

Investigation of heterogeneity and *in vitro* characterization of human thymus-derived regulatory T cells

Hugo Pinto Cardoso Moreira da Cruz

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Faculdade de Engenharia da Universidade do Porto

Instituto de Ciências Biomédicas Abel Salazar (ICBAS) - Universidade do Porto

Center for Regenerative Therapies Dresden, Technische Universität Dresden, Germany

Supervisor: Dr. Anke Fuchs

Co-supervisors: Dr. Yueyuan Hu

Dr. Elva Bonifácio Andrade

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Abstract

Regulatory T cells (Tregs) are one important T cell subset of the immune system, with the mission of controlling the activity of the other immune cells, preventing from immune-related complications, such as graft-versus-host disease, transplant rejection, and other autoimmune disorders. Recent studies and early clinical trials employing peripheral blood as a Treg source for cellular therapy confirmed the success of Treg *in vitro* manufacturing and safety upon adoptive transfer. However, clinical efficacy of Treg cell therapy still needs to be improved, mainly due to the insufficient yield and inhibitory function after *ex-vivo* expansion. Some other data supported the feasibility and reported some advantages of using cord blood as a source of Tregs, in terms of naivety, expansion potential and phenotypic stability. However, the limited number of Tregs that can be isolated from this source remains the main obstacle. Pediatric thymic tissue, routinely removed during heart surgery, has been recently identified as a novel and promising source of Tregs, overcoming the abovementioned limitations associated with blood-derived cells.

FOXP3 has been validated as the gold standard marker for blood-derived Tregs. However, the surface markers generally applied for blood-derived Treg isolation ($CD3^+CD4^+CD8^-CD25^{high}CD127^{low}$) resulted in a large proportion of FOXP3⁻ cells when applied for the sorting of thymus-derived Tregs (thyTregs). It was postulated in literature that CD25⁺FOXP3⁻ cells might serve as precursors for thyTregs. Since FOXP3 is a transcription factor and it is not possible to include it in the above mentioned cell isolation strategies, additional surface markers which are expressed in correlation with FOXP3 in thyTregs will enable a precise identification of the FOXP3⁻ and FOXP3⁺ cells and a further understanding of their phenotype and function.

In this study, we first screened some surface markers to evaluate their expression in correlation to FOXP3 in thymus-derived Tregs, and we identified GPA33⁺ and CD27⁻ as suitable markers to sort Tregs into FOXP3⁺ and FOXP3⁻ subpopulations, respectively. By using those two identified markers together with the abovementioned conventional Treg isolation panel, we sorted three subtypes of Tregs from thymocytes: GPA33⁺, CD27⁻ and bulk Tregs, and cultured those cells for 14 days. Thereafter their expansion capacity, phenotype and suppressive activity along the culture were analyzed.

Our results showed that bulk Tregs had the highest expansion potential, while CD27⁻ Tregs had the lower expansion capacity between three tested donors.

GPA33⁺ and bulk Tregs exhibited comparable phenotypic profiles including the composition of developed CD4⁺CD8⁺ cells 7 and 14 days after expansion, the expression patterns of CD25, CD31, FOXP3 and GPA33. However, for CD27⁻ Tregs, not only CD4⁺CD8⁺ but also a high percentage of CD8⁺ cells were seen after expansion. Data regarding the expression of the other analyzed markers was also significantly different for CD27⁻ Tregs in comparison to GPA33⁺ and bulk Tregs.

The suppressive capacity of those three subsets were similar, as they all significantly suppressed CD4⁺ T effector cells in a allogeneic setting.

Overall, data obtained in this thesis showed that CD27⁻ Tregs, which can represent FOXP3⁻ Tregs, are a different Treg subtype when compared to bulk and GPA33⁺ Tregs. Further functional and cytokine expression analysis of this subtype will help to better design a pure thyTregs isolation panel and understand the developmental program of Treg cells within thymus tissue.

Resumo

As células T reguladoras (Tregs) são uma importante subpopulação de células T do sistema imunitário, cuja missão é controlar a atividade das outras células imunitárias, prevenindo complicações tais como a doença do enxerto contra o hospedeiro, a rejeição de transplantes e outras doenças autoimunes. Estudos recentes e ensaios clínicos preliminares que utilizaram sangue periférico como fonte de Tregs para terapias celulares confirmaram o sucesso do isolamento e expansão *in vitro* de Tregs, assim como a segurança da sua transferência adotiva. No entanto, a eficácia clínica da terapia com Tregs ainda precisa de ser melhorada, principalmente devido ao rendimento e à função supressora insuficientes após a expansão *ex-vivo*. Outras publicações confirmam a viabilidade e referem algumas vantagens do uso de sangue do cordão umbilical como fonte de Tregs, em termos de ingenuidade, potencial de expansão e estabilidade fenotípica. No entanto, o número limitado de Tregs que podem ser isoladas desta fonte continua a ser o principal obstáculo. Recentemente, o tecido tímico pediátrico, removido rotineiramente durante cirurgias cardíacas, foi identificado como uma fonte nova e promissora de Tregs, superando as limitações supramencionadas associadas às células derivadas do sangue.

O fator de transcrição FOXP3 foi validado como o marcador de referência para Tregs derivadas do sangue. No entanto, os marcadores de superfície geralmente aplicados para a isolamento de Tregs derivadas do sangue ($CD3^+CD4^+CD8^-CD25^{high}CD127^{low}$) resultaram numa grande proporção de células FOXP3⁻ quando aplicados no isolamento de Tregs provenientes do timo. Foi proposto na literatura que as células $CD25^+FOXP3^-$ podem ser precursoras de Tregs derivadas do timo. Uma vez que FOXP3 é um fator de transcrição e não é possível incluí-lo nas estratégias de isolamento celular mencionadas anteriormente, marcadores de superfície adicionais que são expressos em correlação com FOXP3 nas Tregs obtidas a partir do timo permitirão uma identificação precisa das células FOXP3⁻ e FOXP3⁺ e uma maior compreensão do respetivo fenótipo e função.

Neste estudo, primeiramente analisámos alguns marcadores de superfície para avaliar a sua expressão em correlação com FOXP3 em Tregs derivadas do timo, e identificámos $GPA33^+$ e $CD27^-$ como marcadores adequados para separar Tregs em subpopulações FOXP3⁺ e FOXP3⁻, respetivamente. Utilizando estes dois marcadores identificados juntamente com o painel convencional de isolamento de Tregs supramencionado, separámos três subtipos de Tregs a partir de timócitos: $GPA33^+$, $CD27^-$ e Tregs totais, e

cultivámos essas células durante 14 dias. Posteriormente, analisámos a sua capacidade de expansão, fenótipo e atividade supressora ao longo do tempo de cultura.

Os nossos resultados mostraram que as Tregs totais tiveram o maior potencial de expansão, enquanto as Tregs CD27⁻ apresentaram a menor capacidade de expansão entre os três dadores testados. As Tregs GPA33⁺ e as Tregs totais exibiram perfis fenotípicos semelhantes, incluindo no que respeita ao desenvolvimento de células CD4⁺CD8⁺ 7 e 14 dias após a expansão e aos padrões de expressão de CD25, CD31, FOXP3 e GPA33. No entanto, para as Tregs CD27⁻, não só as células CD4⁺CD8⁺, mas também uma grande percentagem de células CD8⁺ foram observadas durante a expansão. Os resultados relativos à expressão dos outros marcadores analisados também foram significativamente diferentes para as Tregs CD27⁻ em comparação com as Tregs GPA33⁺ e com as Tregs totais.

A capacidade supressora destes três subtipos de Tregs foi semelhante, já que todas as células suprimiram significativamente as células T CD4⁺ efectoras num contexto alogénico.

No geral, os dados obtidos nesta tese mostraram que as Tregs CD27⁻, que podem representar Tregs FOXP3⁻, são um subtipo diferente das Tregs totais e das Tregs GPA33⁺. Análises adicionais à função e à expressão de citocinas nestas células ajudarão a melhorar o desenho de um painel de isolamento puro de Tregs derivadas do timo e a compreender melhor o programa de desenvolvimento destas células dentro do tecido tímico.

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List of abbreviations

7-AAD – 7-Aminoactinomycin D

APC – Antigen presenting cell

CAR – Chimeric antigen receptor

CCR7 – C-C chemokine receptor type 7

CD – Cluster of differentiation

CTLA-4 – Cytotoxic T-lymphocyte-associated protein 4

DMSO - Dimethylsulfoxid

FACS – Fluorescence activated cell sorting

FcR – Fc receptor

FOXP3 – Forkhead box P3

GARP – Glycoprotein A repetitions predominant

GPA33 – Glycoprotein A33

GvHD – Graft-versus-host disease

HLA – Human leukocyte antigen

HSA – Human serum albumin

HSCT – Hematopoietic stem cell transplantation

IL – Interleukin

LAP – Latency-associated peptide

MACS – Magnetic activated cell sorting

MHC – Major histocompatibility complex

NEAA – Non-essential amino acids

NK – Natural killer

PAMP – Pathogen-associated molecular pattern

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate-buffered saline

PEB – Protein extraction buffer

RT – Room temperature

T1D – Type 1 diabetes

TCR – T cell receptor

Teff – T effector cell

TGF- β – Transforming growth factor β

ThyTreg – Thymus-derived T regulatory cell

TNFR2 – Tumor necrosis factor receptor 2

Treg – T regulatory cell

TregP – Regulatory T cell progenitor

TSRD – Regulatory T cell-specific demethylated region

1 Introduction

1.1 Immune system

Our immune system is responsible for keeping the immune balance along the entire life, being one of the major defense mechanisms against pathogen infections and other external and internal triggers such as damaged cells. Several tissues, cells and molecules have the mission of fighting together against and eliminating them from the human body (Chaplin, 2010; Kondělková et al., 2010).

Physical and physiological barriers are the first line of defense and constitute mechanisms of innate immunity. Besides the skin, they also include the cornea, as well as respiratory, gastrointestinal and genitourinary tracts. After a pathogen or toxin overcomes any of these barriers, some cytokines and other bioactive molecules with antimicrobial properties, together with immune cells such as natural killer cells (NK), macrophages and monocytes, are able to tackle it by recognizing some typical antigens or pathogen-associated molecular patterns (PAMPs). This response is usually very fast in eliminating the target since it does not require the recruitment of highly specific cells (Müller et al., 2019).

However, if innate immunity fails to solve the problem, different immune cell subpopulations initiate an adaptive response. The main cell subtypes with this function are the antibody producers B lymphocytes and also T lymphocytes, contributing to humoral and cellular responses, respectively. T lymphocytes include $CD4^+$ helper and $CD8^+$ cytotoxic T cells, which are activated by the presentation of pathogen-specific antigens by antigen-presenting cells (APCs), through class II or class I major histocompatibility complex (MHC) proteins, respectively. Both B and T lymphocytes are regulated by regulatory T cells (Tregs). Even though the whole process of pathogen-specific cell recruitment takes longer – since it includes T cell activation, expansion and differentiation – it usually leads to a more effective and accurate response, leading to pathogen elimination and the formation of memory cells. These memory cells can react immediately in case of a recurrent infection by the same pathogen (Müller et al., 2019; ten Broeke et al., 2013).

Different immune cells act against different pathogens. NK cells, interferons and cytotoxic T cells elicit an immune response against viruses, while bacteria are mostly targeted by macrophages (J et al., 2019).

In Figure 1, different human immune system components are shown, being responsible for either an innate or adaptive immune response.

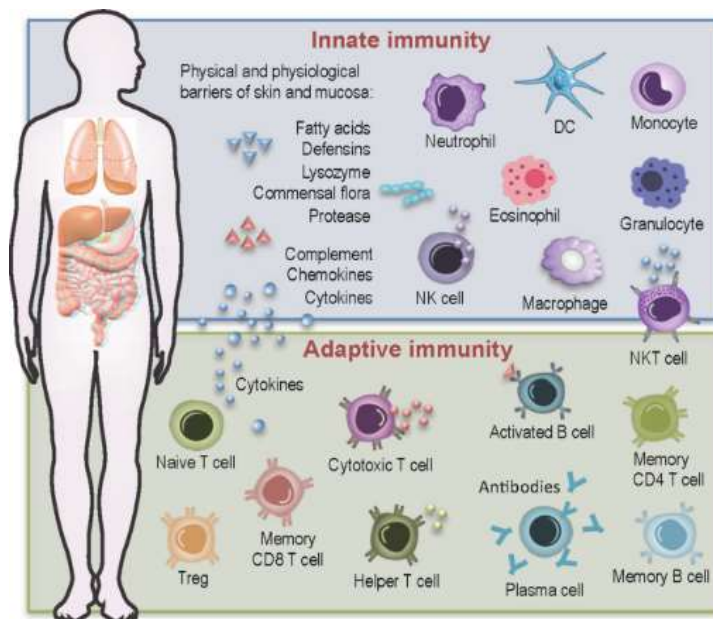


Figure 1. Human immune system components (Müller et al., 2019).

1.2 Immune-related disorders and current treatment options

Since the immune system is responsible for maintaining the immune homeostasis, it is expected that its imbalance can lead to some severe diseases. In fact, autoimmune diseases, which affect 4-8% of the population of developed countries, are characterized by recognition and attack of healthy cells of the patient by immune cells (Bernaldo-de-Quiros et al., 2022; Bluestone et al., 2023). This exacerbated immune response is associated with an inflammatory environment – Cytokine Release Syndrome – and it leads to the dysregulation of the entire immune system and the patient becomes way more vulnerable to any further pathogen infection or external trigger (Shimabukuro-Vornhagen et al., 2018). Other well characterized immune disorders include transplant rejection and Graft-versus-Host disease (GvHD) (Bernaldo-de-Quiros et al., 2022). Transplant rejection happens when immune cells of the recipient react against the external cells from the graft (Bernaldo-de-Quiros et al., 2022). This composes one of the biggest hurdles to organ transplantation, which can ultimately lead to death if the patient cannot find another compatible donor or a different alternative to a failing organ or tissue.

On the other hand, cells from the donor can also attack healthy recipient cells after transplant. In the course of allogeneic Hematopoietic Stem Cell Transplantation (HSCT),

immune cells from the donor are transferred to the recipient, depleting residual malignant cells in a patient with leukemia. However, in every second transplanted patient, cells from the transplanted stem cell graft recognize and react against healthy recipient tissue after HSCT, leading to GvHD (Theil, Tuve, et al., 2015). This is the leading cause of mortality and morbidity after HSCT after relapse (Bernaldo-de-Quiros et al., 2022).

In Figure 2, a schematic representation for both transplant rejection and GvHD is shown.

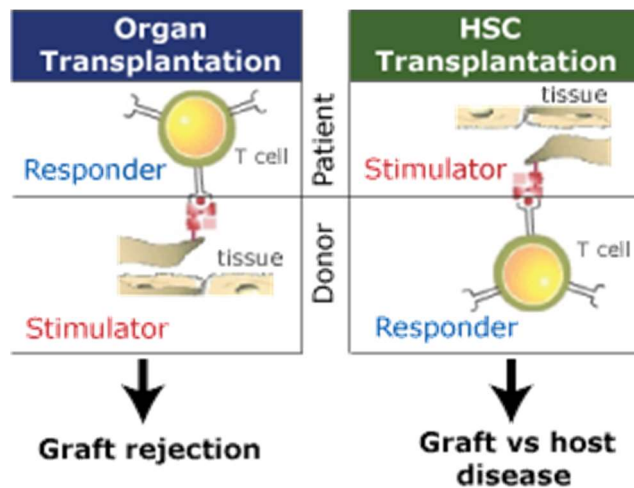


Figure 2. Schematic representation of Graft rejection and GvHD (Faculté de Médecine – Université de Genève).

There are some standard treatment options against those abovementioned immune-related disorders, which currently rely essentially on immunosuppressive drugs with steroids being the first line of treatment. However, many of them have shown some harmful effects. In fact, they systemically suppress the entire immune system, weakening the ability to face infections and tumor cells (Stucker & Ackermann, 2011).

For this reason, novel approaches that could regulate the overacting immune system are needed, enabling the control of immune responses and reducing the risk of tissue damage and infectious complications (Kondělková et al., 2010). In this context, natural cell based suppression of the immune system may be a better alternative than classic immunosuppressants, promoting immune tolerance without compromising the functional integrity of the immune system (Dijke et al., 2016).

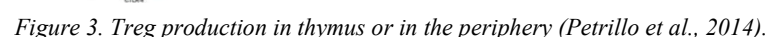
1.3 Regulatory T cells and their role in immune homeostasis

Regulatory T cells are a small subset of T lymphocytes responsible for controlling the activity of the other immune cells, preventing autoimmunity by promoting immune

tolerance. In fact, they can suppress exacerbated immune responses against self and non-self antigens, even under pro-inflammatory conditions (MacDonald, Piret, & Levings, 2019; Sjaastad et al., 2021). Another important feature of Tregs is their ability to promote tissue homeostasis and repair after inflammation, by releasing soluble factors (Arpaia et al., 2015; Sjaastad et al., 2021; Zhang et al., 2017). Their high suppressive capacity is also essential to induce long-term tolerance mechanisms, which are highly relevant during and after transplantation, decreasing the risk of transplant rejection or development of GvHD (Bernaldo-de-Quiros et al., 2022; Gorantla et al., 2010; Miyara et al., 2011; Sakaguchi, 2004).

Moreover, Treg deficiency or even a dysregulation of their mechanisms of action can promote several autoimmune disorders, such as type 1 diabetes (T1D), multiple sclerosis, systemic lupus erythematosus, psoriasis and rheumatoid arthritis (Vojdani & Erde, 2006).

Recently, the scientific community became aware of the presence of subpopulations of Tregs with distinct phenotypes, and with coordinated mechanisms of action (Bernaldo-de-Quiros et al., 2022; Sjaastad et al., 2021). These cells can be activated by T cell receptor (TCR)- and CD28-dependent stimulation, either in the thymus or in the periphery (Morgana et al., 2022; Sjaastad et al., 2021). Indeed, Tregs generated in both of these tissues, as shown in Figure 3, have already shown different but connected immune roles (Haribhai et al., 2011). After these cells get mature, they can be recruited to lymphoid organs and inflamed areas, driven by autoantigens, the local microbiota or other tissue-specific factors (Atarashi et al., 2011; Hofmeister et al., 1999; Lathrop et al., 2011; Samy et al., 2008). After the migration, Tregs can establish their immunosuppressive function, and this immune response is likely to be retained or even promoted by the recruitment of other subsets of immunosuppressive cells (Bluestone et al., 2023).



Tregs have the ability to suppress the activity of several cell types of the immune system, such as CD4⁺ and CD8⁺ T lymphocytes, B lymphocytes, macrophages, dendritic cells, NK cells, granulocytes and osteoclasts (Sakaguchi et al., 2010). Moreover, they can suppress immune responses against both self and non-self antigens, and regulate both innate and adaptive immunity pathways, in lymphoid and non-lymphoid organs (Abbas et al., 2013; Akkaya & Shevach, 2020; Sakaguchi, 2005; Sakaguchi et al., 1995; Vanhanen et al., 2020).

5

et al., 2008). For example, they have the ability to produce immunosuppressive cytokines (such as IL-10 and TGF- β) and promote the cytolysis of T cells by releasing pro-apoptotic enzymes. Moreover, they can also interfere with antigen presentation to immune cells by APCs, as well as uptake some cytokines and growth factors (e.g. IL-2) that are essential for T effector (Teff) cell activation, development and proliferation. In addition, they can promote the disruption of some metabolic pathways, leading to a decrease of the functionality of the other T cell subsets (Bluestone et al., 2023; Nakayama et al., 2021; Sakaguchi et al., 1995; Waldmann, 2014).

In Figure 4, a schematic representation of the main suppressive mechanisms of Tregs over the other immune cells is shown.

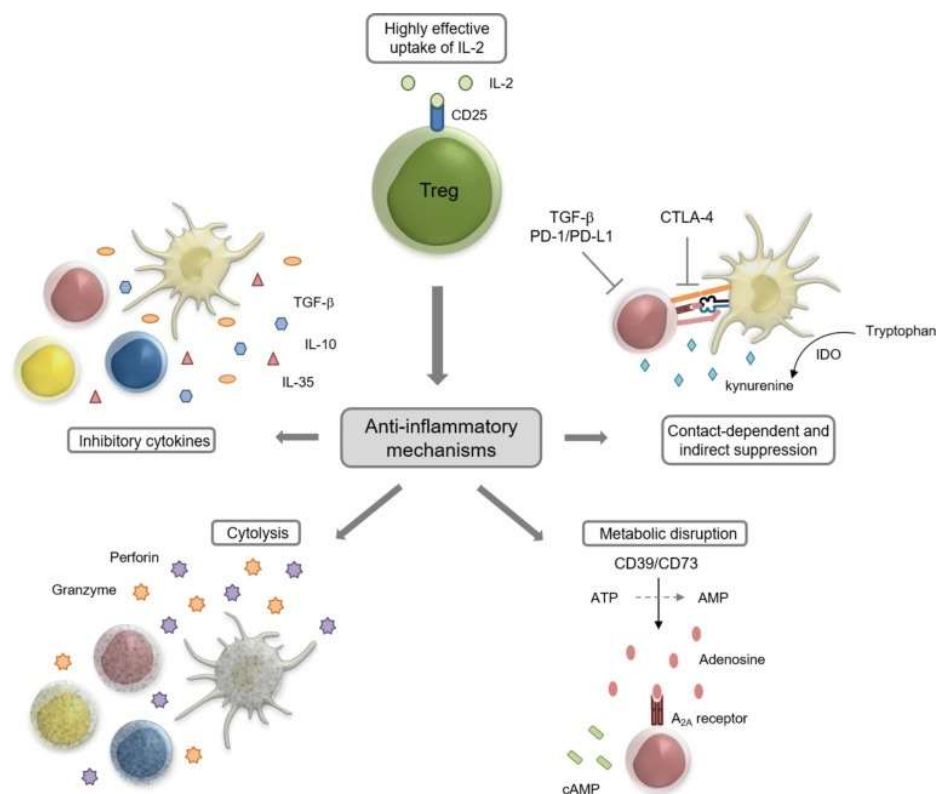


Figure 4. Overview of the principal immunosuppressive strategies of Tregs (Janssens & Cools, 2020).

