Modulating Regulatory T Cells Towards Effective Cancer Immunotherapy

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Declaration of Authenticity

I, the undersigned, declare that this dissertation is my original work, gathered and utilized especially to fulfil the purposes and objectives of this study.

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Oriana Mazzitelli

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Date

Declaration by Supervisor

The undersigned confirms that this dissertation has been undertaken under my supervision and that I approve of its submission for final assessment by the Board of Examiners.

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Professor Pierre Schembri Wismayer

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Date

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LIST OF ABBREVIATIONS

AICD – Activation Induced Cell Death

APC – Antigen Presenting Cells

cAMP – cyclic adenosine monophosphate

CCR4 – Chemokine receptor type 4

CCR6 – Chemokine receptor 6

CD – Cluster of Differentiation

CFSE – Carboxyfluorescein succinimidyl ester

CL097 – water soluble derivative of Resiquimod

CO_2 – carbon dioxide

CpG – cytosine linked, through a phosphate bond, to a guanine

CRTH2 – Chemoattractant receptor-homologous molecule expressed on TH2 cells

CTLA4 – cytotoxic T lymphocyte antigen

CTLs – Cytotoxic T cells

DAMP – Damage-associated molecular patterns

DC – Dendritic Cells

DI – Division Index

DOTAP - Dioleoyl-3-trimethylammonium propane

EAE – Experimental Autoimmune Encephalomyelitis

EDTA - Ethylenediaminetetraacetic acid

ELB – Erythrocyte Lysis Buffer

Eomes – Eomesodermin

FACS – Fluorescent Activated Cell Sorting

FBS – Fetal Bovine Serum

FMO – Fluorescence Minus One

FOXP3 – Forkhead box P3

GITR – Glucocorticoid Induced TNFR family-related protein

HSP – Heat Shock Protein

IDO – Indoleamine 2,3-dioxygenase

IFN-γ – Interferon-γ

IRF – Interferon Regulatory Factor

IL - Interleukin

IPEX – immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

LPS – Lipopolysaccharide

LT – α lymphotoxin α

MAPK – mitogen-activated protein kinase

MDSC - myeloid derived suppressor cells

MFI – Median Fluorescent Intensity

MHC – Major Histocompatibility Complex

mRNA – messenger RNA

MYD88 – Myeloid differentiation primary response 88

NaCl – Sodium Chloride

NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells

NFAT – Nuclear factor of activated T-cells

NK – Natural Killer Cells

NKT – Natural Killer T cells

PAMP – Pathogen-associated molecular patterns

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PD-1 – Programmed cell death protein 1

pDC – Plasmacytoid Dendritic Cells

PEI – Polyethylenimine

Pen-Strep – Penicillin-Streptomycin

PI – Proliferation Index

PolyA – Poly Adenine

PolyG – Poly Guanine

R848 – Resiquimod

RAPA – Rapamycin

ROR- α – Retinoic acid-related orphan receptor α

RRMS – Relapsing Remitting Multiple Sclerosis

Runx – Runt-related transcription factor

SLE – Systematic Lupus Erythematosus

ssPolyU – single stranded polyuridine

ssRNA – single stranded RNA

STAT – Signal transducer and activator of transcription

TAM – Tumour Associated Macrophages

T-bet – T-box transcription factor

TCR – T Cell Receptor

Teff – Effector T cells

TGF-β – Transforming Growth Factor-β

Th – Helper T cells

TIL – Tumour Infiltrating Lymphocytes

TIR – toll/interleukin-1 receptor

TIRAP – TIR domain-containing adaptor protein

TLR – Toll Like Receptor

TNF-α – Tumour Necrosis Factor-α

TRAM – TRIF-related adaptor molecule

Treg – Regulatory T cells

Tresp – Responder T cells

TRIF – TIR domain-containing adaptor inducing interferon-β

VEGF – vascular endothelial growth factor

ABSTRACT

Regulatory T cells (Treg) suppress the immune system and play an important role in the maintenance of immunologic tolerance to self-antigens, and thus in the prevention of autoimmune diseases. However, their function is hijacked by tumours and when released inside the tumour microenvironment, Treg aid cancer cells to evade the immune system promoting tumour growth. For this reason, Treg present a major obstacle to successful immunotherapeutic treatments. There is evidence that suggests that under certain acute inflammatory conditions, induced by immunomodulatory agents, Treg function is decreased or reversed, and sometimes they might also acquire the ability to attain characteristics of other Helper T cells.

Treg were isolated from human PBMC and after expanding the Treg to sufficient working numbers, the Treg cells were treated with selected immunomodulators which include Interferon-γ (IFN-γ), two putative endogenous ligands for Toll-Like Receptors (TLR) 7, 8, and 9 and two synthetic ligands for TLR7 and TLR8.

Flow cytometry using antibodies that identify surface and intracellular markers of different T cell types was used to assess changes in phenotype. Suppression assays of Treg and responder Th co-cultures were carried out to check for immunomodulating effects on Treg function. Cytokine analysis assessed whether the immunomodulator favoured the generation of an anti- inflammatory environment (which promotes Treg) or a pro-inflammatory one (which favours Th1).

Transfection with endogenous nucleic acid ligands, including both human RNA and DNA, did not block Treg suppressor function, with DNA transfection actually enhancing it. The synthetic TLR7/8 ligand CL097 modified Treg phenotype, including augmentation in CD25 marker and the upregulation of Th1 transcription factor T-bet whilst retaining FOXP3 expression. Treatment with CL097 enhanced Treg suppressor function.

The two agents which had the best effects on reducing Treg suppressor function were IFN-γ and TLR7/8 ligand, synthetic single stranded polyuridine, with the former also causing a reduction in Treg signature markers FOXP3 and CD25. Importantly, single stranded polyuridine at lower doses reduced Treg proliferation and suppressor function while high doses of the ligand reversed this effect. Thus appropriate concentrations of these two agents may be developed to control Treg in cancer therapies whilst other agents, such as CL097, may be more useful in the management of autoimmune disease.

1 LITERATURE REVIEW

1.1 The Immune Response: A General Overview

The immune system refers to a group of cells and proteins that function to protect the human body from foreign invading microbes such as bacteria, fungi and viruses, as well as abnormal entities such as cancer cells (Warrington, Watson, Kim, & Antonetti, 2011).

The immune system can be viewed as having two lines of defence, the Innate Immune System and the Adaptive Immune System. The two types of immunity are not mutually exclusive mechanisms of host defence, but rather complement each other (Dunkelberger & Song, 2010).

1.1.1 The Innate Immune System

The first line of defence which an invading pathogen encounters is the innate immune system. This defence mechanism is non-specific and is unable to memorize and recognize the same pathogen in case of recurrent infections. The receptors of cells that belong to the innate immune system are able to detect conserved microbial components that are shared by large groups of pathogens (Turvey & Broide, 2010), hence why it is non-specific. The innate immune response is very fast and occurs within minutes of pathogen exposure. Moreover, the innate immune response plays a central role in activating the second line of defence, which is the adaptive immune system, by a process known as antigen-presentation (Turvey & Broide, 2010) which is discussed further on.

The innate response is performed by cells such as macrophages, neutrophils, dendritic cells, mast cell, eosinophils and natural killer (NK) cells. In this type of immunity, these immune cells are recruited to sites of infection and inflammation through the production of cytokines (Warrington *et al.*, 2011). Cytokines are proteins made and released by one cell that affect the behaviour of other cells and are therefore involved in cell to cell interactions (Molina, Happel, Zhang, Kolls, & Nelson, 2010). Macrophages and neutrophils, display a variety of cell-surface receptors that enable them to recognize and engulf pathogens by a process known as phagocytosis. In addition, neutrophils are granulocytes meaning that they contain granules that, when released, assist in the elimination of pathogenic microbes such as bacteria and fungi. In contrast to neutrophils, macrophages are long-lived cells (Warrington et al., 2011) that besides having a role in phagocytosis, are also involved in antigen presentation to T cells which is discussed further on (Hughes, Benson, Bedaj, & Maffia, 2016).

Dendritic cells and macrophages are differentiated from monocytes. Dendritic cells engulf cells and function as antigen-presenting cells (APCs) to lymphocytes (which form part of the adaptive immune response) and therefore have an active role in both innate and adaptive immunity (Savina & Amigorena, 2007). Mast cells and basophils (being another type of granulocyte) are both involved in acute allergic inflammatory responses, such as those seen in asthma. The difference between the two is that basophils circulate freely in the blood while mast cells are generally found in the connective tissue surrounding blood vessels (Stone, Prussin, & Metcalfe, 2010). A third type of granulocytes called eosinophils, besides from also being involved in allergic inflammatory responses, also have phagocytic properties and play an important role in the destruction of large parasites (Warrington *et al.*, 2011). Natural Killer cells or NK cells

are involved in the destruction of tumour cells and virally-infected cells via the release of granzymes and perforin, which are molecules that induce apoptosis (Stone *et al.*, 2010).

In addition to the hematopoietic cells discussed above, the skin and epithelial cells lining the respiratory, gastrointestinal and genitourinary tracts also play a role in the innate immune response (Turvey & Broide, 2010). The skin forms a physical barrier to infectious agents whereas epithelial cells lining the respiratory and gastrointestinal tracts remove infectious agents through the action of cilia and stomach acid.

Despite this, the range of common pathogens the innate immune system can recognize is limited. The high variability of pathogenic molecular patterns as well as the ability of pathogens to mutate to avoid host detection, has driven the evolution of a second line of defence, the adaptive immune system (Cooper & Alder, 2006).

1.1.2 The Adaptive Immune System

The adaptive immune system comes in action when the innate immune system is ineffective in eliminating infectious agents and the infection is established (Warrington *et al.*, 2011). It is able to recognize pieces of foreign particles such as toxins, chemicals, bacteria, viruses, or other foreign peptides and molecules found in pathogens. These molecules are collectively called antigens. Following this, a specific immune response is generated that eliminates the specific pathogen or any pathogen-infected cells. Additionally, the adaptive immune system will recognize past antigens and quickly eliminate the pathogen should subsequent infections occur (Bonilla & Oettgen, 2010).

There are two types of adaptive immune responses: the cell-mediated and the humoral response. The cells that mediate the adaptive immune system are the lymphocytes. These cells are broadly divided into T cells, which are involved in the cell-

mediated immune response, and B cells which are involved in the humoral immune response.

T cells derive from hematopoietic stem cells in bone marrow and, following migration from the bone marrow, they mature in the thymus. These cells express a receptor on their membrane, known as the T-cell receptor (TCR), and are activated through the action of antigen presenting cells (APCs) such as dendritic cells, macrophages, and B cells (Warrington *et al.*, 2011). T cells are broadly divided into two. These include T cells which express Cluster of Differentiation 4 (referred to as CD4+ T cells) and T cells which express Cluster of Differentiation 8 (referred to as CD8+ T cells). The former act as immune response mediators that produce cytokines to stimulate the activity of other immune cells while the latter are directly involved in cell destruction (Molina *et al.*, 2010; Warrington *et al.*, 2011). Different types of T cells, their roles and functions in the immune system are reviewed in section 1.2.3.

The surfaces of APCs express cell-surface proteins known as the major histocompatibility complex (MHC) which are classified as either class I or class II. Class I MHC are found on all nucleated cells while Class II MHC are found on only certain cells of the immune system such as macrophages, dendritic cells and B cells. The MHC protein binds peptide fragments derived from antigens and displays them on the cell surface for recognition by and activation of T cells (Deseke & Prinz, 2020). Class I MHC are recognized by the TCR of CD8+ T cells while Class II MHC are recognized by the TCR of CD4+ T cells. When they encounter an APC that is displaying antigen fragments bound to its MHC molecules, the T cells are activated. Once the MHC-antigen complex activates the TCR, the T cell secretes cytokines which will determine the type of immune response generated (Warrington *et al.*, 2011). This antigen presentation process stimulates naïve T

cells to differentiate into different T cell lineages to bring about the cell-mediated immune response which will be reviewed in section 1.1.2.2.

B cells also arise from hematopoietic stem cells in the bone marrow but following maturation, they do not migrate to the thymus. B cells display a unique receptor on their membrane which allow them to bind directly to antigens, after which they become activated. Unlike T cells, B cells do not need APCs to activate and can recognize and bind to the antigen directly (Bonilla & Oettgen, 2010). When B cells are activated, they undergo proliferation and differentiate either into plasma cells that secrete proteins called immunoglobulins (also known as antibodies), or into memory B cells. Plasma cells are short lived while the memory B cells are long -lived and continue to express antigen-binding receptors much longer after the infection has subsided (Warrington *et al.*, 2011). These cells can be called upon to respond quickly upon re-exposure to the same antigen, and eliminate it. On the other hand, plasma cells are short-lived cells and undergo apoptosis when the pathogen inducing the immune response is eliminated. This kind of immune response mediated by antibodies is referred to as the Humoral Immune Response and will be described in section 1.1.2.1.

1.1.2.1 Adaptive Immunity: The Humoral Response

Humoral Immunity is mediated by plasma cells that develop from B cells when activated (Bonilla & Oettgen, 2010). The B cell's antigen-binding receptor recognizes and binds directly to a specific part of the antigen called the epitope. This in turn attracts a type of CD4+ T cells, known as T Helper cells (Th cells), which secrete cytokines that help the B cells multiply even more (Chaplin, 2010) and mature into antibody-producing

plasma cells. B cells can recognize a variety of antigens including proteins, polysaccharides, nucleic acids and lipids.

The antibodies that are secreted by the plasma cells bind to the antigens on the surface of pathogens. When this occurs, the antibodies inhibit the toxic effects and infectivity of the pathogen (Payne, 2017). This process is called neutralization.

Moreover, antibodies can also trigger activation of what is known as the complement system. The complement system consists of plasma proteins that attack extracellular forms of pathogens by coating the pathogen with serum molecules called opsonins that render the pathogen more attractive to phagocytes (Dunkelberger & Song, 2010). This process is called opsonization.

Some of the B cells differentiate into memory B cells. The latter quickly respond to previously encountered antigens, giving rise to new plasma cells that produce higher affinity immunoglobulins (Barrington, Pozdnyakova, Zafari, Benjamin, & Carroll, 2002) and bring out an immediate immune response destroying the pathogen before symptoms of the infection arise.

Although immunoglobulins play an important role in containing pathogen proliferation during the acute phase of infection, they will only recognize antigens present outside infected cells or antigens that are freely circulating in the blood. Elimination of pathogens that have entered cells, such as in the case of viral infections, is carried out by the cell-mediated response (Zajac & Harrington, 2014) which is discussed in section 1.1.2.2.

1.1.2.2 Adaptive Immunity: The Cell-mediated Response

The cell-mediated response does not involve antibodies. This type of immunity is involved in the elimination of virus-infected cells as well as defence against fungi, protozoa, intracellular bacteria and cancerous cells. In this kind of immune response, T cells are activated when they encounter a foreign antigen presented to them by antigen presenting cells. T cells (such as cytotoxic T cells) might then kill a virus-infected or cancerous cell that has the same viral or abnormal/mutated antigens on its surface, thereby eliminating the infected/cancerous cell before the virus has had a chance to replicate or before the cancerous cell continues to multiply (Krogsgaard & Davis, 2005; Martínez-Lostao *et al.*, 2015).

Unlike B cells, T cells do not directly bind to the antigens from pathogens. It is the MHC molecules found on the surface of APCs that present the antigens from pathogens to the T cells. T cells can only recognize protein antigens presented to them in the form of peptides (Herzog, Maekawa, Cirrito, Illian, & Unanue, 2005).

CD8+ T cells recognize MHC Class I molecules and when the TCR receptor binds to the antigen-MHC class complex, CD8+ T cells differentiate into cytotoxic T cells (CTLs). CTLs kill infected or cancerous target cells by programmed cell death or apoptosis (Martínez-Lostao, Anel, & Pardo, 2015).

When professional APCs engulf pathogens by phagocytes, they digest the antigens into peptides fragments and display these peptide fragment on their plasma membrane and form the MHC class II-peptide complex. These peptide antigens are then presented to naive CD4+ T cells which differentiate into other kinds of effector T cells called Helper T cells (Th cells). Unlike CTLs, Th cells do not directly kill pathogens, however they release cytokines which further activate cytotoxic T cells and which recruit NK cells

and phagocytes (such as macrophages) to phagocytose the pathogens or the infected cells (Alberts, Johnson, Lewis, *et al.,* 2002). Antigen presentation is further described in sections 1.2.2.

A group of CD4+ T cells do not differentiate into Th. Instead, they differentiate into a group of suppressor cells that regulate immune responses. These cells are referred to as Regulatory T cells and will be the main focus of this study. These cells are fully discussed in a separate section (section 1.3).

Since this study encompasses mainly T cells, which are the main contributors to the cell-mediated adaptive immune response, they will henceforth be the main focus of this literature review.

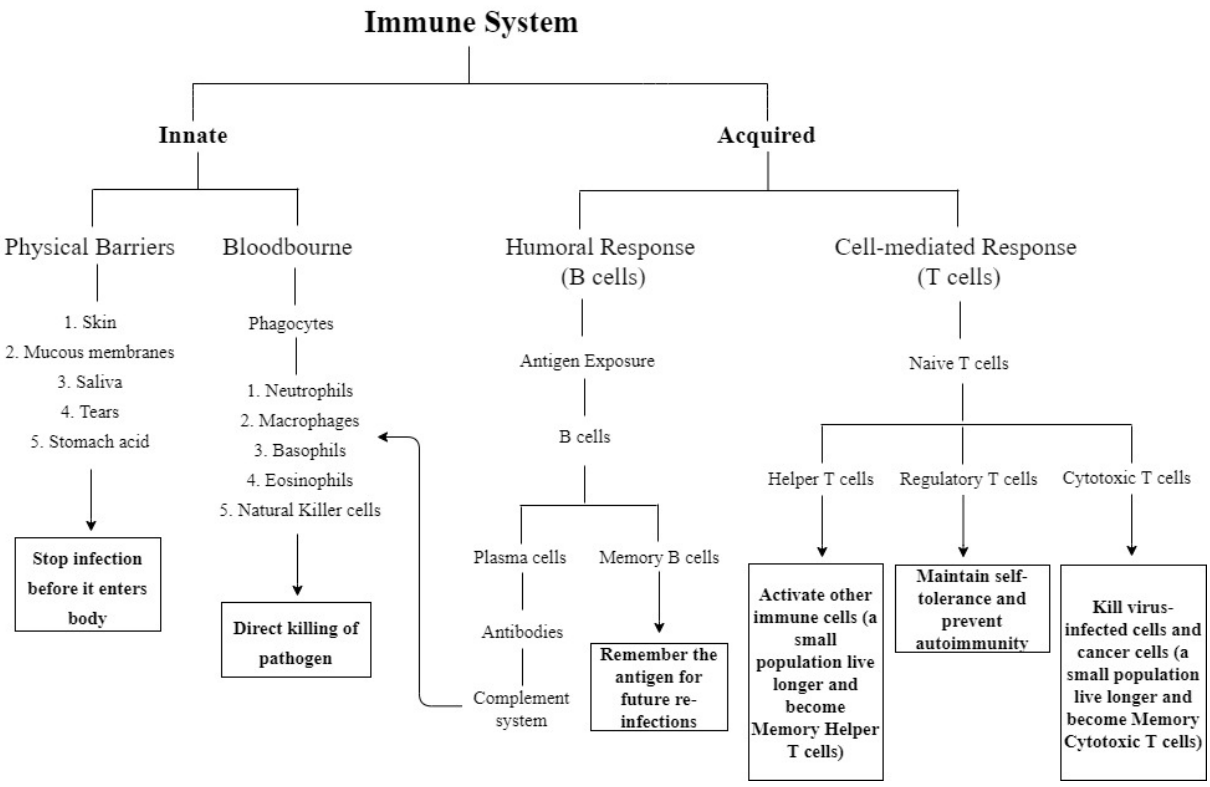


Figure 1.1: The Immune System Structure and Function

1.1.3 Imbalances in the Immune System

An imbalance in the immune system can result in two outcomes. If it fails to protect the body from infectious agents, immunodeficiency results in increased susceptibility to infections. On the other hand, if the immune system is overreactive it might mistake "self" cells for invading pathogens, and a result attack the host's cells and tissue . This results in autoimmunity.

1.1.3.1 Immunodeficiency

Immunodeficiency can arise from a heritable gene mutation. This is referred to as primary immunodeficiency. This is however very rare and immunodeficiency is more often caused secondarily due to pathological conditions such as infection by Human Immunodeficiency Virus (HIV), and blood cancers such as chronic lymphocytic leukaemia and non-Hodgkin Lymphoma (Zabriskie, 2009). Certain forms of therapy such as cytotoxic drugs including chemotherapy, immunosuppressive drugs during organ transplants and radiation can also result in secondary immunodeficiency. An underactive or weakened immune system increases susceptibility to infections and malignancy such as cancer.

Immunodeficiency can be caused both by a defect in the humoral immunity for example by a deficiency in antibody production, or can be caused by a defect in the cell-mediated immunity, for example a lack of functional T cells (Morgan *et al.*, 2011). It can also result from a lack of both functional T and B cells. Cytotoxic drugs for instance can cause severe depletion of CD4+ T cells (and to a lesser extent CD8+ T cell depletion) which might result in a further susceptibility to malignancy. The Human Immunodeficiency Virus (HIV) destroys more CD4+ cells and, if left untreated, it gradually breaks down the

immune system and develops into Acquired Immune Deficiency Syndrome (AIDS) which can result in cancers, infections and eventually death.

Immunodeficiency should not be confused with temporary immunosuppression. The latter means the reduction in the activity, and not the complete lack of the immune system. Such immunosuppression is important to prevent autoimmunity and is discussed under sections 1.3.

1.1.3.2 Autoimmunity

The opposite extreme underactive immune system is an overactive or hyper-responsive immune system.

One of the most striking capacities of the immune system is its ability to discriminate between self and non-self. In a normal person, T cells that are self-reactive are deleted during their development in the thymus in a process known as central tolerance which is discussed under section 1.2.1. Despite this, there is still some leakage of these self-reactive lymphocytes in the periphery. The activation of self-reactive lymphocytes in the periphery is prevented by a process known as peripheral tolerance and is achieved through various mechanisms including anergy (metabolic arrest that leads to cell death), homeostatic control which leads to "switching off" of the cells and regulation by special kind of CD4+ T cells known as Regulatory T cells (Mackay, 2000). However, in people suffering from autoimmunity, central and/or peripheral tolerance fails either due to wrong environmental factors or due to genetic factors (Mackay, 2000).

There are many common diseases that arise from an over-reactive immune system including thyroid diseases such as Hashimoto's thyroiditis and Graves' disease,

rheumatoid arthritis, systemic lupus erythematosus, type I diabetes, multiple sclerosis, myasthenia gravis and many others.

1.2 T Cell Immunity

1.2.1 T Cell Development

T cells are derived from haematopoietic stem cells that are found in the bone marrow. The progenitors of these cells then migrate to the outer cortex of the thymus where they undergo maturation. These cells are referred to as thymocytes.

The earliest developing thymocytes do not express the receptors CD4 or CD8 and are therefore termed double negative (DN) cells. The TCR of these thymocytes interacts with cortical epithelial cells in the thymus that express a high density of MHC class I and class II molecules associated with self-peptides. At this stage, the thymocytes start expressing both CD4 and CD8 and are termed as double positive (DP) cells (Germain, 2002). The fate of the DP thymocytes depends on signalling that is mediated by the interaction of the TCR and these self-peptide–MHC ligands. Signalling that is too weak results in delayed apoptosis referred to as death by neglect. Those cells that engage with the MHC complex with an appropriate affinity survive (Swainson, de Barros, Craveiro, Zimmermann and Tayor, 2013). This process is known as positive selection.

The DP cells than migrate into the medulla of the thymus. Here, they are presented self-antigens by thymic medullary epithelial or dendritic cells. Cells that have survived and that recognize MHC Class I complex stop expressing CD4 and maintain expression of CD8. On the other hand, cells that recognize the MHC Class II complex stop expressing CD8 and maintain expression of CD4. Cells that have survived and bind to the MHC Class I or II too strongly are eliminated by apoptosis (Germain, 2002). This is called

negative selection and is the process that brings about central tolerance and prevent autoimmunity as described in section 1.1.3.2.

The single CD4+ or CD8+ cells that remain exit the thymus and circulate in the periphery.

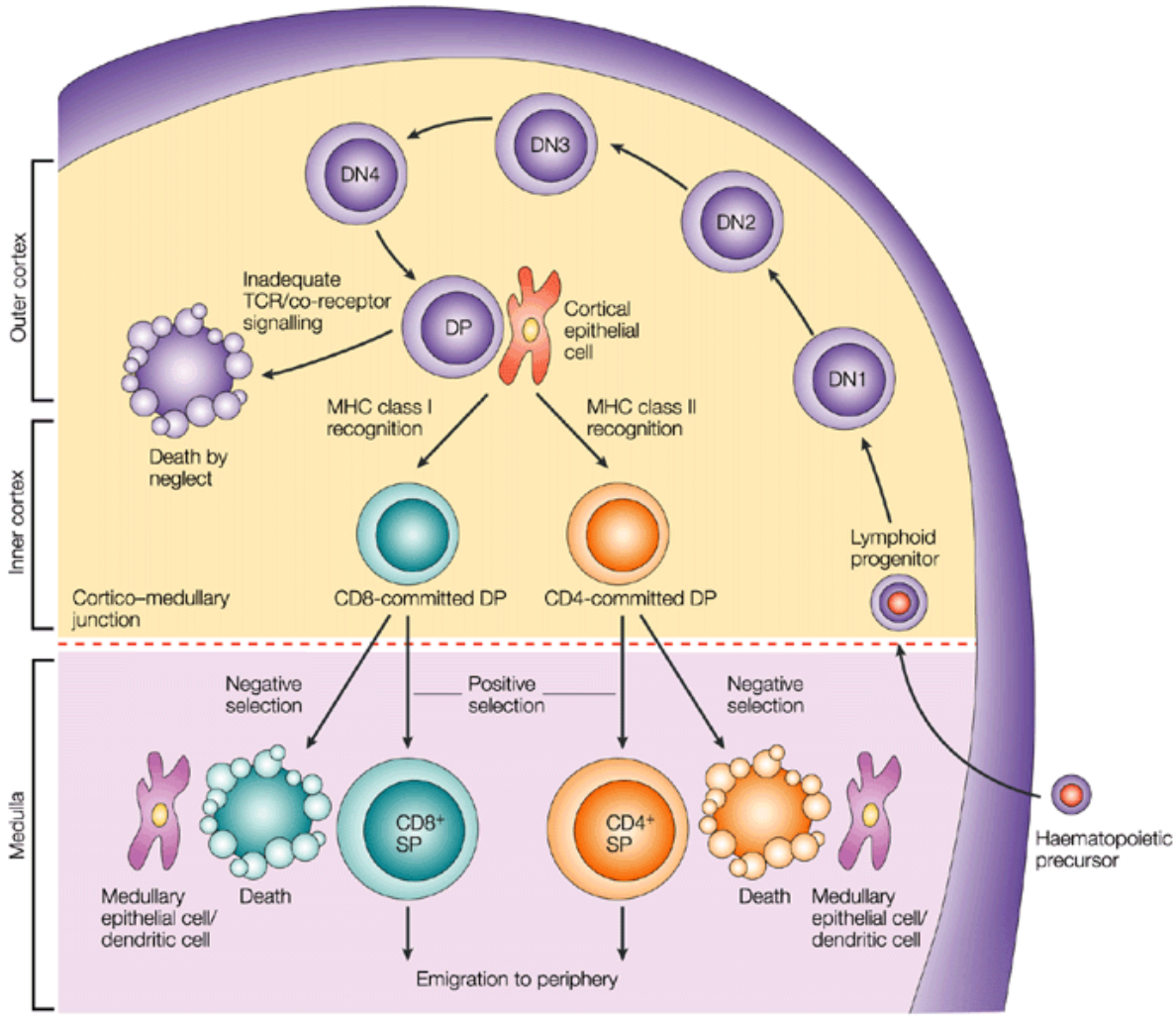


Figure 1.2: T Cell Development and Selection Process in the Thymus. DN – Double negative; DP – double positive; SP – Single positive. Obtained from Germain, R. N. (2002). T-cell development and the CD4–CD8 lineage decision. *Nature Reviews Immunology*, 2(5), 309–322. https://doi.org/10.1038/nri798. *Permission to use the image was obtained from the authors.*

1.2.2 T Cell Activation

When matured T lymphocytes leave the thymus and migrate to the periphery, they are considered naïve until exposed to an antigen. Naïve T cells become activated

upon antigen presentation on the MHC complex of APCs. The events that occur to activate T cells occur at what is referred to as to immunological synapse.

As previously described, T cells recognize peptide antigens that are bound non-covalently to MHC gene products on APCs. T cells are different from B cells in that they do not interact with intact antigens but rather fragments of peptides that have been partly degraded inside the APCs (Robinson & Delvig, 2002).

APCs display three types of protein molecules on their surface that help in the activation of T cells. The first type include MHC proteins, which present the foreign or abnormal antigen to the T cell receptor. The second type are costimulatory molecules, which bind to complementary receptors on the T cell surface and are a requisite for activation. The third type of proteins are cell to cell adhesion molecules which enable a T cell to bind to the APC for as long as required until activation (Alberts, Lewis *et al.*, 2002).

The MHC is a cluster of genes arrayed within a longitudinal stretch of DNA on chromosome 6 in humans and chromosome 17 in mice. The products of the MHC genes plays a central role in the recognition between self and non-self antigens (Andersen, Schrama, thor Straten, & Becker, 2006). There were two kinds of molecules encoded by the MHC which, as previously described in section 1.1.2.2 include the Class I molecules recognized by CD8+ cells and class II molecules which are recognized by CD4+ cells. As also already mentioned, Class I molecules are found on all nucleated cells whereas class II molecules are found only on professional antigen presenting cells, (APCs) including primarily dendritic cells, macrophages and B cells.

Class I MHC molecules are composed of two polypeptide chains, a long α chain and a short β chain while Class II MHC molecules are also composed of two polypeptide

chains but include an α and a β chain of approximately equal length (Wieczorek *et al.*, 2017).

The TCR is a surface molecule found on T cells that will recognize the antigen presented by the MHC class I or class II. The predominant TCR found in lymphoid tissue is the αβ TCR which is a heterodimer composed of one α and one β chain of approximately equal length (Smith-Garvin & Koretzky, 2009). Both chains have a transmembrane region comprised of hydrophobic amino acids which anchor the molecule to the cell membrane. Both chains have a constant region as well as a variable region that determines specificity for an antigen. The variable regions are in contact with the antigen and the MHC (Knapp, Dunbar, Alcala, & Deane, 2017). Each T cell bears a TCR of only one specificity.

The TCR is closely associated with a group of monomeric chains that include γ, δ and two 2 ε chains (Kuhlmann & Geisler, 1993). These proteins are collectively called the CD3 complex. The CD3 complex is necessary for cell surface expression of the TCR during T cell development. It also transduces activation signals to the cell following interaction of the TCR with the antigen presented on the MHC (Smith-Garvin & Koretzky, 2009).

T cell activation required two signals. The first signal is the engagement of the TCR with the antigen-MHC complex on the professional APCs. The other signal, which enhances the first, comes from the interaction of co-stimulatory molecules with their ligands. One of the most important co-stimulatory molecules is Cluster of Differentiation 28 (CD28) on T cells which must interact with CD80 (CD80) or CD81 expressed on APCs (Hünig, Beyersdorf, & Kerkau, 2015). This interaction also enables the T cells and the APCs to adhere together.

Both signals are necessary for T cell activation. Engagement of the TCR with the antigen-MHC complex with no co-stimulation by CD28 results in anergy whilst co-

stimulation on its own has no effect (Budd and Fortner, 2017). Once both signals are received, activation occurs and signals from the cell surface are transmitted to the nucleus via second messengers.

Once activated, CD4+ naïve T cell release interleukin 2 (IL-2) and they start to proliferate and differentiate into CD4+ effector T cells and CD4+ memory T cells. The former can develop into diverse subsets of CD4+ effector T cells as described in section 1.2.3. The latter have the ability to quickly generate more effector T cells if/when the same antigen is encountered in the future.

CD8+ naïve T cell become effector cytotoxic T cells, CTLs, or the longer-lived memory CD8+ T cells. CTLs induce apoptosis in infected or mutated target by releasing cytotoxic proteins including granzymes and perforins. IL-2 also drives cell division and expansion of the activated CD8+ T cells (Boyman & Sprent, 2012).

1.2.3 T cell Types

To recapitulate, T cells are broadly classified as either CD4+ or CD8+ T cells and express only one or the other of these two cell surface markers.

T cells that express the glycoprotein CD8 are just the cytotoxic T cells (CTLs) and are directly involved in cell destruction by apoptosis.

