UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

Expansion and in-depth characterization of antigen-specific regulatory T cells *in vitro* for subsequent clinical translation

Joana Bianchi Marques Bettencourt Gesta

Supervisor: **Doctor João Mascarenhas Forjaz de Lacerda**
Co-supervisor(s): **Doctor Rita Isabel Silva de Azevedo**
Doctor Cláudia Alexandra Martins Lobato da Silva

Thesis approved in public session to obtain the PhD Degree in
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RESUMO

A infusão de células T reguladoras (Treg) tem sido cada vez mais explorada como terapia para a doença do enxerto contra o hospedeiro (GvHD). No entanto, os ensaios clínicos a decorrer atualmente utilizam Treg policlonais, cuja especificidade é incerta. Devido ao vasto leque de especificidades apresentado por estas células, a sua infusão acarreta o risco de que o efeito do enxerto contra leucemia (GVL) seja suprimido simultaneamente com as respostas envolvidas na GvHD. Por esse motivo, a infusão de Treg específicas de antigénio (Ag-sp Treg) seria a solução mais adequada para este fim terapêutico, caso se verifique que a supressão mediada pelas Ag-sp Treg seja específica para a supressão de GvHD e preserve o efeito do GVL. Para obter Ag-sp Treg *ex vivo*, Treg derivadas de um dador são expandidas em cultura com células apresentadoras de antigénio profissionais (APC) que fazem a apresentação de antigénios alogénicos (alo-Ag) de outro dador. No entanto, ainda não há um protocolo definitivo para a expansão de Ag-sp Treg, até porque os protocolos actuais geralmente requerem a utilização de soro humano, o que pode dificultar a sua adaptação para uso clínico. Além disso, muitas vezes a especificidade das Ag-sp Treg resultantes destes protocolos não é clara.

Por estes motivos, o nosso propósito com este trabalho era estabelecer um protocolo definitivo para a expansão de Ag-sp Treg na ausência de soro humano, onde a potência da supressão por elas mediada, a sua especificidade e o seu fenótipo fossem caracterizados em pormenor.

No primeiro capítulo de resultados, focámo-nos em definir um protocolo para a expansão de Ag-sp Treg. Assim, começámos por escolher o protocolo mais adequado para a diferenciação e activação de células dendríticas derivadas de monócitos (moDC), para serem usadas como APC na expansão de Ag-sp Treg. Foi testado o efeito de diferentes meios de expansão e combinações de citoquinas no fenótipo das moDC e, consequentemente, no seu potencial para expandir Treg. De seguida, avaliámos as diferenças na expansão e função das Treg quando alo-Ag eram apresentados directa ou indirectamente pelas APC, quer durante a expansão de Treg e/ou durante os ensaios de supressão subsequentes (SA).

No segundo capítulo de resultados, queríamos esclarecer se diferentes condições de expansão ou de supressão influenciariam a potência e a especificidade, assim como o fenótipo, de Ag-sp Treg expandidas por apresentação directa de alo-Ag. Assim, realizámos experiências em que Ag-sp Treg foram expandidas com uma concentração elevada (100U/mL) ou baixa (10U/mL) de IL-2. A função de ambas as populações de Treg expandidas (eTreg) foi avaliada, separadamente, em SA em que células T convencionais (Tcon) ou células T CD8 foram usadas como respondedoras. Para estes SA, foram usadas como APC

as mesmas moDC que haviam sido utilizadas para a expansão de Treg e, em paralelo, testámos um leque de moDC de dadores com diferentes níveis de emparelhamento do complexo de histocompatibilidade (MHC) quer com o dador das APC de expansão, quer com o dador de Treg. A função de eTreg foi avaliada com base na sua capacidade de suprimir a proliferação das respondedoras em presença de cada tipo de APC, assim como por quantificação da concentração de citoquinas no sobrenadante dos poços dos SA. Desta forma, foi-nos possível obter uma representação bastante abrangente da função das Ag-sp Treg expandidas. Por fim, foram aplicadas técnicas de análise multidimensional para visualizar os fenótipos existentes em populações de Treg no Dia 0 (não expandidas) e após expansão. A utilização destes métodos de visualização permitiu que estimássemos a pureza das eTreg, e possibilitou também a descoberta de diferentes subgrupos fenotípicos dentro de eTreg, que poderão estar relacionados com diferentes mecanismos de supressão.

Concluindo, com base no trabalho descrito nesta Tese, propomos que a especificidade da supressão mediada por Ag-sp Treg depende de: potência das eTreg, em que uma maior potência de supressão pode estar relacionada com uma redução da especificidade; interacções entre Treg e APC, em que a afinidade do receptor das células T (TCR) para o MHC apresentado pelas moDC pode aumentar a supressão das respondedoras; e, por fim, de interacções entre as respondedoras e as APC, onde os sinais fornecidos pelas moDC podem levar a que as respondedoras se tornem resistentes à supressão.

Palavras-chave: Células T reguladoras (Treg); Expansão de Treg específicas de antigénio (Ag-sp Treg); Doença do enxerto contra o hospedeiro (GvHD); Ensaios de supressão; Análise multidimensional

ABSTRACT

The infusion of regulatory T cells (Treg) for the treatment of graft-versus-host-disease (GvHD) has been increasingly investigated. However, the current clinical trials are on the infusion of polyclonal Treg, which have unknown specificity. As a result of the broad specificity of the infused population, there is a risk of suppressing the graft-versus-leukaemia effect (GVL) together with the suppression of GvHD. The infusion of *ex vivo* expanded antigen-specific (Ag-sp) Treg may be more adequate for this clinical purpose, providing that these cells are shown to suppress GvHD without compromising GVL. To generate Ag-sp Treg *ex vivo*, donor-derived Treg need to be expanded with antigen presenting cells (APC) that display recipient-derived allogeneic antigens (allo-Ag). However, there is no definite protocol for the expansion of Ag-sp Treg so far. In fact, current protocols usually require human serum, which is not ideal in the clinical setting. Additionally, the specificity of the resulting Treg is often unclear.

For these reasons, our objective with this work was to establish a protocol for the expansion of Ag-sp Treg in serum-free conditions, where the suppressive potency, specificity and phenotype of the expanded Treg (eTreg) were thoroughly characterized.

In the first chapter of results, we focused on defining a protocol for the expansion of Ag-sp Treg. We started by choosing the most adequate protocol for the differentiation and activation of monocyte-derived dendritic cells (moDC) to use as APC, testing the effect of distinct differentiation protocols (different media and activation cocktails) on the phenotype of moDC and on their potential to expand Treg. We then evaluated the differences in Treg expansion and function when allo-Ag were presented directly or indirectly, during expansion and/or in subsequent suppression assays (SA).

In the second chapter, we wanted to ascertain whether different expansion or suppression milieus could influence the potency and specificity of suppression, as well as the phenotype, of directly expanded Treg. Thus, we performed experiments where Treg were expanded in parallel with high (100U/mL) or low (10U/mL) concentrations of IL-2. An assortment of APC donors was selected for SA, in order to have distinct major histocompatibility complex (MHC) matches to the donor of APC used in expansion or to the donor of Treg. The function of each type of eTreg was then assessed separately in diverse suppression milieus, where Tcon or CD8 T cells were used as responders and the suppression of proliferation and cytokine concentration in the supernatants were measured. This way, we could get a more encompassing representation of Treg function. Finally, by performing a multidimensional analysis of the phenotypes found within eTreg, it was possible to estimate the purity of the final eTreg population, as well as find different phenotypic subsets that may be related to distinct mechanisms of suppression.

Overall, these experiments allowed us to propose that the specificity of Treg depends on: Treg potency, where higher potency may lead to reduced specificity, Treg-APC interactions, where an affinity of the Treg T-cell receptor (TCR) to the MHC of moDC may increase the suppression of responders, and responder-APC interactions, where the co-stimulation provided by moDC may allow for the responders to evade suppression.

Keywords: Regulatory T cells (Treg); Antigen-specific Treg expansion (Ag-sp Treg); Graft *versus* host disease (GvHD); Suppression assays; Multidimensional analysis.

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LIST OF ABBREVIATIONS

aAPC – artificial APC
Ab – antibody
ADP – adenosine diphosphate
Ag –antigen
Ag-sp – antigen-specific
aGvHD – acute graft-*versus*-host disease
Akt – protein kinase B
ALL – acute lymphoblastic leukaemia
Allo – allogeneic
AML – acute myeloid leukaemia
AMP – adenosine monophosphate
APC – antigen presenting cells
ARC – alcohol related cirrhosis
ATP – adenosine triphosphate
ATRA – all-trans retinoic acid
BCL-2 – B cell lymphoma 2
cAMP – cyclic AMP
CAR – chimeric antigen receptor
CCR – chemokine receptor
CD – cluster of differentiation
CD25 – IL-2 receptor alpha chain
CD62L – L-selectin
cGVHD – chronic graft-*versus*-host disease
CM – cytokine-matured
CTL – cytotoxic T lymphocytes
DC – dendritic cell
DC1 – DC-type 1
DC2 – DC-type 2
$_{eff}$Treg – effector Treg
eTreg – expanded Treg
FACS – fluorescence-activated cell sorting
FSC – forward size scatter
G-CSF – granulocyte colony-stimulating factor
GARP – glycoprotein A repetitions predominant
GI – gastrointestinal
GM-CSF – granulocyte-macrophage colony-stimulating factor
GMP – good manufacturing practice
GS – gold standard
GVHD – graft-*versus*-host disease
GVL – graft-*versus*-leukaemia
HLA – human leukocyte antigens
HSCT – hematopoietic stem cell transplantation
ICOS – inducible T cell co-stimulator
iDC – immunogenic DCs
IDO – Indoleamine 2,3-dioxygenase
IFN – interferon
IL – interleukin
iTreg – induced Treg
L – ligand
LAP – latency-associated peptide
mHA – minor histocompatibility antigen
MHC - major histocompatibility complex
MHCII – MHC-class II
MLR – mixed lymphocyte reaction
moDC – monocyte-derived dendritic cells
mRNA – messenger ribonucleic acid
MST – minimum spanning tree
NK – natural killer
nTreg – natural Treg
PB – peripheral blood
PBMC – peripheral blood mononuclear cells
PD – programmed death
PGE2 – prostaglandin E2
PI3K – Phosphoinositide 3-kinase
pTreg – peripheral Treg
rDC – regulatory DC
RT – room temperature
rTreg – resting Treg
SOM – self-organizing map
SSC – side size scatter
T1D – Type 1 diabetes
Tcon – Foxp3$^-$ conventional T cells
TCR – T cell receptor
tDC – tolerogenic DCs
T_{eff} – effector T cells

TGF – transforming growth factor
TNF – tumor necrosis factor
Treg – Regulatory T cells
TSDR – Treg-specific Demethylated Region
UC – ulcerative colitis
UCB – umbilical cord blood

INTRODUCTION

BACKGROUND

1. The problem: Graft versus Host Disease

Blood cancer affects millions of people worldwide. In most diagnosed acute cases, such as acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL), the main course of treatment consists of one (or multiple) rounds of chemotherapy. These treatments target and kill the patient's hematopoietic system, as well as leukaemia cells[1]. As a result, these patients are severely immunocompromised. When remission is achieved (or the patient has undergone too many chemotherapy rounds to no success), consolidation treatment is provided, in the form of hematopoietic stem cell transplantation (HSCT). The transplanted cells may originate from the patient (autologous), an identical twin (syngeneic), or an allogeneic (allo-) donor, who can be either related or unrelated to the patient. Currently, allo-HSCT is the most common type of curative treatment for patients with acute leukaemia. In order to select a donor, the potential donors' and the recipient's ten major human leukocyte antigen (HLA) alleles – HLA-A, -B, -C, -DQ and -DR – within the human major histocompatibility complex (MHC) are genotyped and the pair with highest number of matches is chosen[2]. Alas, even when the donor/recipient pair is fully matched (10/10) at the HLA level, disparity at loci outside the HLA complex– known as minor histocompatibility antigens (mHA) – may be recognized as alloantigens. Minor HA can be found in the patient's tissues, as well as presented by the donor's antigen presenting cells (APC), leading to graft versus host disease (GvHD)[3].

Acute GvHD (aGvHD) manifests mainly in the skin, gastrointestinal (GI) tract and liver, and its incidence is directly associated with the degree of HLA mismatch between donor and recipient, ranging from 35% in HLA-identical related grafts[4] to up to 80% in unrelated donor grafts[5]. Its pathophysiology is associated with prior tissue damage due to infections and/or the conditioning regimen, which triggers innate immune system activation and release of danger signals, like the pro-inflammatory cytokines interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (TNF-α)[6,7]. In this pro-inflammatory microenvironment, donor and recipient APC are activated and increase the expression of MHC molecules of Class I (MHCI) and II (MHCII), presenting tissue mHA, and of co-stimulatory molecules. Furthermore, if there is damage to the GI tract, microbiota-derived signals may translocate and amplify APC activation[7]. Upon engraftment, tissue antigens (Ag) presented by either host APC (direct presentation) or donor APC (indirect presentation) are recognized as foreign by the T-cell receptor (TCR) of donor-derived T lymphocytes[8,9]. These cells differentiate into effector T cells (T_{eff}) and release more pro-inflammatory cytokines, recruiting other T_{eff}, such as T helper (Th) cells and cytotoxic T lymphocytes (CTL), as well as natural killer (NK) cells and macrophages. This exacerbated inflammation milieu leads to target cell lysis and, ultimately, to organ dysfunction[10]. Chronic GvHD (cGvHD) is a more generalized condition resembling severe autoimmune diseases, and it may develop simultaneously or after aGvHD, or even *de novo*. Unlike aGvHD, its pathophysiology is poorly understood, even though it is the leading cause of patient morbidity and non-relapse related mortality[11].

After HSCT, another process may occur, in which there is recognition of mHA present in the patient's malignant cells by the donor's T cells[12,13]. This recognition triggers the elimination of residual malignant cells that could lead to leukaemia relapse if left undisturbed. This graft-versus-leukaemia (GVL) effect is a silver lining to GvHD, as it has been shown that patients who develop GvHD have lower rates of leukaemia relapse[12,13].

The rising incidence of GvHD is partly due to the increased use of unrelated donors and to the use of granulocyte colony-stimulating factor (G-CSF) to mobilize hematopoietic stem/progenitor cells from bone marrow into the peripheral blood (PB), recruiting high numbers of donor T cells that can trigger GvHD[14]. Simultaneously, as the range of GvHD treatment options broadens, there is also the need to find therapies that do not compromise GVL[15].

2. The potential solution: Regulatory T cells

Based on the pivotal role of T cells in GvHD development, partial and complete T cell depletion strategies have been extensively studied as prophylaxis for GvHD. Studies of patients who received T cell depleted[16] or partially depleted[17] HSCT have shown that GvHD incidence can be decreased by these strategies. However, long-term survival was not improved, due to increased complications such as leukaemia relapse and graft failure, so these strategies are not widely used. The current pharmacological approaches for GvHD prophylaxis focus on the immunosuppression of the host immune system by inducing host T cell anergy – reducing severe GvHD incidence and delaying aGvHD onset[18]. Yet, GvHD treatment remains a problem. The current standard of care relies on the control of inflammation through the use of glucocorticoids. Since glucocorticoids are not curative, many serious side effects develop from high dosage and/or prolonged use, such as opportunistic infections, hyperglycaemia, hypertension, cataracts and musculoskeletal system complications[19]. Furthermore, the response rate to glucocorticoids is between 30-40% only. Patients with steroid-refractory cGvHD have inferior survival rates[20], and new therapies for these cases are desperately needed.

Regulatory T cells (Treg) are naturally suppressive cells that account for a very small subset (5-10%) of total $CD4^+$ T cells in peripheral blood (PB)[21]. Despite their low numbers in circulation, Treg are key players in immune tolerance, as their main role is to control peripheral immune tolerance by inhibiting self-reactive T cells[21]. In fact, Treg deficiencies usually lead to the development of auto-immune diseases such as type I diabetes, inflammatory bowel disease and autoimmune gastritis[22]. As for their phenotype, Treg are known to express high levels of the IL-2 receptor α-chain (CD25) together with the fork-head box transcription factor Foxp3, which is essential for their immunosuppressive phenotype[23]. These suppressive capabilities have also been negatively correlated with the expression of high affinity α-chain of IL-7R (CD127)[24]. However, the markers that characterize Treg may also be found in activated T cells, thus, there is still no definite phenotype that undoubtedly defines Treg.

The majority of Treg found in circulation migrate from the thymus and are usually referred to as thymic Treg (tTreg). During their negative selection, tTreg upregulate Foxp3 upon self-Ag recognition, avoiding clonal deletion. At the time of migration into lymphoid tissues, their TCR repertoire is as diverse

as that of Foxp3^{-} conventional CD4 T cells (Tcon), but displays higher affinity for self-Ag ligands[25]. In the HSCT setting, Treg act as immune modulators and provide immune tolerance to the graft. However, it has been shown that in cGVHD patients Treg are present in lower absolute numbers and frequencies than those observed in patients without GVHD and in healthy individuals[26–28]. Overall, it is thought that cGvHD is associated with a severe imbalance between regulatory and effector T cells, resulting in multiple damages to target organs[26,27].These observations have led to many studies envisaging the use of Treg as a therapy for cGVHD. Preclinical studies in mice have shown that the infusion of donor Treg can prevent cGVHD without compromising the graft versus leukemia (GVL) effect[29–31]. In humans, adoptive immunotherapy with donor Treg has been reported to successfully prevent GVHD after haploidentical related[32] and umbilical cord blood unrelated[33] allo-HSCT. Importantly, our group is currently performing a Phase I/II clinical trial assessing the safety and efficacy of Treg infusion in patients with steroid-refractory cGVHD[34].

Albeit promising, these clinical trials leave many issues unaddressed. First and foremost, due to the low numbers of circulating Treg, *ex vivo* expansion may be necessary in order to obtain clinically relevant numbers of Treg for infusion. With that requirement comes the need for an established expansion protocol under GMP conditions. Due to the intracellular nature of Foxp3 expression, this marker cannot be used to select Treg before or after expansion. Moreover, it is now known that the CD25^{high}Foxp3^{+} Treg definition *per se* encompasses many different phenotypes within Treg and, depending on the expansion conditions, Treg may be skewed towards different effector or memory-like phenotypes[35]. Thus, it is crucial to better characterize the population of Treg being infused.

Secondly, there is the concern of maintaining GVL responses upon Treg infusion. Tumor-specific Treg that suppress the activity of Teff have previously been documented in tumor infiltrating lymphocytes[36], so it is possible that donor-derived polyclonal Treg infusions after allo-HSCT include Treg that suppress GVL responses, which could be very detrimental. Some groups are already exploring the advantages of using antigen-specific (Ag-sp) Treg *in vivo*[37]. Ag-sp Treg are obtained by isolating and subsequently expanding donor Treg with APC that present a broad range of the host's antigens. Ag-sp Treg can be easily expanded if the donor and recipient are HLA-mismatched, since the extent of the TCR stimulation is higher. In the HSCT setting, donor/recipient pairs are HLA-matched, thus the donor T cells only recognize the recipient's mHA as foreign. This means that, in an HLA-matched setting, there is the need to expand mHA-specific Treg, which can be harder to detect since the fraction of specific Treg in the initial pool of cells is much smaller and most of the mHA are still unknown.

Finally, the specificity of expanded Ag-sp Treg must be dissected, as it is critical for the successful clinical translation of these therapies. It is known that many mechanisms are involved in Treg-mediated suppression, however, the ways by which each mechanism operates and how they are combined to lend specific responses to different antigenic *stimuli* are still widely unknown[38]. Importantly, none of the groups that are either performing Treg clinical trials or performing studies based on the isolation and expansion of Ag-sp Treg *in vitro* for future GVHD therapies have fully encompassed the immunologic milieu of HSCT, since they do not assess the specificity of those Treg to the patient's disease. The ideal experimental model would be to select and expand *in vitro* a population of Treg that specifically

suppressed responses to broadly expressed host antigens in normal tissues, and that did not suppress donor effector T cell responses against antigens unique to the malignant cells, or (at least) to haematopoietically restricted mHA (**Fig. 1**). If successful, Ag-sp Treg would constitute a very promising approach for the treatment of cGVHD: they would potentially be more effective than polyclonal Treg and would not carry the risk of compromising GVL responses or causing generalized immunosuppression. Hence, it is of the utmost importance to establish protocols for their isolation, expansion and thorough phenotypical and functional characterization prior to clinical translation.

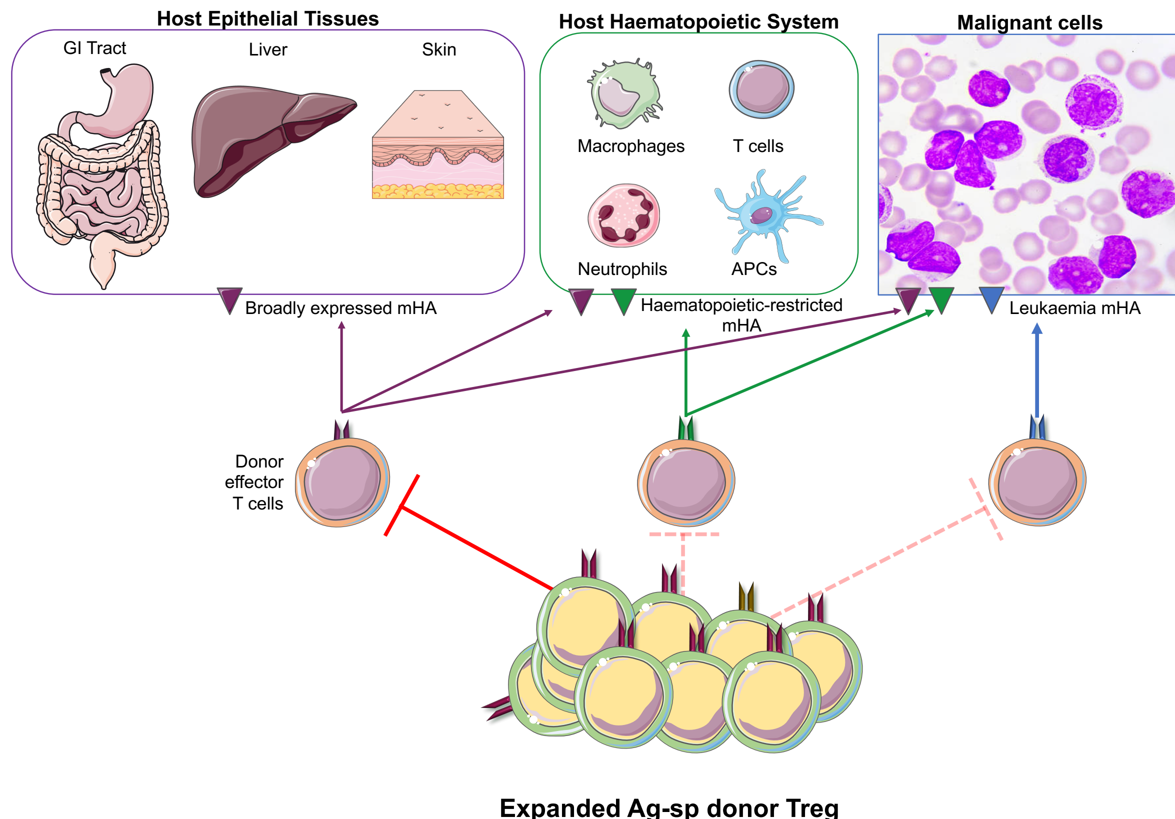

Figure 1: Expansion of antigen-specific regulatory T cells.
Upon engraftment, donor effector T cells react to broadly expressed mHA present in the host's epithelial tissues (purple arrows) as well as to haematopoietic-restricted mHA (green arrows), leading to GvHD. Leukaemia cells also express these mHA, hence the patients benefit from GVL. Since some mHA are unique to malignant cells (blue arrow), when the objective is to suppress GvHD, the ideal strategy would be to expand and select Ag-sp Treg that suppress all effector T cells (red line) but spare the effector T cells reactive to leukaemia-specific or hematopoietic-restricted mHA (dotted red lines). Adapted from Bleakley et al[17].

State of the Art

1. How Can We Expand Treg?

1.1. Circulating Treg

As promising as Treg therapy is, one of its main hurdles to date is the limited numbers of circulating Treg. Hence, the need for adequate *ex vivo* expansion protocols. Within circulating Treg, most are of thymic origin (tTreg), but some may be induced from Tcon following TCR stimulation in favourable milieus – peripheral Treg (pTreg)[39].

Currently, there is a lack of phenotypic markers that unambiguously distinguish tTreg from pTreg populations for isolation and subsequent expansion. We previously defined Treg as $CD4^+$ T cells with high expression of IL-2Rα (CD25) and transcription factor FOXP3, which is necessary for their development and function. Because Foxp3 staining is of intracellular nature, the standardized full panel for Treg isolation is $CD4^+CD25^{high}CD127^{low}$. This strategy results in a population enriched for $Foxp3^+$ cells, with Foxp3 staining being used for characterization purposes only. However, activated T cells may upregulate CD25 and Foxp3 expression to some extent, so contamination of purified Treg populations with activated T cells is still a concern that must be addressed. Finally, the third type of Treg are induced Treg (iTreg), which are derived from Tcon *in vitro*. Induced Treg express the typical Treg markers and have been reported as being suppressive *in vitro*[40,41] and *in vivo*[42]. However, there is the risk that these cells are not stable in the long term *in vivo*. One way to verify iTreg stability is to assess the methylation level of the Treg-specific Demethylated Region (TSDR), which is a sign of stable FOXP3 expression and is usually associated with tTreg and high suppressive capability[43]. Usually, iTreg have a demethylated promotor region with methylated TSDR and may thus transiently express Foxp3[44].

This chapter will focus on circulating Treg, which will be referred to as Treg henceforth. As all $CD4^+$ T cells, Treg can be characterized according to their activation status: resting Treg (rTreg) constitute the majority of Treg in circulation and are similar to naïve T cells in their high expression of homing receptors L-selectin (CD62L) and chemokine receptor (CCR) type 7 (CCR7)[45]. Effector Treg ($_{eff}$Treg) display signs of recent antigen encounter, with upregulation of activation markers such as inducible T cell co-stimulator (ICOS) and several chemokine receptors, and show heterogenous expression profile of CD62L and CCR7[45]. Naïve and memory phenotypes are related to previous antigen priming and conversion from naïve to memory phenotypes is represented by downregulation of the CD45RA and upregulation of the CD45RO isoforms of CD45[46]. There is a fluidity in these subpopulations and their nomenclature, but so far reports indicate that memory Treg can be found within resting and effector compartments[46]. Concurrently, both naïve and memory Treg can be polyclonally expanded, but only the naïve subtype will present a rTreg phenotype after expansion[47].

When it comes to Treg expansion, the first source of protocol-to-protocol variability comes from the isolation of the starting populations – both regarding the markers used and the isolation method *per se*. Although the general consensus is that selecting for $CD4^{+}CD25^{high}CD127^{low}$ will yield a Treg-rich population, the way in which these cells are selected can be very different. The most commonly used options are commercially available immunomagnetic bead-based kits and fluorescence activated cell sorting (FACS). Each of these techniques has pros and cons, which should be weighed according to the aim of the experiment.

Immunomagnetic bead-based commercial kits provide pre-defined marker combinations, which allow for higher reproducibility of the protocols. Within these, clinical-grade kits are optimized for the minimum number of beads to be present in the purified population, by incorporating as many negative selection steps as possible. However, it is not possible to define thresholds for each marker (e.g. choosing only the cells with the highest expression of CD25) on a case-by-case basis. Furthermore, potential markers of interest that have not yet been included in commercial kits cannot be tested for subset isolation. Ultimately, if the isolated cells are to be used in cell therapy, there will be compromises between using positive selection steps (that usually present higher purity) and the need to assess the safety of infusion of any remaining cell-bound beads.

Fluorescence-activated cell sorting (FACS) provides more flexibility in terms of gating stringency, resulting in purer populations. There is also the possibility of sorting distinct subsets at the same time, e.g., naïve Treg and Tcon. Nevertheless, one of the main limitations of this technique is that the sorting protocol may be more stressful for the cells, as they are subjected to very high pressures in the machine. There is also the fact that FACS is very time-consuming. Coupled with the higher purity of the cells, there are fewer cells to work with after isolation. In order to achieve clinically relevant numbers, FACS has to be followed by cell expansion protocols, that present a lot of limitations of their own – as will be discussed below. Finally, it is important to consider that GMP-grade cell sorting is something that is not within reach for most groups, and that the thresholds for cell sorting are defined on a case-by-case basis, thus there may be some donor-derived variability.

1.2. Effector Treg phenotypes

Resting Treg have low turnover rates. Upon activation, they increase proliferation and convert into $_{eff}$Treg subpopulations that may share transcriptional and homing machinery with $CD4^{+}$ T helper cell (Th) effectors[48,49]. These Th-like Treg subsets display suppressive activity *in vitro*, yet express different chemokine homing receptors and may produce both anti- and pro-inflammatory cytokines, enabling them to effectively co-localize and regulate the target Th population.

Duhen *et al.* found four distinct phenotypes within *ex vivo* activated memory Treg: Th1-like Treg expressed CXCR3 and produced IFN-γ; Th2-like Treg were $CCR4^{+}$, yet IL-4 production was not detectable by intracellular staining; Th17-like Treg were $CCR4^{+}CCR6^{+}$ and produced IL-17; and finally Th22-like Treg, which expressed the same homing receptors as Th17-like Treg, plus CCR10, and presented intracellular IL-22 (in opposition to IL-17 in Th17)[48]. Surprisingly, only IFN-γ- and IL-17-producing Treg produced the anti-inflammatory cytokine IL-10. The authors suggested IL-10 could confer immunomodulatory properties to originally pro-inflammatory cytokines such as IFN-γ- and IL-17.

In this work, each subset of effector Treg was associated with selective expression of T lineage-specifying transcription factors, i.e., Th1-like Treg expressed T-bet, Th2-like Treg expressed GATA3, and Th17-like Treg expressed RORγt. Additionally, Halim *et al.* recently reported GATA3-expressing Th2-like Treg and a Th1/17-like Treg population that combined Th1 and Th17-like phenotypes[49]. Interestingly, by cytokine quantification, the authors were able to detect that Th2-like Treg secreted not only IL-4, IL-5 and IL-13, but also more IL-2 and IL-10 than other subsets, contrarily to what had been reported by Duhen *et al.*[48]. Finally, there are reports of IL-17-producing Treg being detected in circulation, suggesting pTreg may acquire the ability to produce IL-17 upon activation[50]. Similarly, the secretion of IL-17 by Treg in response to strong stimuli *in vitro* has been described[51]. Even though all the above-mentioned authors claim that each Th-like Treg is capable of suppressing its effector counterpart, different groups suggest that these Th-like subtypes may contribute to an inflammatory phenotype instead of regulating it [52,53].

The consensus is that $_{eff}$Treg are heterogeneous in their function and that the environment in which antigen presentation occurs may skew them towards one or another effector phenotype. Thus, it is essential that these mechanisms are better understood in order to properly expand and manipulate $_{eff}$Treg for therapeutic usage.

1.3. Signalling for Treg expansion

The immunological fate of Treg is modulated by the signalling received in each milieu. The different mechanisms involved in this modulation are very broad and complex. Briefly, to stimulate Treg proliferation and conversion into $_{eff}$Treg, three different types of signal should be delivered. Signal 1, which requires TCR engagement, is referred to as “antigen-specific”. It is delivered by APC through a peptide-MHC complex and on its own will expand Treg with a memory phenotype, leading to immunological tolerance to that peptide[54]; moreover, mouse studies show that peripheral Treg function and activation is dependent on continuous TCR stimulation[55,56]. Signal 2, or co-stimulation, is usually associated with two main co-stimulation families: B7/CD28 and tumour necrosis factor (TNF)/TNFR. Delivery of Signal 2 usually happens through binding of co-stimulatory molecules CD80 and CD86 in APC to CD28, which is constitutively expressed by T cells, or receptors of the same family, such as ICOS, programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Signal 2 delivery is required to break anergy in Treg, stimulating proliferation and survival[54,57]. Finally, Signal 3 is typically delivered by cytokines produced by activated APC or T_{eff}. As mentioned before, Treg constitutively express high levels of CD25, which forms a trimeric complex with CD122 (β-chain) and CD132 (common gamma chain, γ_c) and has high affinity for IL-2 [58]. Thus, paracrine IL-2 signalling is of great importance for Treg survival and function, as they do not produce it themselves [59]. To expand Treg *ex vivo*, one must make sure that all the correct signals are being delivered. Nevertheless, there is a wide array of protocols to choose from, raising issues concerning assay reproducibility and potentially leading to misunderstandings when it comes to subpopulation nomenclature and characterization after expansion. Regardless of the signalling used to trigger Treg expansion, the conditions in which expansion occurs may also maintain the diversity of their TCR repertoire – polyclonal Treg expansion – or enrich it according to the affinity of their TCR to select antigens, either

known or unknown, generating Ag-sp Treg. The conditions required for one or the other type of expansion are described in the following sections.

1.4. Polyclonal Treg expansion

In the following sub-chapters, the most relevant protocols for Treg expansion will be described, focusing on the source of signalling delivered to T cells and specificity of the resulting Treg population. Starting by polyclonal Treg expansion, most strategies involve artificial APC (aAPC), yet the reported protocols are still very heterogeneous, as can be seen on **Table I**. Bead-based aAPC usually consist of anti-CD3 and anti-CD28 antibody (Ab)-coated beads, where the role of anti-CD3 is to provide unspecific TCR stimulation, delivering Signal 1 to Treg, whilst anti-CD28 delivers costimulatory signals (Signal 2) through CD28. In this setting, high doses of IL-2 are added to the culture media, resulting in a very stimulating environment for T cell expansion that carries the concern of expanding residual contaminating Tcon. Other concerns include loss of potency associated with expanding Treg with undesired specificities. There are other possibilities for exogenous signal 3 delivery, which will be discussed in more detail in the next chapter (**1.5.**).

EX-VIVO **EXPANDED POLYCLONAL TREG**

Expansion stimulus	aAPC	Starting population	References and notes
aCD3/aCD28 beads	Bead-based	$CD4^+CD25^{high}$	Hoffman *et al.*[60] Godfrey *et al.*[61]
		$CD4^+CD25^{high}CD127^{low}$	Hoffman *et al.*[62] Tran *et al.*[63] Putnam *et al.*[64] – T1D
		GMP-grade $CD25^+$	Voskens *et al.*[65] – UC
		GMP-grade $CD8^-CD25^{high}$	Del Papa *et al.*[31] – GvHD Theil *et al.*[66] – GvHD clinical trial Safinia *et al.*[67]- ARC. Velaga *et al.*[68]
		GMP-grade $CD4^+CD25^{high}CD127^{low}$	Bluestone *et al.*[69] – T1D clinical trial
Transduced cell lines	Cell-based	$CD4^+CD25^{high}CD127^{low}$	Golovina *et al.*[57] Hippen *et al.*[70]
		GMP-grade $CD4^+CD25^+$	Brunstein *et al.* – GVHD clinical trial[33,71]

aAPC – artificial APC; APC – antigen-presenting cells; ARC – alcohol related cirrhosis; GMP – good manufacturing practice; GvHD – graft-versus-host disease; T1D – type 1 diabetes; UC – ulcerative colitis.

Table I: Ex-vivo expansion of polyclonal Treg.
Summary of noteworthy papers reporting ex-vivo polyclonal Treg expansion, divided by stimulus used for expansion, type of artificial APC and starting T cell population.

Of note, within expansion protocols using bead-based aAPC, encouraging work on clinical-grade-expanded polyclonal Treg has been published, which is briefly highlighted here. There are reports of successful expansion of Treg from HSCT donors, envisaging GVHD treatment[31,66]. Another group was

able to expand Treg isolated from alcohol related cirrhosis (ARC) patients, in order to use those Treg as prophylaxis for liver rejection [67]. The isolation and expansion of CD25$^+$ cells from patients suffering from ulcerative colitis (UC), envisaging adoptive Treg transfer, has also been published[65]. In these reports, purification of Treg was performed using GMP-approved isolation kits, where there is usually an immunomagnetic CD8-depletion followed by CD25-positive selection. Interestingly, Bluestone *et al.* recently reported good results on a Phase I clinical trial assessing safety of FACS-isolated and polyclonally expanded Treg for Type 1 diabetes (T1D) therapy [69]. Alas, so far there is still no definite GMP-compliant protocol established for *ex vivo* Treg expansion – Velaga *et al.* recently performed a comparative analysis of large-scale expansion protocols that may contribute to this matter[68], whilst Putnam *et al.* have shown that beads from different brands yield very different expansion rates[72].

Conversely, cell-based aAPC are transduced cell lines. This is a much more time-consuming technique with the upside of allowing the user to choose what costimulatory molecules are presented to T cells. This flexibility facilitates the analysis of mechanisms involved in Treg maintenance and expansion. For example, it allowed for Golovina *et al.* to study the importance of CD28 costimulation [57]. Within CD28 co-stimulation, the work developed by Hippen *et al.* highlighted the superior expansion of Treg with CD86-expressing cell-based aAPC (and anti-CD3) when compared to bead-based aAPC [70]. Importantly, Brunstein *et al.* have successfully expanded umbilical cord blood (UCB)-derived donor Treg using cell-based aAPC for subsequent infusion into UCB-derived HSCT recipients, and were able to diminish the risk of them developing aGvHD [33,71].

1.5. Antigen-specific Treg expansion

Ag-sp Treg are expanded from a pool of polyclonal Treg in response to TCR stimulation via target antigens/MHCII complexes in APC. If polyclonal Treg expansion protocols were already diverse, when it comes to Ag-sp Treg expansion the variability increases exponentially, as there are many types of APC suitable for Ag-sp expansion[73]. Some representative protocols for Ag-sp Treg expansion are summarized in **Table II**. *In vivo*, antigen presentation is carried by professional APC such as dendritic cells (DC), macrophages and B cells[74]. Seeing that these cells are present in peripheral blood mononuclear cells (PBMC) and that all other nucleated cells in the body can act as non-professional APC, some groups explored co-culturing allogeneic irradiated total PBMC with Treg for Ag-sp expansion[72,75–77]. Although a simple approach, it required higher Treg:APC ratios to achieve the same or weaker results than with isolated professional APC.

One alternative is to use B cells as APC, which involves stimulating isolated B cells with CD40-ligand (CD40L) expressing cell lines. CD40 engagement stimulated B cell expansion and increased MHCII expression, and expression of co-stimulatory molecules CD80 and CD86[78]. Overall, activated B cells were effective in Ag-sp Treg expansion[72], as well as Ag-sp Treg induction from CD4$^+$CD25$^-$cells[40]. Nevertheless, the steps required to prepare B cells for Ag presentation may thwart the translation of these protocols to the clinical setting.

DC are a heterogenous population that develops from bone-marrow derived progenitors and play a key role in immune regulation. More specifically, DC excel at capturing, processing and presenting antigens via MHCII and through cross presentation on MHCI, delivering Signal 1 to naïve CD4$^+$ and

CD8$^+$ T cells, respectively. During inflammation, DC upregulate CD80 and CD86, costimulatory molecules of the B7 family that work in tandem to deliver Signal 2 through CD28 stimulation, as well as acquire the ability to secrete Signal 3-associated cytokines[79]. The main interactions involved in T cell activation by DC are resumed on **Fig. 2**. For their great potential as immunomodulators and as antigen presenting cells, therapy and vaccination using *ex vivo* DC has been widely studied in the last years, most notably in cancer settings, as reviewed by Palucka *et al.* [80]. Interestingly, it has been shown that conventional DC are the main players in allo-Ag presentation after HSCT in mice[81]. Even though *ex vivo* DC present great plasticity in terms of body location and function, their scarcity hinders their usage as APC for Ag-sp Treg expansion. Notwithstanding, some groups reported expanding donor-specific Treg using allogeneic myeloid DC[77] and donor skin-specific Treg using dermal (myeloid) DC[82].

The terms immature *versus* mature have been widely used to characterize DC subpopulations. However, there is a dichotomy between these terms and the definition of tolerogenic and immunogenic DC, respectively. In accordance with Caetano Reis e Sousa's essay, it is important to describe DC regarding 1) their expression of co-stimulatory molecules and maturation markers and 2) their immunological role[88]. In this thesis, the terms immature or mature will be used in reference to the expression of maturation markers, not effector function.

***EX-VIVO* EXPANDED ANTIGEN-SPECIFIC TREG**

APC Source	Type of APC	Specificity	Starting population	References and notes
autologous	Loaded immature moDC	Allo-peptide	CD4$^+$CD25^{high}	Jiang *et al.*[83]
		allogeneic cell lysate	CD4$^+$CD25^{high}CD127^{low}	Veerapathran *et al.*[75]
syngeneic	Immature moDC	Allo-peptide	CD4$^+$CD25^{high}	Golshayan *et al.*[84] – mice
		sibling mHA	CD4$^+$CD25^{high}CD127^{low}	Veerapathran *et al.*[85]
allogeneic	PBMC	allogeneic donor	CD4$^+$CD25^{high}	Koenen *et al.*[86] Peters *et al.*[87] Veerapathran *et al.*[75] Litjens *et al.*[76]
			CD4$^+$CD25^{high}CD127^{low}	Putnam *et al.*[72] Schoenbrunn *et al.*[77]
	Immature moDC	allogeneic donor	CD4$^+$CD25^{high}	Veerapathran *et al.*[75] Litjens *et al.*[76]
	Mature moDC	allogeneic donor	CD4$^+$CD25^{high}CD127^{low}	Litjens *et al.*[76]
	CD40L-stimulated B cells	allogeneic donor	CD4$^+$CD25^{high}CD127^{low}	Putnam *et al.*[72]
	Myeloid DC	donor skin allo-Ag	CD4+CD25high	Sagoo *et al.*[82]
		allogeneic donor	CD4$^+$CD25^{high}CD127^{low}	Schoenbrunn *et al.*[77]

Ag – antigen; APC – antigen-presenting cells; DC – dendritic cells; mHA – minor histocompatibility antigens; moDC – monocyte-derived dendritic cells; PBMC – peripheral blood mononuclear cells.

Table II: Ex-vivo expansion of antigen-specific Treg.
Summary of noteworthy papers reporting antigen-specific ex-vivo Treg expansion, divided by source of APC, type of APC, specificity of the expanded Treg and starting population.

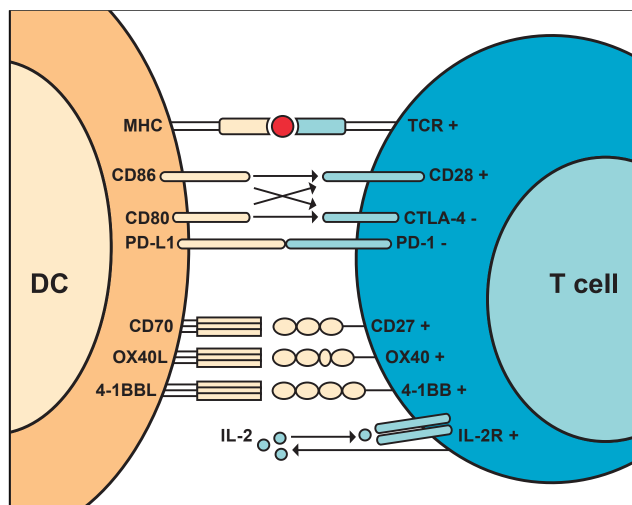

Figure 2: T cell activation by APC.
DC provide activation signals to T cells through MHC-TCR interactions (Signal 1) as well as through the presentation of co-stimulatory molecules i.e. CD80 and CD86, and/or tumour necrosis factor family members, such as CD70,OX40L or 4-1BBL (Signal 2). Cytokines such as IL-2 also play a role in T cell activation (Signal 3), yet may be produced by cells other than APC. *Adapted from Haan et al.*[74].

The possibility of obtaining DC from monocytes *in vitro* was first reported by Sallusto *et al.*, allowing for DC differentiation in high numbers[89]. Monocyte-derived DC (moDC) have since become suitable candidates for clinical use as targeted cell therapies, either as enhancing or suppressive immunomodulators[90]. The differentiation of DC from monocytes *in vitro* usually requires three steps. First, monocytes are isolated from PBMC (obtained from Ficoll density gradients of whole blood), either by plastic adhesion or CD14$^+$ selection using immunomagnetic beads. Next, monocyte differentiation into immature moDC is stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. This stage takes between 3-7 days, in adequate media supplemented with human serum (HS). HS-free expansion of moDC has also been reported, in detriment of final DC yield[91]. During differentiation, moDC lose CD14 expression and increase their expression of CD11c. Immature moDC are lineage negative (CD3$^-$CD19$^-$CD56$^-$) and positive for CD86 and MHC, but there is a high rate of endocytosis and degradation of MHC-peptide complexes [92].

For a long time, it was thought that immature moDC were the *in vitro* equivalent of tolerogenic DC (tDC), which are responsible for inducing T-cell tolerance in the periphery *in vivo*, as well as stimulating Treg induction and expansion [93,94]. That, together with the high antigen uptake and processing ability of immature moDC [88], led many groups to use autologous and syngeneic immature moDC as APC for Ag-sp Treg expansion (see **Table II**). The loaded antigens may derive from peptides[83,84], allogeneic cell lysates[75] (presenting a library of unknown alloantigens) or even XY-specific mHA present in MHC of HLA-matched siblings of different genders[85]. However, it has been shown that tDC may display and require a mature phenotype for optimal signal delivery[95], thus invalidating the hypothesis that immature moDC *in vitro* mirror tolerogenic DC *in vivo*.

For this reason, the last (and optional) step of the protocol for moDC differentiation *in vitro* is activation. Overall, mature moDC differ from immature moDC in their increased expression of the costimulatory factors CD80 and CD86, of the typical DC maturation marker CD83, as well as of MHCI and MHCII. Also, endocytosis is down-regulated during maturation, leading to decreased antigen uptake but conversely to longer permanence of MHC-peptide complexes on the cell surface[92]. There are at least three subpopulations within activated DC, according to their effect on T cells: DC-type 1 (DC1) that skew T cells into a Th1 phenotype; DC-type 2 (DC2), which stimulate a Th2 phenotype, and the previously mentioned tDC, which may cause T cell anergy and stimulate Treg induction [96].

The most common activation factors mimic inflammatory conditions, encompassing microbial compounds such as lipopolysaccharides (LPS), and inflammatory cytokines like interferon (IFN)-γ, TNF-α and prostaglandin E2 (PGE2). Even though LPS induces a stronger activation, the current gold standard (GS) in DC activation for therapeutic applications is a cytokine cocktail of IL-1β, IL-6, TNF-α and PGE2[90]. Of course, this can be adjusted according to the final effector function desired. Changes in activation settings like cocktail type, cytokine concentration and incubation time induce different moDC phenotypes and profiles of Signal 3 cytokine production, which may have different effects on T cells[97]. For example, activation with IFN-γ or LPS stimulated DC1 that induced Th1 polarization[91,98] through the secretion of high amounts of IL-12; PGE2 skewed moDC toward a DC2 phenotype; and IL-10 promoted the activation of tDC[94], which regulated immune responses through the release of indoleamine 2,3-dioxygenase (IDO)[99]. However, the optimal protocol for obtaining each type of DC in culture is still unknown, as it has been shown that even when using the same cytokine, like IFN-γ, there was a shift in phenotype from tDC to DC1 as concentration increased[100].

B7 FAMILY	TNFSF	OTHER MOLECULES
CD80	*CD70*	CD83 (Ig family)
CD86	*OX40L*	CD11c (LFA family)
PD-L1	*4-1BBL*	MHCI
	CD40 (TNFRSF)	***MHCII***

TNFSF – Tumour necrosis factor superfamily;
TNFRSF – TNF receptor superfamily
PD-L1 – programmed death ligand 1;
Ig – immunoglobulin;
LFA – leucocyte function-associated;
MHC – major histocompatibility complex.

Table III: Co-stimulatory and maturation markers expressed by moDC.
MoDC may express a range of molecules that can be involved in their role as APC. This includes a few molecules from the B7 family or the tumour necrosis factor superfamily, together with molecules from other families. The markers in bold are constitutively expressed by moDC, and in italic are the ones that may be relevant for the expansion and specificity of Ag-sp Treg.

Concomitantly, some links between the expression of maturation markers (**Table III**) and Signal 3 production have been reported. For example, increased expression of 4-1BBL in mature moDC was related to IL-12 production[101]. Of note, increased programmed-death (PD)-ligand 1 expression in moDC sustained Foxp3 expression on Treg and could be related to a tDC phenotype[102].

With all the uncertainties associated with using moDC as APC, such as choosing appropriate activation stimuli and target phenotypes and maturation markers, it is pivotal to characterize moDC according to their role as APC. Litjens *et al.* compared allogeneic immature moDC with GS-activated DC as APC for Treg expansion and concluded that both mature moDC and mature skin-derived DC yield more potent Ag-sp Treg than immature moDC[76]. Chung *et al.* reported that the same GS activation protocol generates IDO-producing moDC capable of autologous Treg expansion[99].

In the clinical setting, these details are very relevant because when there is a match in MHC, Signal 1 delivered from APC to Treg will only be as strong as the affinity of the Treg TCR to that Ag, resulting in weaker reactions and possibly a need for increased costimulatory signals and Signal 3 delivery to achieve relevant expansion.

a) TCR-mediated activation

In order to maintain homeostasis *in vivo*, circulating Treg are naturally primed to suppress responses to self-Ag, whilst being anergic to most allogeneic stimuli[103]. However, if subjected to strong activation, Treg anergy can be broken and these cells proliferate and acquire an effector phenotype. In order to expand Treg *in vivo*, whether of polyclonal or Ag-sp nature, proliferation is a requirement. As mentioned above, usually this is achieved by stimulating Treg with a combination of signalling through their TCR (either by anti-CD3 Ab or Ag-MHCII complexes), CD28 (by anti-CD28 Ab or CD80/CD86 co-stimulation) and IL-2R. Co-stimulatory molecules play an important role in the determination of cell fate. However, it is unclear if these conditions mimic the *in vivo* conditions for Treg activation. Finally, since $CD4^+$ T cells are able to detect Ag presented by APC in the context of MHCII, the strength of signalling regarding antigen load and quality of peptide are important not only for Treg expansion[104] but also for the differentiation of $CD4^+$ T cells in general, as summarized by Panhuys *et al.*[105]. Briefly, it was suggested Th1 and Th2 polarization is dependent on Ag strength, with the former requiring either high doses or high affinity Ag and the latter requiring the opposite; yet, Ag concentration predominates over affinity and co-stimulation, facilitating the conversion of Th2 into Th1. The authors also draw a parallelism between Th17 and Treg, suggesting that both populations may be differentiated in the same cytokine milieu, depending only on the strength of TCR signalling. Weak TCR stimulation would then benefit Treg differentiation, and the opposite would be true for Th17. Conversely, IL-2 signalling may weigh on this polarization by inhibiting Th17 differentiation unless there is a strong enough TCR stimulation to overcome it. While these authors propose that Treg may be able to differentiate without TCR stimulation, contradictory studies have been reported. Levine *et al.* showed that, in TCR ablated mice, Foxp3 expression and lineage stability was maintained, but Treg activation and suppression required TCR expression[56]. Also, even though moDC-Treg conjugates were formed in the absence of TCR, there was minimal proliferation and their adhesion was quickly lost. Conversely, using transgenic mice, another group has shown that cognate TCR signalling is not required for Treg suppressive function[106]. Interestingly, Gottschalk *et al.* reported that TCR stimulation with both weak and strong agonist peptides could induce Foxp3 expression *in vivo*, yet, only low doses of strong agonist were able to induce stable and persistent iTreg[107]. Nonetheless, there is a chance that Treg induced from weak agonists may be stabilized by a type of APC or a cytokine milieu different from the ones tested. Because this work focuses on the activation and expansion of stable Ag-sp Treg from purified Treg, it should be considered that the final outcome may depend on the balance of Ag dose *versus* affinity, as well as on an appropriate cytokine milieu.

b) Signal 3 cytokines

Signalling through IL-2R in Treg suppresses apoptosis by upregulating pro-survival protein induced myeloid leukaemia cell differentiation (MCL1), a protein of the B-cell lymphoma 2 (BCL-2) family, and promotes T cell function via phosphorylation of signal transducer and activator of transcription (STAT)-5[108]. Amongst cytokines that signal through IL-2R and phosphorylate STAT5, IL-2 displays the highest affinity, forming a quaternary complex with the three chains of this receptor. IL-15

binds to IL-2Rβ–γ_c, thus forming weaker tertiary complexes. Finally, IL-7 forms the weakest of these bonds, as it only binds to IL-2Rγ_c. Activation of the STAT5 pathway induces Foxp3 up-regulation, so IL-2 is required for pTreg maintenance and homeostasis *in vivo*[109]. In fact, low dose IL-2 therapy has been shown to specifically increase levels of Treg in circulation and improve GvHD clinical features[110] . Conversely, one of the main functions of pTreg is to suppress IL-2 production by Tcon, keeping IL-2 concentrations in check by a feedback loop. When IL-2 concentrations drop, Treg populations contract and IL-2 synthesis is de-repressed on Tcon, thus stimulating pTreg proliferation and enhanced survival[108]. Interestingly, by deleting CD25 in mice Treg, Fan *et al.* have recently demonstrated that continuous IL-2 signalling was required for long-term survival and suppressive function of pre-activated Treg, whilst lineage stability could be maintained by STAT5 phosphorylation induced by IL-15 or IL-7[111]. Similarly, it has been shown that $_{\text{eff}}$Treg in non-lymphoid tissues were insensitive to IL-2 blockade, relying instead on signalling through ICOS for their maintenance[45]. Still, in order to select and expand purified Treg *ex vivo*, there must be antigen presentation/TCR stimulation and co-stimulatory signalling, as well as Signal 3 delivery. Exogenous IL-2 is widely used as a third signal for *ex vivo* Treg expansion; however, when high doses of IL-2 are added, like in most polyclonal expansion protocols, it may lead to outgrowth of contaminating activated Tcon and expansion of iTreg.

Rapamycin, clinically known as sirolimus, blocks a subset of mammalian target of rapamycin (mTOR) functions, inducing apoptosis in rapamycin-sensitive Tcon[112]. Upon STAT5 phosphorylation in T cells, the Phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway is activated, of which mTOR is a downstream target[113]. Because Treg do not activate the PI3K pathway, they are resistant to rapamycin-mediated apoptosis[114], while Tcon are dependent on this pathway for activation and are sensitive to rapamycin[115]. For this reason, several clinical trials using sirolimus have been developed, reporting preserved Treg function and improvement of GvHD manifestations[116]. Based in these reports, rapamycin has been added to Treg expansion protocols as a way to control contamination by Tcon, even though its mechanism of action in Treg is largely unknown. Strauss *et al.* reported that polyclonal Treg expansion with rapamycin and high doses of IL-2 promotes Foxp3 expression and maintains suppression in polyclonally expanded Treg[112]. In a more recent study, rapamycin was suggested to upregulate expression of survival molecules in Treg and of apoptotic molecules in Tcon[115]; yet, the impaired proliferation of Treg in cultures with high concentrations of rapamycin or their delayed expansion was not explained. Besides, STAT5 levels were increased in Treg after only 4 hours incubation with rapamycin, reaching a threshold and declining at higher concentrations[115]. This is suggestive of a STAT5 induction by rapamycin, possibly resulting in increased expression of Foxp3.