1.4 Regulatory T cell therapies to induce immune tolerance

Over the last decades, many researches studying the effect of adoptive transfer of Tregs in animal models have shown encouraging results in suppressing transplantation-related disorders and other immune complications (Joffre et al., 2008; Kelchtermans et al., 2009; Trenado et al., 2003; Weigert et al., 2013).

The early clinical trials infusing Tregs into humans to promote peripheral tolerance already showed to be safe (and some of them showed signs of efficacy) for the treatment of autoimmune diseases such as T1D, for GvHD and along solid organ transplantation (Brunstein et al., 2011; Brusko et al., 2008; Grant et al., 2009; Marek-Trzonkowska et al., 2014; McMurchy et al., 2011; Singer et al., 2014).

Moreover, recent approaches have enabled the production of genetically engineered Tregs with unique antigen specificity, through the introduction of a specific TCR or chimeric antigen receptor (CAR) to target specific tissues, leading to an increased suppressive activity (Bluestone et al., 2023; Elinav et al., 2008; Pierini et al., 2017).

However, challenges to successfully proceed with large-scale applications of Treg therapies still need to be overcome including finding the optimal Treg source, isolation and expansion methods, that lead to a phenotypically stable product with high suppressive ability and low manufacturing costs (Hennessy et al., 2023).

1.5 Overview of different sources for regulatory T cell isolation

Lately, two main sources have been used to isolate Tregs for clinical purposes: adult peripheral blood and umbilical cord blood. Even though adult peripheral blood is relatively easy to collect from a patient or donor, the phenotype of the isolated Tregs is predominately antigen-experienced. This means their expansion potential and lifespan is reduced, and they can become phenotypically unstable after expansion, which is one of the challenges for clinical applications (Bernaldo-de-Quiros et al., 2022; Dijke et al., 2016).

Alternatively, Tregs can be isolated from the umbilical cord blood. Cord blood Tregs usually contain a lower number of memory cells and the vast majority of the cells presents a naïve phenotype (Takahata et al., 2004; Theil, Wilhelm, et al., 2015). Even though this composes an advantage from the clinical perspective, a limited number of Tregs can be isolated from this source, which means that these cells require numerous *in vitro* expansion cycles to meet the desired dosing number for a medical therapy (Bernaldo-de-Quiros et al., 2022; Riley et al., 2009).

1.6 Clinical importance of regulatory T cells isolated from thymus

Recently, some publications reported the isolation of Tregs from discarded pediatric thymus (thyTregs), overcoming some limitations associated with Tregs derived from the abovementioned commonly used sources (Bernaldo-de-Quiros et al., 2022; Dijke et al., 2016). ThyTregs have been showing promising characteristics, encouraging the scientific community about the clinical translation of therapeutic approaches using them.

Thymus is a primary lymphoid gland located in the anterior mediastinum of the human body, above the heart, which plays an essential role in the immune system. The thymus is responsible for the differentiation and expansion of lymphoid precursors, which lead to the formation of many subsets of immune cells (Andreozzi et al., 2023; Ribatti et al., 2006). In the beginning of T cell maturation, CD3⁻CD4⁻CD8⁻ T cell progenitors start expressing CD4 (immature CD4⁺ single positive cells) and then also CD8 (double positive cells), and the α and β loci of their TCR undergo a rearrangement process. After that, if these cells are able to engage self-MHC molecules and if they are responsive to self-peptides presented by this complex, they undergo positive selection in the thymic cortex and differentiate into mature CD4⁺ or CD8⁺ T cells (depending if they bind to MHC class II or class I proteins, respectively). If these cells are reactive against self-antigens, they undergo negative selection in the medulla, being either conducted to apoptosis or differentiated into Tregs. Mature T cells that are not self-reactive leave the thymus as functional cells (Kurd & Robey, 2016; Nunes-Cabaco et al., 2011; Tao et al., 2021).

Besides that, thymic tissue has the ability to self-regenerate after damage and its size starts decreasing gradually after one year of life, facing a natural involution, which means that babies and children have a larger thymus size compared to adults (Gui et al., 2012; Ribatti et al., 2006). Thymus anatomical position within the human body, as well as its shape and composition, are shown in Figure 5.

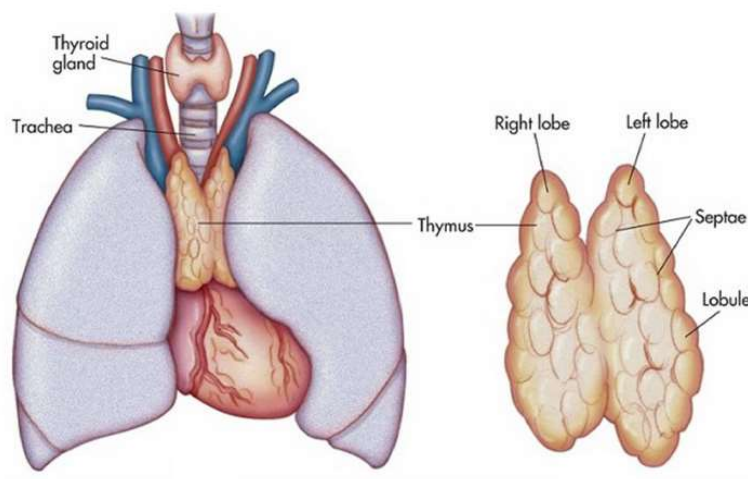


Figure 5. Human thymus - anatomical position, shape and composition (Medika Life, 2020).

During pediatric corrective cardiac surgeries, surgeons sometimes have to remove thymic tissue to gain access to the retrosternal operative field where heart is located (Bernaldo-de-Quiros et al., 2022; Dijke et al., 2016). The removed thymus can then be donated to scientific research studies, after the parents of these children have signed written informed consent, according to the declaration of Helsinki (Bernaldo-de-Quiros et al., 2022; Caramalho et al., 2015; Dijke et al., 2016; MacDonald, Ivison, et al., 2019; Morgana et al., 2022; Nunes-Cabaco et al., 2011; Romano et al., 2021; Vanhanen et al., 2018).

Several advantages of thyTregs have been described, especially when compared to Tregs isolated from peripheral or cord blood. Firstly, thyTregs exhibit a naïve phenotype and longer telomeres than the cells derived from peripheral blood, so they have a greater expansion potential and an increased lifespan, with the possibility of undergoing more cell division cycles in the future without becoming phenotypically unstable (Aubert & Lansdorp, 2008; Bernaldo-de-Quiros et al., 2022; Dijke et al., 2016; Lin et al., 2015). In fact, when these cells are expanded, not only their FOXP3 expression is stable, but they also exhibit high density of CD25, CTLA-4 and Helios expression and a demethylated Treg-specific demethylated region (TSDR) within the FOXP3 gene. Moreover, in contrast to adult blood Tregs, which are more phenotypically unstable, especially under proinflammatory conditions, thyTregs exhibit a more stable suppressive response in this environment (Dijke et al., 2016).

Secondly, it was reported that more Tregs can be isolated from a single infant thymus than from the entire peripheral blood volume of an average-sized adult. Thus, even though due to their early developmental stage their expansion rate is typically slower than in peripheral blood-derived Tregs, long expansion rounds are not required to get enough cells for clinical applications (Dijke et al., 2016; Tang & Lee, 2012).

Thirdly, it was reported that a single CD25⁺ cell enrichment step enabled the isolation of a pure Treg population (Dijke et al., 2016; Wing et al., 2002).

Finally, thymus removal is a common procedure during important pediatric heart surgeries. Thus it is not associated with additional risks or expenses for both patients and physicians, which is also positive from a clinical and ethical point of view.

Overall, many authors believe that human thymus is a promising alternative to commonly used Treg sources, and the abovementioned features of thyTregs are considered very useful for clinical translation (Bernaldo-de-Quiros et al., 2022; Dijke et al., 2016). Moreover, it is possible to cryopreserve thymocytes and thymus-derived Tregs, and several therapeutic doses can be prepared from a single thymus, due to the numerous number of high-quality naïve cells that can be obtained. For this reason, the medical community is now one step closer to get an “off-the-shelf” allogeneic Treg therapy for clinical application (Bernaldo-de-Quiros et al., 2022).

1.7 Isolation of thymus-derived regulatory T cells

After thymocyte isolation from thymic tissue, different methods have been reported to obtain thyTregs. Tregs are usually identified as CD3⁺CD4⁺CD8⁻CD25^{high}CD127^{low}FOXP3⁺ cells (Kondělková et al., 2010; Liu et al., 2006). Since FOXP3 is an intra-nuclear transcription factor and requires cell permeabilization, it cannot be included in the isolation panel for viable Tregs (Fontenot et al., 2005; Karanikas et al., 2008). In general, Magnetic-activated cell sorting (MACS) and/or Fluorescence-activated cell sorting (FACS) are used with this purpose.

Taking advantage of the high percentage of CD25⁺ cells in thyTregs, some studies reported that the magnetic enrichment of a CD25^{high} population was applied for isolation of thyTregs (Bernaldo-de-Quiros et al., 2022; MacDonald, Ivison, et al., 2019). An additional step of cell separation based on CD4 expression was added to the isolation of CD4⁺CD25^{high} Tregs (Dijke et al., 2016; Hoffmann et al., 2006; Liu et al., 2006). In order to obtain a more pure Treg population, a more complete gating strategy such as

CD3⁺CD4⁺CD8⁻CD25^{+/high}CD127^{low} was applied in FACS (57,58). In some studies, thyTregs were obtained with an initial step of magnetic selection of CD25⁺ cells, followed by CD4⁺CD25^{+/high}CD127^{low} cell sorting using FACS (Milward et al., 2019). However, these surface markers are not exclusively expressed by Tregs (Chen & Oppenheim, 2011).

FOXP3 is verified as the gold standard marker for adult and cord blood Tregs. Since it is not possible to include it in the above mentioned cell sorting strategies, additional surface markers which are expressed in correlation with FOXP3 in thyTregs will enable precise identification of the thyTregs.

Furthermore, a representative subpopulation of CD4⁺CD8⁺ double positive cells was found in thyTregs. More studies are required to fully understand the active role of double positive cells for Treg suppressive function (Martín-Gayo et al., 2010; Nunes-Cabaco et al., 2011; Vanhanen et al., 2020).

1.8 Novel regulatory T cell surface markers for Fluorescence-activated cell sorting

Different authors aimed to tackle the abovementioned difficulty related to human Treg isolation by trying to find a Treg-specific surface marker to get a pure population.

In humans, distinct Treg subpopulations can express different surface markers, depending on their location, activation status and functionality, as shown in Figure 6. This Treg heterogeneity opens possibilities to find potential novel markers for thyTregs cell sorting.

For example, HLA-DR⁺ Tregs exhibit higher FOXP3 expression than HLA-DR⁻ Tregs, but the marker is also expressed in about one third of human Teff cells (Baecher-Allan et al., 2006; Chen & Oppenheim, 2011; Santosh Nirmala et al., 2024).

It is reported that the expression of the GARP-encoding gene is much higher in activated human Tregs than in Teff cells, and a subset of CD25⁺GARP⁺ Tregs, also expressing FOXP3, has increased suppressive ability (Chen & Oppenheim, 2011; Santosh Nirmala et al., 2024; Wang et al., 2009). However, its expression is not detected in freshly isolated Tregs and it is unstable in TCR-activated Tregs (Chen & Oppenheim, 2011; Stockis et al., 2009; Wang et al., 2009).

Furthermore, after TCR activation, Latency-associated peptide (LAP) and IL-1 receptors type I (CD121a) and II (CD121b) are transiently expressed in activated and

highly suppressive Tregs, but further studies are still required to confirm these promising features (Santosh Nirmala et al., 2024; Tran et al., 2009).

Another alternative relies on the marker CD49d to exclude CD25⁺ Teff cells, since it is not expressed by human Tregs, so highly pure and functional FOXP3⁺ Tregs can be obtained by excluding CD49d⁺ T cells (Chen & Oppenheim, 2011; Kleinewietfeld et al., 2009).

Moreover, TNFR2 is highly expressed in thymic Tregs and it is not expressed in human Teff cells. Since it is highly correlated with FOXP3 expression, a cell sorting procedure according to the expression of this marker together with CD25 would enable the isolation of suppressive CD25⁺FOXP3⁺ Tregs (Annunziato et al., 2002; Chen & Oppenheim, 2011; Chen et al., 2010).

CD39 is almost exclusively expressed in the majority of FOXP3⁺ cells, with the expression of both markers exhibiting a linear correlation (Borsellino et al., 2007; Chen & Oppenheim, 2011). CD4⁺CD25^{high}CD39⁺ cells are able to suppress IL-17 production and show a typical Treg molecular signature (Chen & Oppenheim, 2011; Dwyer et al., 2010; Fletcher et al., 2009). The number of CD39⁺ Tregs was also highly reduced in the blood of patients with renal allograft rejection of relapsing-remitting multiple sclerosis, while the number of CD39⁻ Tregs remained similar (Borsellino et al., 2007; Chen & Oppenheim, 2011; Dwyer et al., 2010; Fletcher et al., 2009). Additionally, in a study from 2017, sorted CD39^{high} Tregs cultured with IL-1 β and IL-6 kept a stable FOXP3 expression (Gu et al., 2017).

Moreover, Glycoprotein A33 (GPA33) is surface marker found in stable and pure CD45RA⁺ thyTregs, and a study from 2020 reported that CD127⁻GPA33⁺ Tregs expressed FOXP3 and Helios (Opstelten et al., 2020). Even though this marker can potentially help to isolate a pure, stable and suppressive naïve Treg population, its applicability for Treg isolation still requires further studies (Morgana et al., 2022; Opstelten et al., 2020; Santosh Nirmala et al., 2024).

CD27 is a co-stimulatory molecule expressed by a high percentage of CD4⁺ T cells, which can be used to induce, expand and isolate suppressive Tregs. This marker is important for Treg function, and several studies have reported that it is a promising tool to select functional Tregs, with CD27⁻ Tregs expressing low amounts of FOXP3 (Duggleby et al., 2007; Gravestein et al., 1995; Koenen et al., 2005; Muth et al., 2022; Ruprecht et al., 2005; Santosh Nirmala et al., 2024).

Lastly, the secondary lymph node homing receptors CD62L (L-selectin) and C-C chemokine receptor type 7 (CCR7) are expressed in cord blood and adult naïve Tregs, but also in naïve and central memory conventional CD4⁺ T cells. Studies with murine models suggest that these markers are important for Treg migration into secondary lymph organs and for their suppressive function tackling some immune disorders (Ermann et al., 2005; Menning et al., 2007; Santosh Nirmala et al., 2024; Schneider et al., 2007; Seddiki et al., 2006; Taylor et al., 2004; Valmori et al., 2005).

In summary, even though some approaches have been done in this direction and some promising markers were already found, no truly Treg-specific surface marker has been proven ideal for Treg isolation, including for thyTregs. Therefore, the isolation of a pure Treg population remains a challenge (Bluestone et al., 2023; Dijke et al., 2016; Wegrzyn et al., 2022). Additionally, it is proposed that two distinct Treg progenitors (TregP) exist during early stages of Treg development: CD25⁺FOXP3⁻ and CD25⁻FOXP3^{low} Treg progenitors (Dijke et al., 2016; Lio & Hsieh, 2008; Morgana et al., 2022). This argues for the discovery of novel markers expressed exclusively on the surface in correlation to FOXP3 in order to establish an accurate cell sorting procedure according to the typical Treg phenotype already mentioned, and to in-depth understand thyTreg development and function.

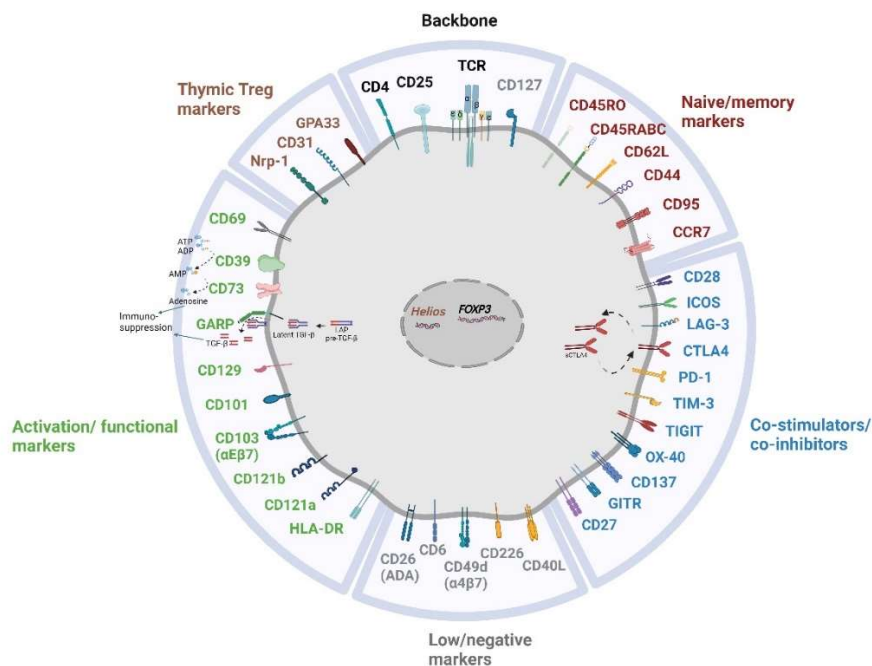


Figure 6. Abundance of Treg markers and heterogeneity of Treg cells (Santosh Nirmala et al., 2024).

1.9 *In vitro* expansion of thymus-derived regulatory T cells

Even though thyTreg do not need extensive expansion to reach a therapeutic dosage, there is still the need to increase the cell number to treat multiple patients in a clinical trial. In fact, there is no exact number of Tregs needed to reach therapeutic efficacy, but some data obtained using mouse model suggest that a high Treg-to-Teff ratio is required for an effective suppressive response (Hoffmann et al., 2002; Taylor et al., 2002).

Treg cells are typically expanded for some days in a culture medium, e.g. TexMACS or RPMI 1640, normally supplemented with serum – either human serum, human albumin serum or fetal calf serum –, cytokines (for example IL-2), among other reagents such as non-essential amino acids, L-glutamine or sodium pyruvate. An antibiotics is often used to prevent from microorganism contamination and consequently increase cell viability during the period of expansion (Bernaldo-de-Quiros et al., 2022; Dijke et al., 2016; MacDonald, Ivison, et al., 2019).

Tregs have to be stimulated through their TCR in the presence of IL-2 to initiate proliferation. Different reagents mimicking TCR engagement with antigen presenting cells through CD3 and CD28 are available, namely α CD3 α /CD28 Dynabeads Treg Xpander (Thermo Fisher Scientific), CD3/CD28/CD2 T Cell Activator (STEMCELL Technologies) or T Cell TransAct (Miltenyi Biotec), which is a polymeric nanomatrix with α CD3/ α CD28 antibodies to mimic APCs (Bernaldo-de-Quiros et al., 2022; MacDonald, Ivison, et al., 2019). Additionally, for a 14-day culture, it is suggested that thyTreg restimulation on day 9 of culture is beneficial to increase overall expansion and growth rate, since these cells achieve their resting size (around 8,5 μ m) on this day and the restimulation should not happen before this milestone (Hippen et al., 2011; Levine et al., 1997; Putnam et al., 2013). On the other side, a second restimulation is prone to promote Treg instability and increase the potential of differentiation into other T cell populations (Hippen et al., 2011; Hoffmann et al., 2009; MacDonald, Ivison, et al., 2019).

2 Aim of the Thesis

The research conducted for this Master's Thesis sought to isolate pure FOXP3⁺ and FOXP3⁻ Treg subpopulations from thymocytes and gain additional *in vitro* insight into their expansion capacity, functionality and phenotypic evolution.

Since FOXP3 is a transcription factor, surface markers needed to be analyzed to determine how they correlated with FOXP3 expression in thymus-derived Tregs. The preliminary data obtained from our lab already proposed four candidates – GPA33, CD62L, CD27 and CCR7 –, which might serve for sorting of distinct FOXP3⁺ or FOXP3⁻ cells within CD25⁺ population. Thus, this project began with the analysis of the expression of those markers in correlation to FOXP3.

After the confirmation of the correlation between GPA33⁺ and FOXP3⁺ expression and between CD27⁻ and FOXP3⁻ populations in thyTregs, a 14-day *in vitro* culture of the sorted GPA33⁺, CD27⁻ and bulk Tregs was performed in order to examine and compare their expansion potential and phenotypic profile. Additionally, their functionality was also accessed, through an analysis of their ability to suppress T effector cells at different timepoints. Moreover, the *in vitro* differentiation of FOXP3⁻ cells was also studied. Since there might be donor variations, the Masters' project confirmed these findings with three thymocyte donors to ensure their validity.

3 Material and methods

3.1 Material

3.1.1 Reagents

All the chemical reagents and solutions used, as well as the respective companies where they were purchased from, are listed in Table 1.

