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The road towards in vitro expansion of Regulatory T cells in an antigen-driven manner

Table of content

Abstract

Current treatments for Rheumatoid Arthritis aim at remission, often rendering patients immunocompromised. Novel treatments options would therefore be beneficial. It is hypothesized that a dysfunction of Regulatory T cells plays a role in the pathology of Rheumatoid Arthritis. Thus, *ex vivo* polyclonal culturing of Regulatory T cells, followed by adoptive cell therapy would be a potential new therapy. Hereby, the number and function of Regulatory T cells can be restored. Although this approach shows potential, it comes with limitations, such as a lack of potency and specificity together with a higher risk of generalized immunosuppression. To overcome these, the use of antigen-specific Regulatory T cells would be more beneficial. However, antigen-specific expansion is challenging due to, among others, low available cell numbers. A method in which the high cell yields of polyclonal expansion are combined with the specificity of antigen-specific Regulatory T cells is therefore needed.

In our research we move towards a method by which Regulatory T cells can be expanded in an antigen-specific manner. To this end, a protocol for the polyclonal expansion of Regulatory T cells is established using low concentrations of TransAct, high concentrations of rhIL-2 and addition of a TNFR2-agonist, leading to high expansion numbers while retaining a stable phenotype. We show that cells expanded under these conditions can potentially be restimulated in an antigen-specific manner with mammalian B29a, a heat shock protein hypothesized to be involved in Rheumatoid Arthritis. This enables us to obtain a high number of B29a-specific Regulatory T cells from which potentially, in future research, TCRs can be isolated.

Layman's summary

Rheumatoid Arthritis is a chronic disease in which the immune system, the defense of the body against pathogens, turns on the body itself. It affects approximately 0.46% of the population worldwide. Current treatment options are aimed at suppression of the immune system as a whole, leading to the patient being more susceptible to disease and infections. Therefore, new methods to treat Rheumatoid Arthritis, in which the immune system can remain operational are preferable. Rheumatoid Arthritis is characterized by the immune system attacking cells of the body, leading to inflammation in the joints, leading to subsequent damage to cartilage and bone. The precise mechanisms by which this happens are not yet fully understood. However, it is hypothesized that one specific cell type of the immune system, the Regulatory T cell, might play a role in this process. Regulatory T cells are the regulators of the defense system and make sure that the response against pathogens remains in balance. However, when these cells are too low in numbers or are not functioning properly, this balance can be distorted. As a result, the immune response can be derailed, which can cause persistent inflammation. Moreover, it can also cause the immune system to respond to and attack cells of the body itself, causing autoimmune disease. Therefore, one potential new way of treatment, would be by restoring the function and number of Regulatory T cells. This can be done by adoptive cell therapy, in which Regulatory T cells are cultured and expanded in the lab. Hereafter, they can be transferred back into the patient. Though this might sound simple, it is difficult to obtain high amounts of cells, which are necessary for such transfer. Moreover, to exert their function properly, they need a certain specificity for the disease and also need to remain Regulatory T cells. Literature shows, that when expanded in the lab, these cells can change to another phenotype, a more proinflammatory one. This can worsen the symptoms of Rheumatoid Arthritis. However, several methods have been developed to overcome these challenges. High cell yields can be obtained with certain expansion methods and addition of factors that improve and promote growth. Secondly, the specificity can be obtained by stimulating the cells with a disease-specific peptide. Lastly, the Regulatory T cell phenotype can be maintained by addition of certain molecular factors, which secure cell fate.

Therefore, in our research we aim to test which of these molecular factors work best to obtain high cell yields and which preserve the Regulatory T cell phenotype after expansion. Once this is known we want to test if we can expand these cells once more in response to a disease specific peptide, to obtain Regulatory T cells with a high specificity. If this were to be successful, the T-cell receptor, a molecule that aids in recognizing the correct antigens, can be isolated from these cells and transferred to Regulatory T cells with less specificity. Hereby, the needed specificity for the adoptive cell therapy can be achieved. To this end, we tried culturing Regulatory T cells

with TransAct in combination with high concentrations of IL-2, a molecule that Regulatory T cells need for growth. Via this combination, we obtained high cell yields which remained high in their expression of specific markers that are related to Regulatory T cells. Moreover, a TNFR2-agonistic antibody and Rapamycin, two molecules hypothesized to ensure a stable Regulatory T cell fate were also tested in our culture. However, Rapamycin lead to a ~50% decrease in cell yield. Addition of the TNFR2-agonist did not affect the expansion. Although the percentage of cells expressing the specific Regulatory T cell markers became higher. This points towards a better maintenance of the Regulatory T cell phenotype and thus proved beneficial to our culture protocol. Once these conditions were established, cells expanded under these methods were restimulated with mammalian B29a, a peptide that is said to be specifically related to Rheumatoid Arthritis. Hereby, we aim to expand Regulatory T cells specific for this peptide and thus for the disease. Preliminary results showed a potential expansion of Regulatory T cells specific for the B29a peptide. Especially, under stimulation with the TNFR2-agonistic antibody. Moreover, the expression of the Regulatory T cell specific markers also seemed to be slightly higher under expansion with the TNFR2-agonist. However, to validate these results, the experiments need to be repeated and optimized to be able to make a stringent conclusion. However, the first steps in establishing a novel protocol for the expansion of Regulatory T cells specific for a disease related peptide have been made.

Introduction

The pathology of Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an autoimmune disease characterized by systemic inflammation, which can lead to swelling of the joints, musculoskeletal pain and additional stiffness as its clinical symptoms [1]. Between 1980 and 2018 the global prevalence was estimated to be 0.46%, affecting approximately 460 out of 100.000 individuals[2]. The common prevalence of RA, together with its progressive disability and comorbidities can lead to early death and high socioeconomic costs[3]. The pathogenesis of RA is complex and includes various processes, such as synovial cell proliferation, fibrosis, vascular membrane formation and erosion of both cartilage and bone. It is hypothesized that one possible mechanism underlying these processes is the fact that self-tolerance is disturbed due to the dysfunction of Regulatory T cells (Tregs). In healthy individuals, Tregs make up approximately 5% of the mature $CD4^+$ $CD8^-$ T cells[4]. They are the regulators of the immune system and maintain immune homeostasis, via several methods[5] . For instance, they suppress other cells of the immune system, either via secretion of anti-inflammatory cytokines (Transforming growth factor (TGF) β, interleukin (IL) 10 and IL-35), expression of specific regulatory cell surface receptors, consumption of IL-2 or via the indirect or direct targeting of T cells and antigen presenting cells (APCs) [6]. However, in autoimmune diseases, such as RA, Tregs are unable to keep the balance of the immune system. This can either be caused by a dysfunctionality in their suppressive activity or in their number. Hereby, an imbalance between Tregs and effector T cells can arise, leading to an abnormal cellular and humoral immunity. Ultimately, this can result in persistence of inflammation and loss of tolerance[7]. The number of Tregs are decreased in the peripheral blood of RA patients. Contradictory, more Tregs are present in the synovial fluid of RA patients. However, these Tregs lack their suppressive function, caused either by the inflammatory environment they reside in or as a result of effector T cells being irresponsive to suppression, rendering them ineffective in their role of maintaining immune homeostasis[1]. Besides effector T cells, other cells from the innate and adaptive immune system, such as mast cells, dendritic cells (DCs), B cells and plasma cells can also be abnormally activated in RA. Activation of these cell types can in response lead to a production of rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), deposition of immune complexes in the synovial tissue and excess production of pro-inflammatory cytokines including IL-6, IL-17 and Tumor necrosis factor (TNF). Ultimately, these processes can lead to persistent synovitis and destruction of the affected joints[1]. In normal situations, these inflammatory processes are counteracted by Tregs, hereby maintaining the immune tolerance. However, it is hypothesized that in RA Tregs cannot exert their function properly and are inefficient in counteracting these inflammatory processes[1]. Furthermore, there are also certain genetic components involved in the pathogenesis of RA. For instance, certain HLA-types contribute to the risk of developing RA and the severity of disease progression. The frequency of the HLA-DR4 is significantly higher in RA patients compared to healthy controls. Moreover, people with HLA-DR4 have up to a 3-fold higher risk of RA development[8]. RA patients

expressing HLA-DR4 often present with a more severe form of RA, characterized by a more aggressive disease progression and high levels of rheumatoid factor (RF)[9,10]. Furthermore, HLA-DR4 positive RA patients also tend to have a more familial pattern of RA occurrence [10].

Currently, autoimmune- or inflammatory diseases such as RA are mostly treated with immunosuppressive drugs, targeting the effector T cells that attack the self-antigens. However, this renders the patient immunocompromised, as the entire immune system is often downregulated[5]. Therefore, research has opted for other treatment options. One potential option is the *ex vivo* culturing of Tregs while subsequently giving them to the patient, hereby restoring the number and/or function of Tregs[6].

Characterization of Regulatory T cells

To study Tregs, both in healthy individuals as well as in disease, they need to be characterized. Tregs circulate in the blood in low amounts and are currently best identified by expression of the surface markers $CD4^{+}CD25^{high}CD127^{low}$, combined with the intracellular expression of Forkhead box P3 (FoxP3) and Helios. Tregs specifically express Foxp3, a transcription factor which is a master regulator of the development of Tregs and their function, while also playing a role in the expression of other genes[1]. Moreover, the expression of FoxP3 on Tregs is regulated via epigenetic-, transcriptional and post-translation mechanisms and plays a key role in the maintenance of the immunomodulatory properties of Tregs[1]. Determination of the methylation status of the FoxP3 locus can also give an important indication of the suppressive function of Tregs. Especially, methylation of the CpG islets at the second intron enhancer site provides information about the stability of FoxP3 expression. In Tregs this region, also known as the major TSDR, is specifically demethylated in contrast to in other cell types. This demethylation subsequently leads to a more stable expression of the transcription factor[11–13]. Moreover, recent evidence has also suggested a role for Helios as a potential marker for the identification of Tregs, as it is highly specific for this cell type. This marker seems to be independently expressed from FoxP3[14,15]. Helios is a zinc-finger transcription factor and a member of the Ikaros family[16], which is said to play a role in the stability of the Treg phenotype. This hypothesis is supported by the fact that Tregs expressing Helios show a better suppressive capacity when compared to Tregs which do not express Helios. Furthermore, it was shown that Tregs lacking Helios expression can downregulate FoxP3 while subsequently upregulating the expression of inflammatory cytokines, converting the Treg to a more Th17-like phenotype[16]. Though these intracellular markers enable good characterization of Tregs, they are not fit for use when Tregs need to be cultured after characterization. Staining for intracellular markers requires permeabilizing and fixation of the cells, leading to their dead. Therefore, only characterization via $CD4^{+}CD25^{high}CD127^{low}$ can be used prior to Treg cultures. Besides these markers, there are also other Treg-associated cell-surface molecules that are upregulated in the presence of Foxp3. These include, amongst others, cytotoxic T cell-associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene/protein (GITR)[17]. However, one limitation of the use of $CD4^{+}CD25^{high}CD127^{low}$ cells expressing FoxP3 and Helios, is that both expression of CD25 and FoxP3 can be transiently upregulated by effector T cells once activated[5].

