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Paula Alonso Guallart

Mixed hematopoietic chimerism and immune tolerance through bone marrow transplantation and infusion of regulatory T cells in a preclinical large animal model

Director/es
BOLEA BAILO, ROSA
DURAN-STRUUCK, RAIMON

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Tesis Doctoral

MIXED HEMATOPOIETIC CHIMERISM AND IMMUNE TOLERANCE THROUGH BONE MARROW TRANSPLANTATION AND INFUSION OF REGULATORY T CELLS IN A PRECLINICAL LARGE ANIMAL MODEL

Autor

Paula Alonso Guallart

Director/es

BOLEA BAILO, ROSA
DURAN-STRUUCK, RAIMON

UNIVERSIDAD DE ZARAGOZA
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DOCTORAL THESIS

MIXED HEMATOPOIETIC CHIMERISM AND IMMUNE TOLERANCE THROUGH BONE MARROW TRANSPLANTATION AND INFUSION OF REGULATORY T CELLS IN A PRECLINICAL LARGE ANIMAL MODEL

Memory presented by PhD candidate **Paula Alonso Guallart**

Directors:
Raimon Duran-Struuck, DMV, PhD
Rosa Bolea Bailo, DVM, PhD

October 2019

D. Raimon Duran-Struuck y **Dña. Rosa Mª Bolea Bailo**, Assistant Professor of Pathobiology from the University of Pennsylvania School of Veterinary Medicine y Profesora Titular del Departamento de Patología Animal de la Facultad de Veterinaria de la Universidad de Zaragoza

INFORMAN

Que Dña. Paula Alonso Guallart ha realizado, bajo nuestra dirección, los trabajos correspondientes a su Tesis Doctoral titulada "**Mixed hematopoietic chimerism and immune tolerance through bone marrow transplantation and infusion of regulatory T cells in a preclinical large animal model**", que corresponde con el plan de investigación aprobado por la Comisión de Doctorado y cumple los requisitos exigidos por la legislación vigente para optar al Grado de Doctor por la Universidad de Zaragoza, por lo que autorizamos su presentación para que pueda ser juzgado por el tribunal correspondiente.

En Zaragoza, a 14 de Octubre de 2019

Fdo.: Raimon Duran-Struuck

Fdo.: Rosa Mª Bolea Bailo

D. Raimon Duran-Struuck y **Dña. Rosa Mª Bolea Bailo**, Assistant Professor of Pathobiology from the University of Pennsylvania School of Veterinary Medicine y Profesora Titular del Departamento de Patología Animal de la Facultad de Veterinaria de la Universidad de Zaragoza

INFORMAN

Que Dña. Paula Alonso Guallart, bajo nuestra dirección, presenta el trabajo correspondiente a su Tesis Doctoral titulada "**Mixed hematopoietic chimerism and immune tolerance through bone marrow transplantation and infusion of regulatory T cells in a preclinical large animal model**" para optar al Grado de Doctor por la Universidad de Zaragoza con "Mención Internacional". Cumpliendo con la legislación vigente, este trabajo incluye dos informes de los siguientes expertos doctores pertenecientes a instituciones extranjeras:

- Dr. Yojiro Kato, Assistant Professor, Department of Surgery, Kidney Center, Tokyo Women's Medical University (Tokyo, Japan). Ph.D. degree obtained through Tokyo Women's Medical University (Tokyo, Japan) in 2012.
- Dr. Vimukthi Pathiraja, Researcher, Center for Transplantation Sciences, Massachusetts General Hospital, Harvard Medical School (Boston, Massachusetts, United States). Ph.D. degree obtained through the University of Melbourne (Melbourne, Australia) in 2016.

Asimismo, avalamos la realización por parte del doctorando de una estancia en una institución de investigación extranjera durante seis meses:

Estancia desde el 3 de enero hasta el 4 de junio del 2018 (152 días) en Columbia Center for Translational Immunology, Columbia University (New York, New York, United States), bajo la supervisión del Dr. Robert J. Hawley, en la que el estudiante de doctorado trabajó en diferentes protocolos para la expansión *in vitro* de células T reguladoras de macacos Cynomolgus y en el desarrollo de un protocolo para la inducción de tolerancia de trasplante *in vivo*. Por tanto, cumple con los requisitos necesarios para optar al Título de Doctor con Mención Internacional.

En Zaragoza, a 14 de Octubre de 2019

Fdo.: Raimon uran-Struuck

Fdo.: Rosa Mª Bolea Bailo

COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK

Mary Jo Shepherd, DVM, CPIA
Executive Director
Institutional Animal Care and Use Committee
154 Haven Avenue, 2nd Floor
New York, NY 10032
Phone: 212-342-1142 Fax: 212-305-5833
Email: ms4387@columbia.edu

October 17, 2019

To Whom it May Concern,

This letter is to confirm that Protocol AAAW1469, Regulatory T cells for promotion of durable mixed hematopoietic chimerism and immune tolerance in a nonhuman primate model, was originally approved by the Columbia University Institutional Animal Care and Use Committee on June 8, 2018. This protocol expires on June 8, 2021.

Please let me know if you require any further information.

Sincerely,

Mary Jo (MJ) Shepherd, DVM, CPIA

FUNDING SUPPORT

Research reported here was performed at the Columbia Center for Translational Immunology (CCTI) and Institute of Comparative Medicine (ICM) at Columbia University. Funding for these studies was provided by National Institutes of Health (NIH) grant R01OD017949, by startup funds from the Columbia University Departments of Medicine and Surgery (to Megan Sykes and Raimon Duran-Struuck), the Banting Foundation (to Megan Sykes), the Columbia University Core Award (to Raimon Duran-Struuck), and the Irving Pilot Translational Science award for new investigators (to Raimon Duran-Struuck). NIH grant R01OD017949 also provided financial support for the researchers. Flow cytometric studies were performed at the CCTI Flow Cytometry Core, supported in part by the Office of the Director financed by NIH grant S10OD020056. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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I. SUMMARY

Summary

I. SUMMARY

Induction of transplantation tolerance to kidney allografts has been achieved through transient mixed hematopoietic mixed in a non-myeloablative approach in both, non-human primates and humans. In order to make this approach applicable to other organs less tolerogenic such as lung or heart, we studied an approach to induce long-term mixed chimerism (instead of transient) trough bone marrow transplantation and infusion of recipient *in vitro*-expanded regulatory T cells (Tregs) in a non-human primate model (Cynomolgus macaque). Immunosuppression monotherapy was discontinued shortly after BMT. Donor-recipient pairs were major-histocompatibility-complex (MHC) mismatched in order to increase the applicability of this approach.

First, we studied the biology of Mauritian Cynomolgus macaque (MCM) Tregs and developed five *in vitro* Treg expansion protocols for translational studies that included the use of artificial antigen presenting cells (aAPCs), donor peripheral blood mononuclear cells (PBMCs) or a pool from different donors of CD40L-stimulated B cells (CD40L-sBc). Tregs from all protocols suppressed the proliferation of anti-CD2CD3CD28 bead-stimulated autologous PBMCs albeit with different potencies, varying from 1:2-1:4 Treg:PBMC ratios, up to >1:32. Treg expansion varied between protocols but at least 1,000 fold expansion was achieved with all of the them, up to >7,000 folds. Reculture of cryopreserved Tregs permitted reexpansion with improved suppressive activity. Occasionally, CD8 contamination was observed and resolved by resorting. Specificity studies showed suppression of PBMCs from autologous cells, cells from the same donor used for stimulation during the Treg cultures and from a third-party PBMC responders, suggestive of the polyclonallity of these Tregs. Similar to humans, the Treg–specific demethylated region (TSDR) within the Foxp3 locus correlated with suppressive activity and expression of Foxp3. Contrary to humans, FoxP3 expression did not correlate with CD45RA or CD127 expression.

We then investigated the efficacy of *ex vivo* expanded Tregs to promote the induction of durable mixed chimerism along with BMT. A total of ten recipients received Tregs with different doses of BM and outcomes were compared to five controls that did not receive Tregs. Prolonged chimerism was observed in Treg-treated recipients that received a high BM dose with infusion of Tregs compared to those that received low-dose BMT or did not received Tregs. Graft-versus-host disease (GVHD) was observed in four recipients, two controls and two animals that received Tregs expanded with CD40L-sBc. In those animals in which prolonged chimerism was observed, a higher number of peripheral Tregs was detected in blood compared to baseline levels and *in vitro*, the anti-donor response was decreased, suggestive of donor tolerance. BM rejection and chimerism loss was associated with an inversion of the CD4 and CD8 ratios and an increase in the CD8 absolute counts. Cytomegalovirus (CMV) was detected in all recipients post-BMT independently of the administration of Tregs. CMV reactivation was associated with an increase in the CD8 counts and with the loss of the BM graft. Therefore, promptly antiviral treatment was stablished for an early CMV control.

In conclusion, Tregs were able to expand the duration of chimerism (albeit transient) when administered with high-dose BMT across MHC barriers without immunosuppression.

II. INTRODUCTION

II. INTRODUCTION

Solid organ transplantation is a procedure that can save and prolong the life of individuals with end-stage heart, lung, liver, kidney, pancreas and small bowel diseases. In many cases, transplantation is the only lifesaving treatment for end-stage organ failure.

II.1. History of transplantation

Based on the latest report released by the National Transplant Organization in August 2018, a total of 135,860 transplants were performed worldwide in 2017 (with 89,823 renal, 30,352 hepatic, 7,626 heart, 5,497 lung, 2,342 pancreatic and 220 intestinal transplants). These numbers represent a 7.25% increase compared to the previous year with 126,670 transplants. Despite the high number of transplants reported, still and most importantly, only less than 10% of patients are transplanted as most die while in the waiting lists due to the shortage of organs.

Transplantation is one of the most expensive procedures available in the medical industry.[2] Interestingly, these costs are even further enhanced while patients wait for a surrogate organ as is in the example of renal failure patients which require weekly dialysis.[3,4]

Improved surgical techniques and the development of new immunosuppressive drugs have led to an increased frequency of transplants performed with excellent results.[5] However, organ recipients have complications, many of which are related to the immunosuppressive therapy required post-transplantation. These include; hypertension, diabetes, renal failure, hypertrichosis, gingival hyperplasia, neoplasia as some of them.

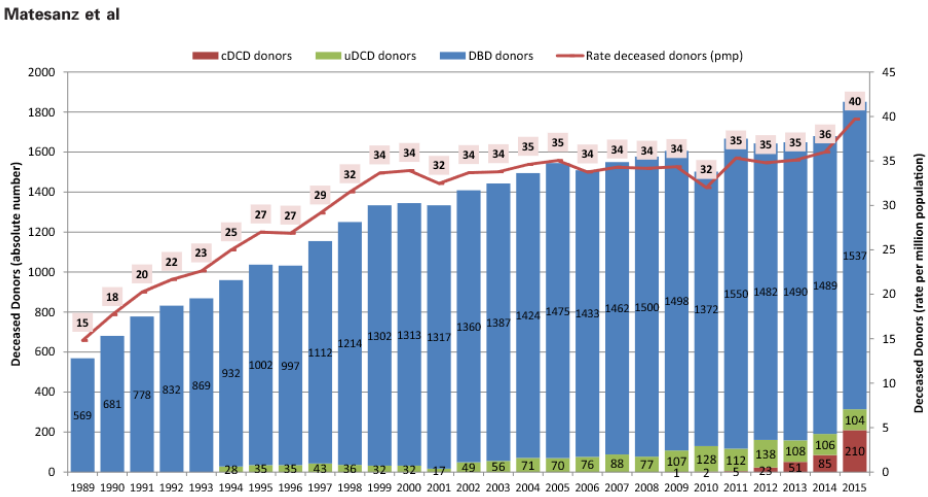
Introduction

The goal of transplantation is not only to prolong the life of patients, but also to offer them a better quality of life. Unfortunately, immunosuppressive drugs are met with significant co-morbidities. A viable drug-free approach would be to induce immunological tolerance to the new organ.

II.2. Spain is a leader in organ transplantation

2018 marked the 27th consecutive year since Spain became the world leader in organ donation and transplantation. During this year, 5,314 transplants were performed, improving the numbers from the previous year (5,259)[6] (data from the National Transplant Organization, ONT), with a transplantation rate of 114 transplants per million population (p.m.p.), which represents the highest rate in the world. Kidney transplants were the most predominant with 3,310 procedures, followed by liver (1,230), lung (369), heart (321), pancreas (82) and intestine (6), resulting in a similar trend as the number of transplants performed worldwide. These data translated into 48 donors p.m.p. with a total of 2,243 donors. The reason for these remarkable numbers stems from the development of the ONT in 1989 by the Spanish Ministry of Health. ONT is an agency that coordinates donations, procurements, and transplantation activities in Spain. ONT follows an organized model to effectively identify donation opportunities and facilitate their transition.[7] In addition, the geographical size of Spain makes it more suitable for organs to be transported within the country relatively fast hence minimizing cold ischemia time leading to improved outcomes. The elements of the Spanish model made Spain double its deceased donation activity in less than a decade (Figure 1).[1]

Figure 1. Diseased donation activity in Spain between 1989 and 2015.



Absolute number and rate per million population of diseased donation activity in Spain between 1989 and 2015. Abbreviations: cDCD, controlled donation after circulatory death; DBD, donation after brain death; pmp, per million population; uDCD, uncontrolled donation after circulatory death (Data from Matasanz et al., American Journal of Transplantation, 2017).[1]

II.3. Immunological tolerance

II.3.1. History and Background

In 1920, studies by Dr. Earl Padgett documented that skin grafts survived longer when transplanted to close relatives.[8] Ten years later, Dr. Barret Brown continued these observations after a skin graft was successfully accepted when transplanted from a patient's identical twin.[9]

Dr. Ray Owen, who was highly interested in bovine blood groups, discovered in 1945 that dizygotic twin pairs had two different sets of red cells. He then linked his findings with Dr. Frank R. Lillie's, who postulated that dizygotic-twin cattle fetuses shared their placental anastomosis with each other. Owen then postulated that dizygotic twin cattle shared their blood supply in utero. Blood transfusions between twin cattle did not cause any reaction despite differences in their blood type, unlike non-twin cattle of different blood type.[10,11]

Introduction

Peter Medawar and his fellow Rupert Billingham, in 1947, tried to differentiate between monozygotic and dizygotic twin cattle through skin allografts. The purpose of this study was to identify the sterile female twin of a male calf (freemartin) as freemartin female bovines were of no use for farmers and breeders. Their findings showed that skin grafts were accepted between related twins independently of the type of offspring but they were rejected between unrelated cattle.

This work led to Medawar, Billingham and the post-graduate student Brent to prove that tolerance could be experimentally induced in mice and chickens.[12-14] When mice received cells from a different inbred strain, these cells were rejected and any posterior infusion of the same cells was quickly destroyed. However, if the inbred-mouse cells were injected into the fetus instead of into an adult mouse, these allogeneic cells were accepted as well as any graft from the same mouse strain.[13] This breakthrough was the beginning of the research field of transplant tolerance.

Early studies by Main and Prehn (1955) showed that irradiated mice receiving an allogeneic HCT accepted skin grafts from the same BM donor[15], hence inferring the importance of a mechanism where BM itself had an influence in tolerance of a solid graft. It was in the 50's that the term "radiation chimera" was developed where the hematopoietic system was from an allogeneic BM donor.[15-21]

II.3.2. Types of tolerance

Transplant tolerance is described as a state of donor-specific unresponsiveness without the need for life-long immunosuppressive therapy.[22] Immunologic tolerance is a state of unresponsiveness to a particular antigen (or set of them) induced by previous exposure to such antigens. Transplantation tolerance has

been considered the ultimate objective in allograft transplantation as it could eliminate the need for long-term immunosuppressive therapy and its many side-effects.

Tolerance is an immunological process divided into two mechanisms, central and peripheral tolerance (Figure 2).

II.3.2.1. Central tolerance

T cells undergo a maturation process that starts with thymocytes (CD3-CD4-CD8- cells) colonizing the thymus and undergoing different maturation stages leading to rearrangements of the T cell receptor (TCR) (Figure 2).

Fist, cells migrate to the thymus cortex and acquire the expression of CD3, CD4 and CD8. Subsequently, they go through a process known as positive selection where immature thymocytes interact with cortical thymic epithelial cells (cTECs). All double positive cells (CD4+CD8+) express a complete αβTCR, however only between 20-25% of the thymocytes are able to interact with major histocompatibility complex (MHC) molecules. Thymocytes will then become either CD8+ or CD4+ single positive T cells as they interact with either MHC class I or II, respectively.[23,24]

Cells surviving this stage will move to the thymic medulla where they undergo negative selection. This selection is mediated by medullary thymic epithelial cells (mTECs). During this step, thymocytes that interact with MHC expressing self-antigen will be eliminated. Thymocytes that escape to the periphery that strongly react against self-antigens and that are not eliminated, can lead to autoimmune diseases, therefore, negative selection is important for the elimination of potentially autoreactive thymocytes by either clonal deletion or

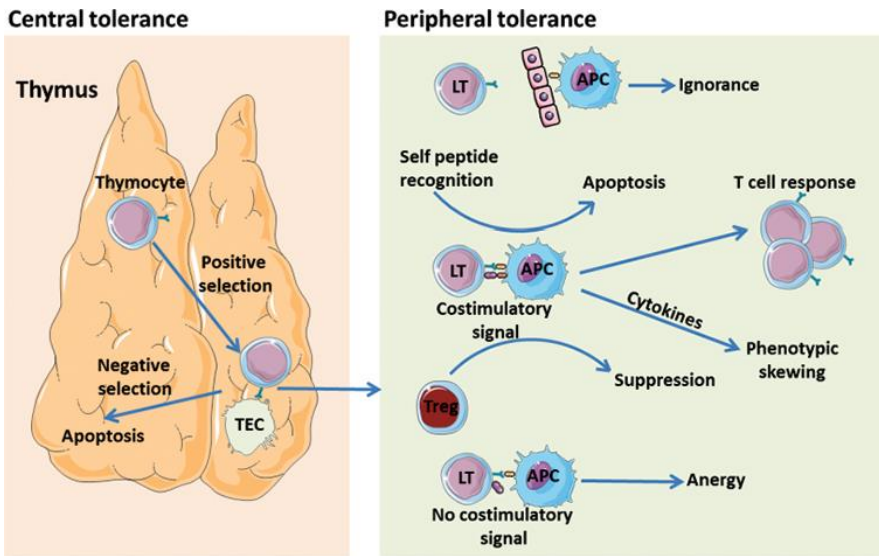
by anergy if the receptor editing fails. In essence, the thymus can be considered a graveyard as most cells never exit to the periphery.[23,24]

mTECs are the only antigen-presenting cells that express ectopic tissue-specific antigens (TSA) which classically has been documented to be controlled by the transcription factor AIRE (Auto-Immune Regulator), however, relatively recently this concept has been challenged. TSA presentation can take place through two different mechanisms:

a. Direct presentation by mTEC, which can be sufficient to induce negative selection in CD4+ and CD8+ cells.
b. Presentation by thymic dendritic cells (DCs) by getting TSAs from mTECs and presenting them to thymocytes.

If thymocytes strongly recognize TSA presented by either mTECs or DCs, they receive a signal to induce apoptosis (programmed cell death). After this process is finalized, about 5% of the total thymocytes can move on to the periphery.[23,24]

Figure 2. Mechanisms of central and peripheral tolerance



Adapted from Janeway's Immunobiology. 8th Edition.

II.3.2.2. Peripheral tolerance

There are several mechanisms aimed to control mature T cells (that have escaped negative selection) with low/moderate affinity TCRs to self MHC/peptide complexes (Figure 2) and which can be responsible for autoimmunity.

II.3.2.2.1. Antigen ignorance

Peripheral tolerance can take place through different pathways, antigen ignorance being the simplest one. T cell ignorance of self-antigens can occur on two different scenarios; the amount of antigens necessary to trigger the T cell response did not reach a given threshold,[25] or self-antigens are sequestered in places that are not easily accessible, also known as immune privilege sites like the testis or the eye.[26,27] T cells can remain as functional circulating cells without being primed by any antigen.[28]

II.3.2.2.2. Anergy

When anergy takes place, the encounter between T cells and self-antigens leads to the functional inactivation of such T cells that are unable to proliferate and produce IL-2 after antigen stimulation.[29,30] Three different mechanisms can induce anergy:

a. Lack of costimulatory signal by APCs to T cells

   Through this mechanism, there is interaction between the TCR and peptide-MHC complex without receiving costimulatory signal. Therefore, T cell activation does not occur.

b. Low affinity of TCR for the antigen[31]

   The interaction between the TCR and the antigen-MHC complex determines the activity and specificity of the T cell. Furthermore, the strength of this interaction can affect the T cell response.[32] Low affinity T cells have a reduced probability of initiating T cell signaling.[33]

c. CTLA-4/B7 interaction

   The interaction between CD28 or CCTLA-4 expressed on T cell surface and CD80/CD86 on APCs regulates the balance between activation and inhibition of T cells, respectively, controlling a potential over-reaction from the immune system leading towards inflammation or autoimmunity.

In addition, Programmed Death-1 (PD-1) interaction with its ligand PDL-1 after antigenic stimulation can induce anergy and reduce IL-2 proliferation. Another important co-stimulatory pathway is the one mediated by CD40-CD40L interaction. CD40 is expressed on APCs, vascular endothelial cells and platelets. CD40L, also known as CD154, is expressed on activated CD4+ T cells, APCs, endothelial cells, basophils and eosinophils. This co-stimulatory pathway is critical for T cell dependent effector function. The disruption of CD40-CD40L interaction can decrease interferon (IFN)-γ and IL-2 production by Th1 T cells and IL-4, IL-5, IL-10 production by Th2 T cells in addition to inhibit the activation of the expression of adhesion molecules by T lymphocytes.

II.3.2.2.3. Peripheral deletion

Through this mechanism, potentially self-reactive clones are removed in the periphery after they escaped central deletion.[34] This process is achieved through apoptosis that can be mediated by Fas/FasL (Fas is expressed in lymphoid, myeloid and non-hematopoietic cells and FasL on T cells) and Bim pathways. Apoptosis is a fundamental mechanism for homeostasis and immune regulation and it is involved in the deletion of clones that are constantly activated when the immune response is no longer necessary. The peripheral deletion is an important mechanism for the induction of immune tolerance since in the absence of donor reactive clones, the response to donor antigens could not be induced.

II.3.2.2.4. Regulation

Cells with regulatory properties are important to limit the effector cell response to pathogens and self-antigens. These are known as regulatory T cells (Tregs) which can acquire their regulatory function during their development (natural Tregs) or later on in the periphery after receiving the right stimuli (induced Tregs). Tregs will be further detailed on an upcoming section.

II.3.3. Strategies to induce immunological tolerance

II.3.3.1. Mixed hematopoietic chimerism

Mixed hematopoietic chimerism is one of the approaches that has been studied to induce tolerance to donor tissues and organs without the requirement of life-long immunosuppressive therapy. Mixed chimerism refers to a state in which a mixture of donor and recipient cells coexist in the recipient.[35] Many protocols have been reported in mice for the induction of transplantation tolerance,[36,37] although the hematopoietic mixed chimerism approach is the only one that has been successfully extended to large animals and to the clinic.

Both, mixed and full donor chimerism approaches have been capable of inducing renal allograft tolerance in the recipient. The full donor chimerism approach presents lower risk of rejection, but the risk of GVHD is elevated, limiting the applicability of this approach. In contrast, tolerance achieved through the mixed hematopoietic chimerism approach has not been associated with GVHD but rejection is harder to predict after the chimerism disappears. It is however extremely difficult to maintain a mixed chimeric state as usually the recipient either rejects the donor graft or the BM graft overtakes the host.[38-42]

II.3.3.1.1. Tolerance studies in mice

Recipient mice received lethal total body irradiation (TBI) and were reconstituted with donor bone marrow that was T cell depleted. This approach

achieved mixed hematopoietic chimerism (without graft loss or GVHD) and *in vitro* data supported the tolerant state.[43-45] Unfortunately, the levels of T cell depletion of either the donor inoculum or the host are hard to achieve, and can be lethal in humans.

GVHD continues to be a major complication in bone marrow transplantation that requires a restrictive HLA matching to minimize comorbidities. Although a low level of GVHD is associated with a desired graft versus leukemia effect,[46] this anti-tumor alloreactivity is not acceptable when using HCT for solid organ transplantation for tolerance induction. Though GVHD can be avoided by depleting T cells from the donor graft, BM loss is common[47-52] requiring heavier conditioning regimen of recipients leading to higher toxicities.

The need for a relatively non-toxic conditioning regimen is key for success of mixed chimerism for solid organ transplantation. Elimination of mature T cells from the recipient is achieved through mild total body and thymic irradiation in addition to T cell depleting therapy.[53] De novo alloreactive T cells from donor and recipient origin are deleted.[54-58] Donor and recipient thymic APCs involved in deletional central tolerance are generated due to the coexistence of HSCs in the marrow from both origins. In this setting, murine studies showed that intrathymic chimerism was enough to maintain tolerance[57] without the need of suppressive mechanisms and peripheral chimerism did not play an essential role.

Hence, protocols were developed that were more applicable to large animals and humans. Because T cells are hard to eliminate and maintenance of T cells is welcomed, co-stimulatory blockade has been shown to avoid the use of T cell depleting antibodies and thymic irradiation,[59] and the use of high bone marrow doses can be replaced for TBI.[60]

The costimulatory blockade approach achieves long-term tolerance through intrathymic deletion after mixed chimerism is achieved[59-61] while preexisting peripheral T cells that recognize alloantigens are deleted and the reminder T cells are spared.[62,63]

In the model that involves BMT and costimulatory blockade with anti-CD40L, long-term tolerance in achieved through central deletion of donor-reactive thymocytes and regulatory mechanisms do not play a significant role.[61-64] On the other hand, models using CTLA-4Ig and anti-CD40L for costimulatory blockade appear to rely on regulatory mechanisms although with less complete deletion of pre-existing donor-reactive T cells.[65-67]

II.3.3.1.2. Tolerance studies in non-human primates

Studies in non-human primates have significant value for the translation of transplantation tolerance protocols into the clinic. The lack of memory cell responses in mice[68-70] (due to their specific-pathogen-free (SPF) status) or the different expression of MHC[71,72] are not representative to outbred large animal species or humans.

Contrary to what was observed in mice, the use of a non-myeloablative regimen failed to induce stable mixed hematopoietic chimerism in non-human primates across MHC barriers.[73,74] In order to enhance the levels of mixed chimerism, several approaches were necessary. Splenectomizing the recipients (for the prevention of humoral rejection[75,76]) and adding a short course of cyclosporine (for one month post-transplant) improved chimerism and outcomes.[77,78] When anti-CD40L (anti-CD154) was added to the regimen (in substitution of splenectomy) the chimerism was further improved however never becoming long-lasting.[76] Kean and colleagues, used busulfan, CD40/CD40L and CD28/B7

co-stimulation blockade. Unfortunately, this regimen failed to induce stable chimerism even in MHC-matched animals.[72,79]

Although stable hematopoietic chimerism failed to be induced, tolerance to MHC-mismatched renal allografts was achieved when the transplant was performed while chimerism was present,[75-78] and it was thought to be regulatory T cell mediated. In addition, the kidney was suspected to have an important role in the induction of tolerance by providing antigen to such Tregs. Moreover, the addition of costimulatory blockade with anti-CD40L instead of splenectomy achieved encouraging results; the eight-transplanted animals had improved chimerism and survived long term.[76] Unfortunately, anti-CD40L has thrombogenic complications[80] but other agents such as CTLA-4Ig have been investigated successfully and without clotting disorders.

This approach has been attempted with other organs less tolerogenic such as the heart, lung and islets. Studies performed on heart transplantation showed that eventually the recipients lost their graft through humoral and cellular rejection.[81] Furthermore, co-transplantation of heart with kidney was performed through the mixed chimerism approach. These experiments suggested that the kidney graft was necessary for the maintenance of tolerance of the other organ after chimerism was lost.[82] If the kidney graft was removed, the heart was subsequently rejected. Similar results were observed with islets, they were accepted without immunosuppression if transplanted under the renal capsule of the BM donor kidney.[83]

In 2007, Kean et al. published a study in which they induced long-lived myeloid chimerism in MHC-matched Rhesus macaques through stem cell transplantation and co-stimulation blockade (busulfan, belatacept and anti-CD40L). Unfortunately, T cell chimerism was not achieved through this protocol and

donor chimerism was eventually lost. In addition, tolerance to skin grafts was not successfully achieved.[84] Later on, they published an adaptation of this strategy with the addition of low-dose total body irradiation achieving multilineage hematopoietic chimerism for up to 24 months (length of the study). These protocols were performed on MHC-matched transplants. In this case, T cell chimerism and acceptance of the donor skin graft were achieved. This was associated with significant infectious diseases (especially CMV reactivation) and end-organ disease[85] due to functional defects in T cell immunity.

Early 2017, Duran-Struuck et al. published a study in which animals received BMT across MHC barriers with or without co-infusion of Tregs. Non-Treg-treated animals had transient chimerism unlike Treg-treated animals, from which 2 out of 5 of the recipients achieved multilineage chimerism including T cells for up to 335 days in one case that displayed recent thymic emigrant phenotype. In addition, these animals accepted the BM-donor kidney four months after the BMT in the absence of immunosuppressive drugs for more than 294 days.[86] This was the first time where such non-myeloablative approach achieved long-lived hematopoietic chimerism across MHC barriers. Similar to Kean's study,[85] CMV reactivation and treatment played an important role in the BM engraftment, as it was associated with failure of durable chimerism, independently of Treg treatment.

Not all the studies performed in large animal models using Tregs have shown positive outcomes. A study conducted by Thomson and his group addressed the capability of *ex vivo* expanded Tregs to induce heart transplant tolerance in NHP across MHC barriers.[87] Tregs were infused while animals were lymphodepleated. Outcomes showed that not only graft function resulted to be inferior compared to control animals but also animals that received Tregs had higher levels of effector memory T cells, interferon (IFN)-γ production by host

CD8+ T cells, pro-inflammatory cytokines and anti-donor antibodies. The authors concluded that the environment in which Tregs are infused during early post-transplantation period and lymphodepletion could impair the therapeutic efficacy of infused Tregs. Differences in the *in vitro* expansion of Tregs may have influenced the outcomes as well as the preparatory regimen.

II.3.3.1.3. Clinical studies

Three clinical studies have been reported up to date in which renal allograft tolerance has been achieved through the mixed hematopoietic chimerism approach:

II.3.3.1.3.1. Stanford approach

Initial studies conducted by Strober et al. reported in 1989 the use of total lymphoid irradiation (TLI) with rabbit ATG (rATG) for the induction of HLA-mismatched renal allograft. TLI consists of the delivery of ionizing radiation to lymphoid organs such as lymph nodes, thymus, and spleen for immunosuppression purposes while non-lymphoid organs remain intact. Originally, the three-transplanted patients achieved kidney tolerance[88] although two of the three patients eventually developed chronic rejection and ureteral stricture that led to the loss of the graft.[89]

Based on this initial experience, mixed chimerism was pursued and HSCT was added to the original regimen with TLI and rATG. The regimen included ten consecutive doses of 80-120 cGy per day starting on Day 1 post-renal transplant. rATG was administered during five days starting on Day 0 post-transplant. Additionally, CD34+ enriched donor peripheral blood stem cells were infused after the last administration of TLI. The immunosuppressive therapy consisted of mycophenolate mofetil (MMF), calcineurin inhibitor (CNI) and steroids.

Three cohorts of patients underwent this regimen:

a. The first group of patients included six recipients that received mismatched renal allografts. Only two of them achieved transient mixed chimerism (during 2 to 3 months) and withdrawal of the immunosuppressive regimen was attempted. Unfortunately, a few months later, mild rejection was detected (Banff I), so immunosuppression was reinstituted.

b. For the next group of patients, the criteria to discontinue the immunosuppressive therapy was strengthened; chimerism had to be present for at least six months, without evidence of GVHD or signs of rejection on the renal biopsy. In addition, all the recipients in this group received HLA-matched kidneys. From the 22 patients, 21 of them developed chimerism and 18 patients met the criteria to discontinue immunosuppression. Sixteen patients were off immunosuppression for up to 66 months.

c. The third group enrolled 10 patients who received HLA-mismatched kidneys. In an attempt to improve the chimerism induction, CD34+ and CD3+ cells doses were increased, being T cells administered in an escalating dose of 3, 10, 20 and 50 million/kg, unlike the patients from the first two groups that received 0.1 to $1x10^6$ T cells/kg. From the ten patients, eight of them developed transient chimerism under six months so immunosuppression was never discontinued. The other two patients developed chimerism for at least 12 months so MMF was discontinued and they currently remain in tacrolimus monotherapy.[90]

As a conclusion of these reports, mixed chimerism was successfully induced in the majority of the patients receiving HLA-matched grafts in which

immunosuppression was able to be discontinued in approximately 70% of the patients, unlike HLA-mismatched recipients.

II.3.3.1.3.2. Northwestern approach

Two different tolerance-induction strategies have been pursued by this group:

a. One approach included fludarabine, cyclophosphamide (pre- and post-transplant) and 200 cGy of TBI on Day -1. The kidney transplant was performed on Day 0 and the HSCT on Day 1 along with an infusion of "facilitating cells". This cell population included tolerogenic CD8+/TCR- and a heterogeneous population composed mainly by plasmacytoid DC.[91,92] The purpose of this cell infusion was to enhance hematopoietic cell engraftment and to reduce the risk of GVHD.[93] In addition, MMF and tacrolimus were administered post-transplant for 6 and 12 months, respectively. From the 31 patients enrolled in this protocol, 30 developed chimerism, and from these patients, 16 discontinued the immunosuppression therapy for up to 65 months, two developed GVHD (despite the decreased incidence of this complication in HLA-mismatched recipients), two had infectious complications and one died.

b. The second protocol included alemtuzumab, donor HSC infusion, MMF and tacrolimus which was converted to sirolimus 3 months later and slowly discontinued by month 24 post-transplant. The recipients received HLA-matched living-donor renal grafts in this regimen. Fifteen patients received this regimen from the original 20 patients that enrolled the study. After 36 months post-transplant, six recipients were able to discontinue the immunosuppressive therapy for up to 64 months.[94]

I.3.3.1.3.3. Massachusetts General Hospital (MGH) approach

Different clinical trials have been performed at MGH to induce allograft tolerance in HLA-matched and HLA-mismatched recipients:

a. Seven patients received HLA-identical combined kidney and BMT for the treatment of renal failure secondary to multiple myeloma. The first six patients received cyclophosphamide in a non-myeloablative conditioning regimen based on a mouse model for separating GVH and GVL.[95-97] After this approach was proven to be safe inducing anti-leukemia effect in patients with hematological malignances in clinical trials, it was translated into patients with multiple myeloma who received an allograft from HLA identical donors.[98-102] Transient and durable mixed chimerism developed and remission of the myeloma was observed with transient mixed chimerism. Immunosuppression was successfully discontinued in the three recipients with transient chimerism and in one of the patients with stable mixed chimerism. In addition, the longest survival of the kidney graft was 14 years. One of the patients with transient chimerism received a second HSCT from the same donor due to myeloma progression and she developed full chimerism so immunosuppression was never discontinued as prophylaxis for GVHD.

b. Extending the previous approach from HLA-matched recipients, the second approach involved HLA-mismatched recipients receiving the renal graft from a living donor.[103,104] The initial conditioning regimen included cyclophosphamide, TI, anti-CD2 mAb and post-transplant calcineurin inhibitor. In addition, rituximab was added later due to humoral rejection development in two patients. The ten patients that enrolled into the study developed transient mixed chimerism and immunosuppression was discontinued in eight of them by 9-14 months after the transplant. One of

them developed acute rejection requiring a second kidney transplant. Four of the seven patients remained off immunosuppression for 6-14 years while three of them restarted treatment at 5-8 years after the kidney transplant due to chronic rejection of disease recurrence.

In addition, acute kidney injury was observed in nine out of these ten patients between Day 10 and 20 post-kidney transplant. This was correlated with hematopoietic cell recovery and early loss of chimerism. Since the acute kidney injury was not observed in the non-human primate recipients that received a low dose of TBI instead of cyclophosphamide, a clinical trial was recently started where cyclophosphamide was substituted for low dose TBI. As result, none of the two patients developed acute kidney injury and one of them remains off immunosuppression for more than three years. Furthermore, clinical trials are pending including belatacept instead of anti-CD2 mAb based on NHP studies[105] since the later one is not Food and Drug Administration (FDA)-approved.

II.3.3.2. Non-chimerism approaches

Different approaches have been taken for the induction of tolerance in humans beside the mixed hematopoietic chimerism approach.

In 2003, Kirk et al. published a clinical trial that involved the use of alemtuzumab, a humanized CD52 antibody, for the induction of tolerance in human renal allografts.[106] This specific monoclonal antibody causes profound T cell depletion in humans reducing the need for maintenance immunosuppression after renal transplantation.

The aim of the study was to determine if tolerance could be induced in renal allografts by performing T cell depletion prior to the transplant with

alemtuzumab. T cell depletion was successfully achieved in blood and secondary lymphoid tissues. On the other hand, tolerance induction to the allograft failed to be achieved as rejection appeared within the first month post-transplant characterized by monocytic infiltrates and low levels of T cells. Rejection was reversed with steroids and/or rapamycin treatment and patients were maintained on low-level immunosuppressants, remaining rejection-free. This study suggested that T cell depletion alone is not enough for tolerance induction.

A subsequent study combined alemtuzumab for T cell depletion with a short course of deoxyspergualin (DSG) which has inhibitory effects on monocytes and macrophages.[107] Unfortunately, and despite significant T cell depletion, tolerance was not achieved. The rejection profile was similar to the one from patients that were treated only with alemtuzumab.