T cells that express the CD4 glycoprotein include the Helper T cells, (Th cells) and the Regulatory T cells (Treg). The former are important as immune response mediators that activate B cells, CTLs and macrophages as well as recruit the latter to sites of infection (Knutson & Disis, 2005; Surman, Dudley, Overwijk, & Restifo, 2000; Zhu & Paul, 2009). There are various subsets of Th cells which are discussed in section 1.2.3.2.1, 1.2.3.2.2 and 1.2.3.2.3.

The more recently-discovered Treg serve to oppose and downregulate immune responses. Treg are discussed in sections 1.2.3.2.4 and 1.3.

1.2.3.1 Activated CD8+ T cells - Cytotoxic T cells

Cytotoxic T cells (CTLs) express the CD8 surface marker. They are very important for immune defence against intracellular pathogens, and for tumour surveillance as they directly kill pathogen-infected or tumour cells. Several cytokines such as interleukin (IL-2), interleukin 12 (IL-12), interleukin 21 (IL-21) and interleukin 27 (IL-27) help induce CTLs from naïve CD8+ T cells (Wang *et al.*, 2017).

When a CD8+ T cell becomes activated, it has three major mechanisms to kill infected or malignant cells. Two of these pathway depend on cell to cell contact while the third one does not.

Direct cell-cell contact can happen in two ways. In one case, the Fas ligand (FasL), which is expressed on the surface of CTLs, binds to the Fas receptor on the target infected or malignant cell (Hassin, Garber, Meiraz, Yael, & Berke, 2011). This binding triggers apoptosis through the classical caspase cascade which releases caspase proteases that cause the cleavage of a number of proteins in a cell and ultimately cell death (Elmore, 2007).

A second cell-cell contact dependent mechanism is the release of the highly cytotoxic proteins, perforin and granzymes by the CTLs. Perforin forms a pore in the membrane of the target cell while granzymes are serine proteases which activate caspases, however they can also cleave many of the proteins leading to cell death even in the absence of caspases. Since it cleaves proteins, granzyme also inhibits viral replication

inside cells. The delivery of the granzymes into the cytosol is mediated by the pores formed by perforin (Andersen *et al.*, 2006; Cullen & Martin, 2008).

In order to avoid apoptosis of by-stander neighbouring cells, the majority of the cytotoxic proteins are pre-synthesized and are therefore secreted for killing only upon encountering a target cell. Additionally, the lytic proteins are packaged in lysosomes which move to the cell surface and expose their content only upon contact with the target cell (Andersen *et al.*, 2006). Moreover, the proteins are released only in the direction of the target cell which is aligned along the immunological synapse.

The third mechanism of cell death mediated by CTLs involves release of the two effector cytokines, Interferon-γ (IFN-γ) and Tumour Necrosis Factor α (TNF-α). TNF-α is a cytokine released by CTLs that binds to the surface of target cells and also triggers apoptosis by the caspase cascade (Janeway, Travers and Walport, 2001). IFN-γ induces transcriptional activation and expression of Fas in target cells and therefore enhance the interaction with FasL on the surface of CTLs thereby leading to Fas- and caspase cascade-mediated apoptosis (Davidson, DiPaolo, Andersson, & Shevach, 2007).

CTLs also can elicit strong cytotoxic effects on tumour cells implementing the perforin as well as the Fas-dependent pathway. However, besides IFN-γ and TNF-α, the cytokine lymphotoxin α (LTα) has been described to directly mediate the cytotoxicity of CTLs and is involved in the initiation of apoptotic cell death in tumour cells, independent of the perforin or Fas mechanism (Yang, Ud Din, Browning, Abrams, & Liu, 2007).

CTL cells can also contribute to an excessive immune response that leads to immunopathology. In particular, CTLs are implicated in organ-specific autoimmune diseases which involves an immune response directed towards an antigen unique to a

single organ. Such autoimmune diseases may involve direct cellular damage mediated by CTLs (Konya, Goronsy & Weyand 2009).

1.2.3.2 Activated CD4+ T cells – Helper T cells

CD4+ Helper T cells are further divided into different subtypes based on the types of cytokines they release. These include T Helper 1 (Th1), T Helper 2 (Th2), T helper 17 (Th17), follicular helper T cells (T_FH) and T-helper 9 (Th9). Differentiation to one lineage depends on specific transcription factors and cytokine signalling (Luckheeram, Zhou, Verma, & Xia, 2012) . These subtypes differentiate from naïve CD4+T cells which are activated after interaction with an antigen-MHC complex as discussed in section 1.2.2 and then differentiate into a specific subtype depending mainly on the cytokines present in the microenvironment (Luckheeram *et al.*, 2012).

1.2.3.2.1 T Helper 1 Cells

T Helper 1 cells, Th1, promote protective immunity against viruses and intracellular bacteria, and are responsible for activating and regulating the development of CTLs cells (Knutson & Disis, 2005; Luckheeram *et al.*, 2012; Prete, 1992). Th1 cells are the most important CD4+ T cells that elicit an anti-tumour immune response as they indirectly kill tumour cells via release of cytokines such as interferon-gamma (IFN-γ), tumour necrosis factor-α, and IL-2 that support CTL function (Disis, 2010). Moreover, they activate tumour antigen-specific CTLs which directly kill the tumour cells (Fallarino *et al.*, 2000).

However, overactivation or misdirected activation of Th1 cells can also lead to organ-specific autoimmune diseases such as multiple sclerosis, type 1 diabetes, rheumatoid arthritis, and delayed-type hypersensitivity responses (Knutson & Disis, 2005; Zhu & Paul, 2009).

The critical cytokines required in the development of Th1 cells include IFN-γ produced by NK cells and T cells (CD4+ Th1 and CD8+ CTLs) and interleukin 12 (IL-12) produced mainly by APCs (Knutson & Disis, 2005; Luckheeram *et al.*, 2012; Trinchieri, Pflanz, & Kastelein, 2003).

The master transcription factors that results into Th1 differentiation is T-bet. Other transcription factors include STAT1, STAT4, Runx3, Eomes and Hlx. (Aune, Collins, & Chang, 2009; Luckheeram *et al.*, 2012). T-bet strongly induces IFN-γ and has an important role in suppressing the development of other T cell subtypes such as Th2 and Th17 (Aune *et al.*, 2009; Lazarevic *et al.*, 2011; Lugo-Villarino, Maldonado-Lopez, Possemato, Penaranda, & Glimcher, 2003). However another study has shown that T-bet also acts as a transcriptional repressor in the later stages of Th1 differentiation by reducing overproduction of IFN-γ in order to avoid an overreactive Th-1 induced immune response (Oestreich, Huang, & Weinmann, 2011). Another transcription factor, GATA3 represses Th1 cells by favouring Th2 development (Aune *et al.*, 2009).

Apart from T-bet expression, Th1 are also strongly induced by the upregulation of CD183 (or CXCR3). CD183 is involved in the trafficking of Th1 (and CD8+ T cells) to peripheral sites of inflammation. It also facilitates the interaction of T cells with APCs leading to the generation of effector and memory cells (Groom & Luster, 2011). However, despite being highly expressed in Th1, CD183 is not solely expressed in Th1 cells.

The main effector cytokine released by Th1 cells is IFN-γ. It is induced through STAT4 by IL-12. IFN-γ can strongly activate macrophages to produce high concentrations of nitric oxide via inducible nitric oxide synthase, iNOS (Niedbala, Cai, & Liew, 2006; Schoenborn JR & Wilson CB, 2007). Upregulation of iNOS enhances phagocytic activity and leads to the killing of intracellular pathogens such as *Leishmania major*, *Listeria monocytogenes* and *Mycobacterium* spp. (Dong, & Martinez, 2010).

Th1 cells also secrete tumour necrosis factor α, TNF-α and LTα. TNF-α stimulates macrophages, NK cells, and CD8+ T cells to promote pathogen clearance. As its name implies TNF-α, is also identified as an anti-tumour agent that induces necrotic cell death in tumours. However, the expression of TNF-α needs to be tightly controlled as systematic overproduction of the cytokine produces excessive inflammatory responses to infection and injury which can result in widespread tissue damage (Thomson and Lotze, 2003). TNF-α is over-produced in individuals with autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (Kassiotis & Kollias, 2001).

LTα formerly known as TNF-β is a member of the Tumour Necrosis Factor (TNF) super family and is structurally similar to Tumour Necrosis Factor α, in that it also binds with high affinity to TNFR1 and TNFR2 (Calmon-Hamaty, Combe, Hahne, & Morel, 2011). The importance of LTα in humans is not clear however mice deficient in LTα are highly susceptible to *Staphylococcus aureus* infections and LTα was also found to be a requirement for resistance against *Mycobacterium*, *Leishmania*, and *Plasmodium* infections in mice (Calmon-Hamaty *et al.*, 2011; Ehlers *et al.*, 2003; Hultgren, Eugster, Sedgwick, Körner, & Tarkowski, 1998). LTα is also associated with inflammation and autoimmune disease such as Th1-dependent rheumatoid arthritis (Calmon-Hamaty *et al.*, 2011; Ott *et al.*, 2014; Suen *et al.*, 1997).

Yet another major cytokine secreted by Th1 cells is Interleukin-2, (IL-2) which stimulates growth, differentiation, and survival of cytotoxic T cells that are essential in eliminating virus-infected cells and tumour cells. Moreover, IL-2 was also found to promote the development of CD8+ memory cells after antigen priming, ensuring a robust immune response in the case of future infections (Williams, Tyznik, & Bevan, 2006).

1.2.3.2.2 T Helper 2 Cells

T Helper 2, Th2, cells are important for immune responses against pathogens that do not directly infect cells, but pathogens that are extracellular parasites such as helminth parasites (Luckheeram *et al.*, 2012).

However, overactivation of these cells has been associated with allergic inflammation and hypersensitivity and indeed Th2 cells are associated with allergic inflammatory diseases such as asthma, allergic rhinitis and atopic dermatitis (Luckheeram *et al.*, 2012; Rui, Liu, Zhu, Cui, & Liu, 2012).

Th2 differentiation is induced by Interleukin-4, IL-4 (in the absence of IL-12 which would otherwise induce Th1), IL-2 and Interleukin-33, IL-33 (Dong *et al.*, 2010; Luckheeram *et al.*, 2012).

The master regulator for Th2 development is GATA3. Other transcription factors include STAT6, STAT5, STAT3, Gfi-1, c-Maf, and IRF4 (Luckheeram *et al.*, 2012). IL-4 induces the transcription factor STAT6 which upregulates the expression of transcription factor GATA3 (Kaplan, Schindler, Smiley, & Grusby, 1996). It is thought that GATA3 in collaboration with STAT6 interacts with and inhibits T-bet to suppress Th1 development (Zhu, Yamane, Cote-Sierra, Guo, & Paul, 2006). However it has also been suggested that

GATA3 downregulates STAT4 (also a transcription factor for Th1) and not T-bet, to suppress Th1 development (Usui, Nishikomori, Kitani, & Strober, 2003). Incidentally, T-bet and Runx3 which, as mentioned in section 1.2.3.2.1, are important transcription factors for differentiation to Th1 cells, inhibit the development of Th2 cells (Luckheeram *et al.*, 2012).

Although not exclusively Th2 preferentially express CCR4 and CCR8. These two are chemoattractant receptors likely to be involved in the recruitment of antigen-specific Th2 cells to sites of allergen exposure (Mikhak *et al.*, 2010).

Recently, a novel surface molecule which is Chemoattractant Receptor homologous molecule expressed on Th2 cells (CRTH2) was described. This receptor was found to be capable of discriminating between Th1 and Th2 cells (Cosmi *et al.*, 2000). This marker was not only found expressed on Th2 cells but was also found to be expressed in blood basophils and eosinophils but is unique in CD4+ T cells suggesting that CRTH2 could be a reliable marker for the identification of Th2 cells from other CD4+ subsets.

The key effector cytokines include the Interleukins 4, 5, 9, 13, 10 and 25 and amphiregulin (Luckheeram *et al.*, 2012). IL-4 stimulates B-cells to produce immunoglobulins. This then stimulates mast cells to release histamine, serotonin, and leukotriene which result in the constriction of airways, intestinal peristalsis and gastric fluid acidification in an attempt to expel helminths parasites (Steinke & Borish, 2001). Since they stimulate the release of immunoglobulins, Th2 cells favour mostly a humoral response as opposed to Th1 which tend to favour the a cell-mediated response. Interleukin-5, IL-5, on the other hand activates eosinophils to attack helminths (Shearer *et al.*, 2003) while Interleukin-9, IL-9, is involved in high secretions of mucus and the release of chemoattractant factors that incidentally also lead to allergic airway inflammation in

asthma (Little, Cruikshank, & Center, 2001). Interleukin-10, IL-10, which is an anti-inflammatory cytokine, makes sure that after pathogen clearance, homeostasis is achieved. IL-10 is an anti-inflammatory cytokine that inhibits Th1 cells as well as other immune cells of the innate system (Couper, Blount, & Riley, 2008). Interleukin-13, IL-13, has a function in combatting gastrointestinal helminthes, helping in the elimination of intracellular pathogens such as *Leishmania* and is also associated with the induction of allergic asthma (Luckheeram *et al.*, 2012). Interleukin-25, IL-25, promotes and intensifies Th2 responses (Kleinschek *et al.*, 2007). IL-25 was found to regulate development of autoimmune inflammation by suppressing Th17 cells as discussed in section 1.2.3.2.3 (Luckheeram *et al.*, 2012). High levels of IL-25 was found to induce pathologies of the lungs and the digestive tract because it enhances the production of IL-13 (Fort *et al.*, 2001). Amphiregulin is a member of the epidermal growth factor (EGF) family and in a mouse study, deficiency of amphiregulin was found to delay the expulsion of the parasitic nematode in mice *Trichuris muris* (Zaiss *et al.*, 2006).

In a murine lung metastases model, Th2 have shown some indirect anti-tumour activity through the eosinophil chemotactic factor, eotaxin and tumour infiltrating-eosinophils (Mattes *et al.* ,2003 as quoted by Ma, Fan and Ribas, 2014). However the role of Th2 effector cells remains unclear with several studies suggesting that Th2 type-inflammation are associated with carcinogenesis and tumour progression (Protti & De Monte, 2012; Kogame *et al.*, 2016)

1.2.3.2.3 T Helper 17 cells

T Helper 17 cells, Th17, promote protective immunity against extracellular bacteria and fungi, mainly at mucosal surfaces. Just like Th1 and Th2, they also may

induce inflammatory and autoimmune diseases, an example of which is multiple sclerosis (Volpe, Battistini, & Borsellino, 2015).

Th17 cells are generated in the presence of Tumour Growth Factor-β, TGF-β and Interleukin-6 (IL-6) and/or Interleukin-21 (IL-21) and are maintained by Interleukin-23 (IL-23), and Interleukin-1 (IL-1) (Dong *et al.*, 2010).

TGF-β is the critical signalling cytokine in Th17 differentiation. However, as will be described in section 1.2.3.2.4, TGF-β also plays an important role in the development of induced Regulatory T cells (iTreg) which act antagonistically to Th17 cells. Only at low concentrations and in the presence of IL-6 does TGF-β induce Th17 differentiation (Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006).

The transcription factors RORϒt and RORα act synergistically together and are important in Th17 cell differentiation and their absence completely aborts the development of Th17 cells (Yang *et al.*, 2008). STAT3 is the major signal transducer for the differentiation of IL-6, IL-21 and IL-23, and is therefore indispensable for Th17 development (Zhu & Paul, 2009). Indeed, deletion of STAT3 also results in complete loss of Th17 cells and was instead found to enhance the expression of transcription factors T-bet and FOXP3 which cause differentiation to Th1 and Treg lineages respectively (Harris *et al.*, 2007; Mathur *et al.*, 2007; Yang *et al.*, 2007). Other suggested important transcription factors for Th17 development include Runx1, Batf, IRF4 and AHR (Luckheeram *et al.*, 2012). Transcription factors that inhibit Th17 differentiation include T-bet, Runx1, Smad3, Runx1 and FOXP3 which incidentally are transcription factors that promote naïve CD4+ T cells to other cell lineages other than Th17.

There is a lack of specific surface markers for Th17, however they are sometimes characterised based on their expression of CCR4 and CCR6 on their surface. CCR4+CCR6+

CD4+ T cells that do not express FOXP3 usually produce IL-17, the main effector cytokine for Th17 (Zhao *et al.*, 2012)

Th17 express IL-17A, IL-17F, IL-21, and IL-22. The former two cytokines are closely related. Both IL-17A and IL-17F are induced by RORYt and their function is to recruit and activate neutrophils during immune responses against extracellular bacteria and fungi (Zhu & Paul, 2009). However, high levels of IL-17A are also associated with several chronic inflammatory diseases including rheumatoid arthritis, psoriasis and multiple sclerosis (Kuwabara *et al.* 2017). Interleukin 21 (IL-21), produced by Th17 cells, acts as the main differentiation cytokine for Th17 development alongside TGF-β. Interleukin-22 (IL-22) is known to mediate the inflammatory response and exhibits tissue protective properties including protecting hepatocytes during acute liver disease and inflammation (Zenewicz *et al.*, 2007).

1.2.3.2.4 Suppressor T cells (Regulatory T cells)

Although as previously described in section 1.2.1 self-reactive T cells are deleted during their development in the thymus in a process known as central tolerance, some self-reactive T cells do overcome negative selection in the thymus and escape in the periphery. The activation and expansion of these escapees is controlled by a class of suppressive CD4+ T cells called Regulatory T cells (Treg) by a process known as immunosuppression (which is not to be confused with immunodeficiency described in section 1.1.3.1). Therefore, Treg have a different function from the Helper T cells and play important role in the maintenance of immunologic tolerance to self-antigens, and thus in the prevention of autoimmunity. After clearance of pathogens, they negatively regulate the immune response by suppressing or downregulating the expansion of further immune

cells, thereby protecting against immunopathology and the development of allergy, asthma, and autoimmune diseases. They also provide protection to commensal bacteria against elimination by the immune system (Shimon, Sakaguchi & Sakaguchi, 2005).

Nonetheless, Treg have also their downside as they play a major role in helping tumour cells evade elimination by the immune system as will be described in sections 1.4.3.

There is extensive evidence from animal models that insufficient Treg cell numbers or impaired Treg function can lead directly to autoimmune conditions and allergy, whereas an over-abundance of these cells can suppress anti-pathogen and anti-tumour immunity (Mcmurchy, Nunzio, Roncarolo, Bacchetta, & Levings, 2009).

Treg constitute about 5–10% and 1-5% of peripheral CD4+ T cells in mice and humans respectively (Gregg *et al.*, 2005; Liu, Kim, Falo, & You, 2009). There are two types of Treg which include the natural Treg, nTreg, and the induced Treg, iTreg. The former originate in the thymus while the latter develop from naïve CD4+ T cells in the peripheral lymphoid organs and spleen in response to environmental antigens (Lin *et al.*, 2013).

High concentrations of TGF-β in the absence of pro-inflammatory cytokines such as IL-6, diverges lineage differentiation of naïve T cells away from Th17 and towards iTreg development, through the induction of the master transcription factor for Treg, forkhead box P3, FOXP3 (Zhou *et al.*, 2008). Deleting TGF-β from Treg cells results in diminished suppressive function and poor Treg survival *in vivo* (Li, Wan, & Flavell, 2007). Both TGF-β as well as Interleukin 2, IL-2, are required for the survival and function of Treg cells even after they have differentiated (Zhu & Paul, 2009).

Transcription factors for Treg include FOXP3, Bcl6, STAT3, Smad2, Smad3, STAT5 and NFAT (Luckheeram *et al.*, 2012). Smad3 is activated by TGF-β while TCR stimulation

activates NFAT. Both Smad3 and NFAT collaborate to promote expression of the master regulator FOXP3 (Burchill *et al.*, 2008; Davidson *et al.*, 2007).

Mutations in FOXP3 in humans will result in immunodysregulation polyendocrinopathy enteropathy X-linked syndrome, IPEX, which is a life-threatening disease caused by a dysfunction in Treg and subsequent autoimmunity (Wildin *et al.*, 2001). Moreover, in humans, a dysfunction in Treg function has been associated, but not always, with various diseases such as myasthenia gravis (Thiruppathi *et al.*, 2012), autoimmune polyglandular syndrome Type II (Kriegel *et al.*, 2004), ulcerative colitis (Uhlig *et al.*, 2006) and multiple sclerosis (Viglietta, Baecher-Allan, Weiner, & Hafler, 2004) as well as graft-versus host disease and allograft rejection. In mice, it results in Scurfy and the mice develop severe lymphoproliferative autoimmune disease attributed to the lack of suppressor Tregs, and results in death between 16 and 25 days of age (Zhu & Paul, 2009). FOXP3 is predominantly a repressor that silences genes that are normally activated after T cell stimulation, especially genes associated with TCR signalling (Marson *et al.*, 2007).

The molecular mechanisms by which Treg exert their suppression and regulatory function has not been fully characterized but a few are discussed in 1.3.3. The main effector cytokines for Treg include IL-10 (which is also secreted by Th2), TGF-β, and the recently characterized Interleukin-35, IL-35 (for the nTreg). IL-10 is a potent inhibitory cytokine with the ability to suppress Th-1 proinflammatory responses and thus limits tissue damage due to inflammation (Asseman, Mauze, Leach, Coffman, & Powrie, 1999). TGF-β together with IL-10, also suppress Immunoglobulin E antibody production thereby showing their important role also in attenuating allergic inflammation (Luckheeram *et al.*, 2012). Section 1.3 shall discuss Treg in more detail.

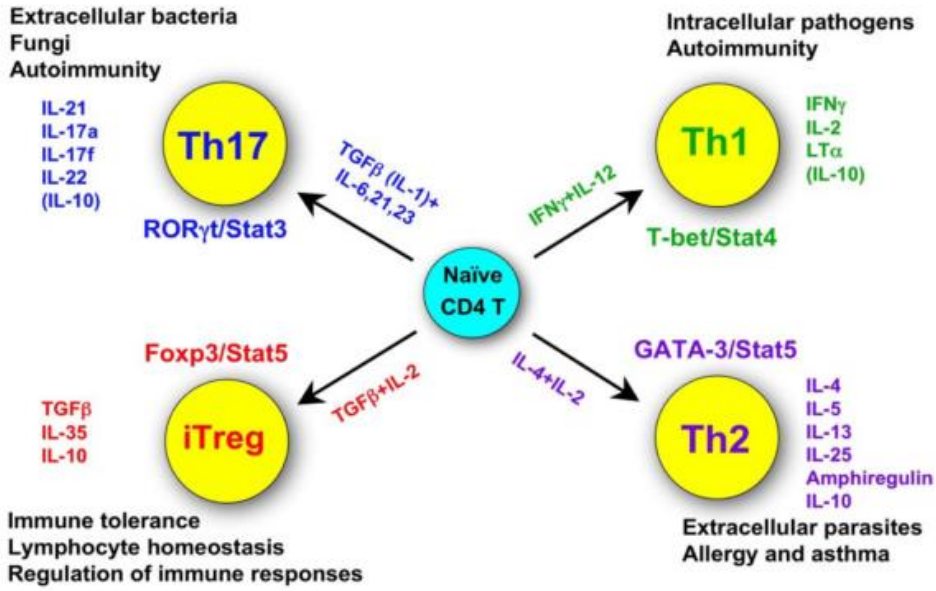


Figure 1.3: Summary of CD4+ T cell fates showing their respective functions, transcription factors that induce them as well as differentiation and effector cytokines. Obtained from Zhu, J., & Paul, W. E. (2009). CD4 T cells : fates , functions , and faults. *Immunobiology*, *112*(5), 1557–1569. https://doi.org/10.1182/blood-2008-05-078154. *Permission to use the figure was obtained from the authors.*

1.3 Regulatory T Cells – A Class of Immunosuppressive T Cells

1.3.1 The role of Regulatory T cells in Immunosuppression

The existence of lymphocytes derived from the thymus that suppressed antigen-induced T cell activation was first reported in 1970 by Gerhson and Kondo (Gerhson & Kondo, 1970). Since their phenotype was not yet characterised, several attempts to isolate these suppressive cells proved unsuccessful. In 1995, Sakaguchi, Sakaguchi, Asano, Itoh and Toda assigned them their first phenotype as cells that express CD4, and high levels of CD25 and have therefore became termed as CD4+CD25+ T cells. Treg suppress many cells including CD4+ and CD8+ T cells, B cells, NK cells, NKT cells, dendritic cells and macrophages, although the immune responses that are critical for Treg-mediated suppression are still unclear.

Treg are critical in that they are involved in controlling autoimmunity, infection, graft-versus-host disease, inflammation, fetal-maternal tolerance, and tumour immunity (Chaudhary & Elkord, 2016).

1.3.2 Regulatory T cell types and their Phenotype

As already mentioned in section 1.2.3.2.4, there are the natural Treg that develop in the thymus and the induced Treg that develop in the periphery. In mice, natural Treg develop in the thymus through the thymic selection process, where the Treg-TCR interacts with the MHC complex and self-peptide. Therefore, intra-thymic generation of Treg would mostly depend on instruction of lineage commitment by self-antigens (Jordan *et al.*, 2001). Development of thymus-derived Treg is known to require strong interaction with MHC-self peptides (Caramalho, Nunes-Cabaço, Foxall, & Sousa, 2015). nTreg recognize self-antigens and are selected in the same way as with other T cell (described in section 1.2.1) which means that those that are selected are the ones where the strength of TCR signalling is above that of positive selection but below that of negative selection. However, it is unclear how Treg expressing a TCR with affinity to self-antigen survive negative selection (i.e. the removal by apoptosis of T cell progenitors that bind excessively to the MHC complex during development in the thymus) while other T cells bearing the same TCR are deleted (Li, Gowans, Chougnet, Plebanski, & Dittmer, 2008). For this reason, human Treg differentiation in the thymus remains poorly understood.

The generation of Treg in periphery is instructed by foreign antigens (Kretschmer, Apostolou, Verginis, & Boehmer, 2008). Indeed, it was found that naïve CD4+ CD25- T cells can be converted into FOXP3+ CD25+ Treg by antigen-specific activation in the

presence of TGF-β and IL-2 in both mice and humans (Apostolou & von Boehmer; 2004 Chen *et al.*, 2003; Fantini *et al.*, 2004;, 2004).

The transcription factors Helios and the cell surface glycoprotein neuropilin-1 are highly expressed in nTreg but poorly expressed in iTreg. Therefore, these two markers are sometimes used to distinguish nTreg from iTreg cells (Li, Li, Tsun, & Li, 2015). Nonetheless, iTreg cells may start to upregulate expression of both factors depending on local inflammation or on the type of antigen-presenting cells and activation signals present (Thornton *et al.*, 2010; Yadav *et al.*, 2012). Therefore, the delineation of these two populations is still difficult due to the lack of specific markers.

There is no unique cell surface marker for Treg but the CD25 surface molecule is highly expressed on the majority of cells that express the master transcription factor of Treg, FOXP3 (Kretschmer *et al.*, 2008). In mice, CD25 is a reliable marker for natural Treg, but in humans activated non-Treg CD4+ effector T cells, especially during an ongoing infection, also express CD25 (Koenen *et al.*, 2008). Although all activated T cells express CD25, Treg cells express the highest levels while expression in other activated CD4+ T cells is intermediate (Koenen *et al.*, 2008). CD25 receptor shows high affinity binding to IL-2 and the high levels of expression of CD25 on Treg cells implicates the importance of IL-2 for the expansion and survival of Treg (Létourneau, Krieg, Pantaleo, & Boyman, 2009).

Characteristically, Treg express CD25, glucocorticoid induced TNFR family-related protein (GITR) and cytotoxic T lymphocyte antigen (CTLA-4), which are all under the control of the transcription factor FOXP3 (Mougiakakos, 2011). However, these markers are only useful for distinguishing Treg cells from naive conventional CD4+ T cells because these markers are also present in conventional T cells once activated.

FOXP3 expression was found to correlate inversely with the cell surface marker CD127. Therefore, nTregs are usually regarded as expressing no or low CD127 levels on their surface. Thus, a combination of low CD127 with high CD25 expression provides better identification and purification of viable Treg.

Considering the above, the intracellular marker FOXP3, encoded by the X chromosome, is probably the most useful way of identifying Treg. Indeed, it is the main method used to estimate the Treg ratios in solid tumour tissue. However, this method of identification still has its limitations, as in humans, FOXP3 can be transiently expressed in activated T cells that are not immunosuppressive Treg (Kretschmer *et al.*, 2005) and, being intracellular, the cells need to be sacrificed in order to detect FOXP3. Nonetheless it is still a reliable method of identification as upregulation of FOXP3 in naïve T cells is what endows Treg with a regulatory function (Jaeckel, Boehmer, & Manns, 2005).

In humans, expression of CD4+CD25highCD127low T cells have been often identified as Treg cells as these cells express high levels of FOXP3. Recently, Treg cells have been further classified into naïve Treg cells that express the cell surface marker CD45RA (CD45RA+) and express low levels of FOXP3 and effector Treg which are CD45RA- (and gain CD45RO) and express higher FOXP3 levels (Li, Li, Tsun, & Li, 2015). Naïve Treg cells can proliferate and differentiate into effector Treg cells after TCR activation (Li *et al.*, 2015).

Treg are unable to produce typical cytokines that other T cells produce such as IL-2. This explains the requirement of IL-2 (produced by other T cells) for Treg to proliferate. They are also less responsive *in vitro* to T-cell receptor (TCR) activation (Shevach, 2006). They do, however, have the ability to produce immunosuppressive cytokines such as IL-10, TGF-β and the recently discovered IL-35.

1.3.3 Mechanisms of Immunosuppression by Regulatory T cells

Tregs can survive for relatively long periods of time as naïve cells but when they encounter their TCR ligand such as through APCs, they will start expressing activation markers and begin to undergo expansion (Klein, Khazaie, & von Boehmer, 2003; Tang *et al.*, 2004; Tarbell, Yamazaki, Olson, Toy, & Steinman, 2004). Such activated cells will accumulate together with other T effector cells in sites of infection and inflammation (Huehn *et al.*, 2004). The co-recruitment of CD4+ and/or CD8+ effector cells with activated Treg determines the specificity of immunosuppression. While Treg may suppress bystander T cell subsets that happen to be in their vicinity and that have not encountered a TCR ligand, this will not result in systematic immunosuppression because the majority of these bystander cells will be distributed throughout the body and were not recruited by antigen presentation to inflamed sites together with the activated Treg (Kretschmer *et al.*, 2008).

There is evidence that Treg cells have Th subset-specific reprogramming to control different immune responses. For example, it is thought that Treg that express T-bet suppress Th1 responses (Koch *et al.*, 2009), GATA-3- and IRF4-expressing Treg suppress Th2 responses (Wang, Su, & Wan, 2011; Zheng *et al.*, 2009) and STAT3 expression in Treg cells suppress Th17 responses (Chaudhry *et al.*, 2015). Indeed, the absence of IRF4 expression in Treg resulted in a spontaneous induction of Th2-mediated inflammation while mice with STAT3-deficient Treg have increased Th17 cells and they develop a fatal colitis (Chaudhry *et al.*, 2015; Y. Zheng *et al.*, 2009). However, the mechanism through which Treg suppress via these transcription factors specific to different T cell lineages is currently unclear (Schmidt, Oberle, & Krammer, 2012).

Mechanisms of immunosuppression by Treg can be grouped into three different modes which include suppression mediated via cytokine secretion, suppression via cell to cell contact, and suppression via metabolic disruption (Li *et al.*, 2015).

Suppression mediated via cytokine secretion involves the production of the inhibitory or anti-inflammatory cytokines IL-10, TGF-β and the recently described IL-35 by Treg to inhibit the action of effector T cells. It is though that IL-10 reduces the production of IFN-γ and IL-2 (Taga & Tosato, 1992) while TGF-β suppresses T cells by reducing the cell-cycle rate and subsequent rounds of cell division rather than inducing apoptosis (McKarns & Schwartz, 2005; Shevach, 2006). However, neutralization of either IL-10 or TGF-β does not stop *in vitro* suppression, suggesting that there are other mechanisms of immunosuppression by Treg (Collison *et al.*, 2007; Kearley, Barker, Robinson, & Lloyd, 2005; Nakamura, Kitani, & Strober, 2001).

Another mechanism of Treg immunosuppression is through direct cell to cell contact. Treg directly kill effector T cells including CD4+ T cells and CTLs but also NK cells or B cells. This cytotoxic action is possible using a granzymes or perforin-dependent mechanism similar to that used by cytotoxic T cells (discussed in section 1.2.3.1). It has been demonstrated that 5-30% of Treg in a tumour microenvironment express granzyme B and these cells lyse NK cells and CTLs using this and perforin cytotoxic molecules (Cao *et al.*, 2010). Another secreted molecule that might play a role in apoptosis of effector cells by Treg is galectin-1 which is a member of a highly conserved family of β-galactoside binding proteins (Garin *et al.*, 2007). Galactin-1 is preferentially expressed on Treg and when these bind to effector T cells they cause cell cycle arrest and apoptosis (Shevach, 2009).