Table 1. Reagents

Reagent name	Supplier
BD FACSClean Solution	BD Biosciences
BD FACSFlow™ Sheath Fluid	BD Biosciences
BD FACSRinse Solution	BD Biosciences
Brilliant Stain Buffer	BD Biosciences
CryoStor® CS10	Sigma-Aldrich, Inc.
CryoSure-DMSO (dimethylsulfoxid)	Wak-Chemie Medical GmbH
Distilled water	Media Kitchen (in-house)
Ethanol ≥70%, denatured	Carl Roth GmbH + Co. KG
FcR Blocking Reagent	Miltenyi Biotec
Lymphoprep™	Serum Bernburg AG
MACSQuant® Rinsing Solution	Miltenyi Biotec
MACSQuant® Running Buffer	Miltenyi Biotec
MACSQuant® Storage Solution	Miltenyi Biotec
MACSQuant® Washing Solution	Miltenyi Biotec
PBS (phosphate-buffered saline)	Media Kitchen (in-house)
Trypan Blue Stain	Gibco
Türk's Solution	Sigma-Aldrich, Inc.

3.1.2 Buffers

Buffers were freshly prepared before use, and the respective composition is described in Table 2.

Table 2. Buffers

Buffer name	Composition	Supplier
PEB (protein extraction buffer)	MACSQuant [®] Rinsing solution + 0,5% Human Serum Albumin (HSA)	Miltenyi Biotec
		Baxalta

3.1.3 Culture media and supplements

Different cell culture media used, as well as the supplements that were added, are listed in Table 3.

Table 3. Media and supplements

Name	Supplier
Benzonase [®] Endonuclease	EMD Millipore
Human AB Serum male	PAN-Biotech GmbH
Human Serum Albumin (HSA)	Baxalta
IL-2 (Proleukin [®])	Clinigen Group
L-Glutamine	Gibco
MEM NEAA	Gibco
Penicillin-Streptomycin	Gibco
RPMI 1640 basal medium	Gibco
RPMI 1640 complete medium	Gibco
Sodium pyruvate	Gibco
T Cell TransAct	Miltenyi Biotec
TexMACS Medium (research grade)	Miltenyi Biotec

3.1.4 Antibodies

Information about the antibodies used during the experiments, including their respective antigen, conjugated fluorochrome, clone and supplier, is summarized in Table 4.

Table 4. Antibodies

Antigen	Conjugate	Clone	Supplier
CD3	FITC	REA613	Miltenyi Biotec
CD4	BUV395	RPA-T4	BD Biosciences
CD8	BUV737	SK1	BD Biosciences
CD25	PE	4E3	Miltenyi Biotec

	PE-Cy5	M-A251	BD Biosciences
CD27	APC	M-T271	BD Biosciences
	PE	LT27	Bio-Rad Laboratories
CD31	BV650	WM59	BD Biosciences
CD45	V500	HI30	BD Biosciences
CD45RA	BV711	HI100	BD Biosciences
CD45RO	BV605	UCHL1	BD Biosciences
CD62L	APC	145/15	Miltenyi Biotec
CD127	BV786	HIL-7R-M21	BD Biosciences
CCR7	BV421	2-L1-A	BD Biosciences
FOXP3	PE-Cy7	PCH101	eBioscience
GPA33	Alexa Fluor [®] 700	402104	R&D Systems
	PE	402104	R&D Systems

3.1.5 Cell viability and proliferation dyes

Different dyes used are listed, together with the respective supplier and function, in Table 5.

Table 5. Cell dyes

Name	Supplier	Purpose
7-AAD staining solution	Miltenyi Biotec	Dead cell exclusion
Cell Proliferation Dye eFluor [™] 450	eBioscience	Tracking of Teff cell divisions
Fixable Viability Dye eFluor [™] 780	eBioscience	Dead cell exclusion

3.1.6 Ready-to-use kits

During these experiments, different ready-to-use kits were used with different objectives – either cell separation, fixation before intracellular staining or preparation of compensation beads for flow cytometry and FACS. Those kits, as well as the respective supplier and function, are listed in Table 6.

Table 6. Ready-to-use kits

Name	Supplier	Purpose
CD4 ⁺ T cell isolation kit	Miltenyi Biotec	Cell separation (MACS): CD4 isolation

CD25 MicroBeads II	Miltenyi Biotec	Cell separation (MACS): CD25 isolation
CliniMACS® CD25 Reagent for cell separation	Miltenyi Biotec	Cell separation (MACS): CD25 isolation
eBioscience™ FcγR3 / Transcription Factor Staining Buffer Set	Invitrogen	Cell fixation for FOXP3 intracellular staining
MACS® Comp Bead Kit, anti-mouse Igk	Miltenyi Biotec	Compensation beads for flow cytometry and FACS
MACS® Comp Bead Kit, anti-rat Igk	Miltenyi Biotec	Compensation beads for flow cytometry and FACS
MACS® Comp Bead Kit, anti-REA	Miltenyi Biotec	Compensation beads for flow cytometry and FACS
MACS® Comp Bead Kit, blank	Miltenyi Biotec	Compensation beads for flow cytometry and FACS

3.1.7 Equipment

Information regarding the technical equipment used to carry out this project is summarized in Table 7.

Table 7. Technical equipment

Name	Specifications	Manufacturer
Cell culture hood	Herasafe™ KS	Thermo Fisher Scientific
Centrifuges (swing-out rotor)	Allegra® X-12R	Beckman Coulter, Inc.
	Avanti J-15R	Beckman Coulter, Inc.
Cryogenic gloves	Cryo-Gloves™ Blue Waterproof	Tempshield
FACS Cell sorter	BD FACSAria™ Fusion	BD Biosciences
Flow cytometers	BD LSRFortessa™	BD Biosciences
	MACSQuant® Analyzer 10	Miltenyi Biotec
Freezing containers	CoolCell™ LX	Corning Inc.
Heating block	Thermoshaker Pro	CellMedia
Lab balance	Adventurer™ Precision	OHAUS Corp.
Incubators	CB 210	BINDER GmbH
	HERAcell 240	Thermo Electron Corp.
Inverted microscope	CKX41	Olympus Corp.
Liquid nitrogen tanks	800 Series -190	Chart Industries, Inc.

MACS magnets and stands	QuadroMACS™ Separator	Miltenyi Biotec
	MACS® MultiStand	Miltenyi Biotec
Microcentrifuge (fixed angle rotor)	MiniStar silverline	VWR International GmbH
Multichannel pipettes	Research® 8-channel pipette, 30-300 µL	Eppendorf
	Xplorer® 12-channel pipette, 15-300 µL	Eppendorf
Pipette controller	Accurpette	VWR International GmbH
Pipettes (0,5-1000 µL)	Single channel pipettes	VWR International GmbH
	Research® plus Single channel pipettes	Eppendorf
Scissors	Microscopy scissors	VWR International GmbH
	Scissors straight	Carl Roth GmbH + Co. KG
Rocking shaker	Stuart™ SSL4 See-Saw Rocker Shaker	Cole-Parmer Instrument Company
Tissue dissociator	genteMACS™ Octo Dissociator with Heaters	Miltenyi Biotec
Tube rack	MACS® 15 mL Tube Rack	Miltenyi Biotec
Ultra low temperature freezer	Innova® U725 Upright Freezer, -80°C	New Brunswick Scientific
Vacuum Hand Operator	VACUBOY	INTEGRA Biosciences
Vacuum pump unit	BVC 21	Vacuubrand GmbH + Co. KG
Vortex shaker	neoVortex® shaker	neoLab
	VORTEX Genius 3	IKA-Werke GmbH & Co. KG
	VV3	VWR International GmbH
Water bath	VWB12	VWR International GmbH

3.1.8 Consumables

Different consumables that were used are listed in Table 8.

Table 8. Consumables

Name	Specification	Supplier
C Tubes	genteMACS™ C Tubes	Miltenyi Biotec
Cell culture flasks	Nunc™ EasYFlask™ 25 cm ² and 75 cm ²	Nunc, Thermo Fisher Scientific

Cell culture plates	Costar® 6, 12, 24, 48 and 96 wells	Corning Inc.
	Nuncclon™ 96 wells	Nunc, Thermo Fisher Scientific
Cell strainers	Falcon™ Cell Strainers, 40 µm and 100 µm nylon	Corning Inc.
Conical tubes	CELLSTTAR® tubes, 15 mL and 50 mL	Greiner Bio-One GmbH
Cryotubes	Cryo.s™, 2 mL	Greiner Bio-One GmbH
Disposable hemocytometer chips	C-Chip	NanoEnTek Inc.
FACS tubes	Falcon™ Polystyrene round bottom tubes 5 ml	Corning Inc.
	Falcon™ Polystyrene round bottom tubes 5 ml with cell strainer snap cap	Corning Inc.
Freezing bags	CryoMACS® Freezing Bags	Miltenyi Biotec
Laboratory wrapping film	Parafilm™ M	Bemis Company, Inc.
MACS column filters	Pre-separation filters, 30 µm	Miltenyi Biotec
MACS columns	LS columns	Miltenyi Biotec
Pasteur pipettes	Disposable Pasteur Pipettes, 1 mL	VWR International GmbH
	Glass Pasteur Pipettes	Media Kitchen (in-house)
Petri dishes	Falcon™ Tissue culture dish	Corning Inc.
Pipette tips (10 µL, 20 µL, 200 µL, 1 mL)	ART® Aerosol Resistant Tip	Thermo Fisher Scientific
	DeckWorks™ pipette tips	Corning, Inc.
	ep Dualfilter T.I.P.S.®	Eppendorf
	Filter Tip	Sarstedt GmbH
	MAXYMum Recovery® pipette tips	Axygen®, Corning Inc.
Reaction tubes	0,5 mL, 1,5 mL	Sarstedt GmbH
	5 mL	Greiner Bio-One GmbH
Reagent reservoirs	25 mL	VWR International GmbH
Separation tubes	Leucosep tubes	Greiner Bio-One GmbH
Serological pipettes	Costar® Stripette® Serological Pipettes, 2 mL, 5 ml, 10 mL, 25 ml and 50 mL	Corning Inc.

3.2 Methods

3.2.1 Cell isolation

3.2.1.1 Thymocyte isolation

Pediatric thymic tissue was obtained from Universitätsklinik für Herzchirurgie, Helios Herzzentrum Leipzig in the course of cardiac surgery in congenital heart disease after written consent of parents (University Dresden ethics board Nr EK-323072023). The tissue was kept in saline solution supplemented with antibiotics and transported to our lab in an ice cooling pack by GO! Express & Logistics Dresden GmbH. After receiving pediatric thymic tissue, the weight was measured and the tissue was transferred into a Petri dish filled with TexMACS GMP medium (without any supplement), as shown in Figure 7. After this initial step, the thymus was firstly cut into two pieces, and then into smaller fragments, which were then transferred to 5 mL tubes with 0.5-1 mL TexMACS GMP medium supplemented with benzonase (15-20 IU/mL) and further chopped using surgical scissors. Those tiny pieces were transferred into gentleMACS™ C Tubes (1-2 g of thymic tissue per C Tube). TexMACS GMP medium with benzonase was added until each C Tube contained approximately 5 mL of total volume. After quickly inverting the C Tubes, they were placed in the heatable gentleMACS™ Octo Dissociator for mechanical digestion for 40 minutes.

After mechanical digestion, cells were gently passed through 100 µm cell strainers and thereafter through 40 µm cell strainers. The filtered cells were resuspended in PEB buffer (CliniMACS rinsing solution with 0,5% human serum albumin) supplemented with benzonase (15-20 IU/mL)), and centrifuged at 300 g for 10 minutes at room temperature (RT). After removing the supernatant, cells were resuspended in PEB buffer with benzonase and counted using the flow cytometer MACSQuant® Analyzer 10 or a hemocytometer as described in Section 3.2.2. The isolated cells were partly processed for further studies and partly cryopreserved.



Figure 7. Thymic tissue before processing for thymocyte isolation.

3.2.1.2 Peripheral Blood Mononuclear Cell isolation

Buffy coat preparations were obtained as by-product from red blood cell donations from the German Red Cross blood donation center in Dresden (DRK Nord-Ost GmbH) after written consent of donors. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from buffy coat followed by the standard gradient isolation procedures. Briefly, 50 mL Leucosep tubes were loaded with 15 mL Lymphoprep™ (lymphocyte separation medium), centrifuged at 1000 g for 1 minute at RT to let Lymphoprep™ pass through the porous barrier. The blood was 1:1 diluted with sterile PBS and 30 mL of diluted blood were carefully overlaid into the Leucosep tube using a serological pipette and centrifuged at 800 g for 40 minutes at RT, with acc 7 and dec 0. After centrifuging, the PBMCs (opaque interphase) were carefully collected and transferred into a new 50 mL falcon tube using a Pasteur pipette, followed by washing with PBS at 500 g for 10 minutes at RT with a subsequent wash with PBS at 200 g for 10 minutes at RT. The cell pellet was resuspended in PBS for cell counting.

3.2.2 Cell counting

The cell viability and concentration were analyzed using either a hemocytometer and/or the flow cytometer MACSQuant® Analyzer 10.

3.2.2.1 Cell counting using hemocytometer

A small aliquot of the cell suspension was diluted in 0.4% trypan blue stain to distinguish the dead cells. Alternatively, when non-nucleated erythrocytes were still present in the sample (for example after PBMC isolation), Türk's solution was used to stain the nucleated cells. To obtain the number of viable nucleated cells in the original cell suspension, the following formula was applied:

$$Viable\ cells = \left(\frac{\sum \text{viable cell nr. in 4 quadrants}}{4} \right) \times \text{dilution factor} \times \text{volume (mL)} \times 10^4$$

3.2.2.2 Cell counting using MACSQuant® Analyzer 10

A 10 µL aliquot of cell suspension was stained with 3 µL 7-AAD in a 96-well plate for 10 minutes in dark for dead cell exclusion. Cells were then diluted 1:5 in PBS before counting. For suspensions with a higher cell concentration, 50 µL were stained with 5 µL 7-AAD and cells were diluted 1:10 in PBS. After setting a gating in live lymphocytes, the cell number per mL and cell viability were measured on MACSQuant® Analyzer 10. To calculate the number of viable cells in the original cell suspension, the following formula was applied:

$$Viable\ cells = \text{nr. viable cells / mL (MACSQuant®)} \times \text{dil. factor} \times \text{volume (mL)}$$

3.2.3 Cell cryopreservation

Cells were pelleted and resuspended in freezing medium such as CryoStor® CS10, or medium containing 90% of a 20% human serum albumin solution and 10% dimethylsulfoxide (DMSO), or 90% human serum and 10% DMSO. The cell suspensions were aliquoted into cryovials, up to 10 Mio cells in 500 µL freezing medium and from 10 Mio to 50 Mio cells in 1 mL freezing medium. Cells were then placed into CoolCell® Containers and thereafter kept at -80°C for more than 1 day. The cryovials were later transferred into liquid nitrogen tanks, with temperatures around -200°C, where they can be stored for several years. Alternatively, cells were resuspended in freezing medium and transferred into freezing bags for higher number of cells (1 billion to 3 billion cells in one bag) and kept in a styrofoam box at -80° C.

3.2.4 Cell thawing

The cryovials were transported in a box containing dry ice and quickly thawed in a 37°C water bath. The pre-warmed thawing medium (RPMI 1640 medium with 5% human serum) was slowly added into the cryovials at 1:1 ratio and mixed gently. The cell suspensions were transferred into a 15 mL conical tube containing 10 mL warm thawing medium and the cryovials were rinsed by 1 mL thawing medium to recover all the cells. Cells were then pelleted at 300 g, at 20°C, for 10 minutes, washed once with PBS, resuspended in culture medium and counted. For thawing thymocytes, RPMI 1640 medium supplemented with 10% human serum and 50.000 U/mL benzonase was used. For thawing thymocytes frozen in cryo bags, the bag was transferred to the water bath, with the tubes and valves above the water level, until a tiny visible ice cube was left. The bag was then transferred into the hood and the middle tubing was cut with sterile scissors. The cell suspension was transferred to a 50 mL conical tube and warm thawing medium was slowly added. The cell suspension was mixed gently and pelleted down at 300 g, at 20°C, for 10 minutes. Cells were then washed once with PBS containing benzonase and 0.5% human serum albumin and counted.

3.2.5 Flow cytometry

Cells were stained with fluorophore-conjugated antibodies specific for markers of interest and analyzed by BD LSRFortessa™ and MACSQuant® Analyzer 10.

3.2.5.1 Staining of extracellular markers and viability dye

0.1-1 million cells from each sample were transferred into 1.5 mL reaction tubes and centrifuged at 300 g, at 20°C, for 10 minutes. The cell pellet was resuspended in 50 µL FcR blocking reagent diluted 1:10 in PBS and incubated for 10-minute at 4°C to prevent from unspecific binding during antibody incubation. 30 µL brilliant stain buffer were then added to each sample and followed by the antibody mix together with eFluor™ 780 viability dye diluted 1:10 in PBS (see Appendix Table 1). After a 30-minute incubation at 4°C, cells were washed twice with 1 mL PEB, at 300 g, at 20°C, for 10 minutes, and the supernatant was discarded. For samples containing 0.1-0.5 million cells, half the amount of the reagents was used. Unstained samples were kept at 4°C until the fixation

step. Cells were resuspended in 300 μ L PEB buffer and analyzed using flow cytometry directly afterwards or proceeded with fixation and intra-nuclear staining.

3.2.5.2 Fixation

The eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set was used according to the manufacturer's instructions. Briefly, Fixation/Permeabilization buffer was prepared by mixing the Fixation/Permeabilization concentrate and diluent at the ratio of 1:3 and the Permeabilization buffer was prepared by diluting the stock solution 1:10 in PBS. 0.3 mL Fixation/Permeabilization solution were added to each sample (including unstained sample). After a 45-minute incubation at RT, 1 mL diluted Permeabilization buffer was added to each sample to stop the fixation process. Cell suspension was pelleted at 500 g, at 20°C, for 5 minutes, and washed again with 1 mL Permeabilization buffer. Cells were then resuspended in 50 μ L Permeabilization buffer and proceeded with intracellular staining. Alternatively, cells were resuspended in 200 μ L PEB and kept at 4°C if the intracellular staining was performed on the following day. The unstained sample was resuspended in 300 μ L PEB, transferred to a FACS tube and kept at 4°C until flow cytometry analysis.

3.2.5.3 Staining of intracellular markers

Cells were resuspended in 50 μ L Permeabilization buffer and intracellular antibodies were added. After a 30-minute incubation at RT, 1 mL Permeabilization buffer was added to the cells and followed by centrifuging at 500 g, 20°C for 5 minutes. The pelleted cells were resuspended in 300 μ L PEB buffer and transferred to FACS tubes and kept at 4°C until analyzing the samples in BD LSRFortessa™.

3.2.5.4 Preparation of compensation controls

To prepare the compensation controls for flow cytometry, 100 μ L PEB was added to each compensation FACS tube. Fluorophore-conjugated antibodies and one full drop of the respective MACS® Comp Bead were added to the respective tube (see volume in Appendix Table 1). A blank compensation tube without antibodies was also prepared. After mixing and incubating for 10 minutes at RT (or 30 minutes at 4°C), 300 μ L PEB were added to the tubes to resuspend the beads. Those compensation tubes were kept at 4°C or on ice until compensation was initiated.

3.2.6 Isolation of Treg and Teff cells

Magnetic-activated cell sorting (MACS) and Fluorescence-activated cell sorting (FACS) were carried out for isolating Tregs from thymocytes. MACS was applied for CD4⁺ Teff cells isolation from PBMCs.

3.2.6.1 Treg isolation from thymocytes

For Treg isolation from thymocytes, the first step was a magnetic enrichment of CD25⁺ cells using CD25 Microbeads, followed by FACS sorting of the population CD3⁺CD4⁺CD8⁻CD25⁺CD127^{low}. Additional sub-populations of interest (CD27⁻ Tregs and GPA33⁺ Tregs) were also sorted based on their fluorescence intensities.

CD25⁺ magnetic enrichment step using the CD25 MicroBeads II kit was performed as the first step to enrich the Treg population. According to the manufacturer's protocol, cells were resuspended in 90 µL PEB and 10 µL MicroBeads per 10 million cells. After a 15-minute incubation at 4°C, cells were resuspended with 1-2 mL per 10 million in PEB buffer and centrifuged at 300 g, at 20°C, for 10 minutes. Cells were resuspended in 0.5 mL PEB per 100 million cells and passed through a 30 µm pre-separation filter placed on the top of each PEB pre-rinsed LS column to avoid the formation of cell aggregates. The flow-through containing the CD25⁻ cell population was collected in 15 ml conical tubes and the columns were washed three times with 3 mL PEB buffer. The washing flow-through was combined with the previously collected CD25⁻ population. Then, each column was removed from the magnetic stand, placed on top of a new 15 mL conical tube and loaded with 5 mL PEB buffer. CD25⁺ cells were released and collected by firmly pushing the provided plunger into the column. To increase the recovery rate of CD25⁺ cells, CD25⁻ cells were loaded onto and passed through new columns (half the number of columns were used as compared to the first step). CD25⁺ cells were collected and combined with the first batch of isolated cells.

In some experiments, the GMP-compatible CliniMACS® CD25 Reagent was used for CD25⁺ cell separation. Briefly, thymocytes were resuspended in 1 mL reagent and 10 mL PEB for up to 3 billion cells. After a 15-minute incubation at 4°C on the rocking shaker (25 rpm), cells were washed with 100 mL PEB buffer, at 300 g, at 4°C, for 10 minutes (deceleration was reduced to 4, according to the manufacturer's protocol), resuspended in 10 mL PEB and isolated using the LS columns as described above.

