Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Biomediche

Ciclo XXVII

Settore Concorsuale di afferenza: 06/D3

Settore Scientifico disciplinare: MED15

In vitro characterisation and expansion of human regulatory T cells for their *in vivo* application in the induction of tolerance in haematopoietic stem cell and solid organ transplantation

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Esame finale 2015

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ABSTRACT

Solid organ transplantation is considered the treatment of choice for many end-stage organ diseases, resulting in marked improvements in both morbidity and mortality. Thus far, short term results are excellent, with patient survival rates greater than 90% one year after surgery, but there are several problems with the long term acceptance and the use of immunosuppressive drugs causing infections, organ failure and cancer.

Hematopoietic Stem Cells Transplantation (HSCT) is an established procedure concerning the infusion of autologous, syngenic or allogeneic stem cells to re-establish acquired and congenital disorders of the hematopoietic system. The success of allogeneic HSCT therapy depends on a multitude of parameters, including the type and stage of the underlying disease, patient age, genetic disparity between donor/host, type and intensity of the pre-transplantation conditioning regimen. The main side effect of HSCT is the Graft versus Host Disease (GvHD) where donor T cells can cause pathology involving the damage of host tissues. Patients undergoing acute or chronic GvHD receive immunosuppressive regimen that is responsible for several side effects.

In summary, the use of immunosuppressive drugs in the setting of solid organ transplantation and GvHD has markedly reduced the incidence of acute rejection and the tissue damage in GvHD. However, the numerous adverse side effects observed, and failure to effectively prevent chronic allograft dysfunction of conventional immunosuppression boost the development of alternative strategies to avoid graft rejection and for the treatment of GvHD.

To this aim, the use of CD4+CD25+FOXP3+ regulatory T cells (Treg) as a cellular therapy is an attractive approach for autoimmunity disease, GvHD and for limiting immune responses to allograft after transplantation. Treg have a pivotal role in maintaining peripheral immunological tolerance, by preventing autoimmunity and chronic inflammation. Animal models have shown how the infusion of freshly isolated or expanded Treg can prevent graft versus host disease (GvHD) and both acute and chronic organ rejection after transplantation. In humans, a few clinical studies have been published reporting the infusion of Treg for the prevention of GvHD and autoimmune disease (Type I diabetes). These are phase-I trials showing safety, but there are no published results testing the efficacy of Treg in solid organ transplantation.

Results of my thesis are divided in three chapters, the first provides the characterization and cell processing of Tregs from healthy controls and patients, the second details the *ex vivo* expansion of freshly isolated Treg from healthy donors and patients in a waiting list for liver transplantation, followed by the investigation of the impact of the main immunosuppressive drugs on viability, proliferative capacity and function of Treg after expansion.

In the first chapter, peripheral blood Treg were enumerated to assess the suitable circulating cells and then isolated for functional characterization. Studied patients have a slightly lower Treg number comparing to healthy donor however cells are functional after *in vitro* analysis.

In the second chapter, it has been developed a protocol, using reagents compatible with Good Manufacture Practices, for the expansion of clinically useful number of functional suppressive Treg cells. CD4+CD25+ T cells were expanded *ex vivo* using rapamycin and then characterized in vitro. After expansion it was possible to recover a high Treg number suitable for the infusion of more than $3x10^6$ cells/Kg, moreover, these cells highly express FOXP3 and are highly suppressive in vitro.

Since ex vivo expanded Treg will be used in a phase I/II clinical trial for promoting allograft tolerance after organ transplantation and for GvHD treatment, the immunosuppressive regimen is still maintained. To date, there are no data showing the effect of these drugs on Treg preparation. To better understand the suitable procedure of Treg infusion, the third chapter of this study investigates the impact of Tacrolimus, Micophenolate-Mofetil and Methyl-Prednisolone on viability, proliferative capacity and function of ex vivo expanded Treg. Results obtained *in vitro* suggest that although immunosuppressive drugs influence the viability of Tregs, their phenotype and mostly suppressive ability is not altered.

At the end it is possible to conclude that *ex vivo* expansion is necessary to infuse a high Treg dose and although many other factors *in vivo* can contribute to the success of Treg therapy, the infusion of Tregs during the administration of the highest dose of immunosuppressants should be carefully considered.

GENERAL INTRODUCTION

1. Targets for cell therapy

1.1 Solid organ transplantation

Solid organ transplantation (SOT) is defined as the artificial transfer of tissues or organs from one individual to another. To date, it is considered the treatment of choice for many end-organ diseases, resulting in marked improvements in both morbidity and mortality. In recent decades, transplantation has saved the lives of thousands of people who otherwise were condemned to death because of their life-threatening diseases. The first successful transplantation of a liver in a human was performed by Thomas Starzl in 1967 and 20 years later liver transplantation was declared as an accepted therapy for end-stage liver disease. The principal indications for liver transplantation in Europe are cirrhosis, cancer, cholestatic diseases and acute hepatic failure. Short term survival is excellent, with patient survival rates greater than 90% one year after surgery, but there are several problem with the long term acceptance and the use of immunosuppresive drugs causing infections, organ failure and cancer. However, comparing this to other solid organs such as the heart or kidney, the liver is an immune privileged organ with a lower incidence of graft rejection. In addition, liver transplants do not require HLA matching of donor to recipients, and it is estimated that about one third of liver transplant recipients with stable function can be totally withdrawn from immunesuppression[1]. Unfortunately the identification of stable patients suitable for withdrawal is complex, and therefore lifelong treatment with immunosuppressive drugs (ID) is required in the majority of patients in order to prevent rejection of the liver graft.

1.2 Graft Rejection

The graft can be syngeneic (between identical individuals like twins) or autologous (auto-transplant). In this situation the recipient is fully tolerant to the transplant and accepts it without a rejection phenomenon. Where there is histo-*in*compatibility, an immune response is elicited against the foreign antigens, the magnitude of which determines acceptance or rejection of the transplanted organ. The response to transplanted organ follows a two step process. "Allorecognition" where the transplanted allogeneic tissues are recognised by the host and the "alloresponse" concerning the effector mechanisms recruited in the reaction to the foreign tissue and the outcome of those effects. To better understand the allorecognition's process it's important to introduce the histocompatibility antigens, a group of genetically determined proteins on the surface of cell membranes that serve to identify a cell as self or non-self. They can be divided in two groups, major and minor, (MHC and mHAg respectively), based on the strength of recipient immune responses engendered by their expression on histo-*in*compatible tissues.

MHC are codificated on the short arm of chromosome 6 and can be divided into two classes (MHC I and II). MHC class I molecules consist of one membrane-spanning α chain (heavy chain), and one β chain (light chain) while MHC class II present two membrane-spanning chains, α and β, of similar size. In each case, the MHC molecule has a groove that binds a peptide, essential for the interaction with T cells and the immune response starting. The two classes of MHC proteins differ not only in their structure, but more importantly in their functional roles within the immune system since they are specialised to present different types of antigens, thereby eliciting different responses. MHC I are located on almost every cell in the body and present endogenous antigens originating from the cytoplasm. They present self-proteins, but also viral proteins. Once degraded, the peptide fragments are transported to the endoplasmic reticulum, where they can bind to MHC I proteins, before being transported via the Golgi apparatus to the cell surface. Once at the cell surface, the membrane-bound MHC I protein displays the antigen for recognition by special immune cells known as cytotoxic T cell lymphocytes. MHC II glycoproteins are only present on specialised antigen-presenting cells

(APC), like macrophages, dendritic and B cells. MHC II proteins present exogenous antigens originating extracellularly. APCs degrade proteins from bacteria and pathogens sequestering these fragments into the endosome so they can be bound to MHC II proteins, before the expression on the cell surface. Once on the cell surface, the membrane-bound MHC II protein displays the antigen for recognition to the helper T lymphocyte (T helper). HLA-A, B and C are the most important MHC-I protein since their products have been well defined as "classical transplantation antigens". Others human class I genes that show sequence homology to classical loci include HLA-E, F, G, H, and a set of five MIC (MHC class I related) genes (MIC A–E). The principal MHC class II proteins consist of six subregions, termed DR, DQ, DP, DO, DN and DM.

Minor histocompatibility antigens are polymorphic peptides consisting of 9–12 amino acids encoded by either autosomal chromosomes or Y-chromosomes. mHAg are recognised by T cells only after the binding to the antigen recognition site of MHC molecules. Thus the occurrence of mHag depends on the presence of specific HLA antigens, which is called the MHC restriction. To date, 28 mHAg have been identified, 18 encoded by autosomal and 10 by Y-chromosomes. On the basis of their tissutal distribution it is possible to find restricted and broad mHag. Most of mHAg encoded by Y-chromosome are present in most tissues, including those crucial for GVHD: skin, intestines, and liver[2]. Most of autosomal and 2 MiHAs encoded by Y-chromosome (B8/HY and B52/HY) appear only in hematologic cells including leukemic cells, dendritic cells, NK, and multiple myeloma cells. Thanks to their restricted distribution all of them may be potentially exploited in immunotherapy.

Allorecognition can proceed via three non-mutually exclusive and concurrent mechanisms (Figure 1):

1. *direct* allorecognition, whereby T cells recognise determinants on intact donor MHC molecules displayed on the surface of transplanted cells ;

2. *indirect*[3] allorecognition in which donor MHC molecules are processed and presented as peptides by self-MHC

3. *semi-direct* allorecognition where trafficking recipient dendritic cells DCs acquire intact donor MHC:peptide complexes from cells of the graft enabling them to stimulate antigen-specific immune responses with both direct and indirect allospecificities[4].

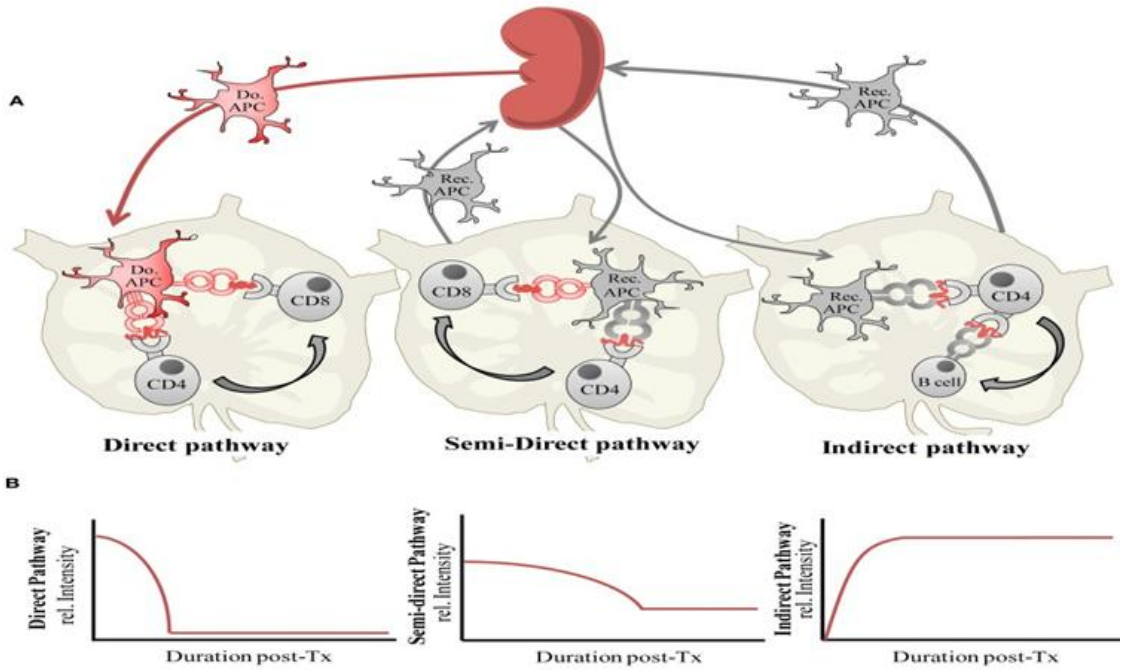

Figure 1 Alloantigen presentation. A) Alloantigen presentation via the direct, semi-direct and indirect pathways following organ transplantation, and **B)** the relative intensity of each antigen-presentation pathway during the post-transplantation (post-Tx) period. From Sagoo et al[5].

Direct allorecognition was long believed to be the only mechanism by which allogeneic antigens could be recognised in the donor graft. This mechanism explain how recipient T cells recognise determinants on intact donor MHC-peptide complexes displayed on the surface of transplanted cells[6]. This was demonstrated in vitro by a mixed lymphocyte reaction or in vivo using *Rag*-/- MHC class II-/- mice reconstituted with syngeneic $CD4^+$ T cells. In this setting mice do not present the capacity to present the antigen via indirect mechanism but they are able to reject cardiac allografts[7]. Under the influence of pro-inflammatory signals engendered by the transplantation procedure, donor DCs traffic to secondary lymphoid tissues of the recipient

initiating a direct responses. To date, as well reviewed by Archbold et al[8], there are two non-mutually exclusive theories that further delineate the molecular characteristics of the high frequency of direct alloreactivity, the "high determinant density" and the "multiple binary complex" models which differ on whether alloreactive T cells directly recognise polymorphisms in allogeneic MHC or presented peptide in the MHC peptide binding groove. The first one explains how T cells are directly able to recognise the exposed polymorphic residues on allogeneic MHC, thus consigning the bound peptide to secondary importance, whilst the latter one proposes that recognition of peptide bound by allogeneic MHC is of primary importance to direct allorecognition in a manner akin to conventional self-restricted responses. It is likely that both mechanisms contribute to direct allorecognition, the overall contribution of each being related to the site and magnitude of the structural differences in MHC molecules between responder and stimulator cells. Where the allogeneic MHC is structurally very disparate from responder MHC, the alloresponse may be directed against residues on the MHC itself whereas where self and foreign MHC are closely matched, the focus of the alloreactivity may be directed towards epitopes of endogenous peptides that are displayed by stimulator but not by responder MHC molecules[9].

The indirect pathway of allorecognition refers to identification of processed peptides of allogeneic antigens presented by self-MHC in a self-restricted manner[3] (Figure 1). Indirect alloantigen presentation results in alloresponses dominated by $CD4^+$ T cells. The requirement for antigen processing in the indirect pathway, despite considerable amplification of this response, naturally correlates with slower responses than those engendered via the direct pathway. Additionally, the lower frequency of T cells in the normal repertoire with indirect compared to direct allospecificity[10] suggests that the direct response dominates the early post-transplant period while the indirect pathway plays a role in long term alloantigen presentation when the donor APC has been exhausted[11]. Although there is significant evidence in support of this assertion, it is important to note that in the absence of direct responses, the indirect pathway alone can also result in rapid acute graft rejection[12]. As showed by Auchincloss et al[12], MHC class I knock out recipient mice could reject skin grafts from MHC class II knock out donor mice. The recipient mice lacked $CD8^+$ cytotoxic T cells capable of recognising donor MHC class I molecules directly, and the $CD4^+$ T cells in the recipient animals could only be stimulated by

recognising donor MHC class I molecules indirectly, presented in the context of recipient MHC class II molecules.

Recently it was discovered that another pathway of allorecognition could provide an alternative explanation for previously published observations. For example, reports of MHC class II deficient recipients rejecting co-stimulation deficient (CD80-/-CD86-/-) allogeneic cardiac grafts as rapidly as wild-type transplants[13] could be attributed to semi-direct presentation of MHC from the graft rather than to trans co-stimulation. Semi-direct presentation provide cell-to-cell contact between donor and recipient APC. This interaction may transfer intact membrane components including intact allo-MHC. Likewise, donor APC can release exosomes containing intact MHC which fuse with the membrane of recipient APCs. The site of MHC transfer is unknown and may occur either within the graft or within regional lymph nodes. If MHC transfer occurs in lymph nodes, it will be between donor and recipient DC but, if within tissues, transfer will be between a non-professional-APC and DC. Recipient APCs, now chimeric for MHC, stimulate direct pathway CD4 and CD8 responses through intact foreign MHC and indirect responses through processing and presentation of peptides of foreign MHC acquired from necrotic and apoptotic cell material. Given that the same APC stimulates both CD4 and CD8

1.3 Hematopoietic Stem Cells Transplantation (HSCT)

HSCT is an established procedure concerning the infusion of autologous, syngenic or allogeneic stem cells to re-establish acquired and congenital disorders of the hematopoietic system, including disorders of the immune system, and enzyme replacement in metabolic disorders. In Europe, 661 of 680 centres in 48 countries reported 37 818 HSCT in 33 678 patients (42% allogeneic and 58% autologous) in the 2013 survey. Main indications are leukaemia, lymphoid neoplasias, solid tumors, and nonmalignant disorders[14].

The success of allogeneic HSCT therapy depends on a multitude of parameters, including the type and stage of the underlying disease, patient age, genetic disparity between donor/host, type and intensity of the pre-transplantation conditioning regimen, and the presence of transplant-

related toxicities. HSCT usually first undergo a conditioning regime that can include chemotherapy and/or radiation and/or T cell depleting antibodies[15]. This conditioning regime eradicates the recipient's own T and B cells leaving the recipient highly immuno-compromised and ready for the engraftment of donor bone marrow. Donor T and B cells present in the graft reconstitute adaptive immune capacity in the recipient[16]. Donor T cells have an additional beneficial effect against any remaining leukaemic or tumour cells within the host, a phenomenon known as graft versus leukaemia (GvL)[16]. However, donor T cells can also cause pathology involving the damage of host tissues called graft versus host disease (GvHD), which is primarily due to varying degrees of donor/host mismatch at both MHC and mHA. In effect, GvHD represents a mirror image of transplant rejection, in that it is the donor allogeneic T cells that recognise the recipient host cells bearing recipient MHC antigen as "foreign".

1.4 GVHD

GVHD is the major complication of HSCT limiting the use of this therapy. It can be compared during the first 100 days after transplantation[17] (acute GvHD) or later (Chronic GVHD). Acute GvHD[18] predominantly affects the skin (81%), gastrointestinal tract (54%), and liver (50%). The severity of acute GVHD is classified on the basis of organ involvement and is graded as follows[19]: grade I (mild); II (moderate); III (severe); and IV (very severe). The development of acute GvHD depends principally on genetic differences between donor and host, donor's sex, and the stem cell source replete with donor T cells. The incidence of acute GvHD in patients receiving fully-matched transplantation is around 40% but it increases greatly with recipients differing of one HLA antigen up to 60-80%[20]. Chronic GvHD is a major cause of morbidity and mortality in long-term survivors of allogeneic stem cell transplantation. There is a classic type without features of acute GvHD and an overlap syndrome with features of both, acute and chronic GvHD. The classic one is a de novo syndrome and the latter one evolves from an acute GVHD. The occurrence of chronic GvHD varies widely between 6-80% interesting different organ like the skin, eyes, mouth, gut, liver, lungs, joints and genitourinary system,

which results in organ failure and decreased survival. According to the severity of organs involved, chronic GvHD is scored as mild, moderate or severe.

The development of acute GvHD occurs in three sequential steps:

1. activation of host APCs;
2. activation, expansion and differentiation, and trafficking of donor T cells;
3. targeted tissue destruction by cellular and inflammatory effectors.

The inflammatory status plus the cytoreductive conditioning causes damage to patient's tissues with the secretion of danger signals, including tumour necrosis factor-α (TNF-α) and interleukins-1 (IL-) and 6. Furthermore, expression of adhesion molecules, costimulatory molecules, and MHC antigens on host APCs is increased. All these factors together result in the activation of host APCs. Following the presentation of antigens to T cells, a strong cytokine response is initiated. These cytokines further promote antigen presentation and the recruitment of effector T cells and innate immune cells, which further augment the pro-inflammatory cytokine milieu. Finally, the effector T cells, natural killer (NK) cells, macrophages and pro-inflammatory cytokines result in end-organ damage, which is clinically recognized as acute GvHD in the skin, lungs, gut and liver. The resulting tissue damage, if not treated, will further amplify the process to more severe stages of GvHD pathology, which are extremely difficult to control[21]

The pathophysiology of chronic GVHD mainly depends on the polarization of $CD4^+$ T cells into T helper 2 (TH2) cells, but there are six hallmarks that are unique to this syndrome as reviewed by Blazar et al[21].

The first feature is the thymus damage by the conditioning regimen or by prior occurrence of acute GvHD. This damage results in decreased negative selection of alloreactive $CD4^+$ T cells (second). Third, there is immune deviation to a TH_2-type cytokine response with the production of IL-4, 5 and 11. This response leads to the release of fibrogenic cytokines principally transforming growth factor-β1 (TGFβ1). All is accompanied by macrophages activation and their production of platelet-derived growth factor (PDGF) and TGFβ1. These molecules induce the proliferation and activation of tissue fibroblasts. Low numbers of Treg cells are the fifth hallmark and finally there is B cell dysregulation which leads to the emergence of autoreactive B

cells and the production of auto-reactive antibodies. It has been suggested that autoreactive B cell activation may be due to the presence of high levels of B cell-activating factor (BAFF) in the lymphoid microenvironment. All these events contribute to an autoimmune-like systemic syndrome that is associated with fibro-proliferative changes.

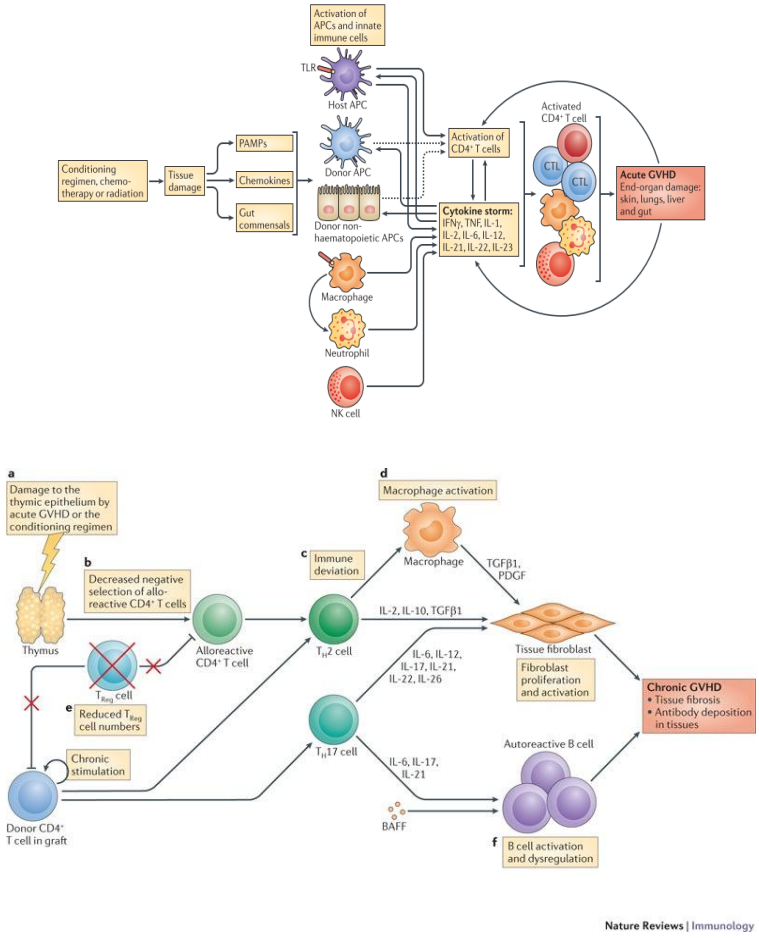

Figure 2 Pathogenesis of acute (top) and chronic (bottom) GVHD. From Blazar et al[21].

1.5 Immunosuppressive Drugs

The transplant outcome depends on a delicate balance between immunosuppression and rejection, for this reason the use of appropriate doses of immunosuppressive drugs (ID) is

extremely important, especially in the first phases after the transplant. During the last 50 years the strategies utilized to prevent acute and graft rejection provide the use of different molecules acting principally to reduce the immune response against the transplanted organ. The first immunosuppressive drugs used were corticosteroids and azathioprine. Later on cyclosporine became the basic ID until a new calcineurin inhibitor, Tacrolimus, was introduced in the 90s. Tacrolimus was more powerful compared to Cyclosporine, but shared a similar spectrum of adverse effects. Tacrolimus is still today the drug of reference for renal transplantation. When used in combination with antiproliferative drugs such as mycophenolate mophetil the frequency of acute rejection episodes was below 20%. On the basis of their mechanisms of action ID are divided in 5 groups as explained in Table 1.

Mechanisms of action of immunosuppressive drugs

Principal mechanism of action	Drug	Target
1. Regulation of gene expression	Glucocorticoids	Glucocorticoid receptors
2. Alkylation	Cyclophosphamide	DNA
3. Kinase and phosphatase inhibitors	Cyclosporin A	Calcineurin, JNK/p38 kinase
	Tacrolimus	Calcineurin, JNK/p38 kinase
	Rapamycin	Cyclin kinase cascade
	Type IV PDE inhibitors	Type IV PDE
	p38 kinase inhibitors	p38 kinase
4. Inhibition of de novo purine synthesis	Azathioprine	Several enzymes
	Mycophenolate mofetil	IMPDH
	Mizoribine	IMPDH
	Methotrexate	Several enzymes
5. Inhibition of de novo pyrimidine synthesis	Leflunomide	DHOD
	Brequinar	DHOD
	Methotrexate	Thymidylate synthase

PDE-cyclic AMP phosphodiesterase.
IMPDH-inosine-5′-monophosphate dehydrogenase.
DHOD-dihydroorotate dehydrogenase.

Table 1 Mechanisms of action of immunosuppressive drugs. Derived from Immunosuppressive drugs: the first 50 years and a glance forward[22].