Also through direct cell to cell contact, Treg deliver a negative signal to responder T cells by upregulating intracellular cyclic Adenosine monophosphate (AMP) which leads to inhibition of T cell proliferation and formation of IL-2 (Shimon Sakaguchi, Wing, Onishi, Prieto-Martin, & Yamaguchi, 2009).

Treg also modulate immune responses through the direct inhibition of DCs which in turn leads to inefficient activation of effector T cells by these antigen presenting cells. As mentioned in section 1.2.3.2.4, Treg highly express CTLA-4 which interacts with CD80 and CD86 that are expressed on the surface of DCs. In turn, this interaction results in the upregulation and secretion of indoleamine 2, 3-dioxygenase (IDO) by DCs. IDO molecule breaks down tryptophan and generates reactive oxygen species (ROS). Depletion of tryptophan obstructs activation and proliferation of T cells while the ROS generated exert cytotoxic effects on effector T cells (Oderup, Cederbom, Makowska, Cilio, & Ivars, 2006; Serra *et al.*, 2003).

As previously discussed in section 1.3.2, Treg cells highly express the IL-2 receptor α-chain, CD25, on their cell surface, which facilitates high binding of IL-2. However, IL-2 is also critical for the proliferation and activation of other effector T cells. As Treg have high binding affinity to IL-2, another mechanism of immunosuppression may be by consuming and depriving effector T cells of local IL-2 (de la Rosa, Rutz, Dorninger, & Scheffold, 2004).

In most scenarios, the mechanism employed by Treg likely depends on the disease setting, which cell type they are targeting, the local inflammatory setting and the anatomical location (Sawant & Vignali, 2014).

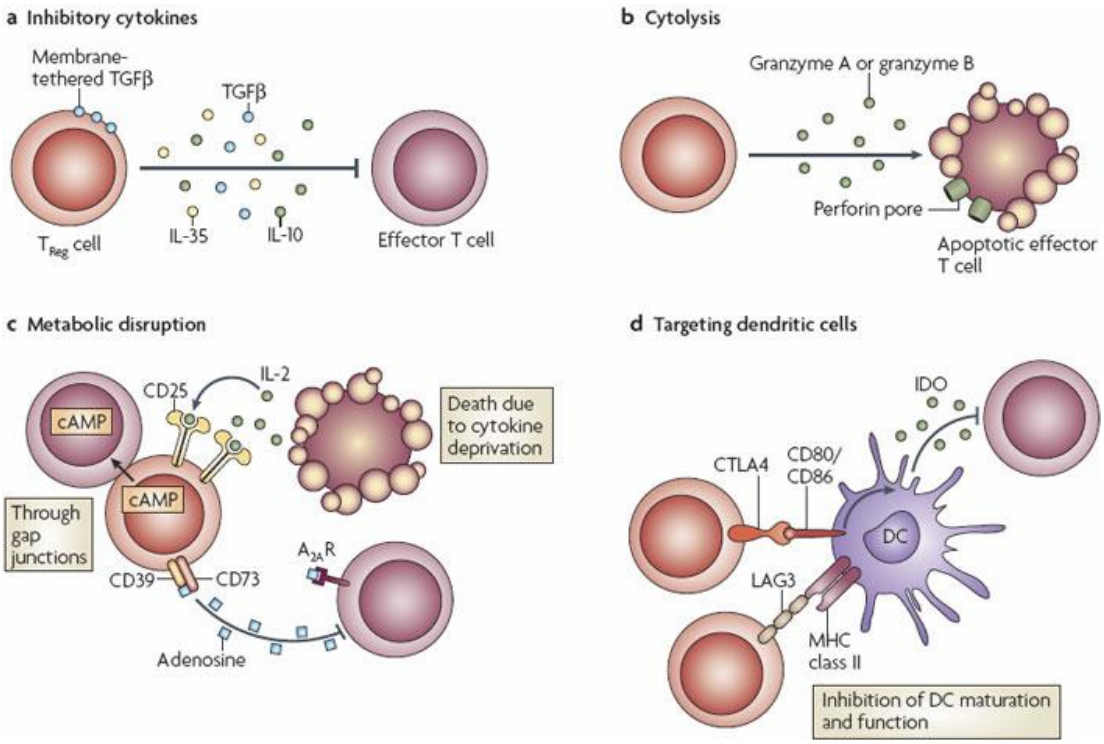


Figure 1.4: Mechanisms of Immunosuppression by Treg. Obtained from Vignali, D. A. A., Collison, L. W., & Workman, C. J. (2008). How regulatory T cells work. *Nature Reviews Immunology*, 8, 523–532. https://doi.org/10.1038/nri2343. *Permission to use image was obtained from the authors.*

1.3.4 Stability and Plasticity of Regulatory T cells

It is largely thought that peripheral Treg are derived mainly from thymic Treg precursors and that conversion in nature is rare (Fontenot *et al.*, 2005). However, extrathymic conversion of naive T cells to Treg is possible under certain environmental conditions (W. Chen *et al.*, 2003; Kretschmer *et al.*, 2005). For instance, as already mentioned in section 1.3.2 when naive peripheral T-cells are activated in the presence of TGF-β and IL-2, expression of FOXP3 is upregulated and the resulting Treg cells are suppressive both *in vitro* and *in vivo* (Chen *et al.*, 2003; Fantini *et al.*, 2004). In addition, all-trans retinoic acid, a vitamin A metabolite, seems to further enhance this conversion and in mice it was found to be important for gut and oral tolerance toward food and environmental antigens (Coombes *et al.*, 2007; Sun *et al.*, 2007). Indeed, TGF-β is found to

be highly enriched in the intestine (Murai, Krause, Cheroutre, & Kronenberg, 2010). TGF-β might be promoting this conversion because it decreases the methylation of the conserved non-coding DNA sequence 2 region (CNS2) next to the FOXP3 promoter in peripheral Treg facilitating the mRNA transcription of FOXP3 (Walker, Kublin, & Zunt, 2014). On the other hand, retinoic acid might indirectly enhance Treg formation by acting directly on effector T cells and inhibiting their production of cytokines, such as IL-4 and IFN-γ, that prevent FOXP3 expression (Hill *et al.*, 2008).

Apart from TGF-β, it was also discovered that FOXP3 expression in $CD4^{+}CD25^{-}$ T cells can also be induced by the main effector cytokine for Th1, IFN-γ but not by other Th1 and Th2 cytokines. In an established animal model of Multiple Sclerosis, Experimental Autoimmune Encephalomyelitis (EAE) which is predominantly mediated via Th1 autoimmunity, Wang *et al*, 2006 found that IFN-γ knock out in mice were associated with impaired CD4+CD25+ Treg function compared with that of wild type mice adding to the severity of EAE in IFN-γ–deficient mice. They also demonstrated that in both human and mouse systems, *in vitro* treatment of CD4+CD25– T cells with IFN-γ led to their conversion to CD4+ Treg as characterized by increased expression of FOXP3 and enhanced regulatory function.

When it comes to conversion of Treg into other types of T cells, as such, nTreg produced in the thymus are a stable lineage with minimal capacity to convert into Th cells. However, iTreg cells may lose FOXP3 expression and convert into Th cells under certain conditions. This could be explained by the fact that the FOXP3 promoter and the CNS2 is highly demethylated in nTreg and this facilitates mRNA transcription of FOXP3 whereas in *in vitro* iTreg, the promoter of FOXP3 and CNS2 is substantially methylated (Zheng *et al.*, 2010).

FOXP3+ iTreg cells can downregulate their expression of FOXP3, lose suppressor functions and in some cases convert into Th subsets. Loss of FOXP3 expression and acquirement of effector Th cell function was observed to occur especially in the presence of inflammatory cytokines such as IL-6 and IFN-γ that induce effector T cells (Shevach & Davidson, 2006). Indeed, in FOXP3-reporter mice, 10-20% of FOXP3+ T cells lost FOXP3 expression and exhibited an inflammatory Th phenotype producing inflammatory cytokines such as IFN-γ, IL-2 and IL-17 in inflamed gut-associated tissues (Zhou, Bailey-Bucktrout, Jeker, & Bluestone, 2009). These observations indicate that FOXP3+ iTreg are plastic and can convert into Th-like cells under certain inflammatory conditions.

However, other mice studies have shown that in general Treg are stable and resistant to conversion into Th cells under the same inflammatory conditions as only a very small fraction of FOXP3+ T cells lost FOXP3 expression (Komatsu *et al.*, 2009; Rubtsov *et al.*, 2010). Komatsu *et al.* found that only the FOXP3+ T-cell population that expressed low levels of CD25 could lose FOXP3 expression and acquire a Th17 phenotype, whereas FOXP3+ T cells that expressed high levels of CD25 remained rather stable.

Interestingly, in contrast to murine Treg, human Treg seem to be less stable. FOXP3+ T cells that express high levels of CD25 and present in human blood could differentiate into cells that produce IL-17 upon TCR activation *in vitro* and in the presence of inflammatory cytokines that includes IL-1b, IL-21 and IL-23 (Koenen *et al.*, 2008; Wang *et al.*, 2009). One possible explanation for this difference between mouse and human Treg stability could be due to mice model differences including different intensities of reporter gene expression and distinct strengths of the TCR and inflammatory signals (Li *et al.*, 2015).

Deknuydt *et al.*, 2009 reported that even stimulation of human nTreg under Th17 polarizing conditions and in the presence of IL-2 converts them into Th17 cells. Conversion of Treg into Th17 cells was shown to occur both from naïve nTregs and, to a higher extent, from memory Tregs. Conversion of Treg into Th17 cells was induced by IL-1β released by monocytes activated by microbial stimuli. This involved downregulation of FOXP3 and suppressive functions.

Komatsu *et al.*, 2014 showed that in autoimmune arthritis, FOXP3+ Treg lose FOXP3 expression and undergo differentiation into Th17 cells which accumulated in inflamed joints. It was found that the conversion was mediated by synovial fibroblast-derived IL-6.

Conversion into other Th types including Th1 and Th2 has been less studied although there are indications that under different inflammatory conditions, FOXP3+ Treg start expressing transcription factors and releasing effector cytokines typical of other Th subsets (Chaudhry *et al.*, 2015; Oldenhove *et al.*, 2010; Y. Zheng *et al.*, 2009). For instance, it was found that IL-12 in a Th1 inflammatory environment triggered by *Toxiplasma gondii* infection may lead to induction of T-bet expression in Treg as well as IL-12 or other inflammatory cytokines and may contribute to the loss of FOXP3 expression when Treg are transferred to lymphopenic mice that lack IL-10 (which is required by Treg). These “ex” Treg were found to release IFN- γ which is typical of Th1 cells (Oldenhove *et al.*, 2009).

The ability to modulate Treg might have an significant implications in the management of autoimmune diseases as well as tumour immune-evasion.

1.4 Inflammation, Cancer and Anti-Tumour Immunity

1.4.1 Acute and chronic inflammation

Inflammation is defined as 'a complex biological response to harmful stimuli and characterized by heat (calor), redness (rubor), pain (dolor), and swelling (tumour)'. There are two stages of inflammation which are acute and chronic. Acute inflammation is an initial stage of inflammation and helps the body to get rid of infections, lasts for a short period of time and is generally regarded as therapeutic inflammation (Aggarwal, Vijayalekshmi, & Sung, 2009). When an inflammation persists for a long period of time, the second stage of inflammation called chronic inflammation sets in (Lin & Karin, 2007). Chronic inflammation is prolonged inflammation due to persistent injury or infection. Monocytes, macrophages, and lymphocytes have an active role in chronic inflammation which is associated with simultaneous fibrosis and tissue healing (Murphy, 2011). Chronic inflammation increases the risks of chronic illnesses, including cancer, cardiovascular diseases, diabetes, obesity, pulmonary diseases, and neurologic diseases (Aggarwal *et al.*, 2009). Symptoms of acute inflammation, such as pain, redness, swelling, warmth, and loss of function are easy to recognize. However, symptoms of chronic inflammation can be hard to detect. Chronic inflammation can be caused by endotoxins, carcinogens such as cigarette smoking, chemotherapeutic agents, hyperglycemia, radiation, inflammatory cytokines and as well as growth factors that promote inflammation (Aggarwal et al., 2009).

The German pathologist Rudolph Virchow (1821- 1902) once remarked that 'chronic irritation which is manifested by a chronic inflammation is a key promoter of cancer'. Indeed, inflammation precedes most cancers and chronic inflammation increases cancer risk (Grivennikov, Greten, & Karin, 2011). Up to 20% of cancers are linked to chronic infections (Aggarwal *et al.*, 2009). Chronic inflammation associated with

persistent infections or autoimmune disease causes tumour development by contributing to the induction of oncogenic mutations, genomic instability, early tumour promotion and enhanced angiogenesis (Grivennikov *et al.*, 2011). Environmental factors such as prolonged exposure to mutagens, and obesity, can also result in low-grade chronic inflammation that results in tumour development.

Over time, chronic inflammation can cause DNA damage leading to the development of tumours. Sustained cell proliferation due to tissue damage and a microenvironment rich in inflammatory cells, growth factors and DNA-damage-promoting agents such as reactive oxygen species released by inflammatory cells, promote the chances of tumour growth.

For instance, people who suffer from chronic inflammatory bowel diseases, such as ulcerative colitis and Crohn disease, have up to a tenfold increased risk of colorectal cancer(M'Koma, 2013). Persistent gastritis induced by *Helicobacter pylori* increases the risk of stomach cancer even by 75% (Eiró & Vizoso, 2012), whereas types B and C hepatitis promote the formation of hepatocellular carcinoma (Salem, Attia, & Galal, 2016).

Carcinogenesis occurs in two steps: it is initiated when the DNA sequence in somatic cells undergoes genetic mutations that remain hidden for many years until another stimulus promotes these mutated cells. This stimulus can be brought about by factors released during chronic inflammation (Korniluk, Koper, Kemona, & Dymicka-Piekarska, 2017).

Once a tumour has grown, inflammation is also involved in tumour progression and metastasis. When the tumour cells in a solid tumour's core become oxygen and nutrient deprived, necrotic cell death occurs that activates cytokine-producing inflammatory cells and create an inflammatory tumour microenvironment referred to as

tumour-associated inflammation (Grivennikov *et al.*, 2011). Various forms of therapy also cause trauma and necrosis and promote tumour-associated inflammation. The cytokines released by the inflammatory cells activate pro-survival genes in residual cancer cells which makes them more resistant to treatments and result in tumour re-emergence. However, necrosis caused by therapy might increase tumour antigen presentation thus stimulating an anti-tumour immune response that improves the therapeutic outcome (Grivennikov *et al.*, 2011). Eventually neo-angiogenesis, which is the creation of new blood vessles to supply oxygen to oxygen-deprived cancer cells, promote tumour progression and finally metastasis by spreading to other regions distant from the primary site of the tumour.

The tumour microenvironment, in addition to the cancer cells and their surrounding stroma, contains cells of the innate immune system such as tumour associated macrophages (TAM) and neutrophils as well cells from the adaptive immune system such as T and B lymphocytes as a result of these inflammatory processes (de Visser, Eichten, & Coussens, 2006). These cells communicate with each other either by direct contact or by means of soluble factors such as cytokines. The abundance and activation state of the different cell types present in the tumour microenvironment as well as the cytokine milieu dictates whether the inflammation will promote tumour growth or whether anti-tumour immunity will result (Lin and Karin, 2007; Smyth *et al.*, 2006).

In contrast to chronic inflammation, it has been demonstrated that acute inflammation can have anti-tumour effects and is sometimes referred to as “therapeutic inflammation” (Korniluk *et al.*, 2017). In 1868, the physicist Burns described cases of severe streptococcal infection during which patients experienced regression of neoplastic disease. A few years later, Coley created a certain microbial preparation made from Gram

Negative bacteria called “Coley’s toxin” which was successfully used to treat cancer. It is thought that the microbial preparation induces activation of the immune system and the bacterial lipopolysaccharides present in the preparation induces the release of TNF-α, which stops tumour growth (Aggarwal & Sung, 2011).

Nowadays, a special vaccine called Bacille Calmet Guerin, originally used against tuberculosis and containing an attenuated *Mycobacterium bovis* strain, is used in the treatment of bladder squamous carcinoma (Grivennikov *et al.*, 2011). Studies are also being conducted on the potential use of *Salmonella typhimurium*-containing vaccines in oncological therapy (Zheng & Min, 2016).

1.4.2 The Role of TH and CTLs in Anti-Tumour Immunity

As mentioned in section 1.2.3.1, CD8+ CTLs exhibit anti-tumour immunity. Indeed, CD8+ CTLs alone or in combination with CD4+ Th1 cells constitute the best adaptive immune response against cancer (Castelli *et al.*, 2000).

It is thought that CD8+ T cells reject tumours by direct cell killing and by inhibiting angiogenesis in an IFN-γ dependent manner (Qin *et al.*, 2003). Effector CTLs promote the apoptotic death of tumour cells, using perforin/granzyme pathway and via direct cell to cell contact using the Fas ligand binding to the Fas receptor mechanism described earlier in section 1.2.3.1 (Maher & Davies, 2004).

CTL are attractive mediators of anti-tumour immunity. Firstly, the widespread expression of MHC class I molecules means that CTLs could, in principle, be deployed against malignancies of diverse origin (Maher & Davies, 2004). Indeed they are extremely sensitive in that even a single peptide can trigger cytolysis. Secondly, they are very

efficient for systematic disease as they continuously recirculate throughout the body to seek out antigens (Sykulev *et al*, 1996).

The number of T-cell epitopes derived from tumour antigens and identified by CTLs exceeds 200 and this number is still increasing (Novellino, Castelli, & Parmiani, 2005). The proteins making up these antigens are divided into four groups: Group I which are patient-specific antigens arising from somatic mutations and which can be expressed by single tumours only or shared among tumours of the same type; Group II consisting of tumour-specific antigens that are shared among cancer patients and can be viral-derived or as a result of mutations; Group III which include shared tumour antigens corresponding to normal tissue-specific gene products, also called differentiation antigens; and Group IV which are antigens consisting of normal proteins that are mainly expressed by tumours but not present in normal tissue (Andersen *et al.*, 2006).

Indeed several immunotherapeutic trials are now using antigen-specific vaccinations to induce anti-cancer CTLs. Cancer-associated antigens that have been studied include 'Cancer-Testis' (CT) antigens expressed in different tumours but not in normal tissues except testis, melanocyte differentiation antigens such as Melan A/MART-1, tyrosinase, gp100/Pmel17, and gp75/TRP-1 (which are expressed both in melanoma and normal melanocytes), point mutations of normal genes such as mutations of the oncogenic proteins p53 and RAS in breast cancer, antigens that are overexpressed in 30% of malignant tissues in breast and ovarian cancer for example HER-2/neu, and viral antigens (Jäger, Jäger, & Knuth, 2001).

CTLs are usually activated and recruited by Th cells. Th cells in turn can be activated by either APCs such as dendritic cells (that have encountered an antigen in the tumour bed) or directly by tumours that express MHC Class II. Examples of tumours that

express MHC Class II include melanoma, lung cancer, breast cancer and osteosarcoma which can directly act as APCs and activate Th cells (Knutson & Disis, 2005).

Th cells can directly activate CTLs through the release of cytokines such as IL-2 (Cheever & Chen, 1997) or by direct cell-to-cell interaction between the Th cell and CTLs through co-stimulatory molecules present on the surface of CTLs such as CD127, CD134 and MHC Class II (Ii, Lu, Kobayashi, Kennedy, & Celis, 2002). Th cells can also activate other cell types that influence CTLs. For instance, IFN-γ released by Th1 cells activate APCs to upregulate molecules that contribute to increased tumour antigen presentation to CTLs. This causes expansion of CTLs which then directly kill the tumour cells. In addition to activating and expanding CTL from the naïve T cells, studies also show that help by Th cells is required for reactivation of memory CTL to a fully functional tumour killer cell (Gao *et al.*, 2002).

Tumour-infiltrating lymphocytes (TILs) are a determinant in the progression and aggressiveness of tumours (Jiang *et al.*, 2017) and a high number of CTLs and Th cells correlate with better survival in some cancers including invasive colon cancer (Galon, 2006), melanoma (Laghi *et al.*, 2009), pancreatic cancer (Swann & Smyth, 2007), head and neck cancer (N. Nguyen *et al.*, 2016), non-small cell lung cancer (Brambilla *et al.*, 2017), colorectal cancer and gastric cancer (Liu *et al.*, 2015).

Similarly, deficiency in T cells or disruption in their cytotoxic programmes can render experimental animals more susceptible to spontaneous or chemical carcinogenesis (Shankaran *et al.*, 2001; Swann & Smyth, 2007)

However, in certain types of cancers, such as early triple-negative breast cancer, T cells are not as effective and despite the substantial high amount of lymphocyte infiltration, tumour progression still occurs (Pruneri *et al.*, 2016). There is also contrasting

evidence that T cell subsets including Th1 cells (Hanada *et al.*, 2006), Th2 cells (Aspord *et al.*, 2007; DeNardo *et al.*, 2009) and Th17 cells (Wang, Wildt, Castro, & Xiong, 2008) found in solid tumours promote tumour progression, or metastasis although the mechanisms by which they do this remains inconclusive. In breast cancer, for example, the presence of tumour infiltrating lymphocytes with high CD4+ to CD8+ ratio and particularly a high Th2 to Th1 ratio correlates with poor prognosis (Kohrt *et al.*, 2005). It has been speculated that this is probably because Th2 cells in general enhance chronic inflammation and are therefore overall tumour-promoting. In contrast, Th1 are primarily anti-tumourigenic as, through IFN-γ, they promote CTLs which eliminate tumour cells.

What makes the same T cell subset anti-tumourigenic in one cancer and pro-tumourigenic in another remains still unknown and may hold the key to the development of successful cancer immunotherapy.

1.4.3 Tumour Immune Evasion by Regulatory T cells

Most tumours elicit an immune response because of their tumour antigens, thus enabling the immune system to identify the tumour from other non-cancerous cells. This means that usually solid tumour are rich with many tumour infiltrating lymphocytes as discussed previously in section 1.4.2. However, tumours suppress host immunity by hijacking normal immunoregulatory mechanisms such as T cell checkpoint molecules and FOXP3+ Treg.

Fujimoto, Greene, & Sehon, 1976 have demonstrated that suppressive lymphocytes were present in tumour-bearing mice which efficiently suppressed tumour rejection by immune cells in methylcholanthrene-induced sarcoma. It could also be demonstrated that these cells were infiltrating tumour tissue and circulating at increased

proportions in patients with lung and ovarian cancer (Woo *et al.*, 2001). It is nowadays acknowledged that these suppressive cells are Treg and that in general they suppress protective anticancer immune responses and promote tumour-immune evasion and hence tumour progression.

Treg preferentially to other types of T cells seem to accumulate in the tumour microenvironment defending and preventing attack of the tumour by other effector T cells. Indeed, tumour Treg numbers are commonly elevated in tumours but also in peripheral blood, or lymphoid organs of many tumour-bearing hosts (Liu *et al.*, 2009). In fact, while Treg normally make up only about 4% of CD4+ T cells in human peripheral blood, near the tumour microenvironment this number can increase from 18% in breast cancer (Ruffell *et al.*, 2012), to 30% in ovarian cancer (Curiel *et al.*, 2004) and up to 40% in pancreatic cancer (Nummer *et al.*, 2017). Indeed, high frequency of Treg in cancer patients has been associated with poor prognosis. The suppressive activity of Treg in the blood of cancer patients is also more enhanced than that of healthy individuals (Yokokawa *et al.*, 2008). It is thought that this occurs because in a tumour microenvironment, Treg are more exposed to tumour-associated antigens which are essentially self-antigens and which Treg detect with a higher affinity than effector T cells (Chaudhary & Elkord, 2016). However, the mechanism by which activated Treg accumulate in the tumour microenvironment is still elusive and varies from cancer to cancer.

Depletion of Treg in the peripheral blood of patients with colorectal cancer exposed the CD4+ responses to tumour antigens whereas on the other hand adoptive transfer of purified Treg inhibited tumour-specific antitumour immunity mediated by CD8+ and NK cells *in vivo* (Ghiringhelli *et al.*, 2005).

Transient Treg depletion resulted in the regression of metastatic lesions in advanced stage melanoma patients (Jacobs, Nierkens, Figdor, de Vries, & Adema, 2012). Treg depletion in breast cancer patients prior to tumour resection and radiotherapy is associated with improved clinical outcome (Rasku *et al.*, 2008). Also, Treg depletion followed by cancer antigen vaccination generated effective and unimpeded CD4+ and CD8+ anti-tumour T-cell responses in metastatic breast cancer patients (Rech *et al.*, 2012).

Tumours may differentiate, expand, recruit, and activate Treg via multiple mechanisms. It is thought malignant cells, bystander fibroblasts, dendritic cells and tumour associated macrophages (TAMs) in the tumour stroma produce and secrete several chemokines that attract Treg and recruits them from blood circulation to the tumour. Pre-existing Treg are then activated and start proliferating upon antigen-specific presentation in the presence of TGF-β and IL-10. These two anti-inflammatory cytokines are usually found at high levels within the tumour microenvironment and are usually released by the myeloid derived suppressor cells (MDSCs) and immature DCs. Additionally, these two cytokines along with antigen presentation that is provided by DCs, TAMs and MDSCs promote the conversion of conventional T cells into suppressive Treg (Mougiakakos, 2011).

Treg suppress tumour-specific CTLs by affecting their function, in particular by stopping them from secreting perforin and/or granzyme that would otherwise kill tumour cells (Kretschmer *et al.*, 2005). In addition, they also hinder dendritic cell function and antigen presentation. It has also been shown that Treg suppress other kinds of cells such as NK cells via TGF-β (Ghiringhelli *et al.*, 2005).

Intra-tumoural Treg also play a role in tumour angiogenesis which is the key step for tumour metastasis. It has been found that in an *in vitro* model of ovarian cancer, Treg under hypoxic conditions similar to an *in vivo* tumour environment, express higher levels of vascular endothelial growth factor-A (VEGF-A) than under normal conditions. VEGF-A is a growth factor which drives the process of angiogenesis (Facciabene *et al.*, 2011).

Given their roles in promoting tumour progression and immune escape, managing Treg offers promising therapeutic targets and to date, cancer-induced immunotolerance mediated by Treg represent a major obstacle to effective cancer immunotherapy.

However, there is also evidence that Treg may play an anti-tumourigenic role under certain circumstances due to their ability to suppress tumour-promoting inflammation in certain types of cancers such as colorectal cancer, head and neck cancer and oesophageal cancers (Erdman & Poutahidis, 2010). Such observations in these specific kinds of cancers can be explained from the fact that Treg are critical in maintaining immune tolerance in areas which are more prone to inflammation such as the airways, the gut and mucosal interfaces (Ladoire, Martin, & Ghiringhelli, 2011; Weiner, da Cunha, Quintana, & Wu, 2011).

1.4.4 Managing and Targeting Regulatory T cells in Cancer

1.4.4.1 Current Existing Therapies

There are two types of therapeutic approaches to Treg. The first approach is to increase their number in patients with autoimmune diseases. The second is to decrease their number in cancer patients without causing systematic autoimmunity. Since this study is concerned with the management of Treg in cancer, we will focus on the second. However, it is of note to say that adoptive transfer of Treg in mice has been successfully

used to prevent and/or reverse pathologies such as Type 1 diabetes (Tang *et al.*, 2004), inflammatory bowel disease (Mottet, Uhlig, & Powrie, 2003), graft versus host disease (Hoffmann, Ermann, Edinger, Fathman, & Strober, 2002; Xia, He, Zhang, & Leventhal, 2006) and rejection of transplanted organs (Graca *et al.*, 2004)

A number of cancer therapies that target Treg have been developed. Some ongoing strategies infuse patients with Treg-depleted donor lymphocytes. Alternatively Treg cell numbers can be decreased *in vivo* using chemical reagents. For instance, the chemotherapeutic drug cyclophosphamide at long term and low dosages selectively eliminates Treg while preserving effector T cells (Ghiringhelli *et al.*, 2004).

Other strategies target Treg directly by binding to the surface molecules that they predominantly express such as CD25. In this way the agent itself binds to the CD25 and prevents binding of IL-2 which is required for Treg expansion. DAB(389)IL-2 (denileukin diftitox, ONTAK) also targets CD25 and is a recombinant protein made up of IL-2 and diphtheria immunotoxin (denileukin diftitox) which displays potent anticancer activity. It was found to result in an efficient reduction of Treg in colorectal cancer patients (Dannull *et al.*, 2005; Morse *et al.*, 2008) and enhances patient survival when CD25 blockade is done prior to adoptive cell therapies, cancer vaccination or other treatment in different cancers (Rech *et al.*, 2012; Yao *et al.*, 2012). Other anti-CD25 monoclonal antibodies (coupled or uncoupled to immunotoxins) such as basiliximab and daclizumab are currently under clinical evaluation. However, there is a downside to these agents due to their short-lasting activities (Mougiakakos, 2011).

Immune checkpoint inhibitors for cancer treatment are recent therapies that block immune checkpoint molecules or their ligands which are over-expressed in the tumour microenvironment and which block immune responses.

Monoclonal antibodies are perhaps the most common examples of immune checkpoint inhibitors and those that are designed to target Treg include anti-CTLA-4 (ipilimumab/tremelimumab) and anti-PD-1 (nivolumab/pembrolizumab) monoclonal antibodies which have been approved by FDA for the treatment of metastatic melanoma, non-small cell lung carcinoma, advanced renal cell carcinoma and Hodgkin's Lymphoma. CTLA-4 is highly expressed on Treg, and as described in section 1.3.3, contributes to the suppressor function of Treg. The generation of Treg cells is also promoted by PD-1 coupled with the presence of TGF-β (Francisco, Sage, & Sharpe, 2010). PD-1 is also highly upregulated on effector T cells that are exhausted, halting the production of IFN-γ and IL-2 (Chaudhary & Elkord, 2016). It is therefore apparent that blocking these immune checkpoint molecules would reduce Treg and their suppressive activity.

These monoclonal antibodies show improved response rates, progression-free and overall survival compared with conventional chemotherapy. Although some immune checkpoint inhibitors might show immune related adverse effects, these are usually manageable and the pros far outweigh the cons.

Promising clinical responses in melanoma patients have been reported when administered ipilimumab or tremelimumab and at the 2010 American Society of Clinical Oncology annual meeting it has been reported that the drugs extended the overall survival in patients with advanced melanoma by 10 months.

However, it has also been revealed that ipilimumab and tremelimumab as monotherapies or in combination have been found to significantly enhance the activation and expansion of effector T cells while leaving the Treg stably maintained or expanded with the Teff and CTLs (Kavanagh *et al.*, 2008; Tarhini *et al.*, 2014). This observation makes anti-CTLA 4 monoclonal antibody treatment somewhat questionable.

Although side effects are usually manageable, a number of serious adverse effects have been associated with the use of ipilimumab including gastrointestinal-immune related adverse effects, primarily colitis (including patients who developed life threatening intestinal perforation), autoimmune hepatitis as well as endocrine immune related adverse effects (Abdel-Wahab, Shah, & Suarez-Almazor, 2016).

Tremelimumab is able to revoke the suppressive activity of healthy donor Treg *in vitro* and treatment of this drug in advanced Stage IV melanoma patients selectively confers resistance of PBMC to Treg (Ménard *et al.*, 2008). This resistance to suppression by Treg is observed even after treatment. It is unclear whether the anti-PD-1 drug nivolumab inhibits the suppressive effects of Treg directly (possibly by downregulating FOXP3 expression) or via activation of effector T cells. The clinical studies investigating the impact of PD-1 blockade on Treg are limited (Gibney *et al.*, 2015; Weber *et al.*, 2013). Adverse effects associated with nivolumab include endocrine immune related adverse effects in particular autoimmune thyroid disease and pneumonitis, although no cases of systematic autoimmune disease or gastrointestinal immune related adverse effects were reported (Abdel-Wahab *et al.*, 2016)

The major challenge of these immune checkpoint inhibitors is how to prevent systematic depletion of Treg which could result in severe autoimmune diseases.

Although this approach has sometimes been successful, they have many downsides from difficult-to-handle side effects, lengthy preparation or treatment times and their cost especially if they are used in combination. Moreover, there are still a significant number of patients that do not respond well to the treatment.

Section 1.4.4.2 describes an alternative approach to managing Treg which is also the aim of this study.

1.4.4.2 Targeting Treg plasticity - The Trojan Horse Approach

It is thought that Treg are able to accumulate within the tumour microenvironment in response to chemokines secreted by tumour cells and innate immune cells. The recruited Treg then start to expand *in situ* and proliferate efficiently in response to tumour derived factors primarily TGF-β and IL-10. In murine models, it has been reported that TGF-β promotes the conversion of non-suppressive $CD25^-$ conventional T cells to suppressive CD25+ Treg, although this has not yet been confirmed in human cancers (Chaudhary & Elkord, 2016; Ondondo, Jones, Godkin, & Gallimore, 2013).

