3K14ac and H3K27me1 were modified only in Tregs $SPEC_{B:9-23}$. It is also important to note that selectivity index (SI) is almost always below 1 for POLY and $UNSPEC_{INS}$ Teffs/Tregs pairs (Tregs more modified than Teffs) and above 1 in other pairs (Tregs less modified than Teffs) (**Table 4**).

Gene expression analysis confirmed that antigen stimulation did not deprive Tregs of the expression of crucial genes. All Tregs subsets had high expression of genes (*FoxP3, IKZF4, IKZF2, CTLA4, IL2RA*) needed for their function and phenotype maintenance. Tregs $SPEC_{B:9-23}$ were characterized by the highest expression but there was not much difference between Tregs POLY and Tregs $SPEC_{B:9-23}$. On the other hand, the analysis has revealed diminished gene expression in Tregs $SPEC_{INS}$. It is known, that *TNFRSF18* (GITR) and *IKZF4* (Eos) are constitutively expressed by $FoxP3^+$ cells and their expression in FoxP3- cells increases during activation (49, 50). In our study, we noticed that Teffs $SPEC_{INS}$ and $SPEC_{B:9-23}$ had high or moderate expression of GITR and Eos, respectively, which confirms a state of activation upon antigen stimulation in these cells. Despite the high level of *FoxP3* mRNA expression, we did not see a correlation between high percentage of $FoxP3^{high}$ cells, presented by Iwaszkiewicz-Grzes et al. (14), and mRNA level of *FoxP3*. Accordingly, other study also confirmed a modest relationship between protein levels and mRNA *FoxP3* expression, indicating a presence of other mechanisms involved in *FoxP3* expression (51). Bjur et al. (52) discovered that mRNA levels may not correlate with corresponding proteins due to post-transcriptional modifications. They noticed that, upon cell activation, changes in translational activity of specific mRNAs occur. Moreover, a recent study suggested that FoxP3 protein is subject to PTMs, which can alter its function, or even its stability (53).

Besides Foxp3 analysis, on the seventh day in our previous study (14), we performed phenotype test of: IKZF2, CD25 and CTLA-4, and observed high expression of each of them. The data on Helios and IL2RA are already published (14). Our current study has shown high percentage of relative gene expression of *CTLA4* in Tregs compared to Teffs. Nevertheless, Teffs cells had detectable *CTLA4* mRNA. At the protein level on day 7 after cell stimulation, Tregs and Teffs CTLA-4 expression on the cell surface was around 95% and 85% respectively. It is confirmed that CTLA-4 is continuously expressed on Tregs and occurs in T effector cells after activation, with maximum peak in proliferating, dividing cells (54). The discrepancies between low mRNA level at day 12 in Teffs and surface protein abundance can be explained by the CD4 activation model. Following TCR stimulation *CTLA4* mRNA is detected after 1 h with its peak around 24-36 h, and depends on mRNA half-life which is within the range from 4,6 h to 8,9 h, according to cell stimulation (55). Furthermore, Chan V. et al. conducted a study on CD4+ cells and observed an increase of mRNA *CTLA4* after 1 h after stimulation, maintained until 18 hours (56). Worth emphasizing is that CTLA-4 surface expression is modulated by many factors including TCR stimulation strength and depends on other mechanisms like CTLA-4 internalization and recycling (57).

Collectively, our results clearly demonstrate that stimulation with antigen-loaded monocytes presenting whole insulin or insulin β chain peptide 9-23 exerts epigenetic changes in Tregs. The type of stimulation determines the level of alterations in global DNA methylation pattern, and specific methylation of TSDR region as well as histone H3 PTMs. Insulin β chain peptide 9-23 promotes mainly Treg-oriented changes, while the phenotype after whole insulin stimulation was less clear. Hence, the pattern of the epigenetic changes may help finding the peptides that shape exclusively Tregs-mediated suppressive response or Teffs-mediated inflammatory response in future cellular drugs. Our observations indicate that antigen-specific Tregs during cell culture remained stable and comprise all Tregs-related features. It strengthens our confidence that our protocol allowing to obtain antigen-specific Tregs is a promising strategy of cell therapy, e.g. in type 1 diabetes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Zenodo and 4442316 doi: 10.5281/zenodo.4442316.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization, DI-G, MG and PT. Methodology, DI-G, MP, MG, ZU-W and PT. Software, DI-G. Validation, DI-G and MP. Formal analysis, DI-G, MP and MG. Investigation, DIG and PT. Writing—original draft preparation, DI-G, MP and MG. Writing—review and editing, PT. Visualization, DI-G. Supervision. PT. Project administration, DI-G and PT. Funding acquisition, DI-G and PT. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.642678/full#supplementary-material

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Review

Regulatory T Cells-Related Genes Are under DNA Methylation Influence

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Abstract: Regulatory T cells (Tregs) exert a highly suppressive function in the immune system. Disturbances in their function predispose an individual to autoimmune dysregulation, with a predominance of the pro-inflammatory environment. Besides *Foxp3*, which is a master regulator of these cells, other genes (e.g., *Il2ra*, *Ctla4*, *Tnfrsf18*, *Ikzf2*, and *Ikzf4*) are also involved in Tregs development and function. Multidimensional Tregs suppression is determined by factors that are believed to be crucial in the action of Tregs-related genes. Among them, epigenetic changes, such as DNA methylation, tend to be widely studied over the past few years. DNA methylation acts as a repressive mark, leading to diminished gene expression. Given the role of increased CpG methylation upon Tregs imprinting and functional stability, alterations in the methylation pattern can cause an imbalance in the immune response. Due to the fact that epigenetic changes can be reversible, so-called epigenetic modifiers are broadly used in order to improve Tregs performance. In this review, we place emphasis on the role of DNA methylation of the genes that are key regulators of Tregs function. We also discuss disease settings that have an impact on the methylation status of Tregs and systematize the usefulness of epigenetic drugs as factors able to influence Tregs functions.

Keywords: Tregs; Tconv; DNA methylation; gene expression; epigenetic modifiers

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1. Introduction

Regulatory T cells (Tregs) are $CD4^+$ suppressor cells that have the capacity to block T effector cells (Teffs). The majority of Tregs-related examinations have confirmed their ability to mediate specific immune system inhibition and their usefulness in many clinical trials. Tregs, as a drug, are used in the treatment of autoimmune diseases and conversely their depletion is considered as a promising tool for cancer patients. Conducting research on regulatory T cells has revealed a great deal of factors that can affect their stability and phenotype, which might be the next step to develop Tregs-related therapy.

Tregs are a heterogeneous population of CD4-positive T cells determined by high expression of CD25 and low expression of CD127. The protein Foxp3 is considered to be the major transcription factor responsible for Tregs function and stability. Besides *Foxp3*, there are many other Tregs-signature genes, coding factors such as *Il2ra* (CD25), *Ctla4* (CD152), *Tnfrsf18* (GITR), *Ikzf2* (Helios), and *Ikzf4* (Eos), which are believed to play an important role in Tregs function [1–4]. Foxp3-positive T cells can be divided into different subpopulations according to their origin: best characterized as, and believed to be highly effective and stable, thymus-derived Tregs (nTregs), peripheral-induced Tregs (pTregs), and in-vitro-generated (iTregs), produced from $CD4^+CD25^-$ naïve T cells. Another division according to phenotype diversity, due to Tregs functional state, allows to distinguish $CD44^{low}CD62L^{high}$ (central, cTregs) and $CD44^{high}CD62L^{low}$ (effector, eTregs). Natural and induced Tregs are characterized by the factors Helios (an Ikaros transcription factor) and Neuropilin-1, which are thought to be specific for Tregs that arose in the thymus [5–7].