Regulatory T cells as a potential therapeutic agent

As Tregs are a promising novel therapeutic agent for the treatment of RA, for example through adoptive cell therapy (ACT), this has been tried in several clinical trials. Results of these trials regarding manufacturing and safety look promising[4,18–20]. However, current approaches still come with some limitations. Such limitations include the instability of the Treg phenotype after expansion. Under specific environments Tregs can divert to a pro-inflammatory, Th17-like phenotype. These transformed Th17 cells show a higher potential to induce osteoclast production and they can accumulate in the joints where they lead to more inflammation[21]. This same phenotypic switch has been observed in *in vitro* expanded Tregs within several studies. Here, these cells showed the potential to express IL-17 and, after repeated rounds of amplification, they had the potential to transform into Th17-like cells[22,23]. As this Th17-like phenotype plays a role in the underlying mechanisms of the inflammatory disease, this cell type is more pathogenic and the switch is a non-desirable effect[1]. Prevention of such a phenotypic switch can be accomplished by addition of different growth factors to the culture of Tregs. One such growth factor is Rapamycin, an immunosuppressant and metabolic inhibitor of the mechanistic target

of Rapamycin (mTOR) kinase. This molecule is often added to Treg cultures to counteract the possible impurity of isolation techniques, as it is said that Rapamycin can inhibit the expansion of other cell types than Tregs. Via this mechanism it can aid in selective expansion of Tregs[24–26]. This is especially of interest, since isolation techniques, such as Magnetic Activated Cell Sorting (MACS) isolation, are often not 100% pure. Thus, small amounts of other cell types such as effector T cells can remain, which is unfavorable as these can aid in the inflammatory response rather than restore homeostasis[27]. Therefore, methods to discard of these cell types and inhibit their expansion are of interest.

Furthermore, a promising role for TNF receptor type 2 (TNFR2) agonists has recently been shown in the maintenance of a stable Treg phenotype. Studies showed that, Tregs with high expression of TNFR2 show a more stable expression of FoxP3. When expression of FoxP3 was more closely studied in these Tregs, hypomethylation of the TSDR of FoxP3 was observed. This points towards a more stable phenotype[28]. Moreover, it was shown that Tregs reverted to Th17-like cells when the TNFR2 was deleted, further confirming the importance of its expression[29]. Lastly, not only does the receptor seem to play a role in the phenotypic stability, as it is said to play a part in Treg metabolism, it is hypothesized to also lead to a higher expansion rate of Tregs[30,31].

Moreover, polyclonal expansion of Tregs still knows several other limitations. Although polyclonal expansion leads to high cell yields with the addition of growth factors, polyclonally expanded Tregs are less potent in their suppressive ability compared to Tregs expanded via an antigen-specific method. Since polyclonally expanded Tregs are not specific to one, or a limited number of antigens, but rather have a wide variety of antigen-recognition, they are less potent in their ability to suppress specific disease phenotypes[6]. An additional limitation of polyclonal Tregs, is that in contrast to antigen-specific Tregs, they seem to provide a higher risk of generalized immunosuppression, as antigen-specific Tregs localize primarily at the site of antigen-presentation while polyclonal Tregs do not. Thus, a method by which endogenous antigen-specific Tregs are expanded and used as a therapeutic agent would seem to be more promising. However, the expansion of these cells is challenging since Tregs that are specific for a defined antigen are infrequent in the periphery[6] and a disease-specific antigen must be known. One such potential disease-related self-antigen connected to the pathology of RA, is mammalian B29a (mB29a). This antigen is an immunodominant heat-shock protein (HSP). HSPs are known to be highly expressed at inflammation sites and can be upregulated in certain stress-rich environments. Here, they can be presented by MHC class II to, amongst others, Tregs [32]. Moreover, recent literature has pointed towards the presence of Tregs specific for mB29a peptide. Specifically HLA-DR4 positive cells show more potential of recognizing and responding to this HSP[32]. Therefore, a method in which the high expansion of polyclonally cultured Tregs and the specificity of mB29a antigen-specific Tregs can be combined would seem to be ideal. To this end, one possible method would be polyclonal expansion of Tregs followed by introduction of synthetic receptors, such as engineered TCRs. In this way, the polyclonal Tregs can be redirected towards an antigen-specific fate[6]. Currently, some efforts have been made towards polyclonal Tregs with engineered TCRs as therapeutic agents. However, in these instances TCRs were mostly isolated from effector T cells rather than Tregs themselves. This poses a possible problem, as TCRs from T effector cells can have differences in intrinsic affinity and their specificity, hereby making them distinctly different from those of Tregs. This can have effects on both the migration and functional activity of these engineered cells.

Therefore, in our research we aim to establish a method by which polyclonally expanded Tregs can be restimulated in an antigen-specific manner, while retaining a stable Treg phenotype. Via this method, we want to obtain antigen-specific Tregs from which, in future research, TCRs can be isolated for cloning purposes and possible transfer to obtain Tregs with an engineered TCR.

Materials and methods

Isolation of (regulatory) T cells

PBMC isolation

Buffy coats from healthy donors were provided by Sanquin, Amsterdam. The peripheral blood mononuclear cells (PBMCs) used for the isolation of Tregs were obtained via density gradient isolation with Ficoll-Paque™ PLUS (1.077 g/mL, Cytivia) following the manufacturers protocol (Figure 1). PBMCs were either further processed for culturing or freezed in TexMACS™ medium (Miltenyi Biotec) at -140 °C after addition of 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich).

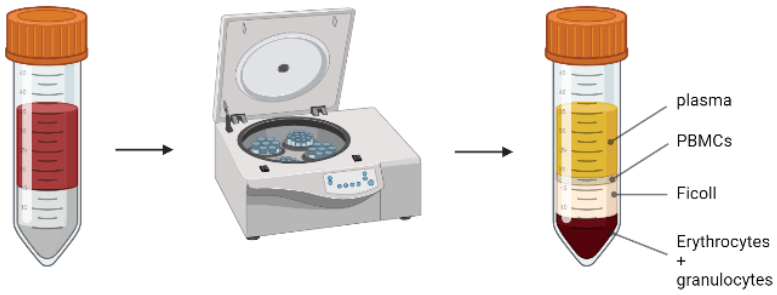

Figure 1 ***Density Gradient Isolation of Peripheral Blood Mononuclear Cells via the use of Ficoll-Paque*****.** Blood from a Buffy coat is loaded on top of a layer of Ficoll within a 50 mL tube. Subsequently, the sample is centrifuged at high speed, creating a Density Gradient. The blood component with the highest density, erythrocytes and granulocytes, are forced through the Ficoll to the bottom of the tube due to the high speed. Hereafter, the Ficoll has the highest density, causing the PBMCs to land on top of this layer. The plasma is the component of the Buffy coat with the lowest density, therefore forming the top layer.

MACS isolation

Isolation of Tregs from PBMCs was either done after thawing frozen vials or by using freshly isolated PBMCs. After thawing or isolation, cells were counted using Trypan Blue. Tregs were isolated via a two-step Magnetic Activated Cell-Sorting (MACS) isolation, using MACS buffer, consisting of 1x DPBS (Corning), 2% NHS and 2 mM EDTA, and the CD4+ CD25+ Regulatory T cell isolation kit (human, Miltenyi Biotec) following the manufacturers protocol. Both the CD4+ CD25+ enriched (Treg) and CD4+ CD25- (non-Treg) fractions were subsequently plated and cultured. The different fractions of the isolation procedure were stained for FACS analysis and run for purity.

Flow cytometry

Phenotypic characterization of cells was done via flow cytometry using a Cytoflex LX (Beckman Coulter) with the corresponding software CytExpert V2.1 (Beckman Coulter). Results were analyzed via the use of Flow Jo data analysis software (Tree star). For characterization of the Treg phenotype the following conjugated mononuclear antibodies (mAbs) were used: CD3 BV510 (clone UCHT1, Biolegend, 1:100), CD4 BV785 (clone SK3, BD Bioscience, 1:200), CD25 FITC (clone M-A251, BD Bioscience, 1:50), FOXP3 PE (clone 236 A/E7, eBioscience, 1:100), Helios AF647 (clone 22F6, Biolegend, 1:50) and Viakrome 808 (Beckman Coulter, 1:1000). For intracellular staining we used the eBioscience™ FOXP3/Transcription Factor Staining Buffer set (Invitrogen) following the manufacturers protocol. In short, cells were first stained for surface markers, followed by a Viakrome staining to distinguish between live and dead cells. Hereafter, cells were fixed with a fixation buffer after which they were permeabilized. Intracellular staining was performed in permeabilization buffer. For consistent analysis between experiments a standard gating strategy was used (Figure 2).