The calcineurine inhibitor Tacrolimus is a macrolide produced by the *Streptomyces tsukubaensis* fungus. Tacrolimus is now preferred to cyclosporine for its potency, essential to reduce the dose and consequentially the related side effects. It becomes biologically active only when complexed with the immunophilin FK binding protein 12 (FKBP-12), that is different from the immunophilin (cyclophilin) to which cyclosporine binds. The complex FKBP-12-tacrolimus

interferes with the transduction pathway of the intracellular calcium-dependent signal, which is a fundamental processes for the activation of T lymphocytes. The biological target of the complex is the calcium/calmodulin-dependent protein phosphatase, calcineurin, a fundamental molecule for the reactions necessary to the synthesis of various cytokines, including IL-2[23]. The tacrolimus-FKBP-12 complex has a strong inhibitory dose-related effect on calcineurin phosphatase activity and consequently on IL-2 expression. T cells activation after the antigen encounter, consist in intracellular calcium release and subsequent activation of the calcium/calmodulin complex. This is essential to form the competent T-cell transcription factor NFAT. Upon activation, NFAT translocates to the nucleus where it associates with other transcription factors, such as members of the activator AP-1 family, and binds to DNA to promote the transcription of IL-2. Inhibition of calcineurin prevents its ability to activate NFAT, affecting the transcription of cytokines important in the immune response. In addition calcineurin is also involved in the activation of NF-κB. It indirectly induces the degradation of a compound known as IκB, which is bound to inactive NF-κB and acts as an inhibitory protein, preventing NF-κB association with its nuclear target genes. The blockade of calcineurin activity by Tacrolimus and cyclosporine affects the ability of NF-κB to exert its action on the genes of the immune system. Recently it was discovered that tacrolimus and cyclosporine are also involved in the inhibition of the mitogen-activated protein kinase (MAPK) pathway. In particular they act on JNK and p38 activated through the MAPK signalling cascade by T cell and CD28 co-stimulatory receptors[24]. The principal adverse effects associated with tacrolimus treatment are dose dependent and include nephrotoxicity, neurotoxicity, disturbances in glucose metabolism, gastrointestinal disturbance and hypertension.

Mycophenolate mofetil (MMF) is an ester of mycophenolic acid (MPA) obtained from a *Penicillum* species acting through the inhibition of inosine monophosphate dehydrogenase, an enzyme involved in the synthesis of nucleotides, leading to B- and T-lymphocyte proliferation inhibition[25]. This is the rate-limiting enzyme in de novo synthesis of guanosine nucleotides. T- and B-lymphocytes are more dependent on this pathway than other cell types are. Moreover, MPA is a fivefold more potent inhibitor of the type II isoform of IMPDH, which is expressed in activated lymphocytes, than of the type I isoform of IMPDH, which is expressed in most cell types. Three other mechanisms may also contribute to the efficacy of MPA in preventing allograft rejection and other applications. First, MPA can induce apoptosis of activated T-

lymphocytes, which may eliminate clones of cells responding to antigenic stimulation. Second, by depleting guanosine nucleotides, MPA suppresses glycosylation and the expression of some adhesion molecules, thereby decreasing the recruitment of lymphocytes and monocytes into sites of inflammation and graft rejection. Third, by depleting guanosine nucleotides MPA also depletes tetrahydrobiopterin, a co-factor for the inducible form of nitric oxide synthase (iNOS). MPA therefore suppresses the production by iNOS of NO, and consequent tissue damage mediated by peroxynitrite[22]. Most authors describe side-effects in about half of the patients treated with mycophenolate mofetil therapy. Specific MMF-related side effects are predominantly of a gastrointestinal nature, including diarrhoea, abdominal pain, decreased appetite with weight loss, nausea and vomiting. These appear dose related and reduce quickly when the drug is reduced. Other adverse reactions reported in literature are leukopenia (similar to azathioprine) and symptomless anaemia that is related to bone marrow toxicity. Infectious complications and malignancies have been observed, although long-term safety data must be awaited. CMV, HZV, HVI-1, Candida, and Aspergillus infections seem to be more common.

Prednisone is a synthetic glucocorticoid obtained by dehydrogenation of cortisone. Its anti-inflammatory, immunosuppressant and mineralcorticoid properties are only exhibited when is converted to prednisolone in the liver. Prednisolone is extensively bound to plasma proteins and is a potent therapeutic agent influencing the immune activation because of the ubiquitous expression of corticosteroid receptors. Corticosteroids inhibit antigen presentation, cytokine production, and proliferation of lymphocytes. It is believed that corticosteroids induce their effects on the cell through a glucocorticoid receptor in the cytoplasm. Corticosteroids induce lymphocytopenia as a result of redistribution of circulating lymphocytes into other lymphoid compartments (spleen and lymph nodes *in primis*). Another effect of corticosteroids is the complete inhibition of T-cell growth[26] factor or IL-2. Therefore, T cells under the influence of corticosteroids lose their ability to proliferate and react to specific antigens. Moreover, it's responsible of a profound monocytopenia and inhibits inflammation by blocking responses to chemotactic factors and macrophage activation factor, phagocytosis and pyrogen production. The side effects of corticosteroids are not specific to lymphocytes and a number of serious adverse events are associated with the prolonged use. Complications of corticosteroid therapy involve any organ system in the body. Short and appropriate use does not have heavy complications however acute adverse effects include central nervous system effects, psychosis,

impaired glucose tolerance, and retention of salt and fluid. Long-term adverse effects are insidious in onset and tend to subside more slowly when therapy is discontinued.

In summary, the use of ID has markedly reduced the incidence of acute rejection and early graft loss. However, the numerous adverse side effects observed, and failure to effectively prevent chronic allograft dysfunction of conventional immunesuppression boost the development of alternative strategies to avoid graft rejection.

2. Regulatory T cells

2.1 Introduction

The immune system has the function to destroy invading microorganisms and stop aberrant outgrowth of tumour cells. In the meantime, it has developed several mechanisms for preventing attack of healthy self tissues. Self-reactive T lymphocytes and B lymphocytes are eliminated during negative selection in the thymus and bone marrow, respectively. However, to block the immune cells escaping this first "barrier", the immune system have developed a series of peripheral mechanisms. For almost 40 years, immunologists have postulated the existence of suppressor T cells that police the immune system to avert unwanted immune responses. However, that phenomenology was cast into doubt as various labs presented unique, hard-to-reproduce systems, each with complexities and idiosyncrasies that raised credibility issues. However, thanks to the identification of a constellation of cell surface, transcriptional and biochemical markers it was easy to find the unique mark of regulatory T cells[27] (Treg). The realisation that Treg cells have a unique surface expression profile incorporating CD25, CD62L together with the identification of the Treg cell–specific transcription factor Foxp3, catapulted Treg from a rare $CD4^+$ T cell subset to what many regard as 'master regulators' of immune homeostasis. After that a big scenario was opened and a myriad subpopulations of Treg cells where discovered, including the $CD4^+CD25^+$ $Foxp3^+$ cells, including IL-10 secreting Tr1 cells[28], TGF- secreting Th3 cells[29], Qa-1 restricted $CD8^+$ cells[30], $CD8^+$CD28- T cells[31], $CD8^+CD122^+$ T

cells[32], $CD8^+CD28-$ cells[33]. Some of these Treg cells, such as the $CD4^+$ $CD25^+Foxp3^+$ cells, originate in the thymus during ontogeny and are referred as natural Treg cells but they can also be induced from naive T cells in the periphery.

2.2 Thimic origins of natural regulatory t cells

Between the late 1960s and early 1980s was observed that thymectomy of mice on the 3rd day of life resulted in organ-specific autoimmune diseases, but that did not occur if neonatal mice were thymectomised on days 1 or 7[34]. In addition, day 3 thymectomised mice could be prevented from developing autoimmunity if they received an infusion of thymocytes[35]. These experiments suggested that autoreactive T cells exit the thymus in the first three days of life followed a few days later by a population of suppressor cells that control the autoreactive cohort. Almost a decade later, Sakaguchi and colleagues identified a small population of $CD4^+$ cells that expressed high levels of the IL-2 receptor -chain, CD25, whose depletion resulted in autoimmune diseases[27] and whose transfer to neonatally day 3 thymectomised mice prevented disease[36]. The subsequent identification of humans and mice deficient in $CD4^+$CD25hi cells which develop severe autoimmune diseases strongly suggests that these cells have a critical role in the maintenance of self-tolerance.

In addition, adoptive transfer of Treg from donor animals lacking ovaries, testes, prostate or thyroid glands did not inhibit organ-specific autoimmune diseases caused by neonatal thymectomy[37], suggesting that antigen specificity is imprinted on Treg during thymic education. As nTreg originate in the thymus, it is thought they initially undergo the same selective pressure and developmental checkpoints as conventional (αβ) T cells (T_{conv}). In order to understand how Treg develop in the thymus, it is important to understand the basis of T cell ontogeny. The two vital checkpoints that occur very close during thymocyte development are positive and negative selection. The former step involves the selection of thymocytes recognising self-MHC while the latter one involve negative selection of T cells with T cell receptors (TCR) at high avidity for class I and class II MHC molecules presenting self-antigens. Thus, duration and avidity of the TCR interaction with self-peptide-MHC complexes on antigen-presenting cells (APC) determine

thymocyte fate. Thymocytes that bind with high avidity undergo programmed cell death in an attempt to limit auto-reactivity in the periphery, while thymocytes with low avidity for self-MHC:peptide are selected as T_{conv}. The interaction with either MHC class I or MHC class II[38] allows for a series of signalling events important for developing respectively $CD8^+$ or $CD4^+$ single positive (SP) T cells. This engagement of TCR/co-receptor with MHC is vital in generating a T cell population that can distinguish self from non-self.

Over 97% of all thymocytes that become DP cells will die by their failure to recognise, at any level, the presence of MHC and therefore will not receive survival signals through the TCR[39]. These cells are said "dead by neglect". The remaining thymocytes are thought to survive initially by the recognition of low-affinity peptides as described in the "kinetic signalling" model. In this model it is proposed that signal intensity allows for positive selection and then signal duration allows for CD4/CD8 lineage commitment. Subsequently, the recognition of self-peptide with high affinity/avidity by the TCR will direct the thymocyte towards controlled apoptosis and deletion[40]. In the thymus, the αβ TCR is expressed on thymocytes entering the DP stage of development. The TCR transmits any intracellular signals from external stimulation by the association with the CD3 complex. Each of the four (ε,γ,δ,ζ) chains in the TCR-CD3 complex contain immunotyrosine activation motifs (ITAMs) that can be phosphorylated by protein tyrosine kinases to recruit downstream signalling molecules. A relatively high number of ITAMs found within the CD3 complex is needed to ensure a critical threshold of signalling possibly through the recruitment and activation of certain downstream signalling molecules such as the tandem Src homology 2 (SH2) domain-containing ζ-chain-associated protein of 70 kDa[41] (ZAP-70). It has been shown that recruitment and activation of the ZAP-70 substrate, linker of activated T cells, is essential in the development of $FOXP3^+$ T cells in the thymus and the periphery[42]. nTreg development takes place solely in the thymus but the complete mechanisms aren't fully understood. It is widely known that FOXP3 expression does not commence until day 3 in neonates and it has been postulated that the thymic architecture is essential to provide a proper co-stimulatory signals for FOXP3 expression[43]. Many co-stimulatory signals have been implicated in the development and lineage commitment of nTreg including: CD28 ligation by CD80/CD86, IL2R, thymic stromal-derived lymphopoietin receptor (TSLPR), CD154, glucocorticoid-induced tumour necrosis factor receptor (GITR), and STAT5 signaling[44]. nTreg are resistant to apoptotic signals, mechanism in part due to the expression of

GITR but even anti- and pro-apoptotic molecules Bim and Bcl2. For example, it was reported that *Bim*$^{-/-}$ mice have an increase in total Treg while there is an increase in the frequency of FOXP3^{+} Treg in Bcl2 transgenic mice. Another co-stimulatory pathway necessary for nTreg development is the of CD28-CD80/CD86 interaction because mice KO for CD28 or CD80/86 have a marked reduction of FOXP3^{+}Treg[45]. IL-2 appears to have a significant role in development and maintenance of Treg in periphery and in thymus as well. IL-2Rβ-deficient mice have a loss of functional Treg[46]. In addition, when there is a lack of IL-2 or the IL-2R, there is a 50% reduction in thymic Treg indicating that this one is only a part of several signals needed for Treg development[44]. Exploiting these discoveries has been introduced a two-step model of nTreg development[47]. When developing thymocytes recognise a self-peptide they upregulate CD25 allowing for their rescue by IL-2- and STAT5-mediated signaling within the medulla and their progression into the Treg lineage. In addition to IL-2R signaling, ligation of TGFβR also appears to be involved in development and maintenance of nTreg. The role of TGFβ became clear when TGFβ receptor I KO-mice were used. In this model was found that numbers of CD4^{+}CD25^{+}FOXP3^{+} thymocytes were greatly reduced in young mice, between days 3 and 5 of age[48]. The reduction in CD4^{+}CD25^{+}FOXP3^{+} thymocytes is only temporary, and the numbers of thymic FOXP3^{+} Treg rapidly recover due to increased production of IL-2. Of note, CD4^{+}CD25^{+}FOXP3^{+} thymocytes were completely lost when mice lacking both TGFβRI and IL-2 were generated. From this work it is apparent that TGFβ is an important upstream mediator of FOXP3 expression although other signals, including TCR signaling, are necessary.

FOXP3 expression within the thymus was facilitated by the generation of FOXP3-GFP reporter mice. Using these experimental models it was clearly established that FOXP3 expression is predominantly seen at the CD4 single positive stage of development. However, a small percentage of GFP^{+} cells were also seen in the double positive, CD8 single positive populations and at the immature double negative stage of thymocyte development. Using GFP as a marker for FOXP3 expression, it was determined that the majority of GFP^{+} cells are found within the medulla of the thymus. In addition, it has been shown that thymic stromal-derived lymphopoietin (TSLP) produced in the medullary region of the human thymus is critical for Treg development[49] giving credence to the notion that nTreg development is medullary. However, thymic dendritic cells (tDCs) also produce TSLP. nTreg develop from the cortical region of the thymus as well, but require other co-stimulatory signals for lineage commitment

and survival. Explaining the T cells generation is important to focus the attention on the autoimmune regulator gene *(Aire)*[50] essential for negative selection. *Aire* is expressed in thymic medullary epithelial cells (mTEC) allowing the expression of many peripheral-tissue antigens that would otherwise be absent during negative selection. It is possible that interactions with AIRE-expressing mTECs could also initiate FOXP3 expression within CD4 SP immature thymocytes[51]. However, AIRE expressing mTECs undergo rapid turnover, allowing for cross-presentation of antigens by traveling tDCs. which then present AIRE antigens in the cortex and the develop of nTreg at the DP stage.

These data indicate that initiation of FOXP3 expression within DP and SP thymocyte populations may be due to a combination, and possibly an accumulation, of signals mediated by the TCR, cytokine receptors and co-stimulatory molecules. From these observations a variation of the two-step model has been proposed[52] where nTreg development begins with a population of pre-Treg generated by unknown mechanisms. After successful rearrangement of the TCR, pre-Treg will recognise a diverse repertoire of self and foreign antigens but only progress to mature nTreg by the accumulation of TCR-dependent and independent signals including a pathway that allows for survival under negative conditions. In this model, TCR dependent and independent signals have variable levels of importance within any given developing nTreg.

2.3 Regulatory T cell markers

Although CD25 expression was the original defining feature of Treg, it is also expressed by antigen-experienced and recently activated conventional T cells. As a result, CD25 is of greatest sensitivity when used to identify Treg from naive T cell populations such as cord blood T cells or antigen-naive animals. As a result, in antigen-experienced mammals, only the top 2% of CD25 expressing $CD4^+$ cells contain genuine Treg[53]. In the description of Treg therefore, a number of additional markers have been described as putative Treg-specifying, but none of which are wholly perfect on their own. Some however, identify subsets of Treg which may have slightly different functions.

2.3.1 FOXP3

The Scurfy mouse, an X-linked mutant strain, described in 1949, exhibits a series of autoimmune features including skin scaliness, diarrhoea and death in association with $CD4^+$ T cell hyper-proliferation, multiorgan $CD4^+$ cell infiltration and over-production of several inflammatory cytokines[54]. This fatal autoimmune lymphoproliferative syndrome was found to map to a gene locus on the X chromosome called *Foxp3*, which was described as a new member of the forkhead/winged-helix family of transcription factors[55]. The *Foxp3* gene is highly conserved between species and a mutation in the human gene, *FOXP3*, was identified as the causative factor responsible for the human equivalent of Scurfy, the Immunodysregulation, Polyendocrinopathy and Enteropathy, X-linked syndrome (IPEX), also known as X-linked autoimmunity and allergic dysregulation syndrome[56] (XLAAD). Both the mouse and human disease lack discrete circulating Treg, suggesting that this gene is essential for Treg development in mice and humans as well. *Foxp3* knockout mice don't present circulating Treg; these animals develop a Scurfy-like syndrome reverted by the adoptive transfer of Treg from a *Foxp3* replete animal[57]. Furthermore, ectopic or over-expression of *Foxp3* in $CD4^+CD25^-$ mouse cells results in development of a Treg phenotype. In mice, Foxp3 expression is a good phenotypic marker of Treg; in humans, however, FOXP3 does not allow the unambiguous identification of Treg[58] as FOXP3 requires several signals for its stable expression and a regulatory phenotype.

A number of signalling pathways triggered by surface receptor-ligand interactions converge to synergistically transcribe the *FOXP3* locus. For example, TCR cross-linking in human cells triggers well-characterised proximal signalling events, including phosphorylation of conserved immunoreceptor tyrosine-based activation motifs (ITAMs) in CD3 chains by members of the Src tyrosine kinase family. Phosphorylation of ITAMs on CD3 allows binding of zeta associated protein 70 (ZAP70) which permits the activation of transmembrane proteins such as Linker of Activated T cells (LAT). Phosphorylated LAT in turn interacts with a number of intermediate signalling proteins. These proximal events lead eventually to the binding of the transcription factors Nuclear Factor of Activated T cells (NFAT) and Activator Protein 1 (AP-1) to the *FOXP3* promoter inducing its transcription. Proximal and distal signalling events are coupled together by Protein Kinase C (PKC) and Calcineurin as murine knockouts of either PKC and Calcineurin have greatly diminished Treg numbers[59]. In mouse, TCR ligation also results in an

increase in intracellular cAMP which leads to the downstream binding of the cAMP-Responsive Element Binding Protein (CREB) and Activating Transcription Factor (ATF) to a TCR-responsive enhancer in the *Foxp3* first intron[43]. In naive peripheral T cells, T activation also results in the propagation of an inhibitory signal to Foxp3 expression. This is mediated by the PI3K Protein Kinase B (AKT) and mammalian target of rapamycin (mTOR) pathway as inhibition of each of these leads to Foxp3 upregulation[60].

Even the CD28 co-stimulation can induce expression of Foxp3 in developing thymocytes in a cytokine-independent manner[61]. However, peripheral co-stimulation through CD28 impairs the conversion of naive T cells to the regulatory phenotype, suggesting that co-stimulation requirements for stable Foxp3 expression differ between nTreg and induced Treg (iTreg). This agrees with previous observations that the conversion of naive T cells to the regulatory lineage is enhanced in the presence of sub-optimal activation[62]. CD28 signalling has overlapping signalling pathways with those activated following TCR stimulation,

Cytokine signalling is very important for Treg differentiation since mice with disrupted IL-2 signalling pathways have no Treg[57], however IL-2 is not the only one. IL-2 signal transduction is mediated by the well-characterised JAK-STAT (Janus Kinase-Signal Transducer and Activator of Transcription) pathway involving JAK1, JAK3 and STAT5. The JAK/STAT pathway is a receptor-coupled signal transduction mechanism linking cytokine-receptor interactions to gene expression. JAK proteins are present in association with the cytoplasmic tails of multimeric cytokine receptors and are activated upon receptor dimerisation. Once activated, STATs dimerise and translocate to the nucleus to regulate gene transcription by binding to specific promoter regions. In IL-2-signalling, phosphorylated STAT5 binds directly to an intronic region in the *FOXP3* gene and induces transcription[63]. As expected, murine knockouts of both *Jak3* and *Stat5* have few or no circulating $Foxp3^+$ cells[64]. TFG-β can induce FOXP3 through two separate but concurrent pathways. Both the transcription factors TGF- β inducible early gene 1 (TIEG1) and Mothers Against Decapentaplegic 3 (SMAD3) bind to promoter regions in the *FOXP3* gene and enhance its expression[65]. There are additional factors which synergise with the TGF- β signalling pathway to promote transcription of the *FOXP3* gene including retinoic acid and Notch-signalling[66], while others, such as GATA3 and IRF1 (Interferon Regulatory Factor 1), inhibit this pathway of Treg differentiation[67].

2.3.1.1 Regulation of the *FOXP3 gene*

Regulation of the FOXP3 locus is critically controlled by epigenetic modifications which determine chromatin structure and alter the accessibility of the gene locus to transcription factors. These modifications include histone acetylation and methylation as well as methylation of CpG dinucleotide-rich regions within promoter sites. In FOXP3 locus it's possible to find three noteworthy non-coding regions which are susceptible to epigenetic modifications (Figure 3). These are: the *FOXP3* promoter, TGF-β sensor and Treg-cell-specific demethylation region (TSDR). *C*pG motifs in the *FOXP3* promoter region are basally demethylated in resting Treg but partially methylated in conventional naive $CD4^+$ T cells[68]. In addition, histones in this region are more acetylated in Treg than in naive T cells[43]. As a result, the *FOXP3* promoter is more accessible for the binding of NFAT and AP1 in Treg than in conventional T cells. Murine experiments suggest that the *Foxp3* promoter region in conventional T cells remains methylated following TCR activation, albeit at a lower level than at baseline[68], and that demethylation requires activation in the presence of TGF-β[43]; these structural effects limit and promote access for induction of Foxp3 transcription respectively. The second, highly conserved region in the *Foxp3* locus contains binding sites for NFAT and SMAD3 and is in an accessible state in nTreg and in naive T cells skewed to a Treg phenotype by TGF-β in the presence of anti-CD3 and anti-CD28 stimulation[65]. For this reason the area is known as the TGF-β sensitive enhancer element. This area has no CpG motifs, therefore the sole epigenetic modification at this locus is through histone acetylation. At baseline level it is not accessible in naive, resting T cells, limiting availability of the *Foxp3* gene for transcription. TGF-β induced SMAD3 binding to this region upregulates *Foxp3* transcription at early time points after stimulation whereas the enhancer effects of NFAT on this region are of a slightly longer duration. A third, highly evolutionarily conserved, CpG dinucleotide-rich region has been identified in both mouse and human cells which is completely demethylated in Treg but methylated in conventional T cells[69]. This area contains a nTreg specific demethylated region and, in addition, acetylated histones[70]. it is characterized by 2 transcription factor binding sites, which in the demethylated state bind STAT5 and CREB/ATF respectively[71]; epigenetic modification at this section are unaffected by TGF-β signalling. The TSDR was initially described as having enhancer activity however to date it is well established that TSDR demethylation is critical for the stability of FOXP3 expression[71].

The signals controlling methylation/demethylation at the TSDR are currently unknown but given the difference between iTreg and nTreg in FOXP3 stability, it is likely that demethylation at this locus is initiated in the thymus.

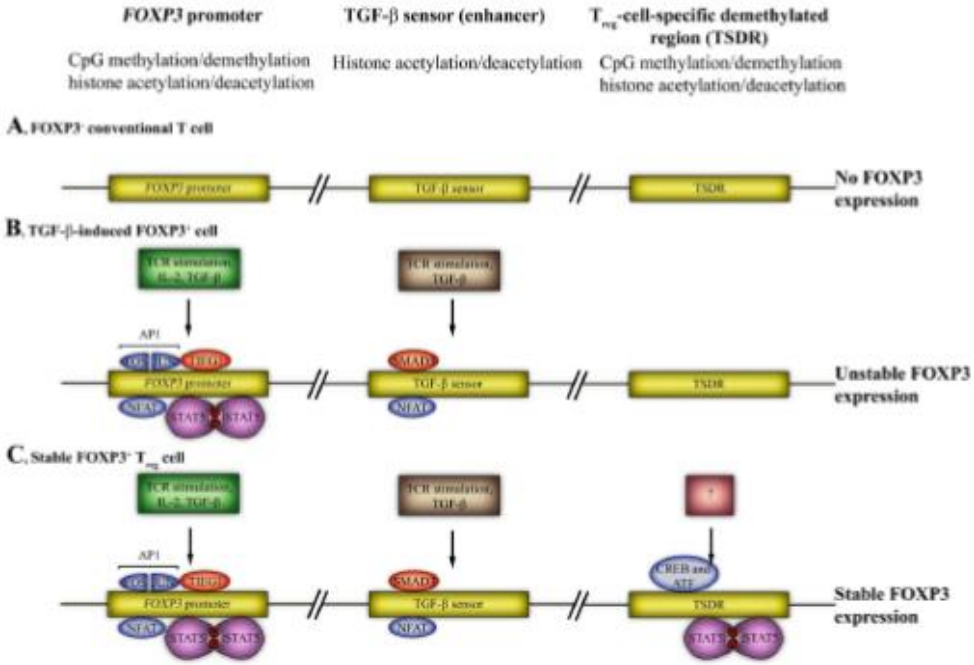

Figure 3. Epigenetic control of the FOXP3 locus. Three distinct regions of the *FOXP3* gene are susceptible to epigenetic modification. These are the *FOXP3* promoter, TGF-β sensor and Treg-cell-specific demethylation region (TSDR). Accessibility of these loci to transcription factors in FOXP3$^-$ Tresps (A), TGF--induced FOXP3$^+$ cells (B) and stable FOXP3$^+$ Treg cells (C) are depicted. Adapted from Huehn et al[70].

2.3.2 Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) and Glucorticoid-Induced TNF Receptor (GITR)

CTLA-4 (CD152) is a receptor related to CD28 that displays approximately 30% homology with CD28 at the protein levels and binds the same ligands, B7-1 and B7-2, on APCs[72]. CTLA-4 is not expressed in the naive and resting state but it is upregulated upon T-cell activation in a CD28-dependent manner. On the other hand it is constitutively expressed in Treg and represents

one of the hallmarks of this cell subset. CTLA-4 KO mice develop a lymphoproliferative disease characterized by massive T-cell activation and expansion that leads to multi-organ infiltration and causes the animal death within two-to-four weeks after birth Then It was showed using mAbs treatments blocking CTLA-4-B7 interactions[73] that CTLA-4 is important for both the induction of anergy and the maintenance of T-cell unresponsiveness. To date is well established that CTLA-4 plays a critical role in the suppressive function of Treg even though compensatory mechanisms exist in CTLA-4-deficient mice allowing the generation of functional Treg that control immune responses through alternative pathways. These compensatory mechanisms are not always sufficient to generate Treg populations capable of controlling effector T-cell function. Indeed, Schmidt *et al*[74] recently reported that ovalbumin-specific CTLA-4-deficient Treg were unable to control disease in an adoptive transfer model of autoimmune diabetes into recipients expressing ovalbumin in pancreatic islets. It is unclear why CTLA-4-deficient Treg cells work in some settings and not others *in vivo*, but it could be due to the model used and/or the mechanisms of action required to control different disease. Moreover, it has recently been suggested that CTLA-4 could dampen the homeostasis of Treg by regulating their expansion since defective CTLA-4 signals following mAbs blockade or in CTLA-4-deficient cells led to amplified proliferation of Treg[74]. In this regard, it is intriguing that a 30–40% increase in the frequency of $CD4^+CD25^+$ Treg was observed in the blood of healthy individuals homozygous for a single nucleotide CTLA-4 polymorphism associated with decreased susceptibility to autoimmune disease[75].