Indeed, Tran *et al.* show that rapamycin enhances Foxp3 purity in culture in detriment of proliferation yield[63]. However, the percentage of IFN-γ and IL-2-producing cells (named Foxp3$^+$ non-Treg by the authors) was the same in Treg cultures with or without rapamycin[63]. In light of recent reports of Th-like Treg, it seems that rapamycin has no effect on the proliferation of these cells[117]. Many authors reported rapamycin-mediated iTreg expansion both *in vitro*[41,118,119] and *in vivo*[42,120]. However, when the initial population comprised total CD4$^+$ T cells, it was unclear whether the final population derives from selectively expanded Treg or iTreg expanded from Tcon. In fact, because activated Tcon phenotypically resemble Treg, rapamycin-treated cultures may blur this distinction further by conferring suppressive

abilities and increasing transient Foxp3 expression in iTreg that may not be stable enough for clinical applications[119]. Besides, Tcon that are spared after initial rapamycin stimulation and become anergic can be rescued by an excess of IL-2[121] or re-stimulation in the absence of rapamycin[118]. Contrarily to a report by Battaglia *et al.*, where it was claimed that IL-2 and rapamycin do not induce Treg from Tcon cultures[118], Long *et al.* demonstrated Treg induction in similar conditions[41]. In the latter work, it was even suggested that rapamycin may skew Foxp3$^-$ rapamycin-resistant cells into Foxp3-expressing cells, resulting in an apparent inhibition of expansion of Foxp3$^-$ cells. Similarly, Valmori *et al.* suggest that increased suppressive ability of rapamycin-expanded populations may be due to Tcon anergy and not to enhanced function of expanded Treg[119].

In a review by Thomson *et al.,* it is suggested that both processes may occur: upregulation of Foxp3 and maintenance of suppressive abilities of "true" Treg, as well as iTreg induction from contaminating Tcon[113]. Because stimulation with rapamycin impairs proliferation of all T cells and may not completely eliminate contaminating Tcon, some authors tried to find the ideal equilibrium for *ex vivo* Treg expansion. One of the proposed solutions is the addition of all-trans retinoic acid (ATRA), a derivative of vitamin A, to the culture. Although addition of ATRA with TGF-β could induce Treg *in vitro*, it did not lead to TSDR demethylation, thus resulting in unstable iTreg[44]; more importantly, addition of ATRA in the presence of IL-1 and IL-6, to mimic inflammatory conditions, seemed to prevent Treg transition into Th1/Th17 cells and stabilize Treg suppressive function[122]. Supplementation with both rapamycin and ATRA *in vitro* in the absence of HS resulted in a highly demethylated TSDR in the final population, enhanced suppressive capacity and abrogation of unstable iTreg[123]. Alas, there was a dose-dependent toxicity to ATRA. In a more recent study, Beerman *et al.* found that neither rapamycin or ATRA, alone or in combination, altered the surface expression of chemokine receptors, allowing for Treg migration to their targets in inflamed organs[124]. However, the problem of impaired proliferation remained and seemed to be aggravated by ATRA plus rapamycin. A different approach by Veerapathran *et al.* was to add another STAT5-stimulating cytokine to the culture, IL-15, along with IL-2, rapamycin and HS[75]. Together with low dose IL-2, IL-15 seemed to rescue Treg proliferation by increasing Bcl-2 protein expression.

Unfortunately, most papers reporting Treg expansion focus merely on fold expansion and suppressive capability of Treg, and not on characterization of $_{eff}$Treg phenotypes. In a very interesting work in mice by Verma *et al.*, different $_{eff}$Treg phenotypes were obtained with different Signal 1 and Signal 3 in culture: IL-2 alone led to polyclonal Treg expansion, whilst IL-2 and Ag presentation generated an intermediate Ag-sp Th2-like phenotype[125]; concurrently, IL-4 and Ag presentation yielded an intermediate Ag-sp Th1-like population[125]. Effector Treg seem to use a different pro-survival programme that requires upregulation of B lymphocyte-induced maturation protein 1 (BLIMP1) for optimal IL-10 production and ICOS expression[108]. Appropriately, it has been reported that $_{eff}$Treg do not require IL-2 for their maintenance[45]. Thus, these intermediate phenotypes could be clonally expanded and converted into Th-like $_{eff}$Treg in response to signals secreted by their Th counterparts.

The appropriate combination of cytokines and APC for Ag-sp Treg expansion most likely varies from mice to human studies. Also, there may be other APC-derived cytokines, such as IDO and IL-12, with different effects on Ag-sp Treg expansion *in vitro*. So far, Litjens *et al.* reported that by using

allogeneic mature moDC as APC there was no need for exogenous IL-2, since addition of IL-15 was sufficient to achieve the same fold expansion in HS-supplemented media[76]. Unfortunately, these authors did not test Treg generated in different conditions regarding their effector phenotype. It seems counter intuitive that cells that require IL-2 for survival *in vivo* may expand and maintain their full potential upon IL-2 starvation *in vitro*. It is a possibility that some cytokines may have been conferred by the HS; these results could have been clearer if the cytokines in HS were quantified, or if HS was not used. On the other hand, Chung *et al.* suggests that if autologous mature IDO-producing moDC are used as APC, there is no need for HS nor exogenous cytokines in Treg expansion from total $CD4^+$ T cells[99]. Once more, Treg source and stability are issues that need to be addressed in this approach.

All in all, there is no definite protocol for Treg expansion as each group focuses on different read-outs and aims, as well as different starting populations. Ideally, one would expand FACS-sorted $CD4^+CD25^{high}CD127^-$ cells with no HS and limited rapamycin and IL-2, in order to reduce contamination and unknown stimulation. To rescue Treg function, IL-15 may be added. Mature moDC can also contribute to Treg activation and stability, while simultaneously presenting Ag to generate Ag-sp Treg. Most importantly, there is the need for more thorough characterization of expanded Treg, focusing on more than just Foxp3 expression and indiscriminate suppressive ability. In the next chapter, we will discuss methods to compare and characterize Treg in depth after expansion.

2. How Can We Characterize Expanded Treg?

2.1. Phenotypic markers of Treg activation, suppressive capability and specificity

Treg are phenotypically highly heterogeneous and there is still no definite classification for distinct subpopulations. So far, some markers have been associated with differentiation sites and trafficking ability, while others can be related with maturity, specificity and expression of suppression-associated molecules within $_{eff}$Treg. This leads to confusion in the field and possibly some overlap between classifications. Furthermore, due to Treg plasticity, these classifications may be inter-changeable depending on the surrounding Treg milieu. Because this work aims for the expansion of Ag-sp Treg *in vitro* from *ex vivo* Treg, this section focuses on reported markers of Treg activation, suppressive capability and specificity. Reported markers of Treg, as well as a few novel markers, can be found on **Table IV,** along with their main roles in Treg function. Edozie *et al.* have also published a comprehensive table of novel markers in human and mice[126].

MOLECULE	MAIN FUNCTION/PHENOTYPE	REF.
	Treg-associated maturation markers	
PD-1	Constitutively expressed co-inhibitory marker Maintain Treg homeostasis via TCR and STAT5 pathway inhibition	127–129
CTLA-4	Constitutively expressed co-inhibitory marker Prevent T_{eff} activation via competition with CD28 for CD80/CD86 ligation	54,130–133
CD39	Downregulate T_{eff} activation through ATP/ADP degradation	117,134–137
	Activation markers found in Treg subsets	
HLA-DR	**DR^{+} Treg**: Early contact-dependent suppression **DR^{-} Treg**: Cytokine-mediated suppression	138,139
ICOS	**ICOS^{+} Treg:** high secretion of IL-10 **ICOS^{-} Treg:** high levels of mTGF-β	140
GARP/LAP	**GARP^{+}/LAP^{+} Treg:** Highly suppressive subset	63,141–143
TNFR2	**TNFR2^{+} Treg:** Highly suppressive subset	144–146
4-1BB (CD137)	**4-1BB^{+}CD40L^{-} Treg**: Ag-sp activated Treg	77,147–149
	Other T cell activation markers	
OX40	Co-stimulatory marker Enhance T cell proliferation Possibly abrogates Treg function	52,150

Table IV: T cell activation markers.
T cells upregulate many different molecules depending on activation stimuli. Some of these molecules are constitutively expressed in Treg and only their membrane expression is upregulated upon activation, whilst the expression of others is dependent on activation stimuli. This table includes markers that may be associated, by one way or the other, to Treg subsets or modalities of function.

As mentioned above, Treg found in circulation can be categorized according to their activation status and/or expression of naïve and memory markers. These cells also constitutively express co-

stimulatory and co-inhibitory molecules, and upregulate the cell surface exposure of those molecules upon activation. Because of their established role in Treg homeostasis and suppression mechanisms, proteins such as PD-1, CTLA-4 and ectonucleoside triphosphate di-phosphohydrolase 1 (CD39) have been widely studied as maturation markers for Treg.

PD-1 is constitutively expressed in Treg and presented on the cell surface of all activated T, B and myeloid cells[102]. PD-1 ligation by PD-1 ligand 1 (PD-L1) and PD-L2 present on APC dephosphorylates effector molecules in the TCR signaling cascade, as well as in the STAT5 pathway[128]. Constitutive PD-1 expression in Treg is part of a homeostatic loop that regulates Treg activation and proliferation by counterbalancing TCR signaling and continuous IL-2 stimulation[127,129]. Recent work by Asano *et al*. further clarified this equilibrium by assessing the role of PD-1 expression during low-dose IL-2 treatment of cGvHD[128]. In this setting, PD-1 expression was increased only in memory Treg. Naïve Treg proliferation depended on PD-1 blockade, which simultaneously increased the expression of apoptotic molecules, confirming the role of PD-1 in halting hyperactivation and preventing T cell exhaustion. Interestingly, the authors suggested that, after starting IL-2 therapy, the rate at which PD-1 expression was increased by Treg when compared to Tcon could be indicative of the treatment outcome.

CTLA-4, another co-inhibitory molecule of the CD28 superfamily, is homologue to co-stimulatory receptor CD28[151]. This molecule is constitutively expressed in Treg and essential for systemic homeostasis, as its abrogation leads to lymphoproliferative disorders and fatal autoimmune disease in mice[152]. Recent studies in mice suggested T cell proliferation was regulated by both CD28 and CTLA-4, with the former triggering proliferative pathways and the latter acting as an intrinsic break[54]. CTLA-4 has also been associated with Treg suppression. Due to its higher affinity and avidity towards co-stimulatory molecules of the B7-family (i.e. CD80 and CD86) compared to CD28, it has been suggested that CTLA-4$^+$ Treg could outcompete Tcon in CD80/CD86 ligation[132]. In this study, interaction of CTLA-4 with CD80 and CD86 downregulated CD80 and CD86 expression in APC, impairing short-term T cell activation by APC.

Finally, CD39 is an ectonucleotidase that hydrolyzes both adenosine triphosphate and diphosphate (ATP and ADP, respectively) into adenosine monophosphate (AMP). It has been shown that this enzyme works in tandem with CD73, inactivating extracellular proinflammatory ATP into adenosine[153]. Within CD4$^+$ T cells, CD39 is almost exclusively expressed in Treg (in frequencies of up to 80%), and its expression is positively correlated with Foxp3 expression, whilst CD73 is broadly expressed in Treg and Tcon[134,137]. Accordingly, some authors have used CD39 expression to distinguish activated Treg subsets. Indeed, in milieus that mimicked pro-inflammatory conditions *in vitro*, CD39$^+$ Treg showed higher resistance to conversion into Th1- and Th17-like subsets in the presence of low-dose IL-2[117]. Furthermore, CD39$^+$ Treg were able to suppress IL-17 production by Th-17 cells[136]. Nevertheless, functional CD39 has been found in non-suppressive CD4$^+$ FoxP3$^-$ T cells within inflamed sites, suggesting that expression of this protein is not sufficient for acquisition of regulatory functions by T cells[135]. Conversely, CD39$^-$ Treg may also suppress proliferation and IFN-y production by Tcon[136], hence CD39 expression alone may not be indicative of a particular Treg subtype.

Even though expression of CTLA-4 and CD39 can be correlated with Treg suppressive function, the list of molecules associated with particular modalities of function is ever growing. In the second part

of this section, T cell activation markers that may define activated Treg subsets with different modalities of function are summarized.

The requirement for TCR stimulation in Treg activation and suppression has been established both in mice[54,55] and in human[56,154] experiments. Usually, TCR stimulation is achieved by interaction with MHC in APC. Interestingly, Baecher-Allen *et al.* found that T cells could express Ag-presenting HLA-DR molecules, and it was suggested T-T cell contact involves TCR engagement by MHCII as well as CD2 engagement by cell adhesion molecules such as lymphocyte function-associated antigen 3 (LFA3) [138]. HLA-DR expression was associated with an activated T cell phenotype; yet, in Treg subsets, it represented different suppression modalities. The authors have shown that even though both HLA-DR-positive and -negative Treg subsets require T-T cell contact for suppression, HLA-DR$^+$ Treg were involved in early contact-dependent suppression, whilst Treg with low expression of HLA-DR were responsible for late cytokine-mediated suppression. Similarly, Ito *et al.* found that expression of ICOS can identify two Treg subsets with different cytokine secretion profiles[140]. While both subsets were anergic and equally suppressive, ICOS$^+$ Treg secreted higher levels of IL-10 and ICOS$^-$ Treg expressed higher levels of the latent version of TGF-β, which was membrane-bound (mTGF-β). Although ICOS expression was upregulated on ICOS$^-$ Treg upon activation, IL-10 secretion was not similarly increased. Interestingly, the authors found that these subsets presented different survival and proliferation mechanisms. Overall, it seems ICOS expression in *ex vivo* Treg may characterize phenotypically distinct subsets more than just differences in activation status.

It has been previously shown that activated Treg and Th clones are capable of producing mTGF-β, yet secretion of the bioactive form of this molecule was only found in activated Treg clones[155]. The same group has also demonstrated that Glycoprotein A repetitions predominant (GARP) and latency associated peptide (LAP) are molecules involved in the expression and activation of TGF-β[141]. LAP binds to mature TGFβ to convert it to its latent form, and GARP anchors latent TGF-β on the cell surface, forming mTGF-β. The authors have also shown that LAP was transiently expressed in activated Treg and Th cells, whilst GARP was upregulated only in Treg after TCR stimulation. Although induced GARP expression was sufficient for cells to bind latent TGF-β, it did not result in active TGF-β secretion. Hence, the means by which Treg are able to secrete active TGF-β are still unknown. In another report, only the expression of GARP was positively correlated with Foxp3 expression and suppressive capacity, yet both GARP$^+$ and LAP$^+$ Treg were highly suppressive[142], hinting at a role for this complex in TGF-β-mediated suppression mechanisms. Furthermore, a GARP$^-$CD25$^+$ subset that included more IL-17-producing cells than their GARP$^+$ counterpart was detected. Interestingly, the authors found different frequencies of CD25$^+$GARP$^+$ and CD25$^+$Foxp3$^+$ subsets in HIV-infected patients, suggesting that GARP allowed for better discrimination between *bona fide* Treg and activated Tcon. Albeit interesting, these differences can be related to low Treg purity in the initial cell population and to the strong mitogenic stimulus used for cell activation. Still, TGF-β-induced Treg did not express LAP[63] nor GARP[142].

Helios is a highly conserved transcription factor that is expressed in a small fraction of human Treg and is reportedly involved in Treg development and maintenance[156]. In a recent study, Elkord *et al.* investigated the correlations between GARP/LAP and Foxp3/Helios on T cells isolated from PB[143]. The authors found GARP/LAP expression could be related to Helios expression, and not to Foxp3

expression, which may help explain the results that had been described for HIV patients' samples. Furthermore, $GARP^+$ cells secreted IL-10, whilst their negative counterparts secreted IFN-γ only or both IFN-γ and IL-10. Because these subsets were found on total activated T cells within PBMC and their suppressive function was not assessed, it is unclear whether the same subsets can be found within Treg.

Activated T cells express a large group of tumor necrosis factor receptor (TNFR) superfamily members. While many are absent in Treg, some are expressed upon appropriate activation, like TNFR2, OX40 and 4-1BB (CD137)[157]. TNFR1 and 2 preferentially bind to soluble TNF and membrane-bound TNF, respectively[158]. It has been shown that TNFR2 is highly expressed in activated Treg, and co-expression of CD25 and TNFR2 identified highly suppressive Treg subsets with high expression of Foxp3 in PB[144]. Interestingly, He *et al.* have shown that *ex vivo* polyclonal expansion of low purity Treg in the presence of a TNFR2-agonist resulted in a homogenous population with a characteristic Treg phenotype and enhanced suppressive ability[145]. However, this expansion was performed in the presence of rapamycin, so there may be rapamycin-induced Treg in the final population. Even though Foxp3 expression seemed stable upon re-stimulation in a pro-inflammatory environment, it was unclear whether suppressive function was maintained. Also, it remains to be determined if this strategy would be of benefit in an antigen-specific expansion setting.

OX40 is primarily described as a costimulatory molecule that provides prosurvival signals to Tcon[150], yet it has been reported that Tcon and Treg may express OX40 upon appropriate activation[157]. There are contradictory reports on the role of OX40 in Treg. Some reports have shown that triggering OX40 leads to Treg expansion and subsequent exhaustion with loss of suppressive function[150], while others claim $OX40^+$ Treg were highly proliferative and able to specifically suppress Th1 responses[52]. Voo *et al.* used mice to develop monoclonal antibodies (mAb) targeting human OX40[150]. Within a pool of plate-bound hOX40 mAb clones tested in parallel, it was shown that only some of them could be potent inducers of T_{eff} proliferation and blocked Treg suppressive function in a dose-dependent manner. Additionally, high doses of hOX40 mAb had a pro-apoptotic effect on Treg, suggesting that the decreased suppressive function reported by the authors could be a result of both lower Treg viability and increased T_{eff} resistance to suppression. Interestingly, some of these mAb clones were able to block Treg function only in the absence of APC. Inversely, Piconese *et al.* assessed existing Treg pools *in vivo* and reported that total Treg frequency could be directly correlated to the frequency of $OX40^+$ Treg[52]. OX40 seemed to play a role in Treg expansion, as expression of OX40 was associated with a proliferating activated phenotype according to expression of Ki-67 and CD39. In addition, $OX40^+$ Treg isolated from PB were more suppressive than their negative counterparts. However, suppression was not dependent on this interaction, as OX40 blockade did not abrogate Treg suppressive function. Interestingly, the authors found that $OX40^+$ Treg specifically suppressed Th1 responses. Overall, OX40-OX40L interaction may influence both Treg proliferation and function, but OX40 does not seem to define a particular Treg subset.

4-1BB (also known as CD137), another member of the TNFR superfamily, has been extensively used in the detection of alloreactive T cells[159]. Litjens *et al.* showed that 4-1BB upregulation in T cells after antigenic challenge was transient, albeit with similar profiles amongst different activation stimuli,

peaking at 24 hours post-stimulation[147]. More importantly, 4-1BB expression was found in a small subset of cytokine-producing cells, and isolated allo-reactive 4-1BB$^+$ cells showed higher proliferative capacity upon re-stimulation. Additionally, Litjens' group and others have assessed the potential of this marker for Ag-sp Treg isolation from blood[148] or after *in vitro* expansion[77]. In order to distinguish activated Treg from Tcon, 4-1BB expression was assessed together with that of CD40L. CD40L is a member of the TNF superfamily and an established activation marker for Tcon[160]. It was described that, in Tcon, CD40L expression peaks first at 6 hours post-activation and then again at 24 hours post-activation; yet, in Treg, it only peaks 12 hours after activation and remains high until 24 hours post-activation[148]. Interestingly, CD40L$^+$ Treg displayed enhanced proliferation and suppressive abilities after re-stimulation with the original Ag. However, these cells also expressed pro-inflammatory cytokines after polyclonal stimulation. In fact, it has been reported that expression of both 4-1BB and CD40L could be a sign of unstable Treg, according to Foxp3 demethylation status[149]. Schoennbrunn *et al.* showed that Treg increased 4-1BB expression in a TCR-dependent way and faster than Tcon, while the pattern of CD40L expression was reversed[77]. Thus, converse expression of 4-1BB and CD40L allowed for the detection and isolation of Ag-sp Foxp3$^+$ Treg in short-term (16h) co-cultures. Isolated and subsequently expanded Ag-sp 4-1BB$^+$CD40L$^-$ Treg mostly maintained a signature Treg phenotype, and were highly suppressive of T_{eff} in response to both original and control stimulators, although slightly more responsive to the original stimuli. This protocol entailed a two-step isolation procedure, in which Treg (CD4$^+$CD25^{high}CD127^{low}) were isolated and co-cultured with CD3-depleted allo-PBMC in 16h co-cultures, after which 4-1BB$^+$CD40L$^-$ Treg were re-isolated and polyclonally expanded. After longer expansion cultures, staining with these markers was not sufficient to distinguish between activated Treg and contaminating Tcon, since there was up to 30% of phenotype overlap between these two cell populations. Thus, the second isolation step was required. In a different approach, Treg were re-stimulated after expansion in order to identify antigen-activated Treg[149]. However, 4-1BB expression was also increased after polyclonal activation, suggesting this is a marker of activation rather than specificity.

Activated Treg may also express other markers, like activation marker CD69[82,161] and co-stimulatory marker CD27[86,162], yet these have not been related to Treg subsets with particular suppressive function nor did they allow for unambiguous discrimination between activated Tcon and Treg. For example, Sagoo *et al.* showed that activated Treg from polyclonal and Ag-sp expansion cultures expressed CD69 and CD71, and the only difference was that the frequency of double positive cells within polyclonally expanded Treg was much higher[82]. Surprisingly, Schoenbrunn *et al.* found that CD69$^+$CD40L$^-$ T cells had much lower Foxp3 expression than 4-1BB$^+$CD40L$^-$ T cells, so it is possible that activated Tcon were present in these cultures[77].

Finally, one thing to keep in mind is that the activation of downstream signaling pathways by different kinases may play a significant part in the function of expanded Treg. Particularly, NIK is a kinase that links, amongst others, TNFR2, OX40 and 4-1BB to the activation of non-canonical NF-κB transcription factor[163]. Interestingly, Polesso *et al.* have shown that constitutive expression of NIK in mouse Treg (NIK-Treg) impaired their suppressive function and, in iTreg, it was completely abrogated[164]. NIK-Treg produced pro-inflammatory cytokines regardless of Foxp3 expression. Contrarily, in human Treg, it has

been shown that stabilization of NIK expression, through TNFR2 ligation and addition of exogenous IL-2, led to increased Treg proliferation and unaltered suppressive function [146]. For this reason, it is vital when characterizing *in vitro* expanded Treg to assess not only their phenotype but also to demonstrate their suppressor function and ability to modulate the cytokine milieu in cocultures with T_{eff}.

2.2. Mechanisms of suppression

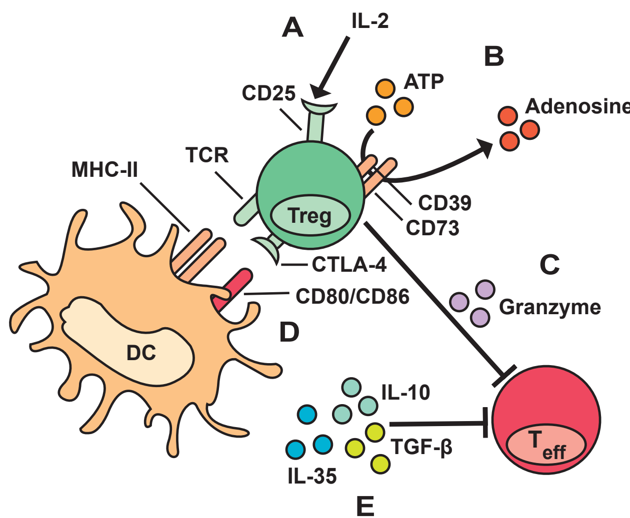

Figure 3: Mechanisms involved in suppression by Treg.
Treg may suppress effector T cells through a number of mechanisms. While some of them may affect the effector T cells directly, namely the consumption of IL-2 (A), the conversion of ATP to adenosine (B) and the production of granzymes (C), others involve the modulation of APC, for example, by downregulation of co-stimulatory molecules (D). Additionally, immunomodulatory cytokines such as IL-10, TGF-*β* and IL-35 (E) may also play a role in suppression of inflammation at target sites.

Because most of the pathways involved in Treg function are still widely unclear, the mechanisms of Treg suppression are currently defined by their outcomes. So far, it is known that Treg may suppress activation, function and/or expansion of a range of immunocompetent cells by targeting responders directly or by modulating APC (**Fig.3**), as reviewed by Shevach *et al.*[165]. Treg activation and/or T-T cell contact is required for optimal Treg suppressive function, yet suppression may be mediated by cell-extrinsic factors, such as TGF-β[166]or IL-10[167]. Due to the difficulty in determining these mechanisms *in vivo*, usually Treg function is assessed by means of mixed leukocyte reactions *in vitro*, commonly called suppression assays (SA). In most SA set-ups, it is possible to ascertain differences in T_{eff} proliferation and cytokine secretion depending on the presence and dilution of Treg. In cases where only Treg and T_{eff} are co-cultured, Ab-coated beads or plate bound Ab are required for mitogenic activation of cells. The most commonly used Ab are anti-CD3 for polyclonal TCR signaling and/or anti-CD2 to mimic T-T cell interactions, together with anti-CD28, which delivers signals to T cell co-stimulatory molecules. It is also possible to perform SA in which APC provide activation in an MHC-dependent way, as well as present target Ag. Whilst in the former SA setting the target of Treg suppression are undoubtedly T_{eff}, in the latter the suppression targets may be APC, T_{eff}, or both.

I*n vitro* SA have allowed for some groups to study the mechanisms employed by Treg for the suppression of inflammatory responses. Here, these mechanisms will be described according to their target, starting by those that target T_{eff} directly, those that modulate APC and finally cell extrinsic and/or infectious tolerance mechanisms. Yet, it should be considered that some mechanisms may have more than one effect or target. Furthermore, it is important to point out that *in vitro* SA may not reflect the myriad of suppression mechanisms employed by Treg *in vivo*.

a) Suppression of effector T cells and immune escape

Suppression of T_{eff} proliferation and cytokine secretion may be achieved through metabolic disruption and induced cytolysis. Because of their high expression of CD25, together with constitutive expression of the other two components of the high affinity IL-2R, it has been proposed that Treg could compete with Tcon for soluble IL-2, acting as a "sink" that removes IL-2 from culture – thus inducing apoptosis of T_{eff} [168]. However, this hypothesis remains controversial. On a report of genetic studies in mice by Chinen *et al.,* it was suggested that IL-2 capture by CD25 was essential for the suppression of $CD8^+$ T cell activation, whilst suppression of Tcon was independent of CD25[169]. These observations are possibly due to the more robust response of $CD8^+$ T cells to high levels of IL-2, which were dispensable for suppression of Tcon. Indeed, it has been shown that pre-activated Treg inhibit both Tcon proliferation and production of IL-2 mRNA[170]. Addition of exogenous IL-2 only partially abrogated this suppression: whilst Tcon proliferation was restored, cytokine mRNA production was still inhibited, suggesting other mechanisms are at play in Treg-mediated suppression.

As mentioned before, IL-2 triggers the STAT5 pathway, which is vital for Foxp3 expression. While IL-2 consumption may not be the most relevant mechanism of Treg-mediated suppression, the presence of IL-2 in suppression assays enhances Treg suppressive potency[111,125].

Treg can also disturb T_{eff} metabolism by catalyzing ATP conversion into ADP through CD39 and further decomposing it into adenosine, an anti-inflammatory molecule that, amongst other functions, reduces cytokine production by T_{eff} and promotes T cell anergy [153]. Recently, it was shown that suppression of T_{eff} proliferation by $CD39^+$ Treg resulted from adenosine signaling to adenosine receptor 2a ($A_{2a}R$), which was upregulated upon T_{eff} activation[134]. Schmidt *et al.* have also reported that Treg were able to modulate calcium (Ca^{2+}) signaling events, decreasing NF-κB pathway activation in Tcon[171]. Inhibition of Ca^{2+}-signaling lead to rapid suppression of cytokine production, which was maintained after removal of Treg from the co-culture. Finally, activated Treg may exhibit cytotoxic functions. Grossman *et al.* found that activated Treg expressed Granzyme A and exhibited perforin-dependent cytotoxicity against activated $CD4^+$ and $CD8^+$ T cells and moDC[172]. Opposingly, other authors found no expression of Granzyme A nor B by Treg, and further suggested Tcon could escape suppression by secreting Granzyme B and consequently inducing apoptosis of highly suppressive Treg subsets[173].

It is possible different results are derived from different SA settings, as well as different Treg pre-activation conditions. It has also been shown that SA conditions may lead to either loss of suppressive function by Treg, Th cell escape from suppression, or both. For example, George *et al.* demonstrated that potent stimulators of Th cell proliferation, such as DC with high levels of co-stimulatory markers and high antigen dose, allowed Th cells to escape Treg-mediated suppression of proliferation[174]. Similarly to what was described for the addition of exogenous IL-2, where only proliferation was restored and not the production of IL-2 mRNA[170], in this report Th cells that escaped suppression of proliferation still secreted lower amounts of cytokines[174].

In conclusion, it seems likely that different mechanisms can result in distinct types of suppression, such as decreased proliferation, inhibited cytokine production and induced apoptosis. Conversely, the same mechanism may have different effects on different types of responders. Finally, the activation

status of APC can modulate Treg activation and suppressive capabilities, but the opposite is also true. In the following section, we describe some mechanisms Treg may employ to modulate APC function.

b) APC modulation by Treg

DC are commonly used in SA to better mimic *in* vivo activation conditions. Modulation of APC function by Treg may result in enhanced suppression by downregulating T_{eff} activation[175], or by triggering cell-extrinsic mechanisms that result in T_{eff} impairment[133]. Early studies reported that co-culture with Treg affected the function of DC as APC[132]. DC co-cultured with Treg were rendered inefficient stimulators of Tcon proliferation, mostly due to their reduced expression levels of co-stimulatory molecules (CD80 and CD86) and of activation markers (CD83). After performing transwell cultures, the authors were able to conclude that downregulation of CD86 was contact-dependent. CTLA-4, a co-inhibitory molecule that binds to CD80 and CD86, is constitutively expressed by Treg and upregulated in all T cells upon activation[176]. Recently, it was shown that constitutive CTLA-4 expression in Treg provides competitive advantage for CD80/CD86 ligation in DC, when compared to Tcon, which require activation for CTLA-4 expression[130,177]. Besides preventing Tcon activation, when Tcon-DC clusters were already formed, CTLA-4$^+$ Treg could physically engage Tcon, as well as disrupt said clusters[130]. Furthermore, CTLA-4 could remove CD86 from DC surface by trans-endocytosis, thus abrogating CD28 costimulation on T_{eff}[131]. Finally, it has been shown that CTLA-4-induced signalling increased IDO release by DC[133], and it was suggested that IDO secretion could lead to Tcon starvation by tryptophan degradation, as well as cell cycle arrest by increasing levels of pro-apoptotic metabolites. Interestingly, IDO secretion by DC has also been described as a way to enhance autologous Treg expansion *in vitro*, as well as expand iTreg[99], so it is possible both mechanisms act together in order to reduce inflammation *in vivo*.

Other mechanism by which Treg may inactivate DC is by transferring cyclic AMP (cAMP) through gap junctions[178]. In a very interesting study with neonatal Treg, it was shown that although neonatal Treg presented higher levels of cAMP, there was lower expression of CTLA-4 when compared to adult Treg. Neonatal Treg were incapable of disrupting Tcon-DC clusters, but decreased DC and Tcon activation[177]. Some subsets of activated Treg may also express the adhesion molecule LAG-3, particularly at tumour sites[179]. LAG-3 is a CD4 homologue that binds to MHCII with high affinity, and has been reported to decrease DC maturation in mice[180].

Besides the direct effects of Treg on T_{eff} and DC, it has also been suggested that Treg may exert suppression through compensatory cell-extrinsic mechanisms, either by their own cytokine production or by stimulating other cells to produce and secrete immunomodulatory cytokines. These mechanisms will be explored in detail below.

c) Cell-extrinsic suppression and induction of tolerance

Immunomodulatory cytokines, like TGF-β, IL-10 and IL-35, can also play a role in the suppression of inflammation at target sites. As previously described, Treg are able to bind and present high amounts of mTGF-β[141] and secrete biologically active TGF-β upon appropriate activation[155]. However, the

requirement for TGF-β in Treg suppression is still controversial. Firstly, no evidence has been found for the secretion of soluble TGF-β by Treg so far[166,181]. Secondly, while TGF-β deficient Treg are incapable of suppressing the development of *in vivo* models of disease such as colitis[166], the addition of TGF-β neutralizing antibodies does not seem to impair Treg suppression *in vitro*[182]. Furthermore, Oberle *et al.* found that while addition of exogenous TGF-β had some inhibitory effect on IL-2 transcription in Tcon upon activation, blockade of mTGF-β signalling did not significantly reduce early Treg-mediated suppression of cytokine production[170] . It is possible different suppression mechanisms can be recruited according to the activation stimuli Treg and/or T_{eff} are submitted to, before or during suppression assays. Some studies have shown that LAP expression could identify highly suppressive activated Treg[63,143], possibly due to its role in the formation of latent TGF-β. Similarly, it has been suggested that presentation of mTGF-β by activated Treg was required for the suppression of activated Tcon proliferation and mediated infectious tolerance by inducing naïve T cell conversion into iTreg with Foxp3 expression and presentation of mTGF-β[181]. Overall, these results suggest immune regulation mechanisms can be mediated by the presentation of the latent form of TGF-β by Treg, but it may not be required for Treg suppressive function.

IL-10 is an anti-inflammatory cytokine that acts primarily on APC, thus reducing T_{eff} activation[183]. IL-10-producing Treg can be found in the *lamina propria* and are thought to play a role in mucosal tolerance *in vivo*[184]. *In vitro* reports have shown that IL-10-deficient Treg were fully suppressive and addition of anti-IL-10 to SA had no effect on Treg suppression of T_{eff} activation[182]. Additionally, no direct role has been found for IL-10 in preventing Th1 activation in SA, albeit *in vivo* production of IL-10 by Treg was required to suppress IFN-γ secretion by T_{eff} in the inflamed skin[185]. One of the ways Treg-derived IL-10 may modulate inflammatory milieus is by tolerizing DC. In a report by Ito *et al.*, expression of CD86 by DC was downregulated upon co-culture with Treg in an IL-10-dependent fashion[140]. Also, the authors reported that circulating $ICOS^+$ Treg presented higher levels of intracellular IL-10 after strong mitogenic activation *in vitro*, whilst $ICOS^-$ Treg expressed higher levels of mTGF-β, suggesting these two subsets had different regulatory modalities. Interestingly, there seems to be a relationship between LAP expression and secretion of IL-10, as activated $GARP^+LAP^+$ Treg were found to secrete the highest levels of IL-10[143]. Because these subsets were detected in response to strong activation stimuli, they may represent iTreg subsets contaminating the cultures, as it has been described that iTreg may secrete IL-10 and modulate DC function[186]. The presence of TGF-β has also been reported to convert Th17 cells into Foxp3-expressing iTreg that secreted IL-10[187]. Studies by Chattopadhyay in mice have further shown that TGF-β-iTreg were able to upregulate March 1, a ligase that promotes the degradation of MHCII and CD86[186]. In this report, CD83, which prevents the association of MHCII with its targets, was effectively downregulated by iTreg-derived IL-10, whilst downregulation of CD80 and CD86 was dependent on the expression of both CTLA-4 and IL-10. Finally, IL-10 may be secreted by DC[132,188]. In this case, Treg were able to modulate anti-inflammatory milieus by increasing IL-10 secretion in DC. Additionally, in mice, incubation of moDC with exogenous TGF-β_2 resulted in moDC with a tolerogenic phenotype, with enhanced IL-10 and IDO secretion profiles[133]. In turn, these moDC increased Treg numbers *in vitro* and *in vivo*. The presence of IL-10 *in vitro* may also induce the

expansion of a suppressive Foxp3⁻ subset, currently known as Tr1, which produce high levels of IL-10 themselves[189]. In conclusion, IL-10 can be secreted by a broad range of cells, yet it seems its primary effect is on APC modulation. Even when there is induction of Treg by IL-10, it stimulates additional IL-10 secretion, which will most likely act on APC.

IL-35 is a recently described immunosuppressive cytokine composed of IL-12α and IL-27β chains, which are encoded by two separate genes[190]. Not only was it shown that, in mice, recombinant IL-35 alone suppressed Tcon proliferation to levels similar to those achieved by activated Treg, but ectopic expression of IL-35 on Tcon was also shown to increase their expression of IL-35 in an antigen-driven proliferation assay, which confers them with regulatory potential[190]. Furthermore, treatment of Tcon with IL-35 *in vitro* induced the expansion of a Foxp3$^-$ subset that was highly suppressive through their own secretion of IL-35[191]. It seems that, like TGF-β and IL-10, this cytokine may be involved in infectious tolerance mechanisms *in vivo*, which are characterized by conversion of non-Treg into iTreg with suppressive capabilities. Still, iTreg induced by IL-35 were able to secrete IL-35 but not TGF-β nor IL-10. Interestingly, in mice, independent subsets within $_{eff}$Treg could be defined by their reciprocal secretion of IL-10 and IL-35[167]. In these subsets, IL-35 expression could be associated with Foxp3 expression, whilst IL-10 secretion could not, yet was associated to granzyme secretion. Ablation of any of these subsets in *in vivo* models of disease led to impaired tolerance, with apparently synergistic effects[167,191].

Differences in Treg suppressive mechanisms may be resultant from disparities in Treg activation, such as source and duration of the stimuli. Another thing to consider is the setting in which Treg are exerting suppression. Baecher-Allan *et al.* have shown that activated Treg displayed distinctive suppressive functions that were suitable for different suppression settings[138]. Treg with different suppressive functions could be identified by their expression of HLA-DR. Briefly, in SA that simulated activation through T-T cell contact using anti-CD2 beads, only HLA-DR$^+$ Treg could exert early suppression of both T_{eff} proliferation and cytokine secretion, suggesting Treg can suppress T_{eff} responses via TCR-MHC interaction. In fact, previous studies had shown that addition of anti-HLA-DR to SA abrogated Treg suppressive function in the absence of APC[192]. Besides, increased levels of Th2 cytokines, such as IL-4, and IL-10 were found in SA that simulated activation through T-T cell contact as well as T-APC cell contact with anti-CD3 beads[138]. Still, increased levels of soluble cytokines were only found in co-cultures with HLA-DR$^-$ Treg. In conclusion, the authors proposed that HLA-DR$^-$ Treg were capable of cytokine and contact-mediated suppression, whereas HLA-DR expression could identify a mature population that exerted only the latter. Recently, these results have been partially validated by Dong *et al.*, who performed single cell analysis on 24-hour aCD3/aCD28-activated Treg and confirmed the heterogeneity of the human Treg compartment[139]. It was verified that within HLA-DR$^-$ Treg, those negative for CD45RA expressed IFNG (although in significantly lower amounts than Tcon), IL4, IL17 and IL10 transcripts. Cytokine transcription was absent after a 2 week-culture. These results indicate that, upon prolonged and/or strong activation stimuli, Treg shift towards a more mature phenotype that relies on cell-cell contact over cytokine secretion to regulate immune responses.

3. What is an "antigen-specific" Treg?

"Antigen-specific" is a very broad term that has been increasingly used in the literature to characterize different types of Treg. In the literal sense, it describes Treg that are specific for the suppression of a particular antigen. However, as it was described above, there are many reports of Treg expanded with APC from allogeneic donors that, on subsequent SA, preferentially suppress T_{eff} proliferation in response to the allogeneic donor used for expansion[72,87,125]. In this case, Ag-sp Treg could be defined as "recipient-specific". It is very difficult to expand Treg that are truly Ag-sp, because most of the peptides that the Treg react to are unknown, let alone the specific sequence that could be identified as an Ag. Additionally, the TCR repertoire of Treg found in circulation is so broad that it is safe to assume that the precursor frequency of Treg specific for a particular Ag would be extremely low. One way around this issue, that was recently explored, is the production of Treg with engineered TCR that are specific for a known Ag[176,193]; however, this approach can only be used when the target Ag is known, which is rarely the case.

When one is envisaging Ag-sp Treg expansion for subsequent clinical translation, it is important to clarify the specificity of Treg-mediated suppression, which requires choosing the appropriate conditions to test their functional ability *in vitro*. It is possible Treg acquire different modalities of suppression according to 1) Treg pre-activation and phenotype, and 2) Presence of TCR stimulation and Ag-presentation during suppression. These concerns have all been addressed separately in the past, and the most interesting results are described here. Most groups worked with mice, which allow for experimental flexibility, yet it is important to keep in mind that validation of the proposed hypothesis *in vitro* using human samples may generate different results.

Activation of freshly isolated T cells by aCD28, aCD3 and/or aCD2 beads during SA leads to polyclonal activation of both Treg and T_{eff}, and may allow for immune escape by T_{eff}[192]. If present, Treg suppression in this setting is nonspecific. Within suppression milieus where there is TCR stimulation, there is also the chance that Treg may suppress responding T_{eff} regardless of their Ag-specificity or MHC-restriction. In fact, studies in mice have shown that freshly isolated Ag-sp Treg may suppress autologous Tcon of different Ag specificity in the absence of cognate Ag[106], albeit in a less effective way than when Treg and Tcon were of the same Ag-specificity. These observations were attributed to Treg constitutive regulatory activity. Because self-DC were used as APC in these SA, there is also the possibility Treg suppression was mediated by recognition of self-MHC in the APC or by cell-extrinsic modalities of suppression. Furthermore, as described in previous chapters, activated Treg increase their expression of MHCII, which was shown to be required for suppression of freshly isolated Tcon[192]; hence, during SA in the absence of APC, it is possible Treg may engage and suppress T_{eff} by productive TCR-MHC interactions in a T-T cell suppression setting. Of note, Thornton *et al.* have shown that, once Treg were activated, suppression through T-T cell interaction was nonspecific[194]. The authors found that T_{eff} of the same or different specificity from Treg were similarly suppressed, as long as the APC used for Treg expansion were also in culture. It would be interesting to ascertain whether MHCII expression would still be required for the suppression of Tcon with different specificities from Treg.

Pre-activation and expansion of Treg with APC by MHC-TCR interaction was demonstrated to enhance Treg suppressive activity[192]. When there is Treg pre-activation (and expansion) by allogeneic or autologous APC loaded with Ag, Treg expansion will be MHC-restricted and/or Ag-specific. Likewise, Ag-sp expanded Treg (eTreg) may present different suppressive functions, according to their specificity towards MHC-Ag complexes presented by the APC in SA. Of note, because activated Treg display enhanced suppressive capabilities, it is common that Ag-sp Treg in high Treg:T_{eff} ratios (such as 1:4 or 1:1) similarly suppress all responses in a nonspecific way[125]. One of the ways to characterize the specificity of expanded Ag-sp Treg populations is to focus on Treg clones. Interestingly, Veerapathran *et al.*[85] expanded mHA-specific Treg clones by co-culturing Treg with allogeneic DCs from an HLA-matched sibling of different gender in a limiting dilution analysis (LDA) setting. LDA allowed for thorough characterization of each subset of Ag-sp Treg that was expanded (almost down to a single-cell level) regarding suppressive modalities and specificity. In this case, each Treg clone was screened for suppression of proliferative responses to known H-Y associated genes, and then expanded *in vitro*, thus obtaining highly specific Treg cell populations. As previously mentioned, this exercise was only possible because the authors had access to MHC-matched siblings of opposite genders and there is a library of known H-Y mHA. However, it is likely that these mHA represent a tiny fraction of the full range of Ag recognized by Treg. Thus, most reports on Ag-sp Treg describe Treg that are specific for a pool of unknown Ag.

Despite the many reports on expansion of Ag-sp Treg[195–197], the underlying mechanisms that shape Treg specificity are still widely unknown, and a basal level of nonspecific suppression can be found in most reports, though most times it is overlooked. Although it is known that Treg may suppress the activation of $CD4^+$ and $CD8^+$ T cells, few reports have focused on the mechanisms that allow for specific suppression of different types of responders. Both $CD4^+$ and $CD8^+$ T cells receive co-stimulation via CD80/CD86 ligation; however, $CD4^+$ T cells detect Ag:MHCII complexes, whilst $CD8^+$ T cells interact with MHCI. It is known that DC present Ag through MHCII and may display Ag:MHCI complexes by cross-presentation, however it is unclear how Treg are able to detect the specificity of $CD8^+$ responders and suppress them accordingly, as Treg TCR is thought to recognize MHCII only. A recent study by Vosken *et al.* proposed that there was no requirement for cell-cell contact in the suppression of $CD8^+$ T cells by polyclonal Treg[65]. One option is that Treg adjust their suppressive modality according to their specificity towards Ag:MHCII complexes in DC, and $CD8^+$ suppression is achieved by nonspecific mechanisms, such as IL-2 consumption[169]. Finally, it has also been reported that $CD8^+$ T cells may present dual MHCI and MCHII recognition[198], so it is possible that there is also some interaction between Treg expressing MHCII and $CD8^+$ T cell's TCR.

In conclusion, when characterizing eTreg, it is vital to assess their phenotype, which may allow for the identification of particular subsets, and their function, which may be complementary to the phenotype. When dealing with Ag-sp Treg, attention should be paid to defining specificity, and how it is present in the suppression of different types of responders. Ideally, the phenotype and function of Ag-sp Treg subsets would reflect that of the subsets identified in polyclonal eTreg, but there is also the chance that different subsets can be found in each setting. Finally, it is likely that different conditions of expansion may also result in populations with different features.

AIMS AND THESIS LAYOUT:

The main aims of this work were to:

1) Establish reproducible protocols for Ag-sp Treg isolation and expansion *in vitro*;
2) Evaluate the degree of specificity in Ag-sp Treg-mediated suppression;
3) Characterize particular sub-phenotypes expressed by expanded Ag-sp Treg.

With these in mind, the Results section has been segmented into two main chapters. In **Chapter 1**, different optimization approaches to consistently expand *in vitro* Ag-sp Treg in good manufacturing practice (GMP) conditions are described. This includes selection and optimization of APC production in serum-free media, as well as different types of antigen presentation (direct/indirect) for Treg expansion and their resulting suppressive function.

In **Chapter 2**, we delve into the functional characterization of expanded Ag-sp Treg. We start by evaluating the specificity of Treg-mediated suppression in response to different levels of HLA matching between donors, according to the type of responders used in mixed lymphocyte reactions (MLR). Treg phenotypical markers are also examined in-depth, by applying pseudo-randomized strategies to identify and characterize Treg sub-populations.

Finally, the Discussion section brings all of the results together, and perspectives for future work are described.

MATERIALS AND METHODS

PBMC isolation and cryopreservation

Buffy coats of healthy donors were provided by the local blood bank (Instituto Português do Sangue e Transplantação, IPST, Lisboa, Portugal) and PBMCs were isolated by density gradient centrifugation using Ficoll® Paque PLUS (GE Healthcare, USA). Typically, on average, 900×10^6 PBMC could be isolated from 50 ml buffy-coats of a healthy donor. Roughly 200×10^6 PBMC from each donor were used for moDC differentiation and the remaining cells were cryopreserved in multiple aliquots. Briefly, PBMC were resuspended in RPMI 1640 (GE Healthcare) with 40% of heat-inactivated human serum (HS; human male AB plasma, Sigma-Aldrich, USA) at a density of around 100×10^6 PBMC/mL One mL of cell suspension was pipetted per 2-mL cryovial, to which 1mL of freezing media containing 40% RPMI 1640, 40%HS and 20%DMSO was added dropwise, to a final concentration of 50×10^6 PBMC/mL in 50% RPMI 1640, 40%HS and 10%DMSO. Where indicated, an aliquot of 5×10^6 PBMC was used to isolate genomic DNA using NZY Blood gDNA Isolation Kit (NZYTech, Portugal), according to the manufacturer's guidelines. DNA samples were sent for high-resolution genotyping of HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQA and HLA-DQB at Centro de Sangue e Transplantação de Lisboa, IPST.

CD4$^+$ cells isolation

When needed, PBMC aliquots were thawed in warm RPMI 1640 with 20% HS and washed. After 3-4 hours rest in RPMI 1640 with 10% HS in a humidified atmosphere at 37°C and with 5% CO_2, PBMC were washed, resuspended in PBS and then incubated with 100 µg/mL of DNase I (Sigma-Aldrich) for 20 min at 37ºC and with 5% CO_2, to prevent clumping. After incubation, dead cells and debris were washed off in PBS. PBMC were counted using a haematocytometer and resuspended in EasySep buffer (PBS with 2%HS and 1mM EDTA) at the recommended concentration (50×10^6 PBMC/mL). CD4$^+$ T cells were separated from PBMCs by positive selection, using EasySep Human CD4 Positive Selection Kit II (StemCell Technologies, Canada), which contains CD4-mAb and dextran-coated magnetic beads.

Treg isolation

In order to measure the proliferation of Treg during expansion co-cultures, we labelled CD4$^+$ cells with carboxyfluorescein diacetate succinimidyl ester (CFSE), using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen™, USA). Briefly, CD4$^+$ cells were counted using a haematocytometer, resuspended in PBS at 5×10^6 cells/mL and mixed with equal volume of CFSE diluted to 1µM, for a final concentration of 0.5µM. The cell suspension was vortexed and then incubated at 37°C and with 5% CO_2 to incorporate the CFSE. After 20 minutes of incubation, the labelled cells were placed on ice for 5 minutes to quench the reaction, washed twice in PBS and then resuspended in standard culture media, consisting of RPMI with 10%HS, 1% Penicillin-Streptomycin (PenStrep, Sigma-Aldrich) and 2mM of L-Glutamine (Sigma-Aldrich).

After an overnight rest at 37°C and with 5% CO_2, CFSE-stained CD4$^+$ cells were washed in PBS, counted using a haematocytometer, resuspended in PBS at 100×10^6 PBMC/mL and labelled for sorting experiments using 25µL/mL of the following antibodies: CD3 PerCP-Cy5.5 (Clone OKT3,

eBioscience™, USA), CD4 BV785 (Clone RPA-T4, Biolegend®, USA), CD25 PE (Clone 2A3, StemCell) and CD127 PE-Cy7 (Clone HIL-7R-M21, BD Biosciences, USA). After a 30-minute incubation at room temperature (RT), the labelled cells were washed, resuspended at 20–25x10^6 PBMC/mL in PBS with 2%HS and sorted under sterile conditions on a BD FACSAria™ II or on a BD FACSAria™ Fusion (BD). Single colour-labelled PBMC were used for marker settings and compensation controls. Treg ($CFSE^+CD3^+CD4^+CD25^{high}CD127^{low}$) and Tcon ($CFSE^+CD3^+CD4^+CD25^-$) were collected into Falcon tubes containing 3 ml of PBS with 20% HS. Treg and Tcon populations were analysed for purity post-sort and determined to be ≥ 99% pure. Typically, on average, the number of sorted Treg was around 0.5-1% of the labelled $CD4^+$ cells.

Phenotypic analysis of Treg

The complete phenotype of Day 0 Treg and Tcon, which were used as a phenotypic control, was evaluated by staining an aliquot the cell surface of each population for 15 minutes at RT with a mix of some or all of the following Ab: CD279 BV605 (PD-1, Clone EH12, BD), CD152 PE-CF594 (CTLA-4, Clone BNI3, BD), CD39 BV650 (Clone Tu66, BD), CD137 APC (4-1BB, Clone 4B4-1, BioLegend), CD154 (CD40L, Clone 24-31, BioLegend) and HLA-DR APCe780 (Clone LN3, eBioscience). For intracellular staining, surface-labelled cells were fixed and permeabilized using FOXP3 Fix/Perm Buffer Set according to the manufacturer's instructions, and then incubated for 30 minutes with FOXP3-eFluor450 (Clone PCH101, eBioscience).

The phenotype of aliquots of expanded Treg and Tcon was analysed using the following mAb: CD3 PerCP-Cy5.5 (Clone OKT3, eBioscience™, USA), CD4 BV785 (Clone RPA-T4, Biolegend®, USA), CD25 PE (Clone 2A3, StemCell), CD127 PE-Cy7 (Clone HIL-7R-M21, BD Biosciences, USA), CD279 BV605 (PD-1, Clone EH12, BD), CD152 PE-CF594 (CTLA-4, Clone BNI3, BD), CD39 BV650 (Clone Tu66, BD), CD137 APC (4-1BB, Clone 4B4-1, BioLegend), CD154 (CD40L, Clone 24-31, BioLegend) and HLA-DR APCe780 (Clone LN3, eBioscience). For intracellular staining, surface-labelled cells were fixed and permeabilized using FOXP3 Fix/Perm Buffer Set according to the manufacturer's instructions, and then incubated for 30 minutes with FOXP3-eFluor450 (Clone PCH101, eBioscience).

Single-color labelled PBMC or Compensation Beads (BD CompBeads, BD) were used for marker settings and compensation controls, and Rainbow Calibration Particles (BD) were used to assure the same settings were applicable to each batch of experiments. Samples were acquired as indicated, either on BD FACSAria™ III, BD LSRFortessa™ II or BD LSRFortessa™ X-20 (experiments with the complete mAb panel), and analysed using FlowJo software (BD). Corresponding Tcon controls were used for gate setting, and marker expression was compared with that of Day 0 Treg and that of Tcon (Day 0 and expanded). Of note, the expression of CTLA-4 was assessed on the cell surface, as it has been shown that it must be relocated to the surface for its inhibitory potential to be achieved[199].

Differentiation and characterization of moDC

Fresh PBMCs were enriched for $CD14^+$ cells using EasySep™ Human CD14 Positive Selection Kit II (StemCell), according to the manufacturer's instructions. The phenotype and purity in monocytes of the isolated $CD14^+$ cells were evaluated at the end the activation culture, with mAb directed against

Lin FITC (CD3 Clone OKT3, CD19 Clone HIB19 and CD56 Clone TULY45, all from eBioscience), CD14 V450 (Clone MφP9, BD), eBioscience), CD11c APC (Clone B-ly6, BD), CD86 PerCPe710 (Clone IT2.2, eBioscience) and HLA-DR APCe780 (Clone LN3, eBioscience). Compensation PLUS Beads (BD CompBeads, BD) were used for marker settings and compensation controls, and Rainbow Calibration Particles (BD) were used to assure the same settings were applicable to each batch of experiments. Samples were acquired on BD LSRFortessa™ II (BD), and analysed using FlowJo software (BD). Corresponding unstained controls were used for gate setting.