II.4. Concepts of bone marrow transplantation: Rejection and Graft-versus-Host-Disease crossing MHC barriers

II.4.1. Hematopoietic stem cell transplantation

Hematopoietic cell transplantation (HCT) is a potentially curative treatment option for malignant and non-malignant diseases. The hematopoietic stem cells (HSC) used for this procedure can be obtained from bone marrow or blood from related or unrelated donors. In addition, umbilical cord blood has been stablished as another potential source. HSC are multipotent so they are able to develop into any of the three types of blood cells; red cells, white cells or platelets.

Two different types of HSC have been identified; long-term and short-term repopulating cells. The long-term repopulating cells (LTRC) have the capacity to maintain self-renewal and multi-lineage differentiation throughout life. Short-

term repopulating cells (STRC) are derived from LTRC and despite that STRC also maintain multipotency, they have a limited self-renewal potential. STRC reconstitute the myeloid and/or lymphoid compartments for about six weeks.

Early experiments demonstrated that irradiated murine recipients could be protected from lethal irradiation by infusing donor syngeneic adult bone marrow into the recipient[108,109] therefore regenerating the irradiation-ablated hematopoietic system.

II.4.2 Sources of HSC

A minimum of $2x10^8$ mononuclear cells per recipient kilogram is necessary for transplantation. Different approaches have been investigated to obtain HSC. They can be harvested from BM through iliac crest aspirates, from peripheral blood or umbilical cord blood (UCB). In BM, around 1 in 100,000 cells are LTRs. Other cells include blood progenitor cells, stromal and stromal stem cells and mature and maturing white and red blood cells. A small number of stem and progenitor cells circulate in blood, therefore HSC need to be mobilized (with granulocyte-colony stimulating factor, IL-3, stem cell factor or AMD-3100) from marrow niches to peripheral circulation prior to collection. This is performed via pheresis with the final product containing 5 to 20% of HSC. Different types of stem and progenitor cells have been found in UCB. Although studies have shown that the number of different hematopoietic progenitors in UCB is about ten times higher than what has been observed in adult blood,[110] the amount of nucleated cells found in one unit of UCB represents only 1/10 and 1/100 compared to bone marrow and peripheral blood allografts, respectively.

II.4.3.Types of transplant

An autograft is a graft that is a “self” graft (i.e. skin graft that is placed from one area of the body to another area). Grafts from genetically identical siblings or

animals, are called syngeneic grafts. When donor and recipient are genetically disparate, grafts are called allogeneic. Finally, xenotransplants are performed between different species.

II.4.4. Relevance of the blood group systems in transplantation

Blood transfusions have been the most established form of cell therapy. The term blood group comprises the red blood cell (RBC) antigens whose specificity is controlled by a group of genes. MHC matching is not required for blood transfusions since red cells and platelets express small amounts of MHC class I molecules and they do not express any MHC class II molecules, so they are not usually targets of the recipient T cells.

In contrast, antibodies against red cells and platelets can induce an anaphylactic response. Blood needs to be matched to avoid the destruction of the transfused red cells by antibodies in the recipient.

Many types of antigens have been observed on the red cell membranes and although 33 blood group systems have been recognized by the International Society of Blood Transfusions,[111] the ABO and Rhesus (Rh) systems are the most commonly studied. These are important for transplantation.

II.4.4.1. ABO system

ABO remains the most important system in blood transfusion and transplantation since any person older than 6 months of age has anti-A and/or anti-B antibodies in the serum. ABO antibodies in the serum are formed naturally, as their production is stimulated when the immune system encounters the “missing” ABO blood group antigens in foods or in microorganisms. Red blood cells in the blood group A express antigen A and the serum contains antibodies against antigen B and vice-versa. Blood group O

contains no A/B antigen on the surface of RBCs and generates antibodies against both, A and B antigens. Lastly, the blood group AB contains red blood cells expressing the antigen A and/or B and the serum does not contain any antibody against those antigens.

II.4.4.2. Rhesus system

The rhesus-system is the second most important blood group system in addition to the most complex as it is highly polymorphic and immunogenic. Currently, the Rh-system consists of 50 defined blood group antigens, being the D antigen (also known as Rh factor) the most important one, followed by the C or c and E or e Rh antigens.[112] The presence of the D antigen on the RBC surface determines the positivity to this blood type. Accordingly, the status is indicated as either Rh-positive (D-antigen present) or Rh-negative (D-antigen absent).

In contrast to the ABO system, anti-Rh antibodies (immunoglobulin G) are normally not present in the blood of individuals with D-negative red blood cells, unless the circulatory system of these individuals has been exposed to D-positive red blood cells. The importance of anti-Rh antibodies relies during pregnancy as the mother will produce anti-Rh antibodies if this antigen is expressed on the RBCs of the fetus, developing hemolytic disease as they can cross the placenta. Prophylaxis is given against Rh immunization using anti-D Ig for pregnant Rh-negative mothers who have given birth to Rh-positive child.

II.4.5. Major Histocompatibility Complex (MHC)

The major histocompatibility complex (MHC) represents a group of highly polymorphic genes that code for proteins present on the cell surface of nucleated cells involved in immunological functions.[113] The name of MHC varies depending on the species, as in humans it is named Human Leukocyte Antigen (HLA). MHC matching is important in organ transplantation. Non-self MHC

molecules play a major role in organ rejection. However, the current advances in immunosuppressive therapies have allowed the performance of solid organ transplantation across MHC barriers. This allowed for a less straightened criteria for MHC matching for most types of solid organ transplantation, but it remains a crucial step in bone marrow transplantation.

There are two large classes of MHCs. Differences of expression are cell dependent. MHC class I proteins are present in all nucleated cells and MHC class II proteins are only present on specialized antigen-presenting cells including macrophages, dendritic and B cells.[114] In humans the MHC system is known as human leukocyte antigen (HLA). HLA-A, HLA-B and HLA-C are class I MHC molecules and HLA-DP, HLA-DQ and HLA-DR are class II. In addition, there are other loci such as E, F, G and H in class I and DM, DN and DO in class II that are much less polymorphic that code for non-classical MHC molecules compared to the ones previously presented. The human genes responsible for MHC are located on the short arm of chromosome 6. The MHC system is highly polymorphic, which implies the existence of more than one allele at the same locus. There are hundreds of variants of MHC proteins, which all of them are relatively present in the population. The differences between the MHC are found in the area of binding to the peptide. This polymorphism is useful when fighting different microorganisms but it is a barrier in organ transplantation.[115,116]

II.4.6. Cell surface antigens target for rejection

II.4.6.1. MIC System (MHC Class I Chain-Related Genes)

Two families of genes, MICA and MICB, conform the MIC system. These are highly polymorphic genes related to MHC class I located on chromosome 6.[117] They are expressed as a response to cellular stress and they code for cell surface proteins not associated with β2-microglobulin and do not bind peptides. NKG2D

is one of their receptors which has been identified on NK cells, γδ and CD8+ T cells. It is suspected that the MICA molecule could be associated with transplant failure since it could be target of antibodies and T cell response.[117]

II.4.6.2. Minor Histocompatibility Antigens (MiHC)

Minor antigens are normal proteins that represent another group of polymorphic proteins different from the MHC system. They are receptors on the cellular surface that can cause an immunological response by the host when the organ is transplanted. These peptides are presented by MHC I and II. These commonly drive GVHD in BMT and rejection for solid organ in MHC matched donor-recipient individuals.[118]

The most described minor antigens are coded by the Y chromosome, and is relevant in sex-mismatched transplants. These responses are known as H-Y and are only seen in female anti-male MiHC.[118] Other antigens are encoded by autosomal genes, HA1-HA8, and have been observed in mitochondrial DNA. They are transmitted by the mother, therefore its name, MTA or maternally transmitted antigens.[119]

II.4.6.3. KIR genes (Natural Killer Cell Immunoglobulin-Type Receptors)

These receptors have been observed on natural killer (NK) cells and in some types of T cells. NK cells assess the MHC class I expression on potential target cells in order to differentiate self from non-self. The genetic region of this receptor is located on chromosome 19 and it is highly polymorphic. This fact is especially important in hematopoietic stem cell transplantation since NK cell alloreactivity has been associated with the improvement of the patient.[120,121] Therefore, after HCT, donor-derived NK cells target recipient HSC, resulting in an anti-leukemia effect and a lower incidence of graft rejection in addition to a decrease incidence of GVHD (because of the targeting of host APCs). The

difference between the KIR receptor on the donor-derived NK cells and the recipient's MHC I is what causes the alloresponse due to the lack of interaction between the inhibitory KIR receptor and self-MHC I molecule eliminating malignant recipient cells by cell lysis.

II.4.7. Antigen presentation

II.4.7.1. Peptides associated with MHC class I

MHC class I presentation occurs in all nucleated cells in the body. MHC class I molecules present peptides to CD8+ T cells. CD8+ T cells recognize the peptide/MHC class I molecule complexes and after activation, CD8+ cells lyse the cells presenting the peptide, which are obtained by proteolytic degradation of cytoplasmic proteins mainly by proteosomes. In order for CD8+ T cells to engage with their target, have to be licensed. This occurs through the contact with an APC presenting the cognate antigen through the TCR and MHC complex engagement and costimulation. The source of these antigens could be a virus or other intracellular pathogens able to synthesize their own proteins during the life cycle. In addition, self- or tumor proteins can also be presented through the same process.[122]

II.4.7.2. Peptides associated with MHC class II

Professional antigen presenting cells (APCs) express MHC class-II. In this case, antigens are captured from outside the cell by endocytosis and they are then presented to CD4+ T cells. The interaction between APCs and CD4+ T cells takes place in the peripheral lymphoid organs. After such interaction, CD4+ T cells become activated acting as effector cells in cell-mediated immunity processes or as classically been documented, they could act as helper T cells providing stimuli to B cells and cytotoxic T cells.[123]

II.4.8. Allorecognition theories

The adaptive immune response in vertebrates is activated when recipient T cells recognize through their TCR antigens in the form of peptides (foreign or self) bound to MHC molecules (peptide-MHC complex). The activation of pro-inflammatory allospecific T cells triggers a cascade of events leading to rejection of the organ. In contrast, inhibition or deletion of alloreactive T cells can lead to acceptance of the graft.[124]

II.4.8.1. Direct presentation

In this case, donor's APCs present alloantigens through their MHC to recipient T cells. Therefore, the activation of allospecific T cells occurs through the interaction of their T cell receptor with intact allogeneic MHC molecules on donor cells. Donor antigen-presenting cells leave the graft, and migrate via lymph to regional lymph nodes where they activate the host T cells. Recipient CD4+ and CD8+ T cells recognize alloantigen presented on intact donor MHC class II and class I, respectively.

II.4.8.2. Indirect presentation

In this case, host T cells are activated by donor peptide presented by host APCs (in the context of solid organ transplantation). Graft alloantigen (typically MHC antigen) is internalized by host APCs that are usually DCs then processed and presented on the recipient MHC for-self restricted recognition by recipient T cells. Therefore, the activation of the recipient T cells occurs through the interaction of self-TCR with donor peptides presented by self-MHC molecules on the recipient APCs. Alloantigen is recognize as self-restricted processed peptide. Although both CD4+ and CD8+ could be activated by this pathway, CD4+ T cells are more significant in rejection of vascularized grafts.

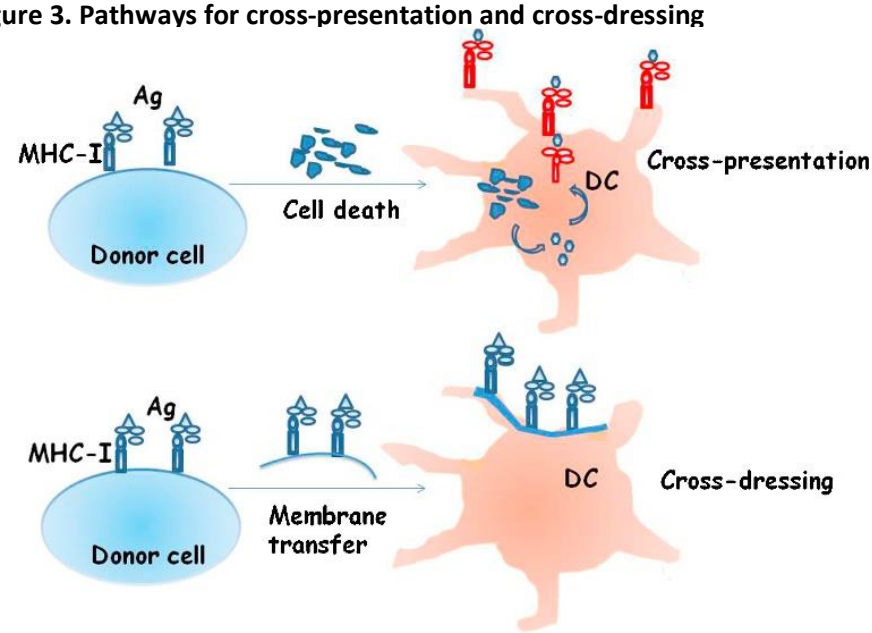
II.4.8.3. Semidirect presentation

Through this pathway, alloreactive recipient T cells are activated by the interaction with allogeneic MHC molecules that have been transferred to recipient APCs.

II.4.8.4. Cross-presentation

In this process, the antigen is synthesized by donor cells and acquired by recipient APCs such as DCs which will process and load the peptides into their own MHC proteins (Figure 3). This implies that peptide-MHC complexes are generated by host DCs themselves. Through this pathway, exogenous antigens are presented through MHC class I and II (Figure 3).

Figure 3. Pathways for cross-presentation and cross-dressing



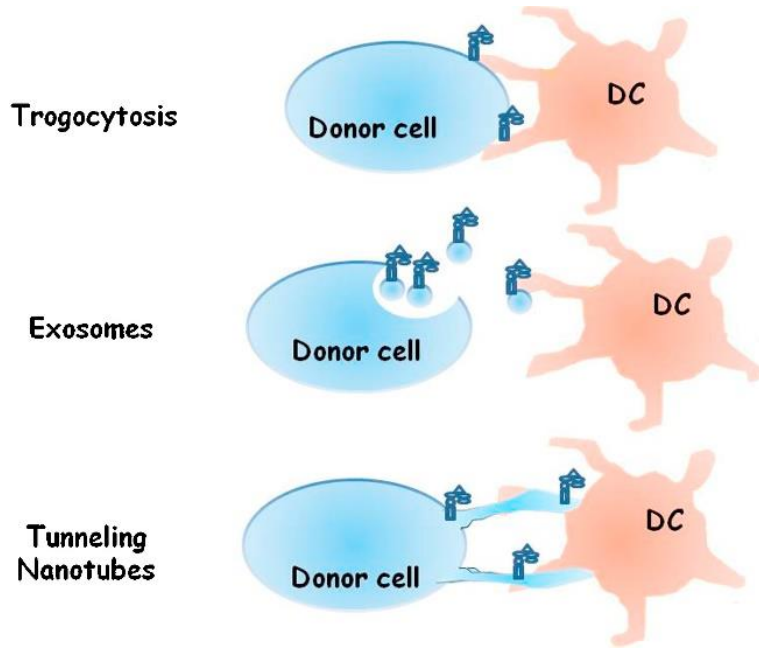
Adapted from Janeway's Immunobiology. 8th Edition.

II.4.8.5. Cross-dressing

Through this process, alloantigen-MHC complexes are transferred from donor to recipient DCs (Figure 3), which present the antigen to recipient T cells without processing.[125] Donor DCs synthesize the antigen peptides and the MHC

molecules and generate the peptide-MHC complexes that will be acquired and presented by recipient DCs to activate recipient T cells. MHC class I and II molecules can be transferred through this mechanism. Different mechanisms have been described for cross-dressing such as trogocytosis, exosome-mediated transfer or tunneling nanotubes (Figure 4).

Figure 4. Mechanisms described for cross-dressing.



Adapted from Janeway's Immunobiology. 8th Edition.

II.4.9. Immunological responses in transplantation

II.4.9.1. Rejection

As previously described, there are different pathways for recipient T cells to recognize alloantigens post-transplantation. Consequently, an effector response is triggered by the host immune system known as alloresponse that activates both, innate and adaptive systems.

II.4.9.1.1. Hyperacute rejection

Transplanted organs ongoing hyperacute rejection are lost within the first 48 hours after the transplant and it occurs only in vascularized grafts. Preformed antibodies in the recipient serum against blood group or polymorphic MHC antigens expressed on the vascular endothelium can cause the rapid rejection of transplanted organs in a complement-dependent reaction characterized by vessel thrombosis leading to graft necrosis that can start within minutes after transplantation.[126]

The encounter of recipient antibodies with donor antigens with high affinity triggers rapid rejection of the vascularized graft. Antibodies react with antigens on the vascular endothelial cells of the graft and activate the complement system and stimulate endothelial cells to secrete Von Willebrand procoagulant factor causing platelet adhesion and aggregation. This reaction causes the vessels of the graft to thrombose. The graft enlarges and becomes hypoxic leading to the death of the organ.[127]

In order to avoid this situation, cross-matching and ABO-matching between donor and recipient are performed routinely in the clinic. Through cross-matching, it can be determined if the recipient has pre-formed anti-donor antibodies that would react with the donor white blood cells. This being the case, transplantation would be highly contraindicated. Desensitization could be performed by treatment with intravenous immunoglobulin, which has been successful in a proportion of patients with preformed antibodies. It is worth to mention that some grafts, such as liver, are less affected by this type of injury.[128]

II.4.9.1.2. Acute rejection

This type of rejection occurs between five days to three months post-transplantation. Acute rejection is the combination of the host humoral and cell-

mediated immune responses against donor antigens. Recipient T cells are stimulated and activated by alloantigens which results in the proliferation of immunocompetent anti-donor T cells. Effector and memory CD4+ and CD8+ T cells are found in histological samples that underwent this kind of rejection.[127] Macrophages form the mainly infiltrating population found in vascular acute rejection. Humoral acute rejection has also been demonstrated through the development of recipient B cells that secrete antibodies (IgM) that bind to donor antigens, especially MHC class I and class II and minor MHC antigens. Consecutively, antibody aggregation causes activation of complement or recruitment of recipient inflammatory cells into the donor graft. Complement activation proteins C3d and C4d can be checked through staining techniques.[124]

II.4.9.1.3. Chronic rejection

This term refers to late graft loss. Chronic rejection is the result of a response to low-grade ongoing injuries to the vascular endothelium of the graft. One of the mechanisms is antibody-dependent complement activation. B cells are known to be involved in this process of late rejection as C4d deposits have been observed simultaneously with donor-specific alloantibodies. Another mechanism is cell arteritis leading to the development of interstitial fibrosis and tubular atrophy.[129,130] Beside the study of C4d deposits, other techniques are evaluated such as ELISPOT assay or intracytoplasmic staining for cytokine measurements, Cylex for measurement of ATP levels or the use of flow cytometry for the study of lymphocyte activation markers.[126,127]

II.4.9.2. Graft versus host response (GVHR)

BMT is the current treatment for hematological diseases, specially malignancies and primary immunodeficiencies. GVHD is a common complication after BMT.[131] This complication implies the activation and proliferation of the donor T cells infused in the BM graft against the recipient proteins. GVHD has been classified

as acute or chronic if it develops before or after 100 days post-BMT, respectively. In addition, the immunological mechanism and clinical development differ from one to another. The targeted organs are mainly the liver, skin and gastrointestinal tract.

Unlike pathogen-specific immunity, in GVHD every APC can present self-peptides. In addition, the APC migration to secondary lymphoid organs is not associated with any pathogen and any primed APC can be responsible for T cell activation. In addition, in this setting, the antigen in unlimited. For these reasons, the alloimmune response is capable of engaging most of the repertoire of adaptive immune mechanisms available.[132,133]

II.5. Regulatory T cells

II.5.1. Introduction

Regulatory T cells, hereafter referred to as Tregs, are naturally occurring cells, present in the immune system. In humans, they are defined by the expression of the extracellular marker CD3, CD4, and CD25 and the lack of CD127 and expression of the intracellular transcription factor forkhead box P3 (FoxP3).[134,135]

The role of Tregs is to maintain self-tolerance and immune homeostasis. Lacking or having dysfunctional Tregs can lead to autoimmune diseases, immunopathology and allergy. In addition, Tregs provide protection from graft-versus-host disease and transplant rejection. In contrast, high number of Tregs allow cancer cells to evade the immune response. Therefore, Tregs play an important role in immune-regulation and they can serve as a tool to manipulate the immune system in the clinic, therefore, Tregs are considered an important therapeutic target. The manipulation of their suppressive capacity of in vivo numbers could have useful implications in the clinic for the treatment of

autoimmune diseases, cancer, prevention or treatment of allograft rejection or GVHD.

Tregs can be classified as natural occurring or induced cells. Natural Tregs are formed in the thymus and induced Tregs derive from conventional CD4+ cells that become Tregs in peripheral sites when exposed to the appropriate stimuli. In addition to CD4+CD25hiCD127-FoxP3+ Tregs, other T cell populations have been shown to possess regulatory properties being able to suppress autoimmune disorders in mice under certain circumstances.[136] These different populations include Treg1 cells (Tr1) which secrete IL-10, T helper 3 (Th3) that secrete transforming growth factor-β (TGFβ) and some CD4-CD8- T cells and CD8+CD28- T cells. These cells are adaptively regulatory, which implies that they can acquire regulatory functions upon specific antigen stimulation, specially cytokines. In contrast, naturally occurring Tregs are, in great majority, developmentally determined as a suppressive T cell population in the thymus.

II.5.2. Background

In 1995, it was observed in mice that cells constitutively expressing CD25 had regulatory properties.[137] Later on, several groups starting in 2001 reported human Tregs as CD4+CD25+[138-143] and in 2003, FoxP3 was described in mice as a gene controlling Treg development and function.[134,135,144] Subsequently, FoxP3 was also confirmed as a specific marker in human Tregs.[145]

Furthermore, in 2006, it was shown that CD25 and FoxP3 could be induced in naïve CD4+ cells in humans,[146] therefore hindering the identification of pure Tregs. This has led to some discrepancy regarding the phenotype, function and stability of Tregs. Studies have shown the lack of stability in Tregs for phenotype, function and gene expression,[147-149] therefore, new markers are necessary for the better understanding of this population.

II.5.3. Identification markers

The identification of accurate surface Treg markers is mandatory for the enrichment of viable Tregs.[150] Human Tregs are characterized to represent the top 2-4% of the CD25+CD4+ T cells.[141] The brightest CD25 expressing CD4+ T cells are considered Tregs and T effector cells also express CD25 upon activation.[138,151]

FoxP3 is a transcription factor intracellularly detected in Tregs.[152-154] Although Tregs consistently express FoxP3, this marker can be upregulated in activated T effector cells when activated,[155] especially in the presence of TGF-β, therefore additional markers are necessary to confidentially identify Tregs. In addition, in order to detect the expression of FoxP3, cells require to be fixed and permeabilized, therefore becoming non-functional for *in vitro* proliferative studies.

Cytotoxic T-lymphocyte antigen 4 (CTLA-4), also known as CD152, is a mainly intracellular protein receptor that acts as an immune checkpoint. CTLA-4 competes with CD28 to bind to CD80 or CD86 on the surface of antigen presenting cells, down-regulating T cell activation. Same as FoxP3, it is continuously expressed in Tregs and transiently expressed in T effector cells after activation.[156] The expression levels correlate with the suppressive capacity of the Tregs. Detection of CTLA-4 by flow cytometry also requires permeabilization of the cells, impairing their use for functional assays.

CD127 is the IL-7 alpha receptor. Human Tregs are characterized for lacking the expression of CD127. Together, the lack of CD127 along with the extracellular expression of CD4 and high levels of CD25 have been used to identify functional Tregs in humans.[157,158] Activated effector T cells express CD127 after activation but they express low CD127 levels in the course of activation,[159] therefore, other

markers such as CD62L have been used to differentiate Tregs from recently activated T effector cells.[160]

CD45RA and CD45RO are expressed by different subsets of CD4+ T cells.[149] The diversity in the expression of these markers allow for the differentiation of naïve/resting Tregs (CD45RA+CD4+CD25+FoxP3low), and activated Tregs (CD45RO+CD4+CD25hiFoxP3hi). Activated Tregs derive from naïve/resting Tregs and present higher suppressive capacity and proliferative response compared to naïve/resting Tregs.[149] In addition, cells that express CD45RO+CD4+CD25+FoxP3low are non-suppressive effector T cells that secrete pro-inflammatory cytokines.

The inducible co-stimulator ICOS, is a co-stimulatory receptor that can be present in both, thymus and periphery Tregs, dividing Tregs into two distinct populations. ICOS+ Tregs present a memory phenotype and suppress DC function through IL-10 excretion and use TGF-β to suppress T cell function, in contrast to ICOS- Tregs that only secrete TGF-β and can express CD45RA (naïve phenotype) or CD45RO (memory phenotype).[148]

The glycoprotein A repetitions predominant (GARP) is critical for tethering TGF-β to the cell surface. Activated Tregs co-express GARP and the latent form of TGF-β.[161,162] The expression of GARP is detected in TCR-activated Tregs with an unstable profile without being detected in freshly isolated Tregs.[163] GARP is expressed 100 fold more by activated human Tregs compared to effector T cells, and its expression has been correlated with FoxP3 expression and suppressive function of Tregs.

The expression of latency-associated peptide (LAP) on activated Tregs represents the latent TGF-β complex.[164] LAP is only expressed on Tregs and it is rapidly expressed for a short period of time after TCR stimulation.

II.5.4. Mechanism of action

Tregs can exert the suppressive function through contact dependent and independent mechanisms through third-party cells or molecules (Figure 5).[165]

II.5.4.1. Suppression by inhibitory cytokines

The cytokines IL-10, TGF-β and IL-35 have been described to possess inhibitory properties through which Tregs exert, in part, their suppressive function. In addition, they have also been described to stimulate the production of induced Tregs.[166,167]

II.5.4.2. Suppression by cytolysis

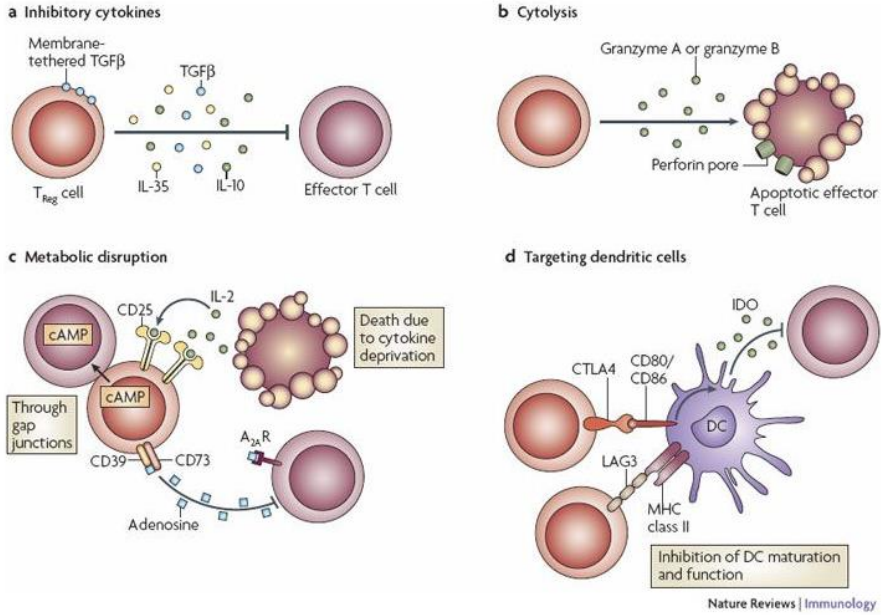
Activated human Tregs have been reported to possess cytolytic properties through the secretion of granzyme A and B and perforin through the adhesion of CD18.[168]

II.5.4.3. Suppression by metabolic disruption

Metabolic disruption of the effector T cell target has been reported in Tregs through the consumption of local IL-2 and deprivation of the target cells resulting in IL2-deprivation-mediated apoptosis.[169] In addition, the expression of CD39 and CD73 on Tregs was shown to generate adenosine nucleosides that suppressed the function of the effector T cells by activating the 2A receptor.[170-172] Furthermore, the binding of adenosine to the receptor A2 also appeared to promote the proliferation of induced Tregs by inhibiting the secretion of IL-6 and promoting the TGF-β secretion.[173] Another pathway that Tregs have shown to exert suppressive activity is by transferring cyclic AMP (cAMP), which is a potent

inhibitory second messenger, to effector T cells through membrane gap junctions.[174]

Figure 5. Modes of action used by Tregs



Suppression by A) inhibitory cytokines, B) cytolysis, C) metabolic disruption, D) modifying DCs. Picture from How regulatory T cells work. Adapted from Vignali et al. Nature Reviews 2008.

II.5.4.3. Suppression by targeting dendritic cells (DCs)

Tregs can modulate the maturation and function of DCs which are required for the activation of effector T cells.[175] The interaction between Tregs and DCs is thought to be through the co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4) which is expressed on Tregs.[176] In addition, Tregs can promote the expression of indoleamine 2,3-dioxygenase (IDO) on DCs, which is a potent regulatory molecule, resulting in the suppression of effector T cells through the interactions between CTLA-4 and CD80/CD86 expressed on effector T cells and DCs, respectively.[177,178] Other studies have shown the capacity of Tregs to downregulate the CD80 and CD86 expression of DCs.[179] Furthermore, other studies have reported the modulation of other APCs, monocytes and

macrophages.[180,181] Other studies have suggested that lymphocyte-activation gene 3 (LAG3 or CD223), expressed on Tregs, may block the maturation of DCs by binding to MHC II.[182,183] Neuropilin-1, expressed on Tregs, has been shown to allow for prolonged interactions between Tregs and DCs, offering an advantage in modulating these APCs over naïve T cells.[184]

II.5.5. *In vivo* experience

Prior to translating Treg therapies to the clinic, it is important to review and study the preclinical models. It has been shown that deletion of CD25+ cells in the recipient through anti-CD25 antibodies has hastened graft rejection.[185-187] Alloreactive Tregs from naïve mice have demonstrated *in vitro* to possess the capacity to suppress responses to alloantigens and when transferred *in vivo,* to avoid allograft rejection.[188]

There are two types of alloreactive Tregs. The direct alloreactive CD4+ T cells occur only in transplantation when the TCR on the Tregs interact with the donor MHC class II presented on donor APCs. In the contrary, the indirect alloreactive T cells are like other T cells in a non-transplant setting and the TCR of the Treg recognize allogeneic antigens presented on the recipients MHC class II molecules on recipient APCs. It appears that like T cells, Tregs interacting through direct recognition are at a higher frequency when compared to indirect recognition. Tregs present in a naïve recipient are not sufficient to prevent allograft rejection despite the high number of direct alloreactive Tregs. Therefore, different approaches have been studied to modify the ratio Treg:Teff favoring the Treg population:[189]

a. One of the approaches has been to promote the expansion and/or conversion of Tregs, therefore increasing the Treg repertoire and favoring the Treg numbers over the effector T cells.

b. A second approach is the administration of Tregs after prior *in vitro* expansion. Different transplant tolerance inducing protocols have studied the use of donor-specific Tregs.

Fifty clinical trials that include the infusion of *ex vivo* expanded polyclonal Tregs (CD4+CD25+CD127-) have been completed or are currently ongoing for different applications such as the prevention of GVHD after BMT, rejection of solid-organ transplantation or autoimmune diseases.[190-195]

II.5.5.1 Prevention of GVHD

Six clinical trials have been reported in BMT for the prevention of GVHD and two for its treatment.[194] Different strategies were followed: isolation from umbilical cord from a third-party donor and then expanded *in vivo*,[191,196,197] Tregs isolated from haploidentical donors without *in vitro* expansion[192] or for the treatment of chronic GVHD.[198] Overall, a delay in the development of GVHD or an improvement of chronic GVHD with Treg infusion was observed although early viral reactivation was detected in one study[197] and some of the patients developed skin tumors in the other study (Table 1).[198]

II.5.5.2 Prevention of solid organ transplant rejection

Several trials are currently ongoing for the prevention of transplantation rejection.[199,200] Most of the clinical trials are planned for kidney transplantation with three of them targeting liver. Most of them are currently in the recruiting phase (Table 1).

II.5.5.3 Treatment of autoimmune diseases

This is the third field that reported the infusion of Tregs in clinical trials. Two studies were focused on the treatment of type 1 diabetes, one pediatric and one in adults.[201-203] In these studies, Tregs were expanded prior to infusion to

increase the protection of the beta cells from the autoimmune attack. Although no serious adverse events were reported, only two of the patients remained insulin independent with a lower dose of insulin required in the rest of the patients.[203]

Another study for the treatment of type 1 diabetes in adults included the infusion of polyclonal Tregs that were infused in escalating doses.[204] No adverse events were observed but only mild improvement in the production of insulin by beta cells was reported with a decline in some cases although not correlating with the Treg doses infused.

In conclusion, overall safety of the use of Tregs for clinical infusions has been reported. As many of the studies are still in an early clinical phase, follow up reports will provide information regarding the efficacy of the infusion of Tregs.

Table 1. Clinical trials with infusion of Tregs

Disease application	Center	Ph	Cell dose	Product	Indication	Effects	Study ID	Ref.
HCT	Gdansk	I	1×10^5–3×10^6/kg	Expanded poly-Tregs	GvHD treatment	Safe/reduced immunosuppression	NKEBN/458-310/2008 (Gdansk ethics board)	(62)
	Minnesota	I	1–30 × 10^6/kg[a]	Expanded CB poly-Tregs	GvHD prophylaxis	Safe reduced acute GvHD, increased infection	NCT00602693[b]	(44, 63)
	Minnesota	I	3–100 × 10^6/kg	Expanded CB poly-Tregs with engineered cell line	GvHD prophylaxis	Safe reduced GVHD and no increased relapse	NCT00602693	(45)
	Perugia	I	2–4 × 10^6/kg	Fresh polyTregs	GvHD prophylaxis	Safe/reduced leukemia relapses/ reduced incidence of GvHD	Protocol No 01/08, CEAS Umbria	(64, 65)
	Regensburg	I	≤ 5 × 10^6/kg	Fresh polyTregs	GvHD prophylaxis	Safe	Treg002EudraCT: 2012-002685-12[c]	(56)
	Milan	I	1–3 × 10^6/kg	Tr1 (*IL-10 DLI* or *DC-10 DLI*)	GvHD prophylaxis	Safe/long-term disease-free survival in 4 patients	ALT-TEN, IS/11/6172/8309/8391	(66)
	Stanford	I/II	0.1–10 × 10^6/kg	Fresh polyTregs	GvHD prophylaxis	Terminated (NCT01050764) Recruiting (NCT01660607)	NCT01050764/ NCT01660607	–
	Dresden	I	0.6–5 × 10^6/kg	Expanded polyTregs	GvHD treatment	Tumors in 2 patients/stable chronic GvHD	Protocol no. EK 206082008	(58)
	Bologna	I/II	0.5–2 × 10^6/kg	Fresh polyTregs	Chronic GvHD prophylaxis	Recruiting	NCT02749084	–
	Minnesota	I/II		Fresh CB polyTregs with IL-2	GvHD prophylaxis	Recruiting	NCT02991898	–
	Boston	I		Fresh polyTregs with IL-2	Steroid refractory chronic GvHD treatment	Recruiting	NCT01937468	–
	Lisbon	I/II	0.5–3 × 10^6/kg	Fresh polyTregs	Steroid refractory chronic GvHD treatment	Recruiting	NCT02385019	–
	Stanford	I		polyTregs	Steroid-dependent/refractory chronic GvHD treatment	Unknown	NCT01911039	–
	Liege	I	0.5 × 10^6/kg	Fresh polyTregs	chronic GvHD treatment	Unknown	NCT01903473	–
	Houston	I/II	1–10 × 10^6/kg	Fucosylated polyTregs	GvHD prophylaxis	Active, not recruiting	NCT02423915	–
	Tampa	I		Donor expanded Tregs	GvHD prophylaxis	Recruiting	NCT01795573	–
	Minnesota	I	3 × 10–100^6/kg	Induced Tregs	GvHD prophylaxis	Active, not recruiting	NCT01634217	–
Organ trans	London, Oxford,	I/II	1–10^6/kg	Expanded polyTregs	Living donor kidney transplant	Recruiting	NCT02129881	(67)
	Berlin	I/II	0.5–3 × 10^6/kg	Expanded polyTregs	Living donor kidney transplant	Recruiting	NCT02371434	(67)
	San Francisco	I/II	4–10 × 10^6/kg[d]	Donor-alloantigen-reactive Tregs	Living donor kidney transplant	Recruiting	NCT02244801	(67)
	Boston	I/II		Belatacept-conditioned Tregs	Living donor kidney transplant	Active, not recruiting	NCT02091232	(67)
	Chicago	I		Expanded polyTregs	Living donor kidney transplant	Active, not recruiting	NCT02145325	–
	Milan	I/II		Antigen-specific Tr1 (T10 cells)	Living donor kidney transplant	Not yet recruiting		(67)
	Moscow	I	3 × 10^6/kg[d]	Expanded polyTregs	Kidney transplantation	Unknown	NCT01446484	–
	Multicenter USA	I/II	6 × 10^6/kg	Donor reactive and polyTregs	Kidney transplantation	Recruiting	NCT02711826	–
	London	I	≤ 4.5 × 10^6/kg	Expanded polyTregs	Liver transplant	Recruiting	ThRIL, NCT02166177	(68)
	Nanjing	I	1 × 10^6/kg	Alloantigen-specific Tregs	Liver transplant	Unknown	NCT01624077	–
	San Francisco	I	7 × 10^5–10 × 10^6/kg[d]	Donor-alloantigen-reactive Tregs	Liver transplant	Recruiting	NCT02188719	–
Other Treg-based trials	San Francisco	I	5 × 10^6/kg[d]	Expanded polyTregs	Subclinical rejection in kidney transplantation	Active, not recruiting	NCT02088931	–
	San Francisco	I	4–7 × 10^6/kg[d]	Donor-alloantigen-reactive Tregs	CNI reduction in liver transplantation	Recruiting	NCT02474199	–
Autoimmunity	Gdansk	I	≤ 30 × 10^6/kg	Expanded polytTregs	Recent T1D	Safe/reduced insulin doses	ISRCTN06128462[e]	(69–71)
	San Francisco	I	7 × 10^4–40 × 10^6/kg[d]	Expanded polyTregs	T1D	Safe	NCT01210664	(72)
	Lille	I/II	1 × 10^4–10 × 10^6/kg	Ovalbumin-specific Tr1	Refractory Crohn's disease	Safe/clinical response in 40% of patients	CATS1	(73)
	Gdansk	I		Expanded polyTregs	Multiple sclerosis	Recruiting		–
	Nanjing	I/II	10–20 × 10^6/kg	Expanded polyTregs	Autoimmune Hepatitis	Not yet recruiting	NCT02704338	–
	Gdansk	II		Expanded polyTregs	Recent T1D	Recruiting		–
	Hunan	I/II	1–5 × 10^6/kg	Expanded third-party CB polyTregs	Recent T1D	Recruiting	NCT02932826/ NCT03011021	–
	Multicenter USA	II		Expanded polyTregs	Recent T1D	Not yet recruiting	NCT02691247	–
	San Francisco	I	3–20 × 10^6/kg	Expanded polyTregs	Recent T1D	Recruiting	NCT02772679	–
	San Francisco	I	1.4–23 × 10^6/kg[d]	Expanded polyTregs	Systemic Lupus erythematosus	Not yet recruiting	NCT02428309	–

[a]*Two infusions day +4 and day +15 after HCT.*
[b]*clinicaltrials.gov.*
[c]*www.clinicaltrialsregister.eu.*
[d]*Converted to cells/kg based on 70 kg average body mass if not stated by study (European standard).*
[e]*www.isrctn.com.*

Clinical application of Tregs. Adapted from Duggleby et al. Frontiers in Immunology 2018.[194]

III. JUSTIFICATION AND OBJECTIVES

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Proof of principle that mixed chimerism can lead to organ allograft tolerance across MHC barriers in NHPs and patients has been achieved using combined kidney and BMT through the induction of mixed chimerism.[77,103] However, the mixed chimerism achieved in these studies was only transient and it was suggested that the kidney itself contributed to the development of the tolerant state in these recipients.[76,77] Tolerance to other organs less tolerogenic than the kidney (such as lung, heart or islets) has not yet been achieved with this approach. Furthermore, kidney transplantation tolerance has been achieved in NHPs in a delayed approach that included infusion of Tregs where the kidney was transplanted four months post-BMT while chimerism was still present.[205] Despite these results are promising, successful results were achieved in a few recipients.