First cloned in 1997 following treatment of a hybridoma T cell line with dexamethasone[76], GITR is a member of the TNF receptor (TNFR) superfamily which is expressed on components of both the innate and adaptive immune systems, including NK and T cells[77]. Its natural ligand, GITRL, is a member of the TNF superfamily (TNFSF) and is expressed on endothelial cells and antigen presenting cells (especially macrophages, DC and B cells). GITR acts as a T cell co-stimulatory molecule upon encounter with GITRL-expressing APC presenting cognate antigen, leading to enhanced T cell proliferation and cytokine production[78], with the ability to substitute for CD28 co-stimulation to rescue T cells from CD3-induced apoptosis. At the beginning GITR was considered a perfect Treg marker cause of its high expression in Treg, abrogation of Treg suppression by GITR neutralisation[79] and its induction upon *Foxp3* transduction of $CD4^+CD25^-$ cells. However the starting enthusiasm diminished after the observations that GITR can be

expressed and up-regulated in non-regulatory $CD4^+$ cells upon activation and that GITRhi $CD4^+CD25^+$ cell lines are not suppressive[79]. This is supported by in vivo data cause Treg from GITR KO-mice still retain suppressive function[78].

2.3.3 IL-7 receptor α-chain (CD127)

IL-7 is a member of a cytokine family which includes IL-2, IL-4, IL-9, IL-15 and IL-21 whose receptor complex utilises a common chain (CD132) in association with a cytokine-specific high affinity chain, for example CD25 for the IL-2 receptor and CD127 for the IL-7 receptor. IL-7 is essential for T cell development since IL-7 knockout mice exhibit severe T cell deficiency[80]. CD127 is a naive T cells marker, lost from the cell surface upon T cell activation and then re-expressed by memory cells[81]. Both naive $CD4^+$ $CD8^+$ cells exiting from the thymus have low expressions of receptors for IL-2 and IL-5 but they are critically dependent on the presence of IL-7 for survival[82]. Recent evidence has also implicated CD127 as a possible biomarker of Treg as the combination of CD4 and CD25 together with low expression of CD127 identifies a group of peripheral blood T cells which are highly suppressive in functional assays and the highest expressers of FOXP3[83]. Indeed, CD127 is a direct FOXP3 target, which may reflect the mechanism of the inverse correlation between levels of CD127 and FOXP3. The utility and drawbacks of CD127 as a unique Treg marker have yet to be fully evaluated; nevertheless, the combination of $CD4^+CD25^+$CD127lo surface markers appear promising as identifiers of Treg populations. For this reason CD127 is very important because it could be used in clinical trials to isolate a high pure Treg population .

2.3.4 Galectins

Galectin are lectins which bind β-galactoside. Using transcriptomic and proteomic assays to identify Treg-markers, two separate groups described expression of members of the galectin family on Treg cells .Galectin-1 is expressed by Treg cells at baseline and upon TCR

engagement in both mice and humans[84]. Galectin-10 was also identified as constitutively expressed by Treg cells[85]. Antibodies blocking this molecule also abrogated Treg suppression *in vitro*. Interestingly, Treg cells from galectin-1 knockout animals are numerically normal but functionally less suppressive than their wild-type counterparts. However, expression of galectin-10 is restricted to intracellular compartments, which renders galectin-10 unsuitable to enrich viable nTreg populations.

2.3.5 Other regulatory T cell markers

Others Treg-associated surface markers have also been described in literature but they generally identify sub-population of Treg. One of this is HLA-DR expressed on a subpopulation of Treg cells including approximately 20-30% of human $CD4^+CD25^{hi}$ T cells[53]. Opposite to the HLA-DR negative counterpart HLA-DR^+ $CD4^+CD25^{hi}$ T cells suppress via an obligate contact-dependent mechanism. However, these Treg populations suppress both proliferation and cytokine production by target T cells. The inducible T cell co-stimulator ICOS (CD278) has been described on a subpopulation of both thymic and peripheral $FOXP3^+$ cells[86]. ICOS is a member of the CD28 and CTLA-4 family of co-stimulatory molecules and it is expressed on the surface of activated T cells where it forms homodimers upon engagement of its ligand, (ICOSL, CD275) playing an import role in cell-cell signalling and immune responses. In murine system, ICOS was found to be an important marker of induced $CD4^+$ Treg[87] and plays indispensable roles in induction and maintenance of immune tolerance besides its function in regulating the differentiation of effector T cells. Murine $CD4^+ICOS^+$ Treg demonstrated better survival, proliferative, and even suppressive abilities than their $ICOS^-$ analogues. The expression of ICOS was recently found to promote the generation, drive the activation and improve the function of human $CD4^+$ Treg as well[88]. In clinic, $ICOS^+CD4^+$ Treg has been identified in type I autoimmune pancreatitis patients and found to help ameliorate the disease severity. The expression of ICOS on human Treg cells seems to direct these cells toward different cytokine profiles as $CD4^+CD25^+ICOS^+$ T cells that produce both IL-10 and TGF-β, whereas $CD4^+CD25^+ICOS^-$ T cells produce only TGF-β[86].

2.4 Regulatory T cells and chemokine receptor

As members of the T cells subset, Treg traffic to different parts of the body at different stages of development, maturation, and activation. This trafficking is mediated by cytokines, integrins, chemokines, and matching receptors. Chemokines are divided into the CXC group and the CC group, so named for the patterns of cystine residues in their binding domains. They are characterised by a basal affinity for a wide range of chemokine receptors, but only few of them display high affinity. Mailloux et al[89] reviewed the stimuli that selectively recruit Treg over conventional effector populations

Chemokine/Integrin	Receptor	Context	Species
CCL19, CCL22	CCR4	Thymus, HC	Human
CCL1	CCR8	Thymus, HC	Human
CCL19	CCR7	Secondary LN	Human
CD62L		Secondary LN	Mouse
	CCR2	Secondary LN	Mouse
	CCR5	Secondary LN	Mouse
CXCL13	CXCR5	B-cell Follicles	Human
CCL4	CCR5	*Paracoccidioides brasiliensis*	Human, mouse
αE (CD103)	E-cadherin	*Leishmania major*	Mouse
CCL28	CCR10	Liver	Human
CXCL12	CXCR4	Bone marrow	Mouse
CCL22	CCR4	Ovarian cancer	Human
CCL17, CCL22	CCR4	Hodgkin's lymphoma	Human
CCL17, CCL22	CCR4	Non-Hodgkin's lymphoma	Human
CCL17, CCL22	CCR4	Gastric cancer	Human
CCL17, CCL22	CCR4	Esophageal squamous cell carcinoma	Human
CCL22	CCR4	Breast cancer	Human
CCL17	CCR4	Breast cancer	Mouse
CCL22	CCR4	Lewis lung carcinoma	Mouse
CCL2	CCR4	Glioma	Human
CCL3, CCL4, CCL5	CCR5	Pancreatic adenocarcinoma	Mouse
CCL20	CCR6	Epstein-Barr virus + Hodgkin's lymphoma	Human

Table 1 Chemokine receptor expressed by Treg. Derived from Regulatory T-cell Trafficking: From Thymic Development to Tumor-Induced Immune Suppression[89]

nTreg emigrate from the thymus into the periphery and secondary lymph tissues so, it is evident that they express different chemokine receptor on the basis on their activation status. In contrast to nTreg, inducible Treg emerge from conventional effector populations in response to stimuli associated with a suppressive or otherwise altered microenvironment. These cells also traffic in response to network of migratory signals, although in many cases the specific factors and receptors involved may be distinct from those for nTreg[90]. Treg development may be accomplished both directly and indirectly by epithelial cells within the Hassal's corpuscles (HC) in the thymus. Epithelial-derived factors such as the EBI1-ligand chemokine (CCL19) and the macrophage-derived chemokine (CCL22) are highly expressed in distinct subsets of the medulla such as the HC, where high levels of Treg congregate. Moreover, HC epithelial cells produce a cytokine milieu that is distinct from thymic stroma, which greatly influences other resident cell populations, in fact, DC activated in this area produce Thymus and activation-regulated chemokine (TARC) and MDC[91]. Both have high affinity for CCR4 and can induce selective chemoattraction of Treg through this receptor. If CCR4 is expressed on developing thymic Treg, its expression is not as prominent as CCR8 which is primarily associated with the chemokine CCL1/I-309. Another chemokine of interest in thymic Treg trafficking is SDF-1 (stromal-derived factor-1) and its cognate receptor CXCR4. However, CXCR4 expression is not limited to thymic Treg since SDF-1 may recruit early thymocytes to the HC, for this reason is not considered selective for Treg. However, more comprehensive Treg-migration studies would be needed to better understand Treg-trafficking signals within the thymus. Polyclonal and highly reactive to self-peptides, naive nTreg emigrate from the thymus and traffic to secondary lymphoid tissues, where they can exert suppressive effects. Chemotaxis studies demonstrate that naive nTreg migrate toward CCL19 in a CCR7-dependent manner, and that this migration results in Treg homing to the T-cell zones in lymph nodes. Essential for Treg role is the CD62L. Animal model using antibody blocking CD62L showed a reduced Treg numbers in draining lymph nodes but not in the spleen[92]. Although not essential for the suppressive ability CD62L is essential to direct Treg in lymph-node where their localisation is critical for the regulation of immune homeostasis. Activated Treg in the periphery trafficked to draining lymph nodes in a CCR2- and CCR5-dependent manner. However CCR2 and CCR5 involvement was not studied in conventional T-cell migration. Upon activation, nTreg lose expression of CCR7 gaining the expression of CXCR5. At this level they are chemotactically responsive to CXCL13[93] essential to recruit B cells to the B-cell zones in lymph nodes[94]. Here Treg are unique among T cells since

they localise in B-cell follicles and can act inhibiting B-cell activation, expansion, and antibody production. Once mature, Treg migrate and take up residence in peripheral tissues.

The majority of studies regarding Treg migration to the periphery are in the context of inflammation, infection, cancer and induction of transplant tolerance. However not a lot is known about the adoptively transferred Treg traffic, or how this subset could be manipulated to maximise their therapeutic potential in the transplant setting because Treg in the wrong place may provide undesirable immune response to the graft, but it may also prevent beneficial immune responses to pathogens. Interference of the CCR7 and CD62L was shown to abrogate tolerogenic function in a mouse cardiac allograft model in which tolerance was induced by donor-specific infusion and anti-CD40 ligand[95], and in an islet allograft model with adoptive transfer of Treg. Animal studies[96] used adoptive Treg transfer in a mouse islet allotransplant model, to show that Treg first migrate into the target tissue, where they become activated, and then traffic to the draining lymph node. The first step is dependent on Treg expression of P-selectin and E-selectin ligands as well as CCR2, CCR4, and CCR5, whereas subsequent migration to lymph nodes requires CCR2, CCR5, and CCR7. Treg trafficking cues in allotransplant tolerance models may vary from model to model in fact a study, using a rat model showed that in kidney allotransplant model tolerant recipients demonstrated a CCL5 gradient between graft and circulation and that (endogenous) Treg expressing the CCL5-receptor CCR5 were markedly more numerous in tolerated grafts[97]. In contrast, using the same rat strain combination, tolerance to cardiac allotransplant appeared not to involve CCL5-dependent Treg recruitment to the graft. Others reports focus their attention on CCR4 and its ligand. Muller *et al.* reported that immobilisation of CCL17, CCR4 ligand, on graft endothelium, was able to enhance recruitment of host Treg to pig islet xenografts[98]. An alternate approach to over-expressing a defined chemokine in the graft is to select subpopulations of Treg expressing subsets of chemokine receptors that will control the trafficking of adoptively transferred cells to specific sites of inflammation. For example, Treg that express the Th1 associated transcription factor TBX21, express CXCR3 and traffic to sites of Th1-mediated inflammation to suppress Th1 cells[99]. Similarly, Treg have been shown to 'imitate' Th2 or Th17 cells in order to suppress their responses[100],[101]. The pertinence of this concept has recently been demonstrated in humans, in that CXCR3-expressing Treg were shown to be more potent than $CXCR3^-$ Treg in suppressing IFN-γ production in mixed lymphocyte reactions. These data suggest that if the major rejection-

driving Th-cell lineage and its chemokine receptor profile are known, then an appropriate subpopulation of Treg might be selected on the basis of the trafficking receptors they express.

2.5 How regulatory T cells Work

Treg are involved in the control of immune tolerance and homeostasis for this reason they have developed several mechanisms to exert their suppressive function on different cell subsets like $CD4^+$ (Th1, Th2 and Th17) $CD8^+$ T cells, macrophages, dendritic cells (DCs) natural killer (NK), NKT cells and B cells. In order to suppress, Treg need to be activated via their TCR but after that the suppressive function is completely antigen non-specific. To date the discussion about Treg-suppressive mechanisms is still opened cause of the different evidences concerning contrasting results obtained from in vivo and in vitro experiments. From a functional perspective the various potential suppressive mechanisms could be divided in four "modes of action": (Figure 4)[102].

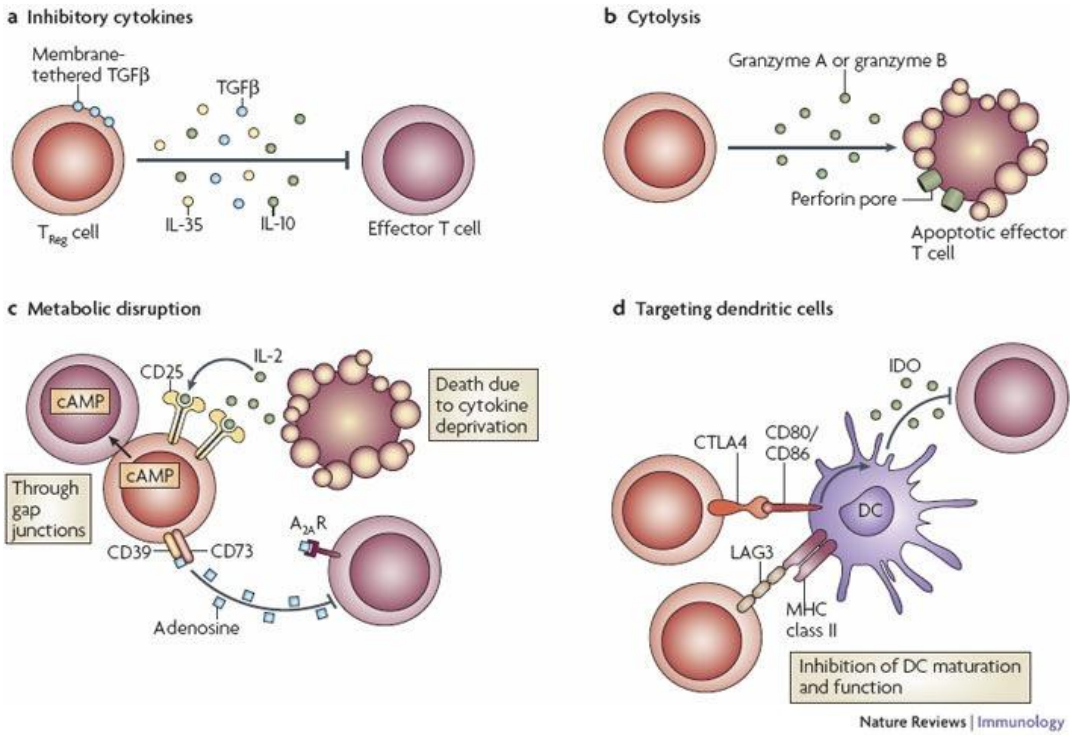

Figure. 4 Mechanisms used by Treg cells. Here are depicted the main mechanisms used by Tregs that could be divided in 4 groups: a) Suppression by inhibitory cytokines, b) suppression by cytolysis, c) suppression by metabolic disruption and d) suppression by targeting DC. From "How regulatory T cells works"

2.5.1 Suppression by inhibitory cytokines.

Immunosuppressive cytokines, IL-10 and TGF-β, were considered essential for Treg function; however to date, the role of these cytokines in Treg-mediated suppression is still incompletely understood. In fact there are contrasting results from *in vivo* and *in vitro* experiment; neutralization of either IL-10 or TGF-β does not abrogate *in vitro* Treg suppression but, in contrast, IL-10 and TGF-β contribute, at least in part, to the *in vivo* suppression of IBD induced in mice by Treg depletion[103]. Moreover it should be considered that cytokine secretion seems to be a mechanism involving principally iTreg suppression activity. In fact Tr1 cells modulate immune responses mainly through the secretion of IL-10 and TGF-β[104] inhibiting T-cell responses by suppressing IL-2 and IFN-γ[105] and by preventing T-cell proliferation[106]. IL-10, locally released by activated Tr1 cells, also acts on APCs, by down modulating co-stimulatory

molecules and production of pro-inflammatory cytokines[107] and on B cells by promoting isotype switching[108]. Some reports shown that in allergy and asthma models both nTreg and iTreg are involved in disease-control using a mechanism involving IL-10 and TGFβ. Two recent studies also delineate that IL-10R signaling is needed in Treg as well as in Th17 cells in order to suppress colonic Th17 responses[109]. In conclusion, the discrepancies found when comparing different studies indicate that the production of IL-10 by Treg and the role of IL-10 in Treg-mediated suppression is most likely dependent on the microenvironment in which the Treg cells are activated but also on the experimental model. Transforming growth factor-β (TGFβ) is an important regulator of T cell tolerance through the induction of Treg cells in the periphery but now a study from Ouyang, et al has shown that it also functions in the thymus where it protects deletion-thymocytes and promotes natural T_{Reg} cell development and homeostasis. When we look at the role of TGF-β in Treg-mediated suppression we can find different results. Several studies, both in mice and humans, have used neutralising antibody against TGF-β or KO mice shown that the suppressive capacity is independent of TGF-β because the immnesuppresive capacity wasn't reversed[110]. Interestingly, the surface bound TGF-β has an essential role in the suppression, possibly by acting directly on the responder T cells or DCs. Nakamura K and colleagues[111] demonstrated that after activation (by plate-bound anti-CD3 and soluble anti-CD28) $CD4^+CD25^+$ T cells produce and express on their membrane high levels of transforming growth factor TGF-β1. This, plus their evidence that a soluble factors doesn't mediate suppression, suggests that $CD4^+CD25^+$ T cells exert immunosuppression by a cell–cell interaction involving cell surface TGF-β1.

IL-35 is a recently discovered cytokine, this is the youngest member of the IL-12 family the only one with a purely immunosuppressive role. IL-35 was identified in the mid-2000s by Dario Vignali and colleagues, and was soon considered a potent mediator of suppression secreted principally by Treg. IL-35 is a heterodimer composed of the p35 and Ebi3 subunits, over-expressed by Treg and not effector cells. Several works show how this cytokine is essential for the maximal suppresive activity of murine Treg since deficiency in one of the two IL-35 chains had reduced suppressive ability *in vitro* and *in vivo* in an IBD model. The difference between this and the other immunosuppresive cytokine concern the ability to differentiate naïve human T cells in a new population called iT_R35 that mediated suppression via IL-35 but did not require IL-10, TGF-β, or Foxp3[112]. Although naïve human Treg did not express high amounts of IL-35,

long-term activation of human Treg led to upregulation of the IL-35 subunits starting at 3 days of activation[113]. These long-term activated Treg exerted contact-independent *in vitro* suppression in an IL-35-dependent manner and also induced iT_R35 cells. Thus, IL-35 has been proposed as one of the infectious tolerance key players.

2.5.2 Suppression by cytolysis.

This process is mediated principally through the secretion of granzymes, a family of serine proteases inducing apoptosis and playing a significant role in the immune defense against viruses, tumours and intracellular bacteria. These are stored in granules that fuse with the plasma membrane of the target cells and release their content which also include perforin. This forms transmembrane pores on the plasma membrane that allows the diffusion of granzyme into the cytosol where they initiate caspase-dependent and independent apoptotic pathways, which rapidly lead to target cells death. These, were considered for a long time a specific products of natural killers cells and $CD8^+$ cytotoxic T lymphocyte (CTLs) till the discovered in 2005 that Treg from grazyme-B-deficent mouse had a reduced suppressive activity in vitro cause a reduced apoptosis granzyme-B dependent[114]. Later this data were confirmed by others studies showing the importance of granzyme-B and perforine secreted by activated Treg in killing B-cells and suppressing the ability of NK and CTLs to kill tumour cells.

Although granzyme and perforin are considered the main molecules involved in Treg-induced apoptosis others mediators are found to be involved in this mechanism for instance tumour-necrosis-factor-related apoptosis-inducing ligand–death receptor 5, galectin-1 and 10.

2.5.3 Suppression by metabolic disruption

To date is well established that IL-2 is essential for T-cell activation, proliferation, and death[115]. In contrast to conventional T-cells, T_{reg} highly express the IL-2R α-chain (CD25), β-

chain (CD122) and γ-chain (CD132) and have STAT5 phosphorylation in the steady-state. For these reasons they are extremely dependent of IL-2 signalling, indeed KO-animals for IL-2, CD25, or CD122 present severe autoimmunity[57]. Since Treg constitutively express CD25, they can sense and capture IL-2 as soon as it is produced, so the presence of Treg could reduce IL-2 availability and limit T responses. Indeed, the presence of Treg leads to substantial competition for IL-2, resulting in impaired proliferation of T cells *in vitro* and *in vivo*[116],[117]. In addition Treg can control IL-2 production acting in the same time on T cells and dendritic cells (DCs). DCs activation is blocked by the high CTLA-4 Treg levels as explained below. Importantly, the amount of CTLA-4 expressed on Treg is again regulated by IL-2 signals. So it's safe to say that Treg control the level of co-stimulation through CD80/CD86 surface expression not only during steady-state[118], but, importantly, also during highly inflammatory processes.

The occurrence of pathological events, such as inflammation, promotes a massive accumulation of extracellular ATP, which is consider a Key danger signal triggering pro-inflammatory responses. After activation by TCR, Treg upregulate CD39 which metabolize ATP in AMP, later this is degraded in adenosine by CD73. Treg-derived adenosine, activates Adenosine 2a (A_{2A}) receptors expressed on T effector cells, which undergo reduced immune activity. In addition, adenosine generation triggers a self-reinforcing loop of Treg function because the stimulation of A_{2A} receptors, expressed on these cells, elicits their expansion and increases their immunoregulatory activity. Treg cells can also transfer the potent second messenger cyclic-AMP to T-effectors via gap-junction reducing their proliferation, differentiation and IL-2 synthesis.

2.5.4 Suppression by modulation of dendritic-cell maturation or function

Treg cells, both in vitro and in vivo, not only act on T cells function but even on their differentiation and activation modulating the antigen presenting cells and consequently T effectors indirectly. Treg cells are the only T-subpopulation that constitutively expresses CTLA-4; this molecule has like target CD80 and CD86, molecules expressed by DCs and important for the co-stimulatory pathway. To date is well established that CTLA-4 interaction with CD80 and

CD86 blocks their increasing expression post DC-activation or even downregulates their expression induced by antigen-specific effector cells[119]. In fact CTLA-4-deficient Treg from mice aren't able to prevent CD80/CD86 expression like the wild-type counterpart[120] while the opposite results is obtained using CTLA4-specific blocking antibody. In this way Treg cells disrupt the CD28-pathway acting on the interaction between T and DC cells. One mechanism that may mediate the down-regulation of CD80 and CD86 expression is trogocytosis, a process in which lymphocytes extract surface molecules through the immunological synapse from the APC to which they are conjugated. Interestingly, CTLA-4 deficiency doesn't alter the development or homeostasis of Treg cells or render them pathogenic but only invalidate their functional activity.

This one is not the only mechanism used by Treg to modulate DCs. Importantly, it was also shown that Treg could modulate the indolamine 2,3 dioxygenase (IDO) a potent immunosuppressive molecule involved in the tryptophan catabolism. IDO activation leads to tryptophan deprivation and the accumulation of kynurenine metabolites. The consequences of IDO activity include restriction of antimicrobial growth and multiple immunoregulatory effects on T cells like inhibition of T cell proliferation, conversion of naïve T cells in Treg and inhibition of the T helper type 17 (Th17) lineage. IDO induction was found to depend on high expression of CTLA-4 on the Treg cells[121].

Recent results have furthermore focused the attention on lymphocyte-activation gene 3 (LAG-3 or CD223). This is a CD4 homolog that binds MHC class II molecules with very high affinity. Binding of LAG-3 to MHC class II molecules expressed by immature DCs induces an ITAM-mediated inhibitory signal which involves FcγRγ and extracellular-signal-regulated kinase (ERK)-mediated recruitment of SRC-homology-2-domain-containing protein tyrosine phosphatase 1 (SHP1) that suppresses DC maturation and immunostimulatory capacity[122].

Fibrinogen-like protein 2 (FGL2) is a protein secreted by T cells, especially regulatory T cells, exerting an immunoregulatory property. Recombinant FGL2 inhibits T cell proliferation in response to anti-CD3 and anti-CD28 and alloantigen stimulation. To support this, Treg from *Fgl2*$^{-/-}$ mice are less suppressive than the WT and a polyclonal anti-FGL2 completely blocked the suppressive activity of Treg cells in a dose-dependent manner.

Neuropilin (Nrp-1) has been proposed to play a role in the interaction of Treg cells with DCs. Nrp-1 is preferentially expressed on Treg cells and can be induced by Foxp3 expression[123]. Nrp-1 promotes long interactions between Treg cells and immature DCs reducing the DCs avaibility. All these mechanisms could be used by Treg but several questions still remain. Do these mechanisms operate synergistically or concurrently? And are they site dependent?

