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Full-length article

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

Dorota Iwaszkiewicz-Grzes[1,6,**,*], Mateusz Gliwinski[1,6,**], Anne Eugster[2], Magdalena Piotrowska[1], Andreas Dahl[3], Natalia Marek-Trzonkowska[4,5,6], Piotr Trzonkowski[1,6,*]

[1] *Department of Medical Immunology, Medical University of Gdansk, Gdańsk, Poland*
[2] *Technische Universität Dresden, DFG-Center for Regenerative Therapies Dresden and the Cluster of Excellence, Dresden, Germany*
[3] *Technische Universität Dresden, DRESDEN-concept Genome Center, Center for Molecular and Cellular Bioengineering, Dresden, Germany*
[4] *Laboratory of Immunoregulation and Cellular Therapies, Department of Family Medicine, Medical University of Gdańsk, Gdańsk, Poland*
[5] *International Centre for Cancer Vaccine Science, University of Gdańsk, Gdańsk, Poland*
[6] *Poltreg S.A., Gdańsk, Poland*

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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 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].

* Dorota Iwaszkiewicz-Grzes and Piotr Trzonkowski, MD, PhD, Department of Medical Immunology, Medical University of Gdansk, Dębinki 7 Street, Bldg 27, II Floor, 80-952 Gdańsk, Poland.
E-mail addresses: dorota.iwaszkiewicz@gumed.edu.pl (D. Iwaszkiewicz-Grzes), ptrzon@gumed.edu.pl (P. Trzonkowski).
** These authors contributed equally to this work.

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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.

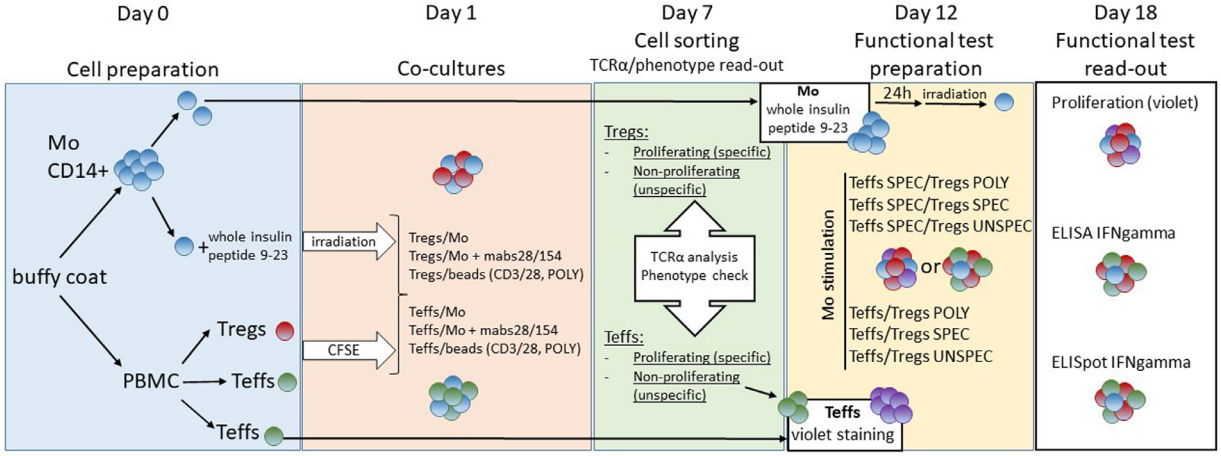

Fig. 1. Experimental overview. Monocytes and lymphocytes are isolated from buffy coats. Tregs (red) and Teffs (green) are isolated from PBMCs. All Tregs and some Teffs are stained with CFSE and stimulated with irradiated autologous monocytes previously loaded with antigen (whole insulin or insulin β 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.