CD25⁺ enriched cells were stained with CD3, CD4, CD8, CD25, CD127, CD27 and GPA33 antibodies (Appendix Table 1), following the extracellular staining procedures described above. Cells were then passed through a 30 µm pre-separation filter to eliminate cell aggregates. Those passed through cells were sorted using FACS for bulk (total) Tregs – CD3⁺CD4⁺CD8⁻CD25⁺CD127^{low} cells –, the CD27⁻ subpopulation of Tregs and the GPA33⁺ subpopulation of Tregs using BD FACSAria™ Fusion. Sorted cells were collected in separated 15 mL conical tubes containing freshly prepared culture medium (TexMACS supplemented with 5% human serum AB male, 1% Penicillin/Streptomycin antibiotic and 1000 U/mL IL-2).

3.2.6.2 CD4⁺CD25⁻ T effector cell isolation from PBMCs

The first step to isolate non-activated CD4⁺CD25⁻Teff cells to be used as responder cells in Treg suppression assays involved using a CD4⁺ T cell isolation kit. Briefly, pelleted PBMCs isolated from buffy coat were resuspended in 40 µL PEB buffer and 10 µL CD4⁺ T cell antibody cocktail per 10 million cells. After mixing well and incubating for 5 minutes at 4°C, 30 µL PEB and 20 µL CD4⁺ T Cell MicroBead Cocktail were added per 10 million cells and incubated for 10 minutes at 4°C. The cells were then loaded onto the already rinsed LS columns and the flow-through was collected as the fraction of CD4⁺ cells. Afterwards, CD25 MicroBeads were used as described above to isolate non-activated CD4⁺CD25⁻ Teff cells.

3.2.7 *In vitro* Treg cell expansion

After taking some cells for phenotypic quality control measurement, the sorted bulk Tregs, CD27⁻ Tregs and GPA33⁺ Tregs were seeded at a concentration of 0.5 Mio/mL in TexMACS cell culture medium supplemented with 5% human AB serum, 1% Penicillin/Streptomycin antibiotics and 1000 U/mL IL-2 (Proleukin®), and cultured for 14 days under 37°C, with 5% CO₂. On days 4, 7, 9 and 11, after monitoring cell morphology under an inverted microscope, cells were counted using the MACSQuant® Analyzer 10. Then, fresh medium was added to dilute the cells to the desired concentration for an optimal growth (0.3 Mio/mL or 0.5 Mio/mL, depending if the next cell counting and feeding day would be 3 or 2 days later, respectively). T cell TransAct (αCD3/αCD28) was added on Day 0 and Day 9 at 1:100 concentration (v/v) for cell

stimulation. Aliquots of cells were taken on days 7 and 14 for phenotyping and suppression assay. Cells were also frozen down during the 14-day culture to keep the culture volume less than 15 mL. A schematic representation of the thyTreg expansion timeline, after MACS and FACS separation, is shown in Figure 8.

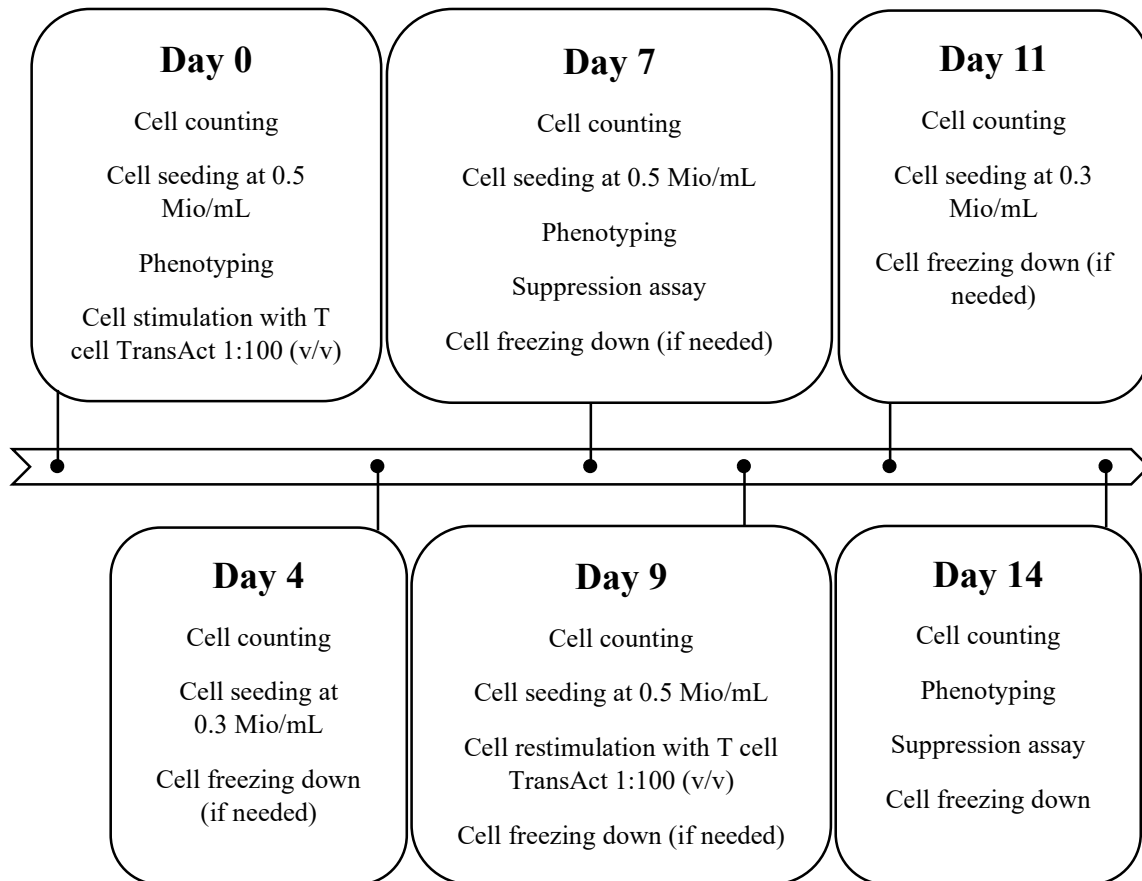


Figure 8. Timeline of thyTreg expansion procedure.

3.2.8 eFluor™ 450 proliferation dye staining of T effector cells

CD4⁺CD25⁻ Teff cells were washed twice with PBS and resuspended in PBS at a concentration of less than 10 Mio/mL. For each 1 mL of cells, 1 mL of eFluor™ 450 staining solution (20 µM) was added to reach a 10 µM final dye concentration. The cells were mixed and incubated at 37°C for 10 min in the dark. Then, the labeling was stopped by adding a 4-to-5 times higher volume of cold complete medium (RPMI 1640 with 10% Fetal Bovine Serum). The Teff cell suspension was kept on ice for 5 min, followed by centrifuging at 300 g, at 20°C, for 10 minutes. The labeled cells were washed once more in complete medium, at 300g, at 20°C, for 10 minutes and used immediately as responder cells in Treg functional suppression assays or frozen down.

3.2.9 Suppression assay

The functionality of CD27⁻, GPA33⁺ and bulk Tregs cultured for 7 and 14 days was tested in a suppression assay measuring the capability of suppressing the proliferation of in-vitro activated CD4⁺CD25⁻ Teff. Tregs and Teff cells were resuspended at a concentration of 0.5 Mio/mL. TransAct was added to the Teff cell suspension at double concentration, to trigger Teff proliferation. Tregs underwent a 1:2 serial dilution by adding 100 µL Tregs into 100 µL culture medium (RPMI 1640, with 10% human AB serum, 1% NEAA, 1 mM sodium pyruvate and 2 mM L-Glutamine) in a round bottom 96-well plate. After that, 100 µL of Teff cells (50.000 cells) were added to each well containing 100 µL of Tregs resulting in Treg-to-Teff cells ratios of 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32. Teff-only control wells with and without TransAct were also included. The cells were then cultured for 4 days at 37°C, with 5% CO₂.

After 4 days of culture, cells were resuspended and 100 µL of the cell suspension was transferred to a new round bottom 96-well plate. 10 µL of the 1:10 diluted 7-AAD solution were added into each well, and incubated for 5-10 minutes in the dark. The samples stained with 7-AAD were then measured on MACSQuant[®] Analyzer 10. The suppression ability was assessed by calculating the proliferating rate and fold expansion of Teff cells.

3.2.10 Statistical analysis

The flow cytometry raw data was analyzed in FlowJo™ X v10.0 and plotted in GraphPad Prism 8.0 and Microsoft[®] Excel[®] 16.0. The statistical analysis was performed in GraphPad Prism 8.0. The significance of two different conditions was compared using the two-tailed unpaired t-test. The significance of three different conditions was compared using two-way ANOVA.

4 Results

4.1 Flow cytometric analysis of thymus-derived Treg markers

To sort different thyTreg subpopulations from thymocytes, the correlation between the expression of the following four surface markers and FOXP3 was tested using flow cytometry: GPA33, CD27, CD62L and CCR7.

Isolated thymocytes were pre-gated on single live cells, and then on the typical Treg markers $CD3^+CD4^+CD25^+$. The preselected cells were further gated based on the low expression of CD127 as a further accepted Treg marker. The pre-gated Tregs were then gated into two subpopulations according to their FOXP3 expression ($FOXP3^-$ and $FOXP3^+$). The gating strategy used for this step is shown in Appendix Figure 1. The percentages of $GPA33^+$, $CD27^-$, $CD62L^-$ and $CCR7^-$ cells in both $FOXP3^-$ and $FOXP3^+$ subpopulations were then analyzed, as shown in Figure 9. For the three tested thymocyte donors, GPA33 was considerably more expressed in $FOXP3^+$ cells than in $FOXP3^-$ cells. Contrastingly, $CD27^-$ cells were mostly present in $FOXP3^-$ cells, with a very small percentage of $FOXP3^+$ cells exhibiting this phenotype. Both $CD62L^-$ and $CCR7^-$ cells showed relatively higher expression levels in $FOXP3^-$ cells for the three donors. For this latter group, the difference was statistically significant, but not observed for all the tested donors.

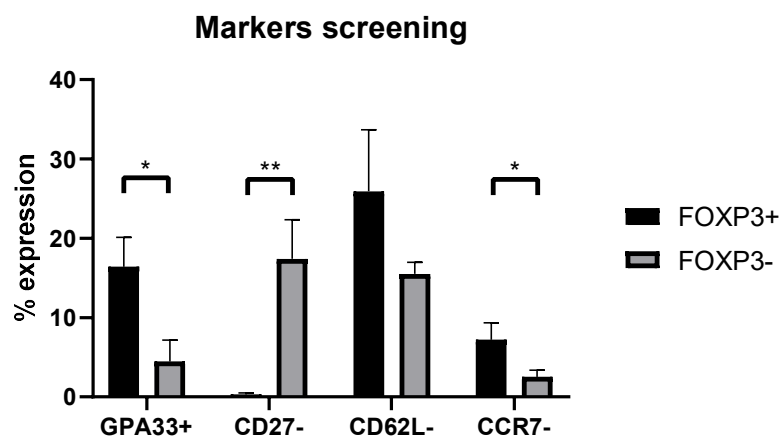


Figure 9. Percentage of $GPA33^+$, $CD27^-$, $CD62L^-$ and $CCR7^-$ cells within $FOXP3^+$ and $FOXP3^-$ Treg subtypes.

Based on these results, we selected GPA33 and CD27 for further experiments, with $GPA33^+$ cells representing a $FOXP3^+$ Treg subpopulation and $CD27^-$ cells, a $FOXP3^-$ Treg subpopulation.

Detailed gating strategies used to study the expression of each marker, as well as the percentage of each subpopulation into FOXP3 positive and negative cells, for each donor, can be found in Appendix Figure 2-Appendix Figure 5.

To sort bulk Tregs, GPA33⁺ Tregs and CD27⁻ Tregs, CD25-enriched thymocytes from three donors (either frozen or freshly isolated) were stained with CD3, CD4, CD8, CD25, CD127, CD27 and GPA33 antibodies, as listed in Appendix Table 1.

Total Tregs were gated as CD3⁺CD4⁺CD8⁻ cells for the first donor, and CD3⁺CD4⁺CD8⁻CD25^{high}CD127^{low} cells for the remaining donors. Based on their CD27 and GPA33 expression, total Tregs were further gated to isolate CD27⁻ Tregs and GPA33⁺ Tregs. The complete gating strategy for cell sorting using FACS is represented in Figure 10.

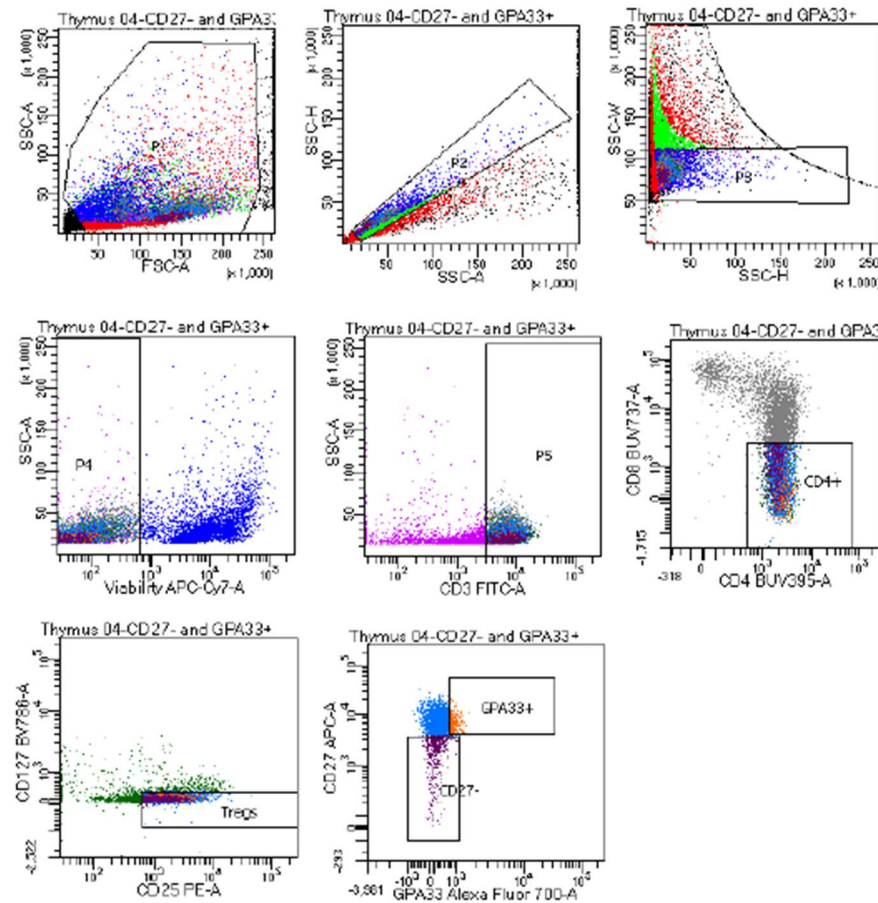


Figure 10. Gating strategy for cell sorting bulk Tregs, CD27⁻ Tregs and GPA33⁺ Tregs.

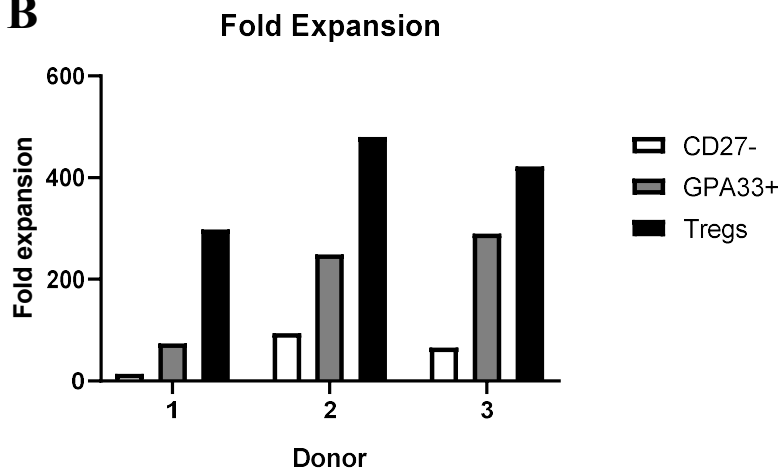
4.2 *In vitro* expansion of thymus-derived Tregs

The sorted cells were cultured for 14 days under controlled conditions as described in Section 3.7. Cells were counted and their fold expansion was calculated (Figure 11). Bulk Tregs reached higher expansion values after 14 days of culture, compared to CD27⁻ and GPA33⁺ Tregs. Between those two Tregs subpopulations, GPA33⁺ Tregs showed higher fold-expansion values. Throughout three tested donors, the CD27⁻ population presented only a limited *in vitro* expansion capacity, especially for donor 1. However, the three subtypes seem to present a similar growth pattern in the second week of expansion, as shown in Figure 11C.

A

Fold expansion	CD27 ⁻ Tregs	GPA33 ⁺ Tregs	Bulk Tregs
Donor 1 (thy02)	14x	74x	298x
Donor 2 (thy03)	94x	249x	480x
Donor 3 (thy04)	66x	290x	422x

B



C

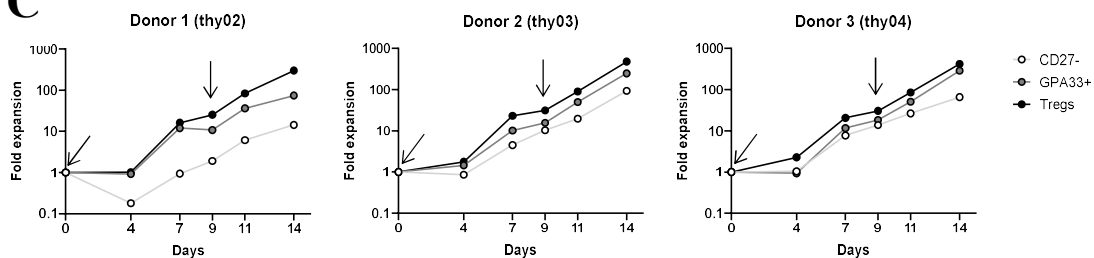


Figure 11. Fold expansion of sorted Treg subtypes (A,B) after 14 days of culture and (C) over time. Black arrows indicate stimulation with T cell TransAct at a 1:100 concentration (v/v).

4.3 Suppressive capacity of thymus-derived Tregs

To test the functional capacity of the three sorted Treg subtypes after expansion for 7-14 days, they were co-cultured with eFluor™ 450 stained CD4⁺CD25⁻Teff cells for 4 days with 1:400 (v/v) TransAct stimulation. After the co-culture, cells were stained with 7AAD and measured using MACSQuant® Analyzer 10. Teff proliferating cells had a reduced signal of the proliferation dye upon consecutive cell division cycles. The percentage of proliferating Teffs was analyzed using FlowJo software and the gating strategy is shown in Appendix Figure 6. To calculate the fold expansion, the number of Teff cells obtained by flow cytometry was divided by the initial seeded Teff cell number. Teff fold expansion and cell proliferation percentage, for each analyzed donor, thyTreg subtype, timepoint and Treg-to-Teff ratio, are shown in Figure 12. A control with Teff cells without co-culture with Tregs was set as the initial fold expansion/proliferation in absence of suppression.

In the test of Tregs after 7 days of expansion, the Teff cells fold expansion and proliferation rate varied between the three experiments, with mean fold expansion values of 1.4, 2.0 and 1.9 and proliferation rate of 54.5%, 89.1% and 42.0% for donors 1-3, respectively. The fold expansion of Teff cells decreased with the increase of co-cultured Treg cell concentration for the three tested thyTreg donors and for the three Treg subtypes. This decrease can be already seen starting from the Treg-to-Teff ratio of 1:32. Thymus-derived Tregs from all those three tested donors showed similar capacity in reducing the Teff fold expansion, except the donor 1 at the Treg-to-Teff ratios of 1:32 and 1:16, with bulk Tregs being significantly less suppressive than GPA33⁺ Tregs (Figure 4A). Teff cell proliferation rate was also assessed, and results showed a similar pattern comparing to their fold expansion, since the proliferation rate decreased with the increase of Treg cell concentration, for all the tested donors and for the three Treg subtypes. For donor 1, GPA33⁺ Tregs also inhibited Teff proliferation more than bulk Tregs at 1:32 and 1:16 ratios, while for donor 3 and 1:16 ratio, Teff cells were significantly more proliferative when co-cultured with bulk Tregs than with CD27⁻ Tregs.