A recent review from Yamaguchi et al[124]. has introduced the hypothesis that Treg can act differently on the basis of the immune status. During the physiological and non-inflammatory state Treg act to sustain natural self-tolerance reducing/eliminating activation signal from responder T cells while, during the inflammatory condition Treg mediate inactivation and killing of T responder cells and APC. So all the mechanisms involved in the CD28 pathway inhibition like CTLA-4-dependent down regulation of CD80/CD86 or IL-2 absorption and CD39/CD73-dependent degradation of ATP are involved to maintain the steady-state condition, and for Treg survival and function. However whit an activated immune system the previous mechanisms are abrogated by a big presence of co-stimuli; in this phase Treg start to produce immuno-suppressive molecules like IL-10, TGF-β, IL-35, granzyme, perforin and cAMP to directly and potently suppress the activation and expansion of responder T cells. Thus, the two modes or phases of Treg-mediated suppression mechanisms are differentially utilised in non-inflammatory and inflammatory conditions. It needs to be determined how these two modes of suppression operate in other strong immune responses such as transplantation of allografts.

2.6 Induced regulatory T cells

As discussed in the previous section, it is now clear that $CD4^+CD25^+$ Treg can be derived from two sources, namely those developing within the thymus and those generated in the periphery. Periphery Treg (iTreg) derive from the conversion of $CD4^+CD25^-$ T cells under specific stimuli including suboptimal dendritic cell activation, sub-immunogenic doses of agonist peptide, mucosal administration of peptide and presence of appropriate cytokines, notably TGF-β and IL-2. There are at present no clear phenotypic markers nor functional differences to distinguish nTreg from iTreg but the epigenetic differences between them could be

used only for their characterisation and not for their isolation. The mechanism(s) by which Treg are generated in the periphery are unknown. However, there are indications that, in the same manner as with Th1/Th2 cell polarisation, the antigenic stimulus may determine the commitment to a Treg phenotype[125]. Amongst the induced Treg one of the most studied population cause of its controversial, is the T helper3 (Th3) subset. This subset was described as an unusual Th2 like regulatory subset secreting TGF-β derived from orally tolerized animals induced by mucosal stimulation with antigen. It has been shown that Th3-mediated suppression is essential for the maintenance of oral tolerance, mechanism described as mediated by TGF-β secreted in response to CTLA4 ligation. Moreover the CTL4 signalling results important for the expression of the gut-homing receptor Lymphocyte Peyer patch adhesion molecule (LPAM) or alpha(4)beta(7) integrin. Th3 cells could be induced through cognate stimulation of pluripotent naïve $CD4^+$ T cells by APC together with CD86 co-stimulation, particularly in the presence of TGF-β and IL-4[29]. However in the gut other co-stimulatory signals are involved. The presence of anti-IL-12 or IL-10 may enhance the expansion of Th3 cells by decreasing Th1-type cells, which can inhibit Th3 expansion[126]. However since Th3-cell function is dependent on TGF-β, and other described regulatory T cells may also function through on TGF-β, there may be a common pathway that leads to a regulatory cell that is TGF-β dependent.

Tr1 cells are another T subset with regulatory function. They were firstly described in severe combined immunodeficient (SCID) patients who had developed long-term tolerance to stem cell allografts, suggesting that these cells might naturally regulate immune responses in humans[127]. IL-10 and Rapamycin Administration *in vivo* was also shown to induce Tr1 cells mediating tolerance in type 1 diabetic mice after pancreatic islet transplantation[128]. In humans, Tr1 are involved in inducing transplantation tolerance, as PBMC from patients who underwent islet transplant and became insulin independent produced significantly higher IL-10 when compared with transplant subjects that continued to be insulin-dependent[129]. Induction of Tr1 cells was also described in patients who spontaneously developed tolerance to kidney or liver allografts[130]. Taken together, these data indicate that IL-10-producing Tr1 cells can be induced under different states of transplantation tolerance and may be involved in inducing tolerance to allotransplants. Moreover, Tr1 cells have the advantage to be inducible *ex vivo* and therefore cells of the desired antigen specificity can be more easily generated. The big issue of this subset is the purity cause they lack of specific cell-surface markers. In fact, even a T-cell line highly enriched for Tr1 can

be contaminated with non-regulatory T cells and this might represent a significant caveat. Furthermore, it is unclear whether the phenotype of Tr1 cells generated *in vitro* is stable once they are transferred *in vivo* in humans. Importantly, Tr1 cells produce the immunosuppressive cytokine IL-10 only after activation with their specific antigen, but once activated, they can mediate some level of bystander suppression.

Maynard et al. have suggested another mechanism in which Tr1 may play a major role. This concern the maintaining of immune homeostasis to the intestinal microbiota[131], which is consistent with the finding that loss of IL-10 results in the development spontaneous enterocolitis in IL-10 deficient mice. To date it is well established that Tr1 cells may play a crucial role in suppressing autoimmunity, not only in experimental autoimmune disease models, but also in many human autoimmune diseases by suppressing tissue inflammation and maintaining self-tolerance.

Collison et al[112] recently demonstrated that natural Treg cells, but not resting or activated T_{conv} cells, express and secrete IL-35. This is a member of the interleukin-12 (IL- 12) cytokine family and is an inhibitory, heterodimeric cytokine having an α chain (a p35 subunit of IL- 12a) and a β chain (an Epstein Barr virus induced gene 3 subunit). Ectopic expression of IL-35 conferred regulatory activity on naïve T cells and recombinant IL-35 suppressed T cell proliferation. IL-35 selectively acts on different T-cell subset populations and is believed to mediate natural Treg cells' suppressive activity and thereby assist Treg cells in immune system homeostasis and tolerance to self-antigens. To date it is possible to culture in vivo or ex vivo an isolated population of T naive cells with an effective amount of exogenous IL-35 until the cells convert to display the regulatory phenotype. The cells can also be cultured with an effective amount of a T cell activating agent, such as an agent that activates a TCR. These cells can suppress T cells proliferation in vitro and act efficiently in different mouse models of *Foxp3*−/− mice, multiple sclerosis and immune bowel disease[112].

3. Regulatory T cells and Cell therapy

3.1 Rapamycin

Rapamycin is a macrolide identified in the early 1970s as a potent antifungal metabolite produced by *Streptomyces hygroscopicus.* This was found to inhibit cell proliferation and to have potent immunosuppressive activity. For this reason, rapamycin is currently used for the prevention of kidney transplant rejection[132] but even for the prophylaxis of graft rejection and GVHD. It acts on the Mammalian target of rapamycin (mTOR) a protein kinase that controls cell growth, proliferation, ribosomal biogenesis, vesicular trafficking, autophagy, cytoskeletal organization and cell size. mTOR is an atypical kinase containing a carboxy-terminal serine/threonine protein kinase domain a FRAP–ATM–TTRAP (FAT) domain and a C-terminal FAT (FATC) domain that might have a role in its structure and stability. Mammalian LST8 associates with the kinase domain of mTOR and is thought to facilitate mTOR signalling, but its precise role has yet to be defined. mTOR can form two distinct complexes; mTOR complex 1 (mTORC1) consisting in mTOR, LST8 and the regulatory associated protein of mTOR (RAPTOR) and mTORC2 formed by LST8 and rapamycin-insensitive companion of mTOR (RICTOR) and possibly MAPKAP1 (mitogen-activated protein kinase-associated protein 1). Rapamycin binding the immunophilin FK506-binding protein 1A (FKBP12) is able to block the *in vitro* and *in vivo* activity of the complex, potentially by disrupting the RAPTOR-mTOR interaction. This activity isn't exerted on mTOR2 even if recent data indicate that prolonged exposure to higher doses leads to inhibition of mTORC2 signalling as well[133].

As mTOR can acts on different cell pathways, I will focus my attention only on its immunological activity (Figure 5). The mTORC1 complex is a regulator of cell growth and other processes downstream of PI3K–AKT, WNT–GSK3 (glycogen synthase kinase 3) and AMP-activated protein kinase (AMPK) signalling. To date is clear how the dimer formed by tuberous sclerosis complex 1 (TSC1) and TSC2 act as upstream inhibitor of mTORC1. In the meantime, factors activating PI3K and subsequently AKT inhibit TSC2, by phosphorylating it, thereby negating the inhibitory effect of TSC2 on mTORC1. Others pathway can regulate negatively TSC1–TSC2 complex like for example RAS–MAPK (mitogen-activated protein kinase)

pathway. The inhibitory activity of the TSC1–TSC2 complex is mediated by inhibiting Rheb (RAS homologue enriched in brain), a RAS-like GTPase and a positive regulator of mTORC1. When mTORC1 inhibition is removed, it acts phosporylating S6 kinase 1 (S6K1) and the eukaryotic initiation factor EIF4EBP1 (eukaryotic translation initiation factor (EIF4E)-binding protein 1). This promotes mRNA translation and cell growth by enhancing the biosynthesis of the translational apparatus in the cell[134]. As rapamycin is a selective mTORC1 blocker we have a good knowledge about its signalling pathway, nevertheless this is not the same for mTORC2 because of the lack of an mTORC2-specific inhibitor. Only in the last few years, using siRNA specific for RICTOR, it has became apparent that mTORC2 is important in mammalian development and several cellular processes like cytosketelon modulation[135].

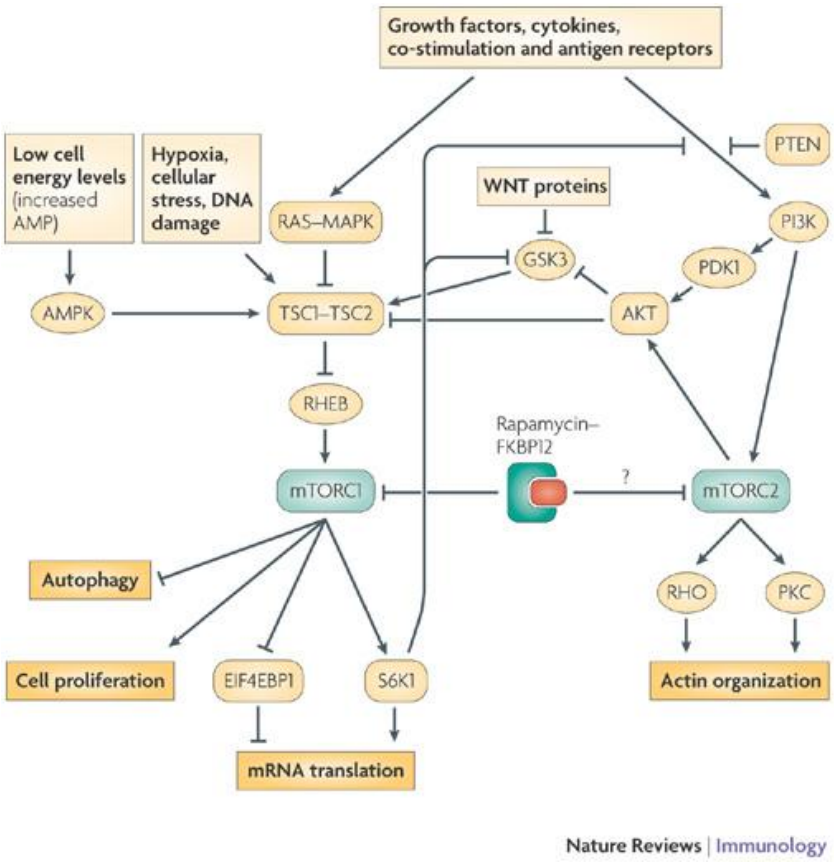

Figure 5. mTor pathway in immune cells. mTORC1 and mTORC2 signals are influenced by growth factors cytokines, co-stimulatory molecules and antigen receptors, WNT proteins, cellular energy levels, as well as hypoxia, cellular stress and DNA damage. Derived from "Immunoregolatory function of mTor"[136]

The mTOR pathway is essential for the activation of different mechanisms in the immune cells. In fact, the mTOR inhibition on DCs maturation and differentiation is well-documented both in vitro and in vivo. Monocyte-derived DCs are not able to up-regulate CD86 when cultivated in presence of rapamycin, and showed a decreased expression of of antigen uptake receptors[137] (such as CD32, CD46, CD91 and CD205).

To explain the potential role of mTOR in $CD4^+$ T cell differentiation, professor JD. Powell's group selectively knocked out mTOR in T cells[138]. Interestingly, KO-mTOR $CD4^+$ T cells fail to differentiate into Th1, Th2, or Th17 effector cells when cultured in appropriate conditions in vitro, rather they differentiate in Treg. Moreover these cells were accompanied by decreased STAT4, STAT3, and STAT6 phosphorylation in response to IL-12, IL-6, and IL-4, respectively. Pharmacological inhibition of mTOR signalling in naive $CD4^+$ T cells by rapamycin treatment also facilitates the development of $Foxp3^+$ Treg, and $Foxp3^+$ CD4 T cells exhibit lower levels of mTOR activity than their effector counterparts. Interestingly, although genetic deletion and pharmacological inhibition of mTOR signalling can result in the induction of a large population of $Foxp3^+$ regulatory CD4 T cells in the absence of high concentrations of exogenous cytokines, this process is still dependent on the low levels of TGF-β found in serum-containing media.

In murine animal model a prolonged rapamycin administration blocks the conversion of thymocytes from double-negative ($CD4^-CD8^-$) to double-positive T cells. However, although the absolute number of T cells is decreased, rapamycin administration does not alter the proportion of $CD4^+$single-positive cells that up-regulate their expression of FOXP3 in the thymus. This indicates that the ontogeny of natural Treg cells is unaffected by mTOR blockade[139]. Like the ontogeny, even the Treg activation is mTOR independent.

When stimulated in periphery by TCR and co-stimulatory molecules, T cells start to proliferate. The first checkpoint is progression from G0 to G1 phase of the cell cycle. For T cells and their progression in G1 phase the 2 activation signals are essential for PI3K and AKT engagement. When activated, they exert their action both on mTORC1 and mTORC2 complexes stimulating four pivotal essential processes for the progression from G0 to G1 phase. These are: increased mRNA translation, increased glycolysis and consequent ATP accumulation,

degradation of the cyclin-dependent kinase (CDK) inhibitor, p27, and promotion of IL-2 and IL-2R expression. The further step, from G1 to S phase, is regulated by CDK proteins. Essential for this step is the stimulation through TCR and CD28, together with autocrine signalling by IL-2 produced during progression through the first checkpoint. Of note, the PI3K–AKT–mTOR pathway is also involved in transmission of this IL-2R signal. So, mTOR-induced signalling might regulate the rate of initial cell cycle entry and the integration of sequential signals that are dictated by TCR and CD28 engagement and IL-2 production. Consequently, rapamycin blocks the T cells-cycle progression. However, it becomes dispensable at later stages, when large quantities of IL-2 are available. In these conditions, cell cycle progression by IL-2 seems to depend on the activity of the serine/threonine protein kinase PIM2[140].

As seen in the mice-thymus, rapamycin treatment doesn't affect Treg cell cycle progression and functional activity as well[141]. Interestingly, in kidney transplant recipients, only patients treated with rapamycin have a markedly increased frequency of $CD4^+CD25^+FOXP3^+$ Treg cells compared with total $CD4^+$ T cell numbers. The observation that rapamycin causes a generalized increase in the frequency of Treg cells is interpreted as the sum of two effects:

1. ability of Treg cells to proliferate in the presence of rapamycin;
2. promotion of FOXP3 expression in peripheral T cells that are then converted into modulators of immune reactivity.

1) The ability of regulatory T cells to proliferate when stimulated in the presence of rapamycin seems to be connected by two effects. First, Treg cells do not down-regulate phosphatase and tensin homologue (PTEN) expression after TCR engagement, which impedes the activation of the rapamycin-susceptible PI3K–AKT–mTOR pathway. Second, FOXP3 drives the expression of PIM2, reinforced by IL-2 and TCR-mediated activation of signal transducer and activator of transcription 5 (STAT5); PIM2 compensates for AKT inactivity and promotes cell cycle progression (Figure 6).

2) When $CD4^+CD25^-$ T cells are stimulated in vitro in presence of TGF-β and IL-2, the result will be the induction of FOXP3 expression[142]. This induction depend on the activation of the transcription factor SMAD3 (mothers against decapentaplegic homologue 3), which, together with TCR-induced NFAT, binds to the enhancer region of *FOXP3* and promotes chromatin

remodelling that is necessary for translation. The AKT–mTOR pathway is a negative regulator of SMAD3, for this reason mTOR inhibition through rapamycin favours FOXP3 upregulation. Interestingly, when rapamycin is present at the time of transduction of constitutively active AKT, the inhibitory effect is lost. This implies that a rapamycin-sensitive signal involving AKT activation (and consequently, mTOR activation) is responsible for the control of peripheral FOXP3 induction. This effect is not restricted to FOXP3 and extends to approximately 50% of the genes that are modulated in response to TGF-β[142], which supports the view that conversion to a regulatory phenotype involves complex genetic reprogramming

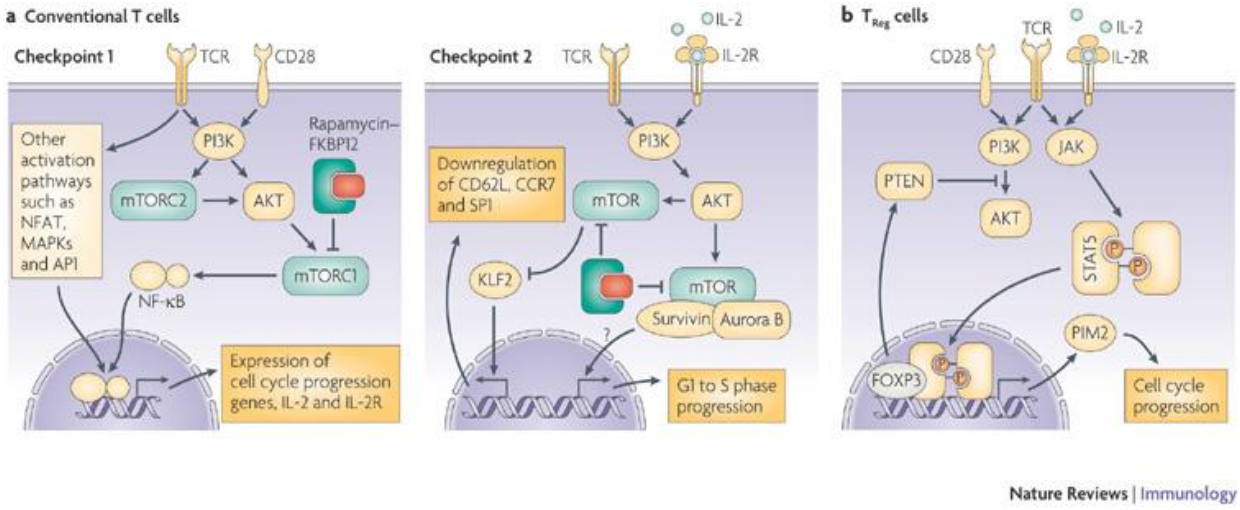

Figure. 6 Influence of mTOR pathway on cell cycle progression of T cells. **a)** In conventional T cells, mTOR integrates TCR and CD28 signals necessary to pass the 2 checkpoints of T cell activation; transition from G0 to G1 phase (checkpoint 1v) and transition from G1 to S phase (checkpoint 2) of the cell cycle. **b)** Ability of Treg to proliferate when stimulated in the presence of rapamycin depends principally by the FOXP3-driven expression of PIM2, reinforced by IL-2- and TCR-mediated activation of STAT5. Derived from "Immunoregolatory function of mTor"[136]

3.2 Pre clinical studies with regulatory T cells

Several preclinical animal studies have established that the adoptive transfer of regulatory T cells can prevent various autoimmune diseases, acute and Chronic Graft versus Host disease and

favour the tolerance of graft transplantation. Autoimmune diseases are characterised by an activation of the immune system against self-antigen and for this reason Treg infusion inhibits ongoing T-cell responses and reverse established pathology. To date several pre-clinical studies are published using Treg for the treatment of autoimmune disease but the most promising strategy include the use of transgenic Treg expressing a T-cell receptor specific for the pathogenic antigen[143],[144]. Overall these data indicate that the suppression of an ongoing autoimmune disease by Treg might only be feasible when these cells are specific for the pathogenic antigen. Alternatively, transfer of polyclonal Treg may cure ongoing disease only in lymphopenic hosts, in which the massive expansion could generate a sufficient number of antigen-specific Treg cells. From experimental data obtained in mice it is clear that Treg represent a potential new therapeutic modality for the control of GvHD. In a model of GvHD prevention using freshly purified Treg, Edinger et al[145] showed a strong inhibition of expansion of donor T cells. However, the addition of freshly purified Treg had minimal or no effect on the acquisition of activation markers and IFN-γ production. In contrast, expanded Treg strongly inhibited the division and expansion of donor T cells and their capacity to express activation markers and produce IFN-γ. It was demonstrated that the in vitro activation of Treg led to an increase of their suppressive activity[146] and probably this could explain why expanded Treg seem to have a more profound suppressive effect on the activation of donor T cells in an HSCT setting. GvHD is due to the early expansion and differentiation of alloreactive donor T cells. Because expanded Treg potently inhibit such activation, this explains their capacity to prevent the disease. There are some differences in the intensity of the effect of donor specific and polyclonal Treg. The former one have better effects rely on their increased ability to rapidly become activated in vivo and exert their immunosuppressive effect early after infusion. In support of this concept, it was recently showed that delaying Treg injection after day 4 was ineffective in treating GvHD[147]. Moreover, they also could display a suppressive effect mainly targeted to alloreactive donor T cells, whereas non-alloreactive donor T cells could be partially spared. The use of donor specific Treg dramatically reduced the expansion/differentiation of donor T cells and the percentage of IFN-γ-producing cells as well. The use of polyclonal vs antigen-specific Treg for the control of GvHD in allogeneic HSCT is an area of debate of intense interest. However, to date, we have only data from animal phase 1 trials using polyclonal expanded Treg. From animal data is possible infer that the use of high numbers of polyclonal Treg might achieve the same level of GvHD inhibition and promotion of immune reconstitution.

In fact, studies by Taylor et al[148] demonstrated that by increasing the number of polyclonal-Treg (three Treg per infused allogeneic T cell), nearly complete inhibition of overt GvHD was durably achieved despite the presence of histopathological disease signs in target organs. Compared with mice receiving 10×10^6 Treg, the injection of 50×10^6 polyclonal Treg improved mainly immune reconstitution. However, in this latter group, immune reconstitution was less efficient as compared with mice receiving 10×10^6 antigen specific Treg. Another aspect that has to be considered is the maintaince of the GVL; mouse model showed that both plyclonal and antigen-specific Treg are able to maintain this essential mechanism[145],[149], however this has to be studied in clinical trials.

Treg are important even in the induction of tolerance to transplanted organs. They are present in tolerated allografts[150], moreover animal data support this assertion through the demonstration that tolerance to a fully-mismatched transplant can be induced in the absence of pharmacological immunosuppression by Treg infusion[151]. The importance of Treg during the encounter with allogeneic tissues is perhaps best highlighted during pregnancy where a significant barrier to successful outcome is foetal carriage of paternally derived histocompatibility antigens. Foetal tissues are tolerated despite the persistence of maternal T cells alloreactive to paternal antigens throughout pregnancy[152]. This is the result of a complex interaction between foeto-maternal immune systems[153] that includes a significant increase in the proportion of Treg, both locally (uterine) and systemically (spleen and lymph node). Since the primary function of Treg is the maintenance of self-tolerance, the question is whether their alloreactivity can confer an advantage in the context of transplantation following presentation of allogeneic antigen via direct, indirect or semi-direct pathway. Donor-specific Treg *can* prevent allograft rejection in murine transplant models[154,151,145] through a predominant effect on the indirect alloresponse[155]. The importance of specificity is suggested by experiments in which allospecific Treg of ABM mice (transgenic for a TCR specific for an intact class II molecule (I-Abm12)) could completely suppress rejection of cardiac allografts bearing their cognate but not third party antigens. These observations are supported by studies in autoimmune models showing that stable associations between Treg and DC precede inhibition of helper T cells[156], suggesting that the APC, and cognate specificity, is central to the *in vivo* function of Treg. Induction of antigen-specific tolerance to fully allogeneic grafts by recruitment of Treg can also be achieved by administration of allogeneic splenocytes or MHC molecules via the mucosal route[157]. Absence of administered

splenocytes from local lymph nodes in these experiments suggests that Treg are recruited in an antigen-specific manner following presentation of the antigen via the indirect (or semi-direct) pathway[158]. While *in vivo* data demonstrate that indirect anti-donor allospecificity is a feature of Treg that mediate transplantation tolerance in experimental animals[159] and in human renal allograft recipients as well[160], *in vitro* observations indicate that Treg cannot be exclusively dependent on the indirect pathway of presentation since they are capable of suppressing the *in vitro* mixed lymphocyte reaction in which only direct allopresentation is available. Recent data resolves some of this dilemma by showing that Treg have both direct and indirect allosopecificity *in vivo* but that their regulatory function is several orders of magnitude more pronounced in allograft responses driven by the indirect pathway[161].

Treg are particularly attractive candidates for adoptive cell therapy to establish clinical transplantation tolerance particularly since they can be selected and expanded for a particular donor specificity *in vitro*[162]. However, human data in stable renal transplant recipients demonstrates that Treg do not significantly contribute to direct pathway hyporesponsiveness[155] and have a suppressive effect predominantly on the indirect anti-donor alloresponse[163] which is not surprising given the three cell model described in section 1.2. As a result, expanded populations of allospecific Treg[162] would not be expected to significantly alter the clinical response to the graft in the short term following transplantation without additional suppression of the direct pathway.

3.3 Clinical Trials with regulatory T cells.

To date, there are only few published results showing safety and feasibility of Treg infusion. However there are several ongoing clinical Trials evaluating the effects of this regulatory subset after solid organ transplantation, autoimmune disease and GvHD.

The strategies used so far include the infusion of freshly isolated Treg and the infusion of ex-vivo expanded Treg. The first clinical trial describing the infusion of expanded Treg was published in 2009[164]. They describe a procedure and first-in-man clinical effects of adoptive transfer of ex vivo expanded $CD4^{+}CD25^{+}CD127^{-}$ cells for the treatment of GvHD. However

they treated only 2 patients affected by acute and chronic GvHD. In the patient with chronic GvHD, the major goal was the waning of steroids, which was supposed to alleviate some adverse effects of these drugs such as diabetes, musculoskeletal pain, and progression of osteoporosis. They achieved not only a reduction of steroids, but also significant improvement in the bronchial obturation to the level that bronchodilatators could be ceased. The results were less optimistic in the case of acute GvHD. However, it has to be highlighted that Treg were administered relatively late.