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

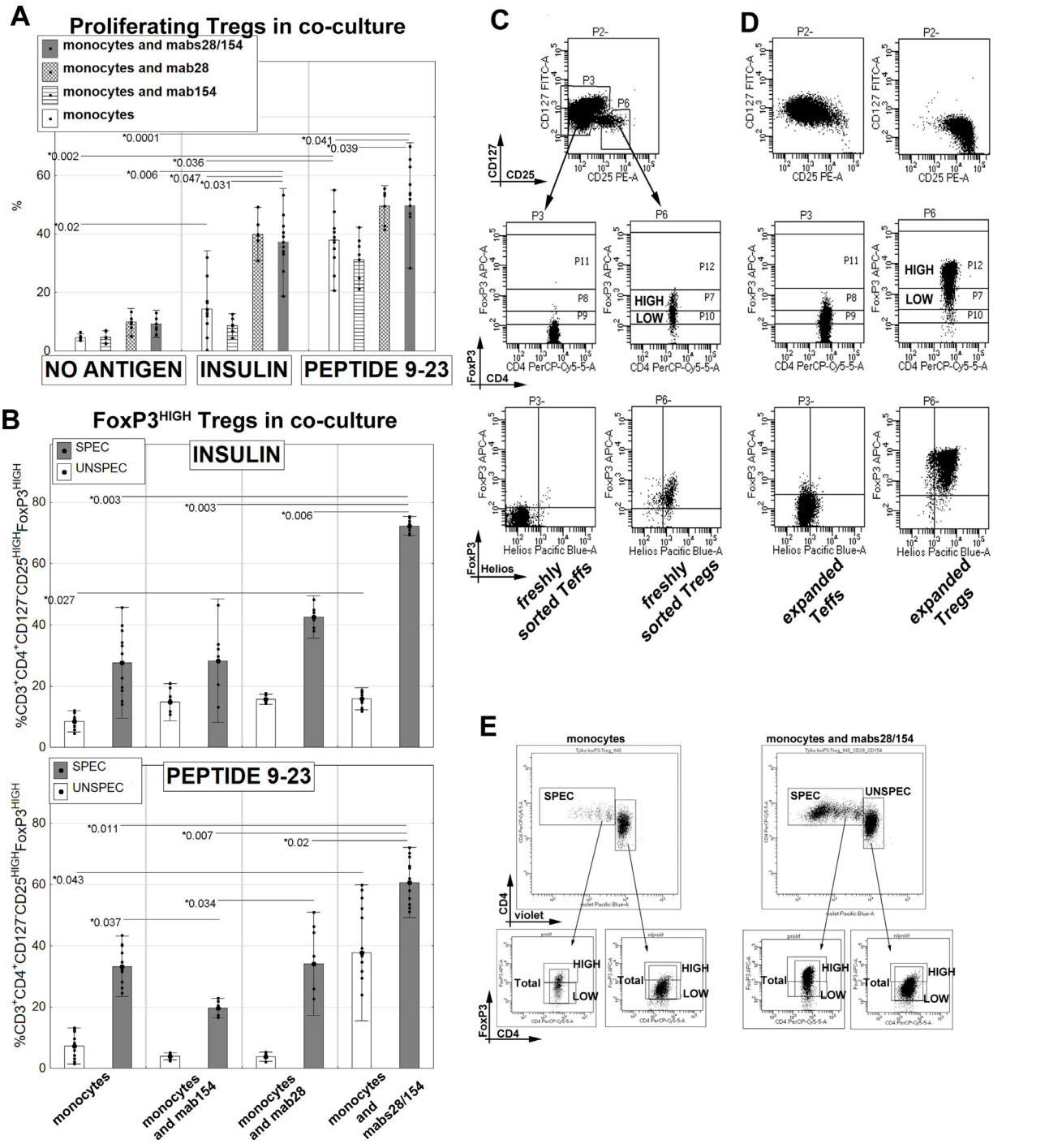

Fig. 2. Percentage of Tregs responding to antigens in co-cultures with monocytes. The percentage of proliferating (antigen-reactive, SPEC) Tregs (A) and Tregs expressing a high intensity of FoxP3 expression (FoxP3^{high}) in specific (SPEC, gray bars) and unspecific (UNSPEC, transparent bars) Tregs (B) 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	36 076
S1		Tregs SPEC	0.7	9.18	12 448
S1		Tregs UNSPEC	1	6.81	139 549
S1		Teff POLY	4.5	14.78	48 070
S1		Teff SPEC	4	5.07	49 172
S2		Tregs POLY	1	14.68	254 832
S2		Tregs SPEC	1	6.86	249 220
S2		Tregs UNSPEC	1	15.89	184 561
S2		Teff POLY	1	14.66	238 868
S2		Teff SPEC	1	16.58	27 654
S3		Tregs POLY	1	25.26	66 161
S3		Tregs SPEC	1	30.3	16 051
S3		Tregs UNSPEC	1	10.6	90 003
S3		Teff POLY	1	18.11	110 327
S3		Teff SPEC	1	19.4	34 000
S3		Teff UNSPEC	1	10.92	167 092
S1	INSULIN PEPTIDE 9-23	Tregs POLY	1	7.34	6722
S1		Tregs SPEC	1	12.5	96 289
S1		Tregs UNSPEC	1	8.03	140 883
S1		Teff POLY	1	8.1	237 344
S1		Teff SPEC	1	11.12	35 229
S1		Teff UNSPEC	1	6.75	65 158
S2		Tregs POLY	1	2.38	16 198
S2		Tregs SPEC	1	5.09	13 991
S2		Tregs UNSPEC	1	6.2	26 385
S2		Teff POLY	1	37.04	78 272
S2		Teff SPEC	1	35.3	53 115
S2		Teff UNSPEC	1	63.44	227 414
S3		Tregs POLY	2	11.04	134 664
S3		Tregs SPEC	2	10.01	3455
S3		Tregs UNSPEC	1	6.99	17 343
S3		Teff POLY	2	8.83	39 143
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, $P =$