Studies in mice indicate that durable mixed chimerism is associated with robust, systemic tolerance that permits survival of the most challenging allografts.[206] Thus, reliable methods of achieving durable mixed chimerism without GVHD, the major complication of HCT in humans, are needed to advance the use of mixed chimerism to achieve organ and islet allograft tolerance.

Therefore, the general objective of the study is to induce durable mixed hematopoietic chimerism and transplantation tolerance (that is independent of early kidney grafting) in NHPs receiving a low-toxicity, non-myeloablative BMT regimen in addition to *in vitro* expanded recipient Tregs. The use of non-specifically expanded recipient Tregs allows generation and cryopreservation of these cells in readiness for transplantation from unknown, cadaveric donors at unpredictable times.

The specific goals of the project include:

- Optimize and develop new strategies for the *ex vivo* expansion of Cynomolgus macaque Tregs including their phenotypic and functional characterization prior to cryopreservation.

- Develop an optimal regimen combining non-myeloablative conditioning, infusion of expanded recipient Tregs, infusion of different doses of donor BM and transient post-transplant immunosuppression with rapamycin to achieve durable mixed allogeneic chimerism in Cynomolgus macaques.

- Monitor chimerism duration in peripheral blood, assess mechanisms of tolerance in mixed chimeras and study the persistence of peripheral Tregs comparing these outcomes between Treg-treated recipients and control animals that did not receive Tregs.

IV. MATERIALS AND METHODS

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IV.1. *In vivo* protocols

IV.1.1. Animals

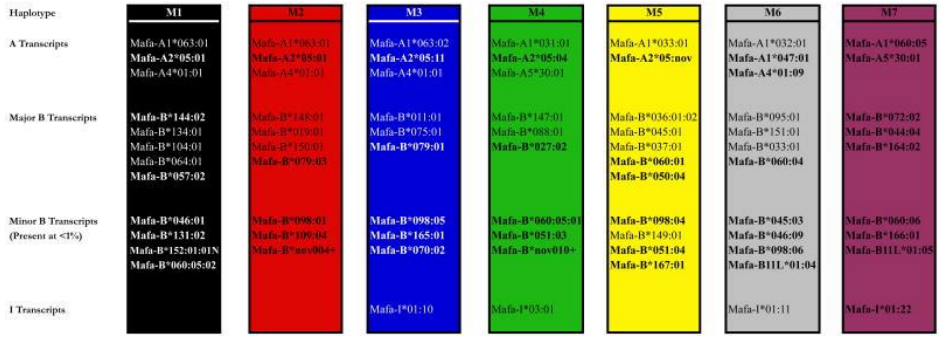
Adult Mauritian origin Cynomolgus macaques were used in this study (Charles River Primates, Wilmington, MA; Sanofi-Synthelabo, Bridgewater, NJ; Bioculture Group, Glenmoore, PA). Recipient and donor pairs were selected for ABO compatibility and mismatching of Cynomolgus leukocyte antigens.[207] Cytomegalovirus serology was assessed prior to transplant (by VRL Laboratories). All animals were negative for B virus, simian T-lymphotropic virus, simian retrovirus, SIV, simian varicella virus and malaria. All macaques were housed at the Institute of Comparative Medicine (Columbia University Medical Center, New York, NY). This facility holds a current USDA assurance and is an AAALAC-accredited institution. All surgical and experimental procedures were performed in accordance with NIH guidelines for the care and use of primates, and approved by the Columbia University Institutional Animal Care and Use Committee.

IV.1.2. Major histocompatibility complex (MHC) genotyping

PBMCs were genotyped at the University of Wisconsin Primate Research Center Laboratory (http://www.primate.wisc.edu/wprc/services/genetics.html). Comprehensive MHC genotypes were determined by the Genetic Services Unit of the Wisconsin National Primate Research Center at the University of Wisconsin-Madison. Genomic DNA isolated from whole blood samples served as templates for PCR with a panel of primers that flank the highly polymorphic peptide binding domains encoded by exon 2 of MHC class I (Mafa-A, -B, -I, -E) and class II (Mafa-DRB, -DQA, -DQB, -DPA and -DPB) loci. These PCR products were generated with Fluidigm Access Arrays that allow all reactions to be multiplexed in a single experiment. After cleanup and pooling, these amplicons were sequenced on an Illumina MiSeq instrument and the resulting sequence

reads were mapped against a custom database of Mauritian Cynomolgus macaque class I and class II.[208]

Figure 6. MCM MHC class I haplotypes



MHC class I A, B, and I transcripts associated with each MCM haplotype. Adapted from Budde et al. Immunogenetics 2018.[207]

IV.1.3. Blood typing

Human A, B, and O blood samples were used as controls. MCM serum was collected and typed on human type O blood. Five milliliters of type O human blood were diluted with 10mL of PBS and spun at 2000 rpm for five minutes at 4C, decanting the supernatant thereafter. This washing step was repeated for three times, resuspending the cells after the final spin in a total volume of 10mL of PBS. From this suspension, 0.5mL were added into a 1.5mL snap top micro tube (one aliquot per animal to be typed) using clear tubes in order to see through it. Tubes were spun at 1000 rpm for 5 minutes at 4C and the supernatant was decanted. From each MCM serum to be typed, 250uL were added to the cell pellet and resuspended. The mix was then incubated at 37C for 30 minutes and spun at 2000 rpm for 5 minutes at 4C. The serum was then transferred to a new tube, getting as much of it as possible and spinning again if necessary, but avoiding any red blood cells. If necessary, it was possible to stop the procedure at this point freezing the serum at -20C.

The next step was to prepare A, B, and O human blood for typing. One milliliter of each human blood type was placed into a 15mL conical brining the final volume to 10mL with PBS. The samples were then spun at 2000 rpm for 5 minutes at 4C. The supernatant was decanted, keeping the red blood cells. This step was repeated for 3 times bringing the final volume to 10mL with PBS. If necessary, it could be stored at 4C. Using a 96 well U-bottom plate, samples were plated as follows:

Table 2. 96 well plate set up for ABO typing

Human Blood Type	A	B	O	A	B	O	A	B	O	A	B	O
	O	**O**	**O**	**O**	**O**	**O**	**O**	**O**	**O**	**O**	**O**	**O**
MCM	#1			#2			#3			#4		

Fifty microliters of each human blood group were added to each well as indicated above. Then 50uL of each MCM serum sample were added to each of the controls. Using a clean tip for each well, content in each well was well mixed and incubated at 37C for 10 minutes. After the incubation, the plate was spun at 1000 rpm for 5 minutes at 4C. Sequentially, wells were resuspended with new pipette tips by mixing the suspension fairly vigorously for about 15-20 seconds. Finally, agglutination was checked. Samples that were agglutinated should be fairly obvious and should not be homogenous after this mixing period. If a sample was questionable, 2-3uL of the suspension was placed on a glass slide and observed under a microscope at 4x magnification. Results were recorded on a chart. The questionable samples should be observed next to the negative control sample (absorbed serum with type O human RBC) in order to get an accurate result.

IV.1.4. Conditioning regimen

Recipients were conditioned with 2.5-3 Gy of total body irradiation divided into two doses on Days -6 and -5, 7 Gy of thymic irradiation on Day -1, T cell depletion with anti-horse ATG (50 mg/kg) on Days -2, -1 and 0, co-stimulation blockade with anti-CD40L (20 mg/kg on Days 0 and 2, and 10 mg/kg on Days 5, 7, 9 and 12) and a short course of immunosuppression post-BMT with rapamycin. BM was administered from a MHC-mismatched donor ± *in vitro* expanded autologous Tregs. Rapamycin levels were maintained between 20-25 ng/mL for 30 days and tapered to 0 thereafter.

IV.1.5. Intravenous (IV) catheter placement

A central catheter was placed in animals undergoing major procedures as it was important for the delivery of drugs and medications. Animals were sedated with ketamine and dexmedetomidine and received isoflurane during the procedure. The neck was exposed laterally and the incision site was blocked with 50:50 1% lidocaine and 0.5% bupivacaine. The external (left or right) jugular vein was approached by using a small 2 cm incision over the jugular groove. The sternocephalicus muscles were isolated after dissected through the platysma. The external jugular was approached, and the fat and fascia cleaned, isolated and ligated with 3-0 silk ties. A 6-French Groshong catheter was inserted and secured with 3-0 silk ties. The distal aspect of the catheter was pushed through the subcutaneous layers and exited between the scapulae. The incision site was closed in a 3-layer pattern with 3-0 PDS. On the back, a connector was used to unite the Groshong and a Tygon tubing. These were ligated and secured over the back of the animal with 2-0 ethilon sutures. Tegaderm was applied over the exit wound. The jacket and tether were placed prior to recovering the animal from anesthesia. The animals received buprenorphine and NSAIDs. The catheter was maintained until the recipient was fully immune reconstituted and in no need of receiving IV medications.

The approach for the removal of the catheter was the same with the exception that the catheter entry site will be identified and the catheter removed, with the closing pattern being the same. The analgesia regimen was also a combination of NSAIDs and buprenorphine.

IV.1.6. Bone marrow transplant (BMT)

Bone marrow (BM) was harvested aseptically from donor iliac bones by multiple percutaneous aspirations or surgically from the vertebrae. BM cells were infused intravenously. CD34+ and T cell content was determined by flow cytometry.

Donor BM harvest was performed in the operating room under ketamine and isoflurane anesthesia. The skin was shaved, followed by the application of local anesthesia with 1% lidocaine and 0.25% bupivacaine. Under aseptic conditions, the BM was aspirated sterilely from the iliac crest. The aspirations were performed with a 15G Illinois BM needle, and 20mL syringes with a Luer-lock containing 2mL of heparin each syringe. Several aspirations at different sites were performed in order to collect sufficient number of cells. Each aspiration yielded 1-5mL of BM. Post-operative management included careful observation of the puncture site for any residual bleeding.

For a non-survival BM donation, the donor underwent catheterization of the external jugular vein and carotid artery (detailed in section III.1.5.). After exsanguination and cardiac arrest, BM was obtained by processing the vertebral bodies.

After BM was processed (protocol detailed in section IV.2.1.) the product was placed in an Intravia bag and administered IV to the recipient through the central catheter over a period of 30 minutes. The recipient was premedicated with anti-histamine prior to the BM infusion.

IV.1.7. Kidney transplantation

Kidneys were transplanted four months after the BMT.

IV.1.7.1. Donor kidney procedure

The kidney donor received a 50:50 mix of 1% lidocaine and 0.5% bupivacaine local anesthesia before the incision was performed. Through a midline incision from the xiphoid to the pubis that was 20-25cm long, the abdomen was explored to assure the presence of two normal-appearing kidneys. The left kidney was exposed, incising the retroperitoneal attachments primarily using cautery. Stay sutures with 2-0 silk ties were placed at the poles of the kidney for manipulation, since undue finger pressure on the kidney could result in vascular spasm and poor post-transplant function. The renal vein was then cleared down to the vena cava. The renal artery was isolated by ligating and dividing the adrenal artery which almost always took off from the proximal renal artery. The renal artery and approximately 1 cm of the adjacent aorta were then cleared. The ureter was dissected from the retroperitoneum, carefully sparing the vascular supply. It was usually necessary to divide the gonadal vein overlying the ureter at some point and continue the isolation of the ureter well into the pelvis to obtain adequate length.

The ureter was then divided, but the kidney was not removed until brisk diuresis was observed from the donor ureter. The distal ureter was ligated with 2-0 black braided silk tie. The small Satinsky clamp was then placed on the aorta. The kidney was perfused with approximately 6-8 mL of cold Ringer's lactate solution containing mannitol (1.25g/500mL). The kidney was perfused uniformly to a pale appearance. When the venous effluent appeared dilute, the vein was clamped with the baby intestinal clamp. The kidney was elevated using the stay sutures. The renal vein was divided. The renal artery was taken with a small patch of donor aorta. The kidney was immediately placed in a sterile dish containing cold Ringer's lactate solution.

The stump of the renal vein was ligated with 2-0 silk tie. The aorta was repaired using 7-0 Prolene. Before skin closure, subcutaneous lidocaine was administered around the incision site for pain management post-operation. The wound was then closed in three layers (muscle to subcuticular) using 2-0, 3-0, and 5-0 running Vicryl. The linea alba was closed with interrupted 2-0 Vicryl. The other layers were closed with a running suture. Following donor nephrectomy, the animals were monitored by observation for clinical status and for blood sampling for renal function and hematologic recovery (at weekly intervals) until these parameters normalized.

IV.1.7.2 Recipient kidney procedure

Recipients underwent unilateral native nephrectomy and ligation of the contralateral ureter on the day of transplant. Incision sites were instilled with a 50:50 mix of 1% lidocaine and 0.5% bupivacaine. Thereafter, the animal's abdomen was opened from the xiphoid to the pubis using a 10-blade. Once the abdomen was entered, the bowels were retracted to expose the left and right retroperitoneum, which was incised using electrocautery to expose the kidney to be removed. The hilum was then dissected using a combination of sharp dissection and electrocautery. The right and left renal arteries and veins were tied off enbloc and subsequently transected, as well as the ureters. The recipient right native kidney was removed from the surgical field and vigilant care was taken to ensure hemostasis. The infrarenal aorta and vena cava were dissected to facilitate application of the vascular clamp. The renal allograft was then brought into the surgical field. The renal artery was anastomosed to the recipient aorta and the renal vein to the recipient cava. Each anastomosis was completed using 8-0 prolene. The kidney was perfused and the anastomotic site was observed for bleeding. Thereafter, the ureter was anastomosed to the bladder using a single, double-armed 8-0 prolene. The recipient's abdomen was closed in three layers, using 3-0 vicryl, 2-0 PDS, and 3-0 monocryl (subcuticular).

IV.1.8. Skin transplant

Donor skin grafts were harvested with a scalpel and then cryopreserved for grafting post-BMT. This procedure was used for confirmation of tolerance induction.

The recipient was placed under anesthesia. The hair on the animal's back was clipped, and the skin was aseptically prepped. Three small 3x4 cm full thickness (epidermal/dermal layer) skin grafts were placed. One graft was a self-graft, one graft was from the bone marrow donor, and a third graft was from a third party animal which was MHC mismatched from both donor and recipient. The skin from this third party animal was from a naïve or healthy animal within the colony. With a 10-blade, the area for the skin graft was created by carefully lifting and removing the hosts' epidermal and dermal layers to accommodate the transplanted skin grafts. As a control, a self-graft was removed and sutured back on. Grafts were fenestrated and sutures placed 1-2 cm apart using 3-0 ethilon in a simple interrupted pattern. The skin grafts were covered with triple antibiotic ointment and dressing to prevent mutilation of the grafts by the physical friction. The animal was recovered and a jacket was placed to cover the skin grafts. The patient received perioperative analgesia including both NSAIDs and opioid analgesics. Skin grafts were checked twice weekly for the first three weeks and once weekly thereafter, taking visual evidences and biopsies.

IV.1.9. Anti-CD40L (anti-CD154) preparation and administration

Anti-CD40L (5C8, Non-human primate reagent resource) is a mouse human chimeric antibody. The proper amount of anti-CD40L was diluted into 50mL of saline solution bag. The neat anti-CD40L was put through a 0.22µm filter (Millex) before being added to the bag. Before administration, recipients were prepped with 1 mg/kg of antihistamine (Benadryl) and 1 mg/kg of ketorolac tromethamine (Hospira) due to the thrombotic effects of anti-CD40L.[209] Once

anti-CD40L was diluted and brought to room temperature, the antibody was administered IV starting at a rate of 20 mL/hr to make sure there was not reaction and increasing slowly thereafter. After the infusion was completed, 20mL of 0.9% NaCl saline solution were added to the bag to flush the line. Appropriate resuscitation equipment was immediately available at all times and animals were monitored throughout the procedure (clinical changes, respiratory effort and respiratory rate). The administered doses were 20 mg/kg on Days 0 and 2, and 10 mg/kg on Days 5, 7, 9 and 12.

IV.1.10. Anti-thymocyte globulin (ATG) preparation and administration

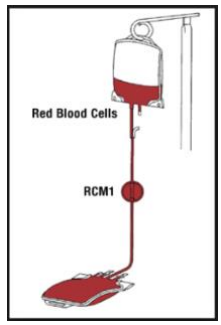
ATG was inspected prior to use to ensure that there was no large granular deposit on the bottom of the bottle, which may occur during storage. Shaking of the bottle was avoided as it may lead to denaturation of the antibody. ATG was gently swirled to mix and diluted to a final concentration no higher than 4 mg/mL. The undiluted ATG was put through a 0.22µm filter (Millex) before being added to the bag. Once ATG was diluted and brought to room temperature, it was administered IV. Before administration, recipients were prepped with 1 mg/kg of antihistamine (Benadryl) and 0.6 mg/kg of methylprednisolone sodium succinate (Solu-Medrol) IV to prevent anaphylaxis. Infusion was started slow and the total dose was administered over 60-90 minutes. Appropriate resuscitation equipment was immediately available at all times and animals were monitored throughout the procedure (clinical changes, respiratory effort and respiratory rate). The administered doses were 50 mg/kg on Days -2, -1 and 0.

IV.1.11. Whole blood banking and blood transfusion

Blood donors were sedated with ketamine and dexmedetomidine and reversed with atipamezole following the NIH guidelines for the care and use of primates. Blood was drawn into syringes with 14% anticoagulant citrate dextrose solution (ACD) (Baxter). Blood was then transferred to an Intravia bag (Baxter) of 150mL

capacity. The Intravia bag was attached to a Neonatal high efficiency leukocyte reduction filter (Haemonetics) which was clamped prior to connecting it to the Intravia bag to avoid air bubbles to get into the filter after blood started flowing. The filter was then unclamped and the Intravia bag was positioned above the collection bag so blood could flow downward. The tube was clamped again before any air entered the filter as letting the filter run empty could lead to contamination of the collected blood with leukocytes. Both ends of the tube connecting the filter to the collection bag were sealed with the Genesis BPS Rapid Seal II (SE330). The tube was then cut above the seals and the filter was detached. The collection bag was properly labeled with the animal's identification number and date of bleeding and stored at 4C. The blood was irradiated before the transfusion (30Gy) using a XRAD 320 irradiator (Precision X-Ray Inc., N. Branford, CT). Non-irradiated blood was banked for up to one month after bleeding and irradiated blood had to be transfused within seven days after collection. Blood was transfused through a 200-micron filter (Hospira).

Figure 7. Schema for blood filtration using a leukocyte reduction filter



Schema of the procedure to filter whole blood prior to irradiation and infusion into the blood recipient.

IV.1.12. Biopsy performance

IV.1.12.1. Renal open biopsy

Open wedge biopsies were taken to evaluate any rise in serum creatinine in the operating room using general anesthesia and local anesthesia.

The right flank was shaved and an IV was started in the saphenous vein if the animal did not have already a central line. The right flank and right abdomen were prepped with betadine and sterilely draped. A 50:50 mix of 1% lidocaine and 0.5% bupivacaine local anesthesia was administered pre-incision. Through a short oblique incision, the skin and flank musculature were opened, exposing the posterior lateral aspect of the transplanted kidney. A 2x4 mm incision extending approximately 1-1.5 mm into the cortex was made with the a 10-size blade. The wedge biopsy was then elevated and excised using iris scissors in the "no touch" technique. Bleeding was controlled with pressure. A pledget of subcutaneous fat or muscle was then sutured into the cortical defect using a single, figure-of-eight, and 5-0 chromic suture. When hemostasis was assured, the wound was closed in layers. Bupivacaine was applied to the final layer closure. After recovery, the animal was returned to the housing area.

IV.1.12.2. Rectal biopsy

This procedure was performed when graft-versus-host disease was suspected. A Kelly or mosquito forceps were used to open the external anal sphincter. The rectal mucosa was gently exteriorized with babcocks and with a 11-blade scalpel, a 1-2 mm thick and 3-4 mm deep rectal sample was taken. Gentle pressure was then applied in the rectal area for 5-10 minutes until bleeding had stopped.

IV.1.13. Ganciclovir (GCV) preparation and administration

A GCV vial was reconstituted with 10mL of sterile water. Each GCV dose (12.5 mg/kg) was diluted into 50mL of 0.9% NaCl saline, therefore two doses of GCV

were added to a 100mL 0.9% NaCl saline bag. Then each bag was labeled indicating the animal's identification number, name of the medication, dose and date. Bags could be prepared a few days in advance and kept in the fridge.

When GCV treatment was administered, the GCV bag was brought up to room temperature before administration in order to minimize air bubbles while infusion. GCV bag was connected to an IV catheter and primed in order to remove the air from the catheter. Then the catheter was attached to the animal's central line after removing the cap from the free port on the stopcock and wiping it with ethanol. The infusion pump was programed at a rate of 50 mL/hr. After the infusion was completed, the catheter was disconnected from the animal's central line and the stopcock was recapped.

IV.1.14. Thaw and infusion of cryopreserved regulatory T cells (Tregs).

Media containing 98% Hank's Balanced Salt Solution (HBSS) and 2% of 25% albumin was prepared (Treg infusion media). Vials containing Tregs, that were kept in a liquid nitrogen freezer, were placed in a bead bath (37C) to thaw. A conical containing 10mL of Treg infusion media was prepared. After the content in the vials defrosted, vials were brought to the hood, after swiping them with ethanol. Sample slowly was dripped into the conical previously prepared at the same time the media in the conical was being gently rotated. Cells we spun at 2000 rpm for 10 minutes at 4C. Cells were resuspended into Treg infusion media and counted using trypan blue exclusion dye. Cells were transferred to a Intravia container (Baxter) and conical was wash twice with 10mL of Treg infusion media that was transferred to the Intravia container thereafter. Cells were infused using a primary blood set (Hospira) on Days 0, 2, 5, 7 and 50 or 2, 4, 7, 9 and 50 depending on the time point of the BMT.

IV.2. *In vitro* protocols

IV.2.1. Bone marrow (BM) processing

After the BM product was placed in BM media (containing 98% RPMI (Life Technologies), 1% Penicillin/Streptomycin (Life Technologies), and 1% DNase (Sigma-Aldrich)), the mixture was placed on the shaker at 700 rpm for 1 hour. The product was then filtered through a 70µm filter and cells were spun down at 2000 rpm for 10 minutes at room temperature. Cells were resuspended in BM infusion media (containing 90% of 0.9% NaCl saline (Baxter) and 10% anticoagulant citrate dextrose solution (ACD) (Baxter)) and filtered again through a 70µm filter brining the final volume to 40mL. Cells were counted with trypan blue exclusion dye lysing the sample first with lysing buffer for one minute to exclude red cells. The BM product was then transferred to an Intravia bag and was infused through a 200-micron filter (Hospira).

IV.2.2. Peripheral blood mononuclear cell (PBMC) Isolation

Blood donors were sedated with ketamine and dexmedetomidine and reversed with atipamezole following the NIH guidelines for the care and use of primates. Heparinized blood was drawn from MCM. Blood was diluted with phosphate buffered saline (PBS) at a ratio 1:2 blood:PBS, overlaid on 60% Percoll (Sigma-Aldrich) and centrifuged for 30 minutes at 3000 rpm at room temperature with the acceleration and break turned off. The buffy coat was collected and contaminating red blood cells were lysed.

IV.2.3. Fluorescence activated cell sorting (FACS)

FACS was performed on the Influx Cell Sorter (BD Biosciences). Tregs were stained with CD4-FITC (L200, BD Biosciences), CD25-PE (BC96, BioLegend), CD127-Alexa Fluor 647 (HIL-7R-M21, BD Pharmingen) and CD8-BV421 (RPA-T8, BD Biosciences). The top 1% of CD25+CD127- population within the CD4+CD8-

gate was sorted and collected in sterile fetal bovine serum (FBS) (Gemini Bio-Products).

IV.2.4. Treg culture

Five different protocols were studied for the expansion of Tregs (Table 3).

Table 3. Treg expansion protocols

Protocol A		**Day 0**	**Day 7**	**Day 14**	**Day 21**			
	IL-2	x	x	x	**Freeze**			
	Anti-CD3	x	x	x				
	Donor PBMCs	-	x	x				
	Artificial APCs	x	-	-				
	Rapamycin	x	-	-				
Protocol B		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**>Day 28**	**>Day 28**
	IL-2	x	x	x	x	x	x	**Freeze**
	Anti-CD3	x	x	x	x	x	x	
	Donor PBMCs	-	x	x	-	-	-	
	Artificial APCs	x	-	-	x	x	x	
	Rapamycin	x	-	-	-	-	-	
Protocol C		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 26**		
	IL-2	x	x	x	x	**Freeze**		
	Anti-CD3	x	x	x	x			
	Donor PBMCs	-	-	-	-			
	Artificial APCs	x	x	x	x			
	Rapamycin	x	-	-	x			
Protocol D		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 26**		
	IL-2	x	x	x	x	**Freeze**		
	Anti-CD3	x	x	x	x			
	Donor PBMCs	-	x	x	x			

	Artificial APCs	x	x	x	x	
	Rapamycin	x	-	-	x	
Protocol E		**Day 0**	**Day 7**	**Day 14**	**>Day 21**	
	IL-2	x	x	x	**Freeze**	
	CD40-sBc	x	x	x		

Tregs grown under Protocol A through D received growth medium that consisted of 85% RPMI (Life Technologies), 10% FBS (Life Technologies), 1% Penicillin/Streptomycin (Life Technologies), 2% Glutamax (Life Technologies), 1% MEM Non-Essential Amino Acids (Life Technologies) and 1% sodium pyruvate (Corning cellgro). In addition, interleukin-2 (IL-2) (NIH/PeproTech), anti-CD3 (SP34-2, BD Pharmingen) and rapamycin (Sigma-Aldrich) were added to the culture as per protocol (Table 3).

These four protocols (A through D) required a mouse fibroblast cell line (L929) transfected with human CD80, CD32 (FcR) and CD58 (LFA3)[210,211] that was used as artificial APCs (aAPCs).[212] aAPCs were irradiated with 50Gy using a XRAD 320 irradiator (Precision X-Ray Inc., N. Branford, CT) and plated 3 to 24 hours before the addition of Tregs. Non-irradiated aAPCs were plated back in culture for further growth with the same medium as the one used for Treg expansion. PBMCs were irradiated with 35Gy prior to plating. MCM PBMC stimulators were MHC mismatched to the Tregs in culture.

Tregs grown under Protocol E received growth medium consisted of 90% X-Vivo 15 (Lonza) or AIM V medium (Gibco) and 10% of human AB serum (Gemini Bio-Products).

Protocol E used a pool of different B cell donors for Treg stimulation. B cells were cultured in a growth medium consisted of 90% X-Vivo 15 (Lonza) or AIM V

medium and 10% of human AB serum (Gemini Bio-Products) with 100 U/mL of insulin (Lilly Products). B cells were stimulated and expanded with CD40L-expressing K562 cells in a medium containing IL-4 (R&D Systems), cyclosporine A (to inhibit T cell proliferation) (Sigma-Aldrich) and ganciclovir (used in human protocols to inhibit the release of Epstein-Barr virus from B cells) (McKesson Corporation) (Table 4). Stimulated B cells were irradiated with 30Gy using a XRAD 320 irradiator (Precision X-Ray Inc., N. Branford, CT) prior to plating with Tregs.

CD40L-expressing K562 cells were maintained in culture with medium consisted of Iscove's Modified Dulbecco's Medium (IMDM) (Gibco), FBS (Gemini Bio-Products), 1% Penicillin/Streptomycin (Life Technologies), and ciprofloxacin (Sigma-Aldrich). Cells were irradiated with 46Gy using a XRAD 320 irradiator (Precision X-Ray Inc., N. Branford, CT) prior to plating for B cell stimulation.

Three protocols were investigated for B cell expansion (Table 4):

Protocol 1: $20x10^6$ PBMCs or splenocytes were plated with CD40L-expressing K562 cells at a ratio 1:2 respectively for 10 days per T150 flask. On Day 0, cells received 40μg of CyA, 16ng of IL-4 and 200μg of GCV in 20mLs of medium. On Days 2 and 4 cells received half of these doses. On Day 7, cells were restimulated and fresh CD40L-expressing K562 cells were added to culture with CyA, IL-4 and GCV at the same doses as on Days 2 and 4. On Day 10, B cells were taken down and cryopreserved. Phenotype analysis was performed on Days 0, 7 and 10.

Protocol 2: Cells were plated in the same manner on Day 0 as described for Protocol 1. On Days 2 and 4, cells received double amount of CyA as Protocol 1 (40μg), maintaining the same doses of IL-4 and GCV. Cells were restimulated on Day 10 (receiving fresh CD40L-expressing K562 cells, CyA, IL-4 and GCV), and

taken down on Day 14. Phenotype analysis was performed on Days 0, 10 and 14.

Protocol 3: This protocol follows Protocol 2 except the restimulation was performed on Day 7 instead of Day 10. Cells were taken down and cryopreserved on Day 14. Phenotype analysis was performed on Days 0, 7 and 14.

Table 4. CD40L-sBc expansion protocols

	Protocol 1	**Protocol 2**	**Protocol 3**
Culture length	10 days	14 days	14 days
Restimulation	Day 7	Day 10	Day 7
CyA dose	2μg/mL (D0), 1μg/mL (D2, 4)	2μg/mL (D0, 2, 4), 1μg/mL (D7, 10)	2μg/mL (D0, 2, 4), 1μg/mL (D7)
IL-4 dose	0.8ng/mL (D0), 0.4ng/mL (D2, 4, 7)	0.8ng/mL (d0), 0.4ng/mL (d2, 4, 7, 10)	0.8ng/mL (d0), 0.4ng/mL (d2, 4, 7)
GCV dose	10μg/mL (D0), 5μg/mL (D2, 4, 7)	10μg/mL (D0), 5μg/mL (D2, 4, 7, 10)	10μg/mL (D0), 5μg/mL (D2, 4, 7)

Five protocols were developed for Treg expansion (Table 3):

Protocol A: Tregs were expanded for 21 days. 200 IU/mL of IL-2 were added to the culture on Days 0, 7 and 14. 100 ng/mL of anti-CD3 was added on Day 0, followed by 1000 ng/ml on Days 7 and 14 and 100 ng/mL of rapamycin on Day 0. aAPCs were used as stimulators on Day 0, but were subsequently replaced with allogeneic PBMCs (1:1 ratio PBMCs:Tregs) on Days 7 and 14. Cells were harvested and frozen on Day 21.

Protocol B: The first 21 days mirrored Protocol A. After Day 21, Tregs were continued in culture with aAPCs (1:10 ratio aAPCs:Tregs) (no PBMCs) for up to

56 days (depending on cell growth). 200 IU/mL of IL-2 were added at the time of restimulation. Anti-CD3 at a dose of 100 ng/mL was used on Day 21 and thereafter.

Protocol C: Cells were cultured with aAPCs (1:10 ratio aAPCs:Tregs). 200 IU/mL of IL-2 and 100 ng/mL of anti-CD3 were added on Days 0, 7, 14 and 21 and 100 ng/mL of rapamycin was added to the culture on Days 0 and 21. Cells were cryopreserved on Day 26. No PBMCs were added to this protocol.

Protocol D: Cells were cultured with both allogeneic PBMCs (1:10 ratio PBMCs:Tregs) and aAPCs (1:10 ratio aAPCs:Tregs) as stimulators. IL-2, anti-CD3 and rapamycin were added as in Protocol C.

Protocol E: Cells were cultured with CD40L-stimulated B cells (CD40L-sBc). B cells were previously expanded with CD40L-expressing K562 cells. The ratio Treg:CD40L-sBc was 1:4. IL-2 was added to the culture at a concentration of 6 U/mL.

IV.2.5. Mixed lymphocyte reaction (MLR)

Host versus graft (HVG) and graft versus host (GVH) responses were tested *in vitro* through a mixed lymphocyte reaction. $0.1x10^5$ responder cells (recipient or donor) were plated in a U-bottom 96-well plate with equal number of stimulators. Self, donor and third-party cells served as stimulator cells that were irradiated at 35Gy prior to plating. Responders with media or anti-CD2CD3CD28 beads (one bead for every two responders) served as negative or positive control, respectively. 1uCi of thymidine was added to each well four days after the initiation of culture and wells were harvested 24 hours after (Tomtec). A plate reader (Perkin Elmer 1450 MicroBeta) was used to measure the thymidine uptake.

IV.2.6. Suppression assays

Ten thousand PBMC responders were plated in triplicate. Tregs were plated with PBMCs starting at 1:1 (Treg:PBMC ratio) and serially diluted up to 1:32 (Tregs:PBMCs). PBMCs were stimulated with anti-CD2CD3CD28 beads (Miltenyi Biotec). 1uCi of thymidine was added to each well four days after plating. Cells were harvested (Tomtec) 24 hours after. A plate reader (Perkin Elmer 1450 MicroBeta) was used to measure the thymidine uptake.

IV.2.7. Cell cryopreservation

Cells were cryopreserved in 95% FBS (Gemini Bio-Products) and 5% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Cells were frozen at a 10 million cells/mL in a step down cell freezer (CryoMed, ThermoFisher Scientific) at a rate of 1C/minute and stored in a liquid nitrogen freezer.

IV.2.8. Cell re-expansion post-cryopreservation

Cells were thawed in a 37C water bath and slowly dripped into RPMI. Group 1 was plated with media; Group 2 received media and 200 U/mL of IL-2; Group 3 was cultured with media, 200 U/mL of IL-2 and 100 ng/mL of anti-CD3; and Group 4 received aAPCs in addition to the same reagents as Group 3. Cells were harvested 48 hours after plating, and the absolute number, phenotype and function were assessed. Group 4 was also further expanded for 10 days and assessed at different time points.

IV.2.9. Flow cytometry staining

Tregs were stained with CD3-PerCP-Cy5.5 (SP34-2, BD Biosciences), CD4-PE (L200, BD Biosciences), CD8-PE-Vio770 (BW135/80, Miltenyi Biotec), CD25-BV421 (BC96, BioLegend), CD45RA-APC-Vio770 (T6D11, Miltenyi Biotec), CD127-Alexa Fluor 647 (HIL-7R-M21, BD Pharmingen) and FoxP3-PE (236A/E7, BD Biosciences and 3G3, Miltenyi Biotec) antibodies.

B cells were stained with HLA-DR-FITC (L243, BD), CD80-PE (L307.4, BD Pharmingen), CD3-PerCP-Cy5.5 (SP34-2, BD Biosciences), MHC I-PE-Cy7 (G46-2.6, BD Biosciences), CD40-Pacific Blue (BioLegend, 5C3) and CD20-APC-Vio770 (LT20, Miltenyi Biotec) antibodies. CD40L-expressing K562 cells were stained with CD154-APC (TRAP1, BD Pharmingen) antibody.