CD14$^+$ cells were seeded at a density of 1-2x10^6/mL in 6-well plates and the differentiation media was Cellgenix GMP DC Medium (Cellgenix GmbH, Germany) or X-Vivo 15 (Lonza, Switzerland) with 1% PenStrep, IL-4 (40 ng/ml; Peprotech, U.K.) and GM-CSF (50 ng/ml; Peprotech). Every two days, half of the media was changed and fresh cytokines were added to reach the initial concentration. After 5-6 days in culture at 37°C and with 5% CO_2, differentiated moDC were activated either with the gold standard cocktail, consisting of IL-1β (10 ng/ml), IL-6 (10 ng/ml), TNF-α (20 ng/ml, all from Peprotech) and PGE_2 (1 µg/ml; Tocris Bioscience, UK) for 48 hours, or with LPS (100ng/mL, Sigma-Aldrich) and IFN-γ (50 ng/mL, Peprotech) for 24 hours. Immature moDC were kept in differentiation media only. Cells that were cryopreserved for later use were removed from wells after 24 hours of activation, cryopreserved according to the protocol described above for PBMC, and upon thawing were incubated for 24 more hours with activation cocktail. The phenotype and viability of moDC were evaluated at the end the activation culture, by staining aliquots of moDC with a mix of the following mAb for 30 min at 4ºC: Lin FITC (CD3 Clone OKT3, CD19 Clone HIB19 and CD56 Clone TULY45, all from eBioscience), CD14 e450 (Clone 61D3, eBioscience), CD11c APC (Clone B-ly6, BD), CD86 PerCPe710 (Clone IT2.2, eBioscience), HLA-DR APCe780 (Clone LN3, eBioscience), CD80 PE (Clone L307, BD), CD83 PE-Cy7 (Clone HB15e, eBioscience), CD274 BV711 (PD-L1, BioLegend). Fixable Viability Dye e506 (eBioscience) was used to stain dead cells. Compensation PLUS Beads (BD CompBeads, BD) were used for marker settings and compensation controls, and Rainbow Calibration Particles (BD) were used to assure the same settings were applicable to each batch of experiments. Samples were acquired on BD LSRFortessa™ II (BD), and analysed using FlowJo software (BD). Corresponding unstained controls were used for gate setting. Live, mature moDC typically consisted of a population of CD14$^-$CD11c$^+$, CD86^{2+}HLADR^{2+}, CD80$^+$CD83$^+$ cells, as described previously [76]. MoDC were irradiated (30 Gy) before being used as APC for expansion co-cultures or suppression assays.

Preparation of lysate-loaded moDC

When indicated, moDC were loaded with lysates of PBMC from an allogeneic donor. Briefly, 10x10^6/mL PBMC were submitted to 3 freeze/thaw cycles in dry ice and at 37ºC, respectively. Afterwards, a spin down was performed and the supernatant was collected, removing cell debris, irradiated at 50Gy and stored at -20ºC. The concentration of protein on each sample was measured using Bradford Reagent (Sigma-Aldrich), per the manufacturer’s protocol. Quantification data was acquired on a microplate reader (Tecan Infinite M200) and analysed on Excel sheets. For allo-Ag and self-Ag loading, 50 µg/mL of cell lysate were added to corresponding moDC before activation. After an

overnight incubation at 37°C and with 5% CO_2, half the media of loaded moDC was replaced by fresh media, supplemented with the gold standard activation cocktail.

In vitro expansion of Treg

CFSE-labelled Treg (75x10^3/well) were expanded in the presence of allo-Ag for 14 d. Treg expanded by indirect allo-Ag or self-Ag presentation were co-cultured with self-moDC (previously loaded with allo-Ag or self-Ag, respectively) at a ratio of 1:1 or 1:4 Treg:moDC, while Treg expanded by direct allo-Ag presentation were co-cultured with allo-moDC at a ratio of 1:10 Treg:moDC. Expansion media was TexMACS Medium (Miltenyi Biotec, Germany) supplemented with 10 U/mL or 100 U/mL of recombinant human IL-2 (R&D Systems, USA), IL-15 (10 ng/ml; R&D Systems) and rapamycin (100 ng/mL, Sigma-Aldrich). Co-cultures were performed in 96-well, round-bottom plates. Starting on day 7, every 2–3 d, fresh culture medium supplemented with IL-2, IL-15 and rapamycin or only IL-2 (as indicated). If necessary, the wells were split into 48-well plates. Kinetics and Treg numbers were compared for the different culture conditions. Tcon (to use as controls) were expanded by direct allo-Ag presentation in the same conditions as Treg, but supplemented only with IL-2.

To assess the number of eTreg after co-culture, cells were removed from wells and 20 µL of cell suspension were added to 300 µL of PBS containing CD3-PerCP (Clone OKT3, eBioscience™, USA) and CD4 APC (Clone RPA-T4, eBioscience). Before acquisition, 50 µL of 123count eBeads Counting Beads (Invitrogen™) were added. Samples were acquired on the same machine as the phenotype tubes. The total number of cells was calculated by a ratiometric enumeration of cells:beads ratio, using the number of counted beads, the known number of beads in 50 µL of reagent, the number of counted $CD3^+CD4^+$ cells in 20 µL of cell suspension and the total volume of cell suspension after culture.

Where indicated, eTreg were labelled for sorting experiments using 25µL/mL of the following antibodies: CD3 PerCP-Cy5.5 (Clone OKT3, eBioscience™, USA), CD4 BV785 (Clone RPA-T4, Biolegend®, USA), CD25 PE (Clone 2A3, StemCell) and CD127 PE-Cy7 (Clone HIL-7R-M21, BD Biosciences, USA). After a 30-minute incubation at room temperature (RT), the labelled cells were washed, resuspended at 20–25x10^6 PBMC/mL in PBS with 2%HS and sorted under sterile conditions on a BD FACSAria™ II (BD). Day 0 settings and compensation controls were used. Proliferated ($CFSE^-$) and unproliferated ($CFSE^+$) were collected into Eppendorf tubes containing 250 µl of PBS with 20% HS. Sorted populations were analysed for purity post-sort and determined to be ≥ 99% pure.

Limiting Dilution Analysis Assays

The frequency of antigen specific Treg within Day 0 and expanded samples was determined by limiting-dilution analysis (LDA) as described previously[75]. Briefly, Treg ($CD4^+CD25^{high}CD127^{low}$) were co-cultured with a fixed concentration of moDC (2 x 10^3). T cells were plated in round bottom 96-well plates at various concentrations, starting at 1500 cells per well (1500, 750, 375, 188, 94, 47, 23, and 12 T cells) in cultures with direct allo-Ag, and at 5000 per well (5000, 2500, 1250, 625, 313, 156, 78, and 39) in cultures with indirect allo-Ag. A minimum of 10-12 replicate wells were seeded per dilution. Culture medium was RPMI 1640 supplemented with 10%HS, 2 mmol/l L-glutamine, 1%PenStrep, IL-2 (10 U/mL), IL-15 (10 ng/mL) and rapamycin (100 ng/mL). Cultures were maintained in a humidified

atmosphere at 37°C and 5% CO_2 for 6 days. At day 5, ^{3}H-thymidine (Perkin-Elmer, USA) was added at 1μCi per well, and after 16-18 hours of incorporation the plates were harvested on a 96 Mach 3-FN Series (TOMTEC, Germany) Harvester and read on a MicroBeta Scintillation and Luminescence Counter (Perkin Elmer). Wells were considered positive if the CPM was greater than the mean plus 3xSD over the sum of negative controls (Treg and APC, cultured separately at the same dilution as Treg co-cultures). Online extreme LDA (http://bioinf.wehi.edu.au/software/elda/index.html) was used to calculate the frequency and 95% confidence interval (95% CI) of responding precursors.

Isolation of Fresh Treg and responders for suppression assays

Fresh Treg and Tcon were isolated from thawed PBMC using EasySep™ Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit (StemCell) per the manufacturer's instructions. Also, $CD8^+$ cells were isolated in parallel using EasySep™ Human CD8 Positive Selection Kit II (StemCell). In order to measure responder proliferation, PBMC or $CD8^+$ cells and/or Tcon were labelled with CellTrace™ Violet Cell Proliferation Kit (CTV, Invitrogen). Briefly, responders were resuspended at $5x10^6$ cells/mL, and mixed with equal volume of CTV at 5μM, for a final concentration of 2.5 μM. After 20 min of incubation at 37ºC, cells were put on ice for 5 min, washed and counted using a haematocytometer. The purity of isolated Fresh Treg was verified with the Treg panel described above. CTV-labelled responders were stained with mAb directed against CD3 PerCP-Cy5.5 (Clone OKT3), CD4 APC (Clone RPA-T4) and CD8a APCe780 (Clone SK1, all from eBioscience). Single-color labelled PBMC were used for marker settings and compensation controls. Samples were acquired on BD LSRFortessa™ II (BD), and analysed using FlowJo software (BD). Purity of Tcon and $CD8^+$ cells was found to be consistently > 95%.

Suppression assays

The suppressive capacity of different Treg fractions was assayed in a mixed leukocyte culture suppression assay. In brief, freshly purified or eTreg were titrated to indicated ratios of Treg:T_{eff} and transferred to duplicate wells of round-bottom 96-well plates. Subsequently, $25x10^3$ CTV-labelled responders (Tcon, PBMC or $CD8^+$ cells) were added to each well. Irradiated moDC (as indicated) were used as APC, at a concentration of $6.26x10^3$/well (1:4 APC:T_{eff}). Where indicated, pre-mixed Ab-coated beads (CD2, CD3 and CD28; Treg Suppression Inspector, Miltenyi Biotec) were used instead of APC at a ratio of 1:1 beads:responders. In vitro suppression assays were conducted in RPMI 1640 supplemented with 10%HS, 2 mM L-glutamine and 1% PenStrep, in a total volume of 200μL. Cells were incubated at 37°C and 5% CO_2 for 6 days, after which wells were washed and stained with a mixture of CD3 PerCP-Cy5.5 (Clone OKT3), CD4 APC (Clone RPA-T4) and CD8a APCe780 (Clone SK1, all from eBioscience) for 20 minutes. Samples were acquired on BD LSRFortessa™ II (BD), and analysed using FlowJo software (BD). Briefly, cells were gated on a forward *versus* side scatter (FSC *vs.* SSC) and then on $CD3^+CD8^+$ or $CD3^+CD4^+$ responders. To exclude Treg from the Tcon analysis, an additional gate was made on labelled CTV cells (on a CTV *vs.* CD4 plot). Within CTV-labelled cells, a CTV histogram of unstimulated responder cells defined the parent population, and proliferation of activated responders was determined by gating on cells with diluted CTV expression. Proliferation of

responders in the presence of Treg is depicted as %Proliferation, corrected for the proliferation of responders in the absence of Treg using the formula:

$$\%Proliferation = \frac{\%CTV^{-}\ \text{responders in presence of Tregs}}{\%CTV^{-}\ \text{responders in absence of Tregs}}.$$

Alternatively, data was transformed into a percentage of suppression using the formula:

$$\%Suppression = 1 - \frac{\%CTV^{-}\ \text{responders in presence of Treg}}{\%CTV^{-}\ \text{responders in absence of Tregs}} = 1 - \%Proliferation.$$

Cytokine quantification

At the end of suppression assays, 100µL of the supernatant of wells was collected before cytometry staining was performed and stored at -20ºC for later cytokine analysis using the Luminex platform. Cytokine determinations were conducted simultaneously for all experiments using the MILLIPLEX MAP Human High Sensitivity T Cell Panel (analytes IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-17A, IL-23 and TNF-α), the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel IV (analyte IL-35) and the MILLIPLEX MAP TGFß1 Magnetic Bead Single Plex Kit (all from Millipore, USA). Samples were acquired on a MAGPIX® System (Luminex) and analysed using the xPONENT® software (Luminex). As the concentration of TGF-β1 was similar on all wells, it could be assumed that the human serum used for these studies contained at least 287 pg/mL TGF-β1. Concentration of cytokines in wells containing Fresh or Day 0 Treg were compared with the concentration measured in wells with responders and no Treg.

Multidimensional analysis of cytometry data

The multidimensional analysis of Treg and Tcon samples was performed in R, using either FlowSOM or k-means clustering algorithms, as previously described[200]. Briefly, after compensating and manually gating on $CD3^{+}CD4^{+}$ cells for all samples, data for equal numbers of cells from each sample was exported to fcs files. FCS files were imported into R and then we concatenated all files to be analysed to a single fcs file in R, using the flowCore package. Fluorescence channels were transformed by optimized Arcsinh[201] (using the 'flowTrans' function) and normalized by scaling. Of note, FlowSOM and k-means clustering algorithms were run on the combined samples to facilitate the identification of small populations that could be overlooked when clustering was performed on each single sample. FlowSOM based unsupervised analysis was then performed[202]. Using the 'BuildSOM' function, the cells in concatenated Treg and Tcon samples were assigned to a self-organizing map (SOM) with a 5x5 grid, based on the expression of CD25, CD127, Foxp3, CD39, HLA-DR, CD137, CD154, CTLA-4 and PD-1. The concatenated Day 0 and eTreg samples were assigned to a SOM with a 5x5 grid, based on the expression of CD39, HLA-DR, CD137, CD154, CTLA-4 and PD-1. Minimum spanning trees (MST) with the concatenated data were built using the 'BuildMST' function and plotted, so that the frequency of cells from each sample allocated to each node could be visualized. Then, cells in a pooled population were separated by sample type (for example, 10U/mL IL-2 eTreg, 10U/mL IL-2 eTcon, Day 0 Treg) and mapped to the respective SOM by using the 'NewData' function. Lastly, MST were built for each sample type using the 'BuildMST' function and plotted, so that the characteristics and the number of cells in each node could be visualized. Due to the pseudo-randomized nature of the FlowSOM algorithm,

multiple MST were generated using the ‘BuildMST’ function, to verify that highly similar clustering was found every time.

For k-means clustering, we used the pre-processed data from the file containing Day 0 Treg and eTreg (10U/mL and 100U/mL of IL-2) samples. K-means clustering was performed with ‘kmeans’ function in R's ‘Stats’ package to divide Treg from each of the three conditions into several subpopulations, based on the expression of CD39, HLA-DR, CD137, CD154, CTLA-4 and PD-1. The optimal number of clusters (k) was determined by plotting sum of squares *versus* number of clusters, and choosing the approximate location of the plot elbow (k was set to 5). The histograms with the characteristics of each cluster were created based on the mean expression of cells within each cluster. Then, for each condition, we calculated the percentages of cells allocated to each of the clusters and compared the distribution of cells to clusters across the three conditions. Statistical analysis of the differences in frequency of cells allocated to each Cluster found between conditions was performed using ANOVA on R. Computational analysis was conducted using RStudio (version 1.1.456) on a MacBook Pro running the OS X El Capitan 10.14.3 with a 2.6 GHz Intel Core i5 processor.

Statistical analysis

Statistical analyses were performed on GraphPad Prism 7.00 software (GraphPad, USA), and values at $P \leq 0.05$ were deemed significant. For comparisons between groups (for example, different APC), Student’s paired t-tests were used, using the Holm-Sidak method. For comparisons within a group (for example, between dilutions), one-way ANOVA was used. Pearson’s correlation coefficients were used to test the relationship between potency and specificity of Treg and between %Suppression and marker MFI on moDC.

RESULTS

1. TREG EXPANSION

1.1. Optimization of serum-free Treg expansion protocols

In the current literature, there is a myriad of Treg expansion protocols with high variability in their methods and outcomes (described in the **State of the Art**). Thus, we set out to establish a definite, reproducible protocol that was focused particularly on the expansion of human Ag-sp Treg from circulating Treg. To streamline the translation of this protocol into the clinical setting, possible sources of contamination were avoided whenever possible, starting by the addition of human serum. The final protocol for Ag-sp Treg expansion and characterization was the result of experiments in which the required methods and protocols were gradually optimized, namely Treg isolation, serum-free moDC differentiation and selection of expansion milieu, where the effect of different Ag presentation modes and IL-2 concentration on Treg expansion and suppressive function were evaluated.

1.1.1. Isolation of circulating Treg

The lack of unique Treg markers, together with the increased expression of CD25 and Foxp3 in activated Tcon, raise the concern of contaminating activated Tcon being present in isolated Treg as well as in expanded Treg (eTreg) after culture. Activated Tcon may skew suppression assays (SA) and, more importantly, in the clinical setting, may have harmful effects on immunocompromised patients upon eTreg infusion. To increase the purity of eTreg populations, it is important to start with a population that is highly enriched in Treg. Therefore, we opted to isolate circulating Treg using FACS, as it is one of the most precise ways to separate rare populations, and it allows for strict cut-off settings. First, total PBMC were separated from buffy coats, and then $CD4^{+}$ T cells were isolated using a positive selection immunomagnetic separation kit. $CD4^{+}$ T cells were labelled with CFSE and left in media overnight to rest prior to cell sorting. During cell sorting, only $CFSE^{+}$ cells with $CD25^{high}CD127^{low}$ expression profiles were selected as Treg, according to the gating strategy described in **Fig.4A-E**. Briefly, cells were gated on lymphocytes **(Fig. 4A)** and singlets **(Fig. 4B)**, followed by gating on $CFSE^{+}$ **(Fig. 4C)** and $CD3^{+}CD4^{+}$ T cells **(Fig. 4D)**. Within $CFSE^{+}CD3^{+}CD4^{+}$ cells, only those with brightest CD25 simultaneously with lowest CD127 expression were selected, and henceforth referred to as Treg (**Fig. 4E**). The strategies used to define cut-offs in CD25 and CD127 expression can be found in **Supplementary Data** (**Fig.S1A-C**). Treg populations were always >90% enriched in $CD25^{high}CD127^{low}$ cells **(Fig.4F)**. $CFSE^{+}CD3^{+}CD4^{+}CD25^{-}$ cells (Tcon) were also sorted and used as a control for Treg phenotype and cell cultures.

Because Foxp3 staining is intracellular, it requires fixation and permeabilization of the cells, so it could not be used as a marker for FACS sorting and was only performed to assess the phenotype of the purified cells. Foxp3 expression in Treg was, on average, 45.14±21.88% **(Fig.4G)**, with high donor-to-donor variability. Decreased Foxp3 expression after cryopreservation had been previously reported on PBMC[203], as well as on isolated Treg samples[204]. This was also verified in our experiments, as Treg isolated from cryopreserved PBMC had significantly lower expression of Foxp3 than those isolated from fresh PBMC (**Fig.5**; 66.34±19.71% for Fresh Treg *vs.* 35.50±15.36% for Thawed Treg).

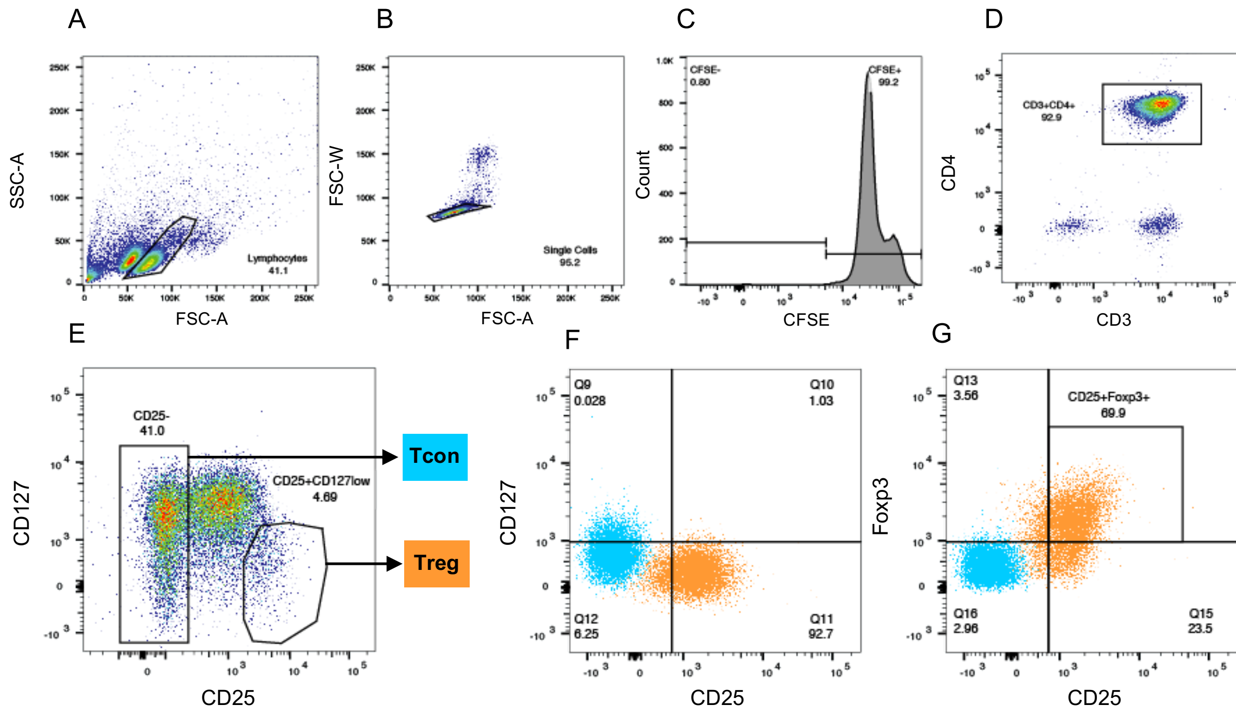

Figure 4: Gating strategy for Treg sorting and Treg purity.

Live lymphocytes were gated on FSC vs. SSC plots (A), which were then cleared of doublets (B). After gating on CFSE⁺ (C) CD3⁺CD4⁺ (D) cells, Treg and Tcon were sorted based on expression of CD25 and CD127 (E). The purity (F) and Foxp3 expression (G) of sorted population aliquots was evaluated a cell analyser after intracellular staining. Overlay of Treg (orange) and Tcon (blue) samples, gated on CFSE⁺CD3⁺CD4⁺ cells, showed that sorted Treg were pure (F). Treg and Tcon had distinct CD25 vs. Foxp3 profiles (G). Data of one representative experiment is shown (n=16).

Nevertheless, these observations may also be related to different specifications of flow cytometers, as samples isolated from fresh PBMC were acquired on a cell sorter (Aria), while thawed Treg samples were acquired on a cell analyser (Fortessa). The cut-off for Foxp3 expression was set with basis on Tcon and Treg overlays, and in Fortessa-acquired samples it was harder to distinguish between positive and negative populations. Thus, by gating only on Treg with higher expression of Foxp3 than the population of Tcon, it is possible some Foxp3⁺ cells were not included, resulting in lower percentages of Foxp3 than expected.

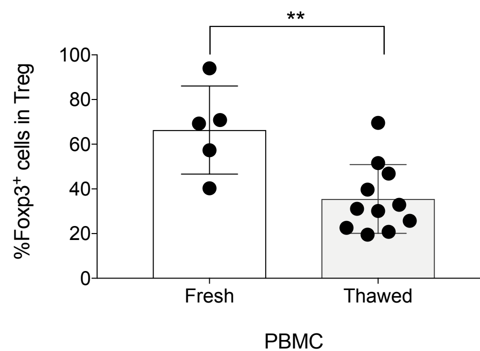

Figure 5: Frequency of Foxp3 positive cells within sorted Treg.

Bar graph shows the frequency of Foxp3⁺ cells within a CD3⁺CD4⁺ gate in Treg samples sorted from fresh (clear bar) or stored and posteriorly thawed (shaded bar) PBMC. Bars represent average values with standard deviation and symbols represent individual measurements. *Statistical analysis was performed by multiple t-tests, by the Holm-Sidak method, with p=0.05. **

1.1.2. Optimization of serum-free moDC differentiation and activation protocols

After Treg isolation protocols were established, we focused on finding appropriate APC for Ag-sp Treg expansion. Monocytes can be easily isolated from PB, and differentiation of monocytes to moDC has been extensively described[73,90,98]. Importantly, moDC can engage the TCR in Treg through MHCII interaction, as well as provide co-stimulatory signaling to varying degrees, depending on their activation status. With that in mind, moDC differentiation and activation protocols were tested in order to find the most suitable moDC to use as APC, with the additional goal of not using human serum. Since purity was not a concern, monocytes were isolated from fresh PBMC using positive selection of $CD14^+$ cells by immunomagnetic separation beads. The resulting population was cultured in parallel with two commercially available serum-free media for moDC differentiation, X-vivo 15 (XV15) and Cellgenix (CG), in the presence of GM-CSF and IL-4. At the end of differentiation (5-6 days), moDC from each condition were activated either with LPS and IFN-γ (referred to as LPS) for 24 hours, or with the current "Gold Standard" (GS) cocktail of cytokines (IL-1β, IL-6, TNF-α and PGE2) for 48 hours. Immature moDC (not activated) were used as a control.

After activation, moDC were collected from wells and counted using a hemocytometer. Viability and phenotype staining of moDC was performed on an aliquot of cells from each condition, and the remaining cells were irradiated at 30 Gy and used as APC in Treg expansion. Differentiation with CG seemed to result in higher percentages of live moDC, when moDC were not activated or activated with GS (**Fig.6A**, % Live cells, full bars). Within cells that were cultured with CG, activation with LPS decreased viability by almost half, while GS did not seem to affect it. The same was not verified for XV15-differentiated cells, where activation did not significantly alter viability and, at most, both activation cocktails seemed to increase cell viability. More importantly, within live cells, the percentage of $CD14^-$ $CD11c^+$ (% moDC) was similar on all conditions and the percentage of monocytes after differentiation was residual (**Fig.6A**, second and third groups, respectively). To assess the potential use of moDC as APC, the frequency and MFI of CD86 and HLA-DR were measured within live cells. Both markers were expressed by >99% of cells in all settings (**Fig.S2)**. The expression of the co-stimulatory marker CD86 was upregulated upon activation on moDC from both media (**Fig. 6B,** left). Due to its bacterial origin, LPS was expected to deliver stronger activation signals than GS to moDC. Interestingly, within moDC cultured in CG, the MFI of CD86 was higher when activation was performed with GS than with LPS (**Fig. 6B,** red bar *vs.* blue bar, respectively). Although monocytes express HLA-DR, it is at much lower intensity than moDC (**Fig.S3**). After differentiation into moDC, the expression of HLA-DR seemed to mirror the trends found in the expression of CD86, albeit at lower MFIs (**Fig. 6B,** right). That is, within CG-cultured cells, there seemed to be higher HLA-DR expression after activation with GS, while in XV15-cultured cells activation with LPS seemed to stimulate higher MFI of HLA-DR.

These experiments showed that moDC differentiation could be achieved in the absence of human serum. Also, the viability of moDC seemed to be affected by the culture media, but similar differentiation yield and phenotypes could be attained from different media by adjusting the activation stimuli.

In order to select the most adequate protocol, it was necessary to evaluate the potential of moDC to expand Treg. We co-cultured isolated Treg with moDC from an allogeneic donor at a ratio of 1:10 moDC:Treg in TexMACS, another commercially available serum-free media, which is designed for T cell expansion. Expansion was carried out in the presence of exogenous IL-2 (100U/mL) and rapamycin, to prevent the proliferation of residual contaminating Tcon. To rescue Treg proliferation in the presence of rapamycin, IL-15 was also added to the culture, as previously described[75]. After 14 days of co-culture, Treg proliferation was assessed by CFSE dilution. As expected, activated moDC (LPS or GS) seemed to stimulate more proliferation in Treg than immature moDC (Control), as measured by the percentage of CFSE$^-$ cells (**Fig.6C**). In fact, these results indicated that Treg proliferation was more dependent on the type of moDC activation (LPS *vs.* GS) than on the media used during moDC differentiation, as little to no variation was seen in the proliferation of Treg co-cultured with moDC differentiated in Cellgenix (green bars) or X-vivo 15 (pink bars). Interestingly, moDC with highest expression of CD86 and HLA-DR (**Fig.6B**, CG+GS and XV15+LPS) did not particularly stimulate more Treg proliferation (**Fig.6C**); in fact, no differences were detected in the proliferation of Treg (%CFSE$^-$ cells) when co-cultured with moDC from CG or XV15 media (**Fig.6C**), despite the different expressions of CD86 (**Fig.6B**). Based on these results, XV15 was used in all subsequent experiments.

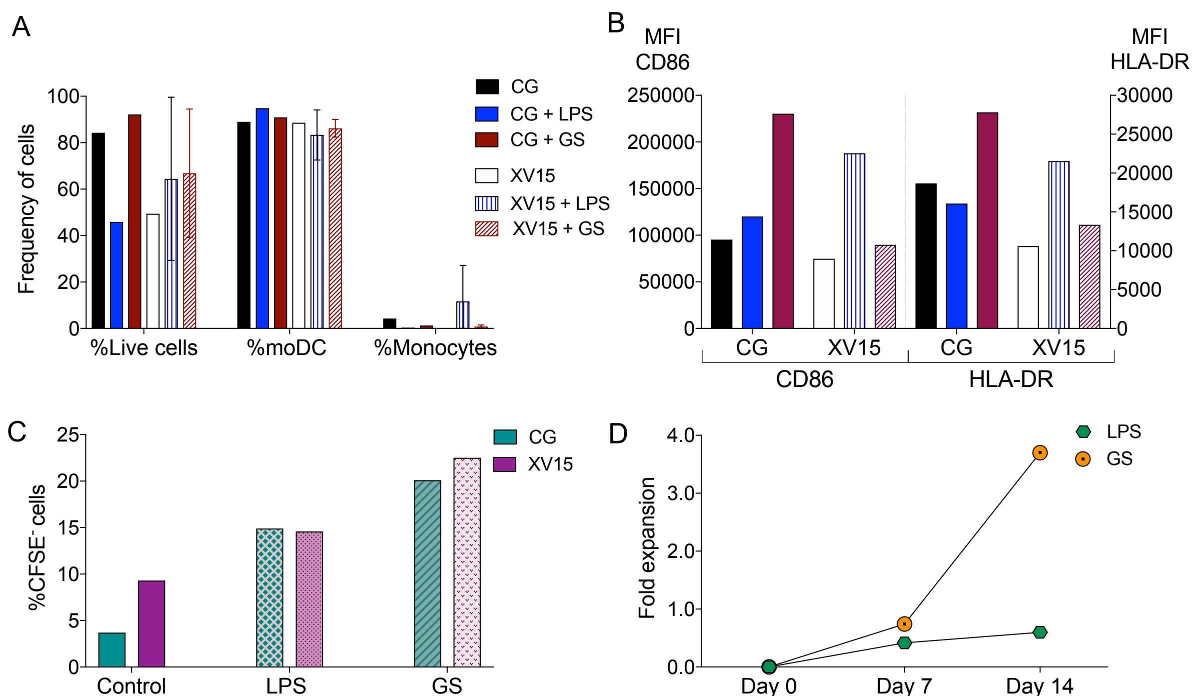

Figure 6: Optimization of moDC differentiation and activation protocol.

CD14$^+$ cells were cultured in Cellgenix (CG) or X-vivo 15 (XV15) media for 5-6 days. After differentiation, LPS or GS activation cocktails were added to the culture for 24 or 48 hours, respectively, and moDC phenotype was assessed by flow cytometry afterwards. Control samples were kept in differentiation media only. (A) Effect of media and activation cocktail on moDC differentiation, as measured by the percentage of live cells (first group) and, within live cells, by the frequency of CD14$^-$CD11c$^+$ cells (%moDC, Second group) and of CD14$^+$ cells (%Monocytes, Third group); (B) Effect of media and activation cocktail on moDC phenotype, as measured by the median fluorescence intensity (MFI) of CD86 and HLA-DR; (C) Effect of media and activation cocktail on Treg proliferation and (D) fold expansion, measured by %CFSE$^-$ cells and fold increase of Treg numbers, respectively. Cumulative data from 2 experiments is presented.

After the media for moDC differentiation was chosen, we focused on identifying an appropriate activation cocktail. Because this protocol for moDC production envisaged their use as APC for Treg expansion, Treg fold expansion was evaluated after co-culture with allo-moDC activated with either LPS (LPS-activated moDC) or the gold standard cocktail (GS-activated moDC). At the end of 14 days in co-culture, GS-activated moDC (from XV15) had stimulated 6 times more expansion of Treg than LPS-moDC (**Fig. 6D;** Day 14**:** 0.6 *vs.* 3.7 in LPS and GS, respectively). Of note, the most significant expansion seemed to occur only after 7 days in culture (**Fig. 6D,** Day 7 *vs.* Day 14).

Using GS-cytokines instead of LPS facilitates the conversion of this protocol to GMP standards, due to the availability of carrier-free cytokines. Thus, all moDC used from this point on correspond to XV-15-differentiated moDC, activated with GS.

After the expansion of Treg, it is important to evaluate the ability of eTreg to suppress responses to allo-moDC from the donor of expansion APC – termed the Original donor. Ideally, the APC for the SA would be the same moDC as the ones used in Treg expansion – the original stimulators – to evaluate the potential of eTreg to suppress responses to the Original donor when presented in the same way. Thus, differentiated moDC from each donor were aliquoted and stored, in order to use similar aliquots for Treg expansion and for the SA. As a result, the storage conditions had to be optimized so that thawed moDC were as similar to fresh moDC as possible. Based on existing literature on DC storage, the tested protocols differed in the activation status of moDC at the time of cryopreservation: cells were either removed from wells right after differentiation (immature), or after 24-hour cultures with GS (semi-mature). Then, upon thawing, immature moDC were pre-cultured for 48 hours with GS, while semi-mature moDC were only cultured for 24 hours before being used as APC. Like in previous experiments, the viability and phenotype of moDC were only assessed after activation (**Fig.7A-B**).

Monocyte-derived DC that were stored in a semi-matured state seemed to present higher viability than immature and fresh moDC (**Fig.7A,** % Live cells); the low viability of fresh moDC was already noticeable in **Fig. 6A**, and may be related to $CD14^+$ cells other than monocytes (such as macrophages) that do not differentiate, undergoing apoptosis. The cryopreservation and thawing protocols involve many washing steps of the cell suspension, so it may result in “cleaner” moDC samples, as long as there is no increase in cell death. Although the frequency of $CD14^-CD11c^+$ cells (% moDC) did not seem to be affected by the activation status of moDC at the time of storage, being similar to that of fresh samples (**Fig.7A,** % moDC), storage of live undifferentiated monocytes within immature or semi-mature moDC suspensions seemed to impair their differentiation after thawing, so higher percentage of monocytes were found in stored moDC (**Fig.7A,** % Monocytes). CD80 and CD83 were included in the moDC staining panel to better assess the level of co-stimulation and activation status of moDC, respectively. The frequency of cells expressing CD86, HLA-DR, CD80 and CD83 was around 100% on moDC from all storage protocols, except for moDC samples stored at an immature stage, which presented slightly lower frequency of $CD80^+$ cells (**Fig.7B**).

Overall, these results indicated that moDC stored in a partially matured stage displayed the most similar phenotype to fresh moDC. From this point onwards, to ensure all moDC were subjected to the same protocol, moDC were differentiated from a batch of donors and cryopreserved before being used as APC for expansion or the SA. Finally, in **Fig. 8,** representative plots of moDC resultant from the

optimized protocols for differentiation and storage can be seen. Despite donor-to-donor variability, most samples had a high frequency of moDC (**Fig.8A**), high MFI and frequency of CD86 (**Fig.8B**), HLA-DR (**Fig.8B**), CD80 (**Fig.8C**), and CD83 (**Fig.8C**). Separate histograms for each marker, overlaid with the corresponding unstained sample, can be found in **Fig.S4**.

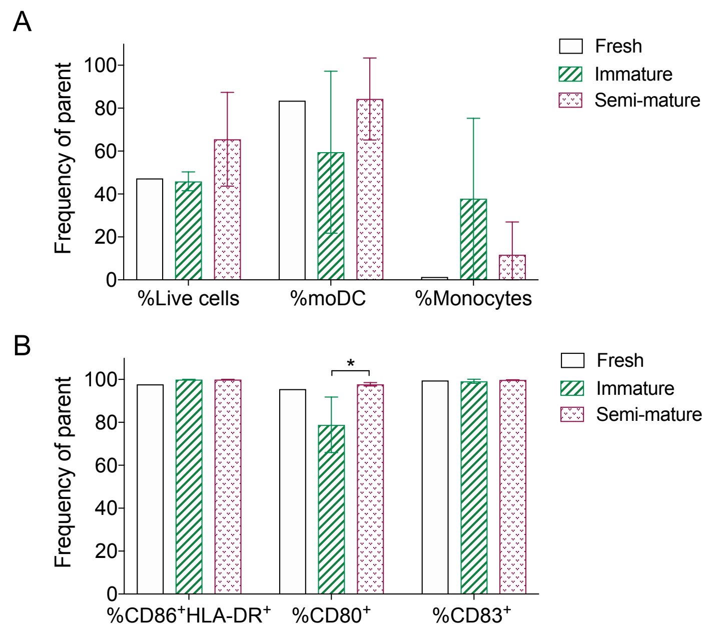

Figure 7: Optimization of moDC storage conditions.
Monocyte-derived DCDC were divided into three groups, where one group was activated with GS for 48 hours and analysed as fresh moDC (fresh, clear bars), the second group was cryopreserved in an immature status, thawed, and then activated with GS for 48 hours (immature, green striped bars) and the third group was activated for 24 hours with GS, thawed, and finally activated with GS for 24 hours more (semi-mature, red dotted bars). (A) The effect of storage conditions on moDC differentiation was assessed by the percentage of live cells (first group of bars) and, within live cells, by the frequency of $CD14^{-}CD11c^{+}$ cells (%moDC, second group of bars) and of $CD14^{+}$ cells (%Monocytes, third group of bars). (B) The effect of storage conditions on moDC phenotype was assessed by the frequency of activation-driven markers, namely the frequency of cells co-expressing CD86 and HLA-DR (first group of bars), the frequency of cells expressing CD80 (second group of bars) and the frequency of cells expressing CD83 (third group of bars). *Statistical analysis was performed using multiple t-tests, by the Holm-Sidak method, with n=2. * represents statistically significant differences, with p<0.05. p=0.035*

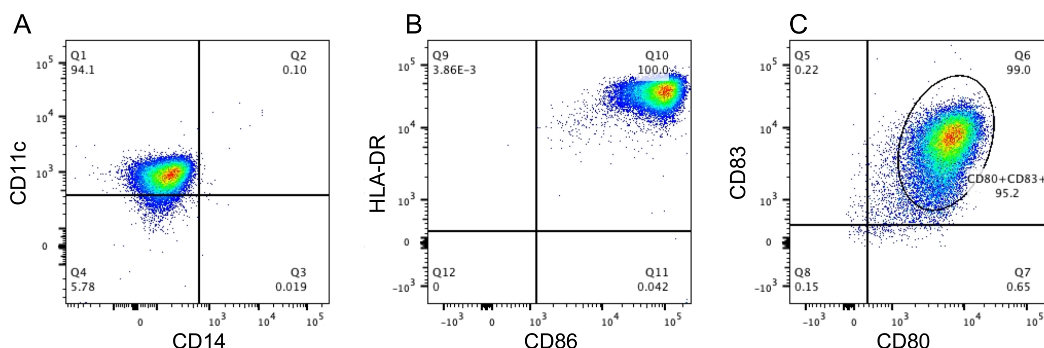

Figure 8: Representative plots of moDC phenotype.
Representative plots of moDC phenotype as used for co-cultures and SA, after differentiation, storage and activation. After gating on live, single cells, moDC phenotype was visualized on dot plots of (A) CD14 *vs.* CD11c, (B) CD86 *vs.* HLA-DR and (C) CD80 *vs.* CD83.

1.2. Expansion of Ag-Sp Treg

During GvHD, donor-derived T_{eff} react to host Ag, which are presented either directly by host APC or indirectly by donor (self) APC after antigen uptake and processing. In order to mimic this scenario *in vitro*, we started by questioning whether Treg would proliferate *in vitro* in response to Ag presented in different modes, and if those eTreg could perform Ag-specific suppression. As described in the **State of the art**, immature moDC have great Ag uptake and processing capabilities. Thus, it is possible to load moDC with target Ag before inducing activation. Based on this, it was possible to design experiments assessing the expansion of Ag-sp Treg in the presence of allo-Ag from the Original donor by different modes of presentation. Direct Ag presentation consisted on using allo-moDC from the Original donor as APC, while the APC in indirect Ag presentation were moDC from the same donor as the Treg donor (self-moDC) loaded with allo-Ag from the Original donor (**Fig.9**). For the loading step of indirect presentation, Allo-Ag from the Original donor were obtained from a PBMC lysate, and loaded onto self-moDC overnight before activation. Co-cultures of Treg with self-moDC loaded with self-Ag (SelfDC) were performed as a control for allo-Ag loading. In short, from now on, nomenclature for APC is as follows: allo-moDC from the Original donor are **OriDC**, self-moDC loaded with allo-lysate from the Original donor are **OriLys** and self-moDC loaded with self-lysate are **SelfDC** (**Fig.9**).

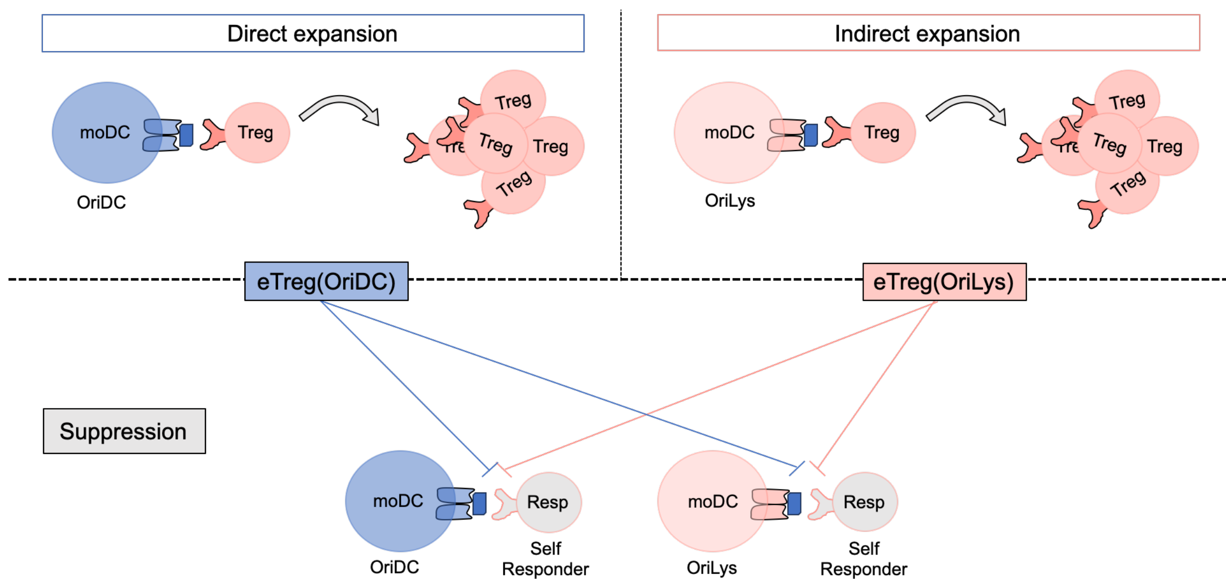

Figure 9: Schematic representation of Treg expansion and characterization with different modes of antigen presentation. Treg from one donor (Self) were expanded by allo-Ag presented either directly by allo-moDC from the Original donor – direct expansion with OriDC – or by allo-Ag presented indirectly by self-moDC, loaded with a PBMC lysate from the Original donor – indirect expansion with OriLys. After expansion, the capability of eTreg(OriDC)and eTreg(OriLys) to suppress responses to allo-Ag from the Original donor presented directly (OriDC) or indirectly (OriLys) was assessed.

1.2.1. Effect of Ag presentation mode on Treg expansion and function

In the experiments described here, the differentiation and storage of moDC were performed according to the optimized protocols, with the addition of a step of overnight Ag-loading before the partial activation performed prior to storage. Treg were isolated as previously described and co-cultured with moDC for 14 days in the presence of IL-2 (100U/mL), IL-15 and rapamycin. After expansion, the fold expansion and phenotype of Treg expanded by either direct – eTreg(OriDC) – or indirect – eTreg(OriLys) - Ag presentation were characterized, as well as control eTreg – eTreg(SelfDC).

a) Treg expansion and phenotype

CFSE-labelled Treg were sorted on Day 0 and co-cultured with OriDC, OriLys or SelfDC. After 14 days, eTreg were almost 100% CFSE$^-$, that is, most live cells had proliferated during culture (**Fig. 10A;** frequency of CFSE$^-$ in eTreg(OriDC) 98.03±1.24%, in eTreg(OriLys) 98.47±0.85% and in eTreg(SelfDC) 97.67±2.48%). It is known that Treg are naturally anergic to self-MHC; hence, proliferation in co-cultures with SelfDC was indicative that high concentration of IL-2 and/or co-stimulation provided by APC were sufficient stimuli to activate and break Treg anergy. Moreover, when the fold expansion of Treg from co-cultures with each APC was compared, the fold expansion of Treg co-cultured with SelfDC seemed similar (**Fig. 10B**, average 18.37±12.02) to that of Treg cultured with allo-Ag presented directly by OriDC (**Fig. 10B**, average 19.73±9.76), yet appeared to be lower than that of Treg co-cultured with indirectly presented allo-Ag by OriLys (**Fig. 10B**, average 24.97±11.87). Interestingly, Treg seemed to be more expanded by allo-Ag presented indirectly by OriLys than by Ag presented directly in OriDC (fold expansion of 24.97±11.87 in OriLys *vs.* 19.73±9.76 in OriDC). It could be expected that Treg would be responsive to allo-Ag presented indirectly by self-MHC, since that would more adequately mimic their expansion milieu *in vivo*. Nevertheless, no statistically significant differences in fold expansion were found between conditions.

When the phenotype of eTreg was assessed after co-culture, CFSE$^-$ eTreg expressed CD25 in higher intensity than CFSE$^+$ eTreg, suggesting that eTreg had proliferated upon activation (**Fig. 10C**). As expected, CD127 expression was unaltered by proliferation (**Fig. 10D**), while Foxp3 expression was only slightly increased in proliferated cells (**Fig. 10E)**. The bulk population of each eTreg (not separated by CFSE dilution) was also compared to *ex vivo* Tcon and *ex vivo* Treg (from Day 0, not placed in culture) regarding the expression of CD25, CD127 and Foxp3 (**Fig. 10F-H)**. Whilst *ex vivo* Treg can be used as a control for eTreg, the phenotype of *ex vivo* Tcon serves as a negative control for CD25 and Foxp3 expression, as well as a positive control for the expression of CD127. Regardless of the APC used for Treg expansion – OriDC, OriLys or SelfDC –, CD25 expression was markedly increased in eTreg (**Fig. 10F**). Surprisingly, a subset of CD25^{low} eTreg, albeit with higher expression of CD25 than Tcon, was found in Treg expanded by direct presentation and in Treg expanded by SelfDC (**Fig. 10F**, red histogram – eTreg(OriDC); orange histogram – eTreg(SelfDC)). This CD25^{low} subset was found mostly within proliferated cells (**Fig. 10C,** red histogram) and was absent in Treg expanded by indirect presentation (**Fig. 10F,** blue histogram – eTreg(OriLys)). Since the starting Treg population is the same,

it could be assumed that activated Tcon, if present, would be found in all conditions, which was not the case. Similarly to what was seen in **Fig.10D**, CD127 expression in bulk eTreg was identical to that of *ex vivo* Treg (**Fig.10G**). Since our initial population was already enriched for Foxp3^{+}cells, no differences were detected between proliferated and unproliferated cells in Foxp3 expression (**Fig.10E**). Nevertheless, Foxp3 expression was upregulated in bulk Treg after expansion (**Fig.10H**, red, blue and orange histograms).

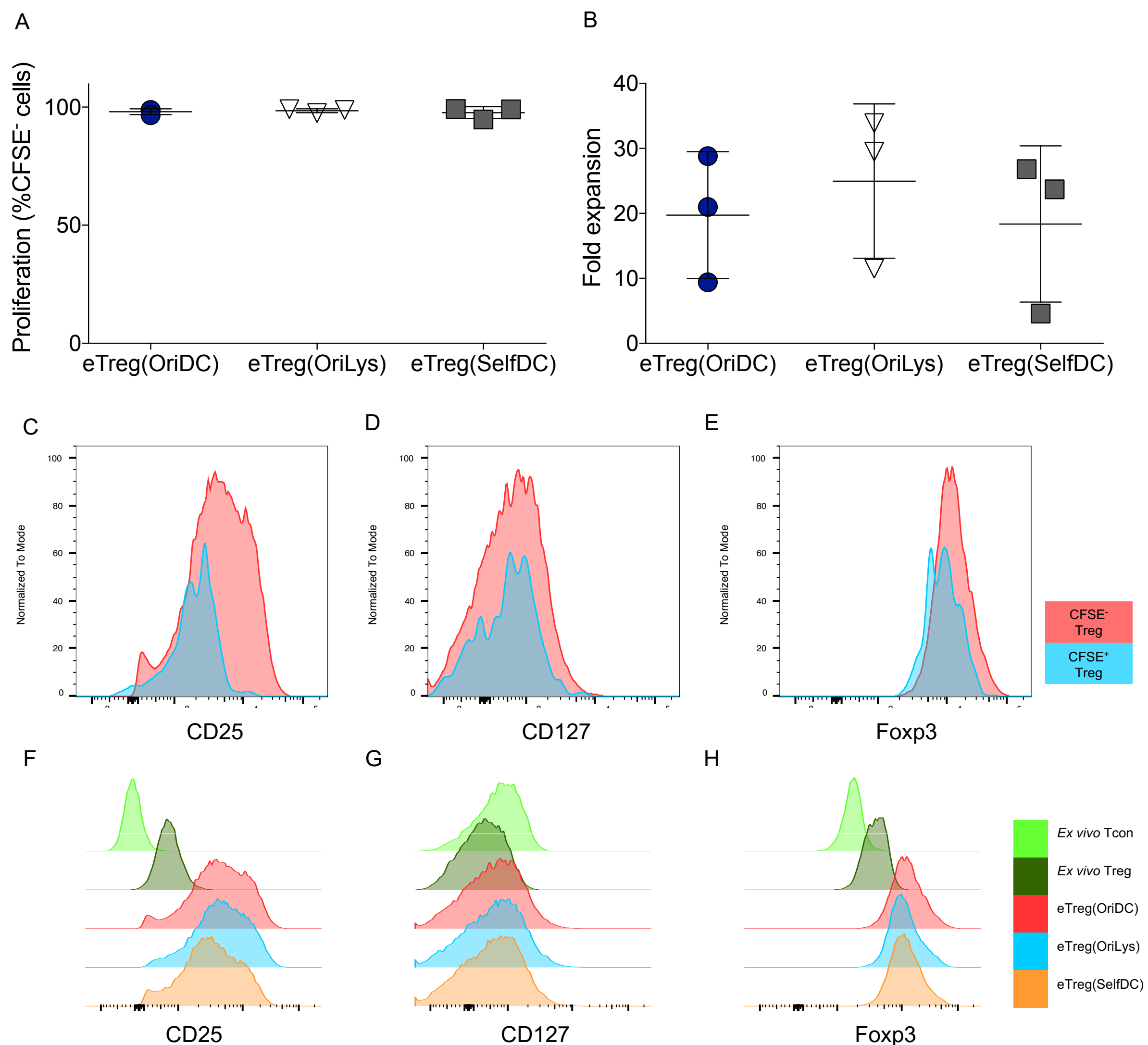

Figure 10: Expansion of Treg by allo-Ag presented direct or indirectly.
Purified *ex vivo* Treg were co-cultured with OriDC, OriLys or SelfDC. (A) Percentage of proliferated (CFSE^{-}) cells within eTreg(OriDC), eTreg(OriLys) and eTreg(SelfDC). Represented by the frequency of parent of CFSE^{-} cells after gating on CD3^{+}CD4^{+} lymphocytes. (B) Treg fold expansion in different conditions. Fold expansion was calculated by dividing the number of CD3^{+}CD4^{+} lymphocytes counted using TruCount beads by the number of seeded Treg on Day 0. (C-E) Histograms of CD25 (C), CD127 (D) and Foxp3 (E) expression with CFSE^{+} and CFSE^{-} eTreg in overlay. (F-H) Offset overlay histograms of CD25 (F), CD127 (G) and Foxp3 (H) expression in *ex vivo* Tcon (light green), *ex vivo* Treg(dark green), eTreg(OriDC) (red), eTreg(OriLys) (blue) and eTreg(SelfDC) (orange). *A-B depicts cumulative data from 3 experiments; C-H: representative data of one out of three experiments. C-E shows data from eTreg(OriDC).*

b) Frequency of Ag-sp Treg

Although it has been shown that after co-culture with APC proliferated (CFSE$^-$) Treg subsets are enriched in Ag-sp Treg[75], the frequency of CFSE$^-$ Treg and of Ag-sp Treg may not be the same. To determine the frequency of Ag-sp Treg within bulk Treg before and after expansion, limiting dilution assays (LDA) were performed. LDA consist of co-cultures of stimulators in fixed numbers (in this case, moDC) with serially diluted responding cells (Treg). Proliferation was quantified by scintillation counts after tritiated thymidine incorporation. By measuring the proliferation of responder cells alone, it was possible to set a threshold for proliferation. The number of positive wells found at each dilution in co-cultures could then be calculated, thus allowing for the quantification of the frequency of responding precursors within a population of responders. LDA frequencies and plots were computed using the online software available at http://bioinf.wehi.edu.au/software/elda/index.html.

In this case, LDA were performed using as responders *ex vivo* Treg, bulk populations of eTreg from each condition or CFSE$^-$ cells sorted from each condition of eTreg. OriDC, OriLys or SelfDC were used as stimulators, thus allowing for the quantification of the frequency of precursors responding to directly presented allo-Ag, indirectly presented allo-Ag or to self-Ag.