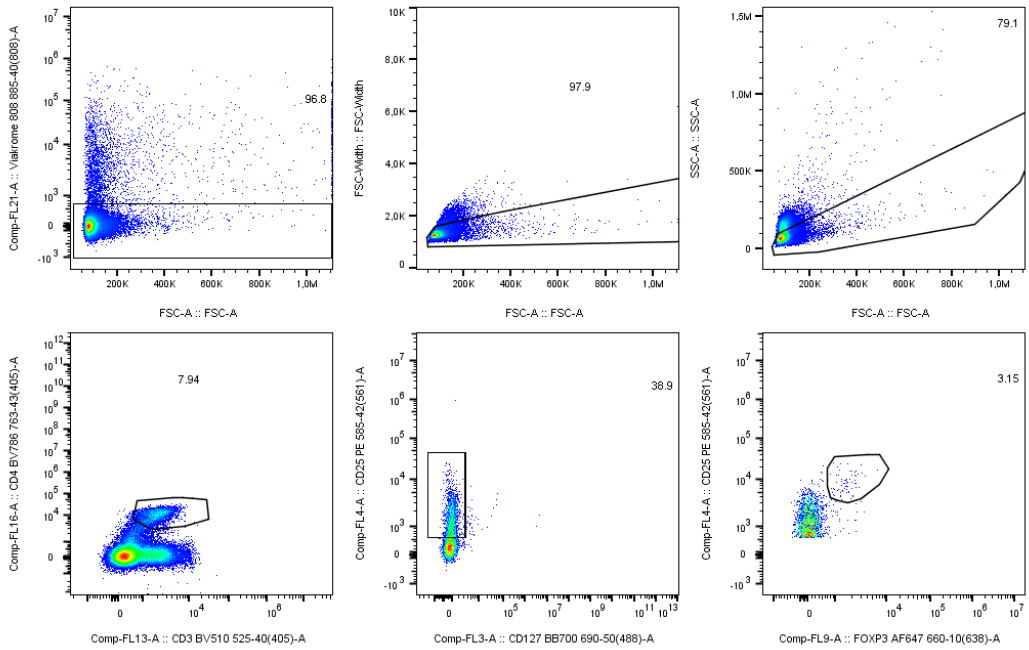

Figure 2 ***Gating strategy of Regulatory T cells with Flow Cytometry analysis.*** Gating starts at the upper left square, where live cells are gated with Viakrome x FSC-A. Moving to the right, first singlets are gated out with FSC-Width x FSC-A after which there is gated on lymphocytes using SSC-A x FSC-A. Hereafter, moving to the bottom left square, CD3+ CD4+ T cells are gated out using CD4 x CD3 selection. Next, T cells are gated on CD127 low and high expression of CD25 using CD25 x CD127, after which lastly Regulatory T cells are gated with high expression of both CD25 and FoxP3.

In vitro (regulatory) T cell culture

PBMCs and/or MACS isolated T cells were cultured in TexMACS™ medium (Miltenyi Biotec) supplemented with penicillin and streptomycin (P/S) at 37°C, 7% CO2. Cells were cultured under different conditions with T cell TransAct (Miltenyi Biotec), rhIL-2 (Proleukin, Novartis) and either TNFR2-agonistic antibody (2.5 µg/mL, mAb MR2-1, Hycult), Rapamycin (Pharmacopoeia standard) or a combination of the aforementioned. PBMCs were either thawed or obtained from a buffy coat and subsequently stained with CFSE to track the expansion. To this end 0,5 µM CFSE was added and cells were incubated for a maximum of 10 minutes in a 37°C waterbath. The reaction was quenched by adding either TexMACS + P/S + 5% Human serum or MACS buffer. Subsequently, cells were placed in culture or Tregs were first isolated via MACS. Cells were added to a 96-wells U-bottom plate in an end-concentration of 100.000 cells in 200 microliters. Cells were cultured for 7 days, after which they were rested. To this end the supernatant was removed and cells were resuspended in fresh TexMACS . On day 8 the cells were harvested and stained for Flow Cytometry.

Antigen specific restimulation of Regulatory T cells

For antigen-specific restimulation, polyclonally expanded Tregs were co-cultured with autologous irradiated PBMC (feeder cells) and peptide. Mammalian B29a-peptide (VLRVINEPTAAALAY) (GenScript, New Jersey, USA), Tetanus Toxoid (TT) peptide (IntraVacc, Bilthoven, The Netherlands) or no peptide was used in the presence of rhIL-2 together with or without TNFR2-agonist in TexMACS medium. PBMCs from two HLA-typed donors, both HLA-DR4, were used. PBMC feeder cells from the same donor were used and labeled with 0,5 µM CFSE. Cells were brought to a concentration of $10*10^6$ cells per mL, after which they were irradiated with 30 Gy at the UMC, Utrecht. Irradiated feeder cells were added at a 1:1 ratio to Tregs, thus 100.000 cells were added to each well. Subsequently, Tregs were cultured for another 7 days. On day 4 and 6 of the restimulation the medium was refreshed and supplemented with new rhIL-2. On day 7, cells were stained and analyzed with Flow Cytometry.

Results

Lower TransAct stimulation seems to favor the expansion of Regulatory T cells

As a prerequisite for the culturing of Tregs, we isolated Tregs from healthy donor PBMCs via MACS-isolation. During the course of the project, we performed a total number of seven Treg isolations. This resulted in an average of ~61,6 % purity of $CD25^{+}FoxP3^{+}$ cells, ranging from ~50% to 70% (Figure 3). Furthermore, an average of 0,54% of Tregs was isolated out of the initial starting material of PBMCs. For example, when $300*10^{6}$ PBMCs were thawed and used for MACS-isolation, this resulted in an average of ~2 million Tregs.

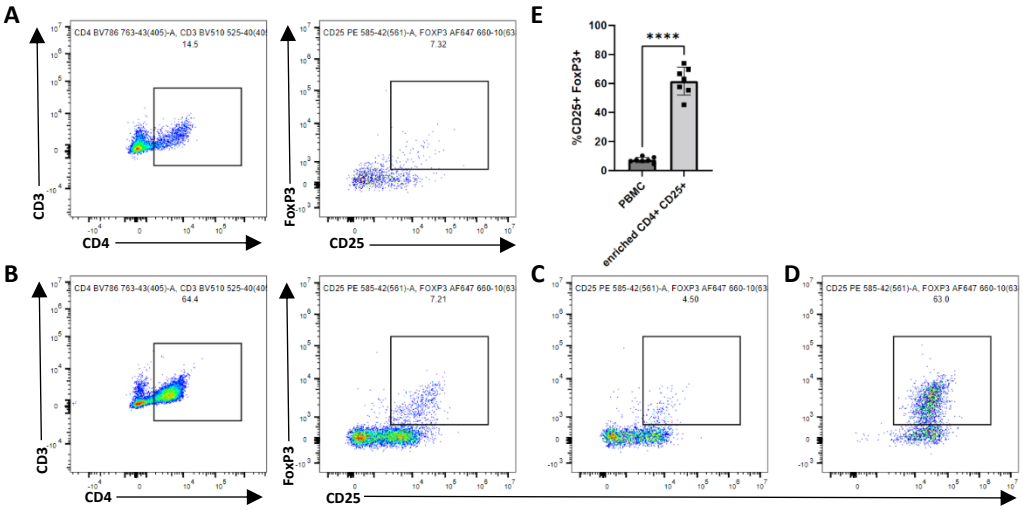

Figure 3 ***The purity of regulatory T cell isolation via MACS of PBMCs.*** Tregs were isolated with a two-step MACS isolation using a CD4+ CD25+ Regulatory T cell isolation kit (Miltenyi Biotec) and subsequently the different fractions were stained for flow cytometry analysis as described previously. **A)** preMACS fraction gated on CD3+ CD4+ T cells on the left and CD25+ FoxP3+ Tregs on the right. **B)** CD4+ fraction of the MACS isolation gated on CD3+ CD4+ T cells on the left and CD25+ FoxP3+ Tregs on the right. **C)** CD25+ FoxP3+ Treg cells present in the CD25- fraction of the MACS isolation. **D)** Percentage of CD25+ FoxP3+ Treg cells in the CD25+ fraction of the MACS isolation. **E)** Average percentage of CD25+ FoxP3+ cells in PBMCs versus in enriched CD4+ CD25+ cells out different (n=7) MACS isolations.

The first step in finding a method for high expansion of Tregs is determining how to effectively activate them so that they start expanding at high rates. To this end, we activated isolated Tregs through stimulation with CD3 and CD28 via the use of TransAct. However, a high concentration of stimulation is not directly equal to the highest percentage of expansion. Overstimulation of Tregs can lead to loss of their regulatory phenotype. Thus, the concentration of stimulation cannot be too high nor too low. Therefore, different concentrations of TransAct stimulation were tested. First, an idea of the range of TransAct dilutions was obtained by culturing PBMCs under different stimulation dilutions, to this end dilutions of 1:100, 1:200, 1:500 and 1:1000 were tested, next to no stimulation (Figure 4B).

From these experiments it became clear that for the culturing of PBMCs a 1:1000 dilution was too little stimulation, as there was almost no expansion. However, both the 1:100 and 1:500 dilutions showed promising expansions of 79.0% and 72.0%, respectively (Figure 4C). Subsequently, these dilutions were tested in a Treg culture. Here, the same trend was observed (Figure 4C). There was little to no expansion in the cells stimulated with a 1:1000 dilution, while almost all the cells expanded with 1:100 stimulation. Moreover, both the 1:200 and 1:500 stimulations showed similar 4-fold expansion rates (Figure 4D) and had similar viabilities of around 60% (Figure 4E).