Whole blood was lysed and labeled with a combination of Bw6-FITC (REA143, Miltenyi Biotec), MHC I-PE-Cy7 (G46-2.6, BD Biosciences), CD56-PE (NCAM16.2, BD), CD20-APC-Vio770 (LT20, Miltenyi Biotec), CD11b-VioGreen (M1/70.15.11.5, Miltenyi Biotec), CD31-PE (WM59, BD Pharmingen), CD45RA-APC-Vio770 (T6D11, Miltenyi Biotec), CD25-BV421 (BC96, BioLegend), CD3-PerCP-Cy5.5 (SP34-2, BD Biosciences), CD4-BV510 (L200, BD Biosciences), CD4-APC (L200, BD Biosciences), CD8-APC (RPA-T8, BD Pharmingen), CD8-BV421 (RPA-T8, BD Biosciences), CD95-PE (DX2, BD Biosciences), CD28-Pacific Blue (CD28.2, BioLegend), CD197 (CCR7)-FITC (G043H7, BioLegend), CD34-APC (563, BD Biosciences) and CD127-Alexa Fluor 647 (HIL-7R-M21, BD Pharmingen) antibodies.

Gates were drawn based on the isotype controls (IS11-3B2.2.3, Miltenyi Biotec). *In vitro* cultured Tregs and cells from whole blood were permeabilized with the BioLegend FoxP3 Fixation/Permeabilization Buffer Set according to the manufacturer's protocol.

Data was collected on a FACSCantoII or Fortessa (BD Bioscience) and analyzed using Flowjo VIX and VX (Tree Star) or FCS Express (De Novo Software).

IV.2.10. Treg-specific demethylated region (TSDR) studies of MCM Tregs

qPCR for the Foxp3 region was performed by Epiontis. Using a standard dilution curve, the number of demethylated and methylated genomic regions was

determined, referred as TpG and CpG plasmid units, respectively. The Treg content is calculated by dividing the number of TpG plasmid units by the sum of all Foxp3 regions in the sample; TpG/ (TpG+CpG).

IV.2.11. Anti-donor antibody testing

Detection of anti-donor IgG and IgM antibodies in serum was performed by incubating donor PBMCs with serum samples from the recipient collected at different time points post-transplant. Serum was decomplemented prior to incubation by placing the samples in a heat bock at 56C during 35 minutes to destroy heat-labile proteins such as complement and avoid immunoglobulin aggregation. Fc receptors on donor PBMCs were blocked with human serum prior to incubation to reduce non-specific binding and decrease background. Detection of IgG and IgM against the donor were detected by flow cytometry by staining donor PBMCs with the secondary antibodies anti-IgG and anti-IgM. Other extracellular stains such as CD3 and CD20 were added in order to differentiate antibodies against MHC class I and class II.

IV.2.12. Development of Cynomolgus cytomegalovirus (CMV) quantitative polymerase chain reaction (qPCR)

A quantitative PCR assay was designed to detect a fragment of the DNA polymerase gene of cynomolgus CMV. Using Primer3 software, ten primer pairs were designed and tested in a gel-based assay. To generate template material for testing, we used the EasyMag extaction platform (Biomerieux) to extract DNA from serum and brain of a Cynomolgus macaque that succumbed to CMV. From the initial tests, we selected one optimal primer pair, consisting of primers CMV-Forward-GATGGGACCGCTCAAGTTTC, CMV-Reverse-TGACGGTAGCGAGGAGACAA, and CMV-Probe-(Fam) GGTCGATGGGGTTTTGACTCACGA (Tam). To generate a quantified standard for the assay, we cloned a PCR product containing the primer and probe binding

sites into the PGem T-easy (Promega) ligation and vector system. Plasmid were isolated and purified using the PureLink plasmid miniprep kit (ThermoFisher). The concentration of the standard was quantified, and then serially diluted. These serial dilutions were used to optimize the qPCR assay using TaqMan Universal PCR Master Mix (ThermoFisher) and to test the sensitivity of the primers and probe. The assay has an efficiency of 95% and a sensitivity of $\geq$5 copies.

For CMV testing, 200µl of serum were extracted on the EasyMag platform and eluted in 40µl of DNA. PCR reactions were run in triplicate using 4µl of DNA along with serially diluted standards and a no-template controls.

IV.2.13. Subcutaneous rapamycin preparation

Rapamycin (LC Laboratories, Woburn, MA) was reconstituted prior to *in vivo* administration. The Columbia University Medical Center Pharmacy formulated a parenteral rapamycin solution (2 mg/mL) based on previous studies.[213] The rapamycin formulation consisted of 1800mg of rapamycin that were diluted into 900mL of reconstitution buffer (Table 5). The rapamycin reconstitution buffer consisted of 10% ethanol, 40% propylene glycol, 1.5% benzyl alcohol and 5% benzoate buffer (consisting of benzoic acid (250mg) and sodium benzoate (290mg) that were reconstituted into 100mL of sterile water). The final concentration was 2 mg/mL.

Table 5. Rapamycin reconstitution buffer

Compounds	Amount	Volume
Ethanol	10%	90mL
Propylene glycol	40%	360mL
Benzyl alcohol	1.5%	13.5mL
Benzoate buffer	5%	45mL
Sterile water	43.5%	391.5mL

The formulation was filtered through a 0.2mm filter. HPLC analysis (PharmD) were performed to verify the drug concentration using a standard curve of rapamycin spike in comparison to different concentrations (0.05, 0.1, 0.25, 0.5 mg/mL). Stability studies consisted of HPLC analysis at time zero (t=0) and after 1-month storage at different temperatures. A portion of the remaining volume was held at 4C for 6-month stability studies, again showing stability of this initial formulation at 4C. Endotoxin was measured prior to rapamycin use with Limulus Amebocyte Lysate QCL-1000H kits (Cambrex, Watersville, MD) and results showed negligible levels.

IV.2.14. Statistics

Data was analyzed using paired Student's T-test. P values of ≤0.05 were considered statistically significant (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$).

V. RESULTS

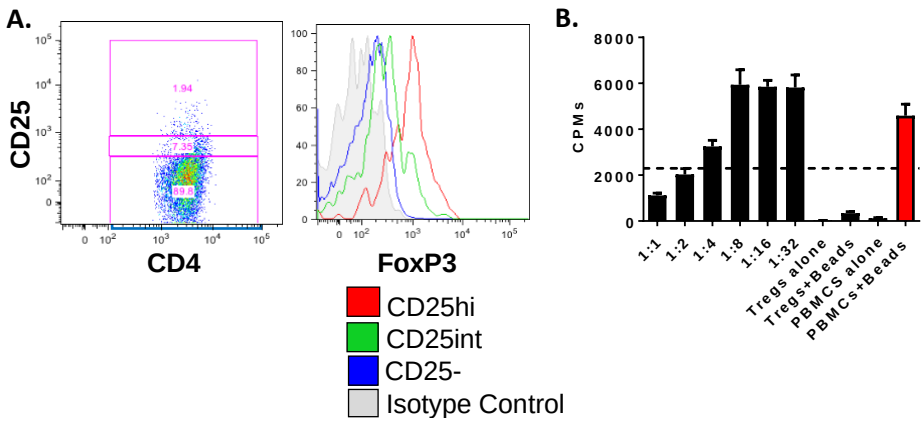
V. RESULTS

V.1. *In vitro* expansion of Regulatory T cells (Tregs)

V.1.1. Characterization of the Treg phenotype in the Mauritius Cynomolgus Macaque (MCM)

We first characterized the phenotype of Tregs in the MCM. Similar to what has been published in Indian origin Cynomolgus macaque and human Tregs, MCM CD4+ Tregs co-express CD25 (the alpha chain of the IL-2 receptor) and the transcription factor FoxP3.[214] We observed that as the cell surface expression of CD25 increased via flow cytometry, so did FoxP3 expression (Figure 8A). Freshly sorted Tregs (top 1% of CD4CD25+ cells) consistently suppressed the proliferation of anti-CD2CD3CD28 bead-stimulated autologous PBMCs by at least 50% (of maximum) at a 1:2 Treg:PBMC ratio (Figure 8B). CD25hiFoxP3+ Tregs comprised 2.4±0.4% of CD4+CD3+ T cells,[215] ranging from 2-5% (n=30) (Figure 8A, representative plot).

Figure 8. Phenotype and function of Cynomolgus macaque Tregs

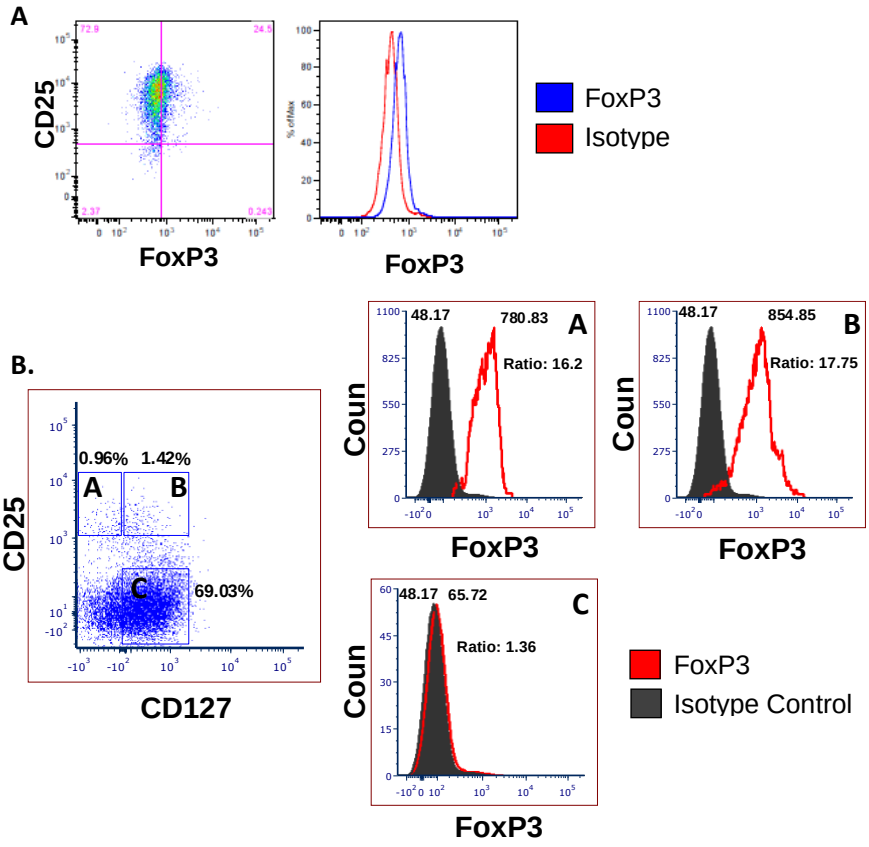


A. FoxP3 levels based on CD25 expression in CD4+ T cells. **B.** *In vitro* suppression of anti-CD2CD3CD28 bead-stimulated autologous-PBMCs by freshly isolated CD4+CD25hi Tregs.

Human activated T cells can up-regulate both CD25 and FoxP3 (albeit at lower levels than Tregs).[216] We cultured CD3+CD4+CD25low/negative sorted MCM T

cells with anti-CD2CD3CD28 beads for seven days. Similar to human Tregs, we observed an upregulation of FoxP3 in MCM T cells (Figure 9A). In humans, CD127 is expressed on effector CD4+ T cells and not on Tregs.[217,218] Hence, we assessed the expression of CD127 in unstimulated MCM PBMCs (n=4). Contrary to human studies, FoxP3 in the MCM was not expressed at higher levels in the CD127- cells compared to CD127+ T cells that had similar CD25 expression (Figure 9B). This suggests that CD127 may not be a reliable marker to differentiate Tregs from effector T cells in MCM.

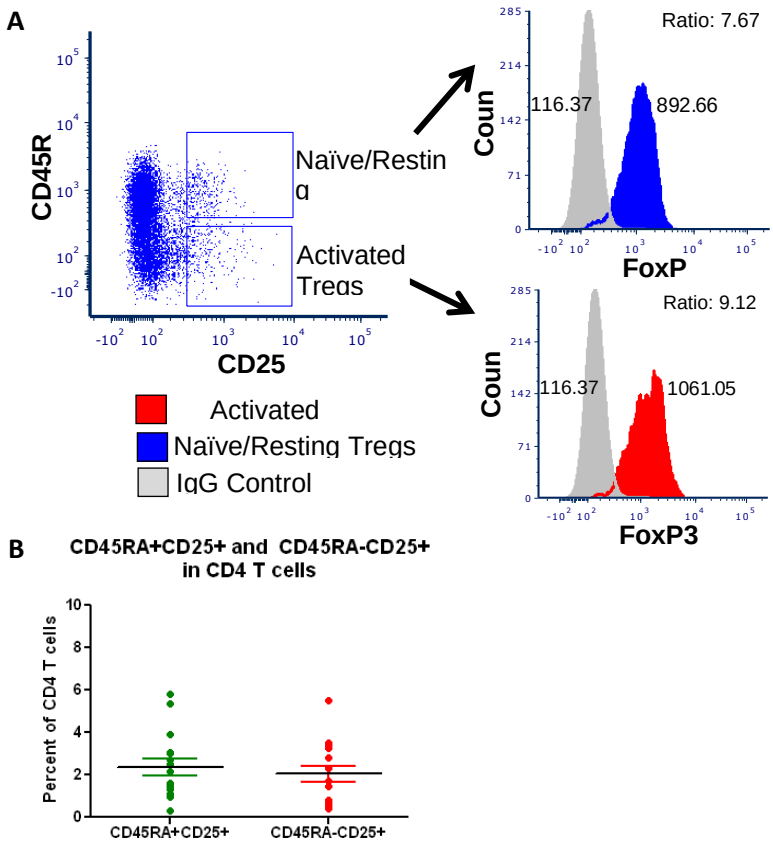
Figure 9. FoxP3 expression on activated T effector cells and CD127 expression on MCM Tregs



A. FoxP3 expression of CD3+CD4+CD25low/negative sorted T cells after seven days of incubation with anti-CD2CD3CD28 beads. **B.** FoxP3 expression in relation to CD25 and CD127 in unstimulated PBMCs (representative figure of n=4).

We also assessed the expression of CD45RA (a marker of naïve T cells in humans[219]) within the MCM Treg populations. Based on their CD45RA expression, human Tregs have been divided into naïve/resting (rTregs; CD45RA+CD25+FoxP3lo) and activated subsets (aTregs; CD45RA-CD25hiFoxP3+).[220] In the MCM peripheral blood we observed 2.0 ± 0.38% and 2.4 ± 0.39% expression of Foxp3 within CD45RA+CD25hi and CD45RA-CD25hi T cells respectively (n=16). Unlike human cells, these MCM populations expressed comparable levels of FoxP3 regardless of CD45RA expression (Figure 10A). CD45RA positive and negative cells and CD4CD25hi MCM cells were equally distributed (Figure 10B).

Figure 10. CD45 expression on peripheral blood MCM Tregs

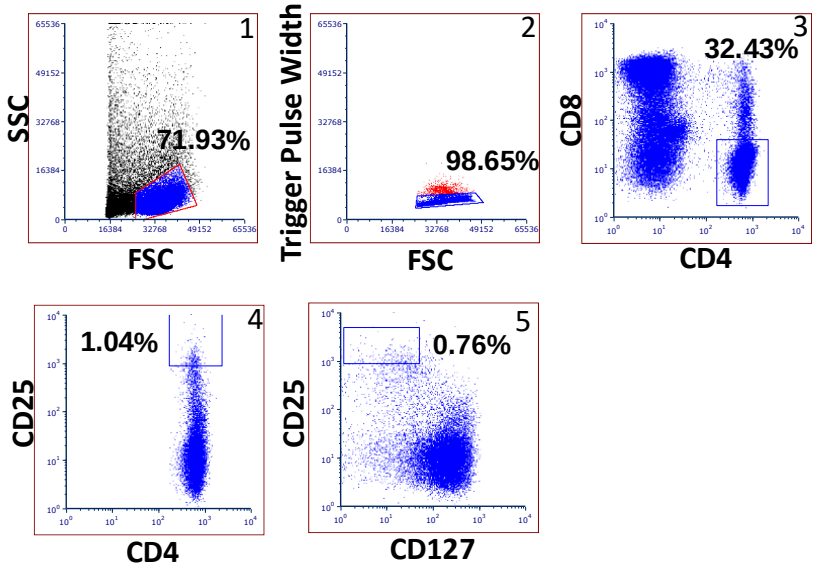


A. Analysis of FoxP3 expression in naïve/resting and activated Tregs in whole blood. **B.** Average number of Tregs within the blood based on the expression of CD25 and CD45RA (n=16).

V.1.2. Expansion, phenotype and suppression of polyclonal Tregs *in vitro*

We developed five protocols for the expansion of MCM Tregs (Table 3). Our gating strategy selected the top 1% of CD25+ and CD127- cells within CD4+ T cells (Figure 11).

Figure 11. Isolation of Tregs via FACS

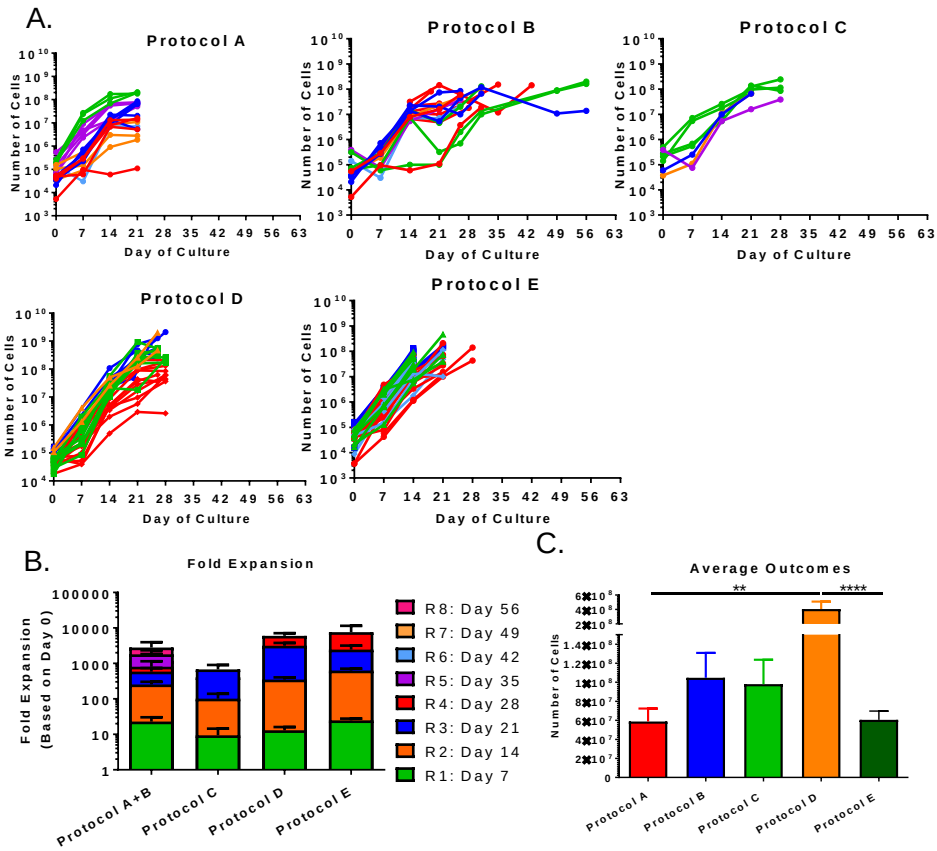


Sorting schema for the MCM Tregs. The number on the top right side of each quadrant indicates the gating order.

Expansion of Tregs varied between protocols (Figure 12A), but at least 1,000-fold expansion was achieved with the five protocols (Figure 12B). Protocol E achieved the highest Treg expansion with an average of 7,599.4 ± 5,504.1 if cells were cultured for 28 days, followed by Protocol D that expanded Tregs 3,338 ± 1,143 folds, followed by Protocol B, with 2,242 ± 400.8-fold expansion (Figure 12B). Protocol E received stimulation from a combination of MHC-mismatched CD40L-stimulated B cells. Protocols B and D received a combined stimulation of aAPCs and allogeneic PBMCs. In contrast, when allogeneic PBMCs alone were used as stimulators from days 7-21 (Protocol A) or aAPCs were used alone as the only stimulation source (Protocol C), fewer fold expansions were obtained

(687.9 ±1 49.5 for Protocol A and 716.2 ± 220.6 for Protocol C) (Figure 12B). When final cell numbers (prior to cryopreservation) were compared across the five protocols, significant differences were found between protocols as described below. When final cell numbers (prior to cryopreservation) were compared between protocols, A and D ($p=0.0044$) and protocols E and D ($p<0.0001$) showed the greatest statistical significant differences (Figure 12C).

Figure 12. In vitro Treg expansion under different approaches



A. Growth curves of Tregs cultured under four different protocols over time. Each color represents a different animal. **B.** Average fold-expansion in each restimulation. Protocols A and B are identical during the first 21 days, and are therefore combined until day 21. "R" is indicative of the number of restimulations. **C.** Average yield of Tregs obtained at the end of each protocol.

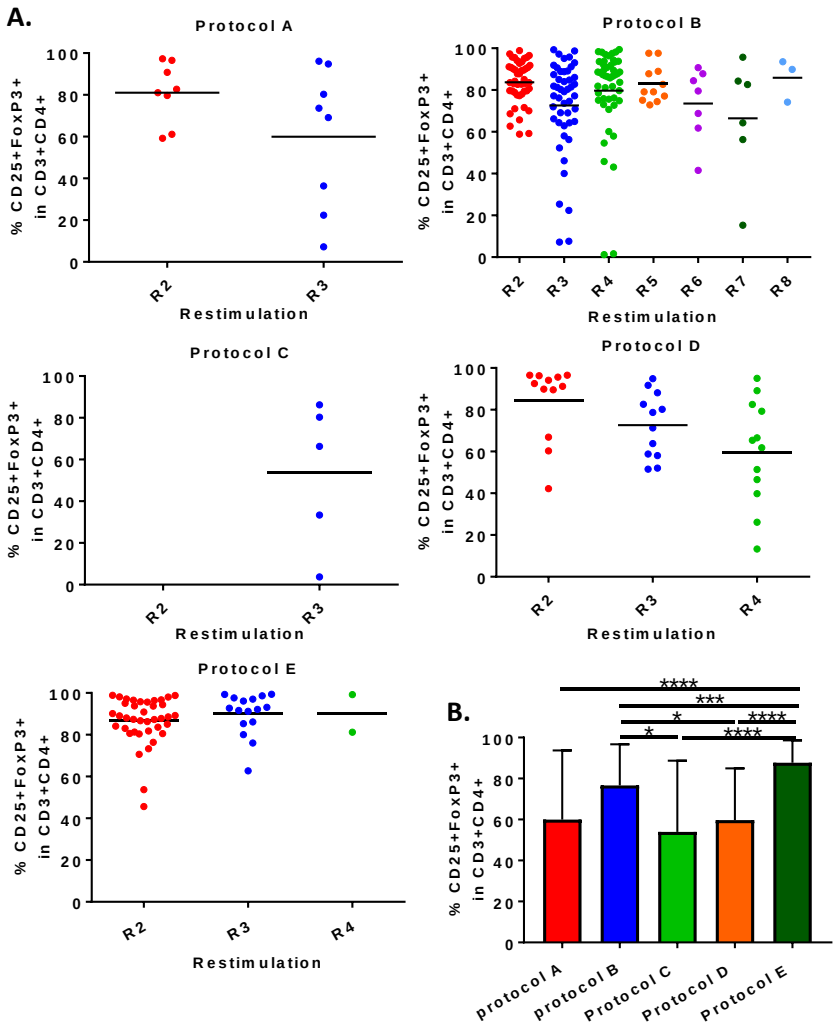
Results

We then compared the FoxP3 expression prior to cryopreservation. We observed that protocol E retained the highest and most stable FoxP3 expression at different time points, followed by protocol B (Figure 13A). Significant differences were found by Student's t-Test between protocols B and C ($p=0.0294$) and B and D ($p=0.0158$) (Figure 13B). In addition, significant differences were observed between protocol E and the other four protocols (E vs A, $p<0.0001$; E vs B, $p=0.0005$; E vs C, $p<0.0001$; E vs D, $p<0.0001$) (Figure 13B).

We also studied the *in vitro* suppressive capacity of Tregs before cryopreservation (Figure 14). Tregs cultured with protocol E showed the best suppression of bead-stimulated autologous PBMC proliferation compared to the other four protocols (Figure 14E). A 1:16 Treg:PBMC ratio suppressed PBMC proliferation with protocol E in average, followed by protocol A and B (Figure 14A and 14B) that achieved an average of 1:4 to 1:8 for protocol A and 1:2 to 1:4 for protocol B, in contrast to protocols C and D, in which 1:1 to 1:2 Treg:PBMC dilution produced 50% suppression (Figure 14C and 14D).

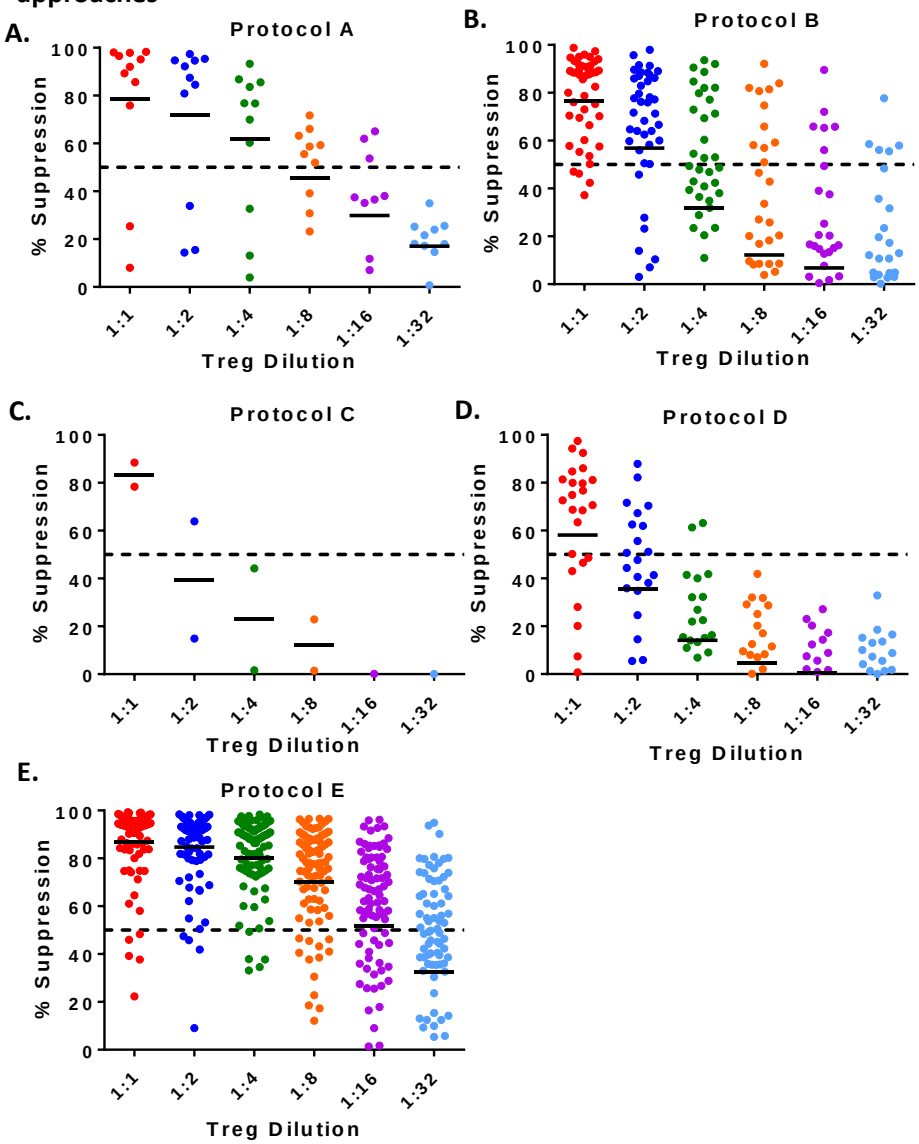
In summary, Protocol E had the best FoxP3 expression and suppressive capacity followed by Protocol A and B. In contrast, these three protocols generated moderate expansion of Tregs compared to Protocol D that achieved the highest numbers overall. Protocol E was able to achieve the highest fold expansion if Tregs were cultured for 28 days but due to the high number of CD40L-stimulated B cells needed, most of the lines were cryopreserved by Day 14 of culture resulting in moderate Treg expansion compared to Protocol D in which Tregs were consistently expanded for 26 days.

Figure 13. FoxP3 expression in Tregs expanded under different conditions



A. Expression of FoxP3 among CD4+CD25hi cells in each culture condition. **B.** Average of FoxP3 expression at the end of the culture for each Treg

Figure 14. Suppressive capacity of MCM Tregs cultured under different approaches

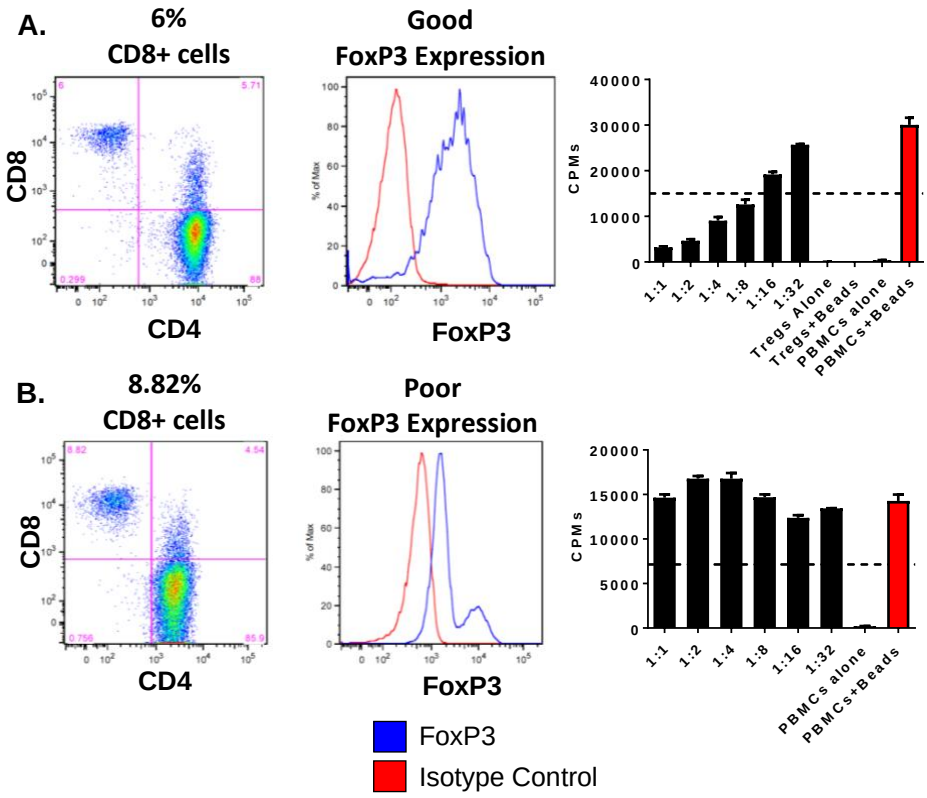


Suppressive function of Treg lines. Suppression was measured by the ability to prevent the proliferation of anti-CD2CD3CD28 bead-stimulated-PBMCs. The dashed line denotes 50% suppression. Phenotype and function were tested in most (but not all) restimulations. With the exception of R4 in protocols C and D on day 26, restimulations occurred every seven days.

V.1.3. Management of outgrowth of CD8+ T cells in Treg cultures

CD8+ T cells have been reported to contaminate cultured Tregs[221]. In agreement with published literature, we occasionally observed the outgrowth of CD8+ T cells in our cultures. Because host effector CD8+ T cells are undesirable in transplant studies because of their potential to induce rejection, we developed methods aimed to prevent CD8+ T cells from contaminating our cultures (Figure 15 and 16).

Figure 15. CD8 and FoxP3 expression along with the suppressive capacity of two Treg lines with comparable CD8 contamination



CD8, FoxP3 expression and *in vitro* suppressive capacity of two different Treg lines (**A** and **B**) with comparable CD8 contamination and different FoxP3 expression.

V.1.3.1. Use of anti-CD8 antibody at the initial FACS

Our first approach attempted to exclude CD8+ cells by using anti-CD8 antibodies at the initial FACS (Figure 11). This approach was not always successful, as CD8+ T cells were still detected in the cultured cell lines (Figure 15A and 15B).

We also assessed the suppression by Treg cultures that had comparable CD8 contaminations. Differences in suppression were directly related to the levels of FoxP3 expression of the CD4+ T cells (Figure 15A and 15B), indicating that the Treg stability/purity could override the proliferation of CD8+ T cells (Figures 15A and 15B).

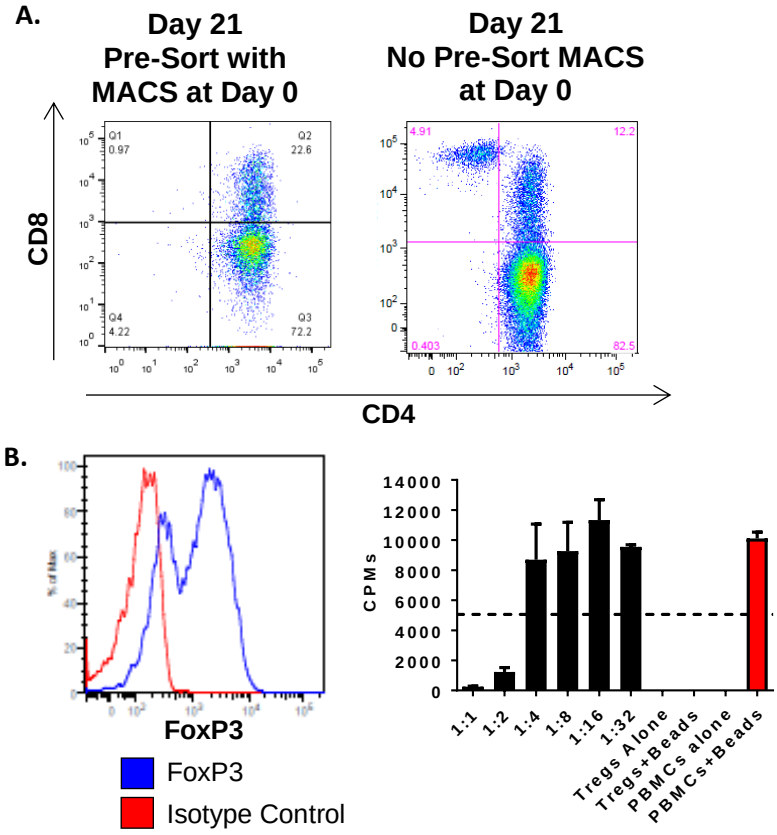
V.1.3.2. Pre-enrichment of CD4+ cells through MACS

Pre-enrichment of CD4+ T cells from PBMCs by magnetic-activated cell sorting (MACS) prior to FACS was shown to efficiently remove CD8+ T cells (Figure 16A), but Treg lines grown following this technique consistently failed to grow or maintain FoxP3 expression and suppressive activity (Figure 16B). Hence, in our hands, MACS may have removed or damaged natural Tregs.

V.1.3.3. CD8 depletion by FACS early in culture to eliminate CD8 contamination

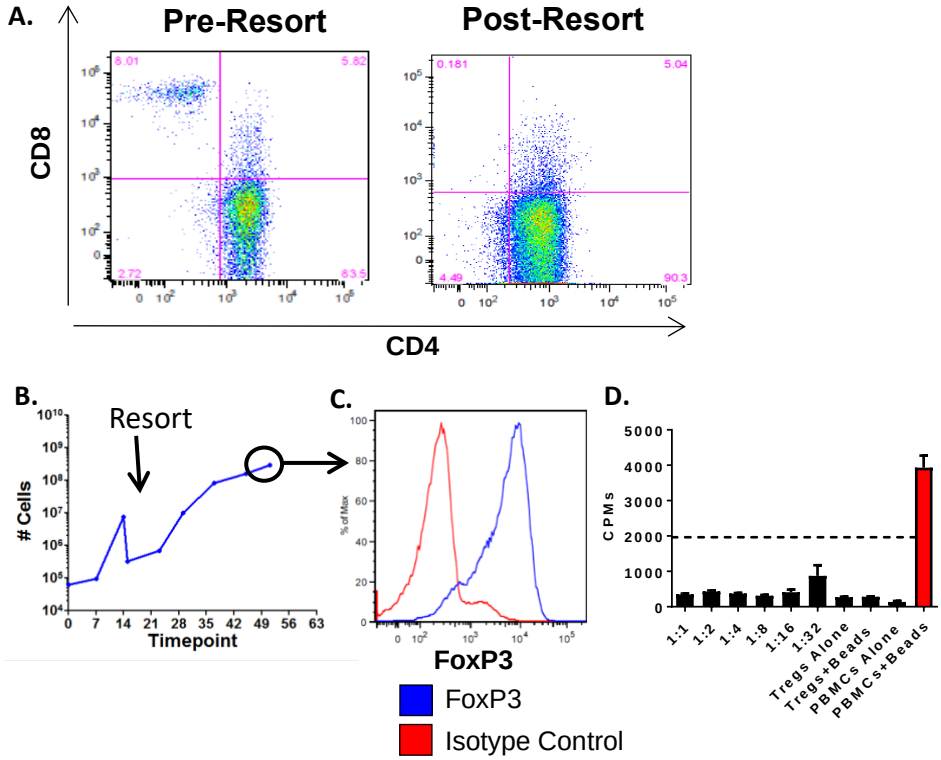
We subsequently attempted to resort Tregs mid-way through the culture period. Lines were resorted when >4% of CD8+ T cells were detected. This approach proved to be successful in eliminating CD8+ T cells (Figure 17A), but was associated with significant loss of Tregs, thereby prolonging the culture period (Figure 17B). However, despite culturing Tregs for >50 days (protocol B), expression of FoxP3 and suppressive function was maintained at high levels (Figure 17A and 17B).

