Modulation of T-Regulatory Cells by Small Molecules: Potential Therapy for GvHD

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Declarations

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

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Abstract

Allogeneic haematopoietic stem cell transplantation (alloHSCT) is often curative for patients suffering from blood cancers. Nonetheless, significant morbidity and mortality are still prevalent. One major complication is graft versus host disease (GvHD) which occurs in 30-70% of patients. In this disease donor T-cells recognise the host as foreign and subsequently expand and attack the host's tissues and organs. T-regulatory cells (Tregs) are a subpopulation of T-cells which promote anti-inflammatory immune responses and suppress T-cell activation and proliferation. It has been demonstrated that a high proportion of Tregs in the donor graft significantly reduce the occurrence of acute GvHD and increases overall survival. However, modern techniques have failed so far to routinely expand Tregs in the clinic due to many reasons including lineage instability and cost. This thesis explores the repurposing of two drugs, Atorvastatin and Vitamin D, which have an excellent safety profile, are cheap and easy to use, to boost Treg numbers and modulate distinct immune cell populations. The drug combination efficiently elevates Treg numbers, inhibits pathogenic CD4+ T-cells and suppresses T-cell proliferation. In addition, it induces a tolerogenic phenotype, at the transcriptional and translational level, in monocyte-derived dendritic cells. Atorvastatin and Vitamin D treatments are a viable approach to boost Treg numbers in the donor as well as combating GvHD in the recipient.

Abbreviations

AE	Adverse Events
ALDH	Aldehyde Dehydrogenase
ALL	Acute Lymphoblastic Leukaemia
alloHSCT	Allogeneic Haematopoietic Stem Cell Transplantation
AML	Acute Myeloid Leukaemia
APC	Antigen Presenting Cell
AT	Atorvastatin
ATG	Antithymocyte Globulin
BMT	Bone Marrow Transplantation
CNS	Conserved Non-coding Sequence
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
DAMPs	Damage Associated Molecular Patterns
DBP	Vitamin D Binding Protein
DC	Dendritic Cell
DUB	Deubiquitinases
EAE	Experimental Autoimmune Encephalomyelitis
FOXP3	Forkhead Box P3
GFP	Green Fluorescent Protein
GI	Gastrointestinal
GMP	Good Manufacturing Practice
GvHD	Graft versus Host Disease
GvL	Graft versus Leukaemia
HDAC	Histone Deacetylase
HLA	Human Leukocyte Antigen
HSCT	Haematopoietic Stem Cell Transplantation
IDO	Indoleamine 2,3-Dioxygenase

IEC	Intestinal Epithelium Cell
IFN-γ	Interferon-γ
IL	Interleukin
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked
IRF	Interferon Regulatory Factor
KDAC	Lysine Deacetylase
LPL	Lamina Proporia Lymphocytes
MDS	Myelodysplastic Syndromes
MLN	Mesenteric Lymph Node
MS	Multiple Sclerosis
NFAT	Nuclear Factor of Activated T-cells
NHL	Non-Hodgkin Lymphoma
NK	Natural Killer Cells
NRM	Non-relapse Mortality
OS	Overall Survival
PAMPs	Pathogen-associated Molecular Patterns
PBSC	Peripheral Blood Stem Cells
PFS	Progression Free Survival
PPAR	Peroxisomal Proliferator Activated Receptor
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Element
RXR	Retinoic X Receptor
SCFA	Sort Chain Fatty Acid
SLE	Systemic Lupus Erythematosus
STAT	Signal Transducer and Activator of Transcription
TBI	Total Body Irradiation

TCR	T-cell Receptor
TNF	Tumour Necrosis Factor
TRM	Treatment Related Mortality
TSDR	Treg Specific Demethylation Region
UCB	Umbilical Cord Blood
USP	Ubiquitin-specific Peptidase
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
WT	Wild Type

CHAPTER 1-6

Introduction

1. History and Current Allogeneic HSCT

Haematopoietic stem cell transplantation (HSCT) is an intervention used to treat haematological malignancies, congenital or acquired disorders of the haematopoietic system - such as blood cancers like leukaemia or lymphoma - and may be curative for these patients. However, the application of this therapy is limited by the considerable acute and chronic side effects of treatment which may cause up to 20% transplant-related mortality. In addition, these side effects and their sequelae may cause significant reduction in the quality of life of transplant recipients.

The principle of this therapy is transplantation of haematopoietic stem cells from a healthy donor into an immunosuppressed host for immunological reconstitution and to generate a new and functional haematopoietic system. During bone marrow transplantation, a donor inoculum is given to a recipient which can be harvested either directly from the bone marrow, from peripheral blood or cord blood and placenta. The inoculum contains pluripotent haematopoietic stem cells, as well as more mature haematopoietic cells arising from the myeloid, lymphoid, and erythroid lineages. These pluripotent stem cells form the basis of lymphoid or myeloid progenitors which reconstitute the patient's immune system. However, other donor cells types might be present such as T-cells or B-cells which can have detrimental effects on the receiving patient. Yet one cell type, T-regulatory cells, have been shown to be immensely beneficial in the donor's graft outcome of bone marrow transplantation.

According to the European Bone Marrow Transplant (EBMT) registry data over 47,000 HSCTs took place in 2018 in 50 countries (Passweg *et al.* 2020). Of these, nearly 28,000 were autologous (~60%) and nearly 20,000 were allogeneic (~40%). Around 70% of the first HSCTs treated leukaemia followed by lymphoproliferative disorders and bone marrow failure (Passweg *et al.* 2018). On average around 110 people a day or every 14

minutes are diagnosed with blood cancer or related disease worldwide making research into this field indispensable to provide the best quality of life for patients (Anthony Nolan).

1.1 History of Allogeneic Haematopoietic Stem Cell Transplantation

With the beginning of the atomic age and radiation damage among atomic bomb survivors, Jacobsen and colleagues established that radiation damage can be reduced by shielding the spleen or femur of mice with lead (Jacobson *et al.* 1949). Shortly after it was demonstrated, that mice and guinea pigs can be protected from radiation by infusing bone marrow cells (Lorenz *et al.* 1951). This was a crucial point in the development of haematopoietic stem cell transplantation (HSCT) as it demonstrated that intravenous transfusion of bone marrow cells can rescue mice from lethal radiation. Initially, investigators were not aware that the rescue effect was due to the transfer of cells, instead it was hypothesised that some humoral factor(s) in spleen or bone marrow stimulated haematopoietic cells that survived after irradiation. During the following years, it was shown that the transfer of bone marrow cells protected mice from the lethal radiation effects (Kraft 2009). Researchers started to understand the potential of this transfer of bone marrow cells to treat haematological malignancies, congenital or acquired disorders of the haematopoietic system.

This led to the principle of allogeneic HSCT: transfer of stem cells from allogeneic donors is accompanied by high dose radiation/chemotherapy that will destroy the patient's immune system and to keep the patient's immune systems in an immunosuppressive state which allows the donor's stem cell to engraft.

In the early 1950's human allogeneic HSCTs were mostly unsuccessful due to failed engraftment or Graft versus Host Disease (Bortin 1970). But in 1957, Thomas and colleagues reported the first transient engraftment in two patients with the eventual relapse of leukaemia (Thomas *et al.* 1957). Only two years later, Thomas and colleagues were able to successfully treat a leukaemia patient with a bone marrow graft from their identical twin (Thomas *et al.* 1959). In fact, a year earlier, the French immunologist and oncologist Georges Mathé had performed the first successful human allogeneic bone

marrow transplantation in six Yugoslavian nuclear reactor workers accidentally exposed to radiation (Mathe *et al.* 1959). Again, an initial successful engraftment was subsequently reported to have been rejected by their hosts. Regardless, four workers survived. Around the same time a crucial discovery was made in the form of the human leukocyte antigen (HLA) genes encoding MHC class I and II which present antigens to T-cells and discriminate self-antigens from foreign, leading to increased recognition of HLA-matching to support engraftment and prevent Graft versus Host Disease (GvHD) (Dausset and Brecy 1957). GvHD, in which the donor's graft attacks the host's immune system, is to date one of the major complications of allogeneic transplantation. At this time Georges Mathé coined the term Graft versus Leukaemia (GvL) effect through early observations in mice made by Barnes and Loutit.

In 1965 the next milestone was accomplished when the first successful allogeneic bone marrow transplantation was reported in a leukaemia patient (Mathe *et al.* 1965). The leukemic patient received an infusion of bone marrow from six close relatives (mother, father and four siblings), and a sibling's bone marrow graft was initially successful. Despite successful engraftment, achieving remission and survival for around 20 months, the patient later died of GvHD.

Despite early success in both animal and clinical studies the belief of leading immunologists was that graft rejection would never be resolved and this field reached a dead end (Bekkum and Vries 1967). In retrospect it seems clear that the rush to translate animal observations into a clinical context was attempted too early, without the basic knowledge required to understand why some grafts were successful and so enable transfer of animal models to successful clinical therapy. The basis of histocompatibility matching of donor and recipient was not understood. Particularly, these early transplants were performed with a poor understanding and experience in histocompatibility matching (HLA) of donor and recipient, conditioning regimens and control of GvHD (Mc *et al.* 1959,

Thomas *et al.* 1959, Mathe *et al.* 1965). Out of 200 registered bone marrow transplants conducted between 1957 to 1967 no patient survived long-term (Bortin 1970).

The development of immunosuppressive drugs heralded a new era in bone marrow transplantation and led to resumption of clinical studies. Animal models had demonstrated that the administration of such drugs, particularly the folic acid antagonist Methotrexate, can significantly reduce GvHD post-transplantation (Thomas *et al.* 1962, Storb *et al.* 1970). In addition, more attention was given to HLA matching as DLA-matched dogs without conditioning survived significantly longer than mismatched (Epstein *et al.* 1968). Regimens were refined and histocompatibility matching was better understood and improved; and it was recognised and confirmed to be of pivotal importance in preventing GvHD (Thomas *et al.* 1975). As a result, in 1968, a 5-month old boy with primary immune deficiency disorder (SCID) underwent the first successful allogeneic HSCT using bone marrow and peripheral blood cells from their incompletely HLA-matched sister without a conditioning regimen (Gatti *et al.* 1968). In 1973, another great leap was taken as a 5-year old boy in New York, USA with SCID underwent the first unrelated bone marrow transplantation using stored cells from a Danish blood bank. Finally, in 1979, the first unrelated allogeneic bone marrow transplantation for a leukaemia patient occurred (Thomas *et al.* 1979). At this time most patients treated by stem cell transplantation had advanced haematological malignancies and were often in poor clinical condition after their transplant which drove development of intensive care of cytopenic patients using blood and platelet transfusions, prophylaxis or treatment for bacterial, fungal and viral infections.

In the late 1970s and 1980s the first national and international bone marrow registries were established which led the way for more successful matching of HLA compatible donor and recipient. It was also recognised that stem cells could be harvested, not only from the bone marrow, but also from peripheral and cord blood (see 1.2.3) (Juttner *et al.* 1988, Gluckman *et al.* 1989, Thomas and Blume 1999). Over the last 15 years

tremendous progress has been made allowing the therapy of allogeneic HSCT to not just to younger patients with immunodeficiency but also medically fit older patients who have the highest prevalence of haematological malignancies. In addition, in recent years autoimmune diseases, especially multiple sclerosis, are being increasingly treated by HSCTs even though the great majority are yet autologous HSCTs.

1.2 Autologous Haematopoietic Stem Cell Transplantation

In the preceding section I have described the many discoveries that have led to today's principle of allogeneic HSCT. It is important to note that at the same time discoveries were made that contributed to autologous HSCT (autoHSCT). The principle of autologous HSCT is the infusion of self-haematopoietic stem cells to reconstitute the blood and the immune systems. Over the last 20 years autologous HSCT are increasingly used to treat autoimmune diseases such as multiple sclerosis (MS) and are now the fastest growing indication for HSCT (Passweg *et al.* 2018). Autologous HSCT are preferred for the treatment of autoimmune diseases due to their much lower treatment-related mortality, mainly due to the absence of graft versus host disease.

In the early 1990s it was noted that rodents with adjuvant arthritis responded just as well to autologous or syngeneic bone marrow transplantations as to grafting with allogeneic bone marrow (Knaan-Shanzer *et al.* 1991). Soon thereafter, several groups reported that experimental autoimmune encephalomyelitis (EAE), an inducible autoimmune demyelinating disease characterized by the infiltration of immune cells into the central nervous system and brain, could be rescued by grafting of autologous or syngeneic bone marrow (Karussis *et al.* 1992, van Gelder *et al.* 1993). These early animal studies not only showed the potential of autologous HSCT but also limitations as animals treated early could be rescued from EAE but animals treated in the chronic phase of EAE did not experience any disability improvements (Burt *et al.* 1998). These encouraging results were first tested in a small clinical trial of primary and secondary progressive MS patients

due to the similarity between EAE and MS in disease pathophysiology. The treatment regimen was deemed relatively safe, however, and as other groups in the following years reported, outcome was disappointing as many patients continued to deteriorate (Fassas *et al.* 1997, Fassas *et al.* 2000, Krasulova *et al.* 2010). The turning point came when relapsing-remitting MS (RRMS) patients were treated with autologous HSCTs. RRMS patients are diagnosed earlier with more brain rather than spinal cord lesions and with more frequent inflammatory relapses. In 2009 two independent research groups reported that autoHSCT could completely abrogate disease activity in RRMS patients leading to the consensus that autoHSCT should be considered as a therapeutic option (Burt *et al.* 2009, Fagius *et al.* 2009). Due to the prevalence of MS (~2.3 million people worldwide and ~100,000 people in the UK) the majority of reports have focused on this disease, however, other neurological and autoimmune disease, such as Crohn's disease, are now being treated with autoHSCTs (Snowden *et al.* 2018).

Of note, a recent clinical trial (NCT00273364) of RRMS patients compared the effectiveness of autoHSCT against current disease modifying therapies and concluded that autoHSCT is safe and very effective, however, further research is needed (Burt *et al.* 2019). In addition, despite the notion that CNS-compartmentalized inflammation is mediated by a wide range of immune cells (e.g. T-cells, B-cells and monocytes), including CD4+ T-cells which secrete a range of proinflammatory cytokines (e.g. IFN-γ and IL-17A), it is poorly targeted by currently available disease modifying therapies (Lassmann 2017). In fact, relapses seen in EAE disease models and MS patients are principally mediated by aberrant activated CD4+ and CD8+ T-cells (Dendrou *et al.* 2015, Baecher-Allan *et al.* 2018). One potential cause is the insufficiency of Tregs, decreased expression levels of FOXP3 and regulatory-deficient Tregs (Viglietta *et al.* 2004, Venken *et al.* 2008, Frisullo *et al.* 2009, Kaskow and Baecher-Allan 2018, Kitz *et al.* 2018).

1.3 Current Allogeneic Haematopoietic Stem Cell Transplantation

Today a vast variety of allogeneic haematopoietic stem cell transplantations (alloHSCT) are performed for hematologic malignancies such as acute myeloid leukaemia (AML, 35%), acute lymphoblastic leukaemia (ALL, 15%), myelodysplastic syndromes (MDS, 12%) and non-Hodgkin lymphoma (NHL, 7%) (Passweg *et al.* 2020). Despite huge progress the overall 5-year survival after allogeneic HSCT for haematological malignancy is 40-60%, where approximately 20-30% of recipients suffer relapses and 20-30% of recipients die of transplant-related complications. Overall survival depends on a number of factors including the stage of disease, age of the patient as well as the particular type of disease being treated. For example, whereas for ALL the 5-year overall survival in patients aged 14 or younger is over 90% this drops to 40% in patients between 25 and 65 years and for patients aged over 65 it is only 15% (Cancer Research UK). Every year more HSCT are performed in Europe with the same trend for autologous as well allogeneic HSCT. Even though the trend to use unrelated, but HLA-matched, donors has slowed it is still by far the most used therapeutic option. In recent years, the use of a haploidentical donor has become popular, this transplant is identified from a half-matched HLA family member, and although this increases the chances of finding a compatible match it increases the requirement for appropriate control of GvHD and related complications. In addition, autoimmune diseases, predominantly multiple sclerosis, are increasingly being treated by (autologous) HSCT, with transplantations increasing from around 50 in 2005 to more than 350 in 2016 to nearly 600 in 2018 (Passweg *et al.* 2018, Passweg *et al.* 2020).

1.3.1 Pre-transplant Conditioning

Prior to alloHSCT, transplant recipients have to undergo preparative conditioning with the most common form being chemotherapy but others forms exist such as radiotherapy or immunotherapy. This has several important purposes. Firstly, it has a myeloablative

effect which destroys the recipient's residual haemopoietic system, creating a space in the bone marrow niche for the incoming graft and reduces any tumour burden in the recipient. Secondly, it reduces the risk of rejection of the incoming graft as the therapy has severe immunosuppressive effects on the immune system of the recipient. The exact process of the conditioning regimen depends upon the recipient (e.g. age and status), the disease being treated (e.g. tumour burden; risk or relapse), the risk of rejection (e.g. HLA matched/mismatched, sibling or unrelated donor) as well as preference of the performing centre. Conditioning regimens can vary greatly in many ways (e.g. dose, dose rate, fractionation, interval between fractions or source of radiation) but the most common form is a radiation-based regimen consisting of total body irradiation (TBI) in combination with immunosuppressive drugs cyclophosphamide (CY) or cyclophosphamide and busulfan (BU). Simply said, the higher the genetic disparity, the stronger the immune suppression has to be and as higher the aggressiveness of the malignancy, the stronger the myelosuppression treatment. TBI and CY/BU are known as high-dose conditioning regimens and are most commonly used in patients undergoing alloHSCT for haematological malignancies due its immunosuppressive capacity, its effectiveness against most leukaemia's and lymphomas, and its ability to penetrate to sanctuary sites (Gyurkocza and Sandmaier 2014). Although these regimens have strong anti-tumour effects, they cause considerable damage and toxicities to many organs. This has to be carefully considered as a randomized trial of AML patients has shown that high doses had decreased relapse rates compared to patients receiving lower doses but also increased transplant-related deaths resulting in similar survival rates between both groups (Clift *et al.* 1990, Clift *et al.* 1998). As there is an increasing trend in haploidentical and less well HLA-matched donors it follows that more aggressive conditioning regimens are required at the cost of increased toxicities. New drugs are incorporated into regimens to lower toxicity such as treosulfan which is approved for treatment of advanced ovarian cancer. It is now also being trialed for alloHSCT in combination with fludarabine with or without low-dose TBI and has shown promising results as toxicity was significantly

lowered, transplant-related mortality was reduced and improvements shown in patient survival (Nemecek *et al.* 2011, Gyurkocza *et al.* 2014). Fludarabine in combination with cyclophosphamide and antithymocyte globulin (ATG) is now successfully used in patients undergoing allogeneic HSCT with severe aplastic anaemia and has significantly improved survival rates in comparison to cyclophosphamide and busulfan regimens (Bejanyan *et al.* 2019).

1.3.2 Donor Selection

The elucidation of HLA proteins has greatly advanced our understanding in how the immune system discriminates foreign from self-peptides. HLA proteins have a critical function in the immune system such as presentation of peptides to T-cells or B-cell differentiation into antibody-producing plasma cells. HLA class I molecules (A, B and C) are expressed on every nucleated cell whereas class II (DP, DQ and DR) are only expressed on B-lymphocytes, activated T-lymphocytes and antigen-presenting cells. The importance of HLA molecules was demonstrated by Zinkernagel and Dougherty in 1974 as T-lymphocytes must have the same HLA molecules as APCs to induce an immune response (Zinkernagel and Doherty 1974). In addition, HLA molecules recognise foreign antigens presented to them and induce an immune response (Montgomery *et al.* 2018). This is critical as a significant proportion of circulating T-cells are alloreactive i.e. recognise foreign or 'non-self' HLA. Due to structural difference encoded by sequence polymorphisms in HLA genes and minor histocompatibility antigens presented by HLA induce T-cell activation and immune reactions mediated by alloreactive T-cells and B-cells producing anti-HLA antibodies (Erlich *et al.* 2001). The polymorphic residue that define each unique HLA molecule are located within or very near the peptide binding groove and these genetic differences are recognised by alloreactive T-cells and distinguish between donor and recipient (Parham 1992, Erlich *et al.* 2001). In the context of bone marrow transplantation, alloreactive donor T-cells will

recognise and directly attack recipient cells bearing this foreign molecule or can attack donor cells with self HLA that have bound a recipient peptide (Erlich *et al.* 2001). In relation to alloHSCT, donor and recipient are HLA typed and ideally all 12 HLA antigens should match. Not all HLA antigens (e.g. HLA-DQ and HLA-DP) are as critical for successful alloHSCT than others (e.g. HLA-B) (Flomenberg *et al.* 2004, Passweg *et al.* 2015, Lazaryan *et al.* 2016). However, the greater the disparity in HLA matching, the greater the risk for complications and mortality (Lee *et al.* 2007). Another factor is the availability and choice of donor and this primarily is determined by family composition as full siblings have a one in four chance to be matched unless they are genetically identical twins. If a sibling is not available the other option is a HLA matched unrelated donor (MUD) through national or international bone marrow registries. Unfortunately, ethnicity plays a remarkable factor as the chance for a full match for Caucasian/European-origin patient is approximately 70-75%, whereas this number is 16-18% for African-Americans/Africans. One or two HLA-antigen mismatched transplants have been carried out in certain situations, however, GvHD and graft failure are more common (Buck *et al.* 2016, Singh and McGuirk 2016). Cord blood and partially HLA-matched related (haploidentical) donors is one option to overcome HLA mismatch, however these approaches come with serious complications which have yet to be overcome. The best option, if no HLA matched donor is available, is umbilical cord blood (UCB) of which many UCB banks exist worldwide. Challenges still remain as usually the sample size is small and engraftment and immune reconstitution is delayed in comparison to conventional transplants (Brunstein *et al.* 2010). However, from a HLA-matching point of view, this procedure is superior as lymphocytes in cord blood have minor immunological reactivity in comparison to lymphocytes from older donors (Krishnamurti *et al.* 2003). Both approaches may be appropriate for high risk disease or when no other option is available. Donor age is now considered to be the most non-HLA related influential factor in relation to overall survival. A study in fully matched URD has shown that 2-year overall survival rate can be increased by 3% when a donor is 10 years younger. This is also

reflected in the steady rise of selected donors under the age of 30 (36% in 1988-2006, 51% in 1999-2011 and 69% in 2012-2014) (Shaw *et al.* 2018).

1.3.3 Stem Cell Sources

Three options are available for the harvest of stem cells with many differences in cellular constituents and biological and immunological properties. The first one is the traditional bone marrow (BM) harvest which carried out under general anaesthesia and bone marrow is taken from the donor's hip (posterior superior iliac crests). While providing sufficient stem cells there is a small risk of wound infection, bleeding, pain at the site of collection and the requirement for general anaesthesia. The second option is peripheral blood stem cell (PBSC) harvest, this is preferred by most donors as it only requires withdrawal of blood from a peripheral vein making it less invasive than bone marrow harvest. However, to collect enough CD34+ stem cells from the blood for transplant, donors have to undergo repeated injections of granulocyte-colony stimulating factor (G-CSF) 4-7 days prior to harvest to mobilise stem cells from the bone marrow into the blood. This procedure is associated with low risks of infection, while there is a chance of thrombosis and failing to mobilise enough stem cells with a general lower yield of CD34+ cells (Siddiq *et al.* 2009, Cheuk 2013). Importantly, a greater risk of GvHD is associated with PBSC than with BM (Cheuk 2013). BM as well as PBSC allows the harvest of additional stem cell types from the donor if needed. The third option is cord blood (CB) which is donated from the blood of the placenta and umbilical cord of a new-born infant. However, if more stem cells are needed at a later time-point it is impossible to acquire but due to its immature state of lymphocyte this procedure is associated with the lowest risk of GvHD (Cheuk 2013). Today, most stem cell harvests are from PBSC harvests (95% of all autoHSCTs and 70% of all alloHSCTs) due to time and cost constraints (Passweg *et al.* 2016).

1.3.4 Graft Manipulation

To reduce the risk of GvHD and preserving GvL different approaches have been developed to manipulate the graft. Traditionally, pharmacological immunosuppressive drugs were used but are associated with serious complications such as acute and long-term toxicity, lack of antigen specificity and the requirement for long-term use. Therefore, novel approaches are continuously developed and tested to circumvent these problems. In addition, to make the graft more suitable to the recipient, several procedures can be implemented prior to engraftment. These include volume reduction, washing to reduce antibodies, anticoagulants or depletion of erythrocytes due to blood group incompatibility.

CD34+ stem cell enrichment is a common graft manipulation and, alongside depletes T-cell numbers, which reduces the chances of developing GvHD (Aversa *et al.* 1998, Ringhoffer *et al.* 2004). The process of CD34+ enrichment has been refined over the years and other cell types can be added to the depletion/enrichment process. Another approach is CD3+ T-cell depletion which is technically more demanding and even low numbers of contaminating T-cell can endanger the success of the manipulation. Depletion of CD3+ has been demonstrated to be effective with reduced treatment related mortality (TRM) rates even when combined with reduced toxicity and intensity conditioning regimens (Federmann *et al.* 2012, Lang *et al.* 2014). Alternatively, TCRαβ lymphocyte depletion can be used which has an even higher depletion efficacy than CD3+ depletion. Clinical trials have proven that this procedure results in very low incidences of GvHD, favourable engraftment and TRM rates (Kaynar *et al.* 2017, Locatelli *et al.* 2017). Often combined with T-cell depletion is B-cell depletion (CD19+) to further lower the risk of GvHD. Alternatively, B-cell depletion can be done *in vivo* by using anti-CD20 monoclonal antibodies (Locatelli *et al.* 2017). T-cell and B-cell depletion have the advantage that they preserve large numbers of NK cells in the graft. NK cells are critical as they provide immediate reactivity against viral infections and tumours. T-cells

may be added (donor lymphocyte infusion/DLI) to the graft or after transplantation to provide T-cell immunity in certain situations. However, care is to be taken as these strategies significantly increase the risk of infections and disease relapse. Which strategy is used depends on disease, donor and recipient, HLA-matching and transplant centre preferences.

1.4 Engraftment and Immune Reconstitution

The aim of alloHSCT is to reconstitute the recipient's immune system with that of the donor. For successful engraftment many factors play an important role, these include HLA-matching between donor and host, the amount of CD34+ stem cells transplanted and suppression of the donor's immune system to prevent graft rejection. Depending on the pre-conditioning regimen, myeloablative (irreversible cytopenia with requirement for stem cell support) versus non-myeloablative (minimal cytopenia with case-dependent requirement for stem cell support), a proportion of the host haematopoietic system may be present and in the initial phase of reconstitution mixed chimerism of host and donor peripheral blood cells is seen. Reconstitution of different cell types occur at different time-points starting with neutrophils between 14 to 30 days post-engraftment depending on the stem cell source (Seggewiss and Einsele 2010, Danby and Rocha 2014). NK cells are reconstituted within 30-100 days, T-cells around 6-12 months depending on age and CD19+ B-cells between 1-2 years (Seggewiss and Einsele 2010, Danby and Rocha 2014). CD4+ T-cells recover later than CD8+ T-cells as they depend more on thymic generation of CD4+CD45RA+ naïve T-cells leading to an imbalance of CD4+/CD8+ ratio (Fry and Mackall 2005, Huttunen *et al.* 2015). Recovery of CD4+ T-cells is critical as they encompass CD4+ Tregs which have been shown to improve outcome of alloHSCT by inducing immune homeostasis and suppress GvHD (Edinger and Hoffmann 2011). Patients receiving alloHSCT graft with a high Treg:CD4+ T-cell ratio had substantially reduced non-relapse mortality and increased overall survival (Rezvani *et al.* 2006,

Pastore *et al.* 2012, Danby *et al.* 2016). Further evidence to support the protective role of Tregs in HSCT is provided by a meta-analysis study by *Fisher, Roberts, Danby and colleagues* (Fisher *et al.* 2017). Here, 14 studies of HSCT with named Treg composition were analysed and clearly identified the benefits of a high proportion of Tregs in reduced acute GvHD and improved overall survival.

1.5 Graft versus Host Disease (GvHD)

Transplanted organs that are harvested from genetically non-identical donors to the recipient, which is the vast majority of transplants, elicit immune responses that are directed against foreign tissue antigens. This can have severe consequences and may ultimately result in the grafts rejection or, conversely, lead to Graft versus Host Disease (GvHD) in which the donor's immune cells attack the host. GvHD is commonly observed in allogeneic haematopoietic stem cell transplantation and is classified into two (overlapping) branches: acute and chronic. The biggest risk to GvHD is HLA-disparity and, the greater this disparity, the higher the probability of developing increasingly more severe GvHD. Other risk factors are patient age with a trend of ever older patients being treated resulting in more cases of GvHD, opposite sex in donor and recipient and nature of GvHD prophylaxis. In addition, polymorphisms in genes including cytokines are now appreciated to be associated with GvHD (Dickinson and Holler 2008, Gam *et al.* 2017). Acute GvHD is despite much progress in treatment very prevalent as around 30-50% of all patients undergoing alloHSCT develop aGvHD and 30-70% develop chronic GvHD with 14% develop severe cGvHD (grade III-IV) (Lee and Flowers 2008, Martin *et al.* 2012, Jagasia *et al.* 2015, Zeiser and Blazar 2017). Not only is GvHD a risk for mortality but also for morbidity.

1.5.1 Acute Graft versus Host Disease

Definitions of acute and chronic GvHD have changed over the years due to new techniques being used in processing stem cells, the use of new regimens to treat GvHD and new knowledge gained. In 2014 the National Institute of Health (NIH) proposed a revision of the 2005 NIH chronic GvHD (cGvHD) criteria even though it was found that the 2005 guidelines are clinically reliable measures (Kerep *et al.* 2019). This revision was intended to reflect the accumulated scientific evidence and clinical experience since the initial 2005 consensus meeting. Acute GvHD (aGvHD) (characterized by erythema,

vomiting, anorexia, profuse diarrhoea, maculopapular rash, nausea, ileus anorexia, profuse diarrhoea, or cholestatic liver disease; see Table 1) was divided into classic aGvHD occurring within 100 days after transplantation, persistent (extends after 100 days), recurrent (both before and after 100 days) and late onset (starts after 100 days). A further classification is overlap where one or more symptoms of aGvHD are present in a patient who is diagnosed with cGvHD (Jagasia *et al.* 2015).

Classically, aGvHD develops in three phases (Ball *et al.* 2008):

1. The afferent phase. A normal but strong immune response is induced leading to the activation of APCs. The pre-transplant conditioning regimen for alloHSCT leads to tissue damage and consequently excessive activation and secretion of pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 which in turn up-regulate MHC antigen presentation and cell surface adhesions molecules on host cells. Tissue damage leads to the release of damage-associated molecular patterns (DAMPs) into the extracellular space, such as ATP or uric acid, which can cause the activation of the Nlrp3 inflammasome which in turn activates IL-1β and enhances aGvHD (Jankovic *et al.* 2013). Intestinal epithelium damage releases bacteria and alteration to the microbiome further elevating the elicited immune response (Qayed and Horan 2015, Teshima *et al.* 2016). Release of pathogen-associated molecular patterns (PAMPs) and luminal damage-associated molecular patterns from damaged tissue also results in activation of APCs.
2. The efferent phase: The pro-inflammatory environment leads to the full activation of all APCs of the host as well as donor and present alloantigens to the donor's T-cells which is followed by T-cell activation, trafficking and expansion (Paczesny *et al.* 2010, Blazar *et al.* 2012). Donor naïve CD4+ cells differentiate into pro-inflammatory Th1, Th2 and Th17 effector cells releasing cytokines such as IL-2 and IFN-γ, IL-4, and IL17 and IL-21, respectively. It is thought that aGvHD is

mainly driven by CD4+ T-cells. Donor naïve CD8+ T-cells differentiate into cytotoxic T-lymphocytes and target tissues.

3. The effector phase: The pro-inflammatory response causes a complex cascade of migration and activation of many other cells types including macrophages, NK cells and neutrophils promoting further tissue damage, cell death and continual propagation of this pathway (Tanaka *et al.* 2012, Morris *et al.* 2013).

Clinical manifestations of acute GvHD is graded and staged in severity from grade I to IV (see Table 1). It most often involves the skin, liver, and gastrointestinal tract (GI) and occasionally the eye and oral mucosa. It is often characterised by skin rash, diarrhoea, elevated bilirubin and is also associated with recurrent infections. The severity of aGvHD depends on, among other factors, HLA-matching between donor and recipient, haematological malignancies and age. To reduce severity of aGvHD careful donor selection is required and manipulation of the graft (e.g. T-cell and B-cell depletion) and intensity of recipient conditioning (myeloblative vs non-myeloblative). There is a trend in alloHSCT to reduce the intensity of pre-conditioning but a recent phase III trial in young patients with AML or MDS has shown that intensive conditioning regimen patients were far less likely to experience disease relapse than patients who received less intensive regimens (Scott *et al.* 2017).

Clinical Stage	**Lower GI** (ml/d)	**Upper GI**	**Liver** (bilirubin level in mg/dL)	**Skin** (% rash of body surface arena BSA)
1	Diarrhoea 500-1000	Nausea/ vomiting	2-3	<25%
2	Diarrhoea 1000-1500		3-6	25-50%
3	Diarrhoea 1500-2000		6-15	Generalized erythroderma
4	Diarrhoea >2000 or pain or ileus		>15	Bullae/Desquamation

Clinical Grade	**Lower GI**	**Upper GI**	**Liver**	**Skin**
I	0	0	0	1-2
II	1	1	1	3
III	2-3		2-4	-
IV	4		-	4

Table 1. Clinical Manifestations and Grading System according to Glucksberg *et al*. and Prezepiorka *et al*. (1994 Consensus Conference on acute GVHD Grading) (Glucksberg *et al.* 1974, Przepiorka *et al.* 1995)

Despite conditioning aGvHD can often develop and pharmacological immunosuppression is required. There is no standardized preventive measure for aGvHD but most common agents used are a combination of a calcineurin inhibitor (cyclosporine or tacrolimus) and methotrexate. Calcineurin inhibitors suppress the transcription factor NFAT in T-cells which result in less IL-2 transcription and secretion (Dumont 2000, Matsuda and Koyasu 2000). IL-2 is required for T-effector activation and expansion and this reduces T-cell mediated responses at the cost of anti-inflammatory IL-2 dependent Tregs. To circumvent this dilemma, a new agent – cyclophosphamide − has been recently implemented to successfully prevent GvHD by depleting conventional T-cells and preserving Tregs (Luznik *et al.* 2010, Ganguly *et al.* 2014). The combination of a calcineurin inhibitor and methotrexate has been proven to be effective in the prevention of aGvHD (Chao *et al.* 1993, Ram *et al.* 2009). Other prophylaxis regimens include the use of anti-thymocyte globulin (ATG), an polyclonal antibody raised in rabbit (Sanofi Genzyme, USA and Neovii, Switzerland) and horse (Pfizer, USA and Bharat

Serums and Vaccines Ltd., India) and directed against T-cells, which have shown to reduce aGvHD as well cGvHD without compromising overall survival (Finke *et al.* 2009, Kroger *et al.* 2016, Siddiqui *et al.* 2019). Many other regimens/drug combinations are used depending on disease and patient status.

One novel approach is a Treg based treatment that has shown promising results in the reduction of aGvHD. In a clinical trial, patients were given donor lymphocytes cultured with IL-10-treated host APCs to induce IL-10 and TGF-β Treg-like cells (Tr1) 1-2 months after alloHSCT and half of these patients showed enhanced immune reconstitution (Bacchetta *et al.* 2014). Another prophylaxis approach is the adoptive transfer of Tregs and this also results in reduced aGvHD and immune reconstitution (Martelli *et al.* 2014, Brunstein *et al.* 2016). Other approaches include the inhibition of α-GalCer, a glycolipid, that expands and activates NKT-cells to subsequently expand Tregs in patients or the use of HDACs inhibitors which are known to play a key role in cytokine pathways in Treg biology (Choi *et al.* 2014, Chen *et al.* 2017). Many other novel approaches have been tested in pre-clinical models that focus on expanding and increasing the efficacy of Tregs. Such examples include Treg stimulation with a fusion protein that ligates the TNF receptor superfamily 25 (TNFRSF25) to trigger Treg expansion, agonistic TNFR2 antibody and agonistic DR3 antibody (Chopra *et al.* 2016, Nishikii *et al.* 2016, Wolf *et al.* 2017).

In recent years a lot of attention has been given to the human gut microbiome which consists of hundreds of bacterial species and an estimated 100 trillion microbial cells or 10 microbial cells for every host cell (Belkaid and Hand 2014). The intestine is one of the most effected organs in GvHD and bacteria are critical for its homeostasis and induction of Tregs through short chain fatty acids (SCFA). Antibiotics are a key line of defence in the clinic and infectious diseases represent one of the most frequently and feared complications of HSCT. However, reports link antimicrobial treatments to an increase in GvHD and associated mortality probably due to disequilibrium within the GI tract and its

microbiota-derived metabolites (Shono *et al.* 2016). For example, bacteria-derived SCFA butyrate can induce Tregs and transfer of selected human bacterial strains, such as *Clostridia* known to produce SCFA butyrate, into mice resulting in increased Treg frequency (Arpaia *et al.* 2013, Atarashi *et al.* 2013). In addition, butyrate driven histone acetylation is important for intestinal epithelial cells integrity and mitigation of GvHD which may be in part through Treg induction (Mathewson *et al.* 2016). Indeed, butyrate given via the GI tract also resulted in elevated Treg numbers, however, with the disadvantage that repeated injections would be necessary over the course of GvHD (Taylor *et al.* 2002, Edinger *et al.* 2003).

Tregs can be isolated on large scale from peripheral blood and umbilical cord blood and have been used in the clinic (Brunstein *et al.* 2011, Safinia *et al.* 2016). Isolation of Tregs by GMP (Good Manufacturing Practice) approved CliniMACS has been proven difficult as contaminating cells reduce purity of FOXP3 (Forkhead box P3; for detailed explanation see chapter 3) expressing cells to 80% which can have deleterious consequences (Di Ianni *et al.* 2012). A similar approach is the sorting of Tregs by flow cytometry which achieves purity of ≥98% CD4+CD25+CD127dim Tregs. Tregs were expanded using anti-CD3/CD28 and IL-2, achieving >90% FOXP3+ Tregs (Marek-Trzonkowska *et al.* 2014, Bluestone *et al.* 2015). In addition, TSDR (Treg-specific demethylation region) methylation status confirmed purity and lineage stability of expanded Tregs (Bluestone *et al.* 2015). Interestingly, expanded Tregs supressed *in vitro* to a much greater extent than freshly isolated Tregs from the same individual (Bluestone *et al.* 2015). Another complication is the short half-life of *ex vivo* expanded Tregs, however, only one study used deuterium to track infused Tregs (Bluestone *et al.* 2015). One approach to circumvent this might be the isolation of CD4+CD25+CD127dimCD45RA+ Tregs, which, due to their epigenetic programming, express stable FOXP3 and their naïve phenotype avoids isolation of activated T-cells which express intermediate CD25 levels (Canavan *et al.* 2016). However, a large number

of PBMCs have to be processed which requires CD4+ pre-enrichment and considerably increases the cost of a single preparation. To date, no clinical trial within the EU has utilized this method due to GMP constraints but flow cytometers and antibodies are in development to comply with GMP requirements (Romano *et al.* 2019).

New clinical protocols are developed for Treg *ex vivo* expansion as Treg numbers in peripheral blood are low. One example is the use of rapamycin, in combination with anti-CD3/CD28 and IL-2, which efficiently suppresses the proliferation of contaminating Teffector cells, favours the upregulation of FOXP3 and confers their suppressive capacity (Battaglia *et al.* 2006, Thomson *et al.* 2009, Safinia *et al.* 2016). Other protocols involve the use of TGF-β and/or Retinoic acid to expand Tregs (Schiavinato *et al.* 2017, Mathew *et al.* 2018). Use of retinoic acid has the advantage of inducing gut homing receptors (CCR7 and α4β7) on Tregs which might ameliorate gut GvHD.

Ex vivo expanded Tregs have been successfully used in a few clinical trials treating haematological malignancies and GvHD. In addition many phase I/II trials are ongoing for the treatment of autoimmune diseases as well as liver and kidney transplants (Romano *et al.* 2019). Brunstein *et al.* (2011) used *ex vivo* expanded cryopreserved UCB Tregs in 23 patients with advanced or high-risk haematological malignancies undergoing allogeneic double UCB transplantation. No toxicities were observed during infusion and grade II-IV aGVHD was significantly reduced in comparison to historical data with no deleterious effect on risks of infection, relapse or early mortality (Brunstein *et al.* 2011). A study investigating the effect of *ex vivo* expanded Tregs in HLA-haploidentical stem cell transplantation of high risk acute leukaemia found that relapse could be significantly reduced (Martelli *et al.* 2014). However, no statistically significant effect on GvHD could be detected in comparison to historic cohort which might be attributed to cohort receiving extensive T-cell depleted grafts and ATG for *in vivo* T-cell depletion.

Despite the best efforts to prevent aGvHD with prophylaxis therapies this disease has a high incidence after alloHSCT. Grade I aGvHD is usually treated with topical steroids

alone and no systemic treatment is required. However, higher severity requires systemic treatment and mainstay treatment is high-dose methyl-prednisolone (glucocorticoid) which is gradually lowered over time. Prolonged high doses of steroids are very immunocompromising and make it challenging to control infections. Glucocorticoids are synthesised and released by the adrenal gland and are produced in the response to stress and physiological cues. These steroid hormones signal through genomic and non-genomic pathways and genomic actions are mediated by the glucocorticoid receptor, which in the absence of the hormone is mainly found in the cytoplasm, but on ligand binding rapidly shuttles into the nucleus and binds to glucocorticoid response element or by tethering to other transcription factors which results in the activation and repression of genes (Ramamoorthy and Cidlowski 2016). Corticosteroids have been shown in randomized controlled trials to be the best option and patients who are refractory to corticosteroids have only a 5-30% long term overall survival prognosis (Mielcarek *et al.* 2015). If corticosteroids fail, then second-line therapy is required which is usually monoclonal antibodies against T-cells such as alemtuzumab which recognises the T-cell maker CD52. Unfortunately, after initial control of aGvHD by alemtuzumab and related drugs, patients usually relapse with 80% mortality (Holler *et al.* 2019). Many novel targets are tested to alleviate or prevent aGVHD such as down-stream signals of cytokine pathways (JAK1/2) or co-stimulatory pathways (CD28) (Hill *et al.* 2018).

1.5.2 Chronic Graft versus Host Disease

Chronic Graft versus Host Disease (cGvHD) manifests as a multi-systemic disorder and historically has been defined to occur later than 100 days post-transplant. New diagnostic features outlined in the NIH consensus criteria (2014) established that distinct cGvHD clinical features may occur before day 100 and may occur simultaneously with aGvHD (Jagasia *et al.* 2015). Clinical features and severity score of cGvHD are summarized in Tables 2 and 3. How each individual organ is scored is beyond the scope of this section,

however, unified documents exist for each organ to calculate a score based on certain criteria. Whereas aGvHD is limited in most cases to a few organs with distinct features chronic GvHD can affect many more organs and can imitate almost any autoimmune disease.

Chronic GvHD is a major cause of morbidity and non-relapse mortality (NRM) after alloHSCT. Most deaths after HSCT occur within 2 years after transplant as a result of relapse, infection, acute or chronic GvHD or other transplant-related toxicities, although mortality and morbidity varies significantly between malignancies and studies (Wingard *et al.* 2011). Interestingly, mild forms of cGvHD can have protective effects with a lower recurrence of their underlying disease and even patients with moderate cGvHD have similar survival rates than patients without any cGvHD (Kuzmina *et al.* 2012, Grube *et al.* 2016). On the other hand, severe cGvHD has a mortality rate as high as 50%, but by 2 years post-engraftment death becomes more infrequent (Csanadi *et al.* 2019). In fact, 80-92% of 2-year post-engraftment patients survived for 10 years or longer with the main risk for later deaths associated with age and GvHD (Wingard *et al.* 2011). A large multi-centre study found that around 50% of all HSCT patients develop cGvHD (Arora *et al.* 2016). Reasons for this trend in cGvHD cases is the source of stem cells as (1) less well HLA-matched donors are more often used, (2) an increase of PBSC over BM donors and (3) older recipients (Flowers *et al.* 2011). Haploidentical donors have emerged as increasingly used donor source but due to the lower HLA match cGvHD is more frequent. (Passweg *et al.* 2015, Wang *et al.* 2015). A randomized trial investigating the effect between PBSC and BM on recipient found that PBSC leads to accelerated engraftment and fewer graft failures but at the cost of increased incidence of cGvHD (Stem Cell Trialists' Collaborative 2005, Anasetti *et al.* 2012). The strongest predictor of chronic GvHD is the severity of acute GvHD. First-line treatment of cGvHD is similar to that of aGvHD with prolonged treatment periods.

Organ or Site	**Diagnostic** (Sufficient to Establish the Diagnosis of Chronic GvHD)	**Distinctive**[1] (Seen in chronic GvHD, but Insufficient Alone to Establish a Diagnosis)	**Other Features or Unclassified Entities**[2]	**Common**[3] (Seen with Both Acute and Chronic GvHD)
Skin	Poikiloderma Lichen planuse-like features Sclerotic features Morphea-like features Lichen sclerosus-like features	Depigmentation Papulosquamous lesions	Sweat impairment ichthyosis Keratosis pilaris Hypopigmentation Hyperpigmentation	Erythema Maculopapular rash Pruritus
Nails		Dystrophy Longitudinal ridging, splitting or brittle features Onycholysis Pterygium unguis Nail loss (usually symmetric, affects most nails)		
Scalp and body hair		New onset of scarring or nonscarring scalp Alopecia (after recovery from chemoradiotherapy) Loss of body hair Scaling	Thinning scalp hair, typically patchy, coarse or dull (not explained by endocrine or other causes) Premature gray hair	
Mouth	Lichen planus-like changes	Xerostomia Mucoceles Mucosal atrophy Ulcers Pseudomembranes		Gingivitis mucositis Erythema Pain
Eyes		New onset dry, gritty, or painful eyes	Photophobia	

		Cicatricial conjunctivitis KCS Confluent areas of punctate keratopathy	Periorbital hyperpigmentation Blepharitis (erythema of the eyelids with odemaoodema)	
Genitalia	Lichen planus-like features Lichen sclerosus-like features	Erosions Fissures		
Females	Vaginal scarring or clitoral/labial agglutination	Ulcers		
Males	Phimosis or urethral/meatus scarring or stenosis			
GI Tract	Oesophageal web Strictures or stenosis in the upper to mid third of the oesophagus		Exocrine pancreatic insufficiency	Anorexia Nausea Vomiting Diarrhoea Weight loss Failure to thrive (infants and children)
Liver				Total bilirubin, alkaline phosphatase > 2 x upper limit of normal ALT > 2 x upper limit of normal
Lung	Bronchiolitis obliterans diagnosed with lung biopsy BOS[4]	Air trapping and bronchiectasis on chest CT	Cryptogenic organizing pneumonia Restrictive lung disease[5]	
Muscles, fascia, joints	Fasciitis Joint stiffness or contractures	Myositis or polymyositis[6]	Odema Muscle cramps Arthralgia or arthritis	

Heamatopoietic and Immune			Thrombocytopenia Eosinophilia lymphopenia Hypo- or hyper-gammaglobulinemia Autoantibodies (AIHA, ITP) Raynaud's phenomenon	
Other			Pericardial or pleural effusions Ascites Peripheral neuropathy Nephrotic syndrome Myasthenia gravis Cardiac conduction abnormality or cardiomyopathy	

Table 2. NIH Consensus 2014 Criteria for chronic Graft versus Host Disease (cGvHD).

[1] In all cases, infection, drug effect, malignancy, or other causes must be excluded.

[2] Can be acknowledged as part of the chronic GvHD manifestations if diagnosis is confirmed.

[3] Common refers to shared features by both acute and chronic GvHD.

[4] BOS can be diagnostic for lung chronic GvHD only if distinctive sign or symptom present in another organ (see text).

[5] Pulmonary entities under investigation or unclassified.

[6] Diagnosis of chronic GvHD requires biopsy.

Taken from: Jagasia, M. H., *et al.* (2015). "National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group report." Biol Blood Marrow Transplant **21**(3): 389-401 e381.

Severity score of chronic GvHD
Mild chronic GvHD 1 or 2 Organs involved with no more than score 1 plus Lung score 0
Moderate chronic GvHD 3 or more organs involved with no more than score 1 OR At least 1 organ (not lung) with a score of 2 OR Lung score 1
Severe chronic GvHD At least 1 organ with a score of 3 OR Lung score of 2 or 3

Table 3. NIH Consensus 2014 Scoring of Severity for chronic Graft versus Host Disease (cGvHD).

2. Aldehyde Dehydrogenase and Graft versus Host Disease

Aldehyde dehydrogenases (ALDH) have many important functions such as protection against oxidative stress, cellular detoxification, mitochondrial permeability, amino acid metabolism or production of retinoic acid (RA) and at least one family member of this large superfamily is found in the majority of mammalian tissues (Brocker *et al.* 2010, Seitz and Stickel 2010, Jackson *et al.* 2011). Besides these crucial protective tasks ALDH activity is involved not only in tumour pathogenesis in various carcinomas but also resistance to cancer therapies (Marcato *et al.* 2011). Overexpression of many different ALDH isozymes in tumour tissues have been associated with poor prognosis. Cancer stem cells have elevated ALDH expression and are known to drive tumour proliferation, repopulation after various insults (e.g. chemotherapy, targeted therapy, radiation) and metastasis (Muzio *et al.* 2012, Li *et al.* 2015, Vlashi and Pajonk 2015). Furthermore, cancer stem cells are resistant to many therapies and can cause relapse and progression to more severe disease (Muzio *et al.* 2012). Consequently, inhibition of ALDH activity has been a promising and active field of cancer research. However the role of ALDH in disease is complicated as several murine studies have demonstrated a protective role of ALDH activity and retinoic acid in autoimmune diseases such as multiple sclerosis, systemic lupus, collagen-induced arthritis or EAE (see 2.1) (Racke *et al.* 1995, Kinoshita *et al.* 2003, Perez de Lema *et al.* 2004, Xiao *et al.* 2008, Kwok *et al.* 2012). However, conflicting evidence has been published of RA involvement in GvHD as murine studies have demonstrated that ALDH activity and RA can exacerbate GvHD (see 2.2).

Aldehyde dehydrogenases are a superfamily of 19 NAD(P)+-dependent enzymes which oxidize a wide range of exogenous and endogenous aldehydes to their corresponding carboxylic acids (Marchitti *et al.* 2008). Vitamin A, an essential micronutrient, is stored as retinyl esters in hepatic stellate cells and is subsequently converted into retinol and then into retinal. Biosynthesis of retinal into biologically active retinoic acid (RA) is performed by family members of the ALDH superfamily ALDH1A1 (RALDH1 in mice,

predominantly expressed by intestinal stromal cells and IECs), ALDH1A2 (RALDH2, predominantly expressed by DCs) and ALDH1A3 (RALDH3) (Yokota *et al.* 2009, Vicente-Suarez *et al.* 2015). RA is catabolized by enzymes such as cytochrome P450 – mainly from the CYP26 family which predominantly include CYP26A1, CYP26B1 and CYP26C1 and are widely expressed by immune cells (Guo *et al.* 2015). To exert function RA binds to either nuclear retinoic acid receptor (RAR) or retinoid x receptor (RXR) which can form homo- or heterodimers which shuttle into the cells nuclei and bind to the retinoic acid response element (RARE) (Bushue and Wan 2010). Especially RXR can bind a variety of different receptors to activate or silence its binding partner which in turn regulates gene expression. In addition, it has been demonstrated that RA can also regulate non-genomic actions through modulating transcription factors such as NF-κB, IFN-γ and TGF-β (Connolly *et al.* 2013). Binding partners include among others fatty acid receptor peroxisomal proliferator activated receptor (PPAR), vitamin D receptor (VDR) or RARs (Bushue and Wan 2010).

2.1 Retinoic Acid and T-cell Differentiation

Professional antigen presenting cells, such as dendritic cells (DCs), are critical players in T-cells immune responses and in bridging innate and adaptive immunity. Dendritic cells have vast roles in immunity which include priming and differentiation of naïve to effector CD4+ T-cells or promotion of B-cell antibody responses. In addition, DCs play a critical role in maintenance of immune homeostasis as they phagocytose self-antigen derived from apoptotic cells. DCs also recognise many inflammatory stimuli including pathogen-associated molecular patterns (PAMPs), endogenous cytokines or damage-associated molecular pattern molecules (DAMPs) which activate survival and inflammatory genes, induce DC maturation and enhance antigen presentation (Wehr *et al.* 2019).

Dendritic cells also play a key role in the induction of peripheral Tregs (pTregs) and it has been demonstrated that up-regulation of ALDH in DCs and production of RA increases their ability to induce pTregs (Bazewicz *et al.* 2019). One of the main organs mediating this specific interaction is the gastrointestinal (GI) tract which is also one of the classical organs affected in acute and chronic GvHD. The GI tract is exposed to a vast variety of beneficial and non-harmful antigens derived from ingested substances and organisms and induction of pTregs is crucial in keeping intestinal homeostasis (Steele *et al.* 2012, Kim *et al.* 2016). For example, lamina propria (LP) DC capture antigens in the intestine migrate to the mesenteric lymph node (MLN) where antigen presentation to T-cells can induce pTregs (Shiokawa *et al.* 2017). DC mediated induction of Tregs can be potentiated by several factors such as RA, TGF-β or IDO and DCs isolated from LP or MLN showed high levels of *Aldh1a2* expression (Matteoli *et al.* 2010, Shiokawa *et al.* 2017). CD4+CD25- T-cells cultured with MLN or splenic DCs in combination with TGF-β and RA induced high levels of Fopx3+ cells, whereas either combination alone failed to do so. This effect was also seen on the induction of CD8+ T-cells which implies that the synergistic effect of TGF-β and RA might not be restricted to Tregs (Mucida *et al.* 2007). To test if *in vitro* TGF-β and RA induced Fopx3+ Tregs are suppressive *in vivo*, naïve CD4+ T-cells, which induce colitis in immune-deficient mice, were co-transferred with differentially treated CD4+ T-cells. Mice who received CD4+ cells activated without cytokine conditioning developed severe colitis whereas TGF-β treated CD4+ cells partly protected and TGF-β and RA activated CD4+ cells fully protected against colitis induction (Mucida *et al.* 2007). In addition, RA produced by intestinal DCs can induce the gut-homing receptors CCR9 and α4β7 on Tregs (Benson *et al.* 2007). Recent findings have illustrated the important relationship between the gut microbiome and immune homeostasis. One such study has demonstrated that the bacteria *lactobacillus planatarum* enhances ALDH activity in DCs which, in turn, drives pTreg induction (Yoshida *et al.* 2013). Other cells types have been shown to express

ALDH such as LP macrophages which also contribute to pTreg differentiation (Denning *et al.* 2007).

Inhibition of Teffector differentiation and cytokine secretion is key in controlling autoimmune diseases. Many autoimmune diseases are driven by differentiation of naïve CD4+ cells into Th1 or Th17 which in turn secrete pro-inflammatory cytokines. TGF-β drives differentiation of naïve CD4+ cells into pTregs however in the combination with IL-6, Th17 differentiation is favoured (Korn *et al.* 2009). Addition of RA to TGF-β and IL-6 inhibits the differentiation of naïve CD4+ to Th17 cells and secretion of IL-17 *in vitro* (Mucida *et al.* 2007, Xiao *et al.* 2008). However, RA alone has no effect on Th17 differentiation. Interestingly, TGF-β but not IL-6 nor IL-21 up-regulates IL-6α receptor and addition of RA inhibits this upregulation to block IL-6 signalling and consequently Th17 differentiation (Xiao *et al.* 2008). Activated CD4+ cells treated with TGF-β, IL-6 and RA led to significantly higher levels of *Smad3* but not *Smad2* mRNA expression in comparison without RA. In addition, Smad3 and phosphorylated Smad3 protein levels were significantly increased, whereas Smad2 was unaffected when RA was added (Xiao *et al.* 2008). Smad3 signalling is known to be involved in the upregulation of FOXP3 and Smad3 deficient mice have enhanced Th17 differentiation suggesting a skew in Treg:Th17 ratios (Martinez *et al.* 2009). STAT3 and Interferon regulator factor 4 (IRF4), being key mediators of Th17 differentiation of naïve CD4+ T-cells, are likely to play a role in this process. Naïve CD4+ cells activated by IL-6 secrete IL-21 through STAT3 dependent signalling and IL-21 in combination with TGF-β induce the differentiation of naïve CD4+ T-cells to RORγt Th17 cells (Xiao *et al.* 2008). IL-21, which amplifies its own production by an autocrine feedback loop, also acts upstream of RORγt mainly through STAT3 to induce the expression of IL-23R. This signalling stabilizes the Th17 phenotype further as engagement of IL-23 with its cognate receptor induces RORγt expression (Bettelli *et al.* 2007, McGeachy *et al.* 2007). Addition of RA to TGF-β and IL-6 reduces the phosphorylation of STAT3, however RA alone does not reduce phosphorylation of

STAT3 induced by IL-6 or IL-21 indicating that RA does not affect IL-6/IL-21 signalling directly (Xiao *et al.* 2008). Furthermore, IRF4 expression was significantly reduced when RA was added to TGF-β with or without IL-6 or IL-21, but could not reduce IRF4 expression induced by IL-6 and IL-21 alone. These findings suggest that a mode of action of RA is through modulating TGF-β function. Experimental autoimmune encephalomyelitis (EAE) is a widely used murine autoimmune disease model and pathology is mediated by Th1 and Th17 responses. *In vivo* experiments using the EAE murine model confirmed that RA administration reduces Th17 and Th1 responses (Xiao *et al.* 2008)

2.2 Aldehyde Dehydrogenase and Graft versus Host Disease

In contrast to the beneficial effects of RA described in autoimmune disease models, opposing observations have been documented in GvHD where inhibition of RA results in a better clinical outcome. Several studies reported that RA signalling influences T-cell differentiation to a pro-inflammatory phenotype to drive GvHD progression, inhibition of RA signalling or vitamin A deficiency ameliorated GvHD in mice (Aoyama *et al.* 2013, Chen *et al.* 2013, Dodge *et al.* 2016, Thangavelu *et al.* 2019).

Recently, Aoyama *et al.* (2013) demonstrated that inhibition of RA ameliorates GvHD by modifying T-cell differentiation and intestinal migration in a murine bone marrow transplantation (BMT) model. RA metabolism was analysed 7 and 14 days post-BMT using mice expressing a retinoic acid responsive element (RARE) luciferase reporter. Mice showed increased levels of RARE activity within organs classically affected by GvHD, the small intestine and colon, but not in the lung and liver. Furthermore, RA levels were measured in these tissues and showed significantly increased levels in the colon (Aoyama *et al.* 2013, Chen *et al.* 2013). To investigate the role of RA signalling on donor T-cells in a GvHD model, bone marrow (BM) with or without T-cells expressing RARE luciferase reporter were transplanted and demonstrated that donor T-cells could sense

heightened RA levels in GvHD as demonstrated by increased RARE activity as GvHD progressed. At 14 days post-BMT over 90% of haematopoietic cells were donor derived in mesenteric lymph node (MLN) and in the small intestine and, not surprisingly, donor DCs and macrophages from MLN and lamina propria lymphocytes (LPL) showed significantly increased aldehyde dehydrogenase (ALDH) activity, the enzyme which drives RA production, in comparison to non-GvHD mice. Crucially, RA administration in post-transplantation mice significantly increased GvHD lethality. Nuclear receptor RARα is expressed in CD4+ and CD8+ T-cells and is critical for RA mediated signalling (Hall *et al.* 2011, Guo *et al.* 2012). Mice who received dominant-negative (dn) RARα donor CD4+ T-cells (RAR signalling deficiency restricted to CD4+ cells) to induce GvHD showed signs of GvHD but survived >100 days post-BMT with significantly lower GvHD scores for lung, spleen, liver, small intestine and colon tissues whereas all control and WT mice died clearly demonstrating the effect of RAR signalling on T-cell function. In addition, dnRARα-CD4+ mice showed lower levels of IFN-γ and higher levels of IL-4 and IL-17 secreting CD4+. Consistent with the improved GvHD score, dnRARα-CD4+ mice showed lower levels of infiltrating gut homing CXCR3+, α4β7+ or CCR9+ CD4+ cells into tissues post-BMT reducing the capability to migrate and initiate GvHD. This was reflected in a significant overall reduction in CD4+ and CD8+ cells in LPL. These observations are in line with a recent report which demonstrated that vitamin A deficiency reduced intestinal GvHD (Koenecke *et al.* 2012). RA has been demonstrated to promote TGF-β mediated conversion of naïve CD4+ cells into Foxp3+ cells (see 2.1). As expected, i*n vitro* experiments showed a reduced ability of dnRARα-CD4+ T-cells to differentiate into FOXP3+ cells, but, surprisingly, *in vivo* quantification of post-BMT T-cell composition indicated significantly higher Fopx3+ cells in recipient mice receiving dnRARα-CD4+ T-cells. Importantly, RARα deficient CD4+ T-cells did not compromise GvL effect but ameliorated GvHD in mice.