The fact that there is evidence, as described in section 1.3.4, that Treg are plastic and can be converted into Th cells after being exposed to an inflammatory environment, has important implications for cancer therapy as infiltrated and/or converted Treg in solid tumour microenvironments impeding anti-tumour immunity are reduced whilst the newly converted Th cells (which would be already present within the tumour and exposed to tumour antigens), through the release of cytokines, recruit CTLs which would then attack the tumour tissue. The aim of this 'Trojan Horse Approach' is to try and stimulate, using inflammatory agents, the conversion of Treg that have infiltrated inside the solid tumour to Th, without affecting those in periphery in order to reduce the risks of autoimmunity. Moreover, the inflammatory response generated would be acute, and not chronic thereby acting as anti-tumourigenic rather than pro-tumourigenic effects. This is similar to the kind of response that Coley's toxin generated but with a focus on Treg.

This approach has the advantage that the inflammatory mediators that will be used are cheaper than the monoclonal antibodies used as immune checkpoint inhibitors. Moreover, monoclonal antibodies are only meant to target a specific molecule, whereas

the inflammatory response triggered by inflammatory mediators will result in the release and suppression of many molecules with multiple effects. Additionally, only Treg present within the tumour are affected, thus preventing any systematic side effects.

As discussed in section 1.3.4, conversion of Treg into Th17 cells has already been achieved. However, Th17 cells are not the best weapon against tumour cells. Neither are Th2 as they are usually considered as being pro-tumourigenic. The focus of this study will be to investigate whether it is possible to convert Treg into other subsets of Th cells, focusing particularly on Th1 cells, since they are the main Th subset involved in anti-tumour immunity and recruitment of CTLs.

Should this be achieved, future treatment could involve injecting the Treg-rich tumour with these inflammatory agents to improve the anti-tumour response prior to surgical removal of the cancer as a way to shrink the tumour (rather than relying on current used methods such as radiotherapy) and to prevent metastases by lingering cancer cells.

The agents investigated in this study to try and drive Treg modulation include IFN-γ and Toll-like Receptor (TLR) ligands which have been recently proposed as potential anti-cancer immune-therapeutics. A review of them is given in the next section.

1.5 Interferon-γ in Cancer Immunotherapy

Interferon gamma, IFN-γ is the main cytokine released by Th1 cells and CTLs and it is also the main principal effector cytokine of cell-mediated immunity (Refaeli, Parijs, Alexander, & Abbas, 2002). IFN-γ was first identified as having anti-viral properties, however recently it has also been shown to play a role in cancer immunology. The main source of IFN-γ in tumours are predominantly the tumour infiltrating lymphocytes and

release of this cytokine was shown to be of importance in cancer immunosurveillance (Ni & Lu, 2018). IFN-γ is known to induce cell cycle arrest in tumour cells, apoptosis and necroptosis (a programmed form of necrosis or inflammatory cell death) thus decreasing tumour cell growth. In cancer such as breast cancer (Kochupurakkal, Wang, Hua, & Culhane, 2015), colorectal cancer (Lu Wang *et al.*, 2015) and hepatocellular cancer (Li *et al.*, 2012), IFN-γ enhances the production of cell cycle inhibitor proteins which in turn inhibit the proliferation of tumour cells. It has also been shown that IFN-γ slows down tumour growth by reducing tumour vasculature and inducing tumour ischemia (Kammertoens *et al.*, 2017).

IFN-γ was found to play a role in determining the efficacy of ionizing radiation therapy in cancer. Gerber *et al.*, 2013 observed that in IFN-γ-knockout mice, ionizing therapy had no effect on tumour growth in a mouse colon cancer model, however wild type mice were responsive to the treatment. T cells treated with ionisation radiation have an increased capacity to lyse tumour. This is because irradiation damages and kills cells which in turn induces an inflammatory signal via the secretion of cytokines in the microenvironment which includes the release of IFN-γ by Th1 and CTLs. Hence, IFN-γ is important in meditating the anti-tumour effects of radiation therapy.

IFN-γ however does not only act directly on tumour cells but also indirectly on immune cells in the tumour micro-environment. IFN-γ also activates APCs to upregulate cytokines such as IL-12 and IL-18 (which further enhances IFN-γ production) and co-stimulatory molecule CD28 to promote Th1 and CTLs which are the T cells with anti-tumour activity (Aqbi, Manjili, Wallace, Sappal, & Payne, 2018; Kursunel & Esendagli, 2016). It also directly induces signals in T cells to function efficiently and promotes tumour elimination by inhibiting the function of Treg. Recently Overacre-delgoffe *et al.*, 2017 found that deletion of neutropilin-1 in Treg, (a transmembrane protein required for

their stability and function in tumours but is not critical for peripheral tolerance) in a mouse model of melanoma, produce IFN-γ and result in tumour elimination. This is because the IFN-γ produced induces fragility in Treg (described as Treg that retain FOXP3 expression but lose their suppressive activity), boosts the anti-tumour response and thus facilitates tumour clearance. They further showed that Treg fragility induced by IFN-γ enhanced the response to PD-1 targeted immunotherapy, which, as described in section 1.4.4.1 is one of the immune checkpoint blockade therapies used to target Treg in cancer immunotherapy.

Several publications have also demonstrated that anti-CTLA-4 (ipilimumab/tremelimumab) and anti-PD-1 (nivolumab/pembrolizumab) that target Treg also result in an increase in IFN-γ production, which in turn leads to the elimination of cancer cells (Chen *et al.*, 2009; Peng *et al.*, 2012). Indeed, Manguso *et al.*, 2017 confirmed that resistance to immunotherapy is attributed to defects in IFN-γ signalling. These indicate that cancer immunotherapy in these instances can be improved by IFN-γ treatment.

Although Overacre-delgoffe *et al.* showed that FOXP3 expression in Treg was retained in the presence of IFN-γ, other studies have shown that when IFN-γ levels are elevated during acute inflammation, Treg are observed to lose FOXP3 expression in the presence of IFN-γ (Shevach & Davidson, 2006). Similarly, Wei *et al.*, 2007 also demonstrated that the addition of IFN-γ (or IL-12) to cultures of naïve CD4+ T cells resulted in a substantial reduction in the percentage of FOXP3 expression. *In vitro* differentiation studies by Chang et al, 2009 have demonstrated, that conversion of naïve T cells into FOXP3+ induced Treg was significantly inhibited by IFN-γ. Therefore, these studies provide evidence that IFN-γ plays a role in anti-tumour immunity.

However, IFN-γ, with the exception of malignant osteoporosis, has not been approved by FDA to treat patients with a variety of cancer types. This could be explained by the evidence that IFN-γ also plays a role in tumour evasion (Aithal *et al.*, 2018; Ni & Lu, 2018).

Morgado *et al.*, 2016 have shown that IFN-γ-signalling, coupled to TNF-α promotes tumourigenesis via the transmembrane mucin MUC16 using the NFκB pathway. MUC16, which is over-expressed and is used as a biomarker in cancer, in turn suppresses NK cell killing capacity and reduces the sensitivity of tumour to drug therapy (Ni & Lu, 2018).

Although it has been demonstrated that IFN-γ slows down tumour growth by reducing neovascularisation, Lu *et al.*, 2014 showed that, in ovarian cancer clinical specimen, IFN-γ suppressed TNFSF15, a pro-inflammatory cytokine belonging to the TNF cytokine family. TNFSF15 inhibits neovascularisation so its suppression leads to angiogenesis in tumours.

Also, although it has been implied that anti-CTLA-4 and anti-PD-1 therapy improves in the presence of IFN-γ, the cytokine also itself induces the expression of CTLA-4 and PD-1 in tumour cells. Mo *et al.*, 2018 reported than in melanoma, IFN-γ-induced melanocytes to express the CTLA-4 gene which was dependent on IFNGR/STAT1 signalling pathways.

Under normal conditions, IFN-γ induces PD-1 expression on APCs which is required to control T cell activation and avoid tissue damage. However, under cancer conditions, tumour cells express PD-L1 in order to escape anti-tumour immunity. Bellucci *et al.*, 2015 showed that increased expression of PD-L1 by tumour cells resulted in enhanced resistance to elimination by NK cells and blockade of IFN-γ led to higher tumour death by NK cells.

Apart from CTLA-4, IFN-γ also induces the expression of IDO in tumour cells including renal cell carcinoma and murine renal cell adenocarcinoma and high levels of IDO are correlated with poor prognosis (Trott *et al.*, 2016). As described in section 1.3.3, IDO impedes activation and proliferation of T cells.

Some studies have also shown that IFN-γ has also been shown to increase Treg. Wang *et al.*, 2006 have shown that IFN-γ, in the presence as well as the absence of TCR co-stimulation, leads to the conversion of CD4+CD25- T cells into CD4+CD25+ Treg as shown by increased FOXP3 expression in both mouse and human experimental systems. Indeed, experimental autoimmune encephalomyelitis (mouse experimental model for multiple sclerosis) was suppressed by adoptive transfer of IFN-γ-treated CD4+CD25- T cells. It is thought that during inflammation, where substantial quantities of IFN-γ are released, a controlling mechanism is triggered which increases the number of Treg in order to regulate effector T cell numbers and thus prevent autoimmunity.

Therefore, the role of IFN-γ in cancer therapy remains contradictory. This study will explore the capacity of IFN-γ to modulate Treg phenotype and function, and will evaluate whether it will primarily favour anti-tumour immunity or not.

1.6 Toll-Like Receptors, their Ligands and Roles in Immunity

1.6.1 Toll Like Receptors – Introduction

Toll-like receptors (TLR) are a family of proteins that recognize and respond to conserved molecular patterns present on pathogens such as bacteria, viruses, fungi and protozoa known as Pathogen-Associated Molecular Patterns (PAMPs). PAMPs that are recognized by TLRs activate intracellular signalling pathways that lead to the induction of inflammatory molecules (such as IL-6, TNF-α and IL-12), chemokines and interferons, the

latter of which elicit anti-viral responses. TLR signalling also causes the upregulation of costimulatory molecules in DCs that in turn facilitate activation of the adaptive immune response (Van Maren, Jacobs, De Vries, Nierkens, & Adema, 2008).

TLRs have two domains: the first is an ectodomain which recognizes the PAMPs and is composed of leucine-rich repeats while the second one is a cytoplasmic domain, which is required for downstream signalling and which is homologous to the IL-1 receptor and therefore called toll/interleukin-1 receptor (TIR) domain (Akira, Uematsu, & Takeuchi, 2006).

Apart from PAMPs, TLRs also recognize self/endogenous ligands. Matzinger, 2012 proposed that the immune system is more concerned with tissue damage than with the origin of the antigens (i.e. whether self or non-self). In her 'danger theory' she suggested that during tissue stress or damage, endogenous molecules are released which initiate an inflammatory response and empower APCs to activate the adaptive immune response. These molecules are collectively known as Damage-Associated Molecular Patterns, DAMPs (Amarante-Mendes *et al.*, 2018). Similarly to PAMPs, DAMPs could also engage as TLR ligands.

There are 10 human TLRs and 12 murine functional TLRs that have been identified so far and that recognize distinct conserved PAMPs or DAMPs. TLRs are generally divided into three groups and include those that recognize microbial lipids and lipoproteins (TLRs 1, 2, 4 and 6), those that recognize microbial proteins (TLR5 and murine TLR11) and those that recognize nucleic acids (TLR 3, 7, 8 and 9) (Kawai & Akira, 2007). Another, TLR, TLR10 is only found in humans while TLR11, TLR12 and TLR13 are only present in mice (Pendergraft & Means, 2016). The ligands for TLR10, TLR12 and TLR13 have not been discovered so far (Guangwei Liu & Zhao, 2007).

These TLRs differ from one another in the cell types in which they are expressed, their ligand specificity, the signalling adaptors they utilize, and the cellular responses they induce.

TLRs 1, 2, 4, 5 and 6 are located on the plasma membrane where they interact with microbial PAMPs that come into contact with host cells. On the other hand, TLRs 3, 7, 8 and 9 are situated inside cells in the membranes of endosomes and recognize pathogen nucleic acids from pathogens such as viruses that infect cells or that have been internalized into host cells following phagocytosis (Van Maren *et al.*, 2008). TLR , 7, 8 and 9 are also capable of recognizing self-nucleic acids. Discrimination between self and non-self is straightforward for receptors that detect unique microbial products such as TLRs 1, 2, 4, 5 and 6, but is more complex for nucleic acid sensors such as TLRs 3, 7, 8 and 9 because nucleic acids are not unique to pathogens (Stetson & Medzhitov, 2006).

Endosomes are organelles that break down pathogens inside host cell which results in the exposure of pathogen nucleic acids that then bind to the endosomal TLRs to induce their activation (Trivedi & Greidinger, 2009).

TLRs are expressed by a number of cell types, including macrophages, dendritic cells (DCs), B cells, mast cells, NK cells, monocytes, neutrophils, basophils, regulatory and helper T cells, as well as respiratory and intestinal epithelial and endothelial cells (Makkouk & Abdelnoor, 2009).

Human T cells isolated from peripheral blood have been reported to express mRNA for most TLRs although studies have reported different expression levels (Hornung *et al.*, 2002; Mansson, Adner, & Cardell, 2006; Zarember, Godowski, & Alerts, 2002). TLR1, TLR2, TLR5, TLR9 and TLR10 however predominated (Mansson *et al.*, 2006). Expression levels depends on their activation status. In fact, naïve T cells express low levels of TLR1, TLR2

and TLR4 on their surface but appreciable levels are detected when the T cells are activated or when they become memory T cells (Guangwei Liu & Zhao, 2007)

Mansson, Adner, & Cardell, 2006 observed that TLR1 and TLR9 mRNA was expressed to a greater extent in CD4+ cells than CD8+ T cells, whereas expression of TLR3 and TLR4 mRNA was higher in CD8+ cells than in CD4+ T cells.

Among the CD4 + cells, studies reported that murine and human $CD4^{+}CD25^{+}$ Treg express higher levels of TLR4, TLR5, TLR7 and TLR8 in comparison with $CD4^{+}CD25^{-}$ T cells (Caramalho *et al.*, 2003; Kabelitz, 2007; Komai-Koma, Jones, Ogg, Xu, & Liew, 2004). TLR1, TLR2, TLR3 and TLR6 appear to be more widely expressed on CD4+ T cells and not confined in higher numbers to CD4+CD25+ Treg (Guangwei Liu & Zhao, 2007) .

Engagement of TLRs by PAMPs or DAMPs triggers intracellular signalling cascades through a set of TIR-domain containing adaptors. These include myeloid differentiation response protein 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing interferon (IFN)-β (TRIF) and TRIF-related adaptor molecule (TRAM) (Kawai & Akira, 2007). These TIR-domain containing adaptor proteins initiate signal transduction pathways that ultimately result in the activation of NF-κB or IRFs (which are transcription factors), or MAP kinases that regulate the expression of inflammatory cytokines, chemokines, and type I IFNs that ultimately protect the host from microbial infection (Kawasaki & Kawai, 2014).

With the exception of TLR3, MyD88 is a universal adaptor in all TLRs. It activates MAP kinases and the transcription factor NF-κb to control the expression of inflammatory cytokine genes. TIRAP mediates the activation of a MyD88-dependent pathway downstream of TLR2 and TLR4. TRIF is involved in TLR3 and TLR4-signalling and activates a MyD88-independent pathway (also known as the TRIF/TRAM-dependent pathway) that

leads to the activation of NF-ƙb, MAPK and the transcription factor IRF-3. The latter is important for the induction of Type I interferons. TRAM selectively participates in the activation of the TRIF-dependent pathway downstream of TLR4, but not TLR3 (Kawai & Akira, 2007). Figure 4 gives an illustration of the signal transduction pathways that different TLRs engage in.

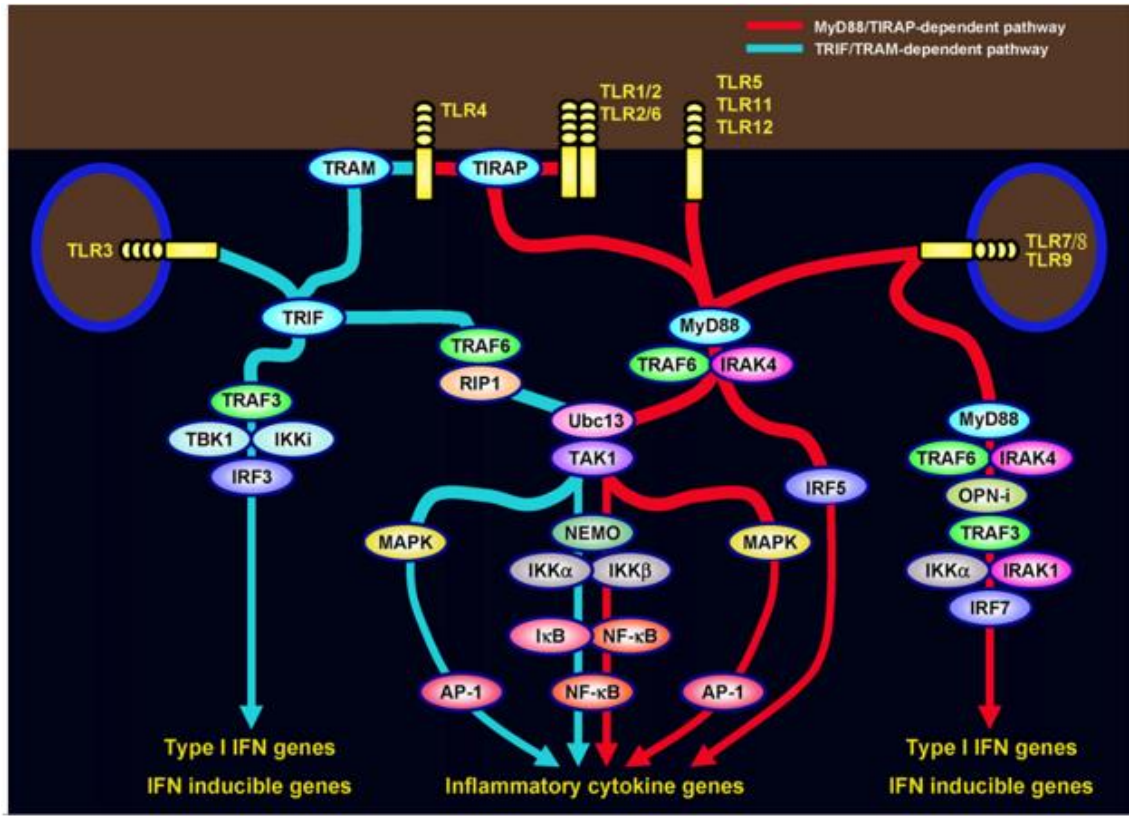


Figure 1.5: Signalling cascading pathways in different TLRs. The MyD88/TIRAP-dependent pathway occurs in all TLRs excluding TLR3. The TRIF/TRAM-dependent pathway occurs in TLR3 and TLR4 only. Modified from Kawai, T., & Akira, S. (2007). TLR signalling. *Seminars in Immunology*, *19*, 24–32. https://doi.org/10.1016/j.smim.2006.12.004. *Permission to use figure has been obtained from authors.*

As already described, TLRs are capable of identified microbial derived molecular patterns as well as endogenous ligands including host nucleic acids released from dying cells. The ligands for the different TLRs are described next.

1.6.2 Natural Microbial and Endogenous Toll-Like Receptor Ligands

TLR1 and TLR2 are found on the cell surface and recognize bacterial lipoproteins (Guangwei Liu & Zhao, 2007). TLR6 (in association with TLR2) recognizes diaceyl lipopeptides (Takeuchi *et al.*, 2001). Specifically, TLR1/2 and TLR2/6 can discriminate triacyl- and diacyl-lipopeptide, respectively (Akira *et al.*, 2006). The ligand for TLR11 (which is present only in mice) is profilin, an actin-binding protein involved in the restructuring of the cytoskeleton and found in the protozoan parasite *Toxiplasma gondii* (Yarovinsky *et al.*, 2005). In humans, TLR11 is non-functional due to the presence of a stop codon in the gene (Akira *et al.*, 2006).

TLR4 and TLR5, which are also localised on the cell membrane recognize lipopolysaccharides in the cell wall of Gram negative bacteria, and flagellin found in bacterial flagella respectively. Heat shock proteins (HSP) 60 and 70, released by cells under stressful conditions act as endogenous ligands for TLR 2 and TLR 4 (Ohashi, Burkart, Flohe, & Kolb, 2000; Vabulas *et al.*, 2001)

The TLRs that recognize pathogen nucleic acids are localised intracellularly in endosomes. TLR 3 was shown to recognize double stranded RNA (dsRNA) produced by viruses during replication (Kawai & Akira, 2007) and plays a key role in antiviral immunity. However, evidence has shown that TLR3 is also capable of recognizing endogenous messenger RNA released from necrotic cells (Kariko, Ni, Capodici, Lamphier, & Weissman, 2004).

TLR7 and TLR 8 recognize uridine-rich or guanosine-rich single stranded RNA (ssRNA) of viral origin. Many enveloped viruses enter cells through the endosomal compartment. Under acidified conditions and the presence of degradation enzymes, viral particles are damaged leading to the release of ssRNA which activate TLR 7 and 8.

Moreover, when virus-infected apoptotic cells are engulfed by phagocytes, viral RNA is released into the endosomes of the phagocytes.

TLR 7 and 8 can also recognize uridine or guanosine-rich single stranded RNA of host origin as well as RNA found in immune complexes (made up from RNA and autoantibodies). However, induction of TLRs 7 and 8 by endogenous RNA is a rare event since extracellular RNA is usually degraded by extracellular RNases before they can make it to the endosomal compartment of immune cells (Akira *et al.*, 2006). However, significant amount of RNA immune complexes found in the serum of individuals suffering from SLE have been implicated in the pathology of the autoimmune diseases (Barrat *et al.*, 2005). It is thought that although TLR7 and TLR8 are able to distinguish between self and viral RNA, they seem to be able to distinguish the presence of viral RNA by detecting their abnormal localization in the endosome rather than a particular RNA motif (Diebold, Kaisho, Hemmi, Akira, & Sousa, 2004).

Bacterial genomic DNA is also an immunostimulant and is recognized by TLR9 (Hemmi *et al.*, 2000). The immunostimulatory properties of bacterial DNA is due to the presence of unmethylated CpG-DNA. These are regions of DNA where the cytosine nucleoside is followed by a guanosine nucleoside. Although these are also present in the mammalian genome, CpG DNA found in mammals is methylated (Akira *et al.*, 2006). Bacterial DNA is delivered into the endosomal intracellular compartment where acidic and reducing conditions degrades bacterial double stranded DNA into multiple single stranded CpG DNA motifs that activate TLR9 (Ahmad-nejad *et al.*, 2002). Bacterial unmethylated CpG DNA induces the production of inflammatory cytokine production and Th1 immune responses.

TLR 9 can also in some instances, recognize human self-DNA found in immune complexes (made up from DNA and autoantibodies) from individuals with autoimmune diseases, as well as chromatin (Bhardwaj, Gnjatic, & Sawhney, 2010). Cell free DNA released from dead or dying cells is usually cleared off by phagocytes and DNases, however this system may not be as efficient in disease states where there is a high amount of cell death that exceeds the clearance capacity (Mittra, I., Kumar Nair, 2012; Vaart & Pretorius, 2008). This raises the question of whether the presence of unmethylated CpG DNA motifs is an obligatory requirement for the activation of TLR 9 (Barton, Kagan, & Medzhitov, 2006).

TLR9 is also capable of recognising viral DNA including that from herpes simplex virus 1 (HSV-1), HSV-2, and murine cytomegalovirus (MCMV) that contain genomes that are rich in CpG-DNA motifs (Heil, Hemmi, & Hochrein, 2004b; Lund *et al.*, 2004). By binding to TLR9, these ligands stimulate the secretion of inflammatory cytokines and type I IFN with anti-viral properties.

Since TLRs endosomal TLRs can also recognize host nucleic acid, their location inside endosomal compartments (and not on the cell surface) might be important for preventing recognition of self-DNA and thus preventing the generation of a sterile inflammatory response (i.e. inflammation without infection).

1.6.3 Synthetic Toll Like Receptor Ligands

Since natural TLR ligands can enhance the innate as well as adaptive immune response, synthetic TLR ligands, with varying degrees of similarity to natural ligands, have been developed. A few of these synthetic ligands have been approved or are currently undergoing clinical trials as therapeutic drugs in the management or treatment of cancer, allergies and

infectious diseases. Kanzler, Barrat, Hessel, & Coffman, 2007 give a detailed review of these synthetic TLR ligands.

Pam3Cys-SK4 is a synthetic analogue of the triacylated *N*-terminal part of bacterial lipoproteins and therefore is a ligand for TLR 1 and 2.

TLR3 recognizes the synthetic ligands Polyinosinic–polycytidylic acid (Poly I:C) and Polyadenylic–polyuridylic acid (PolyA:U) which are double-stranded homopolymers of double stranded RNA (Kanzler *et al.*, 2007).

Synthetic oligoribonucleotides with Poly(U) and GU-rich- motifs are synthetic ligands for TLR7 and TLR 8. Additionally, TLR7 also recognises small synthetic anti-viral molecules called imidazoquinolines which include Imiquimod and Resiquimod (R848), as well as guanine nucleotide analogues such as Loxoribine (Hemmi *et al.*, 2002). TLR8 also recognises Resiquimod.

Synthetic deoxynucleotides with unmethylated CpG motifs are synthetic ligands for TLR9. The CpG motif are equivalent to bacterial DNA in their immunostimulatory activity (Akira *et al.*, 2006).

There are no synthetic ligands developed yet for TLR5 and TLR10 (Kanzler *et al.*, 2007).

A number of recent studies have shown that different TLRs exhibit different effects on Treg and responder non-Treg T cells. TLRs can stimulate antigen-presenting cells which in turn either stimulate Treg proliferation and function or inhibit Treg suppression. They can also act by rescuing responder T cells from Treg-mediated suppression. Since T cells also express TLRs, ligands for TLRs can also act directly on T cells. A few of these studies are described in the next section.

1.6.4 The Role of Toll Like Receptors in Anti-Cancer Immunity and T cells

Researchers have developed drugs that act as TLR agonists and have used them with radiation, cytotoxic drugs and monoclonal antibodies. In this review, focus will be made on TLR ligands that have been applied in cancer treatment and their direct and indirect effects on T cells will also be discussed.

1.6.4.1 The effect of Cell Surface Toll-Like Receptors

TLR2 activation using the synthetic ligand Pam3Cys-SK4, in combination with TCR triggering, and IL-2, can induce Treg proliferation whilst at the same time resulting in their temporary loss of suppression on effector T cells (Sutmuller, Morgan, Netea, Grauer, & Adema, 2006). Lui *et al.*, 2006 found that both CD4+CD25- and CD4+CD25+ functionally express TLR2. They also observed that the same synthetic TLR ligand rendered the effector T cells resistant to Treg-suppression by increasing IL-2 secretion and by suppressing the induction of FOXP3 mRNA. This effect is however only transient (Liu, Komai-Koma, Xu, & Liew, 2006). Similarly, they also observed that the ligand causes an increase in Treg proliferation. They therefore hypothesized that during acute bacterial infection (simulated by the addition of the Pam3Cys-SK4), the TLR2 ligand rapidly increases the host's adaptive immunity to fight off the infection by expanding effector T cells and making them refractory to Treg-mediated immunosuppression. However, in the process, the TLR2 ligand also expands the Tregs, which recover their suppressive activity when the infection has subsided, in order to prevent auto-immunity that might result from the overactivation of effector T cells (Liu, Komai-Koma, Xu, & Liew, 2006).

Apart from its importance in infectious disease, the temporary downregulation of FOXP3 expression and decrease in suppressive activity of Treg can also be advantageous

in a tumour microenvironment. An acute inflammatory response might trigger off an anti-tumour response required for tumour clearance but it is also important that the Treg eventually then regain their suppressive function in order to prevent tissue damage and chronic inflammation.

In contrast, the endogenous TLR2 ligand Hsp60 was found to have the opposite effect on Treg and actually enhance the ability of the Treg to down-regulate both CD4+ effectors as well as CD8+ cytotoxic T cells by cell-to-cell contact as well as the secretion of anti-inflammatory cytokines such as TGF-β and IL-10 (Zanin-zhorov *et al.,* 2006). Therefore, it is suggested that ligands for the same TLR can elicit different responses and the effects of TLR2 on Treg remains uncertain.

Lipopolysaccharides (LPS) are naturally derived components from the cell membrane of gram-negative bacteria and have profound effects on CD4+ T cells. LPS is recognized by TLR4, which is expressed predominantly by APCs such as macrophages and dendritic cells but is also weakly expressed on the surface of naïve T cells and upregulated on activated or memory T cells (Liu, Zhang, & Zhao, 2010).

TLR4 is known to induce T cell mitogenesis *in vitro* either through direct stimulation or through signals derived from APCs. This is known to occur via the release of cytokines that induce inflammation, and via the upregulation of co-stimulatory molecules on APCs. This causes the CD4 cells to accumulate and expand in large numbers and become programmed for long-term survival (McAleer & Vella, 2008).

Studies have shown that LPS tends to favour Th1 responses (McAleer & Vella, 2008). Indeed, when human CD4+ T cells are stimulated with LPS from *Brucella abortus* they produce higher levels of IFN-γ and negligible levels of Th2 cytokines such as IL-5,

when compared to activation of T cells by other stimulating agents such as anti-CD3 and anti-CD28 alone (Koch *et al.*, 2007).

Similarly, other studies have shown that Lipid A, which is the component in LPS that activates TLR4, inhibits Th2 differentiation since levels of IL-4 were reduced when naïve T cells were stimulated in the presence of LPS (Watanabe *et al.*, 1999).

In contrast, another study found that LPS from *Porphyromonas gingivalis* promotes Th2 differentiation and the release of Th2-assoicated cytokines IL-13, IL-5, and IL-10 but lower levels of IFN-γ (Pulendran *et al.*, 2001). This was not the case when the LPS used was derived from *Escherichia coli* suggesting that LPS derived from distinct bacteria also results in different immune responses.

Recently, LPS has also been found to induce *in vitro* the differentiation of naïve T cells to Th17. Indeed, when naïve CD4+ T cells were cultured with medium from LPS-*in vitro* stimulated DCs, anti-CD3- and anti-CD28-coated beads, and TGF-β, the naïve T cells differentiated into Th17 (Veldhoen *et al.*, 2006).

In contrast, other studies have also shown that LPS negatively regulates Th effector function. Pre-treating human PBMCs with LPS decreased TNF-α and IFN-γ production as well as T cell proliferation in response to TCR stimulation. It is thought that this is due to the induction of FOXP3 expression in $CD4^{+}CD25^{-}$ T cells induced by monocytes that were stimulated by LPS (Bryn *et al.*, 2008). Moreover, directly treating human Treg with LPS enhanced their ability to suppress other immune cells, although FOXP3 expression was unexpectedly reduced (Lewkowicz, Lewkowicz, Sasiak, & Tchorzewski, 2006). In another study using mice, transferring LPS-activated DCs that produce IL-10 increased the number of FOXP3-positive cells and for this to occur MHC class II antigen presentation was required (Lau, Biester, Cornall, & Forrester, 2008).

Caramalho *et al.*, 2003 reported that LPS-activated CD4+CD25+ Treg cells were found to have better immunosuppressive abilities by 10-fold and this did not require activation by APCs (Caramalho *et al.*, 2003).

On the contrary, a study conducted by Pasare and Medzhitov found that conditioned medium from LPS-stimulating DCs caused effector CD4+ T cells to overcome Treg-mediated proliferative suppression *in vitro* (Pasare & Medzhitov, 2003).

All of these studies suggests that LPS has contrasting roles in T cell differentiation and function. These differences can be the result of different LPS doses or bacterial species from which the LPS is derived, and the cytokines environment present.

TLR5 is expressed in both CD4+CD25+ and CD4+CD25- and in comparable amounts to DC and monocytes (Guangwei Liu & Zhao, 2007). When CD4+CD25- T cells were stimulated with anti-CD3 and the natural TLR5 ligand flagellin, proliferation of the cells was enhanced and IL-2 was produced at levels that were comparable to those achieved by co-stimulation with CD28. However, co-stimulation with flagellin, despite not enhancing Treg proliferation, increased the immunosuppressive capabilities of CD4+CD25+ Treg and upregulated the expression of FOXP3 (Crellin *et al.*, 2005).