Ex vivo Treg populations included Treg that were responsive to allo-Ag from the Original donor, whether directly presented by OriDC or indirectly presented by OriLys, as well as to Self-Ag, presented by SelfDC, since positive wells for proliferation were found in each of these co-cultures. After the precursor frequency was computed and plotted, it could be seen that the frequency of precursors responsive to each APC was different, which was represented by the different slopes amongst straight lines (**Fig.11A,** black line - frequency of precursors responsive to OriDC, red line – frequency of precursors responsive to OriLys, green line – frequency of precursors responsive to SelfDC). The frequency of Treg precursors responsive to directly presented allo-Ag – OriDC – was the highest, with an average of 1.40±1.19% responsive cells (**Fig.11B**). The frequency of cells responsive to indirectly presented allo-Ag – OriLys – within *ex vivo* Treg was 0.68±0.42%. Finally, there was 0.36±0.12% of *ex vivo* Treg that were responsive to self-Ag – SelfDC. It makes sense that the specificity for self is somewhat constant throughout experiments since only one donor is used in these conditions (**Fig.11B**, each experiment is represented by the same shape); when there are allo-Ag being presented, in a direct or indirect way, two different donors (Treg and Original donor) are required for each experiment, thus increasing the variability. Since each dilution of the LDA is performed in 10-12 replicates on each experiment, the statistical analysis represented in **Fig.11A** pertains to each experiment. Nevertheless, the same statistically significant trends were found in 2 out of 3 individual experiments. Despite the differences found on day 0, after expansion, bulk eTreg from co-cultures with OriDC, OriLys and SelfDC had significantly higher frequency of precursors specific for the APC used in their expansion (**Fig.11C**). That is, Treg that were co-cultured with OriDC, for example, had increased precursor frequency for OriDC-sp Treg after expansion. Furthermore, there was an enrichment in precursor frequency within sorted CFSE$^-$ Treg, when compared to bulk eTreg (**Fig.11C)**. Since Treg are expected to proliferate upon activation, it makes sense that, within proliferated cells, there is a higher frequency of cells responsive to the same stimuli used for the initial activation. Thus, the remaining LDA were performed using only CFSE$^-$ eTreg. Finally, we wanted to assess the specificity of Treg expanded by allo-Ag

presented indirectly by OriLys. To do so, we sorted CFSE⁻ eTreg after co-culture with OriLys, and performed LDA using OriDC, OriLys or SelfDC as APC. This way, it was possible to quantify the frequency of precursors responding to each moDC (**Fig.11D**). Surprisingly, we found that within the proliferated fraction of eTreg(OriLys), the highest precursor frequency was for Treg responsive to self-Ag (SelfDC), which was verified in two independent experiments. Moreover, within these fractions of Treg expanded by indirect allo-Ag presentation, the frequency of precursors responsive to allo-Ag directly presented (OriDC) was significantly higher than the frequency of precursors responsive to indirectly presented allo-Ag (OriLys). In fact, looking at each independent experiment with CFSE⁻ eTreg(OriLys), the lowest precursor frequency was always the frequency of precursors responsive to OriLys (**Fig.11D**, different colours represent different experiments). Still, this frequency was significantly higher than that found within *ex vivo* Treg, where the average precursor frequency for OriLys was 0.76±0.55%, as opposed to 1.58±0.13% in CFSE⁻ eTreg(OriLys). Within each APC, no statistically

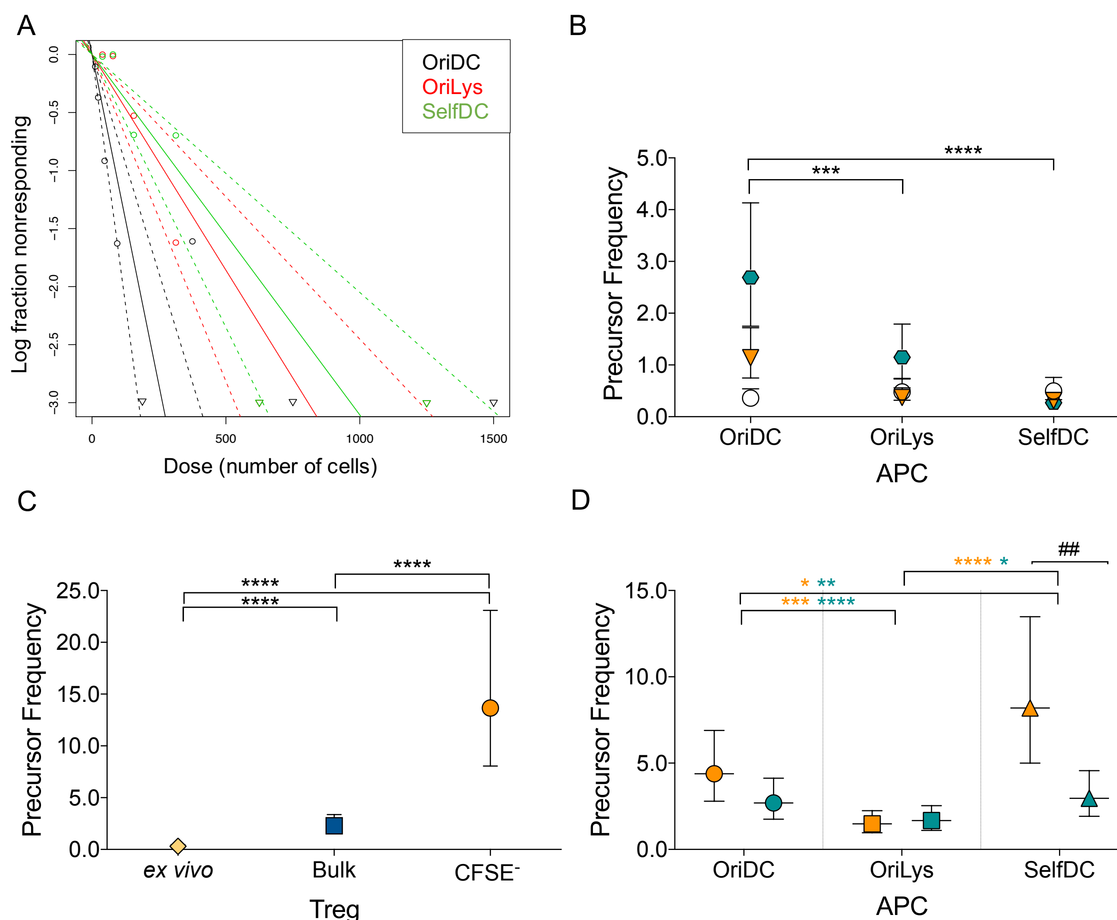

Figure 11: Frequency of precursors responsive to OriDC, OriLys and SelfDC before and after Ag-sp Treg expansion. (A) Representative LDA plot of responding precursors within *ex vivo* Treg, in co-cultures with OriDC (black line), OriLys (red line) and SelfDC (green line). Dotted lines represent the lower and upper confidence limits in each condition. (B) Frequency of precursors responsive to each APC in *ex vivo* Treg. Each coloured symbol represents the estimated frequency of Treg from one experiment, with corresponding lower and upper limits. (C) Frequency of precursors responsive to the same APC before expansion (*ex vivo* Treg), after expansion (Bulk eTreg), and after sorting proliferated eTreg (CFSE⁻ eTreg). Data shown here is from eTreg(SelfDC), yet it is representative of all conditions for each experiment. (D) Frequency of precursors responsive to each APC, within the proliferated subset of eTreg(OriLys). Orange and green symbols represent data from one experiment each. Statistically significant differences within experiments are illustrated by the respective colours. * - *Statistically significant differences found between conditions, within each experiment; # - statistically significant differences between experiments. All data was analysed with pair-wise tests on LDA online software. N=3 in A-C and n=2 in D.*

significant differences in precursor frequency were found between experiments, except in SelfDC, where one experiment had significantly higher frequency of responding precursors than the other. Since the donors of each experiment were different, some variability in responsiveness to self was considered normal.

In sum, the proliferated subset of Treg that were indirectly expanded by OriLys – CFSE^{-} eTreg(OriLys) – presented higher frequency of precursors responsive to Self-Ag (SelfDC) and to directly presented allo-Ag (OriDC) than to indirectly presented allo-Ag (OriLys). This indicates that there was Ag-sp expansion by indirectly presented allo-Ag in OriLys, yet, upon re-stimulation, the Ag-sp Treg expanded by indirect Ag presentation are more responsive to either self-MHC or directly presented allo-Ag from the Original donor. Furthermore, even within CFSE^{-} cells, the frequency of responsive cells was always less than 10%. This suggests that, within Treg expanded in an Ag-sp setting, there is always a substantial fraction cells that proliferates yet does not display specificity towards the Ag being presented. Finally, it could also be concluded that indirect expansion by OriLys is a good alternative for the expansion of Ag-sp Treg envisaging their clinical application, as the resulting donor-derived eTreg would be more responsive to either donor APC (derived from the initial graft) or host APC (such as tissue-resident DC). If the host's leukaemia Ag were to be presented indirectly by donor-derived APC, it is possible GVL could be maintained. Nevertheless, precursor frequency is only representative of the percentage of proliferating eTreg upon re-stimulation. Thus, the suppressor function of those eTreg still needs to be determined. Because the cells used in LDA are lost during acquisition, the suppressive function of cells from wells containing responding precursors could not be assessed. Instead, SA were performed with either bulk or CFSE^{-} eTreg from co-cultures with each APC. Another option, which we intend to explore in the future, would be to serially dilute eTreg, expand Treg clones in the absence of APC, and then measure for each clone the frequency of precursors responsive to each APC and their suppressive function.

c) Potency and specificity of suppression

After the frequency of precursors responsive to APC used in each Ag-sp Treg expansion was calculated, the function of eTreg had to be assessed. One hypothesis is that the suppressive function of eTreg may be unrelated to precursor proliferation upon re-stimulation with APC, in which case, LDA would not be the most accurate assay to evaluate eTreg function. Additionally, eTreg that do proliferate upon re-stimulation with APC, which are identified as Ag-sp Treg precursors, may confer signals to modulate the non-responding eTreg cells during SA. The suppressive function of eTreg can be defined by the potency they display in suppressing the proliferation of responder cells. Also, suppression may be achieved in different potencies when different APC are used in SA – thus, denoting the specificity of suppression by eTreg. As a side note, when the APC used for the SA are the same as the ones used for the expansion of the tested eTreg, they are termed as original stimulators. In these experiments, the original stimulators are either moDC from the Original donor (OriDC) or moDC from the Treg donor, loaded with a lysate from the Original donor (OriLys).

The function of bulk, sorted CFSE^{+} and sorted CFSE^{-} eTreg from co-cultures with direct allo-Ag presentation – eTreg(OriDC) – was measured in parallel in SA where the original stimulators (OriDC)

were used as APC. Proliferation of responders (Tcon) was measured by CTV dilution and normalized to control wells (co-cultures of responders and OriDC only, in the absence of eTreg). There was similar suppression of responses to OriDC by eTreg in bulk and by the subset of proliferated ($CFSE^-$) eTreg (**Fig.12A**). Bulk eTreg and proliferated $CFSE^-$ eTreg displayed high potency of suppression, as Tcon proliferation was decreased to less than 50% of the control (0:1) at a ratio of 1:10 Treg:T_{eff}. Moreover, at a ratio of 1:2 Treg:T_{eff}, Tcon proliferation was suppressed to 25.4% and 18.4% by Bulk eTreg and by $CFSE^-$ eTreg, respectively. Of note, the subset of unproliferated ($CFSE^+$) eTreg did not suppress Tcon proliferation. Since the percentage of $CFSE^-$ cells within bulk eTreg was almost 100% (**Fig.10A**), it was normal that no major difference in the suppressive potency of these populations was detected in SA. Next, we assessed the potency of Ag-sp Treg expanded by direct or by indirect allo-Ag presentation to suppress allo-Ag from the Original donor, when presented directly by OriDC. Proliferated ($CFSE^-$) subsets were sorted from Treg expanded through direct presentation – eTreg(OriDC) – or through indirect presentation – eTreg(OriLys) –, and SA were performed in parallel with OriDC as stimulators. Tcon proliferation seemed more suppressed by directly expanded Treg – eTreg(OriDC) – than by indirectly expanded Treg – eTreg(OriLys) – at all ratios (**Fig.12B**). At the highest Treg:T_{eff} ratios, Tcon proliferation was decreased to 18.4% by eTreg(OriDC) as opposed to 38.1% by eTreg(OriLys). In short, these results suggest that Treg expanded by allo-Ag presented indirectly – eTreg(OriLys) – can suppress responses to directly presented allo-Ag from the Original donor, albeit to a lower extent than directly expanded Treg – eTreg(OriDC).

Furthermore, when we assessed the potency of indirectly expanded Treg (in bulk) to suppress responses to allo-Ag from the Original donor, either direct or indirectly presented by OriDC and OriLys, respectively, there was similar suppression of responses to both forms of allo-Ag presentation (**Fig.12C**). Particularly, indirectly expanded Treg seemed more potent suppressors of responses to indirectly presented allo-Ag (OriLys) than to directly presented allo-Ag (OriDC), as at 1:50 Treg:T_{eff} Tcon proliferation was decreased to 42.0% in SA with OriLys, while it was still 92.5% in SA with OriDC. The higher suppression of responses to OriLys was expected, as these APC are the original stimulators of this expansion. Conversely, it was surprising to observe that when the number of eTreg in SA was increased (higher Treg:T_{eff} ratios), the suppression of responses to OriLys was not increased, as Tcon proliferation seemed constant at all ratios. Indeed, at 1:2 Treg:T_{eff}, Tcon proliferation was still 34.4% of the control. On the other hand, in SA with OriDC, increasing numbers of eTreg seemed to result in higher suppression, as Tcon proliferation was decreased to 42.8% at 1:10 Treg:T_{eff}, and to 22.4% at a ratio of 1:2 Treg:T_{eff}.

As mentioned before, Tcon proliferation was measured by CTV dilution (data not shown). To do so, at the end of SA, we gated on $CD3^+CD4^+$ cells and then measured the frequency of CTV^- cells within this population. However, since Treg are also $CD3^+CD4^+$, it was hard to set thresholds that distinguished between Treg (not labelled with CTV) and proliferated Tcon responder cells (CTV^-). The addition of CD25 or Foxp3 to the cytometry panel would not solve this issue, as activated Tcon increase their expression of these markers. Since $CD8^+$ T cells may be activated and proliferate in response to moDC, and are also targeted by Treg suppression in inflammatory milieus, it was decided to use total PBMC as responders for the following experiments, where the proliferation of $CD8^+$ T cells was quantified by

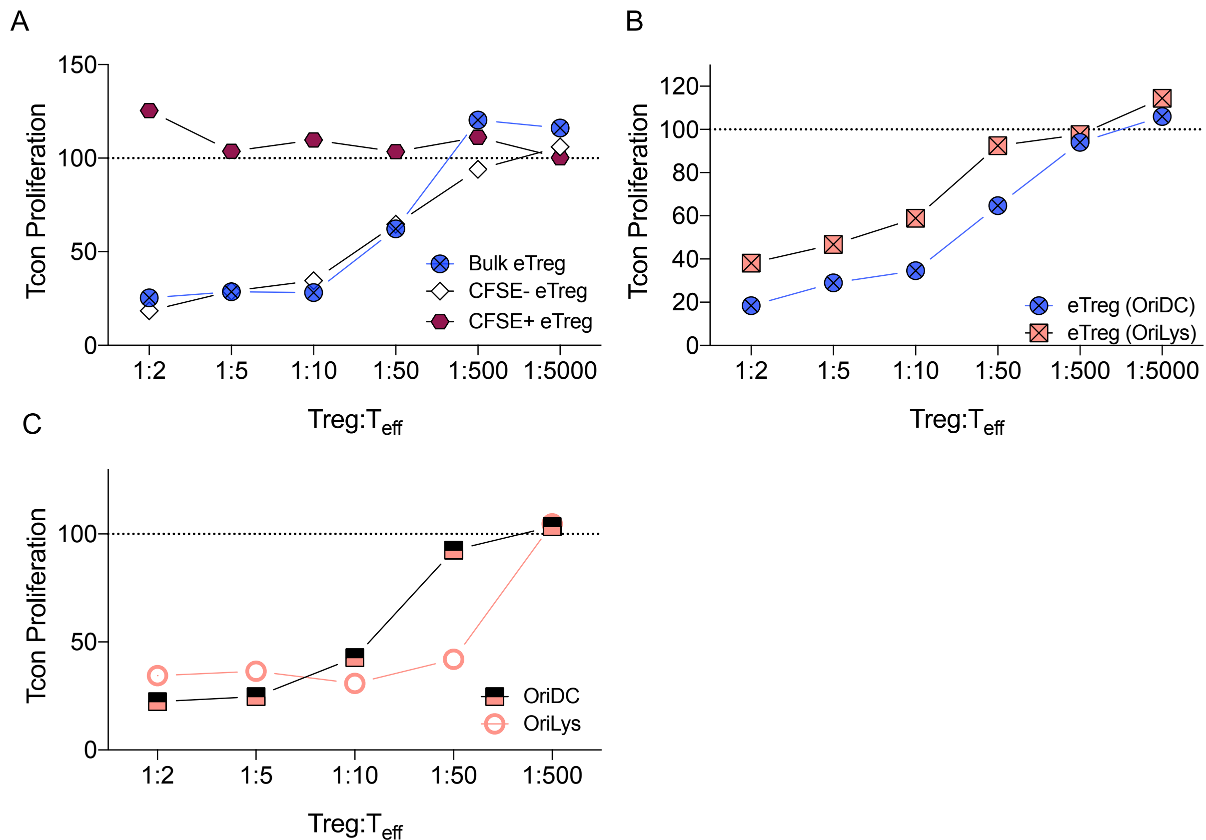

Figure 12: Potency of suppression of Tcon proliferation by Treg expanded with allo-Ag presented direct or indirectly. Proliferation of Tcon was measured by CTV dilution and normalized to proliferation in the absence of eTreg. (A) Proliferation of Tcon in response to directly presented allo-Ag (OriDC), in the presence of Treg expanded by directly presented allo-Ag – eTreg(OriDC). The suppressive function of bulk eTreg (Blue circles), proliferated ($CFSE^-$) eTreg (White diamonds) and unproliferated ($CFSE^+$) eTreg (Red hexagons) was evaluated at several dilutions. (B) Proliferation of Tcon in response to directly presented allo-Ag (OriDC), in the presence of several dilutions of proliferated ($CFSE^-$) subsets sorted from Treg either expanded by direct allo-Ag presentation – eTreg(OriDC), blue circles with cross – or by indirect allo-Ag presentation – eTreg(OriLys), orange squares with cross. (C) Proliferation of Tcon in response to either directly presented allo-Ag by OriDC, Orange and black squares) or indirectly presented allo-Ag by OriLys (Pink circumferences), in the presence of indirectly expanded Treg– eTreg(OriLys) – in bulk, at several dilutions. *Data from one experiment.*

gating on $CD3^+CD8^+$ cells. As $CD8^+$ cells do not express CD4, as activated Tcon do, this strategy allows for Treg exclusion by selecting only $CD3^+CD8^+$ cells within a $CD4^-$ gate.

Ex vivo Treg were expanded in the same conditions as before, and SA were performed with the same settings. First, the suppression of responses to Ag presented directly (by OriDC) by subsets of Treg previously expanded with the same APC – eTreg(OriDC) – was assessed (**Fig.13A**). Both bulk eTreg and the proliferated ($CFSE^-$) subset of eTreg suppressed $CD8^+$ proliferation to a similar extent, as we had observed with Tcon as responders. Proliferation was decreased to a minimum of 21.1% and 15.5%, by bulk and $CFSE^-$ eTreg, respectively (**Fig.13A**). When Ag from the Original donor were directly presented by OriDC, $CD8^+$ cells proliferation was more suppressed by the proliferated ($CFSE^-$) subset of directly expanded Treg – eTreg(OriDC) – than by the proliferated ($CFSE^-$) subset of indirectly expanded Treg – eTreg(OriLys) (**Fig.13B**). In fact, at dilutions as low as 1:50 Treg:T_{eff}, the proliferation of $CD8^+$ cells was suppressed to 50.17±20.85% by $CFSE^-$ eTreg(OriDC). Furthermore, at 1:2 Treg:T_{eff}, $CD8^+$ cells proliferation was decreased to 15.47±10.62% by the same subset of eTreg (**Fig.13B**). Conversely, the proliferated subset of indirectly expanded Treg – eTreg(OriLys) –only decreased $CD8^+$

cells proliferation to less than 50% of the control at high concentrations (at 1:2 Treg:T_{eff}, proliferation was 43.10±20.93%). Although the proliferation of $CD8^+$ cells in response to directly presented allo-Ag by OriDC seemed to be more suppressed by the proliferated subset of directly expanded Treg – eTreg(OriDC) – than by the proliferated subset of indirectly expanded Treg – eTreg(OriLys) – at all dilutions, significantly lower $CD8^+$ cells proliferation in response to OriDC was found only at 1:10 Treg:T_{eff}, (p=0.0395). This trend for higher suppression of responses to directly presented allo-Ag by Treg derived from direct expansion had also been detected in SA with Tcon as responders (**Fig.12B**).

Finally, we compared the potency of bulk populations of Treg expanded by indirect presentation of allo-Ag– eTreg(OriLys) – to suppress allo-Ag from the Original donor, when they were presented directly by OriDC or indirectly by OriLys (**Fig.13C**). $CD8^+$ cell proliferation seemed more suppressed in the presence of OriLys, which are the same APC used during expansion. In this condition, $CD8^+$ cell proliferation was decreased from 75.32±8.51% at 1:500 Treg:T_{eff} to 10.94±7.87% at a ratio of 1:2 Treg:T_{eff}. Concurrently, in the presence of OriDC, $CD8^+$ cell proliferation seemed increased by the

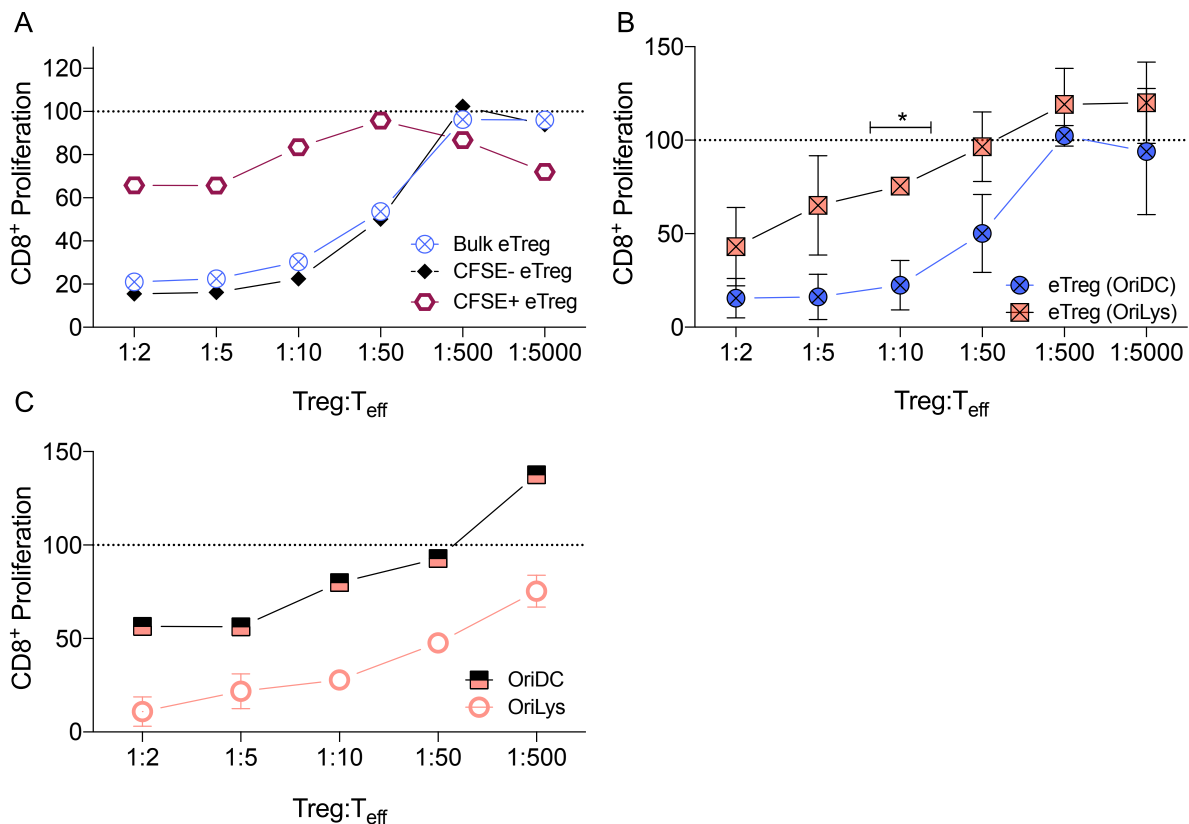

Figure 13: Potency of suppression of $CD8^+$cells proliferation by Treg, after expansion with Ag presented direct or indirectly.

Proliferation of $CD8^+$ cells was measured by CTV dilution and normalized to proliferation in the absence of eTreg. (A) Proliferation of $CD8^+$ cells in response to OriDC, in the presence of bulk eTreg(OriDC) (Blue circles with square), sorted $CFSE^-$ eTreg(OriDC) (Black diamonds) and sorted $CFSE^+$ eTreg(OriDC) (Red empty hexagons) at several dilutions. (B) Proliferation of $CD8^+$ cells in response to OriDC, in the presence of sorted $CFSE^-$ eTreg(OriDC) (Blue circles with cross) and sorted $CFSE^-$ eTreg(OriLys) (Orange squares with cross) at several dilutions. (C) Proliferation of $CD8^+$ cells in response to OriDC (Orange and black squares) and OriLys (Pink circumferences), in the presence of bulk eTreg(OriLys) at several dilutions. A: representative data from one experiment; B-C: *data from three experiments. Statistical analysis was performed using multiple t-tests, by the Holm-Sidak method. *- statistically significant difference in proliferation, with p<0.05.*

presence of very low numbers of eTreg (1:500, 137.6%), and was only decreased to 56.5% at the highest eTreg concentration.

The suppression of $CD8^+$ cell proliferation by eTreg seemed specific for the APC used during expansion; that is, eTreg(OriDC) were more potent suppressors of responses to OriDC, and eTreg(OriLys) seemed more suppressive in the presence of OriLys. In other words, Treg expanded by direct presentation of allo-Ag from the Original donor seemed more suppressive of responses to directly presented allo-Ag from that same donor. Similarly, Treg expanded by indirect presentation of allo-Ag from the Original donor seemed more suppressive of responses to indirectly presented allo-Ag from that donor. In a previous experiment using Tcon as responders, there seemed to be specific suppression of all responses to the Original donor by Treg expanded through indirect Ag presentation, regardless of the Ag presentation mode (OriDC or OriLys) (**Fig.12C**). When $CD8^+$cells were used as responders, the specificity of suppression by indirectly expanded Treg seemed limited to indirect Ag presentation. In other words, it seems that Treg expanded through indirect presentation specifically suppress Tcon in the presence of Ag from the Original donor presented direct or indirectly, while suppression of $CD8^+$ cells is specific only for indirectly presented Ag from the Original donor.

Of note, in the experiments of **Fig.13**, although only $CD8^+$ proliferation was quantified, the number of T_{eff} plated refers to total PBMC, so the Treg:T_{eff} ratio refers to the number of Treg plated in relation to total PBMC. Because $CD8^+$ T cells are only a subset of PBMC (with variable frequency among donors), the ratio of Treg:$CD8^+$ cells is most likely different from the Treg:Tcon ratio, which refers to exact numbers of purified Tcon. Thus, it is not possible to compare between the suppression detected in SA with $CD8^+$ cells to the suppression detected with Tcon.

In conclusion, these experiments show that highly suppressive Ag-sp Treg can be expanded by allo-Ag presented direct and indirectly by moDC. Still, a few concerns remained unaddressed and should be pondered to further characterize eTreg function. The first concern was that high concentrations of IL-2 (100U/mL) were used during expansion. If, on one hand, this allowed for high fold expansions, on the other hand, it may skew eTreg potency and specificity by enforcing polyclonal proliferation in the absence of TCR affinity. Also, fresh rapamycin was added at every media change (every 2/3 days), as a way to impair residual Tcon proliferation. However, as described in the **State of the Art**, rapamycin may also increase the expression of Foxp3 and CD25 in Tcon and confer suppressive function to these cells, thus muddling the characterization of eTreg phenotype and function. Additionally, using total PBMC as responders in SA not only leads to variable Treg:T_{eff} ratios, but may also include the addition of confounding subsets, such as fresh Treg. Finally, the specificity of eTreg-mediated suppression, measured in SA with a 3rd party donor as APC, remained to be assessed. To validate these results and tackle the described concerns, new experiments were performed, where a few changes were introduced. The respective results will be described next.

1.2.2. Effect of low IL-2 concentration on Ag-sp Treg expansion and suppressive modalities

In the following experiments, the goal was to assess the effect of low IL-2 concentration (10U/mL) during expansion on Ag-sp eTreg expansion and function. Since expansion was to be performed in the

presence of low dose IL-2, the risk of expanding residual Tcon was much smaller; hence, rapamycin and IL-15 were only added at Day 0 of co-cultures. That is, when there were media changes, starting on Day 7, only fresh IL-2 was added. As a result, it was expected to increase Treg suppressive potency and, more importantly, specificity – possibly in detriment of fold expansion. To better characterize eTreg specificity, the suppression of responses to APC from 3rd party donors who were HLA-mismatched to the Treg and Original donors was measured in parallel.

a) Treg expansion and phenotype

The use of a lower concentration of IL-2 during expansion not only increased the standard deviation between experiments but also seemed to decrease the overall frequency of proliferated cells found at the end of culture (**Fig.14A**, as opposed to **Fig.10A**). Nevertheless, expansion with OriDC seemed to stimulate the highest Treg proliferation (79.4±24.3% of CFSE$^-$ Treg). In fact, in each independent experiment, the highest frequency of proliferated Treg was found in co-cultures with OriDC (**Fig.S5**). Besides presenting lower frequencies of proliferated cells, conditions that involved stimulation by self-MHC in the presence of Ag (OriLys) or not (SelfDC) had higher variation of frequency from experiment to experiment, as determined by the standard deviation of average frequencies. Such was the case of proliferated cells within eTreg(OriLys) and eTreg(SelfDC) (45.2±43.4% *vs.* 57.7±48.1%, respectively). Interestingly, fold expansion was similar on all conditions (**Fig.14B)**, which could suggest that, on conditions with higher proliferation there was also more cell death whereas, in conditions with lower proliferation, viability was maintained. The average fold expansion achieved with OriDC was 0.70±0.47, followed by co-cultures with OriLys (0.52±0.30) and finally co-cultures with SelfDC had an average fold expansion of 0.76±0.84. Nonetheless, proliferated Treg (CFSE$^-$ eTreg) had higher CD25 expression than unproliferated cells (CFSE$^+$ eTreg) (**Fig.14C**), while expression of CD127 was maintained (**Fig.14D**). Foxp3 MFI was slightly higher in proliferated cells as well (**Fig.14E**).

The expression of CD25, CD127 and Foxp3 was compared on offset overlays of samples from *ex vivo* Tcon*, ex vivo* Treg and bulk populations of Treg expanded with OriDC, OriLys or SelfDC (**Fig.14F-H**). There was always increased CD25 expression in eTreg, regardless of the APC used, when compared to *ex vivo* Treg (**Fig.14F**). Furthermore, although there was a slight increase in CD127 expression when *ex vivo* Treg were compared to eTreg, CD127 expression was still higher on *ex vivo* Tcon than in all Treg populations (**Fig.14G**). Finally, similarly to the expression of CD25, Foxp3 expression was increased on all eTreg (**Fig.14H**). Overall, this phenotype showed that despite the lower fold expansion and proliferation, Treg were still activated by moDC and proliferated in the presence of low concentration of IL-2.

Since lower fold expansion resulted in lower numbers of Treg after expansion, together with the fact that previous experiments had shown that LDA were not conclusive regarding eTreg function (potency and specificity of suppression), it was decided not to perform LDA to quantify the frequency of precursors in these experiments. Instead, at the end of expansion, a small aliquot of eTreg was separated for intracellular Foxp3 staining and the majority of eTreg were used to perform different SA conditions.

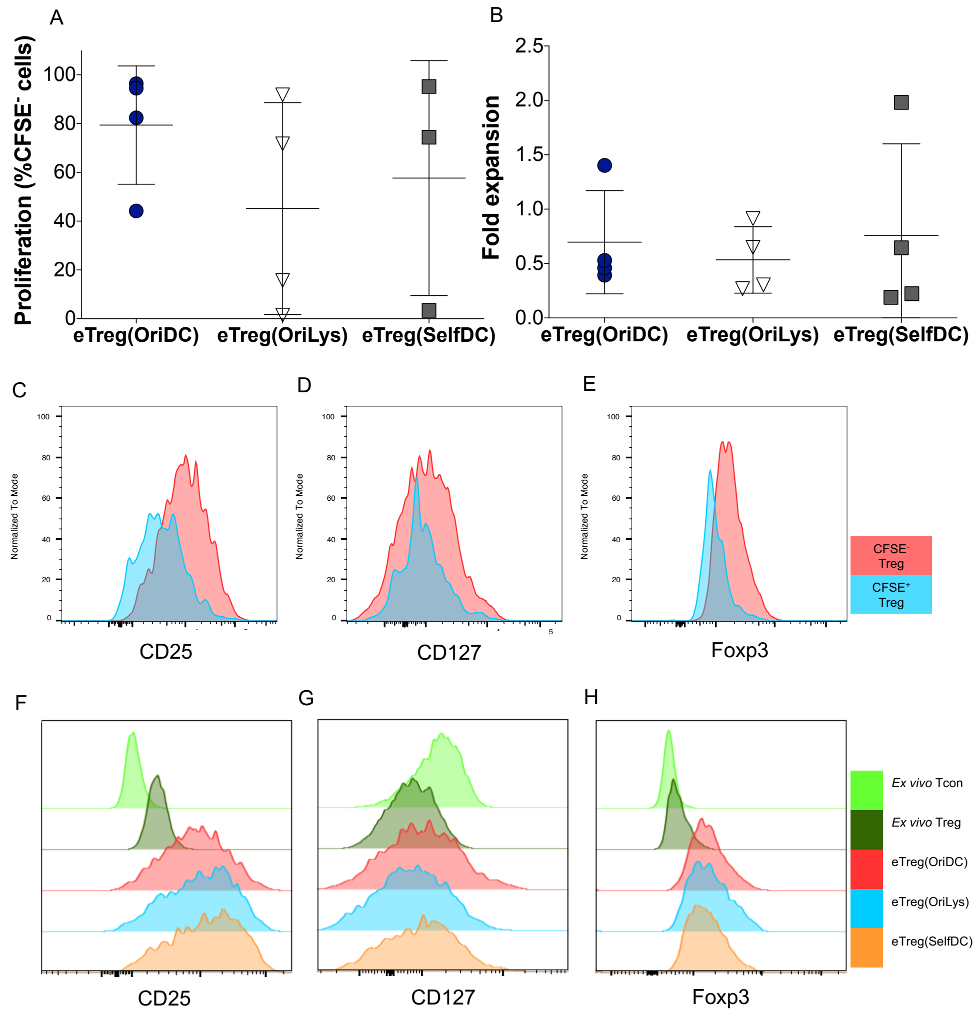

Figure 14: Expansion of Treg with low IL-2 in the presence of allo-Ag presented direct or indirectly.
Purified *ex vivo* Treg were co-cultured with OriDC, OriLys or SelfDC at a reduced concentration of IL-2 (10U/mL). (A) Percentage of proliferated (CFSE$^-$) cells after co-culture with OriDC, OriLys or SelfDC. Measured by the frequency of parent of CFSE$^-$ cells after gating on CD3$^+$CD4$^+$ lymphocytes. (B) Treg fold expansion after co-culture with OriDC, OriLys or SelfDC. Fold expansion was calculated by dividing the number of CD3$^+$CD4$^+$ lymphocytes counted using TruCount beads by the number of seeded Treg on Day 0. (C-E) Histograms of CD25 (C), CD127 (D) and Foxp3 (E) expression with CFSE$^+$ and CFSE$^-$ eTreg in overlay. (F-H) Offset overlay histograms of CD25 (F), CD127 (G) and Foxp3 (H) expression in *ex vivo* Tcon (light green), *ex vivo* Treg(dark green), eTreg(OriDC) (red), eTreg(OriLys) (blue) and eTreg(SelfDC) (orange). *A-B depicts cumulative data from 3 experiments; C-H: representative data of one out of three experiments. C-E shows data from eTreg(OriDC).*

b) Specificity of suppression

One of the aims in reducing the concentration of IL-2 during expansion was to select for eTreg that, during SA, specifically suppressed responses to the same APC they were expanded with – original stimulators. Specificity of suppression was determined by comparing the potency of suppression in the presence of the original stimulator with the potency of suppression in the presence of moDC from an unrelated 3rd party donor. To assure that each 3rd party donor had minimal MHC-matches with the Treg and Original donor, high resolution HLA sequencing (HLA-A, -B, -C, -DR, -DQB1 and -DQB2) was performed for all donors (**Table S- I**), after which the 3rd party donor was chosen for each experiment. The suppression of Tcon proliferation by bulk populations of eTreg(OriDC) (**Fig.15A**) or by eTreg(OriLys)

(**Fig.15B**) was measured using, as stimulators, APC from the original stimulator of each co-culture (OriDC or OriLys, respectively) or 3rd party DC.

Treg that were expanded by direct allo-Ag presentation with OriDC – eTreg(OriDC) – similarly suppressed Tcon proliferation when the stimulators were OriDC and 3rd party DC (**Fig.15A**). Maximum suppression was seen with 1:5 Treg:T_{eff} in both conditions, where Tcon proliferation was decreased to 26.34±17.71% in the presence of OriDC and 28.88±6.12% with 3rd party DC. With lower numbers of eTreg (from 1:50 to 1:5000 Treg:T_{eff}), responses to OriDC seemed more suppressed than responses to 3rd party DC yet, at higher Treg:T_{eff} ratios, suppression seemed non-specific, as responses to the original stimulator and to the 3rd party donor were highly suppressed. On the other hand, there seemed to be specific suppression of Tcon proliferation by indirectly expanded Treg – eTreg(OriLys) at all concentrations. That is, proliferation of Tcon was higher in conditions with 3rd party DC than in conditions with OriLys, which was the original stimulator of these cells' expansion (**Fig.15B**). Again, lowest Tcon proliferation was achieved at 1:5 Treg:T_{eff}, where it was reduced to 18.02±9.38% with OriLys and 42.21±12.61% with 3rd party DC. Nevertheless, no statistically significant differences were found amongst Tcon proliferation in response to different APC, therefore it could not be confirmed that the suppression of Tcon by eTreg was specific for the original stimulator.

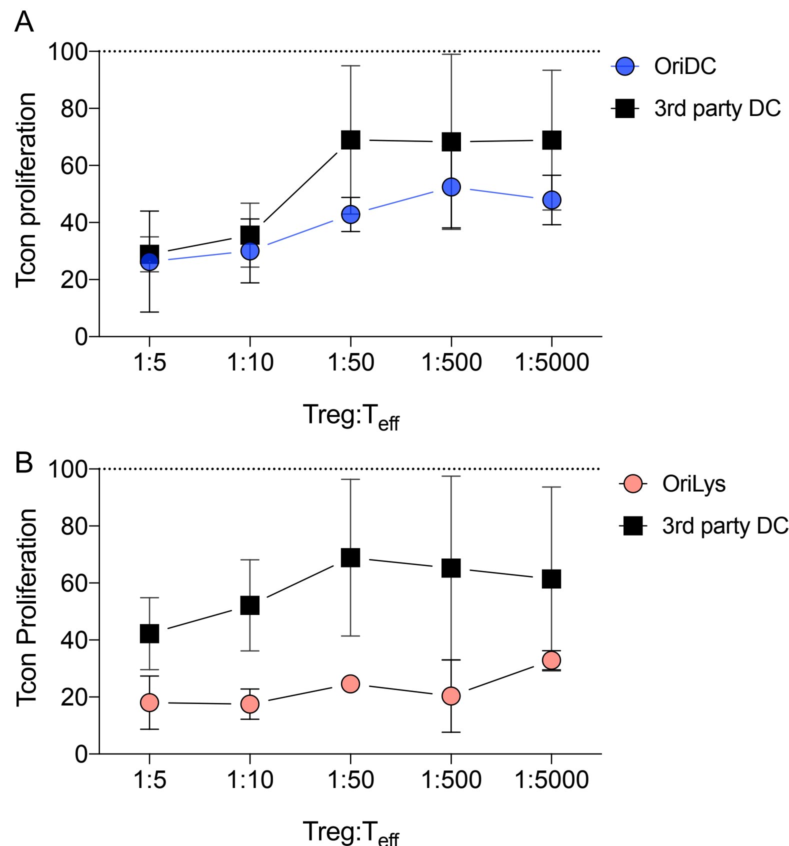

Figure 15: Specificity of suppression of Tcon proliferation by Treg after expansion with Ag presented direct or indirectly.
Proliferation of Tcon, as measured by CTV dilution, normalized to proliferation (%CTV- cells) in the absence of eTreg. (A) Proliferation of Tcon in SA with eTreg(OriDC) at several dilutions, in the presence of OriDC (Blue circles) or 3rd party DC (black squares). (B) Proliferation of Tcon in SA with eTreg(OriLys) at several dilutions, in the presence of OriLys (Pink circles) or 3rd party DC (black squares). D*ata from 3-4 experiments. Statistical analysis was performed using multiple t-tests, by the Holm-Sidak method.*

In brief, responses to 3rd party DC appeared more suppressed by directly expanded Treg – eTreg(OriDC) – than by Treg expanded by indirect presentation of allo-Ag – eTreg(OriLys). Still, eTreg(OriLys) seemed to potently suppress responses to their original stimulator, when compared to the control (absence of eTreg), although there was no effect on Tcon proliferation of increasing the number of eTreg(OriLys). Overall, expansion by indirectly presented Allo-Ag seemed to increase eTreg specificity, when compared to eTreg expanded with OriDC. Nevertheless, eTreg from both expansion milieus seemed highly suppressive of their original stimulators. Next, we assessed the potency of each type of eTreg to suppress Tcon proliferation when allo-Ag from the Original donor were presented direct or indirectly.

c) Potency of suppression

In the same SA where specificity was determined, the potency of each type of eTreg to suppress responses to allo-Ag from the Original donor, in different presentation modes, was also assessed. That is, we wanted to determine the potency of Treg expanded with allo-Ag presented directly – eTreg(OriDC) – or indirectly – eTreg(OriLys) – to suppress Tcon proliferation when the stimulators were OriDC (**Fig.16A)** or OriLys (**Fig.16B**).

Treg that were expanded by directly presented allo-Ag (OriDC) seemed more potent than Treg expanded by indirectly presented allo-Ag (OriLys) in the suppression of Tcon proliferation in the presence of OriDC (**Fig.16A**). Indeed, at a ratio of 1:5 Treg:T_{eff}, Tcon proliferation was reduced to 26.34±17.71% by eTreg(OriDC) and only to 57.97±19.01% by eTreg(OriLys). Since OriDC was the original stimulator of eTreg(OriDC), it was expected that they would be more potent suppressors of responses to it. Furthermore, whilst Tcon proliferation at 1:5000 Treg:T_{eff} was already decreased to 47.94±8.71% by eTreg(OriDC), it was still 80.69±36.10% in SA with eTreg(OriLys). Regarding the suppression of Tcon proliferation in the presence of indirectly presented allo-Ag by OriLys, eTreg from both expansion milieus had similar potency (**Fig.16B**). At 1:5 Treg:T_{eff}, Tcon proliferation was reduced

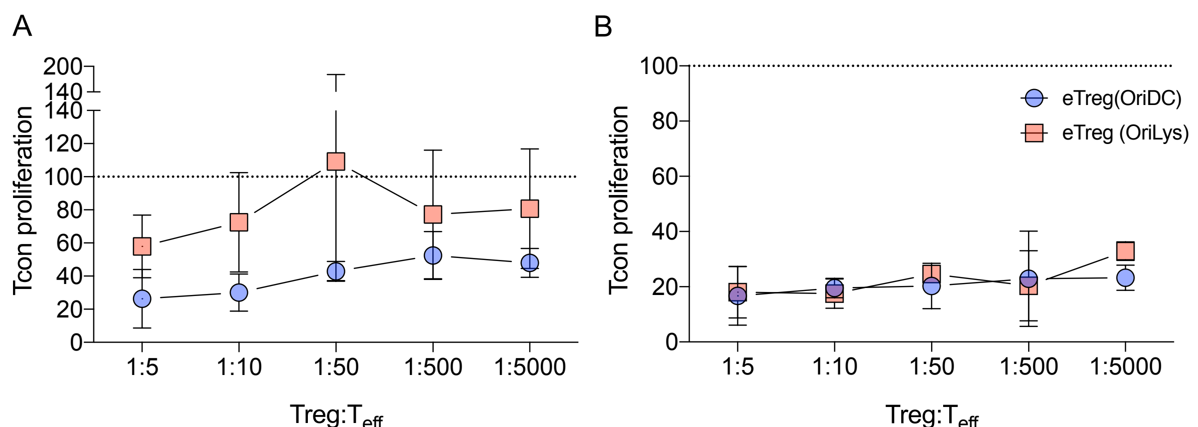

Figure 16: Potency of suppression of Tcon proliferation by Treg after expansion with OriDC or OriLys, in the presence of the same stimulators.

Proliferation of Tcon, as measured by CTV dilution, normalized to proliferation (%CTV^- cells) in the absence of eTreg. (A) Proliferation of Tcon in response to OriDC, in the presence of directly expanded Treg – eTreg(OriDC), blue circles – or indirectly expanded Treg – eTreg(OriLys), pink squares – at several dilutions. (B) Proliferation of Tcon in response to OriLys, in the presence of directly expanded Treg – eTreg(OriDC), blue circles – or indirectly expanded Treg – eTreg(OriLys) – at several dilutions (pink squares). D*ata from 3-4 experiments. Statistical analysis was performed using multiple t-tests, by the Holm-Sidak method, with p=0.05.*

to 16.70±10.60% by eTreg(OriDC), and to 18.02±9.38% by eTreg(OriLys). Surprisingly, the suppression of responses to OriLys was not increased by increasing eTreg numbers, as at 1:5000 Treg:T_{eff} Tcon proliferation was already decreased to 23.29±4.62% and 32.92±3.41%, respectively.

If, on one hand, these results indicate that directly expanded Treg – eTreg(OriDC) – could be more potent suppressors of any responses to allo-Ag from the Original donor (presented direct or indirectly), on the other hand, it was puzzling that, in all conditions, there was low Tcon proliferation at all Treg:T_{eff} ratios. Moreover, Tcon proliferation did not seem decreased by increasing the number of eTreg, suggesting the low proliferation may not be a result of suppression by eTreg. The only exception was found in the suppression of OriDC by indirectly expanded Treg – eTreg(OriLys) – where there was a slight decrease in Tcon proliferation with increasing Treg numbers, yet Tcon proliferation remained higher than in the presence of OriLys at all ratios. This observation might indicate that albeit less potent suppressors of Tcon proliferation, these cells may be more specific to their original stimulator (OriLys).

Finally, we wanted to validate if the suppression of responses to the Original donor by eTreg(OriDC) was independent of the type of allo-Ag presentation, and if similar observations could be found using Tcon or $CD8^+$ cells as responders. Thus, the function of Treg expanded by direct allo-Ag presentation - eTreg(OriDC) – was investigated in SA where both types of stimulators were used in parallel (OriDC and OriLys), and Tcon (**Fig.17A**) or sorted $CD8^+$ cells (**Fig.17B**) were used as responders. Tcon proliferation was very low in the presence of indirectly presented allo-Ag (OriLys), even at ratios where few eTreg(OriDC) were present, such as 1:5000 Treg:T_{eff} (**Fig.17A**, 47.94±8.71% with OriDC *vs.* 23.30±4.62% with OriLys). In the presence of directly presented allo-Ag (OriDC), Tcon proliferation was already 47.94±8.71% of the control at 1:5000 Treg:T_{eff} (**Fig.17A**). However, with increasing eTreg numbers, Tcon proliferation in response to directly presented allo-Ag (OriDC) was suppressed while, with indirectly presented allo-Ag (OriLys), the proliferation of Tcon remained constant, and low. Thus, at higher Treg:T_{eff} ratios, proliferation of Tcon in conditions with direct allo-Ag presentation was decreased to similar levels as found in conditions with indirect allo-Ag presentation (1:5 Treg:T_{eff}: OriDC 26.34±17.71%, OriLys 16.70±10.59%). Interestingly, in an exploratory experiment, we verified that in

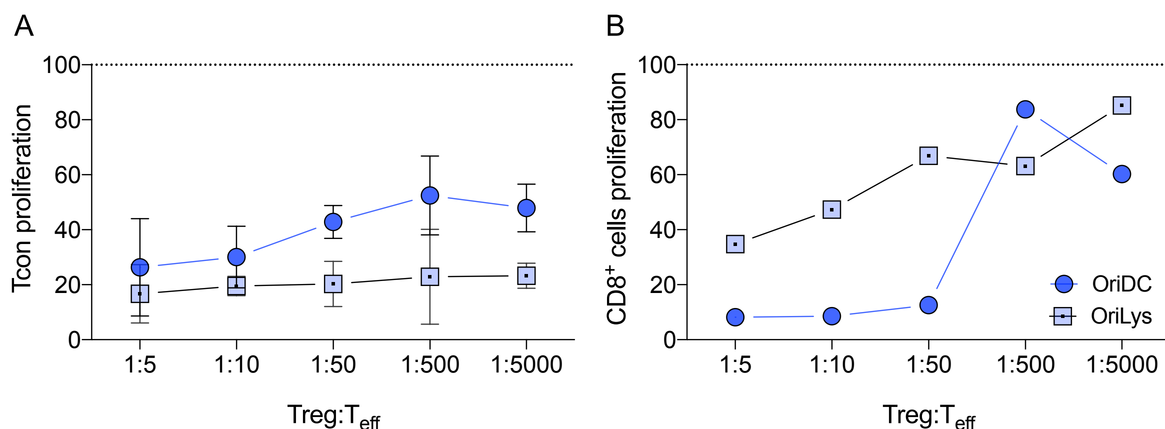

Figure 17: Suppression of responses to Original donor by eTreg(OriDC).
Proliferation of responders was measured by CTV dilution, normalized to proliferation (%CTV^- cells) in the absence of eTreg. (A) Proliferation of Tcon in the presence of eTreg(OriDC) at several dilutions, with stimulation by OriDC (Blue circles) or by OriLys (Light blue squares). (B) Proliferation of $CD8^+$ cells in the presence of eTreg(OriDC) at several dilutions, with stimulation by OriDC (Blue circles) or OriLys (Light blue squares). A: D*ata from 3 experiments; B: Data from 1 experiment.*

the presence of allo-Ag from the Original donor, presented directly by OriDC or indirectly by OriLys, suppression of CD8+ cell proliferation was increased by increasing eTreg numbers (**Fig.17B**). Furthermore, suppression of CD8+ cells seemed more potent in response to directly presented allo-Ag (OriDC), thus there seemed to be specific suppression by directly expanded Treg – eTreg(OriDC) – in this setting. For example, at a ratio of 1:5 Treg:T_{eff}, CD8+ cells proliferation was decreased to 8.17% in the presence of OriDC as opposed to 34.80% in the presence of OriLys.

Trying to understand why increased eTreg numbers had different effects on Tcon suppression, particularly when there was indirect allo-Ag presentation by OriLys – where proliferation did not seem to be decreased by eTreg – we looked at the basal proliferation of Tcon and CD8+ cells in the presence of stimulators only (0:1 Treg:T_{eff}). The basal proliferation of Tcon was significantly lower in the presence of indirectly presented allo-Ag (OriLys) than in the presence of directly presented allo-Ag (OriDC) (**Fig.18**). Hence, it could be easier for eTreg to suppress Tcon responses to indirectly presented allo-Ag from the Original donor than to directly presented allo-Ag from the same donor. These results suggested that Treg expanded by indirect allo-Ag presentation – eTreg(OriLys) – were weak suppressors, capable of suppressing responses to weak stimuli only. Simultaneously, Treg expanded by direct allo-Ag presentation - eTreg(OriDC) – were apparently potent suppressors, yet unspecific in their suppression of Tcon proliferation. Interestingly, directly expanded Treg – eTreg(OriDC) – were also able to suppress CD8+ cells, and in these conditions (**Fig.17B**) there seemed to be a gradual effect of increased eTreg number in increasing suppression. So, while in the suppression of Tcon an important role seemed to be played by the basal proliferation of responders, which could be related to the strength of the stimulator (**Fig.17A**), suppression of CD8+ cells by directly expanded Treg – eTreg(OriDC) – seemed specific to their original stimulator (OriDC). One of the main differences between OriDC and OriLys as APC is the fact that the former present allo-Ag through an allo-MHC, forming a completely allogeneic Ag-MCH complex. In the latter (OriLys), there is allo-Ag presentation through a self-MHC. Thus, the specificity of directly expanded Treg may stem from a specificity of the eTreg TCR to the whole allo-Ag-MHC complex (**Fig.17B**).

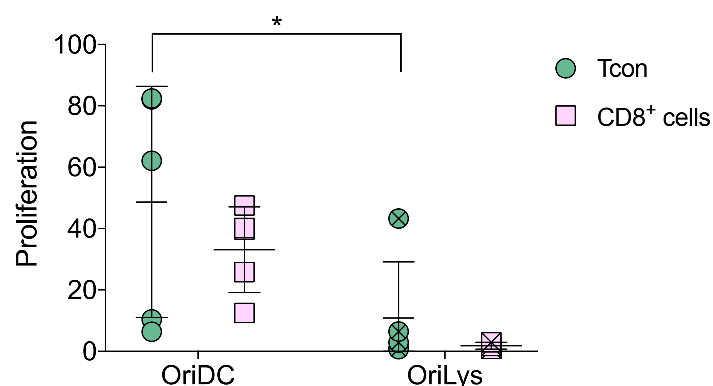

Figure 18: Proliferation of Tcon and CD8+ cells after stimulation by OriDC or OriLys.
Proliferation of responders measured by CTV dilution (%CTV- cells) in the absence of eTreg. Proliferation of Tcon after stimulation by OriDC (green circles) or by OriLys (green circles with cross), and proliferation of CD8+ cells after stimulation by OriDC (pink squares) or by OriLys (pink squares with cross). D*ata from 4 experiments. Statistical analysis was performed using multiple t-tests, by the Holm-Sidak method.* * represents statistically significant differences, with *p=0.0471*

Overall, we concluded it would be interesting to validate these observations not only by repeating the experiments but also by assessing all of these conditions in parallel. This way, it would be possible to assess, for each experiment, the effect of the concentration of IL-2 in eTreg expansion and function, as well as directly compare the potency and specificity of suppression of Tcon with the potency and specificity of suppression of $CD8^+$ cells for each expansion millieu. The results of these experiments will be described in the next chapter.

2. CHARACTERIZATION OF AG-SP TREG FUNCTION

In the second part of this work, the main goal was to characterize in-depth the function of Ag-sp Treg after expansion in serum-free conditions. To fully characterize the potency of suppression of eTreg, it was required that the APC used in SA elicited similar proliferation of responders. Thus, according to the results described in the previous chapter, it was decided to use APC with direct allo-Ag presentation only. Consequently, the expansion of Ag-sp Treg was only performed in the presence of directly presented allo-Ag from the Original donor (OriDC). This way, it was possible to assess in parallel the expansion of Treg in low or high concentrations of IL-2 (10U/mL or 100U/mL, respectively), as well as the suppressive function of eTreg from each expansion milieu.