Further evidence is provided by an elegant study conducted by Thangavelu *et al.* (2019) that specifically investigated which RA producing and catabolizing cells influence GvHD. Overexpression of catabolizing enzyme CYP26A1 in haematopoietic cells such as DC but not in non-haematopoietic cells such as IECs significantly reduced the capability of RA production. Recipient mice with this phenotype undergoing fully mismatched BMT displayed significantly improved overall survival and clinical score (Thangavelu *et al.* 2019). Conversely, experiments in which donor haematopoietic cells overexpressed CYP26A1 only showed modest amelioration of GvHD with no recipient surviving beyond 2 months post-BMT. DCs are sufficient to induce GvHD-related mortality and recipient mice deficient of DC RALDH2 showed, as expected, significantly reduced initial RALDH2 activity after BMT but donor DCs brought activity back to normal levels in progressive days. However, recipient RALDH2-deficient mice undergoing BMT had significantly improved overall survival and reduced clinical scores. To support these findings, complementary experiments showed RALDH2-deficient donor DCs modestly ameliorated GvHD and survival. Importantly, the same experiments were conducted for IECs overexpressing RA catabolizing enzyme CYP26A1 or IECs-deficient of RA producing enzyme RALDH1 and showed no beneficial effect on GvHD. This clearly demonstrates the importance of DCs for GvHD initiation, progression and lethality. Recipient RALDH2-deficient mice had significantly lower levels of IFN-γ expressing CD4+ and CD8+ as well as lower numbers of TNF-α expressing CD8+ T-cells in spleen and intestine. In addition, donor CD4+ cells upregulated co-inhibitory molecules PD-1 and Lag3, and donor T-cells had reduced gut-homing receptor expression in RALDH2-deficient DC recipient mice, all these factors are likely to contribute to the reduction in GvHD. As Aoyama *et al*. (2013) reported in their study the same was seen with RA administration that significantly accelerated GvHD and overrode the previously observed RALDH2-deficient DC outcome. Furthermore, Treg frequency was increased in RALDH2-deficient DC recipient mice post-BMT in comparison to WT. RALDH2-deficient DC recipient mice were infused with either BM + intact T-cells or BM + CD4+CD25- and

both groups survived significantly longer than their control littermates. This suggests that the increased Treg frequency is due to *in vivo* pTreg generation rather than expansion of the few remaining thymic-derived Tregs from the donor.

Despite many reports that RA has beneficial effects on autoimmune diseases, it appears detrimental to a favourable BMT and promotes GvHD. These data also provide insights into the critical pathways for the pathogenesis of aGvHD. Although many immune cells types help drive GvHD, DCs appear essential for GvHD initiation while macrophages and B-cells are not (MacDonald *et al.* 2010, Matte-Martone *et al.* 2010). IECs are capable of driving GvHD, however, in the context RA signalling IECs do not appear to play a significant role (Koyama *et al.* 2015). One explanation could be that other cell types such as villin cells or DCs may act as a source of RA or that RA is simply associated with GvHD and not causal. On the other hand, production of RA by DCs plays a critical role in GvHD lethality as well in influencing T-helper subsets as the T-cells are skewed less towards pro-inflammatory Th1 cells. Despite many reports that RA signalling in DC or gut bacteria leads to pTreg induction, Tregs frequencies were higher despite RALDH2 deficiency in DCs. This could be due to reduced numbers of expanding donor T-cells and secretion of cytokines such as IL-21 which create a favourable environment for Treg induction as well as highlighting the effect of RA on immune cells might be highly context specific. In conclusion, ALDH (RALDH) inhibition seems to be a promising therapeutic target in prevention of GvHD and deserves further investigation. So far no direct inhibitor of ALDH activity has been investigated in a clinical trial particularly not for the treatment of GvHD. However, in cancers (e.g. breast cancers) many different agents are trialled against various ALDH isoforms in animal studies but none has been investigated in a clinical trial yet (Dinavahi *et al.* 2019).

3. Regulatory T-cells

T-regulatory cells (Tregs) are an important component of adaptive immune responses and play a critical role in suppressing autoimmunity and orchestrating peripheral immune tolerance. The existence of such a population was proposed over 50 years ago, however, it took until 1995 to definitively prove the existence of such a population due to a lack of phenotypic markers. In the late 1960s first evidence emerged that adaptive immunity was the product of two major classes of lymphocytes – B-cells and T-cells. Shortly after, evidence emerged that T-lymphocytes not only drive immune responses but also can suppress them (Allison *et al.* 1971, Droege 1971, Gershon and Kondo 1971, Rich and Pierce 1973). These cells were termed 'suppressor cells' and amid the logic of the argument that the immune system needs cells to control excessive inflammation in combination with emerging experimental evidence led to quick acceptance of this population (Germain 2008). The arrival of new techniques including detailed phenotyping using monoclonal antibodies, molecular cloning or genomic sequencing started to fracture the field as many previous results were identified as false or flawed, raising major concerns over the so called 'suppressor cells', which led to loss of momentum and basically to a complete hold of research into this field (Germain 2008).

Studies of autoimmunity in thymectomized mice suggested that a subset of T-cells was critical to immune homeostasis and control of T-effector populations (Nishizuka 1982). Observations that transfer of T-cells with certain activation/memory markers (CD4+CD45RBhigh) into immunodeficient hosts led to autoimmunity and that addition of certain T-cell subsets (CD4+CD45RBlow or unfractionated CD4+) could prevent disease provided further evidence of the existence of 'suppressor cells' within the CD4+ T-cells (Powrie and Mason 1990, Powrie *et al.* 1993). In 1995, Sakaguchi and colleagues described a specific murine population of CD4+CD25- T-cells that when isolated from BALB/c nu/+ mice (immunocompetent) and transferred into BALB/c nu/nu mice (immunodeficient, athymic) introduced widespread autoimmunity but could be rescued

by co-transfer of donor CD4+CD25+ (Sakaguchi *et al.* 1995). These cells would become known as T-regulatory cells and were subsequently shown to exist in humans (Shevach 2001, Stephens *et al.* 2001). There has been extensive interest and research in Treg biology as potential therapeutic targets in diverse diseases such as autoimmune diseases or cancers but Tregs have very distinct roles in respective pathophysiology of these conditions. While in autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis or transplantation-related diseases such as Graft versus Host Disease (GvHD) a high ratio of Tregs to effector cells may suppress activated pathological T-effector cells the opposite is true for cancer as Tregs may hinder anti-tumoral responses. For example a high ratio of Tregs to cytotoxic CD8+ T-cells in tumours is an indicator of poor prognosis in cancer (Nishikawa and Sakaguchi 2014).

3.1 Treg Phenotypic Markers

In humans and mice, Tregs are defined as CD4+ T-cells with constitutive expression of the FOXP3 protein. This forkhead transcription factor is often referred to as the master regulator of this lineage as it is critical for Treg development, function and stability (Fontenot *et al.* 2003, Hori *et al.* 2003, Lu *et al.* 2017). Scurfy mice have functional mutations and/or deficiency of *Foxp3*, causing a deficiency in natural Tregs and a severe and life-threating development of autoimmune diseases, demonstrating the crucial importance of Foxp3 to Treg function (Brunkow *et al.* 2001, Fontenot *et al.* 2003).

The scurfy mice phenotype arose from a spontaneous mutation in mice at the Oak Ridge National Laboratory (USA) in 1949 and these mice were characterized by scaly and ruffled skin, reddened eyes, enlargement of the spleen and lymph nodes and premature death at around 3 weeks after birth. It was the first X-linked disease to reported in mice and is caused by a lethal recessive mutation (Russell *et al.* 1959). Decades past before the first immunological studies investigated scurfy mice, describing the dramatic infiltration of lymphoid and myeloid cells into multiple organs and the pronounced

autoimmune pathology (Godfrey *et al.* 1991, Godfrey *et al.* 1991). Over the years several groups investigated further and demonstrated the absolute T-cell dependence of this phenotype (Blair *et al.* 1994, Ramsdell and Ziegler 2014). In addition, researchers noticed that scurfy mice resembled mice that failed to express key T-cell suppressor molecule cytotoxic T lymphocyte antigen 4 (CTLA-4) (Tivol *et al.* 1995, Waterhouse *et al.* 1995). This observation led researcher to attempt to map and clone the gene responsible for the scurfy phenotype. A team led by Mary Brunkow identified several open reading frames in part of the sequenced critical X-chromosome region and all exons for each gene were sequenced in both normal and scurfy mice. One of these genes was termed *Foxp3* and was the only coding mutation found in the critical region of scurfy mice (Brunkow *et al.* 2001). To confirm that *Foxp3* was the casual gene, transgenic mice expressing varying levels of wild-type (WT) *Foxp3* gene were backcrossed onto scurfy mice. All mice carrying both the WT *Foxp3* gene and scurfy mutation appeared normal and viable and thus confirmed that the 2-bp mutation found in *Foxp3* was responsible for the scurfy phenotype (Brunkow *et al.* 2001).

Sakaguchi and colleagues had demonstrated that in mice CD4+CD25+ T-cells were responsible for the suppression of CD4+CD25- Teffector cells. It was then demonstrated that, in mice, only CD4+CD25+ cells would express Foxp3 and the greater the Foxp3 expression in transgenic mice the smaller the T-cell compartment (Khattri *et al.* 2001, Khattri *et al.* 2003). Around the same time, the Treg *in vitro* suppression assay was developed to measure Treg suppressive capacity (Takahashi *et al.* 1998, Thornton and Shevach 1998). CD4+CD25+ T-cells were co-cultured with CD4+CD25- T-cells and stimulated with CD3-specific monoclonal antibodies. To establish if CD4+CD25+FOXP3+ represent an own T-cell lineage, an *in vivo* model was developed that expressed green fluorescent protein (GFP) under the control of the *Foxp3* locus (Fontenot *et al.* 2005). This study demonstrated that only a small subset of αβ T-cell receptor (TCR) expressing T-cells express Foxp3. Furthermore, loss of *Foxp3* in

conventional CD4+CD25- T-cells had no impact on CD4+CD25+ Treg cell function. In addition, expression of *Foxp3* correlated with Treg cell function irrespective of CD25 expression levels, establishing CD4+CD25+FOXP3+ Treg cells as an independent T-cell lineage.

Already in early 1980s several isolated human cases of an X-linked autoimmune syndrome were investigated, however, the causal gene for this disease was undetermined. The syndrome was given the name immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) due to the general characteristic seen in patients (Powell *et al.* 1982). After the discovery of *Foxp3* gene in scurfy mice, the IPEX syndrome was re-examined in several affected families and their *FOXP3* gene sequenced. Several mutations in the *FOXP3* gene were identified and all mutations were classified as deleterious (Bennett *et al.* 2001, Wildin *et al.* 2001).

In contrast to mice, where Foxp3 expression is restricted to natural Tregs, in humans, many cell types (e.g. conventional T-cells) can express this transcription factor and therefore cannot be used alone to identify Tregs. It has been demonstrated that *in vitro* activated CD4+CD25- T-cells show transient expression of FOXP3 at the transcriptional, as well as translational, level and that these cells, despite their FOXP3 expression, cannot suppress proliferation of autologous CD4+CD25- T-cells (Pillai *et al.* 2007, Wang *et al.* 2007). In addition, these FOXP3+ cells can secrete pro-inflammatory cytokines such as IL-2 and IFN-γ (Pillai *et al.* 2007).

Besides the expression of FOXP3, human and murine Tregs express very high levels of the interleukin-2 receptor α-chain (CD25) which allow Tregs to scavenge IL-2 from other cellular sources, a crucial trait of this cell type as Tregs do not produce their own supply of this survival and expansion-promoting cytokine (Sakaguchi *et al.* 2007). Other cytokine receptors have been associated with Tregs. Tregs express low levels of the interleukin-7 receptor α-chain (CD127), and this allows CD25+CD127- Tregs to be distinguished from activated CD4+ T-cells expressing CD25+CD127+ (Liu *et al.* 2006).

Other cell markers which are critical for Treg function cannot be used to identify Tregs as these markers are not specific. One example would be cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which is a key suppressive molecule for Treg cell function. However, CTLA-4 can also be upregulated in activated T-cells or antigen presenting cells (APCs) such as dendritic cells. Germline deletion or specific loss of CTLA-4 in Treg cells leads to fatal autoimmunity similar to that seen in scurfy mice (Waterhouse *et al.* 1995, Wing *et al.* 2008). More recently, studies have shown that Treg-specific deletion of CTLA-4 in adult mice leads to severe autoimmune disease with extensive T-cell activation, although symptoms are less serve than in scurfy mice (Paterson *et al.* 2015, Klocke *et al.* 2016). The importance of CTLA-4 expression of Tregs was also documented in humans as family groups with autoimmune diseases had heterologous mutations at the CTLA-4 gene (Kuehn *et al.* 2014, Schubert *et al.* 2014).

Another marker which seems to correlate with suppressive function of Tregs is Helios, an Ikaros family transcription factor. *In vitro* experiments have shown that human FOXP3+Helios- cells under inflammatory conditions can secrete pro-inflammatory cytokines and have reduced suppressive capacity compared to FOXP3+Helios+ cells (Bin Dhuban *et al.* 2015). Not surprisingly, FOXP3+Helios- cells show a less de-methylated Treg-specific de-methylated region (TSDR), a region important FOXP3 maintenance (see 3.2.2) (McClymont *et al.* 2011, Raffin *et al.* 2013).

Many other markers have been proposed to identify Tregs such as TIGIT which correlates with Helios expression, ICOS or CD15s, although none of them seem to be uniformly expressed on Treg cells. The expression and function of programmed cell death 1 (PD-1; CD279) receptor and its ligands PD-L1 (CD274) and PD-L2 (CD273) have been also been widely investigated and PD-L1 is widely expressed on many cell types including T- and B-lymphocytes, DCs or macrophages (Yamazaki *et al.* 2002). It has been shown, that PD-1 can downregulate self-reactive T-cells during self-antigen presentation to DCs, inhibit maturation of DCs, generate tolerogenic DCs and promote

IL-10 secretion by T-cells (Dong *et al.* 2003, Kuipers *et al.* 2006, Keir *et al.* 2007). PD-1 blockage has been successfully employed in cancers and oncotherapy has PD-1 signalling inhibits active Teffector proliferation and reduces anti-tumour activity (Mahoney *et al.* 2015).

3.2 FOXP3

3.2.1 FOXP3 Protein Interactions

The FOXP3 (forkhead box P3) gene is located on the p-arm of the X chromosome and is highly conserved between human and mouse. FOXP3 has been identified as the master regulator of Treg gene expression and function, and has been shown to be capable of binding more than 2800 genomic sites which corresponds to approximately 700 to 1400 genes important in the development and establishment of Tregs (Marson *et al.* 2007, Zheng *et al.* 2007, Samstein *et al.* 2012). FOXP3 is involved in the initiation, maintenance and function of the Treg cell phenotype. Interestingly, only 6-10% of genes bound by FOXP3 are under its direct control (Zheng *et al.* 2007). Hence, FOXP3 controls the activity of many transcriptional targets indirectly by recruiting many co-factors. FOXP3 and its binding partners form many different multi-protein complexes in the region of 400-800 kDa and larger with over 360 possible identified binding partners including different transcription factors and epigenetic modifying factors (Hori 2012, Rudra *et al.* 2012). Some key transcription factors that interact with FOXP3 include nuclear factor of activated T-cells (NFAT) and Runt-related transcription factor 1 (RUNX1) and disruption of either of these transcription factors results in reduced Treg function (Wu *et al.* 2006, Ono *et al.* 2007).

Interferon regulatory factor 4 (IRF4) is another crucial FOXP3 binding partner that regulates many Treg genes (it is estimated to be around 20% of all Treg genes) and its importance is demonstrated in murine IRF4-deficient Tregs which lose their ability to

suppress the production of T-helper 2 cell (Th2) cytokines IL-4 and IL-5 (Zheng *et al.* 2009). Furthermore, it is thought that IRF4 is linked to the suppression of T-helper 17 (Th17) cells in a mouse arthritis model (Darce *et al.* 2012).

Other key interactions of FOXP3 that mediate negative regulation of alternative T-cell differentiation pathways include GATA-3, the key transcription factor for Th2 development, and RORγt, the key transcription factor in Th17 development (Zhang *et al.* 2008, Rudra *et al.* 2012). GATA-3 has been identified as a prominent FOXP3-associated factor. Under steady-state conditions only a small proportion of *ex vivo* isolated Treg cells express GATA-3, however, upon *in vitro* TCR stimulation in the presence of IL-2 a large proportion upregulates GATA-3 (Rudra *et al.* 2012). Co-immunoprecipitation confirmed interaction of Foxp3 and GATA-3 under these conditions. Foxp3 binds the promotor and intronic regions of the *Gata3* gene while GATA-3 binds to the TSDR of the *Foxp3* gene highlighting that Foxp3 and GATA-3 not only interact on the protein level but also bind genetic loci to modulate Treg function. To test the functional impact, mice deficient of GATA-3 in Treg cells were generated (*Gata3*$^{fl/fl}$*Foxp3*-YFP-Cre). These mice developed intestinal pathology and dermatitis after 6 months which was accompanied by a marked increase in activated CD4+FOXP3- cells and IL-4, IL-5 and IL-13 producing GATA-3+ Th2 cells (Rudra *et al.* 2012) and IL-17 production was increased in FOXP3+ cells of *Gata3*$^{fl/fl}$*Foxp3*-YFP-Cre mice (Wang *et al.* 2011, Rudra *et al.* 2012). Interestingly, the observed phenotype was not due to fewer Treg cells but the loss of function of GATA-3 deficient Treg cells to control Th2 Teffector cells. This reiterates the importance of Foxp3 interaction with its binding factors in Treg function.

For FOXP3 to selectively activate or repress genes, epigenetic remodelling is required at the target loci including of (de)acetylation and (de)methylation. To fulfil these functions, FOXP3 can recruit different epigenetic modifying molecules such as FOXP3-associating histone acetyltransferases 60 kDa Tat-interactive protein (TIP60), p300 and histone deacetylase 7 (HDAC7) or histone-lysine N-methyltransferase (EHMT2) and histone

methyltransferase enhancer of zeste homologue 2 (EZH2) (Li *et al.* 2007, Tao *et al.* 2007, Arvey *et al.* 2014). One example of FOXP3's ability to repress genes is through deacetylation of histone H3 at promoters of IL-2 and IFN-γ encoding genes (Tao *et al.* 2007). These FOXP3 interactions ultimately provide signals for silencing of active transcription of genes. Other partners of FOXP3 suppress the expression of the expansion-stimulating cytokine IL-2. EOS binds to FOXP3 and recruits further factors such as carboxyl-terminal binding protein 1 (CTBP1) and EHMT2 to the *Il2* locus to prevent the histone trimethylation (H3K4), which promotes active transcription, and acetylation. Furthermore, methylation of histone H3K9, a signal for transcriptional silencing, and methylation of CpG dinucleotides, a signal for transcriptional silencing at the *Il2* promotor are promoted by EOS (Pan *et al.* 2009).

In contrast, FOXP3 interaction with the genes glucocorticoid-induced tumour necrosis factor (TNF) receptor-related protein (*GITR*; also known as TNFRSF18), *CD25* and *CTLA4* leads to histone acetylation and expression of these genes (Chen *et al.* 2006). In this complex network of binding partners and factors of FOXP3, there is considerable redundancy in the co-factors of FOXP3 required for Treg suppressive function. This could be due to functional overlap of co-factors or some functions only be needed in specific circumstances and so contribute to resilience of the ability of FOXP3 to regulate Treg function.

3.2.2 Control of *FOXP3* Gene Expression

Many signals for the activation and repression of FOXP3 gene expression are integrated at the promotor. Signalling via TCR and co-stimulation pathways leads to the activation of transcription factors such NFAT and AP-1 which subsequently bind to the *FOXP3* promotor (Josefowicz *et al.* 2012). Many other transcription factors such as the forkhead box O proteins 1 (FOXO1) and 3 (FOXO3) or cAMP-responsive element-binding protein (CREB)–activating transcription factor 1 (ATF1) complexes have been reported to bind

to the promotor and other regulatory elements of the *FOXP3* gene to drive *FOXP3* transcription (Kim and Leonard 2007, Ouyang *et al.* 2010). It is important to note that the *FOXP3* promotor has been identified to have low *trans*-activating potential and therefore relies heavily on conserved enhancer regions in order to be transcribed (Josefowicz *et al.* 2012). The *FOXP3* gene has three important conserved non-coding sequences (CNS1-3) which are critical for initiation and maintenance of *FOXP3* transcription (Zheng *et al.* 2010). CNS3 is located between exon 1 and 2 and controls *FOXP3* transcription by binding of REL (member of the nuclear factor κB family), a key transcription factor in Treg development in the thymus. This region is indispensable for initiation of *FOXP3* transcription although dispensable for the maintenance of FOXP3 expression (Ruan *et al.* 2009, Feng *et al.* 2015). Arguably, the most important regulatory region is CNS2, also known as Treg-specific demethylated region (TSDR), which is critical in maintaining stable *FOXP3* transcription and Treg suppressive function even under inflammatory conditions. Hypomethylation of the CpG motifs within CNS2 enables the binding of several transcription factors such as REL, CREB–ATF1, RUNX1–core-binding factor subunit-β (CBFβ), ETS1 and signal transducer and activator of transcription 5 (STAT5), as well as FOXP3 itself (Zheng *et al.* 2010, Josefowicz *et al.* 2012). Hypomethylation of CNS2 allows the differentiation of natural Tregs (nTregs) from *in vitro* induced Tregs (iTregs) as these iTregs have a less stable expression of FOXP3 than nTregs due to significant hypermethylation at this region (Polansky *et al.* 2008). However, peripherally induced Tregs (pTregs) are generally thought to have epigenetic profiles that resemble those of nTregs with stable FOXP3 expression (Ohkura *et al.* 2012). In order to maintain the hypomethylation status, CNS2 recruits and binds factors such as Methyl-CpG-binding domain protein 2 (MBD2) which in turn recruits ten-eleven translocation (TET) demethylases. The importance of MBD2 has been demonstrated as mbd2-deficient mice have reduced Treg number and function due to lower levels of hypomethylation at CNS2 (Wang *et al.* 2013).

Cytokine signalling can also potentiate methylation or hypomethylation of CNS2, for example the pro-inflammatory cytokine IL-6 can trigger DNA methyltransferase 1 (DNMT1) dependent methylation at CNS2 region (Nair *et al.* 2016, Yue *et al.* 2016). This can be counteracted by transforming growth factor-β (TGF-β) signalling which has been shown to suppress DNMT1 expression or by IL-2 and Vitamin C which mobilise TETs to maintain hypomethylation at CNS2 (Li *et al.* 2013, Nair *et al.* 2016, Yue *et al.* 2016). CNS1 is essential for the induction of iTregs and pTregs. Naïve CD4+ T-cells can differentiate into pTregs and exert suppressive function but critically can convert back into a Teffector phenotype and secrete pro-inflammatory cytokines. Experiments have shown that Foxp3 can be induced by TGF-β mediated Smad signalling. Smad3-Smad4 heterodimers binding to CNS1 is a key event in the induction pTregs and iTregs but not that of nTregs (Schlenner *et al.* 2012). Accordingly, CNS1-deficent mice have a defective pTreg cell compartment that fails to maintain immune tolerance at barrier sites such as the gut, but these mice do not succumb to autoimmunity (Josefowicz *et al.* 2012, Schlenner *et al.* 2012). In addition, all-trans retinoic acid (RA) is known to drive pTreg and iTreg differentiation and is likely due to RA ability to enhance TGFβ-driven Smad3 phosphorylation and/or the suppression of pro-inflammatory cytokines that interfere with *Foxp3* transcription (Hill *et al.* 2008, Xiao *et al.* 2008). Besides the ability of RA to potentiate signalling pathways, RA can induce functionally significant epigenetic changes by driving histone acetylation at the *FOXP3* locus (Kang *et al.* 2007).

3.2.3 Post-translational Modifications of the FOXP3 Protein

The *FOXP3* gene is not only tightly regulated and modified on the transcriptional level but the FOXP3 protein also undergoes extensive post-translational modifications including acetylation, phosphorylation or ubiquitylation. To date, 15 residues have been identified on the FOXP3 protein that undergo post-translational modifications to

predispose FOXP3 to degrade or stabilise the protein or enhance the functional effects of the protein (Lu *et al.* 2017).

Attachment of acetyl groups to lysine residues by lysine acetyltransferases, TIP60 and p300, improves the FOXP3 protein stability in comparison to non-acetylated FOXP3 as acetylation protects against the degradation mechanism of ubiquitylation (van Loosdregt *et al.* 2010). Deletion of these acetyltransferases has profound effects on Treg fitness and function, in some circumstances, such as anti-tumour responses, antagonism of FOXP3 acetylation is beneficial but in other circumstances, such as autoimmunity, it can drive disease pathology (Liu *et al.* 2013, Xiao *et al.* 2014, Wang *et al.* 2016). The opposite mechanism to attachment of acetyl groups is driven by lysine deacetylases (KDACs) and histone deacetylases (HDACs). One example of a KDACs is Sirtuin 1 (SIRT1) which upon TCR stimulation is upregulated in conventional T-cells but suppressed in Treg cells. Forced expression of lysine deacetylases in Treg cells greatly reduces acetylation of the FOXP3 protein and increases polyubiquitylation and consequently its degradation by the proteasome (van Loosdregt *et al.* 2011). As expected, Treg-specific deletion of SIRT1 greatly enhances acetylation, increases FOXP3 expression and Treg function and increased allograft tolerance (Beier *et al.* 2011). Bacterially derived short-chain fatty acids (SCFAs) within the gut, such as butyrate, have also been found to increase acetylation of FOXP3 by inhibition of HDACs and as a result induce pTregs (Arpaia *et al.* 2013).

Polyubiquitylation is a well-known signal for proteasomal degradation and several studies have demonstrated that FOXP3 can be modified in this manner with negative effects on Treg function. Acetylation and ubiquitylation have an inverse relationship as many lysine residues can only be modified by one of these post-translational marks at any one time. FOXP3 protein has a relatively short half-life of around 4-6 hours with constant turnover due to polyubiquitylation-induced proteasomal degradation (Chen *et al.* 2013, van Loosdregt *et al.* 2013). One protein that mediates the polyubiquitylation is

the heat shock 70kDa protein (HSP70) which recruits the U-box domain type E3 ubiquitin ligase STUB1 and degrades important transcription factors for Treg function including FOXP3, although the exact mechanism of degradation remains unclear (Chen *et al.* 2013). Conversely, STUB1-deficient Treg cells have stabilized FOXP3 expression and increased suppressive function. As with every mechanism there is a counter measure to ubiquitylation in the form of deubiquitinases (DUBs) and several DUBs have been identified to stabilize FOXP3 expression. A well-known DUB in Treg cells is ubiquitin-specific peptidase 7 (USP7) which has been shown to be crucial for Treg function and phenotype as knock-down of USP7 or general DUB inhibition results in reduced FOXP3 levels and Treg suppressive capacity with the opposite effects when overexpressing USP7 (van Loosdregt *et al.* 2013). The importance of USP7 was demonstrated in conditional deletion of USP7 in mouse Treg cells which resulted in lethal autoimmunity within a month after birth (Wang *et al.* 2016). This study produced some interesting results as the USP7 deficiency did not result in reduced overall Treg numbers or FOXP3 expression in the thymus but profoundly disrupted Treg function highlighting the need for Tregs to restrain polyubiquitylation. Recently, USP21 was found to be highly expressed in mouse and human Tregs and deletion of this DUB in FOXP3+ cells resulted in significantly upregulated secretion of pro-inflammatory cytokines IFN-γ by CD4+ Teffector cells (Li *et al.* 2016).

Taken together, maintaining appropriate function of Tregs requires not only regulation of *FOXP3* gene regulation but also precise regulation of FOXP3 protein levels.

3.3 Treg Heterogeneity

As the understanding of Treg biology has improved it became apparent that, as in most cell populations, there is extensive heterogeneity of Treg function and tissue-specificity. When defining Treg lineages it is important to consider the difference between the loss of Treg identity (lineage instability) and a partial shift towards Teffector characteristics while maintaining some core Treg characteristics (functional adaptability). An elegant FOXP3-fate reporter experiment in mice have demonstrated that Tregs are mostly a highly stable lineage (Rubtsov *et al.* 2010). Mice expressing enhanced green fluorescent protein (eGFP) under control of the *Foxp3* gene were bred with mice that express constitutively yellow fluorescent protein (YFP) tagged to Foxp3+ cells when treated by tamoxifen. This allows identification of Foxp3+ cells by the expression of eGFP and tracking Foxp3+ protein by YFP+. Under basal and inflammatory conditions the overwhelming majority of YFP+CD4+ remained eGFP+ demonstrating incredible lineage stability of natural Tregs (Rubtsov *et al.* 2010).

Lineage instability is observed in certain settings in which previously FOXP3+ cells convert to an FOXP3- Teffector phenotype. One explanation for this phenomenon is that these FOXP3 expressing cells only transiently express FOXP3 and lack the full epigenetic program and characteristics of a true Treg cell which then allows them to convert back to a Teffector phenotype. On the other hand, there is now considerable evidence that Tregs can gain some characteristics of Teffector populations by induced expression of certain transcription factors and chemokine receptors (Wing *et al.* 2019). However, the epigenetic programming of a Treg cell hinders conversion to a fully pathogenic Teffector cell. For example, repressive histone markers at the *IL12Rβ2* promotor impairs Treg responsiveness to IL-12 but still forces Tregs into an Th1 phenotype with the development of T-bet and CXCR3 expression but with impaired IFN-γ expression (Koch *et al.* 2012). Another example of Treg epigenetic safety mechanisms that safeguards from Teffector phenotype switching is the transcription factor B

lymphocyte-induced maturation protein-1 (BLIMP-1/PRDM1). Tissue-specific RORγt+FOXP3+ Tregs, known to control homeostasis in the intestinal mucosa, preferentially express BLIMP1. Despite the expression of RORγt expression these cells retained their suppressive and regulatory capacity (Ohnmacht *et al.* 2015, Sefik *et al.* 2015). One mechanism for this phenomena is BLIMP1-binding to the *IL-17* locus which results in the induction of inhibitory histone modifications that prevent the expression of IL-17 and a complete switch to a Th17 RORγt+ Teffector cell (Ogawa *et al.* 2018).

According to Wing *et al.* (2019) and Miyara *et al.* (2009) human peripheral blood CD4+FOXP3+ T-cells can be subdivided into three distinct fractions by the use of markers FOXP3, CD25 and CD45RA. Fraction 1 is defined as CD45RA+FOXP3low/CD25low and are resting or naive Treg (nTreg) cells, Fraction 2 CD45RA-FOXP3high/CD25high are effector Treg (eTreg) cells, and Fraction 3 CD45RA-FOXP3low/CD25low cells, of which the majority are not Treg cells.

Fraction 1 encompasses thymic-derived Tregs (nTregs) with a fully demethylated TSDR and consequently stable FOXP3 expression and suppressive function. Phenotypic markers include: CD45RA, a marker for naïve T-cells; CCR7, a marker with various function but among other things required for negative selection of auto-reactive thymocytes in the thymic medulla and guiding cells from peripheral tissue to the lymph node and intestinal Peyer's patch (Forster *et al.* 1999, Bromley *et al.* 2005, Debes *et al.* 2005); CD62L, a marker for resting T-cells which is lost upon cell activation (Yang *et al.* 2011) and the lack of CD45RO, a marker for memory T-cells. CD45RA+ nTregs have not experienced any proliferation, are found predominately in cord blood but can be detected in peripheral blood and maintained throughout life, however, numbers drop with age. They also express higher levels of CD31, a T-cell marker for thymic egress, in comparison to eTregs highlighting the naïve Treg characteristics (Haas *et al.* 2007, Miyara *et al.* 2009). Under physiological conditions nTregs are not proliferative, but once activated these cells convert into an eTreg type and become highly proliferative.

However, Fraction 1 is not a homogenous population as chemokine receptor CXCR5, that mediates the homing of both B-cell and follicular helper T-cells into follicles of secondary lymphoid organs, is expressed on a considerable number of peripheral nTregs (Lim *et al.* 2006, Fonseca *et al.* 2017). This suggests that this population is not a true naïve Treg subset, but the sub-populations of nTregs require further investigation and characterisation.

Although significantly different in function, Fraction 2 and 3 can be broadly characterized as CD45RA-CD45RO+. Phenotypically Fraction 2 shows higher levels of CD25 and FOXP3 than Fraction 3 and expresses a range of effector Treg molecules, such as CTLA-4 or Helios, in order to exert their suppressive function. Importantly, the TSDR is demethylated allowing a stable expression of FOXP3 and high suppressive activity *in vitro* as seen with Fraction 1. eTregs are also highly proliferative, under physiological conditions (i.e. under no pathological conditions), in comparison to Fraction 1 or 3 and were also unable to secrete pro-inflammatory cytokines IL-2 and IFN-γ.

This is in contrast to Fraction 3, characterized by low levels of CD25 and FOXP3, which possess only intermediate levels of demethylation at the TSDR. This population contain cells that secrete pro-inflammatory cytokines and show low to no suppressive capacity *in vitro* (Miyara *et al.* 2009, Cuadrado *et al.* 2018). In conclusion, Fraction 3 is a heterogeneous population of Tregs and non-Tregs which can be subdivided into more subpopulations using markers such as CD127, CD25, chemokine receptor CCR4 and α-integrin subunit CD49d to allow differentiation between Tregs and pro-inflammatory secreting T-cells, with recent reports estimating that the true Treg proportion of this fraction is ~30% (Ferreira *et al.* 2017, Wing *et al.* 2017, Cuadrado *et al.* 2018). One of the key findings by Miyara *et al.* (2009) was that most eTregs were derived from nTregs and that only a very small number of FOXP3-CD4+ cells are converted into eTregs *in vivo*. However, it is important to note that this observation is concluded from an 18-month interval tracing experiment but with only a single individual. Beyond protein markers,

these populations also had distinct gene expression patterns with Fraction 3, as expected, displaying the most pro-inflammatory pattern.

3.4 T-cell and Treg Development

In order to appreciate Treg development it is first necessary to understand T-cell development in the thymus as they are closely interlinked. Development of a T-cell begins with committed lymphoid progenitors in the bone marrow that migrate via the blood to the thymus. In the thymus these progenitors lose their potential to differentiate to B-cells and/or NK cells and are committed to a CD4+ or CD8+ T-cell fate (Pui *et al.* 1999, Radtke *et al.* 1999, Michie *et al.* 2000, Wilson *et al.* 2001). The first step in their development is the double negative (DN) stage in which the progenitor does not express either CD4 or CD8. These progenitors undergo another four stages of differentiation defined by the expression of CD44 and CD25: DN1 to DN4 (Godfrey *et al.* 1993).

The next stage in T-cell development is the commitment to either the αβ or γδ T-cell receptor. In humans 95% of all T-cells commit to the αβ lineage while the remainder commit to a γδ lineage. Commitment to αβ TCR means that DN3-stage cells are expressing a pre-TCR-α chain that pairs with a pre-TCR-β chain (von Boehmer and Fehling 1997, Aifantis *et al.* 1999), a product of somatic DNA rearrangement that requires the expression of recombination-activating gene 1 (RAG1) and 2 (RAG2) (Mombaerts *et al.* 1992, Shinkai *et al.* 1993). The pre-TCR-αβ interacts at the cell surface with various proteins (the CD3/ζ complex) and undergoes active signalling for further T-cell maturation and TCR-β chain selection in order to exclude mutations that prevent the expression of key enzymes or adaptors that mediate the generation of intracellular second messengers by the pre-TCR complex. This active signalling causes maturational arrest of the DN3 stage (van Oers *et al.* 1996, Clements *et al.* 1998). After some cell divisions a mature αβ antigen receptor appears and the expression of the pre-TCR is lost with T-cells expressing progressively lower levels of the αβ TCR. Thymocytes begin

to express co-receptors CD8 (MHC-I) and CD4 (MHC-II), usually starting with CD8 followed by CD4, yielding a large double positive population (DP; CD8+CD4+) of αβ TCR expressing immature T-cells (Robey and Fowlkes 1994). The next stage is the selection of the appropriate immature T-cells which undergo a series of processes. In the first stage T-cells interact with their self-peptide MHC ligands of both class I and II to generate intracellular signals to sustain viability and if they react poorly this signal is not generated and the cell undergoes death by neglect, a form of cell death. However, some T-cells interact vigorously with their self MHC peptides and could cause autoimmunity on egress from the thymus so it is imperative these are negatively selected and undergo apoptotic death (negative selection). If the immature T-cell survives both selection processes then a complex multi-step process is initiated known as positive selection, driven by transcription factors such as nuclear factor of activated T-cells (NFAT) and mitogen-activated protein kinase (MAPK) pathways, which yields lineage-specific CD4+ or CD8+ mature T-cells which leave the thymus.

Evidence for the commitment of developing T-cells to the Treg lineage becomes apparent at the CD4+ SP stage as a Treg-specific epigenetic pattern is observed in some of these cells (Ohkura *et al.* 2012). Furthermore, mice experiments have shown that a Treg-specific DNA hypomethylation profile is initiated without FOXP3 expression and has global effects on the cell's transcriptional programme (Ohkura *et al.* 2012). The answer of Treg cell fate lies in the quality and quantity of TCR stimulation a developing T-cell receives. Firstly, the appropriate intensity of TCR stimulation leads to rapid induction of FOXP3 expression and is stronger than that of conventional T-cells but not too strong as these developing Treg cells still avoid negative selection (Jordan *et al.* 2001, Kawahata *et al.* 2002). T-cells that ubiquitously express self-antigens are likely to be negatively selected but tissue-restricted self-antigens avoid this fate and are the main source of Treg generation (Legoux *et al.* 2015, Malhotra *et al.* 2016). Then, the establishment of Treg-specific DNA hypomethylation relies on the duration of TCR

stimulation (Ohkura *et al.* 2012). It is thought that thymic-derived Tregs (nTregs) only develop into nTregs if the epigenetic landscape is permissive to transcriptional *FOXP3* induction to allow development into a stable FOXP3+ Treg cell.

3.5 Treg Function

Treg cells have multiple mechanism of action to suppress the proliferation and activation of Teffector cells. These can be classified into four groups: suppression mediated by cell-cell contact either mediated through antigen-presenting cells (APCs) or directly by Tregs, cytokine secretion, cytolysis and metabolic disruption.

One core mechanism of Treg suppressive capacity is mediated through CTLA-4 as binding of CTLA-4 facilitates the downregulation and degradation (by a mechanism called trans-endocytosis) of CD80 and CD86 on APCs which are co-stimulatory molecules required for T-cell activation via CD28 (Qureshi *et al.* 2011). The importance of this mechanism has been demonstrated in murine models either by CTLA-4 specific blocking antibodies or by Treg-specific *Ctla-4* knock-out as these mice developed autoimmune diseases with decreased Treg suppressive capacity and pronounced activation and expansion of dendritic cells and Teffector cells (Read *et al.* 2000, Wing *et al.* 2008). In addition, CTLA-4 binding to CD80/CD86 upregulates the expression of enzyme indoleamine 2,3-dioxygenase (IDO) which catalyses the essential amino acid tryptophan into immunosuppressive kynurenine which, for example, induces tolerogenic dendritic cells (Belladonna *et al.* 2007).

Another mechanism of Treg suppressive function is the ability to secrete inhibitory cytokines such as IL-10 and TGF-β. The immunosuppressive effect of IL-10 is undisputed although its contribution on nTregs function is matter of debate. Models of allergy and asthma models have demonstrated that Tregs control disease, at least in part, by secretion of IL-10 and this effect could be reversed by administration of anti-IL-10 antibodies (Hawrylowicz 2005, Kearley *et al.* 2005). However, adoptive transfer of IL-

10-deficient Tregs into these models, retained the ability to suppress disease pathologies suggesting that Treg-derived IL-10 is not required for Treg dependent immunosuppression. In contrast, many other disease models, such as inflammatory bowel disease (IBD) or various infections, have clearly demonstrated the need for Treg-derived IL-10 to prevent the onset of disease pathologies (Asseman *et al.* 1999, Anderson *et al.* 2007, Jankovic *et al.* 2007, Kursar *et al.* 2007). Another key cytokine is TGF-β which has also been shown to be important in the control of infection, allergic responses or prevention of colitis by controlling the differentiation of Th1 and Th17 cells (Joetham *et al.* 2007, Kursar *et al.* 2007, Li *et al.* 2007). Critical to Treg function is the pleiotropic cytokine IL-2 which plays a role in Treg development and maintenance as well as enhancing expression of CTLA-4 or by driving the phosphorylation of STAT5 which is critical for FOXP3 expression (Bayer *et al.* 2007, Barron *et al.* 2010). IL-2 is predominantly produced by activated CD4+ cells and, to some extent, by CD8+ cells as well as activated DCs, natural killer (NK) cells, NKT cells and B-cells but not Tregs. Therefore, Tregs rely on paracrine secretion of IL-2 from other sources to bind IL-2 receptor (CD25) which is highly expressed on the surface of Tregs (Harris *et al.* 2000). This abundant CD25 expression makes Tregs effective IL-2 scavengers and they readily deplete IL-2 available for other immune cells which can result in BIM-mediated apoptosis of CD4+ effector cells (Pandiyan *et al.* 2007, Busse *et al.* 2010). This mechanism is an example of how Tregs can cause metabolic disruption of the adaptive immune response. In addition, IL-2 can work in cohort with TGF-β and anti-CD3 stimulation to induce pTregs and iTregs (Davidson *et al.* 2007, Zheng *et al.* 2007).

IL-35 is another key inhibitory cytokine secreted by Tregs which is needed for full suppressive capacity (Collison *et al.* 2007, Bettini *et al.* 2012). IL-35 is a member of the IL-12 heterodimeric family and is formed by pairing p35, which is also known as *Il12a*, and Epstein–Barr virus-induced gene 3 (*Ebi3*). Interestingly, *Ebi3* and *Il12a* are preferentially expressed in mouse FOXP3+ cells but not by resting or activated CD4+

Teffector cells and is significantly upregulated in actively suppressing Tregs (Collison *et al.* 2007, Gavin *et al.* 2007). As expected, Treg-specific knock-out of either *Ebi3* or *Il12a* significantly reduces Treg suppressive function *in vivo*. A recent publication has also provided evidence of IL-35 limiting anti-tumour immunity by using a model of Treg-specific Ebi3 knock-out mice (Turnis *et al.* 2016).

3.6 CD4+ T-helper Populations

Over 30 years ago, Mossman *et al*. (1986) made the observation that murine T-helper (Th) clones could be distinguished based on their cytokine profile, significantly advancing our understanding of how the immune system can tailor a response based on a specific pathogenic challenge. Since this initial discovery many more T-helper sub-populations have been characterised further illustrating the multifaceted nature of the T-cell immune response.

One of the first identified T-cell populations were the Th1 population which are important mediators of inflammation and immunity against intracellular pathogens. This sub-population is defined by the expression of the transcription factor T-bet and by the secretion of signature cytokines, IL-2 and IFN-γ. Other cytokines have been reported to be secreted by Th1 cells including TNF or the unique secretion of lymphotoxin. Aside from strong TCR signalling, other pathways important in driving Th1 differentiation are IFN-γ/STAT1, IL-2/STAT5 and IL-12/STAT4. Critical to their function is the expression of the IFN-γ receptor, consisting of two heterodimers: IFN-γR1 and IFN-γR2. While IFN-γR1 is ubiquitously expressed on almost every lymphoid and non-lymphoid lineage, IFN-γR2 expression level vary from cell to cell type and are dependent on activation status (Tau *et al.* 2000, Bernabei *et al.* 2001). In T-cells, IFN-γ binding to its receptor leads to the activation of the JAK/STAT1 signalling pathway and subsequent upregulation of transcription factor T-bet which results in the impairment of the function of transcription

factors GATA-3 and RORγt to allow full maturation into Th1 cells (Hwang *et al.* 2005, Djuretic *et al.* 2007, Lazarevic *et al.* 2011).

IFN-γ is involved in many autoimmune diseases such as type 1 diabetes, multiple sclerosis and rheumatoid arthritis (Skurkovich and Skurkovich 2005, Raphael and Forsthuber 2012), so unsurprisingly, important immune responses are driven by this pro-inflammatory cytokine which includes the upregulation of MHC class I and II for antigen processing, promoting immunoglobulin (Ig)G class switching, macrophage activation, induction of chemokine secretion or expression of toll-like receptors (TLR) by innate immune cells (Raphael *et al.* 2015). However, other experiments have shown that IFN-γ can also reduce lymphocyte trafficking to draining lymph nodes, is important for the induction of anti-inflammatory enzyme IDO and can suppress the differentiation of naïve CD4+ cells into more pathogenic Th17 cells in a murine EAE model indicating that this cytokine can also play an anti-inflammatory role (Flaishon *et al.* 2002, Komiyama *et al.* 2006, Curran *et al.* 2014). IFN-γ production by T-cells can be induced by cytokines IL-12 and IL-18 while cytokines IL-4, IL-10 and TGF-β downregulate its production (Lu and Waller 2009). Interestingly, while IFN-γ KO mice develop a normal immune system, these mice appeared to be more susceptible to bacterial, parasitic and viral infections indicating the importance of IFN-γ in cellular immune responses (Dalton *et al.* 1993).

Amongst their diverse functions, Th2 cells play an important role in the host's defence against multicellular parasites, allergies, atopic illnesses and infections that predominantly affect the intestinal tract, epithelial tissues and lungs (Raphael *et al.* 2015). Differentiation of Th2 cells is driven by its master transcription factor GATA-3 (which is downregulated in Th1 differentiation) and IL-4 driven STAT6 signalling, and these cells are potent secretors of IL-4, IL-5 and IL-13 as well as IL-9. However, it is now widely accepted that these cytokines can also be derived from other cellular sources as well. Interestingly, while Th1 and Th17 differentiation require DC production of polarizing cytokines during an immune response, it is thought that Th2 differentiation is a default

pathway without the need of DC support (MacDonald and Maizels 2008), although other studies suggest that DC priming is required for optimal *in vivo* Th2 polarisation (van Rijt *et al.* 2005, Phythian-Adams *et al.* 2010). Among several distinct functions, Th2 cytokines help to drive B-cell proliferation and IgE class switching, establish an anti-inflammatory M2 macrophage phenotype, promote eosinophilia and mastocytosis and goblet cell hyperplasia (Fallon *et al.* 2002, Zhu and Paul 2008). Th2 cells play an important role in autoimmune diseases as it is thought that the shift from Th1 to Th2 protects in disease models such as EAE or MS (Genain *et al.* 1996, Antel and Bar-Or 2003). In contrast, other reports demonstrate that Th2 can induce EAE but with delayed onset and lower severity (Lafaille *et al.* 1997, Jager *et al.* 2009).

Another key subpopulations of Teffector cells are the Th17 cells named after the secretion of pro-inflammatory cytokine IL-17 which are involved in the host defence against fungi, extracellular bacteria and other eukaryotic pathogens as well as a key mediator of autoimmunity. IL-17 secretion is not exclusive to Th17 cells as γδT-cells, NK cells, NKT-cells and macrophages have all been shown to secrete this cytokine (Cua and Tato 2010, Gu *et al.* 2013). Differentiation of naïve CD4+ cells into Th17 is driven by the transcription factor RORγt as experiments have demonstrated (Ivanov *et al.* 2006). RORγt-deficient cells produce very little IL-17 and RORγt-deficient mice are partially resistant to EAE (Ivanov *et al.* 2006). Further, Stat3 signalling, driven by cytokines IL-6, IL-21 and IL-23, is indispensable for Th17 induction as it binds to the *Il17* and *Il21* locus to mediate their transcription and is responsible for RORγt and IL-23R induction (Chen *et al.* 2006, Wei *et al.* 2007, Yang *et al.* 2007). Mutations or loss of Stat3 or Irf4 expression results in the loss of Th17 cells and a failure to induce EAE (Brustle *et al.* 2007, Harris *et al.* 2007, Mathur *et al.* 2007). IL-17 has been noted to be a major cytokine in many inflammatory and autoimmune diseases such as multiple sclerosis (MS) (Lock *et al.* 2002), rheumatoid arthritis (Kotake *et al.* 1999), systemic lupus erythematosus (SLE) (Wong *et al.* 2008) and airway inflammatory disease (Linden *et al.* 2000) leading

to major interests in Th17 biology. One mechanism in which Th17 can drive pathogenesis is by promoting B-cell survival and autoantigen production. For example, IL-17 has been shown in an murine SLE model to reduce apoptosis and enhance proliferation of B-cells and, in addition, enhance their maturation to antibody producing plasma cells (Doreau *et al.* 2009). IL-17 can also influence the chemotaxis of cells as demonstrated in an RA model in which IL-17 led to the infiltration of monocytes and neutrophils into the synovial fluid (Shahrara *et al.* 2009). The widely used model of autoimmunity EAE also demonstrated the importance of Th17 cells as either adoptive transfer of IL-17-deficient Teffector cells or induction of disease in Th17-deficient mice resulting in delayed disease onset, as well as reduced severity and early recovery from the illness (Komiyama *et al.* 2006). Despite all the evidence that Th17 are very potent pro-inflammatory cells under certain conditions Th17 can be anti-inflammatory by producing IL-10 (Zielinski *et al.* 2012).

Other Teffector populations include Th9 cells, important in activation of mast cells and mucus production, or Th22 cells which play a role in skin pathologies (Raphael *et al.* 2015). In conclusion, the immune systems seems to favour a balance between protective and pathogenic T-helper cells with different diseases requiring specific T-helper responses.

3.6.1 CD4+ T-helper populations in GvHD

GvHD has a complex disease pathophysiology to which many different cell populations contribute. Nonetheless, T-cells are at the centre in the development and aggravation of acute GvHD. As previously described (see 1.4), acute GvHD can be thought of in 3 phases. The conditioning regimen leads to tissue damage and the release of, among others, pro-inflammatory cytokines (e.g. IL-6) and PAMPS (e.g LPS) which activate host antigen presenting cells and enhance alloantigen presentation (phase 1). Transplanted donor CD4+ and CD8+ T-cells recognize alloantigens presented by host antigen

presentation cells and consequently activate, expand and differentiate into effector T-cells (phase 2). As a result donor effector T-cells secrete large amounts of pro-inflammatory cytokines (e.g. IFN-γ, TNF-α, IL-2 and IL-17A) and cause significant tissue damage and apoptosis in the recipient (phase 3). Pathogenic Th1 (IFN-γ) and Th17 (IL-17A) T-cells are mainly responsible for host cell elimination and tissue damage but also promote engraftment while other T-cell populations may play a role in acute GvHD but are regarded as less relevant in the pathophysiology (Zeiser and Blazar 2017).

Early preclinical and clinical studies have shown significant IFN-γ expression in GvHD target organs (Roy *et al.* 1995, Krenger and Ferrara 1996) and clinical studies have found a positive correlation between IFN-γ and TNF-α cytokine levels and GvHD severity (Dickinson *et al.* 1994, Yang *et al.* 2005). While many studies point towards a pathogenic role, other studies also show that IFN-γ can limit GvHD pathology. In this context, preclinical studies have demonstrated that the transfer of IFN-γ -deficient donor T-cells or anti-IFN-γ antibody therapy results in exacerbated GvHD lethality due to increased lung injury but also attenuated GvHD within the GI tract (Burman *et al.* 2007, Varelias *et al.* 2015). Interestingly, a murine BMT study found that high levels of exogenous IFN-γ immediately after BMT for 5 weeks is crucial in the prevention of GvHD while a shorter duration of treatment with a higher dose did not result in attenuated GvHD and increased survival, highlighting the importance of timing and intensity of treatment (Brok *et al.* 1998). In addition, IFN-γ has been demonstrated to mediate the induction of IDO which blocks T-cell activation and proliferation (Jasperson *et al.* 2008, Jasperson *et al.* 2009). One explanation for IFN-γ protective role is that it limits donor CD4+ mediated GvHD. This paradoxical observation is in contrast to, for example, IFN-γ mediated apoptosis of intestinal epithelial crypt cells and stimulation of pro-inflammatory cytokines from innate immune cells in the gut resulting in exacerbated GvHD (Ellison *et al.* 2003). However, transfer of CD4+ T-cells along with bone marrow from IFN-γ-deficient mice into lethally irradiated recipient mice lead to mortality within 3 weeks while lethally irradiated mice

receiving WT CD4+ T-cells and bone marrow survived long-term (Welniak *et al.* 2000). Nevertheless, a more recent BMT murine model demonstrated that the inhibition of the IFN-γ receptor significantly reduced GvHD and in combination with anti-IL-6 receptor antibodies resulted in near-complete inhibition of GvHD. Pharmacological inhibition of JAK1/JAK2, downstream targets of IFN-γ-R and IL-6R, completely inhibited GvHD, expanded Tregs, downregulated co-stimulatory molecules on APCs while preserving and enhancing GvL effects (Choi *et al.* 2018). These observations highlight the complexity of IFN-γ signalling and its detrimental and protective roles in GvHD.

Pathogenicity of Th17 cells and IL-17 is well documented as experiments in murine, nonhuman primates and clinical trials have demonstrated their emergence and persistence in acute GvHD (Carlson *et al.* 2009, Dander *et al.* 2009, Kappel *et al.* 2009, Bossard *et al.* 2012, Furlan *et al.* 2016). Injection of *in vitro* generated Th17 cells into a BMT murine model lead to the development of lethal GvHD while transfer of T-cells from IL-17-deficient mice resulted in delayed occurrence of GvHD in comparison of transfer of WT T-cells (Carlson *et al.* 2009, Kappel *et al.* 2009). Other reports demonstrated that the lack of IL-17 resulted in increased IFN-γ levels and Th1-driven GvHD (Yi *et al.* 2008, Yu *et al.* 2011). Clinical trials have shown that patients with mild and severe aGvHD have significantly higher levels of Th17 cells and significantly lower levels of Tregs than time matched controls who did not develop aGvHD after alloHSCT (Liu *et al.* 2013). Th17 cells were found early on after alloHSCT which suggest that Th17 cells are critical for the initiation of aGvHD (Ratajczak *et al.* 2010). This event most likely attracts other T-cell populations and research suggests that crosstalk between Th17 and Th1 occurs and subsequently Th1 cells overshadow the Th17 immune response and causing the main tissue damage. Further evidence of crosstalk between Th1 and Th17 populations is demonstrated by the absence of IFN-γ as differentiation of Th17 cells was enhanced, resulting in augmented GvHD, while lack of IL-17 resulted in enhanced IFN-γ production and augmented Th1-driven GvHD (Yi *et al.* 2008, Yi *et al.* 2009).

Pro-inflammatory cytokine IL-2 is primarily secreted by T-cells and in particular by CD4+ T-cells and is required for their survival, activation and expansion. Several GvHD treatments (e.g. calcineurin inhibitors) inhibit IL-2 mediated transcription and consequent activation and differentiation of T-cells but also impair Treg development.

More than a dozen clinical studies to date investigate different strategies to block pro-inflammatory cytokine mediated GvHD. In addition, several clinical studies also investigate strategies to modulate costimulatory molecule mediated GvHD. The reader is referred to this review for a more detailed list of the above mentioned clinical trials (Hill and Koyama 2020).

4. Atorvastatin in T-cell Biology and Graft versus Host Disease

Statins have been used clinically for decades and are the most prescribed group of drugs in the world. This is due to several reasons; firstly, statins have well documented benefits in many cardiovascular diseases – the highest single cause of deaths in the world. Secondly, statins are well tolerated making them versatile drugs as they can be used in both paediatric and geriatric patients with moderate to high cardiovascular risk (Smith 2000, Davidson 2001). In addition, Atorvastatin has a well proven safety track record (Newman *et al.* 2003). Clinical trials have shown in middle aged men, at higher risk for cardiovascular diseases, the benefits of statins in improving both morbidity and mortality outweigh the costs of extended prophylactic treatment (The Scandinavian Simvastatin Survival Study 1994, Long-Term Intervention with Pravastatin in Ischaemic Disease Study 1998, Criqui and Golomb 2004).

The mode of action of statins is through inhibition of 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the conversion of HMG-CoA into mevalonic acid within the mevalonate pathway. This pathway yields many products besides cholesterol including coenzyme Q10, heme-A and isoprenylated proteins (Buhaescu and Izzedine 2007). Cholesterol is not the final product in this pathway but is a critical precursor for products such as corticosteroids, sex steroids, Vitamin D (colecalciferol) and bile acids (Mol *et al.* 1989, Hyyppa *et al.* 2003). Over the years statins have been shown to be a versatile group of drugs that goes much beyond the lipid-lowering effect (including reduction in low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides) for which they were initially prescribed. Other benefits include effects on polyunsaturated fatty acids, inflammatory markers and nitric oxide and positive effects on autoimmune diseases such as EAE (Youssef *et al.* 2002, Harris *et al.* 2004, Cimino *et al.* 2007)

Many different statins exist such as Atorvastatin, Lovastatin, Pravastatin or Rosuvastatin, which have different chemical structures and pharmacokinetic properties. Whereas some statins are fungal derived (e.g. Pravastatin or Lovastatin) others are fully synthetically derived (e.g. Atorvastatin or Rosuvastatin) (Manzoni and Rollini 2002, Schachter 2005). Statins are remarkable class of drugs in lowering serum low-density lipoprotein cholesterol (LDL-C) and triglycerides. For example, Atorvastatin reduces 50% and 29%, respectively, while Pravastatin reduces 34% and 24%, respectively (Schachter 2005). Atorvastatin's success in the clinic is partly owned to its long elimination half-life (14h) in comparison to many other statins (e.g. Lovastatin, Pravastatin or Simvastatin <3h) (Schachter 2005).

Many studies have investigated the effect of *in vitro* and *in vivo* administration of Atorvastatin on T-helper cells and Treg cell function (human studies on Tregs are summarized in Table 4). Several mechanisms have been proposed to explain how statins exert their anti-inflammatory potential including how Tregs can modulate the metabolism of lipoproteins and inhibit the development of atherosclerosis (Meier *et al.* 2008). Depletion of Foxp3+ Tregs in transgenic mice results in elevated levels of plasma cholesterol and consequently these mice develop atherosclerosis (Klingenberg *et al.* 2013). In addition, a murine ischemia-reperfusion myocardial injury model demonstrated the effect of statins on Treg induction (Ke *et al.* 2013). In this study, rats treated with statins had a significant increase of Foxp3 expression in heart and spleen accompanied with increased Treg accumulation in the heart and reduced infarct size compared to control rats. This effect was completely reversed when mevanolic acid was added suggesting that Tregs were induced by statin treatment. In addition, accumulation of inflammatory cells were negative correlated with Foxp3 expression and Treg accumulation (Ke *et al.* 2013).

4.1 Effect of Atorvastatin on Tregs

Several studies have shown that statins can directly induce Tregs (iTregs) in murine models as well as humans. An initial study by Mausner-Fainberg *et al.* (2008) showed that not all statins have the same capability to induce Tregs. Human peripheral blood mononuclear cells (PBMC) *in vitro* studies demonstrated that Atorvastatin significantly increased Tregs but Pravastatin and Mevastatin failed to do so, while, *in vivo* murine experiments failed to confirm these *in vitro* findings. Furthermore, a clinical trial with hypercholesterolemia patients, Pravastatin did elevate CD4+CD25high Tregs and increased FOXP3 expression in PBMCs (Mausner-Fainberg *et al.* 2008). In addition, Treg suppression assay revealed that Atorvastatin, but not Pravastatin, can significantly inhibit Teffector cell proliferation. Since then many other clinical trials or human *in vitro* studies (see Table 4) have confirmed the effect of Atorvastatin on the induction of Tregs as well its ability to significantly increase Treg suppression function. It has been demonstrated that Atorvastatin upregulates *FOXP3* mRNA in PBMCs (Zhang *et al.* 2011, Wang *et al.* 2015). To identify a mechanism for the demonstrated iTreg generation and upregulation of FOXP3, Tang *et al*. (2011), probed *in vitro* Atorvastatin-treated CD4+CD25- T-cells for several pathways involved in FOXP3 signalling. PI3K-Akt-mTor and ERK signal transduction pathways negatively regulate FOXP3 expression and Atorvastatin inhibits phosphorylation of these pathways. Other signalling molecules involved in FOXP3 expression such as STAT5, SMAD3 or JNK were unaffected. Another potential mechanism may be the TSDR methylation status which is critical to maintenance of FOXP3 expression and Treg phenotype, although PBMCs-treated with Atorvastatin did not show any increase in FOXP3 TSDR demethylation yet Simvastatin did (Rodriguez-Perea *et al.* 2015). Moreover, CD4+ cells isolated from Atorvastatin-treated individuals showed increased *TGF-β* mRNA levels and it is known that TGF-β drives FOXP3 expression in a SMAD3-dependent manner, although transcription of other anti-inflammatory mediators, measured by *IL-10* and *IDO* mRNA levels, were

unaffected (Rodriguez-Perea *et al.* 2015). Consistent with the upregulation of Treg numbers and/or function by statins, it has been demonstrated that inhibition of geranylgeranylation (GGPP) but not farnesylation (FPP), down-stream products of the mevalonate pathway and catalyzed by the enzymes GGTase and FTase, respectively, potentiates the effect of TGF-β in the generation of iTregs and increases their suppressive function *in vitro* (Zhang and Casey 1996, Kagami *et al.* 2009). This effect was confirmed in an *in vivo* colitis model in which adoptive transfer of CD4+CD25- T-cells treated with TGF-β and GGTase inhibitor significantly reduced severity of colitis in comparison to TGF-β alone (Kagami *et al.* 2009).