In 2011 a clinical study were published by Brunstein et al.[165]. This could be considered the first phase 1 clinical study where the safety profile of umbilical cord blood (UCB) Treg was evaluated in 23 patients with acute GvHD. Patients received a dose of 0.1-30 × 10^5 UCB Treg/kg. No infusional toxicities were observed after infusion, and Treg were detected for 14 days, with the greatest proportion of circulating $CD4^+$ $CD127^{low}$ $FoxP3^+$ cells observed on day +2. Although this was only a phase I trial the authors affirmed that, compared with identically treated 108 historical controls, there was a reduced incidence of grade II-IV aGVHD (43% vs 61%, P = .05) with no deleterious effect on risks of infection, relapse, or early mortality.

In 2011 another trial was published by Di Ianni group[166]. The aim of this study was the infusion of freshly isolated Treg before HSCT to avoid the extensive ex vivo T-cell depletion of the graft, a mandatory procedure to prevent acute and chronic GvHD. This study demonstrated for the first time that adoptive immunotherapy with freshly purified $CD4^+CD25^+$ Treg counteracts the GvHD potential of a high number of donor Tcons in HLA-haploidentical HSCT. The surprising finding was the absence of GvHD in patients who received up to 1 × 10^6 Tcons/kg after an infusion of 2 × 10^6 Treg.

A new clinical trial from the University of Liegi (ClinicalTrials.gov Identifier: NCT01903473) is trying to assess the safety of the combination of donor Treg infusion and rapamycin administration (a non-standard immunosuppressor for this disease) in patients with steroid-refractory chronic GvHD. Patients who have refractory chronic GvHD will be eligible. They will be firstly treated with Rapamycin and after 3-4 weeks one infusion of $\geq$ 0.5 x10^6 Treg/kg is administrated. They are trying to take advantage of the rapamycin positive effects on Treg subsets increasing the Treg numbers usually reduced in c GvHD setting.

Treg adoptive infusion has been tested in patients affected by autoimmune disease since this subset of pathologies is characterised by a reduced number and/or defective Treg activity. In 2014 a study testing the infusion of ex-vivo expanded Treg in diabetes Mellitus type 1 has been published by a group from Poland[167]. They presented a prospective, non-randomised safety and efficacy pilot study of cellular therapy with *ex vivo* expanded $CD4^{+}CD25^{high}CD127$- Treg. The study aim was the restoring regulation in the immune system and the stop or at least delay the disease progress. Twelve Caucasian children with recently diagnosed diabetes were treated with *ex vivo* expanded autologous Treg. The general health and metabolic status of treated individuals were followed and compared at 4 and 12 months after infusion with 10 non-treated control patients matched for age, sex and disease duration. The administered Treg doses were: 10×10^6 Treg/kg in a single infusion (3 patients), 20×10^6 Treg/kg in a single infusion (3 patients), and 30×10^6 Treg/kg in two infusions (6 patients). No severe adverse effects were observed in all the patients and the infusion was followed by an increase in peripheral blood Treg number. Interestingly, in 8 patients, Treg administration resulted also in lower requirement for exogenous insulin with two children completely insulin independent at one year.

Treg are particularly attractive candidates even for adoptive cell therapy to establish clinical transplantation tolerance as well shown from pre-clinical studies. To date the most important clinical trials using Treg in solid organ transplantation are THE ONE STUDY (ClinicalTrials.gov Identifier: NCT02129881) and Thril (ClinicalTrials.gov Identifier: NCT02166177). They are a phase I clinical trials testing the safety and feasibility of Treg infusion in patients receiving respectively kidney and liver transplantation.

THE ONE STUDY is a cooperative project to establish whether purified cells with regulatory activity can be used therapeutically to reduce the level of pharmacologic immunosuppression needed by recipients of transplanted organs. To assess this, several regulatory cell products will be tested: natural ex vivo expanded Treg, Mregs, Tr1 cells and DCs. In detail, the section of this project involving the use of nTreg is coordinated by the Immunereguation laboratory of King's College London. Autologous regulatory T cells will be grown under GMP conditions and re-infused (1-10 million cells/kg) intravenously 5 days post renal transplantation. Recipients also receive prednisolone, mycophenolate mofetil, and tacrolimus.

ThRIL is an open label, randomised, controlled and single ascending dose study, investigating the safety and tolerability of Treg immunotherapy after liver transplantation. Patients will be treated with ATG at time of transplantation, followed by Tacrolimus. Rapamycin will be started at 2 months, with an infusion of Treg at 3 months post transplantation. Two Treg doses will be tested, a low, $1.0x10^6$cells/kg and high, $4.5x10^6$cells/kg. The cohort of patients receiving the dose which is safe and well tolerated will be expanded to investigate an efficacy signal, providing the evidence required to take the development of Treg immunotherapy towards a larger Phase II/III study.

ISOLATION, CHARACTERIZATION AND REGULATORY T CELLS MANAGEMENT

1. Introduction and Aims

Solid organ transplantation represents a life-saving treatment for patients affected by end stage organ disease. Thought patient and graft survival rates for liver transplantation (LT) have reached 90% after 1 years, there are some side effects due to long-term immunosuppression. Although modern immune suppressive (IS) drugs have progressively reduced the incidence of acute rejection and transplantation-related mortality, their administration is associated with major side effects and potentially lethal toxicity, such as opportunistic infection, damage of transplanted organs and secondary cancer. Indeed, secondary malignancies are one of the leading causes of late mortality in LT recipients. Therefore, new treatment strategies aiming to improve long-term graft survival, decreasing IS therapy, and, ideally, inducing tolerance are needed. In the last years, immunomodulatory cell therapy has emerged as a promising therapy for the induction of tolerance after SOT. The major challenge is to achieve operational tolerance, defined as the absence of pathologic immune response against a graft, in an immunocompetent host, without maintaining protective responses against third party antigens. In this regard, regulatory T cells (Treg) have emerged as promising candidates for immunoregulatory cell therapy Treg have a pivotal role in maintaining peripheral immunological tolerance, by preventing autoimmunity and chronic inflammation. In animal models, several groups have shown that the infusion of freshly isolated or expanded Treg can prevent graft versus host

disease (GvHD) and both acute and chronic organ rejection after solid OT. In humans, a few clinical studies have been published reporting the infusion of Treg for the prevention of GVHD and autoimmune disease [166],[167]. These are phase-I trials showing safety of Treg infusion, however there are no published results testing the efficacy of Treg in OLT. In addition, even though liver is a privileged organ and differs from the others cause lower incidence of rejection, immunosuppressive regimens are still required. Recent data have shown that IS withdrawal coincide with the increase in the frequency of $CD4^{+}CD25hi$ T cells and FOXP3 transcripts in operationally tolerant liver transplant recipients. However, to date, there aren't published results about freshly-isolated or ex-vivo expanded Tregs for the induction of tolerance in setting of solid organ transplantation. Moreover, the possibility to infuse cryopreserved Treg would highly improve the transplantation management and allow the use of these cells, for patients undergoing solid organ transplantation. So far, different cryopreservation and medium strategies have been reported in the literature, leading to inconclusive results[168,169,170]. For these reasons, the aim of this chapter is to characterize the circulating Treg in healthy donors and in patients with end stage liver disease from a waiting list for liver transplantation.

2. Material and Methods

2.1 PBMC separation

Peripheral blood (PB) of 8 healthy donors (HD) were provided by the "Centro Trasfusionale, Policlinico Sant'Orsola Malpighi" while samples from 12 patients (Pts) were provided from the liver transplant waiting list at the Sant'Orsola Malpighi Hospital (Bologna). Patients with autoimmune liver disease, HIV or RNA-positive Hepatitis C Virus infection were excluded. Blood was diluted 1 in 2 with PBS, before layered on top of 15mL of Ficoll-Hypaque (Amersham Bioscience, Piscataway, NJ), followed by centrifugation at 2000 rpm at 20ºC for 20 minutes with slow acceleration and no brake. Peripheral blood mononuclear cells (PBMC) were

derived from the harvesting of interface cells, followed by three washes with PBS at 2000 and 1800 rpm twice respectively for 10 minutes at 4 ºC with normal acceleration and deceleration.

2.2 Isolation of $CD4^+CD25^+$ T cells

$CD4^+CD25^+$ regulatory T cells were isolated using miniMACS $CD4^+CD25^+$ T Regulatory Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The kit contains a cocktail of biotinylated antibodies and anti-biotin micro-beads for depletion of non-$CD4^+$ T cells, and CD25 micro-beads for subsequent positive selection of the $CD4^+CD25^+$ regulatory T cells. PBMC were incubated with 90µl Staining buffer and 10µl of biotin antibody-cocktail /10^7 cells for 10 minutes, followed by 15 minutes incubation with anti-biotin micro-beads at 4 ºC. The biotin antibody-cocktail contained biotin-conjugated monoclonal anti-human antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and CD235a (glycophorin A). The cells were then washed once with Staining buffer (centrifugation 1800 rpm for 5 minutes at 4 ºC) and resuspended in 500µl Staining buffer/10^8 cells. An LD MACS separation column was prepared by a single rinse with 2mL Staining buffer and placed on a MACS separator magnet. The resuspended cells were passed over the column and the negative fraction (unbound $CD4^+$ cells) were eluted with a series of two x 1mL Staining buffer washes. Eluted cells were centrifuged and resuspended in 90 µl Staining buffer and 10 µl of $CD25^+$ micro-beads/10^7 cells and incubated in the dark for 15 minutes at 4 ºC. These were then washed once with Staining buffer (centrifugation 1800 rpm for 5 minutes at 4 ºC) and resuspended in 500 µl Staining buffer /10^8 cells. The cells were passed through an MS column and the negative selection ($CD4^+CD25^-$) eluted with a series of three x 500 µl Staining buffer washes. The column was removed from the magnet and the CD25-microbead-positive fraction, the $CD4^+CD25^+$ regulatory T cells, were eluted with 1mL of Staining buffer and firm column pressure from the plunger. After purification, the two separate fractions of $CD4^+CD25^-$ (Tresp) and $CD4^+CD25^+$ (Treg) were resuspended in RPMI 1640 medium (Lonza, Milan, Italy) supplemented with 10% heat-inactivated FBS (Life Technologies-Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 100 U/ml

penicillin, and 100 μg/ml streptomycin (MP Biomedicals, Verona, Italy). Treg were characterized by cytofluorimetric, functional and molecular analysis.

2.3 Freezing and thawing

Treg and Tresp were frozen in medium containing 50% AB plasma (healthy blood donor from Policlinico Sant'Orsola Malpighi), 40% CliniMacs buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) and 10% dimethyl sulfoxide (DMSO). Cells were cryopreserved using Planer Kryo 560-16 (Planer Middlesex United kingdom) in a controlled-rate cryo-freezer and stored at -196° C in liquid nitrogen as previously reported[171]. After one month, samples were thawed rapidly at 37°, transferred into a tube containing RPMI 1640 and then centrifugated once to remove DMSO. Viability was determined upon thawing by Propidium Iodide (PI) assay. Briefly, cells were stained with PI, a fluorescent dye that binds to the DNA of dead cells, for 10 minutes at 4 °C. According to cytofluorimetric analysis, the yield of viable Treg were calculated. Finally, cell concentration was adjusted to 10^6 cells/mL in RPMI 1640.

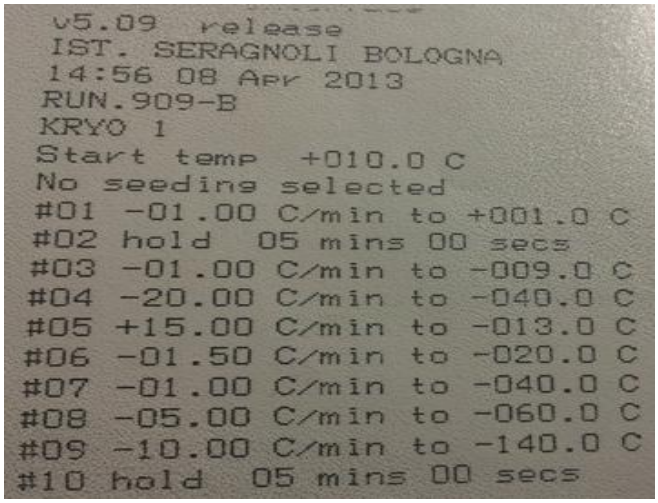

Figure. 1 Scheme of the cycle used to freeze Treg. The temperature goes down following the 10 steps procedure starting from $^{+}10°$ till -140°C.

2.4 Phenotypic analysis of regulatory T cells

The phenotype of Treg cells was analyzed by flow cytometry FACSCantoII (Beckton Dickinson). For cell-surface staining, the following conjugated monoclonal antibodies were used: anti-CD45 Per-CP (HI30), anti-CD25 Biotin (4E3), anti-Biotin APC; anti-CD4 FITC (MT-466), anti-CD127 PE (clone: MB15-18C9). All antibodies were purchased from Miltenyi Biotech. For intracellular staining, anti-human FoxP3 (PCH101) Staining Set PE Kit was used (eBiosciences), according to the manufacturer's instructions. Isotype control rat IgG2 PE was used as a control (eBiosciences). Briefly, cells were surface stained if required, washed once in PBS and then vortexed and incubated in 1mL of Fixation/Permeabilization solution for 30 minutes at 4ºC in the dark. Cells were then washed twice with Permeabilization buffer 1x and incubated with anti-human FOXP3 antibody for 30 minutes at 4 ºC in the dark. The cells were washed once with Permeabilization buffer 1x and resuspended in the same solution for FACS analysis. This phenotypic analysis was carried out on the PB of healthy donors and patients to evaluate number and percentage of circulating Treg. A lysis buffer from BD was used in order to lysate red blood cells. The same analysis was used on the 2 purified cell fractions after

immunomagnetic isolation, on both freshly and frozen cells to confirm the selected population. Lymphocytes were gated based on their forward and side scatter profile. The percentage of Treg was acquired by calculating the percentage of FoxP3^{+}cells within the CD4^{+} CD25^{+} double-positive cells. Recently, it was demonstrated that the IL-7 receptor CD127 is a reliable surface marker for Treg identification and isolation. CD127 is downregulated on all T-cells after activation, but not in effector and memory T-cells. For this reason, we can identify Treg with two different protocols. The first one allows the identification of CD4^{+} CD25^{+} FoxP3^{+} cells, while the second one avoids the need for intracellular staining and recognises the CD4^{+} CD25^{+}CD127^{-} populations.

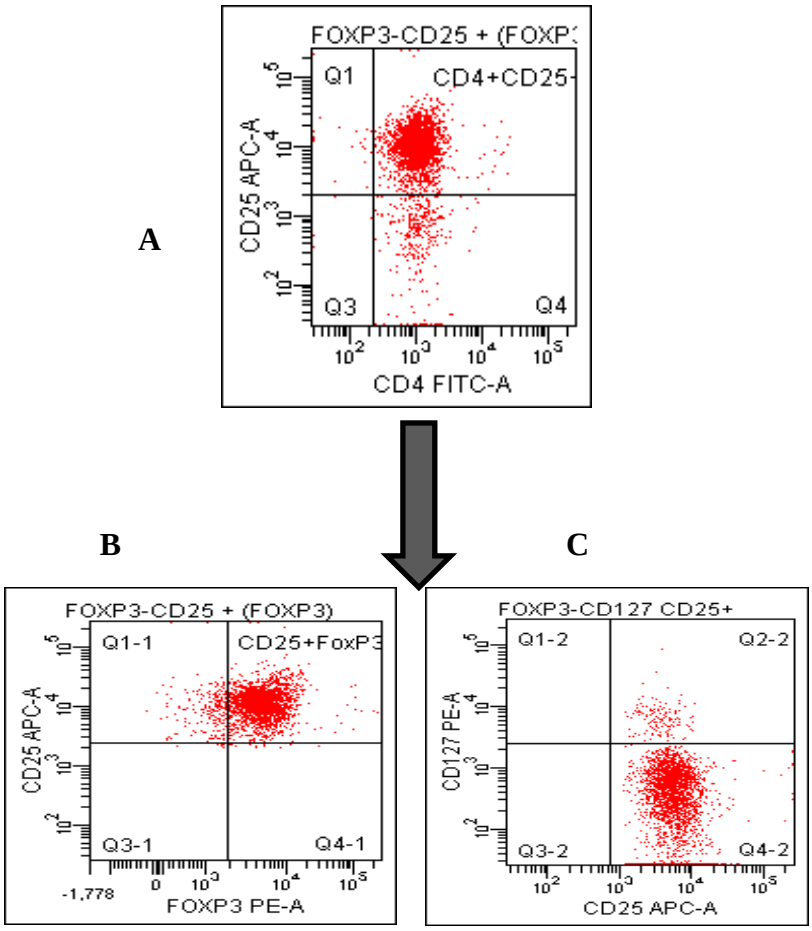

Figure. 2 Gating strategy used to characterize Treg cells pre and post thawing One representative experiment showing: **A)** Dot-plot gating CD4 and CD25 suface markers; **B)** Dot-plot gating CD25 and FOXP3; **C)** Dot-plot gating CD25 and FOXP3

2.5 Suppression assay

To assess the inhibitory capacity of freshly-isolated and cryopreserved Treg, we set up a Mixed Leukocyte Reaction (MLR). 10^5 Tresp were labelled with 5µ/mol Carboxyfluorescein succinimidyl ester (CFSE), according to the standard protocol. Next, they were co-cultured, for 4 days, alone or with autologous and irradiated (3000 cGy) Treg at different Tresp:Treg ratios (1:2, 1:4, 1:5, 1:10, 1:25). MLR was set up in flat-bottom 96-well plates pre-coated with anti-CD3 mAb (5 µg/ml, clone UCHT1; BioLegend, San Diego, CA) in the presence of soluble anti-CD28 mAb (5 µg/ml, clone CD28; BioLegend). The suppressive capacity of fresh and thawed Treg was evaluated concomitantly. CFSE covalently binds the cytoplasmic components of cells and upon cell division is distributed equally between daughter cells. This feature is exploited to assess cell division by flow cytometry FACSCantoII (Beckton Dickinson). The capacity of Treg to modulate Tresp proliferation was analyzed using ModFit LT™ 3.1 calculating the Upper generation Proliferation Index, a measure of the increase in cell number over the course of the experiment for cells that are really proliferating (Figure 3).

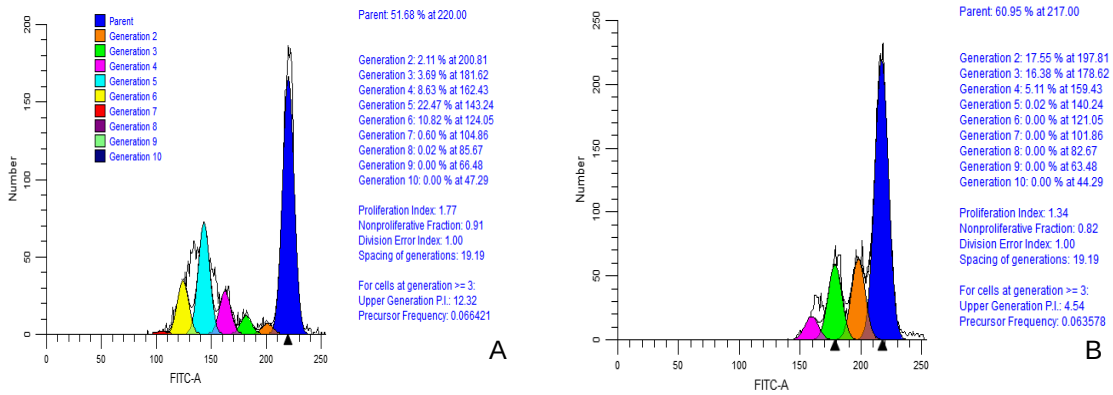

Figure. 3 Calculation of cell proliferation by the CFSE staining method. Representative histogram of CFSE fluorescence analyzed using ModFit LTTM 3.1. Every peak represent a cell generation as explained in the legend. A) Tresp in culture without Treg; B) Tresp in culture with Treg (1/4 Treg/Tresp ratio)

2.6 Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad software Inc., USA). Parametric and non-parametric data were calculated as the mean±s.d. and median (interquartile range, IQR) respectively. For comparison of parametric and non-parametric data, t-test, one- or two-way ANOVA with Bonferroni test comparison and Kruskal-Wallis test were used where appropriate.

3. Results

3.1 Enumeration of regulatory T cells in Peripheral blood

Clinical applications based on Treg infusions require high numbers of active and suppressive cells. For this reason the assessment of the $CD4^{+}CD25^{high}$ $FoxP3^{+}$ population will be useful to better understand the starting number of cells present in PB and yields after cell-processing. Moreover assessing the number of circulating Treg will be useful in order to choose the best cell source from either PB or leukapheresis.

First, we compared percentages and absolute numbers of circulating Treg in both healthy donors and patients waiting for LT. One million cells from PB were stained (*see material and methods*) and the percentages of lymphocytes, $CD4^{+}CD25^{+}$, $CD4^{+}CD25^{high}$ and $CD4^{+}CD25^{high}$ $FOXP3^{+}$ T cells were evaluated. As shown in Tables 1 and 2 the mean percentage of $CD4^{+}CD25^{high}$ $FOXP3^{+}$ T cells was not significantly different between patients (0.55%±0.23 *n=12)* and HD (0.44±0.13 *n=8*). Although the relative percentage of Treg in white blood cells (WBC) tends to be slightly higher in patients, their cognate absolute numbers are actually lower (29.8±5.61 vs 25.13±9.92 cells/μL) than those retrieved in healthy donors. This is due to the leukopenic status of these patients (7050±1220 vs 4950±2010 WBC/μL) (Figure.4). When we identified Treg by the use of CD127 surface marker, once again the mean percentage of $CD4^{+}CD25^{+}CD127^{-}$ cells of patients (0.74±0.27%) was not significantly different from the normal counterparts (0.85±0.26%).

HD	Lymphocyte %	$CD4^+CD25^+$ %	$CD4^+CD25^{high}$ %	$CD4^+CD25^{high}$ $FOXP3^+$ %
1	38.6	3.1	0.7	0.6
2	29	4.7	0.9	0.5
3	22.5	4.7	0.6	0.3
4	29	4.8	0.9	0.3
5	30	3	0.8	0.5
6	32	5.7	0.9	0.3
7	22.4	3	0.7	0.6
8	27.6	3.3	0.6	0.4
Mean	**28.89**	**4.04**	**0.76**	**0.44**
St. Dev	**5.21**	**1.06**	**0.13**	**0.13**

Table. 1 Peripheral Blood evaluation in healthy donors. Percentage of lymphocytes, $CD4^+CD25^+$, $CD4^+CD25^{high}$, $CD4^+CD25highFOXP3^+$ in the circulating white blood cells fraction was evaluated by flow cytometry analysis.

Liver Patients	**Lymphocyte %**	**$CD4^+CD25^+$ %**	**$CD4^+CD25^{high}$ %**	**$CD4^+CD25^{high}$ $FoxP3^+$ %**
1	39	9.7	1.5	0.9
2	14	2	0.4	0.2
3	24.5	3.6	0.7	0.4
4	28.6	7	1	0.7
5	23	7.5	0.9	0.6
6	15	2.6	0.3	0.3
7	20	3.7	0.6	0.5
8	18	5.6	0.7	0.4
9	30	6.8	0.8	0.4
10	20	5.9	1	0.9
11	15.5	7	0.6	0.5
12	4.9	4	0.8	0.8
Mean	**21.04**	**5.45**	**0.78**	**0.55**
St. Dev	**8.86**	**2.29**	**0.31**	**0.23**

Table. 2 Peripheral Blood evaluation in patients from waiting list for liver transplantation.
Percentage of lymphocytes, $CD4^+CD25^+$, $CD4^+CD25^{high}$, $CD4^+$CD25high$FOXP3^+$ in the circulating white blood cells fraction was evaluated by flow cytometry analysis.

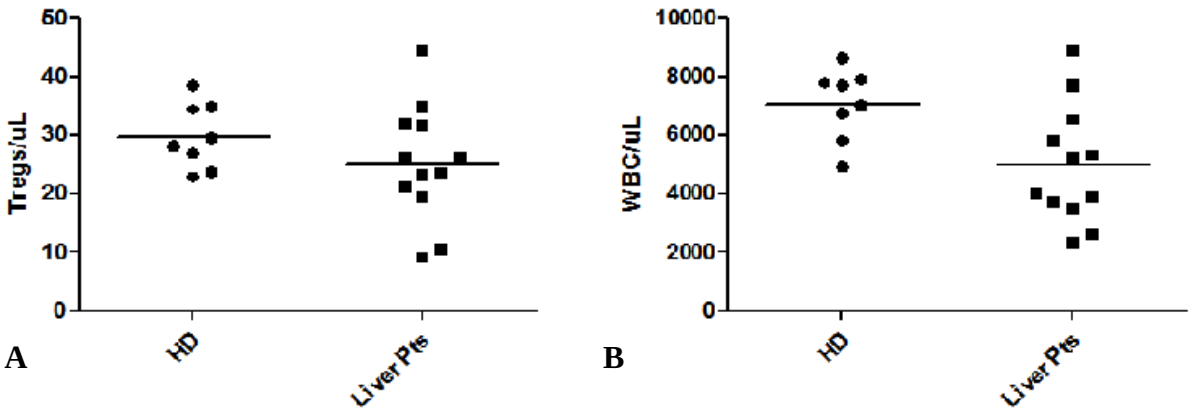

Figure. 4 Circulating cells. Number of circulating **A)** Treg and **B)** WBC in healthy donors and liver patients.

3.2 Isolation of $CD4^+$ $CD25^+$ cells.

Subsequently, we isolated Treg from PBMCs by a two-step immunomagnetic selection. First, $CD4^+$ cells were separated by depletion from PBMCs followed by enrichment in $CD25^+$ fraction. After selection, cell purity was assessed by flow cytometry. In healthy donors, 90.69±3.62% were $CD4^+CD25^+$ cells with 89.6±4.72% of cells expressing FOXP3, while in patients this was 88.61±4.99% and 91.9±4.37%, respectively (Figure 5). Analysis of the Tresp fraction showed a percentage of $FoxP3^+$ cells < 8% in both patients and healthy donors (6.44±3.3% and 5.03±0.16%) respectively.