Figure 16. CD8 contamination comparison on day 21 of culture between a line that underwent MACS purification (left panel) and one that did not (right panel)



A. CD8 single positive (SP) contamination on day 21 of culture between a Treg line that underwent MACS purification (left panel) and one that did not (right panel). **B.** FoxP3 expression and suppressive capacity at the end of the culture period of Tregs that underwent CD8 MACS depletion prior to FACS sorting on day 0.

Figure 17. CD8 FACS depletion of a Treg line early in culture, cell growth, FoxP3 expression and suppressive capacity at the end of the culture (day 56)



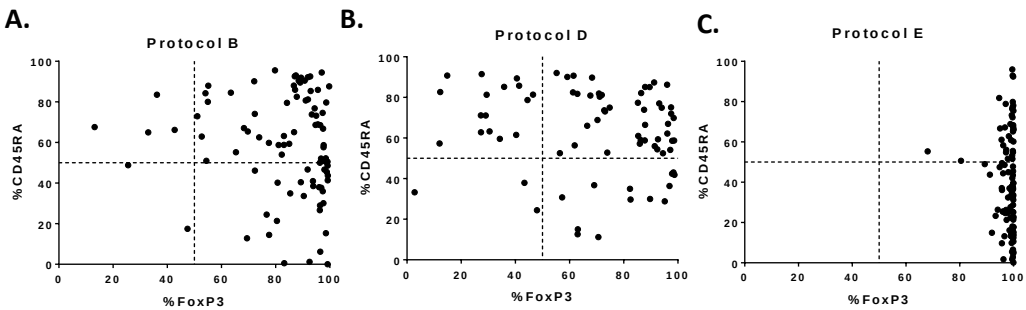
A. CD8 depletion by FACS early in culture to eliminate CD8 contamination. **B.** Cell growth of a Treg line that underwent FACS on day 15 of culture to eliminate CD8 T cells. **C.** FoxP3 and **D.** suppressive capacity of a Treg line at the end of the culture period (day 56) after undergoing FACS on day 15 due to CD8 T cell contamination.

V.1.4. FoxP3 expression is maintained in CD45RA+ and CD45RA- cells in long-term cultures

Human Treg studies suggest that CD45RA+ and not CD45RA- Tregs maintain the expression of FoxP3 during prolonged cultures.[222] We thus examined the correlation between CD45RA and FoxP3 in our *in vitro* expanded Tregs cultured under protocols B (Figure 18A), D (Figure 18B) and E (Figure 18C). Regardless of the protocol, the expression of CD45RA in the cultured cells did not correlate with FoxP3 expression. CD45RA intermediate/low cells also expressed high levels of FoxP3 in some cases.

Therefore, and contrary to human studies, reduced FoxP3 was not associated with loss of CD45RA expression in MCM.

Figure 18. CD45RA expression in cultured Tregs

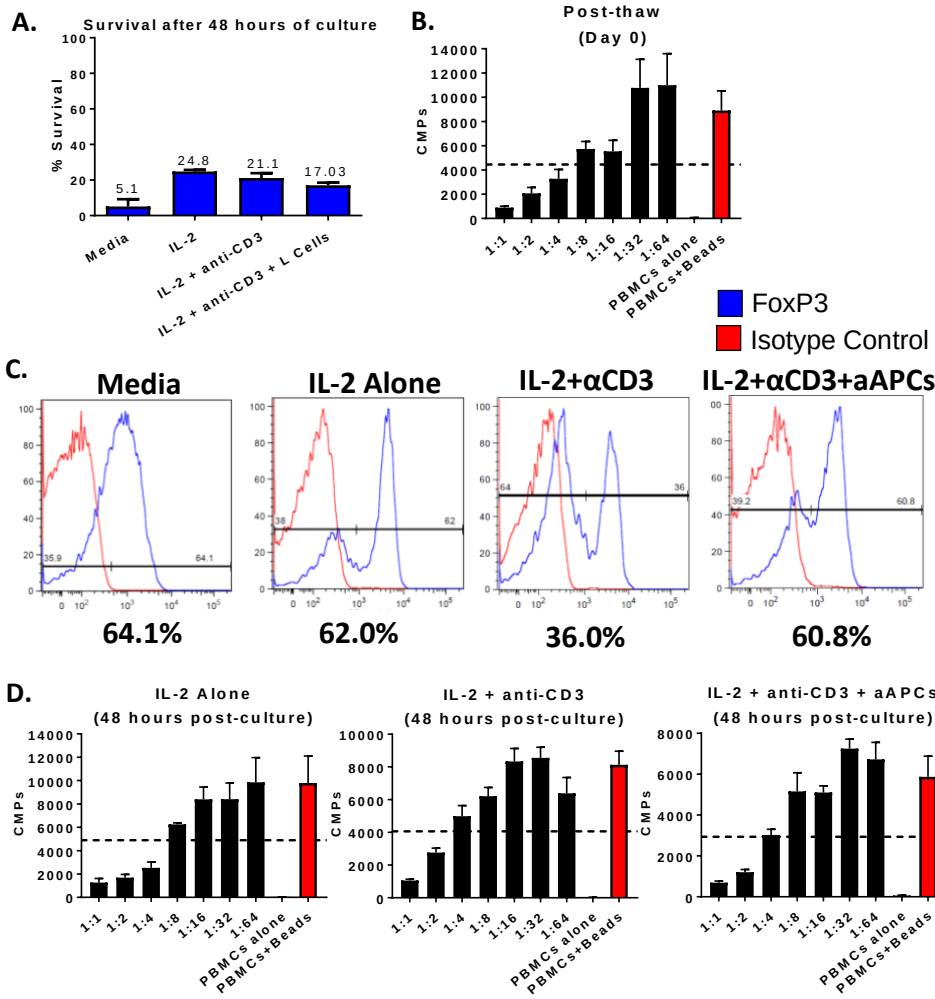


A. Correlation between FoxP3 and CD45RA expression in protocol B. **B.** Correlation between FoxP3 and CD45RA expression in protocol D. **C.** Correlation between FoxP3 and CD45RA expression in protocol E.

V.1.5. Recovery of Tregs after cryopreservation

In order to be used as an "off-the-shelf" product, Tregs must be functional after thawing. However, cryopreservation has been reported to impact Treg phenotype and suppressive activity.[223] We thus studied the effects of cryopreservation on MCM Tregs expanded under protocol B. Cryopreserved and thawed Treg lines were plated under four culture conditions (see Materials and Methods). Regardless of condition, the viability was low after 48 hours, ranging from 5.1 to 24.8% (Figure 19A). A distinct FoxP3 negative population was observed in all the conditions except the media alone (Figure 19C). Freshly thawed cells had 50% suppression at ratios of 1:4-1:8 Tregs:PBMCs (Figure 19B), whereas suppression 48 hours post-reculture varied from 1:2 to 1:8 (Figure 19D). Thus, reculturing Tregs for 48 hours (regardless of condition) did not improve FoxP3 expression or suppression.

Figure 19. Cryopreservation and recovery of Tregs under different conditions

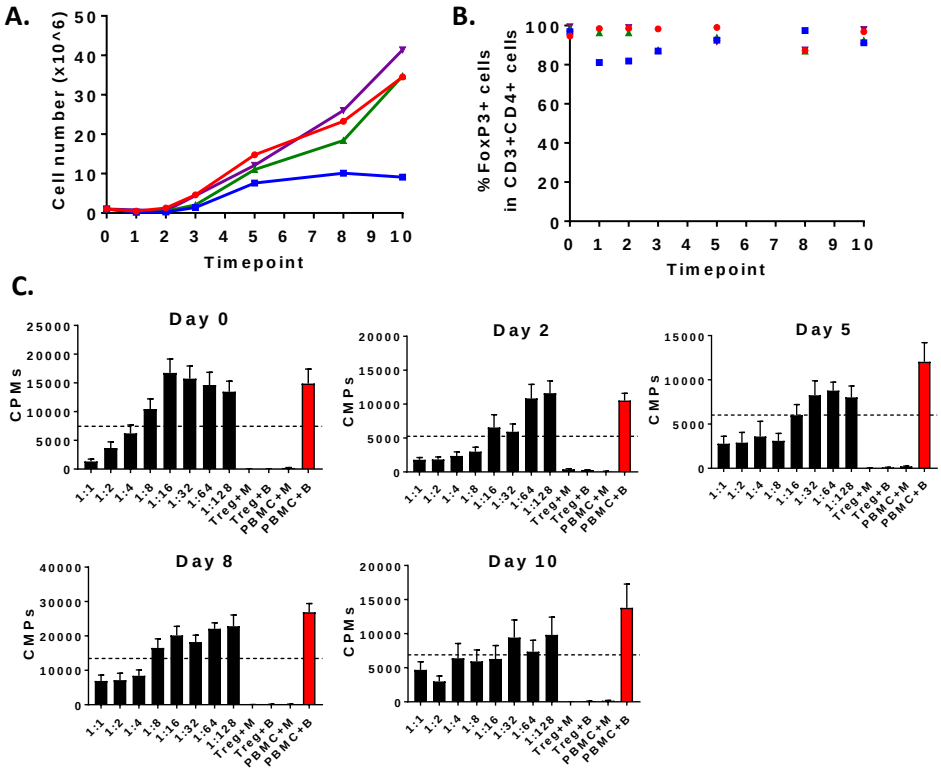


A. Average cell survival after 48 hours of culture post-thaw (n=3). Cell death was assessed by trypan blue and propidium iodide (PI). **B.** Average suppressive activity of freshly thawed Tregs. **C.** FoxP3 expression after two days of culture under various conditions. **D.** Average suppressive activity of Tregs 48 hours after culture (n=3).

We then studied whether longer periods of culture post-thaw would enhance the purity and number of Tregs beyond that observed after 48 hours. Cells were cultured with IL-2, anti-CD3 and aAPCs. During the first 24-48 hours of culture, the majority of cells died again (Figure 20A). However, maintaining cells *in vitro* for a longer period allowed them to expand to the original number by Day 3, by a log by Day 5 and 30±7.1 fold by the end of the culture on Day 10 (Figure 20A).

FoxP3 expression was maintained throughout the culture period post-cryopreservation (Figure 20B). In addition, Treg function was improved compared to the thawing day (Figure 20C). These results suggest that a minimum of 3-day re-culture period can improve function and re-expand Treg numbers under the described conditions.

Figure 20. Cryopreservation and recovery post-thaw of Tregs after 10 days of culture

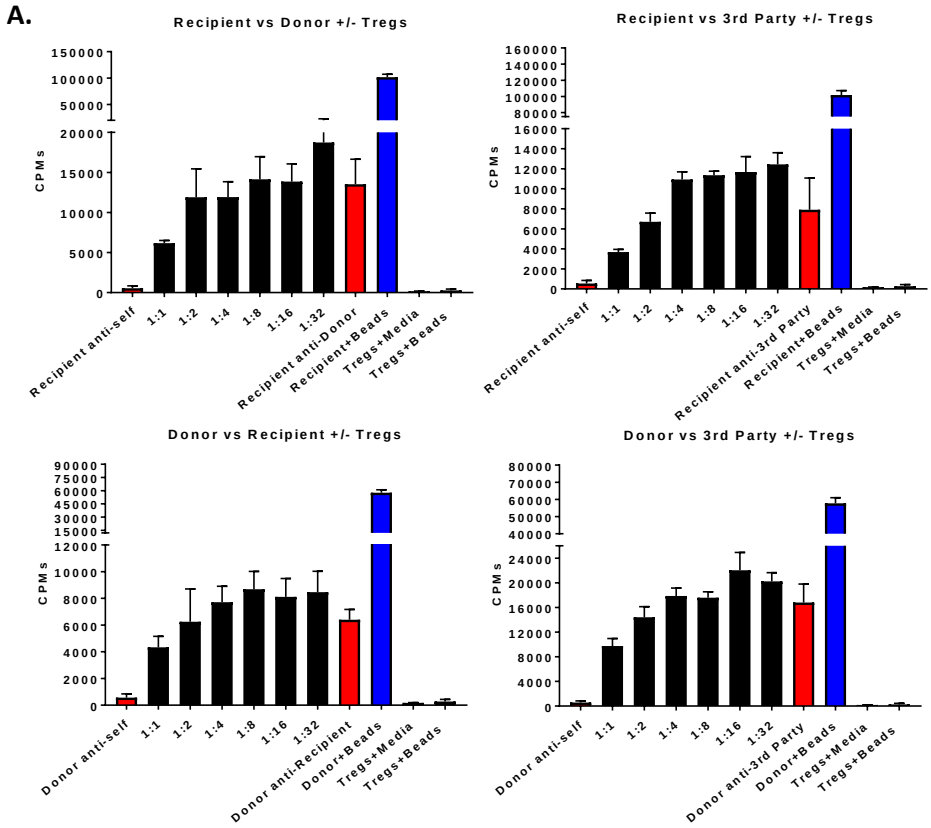


A. Cell growth of thawed Tregs plated with IL-2, anti-CD3 and aAPCs for 10 days (n=4). **B.** FoxP3 expression of freshly thawed Tregs (Day 0) and over the course of the culture (n=4). **C.** Suppressive capacity of freshly thawed Tregs (Day 0) and over the course of the culture (n=4).

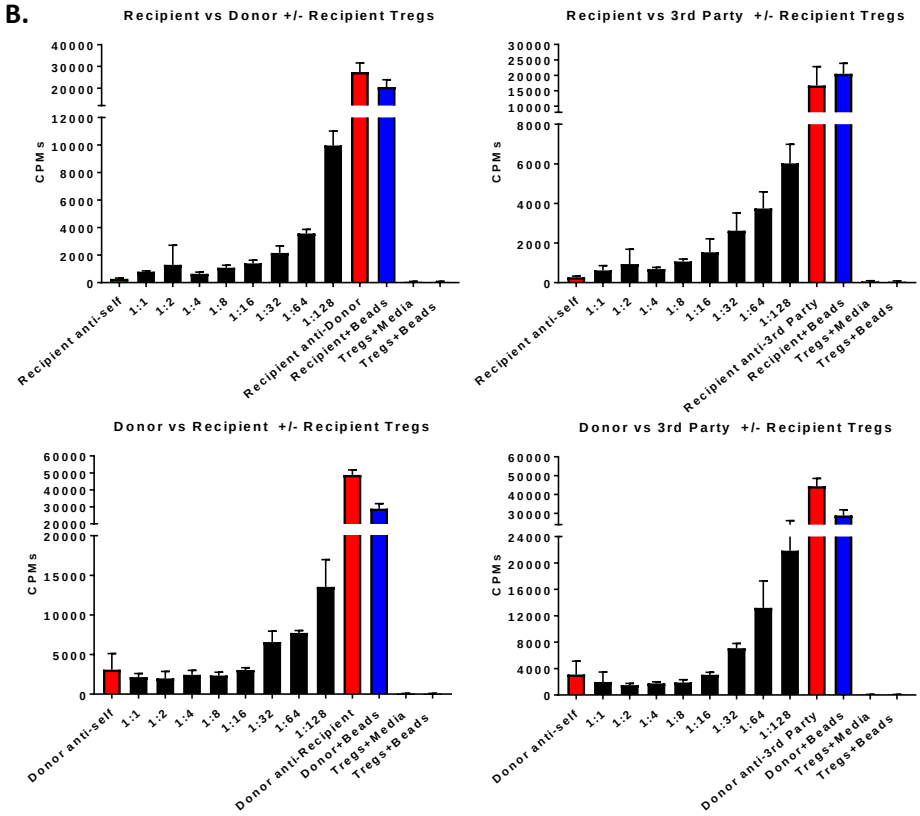
V.1.6. Tregs were able to suppress HVG and GVH responses in support of their polyclonal function

We assessed the specificity of suppression of polyclonal Tregs after expansion under Protocol B and E, which provided the best outcomes (Figure 21).

Figure 21. Specificity study of *in vitro* expanded Tregs



We observed comparable suppression by host Tregs when cultured in a mixed lymphocyte assay against three different responders. Tregs were mixed with autologous, donor (previously seen during cell expansion) or MHC-mismatched third-party T cells which were not stimulated with beads but instead with either autologous (syngeneic), donor or third-party APCs.



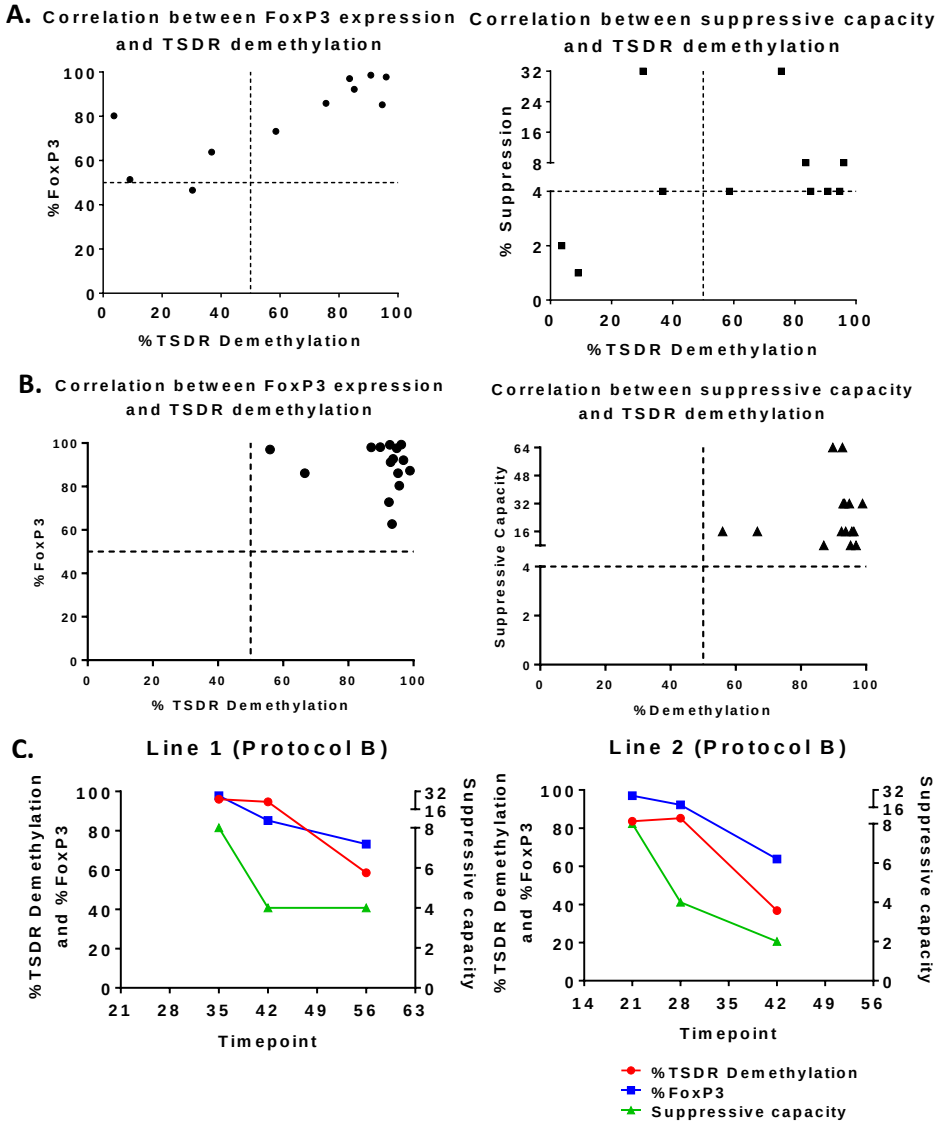
A. Host PBMC responders stimulated with donor and third party PBMCs and donor PBMC responders stimulated with host and third party PBMCs. Tregs were cultured following protocol B. **B.** Host PBMC responders stimulated with donor and third party PBMCs and donor PBMC responders stimulated with host and third party PBMCs. Tregs were cultured following protocol E. Donor and third party PBMCs are full-MHC mismatched to host. Donor and third party PBMCs do not share any MHC.

Autologous, stimulator-type (MHC-mismatched PBMCs used for stimulating the Treg culture) or third-party (MHC-mismatched to both Tregs and stimulator PBMCs) cells were used as responders. At the time of analysis, Tregs had been exposed to stimulator-type PBMCs for two weeks. Tregs were successful at suppressing the responses of PBMCs from any given responder, either previously seen or in contact with for the first time (Figure 21A and 21B). In conclusion, expanded Tregs proved to be polyclonal as they not only suppressed both HVG and GVH responses, but also powerfully suppressed responses to a third-party to which Tregs were never exposed before.

V.1.7. Treg-specific demethylated region (TSDR) levels correlate with high FoxP3 expression and good suppressive activity in MCM

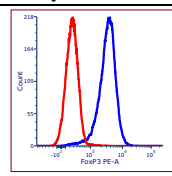
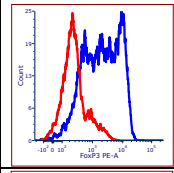
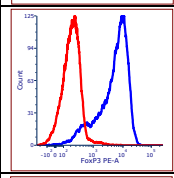
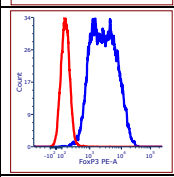
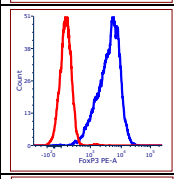
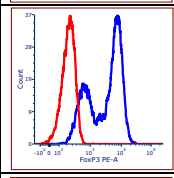
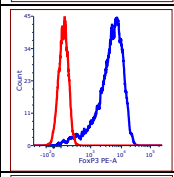
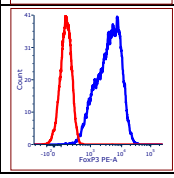
Demethylation of the TSDR has been shown to identify stable Tregs.[224] We therefore analyzed the TSDR of MCM Tregs that were grown under protocols A, B, D, (Figure 22A and Table 6) and E (Figure 22B). Treg lines that concurrently had ≥50% suppression at a 1:4 or lower Treg:PBMC ratio and FoxP3 expression >80% (as a single peak) all had TSDR demethylation >75%. Tregs from cultures which lost FoxP3 expression and were less suppressive in some cases had greater TSDR methylation (Figure 22A and Table 6). One Treg line with high FoxP3 expression (80%) showed a low percentage of TSDR demethylation, possibly reflecting two peaks of FoxP3 expression (Table 6; Line 2). A second outlier had good suppressive capacity with a low percentage TSDR demethylation, possibly due to a high percentage of CD8+ T cells in this line (9.6%). In addition, in two cell lines that were studied over time, we observed a decrease in the demethylation of TSDR in association with a decrease in FoxP3 expression and suppressive capacity (Figure 22C). In conclusion, all the aforementioned protocols were able to generate highly demethylated Tregs. These results mirror what has been found in humans[225] and further support the MCM as a good model for Treg studies.

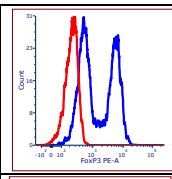
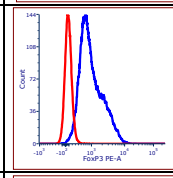
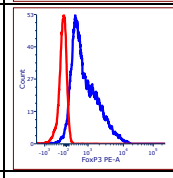
Figure 22. Demethylation status of the TSDR of *in vitro* expanded Tregs.



Demethylation status of the TSDR of *in vitro* Tregs cultured under **A)** protocol A, B and D and **B)** protocol E in correlation with the FoxP3 expression and suppressive capacity. **C)** Study of the correlation between the TSDR demethylation status, FoxP3 expression and suppressive capacity of two Treg lines cultured under protocol B over time.

Table 6. Study of methylation status of the TSDR

Protocol	Line	FoxP3 expression	Suppressive capacity	Culture duration	%Demethylation
B	1		1:4	30	90.8
B	2		1:32	56	30.3
B	3		1:32	51	75.6
B	4		1:8	35	96.0
B	5		1:4	42	94.7
B	6		1:4	56	58.6
A	7		1:8	21	83.6
B	8		1:4	28	85.2

B	9		1:2	42	36.8
D	10		1:2	26	3.7
D	11		<1:1	26	9.1
Control	CD4+CD8-CD25high	NA	NA	0	92.6
Control	CD4+CD8-CD25intermediate	NA	NA	0	9.6
Control	CD4+CD8-CD25low	NA	NA	0	0.6

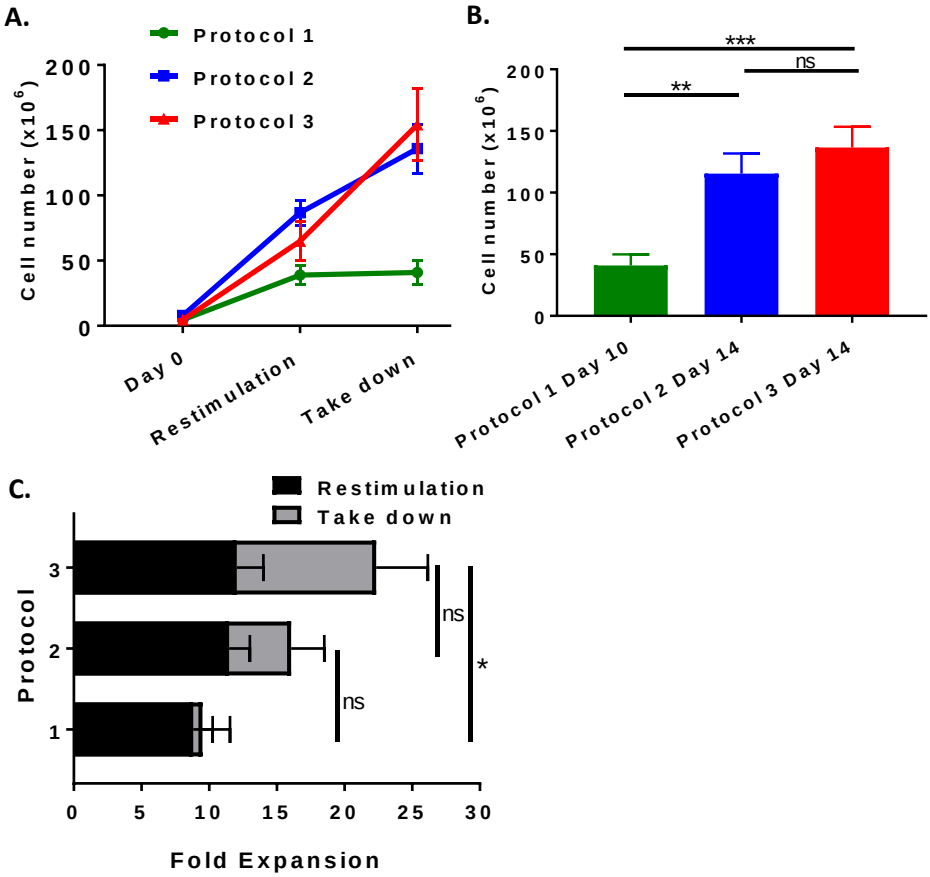
Blue histogram represents FoxP3 expression and red histograms are controls.

V.1.8. Expansion of MCM CD40L-Stimulated B cells (CD40L-sBc)

Expanded and CD40L-activated B cells (CD40L-sBc) were used as stimulators for Treg expansion in Protocol E instead of PBMCs or aAPCs as the other protocols (A through D). This approach was an adaptation from a previously published work for the manufacturing of donor specific Tregs for human clinical trials.[226] To extend this strategy to deceased donation (as the donor HLA is unknown in advance), we aimed for the expansion of polyclonal Tregs.

Tregs were cultured with a pool of CD40L-sBc expanded from MCM donors with disparate MHC at a ratio 1:4 Treg:CD40L-sBc.

Figure 23. B cell growth comparison between three different approaches.



A. CD40L-sBc growth average comparison between protocol 1 (n=10), 2 (n=14) and 3 (n=18) over time. **B.** Averages of the CD40L-sBc numbers at the end of the culture for protocol 1, 2 and 3. **C.** Fold expansion of each CDD40L-sBc protocol at the restimulation and harvest time point.

We studied three different approaches for the expansion of MCM B cells. Freshly isolated or thawed PBMCs or splenocytes were cultured under different conditions with CD40L-expressing K562 cells for either 10 or 14 days depending on the protocol (Table 4). K562 cells were generated through lentiviral vectors encoding human CD40L, CD64, DRA and DRB0401.[226] In addition, IL-4 for B cell stimulation, cyclosporine A (CyA) to avoid the growth of T cells and ganciclovir (GCV) for the control of Epstein-Barr virus (as these approaches were based on a human protocol) were added to culture.

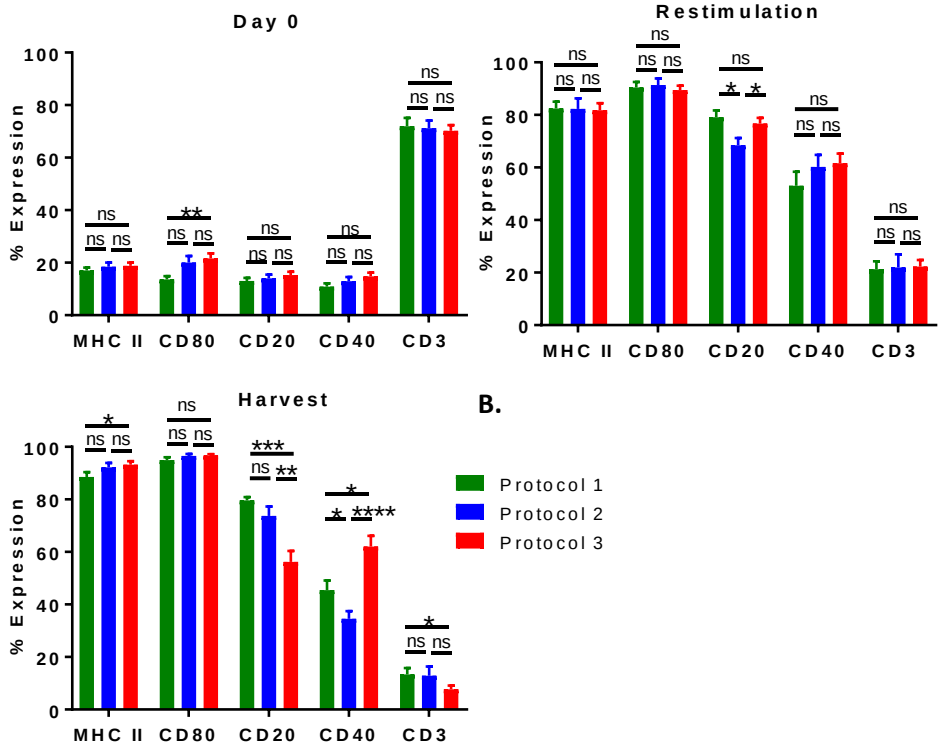
An average of $6.2x10^6 \pm 3.5x10^6$ B cells (based on the percentage of CD20+ cells in PBMCs/splenocytes) was plated on Day 0. Protocol 1 was based on previous human studies[226]. Cells were in culture for 10 days. A slower cell growth was observed with this protocol compared to Protocol 2 and 3 (Figure 23A). At the end of the culture, an average of $40.88x10^6 \pm 9.1x10^6$ B cells was obtained (Figure 23B and 23C). In order to improve the yield, we increased the culture time to 14 days for Protocol 2 and 3. With this approach, final cell yields increased to $115.4x10^6 \pm 16.34x10^6$ and $136.6x10^6 \pm 16.78x10^6$ for Protocol 2 and 3, respectively, with significant difference between these two protocols and Protocol 1 (Figure 23B; p=0.0017 (Protocol 1 vs 2), p=0.0004 (Protocol 1 vs 3)). Since the final number of cells is affected by the starting number of cells, we calculated their fold expansion in order to normalize the cell growth to the number of B cells plated on Day 0. The highest average fold expansion was achieved with Protocol 3 (22.3 fold), compared to Protocol 2 (16.1 fold) and Protocol 1 (9.5 fold) (Figure 23C) with significant difference found between Protocol 1 and 3 (p=0.02).

To ascertain if the different protocols resulted in differences in quality or purity of CD40L-sBc, we followed the level of B-cell marker expression over time as well as the percentage of T cells in culture (Figure 24). Cells expressed low levels of the B cell markers at the beginning of the culture (Day 0) with a high percentage of T cells, as expected. The concentration of CyA was increased in Protocols 2 and 3 on Days 2 and 4 in order to decrease the number to contaminating T cells. In addition, the restimulation with fresh CD40L-expressing K562 cells was performed on Day 10 for Protocols 1 and 2, and on Day 7 for Protocol 3. Over time, the expression of B cell markers increased and the T cell counts decreased with all protocols (Figure 24). The three approaches produced cells with comparable MHC II and CD80 levels. Cells from Protocol 1 and 2 had

higher CD20 expression (p=0.0003, Protocol 1 vs 3; p=0.0043, Protocol 2 vs 3). In contrast, Protocol 3 produced cells with the highest level of CD40 (p=0.0118, Protocol 1 vs 3; p<0.0001, Protocol 2 vs 3), and with the lowest T-cell contamination, with significant difference between Protocol 1 and 3 (p=0.0348) (Figure 24).

Due to the higher yield, the greater CD40 expression and the lower T-cell contamination observed in B cells cultured under Protocol 3, we utilized this approach to generate the CD40L-sBc in the experiments to follow.

Figure 24. Phenotype on expanded B cells over time.



Comparison of the expression of B cell markers between protocol 1, 2 and 3 at different time points.

V.2. NHP studies in tolerance induction

We investigated whether the infusion of polyclonal Tregs could achieve long-lasting chimerism as a marker of tolerance after BMT compared to control recipients that did not receive Tregs. Five animals received a non-myeloablative conditioning (Table 7 and Figure 25) followed by BMT. In contrast, ten BMT recipients received Tregs split into several infusions (Table 8). Among those, three Treg-treated animals received low-dose BM from donor iliac crest BM aspirates while the rest of the recipients received high-dose (megadose) BM infusions (BM harvested from the donor iliac crest and vertebral bodies) (indicated in Table 8).

Table 7. TBI dose, cell dose administered, chimerism duration and cause of death of control recipients.

Control Animals						
Animal ID	**POD BMT**	**TBI**	**CD34+ cell/ CD3+ cell/ Treg dose (x10^6/kg)**	**Treg expansion protocol**	**Chimerism duration (Days)**	**Cause of euthanasia**
AH260F (C1)	0	250	7.7/47.3/NA	NA	36	Scientific endpoint
AT468G (C2)	2	250	7.9/141.8/NA	NA	>50	Renal failure/ sepsis
BY648F (C3)	2	250	50.4/67.4/NA	NA	37	Scientific endpoint
AP532B (C4)	2	250	8.4/73.5/NA	NA	>92	GVHD
F813M (C5)	2	250	15.8/54.2/NA	NA	>65	GVHD

"C" represents control animals that did not receive Treg infusions. Five animals were performed in this group. ">"time point when recipients were euthanized due to secondary complications. "Scientific endpoint" represents euthanasia after the skin transplant reaching the end of the experiment. "GVHD" represents euthanasia due to graft versus host disease.

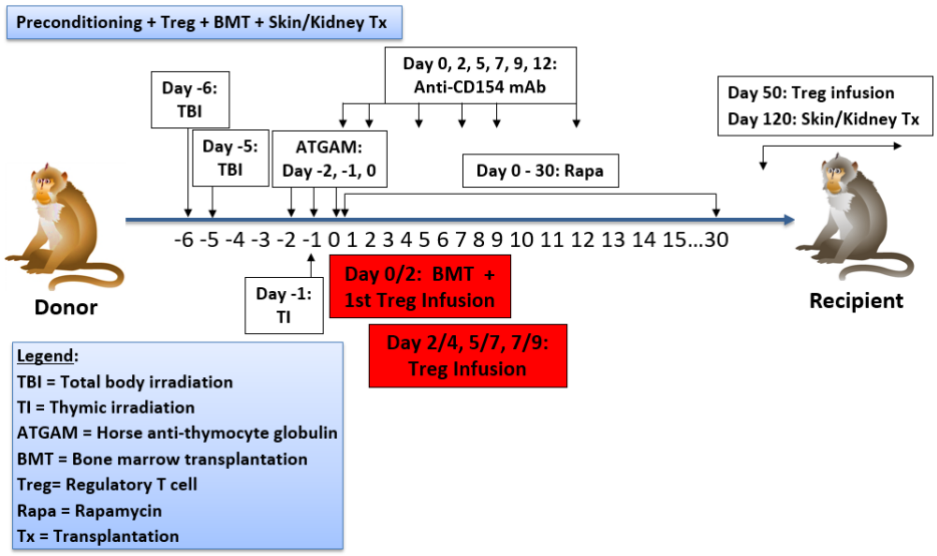
Table 8. TBI dose, cell dose administered, Treg expansion protocol, chimerism duration and cause of death of Treg-treated recipients.

Treg-Treated Animals						
Animal ID	**POD BMT**	**TBI**	**CD34+ cell/ CD3+ cell/ Treg dose ($x10^6$/kg)**	**Treg expansion protocol**	**Chimerism duration (Days)**	**Cause of euthanasia**
SA196A (T1)	0	300	0.97/19.1/94.7	D	57	Scientific endpoint
AJ606D (T2)	2	250	1.2/36.6/75	E	34	Scientific endpoint
AN113D (T3)	2	250	2/33.5/54.4	E	55	Scientific endpoint
AM538C (T4)	0	250	19.5/58.6/32.1	B	110	Scientific endpoint
AK746F (T5)	2	250	7.3/40.8/58	B	33	Scientific endpoint
BF418F (T6)	2	250	11.2/58.8/43	B	98	Scientific endpoint
AG531J (T7)	2	250	12/39.6/46.2	B	69	Scientific endpoint
BM12B (T8)	2, 4	250	7.1/37.2/37.5	E	37	Scientific endpoint
AF201G (T9)	2	250	8/70/41.7	E	>62	GVHD
V59M (T10)	2	250	11.9/105/59.2	E	>78	GVHD

"T" represents Treg-treated animals. Ten animals were performed in this group. ">" time point when recipients were euthanized due to secondary complications. "Scientific endpoint" represents euthanasia after the skin or kidney transplant reaching the end of the experiment. "GVHD" represents euthanasia due to graft versus host disease.