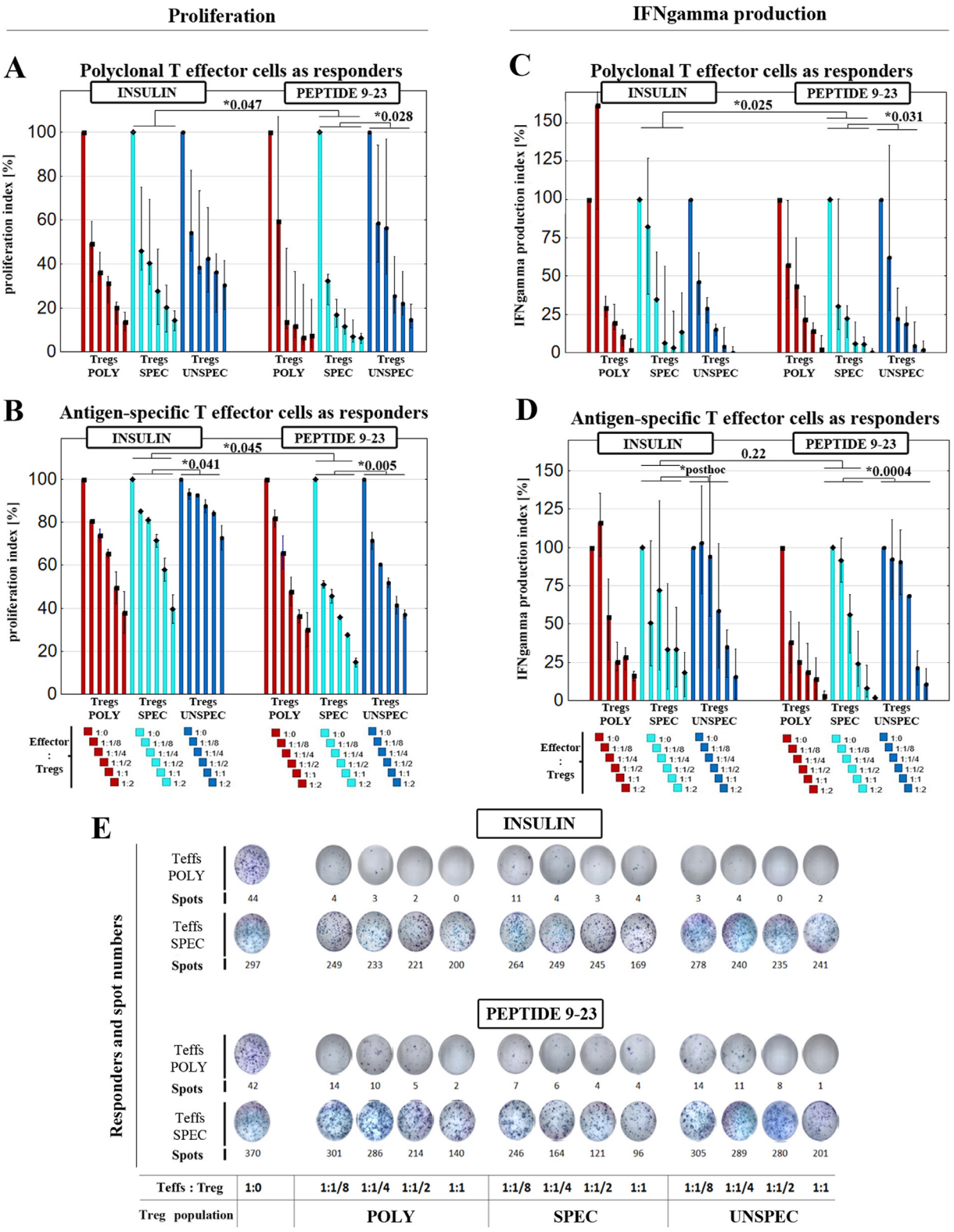

Fig. 3. Functional tests, inhibition of Teff lymphocyte proliferation and IFNγ production. POLY, SPEC (toward insulin or insulin β chain peptide 9–23) and UNSPEC Tregs were co-cultured with autologous Teff cells as responders. We used polyclonal (not stimulated with antigen or bead anti-CD3/anti-CD28) (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.