Epigenetics describe changes in gene expression not caused by alterations in the DNA nucleotide sequence. The most important and well known are DNA methylation,

histone posttranslational modifications, and inhibition by non-coding RNA (ncRNA). Each epigenetic mark is responsible for maintenance of DNA availability and results in changes in gene expression as well as in chromatin structure. Subsequent chromatin remodeling has an impact on the production of many crucial proteins for the proper action of the immune system [8,9].

DNA methylation is a process of cytosine conversion into 5-methylocitosine catalyzed by specific enzymes called DNA methyltransferases (DNMTs), which use S-adenosylmethionine as a substrate. DNMTs are a group of enzymes—DNMT3A, DNMT3B, and DNMT1—that transfer the methyl group during de novo methylation or during cell replication. This reaction is presented at CpG islands, highly enriched in CG content. When CpG islands are methylated, it is believed that gene expression is silenced, and the gene is repressed. TET enzymes (Ten-Eleven Translocation family enzymes) are able to reverse the process and induce a demethylation process that leads to an open chromatin structure, and eventually to gene expression [10,11].

In the case of regulatory T cells, DNA methylation is particularly important. Several studies revealed more than 100 differentially methylated regions (DMRs) in Tregs compared to Teffs. CpG hypomethylation in Tregs-related genes, such as *Ctla4* exon 2, *Foxp3* intron 1, *Tnfrsf18* exon 5, *Ikzf4* intron 1b, and *Il2ra* intron 1a, is limited to Tregs and persists after cell stimulation [12,13]. It is worth emphasizing that Tregs-specific hypomethylation of critical genes is a dynamic process during Tregs development. It begins at the precursor stage and continues until full cellular maturity [14].

In this review, we discuss DNA methylation patterns in genes crucial for regulatory T cells, such as *Foxp3*, *Ctla4*, *Il2ra*, *Tnfrsf18*, *Ikzf2*, and *Ikzf4*. Moreover, we will give a brief overview on the role of DNA methylation in specific gene regions and its diversity in Tregs function and stability. The main objectives are presented in Figure 1.

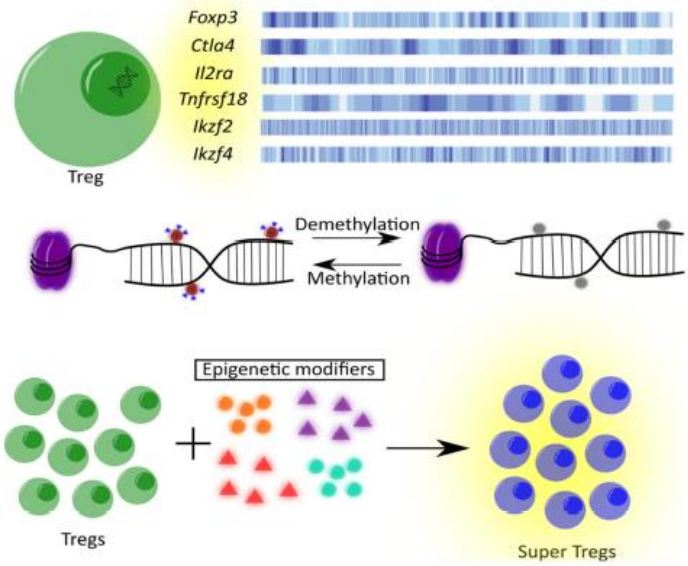

Figure 1. The main purpose of the article is shown. Genes that are under discussion are presented as well as the role of DNA methylation and epigenetic modifiers in regulatory T cells.

2. DNA Methylation Overview

DNA methylation is a post-replicative process occurring in CpG islands in mammalian cells that is passed onto daughter cells and provides cell memory [1,2]. The definition of

CpG islands said that these 200 bp regions comprise over 50% CG nucleotides. However, Takai and Jones proposed new criteria that require at least a 55% CG content and extended the length to 500 bp [15,16]. While disturbances in DNA methylation patterns predispose to the development and progression of cancer, it is highly important to preserve proper mechanisms allowing DNA methylation [3,4]. DNA methylation occurs with the presence of DNMTs having capacity to transfer a donor group (S-adenosyl-L-methionine) into the fifth carbon of cytosine residues in DNA. Such enzymes are divided into subgroups depending on their mechanism of action [17]. The first—DNA methyltransferase 1 (DNMT1)—prefers hemi-methylated DNA and enables it to maintain DNA methylation during cell divisions. The major function of DNMT1 was confirmed in the study of inheritance neurodegeneration, where mutations in the DNM1 gene caused hereditary sensory and autonomic neuropathy type 1 disease (HSAN1) [18,19]. On the other hand, DNMT3 isoforms are responsible for de novo methylation and consist of the catalytically active DNMT3A and DNMT3B and the inactive DNMT3L. During development and cell proliferation, mutations in DNMT3B contributes to immunodeficiency–centromeric instability–facial anomalies syndrome (ICF syndrome), which is an immunodeficiency disease with the presence of centromeric instability and facial anomalies. While mutations in DNMT3A act as negative prognostic factor in patients with acute myeloid leukemia (AML) [20,21], DNTM1 ablation in Teffs and its inhibition by DNMTs inhibitor 5-aza-2′-deoxycytidine (Aza) lead to an increase in *Foxp3* expression in these cells. On the contrary, deletion of DNMT1 in developing thymic Tregs results in lethal autoimmunity [8,22,23]. Moreover, the study on Uhrf1 proteins, which recruits DNMT1 and promotes DNA methylation in Tregs, revealed that mature Tregs lacking Uhrf1 have proinflammatory capacities [24].

The DNA demethylation process occurs in a passive and active manner. Passive global DNA demethylation due to cell proliferation is caused by insufficient inheritance of the DNA methylation pattern that can be restored by DNMT1 [25]. Active DNA demethylation is mediated by the three mammalian members of the TET family enzymes that are able to convert 5 mC into 5 hmC (5-hydroxymethylcytosine), 5 fC (5-formylcytosine), and 5 caC (5-carboxylocytosine) [26]. Elevated TET enzyme levels from low oxygen conditions promotes CNS2 demethylation and *Foxp3* stability [27]. Reports have shown that DNA methylation is a repressive mark and switches off the transcriptional machinery [28]. DNA methyltransferases, as was discussed above, play a crucial role in such mechanisms. Unfortunately, subsequent silencing of gene expression is a multidimensional process. A group of proteins (MeCP1-2, MBD1-4) possessing domains for methylated DNA (MBD—methyl-CpG-binding domain) are connected with gene repression owing to their capacity to recruit histone protein-modifying enzymes and chromatin remodeling. For example, MeCP2 silences gene expression through recruitment of histone deacetylase (HDAC) and allows lysine 9 in H3 methylation. Moreover, MeCP2 interacts with HP1 (heterochromatin protein 1), together leading to gene repression [15,16,29]. Furthermore, many transcription factors (TFs) are unable to bind to their ligands when methylated. For example, binding of reduced NFAT (Nuclear Factor of Activated T cells) in the *Ctla4* promoter was diminished due to enrichment in methylation level, suggesting a higher binding affinity for demethylated regions. In another study on NRF1 (Nuclear Respiratory Factor 1), it was also confirmed that this TF prefers binding to unmethylated promoters [30,31].