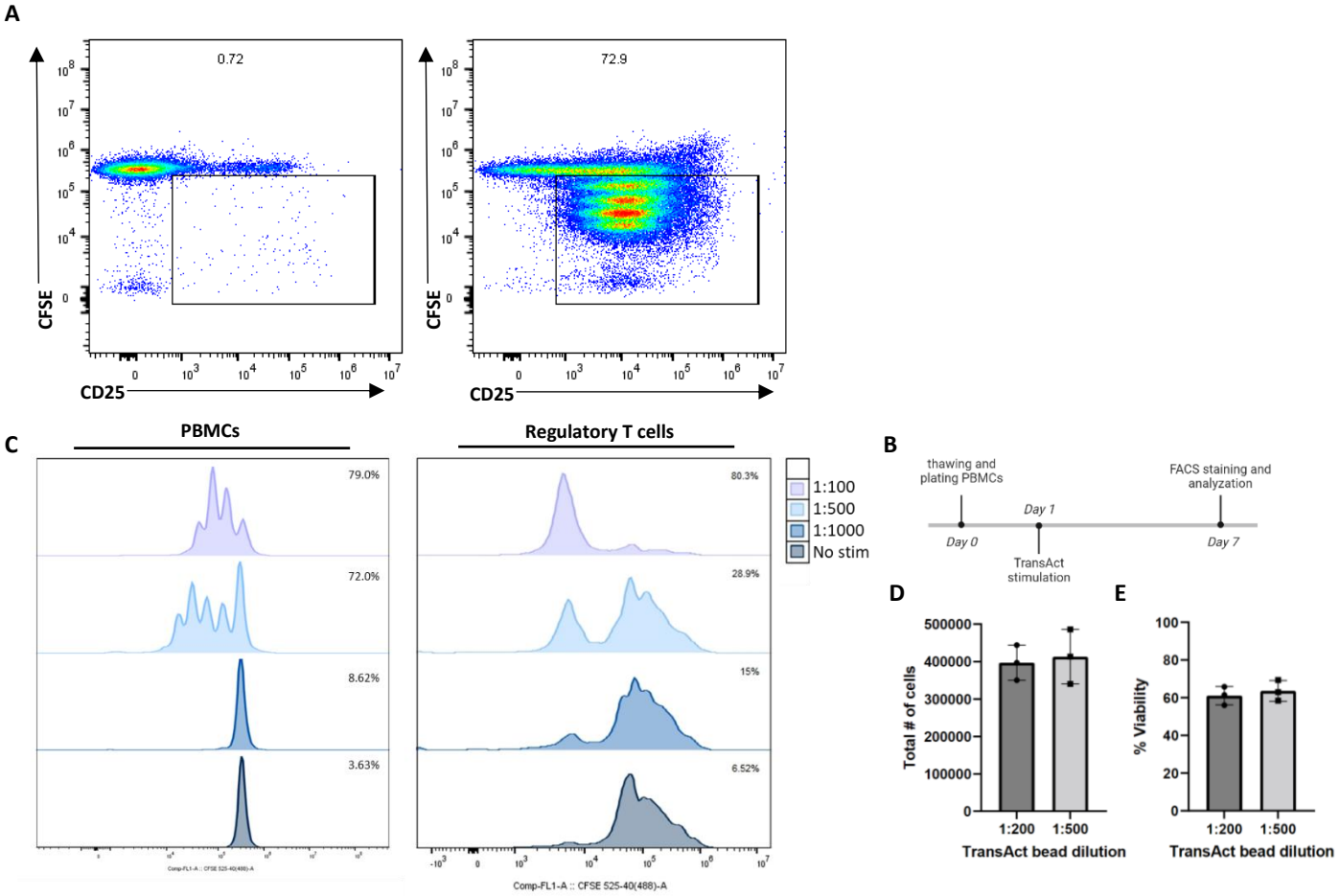

Figure 1 ***Stimulation of (regulatory) T cells with different dilutions of TransAct bead stimulation.*** **A)** CFSE-staining of a $CD3^+$ $CD4^+$ cell population, cells are gated on CFSE x FSC-A. Cells were cultured for 7 days without stimulation on the left and with 1:200 TransAct stimulation on the right. Stimulation results in expansion of T cells as can be seen by the dilution of CFSE-signal. **B)** PBMCs or MACS-isolated Tregs were labeled with CFSE on day 0 and stimulated with different dilutions (1:100, 1:200, 1:500 or 1:1000) of TransAct on day 1. Cells were cultured for 7 days after which they were stained for flow cytometry, counted and analyzed. **C)** Expansion of PBMCs (left panel) versus regulatory T cells (right panel) under different TransAct stimulations as shown by CFSE-dilution (Dark blue = no stimulation, blue = 1:1000 dilution, light blue = 1:500 dilution, purple = 1:100 stimulation). **D)** Total number of cells after 7 day culture of regulatory T cells with 1:200 versus 1:500 TransAct stimulation (n=3), 100.000 cells per well were plated at start of culture. **E)** Percentage of viability of cells after 7 day culture of regulatory T cells with 1:200 versus 1:500 TransAct stimulation (n=3).

Furthermore, when the phenotype within the CD4+ CD25+ enriched Treg cell culture was compared to the culture of non-Tregs, we observed that the non-Tregs cells also produced high percentages of $CD25^+FoxP3^+$ cells under the different expansion conditions, against our expectations. Thus, due to this transient upregulation of CD25 and FoxP3, it became of importance to find a method to limit this transient upregulation while maintaining a high expansion and $CD25^+FoxP3^+$ percentage in the CD4+CD25+ enriched Tregs. Therefore, in the following experiments the 1:100, 1:200 and 1:500 conditions were taken forward for further testing while also paying more attention to phenotypic changes.

Regulatory T cells prefer a high amount of rhIL-2 during culture

One cytokine important for the activation process of Tregs is IL-2, which binds to CD25. CD25 is the high affinity interleukin 2 (IL-2) receptor. This receptor is expressed at high levels on Tregs and binding of IL-2 induces cell

proliferation[1]. IL-2 is necessary for the sustained expression of both CD25 and Foxp3. Moreover, IL-2 seems to influence the immune system via a negative feedback loop. First, activated T cells produce IL-2 which can subsequently bind to its receptor on Tregs, leading to their expansion and activation. Subsequently, when Tregs get activated and maintained by IL-2, they can exert their function and limit the expansion of effector T cells[17].

In current Treg culture protocols, the used concentration of rhIL-2 varies, therefore we aimed to test which concentration is most beneficial in obtaining a high expansion rate. To this end we tested a range of rhIL-2 concentrations, 100, 250 and 500 U/mL, in parallel to the TransAct dilutions. To this extent, Tregs were cultured as mentioned previously (Figure 5A). During these experiments it became evident that, in contrast to general T cell cultures, CFSE staining did not prove useful for tracking the expansion. With addition of rhIL-2, almost all cells had expanded at the end of the culture. Thus, the entire CFSE staining had shifted to the left, making it difficult to observe the percentage or rounds of expansion through this method. Therefore, it proved more useful to look at the number of cells in following experiments.

Analysis of flow cytometric data on day 8 showed that a higher concentration of rhIL-2 leads to a better expansion of $CD25^{+}FoxP3^{+}$ cells in the CD4+ CD25+ enriched Treg fraction. The concentration of rhIL-2 had little to no influence on the expansion of this phenotype in the non-Treg fraction (Figure 5C). In the Treg culture, the percentage of Tregs expanded to 33.1% when cultured with 500 U/mL while this was a only 17.9% when cultured with 100 U/mL with a 1:200 bead stimulation (Figure 5B).

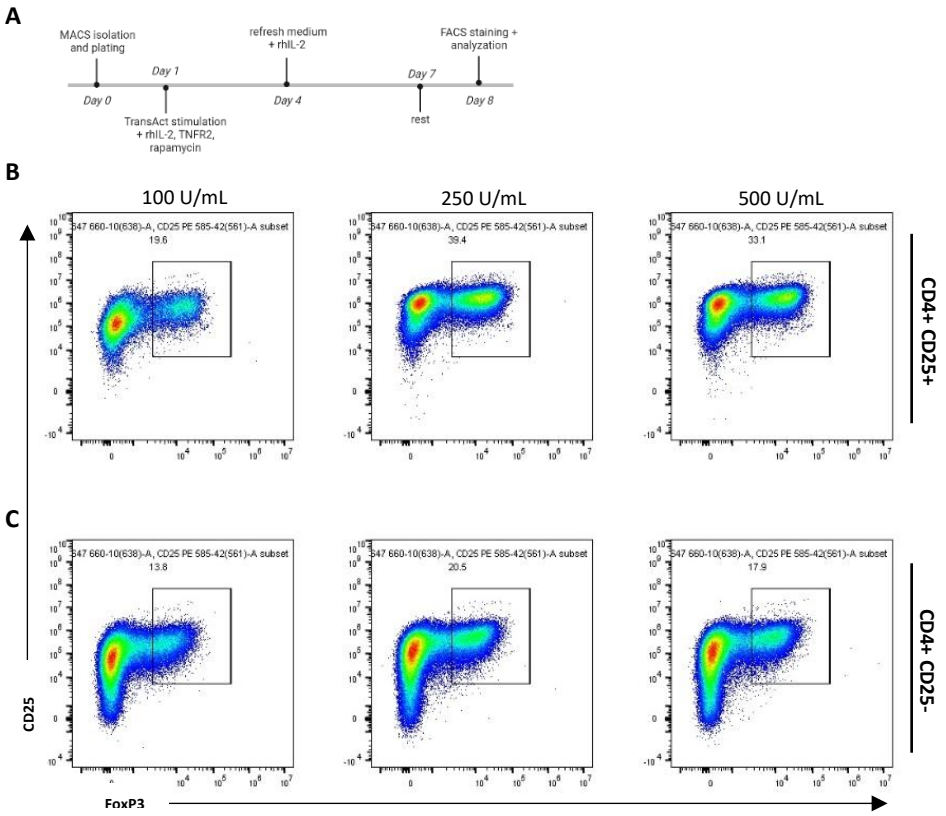

Figure 2 ***Addition of rhIL-2 to the culture of Regulatory T cells increases the percentage of CD25+ FoxP3+ cells.*** **A)** MACS-isolated Tregs were plated on day 0 in TexMACS medium and stimulated with 1:200 dilution of TransAct with the addition of various rhIL-2 concentrations on day 1. On day 4 medium was refreshed and new rhIL-2 was added corresponding to the previous concentrations. On day 7 cells were rested. Subsequently on day 8 cells were stained for flow cytometry and analyzed. **B)** Percentage of co-expression of CD25 and FoxP3 in CD4+ CD25+ enriched (Tregs) cells cultured under increasing rhIL-2 concentrations from left to right 100, 250 and 500 U/mL, respectively. **C)** Percentage of co-expression of CD25 and FoxP3 in CD4+ CD25 – (non-Treg) cells cultured under increasing rhIL-2 concentrations from left to right 100, 250 and 500 U/mL, respectively.