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

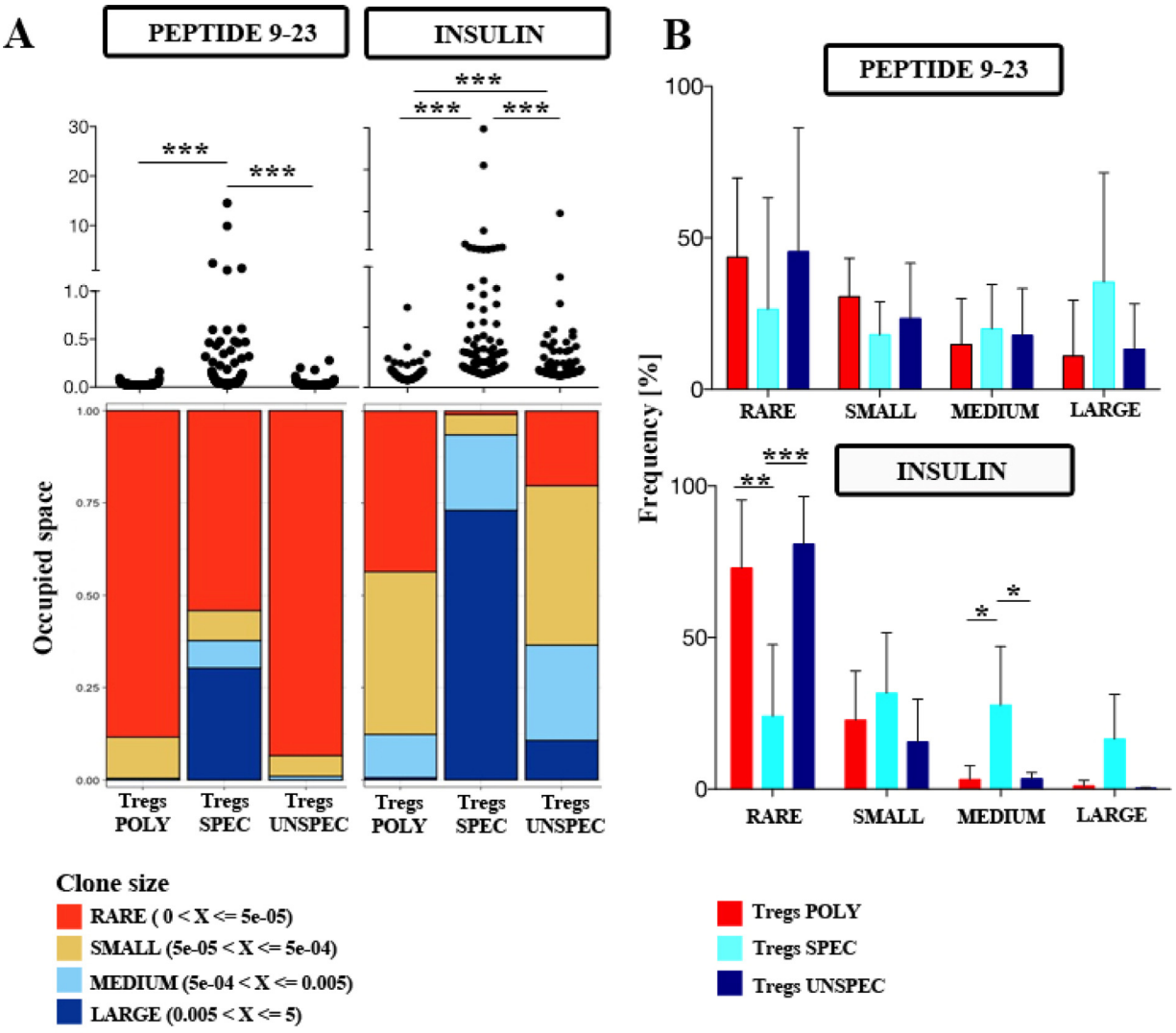

Fig. 4. Clonotype distribution in stimulated Tregs repertoires. High-throughput TCRα chain sequencing was used to analyze clonotype frequencies in Tregs (reactive, polyclonal or unreactive). (A) Relative frequencies (%) of the 100 most abundant clonotypes are shown (top). The proportion of space occupied 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. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