3. *Foxp3* Gene

3.1. Foxp3 Gene Structure

Human *Foxp3* gene is located on the X chromosome (Xp11.23). Its transcript contains 2264 bps and encodes 431 amino acids [32]. It consists of 12 exons (11 coding and 1 non-coding). Moreover, the 5′ part of exon 2 and 3′ part of exon 12 represent non-coding fragments. Mantel et al. using 5′-PCR RACE, have reported that the promoter region appears upstream approximately −6 kbp from the translation start site, and is conserved in humans, mice, and rats. They have also revealed the basal promoter targeting cell specificity, which is placed within the first 500 bp and is bound to AP-1 and NFAT transcription

factors [33]. Typical *cis*-regulatory DNA components, such as enhancers, play a pivotal role in *Foxp3* regulation. Analysis of ~30 kb of mouse genomic DNA revealed an upstream enhancer −4970 to −6021 bp from the transcriptional start site (TSS) and confirmed its 92% conservativeness with human DNA [8]. Recent studies have revealed that analogous human upstream enhancer is about 800 bp and is placed approx. −6100 to −5300 bp from the TSS [34]. Another one, named CNS0 (conserved non-coding sequence), is placed ~8000 bp upstream of the TSS. This sequence is considered as a super-enhancer (SE), highly enriched in a massive amount of TFs that plays a pivotal role in gene expression, controlling the cell identity and fate [35]. Besides upstream promoter enhancers, there are downstream regulatory elements, located in intronic DNA sequences, referred to as CNS1-3 (according to their distance from TSS). CNS1 and CNS2 are located in the intronic sequence 1, while the CNS3 is placed in intron 2, between exon 1 and exon 2 [36].

3.2. *Foxp3* DNA Methylation

As mentioned, methylation occurs in CpGs, which are included in *cis*-regulatory elements such as enhancers and promoters. The master regulator of Tregs is *Foxp3*, and its regulation is crucial for maintaining immune homeostasis. However, *Foxp3* expression alone is not sufficient to achieve full Tregs suppressive function and its coaction with CpG hypomethylation is required for Treg cells development. Okhura et al. confirmed that *Foxp3* expression alone was not enough for Tregs lineage generation and the presence of a genome-wide nTregs cell-type CpG hypomethylation pattern was crucial, and depended on T cell receptor (TCR) signaling [12].

The newly discovered CNS0 acts as an SE and is bound by a pioneer factor Satb1, triggering Tregs differentiation due to *Foxp3* induction. Disruption of Satb1-mediated SE activation not only leads to impaired *Foxp3* induction but also diminishes DNA demethylation in Tregs-specific demethylation regions (TSDRs) [35]. This region is also bound by STAT5, and cooperatively with Il-2 enables *Foxp3* induction in Tregs precursors, while its depletion together with *Aire* resulted in a worsened autoimmune disease [37]. Moreover, both naïve and activated Tregs comprise the Tregs-SEs, which correlate with Tregs-DRs (demethylated regions), affecting gene expression. What is more, autoimmune-disease-associated SNPs (single nucleotide polymorphisms), which affect Tregs function, are abundant in Treg-specific demethylated regions [37,38].

Another upstream enhancer element (downstream CNS0), located −4970 to −6021 in mouse DNA, comprises a CpG island with ~67% GC content. Its detailed analysis shows a sequence from −5786 to −5558, which includes 23 CpG, revealing that this region was highly demethylated in naturally occurring Tregs, while it was methylated in TGFβ-induced Tregs and conventional T cells (Tconv cells). The −5 kb upstream enhancer is also important in *Foxp3* induction in natural Tregs. In naïve $CD4^+CD25^-$ T cells, artificial demethylation by DNMTs inhibitor Aza enhances Foxp3 protein production, and can be boosted by addition of TGF-β to cell culture [8]. The aforementioned human homologue that includes 28 CpGs has different methylation levels between Tregs and Tconv cells, with substantial demethylation in the former. Furthermore, in patients with rheumatoid arthritis, some differences in the upstream enhancer region were observed, as well as in the level of DNMTs. Research has revealed that this region's methylation was negatively correlated with *Foxp3* mRNA expression levels, and in vitro methylation diminishes its activity [34]. These observations were confirmed in another study including 8 CpGs located from −5835 to −5794. In addition to weakening *Foxp3* expression, Tregs with upstream enhancer methylation had impaired suppressive functions [39].

With reference to the upstream enhancer methylation pattern, the discrepancies between the regulatory T cells and other CD4+ cells that are capable of transient Foxp3 production can be revealed by the examination of the *Foxp3* promoter region. By comparing the different CpG sites in the human putative promoter region, it was shown that positions −113, −77, −65, and −58 were significantly diversely methylated between $CD4^+CD25^{high}Foxp3^+$ and $CD4^+CD25^{low}$ cells. Moreover, the CpG site −77 was the most

differently demethylated between these two populations. Therefore, it was considered as a pivotal region for cell differentiation. In reference to stable *Foxp3* expression, only Tregs that had almost complete promoter demethylation were committed to the Tregs population and remained functionally stable [40]. Another study on the methylation status of 10 CpG sites within the 451 bp region of *Foxp3* promoter has disclosed complete demethylation in Tregs and substantial methylation in $CD4^+$ conventional cells [41]. In studies of patients with diseases that are connected with impaired Tregs function, such as biliary atresia, systemic sclerosis, fulminant type 1 diabetes (T1D), and recurrent spontaneous abortion, it was noted that the level of mRNA *Foxp3* was negatively correlated with higher promoter methylation. This relation might be responsible for diminished suppressive function of Tregs and further stand behind the pathogenesis of many diseases [42–45].

Furthermore, the DNA methylation pattern has been extensively studied at the *Foxp3* gene locus on several downstream intronic enhancers, described as CNSs: 0–3, according to their distance from the TSS.

CNS1, containing binding sites for SMAD and NFAT, is necessary for the induction of peripheral Tregs, and its depletion causes spontaneous abortion of embryos [36,46,47]. Peripheral Tregs generation in mice lacking the CNS1 region is attenuated, and leads to impairment of the Tregs-related mechanisms [48]. The importance of DNA methylation in this region was unleashed through the study on TET family proteins, where the loss of TETs led to diminished suppressor function of Tregs due to impairment of CNS1 demethylation [49]. Similar conclusions were reached in another study on a mouse model. It was shown that TET2 and TET3 were enzymes mediating CNS1 demethylation during cell development, particularly this TET-dependent demethylation occurred gradually in Tregs after the CD4 SP stage. Additionally, the study on 4 CpGs in CNS1 has shown that WT mice were highly unmethylated, while Tet2/3 DKO mice had substantial methylation (20–30%), which resulted in instability in cell culture and lower suppressor function in vitro [26].

Extensive research on induced Tregs has revealed their instability in cell culture and diminished suppressor function in vivo, which might be connected with the overall increase in CNS1 methylation. In order to improve their function, many substances are believed to upregulate their properties, e.g., vitamin C [50,51]. The addition of vitamin C in mouse, as well as human, iTregs caused CNS1/2 demethylation, leading to an increase in suppressor functions [26].

Furthermore, another locus—CNS2—is also under TET regulation [49]. In the research on 11 CpGs methylation in mice Tregs precursors, $CD25^+Foxp3^-$ and $CD25^-Foxp3^+$, it was confirmed that progressive CNS2 demethylation occurred in the latter [26]. This progression was disturbed when the TET2/3 enzymes were depleted from the cell precursors. In mice models, it was revealed that TET2/3-deficient-Tregs were less stable and more likely converted into Th17 cells, having higher methylation in the CNS2 locus [26,52].