Moreover, the amount of rhIL-2 had more effect on enhancing the expansion of Tregs in the CD4+ CD25+ enriched Treg culture, while the percentage of cells co-expressing CD25 and FoxP3 in the non-Treg culture did not increase (Figure 5B and C). This difference in percentages was further increased when lower stimulations of TransAct were used. When a 500 U/mL IL-2 concentration was used together with a 1:500 bead dilution, the percentage of Tregs was 44.7% in the Treg culture and 15.6% in the non-Treg culture (Figure 6B). When the same rhIL-2 concentrations were tested under stimulation with a 1:100 bead dilution, the percentage of double positive cells was 41.0% and 23.5% respectively (Figure 6A). These results provided us with further information for the appropriate TransAct dilution. Thus, either dilutions of 1:200 or 1:500 stimulation seems to work best in our hands, even more so in combination with high amounts of rhIL-2. Thus, in following experiments the conditions of 250 and 500 U/mL rhIL-2 were taken along, in combination with 1:200 and 1:500 TransAct stimulation, while also giving attention to in which conditions the greatest difference in expansion between the non-Treg and Treg cultures could be observed.

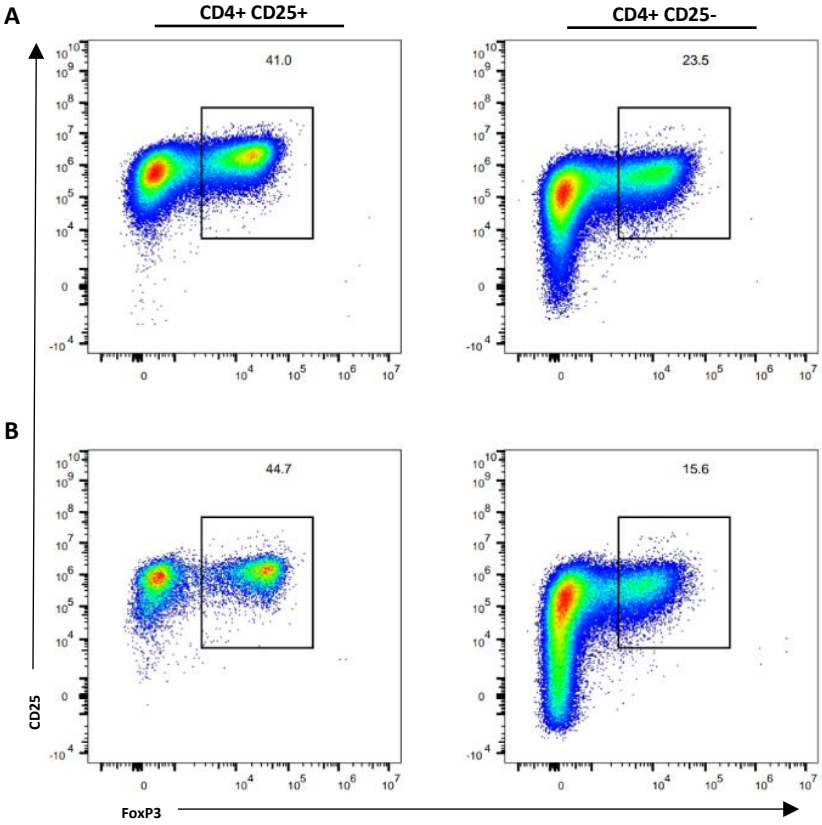

Figure 6 ***The percentage of CD25+ FoxP3+ cells goes down in the CD4+CD25- cell fraction in comparison to the CD4+CD25+ enriched cells when low TransAct stimulation is combined with high amounts of rhIL-2.*** **A)** Percentage of CD25+ FoxP3+ cells within CD4+CD25+ enriched (Treg) cells (left) and CD4+CD25- (non-Treg) cells (right) stimulated with 1:100 TransAct dilution in combination with addition of 1:500 U/mL rhIL-2. **B)** Percentage of CD25+ FoxP3+ cells within CD4+CD25+ enriched (Treg) cells (left) and CD4+CD25- (non-Treg) cells (right) stimulated with 1:500 TransAct dilution in combination with addition of 1:500 U/mL rhIL-2

Addition of TNFR2-agonistic antibody improves expansion of true Regulatory T cells

Another variable that caught our attention for addition to the culture of Tregs was a TNFR2-agonistic antibody. X. He *et al*., (2016)[33] showed that when an agonistic antibody against TNFR2 was added during the culture of Tregs, the percentage of $CD25^{+}FoxP3^{+}$ but also the percentage of $CD25^{+}Helios^{+}$ cells increased, overall pointing towards a more stable Treg phenotype. With this in mind, we decided to add TNFR2-agonistic antibody to the cell cultures, to see if this would influence the expansion of $CD25^{+}FoxP3^{+}$ cells in comparison to no added TNFR2-agonistic antibody. Another potent marker for Tregs is Helios[33]. We added this marker to the FACS staining panel, to investigate if this would allow for a better discrimination between true Tregs and transient upregulation of FoxP3 within non-Tregs.

With addition of TNFR2-agonistic antibody, we first observed that these cultures formed more distinct cell clusters compared to wells without the agonist under the microscope. Wells without the agonist formed more dispersed and lighter clusters (data not shown). This suggests that the TNFR2-agonist possibly leads to higher expansion. However, TNFR2-agonist did not lead to a significant increase in cell expansion after 8 days of culture (Figure 7B). Analysis by flow cytometry suggested that TNFR2-agonistic antibody may lead to expansion of Tregs within the Treg population. This same effect was not observed in the non-Treg population. This difference in percentage of double positive cells increased when TNFR2-agonist was added. This lead to more $CD25^{+}FoxP3^{+}$ cells in the Treg cell culture (Figure 7C). Moreover, when the results were gated on cells double positive for FoxP3 and Helios, almost no cells co-expressing these markers were present in the non-Treg cell culture as compared to the CD4+ CD25+ enriched Tregs. Moreover, addition of TNFR2-agonist increased the percentage of $FoxP3^{+}Helios^{+}$ cells in the Treg culture. Thus, it seems that addition of TNFR2-agonist with subsequent gating on FoxP3, Helios double-positive cells enables us to better differentiate and expand true Regulatory T cells. Therefore, we decided to add TNFR2-agonistic antibody to our culture protocol for future experiments.

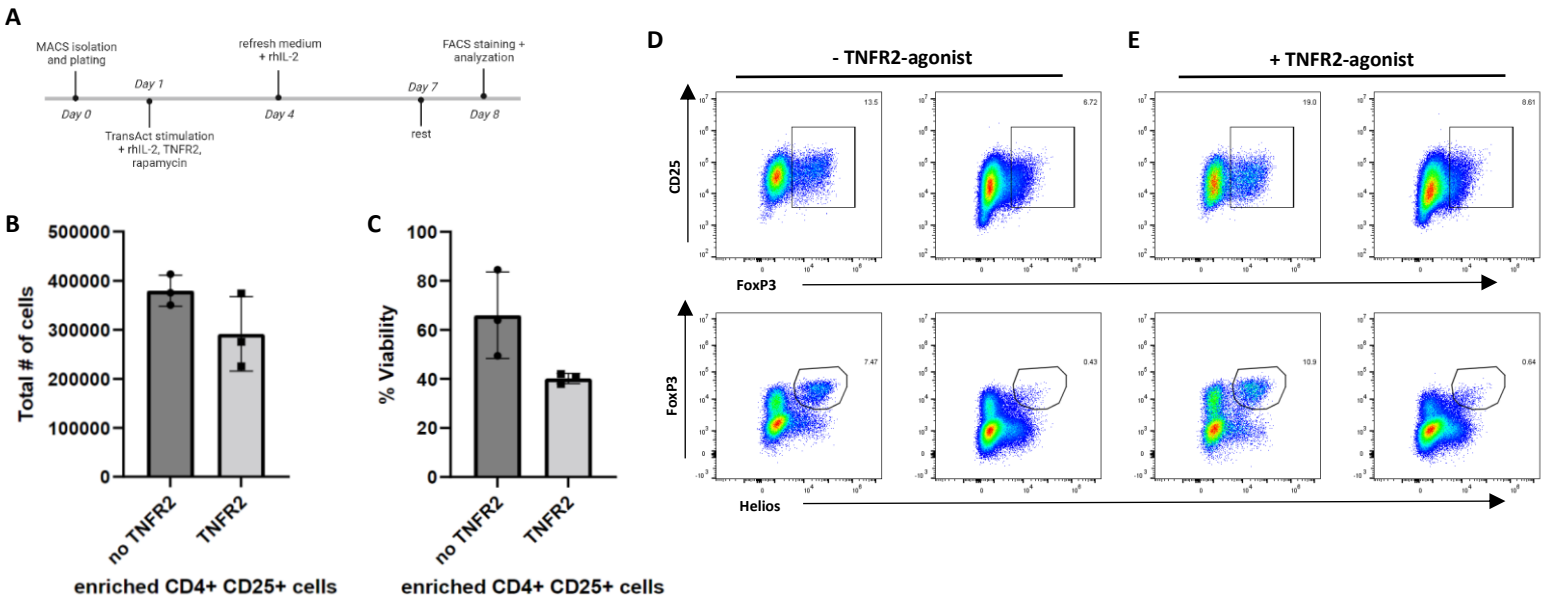

Figure 7 ***TNFR2-agonistic antibody possibly lowers overall expansion but increases the percentage of CD25+ FoxP3+ co-expressing cells.*** **A)** MACS isolated Tregs were plated and cultured on day 0 in TexMACS medium and stimulated with 1:500 TransAct on day 1 with addition of 500 U/mL rhIL-2 and 0 or 2.5 µg TNFR2-agonistic antibody. On day 4 the medium was replaced and new rhIL-2 was added. On day 7 the culture was rested after which on day 8 cells were stained for flow cytometry, counted and analyzed. **B)** The number of cells of CD4+ CD25+ enriched cells cultured with no agonist versus with agonist (n=3), 100.000 cells per well were plated at the start of the culture. **C)** Percentage of viable cells within CD4+ CD25+ enriched cells cultured without agonist versus with agonist (n=3). **D)** Percentage of CD25+ FoxP3+ and FoxP3 + Helios + cells within CD4+ CD25+ enriched cells cultured without TNFR2-agonistic antibody on the left versus within CD4+ CD25- cells cultured without agonist on the right. **E)** Percentage of CD25+ FoxP3+ and FoxP3 + Helios + cells within CD4+ CD25+ enriched cells cultured with TNFR2-agonistic antibody on the left versus within CD4+ CD25- cells cultured with agonist on the right.