MCM recipients were conditioned with total body irradiation (TBI) (1.25-1.5Gy, Days -6, -5), thymic irradiation (TI) (7Gy, Day -1), horse-antithymocyte globulin (ATG) (Days -2, -1, 0), anti-CD40L (Days 0, 2, 5, 7, 9, 12) and rapamycin for 30 days (Day -2 to 28 or Day 0 to 30 if BMT was performed on Day 0 or 2, respectively) aiming for a level of ~20 ng/mL that was gradually decreased over a three week period (15 mg/mL for first week, 10 ng/mL during the second week and 5 ng/mL for the third week) (Figure 25). MHC-mismatched BM (Figure 26) was infused either on Day 0 or Day 2 (Table 7 and 8) along with the first dose of polyclonal recipient Tregs. Consecutive Treg doses were administered on Day 2, 5 and 7 or 4, 7 and 9 depending on the BMT time point, either on Day 0 or 2, respectively. A fifth Treg infusion was administered on Day ± 50 or earlier in some (not all) animals if chimerism decreased or *in vivo* CD8+ T cell counts increased rapidly (and cryopreserved *in vitro* expanded Tregs were available) (Table 9). Tregs were expanded with either aAPC and donor PBMCs (Protocol B and D) or CD40L-sBc (Protocol E) (Table 8).

Figure 25. Tolerance induction protocol.



Transplant protocol with BMT and first Treg infusion performed on day 0 or day 2.

Table 9. Time points for Treg administration in Treg-treated animals

Animal ID	POD for early Treg infusions	POD for 5th Treg infusion
SA196A (T1)	0, 2, 5, 7	48
AJ606D (T2)	2, 4, 7, 9	50
AN113D (T3)	2, 4, 7, 9	30
AM538C (T4)	0, 2, 5, 7	NA
AK746F (T5)	2, 4, 7, 9	34
BF418F (T6)	2, 4, 7, 9	36
AG531J (T7)	2, 4, 7, 9	46
BM12B (T8)	2, 4, 7, 9	35
AF201G (T9)	2, 4, 7, 9	50
V59M (T10)	2, 4, 7, 9	50

We investigated the ability of *ex vivo* expanded polyclonal Tregs to enhance BM engraftment with the goal to extend the duration of donor-derived chimerism without GVHD and achieve donor-specific tolerance to any grafts (cells such as islets or solid organs such as heart, liver or lung). Transplants were performed across major histocompatibility complex (MHC) barriers and without long-term immunosuppressive drugs. Donor and recipient pairs were haploidentical or full mismatched (Figure 26).

Five recipients received high-dose BMT without Treg infusions (C1 through C5, Table 7 and 10). A total of ten BMT recipients received Treg infusions (T1 through T10, Table 8 and 10). Among the Treg-treated animals, three of the recipients received low-dose BM harvested from the donor iliac crest (IC) (T1 through T3) and seven Treg-treated animals received high-dose (megadose) donor BM (T4 through T10).

Table 10. Relation between bone marrow dose and infusion of Tregs

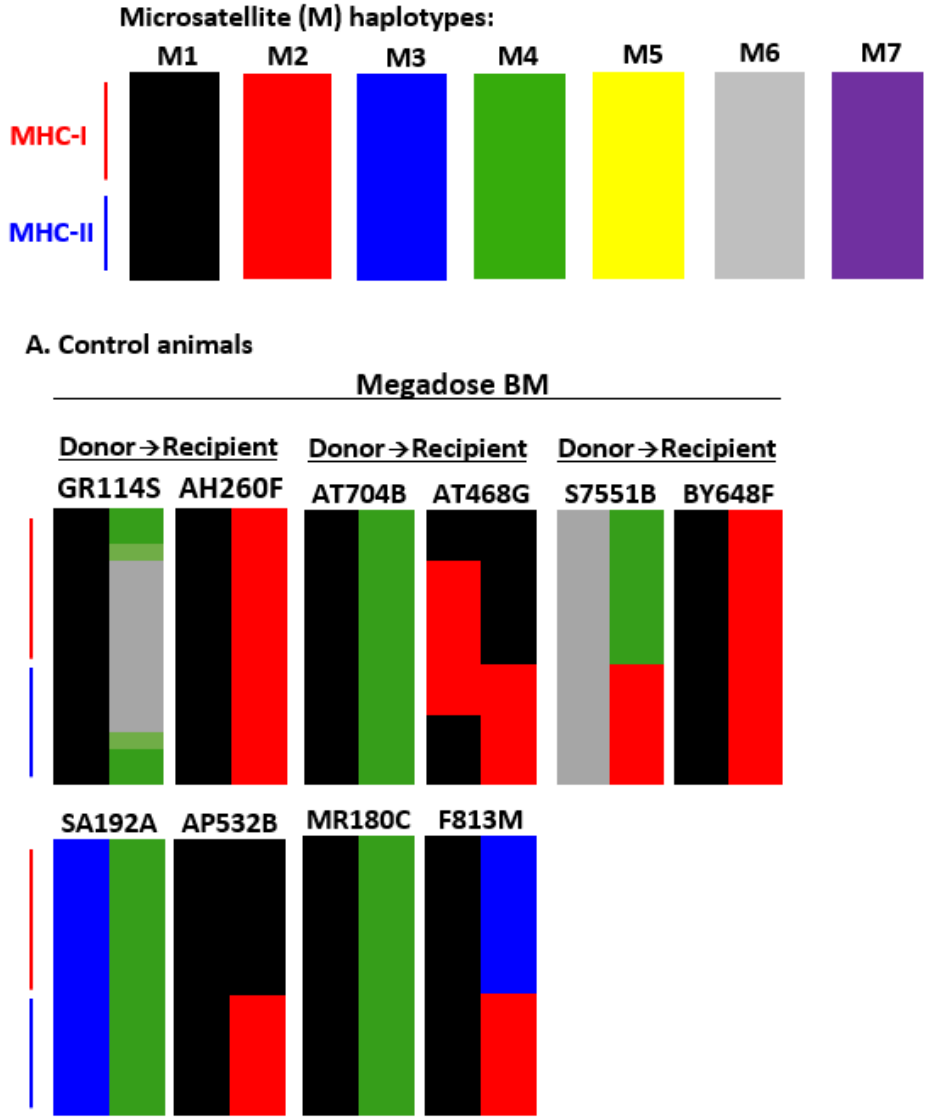
Animal ID	BM dose	Treg administration
AH260F (C1)	High	No
AT468G (C2)	High	No

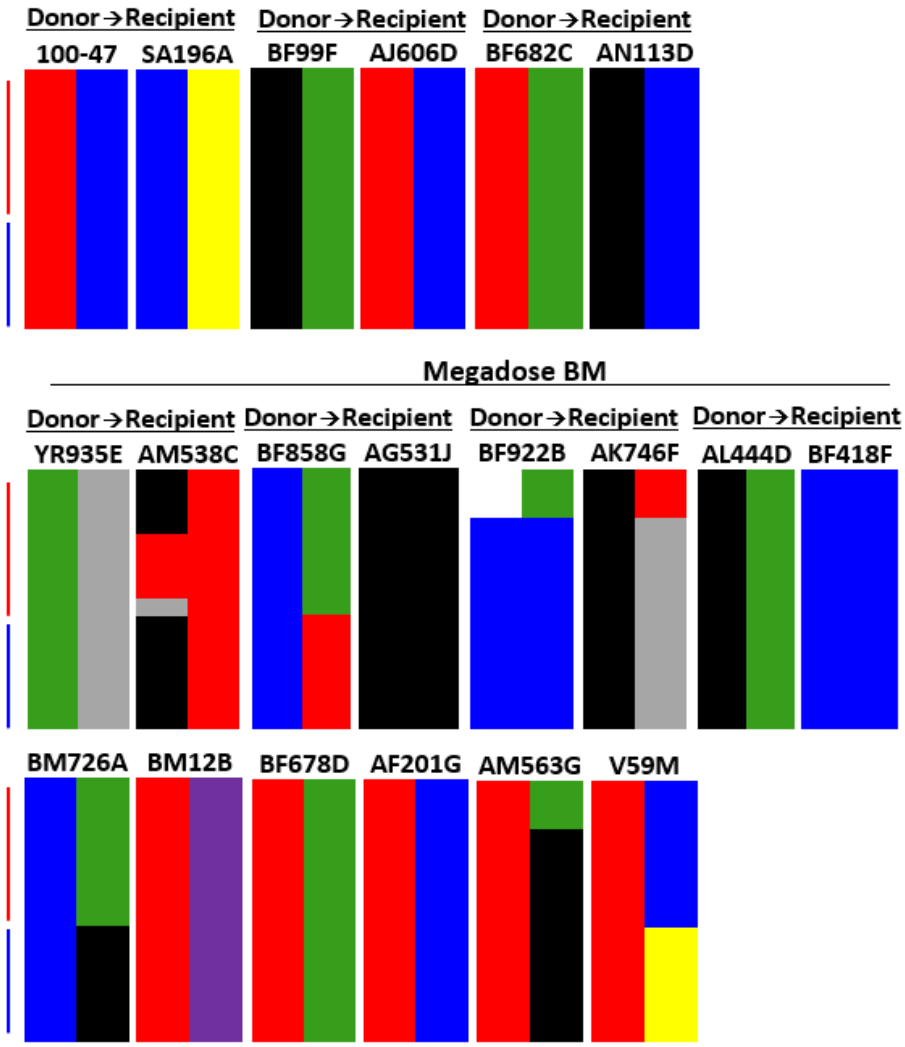
BY648F (C3)	High	No
AP532B (C4)	High	No
F813M (C5)	High	No
SA196A (T1)	Low	Yes
AJ606D (T2)	Low	Yes
AN113D (T3)	Low	Yes
AM538C (T4)	High	Yes
AK746F (T5)	High	Yes
BF418F (T6)	High	Yes
AG531J (T7)	High	Yes
BM12B (T8)	High	Yes
AF201G (T9)	High	Yes
V59M (T10)	High	Yes

Among the Treg-treated recipients, Tregs cultured under Protocol D were infused into animal T1. As previously shown, Tregs grown under this approach appeared to be less suppressive than Tregs cultured under the Treg expansion Protocol B or E, therefore, T4, T5, T6 and T7 received Tregs cultured under Protocol B and T2, T3, T8, T9 and T10 received Tregs cultured under Protocol E (Table 8).

Overall, we observed a prolonged chimerism in those Treg-treated animals that received megadose BMT and infusion of Tregs compared to those receiving low (IC) BM dose that also received Tregs (Figure 29 and 30). Control animals without Tregs lost the chimerism early post-BMT after the discontinuation of rapamycin regardless of receiving high-dose BMT (Figure 32). GVHD was observed in a total of four recipients, two that received megadose BMT and Tregs cultured under protocol E (with CD40L-sBc) and two controls (Figure 31 and 32).

Figure 26: Donor:recipient MHC I and II alleles





A. Donor:recipient MHC I and II alleles from control animals (no Tregs were infused). **B.** Donor:recipient MHC I and II alleles from Treg-treated animals. Megadose BM represents donor bone marrow harvested from iliac crest and vertebral bones; IC BM represents bone marrow collected exclusively from donor iliac crest.

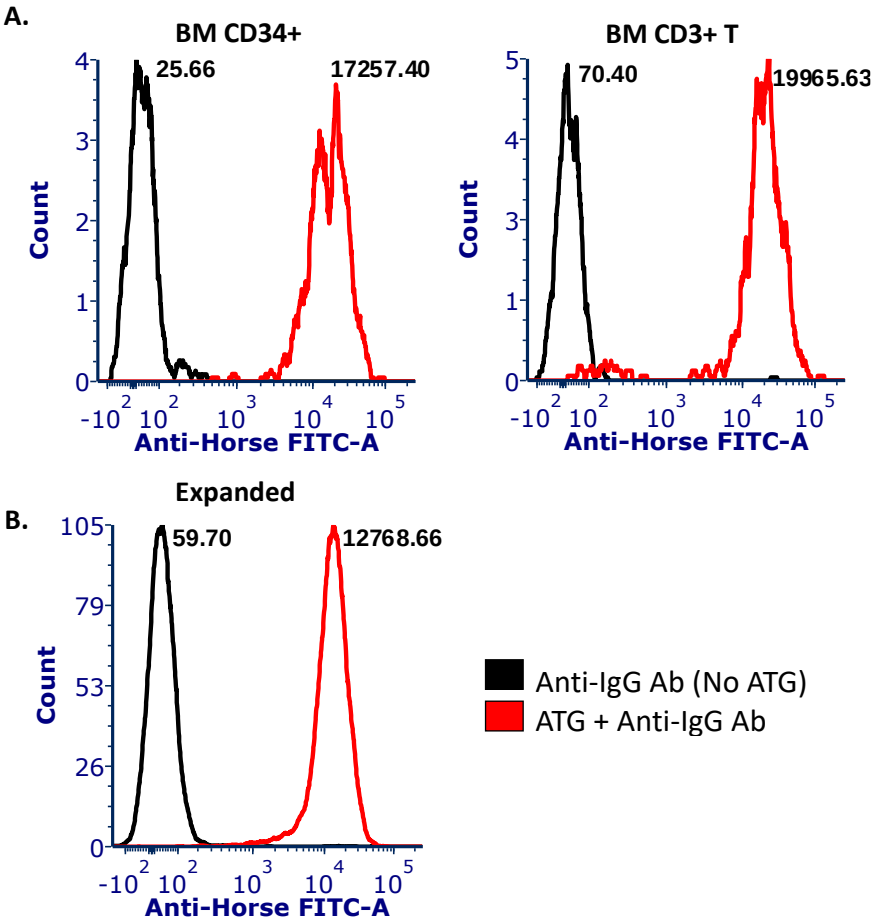
V.2.1. Day of BMT infusion did not affect the chimerism outcomes

ATG, used for T cell depletion during the conditioning regimen, is a polyclonal antibody. As such, we studied if ATG could affect the CD34+ and CD3+ cells within the donor BM graft or the *in vitro* expanded Tregs, therefore, possibly interfering with Treg function and BM engraftment leading to transient (instead

of durable) mixed chimerism. To this end we tested whether delaying the BM infusion would benefit engraftment outcomes.

In addition to CD3+ T cells, ATG was found to bind CD34+ BM cells (Figure 27A), and Tregs (Figure 27B) supporting the hypothesis that ATG could interfere with BM engraftment.

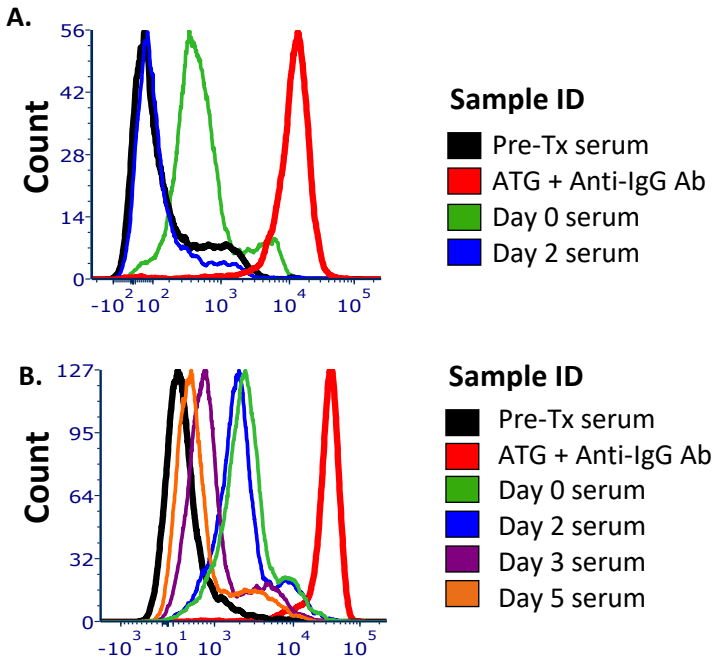
Figure 27. ATGAM binding activity to CD34+ and CD3+ T cells from BM and *in vitro* cultured Tregs



Mean fluorescence intensity (MFI) of the ATG binding activity to **A)** CD34+ and CD3+ cells from the donor BM graft and **B)** recipient *in vitro* expanded Tregs.

We decided to delay the infusion of BM cells to prevent ATG from interfering with engraftment. In order to decide the timeframe of the BM infusion, we assessed for the presence of ATG at different time points post-BMT (which could bind to hematopoietic cells) in the serum in animals that had received the BM on Day 0 (Figure 28). CD34+ cell-binding was not observed at the 48-hour time point (Figure 28A). We also assessed the levels of ATG in serum from animals that received the BM inoculum on Day 2 (Figure 28B). To our surprise, they had ATG levels up to Day 5 (i.e. 2-3 days post-BM). Though the intention was to prevent binding of the ATG when the BM was given on Day 2, these results suggest that ATG remains at high levels in the serum and only after the infusion of BM (i.e. by sequestering ATG) that leads to its disappearance from the circulation.

Figure 28. Assessment of the clearance of ATG in the serum of BMT recipients



Mean fluorescence intensity (MFI) of the detection of ATG in serum at different time points in a recipient that received BMT **A)** on Day 0 or **B)** on Day 2 of the protocol.

Despite these efforts, we did not observe a prolonged chimerism in those animals that received BMT on Day 2 as T1 that received BMT on Day 0 had the longest multilineage chimerism from all the recipients that did not develop GVHD. In conclusion, the time point at which BMT and the first Treg infusion were administered did not affect the chimerism outcomes.

V.2.2. Chimerism duration in bone marrow transplant recipients

V.2.2.1. Low-dose BMT recipients with infusion of Tregs

First, three Treg-treated animals received a low-dose BM from donor iliac crest (T1 through T3). We started with the lower BM dose to study if such dose (which could be easily obtained in the clinic) was sufficient to induce durable chimerism in our tolerance induction protocol with rapamycin.

The CD34+ cell dose infused in the BM graft ranged between 0.97x10^6 to 2x10^6 CD34+ cells/kg with an average of 1.4x10^6 CD34+ cells/kg. The T cell dose infused ranged between 19.1x10^6 to 36.6x10^6 T cells/kg. On average, 30x10^6 T cells/kg were infused (Table 8 and 11). Recipient T1 received Tregs cultured under Protocol D and recipient T2 and T3 received Tregs cultured under Protocol E (Table 8).

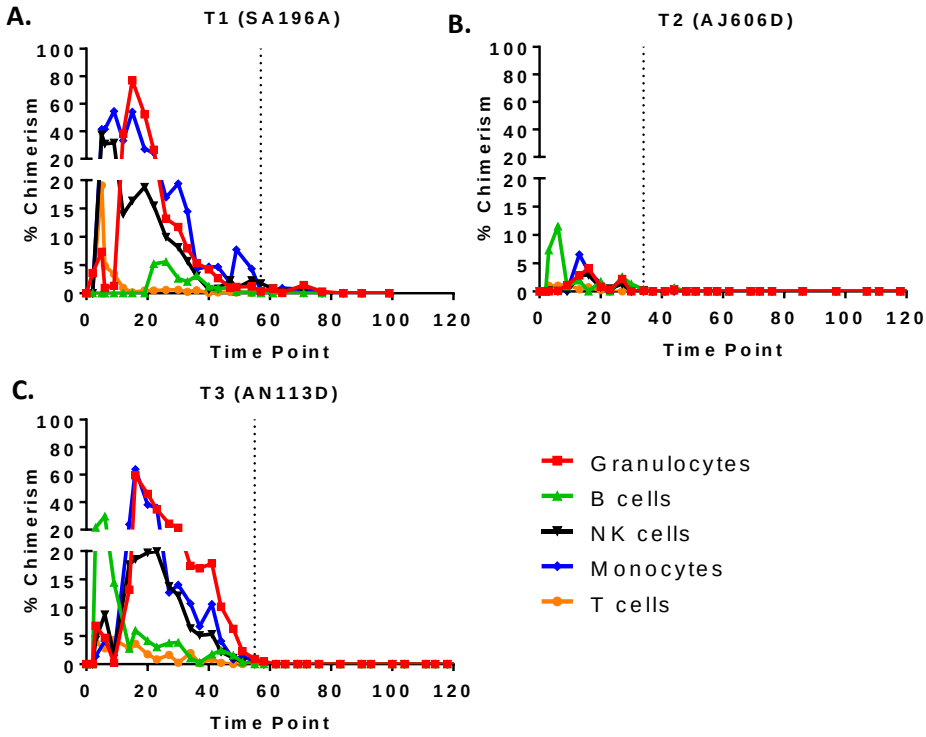
Outcomes showed transient multilineage chimerism for up to 57 days (Figure 29A), followed by 55 and 34 days (Figure 29C and 29B, respectively) that included T cell chimerism.

The first recipient, T1, received 300 cGy of TBI based on previous studies[76-78,205]. However, irradiation was modified to avoid the severe leukopenia, thrombocytopenia and anemia that ensued in such animals (which also required blood transfusions). The next recipients in this study received a total TBI dose of

250 cGy (instead of 300 cGy). Modifying this dose aligned the pancytopenia and recovery to responses from previous studies[76-78] (Table 8).

In summary, low-dose BM infusions did not lead to high levels of chimerism. This could have been (in part) due to the remaining level of host immunity leading to graft loss.

Figure 29. Chimerism from Treg-treated recipients that received donor iliac crest BM



Percentage of donor chimerism for **A)** T1, **B)** T2, and **C)** T3.

V.2.2.2. High-dose BMT recipients with infusion of Tregs

Based on the results from low-dose BMT (T1 through T3), we hypothesized that by giving a higher BM dose (megadose), and therefore a higher CD34+ and T cell dose (T cells favor engraftment[227]), we would achieve prolonged chimerism.

Animals T4 through T7 received a megadose BMT by harvesting the donor BM from iliac crest aspirates and vertebral bodies (Table 8). In addition, the Tregs

infused into this group of animals were cultured under Protocol B. Animals in this group received on average a CD34+ cell dose of $12.5x10^6$ cells/kg that ranged between $7.3x10^6$ to 19.5x10^6 cells/kg (9x the CD34 dose given to animals T1-T3). In addition, the CD3+ cell dose ranged between $39.6x10^6$ to 58.8x10^6 cells/kg with an average of $49.45x10^6$ cells/kg (Table 8 and 11) (1.6x from animals T1-T3).

In addition, the total Treg dose ranged between $32.1x10^6$ cells/kg for the animal that received the lowest dose to $58x10^6$ cells/kg for the highest dose, receiving on average $44.8x10^6$ Tregs/kg.

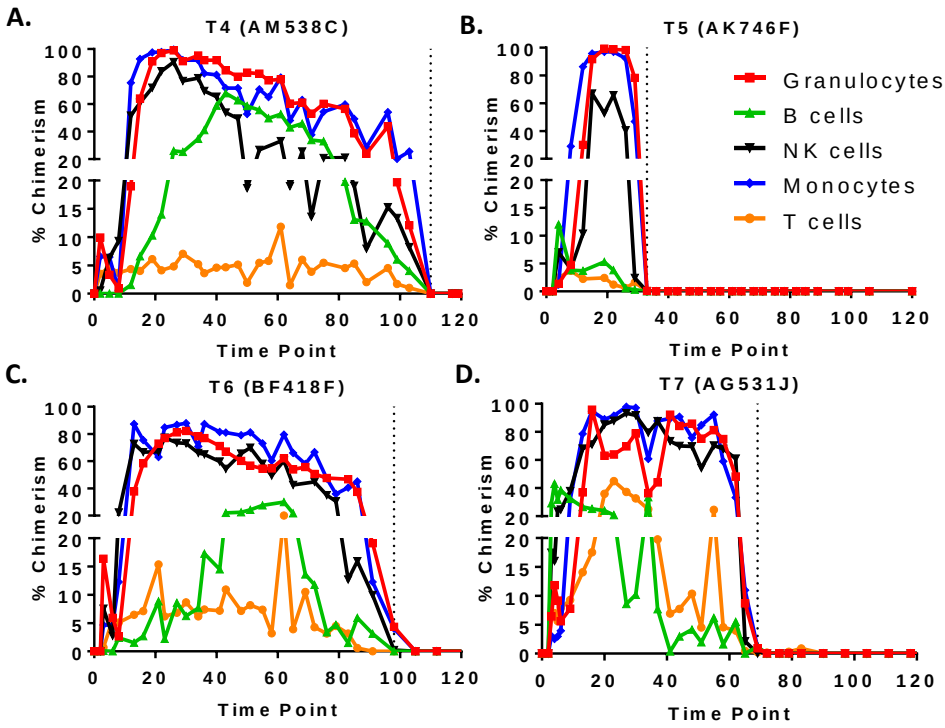
Overall, recipients that received megadose donor BM with infusion of Tregs cultured under Protocol B achieved a higher level with a more prolonged chimerism (Figure 30) when compared to the IC BMT Treg-treated recipients (Figure 29). T cell chimerism was present in all the recipients from this group at higher levels compared to the IC BMT recipients. T4 achieved the longest multilineage chimerism for up to 110 days (Figure 30A), followed by recipient T6, which chimerism became undetectable on Day 98 (Figure 30C) and T7, whose macrochimerism lasted for up to 69 days (Figure 30D). In contrast, recipient T5 lost the chimerism early post-transplant (Figure 30B), likely as a result of an unexpected early CMV reactivation, with peak titers >40,000 copies/mL.

Due to the significant improvement that we observed in the duration and levels of chimerism with the increased CD34 and T cell doses without the development of GVHD, we hypothesized that increasing the number and potency of Tregs could further improve the chimerism outcomes. We tested whether the infusion of Tregs cultured under Protocol E (more potent immunosuppressors) would improve the duration of chimerism. Under this approach, three recipients

received megadose BMT with infusion of Protocol-E Tregs (T8 through T10) (Figure 31).

On average, these recipients received $9x10^6$ CD34+ cells ranging from $7.1x10^6$ to $12x10^6$ CD34+ cells/kg (slightly lower than T4-T7). In addition, $70.7x10^6$ T cells/kg were infused on average (ranging from $37.2x10^6$ T cells/kg to $105x10^6$ T cells/kg) (1.4x compared to T4-T7 and 2.4x compared to T1-T3). An average of $46.1x10^6$ Tregs were infused ranging from $37.5x10^6$ to $59.2x10^6$ Tregs/kg (Table 8) which were comparable to T4-T7. In essence, T8-T10 received similar Treg doses, lower number of CD34+ cells and higher T cell doses when compared to T4-T7 (Table 11).

Figure 30. Chimerism from Treg-treated recipients that received donor megadose BM



Percentage of donor chimerism for **A)** T4, **B)** T5, **C)** T6, and **D)** T7.

Table 11. CD34 and T cell doses infused into Treg-treated recipients

Regimen	Average CD34+ cell dose ($x10^6$/kg)	CD34+ cell range ($x10^6$/kg)	Average CD3+ cell dose ($x10^6$/kg)	CD3+ cell range ($x10^6$/kg)
Lower BM	1.4	0.97 - 2	30	19.1 - 36.6
Megadose BM and protocol-B Tregs	12.5	7.3 - 19.5	49.45	39.6 - 58.8
Megadose BM and protocol-E Tregs	9	7.1 - 12	70.7	37.2 - 105

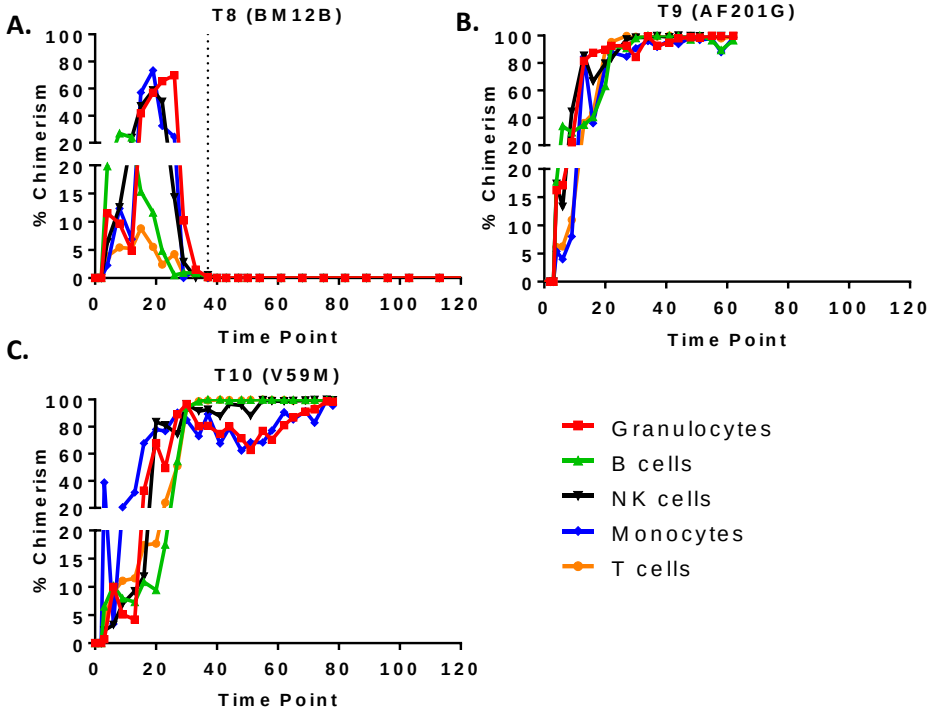
T8 is the first animal that received Tregs expanded with CD40L-stimulated B cells (CD40L-sBc). To preserve the polyclonal nature of the Tregs, we used CD40L-sBc from multiple animals in our colony, including the BMT donor, to provide a broader antigen stimulation repertoire during Treg expansion. The first recipient in this group, T8, received two additional changes. Analysis of the serum of previous recipients demonstrated that ATG was present in circulation until day 1-3 post-BMT (Figure 28). To this end, we decided to split the BM infusion, giving two-thirds of the total BM on Day 2 and the remaining (one-third) BM on Day 4 to avoid potential ATG binding to CD34+ cells and Tregs.

It has been shown that IL-6 is indispensable for the differentiation of naïve CD4+ T cells into Th17 cells[228] in addition to promote the proliferation of CD8+ T cells[229]. In addition, IL-6 inhibits the differentiation of TGF-β induced Tregs[228]. We administered six weekly infusions of anti-interleukin 6 (IL-6) receptor antibody starting on Day 30 in an attempt to limit late immune activation via the Th17 pathway.

This animal developed multilineage donor chimerism for 37 days (Figure 31A). In this case, unlike any other recipient that rejected the donor BM, chimerism disappearance was not associated with the inversion of the CD4:CD8 ratio.

The following two recipients (T9 and T10) received megadose BM with co-infusion of Protocol-E Tregs. BM was infused in a single dose and anti-IL6 receptor antibody was not administered as we did not observe an improvement in recipient T8 that received these modifications. T9 and T10 developed full donor chimerism (Figure 31B and 31C) that translated into GVHD. In these recipients, the T cell doses infused in the BM graft were 70 and 105 millions/kg for T9 and T10, respectively, which were the highest doses among the Treg-treated recipients. In summary, full donor chimerism was achieved with this protocol.

Figure 31. Chimerism from Treg-treated recipients that received donor megadose BM



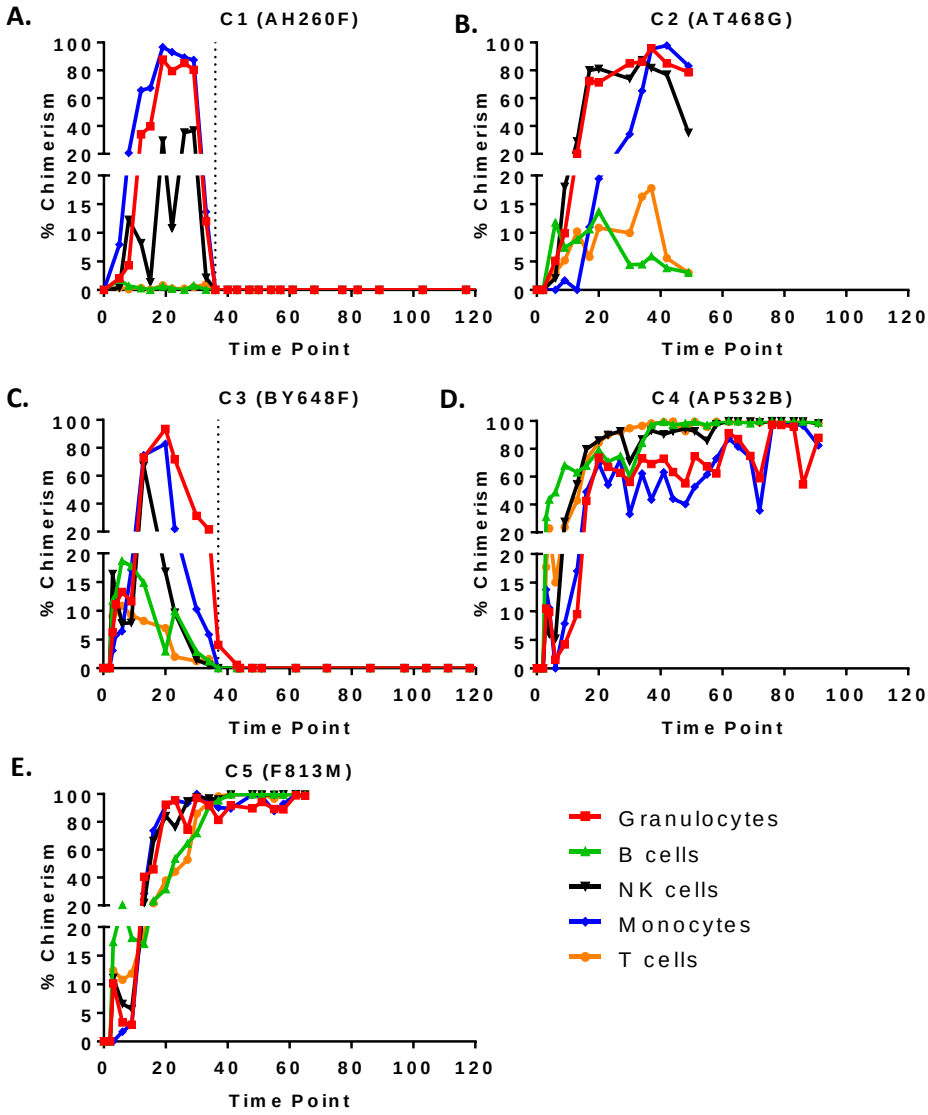
Percentage of donor chimerism for **A)** T8, **B)** T9, and **C)** T10.

V.2.2.3. Control BMT recipients (without Treg infusions)

Two of the animals that received BMT without infusion of *in vitro* cultured Tregs (C1-C5) showed an early loss of chimerism post-transplant (Figure 32A and 27C) and two recipients developed GVHD (Figure 32D and 32E). Recipient C2 developed renal failure that resulted in an early experimental endpoint. Although mixed chimerism could not be monitored passed 50 days post-BMT, it started to decrease at the time of euthanasia (Figure 32B). We hypothesize that C2 would have rejected the BM graft as C1 and C3, although these results cannot be confirmed.

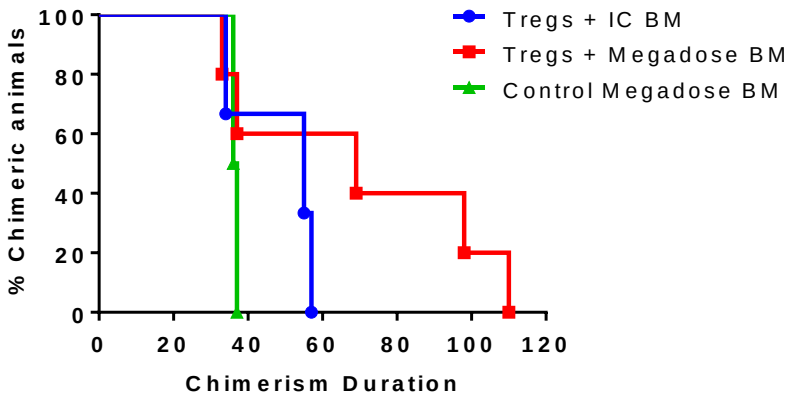
If the duration of chimerism is compared across the different groups, a prolonged chimerism was observed in those animals that received megadose BMT with infusion of *in vitro* expanded Tregs followed by Treg-treated animals that received low-dose BM. In contrast, control recipients (that only received BM without Tregs) had the shortest chimerism duration (Figure 33). This indicates that the chimerism can be prolonged with the administration of Tregs and further enhanced with high BM doses. Animals that developed GVHD received CD34+ and high doses of T cells (comparable to T4-T10) either with Tregs cultured under Protocol E or without Tregs (control recipients). It is interesting to note that animals that received T cell doses >55 millions/kg developed GVHD unless they received Tregs from Protocol B. Animals that received Tregs from Protocol E in conjunction with such high T cell doses were unable to control GVHD hence questioning whether their potency *in vivo* did not align with their *in vitro* results.

Figure 32. Chimerism from control recipients (no Tregs) that received donor megadose BM



Percentage of donor chimerism for **A)** C1, **B)** C2, **C)** C3, **D)** C4, and **E)** C5.