CNS2 contains binding sites for the NFAT, c-Rel, CREV, Runx1, Ets-1, GATA3, and STAT3 transcription factors, ensuring stable expression of Foxp3. As the presence of each of these TFs is crucial, studies using specific knockdown models have shown that CNS2-related TFs deprivation did not affect Tregs maturation, but rather contributed to Tregs instability in cell culture due to decreased Foxp3 expression [53–57]. Therefore, DNA demethylation in the CNS2 region of the *Foxp3* gene is believed to be an important element ensuring Tregs stability and functionality and called the Major Treg-Specific Demethylated Region (TSDR). The importance of TSDR demethylation is indicated by instability of the in vitro-generated Tregs, which easily lose their phenotype and function due to methylation in CNS2. In addition, the previously mentioned TFs can bind to this region only when they are unmethylated, triggering Foxp3 expression [58,59]. From many TFs, the Runx1–Cbfβ–Foxp3 complex seems to be the most important. Runx1 and its cofactor Cbfβ together interact with demethylated CNS2 and allow continuous production of Foxp3 in a mechanism called a feed-forward loop. Moreover, DNA demethylation in TSDR provides 'cell memory' and ensures regulatory T cell stability upon pro-inflammatory conditions. Cells that have DNA demethylation in TSDR, and partly have lost Foxp3

expression under certain conditions, are able to restore Foxp3 expression due to epigenetic recollection [59,60]. It is believed that the TSDR methylation status, more than *Foxp3* expression, is a reliable factor that can predict the quality of regulatory T cells during cell culture because of the fact that the mRNA and protein levels are unstable parameters [61,62]. Likewise, TSDR demethylation is an exclusive marker of regulatory T cells that allows discriminating Tregs from activated effector cells. This is because T effectors may acquire transient Foxp3 expression upon activation [63]. Considering the Tregs populations, nTregs acquire stable and profound TSDR demethylation, while iTregs remain almost completely methylated, which makes them less stable during cell culture [64]. In addition, Tregs that are generated in vivo have similar, albeit lower TSDR demethylation compared to nTregs [65].

The proper methylation pattern in the CNS2 locus is promoted by Blimp1. This protein restricts DNMT3a from mediating the methylation of TSDR and consequently leads to cell stability [66]. Another protein—Mbd2—is considered as a factor that promotes DNA methylation; conversely, in Tregs it contributes to the maintenance of TSDR demethylation and its targeting is associated with impaired Tregs function [67]. A recent study by the Sakaguchi research group has revealed that stable Tregs could be generated from Tconv cells upon abrogation of the CD28 signaling pathway. This mechanism blocked the CD28–PKC–NF-kB axis and enabled demethylation in the CNS2 locus in Tregs generated in vitro, which made them stable and effective [68].

The last sequence that controls the *Foxp3* locus—CNS3—controls the tTregs and pTregs number. It was suggested that this region was not crucial for the maintenance of Foxp3 expression, rather than for early modification of the *Foxp3* locus. Thus, this pioneer element allows the poised state of the *Foxp3* promoter, enabling responsiveness to TCR stimuli [69]. Due to the fact that this region binds c-Rel, a TF highly important for *Foxp3* induction, it is believed that cooperation with c-Rel-CNS2-CNS3 enables recruitment of other TFs and eventually leads to stable demethylation of CNS2 in nTregs [36].

3.3. Epigenetic Modifiers of the Foxp3 Locus

Due to massive participation of DNA methylation in Tregs regulation and development, and the reversibility of epigenetic changes, there is a possibility to mediate Tregs function by epigenetic pharmaceutics, such as cytosine modifiers. DNMTs inhibitors (DNMTIs), such as azacytidine, decitabine, and zebularine, are a group of suitable candidates. The first two are approved by the FDA (Food and Drug Administration) for the treatment of hematologic disorders, but the last one, until now, has no approval [70,71]. Besides the impact of DNMTIs on cancer, their properties are used to modulate regulatory T cell function [8].

The DNA methyltransferase inhibitor azacytidine (AZA) is used in the treatment of AML (acute myeloid leukemia) or MDS (myelodysplastic syndromes) after allo-SCT (allogenic blood stem cell transplantation). The treatment results in diminished induction of graft-versus-host disease (GvHD) due to enhancement of Foxp3 expression and further Tregs expansion [72,73]. The profitable effect on 5-AzaC was observed in patients with MDS, in which the overall Tregs number increased because of CD25- cells' conversion into regulatory T cells. Additionally, Tregs cultured with AZA had lower promoter methylation compared to the control [74]. Mouse research on an AZA derivative, decitabine, has led to similar conclusions, where naturally occurring Tregs played an essential role in protecting from GvHD after exposure to AZA [74,75]. Consistent with the data, treatment with a low dose of AZA augmented *Foxp3* RNA expression in $CD4^+Foxp3^-$ cells, and increased the total amount of $CD4^+CD25^+$ cells, leading to mitigated T effector cells activation. Altogether, these effects prevented experimental autoimmune encephalomyelitis [76]. Moreover, Tregs that have been modulated by azacytidine during inflammatory conditions in mice were highly effective, and TSDR demethylation in iTregs was about 80%, while iTregs without AZA had about 5% of TSDR demethylation. Given the fact that iTregs are highly unstable, the addition of AZA might be a promising tool for iTregs improvement [77].

Similar results were obtained by another research group, which have revealed a positive effect of another DNA methyltransferase inhibitor—decitabine (5-aza-2′-deoxycytidine)—on Tregs populations. They found a trend of diminished TSDR methylation in Tregs after decitabine (DAC) treatment. In mice models, it was established that DAC-treated Tconv cells have acquired the ability to produce Foxp3, mediated suppressor functions, and revealed strong expression of Tregs-related molecules [78,79]. Nevertheless, mice treated with DAC consisted of a higher percentage of Foxp3-positive cells in the thymus, with lower intron 1 and promoter methylation, which in turn prevented cyclophosphamide-induced T1D [79]. Although suppressor functions of DAC-treated effector cells were achieved in the mouse model, studies on human cells did not confirm this. Human $CD4^+CD25^-$ cells stimulated with DAC possessed only partial TSDR demethylation and were able to produce Tregs-specific genes but in smaller amounts compared to nTregs. Despite having some features of nTregs, DAC-induced Tregs did not have any functional activity [80]. In addition, patients infected with HBV and subjected to DAC treatment had higher percentages of Tregs and *Foxp3* [81].

On the other hand, zebularine (ZEB)—a less toxic reagent, which has significant demethylating properties—has not been yet approved by the FDA [82]. Similarly, research in mice models on azacitidine and decitabine function found that ZEB promoted *Foxp3* expression and CpGs demethylation of the Tregs' upstream enhancer. In addition, human $CD4^+$ ZEB-mediated cells had diminished INF-γ and IL-17A expression [83].

Furthermore, rheumatoid arthritis (RA) patients treated with methotrexate (MTX), which reduces the level of DNMT1, were characterized by restored Tregs functions and higher *Foxp3* expression caused by a decrease in upstream enhancer methylation [84].

The second group of epigenetic modifiers are factors that influence TET-mediated active DNA demethylation, such as vitamin C, which has a profound effect on iTregs. Vitamin C, a known coactivator for TET proteins, upregulates these proteins in iTregs. The stability and suppressive function of these cells are increased due to the lower methylation of CNS1 and CNS2 [26]. Despite the fact that pTregs are under vitamin-C influence, vitamin-C deprivation does not influence nTregs, because these cells have substantial TET expression, keeping CNS2 demethylated [85]. Low oxygen culture conditions, alone or in combination with vitamin C, can induced a stable phenotype in TET-transduced iTregs. These murine iTregs not only had demethylation in Treg-related genes but also were more suppressive compared to normal iTregs [27]. The research conducted on mice has shown a beneficial effect of stimulated iTregs on the prevention of GvHD or allograft rejection [86,87]. More complex research on iTregs, including the cooperation of three epigenetic modifiers, including vitamin C, has disclosed a deep CNS2 demethylation in stimulated cells with high stability and usefulness in therapeutic trials [64].