A recent publication by Rodriguez-Perea *et al*. (2019) took another approach to previous studies and investigated the effect of Atorvastatin on Tregs. FACS-sorted Tregs (CD4+CD25highCD127dim) were incubated with Atorvastatin (0.5-10μM) for 48h, washed and then incubated with T-effector cells rather than incubating Tregs and responder cells together with Atorvastatin. Results showed that low concentrations of Atorvastatin (0.5-1μM) did not have any effect on suppressive capacity in comparison to control Tregs and that high concentrations (2-10μM) significantly reduced Treg suppression. In addition, key suppressive markers of Treg function were investigated by stimulating Tregs with anti-CD3/CD28 and subsequent treatment with Atorvastatin. High Atorvastatin (10μM) reduced the expression of FOXP3, CTLA-4 and PD-1 but not CD25. Under certain circumstances Tregs can gain an inflammatory phenotype and secrete pro-inflammatory cytokines, to test this possibility, isolated Tregs were initially stimulated with Atorvastatin and then re-stimulated with phorbol myristate acetate (PMA) and ionomycin. Atorvastatin-treated Tregs secreted, in a dose-dependent manner, lower levels of pro-inflammatory cytokine IFN-γ but increased levels of TNF-α, while IL-10 secretion was unaffected.

Atorvastatin may alter Treg numbers indirectly via the modulation of DCs. Several papers have shown that *in vitro* Atorvastatin treatment of murine bone marrow-derived dendritic cells (BMDC) generates a tolerogenic phenotype by downregulating CD80, CD86 and MHC-II (Xu *et al.* 2014, Li *et al.* 2015, Chen *et al.* 2018). Atorvastatin-treated DCs were administered *in vivo* which resulted in a reduced clinical score in several CD4+ T-cell mediated disease models such as experimental autoimmune neuritis (EAN) (Xu *et al.* 2014), experimental autoimmune myasthenia gravis (EAMG) (Li *et al.* 2015) and EAE (Chen *et al.* 2018). Interestingly, whereas Xu *et al.* (2014) and Chen *et al.* (2018) reported an increase in Foxp3+ Tregs, Li *et al.* (2015) did not.

Dose of Atorvastatin	Subjects and approach	Possible mechanism involving Treg	Reference
In vivo: 10mg/d for 2 weeks	Peripheral blood samples from ACS patients; Baseline vs 2 weeks (n=48)	↑ CD4+CD25+ Tregs ↑ Suppressive function of Tregs ↑ IL-10, ↑ TGF-β, ↓ IFN-γ	Hu *et al.* (2007)
In vitro: 2-50μM	PBMC culture (n=5)	↑ CD4+CD25high Tregs ↑ CD4+CD25highFOXP3+ Tregs ↑ Suppressive function of Tregs	Mausner-Fainberg *et al.* (2008)
In vivo: 20mg/d for 4 weeks	Peripheral blood of renal transplant recipients and hypercholesterolemic patients; Baseline vs 4 weeks (n=50)	↔ No increase in CD4+CD25+FOXP3+ Tregs ↔ No sign. change in IL-2, IFN-γ, IL-10 [1]	Guillen *et al.* (2010)
In vitro: 1-10μM *In vivo*: 20mg/d for 12 weeks	*In vitro*: CD4+CD25- from patients with RA *In vivo*: Baseline vs 12 weeks (n=97)	↑ CD4+CD25+FOXP3+ Tregs ↑ Suppressive function of Tregs ↓ Phosphorylation of Akt, mTor and ERK	Tang *et al.* (2011)
In vivo: 80mg after diagnosis + 20mg/d after PCI for 12 weeks	Blood samples obtained from the ICA and a peripheral vein, STEMI patients (n=112)	↑ CD4+CD25+ Tregs ↑ *FOXP3* mRNA in PBMC ↑ TGF-β, ↓ IFN-γ	Zhang *et al.* (2011)
In vivo: 20mg/d for 4 weeks	Peripheral blood samples from ACS patients + healthy volunteers.; Baseline vs 4 weeks (n=180)	↑ CD4+CD25+ Tregs ↑ CD4+CD25+FOXP3+ Tregs [2] ↑ *FOXP3* mRNA in PBMC ↑ Suppressive function of Tregs ↑ IL-10, ↑ TGF-β, ↓ IFN-γ	Wang *et al.* (2015)

In vivo: 20mg/d for 45 days	Peripheral blood of healthy male volunteers (n=21)	↑ CD4+FOXP3+ Tregs ↓ CD4+FOXP3+CD25+ Tregs ↑ *FOXP3* mRNA in PBMC ↑ TGF-β and CTLA-4 in CD4+FOXP3+ Tregs	Rodriguez-Perea *et al.* (2015)
In vitro: 5-20μM	CD4+ T-cells from ITP patients	↑ CD4+CD25+FOXP3+ Tregs ↓ CD25 expression on CD4+ cells ↓ CD4+CD25+CD45RA+ ↓ CD4+CD25+CD45RO+ ↓ CD4+ proliferation	Xu *et al.* (2018)
In vitro: 0.5-10μM	CD4+ T-cells from healthy volunteers	↓ Suppressive function of CD4+CD25+CD127dim Tregs at high AT conc. [3] ↓ FOXP3, CTLA-4 and PD-1 of Tregs at high AT conc. [3]	Rodriguez-Perea *et al.* (2019)

Table 4. Clinical Trials or Human *In Vitro* Studies that Investigated the Effect of Atorvastatin on T-Regulatory Cells.

The table summarizes clinical trials or human *in vitro* studies (as of October 2020) that investigated the effect of Atorvastatin on Tregs and closely related assays and cytokines.

[1] Whole blood of renal transplant recipients was stimulated with concanavalin (ConA) for 48h. Supernatant was then analysed.

[2] Data not shown for CD4+CD25+FOXP3+ Tregs but mentioned in text that a similar trend was seen as with CD4+CD25+ Tregs

[3] Tregs were incubated with Atorvastatin for 48h, then washed and incubated with responder cells and activated without Atorvastatin.

Adapted and modified from Forero-Pena *et al.* (2013)

4.2 Effect of (Atorva)statin on T-helper Populations

One of the first studies investigating the effect of Atorvastatin on an anti-inflammatory phenotype was conducted by Youssef *et al.* (2002) that demonstrated Atorvastatin could inhibit and reverse chronic and relapsing EAE in a murine model. They attributed this effect to a shift from Th1 cells, known to mediate EAE, to Th2 cells based on cytokine profile changes from IL-2, IL-12, IFN-γ and TNF-α to IL-4 and IL-5 as well as IL-10 and TGF-β. Protein extracts from draining lymph nodes confirmed this finding as Stat4 phosphorylation, a major driver of pro-inflammatory cytokine production, was abolished while a prominent stimulator of anti-inflammatory cytokines, as measured by Stat6 phosphorylation, was increased. It was concluded that atorvastatin can promote Th2 differentiation at the expense of the Th1 lineage. In addition, naïve Th0 Atorvastatin-treated cells inhibited T-cell proliferation *in vitro* and this effect could be reversed by mevalonic acid. Furthermore, professional APCs from Atorvastatin-treated mice had reduced ability to activate T-cells *in vivo* and *in vitro* and primary macrophages activated by IFN-γ had down-regulated expression of MHC-II and co-stimulatory molecules CD40, CD80 and CD86. Another key finding was that adoptive transfer of atorvastatin-treated CD4+ cells protected against induction of EAE in recipient mice but CD8+ cells did not. Clinical trials confirmed Atorvastatin's effect on Th1 differentiation and cytokine secretion. For example, Hu *et al*. (2007) and Wang *et al*. (2015) found that in acute coronary syndrome patients Atorvastatin reduces IFN-γ secretion and elevates anti-inflammatory cytokines IL-10 and TGF-β.

Another statin, Simvastatin, which is closely related to Atorvastatin, was shown to inhibit Th17 differentiation under polarising conditions and this was accompanied with markedly decreased *in vitro* IL-17 production (Kagami *et al.* 2009). In line with other studies, IFN-γ production and proliferative responses of CD4+ cells are not affected, while inhibition of Th17 differentiation can be reversed by mevalonic acid. As mentioned above the inhibition of geranylgeranylation is beneficial for iTreg generation, and GGTase I

inhibitor, but not FTase inhibitor, decrease Th17 differentiation and IL-17 production indicating that protein geranylgeranylation is critical for Th17 differentiation and for the balance between Th17 cells and Tregs. In addition, GGTase I inhibitor-treated CD4+ cells have reduced IL-6 mediated *in vitro* STAT3 phosphorylation, a critical pathway for the induction of RORγt expression and IL-17 production (Kagami *et al.* 2009).

Protein prenylation (15-carbon farnesyl or 20-carbon geranylgeranyl) is a post-translational modification that possesses regulatory capacity in cell signalling and function and mediates cell membrane association with many GTPases. Prenylation is a downstream target of the mevalonate pathway and is inhibited by statins.

It has been shown that protein farnesylation favours Th1 differentiation and IFN-γ production in a manner that does not affect T-cell proliferation but rather inhibits Th2 differentiation and cytokine production, and that this mechanism is inhibited by Atorvastatin in favour of Th2 differentiation and a significant reduction in T-cell proliferation (Dunn *et al.* 2006). As expected, T-cells treated with a farnesylation inhibitor rather than statins show increased GATA-3 and decreased T-bet expression, while inhibition of geranylgeranylation did not affect T-bet expression nor IFN-γ secretion in comparison to controls but instead increased IL-4 secretion (Dunn *et al.* 2006). Importantly, neither prenyltransferase inhibitors used in this study achieved the increased levels of IL-4 secretion induced by Atorvastatin suggesting that repression of both farnesylation and geranylgeranylation is required for full Th2 differentiation. Consistent with this finding, several farnesyltransferase inhibitors were able to reduce the secretion of pro-inflammatory cytokines in *in vitro* anti-CD3/CD28 stimulated murine CD4+ cells (Marks *et al.* 2007). This result was attributed not to interference of signalling transducers such as ERK, JNK or MAP nor the suppression of cytokine mRNA but inhibition of T-cell activation at the post-translational level. To elucidate a more defined molecular mechanism on how isoprenylated proteins induce a Th differentiation responses, an antigen-driven mouse model was used (MBP Ac1-11 TCR Tg mice), as

farnesylated Ras and geranylgeranylated Rac1, RhoA, and Rap are the only prenylated proteins known to be situated in antigen-activated signalling pathways that modulate T-helper differentiation (Cantrell 2003). Atorvastatin-treatment leads to an accumulation of Ras, Rap and RhoA but not Rac1 in the cytosol of the draining lymph node accompanied with decreased Ras and RhoA at the membrane (Dunn *et al.* 2006). This is important as strong TCR signalling activates Ras and ERK leading to increased c-fos activity to cause c-fos and jun interactions that form an AP-1 heterodimer that ultimately transactivates the *IFN-γ* promoter and represses the *IL-4* promoter (Badou *et al.* 2001, Jorritsma *et al.* 2003, Dunn *et al.* 2006). As Atorvastatin decreases available Ras and RhoA at the membrane it may skew T-cell polarisation towards Th2 differentiation through reduced TCR signalling. TCR signalling targets p38 and ERK and transcription factors c-fos (a target of p38 and ERK), ATF-2 (a target of p38 and JNK) and c-jun (target of Rac1 and JNK) would all be affected by a reduction in membrane bound Ras and RhoA. Consistent with the effects on Ras and RhoA, *in vivo* Atorvastatin treatment reduces the TCR-induced activation of ERK and p38 as well as decreasing the DNA-binding of c-fos and ATF-2 whereas Rac1 target c-jun appears unaffected. This represses *IFN-γ* promoter activity and activates Rac1-JNK mediated *IL-4* promoter activity. To confirm that Atorvastatin-mediated inhibition of Ras-ERK and RhoA-p38 signalling was responsible for Th2 differentiation, upstream targets of these pathways were inhibited resulting in reduced IFN-γ and increased IL-4 production in CD4+ cells (Dunn *et al.* 2006). Interestingly, administration of farnesylation inhibitors results in delayed onset of EAE in mice without significantly reducing clinical score. Isolated and stimulated splenocytes significantly secrete less IFN-γ *in vitro* yet fail to induce an increase IL-4 production nor decrease TNF-α production. Collectively, these studies suggest that both farnesylation and geranylgeranylation are required for full Th2 commitment.

4.3 Atorvastatin and Graft versus Host Disease

There is considerable evidence that Atorvastatin is beneficial in the treatment of autoimmune diseases such as Graft-versus-Host Disease (GvHD). Zeiser *et al*. (2007) demonstrated in a comprehensive study that Atorvastatin can protect against GvHD and permit GvL in an MHC-mismatch allogeneic bone marrow transplantation (BMT) murine model which has laid the foundation for many clinical studies. Adoptive transfer of T-cell depleted bone marrow (TCD-BM) and T-cells into recipient mice causes development of acute GvHD (aGvHD) with death within 20 days after allogeneic BMT. In contrast, T-cells from donor mice fed Atorvastatin were associated with a significantly reduced mortality rate in the respective recipient mice (Zeiser *et al.* 2007). This effect was attributed to extensive reduction in donor T-cell expansion and lower levels of IFN-γ and TNF production in combination with elevated IL-10 and IL-4 secretion. Co-stimulatory molecules CD40, CD80, CD86, and MHC-II on APCs derived from liver, spleen and lymph nodes of Atorvastatin-fed recipient mice were analysed and displayed vastly reduced expression compared to controls. As these markers are critical for the induction of aGvHD, it appears that this may be one pathway for Atorvastatin to exert a protective effect in this disease. Moreover, Atorvastatin-fed recipient mice in this study also displayed increased levels of IL-4 and IL-10 secreting T-cells further strengthening the notion that Atorvastatin is protective against aGvHD particularly as these mice have a significantly higher survival rate in comparison to non Atorvastatin-fed recipient mice. Combining both approaches, Atorvastatin treatment of both donor and recipient resulted in even higher overall survival rate.

Stat6-deficient mice were studied in this GvHD setting to investigate the underlying molecular mechanisms that cause the shift from pathology-driving Th1 cells to protective Th2 cells. As Stat6 is required for IL-4 dependent Th2 lineage commitment, Stat6-deficiency led to a decrease in Atorvastatin-mediated protection but did not completely abrogate the effect on aGvHD lethality. Consistent with this mortality rate, T-cell

proliferation was significantly reduced in Atorvastatin-treated Stat6-deficient mice in comparison to control group but also significantly increased in comparison to Atorvastatin-treated wild-type mice. This was also reflected in the overall GvHD histopathology score as it was significantly reduced in Stat6-deficient mice in comparison to control group but increased to Atorvastatin-treated WT mice.

Prenylation is important in TCR-mediated signalling and T-cells treated with Atorvastatin and alloantigen showed reduced levels of RAS, RAP-1 and Rho-B prenylation compared to T-cells only stimulated with alloantigens. Atorvastatin-treated donor CD4+ T-cells were analysed before injection into recipient mice and showed reduced levels of Stat4 and Stat1 phosphorylation and slightly increased Stat6 phosphorylation, key mediators of Th1 and Th2 differentiation, respectively. Donor CD4+ cells were also analysed in recipient mice 3 days after BMT and showed that the induced phenotype by atorvastatin is preserved as Stat4 and Stat1 phosphorylation was still significantly reduced, and that the Th17 signalling mediator more Stat3 was slightly reduced than in control groups, while Stat6 was significantly upregulated. T-bet, a lineage factor for Th1-commitment, was significantly reduced and GATA-3 and c-MAF, two lineage factors for Th-2 commitment, were significantly elevated 3 days post-BMT. Taken together, these findings demonstrate how, at the molecular level, Atorvastatin induces polarisation of Th2 and suppression of Th1/Th17.

A successful alloHSCT/BMT requires not only the prevention of GvHD but a permissive environment for the Graft-versus-Leukaemia effect. Animals who received TCD-BM and leukemic cells achieved tumour control but died of aGvHD. Atorvastatin-treated recipient mice receiving Atorvastatin-treated leukemic cells achieved tumour control but importantly did not die of aGvHD, strongly indicating an intact cytotoxic T-lymphocyte response against the leukaemia cells. Histology and flow cytometry of bone marrow 7 days after BMT demonstrated total clearance of leukemic cells. Consistent with other studies, isolated and CD8+ T-cells from donor animals did not exhibit increased *in vitro*

cytolytic activity against lymphoma cell lines when treated with Atorvastatin. Despite the successful prevention of GvHD, and other studies demonstrating Atorvastatin's effect on pTreg generation, this study could not observe any increase in Foxp3+ cells.

Few studies investigated the effect of Atorvastatin on GvHD but other studies investigated the effect of other statins such as Simvastatin or Lovustatin on GvHD. Wang *et al.* (2009) demonstrated that Lovustatin reduces mortality and morbidity in an murine BMT model. This effect was attributed to reduced T-lymphocyte homing to the lymph node and Peyer's patch and consequently reduced GvHD (Wang *et al.* 2009). A more recent study investigated the effect of Simvastatin on GvHD and angiopoietins (Zheng *et al.* 2017). Angiopoietins play an important role in vascular endothelial function and endothelial damage is a key pathogenesis seen in GvHD (Leonhardt *et al.* 2013, Riesner *et al.* 2017). Simvastatin could modulate angiopoietins *in vitro* and *in vivo* and ameliorate aGvHD and survival in mice.

A retrospective analysis investigating the prophylaxis effect of statins on GvHD in haematological malignancies in a clinical setting demonstrated that donor use (n=75, 65% used Atorvastatin) reduced grade III-IV aGVHD in recipient (Rotta *et al.* 2010). Interestingly, statin use by recipient only (n=16) did not confer GvHD protection. Hamadani *et al.* (2013) (NCT01175148) investigated the prophylactic effect of Atorvastatin on donor as well as recipient in combination with standard tacrolimus and methotrexate treatment of matched sibling allogeneic HSCT (Hamadani *et al.* 2013). Acute GvHD prevalence was low, especially as this study included many high-risk patients for acute GvHD, yet chronic GvHD was comparable to historical data. Nonetheless, the authors concluded that Atorvastatin is an effective strategy against acute GvHD. These results were replicated in a more recent alloHSCT trial (NCT01665677) which included matched unrelated donors (MUD) although only recipients received Atorvastatin treatment (Kanate *et al.* 2017). The authors noted that the cumulative incidence rate for aGvHD in matched related donors (MRD) were low in

comparison to historical data but higher than in Hamadani *et al.* (2013) study which suggests that donor treatment with Atorvastatin might be beneficial. Again, cGVHD were comparable to historical cohorts. A clinical trial conducted by Efebera *et al.* (2016) (NCT01491958) investigating the prophylactic effect of Atorvastatin on MRD undergoing alloHSCT reported no beneficial effect of Atorvastatin treatment on aGVHD incidence. Analysis revealed unusually high upper gastrointestinal (UGI) aGVHD (42% of grade II-IV) which explained the high incidence rate despite Atorvastatin treatment. This is in stark contrast to two large retrospective studies that reviewed the incidence rate for high upper gastrointestinal aGvHD in alloHSCT and found that 6.7% for MRD, MUD and mismatched unrelated donors (MacMillan *et al.* 2015) (n=1723) and 1.9% and 3.4% for MRD and unrelated donors, respectively, (Nikiforow *et al.* 2018) (n=8567) develop UGI aGVHD. Nonetheless 1-year OS was significantly increased (82% vs 58%).

Current Phase II clinical trials (see Table 5) investigating the effect of Atorvastatin alone or in combination with standard treatment (tacrolimus and methotrexate) on GvHD in different transplantation settings have shown promising results in the prevention or reduction of aGvHD and increase in the overall survival rate. No toxicity has been associated with Atorvastatin and it has been deemed to be safe in this setting. Due to these promising results a phase III study (NCT03066466; recruitment stage as by clinicaltrial.gov) has been planned in the United States. To date no clinical trial is investigating the effect of Atorvastatin on GvHD in Europe including the United Kingdom.

Regimen	Trial and Patients	Outcome	Reference
≥40mg/d statin [1]	The impact of HMG-CoA reductase inhibition on the incidence and severity of GVHD in patients with acute leukaemia undergoing allogeneic transplantation Study Location: USA	1. Sign. reduction in Gr II-IV of aGvHD 2. No difference in cGvHD 3. All patients engrafted 4. Kaplan-Meier estimates of PFS at 3years in AML patients with or without statin use were 54% and 28%, respectively 5. Strong indication that GvL is preserved under statin regimen	Hamadani *et al.*, Blood (2008)
40mg/d AT in combination with standard tacrolimus and short-course methotrexate	**Phase II Trial** (NCT01175148); Sibling Donor and Recipient Immune Modulation With Atorvastatin for the Prophylaxis of Acute Graft-Versus-Host Disease Study Location: USA	1. AT administration was not associated with any Gr III-IV AE 2. aGvHD: Day 100 and 180: (CI) 3.3% and 11.1%, respectively 3. Viral and fungal infections were infrequent 4. One-year NRM 9.8%, relapse 25.4%, one-ear OS 74%, PFS 65% 5. AT administration was associated with increase in IL-10 6. **Conclusion**: Administration in both donors and recipients of matched sibling allogeneic HCT seems to be a feasible, safe, and potentially effective strategy to prevent acute GvHD.	Hamadani *et al.*, J Clin Oncol. (2013)

Regimen	Trial and Patients	Outcome	Reference
40mg/d AT in combination with standard tacrolimus and micro-dose methotrexate	**Phase II Trial** (NCT01665677); Prospective, Multicenter Clinical Trial of Atorvastatin-Based aGVHD Prophylaxis in Recipients of HLA-Matched Related Donor (MRD) and Matched Unrelated Donor (MUD) alloHCT Study Location: USA	1. All patients engrafted. 2. Day100: CD33+ cell chimerism in both cohorts was 100% 3. aGvHD: Day 100: (CI) MRD: Gr I-II 9.9%, Gr III-IV 3.4% MUD: Gr I-II 29.8%, Gr III-IV 18.3% aGVHD: Day 180: (CI) MRD: Gr III-IV 3.4% MUD: Gr III-IV 21.4% 4. cGvHD (1 year): moderate/severe (CI) MRD: 35.6% MUD: 43.5% 5. 1-year NRM 3.3% and 9.4%; relapse 43% and 19%, PFS 50% and 71% and OS 65% and 76% respectively for MRD and MUD. 6. **Conclusion**: Addition of AT to standard GvHD prophylaxis in MRD and MUD HCT appears to be feasible, safe and effective.	Kanate *et al.*, BBMT (2017)
AT in combination with Fludarabine Phosphate and Mycophenolate Mofetil	**Phase II Trial** (NCT01527045); Donor Atorvastatin Treatment in Preventing Severe Acute GVHD After Nonmyeloablative Peripheral Blood Stem Cell Transplant in Patients With Hematological Malignancies Study Location: USA	Study not yet completed.	

Regimen	Trial and Patients	Outcome	Reference
AT daily	**Phase II Trial** (NCT01525407); Donor Atorvastatin Treatment for Preventing Severe Acute Graft-Versus-Host Disease in Patients Undergoing Myeloablative Peripheral Blood Stem Cell Transplantation Study Location: USA	Study not yet completed.	
40mg/d AT in combination with standard tacrolimus and micro-dose methotrexate	**Phase III Trial** (NCT03066466); Randomized Study: Standard of Care With or Without Atorvastatin for Prevention of GvHD for Matched Unrelated Donor BMT Study Location: USA	Study not yet completed.	

Table 5. Clinical Trials to Date Investigating the Effect of Atorvastatin Alone or Being Part of a Drug Regime in the Prevention of GVHD. Clinical trials only included when listed on clinicaltrials.gov (as of August 2020) with the exception for the study conducted by Hamadani *et al.* (2008).

5. Vitamin D in T-cell Biology and Graft versus Host Disease

Vitamin D is a versatile molecule which influences many aspects of the immune system beyond its classical role in calcium homeostasis and bone metabolism. Although there is no common agreement on the threshold levels for Vitamin D deficiency, a growing interest in Vitamin D biology has led to the estimation that a significant portion of the world's population are likely to suffer from hypovitaminosis D (Hagenau *et al.* 2009, Mithal *et al.* 2009). This is a worrying observation as Vitamin D deficiency is known to cause osteoporosis and muscle weakness, increasing the risk for fractures due to falls, which is particularly concerning for the elderly. Given that life expectancy in many developed countries are progressively becoming higher, age related diseases or injuries will become increasingly prevalent (Janssen *et al.* 2002). Hypovitaminosis D is involved in many disease states including not only metabolic and cardiovascular disease but also immunological pathologies such as autoimmune disease (Judd and Tangpricha 2009, Pilz *et al.* 2009, Shoenfeld *et al.* 2009).

In humans, Vitamin D can be derived from two sources: by sunlight, through synthesis in the skin, or through the diet. Sunlight-derived Vitamin D is the most important source and the amount of Vitamin D produced depends on the intensity of ultraviolet irradiation which, in turn, is dependent on season, latitude and region. Vitamin D_3 from the skin (provitamin D_3) is not biologically active and undergoes a multi-step process to be activated. Vitamin D_3 is transported via the blood by Vitamin D binding protein (DBP) to the liver where it is hydroxylated to produce 25-hydroxyvitamin D_3 ($25(OH)D_3$) and this is the major circulating form in the blood. In addition, this metabolite is used routinely as a biomarker to test for Vitamin D concentrations in serum as it has a long half-life of around 2-3 weeks. 25-hydroxyvitamin D_3 is then transported by DBP to the kidneys where it is further hydroxylated to its biologically active form, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$ also called calcitriol). $1,25(OH)_2D_3$ can exert biological effects in a non-genomic or genomic manner. Non-genomic effects of $1,25(OH)_2D_3$ follow binding to

membrane-associated receptors such as the rapid-response steroid-binding protein receptor (1,25D_3-MARRS) to influence signalling pathways directly (Haussler *et al.* 2011, Larriba *et al.* 2014). Genomic action of calcitriol is exerted by binding to the nuclear vitamin D receptor (VDR) which is found in many different cell and tissue types (Pike and Meyer 2012). VDR forms both homodimers or a heterodimer with the retinoic x receptor (RXR) and both can bind to the vitamin D response element (VDRE) in order to regulate more than 900 genes involved in innate and adaptive immunity as well as act through other physiological pathways (Bizzaro *et al.* 2017).

5.1 Vitamin D and T-Regulatory Cells

Initial evidence that Vitamin D may be involved in T-cell responses and immunity came with the observations that activated T-cells express VDR (Bhalla *et al.* 1983). Vitamin D treatment of monocytes differentiating to DCs leads to an immature DC phenotype (Unger *et al.* 2009). In addition, these tolerogenic DC had increased capacity to convert naïve CD4+ T-cells into IL-10 producing Tregs. It was suggested that PD-L1 expression was important in mediating this Treg transition as suppression of PD-L1 on DCs leads to the induction of IFN-γ, rather than IL-10, producing T-cells. Furthermore, Vitamin D has been shown to induce the transcription of *FOXP3* which is critical for the induction of Tregs as well as expression of CTLA-4 (Jeffery *et al.* 2009, Joshi *et al.* 2011). An anti-inflammatory environment is further enhanced by Vitamin D acting on pro-inflammatory T-cells through inhibiting Th17 cell differentiation and IL-17 secretion by inhibiting NFAT binding to the *IL-17* promoter and subsequent reduction in IL-17 transcription (Liu *et al.* 2004). VDR/RXR binding to the *IL-17* promoter at common binding sites, for either VDR/RXR or NFAT, blocks NFAT in a competitive manner and thereby inhibiting NFAT-mediated IL-17 transcription (Joshi *et al.* 2011). This is in agreement with a recent report which showed that Vitamin D significantly reduces T-cell capacity to secrete a variety of pro-inflammatory cytokines including IL-13, IL-9 and IL-17, while IFN-γ and TNF-α were

reduced in comparison to controls (Van Belle *et al.* 2014). Tregs are key in controlling autoreactive T-cells and Vitamin D-modulated tolerogenic DCs could induce antigen-specific Tregs that potently suppress Teffector cells (Kleijwegt *et al.* 2011). Clinical trials have investigated the effect of Vitamin D supplementation on Treg frequency and have found that Vitamin D increases Tregs. Two 12-week studies in healthy volunteers reported that Treg frequency significantly increased over the course of the trial and had no effect on other immune cell frequencies or toxicity (Bock *et al.* 2011, Prietl *et al.* 2014) although no increase in Treg suppression could be measured *in vitro* (Prietl *et al.* 2014). This is in contrast to a study in young new-onset type 1 diabetes patients in which no increase in Tregs could be measured but a significant increase in Treg suppressive capacity was observed (Treiber *et al.* 2015). An 18-month randomized trial also reported a slight increase in Tregs (Gabbay *et al.* 2012) associated with a non-significant reduction on pro-inflammatory cytokines IL-12 and TNF and anti-inflammatory cytokine IL-10 but no effect on IL-12, IL-6, IL-1β, IL-8. Only CCL2 was significantly increased in the Vitamin D treatment group which has been reported to be an important factor in the differentiation of naïve CD4+ cells to the Th2 phenotype (Gu *et al.* 2000, Roep 2003). Vitamin D treatment in SLE patients, who often have low Vitamin D levels, revealed a non-significant increase in IL-4 among CD4+ and CD8+ T-cells and a slight reduction in the expression of IFN-γ and IL-17 in association with an increase in Tregs (Piantoni *et al.* 2015). A systematic review by *Fisher, Roberts, Lamikanra and colleagues* analysing 8 studies with known Treg content confirmed the beneficial role of Vitamin D treatment on Treg frequency (Fisher *et al.* 2019).

5.2 Vitamin D and Graft versus Host Disease

It is widely accepted that Vitamin D deficiency is a common side effect in HSCT resulting from many factors associated directly with this intervention, these include long hospitalisation and related lack of sun exposure, variable degrees of recovery, which is often associated with nutritional compromise as well as multisystem organ damage due to GvHD, or infections (Ros-Soto *et al.* 2019). The majority of studies agree that Vitamin D levels decrease post-HSCT due to leukopenia and subsequent reduction in local Vitamin D production (Kreutz *et al.* 2004). In addition, Vitamin D deficiency, or a reduction in Vitamin D levels, is more prevalent post- than pre-HSCT (Sproat *et al.* 2011, Beebe *et al.* 2018). Reduced Vitamin D levels can have significant effects on overall survival as several studies have demonstrated. A retrospective study by von Bahr *et al*. in 166 adults showed that the 2-year overall survival was 13% lower in patients with Vitamin D deficiency (von Bahr *et al.* 2015). In addition, patients with lower Vitamin D levels pre-HSCT had lower overall survival and higher relapse rates in comparison to patients with higher Vitamin D levels (Hansson *et al.* 2014, Wallace *et al.* 2015, Beebe *et al.* 2018). In contrast, other studies have not found a correlation between Vitamin D status and overall survival or relapse (Glotzbecker *et al.* 2013, Campos *et al.* 2014, Caballero-Velazquez *et al.* 2016).

Acute GvHD is common in patients undergoing HSCT and its severity is often an indicator for the development of chronic GvHD. Kreutz *et al*. demonstrated that most patients had Vitamin D levels in the normal range before transplantation with many patients experiencing a marked decrease after transplantation. Interestingly, patients who developed grade III-IV aGvHD have lower Vitamin D serum levels than those patients who developed grade I-II aGvHD, these findings suggest that Vitamin D influences the development of aGvHD (Kreutz *et al.* 2004). A more recent study confirmed these observations (Urbain *et al.* 2012) while other studies have demonstrated that patients with lower Vitamin D levels at the time of transplant were much more likely

to develop chronic GvHD (Glotzbecker *et al.* 2013, von Bahr *et al.* 2015). However, other studies failed to find an association between acute or chronic GvHD and Vitamin D deficiency (Robien *et al.* 2011, Campos *et al.* 2014, Wallace *et al.* 2015, Beebe *et al.* 2018). To date (October 2020), only three clinical studies (NCT02600988, NCT03759262 and NCT03093805) have investigated Vitamin D in the context of alloHSCT and GvHD. One multicenter trial (NCT02600988) found that daily doses of Vitamin D (1000 or 5000 IU per day, days -5 to +100) did not ameliorate incidence rate of acute GvHD but significantly lowered cumulative incidence rate of chronic GvHD (Caballero-Velazquez *et al.* 2016), while the other two clinical trials are ongoing. It has also to be noted that in this trial patients did not undergo any *ex vivo* or *in vivo* T-cell depletion as GvHD prophylaxis.

6. Aims and Hypothesis

Acute and chronic GvHD are very prevalent in many patients undergoing allogeneic HSCT and cause significant morbidity and mortality. Key mediators of pathology in GvHD are activated donor T-cells which secrete pro-inflammatory cytokines and cause severe damage in immunosuppressed patients. On the other hand, T-regulatory cells have been demonstrated to be a significant determinant in the outcome of alloHSCT and a high ratio of Tregs:CD4+ T-cells in the donor graft improves survival rates considerably.

Statins have been used for decades in the clinic as lipid-lowering drug and hasve proven their excellent safety profile. In addition, it is demonstrated that Atorvastatin can induce anti-inflammatory responses, suppress T-cell mediated immune responses and induce Tregs. Furthermore, many patients undergoing alloHSCT are Vitamin D deficient, especially post-alloHSCT, and several studies have associated this deficiency with reduced overall survival and increased relapse rates. Vitamin D also has been demonstrated to modulate immune responses by inhibiting pro-inflammatory T-cells and to induce Tregs.

Several studies have investigated the effect of Atorvastatin on human cells *in vitro* but used supraphysiological levels of Atorvastatin (>1-50μM). Yet, the highest acceptable dose given is 80mg (daily) which is approximately equivalent to a mean serum concentration of 80-100nM with a C_{max} of 200-250nM in a healthy subject (Cilla *et al.* 1996, Stern *et al.* 2000, Bjorkhem-Bergman *et al.* 2011). Furthermore, clinical studies to date (see Table 5) use lower doses (40mg) of Atorvastatin. In addition, several human studies investigating Tregs *in vitro* and *in vivo* failed to define them as CD4+CD25highCD127dimFOXP3+, which is accepted in the literature as a valid standard phenotypic definition of a Treg cell. These studies (see Table 4) either defined Tregs without FOXP3, which gives the possibility to include T-cells which do not have the epigenetic programming of a Treg cell and, hence, have no regulatory capacity, or

without CD127 which allow to include activated T-cells which upregulated FOXP3 but, again, do not have the regulatory capacity of a Treg cell.

Many completely novel strategies are being developed to boost Treg numbers and to prevent or manage GvHD. Such novel approaches require rigorous and extensive testing to assess their effectiveness and safety before they can be used in the clinic. The aim of this thesis was to test a simple but novel strategy to understand if Vitamin D (calcitriol) and statins (Atorvastatin), that have already have regulatory approval, can be repurposed alone or in combination to inhibit T-cell responses and/or boost Treg numbers.

Therefore, the overall aim of the thesis was to test the hypothesis that the combination of Atorvastatin and Vitamin D, at physiological levels, is effective in inhibiting T-cell responses and in inducing T-regulatory cells *in vitro*.

The primary objective of the thesis was to test if physiological concentrations of Atorvastatin and Vitamin D can (1) induce CD4+CD25highCD127dimFOXP3+, (2) modulate Th1/Th2/Th17 Teffector populations, (3) enhance Treg suppressive function and (4) induce FOXP3 at the transcriptional and translational level.

An additional objective was to investigate the possibility if Atorvastatin and Vitamin D can induce demethylation of the TSDR in CD3+ T-cells and increase Treg lineage stability. Naive Tregs (nTregs) and peripheral Tregs (pTregs) exhibit a fully demethylated TSDR which confers their FOXP3 expression and stabilizes their Treg function. In contrast, *in vitro* induced Tregs (iTregs) do not express a fully methylated TSDR and consequently are unstable in their FOXP3 expression and Treg function.

Dendritic cells are critical in mounting an effective immune response and contribute to GvHD. Monocyte-derived dendritic cells were generated with the objective of investigating if Atorvastatin and Vitamin D can induce a tolerogenic phenotype and skew cytokine secretion to be anti-inflammatory. Furthermore, we tested whether Atorvastatin

and/or Vitamin D generated monocyte-derived dendritic cells enhance suppressive function of Tregs. A final objective was to investigate aldehyde dehydrogenase (ALDH) activity in monocyte-derived dendritic cells as ALDH significantly contributed to the initiation and progression of GvHD in murine bone marrow transplantation models.

CHAPTER 7

Materials and Methods

7. Materials and Methods

7.1 Research Ethics

Ethical approval was given by the National Research Ethics Committee (REC reference 15/SC/0514). All blood donors gave written informed consent to allow their donation to be used for research.

7.2 Chemicals and Plasticware

Chemical	Supplier	Catalog Number
BSA	Sigma	A9418-100G
Chloroform	Sigma	C2432-500ML
DMSO	Sigma	D2650-100ML
Ethanol	ThermoFisher	E/0665DF/17
DPBS	Gibco	14190-094
Distilled Water	Gibco	15230-089
15ml Falcon Tube	Corning	352096
50ml Falcon Tube	Corning	352070
FACS Tube	Corning	352052

7.3 Reagents

7.3.1 Fresh whole blood samples

Samples were obtained from anonymous volunteer human donors (National Health Service Blood and Transplant, Filton, UK).

7.3.2 MACS Buffer with 0.5% BSA

MACS buffer (MiltenyiBiotech #130-091-222) was supplemented with 0.5% BSA and filtered through a 0.22µm filter system (Corning #430769) and stored at 4°C. This buffer was used for flow cytometry staining and washing.

7.3.3 RPMI-1640 Supplementation (RP-10)

RPMI-1640 (Gibco #31870-025) was supplemented with 10% FBS, 5ml glutamine (final conc. 2mM/ml) (Sigma #7513) and 5ml Penicillin-Streptomycin (final conc. Penicillin 100 units and 100µg Streptomycin per ml) (Lonza #DE17-602E).

7.3.4 human recombinant (r)IL-2

Human rIL-2 (Proleukin) was diluted in DPBS to 10^6 IU/ml, aliquoted and stored at -80°C.

7.3.5 human recombinant (r)IL-4

Human rIL-4 (R&D #204-IL) was diluted in DPBS with <0.5% BSA to 1450 IU/ml, aliquoted and stored at -80°C.

7.3.6 human recombinant (r)GM-CSF

Human rGM-CSF (R&D #215-GM) was diluted in DPBS with <0.5% BSA to 1500 IU/ml, aliquoted and stored at -80°C.

7.3.7 human recombinant (r)IFN-γ

Human rIFN-γ (R&D #285-IF) was diluted in DPBS with <0.5% BSA to 2000 IU/ml, aliquoted and stored at -80°C.

7.3.8 human Calcitriol

Human Calcitriol (Sigma #C0225000) was diluted to 100mM in 100% ethanol (stock solution) and stored at -20°C. Each week a second dilution was prepared by adding 10µl of the stock solution to 100% ethanol to make a 1mM solution which was stored at 4°C and used for all experiments that week.

7.3.9 Atorvastatin

Atorvastatin (Sigma #Y0001327) was diluted to 10mM in DMSO (stock concentration), aliquoted and stored at -80°C. For each experiment a new aliquot was used.

7.3.10 Mevalonic Acid

Mevalonic acid (Sigma #50838) was diluted to 1M in DPBS (stock concentration), aliquoted and stored at -80°C. For each experiment a new aliquot was used.

7.3.11 (R)-β-Hydroxybutyric acid sodium salt

R-β-Hydroxybutyric acid sodium salt (Sigma #298360) was diluted to 1M in DPBS (stock concentration), aliquoted and stored at -80°C. For each experiment a new aliquot was used.

7.3.12 Sodium Butyrate

Sodium butyrate (Sigma #B5887) was diluted to 1M in DPBS (stock concentration), aliquoted and stored at -80°C. For each experiment a new aliquot was used.

7.3.11 Phorbol 12-myristate 13-acetate (PMA)

PMA (Sigma #P1585) was diluted to 1µg/ml in 100% ethanol and stored at -20°C.

7.3.12 Ionomycin

Ionomycin (Sigma #I0634) was diluted to 1µg/ml in 100% ethanol and stored at -20°C.

7.3.13 Freezing Mix

Cells were frozen down for storage in 90% FBS and 10% DMSO. Vials were put into a Mr Frosty freezing container (ThermoFisher) and stored at -80°C for a minimum of 24h; vials were then transferred from the freezing container into cryo-storage box and stored at -150°C.

7.4 *In Vitro* Cell Culture Experiments

7.4.1 Isolation of human PBMC

PBMCs were isolated by density gradient centrifugation. Leukocyte cone blood (around 8ml) was diluted in DPBS (1:4) and layered onto 10ml of Ficoll-Paque (Sigma #GE17-1440-02) in a 50ml Falcon tube. Density centrifugation was used at 1200xg (Heraeus Megafuge 40R) for 20min with no brake at RT to give rise to the intermediate layer which contains the PBMCs and is collected using an individually wrapped plastic Pasteur pipette. PBMCs were washed twice in DPBS at 1300 RPM (280xg, Hettich Universal 320R) at 4°C. Supernatant was aspirated to waste, effectively removing platelets, and cells were resuspended in DPBS and counted using an automated cell counter (1:10 dilution) (Vi-cell XR, Beckman Coulter).

7.4.2 Isolation of human CD14+ cells

CD14+ cells were isolated from PBMCs (as described in 7.4.1) according to manufacturer's instructions. In general, $1x10^8$ PBMCs were transferred into a new 50ml falcon tube (Corning #352070) and washed once in MACS buffer at 1,300 RPM (280xg, Hettich Universal 320R) for 5min at 4°C. Supernatant was aspirated to waste and cells re-suspended in 800µl MACS buffer (80µl per 10^7 cells; MiltenyiBiotec #130-091-221) and 200µl CD14 MicroBeads (20µl per 10^7 cells; MiltenyiBiotec #130-050-201) for 15min at 4°C to magnetically label CD14+ cells. Cells were washed in MACS buffer and re-suspended in 2ml MACS buffer. LS column (MiltenyiBiotec #130-042-401) and 30µM pre-separation filter (MiltenyiBiotec #130-041-407), to avoid blocking of the column, were positioned into a separator magnet and prepared by running 2ml MACS buffer through it. Cells were run over an LS column in 1ml intervals. LS column and filter were washed with 2 x 1ml MACS buffer re-suspended in the tube containing incubated PBMCs to maximize recovery and 1 x 1ml fresh MACS buffer. The flow through containing the unlabelled CD14- fraction was not required and discarded. Magnetically labelled CD14+

cells were eluted from the LS column in 4ml RP-10 by removing the column from the magnet and pushing the plunger into the column. CD14+ cells were washed and cells were re-suspended in 1ml RP-10. Cells were counted using an automated cell counter (1:5 dilution) (Vi-cell XR, Beckman Coulter) and a small proportion of each fraction was collected into a FACS tube for purity analysis by flow cytometry.

7.4.3 Generation of Monocyte-derived Dendritic Cells

CD14+ monocytes were differentiated into monocyte-derived dendritic cells (moDC) over 6 days by IL-4 and GM-CSF and then matured for 24h by IFN-γ and LPS.

Isolated CD14+ cells were seeded at a density of 0.8-1x10^6/ml in a 12-well plate (Costar #3513) in RP-10 containing 800 IU/ml GM-CSF (R&D #215-GM) and 500 IU/ml IL-4 (R&D #204-IL). Fresh media was added on day 3 with RP-10 containing 800 IU/ml GM-CSF and 500 IU/ml IL-4. On day 6, cells were matured for 24h in 1ml RP-10 containing 1000 IU/ml IFN-γ (R&D 285-IF) and 100ng/ml LPS (InvivoGen #tlrl-eklps). Immature moDC received only RP-10 on day 6.

Differentiation of monocytes with Atorvastatin and/or Vitamin D: CD14+ monocytes were differentiated into moDC with Atorvastatin (50nM, 250nM, 500nM or 1μM) and 100nM Calcitriol, without Atorvastatin and Calcitriol, or with Calcitriol only. See Table 6 for details.

On day 6 differentiation media containing moDCs (2ml) was transferred into a 15ml falcon tube and centrifuged at 1,200 RPM for 5min at RT. 200μl of the supernatant was collected and transferred into a separate tube and stored at -20°C for cytokine analysis. The supernatant was aspirated and 500μl of the maturation media was added to re-suspend any pelleted cells. These cells were then transferred back into their original well that contained 500μl maturation media. After 24h the maturation media was collected once more and centrifuged at 1,300 RPM (280xg, Hettich Universal 320R) for 5min at 4°C. Again, 200μl were collected and transferred into a separate tube and stored at -20°C for cytokine analysis. 500μl of dissociation buffer (Gibco #13151014) were added

to each well and placed for 10min into the incubator (37°) to dissociate any adherent cells, these cells were combined to the cells that were pelleted during the previous centrifugation step.

Time point	Volume	
Day 0	250µl	4X Differentiation media containing 3200 IU/ml GM-CSF and 2000 IU/ml IL-4.
	250µl	4X Atorvastatin E.g. to achieve 1µM concentration in the well a 4µM concentration was added.
	250µl	4X Calcitriol (400nM/ml) or RP-10.
	250µl	CD14+ Monocytes (3.2-4x10^6/ml).
Day 3	500µl	4X Differentiation media containing 3200 IU/ml GM-CSF and 2000 IU/ml IL-4.
	250µl	8X Atorvastatin E.g. to achieve 1µM concentration in the well a 8µM concentration was added.
	250µl	8X Calcitriol (800nM/ml) or RP-10.
Day 6	1000µl	Maturation media containing 1000 IU/ml IFN-γ and 100ng/ml LPS.
Day 7		Phenotypic analysis by flow cytometry and cells used in MLR.

Table 6. Generation of monocyte-derived Dendritic Cells with Atorvastatin and/or Vitamin D.

7.4.4 Isolation of human CD4+ cells

Leukocyte cone blood was eluted into a 50ml falcon tube and 300µl were transferred into a new tube for a blood count on a Sysmex (XN-1000, Sysmex). Human blood was diluted to 50x10^6/ml WBC with DPBS. 10-20ml of diluted blood were transferred into a new 50ml falcon tube and 0.5-1.0ml of RosetteSep CD4+ cocktail (Stemcell Technologies #15062) was added. The tube was placed on an orbital shaker (70 RPM, Labnet Reciprocal 30) for 20min at RT to allow crosslinking of red blood cells with non-CD4+ cells. 10-20ml of 2% FBS/DPBS were added and the blood was layered onto 10ml Ficoll-Plaque. Density

centrifugation was used at 1200xg (Heraeus Megafuge 40R) for 20min with no brake at RT to resolve an intermediate layer which contained the CD4+ cells, this was subsequently collected using an individually wrapped plastic Pasteur pipette (Alpha Laboratories #LW4638) and transferred to a clean tube. Cells were washed twice with DPBS and counted using an automated cell counter (1:5 dilution) (Vi-cell XR, Beckman Coulter). A small proportion of the CD4+ fraction was collected into a FACS tube for purity analysis by flow cytometry.

7.4.5 Isolation of human CD4+CD25- and CD4+CD25high cells

Isolated human CD4+ cells were counted and washed twice with DPBS at 1,300 RPM (280xg, Hettich Universal 320R) for 5min at 4°C. To magnetically label cells for CD25 isolation, cells were re-suspended in the appropriate amount of MACS buffer (90µl per 10^7 cells; MiltenyiBiotec #130-091-221) and CD25 MicroBeads (10µl per 10^7 cells; MiltenyiBiotec #13-0092-983) for 10min at 4°C according to manufacturer's instructions. LS column (MiltenyiBiotec #130-042-401) and 30µM pre-separation filter (MiltenyiBiotec #130-041-407), to avoid blocking of the column, were positioned into a separator magnet and prepared by running 2ml MACS buffer through it. Incubated CD4+ cells were washed once in MACS buffer at 1,300 RPM (280xg) for 5min at 4°C. Supernatant was aspirated off and cells were re-suspended in 2ml MACS buffer and run over the LS column and pre-separation filter in 1ml intervals. The LS column and filter were washed with 2 x 1ml MACS buffer re-suspended into the tube containing the CD4+ to maximize recovery of all CD4+ cells and 1 x 1ml of fresh MACS buffer. The flow through containing the unlabelled CD25- fraction, was used as Teffector cells in subsequent experiments. The retained fraction inside the LS column, the magnetically labelled CD25+ fraction, was eluted using 4ml MACS buffer by removing the column from the magnet and pushing the plunger into the column. The CD25+ fraction was washed and cells were re-suspended in 1ml MACS buffer and run over an MS column (MiltenyiBiotec #130-042-201) at 500µl intervals. The MS column was positioned into a smaller separator magnet and

equilibrated by running 1ml of MACS buffer through it. The MS column was washed with 2 x 500µl MACS buffer added to the tube containing the CD25+ cells from the LS column to maximize recovery from the tube and 2 x 500µl fresh MACS buffer. The CD25+ magnetically labelled fraction of the MS column was eluted by removing the column from the magnet and the plunger pushed into the column using 2ml RP-10 followed by another 1ml RP-10 to maximize recovery of CD25+ cells. This fraction was used as Tregs for subsequent assays and the flow through discarded. Teffector cells were counted on an automated cell counter (1:5 dilution) (Vi-Cell XR, Beckman Coulter) whereas Tregs were counted by haemocytometer with trypan blue (1:2 dilution). A small proportion of each fraction was collected into a FACS tube for purity analysis by flow cytometry. Preparations were often ≥85% Tregs.

7.4.6 *In vitro* Treg Suppression Assay

Tregs and autologous Teffector cells were incubated at a ratio of 1:1 for 5 days and stimulated at 1:8 bead-to-Tcell ratio with anti-CD3/CD28 dynabeads (ThermoFisher #11131D). These conditions were used after optimisation steps described in 8.5. Teffector cells were stained with CellTrace Blue reagent (Invitrogen #C34568) which tracks the division of cells as half of the dye is passed from the mother onto a dividing daughter cell. CellTrace Blue reagent readily diffuses into cells to covalently bind intracellular amines that results in a stable, non-toxic and well-retained fluorescent stain. Any excess reagent can passively diffuse out of cells to be quenched by proteins within the media.

7.4.6.1 CellTrace Blue Staining

Teffector cells were stained with CellTrace Blue (Invitrogen #C34568) according to manufacturer's instructions. An appropriate amount of Teffector cells (5-10x10^6) were centrifuged at 1,300 RPM (280xg, Hettich Universal 320R) for 5min at RT and the supernatant was completely taken off as proteins can inhibit the subsequent reaction.

Teffector cells were re-suspended in 1ml DPBS and 2µl of 5µM CellTrace Blue reagent was added and mixed promptly to ensure equal dispersion. Cells were incubated for 20min at RT in the dark. 5ml RP-10 were added to the tube and allowed to incubate for at least 10min in an incubator (37°C) to quench any free dye in the solution. Stained cells were then centrifuged at 1,300 RPM (280xg) for 5min at RT, the supernatant was removed and cells were re-suspended in 1ml RP-10 prior to being counted on an automated cell counter (1:5 dilution) (Vi-Cell XR, Beckman Coulter).

7.4.6.2 Anti-CD3/CD28 Dynabeads

Anti-CD3/CD28 dynabeads (ThermoFisher #11131D) closely mimic the *in vivo* T-cell activation provided by APCs. As dynabeads are three-dimensional and similar in size to APCs, this approach to stimulate T-cells is more physiological than equivalent monoclonal antibodies.

A specific amount of anti-CD3/CD28 dynabeads were transferred from the original container to 1.5ml Eppendorf tube in order to wash off their conservation media. 1ml DPBS was added to the tube and vortexed for a few seconds. The tube was then held against a magnet and fluid was carefully removed. The tube was quickly centrifuged to collect any residual liquid as it is important to achieve the original volume of dynabeads to ensure the correct dynabeads-to-cell ratio. The same volume of RP-10 is added to the tube as initially taken from the manufacture's container.

7.4.6.3 *In vitro* Suppression Assay

Stained CellTrace Blue (CTB) Teffector cells were mixed at a ratio of 1:1 with Tregs (i.e. 10,000 Tregs + 10,000 Teffectors) or incubated alone (i.e. 20,000 Teffectors) in a total volume of 200µl of RP-10 in a U-shaped 96-well plate (Costar #3799). Anti-CD3/CD28 dynabeads were added at a ratio of 1:8 bead-to-Tcell for stimulation. In some experiments, Atorvastatin (0-10µM) and/or Calcitriol (100nM) were added on day 0. In addition, in some experiments the effect of Atorvastatin was reversed by adding 1mM

Mevalonic Acid on day 0. Cells are harvested after 5 days and analysed by flow cytometry (LSRII). In order to avoid autofluorescence of the dynabeads during flow cytometry analysis the 96-well plate was placed on a 96-well plate magnet (Invitrogen #12027) allowing for media transfer into a FACS tube without the carry-over of contaminating dynabeads.

Suppression assay with Atorvastatin and/or Vitamin D: Tregs-Teffector 1:1 and Teffector without Tregs were incubated with Atorvastatin and/or Calcitriol and stimulated with anti-CD3/CD28 dynabeads.

The assay was set-up in the following way (see Table 7): Atorvastatin and/or Calcitriol were added to the appropriate well in the U-shaped 96-well plate. In some experiments 1mM mevalonic acid was also added. Lastly, Tregs + Teffectors + anti-CD3/CD28 dynabeads or Teffectors + anti-CD3/CD28 dynabeads were added. To achieve the correct volume and ratio of Tregs + Teffectors 1:1, first Tregs were added to a 15ml falcon tube. This tube was centrifuged at 1,200 RPM for 5min at 4°C and the supernatant was aspirated to waste. Then CTB treated Teffector cells were added so that a ratio of 1:1 to Tregs is achieved. Volume was topped up with RP-10 to achieve a cell density of $0.4x10^6$/ml (or 20,000 cells per 50µl). Lastly, the correct amount of anti-CD3/CD28 dynabeads (2µl per 80,000 cells for 1:1 dynabeads-to-cell ratio) were added and mixed by inverting the tube. Finally, wells were mixed by pipetting up and down a few times with a multi-pipette to ensure even distribution of Tregs and Teffector cells as well as anti-CD3/CD28 dynabeads. In the case for Teffectors alone, Teffectors were added to a 15ml falcon tube and the tube was not centrifuged. Instead, volume was topped up with RP-10 to achieve a cell density of $0.4x10^6$/ml (or 20,000 cells per 50µl). Same amount of anti-CD3/CD28 dynabeads were added as for Tregs-Teffector 1:1.

Time point	Volume	
Day 0	50µl	4X Atorvastatin E.g. to achieve 1µM concentration in the well a 4µM concentration was added.
	50µl	4X Calcitriol (400nM/ml) or RP-10
	50µl	4X Mevalonic Acid (4mM/ml) or RP-10
	50µl	Tregs + Teffectors + anti-CD3/CD28 dynabeads or Teffectors + anti-CD3/CD28 dynabeads
Day 5		Analysis by flow cytometry

Table 7. Set-up of *In Vitro* Suppression Assay with Atorvastatin and/or Vitamin D.

7.4.6.4 *In vitro* Suppression Assay Calculations

To estimate the suppression mediated by Tregs on Teffector proliferation the following calculation was used:

[Undivided cells Tregs-Teff 1:1] - [Undivided cells Teffector alone]

= Suppression mediated by Tregs

To estimate the suppression mediated by Atorvastatin on Teffector proliferation the following calculation was used:

[Undivided cells Tregs-Teff 1:1 + Atorvastatin] - [Undivided cells Tregs-Teff 1:1 + Mevalonic Acid]

= Suppression mediated by Atorvastatin

To estimate the suppression mediated by Vitamin D (Calcitriol) on Teffector proliferation the following calculation was used:

[Undivided cells Tregs-Teff 1:1 + Vitamin D] - [Undivided cells Tregs-Teff 1:1]

= Suppression mediated by Vitamin D

7.4.7 *In vitro* Mixed Lymphocyte Reaction (MLR)

Tregs and Teffector were treated in the same way as in 7.4.6. In addition, moDC were added in a 1:5 DC-to-Tcell ratio (4,000 DCs). These moDC were either differentiated with Atorvastatin and/or Calcitriol and then incubated with Tregs-Teff 1:1 or Teffector alone, or the moDC were differentiated without Atorvastatin and/or Calcitriol and then incubated with Tregs-Teff 1:1 or Teffector alone in the presence of Atorvastatin and/or Calcitriol. After 5 days the cells were harvested and analysed by flow cytometry.

One week prior to Treg and Teffector isolation, CD14+ cells were isolated (see 7.4.2) and differentiated into moDC (see 7.4.3) and were subsequently harvested into 15ml falcon tubes and a proportion transferred to FACS tube for phenotyping. The rest of moDC were centrifuged at 1,300 RPM for 5min at RT and the supernatant was aspirated off. Cells were re-suspended in 1ml of RP-10 and counted on an automated cell sorter (1:5 dilution) (Vi-Cell XR, Beckman Coulter). moDC were adjusted to a cell density of $8.0x10^4$ to give a 1:5 DC-to-Tcell ratio. Isolated Tregs and CTB stained Teffector cells (see 7.4.5 and 7.4.6.1) were added to a 15ml falcon tube at a ratio of 1:1 and anti-CD3/CD28 dynabeads were added at a ratio of 1:8 beads-to-T-cell.

MLR with Atorvastatin and/or Vitamin D: moDC were differentiated without Atorvastatin and/or Calcitriol, incubated with Tregs-Teffector 1:1 or Teffector alone + treatment and stimulated with anti-CD3/CD28 dynabeads.

The assay was set up in the following way (see Table 8): Atorvastatin and/or Calcitriol were added to the appropriate well in the 96-well plate. Then, moDCs were added to each well except to the control well without moDCs. Lastly, 50µl of Tregs-Teffector 1:1 + anti-CD3/CD28 dynabeads or Teffector + anti-CD3/CD28 dynabeads were added. Cells were mixed by a multi-pipette to ensure even distribution of cells and anti-CD3/CD28 dynabeads.

Time point	Volume	
Day -7		Isolation of CD14+ monocytes and differentiation with GM-CSF and IL-4
Day -1		Maturation of moDC with IFN-γ and LPS
Day 0	50µl	4X Atorvastatin E.g. to achieve 1µM concentration in the well a 4µM concentration was added.
	50µl	4X Calcitriol (400nM/ml) or RP-10
	50µl	moDC ($8.0x10^4$/ml)
	50µl	Tregs + Teffectors + anti-CD3/CD28 dynabeads or Teffectors + anti-CD3/CD28 dynabeads
Day 5		Analysis by flow cytometry

Table 8. Set-up of *In Vitro* Mixed Lymphocyte Reaction with Atorvastatin and/or Vitamin D.

MLR with Atorvastatin and/or Calcitriol differentiated moDC: moDC were differentiated with Atorvastatin and/or Calcitriol, incubated with Tregs-Teffector 1:1 or Teffector alone and stimulated with anti-CD3/CD28 dynabeads.

The assay was set-up in the following way as depicted in Table 9.

Time point	Volume	
Day -7		Isolation of CD14+ monocytes and differentiation with GM-CSF and IL-4 and Atorvastatin and/or Calcitriol
Day -1		Maturation of moDC with IFN-γ and LPS
Day 0	50µl	Atorvastatin and/or Calcitriol differentiated moDC ($8.0x10^4$/ml)
	50µl	Tregs + Teffectors + anti-CD3/CD28 dynabeads or Teffectors + anti-CD3/CD28 dynabeads
	100µl	RP-10
Day 5		Analysis by flow cytometry

Table 9. Set-up of *In Vitro* Mixed Lymphocyte Reaction with Atorvastatin and/or Vitamin D differentiated moDC.