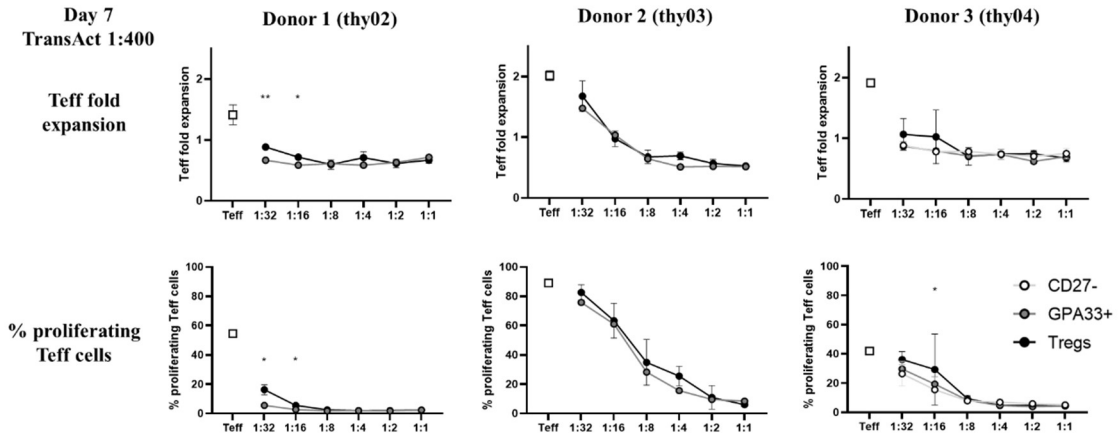
For tests to study the suppressive capacity of Tregs after a 14-day expansion, Teff control samples showed a 1.2, 2.0 and 1.0 fold expansion and a proliferation rate of 37,8%, 73.0% and 50.5% for donors 1-3, respectively. Data from day 14 suppression assay showed more statistically significant differences between subtypes. Starting with Teff fold expansion, for donor 1, CD27⁻ Tregs showed increased suppressive ability, leading

to a significant reduced Teff fold expansion when compared to bulk Tregs (1:32 and 1:16 ratios) and to GPA33⁺ Tregs (1:32 ratio). For donor 2, GPA33⁺ Tregs were significantly more suppressive comparing to bulk Tregs at 1:32 ratio. For donor 3, Teff fold expansion after co-culture with bulk Tregs was significantly lower when compared to the cells co-cultured with GPA33⁺ Tregs, and even more with CD27⁻ Tregs at 1:32 ratio. The difference between data from these two latter groups was also statistically significant. Data regarding the percentage of Teff cell proliferation was in accordance with Teff cell fold expansion. For donor 1, bulk Tregs exhibited the lowest suppressive ability, with Teff cells being significantly more proliferative than the ones co-cultured with the other Treg subtypes for Treg-to-Teff ratios from 1:32 to 1:8, except that at 1:32 ratio GPA33⁺ Tregs showed similar capacity comparing to bulk Tregs. For donor 2, GPA33⁺ Tregs were more suppressive than the other Treg subtypes at the ratios 1:32 to 1:8. Additionally, for 1:8 ratio, bulk Tregs showed significantly higher suppressive capacity comparing to CD27⁻ Tregs. Lastly, for donor 3, the three tested Treg subtypes showed a similar suppressive pattern, with a decrease of the proliferation rate with the increase of Treg cell concentration for all the three Treg subtypes. Only for 1:32 ratio, CD27⁻ Tregs were significantly less suppressive than the remaining cells.

Between day 7 and day 14 data, no clear differences seem to be observed, except that GPA33⁺ Tregs (for lowest Treg concentration values) and bulk Tregs from donor 1 seem to have been more suppressive on day 7.

Overall, no remarkable differences were observed between the suppressive pattern of the three experimental groups. On day 7, bulk Tregs were more suppressive than GPA33⁺ Tregs for donor 1, as it can be seen at lower Treg-to-Teff ratios. On day 14, for lower Treg concentration, CD27⁻ Tregs were more suppressive than bulk Tregs for donor 1, GPA33⁺ Tregs were the most suppressive cells for donor 2 and CD27⁻ Tregs were the least suppressive cells for donor 3.

A



B

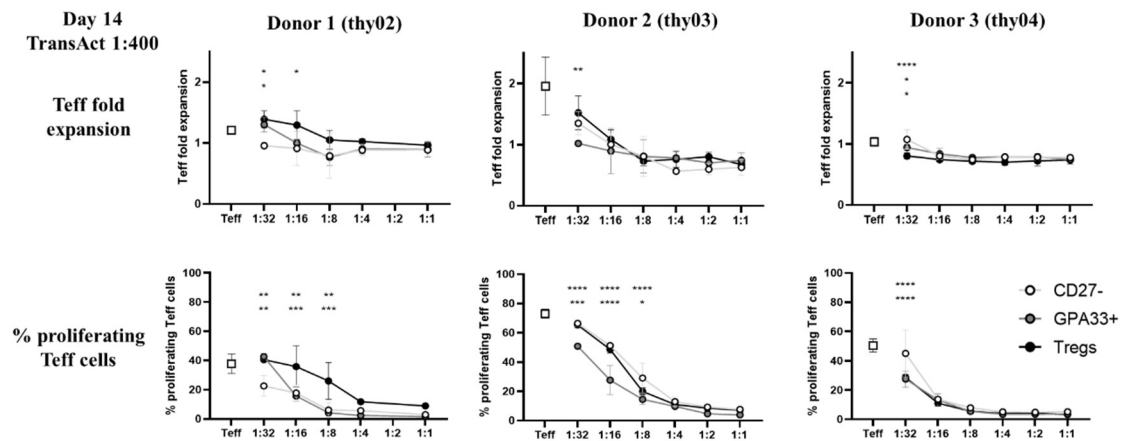


Figure 12. Suppression assay. Representation of Teff fold expansion and percentage of proliferating Teff in co-culture with thyTregs expanded for (A) 7 days and (B) 14 days. TransAct was added to the co-culture for cell stimulation at a concentration of 1:400 (v/v) on day 0.

4.4 Phenotypic profile of thymus-derived Tregs

Flow cytometry was used to study the phenotype of the three sorted Treg subtypes before being seeded for expansion, and also after 7 and 14 culture days. Cells were stained with antibodies specific for surface markers and later fixed and proceeded with intracellular markers staining before flow cytometry analysis. The CD3⁺ total T cell population was gated for further analysis. The gating strategy used for CD3⁺ cells was similar to the one applied for Section 4.1 (see Appendix Figure 1).

4.4.1 CD4 and CD8 expression

According to the expression of CD4 and CD8, pre-gated CD3⁺ cells were then gated into a CD4⁺ single positive, a CD8⁺ single positive and a CD4⁺CD8⁺ double positive subpopulation, as shown in Figure 13.

On day 0, the three sorted subtypes presented a high CD4⁺ purity for donor 2 (Figure 5). However, after 7 days of expansion, other CD3⁺ cell subsets such as CD4⁺CD8⁺ and CD8⁺ populations appeared. The CD4⁺CD8⁺ double positive population constituted 27.3%, 35.1% and 34.2% of the total CD3⁺ cells for CD27⁻, GPA33⁺ and bulk Tregs, respectively. On the other hand, the CD8⁺ population constituted 7.6% of the total CD3⁺ cells for CD27⁻ Tregs but was almost absent in GPA33⁺ and bulk Tregs ($\leq 0.2\%$).

After 14 days of expansion, the number of double positive cells was still significant, but the respective percentage was reduced comparing to day 7. Additionally, in CD27⁻ Tregs, the CD8⁺ population increased from 7.6% on day 7 to 38.6% on day 14. The percentage of CD8⁺ cells was minimal for both GPA33⁺ Tregs and bulk Tregs.

Similar data was obtained for the other two donors, as shown in Appendix Figure 7. For both of them, a higher CD4⁺ cell purity was observed on day 0 in all 3 subtypes. Double positive cells appeared on day 7 and they were also seen on day 14 in all 3 subtypes. On the other side, CD8⁺ cells appeared on day 7 only for CD27⁻ Tregs and their expression increased on day 14. Additionally, CD8⁺ cell percentage varied from different donors, representing 47.1%, 7.6% and 2.6% of CD3⁺ cells from donors 1-3 on day 7, and 87.6%, 38.6% and 12.3% on day 14, respectively.

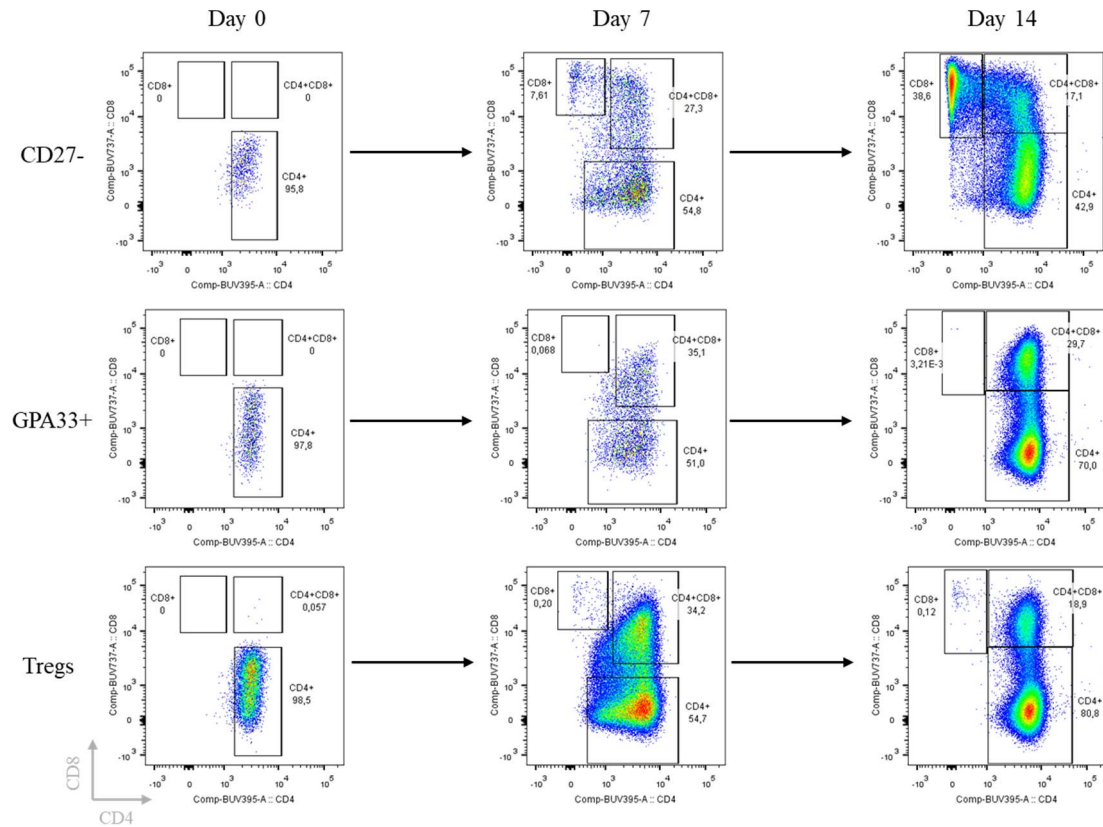


Figure 13. Representative dotplots for CD4⁺, CD8⁺ and CD4⁺CD8⁺ cells within Tregs directly after sorting, 7 days or 14 days post expansion (data shown for donor 2, pre-gated on live CD3⁺ cells).

4.4.2 CD25 expression

The expression of the marker CD25 in CD4⁺ cells was further analyzed, as represented in Figure 14A.

As shown in Figure 14B, for donor 1 (whose sorting gating did not include the selection of CD25^{high}CD127^{low} cells), CD4⁺ cells from the three subtypes had a low CD25 expression on day 0, especially CD27⁻ Tregs. On day 7, this expression increased to a value close to 100% for the three groups (likewise observed for the other donors), but on day 14 this value was lower for CD27⁻ Tregs and GPA33⁺ Tregs, with a more abrupt decrease being observed for the first group.

For donors 2 and 3, after sorting, almost all CD4⁺ cells of GPA33⁺ and bulk Tregs expressed CD25 on expansion days 0, 7 and 14 (>98%). In CD27⁻ Tregs from donor 2, CD25⁺ expression was lower on days 7 and 14, but for donor 3 this value remained close to 100% for the three tested time points.

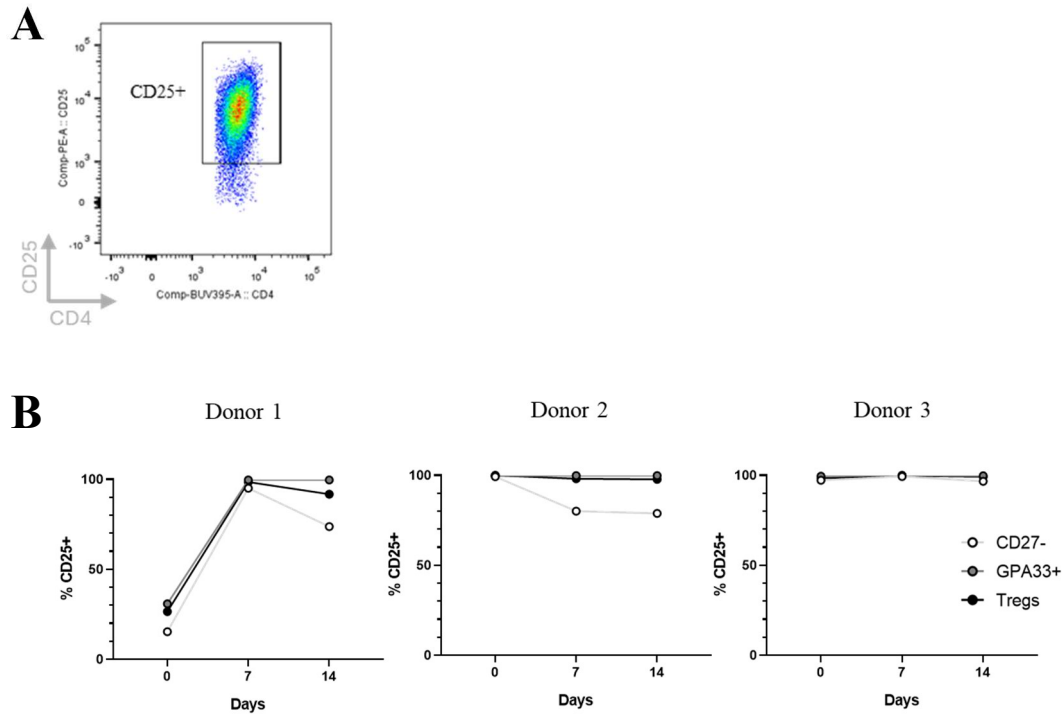


Figure 14. Analysis of CD25 expression in CD4⁺ cells. (A) Representation of CD25⁺ gating on CD4⁺ cells. (B) Percentage of CD25 expression in CD4⁺ cells at different culture time points.

4.4.3 CD31 expression

CD31 is expressed in the surface of Tregs recently migrated from thymus. The expression of this marker on CD4⁺CD25⁺ cells was further analyzed, as shown in Figure 15A. The percentage of CD31⁺ cells among the CD4⁺CD25⁺ cells for each Treg subtype, donor, and for culture days 0, 7 and 14, is represented in Figure 15B.

On day 0, a high percentage of CD31⁺ cells was observed for the three Treg subtypes from all three donors (>70%), and this value was considerably lower on day 7, especially for CD27⁻ Tregs, as represented in Figure 15C.

On day 14, for donors 1 and 3, the percentage slightly decreased further from day 7 for every Treg subset, while for donor 2, it slightly increased. Overall, the percentage of CD31⁺ cells was lower than on day 0, for all the analyzed CD25⁺ cells.

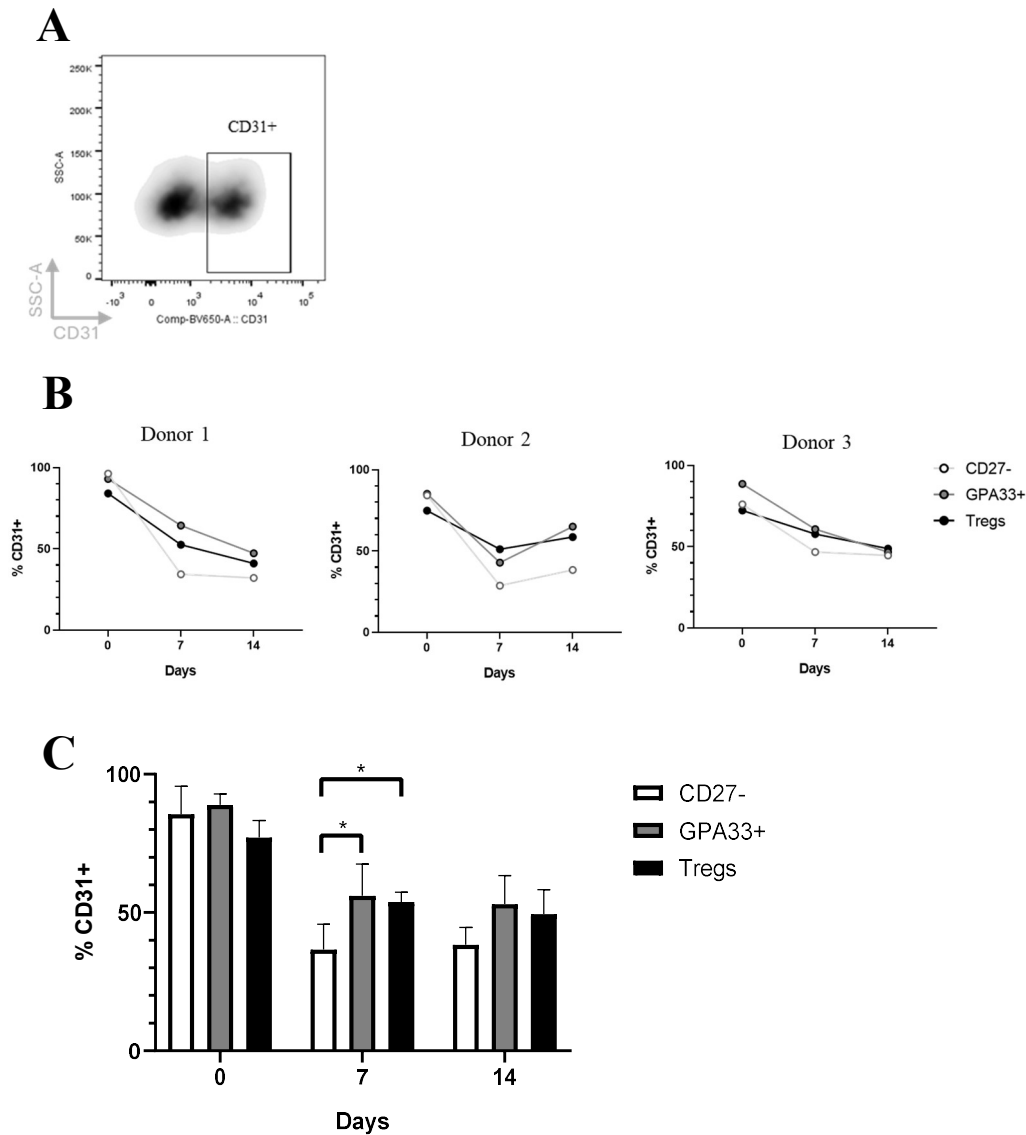


Figure 15. Analysis of CD31 expression on $CD4^+CD25^+$ cells. (A) Representation of $CD31^+$ gating on $CD25^+$ cells. (B) Percentage of CD31 expression in $CD25^+$ cells at different culture time points. (C) Comparison between percentage of $CD31^+$ cells between Treg subtypes, for each time point.

4.4.4 CD45RA and CD45RO expression

CD45RA is a T cell naivety marker, while CD45RO is associated with a memory phenotype. The expression of these surface markers in $CD25^+$ cells was then studied to gain an insight into the developmental state of the different Treg subtypes over the culture period. $CD25^+$ cells were gated into $CD45RA^+$ or $CD45RO^+$, according to Figure 16A.

As shown in Figure 16B, $CD27^-$ Tregs expressed low levels of CD45RA and high levels of CD45RO for every tested time point.

$GPA33^+$ Treg and bulk Treg subsets presented an overall increase of the percentage of $CD45RO^+$ cells during expansion and the percentage of $CD45RA^+$ cells was

conversely decreased. GPA33⁺ Tregs exhibited a high percentage of CD45RA⁺ cells on day 0, with 75.8%, 69.4%, and 76.9% for donors 1-3, respectively. This percentage decreased drastically on day 7 to 18.9%, 2.7% and 6.7%, respectively. On day 14, the percentage of CD45RA⁺ cells was similar than that on day 7. Bulk Tregs presented identical percentage of CD45RA⁺ and CD45RO⁺ cells on day 0 for donor 1, while 37.1% and 41.4% of CD45RA⁺ for donors 2 and 3, respectively. On day 14, the percentage of CD45RA⁺ cells was similar than that on day 7.

On day 0, the percentage of CD45RA⁺ cells was significantly lower in CD27⁻ Tregs and significantly higher in GPA33⁺ Tregs (Figure 16C). On both days 7 and 14, this percentage was similar for the three Treg subtypes.