The high purity of the Treg fraction was confirmed by CD127-staining since the percentage of $CD4^+CD25^+CD127^-$ was 83.8±3.96% and 81.3±8.7% in healthy donors and patients respectively (Figure 5).

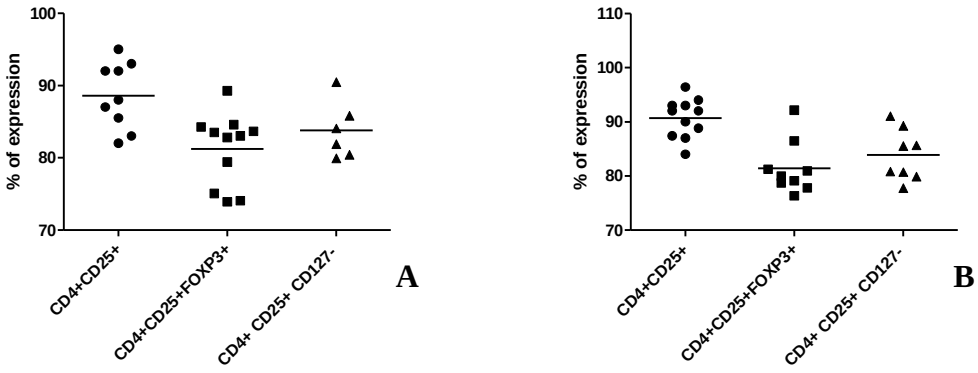

Figure. 5 Phenotypic evaluation of Treg after immunomagnetic isolation. Each dot represents one evaluation showing the $CD4^+CD25^+$, $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^+CD127^-$ expression in healthy donors, panel A, and patients, panel B, obtained using flow cytometry.

3.3 Regulatory T cells are viable and maintain their phenotype after thawing

Highly purified Treg and Tresp fractions were stored using a controlled rate cryo-freezer and afterwards stored in liquid nitrogen. After a month, cells were thawed at 37° followed by a centrifugation in RPMI 1640 to eliminate DMSO and re-stained to assess viability, as well as FOXP3 and CD127 expression.

Viability was evaluated by PI test. Viable cells were 88.17±5.41% in healthy donor Treg, while in patients the average percentage was 86.57±4.24%.

The second steps provided the FOXP3 evaluation because previous studies[168,169,170] reported decreased expression of this marker post-thawing.

In this experimental system Treg maintain their phenotype based on the expression of FOXP3 marker. In fact no significative difference in FOXP3 expression was founded between fresh and thawed cells. (Figure 6)

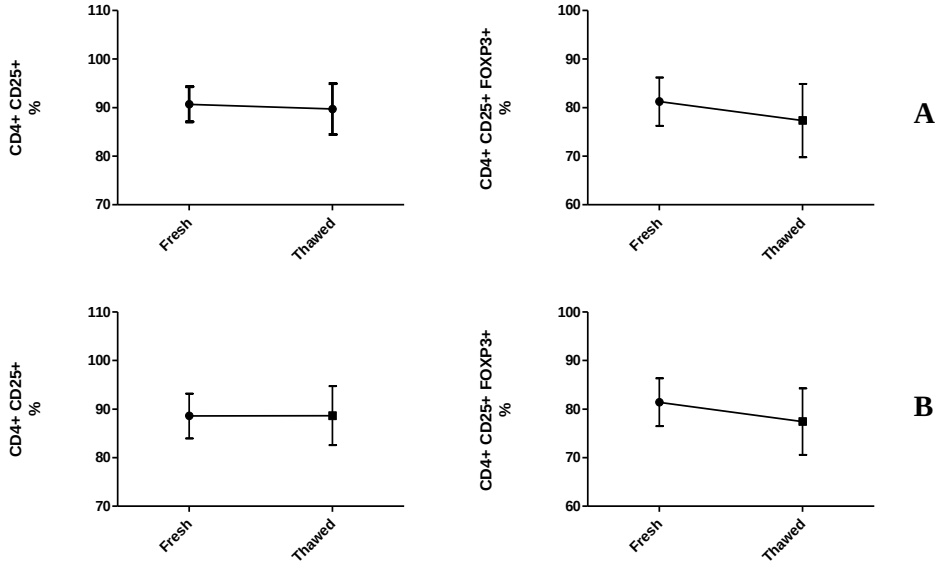

Figure. 6 Phenotypic evaluation of Treg pre and post thawing. $CD4^+CD25^+$ and $CD4^+$ $CD25^+$ $FoxP3^+$ expression determinated by flow cytometry in freshly isolated and thawed Treg fractions. Results show the mean percentage of $CD4^+CD25^+$ and $CD4^+$ $CD25^+$ $FoxP3^+$ pre e post thawing from healthy donors, panel A, and patients, panel B

Moreover, no significant differences were found when the mean intensity of fluorescence (MFI) of FOXP3 between fresh and thawed cells was compared. In detail, in healthy donors the MFI is 5109±2642 in fresh samples and 4163±2109 after thawing, while in patients this is 8091±2820 and 5768±1456 respectively. To confirm this data Treg were analysed for CD127 expression. Once again, no significant differences were observed between fresh and thawed cells as shown in figure 7.

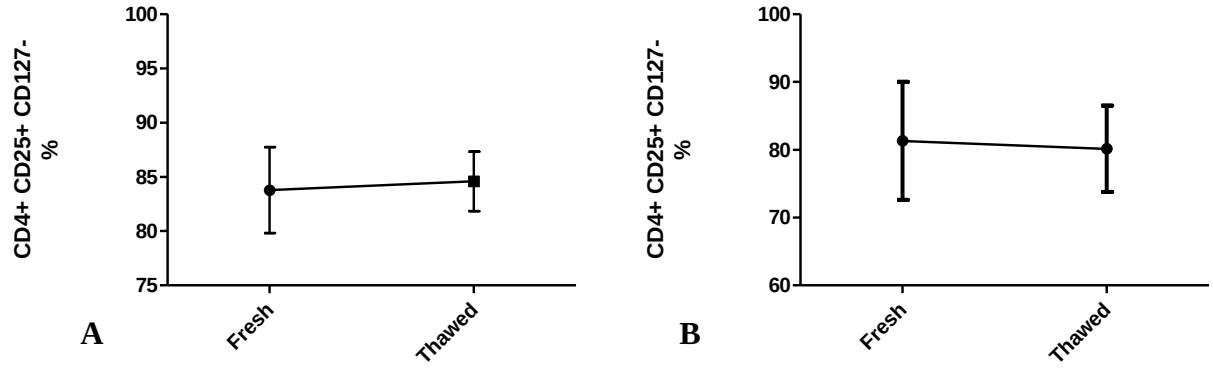

Figure.7 Phenotypic evaluation pre and post thawing. $CD4^+$ $CD25^+$ $CD127^-$ expression determinated by flow cytometry in freshly isolated and thawed Treg. Results showt he mean percentage of $CD4^+CD25^+$ $CD127^-$ pre and post thawing from healthy donors, panel A, and patients, panel B

3.4 Evaluation of suppressive ability

To assess the function of isolated Treg before and after cryopreservation, we set up MLR. Autologus Tresp labelled with CFSE and stimulated with anti-CD3 and anti CD28 were cultured alone or with different ratios of Treg for 5 days. At the end of the co-culture cells were harvested and fluorescence intensity was analysed by flow cytometry. Fresh and thawed Treg activity was tested using a fresh autologous Tresp population at different Treg/Tresp ratio.

As shown in figure 8 freshly isolated Treg from patients and healthy donors efficiently reduced Tresp proliferation in a dose-dependent manner. Even though the differences are not significant. Treg from patients at a high ratio (Treg/Tresp 1/4) showed a reduced suppressive ability compared to healthy donors.

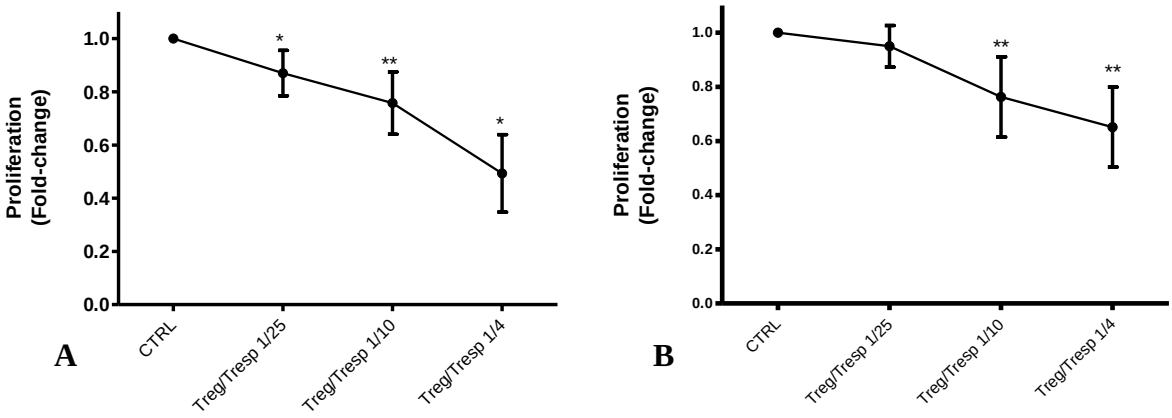

Figure 8 Suppressive ability of freshly isolated Treg. Co-culture (4 days) of autologous Treg with $CD4^+$ CD25- (Tresp) stimulated with anti-CD3 and anti CD28 (5ug/ml) and labeled with CFSE. FOLD-CHANGE was calculated as ratio between the" upper generation proliferation index" of Tresp cultured in the presence of increasing ratios of Treg and the" upper generation proliferation index" of CTR culture where no Treg were added. Panel A and B show the suppressive function of freshly isolated Treg from HD and patients respectively; ** $p< 0.01$ * $p< 0.05$ referred to CTRL condition

The next step was the evaluation of the suppressive ability of Treg after thawing. Samples were thawed rapidly at 37°C, transferred into a tube containing RPMI 1640 and then centrifugated once to remove DMSO. Subsequently cells were cultured at different Treg/Tresp ratio.
Treg from healthy donors, had a reduced suppressive ability compared to freshly isolated Treg, especially when high ratios were compared (proliferation fold change at a 1/4 ratio were 0.49±0.15 vs 0.79±0.04; $p<0.05$, respectively). Thawed Treg from patients were unable to suppress the proliferative activity of Tresp, even at increased Treg/Tresp ratio. (Figure 9)

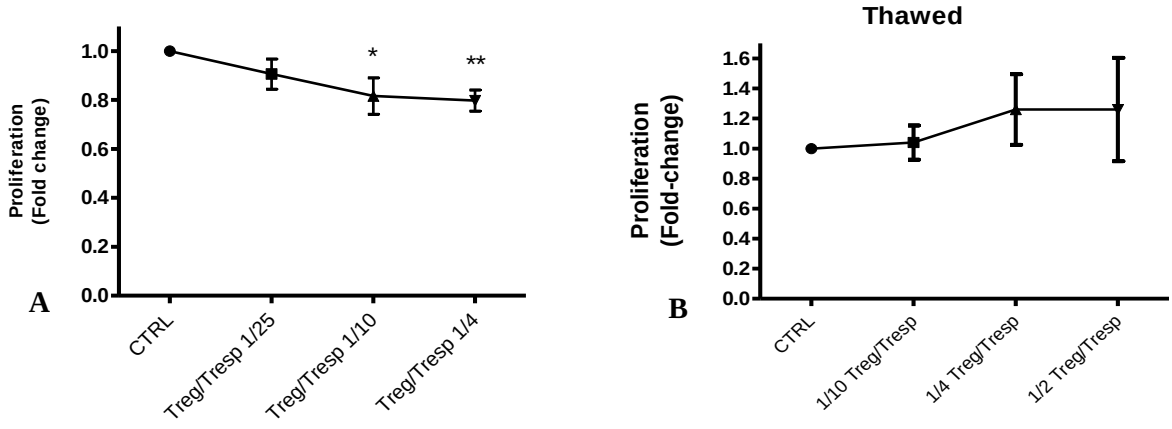

Figure 9 .Suppression abibity of thawed Treg. Co-colture (4 days) of autologous Treg with $CD4^+$ CD25- (Tresp) stimulated with anti-CD3 and anti CD28 (5ug/ml) and labeled with CFSE. FOLD-CHANGE was calculated as ratio between the" upper generation proliferation index" of Tresp cultured in the presence of increasing ratios of Treg and the" upper generation proliferation index" of CTR culture where no Treg were added. Panel A and B show the suppressive function of thawed Treg from HD and patients respectively; ** $p< 0.01$ * $p< 0.05$ referred to CTRL condition

3.5 Yields

Given the low Treg numbers present in PB it is very important to understand how many cells we recover during the different steps of cell processing. For this reason, in compliance with the cytofluorimetric analysis we calculate the number of recovered cells after thawing to get the final yield and understand the feasibility of our method.

After the immunomagnetic-selection, cells were counted and cell number was adjusted in compliance with viability and the percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ cells assessed by flow cytometric analysis. To test the cryopreservation protocol cells were frozen using a controlled rate cryo-freezer. Controlled-rate freezing before long-term sample storage is a vital step in the cryopreservation procedure, since the rate at which cells are cooled has an extremely significant influence on cell survival. Yield was then calculated considering the numbers of recovered cells, according to $CD4^+$ $CD25^+FOXP3^+$ and viability analysis. Yield after thawing is 75 ±12.5% in HD but dropped to 59±10% in patients. (Figure 10)

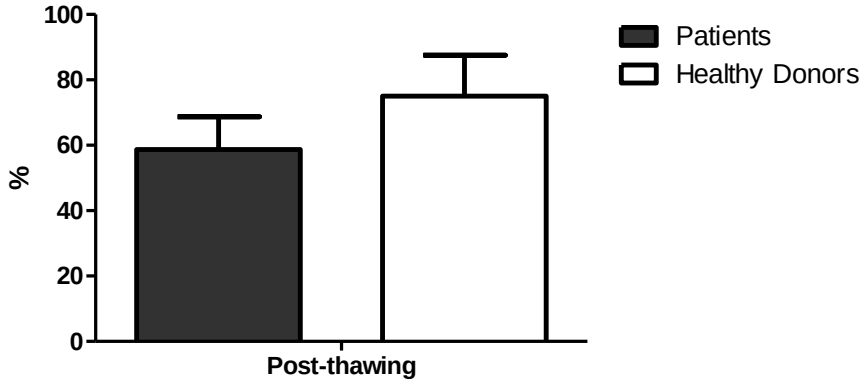

Figure. 10 Yield after thawing. Mean percentage of recovered cells after thawing calculated considering the numbers of stored and recovered cells according to $CD4^+$ $CD25^+FOXP3^+$ and viability analysis.

4. Discussion

Cell therapy has emerged in the last years as a promising therapy for the induction of tolerance after organ transplantation. Major challenge is to determine whether cellular therapy makes the immune microenvironments permissive to graft tolerance and, specifically, whether it can improve solid organ and HSCT outcomes through anti-inflammatory effects and the induction of immune tolerance.

The use of CD4+CD25+FOXP3+ regulatory T cells (Treg) as a cellular therapy is an attractive approach for autoimmunity disease, GvHD and for limiting immune responses to allograft after transplantation. Possibility of avoiding or minimizing the use of immunosuppressive drugs will positively impact on most of treatment-related complications, such as infections, cardiovascular diseases, diabetes, renal failure, neurological complications and secondary cancers.

Cell therapy using Treg is not yet fully accomplished cause of several problem regarding: selection of a pure cell fraction, effective cell dose, use of freshly isolated or ex-vivo expanded cells, possibility to cryopreserve the purified product or expanded Treg-fraction and use of alloantigen-reactive or polyclonal Tregs.

The first question of this work concerned the evaluation of the amount of Treg available for cell therapy in PB of studied group. In the analysed set of patients the percentages of Treg tend to be slightly higher than HD (0,55% vs 0,44%, Table 1 and 2) nevertheless the absolute number is lower because of their leukopenic status (29.8±5.61 vs 25.13±9.92 cells/μL) (figure 4). This result confirms what Julia Almeida et al. have found in patients with alcohol-related disease[172]. They analyzed patients with alcoholic related hepatitis correlating the Treg reduction with the dysregulation of cytokine modulating proliferation and/or Treg survival.

These results show that the amount of Treg present in PB and leukapheresis are not enough for a clinical trials with high cell numbers. Normally after a leukapheresis procedure (without mobilization with G-CSF) the amount of white blood cells is around $10x10^9$; considering the percentages of Tregs previously reported the number of cells available for cell therapy will be around $50x10^6$ therefore not enough for a clinical trial.

A possible solution is provided by PB mobilization with G-CSF as shown by Ukena SN et al in Hematopoietic Stem Cell donors[173]. Low Treg numbers can be implemented, exploiting the capacity of G-CSF application to lead a numerical increase of circulating Tregs without modification in their suppressive activity and phenotype. However, this strategy does not appear to be enough to recover a number of cells necessary for the infusion of more than $3x10^6$ cells/Kg, leaving ex vivo expansion as the only possible strategy.

In a cell therapy protocol with Treg, cryopreservation is important because it can overcome several issues. This approach would allow for more flexibility in terms of planning the therapy, timing of the infusions, and also allow for subsequent Treg infusions, regardless of pharmacological treatment applied after the first infusion.

For this reason, Treg stored in advance can be used when necessary, and this possibility can be exploited during the follow-up of forthcoming clinical trials to stock samples for future analysis. Therefore, in this research work phenotype and function of freshly isolated Treg, before and after cryopreservation has been tested. Data show that thawed cells, both in controls and patients, maintain their phenotype (figure 6 and 7), while only thawed Treg from patients lose their ability to suppress proliferation *in vitro*. To date, contrasting results have been published about the feasibility of Treg cryopreservation. The first evaluation on cryopreserved Treg, made by Dr. Elkord[168], found a significant reduction of $CD4^+CD25^+FOXP3^+$ cells after thawing. Conversely, Venet and Van Hemelen have shown that cryopreservation did not alter the percentage of Tregs in HIV-patients and HD[169174]. In these reports, PBMC freezing protocols included different

cryopreservation media and Treg detection strategies; Elkord's group evaluation was made on 6 HD using a medium composed by 90% FBS and 10% of DMSO. In contrast, Venet's group analyzed 17 HIV-infected patients and no significant differences in the percentage of CD4+ CD25+ CD127- cells were founded, however they used a different medium, consisting in 60% human serum AB, 30% RPMI 1640 and 10% of DMSO.

In this project a controlled rate cryo-freezer has been used and a new medium for cryopreservation (GMP compliant) composed by 50% human AB serum, 40% CliniMacs buffer and 10% DMSO, was purposed. After thawing, cell viability was 88.2%±5.41 in HD and 86.6±4.24 in patients, and, most importantly, in both groups a high expression of FOXP3 was preserved in thawed Treg cells (figure 6). However when patient Treg were tested in vitro they were not able to suppress proliferation of CD4+ CD25- (figure 9). This result confirmed what Peters et al. found in Treg isolated via CliniMACS and then stored in liquid nitrogen[175]. After thawing, Treg showed 70–80% viability but their suppressive capacity was impaired and not regained by cell resting for up to seven days in culture medium. Only Treg expansion was able to restore their suppression ability.

In fact, a possible solution might be the use of rapamycin-expanded Treg because, compared to freshly isolated fraction, they are more suppressive both in vitro and in vivo. A study comparing freshly isolated Treg with the expanded counterpart showed that after expansion, Treg increased the intracellular CTLA-4 and GITR expression.

Moreover the expansion procedure breaks the anergic Treg-status by the use of high IL-2 doses and antiCD3/CD28 beads and rapamycin, with its capacity to select the regulatory population, is essential for Treg function. In fact, in type 1 diabetic patients, freshly isolated CD4+CD25+ Tregs are characterized by a reduced suppressive ability that is recovered after expansion in the presence of rapamycin[128]. Previous works studying Treg management found that freezing and thawing did not adversely affect the function of expanded third-party Tregs since they effectively prolonged embryonic pig pancreatic graft survival and function in C57BL/6 mice for up to 8 weeks post-implantation, similarly to freshly isolated Tregs[176]. A more detailed study showed that human Tregs, which were massively expanded by stimulation with APCs and subsequently frozen, maintained suppressive function in vitro after thawing[177].

In conclusion these results show that Treg expansion is a necessary procedure especially for patients; it increases the Treg number available for adoptive cell therapy and solves the problem due to freezing procedure.

EXPANSION OF REGULATORY T CELLS UNDER GOOD MANUFACTURE PRATICE CONDITION

1. Introduction and Aims

The above described results have been important to demonstrate that the isolation of Tregs from PB will not be sufficient to obtain the numbers of Tregs necessary for a clinical trial, both in solid organ transplantation and GvHD. In addition, after cryopreservation, the yield was high and the cells maintained their phenotype, even though reducing their suppressive capacity. For this reason, the ex vivo expansion of regulatory T cells is needed to obtain the sought cell product. Thus far is possible to find in literature only works showing Tregs expansion in healthy donors using antiCD3/antiCD28 beads in presence of rapamycin and IL-2[178] for this reasons the aim of this part was to develop a protocol for HD and liver patient Treg using reagents respecting the Good manufacture Practice (GMP). The expansion will be useful to obtain a therapeutic Treg number (more than $3x10^6$ cells/kg) that could be used for the infusion in clinic after solid organ transplantation or for the treatment of GvHD.

2. Material and Methods

2.1 $CD4^+CD25^+$ Isolation

The isolation of the $CD4^+CD25^+$ population has been effectuated in 2 steps, CD8 depletion and subsequent CD25 enrichment. This procedure differs from that one used in the previous section because no GMP compliant biotin antibody cocktail and anti-biotin antibody cocktail are available on the market.

Peripheral blood (100 mL) (PB) of 3 healthy donors were provided by the "Centro Trasfusionale, Policlinico Sant'Orsola Malpighi" while samples from 3 patients from the liver transplant waiting list at the Sant'Orsola Malpighi Hospital (Bologna) were provided by the Surgical Unit. PBMCs were isolated as described in paragraph 2 section 2.1 and then resuspended in MACS buffer, at 10 ml/10^9 total cells. Afterwards they were incubated with CD8 microbeads, 750 μL/109 total cells, for 30 minutes in the dark at room temperature, on a roller. Post incubation, the bead bound cells were washed in MACS buffer by adding 1-2ml of MACS buffer for 10^7 cells and centrifuged at 1800 rpm for 10 minutes. Subsequently the supernatant was completely aspirated. The cells were next resuspended in 500μl of MACS buffer per 10^8 cells and subsequently passed through MACS LS columns contained within a magnet to remove the CD8 microbead labeled cells. Of note, the LS columns were prepared prior the application of the cell suspension, by rinsing with 3ml of MACS buffer. Post application of the cell suspension onto the column, the unlabeled cells were collected below and the column washed three times with 3 ml of MACS buffer. The effluent, containing the unlabeled cells, was collected and washed with MACS buffer. After that, cells were resuspended in MACS buffer (20 mL/10^9 total cells) and incubated with CD25 microbeads (750 μL/10^9 total cells) for 15 mins at 4oC in the dark. Cells were next washed with MACS buffer, by adding 1-2ml of buffer per 107 cells and centrifuged at 1800 rpm for 5 minutes. The cells were next resuspended in 500μl of MACS buffer per 108 cells and passed through MACS LS columns contained within a magnet, to isolate CD25 microbead labeled cells. Of note, prior to the application of the cell suspension, the column was placed in the magnetic field of a suitable MACS separator and rinsed with 3ml of MACS buffer. Post cell application of the cell suspension onto the column, the column was washed with 3ml of MACS buffer three times. Subsequently the column was removed from the magnet and placed on a suitable collection tube. The labeled cells within the column were plunged through with 2 mL of MACS buffer. The CD25 microbead labeled cells were subsequently washed in staining buffer and purity assessed by flow cytometry.

2.2 Regulatory T cells expansion using GMP reagents

Tregs were isolated as previously described (section 2.1). $CD4^+CD25^+$ Tregs were then plated at 1 x 10^6 cells/ml, on 24-well flat bottom plates in culture media, TexMACS GMP Medium (Miltenyi Biotech), supplemented with 5% human AB serum (HS), and expanded in presence of 100 nM GMP rapamycin (Miltenyi Biotech). Cells were polyclonally activated with anti-CD3 and anti-CD28 coated beads (Miltenyi Biotech) at a 4:1 bead:cell ratio and cultures supplemented with IL-2 (1000 IU/mL) 2 days post-activation and replenished every 2 days thereafter. Cells were restimulated every 7 days by magnetically removing the activation beads and adding fresh beads (1:1 bead:cell ratio), rapamycin and IL-2. The expansion period was limited to 21 days with concurrent assessment of Treg phenotype and function (described in paragraph 1 section 2.4)

2.3 Flow Cytometry analysis

The following monoclonal antibodies were used: anti-CD25 Biotin (4E3), anti-Biotin APC; anti-CD4 FITC (MT-466), anti-CD127 PE (clone: MB15-18C9), anti-CD8 (SK1). anti-CD196 (11A9), anti-CD161 (DX12), anti-CD3 (Leu-4), anti-CD56 (NCAM-16), anti-CD19 (4G7), anti-human FoxP3 (PCH101) and anti-CD14 (MΦP9)

3. Results

3.1 Regulatory T cells expansion in patients and healthy donors

After isolation cells were resuspended at 10^6/mL in TexMacs and stimulated with antiCD3/CD28 beads plus rapamycin (100nM) and IL-2 (1000U/mL). At the end of every cycle they were counted, re-stimulated with beads (beads/cells ratio 1:1) and re-plated in the presence of IL-2 and rapamycin. Analysis of the Fold increase (Figure 1B), demonstrated that cell

expansion reached a peak between the second and third re-stimulation. The same analysis showed that the mean fold increase expansion after 21 days of culture is 559 (342-826) with a great donor variability. Of note, a therapeutic cell numbers was reached in every sample (figure 1A)

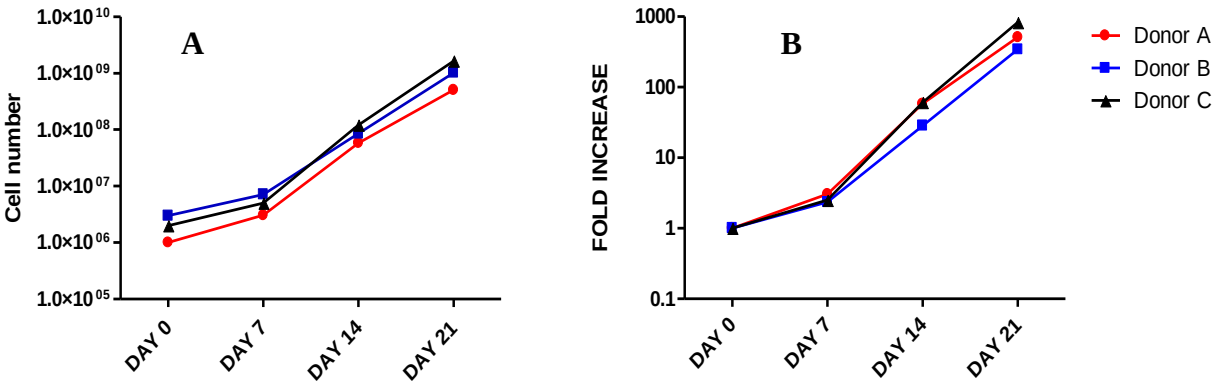

Figure. 1 *Ex vivo* Treg expansion from healthy donors. A) Treg cell lines were stimulated with anti-CD3/CD28 beads and IL-2 (1000 IU/mL) in the presence of Rapamycin (100nM). Cell counts were performed at weekly intervals (DAY) throughout culture period. **B)** The in vitro expansion of 3 different Treg cell lines was calculated as Fold increase (number of cells at each time point divided for starting Tregs number).