Another reagent—hydrogen sulfide (H2S), enables TET1/2 binding to promoter region, and its deficiency abrogates Tregs function. Similar to TET-deleted mouse Tregs, an insufficient H2S level results in high methylation of promoter and CNSs regions [88]. The inhibition of TET activity by 2-hydroxyglutarate can be resolved using aminooxyacetic acid (AOA). This reagent selectively shifts the balance towards iTregs, blocking Th17 differentiation, and consequently mitigates experimental autoimmune encephalomyelitis (EAE) in mice. Namely, AOA reduces *Foxp3* methylation in the promoter region in TH17 cells, simultaneously decreasing CNS2 methylation in iTregs and TH17 cells [89].

The indisputable effect of IL-2 on Tregs functionality is connected to the epigenetic signature. Namely, mice Tregs lacking IL-2 had diminished expression of TET and lower TSDR demethylation [90]. During cell expansion, IL-2 allows stable demethylation and preserves the function of the iTregs [91].

CRISPR–dCas9-based technology, which allows for the manipulation of the gene expression in a specific DNA locus, is a promising tool to target the *Foxp3* gene. Okada et al. conjugated guide RNA (gRNA) with a catalytic domain of TET1 and showed partial demethylation in CNS2 in primary T cells. Even so, they did not see stabilization of Foxp3 expression [92]. Similar data were obtained in another study, where the CRIPSR

method caused TSDR demethylation with subsequent *Foxp3* expression but no typical Tregs properties were observed [93].

Moreover, other known factors can mediate alteration in the methylation status of the *Foxp3* gene. One of them—an active metabolite of vitamin A, ATRA—is connected with Tregs promotion by targeting methylation at the promoter of the *Foxp3* gene. $CD4^+$ cells from patients with systemic sclerosis subjected to ATRA were more abundant in *Foxp3* at the mRNA and protein level. However, the mechanism of ATRA-mediated demethylation remains unknown [94]. On the other hand, this metabolite of vitamin A does not cause any differences in TSDR methylation in iTregs and thus is not able to enhance iTregs stability through DNA demethylation [12]. Although, enhancement of iTregs generation and stability through co-action of ATRA and TGF-β can be achieved by modulation of histone acetylation [95]. Similarly, Tregs stimulated with rapamycin (RAPA) alone or together with ATRA after expansion were more stable, with sustained TSDR demethylation and better suppressive function of these cells [62]. Nevertheless, RAPA alone or with TGF-β, failed to induce TSDR demethylation in iTregs in all 11 analyzed CpGs [96].

Looking into other manipulations, TNFR2 agonist or TNF1 antagonists can be used to enhance Tregs function by promoting demethylation in the proximal promoter of the *Foxp3* gene [97]. It can be also achieved through blocking of PIAS1 (SUMO E3 ligase), which recruits DNMTs and leads to chromatin repression of the *Foxp3* locus. The study on PIAS1-depleted mice has shown reduced methylation of the *Foxp3* promoter in developing $CD4^+CD25^-$ cells, which might be related with higher production of $Foxp3^+$ cells from cell precursors [98]. An overview of the abovementioned is shown in Figure 2A.

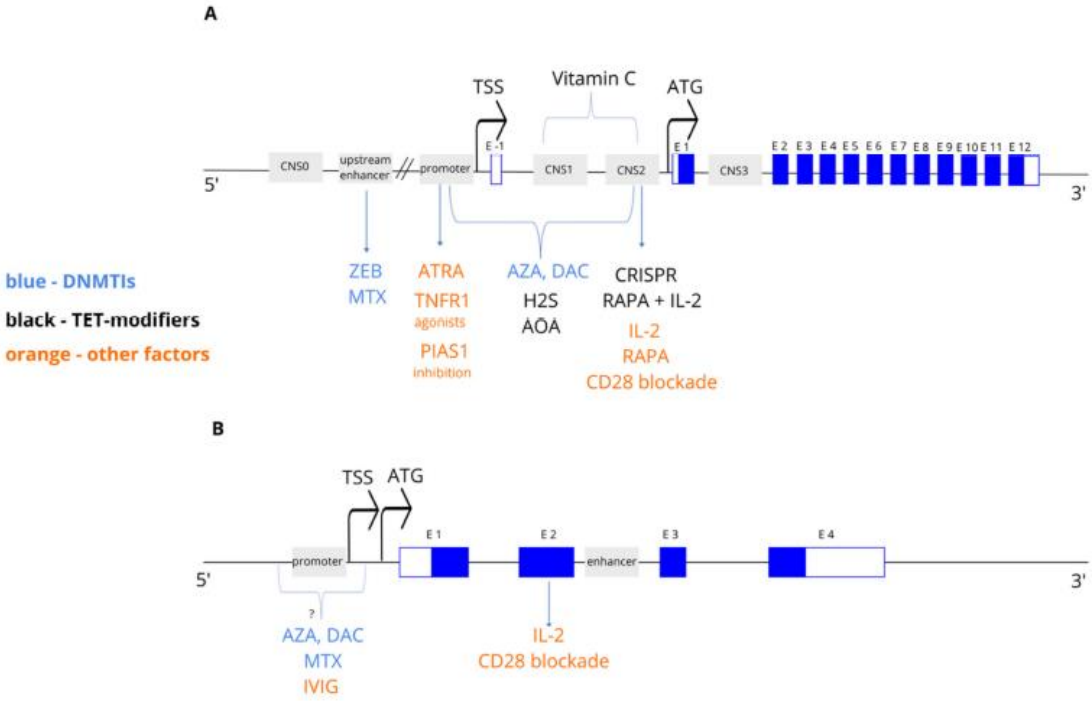

Figure 2. Overview of the structure of the *Foxp3* (**A**) and *Ctla4* (**B**) genes and the location of the epigenetic modifiers' action. CNS0-1—conserved-non-coding elements; E—exon; TSS—transcription start site; ATG—translation initiation codon; ZEB—zebularine; AZA—azacytidine; DAC—decitabine; MTX—methotrexate; ATRA—all-trans retinoic acid; H2S—hydrogen sulfide; AOA—aminooxyacetic acid; RAPA—rapamycin; IVIG—intravenous immune globulin.

4. *Ctla4* Gene

4.1. *Ctla4* Gene Structure

Ctla4 is located in the long arm of chromosome 2 (2q33.2) [99]. It has a 76% overall homology with the murine gene, and consists of 4 exons separated by 3 introns [100]. Its transcript is 1997 bp long and encodes a 223 aa protein. In Tconv cells, the gene is activated after cell stimulation, but in Tregs *Ctla4* expression is constant [101,102]. The human proximal promoter, located 5′ upstream from the UTR (untranslated region), comprises a pivotal regulatory sequence located from −200 to −330 bp. This sequence binds with a key transcription factor, NFAT, leading to *Ctla4* gene upregulation [103]. In the case of Tregs, the promoter of *Ctla4* is bound by the Foxp3:NFAT1 complex. The cooperation through stable *Foxp3* expression accelerates gene expression and stabilizes the *Ctla4* promoter [104]. Another transcription factor, *Foxp1*, binds the *Ctla4* promoter region and coordinates the binding of *Foxp3* to the promoter region [105]. The murine gene sequence that controls gene expression is located 335 bp upstream of the *Ctla4* sequence, with the key regulatory element placed between −238 and −167 from the transcription start site [101]. Using ChIP-Seq gene analysis of the H3K4me3 and H3K4me1 peaks, an enhancer element was revealed in the human *Ctla4* gene located in intron 2 [106]. Moreover, *Ctla4* SEs, illustrated by H3K27ac abundance, were found in Tregs and conventional cells [38]. Treg-specific SEs in the *Ctla4* locus were observed from the pre-tTreg (precursor) developmental stage [35].

4.2. *Methylation of Ctla4*

Consistent with the previous notion that CpG methylation affects gene expression, the pattern of DNA methylation in particular locations has an impact on cell functionality. During Tregs development, precursor cells subsequently acquire *Ctla4* demethylation, and this process can be enhanced by IL-2 [14].