Rapamycin dampens the expansion of Regulatory T cells

Because of the persistence of the high percentage of FoxP3 positivity in the non-Treg culture it was decided that addition of the metabolic inhibitor Rapamycin could also prove beneficial. Rapamycin is said to keep Tregs more stable in regards to the expression of FoxP3 and its methylation status. Furthermore, in literature it was found that the effect on inhibition of the metabolism, and thus inhibition of expansion, is greater in non-regulatory T cells, rather than in Tregs[34]. Therefore, we hypothesized that Rapamycin could possibly solve part of the issue with the observed transient upregulation of FoxP3 expression in the non-Treg culture.

Thus, in the following experiment MACS-isolated Tregs were cultured as previously described with the addition of 1 µM Rapamycin or no added Rapamycin (Figure 8A). We observed that the expansion of Tregs was less when Rapamycin was added compared to when no rapamycin was present. We observed this effect in both the CD4+ CD25+ enriched Treg culture and non-Treg cell culture (Figure 8B). Moreover, when the cultured cells were analyzed with flow cytometry, we observed little effect of Rapamycin on the phenotype of Tregs. The percentage of $CD25^{+}FoxP3^{+}$ expressing cells even seemed to be slightly higher in the cells cultured without Rapamycin. While the percentage of cells co-expression FoxP3 and Helios was similar in both conditions (Figure 8C and D). Thus, Rapamycin seems to not make a difference between expansion of Treg- or non-Treg cultures nor did it seem to have a positive effect on the stability of the Treg phenotype in our hands. One benefit however, of Rapamycin, was that it caused a slightly higher viability compared to when no rapamycin was added (Figure 8B). Although this is also important, our main goal is the obtainment of a large expansion rate and as this was inhibited by the addition of Rapamycin, it was decided that the benefit of a higher viability did not weigh up to the disadvantages. Thus, Rapamycin was therefore not added to our culture protocol in future experiments.

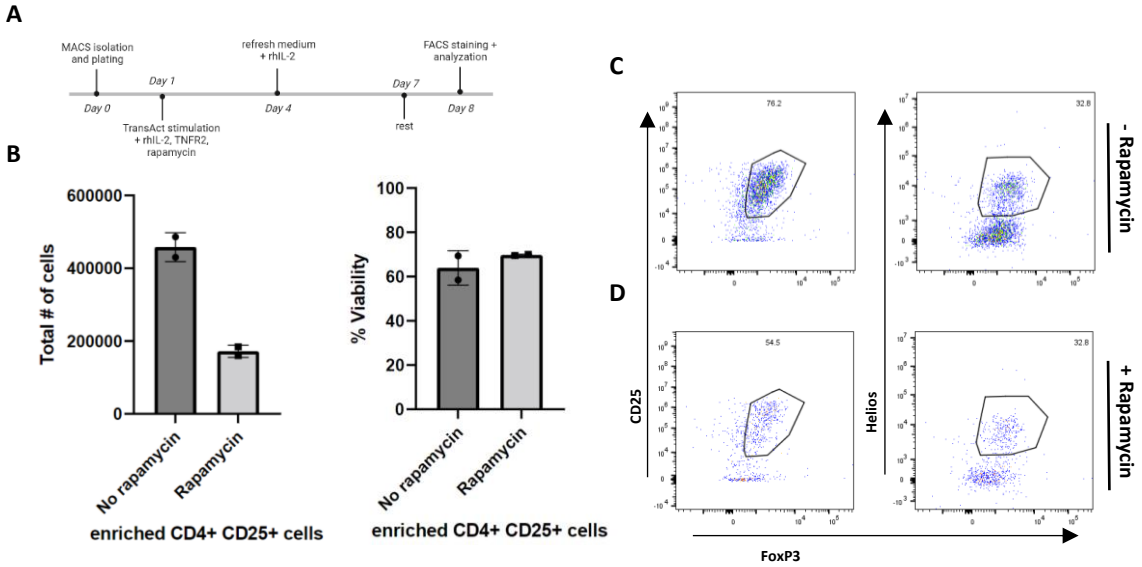

Figure 8 ***Rapamycin has a negative effect on the expansion of Regulatory T cells and little influence on the phenotype.*** **A)** MACS isolated Tregs were plated and cultured on day 0 in TexMACS medium and stimulated with 1:500 TransAct on day 1 with addition of 500 U/mL rhIL-2 and 0 or 1 µM Rapamycin. On day 4 the medium was replaced and new rhIL-2 was added. On day 7 the culture was rested after which on day 8 cells were stained for flow cytometry, counted and analyzed. **B)** Total number of cells of CD4+ CD25+ enriched cells culture with (left) or without (right) Rapamycin. The starting number of cells was 100.000 per well (n=2). **C)** Percentage of viable cells within CD4+ CD25+ enriched cells cultured without Rapamycin or with Rapamycin (n=2). **D)** Percentage of CD25+ FoxP3+ cells versus FoxP3+ Helios+ cells within CD4+ CD25+ enriched cells without Rapamycin (upper two squares) or without rapamycin (bottom two squares).

Restimulation of expanded Regulatory T cells in an antigen-specific manner

Having obtained a protocol by which we could expand Tregs, we sought to test if restimulation of polyclonally expanded Tregs in an antigen-specific manner proved possible. To this end, MACS-isolated Tregs were cultured as before with 1:500 TransAct stimulation, 500 U/mL rhIL-2 and 0 or 2.5 µg TNFR2-agonist (Figure 9A). On day 8 cells were counted with Trypan Blue and duplicate wells were pooled to obtain a higher number of cells for restimulation. Subsequently, Tregs were restimulated with autologous PBMC irradiated feeder cells together with either mB29a-, Tetanus Toxoid (TT)- or no peptide. When observing the wells under the microscope, we saw clear differences regarding medium discoloration and cell expansion during culture. Especially in the wells where TNFR2-agonist was added during the restimulation, the medium had discolored more towards yellow (data not shown). Furthermore, under the microscope more distinct and larger groups of cells were observed in these same conditions (data not shown). During analyzation it appeared that addition of TNFR2-agonist during the restimulation, regardless of its presence during the first culture, increased the expansion of CD4+ CD25+ enriched Tregs restimulated with mB29a peptide (Figure 9B). This same trend was not observed in the CD4+ CD25+ enriched Tregs restimulated with TT-peptide. However, within the CD4+ CD25+ enriched Tregs

restimulated with no peptide, expansion also could be observed, making it more difficult to determine the results.

Analysis of the flow cytometric results of the cell cultures showed that both the percentage of FoxP3$^+$ and of FoxP3$^+$Helios$^+$ cells seemed higher when cells where cultured with TNFR2-agonist. However, this effect did not require TNFR2-agonist to be present during antigen restimulation, since we observed the same effect when TNFR2-agonist was only added during polyclonal expansion. We observed an upward trend, where addition of both TNFR2-agonist in the polyclonal expansion and during the restimulation lead to highest percentages of double-positive cells and FoxP3$^+$ cells (Figure 10). However, addition of TNFR2-agonist seemed to have a similar influence on expansion in both the mB29a-, TT-peptide restimulation as well as in the culture with no added peptide. This was in contrast to the observed effects of the TNFR2-agonist on cell expansion. Responses also differed between the two donors, where donor #1 had an overall higher percentage of FoxP3$^+$ Helios$^+$ cells within the CD4+ CD25+ enriched Tregs, except for the percentage of FoxP3 positive cells, which was overall higher in donor #2 (Figure 10). Lastly, when in the Treg culture only Helios$^+$ cells were taken into account, we observed the same trend as within the number of cells (Figure 9B). Here, addition of the TNFR2-agonist seemed to increase the percentage of Helios$^+$ cells more when added during the restimulation rather than during the polyclonal expansion. However, in this instance this trend was similar over the different stimulation conditions and not mutually exclusive for the mB29a-peptide restimulation (Figure 10).

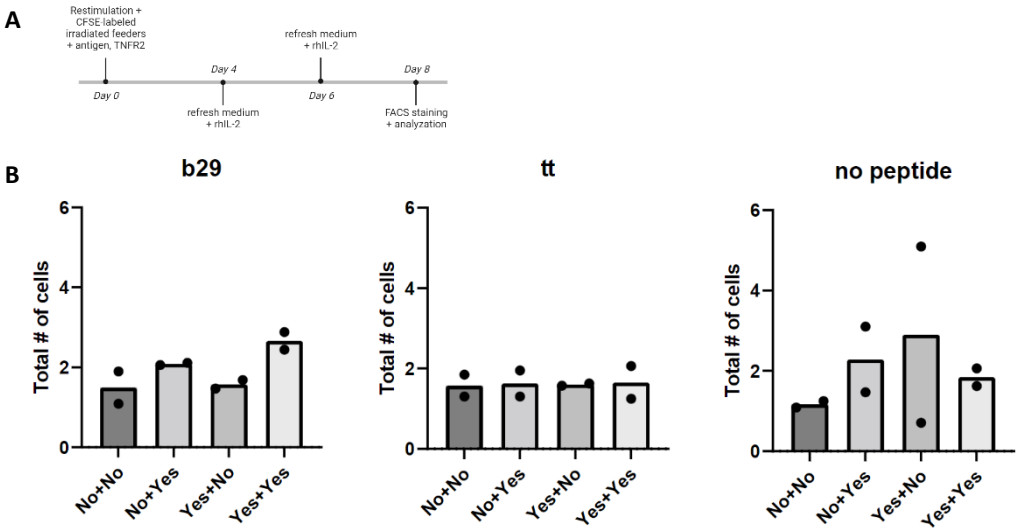

Figure 9 ***The effect of TNFR2-agonist addition on antigen-specific restimulation of Regulatory T cells.*** **A)** CD4+ CD25+ enriched cells were cultured as in previous experiments for 8 days after which they were restimulated on day 0 with CFSE-labeled irradiated CD4 feeder cells, either mB29a-, TT- or no peptide in the presence or absence of 2.5 µg TNFR2-agonist in TexMACS medium supplemented with 500 U/mL IL-2. On day 4 and day 6 the medium was refreshed with new rhIL-2. On day 8 cells were stained for flow cytometry analysis. **B)** Total number of cells after 8 day culture with mB29a-, TT- or no peptide. There was discriminated between TNFR2-agonist presence in the first culture or during the restimulation culture as is indicated by the – and + in the table.