Subsequently the expansion ability of patient Treg was evaluated. Following the same expansion procedure cell numbers and Fold Increase were evaluated. Although Treg from patients were able to expand in culture (Figure 2A and B), they did not reach the same expansion rate when compare with the HD counterpart (Mean fold increase 559 vs 255 respectively in HD and Pts).

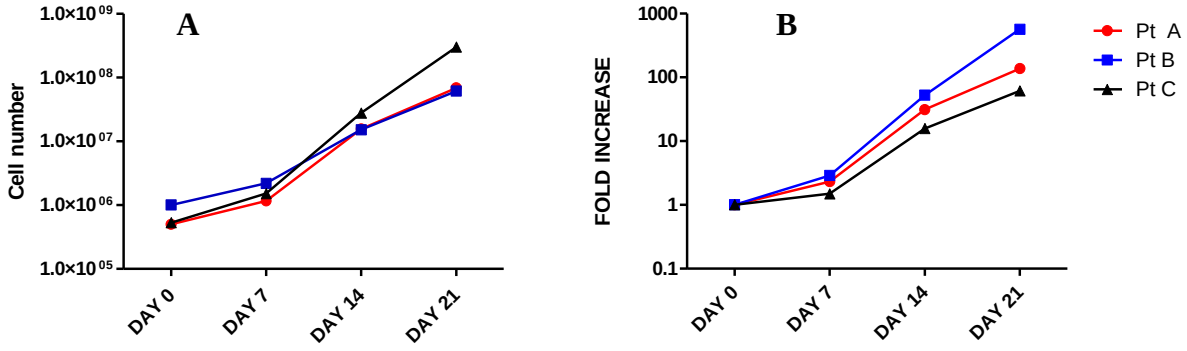

Figure. 2 Ex vivo expansion of Treg from Patients in waiting list for liver transplantation. A) Treg cell lines were stimulated with anti-CD3/CD28 beads and IL-2 (1000 IU/mL) in the presence of Rapamycin (100nM). Cell counts were performed at weekly intervals (DAY) throughout culture period **B)** The in vitro expansion of 3 different Treg cell lines was calculated as Fold increase (number of cells at each time point divided for starting Tregs number).

3.2 Phenotypic evaluation of expanded cells

Human Treg cells have been shown to be an heterogeneous population in the expression of specific cell markers. In order to investigate if the expansion procedure was effective in promoting phenotypic signatures on these cells, the percentage of $CD4^+CD25^+$, $CD4^+CD25^+FOXP3^+$ and $CD4^+CD25^+CD127^-$ fraction was evaluated at day 0 and at each time point both in patients and healthy donors. Flow cytometric analysis in figure 3 shows that at day 0 the population is not highly pure because the mean percentages of $CD4^+CD25^+FOXP3^+$ cells were 65.3±13.3 and 61.4±4.6 respectively in HD and Pts. Data confirmed by CD127 analysis (figure 3). However after the 21 days expansion procedure all the cells were $CD4^+CD25^+FOXP3^+$ and $CD4^+CD25^+CD127^-$.

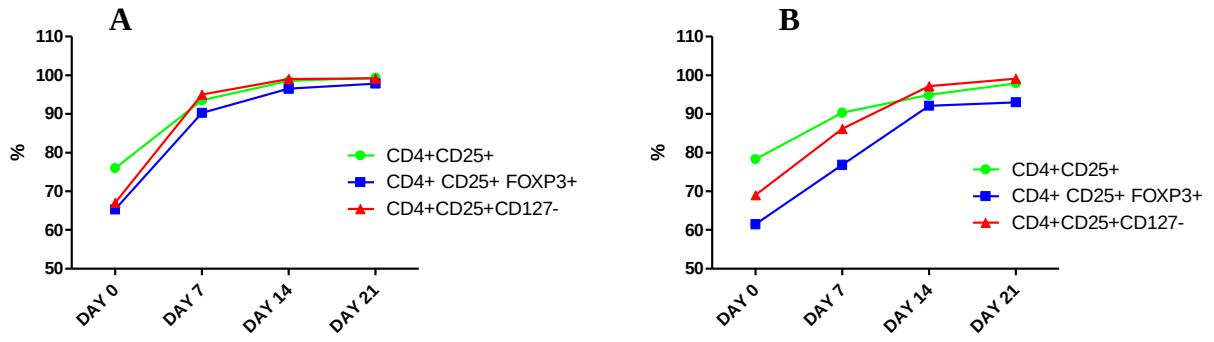

Figure. 3 Phenotypic analysis of Treg cells from healthy donors and patients during 21 days expansion. Percentage of expression of $CD4^{+}CD25^{+}$, $CD4^{+}CD25^{+}FOXP3^{+}$ and $CD4^{+}CD25^{+}CD127$- was calculated by flow cytometry at Day 0,7,14 and 21 in HD (panel A) and Pts (Panel B) respectively.

Since at Day 0 cell fractions didn't show an high purity the evaluation of cell contaminants was necessary in order to understand the components of the cell product. Cells at day 0 and at each time point were stained with markers specific for monocyte (CD14), T helper 17 (CD4, CD161 and CD196), CD8, B lymphocyte (CD19) and T NK (CD3 and CD56). As Shown in figure 4, both cells from healthy donors (Panel A) and patients (Panel B) present contamination, especially Th17 and B lymphocyte. However at the end of expansion no contaminants were observed.

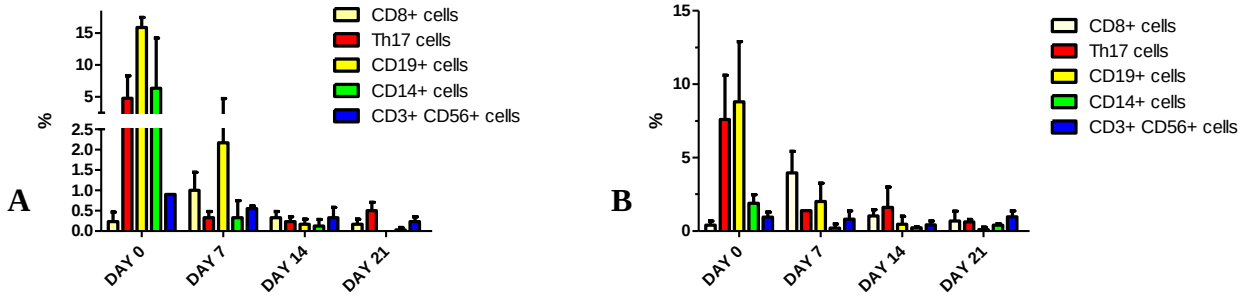

Figure. 4 Phenotypic analysis of freshly isolated and ex vivo expanded Treg from HD and patients. Percentages of CD14, CD8, CD19, Th17 and T NK cells were calculated by flow cytometry analysis at Day 0,7,14 and 21 in HD (panel A) and Patients (Panel B).

3.3 Functional evaluation of expanded cells

To compare the regulatory function of each Treg cell line preparation, responder $CD4^+CD25^-$ T cells were stained with CFSE as described in Material and Methods (Paragraph 1 section 2.5) and stimulated with anti-CD3/CD28 antibody in the presence or absence of expanded Treg at different ratios. After 4 days of co-culture, the ability of the different Treg cell lines to suppress a polyclonal stimulus was determined by CFSE dilution using flow cytometry. As depicted in figure 5, after 21 days of expansion both Treg from healthy donors and patients were able to suppress Tresp proliferation and no significant differences were observed between patients and controls.

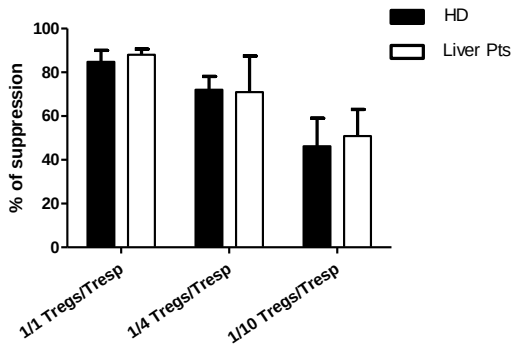

Figure 5 Suppressive Treg ability after 21 days expansion. Co-culture (5 days) of $CD4^+$ CD25- (Tresp) stimulated with anti-CD3 and anti CD28 (5ug/ml) and labeled with CFSE with different Treg ratio. Data are expressed as average percent of suppression of Tresp proliferation ±1SD.

4. Discussion

Treg expansion is the best choice in clinical trials using highly suppressive T-cell[179]. However, to date, there are some issues concerning the use of antigen-specific or polyclonal Treg. Antigen-specific Treg, as shown in literature, are known to be effective at lower cell doses and thus may be preferable for tolerance induction strategies however the develop of a clinical protocol for cell therapy with these cells is complex. A possible strategy could be the use of antigen-presenting cells to differentiate an antigen-specific Treg subset *ex vivo*. This procedure, already published, cannot be used in clinic due to the lack of a GMP-compliant isolation procedure able to select a pure Treg population[180].

For this reason, approaches using polyclonal Treg are likely to be more universally applicable.

To date, adoptive transfer of freshly isolated human Tregs has prevented GvHD in patients treated with allogeneic hematopoietic stem cell transplantations partially depleted of conventional T cells. However, T cell–replete grafts are more commonly used, and prevention of GvHD after T cell–replete transplantations require the transfer of a higher number of Tregs that can only be obtained through ex vivo expansion.

Here it has been shown how Treg can be expanded by more than 500-fold (figure 2) by anti-CD3 and anti-CD28 coated beads, IL-2 and rapamycin. Despite high variability in liver patients, the resulting fold increase is useful to set up a clinical trial using more than $3x10^6$ cells.

Phenotypic analysis demonstrates that the cell profile changes during expansion; the contaminants found at day 0 were principally CD19+ and CD14+ cells. However, their expression was already reduced after 7 days and at the end of expansion the percentages of all the non-regulatory T-cells was less than 1 % (figure 4). The achievement of this purity, obtained exploiting the positive effects of rapamycin, is mandatory for a cell therapy protocol inducing tolerance. Rapamycin is essential to obtain a pure and highly suppressive population. As previously explained (section 3.1; general introduction), only Treg are able to proliferate when rapamycin is added in culture, because they are not dependent on the PI3K-AKT-mTOR pathway for cell cycle progression. Moreover, mTOR-blocking promotes FOXP3 expression in T-cells, which are then converted into modulators of immune reactivity.

Data from this thesis show that expanded cell, from both control and patients, highly express the FOXP3 marker. In addition, Treg purity was further confirmed by CD127 evaluation and after 21 days of expansion, cells do not express CD127 (figure 3). Phenotypic evaluation is strengthened by the functional analysis where Treg from HD and patients are highly suppressive (figure 5)

The results obtained in this thesis confirm what several published works have previously shown in healthy donors and patients with autoimmune disease about the positive effect of expansion on Treg extending the possibility to expand Treg from patients with liver diseases.

IMPACT OF IMMUNOSUPPRESSIVE DRUGS ON EX VIVO EXPANDED REGULATORY T CELLS

1. Introduction and Aims

Immune suppressive regimen in clinical transplantation and GvHD plays the crucial role to dampen down the immune response and allow graft survival. These powerful immunosuppressive drugs efficiently control acute graft rejection. However, because of their non-specificity the constant use of this therapeutic approach presents adverse effects and fails to control chronic rejection. To this aim various compounds have been developed and selected according to their ability to control lymphocyte activation.

Conventional immunosuppressive therapy employs a calcineurin inhibitor such as cyclosporine (CsA) or tacrolimus (Tac), as well as corticosteroids and mycophenolate mofetil (MMF) to control immune responses. At the moment, successful organ transplantation still requires long term treatment with diverse combinations of these drugs responsible of several side effects. Although their precise molecular mechanism of action is well characterized, little is known regarding the effect on specific T cell subsets and ultimately their capacity to influence Treg viability and function.

Current evidence suggests that calcineurin inhibitors have markedly negative effects on Treg, mainly by interfering with the IL-2 production and therefore affecting Treg function and survival. Conversely, corticosteroids have been reported to increase Treg frequency and FOXP3

expression in patients with autoimmune diseases. Finally, patients receiving MMF show significantly higher $CD4^{+}CD25^{hi}FOXP3^{+}$ Treg compared to patients on other treatments. Furthermore, studies on patients with renal impairment showed that MMF may reverse the effect of anti-calcineurin treatment on Treg, maintaining their functional activity in vitro.

Ex vivo rapamycin expanded Treg are a potential tool for promoting allograft tolerance after organ transplantation and for GvHD treatment. However, all the in-vivo studies published so far and in the ongoing clinical trial of solid organ transplantation these cells will be injected in combination with the immonusuppressive drugs.

Although none of the immunosuppressive drugs have been specifically designed to influence Treg, they nevertheless do so through various pathways. In addition, there are no data showing the effect of the immunosuppressive regimen on Tregs expanded in vitro. In this chapter the effects of Tac, MMF and methyl-prednisolone on viability, proliferative capacity and function of in vitro expanded Tregs has been investigated.

2. Materia land Methods

2.1 Cell isolation and separation

PBMCs from healthy donors were obtained from anonymized human leukocyte cones supplied by the National Blood Transfusion Service (NHS blood and transplantation, Tooting, London, UK). Human studies were conducted in accordance with the Helsinki Declaration and approved by the Institutional Review Board of Guy's Hospital. (reference 09/H0707/86). Informed consent was obtained from all healthy donors prior to enrolment into the study. PBMC were isolated by Lymphocyte (PAA, Austria) density gradient centrifugation. $CD4^{+}CD25^{+}$ T cells were purified as described in paragraph 2 section 2.2. The purity of $CD4^{+}CD25^{+}$ T cells was between 90-98%.

2.2 Expansion of Treg cell lines for Drugs experiment

Healthy human $CD4^+CD25^+$ T cells, were plated at $1x10^6$/ml in X-Vivo 15 supplemented with 5% HS and Rapamycin (100nM). Cells were initially stimulated with anti-CD3/CD28 beads *(Invitrogen)* at a bead: cell ratio of 1:1. IL-2 (1000IU/mL) was added at day 2 post-activation and replenished every 2 days. Beads were removed by magnetic adherence every 7 days post-activation and fresh anti-CD3/CD28 beads (1:1 ratio), Rapamycin, and IL-2 (1000 IU/ml) were added as well. Cells were cultured for 3 weeks prior to use.

2.3 Drugs experiment

After 21 days of expansion, beads were removed and different doses of immunosuppressive drugs, tacrolimus, mycophenolate mofetil and methyl-prednisolone were tested one by one or in combination. Treg were activated with anti-CD3/CD28 beads (1:2 ratio) and IL-2 (20 IU/ml) in X-Vivo 15 plus the 3 drugs at different concentration. Tacrolimus range was 0-20 ng/ml, mycophenolate's one 0-2.5 ug/ml and for methylprednisolone the range was 0-4 ug/ml. Cells remained in culture for 5 days after that suppression ability and phenotype were analyzed.

2.4 Suppression assay

For functional assay, cryopreserved responder T cells (Tresp cells) were thawed and labeled with CFSE as follows. Cells were washed twice in PBS, before being stained with 2.5 μM CFSE in PBS at $1x10^7$/mL. After 3 minutes at room temperature in the dark, the reaction was stopped by washing three times the cells with X-Vivo 15 supplemented with 5% HS. The cells were then resuspended in the same medium at the required concentration. In order to quantify differences in susceptibility to suppression, Tresp cells, labeled with CFSE, were cultured alone or at different ratios with Treg cells treated with drugs. In each case, the number of Tresp cells was kept constant ($1x10^5$) and the number of Treg cells titrated down (Treg:Tres ratio= 1:1, 1:3 and 1:10) in X-Vivo15 supplemented with 5% HS. The cells were stimulated with anti-CD3/CD28 beads in U-bottom 96-well plates and incubated at 37°C, 5% CO_2 for 5 days. To test the drugs

influence, suppression assays were setted up even in presence of drugs during the 5 days. After harvest, qualitative CFSE dilution was assessed by flow cytometry exluding dead cells using Live and Dead Fluorescent dying. The percentage of suppression was calculated using FlowJo software (TreeStarInc, USA).

2.5 Antibodies for flow cytometry

The following monoclonal antibodies were used: CD4-FITC/APC (OKT4), CD62L-FITC (Greg-56) Live and Dead Fixable Yellow Dead Cell Stain kit from Invitrogen, UK; CD25-PE (4E3), CD152-PE (14D3), CD127-PE-Cy7 (eBioRDR5), GITR-APC (eBioAITR), ICOS-PE-Cy7 (ISA-3), CD39-FITC (eBioA1), IFN-γ-FITC (4S.B3), IL-17-PE (eBio64DEC17) and FOXP3-eFluor 660 (PCH101) from eBioscience, USA; Integrin β7-PE-Cy5 (FIB504) from BD-Bioscience, UK; HLA-DR-APC (L243), CLA-FITC (HECA-452), CCR4-PerCP-Cy5.5 (TG6/CCR4) from Biolegend, USA. Appropriate isotype controls from mouse or rat were used. Prior to use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions.

2.6 Cytometric beads array

The concentration of cytokines was measured using a Cytokines cytometric bead array assay kit (BD Biosciences, San Diego, CA, USA) analysed on a BD Fortessa flow cytometer (BD Biosciences, San Diego, CA, USA). In this test we used three populations of beads with different fluorescence intensity, giving a discrete peak on the flow cytometer. Each bead population was coated with a specific antibody against a human cytokine protein. This allowed the simultaneous measurement of different cytokines in a single multiplexed assay. The cytokines measured were human IL-10, IL-17 and INF-gamma. The beads, detection antibodies and samples or standards were incubated together, forming sandwich complexes, followed by aspiration into the flow cytometer for particle quantitation. Similarly to ELISA, in this assay

there is a direct correlation between fluorescence of coated antibodies and cytokine concentration. However, the main advantage of this kit is to require small sample volume to test many different cytokines in only one single assay.

2.7 Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad software Inc., USA). Parametric and non-parametric data were calculated as the mean±s.d. and median (interquartile range, IQR) respectively. For comparison of parametric and non-parametric data, t-test, one- or two-way ANOVA with Bonferroni test comparison and Kruskal-Wallis test were used where appropriate.

3. Results

3.1 Understanding the optimal dose for in vitro experiment

To determine the direct effect of immunosuppressive regimen on Treg preparations, $CD4^+CD25^+$ T cells were purified and in vitro expanded in the presence of rapamycin. Treg culture and expansion was performed as described in material and methods. Tacrolimus, methyl-prednisolone and mycophenolate mofetil were tested as single therapeutic treatment or in combination on 21-day expanded Treg. To establish the right drug concentrations to replicate in vitro what happens in vivo with patients, a wide range of concentration, including the trough levels described in patient's sera and reported in literature, were tested. Different range for tacrolimus (0-20 ng/ml) and mycophenolate mofetil (0-2.5 ug/ml) were used. Due to the lack of data on serum concentration of methylprednisolone in humans, a range of 0-4 ug/ml was used.
The first experiment was the evaluation of cell viability in presence of the 3 drugs together (MIX) and separately. Cells were stimulated with anti CD3/CD28 coated beads and IL-2 (20 IU/ml) and cultured for 3 days with the drug-range indicate above. Data in Figure 1 show the effect of the three different drugs on cell viability. Dose response curves were plotted to obtain the concentration of immunosuppressant leading to 50% of Treg viability. We calculated the half maximal effective concentration (EC50) of the single drugs inducing 50% survival in Treg activated for 3 days. Data showed that EC_{50} of tacrolimus, mycophenolate mofetil and methylprednisolone were 2 ug/mL, 1 ng/ml and 5 ug/ml respectively (Figure 1, Panels B-D). These concentrations were used in the setting of all in vitro experiments showed in this study.

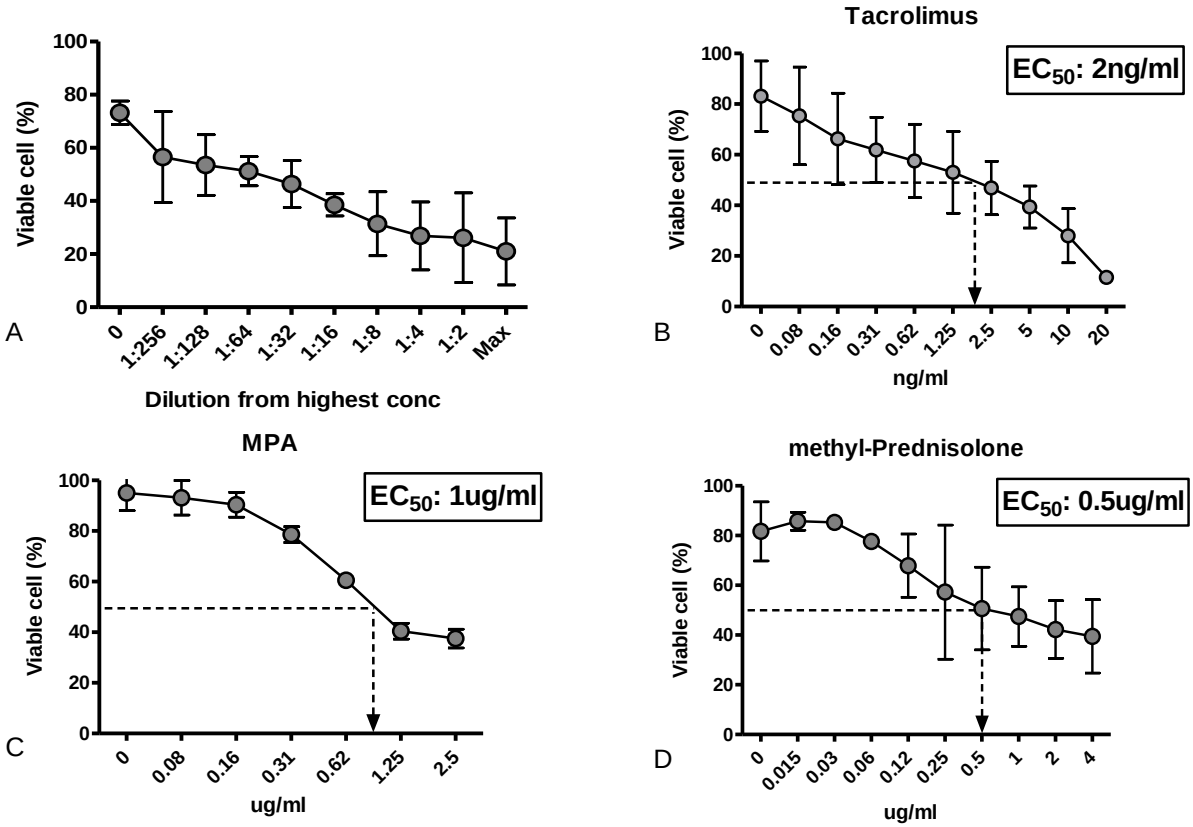

Figure 1. Dose response curves to derive the concentration of immunosuppressant. Ex vivo expanded Treg were activated with anti-CD3/CD28 coated beads (bead:cell ratio = 1:2) and IL-2 (20 IU/ml) for 3 days in the presence of different concentration of drugs. Data in panel A show the Treg viability in presence of different concentrations of the 3 drugs together. Panel B,C and D show the concentration of tacrolimus, mycophenolate mofetil and methyl-prednisolone respectively leading to 50% of Treg viability.

3.2 Drug Effects on regulatory T cells expansion

The presence of immunosuppressive treatment during cell therapy could also influence Treg proliferation. To this aim cells were activated with anti CD3/CD28 coated beads, IL-2 (100 IU/ml) and cultured with the single treatments or the combination of drugs for 5 days to investigate the effect of tacrolimus, mycophenolate mofetil and methylprednisolone on their proliferative capacity. Cell counts at the end of culture showed that any immunosuppressive agent significantly reduced the proliferation of Treg (Figure 2, Panel A). The further analysis of

cell division, using *Cell Trace Violet* dilution profiles, revealed that Treg were more severely inhibited by tacrolimus, mycophenolate mofetil and the combination of drugs compared with methylprednisolone (Figure 2, Panel B). These conditions not only affected the final number of cells, but also the number of divisions that each cell had undergone (Figure 2 , Panel B and C).