Figure 33. Comparison of the duration of chimerism between Treg-treated recipients and control animals

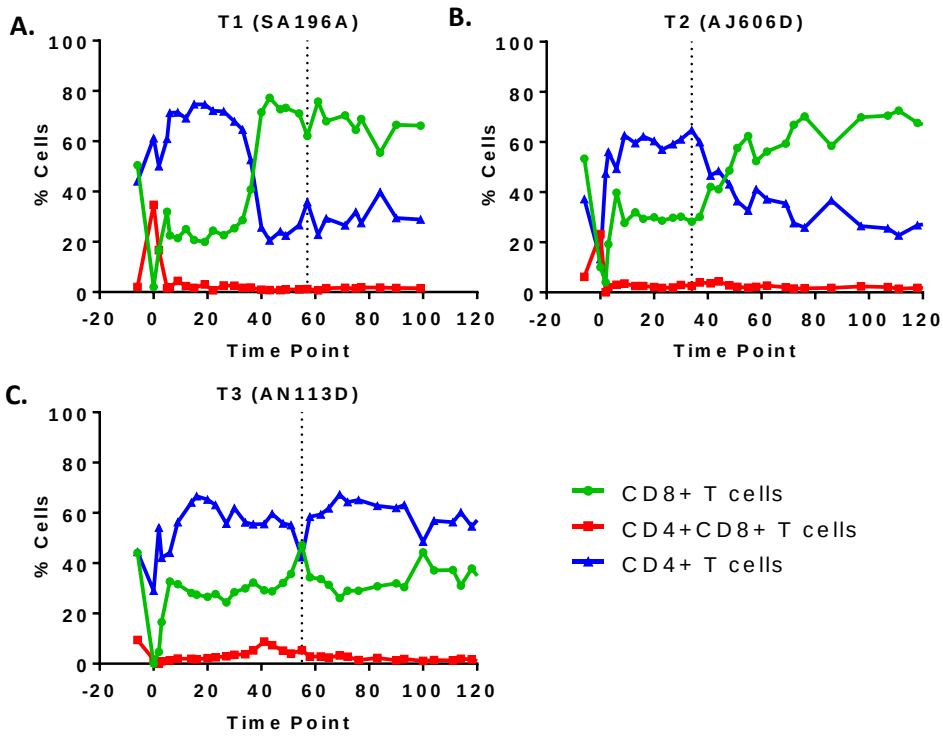


Time point when donor chimerism became undetectable in peripheral blood in recipients that received Tregs and low BM dose (blue line; T1, T2, T3), Tregs and high BM dose (red line; T4, T5, T6, T7, T8) and high BM dose without Tregs (green line; C1, C3). Recipients that were sacrificed while chimeric due to GVHD (T9, T10, C4, C5), or clinical complication (C1) were excluded from this graph as the duration of chimerism could not be assessed.

V.2.3. CD4 and CD8 ratio is predictive of BM graft and chimerism loss

CD4 and CD8 T cell kinetics were monitored in the peripheral blood of the recipients after BMT. Pre-transplant CD4+ T cells were more abundant than the CD8+ T cells (with a 2:1 ratio). The loss of mixed hematopoietic chimerism in blood usually correlated with the inversion in the percentages of CD4+ and CD8+ T cells, with CD8+ T cells becoming more prominent than CD4+ T cells. This was observed in all the groups, independently of the BM dose or the administration of Tregs(Figure 34-37).

Figure 34. CD4 and CD8 percentage from Treg-treated recipients that received iliac crest donor BM

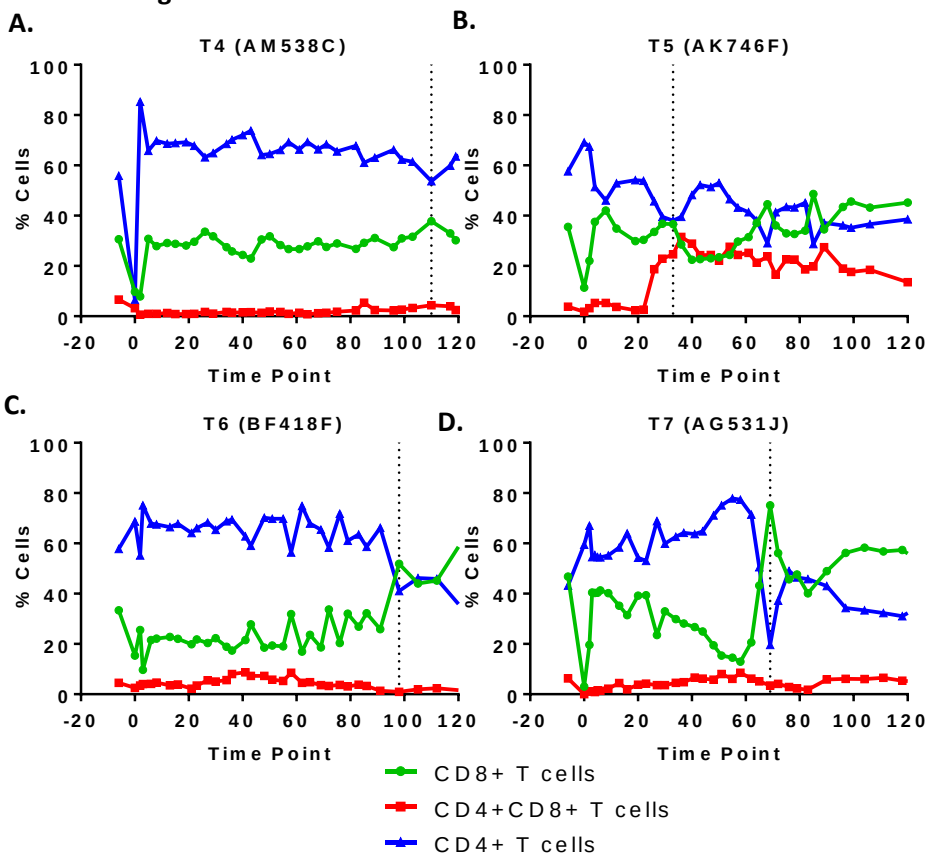


Percentages of T cell populations (CD4+ T cells, blue; CD4+CD8+ T cells, red; CD8+ T cells, green) for **A)** T1, **B)** T2, and **C)** T3. Dotted line represents the time point for the loss of chimerism.

An interesting finding, and unlike what was observed in the previous group, animal T4 (which received megadose BMT with infusion of Tregs cultured under protocol B) maintained a stable CD4:CD8 ratio even when the BM graft was lost on Day 110 (Figure 35A). This animal represents the only recipient in this group without an inversion of the CD4+ and CD8+ T cell percentages at the time of chimerism loss. This provides potential evidence that survival of donor stem cells may not be as robust as expected. Further research will be needed to understand the cause of graft loss if it is not secondary to rejection by host versus graft responses.

T8 is another interesting recipient that did not follow the classical CD4:CD8 inversion at the time of graft loss. This animal was the first recipient that received megadose BMT with Tregs grown under Protocol E (Figure 36A). A potential explanation could be the administration of anti-IL-6 receptor antibody, which first dose was administered on day 30 around the time when the chimerism was lost, as IL-6 has been shown to promote the proliferation of CD8+ cells[229]. Therefore, by blocking the effects of IL-6, the proliferation of CD8+ cells might have been stopped. A total of six doses (10 mg/kg) were administered once a week for six weeks. No changes in chimerism were observed but the

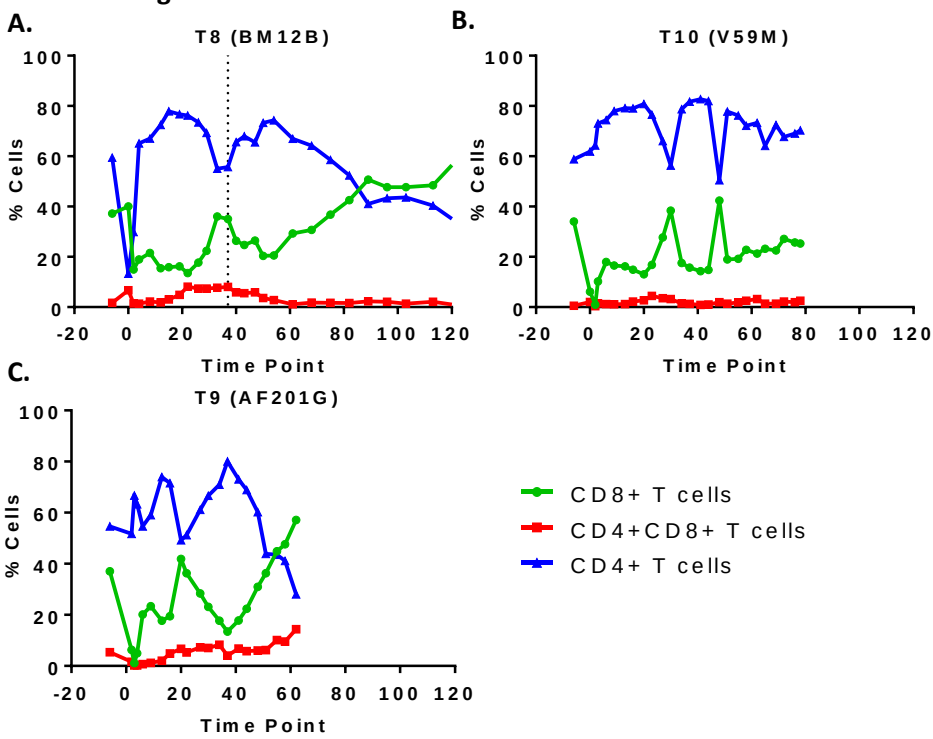
Figure 35. CD4 and CD8 percentage from Treg-treated recipients that received megadose donor BM



Percentages of T cell populations (CD4+ T cells, blue; CD4+CD8+ T cells, red; CD8+ T cells, green) for **A)** T4, **B)** T5, **C)** T6 and **D)** T7. The dotted lines represent the time of loss of chimerism.

CD4:CD8 ratio decreased between day 80 and 90, soon after the anti-IL6 receptor treatment was discontinued.

Figure 36. CD4 and CD8 percent from Treg-treated recipients that received megadose donor BM

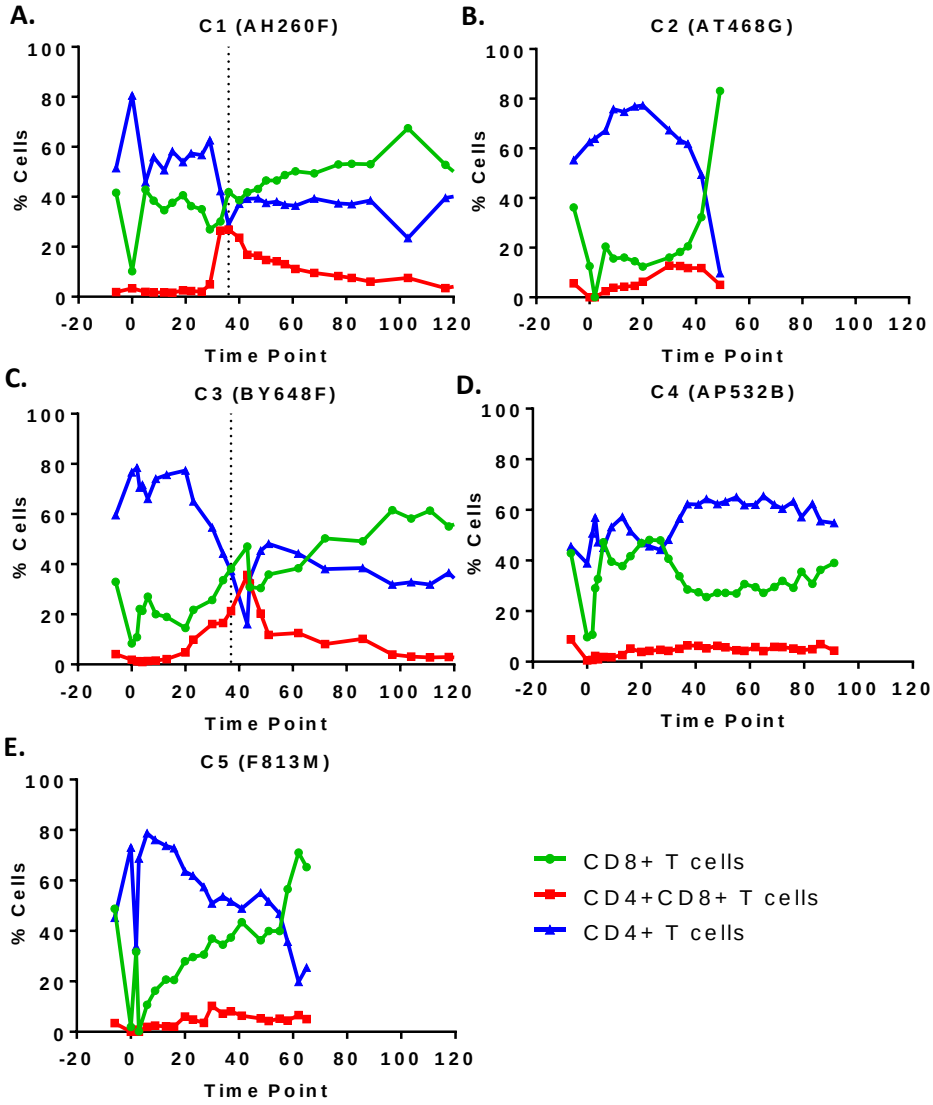


Percentages of T cell populations (CD4+ T cells, blue; CD4+CD8+ T cells, red; CD8+ T cells, green) for **A)** T8, **B)** T9, and **C)** T10. The dotted lines represent the time of loss of chimerism.

The animals that developed GVHD (Treg-treated, Figure 36B and 36C, and controls, Figure 37D and 37E) did not show this correlation, as the CD4:CD8 ratio decreased in all of them but with no clear pattern in time.

The decrease in the CD4:CD8 ratio was also observed in the control recipients C1 and C3 that lost the chimerism early post-transplant (Figure 37A and 37C). The CD4:CD8 ratio decreased in C2 at the time of euthanasia when the chimerism started to decrease (Figure 37B). We hypothesize that recipient C2 was following the same pattern as C1 and C3

Figure 37. CD4 and CD8 percentage of control recipients that received megadose donor BM

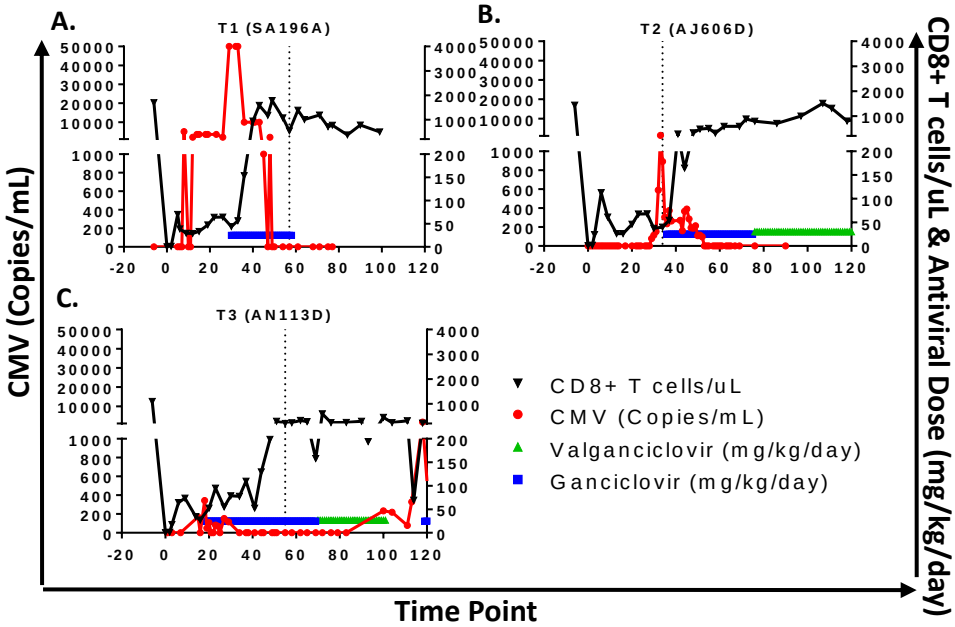


Percentages of T cell populations (CD4+ T cells, blue; CD4+CD8+ T cells, red; CD8+ T cells, green) for **A)** C1, **B)** C2, **C)** C3, **D)** C4 and **E)** C5. The dotted lines represent the time of loss of chimerism.

V.2.4. CMV reactivation interferes with immune reconstitution

Cytomegalovirus (CMV) infection is a serious complication in patients with impaired T cell immunity as observed in BMT patients. Because this is a clinically-relevant side effect of immunosuppression which may have an impact in BM engraftment, we assessed its impact in immune reconstitution.

Figure 38. CMV in Treg-treated recipients receiving IC BMT

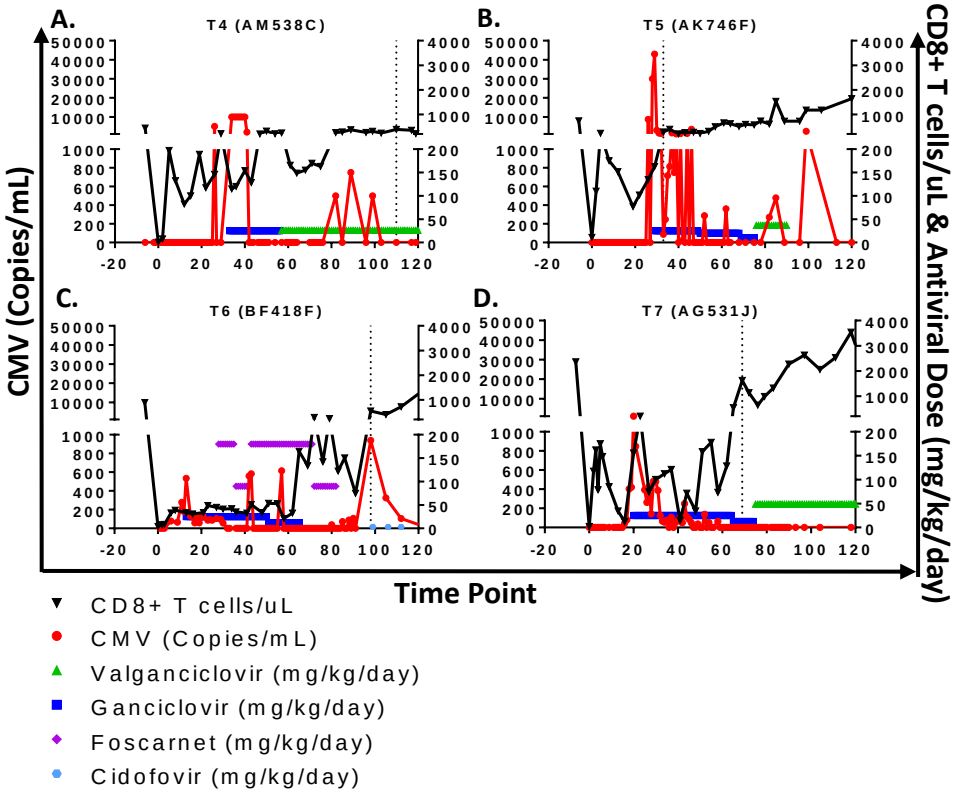


CMV viremia, CD8 counts and antiviral treatment for **A)** T1, **B)** T2, and **C)** T3. CMV viremia (Copies/mL) is plotted on the left axis, and total CD8 counts (Cells/uL) and antiviral dose (mg/kg/day) are plotted on the right axis.

We monitored CMV viremia via quantitative polymerase chain reaction (qPCR). On average, rapamycin-treated recipients reactivated CMV on Day 16 post-BMT and if left untreated, CMV reached high levels that could cause CMV-related immune-activation, graft loss or even death (Figure 38-41).

As observed in Figure 38 through 41, all recipients reactivated CMV post-BMT independently of the administration of Tregs. CMV reactivation correlated with an increase in the absolute counts of CD8+ T cells and was associated with the loss of chimerism, as marked by dotted lines (i.e. Figure 39C). We observed that when CD8 counts were stable over time and maintained under 500 cells/uL, the chimerism tended to be prolonged[230] (Figure 39A).

Figure 39. CMV in Treg-treated recipients receiving megadose BMT

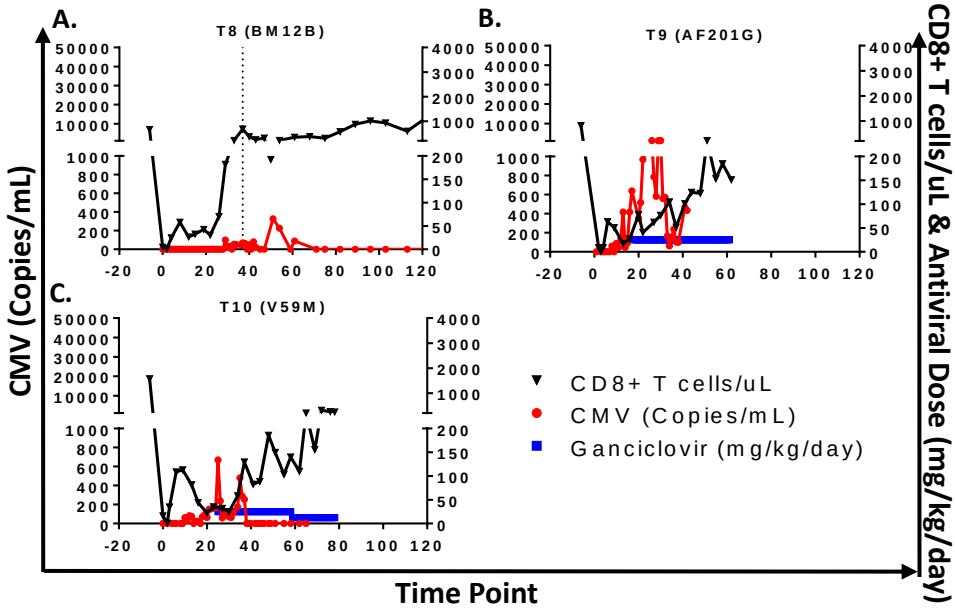


CMV viremia, CD8 counts and antiviral treatment for **A)** T4, **B)** T5, **C)** T6 and **D)** T7. CMV viremia (Copies/mL) is plotted on the left axis, and total CD8 counts (Cells/uL) and antiviral dose (mg/kg/day) are plotted on the right axis.

Upon CMV reactivation, antiviral treatment with ganciclovir was started followed by oral valganciclovir. This treatment effectively controlled CMV viremia and prevented the most commonly observed clinical signs (neurological effects). Treatment with antivirals came with a downside, bone marrow

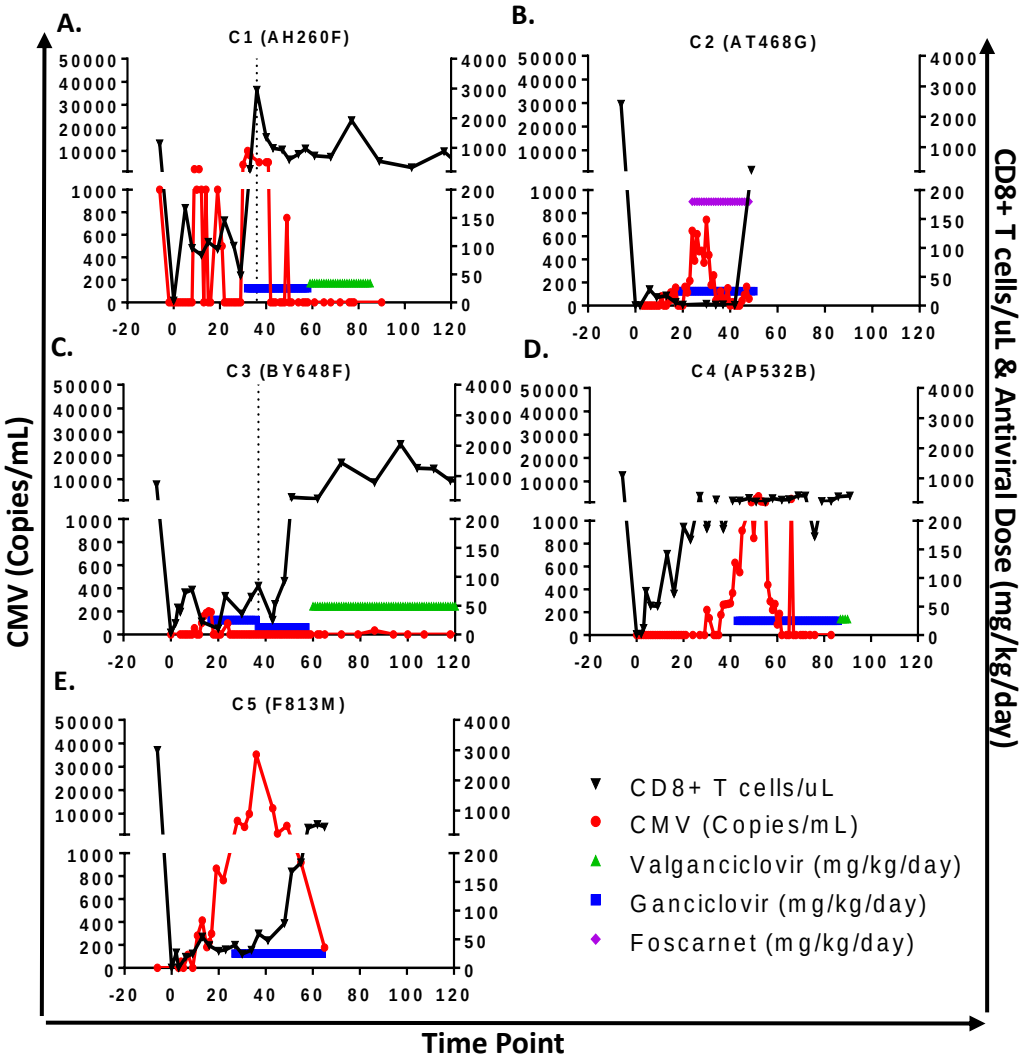
suppression (as been described in Duran-Struuck et al 2017)[205]. Hence, prophylaxis was avoided as it has been known to interfere with BM engraftment. However, despite our efforts at quickly treating CMV upon activation, CMV viremia often escaped our attempts to control it leading to increases of CD8 counts and loss of chimerism (Figures 38-41).

Figure 40. CMV in Treg-treated recipients receiving megadose BMT.



CMV viremia, CD8 counts and antiviral treatment for **A)** T8, **B)** T9 and **C)** T10. CMV viremia (Copies/mL) is plotted on the left axis, and total CD8 counts (Cells/uL) and antiviral dose (mg/kg/day) are plotted on the right axis.

Figure 41. CMV in control recipients receiving megadose BMT

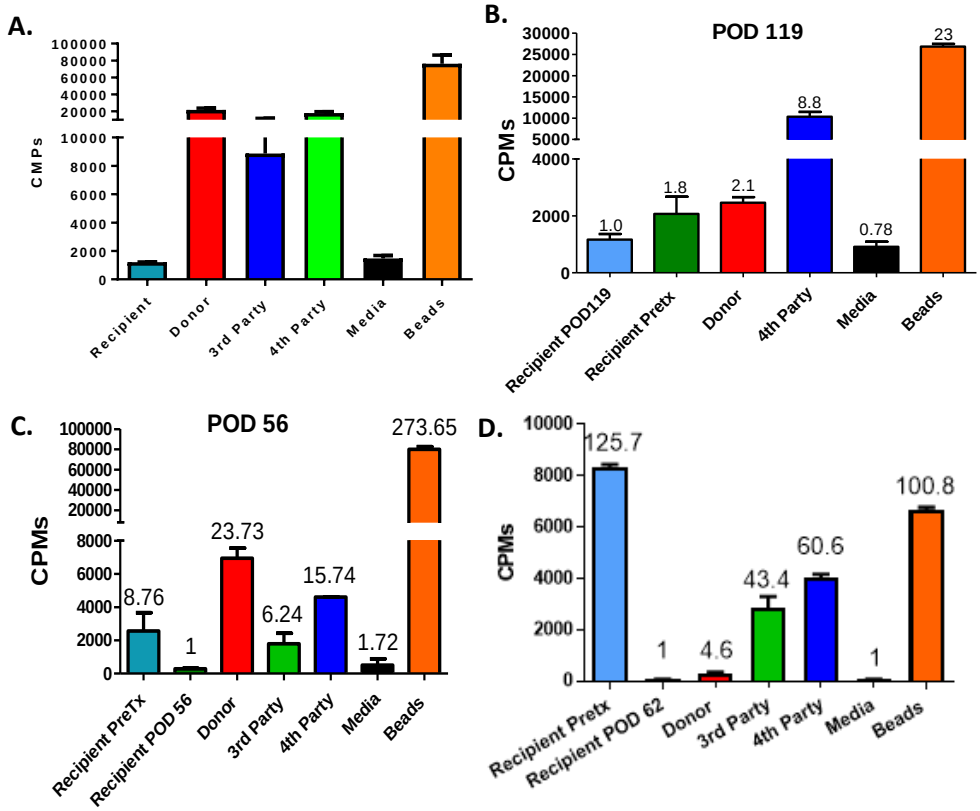


CMV viremia, CD8 counts and antiviral treatment for **A)** C1, **B)** C2, **C)** C3, **D)** C4, and **E)** C5. CMV viremia (Copies/mL) is plotted on the left axis, and total CD8 counts (Cells/uL) and antiviral dose (mg/kg/day) are plotted on the right axis.

V.2.5. *In vitro* donor-specific hyporesponsiveness was associated with prolonged hematopoietic chimerism

The recipients' responsiveness to donor before and after transplant was tested *in vitro* using mixed lymphocyte reactions (MLR) (Figure 42).

Figure 42. Test of recipient responsiveness *in vitro* against different conditions through mixed lymphocyte reaction (MLR)



A. Representative MLR of pre-transplant recipient PBMCs showing *in vitro* donor hyper-responsiveness. **B.** Representative MLR of PBMCs from recipients that showed prolonged chimerism with *in vitro* donor hypo-responsiveness. **C.** Representative MLR of PBMCs from recipients that lost the chimerism early post-BMT with *in vitro* donor hyper-responsiveness. **D.** Representative MLR of PBMCs from recipients that developed full donor chimerism (with GVHD in some cases) with *in vitro* "pre-transplant self" hyper-responsiveness.

As expected, all recipients showed pre-transplant *in vitro* anti-donor responsiveness (Figure 42A, representative figure). After BMT, recipients that

showed prolonged donor chimerism such as T4, T6, and T7 for 110, 98 and 69 days respectively, showed hyporesponsiveness against the BM donor when compared to recipient's responses against a third-party stimulator (Table 12 and Figure 42B, representative figure).

Table 12. Summary of MLR responses

Animal ID	**MLR response**
AH260F (C1)	Anti-donor
AT468G (C2)	NA
BY648F (C3)	Anti-donor
AP532B (C4)	Anti-pretransplant recipient
F813M (C5)	Anti-pretransplant recipient
SA196A (T1)	Anti-donor
AJ606D (T2)	Anti-donor
AN113D (T3)	Anti-donor
AM538C (T4)	Donor hyporesponsive
AK746F (T5)	Anti-donor
BF418F (T6)	Donor hyporesponsive
AG531J (T7)	Donor hyporesponsive
BM12B (T8)	Anti-donor
AF201G (T9)	Anti-pretransplant recipient
V59M (T10)	Anti-pretransplant recipient

In contrast, recipients that lost donor chimerism early post-transplant after discontinuation of immunosuppression, showed a stronger anti-donor response compared to a third-party control (Table 12 and Figure 42C, representative figure). Among these animals, controls C1 and C3 lost the chimerism on Day 36 and 37, respectively, which was expected as these recipients did not receive infusions of Tregs.

The three Treg-treated recipients that received CD34+ cells from the iliac crest had transient chimerism that disappeared early, with T1 exhibiting the longest duration of 57 days. Similar to controls that had lost chimerism, these Treg recipients that received iliac crest CD34+ cells also had higher anti-donor responses (Figure 42C, representative figure).

CMV reactivation leading to loss of chimerism was observed in several animals. T5 is a clear example of an animal where chimerism loss and return of anti-donor responses were associated with CMV reactivation (documented on Day 56). Such findings support the hypothesis of BM host-anti donor responses being responsible for graft rejection (Figure 42C, representative figure).

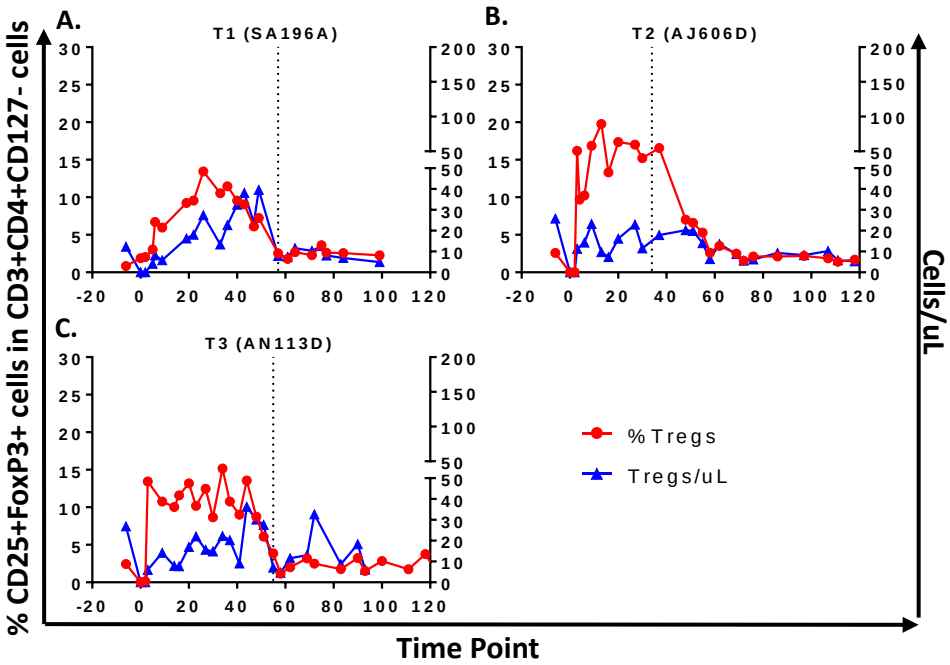
Acute GVHD is a major complication after BMT that occurs in 35 to 50% of patients[231]. Unfortunately, we also observed GVHD in four recipients (T9, T10, C4, and C5). In all four cases, the MLR responses of the full-donor chimeras showed potent proliferation against pre-transplant autologous stimulators while they were hypo-responsive to donor PBMCs (Figure 42D, representative figure).

In conclusion, *in vitro* findings correlate with the duration and course of chimerism. In those animals that donor BM was rejected early post-transplant, higher *in vitro* responses against the donor were observed. In contrast, prolonged chimerism lead to donor unresponsiveness. Lastly, those animals that converted to full (100%) donor chimerism demonstrated proliferation against the pre-transplant "self" which in the four recipients, correlated with the development of GVHD. In these full-donor recipients, anti-donor *in vitro* responses were significantly reduced.

V.2.6. Kinetics of CD3+CD4+FoxP3+ cells in the circulation following BMT and Treg infusions

We studied the kinetics of the peripheral Tregs based on the percentage of CD25+FoxP3+ cells within CD3+CD4+CD127- cells and the absolute Treg counts. Overall, we observed higher Treg levels while the recipients were chimeric. The animals that received iliac crest BM appeared to have higher levels of Tregs compared to the baseline (pre-transplant) during the first 50 days post-BMT that decreased around the time when the chimerism was lost (Figure 43A and 43C). T2, that lost the chimerism before the Treg levels returned to baseline (Figure 43B) received a total Treg dose of 75x10^6 Tregs/kg, which was higher than the average infused to the rest of the recipients, which could have prolonged the high Treg level despite the lack of chimerism.

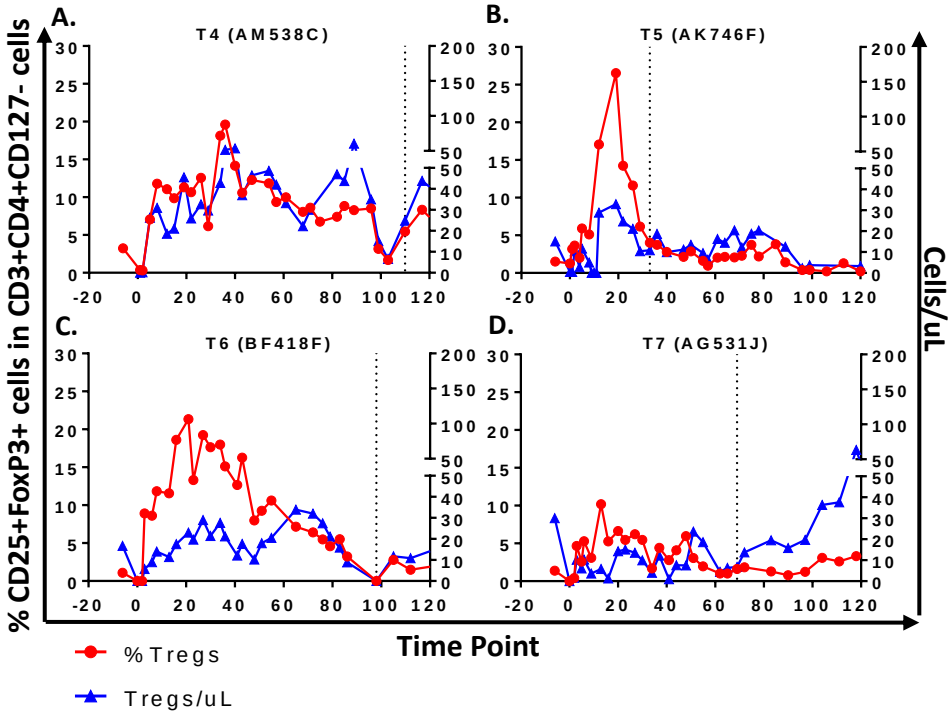
Figure 43. Peripheral Tregs in Treg-treated recipients receiving IC BMT



Percentage (red) and absolute counts (blue) of total peripheral Tregs (donor and recipient) of **A)** T1, **B)** T2 and **C)** T3. The dotted lines represent the time of chimerism loss.