A *Ctla4* promoter analysis in patients with colorectal cancer and melanoma has shown that CTLA-4 overexpression was caused by promoter hypomethylation. The mechanism behind this is the upregulation of TETs (TET1, TET2, and TET3) and reduced amount of DNMTs. The subsequent Tregs over-activity is used by tumor cells to escape from immune response, leading to a poorer prognosis. Thus, the examination of methylation status of promoter genes can be used as a predictive marker for cancer therapy [107,108]. Similar results have been observed in patients with breast cancer, in which enzymes that target DNA methylation were differentially expressed compared to the control [109]. Higher methylation status (33.98% vs. 19.81%) of the promoter gene, lower *Ctla4* mRNA, and increased DNTMs expression were detected in patients with myasthenia gravis (MG), causing a reduction in Tregs-related cytokines (TGF-β and IL-10) [110].

Contrary to this study, no differences have been observed in the level of DNMTs in RA patients, who possessed a higher promoter methylation pattern. However, the analysis of 10 CpGs in the promoter region has revealed the methylation discrepancies between RA patients and healthy individuals in two CpGs, −658 and −793, with statistical significance in the former. What is more, the researchers confirmed that Tregs had a similar demethylation pattern to T effector cells in the promoter region, with the exception of two CpGs that have been differentially methylated in RA patients. This research also revealed the mechanism by which NFAT2 binds to the −658 promoter region, only when this region is demethylated [30]. Similar data were obtained in the research on promoter methylation in MG patients where statistically significant hypermethylation occurred at −658 and −793 CpGs [111]. It confirms the inverse correlation between promoter methylation and gene expression.

Apart from the promoter, *Ctla4* exons have different patterns between the regulatory and Tconv cells. Exon 2 was considered as a Tregs-specific region defining cell commitment and stability upon activation. However, $CD4^+CD25^-$ cells cultured with polarized conditions towards iTregs did not acquire demethylation in this region [12,16]. Research on mouse models has shown that alloantigen-specific iTregs generated in the presence of DCs had Treg-specific epigenetic marks, including *Ctla4*, and were able to mediate

cell suppression [112]. Consistent with this study, antigen stimulation led to sustained demethylation in both iTregs and pTregs [113]. In another study, naïve T cells were almost completely methylated in exon 2 but effector memory T cells were demethylated at around 28%, while Tregs were demethylated in 92% [114].

Apart from the above, similar to SEs in the *Foxp3* locus, the *Ctla4* gene comprises specific enhancer elements, enriched in the hypomethylated regions. These CpG regions include the SNPs responsible for autoimmune diseases, e.g., T1D [38].

4.3. *Ctla4* Modifications through Changes in DNA Methylation

The previously mentioned AZA not only acts on TSDR methylation in the *Foxp3* locus but also contributes to stabilization of *Ctla4* gene expression, which lasts after cell expansion. Enhanced conversion of human $CD4^{+}CD25^{-}$ cells into iTregs by AZA increases CTLA-4 expression. However, the combination of azacytidine and low-dose panobinostat (HDACi) decreased the Tregs function and cell population size due to lower production of Foxp3 and CTLA-4 [115,116].

As mentioned, these epigenetic modifiers, e.g., AZA, are widely used in cancer treatment strategies [117,118]. Nevertheless, it is worth emphasizing that DNA hypomethylating agents (DHAs) can upregulate immune checkpoint molecules, such as PD-L1, PD-L2, and CTLA-4. The increased expression of CTLA-4 can be connected with poorer response to cancer treatment. For this reason, many clinical trials, which include AZA or DAC therapy, combine the therapy with CTLA-4 or PD-L1/2 inhibitors, in order to improve the outcome of the treatment [119–121]. Apart from AZA and DAC, another DHA—guadecitabine—increases the level of *Ctla4* in melanoma and hematological cancer cells [122]. MTX also increases *Ctla4* expression indirectly via its action on the *Foxp3* methylation level and DNMT1 reduction. Like in the case of DHAs, it was responsible for suppressor activity of the Treg cells [84]. Despite the unequivocal effect of DNMTIs on upregulation of CTLA-4 expression, there are no data revealing the methylation status of the *Ctla4* gene in DNMTI-treated cells. However, DNMTIs promote demethylation in *Foxp3* and the *Gitr* promoter, leading to increasing gene transcription; it is possible that the same mechanism targets the *Ctla4* locus.

Another study has revealed the positive effect of intravenous immunoglobulins (IVIG) on CTLA-4 function. Tregs that have been expanded by DCs in response to IVIG had restored their suppressor abilities. It was reported that IVIG therapy diminished methylation in two CpGs in the promoter of the *Ctla4* gene that were highly methylated in non-treated MG patients [111]. The properties of IVIG to change the methylation level were confirmed by another study. However, the mechanism underlying the effect of IVIG on CpGs is poorly understood [123].

As previously mentioned, deprivation of the CD28 costimulatory molecule in Tregs resulted in high demethylation in the *Ctla4* locus compared to cells that expressed CD28, which might be another therapeutic maneuver [68].

On the other side, no beneficial effect on the *Ctla4* demethylation signature was observed in cells treated with vitamin C or retinoid acid [12,27]. An overview of the above mentioned is shown in Figure 2B.

5. *Il2ra* Gene

5.1. *Il2ra* Gene Structure

The human *Il2ra* gene is encoded in chromosome 10 (10p15.1). The transcript is 3218 bp and creates a 272 aa protein. It contains 8 exons, from which 6 are coding, while exon 8 and the 5′ fragment from exon 1 are noncoding [124]. A pivotal enhancer element (positive regulatory region—PRRI) in the promoter region is located from −299 to −228 relevant to the TSS and binds NF-κB [125]. Additionally, another human enhancer element (PRRII), comparable to the murine gene, is located between nucleotides −137 and −64, and comprises binding sites for Elf-1 and HMG-I(Y), and is believed to have basal promoter activity [126]. Third enhancer element (PRRIII), located ~3.7 kb from the TSS (−3700 to

−3703), binds the Elf-1, Stat5, and GATA proteins. Its equivalent in the murine gene is a sequence between −1376 and −1304 [127]. PRRIV, on the other hand, is located within intron 1 (+3389 to +3596), both in humans and mice, while PRRV binds SMAD and CREB and is placed ~7.6 kb 5′ from the TSS [128,129]. The last one—PRRVI—is placed ~8.5 kb 5′ from the initiation transcription site, and is connected with the response to CD28 stimulation [130]. There are also two negative regulatory elements (NRE1/2) placed between −401 to −367 and −341 to −308, respectively [131].

The SEs stretched between the body gene and the sequence upstream of the TSS in the Il2ra gene binds STAT5 after cell activation by IL-2. CRISPR–Cas9-mediated deletion of the STAT5-binding sites within *Il2ra* SEs contributed to the diminished STAT5 binding and consequently resulted in lower IL2RA protein expression in T cells after 4 days of IL-2 stimulation [132].

5.2. Il2ra Methylation

Naturally occurring Tregs compared to Tconv cells have substantial demethylation in intron 1a in the *Il2ra* gene. Moreover, this Tregs-enhancer element is demethylated in freshly isolated cells, as well as cells subjected to the expansion procedure [13]. Upon stimulation, this region undergoes progressive demethylation in Tconv cells, indicating that demethylation in the intronic element of *Il2ra* is not restricted to the Tregs population. Additionally, $CD4^+CD25^-$ stimulation with TGF-β also leads to changes in methylation in the intronic region [12].