7.4.7.1 Mixed Lymphocyte Reaction Assay Calculations

To estimate the suppression mediated by Tregs, in the presence of monocyte-derived dendritic cells, on Teffector proliferation the following calculation was used:

[Undivided cells Tregs-Teff 1:1 + DC] - [Undivided cells Teffector alone + DC]

= Suppression mediated by Tregs

7.4.8 Aldehyde Dehydrogenase Assay

Aldeflour reagent, BODIPY-aminoacetaldehyde (BAAA), is a fluorescent non-toxic substrate for aldehyde dehydrogenase (ALDH) which, freely diffuses into viable cells. Once BAAA is activated at 37°C it reacts with ALDH which leads to the conversion of BAAA to BODIPY-aminoacate (BAA) which is retained inside the cell. The amount of fluorescence emitted by BAA is proportional to the ALDH activity. To control for background ALDH fluorescence a specific inhibitor of ALDH activity, diethylaminobenzaldehyde (DEAB), is used.

Assay was performed according to manufacturer's instructions with some small modifications outlined below (Stemcell Technologies #01700). Cells were split between two tubes and centrifuged at 1,300 RPM for 5min at RT. Cells were then re-suspended in 0.4ml instead of 1ml of Aldeflour Assay buffer. Accordingly, instead of using 5µl Aldeflour reagent and DEAB reagent, 2µl of each was used. Cells were incubated at 37°C for 45min in a water bath, centrifuged and re-suspended in fresh 150µl Aldeflour Assay buffer. Analysis was performed by flow cytometry (LSRII) using the FlowJo software.

7.4.9 T-cell Activation Assay

CD4+ T-cells were isolated as described in 2.4.4. CD4+ T-cells ($1x10^6$/ml) were activated for 5 days with anti-CD3/CD28 dynabeads at a ratio of 1:2 in a U-shaped 96-well plate (Costar #3799) in the presence of 30 IU/ml rIL-2. Cells were harvested and anti-CD3/CD28 dyanbeads removed. Cells were re-stimulated with 50ng/ml PMA and 1µg/ml

ionomycin in the presence of Brefeldin A (1µl/ml; BD, #555029) for 4h in a new U-shaped 96-well plate. Cells were then analysed by flow cytometry for cytokine production. For western blot analysis, cells were not restimulated by PMA and ionomycin.

7.4.10 Treg Induction Assay

PBMCs were isolated as described in 7.4.1. PBMCs were cultured in a 48-well plate (ThermoFisher #150687) for 3 days in the presence of 30 IU/ml rIL-2 and 50nM Atorvastatin and/or 100nM Calcitriol. Cells were harvested and used for DNA, RNA and flow cytometry analysis.

7.4.11 Irradiation of Karpas299

Karpas299 cell line was irradiated at 50 Gy at the John Radcliffe Hospital (NDS) irradiation facility.

7.5 Flow Cytometry

7.5.1 Antibody Staining

Antibodies were titrated on an LSRII (BD) flow cytometer and the staining index calculated using FlowJo software. The concentration with the greatest staining index got used in experiments. Staining volume was usually 20µl except for intracellular staining which was 40µl. See Table 12 for conjugated antibodies used in experiments.

Cells were collected in FACS tubes, washed in cold DPBS (when cells were incubated in a Live/Dead fixable cell stain to assess cell viability) or MACS buffer with 0.5% BSA as appropriate and re-suspended in appropriate volume MACS buffer with 0.5% BSA for staining. Conjugated surface antibodies were mixed together in an antibody cocktail and the appropriate amount was added to the FACS tube. Cells were incubated for 30min at 4°C. Cells were washed once with MACS buffer + 0.5% BSA. If cells needed fixation and permeabilisation (MiltenyiBiotec #130-093-142 and Invitrogen #00-8333-56, respectively), cells were first fixed for 30min at 4°C, washed twice with MACS buffer + 0.5% BSA and then washed once with 1X permeabilisation buffer. Cells were then blocked with human FcR blocking reagent (MiltenyiBiotec #130-059-901) in permeabilisation buffer for 5min at 4°C and then stained for intracellular markers for 30min at 4°C in appropriate volume of permeabilisation buffer. Cells were washed twice in permeabilisation buffer to wash out any excess intracellular antibody. Cells were re-suspended in 150-250µl of MACS + 0.5% BSA for flow cytometry analysis.

Cytokines were analysed using the Fixation/Permeabilisation Solution Kit with GolgiPlug (BD #555028). Cells were washed in MACS + 0.5% buffer and re-suspended in 100µl fixation/permeabilisation buffer for 20min at 4°C. Cells were washed twice in permeabilisation/wash buffer and re-suspended in 40µl permeabilisation/wash buffer. Cells were incubated with conjugated antibodies for 30min at 4°C, washed twice with permeabilisation/wash buffer and re-suspened in MACS + 0.5% BSA buffer for flow

cytometry analysis. Antibodies were used at recommended concentration and as single stain.

7.6 Molecular Biology

7.6.1 RNA Extraction

Cells were harvested and re-suspended in 600µl TRI reagent solution (Ambion #AM9738) and frozen down at -150°C until needed for RNA isolation. RNA was isolated to manufacturer's instructions (Qiagen #217004).

7.6.2 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

250-500ng total RNA was reverse transcribed to cDNA using SuperScript IV Reverse Transcriptase (Invitrogen #18090050) by following the manufacturer's instructions. Briefly, 1µl of 50µM random hexamers (Invitrogen #N8080127) and 1µl of 10mM dNTP mix (Invitrogen #AM8228G) were added to the reaction mix in a PCR tube (Simport #T320-2) and incubated in a TProfessional Thermocycler (Biometra) at 65°C for 5min followed by at least 1min on ice. In the next step, instead of the recommended 1µl reverse transcriptase, 0.5µl reverse transcriptase was used and, consequently, 1.5µl instead of 1µl nuclease free water (Ambion #AM9937) was added to maintain the same recommended volume. The reaction mix was heated up to 23°C for 10min, followed by 55°C for 10min and inactivation at 80°C for 10min using a TProfessional Thermocycler (Biometra). cDNA was either used immediately, diluted 1:2 in dH_2O or stored at -20°C.

7.6.3 Quantitative Real Time Polymerase Chain reaction (qRT-PCR)

Gene targets of interest were analysed using the TaqMan platform. The principle of the TaqMan Assay is a pre-designed pair of unlabelled primers and pre-designed probe, consisting of a 5'-end FAM or VIC labelled dye and a non-fluorescent quencher (NFQ) on the 3'-end, which binds covalently to your target sequence of interest. At the start of the PCR reaction, the double stranded DNA is denatured and the signal of the dye is quenched by the NFQ. When the temperature is lowered it allows primers and probe to

anneal to the single stranded DNA at their specific target sequence. The TaqMan DNA polymerase will synthesize a new strand using the unlabelled primers bound to the template and once it reaches the probe it will cleave off the 5'-end dye from the NFQ. Each PCR cycle will release more dye molecules with the dye intensity measured being proportional to the amount of gene target of interest present.

TaqMan assay was set up according to manufacturer's instructions using the TaqMan fast Advanced Master Mix (Applied Biosystems #4444557) on a 384-well plate (Applied Biosystems #4309849) in a total volume of 10µl. Each condition was plated as biological duplicates and technical duplicates. When multiple targets were analysed, FAM and VIC labelled gene targets were used in a multiplex reaction. To have an equal amount of primers and probe in each well (TaqMan Gene Expression Assay, 0.5µl per gene target/reaction, see Table 11), primers and probe were mixed together with the TaqMan Fast Advanced Master Mix (5µl per reaction) for all reactions and distributed to appropriate tubes. In a second tube, cDNA (1µl per reaction, 2µl for FOXP3) and nuclease free water (2.5-3.5µl) were mixed together and added to appropriate tubes containing the primer and probe mix. This solution was then used to fill the 384-well plate. Analysis was performed using a Viia7 Real-Time PCR machine and Viia7 software (Applied Biosystems). The following settings were used: Fast-Run, 384-well standard, 10µl reaction volume. Reaction mix was heated up to 95°C for 20 seconds followed by 50 cycles of 95°C for 1 second and 60°C for 20 seconds.

For the analysis of TET1, TET2 and RPL13A, the SYBR Green platform was used. The principle of this platform is the use of a ready-to-use cocktail containing all components, except primers and cDNA, for the amplification and detection of DNA. Primers are designed by the researcher and added to the solution. SYBR Green will bind to any minor groove of double-stranded DNA. Consequently, as more double-stranded amplicons of your target are produced, the more SYBR Green will bind and the intensity of the fluorescent signal will increase.

SBYR Green assay (Invitrogen #11733) was set-up to manufacturer's instructions on a 384-well in a total volume of 10µl. Each condition was plated as a single biological replicate and technical triplicate. To have an equal amount of primers (0.5µl per reaction at 1µM concentration; for primer sequences see Table 10) in each well, primers were mixed together with the SYBR Green SuperMix (5µl per reaction) for all reactions and distributed to appropriate tubes. In a second tube, undiluted cDNA (1µl per reaction) and nuclease free water (3.5µl) were mixed together and added to the appropriate tubes containing the primer mix. This solution was then used to fill the 384-well plate. Analysis was performed using a Viia7 Real-Time PCR machine and Viia7 software (Applied Biosystems). The following settings were used: Fast-Run, 384-well standard, 10µl reaction volume. Reaction mix was heated up to 50°C for 2 minutes followed by 95°C for 2 minutes. This was then followed by 50 cycles of 95°C for 3 second and 60°C for 30 seconds followed by melt curve analysis.

Amplicon	Forward	Reverse
RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA
TET1	CCGAATCAAGCGGAAGAATA	ACTTCAGGTTGCACGGTCTC
TET2	AGCCCCATCACGTACAAAAC	TGTGGTGGCTGCTTCTGTAG

Table 10. PCR Primers used for SYBR Green assay. TET1 and TET2 sequences were taken from (Lin *et al.* 2017).

7.6.4 TSDR Methylation Assay

7.6.4.1 DNA Extraction

Cells were harvested and frozen down as a cell pellet at -150°C until needed for DNA extraction. DNA was isolated using a blood and tissue DNA kit (Qiagen #96504) by following the manufacturer's instructions. DNA was eluted in two 15µl dH_2O steps to minimize DNA retention in the filter and maximize recovery.

7.6.4.2 Bisulphite Conversion

500ng DNA (see 7.6.4.1) per sample was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research #D5006) according to manufacturer's instructions. 40µl DNA was added to 110µl CT conversion reagent (single vial of CT conversion reagent was reconstituted in 700µl dH_2O, 300µl M-Dilution buffer, and 50µl M-Dissolving buffer). Tubes were placed into a thermal cycler (T3000 thermocycler, Biometra) with the following programme: 98°C for 10 minutes, 64°C for 2.5 hours and then kept at 4°C till ready to use. A Zymo-Spin IC column was placed into a collection tube and 600µl of M-binding buffer was added to the spin column. The sample was then added to the spin column and mixed with the M-binding buffer by pipetting up and down followed by centrifugation at 10,000g for 30 seconds. Flow-through was then aspirated. 100µl of M-Wash buffer was added and the spin column was centrifuged at >10,000g for 30 seconds. Then, 200µl of M-Desulphonation buffer was added and the spin column was placed for 15min into a heat block set at 30°C. Afterwards, the spin column was centrifuged at >10,000g for 30 seconds. 200µl of M-Wash buffer was added to the column and centrifuged at >10,000g for 30 seconds. This step was repeated another time followed by a dry spin in a new collection tube. The spin column was then placed into a fresh 1.5ml collection tube and 20µl of M-Elution buffer was added. Bisulfite converted DNA was eluted by a final spin of >10,000g for 30 seconds and tubes kept on ice for quantification using a Nanodrop with an absorption coefficient at 260nM of 40µg/ml. Bisulfite converted DNA were stored at 4°C when used within a week or stored at -20°C.

7.6.4.3 PCR Primers and Probes

Bisulfite-specific (BS) and methylation-specific (MS) primers and probes were tested and established at the Blood Research Laboratory by Dr Robert Danby (see Table 10 and Appendix 13.1). The BS primers for FOXP3 were published by Baron *et al.* (2007). The BS primers for CD3 were designed by Dr Robert Danby (former member of the Blood

Research Laboratory) using Primer 3 (Copyright Untergasser A., Nijveen H., Rozen S. and Skaletsky H. 2006-2011). All other primers and probes were published by Sehouli *et al.* (2011). Probes were labelled with 5' 6-FAM and 3' black hole quencher and were purchased from ThermoFisher.

Amplicon	Specificity	Forward	Reverse
FOXP3	BSMS (TpG)	GTTTTTGATTTGTTTAGATTTTTTTGTT	CCTCTTCTCTTCCTCCATAATATCA
	BSMS Pr (TpG)	ATGGTGGTTGGATGTGTTGGGTT	
	BSMS (CpG)	GTTTTCGATTTGTTTAGATTTTTTCGTT	CCTCTTCTCTTCCTCCGTAATATCG
	BSMS Pr (CpG)	ATGGCGGTCGGATGCGTC	
CD3	BSMS (TpG)	TTTAAATATTGTTATATTTTGA	AAAAATCTAACTACTACAACTTAC
	BSMS Pr (TpG)	GAGAAGTTGTTGGTTTTATAGTGTAAG	
	BSMS (CpG)	TTAAATATTGTTATATTTCGA	AAATCTAACTACTACGACTTAC
	BSMS Pr (CpG)	TCGTCGGTTTTATAGCGT	

Table 10. PCR Primers and Probes used for TSDR Methylation Assay.

7.6.4.4 Methylation-specific Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

TpG and CpG standards were prepared and aliquoted by Dr Robert Danby and colleagues (Blood Research Laboratory) and stored at -40°C. Standards were prepared in the following way: 30,000 copies, 3000, 300, 30, 10 and 3 of TpG or CpG. All samples were run in as technical quadruplicates. MSRT-qPCR reactions were set up using 22µl 2X Epitect Methylight mastermix (Qiagen), 0.88µl 50X Rox dye, 4.4µl 10X primer mix (forward and reverse), 14.72µl nuclease free water and 2µl of the probe per sample, and TpG and CpG. 41.8µl sample mastermix was then added to the correct tubes and 2.2µl of bisulfite converted DNA (template) was added to give an overall volume of 44µl. 59.2µl of the mastermix were added to the correct tubes designated for the standards and 6.8µl of the correct standard (either TpG or CpG) were added to designated tube. Standards

were kept on ice for the next plate (one plate for FOXP3 and one for CD3). Tubes were flicked and centrifuged at 1500 RPM. 10µl were added to a 384-well plate placed on a cold metal block. Left-over of the mastermix was used as non-template control. Plate was sealed, centrifuged at 1500 RPM at RT, and run on a Viia7 Real-Time PCR machine (Applied Biosystems) under cycling conditions: 95°C for 5min, followed by 50 cycles of 95°C for 15s and 53°C for CD3 TpG and CpG or 60°C for FOXP3 TpG and CpG.

7.6.5 ELISA

ELISA was performed as by manufacturer's instructions (ThermoFisher; IL-10 #88-7106 and IL-12p70 #88-7126). ELISA plates were coated with coating antibody overnight at 4°C on an orbital shaker (Luckham R100). Sample was incubated overnight at 4°C on an orbital shaker for maximum sensitivity. ELISA plate was analysed using a iMark plate reader (Bio-Rad).

7.6.6 Immunoblotting

7.6.6.1 Nuclear Extraction

Cultured CD4+ T-cells (see 7.4.9) were either used immediately or frozen down at -80°C as cell pellets for subsequent nuclear extraction. Nuclear extraction was performed using NE-PER nuclear extraction kit (ThermoFisher #78833) by following the manufacturer's instructions. Protease inhibitor (Roche #11836153001) was added to reagents, CER I and NER, prior to use. Pelleted cells were re-suspended in appropriate amount of ice-cold CER I and incubated on ice for 10 min. Appropriate amount of ice-cold CER II was added and samples were vigorously vortexed and incubated for 1min on ice. Samples were centrifuged at 16,000xg for 5min at 4°C. Supernatant (cytoplasmic fraction) was transferred to a new 1.5ml Eppendorf tube. The pellet, containing nuclear proteins, was re-suspended in appropriate amount of ice-cold NER, vigorously vortexed and incubated on ice. Vortexing was repeated every 10min for a total of 4 times. Samples were centrifuged at 16,000xg for 10min at 4°C. Supernatant (nuclear fraction) was transferred

to a new 1.5ml Eppendorf tube. Cytoplasmic and nuclear fractions were stored at -80°C until needed for western blot analysis.

7.6.6.2 Western Blot

Protein concentration were determined with reference to a standard curve using a micro BCA protein assay kit (ThermoFisher #23345) and spectrophotometry performed on a iMark microplate reader (Bio-Rad). Loading dye (Bio-Rad #161-0791) with 10% β-merceptaethanol (Bio-Rad #161-0710) was added to samples and then incubated for 5min at 90°C to denature complex protein structures. Samples were resolved on a SDS-PAGE gradient gel (5-14%; Bio-Rad #5671083) using TGS (Bio-Rad #1610732) running buffer at 150V. Gels were transferred onto a nitrocellulose membrane (0.2uM pore size; ThermoFisher #88013) using a wet transfer method. Transfer buffer was composed of 25 mM Tris (Sigma #T1503), 192 mM glycine (Sigma G7126), 20% (v/v) methanol (pH 8.3) and proteins were transferred at 4°C at 100V for 45 minutes. Ponceau S (Sigma #P7767) was used to verify that transfer was successful and protein loading was equal between samples. Blots were blocked with gentle agitation in PBS based Odyssey blocking buffer (Li-Cor #927-40000) for 1 hour at room temperature and then incubated overnight with gentle agitation at 4°C in primary antibody diluted in PBS based Odyssey blocking buffer (Li-Cor). After washing in 0.01% Tween-20 (Sigma #P9416) DPBS, secondary anti-mouse and anti-rabbit antibodies conjugated to infrared tags, either IRDye 680RD and 800CW (Li-Cor #926-68071 and #926-32210 respectively) were diluted 1:10,000 – 1:5000 in PBS based Odyssey blocking buffer and incubated at room temperature for 1 hour with gentle agitation. Blots were washed once more then visualised using an Odyssey CLx infra-red imager (Li-cor). Protein quantification of blots were performed using the software suite Image studio 2.1. Antibodies used in western blot analysis were T-bet (1:1000, Cell Signalling Technologies #13232), GATA-3 (1:1000, CST #5852), FOXP3 (1:200, eBiosciecne #14-4777-82), VDR (1:2000, ThermoFisher #MA1-710), TET1 (1:1000, GeneTex #GT465), TET2 (1:1000, GeneTex

#GT649), GAPDH (1:1000, Abscitech #AB21612) and Histone H3 (1:10,000 Abcam #ab1791).

7.7 Statistical Analysis

Statistical analysis was performed using an appropriate method of analysis. If not otherwise indicated, a two-way ANOVA corrected for multiple comparison was performed using Prism8 software (GraphPad software). Data is usually shown with the standard error of the mean (SEM). Number of experiments is indicated for each data set.

Target	Assay ID	Catalog Number
RPL13A	Hs04194366_g1	4331182
ACTB	Hs01060665_g1	4331182
IL-10	Hs00984148_m1	4448489
IL-12A	Hs01073447_m1	4331182
IFNG	Hs00989291_m1	4448489
IFNA2	Hs00265051_s1	4448489
IFNB1	Hs01077958_s1	4448489
IDO1	Hs00984148_m1	4448489
CTLA-4	Hs00175480_m1	4331182
TGFB1	Hs00998133_m1	4331182
ALDH1A1	Hs00946916_m1	4331182
ALDH1A2	Hs00180254_m1	4448489
FOXP3	Hs01085834_m1	4331182
DNMT1	Hs0094589_g1	4331182

Table 11. TaqMan (ThermoFisher) Primers and Probes used in qRT-PCR.

Target	Company	Conjugate	Clone	Catalog Number
Lineage Panel				
CD3	BD	FITC	UCHT1	555916
CD4	BD	PerCp.Cy5.5	RPA-T4	560650
CD8	BD	BUV395	RPA-T8	563795
CD14	BD	PE-Cy7	M5E2	557742
CD19	BD	APC	HIB19	555415
CD56	BD	PE	B159	555516
DC Panel				
CD1a	BD	BB700	HI149	746202
CD1c	BD	BUV395	F10/21A3	742751
CD11c	BD	PE-CF594	MAB	562393
CD14	BD	PE-Cy7	M5E2	557742
CD80	BD	PE	L307.4	557227
CD86	BD	BUV737	2331	612784
CD141	BD	APC	1A4	564123
HLA-DR	BD	BB515	G46-6	564516
Live/Dead – Near IR	ThermoFisher			L10119
Treg Panel				
CD3	BD	FITC	UCHT1	555916
CD4	BD	PerCp.Cy5.5	RPA-T4	560650
CD25	BD	PE	M-A251	555432
CD127	BD	PE-Cy7	HIL-7R-M21	560822
FoxP3	eBioscience	APC	PCH101	17-4776-42
Live/Dead – UV	ThermoFisher			L23105

Table 12.1. Conjugated Antibodies used in Flow Cytometry.
Conjugated antibodies were used in combination as shown for each panel.

Target	Company	Conjugate	Clone	Catalog Number
Cytokine Antibodies				
IL-4	BD	PE-Cy7	8D4-8	560672
IL-10	BD	PE	JES3-19F1	559330
IL-17A	BD	PE	N49-653	560486
IFN-γ	BD	PE	4S.B3	559326
TGF-β1	BD	PE-CF594	TW4-9E7	562422
TNFα	eBioscience	PE	Mab11	12-7349-41

Table 12.2. Conjugated Antibodies used in Flow Cytometry.
Conjugated antibodies were used as single stain to analyse cytokines.

CHAPTER 8

Flow Cytometry

8. Flow Cytometry

Flow Cytometry was performed using a LSRII Flow Cytometer (BD Bioscience) equipped with a blue argon-ion laser (488nm), a red helium-neon laser (635nm) and a solid state UV laser (355nm). Each day the flow cytometer was calibrated using CST calibration beads.

8.1 Antibody and Fluorochrome Selection

Fluorochromes were chosen on the following basis:

1) Primary: Well-characterized target, usually identify parent populations and expression pattern is contained to certain populations.
2) Secondary: Well-characterized target with high expression pattern.
3) Tertiary: Might be an uncharacterized target and might requires intracellular staining. In addition, low expression pattern.

Tertiary targets require the brightest fluorochromes with the least spectral spill over. Next in line are secondary targets which require also fluorochromes with little spectral spill over. Dimmer fluorochromes with possible spill over are assigned to primary targets.

Brightness of fluorochromes depends on laser availability to excite certain fluorochromes and what flow cytometer is available. The used flow cytometer LSRII (BD Bioscience) can analyse in theory up to 11-12 targets however we found that a maximum of 9 targets is possible without significant spill over. See Table 13 for available fluorochromes used on LSRII and Table 12.1 and 12.2 for assigned conjugated antibodies for designed panels.

Laser	Fluorochrome	Brightness Index
Blue (488nm)	PE	Very bright/Bright
Blue (488nm)	PE-CF594	Bright
Blue (488nm)	PE-Cy7	Bright
Blue (488nm)	FITC / BB515	Moderate
Blue (488nm)	PerCp-Cy5.5 / BB700	Moderate
Red (633nm)	APC	Very bright/Bright
Red (633nm)	AlexaFluor647	Moderate
Red (633nm)	Alexa Flour 700	Dim
UV (355nm)	BUV737	Bright
UV (355nm)	BUV395	Moderate

Table 13. Brightness Index for available Fluorochromes on LSRII Flow Cytometer. Staining index ranks fluorochromes from the highest brightness (very bright) to lowest brightness (dim).

8.2 Antibody Titration

After having assigned conjugated antibodies to target of interest, the next step is the titration of each individual antibody to find the optimal antibody concentration. Each antibody is supplied with a recommended staining volume however each antibody was tested for:

1) That the antibody concentration with the highest separation index (SI) was chosen. The separation index is defined as following:

$$Separation\ Index = \frac{Median\ Positive - Median\ Negative}{(84\%\ Negative - Median\ Negative)/0.995}$$

2) To ensure that the antibody does not have a high non-specific background staining.
3) To ensure the most economically staining volume is used without compromising resolution.

8.3 Multi-colour Panel Compensation Optimization

For multi-colour panel flow cytometry the photomultiplier tube (PMT) for each fluorochrome was determined by automatic compensation to decrease emission of one fluorochrome into an inappropriate detector. However, manual compensation was used in order to minimize spill over from one fluorochrome into another and maximize resolution between populations. Fluorescence minus one (FMO) controls were used to determine the spill over of all other fluorochrome in the panel into the missing fluorochrome's channel. After gating on the population of choice (e.g. lymphocytes within PBMCs but not a specific population within lymphocytes) a new dot-plot graph was opened. On the y-axis the missing fluorochrome (e.g. FITC) of the FMO was chosen and on the x-axis one of the other fluorochromes (e.g. PE) was plotted. If no spill over was observed from PEs into FITCs channel then a straight horizontal line will appear. However, if the line is pointing upwards then spill over from PE into FITCs channel has occurred (under-compensation) and compensation of PE's channel to FITC's channel has to be increased. In contrast, when the line is pointing downwards then over-compensation is observed and the compensation of PE's channel into FITC's channel has to be reduced. This has to be repeated for all fluorochromes of the FMO. It is important to note that a perfect compensation is not achievable for every fluorochrome. Once finished, the spectral overlap is copied onto the next FMO and the procedure is repeated. Once all FMOs have been optimized the last spectral overlap is copied onto all samples.

Antibody staining controls used in each experiment were:

Unstained control: Control without any antibody staining. Allows to identify autofluorescence of cell population.

Fluorescence minus one (FMO): This control consists of all antibodies used in a multi-colour panel except one. This provides accurate gate setting for the fluorochrome of interest allowing for discrimination between positive and negative cells. As some fluorochromes will have spill over into other channels FMO optimisation allows identification of false-positives which would otherwise have been counted as positive cells.

8.4 Validation of Treg Panel

To analyse specific T-cell populations a multi-colour flow cytometry panel was used to quantify isolated populations as well as for the Treg induction assay. The following conjugated antibodies were used: CD3-FITC, CD4-PerCp-Cy5.5, CD25-PE, CD127-PE-Cy7 and FOXP3-APC. CD3 and CD4 were chosen to identify the parent CD4+ T-cell population. CD25 is highly expressed on Tregs and is to date, in combination with FOXP3, the most reliable marker to identify Tregs. CD4+ T-cells found expressing the highest level of CD25 are known to have the greatest expression of FOXP3 (Magenau *et al.* 2010). In addition, CD127 expression has been found to be inversely related to FOXP3 and allows further discrimination between Tregs (CD127dim) and activated T-cells (CD127+). Therefore, Tregs were defined as CD3+CD4+CD25highCD127dimFOXP3+. It is acknowledged that the phenotypic definition of Tregs varies between investigators, but the approach described to identify this T-cell subtype is well accepted in the field.

Tregs (CD4+CD25highCD127dim) and Teffectors (CD4+CD25-) were isolated from a pure CD4+ T-cell population (see 7.4.4 and 7.4.5). The main aim was to isolate a pure population of Tregs (>90% CD25high) and an autologous responder Teffector population (CD25low). Isolation of Tregs by commercially available T-regulatory isolation kits, such as MiltenyiBiotec's CD4+CD25+CD127dim/- Regulatory T Cell Isolation Kit II (#130-094-775), allows for the isolation of the highest purity of Tregs, however, this is a very expensive method of purification. In addition, autologous responder Teffector cells have to be isolated separately by CD4+ isolation followed by CD25 depletion. Therefore, it was felt that the best approach is to initially isolate CD4+ cells from which CD25high cells can be subsequently isolated and used as Tregs. This is a more economical approach which allows for a significantly higher number of experiments to be performed with only minimal compromise on Treg purity.

Purity of isolated Treg (CD4+CD25highCD127dim) and Teffector cells (CD4+CD25low) populations were quantified by flow cytometry after each isolation. Despite not depleting for CD127+ cells from the Treg population isolated Tregs expressed very little to no CD127. In addition, most isolated Tregs expressed the transcription factor FOXP3.

Target	Company	Conjugate	Clone	Catalog Number
CD3	BD	FITC	UCHT1	555916
CD4	BD	PerCp-Cy5.5	RPA-T4	560650
CD25	BD	PE	M-A251	555432
CD127	BD	PE-Cy7	HIL-7R-M21	560822
FOXP3	eBioscience	APC	PCH101	17-4776-42
Live/Dead – UV	ThermoFisher	(DAPI)		L23105

Table 14. Treg Flow Cytometry Panel.
Conjugated antibodies used for phenotypic analysis of T-cells.

8.4.1 Antibody Titration for Treg Panel

The Treg flow cytometry panel was already established at the Blood Research Laboratory and therefore did not require antibody titration.

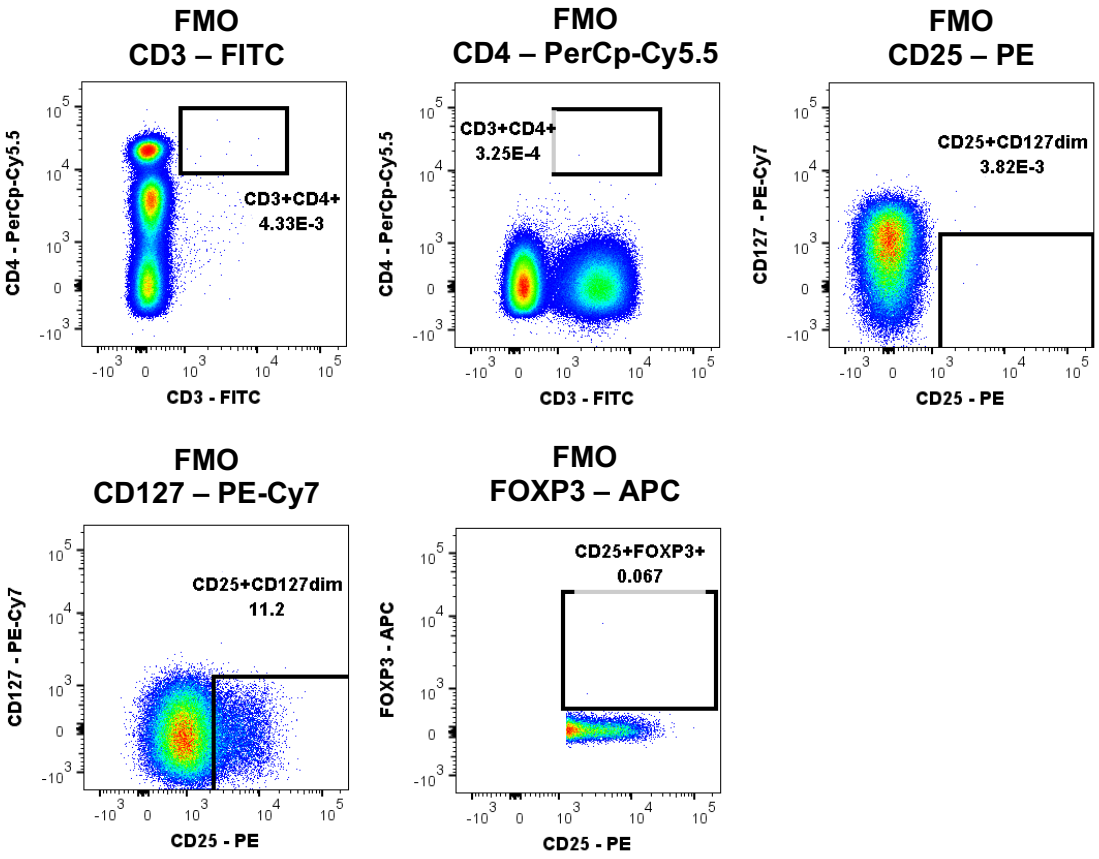
Figure legend for 8.4.1 on the next page

Fig 1. Staining Control for Treg Panel.
PBMCs were stained with the Treg panel antibodies (anti-CD3 – FITC, anti-CD4 – PerCp-Cy5.5, anti-CD25 – PE, anti-CD127 – PE-Cy7 and anti-FOXP3 – APC). Negative/positive boundaries were established using FMO controls.

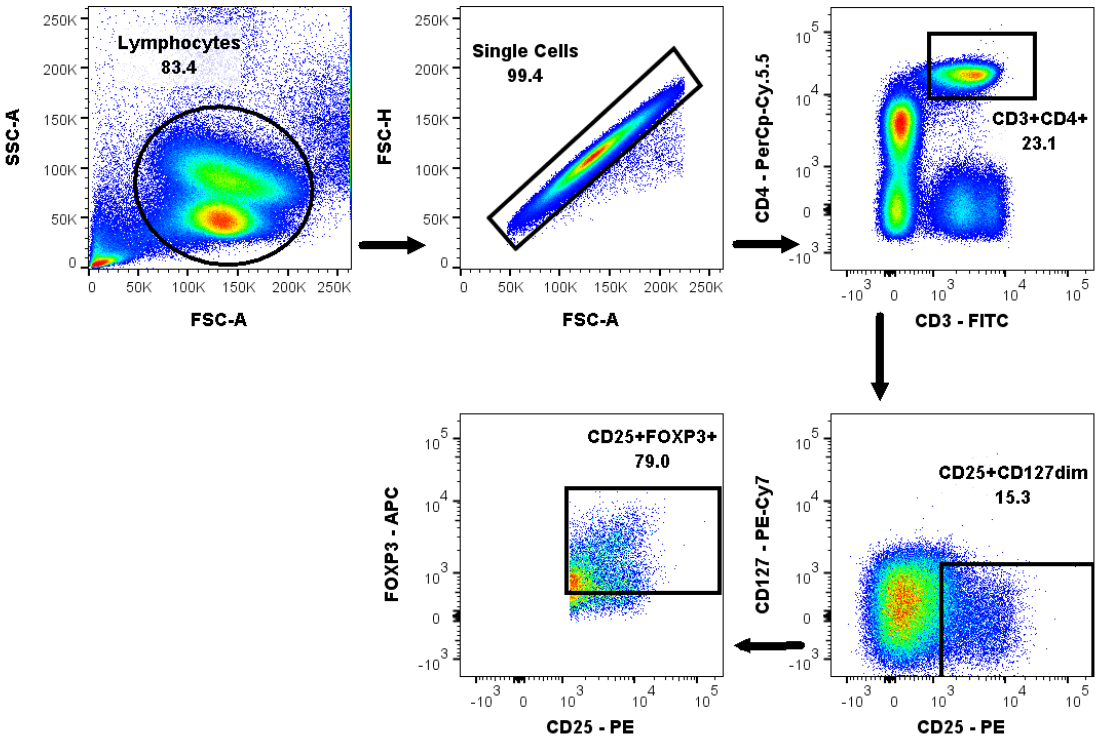
Figure legend for 8.4.2 on the next page

Fig 2. Gating Sequence to identify Tregs.
PBMCs were analysed for their expression of CD3+CD4+ (conventional T-cells), followed by the identification of CD25+CD127dim T-cells. To identify Tregs, FOXP3 expression was analysed.

8.4.1 FMO for Treg Panel



8.4.2 Gating Sequence for Treg Panel



8.5 Validation of *In Vitro* Treg Suppression Assay

To assess the functionality of Tregs the most used assay is the *in vitro* Treg suppression assay. If isolated Tregs possess suppressive capacity then they will reduce the anti-CD3/CD28 driven proliferation of isolated Teffector cells. In this assay, isolated Tregs (CD4+CD25highCD127dim) were incubated with autologous responder Teffector cells (CD4+CD25low) at a ratio of 1:1. To find the optimal stimulation ratio, T-cells were incubated at different ratios of anti-CD3/CD28 dynabeads (1:1 anti-CD3/CD28-to-Tcells, 1:2, 1:8, 1:16 and 1:32) for 4 or 5 days and analysed by flow cytometry. Furthermore, no IL-2 was used to drive Teffector proliferation or Treg survival to exclude any possibility of exogenous IL-2 driven mechanisms. Teffector cells were stained with CellTrace Blue (CTB) to trace the amount of division Teffector cells undergo (an indicator of proliferation) and to identify undivided ('suppressed') cells. Suppressed Teffector cells are determined by the proportion of the highest CTB peak intensity. Unstimulated labelled CTB Teffector cells and Tregs incubated without Teffector cells were used as a negative control and to set accurate gates in flow cytometry analysis.

To determine the suppression effect of Tregs the following calculation was used:

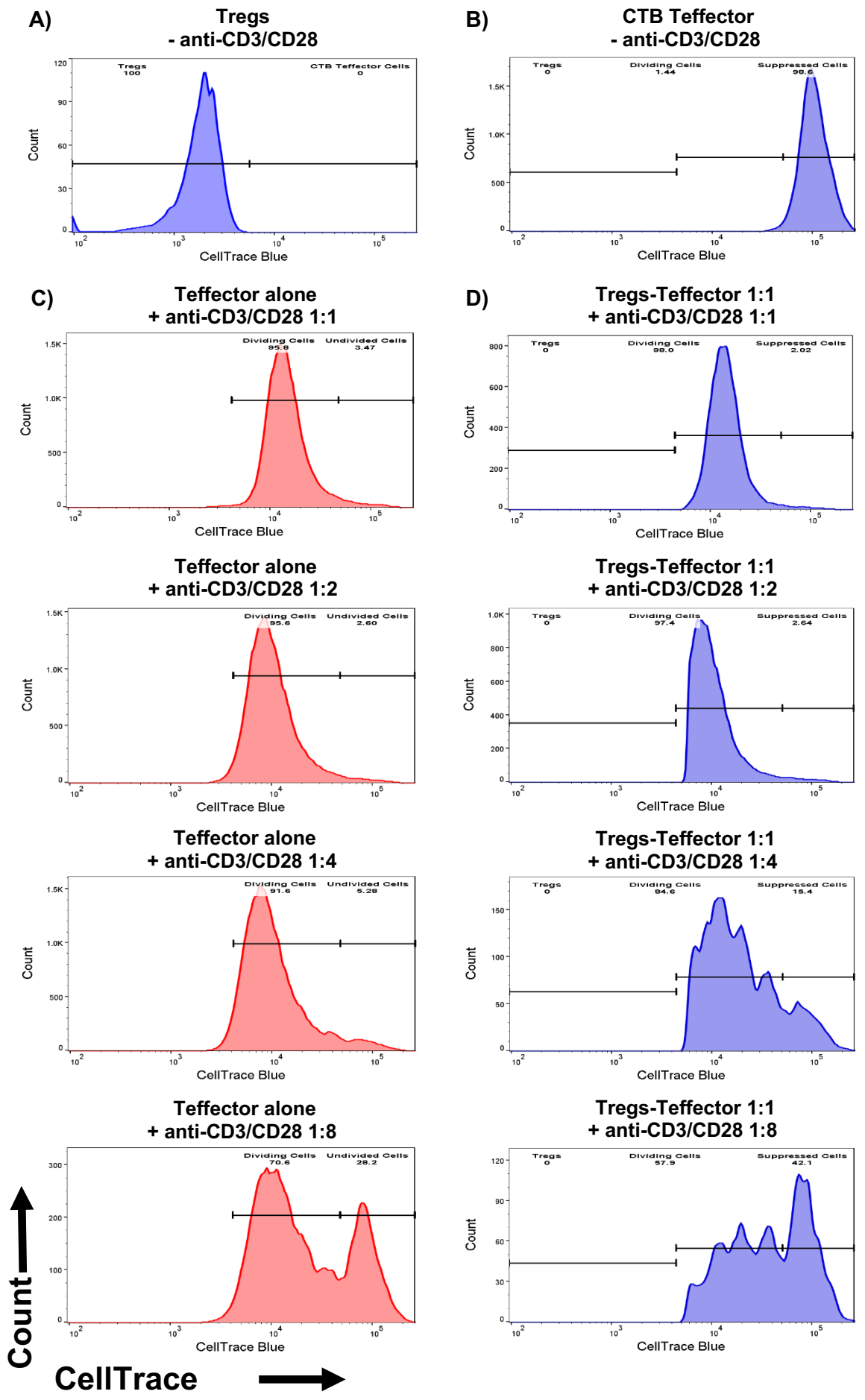
[Undivided cells Tregs-Teff 1:1] - [Undivided cells Teffector alone]
= Suppression induced by Tregs

The aim of this validation was to identify the optimal anti-CD3/CD28 stimulation giving rise to the largest difference in suppression between Tregs-Teffector 1:1 and Teffector alone. Further, only 20,000 T-cells in total should be incubated per well (i.e. for Tregs-Teffector 1:1 it would be 10,000 Tregs and 10,000 Teffector).

To exclude autofluorescence of the unlabelled Treg population, that can confound identification of the labelled Teffector pool, a negative gate was set (Fig. 3A). Unstimulated, labelled Teffector cells were used to set a gate for 'suppressed' cells (Fig. 3B). Figure 3C (in red) shows representative graphs of 1:1 to 1:32 anti-CD3/CD28-to-

Tcell stimulation for Teffector stimulated in the absence of Tregs after 5 days of stimulation. Figure 3D (in blue) shows representative graphs of 1:1 to 1:32 anti-CD3/CD28-to-Tcell stimulation for Tregs-Teffector 1:1 after 5 days of stimulation.

There is no difference in suppression between Tregs-Teffector 1:1 and Teffector alone at day 4 (Fig 4A), however differences in Teffector proliferation become apparent at day 5 (Fig 4B) with the greatest difference at an anti-CD3/CD28-to-Tcell ratio of 1:8 (Fig. 4C). Therefore, for subsequent experiments, a 1:8 stimulation was used over 5 days.


A)
Tregs
- anti-CD3/CD28
Tregs 100
CTB Teffector Cells 0
B)
CTB Teffector
- anti-CD3/CD28
Tregs 0
Dividing Cells 1.44
Suppressed Cells 98.6
C)
Teffector alone
+ anti-CD3/CD28 1:1
Dividing Cells 95.8
Undivided Cells 3.47
D)
Tregs-Teffector 1:1
+ anti-CD3/CD28 1:1
Tregs 0
Dividing Cells 98.0
Suppressed Cells 2.02
Teffector alone
+ anti-CD3/CD28 1:2
Dividing Cells 95.6
Undivided Cells 2.60
Tregs-Teffector 1:1
+ anti-CD3/CD28 1:2
Tregs 0
Dividing Cells 97.4
Suppressed Cells 2.64
Teffector alone
+ anti-CD3/CD28 1:4
Dividing Cells 91.6
Undivided Cells 5.28
Tregs-Teffector 1:1
+ anti-CD3/CD28 1:4
Tregs 0
Dividing Cells 84.6
Suppressed Cells 15.4
Teffector alone
+ anti-CD3/CD28 1:8
Dividing Cells 70.6
Undivided Cells 28.2
Tregs-Teffector 1:1
+ anti-CD3/CD28 1:8
Tregs 0
Dividing Cells 57.9
Suppressed Cells 42.1
Count
CellTrace Blue
Count
CellTrace

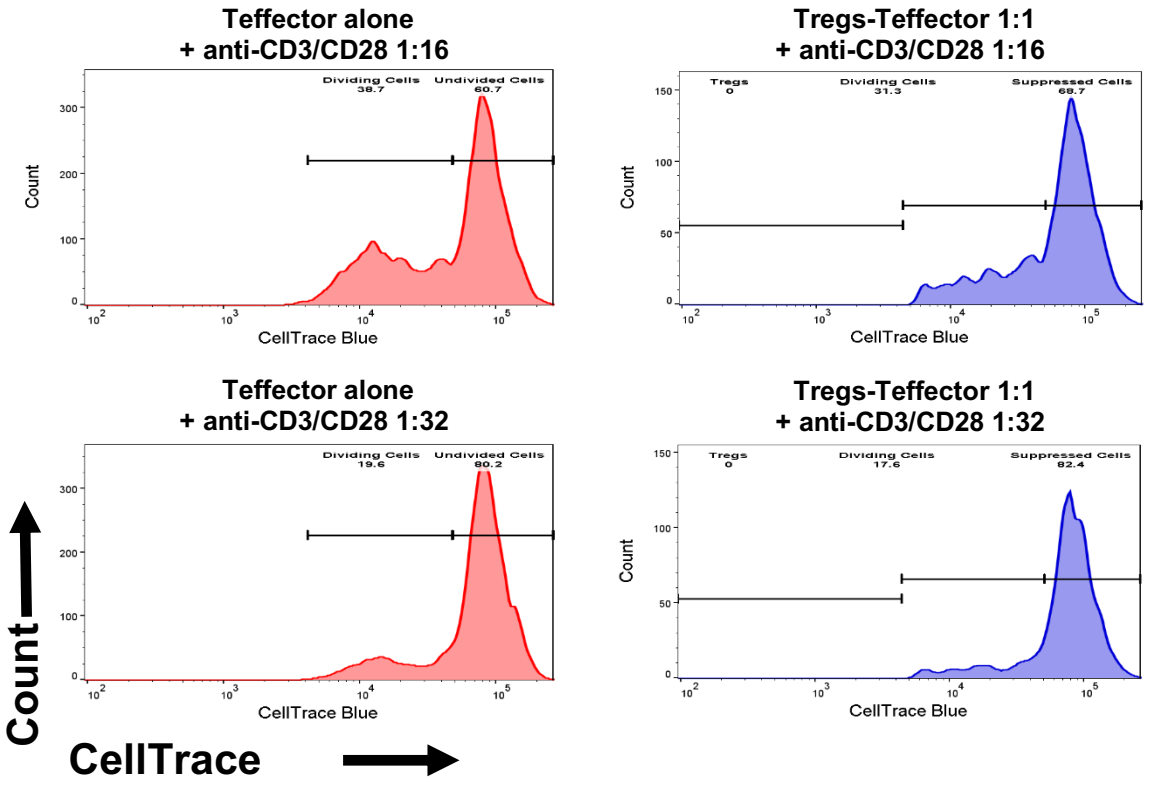


Fig 3. Validation of *In Vitro* Treg Suppression Assay

CellTrace Blue (CTB) labelled CD4+C25- Teffector cells were activated by anti-CD3/CD28 dynabeads for 4 or 5 days (5 days shown here) in the presence (blue graphs) or absence (red graphs) of autologous Tregs (CD4+CD25+CD127dim). Flow cytometry plots show CellTrace Blue staining for (A) unlabelled autofluorescence CD4+CD25+CD127dim Tregs used to set the gate for Teffector cells; (B) unstimulated CellTrace Blue stained Teffector cells (CD4+CD25-; no anti-CD3/CD28) used to set the gate for undivided/suppressed cells; (C) CellTrace Blue stained Teffector cells were incubated without Tregs with anti-CD3/CD28-to-Tcell ratios of 1:1 to 1:32 respectively; (D) Tregs and CellTrace Blue stained Teffector cells were incubated at a ratio of 1:1 with anti-CD3/CD28-to-Tcell ratios of 1:1 to 1:32 respectively. Data shown are from a single experiment that is representative of four independent experiments performed.

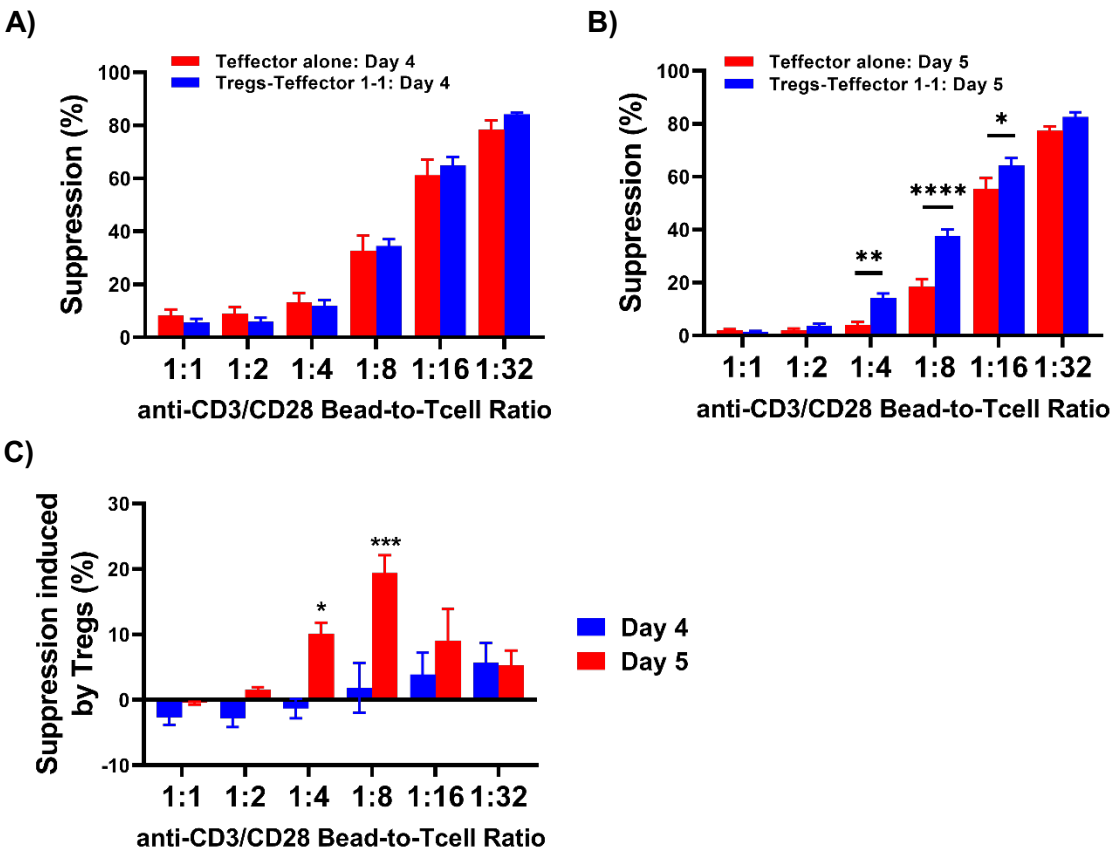


Fig 4. Validation of *In Vitro* Treg Suppression Assay

(A + B) Suppression measured for Tregs-Teffector 1:1 and Teffector alone at day 4 (A) and day 5 (B) at decreasing ratios of anti-CD3/CD28 beads to total T-cells. (C) Suppression induced by Tregs calculated by the difference in Teffector suppression between Tregs-Teffector 1:1 and Teffector alone at each anti-CD3/CD28 ratio.

n=4 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

8.6 Validation of Cell Lineage Panel

To analyse different cell populations a multi-colour flow cytometry panel was designed with key lineage markers identifying following populations: CD3–FITC, CD4–PerCp-Cy5.5 and CD8-BUV395 to identify conventional and cytotoxic T-cells respectively; CD14–PE-Cy7 to identify monocytes; CD19–APC to identify B-cells; and CD56–PE to identify NK cells and in combination with CD3 it allows to identify NKT-cells. It is acknowledged that, whilst this might be an imperfect phenotypic definition of certain populations, chosen lineage markers are considered as a valid approach to identify each respective lineage.

Target	Company	Conjugate	Clone	Catalog Number
CD3	BD	FITC	UCHT1	555916
CD4	BD	PerCp.Cy5.5	RPA-T4	560650
CD8	BD	BUV395	RPA-T8	563795
CD14	BD	PE-Cy7	M5E2	557742
CD19	BD	APC	HIB19	555415
CD56	BD	PE	B159	555516

Table 15. Cell Lineage Flow Cytometry Panel.
Conjugated antibodies used to phenotype different lineages of cells.

8.6.1 Antibody Titration for Cell Lineage Panel

Each antibody was titrated using PBMCs; starting with one dilution factor above the recommended dilution factor and three dilution factors below the recommended one. No titration was conducted for CD3 – FITC and CD4 – PerCp-Cy5.5 as dilution factor was already established at the laboratory. CD14 – PE-Cy7 dilution factor was derived from the dendritic cell phenotype panel antibody titration.

Fluorochrome	Recommended volume per test (µl)	Recommended Dilution Factor	Used Dilution Factor
CD3–FITC	20	1:5	1:20
CD4–PerCP-Cy5.5	5	1:20	1:80
CD8–BUV395	5	1:20	1:20
CD14–PE-Cy7	5	1:20	1:40
CD19–APC	20	1:5	1:10
CD56–PE	20	1:5	1:5

Table 16. Selected Fluorochromes Concentrations for Cell Lineage Panel.
For each fluorochrome-conjugated antibody the recommended manufacture's working volume (µl) and the selected volume for a total staining volume of 100µl is shown.

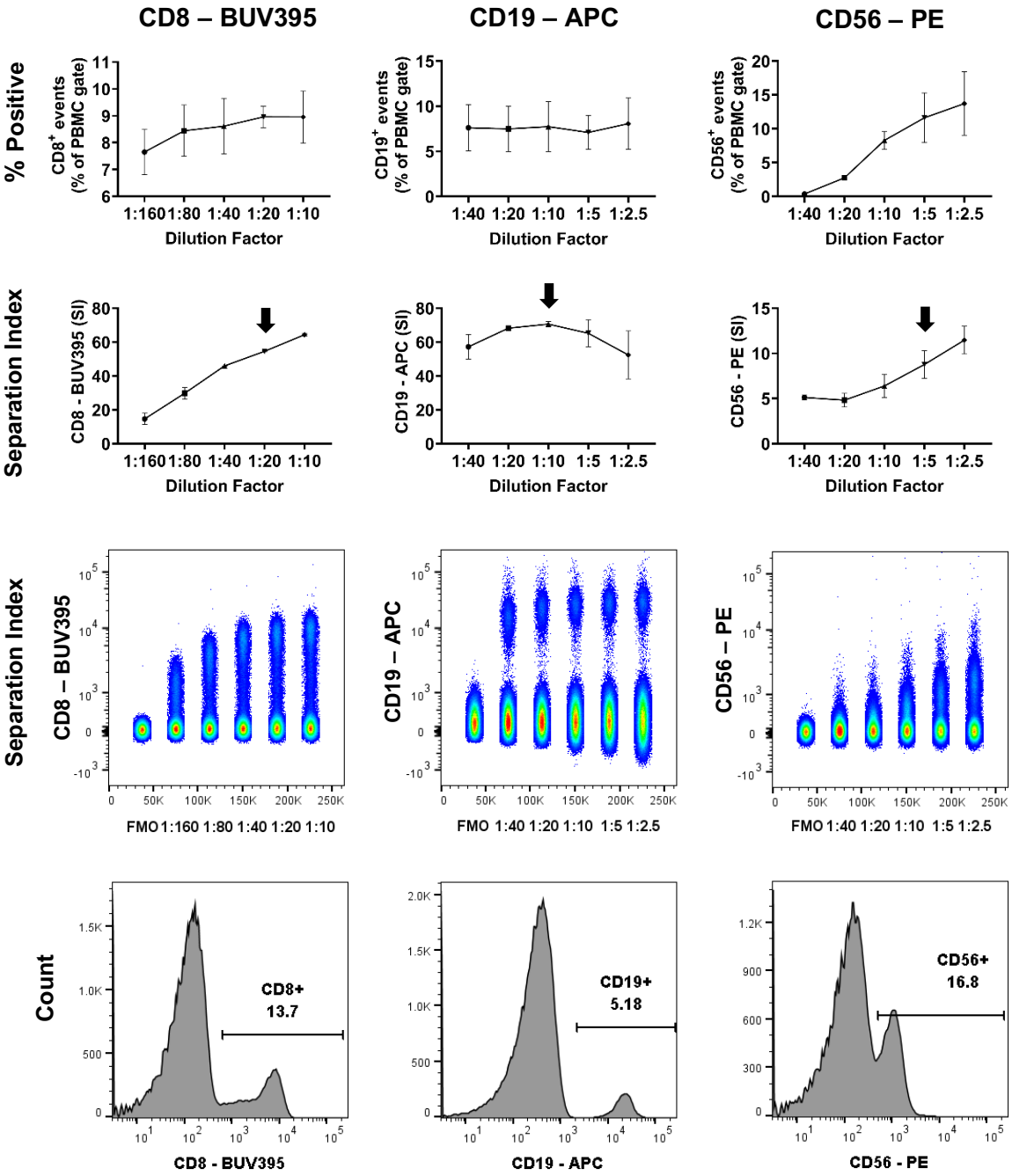


Figure 5. Anti-CD8–BUV395, anti-CD19–APC and anti-56–PE Titration.

PBMCs were isolated from leukocyte cones using density gradient centrifugation. 1x10^6 PBMCs were stained with anti-CD8–BUV395, anti-CD19–APC or anti-CD56–PE at 1:2.5 to 1:160 dilution factor ratio. Antibody concentration (x-axis) against % positive cells (top), separation index (middle), and histogram (bottom) showing separation of positive and negative population at used antibody concentration (↓) in lineage panel.

n=2 ± SEM

8.6.2 FMO for Cell Lineage Panel

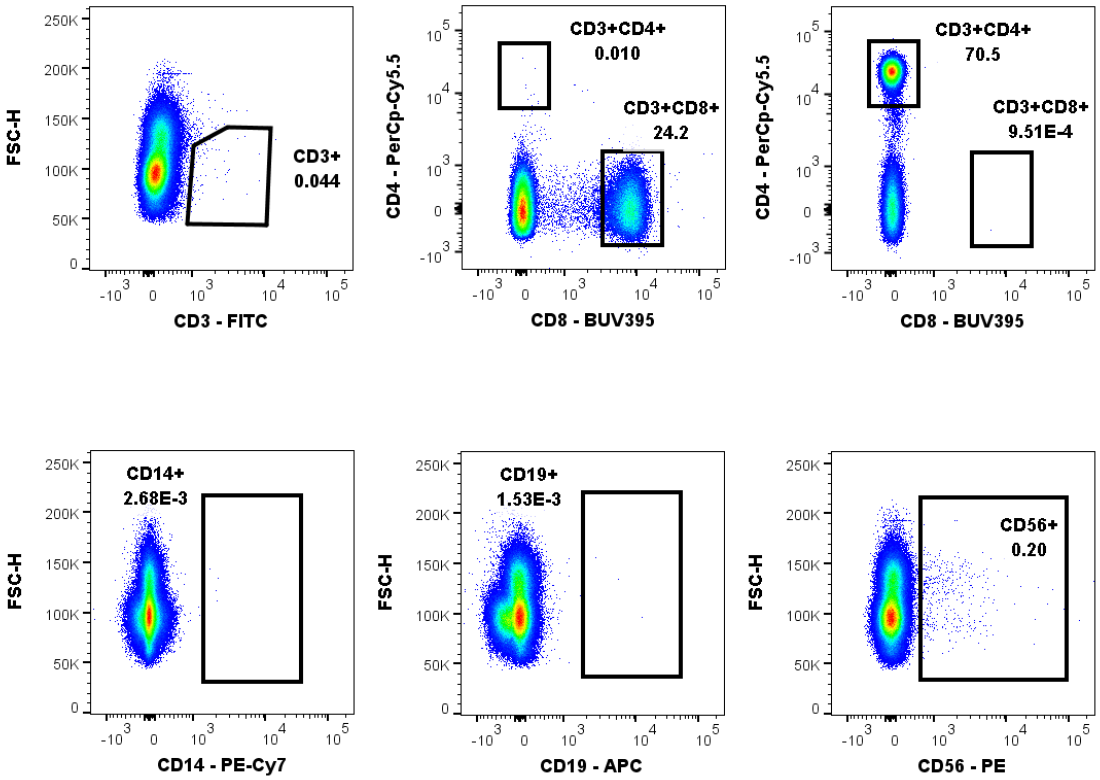


Fig 6. Staining Control for Cell Lineage Panel.
PBMCs were stained with the Lineage Panel (anti-CD3 – FITC, anti-CD4 – PerCp-Cy5.5, anti-CD8 – BUV395, anti-CD14 – PE-Cy7, anti-CD19 – APC and anti-CD56 – PE). Negative/positive boundaries were established using FMO controls.

8.6.3 Gating Sequence for Cell Lineage Panel

The gating sequence used to analyse the flow cytometry data to quantify lymphocyte populations are shown in figure. Lymphocytes were first gated using FSC/SSC to exclude cellular debris. In addition, to exclude any clumping cells or coincident events, which could interfere with analysis, a single cell gate was set. PBMCs were then analysed for their expression of CD3 followed by their expression of CD3+CD4+ (conventional T-cells), CD3+CD8+ (cytotoxic T-cells) or CD3+CD56+ (NKT-cell), expression of CD14+ (monocytes), expression of CD19+ (B-cells) and expression of

CD56+ (NK cells). The negative control to ascertain the negative/positive boundaries were FMO controls.