The function of Treg from each expansion millieu was characterized regarding their potency of suppression in SA with APC from the Original donor, as well as regarding the specifcity of suppression. Furthermore, each condition was assessed in SA not only with Tcon but also with $CD8^+$ cells as responders. Finally, we quantified the levels of cytokines in SA supernatants and the co-stimulation provided by APC to both eTreg and responders during SA. The objective of this analysis was to identify possible mechanisms involved in the suppression of proliferation performed by eTreg. Of note, the phenotype of eTreg from both expansion milieus was also analysed in-depth using novel computational methods, and those results will be described in **Chapter 2.3**.

2.1. Effect of expansion milieu on Treg expansion

Treg were sorted by FACS and co-cultured with moDC from the Original donor (OriDC) in TexMACS medium at a ratio of 4:1 Treg:APC, as described in **Materials and Methods**. To assure all experiments were as similar as possible, the HLA of Original and Treg donors were sequenced and Treg-OriDC donors were paired based on HLA typing (detailed HLA-typing can be found in **Annexes, Table S-II**). Of note, the level of matching between donors was the same for MHCI and MHCII, and was limited to 2 out of 6 matches in both classes. Half of the wells were supplemented with exogenous IL-2 at a concentration of 10U/mL, and the other half of the wells were supplemented with 100U/mL IL-2. At the time of the first media change, on Day 7, the concentration of IL-2 was kept at the same concentration as on Day 0. Exogenous IL-15 and rapamycin were added to wells from both conditions on Day 0 only.

Cells were kept in culture for 14 days, after which eTreg were removed from wells and counted using a hemocytometer. Treg fold expansion was, on average, 1.60±0.62 with 10U/mL IL-2 and 2.05±0.86 when IL-2 concentration was kept at 100U/mL (**Fig.19A**). While a trend for higher fold expansion in cultures with more IL-2 could be detected, these differences were not statistically significant. Also, the percentage of proliferated Treg was similar in both conditions, as there were 72.40±27.79% of $CFSE^-$ cells after expansion with 10U/mL IL-2 and 73.97±32.64% with 100U/mL IL-2 (**Fig.19B**). This observation may indicate that Treg have higher survival rates in the presence of higher levels of IL-2, which would result in higher fold expansion.

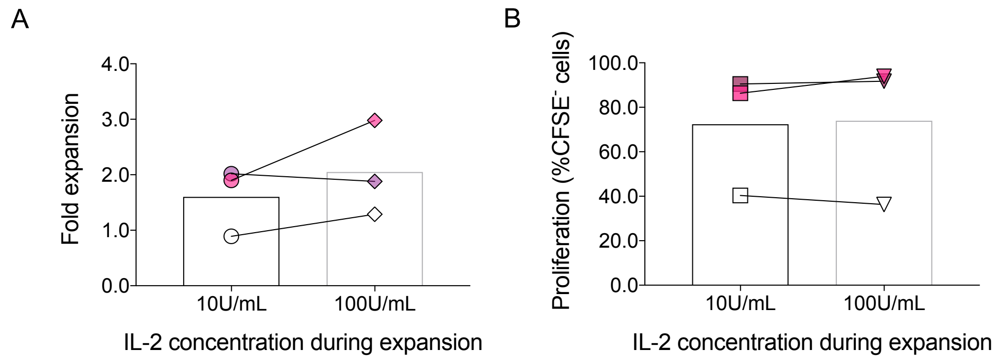

Figure 19: Effect of IL-2 concentration during expansion on Treg fold expansion and proliferation.
(A) Treg fold expansion after 14 days in co-culture with OriDC and IL-2 at a concentration of 10U/mL (circles and black bar) or 100U/mL (diamonds and grey bar). Fold expansion was calculated by dividing the number of counted live cells by the number of seeded cells on Day 0; (B) Treg proliferation after 14 days in co-culture with OriDC and IL-2 at a concentration of 10U/mL (squares and black bar) or 100U/mL (inverted triangles and grey bar). Proliferation was measured by gating on $CD3^{+}CD4^{+}$ cells and then on $CFSE^{-}$ cells. *Bars represent average numbers, and each experiment is represented by a colour. N=3*

2.2. Effect of expansion milieu on Treg function

2.2.1. Potency of suppression of responses to the original stimulator

The capability of eTreg from different expansion milieus to suppress Tcon and $CD8^{+}$ T cell proliferation was evaluated first in the presence of moDC from the Original donor (OriDC), which was the original stimulator of their expansion. Tcon (**Fig.20A**) and $CD8^{+}$ T cell (**Fig.20B**) proliferation was significantly decreased by eTreg, as the proliferation of responders in the presence of eTreg at a ratio

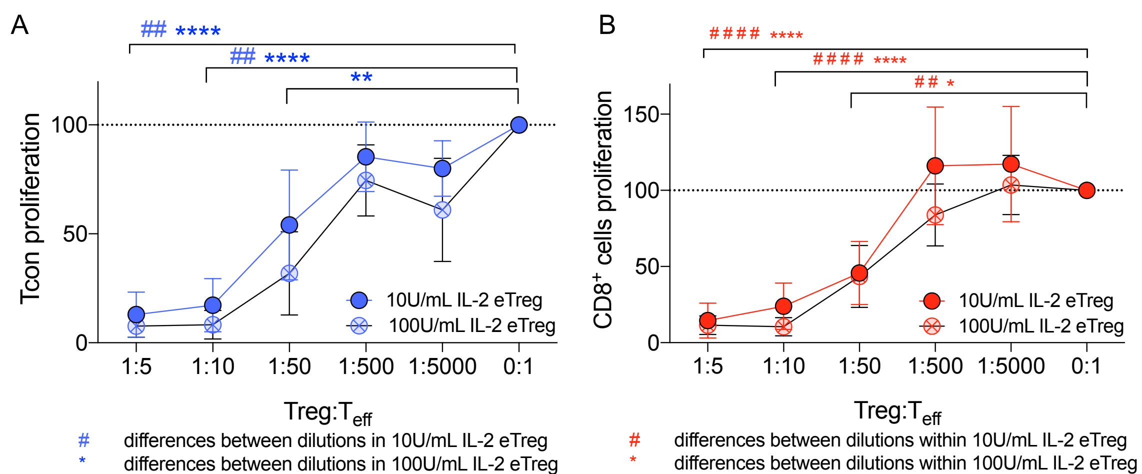

Figure 20: Potency of Treg expanded with 10U/mL or 100U/mL of IL-2 in the suppression of Tcon and $CD8^{+}$ cells proliferation, in response to OriDC.
(A) Proliferation of Tcon in response to OriDC, in the presence of Treg expanded with 10U/mL IL-2 (blue circles) or 100U/mL IL-2 (light blue circles) at several dilutions. (B) Proliferation of $CD8^{+}$ cells in response to OriDC, in the presence of Treg expanded with 10U/mL IL-2 (red circles) or 100U/mL IL-2 (light red circles) at several dilutions. *N=3. Statistical analysis of differences between dilutions was performed with one-way ANOVA and differences between conditions were evaluated with multiple t-tests, by the Holm-Sidak method. Statistical significance was assumed when p<0.05.*

of 1:10 and 1:5 Treg:T_{eff} was significantly lower than in the absence of Treg (0:1). Interestingly, Treg expanded with more IL-2 (100U/mL IL-2 eTreg) seemed slightly more suppressive of Tcon proliferation than those expanded with less IL-2 (10U/mL IL-2 eTreg), particularly in higher Treg dilutions, such as 1:50 Treg:T_{eff} (**Fig.20A**). Yet, no difference was found in Tcon proliferation with eTreg from different conditions. There was significant suppression of $CD8^+$ T cell proliferation by eTreg from both conditions, starting at 1:50 Treg:T_{eff} (**Fig.20B**). Since eTreg from both conditions similarly suppressed $CD8^+$ cells proliferation, there seemed to be no effect of the expansion milieu on eTreg potency of suppression of $CD8^+$ cells proliferation. In sum, eTreg from both expansion milieus were potent suppressors of responder proliferation in the presence of their original stimulator (OriDC), decreasing Tcon and $CD8^+$ T cell proliferation to around 10% of the control (0:1) at a ratio of 1:5 Treg:T_{eff}.

2.2.2. Potency of suppression of responses to non-specific stimuli

A commonly used method to assess eTreg potency of suppression in SA is by using Ab-coated beads (Beads) as aAPC. The commercially available combinations of Ab-coated beads usually include aCD2, aCD3 and aCD28 and provide mitogenic stimuli to T cells, thus allowing for the evaluation of full potential of suppression by eTreg in the absence of any cell-based APC. Treg expanded with 10U/mL IL-2 (**Fig.21A and Fig.21C**) or 100U/mL IL-2 (**Fig.21B and Fig.21D**) at various ratios were co-cultured with fixed numbers of Tcon (**Fig.21A-B**) and $CD8^+$ cells (**Fig.21C-D**). Suppression of proliferation in the presence of beads was compared to the suppression exerted by eTreg in the presence of OriDC.

In each setting, the proliferation of responders with either OriDC or Beads was always significantly suppressed by Treg (**Fig.21A-D**). However, there seemed to be less suppression of proliferation when the stimulators were beads, except when $CD8^+$ cells were used as responders for Treg expanded with 10U/mL IL-2 (**Fig.21C**). Nevertheless, within each of the four settings, no differences were found between the proliferation with OriDC or Beads. When the potency of suppression by Treg expanded with 10U/mL of IL-2 (10U/mL IL-2 eTreg) was compared to the potency of Treg expanded with 100U/mL (100U/mL IL-2 eTreg), there seemed to be no effect of increasing IL-2 concentration during expansion on the potency of suppression of Tcon (**Fig.21A,** compared to **Fig.21B)** or of $CD8^+$ cells (**Fig.21C**, compared to **Fig.21D**). Simultaneously, Treg expanded with 10U/mL of IL-2 similarly suppressed both types of responders (**Fig.21A,** as compared to **Fig.21C**), and so did Treg expanded with 100U/mL of IL-2 (**Fig.21C**, compared to **Fig.21D**).

Overall, Treg from both expansion milieus were potent suppressors of Tcon and $CD8^+$ cells proliferation not only in the presence of the moDC from the Original donor (OriDC), but also when a mitogenic stimulus was provided (Beads). However, the fact that responses to non-specific stimuli seemed as suppressed as those to the Original donor confirms the need for additional specificity assays.

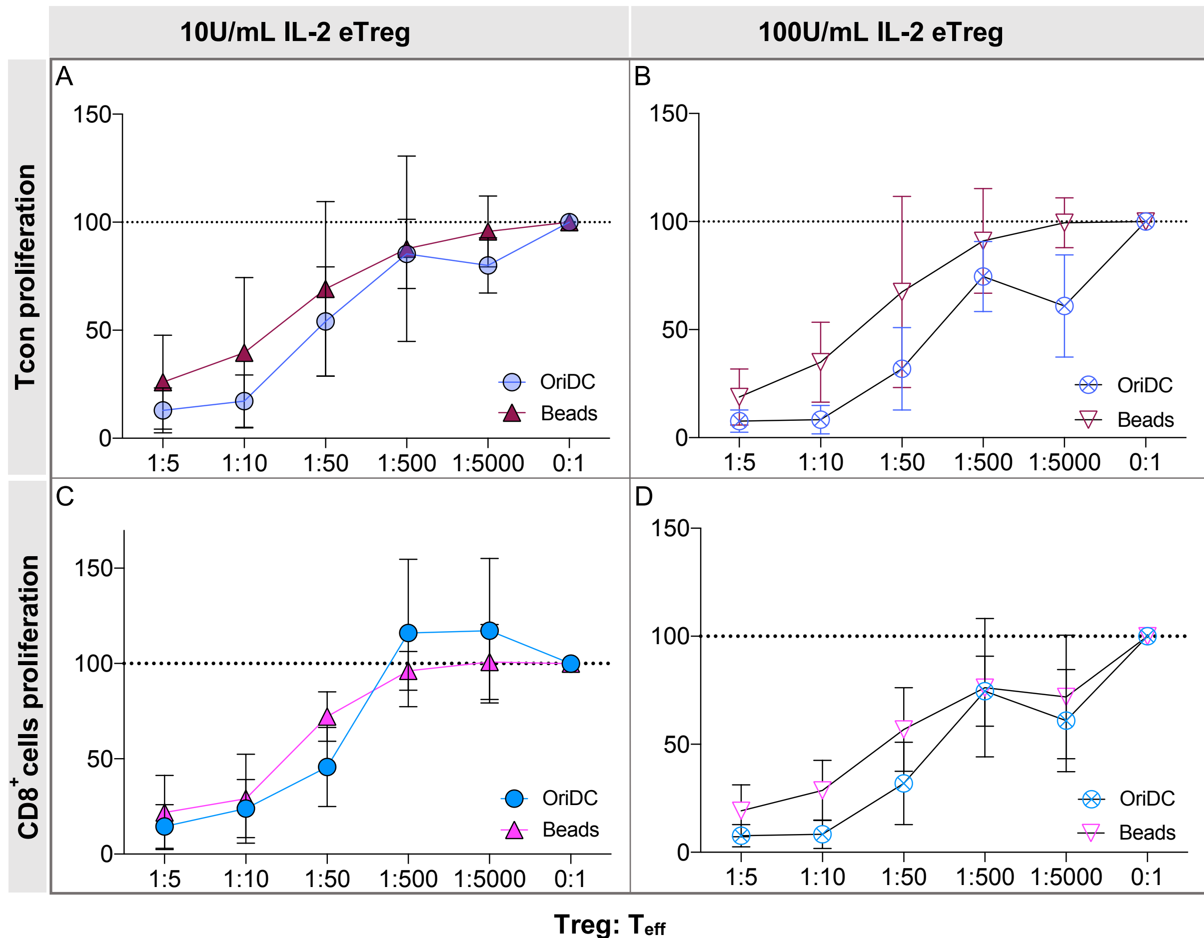

Figure 21: Potency of Treg expanded with 10U/mL or 100U/mL of IL-2 in the suppression of Tcon and CD8⁺ cells proliferation, in response to inspector beads.
(A) Proliferation of Tcon in the presence of Treg expanded with 10U/mL IL-2 at several dilutions, in response to OriDC (blue circles) or inspector beads (red triangles). (B) Proliferation of Tcon in the presence of Treg expanded with 100U/mL IL-2 at several dilutions, in response to OriDC (empty blue circles) or inspector beads (empty red triangles). (C) Proliferation of $CD8^+$ cells in the presence of Treg expanded with 10U/mL IL-2 at several dilutions, in response to OriDC (blue circles) or inspector beads (pink triangles). (D) Proliferation of $CD8^+$ cells in the presence of Treg expanded with 100U/mL IL-2 at several dilutions, in response to OriDC (empty blue circles) or inspector beads (empty pink triangles). *N=3. Statistical analysis of differences within conditions (between dilutions) was performed with one-way ANOVA and differences between conditions were evaluated with multiple t-tests, by the Holm-Sidak method, with $p<0.05$.*

2.2.3. Specificity of suppression

After verifying that eTreg were highly suppressive of responses to their original stimulator (OriDC) as well as to mitogenic stimuli (Beads), the next step was to assess their specificity, i.e., whether those eTreg would suppress proliferation regardless of the APC presented in the suppression milieu. To do this, moDC from a 3rd party donor were used as APC in SA. Importantly, a 3rd party donor for each independent experiment was selected so that there would be no matching of the donor's HLA to the HLA of the Treg donor or to the HLA of the Original (APC) donor. The high-resolution HLA-typing of 3rd party donors can be found in **Annexes, Table S-III**, and the SA conditions with moDC differentiated from these donors will be referred to as "Mismatch 3P".

Treg that were expanded with 10U/mL of IL-2 (10U/mL IL-2 eTreg) did not suppress Tcon proliferation upon stimulation with moDC from a mismatched 3rd party donor (Mismatch 3P, **Fig.22A**). In fact, at 1:5 and 1:10 Treg:T_{eff}, there was significantly lower proliferation of Tcon in the presence of OriDC than in the presence of Mismatch 3P (for 1:5, proliferation was 12.93±10.37% with OriDC *vs.* 79.92±45.71% with Mismatch 3P, p=0.0014; at 1:10, proliferation was 17.24±12.16% with OriDC *vs.* 90.91±45.67% with Mismatch 3P, p=0.007). That is, at these ratios of Treg:T_{eff}, Tcon proliferation in response to the Original donor was significantly more suppressed than the proliferation of Tcon in response to moDC from a mismatched 3rd party donor.

In SA with Treg that were expanded with 100U/mL of IL-2 (100U/mL IL-2 eTreg), Tcon proliferation was significantly lower in the presence of OriDC than of Mismatch 3P at all eTreg dilutions (**Fig.22B**). That is, Tcon proliferation was significantly more suppressed by eTreg when moDC from the Original donor were present than when the stimulators derived from a mismatched 3rd party donor. Nevertheless, Tcon proliferation was reduced to 49.07±30.12% of the control (0:1) at the highest Treg:T_{eff} ratio – 1:5. Interestingly, the expansion milieu of Treg seemed to affect the suppression of Tcon proliferation in the presence of moDC from a mismatched 3rd party donor (Mismatch 3P**, Fig.22A**, compared to **Fig.22B**). Apparently, there was less proliferation of Tcon when cultured with Treg expanded with 100U/mL of IL-2 than with Treg expanded with 100U/mL of IL-2. Since these differences were not detected in the suppression of responses to moDC from the Original donor, there is the risk that Treg expanded with 100U/mL of IL-2 may be more potent suppressors of responses to APC from unrelated donors and thus result in unspecific suppression.

The proliferation of $CD8^+$ cells was also measured in the same conditions (**Fig.22C-D**). In SA using Treg expanded with 10U/mL of IL-2 (10U/mL IL-2 eTreg), there was no statistically significant difference between the proliferation of $CD8^+$ cells in response to moDC from the Original donor (OriDC) and the proliferation of $CD8^+$ cells in response to a mismatched 3rd party donor (Mismatch 3P, **Fig.22C**). In fact, there was statistically significant suppression of $CD8^+$ cells proliferation in response to both stimulators, when compared to the control (0:1). Nevertheless, starting at 1:50 and at higher Treg:T_{eff} ratios, $CD8^+$ cells proliferation seemed less suppressed in the presence of moDC from a mismatched 3rd party donor (Mismatch 3P) than in the presence of the moDC from the Original donor (OriDC). For example, at a ratio of 1:5 Treg:T_{eff}, proliferation was suppressed to 14.52±11.42% with OriDC, as opposed to 41.01±23.08% with Mismatch 3P. Similar suppression profiles could be observed in presence of Treg expanded with 10U/mL of IL-2 (10U/mL IL-2 eTreg, **Fig.22C**) and Treg expanded with 100U/mL of IL-2 (100U/mL IL-2 eTreg, **Fig.22D**). In SA with Treg expanded with 100U/mL of IL-2 at a ratio of 1:5 and 1:10 Treg:T_{eff}, the proliferation of $CD8^+$ cells was significantly lower in the presence of moDC from the Original donor (OriDC) than in the presence of moDC from a mismatched 3rd party donor (Mismatch 3P, **Fig.22D**). For example, at 1:5 Treg:T_{eff} proliferation of $CD8^+$ cells was reduced to 11.51±6.15% in cultures with OriDC and to 27.48±5.39% in cultures with Mismatch 3P (p=0.0031); at a ratio of 1:10 Treg:T_{eff}, proliferation was already decreased to 10.51±6.00% with OriDC while there was still 39.54±12.18% of proliferation with Mismatch 3P (p=0.0026). Overall, these results point to the fact that eTreg can highly suppress $CD8^+$ T cell proliferation, yet the potency of suppression may limit its specificity, if not render it unspecific. Interestingly, Treg expanded with 100U/mL of IL-2

seemed more suppressive of CD8⁺ cells proliferation in response to moDC from a mismatched 3rd party donor (Mismatch 3P) than Treg expanded with 10U/mL of IL-2, indicating that 100U/mL IL-2 eTreg may be more potent suppressors overall. This observation contradicts what had been shown in SA plots with moDC from the Original donor only (OriDC, **Fig.20**), where the potency of suppression of CD8⁺ cells proliferation did not seem to vary with the expansion milieu. Conversely, a similar trend for the suppression of responses to moDC from a mismatched 3rd party donor was detected in the suppression of Tcon proliferation. In other words, when the stimulators in SA were moDC from a mismatched 3rd party donor, the proliferation of Tcon and CD8⁺ cells seemed more suppressed by Treg expanded with 100U/mL of IL-2 than by Treg expanded with 10U/mL of IL-2.

To conclude, the results described here indicate that Treg expanded with higher concentration of IL-2 may be, in general, more potent suppressors. On the other hand, suppression of Tcon proliferation by Treg expanded with 10U/mL of IL-2 seemed to be limited to the presence of moDC from the Original donor, in what could be described as a typically specific suppression. Still, it could be argued that suppression of responses to an unrelated 3rd party donor does not imply that there is no specificity, as there was always more potent suppression in the presence of moDC from the Original donor.

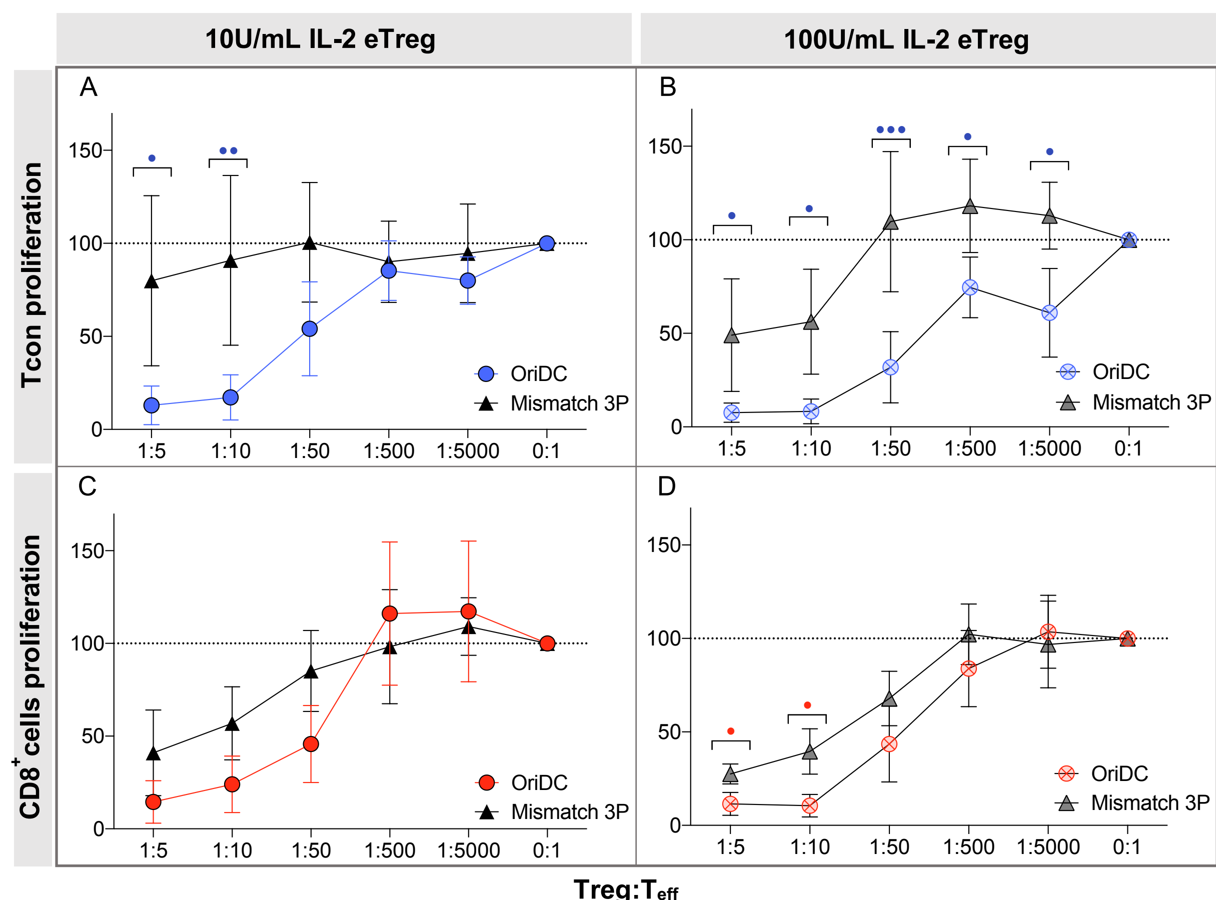

Figure 22: Specificity of suppression of Treg expanded with 10U/mL or 100U/mL of IL-2 in the suppression of Tcon and CD8⁺ cells proliferation.
(A) Proliferation of Tcon in the presence of Treg expanded with 10U/mL IL-2 at several dilutions, in response to OriDC (blue circles) or Mismatch 3P (black triangles). (B) Proliferation of Tcon in the presence of Treg expanded with 100U/mL IL-2 at several dilutions, in response to OriDC (blue circles) or Mismatch 3P (grey triangles). (C) Proliferation of CD8⁺ cells in the presence of Treg expanded with 10U/mL IL-2 at several dilutions, in response to OriDC (red circles) or Mismatch 3P (black triangles). (D) Proliferation of CD8⁺ cells in the presence of Treg expanded with 100U/mL IL-2 at several dilutions, in response to OriDC (red circles) or Mismatch 3P (black triangles). *N=4. Statistical analysis of differences within conditions (between dilutions) was performed with one-way ANOVA and differences between conditions were evaluated with multiple t-tests, by the Holm-Sidak method, with $p<0.05$*

2.2.4. Relationship between eTreg potency and specificity of suppression

Judging by the previous SA, it seemed that when the potency of suppression by eTreg was altered by the expansion milieu, there could be an effect on the specificity of suppression. Thus, it was hypothesized that the potency of suppression by eTreg could be related to its specificity. First, the difference in specificity (∆Specificity) displayed by Treg expanded with 10U/mL of IL-2 or expanded with 100U/mL of IL-2 was calculated. For each independent experiment, Treg from each expansion milieu had two corresponding values of ∆Specificity: one relative to Tcon and one relative to $CD8^+$ cells. To calculate each ∆Specificity, the relative proliferation of responders in SA with either OriDC or Mismatch 3P was calculated first, as follows:

$$\text{Relative proliferation } (OriDC/Mismatch\ 3P) = \frac{Proliferation\ with\ APC}{Proliferation\ with\ Beads}.$$

Of note, *Proliferation with APC* corresponds to the proliferation (% CTV^- cells) of responders at a ratio of 1:5 Treg:T_{eff} in the presence of OriDC or Mismatch 3P, normalized to the appropriate control (% CTV^- cells co-cultured with the respective APC, in the absence of eTreg – 0:1). That is, $Proliferation\ APC = \frac{\%CTV^-cells\ at\ 1{:}5\ Treg{:}T_{eff}}{\%CTV^-cells\ at\ 0{:}1\ Treg{:}T_{eff}}$.

Briefly, relative proliferation corresponds to the proliferation (normalized to 0:1) of responders in SA with 1:5 Treg:T_{eff} and either OriDC or Mismatch 3P as stimulators, divided by the proliferation (normalized to 0:1) of the same responders in SA with 1:5 Treg:T_{eff} and Beads as stimulators. This way, it was possible to normalize the responses found in each independent experiment to responses to a consistent stimulator, which are the Beads, thus reducing deviations due to donor-to-donor variability.

Finally, each ∆Specificity represented the difference between the relative proliferation with OriDC and the relative proliferation with Mismatch 3P, divided by the relative proliferation with OriDC:

$$\Delta\text{Specificity} = \frac{Relative\ proliferation\ OriDC - Relative\ proliferation\ Mismatch\ 3P}{Relative\ proliferation\ OriDC}$$

As a result, higher ∆Specificity meant that there was a bigger difference between the potency of suppression of the original stimulator (OriDC) and the potency of suppression of moDC from a mismatched 3rd party donor. Lower ∆Specificity, on the other hand, meant that there was a smaller difference in the suppression of responders with both stimulators. Nevertheless, a small ∆Specificity, for example, does not indicate if there is high suppression of responses to both stimulators, or if there is no suppression of responses to either.

Accordingly, suppression of responses to the original stimulator (OriDC) at a ratio of 1:5 Treg:T_{eff} was calculated by applying the formula:

$$Suppression = 1 - Normalized\ proliferation\ with\ OriDC$$

With the corresponding data of ∆Specificity and Suppression for each condition, it was possible to assess if there was any correlation between suppression and specificity in SA with either Tcon or $CD8^+$ cells, when the suppressors were Treg expanded with 10U/mL of IL-2 and when the suppressors were Treg expanded with 100U/mL of IL-2 (**Table V**).

	∆Specificity	
	10U/mL IL-2 eTreg	100U/mL IL-2 eTreg
Tcon suppression	-0.937(0.063)	-0.943(0.057)
CD8+ cells suppression	0.152(0.848)	0.908(0.092)

Table V: Correlation between suppression and specificity.
∆Specificity and Suppression were calculated for each type of responder used in SA with either Treg expanded with 10U/mL of IL-2 or 100U/mL of IL-2. N=4; p values for each correlation within brackets.

Negative correlations were found between the suppression of Tcon and the ∆Specificity of Treg expanded with 10U/mL of IL-2, as well as between the suppression of Tcon and the ∆Specificity of Treg expanded with 100U/mL of IL-2. That is, more suppression of Tcon proliferation in the presence of moDC from the Original donor was related to a lower ∆Specificity. Therefore, when Tcon were used as responders, more potency of suppression could be related to less specificity. Since these correlations were nearly significant in eTreg from both expansion conditions (p=0.063 in 10U/mL IL-2 eTreg, and p=0.057 in 100U/mL IL-2 eTreg), we can assume that this trend would be made significant with more replicates. Additionally, these results matched what was shown in **Figure 22,** where Treg expanded with 100U/mL of IL-2 seemed more potent suppressors of responses to moDC from the Original donor and to moDC from a mismatched 3rd party donor than Treg expanded with 10U/mL of IL-2. As a result, at a ratio of 1:5 Treg:T_{eff}, there was a smaller difference between the proliferation in the presence of the original stimulator (moDC from the Original donor) and the proliferation in the presence of moDC from a mismatched 3rd party donor. Nevertheless, there was still significantly less Tcon proliferation with the original stimulator, thus specificity was not lost.

Conversely, no relation was detected between the suppression of $CD8^+$ cells and ∆Specificity, since the values for correlations were very different in Treg expanded with 10U/mL of IL-2 and Treg expanded with 100U/mL of IL-2. Moreover, none of these correlations was statistically significant, thus the hypothesis that the potency of suppression of $CD8^+$ cells was related to its ∆Specificity was rejected. In other words, higher potency of suppression of responses to the original stimulator was not related to a smaller difference between the proliferation with the original stimulator and the proliferation with moDC from a mismatched 3rd party donor.

Finally, the suppression and ∆Specificity of all experiments were plotted on a XY graph as individual points (**Fig.23**). Data from Tcon suppression by Treg expanded with 10U/mL of IL-2 (diamonds) and by Treg expanded with 100U/mL of IL-2 (circles) was fit into linear regressions, with an R^2 of 0.8782 in the former setting and 0.8889 in the latter (**Fig.23A**). Inversely, data from SA with $CD8^+$ cells did not seem to fit into linear regressions, as the previous correlations had indicated (**Fig.23B**).

In summary, the potency and specificity of eTreg in the suppression of Tcon were interdependent, indicating that eTreg displaying higher potency would also be more suppressive of responses to unrelated APC. This higher suppressive potency may be a result of cell-extrinsic mechanisms that do not require cell-cell contact, such as infectious tolerance mechanisms, so specificity may be lost. Simultaneously, the fact that these features do not seem to be related in the suppression of $CD8^+$ cells proliferation, yet specific suppression of these responders was detected, suggests that an increased

potency of suppression of responses to the original stimulator is a result of a suppression mechanisms that do not increase unspecific suppression. Therefore, it could be concluded that the proliferation of each type of responder is suppressed by different mechanisms. Since specificity may be a result of eTreg-APC and/or responder-APC interactions, we then focused our investigation on the role of APC in the potency of suppression.

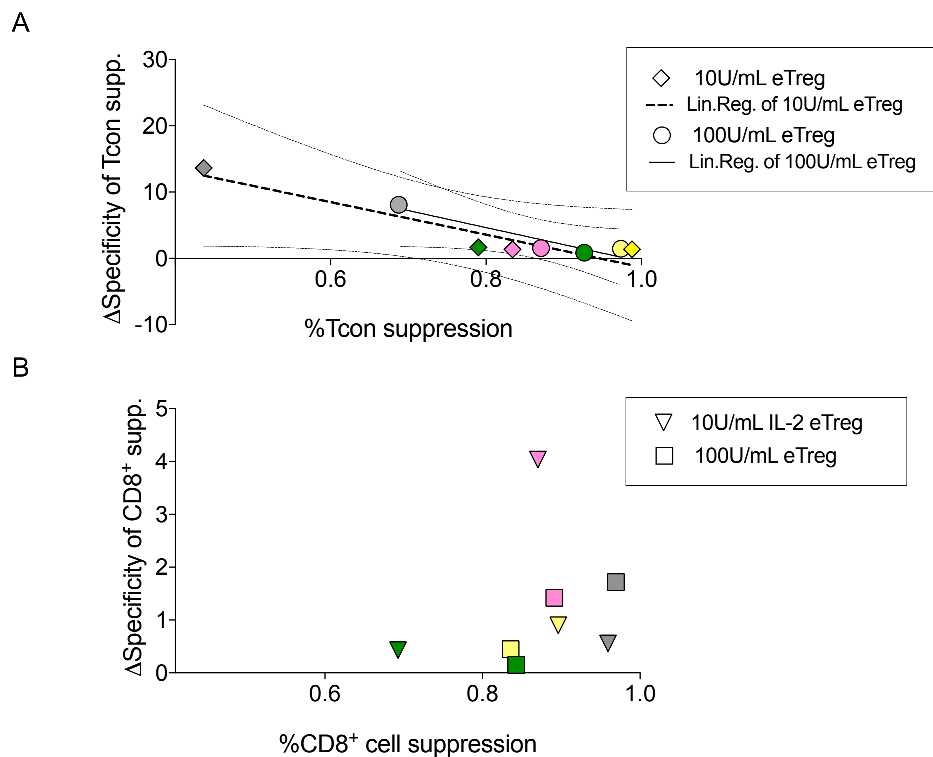

Figure 23: XY-plots of potency vs. specificity of Tcon and $CD8^+$ T cell suppression

(A) The percent suppression of Tcon by 1:5 Treg:T_{eff} in SA with the Original donor was plotted against ΔSpecificity calculated for the respective eTreg. Each color represents one independent experiment; diamonds represent values for Treg expanded with 10U/mL of IL-2 and circles represent values for Treg expanded with 10U/mL of IL-2. Straight lines represent the linear regressions of suppression *vs.* ΔSpecificity with standard error, in Treg expanded with 10U/mL of IL-2 (dotted line) and in Treg expanded with 100U/mL of IL-2 (black line). (B) The percent suppression of $CD8^+$ cells by 1:5 Treg:T_{eff} in SA with the Original donor was plotted against ΔSpecificity calculated for the respective eTreg. *Each color represents one independent experiment; diamonds represent values for Treg expanded with 10U/mL of IL-2 and circles represent values for Treg expanded with 10U/mL of IL-2. Statistical analysis of data was performed on GraphPad.*

2.2.5. Effect of MHC-matching on potency of suppression

In order to ascertain the role of APC in the potency of suppression after expansion, 3rd party moDC donors with distinct MHC-matching to either the Treg or Original donor were included in SA as additional conditions (**Fig.24**). Along with moDC from the Original donor (OriDC) and moDC from a mismatched 3rd party donor, the full panel of stimulators used in SA comprised moDC from a donor with matching in MHCI and MHCII to the Original (APC) donor and no match to the Treg donor (DC-match 3P), and moDC from a donor with MHCII matches to the Treg donor and none to OriDC donor (Treg-match 3P). The high-resolution typing of all donors used in each experiment can be found in **Annexes, Table S-IV**.

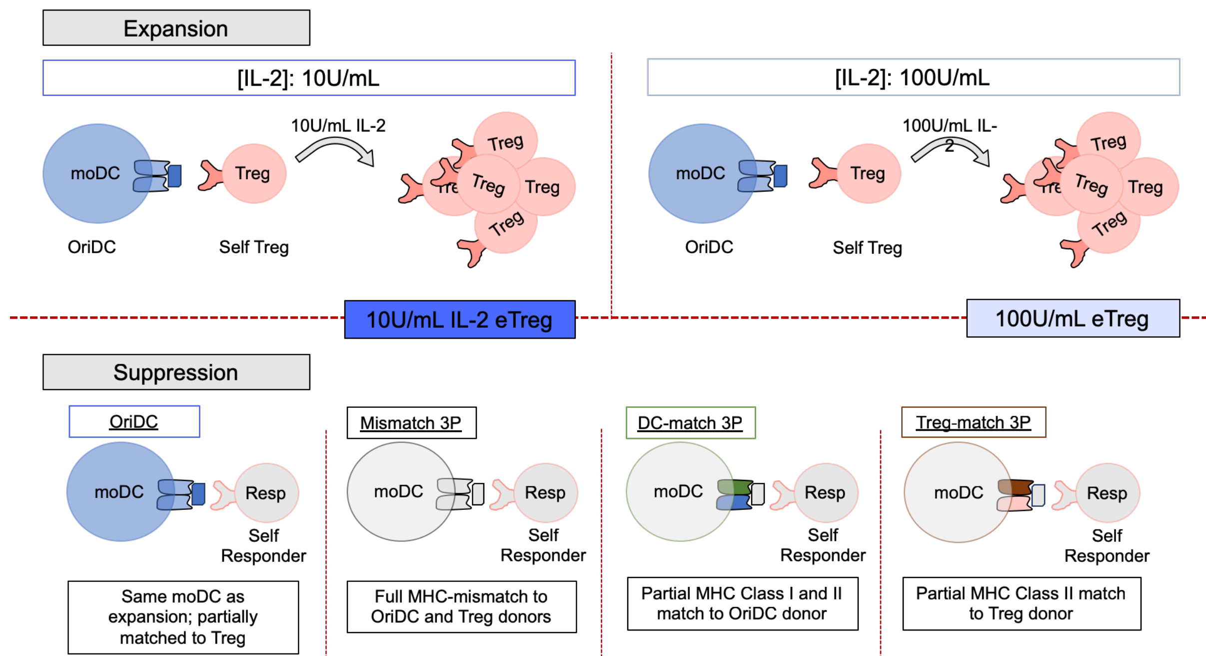

Figure 24: Schematic representation of experiments to characterize the function of Ag-sp Treg from different expansion milieus.
Isolated Treg were expanded in co-cultures with allo-moDC from the Original donor (OriDC) supplemented with 10U/mL or 100U/mL of IL-2. After 14 days, SA were performed with Treg from each expansion milieu, where their potency of suppression of Tcon and $CD8^+$ cells was evaluated. The stimulators for SA were moDC from the Original donor (OriDC), moDC from a 3rd party donor with no MHC-matches to the Treg nor the Original donor (Mismatch 3P), moDC from a 3rd party donor with MHC-matches to the Original donor (DC-match 3P) and moDC from a 3rd party donor with MHC-matches to the Treg donor (Treg-match 3P).

Once again, it is important to highlight that each type of eTreg was evaluated regarding their suppression of both Tcon and $CD8^+$ cells proliferation (in parallel), in response to the full panel of stimulators. Although the potency of suppression of responses to the Original donor (OriDC) and to the fully mismatched 3rd party donor (Mismatch 3P) were already described, in order to properly evaluate the suppression of responses to differently matched 3rd party donors, it was important to use OriDC and Mismatch 3P as positive and negative controls, respectively, for data analysis.

The proliferation of Tcon was significantly suppressed by Treg expanded with low concentration of IL-2 – 10U/mL IL-2 eTreg – in the presence of all stimulators except moDC from the completely mismatched 3rd party donor (Mismatch 3P, **Fig.25A**). Indeed, the proliferation of Tcon was significantly higher in the presence of moDC from a mismatched 3rd party donor (Mismatch 3P) than in the presence of moDC from the Original donor (OriDC), as had been described before (**Fig.22A**). Furthermore, starting at 1:50 Treg:T_{eff} and at higher Treg numbers, the potency of suppression of Tcon displayed by eTreg seemed to differ with the stimulator present in each condition (**Fig.25A**). Namely, responses to moDC from a donor partially matched to the Original donor (DC-match 3P) seemed more suppressed than responses to moDC from a donor partially matched to the Treg donor (Treg-match 3P), while responses to moDC from the Original donor (OriDC) remained the most suppressed ones. However, due to the standard deviation of the experiments, statistical significance was not found.

In the previous representation of Tcon proliferation in response to moDC from the Original donor and in response to moDC from a mismatched 3rd party donor, Treg expanded by 100U/mL of IL-2 (**Fig.22B)** were apparently more potent suppressors of proliferation than those expanded by 10U/mL of IL-2 (**Fig.22A**). As a result, suppression of Tcon in the presence of moDC from a mismatched 3rd party donor (Mismatch 3P) seemed similar to the suppression achieved in the presence of moDC from a donor that was partially matched to the Treg donor (Treg-match 3P, **Fig.25B**). However, Tcon proliferation was significantly higher in the presence of moDC from a donor partially matched to the Treg donor (Treg-match 3P) than in the presence of moDC from the Original donor (OriDC) at 1:10 and 1:50 Treg:T_{eff} only, whilst there was significantly higher proliferation of Tcon with moDC from mismatched 3rd party donor than with moDC from the Original donor (OriDC) at all ratios (**Fig.25B**, brown squares - statistically significant difference between proliferation with OriDC and Treg-match 3P, blue dots - statistically significant difference between OriDC and Mismatch 3P). In other words, in conditions with Treg expanded by 100U/mL of IL-2, the responses to a mismatched 3rd party donor were always significantly less suppressed than the responses to the Original donor. Additionally, the suppression of responses to a donor that was partially matched to the Treg-donor was similar to the suppression of responses to a mismatched 3rd party donor, but only at lower Treg:T_{eff} ratios. At higher Treg:T_{eff} , the responses to a donor that was partially matched to the Treg-donor seemed more suppressed than the responses to a mismatched 3rd party donor. Similarly to what was observed in SA with Treg expanded by 10U/mL of IL-2, in SA with Treg expanded with 100U/mL of IL-2 – 100U/mL IL-2 eTreg –, the suppression of responses to moDC from a donor partially matched to the Original donor (DC-match 3P) seemed higher than the suppression of responses to moDC from a donor partially matched to the Treg donor (Treg-match 3P), yet the Original donor remained the most suppressed stimulator (**Fig.25B**).

When $CD8^+$ cells were used as responders to Treg expanded by 10U/mL of IL-2 – 10U/mL IL-2 eTreg, **Fig.25C** – there was similar suppression of responses to moDC from a mismatched 3rd party donor (Mismatch 3P) and to moDC from a donor partially matched to the Treg donor (Treg-match 3P). At the same time, suppression in the presence of moDC from a donor partially matched to the Original donor (DC-match 3P) was comparable to the suppression in the presence of moDC from the Original donor (OriDC). In fact, at 1:10 Treg:T_{eff}, $CD8^+$ cells proliferation in the presence of moDC from a donor partially matched to the Original donor (DC-match 3P) was significantly lower than that in the presence of moDC from a mismatched 3rd party donor (Mismatch 3P, green star - statistically significant difference between proliferation with Mismatch 3P and DC-match 3P) and significantly lower than in the presence of moDC from a donor partially matched to the Treg donor (Treg-match 3P, green diamond - statistically significant difference between proliferation with Treg-match 3P and DC-match 3P). Conversely, at 1:50 Treg:T_{eff}, there was significantly more $CD8^+$ cell proliferation in conditions with moDC from a donor partially matched to the Treg donor (Treg-match 3P) than in conditions with moDC from the Original donor (OriDC, brown square - statistically significant difference between proliferation with OriDC and Treg-match 3P). Surprisingly, no differences were detected in the proliferation of $CD8^+$ cells in response to moDC from the mismatched 3rd party donor as opposed to moDC from the Original donor, suggesting

that, at some Treg:T$_{eff}$ ratios, the responses to moDC from a donor partially matched to the Treg donor may be even less suppressed than the responses to a mismatched 3rd party donor.

In brief, these results could suggest that Treg expanded with 10U/mL of IL-2 suppress CD8$^+$ cells in a way that is specific for the Original donor, thus, APC from a donor that has matches to that donor may be equally suppressed. These observations were also valid for SA with Treg expanded by 100U/mL of IL-2 – 100U/mL IL-2 eTreg, **Fig.25D)**. As a matter of fact, CD8$^+$ cell proliferation was significantly higher in cultures at 1:5 and 1:10 Treg:T$_{eff}$ with moDC from a mismatched 3rd party donor (Mismatch 3P) and at 1:10 and 1:50 Treg:T$_{eff}$ with moDC from a donor partially matched to the Treg donor (Treg-match 3P) than with moDC from the Original donor (OriDC, **Fig.25D**). Furthermore, when there were very few eTreg (1:500 Treg:T$_{eff}$), responses to moDC from a donor partially matched to the Original

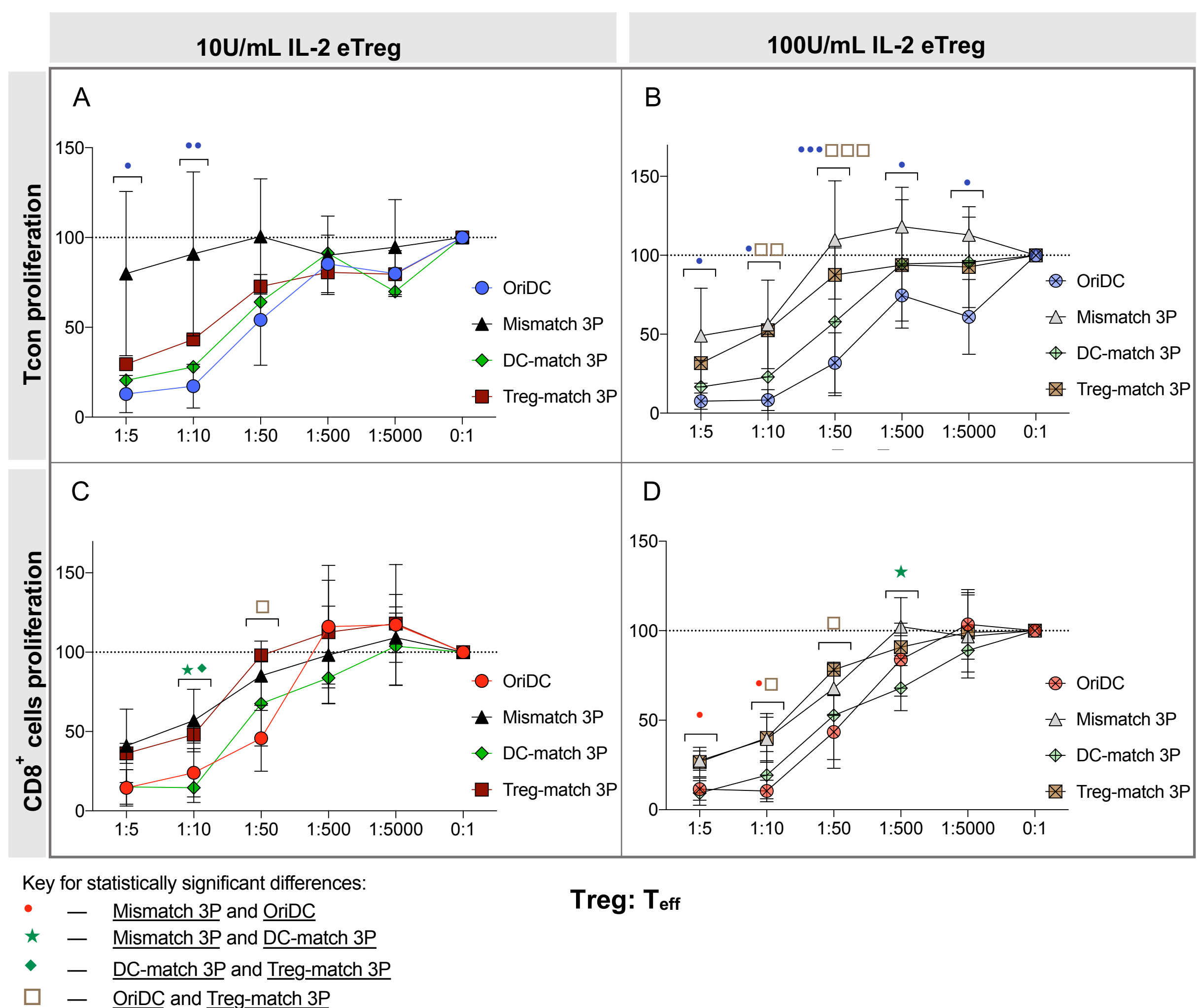

Figure 25: Specificity of suppression of the proliferation of Tcon or CD8$^+$ cells by Treg expanded with 10U/mL or 100U/mL of IL-2, in the presence of MHC-mismatched APC.
(A-B) Proliferation of Tcon in cultures with several dilutions of Treg expanded with 10U/mL IL-2 (A) or 100U/mL of IL-2 (B), in the presence of OriDC (blue circles), Mismatch 3P (black triangles), DC-match 3P (green diamonds) or Treg-match 3P (brown squares) as stimulators. (C-D) Proliferation of CD8$^+$ cells in cultures with several dilutions of Treg expanded with 10U/mL IL-2 (C) or 100U/mL of IL-2 (D), in the presence of OriDC (blue circles), Mismatch 3P (black triangles), DC-match 3P (green diamonds) or Treg-match 3P (brown squares) as stimulators. *N=4. Statistical analysis of differences within conditions (between dilutions) was performed with one-way ANOVA and differences between conditions were evaluated with multiple t-tests, by the Holm-Sidak method. Differences were considered statistically significant when $p<0.05$.*

donor (DC-match 3P) were already significantly more suppressed than those to moDC from a mismatched 3rd party donor (Mismatch 3P).

To conclude, eTreg from both expansion milieus apparently displayed similar trends in regards to the suppression of responses to donors partially matched to the Original donor (DC-match 3P), which were observed when either Tcon or $CD8^+$ cells were used as responders: that is, despite differences in suppressive potency between eTreg, there was specific suppression of responses to the Original donors and, possibly, to the donors that were matched to them (DC-match 3P). Of note, it seems that Tcon were also more suppressed in the presence of moDC from donors partially matched to the Treg donor (Treg-match 3P).

To better visualize the impact of each stimulator in the suppression of Tcon or of $CD8^+$ cells, we focused only on the suppression (inverse of the proliferation) found at the higher Treg:T_{eff} ratio (1:5), where it was shown to be the highest (**Fig.26**). As previously described, Tcon seemed more suppressed in the presence of moDC from the Original donor (OriDC, **Fig.26A**, blue circles), where 87.07±10.37% of Tcon proliferation was suppressed by Treg expanded with 10U/mL of IL-2 and 92.36±5.14% of Tcon proliferation was suppressed by Treg expanded with 100U/mL of IL-2.

There was also high suppression in conditions with moDC from donors partially matched to the Original donor (DC-match 3P, **Fig.26A**, grey triangles), with 79.45±28.71% of Tcon proliferation suppressed by Treg expanded with 10U/mL of IL-2 and 83.36±16.90% of Tcon proliferation suppressed by Treg expanded with 100U/mL of IL-2. Suppression in the presence of moDC from donors partially matched to the Treg donor (Treg-match 3P, **Fig.26A**, green diamonds) was slightly lower than that of DC-match 3P, as Tcon proliferation was reduced by 70.49±13.06% with Treg expanded with 10U/mL of IL-2 and by 68.33±14.22% with Treg expanded with 100U/mL of IL-2. In both SA with eTreg, the lowest suppression of Tcon was in the presence of moDC from a completely mismatched 3rd party donor (Mismatch 3P, **Fig.26A**, brown squares) where there was only 20.08±45.71% of suppression by Treg expanded with 10U/mL of IL-2 and 50.93±30.12% of suppression by Treg expanded with 100U/mL of IL-2.

When $CD8^+$ cells were used as responders, the suppression in conditions with moDC from the Original donor (OriDC, 85.4±11.42% with 10U/mL IL-2 eTreg and 88.49±6.15% with 100U/mL IL-2 eTreg) was similar to conditions with moDC from donors partially matched to the Original donor (DC-match, 84.87±10.91% with 10U/mL IL-2 eTreg and 90.61±6.88% with 100U/mL IL-2 eTreg).

Conversely, suppression of $CD8^+$ cells in the presence of moDC from donors partially matched to the Treg donor (Treg-match 3P, 63.74±6.38% with 10U/mL IL-2 eTreg and 73.34±8.24% with 100U/mL IL-2 eTreg) was lower than the suppression of Tcon with the same APC. In fact, the suppression of $CD8^+$ cells in the presence of moDC from donors partially matched to the Treg donor was similar to the suppression found with moDC from a completely mismatched 3rd party donor (Mismatch 3P, 58.99±23.08% with 10U/mL IL-2 eTreg and 72.52±5.39% with 100U/mL IL-2 eTreg).

The specific suppression by eTreg of responses to a donor that was partially matched to the Original donor (DC-match 3P) may derive from the recognition of allo-Ag present in the MHC of moDC from both the Original donor and the donor partially-matched to it. On the other hand, the specific suppression of Tcon responses to a donor matched to the Treg donor may suggest that either eTreg or

Tcon are detecting self-MHC. Since CD8+ cells only bind to APC through MHCI, they would not recognize any Ag in the Treg-matched donor as self, as these donors are only matched to the Treg donor in MHCII, so it makes sense that there is less suppression of CD8+ cells than of Tcon proliferation in that setting, although eTreg could still detect self-MHC. These observations indicated that the potency and specificity of suppression by eTreg may be influenced by the stimuli provided directly by the APC to the responders. Thus, the phenotype of all stimulators was analysed, and compared to the suppression achieved in the presence of each stimulator.