In the study on *Il2ra* methylation, including 18 CpGs in three $CD4^+$ subpopulations, the trend towards diminished methylation in Tregs compared to naïve and memory $CD4^+$ cells was revealed. Moreover, the CpG methylation pattern did not differ between the T1D cells and control groups, and upon activation, the naïve T cells underwent demethylation in PRRVI and +3502 CpG [133]. Samples analysis of whole blood from patients with autoimmune thyroid diseases (AITDs), in this case Hashimoto thyroiditis or Graves' disease, have shown general hypomethylation in the promoter region in the latter [134]. Similar results from PBMC-derived samples have shown hypomethylation of the *Il2ra* promoter in children with obesity-associated asthma, which could be connected with inflammation in these patients [135]. In another study, differences in the methylation pattern in PBMC-derived DNA samples between the study and control group could not be seen due to indiscernibility in the mixed cellular population [136]. Consistent with that, the analysis was extended to discrimination of five subsets of cells—T and B-lymphocytes, neutrophils, natural killer, and monocytes—using population-specific expression analysis (PSEA). Such an action revealed that T cells were the only cells with a hypomethylated promoter region in patients with multiple sclerosis (MS) compared to healthy individuals. Moreover, these cells had higher *Il2ra* expression than the control group, indicating a link between methylation in the promoter region and gene expression [136]. The methylation pattern in the proximal promoter in 6 CpGs, −459, −456, −373, −356, −272, and −241, varies between different subsets of immune cells. Moreover, some alterations of the level of methylation in particular CpGs occur. The least methylated CpGs are −241, −272, and −256, while the most are −456 and −459. However, −373 has an intermediate methylation level. What is more, Tregs compared to other cells had lower methylation in −459, −456, and −373 CpGs. The overall methylation did not alter between the T1D patients and control group, with the exception of −456 and −373, which were more methylated in the T1D group [137].

Like in the case of *Foxp3* and *Ctla4*, many DRs are found in SEs in the *Il2ra* gene, in which autoimmune-disease-associated SNPs (e.g., T1D and MS) are located.

5.3. Il2ra Methylation Modifications

A positive correlation between mRNA *Il2ra* and its protein CD25 was confirmed, which can be used to estimate the effect of methylating agents on their levels [138]. Previously mentioned dynamic demethylation in the *Il2ra* locus, which can be achieved through T

cell activation or CD25$^-$ cells' stimulation with TGF-β, is a potent mechanism targeting cell functionality [12]. Consistent with this, AZA or DAC promotes conversion of CD25$^-$ cells into CD25-expressing Tregs, which have a methylation pattern similar to nTregs; these reagents should mediate demethylation in the *Il2ra* locus [74–77]. The positive effect of other hypomethylating reagents, such as vitamin C, on the conversion of CD25 negative cells into positive cells was observed in humans and mice [139,140]. In humans, vitamin C enhances transformation of γδ T cells into Treg-like cells, leading to alterations in global DNA methylation, such as a decreased level in the *Il2ra* gene [140]. As Il-2 is a homeostatic regulator for Tregs, resulting in exaggeration of cell expansion, the therapy with a low dose of Il-2 is another factor affecting these cells [141–143]. From regulatory elements in the *IL2ra* gene, PPRIV—especially one CpG (+3502)—correlates with T cell activation. The higher level of IL-2 in supernatants of CD4$^+$ cell cultures was connected with lower DNA methylation and exaggerated *Il2ra* gene expression [133]. In addition, mice treated with combination therapy using RAPA and IL-2 had an increased number of nTregs, with the highest peak on Day 28, when TSDR was profoundly demethylated [144]. Research with non-human primates receiving low dose IL-2 or engineered IL-2 molecules showed excessive Tregs proliferation. These cells after in vivo expansion maintained high demethylation in exon 2 of the *Ctla4* gene and *Foxp3*-TSDR [145]. However, another study revealed no impact of IL-2 deprivation on TSDR methylation after 36-h stimulation [146]. In opposition, therapies using anti-CD25 blockade, such as daclizumab, result in Tregs depletion. Nevertheless, the remaining cells comprised the Treg-specific epigenome and were not much altered after anti-CD25 monoclonal antibodies treatment [147].

6. *Tnfrsf18* Gene

6.1. *Tnfrsf18* Gene Structure

The human *Tnfrsf18* gene on chromosome 1 (1p36.33) encodes a protein called GITR. The 1083 bp-long transcript codes a 241 aa protein, and consists of 5 exons, separated with 4 introns. The 5′ of exon 1 and 3′ of exon 5 are non-coding elements. The homology between the human and murine gene is around 55% [148,149]. Analysis of the *Tnfrsf18* promoter region has shown that *Foxp3* binds to this region between −227 and −19 from the TSS, leading to enhanced histone acetylation, and thus higher gene expression [150].

In addition, research on the mice gene has revealed an enhancer element that is responsible for NF-κB binding, located ~5 downstream of the promoter region [151].

6.2. *Tnfrsf18* Methylation

As TNFRSF18 expression on Tregs is constitutive. It was revealed that after CD4$^+$CD25$^-$ cells activation, substantial amounts of GITR are present on the cell surface [152]. The analysis of two CpGs: −121 bp to +125 bp and in exon 4 in the human *Gitr* gene, has confirmed total methylation in Tconv due to activation of DNMT1. As expected, Tconv possessed lower *Gitr* mRNA compared to Tregs and higher binding of DNMT1 and MBD4 to the promoter region [153]. During Tregs thymic maturation, illustrated by the decrease in CD24, it was reported that these cells underwent successive demethylation in the *Gitr* locus, which can be upregulated by the presence of IL-2 [14]. Regarding Tregs-specific hypomethylation regions, exon 5 in *Tnfrst18* is considered as a pivotal region, which remains demethylated upon cell activation. In addition, upon iTregs generation with the use of TGF-β, no reduction in *Gitr* methylation was observed [12]. Moreover, pTregs, generated in the tumor microenvironment, had reduced suppressive function and were hypermethylated in the Treg-related loci, e.g., *Tnfrsf18* exon 5, compared to nTregs [154]. In an animal model study, generated antigen-specific iTregs were able to acquire Tregs-specific hypomethylation in *Gitr* in exon 5, and prevented skin-graft rejection [113].

6.3. Tnfrsf18 Modifications

Gitr promoter hypermethylation was observed in patients with multiple myeloma, resulting in tumor progression. However, the treatment with AZA restored *Gitr* expression due to promoter hypomethylation [155].

Aza upregulation of the Tregs function has also been connected with higher expression of GITR on the cell surface, which can be done by demethylation in this region [77]. Consistent with these data, DAC was the agent that upregulated GITR in $CD4^+CD25^+$ cells [78]. Furthermore, miRNA-mediated knockdown of DNMT1 in Tconv has contributed to demethylation in regions specific to Tregs, including *Tnfrsf18* exon 5, as well as other commonly hypomethylated regions, such as exon 1 and −700 [12].

Despite the positive role of TET retroviral induction in iTregs on *Foxp3* CNS2 and *Il2ra* demethylation, no differences were observed in the *Tnfrsf18* locus. The addition of vitamin C with the combination of a low-oxygen cell culture, did not cause *Tnfrsf18* demethylation, indicating that this region is resistant to such a modification [12,85]. In another study, on alloantigen-induced Tregs, *Gitr* expression was detected but the addition of vitamin C did not alter the methylation level of this gene [86]. Another study revealed a little demethylation due to vitamin C. Nevertheless, it was not so profound as in other genes [86,87].