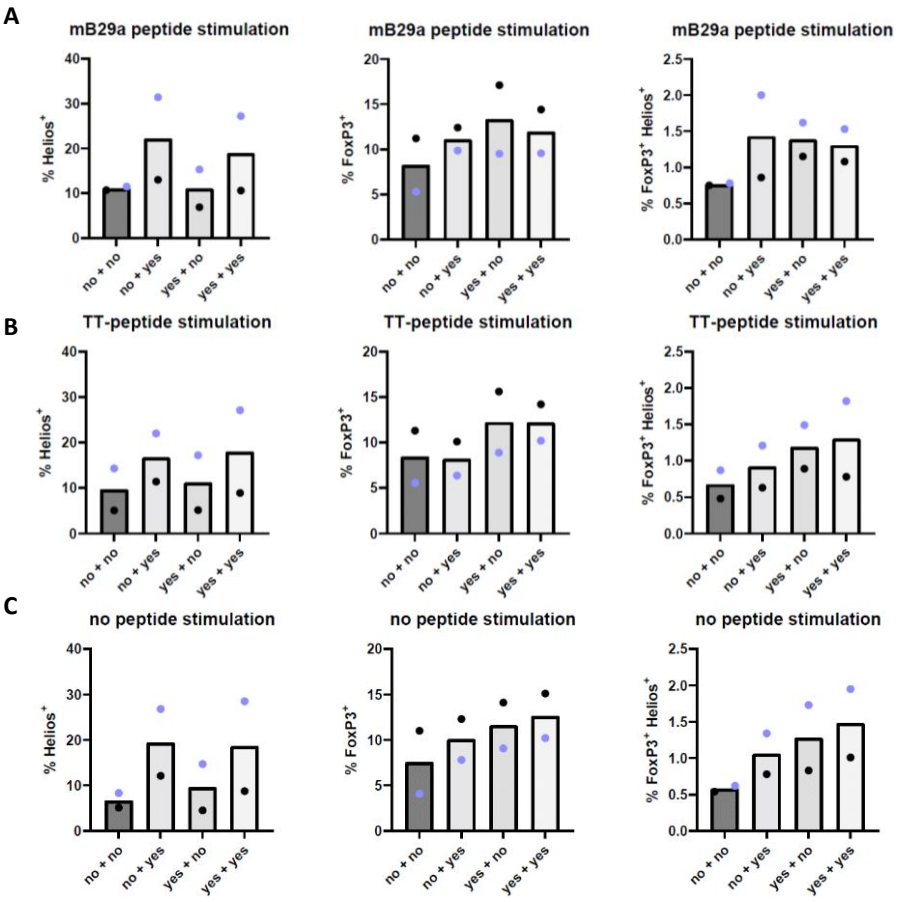

Figure 10 ***Phenotypic stability of cultured CD4+ CD25+ enriched cells after antigen-specific restimulation with or without TNFR2-agonistic antibody.*** **A)** Percentage of Helios- (left), FoxP3- (middle) and double positive (right) cells within the CD4+ CD25+ enriched culture restimulated with mB29a peptide as previously described. **B)** Percentage of Helios- (left), FoxP3- (middle) and double positive (right) cells within the CD4+ CD25+ enriched culture restimulated with TT-peptide as previously described. **C)** Percentage of Helios- (left), FoxP3- (middle) and double positive (right) cells within the CD4+ CD25+ enriched culture restimulated with no peptide as previously described. *Experiment was carried out with n=2, blue dots indicate donor #1 while black dots indicate donor #2*

Discussion

Although many advances have recently been made regarding the potential of Tregs as an ATC for the treatment of autoimmune diseases, such as RA, current techniques still know some limitations. As it still remains difficult to expand highly specific and potent antigen-specific Tregs, in this research we tried to establish a novel culture method for the antigen-specific expansion of Tregs. Our goal was to be able to restimulate polyclonally expanded Tregs in an antigen-specific manner, to allow subsequent identification of TCRs. These TCRs may in the future be transduced into Tregs, thereby redirecting polyclonally expanded Tregs to a defined antigen-specificity while also obtaining high cell yields.

In this study we showed that when stimulating Tregs in a polyclonal manner using TransAct, high cell yields are best established with relatively low stimulation concentrations in combination with high concentrations of rhIL-2. The purity of our MACS-isolated Tregs was ~61,6% and thus contained around 40% of non-Tregs. Therefore, we wanted to make sure that with our culture methods and gating strategy we could make a good distinction between true Tregs and non-Tregs. To this end, we also took CD4+CD25- cells (non-Tregs) along in separate cultures. When these cells were analyzed, it became evident that under bead stimulation they upregulate CD25 but also transiently upregulate FoxP3. Therefore, gating on $CD4^{+}CD25^{high}FoxP3^{+}$ cells was not exclusive for Tregs only. To compensate for this transient upregulation, cells in cultures were rested for one day to possible lower the transient upregulation in non-Tregs. Moreover, when stimulating Tregs with TransAct™, we observed that although a high stimulation of a 1:100 TransAct dilution lead to ~80% expansion, a high percentage of $CD25^{+}$ $FoxP3^{+}$ cells was also observed in the non-Treg culture. In contrast, when lower concentrations of TransAct stimulation where used in combination with high amounts of rhIL-2, the difference in percentage of $CD25^{+}$ $FoxP3^{+}$ cells between cultured Tregs and non-Tregs became larger. This combination of low TransAct and high rhIL-2 lead to a decrease in the co-expression of CD25 and FoxP3 in the non-Treg culture, which was favorable for the culturing of true Tregs. Moreover, as still some transient upregulation of CD25 and FoxP3 was seen in the non-Treg culture, Helios was added as a marker to the panel to better distinguish between non-Tregs and true Tregs. Addition of Helios proved to enable us to better differentiate between these different cells types, as co expression of FoxP3 and Helios was more exclusive to true Tregs, as little to no co-expression was observed in the non-Treg cell cultures. These results show that, despite the conflicting literature surrounding the use of Helios as a marker for Treg stability and definition, in our hands it proved beneficial for these purposes and lead to a better distinguishment between true Tregs and non-Tregs.

However, the relatively low average purity of ~61,1% that was obtained during our experiments, still remained unfavorable. Ultimately contamination with non-Tregs, such as T effector cells can influence ACT in a negative manner. Therefore, in future experiments the purity of isolation can either be improved by using a higher concentration of beads in the different the steps of the MACS procedure. Secondly, it was observed that a lower purity often was accompanied by cell clumps in the column during MACS-isolation. As clumps are often the results of cells dying, an improvement of viability during the MACS-isolation could also aid in improving the purity. Lastly, isolation purity could be improved by using a different isolation technique altogether. Techniques that have proven to result in a higher purity of isolated Tregs are for example isolation through clinical grade systems or via the use of fluorescence activated cell sorting (FACS).

Moreover, in our hands, taking both the expansion rate and phenotypic stability into account, stimulation with a 1:500 bead dilution lead to the best results. Under these conditions, the Treg phenotype remained relatively stable with high expression of CD25 and FoxP3, while the expansion still increased ~4-fold. Furthermore, our findings showed Tregs expand best when supplemented with high amounts of rhIL-2 throughout the culture period. Addition of 500 U/mL rhIL-2 at the start of and during the culture lead to the highest observed expansion of $CD25^{+}FoxP3^{+}$ cells. Moreover, this same increase in percentage of $CD25^{+}FoxP3^{+}$ cells was not observed in the non-Treg culture. This also suggests, that adding high amounts of rhIL-2 to Treg cultures increases the phenotypic stability while it does not lead to an increase of the transient upregulation of CD25 and FoxP3 in non-Treg cells. These results were partially in line with published culture protocols, where often high amounts of rhIL-2 are described as most favorable for expansion of Tregs. However, often little is known in these protocols regarding

transient upregulation of Treg specific markers by non-Tregs and how different concentrations of bead stimulation or rhIL-2 influence this.

Transient upregulation of Treg specific markers remained an issue regarding identification of true Tregs after culture. Therefore, we explored the addition of Rapamycin to our cultures. This mTOR-inhibitor is often used in other Treg culture protocols where it shows to improve phenotypic stability in Tregs while decreasing expansion of non-Treg contaminants. However, contradictory to other studies, in our research addition of Rapamycin had little effect on the percentage of $CD25^{+}FoxP3^{+}$ cells in the CD4+CD25+ enriched Tregs. Percentages of cells co-expressing CD25 and FoxP3 were even slightly higher in cell cultures without Rapamycin than when the mTOR-inhibitor was added. Moreover, addition of Rapamycin lead to a large decrease in cell expansion. Tregs cultured with Rapamycin showed ~2-fold expansion, while cells cultured without Rapamycin showed an ~4-fold expansion. As one of our main goals was to obtain the highest possible expansion, these results together with the little effect we observed of Rapamycin on the Treg phenotype, lead us to conclude to not add Rapamycin to our culture protocol in further experiments.