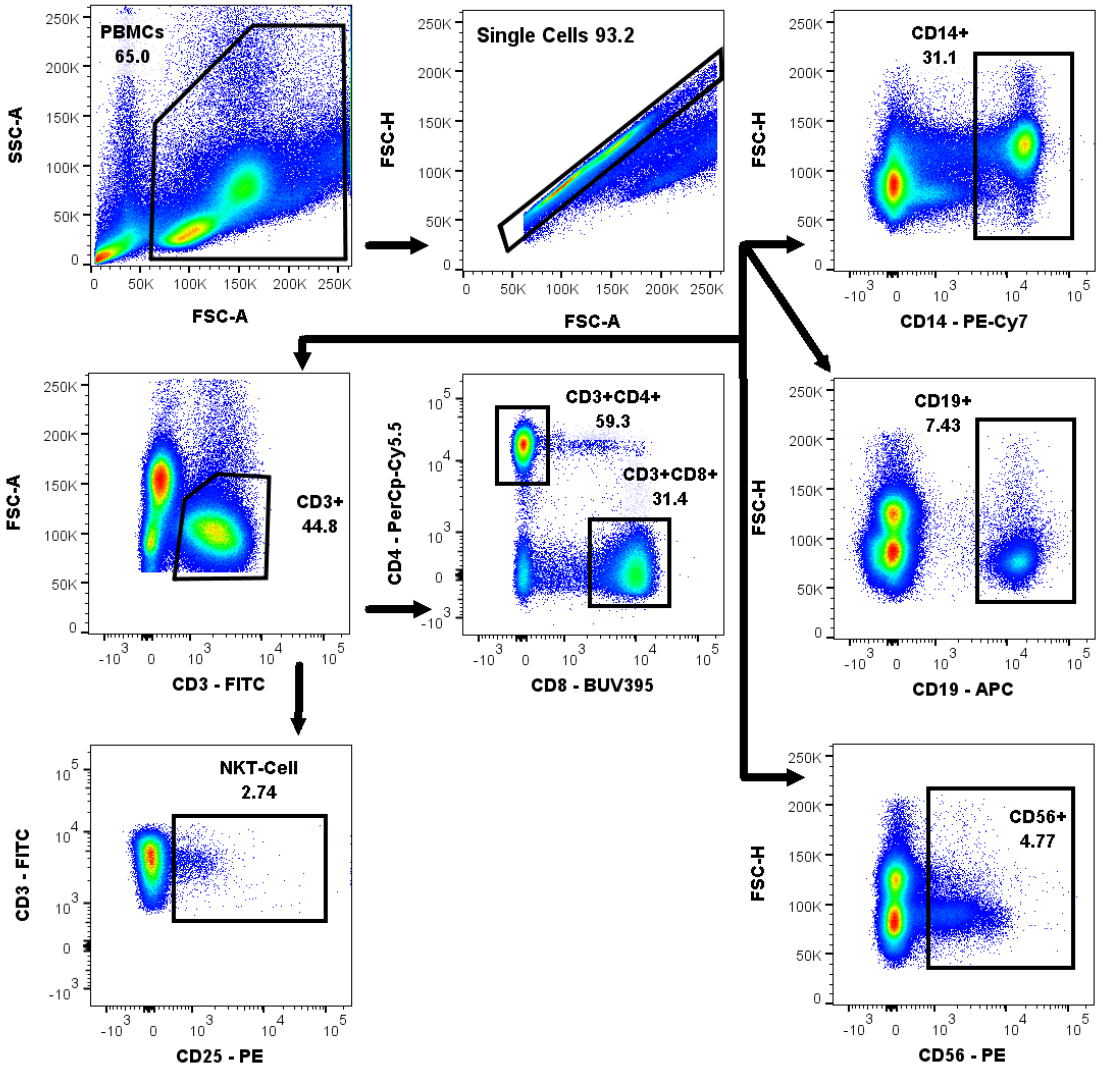


Fig 7. Gating Sequence to identify Cell Lineages.
PBMCs were analysed for their expression of CD3 followed by their expression of CD3+CD4+ (conventional T-cells), CD3+CD8+ (cytotoxic T-cells) or CD3+CD56+ (NKT-cell), expression of CD14+ (monocytes), expression of CD19+ (B-cells) and expression of CD56+ (NK cells).

8.7 Validation of Dendritic Cell Phenotype Panel

To analyse the phenotype of *in vitro* generated monocyte-derived dendritic cells (moDC) a multi-colour flow cytometry panel was designed with the following conjugated antibodies: CD1a–BB700, CD1c–BUV395, CD11c–PE-CF594, CD14–PE-Cy7, CD80–PE, CD86–BUV737, CD141–APC and HLA-DR–BB515. Human blood dendritic cells are derived from haematopoietic stem cells that belong to the lineage of HLA-DR+ cells and are distinguished from blood monocytes by their lack of CD14 cell surface expression (Clark *et al.* 2019). In addition, blood DCs can be ontologically divide into CD11c+ classical DCs (cDC) and CD11c- plasmacytoid DCs (pDC) (MacDonald *et al.* 2002). Transcriptomic data allows the classification of up to 6 different blood DC populations with unique phenotypic markers that have also confirmed established DC markers (Villani *et al.* 2017). Due to limited panel size we decided to focus on classical DCs defined by established markers CD141+ (cDC1) and CD1c+ (cDC2) in combination with CD11c+. The aim was to quantify the maturation status of moDC under different treatment conditions and if tolerogenic DC can be induced. Therefore, CD80 and CD86 were used to quantify T-cell co-stimulatory potential as well HLA-DR to quantify antigen presentation to the TCR. In addition, CD1a was added to the panel as it is considered a marker of *in vitro* generated moDC and is also involved in antigen presentation. Lastly, to identify viable cells a fixable live/dead stain was used. It is acknowledged that whilst this might be an imperfect phenotypic definition of a dendritic cell subpopulation it is a valid approach to quantify classical DCs. In addition, *in vitro* generated monocyte-derived dendritic cells are known to behave phenotypically and functionally different than to DC isolated from fresh venous blood (Clark *et al.* 2019).

Table legend for 8.7 on the next page

Table 17. DC Phenotype Flow Cytometry Panel.
Conjugated antibodies used to phenotype monocyte-derived dendritic cells.

Target	Company	Conjugate	Clone	Catalog Number
CD1a	BD	BB700	HI149	746202
CD1c	BD	BUV395	F10/21A3	742751
CD11c	BD	PE-CF594	MAB	562393
CD14	BD	PE-Cy7	M5E2	557742
CD80	BD	PE	L307.4	557227
CD86	BD	BUV737	2331	612784
CD141	BD	APC	1A4	564123
HLA-DR	BD	BB515	G46-6	564516
Live/Dead – Near IR	ThermoFisher			L10119

8.7.1 Antibody Titration of Dendritic Cell Phenotype Panel

Each antibody was titrated using PBMCs; starting with one dilution factor above the recommended dilution factor and three dilution factors below the recommended one.

Fluorochrome	Recommended volume per test (µl)	Recommended Dilution Factor	Used Dilution Factor
CD1a–BB700	*	*	1:20
CD1c–BUV395	*	*	1:40
CD11c–PE-CF594	5	1:20	1:40
CD14–PE-Cy7	5	1:20	1:40
CD80–PE	20	1:5	1:20
CD86–BUV737	5	1:20	1:40
CD141–APC	5	1:20	1:40
HLA-DR–BB515	5	1:20	1:20
Live/Dead–Near I/R	1:1000	1:1000	1:1000

Table 18. Selected Fluorochromes Volumes for Dendritic Cell Phenotype Panel.
For each fluorochrome-conjugated antibody the recommended manufacture's working volume (µl) and recommended dilution factor is shown. In addition, the actual used dilution factor for each antibody is shown. * No volume is recommended by manufacturer.

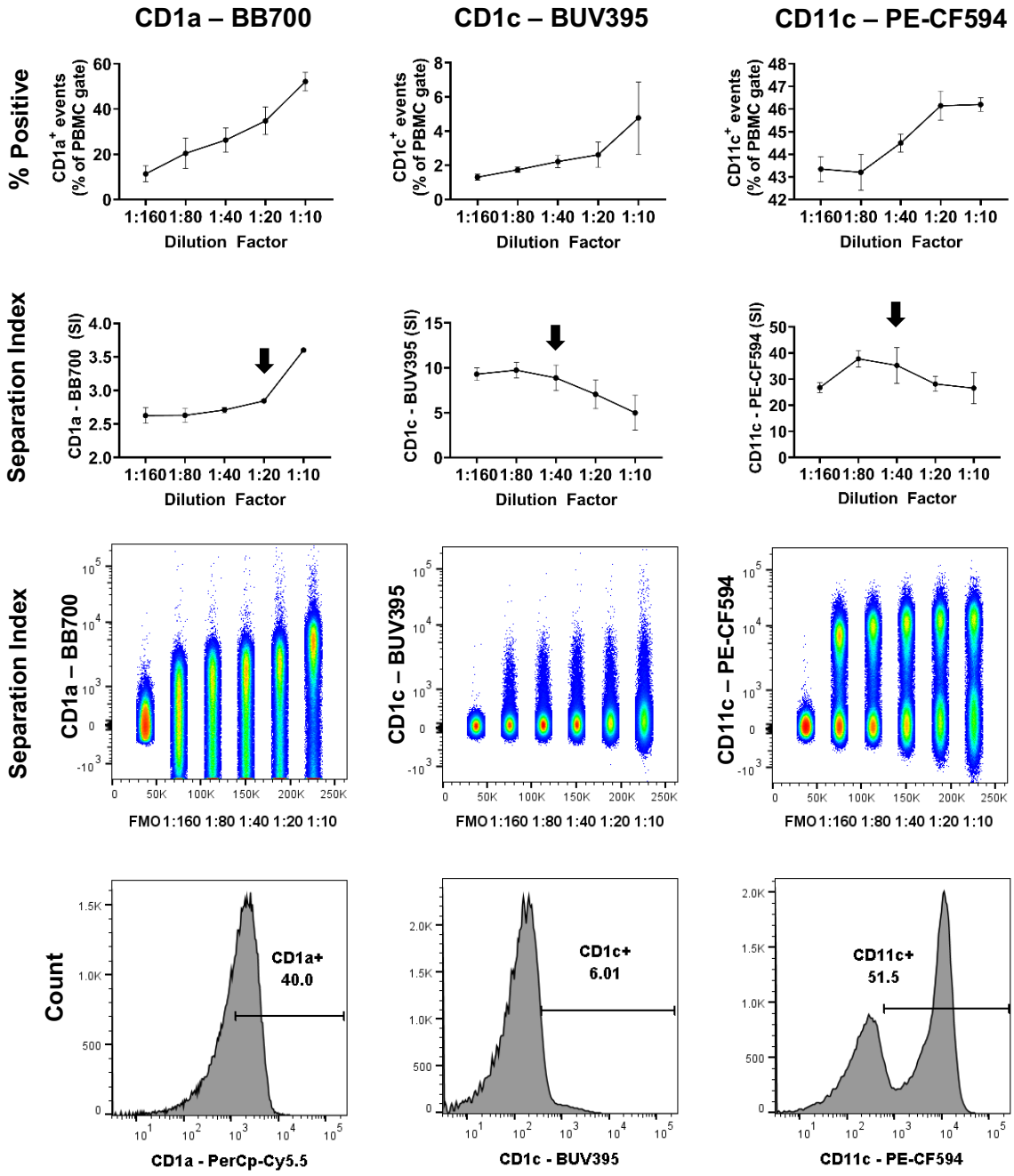


Fig 8.1. Anti-CD3–BB700, anti-CD1c–BUV395 and anti-CD11c–PE-CF594 Titration. PBMCs were isolated from leukocyte cones using density gradient centrifugation. 1x10^6 PBMCs were stained with anti-CD1a–BB700, anti-CD1c–BUV395 or anti-CD11c–PE-CF594 at 1:10 to 1:160 dilution factor ratio. Antibody concentration (x-axis) against % positive cells (top), separation index (middle), and histogram (bottom) showing separation of positive and negative population at used antibody concentration (↓) in dendritic cell phenotyping panel.

n=2 ± SEM

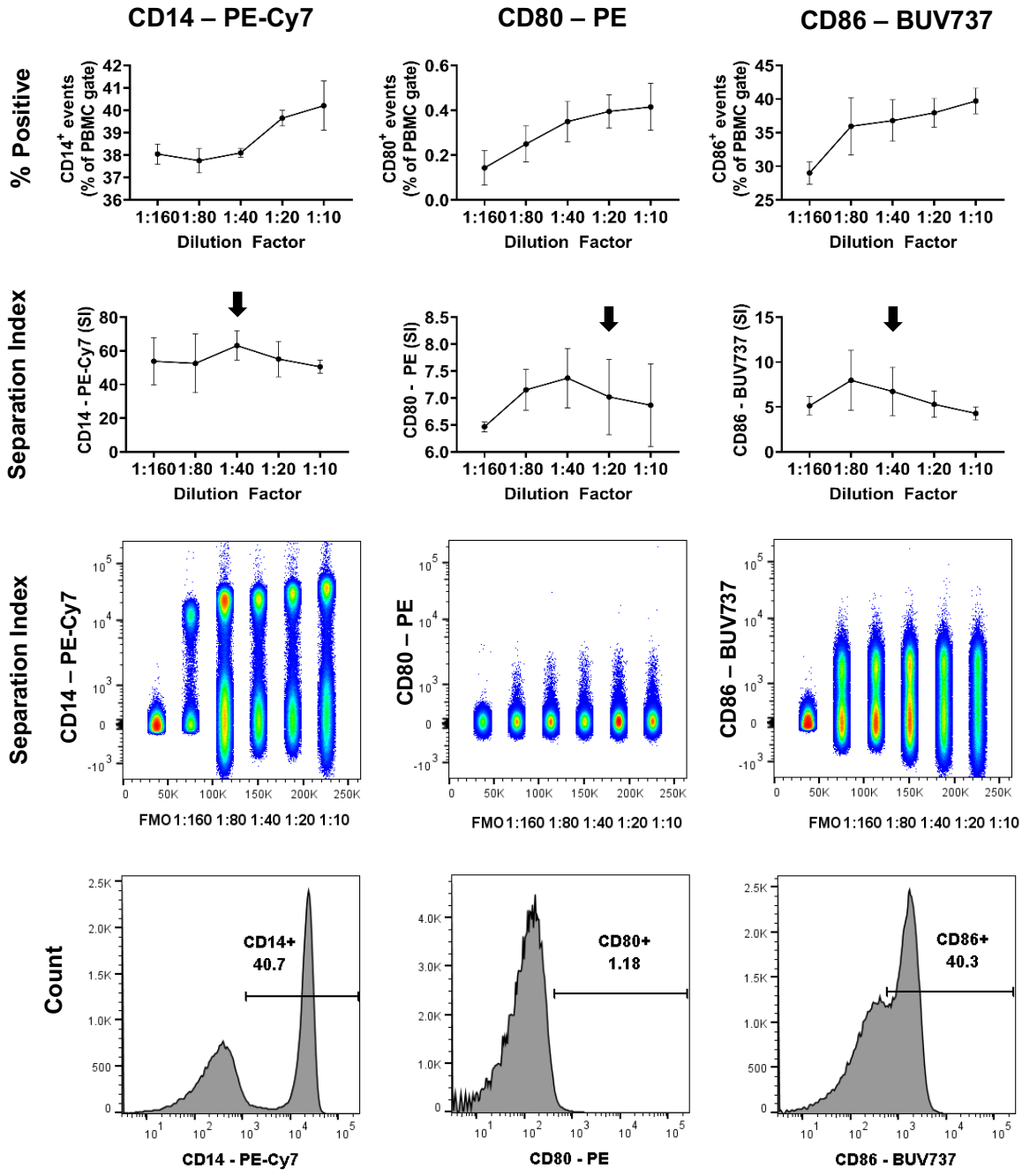


Fig 8.2. Anti-CD14–PE-Cy7, anti-CD80–PE and anti-CD86–BUV737 Titration. PBMCs were isolated from leukocyte cones using density gradient centrifugation. 1x10^6 PBMCs were stained with anti-CD14–PE-Cy7, anti-CD80–PE or anti-CD86–BUV737 at 1:10 to 1:160 dilution factor ratio. Antibody concentration (x-axis) against % positive cells (top), separation index (middle), and histogram (bottom) showing separation of positive and negative population at used antibody concentration (↓) in dendritic cell phenotyping panel.
n=2 ± SEM

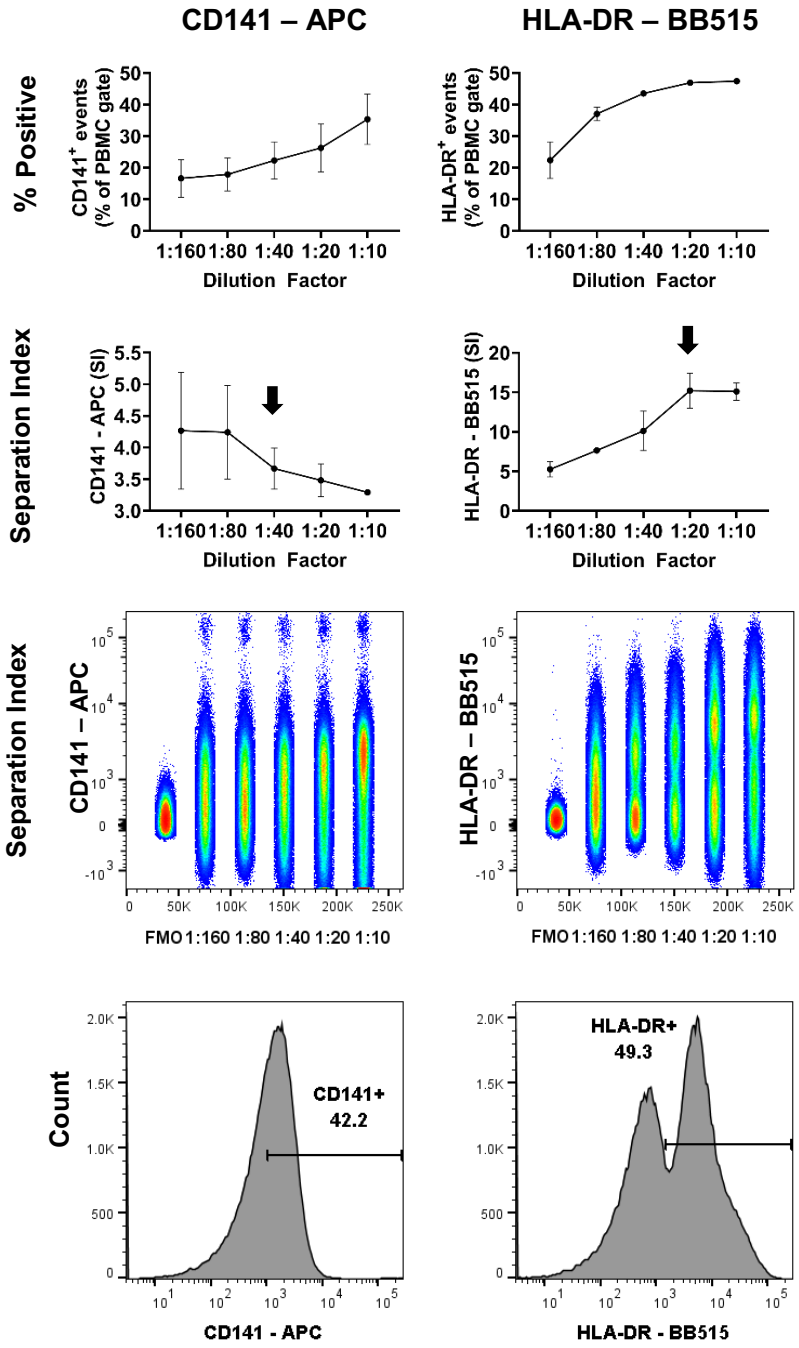


Fig 8.3. Anti-CD141–APC and anti-HLA-DR–BB515 Titration.

PBMCs were isolated from leukocyte cones using density gradient centrifugation. 1x10^6 PBMCs were stained with anti-CD141–APC or anti-HLA-DR–BB515 at 1:10 to 1:160 dilution factor ratio. Antibody concentration (x-axis) against % positive cells (top), separation index (middle), and histogram (bottom) showing separation of positive and negative population at used antibody concentration (↓) in dendritic cell phenotyping panel.

n=2 ± SEM

8.7.2 FMO for Dendritic Cell Phenotype Panel

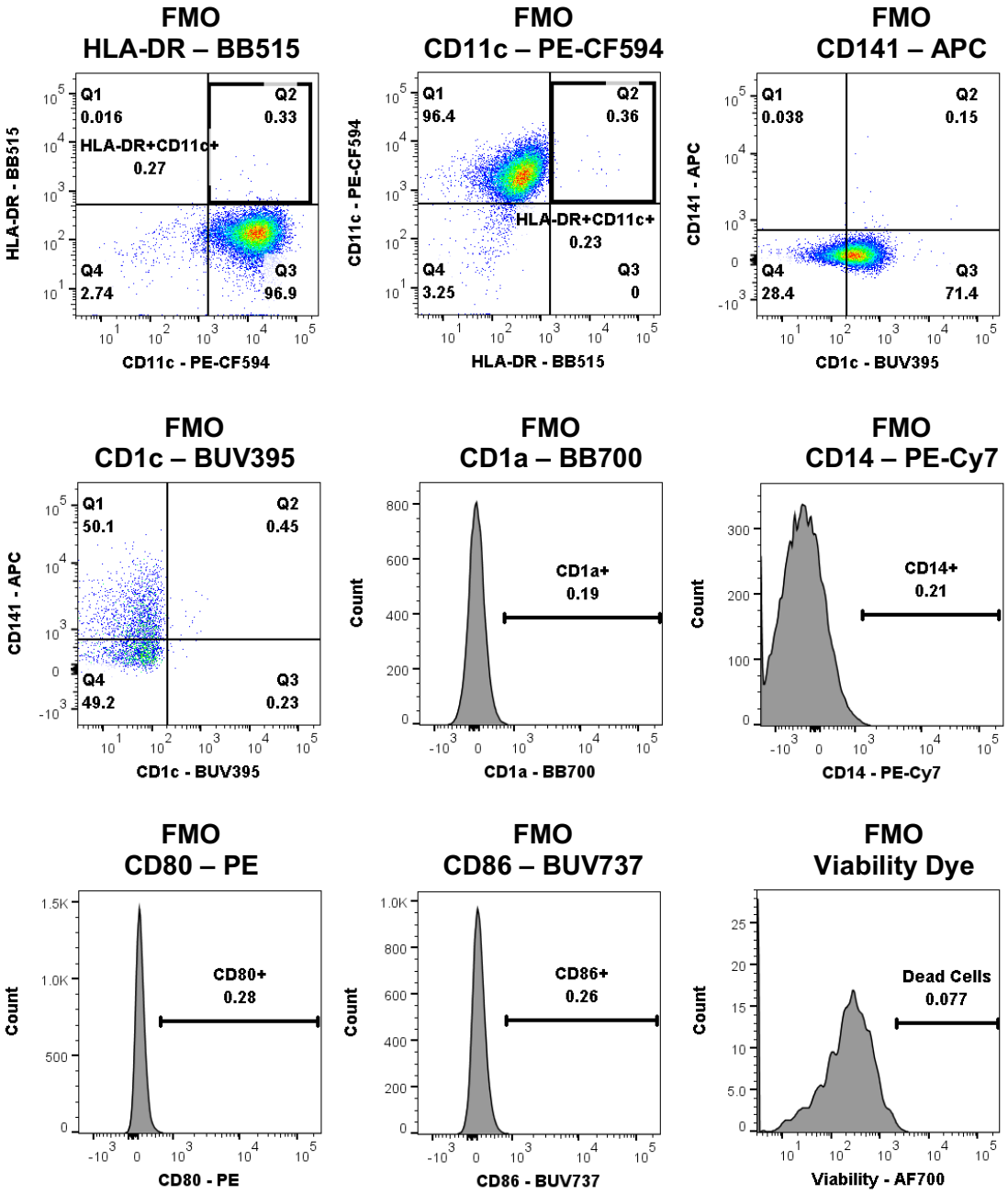


Fig 9. Staining Control for Dendritic Cell Phenotype Panel.

Monocyte-derived dendritic cells were stained with the Dendritic Cell Phenotype Panel (anti-CD1a – BB700, anti-CD1c – BUV395, anti-CD11c – PE-CF594, anti-CD14 – PE-Cy7, anti-CD80 – PE, anti-CD86 – BUV737 and viability dye – AlexaFluor700). Negative/positive boundaries were established using FMO controls.

8.7.3 Gating Sequence for Dendritic Cell Phenotype Panel

The gating sequence used to analyse the flow cytometry data to quantify the phenotype of *in vitro* generated monocyte-derived dendritic cells is shown in Figure 10. Firstly, a FSC/SSC gate was set to include myeloid cells and exclude cellular debris. Then cells were analysed for their viability by using a fixable live/dead stain. To exclude any clumping cells or coincident events, which could interfere with analysis, a single cell gate was set. Cells were then analysed for their expression of HLA-DR and CD11c. HLA-DR+CD11c+ moDC were analysed for their expression of lineage markers CD1c+ and CD141+. In addition, HLA-DR+CD11c+ moDC were also analysed to determine expression of maturation markers CD1a, CD80, CD86 and HLA-DR as well as CD1c, CD14 and CD141 expressed as mean fluorescence intensity (MFI). The negative control to ascertain the negative/positive boundaries were FMO controls.

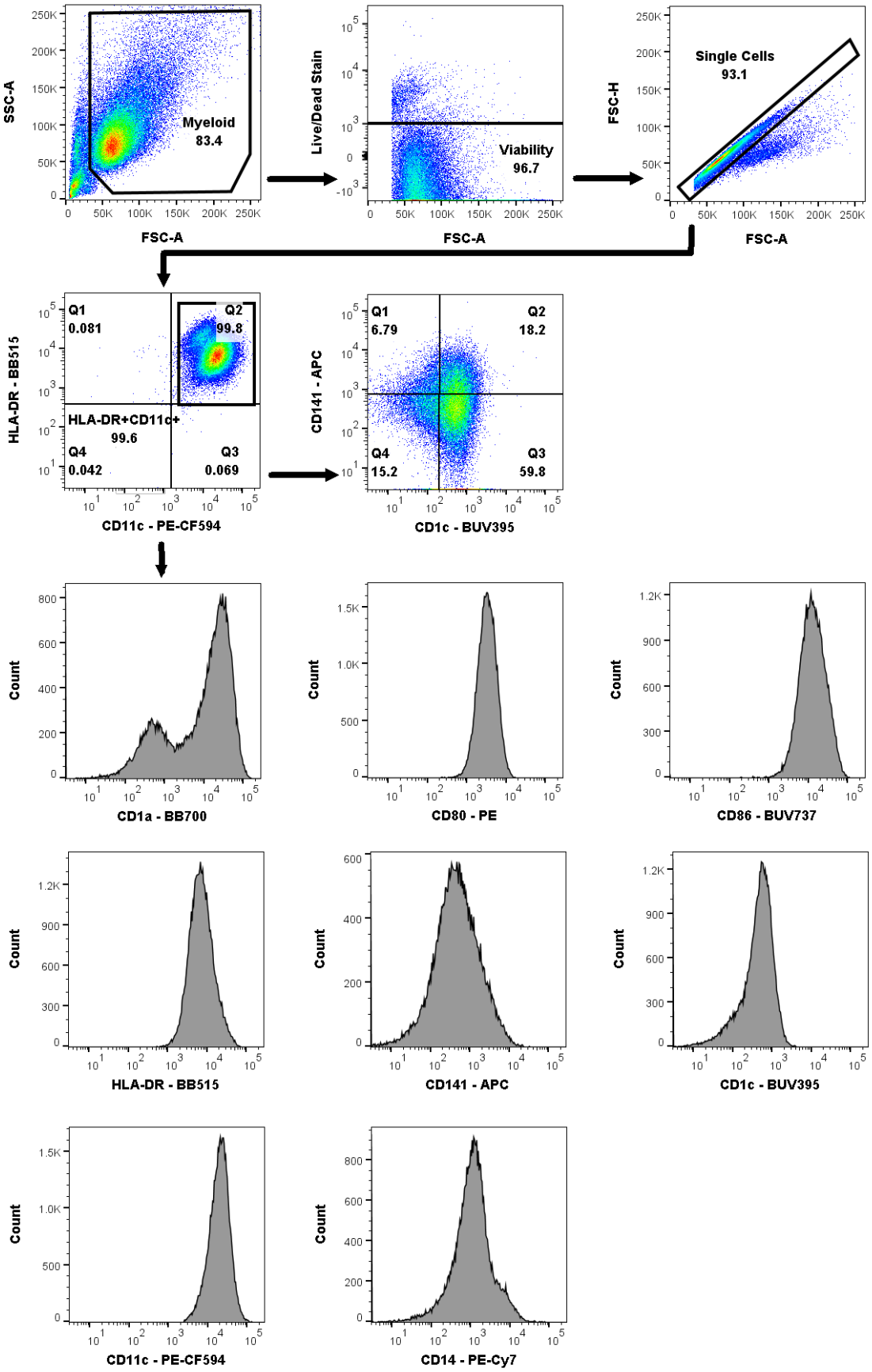


Fig 10. Gating Sequence for Phenotypic Analysis of Monocyte-derived Dendritic Cells.

8.8 Validation of Phenotype of *In Vitro* generated Human Monocyte-derived Dendritic Cells

Human dendritic cells can be isolated directly from peripheral blood, but their frequency is very low (<1% of leukocytes) making isolation of enough DCs to conduct an *in vitro* experiment challenging. An alternative option is to generate dendritic cells from monocytes, which are defined as CD14+, through cytokines IL-4 and GM-CSF stimulation. Monocyte-derived dendritic cells can then be matured with LPS and IFN-γ.

The most economical and simplest option to isolate monocytes, which are abundant in the blood, is to use PBMCs and exploit the adherence properties of monocytes to plastic. However, the more precise method is to isolate CD14+ cells from PBMCs (Fig. 11A). To determine the effect of the cytokines GM-CSF + IL-4 and IFN-γ + LPS on CD14+ cells, a negative control was introduced which did not receive any treatment over the course of the experiment (termed 'CD14+'). A second negative control was used to determine the effect of LPS and IFN-γ on monocyte-derived dendritic cells which were differentiated with GM-CSF and IL-4 but matured without LPS and IFN-γ (termed '-LPS/IFN-γ').

Differentiation of CD14+ monocytes with GM-CSF and IL-4 yields are heterogeneous population of single CD1c+ or CD141+ positive cells as well as double positives (Fig. 11B). In addition, on the flow cytometry plot no clear boundaries are seen between single positive and double positives. This is in contrast to venous blood were classical dendritic cells are either CD1c+ or CD141+. However, *in vitro* monocyte-derived dendritic cells express classical lineage markers HLA-DR+CD11c+ (Fig. 11C). Differentiation or maturation step does not introduce any significant cell death (Fig 11D). Lineage markers CD1c and CD11c are upregulated under maturation conditions (Fig. 11E). While CD1c is not induced when GM-CSF and IL-4 are not present, interestingly CD141 is observed in the 'CD14+' condition. As expected, CD14 expression is significantly down-regulated when monocytes are differentiated into dendritic cells (Fig. 11E). Maturation with LPS and IFN-γ induced the upregulation of CD1a, CD80, CD86 and HLA-DR with all being

statistically significant in comparison to immature moDC (-LPS/IFN-γ) with the exception of CD1a (Fig. 11F).

In conclusion, monocytes could be differentiated successfully into monocyte-derived dendritic cells. This was confirmed by flow cytometry as well as by light microscopy as monocytes changed their morphology from a round cell type into a branched projections (‘dendrites’) morphology.

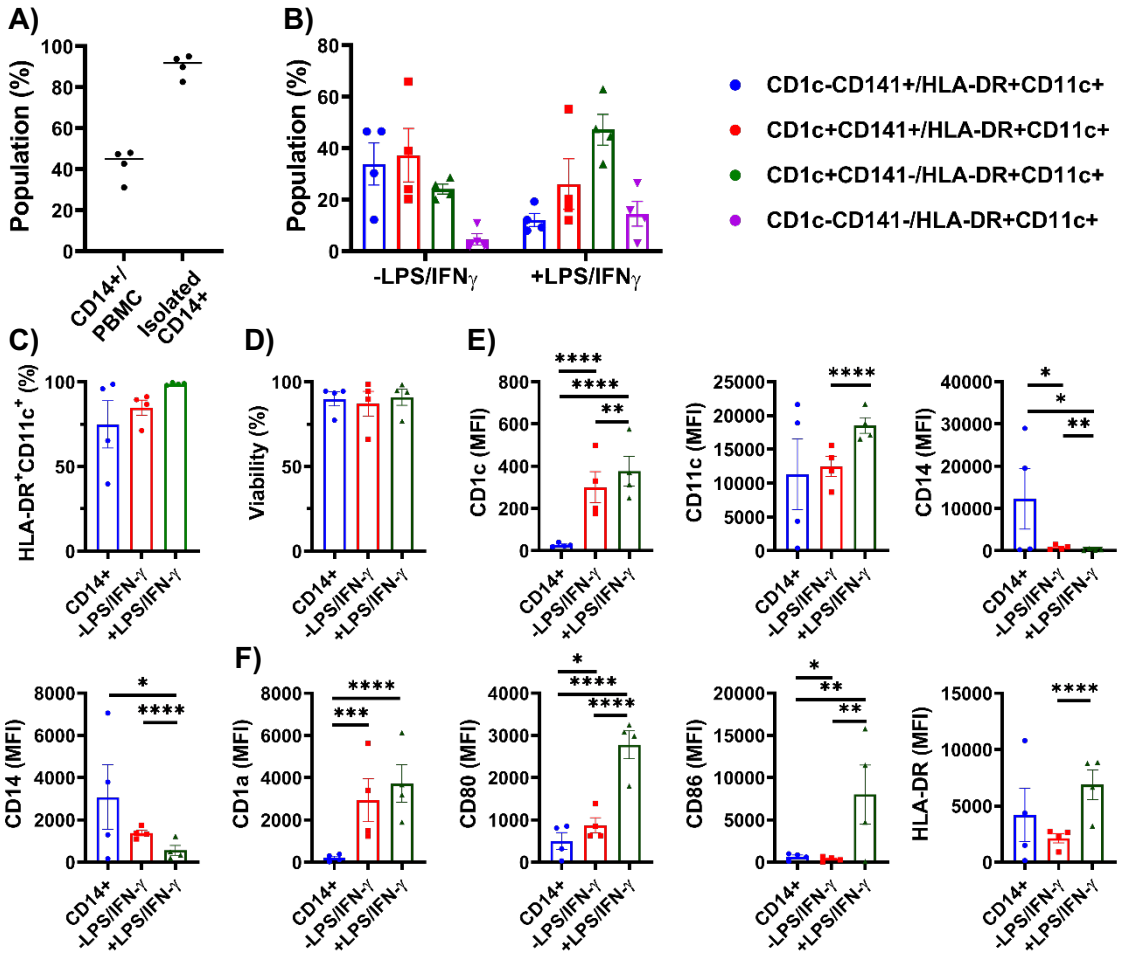


Fig 11. Phenotypic Analysis of Monocyte-derived Dendritic Cells.
Monocytes were differentiated in the presence of GM-CSF and IL-4 and matured with LPS and IFN-γ (‘+LPS/IFN-γ’) or without (‘-LPS/IFN-γ’) or were not treated at all (‘CD14+’). (A) Purity of isolated CD14+ monocytes from PBMCs used for differentiation to moDC. (B) Expression of CD1c+ and CD141+ of HLA-DR+CD11c+ moDC. (C) Percentage of moDC expressing HLA-DR+CD11c+. (D) Viability of moDC. (E) Expression (MFI) of lineage markers of classical dendritic cells. (F) Expression (MFI) of maturation markers.
n=4 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

8.9 Validation of Mixed Lymphocyte Reaction (MLR)

The principle of a mixed lymphocyte reaction (MLR) is the co-incubation of responder cells with stimulator cells. In the case of this assay, isolated responder cells were Teffector (CD4+CD25-) cells and Tregs (CD4+CD25highCD127dim) and the stimulator cells were allogeneic *in vitro* generated and matured monocyte-derived dendritic cells (HLA-DR+CD11c+).

To determine the best ratio of dendritic cells to T-cells different ratios of dendritic cells were incubated with Tregs-Teffector 1:1 or Teffector alone over 4 or 5 days. Ratios of 1:1 DC-to-Tcells, 1:2, 1:5 and 1:10 were chosen in combination with or without anti-CD3/CD28 beads for T-cells stimulation. Isolated CD14+ monocytes, used to generate moDC, were analysed by flow cytometry and had a purity of ≥90% (Fig 12A). All matured, viable moDC expressed classical dendritic cell lineage markers HLA-DR+CD11c+ (Fig. 12C-E). Maturation with LPS and IFN-γ showed a significant upregulation of maturation markers CD80, CD86 and a small increase in HLA-DR in comparison to immature control moDC (Fig 12F). Isolated Tregs were analysed and showed a phenotype of ≥90% CD25highCD127dim with over 95% of Tregs expressing FOXP3 (Fig. 12B).

Incubation of matured moDC with Tregs-Teffector 1:1 or Teffectors in the absence of Tregs without anti-CD3/CD28 stimulation lead to dendritic cell driven proliferation of Teffector cells in a ratio-dependent manner (Fig 13A + B). Tregs induce a small positive difference in Teffector proliferation between Tregs-Teffector + moDC and Teffector alone + moDC and this is only apparent on day 5 (Fig. 13C + D) which is in line with suppression observed in the Treg suppression assay. As expected there is no Teffector proliferation without dendritic cells and anti-CD3/CD28 beads.

Incubation of moDC with Tregs-Teffector 1:1 stimulated by anti-CD3/CD28 the opposite pattern was observed than reported above. Instead of moDC driving Teffector proliferation it suppresses proliferation in a dose-independent manner (Fig. 13E). In

addition, no difference in suppression is measured between day 4 and 5 for Teffectors incubated in the absence of Tregs. Again, the same trend is measured however suppression induced by moDC is ratio-dependent. A statistical significant difference in suppression is measured between Tregs-Teffector + moDC and Teffector alone + moDC at every DC-to-Tcell ratio when anti-CD3/CD28 stimulation is provided (Fig. 13G + H). In addition, a statistical difference in suppression is measured between Tregs-Teffector and Teffector alone without dendritic cells when anti-CD3/CD28 stimulation is provided (36.8% ± 1 and 7.4% ± 0.4, respectively).

In conclusion, addition of dendritic cells to Tregs-Teffector leads to an increase in suppression and is ratio-independent given that anti-CD3/CD28 stimulation is provided. Even though both time points exhibit equal statically significance between both groups and days, day 5 was picked as a time point for subsequent experiments as it makes the assay more comparable to the *in vitro* Treg suppression assay. Furthermore, anti-CD3/CD28 stimulation for T-cells is required. A DC-to-Tcell ratio of 1:5 was deemed to be optimal and was used in subsequent experiments.

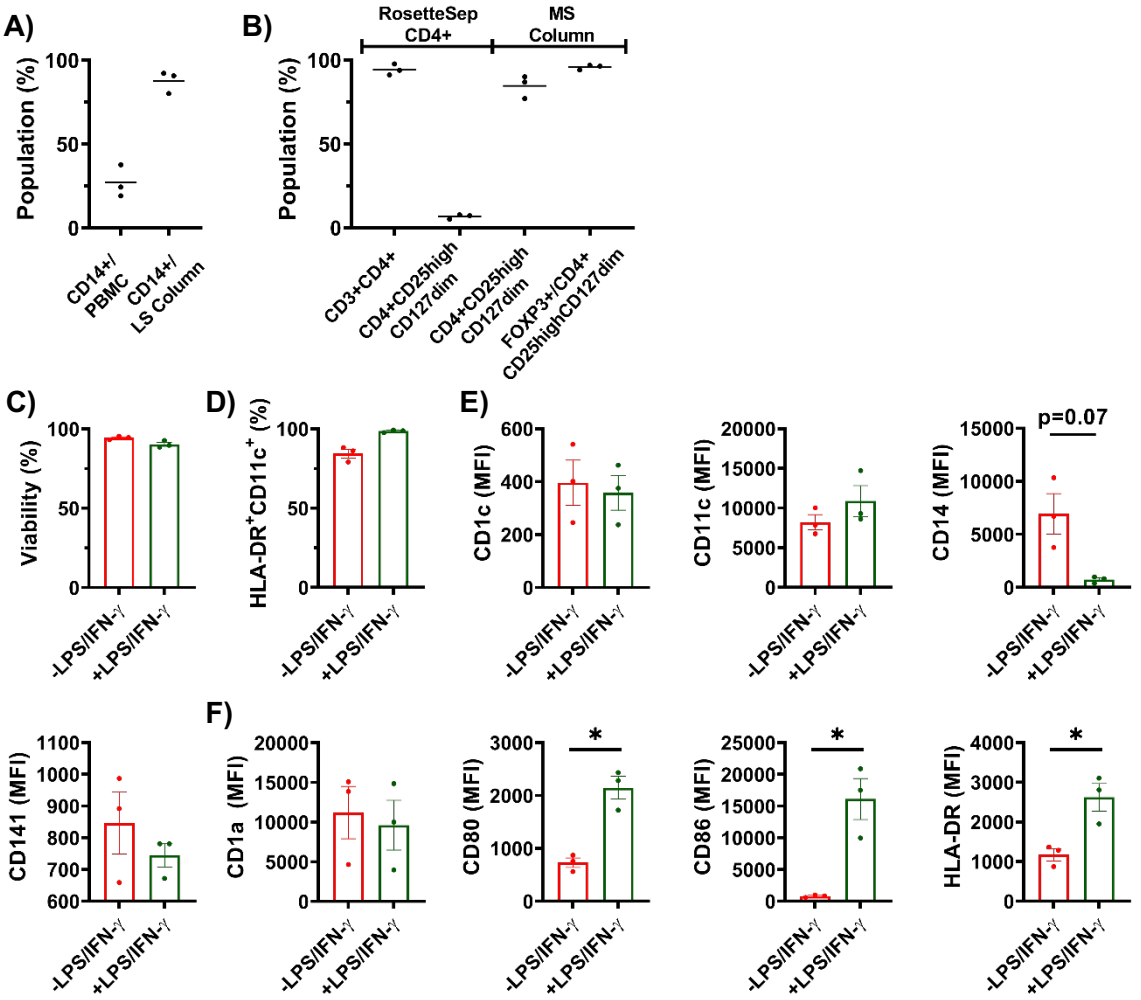


Fig 12. Phenotypic Analysis of Monocyte-derived Dendritic Cells for MLR.

(A) Purity of isolated CD14+ monocytes from PBMCs used for differentiation of moDC. (B) Purity of isolated CD4+ T-cells (CD3+CD4+) from human blood by RosetteSep. Percentage of CD4+CD25highCD127dim cells in isolated CD4+ cells. Purity of isolated CD4+CD25highCD127dim cells. FOXP3 expression of isolated CD4+CD25highCD127dim cells. (C) Viability of moDC. (D) Percentage of moDC expressing HLA-DR+CD11c+. (E) Expression (MFI) of lineage markers of moDCs. (F) Expression (MFI) of maturation markers of moDCs.

n=3 ± SEM; paired t-test, *=p<0.05.

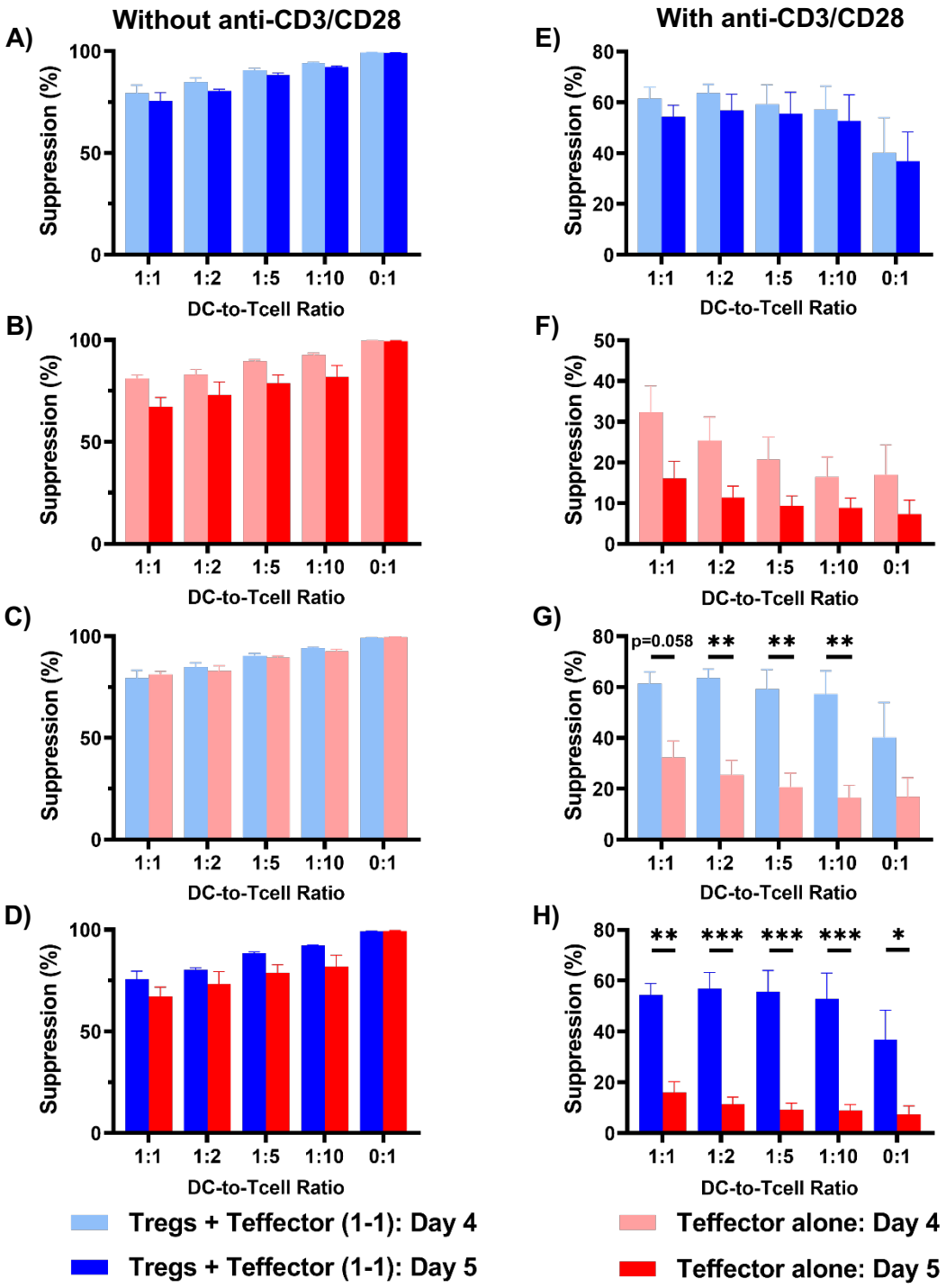


Fig 13. Validation of Mixed Lymphocyte Reaction.

(A + B) MLR without anti-CD3/CD28 stimulation. Tregs-Teffector (1:1) (A) or Teffector alone (B) were incubated for 4 or 5 days at different ratios with matured dendritic cells. (C + D) Comparison of proliferation between Tregs-Teffector (1:1) and Teffector alone at day 4 (C) and 5 (D). (E + F) MLR with anti-CD3/CD28 stimulation. (G + H) Comparison of proliferation between Tregs-Teffector (1:1) and Teffector alone at day 4 (G) and 5 (H) when anti-CD3/CD28 stimulation is provided.

n=3 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002 and ***=p<0.0002.

8.10 Validation of Treg Cell Line – Karpas299

Karpas299 is a human T-cell lymphoma cell line established from the peripheral blood of a 25-year-old man with T-cell non-Hodgkin's lymphoma in 1986 by Dr Karpas; now classed as CD30+ anaplastic large cell lymphoma (ALCL). There have been reports that these cells are phenotypically and characteristically like Tregs. Karpas299 could be used as a substitute to isolated Tregs. Karpas299 are easy to culture and divide quickly (around every 30 hours) which would have the benefit of being abundant in numbers and no requirement of expensive isolation procedure.

Karpas299 have a very similar phenotype as human Tregs as analysed by flow cytometry (Fig. 14A). They are slightly larger cells than human lymphocytes, as seen in FSC/SSC, but express the T-cell receptor CD3 as well as high levels of co-stimulatory molecule CD4. In addition, very high levels of CD25 and low levels of CD127 are expressed. FOXP3 is also strongly expressed leading to the conclusion that this cell line has all the phenotypic characteristic to be a functional substitute for human Tregs. However, tested in an *in vitro* Treg suppression assay no suppression of Teffector proliferation by KCL299 could be measured (Fig. 14B). Increasing the ratio of KCL299 to Teffector cells did not result in greatly elevated suppression. One of the possible reasons for the lack of Teffector suppression could be the methylation status of the Treg-specific demethylation region (TSDR) which is critical for Treg development and function. De-methylation status of TSDR is directly proportional to FOXP3 expression in Tregs. Whereas the TSDR is mostly de-methylated for true Tregs it is fully methylated in the Karpas299 cell line (30.5±6.4% and 99.6±0.03%, respectively) which is in contradiction to Karpas299 high FOXP3 expression level (Fig. 14C). In conclusion, this cell line has many characteristics of Tregs, but crucially lacks suppressive capacity which makes it an unsatisfactory substitute for Tregs purified from PBMCs.

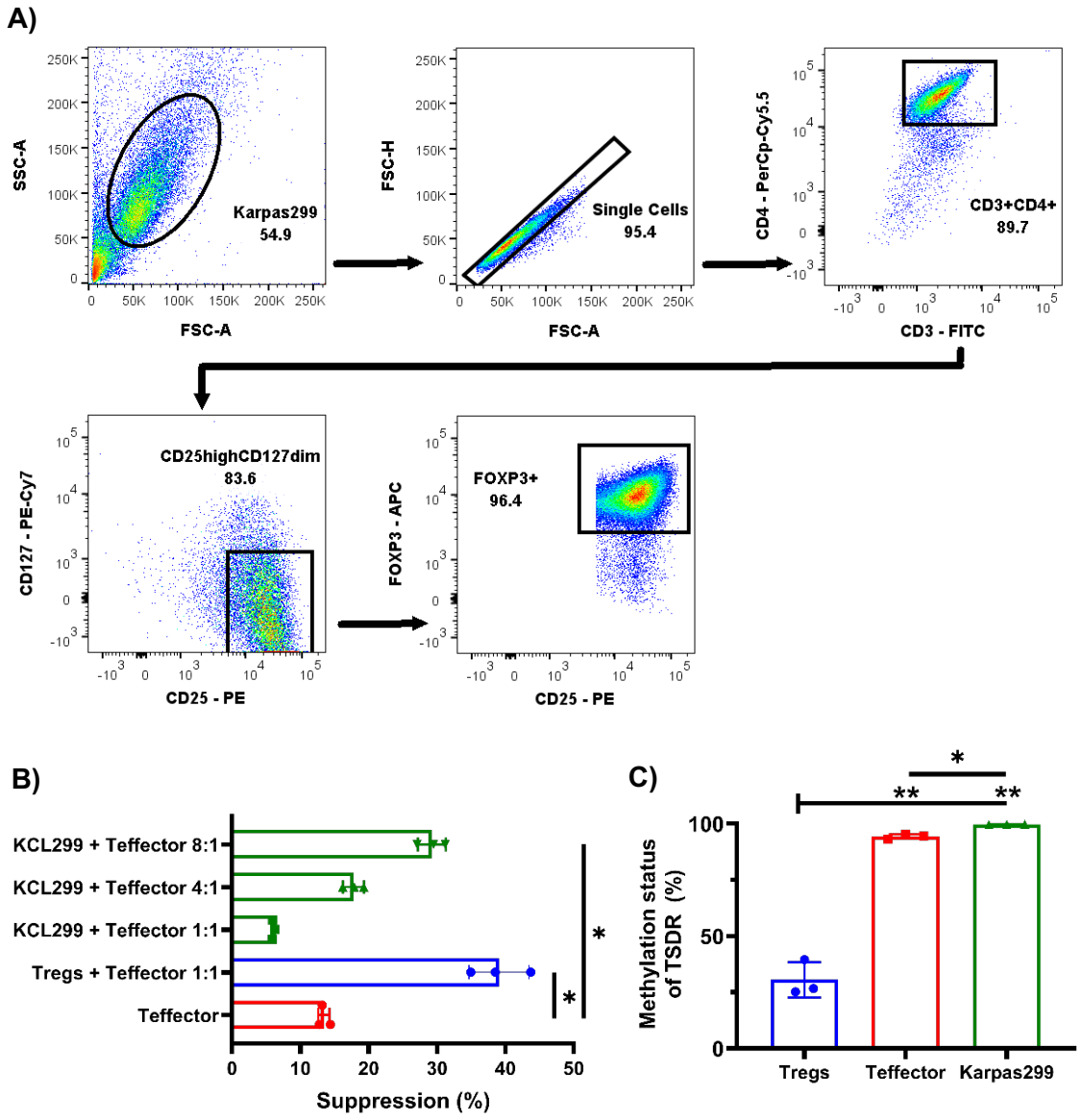


Fig 14. Phenotypic and Functionality Analysis of Treg-like Cell Line Karpas299.
(A) Gating sequence and phenotypic analysis of Karpas299 cells (KCL299). (B) *In vitro* suppression assay with Karpas299 cells as a substitute to Tregs in comparison to conventional Treg suppression assay and Teffector alone. (C) Methylation status of TSDR. Isolated DNA from Tregs (CD4+CD25highCD127dim), Teffectors (CD4+CD25-) and Karpas299 (CD4+CD25highCD127dim) was bisulfite converted and investigated for FOXP3 TSDR methylation.
n=3 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

8.11 Principle of Methylation-specific RT-qPCR

DNA methylation is an epigenetic mechanism to repress gene transcription by limiting chromatin accessibility. DNA methylation takes principally place at the fifth carbon of a cytosine base (Gardiner-Garden and Frommer 1987) and occurs more frequently in the sequence of a genome when a guanine (G) nucleotide follows on a cytosine (C) nucleotide (Jaenisch and Bird 2003). In some regions of the genome this pattern can be up to 10 times greater than the average and these regions are called CpG island with a GC percentage of 50% and greater (Bird 1986). In general, CpG sites inside an CpG island are mostly unmethylated whereas CpG sites outside an CpG island are mostly methylated (Takai and Jones 2002). In fact, around 80% of CpG dinucleotdies in the mammalian genome are methylated and the majority of the remaining 20% are unmethylated CpG dinucleotides and mostly found in promotor regions (Deaton and Bird 2011).

Natural Tregs cannot be distinguished from activated conventional T-cells or induced Tregs on the basis of their FOXP3 expression as it has been shown that these populations can also transiently express FOXP3. However it has been demonstrated that a specific region within the *FOXP3* gene (CNS2/TSDR) is fully demethylated in nTregs whereas it is partially methylated in iTregs and fully methylated in conventional T-cells and allows to distinguish these populations on the basis of their TSDR methylation status (Baron *et al.* 2007).

To allow quantification of the methylation context in a cell population, extracted DNA has to be bisulfite converted. In this method, DNA is treated with bisulfite which converts unmethylated cytosines into uracil, while methylated cytosines remain unchanged during the bisulfite treatment. PCR amplification of the bisulphite-treated DNA replaces the converted uracil to thymine and generates a TpG in the position of the original unmethylated CpG and a CpG remains in the position of the methylated CpG. TpG-

specific oligonucleotides will be complementary to the bisulfite-converted DNA of Tregs and T-cells. CpG-specific oligonucleotides will amplify methylated DNA and thus indicates non-Tregs and non-T-cells.

CHAPTER 9

Modulation of CD4+ T-cell Populations by Atorvastatin and Vitamin D

9. Atorvastatin and Vitamin D

A series of *in vitro* and *in vivo* observations have highlighted the anti-inflammatory properties of Atorvastatin which could be mediated, at least in part, through the induction of Tregs. However, one caveat of these *in vitro* experiments is the use of supra-physiological concentrations (1-50μM) which cannot be used in a clinical setting. It is estimated that the highest dose of Atorvastatin (80mg) that is considered safe for patient administration can reach a mean serum concentration of 80-100nM with a C_{max} of 200---250nM (Bjorkhem-Bergman *et al.* 2011) and, in most clinical settings, 40mg is often used. Therefore, the effects of Atorvastatin in a more clinically relevant range (50-500nM) was investigated in this chapter. The biologically active form of Vitamin D, Calcitriol, is known to influence many physiological and cellular functions including inflammatory responses as well as boosting Treg numbers. Circulating Vitamin D levels vary significantly but on average fall within a 25-100nM range in healthy adults. Therefore, 100nM Calcitriol was chosen as a physiologically relevant concentration to use in *in vitro* experiments.

In this chapter a series of studies were performed to determine if combinations of Atorvastatin and Vitamin D can induce anti-inflammatory responses in primary human immune cell cultures, if the combination has additive effects, and if the combination is superior in modulating immune responses in comparison to Atorvastatin alone.

9.1 Atorvastatin and Vitamin D induce Tregs

Tregs play a critical role in ameliorating and preventing GvHD. To investigate if Atorvastatin and Vitamin D can induce CD4+CD25highCD127dimFOXP3+ Tregs *in vitro*, either PBMCs or purified CD4+ T-cells were incubated with Atorvastatin, Vitamin D or a combination of both and cultured immune cells were subsequently analysed by flow cytometry.

Treatments of PBMCs revealed a significant increase in Tregs with both Atorvastatin and Vitamin D and an additive but non-significant effect of both drugs (Fig. 15A). Increasing concentrations of Atorvastatin had no effect on Treg induction. No increase in Tregs could be measured after treatments in isolated CD4+ T-cells with the exception of 50nM Atorvastatin + Vitamin D (Fig. 15B).

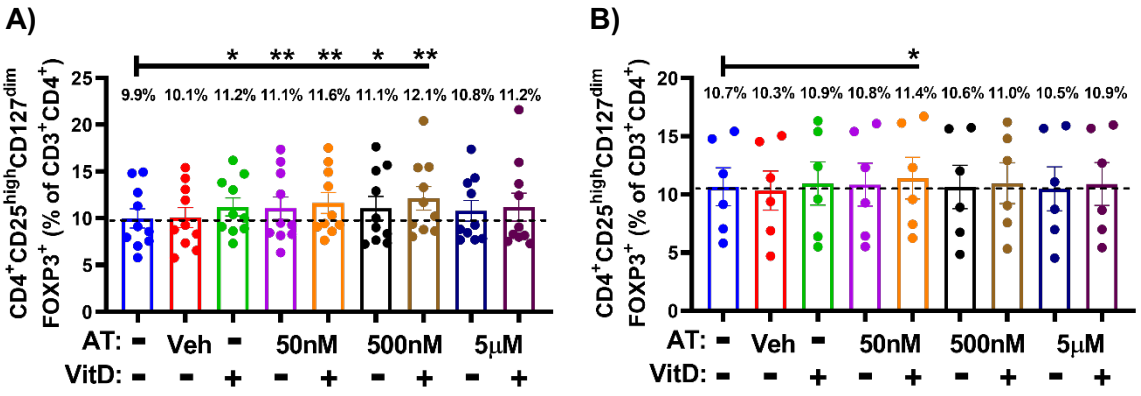


Fig 15. Atorvastatin and Vitamin D induce T-Regulatory cells.
(A) PBMCs or (B) CD4+ T-cells were incubated with Atorvastatin and Vitamin D in the presence of IL-2 for 3 days. CD3+CD4+CD25highCD127dimFOXP3+ Tregs were analysed by flow cytometry. Vehicle represent the equivalent amount of DMSO used at 5µM Atorvastatin.
(A) n=10 ± SEM; 1-way ANOVA, *=p<0.05, **=p<0.002. (B) n=5 ± SEM; 2-way ANOVA, *=p<0.05

The transcription factor FOXP3 drives a transcriptional programme critical for suppressive immune Treg function. In this way, it is predicted that an increase in FOXP3 expression and/or activity would be a good indicator of increased Treg dependent

suppressive activity. Interestingly, despite an increase in Treg numbers after treatment of Atorvastatin and Vitamin D, a dose-dependent decrease in FOXP3 protein levels were measured by flow cytometry. This effect was more pronounced in the overall CD3+CD4+ T-cell population (Fig. 16A and C) than in the Treg population (Fig. 16B and D). To investigate if the decrease in FOXP3 protein levels is a result of reduced transcriptional activity of the *FOXP3* gene, RNA was isolated from PBMCs after treatment by Atorvastatin and Vitamin D. Despite the decrease in FOXP3 protein expression within PBMCs after treatments, no statistically significant decrease in *FOXP3* mRNA levels was observed (Fig. 16E) and this was coupled with no detectable changes in TSDR DNA demethylation responsible for continuous expression of the *FOXP3* gene (Fig. 16F).