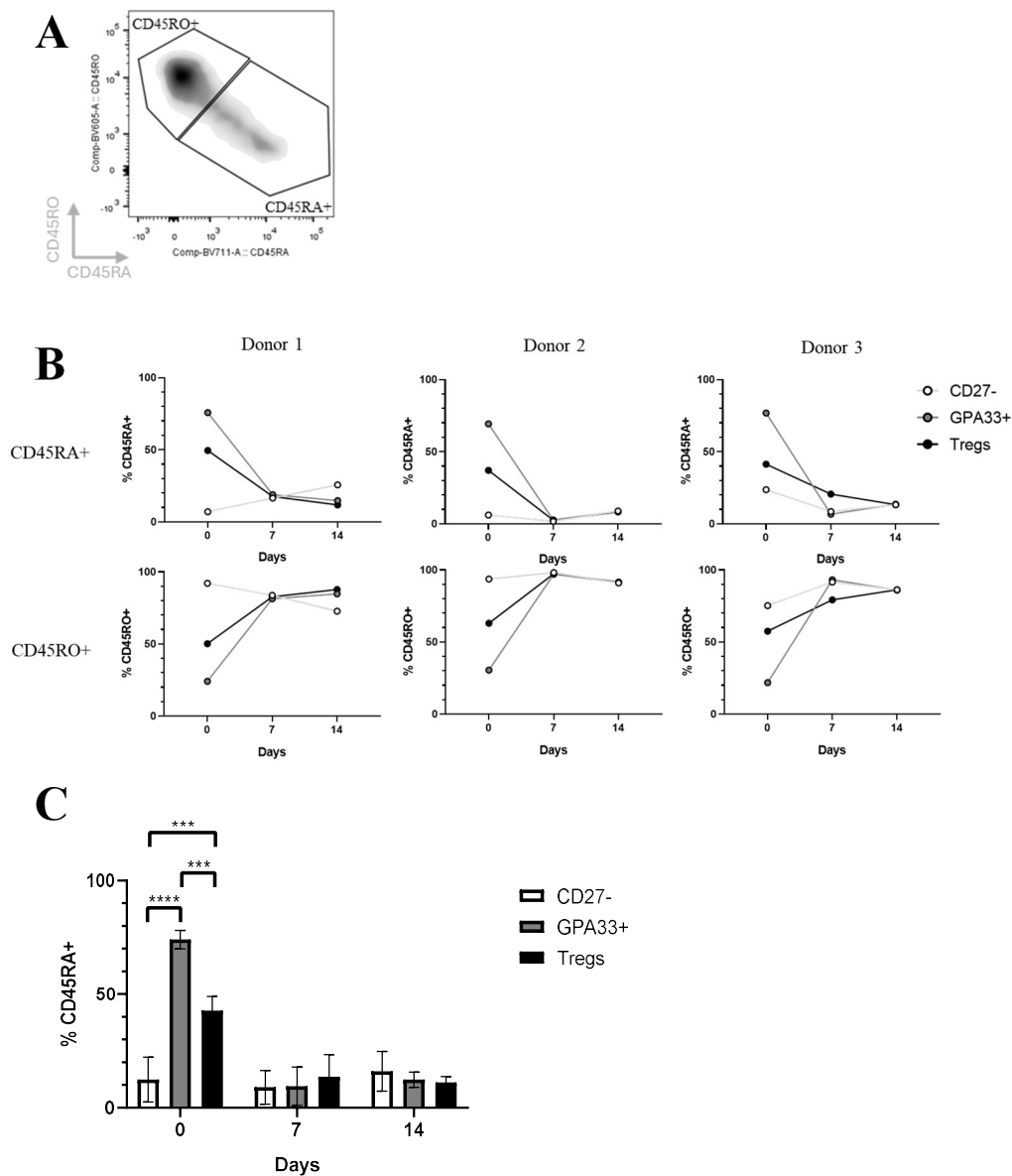
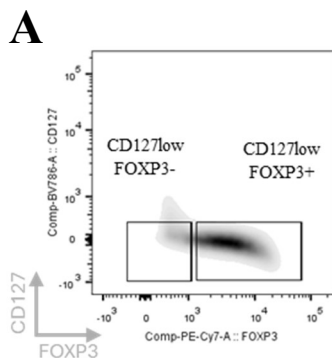


Figure 16. Analysis of CD45RA and CD45RO expression in CD25⁺ cells. (A) Representation of CD45RA⁺ and CD45RO⁺ gating, for CD25⁺ cells. (B) Percentage of CD45RA and CD45RO expression in CD25⁺ cells at different culture time points. (C) Comparison between percentage of CD45RA⁺ cells between Treg subtypes, for each time point.

4.4.5 FOXP3 expression

To study the expression of the transcription factor FOXP3 in Tregs of the three tested subtypes, CD25⁺ cells were gated according to the expression of CD127 and FOXP3, into two subsets: CD127^{low}FOXP3⁻ and CD127^{low}FOXP3⁺ (Figure 17A). Data shown in Figure 17B reveals that for donor 1, the three Treg subsets presented low levels of CD127^{low}FOXP3⁺ and CD127^{low}FOXP3⁻ cells on day 0, meaning that the majority of the CD25⁺ cells were CD127^{high}. On day 7, the percentage of CD127^{low}FOXP3⁺ cells increased drastically, and it slightly decreased on day 14. For donors 2 and 3, bulk and GPA33⁺ Tregs presented more than 80% of CD127^{low}FOXP3⁺ cells on day 0 and this percentage was consistent after 7 days and 14 days culture, while for CD27⁻ Tregs, the percentage increased.

The percentage of CD127^{low}FOXP3⁻ cells for donor 1 increased for the three Treg subtypes both on day 7 and on day 14, especially for CD27⁻ Tregs, reaching the value of 40.7% (and it was below 0.3% on day 0). For donors 2 and 3, GPA33⁺ and bulk Tregs expressed a low percentage of CD127^{low}FOXP3⁻ for these Treg subtypes on days 0, 7 and 14. However, CD27⁻ Treg subset exhibited a distinct expression pattern, with 85.5% and 43.8% of CD25⁺ cells being CD127^{low}FOXP3⁻ on day 0 for donors 2 and 3, respectively. This percentage dropped to 23.3% and 2.6% on day 14, respectively.



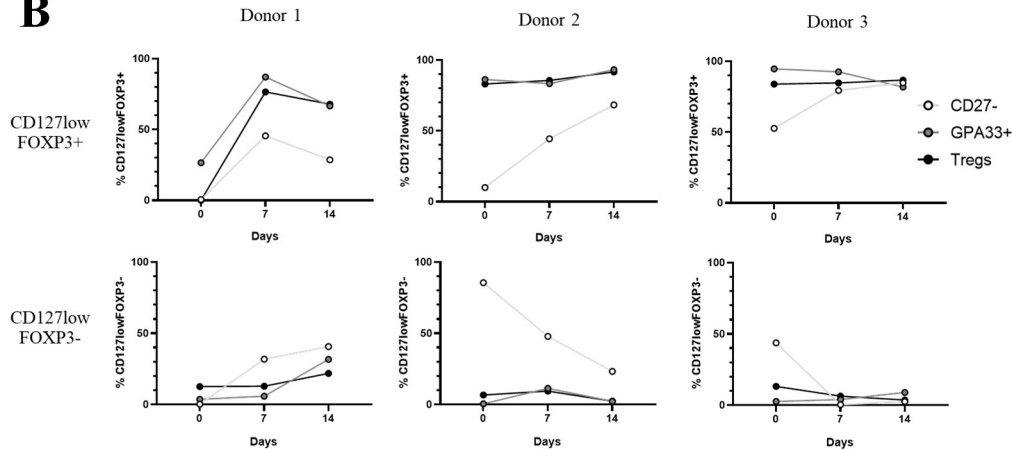
B

Figure 17. Analysis of FOXP3 expression in CD25⁺CD127^{low} cells. (A) Representation of CD127^{low}FOXP3⁻ and CD127^{low}FOXP3⁺ gating on pre-gated CD25⁺ cells. (B) Percentage of CD127^{low}FOXP3⁺ and CD127^{low}FOXP3⁻ cells in CD25⁺ cells at different culture time points.

4.4.6 CD27 expression

CD27 expression was analyzed on pre-gated CD25⁺ cells for CD27⁻ Tregs and bulk Tregs. The pre-gated CD25⁺ cells were further gated in CD127^{low}CD27⁻ cells, as shown in Figure 18A. The percentage of CD127^{low}CD27⁻ in CD25⁺ cells was plotted for the three donors, as shown in Figure 18B.

Bulk Tregs from the three tested donors showed lower percentage of CD127^{low}CD27⁻ cells on day 0 in comparison to CD27⁻ Tregs. A low percentage remained on both days 7 and 14, though it was slightly increased for donor 1 on days 7 and 14.

In contrast, CD27⁻ Tregs from donors 2 and 3 exhibited high percentages of these cells on day 0 (95.4% and 87.0%, respectively), but this percentage drastically decreased to 23.0% and 6.9% on day 14, respectively. For donor 1, the percentage of CD127^{low}CD27⁻ cells on day 0 was residual, it drastically increased 56.9% on day 7, and then slightly decreased to 39.8% on day 14.

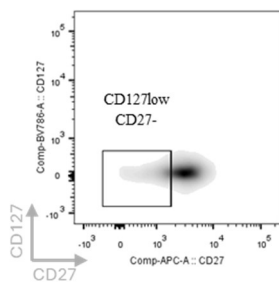
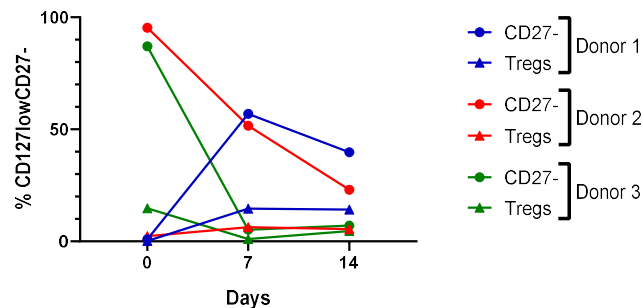
A**B**

Figure 18. Analysis of CD27 expression in CD25⁺CD127^{low} cells. (A) Representation of CD127^{low}CD27⁻ gating, for CD25⁺ cells. (B) Percentage of CD127^{low}CD27⁻ cells within CD25⁺ cells at different culture time points.

4.4.7 GPA33 expression

The expression of GPA33 on the pre-gated $CD25^+$ cells for $GPA33^+$ and bulk Tregs was analyzed (Figure 19A). The percentage of $CD127^{low}GPA33^+$ in $CD25^+$ cells was plotted for the three donors, as shown in Figure 19B.

The result shows that a low percentage of $CD25^+$ were $CD127^{low}GPA33^+$ for both of the $GPA33^+$ and bulk Tregs, for the three donors and three time points.

Additionally, no relevant differences seem to be detected between $GPA33^+$ Tregs and bulk Tregs, except for donor 1, whose $GPA33^+$ Tregs expressed the highest amounts of GPA33 on days 7 and 14 among all tested groups (respectively 31.2% and 26.9% of $CD127^{low}FOXP3^+$ cells, versus 16.6% and 13.1% for bulk Tregs).

However, an additional anti-GPA33 PE antibody was tested on day 14 for donor 3. The result shows that the percentage of $CD127^{low}GPA33^+$ cells using the new GPA33-PE antibody was close to 100% for both $GPA33^+$ Tregs and bulk Tregs, unlike it was observed for the same donor using the previous antibody, GPA33-Alexa Fluor[®] 700 (4.9% and 3.8%, respectively). This argues for the low stability of the antibody conjugation with Alexa Fluor[®] 700, and further research is required to confirm this.

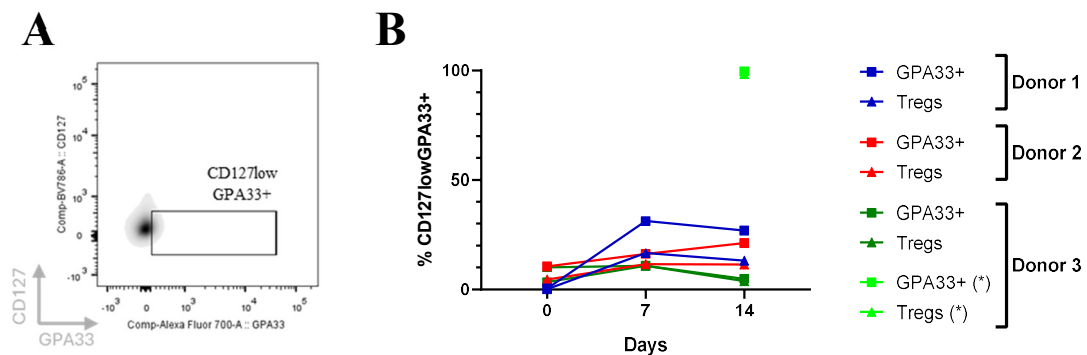


Figure 19. Analysis of GPA33 expression in $CD25^+CD127^{low}$ cells. (A) Representation of $CD127^{low}GPA33^+$ gating, for $CD25^+$ cells. (B) Percentage of $CD127^{low}GPA33^+$ cells in $CD25^+$ cells at different culture time points. “*” means that the respective group was analyzed using an anti-GPA33 PE antibody (instead of an anti-GPA33 Alexa Fluor[®] 700 antibody used for the other groups).

5 Discussion

5.1 Flow cytometric analysis of thymus Tregs markers

It was reported that CD25⁺ cell enriched thymocyte population contains a high percentage of Tregs (Bernaldo-de-Quiros et al., 2022). However, in the enriched CD25⁺ population there are both FOXP3⁺ and FOXP3⁻ cells. Additionally, it was proposed that CD25⁺FOXP3⁻ cells exist during early stages of the Treg development as one of the Treg progenitors (Dijke et al., 2016; Lio & Hsieh, 2008; Morgana et al., 2022). Thus it is important to study the difference between FOXP3⁻ and FOXP3⁺ cells within CD25⁺ population. Since FOXP3, the gold standard marker for Tregs, is a transcription factor, a strategy using surface markers to sort cells with high purity to represent FOXP3⁻ or FOXP3⁺ populations is needed.

Based on profound markers screening previously done in our lab, it was found that GPA33, CD27, CD62L and CCR7 might serve as the surface markers in correlation to FOXP3 expression (data not shown). As described in Section 1, all those four markers (GPA33, CD27, CD62L and CCR7) are playing significant roles for Treg functional activity. For this reason, GPA33, CD27, CD62L and CCR7 expression was analyzed together with the standard Treg markers (CD3⁺CD4⁺CD25⁺CD127-FOXP3⁺).

Data from Figure 9 shows that GPA33⁺ was considerably more expressed in FOXP3⁺ Tregs than in FOXP3⁻ Tregs, which means that GPA33⁺ cells could represent a FOXP3⁺ population for thyTregs, as it was previously proposed. In fact, a representative population of FOXP3⁺ cells expressed this surface marker (around 12-20%). However, looking into the FOXP3⁻ population, some of these cells also expressed GPA33⁺ (and for example, for donor 2, the percentage of expression was 7.58%). This means that, even though a correlation could be established between the positive expression of GPA33 and FOXP3 in thymus-derived Tregs, the sorted GPA33⁺ cells might not represent a pure FOXP3⁺ population.

Regarding CD27 expression, 14-24% of FOXP3⁻ Tregs were CD27⁺ and less than 0.5% of FOXP3⁺ cells expressed this marker, meaning that a cell subpopulation sorted on CD27⁺ population would represent a FOXP3⁻ subset. Additionally, this sorted subpopulation should theoretically be more pure than the FOXP3⁺ subpopulation obtained after GPA33-based cell sorting.

Moreover, the percentage of CD62L⁻ cells was higher in FOXP3⁺ cells (17-32% of these cells were CD62L⁻), in opposition to the initial referred hypothesis. However, 14-18% of FOXP3⁻ cells were also lacking the expression of this marker, so a subpopulation sorted based on the absence of CD62L expression would not be pure, due to its similar expression in FOXP3⁺ and FOXP3⁻ cells (for example, for donor 3, this marker was expressed for 17.0% of FOXP3⁺ Tregs and 14.2% of FOXP3⁻ cells).

Similarly, a CCR7⁻ population was also unexpectedly more representative in FOXP3⁺ Tregs, but the percentage of expression was below 10% for every tested groups. Besides that, clear differences of expression were not observed for every donor, with 4.9% of FOXP3⁺ cells and 3.3% of FOXP3⁻ cells from donor 3 lacking CCR7 expression, for example.

For these reasons, CD62L and CCR7 were excluded from the staining panel for cell sorting using FACS. In fact, results regarding these markers were not expected since secondary lymph nodes typically present a high pool of naïve Tregs (Eagar & Miller, 2022; Khantakova et al., 2022). However, it would be very useful to conduct further research to analyze why these CD4⁺FOXP3⁺ Tregs presented a higher proportion of cells with a typical Teff memory cell phenotype and lacking the expression of those important secondary lymph nodes homing receptors.

Taking all these findings in consideration, a cell sorting gating strategy was defined as shown in Figure 10, with CD3⁺CD4⁺CD8⁻CD25⁺CD127^{low} isolated cells representing bulk Tregs. Furthermore, in an independent cell sorting procedure, the use of an additional gating step led to the sorting of this subpopulation into CD27⁻ Tregs and GPA33⁺ Tregs. However, for donor 1, the gating step of CD25⁺CD127^{low} cells was mistakenly excluded, meaning that bulk Tregs were directly isolated based on CD3⁺CD4⁺CD8⁻ expression, and consequently, the two other Treg subpopulations were also not sorted according to the positive expression of CD25 and a low expression of CD127. This could have led to different results for this donor, mainly regarding cell fold expansion and phenotype, as it will be further discussed in the respective sections.

5.2 *In vitro* expansion of thymus-derived Tregs

Regarding Treg fold expansion, cells showed a limited growth between day 0 and day 4 of culture, as it was expected. Before being seeded in a well plate, cells were sorted using FACS, which was a very harsh process for them, even more after they were washed

in the end. For these reasons, after cells were plated, they needed some days to recover from these stress and reach their full expansion potential (Pfister et al., 2020).

Cells were seeded at a concentration of 0.5 Mio/mL, and even though they were stimulated with T cell TransAct on the seeding day, it was expected that cells death surpassed their expansion in the first few days, leading to a drop of the fold expansion value. After that, this value was expected to start growing around day 2 or 3, when cells were already functionally recovered from all the stress and already got their full expansion capacity. However, this was not seen in this project, since the first cell counting timepoint after the day of seeding was on day 4, but this explanation is in accordance with the results provided on Figure 11C, since cells on day 4 showed a fold expansion value close to 1, possibly because this value dropped initially and then cells were recovering their growth capacity. The only exception was observed for CD27⁻ Tregs from donor 1, with a very low fold expansion value at this timepoint. However, as it was already mentioned, these cells might not represent pure Tregs after sorting. Moreover, they expressed low amounts of FOXP3 during the first week of expansion (Figure 17B), and since this is a transcription factor associated with Treg *in vitro* activation, it is not surprising that they were not growing yet at this timepoint (Duhén et al., 2012; Marson et al., 2007; Y. Zheng et al., 2007). This also explains why this Treg subpopulation presented the most limited overall fold expansion for the three tested donors.

Furthermore, from day 4 to day 7, cells showed an exponential growth, since they already achieved their exponential expansion phase, and during this period the majority of the tested groups reached their highest expansion rate, which can be seen by the highest slope of the plot. On day 7, bulk Tregs already presented the highest fold expansion among the three donors, while CD27⁻ Tregs had a lower expansion rate. This order did not change during the second week of expansion, with little differences regarding the fold expansion pattern of the three tested subpopulations for each donor.

From day 7 to day 9, the fold expansion value for all the tested groups was increasing slightly lower. This could be due to the long period had passed after they were stimulated with TransAct on day 0, thus these cells were losing their expansion capacity.

For this reason, a cell restimulation with TransAct at the same concentration (1:100 (v/v)) at day 9 promoted an increase of the expansion rate of almost every tested group until day 11 (except CD27⁻ Tregs from donor 2) (MacDonald, Ivison, et al., 2019). With this restimulation, cells became active again, reaching an exponential growth, with the

respective slope being more similar to the observed for the period between days 4 and 7 of culture.

Lastly, from day 11 to day 14, cells still exhibited great expansion rates, with some groups showing a slight growth reduction, while others kept expanding at the same rate as observed right after cell restimulation.

After 14 days of culture, CD27⁻ Tregs presented the lowest overall fold expansion values for all the three donors, similarly to the data already observed on day 4 for donors 1 and 2, and on day 7 for donor 3. This was possibly related to the absence of FOXP3 expression (Figure 17B), since FOXP3 plays an important role for Treg *in vitro* activation (Duhon et al., 2012; Marson et al., 2007; Y. Zheng et al., 2007). On the other side, bulk Tregs showed higher expansion after 14 days when compared to GPA33⁺ Tregs, and that was already visible on day 4. However, data in Figure 9B shows that no big difference was observed for FOXP3 expression between these two groups, even though the GPA33⁺ Tregs was expected to represent a FOXP3⁺ population. Further studies are required to address the reason of this slower growth of sorted GPA33⁺ Tregs.

Finally, comparing the obtained data from the three donors, cells exhibited different expansion capacity, possibly due to donor variation. Cells from donor 1 exhibited the lowest expansion rate, which could be partially because of the different gating used for cell sorting, so that the sorted cells might include cells with less expansion capacity, especially for CD27⁻ Tregs.

5.3 Suppressive capacity of thymus-derived Tregs

Since FOXP3 is associated with Treg functional capacity, it would be expected that cells expressing higher amounts of FOXP3 were more suppressive. (Hori et al., 2003; Kondělková et al., 2010; Sjaastad et al., 2021; Vanhanen et al., 2018). This means that GPA33⁺ Tregs should theoretically be the most suppressive subpopulation, followed by bulk Tregs and CD27⁻ Tregs. However, the obtained data did not confirm this hypothesis, as shown in Figure 12.

On day 7, the three tested subpopulations showed a similar tendency regarding Teff fold expansion and proliferation, with an increase of Treg concentration promoting a decrease of Teff proliferation, even for lower Treg-to-Teff ratios. More specifically for donor 1, GPA33⁺ Tregs were more suppressive than bulk Tregs (at 1:32 and 1:16 ratios).

For donor 3, CD27⁻ Tregs seem to represent the most suppressive population for Treg-to-Teff ratio of 1:16.

On day 14, the suppression trend of all tested donors was similar to the one on day 7. However, a different suppression capacity was seen between those three tested Treg subtypes and the three donors. For donor 1, bulk Tregs were the least suppressive cells, and CD27⁻ Tregs showed the highest suppressive capacity. Regarding donor 2, GPA33⁺ Treg subtype were the most suppressive one, while for donor 3, CD27⁻ Tregs presented the lowest suppression capacity for 1:32 ratio.

Comparing between Tregs from day 7 and 14 after expansion, there is no clear suppression difference. Regarding different donors, cells from donor 2 seem to be the least suppressive ones, with the need of a higher Treg concentration to suppress Teff expansion. However, Teff control without Tregs also showed higher fold expansion and proliferation values for this donor, which indicates that the Teff cells were more activated. This could explain the need for a higher Treg-to-Teff ratio to suppress Teffs. Moreover, it is also important to note that a pipetting error was committed for both days 7 and 14 for the donor 1 (a serial dilution performed with 20% more volume of Treg suspension), leading to the seeding of more Tregs in each well for all conditions. This means that the real Treg-to-Teff ratios for this donor were higher than represented, which might have contributed to a slightly enhanced suppressive capacity of on day 7, even though no clear differences were observed on day 14.