1.6.4.2 Endosomal Toll-like Receptors

TLR3, TLR7, TLR8 and TLR 9 are all expressed as endosomal TLRs. A number of synthetic ligands for endosomal TLRs have been approved or are currently under clinical trials to treat various forms of cancer. For instance, TLR7 imidazoquinoline ligands are used for the treatment of basal cell carcinoma (Aldara imiquimod cream) and melanoma (852A). By triggering cytokine production such as IFN-γ, TLR7 ligands like imiquimod

enhance the ability of antigen-presenting cells (APCs) to present viral or tumour antigens to reactive T cells and amplify the Th1-mediated immune response (Wang *et al.*, 2014). Moreover, it has been suggested that imiquimod and resiquimod may be suitable adjuvants for therapeutic DNA vaccines as, besides producing Th1-biased immune responses, they also induce potent cytotoxic T cell responses (Thomsen, Topley, Daly, Brett, & Tite, 2004).

A TLR3 ligand (IPH 31XX) has also been developed for the treatment of breast cancer.

Mesenchymal stromal cells (MSC) that were treated with the synthetic TLR3 ligand Poly (I:C) (and TLR4 ligand LPS) enhanced the generation of Treg in human co-cultures composed of CD4+ T cells and MSC (Rashedi, Gómez-Aristizábal, Wang, Viswanathan, & Keating, 2017). However, no studies investigated the direct effects of TLR3 synthetic or endogenous ligands on CD4+CD25+ Treg (in the absence of MSC) which might be worth-while in the future.

Exogenous and endogenous TLR7 ligands (present during infection or autoimmune diseases respectively) directly activate DCs and B cells as well as block immunosuppression by Treg. A study in naïve murine T cells has shown that the TLR7 ligand imiquimod reduces the number of Treg generated both *in vitro* and *in vivo*. In the presence of TLR7-activated splenic DCs, FOXP3 expression was transiently induced in naïve T cells by TGF-β but was later downregulated after 3 days. This FOXP3 downregulation was probably responsible for the abrogation of T-reg immunosuppression and was thought to occur via the accumulating IL-6 produced by DCs that were stimulated with TLR 7 ligands (Hackl, Loschko, Sparwasser, Reindl, & Krug, 2011).

In another study, synthetic RNA oligonucleotides, which also act as TLR7 ligands, and which possessed immunostimulatory motifs (the RNA was delivered inside cells using

DOTAP) also blocked suppression by Treg and similarly, this effect was dependent on IL-6 production by dendritic cells.

Peng *et al.*, 2005 also observed that TLR8 signalling induced by ssRNA reversed the suppressive function of Treg cells. However, they also observed that this effect is independent of DCs, but, rather dependent on TLR8-MyD88-IRAK4 signalling (explained in section 1.6.1) in Treg cells. Moreover, adoptive transfer of TLR8-ligand-stimulated Treg cells into tumour-bearing mice enhanced anti-tumour immunity (Peng *et al.*, 2005). The suppression of CD4+CD25+ T cells induced by TLR signalling was due to a direct effect on the CD4+CD25+ Treg and not on CD4+CD25- effector T cells probably because Treg express higher levels of TLR8 when compared to conventional CD4+ T cells. Therefore, this study proves that some TLR ligands also act directly on Treg without the intervention of DCs. Nonetheless, studies on the direct effects of TLR7 and TLR 8 ligands on Treg are still lacking. TLR8 agonists are also particularly effective at inducing Th1-polarizing responses from human monocytes and myeloid dendritic cells, with the magnitude of response substantially exceeding that induced by ligands of other TLRs.

Blockade of Treg-mediated immunosuppression as a result of TLR7 or TLR8 signalling has been implicated in autoimmune renal disease in patients with SLE. These individuals have high levels of immune complexes containing uridine-rich small nuclear RNA in their serum which act as endogenous TLR7/8 ligands. This continuous TLR-signalling over-activates T cells and leads to the progression of the autoimmune disease (Pendergraft & Means, 2016).

TLR 9 ligands, have been used for the treatment of non-small cell lung carcinoma (PF3512676), Non-Hodgkin lymphoma (1018 ISS), Renal-cell carcinoma, (IMOxine) and colon cancer (1018 ISS).

In a study by Chiffoleau *et al.*, 2007, the TLR9 ligand CpG-ODN induced the proliferation of both rat CD4+CD25- and CD4+CD25+ T cells. However, the TLR9 ligand was found to block the immunosuppressive capabilities of Treg. Contrary to signalling by TLR8 ligands, this abrogation might have had a partial direct effect on effector T cells (rather than directly target Treg) in that they were rendered more resistant to Treg-mediated suppression. When protein expression analysis was carried it was revealed that FOXP3 expression was not altered in Treg treated with CpG-ODN (Chiffoleau *et al.*, 2007). Secretion of cytokines IL-2, IFN-γ and IL-10 was also not affected by the addition of the TLR9 ligand. Therefore, the mechanism of how Treg-mediated suppression was reduced is not entirely understood. No published studies on the direct effect of TLR9 signalling on Treg using endogenous (rather than microbial or synthetic) ligands are available.

In view of the above, a general trend is observed where ligands for TLR3, TLR4, TLR7, TLR8 and TLR9 preferentially favour Th1-bias while TLR4 and TLR5 preferentially favour Th2 (Toussi & Massari, 2014). Moreover, despite the disagreement in some studies, the general trend shows that TLR 2, TLR 7 or TLR 8 ligands reverse the immunosuppressive function of Treg, whereas TLR 4 or TLR 5 and some TLR2 ligands seems to enhance CD4+ CD25+ Treg cell-mediated immunosuppressive capacity. Nonetheless, different studies have shown that the type of TLR ligand (and not just the receptor) can result in different outcomes.

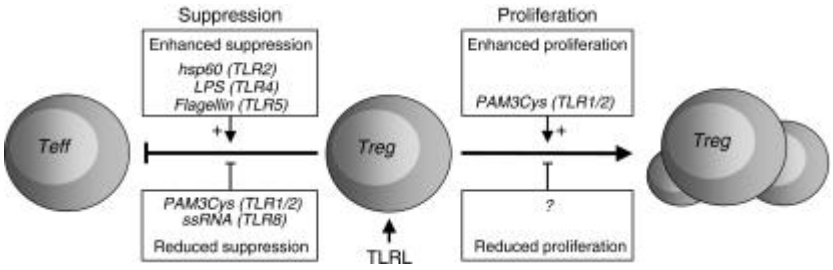


Figure 1.6: Effects of various TLR ligands (TLRL) on T-reg mediated suppression on effector T cells. Hsp60 (TLR2), LPS (TLR4) and Flagellin (TLR5) enhance immunosuppression by Treg. Pam3Cys (TLR2) and ssRNA (TLR7/8) inhibit immunosuppression yet PamCyS induces proliferation of Treg. Obtained from Van Maren, W. W. C., Jacobs, J. F. M., De Vries, I. J. M., Nierkens, S., & Adema, G. J. (2008). Toll-like receptor signalling on Tregs: To suppress or not to suppress? *Immunology*, 124(4), 445–452. https://doi.org/10.1111/j.1365-2567.2008.02871.x. *Permission to use figure was obtained from authors.*

2 AIMS AND OBJECTIVES

2.1 Aim

As described in the literature review, tumour immune-evasion mediated by Treg represents a major obstacle for successful cancer immunotherapy as they suppress the anti-tumour responses induced by the treatment. Hence, depletion or functional modulation of these cells is a possible way to restore immunity. Therapies that target Treg have been developed but these are either cytotoxic, cause severe immune-related side effects, are expensive or are sometimes ineffective.

The stability of Treg is still under debate as evidenced by disparate results obtained in different studies. Nevertheless, a number of studies have shown that Treg under certain inflammatory conditions can attain characteristics of other Th cells. Yet, to date there is less conclusive and documented evidence whether it is possible to convert Treg into Th1 or Th2 cells. This study aims at modulating Treg, using immunomodulatory agents, by targeting their plasticity with the intent of reducing their suppressor function and possibly induce a flip to other T cell types. The ideal outcome would be to convert the Treg into Th1-like cells that secrete Th1-specific cytokines. This is because Th1 recruit CTLs that would kill off cancer cells. This study is therefore a preliminary *in vitro* attempt to the “Trojan Horse Approach”.

Should the aim be achieved, it would have important implications in cancer treatment especially if this scenario could be further replicated *in vivo* through direct injection in the tumour. Being able to modulate Treg within the tumour microenvironment would indeed be a great accomplishment as it is expected to improve

immunotherapeutic attempts. This alternative method of modulating Treg would also be cheaper and should boost the anti-tumour response.

2.2 Objectives

The objective of this study is to use immunomodulating agents, such as IFN-γ and TLR ligands, to try and cause a shift from a suppressive phenotype characteristic of FOXP3+ Treg (that reduce anti-tumour immunity) to an effector phenotype characteristic of FOXP3- Th cells that display anti-tumour properties. Apart from a shift in the phenotype, the second objective is to abrogate or at least reduce the Treg suppressor function on effector Th cells. The third objective would be to induce a shift from an anti-inflammatory, suppressive cytokine environment to a pro-inflammatory cytokine environment which is required to induce a proper anti-tumour response and ameliorate immunotherapeutic treatments.

The direct effects of IFN-γ and four TLR ligands on Treg phenotype will be investigated. Moreover, the functional suppressive capabilities of Treg in a co-culture of Treg and Th cells will be investigated by monitoring cell proliferation and cytokine secretion in Tresp and Treg co-cultures whereby the latter had been treated with the selected immunomodulatory agents.

IFN-γ was selected because it is the main effector cytokine for Th1 and has been shown to promote an anti-tumour response by inducing Th1 and CTLs whilst also inhibiting Treg function.

The effects of two endogenous and two synthetic TLR ligands will also be investigated. All of the TLR ligands selected target endosomal TLR ligands including TLRs

7, 8 and 9. From the literature review, it is evident that in general there is a common agreement between studies that ligands for such TLRs reduce Treg function but the exact mechanism is not known. There is less agreement on the effects of ligands for cell surface TLRs. Moreover, most previous studies have reported that modulation of Treg is dependent on DCs that respond to TLR ligands and, although there is evidence that Treg cells respond directly to microbial or endogenous TLR ligands, studies on the direct effects have been largely overlooked. Investigations on the direct involvement of TLRs in Tregs cells are vitally required and will be the focus of this study.

Since studies are lacking, part of this study will look into the effects of endogenous TLR ligands, such as cell-free human RNA and DNA on T cells, to test whether these possess immunostimulatory properties, and if so, whether they elicit different effects from their exogenous microbial or synthetic ligand counterparts.

Two synthetic widely used TLR 7 and 8 ligands will also be used. These include single stranded Poly Uridine (synthetic RNA single strand), and CL097, which is a resiquimod derivative and therefore belongs to the imidazoquinoline group of compounds. Single stranded polyuridine and CL097 have been reported to reduce Treg suppression in the presence of DCs, but their direct effect on Treg has been poorly investigated.

3 METHOD

3.1 Isolation, Purification and Culturing Of T Cells

3.1.1 Isolation of Lymphocytes From Human Peripheral Blood

Blood buffy coats were retrieved from the National Blood Transfusion Services (NBTS) centre in Pieta'. Since human samples were being used, ethics approval (Refer to Appendix I – Ethical Consent) was sought and granted from the Faculty Research Ethics Committee (FREC) and the University Research Ethics Committee (UREC). Each buffy coat collected came from 500ml blood from a single donor. At the NBTS, the whole blood from donors is left standing for 3 to 20 hours. The blood is then centrifuged at 3770rpm for 12.5 minutes. After centrifugation, three layers are obtained: top layer containing plasma, middle layer containing the buffy coat while the red cell concentrate collects at the bottom layer. The NBTS remove most of the top plasma part and the bottom red cell concentrate. The top plasma layer is also used to make pooled platelets. What is left i.e. the buffy coat layer (which NBTS do not require) was kindly provided for this study.

The buffy coats contained the anti-coagulant Citrate phosphate dextrose (CPD). Once blood bags were collected, isolation of leucocytes was carried out by density gradient centrifugation as described below on the same day of collection.

Blood from the collection bags was dispensed in 50ml sterile conical centrifuge tubes and diluted with Phosphate Buffered Saline, PBS, (Sigma-Aldrich, USA) at a ratio of 1:3 (v/v) blood:PBS. In separate sterile 50ml centrifuge tubes, 15ml of Histopaque®-1077 (Sigma-Aldrich, USA) were added and the diluted blood was layered cautiously on the Histopaque®-1077. The tubes containing the blood layered on top of the Histopaque®-

1077 were then centrifuged at 450 × g for 30 minutes, making sure that the centrifuge was set on “brake off” position in order for the deceleration to be gradual, preventing the density gradient separated layers from being disturbed.

After centrifugation, the uppermost layer containing plasma was discarded and the white hazy layer (containing the leucocytes) between the plasma and the Histopaque was collected in a new sterile 50ml conical centrifuge tube while being careful not to collect any red blood cells from the bottom most layer. The white cells collected in the separate tubes were then washed with PBS and centrifuged at 250 × g for 5 minutes.

If the resulting pellet had a red tinge to it due to contamination by red blood cells, the pellet was resuspended in Erythrocyte Lysis Buffer (ELB) prepared in-house (Refer to Appendix IV: Recipes of Buffers and Solutions) and PBS at a 3:1 ratio (v/v) respectively and incubated for 10 minutes at 37°C. The mixture was then centrifuged, the supernatant discarded and the white cell pellet was re-suspended in RPMI-1640 medium (Life Technologies, USA) supplemented with 10% Fetal Bovine Serum, FBS, (Gibco, USA), 1% Penicillin Streptomycin antibiotic, Pen-strep (Sigma-Aldrich, USA) and 1% anti-fungal Amphotericin B (Sigma-Aldrich, USA). The isolated leucocytes were then placed in an incubator at 37°C and 5% CO_2 in sterile T75 flasks.

After approximately four hours, the non-adherent lymphocytes were separated from the adherent monocytes by transferring the liquid RPMI-1640 medium containing the suspended lymphocytes to a new sterile T75 flask and incubating at 37°C and 5% CO_2. The cells were further processed the next day or cryopreserved as described in Appendix V: Protocol for the Cryopreservation and Thawing of Lymphocytes.

3.1.2 Measuring Cell Count and Viability

A 10µl cell suspension was withdrawn and stained with an equal volume of Trypan Blue (Sigma-Aldrich, USA) and left to incubate for five minutes at room temperature. If the cell suspension was very concentrated, the volume of cell suspension withdrawn was diluted tenfold, or sometimes even hundred fold, using PBS. Then, 10µl of the cell/dye mixture was injected inside a FastRead102 disposable counting slide (Immune Systems Limited, UK) and viewed under the microscope at a magnification of × 100. Trypan blue allowed the identification of viable cells as these are impermeable to the blue dye whilst dead cells are not.

The number of cells inside *n* large 4×4 grids were counted. Only cells that touched the top and right vertical perimeter were counted to make sure that no cells were counted more than once. Cells that were clear were considered as viable while cells that appeared blue were considered as dead.

The total number of viable cells was calculated as follows:

% viable cells = × 100

The number of viable cells per ml in the sample was determined as follows:

No. of viable cells/ml = × 10^4 × 2 × dilution factor (if applicable)

Where:

10^4 = volume conversion to 1ml

2 = takes into account the 1:1 dilution of the sample with Trypan blue

Dilution factor = takes into account any dilution of the original sample before placing in haemocytometer.

To obtain the total number of cells present in the sample the concentration of viable cells/ml was multiplied by the total volume of medium inside the flask.

3.1.3 Isolation of CD4+ T Cell Populations By Magnetic Beads

The next step was to separate the CD4+ lymphocytes from the rest of the mononuclear non-adherent cells and further separately isolate the CD4+CD25+ phenotype (representing the Treg) from those T cells with a CD4+CD25- phenotype (representing the CD4+ non-Treg cells). This was first achieved by magnetic isolation.

Three antibodies were used in order to isolate CD4+CD25+ Treg and CD4+CD25- non-Treg cells. These antibodies include CD4, CD25 and CD127 the latter of which is expressed in very low quantities in Treg cells and in moderate to high quantities in non-Treg CD4+ T cells. This magnetic isolation was carried out using the EasySep^{TM} Human CD4+CD127lowCD25+ Regulatory T Cell Isolation kit (STEMCELL Technologies, USA, Catalog #18063).

In this procedure, CD4+CD25+ cells are first magnetically separated from non-Teg cells by positive selection using CD25 monoclonal antibodies. EasySep™ Releasable RapidSpheres™, which is a suspension of magnetic particles in water, is then added followed by a $CD4^{+}$ T cell Enrichment Cocktail.

Here, the CD4+CD25+ Treg cells bind to CD25 monoclonal antibodies which bind to the Releasable Rapidspheres magnetic particles while the CD4+CD25- non-Treg T cells, CD8+ and B cells do not and are instead collected in the supernatant once the sample was placed in a magnet.

After that, the CD4+CD25+ Treg are detached from the EasySep™ Releasable RapidSpheres™ using a release buffer provided with the kit and separated from any unwanted non-Treg cells still in the tube by negative selection using the CD127 monoclonal antibodies and EasySep™ Dextran RapidSpheres^{TM}. Here, the non-Treg cells that are CD4+CD25-CD127high bind to the CD127 antibodies which in turn bind to and remain bound to the Dextran RapidSpheres^{TM} beads while the CD4+CD25+CD127low fraction are then collected as the supernatant.

The CD4+CD25- non Treg cells can also be retrieved and separated by negative selection from the B cells and CD8+ cells by adding Dextran RapidSpheres. Since the CD4+ T cell Enrichment Cocktail will be previously added, the non-CD4+ cells (i.e. B cells and CD8+ cells) will attach to the antibody mix in the CD4+ T cell enrichment cocktail which then bind to the Dextran RapidSpheres. B cells and CD8+ cells remain attached to the tube while the CD4+CD25- are collected as the supernatant after placing the tube in the magnet.

3.1.3.1 Enrichment of CD4+CD127$_{low}$CD25+T Cells

Lymphocytes were re-suspended in Isolation Buffer prepared in-house (PBS containing 2% FBS and 1mM EDTA) at a concentration of 5×10^7 cells/ml and transferred to a sterile 5ml (12 × 75mm) round-bottomed polypropylene tube. The final volume ranged between 0.25ml and 2ml.

To the tube, 50µl of CD25 Positive Selection Cocktail per ml of lymphocytes were added, mixed and incubated for 5 minutes at room temperature.

Releasable RapidSpheres™ beads were vortexed for 30 seconds and 30µl of the beads were added per ml of lymphocytes, followed by 50µl of CD4+ T cell Enrichment Cocktail per ml of lymphocytes. The contents were mixed and incubated for 5 minutes at room temperature. The tubes were then topped up to 2.5ml with isolation buffer, mixed gently by pipetting and the tube without lid was placed in an EasySep™ magnet (STEM CELL TECHNOLOGIES, USA, Catalogue #18000) and incubated for 10 minutes at room temperature.

After the incubation time, the magnet was inverted in one continuous motion pouring the supernatant into a new sterile 5ml tube. The supernatant was set aside for isolating CD4+CD25- non Treg T cells (Section 3.1.3.2).

The tube was removed from the magnet and the contents were topped up to 2.5ml with isolation buffer and mixed by gently pipetting up and down. The tube was placed again without lid into the magnet, incubated for 5 minutes at room temperature and in one continuous motion the magnet was inverted and the supernatant poured out and discarded. The steps in this paragraph were repeated twice in order to make sure that all non-CD4+CD25+ are removed.

The tube was removed from the magnet and topped up with the same volume of isolation buffer as the original starting volume i.e. 0.25ml - 2ml. 100µl of Release Buffer (provided with the kit) were then added per ml of lymphocytes and the tube contents mixed by vigorously pipetting up and down more than 5 times. To the tube, 50µl of CD127high Depletion Cocktail were added per ml of lymphocytes, mixed and incubated for 5 minutes at room temperature. Dextran RapidSpheres™ beads were vortexed for 30

seconds and 10µl of the beads were added per ml of lymphocytes, mixed and incubated for 5 minutes at room temperature.

The sample was topped up to 2.5ml with isolation buffer and mixed gently by pipetting up and down. The tube was placed without lid into the magnet and incubated for 5 minutes at room temperature. The magnet was then inverted in one continuous motion pouring the enriched cell suspension into a new tube. These cells contained a purified fraction of CD4+CD127lowCD25+ Treg cells.

3.1.3.2 Enrichment of CD4+CD127$_{high}$CD25- T Cells

The Dextran RapidSpheres™ were vortexed for 30 seconds and 90µl of the beads per ml of lymphocytes were added to the supernatant obtained in section 3.1.3.1 that contained the CD4+CD127highCD25- T cells. The tube was mixed and incubated for 5 minutes at room temperature. The tube was placed into the magnet without lid and incubated for 5 minutes at room temperature.

The magnet was then inverted in one continuous motion and the supernatant was poured inside a new sterile 5ml tube. The tube was removed from the magnet, and the tube containing the supernatant with the enriched cells was placed inside the magnet and incubated for 1 minute to make sure that all the beads had been removed from the supernatant. The magnet was inverted in one continuous motion and the supernatant poured inside a new sterile tube. The collected supernatant now contained pure CD4+CD127highCD25- non-Treg T cells.

3.1.4 Purification of T Cell Populations By Fluorescent Activated Cell Sorting

The purity of the sorted cell populations obtained with magnetic sorting is not always satisfactory. There are other available cell sorting technologies such as Fluorescent Activated Cell Sorting (FACS) which yield higher purities. For this reason, the enriched T cell populations obtained after magnetic sorting were further purified by FACS.

Enrichment of the CD4+CD25+ population prior to FACS by magnetic isolation is still recommended (and was still carried out each time). Since CD4+CD25+ Treg represent only a very small percentage of the total CD4+ T cells, FACS alone is sometimes problematic to isolate "rare" populations from a huge quantity of non-Treg lymphocytes (Cossarizza *et al.*, 2017), hence why enrichment by magnetic sorting prior to FACS is recommended.

In FACS, particles including cells are introduced into a column of pressurized sheath fluid and as they emerge from a nozzle, they pass through one or more laser beams. The technology is based on the principle that as a cell passes through the laser beam, light is scattered in all directions, and any fluorochromes (which would be conjugated to antibodies specific to receptors on/in cells of interest) that bind to cells are excited and they emit light of a higher wavelength. Scattered and emitted light is collected by two lenses: one lens is in front of the light source (this is known as the forward scatter) while the other one is set at right angles to it. By a series of beam splitters, optical filters and detectors, the wavelengths of light emitted by particular fluorochromes can be isolated and quantified. At this point, which is called the moment of analysis, the cytometer gathers information about the fluorescence characteristic of the particle and it is at this stage that cell populations are identified and gated.

Sorting is then accomplished by the electrostatic deflection of charged particles. This technique is based on the principle of droplet formation from the fluid stream of cells. This is called the breakoff point. Once the droplet is formed, it is then charged at the instance (moment of charging). Charged drops are then passed through deflection plates and are deflected into collection vessels or sent to waste. The theory of operation of FACS in more detail is well documented (Ormerod, 2000; Givan, 2001; Shapiro, 2003).

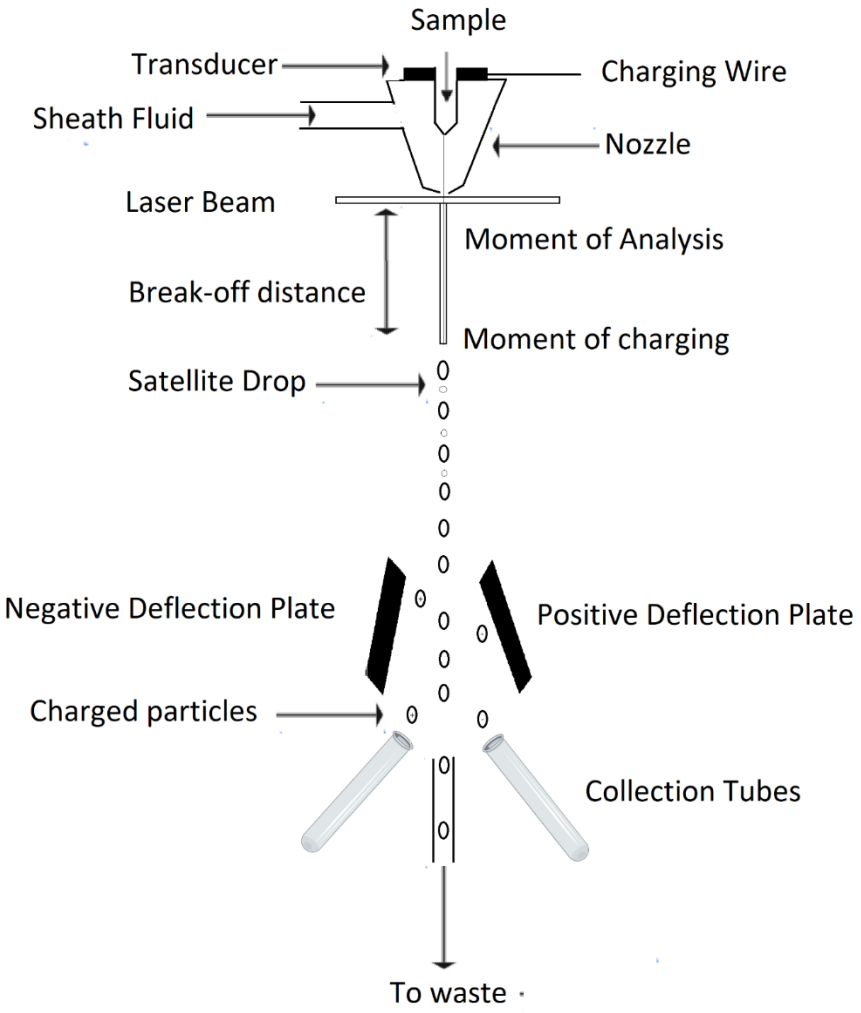


Figure 3.1: The technology behind Fluorescent Activated Cell Sorting. Modified from Davis, D (2007) Cell Sorting by Flow Cytometry. In: Macey, M.G. (eds) Flow Cytometry: Principles and Applications. Humana Press Inc., Totowa, NJ, USA

3.1.4.1 Procedure for Antibody Surface Staining

The CD4+CD25+ and CD4+CD25- T cell populations separated by magnetic sorting (methods described in section 3.1.3) were transferred into a sterile round-bottom 5ml (12mm × 75mm) polypropylene tube, centrifuged at 250 × g for 5 minutes and the supernatant discarded. The cells were re-suspended in 500µl of a blocking agent prepared in-house (PBS, 2mM EDTA, 10% FBS) for 10 minutes. The blocking agent serves to block binding of the test antibodies to Fc receptors which results in non-specific binding giving false positives. Non-specific binding happens because the antibodies bind to other sites rather than the antigen specific fragment or the antigen binding (Fab) domain. The high concentration of serum in the blocking agent binds to these non-specific sites, reducing the chances of the antibody binding to these non-specific sites.

After the cells were incubated for 10 minutes in blocking agent, the tube was centrifuged at 250 × g for 5 minutes and the supernatant decanted.

The cells were then stained for CD4, CD25 and CD127 as described below. Sorting of Treg from other CD4+ non-Treg T cells was performed on the basis that Treg are CD4+CD25highCD127-/low while naïve non-Treg CD4+ T cells are CD4+CD25-CD127+. Although the master transcription factor of Treg, FOXP3, provides a more reliable discrimination between Treg and non-Treg cells, it was not possible to sort viable cells using this marker since being an intracellular marker, fixation and permealisation of the cells meant killing the cells in the process.

The following procedure was performed in the dark. A master mix was prepared in a sterile tube to give the quantities and volumes per test as given in Table 3.1. Note that to prepare the mastermix, the volume per test and total volume per test given in the table

were multiplied by the total number of tubes to be sorted. All antibodies were purchased already conjugated to the fluorochromes from BD Biosciences, USA.

Reagent	Fluorochromes	Purchased Concentration	Quantity	Volume per test (in 100µl total volume)	Concentration per test
		(µg/ml)	(µg)	(µl)	(µg/ µl)
Human Anti-CD4 Mouse IgGκ1	FITC	50	1.00	20	0.010
Human Anti-CD25 Mouse IgGκ1	PerCP-Cy5.5	50	0.25	5	0.003
Human Anti-CD127 Mouse IgGκ1	PE	200	0.50	2.5	0.005
Staining Buffer (PBS, 2% FBS, 1mM EDTA)				72.5	

Table 3.1: Extracellular staining of CD4, CD25 and CD127 for fluorescent activated cell sorting of Treg and non-Treg cells. The quantities and volumes of antibodies shown are those used per test. The master mix was prepared by multiplying the volume per by the number of samples. CD4-FITC, CD25-PerCP-Cy5.5 and CD127-PE antibodies were purchased from BD Biosciences.

From the master mix, 100µl were transferred to each tube and the mixture incubated on ice for 30 minutes in the dark.

After that, the tubes were centrifuged at 250 × g for 5 minutes and the supernatant discarded. The cells were washed twice to three times with PBS, centrifuged at 250 × g for 5 minutes and then re-suspended in a suitable amount of staining buffer (PBS, 2% FBS, 1mM EDTA). The cells were stored at 4°C in the dark until further use.

3.1.4.2 Pre-Sort Analysis and Gating Strategy

Before loading the cells in the cell sorter, a number of dot plots were plotted using BD FACSDiva™ software as shown in Figure 3.2. A dot plot of area of forward scatter (FSC-A) versus area of side scatter (SSC-A), panel A on Figure 3.2, was first plotted and the cells

gated on the singlet lymphocyte population. Forward scatter is a measure of cell size while side scatter is a measure of granularity/cell complexity. When cells clump together they form doublets and chances are that they are not properly labelled with antibody during the staining process. Therefore, one must exclude doublets. Doublets are expected to have a high FSC vs. SSC. On the other hand, events with very low FSC vs. SSC should also be excluded from the gating strategy as these are usually dead cells or cell debris.

Another way how to discriminate between singlets and doublets is by plotting two other dot plots as shown in Panel B and Panel C in Figure 3.2. Panel B shows the area of forward scatter (FSC-A) versus height of forward scatter (FSC-H), while Panel C shows the side scatter area (SSC-A) versus side scatter height (SSC-H). The basics of Area vs. Height strategy is that assuming that the instrument is correctly set, the Area is equal to the Height when correlating the same diameter so the same cell will have the same or very similar value on both axis. Therefore, all singlet events will be presented as a more or less diagonal line (Cossarizza *et al.*, 2017). This is not the case for doublets. Therefore only cells that were present along the diagonal line were gated in order to eliminate doublets. Populations P2 and P3 in Panels B and C represent singlet lymphocyte populations.

A dot plot of CD4 vs. CD25, like the one shown in Panel D in Figure 3.2, was also plotted. Two gates were created on the singlet lymphocytic population, P3. These included a gate for singlet cells from P3 that were CD4+CD25-/low and another gate for singlet cells from P3 that were CD4+CD25high. Lastly, two other dot plots of CD25 vs. CD127 and CD4 vs. CD127, panel E and F respectively in Figure 3.2, were plotted. From the singlet population P3 , two gates in Panel E were included, one for cells that were either CD25-/lowCD127+ or CD25+CD127low while another two gates in Panel F were

included representing the population of cells that were either CD4+CD127low or CD4+CD127+.