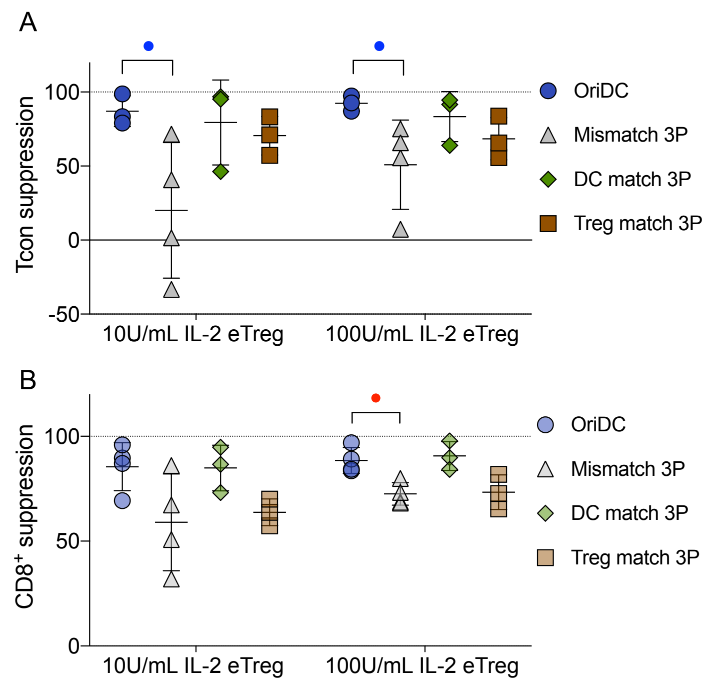

Figure 26: Suppression of Tcon or CD8+ cells by Treg expanded with 10U/mL or 100U/mL of IL-2, in the presence of differently MHC-mismatched APC.
Suppression of Tcon (A) or CD8+ cells (B) by Treg expanded with 10U/mL IL-2 or 100U/mL of IL-2 at a ratio of 1:5 Treg:T_{eff}, in the presence of OriDC (blue circles), Mismatch 3P (grey triangles), DC-match 3P (green diamonds) or Treg-match 3P (brown squares) as stimulators. *N=4. Statistically significant differences between conditions were evaluated with multiple t-tests, by the Holm-Sidak method. Differences were considered statistically significant when $p<0.05$.*

2.2.6. Role of APC co-stimulation in suppression assays

The phenotype of each stimulator used in SA was analysed by flow cytometry before irradiation and culture. Besides the typical markers used to identify live moDC (Viability dye, CD14 and CD11c), the full panel for moDC characterization included activation markers such as CD83 and HLA-DR, as well as markers for co-stimulatory molecules, namely CD80, CD86, and PD-L1. For each APC, there was a corresponding percentage of suppression exerted by eTreg on Tcon or CD8+ cells proliferation. Thus, it was possible to determine the correlation between the MFI of each of these markers and the degree of suppression achieved by Treg expanded with 10U/mL or 100U/mL of IL-2 – 10U/mL IL-2 eTreg or 100U/mL IL-2 eTreg, respectively. The most relevant correlations found are presented in **Table VI**. Tcon suppression was negatively correlated to CD86 and CD83 MFI; that is, when the APC displayed higher MFI of CD86 or CD83 at the start of the SA, there was lower suppression of Tcon.

The trend in this correlation and its statistical significance were found in SA with Treg expanded with 10U/mL and with 100U/mL of IL-2. Statistically significant negative correlations were also found between the suppression of CD8$^+$ cells and the MFI of CD86, for SA with Treg expanded with 10U/mL and with 100U/mL of IL-2. Surprisingly, there seemed to be no relation between the suppression of CD8$^+$ cells and the MFI of CD83.

	Tcon suppression		**CD8$^+$ cells suppression**	
	10U/mL IL-2 eTreg	100U/mL IL-2 eTreg	10U/mL IL-2 eTreg	100U/mL IL-2 eTreg
CD86 MFI	**-0.6699****	**-0.5794***	**-0.7179****	**-0.5713***
CD83 MFI	**-0.6207***	**-0.7978*****	-0.1159	-0.2379

Table VI: Correlation between co-stimulatory marker expression in moDC and suppression of Tcon and CD8$^+$ cells. Pearson values of statistically significant correlations are presented in bold, with * representing statistically significant correlations (with $p<0.05$).

Even though these correlations did not fit into linear regressions, XY-plots of CD86 MFI *vs.* Tcon suppression (**Fig.27A**), CD83 MFI *vs.* Tcon suppression (**Fig.27B**) or CD86 MFI *vs.* CD8$^+$ cells suppression (**Fig.27C**) allowed for the visualization of each individual dot. This way, it was possible to identify APC with higher suppression and lower MFI and vice-versa. Of note, suppression by Treg expanded with 10U/mL of IL-2 is represented by circles, and the suppression by Treg expanded with 100U/mL of IL-2 is represented by triangles. Interestingly, when each APC was traced back to its donor (OriDC, Mismatch 3P, DC-match 3P or Treg-match 3P) it was verified that moDC from donors partially matched to the Original donors (DC-match 3P) were amongst the APC with lowest MFI of CD86 (**Fig.27A and Fig.27C**, green symbols) and CD83 (**Fig.27B**, green symbols). On the other hand, moDC from completely mismatched 3rd party donors (Mismatch 3P, red symbols) presented a wider diversity of CD83 and CD86 expression. This observation was very curious, as all moDC were subjected to the same protocol, and their phenotype was analysed prior to any co-culture that could affect the expression of these markers.

Knowing that the initial phenotype of the stimulators used in SA was related to the suppression of proliferation achieved by eTreg in such a strong way, the last step was to ascertain whether there was a role played by cytokines in these SA. The involvement of cytokines in SA may be diverse: they may act as cell-extrinsic mechanisms of suppression when produced by eTreg or by APC, and/or there may be pro- or anti- inflammatory cytokine production by responders after co-culture with APC. If the phenotype of APC is related to the suppression of proliferation, it could be expected that it would also affect the production of cytokines by responders. In any case, it was important to assess if the presence of eTreg would affect cytokine secretion by responders or APC, thus modulating the cytokine profile found in the suppression milieu. Moreover, it was possible that eTreg were also secreting cytokines as

a cell-extrinsic mechanism of suppression. With that in mind, the results of cytokine quantification in the supernatant of SA are presented next.

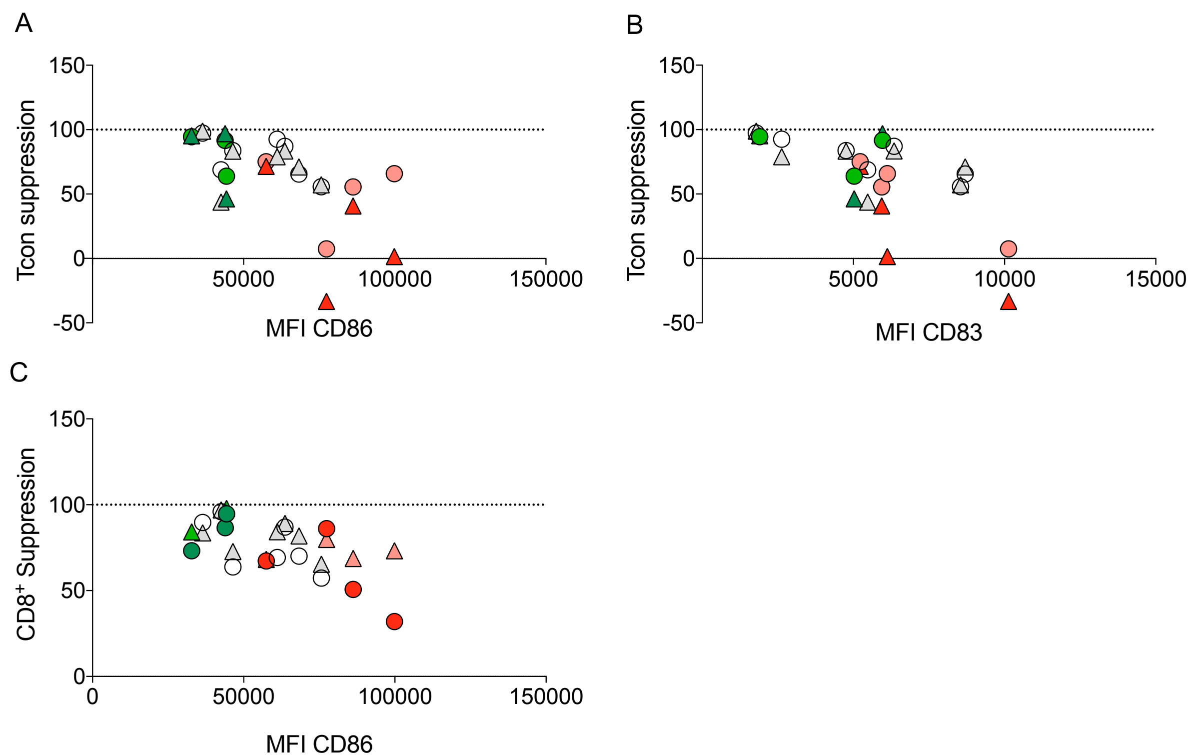

Figure 27: XY-plots of marker MFI vs. suppression of Tcon and $CD8^+$ cells suppression
(A) The MFI of CD86 for each APC was plotted against the percent suppression of Tcon by Treg expanded with 10U/mL of IL-2 (triangles) or Treg expanded with 100U/mL of IL-2 (circles) at 1:5 Treg:T_{eff}. (B) The MFI of CD83 for each APC was plotted against the percent suppression of Tcon by Treg expanded with 10U/mL of IL-2 (triangles) or Treg expanded with 100U/mL of IL-2 (circles) at 1:5 Treg:T_{eff}. (C) The MFI of CD86 for each APC was plotted against the percent suppression of $CD8^+$ cells by Treg expanded with 10U/mL of IL-2 (triangles) or Treg expanded with 100U/mL of IL-2 (circles) at 1:5 Treg:T_{eff}. *Red symbols are moDC from mismatched 3rd party donors and green symbols are moDC from Original-matched donors.*

2.2.7. Role of cytokines in suppression assays

As described in the **State of the Art**, cytokines may play many roles in the suppression of inflammation. On the one hand, there may be anti-inflammatory cytokine production by eTreg or APC; on the other, pro-inflammatory cytokines may be secreted by responders after stimulation by APC. By measuring the concentration of cytokines in the supernatant of SA wells, it is possible to assess which, if any, cytokines are being secreted and if the presence of Treg alters the concentration of soluble cytokines in the supernatant. Furthermore, similarly to the proliferation of responders in the presence of APC only, cytokine production by responders in co-culture with APC only (in the absence of Treg) may also be an indicator of their basal response to different APC. To extract the maximum amount of information from each sample, commercially available Multiplex kits were used, which allow for the simultaneous quantification of a high number of cytokines within low volumes of sample. This way, it was possible to simultaneously screen for cytokines commonly associated with Th1, Th2, Treg and even DC (**Table VII**).

Th1-associated cytokines	IL-2	TNF-α	IFN-γ	
Th2-associated cytokines	IL-4	IL-5	IL-10	
Treg-associated cytokines	*IL-10*	TGF-β	IL-17A	IL-35
DC-associated cytokines	IL-12p70	IL-23		

Table VII: Cytokine screening in SA
The concentration of cytokines present in the supernatant of wells from SA was measured by Multiplex kits. The concentration of Th1-, Th2-, Treg- or DC- associated cytokines was assessed on each sample.

The cytokines present in the supernatant of wells from SA with the highest number of Treg (1:5 Treg:T_{eff}) were quantified, as it could be expected the highest suppression of cytokine production by T_{eff} and/or the highest concentration of cytokines secreted by eTreg would be detected in these wells. The concentration of each cytokine was measured in wells from SA with Treg expanded by 10U/mL of IL-2 – 10U/mL IL-2 eTreg – and Treg expanded by 10U/mL of IL-2 – 100U/mL IL-2 eTreg, in the presence of each type of stimulator, as well as in the presence of aAPC (Beads). Additionally, the concentration of each cytokine in wells from SA with Fresh Treg (not expanded before SA) was used as a control for eTreg. Finally, wells containing only responders with APC (Tcon alone or $CD8^+$ cells alone) were used as controls for the effect of Treg in the suppression milieu.

In control wells of Tcon with APC only (Tcon alone), there was high concentration of IL-2 (**Fig.28A**) when the stimulators were moDC from the Original donor (OriDC), moDC from a mismatched 3rd party donor (Mismatch 3P) and moDC from a donor partially matched to the Treg donor (Treg-match 3P). Interestingly, in control wells (Tcon alone) where there was high concentration of IL-2 in the supernatant, there was correspondingly high concentration of the pro-inflammatory cytokine TNF-α (**Fig.28B**). Additionally, there was high concentration of IFN-γ in control wells where the stimulators were moDC from the Original donor (OriDC) or moDC from a donor partially matched to the Treg donor (Treg-match 3P), albeit at a lower concentration in the latter (**Fig.28C**). However, the concentration of IFN-γ was not increased in control wells with moDC from a mismatched 3rd party donor. Since the donors of OriDC and of Treg-match 3P are partially matched to the Treg donor, it may be the case that IFN-γ secretion by Tcon is higher in settings where there is self-Ag recognition, whilst IL-2 and TNF-α secretion seem triggered by other stimulation provided by APC. Thus, it seems fitting that moDC from a donor partially matched to the Original donor (DC-match 3P) did not stimulate as much pro-inflammatory cytokine secretion as the remaining APC, since it has been shown that these moDC display the lower MFI of CD86 and CD83 (**Fig.28A-B**).

Surprisingly, stimulation with Beads, which provide a mitogenic stimulus to responders (through CD2, CD3 and CD28), did not stimulate the secretion of Th1-associated cytokines, suggesting that aAPC do not adequately mimic the stimulation provided by activated moDC. Furthermore, if IFN-γ secretion does result from self-Ag recognition, it would also make sense that the Beads did not increase the secretion of this cytokine (**Fig.28C**). Overall, these results suggest that the secretion of Th1-

associated cytokines by Tcon may be dependent on the degree of stimulation provided by the APC and, at least in the case of IFN-γ secretion, on the degree of affinity of Tcon TCR to moDC MHC.

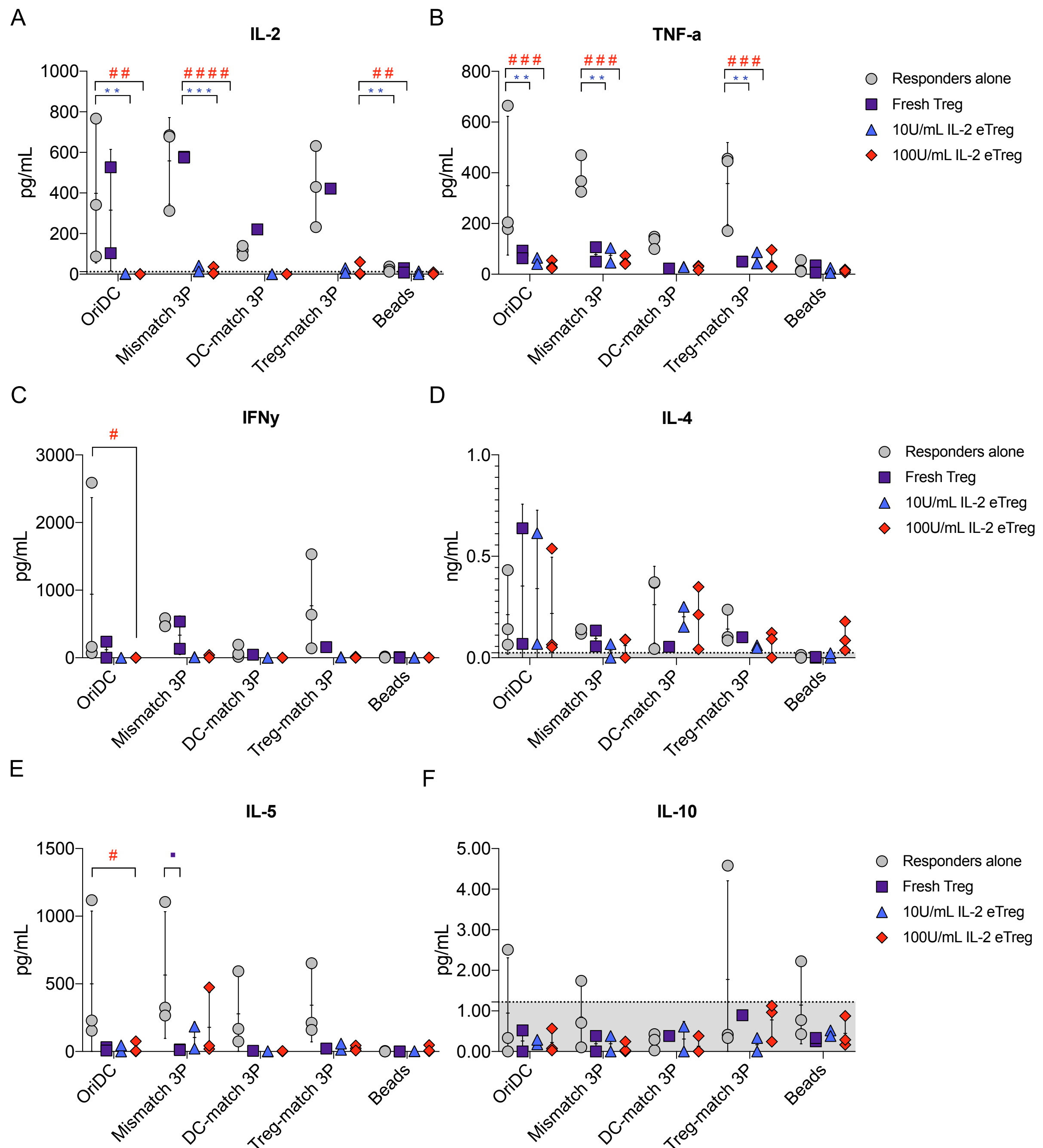

Figure 28: Concentration of Th1 and Th2-associated cytokines in the supernatant of SA with Tcon responders. *(*A-F) Concentrations found in each independent experiment are represented by symbols, with average and standard deviation values. Values below the detection limit of the kit were extrapolated and plotted in the grey area. For each cytokine, data was organized by APC, according to the presence of moDC from the Original donor (OriDC), from a mismatched 3rd party donor (Mismatch 3P), from a donor partially matched to the Original donor (DC-match 3P) or from a donor partially matched to the Treg donor (Treg-match 3P). Supernatant from wells with Tcon and APC only (Tcon alone, grey circles), Tcon with APC and freshly isolated Treg at 1:5 Treg:T_{eff} (Fresh Treg, purple squares), Tcon with APC and Treg expanded by 10U/mL of IL-2 at 1:5 Treg:T_{eff} (10U/mL IL-2 eTreg, blue triangles) and Tcon with APC and Treg expanded by 100U/mL of IL-2 at 1:5 Treg:T_{eff} (100U/mL IL-2 eTreg, red diamonds) was quantified. Th1-associated cytokines include IL-2 (A), TNF-α (B) and IFN-γ (C). Th2-associated cytokines are IL-4 (D), IL-5 (E) and IL-10 (F). *Statistical analysis was performed by multiple t-tests, by the Holm-Sidak method. Differences were considered statistically significant when p<0.05. Statistically significant differences in cytokine concentration between Tcon alone and Fresh Treg are represented by ■, differences in cytokine concentration between Tcon alone and 10U/mL IL-2 eTreg are represented by * and differences in cytokine concentration between Tcon alone and 100U/mL IL-2 eTreg are represented by #. N=3 except for Fresh Treg conditions, where n=2.*

As for the wells with Treg, it seemed that fresh Treg were not capable of statistically significant suppression of Th1 cytokines; particularly, the concentration of IL-2 in the presence of fresh Treg was similar to that found in wells with Tcon and APC only (**Fig.28A**). However, the concentration of TNF-α and IFN-γ seemed suppressed by the presence of Fresh Treg (**Fig.28B-C**). Conversely, the concentration of IL-2 (**Fig.28A**) and TNF-α (**Fig.28B**) was significantly lower in wells with Treg expanded by either 10U/mL of IL-2 or 100U/mL of IL-2, being suppressed to concentrations of nearly zero, and the same trend seemed true in the concentration of IFN-γ (**Fig.28C**). Although it has been shown that the suppression of Tcon proliferation by eTreg varies with the stimulators present in the SA (**Fig.26A**), cytokine secretion was always suppressed, in a way that seemed independent of the stimulators present in the SA. In short, it seems that the specificity displayed by eTreg in the suppression of Tcon proliferation is not mirrored in the suppression of Th1-associated cytokine secretion, where the concentration of cytokines is always reduced.

If, so far, it was simple to attribute the production of pro-inflammatory cytokines to Tcon, since the concentration of these cytokines was elevated in wells containing Tcon with APC only, the same reasoning could not be followed for Th-2-associated cytokines. Elevated IL-4 concentration was detected in wells containing Tcon alone (with APC) and also Tcon with eTreg, in the presence of all stimulators but moDC from a mismatched 3rd party donor (Mismatch 3P, **Fig.28D**). Thus, it was unclear whether eTreg could not suppress IL-4 secretion by Tcon, or if IL-4 could be secreted by eTreg. In fact, in wells with aAPC (Beads), the concentration of IL-4 was increased only in the presence of Treg expanded by 100U/mL of IL-2 – 100U/mL IL-2 eTreg – and not in wells with Tcon alone, suggesting a role for eTreg in the modulation of IL-4 concentration. Furthermore, the fact that IL-4 concentration was not increased in any wells with moDC from mismatched 3rd party donors (Mismatch 3P), where it has been shown suppression is the highest, indicate that IL-4 may be involved in one of the mechanisms of suppression by eTreg. On the other hand, IL-5 was apparently secreted by Tcon only, as its concentration was increased in control wells with Tcon and all APC in the absence of Treg (Tcon alone), and it seemed decreased by the presence of either fresh or eTreg (**Fig.28E**). Particularly, in conditions with moDC from the Original donor, the concentration of IL-5 was significantly lower in the presence of Treg expanded with 100U/mL of IL-2 eTreg – 100U/mL IL-2 eTreg – than in control wells. Fresh Treg also seemed suppressive of IL-5 secretion, albeit statistically significant differences in the concentration of IL-5 between Tcon alone and Fresh Treg were only found in the presence of moDC from mismatched 3rd party donors (Mismatch 3P). Interestingly, eTreg did not seem to suppress IL-5 production in the presence of moDC from mismatched 3rd party donors (Mismatch 3P) as much as Fresh Treg, nor as much as in the presence of other APC. It seems that, in SA with eTreg, when there was less suppression of Tcon proliferation (**Fig.26A**), there was also less suppression of IL-5 secretion (**Fig.28E**). It is a possibility that the concentration of IL-5 is not reduced further in the presence of moDC from mismatched 3rd party donors (Mismatch 3P) because there are more proliferating Tcon secreting IL-5. However, in the case of Th1-associated cytokines, the concentration of cytokines was decreased by eTreg in wells with moDC from mismatched 3rd party donors (Mismatch 3P) (**Fig.28A-C**), suggesting that there may be suppression of cytokine secretion independently of suppression of proliferation. Thus, it seems that the suppression of proliferation is particularly linked to the suppression of IL-5 secretion,

which could suggest that similar mechanisms regulate the specific suppression of Tcon proliferation and IL-5 secretion.

Finally, it was surprising to find that IL-10 concentration was only increased in wells with Tcon and APC, in the absence of Treg (Tcon alone**, Fig.28F**). This immunomodulatory cytokine has been described as playing a major role in suppression by Treg[138], yet here it was apparently secreted by Tcon only, although at low concentration, and its concentration was decreased to concentrations below the detection limit in cultures with any kind of Treg. Still, it cannot be excluded that Treg were producing IL-10 and presenting it in its membrane-bound form, in which case IL-10 would not be detected in the supernatant.

It should be noted, once again, that using aAPC (Beads) as APC does not seem to appropriately mimic the effect of using moDC. There was only increased secretion of IL-10 by Tcon alone in conditions with Beads, as opposed to conditions with moDC, where there were detectable concentrations of IL-2, TNF-α, IFN-γ, IL-4, IL-5 and IL-10. Surprisingly, Beads seemed to stimulate IL-4 (**Fig.28D**) and IL-17A (**Fig.S6**) secretion only in wells with Treg expanded by 100U/mL of IL-2 – 100U/mL IL-2 eTreg, suggesting that only this kind of mitogenic stimuli leads to Treg conversion into IL-17 secreting Th17-like cells. Also, none of the other Treg- and DC-associated cytokines (IL-35, IL-12p70 and IL-23) were detected in the supernatant of SA, except for TGF-β that was found in constant concentration in all conditions (**Fig.S6** and **Fig.S7**).

The concentration of cytokines in the supernatant of wells of SA with CD8$^+$ cells as responders was also measured; however, only conditions with Treg expanded by 100U/mL of IL-2 – 100U/mL IL-2 eTreg – or control conditions, of CD8$^+$ cells with APC, in the absence of Treg, were assessed. Primarily, all cytokines were found in much lower concentrations. Nevertheless, trends similar to those found in SA with Tcon could be detected, such as the secretion of IL-2 and IFN-γ by CD8$^+$ cells and its consequent suppression by 100U/mL IL-2 eTreg (**Fig.29A and C**). Interestingly, the concentration of TNF-α was constant in the supernatant of all tested conditions (**Fig.29B**). The concentrations of IL-4, IL-5 and IL-10 were very low (**Fig.29D-F**), and IL-10 concentration, particularly, was always below the detection limit. These results were expectable, as besides the production of IL-2 and IFN-γ[205], which were suppressed by eTreg, the main mechanism of action of CD8$^+$ cells is through the production of cytotoxins, such as perforin[206] and Granzyme B[207].

In brief, these results have shown that eTreg were capable of suppressing cytokine production by Tcon, more so than fresh Treg. By quantifying cytokines in SA supernatants with a range of APC, it was noticeable that other factors may influence eTreg-mediated suppression, such as the level of co-stimulation or the affinity of the TCR to the MHC complex displayed by APC. For example, it was curious to find that DC-match 3P apparently stimulated Tcon to produce only IL-4 and IL-5, contrarily to other APC that stimulated the production of pro-inflammatory cytokines. Since the Tcon donor is the same as the Treg donor, who does not have any match in HLA with either the mismatched 3rd party donor or the donor partially matched to the Original donor, similar responses of Tcon alone to both these APC were expected. Thus, it seems that the effect of stimulation provided by the APC affects not only the level of suppression by eTreg but also the phenotype acquired by the effector cells.

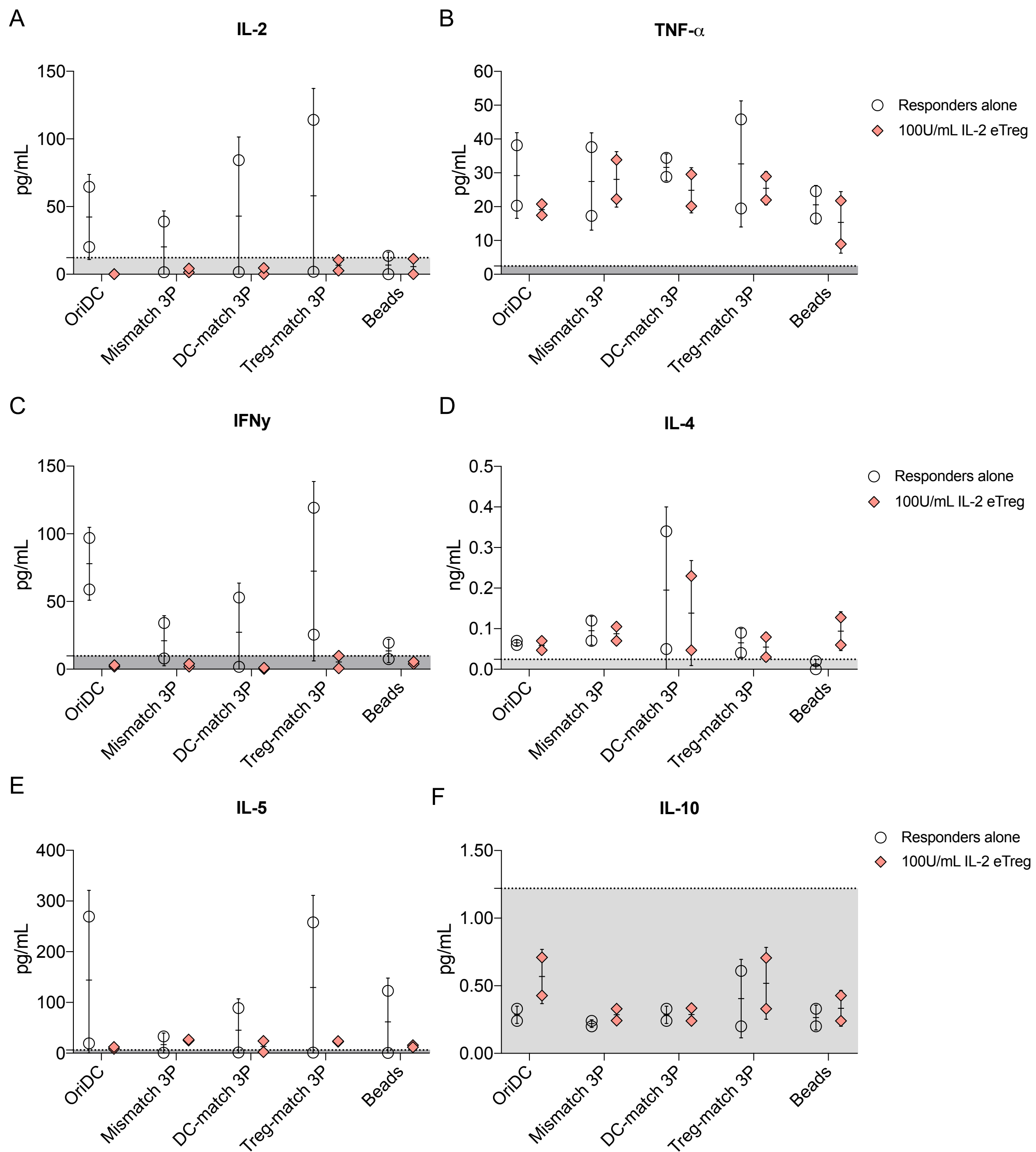

Figure 29: Concentration of Th1 and Th2-associated cytokines in the supernatant of wells from SA with CD8$^+$ cells responders.

*(*A-F) The concentration of all cytokines was measured on each sample. Concentrations found in each independent experiment are represented by symbols, as well as the average and standard deviation. Values below the detection limit of the kit were extrapolated and plotted in the grey area. Within each cytokine, data was organized by APC, according to the presence of moDC from the Original donor (OriDC), from a mismatched 3rd party donor (Mismatch 3P), from a donor partially matched to the Original donor (DC-match 3P) or from a donor partially matched to the Treg donor (Treg-match 3P). Supernatant from wells with CD8$^+$ cells and APC only (CD8$^+$ cells alone, empty circles) and CD8$^+$ cells with APC and Treg expanded by 100U/mL of IL-2 at 1:5 Treg:T_{eff} (100U/mL IL-2 eTreg, pink diamonds) was quantified. Th1-associated cytokines include IL-2 (A), TNF-α (B) and IFN-γ (C). Th2-associated cytokines are IL-4 (D), IL-5 (E) and IL-10 (F). *Statistical analysis was performed by multiple t-tests, by the Holm-Sidak method. N=2.*

2.3. Effect of expansion milieu on Treg phenotype

In the last section of this work, we wanted to provide an in-depth charaterization of the phenotype of Treg after expansion, which could possibly lead to the identification of novel eTreg subsets that could be related to particular functions. Treg were characterized with an 11-color antibody panel, where the expression of Treg-associated molecules (CTLA-4, PD-1), as well as activation markers (CD39, HLA-DR, 4-1BB and CD40L), were measured, in addition to the commonly used markers for Treg identification (CD3, CD4, CD25, CD127, Foxp3). Visual representation with FlowJo software is limited to the coexpression of only 2 markers at a time and multidimensional representation of data is not feasible. Additionally, some yet unknown subsets may be missed if the data is analysed on FlowJo, by manually gating only on known subsets. As a result, there is currently a big focus on using automated analysis of flow cytometry data[208]. In fact, recent publications in the field have used multidimensional analysis for the identification of novel subpopulations using new markers by flow [200,209] or mass cytometry[210]. Hence, in this work, two multidimensional analysis algorithms were used to evaluate Treg populations using the high-dimensional data obtained from flow cytometry.

FlowSOM is a visualization technique designed for the unsupervised analysis and organization of cytometry datasets that allows for the visualization of the full range of phenotypes within a sample[202]. This tool is available as an R script or as a plugin for FlowJo. Since the data visualization options in the plugin were not as costumizable as on the R script, we opted to run the script in R. In short, this algorithm maps out each cell to a node within a NxN grid, according to the expression of all markers on that cell. The next cell will be allocated to the node with the most similar phenotype, and so on, for all cells. In the end, a self-organizing map (SOM) is formed. The nodes in a SOM are then connected in a minimum spanning tree (MST), where each node is placed based on its similarity to the surrounding nodes. In our case, a 5x5 grid was used to build the SOM, so 25 nodes were visualized in the MST. Usually, the SOM is built using concatenated data from all the samples we wish to visualize, so that all of the phenotypes are represented in the SOM. Afterwards, to visualize the phenotype of only one sample, the corresponding data is mapped onto the previously built SOM and the resultant MST is plotted, where each node has pie charts that display the expression of each marker in that node. Also, the diameter of the node is proportional to the number of cells assigned to it.

The other algorithm used for data analysis was k-means clustering. This tool, which is available as a function of R's Stats package, is similar to FlowSOM in the fact that each cell is assigned to a subpopulation, within a defined number of clusters. The optimal number of clusters is determined by plotting sum of squares versus number of clusters (k), and k is chosen based on the location of the elbow in the curve. In our case, k was set to 5. Since there are 25 nodes in the FlowSOM, it is normal that each cluster comprises more cells than the nodes used for FlowSOM. Thus, this algorithm is more adequate to find new subsets of cells, as the marker expression of each cluster can be translated into discrete units (ie, positive, negative, low), whereas in FlowSOM the transitions between nodes are more fluid, thus harder to translate.

Before any multidimensional analysis, each sample was compensated on Diva and manually gated on $CD3^{+}CD4^{+}$ lymphocytes. Afterwards, all of the data generated in 3 independent experiments

was concatenated and exported to R, where the intensity of fluorescence from each channel was transformed and normalized by scalling. Finally, the data was analysed, depending on whether we wanted to compare samples based on how all markers were behaving on all cells, that is, by visualizing the entire range of phenotypes present in each sample – where FlowSOM was best suited – or if we wanted to organize the phenotypes found in each sample into clusters, which could allow for the identification of new Treg subsets – where k-means clustering was more adequate.

2.3.1. Purity of expanded Treg

First of all, we wanted to ascertain whether contaminating activated Tcon were present in the final eTreg population, a possibility that could not be excluded up to this point. Therefore, in each of 3 independent experiments, we expanded Tcon in parallel with Treg in the same conditions (except for the presence of IL-15 and rapamycin). After expansion, the phenotype of Tcon was acquired with the same panel of markers as eTreg in a Fortessa X-20. Of note, eTreg from these experiments were the same as those used in SA described in the previous sub-chapter. Automated compensation was performed on Diva, after which the fcs files were exported to FlowJo, where it was manually verified. Lymphocytes were gated on FSC vs. SSC dotplots, followed by gating on $CD3^{+}CD4^{+}$ cells. Then, data from all samples (Tcon and Treg expanded with 10U/mL or 100U/mL of IL-2) was exported and concatenated into one file that was used as input to design the SOM. Our hypothesis was that by building a common SOM that encompassed all of the phenotypes found in expanded Tcon (eTcon) and in eTreg, upon projecting the concatenated data (from 3 experiments) from eTcon onto the SOM, we would be able to identify in the resulting MST the nodes representing activated Tcon. This way, it would be possible to assess if these nodes were present in eTreg samples, thus validating the purity of those samples. In order to encompass the full range of phenotypes present in each sample, all Treg-associated and activation markers were used to build this SOM (CD25, CD127, Foxp3, CTLA-4, PD-1, CD39, HLA-DR, 4-1BB and CD40L) and the respective MST.

In the MST presenting the cumulative data from all samples, each node has a pie chart displaying the frequency of cells from each sample that express that phenotype (**Fig.30A**). In this MST, it was already noticeable that eTreg and eTcon displayed distinct phenotypes, as eTreg were allocated to nodes in the top section of the tree (white and blue slices) and eTcon were mostly allocated to nodes in the lower-right section (red and green slices). It was also possible to observe that, within each type of cell (Treg or Tcon), different concentration of IL-2 during expansion did not result in different phenotypes, as cells from both expansion millieus were allocated to the same nodes. The biggest difference found between expansion conditions seemed to be the frequency of cells allocated to each node. That is, cells expanded with 10U/mL or 100U/mL of IL-2 were encompassed in the same range of phenotypes, although some of those phenotypes were more prevalent in cells from one of the two expansion conditions. Interestingly, eTcon presented a more diverse range of phenotypes, since even in nodes that were comprised mostly of eTreg there were cells derived from eTcon samples. In other words, this may indicate that, during expansion, Tcon acquire a more broad range of phenotypes, overlapping with Treg on some of them. This information is not novel, as the phenotypic overlap

between activated Tcon or even induced Treg with the natural Treg phenotype has been extensively described in the literature[39]. Nevertheless, this visualization method is novel in the way that it allows us to observe all of the phenotypes acquired by eTcon after expansion at the same time.

In fact, when only the data obtained from Tcon expanded by 100U/mL of IL-2 was projected onto the same SOM, we could identify nodes that possibly represented non-activated Tcon (**Fig. 30B**, red arrow), according to their lack of expression of CD25 or Foxp3 and high expression of CD127. Additionally, a region of nodes where all activation markers were increased could be attributed to activated Tcon (black arrow). Tcon expanded with the highest concentration of IL-2 were used to identify these nodes, as these cells received more activation stimulation and thus were expected to include the highest frequency of activated cells. On the other hand, almost no eTreg were found in nodes comprising mostly eTcon, indicating that eTreg do not present overlapping phenotypes with Tcon, which

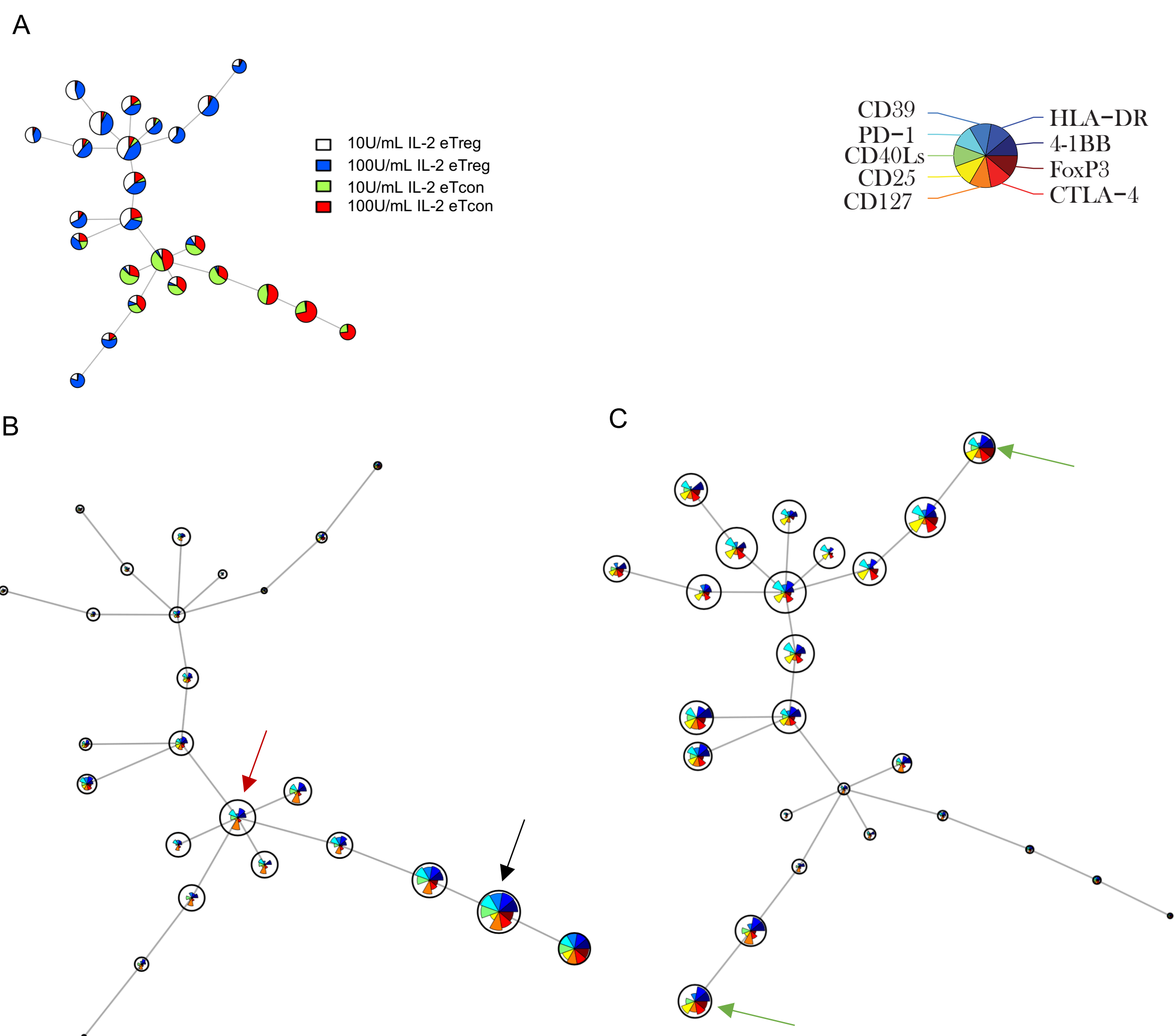

Figure 30: Minimum spanning tree of eTcon and eTreg samples.
(A) Minimum spanning tree of the self-organizing map built using cumulative data of all phenotypes expressed by Treg and Tcon expanded with 10U/mL of IL-2 or 100U/mL of IL-2. The diameter of each node is proportional to the total number of cells attributed to it, and each node displays the frequency of cells from each sample. (B) Minimum spanning tree displaying the phenotypes expressed by Tcon expanded with 100U/mL of IL-2, and (C) phenotypes expressed by Treg expanded with 100U/mL of IL-2. Each node displays the relative intensity of all markers on that node. The legend for markers can be found in the pie chart on the top-right corner.

could suggest that our populations were free from activated Tcon (**Fig.30A**). Interestingly, when data from Treg expanded with 100U/mL of IL-2 was projected onto the SOM, the resulting MST showed that there were no cells allocated to any of the nodes identified in eTcon, which was represented by the diameter of the nodes (**Fig.30C**). This means that, within eTreg, there were no cells expressing a phenotype that was similar to that of non-activated or activated Tcon, which may be taken as an indication that our eTreg populations were mostly pure. Of note, all of the nodes in this MST presented high expression of CD25 (yellow slice) and CTLA-4 (red slice). Finally, two distinct nodes representing phenotypes with increased expression of most activation markers, but converse expression of CD39 and PD-1, could be pin-pointed (green arrows), suggesting that there may be more than one phenotype presented by eTreg upon activation. As such, the characterization of subsets within eTreg was performed next. Since eTreg seemed free of contaminating eTcon and the expression of CD25, CD127 and Foxp3 in eTreg subsets seemed constant, the compartmentalization of eTreg into subsets was performed based only on the expression of other Treg-associated markers, namely PD-1 and CTLA-4, and activation markers (CD39, HLA-DR, 4-1BB and CD40L).

2.3.2. Identification of eTreg subsets

Based on the possibility that there could be more than one subset of activated Treg, the next logical step was to compile all of the phenotypes expressed by Treg, before and after expansion, and then divide them into clusters using unsupervised learning tecniques. Finally, each cluster could be characterized by its phenotype. To generate clusters that encompassed all phenotypes expressed by Treg, flow cytometry data from samples of Day 0 Treg (not expanded) and Treg expanded by 10U/mL of IL-2 and 100U/mL of IL-2 were concatenated and processed as described before (transformed and scaled). Each sample type was already represented by cumulative data from 3 independent experiments. Since by now we felt it could be assumed that these populations were reasonably pure and to simplify the characterization of each cluster's phenotype, this analysis was performed based on Treg-associated (PD-1 and CTLA-4) and activation markers (CD39, HLA-DR, 4-1BB and CD40L) only. First, k-means clustering was performed, where k was set to 5, and each cell was allocated by the unsupervised algorythm to the best-fitting cluster, according to its phenotype. After all cells were allocated, the stacked density plots of each marker could be seen (**Fig.31**). Additionally, the characteristics of each cluster could be manually translated from the density plots onto a table, allowing us to define each cluster (**Table VIII**). Since the MFI of each marker was transformed and normalized by scaling, it is important to note that the intensity of each marker was always relative to the intensities present in the samples used. That is, a cluster that has "negative" expression of CTLA-4, for example, is, simply put, the cluster where cells have the "least positive" expression of that marker.

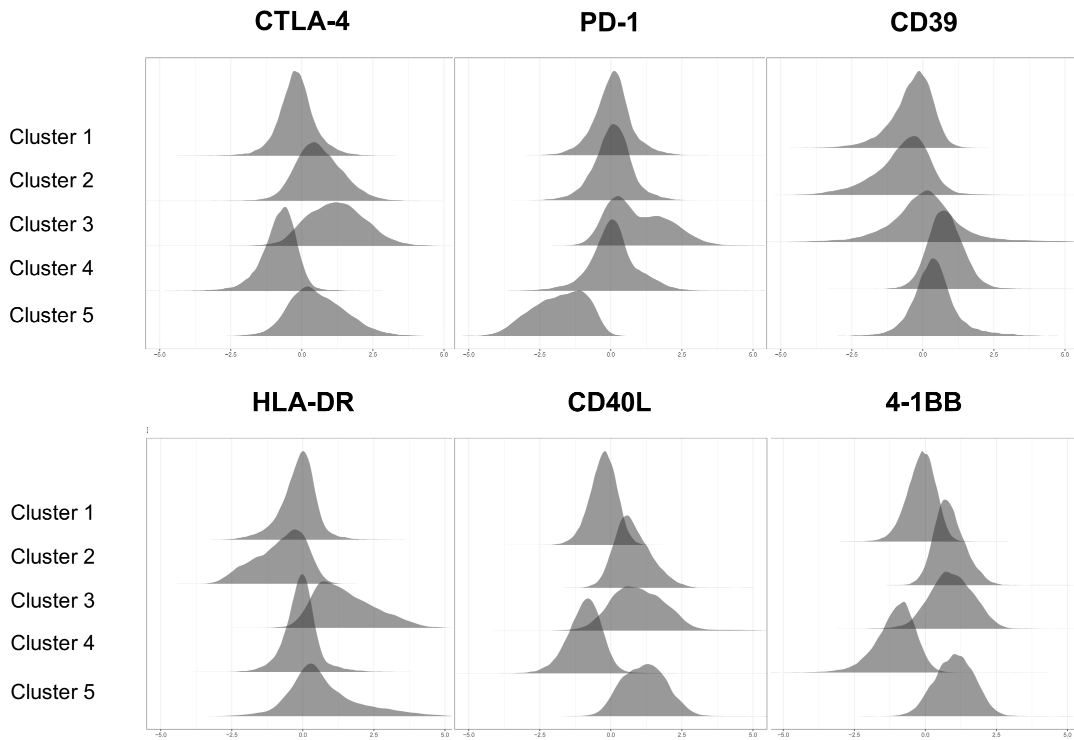

Figure 31: Histograms of CTLA-4, PD-1, CD39, HLA-DR, CD40L and 4-1BB expression on each cluster.
After performing k-means clustering of the phenotypes displayed by Treg before and after expansion, the expression of markers was displayed on stacked density plots for every cluster. The expression of each marker was transformed and normalized by scaling before k-means clustering, thus it is centred on 0.

	CTLA-4	PD-1	CD39	HLA-DR	CD40L	4-1BB
Cluster 1	-	low	-	low	-	low
Cluster 2	+	low	-	-	+	+
Cluster 3	++	+	low	+	++	+
Cluster 4	-	low	+	low	-	-
Cluster 5	+	-	+	+	+	+

Table VIII: Characterization of cluster phenotypes.
Each cluster was characterized based on the relative expression of each marker. Since the histograms are centred on 0, we defined marker expression as - when the peak of the curve was below 0, low when the peak of the histogram was on 0 and + when the peak was more than 0. The symbol ++ was used to distinguish clusters with the highest expression of that marker.

By defining the characteristics of each cluster, it was possible to identify some interesting marker combinations, which may be used to characterize Treg subsets with distinct functions. For example, the phenotype of cells allocated to Cluster 1 could be described by a low expression of all of the analysed markers. This may suggest that the cells assigned to this Cluster display a non-activated phenotype. The cells assigned to Cluster 2 had high expression of CTLA-4 and intermediate PD-1, which are typical Treg markers, as well as high expression of CD40L and 4-1BB, which are activation markers. Thus, it could indicate that this cluster represents a subset of Treg with an activated phenotype. Cluster 3, on the other hand, was characterized by the highest expression of all markers but CD39 (an ectoenzyme involved in one of Treg's mechanisms of suppression[137]). Since PD-1 expression was also at its highest in this cluster, the subset of cells in this cluster could be activated cells (hence the

upregulation of most activation markers) that were now displaying signs of exhaustion (high PD-1 levels). Cluster 4 differs from Cluster 1 only on the higher expression of CD39 and lower expression of 4-1BB. Hence, it may suggest that although the subset of cells in cluster 4 were not expressing an activated phenotype, they could present some non-specific suppressive function as a maintenance mechanism. Finally, Cluster 5 was characterized by high expression of all markers but PD-1. Although overall this is suggestive of a highly activated phenotype, similarly to the phenotype of Cluster 3, the downregulation of PD-1 may in turn suggest that this subset of cells may have increased proliferative capacity, and the converse expression of CD39 may reveal distinct suppressive profiles. From this analysis, it could be speculated that the subset of cells within Cluster 5 would be more suppressive than those in Cluster 3 or Cluster 1; alas, because this analysis cannot be performed live on a FACSorter and, more importantly, due to the limited numbers of cells in each cluster, it would be very difficult to verify the function of each of these clusters independently. A more realistic approach would be to characterize each sample according to the distribution of cells by each cluster, and then drawing a parallel to their suppressive function.

With that in mind, the percentage of cells assigned to each cluster was plotted on polar charts (**Fig.32A**) and bar charts (**Fig.32B**) for each sample. It could be seen that *ex vivo* Treg (Day 0 Treg) expressed different phenotypes than eTreg (**Fig.32A**). Specifically, around 60% of the cells in Day 0 Treg samples were allocated to Cluster 4, and the frequency of cells expressing this phenotype was significantly reduced to residual levels on both eTreg samples (Day 0 Treg, **Fig.32B**). The remaining cells from Day 0 Treg expressed the phenotype of Cluster 1. Interestingly, the percentage of cells allocated to Cluster 1 was identical on all samples. On the other hand, the percentage of cells assigned to Cluster 2 was increased from residual values on Day 0 Treg to more than 50% of cells present in Treg expanded by 10U/mL of IL-2 – 10U/mL IL-2 eTreg –, reaching near-significance ($p=0.057$). Within Treg expanded by 100U/mL of IL-2 – 100U/mL IL-2 eTreg – there were less cells allocated to Cluster 2 than in Treg expanded by 10U/mL of IL-2. However, there was a higher percentage of cells expressing the phenotype of Cluster 3 and Cluster 5 in Treg expanded by 100U/mL of IL-2.

Overall, this analysis showed that Treg can express different phenotypes upon activation and subsequent expansion; however, it seems that after expansion, only a fraction of the cells acquired a different phenotype, whilst a fraction of the cells still displayed a phenotype that was already present on *ex vivo* Treg (Cluster 1). Within cells that did express a different phenotype after expansion, the same three clusters could be found despite being expanded by 10U/mL of IL-2 or by 100U/mL of IL-2. In fact, the main difference between eTreg samples seemed to be in the percentage of cells assigned to each cluster (**Fig.32A-B**). Briefly, it seemed that both samples displayed the same diversity of phenotypes, albeit expressed at different frequencies.

Lastly, the phenotype of Day 0 Treg (*ex vivo*), Treg expanded by 10U/mL of IL-2 and Treg expanded by 100U/mL of IL-2 was characterized using FlowSOM, to assess if similar profiles could be detected in the phenotype of expanded Treg. A SOM was built from treated data derived from concatenated tubes of Day 0 Treg, 10U/mL IL-2 eTreg and 100U/mL IL-2 eTreg, using the same markers as the ones used for k-means clustering, and a 5x5 grid. In the resulting MST with data from all samples, it was already noticeable that each node had varying frequencies of cells from each sample

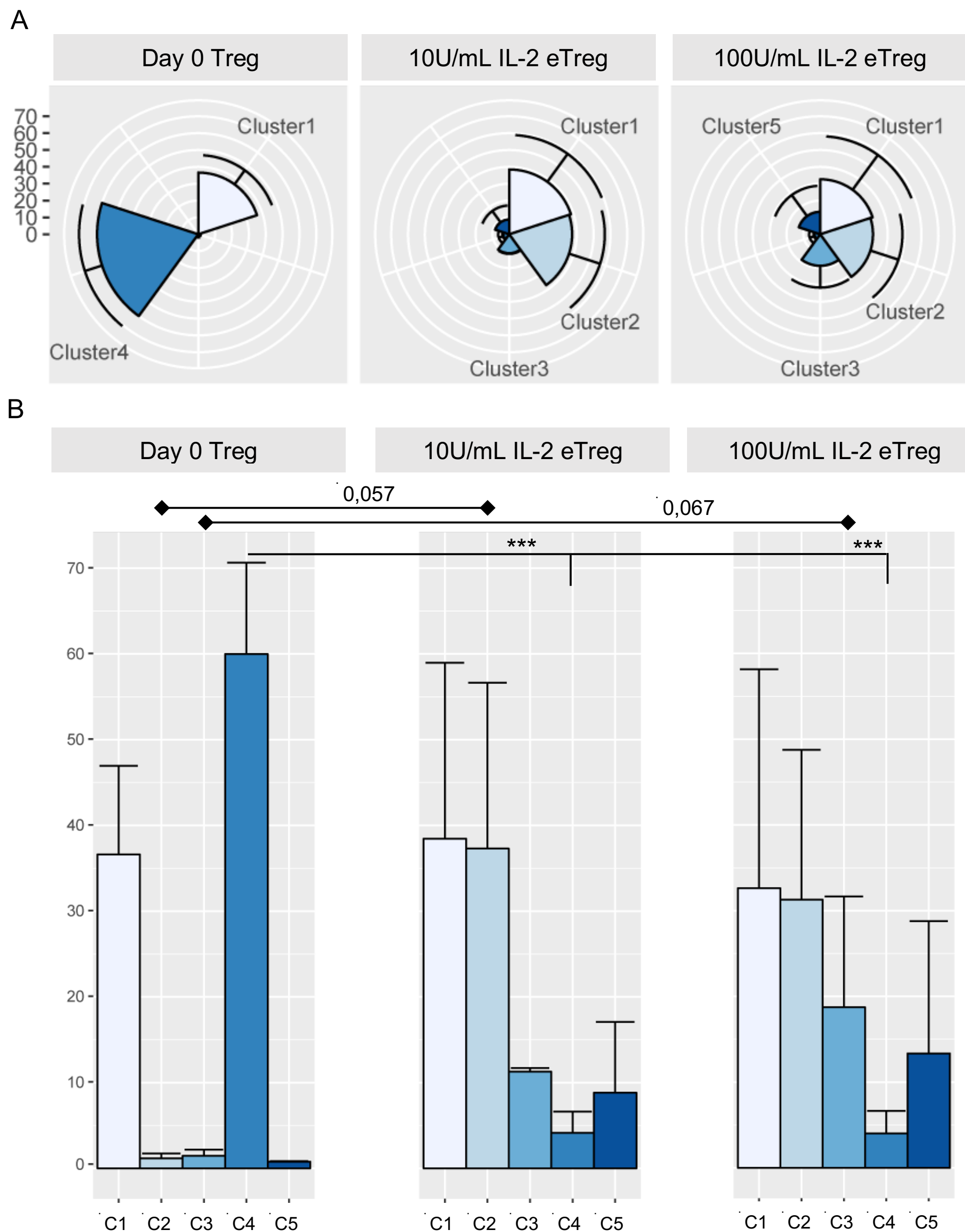

Figure 32: Frequency of cells allocated to each Cluster, aggregated by sample.
(A) The distribution of Treg subsets to clusters was aggregated by sample and displayed as polar plots (B) or bar graphs. The radius of a Cluster or the height of the bar represents the mean percentage of cells allocated to that cluster and the error bar represents standard deviation. Statistical analysis was performed by multiple t-tests on R's Stats package. N=3

(**Fig.33A**). Some nodes were mainly composed of Day 0 Treg cells (black arrow), others had equal parts of all samples (green arrows) and finally, a few nodes comprised cells from eTreg only (blue arrows). This confirmed that some phenotypes could be found in all samples, whilst others were more prevalent in Day 0 Treg or eTreg; furthermore, it seemed that according to the expansion millieu, the same phenotypes would be expressed by different frequencies of cells, which was in accordance to what had been found in k-means clustering analysis. In the MST of Day 0 Treg samples, most cells were allocated to the central nodes of the tree (**Fig.33B**). Conversely, the phenotypes present in eTreg were displayed in outter nodes (**Fig.33B-C**). Treg expanded by 100U/mL of IL-2 – 100U/mL IL-2 eTreg – had a higher frequency of cells in nodes of the lower section of the MST (**Fig.33C**). The outtermost

nodes, due to their smaller amount of connections to nearby nodes, can be assumed to represent the most distinct phenotypes. These nodes also displayed the highest expression of most markers – thus, these nodes possibly represent the phenotype of more activated subsets. Hence, it seemed fitting that the MST of Treg expanded by 100U/mL of IL-2 – 100U/mL IL-2 eTreg , **Fig.33D** – had more cells (larger diameter of nodes) in these nodes than Treg expanded by 10U/mL of IL-2 (10U/mL IL-2 eTreg, **Fig.33C**).