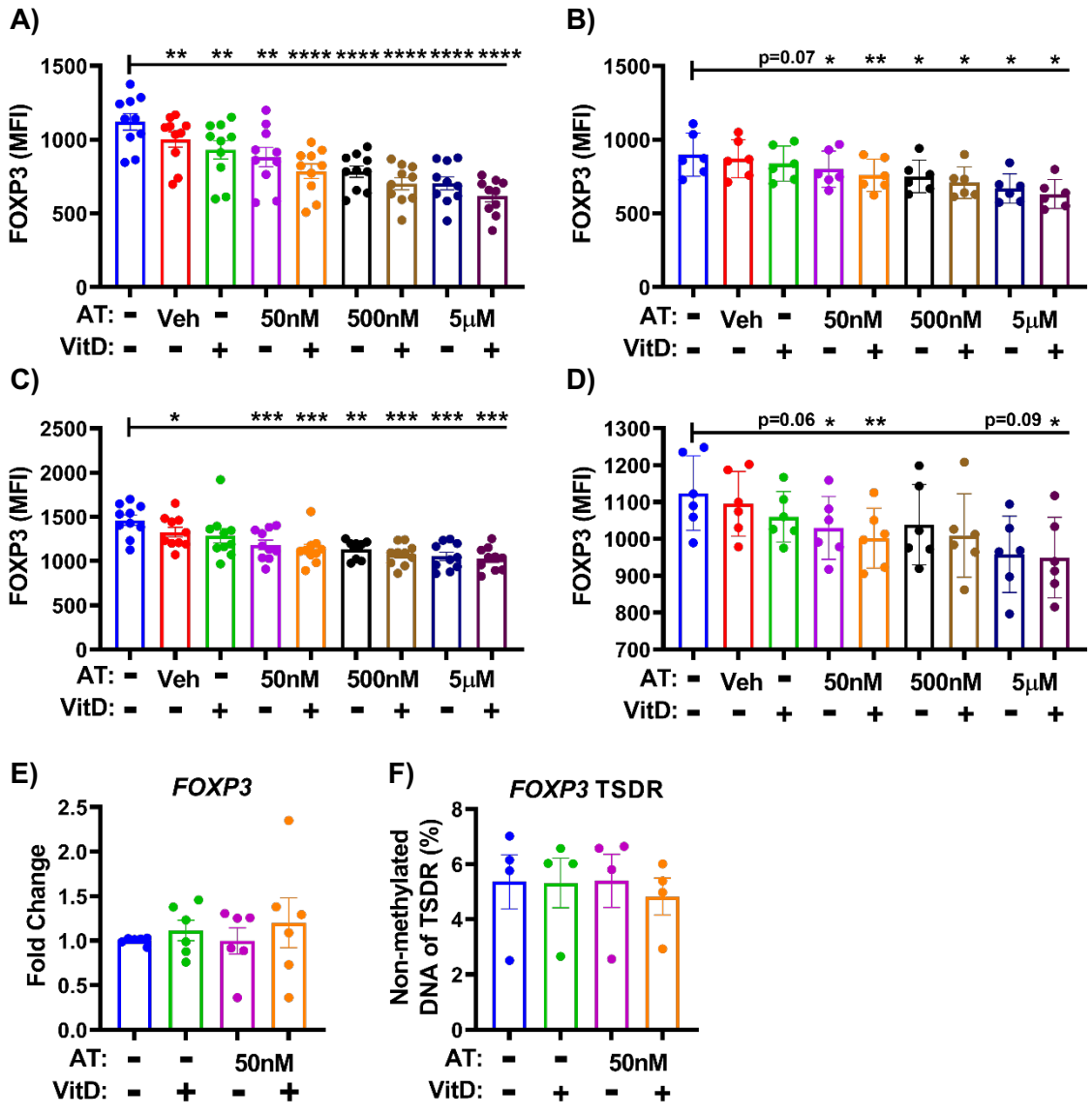


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Fig 16. Atorvastatin and Vitamin D affect FOXP3.
(A + B) PBMCs or (C + D) CD4+ T-cells were incubated with Atorvastatin and Vitamin D in the presence of IL-2 for 3 days. (A + C) CD3+CD4+ and (B + D) CD3+CD4+CD25highCD127dimFOXP3+ Treg FOXP3 protein level were analysed by flow cytometry and represented as MFI. Vehicle represent the equivalent amount of DMSO used at 5μM Atorvastatin. (E) RNA was isolated from PBMCs incubated with Atorvastatin and Vitamin D and probed for *FOXP3* mRNA levels. (F) DNA was isolated from PBMCs incubated with Atorvastatin and Vitamin D and probed for *FOXP3* TSDR DNA methylation level.
(A + C) n=10 ± SEM; 1-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001. (B + D) n=5 ± SEM; 2-way ANOVA, *=p<0.05 and **=p<0.002.

9.2 Atorvastatin and Vitamin D modulate T-helper Populations

Activated (donor) T-cells play a critical role in the initiation and progression of GvHD by differentiating into effector T-cells and causing host tissue damage. Effector T-cell populations secrete pro-inflammatory cytokines that drive innate and adaptive immune responses and Th1 (IFN-γ) and Th17 (IL-17A) T-cells are known to be critical in mediating GvHD pathology. *In vitro* experiments were performed to investigate the effects of Atorvastatin and Vitamin D on both effector T-cell numbers and the ability of this T-cell subtype to secrete cytokines. Purified CD4+ T-cells were activated by anti-CD3/CD28 and re-stimulated with PMA and ionomycin. Brefeldin A was also added to the culture media to inhibit protein transport from the endoplasmic reticulum to the golgi apparatus resulting in accumulation of cytokines within cells, allowing for easier detection by flow cytometry.

Atorvastatin and Vitamin D affect Th1/Th2 balance by promoting IL-4 production and significantly inhibiting IFN-γ production respectively (Fig. 17A + B). Atorvastatin treatment elevates IL-4 secreting CD4+ T-cells in a dose-dependent manner and this effect was reversed by Mevalonic Acid, while Vitamin D significantly inhibited IFN-γ

production. Atorvastatin did not have any effect on IFN-γ production expect for the highest supraphysiological concentration (5μM) which also fully inhibited CD4+ T-cell proliferation (see 10.1). Th17 cells are one of the main mediators of GvHD by secreting pro-inflammatory cytokines such as IL-17A. Several pre-clinical studies have confirmed the pathogenic role of Th17/IL-17A in the induction and progression of GvHD and IL-17A or RORγt deficient T-cells attenuate GvHD (Kappel *et al.* 2009, Yu *et al.* 2011, Fulton *et al.* 2012, Wu and Yu 2018). In contrast, other reports indicate a protective role of IL-17A by repressing IFN-γ-secreting Th1 cells (Yi *et al.* 2008, Cai *et al.* 2018). The results of this current study showed that Vitamin D significantly reduces IL-17A production in CD4+ T-cells and has an additive inhibitory effect in combination with Atorvastatin (Fig. 17C). Pro-inflammatory cytokine TNF-α is involved in all stages of GvHD is and the production of this cytokine is reduced by Atorvastatin in a dose-dependent manner as well as 100nM Calcitriol (Vitamin D) (Fig. 17D). In addition, an additive effect in combination of both drugs can be detected.

Anti-inflammatory cytokines such as IL-10 or TGF-β1 are important mediators of immune tolerance and clinical studies have shown that *IL-10* polymorphisms or IL-10 levels prior to HSCT influence outcome and severity of GvHD (Hempel *et al.* 1997, Holler *et al.* 2000, Lin *et al.* 2003). On the other hand, pre-clinical studies indicate that IL-10 not only has the ability to suppress but also enhance GvHD depending on dose and timing (Blazar *et al.* 1995, Zeller *et al.* 1999, Abraham *et al.* 2015). In this current study, neither Atorvastatin nor Vitamin D appeared to affect IL-10 or TGF-β1 production in CD4+ T-cells (Fig. 17E + F). *TGFB1* transcripts were unaffected, complementing no changes observed at the protein level for this cytokine in Vitamin D treated cultures. In contrast, *IL10* mRNA levels were significantly upregulated, which was in conflict with no changes in IL-10 protein levels. These differences could be due to pro- and anti-inflammatory cytokines being differentially affected by cytokine trapping in the golgi apparatus by chemical blockade with the latter being negatively regulated. Equally, *IL10* mRNA may

accumulate but are not reflected at the protein level due to a lack of increased translation of these transcripts. Furthermore, *CTLA4*, critical to inhibitory function of Tregs, was upregulated by Vitamin D treatment but did not reach statistical significance (Fig. 17G).

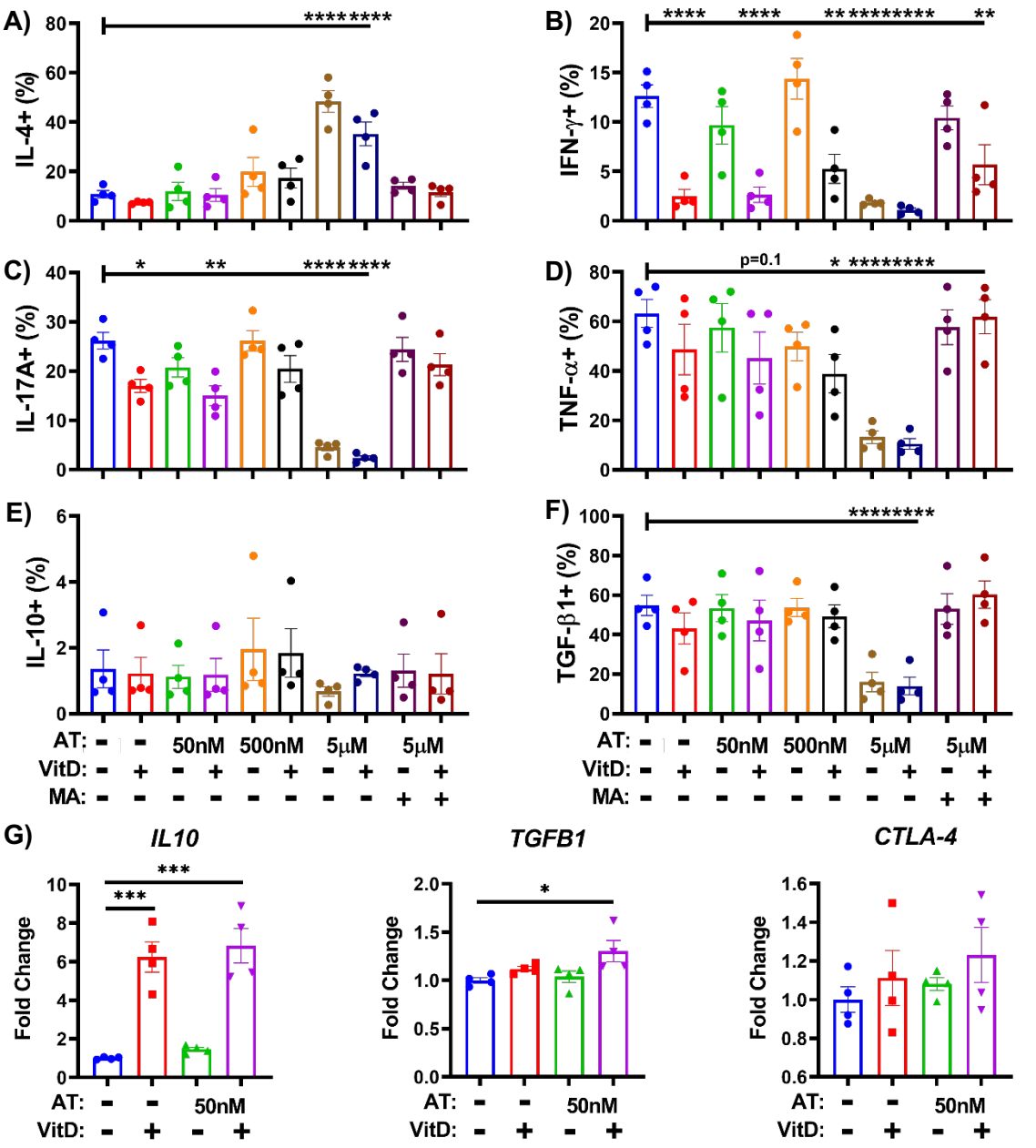


Fig 17. Atorvastatin and Vitamin D modulate T-helper cell populations.
(A-F) CD4+ T-cells were activated by anti-CD3/CD28 beads and IL-2 in the presence of Atorvastatin (AT), 100nM Vitamin D (VitD) and/or 1mM Mevalonic Acid (MA) and restimulated by PMA and ionomycin in the presence of Brefeldin A. Cytokine production was analysed by flow cytometry. (G) RNA of activated CD4+ T-cells was analysed for *IL10*, *TGFB1* and *CTLA4* expression.
n=4 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001

To confirm these findings, western blot analysis was performed for T-cell subset defining transcription factors (Fig. 18A + B). Investigated transcription factors are critical for the development and function of respective T-helper subset lineages including cytokine production. While no decrease in nuclear T-bet expression levels could be measured, Vitamin D treatment led to a significant increase nuclear GATA-3 protein expression. This is a paradoxical observation as significant reduction in IFN-γ can be measured and T-bet expression is driving IFN-γ production (Szabo *et al.* 2000, Szabo *et al.* 2002). Analysis of protein expression was performed at physiological concentrations of Atorvastatin (50nM) and thus had no effect on GATA-3 which is in line with observed IL-4 cytokine production. IL-17A production in Th17 cells is regulated by RORγt (Ivanov *et al.* 2006). Nuclear RORγt expression is reduced by Vitamin D, reflecting flow cytometry measured cytokine levels. Secretion of IL-10 and TGF-β1 was unaffected by Vitamin D and Atorvastatin treatment, nonetheless, nuclear FOXP3 expression level was significantly elevated by Vitamin D treatment in activated T-cells while cytoplasmic FOXP3 was unaffected or slightly but statistically non-significantly increased (Fig. 18A + B). To further explore the mechanisms underlying an increase in FOXP3 expression, the methylation status of the TSDR within the *FOXP3* gene, responsible for continuous expression of FOXP3, and a binding target of FOXP3 to establish an autoregulation feedback loop, was investigated. Interestingly, a decrease in de-methylated TSDR DNA was measured in Vitamin D treated T-cells, while Atorvastatin had no effect (Fig. 18C). Despite the decrease in demethylation measured by bisulfite sensitive qPCR, a significant increase in *FOXP3* mRNA could be detected (Fig. 18D). To confirm that Vitamin D led to nuclear VDR translocation, VDR expression was analysed by western blot and showed significant increase in nuclear VDR accumulation in activated CD4+ T-cells. TET proteins (TET1/2/3) are responsible for the demethylation of methylated cytosines and are key in the process of TSDR demethylation. Western blot analysis revealed a significant increase in nuclear TET1 and TET2 protein levels in Vitamin D treated cultures, however, only *TET1* reached statistical significance at the mRNA level.

DNA methyltransferase 1 (*DNMT1*), responsible for methylation of CpGs on hemimethylated DNA strands after replication, was unaffected by individual treatments, however the combination of Vitamin D and Atorvastatin led to a statistically significant increase in *DNMT1* mRNA transcripts (Fig. 19C).

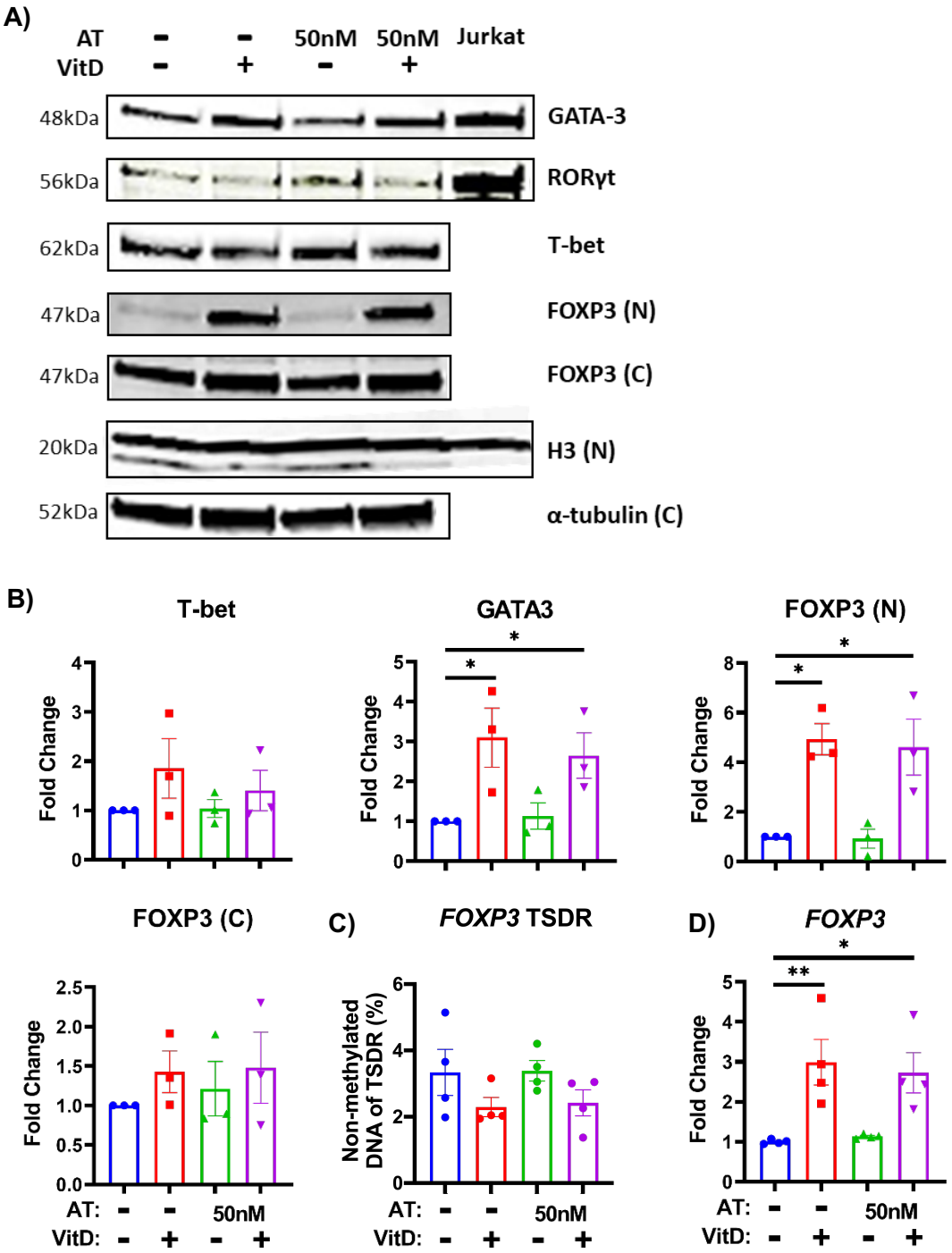


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Fig 18. Atorvastatin and Vitamin D modulate T-helper cell populations.
(A-D) CD4+ T-cells were activated by anti-CD3/CD28 beads and IL-2 in the presence of Atorvastatin (AT) and/or 100nM Vitamin D (VitD). (A) Western blot analysis of key transcription factors orchestrating the differentiation and function of effector cell lineages. For all experiments the nuclear protein fractions were used, where both fractions were used, nuclear (N) and cytoplasmic (C), it is indicated. Jurkat cells were used as a control for GATA-3 and RORγt expression. Histone H3 was used as loading control for nuclear and α-tubulin for cytoplasmic fractions. (B) Quantification of western blot bands for respective transcription factors. (C) DNA demethylation status of *FOXP3* TSDR. (D) mRNA expression of *FOXP3*.
Shown blots are representative of 3 independent experiments.
n=3 ± SEM; 2-way ANOVA, *=p<0.05 and **=p<0.002

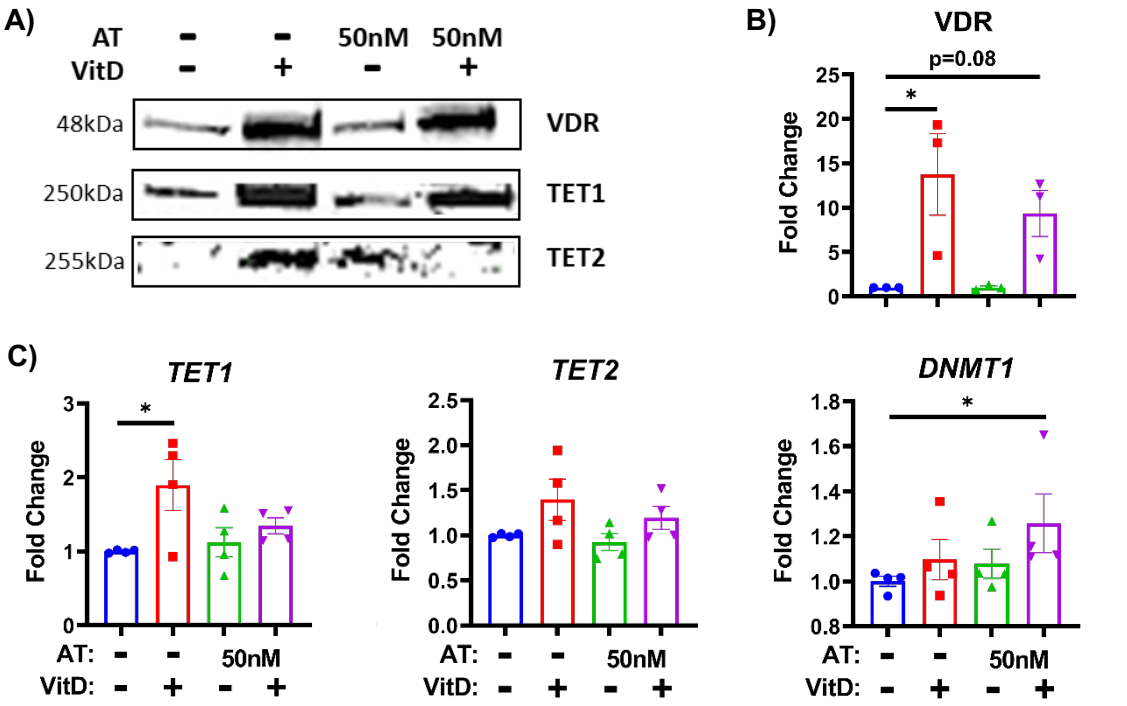


Fig 19. Atorvastatin and Vitamin D modulate T-helper cell populations.
(A-D) CD4+ T-cells were activated by anti-CD3/CD28 beads and IL-2 in the presence of Atorvastatin (AT) and/or 100nM Vitamin D (VitD). (A) Western blot analysis of Vitamin D receptor (VDR) and TET1 and 2. (B) Quantification of western blot bands for VDR. (C) mRNA expression of *TET1*, *TET2* and *DNMT1*.
Shown blots are representative of 3 independent experiments.
n=3-4 ± SEM; 2-way ANOVA, *=p<0.05

9.3 Discussion

T-regulatory cells (Tregs) are essential in the prevention, as well as treatment, of GvHD. Recipients who receive HSC grafts with a high proportion of CD4+ Tregs have significantly increased overall survival and reduced aGvHD occurrence. Tregs modulate immunity by inhibiting T-cell mediated immune responses and by modulating the function of APCs that are critical for T-cell activation and T-helper differentiation. This chapter determined the therapeutic benefits of both Atorvastatin and Vitamin D on T-cell populations in general and Tregs in particular.

Phenotypically an increase in CD4+CD25highCD127dimFOXP3+ Tregs was measured in PBMCs, but not CD4+ T-cells, when incubated with Atorvastatin and Vitamin D. This indicates that other cell types such as APCs (e.g. dendritic cells) are critical in the induction of Tregs and that Atorvastatin and Vitamin D can modulate these populations. However, this is in contrast to other studies that have reported an increase in isolated CD4+ T-cell populations (Tang *et al.* 2011, Xu *et al.* 2018). Despite FOXP3 being critical for the induction of iTregs and maintenance of Treg suppressive function, in this current study a decrease in FOXP3 protein abundance was observed. Interestingly, *FOXP3* mRNA level were unaffected by Atorvastatin and Vitamin D which indicates that FOXP3 gene and protein expression are differentially controlled by these treatments that culminate in unaffected *FOXP3* mRNA but decreased FOXP3 protein levels. It is known that FOXP3 undergoes many post-translation modifications, such as acetylation and phosphorylation, which protect this protein from proteasomal degradation and Atorvastatin and Vitamin D could modulate FOXP3 protein stability by altering the levels of FOXP3 modifications (Lu *et al.* 2017).

DNA methylation is a fundamental epigenetic mechanism by which gene expression can be regulated. CpG methylation represses transcription by preventing the binding of transcription factors necessary for active gene transcription. Critical to the maintenance

of FOXP3 is the TSDR/CNS2 region within the *FOXP3* gene which is fully demethylated in thymic-derived Tregs and allows stable FOXP3 expression and confers the Treg phenotype. However, no increase in demethylated TSDR DNA by either treatment was observed in resting PBMCs, indicating that the measured increase in Tregs might be only transient and could convert back into an effector T-cell phenotype under inflammatory conditions due to instable *FOXP3* expression.

Atorvastatin and Vitamin D differentially affect cytokine production in anti-CD3/CD28 stimulated CD4+ T-cells. While Atorvastatin strongly effects Th2 cytokine IL-4, Vitamin D affects a whole range of cytokines but not IL-4. This allows for the possibility that Vitamin D and Atorvastatin co-treatments would have greater benefits in safeguarding from GvHD than either treatment alone. Cytokines production of T-cells are critical to manifestation of GvHD. Alloantigen-exposed donor CD4+ T-cells differentiate into pathogenic Th1 and Th17 which promote tissue damage and GvHD. Although other T-cells lineages such as Th2 have a role in (acute) GvHD they are regarded as less relevant.

Th1 cytokine IFN-γ, classically described as a pro-inflammatory cytokine that drives effector function, has been demonstrated to both exacerbate and protect against GvHD (Wang and Yang 2014). While IFN-γ deficiency accelerates lung GvHD, it promotes GI tract GvHD (Burman *et al.* 2007, Varelias *et al.* 2015). One reason for the conflicting outcomes might be the wide expression of the IFN-γ receptor which is found on almost every cell lineage including T-cells, B-cells, NK-cells or APCs. Furthermore, despite IFN-γ being critical in priming, proliferation, effector function development and memory generation of T-cells (Whitmire *et al.* 2007), it is also responsible for Fas-mediated cell death, inhibition of CD4+ T-cell activation by caspase-8-dependent apoptosis or PD-L1 mediated apoptosis (Novelli *et al.* 1997, Refaeli *et al.* 2002, Blazar *et al.* 2003). The relative importance of these distinct modes of IFN-γ could be due to the milieu of cells

present within a graft site which is likely to differ between organs and tissue types as well as between donors.

IL-17A is a well-established pathogenic cytokine in GvHD but also protective in maintaining mucosal integrity and microbiome diversity or repressing IFN-γ secreting Th1 cells (Yi *et al.* 2008, Varelias *et al.* 2017). Nonetheless targeting IL-17 is a good therapeutic target as it mediates GvHD but not GvL (Gartlan *et al.* 2015). To fully understand how Atorvastatin and Vitamin D affect cytokine production it would be useful to establish if certain cytokines produced by T-helper populations are inhibited or if the development of cells that produce these molecules are repressed. Another promising therapeutic target is pro-inflammatory TNF-α which is released during pre-transplant conditioning as damaged host tissue release danger signals including TNF-α as well as during active phases of GvHD. Th1 cells release large amounts of TNF-α and significantly exacerbates GvHD especially in the GI tract by TNF-α mediated activation of APCs and recruitment of effector cells (Ferrara *et al.* 2009). It is therefore favourable that the drug combination of Atorvastatin and Vitamin D significantly reduces the production of TNF-α by CD4+ T-cells. Several drugs (e.g. etanercept or infliximab) have been developed to block TNF-α and significantly reduced GvHD severity in clinical trials (Reddy and Ferrara 2003, Levine *et al.* 2008). Targeting anti-inflammatory IL-10 and TGF-β as a therapy will be challenging due to the small therapeutic window and dose-dependent effects.

Interestingly, western blot analysis revealed no decrease in T-bet expression despite significantly reduced IFN-γ levels in Vitamin D treated CD4+ T-cells, but an increase in GATA-3 which is responsible for the induction of Th2 cells and IL-4 production. However no increase in IL-4 production was measured in Vitamin D treated T-cells. This is in line with other reports who demonstrated that Vitamin D treatment results in upregulation of GATA-3 in activated T-cells (Boonstra *et al.* 2001, Sloka *et al.* 2011). Sloka *et al.* also demonstrated that despite a significant reduction in IFN-γ production in T-cells, T-bet

expression was unaffected by Vitamin D treatment. FOXP3 is often described as the single best marker to identify Tregs as expression is necessary for its regulatory function but activated T-cells also transiently upregulate FOXP3. However, subcellular localization of FOXP3 differs between Tregs and activated Teffector cells. While in activated Teffector cells FOXP3 is predominantly found in the cytoplasm, in Tregs it is mostly nuclear (Magg *et al.* 2012). FOXP3 protein expression could be detected in the nuclear fraction of activated CD4+ T-cells and Vitamin D treatment led to significant higher expression levels suggesting that Vitamin D potentiates nuclear FOXP3 expression but this was not translated into increased levels of IL-10 and TGFβ1 production. Increase in GATA-3 despite no increase in Th2/IL-4 secreting cells by Vitamin D might be explained with its relationship with FOXP3. Murine studies have shown that GATA-3 is not only upregulated in TCR stimulated Tregs but also is required for optimal function particularly under inflammatory conditions as GATA-3-deficient Tregs expressed lower levels of Foxp3 and expressed effector cytokines (Wang *et al.* 2011, Wohlfert *et al.* 2011). Vitamin D ($1,25(OH)_2VD_3$) binds to nuclear VDR which in turn binds to VDRE within DNA and several VDREs are found within the *FOXP3* gene (Kang *et al.* 2012) and murine and human studies have demonstrated that Vitamin D treatment results in elevated FOXP3 in activated T-cells (Daniel *et al.* 2008, Jeffery *et al.* 2009). However, the exact molecular mechanism how Vitamin D upregulates FOXP3 is unclear. Nonetheless, it was demonstrated that nuclear FOXP3 expression is required for acquiring regulatory function in T-cells, while cytoplasmic expression has no effect (Magg *et al.* 2012). Vitamin D treatment of activated CD4+CD25- Teffector cells in the absence of Tregs resulted in elevated suppression (see 10.1) which suggest that some suppression of Teffector cells is mediated by Teffector cells acquiring regulatory function by Vitamin D induced nuclear FOXP3. This notion is further strengthened by the fact that Vitamin D induced Tregs in resting PBMCs (see 9.1), significantly reduced ability of activated CD4+ T-cells to produce pro-inflammatory cytokines (see 9.2) and significant upregulation of FOXP3 in activated CD4+ T-cells (see 9.2). Recently, Vitamin C has

been shown to facilitate demethylation of the TSDR/CNS2 under iTreg polarizing conditions (anti-CD3/CD28, IL-2 and TGF-β) resulting in stable expression of *in vitro* and *in vivo* Foxp3 (Sasidharan Nair *et al.* 2016, Yue *et al.* 2016). This increase was dependent on TET2, an enzyme of the family of dioxygenases that catalyses the oxidation of 5mC into 5hmC (and subsequently to 5fC and 5caC) which can eventually yields an unmethylated cytosine (Tahiliani *et al.* 2009, Pastor *et al.* 2013, Sasidharan Nair *et al.* 2016). Anti-CD3/CD28 and IL-2 activated T-cells in the presence of Vitamin D resulted in significant increase of nuclear FOXP3 which raised the question if Vitamin D could facilitate the increase in FOXP3 in a similar manner to Vitamin C. Unexpectedly, a decrease in TSDR demethylation was measured. This finding was accompanied with an increase in *TET1* and *TET2* mRNA and nuclear protein expression while *DNMT1*, responsible for TSDR methylation, was unaffected. One simple explanation for the apparent increase in 5mC could be the inability of bisulphite sequencing to distinguish between 5mC and 5mC oxidized intermediates (5hmC, 5fC and 5caC), which might conceal a potential increase in demethylation. In support of this view, peripheral and thymic-derived Tregs possess oxidized methylcytosine intermediates at their CpG sites within the TSDR region (Yue *et al.* 2016). Of note, recent advances in understanding the sequential oxidative events in TET-mediated chromatin reprogramming in mouse induced pluripotent stem cells (iPSC) revealed that 5hmC generation alone is insufficient to drive the epigenetic changes observed in iPSC reprogramming. Instead, the fC/caC pathway promoted rapid DNA demethylation at the reprogramming loci (Caldwell *et al.* 2020). Further evidence of the ability of Vitamin D to induce regulatory function via TET2-mediated demethylation is provided by a study investigating DC tolerogenesis (Catala-Moll *et al.* 2020). This study demonstrated the occurrence of vitamin D-specific DNA demethylation and transcriptional activation at VDR binding sites associated with the acquisition of tolerogenesis. Furthermore, it would be interesting to know if other methyltransferase such as DNMT3a, DNMT3b and MBD2 are affected by Vitamin D treatment which play a critical role in FOXP3 expression. It has been demonstrated that

there are significant differences in expression level of methyltransferases between naïve CD4+ T-cells and Tregs, however, CD3 stimulation in combination with IL-2 did not affect expression level in naïve CD4+ T-cells (Lal *et al.* 2009).

While the TSDR is critical for the maintenance of *FOXP3* expression, it is dispensable for its transcriptional initiation. Initiation of *FOXP3* transcription in pTregs and iTregs is mediated by CNS1 which also possess CpG sites and is demethylated in Tregs but methylated in CD4+CD25- T-cells (Baron *et al.* 2007, Yue *et al.* 2016). Tet2/3 KO-mice have shown a substantial increase in methylation at CNS1 and CNS2, accompanied with a significant decrease in Tregs (Yue *et al.* 2016). Vitamin D treatment might facilitate the increase in FOXP3 transcription and translation via demethylation of CNS1. On the other hand, it has been shown that under non-polarizing conditions (i.e. anti-CD3/CD28 and IL-2 only), Vitamin C had no effect on demethylation of CNS1 and 2 in iTregs (Yue *et al.* 2016). Without further investigation this data is difficult to interpret, however, it suggests either (1) a concealed increase in de-methylation of TSDR due to the inability of bisulfite conversion to distinguish between 5mC and oxidised intermediates or (2) an increase in transient FOXP3 expression mediated via CNS1 but no increase de-methylation at CNS2. In resting CD4+ T-cells, FOXP3 expression was significantly reduced despite Vitamin D treatment. It would be useful to investigate if activated CD4+ T-cells cultured with Vitamin D would sustain their FOXP3 expression, once stimulation and treatment is removed.

Inflammation plays a prominent role in FOXP3 regulation and pro-inflammatory cytokines downregulate FOXP3 expression to allow effective immune responses to occur during inflammation (Littman and Rudensky 2010, Sakaguchi *et al.* 2013, Li *et al.* 2014). Vitamin D treated T-cells secrete significantly lower levels of pro-inflammatory cytokines which likely contribute to the increase in FOXP3 expression. Modern techniques allow to track Foxp3 expression in real time and has demonstrated a dynamic profile of FoxP3 transcription. While during inflammation the rate of Foxp3 transcription was increased, it

was reduced in non-inflammatory conditions (Bending *et al.* 2018). In the case of *in vitro* activated T-cells both of the above statements can apply as TCR stimulation is provided which triggers FOXP3 expression and in combination with reduced pro-inflammatory cytokines might has a synergistic effect on FOXP3 expression. Larger proteins (~ >40kDa) require active transport for nucleocytoplasmic transport due to size limitation of nuclear pores (Nigg 1997). The FOXP3 protein has several nuclear import signals which are required for signal-mediated transport across the nuclear envelope (Lopes *et al.* 2006, Hancock and Ozkaynak 2009, Magg *et al.* 2012). To exclude the possibility that increased nuclear FOXP3 in Vitamin D treated T-cells was due to nuclear import, the cytoplasmic fraction was probed for FOXP3 expression and no significant changes between Vitamin D-treated and non-Vitamin D treated T-cells could be measured, further strengthen the hypothesis that Vitamin D treatment results in increased FOXP3 expression and it is possible that the *de novo* FOXP3 protein is preferentially imported to the nucleus.

Of note, low levels of Vitamin D are found in patients with autoimmune diseases including rheumatoid arthritis and SLE, suggesting a role of Vitamin D in self-tolerance and autoimmunity (Muller *et al.* 1995, Huisman *et al.* 2001, Arnson *et al.* 2007, Kerr *et al.* 2011).

It is promising that Vitamin D significantly inhibits IFN-γ as well as IL-17A. Murine models of alloHSCT have demonstrated that inhibition of Th1 and Th17 cell differentiation significantly reduce the severity of acute and chronic GvHD (Wu *et al.* 2015). Inhibiting both Th1 and Th17 populations will be more effective in controlling GvHD. Furthermore, FOXP3 expression was significantly upregulated in Vitamin D treated activated T-cells, suggesting a strong autoimmune inhibitory potential. In combination with elevated Treg numbers, Atorvastatin and Vitamin D, could prove to be an effective strategy to attenuate GvHD pathology in the recipient. In addition, the drug combination could be used to boost

Treg numbers in the donor and graft due to its non-toxic and proven safety profile without any expected adverse effects on the donor.

CHAPTER 10

Immunosuppressive Effect of Atorvastatin and Vitamin D on CD4+ T-cells and Monocyte-derived Dendritic Cells

10. Immunosuppressive Effect of Atorvastatin and Vitamin D on CD4+ T-cells and Monocyte-derived Dendritic Cells

Expanding pathogenic donor T-cells have been identified as the main mediators of (acute) GvHD pathology. Their suppression, either directly or through other cells such as Tregs, is indispensable in ameliorating GvHD. A reliable assay to estimate the suppressive capacity of Tregs or drug treatments such as Atorvastatin and Vitamin D is the *in vitro* suppression assay in which autologous Teffector cells are co-cultured with Tregs. Dendritic cells also regulate T-cell proliferation and Treg function. Their suppressive ability can be estimated in an *in vitro* mixed lymphocyte reaction in which allogeneic DCs are co-cultured with autologous T-cells. This chapter explores the immunosuppressive effects of Atorvastatin and Vitamin D on CD4+ T-cells and monocyte-derived dendritic cells using both assays. Previous studies have reported that these drug have anti-inflammatory properties, however, others have reported that Atorvastatin, at least at high concentrations, deteriorates Treg suppressive capacity.

10.1 Suppression Assay with Atorvastatin and Vitamin D

Host APC interaction with donor T-cells is key in initiating GvHD. Expanding donor T-cells then drive GvHD principally through differentiation into pathogenic effector T-cells, therefore, limiting T-cell proliferation is essential in ameliorating pathologies associated with immune cell grafts. In this chapter, the capacity of Atorvastatin and Vitamin D to suppress T-cell proliferation in isolated CD4+CD25- Teffector cells were assessed *in vitro* by increasing concentration of Atorvastatin (50nM - 10μM) and 100nM Vitamin D (calcitriol) in the presence and absence of Tregs.

Atorvastatin induced Teffector suppression in a dose-dependent manner from no additional suppression measured at the nano molar concentrations to excellent suppression at the low micro molar concentrations (Fig. 20A-C; for equations see 7.4.6.4). Incubation of Atorvastatin with Tregs resulted in a significant additional suppression at 5 and 10μM (10.8 ± 21.9% and 34.3 ± 22.8% respectively; mean ± SD) highlighting the importance of Treg function in Teffector suppression. However, at such high concentrations of Atorvastatin, Teffector proliferation is suppressed to such an extent that Tregs become redundant (Fig. 20A). This becomes apparent as significant Teffector suppression is observed at 2.5, 5 and 10μM Atorvastatin in the absence of Tregs (11.3 ± 16.3%, 37.5 ± 25.4% and 60.4 ± 21.3% respectively; mean ± SD) (Fig. 20C). Vitamin D induced a steady increase in Teffector suppression, although, in the presence of high doses of Atorvastatin, it becomes negligible (Fig. 20D + E). Regardless, it is important to note that Atorvastatin and Vitamin D have a small additive effect on Teffector suppression, especially at lower Atorvastatin concentrations (Fig. 20A).

To determine if the effect of Atorvastatin is mediated through inhibition of the mevalonate pathway, 1mM mevalonic acid was added to the culture, to circumvent the inhibition of HMA-CoA reductase by Atorvastatin and subsequent inhibition of mevalonate. As expected, mevalonic acid reversed the suppression induced by Atorvastatin completely and had no adverse effects on suppression (Fig. 20F + G). Importantly, mevalonic acid

could not reverse the suppressive effect of Vitamin D nor affected suppression mediated by Tregs suggesting that Vitamin D suppresses through an alternative manner than inhibition of the mevalonate pathway.

Atorvastatin is a potent suppressor of CD4+CD25- Teffector proliferation at micro molar concentrations but had no effect at nano molar and low micro molar (≤2.5μM) concentrations, while physiological levels of Vitamin D also inhibited Teffector proliferation, albeit to a lower extent than Atorvastatin. However, the combination of Atorvastatin and Vitamin D could prove to be of value in a clinical setting, where Atorvastatin is used at nano molar concentrations. As expected, Tregs play an important role in the suppression of Teffector proliferation, especially, at more physiologically relevant Atorvastatin levels but become redundant at higher concentrations. In support of this view, it is estimated that the highest acceptable dose of Atorvastatin given to patients, 80mg, results in around 80-100nM mean serum concentration (Bjorkhem-Bergman *et al.* 2011). Therefore, it is very likely that Treg suppression is indispensable to control Teffector proliferation in a clinical setting and Atorvastatin and Vitamin D further limit proliferation and might reduce donor T-cell expansion and ameliorate GvHD.

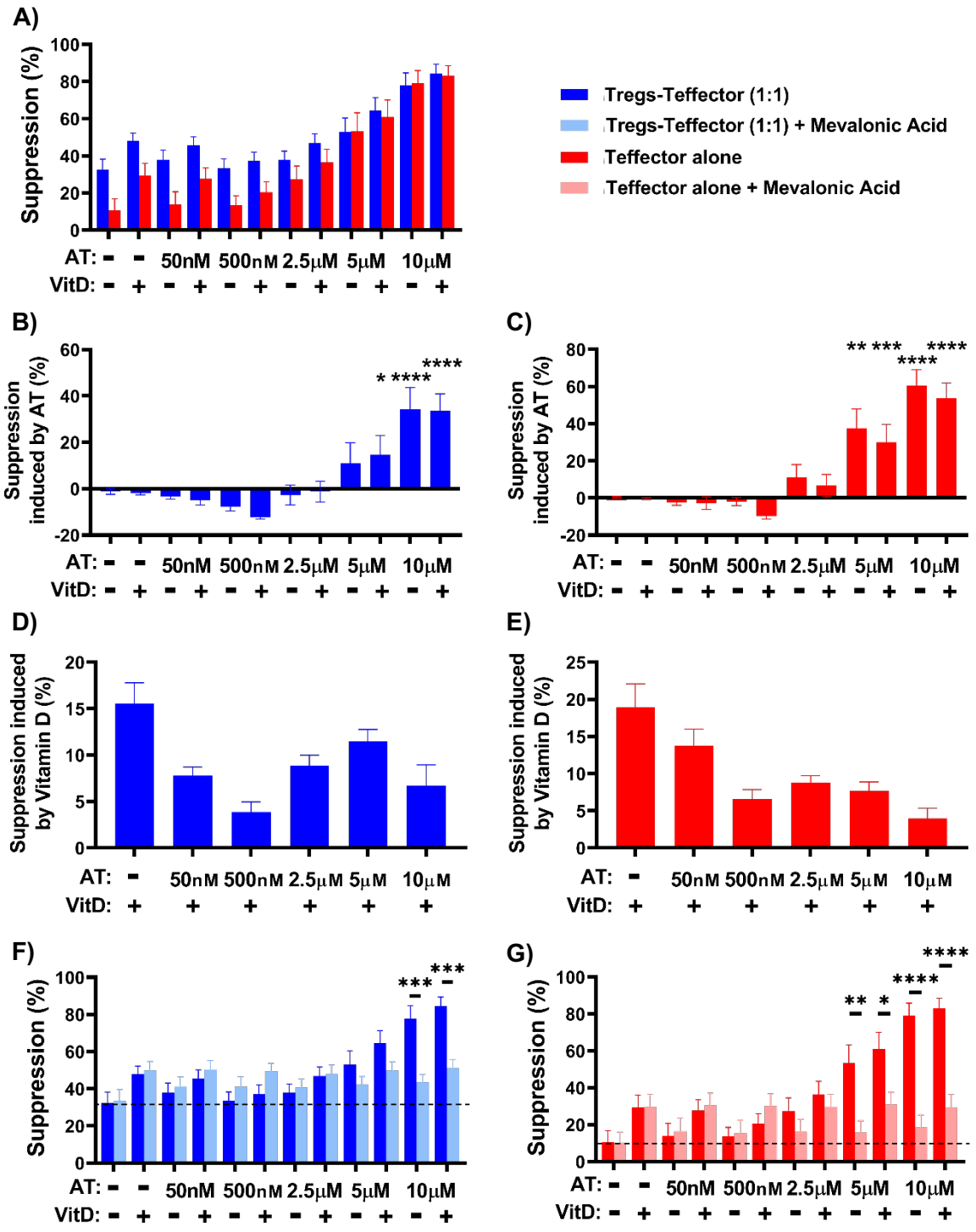


Fig 20. *In vitro* Suppression Assay with Atorvastatin and Vitamin D.
(A) Treg suppression assay in comparison to Teffector alone. (B + C) Suppression induced by Atorvastatin. (D + E) Suppression induced by Vitamin D (Calcitriol). (F + G) Suppression assay with Mevalonic Acid to reverse the effect of Atorvastatin.
n=6 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

10.2 Mixed Lymphocyte Reaction with Atorvastatin and Vitamin D

10.2.1 Monocyte-derived DCs incubated with Atorvastatin and Vitamin D

Dendritic cells (DCs) are professional antigen presenting cells essential in mounting a targeted and effective T-cell immune response. Preclinical and clinical studies have demonstrated the central role of DCs in GvHD. While some DCs induce alloreactive T-cell responses that mediate host tissue damage, other DC subsets induce donor T-cell tolerance against the host tissue (Yu *et al.* 2019). In the setting of alloHSCT, host-derived DCs are essential in donor T-cell mediated GvHD (Shlomchik *et al.* 1999). Furthermore, while Atorvastatin and Vitamin D treatment was unable to induce Tregs in isolated CD4+ T-cells, an increase in Tregs could be detected in PBMCs (see 9.1) which indicates that other cell types such as DCs are critical in resolving disease pathology.

Initial studies in this chapter determined the effect of Atorvastatin and Vitamin D on CD4+CD25- Teffector proliferation in an *in vitro* suppression assay and found that Teffector proliferation could be fully inhibited by Atorvastatin at low micromolar concentrations (see 10.1). To further investigate the effect of Atorvastatin and Vitamin D on T-cell proliferation, anti-CD3/CD28 stimulated CD4+CD25- Teffector cells, in the presence or absence of Tregs, were incubated with monocyte-derived dendritic cells. A lower concentration of Atorvastatin (50, 250 and 500nM) was chosen to model a more physiological range and 100nM Calcitriol was used as in previous experiments.

A pure population of CD14+ monocytes (>95%) were differentiated into monocyte-derived dendritic cells (Fig. 21A) and matured by LPS and IFN-γ. Monocyte-derived DCs used in these assays were viable (Fig. 21C) and expressed classical DC lineage markers HLA-DR+CD11c+ (Fig. 21D). MoDC were phenotyped by flow cytometry to confirm their maturation status in comparison to control immature moDC (Fig. 21E + F), while isolated

Tregs (CD4+CD25highCD127dim) were also phenotyped to confirm purity of the Treg population (Fig. 21B).

Incubation of matured moDC with Tregs-Teffector (1:1) led to a significant increase in Teffector suppression in comparison without moDC (Fig. 21G). To estimate maximum possible induced suppression by moDC immature moDC were used as a reference. It was then determined if Atorvastatin and/or Vitamin D could potentiate the effect of moDC on Teffector proliferation. Indeed, addition of 100nM Vitamin D induced additional Teffector suppression in comparison to untreated cultures but did not reach statistical significance, while Atorvastatin had little to no effect on suppression. Furthermore, no additive effect could be detected between Atorvastatin and Vitamin D treatment. Nonetheless, moDC significantly increased Teffector suppression, while addition of Vitamin D to Tregs-Teffector (1:1) + moDC had a small but non-significant effect on suppression.

Frequency of Tregs can vary significantly between individuals, in light of this, it is important to establish if significant T-cell suppression can be induced in the absence of Tregs. This can be achieved by direct inhibition of CD4+CD25- Teffector cells or by boosting suppressive capacity of other cell types. Matured moDC were incubated with CD4+CD25- Teffector cells but were unable to mediate significant suppression (Fig. 21H). However, incubation of Teffector cells with moDC and Vitamin D caused significantly increased suppression, while Atorvastatin had a small but non-significant effect (Fig 21H). The combination of 100nM Vitamin D and 50nM Atorvastatin with moDCs induced a small additive, but statistical non-significant, suppressive effect. Interestingly, Vitamin D alone or in combination with Atorvastatin induced a higher suppression on Teffector proliferation than immature moDC.

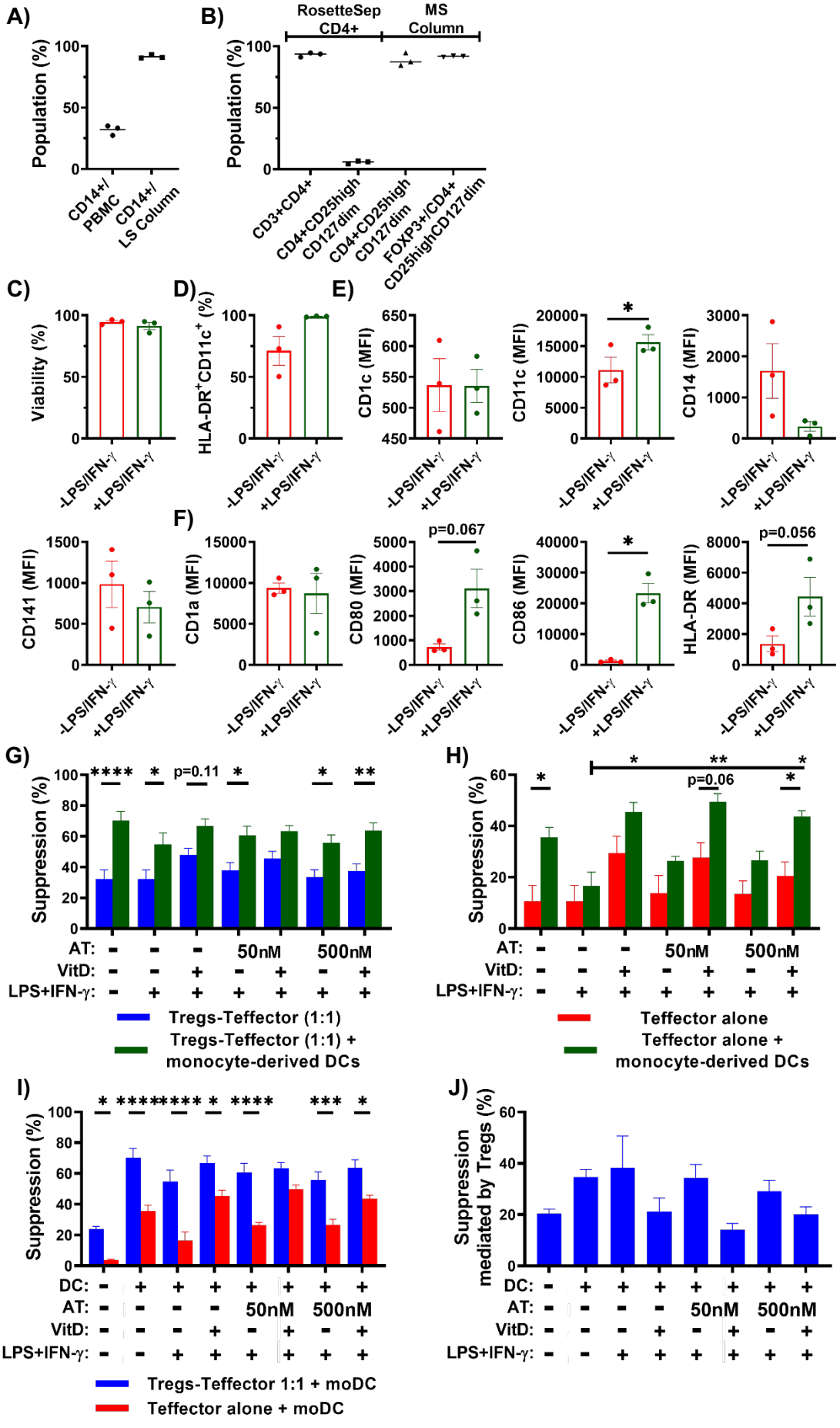
The ability of Tregs to inhibit CD4+CD25- Teffector proliferation is of great therapeutic potential and interventions that can improve Treg function would be beneficial in combating GvHD. To estimate if Treg suppressive capacity can be improved either by moDC or directly through treatment with Atorvastatin and Vitamin D, Tregs-Teffector

(1:1) and Teffector alone were incubated with moDC and compared for each treatment group (Fig. 21I). Whereas for Tregs-Teffector the same level of suppression was reached for each treatment, moDC incubated with Teffector in the absence of Tregs exhibited higher levels of suppression when Vitamin D was present. Indeed, Treg suppressive capacity can be improved by immature, as well as mature, moDC (34.7 ± 5.9 % and 38.2 ± 24.7 %, respectively; mean ± SD). Treatment of Vitamin D reduces reliance on Treg suppression which can be further reduced when Atorvastatin is added (Fig. 21J).

Importantly, this assay used matured monocyte-derived dendritic cells that expressed high levels of cell-surface co-stimulatory molecules CD80 and CD86 as well as HLA-DR which drive T-cell activation and proliferation (see 11.1 for more details). Regardless, moDC mediated Teffector suppression was slightly elevated by Atorvastatin and Vitamin D. This indicates that other mechanism, such as secretion of anti-inflammatory cytokines, might mediate the suppression or other surface proteins are responsible for the suppression. Combining moDC with Tregs further increased suppression and Tregs were indispensable to achieve maximum suppression. Overall, dendritic cells play an important role in shaping a T-cell response and 100nM Vitamin D and physiological levels of Atorvastatin increased moDC mediated suppression.

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Fig 21. Mixed Lymphocyte Reaction with Atorvastatin and Vitamin D.
(A) Purity of isolated CD14+ monocytes used for differentiation of moDC. (B) Purity of isolated CD4+ T-cells (CD3+CD4+) from leukocyte cones. Percentage of CD4+CD25highCD127dim Tregs in isolated CD4+ T-cells. Purity of isolated Tregs. FOXP3 expression of isolated Tregs. (C) Viability of moDC. (D) Percentage of moDC expressing classical DC lineage markers HLA-DR+CD11c+. (E) MFI of lineage markers. (F) MFI of maturation markers. (G-H) Comparison of *in vitro* suppression assay to MLR. (I) Comparison of suppression induced by Tregs-Teffector (1:1) + moDC against Teffector alone + moDC. (J) Estimation of Treg mediated suppression which was estimated by calculating the difference in suppression between Tregs-Teffector (1:1) + moDC and Teffector alone + moDC.
n=3 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.


A)
Population (%)
CD14+/ PBMC
CD14+/ LS Column
B)
RosetteSep CD4+
MS Column
Population (%)
CD3+CD4+
CD4+CD25high CD127dim
CD4+CD25high CD127dim
FOXP3+/CD4+ CD25highCD127dim
C)
Viability (%)
D)
HLA-DR+CD11c+ (%)
E)
CD1c (MFI)
CD11c (MFI)
CD14 (MFI)
-LPS/IFN-γ
+LPS/IFN-γ
F)
CD141 (MFI)
CD1a (MFI)
CD80 (MFI)
p=0.067
CD86 (MFI)
HLA-DR (MFI)
p=0.056
G)
Suppression (%)
p=0.11
AT:
VitD:
LPS+IFN-γ:
50nM
500nM
Tregs-Teffector (1:1)
Tregs-Teffector (1:1) + monocyte-derived DCs
H)
p=0.06
Teffector alone
Teffector alone + monocyte-derived DCs
I)
DC:
Tregs-Teffector 1:1 + moDC
Teffector alone + moDC
J)
Suppression mediated by Tregs (%)

10.2.2 Atorvastatin and Vitamin D differentiated Monocyte-derived Dendritic Cells

Monocyte-derived dendritic cells can induce considerable Teffector suppression and Atorvastatin and Vitamin D can further elevate suppression (see 10.1). In addition, differentiation of monocytes with Atorvastatin and Vitamin D can induce a tolerogenic dendritic cell phenotype (see 11.1). To test if this tolerogenic phenotype is translated into suppression of Teffector proliferation, Atorvastatin and/or Vitamin D differentiated monocyte-derived dendritic cells were incubated with anti-CD3/CD28 stimulated Teffector cells in the presence and absence of Tregs.

Phenotypic analysis of differentiated moDC confirmed their maturation status (Fig. 22). Vitamin D treatment induced a shift in lineage marker expression by inhibiting CD1c and significant upregulation of CD141 expression. In addition, co-stimulatory marker CD80 were slightly but statistical significantly reduced by Atorvastatin treatment, whereas the combination of Atorvastatin and Vitamin D treatment did not reach statistical significance. However, in comparison to the phenotype of moDC in initial experiments conducted in June/July (see 11.1), moDC differentiated in January/February expressed higher levels of CD80 and CD86 as well as HLA-DR despite identical isolation technique and treatment method. In line with previous (summer) experiments, expression of CD1a was fully inhibited by Vitamin D treatment.

Nonetheless, incubation of these moDC with Tregs-Teffector (1:1) induced significant Teffector suppression compared to no moDC (Fig. 23E). Interestingly, despite their mature phenotype, induced suppression is comparable to the positive control of immature moDC (86.1 ± 5.1 % and 67 ± 6.4 %; mean ± SD; p=0.25). Differentiation of moDC with Atorvastatin and/or Vitamin D has no beneficial effect on moDC capacity to suppress Teffector cells (Fig. 23F).

To estimate the suppressive effect mediated by Tregs, Teffector cells incubated in the presence of Tregs and moDCs were compared to Teffectors incubated with moDCs but

in the absence of Tregs (Fig. 23F). Despite moDC having a mature phenotype, and no Atorvastatin and/or Vitamin D present to aid in Teffector inhibition, suppression was comparable to the previous assay (see 10.2.1). Monocyte-derived dendritic cells appear to increase Treg suppressive capacity (Fig. 23G + H). Differentiation of moDC with Vitamin D reduced dependency of Treg mediated suppression as it increased suppression mediated by moDC.

In conclusion, matured moDCs can significantly induce suppression of CD4+CD25- Teffector proliferation. Incubation of moDC with physiological relevant concentrations of Atorvastatin and 100nM Vitamin D slightly increased suppression and appeared to have a direct effect on Teffector cells rather than Treg mediated inhibition as significant suppression was also detected in the absence of Tregs. On the other hand, differentiation of moDC with Atorvastatin and Vitamin D induced comparable suppression on Teffector cells but it appeared to be mostly mediated by enhancing Treg suppressive capacity as Teffector proliferation could not be inhibited by moDC in the absence of Tregs. Atorvastatin has a small effect on Teffector suppression which is mostly noticeable when directly added to the culture media. In contrast, Vitamin D has a considerable and consistent effect on suppression in both treatment conditions. Dendritic cells are essential in anti-inflammatory responses and their inhibitory potential of activated CD4+CD25- Teffector cells is demonstrated in this chapter. Direct incubation of Teffector cells and moDCs with Atorvastatin and Vitamin D appeared to be more effective rather than indirectly through differentiation of moDC with these drugs as Teffector cells could be significantly suppressed in the absence of Tregs by Atorvastatin and Vitamin D and this effect was further potentiated by moDCs. Nonetheless, Tregs are crucial in the inhibition of Teffector proliferation and Vitamin D, and to a lesser extent Atorvastatin, can enhance this inhibition. In addition, DCs not only modulate T-cells responses by activating or repressing T-cell proliferation but also by inducing Tregs from naïve CD4+ T-cells or by boosting Treg suppressive function.