Overall, the three tested Treg subpopulations did not show obvious differences regarding their suppressive capacity, for the three different donors, even though CD27⁻ Tregs expressed lower levels of FOXP3⁺. This could be due to the development of other cell subsets during expansion of CD27⁻ Tregs, e.g. CD25⁺CD8⁺ and CD25⁺CD4⁺CD8⁺ (Figure 13). Further functional studies about those two emerged cell types are needed to confirm this hypothesis.

Additionally, it was surprising that Teff control samples with TransAct stimulation showed so different results for distinct donors and timepoints, even when the same TransAct batch and the same Teff cell donor were used. For this reason, it would be important to troubleshoot this problem with the company, in order to understand what could have caused these differences. Alternatively, different T cell stimulators could be used instead, such as Dynabeads Treg Xpander or CD3/CD28/CD2 T Cell Activator (MacDonald, Ivison, et al., 2019).

5.4 Phenotypic profile of thymus-derived Tregs

In addition to the backbone panel of Tregs ($CD3^+CD4^+CD25^+CD127^{low}FOXP3^+$), CD31, CD45RA and CD45RO were analyzed for all the three sorted Treg subtypes: bulk Tregs, GPA33⁺ Tregs and CD27⁻ Tregs, directly after sorting, 7 days and 14 days post expansion. Moreover, CD27 and GPA33 were also included in order to study the sorting efficiency and the correlation of those two markers with the others. The results show that those three Tregs subtypes exhibited different phenotypes not only in the composition of CD4⁺, CD8⁺ and CD4⁺CD8⁺ cells, but also regarding the expression levels of all the remaining markers. This already highlights that those three subtypes have a significant difference between them.

5.4.1 CD4 and CD8 expression

Directly after sorting, all the three Treg subtypes were mostly CD4⁺CD8⁻, for the three tested donors (>90%). However, after expansion for 7 or 14 days, other two populations appeared: CD4⁻CD8⁺ and CD4⁺CD8⁺.

This was in accordance with previous findings (Martín-Gayo et al., 2010; Nunes-Cabaco et al., 2011; Vanhanen et al., 2020) showing that some thymus-derived Tregs developed a subset of CD4⁺CD8⁺ cells within some days of expansion, but these cells had a lower *in vitro* expansion rate than CD4⁺ Tregs (which might help to explain why on day 14 this population was not as representative as on day 7). Until now the suppressive function of these double positive cells is not yet fully addressed. Some previous data suggested that they could be precursors for CD4⁺ Tregs (Bremer et al., 2021; Martín-Gayo et al., 2010). However, more studies are still required to further confirm this hypothesis. Bulk Tregs and GPA33⁺ Tregs showed similar compositions, which might be correlated with their similar FOXP3 expression. Additionally, the CD8⁺ population that appeared largely in CD27⁻ Tregs reduced even more the CD4⁺ purity among the CD3⁺ cells. This data suggests that FOXP3⁻ Tregs are more prone to differentiate into CD8⁺ T cells after expansion. A sophisticated study on those CD8⁺ cells derived from CD27⁻ Tregs is needed to further address its function, and then decide whether FOXP3⁻ cells should be excluded in the sorting of pure thyTregs.

These results might be related to the plasticity of the thymus-derived Tregs, which led to phenotype alterations during the expansion, with the development of CD8⁺

CD4⁺CD8⁺ cell subsets, after starting with a pure CD4⁺ population on day 0 (Qiu et al., 2020).

5.4.2 CD25 expression

Some studies reported that a single-step CD25⁺ enrichment was sufficient to isolate a high percentage of CD4⁺CD25⁺FOXP3⁺ Tregs (Bernaldo-de-Quiros et al., 2022). However, in this study, the lack of a selection of CD25⁺CD127^{low} cells (donor 1) during the sorting of CD25⁺ pre-enriched thymocytes resulted in a low CD25⁺ percentage (<35% on day 0) (Figure 14). Thus, an efficiency test of CD25 enrichment before sorting is required. After 7 days expansion, >95% cells were CD25⁺ for this donor, which was expected as a result of TransAct stimulation. After 14 days, the percentage of CD25⁺ cells significantly decreased in CD27⁻ Tregs.

When the CD25⁺CD127^{low} gating was included during the sorting for donors 2 and 3, the percentage of CD25 expression was high on day 0 (close to 100%), confirming that those two sorting procedures were efficient. On days 7 and 14, the percentage of CD25⁺ cells dropped in CD27⁻ Tregs for donor 2. The reason for the decrease of CD25 expression in CD27⁻ Tregs over time still needs to be further addressed.

When analyzing those CD8⁺ cells within CD27⁻ Tregs, it was confirmed that their CD25 expression was high on day 7 but it decreased on day 14 (data not shown).

Some published data reported that CD8⁺FOXP3⁺ Tregs also express CD25 (Niederlova et al., 2021; Vieyra-Lobato et al., 2018). For this reason, it is important to analyze the FOXP3 expression of those CD8⁺ cells (discussed in Section 5.4.5).

5.4.3 CD31 expression

CD31 is expressed in Tregs recently migrated from thymus (Booth et al., 2010; Santosh Nirmala et al., 2024), which explains the high levels of expression of this marker on day 0, when the tested Tregs had not undergo expansion cycles. As expected, CD31 expression dropped on day 7, especially for CD27⁻ Tregs. On day 14, CD25⁺ cells from both donors 1 and 3 showed the same tendency to express a lower percentage of CD31, but for donor 2, this percentage unexpectedly slightly increased. This could be due to the independent staining procedures and the gating-derived variations.

5.4.4 CD45RA and CD45RO expression

The analysis of CD45RA and CD45RO expression can provide useful information regarding the developmental state of thyTregs. CD45RA is usually considered as a T cell naivety marker, but during the complex process of Treg development from thymocytes, its expression starts in a later developmental state than CD45RO, which is a marker related to an experienced phenotype (Fujii et al., 1992; Santosh Nirmala et al., 2024; Valmori et al., 2005).

Our data shows the similar result that on day 0 a large proportion of cells expressed CD45RO. CD27⁻ Tregs presented the lowest proportion of CD45RA⁺ cells, supporting the previous hypothesis that those CD27⁻ Tregs, representing FOXP3⁻ Tregs, are in an earlier developmental state when comparing to FOXP3⁺ Tregs. Conversely, GPA33⁺ Tregs expressed more CD45RA, suggesting a more advanced developmental state.

On days 7 and 14, most of the cells were CD45RA⁻, which was expected since those cells were already intensively stimulated via TCR signaling by T cell stimulator TransAct.

However, it will be very useful to analyze more intermediate timepoints (e.g. days 1 and 2), to check the thyTregs CD45RA/CD45RO developmental dynamics. To further gain a precise conclusion regarding Treg developmental state, more associated markers such as CD69 and CCR7 should be added into the study panel.

5.4.5 FOXP3 expression

For donor 1, the predominant CD127^{high} phenotype on day 0 can be explained by the lack of a selection of CD127^{low} cells during cell sorting, as already mentioned. However, upon activation, a large proportion of CD4⁺CD25⁺ cells became CD127^{low}FOXP3⁺ on day 7, but on day 14 this percentage slightly decreased. This data is similar to the results obtained for CD25 expression in this donor discussed in Section 5.4.2. This also suggests a correlation between the expression of CD25 and FOXP3, as two key factors for FOXP3⁺ Treg suppressive capacity, as it was already reported by several publications (Camperio et al., 2012; Chatenoud, 2011).

For donors 2 and 3, the three thyTreg subtypes were sorted based on a CD25⁺CD127^{low} phenotype. Thus GPA33⁺ and bulk Tregs showed a high percentage of FOXP3⁺ cells on day 0, and these values remained high after 7 and 14 days of expansion.

More specifically, GPA33⁺ Tregs seem to include slightly more FOXP3⁺ cells than bulk Tregs of donors 1 and 3 on day 0, but this difference decreased on 7 and disappeared on day 14. This can be explained by the correlation of GPA33⁺ to FOXP3⁺ as shown in Figure 9. The slightly higher percentage of FOXP3⁺ in GPA33⁺ Tregs might also help to explain their relatively higher suppression ability compared to bulk Tregs (Figure 12).

On the other side, CD27⁻ Tregs exhibited a low percentage of FOXP3⁺ cells for donor 2 on day 0. This is in correlation with CD27⁻ representing a FOXP3⁻ population. For donor 3, FOXP3 expression for CD27⁻ Tregs was unexpectedly higher than for donor 2 on day 0, probably due to the gating strategy applied for cell sorting of CD27⁻ Tregs, which might have led to the isolation of a non-pure FOXP3⁻ Treg subpopulation. For both of these donors, FOXP3 expression increased over time for CD27⁻ Tregs, as a result of Treg activation and expansion. However, the lower FOXP3 expression of CD27⁻ Tregs does not seem to match its higher suppressive capacity in donor 1 (day 14) and donor 3 (day 7) (Figure 12). A deeper analysis into the phenotype of these cells is needed to fully understand their suppressive function.

Summing up, when taking a look into CD4⁺ cells, it is interesting to notice that CD27⁻ Tregs (representing FOXP3⁻ Tregs) differentiated into FOXP3⁺ cells, but further studies are required to understand if these FOXP3⁻ Tregs are precursors for CD4⁺FOXP3⁺ Tregs.

Further analysis was also done for CD4⁺CD8⁺CD25⁺ cells, showing that on day 7, they expressed high levels of FOXP3 for both GPA33⁺ Tregs and bulk Tregs from the three donors, and for CD27⁻ Tregs from donor 3 (data not shown). For donors 1 and 2, CD27⁻ Tregs expressed low amounts of FOXP3, likewise observed for CD4⁺CD25⁺ cells. On day 14, high levels of FOXP3 were detected for GPA33⁺ and bulk Tregs from donors 2 and 3. Similarly, analysis was also done on CD8⁺CD25⁺ cells, and the proportion of FOXP3⁺ cells was low on day 7 and increased on day 14 (except for donor 1, whose cells expressed a lower percentage of FOXP3 on day 14, as also observed for CD4⁺CD25⁺ cells) (data not shown). These changes could have been influenced by the differentiation and activation status of these newly developed cells, which still requires further research.

5.4.6 CD27 expression

For donor 1, the major part of the sorted cells were CD127^{high} (as it was already explained in Section 5.4.5), thus both CD27⁻ Tregs and bulk Tregs presented a really low percentage of CD127^{low}CD27⁻ cells on day 0. On days 7 and 14, this percentage increased

largely for bulk Tregs and even more for CD27⁻ Tregs. This might be due to the activation with T cell TransAct and further studies are required to understand the correlation between their activation status and CD27 expression.

For donors 2 and 3, a high number of CD27⁻ cells was detected in CD27⁻ Tregs on day 0, as expected. A lower amount of these cells was detected for donor 3, which is in correlation with the higher percentage of FOXP3⁺ cells. A higher FOXP3 expression can also justify the lower CD27 expression on days 7 and 14 for these donors. On the other side, bulk Tregs showed a low percentage of CD27⁻ cells for every tested culture timepoint as expected, due to their high FOXP3 expression (Figure 17B).

Further analysis was done on the gated CD25⁺CD127^{low}CD27⁻ cells from the three donors (Appendix Figure 8). For donor 1, the percentage of FOXP3⁻ cells slightly increased for both groups on day 14 compared to day 7, reaching 78.3% for CD27⁻ Tregs. For this donor, the analysis of FOXP3 expression on day 0 was not possible since a low number of these cells was obtained (Figure 14B and Figure 18B). For donors 2 and 3, the percentage of FOXP3⁻ cells was high on day 0, but it surprisingly gradually decreased on days 7 and 14. It was expected that these CD27⁻ cells would keep a higher population of FOXP3⁻ cells, due to the correlation between these two populations confirmed by Figure 9. However, to fully understand this phenomenon, further studies will be required to address the correlation between CD27 and FOXP3 expression after stimulation.

5.4.7 GPA33 expression

The percentage of CD127^{low}GPA33⁺ cells among CD25⁺ cell were analyzed. The result shows that the percentage of these cells was very low (<15%) for both sorted GPA33⁺ and bulk Tregs on day 0, for all donors. On days 7 and 14, a similar percentage was observed, even though a slight increase was seen in GPA33⁺ Tregs for donor 1 on day 7 (31.2%) and for donor 2 on day 14 (21.2%) (Figure 19).

Data from Figure 17B shows that a high percentage of CD25⁺CD127^{low} cells was CD127^{low}FOXP3⁺ (except on day 0 for donor 1), but surprisingly no significant number of CD127^{low}GPA33⁺ cells was observed for these two thyTreg subtypes. Further analysis on CD25⁺CD127^{low}GPA33⁺ cells from donors 2 and 3 showed that they were mainly FOXP3⁺ (Appendix Figure 9), likewise observed for CD25⁺CD127^{low} cells (Figure 17B).

These results were not as expected for GPA33⁺ Tregs especially on day 0, right after they were sorted on GPA33⁺ population. Our study already showed that GPA33

expression was low in CD25⁺CD127^{low} thymocytes for both FOXP3⁺ (<20%) and FOXP3⁻ (<10%) populations (Figure 9), so it seems that the sorting based on GPA33 was not efficiently achieved.

During the sorting for the third donor, we noticed that GPA33 signal decreased in about 3 hours during the sorting procedure (4 °C) and vanished 1 day after sorting when the cells were kept at 4°C in the dark (Appendix Figure 10). This might indicate that the conjugation of the fluorophore with the antibody (GPA33-Alexa Fluor[®] 700) was not stable. Thus we included another antibody (GPA33-PE) for analyzing the day 14 sample for donor 3. The result showed that the percentage of GPA33⁺ cells was surprisingly more than 95% for both GPA33⁺ and bulk Tregs, in comparison with the staining with GPA33-Alexa Fluor[®] 700 antibody with percentages below 5%.

For this reason, further research should be carried out to confirm if this GPA33-PE antibody is suitable for sorting of GPA33⁺ cells with high purity, and thereafter perform phenotypical and functional studies for those sorted GPA33⁺ cells.

6 Conclusions and future perspectives

Summing up, this project showed that the incorporation of the surface marker CD27 into the conventional Treg isolation panel enabled thymocyte sorting into a FOXP3⁻ subpopulation, which exhibited distinct proliferation potential, phenotypic profile and developmental state in comparison to the FOXP3⁺ Tregs. The obtained data showed that other subpopulations such as CD8⁺ and CD4⁺CD8⁺ also emerged after expansion for CD27⁻ Tregs and that some of these cells developed into FOXP3⁺ cells, which might argue for their earlier developmental state in comparison with the FOXP3⁺ Tregs.

However, cells that were sorted based on GPA33⁺ expression had a similar phenotype and suppressive ability when compared to bulk Tregs, though with relatively less expansion capacity. The similar GPA33 and FOXP3 expression of those two sorted subtypes indicated the inefficient sorting of GPA33⁺ cells using the GPA33-Alexa Fluor[®] 700 antibody. A preliminary test of another antibody, GPA33-PE, led to a higher detection of the GPA33 expression. It will be important to further validate the GPA33 sorting in order to isolate a more pure FOXP3⁺ population and afterwards compare the sorted GPA33⁺ Tregs with bulk Tregs.

Furthermore, more surface markers should be screened regarding their suitability to be used for cell sorting of a FOXP3⁺ or a FOXP3⁻ Treg subset, in order to achieve a more pure thyTreg isolation.

Additional extensive studies such as functional and cytokine production assays for the newly developed CD8⁺ and CD4⁺CD8⁺ populations will be useful to understand their function and also thyTreg development, and then decide whether FOXP3⁻ cells should be excluded in the sorting of pure thyTregs for clinical translation.

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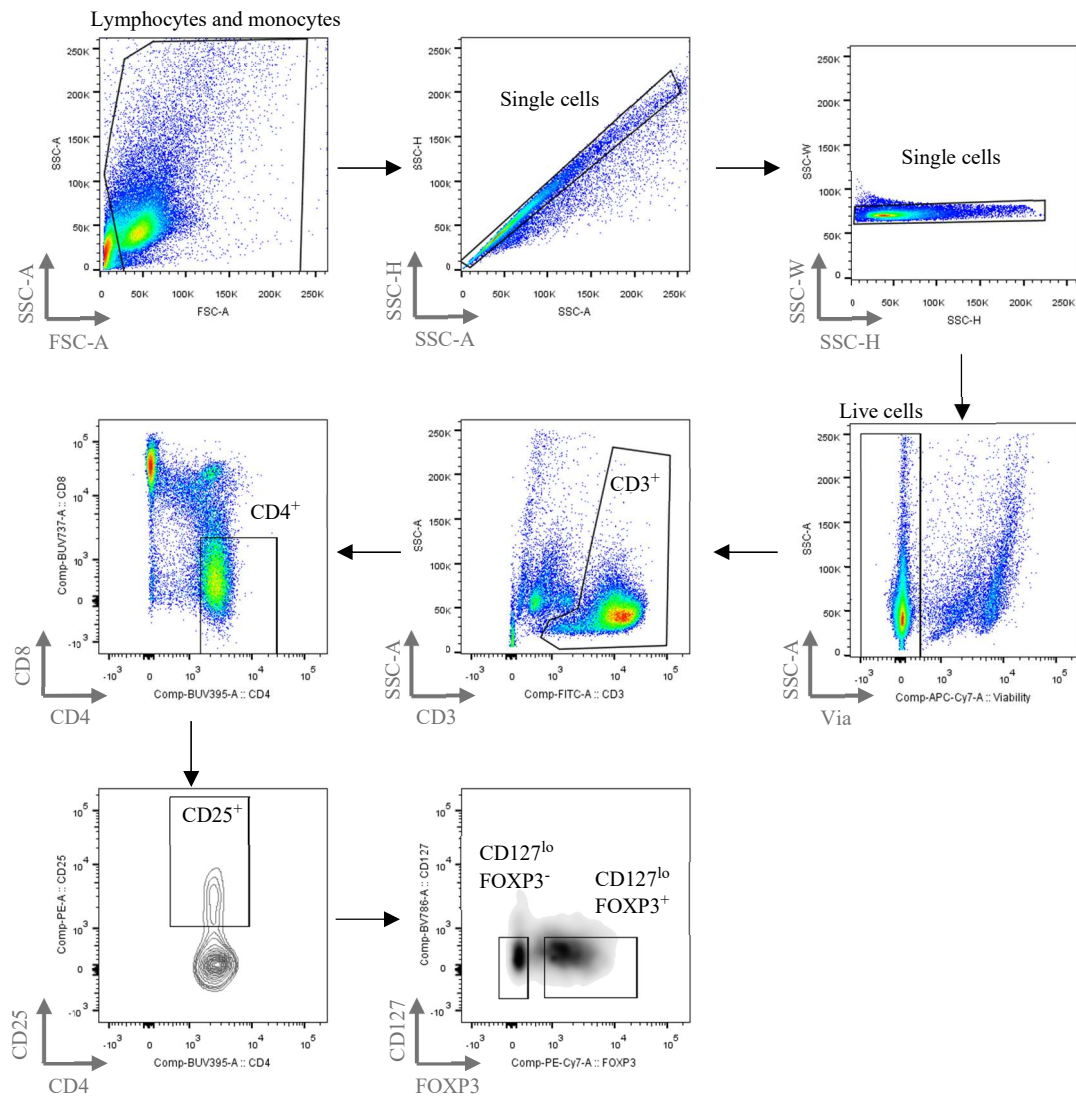
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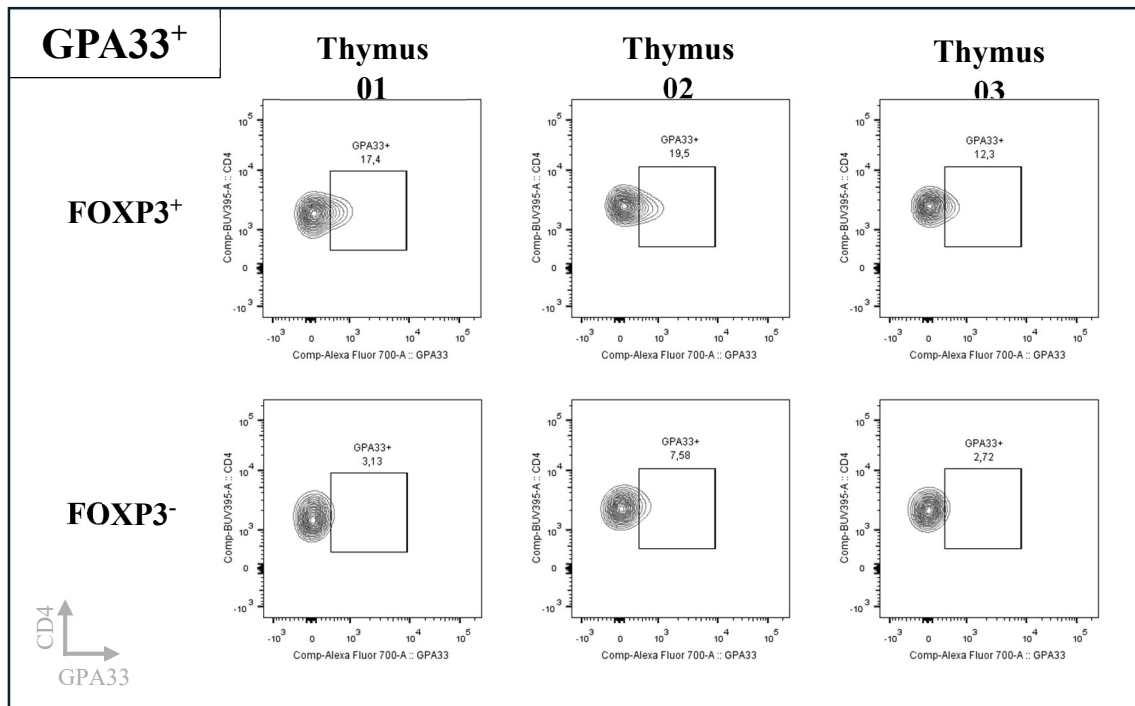
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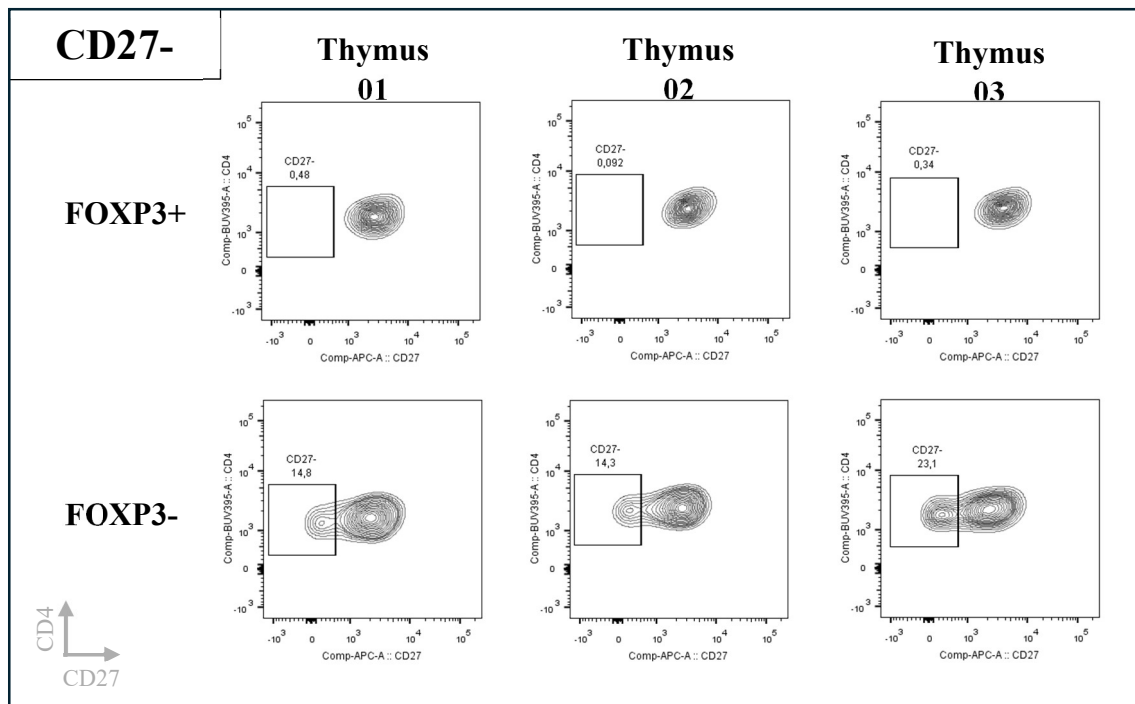
Appendix