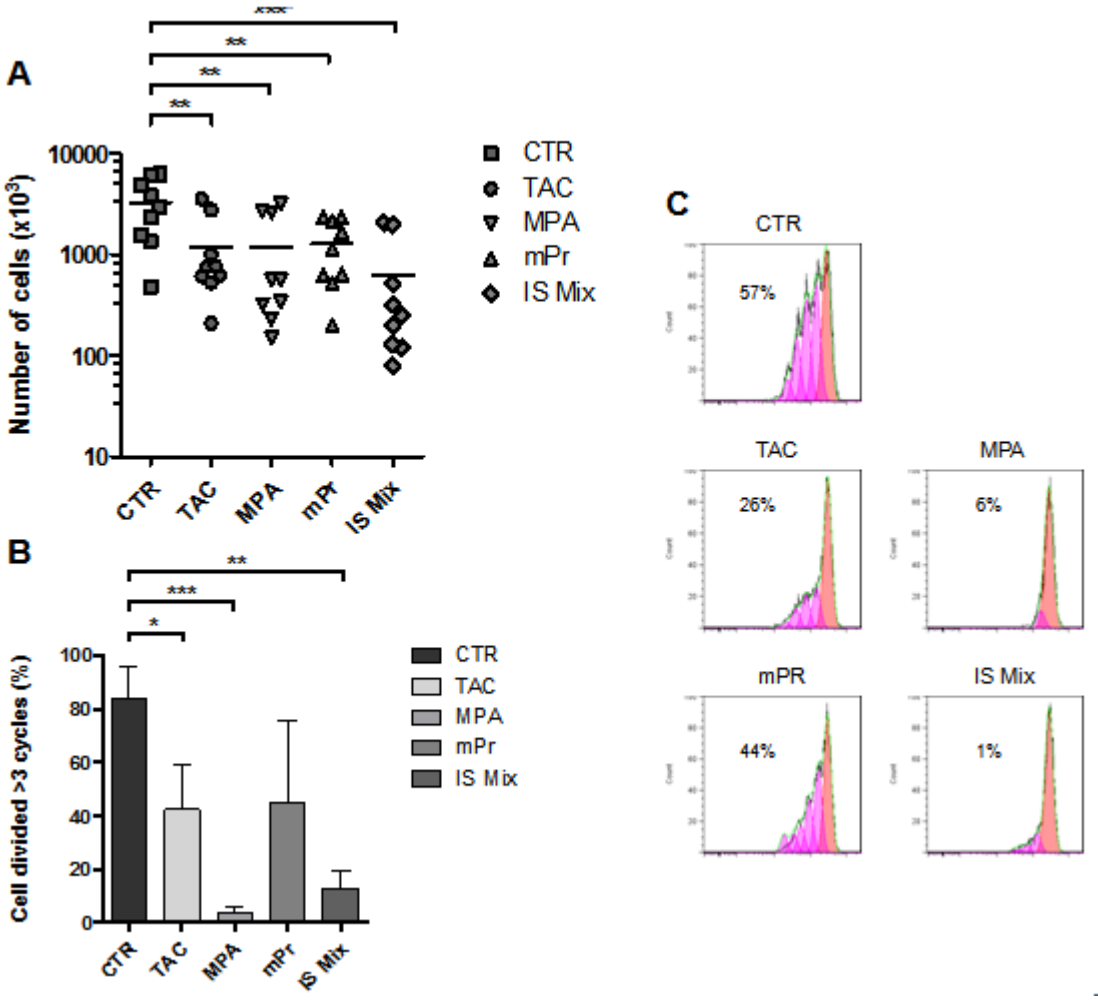

Figure 2. Proliferative capacity of Treg in the presence of immunosuppressants. Panel A, total number of ex vivo expanded and rapamycin treated Treg activated with anti-CD3/CD28 coated beads (bead:cell ratio = 1:2) and IL-2 (100 IU/ml) and cultured for 5 days with only medium (CTR), single immunosuppressive agent at EC_{50} (TAC, MPA and mPR) or their combination (IS Mix) (n=9). Panel B, percentage of Treg determined by CTV dilution profile with more than three cycles of cell division determined in the same conditions described above (n=3). Data are presented as mean ± SD. Panel C, representative histograms showing the CTV dilitution profile of Treg cultured in the presence of drugs. * $p<0.05$, ** $p<0.01$; *** $p<0.001$.

3.3 Drug Effects on main regulatory T cells markers and homing receptor

To better understand if the immunosuppressant regimen can affect Treg in vivo, the analysis proceeded evaluating the main Treg markers essential for their in vivo action. The first evaluation concerned FOXP3 and CD25, followed by CD39, CTLA4, PD-L1, HLA-DR, GITR, ICOS and TGF-β (LAP). Cells were first incubated for 5 days in presence of tacrolimus, mycophenolate mofetil, methyl prednisolone and the combination of 3 drugs together and then stained with the cognate antibody. Phenotype was evaluated using flow cytometry.

A complete analysis of these markers revealed that cells did not change their expression retaining the same phenotype (Figure 3 Panel A). The same analysis showed that, in presence of immunosuppressants, the percentages of Treg expressing both FOXP3 and CD25 molecules did not change, however a reduction in the MFI for CD25 was observed (Figure 3B and C).

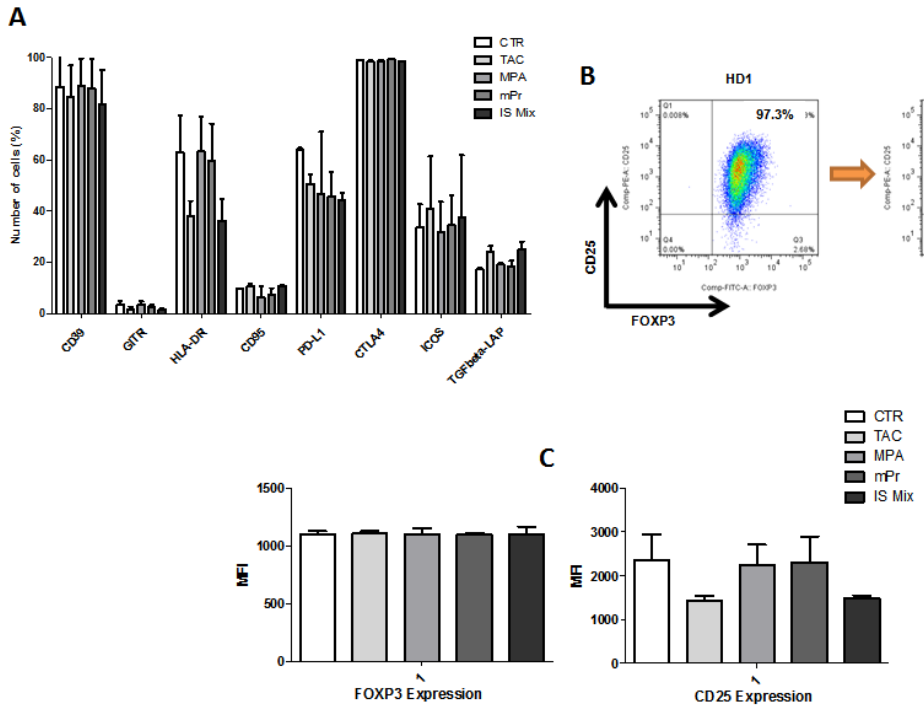

Figure 3. Phenotype of Treg cultured with immunosuppressive agents. Panel A, Expression of typical Treg markers, panel A, on cells treated for 5 days with only medium (CTR), single immunosuppressive agent (TAC, MPA and mPR) or their combination (IS Mix) (n=5). Panel B, representative dot plots from 9 different Treg preparations of FOXP3 and CD25 expression on Treg cultured 5 days with either only medium (CTR) or with the combination of 3 drugs (IS Mix). Panel C, mean fluorescence intensity of FOXP3 and CD25 on Treg treated in the same way described above

Rapamycin has the ability to modulate the expression of specific homing receptor. For this reason the expression of chemokine receptors like CCR4, CLA, CCR10 (skin-homing) integrin α4β7 and CXCR3 (gut/liver-homing) were evaluated following the in vitro treatment with drugs for 5 days. The expression of CCR4 and CLA was significantly reduced by tacrolimus and the combination of drugs. Similarly, in the same culture conditions Integrin α4β7 was up-regulated (Figure 4). This evidence suggests that tacrolimus alone or in combination with the other drugs might have an effect on homing capacity favoring Treg to traffic into the gut.

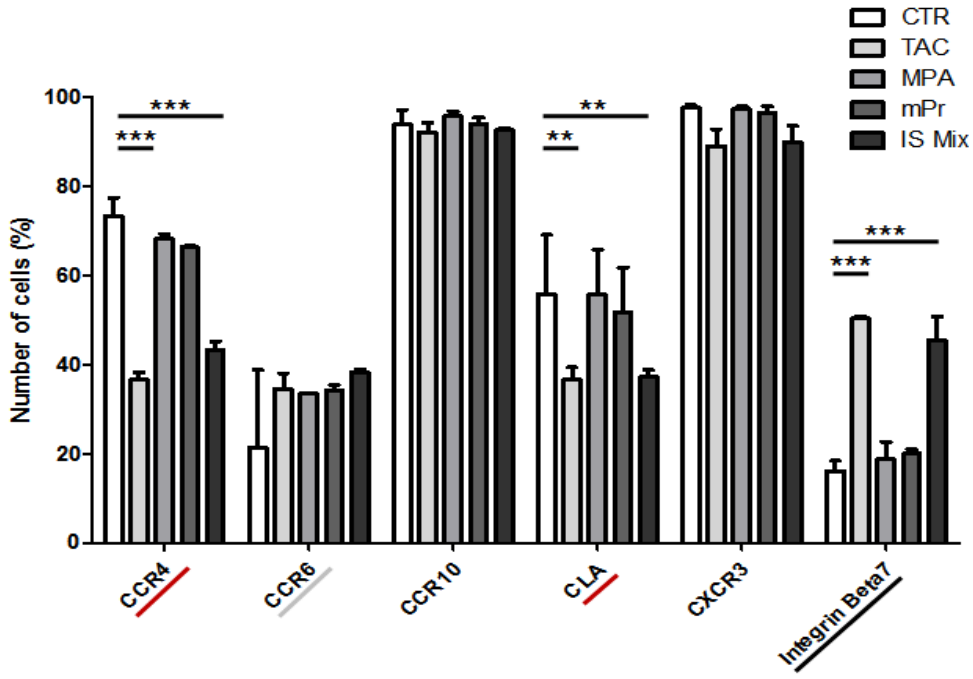

Figure. 4 Expression of homing receptor in Treg cultured with immunosuppressive agents Expression of homing receptor on Treg treated for 5 days with only medium (CTR), single immunosuppressive agent (TAC, MPA and mPR) or their combination (IS Mix) (n=5) ** $p<0.01$; *** $p<0.001$.

3.4 Immune suppressive treatment doesn't affect regulatory T cells suppressive ability

Next step was the evaluation of functional Treg activity in vitro. As shown in previous reports[178], rapamycin treated nTreg displayed a potent suppression of T cell proliferation. However, the

presence of immunosuppressive drugs could affect this suppressive ability. We tested whether pre-treatment of Treg with the combination of drugs or the single dose of tacrolimus, mycophenolate mofetil and methylprednisolone could impair suppressive function. For this reason Treg were activated with anti-CD3/CD28 coated beads (bead:cell ratio = 1:2) and IL-2 (20 IU/ml) for 7 days in presence of the immunosuppressive drugs and then the suppressive ability was tested. As shown in figure 5, the suppressive ability of immunosuppressive drugs (alone or in combination) pre-treated Tregs were not significantly different from that of the untreated cells (CTR).

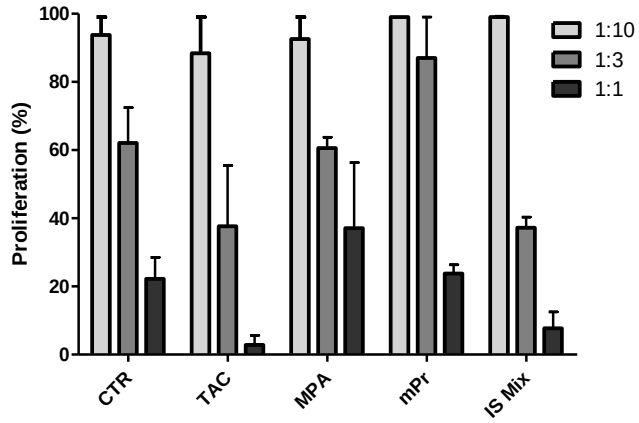

Figure. 5 Effect of immunosuppressants on Treg suppressive ability. . Suppressive ability of Treg activated with anti-CD3/CD28 coated beads (bead:cell ratio = 1:2) and IL-2 (20 IU/ml) and pre-cultured for 7 days with only medium (CTR), single immunosuppressive agent (TAC, MPA and mPR) or their combination (IS Mix) (n=5).

However, to better understand whether the suppressive function was affected in the presence of immunosuppressive drugs, we performed a suppression assay adding the single drugs or the combination of the drugs into the culture condition. To discriminate between the suppressive function of Treg and the anti-proliferative effect of drugs, we separately evaluated the proliferation of effector T cells in the presence of only drugs or drugs plus Treg at different ratio. Data in figure 6 show that although effector T cell proliferation is reduced by immunosuppressants, Treg were no affected in their function and preserved their full suppressive capacity

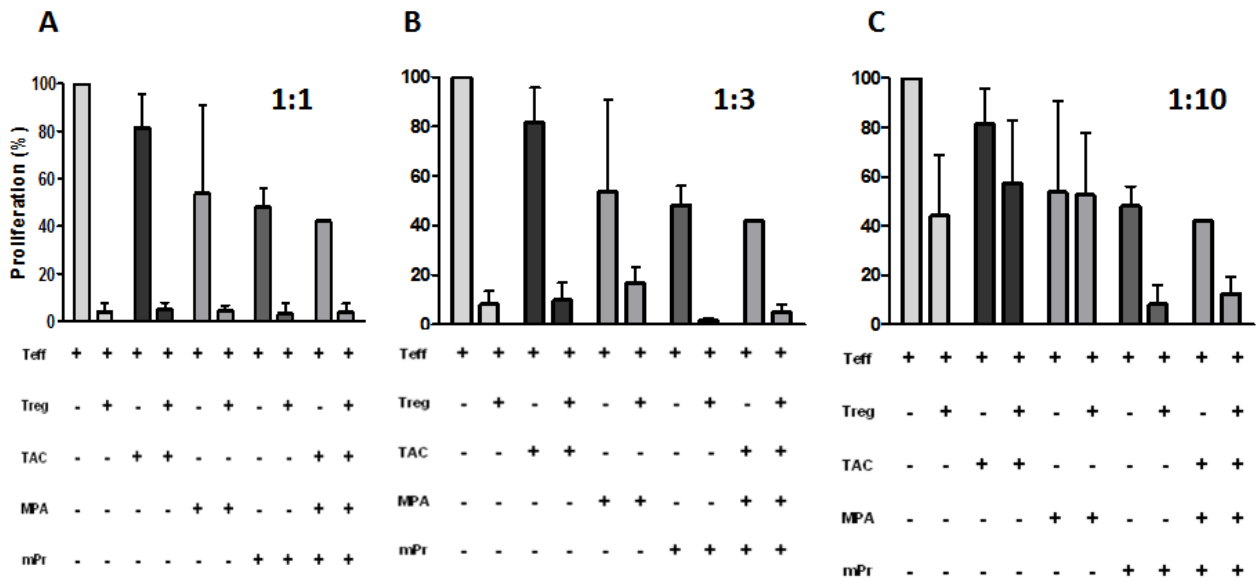

Figure. 6 Effect of immunosuppressants on Treg suppressive ability. Proliferative response of Teff cultured alone or in co-culture with Treg. Panel A B and C show ratio the 1:1, 1:3 and 1:10 Treg/Tresp ratio respectively in presence of different immunosuppressive agents as described above (n=5).

3.5 Immunosuppressant drugs inhibit IL-10 production by Regulatory T cells

Rapamycin inhibits proliferation of non-Treg cells, selectively favors the expansion of Treg and reduces the frequency of cells secreting pro-inflammatory cytokines such as IL-17 and IFN-γ[178]. A further analysis of the cytokine production profile of rapamycin treated Treg revealed that in this setting cells produce amount of IL-10 but nothing is known about the influence on cytokine production after immunosuppressant regimen. To this aim, rapamycin treated Treg were activated in the presence of tacrolimus, mycophenolate mofetil, methyl-prednisolone or their combination. After 5 days, culture supernatants were harvested and tested for the presence of cytokines. The amount of cytokine was normalized to the total number of cells at the end of culture. Data revealed that immunosuppressive treatments have no effect on IL-17 and IFN-γ production (Figure 7). However, the analysis of IL-10 production showed that tacrolimus and the combination of drugs strongly reduced this cytokine (Figure 7 panel C). This evidence suggests that although the suppressive capacity of Treg was not impaired, any treatment including

tacrolimus affects cytokine production including those that can lead to the recruitment and expansion of tolerogenic cells.

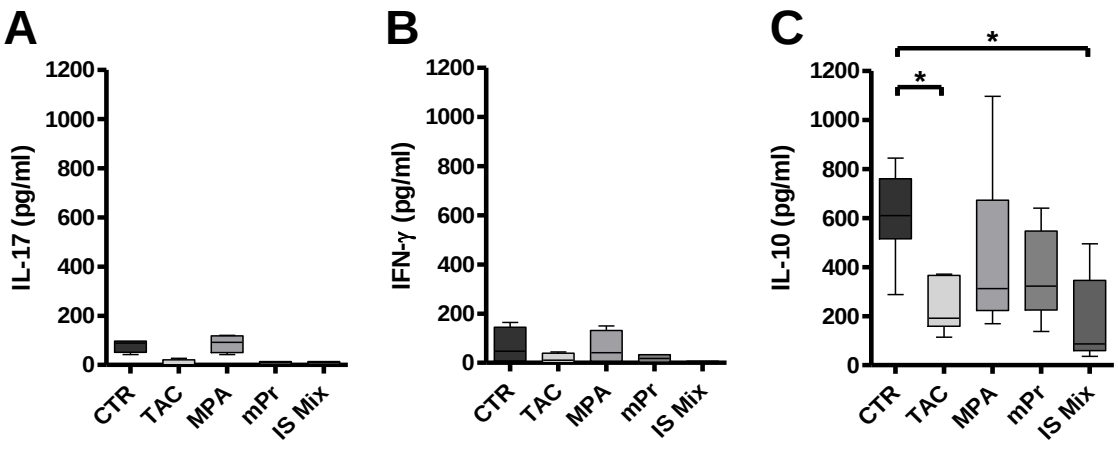

Figure. 7 Effect of immunosuppressants on Treg cytokine production. Interleukin-17 (panel A), IFN-γ (panel B) and IL-17 (panel C) concentration of 5-day culture supernatant collected from Treg activated with anti-CD3/CD28 coated beads (bead:cell ratio = 1:2) and IL-2 (20 IU/ml) and treated with single immunosuppressive agent (TAC, MPA and mPR) or their combination (IS Mix) (n=3). * $p<0.05$

4. Discussion

Making the use of adoptive Treg therapy effective as a practical tool for preventing allograft rejection or for GvHD treatment requires supplemental considerations about the possible presence of concurrent immunosuppressive therapies. Administration of drugs which target and dampen excessive immune response can potentially prove harmful to adoptively-transferred Treg. However, it would be unethical and medically inappropriate to test the efficacy of infused Treg preparations in patients without support from conventional treatments, especially during phase I and II trials where Treg efficacy is not tested. As such, it is necessary to obtain pre-clinical data showing the influence of a concurrent conventional therapy on Treg.

This study is mainly focused on tacrolimus, mycophenolate-mofetil and methyl-prednisolone, investigating their effect on Treg preparations that potentially can be infused in a program of adoptive cell therapy. Data show that immunosuppressive drugs partially influence the viability and the proliferative capacity of Tregs, however their phenotype and most of its suppressive ability is not altered.

Studying the influence of immunosuppressive regimen on either in vitro or animal model is essential to establish the appropriate concentrations of drugs which simulate the outcome seen in humans, as closely as possible. Several studies have extensively reported the correct drug concentrations administered to mice coping the efficacy of immunosuppressive regimen in human transplantation. However, some considerations are required about the in vitro use of drug trough level to evaluate the impact of the treatment on human Treg.

Several studies revealed that trough level may not correspond to the actual concentration active on cells in circulation. In fact, tacrolimus results mainly associated with erythrocytes (>70%), plasma proteins (>20%) and only a small fraction with lymphocytes (about 1%)[181]; mycophenolate is mainly bound to serum albumin (>97%) and only the free fraction is thought to be responsible for the immunosuppressive effect[182].

To this aim, a wide range of concentrations was tested (Figure 1) to establish the most appropriate to investigate the effect of immunosuppression on ex vivo expanded Treg.

Data show that mycophenolate-mofetil, methyl-prednisolone and tacrolimus can, as either single treatment or in combination, decrease the number of Treg cell divisions (Figure 2).

These results were in part expected considering the mechanism of action of immunosuppressant. The survival and suppressive activity of Tregs depend on an exogenous supply of IL-2[183,184]. Tacrolimus, inhibiting calcineurin pathway, blocks IL-2 production from T cells and in this way explains part of the inhibitory effect on Tregs[185]. Previous studies provided only indirect evidence of this effect on Tregs showing calcineurin inhibitors to be potentially harmful[186]. Studies in animal model reported that Cyclosporins A, another calcineurin inhibitor, reduces Foxp3 expression in natural Tregs[187,184], diminishes the frequencies of CD4+CD25+Foxp3+ T cells[188] and fails to support the differentiation of the highly suppressive CD4+CD25+CD27+ subset upon alloantigen stimulation[139].

Moreover, these data reveal that the detrimental effect of immunosuppressive regimen on Tregs might be extended to mycophenolate. In literature the effect of this drug on Tregs has not been extensively studied and data are controversial. Mycophenolate inhibits inosine 5'-monophosphate

dehydrogenase, a rate-limiting enzyme in the de novo synthesis of guanine nucleotides, thereby affecting DNA synthesis and cell replication of both conventional T cells and Tregs[189]. Data in literature support the idea that the influence of this drug on cell division may alter the expansion of antigen-specific Treg and prevent the settlement of a long term tolerance[190]. Similarly, Lim and collaborators showed that mycophenolate adversely affected the therapeutic effectiveness of adoptively-transferred Tregs, reducing the allograft survival in recipient mice from 21 to 16 days[191]. Nevertheless, results in murine models are contrasting. Zeiser and collaborators demonstrated in a model of GvHD that the concurrent administration of freshly isolated Tregs and mycophenolate did not significantly inhibit Treg expansion, suppressive ability and eventually extended the overall survival[184].

Likewise the other drugs, *in vitro* data showed that methyl-prednisolone does not affect phenotype and function of Tregs (figure 3,5,6). Although the single treatment provided no additional benefit to cell preparation, at a certain dose, it influenced the expansion reducing cell division of Tregs. This result seems to be in conflict with data in literature. Many authors described positive effects of steroids in regard to the maturation and expansion of Tregs in autoimmune diseases. They have been described to amplify the IL2-dependent expansion of FOXP3+CD4+CD25+ T cells in vivo[192], increase FOXP3 expression of Tregs in patients affected by asthma[193] and restore the impaired suppressive function of Tregs in patients with relapsing multiple sclerosis[194]. However, both difficulties of in vivo steroid measurement and lack of data in literature showing the trough level of this medication make it hard to copy the effect of this treatment in vitro.

In summary, this study shows that although immunosuppressive drugs partially influence the viability and the proliferative capacity of Tregs, their phenotype and most of its suppressive ability is not altered. Some concerns arise about the administration of high doses of immunosuppressive therapy during concurrent adoptive transfer of Tregs. Mainly in vitro data showed that the presence of drugs may reduce the proliferative capacity of Tregs and influence either the expression of homing receptors or tolerogenic cytokine production, such as IL-10. Thus, although many other factors in vivo can contribute to the success of a Treg therapy, the infusion of Tregs during the administration of the highest doses of immunosuppressants should be carefully considered or, at least, plan to infuse Tregs in multiple-dosing regimen.

General conclusion

My research work as PhD student was principally devoted to the study of Treg as a tool for cellular therapy in patients undergoing organ or hemopoietic stem cell transplantation. Specifically, my work has been done at the Laboratory of Cellular Therapy of the Institute of Hematology "L. e A. Seràgnoli" of the University of Bologna and the laboratory of Immunoregulation at King's College in London.

Both laboratories are highly experienced in cell therapy and regulatory T cells characterization. This gave me the opportunity to exploit different backgrounds and knowledge to study the infusion of regulatory T cells to reduce/avoid graft rejection and for the treatment of GvHD

In conclusion, it is possible to affirm that the use of expanded Tregs is necessary both in HD and patients, even when leukapheresis products are used as cell source. The high suppressive ability and the possibility to freeze the expanded fraction without problems will improve transplantation management, and allow the use of these cells for patients undergoing liver transplantation. Moreover, Treg expansion obtained in the presence of rapamycin confirms its importance as a drug improving Treg phenotype and function. The double-positive action of rapamycin, in vivo and in vitro, could be exploited to set up clinical trials in which this drug will be used for the *ex vivo* expansion and the *in vivo* retention of the infused cell product.

Therapeutic strategies using Treg have to take into account that these cells not only need potent suppressive function, but also need appropriate tissue trafficking to enable contact with their target cells. Therefore, if the Treg are injected via a peripheral vein then it is important that they express molecules such as CD62L and CCR7 that are crucial for their migration to the lymph nodes and other chemokine receptors, e.g. CXCR3 for liver homing.

Another important question concerns the number of cells needed for the infusion. To date, Treg therapy in the context of bone marrow transplantation has been very important to understand the tolerated and safe Treg doses. However, the first trials of Treg therapy in solid organ transplantation will need to start with a dose escalation study to assess the safety and tolerability of Tregs in this settings. In addition, whether a single injection or multiple injections are required is a matter of debate and may be determined in a Phase II efficacy study, where patient outcomes should also be measured. When a bigger number of patients will be analysed

the use of molecular diagnostic tools can help to assess the increased/reduced expression of either biomarkers of operational tolerance or rejection in patients receiving Treg therapy. Furthermore, phenotypic analysis of patient PBMCs, using flow cytometric analysis, can determine differences of the T cell compartment or cytokine profile as result of the adoptive cell therapy.

As the infusion schedules are essential in clinical trials using Treg, the possibility of a single or double infusion and their timing should be carefully considered. The last section in this thesis showed how the presence of immunosuppressive drugs may reduce the proliferative capacity of Tregs, and how they may influence either the expression of homing receptors or the tolerogenic property of Tregs, for example trough the production of cytokine, such as IL-10. Thus, although many other factors in vivo can contribute to the success of Treg therapy, the infusion of Tregs during the administration of the highest doses of immunosuppressants should be carefully considered or, at least, plan to infuse Tregs in multiple-dosing regimen.

Together these studies contribute to the large body of literature that demonstrates the importance of using Treg cells as therapy in various transplant models. Although much work is still to be done in this regard, there is now concrete evidence to support the translation of this approach to the clinical arena.

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