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

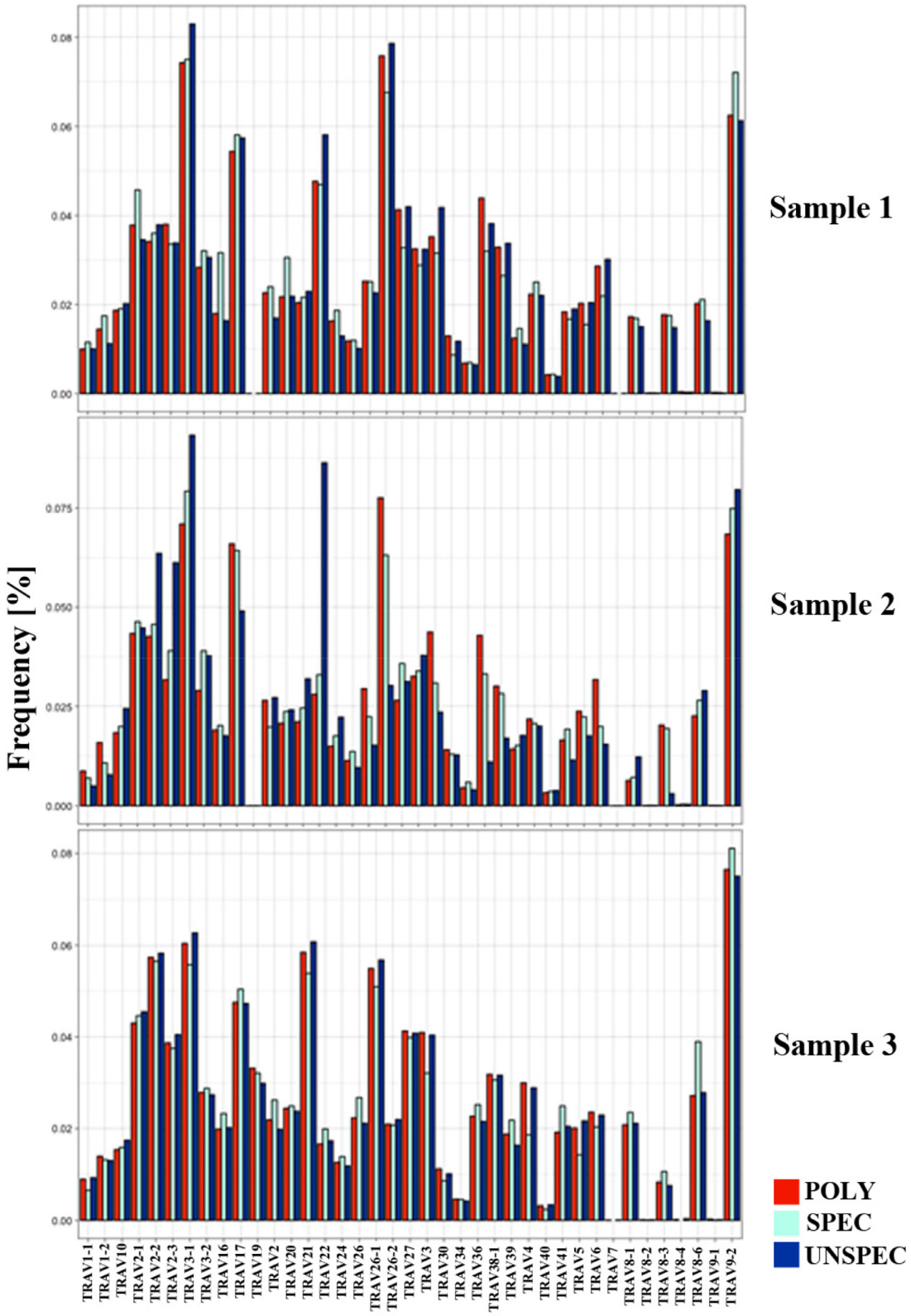

Fig. 5. *TRAV* gene usage of stimulated Tregs repertoires. High-throughput TCRα chain sequencing was used to compare *TRAV* gene usage of Tregs in SPEC, POLY and UNSPEC cells. Shown are the frequencies (%) of the individual T-cell receptor alpha variable (*TRAV*) genes for 3 samples tested.