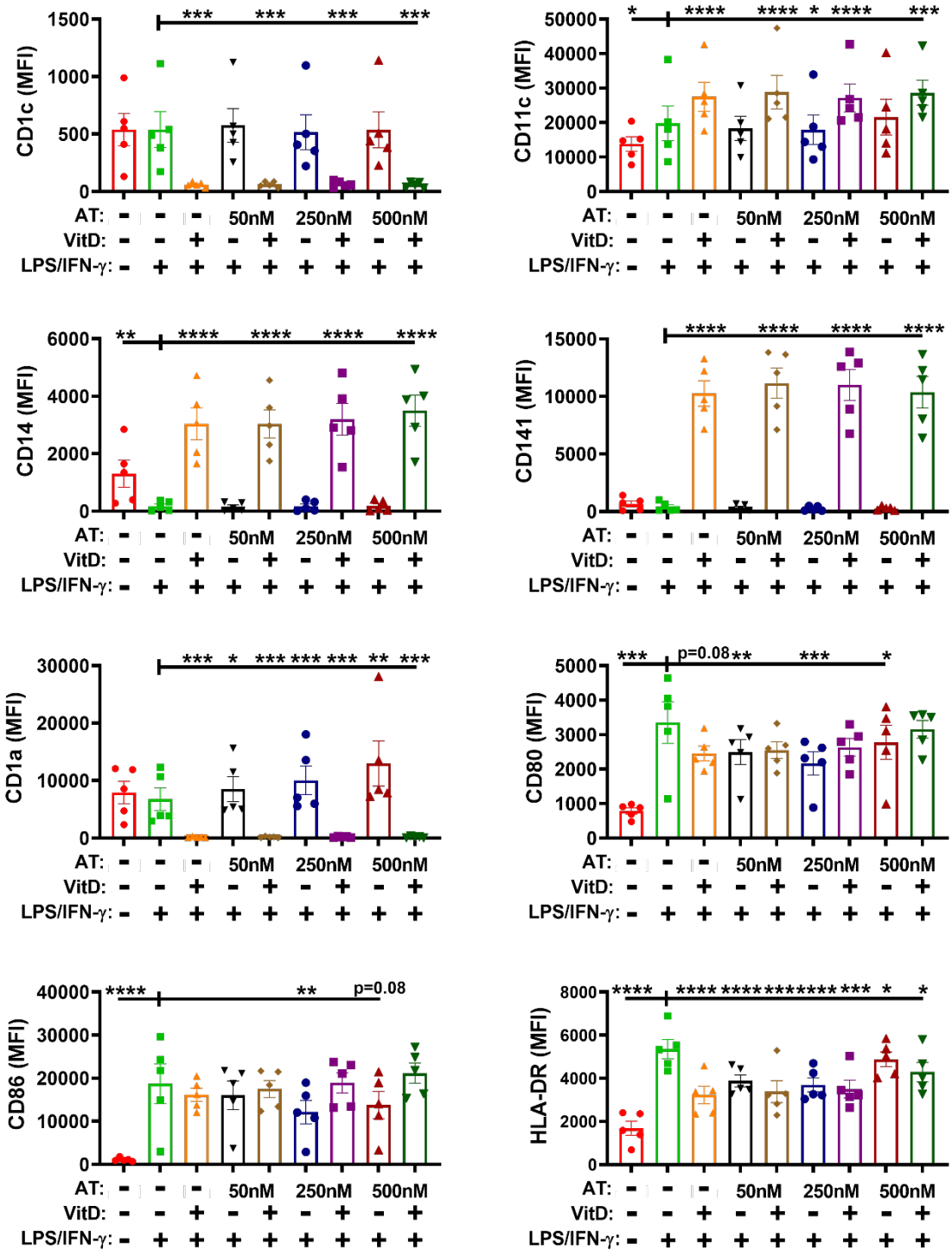


Fig 22. Phenotypic Analysis of Monocyte-derived Dendritic Cells used in MLR. CD14+ monocytes were differentiated with Atorvastatin and/or 100nM Vitamin D and matured with LPS and IFN-γ. Phenotype of monocyte-derived dendritic cells were analysed by flow cytometry.

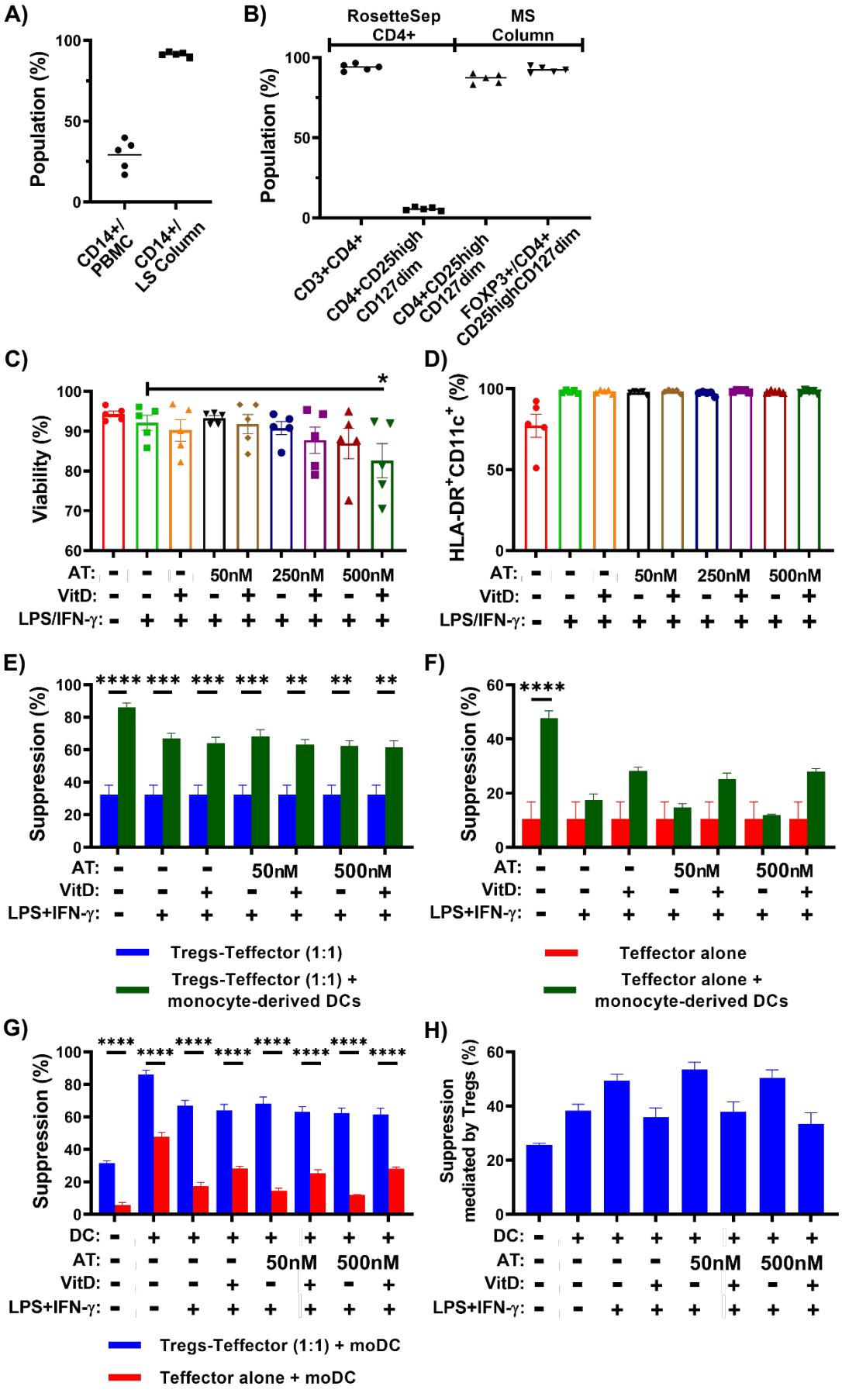
n=5 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and

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Fig 23. Mixed Lymphocyte Reaction with Atorvastatin and Vitamin D differentiated Monocyte-derived Dendritic Cells.

(A) Purity of isolated CD14+ monocytes used for differentiation of moDC. (B) Purity of isolated CD4+ T-cells (CD3+CD4+) from leukocyte cones. Percentage of CD4+CD25highCD127dim Tregs in isolated CD4+ T-cells. Purity of isolated Tregs and FOXP3 expression of isolated Tregs. (C) Viability of moDC. (D) Percentage of moDC expressing classical DC lineage markers HLA-DR+CD11c+. (E + F) Comparison of *in vitro* suppression assay to MLR. (G) Comparison of suppression induced by Tregs-Teffector (1:1) + moDC against Teffector alone + moDC. (H) Estimation of Treg mediated suppression which was estimated by calculating the difference in suppression between Tregs-Teffector (1:1) + moDC and Teffector alone + moDC.

n=5 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.


A)
Population (%)
CD14+/ PBMC
CD14+/ LS Column
B)
RosetteSep CD4+
MS Column
Population (%)
CD3+CD4+
CD4+CD25high CD127dim
CD4+CD25high CD127dim
FOXP3+/CD4+ CD25highCD127dim
C)
Viability (%)
AT:
VitD:
LPS/IFN-γ:
50nM
250nM
500nM
D)
HLA-DR+CD11c+ (%)
E)
Suppression (%)
Tregs-Teffector (1:1)
Tregs-Teffector (1:1) + monocyte-derived DCs
F)
Suppression (%)
Teffector alone
Teffector alone + monocyte-derived DCs
G)
Suppression (%)
DC:
Tregs-Teffector (1:1) + moDC
Teffector alone + moDC
H)
Suppression mediated by Tregs (%)

10.3 Discussion

As demonstrated in this chapter Atorvastatin efficiently inhibits anti-CD3/CD28 stimulated CD4+CD25- Teffector cells at the micromolar range (5-10μM) in the presence or absence of Tregs. These findings indicate that at high micromolar concentrations of Atorvastatin Tregs are not required to achieve Teffector suppression and directly inhibit Teffector cells. However, in the physiological relevant nanomolar range (50-500nM) Atorvastatin did not induce any significant additional suppression and Tregs were necessary to significantly decrease Teffector proliferation. Atorvastatin doses given in a clinical setting will reach a maximum mean serum concentration in the low nanomolar range (~80-100nM). Of note, 50nM Atorvastatin induced higher suppression levels than 500nM and 2.5μM. One possible explanation could be that 50nM boost Treg function whereas higher concentrations have adverse effects on Treg fucntion as Rodriguez-Perea *et al.* (2019) has reported.

Vitamin D induced a steady increase in suppression (~10-20%) in the presence or absence of Tregs which demonstrates a direct inhibitory effect on Teffector cells but doesn't exclude the possibility that Vitamin D also boost Treg function. A direct effect might prove to be of therapeutic value when Tregs are sparse but peripheral tolerance is needed to control GvHD pathology. Danby *et al.* (2016) reported that there is considerable difference in Treg content within the graft (range, 0.81-8.56% of CD4+ T-cells) and that a higher proportion is associated with reduced occurrence of aGvHD and increased survival.

The combination of Atorvastatin and Vitamin D has a small and non-significant additive inhibitory effect. Nonetheless, in the physiological nanomolar range the combination can provide additional suppression and in the presence of Tregs might be effective in controlling GvHD.

Dendritic cells (DCs) are pivotal in instructing T-cell responses and in mediating GvHD and consequently, play a non-redundant role in resolving GvHD. Host DCs have been

demonstrated to be critical to initiate GvHD and that donor DC can intensify GvHD (Shlomchik *et al.* 1999, Matte *et al.* 2004). Preparative conditioning for alloHCST and inflammatory conditions lead to the secretion of pro-inflammatory cytokines (e.g. IL-12) and up-regulation of receptors (e.g. CD80 and CD86) by dendritic cells which drive expansion and differentiation of antigen-activated T-cells (Yu *et al.* 2019). Furthermore, monocytes play a predominant role in GvHD by inducing pro-inflammatory subsets such as Th17 cells (Reinhardt-Heller *et al.* 2018). It is therefore crucial to modulate dendritic cell ability by inducing a tolerogenic phenotype to inhibit T-cell proliferation and differentiation.

Incubation of matured monocyte-derived dendritic cells (moDCs), activated by LPS and IFN-γ, with Tregs and Teffector cells significantly increased Teffector suppression. Addition of 100nM Vitamin D or 50nM to the culture media further increased Teffector suppression. Interestingly, in the absence of Tregs incubation of Teffector cells with immature moDCs resulted in an significant increase in suppression however incubation with matured moDCs and Vitamin D results in even greater increase in suppression highlighting the immunosuppressive ability of Vitamin D (35±7.8% and 45±5%, respectively; mean ± SD). Incubation of 50nM Atorvastatin also increased suppression albeit lower than Vitamin D whereas the combination had a small additive effect (49±5%; mean ± SD). From this data it cannot to be derived if the increase in suppression is mediated by boosting dendritic cell function or directly by inhibiting Teffector proliferation. For example, dendritic cells could acquire a tolerogenic phenotype by downregulating CD80/CD86 which interacts with CD28 to drive Teffector proliferation. Dendritic cells could also secrete anti-inflammatory cytokine such as IL-10 to inhibit Teffector proliferation. On the other hand, as demonstrated previously, Vitamin D can directly inhibit Teffector proliferation.

Consequently, the hypothesises that differentiation of monocytes with Atorvastatin and Vitamin D leads to a tolerogenic dendritic cell phenotype and this results in increased suppression capacity was tested in this chapter. However no measurable increase in

suppression with Atorvastatin was detected but with Vitamin D treatment. The increase in suppression by Vitamin D differentiated moDCs was only detectable when incubated with Teffector cells in the absence of Tregs while in the presence of Tregs no increase could be measured. One possible explanation could be that in a high suppressive environment the additional suppression provided by these moDCs is too small to detect. In this current chapter we have incubated distinct immune cell types that are known to contribute to GvHD, specifically Tregs, Teffectors and DCs. However, it is important to note that, although useful, *in vitro* experiments are limited and do not fully recapitulate the cellular environment within a graft or recipient. In light of this, other immune and non-immune cells that contribute to GvHD are not present but could contribute to graft rejection or host tissue damage through generation of pro-inflammatory cytokines or other means.

Physiological level Atorvastatin and Vitamin D led to enhanced, but non-significant increase in suppression of anti-CD3/CD28 stimulated Teffector cells in the presence of Tregs. Interestingly, the drug combination had a greater impact in the absence of Tregs indicating it has a direct effect on Teffector proliferation. Dendritic cells can significantly enhance suppression and Vitamin D can elevate this effect. Taken together, Atorvastatin and Vitamin D can limit pathogenic CD4+ T-cell proliferation and consequently make it a suitable therapeutic option for the treatment of GvHD.

CHAPTER 11

Modulation of Monocyte-derived Dendritic Cells by Atorvastatin and Vitamin D

11. Characterisation of Monocyte-derived Dendritic Cells by Atorvastatin and Vitamin D

Dendritic cells were discovered many decades ago but were met with scepticism that these cells should have a unique role in the immune system. Now it is fundamentally accepted that dendritic cells are specialized antigen presenting cells that orchestrate adaptive immunity to foreign antigens. Also, it becomes more evident that their contribution of tolerance to self-antigens is critical. As a result, modulating dendritic cells is likely to be of therapeutic benefit in many suppressive therapies including autoimmune diseases and GvHD. Dendritic cells develop in a multi-step process from the bone marrow and their frequency of total leukocytes in the periphery is very low (<1%). To be able to study dendritic cells in this chapter, monocyte-derived dendritic cells were used which have dendritic cell like properties. This chapter explores the ability of Atorvastatin and Vitamin D to modulate dendritic cell differentiation on a transcriptional and translational level. Host antigen presentation to donor T-cells by dendritic cells is a key event in the induction of GvHD. Consequently, modulation of their antigen presentation machinery, including MHC class II, co-stimulatory molecules, and pro-inflammatory cytokines would be of high therapeutic value.

11.1 Characterisation of Atorvastatin and Vitamin D treated Monocyte-derived Dendritic Cells

Human CD14+ monocytes were differentiated with varying concentrations of Atorvastatin or in combination with 100nM Vitamin D (Calcitriol). After 6 days of differentiation, moDC were matured with the bacterial derivative LPS and the pro-inflammatory stimulus IFN-γ to model extracellular as well as intracellular derived stimuli.

Atorvastatin alone, as well as in combination with Vitamin D, does not compromise the viability of moDC except for at the highest Atorvastatin concentration (Fig. 24A). Therefore, subsequent experiments used up to 500nM Atorvastatin. Whereas 1µM Atorvastatin was included in the first round of experiments (June/July, n=4) it was excluded from the second round (January/February, n=5). All matured moDC expressed classical lineage markers HLA-DR+CD11c+ confirming their commitment to a dendritic cell lineage. The classical lineage marker CD11c was not affected by Atorvastatin or Vitamin D treatment. Vitamin D treatment has a profound effect on the expression of lineage markers CD1c and CD141. Whereas CD1c is profoundly downregulated, the contrary is observed for CD141 which is substantially increased in Vitamin D treated moDC irrespective of Atorvastatin treatment and concentration (Fig. 24B). In contrast, Atorvastatin did not significantly alter the expression of CD1c or CD141 suggesting that Vitamin D and Atorvastatin have distinct effects on moDC. Whereas Vitamin D drives dendritic cells into the specific CD141+ phenotype, Atorvastatin does not induce a specific phenotype but rather stimulates a heterogeneous population of single positives, CD1c+ or CD141+, as well as double-positive CD1c+CD141+ cells. Several maturation markers were assessed that are important in T-cell activation, for example, CD1a and HLA-DR which were significantly downregulated by Vitamin D treatment while Atorvastatin alone had only minimal effects on the expression levels of these markers (Fig. 24C). Particularly, CD1a expression was completely inhibited by Vitamin D, while

expression of CD80 and CD86 were, on average, mostly unaffected by Atorvastatin or Vitamin D.

It is well documented that seasonal changes can affect the immune system (Dopico *et al.* 2015, Onishi *et al.* 2020). Some experiments were conducted during the summer months of June and July (n=4) and others during the winter months of January and February (n=5). Interestingly, there are marked differences in expression for some of the assessed DC markers. The expression of lineage markers CD1c, CD11c and CD141 had a tendency to be expressed more highly in winter than in summer but did not reach statistical significance with the exception for 500nM Atorvastatin + Vitamin D (Fig. 25). This contrasts with maturation markers CD1a, CD80 and CD86 which tended to be expressed higher in winter than in summer. Whereas for CD80 and CD86 a dose-dependent decrease can be measured in the summer months in response to Atorvastatin and Vitamin D the opposite is observed in the winter with increases evident for both markers. In terms of CD86 a large seasonal difference in expression is detected with levels the highest in the winter (Fig. 25). Interestingly, the opposite trend is measured for HLA-DR where summer has higher levels of expression than winter.

A study conducted by *Lamikanra, Danby, Roberts and colleagues* investigated the seasonal change on immune cells of an English cohort and correlated them with known seasonal affected metabolites Vitamin D and cortisol (Fisher *et al.* 2019). White blood cells (WBC), lymphocytes, monocytes or neutrophils counts, measured by a haematological analyser, did not change during a one year period (January to December). Analysis of leukocyte cones used for the isolation of cells for this project for the same period in 2019 confirmed their findings. (Fig. 26). However the study observed significant changes for absolute Treg (CD3+CD25+FOXP3+CD127dim) numbers which were the lowest in January/February and November/December, but increased during March/April and peaked during July/August. Interestingly, for CD4+ Tregs the seasonal change were not as profound. Further analysis of leukocyte cones used for this project

did not find any statistically significant changes for WBC, lymphocytes, monocytes or neutrophils between summer (n=49; 2019 n= 28, 2020 n=21) and winter (n=59; 2019 n=22, 2020 n=37) (Fig. 27A). This was also confirmed by flow cytometry (summer n=9; winter n=7) as no statistically significant difference could be measured for total CD3+, conventional CD3+CD4+ and cytotoxic CD3+CD8+ T-cells but with a trend towards an increase in total CD3+ and CD3+CD4+ T-cells and a decrease in CD3+CD8+ T-cells in winter (Fig. 27B). In addition, no difference was observed for CD19+ B-cells, CD56+ NK cells and CD3+CD56+ NKT-cells. However, CD14+ monocytes were significantly reduced in winter. Furthermore, no difference was observed for CD4+ Tregs (CD25+FOXP3+CD127dim) between summer and winter (Fig. 26C). No measurement could be taken for overall CD3+ Tregs as Tregs were isolated from a pure CD4+ population rather than PBMCs.

Vitamin D levels correlated well with total sunlight hours per month with the lowest in January/February and November/December, increasing in the months from March to June and peaking in July/August. A strong relationship between Treg numbers and Vitamin D level was described in chapter 7.

The winter to summer changes in maturation marker expression of monocyte-derived dendritic cells described in this chapter could be casual factors determining seasonal Treg changes that have been previously documented (Anderson *et al.* 2009, Fisher *et al.* 2019). Tregs mediate immune homeostasis and facilitate an anti-inflammatory environment and are more prevalent in summer. Monocyte differentiated in summer express significantly lower levels of CD80 and CD86 than their counterparts in winter. It could be that monocytes isolated in summer are poised to express lower levels of activation markers such as CD86 to facilitate a more anti-inflammatory environment and this is due to seasonal rhythm. In support of this view, a clinical study in a German cohort found that over 5000 genes are significantly affected by seasonal changes including *CD86* mRNA transcripts (Dopico *et al.* 2015).

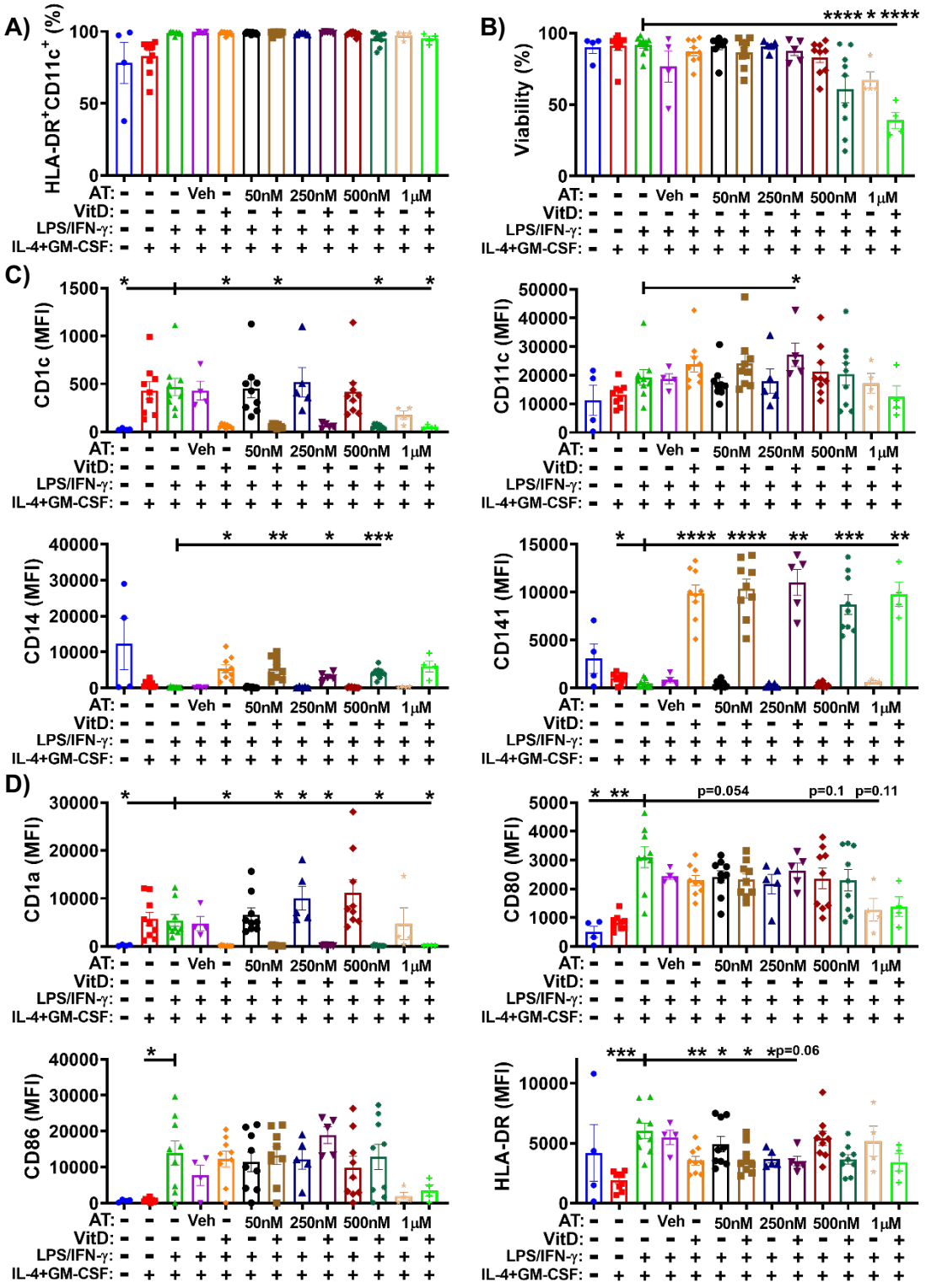


Fig 24. Phenotypic Analysis of Atorvastatin and Vitamin D differentiated Monocyte-derived Dendritic Cells.

(A) Monocyte-derived dendritic cells commit to the classical dendritic cell lineage HLA-DR+CD11c+. (B) Viability of moDC at the end of the experiment. (C + D) Expression level of lineage (C) and maturation (D) markers of analysed dendritic cells. n=9 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

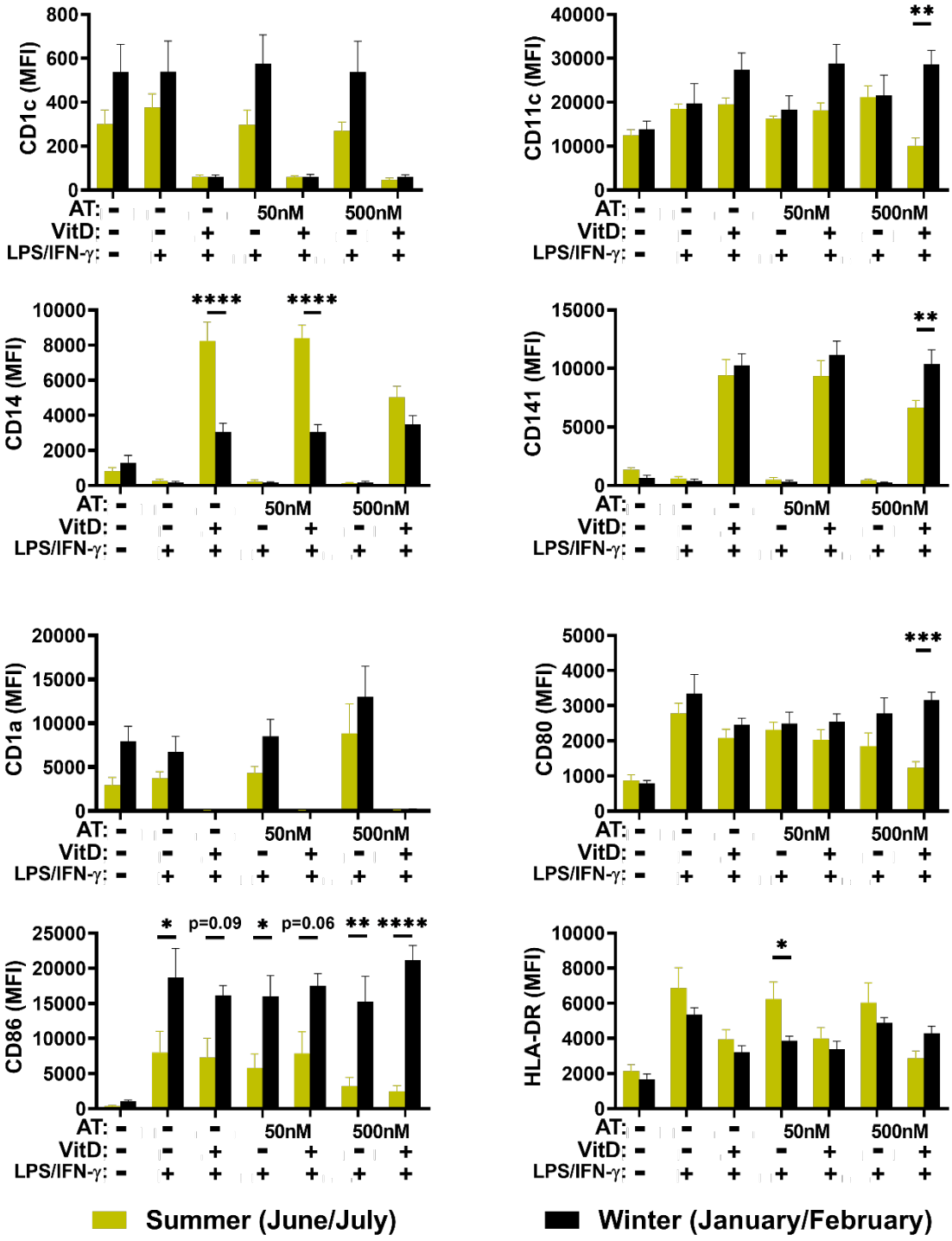


Fig 25. Phenotypic Analysis of Atorvastatin and Vitamin D differentiated monocyte-derived Dendritic Cells in Summer and Winter.

Monocyte-derived dendritic cells were cultured in summer (June/July) and winter (January/February) and their phenotype analysed by flow cytometry.

n=9 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

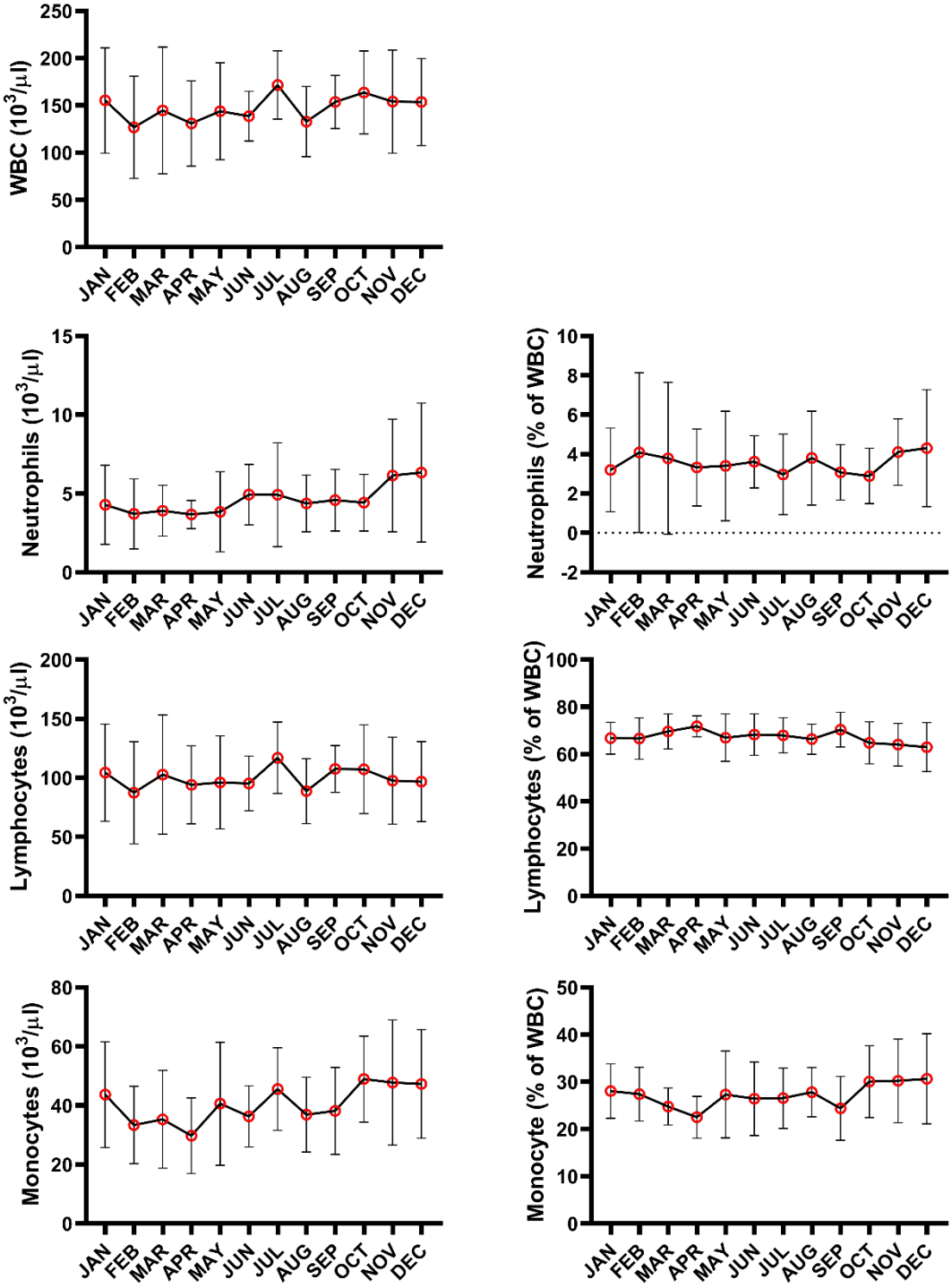


Fig 26. Blood Count Analysis of Leukocyte Cones in 2019.

Leukocyte cone blood count was analysed in 2019 (January – December) by using a Sysmex haematology analyser. January n=12, February n=10, March n=8, April n=5, May n=11, June n=10, July n=18, August n=8, September n=8, October n=16, November n=22 and December n=12

Mean ± SD; 2-way ANOVA, *=p<0.05

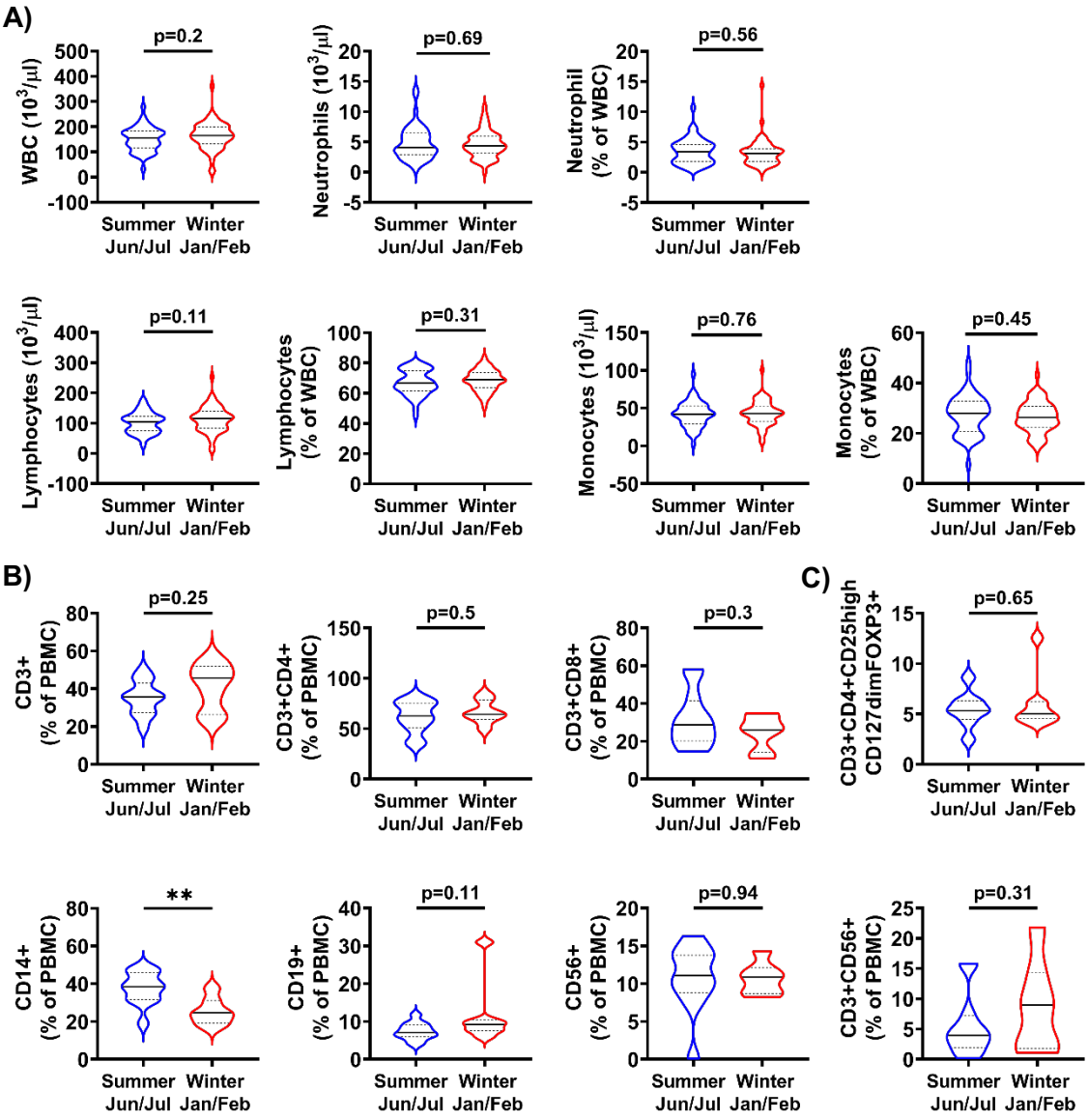


Fig 27. Blood Count and Lymphocyte Analysis of Leukocyte Cones in Summer and Winter.

(A) Leukocyte cone blood count was analysed in summer (n=49) and winter (n=59) by using a Sysmex haematology analyser. (B) Isolated PBMCs from leukocyte cones were analysed in summer (n=9) and winter (n=7) for different immune cell lineages by flow cytometry. (C) Treg content in isolated CD4+ cells in summer (n=8) and winter (n=16)

Median ± quartiles; unpaired t-test, *=p<0.05, **=p<0.002, ***=p<0.0002

11.2 Transcriptional Analysis of Atorvastatin and Vitamin D differentiated Monocyte-derived Dendritic Cells

Dendritic cells are pivotal in orchestrating a T-cell immune response through a number of distinct mechanisms including antigen presentation, secretion of anti- or pro-inflammatory cytokines and by the production of immunometabolites such as retinoic acid (RA). To determine the effect of Atorvastatin and/or Vitamin D on the expression profile of a distinct set of immune related genes qRT-PCR assays were performed. Monocyte-derived dendritic cells were differentiated with Atorvastatin and Vitamin D and RNA from these cells were used to measure transcript levels of pro-inflammatory IFNs (*IFNA2*, *IFNB1* and *IFNG*) and IL-12 (*IL-12A*) and anti-inflammatory molecules IL-10 (*IL-10*), TGF-β1 (*TGFB1*), IDO1 (*IDO1*) and CTLA-4 (*CTLA4*). In addition, transcripts involved in RA metabolism, *ALDH1A1* and *ALDH1A2*, were analysed.

Cytokines such as IL-10 or TGF-β play an important role in anti-inflammatory response of DCs and induction of Tregs (pTregs) from naïve CD4+ T-cells. In addition, versatile enzymes such as IDO have been shown to potentiate DC capacity to induce Tregs or to produce anti-inflammatory kynurenine from tryptophan to inhibit T-cell responses such as the differentiation of naïve CD4+ T-cells to Th17 cells. Dendritic cells also secrete pro-inflammatory cytokines such as IL-12 or IFNs. IFNs are a large family and can be classified into three types: Type I IFN (such as IFN-α and IFN-β), type II (IFN-γ) and type III (IFN-λ). The most intensely studied IFN is IFN-γ, which is secreted on activation by mitogens and cytokines such as IL-12 or IL-18, and plays an important role in mediating inflammation and the elimination of intracellular pathogens (Seder *et al.* 1993, Parker *et al.* 2016). IFNs type II and III are activated by DAMPs, which are key players in the initiation and maintenance of GvHD, rather than cytokines and have been shown to have important roles in homeostasis and anti-tumour immunity (Parker *et al.* 2016). Secretion of IL-12 by DCs drives the conversion of naïve CD4+ T-cells into the pro-inflammatory Th1 phenotype, a T-cell subtype that is known to drive GvHD (Heufler *et al.* 1996). One

of the main mechanisms of Treg-mediated inhibition is the engagement of CTLA-4 with molecules CD80 and CD86 on APCs. However, it has been demonstrated that monocyte-derived matured DC also express CTLA-4 and engagement of this molecule results in increased IL-10 production and decreased IL-8 and IL-12 release (Laurent *et al.* 2010).

Differentiation of monocytes into moDCs with 100nM Vitamin D induces significant upregulation of *IL-10* and *TGF-β1* (Fig. 28). Interestingly, immature moDC do not upregulate *IL-10* but have evident increases in *TGF-β1,* albeit less than matured moDC, when differentiated with Vitamin D. Atorvastatin has no effect on *IL-10* or *TGF-β1* expression. Moreover, Vitamin D significantly downregulates *IL-12A*, which encodes the rate-limiting subunit p-35 of the biologically active heterodimeric cytokine IL-12p70. IL-10 and IL-12p70 were also measured in the culture media by ELISA and correlated well with qRT-PCR data (Fig. 29). Furthermore, Vitamin D completely inhibits *IFNG* expression, while, Atorvastatin had no effect on the expression levels of either *IL-12A* or *IFNG*. Interestingly, Atorvastatin increased type I *IFNA* and *IFNB* whereas Vitamin D reduced the expression of these transcripts and inhibited this Atorvastatin induced upregulation. Expression of anti-inflammatory enzyme *IDO1* is increased by Vitamin D and, surprisingly, it is fully downregulated in immature DCs. In addition, *CTLA-4* expression is upregulated by Vitamin D, however, not every donor's monocytes responded uniformly to the treatment with some donors responding well to Vitamin D treatment whereas cells from other donors do not.

Atorvastatin, at the concentration tested, has no effect on the gene expression of moDC. This is in contrast to Vitamin D which induces a strong tolerogenic phenotype indicated by the downregulation of many pro-inflammatory genes and up-regulation of genes encoding anti-inflammatory cytokines and enzymes.

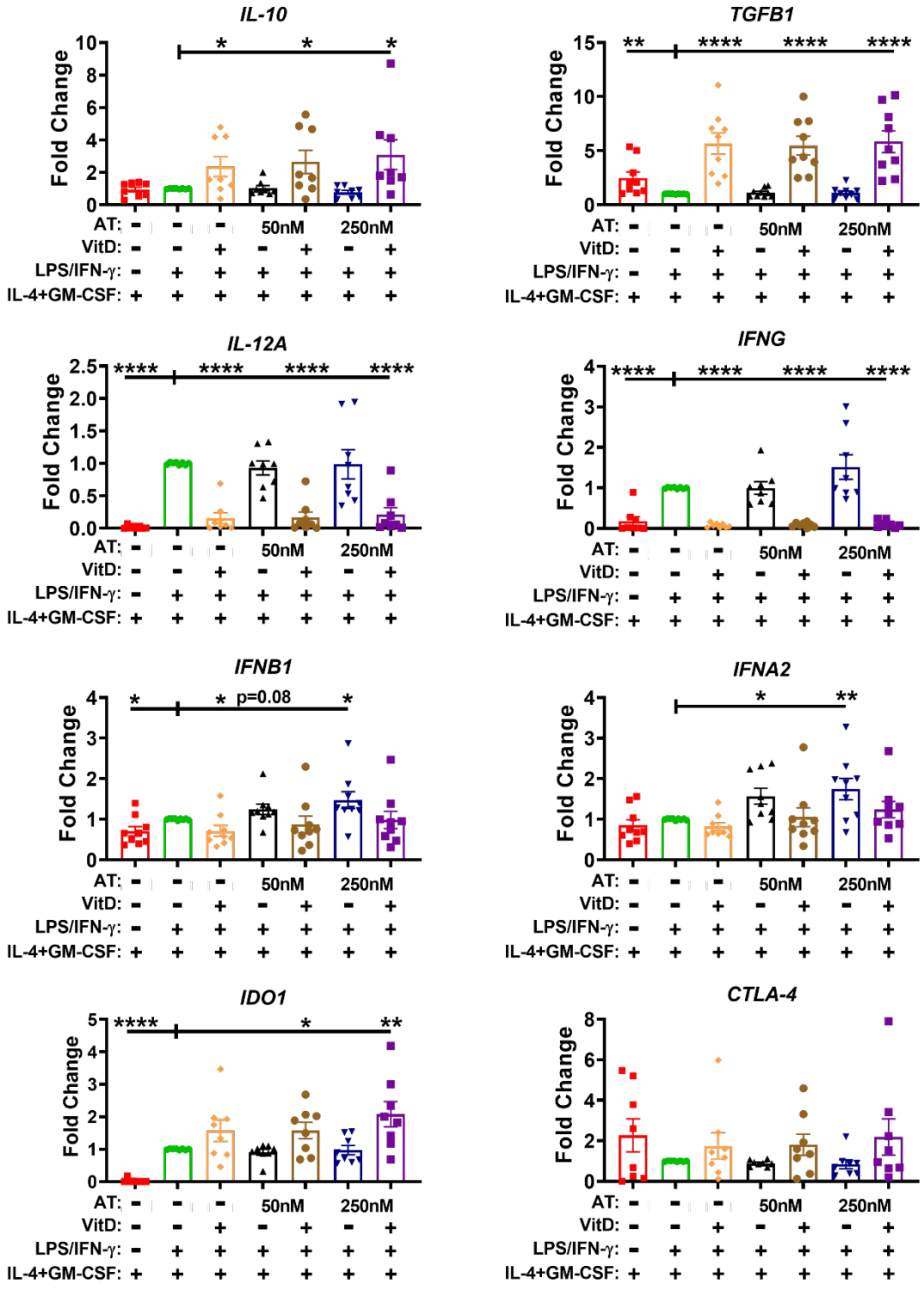


Fig 28. RT-qPCR on Monocyte-derived Dendritic Cells differentiated with Atorvastatin and 100nM Vitamin D.

Monocytes were differentiated with GM-CSF and IL-4 in the presence of Atorvastatin and/or 100nM Vitamin D and matured with LPS and IFN-γ. RNA was then isolated and genes quantified using the Taqman Platform.

n=8 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

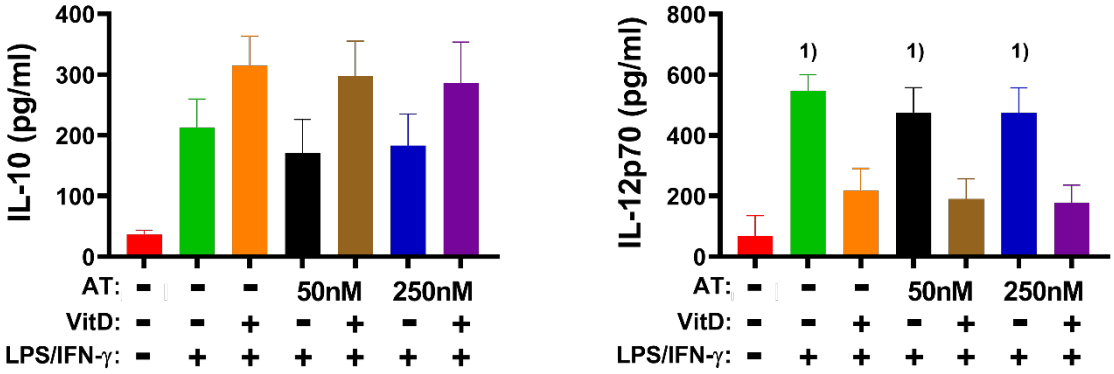


Fig 29. ELISA on Monocyte-derived Dendritic Cells differentiated with Atorvastatin and 100nM Vitamin D.

Monocytes were differentiated with GM-CSF and IL-4 and in the presence of Atorvastatin and/or 100nM Vitamin D. Culture media was collect after the moDC were matured with IFN-γ and LPS for 24h.

1) Several OD values were significantly above detection limit and where plotted as 600 pg/ml as it represent the detection limit by standard curve. See appendix 13.2 Table 1 and 2 for more information.

n=8 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

11.3 Aldehyde Dehydrogenase Activity in Atorvastatin and Vitamin D Differentiated Monocyte-derived Dendritic Cells

It has been documented that mouse bone marrow and splenic dendritic cells cultured with IL-4 and GM-CSF synergistically induce ALDH expression whereas most other cytokines such as IFN-γ or IL-10 do not (Yokota *et al.* 2009). This suggests that monocyte-derived dendritic cells resident in these tissues have higher levels of ALDH activity compared to their natural counterparts found in the blood. In the context of GvHD, ALDH has a controversial role as, on one hand, many studies have observed an increase in Tregs in the presence of *in vitro* and *in vivo* RA and a high Treg proportion to total CD4+ cells which is beneficial in ameliorating GvHD but, on the other hand, it has been demonstrated that ALDH activity and RA exacerbates GvHD pathology and mortality in preclinical studies (see 2.2).

Maturation of moDC with LPS and IFN-γ stimulation does not induce ALDH activity in comparison to immature moDC (Fig. 30A). However, differentiation of monocytes to moDC in the presence of Atorvastatin leads to a dose-dependent decrease in ALDH activity (-16.2% and -50.2%, respectively, in comparison to +LPS/INF-γ). This is in contrast to moDC differentiation in the presence of 100nM Vitamin D (Calcitriol) which results in a significant increase in ALDH activity in moDC (+202%). This finding suggests that Vitamin D activated VDR-RXR heterodimers, a potential downstream target of aldehyde dehydrogenase produced RA, play an important role in Vitamin D mediated ALDH stimulation or signalling. The combination of Atorvastatin and Vitamin D reduces ALDH activity in a dose-dependent manner, although 50 or 250nM Atorvastatin did not override Vitamin D induced ALDH activity (+183% and +112%, respectively).

Conversion of retinal to RA in humans is performed by three isozymes encoded by *ALDH1A1*, *ALDH1A2* and *ALDH1A3*. We focused on *ALDH1A1* and *ALDH1A2* as these have been reported to play a critical role in GvHD (see 2.2). While *ALDH1A2* expression is unaffected by Vitamin D treatment, *ALDH1A1* shows an increase in expression, and

although not reaching statistical significance (Fig. 30B), suggests that ALDH1A1 may be the major isozyme contributing to ALDH activity and possible RA production.

Atorvastatin and Vitamin D have opposing effects on ALDH function in moDC. Atorvastatin reduces, while Vitamin D significantly elevates, ALDH activity. In terms of the therapeutic value of using both Atorvastatin and Vitamin D in the treatment of GvHD, it could be speculated that a combination could drive GvHD rather than ameliorate it. It has been shown that DC ALDH activity and RA production is critical in the induction of GvHD, although it is plausible that low ALDH activity could be beneficial by inducing Tregs but not sufficiently high to drive GvHD. However, as discussed in section 5.2, there is currently no evidence that Vitamin D exacerbates GvHD but is rather beneficial in the treatment of it. These observations support the notion that Vitamin D can stimulate Tregs through a ALDH dependent mechanism that could be important in successful engraftments.

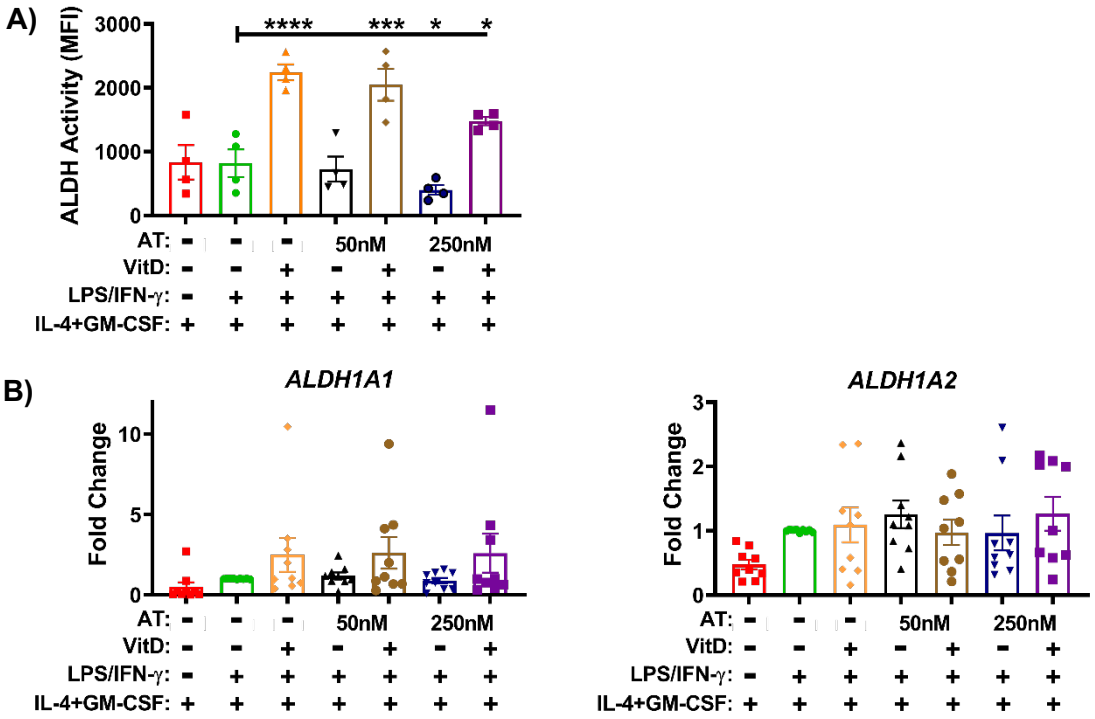


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Fig 30. Aldehyde Dehydrogenase Activity in Monocyte-derived Dendritic Cells differentiated with Atorvastatin and Vitamin D.

Monocytes were differentiated with IL-4 and GM-CSF in the presence of Atorvastatin and/or Vitamin D into monocyte-derived dendritic cells and matured with LPS and IFN-γ. ALDH activity was then measured by flow cytometry. (A) ALDH Activity and (B) expression level of *ALDH1A* genes in moDCs.

n=4 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

11.4 Discussion

Vitamin D and Atorvastatin induce a tolerogenic phenotype at the transcriptional and translational level in monocyte-derived dendritic cells. Particularly Vitamin D which is effective at polarising dendritic cells into a tolerogenic state despite challenging moDCs with pro-inflammatory stimuli LPS and IFN-γ. Several pro-inflammatory genes display significantly reduced expression levels while multiple anti-inflammatory genes were upregulated. In addition, the increase (IL-10) or decrease (IL-12A) in gene expression was reflected in the quantity of cytokines in the culture media. Furthermore, important receptors in T-cell activation (such as HLA-DR and CD80/86) were downregulated but were subject to seasonal change. Dendritic cells are, as previously discussed, a key player in the induction of acute GvHD by presenting host antigens to donor T-cells. However, it would be useful to investigate if matured monocyte-dendritic cells would respond in a similar manner to the drug treatment after differentiation.

Monocytes also play a central role in inflammation, tissue repair, fibrosis and monocyte-activating alarmins such as S100A8/9, which have been detected in stool, serum, and bowl tissue of patients with GvHD (Foell *et al.* 2008, Reinhardt *et al.* 2014), are endogenous ligands of TLR4 (as is LPS) on monocytes resulting in the release of pro-inflammatory cytokines and increased monocyte mediated development of Th17 cells (Reinhardt *et al.* 2014). Another study found a strong correlation between (intermediate) monocytes and patients with GvHD (Reinhardt-Heller *et al.* 2017). One explanation for this phenomenon could be the positive correlation between numbers of intermediate monocytes and Th17 cells. As monocytes differentiated with Vitamin D lead to a tolerogenic dendritic cell phenotype it is tempting to speculate that Vitamin D would also induce a tolerogenic phenotype in monocytes.

Most *in vivo* DCs derive from a precursor termed pre-DC, are distinct from monocytes, but nonetheless monocytes also serve as precursors *in vivo*. Numerous murine and

human studies have subsequently shown that monocytes are recruited predominately to inflammatory sites and differentiate *in situ* into DCs (Tang-Huau and Segura 2019). These *in vivo* monocyte-DCs efficiently activate CD4+ and CD8+ T-cells and secrete pro-inflammatory cytokines. Although data is limited on this subpopulation, studies suggest that these monocyte-DCs drive inflammation and tissue damage (Tang-Huau and Segura 2019). Monocyte-DCs from rheumatoid arthritis and psoriasis patients showed that these cells are potent Th17 inducers *ex vivo* (Zaba *et al.* 2009, Segura *et al.* 2013), one of the main T-cell subpopulations responsible for acute GvHD. It is therefore beneficial that the drug combination tested in this thesis induced a tolerogenic phenotype in monocyte-derived dendritic cells.

Aldehyde dehydrogenase (ALDH) activity has been demonstrated in murine BMT models to significantly drive GvHD pathology and mortality and that dendritic cells rather than intestinal epithelial cells are crucial in mediating this effect. Consequently, we investigated if Atorvastatin and Vitamin D affect ALDH activity. Interestingly, Atorvastatin and Vitamin D have opposite effects on ALDH activity. One possible mechanism could be differentially affected transcriptional activity, however, analysis of gene expression of *ALDH1A1* and *ALDHA1A2* did not give a conclusive answer albeit *ALDH1A1* expression was increased with Vitamin D treatment. On the other hand, no significant decrease in *ALDH1A1* gene expression was measured for Atorvastatin despite significant decrease in ALDH activity. We focused on these two isozymes as it is reported that they are the main enzymes responsible in RA conversion but it cannot be excluded that *ALDH1A3* could mediate the increase in ALDH activity. It is possible that ALDH activity is regulated at the posttranslational level rather the transcriptional level. Indeed, aldehyde dehydrogenase undergoes posttranslational modifications which affect its catalytic activity (Song *et al.* 2011).

CHAPTER 12

General Discussion

12. General Discussion

Allogeneic haemopoietic stem cell transplantation (alloHSCT) is often curative for blood cancers such as leukaemia. Many advances in the treatment of this disease over the last few decades have made alloHSCT accessible to a wide range of patients and significantly increased survival of recipients. Nonetheless, considerable morbidity and mortality is still prevalent in patients suggesting that further improvements in this treatment are required. One major complication in allogeneic HSCT is the life-threating graft versus host disease (GvHD) in which donor T-cells recognise the host as foreign and attack the recipient's tissue and organs. This is further complicated by an increasing trend towards using haploidentical and mismatched HLA-donors which considerably increase chances of developing GvHD. Many potential therapeutic strategies have been developed to ameliorate GvHD by either directly targeting T-cells and their epigenetic programming (e.g. anti-thymocyte globulin or cyclosporin) or cell replication in general (e.g. methotrexate). However, many preventive measures come with considerable toxicity and adverse effects and despite these measures acute GvHD develops in around 30-50% of all patients and 30-70% develop chronic GvHD. Therefore, there is an urgent need to develop novel and quickly deployable strategies to prevent GvHD.

Development of acute GvHD is driven by the interaction of donor T-cells with host antigen presenting cells resulting in expanding donor T-cells causing cell death and tissue damage in the recipient. It is, therefore, crucial to limit the expansion of donor T-cells and minimize the ability of antigen presenting cells to present host peptides in combating GvHD.

In the majority of patients undergoing alloHSCT, acute GvHD is managed in the recipient by prophylaxis treatment with a calcineurin inhibitor and most often with the folate antagonist methotrexate. In recent years it was demonstrated that Tregs within the graft significantly influence the frequency of (acute) GvHD in the recipient and that a high portion of Tregs is associated with reduced aGvHD and increased survival. It is,

therefore, crucial not only to treat the recipient but also to condition the donor to maximize the ability to reduce severity of GvHD. This would shorten the time to engraftment, reduce the use of immunosuppressive therapy for GvHD and reduce opportunistic infection to improve overall survival of recipients after transplantation. In this thesis a novel non-invasive, non-toxic drug combination, Atorvastatin and Vitamin D (Calcitriol), was tested on their ability to increase Treg frequency, modulate CD4+ T-helper populations and suppress T-cell proliferation *in vitro*. The highest acceptable dose of Atorvastatin (80mg/day) can be safely administered for up to 8 weeks without any serious adverse events. There are no known adverse effects of Vitamin D and a single dose of up to 250,000 IU can safely maintain 25-hydroxyvitamin D3 levels above 50 nmol/L for up to 6 weeks (Ilahi *et al.* 2008, Prietl *et al.* 2010, Kearns *et al.* 2014, Kearns *et al.* 2015). In addition, Atorvastatin is associated with myalgia and Vitamin D can reduce this side-effect, suggesting that this drug combination can have benefits beyond GvHD (Michalska-Kasiczak *et al.* 2015). There is good evidence in the literature that the combination is effective in increasing Treg numbers and ameliorating GvHD and is deemed safe for the use in donors. Nonetheless further research and clinical trials are required to make Atorvastatin and Vitamin D a standard therapy in the treatment of GvHD.

Atorvastatin and Vitamin D modulate CD4+ T-cell Populations

Physiological relevant doses of Atorvastatin and Vitamin D induce CD4+ Tregs and have an additive but non-significant effect in combination. While Atorvastatin induced Th2 cytokine IL-4 in activated CD4+ T-cells, Vitamin D significantly inhibited the production of Th1 cytokine IFN-γ. Furthermore, Vitamin D also significantly reduced the expression of pro-inflammatory cytokines IL-17A and TNF-α, while neither drugs had an effect on anti-inflammatory cytokines IL-10 and TGF-β. Th1 and Th17 populations are the main mediators of acute GvHD and their combined inhibition will significantly limit the severity of GvHD. In addition, increased Treg numbers mediated by Atorvastatin and Vitamin D

will further limit development of pathogenic T-cell populations. Despite the increase in numbers, no increase in *FOXP3* mRNA nor FOXP3 TSDR demethylation but a decrease in FOXP3 protein levels was detected. This might indicate a transient Treg phenotype which possibly converts back into a Teffector phenotype under inflammatory conditions. However, in activated CD4+ T-cells, incubated with Vitamin D, FOXP3 was significantly elevated on the mRNA and protein level indicating that some CD4+ T-cells acquire regulatory function. Dividing donor T-cells are key in initiation and progression of acute GvHD, and Atorvastatin and Vitamin D further limit their proliferation by directly inhibiting cell division. However, for significant Teffector suppression Tregs are indispensable.