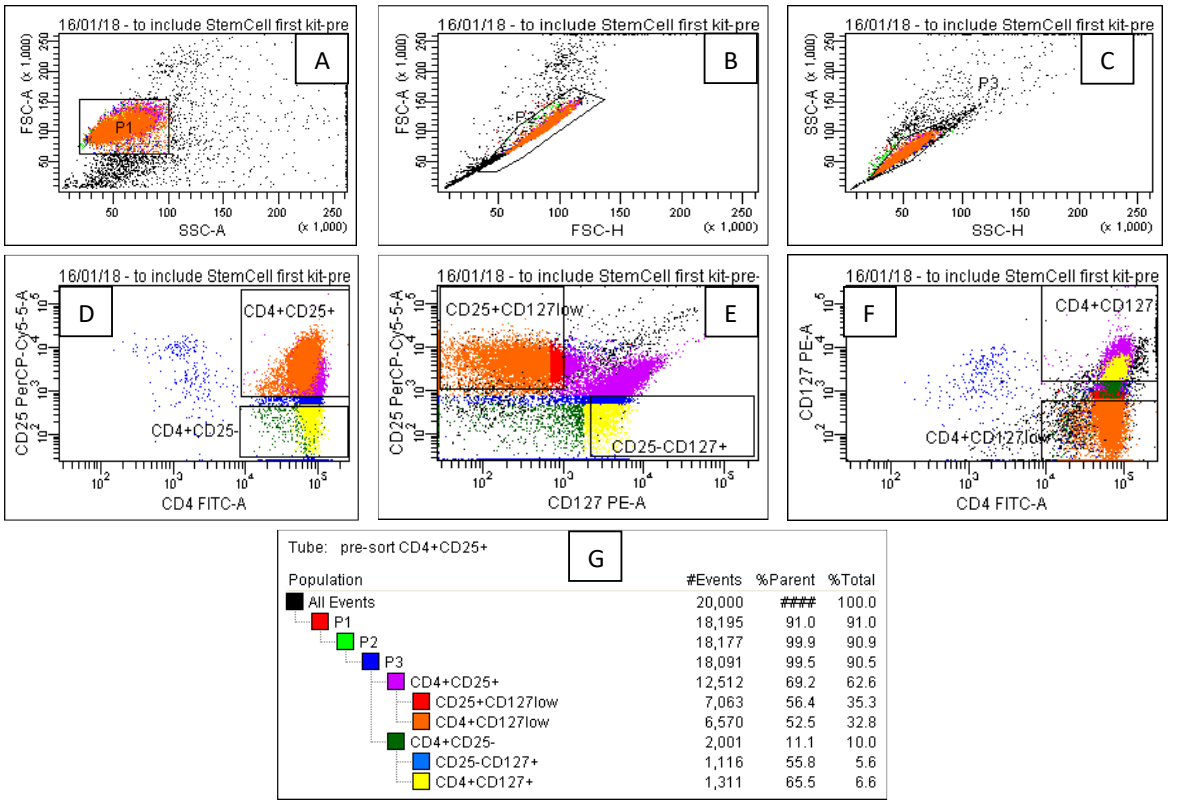


Figure 3.2: Sorting strategy. (A): Dot plot of area of forward scatter vs. area of side scatter. The gated population was designated as P1; (B): Dot plot of area of forward scatter vs. height of forward scatter. The gated population from P1 was designated as P2; (C): Dot plot of area of side scatter vs. height of side scatter. The gated population from P2 was designated as P3; (D): Dot plot of CD4 vs. CD25. The two gated population from P3 was designated as CD4+CD25+ and CD4+CD25-; (E): Dot plot of CD25 vs. CD127. The gated population from the $CD4^{+}CD25^{+}$ was designated as CD25+CD127low and the gated population from CD4+CD25- was designated as CD25-CD127+; (F): Dot plot of CD127 vs CD4. The gated population from as CD4+CD25+ was designated as CD4+CD127low while the gated population from CD4+CD25- was designated as CD4+CD127+; (G): Population Hierarchy used for gating

3.1.4.3 Sorting

The labelled lymphocytes were loaded onto a BD FACSAria™ III system (BD Biosciences, USA) and analysed using BD FACS Diva™ software. Quality Control of the instrument's optics, electronics, fluidics and detectors' sensitivity was routinely carried out using the BD™ CS&T Beads (BD Biosciences, USA). Fluorescence compensation was performed before sample acquisition to correct for the spectral overlap of the fluorochromes using BD™ CompBeads (BD Biosciences, USA). The method for fluorescence compensation is described in Appendix VII: Setting up Compensation. The drop delay (the time between the point at which the cell passes through the laser beam and is analysed until its inclusion in a droplet as the breaks from the stream) was set before sorting the samples were loaded using BD™ FACS Accudrop beads (BD Biosciences, USA).

The cells were sorted through an 85µm nozzle. The gated sub-P3 populations of cells that were CD4+CD25high, CD25highCD127-/low cells and CD4+CD127-/low, all representing a Treg phenotype, were collected in one tube as described below. The gated P3 population of cells that were CD4+CD25-/low, CD25-/low CD127+ and CD4+ CD127high, all representing a non-Treg CD4+ T cell phenotype were collected in a separate tube as described below.

The sorted cells were collected in sterile 12 × 75-mm 5ml round bottomed tubes which were previously filled and coated with FBS in order to prevent cells from adhering to the sides of the tubes. The sorted cells were collected in RPMI-1640 medium supplemented with 10% FBS, 1% Pen-Strep and 1% Amphotericin B. The flow rate was set to 800-2000 events/s. The sort was paused periodically and the collection tubes were

gently inverted to ensure that cells are in suspension. The sorted cells were then centrifuged at 250 × g for 5 minutes and post-sort analysis was carried out as described in section 3.1.4.4. After that, the cells were left rest overnight in an incubator set at 37°C and 5% CO_2 before being used/manipulated further since FACS stresses the cells.

3.1.4.4 Post-Sorting Analysis

To check the sorting efficacy, a fraction of the sorted cell populations and at least 10,000 events were acquired on the BD FACSAria™ III. The events were gated using the same gating strategy as for the sort described in section 3.1.4.2. Before loading the cells, the sample line was back-flushed from cells of the previous sample using the *Sample Line Backflush* option in the cleaning modes built-in feature of the machine.

Purity was assessed by looking at the percentage of CD4+CD25highCD127-/low in the CD4+ Treg sorted samples and the percentage of CD4+CD25-/lowCD127+ in the non-Treg CD4+ T cells sorted samples. If the percentage of cells was > 90%, the sorted cells were considered as having optimal purity.

3.1.5 Confirming The Purity of Sorted T Cell Populations

As part of method optimization, following FACS, a further check to ascertain the purity of the sorted Treg and non T-reg populations was done by sacrificing a small fraction of sorted cells and looking for the expression of intracellular FOXP3 in Treg cells and its absence in non-Treg T cells. Together with FOXP3, extracellular markers CD25 and CD127 were also used for this discrimination. Treg should be FOXP3+CD25highCD127-

/low while naïve non-Treg T cells should be FOXP3-CD25-/lowCD127+. The CD4 marker was omitted at this stage since both population of cells express it.

3.1.5.1 Extracellular Staining with CD25 and CD127

At least 1 × 10^5 cells from each sample were collected, placed in a 5ml round bottomed 12 x 75mm polypropylene tube, centrifuged at 250 × g for 5 minutes and the supernatant decanted. The pellet was then washed in PBS, centrifuged at 250 × g for 5 minutes and the supernatant decanted.

To the pellet, 500µl of Blocking Agent prepared in-house (PBS, 2mM EDTA, 10% FBS) were added to the cells, vortexed and incubated for 10 minutes at room temperature.

Each sample was split into three new BD Falcon™ 12x75mm Polystyrene tubes: one to serve as the unstained sample, one as the antibody-stained sample and another that would serve as the isotype-stained sample. The tubes were centrifuged at 250 × g for 5 minutes to remove the blocking agent. The cells were then washed with PBS and centrifuged again at 250 × g.

A test antibody mastermix and an isotype antibody mastermix were prepared in the dark. Isotype controls were used to discriminate between specific and non-specific binding. They are primary antibodies conjugated to the same fluorochrome but that lack specificity to the target (i.e. they lack the specific Fab region), but match the immunoglobulin class and the antibody host. Since isotype controls would not bind to the specific target, any fluorescence detected resulting from the isotype control (which does not overlap with autofluorescence) is due to non-specific protein binding.

The unstained controls were set up to identify fluorescence that is not due to the fluorochromes, but is autofluorescence (background fluorescence coming from the cells themselves). Unstained controls were also important to set the appropriate PMT voltages on the instrument.

The final concentrations of antibodies used per sample are given in Table 3.2. To prepare the mastermix, the volumes of antibodies/isotypes used per test given in the table were multiplied by the total number of antibody or isotype samples (excluding the unstained samples). The final concentration of the isotype controls was always equal to that of its respective antibody. All fluorochrome-conjugated antibodies and isotypes were purchased from BD Biosciences, USA. The unstained samples were prepared by re-suspending unstained cells in 100µl staining buffer only.

	Reagent	Fluorochrome	Purchased Concentration (µg/ml)	Quantity/test (µg)	Volume/test (in 100µl total volume) (µl)	Final Concentration/test (µg/ µl)
Antibody	Human Anti-CD25 Mouse IgGκ1	PerCP-Cy5.5	50.0	0.25	5.0	0.0025
	Human Anti-CD127 Mouse IgGκ1	PE	200.0	0.50	2.5	0.0050
	Staining Buffer (PBS, 2% FBS, 1mM EDTA)				92.5	
Isotype	Mouse IgGκ1	PerCP-Cy5.5	12.5	0.25	20.0	0.0025
	Mouse IgGκ1	PE	50.0	0.50	10.0	0.0050
	Staining Buffer (PBS, 2% FBS, 1mM EDTA)				70.0	
Unstained	Staining Buffer (PBS, 2% FBS, 1mM EDTA)				100	

Table 3.2: Extracellular staining of CD25 and CD127 - Quantities and volumes of reagents used per test. Note that while preparing the master mix, the volumes of antibodies/isotypes used per test was multiplied by the number of antibody/isotype samples.

The antibody mastermix was vortexed and distributed equally among the antibody designated tubes which now contained the pellet of cells. The Isotype master mix was also vortexed and distributed equally among the isotype designated tubes which also contained the pellet of cells. The tubes were placed in a vortex and then incubated on a rotating platform for 30 minutes in the dark. After incubation, the tubes were washed with PBS, centrifuged at 250 × g for 5 minutes and stained for intracellular FOXP3 following the procedure in section 3.1.5.2 and 3.1.5.3.

3.1.5.2 Fixation and Permeabilization for Intracellular Staining

Fixation and permeabilization of cells for intracellular staining of FOXP3 was carried out using the Human FoxP3 Buffer Set (BD Biosciences, USA, Catalogue No.: 560098) which contained the fixative and the permeabilization buffer.

To fix the cells, 2ml of 1X Human FoxP3 Buffer A was added to the pellet of cells in each tube. Human FoxP3 Buffer A is a fixative made up of a mixture of diethylene glycol, formaldehyde and methanol and was prepared by diluting 10X Human FoxP3 Buffer A supplied with the kit at a ratio of 1:10 with room temperature deionized water. Once the fixative was added to the cells, the tube was vortexed and incubated for 10 minutes at room temperature protected from light.

The tube was then centrifuged at high speeds of 500 × g for 5 minutes and the fixative was removed by decanting, taking care not the lose the pellet which becomes buoyant after fixation. The pellet was then washed with PBS and centrifuged again at 500 × g for 5 minutes to make sure that the fixative had been completely removed.

The cells were then permeabilized in order to allow the intracellular marker antibody to penetrate the cells. This was achieved by gently re-suspending the cells in 500µl of 1X working solution Human FoxP3 Buffer C for each tube. Human FoxP3 Buffer C is a permeabilising agent made up of saponins which interact with the cholesterol in the cell membrane leaving holes in the membrane. It was prepared by diluting 50X Human FoxP3 Buffer B supplied with the kit with 1X FoxP3 Buffer A at a ratio of 1:50 (Buffer B: Buffer A). The tubes were then vortexed and incubated for 30 minutes at room temperature and protected from light. The cells were then washed in PBS and centrifuged at 500 × g for 5 minutes at room temperature. The permeablising agent was decanted and the washing step was repeated again. The pellet was then stained with FOXP3 antibody (or its isotype) as described in the next section.

3.1.5.3 Intracellular Staining with FOXP3

Two master mixes were prepared in tubes, one composed of the antibody mix and the other composed of the isotype mix. The compositions of each master mix are given hereunder.

The final concentrations of antibodies and isotypes used per sample are given in Table 3.3. To prepare the master mix, the volumes of antibodies/isotypes per test given in the table were multiplied by the total number of antibody or isotype samples (excluding the unstained samples). The final concentration of the isotype control was always equal to that of its FOXP3 antibody. The fluorochrome-conjugated antibody and isotype were purchased both from BD Biosciences, USA or Biolegend, USA.

	Reagent	Fluorochrome	Purchased Concentration (µg/ml)	Quantity (µg)	Volume per test (in 100µl total volume) (µl)	Concentration per test (µg/µl)
Antibody	Human Anti-FOXP3 Mouse IgGκ1	Alexafluor® 488	25	0.5	20	0.005
	Staining Buffer (PBS, 2% FBS, 1mM EDTA)				80	
Isotype	Mouse IgGκ1	Alexafluor® 488	100	0.5	5	0.005
	Staining Buffer (PBS, 2% FBS, 1mM EDTA)				95	
Unstained	Staining Buffer (PBS, 2% FBS, 1mM EDTA)				100	

Table 3.3: Intracellular staining of FOXP3 - Quantities and volumes of reagents used per test. Note that when preparing the master mix the volume per test was multiplied by the number of samples.

The antibody master mix was vortexed and distributed equally (100µl per tube) among the tubes which contained cells that had been previously stained with the extracellular markers CD25 and CD127. The Isotype master mix was also vortexed and distributed equally (100µl per tube) among the tubes which had been previously stained with the extracellular isotypes for CD25 and CD127. Again, the unstained cells were just re-suspended in 100µl staining buffer.

The tubes were then vortexed and then incubated on a rotating platform for 30 minutes in the dark. The tubes were then washed with PBS and centrifuged at 500 × g for 5 minutes. The washing step was performed twice and the pellet was then re-suspended in 500µl of staining buffer (PBS, 2% FBS, 1mM EDTA). The samples were then run on BD FACSCalibur™ (BD Biosciences, USA) immediately and analysed on BD CellQuest Pro™ software (BD Biosciences, USA).

If for some reason, the cells were not analysed on the day of staining, the cells were fixed with 300µl of 1% paraformaldehyde in PBS, stored at 4°C and analysed the following day.

3.1.5.4 Flow Cytometric Analysis to Determine Purity Of Populations

A dot plot of FSC-H versus SSC-H was plotted. Three histograms of cell count versus mean fluorescent intensity (MFI) of a) FOXP3 b) CD25 and c) CD127 were plotted. Also, three other dot plots were plotted: FOXP3 versus CD25, FOXP3 versus CD127 and CD25 versus CD127.

A FACS tube containing unstained cells was loaded into the BD FACSCalibur™, and 15,000 – 30,000 total events were collected with the instrument flow rate set on LOW. The instrument voltage settings were set such that the peak of the unstained sample (attributed to background autofluorescence) was set at the far left side of the histogram. The tube containing the isotype-stained cells followed by the tube containing the antibody-stained cells were then sequentially loaded and read on the instrument.

The .fcs files were exported from the machine and the files analysed on FlowJo™ Version 7 (TreeStar).

Using the dot plot of FSC-H versus SSC-H, the singlet lymphocyte population was identified and gated. Unfortunately, contrary to BD FACSAria™ III software, BD FACSCalibur™ CellQuest Pro™ software does not allow the option to plot FSC-A vs. FSC-H or FSC-A vs. FSC-H to help in distinguishing singlets versus doublets.

Once the singlet population was identified, the percentage specific binding was determined by overlaying the histogram of the isotype-stained sample and the unstained sample onto those of the antibody-stained sample. The percentage of cells positive for the markers (i.e. the non-overlapping area of the histogram to the right) was then automatically calculated by the software itself once an interval gate was manually set.

In turn, the histograms were then used to delineate the quadrants on the FOXP3 vs. CD25, FOXP3 vs. CD127 and CD25 vs. CD127 dot plots drawn, namely to identify the following populations: a) FOXP3highCD25high, b) FOXP3-/low CD25-/low, c) FOXP3highCD127-/low, d) FOXP3-/low CD127+, e) CD25highCD127-/low, f) CD25-/lowCD127+. The percentage of cells falling in each of the quadrants was generated automatically by the software.

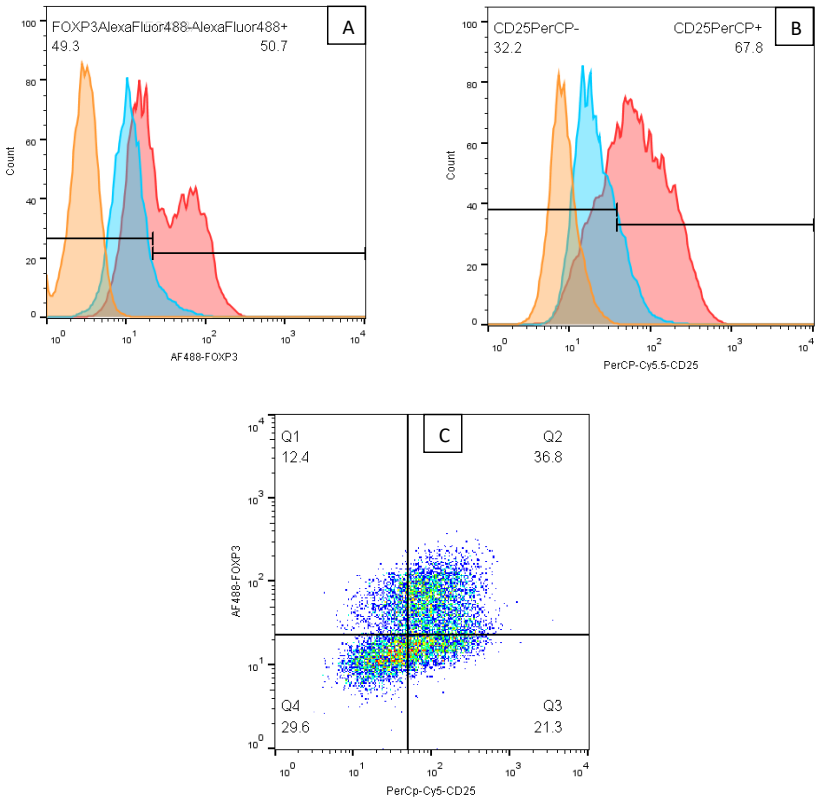


Figure 3.3: Gating strategy used to determine percentage of cells positive (or not) for a marker using FlowJo Version 7. Histograms A and B show the antibody-stained sample (red peak) overlain on top of the isotype-stained sample (blue peak) and unstained sample (orange peak). An interval gate is set and the percentage positive cells is shown to the right of the histogram. The histograms were then used to delineate the quadrants in dot plot C. Quadrant Q2 gives the percentage of cells that are positive for both FOXP3 and CD25. This gating strategy to determine percentage positivity was adopted for all extracellular and intracellular markers used throughout the study.

3.1.6 Activation and Expansion of T Cells

Since Tregs compromise only 4% of CD4+ T cells, the number of isolated Treg was generally very low. Therefore, the Treg needed to be expanded into sufficient numbers for downstream experiments. Activation was achieved using soluble anti-CD3/CD28 monoclonal antibodies.

The ImmunoCult™ Human CD3/CD28 T Cell Activator (STEMCELL Technologies, USA, Catalogue No.: 10991) was used. Soluble monoclonal antibodies against CD3 and CD28 were used to activate the T cells. The advantage of using soluble rather than magnetic bead-coupled anti-CD3 and anti-CD28 is that, apart from the extra step of having to remove the beads magnetically before using the cells, cell loss during magnetic bead removal due to strong adherence of the cells to the beads, is avoided.

To activate the T cells using soluble anti-CD3/CD28, the cells were re-suspended, usually in a 96 well plate or 24 well plate, at a concentration of 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% FBS, 1% Pen-Strep and 1% Amphotericin. 25µl of ImmunoCult™ Human CD3/CD28 T Cell Activator per ml of cells was added, followed by 1000U/ml of recombinant human IL-2 (Life Technologies, USA). The cells were incubated at 37°C and 5% CO_2 for 10-14 days.

On Day 1, 1000U/ml of IL-2 were re-added. The cells were examined daily and if the cells were observed as highly confluent under the microscope or the medium appeared yellow, the cells were split by transferring the suspended cells in adjacent wells and topped up with fresh complete medium supplemented with 1000U/ml of IL-2 to yield a final concentration of approximately $1\text{-}5 \times 10^6$ cells/ml.

The same procedure as described above was followed to expand non-Treg CD4+ T cells with the difference that only 100U/ml (instead of 1000U/ml) of IL-2 were added since CD4+CD25- non-Treg cells produce their own IL-2. Again, when splitting the cells due to confluency, the wells were topped up with fresh medium supplemented with 100U/ml of IL-2.

Cell count/ well	**2×10^5**		**1×10^6**	
Cell type	Treg	Non T-reg	Treg	Non T-reg
Culture plate	96-well		24-well plate	
ImmunoCult™ Human CD3/CD28 T Cell Activator	25µl/ml	25µl/ml	25µl/ml	25µl/ml
IL-2	1000U/ml	100U/ml	1000U/ml	100U/ml
Total seeding volume (topped up RPMI-1640 + 10% FBS + 1% Pen-Strep + 1 % Amphotericin B)	200µl	200µl	1000µl	1000µl

Table 3.4: Specifications for the activation and expansion T cells using ImmunoCult™ Human CD3/CD28 T Cell Activator

3.1.7 Immunosuppression Assays to Confirm Treg Function

A key feature of Treg is their ability to inhibit the proliferation of responder T cells (Tresp) *in vitro* and therefore the most prevalent evaluation method to assess the functional potential of Treg is to assess their ability to suppress proliferation of responder T cells in an immunosuppression assay. In the procedure described below, Treg cells were co-cultured with responder T cells at different ratios ranging from 1:1 to 1:32 Treg to responder T cells respectively and the percentage suppression of Tresp proliferation at these different Treg:Tresp ratios calculated. This step formed part of method validation to check whether Treg cells extracted using the methods outlined so far were suppressive and up to which Treg:Tresp ratios.

To monitor lymphocyte proliferation, cell proliferation dyes were used. Cell-tracking assays using cell proliferation dyes are based on dilution of the labelling dye with subsequent cell divisions. When the labelled cell divides, the fluorescence is distributed equally to the daughter cells (halved), so a decrease in fluorescence intensity is observed with every successive cell division. To monitor cell proliferation of Tresp cells in the absence or presence of Treg, Carboxyfluorescein diacetate succinimidyl ester, CFSE (Biolegend, USA) was used. Labelling of cells with CFSE is described in the next section. The decrease in fluorescent intensity was monitored by flow cytometry.

3.1.7.1 Labelling Tresp with CFSE

The Tresp cells were spun down at 250 × g for 5 minutes to remove the medium. The cells were then washed twice with PBS to remove any remaining medium containing serum which may interfere with the staining procedure. In the meantime, a 5µM CFSE working solution was prepared by adding 1µl of 5mM CFSE stock solution (BioLegend®, USA) in 1ml PBS. The pellet obtained after the two washing steps, was re-suspended in the CFSE working solution to a concentration of 1-10 × 10^7 cells/ml. The cells were then placed for 20 minutes in an incubator at 37°C protected from light. The staining was quenched by adding 5ml of cell culture medium containing 10% FBS. The cells were pelleted by centrifugation at 250 × g for 5 minutes, re-suspended in pre-warmed cell culture medium and incubated at 37^{o}C with 5% CO_2 until further use.

3.1.7.2 Co-Culturing Regulatory T Cells and Responder T Cells.

The Tresp cells (1 × 10^7 cells) were labelled with 5µM CFSE (Biolegend®, USA) following the procedure described in 3.1.7.1 . The labelled cells were re-suspended in RPMI-1640 medium complete with 10% FBS,1% Pen-strep and 1% Amphotericin B.

In a volume of 150µl complete RPMI-140 medium, 5 × 10^5 Treg cells were plated in 96 well plates and 50µl of cells were withdrawn from this well (Well A) and transferred to an adjacent well (Well B). To this well, 50µl of complete RPMI 1640 medium was added to achieve half the cell concentration found in well A. From Well B, 50 µl of cells were transferred to another adjacent well, Well C, and to this well 50µl of complete RPM1640 medium were added. Three other such serial halving dilutions (Well D – F) were carried out in the same way to give a total of six wells.

Following this, 5×10^5 CFSE-labelled Tresp cells (in 100µl) were added to each of the six well to achieve final Treg:Tresp ratio of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 starting from Well A down to Well F respectively.

To activate the cells, 25µl/ml of ImmunoCult™ Human CD3/CD28 T Cell Activator and 100U/ml of IL-2 were added to each well. RPMI-1640 medium was then added to each well to achieve a final concentration of 1×10^6 total cells per ml.

A negative control for immunosuppression (normally/fully proliferative) was set up which consisted of 5×10^5 Tresp cells alone in the absence of Treg in a total seeding volume of 500µl and supplemented with 25µl/ml of ImmunoCult™ Human CD3/CD28 T Cell Activator and 100U/ml of IL-2. In the absence of Treg, full proliferation of Tresp was expected.

A positive control for immunosuppression (strongly suppressive) was also set up. This consisted of 5×10^5 Tresp cells alone in the absence of Treg in a total seeding volume of 500µl and supplemented with 25µl/ml of ImmunoCult™ Human CD3/CD28 T Cell Activator, 100U/ml of IL-2 and 300ng/ml of Rapamycin (Cayman Chemicals, USA). Rapamycin is an immunosuppressive drug that inhibits proliferation of responder T cells (Battaglia, Stabilini, Roncarolo, View, & Gladbach, 2005).

A culture of Tresp cells that had not been activated with ImmunoCult™ Human CD3/CD28 T Cell Activator was also set up. It consisted of 5×10^5 Tresp cells alone in the absence of Treg in a total seeding volume of 500µl and without ImmunoCult™ Human CD3/CD28 T Cell Activator and IL-2.

An unlabelled, activated sample of Tresp cells was used to account for autofluorescence during flow cytometric analysis as well as to set up the adequate PMT

voltages of the instrument. This sample consisted of unlabelled 5×10^5 Tresp cells alone in the absence of Treg in a total seeding volume of 300µl and supplemented with 25µl/ml ImmunoCult™ Human CD3/CD28 T Cell Activator and 100U/ml of IL-2. The table below summarizes the contents of each well.

Well	No. of Treg per 300µl	No. of Teff per 300µl	5µM CFSE	25µl/ml ImmunoCult™ Human CD3/CD28 T Cell Activator	100U/ml IL-2	300ng/ml Rapamycin
Unstained ctrl	×	5×10^5	×	✓	✓	×
Un-activated ctrl	×	5×10^5	✓	×	×	×
Uninhibited (negative) ctrl	×	5×10^5	✓	✓	✓	×
Suppressive (positive) ctrl	×	5×10^5	✓	✓	✓	✓
1:1 Treg:Teff	5.0×10^5	5×10^5	✓	✓	✓	×
1:2 Treg:Teff	2.50×10^5	5×10^5	✓	✓	✓	×
1:4 Treg:Teff	1.25×10^5	5×10^5	✓	✓	✓	×
1:8 Treg:Teff	6.20×10^4	5×10^5	✓	✓	✓	×
1:16 Treg:Teff	5.0×10^4	5×10^5	✓	✓	✓	×
1:32 Treg:Teff	1.5×10^4	5×10^5	✓	✓	✓	×

Table 3.5: Immunosuppression Assay – Components of each well.

The cells were examined daily and if the cells were observed to be highly confluent under the microscope or the medium appeared yellowish, indicating extensive cell growth, the cells were split by transferring a fraction of the well contents to adjacent wells and topped up with fresh complete medium supplemented with 100U/ml of IL-2 to yield a final concentration of approximately 1×10^6/ml.

The cells were co-cultured for 96 hours after which cell proliferation of Tresp cells was analysed by flow cytometry as described in the next section.

3.1.7.3 Data Acquisition and Analysis Of Cell Proliferation

The percentage suppression in the proliferation of Tresp cells by Treg cells was analysed by flow cytometry at 96 hours post activation. The cells were loaded onto the BD FACS Calibur and information on the fluorescence emitted by CFSE by Tresp acquired using BD CellQuest™ Pro software.

The cells were transferred from the wells to non-sterile 12x75mm polypropylene 5ml tubes, washed with PBS and centrifuged at 250 × g for 5 minutes. The cells were then washed again with PBS, centrifuged at 250 × g for 5 minutes and re-suspended in 500µl of staining buffer.

Prior to loading the cells into the BD FACS Calibur™, a plot of SSC-H versus FSC-H was plotted using BD CellQuest™ Pro in order to help identify the singlet lymphocyte populations. The CFSE dye is excited by the blue laser and detected by the same channel as the FITC fluorochrome (FL-1 channel on the BD FACS Calibur™). A histogram of cell count versus fluorescent intensity of CFSE was plotted.

The unstained sample of cells was then loaded into the machine and the cells gated on the singlet cell population in the same way as described in previous sections. The instrument PMT voltages were adjusted such that the peak signal from the unstained sample (if present, attributed to autofluorescence) was set at the far left side of the histogram. Next, the labelled Tresp cells which were not activated were run, followed by the positive (suppressive) control (labelled Tresp cells in the presence of rapamycin) and the PMT voltages adjusted to set their histogram peaks at the far right side of the histogram, since these non-dividing cells should retain the highest fluorescence intensity. The peaks of the co-culture of Tresp and Treg were expected to be found somewhere in

between the peaks of the unstained sample and the not activated/positive (suppressive) control. All the data was saved as .fcs files and exported on FlowJo Version 7 to analyse the data as described in the next section.

3.1.7.4 Calculation of Percentage Suppression of Tresp Proliferation

To obtain the percentage of cells which have undergone cell division, each histogram was overlain onto the histograms generated from the positive (suppressive) control and the cells which were not activated. An interval gate as shown in Figure 3.4 was set up in order for the software to automatically generate the percentage of divided cells, by comparing the areas under the graph peaks between the undivided peak and the peaks shifted to the left.

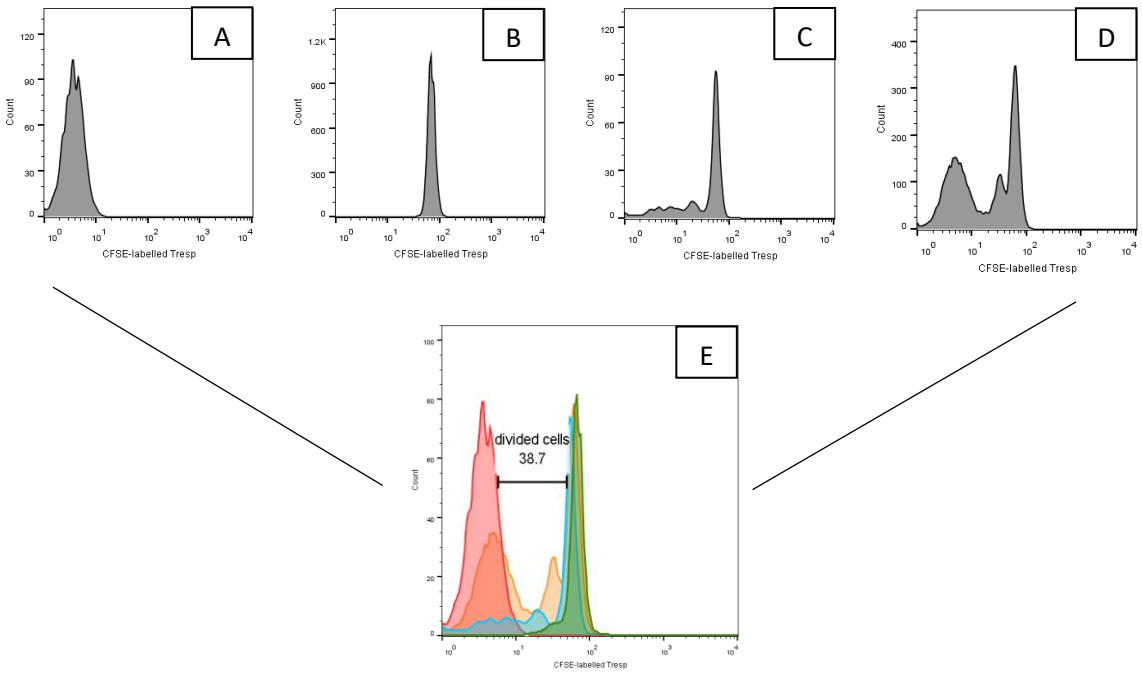


Figure 3.4: How to determine percentage of proliferated cells using FlowJo software Version 7. Panel A-D shows the separate histograms as follows: Panel A - unstained sample, Panel B – sample of cells which have not been activated, Panel C - positive control (i.e. Tresp activated with anti-CD3/CD28 and 100U/*ml* IL-12 in the presence of 300ng/*ml* Rapamycin), Panel D co-culture of Treg and Tresp at a 1:1 activated with anti-CD3/CD28 and 100ng/*ml* IL-2. Panel E shows the overlain histograms (pink peak – unstained sample, green peak, not activated sample, blue peak – positive control, orange peak 1:1 Treg:Tresp co-culture). An interval gate was manually set up for the software to automatically calculate the percentage of divided cells. In this example, only 38.7% of the Tresp co-cultured with Treg at a ratio of 1:1 underwent cell division.

The percentage suppression of Tresp proliferation by Treg was then manually calculated using the following formula:

$$\% \text{ suppression (of Tresp proliferation)} = 100 - \left(\% \textit{ divided in the presence of } Treg / \% \textit{ divided in the absence of } Treg\right) 100$$

Equation 3.1: Equation used to calculate percentage immunosuppression using the conventional gating method (Mcmurchy & Levings, 2012).

3.2 Investigating The Effect Of Interferon-ϒ And Toll-Like Receptor Ligands On Treg Phenotype, Suppressor Function And Cytokine Production Profile

3.2.1 Cell Culturing For Phenotype Assays

The CD4+CD25+ Treg cells that were extracted from peripheral blood and expanded following the procedures described in section 2.1 were used for this analysis. If these cells had already been activated with anti-CD3/CD28 to expand them to sufficient working numbers, they were allowed to rest and not re-activated prior to 12 days after the first activation. After the 10^{th} day post-activation, the cells were starved from IL-2 to slow down the rate of expansion. The procedure described below is a general one that was used when preparing Treg for phenotype analysis and was carried out when investigating the effects of Interferon γ and Toll Like Receptor ligands.