Same correlation was observed in the next group of animals, that received megadose BM and Tregs cultured under protocol B with donor PBMC and aAPC stimulation. In this case, Tregs reached high levels during longer time compared to the previous group. Multilineage mixed chimerism was detected in the recipient T4 for 110 days. Around Day 100, the Treg level started to decrease followed by the chimerism loss (Figure 44A). Similar kinetics were observed in recipient T5, T6 and T7, which recipients lost the chimerism on Day 47 (Figure 44B), 98 (Figure 44C) and 69 (Figure 44D), respectively. Therefore, the chimerism loss was associated with a decrease of peripheral Treg levels. In general, the percentage of Tregs in the CD4+ population is below 5%, and during the periods of chimerism, all animals had a Treg percentage of ~10 or above.

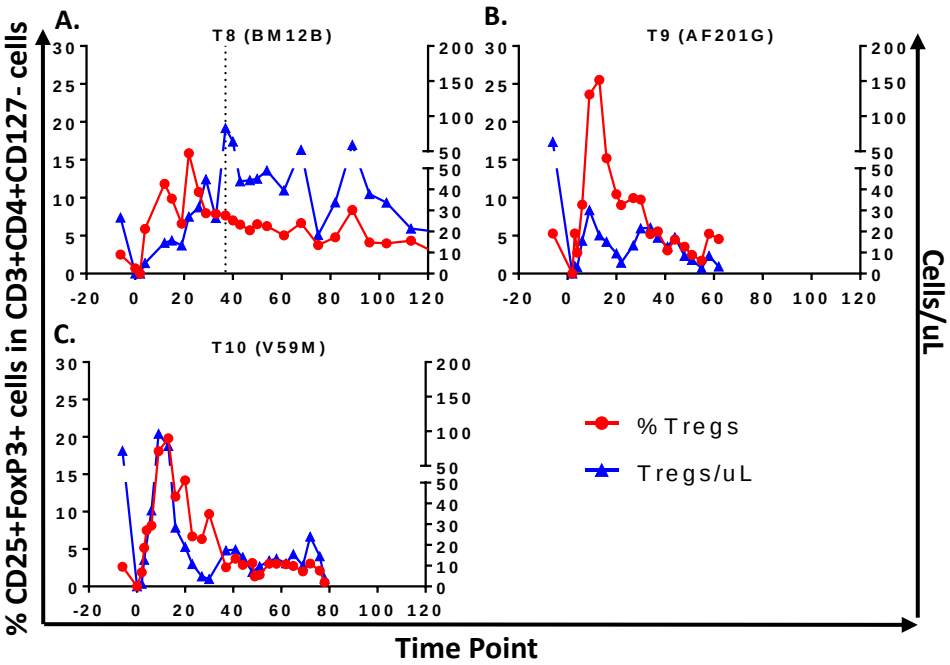
Figure 44. Peripheral Tregs in Treg-treated recipients receiving megadose BMT



Percentage (red) and absolute counts (blue) of total peripheral Tregs (donor and recipient) of **A)** T4, **B)** T5, **C)** T6 and **D)** T7. The dotted lines represent the time of chimerism loss.

Among the recipients that received megadose BMT with Tregs cultured under protocol E with CD40L-sBc stimulation, there were two animals that developed GVHD. Around the time of the appearance of the disease (Day 40), a decrease in the level of Tregs was observed for both recipients T9 (Figure 45B), and T10 (Figure 45C). In contrast to these findings, the third recipient in this group (T8) followed a similar patter as the previous groups, where the Treg level dropped at the time when the chimerism disappeared (Figure 45A).

Figure 45. Peripheral Tregs in Treg-treated recipients receiving megadose BMT

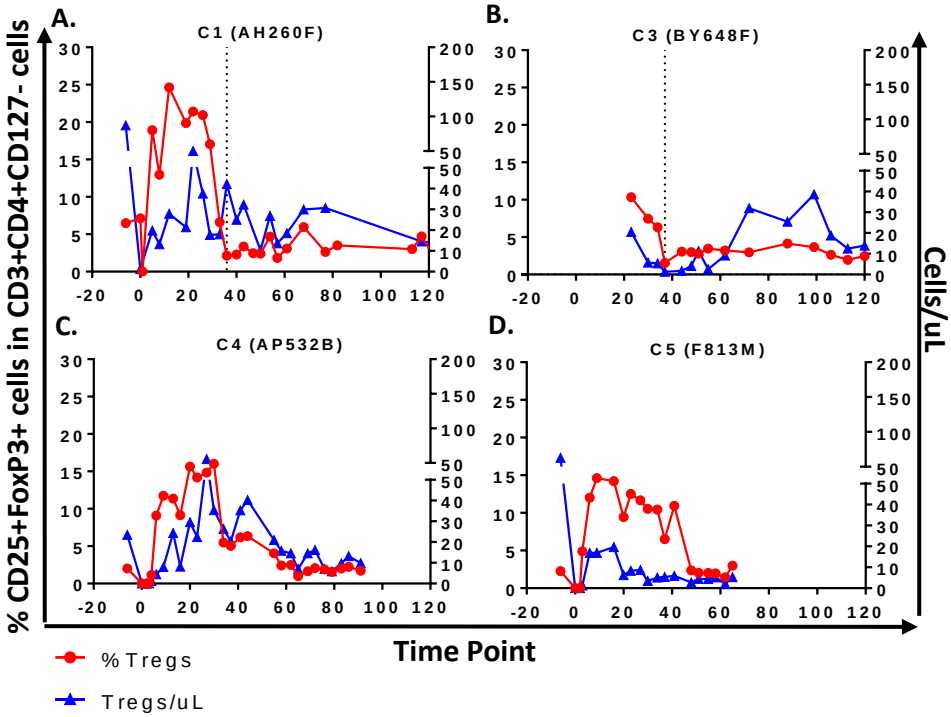


Percentage (red) and absolute counts (blue) of total peripheral Tregs (donor and recipient) of **A)** T8, **B)** T9 and **C)** T10. The dotted lines represent the time of chimerism loss.

Similar pattern was observed in the control recipients that lost the chimerism early post-BMT, C1 and C3, where the Treg levels decreased around the same time as the chimerism loss occurred (Figure 46A and 46B). In the contrary, the two control recipients that developed GVHD presented low Treg levels at the

time of the disease, in accordance of what was observed in the GVHD Treg-treated animals T9 and 10 (Figure 46C and 46D). The data for control C2 is not available.

Figure 46. Peripheral Tregs in control recipients receiving megadose BMT



Percentage (red) and absolute counts (blue) of total peripheral Tregs (donor and recipient) of **A)** C1, **B)** C3, **C)** C4 and **D)** C5. The dotted lines represent the time of chimerism loss.

In summary uncontrolled graft-versus-host or host-versus-graft responses leading to GVHD or graft loss were highly associated with a decrease of Treg levels in the peripheral blood.

VI. DISCUSSION

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VI.1. *In vitro* expansion of MCM Tregs

Pre-clinical and clinical studies support the safe and efficacious use of Tregs for the treatment of autoimmune diseases, prevention of rejection and GVHD in allotransplantation.[201,203,232-234] Their low number in the circulation continues to be a major limitation for their widespread therapeutic use. Development of efficient and consistent Treg expansion protocols has been a major focus of many groups.[204,226,235,236] Here, we have provided our experience with MCM Tregs and documented five protocols aimed to use *ex-vivo* for the expansion of Tregs which can be potentially translated to the clinic.

MCM Tregs showed a direct correlation between FoxP3 and CD25 expression in CD4+ T cells, comprising 2.4±0.4% of the CD4+ T cells.[149] This parallels what has been observed with human Tregs. However, some differences were also identified. In MCM, the pattern in expression of CD127 and FoxP3 did not mirror to what has been demonstrated in published human studies.[157] MCM Tregs that were CD25hi had an equal expression of CD127, which did not reliably correlate with what has been documented in humans. Future studies will need to address the IL-7R expression and its biology in MCMs. CD45RA has been utilized to describe naïve versus memory subsets of Tregs in humans. In our hands, CD45RA and FoxP3 expression in MCM also did not correlate with what has been documented in humans whereby CD45RA is a robust marker of natural Tregs and highly desired for expansion. In the contrary, in our *in vitro* expanded MCM Tregs, CD45RA was not a reliable marker of long-lived Tregs. We observed that the composition of Tregs long term after culture (i.e. the survival of Tregs that were 1% CD25hi sorted in Day 0) were equally distributed two months post-culture ($\pm$ 50% of Tregs were CD45RA+ and 50% were CD45RA-). Though this is suggestive that survival of Tregs in NHPs is not dependent on CD45RA expression, we never assessed the long-term survival of CD45RA+ or CD45RA-

populations sorted on Day 0. Further functional studies will be required to better understand the relevance of such differences.

CD8+ T cells readily contaminated our cultures. This has also been observed in cultured human Tregs.[221] Though the inclusion of the CD8 marker in our sorting strategy minimized the contamination of such cells, it did however not eliminate them. Attempts to pre-select CD4+ cells through MACS prior to sorting prevented CD8 contamination, however these Tregs proved to be poor suppressors, suggesting a potential loss (or damage) of highly suppressive natural Tregs. We controlled CD8 growth by re-sorting (in selected cultures) early (by day 14) by FACS. This approach removed CD8+ T cells however, Treg cultures needed to be prolonged due to significant cell loss after re-sorting.[205] Bead selection may be favored in the clinic due to the established Miltenyi's cliniMACS. However, an alternative approach (and which could also be less costly) would be to use clinical-grade irradiated aAPCs. There is precedence for the use of aAPCs clinically such as K562 cells in vaccine studies (such as the GVAX trial).[237] Based on our studies, if the desire remains to use beads, we recommend that the quality of human Tregs expanded with such approach be properly vetted prior to their use in the clinic.

We studied five expansion Treg approaches. We were able to expand this population from all five protocols, however we found significant differences between them. Protocol A and B used a combination of donor PBMCs with aAPCs while protocol E used a combination of stimulated B cells (from several donors with disparate MHC). Protocol E obtained the most potent Tregs when tested *in vitro*. The use of aAPCs alone did not generate highly suppressive MCM Tregs (protocol C). Since protocol A was limited by the dearth of cells harvested, it was not pursued for *in vivo* studies. In contrast, protocol B and E allowed for the expansion of meaningful number of Tregs with robust suppression. Based

on these findings, Tregs cultured from these two protocols were selected for infusion to monkeys.

Expansion of MCM B cells for the expansion of Tregs was borrowed from human clinical trials[226] with the rationale that it would be a well-received approach for translation. In order to generate polyclonal Tregs, B cells from MHC-disparate donors were used for Protocol E. This approach is thought to be more attractive than Protocol B as it does not use rodent-based aAPCs.

We aimed to generate B cells with high expression of MHC-II, CD80 and CD40 as these have been shown to expand Tregs well.[226] Three different approaches were studied. Protocol 1 (based on clinical studies[226]) had around 50% expression of CD40 with high T cell counts in some cases on Day 10 of culture. Unfortunately, the total cell numbers obtained were not sufficient for subsequent Treg expansion. When the culture time was increased to 14 days and with additional cyclosporine to mitigate the expansion of T cells as in Protocol 2 and 3, higher numbers of B cells were achieved. Culturing the B cells with CD40L-K562 cells for 7 days instead of 4 days (B cell Protocol 3) before their harvest forced a higher expression of CD40 on B cells, with the additional benefit of obtaining higher B cell counts with less contaminating T cells.

Studies using extended human Treg cultures have shown the loss of FoxP3 expression and suppressive function. MCM Tregs grown under Protocol B maintained potent suppression and high FoxP3 levels after prolonged culture periods (up to 2 months). However, protocols that used aAPCs throughout the 26-day culture period lost FoxP3. These Tregs may have been too aggressively stimulated or overtaken by contaminating conventional T cells. A possible explanation is a potential stronger stimulation of Tregs provided by aAPCs compared to PBMCs or B cells (unmodified APCs).

Because we aimed to develop an "off-the-shelf" protocol to generate Tregs potent enough to prevent organ rejection to any donor, we tested the specificity characteristics of our polyclonal Tregs that were cultured under either Protocol B (with aAPCs and donor PBMCs) or Protocol E (cultured with a combination of MHC-mismatched B cells from multiple donors). Tregs cultured under Protocol B are suspected to be more polyclonal than Tregs expanded under Protocol E as Protocol-B Tregs were able to suppress equally anti-donor and anti-third-party responses. In contrast, Tregs cultured with Protocol E showed a more potent suppression of T cells stimulated by APCs whose MHC had been previously seen by the expanding Tregs. These outcomes could be explained because aAPCs may have stimulated all Tregs present in culture while this was not the case with Protocol E. Therefore, despite that Protocol E was able to generate potent *in vitro* Tregs, these might have been limited to exposed MHC antigens. Therefore, we argue that if Protocol E is desired to generate polyclonal Tregs, a wider array of B cells expressing multiple MHCs might be necessary to stimulate Tregs. To better understand the polyclonality of Tregs, TCR repertoire studies will be needed from our NHP Tregs.

Despite some contradictory reports,[223] we demonstrated that Tregs can improve their suppressive function and numbers after cryopreservation if allowed to expand for a minimum of 3 days post-thaw. This may be valuable in situations where additional Tregs are required from a frozen stock.

Our studies showed a correlation between FoxP3 expression, suppressive capacity and demethylation of the TSDR. Long culture periods (in general) decreased the demethylation percentage in correlation with FoxP3 expression and the suppressive activity of the Tregs. These observations raise questions about the utility of culturing the lines longer for the purpose of achieving higher

cell yields. Hence, the methylation status of MCM Tregs paralleled what has been observed in humans.

Despite the beneficial outcomes that Tregs have shown in transplant tolerance protocols in different reported models,[205,206] one NHP heart transplant study by Thomson's group in Pittsburg reported in their ATG-lymphodepleted model that Tregs lead to increased effector memory T cells and anti-donor antibodies[238] in addition to an eventual decrease in graft survival and function compared to non-Treg recipients. Although Tregs *in vitro* were shown to be potent suppressors, this is the first report to argue against their use. It is important to mention that this model was in the context of severe lymphodepletion with cardiac allografts which historically have been shown to be difficult to tolerize.[81,82] Our studies were different by which recipients received MHC-mismatched donor BM prior to the allograft (kidney) under a non-myeloablative conditioning regimen aiming to harness the benefits of mixed chimerism.[205] Though differences in the preparative regimens may have influenced the outcome of both studies (by impacting the survival of the Tregs) we remind mindful that these studies are not innocuous and protocol differences can lead to unwanted outcomes. Besides Thompson's study, our studies and other clinical trials support that Tregs are safe and the improvement of their potency and dosing will likely further lead to enhanced outcomes.

In summary, MCM Tregs operate similarly to human Tregs. We demonstrated the feasibility of expanding large number of MCM Tregs *in vitro* for pre-clinical studies. We have generated five protocols, all of which expanded MCM Tregs; however, Protocol B and E yielded the most suppressive Tregs with acceptable cell numbers. Tregs could be safely re-expanded after cryopreservation and maintained robust suppressive function. The culture method is key and careful quality assessment is necessary prior to infusion. Our most successful Treg

expansion protocols (B and E) allowed Tregs to suppress robustly host or donor responder T cells. Furthermore, MHC-mismatched third-party effector cells could be also suppressed, encouraging their potential use in patients receiving brain-dead donor grafts. Our approach is safe and allows Tregs to maintain their phenotype and function over long-culture periods demonstrated by the TSDR stability confirming their "natural" thymic origin. Finally, though MCM Tregs did not share all phenotypic characteristics with human Tregs, overall they very closely resemble their biology, which argues in favor of their utility in key proof of concept, large animal pre-clinical studies.

VI.2. *In vivo* studies for the induction of transplantation tolerance

Despite great progress has been developed in the past decades with immunosuppressive therapies improving BMT and solid organ transplantation outcomes, patients continue to be met with secondary side-effects such as diabetes mellitus, osteoporosis, hypertension, and infections.[239] In addition, titration for the optimal clinical dose remains a challenge. High doses can lead to cancer and infection while insufficient drug levels lead to rejection of the graft. Because current standard of care immunosuppressive agents suppress the immune system non-specifically, more selective approaches to control anti-donor responses are in great need.[240] Therefore, the induction of transplantation tolerance (as we are aiming) is an attractive approach that is specific and is not burdened by life-threatening side effects.

Different strategies have succeeded in the induction of transplantation tolerance in murine models. However, when attempted in non-human primates or humans, rodent approaches were challenging to achieve (Table 13). Heterologous immunity could be one possibility. Because mice used for research remain in selective pathogen-free environments since birth, memory responses do not develop. In contrast, non-human primates or humans carry an immune

history (i.e. immunological memory) which classically have been the most resistant cell-subsets to immunosuppress and responsible for rejection. As an example, CMV-specific CD8+ central memory T cells[241] have been shown to cross-react and be responsible for rejection.[242]

VI.2.1. Induction of transplantation tolerance through the mixed hematopoietic chimerism (MC) approach

Mixed hematopoietic chimerism (MC) is defined as the coexistence of donor and recipient cells within the recipient's immune system. MC has been studied as a potential approach for tolerance induction for allogeneic[243,244] and xenogeneic transplantation.[245] Along with other approaches (Table 13),[246] transplantation tolerance has been successfully achieved in rodent models, but as expected, it has been more difficult to achieve in outbred (non-specific pathogen free) species. However, the MC approach has to date been the most successful approach across models (Table 13).[246]

Table 13: Methods for the induction of transplantation tolerance

Method	Mice	Primates and humans
Enhancement	+	-
Donor-specific transfusion	+	-
Peptides	+	-
Anti-MHC mAB's	+	-
Calcineurin inhibitors	+	-
Antilymphocyte serum	+	-
Anti-CD24	+	-
Anti-CD25	+	-
Total lymphoid irradiation	+	+/-
Anti-CD3 toxin	+	+/-
Costimulatory blockade	+	+/-

Chimerism	+	+

Adapted from Sachs, 2003, J Clin Inv[246]

Kean et al have shown transplantation tolerance in NHPs, however their approach used MHC matched (minor antigen mismatched) rhesus macaques.[247] Her approach included busulfan combined with belatacept, anti-CD40L and low-dose total body irradiation with a tapered immunosuppression. Three of the nine recipients showed MC for up to 24 months with a prolonged survival of the donor skin graft. Approaches using mixed chimerism by Dr. Kawai et al have been met with transient chimerism and 40% of the animals would eventually lose their kidney grafts. Proof of concept that Tregs are capable to induce immunological tolerance with the Kawai non-myeloablative approach[205] has provided enthusiasm to further refine the model. Our results build from such studies with additional improvements, such as the use of rapamycin and the generation of further refined Tregs.

We substituted the use of cyclosporine for rapamycin (partially) for its antiviral properties.[248] Duran-Struuck studies had been met with significant mortality/morbidity due to the reactivation of CMV, and evidence that rapamycin can prevent CMV reactivation has been well-documented in the kidney transplant field.[249,250] To this end, our animals had a decreased incidence of CMV as shown in our result section and recently publication.[230]

A second reason that strongly justified the use of rapamycin in our studies was related to its effects on Tregs. Rapamycin selectively facilitates the expansion of Tregs[251,252] while controlling effector T cells.

Our Treg expansion protocol was also improved.[253] Studies performed by Duran-Struuck at al. reported the infusion of Tregs that were cultured under Protocols

A and D.[205] Our protocols instead allowed for the production of even more potent Tregs (Protocol B and E)[253] and a higher yield as shown in figure 12 and 14 in result section.

VI. 2.2. Impact of the BM source: iliac-crest versus megadose.

Human BMT studies have shown that the number of donor T cells is important for optimal engraftment of the BM graft in allogeneic HCT.[227] We hypothesized that increasing the BM dose (with higher stem and T cell doses) would improve the BM engraftment and increase donor chimerism. We thus studied recipients of low and high BM doses with the addition of Tregs that were cultured under two different protocols, B and E. We hypothesized that the risk of GVHD would be minimal because Tregs would enhance engraftment while controlling unchecked GVH responses. Our protocol, as predicted, led to prolonged and higher (however still transient) MC in animals that received the high BM doses ($12.5x10^6$ CD34+ and $49.45x10^6$ CD3+ cells/kg). Interestingly, recipients that received Tregs cultured under Protocol B (with aAPCs and donor PBMCs) never developed GVHD . One (of four) recipients in this group lost chimerism early post-BMT due to acute CMV reactivation. In contrast, two of the three animals that received protocol E-Tregs (that were cultured with a pool of B cells from three to four mismatched donors and without aAPCs) developed GVHD (after converting to full-donor chimeras). These findings argues that even though Protocol-E Tregs seemed to be highly suppressive *in vitro*, they were unable to control GVHD *in vivo*. The third animal under this group lost the chimerism early post-BMT (one month after BMT) but had also additional protocol modifications such as anti-IL-6 receptor antibody infusions, which difficult the interpretation of this animal.

VI. 2.3. Kinetics associated with BM rejection and loss of MC

The inversion of the CD4+ and CD8+ T cell ratio and the increase in the CD8 absolute counts consistently correlated with the loss of chimerism. We hypothesized that as previously documented[205] BM rejection would occur. To this end, a fifth Treg infusion (originally scheduled on Day 50 of the protocol) was delivered to prevent the loss of the donor graft. Unfortunately, infusions of Tregs consistently failed to prevent graft loss (Table 9). It is hard to predict if CD8 rises occur from recovery after lymphodepletion from the conditioning regimen or due to CMV reactivation which induces CD8 proliferation. In order to test whether CMV reactivation triggered the proliferation of CD8+ T cells, CMV tetramers would be required to identify CMV-specific CD8+ T cells.

Reactivity against the donor was tested *in vitro* through a mixed lymphocyte reaction assay. We observed that those animals that developed prolonged chimerism were hyporesponsive to the BM donor in comparison to a mismatched third-party control even after the chimerism was lost as observed in animal T4 (Figure 42A), and even after discontinuation of immunosuppression. The early loss of chimerism correlated with *in vitro* anti-donor responses that were in general more vigorous than an anti-third party response.

VI. 2.4. Kinetics of peripheral Tregs after BMT

We monitored the kinetics of peripheral Tregs in the recipients after BMT. In naïve MCM, the percentage of Tregs in blood ranges between 2 to 4%[253] while we observed an increase after BMT and Treg infusions, in some cases, reaching levels as high as 25% of CD25hiFoxP3+ cells within CD3+CD4+ cells (Figure 44B). Overall, the high Treg numbers persisted during the first month post-BMT for all the recipients while the MC was present and rapamycin was being administered. This could have been influenced by the use of rapamycin since animals that

received BMT (and not Tregs) with rapamycin monotherapy had high level of Tregs during the rapamycin administration period. Animals resumed their basal levels of Tregs when chimerism was lost (in control recipients at the end of the rapamycin treatment and in animals that received Tregs at the time that lost chimerism regardless of the time of rapamycin discontinuation). Therefore, mixed chimerism and tolerance correlated with higher levels of Tregs. This significant Treg increase during rapamycin treatment can be argued to be related to the decrease of the CD4+ effector T cells (partially, due to the lymphodepletion derived from the conditioning regimen). Importantly, animals that developed GVHD showed lower Treg levels at the time of the disease, independently of the previous administration of cultured Tregs.

VI. 2.5. Impact of the Treg dose

Although we tried to standardize the number of Tregs infused into the recipients across the iliac-crest and megadose group, on average, $45.5x10^6$ Tregs/kg were infused into recipients that received megadose BMT, compared to the lower-dose IC BM recipients that received an average of $74.7x10^6$ Tregs/kg. As previously discussed, the duration of MC varied between the different groups, with the Treg-treated recipient achieving the longest MC duration compared to control recipient that did not receive Tregs. Among the Treg-animals, those that received a megadose BM, showed better outcomes. This suggests that the donor CD34+ cell and T cell doses infused at the time of the transplant play an important role in the induction of BM engraftment as it has been previously shown.[227]

VI. 2.6. Impact of Treg expansion protocol

Treg specificity assays were performed in order to study the function of the expanded Tregs aimed to be used as an “off-the-shelf” product for brain-dead donors across MHC barriers. Mixed lymphocyte reactions showed that Tregs

that were cultured under Protocol B and E were potent suppressors to multiple MHC (self, PBMCs expressing antigens from the same BMT donor during the culture expansion and full-mismatched third-party responders). If Protocol E were translated to the clinic, and if a truly polyclonal population is desired, multiple B cell donors would need to be used as stimulators (or artificial APCs could be used if deemed safe) like protocol B-Tregs. Because aAPCs used in Protocol B were of mouse origin, some Tregs may have been selected to mouse antigens. We hypothesized that the reason why none of the recipients that received megadose BMT with Tregs cultured under Protocol B developed GVHD is because of the potential polyclonal background of these cells. As Treg infusions are delivered during the first week before chimerism spikes (if the function of our Protocol-E Tregs depends on indirect presentation), there might not be enough donor APCs present yet (as chimerism tents to spike after Day 13) to stimulate the infused Tregs and mediate GVH and HVG responses. Labeling studies would be required to answer these hypothesis and further understand the homing properties of these Tregs.

Another question would be the suppressive capacity of these Tregs under inflammatory conditions (such as GVHD) or CMV reactivation. Answering this question through *in vitro* assays would require responders isolated from recipients during this inflammatory phase post-transplant (or T cells activated *in vitro* prior to the suppression assay) and ideally, blood Tregs from recipients with GVHD and assess whether the lack of suppression was conserved (or lost) *in vitro*, informing us to whether other potential site-specific factors could be preventing the function of Tregs.

VI. 2.7. Impact of delayed BM infusion

Because horse-antithymocyte globulin (ATG) is administered in our conditioning regimen for T cell depletion before BMT we hypothesized that Tregs and BM

stem cells may have been impacted (or killed) by it. We therefore studied if ATG could have bound to either the CD34+ cells in the BM graft and/or to the *in vitro* expanded infused Tregs, thus interfering with BM engraftment. If this is the case, MC loss could have been due to non-immunological causes instead of rejection. Our studies support that ATG was able to bind to both populations and that it was present at least until two days after the last ATG dose. We decided to delay the BMT two days after the last ATG administration to prevent this potential side effect, but when serum samples were tested post-transplant we also observed that ATG was still sometimes present until 2-3 days after the BMT. These data suggest that ATG may be cleared from the system (by being absorbed) by binding to the donor-BM cells.

In conclusion, additional BMTs would be required in recipients receiving Tregs expanded under protocol B as this protocol has shown the best *in vivo* outcomes with prolonged MC and without development of GVHD. In contrast, recipients that received protocol E-Tregs, which originally appeared to produce Tregs with more potent *in vitro* suppressive capacity, developed GVHD in 66% of the cases. Peripheral blood mobilized cells harvested via leukapheresis can be an alternative to obtain high BM numbers.

VI.2.8. Effect of CMV in immunocompromised transplant recipients

Human CMV is a ubiquitous virus, highly prevalent among humans.[254,255] CMV remains the most important opportunistic pathogen in immunocompromised individuals such as BMT recipients.[256-259] The CMV status of the donor and recipient plays a major role in the development of viremia, with seronegative recipients receiving transplants from seropositive donors having the highest risk of disease. In addition, the intensity of recipient immune system manipulation has a major impact on the development of opportunistic infections. Despite the highly species-specific nature of each CMV strain, non-human primate CMV

contains most of the human CMV gene families, facilitating mechanistic studies of viral pathogenesis and its effect on the host immune response.[260-262]

Our conditioning protocol, including ATGAM-based T-cell depletion, uniformly resulted in substantial CD4+ and CD8+ T cell reduction. It was during this lymphopenic period that CMV reactivation occurred. We found that T cell recovery, predominantly by CD8+ T cells, started at the peak of CMV viremia and progressed rapidly until the virus became undetectable in the serum. There are several potential explanations for this pattern of T cell expansion. First, lymphopenia-driven expansion is known to drive the division of T cells that then assume a memory phenotype. However, CMV is also known to drive T cell recovery after T cell depletion. In a clinical study of kidney recipients that received rabbit ATG, when T cell recovery was compared between recipients based on their CMV serostatus, CD8+ T cells were shown to repopulate faster (1-2 months) in those patients that reactivated CMV compared to seronegative recipients (taking up to two years).[263] These findings could suggest that CMV infection promoted T cell expansion that in turn caused immune activation against CMV and potentially the graft due to heterologous immunity. Consistent with this possibility, we previously reported that the only Cynomolgus recipient that remained CMV negative in a prior study of BMT achieved a markedly increased level and of duration chimerism compared to the rest of the group.[205] However, in order to rigorously study the effect of CMV on BM engraftment and rejection, we would require a large CMV seronegative control group. Unfortunately, CMV-seronegative MCM are extremely rare making a study of this nature infeasible.

We found that GCV at 12.5 mg/kg BID was able to control CMV infection if instituted soon after viremia was detected (1,000 copies/mL) and before the viremia reached 10,000 copies/mL. Lower GCV doses were not sufficient to clear

the viremia. Long-term CMV control was maintained when VGC was given once the viremia cleared and CD8 counts were >500 cells/µL. Thus, we show here in our MCM model that CMV can be controlled by high doses of GCV followed by VGC after viremia clears and CD8+ T cell counts recover. This strategy is also adopted in the clinic in BMT recipients, where antiviral treatment is started after CMV detection. Unlike solid organ transplantation, where CMV prophylaxis is an option, the early use of antivirals is avoided in BMT recipients due to the myelosuppressive component of these drugs. We propose that for solid organ transplantation studies in macaques, prophylactic dosing with GCV be administered if T cell depletion is utilized. For BMT studies, we propose a rigorous monitoring protocol for CMV reactivation and initiation of GCV at 12.5 mg/kg BID once viremia exceeds 1,000 copies/mL. Due to the myelotoxicity of antivirals and to protect the infused BM during the engraftment period, we opted for treating CMV after reactivation instead of choosing a preemptive strategy. Other groups that study BMT in NHPs have used cidofovir prophylaxis during the peri-transplant period. They found a significant incidence of CMV reactivation, consistent with our experience that it was unable to suppress viremia completely, and therefore frequent monitoring for viremia is still required.[247] Recently, a new CMV prophylaxis approach that lacks the toxic effects of the currently available antiviral drugs, letermovir, has been developed.[264,265] Letermovir inhibits CMV replication by binding to components of the terminase complex (UL51, UL56 or both). It is now clinically available and clinical trials showed that prophylaxis with letermovir resulted in a significant lower risk of clinically CMV infection compared to those that received placebo.
Lastly, and importantly, we observed decreased incidence of CMV reactivation when using rapamycin over calcineurin inhibitors, as shown in the study by Duran-Struuck et al[205] where recipients received cyclosporine instead of rapamycin as immunosuppression monotherapy post-BMT and reactivated CMV during the first week after transplant. A comparison between the time point of

CMV reactivation on animals receiving cyclosporine versus rapamycin under the same approach has shown a delayed reactivation in the latter group.[230] Further studies in MCM are necessary to establish its potential in this model.

In our studies, we found that close monitoring of CMV and promptly treatment after reactivation allowed for the development of multilineage chimerism as observed in T4, T6 and T7, recipients that developed MC for up to 110 days, all of them with viremia. CMV reactivation was accompanied by an increase in the CD8 counts, possible as a result of immune activation, leading to BM rejection. Therefore, we hypothesize that lack of CMV viremia could have achieved much improved outcomes, although not as realistic due to the high prevalence of CMV among the human population. In the contrary, if CMV was left untreated and reached high levels (>10,000 copies/mL), immune activation occurred much earlier and faster, as observed in recipient T5.

Novel approaches are currently being studied for the prevention and control of CMV to avoid or decrease the requirements for conventional antiviral therapies. Adoptive transfer of donor CMV-specific T cells offers an alternative to restore CMV immunity that would reduce the need for the conventional antiviral treatments and their side effects.[266-268] In addition, they provide an alternative in the face of CMV drug-resistance, which is currently a growing problem.[269,270] One potential pitfall of this strategy is the possibility of heterologous immunity of anti-CMV T cells and precipitation of rejection by the use of cellular immunotherapy.[271] Alternatively, CMV vaccines represent an approach being investigated for CMV prevention.[272] Although there are currently no licensed CMV vaccines, there is an increasing interest in this approach and vaccines are under clinical development. CMV-specific CD8+ and CD4+ T cell recovery is associated with protection against CMV.[273] Therefore, monitoring the CMV-specific T cell population might be a strategic approach to predict CMV

reactivation in BMT recipients and improve the timing for the initiation of antiviral treatment.[274,275] The use of tetrameric complexes of HLA molecules loaded with a CMV peptide has been investigated in human BMT recipients to monitor the recovery of CMV-specific CD8+ T cells post-transplant.[276] Findings support the need for CMV-specific CD8+ T cells for CMV protection and suggest that graft-origin CMV-specific memory T cells contributed to CMV protection. The development of non-human primate tetramers and other assays for the study of these populations in MCM transplant models would facilitate the determination of whether or not CMV represents a barrier to the induction of transplantation tolerance.

In conclusion, CMV infection remains a challenge for BMT recipients. Viremia and antiviral therapy not only interfere with BM engraftment, hindering successful outcomes, but also cause morbidity and mortality in immunocompromised recipients. Similar results were observed in our Cynomolgus macaque transplant tolerance induction protocol, where recipients developed CMV viremia that progressed to clinical CMV disease if not treated promptly. Additionally, the immune response to CMV may contribute to immune activation, thus preventing the development of chimerism and donor-specific tolearance.[205] Newer antivirals without bone marrow toxicity are a promising approach to avoid CMV reactivation and its consequent adverse effects.

VII. CONCLUSIONS

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1. *In vitro,* Tregs cultured under Protocol A, B and E showed the best FoxP3 expression and suppressive capacity compared to the rest of the protocols, with higher numbers being achieved with the last two approaches.
2. Treg specificity assays showed that Tregs cultured under Protocol B and E were polyclonal as they were able to suppress T cell responses from the "donor" that provided stimulation during Treg expansion and from a third-party responder not previously seen.
3. Proof of concept (under some situations) that BMT and infusion of *in vitro* expanded Tregs resulted in prolonged levels and duration of multilineage MC (with T cell chimerism) in a Cynomolgus macaque model with a non-myeloablative regimen across MHC barriers.[205]
4. MC could be further improved by administering higher stem cell and T cell doses in the BM graft.
5. GVHD was observed in control animals without Treg infusions as well as in Treg-treated recipients that received sBc-Tregs (protocol E) suggesting that those Tregs were unable to control such potent alloresponses.
6. None of the recipients that received megadose BMT and protocol-B Tregs (stimulated with aAPCs and donor PBMCs) developed GHVD and showed more prolonged mixed chimerism, which suggest that those Tregs may be more functional (and protective).
7. A correlation between the loss of MC, the percentage of CD4+ and CD8+ T cells inversion and the increase in the absolute CD8 counts was observed, that also correlated with CMV reactivation.
8. CMV reactivation occurred in 100% of infected recipients secondary to lymphopenia.
9. The percentage and absolute counts of Tregs in blood were increased in all the recipients during the MC duration (and rapamycin administration), that in the controls animals returned to base line levels after the discontinuation

of rapamycin. In some of the Treg-treated animals, the higher Treg levels lasted past the time of rapamycin administration while the MC was present.

10. Overall, the loss of chimerism correlated with a decrease in the Treg percentage *in vivo*.
11. Lower Treg levels were observed in the recipients that developed GVHD independently of the administration of cultured Tregs.
12. Anti-donor *in vitro* hyporesponsiveness was observed in the Treg-treated recipients that had prolonged chimerism surpassing the immunosuppression period. All of the control recipients showed higher anti-donor response.
13. ATG bound to all tested cell populations and took over 48 hours for clearance after BM infusion. Clearance from circulation was likely to being absorbed by the donor BM graft.

VIII. FUTURE DIRECTIONS

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Based on our outcomes, additional BMT experiments with recipients receiving mega BM doses and Tregs cultured under Protocol B would be needed to confirm that this is the most promising approach in our study. In addition, repeating experiments that include the infusion of Tregs expanded under Protocol E would help confirm our hypothesis regarding the control of GVHD in these recipients. *In vivo* confirmation of tolerance would be required through a solid organ transplant performed four months after BMT. In order to maintain the donor alive after the BM collection, an alternative option to obtain high BM cell numbers would be through the mobilization of stem cells in peripheral blood through leukapheresis. In addition, and due to the high impact that CMV reactivation has over transplantation and BM engraftment, new alternatives, such as less toxic and newly developed antivirals (letermovir) would be an alternative for the control of CMV and to avoid the raise of CD8+ T cells post-BMT. Further studies could include the addition of other modulatory cell populations, such as CAR Tregs, if proved to have beneficial effects for the suppression of alloresponses in these model.

IX. REFERENCES

IX. REFERENCES

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X. ACRONYMS

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A

Ab	**Antibody**
ADA	Anti-donor antibody
APC	Antigen presenting cell
aAPC	Artificial antigen presenting cell
ATG	Horse anti-thymocyte globulin

B

BM	Bone marrow
BMT	Bone marrow transplant

C

CD40L-sBc	CD40L-stimulated B cells
CMV	Cytomegalovirus

F

FACS	Fluorescence-activated cell sorting

G

GCV	Ganciclovir
GVH	Graft-versus-host
GVHD	Graft-versus-host disease
Gy	Gray

H

HLA	Human leukocyte antigen
HVG	Host-versus-graft

I

IM	Intra-muscular
IV	Intra-venous

K

KG	Kilogram

M

Acronyms

MACS	Magnetic-activated cell sorting
MCM	Mauritius Cynomolgus macaque
MHC	Major histocompatibility complex
ML	Milliliter
MLR	Mixed lymphocyte reaction
N	
NHP	Non-human primate
NIH	National Institute of Health
P	
PBMC	Peripheral blood mononuclear cell
Q	
qPCR	Quantitative polymerase chain reaction
R	
Rapa	Rapamycin
T	
Tcon	Conventional CD4+ T cell
Treg	Regulatory T cell
Tx	**Transplant**
V	
VGC	Valganciclovir
T	
TBI	Total body irradiation
TI	Thymic irradiation
TNC	Total nucleated cells
TSDR	Treg-specific demethylated region