7. *Ikzf2* and *Ikzf4* Overview

Both *Ikzf2* and *Ikzf4* belong to the Ikaros family zinc finger protein 2. Based on the Ensembl database, the *Ikzf2* gene encodes a Helios protein and is located in chromosome 2 (2q34). Its 3888 bp transcript contains 9 exons, from which 7 are coding and encodes a 526 aa-long protein. *Ikzf4* encodes an Eos protein. It is placed on chromosome 12 (12q13.2). A 5314 bp transcript consists of 8 coding exons and creates a 585 aa-long protein. These two genes comprise a specific methylation pattern, which differs between nTreg and Tconv cells. Bisulfite sequencing of the *Ikzf2* gene revealed substantial demethylation in intron 3a and exon 6 in nTregs compared to Tconv cells, and a specific Tregs demethylated region in intron 1b of the *Ikzf4* gene. Moreover, in vitro-generated Tregs with or without retinoic acid or TGF-β and Foxp3-transduced Tconv cells do not acquire demethylation in *Ikzf2/4* genes (12). In addition, TGF-β deficiency in the environment of tumor-infiltrating cells did not much alter the methylation signature, including the *Ikzf4* region [156]. The discrepancies between the Tregs population were observed in another study, where pTregs and tTregs had a different methylation percentage of *Ikzf2* (complete methylation in the former) [157]. *Ikzf2* and *Ikzf4*, similar to the other abovementioned genes, also contain SEs, which are enriched in DRs. Regarding *Ikzf4*, these DRs are enriched in SNPs connected with autoimmune diseases. Development of $Foxp3^-$ thymic precursor cells is impaired in Satb1-deprived mice, which is indicated by the lack of demethylation in Tregs-related genes, e.g., *Ikzf2* and *Ikzf4* [35,38]. Consistent with the previously mentioned role of TET proteins in the demethylation process, this enzyme is highly important in demethylation in intron 1b of the *Ikzf4* gene. The analysis of 6 CpG sites in mice lacking TET2 and TET3 proteins has revealed higher methylation in all examined CpGs compared to wild-type mice [49].

Despite the lack of influence of retinoic acid or vitamin C in the demethylation of the *Ikzf2* gene, the beneficial effect of the BCG vaccine on *Ikzf2/4* demethylation was confirmed in a study on T1D. Significantly higher demethylation in 8 out of 11 CpGs, due to the BCG treatment, was found in Tregs-related genes [27,158].

Moreover, CD28 blockade is another approach that can be used to manipulate the methylation status of these genes. It was shown that $CD28^-$ iTregs had substantial demethylation in intron 1 of the *Ikzf4* gene and intron 3a of the *Ikzf2* locus [68].

8. Conclusions and Future in Tregs Applications

Therapies using Tregs have been extensively developed over the last years. Tregs' suppressive potential was used in the treatment of GvHD after bone marrow transplanta-

tion [159], in solid-organ transplantation [160], and in autoimmune diseases, such as T1D or MS [161].

In current clinical trials, polyclonal Tregs are utilized, whose positive effect and safety has been confirmed [162]. Polyclonal Tregs possess specificity towards multiple antigens, which decreases their tissue-specific effectiveness. For this reason, researchers are searching for a method to generate Tregs that would migrate precisely to the site of ongoing infection. Nowadays, several methods for generating antigen-specific (Ag-spec) Tregs are known.

Tang et at. in their study on Ag-spec Tregs in transgenic mice have shown higher suppressive capacity of these cells compared to polyclonal Tregs. They have also indicated better migration properties and duration in the target tissue [163]. Another approach to generate specific Tregs is based on in vitro expansion with antigen-presenting cells (APCs) derived from donors. It has been found that the exposure to alloantigens presented by DCs of B cells resulted in specific Tregs expansion [164,165]. Moreover, our research team has developed a method that uses monocytes presenting antigens to create Ag-specific Tregs. We have shown that Ag-spec Tregs were stable during cell culture and possessed higher suppressive properties than polyclonal cells [166,167]. Other approaches use transfection of viral vectors encoding TCRs or chimeric antigen receptors (CARs). In murine models, tumor-specific Tregs generated by lentiviral transfer of TCRs suppressed tumor-specific Teffs, which was indicated as tumour growth [168]. In reference to retroviral CAR transfection, lung epithelial-directed Tregs were able to diminish the immune response in a murine airway hyper-reactivity model [169]. The CRISPR–Cas9 method, based on gene knock-in and knock-out, is another that may improve the therapeutic effects of the cells via upregulation of the genes crucial for Tregs [170,171].

These methods are believed to be a potential tool for providing effective and safe Tregs therapies. In addition, the previously mentioned substances, the so-called epigenetic modifiers, could be another factor boosting Tregs function (Table 1).

Table 1. The discussed factors that can mediate changes in the DNA methylation pattern are listed with the mechanism of action and the direct role on the function of regulatory T cells.

Epigenetic Modifier	Affected Genes	Direct Effect on Gene	Direct Effect on Tregs	Reference
Vitamin C	*Foxp3, Ctla4, Il2ra, Ikzf4*	-increased CNS1/2 demethylation in iTregs in *Foxp3* gene -decreased methylation of Tregs DMRs in all genes	-enhancement of iTregs stability during cell culture -boosting *Foxp3* mRNA production -improving iTregs function and usefulness in therapies	[27,64,86,87]
IL-2	*Foxp3, Ctla4, Tnfrsf18, Ikzf4*	-decreased methylation of Tregs DMRs	-induction of demethylation in Treg precursors -preserving cell stability upon expansion	[43]
H2S	*Foxp3*	-higher promoter demethylation	-maintenance of Tregs properties	[88]
AOA	*Foxp3*	-increasing demethylation of promoter and CNSs	-promotes Treg-like polarization	[89]
Vitamin A	*Foxp3*	-demethylation in promoter of *Foxp3*	-increasement of mRNA and protein level of Foxp3 -enhanced pTregs production	[94]
MTX	*Foxp3, Ctla4*	-lower upstream enhancer methylation in *Foxp3* gene	-higher Foxp3 expression in cells from RA patients -higher CTLA-4 expression	[84]
RAPA	*Foxp3*	-stabilizes TSDR demethylation upon expansion	-protect Tregs phenotype and functional stability during cell expansion	[62]

Table 1. *Cont.*

Epigenetic Modifier	Affected Genes	Direct Effect on Gene	Direct Effect on Tregs	Reference
AZA DEC ZEB	*Foxp3, Ctla4, Tnfrsf18*	-decreasing promoter and TSDR demethylation in *Foxp3* gene -lower methylation of promoter in GITR gene	-increasing overall pTregs number -conversion of CD4+CD25- cells into Tregs -enhanced Foxp3, CTLA-4 and GITR expression	[35,74–80,83,116,117,119–122]
CD28 signaling pathway blockade	*Foxp3, Ctla4, Ikzf2/4*	-decreased methylation of Tregs DMRs in all genes	-generation of stable iTregs having comparable epigenome to nTregs	[68]
TNF1 antagonists	*Foxp3*	-lower promoter methylation	-increasing Tregs stability upon inflammatory environment	[97]
PIAS1 deletion	*Foxp3*	-lower promoter methylation	Higher production of Foxp3+ cells	[98]
CRISPR-dCas9-TET1CD	*Foxp3*	-induction of TSDR demethylation	-Foxp3 production in primary human T cells	[92,93]
IVIG	*Ctla4*	-demethylation of promoter	-upregulation of CTLA-4 transcript and protein level	[111]
BCG	*Foxp3, Il2ra, Tnfrsf18, Ikzf2, ikzf4*	-reduction of DNA methylation at multiple CpGs	-higher production of mRNA	[158]

ZEB—zebularine; AZA—azacytidine; DAC—decitabine; MTX—methotrexate; ATRA—all-trans retinoic acid; H2S—hydrogen sulfide; AOA—aminooxyacetic acid; RAPA—rapamycin; IVIG—intravenous immune globulin.

The possibility to create mixed protocols to create antigen-specific Tregs, with the addition of epigenetic modifiers, is a promising idea for future clinical trials. However, it is crucial to remember that these substances drive changes in non-Tregs too. For this reason, not only efficacy but also safety studies should be performed before wider clinical applications.

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