One last growth factor that sparked our interest with regards to obtaining high cell yields with a stable phenotype, was a TNFR2-agonistic antibody. Addition of this antibody to our cultures, did not affect the expansion rate of the Tregs. However, the TNFR2-agonistic antibody did seem to affect the stability of the Treg phenotype, in line with recent literature. Tregs cultured with TNFR2-agonist showed higher percentages of CD25 and FoxP3 expression and also increased the percentage of $Helios^{+}FoxP3^{+}$ cells. Moreover, especially the difference of these percentages between $CD4^{+}CD25^{+}$ enriched Tregs versus $CD4^{+}CD25^{-}$ non-Tregs cultures was promising. In contrast to the Treg culture, we observed no increase in $Helios^{+}FoxP3^{+}$ cells in the non-Treg culture when TNFR2-agonistic antibody was added. A TNFR2-agonistic antibody therefore seems a promising stabilizer of the Treg phenotype and was therefore included in our culture protocol.

Lastly, we tested if Tregs that were stably expanded from HLA-DR4 typed donors using rhIL-2 and/or TNFR2-agonist, could be restimulated in an antigen-specific manner. In these experiments, preliminary results suggest antigen-driven expansion of mB29a-antigen specific Tregs, but not of TT-peptide specific Tregs. This preliminary data suggest that it might be possible to restimulate polyclonally expanded Tregs in an antigen-specific manner following our culture protocol. Especially, addition of TNFR2-agonistic antibody increased the expansion of mB29a-antigen specific Tregs. However, in our control condition where no peptide was added to the culture, we also observed increased expansion of Tregs when TNFR2-agonistic antibody was added. Further experiments are needed to clarify these results.

Prospects

Once the protocol is more established and if Tregs cultured via this method can indeed be restimulated in an antigen-specific fashion, the next step would be to test if TCRs can be isolated from this pool of antigen-specific Tregs. If possible, we would be one step closer to engineering polyclonally expanded Tregs with a relevant TCR. However, to this end it would also be beneficial to further look into the stability of the cultured Tregs to have a better understanding of their true suppressive capacity. To this end, it would be interesting to observe the cytokine production of the cultured cells. This can be done on protein level, via detection of cytokines in the medium, or on the transcription level with the use of qPCR. Cytokines that would be of interest to test for are TGF-beta, IL-10 and/or IL-35 which correspond to the anti-inflammatory Treg phenotype. On the other hand, IFN-gamma, IL17, IL-21 and/or IL-22 would inform us that a Th17-like phenotypic switch might have occurred or that there are still T effector cells present in the culture. Moreover, the stability of the phenotype could be further tested by looking at the methylation status of the TSDR of FoxP3.

Lastly, if antigen-specific expansion of Tregs under the conditions described in this research proves possible, it would be an important step to test their suppressive function *in vitro* to observe if they still hold their regulatory potential. This could be tested by performing a suppressor assay, to establish if the expanded Tregs have the ability to suppress effector T cells *in vitro*. As sometimes in literature, it is said that expanded Tregs can lose their

suppressive function, this would be an important check before further steps can be taken towards using these cells for ACT.

Taking all this into account, the research in this paper forms a first step towards a possible new protocol for the antigen-specific expansion of polyclonally expanded Regulatory T cells, specific for mB29a-peptide which is associated with the pathology of RA. Once further research has been done, this study could provide a possible method for the isolation of Treg specific TCRs which could subsequently be engineered and transferred back to polyclonally expanded Tregs. Via these methods, ACT as a therapy for RA could be closer to being an effective new treatment option. Though, to realize this, many more steps need to be taken.

References

1. Jiang, Q., Yang, G., Liu, Q., Wang, S. & Cui, D. Function and Role of Regulatory T Cells in Rheumatoid Arthritis. *Front. Immunol.* **12**, 1–12 (2021).
2. Almutairi, K., Nossent, J., Preen, D., Keen, H. & Inderjeeth, C. The global prevalence of rheumatoid arthritis: a meta-analysis based on a systematic review. *Rheumatol. Int.* **41**, 863–877 (2021).
3. McInnes, I. B. & Schett, G. The Pathogenesis of Rheumatoid Arthritis. *N. Engl. J. Med.* **365**, 2205–2219 (2011).
4. Ferreira, L. M. R., Muller, Y. D., Bluestone, J. A. & Tang, Q. Next-generation regulatory T cell therapy. *Nat. Rev. Drug Discov. 2019 1810* **18**, 749–769 (2019).
5. Romano, M., Fanelli, G., Albany, C. J., Giganti, G. & Lombardi, G. Past, present, and future of regulatory T cell therapy in transplantation and autoimmunity. *Front. Immunol.* **10**, 43 (2019).
6. Raffin, C., Vo, L. T. & Bluestone, J. A. T reg cell-based therapies: challenges and perspectives. *Nat. Rev. Immunol.* **20**, 158–172 (2020).
7. Sparks, J. A. Rheumatoid Arthritis. *Ann. Intern. Med.* **170**, 1–15 (2019).
8. McMichael, A. J., Sasazuki, T., McDevitt, H. O. & Payne, R. O. Increased Frequency of HLA-Cw3 and HLA-Dw4 in Rheumatoid Arthritis. *Arthritis Rheum.* **20**, 1037–1042 (1977).
9. Roitt, I. M. *et al.* HLA-DRW4 and prognosis in rheumatoid arthritis. *Lancet (London, England)* **1**, 990 (1978).
10. Gran, J. T., Husby, G. & Thorsby, E. The association between rheumatoid arthritis and the HLA antigen DR4. *Ann. Rheum. Dis.* **42**, 292–296 (1983).
11. Feng, Y. *et al.* Control of the inheritance of regulatory T cell identity by a cis element in the Foxp3 locus. *Cell* **158**, 749–763 (2014).
12. Kitagawa, Y., Ohkura, N. & Sakaguchi, S. Molecular Determinants of Regulatory T Cell Development: The Essential Roles of Epigenetic Changes. *Front. Immunol.* **4**, 1–10 (2013).
13. Sekiya, T., Nakatsukasa, H., Lu, Q. & Yoshimura, A. Roles of transcription factors and epigenetic modifications in differentiation and maintenance of regulatory T cells. *Microbes Infect.* **18**, 378–386 (2016).
14. Hill, J. A. *et al.* Foxp3 Transcription-Factor-Dependent and -Independent Regulation of the Regulatory T Cell Transcriptional Signature. *Immunity* **27**, 786–800 (2007).
15. Sugimoto, N. *et al.* Foxp3-dependent and -independent molecules specific for CD25+CD4+ natural regulatory T cells revealed by DNA microarray analysis. *Int. Immunol.* **18**, 1197–1209 (2006).
16. Lam, A. J., Uday, P., Gillies, J. K. & Levings, M. K. Helios is a marker, not a driver, of human Treg stability. *Eur. J. Immunol.* **52**, 75–84 (2022).
17. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T Cells and Immune Tolerance. *Cell* **133**, 775–787 (2008).
18. Duggleby, R., Danby, R. D., Madrigal, J. A. & Saudemont, A. Clinical grade regulatory CD4+ T cells (Tregs): Moving toward cellular-based immunomodulatory therapies. *Front. Immunol.* **9**, 1–13 (2018).
19. Tang, Q. & Vincenti, F. Transplant trials with Tregs: perils and promises. *J. Clin. Invest.* **127**, 2505–2512 (2017).
20. Bluestone, J. A. *et al.* Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci. Transl. Med.* **7**, 1–14 (2015).
21. Komatsu, N. *et al.* Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat. Med.* **20**, 62–68 (2014).
22. Zhou, X. *et al.* Foxp3 instability leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* **10**, 1000–1008 (2009).
23. Hoffmann, P. *et al.* Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon

repetitive in vitro stimulation. *Eur. J. Immunol.* **39**, 1088–1097 (2009).

24. Scottà, C. *et al.* Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4+CD25+FOXP3+ T regulatory cell subpopulations. *Haematologica* **98**, 1291–1299 (2013).

25. Strauss, L. *et al.* Selective Survival of Naturally Occurring Human CD4+CD25+Foxp3+ Regulatory T Cells Cultured with Rapamycin. *J. Immunol.* **178**, 320–329 (2007).

26. Battaglia, M., Stabilini, A. & Roncarolo, M. G. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* **105**, 4743–4748 (2005).

27. Fraser, H. *et al.* A Rapamycin-Based GMP-Compatible Process for the Isolation and Expansion of Regulatory T Cells for Clinical Trials. *Mol. Ther. - Methods Clin. Dev.* **8**, 198–209 (2018).

28. Santinon, F. *et al.* Involvement of Tumor Necrosis Factor Receptor Type II in FoxP3 Stability and as a Marker of Treg Cells Specifically Expanded by Anti–Tumor Necrosis Factor Treatments in Rheumatoid Arthritis. *Arthritis Rheumatol.* **72**, 576–587 (2020).

29. Tseng, W. Y. *et al.* TNF receptor 2 signaling prevents DNA methylation at the Foxp3 promoter and prevents pathogenic conversion of regulatory T cells. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 21666–21672 (2019).

30. Mensink, M. *et al.* TNFR2 Costimulation Differentially Impacts Regulatory and Conventional CD4+ T-Cell Metabolism. *Front. Immunol.* **13**, 2628 (2022).

31. de Kivit, S. *et al.* Stable human regulatory T cells switch to glycolysis following TNF receptor 2 costimulation. *Nat. Metab.* **2**, 1046–1061 (2020).

32. De Wolf, C. *et al.* An Arthritis-Suppressive and Treg Cell-Inducing CD4+ T Cell Epitope Is Functional in the Context of HLA-Restricted T Cell Responses. *Arthritis Rheumatol. (Hoboken, N.J.)* **68**, 639–647 (2016).

33. He, X. *et al.* A TNFR2-Agonist facilitates high purity expansion of human low purity treg cells. *PLoS One* **11**, (2016).

34. Howden, A. J. M. *et al.* Quantitative analysis of T cell proteomes and environmental sensors during T cell differentiation. *Nat. Immunol.* **20**, 1542–1554 (2019).