Interestingly, when the complete phenotype of each sample was visualized in the corresponding MST, nodes with similar characteristics to the clusters found by k-means clustering could be identified, with the guidance of the table of characteristics of each cluster (**Table VIII**). In fact, a node with similar phenotype to Cluster 1 was found in all samples (**Fig.33B-D**, grey arrow), whilst a nodal region with similar phenotype to Cluster 4 was only present in Day 0 Treg (**Fig.33B**, blue arrow). Also, in both eTreg samples , a nodal region similar to Cluster 2 could be identified, and seemed to include similar frequency of cells in both samples (**Fig.33C** and **Fig.33D**, black arrows). Finally, the nodes with a phenotype that was similar to that of Cluster 3 and of Cluster 5 were larger in Treg expanded by 100U/mL of IL-2 than in Treg expanded by 10U/mL of IL-2 (**Fig.33C-D**; Cluster 3 – red arrow, Cluster 5 – green arrow), thus more cells expressed this phenotype, as had been previously shown in **Fig.33B**.

In conclusion, these multidimensional analysis and visualization methods for the characterization of eTreg allowed us to define phenotypic subsets that may be related to Treg displaying particular mechanisms of function or different activation status. We propose that Treg that were not expanded were more enriched in cells with a non-activated phenotype that were possibly also weaker suppressors (Cluster 1). Cells with a non-activated phenotype, but possibly more suppressive through increased expression of CD39, were also found in *ex vivo* Treg (Cluster 4). After expansion, less than half of the cells found in culture still displayed a non-activated phenotype (Cluster 1), but there were no cells with a non-activated phenotype and high expression of CD39 (Cluster 4). Logically, subsets with increased expression of activation markers were more prevalent after expansion. Activated Treg were assigned to three clusters with distinct phenotypes: Cluster 2, where the expression of only a few markers was increased, Cluster 5, that had high expression of all activation markers and, we speculate, included cells with high proliferative capability, and Cluster 3, which had high expression of all activation markers but CD39, and could possible include exhausted cells. Finally, Treg expanded with higher concentration of IL-2 had higher frequency of cells with a phenotype suggestive of high activation and proliferative capacity (Cluster 5), as well as cells with an activated phenotype but possibly displaying signs of exhaustion (Cluster 3), while the subset of activated Treg (Cluster 2) was found in similar frequencies on both expansion milieus.

It also seems important to highlight that none of these subsets could be distinguished by their expression of only one marker, validating the importance of these novel methodologies for data visualization in the identification of populations of cells that have yet to be described. Nevertheless, we hypothesize that by separating eTreg into two subsets, based on the converse expression of CD39 and PD-1, it would result in two subsets of highly activated cells, perhaps displaying different mechanisms of suppression. We speculate that one would be enriched in highly activated, highly suppressive, cells ($CD39^{+}PD\text{-}1^{low}$) and the other would be enriched in highly activated, possibly exhausted cells ($CD39^{low}PD\text{-}1^{high}$). Since, even after expansion, there is limited availability of Treg, another way in which

this type of analysis could prove enlightning would be in the characterization of infused Treg. In the impossibility of pre-selecting a particular Treg subset before infusion, the in-depth characterization of Treg phenotypes present in the infusion product could serve as an indicator of the outcome of these therapies.

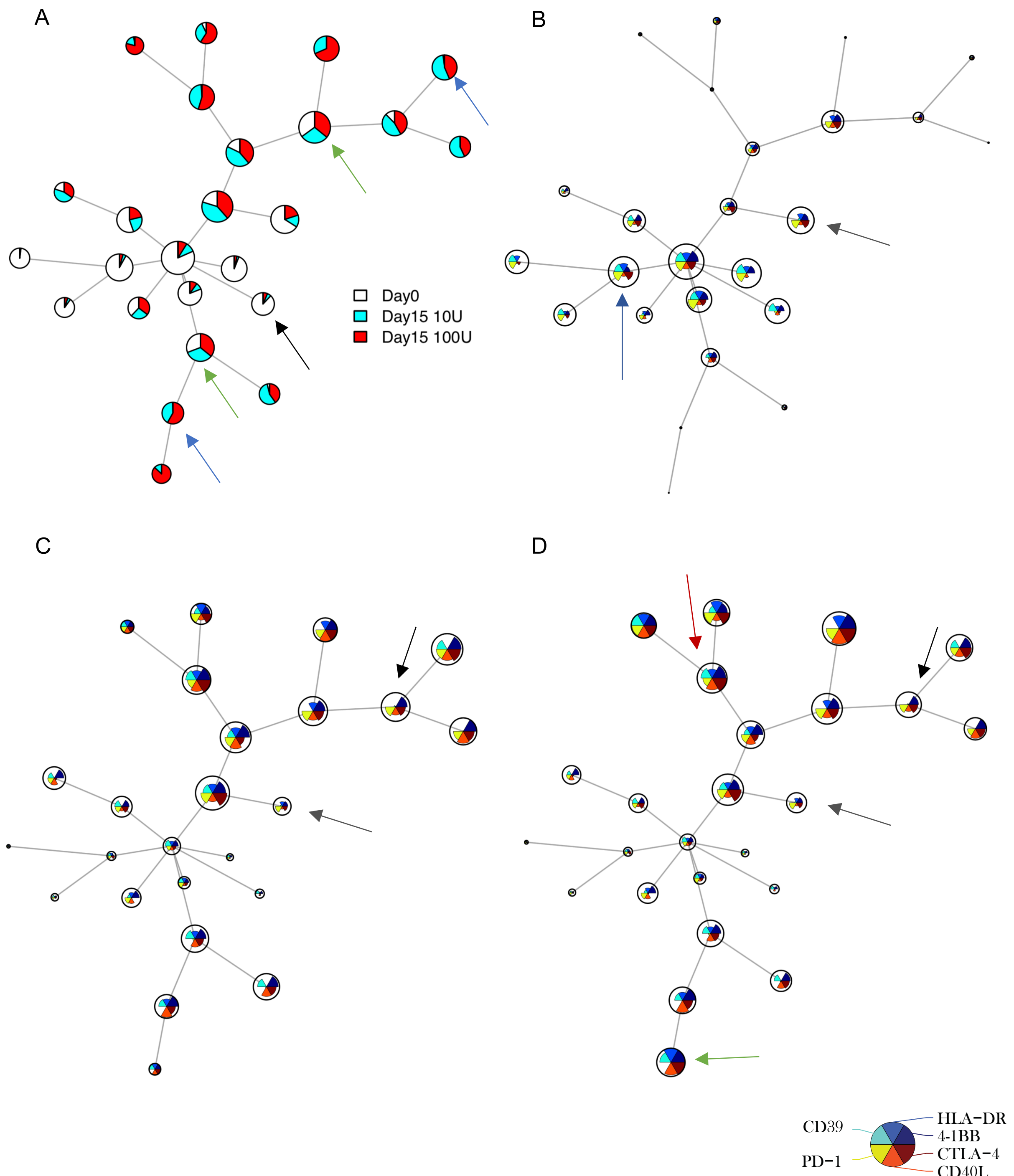

Figure 33: Minimum spanning tree of Treg phenotypes before and after expansion with 10U/mL or 100U/mL of IL-2. (A) Minimum spanning tree of the self-organizing map built using cumulative data of all phenotypes expressed by Treg before and after expansion. The diameter of each node is proportional to the total number of cells attributed to it, and each node displays the frequency of cells from each sample. (B) Minimum spanning tree displaying the phenotypes expressed by Day 0 Treg, (C) Treg expanded with 10U/mL of IL-2, and (D) Treg expanded with 100U/mL of IL-2. Each node displays the relative intensity of all markers on that node. The legend for markers can be found in a pie chart in the lower right corner.

DISCUSSION AND CONCLUSIONS

The primary aim of this work was the establishment of a reproducible protocol for Ag-sp Treg isolation and expansion *in vitro*. Additionally, we evaluated the specificity of suppression by expanded Ag-sp Treg and characterized phenotypic subsets within Ag-sp Treg. In the future, our goal is to apply the knowledge acquired with this work to the production of Ag-sp Treg suitable for clinical application.

In the first chapter of this work, we tested a few variations of Ag-sp Treg expansion protocols. This included the optimization of moDC differentiation protocols and the evaluation of the effect of the mode of Ag presentation and concentration of IL-2 during expansion on the final Ag-sp Treg population. Of note, both the differentiation of moDC and the subsequent Treg expansion were performed in the absence of human serum, which we believe could facilitate the translation of the final protocol to a GMP-compliant clinical setting.

Treg isolation was performed by FACSorting and resulted in an initial Treg population with high purity of $CD25^{high}CD127^{low}$ cells (>90%). Due to the intracellular location of the Foxp3 protein, it was not possible to sort cells based on this marker. Nevertheless, it has been shown that CD127 expression is inversely correlated with Foxp3[24], which was confirmed by our data, as the initial Treg population was enriched in $Foxp3^+$ cells. The advantage of using a FACSorter for Treg isolation, as opposed to commercially available magnetic Ab-bound bead kits, is that it allows for fine-tuning of marker cut-offs, whereas with magnetic separation kits the thresholds for selection of CD25 and CD127 can only be adjusted by modifying the number of beads added to the cell suspension. FACSorting also provides greater freedom in regards to the markers used for cell selection, whereas with magnetic separation the combination of markers used are restricted to the commercially available kits. However, it is important to point out that few GMP-grade cell sorters are available worldwide, whilst GMP-approved magnetic cell separation kits are already commercially available, so it is possible that this step of our protocol has to be re-optimized prior to clinical translation.

In order to achieve Ag-sp Treg expansion, it was important to select APC that could not only overcome Treg anergy and trigger their proliferation, but also present target Ag to Treg in a way that would lead to the enrichment of Ag-sp Treg within expanded cells. Since moDC are easy to obtain and were shown to be suitable APC for Ag-sp Treg expansion[75,76,211], a protocol for differentiation of moDC in serum-free conditions was optimized. To select between commercially available serum-free media, the viability and efficacy of differentiation of moDC were assessed. As no major differences in the viability and frequency of differentiated moDC were detected between Cellgenix and X-vivo 15, the latter was selected for our protocol. Many reports of Ag-sp Treg expansion use immature moDC (as described in **Table II**), possibly because it was believed for a time that these cells were tolerogenic. However, tolerogenicity cannot be attributed to a specific phenotype alone[88]. For example, immature DC with low to intermediate expression of co-stimulatory markers, as well as MHC-I and II, could induce Treg from naïve $CD4^+$ T cells[212]. Simultaneously, it has been proposed that the expansion and maintenance of Treg requires moDC with high CD86 and HLA-DR expression[76]. In fact, it has already been shown that activated moDC were better than immature moDC in Treg expansion[76]. Since there was no established protocol for Ag-sp Treg expansion using moDC, we set out to compare the effect of activated moDC with immature moDC (Control) for Treg proliferation and expansion. Currently, a cytokine cocktail with IL-1β, IL-6, TNF-α and PGE-2 is known as the gold-standard (GS) for moDC

activation. While moDC activated with this cocktail have been described as adequate APC for in Treg expansion[76], these APC have also been used for the induction of Treg from naïve T cells[94,99]. Similarly, the use of LPS-activated moDC has been described in Treg expansion[211], but the combination of LPS with IFN-γ in moDC activation has been described as yielding highly immunogenic moDC[91,100,213]. Thus, both GS and a combination of LPS with IFN-γ were tested for moDC activation. As expected, we found that both types of activated moDC stimulated more proliferation of Treg than immature moDC. Interestingly, we found that Treg fold expansion was much higher in the presence of GS-activated moDC than in the presence of LPS-activated moDC, although the frequency of proliferated cells was similar in both settings. Seeing that LPS-activated moDC displayed the highest MFI of CD86, and higher expression of CD86 after activation with LPS and IFN-γ has been reported to impair the suppressive function of *ex vivo* Treg[175], one could hypothesize that moDC subjected to stronger activation stimuli (LPS) could lead to Treg death during expansion. Alas, in the studies that claimed loss of suppression potency in this setting, the method for quantification of responder proliferation was thymidine incorporation[175]. This methodology does not allow for the discrimination between Treg or T_{eff} proliferation, so it is unknown whether the absence of suppression (increased proliferation) detected in this setting was a result of Tcon proliferation due to Treg death or loss of function, or even a result of Treg proliferation. Either way, in our final protocol, X-Vivo 15 media and the gold standard cocktail of cytokines were used for moDC differentiation and activation, respectively.

In addition to the stimulation provided by APC to Treg, namely, Signal 1 through TCR-MHC interaction and Signal 2 through CD28-CD80/CD86 interaction, the delivery of a third signal can make for optimal Treg expansion[214]. Since stimulation through the IL-2R is required for Treg expansion[76], as well as *in vivo* survival and suppressor function[59], the addition of IL-2 is a common practice in Ag-sp Treg expansion[125]. In the first experiments of Chapter 1, expansion co-cultures were supplemented with 100U/mL of IL-2 which, according to the literature, was within the intermediate range of concentrations used for Treg expansion[64,67,76,211]. Even though our initial cell population was pure in $CD4^+CD25^{high}CD127^{low}$ cells, which is the phenotype commonly used to select Treg, the possibility of residual activated Tcon being present in this population, displaying the same phenotype, could not be overruled. Therefore, it was a possibility that contaminating activated Tcon could be expanded simultaneously with Treg. Hence, rapamycin was added to the expansion media as a way to impair Tcon proliferation, as previous reports had suggested[115]. Nonetheless, it is important to keep in mind that rapamycin has also been shown to expand iTreg from Tcon[41], and that rapamycin together with IL-2 were shown to skew the phenotype of Tcon towards that of Treg in cultures of $CD4^+$ cells[119]. These observations reinforced the need for an initial Treg population of high purity, albeit in cultures of pure Treg rapamycin was shown to impair Treg proliferation. This impairment of Treg proliferation by rapamycin was more noticeable in the first week of culture, and occurred both in the presence[124] and absence[123] of human serum. The addition of IL-15, together with IL-2, has been shown to rescue Treg proliferation in the presence of rapamycin[75]. However, this has not yet been demonstrated in the absence of human serum. Additionally, Litjens *et al.* showed that IL-15 and/or IL-2 were needed for optimal Treg expansion in the presence of mature moDC[76]. Accordingly, it was decided to also supplement our expansion media with IL-15. Interestingly, it has been suggested that expansion of Treg

in the presence of rapamycin and IL-2 in serum-free conditions limited the expansion of cells with transient Treg phenotype and function, and serum-free expanded Treg were shown to have more stable suppressive capacity[123]. Therefore, the optimization of a protocol for the expansion of Ag-sp Treg in serum-free conditions may not only be relevant for its ease of translation into the clinical setting, but may also help assure the purity and stability of the final population.

After this key protocol was defined, we set out to evaluate the effect of different modes of Ag presentation on Treg expansion and function. This was of importance to us because, during GvHD, the donor's effector T cells react to patient derived-Ag that may be presented by patient APC (direct allo-presentation) or by the donor's APC, after uptake and processing of patient's Ag (indirect presentation)[8]. To generate APC presenting allo-Ag direct or indirectly, moDC were differentiated from an allogeneic donor (Original donor) or from the same donor as the Treg donor (self-moDC). Direct allo-Ag presentation was provided by moDC from the Original donor (OriDC); indirect allo-Ag presentation was conveyed by self-moDC loaded with a lysate of PBMC from the Original donor (OriLys). As a control for indirect allo-Ag presentation, Treg were co-cultured with self-moDC loaded with self-lysate (Self-DC). Of note, at the end of co-cultures, virtually all Treg had proliferated, regardless of the mode of Ag presentation. There was an average expansion of approximately 20 fold, which is within the same range as what had been demonstrated in other protocols of Ag-sp Treg expansion with mature moDC[76]. Thus, the main differences in our protocol, such as the absence of serum and the addition of rapamycin, did not seem to affect Treg expansion. It is possible that the negative effect of rapamycin on Treg proliferation was rescued by IL-15 in the absence of human serum, as had been previously described in the presence of human serum[75,76]. Surprisingly, Treg that were co-cultured with control moDC (self-DC) displayed similar proliferation to Treg expanded in the presence of allo-Ag. These results suggested that proliferation of Treg was a result of the elevated concentration of IL-2 (100 U/ml) and co-stimulation provided by APC, similarly to what had been reported by Verma *et al.*[125]. Together with the fact that Treg proliferation and fold expansion were similar in all co-culture settings, this raised the possibility that Treg were proliferating independently of the specificity of their TCR.

To verify the specificity of the Treg TCR before and after expansion, the frequency of cells responsive to each APC was evaluated within *ex vivo* Treg and for each type of eTreg by limiting dilution assays (LDA). Within *ex vivo* Treg, the highest frequency of precursors was responsive to directly presented allo-Ag, while the frequency of precursors responsive to indirectly presented allo-Ag was significantly lower than that. Moreover, there were fewer precursors responsive to Self-DC than to the other APC. These results were predictable, as Treg are naturally anergic to self-MHC, so no proliferation was expected. Still, this also seemed to validate the hypothesis that non-specific Treg were proliferating alongside with Ag-sp Treg in expansion cultures, reaching similar levels of expansion across all of the APC. Still, eTreg were enriched for Ag-sp Treg, as the frequency of cells responding to each APC was increased after expansion with the corresponding stimulator. Particularly, the proliferated fraction of eTreg was the most enriched in cells responding to the same APC as the ones present during their expansion. This made sense, as Treg responsive to APC would be activated and proliferate during expansion, and continue to proliferate upon re-stimulation with the same APC. Nevertheless, even within proliferated eTreg, the frequency of precursors responsive to the APC present in their expansion

was much lower than the frequency of proliferated (% CFSE$^-$) cells. This way, it could be concluded that not all Treg that proliferate in an Ag-sp setting are responding specifically to the presented Ag. This conclusion was disparate from what had been described by Veerapathran *et al.*, who assumed that the frequency of proliferated cells and the frequency of responsive precursors could be assumed to be similar[75]. Actually, we verified that Treg expanded by indirect allo-Ag presentation had higher frequency of precursors responsive to directly presented allo-Ag and of precursors responsive to self-moDC (without allo-Ag presentation) than of precursors responsive to indirectly presented Ag, unlike what was expected.

One of the ways to expand Treg of distinct specificities would be to separately expand Treg clones and then assess the suppressive function of each clone, as Veerapathran *et al.* described in a more recent work[85]. However, this type of experiment would require for the target Ag to be known, which was not possible in our setting. In fact, it could be expected that each target APC displayed a pool of unknown Ag, so it would be likely that more than one clone of different TCR specificity could be responsive to the same APC. Since our aim was to expand Ag-sp Treg suitable for clinical application, it was more important for us to ascertain if they would be suppressive of all responses to one donor than to verify their individual specificity. In other words, if we verified that eTreg could specifically suppress all responses to the same allo-Ag they were expanded with, these cells could be of value in the clinical setting. Furthermore, the frequency of responsive cells in LDA assays was calculated with basis on the proliferation of eTreg measured after re-stimulation with APC. Since all Treg had already proliferated during expansion, their proliferation in the LDA may not be indicative of the specificity of their TCR. Finally, it is not known whether the frequency of responsive cells can be directly linked to the suppressive potential of eTreg as a whole.

For all of these reasons, it was decided to perform SA parallelly to LDA. Both Treg expanded by direct allo-Ag presentation and Treg expanded by indirect allo-Ag presentation suppressed the proliferation of Tcon and CD8$^+$ cells in the presence of directly presented allo-Ag from the Original donor. These results indicated that Treg could be expanded in response to indirect allo-recognition, without compromising the suppression of responses to allo-Ag presented directly. Nevertheless, directly expanded Treg seemed more suppressive of responses to directly presented allo-Ag from the Original donor than indirectly expanded Treg. Consequently, higher numbers of indirectly expanded Treg may be required to achieve the same degree of suppression as that mediated by directly expanded Treg. Still, in the impossibility of expanding Ag-sp Treg directly, indirectly expanded Treg may provide a suitable solution for the expansion of Ag-sp Treg envisaging therapeutic application. This may prove useful, for example, when the patient's PBMC are poor in monocytes. Of note, the expansion of Ag-sp Treg by indirectly presented allo-Ag had been previously reported, though in the presence of human serum[75]. More importantly, the suppressive function of those cells had not been verified before. Here, we showed that Treg expanded through indirect allo-recognition seemed capable of suppressing responses not only to allo-Ag presented indirectly but also to allo-Ag presented directly. We even went as far as to show that Tcon proliferation was similarly suppressed in the presence of APC with direct and indirect allo-Ag presentation, while the proliferation of CD8$^+$ cells was more potently suppressed in the presence of APC with indirect allo-Ag presentation. Since, in SA with each type of eTreg, the

proliferation of CD8+ cells seemed more suppressed when the mode of allo-Ag presentation by APC was the same as in Treg expansion, it could be hypothesized that there is more specificity in the suppression of CD8+ cells. Conversely, in SA with Tcon, this trend was not evident, as responses to APC with different modes of allo-Ag presentation were similarly suppressed. Nevertheless, we believe it would not be accurate to directly compare the suppression of different responders. This is not only because they result from distinct experiments, but also because the actual Treg:T_{eff} ratios in SA with CD8+ cells were unknown, as total PBMC were used as responders.

Since the frequency of cells responding to the APC used in expansion was minute in both eTreg populations, in opposition to the frequency of proliferated cells (nearly 100%), it could be suggested that most of the proliferated cells were polyclonally expanded in response to elevated concentrations of IL-2 – in which case, specific suppression of responses to the original stimulators (with the same mode of allo-Ag presentation) could not be expected. Thus, the observed similar suppression of responses to allo-Ag, regardless of the mode of Ag presentation, could be an indicator of lack of specificity instead of an indicator of specificity for the Original donor. Last but not least, the differing frequencies of responding cells *vs.* proliferated cells also raised the possibility that iTreg could be expanded from contaminating Tcon, as a result of the presence of fresh rapamycin and IL-2 during the entire expansion period, as had been shown in previous reports[41]. In this case, it is possible that the TCR stimulation provided by APC could contribute to this induction as well[107]. It has been described, in experiments with 100U/mL of IL-2, that a low dose of a strong agonist induces Foxp3 expression in Tcon that persists *in vivo*, while high doses of weak agonist lead only to transient Foxp3 expression in Tcon[107]. Thus, it may be the case that the enhanced potency of suppression found in directly expanded Treg (hence, with stronger TCR stimulation) also reflects the contribution of stable iTreg. However, it has been described that there is selective survival of Treg over Tcon after TCR and IL-2 stimulation in the presence of rapamycin[112]. The authors showed that Tcon were apoptotic in the presence of rapamycin, while Treg were expanded and even had enhanced suppressive function. Yet, similar concerns can be raised from that report and from ours: the possibility that activated Tcon were present on the initial Treg population, being induced to express a Treg-like phenotype after activation in the presence of rapamycin, which would render them undistinguishable from Treg. It could be argued that if the bulk population of eTreg is proven to be stable and functional, these cells should still prove useful for clinical applications, regardless of the presence of Treg alone or alongside iTreg. Additionally, it has been reported that rapamycin-expanded polyclonal Treg can induce infectious tolerance[123], and that differentially stimulated Treg exhibit distinct suppressive abilities[215]. Therefore, it would be impossible to ascertain whether the enhanced suppression displayed by directly expanded Treg results from the influence of iTreg generated during expansion or from different mechanisms of suppression being triggered in eTreg by distinct expansion stimuli.

Nevertheless, we wanted to clarify the ways in which the function of eTreg could be modulated by manipulating the expansion milieu. By reducing the concentration of IL-2 (10U/mL) and adding rapamycin and IL-15 only on Day 0, we expected the phenotype of expanded cells at Day 14 to no longer be muddled by the influence of rapamycin. This way, we could unravel the "real" phenotype of eTreg. We focused particularly on the suppressive potency and specificity of eTreg, taking into

consideration the effect of the different modes of allo-Ag presentation during expansion. Judging by previous reports of Ag-sp Treg expansion in the presence of low concentration of IL-2, inferior proliferation and fold expansion could be expected in these experiments[75]. The absence of IL-15 could also hamper Treg proliferation[76]. Yet, at this stage, our focus was on achieving a population that was highly suppressive, and specific for its original stimulator, so these seemed to be the best conditions to prevent the expansion of polyclonal Treg as well as of contaminating Tcon. With lower concentrations of IL-2, it was expected that Treg could be more specific after expansion, as proliferation was only expected to occur in response to the signaling provided through TCR-MHC interactions.

Surprisingly, in this new expansion milieu, no differences were found between the proliferation and fold expansion of Treg from co-cultures with different APC. Among Treg expanded with self-moDC, there was similar proliferation and fold expansion in the presence or absence of allo-Ag. These observations were unexpected, as Veerapathran *et al.* had reported that Treg co-cultured with self-moDC loaded with self-Ag displayed significantly less Treg proliferation than in co-cultures with self-moDC loaded with allo-Ag, in the presence of the same concentration of IL-2[75]. On the one hand, that report measured proliferation by scintillation counts after a 16-hour period of tritiated thymidine incorporation while we assessed proliferation through CFSE dilution – hence, their proliferation referred only to that 16-hour span, while we identified the total frequency of proliferated cells, regardless of the moment of proliferation. On the other hand, the authors measured proliferation on Day 6, while we did so at Day 14. Thus, it is possible that the differences in proliferation were diluted in longer cultures. Additionally, the authors used immature moDC as APC, while we used activated moDC, which have been shown to result in higher Treg proliferation[76]. It is worth mentioning that the authors reported a 2000-fold expansion of Ag-sp Treg on Day 27, yet this value was calculated by dividing the total number of Ki-67$^+$ (proliferating) cells in culture on Day 27 by the number of specific precursors quantified by LDA on Day 0[75]. In our opinion, this method does not seem adequate, as it has been shown by them and also in our present work that not all proliferated cells are Ag-sp. In fact, when one looks at the total number of Treg in their cultures, it seems that it was maintained throughout the entire period of co-culture[75]. This indicates that the fold expansion of Treg in indirect allo-Ag presentation cultures was comparable in our protocol and the previously reported one[75].

Furthermore, in our experiments, Treg seemed to proliferate more in response to directly presented allo-Ag than in response to indirectly presented allo-Ag. It could be speculated that this was a result of a bigger weight of the affinity of Treg TCR to Ag-MHC complexes during Treg expansion with low concentrations of IL-2. That is, directly expanded Treg proliferated more in response to allo-MHC, which could be assumed to provide a stronger stimulus than self-MHC. Indirectly expanded Treg were only responding to allo-Ag presented by self-MHC, which could be assumed to provide a weaker stimulus. Of note, the DC:Treg ratio was increased from 1:10 in cultures with directly presented allo-Ag to 1:4 in cultures with indirectly presented allo-Ag. Increased DC:Treg ratios have been shown to increase Treg fold expansion[76], but a ratio of 1:10 DC:Treg had been described as sufficient for Treg expansion with allo-moDC[75]. Thus, the fact that there still seemed to be higher Treg expansion with direct allo-recognition even at lower DC:Treg ratios could indicate that higher stimulation through the TCR trumps increased co-stimulation in settings with lower TCR affinity. Conversely, Long *et al.* have

reported that while *de novo* Treg induction was enhanced by low dose Ag, nTreg proliferation was not affected by Ag dose[216]. Whether a parallelism can be drawn between low/high dose Ag and indirect/direct presentation of allo-Ag is unknown, but it must be noted that studies on the effect of the strength of TCR signaling usually focus solely in the induction of Treg and rely on signaling via the AKT/mTOR pathway[217]. In the presence of rapamycin, this pathway is interrupted, thus it could lead to different results. Of note, higher variation in Treg proliferation and expansion was detected among experiments with low concentration of IL-2 than in experiments performed with intermediate concentration of IL-2. This could also mean that, in this setting, casual variations in antigen load and quality of peptide could heavily influence Treg proliferation.

Despite the differences in proliferation and expansion, the phenotype of eTreg was comparable in Treg expanded with 10 or 100U/mL of IL-2, which suggested that the same type of cells was present in both cases. Surprisingly, when the suppression of Tcon proliferation in response to the original stimulator (used during expansion) was compared to the suppression of responses to a 3rd party donor who had no MHC matches to either the Treg or the Original donor, it seemed that directly expanded Treg were only more suppressive of the original stimulator, read specific, at low ratios. With higher Treg numbers, responses to both the Original and the 3rd party donor were similarly suppressed. Inversely, indirectly expanded Treg seemed to specifically suppress Tcon proliferation in the presence of the original stimulator at all ratios. Two suggestions could be drawn from these observations: first, directly expanded Treg seemed more potent suppressors of responders, regardless of the APC present. Second, indirectly expanded Treg seemed less potent suppressors of unspecific Tcon proliferation, yet were more potent in the suppression of responses to indirectly presented allo-Ag from the Original donor. Overall, these results suggested that the lower proliferation found in expansion with 10U/mL of IL-2 only resulted in specific eTreg in the case of indirectly expanded Treg.

It is true that some groups reported that Treg expanded with low concentration of IL-2 could specifically suppress alloantigen-specific responses[72,75]. However, it should be kept in mind that the definition of specificity in the field is fickle, not to mention that there are many differences in methodology from study to study, which may also contribute to the uncertainties around this topic. After reading the work of other groups with settings similar to ours (where Ag specificity was unknown), specific Treg were usually defined by the different potencies of suppression found in the presence of the original stimulator and in the presence of a 3rd party donor[72,75]. Even within this definition, most reports are unclear. For example, Veerapathran *et al.* have described higher potency of suppression by Treg in the presence of the original stimulator than in the presence of a 3rd party donor only up to a ratio of 1:20 Treg:T_{eff}[75]. Conversely, Putnam *et al.* showed that at higher Treg:T_{eff} ratios, such as 1:3 or 1:1, there was similar suppression of responses to the original stimulator or to a 3rd party donor[72]. Interestingly, both authors claimed to have expanded Ag-sp Treg. Additionally, Litjens *et al.* portrayed Ag-sp Treg as Treg that were more suppressive of the original stimulator than polyclonally expanded Treg, without further comparison with a 3rd party donor[76]. Since eTreg are expected to be highly suppressive, it seems acceptable that at high Treg:T_{eff} ratios, proliferation in response to a 3rd party donor may be decreased through non-specific mechanisms. These mechanisms may possibly be involved in infectious tolerance[218], where there is an induction of responders with suppressive function[181]. Thus, it remains to

be defined where do we set the threshold to distinguish between highly suppressive, *specific,* Treg and highly suppressive, *non-specific,* Treg. We advocate that in order to define the specificity of Treg expanded through a particular protocol, one should focus not only on the potency of suppression displayed by those eTreg at several ratios, but also on their potential to suppress different responders.

Most studies on the role of TCR stimulation during Treg expansion focus on the effect it has in Treg function[56,215]. Similarly, we wanted to ascertain if the mode of Ag presentation could influence the suppression of allo-Ag from the Original donor. Thus, we compared the potency of suppression displayed by directly expanded Treg with the potency of suppression displayed by indirectly expanded Treg, when the stimulators presented allo-Ag from the Original donor either direct or indirectly. We found that Tcon proliferation in response to directly presented allo-Ag was more suppressed by directly expanded Treg than by indirectly expanded Treg. Conversely, responses to indirectly presented allo-Ag were similarly suppressed by both types of Treg. It has been described that weakly stimulated Tcon are more susceptible to suppression, while strongly activated Tcon are more resistant to suppression[215]. Thus, it could be the case that the suppression profiles found in these experiments do not reflect Treg specificity but instead result from a particular susceptibility of Tcon to be suppressed when stimulated by indirectly presented allo-Ag. Interestingly, when we performed an exploratory experiment with directly expanded Treg to compare the potency of suppression of Tcon to the potency of suppression of $CD8^+$ cells, this susceptibility was more evident in Tcon, as these responders were more suppressed in the presence of indirectly presented allo-Ag than in the presence of directly presented allo-Ag. Conversely, $CD8^+$ cells were more suppressed in the presence of directly presented allo-Ag than in the presence of indirectly presented allo-Ag.

In these experiments we could verify that directly expanded Treg were more suppressive of Tcon proliferation than indirectly expanded Treg, in response to directly presented allo-Ag, which suggested that directly expanded Treg were overall more potent suppressors. However, these cells were also more suppressive of Tcon proliferation in the presence of indirectly presented allo-Ag than in the presence of directly presented allo-Ag, which could indicate that the suppression of Tcon proliferation was not specific for the same mode of Ag presentation present during expansion. On the contrary, the suppression of $CD8^+$ cells seemed specific for the same mode of Ag presentation present during expansion.

Hence, it remained to be ascertained if directly expanded Treg were more potent suppressors, albeit only specific in the suppression $CD8^+$ cells, or if indirectly expanded Treg were more specific suppressors, or simply weaker suppressors. Finally, the differences detected in the suppression of Tcon and $CD8^+$ cells could indicate that Tcon and $CD8^+$ cells react differently to the mechanisms of suppression employed by eTreg. However, conclusions could not be drawn from these observations as they derive from different experiments, with high variability among them.

In conclusion, the results described here indicated that the milieu of Treg expansion might alter eTreg function, possibly by eliciting different mechanisms of suppression by Treg, as different potency and specificity of suppression were detected. The potency and specificity of suppression displayed by eTreg were also dependent on the suppression millieu, that is, on the type of responders present and their basal activation in response to APC (in the absence of eTreg). Because experiments with high

and low concentration of IL-2 were performed separately, direct comparison of Treg function after expansion with different concentrations of IL-2 was not feasible. Thus, conclusions on the effect of IL-2 concentration during expansion in Treg function could not be surely drawn. This was also true for comparisons between responders, as the suppression of Tcon and $CD8^+$ cell proliferation was not measured parallelly in all experiments. Another issue we faced in the previous work was experimental variability. We hypothesize it could be related to donor-to-donor variability, and to varying degrees of MHC-match amongst the Treg donor and Original donor. Nevertheless, at this point, all of the protocols required to perform more complex experiments were duly optimized. With that in mind, from this point on, high-resolution HLA genotyping of all BC donors was performed, and Treg donors were paired with Original donors in a way that Treg expansion would always occur under similar levels of MHC-mismatch. Furthermore, in experiments with indirect allo-Ag presentation, the quantity and quality of allo-Ag being presented during expansion could not be measured, so variability from experiment to experiment was inevitable. Plus, self-moDC (with or without allo-Ag) were very weak stimulators of responder proliferation, even at increased DC:T cell ratios (1:1, as opposed to 1:4). Thus, the full potential of suppression by eTreg was not assessed in this setting. Since these details would impair a confident characterization of eTreg potency and specificity of suppression, experiments with Ag-loaded self-moDC were not repeated. Nevertheless, it is important to retain that indirectly expanded Treg could specifically suppress responders in a setting of indirect allo-recognition, while directly expanded Treg were capable of performing suppression in settings of direct and of indirect allo-recognition.

These differences could be explored for future clinical applications, as it is possible that indirectly expanded Treg would be less capable of generalized suppression yet sufficient for the suppression of responses to Ag presented indirectly (e.g. patient-derived tissue Ag presented by donor APC). Directly expanded Treg could be more potent suppressors of donor-derived responses to Ag presented directly by patient-derived APC, being more efficient in the suppression of GVHD, albeit perhaps at the risk of also suppressing donor responses to patient-derived tumour-Ag (regardless of their presentation mode). It should be noted that in the clinical setting, there would be a high level of MHC-matching between the Treg donor and the Original donor, so the function of directly expanded Treg may be more similar to that of indirectly expanded Treg in a mismatched setting, such as the one we described.

In the future, we intend to explore this hypothesis, using stored PBMC samples from HSCT donor-recipient pairs. We plan to isolate and expand donor Treg in the presence of recipient-derived APC, and then evaluate their suppressive function in SA with recipient-derived APC, donor-derived APC loaded with a lysate of the recipient's PBMC and donor-derived APC loaded with a lysate of the recipient's leukemic cells. Ideally, we would like to ascertain whether eTreg could suppress responses to recipient-derived APC and donor-derived APC presenting recipient-derived Ag, making them possible suppressors of GvHD responses, without suppressing responses to donor-derived APC presenting tumour Ag, possibly preserving the GVL effect. Since donor-recipient pairs usually have high number of HLA-matches, it is possible that some of the recipient's Ag found in lysates of PBMC are haematopoietic-resctricted mHA, thus leukemic cells could also express those mHA[9]. Hence, it would be interesting to assess the suppressive function of eTreg in the presence of tissue-derived mHA. As it

has been described that mHA found in skin tissues play a role in GvHD[219], we would like to evaluate the function of eTreg in the suppression of responses to either recipient-derived skin DC or to donor-derived APC loaded with lysates of cells from punch biopsies. Furthermore, upon activation by APC, responder T cells are expected to differentiate into effector cells with specificity towards those stimulators. Since it has been shown that suppression by Treg may also differ with the specificity of Th cells[174], it would also be interesting to perform SA where the suppression of fresh responders, like those used in this work, was compared to the suppression of responders previously activated with each target stimulator. This way, the full potential of suppression by eTreg in the presence of each APC could be verified. Thus, it would be possible to obtain a more complete picture of the suppressive profile of eTreg, in regards to the suppression of responses that cause GvHD and the maintenance of GVL effects, unlike any other human study performed so far. We believe this type of analysis could prove very enriching not only for the development of future therapies for GVHD but also provide clues in regards to the outcome of Treg infusion therapies currently being studied.

Coming back to the present work, there was still the need for a more accurate comparison of the effect of IL-2 concentration on Treg expansion and function, in order to define the most suitable protocol of Ag-sp Treg expansion. Additionally, despite the broad range of reports assessing the potency and specificity of Ag-sp Treg, there was a lack of reports that compared in parallel the suppression of different types of responders within each experiment. More importantly, there were no studies that compared the suppression performed by expanded Ag-sp Treg in the presence of stimulators with varying degrees of MHC-matching. This type of analysis could provide very interesting insights on the mode by which specific suppression is accomplished. Since every condition was tested on each experiment, we could more confidently compare between conditions, as general conclusions could be supported by validating each independent experiment. Briefly, for each Treg and Original donor pair, we compared the effect of IL-2 on Treg function and the suppression of Tcon and $CD8^+$ cells in the presence of a range of APC. Because the design of these experiments was so robust, we could look for clues on the mechanisms involved in suppression, focusing not only on the phenotype of moDC but also on the presence of cytokines in the supernatant of SA. Last but not least, by performing in-depth multidimensional analysis of the phenotype of Treg before and after expansion, we could infer on the purity of expanded cells, and, more importantly, try to identify Treg subsets with particular combinations of markers that may be related to their functional status and suppressive function. Thanks to the cohesiveness in these experiments, and due to their complexity, the entire second chapter of results derives from three independent experiments only.

It is important to point out that we were dealing with human samples randomly provided, upon request, by Instituto Português do Sangue e Transplantação (IPST). This means that to perform three independent experiments, we had to process the buffy-coats of 15 different donors, in total, and sequence their HLA. With these data, we first identified candidate pairs of Treg-Original donors that had the same number of mismatches in HLA-I and HLA-II. Then, we excluded every Treg-Original donor pair for which we did not find a 3rd party donor that was fully mismatched to both the Treg and the Original donor. For each of the remaining Treg-Original donor pairs, we screened all donors for one that was partially matched in HLA-I and HLA-II to the Original donor – to use as DC-matched donor –

and for a donor that was partially matched in HLA-II only to the Treg donor – to use as Treg-matched donor. Those Treg-Original donor pairs for which we did not find suitable 3rd party candidates were also excluded from the pool of candidates. In the end, we chose three Treg-Original donor pairs that had similar degree of mismatch between the Treg and the Original donor amongst each other. In order to fulfill all of these criteria in each experiment , some donors had to be used in more than once, albeit with different roles (for example, the Treg donor of one experiment could be used as a mismatched 3rd party donor in another experiment). Consequently, we only used cells from a total of 9 out of 15 donors. As a side note, for the entirety of the work described in this thesis, we had to process the buffy coat and sequence the HLA of a total of 79 donors.

The experiments described next resulted from the serum-free expansion of Treg in co-cultures with directly presented allo-Ag from the Original donor, in the presence of either 10U/mL or 100U/mL of IL-2, thus allowing for the direct assessment of the effect of IL-2 on Treg expansion, function and phenotype. Since Treg fold expansion seemed increased in cultures with 100U/mL of IL-2, but the frequency of proliferated cells was seemingly similar in co-cultures with either concentration of IL-2, it could be speculated that a higher concentration of IL-2 is more adequate for the maintenance of proliferated Treg in serum-free expansion millieus. Of note, when the frequency of proliferated cells was measured in independent experiments with either 10U/mL or 100U/mL of IL-2, it seemed that there was a higher frequency of proliferated cells in expansion cultures with higher concentration of IL-2, which was not verified in these experiments. This observation highlights the importance of comparing between conditions within each experiment, instead of performing separate experiments. As Treg from both expansion millieus were highly suppressive of responses to the Original stimulator, reaching similar degrees of suppression, it could be concluded that the expansion millieu did not significantly affect the potency of suppression by Treg. Furthermore, this could also indicate that the increased fold expansion verified in Treg expanded in the presence of 100U/mL of IL-2 was related only to the maintenance of eTreg and not to the expansion of residual activated Tcon.

Conversely, the suppression of responses to moDC from a mismatched 3rd party donor seemed affected not only by the expansion millieu of Treg, but also by the type of responders present in the SA. Briefly, the responses to moDC from a mismatched 3rd party donor were always significantly less suppressed than the responses to the Original donor. However, on the one hand, the proliferation of Tcon seemed more suppressed by Treg that were expanded with high (100U/mL) concentration of IL-2 than by Treg that were expanded with low (10U/mL) concentration of IL-2. On the other hand, there seemed to be no difference between the suppression of $CD8^+$ cells by Treg expanded with 10U/mL of IL-2 and the suppression of $CD8^+$ cells by Treg expanded with 100U/mL of IL-2. Moreover, the suppression of $CD8^+$ cells seemed to be generally more unspecific than that of Tcon, since the differences between the suppression of responses to a mismatched 3rd party donor and the suppression of responses to the Original donor seemed smaller when $CD8^+$ cells were used as responders. Using TCR-transgenic Treg and Th, George *et al.* have shown that potently stimulated Treg of one specificity could suppress Th cells proliferating in the presence of stimulators of a distinct specificity[174]. Nevertheless, the authors also showed that Th that are highly activated become resistant to suppression when Treg are of a different specificity. These observations are in accordance with what

we found for the suppression of a 3rd party donor, since moDC from this donor are likely to activate responders of different specificity than that of eTreg.

Additionally, when we calculated the degree of specificity of suppression by eTreg from each expansion millieu and correlated it with the potency of suppression of Tcon or CD8+ cells, it seemed that the specificity and the potency of suppression were inversely correlated. However, this only seemed valid for the suppression of Tcon, while the potency of suppression of CD8+ cells seemed unrelated to the degree of specificity achieved. In other words, our results indicated that Ag-sp expanded Treg that were capable of more potent suppression of Tcon proliferation in response to the Original stimulator were in turn less specific, that is, were also more suppressive of Tcon proliferation in response to a mismatched 3rd party donor. This trend was detected separately in Treg expanded with 10U/mL IL-2 and in Treg expanded with 100U/mL IL-2, reaching near-significant p-values in both cases. Since the correlation was made statistically significant when the data was not separated by expansion millieu (data not shown), it could be inferred that these conclusions are applicable to eTreg of both expansion millieus, and that statistical significance for the separate correlations could be reached by including data from more replicates. When comparing between expansion millieus, the differences in Treg potency could be attributed to the increased concentration of IL-2 during expansion. Nevertheless, these results indicated that differences in Treg potency could be found within each expansion millieu (albeit small, as could be seen in the standard deviation of **Fig.22**). In fact, these small differences seemed sufficient to correlate negatively with the suppression of unspecific responses. We suggest that these differences in eTreg potency could result from different affinities of Treg TCR to the MHC of moDC from the Original donor, as it has been shown that mismatches in different alleles can generate stronger or weaker responses from T cells[3,5,220]. Interestingly, these responses have been associated with distinct outcomes of HSCT[3,5,220]. It has also been shown by Putnam *et al.* that the frequency of responding cells within responders and Treg is increased when the number of mismatches in HLA-AB (for CD8+ cells) and in HLA-DR (for Tcon and Treg) is also increased[72]. Moreover, in that report, the precursor frequency found within Treg and Tcon seemed to be highly variable from sample to sample, even when the number of mismatches was the same, which validates the hypothesis that mismatches in different alleles can result in distinct responsiveness of T cells. Since only the number of mismatches was controlled in our experiments, but it was not feasible to control the location of the mismatches, it could be the case that some Treg-Original donor pairs generate more potent Treg than others.

Finally, the absence of a statistically significant correlation between the potency and specificity of Treg in the suppression of CD8+ cells may imply that the mechanisms involved in the suppression of Tcon and of CD8+ cells may not be the same. Yet surprisingly, when we evaluated the suppression of proliferation in response to moDC from 3rd party donors with different degrees of MHC-mismatch to either the Treg or the Original donor, we found that the potency of suppression displayed by eTreg was not very different when responders were Tcon or CD8+ cells. Besides the fact that responses to moDC from a mismatched 3rd party donor were significantly less suppressed than responses to moDC from the Original donor, we also found that responses to moDC from a 3rd party donor partially MHC-matched to the Original donor (DC-matched) were nearly as suppressed as the responses to the Original donor. Indeed, particularly in the suppression of CD8+ cells, responses to the DC-matched donor seemed as

suppressed as those to the Original donor. Furthermore, the responses to this DC-matched donor were more suppressed than those to a 3rd party donor partially matched in MHCII to the Treg (Treg-matched). While in SA with $CD8^+$ cells responses to the Treg-matched donor were as suppressed as those to a mismatched 3rd party donor, in SA with Tcon the responses to this Treg-matched donor were more suppressed than those to a mismatched 3rd party donor. Since the Treg donor is also the donor of responders, both eTreg and responders are partially matched in MHCII to the Treg-matched donor. Thus, Tcon and Treg may recognize some MHCII allelles presented by moDC from the Treg-matched donor as Self. Seeing that there were no matches in MHCI between the Treg donor (also the $CD8^+$ cell donor) and the Treg-matched donor, it could be speculated that in SA with moDC from a Treg-matched donor, the increased suppression of Tcon is a result of an increased susceptibility to suppression upon recognition of self-MHCII by Tcon, which does not happen with $CD8^+$ cells. Interestingly, at a ratio of 1:5 Treg to T_{eff}, these differences were no longer as noticeable, which highlights the importance of thoroughly characterizing suppression by Treg in several conditions.

Considering that, upon co-culture with moDC, $CD8^+$ cells are expected to interact with MHCI and Tcon are expected to engage with MHCII, it was unlikely that $CD8^+$ cells and Tcon were responding to the same allo-Ag in these SA. In our SA setting, three axes of interaction may simultaneously be at play, as there may be T-T cell (Responder-Treg), responder-APC and Treg-APC interactions. One could speculate that the differences found in the potency of suppression in response to different APC resulted from a specificity of Treg to APC (Treg-APC interaction), as Treg and responders were the same in each condition. Hence, the weight of T-T cell interactions would be constant. In this scenario, our hypothesis was that Treg were responding to APC by triggering similar mechanisms of suppression regardless of the type of responder present, but then each responder would be differently modulated. Nevertheless, there is still the influence of responder-APC interactions to be accounted for, and we have in fact shown that the degree of stimulation provided by the APC to responders could not be overlooked as a factor in the outcome of SA. Particularly, when the phenotype of each moDC was correlated to the potency of suppression displayed by eTreg in the presence of those moDC, we found that the initial MFI of CD86 in moDC was negatively correlated to the degree of suppression of Tcon and $CD8^+$ cells.

The role of CD86 in the function of Treg has been widely described, not only from the point of view of the co-stimulation provided to Treg[54] and responders[221], but also as part of a mechanism of suppression by Treg[99,174,175]. Particularly, George *et al.* suggested that simultaneous blockade of CD80 and CD86 expression on APC by mAb impaired the capability of Th to escape suppression[174]. In a later study, CD80 and CD86 were independently blocked by mAb and it was shown that blockade of CD86 in moDC increased the suppression of Tcon proliferation, while in contrary, blockade of CD80 impaired suppression[175]. In the latter study, the authors suggested that these molecules interacted with Treg through CTLA-4 and CD28, respectively, which had opposing effects on the function of Treg. This premise was further supported by Qureshi *et al.,* who showed that T cells were capable of transendocytosis of CD86 from DC via a CTLA-4 dependent mechanism, thus decreasing the stimulation provided to responders[131]. Together with our results, it can be suggested that higher initial expression of CD86 by APC leads to less suppression of responders. What remains to be ascertained

is whether this is a result of a limiting number of molecules that can be acquired or ligated to by Treg, decreasing the stimulation delivered to responders only up to a certain point, or if the increased expression of CD86 causes the responders to escape suppression. In the future, we intend to perform SA with eTreg in which the expression of CD86 by moDC is measured at the beginning and at the end of the suppression assays. Additionally, we would like to evaluate if these measurements would be affected by blocking CTLA-4 on eTreg, as performed by Qureshi *et al.* on fresh Treg[131]. This way, it would be possible to clarify if the initial MFI of CD86 affects suppression by reaching a limit in down-modulation by Treg or if it has a direct effect on the responders' susceptibility to suppression. Surprisingly, no correlation was found between the initial CD80 MFI in moDC and the potency of suppression by eTreg, although it has been reported that CD80 was required in suppression by Treg[175]. Since both CD80 and CD86 provide co-stimulatory signals to T cells through CD28, this observation could indicate that the decreased suppression found with increased MFI of CD86 was not due to the immune escape of responders as a result of enhanced co-stimulation since, in that case, it would be expected to find a similar relation of CD80 MFI to the potency of suppression.

Interestingly, we also found that the MFI of CD83 was inversely correlated with the suppression of Tcon, but not of $CD8^+$ cells. While this has yet to be validated in human studies, it has been described that Treg may down-regulate CD86 and MHCII expression by increasing mRNA expression of MARCH1 on DC, which leads to the degradation of the β-chains of those molecules[186]. CD83, in turn, inhibits the association of MHCII with MARCH1, thus preventing their degradation. While Chattopadhyay *et al.* have shown that iTreg may suppress the upregulation of CD83 in DC via an IL-10 dependent mechanism[186], in our study, higher initial MFI of CD83 on moDC was enough to correlate with decreased suppression of Tcon. We propose that APC with more CD83 are more resistant to the degradation of MHCII, and thus are more potent stimulators of Tcon proliferation, allowing for them to escape suppression by Treg. Although CD83 expression on APC has been shown to modulate immune responses by increasing IFN-γ production on tumor infiltrating lymphocytes and $CD8^+$ cells[222], in our work, higher initial CD83 expression was unrelated to $CD8^+$ cell suppression. This observation somewhat reinforces our theory that the initial expression of CD83 is relevant for the maintenance of HLA-DR expression in moDC, which would only affect the stimulation of Tcon and not of $CD8^+$ cells. Surprisingly, we found no relation between the potency of suppression by eTreg and the initial MFI of HLA-DR expression. However, in future experiments, it would be interesting to evaluate whether the expression of HLA-DR at the beginning and at the end of suppression cultures is related to the initial MFI of CD83, as well as to ascertain if blocking HLA-DR in moDC would abrogate the significance of the correlation between the potency of suppression of Tcon and the initial MFI of CD83.

Interestingly, when we identified each moDC donor on scatter plots of suppression *vs.* CD86 and suppression *vs.* CD83, we detected that moDC from DC-matched 3rd party donors were among the moDC with lowest expression of CD86 and CD83. On the contrary, within fully mismatched 3rd party donors, most of the moDC had high CD86 expression. Lastly, there were moDC from the Original donors that were more similar in CD86 and CD83 expression to moDC from DC-matched 3rd party donors, while others were more similar to moDC from fully mismatched 3rd party donors. Yet, responses to moDC from the Original donors were always significantly more suppressed than responses to moDC

from fully mismatched 3rd party donors. Therefore, the differences in potency of suppression by eTreg could not be entirely attributed to the effect of APC phenotype, as in that case we would not find the responses to the Original donor to always be the most suppressed.