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

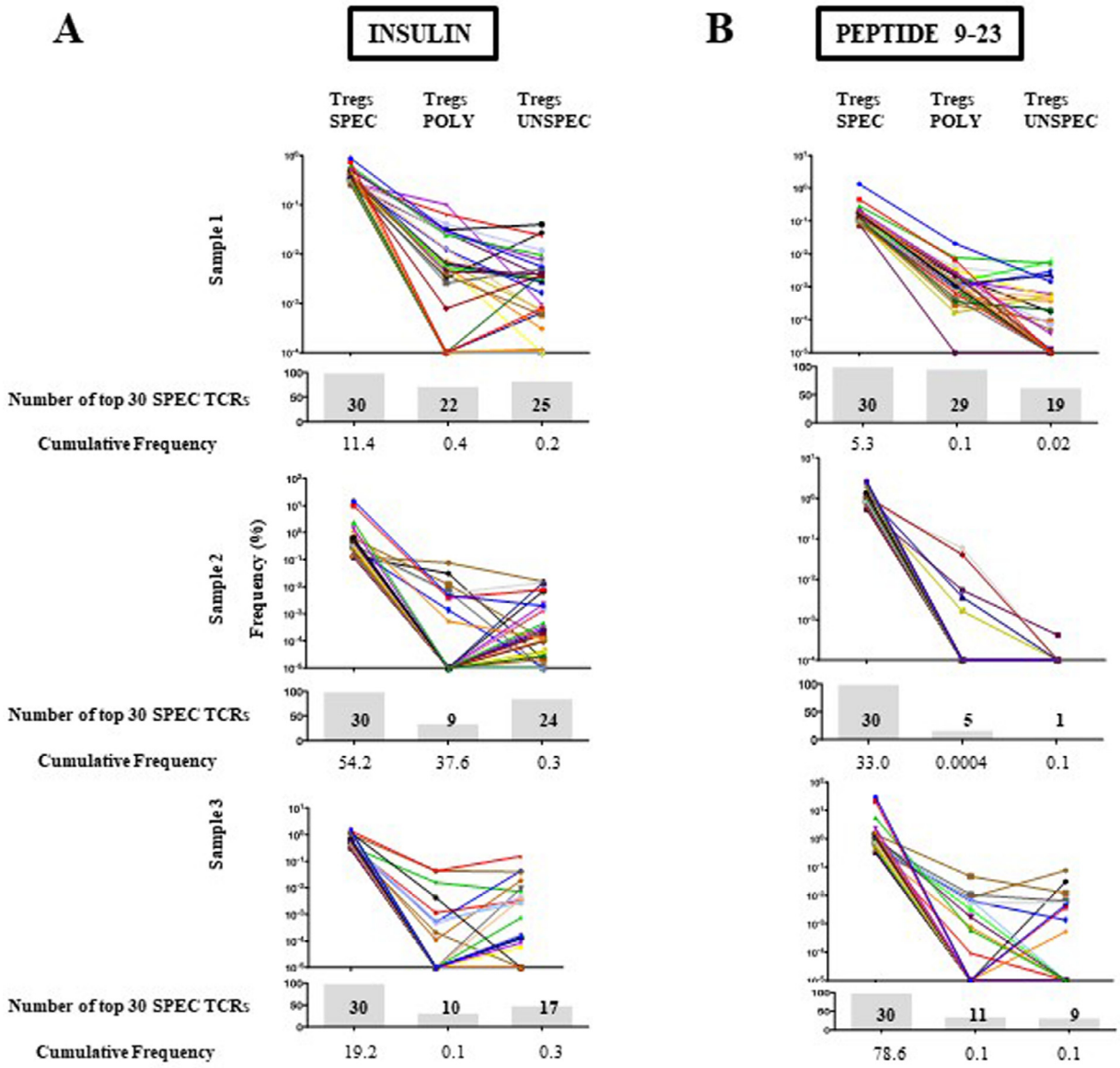

Fig. 6. Sharing of the 30 most frequent reactive Tregs clonotypes. Tracking the 30 most frequent clonotypes of whole insulin (A) and insulin β chain peptide 9–23 (B) reactive Tregs repertoires from 3 samples in the repertoires of polyclonally and unspecifically stimulated Tregs obtained by high-throughput 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.

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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