Once the number of CD4+CD25+ Treg cells were expanded (if required) to sufficient numbers, they were seeded in 96 well plates at a maximum of 2×10^5 cells in RPMI-1640 medium complete with 10% FBS, 1% Pen-Strep and 1% Amphotericin in a total well volume of 200µl and therefore a starting concentration of 1×10^6 cells/ml. To activate the Treg, 7µl of ImmunoCult™ Human CD3/CD28 T Cell Activator (STEMCELL TECHNOLOGIES, Canada) were added to each well as well as 1000U/ml of IL-2 (Life Technologies, USA). The 96-well plate was placed in a plate shaker and shaken at 600rpm for 5 minutes to mix the contents in the wells. The cells were then placed in a humidified incubator at 37°C with 5% CO_2. The agents to be tested were also added on the same day at the required concentration (Refer to dedicated sections for information on concentrations used etc).

On Day 1 following activation, 1000U/ml of IL-2 were added again. Following activation and treatment, the cells were cultured for a total of 6 days post-activation and phenotype analysis was carried out as described in section 3.2.4

3.2.2 Cell Culturing For Functional Assays

The procedure described below is a general one that was used when preparing Treg and Tresp for functional and proliferation analysis and was carried out when investigating the effects of Interferon γ and Toll Like Receptor ligands.

To monitor proliferation of Treg as well as CD4+ non-Treg cells, the cells had to be stained with two different proliferation dyes. The CD4+ non-Treg cells will henceforth be referred to as Responder T cells or Tresp. In the case of Treg, they were first stained with eBioscience™ Cell Proliferation Dye eFluor™ 670 (ThermoFisher Scientific, USA) following the procedure described in section 3.2.2.1. An unstained sample of cells was also retained to be used later on for setting up the PMT voltages on the flow cytometer.

After staining with the cell proliferation dye, the cells were activated (or reactivated 12 days after the first activation) with anti-CD3/CD28 and IL-2, as described in section 3.2.1 above. A well containing 2×10^5 stained cells in 200µl total volume (giving a concentration of 1×10^6 cells/ml) was left inactivated by omitting the addition of ImmunoCult™ Human CD3/CD28 T Cell Activator and IL-2. This sample was required as a no-proliferation control. The Interferon-γ or TLR ligands were added to the Treg on the same day of activation. The 96-well plate was placed in a plate shaker at low speed for 5 minutes to mix the contents of the well. Then, the plate was placed in a humidified incubator at 37^{o}C with 5% CO_2 and left untouched for 2 days.

To determine the functional and suppressive capabilities of Treg, CD4+CD25- Tresp cells were co-cultured with CD4+CD25+ Treg. For this reason, Tresp cells obtained from the same blood donor as the Treg were activated using anti-CD3/CD28 and IL-2. Before activation, Tresp cells were stained with another cell proliferation dye, CFSE (BioLegend®, USA) following the procedure described in section 3.1.7.1. Similarly, an unstained sample of cells was also retained in order to set up the PMT voltages on the flow cytometer.

After staining, the Tresp cells were seeded in 96 well plates at 2×10^5 in RPMI-1640 medium complete with 10% FBS, 1% Pen-Strep and 1% Amphotericin in a total volume of 200µl and therefore a starting concentration of 1×10^6 cells/ml. To activate the cells, 7µl of ImmunoCult™ Human CD3/CD28 T Cell Activator and 100U/ml of IL-2 were added to each well containing the Tresp. An inactivated sample of 2×10^5 stained cells was also prepared by omitting ImmunoCult™ Human CD3/CD28 T Cell Activator and IL-2. The 96-well plate was placed in plate shaker at low speed for 5 minutes to mix the contents of the well. The cells were then placed in a humidified incubator at 37°C with 5% CO_2.

On day 2 post-activation of both the Treg and the Tresp cells, the two separate plates containing the Treg and Tresp were centrifuged at 250 × g for 5 minutes, half of the supernatant in each well (100µl) discarded and the cells resuspended in the remaining supernatant (100µl) by gentle pipetting. The Tresp were then added to the Treg to obtain a co-culture of Treg and Tresp at a ratio of 1:1 and a hence a total of approximately 4×10^5 cells (2×10^5 cells of each). The wells now contained the co-culture in a total volume of 200µl and a concentration of 2×10^6 cells/ml. Since this cell concentration was too high, the co-cultures were transferred to a bigger 48 well plate and each well topped up to a total of 400µl with complete RPMI 1640 medium to return the concentration to 1×10^6 cells/ml. A Tresp:Treg ratio of 1:1 was chosen (and was the only ratio used in all experiments following

method validation) because it was hypothesized that if the treatment affected Treg-mediated immunosuppression at such high Treg:Tresp ratios, it would also do so at the lower Tresp: Treg ratios typically observed in tumours.

The number of wells containing the co-cultures depended on the number of treatment conditions set up. These different treatment conditions are described in section 3.2.3. In all experiments, however, some wells were reserved for a monoculture of Treg and a monoculture of Tresp. These monocultures consisted of 2×10^5 of Treg or Tresp cells in 200µl of RPMI-1640 medium (i.e. starting concentration of 1×10^6 cells/ml). One monoculture well of Tresp was left untreated while the others were treated with the agents at the same concentration used for Treg. This was done in order to investigate the effects of the agents also on Tresp.

The two wells which contained the unactivated Treg and Tresp cells were also retained as monocultures and cultured in 96 well plates in a total of 200µl of complete RPMI-1640 medium only. Additionally, a positive control for immunosuppression was also set up. This well consisted of a monoculture of Tresp cells previously activated with ImmunoCult™ Human CD3/CD28 T Cell Activator with Rapamycin (Cayman Chemical, USA) added on the day of activation to a final concentration of 300ng/ml. The table below summarizes the contents of each mono/co-culture. The cells were kept in a humidified incubator at 37^{o}C and 5% CO_2 and cultured for a total of 7 days after activation (or 5 days after the co-cultures were prepared).

	Proliferation Dye[i]	Anti-CD3/CD28 (7ul)	IL-2 (100-1000U/ml)	Treatment (IFN-γ or TLR Ligand)[iv]	Treg (2×10^5 cells)	Tresp (2×10^5 cells)	300ng/ml Rapamycin	Total seeding volume[iii]
Stained Non-activated Treg monoculture	✓	x	x	x	✓	x	x	200
Stained Non-activated Tresp monoculture	✓	x	x	x	x	✓	x	200
Stained Activated Treg monoculture (-ctrl)	✓	✓	✓	x	✓	x	x	200
Stained Activated Tresp monoculture, untreated	✓	✓	✓	x	x	✓	x	200
Stained Activated Tresp monoculture, treated	✓	✓	✓	✓	x	✓	x	200
Stained Activated Tresp monoculture (+ ctrl for immunosuppression)	✓	✓	✓	x	x	✓	✓	200
Stained Activated co-culture, untreated	✓	✓	✓	x	✓	✓	x	200
Stained Activated co-culture, treated [ii]	✓	✓	✓	✓	✓	✓	x	400
Unstained activated co-culture	x	✓	✓	x	✓	✓	x	200

Table 3.6: Contents of each well type for functional analysis.

[i] Treg were stained with eBioscience™ Cell Proliferation Dye eFluor™ 670 while Tresp were stained with CFSE.

[ii] Number of wells with stained activated co-culture depends on the number of treatment conditions tested. The Tresp were added to the Treg 2 days post-activation.

[iii] Starting concentration (before proliferation) was 1×10^6 cells/ml in each well

[iv] The agents were added to the cells on the same day of activation.

3.2.2.1 Labelling Treg With Cell Proliferation Dye Efluor™ 670

Cell Proliferation Dye eFluor® 670 (Thermo Fisher Scientific USA) is a red fluorescent dye that can be used to monitor individual cell proliferation for up to 6 generations. It has a peak excitation of 647nm and is excited by the red laser (633nm). It has a peak emission of 670nm and can be detected with a 660/20 band pass filter.

To label the Treg cells with the dye, the cells were washed with PBS and centrifuged at 250 × g for 5 minutes to remove any serum which might interfere with the staining procedure. This washing step was repeated twice. The cells were then resuspended in PBS at a concentration of 5×10^5 - 10×10^6 cells/ml. While vortexing the cells, 1µl per ml of 5mM of Cell Proliferation Dye eFluor® 670 was added to give a final concentration of 5µM of the dye in PBS. The cells were incubated for 10 minutes at 37°C in the dark. Labelling was stopped by adding 4-5 times volumes of RPMI-1640 supplemented with 10% FBS and

incubated on ice for 5 minutes. The cells were then washed 3 times with complete RPMI-1640 medium. The supernatant was decanted and the cells re-suspended in RPMI-1640 medium supplemented with 10% FBS, 1% Pen-Strep and 1% Amphotericin and incubated at 37^{o}C with 5% CO_2 until further use.

3.2.3 Treatments

3.2.3.1 Interferon - γ

For phenotype analysis, four wells per replicate were prepared and the Treg cells were activated as described in section 3.2.1. No IFN-γ was added to one of the wells. This well served as the untreated control. To another well, 25ng/ml of IFN-γ (Life Technologies, USA) were added on the day of activation. To a third well, 50ng/ml of IFN-γ were added. The 96-well plate was then placed in plate shaker at low speed for 5 minutes to mix the contents of the wells and the cells placed in a humidified incubator at 37^{o}C with 5% CO_2 . The cells were cultured for a total of 6 days. If the wells were observed as highly confluent due to proliferation of cells, the contents of the wells were split in half and topped up with fresh complete medium supplemented with IL-2. After 6 days, the cells were labelled for surface and intracellular marker and analysed by flow cytometry as described in 3.2.4. A total of three biological replicates (each with the same sets of treatment conditions) were set up.

For functional analysis, the monocultures and co-cultures of Tresp and Treg were prepared and stained with the proliferation dyes as described in section 3.1.7.1. Two monocultures of Tresp were treated with 25ng/ml and 50ng/ml IFN-γ on the same day of activation while one monoculture was left untreated.

Also on the day of activation, three wells containing Treg were prepared where one was left untreated and the other two were treated with 25ng/ml and 50ng/ml of IFN-γ. Two days later, the Treg were added to the Tresp.

Upon addition, the plates were placed in a plate shaker at low speed for 5 minutes. The plates were then placed in a humidified incubator at 37°C with 5% CO_2 and cultured for a further 5 days. If during this five day period, the wells were observed as highly confluent due to proliferation of cells, the contents of the wells were split in half and topped up with fresh complete RPMI-1640 medium supplemented with IL-2 (excluding the non-activated samples). No IFN-γ was added once the Treg were added to the Tresp (only prior). After 5 days co-culturing (and a total of 7 days since activation), the proliferation of the cells was analysed by flow cytometry as described in section 3.2.5. The cell supernatant (conditioned medium) of both monocultures and co-cultures was retained and stored at -20°C for cytokine analysis (Refer to section 3.2.6). A total of three biological replicates (each with the same sets of treatment conditions) were set up.

3.2.3.2 Endogenous TLR7/8 Ligand: Human Cell-Free RNA

Extraction of endogenous RNA from human cells

The RNeasy Mini Kit (Qiagen, Germany, Catalogue number 74106) was used to extract endogenous RNA from human lymphocytes using spin column technology.

This kit allows the purification of RNA molecules/fragments containing more than 200 nucleotides which would mainly consist of messenger RNA (as other RNA types are usually less than 200 nucleotides).

In a 15ml sterile centrifuge tube, 10^7 human lymphocytes in cell culture medium were centrifuged at 350 × g for 5 minutes and the supernatant was decanted. It was made sure that all the cell culture medium was removed as incomplete removal would have inhibited lysis and diluted the lysate affecting the conditions for binding of RNA to the RNeasy membrane. The cell pellet was then resuspended completely in 350µl QIAzol Lysis Reagent (QIAGEN, Germany) for cell lysis using sterile filter tips and was frozen at -80°C. This procedure was repeated following 6 hours and 12 hours incubation. QIAzol is a reagent that contains guanidine isocyanate which is strong protein denaturant which, when used at high concentrations, is useful in isolating intact RNA. It also removes DNA and inhibits RNases from breaking down RNA.

Cell lysates were thawed on ice and 140µl of chloroform (Sigma-Aldrich®, USA) were added and mixed by vortexing. The tubes were placed on the benchtop at room temperature and then centrifuged at 12,000 × g for 15 minutes at 4 °C. Two layers are obtained: an upper aqueous layer, an interphase and a lower organic layer. RNA collects in the upper aqueous layer, proteins collect in the lower layer or interphase while DNA collects at the interphase. The upper aqueous phase was then carefully transferred to new 2ml microcentrifuge tube taking care that no contamination with the interphase layer occurred as this would have decreased RNA purity. To help the RNA bind to the silica-based membrane on the spin column, 350µl of absolute ethanol (Schalau, Spain) were added. The contents were mixed using a sterile pipette tip in order to ensure complete precipitation of RNA. The sample, and any formed precipitate were transferred in an RNeasy mini spin column fitted with a 2ml collection tube provided with the kit and was centrifuged at 16°C for 15 seconds at 20,000 × g for 2 minutes. The flow-through was discarded and 700µl of Buffer RW1 provided with the kit was added to the RNeasy spin column to remove non-

specifically silica bound proteins, carbohydrates and fatty acids. The RNeasy spin column was then centrifuged at 20,000 × g for 15 seconds at room temperature. For salt removal, 500µl of Buffer RPE provided with the kit were added to the RNeasy spin column. Centrifugation was performed at room temperature for 15 seconds at 20,000 × g and the flow through was discarded. This latter step was performed for another time. The RNeasy spin column was then placed in a new 2ml collection tube provided with the kit. Centrifugation was performed at room temperature for 1 minute at 20,000 × g to dry the column and the collection tube and the flow-through was discarded. The RNeasy spin column was then placed in a new 1.5ml collection tube provided with the kit and 30µl RNase free water also provided with the kit was pipetted directly onto the spin column. Centrifugation was performed at room temperature for 1 minute at 20,000 × g to elute the RNA. The spin column was removed and discarded, while the collection tube contained the eluted RNA.

The concentration in ng/µl was calculated and the purity of extracted RNA was determined by measuring the A230/A280 ratio on a Nanodrop 2000 UV-Vis spectrophotometer (Life Technologies, USA). The RNA was then stored at -80^{o}C until further use.

Complexing RNA to Polyethylenimine Transfection Reagent

Since TLR 7 and 8 are found localised on intracellular cytoplasmic endosomes, the RNA had to be complexed to a transfection agent for entry inside Treg and to reach the endosomes. Transfection was achieved using Transporter™ 5 transfection reagent (Polysciences, USA Inc. Catalogue No: 26008-5). This is a ready-to-use form of a proprietary linear polyethylenimine (PEI) derivative which is a cationic polymer widely

adopted in non-viral gene delivery of nucleic acids both *in vitro* and *in vivo* due to its high transfection efficiency and protection from nucleases (Jonker *et al.*, 2017) PEI is capable of condensing nucleic acids by forming ionic bonds with the phosphate backbone. In this way complexes are formed that are internalized into cells by endocytosis and PEI delivers the cargo into endosomal and cytosolic compartments in cells (Wegmann *et al.*, 2013).

Extracted RNA was added to the diluent (150mM NaCl solution in sterile deionized water) in a polypropylene tube. The volume of diluent added was 10% the total seeding volume. The solution was briefly vortexed and 4µl of Transporter™ 5 added to the mixture per µg of RNA (ratio of RNA to Transporter™ 5 must be 1:4 according to manufacturer's instructions). The solution was then vortexed for 5 seconds. The solution was further allowed to sit for 20 minutes in a hooded environment to allow the Transporter™ 5-RNA complexes to form. Following this, the transfection solution was mixed by gently pipetting up and down 3 times and the transfection solution was set aside.

Transfecting cells with RNA

For phenotype analysis, three wells of activated Treg were prepared as described in section 3.2.1. The cells in these three wells were to be exposed to three different conditions. No RNA /PEI complex was added to one of the wells, which served as the untreated control. The cells in the other two wells were transfected with 1 or 10 µg/ml RNA as described below. A total of three biological replicates (each with the same sets of conditions) were set up.

On the same day of activation, the Treg cells to be transfected were centrifuged at 250 × g for 5 minutes and the supernatant transferred into a new sterile tube and kept aside. The pellet of cells from each well were resuspended in 200µl of RPMI 1640, free of serum. It was important to carry out transfection in serum-free medium as serum might have decreased transfection efficiency by inhibiting Transporter™ 5.

An adequate volume of transfection solution prepared as described above was added to two separate wells to yield a final concentration of 1µg/ml and 10µg/ml of RNA complexed to Transporter™ respectively. Upon addition, the 96-well plate was placed in plate shaker at 650rpm for 3 hours inside a 37°C incubator to allow for transfection. After that, the cells were washed and centrifuged at 250 × g for 5 minutes to remove any residual RNA and PEI which had not entered the cells. They were then resuspended in their own complete conditioned medium (previously set aside).

The cells were then kept in a humidified incubator at 37°C with 5% CO_2 for a further 6 days. Cells in wells observed to be highly confluent, were split in half and topped up with fresh complete medium supplemented with IL-2. Six days post-addition of RNA, the cells were stained with surface and intracellular markers and analysed by flow cytometry as described in section 3.2.4. A total of three biological replicates (each with the same sets of treatment conditions) were set up.

For functional analysis, the monocultures and co-cultures of Tresp and Treg were prepared and stained with the proliferation dyes as described in section 3.1.7.1 and 3.2.2.1. Additionally, two monocultures of Tresp were prepared and transfected with 1µg/ml and 10µg/ml RNA complexed to PEI on the same day of activation while one

monoculture was left untreated and no transfection solution was added. Also on the day of activation, three wells containing Treg were prepared where one was left untreated and the other two were transfected with 1µg/ml and 10µg/ml RNA complexed to PEI as described in the previous paragraphs for phenotype analysis. Two days later, the Treg were added to the Tresp.

The cells were cultured in a humidified incubator at 37°C with 5% CO_2 for a further 5 days after co-culturing (total of 7 days after activation). If during these days of culture, the cells in the wells were observed as highly confluent, they were split by transferring half the volume in a new well and topped up with fresh complete RPMI medium supplemented with IL-2 (excluding the non-activated samples). The proliferation of cells at 7 days post-activation was then analysed by flow cytometry as described in section 3.2.5. The cell supernatants (conditioned medium) of the monocultures and co-cultures were retained and stored at -20^{o}C for cytokine analysis (Refer to section 3.2.6). A total of three biological replicates (each with the same sets of treatment conditions) were set up.

3.2.3.3 Endogenous TLR9 Ligand: Human Cell-free DNA

Extraction of endogenous DNA from human cells

DNA from human lymphocytes was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea, Catalogue No.: K-3032R).

Approximately 1 × 10^7 cells cultured in complete RPMI-1640 medium were centrifuged at 250 × g for 5 minutes. The supernatant was discarded and the pellet re-suspended in 200µl PBS.

To a sterile 1.5ml tube, 20µl of Proteinase K supplied with the AccuPrep® Genomic DNA Extraction Kit was added and the 200µl of cell suspension was added to this tube.

A volume of 200µl of binding buffer supplied with the kit was added to the sample and mixed on a vortex mixer making sure that the sample was completely re-suspended. The sample was then incubated at 60°C for 10 minutes and after this time 100µl of isopropanol was added and everything mixed well by pipetting up and down.

After this step, the tube was centrifuged briefly to get the drops clinging under the lid. The lysate was transferred into the upper reservoir of a Binding column tube supplied with the kit, fitted in a 2ml tube also supplied with the kit, closed and the tube was centrifuged at 6797 × g for 1 minute. If the lysate was not completely passed through the column after centrifugation, the tube was centrifuged at higher speeds until the binding column tube was empty.

The tube was then opened and the Binding column transferred to a new 2ml tube supplied with the kit for filtration. Following this, 500µl of Washing Buffer 1 supplied with the kit were added without wetting the rim, the tube was closed, and centrifuged at 10,621 × g for 1 minute.

The tube was opened and the solution was discarded from the 2ml tube. Then 500µl of Washing Buffer 2 supplied with the kit were carefully added to the tube with the binding column without wetting the rim, the tube closed and centrifuged at 10,621 × g for 1 minute.

The tube was centrifuged once more at 15,294 × g for 1 minute to completely remove any ethanol (part of Washing Buffer 2). The Binding column tube was transferred to a new 1.5 ml tube supplied with the kit for elution. In order to elute, 50µl of Elution

buffer supplied with the kit were added onto the Binding column tube and left for 5 minutes at room temperature (15-25 ℃) until all the elution buffer was completely absorbed into the glass fibre of the Binding column tube. The tube was then centrifuged at 10,621 × g for 1 minute to elute. A repeat elution was performed with 10-20µl of Elution buffer being added again onto the Binding column, and left for 5 minutes again at room temperature before centrifuging at 10,621 × g for 1 minute.

The concentration in ng/µl was calculated and the purity of extracted DNA was determined by measuring the A260/A280 ratio on a Nanodrop 2000 UV-Vis spectrophotometer (Life Technologies, USA). The cell-free DNA was then stored in elution buffer at -20^{O}C until further use.

Complexing cell free DNA to Polyethylenimine

Since TLR9, like TLR 7 and 8, is found localised on intracellular endosomes, the DNA also had to be complexed to a transfection reagent. As with RNA, the cell free DNA was complexed to Transporter™ 5 transfection reagent (Polysciences Inc., USA Catalogue No: 26008-5). The same procedure described in section 3.2.3.2 was used.

Transfecting cells with cell-free DNA

Three wells of activated Treg were prepared as described in section 3.2.1 (for phenotype analysis) and section 3.2.2 (for functional analysis). The cells in each well were exposed to three different conditions. One well was left untreated and no DNA/ PEI complex was added. The other two wells were transfected with1µg/ml and 5µg/ml of

DNA/PEI complex respectively. Other than that, the same procedure used to transfect cells with endogenous RNA (both for phenotype and functional analysis) was followed and is described in section 3.2.3.2. A total of three biological replicates (each with the same sets of treatment conditions) were set up.

3.2.3.4 Synthetic TLR 7 and 8 Ligand: CL097 Imidazoquinoline Compound

CL097 is a highly water-soluble derivative of the imidazoquinoline compound Resiquimod or R848. Similarly to R848, CL097 in humans acts as an agonist to TLR7 and, to a smaller extent, TLR8.

For phenotype analysis, seven wells of activated Treg were set up as described in section 3.2.1. The cells in these seven wells were to be exposed to different conditions. To one of the wells no CL097 was added. This well served as the untreated control. To another well, 2µg/ml of CL097 (InvivoGen, USA) were added on the day of activation. To a third well, 5µg/ml of CL097 were added. According to the manufacturer's instructions, stimulation of TLR7 is achieved with 50ng - 5µg/ml of CL097 and stimulation of TLR8 is achieved with 0.5 - 5µg/ml of CL097, hence why these two concentrations within this range were selected.

Napolitani, Rinaldi, Bertoni, Sallusto, & Lanzavecchia, 2005 have shown that TLR4 and TLR8 worked in synergy to increase the production of IL-12, the differentiation cytokine for Th1. Therefore it was of interest to investigate this interaction and therefore, to the fourth well, 5µg/ml of CL097 together with 100ng/ml of bacterial LPS (an agonist for TLR4) derived from *E.coli* (Sigma-Aldrich, USA) were added. The same study by Napolitani *et al.*, 2005 had also shown that TLR8 stimulation and the simultaneous addition of IL-12

induced Th1 polarizing capacity so in order to also investigate this interaction, 5µg/ml of CL097 together with 1ng/ml of IL-12 (InvivoGen, USA) were added to the fifth well. The effect of LPS and IL-12 in the absence of CL097 was also investigated by treating the Treg in the sixth and seventh well with 100ng/ml LPS and 1ng/ml IL-12 only respectively (without adding CL097). The table below gives a summary of the composition of each of the seven wells.

Well	Treatment	Anti-CD3/CD28 (7µl)	IL-2 (1000U/ml)	CL097	LPS (100ng/ml)	IL-12 (1ng/ml)
1	Untreated	✓	✓	x	X	x
2	Treated w/ 2µg/ml CL097	✓	✓	✓ (2µg/ml)	x	x
3	Treated w/ 5µg/ml CL097	✓	✓	✓ (5µg/ml)	x	x
4	Treated w/ 5µg/ml CL097 + LPS	✓	✓	✓ (5µg/ml)	✓	x
5	Treated w/ 5µg/ml CL097 + IL-12	✓	✓	✓ (5µg/ml)	x	✓
6	Treated w/ LPS only	✓	✓	x	✓	x
7	Treated w/ IL-12 only	✓	✓	x	x	✓

Table 3.7: Investigating the effect of CL097on Treg phenotype - Composition of each well for phenotype analysis

Upon completion, the 96-well plate was placed in plate shaker at low speeds for 5 minutes and the cells placed in a humidified incubator at 37°C with 5% CO_2 and cultured for a further 6 days. If cells in the wells were highly confluent, the contents of the wells were split in half in adjacent wells and topped up with fresh complete medium supplemented with IL-2. After these 6 days, the cells were stained with surface and intracellular marker and analysed by flow cytometry as described in section 3.2.4. A total of three biological replicates (each with the same set of treating conditions) were set up.

For functional analysis, the monocultures and co-cultures of Tresp and Treg were prepared and stained with the proliferation dyes as described in section 3.2.2. Two monocultures of Tresp were treated with 2µg/ml and 5µg/ml of CL097 on the same day of activation while one monoculture was left untreated with no CL097 added. Also on the day of activation, seven wells of Treg were prepared as in Table 3.7. Two days later, the Treg were added to the Tresp.

Upon addition, the plates were placed in plate shaker at a low speed for 5 minutes. If the cells were observed to be highly confluent, they were split in half and topped up with fresh complete medium supplemented IL-12. Five days following co-culturing (and a total of 7 days post-activation), the proliferation of cells was analysed by flow cytometry as described in 3.2.5. The cell supernatant (conditioned medium) of both monocultures and co-cultures was retained and stored at -20^{o}C for cytokine analysis. A total of three biological replicates (each with the same set of conditions) were set up.

3.2.3.5 Synthetic TLR7 and TLR8 Ligand: Single Stranded Poly-Uridine

Single Stranded Poly-Uridine or ssPolyU is a synthetic single stranded RNA which mimics viral RNA and is an ligand for TLRs 7 and 8.

The lyophilized ssPolyU which was used in this study was purchased from InvivoGen, USA and was already complexed with the patented cationic lipid LyoVec^{TM} to protect is from degradation and facilitate its uptake inside cells. Therefore, there was no need to add a transfecting reagent and the cells were directly treated with the ssPolyU once reconstituted following manufacturer's instructions.

For phenotype analysis, eight wells of activated Treg were prepared in 96 well plates as described in section 3.2.1. The cells in each well were exposed to different conditions. To one of the wells no ssPolyU/LyoVec™ was added. This well served as the untreated control. To a second well, 1µg/ml of ssPolyU complexed with LyoVec™ (InvivoGen, USA) were added on the same day of activation. To a third well, 10µg/ml of ssPolyU complexed with LyoVec™ were added. According to the manufacturer's instructions, stimulation of TLR7 and TLR8 is achieved with 1-10µg/ml of ssPolyU/LyoVec™, hence why these two concentrations at the extreme of the range were selected.

To investigate the combined effect of this TLR8 ligand with a TLR4 one, ssPolyU complexed with LyoVec™ was added to the fourth well at a concentration of 10µg/ml together with 100ng/ml LPS dervied from *E.coli* (Sigma-Aldrich, USA). To test the combined effect of this TLR8 agonist and the simultaneous addition of IL-12, to the fifth well, 10µg/ml of LyoVec™ complexed to ssPolyU together with 1ng/ml IL-12 (InvivoGen, USA), were added. The triple combined effect of ssPolyU, LPS and IL-12 was tested in the sixth well by adding ssPolyU, LPS and IL-12 to yield final concentrations of 10µg/ml, 100ng/ml and 1ng/ml respectively.

The Treg in the seventh and eighth well were only treated with 100ng/ml LPS and 1ng/ml IL-12 respectively. The table below gives a summary of the composition of each well.

Well	Treatment	Anti-CD3/CD28 (7µl)	IL-2 (1000U/ml)	ssPolyU /LyoVec™	LPS (100ng/ml)	IL-12 (1ng/ml)
1	Untreated	✓	✓	x	x	x
2	Treated w/ 1µg/ml ssPolyU/LyoVec™	✓	✓	✓ (1µg/ml)	x	x
3	Treated w/ 10µg/ml ssPolyU/LyoVec™	✓	✓	✓ (10µg/ml)	✓	x
4	Treated w/ 10µg/ml ssPolyU/LyoVec™ + LPS	✓	✓	✓ (10µg/ml)	✓	x
5	Treated w/ 10µg/ml ssPolyU/LyoVec™ conc) + IL-12	✓	✓	✓ (10µg/ml)	x	✓
6	Treated w/ ssPolyU/LyoVec™ + LPS + IL-12	✓	✓	✓ (10µg/ml)	✓	✓
7	Treated w/ LPS only	✓	✓	x	✓	x
8	Treated w/ IL-12 only	✓	✓	x	x	✓

Table 3.8: Investigating the effect of ssPolyU on Treg phenotype - Composition of each well for phenotype analysis

Upon completion, the 96-well plate was placed in plate shaker at low speed for 5 minutes and the cells placed in a humidified incubator at 37°C with 5% CO_2 and cultured for a further 6 days. If the cells in the wells were highly confluent, the contents were split in half into adjacent wells and topped up with fresh complete medium supplemented with IL-2. After 6 days in culture, the cells were stained with surface and intracellular marker and analysed by flow cytometry as described in 3.2.4. A total of three biological replicates (each with the same set of treatment conditions) were set up.

For functional analysis, the monocultures and co-cultures of Tresp and Treg were prepared and stained with the proliferation dyes as described in section 3.2.2. Two monocultures of Tresp were treated with 1µg/ml and 10µg/ml of ssPolyU on the same day of activation while one monoculture was left untreated with no ssPolyU being added. Also on the day of activation, eight wells of Treg were prepared as in Table 3.8. Two days later, the Treg were added to the Tresp.

Upon completion, the plates were placed in a plate shaker at low speeds for 5 minutes. If the cells in wells were highly confluent, the contents of the wells were split in half into adjacent wells and topped up with fresh complete medium supplemented with IL-2.

After 5 days post-co-culturing (total of 7 days in culture), the proliferation of cells was analysed by flow cytometry as described in 3.2.5. The cell supernatant (conditioned medium) of both monocultures and cocultures was retained and stored at -20°C for cytokine analysis. A total of three biological replicates (each with the same set of treatment conditions) were set up.

3.2.4 Flow Cytometric Phenotype Analysis

One way to test the effect of the IFN-γ and TLR agonists on Treg was to monitor phenotypic changes, if any, of treated versus untreated samples. The intent was to check whether treated samples exhibited a flip from a Treg phenotype to that of other CD4+ non-Treg cell phenotypes, namely Th1, Th2 or Th17. This was achieved by staining the cells with fluorochrome-labelled antibodies specific to cell surface and intracellular markers that characterise different CD4+ T cell populations and then carry out analysis by flow cytometry as described in the next sections.

3.2.4.1 Selection of Markers

Table 3.9 below shows the antibody panel that was used for phenotype analysis. The untreated and treated cells, prepared as described in previous sections, were stained with

these antibodies after a total of 6 days in culture post-activation and analysed on BD FACS Aria III (BD Biosciences, USA).

The presence (or absence) of a combination of markers was used to strengthen discrimination between the different populations. Each marker was conjugated to fluorochromes that do not overlap with each other in their emission spectrum such that the whole antibody panel could be run simultaneously in parallel. Treg cells were identified using the two characteristic markers including CD25, which is the extracellular IL-2 receptor and is highly expressed on Treg, and the master transcription factor FOXP3. To check for a switch towards a Th1 phenotype, two markers were used – CD183 (or CXCR3) which is highly expressed on Th1 cells, and the intracellular transcription factor, T-bet that is essential for differentiation of naïve CD4+ T cells to Th1 but not Th2 cells (Kanhere *et al.*, 2012). To check for a switch towards a Th2 phenotype, the extracellular marker CCR4 (not uniquely expressed in Th2) and CRTH2 were used. The latter is a very reliable marker for Th2 cells as it is thought to be absent in other types of CD4+ T cells (Cosmi *et al.,* 2000). There is no unique marker for Th17 cells so switching towards this population of CD4+ T cells was checked using markers CCR6 and CCR4, both of which are also expressed on Treg, and by the absence of FOXP3 as this is only expressed in Treg.