In conclusion, we propose that the degree of suppression mediated by Ag-sp eTreg may result from a combination of stimuli from at least three axes of interaction. We suggest that Treg may suppress responders in a non-specific way, as seen in SA with mismatched 3rd party donors, as a result of T-T cell interactions only. This could be indicative of the "basal" suppression Treg are capable of. Thus, we recommend that specificity should be evaluated by comparing the suppression of responses to the Original donor or MHC-matched donors with the suppression of responses to the mismatched 3rd party donor. Nevertheless, it should be kept in mind that even when responses to MHC-matched donors are more suppressed than those to a mismatched 3rd party donor, it is still possible that this suppression in not specific. We propose that the degree of suppression by eTreg is also mediated by a) responder-APC interactions, where the degree of co-stimulation (through CD86) provided by moDC may influence the potency of suppression by allowing the responders to escape suppression, and b) Treg-APC interactions, where Treg may detect Ag-MHC complexes and, according to the affinity of their TCR, possibly trigger distinct mechanisms of suppression.

This way, responders that would normally escape suppression in result of high co-stimulation from moDC can be specifically suppressed by Treg, as shown in SA with moDC from the Original donor. Since there were hints of different mechanisms being employed by eTreg for the suppression of responder's proliferation, we proceeded to evaluate if some of this suppression was mediated by cell-extrinsic mechanisms. Additionally, we wanted to ascertain whether the presence of Treg would not only decrease the proliferation but also modulate the production of cytokines by responders, as it has been described that Treg may impair cytokine transcription on T_{eff}[170].

Commercially-available multiplex kits have the great advantage of allowing the quantification of several cytokines using only a small volume of sample. This way, it was possible to simultaneously quantify the concentration of a wide range of cytokines in the supernatant of each well from SA. However, it has some drawbacks, which were taken into consideration. The first drawback is that this technique does not allow for us to identify which cells are producing the detected cytokines. However, by using the supernatant from wells with responders and APC only (in the absence of Treg) as controls, and by comparing among controls, we could estimate whether cytokines seemed produced by responders, APC or Treg. Secondly, some cytokines can be found in soluble or membrane-bound forms, such as TGF-β[141]. While the former would be detected in these assays, the latter would not. Hence, the lack of detection of a cytokine may not indicate that it is not being produced, only that it was not detected with this technique. Similarly, it may be the case that cells are producing cytokines that are either under the limit of detection or are not being secreted into the supernatant. The quantification of mRNA would allow for a wider picture of these mechanisms, but would require for cells to be sorted after SA in order to separate Treg from responders and APC, which was not feasible with such minute numbers of cells. Flow cytometry would also allow to pinpoint the producing cells, but the detection threshold for cytometry is much higher, thus the quantification of cytokines would be much less sensitive. Besides, it would be impossible to quantify all of the tested cytokines simultaneously on each sample, like it was

done here. At last, performing cytometry and/or mRNA quantification studies for all of the tested cytokines would require a much higher number of cells than those available. For all of these reasons, this method was the most adequate candidate to assess this plethora of cytokines in an initial screening. Nevertheless, in the future, it would be interesting to select some target cytokines and assess not only which cells are producing them, but also what is the frequency of cytokine-secreting cells within each cell type. Still, it would be likely that the production of some cytokines would not be quantifiable by flow cytometry, due to their reduced concentration. The quantification of mRNA production by Treg and responders would complement the results obtained by flow cytometry and multiplex analysis, as well as provide further information in regards to the functional phenotype of these cells during SA, if the expression of transcription factors is analysed in parallel with that of cytokines.

Starting by the wells from SA with Tcon as responders, it could be seen that the concentration of IL-2 was significantly lower than in the control wells in the presence of eTreg. It has been widely described that one the mechanisms of suppression by Treg is to capture IL-2, decreasing its concentration, which in turn suppresses Tcon proliferation[169,170]. Additionally, these reports have shown that both fresh and pre-activated Treg suppress mRNA production of IL-2 by Tcon, even in the presence of exogenous IL-2. As Tcon and Treg consume IL-2, upon suppression of further production by Tcon, it seemed fitting that in our results the concentration of IL-2 was reduced in wells containing eTreg. However, our data seem to contradict what was described by Oberle *et al.*[170], because the concentration of IL-2 was not reduced in wells with fresh Treg. It should be noted that in our assay the ratio of Treg:T_{eff} was of 1:5, while Oberle *et al.* performed the experiments at a ratio of 1:1 Treg:T_{eff}. As we (data not shown) and the authors have verified, fresh Treg are not as suppressive of Tcon proliferation as pre-activated Treg, thus it is possible that they are equally less suppressive of IL-2 production. Thus, it would be required to have higher numbers of fresh Treg than of eTreg in order to reach similar levels of suppression of IL-2 secretion. The kinetics of TNF-α production and suppression by Treg were similar to those of IL-2. This was in accordance with Oberle *et al.*, who claimed that Treg suppressed TNF-α concentration in co-cultures[170]. However, in a paper by Barsoumian *et al.*, it was shown that while IL-2 concentration was decreased by the presence of fresh Treg and APC with soluble aCD3 or with plate-bound aCD3, the concentration of TNF-α remained unchanged (even at 1:1 Treg:T_{eff})[223]. Since the type of stimulation provided to cells during these SA was different, it is hard to draw direct comparisons between these studies and ours.

Interestingly, the production of IFN-γ by Tcon seemed restricted to conditions where there were any MHC-matches between the donor of APC (namely, Original donor and Treg-matched 3rd party) and of responders (Treg donor). In the conditions where there was production of IFN-γ, its concentration was also decreased in the presence of Treg. Previous studies have shown that Treg could suppress IFN-γ mRNA production on Tcon[170], yet murine studies suggested that this did not impair lineage commitment of Tcon into Th1[185]. Since it seems that Tcon were polarized into a Th1 phenotype upon stimulation by our APC, the effect of these Ag-sp eTreg on the control of inflammation still needs to be verified *in vivo*. Nevertheless, the fact that eTreg were capable of suppressing IL-2, TNF-α and IFN-γ production by Tcon, as well as their proliferation, may be taken as a good indication. Also, there are reports of IL-2-expanded Ag-sp Treg being capable of specific suppression in *in vivo* studies already[125].

Importantly, in our assays, the expansion milieu of Treg did not seem to affect their mechanisms of suppression.

The production of IL-2 and IFN-γ by CD8+ cells was detected too, which was similarly suppressed in the presence of eTreg. Chinen *et al.* have shown that IL-2 capture by Treg was essential for the suppression of murine CD8+ cell proliferation and cytokine production, as these responders were more sensitive to IL-2 induced stimulation than Tcon[169]. Furthermore, it had already been described in an older study that IL-2 responsiveness was a key factor for IFN-γ production by CD8+ cells[205]. Therefore, not only is our data in accordance with these reports, but it may also provide a more encompassing view of the cytokines involved in suppression by Treg.

Ipso facto, due to the contradictory reports available on the role of IL-10 in suppression by Treg, it was interesting to find that the concentration of IL-10 was higher in the supernatant of wells with Tcon and APC only, and seemed suppressed by the presence of Treg. On the one hand, Oberle *et al.* have shown that while the concentration of IL-10 was not decreased in co-cultures of Tcon and responders, the production of IL-10 mRNA was decreased on Tcon. Moreover, the addition of anti-IL10R did not affect suppression by Treg[170]. On the other hand, Baecher-Allan *et al.* have shown that activated HLA-DR- Treg could not only produce IL-10 but also mediate suppression through this cytokine[138]. While these studies indicated that eTreg may be able to produce IL-10, Litjens *et al.* have shown that this production may be affected by the type of APC present, as the authors only found IL-10 production in aCD3/aCD28 expanded Treg[76]. Looking at the current literature, it seems that IL-10 is usually found only in the supernatant of SA with iTreg[94,186], while studies with nTreg usually assess the production of this cytokine by Treg in flow cytometry assays or by mRNA quantification[76,138]. Similarly to studies with iTreg, when IL-10 is produced by Tcon during SA, it may still be found in the supernatant[138]. Finally, it has been suggested that Treg only produce IL-10 in response to strong TCR stimulation (measured by the increased expression of ICOS)[140], thus different results may derive from different modes of stimulation and activation status of Treg. Interestingly, we found that the concentration of most of the tested cytokines was lowest when aAPC beads (aCD2/aCD3/aCD28) were used instead of APC. This observation could also mean that aAPC may not be the most adequate APC to characterize Treg suppression mechanisms, which would have a severe impact on what little knowledge we have on the function of Treg, as most studies of suppression so far were performed using some type of beads as aAPC. For example, it has been described that strong pro-inflammatory stimulus may polarize Treg into IL17-producing Treg[50,51] and, in our studies, IL-17 concentration was only increased in co-cultures of Treg expanded with the highest concentration of IL-2 with beads. What remains to be ascertained is whether the cytokine secretion profiles detected in Treg stimulated with aAPC will be replicated in any *in vivo* situation, or if these extreme conditions serve only as mechanistic tests of Treg function and are of no value in the clinical field.

Of note, in their study of Treg subpopulations, Baecher-Allan *et al.* had tested different combinations of beads for the stimulation of IFN-γ, IL-4 and IL-10 production by Tcon and subsequent suppression by Treg[138]. In that report, IFN-γ was more suppressed by Treg in the presence of aCD3/aCD2 beads than in the presence of aCD3/aCD28 beads. Also, IL-10 and IL-4 secretion did not seem suppressed by Treg. In fact, it seemed that HLA-DR+ Treg were also producing IL-10 and IL-4 in

the presence of aCD3/CD28 and aCD3/aCD2 beads. While we did not find an increase in the production of most cytokines in wells with beads, the concentration of IL-4 did seem increased in wells with fresh and expanded Treg, particularly in the presence of moDC from the Original donor, and in wells with eTreg in the presence of moDC from DC-matched donor. Conversely, the concentration of IL-5 seemed increased in wells with eTreg in the presence of moDC from the mismatched 3rd party donor. Since these observations were applicable to SA with Tcon and CD8+ cells, it may be speculated that Treg modulate suppression through the secretion of IL-4 and IL-5.

We further suggest that these cytokines may play opposing roles as, in the presence of Treg, IL-4 was increased in conditions where there was more suppression and IL-5 was increased in conditions where there was less suppression. In a very interesting murine study, Verma *et al*. have described that Ag-sp Treg activated by IL-2 during short-term cultures expressed more il-5 mRNA than fresh or IL-4 activated Treg[125]. Additionally, IL-4-activated Treg expressed more il-5rα mRNA, which the authors proposed was a marker of Ag-sp Treg. Nevertheless, both IL-2 and IL-4-activated Treg specifically inhibited the rejection of an allograft from a specific donor. Moreover, the expression of il-4 mRNA was similar in fresh and activated Treg, however, activated Treg had increased expression of il-4rα. Besides, Treg proliferated more in co-cultures with allo-APC and IL-4 than with IL-5. In a more recent study that included human Treg, it was shown that IL-4 increases il-5rα mRNA expression in the presence of alloantigen, resulting in Ag-sp Treg that may later be expanded by IL-5[224]. Thus, we hypothesize that in our Ag-sp settings, suppression mediated by eTreg involves IL-4, which may increase Ag-sp eTreg proliferation, hence specifically increasing suppression. Conversely, in a non-specific SA setting, eTreg produce IL-5, which on its own has been shown to promote allograft survival by inducing tolerance[224], thus explaining the occurrence of non-specific suppression. However, the requirement for IL-4 and IL-5 in specific suppression by eTreg and the source of these cytokines would have to be validated in future studies, using neutralizing mAb and either cytokine mRNA quantification or flow cytometry analysis of Treg and responders, respectively.

The last methodology we used to characterize expanded Treg, as well as to ascertain some of the possible mechanisms involved in their function, was the acquisition and analysis of data from a 12-color flow cytometry panel. To do so, in all independent experiments, we separated an aliquot of each eTreg prior to SA, which was then used for cytometry staining. In addition to CFSE, this panel included T cell (CD3 and CD4) and Treg identification markers (CD25, CD127 and Foxp3), as well as Treg-associated molecules (CTLA-4, PD-1) and activation markers (CD39, HLA-DR, 4-1BB and CD40L) to provide further insight on the function of Treg. Upon generating such a large amount of data for each sample, we felt that multidimensional analysis using pseudo-randomized algorithms would allow for a more thorough characterization of phenotypes, as this way the expression of all markers on all cells (organized by nodes or clusters) could be easily visualized. Since activated Tcon upregulate most of these markers upon activation, at present, no definite way to distinguish between the phenotype of human natural Treg, induced Treg and activated Tcon has been described. Although, so far, our results indicated that Treg expanded in serum-free conditions using our protocol were mostly clear of contaminating Tcon, we felt that this type of analysis could provide further indication of the purity of eTreg. Thus, we started by comparing the phenotypes found in expanded Treg to those of expanded

Tcon, and found that cells from Tcon and Treg samples were not allocated to the same clusters. While by conventional flow cytometry analysis it could be very hard to discriminate between activated Tcon and expanded Treg, especially by visualizing only two markers at a time, by applying FlowSOM analysis there were strong indications that our eTreg samples were mostly free from contaminating activated Tcon. Although this analysis did not highlight a particular combination of markers that could be used to discriminate between eTreg and eTcon, it may prove very useful in the validation of GMP-approved protocols for clinical applications.

Interestingly, we also found that the range of phenotypes presented by fresh and expanded Treg could be organized into 5 distinct clusters that presented different combinations of marker expression, which could represent Treg subsets. More importantly, we were able to show that Treg expansion altered Treg phenotype. Briefly, it could be seen that fresh Treg were enriched in cells allocated to the two subsets with the lowest expression of CTLA-4, PD-1, HLA-DR, 4-1BB and CD40L, and the main difference between these subsets seemed to be their converse expression of CD39. CD39 expression on fresh Treg has been recently described as a marker for highly suppressive Treg[134]. However, no cells were allocated to this subset (Cluster 4) after expansion. On the contrary, there was a similar frequency of cells allocated to the subset with the lowest expression of all tested markers within fresh and expanded Treg. If we hypothesize that CD39^{+} cells are more susceptible to activation than CD39^{-} cells, this would mean that it would lead to an upregulation of other activation markers and thus the same cells would be allocated to a different subset after expansion. In fact, we showed that expanded Treg had higher frequency of cells allocated to subsets with higher expression of activation markers, and it could be seen that the increased concentration of IL-2 during expansion did not change the range of phenotypes presented by cells, but yet altered the frequency of cells allocated to each subset. Answering Edozie *et al.*'s plee, that claimed "little attention has been paid to subpopulations"[126], we tried to focus on the phenotipic characteristics of each subset, and speculate whether these phenotypes could be related to distinct suppression mechanisms and specificity of eTreg. We already mentioned that CD39 expression could be linked to increased suppressive function of fresh Treg. Another study has shown that, after expansion, Treg that are CD39^{+} were more suppressive than their couterparts[117]. It could be hypothesized that these cells would, in our analysis, be allocated to Cluster 5, which not only has high expression of CD39 but also of CTLA-4, HLA-DR, CD40L and 4-1BB. At the same time, the highest expression of CTLA-4 was found on Cluster 3, where Treg have lower expression of CD39, but higher expression of PD-1 and CD40L than in Cluster 5. CTLA-4 is known to interact with CD80/CD86 on DC and modulate Treg homeostasis[54] and function[225]. Treg that were expanded with 100U/mL of IL-2 had more cells with high expression of CTLA-4. If an increased expression of CTLA-4 means that Treg are capable of capturing or competing for more CD86 on APC, this could justify why the correlation between the potency of suppression by these cells and the expression of CD86 was less negative than the same correlation using data from SA with Treg expanded with 10U/mL of IL-2. Similarly to CTLA-4, PD-1 has been reported to modulate Treg homeostasis. It has been shown that increased IL-2 increases PD-1 expression on Treg, although these cells quickly became pro-apoptotic[128]. Thus, it could be speculatd that cells allocated to Cluster 3 would not be as stable as those expressing a phenotype of Cluster 5, which also expressed high CTLA-4, but

had lower expression of PD-1. Generally speaking, Treg that were expanded with higher concentration of IL-2 had more cells allocated to subsets with high expression of activation markers (Cluster 3 and 5), namely CD40L and 4-1BB, hence leading us to conclude that those cells were more activated than those expanded with lower concentration of IL-2. Indeed, it has been shown that IL-2 upregulates 4-1BB expression on Treg, which in turn increases suppression by Treg[214]. Interestingly, Cluster 3 and Cluster 5 were also the clusters with the highest expression of HLA-DR, which has been associated with early contact-dependent suppression and lower IL-10 secretion[138]. Overall, this analysis provides some clues as to the higher potency of suppression displayed by Treg expanded with higher concentration of IL-2. Of note, similar clusters were identified in FlowSOM and k-means analysis, performed independently, which seems to validate the robustness of these techniques as visualization tools.

We believe that as technology advances and more data is generated from each experiment, it is of great importance to explore novel ways for big data visualization. By doing so, we were able to identify 5 novel Treg subsets that had not been described before, and that may be related to Treg function. Particularly, within expanded Treg, we found two subsets with an activated phenoype, and different expression of PD-1 and CD39. The separation of eTreg sub-populations according to the expression of those markers could be a suitable strategy to characterize the suppressive function of these subsets, provided there are enough cells. More importantly, this type of multidimensional analysis may prove very useful for clinical applications. In the field of HSCT and GvHD, this would allow for us and others to have a more comprehensive picture of the phenotypes present during immune allo-reconstitution after HSCT, as well as of the phenotypes present in Treg infusions for GvHD treatment. Ultimately, these techniques may lead to the discovery of new indicators of treatment outcome, and to the establishment of new inclusion/exclusion criteria for the isolation of Treg before infusion.

In conclusion, we feel that our primary goal of establishing a reproducible protocol for *ex vivo* Ag-sp Treg isolation and expansion in serum-free conditions was achieved in this work. We were able to show that Treg can be expanded through direct and indirect allo-recognition, as well as suppress the proliferation of responses to allo-Ag presented direct or indirectly. Furthermore, by evaluating the specificity of suppression by expanded Ag-sp Treg, we are now able to propose that suppression by Ag-sp Treg depends on: a) Treg potency, where higher potency of suppression may implicate less specificity in Tcon suppression; b) Co-stimulation provided by APC, where higher co-stimulation may lead to reduced suppression of Tcon and $CD8^+$ cells; and c) Interaction between TCR-Ag-MHC complexes, where the affinity of Treg TCR to Ag-MHC in APC may lead to specific suppression of responders.

Finally, by providing an in-depth look at the phenotypes expressed by Treg, we concluded that the phenotype of *ex vivo* Treg is altered as a result of expansion. Conversely, the use of different concentrations of IL-2 during expansion does not change the range of phenotypes present after expansion, yet influences the frequency of cells allocated to each phenotypic subset. Additionally, if the bulk population of eTreg is more enriched for cells of a particular phenotype, it may impact their suppressive function.

One assay we have yet to perform is the evaluation of the Treg TCR diversity before and after expansion. The analysis of the diversity of the Treg TCR will allow for us to validate if Treg expanded by this protocol present a decreased TCR diversity as a result of Ag-sp expansion, as would be expected. Since we already have DNA samples stored from the experiments described in Chapter 2, this evaluation would first be performed by next-generation sequencing of those samples. In the future, this analysis may be performed on a single-cell level through deep sequencing, which would allow for us to link individual TCR sequences with functional phenotypes. This methodology has been described in a very interesting study of tissue infiltrating lymphocytes[226], where for each T cell, the expression of multiple functional genes (e.g. cytokines and transcription factors) was analysed in parallel to the sequencing of TCR chains.

In the future, we would like to perform a few more studies of the mechanisms involved in the specificity of suppression by Ag-sp eTreg, which will be described next. We will be focusing on the mechanisms involved in the determination of specificity and subsequent suppression of Tcon and CD8$^+$ cells. If possible, the SA will be performed with the same range of APC, namely, moDC from the Original donor and moDC from differently MHC-mismatched 3rd party donors. Since we found that Treg expanded with 100/mL of IL-2 were more enriched in activated subsets, we plan on performing these studies only on Treg expanded with that concentration of IL-2, in the presence of allo-moDC.

Firstly, we intend to perform blocking assays where we would evaluate the role of the TCR in the specificity of suppression by Treg. There are plenty of murine studies assessing the requirement for TCR signalling in Ag-sp Treg-mediated suppression, using TCR transgenic[106] or TCR-ablated[56] mice. However, studies of the role of TCR in Ag-sp suppression by human Treg are more scarce, due to the impossibility of blocking the TCR without triggering Treg activation. In order to test the role of TCR signalling in our SA setting, our plan is to use mAb to selectively block HLA-I or HLA-II on moDC. However, there is a risk that this will result in reduced basal proliferation of the responders, as Treg proliferation has been shown to be affected by HLA-II blockade. Case in point, Sagoo *et al.* have demonstrated that the addition of HLA-DR-blocking Ab prevented the activation of Treg during co-cultures with HLA-DRß1-mismatched DC, suggesting that Treg activation was dependent on the recognition of intact allogeneic HLA molecules[82]. Similarly, Litjens *et al.* have demonstrated that by blocking HLA-II in moDC, Treg expansion was impaired[76]. Considering that we found the suppression of CD8$^+$ cells by eTreg to be specific, we intend to perform these studies using CD8$^+$ cells as responders. That way, the blockade of HLA-I in moDC is expected to affect only CD8$^+$ cells proliferation, while a blockade of HLA-II in moDC may affect the specificity of suppression by eTreg.

In parallel to these conditions, it would also be interesting to block CTLA-4 on Treg using anti-CTLA-4 mAb or CD86 on moDC using anti-CD86 mAb, to see if the suppression of Tcon and CD8$^+$ cells is differentially affected. This study is somewhat similar to a recent study of the role of CD28/CTLA-4 signalling on Treg homeostasis[54], but applied to the analysis of suppression by eTreg. However, we plan on using anti-CD86 instead of CTLA-4-Ig (which blocks CD80 and CD86), as it has been previously demonstrated to selectively block CD86 on human moDC[175]. Since CD86 was inversely correlated with the potency of suppression of Tcon and CD8$^+$ cells, this type of blockade could allow for us to ascertain

whether the reduced suppression with increased initial MFI of CD86 is a result of a limit in downregulation of CD86 by CTLA-4 on Treg and/or of immune escape of responders.

In this setting, it is unlikely that Treg are affected by a blockade of CD86, as Ito *et al.* have demonstrated that the proliferation of *ex vivo* ICOS$^+$ (activated) Treg was independent of CD80/CD86[140]. In fact, the authors suggested that ICOS$^+$ Treg decreased CD86 expression on DC in an IL-10 dependent way. Hence, besides measuring the suppression of proliferation in these conditions, we also plan on quantifying the expression of CD86 on moDC before and after SA. Finally, to assess if the downregulation of CD86 is mediated by CTLA-4, IL-10, or both, we plan on having one SA condition where CTLA-4 is intact, yet anti-IL-10 is added, to verify if the expression of CD86 is restored. Similarly, a study on the role of IL-10 has been performed in the past, where it was shown that in the presence of anti-IL-10, iTreg were not capable of down-regulating CD86 nor CD83[186]. We have shown here that CD83 was inversely correlated with the suppression of Tcon, which is possibly due to the role of CD83 in the prevention of HLA-DR degradation in moDC. Besides assessing the effect on anti-IL-10 in SA cultures, we intend to quantify the expression of HLA-DR before and after SA. The quantification of HLA-DR on moDC in control conditions and conditions with anti-CTLA-4 or anti-IL-10 can be performed simultaneously with the quantification of CD86, and may provide a better insight into the mechanisms of suppression mediated by eTreg.

Nevertheless, it must be kept in mind that we did not find IL-10 in the supernatant of SA, thus other cytokines may be mediating the downregulation of CD83 and, consequently, increasing the degradation of HLA-DR on moDC. Therefore, during the acquisition of data from SA cultures, we plan on sorting Treg (based on the expression of CD3, CD4 and CFSE) to quantify their mRNA expression of IL-10, IL-4 and IL-5 on each condition.

Finally, HLA-DR expression on Treg has been shown to provide T-T cell stimulation[138], and has also been described to affect the Treg suppression only in a specific setting[192]. Since there is increased HLA-DR expression on Treg, it would be very interesting to see the effect of an HLA-II blockade on the specificity of suppression by eTreg. Ideally, all of these blockade experiments would be performed in parallel using the same eTreg, and then repeated for three independent experiments. To do so, it may be necessary to select only one or two Treg:T_{eff} dilutions, in order to have sufficient Treg for all of these conditions. We plan on using 1:10 and 1:50 Treg:T_{eff}, which would reflect the mechanisms of suppression by eTreg in high or low numbers, respectively.

While these studies may provide a better comprehension of the mechanisms involved in Treg suppression, the fact that the safety and efficacy of polyclonal Treg infusions are already being assessed in the treatment of GvHD by our group[34] and others[66] raised the concern that the GVL effect may also be ablated in the process. Thus, we believe it is of great urgency to perform functional studies on Ag-sp Treg, to ascertain whether these cells would be more suitable for the treatment of GvHD without impairing the GVL effect. So far, these studies have only been performed in mice[31], possibly due to the limited availability of suitable human samples. Because our group is currently part of a prospective study that focuses on cGvHD development after HSCT[26], we are in a privileged position where we can biobank samples from patients at the time of the diagnosis and during remission, as well as samples of the HLA-matched donor-derived grafts. Using these samples, we plan to isolate donor-

derived Treg and expand them in the presence of recipient-derived moDC, using the protocols defined in this work. This would result in recipient-sp Treg, which can then be used to perform SA with donor-derived *ex vivo* responders, in the presence of recipient-derived moDC, recipient-derived skin DC, or donor-derived moDC loaded with recipient's tumour lysates. This way, we will be able to ascertain the potential of recipient-sp Treg to suppress responses to the recipient's hematopoietic mHA and skin mHA, which can mimic the suppression of cGvHD, and to the recipient's tumour Ag, which can mirror the suppression of GVL *in vivo*. Since, in a cGvHD setting, recipient-sp Treg are expected to find donor-derived activated T cells, it would also be interesting to expand donor-derived Tcon in the presence of the same recipient-derived moDC, and to assess to potency of eTreg to suppress these responders in the presence of the cGvHD and GVL-mimicking APC.

Last but not least, we believe another project could be derived from the multidimensional analysis assays, where data from Treg infusion therapies would be analysed. The goal would be to analyse the infused Treg and assess if the frequency of cells allocated to each cluster could be an indicator of the outcome of these therapies. Furthermore, if the frequency of cells allocated to a particular cluster is found to be a strong indicator of the therapy outcome, it may be interesting to select a combination of two (or more) markers to partially define that cluster and perform FACSorting of donor Treg based on those markers. This way, the sorted cells will be enriched in cells expressing the phenotype of the cluster of interest. By evaluating their function and mRNA expression, we may be able to recommend whether the expression of the selected markers should be used as exclusion/inclusion criteria in future therapies.

Overall, we believe the work developed in this project is of great interest for the scientific community, as it tackles several questions about the function of Ag-sp eTreg that had not been explored before, such as the specificity of suppression by indirectly expanded Treg and the potential of Ag-sp eTreg to suppress responses to differently MHC-mismatched APC donors. More importantly, we believe the work described here provides the foundation for subsequent studies on very relevant topics, such as the mechanisms required for specific suppression of responses by eTreg, and how can we best expand and select Ag-sp Treg that are capable of suppressing cGvHD without compromising the GVL effect.

SCIENTIFIC OUTPUT

- Poster and oral presentation at Immunology 2019 in San Diego, 9-13th May 2019
- Poster presentation at FEBS Immunology Summer School in Hvar, 2018
- Paper (in preparation): "*Phenotypic and functional characterization of expanded antigen-specific regulatory T cells*"

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SUPPLEMENTARY DATA

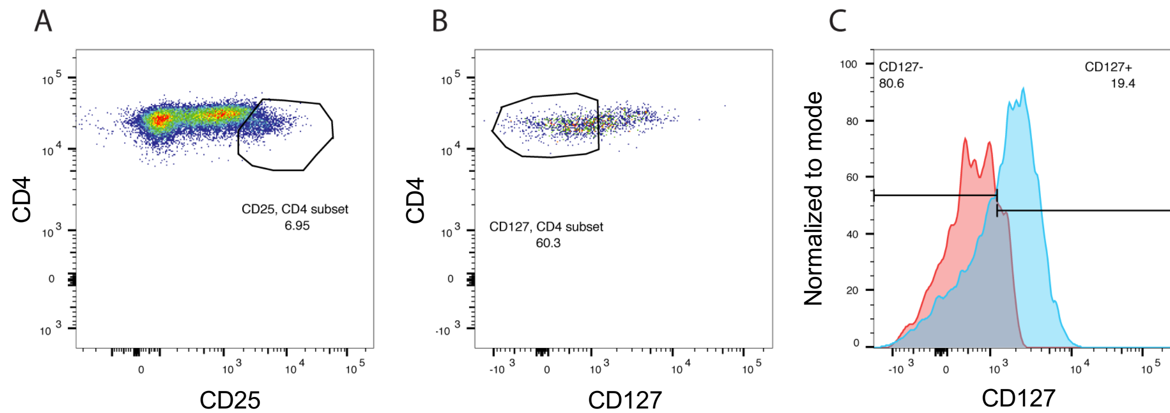

Figure S-1: Strategy for validation of CD25^{bright} CD127^{low} gate for Treg FACSorting.
The Treg gate was verified by comparing the frequency of cells within a CD25^{bright} CD127^{low} gate with the frequency of cells within a (A) CD4^{+}CD25^{bright} gate followed by a (B) CD4^{+}CD127^{low} gate. (C) The cut-off for CD127 expression was set on overlay histograms of CD127 expression on CD4^{+}CD25^{bright} cells (red histogram) and CD4^{+}CD25^{-} cells (blue histogram).

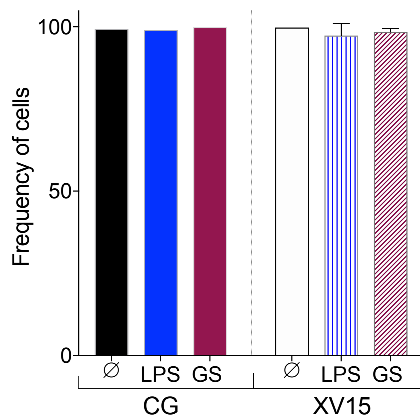

Figure S-2: Effect of media and activation cocktail on moDC phenotype.
Frequency of cells co-expressing CD86 and HLA-DR, within live cells.

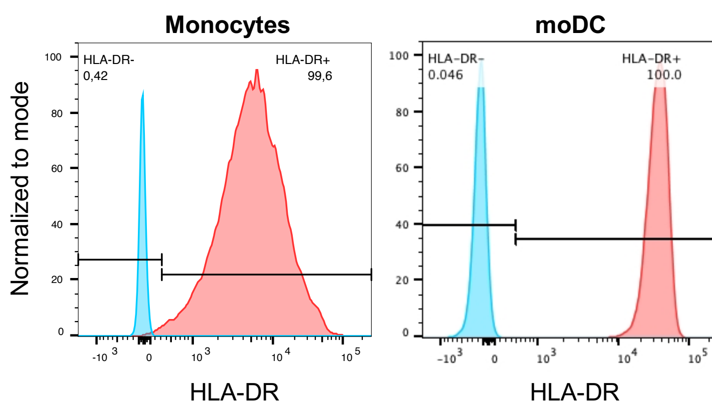

Figure S-3: Expression of HLA-DR on monocytes and moDC.
Overlay histograms of HLA-DR expression on unstained (blue histograms) *vs.* stained (red histograms) samples of monocytes and moDC.

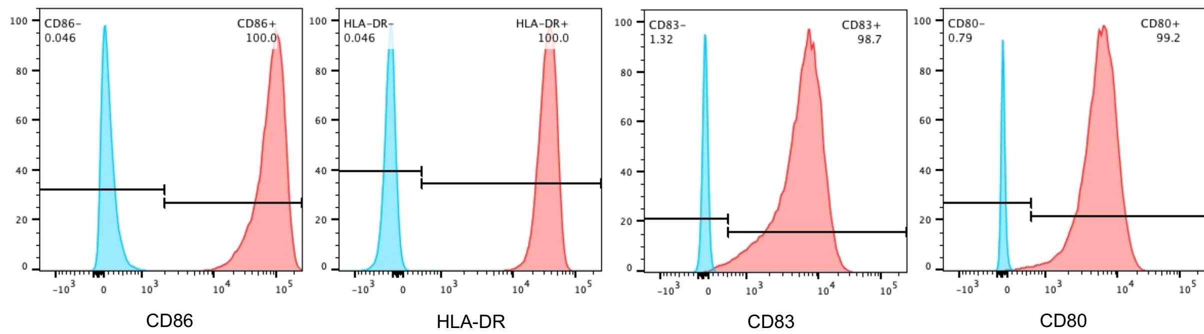

Figure S-4 : Expression of surface markers on unstained moDC.
Overlay histograms of CD86, HLA-DR, CD83 and CD80 expression on unstained (blue histograms) *vs.* stained (red histograms) samples of moDC.

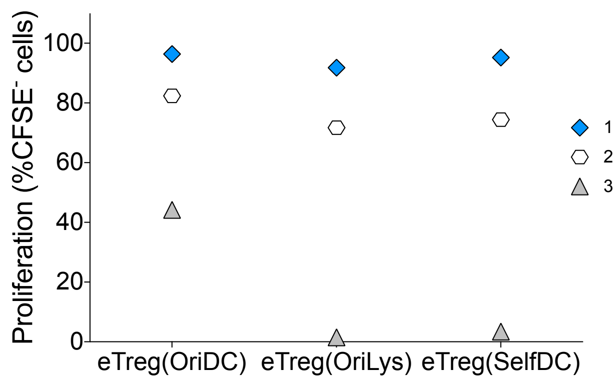

Figure S-5: Proliferation of Treg on independent experiments
The frequency of CFSE⁻ Treg (proliferated) at the end of expansion cultures is represented by different symbols for each independent experiment. Within each experiment, the proliferation of Treg in the presence of directly presented allo-Ag by OriDC, indirectly presented allo-Ag by OriLys and in the presence of self-moDC is shown.

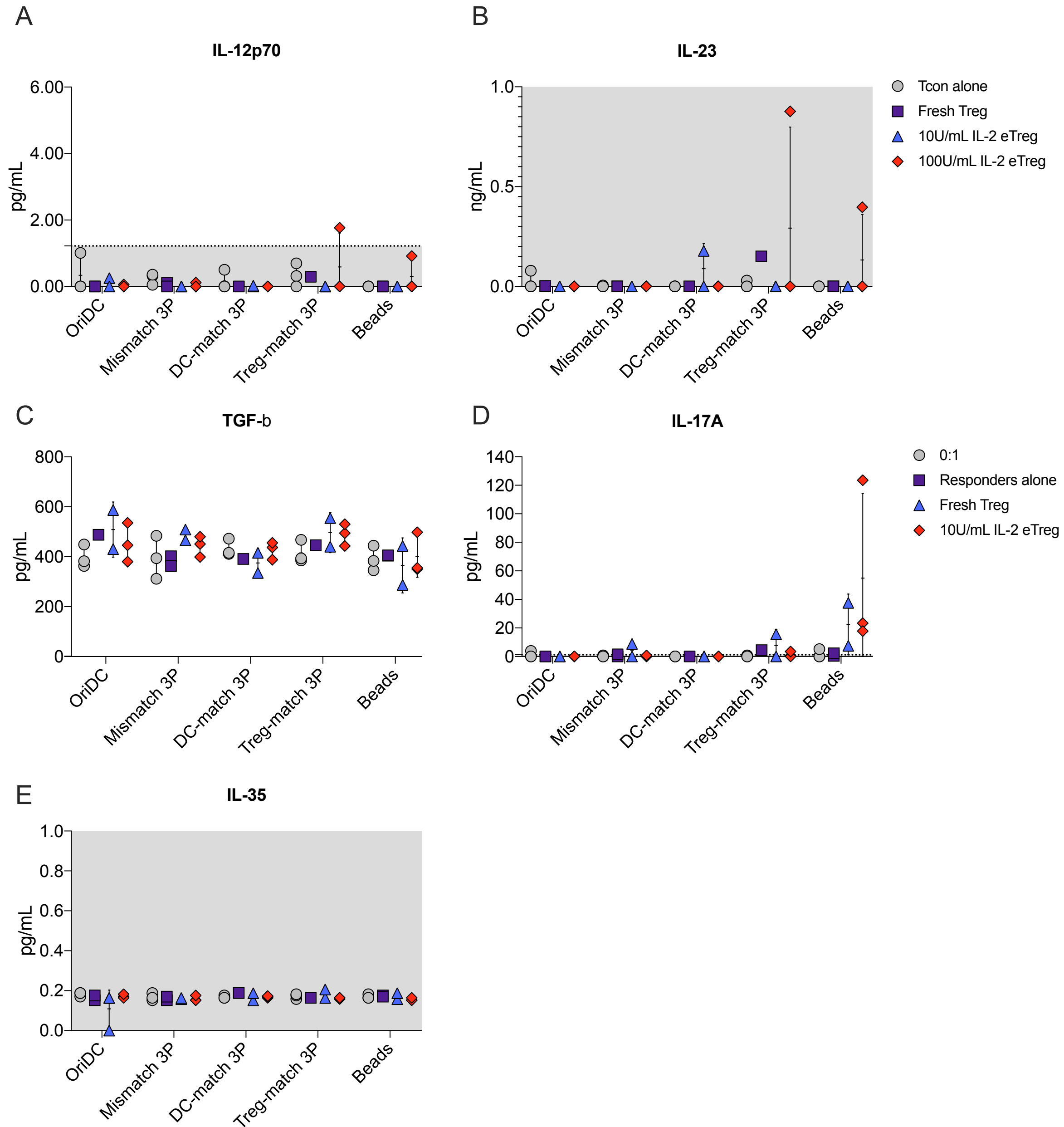

Figure S- 6: Quantification of DC-associated and Treg-associated cytokines on SA with Tcon
*(*A-E) Concentrations found in each independent experiment are represented by symbols, with average and standard deviation values. Values below the detection limit of the kit were extrapolated and plotted in the grey area. For each cytokine, data was organized by APC, according to the presence of moDC from the Original donor (OriDC), from a mismatched 3rd party donor (Mismatch 3P), from a donor partially matched to the Original donor (DC-match 3P) or from a donor partially matched to the Treg donor (Treg-match 3P). Supernatant from wells with Tcon and APC only (Tcon alone, grey circles), Tcon with APC and freshly isolated Treg at 1:5 Treg:T_{eff} (Fresh Treg, purple squares), Tcon with APC and Treg expanded by 10U/mL of IL-2 at 1:5 Treg:T_{eff} (10U/mL IL-2 eTreg, blue triangles) and Tcon with APC and Treg expanded by 100U/mL of IL-2 at 1:5 Treg:T_{eff} (100U/mL IL-2 eTreg, red diamonds) was quantified. DC-associated cytokines include IL-12p70 (A) and IL-23(B). Possible Treg-associated cytokines are TGF-β (C), IL-17A (D) and IL-35 (E). *Statistical analysis was performed by multiple t-tests, by the Holm-Sidak method. Differences were considered statistically significant when p<0.05. N=3 except for Fresh Treg conditions, where n=2.*

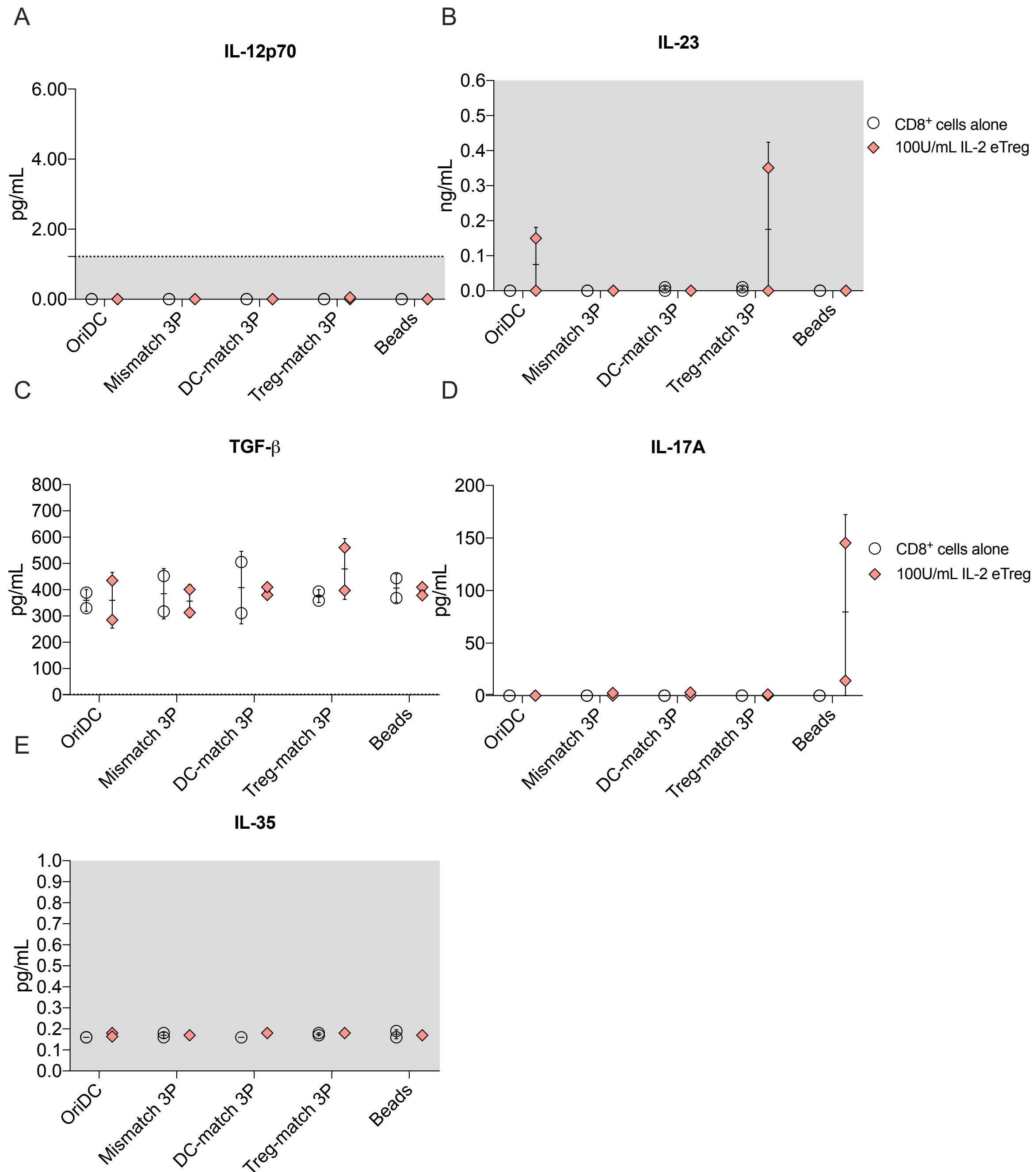

Figure S- 7: Quantification of DC-associated and Treg-associated cytokines on SA with CD8⁺ cells

(A-E) Concentrations found in each independent experiment are represented by symbols, with average and standard deviation values. Values below the detection limit of the kit were extrapolated and plotted in the grey area. For each cytokine, data was organized by APC, according to the presence of moDC from the Original donor (OriDC), from a mismatched 3rd party donor (Mismatch 3P), from a donor partially matched to the Original donor (DC-match 3P) or from a donor partially matched to the Treg donor (Treg-match 3P). Supernatant from wells with $CD8^+$ cells and APC only ($CD8^+$ cells alone, empty circles) and $CD8^+$ cells with APC and Treg expanded by 100U/mL of IL-2 at 1:5 Treg:T_{eff} (100U/mL IL-2 eTreg, pink diamonds) was quantified. DC-associated cytokines include IL-12p70 (A) and IL-23 (B). Possible Treg-associated cytokines are TGF-*β* (C), IL-17A (D) and IL-35 (E). *Statistical analysis was performed by multiple t-tests, by the Holm-Sidak method. Differences were considered statistically significant when p<0.05. N=2.*

EXP.1	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*03:01 A*29:01	B*35:01 B*44:02	C*04:01 C*07:02	DRB1*07:01 DRB1*13:01	DQA1*01:03 DQA1*02:01	DQB1*02:02 DQB1*06:03
DC donor	A*24:02 A*26:01	B*14:02 B*44:03	C*02:02 C*04:01	DRB1*01:02 DRB1*07:01	DQA1*01:01 DQA1*02:01	DQB1*02:02 DQB1*05:01
3rd party donor	A*02:11 A*11:01	B*07:02 B*48:02	C*04:01 C*07:01	DRB1*09:01 DRB1*12:01	DQA1*03:02 DQA1*05:05	DQB1*03:01 DQB1*03:03
EXP.2	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*02:01 A*11:01	B*27:03 B*35:01	C*02:02 C*04:01	DRB1*08:01 DRB1*13:01	DQA1*01:03 DQA1*04:01	DQB1*04:02 DQB1*06:03
DC donor	*A*02:01* *A*24:02*	*B*07:02* *B*51:01*	*C*07:02* *C*14:02*	*DRB1*07:01* *DRB1*15:01*	*DQA1*01:02* *DQA1*02:01*	*DQB1*02:02* *DQB1*06:02*
3rd party donor	*A*02:11* *A*11:01*	*B*07:02* *B*48:02*	*C*04:01* *C*07:01*	*DRB1*09:01* *DRB1*12:01*	*DQA1*03:02* *DQA1*05:05*	*DQB1*03:01* *DQB1*03:03*
EXP.3	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*02:01 A*26:01	B*38:01 B*53:01	C*04:01 C*12:03	DRB1*04:01 DRB1*10:01	DQA1*01:01 DQA1*03:01	DQB1*03:02 DQB1*05:01
DC donor	A*11:01 A*24:02	B*35:01 B*35:03	C*04:01 C*12:03	DRB1*01:03 DRB1*03:01	DQA1*01:01 DQA1*05:01	DQB1*02:01 DQB1*05:01
3rd party donor	A*03:01 A*32:01	B*15:01 B*40:01	C*03:04 C*03:04	DRB1*04:01 DRB1*13:02	DQA1*03:02 DQA1*05:01	DQB1*03:01 DQB1*03:01
EXP.4	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*02:01 A*68:02	B*44:02 B*55:01	C*03:03 C*05:01	DRB1*04:06 DRB1*11:01	DQA1*03:02 DQA1*05:05	DQB1*03:01 DQB1*04:02
DC donor	A*02:01 A*30:01	B*08:01 B*44:02	C*05:01 C*07:01	DRB1*03:01 DRB1*04:02	DQA1*03:01 DQA1*05:01	DQB1*03:02 DQB1*03:02
3rd party donor	A*01:01 A*32:01	B*44:03 B*50:01	C*04:01 C*06:02	DRB1*01:01 DRB1*07:01	DQA1*01:01 DQA1*02:01	DQB1*02:02 DQB1*05:01
EXP.5	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*01:01 A*23:01	B*08:01 B*44:03	C*04:01 C*07:01	DRB1*03:01 DRB1*04:03	DQA1*03:01 DQA1*05:01	DQB1*02:01 DQB1*03:04
DC donor	A*02:01 A*03:01	B*07:02 B*13:02	C*06:02 C*07:02	DRB1*13:01 DRB1*15:01	DQA1*01:02 DQA1*01:03	DQB1*06:02 DQB1*06:03
3rd party donor	A*24:02 A*26:01	B*14:02 B*44:03	C*02:02 C*04:01	DRB1*01:02 DRB1*07:01	DQA1*01:01 DQA1*02:01	DQB1*02:02 DQB1*05:01

Table S-I: HLA-typing of donors used for experiments with 10U/mL IL-2 described in Chapter 1.

EXP.1	HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQA1	HLA-DQB1
Treg donor	A*03:01 A*29:01	B*15:01 B*44:03	C*04:01 C*16:01	DRB1*07:01 DRB1*08:01	DQA1*02:01 DQA1*04:01	DQB1*04:02 DQB1*04:02
OriDC donor	A*23:01 A*25:01	B*18:01 B*44:03	C*04:01 C*12:03	DRB1*04:01 DRB1*07:01	DQA1*02:01 DQA1*03:01	DQB1*02:02 DQB1*03:02
EXP.2	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*02:01 A*68:01	B*37:01 B*52:01	C*06:02 C*12:02	DRB1*13:03 DRB1*15:02	DQA1*01:03 DQA1*05:05	DQB1*06:01 DQB1*06:01
OriDC donor	A*02:05 A*30:02	B*18:01 B*50:01	C*05:01 C*06:02	DRB1*07:01 DRB1*13:01	DQA1*01:03 DQA1*02:01	DQB1*02:01 DQB1*06:03
EXP.3	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*11:01 A*29:01	B*44:03 B*52:01	C*12:02 C*16:01	DRB1*07:01 DRB1*15:02	DQA1*01:03 DQA1*02:01	DQB1*02:02 DQB1*06:01
OriDC donor	A*29:01 A*29:01	B*51:01 B*51:01	C*02:02 C*14:02	DRB1*01:01 DRB1*11:03	DQA1*01:01 DQA1*05:05	DQB1*05:01 DQB1*06:01
EXP.4	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*02:01 A*23:01	B*18:01 B*49:01	C*07:01 C*07:01	DRB1*04:03 DRB1*04:05	DQA1*03:01 DQA1*03:03	DQB1*03:02 DQB1*03:02
OriDC donor	A*23:01 A*25:01	B*18:01 B*44:03	C*04:01 C*12:03	DRB1*04:01 DRB1*07:01	DQA1*02:01 DQA1*03:01	DQB1*02:02 DQB1*03:02

Table S-II: HLA-typing of donors used for experiments described in Chapter 2.

EXP.1	HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQA1	HLA-DQB1
Mismatch 3P donor	A*02:01 A*68:01	B*37:01 B*52:01	C*06:02 C*12:02	DRB1*13:03 DRB1*15:02	DQA1*01:03 DQA1*05:05	DQB1*06:01 DQB1*06:01
EXP.2	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Mismatch 3P donor	A*01:01 A*33:01	B*08:01 B*14:02	C*07:01 C*08:02	DRB1*01:02 DRB1*03:01	DQA1*01:01 DQA1*05:01	DQB1*05:01 DQB1*05:01
EXP.3	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Mismatch 3P donor	A*02:01 A*02:01	B*07:02 B*07:02	C*07:02 C*07:02	DRB1*08:01 DRB1*15:01	DQA1*01:02 DQA1*04:01	DQB1*04:02 DQB1*04:02
EXP.4	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Mismatch 3P donor	A*32:01 A*33:01	B*14:02 B*14:02	C*08:02 C*15:05	DRB1*13:01 DRB1*16:01	DQA1*01:02 DQA1*01:03	DQB1*05:02 DQB1*06:03

Table S-III: HLA-typing of mismatch 3P donors used for experiments described in Chapter 2.

EXP.1	HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQA1	HLA-DQB1
Treg donor	A*03:01 A*29:01	B*15:01 B*44:03	C*04:01 C*16:01	DRB1*07:01 DRB1*08:01	DQA1*02:01 DQA1*04:01	DQB1*04:02 DQB1*04:02
OriDC donor	A*23:01 A*25:01	B*18:01 B*44:03	C*04:01 C*12:03	DRB1*04:01 DRB1*07:01	DQA1*02:01 DQA1*03:01	DQB1*02:02 DQB1*03:02
Mismatch 3P donor	A*02:01 A*68:01	B*37:01 B*52:01	C*06:02 C*12:02	DRB1*13:03 DRB1*15:02	DQA1*01:03 DQA1*05:05	DQB1*06:01 DQB1*06:01
DC-match 3P donor	A*02:01 A*23:01	B*18:01 B*49:01	C*07:01 C*07:01	DRB1*04:03 DRB1*04:05	DQA1*03:01 DQA1*03:03	DQB1*03:02 DQB1*03:02
Treg-match 3P donor	A*02:01 A*02:01	B*07:02 B*07:02	C*07:02 C*07:02	DRB1*08:01 DRB1*15:01	DQA1*01:02 DQA1*04:01	DQB1*04:02 DQB1*04:02
EXP.2	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*02:01 A*68:01	B*37:01 B*52:01	C*06:02 C*12:02	DRB1*13:03 DRB1*15:02	DQA1*01:03 DQA1*05:05	DQB1*06:01 DQB1*06:01
OriDC donor	A*02:05 A*30:02	B*18:01 B*50:01	C*05:01 C*06:02	DRB1*07:01 DRB1*13:01	DQA1*01:03 DQA1*02:01	DQB1*02:01 DQB1*06:03
Mismatch 3P donor	A*01:01 A*33:01	B*08:01 B*14:02	C*07:01 C*08:02	DRB1*01:02 DRB1*03:01	DQA1*01:01 DQA1*05:01	DQB1*05:01 DQB1*05:01
DC-match 3P donor	A*23:01 A*25:01	B*18:01 B*44:03	C*04:01 C*12:03	DRB1*04:01 DRB1*07:01	DQA1*02:01 DQA1*03:01	DQB1*02:02 DQB1*03:02
Treg-match 3P donor	A*29:01 A*29:01	B*51:01 B*51:01	C*02:02 C*14:02	DRB1*01:01 DRB1*11:03	DQA1*01:01 DQA1*05:05	DQB1*05:01 DQB1*06:01
EXP.3	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQA1**	**HLA-DQB1**
Treg donor	A*11:01 A*29:01	B*44:03 B*52:01	C*12:02 C*16:01	DRB1*07:01 DRB1*15:02	DQA1*01:03 DQA1*02:01	DQB1*02:02 DQB1*06:01
OriDC donor	A*29:01 A*29:01	B*51:01 B*51:01	C*02:02 C*14:02	DRB1*01:01 DRB1*11:03	DQA1*01:01 DQA1*05:05	DQB1*05:01 DQB1*06:01
Mismatch 3P donor	A*02:01 A*02:01	B*07:02 B*07:02	C*07:02 C*07:02	DRB1*08:01 DRB1*15:01	DQA1*01:02 DQA1*04:01	DQB1*04:02 DQB1*04:02
DC-match 3P donor	A*01:01 A*33:01	B*08:01 B*14:02	C*07:01 C*08:02	DRB1*01:02 DRB1*03:01	DQA1*01:01 DQA1*05:01	DQB1*05:01 DQB1*05:01
Treg-match 3P donor	A*02:05 A*30:02	B*18:01 B*50:01	C*05:01 C*06:02	DRB1*07:01 DRB1*13:01	DQA1*01:03 DQA1*02:01	DQB1*02:01 DQB1*06:03

Table S-IV: High-resolution HLA sequencing of donors in MHC-matched specificity assays from Chapter 2.
Of note, in EXP.3, DC-match 3P is only matched to DC in MHCII.