Atorvastatin and Vitamin D induce tolerogenic Dendritic Cells

Antigen presenting cells present host peptides to donor T-cells and subsequently drive their activation and expansion which is a hallmark of GvHD. In addition, monocytes can differentiate into DCs *in situ* and drive inflammation and tissue damage in humans. Atorvastatin and Vitamin D downregulate co-stimulatory molecules CD80 and CD86 on monocyte-derived dendritic cells (moDCs) required for lowering TCR activation threshold via CD28 on T-cells. HLA-DR is also downregulated by both drugs limiting DCs ability to present peptides to the TCR ultimately reducing activation of pathogenic T-cells. Interestingly, a seasonal variability was detected with decreasing protein surface expression in the summer and increasing levels in the winter. Accumulating evidence highlights that seasonal change can influence the immune system and a cohort of young individuals reported significant changes for CD86 during summer and winter (Dopico *et al.* 2015). Monocyte-derived dendritic cells differentiated alongside Atorvastatin and Vitamin D demonstrated a tolerogenic gene expression profile with increased expression of *IL-10*, *TGFB1* and decreased *IFNG* and *IL-12A*. ELISAs for IL-10 and IL-12p70 demonstrated that observed increase in gene expression was associated with stimulated secretion of these cytokines. Furthermore, the significant increase in *IDO1* gene expression, critical in tryptophan metabolism and inhibition of alloreactive T-cells, was

significantly elevated. In this thesis moDCs were incubated in a mixed lymphocyte reaction which had a limited tolerogenic protein surface phenotype. Nonetheless, addition of matured moDCs significantly increased suppression of CD4+CD25- Teffector cells in the presence and absence of Tregs. Addition of Atorvastatin or Vitamin D further increased suppression especially in the absence of Tregs. However, addition of Atorvastatin and Vitamin D differentiated moDCs had only little effects on Teffector suppression. While Tregs are essential in suppressing activated Teffector cells, dendritic cells can further limit their proliferation and Atorvastatin and Vitamin D are capable of inducing a tolerogenic phenotype at the transcriptional as well as translational level.

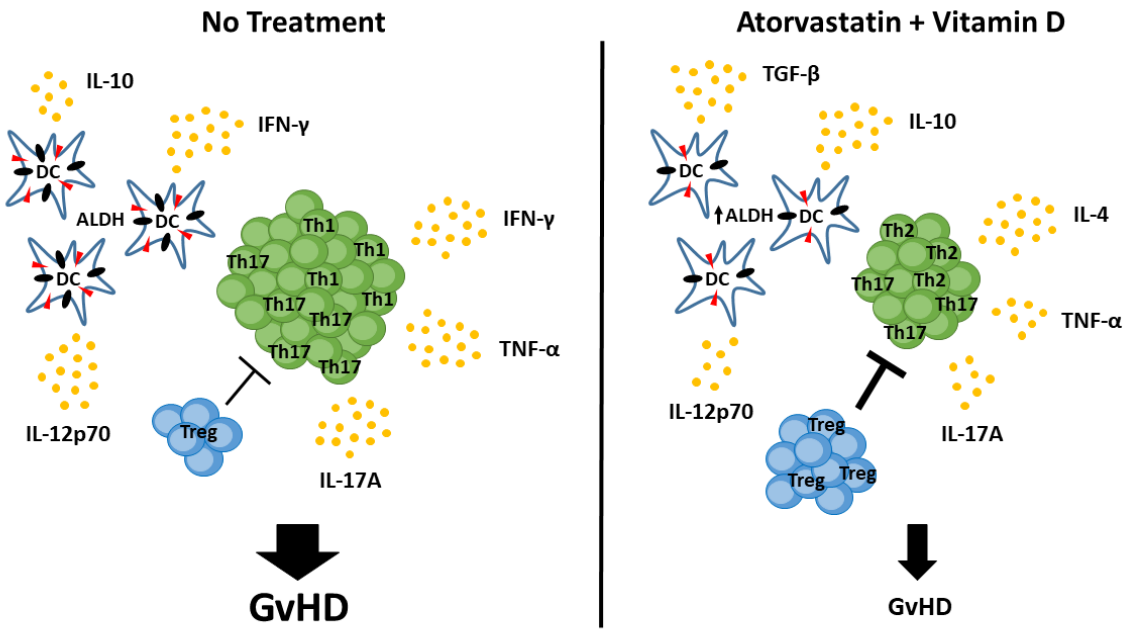


Fig 30. Graphic Depicting Immunomodulatory Effects of Atorvastatin and Vitamin D. Atorvastatin and Vitamin D modulate CD4+ T-cell populations by increasing Treg frequency and inhibiting Th1 and Th17 differentiation, resulting in subsequent change in cytokine production. In addition, activated Teffector proliferation is suppressed by the drug combination. Monocytes-derived dendritic cells differentiated in the presence of both drugs take on a tolerogenic phenotype by down-regulating co-stimulatory molecules CD80 and CD86 (indicated as ▼) as well as MHC class II (HLA-DR; indicated as ⬮). Furthermore, a change in gene expression occurs with upregulation of anti-inflammatory and a reduction in pro-inflammatory genes which is translated into subsequent cytokine secretion. Taken together, this should result in reduced GvHD severity and pathology.

Atorvastatin and Vitamin D have opposing Effects on ALDH Activity

Preclinical studies have demonstrated that aldehyde dehydrogenase (ALDH) activity, especially in dendritic cells, is in part responsible for GvHD pathology. While increased catabolism or pharmacologic inhibition of RA ameliorates GvHD, administration of RA significantly exacerbate GvHD. Differentiation of monocytes to dendritic cells in the presence of Atorvastatin and Vitamin D revealed that Vitamin D significantly elevated, while Atorvastatin decreased, ALDH activity. Combing both drugs also resulted in a significant increase in ALDH activity, albeit to a lesser extent than Vitamin D alone. It is plausible that administration of Atorvastatin and Vitamin D would enhance GvHD due to increased ALDH activity, however, it seems to be unlikely as several clinical studies (see 5.2) have linked Vitamin D deficiency with exacerbated GvHD. In addition, many autoimmune diseases (such as EAE) have similarities with GvHD as these pathologies are often the result of expanding pathogenic CD4+ T-cells and RA is beneficial in these settings. To date, only three clinical studies (NCT02600988, NCT03759262 and NCT03093805) have investigated Vitamin D in alloHSCT and GvHD. One multicenter trial (NCT02600988) found that daily doses of Vitamin D (1000 or 5000 IU per day, days -5 to +100) did not ameliorate incidence rate of acute GvHD but significantly lowered cumulative incidence rate of chronic GvHD (Caballero-Velazquez *et al.* 2016), while the other two clinical trials are ongoing. Additionally, patients in this trial did not undergo any *ex vivo* or *in vivo* T-cell depletion as GvHD prophylaxis. It, therefore, seems feasible that Vitamin D, despite leading to an increase in ALDH activity, is a safe and effective treatment for GvHD.

Prospective Study

There is good evidence that this treatment regimen could improve the outcome of alloHSCT by reducing acute GvHD. Naturally, after successful *in vitro* studies are either *in vivo* studies and/or phase 1 clinical trials to test the hypothesis in a more relevant and challenging setting. With access to pre- and post-transplant blood samples of recipients

treated with Atorvastatin and Vitamin D a much more accurate picture of this regimen could be drawn. Cytokine burden could be investigated, metabolites of interest measured, T-helper and other relevant immune populations explored, and transcriptional and translational changes studied (e.g. genome-wide association studies and RNA-sequencing). Importantly, molecular insights gained could be directly correlated with GvHD pathology seen in the patient. Furthermore, other complications and disease states associated with alloHSCT (e.g. opportunistic infections and cardiovascular diseases) could be scored as well. Taken together, this would allow to draw a more conclusive picture and path the way for further larger clinical trials to establish this regimen in Europe as a standard (prophylaxis) treatment for acute GvHD.

Economical Burden of HSCT

Haematopoietic stem cell transplantation is a complex and costly intervention with prolonged hospital stays and care responsibilities. A significant amount of NHSBTs expenditure is related to transplantation activities such as HSCTs (https://nhsbtdbe.blob.core.windows.net/umbraco-assets-corp/16680/nhsbt-2019-24-summary-5-year-plan.pdf). Advanced and novel therapies usually require expensive GMP and GLP approved equipment and requires experienced and qualified personal to operate and prepare which would further increase the cost of HSCT and the burden on the health system, an example would be the expansion of *ex vivo* Tregs. An alternative to this strategy is to identify therapeutics that can stimulate *in vivo* Tregs in recipients, donors or both. The drug combination tested in this thesis has been shown to expand Tregs in a cheap and effective manner, additionally, Atorvastatin (Liptor) and Vitamin D are off patent and, therefore, generically available and can be easily and legally administrated by medical staff into donors and recipients.

Conclusion

Atorvastatin and Vitamin D are potent immunosuppressive molecules which significantly modulate CD4+ T-cell populations and monocyte-derived dendritic cells and suppress

activated CD4+CD25- Teffector cells. Danby *et al*. (2016) demonstrated the significant impact Treg content within the graft has on acute GvHD and overall survival in the recipient in alloHSCT. The drug combination efficiently elevates Tregs and is consequently a viable approach to increase Tregs within the donor and graft. One the other hand, due to its considerable immunosuppressive properties, it is also a viable approach to be used as GvHD prophylaxis treatment in the recipient.

CHAPTER 13

Appendix

13. Appendix

13.1 TSDR Methylation Assay

This section shows the genomic DNA location and sequence of the two gene loci tested in the TSDR methylation assay (*FOXP3* and *CD3;* e!Ensembl release 101, November 2020, www.ensembl.org). In addition, the sequences for the PCR primers and probes are shown. CpG dinucleotides are highlighted in yellow. (Bisulphite and methylation specific – BSMS, Probes – Pr, unmethylated – TpG and methylated – CpG).

In addition, the bisulphite treated DNA sequences for unmethylated (Tregs and T-cells) and methylated (non-Tregs and non-T-cells) versions are also shown. BSMS primers are indicated in red and BSMS probes are underlined.

FOXP3 and *CD3* CpG and TpG standards were generated by using plasmid DNA of unmethylated and methylated *FOXP3* and *CD3* DNA. A serial dilution of the plasmid DNA (i.e. 30,000, 3000, 300, 30, 10 and 3 template copies per reaction) was used to generate the standard curve used to quantify unmetylated FOXP3 (nTregs), methylated FOXP3 (non-Tregs), unmethylated CD3 (T-cells) and methylated CD3 (non T-cells). The standards used in this assay were aliquots of the original batch.

Specificity and sensitivity of primers and probes, plasmid DNA sequence and standards were checked and confirmed by Dr Robert Danby and colleagues.

The ratio of nTregs was calculated by the following equation:

$$\mathrm{nTregs} = \frac{\mathrm{TpG\ FOXP3} \div (\mathrm{TpG} + \mathrm{CpG\ FOXP3})}{\mathrm{TpG\ CD3} \div (\mathrm{TpG} + \mathrm{CpG\ CD3})}$$

FOXP3 TSDR

Amplicon	Specificity	Forward	Reverse
FOXP3	BSMS (TpG)	GTTTTTGATTTGTTTAGATTTTTTTGTT	CCTCTTCTCTTCCTCCATAATATCA
	BSMS Pr (TpG)	ATGGTGGTTGGATGTGTTGGGTT	
	BSMS (CpG)	GTTTTCGATTTGTTTAGATTTTTTCGTT	CCTCTTCTCTTCCTCCGTAATATCG
	BSMS Pr (CpG)	ATGGCGGTCGGATGCGTC	

Genomic DNA (GRCh38:X:49260595-49260930)

5:TGTCTGGGGGTAGAGGACCTAGAGGGCCGGCTGGGCAGCCGGCTTCCTGCACTGTC
TGTTGGGACGTCCCTTTCTGACTGGGTTTCTCAGAAGCTGAATGGGGGATGTTTCTGGG
ACACAGATTATGTTTTCATATCGGGGTCTGCATCTGGGCCCTGTTGTCACAGCCCCCGA
CTTGCCCAGATTTTTCCGCCATTGACGTCATGGCGGCCGGATGCGCCGGGCTTCATCGA
CACCACGGAGGAAGAGAAGAGGGCAGATACCCCACCCCACAGGTTTCGTTCCGAGAACT
GGCTGCCCTGTCCTGCAGCAGGCTTGGCCCAGGTGGGGTGACA:3

Bisulphite Treated DNA from Tregs (TpG specific)

5:TGTTTGGGGGTAGAGGATTTAGAGGGTTGGGTTGGGTAGTTGGTTTTTTGTATTGTT
TGTTGGGATGTTTTTTTTTGATTGGGTTTTTTAGAAGTTGAATGGGGGATGTTTTTGGG
ATATAGATTATGTTTTTATATTGGGGTTTGTATTTGGGTTTTGTTGTTATAGTTTTTGA
TTTGTTTAGATTTTTTTGTTATTGATGTTATGGTGGTTGGATGTGTTGGGTTTTATTGA
TATTATGGAGGAAGAGAAGAGGGTAGATATTTTATTTTATAGGTTTTGTTTTGAGAATT
GGTTGTTTTGTTTTGTAGTAGGTTTGGTTTAGGTGGGGTGATA:3

Bisulphite Treated DNA from Non-Tregs (CpG specific)

5:TGTTTGGGGGTAGAGGATTTAGAGGGTCGGGTTGGGTAGTCGGTTTTTTGTATTGTT
TGTTGGGACGTTTTTTTTTGATTGGGTTTTTTAGAAGTTGAATGGGGGATGTTTTTGGG
ATATAGATTATGTTTTTATATCGGGGTTTGTATTTGGGTTTTGTTGTTATAGTTTTCGA
TTTGTTTAGATTTTTTCGTTATTGACGTTATGGCGGTCGGATGCGTCGGGTTTTATCGA
TATTACGGAGGAAGAGAAGAGGGTAGATATTTTATTTTATAGGTTTCGTTTCGAGAATT
GGTTGTTTTGTTTTGTAGTAGGTTTGGTTTAGGTGGGGTGATA:3

CD3

Amplicon	Specificity	Forward	Reverse
CD3	BSMS (TpG)	TTTAAATATTGTTATATTTTGA	AAAAATCTAACTACTACAACTTAC
	BSMS Pr (TpG)	GAGAAGTTGTTGGTTTTATAGTGTAAG	
	BSMS (CpG)	TTAAATATTGTTATATTTCGA	AAATCTAACTACTACGACTTAC
	BSMS Pr (CpG)	TCGTCGGTTTTATAGCGT	

Genomic DNA (GRCh38:11:118342846-118343078)

5:GGCAGAGAATATGGAAAAGGTGGCCTGAACTTTAGCGTCCCTATTGACAATGCAACC
ACATTTACACACAACCTAAACACTGCCACATCTCGAAGCCCCTTGAGAGAAGCCGTCGG
CCCCATAGCGCAAGCCGTAGCAGCTAGATTTCTCATGGAGGCTGATCTTTCTCAGGACC
CTTCACTAGGCAGCCAGGGACACCAGATCTAGCAGCTTCTTGTCAGTGGGAGGTTGGG:
3

Bisulphite Treated DNA from T-cells (TpG specific)

5:GGTAGAGAATATGGAAAAGGTGGTTTGAATTTTAGTGTTTTTATTGATAATGTAATT
ATATTTATATATAATTTAAATATTGTTATATTTTGAAGTTTTTTGAGAGAAGTTGTTGG
TTTTATAGTGTAAGTTGTAGTAGTTAGATTTTTTATGGAGGTTGATTTTTTTTAGGATT
TTTTATTAGGTAGTTAGGGATATTAGATTTAGTAGTTTTTTGTTAGTGGGAGGTTGGG:
3

Bisulphite Treated DNA from Non T-cells (CpG specific)

5:GGTAGAGAATATGGAAAAGGTGGTTTGAATTTTAGCGTTTTTATTGATAATGTAATT
ATATTTATATATAATTTAAATATTGTTATATTTCGAAGTTTTTTGAGAGAAGTCGTCGG
TTTTATAGCGTAAGTCGTAGTAGTTAGATTTTTTATGGAGGTTGATTTTTTTTAGGATT
TTTTATTAGGTAGTTAGGGATATTAGATTTAGTAGTTTTTTGTTAGTGGGAGGTTGGG:
3

13.2 ELISA OD Values

IL-10	STD	STD	+LPS/IFN-γ	-LPS/IFN-γ	100nM VitD	50nM AT	50nM AT + VitD	250nM AT	250nM AT + VitD
Donor 1	2.511	2.469	(+)	0.315	(+)	(+)	(+)	3.204	(+)
Donor 1	1.368	1.355	(+)	0.334	(+)	(+)	(+)	3.33	(+)
Donor 2	0.712	0.632	2.513	0.191	2.928	2.952	2.935	1.962	3.038
Donor 2	0.394	0.369	2.506	0.193	2.923	3.056	2.984	2.021	3.153
Donor 3	0.248	0.224	1.514	0.655	3.427	1.168	(+)	1.326	(+)
Donor 3	0.152	0.143	1.554	0.663	3.4	1.162	(+)	1.277	3.381
Donor 4	0.115	0.101	(+)	0.49	2.766	(+)	3.199	(+)	3.474
Donor 4	0.122	0.109	(+)	0.552	2.901	(+)	3.322	(+)	(+)
Donor 5	2.572	2.499	1.988	0.401	2.226	0.404	1.871	0.237	1.612
Donor 5	1.308	1.293	2.001	0.41	2.303	0.411	2.004	0.242	1.616
Donor 6	0.698	0.686	1.552	0.44	(+)	1.429	(+)	1.308	(+)
Donor 6	0.386	0.37	1.558	0.426	(+)	1.19	(+)	1.292	(+)
Donor 7	0.225	0.221	2.966	0.341	3.489	2.541	3.388	2.515	3.326
Donor 7	0.145	0.144	2.968	0.344	(+)	2.615	3.401	2.547	3.28
Donor 8	0.109	0.103	0.272	0.132	0.864	0.292	0.869	0.32	0.577
Donor 8	0.131	0.118	0.317	0.174	0.903	0.32	0.912	0.368	0.65

Table A1. IL-10 ELISA OD Values of Monocyte-derived Dendritic Cells' Culture Media.

Table shows the OD values for IL-10 ELISA of Atorvastatin (AT) and 100nM Vitamin D (VitD) differentiated monocyte-derived dendritic cells (moDCs). Each condition was tested in duplicate. All moDCs were matured with LPS and IFN-γ except for immature moDCs (-LPS/IFN-γ; row 5). (+) indicates an OD value above the detection limit of 3.5. Donor 1-4 and donor 5-8 were run on separate ELISA plates with their own standard curve (STD, r^2=0.99).

IL-12p70	STD	STD	+LPS/IFN-γ	-LPS/IFN-γ	100nM VitD	50nM AT	50nM AT + VitD	250nM AT	250nM AT + VitD
Donor 1	2.189	2.035	(+)	0.077	0.765	(+)	0.781	(+)	0.832
Donor 1	1.003	1.037	(+)	0.065	0.735	(+)	0.777	(+)	0.828
Donor 2	0.58	0.553	(+)	0.062	1.142	(+)	1.314	(+)	1.648
Donor 2	0.347	0.297	(+)	0.061	1.108	(+)	1.252	(+)	1.647
Donor 3	0.212	0.181	(+)	3.285	1.529	(+)	1.387	(+)	1.318
Donor 3	0.153	0.119	(+)	3.341	1.52	(+)	1.43	(+)	1.306
Donor 4	0.129	0.098	(+)	0.059	0.355	(+)	0.396	(+)	0.364
Donor 4	0.153	0.123	(+)	0.1	0.387	(+)	0.409	(+)	0.407
Donor 5	2.011	2.093	3.35	0.085	0.357	0.426	0.325	0.417	0.181
Donor 5	1.044	0.981	3.458	0.097	0.382	0.437	0.348	0.448	0.193
Donor 6	0.565	0.555	0.786	0.059	0.06	0.517	0.066	0.49	0.058
Donor 6	0.298	0.295	0.746	0.054	0.059	0.497	0.063	0.46	0.057
Donor 7	0.185	0.178	(+)	0.061	0.164	(+)	0.146	(+)	0.13
Donor 7	0.119	0.117	(+)	0.063	0.167	(+)	0.145	(+)	0.133
Donor 8	0.097	0.087	(+)	2.187	2.342	(+)	2.254	(+)	1.644
Donor 8	0.105	0.093	(+)	2.245	2.297	(+)	2.237	(+)	1.656

Table A2. IL-12p70 ELISA OD Values of Monocyte-derived Dendritic Cells' Culture Media.

Table shows the OD values for IL-12p70 ELISA of Atorvastatin (AT) and 100nM Vitamin D (VitD) differentiated monocyte-derived dendritic cells (moDCs). Each condition was tested in duplicate. All moDCs were matured with LPS and IFN-γ except for immature moDCs (-LPS/IFN-γ; row 5). (+) indicates an OD value above the detection limit of 3.5. Donor 1-4 and donor 5-8 were run on separate ELISA plates with their own standard curve (STD, r^2=0.99).

13.3 Ketone-Treg Clinical Trial

As part of this D.Phil a clinical study was set up to investigate the effect of a ketogenic supplement on immune cells in healthy volunteers with a focus on T-cells and Tregs. This project was planned with the help of Dr Abigail Lamikanra. Prof David Roberts was the principal investigator. The proposed start date was Spring 2020, however, due to the emergence of the Sars-Cov-2 pandemic all clinical trials were suspended in the UK. Consequently, this project could not anymore to be implemented as part of this D.Phil.

This clinical trial received ethical approval by the East Midlands Nottingham 1 Research Ethics Committee **(REC reference: 20/EM/0017)**.

In order to submit the clinical trial for ethical approval by the Clinical Trials and Research Governance (CTRG), University of Oxford and REC, following documents had to be submitted:

1. Study Protocol
2. Participant Information Sheet (PIS)
3. Tolerability Form
4. Dietary Log
5. Contact Card
6. Consent Form
7. Return Slip
8. Advertisement

13.3.1 Synopsis

Study Title	A Study on the Influence of Ketogenic Drinks on Immune Cells in Healthy Adults.		
Internal ref. no. / short title	Ketones and Tregs.		
Study registration	Not applicable.		
Sponsor	University of Oxford, Clinical Trials and Research Governance (CTRG), Joint Research Office, Boundary Brook House, Churchill Drive, Oxford, OX3 7GB.		
Funder	Nuffield Division of Clinical Laboratory Science, University of Oxford Dr Mark Evans Senior Administrator Level 4, John Radcliffe Hospital, OX3 9DU. Tel: 01865 220355 Email: mark.evans@ndcls.ox.ac.uk		
Study Design	Open label, 42-day, controlled trial		
Study Participants	Healthy adults		
Sample Size	50 healthy volunteers		
Planned Study Period	7 weeks; of which 1 week is to screen for eligibility; 4 weeks are treatment and the remaining 2 weeks are follow up.		
Planned Recruitment period	20th January 2020 – 26th February 2021		
	Objectives	Outcome Measures	Timepoint(s)
Primary	To determine the change in frequency of FOXP3+ Tregs after daily ingestion of a ketogenic dietary supplement (e.g. ΔG®) compared to a Low glucose placebo drink for 28 days.	The change in ratio of Tregs defined as (CD4+CD25highFOXP3+CD127-) to CD4+ T cells.	Baseline (0), 14, 28, 42 days after ingestion of the supplement.
Secondary	To determine after daily ingestion of a ketogenic dietary supplement (i.e. ΔG®) or Low glucose placebo drink for 28 days:	1. Treg sub-type: CD4+ T cell ratio. 2. Treg suppression assay.	Baseline (0), 14, 28, 42 days after ingestion of the supplement.

	1. The change in Treg sub-types 2. The change in Treg suppressive function. 3. The changes in immune cell composition. 4. The change of blood ketone metabolites 5. Secondary or adverse effects or events. 6. The molecular basis of altered Treg function.	3. Myeloid cell (e.g. dendritic cells) and T-cell sub-types. 4. Beta-hydroxybutyrate (ketone) levels in the blood 5. Liver and Kidney Function; Glucose and Ferritin levels; stomach discomfort 6. Differential expression of Treg genes.	
Intervention (s)	Ketogenic supplement (i.e. ΔG®)		
Comparator	Low glucose placebo drink that does not contain the ketogenic supplement. Baseline before ingestion of the supplement to provide an internal control within each participant.		

13.3.2 Background and Rationale

Background

We have previously shown that donor T-Regulatory cells (Tregs) affect clinical outcome following haematopoietic stem cells transplantation. Our data and a systematic review have shown that Tregs improve survival (Danby *et al.* 2016, Fisher *et al.* 2017). One possible modulator of Tregs in donors is diet and high levels of naturally occurring ketones can increase levels and function of circulating Tregs. In this study, we will study the effect of exogenous ketone supplements on the number and function of circulating Tregs in volunteer donors.

Blood cancers like leukaemia or lymphoma are the fifth most common cancer in the UK and one of the most prevalent cancers worldwide. Haematopoietic stem cell transplantation (HSCT) is often curative for people suffering hematologic malignancies, congenital or acquired disorders of the haematopoietic system (such as leukaemia) and is often the last hope for cure for these patients.

Haematopoietic stem cells (HSCs) have to be either mobilised from the patient (autologous) or from a matched donor (allogeneic). Allogeneic HSCTs carry the highest risk of mortality; yet their use continues to rise to be one of the most common interventions in the future (less than 10% allogeneic HSCTs were performed in 1992 compared to over 40% in 2006 and this number continues to rise (Hatzimichael and Tuthill, 2015). Moreover, the age of patients undertaking stem cell transplantation has risen (Balassa and Pawson, personal communication) which adds to the challenges of this procedure. Therefore, continued research into this field is required to address future challenges and improve patient outcomes following HSCT.

The donor's haematopoietic stem cells are transplanted to the recipient to allow haematopoietic and lymphoid reconstitution in the recipient. The effectiveness of the procedure relies on the graft versus leukaemia (GvL) effect where a subset of the donor's CD3+ T-cells can attack and destroy residual recipient leukaemia cells.

However, this procedure is associated with 40-60% mortality overall. This mortality rate can be due in large part to one or more complications that include growth and stimulation of donor T cells that attack the skin, liver, or gut cells of the patient, and cause graft versus host disease (GvHD).

Surprisingly little is known about how the total cell composition of the graft effects the development of this serious complication. In experimental models CD3+CD4+ T-cells called T-regulatory cells can improve survival while permitting function of the GvL effector cells and thus suppression of leukemic cells.

We have previously carried out a prospective study to determine if Tregs affect clinical outcome following HSCT. Here, different cell types within the graft were labelled, identified and counted by flow cytometry. We found that Tregs improve survival (Danby *et al.* 2016, Fisher *et al.* 2017) and this effect was sustained and profound and patients receiving HSCT allograft with higher than median Treg:CD4+ T-cell ratio had:

- substantially reduced non-relapse mortality, hazard ratio, 0.36 (95% CI 0.14-0.96) (p=0.04)
- substantially increased overall survival, hazard ratio, 2.21 (95% CI 1.12-2.21) (p=0.02)

We also found that the proportion of Treg cells varied considerably in the graft, ranging from 2% to 8% but the causes of such wide variation in grafts, and by implication in donors, is not understood.

Dietary modulation of immune cells to increase Treg frequencies

One possible modulator of Tregs in donors is diet. The gut-bacteria that break down the non-digestible fibre in the colon produce fermentation products called short chain fatty acids (SCFAs). SCFAs such as butyrate, can increase the numbers and function of Tregs in the gut (Arpaia *et al.* 2013, Smith *et al.* 2013). Other small molecules that act as diet modulators are ketones. Ketones (D-β-hydroxybutyrate, Acetoacetate and Acetone) are

primarily produced as a response to starvation in the liver from fatty acids. Historically ketones were viewed as a simple source of energy. However, nowadays it is increasingly appreciated that ketones are much more than an alternative energy source but may be mediators of signalling, post-translational modifications and potentially modulators of inflammation and oxidative stress.

During periods of fasting the body utilizes fat stores as a source of energy rather than carbohydrates (Cahill 2006). This produces ketone bodies such as D-β-hydroxybutyrate (D-β-HB; most abundant ketone) that provide sources of carbon energy to the brain and heart. In humans, basal serum level of ketones are in the low micromolar (μM) range, but begin to rise quickly after >12h fasting, reaching 1-2mM after 2 days (Cahill *et al.* 1966, Robinson and Williamson 1980). Prolonged starvation can increase ketone levels further and intense exercise can elevate D-β-HB into the 1-2mM range as well (Koeslag *et al.* 1980, Cahill 2006). Randomised controlled trials have shown that low carbohydrate, high fat (i.e. ketogenic) diets are safe and effective (Accurso *et al.* 2008, Westman *et al.* 2008). However, enforcement of high fat intake plus strict carbohydrate restriction (<20 g/day) is required to raise endogenous ketone levels, thereby making the regimen unpleasant, resulting in constipation and, all too often, lack of adherence (Denke 2001).

Ketone Supplements

Circulating ketone levels can increase during periods of calorific deprivation, with β-hydroxybutyrate levels reported to be 4–5 mM in the blood following a 5- to 8-day fast (Owen *et al.* 1967, Mensink and Katan 1992, VanItallie and Nufert 2003, Cahill 2006). Serious adverse events associated with ketoacidosis that increase permeability of the brain microvascular endothelium, and cause cerebral oedema are observed when levels of ketones in the blood reach supra-physiological levels (10–20 mM or higher) (Laffel 1999, Cahill and Veech 2003).

Since fasting is impractical, and a ketogenic diet is difficult, a ketone ester, (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, called ΔG®, has been developed that is broken

down slowly by gut esterases to directly raise circulating D-β-HB levels (Clarke *et al.* 2012). Studies, in healthy human subjects, support the safety and tolerability of ketone drinks for more than 6 weeks. In collaborations with UK Sport, many studies on approximately 250 elite athletes have determined the effects of ketone supplements (ΔG®) on physical endurance and cellular metabolism without reporting problems. For example, the ΔG® product has FDA approval and is commercially available in the United States as a sports supplement.

The safety and tolerance to the ketogenic supplement under investigation here has previously been described (Clarke *et al.* 2012). Many of the participants administered the highest dose in this repeated-dose study developed gastrointestinal discomfort which was considered mild to moderate in severity and resolved spontaneously by the end of the study. The gastrointestinal side effects were attributed more to the consumption of large volumes (~1.1 L) of a milk-based drink used to dilute the supplement within a short time-frame, rather than an adverse effect produced from elevated ketone levels.

Importantly, ingestion of 26.8g of the ketogenic supplement (ΔG®) 3 times daily is safe and tolerable in a more recent 28-day trial with no serious adverse events being reported (Soto-Mota *et al.* 2019). Here levels of the β-hydroxybutyrate ketone in blood increased from 0.1mM ± 0.01 to 4mM ± 1 (mean, SD, n=27) within 30 minutes after ingesting 26.8g of ΔG®.

Thus in this study, ingestion of 25g as described by Soto Mota *et al.* (2019) will safely increase levels of β-hydroxybutyrate ketone. We would like to determine whether this safe increase of β-hydroxybutyrate ketone is sufficient to increase Tregs.

The mild ketosis induced by consumption of the ketone monoester can be considered similar to the physiological response observed during fasting states in humans and it is unlikely that the study methodology described in this protocol could be associated with any serious adverse events. Nonetheless, a procedure will be in place to report adverse

events in accordance with the schedule of study procedures. To date the direct effect of ketones on the immune system in man is unknown. By utilizing the ketogenic drink, ketones are raised directly, allowing exclusion of other factors which might influence the outcome.

Experiments in the laboratory suggest a fasting diet or ingestion of butyrate could ameliorate the symptoms of inflammatory disease by enhancing the numbers of non-inflammatory Tregs and by reducing the numbers of pro-inflammatory T-cells (Choi *et al.* 2016, Chen *et al.* 2017). A recent clinical study has shown that a ketogenic diet of high lipid and high protein for 7 days can increase the frequencies of protective Tregs but decrease the frequency of non-Treg T-cells known to contribute to inflammation (Ni *et al.* 2016). Comparison of a ketogenic diet (0.5 – 1mM ketone bodies in serum) against a low-fat diet in 40 overweight men and woman in a clinical trial revealed a reduction in many pro-inflammatory cytokines (e.g. IL-6, IFN-γ and TNF-α) independent of weight loss (Forsythe *et al.* 2008).

Our own preliminary data show the modulation of professional antigen presenting cells – called dendritic cells – by D-β-hydroxybutyrate into a more tolerogenic phenotype (see 13.3.3). After pre-incubation with ketones, the expression of MHC-II receptor (HLA-DR) is down-regulated so reduce the ability of these APCs to activate T-cells. In this environment, Tregs are maintained and the proportion of Tregs in the T-cell population increased.

Our study is designed to determine if 25g of ΔG® ingested as described by Soto-Mota *et al.* (2019) can alter the composition of immune cells and enhance the number of Tregs or alter the functional properties of Tregs. Since Tregs and other immune subsets can be influenced by environmental factors (Druzd *et al.* 2017, Lamikanra *et al.* 2020) we will employ a matching placebo group that will ingest a drink of similar calorific value and volume as the ketogenic supplement.

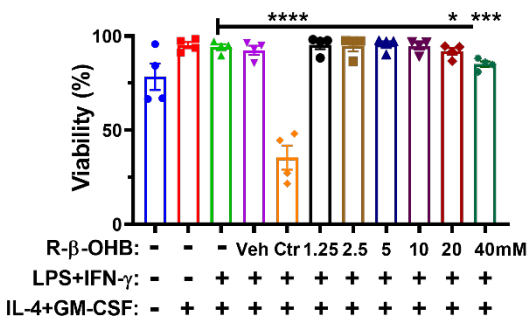
13.3.3 Modulation of Monocyte-derived Dendritic Cells by R-β-Hydroxybutyrate

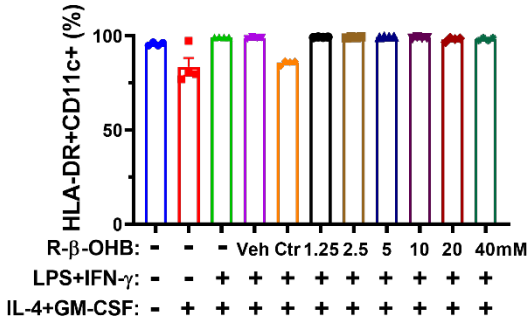
The ketone R-β-Hydroxybutyrate and butyrate receptor, GPR109a (also known as HCA2), is expressed on various human immune cells such as monocytes, dendritic cells, macrophages and neutrophils, however, is not expressed on T- and B-cells and NK cells (Maciejewski-Lenoir *et al.* 2006, Singh *et al.* 2014). To investigate the effect of the most abundant ketone, D-β-Hydroxybutyrate, on immune cells, monocytes were differentiated to dendritic cells in the presence of various concentrations of R-β-Hydroxybutyrate. It is known that butyrate has strong anti-inflammatory effects and was used as a positive reference.

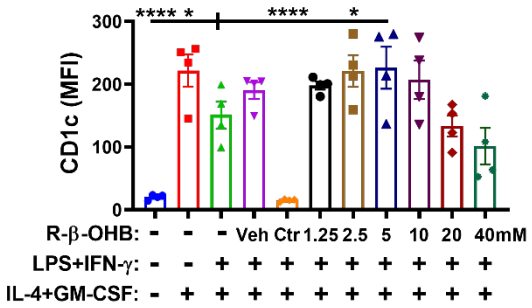
While most investigated markers were unaffected by R-β-Hydroxybutyrate, activation marker CD1a and HLA-DR were significantly downregulated in a dose-dependent manner on monocyte-derived dendritic cells (Fig. A1). Sodium butyrate, which can signal through GPR43 as well as GPR109a, was significantly more potent in downregulating activation markers with fully inhibiting expression of CD80 and CD86 in addition to CD1a and HLA-DR (Fig. A1).

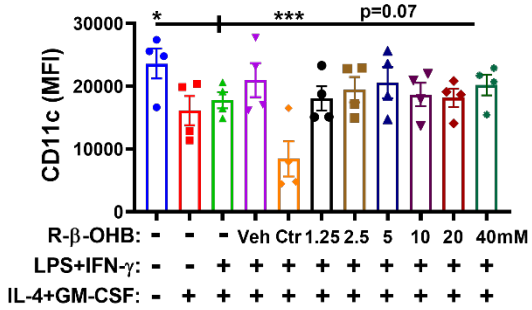
Figure legend for graph on the next page

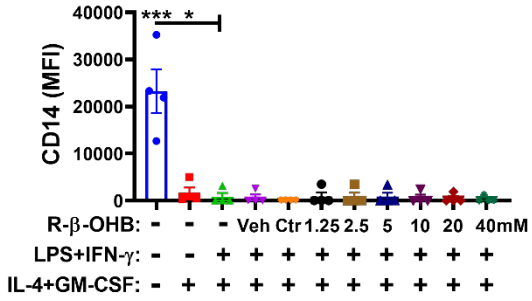
Fig A1. Differentiation of Monocyte-derived Dendritic Cells with R-β-Hydroxybutyrate. Monocytes were differentiated with IL-4 and GM-CSF in the presence of increasing concentrations of R-β-Hydroxybutyrate (R-β-OHB) into dendritic cells. Monocyte-derived dendritic cells were matured with LPS and IFN-γ. Vehicle represents equal amount of DPBS used at the highest R-β-OHB concentrations. Positive control (Ctr) represents 0.5mM sodium butyrate.
n=4 ± SEM; 2-way ANOVA, *=p<0.05, **=p<0.002, ***=p<0.0002 and ****=p<0.0001.

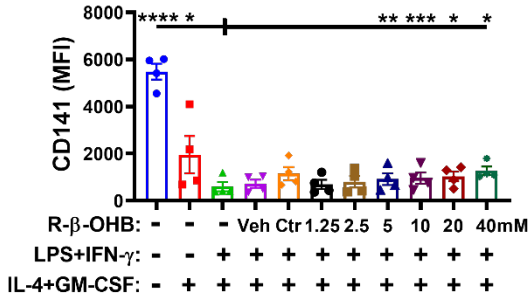

Viability (%)
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +

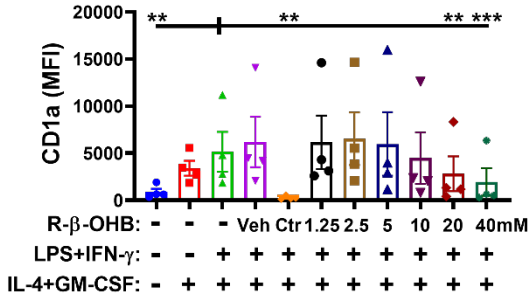

HLA-DR+CD11c+ (%)
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +

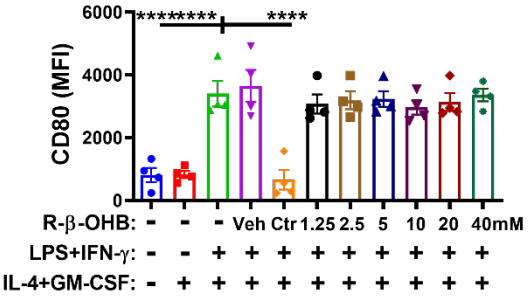

CD1c (MFI)
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +

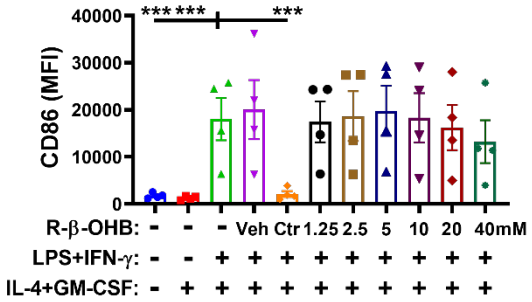

CD11c (MFI)
p=0.07
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +

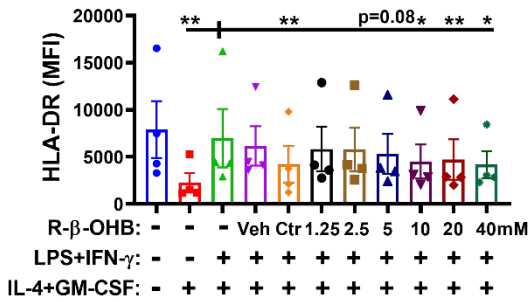

CD14 (MFI)
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +


CD141 (MFI)
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +


CD1a (MFI)
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +


CD80 (MFI)
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +


CD86 (MFI)
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +


HLA-DR (MFI)
p=0.08
R-β-OHB: - - - Veh Ctr 1.25 2.5 5 10 20 40mM
LPS+IFN-γ: - - + + + + + + + + +
IL-4+GM-CSF: - + + + + + + + + + +

13.3.4 Competitive Funding – HIDI

The Human Immunology Discovery Initiative (HIDI) is an initiative which funds platforms across the University of Oxford to analyse immunologic targets. We successfully applied to this fund to facilitate part of the analysis of this clinical trial.

Two platforms would have been used in this study:

1. Oxford Flow Cytometry Facility, John Radcliffe Hospital for deep immune phenotyping
2. Wellcome Centre for Human Genetics for RNA-Sequencing

We designed two extensive flow cytometry panels with Dr Helen Ferry (Oxford Flow Cytometry Facility) to deep immune phenotype the effects of the ketogenic supplement on immune cells of healthy volunteers at different time points (see Fig. A2). One panel (Fig. A3 and Table A2) investigated general immune cell populations (e.g. T-cells, B-cells, DCs, NK, monocytes, and haematopoietic stem cells) and regulatory populations (e.g. Tregs and Bregs) while the other panel (Fig. A4 and Table A3) investigated in detail T-cells populations and their migratory properties.

In addition, we intended to RNA-sequence Tregs at the start of the study and after 4 weeks of ketogenic supplement to investigate any changes at the transcriptional level of Tregs.

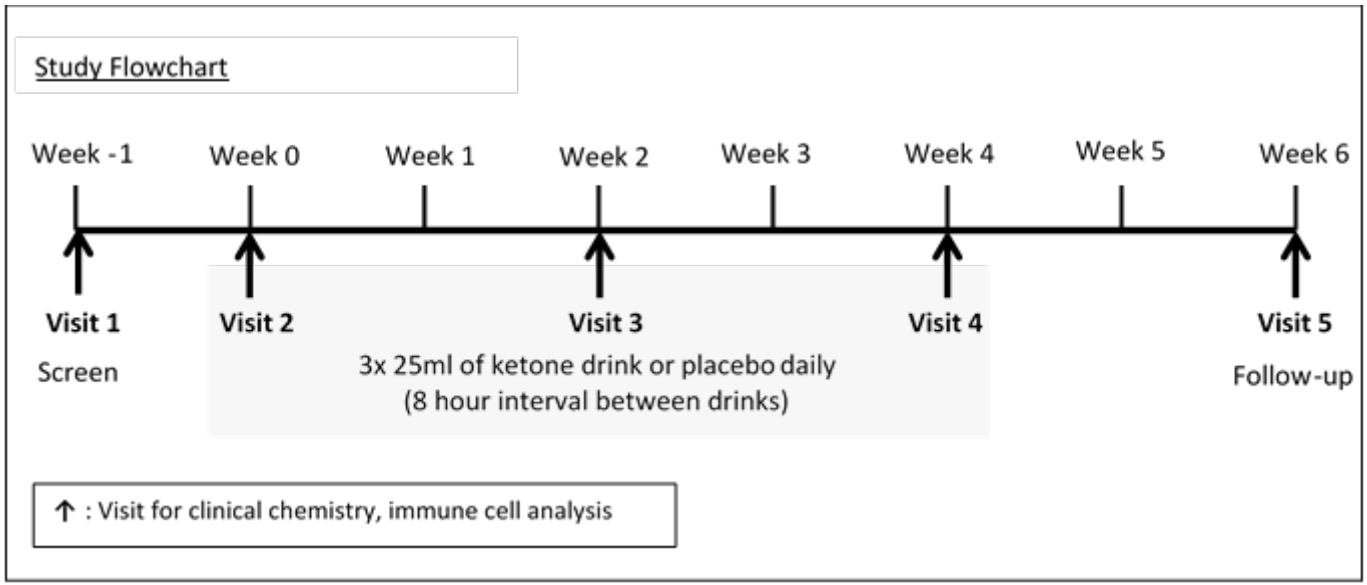


Fig A2. Schematic of Study Design.

Healthy volunteers would come to the study centre at least one week prior to the study start (visit 1) to allow enough time to check their health status including liver function, explain the study and answer any questions and concerns regarding the study. Visit 2 and 3 was intended to supply volunteers with a 14-day supply of the ketogenic supplement and to draw a blood sample. At visit 4 a further blood sample would be drawn and also indicates the end of the treatment period with the ketogenic supplement. Visit 5 is a follow-up visit and a final blood sample is drawn and concludes the study. See Table A1 for procedure details at each visit.

Procedures	Visits (5 visits in total)				
	Visit 1 (week -1)	**Visit 2 (week 0)**	**Visit 3 (Week 2)**	**Visit 4 (Week 4)**	**Visit 5 (Week 6)**
	Screening	**Baseline**	**t= 14±2 days**	**t=28±2 days**	**t= 42±2 days**
Informed consent	x				
Eligibility assessment	x				
Demographics	x				
Medical history	x				
Physical examination	x	x	x	x	x
Laboratory tests	x	x	x	x	x
Allocation to ketogenic drink or the control drink *		x			
Provide ketogenic drink or the control drink		x		x	
Keto-meter measurements		x	x	x	x
Urine dipstick		x	x	x	x
Both groups complete dietary log and tolerability questionnaire			x	x	x
Review of the dietary log and tolerability questionnaire for both groups			x	x	x
Adverse event assessments for both groups			x	x	x

Table A1. Procedures performed at Study Visits

Table indicates planned procedures to be performed at each study visit.

* Allocation is performed after eligibility to participate has been confirmed at baseline.

There will be no visit for randomisation or allocation to each group.

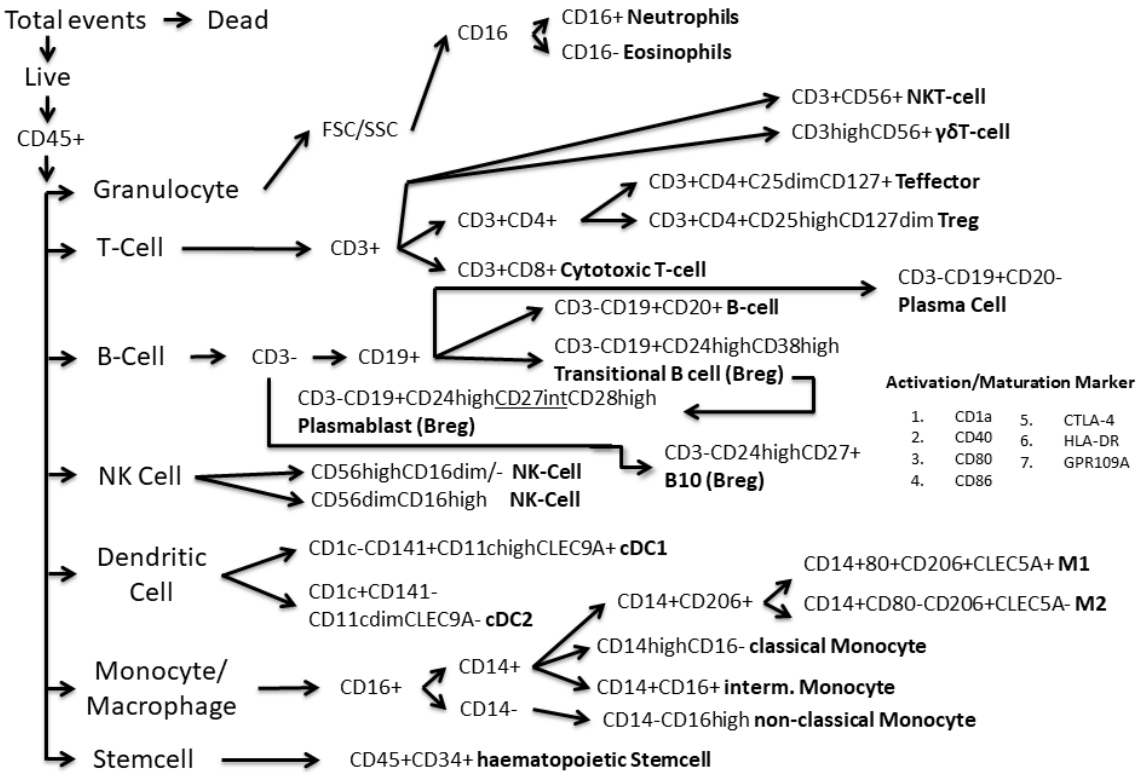


Fig A3. Immune Cell Flow Cytometry Panel.

Shown figure illustrates gating sequence to analyse the effect of ketogenic supplement on immune cell populations of healthy volunteers using multi-colour flow cytometry. Major immune cell populations and regulatory populations of interest are indicated with markers used to identify them. In addition to markers used to identify cell populations, activation markers are indicated to analyse activation status of different populations.

CD45	BUV395	BD	HI30
Viability	L/D Blue	ThermoFisher	(L23105)
CD4	BUV496	BD	SK3
CD19	BUV563	BD	HIB19 or SJ25C1
CD56	BUV615	BD	NCAM16.2
CD206	BUV661	BD	19.2
CD24	BUV737	BD	ML5
CD16	BUV805	BD	3G8
CLEC9A	BV421	BD	3A4/Clec9A
CD25	SB436	ThermoFisher	BC96
CD86	BV480	BD	2331(FUN-1)
CD20	BV510	BioL or BD	2H7
CD14	BV570	BioL	M5E2
CD8	BV605	BioL or BD	SK1
CD40	BV650	BioL	5C3
CD1c	BV711	BioL	L161
CD27	BV785	BioL or BD	L128
CD80	BB515	BD	L307.4
CD34	AF488	BioL	561 or 581
CD3	SP550	BioL	SK7
CLEC5A	PE	R&D	283834
CD11c	PE-Dazzle	BioL	3.9 or Bu15
CD1a	PE-Cy5	BioL or BD	HI149
CD141	BB700	BD	1A4
CD38	PE-Cy7	BioL	HB-7
CTLA-4	APC	BioL or BD	BNI3
GPR109A	AF647	ThermoFisher	4NZBRGO
CD127	APC-R700	BD	HIL-7R-M21
HLA-DR	APC-Fire750	BioL	L243

Table A2. Assigned Conjugated-Antibodies for Immune Cell Panel.
Assigned conjugated-antibodies to be used in a multi-colour panel on a Cytek Aurora spectral flow cytometer equipped with a UV (355nM), violet (405nM), blue (488nM) and red (638nM) laser. Left column shows antibody targets, second column from the left shows assigned fluorochromes to antibody, second column from the right shows manufacturer (BioL – BioLegend and BD) and the right column shows antibody clone.

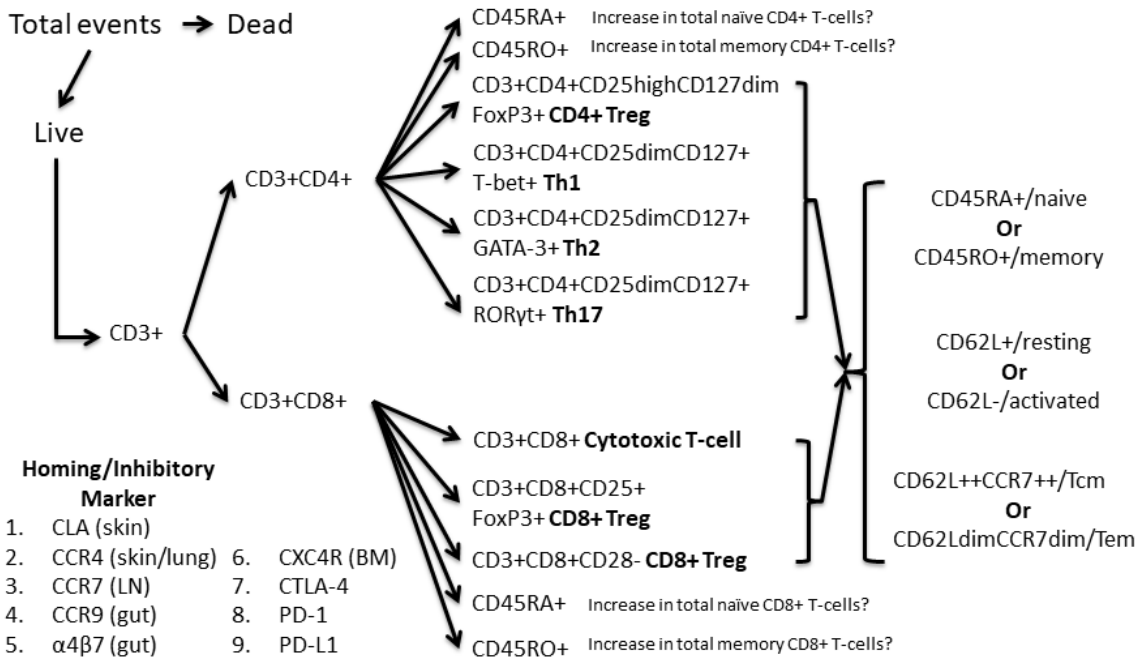


Fig A4. T-Cell Flow Cytometry Panel.

Figure illustrates gating sequence to analyse the effect of ketogenic supplement on T-cell populations of healthy volunteers using a multi-colour flow cytometry panel. Major T-cell populations and regulatory populations of interest are indicated with used markers to identify them. In addition to markers used to identify cell populations, homing and inhibitory markers are indicated to analyse migratory (lymph node – LN and bone marrow – BM) and inhibitory potential of different populations. Further, activation status of T-cell population is analysed (CD62L) and memory status (central memory T-cell – Tcm and effector memory T-cell – Tem).

CD3	BUV395	BD	UCHT1
Viability	L/D Blue	ThermoFisher	(L23105)
CD4	BUV496	BD	SK3
CD45RA	BUV563	BD	HI100
CD25	BUV661	BD	M-A251
CD8	BUV805	BD	SK1
PD-L1	BV421	BioL or BD	MIH5
CLA	PB	BioL	HECA-452
PD-1	BV480	BD	EH12.1
CD45R0	BV570	BioL	UCHL1
CCR4	BV605	BioL or BD	1G1
CCR7	BV650	BioL or BD	G043H7
CD28	BV785	BioL or BD	CD28.2
CTLA-4	Vio-515	Miltenyi Biotec	REA1003
RORgT	PE	BD	Q21-559
T-bet	PE-Dazzle	BioL	4B10
Foxp3	PE-Cy5	ThermoFisher	PCH101
GATA-3	BB700	BD	L50-823
CCR9	PE-Cy7	BioL	L053E8
CXCR4	APC	BioL or BD	12G5
α4β7	AF647	R&D	Hu117
CD127	APC-R700	BD	HIL-7R-M21
CD62L	APC-Fire750	BioL	DREG-56

Table A3. Assigned Conjugated-Antibodies for T-Cell Panel.
Assigned conjugated antibodies to be used in a multi-colour panel on a Cytek Aurora spectral flow cytometer equipped with a UV (355nM), violet (405nM), blue (488nM) and red (638nM) laser. Left column shows antibody targets, second column from the left shows assigned fluorochromes to antibody, second column from the right shows manufacturer (BioL – BioLegend and BD) and the right column shows antibody clone.

13.4 Plasma Studies of Sars-Cov-2

Prof David Roberts is co-leading two clinical trials for the treatment of Sars-Cov-2 patients with convalescent plasma in the UK:

1. The Randomised, Embedded, Multi-factorial, Adaptive Platform for Community-Acquired Pneumonia (REMAP-CAP) is an international trial which is examining the effects of providing convalescent plasma for adults in intensive care with COVID-19.
2. The Randomised Evaluations of COVID-19 Therapy (RECOVERY) trial is a national trial to evaluate existing treatments that may help people, of any age, hospitalized with COVID-19.

Due this new situation I helped in several aspects to facilitate part of these studies:

1. **Negative Reference Plasma:** During the early phase of the pandemic (March-June 2020,) I helped to aliquot several hundred plasma bags (~300) donated prior to July 2019 and designated seronegative for Sars-Cov-2. These were used as negative reference plasma in various assays. These samples were used amongst others in the following publication:
    a. Adams, ER., et al. (2020). “Antibody testing for COVID-19: A report from the National COVID Scientific Advisory Panel”. Wellcome Open Res **5**:139
2. **Convalescent Plasma:** Convalescent plasma bags (~200) of patients surviving COVID-19 infection were aliquoted, stored and sent to relevant laboratories to perform various assays on these samples.
3. **RECOVERY Samples:** As part of the RECOVERY trial plasma samples were sent from participating hospitals to Prof Roberts laboratory where these samples were relabelled, fed into a databank, aliquoted and stored (see Fig A5). Subsequently aliquots were retrieved and sent to relevant laboratories to perform assays on.

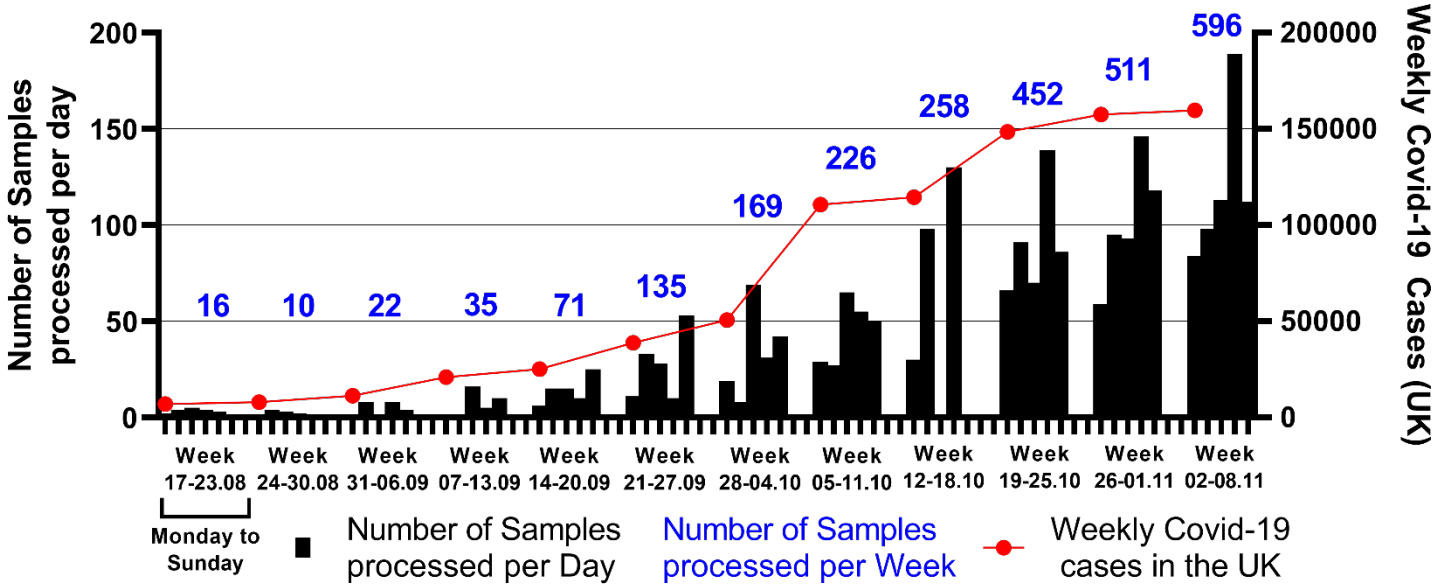


Fig A5. RECOVERY Samples processed at BRL.

RECOVERY samples were received every day (Monday-Friday) at the laboratory. There samples were removed from of their primary postage packaging, so that only a small box containing the sample and sample tracking form is retained. Samples are removed from their protective box and protection tube, and sample number on tube (SST or serum tube) is compared to sample number on accompanying sample tracking form. Sample tubes are then relabelled with a scannable ID ('primary ID') to anonymise. Sample number is manually transferred into the database with corresponding scan able ID ('primary ID') in order to allow to trace back of aliquoted samples to patient. Serum containing tubes are centrifuged and aliquoted in individually coded fludiX tubes in a coded 96-well rack which are linked in the database to the primary ID. Racks are stored at -80°C.

CHAPTER 14

References

14. References

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