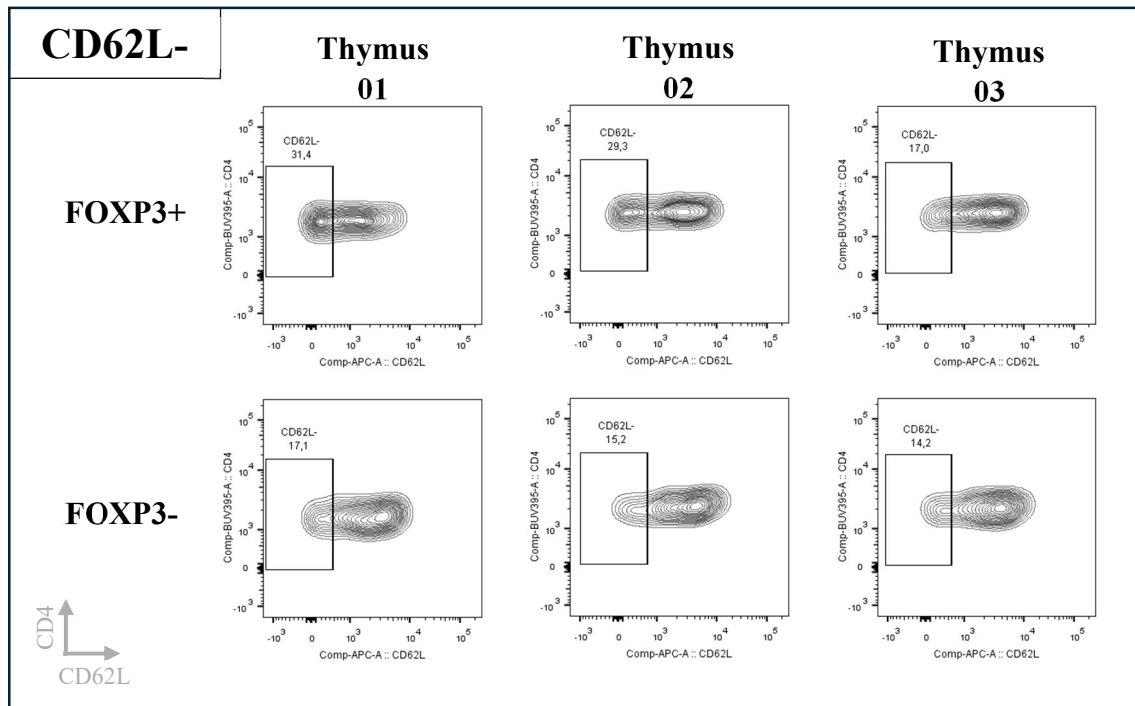
Appendix Figure 1. Treg gating strategy for markers screening.



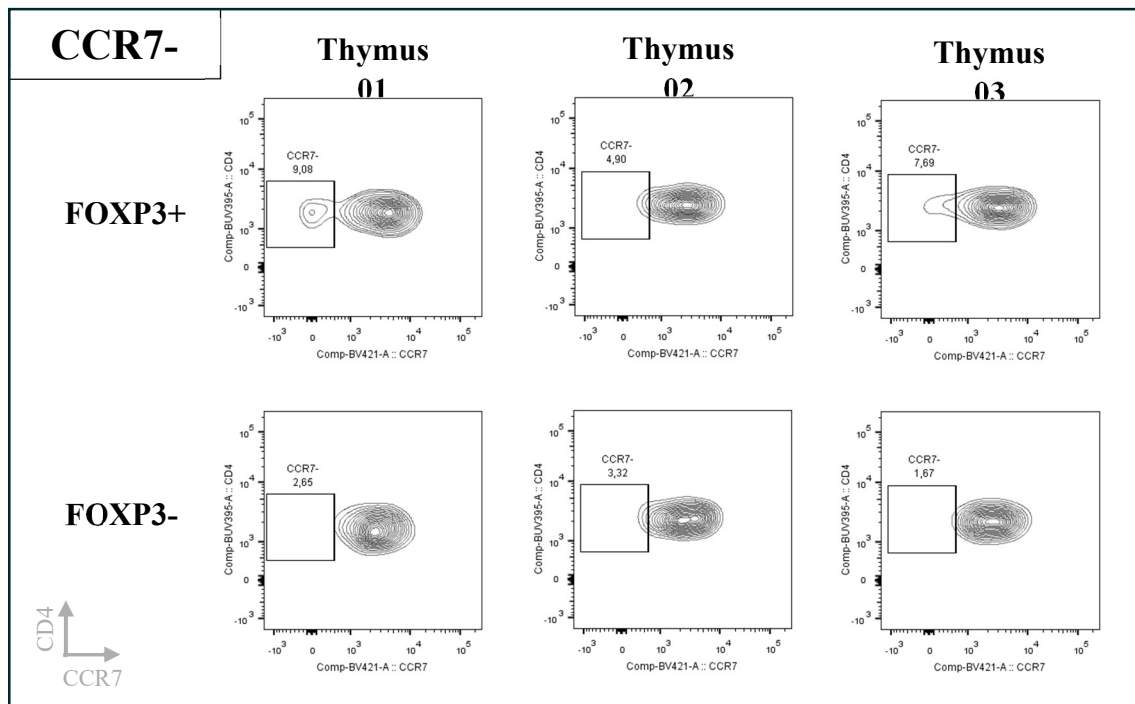
Appendix Figure 2. GPA33⁺ cells in CD127^{low}FOXP3⁺ and CD127^{low}FOXP3⁻ Treg subtypes.



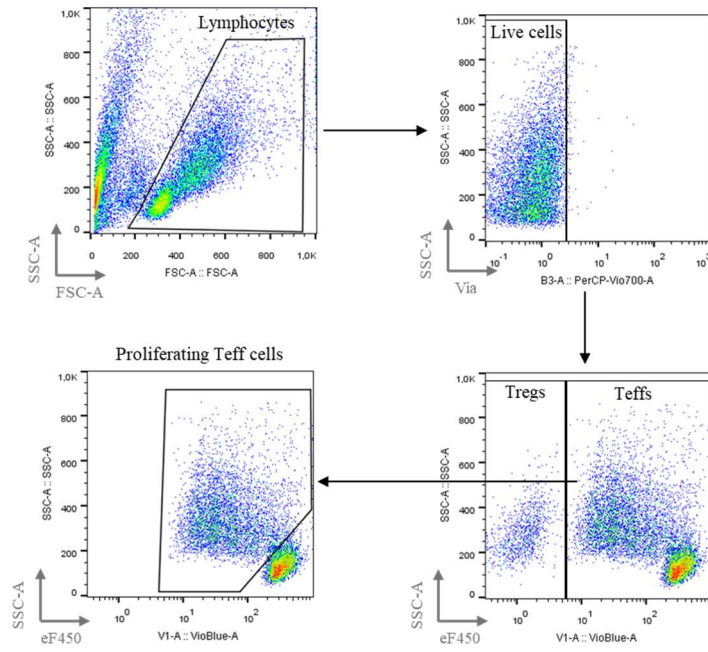
Appendix Figure 3. CD27⁻ cells in CD127^{low}FOXP3⁺ and CD127^{low}FOXP3⁻ Treg subtypes.



Appendix Figure 4. CD62L⁻ cells, on CD127^{low}FOXP3⁺ and CD127^{low}FOXP3⁻ Treg subtypes.

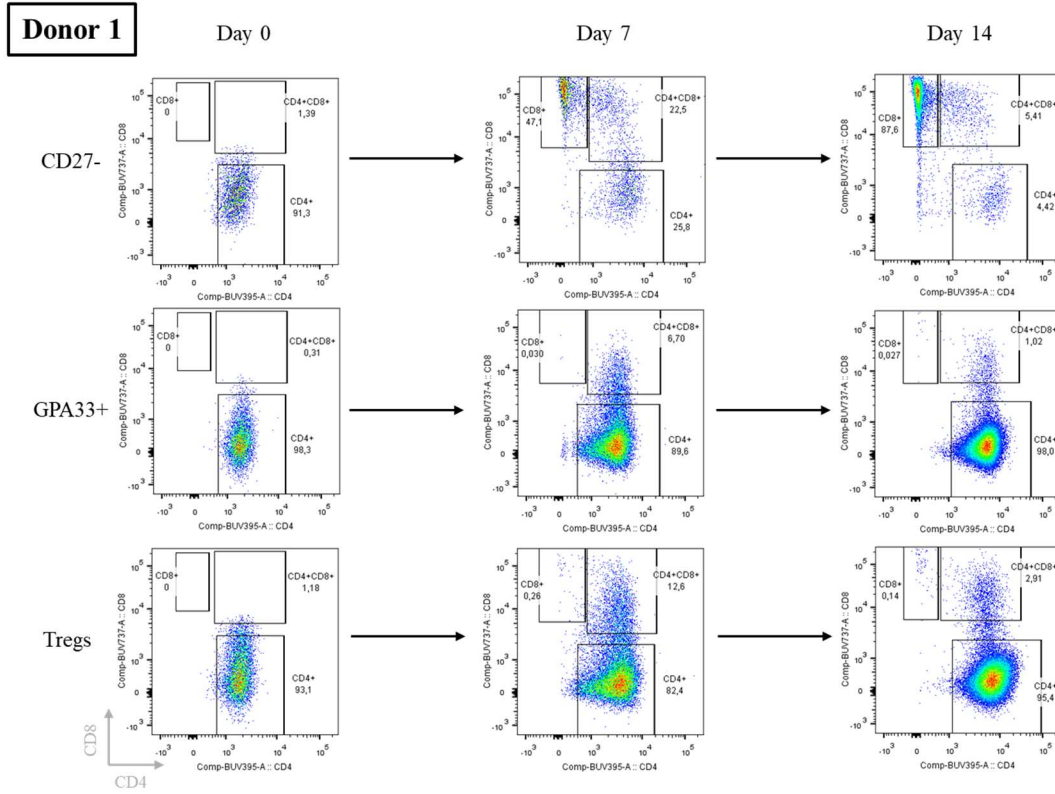


Appendix Figure 5. CCR7⁻ cells, on CD127^{low}FOXP3⁺ and CD127^{low}FOXP3⁻ Treg subtypes.

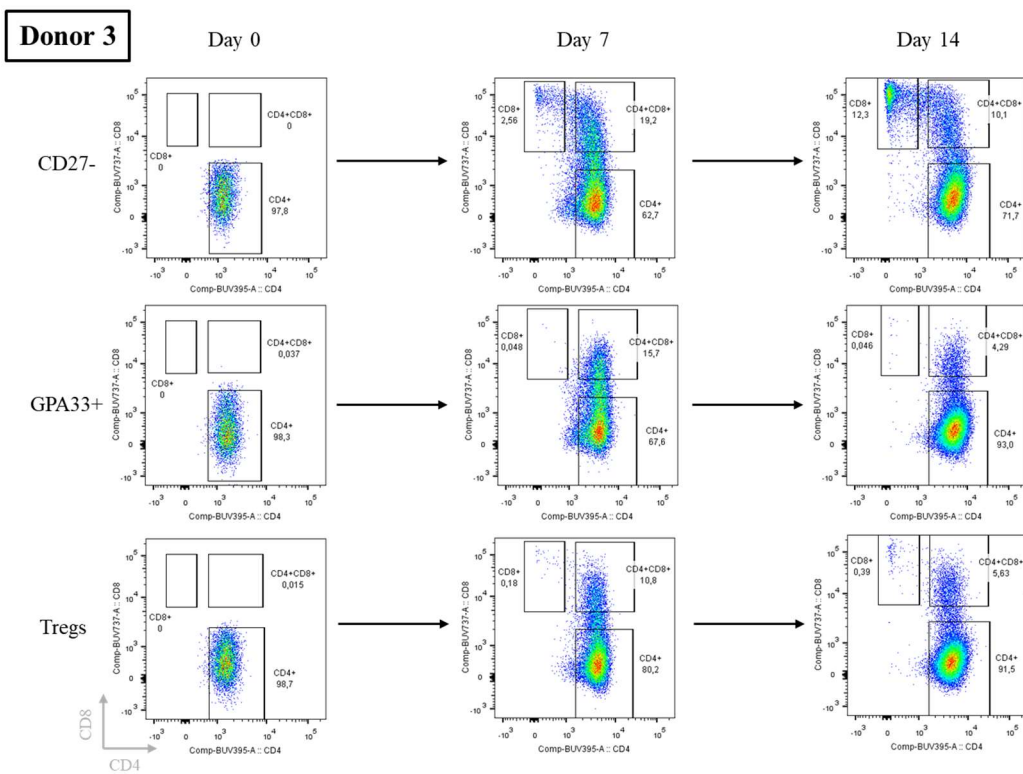


Appendix Figure 6. Gating strategy to obtain the number of Teff cells and the percentage of proliferating Teffs.

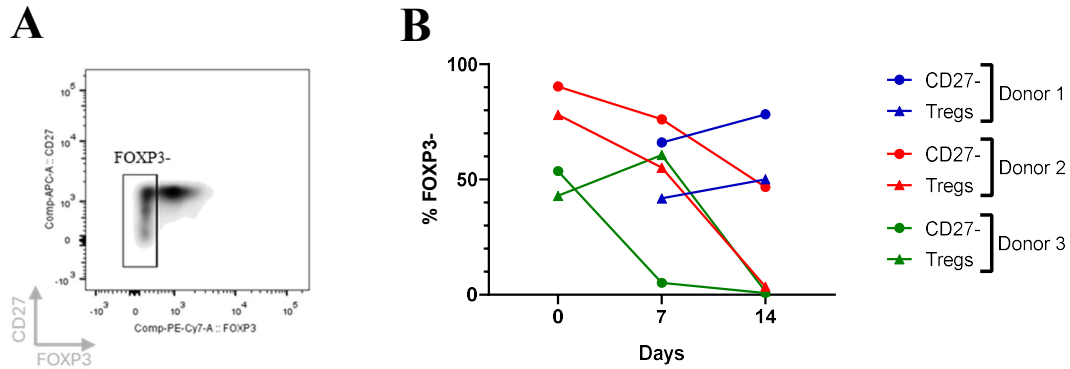
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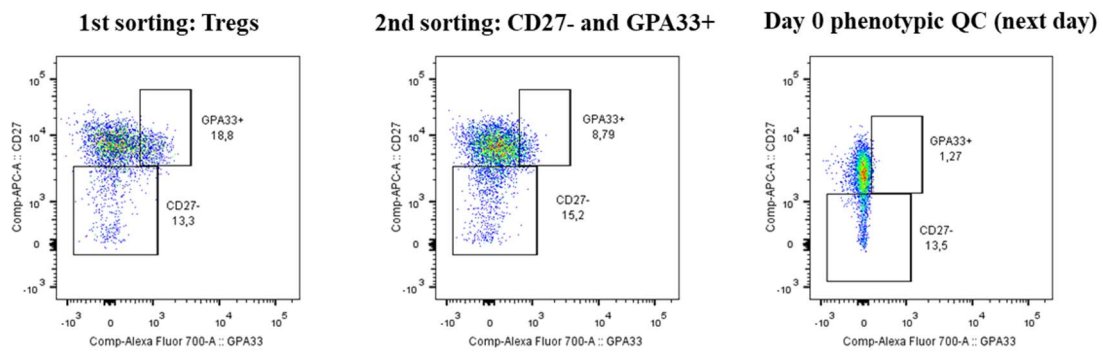
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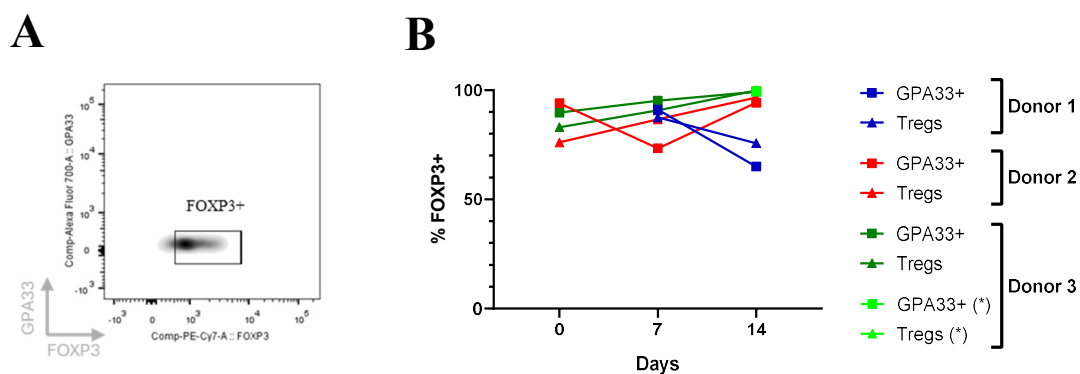
Appendix Figure 7. Gating on CD4⁺, CD8⁺ and CD4⁺CD8⁺ cells for phenotyping of sorted Treg subtypes, for (A) donor 1 and (B) donor 3 (gated on live CD3⁺ cells).



Appendix Figure 8. Analysis of FOXP3 expression in $CD25^+CD127^{low}CD27^-$ cells. (A) Representation of FOXP3- gating, for $CD25^+CD127^{low}CD27^-$ cells. (B) Percentage of FOXP3- cells in $CD25^+CD127^{low}CD27^-$ cells at different culture time points.



Appendix Figure 9. Comparison between the percentage of GPA33+ and CD27- Treg subpopulations in CD25-enriched cells (A) in the beginning of the first cell sorting step for bulk Tregs, (B) in the beginning of the second cell sorting step for CD27- Tregs.



Appendix Figure 10. Analysis of FOXP3 expression in $CD25^+CD127^{low}GPA33^+$ cells. (A) Representation of FOXP3+ gating, for $CD25^+CD127^{low}GPA33^+$ cells. (B) Percentage of FOXP3+ cells in $CD25^+CD127^{low}GPA33^+$ cells at different culture time points. “*” means that the respective group was analyzed using an anti-GPA33 PE antibody (instead of an anti-GPA33 Alexa Fluor® 700 antibody used for the remaining groups).

Appendix Table 1. Antibodies and dyes used for flow cytometry and FACS

Antigen/ dye	Conjugate/ channel	Flow cytometry volume / 1 Mio cells (µL)	FACS volume / 1 Mio cells (µL)	Volume for compensation (µL)
CD3	FITC	2	1	1
CD4	BUV395	2	1	1
CD8	BUV737	2	1	1
CD25	PE	4	2	1
	PE-Cy5	10	5	3
CD27	APC	10	5	3
	PE	-----	2,5	1
CD31	BV650	4	-----	1
CD45	V500	4	-----	1
CD45RA	BV711	2	-----	1
CD45RO	BV605	4	-----	1
CD62L	APC	1	-----	1
CD127	BV786	4	2	1
CCR7	BV421	2	-----	1
FOXP3	PE-Cy7	2	-----	1
GPA33	Alexa Fluor® 700	4	2	1
	PE	10	-----	3
eF780 viability dye	APC-Cy7	2	1	2