	Name	Fluorochrome	Laser	Collection filter	Name	Fluorophore	Filter	Collection filter
	Th1 CD183+ T-bet+				**Treg FOXP3+CD25+CCR4+CCR6+**			
Extracellular marker	CD183/CXCR3	Alexafluor® 488	Blue (488nm)	530/30	CD25	APC-Cy7	Red (633nm)	780/60
Intracellular transcription factor	T-bet	Alexafluor® 647	Red (633nm)	660/20	FOXP3	Brilliant Violet 421	Violet (405nm)	450/50
	Name	Fluorophore	Laser	Collection filter	Name	Fluorophore	Laser	Collection filter
	Th17 CCR4+CCR6+FOXP3-				**Th2 CCR4+CRTH2+**			
Extracellular marker	CCR6/CD196	PE	Yellow-Green	582/15	CCR4	Per CP-Cy5.5	Blue (488nm)	695/40
	CCR4	Per CP-Cy5.5	Blue (488nm)	695/40	CRTH2	Brilliant Violet 510	Violet (405nm)	510/50

Table 3.9: Table showing antibody panel used for phenotypic analysis by flow cytometry. Each antibody was purchased conjugated to fluorochromes also given in the table. The laser and wavelength used to excite each fluorochromes are given. The table also shows the wavelength emissions of each fluorochrome and the collection filter used to collect their emission. It was made sure that during selection of the fluorochromes, none overlapped with each other.

3.2.4.2 Extracellular Staining

From each well, 1×10^6 cells were withdrawn and transferred in BD FACS flow tube. The cells were first centrifuged 250 × g for 5 minutes and the media decanted. The pellet was washed with PBS and centrifuged again at 250 × g for 5 minutes removing the supernatant.

To each tube, 500µl of Blocking Agent prepared in house (PBS + 10% FBS) was added, the tubes were vortexed and incubated at room temperature for 10 minutes. Each sample was then divided into two by transferring 250µl in a new tube. After that, the tubes were centrifuged at 250 × g for 5 minutes and the Blocking Agent decanted off.

The following steps were performed in the dark to prevent fluorophore bleaching. An antibody and isotype master mix was prepared in labelled microcentrifuge tubes. The quantities (in µg), volumes and final concentrations of antibodies used per sample are given in Table 3.10. The quantity (in µg) of isotypes used were equal to the quantities of their respective antibodies. Volumes given in the table were multiplied by the number of tubes to prepare the final master mix. All conjugated antibodies and isotypes were purchased from BD Biosciences, USA or BioLegend®, USA. The staining buffer used to prepare the master mixes was prepared in-house (PBS, 1mM EDTA, 2% FBS).

	Antibody/Marker	Fluorochrome	Purchased Concentration	Quantity per tube	Volume per tube*	Concentration per tube
			(µg/ml)	(µg)	(µl)	(µg/ ml)
Extracellular markers	**Human Anti-CD25**	APC-Cy7TM7	50	0.125	2.5	1.25
	Human anti-CD183	Alexafluor®488	200	0.25	1.25	2.5
	Human Anti-CCR4	PerCP-CyTM5.5	50	0.125	2.5	1.25
	Human Anti-CCR6	PE	100	0.25	2.5	2.5
	Human Anti-CRTH2	Brilliant Violet 510	300	0.75	2.5	7.5
	Staining buffer				88.75	
	TOTAL VOLUME PER TEST (µl)				100	
Isotype	**Mouse IgG1κ**	APC-Cy7TM7	200	0.125	0.625	1.25
	Mouse IgG1κ	Alexafluor®488	100	0.25	2.5	2.5
	Mouse IgG1κ	PerCP-CyTM5.5	200	2	10	20
	Mouse IgG2bκ	PE	50	0.25	5	2.5
	Rat IgG2aκ	Brilliant Violet 510	100	0.75	7.5	7.5
	Staining buffer				74.4	
	TOTAL VOLUME PER TEST (µl)				100	

∗Volumes used were multiplied by the number of tubes to prepare the master mix.

Table 3.10: Extracellular staining of CD25, CD183, CCR4, CCR6 and CRTH2 - Quantities and volumes of Antibody/Isotype quantities (µg), volumes and concentration used per tube containing 1×10^6 cells. Quantity of isotype used was equal to the quantity used of its respective antibody

The master mix of antibody or the isotype master mix was vortexed and distributed equally (100µl) among respective test tubes. The tubes were then placed in a vortex for 5 seconds and then incubated in the dark on a rotating platform for 30 minutes on ice.

3.2.4.3 Fixation and Permeabilization

After staining for extracellular markers, the cells were fixed and permeabilised to allow for staining of intracellular markers.

Fixation and Permeabilization was carried out using True-Nuclear™ Transcription Factor Buffer Set (BioLegend®, USA). One ml 1X True-Nuclear™ 1X Fix Concentrate (prepared by diluting True-Nuclear™ 4X Fix Concentrate with True-Nuclear™ Fix Diluent at a 1:4 ratio respectively) were added to each tube. This solution, prepared using reagents

provided in the kit contained formaldehyde which acted as the fixative. Each tube was vortexed and incubated at room temperature in the dark for at least 60 minutes.

Without washing, 2ml of the True-Nuclear™ 1X Perm Buffer (prepared by diluting True-Nuclear™ 10X Perm Buffer with deionized water at a 1:10 ratio respectively) were added to each tube. This solution, also prepared using the reagent provided with the kit, contained saponins which served as the permeabilising agent. The tubes were centrifuged at 400 × g for 5 minutes and the supernatant discarded. Once again, 2ml of the True-Nuclear™ 1X Perm Buffer were added to the resultant pellet and the tubes centrifuged at 400 × g for 5 minutes and the supernatant discarded.

The cells were then resuspended in 100µl of True-Nuclear™ 1X Perm Buffer and the cells stained for intracellular markers as described next.

3.2.4.4 Intracellular staining

Cells re-suspended in 100µl True-Nuclear™ 1X Perm Buffer were stained for intracellular markers FOXP3 and T-bet. These steps were performed in the dark. An antibody and isotype master mix were prepared in labelled microcentrifuge tubes. The quantities (in µg), volumes and final concentrations of antibodies used per sample are given in Table 3.11. The quantity (in µg) of isotypes used were equal to the quantities of their respective antibodies. Antibodies and isotype were diluted in True-Nuclear™ 1X Perm Buffer. Volumes given in the table were multiplied by the number of tubes to prepare the final master mix. All conjugated antibodies and isotypes were purchased from BioLegend®, USA.

	Antibody/Marker	Fluorochrome	Purchased Concentration	Quantity per tube	Volume per tube*	Concentration per tube
			(µg/ml)	(µg)	(µl)	(µg/ ml)
Intracellular markers	**Human Anti-FOXP3**	Brilliant Violet 421	100	0.25	2.5	2.5
	Human anti-T-bet	Alexafluor®647	500	0.5	1	5
	True-Nuclear™ 1X Perm Buffer				96.5	
	TOTAL VOLUME PER TEST (µl)				100	
Isotype	**Mouse IgG1κ**	Brilliant Violet 421	100	0.25	2.5	2.5
	Mouse IgG1κ	Alexafluor®647	100	0.5	5	5
	True-Nuclear™ 1X Perm Buffer				92.5	
	TOTAL VOLUME PER TEST (µl)				100	

*Volumes used were multiplied by the number of tubes to prepare the master mix.

Table 3.11: Intracellular staining of FOXP3 and T-bet - Quantities and volumes of Antibody/Isotype quantities (µg), volumes and concentration used per tube containing 1 × 10^6 cells. Quantity of isotype used was equal to the quantity used of its respective antibody

The master mix of antibody and the isotype master mix was vortexed and distributed equally (100µl) among respective testing tubes. The tubes were then placed in a vortex for 5 seconds and then incubated in the dark at room temperature on a rotating platform for 45 minutes.

After incubation, 2ml of True-Nuclear™ 1X Perm Buffer was added to each tube. The tubes were then centrifuged at 400 × g for 5 minutes and the supernatant discarded. Two ml of staining buffer were added and the tubes centrifuged again at 400 × g for 5 minutes and the supernatant discarded. The cells were then re-suspended in 500µl staining buffer and the tubes analysed, preferably on the same day, by flow cytometry as described next.

3.2.4.5 Data Acquisition and Analysis of Phenotype

Before loading the cells in the flow cytometer, a number of histograms and dot plot templates were prepared using BD FACSDIVA™ software (BD Biosciences, USA). Dot plots

of FSC-A versus SSC-A, FSC-A versus FSC-H and SSC-A versus SSC-H were first plotted and the cells gated on the singlet lymphocyte population (as described in section 3.1.4.2).

A histogram of cell count versus fluorescent intensity for each fluorochrome-conjugated antibody used was plotted.

A number of dot plots were plotted to identify different T cell populations according to phenotype. These included:

- FOXP3 versus CD25 (high expression of both is indicative of a Treg phenotype)
- CD183 versus T-bet (high expression of both is indicative of a Th1 phenotype)
- CCR4 versus CRTH2 (high expression of both is indicative of a Th2 phenotype)
- CCR4 versus CCR6 (high expression of both is indicative of a Th17 phenotype if negative for FOXP3).

First, the unstained sample was loaded and run. The PMT voltages were adjusted from the BD FACS Diva™ software such that the peak for the unstained sample was to the far left of every histogram. The isotype-labelled samples were then loaded followed by the antibody-stained samples. Fluorescence compensation was performed beforehand to correct for the spectral overlap of the fluorochromes using BD™ CompBeads (BD Biosciences) as were the FMO controls (described in Appendix VII: Setting up Compensation and Appendix VI: Determining Positive Expression using Fluorescent Minus One (FMO) Controls).

A total of 15,000 - 30,000 events were collected with a flow rate of 800-2000 events/s. The instrument settings were kept constant for the samples derived from the same biological replicate. After obtaining data for all the tubes, the .fcs files were exported and analysed on FlowJo™ software (TreeStar).

Using FlowJo, the .fcs files were loaded onto the interface and the histograms plots of the unstained and isotype-stained sample were overlain onto those of the antibody-stained sample to show specific binding. Interval gates were set to delineate the percentage of cells generated by the software itself that were negative and positive in expressing the markers. Using the same interval gates drawn for the histograms, quadrants were drawn onto the dot plots and the software generated the percentage of cells that were positive for both markers, positive for just one marker and negative for both. The former was especially of interest. The same gates were used when analysing data derived from the same biological replicate. For each of the four phenotypes, box and whisker plots were plotted to show percentage double positive cells for each treatment. When required, the Median Fluorescent Intensities (MFI), calculated by the software itself, were used to show shifts in the expression of the markers. The MFIs increase as the expression of the markers increase. MFIs of 6 days post-activation were normalized and divided by the MFI of the same marker at Day 0 i.e. prior to activation. Box and whisker plots of normalized MFI versus treatments could then be plotted. All graphs were plotted using GraphPad Prism 6 (GraphPad Software).

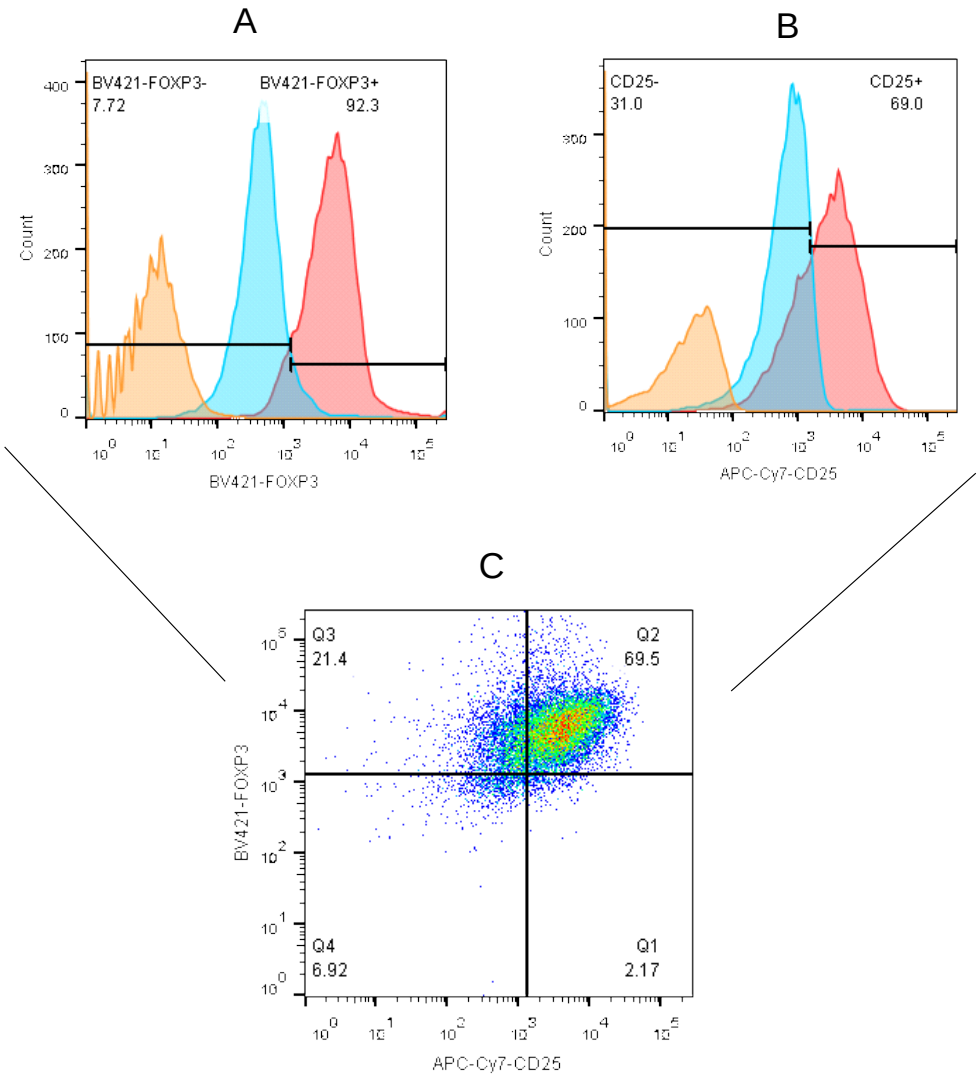


Figure 3.5: Gating strategy used to determine percentage of cells expressing the markers. (A) Overlain histograms for FOXP3 marker (orange – unstained sample, blue – isotype-stained sample, red – antibody-stained sample; (B) Overlain histograms for CD25 marker (orange – unstained sample, blue – isotype-stained sample, red – antibody-stained sample; (C) Histograms were used to delineate quadrants in dot plot of antibody-stained sample to determine percentage cells positive for both markers (Q2)

3.2.5 Proliferation And Suppression Assays

Cell proliferation analysis was carried out in order to investigate the effect of treatment on the immunosuppressive capabilities of Treg on CD4+ Tresp cells in comparison to untreated samples. Tresp cells had been previously stained with CFSE

while Treg had been stained with eBioscience™ Cell Proliferation Dye eFluor™ 670 as described in sections 3.1.7.1 and 3.2.2.1 respectively.

3.2.5.1 Data Acquisition and Analysis for Functional Assays

After 5 days post-co-culturing (total of 7 days in culture), the CFSE-labelled Tresp and eFluor™ 670-labelled Treg monocultures and cocultures were transferred into 5ml FACS tubes and analysed on BD FACS Aria III (BD Biosciences, USA).

Before loading the cells in the flow cytometer, a number of histogram and dot plot templates were prepared using BD FACSDIVA™ software (BD Biosciences, USA). Dot plots of FSC-A versus SSC-A, FSC-A versus FSC-H and SSC-A versus SSC-H were first plotted and the cells gated on the singlet lymphocyte population as 3.1.4.2

A histogram of cell count versus CFSE fluorescent intensity and of cell count versus eFluor™ fluorescent intensity were plotted.

First, the unstained sample was loaded onto the BD FACS Aria III and run. The PMT voltages were adjusted from the BD FACS Diva™ software such that the peak for the unstained sample was to the far left of both CFSE and eFluor™ histograms. The tubes containing the Treg and Tresp un-activated (and thus non-proliferative) monocultures as well as the tubes containing the Tresp monoculture treated with 300ng/ml Rapamycin (suppressive positive control) were then loaded and PMT voltages adjusted such that the peaks were to the far right of the CFSE and eFluor™ histogram. The stained monocultures of Tresp alone (negative control for suppression) was then loaded followed by all other tubes.

A total of 15,000 - 30,000 events were collected with a flow rate of 800-2000 events/s. The instrument settings were kept constant for samples coming from the same biological replicate. After obtaining data for all the tubes, the .fcs files were exported and analysed on FlowJo™ software (TreeStar).

Data Analysis – Dye Dilution Method

Using FlowJo, the .fcs files were loaded onto the interface. The singlet cells were identified from the dot plot of FSC-A versus FSC-H. From this singlet population, the MFI emitted by CFSE and eFluor™ of every sample was generated from the software. The MFI of Day 0 was divided by every MFI emitted by cells under different treatments in order to calculate the dilution of the dye. The higher the dilution, the higher the proliferation of the cells. The method was obtained from Montcuquet *et al.*, 2008. Box and whisker plots of CFSE or eFluor™ dye dilution by cells under different treatment conditions were plotted using GraphPad Prism 6 (GraphPad Software).

Data Analysis – Proliferation Modelling Method

The dye dilution method described above has its limitations. Although it is suitable to provide a general picture of relative proliferation to compare between samples, it does not provide information on the number of cell divisions that the cells undergo. Therefore this method should not be used solely to assess proliferative capacity. A second method to analyse patterns in cell proliferation was used which involved the use of flow cytometry analysis software that included a proliferation modelling platform. This platform can perform certain statistical on proliferation data. These statistics can reveal information that the dye dilution method does not.

The Proliferation Tool included in FlowJo (Version 10.5.3) was used. The original undivided population was manually gated and the software then modelled the number of cell divisions by looking for peaks with diminishing fluorescence with an approximate ratio of 0.5 per generation (or otherwise). The software was capable of generating two valuable statistical indices which FlowJo defines as below. It should be noted that apart from FlowJo, there are other software packages available that offer proliferation modelling and the indices below might be defined in a different way.

The *Proliferation Index* - the average number of cell divisions that the dividing cells in the population undergo. This statistic ignores the population of cells that did not undergo any cell division. It is calculated by dividing the total number of divisions by the number of cells that went into division (Roederer, 2011).

The *Division Index* - the average number of divisions that all cells in the starting population have undergone. It is different from the proliferation index in that this statistic includes also the cells that never divided. It is calculated by dividing the total number of divisions by the number of cells at the start of culture. The division index will always be lower than the proliferation index if there were cells which did not undergo any cell division (Roederer, 2011).

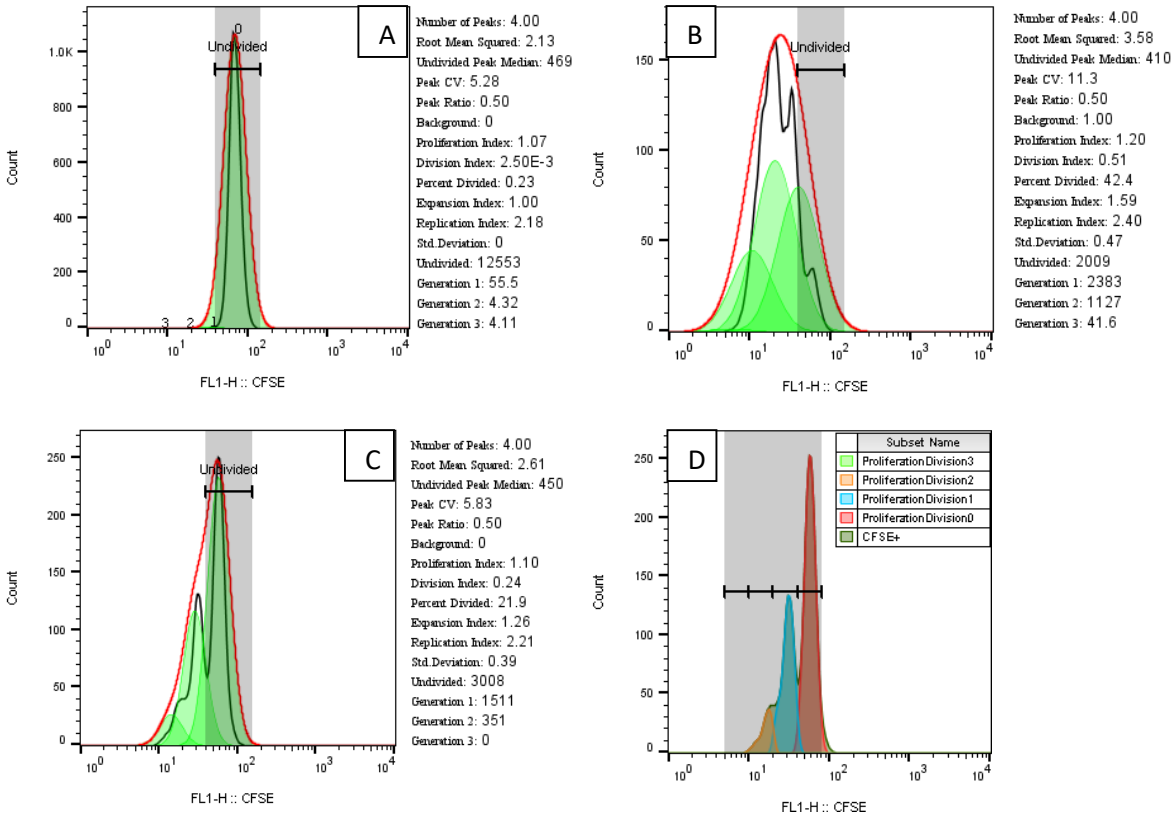


Figure 3.6: An example of Proliferation Modelling using FlowJo (Version 10). Panel A shows population of cells that were not activated with anti-CD3/CD28. Panel B represents the population of anti-CD3/CD28 activated Tresp cells without Treg, Panel C represents the population of anti-CD3/CD28 activated Tresp cells co-cultured with Treg at a 1:1 ratio. The different generations created by the software are represented as green peaks and the statistics including the proliferation and division indices are listed to the right for Panels A, B and C. Panel D shows in different colours the four generations created by the software for the 1:1 co-cultured populations. When using this method, the percentage of Tresp cells that divided in the co-culture was estimated to be less than that estimated using the conventional method (21.9% instead of 38.85).

Not all the models automatically generated by FlowJo will fit the acquired data properly. Therefore, additional input from the user would be required. This is because in some experiments the cell cycle might deviate from the “standard” shape of a proliferation curve. For this reason, the model was sometimes adjusted manually to fit better the data by applying constraints to the model parameters. These model parameters include the Ratio, the Coefficient of Variation and the Background. However,

in order to allow a fair comparison between samples, the same proliferation node with the same model parameters (i.e. same values for ratio, coefficient of variation and background) were applied to all the samples in one experiment.

The Ratio (R) of Diminishing Fluorescence is the ratio of fluorescence between subsequent peaks. The software itself by default sets the ratio of the fluorescence to 0.5, indicating perfect conservation of the proliferation dye and that exactly 50% of the dye is passed onto the daughter cells. However, this is sometimes not always the case (due to loss of fluorescence not owing to cell proliferation) and the value of this ratio was sometimes decreased to improve the fitting of the model to the data.

The Coefficient of Variation (CV) is the ratio of the standard deviation to the mean fluorescent intensity. In other words, this value represents the width of each peak. It can also be changed from the default settings the software sets.

The Background value (B) can be changed to account for autofluorescence. The amount inputted is subtracted as background from every cell. The model assumes that fluorescence of each generation is equal to the fluorescence of the previous generation multiplied by the ratio, adjusted for background noise. Expressed mathematically the fluorescence is:

$$F(n) = [\, F_{(n-1)} - B] \times r + B$$

Equation 3.2: Equation FlowJo's Proliferation Modelling Platform uses to calculate fluorescence

Where:

F(n) = fluorescence of the n^{th} generation

R = ratio

B = background

However, an adequate value for background was sometimes adjusted by inputting the mean fluorescent intensity of the unstained sample which accounts for background fluorescence.

The Root Mean Square Error Statistic (RMS) was used to quantify how adequate the model fits the data acquired. It is the square root of the squared distance of the composite model line from the histogram. A smaller RMS therefore indicates a better fit. The RMS is not appropriate to compare between experiments as the experimental condition will play an important role in determining what a good RMS is. The RMS is only suitable to see which model fits the data tighter, and whether a constraint inputted by the user itself improved the fit or not when compared to that of the model that the software itself automatically generates.

Proliferation and division indices were generated for each and every sample and box and whisker plots of the indices versus treatment were plotted using GraphPad Prism 6.

3.2.6 Multiplex Cytokine Analysis

Cytokine analysis was carried out using a Custom-made Human LEGENDplex™ Multi-Analyte Flow Assay kit (BioLegend®, USA). This kit allowed the simultaneous quantification of nine different analytes and is an alternative to the traditional ELISA method. The assay is a bead-based immunoassay using the same principle as sandwich immunoassays.

The beads come in two different sizes levels of APC fluorescence, so that they can be distinguished from each other. Each bead is conjugated with a specific antibody on the surface and serves as a capture bead for a particular analyte of interest. When the capture beads are incubated with the sample containing the target analytes, each analyte

will bind to its specific capture bead. After washing, biotinylated detection antibodies are added and each detection antibody will bind to its specific analyte which in turn is bound to the capture beads. This forms the capture bead-analyte-detection antibody sandwiches. Streptavidin-phycoerythrin (SA-PE) is subsequently added, which will then bind to the biotinylated detection antibodies, providing fluorescent signal with intensities in proportion to the amount of bound analyte. For each bead population, the PE signal fluorescence intensity is then quantified by flow cytometry and, using the LEGENDplex™ data analysis software, the concentration of a particular analyte is determined based on a standard curve generated in the same assay.

The custom kit that was designed to target the following pro-inflammatory and anti-inflammatory molecules:

- Human IL-12 (p70) – Differentiation cytokine for Th1 (pro-inflammatory)
- Human TNF-α – Effector cytokine for Th1 (pro-inflammatory)
- Human IL-4 – Differentiation cytokine for Th2 cells and inhibits Th1 (anti-inflammatory)
- Human TGF-β – Effector cytokine for Treg and differentiation cytokine for Treg and Th17 (anti-inflammatory)
- Human IL-10 – Effector cytokine for Treg and Th2 (anti-inflammatory)
- Human IFN-γ – Effector cytokine for Th1 (pro-inflammatory)
- Human IL-17A – Effector cytokine for Th17 (pro-inflammatory)
- Human Granzyme A – released by Treg (and cytotoxic T cells) to induce apoptosis in other effector T cells (can act as both pro-inflammatory and anti-inflammatory)
- Human Granzyme B - released by Treg (and cytotoxic T cells) to induce apoptosis in other effector T cells (can act as both pro-inflammatory and anti-inflammatory)

3.2.6.1 Standards Preparation

Prior to use, the lyophilized panel standard provided with the kit was reconstituted with 250µl Assay buffer also provided with the kit. The vial was then allowed to sit at room temperature for 10 minutes and then the standard was transferred in a labelled polypropylene microcentrifuge tube. This tube served as the top standard, C7, as indicated in table 13.

To 6 polypropylene microcentrifuge tubes labelled C6, C5, C4, C3, C2 and C1, 75µl of Assay buffer were added. A volume of 25µl of C7 were transferred to C6 to prepare a 1:4 dilution, and the tube mixed well. In the same manner, serial 1:4 dilutions were performed to obtain C5, C4, C3, C2 and C1 standards. Another tube representing the blank and labelled as C0 was prepared by adding just 75µl Assay buffer. The table below shows how the serial dilutions were prepare in order to prepare the standards.

Standard	Dilution	Volume of Assay Buffer (µl)	Volume of Standard (µl)	Resultant Concentration (pg/ml)
C7	--	--	--	10,000
C6	1:4	75	25µl of C7	2,500
C5	1:16	75	25µl of C6	625
C4	1:64	75	25µl of C5	156.3
C3	1:256	75	25µl of C4	39.1
C2	1:1024	75	25µl of C3	9.8
C1	1:4096	75	25µl of C2	2.4
C0	--	75	--	0

Table 3.12 Preparation of standards by serial dilutions

3.2.6.2 Assay Procedure

Standards and samples were run in duplicates and the assay was performed using a V-bottomed plate supplied with the kit. First, 25µl of Assay Buffer, supplied with the kit, were added to all wells. From each of standard C7 to C0 (run in duplicate), 25µl were added to the wells designated for the standards. A volume of 25µl of each sample was then added to the wells designated for the samples. The samples used were the conditioned media from the monocultures and co-cultures of Treg:Tresp frozen at -20°C during the functional assays.

The pre-mixed Antibody-Immobilized beads provided in the kit were vortexed for 1 minute and 25µl of the beads were added to each well to give a final volume of 75µl in each well. The plate was then sealed with a plate sealer and the entire plate covered with aluminum foil. The plate was shaken at 800rpm on a plate shaker for 2 hours at room temperature.

The plate was then centrifuged at 250 × g for 5 minutes. The supernatant was discarded, taking care not to dislodge any of the beads from the bottom. The plate was then washed by dispensing 200µl of 1X Wash buffer (prepared by diluting 20X Wash

buffer provided with the kit with deionized water) into each well. The plate was shaken at 800rpm for 1 minute, centrifuged at 250 × g for 5 minutes and the supernatant discarded.

Following this, 25µl of Detection Antibodies provided with the kits were added to each well, the plate sealed with a plate sealer, covered with aluminum foil and the plate shaken at 800rpm for 1 hour at room temperature.

Without washing the plate, 25µl of SA-PE provided with the kit was added directly to each well. The plate was sealed with a plate sealer, covered with aluminum foil and shaken at 800rpm for 30 minutes at room temperature.

The plate was then centrifuged at 250 × g for 5 minutes, the supernatant discarded and 150µl of 1X Wash Buffer was added to each well. The beads were re-suspended by shaking the plate at 800rpm for 5 minutes at room temperature. The contents in each well were transferred in separate 5ml round-bottomed (12 x 75mm) polypropylene tube and the tubes analysed on the FACS Aria™ III (BD Biosciences).

3.2.6.3 Data Acquisition and Analysis of Cytokine Data

The flow cytometer was set up properly as described in the LEGENDplex™ Custom Human Assay Manual (BioLegend®, USA). Each sample was vortexed for 5 seconds before analysis. The flow rate was set to low and the number of beads (i.e. the number of events) acquired on the machine was set to 3000 (300 per analyte) as per manual's instruction. The samples were then loaded in order (row by row) as they were arranged in the plate (i.e. A1, A2, A3,… B1, B2, B3,…). Data was exported in .fcs format and the data analysed using LEGENDplex™ Data Analysis software following the instructions found in the LEGENDplex™ Data Analysis Software Version 8 User Guide (BioLegend®, USA).

Box and whisker plot of cytokines' concentration versus treatment were plotted using GraphPad Prism 6 (GraphPad Software).

3.2.7 Statistical Analysis

In order to check whether the differences observed in a) phenotype b) proliferation c) cytokine production between different treatments were statistically significant and were not due to chance, statistical analysis was carried out as described in the following sections. All statistical analysis was carried out using IBM SPSS Statistics Version 20 software (IBM).

3.2.7.1 Testing for Normality

First a normality test was performed to check for the sample distribution of the data. Sample distribution can be either normal or non-normal. In normally distributed data, the data peaks in the middle and is symmetrical about the mean. However, this is not the case with non-normally distributed data therefore presenting the data as the mean is not adequate and in this case displaying the data as the median with 25^{th} and 75^{th} quartiles would provide a better representation.

In normality testing, the Null Hypothesis (H_0) states that the data is normally distributed and that it does not deviate from Gaussian distribution. If the Null Hypothesis is rejected, then the Alternative Hypothesis is accepted which states that the data is not normally distributed and deviates from Gaussian distribution.

Since the sample size was smaller than 50, the Shapiro-Wilk test was chosen to determine normality instead of the Kolmogorov-Smirnov test (Ghasemi & Zahediasl,

2012). If the *p* value was more than 0.05, then the Null Hypothesis was accepted and one can conclude that the data was normally distributed. Otherwise, if the *p* value was less than or equal to 0.05 then the Null Hypothesis was rejected and the Alternative Hypothesis accepted meaning that the data was not normally distributed (Goodman & Royall, 1988).

For comparison purposes within the same set of experiments, if some data was normally distributed and some was non-normally distributed, all of the data in box and whisker plots was represented as one would normally do for non-normal data. Non-normally distributed data are usually represented as box and whisker plots with median, 25^{th} and 75^{th} quartile ranges (as compared to normally distributed data which are usually represented using mean and standard deviations).

Box and whisker plots (also called box plots) are a convenient way of visualising and displaying the distribution of data between data sets. For non-normally distributed data, they summarise five statistics from the data set which include the minimum value, the 25^{th} (or first quartile, Q_1), the median (or second quartile, Q_2) , the 75^{th} quartile (or third quartile, Q_3), and the maximum value.

Minimum value – is the smallest value from the data set

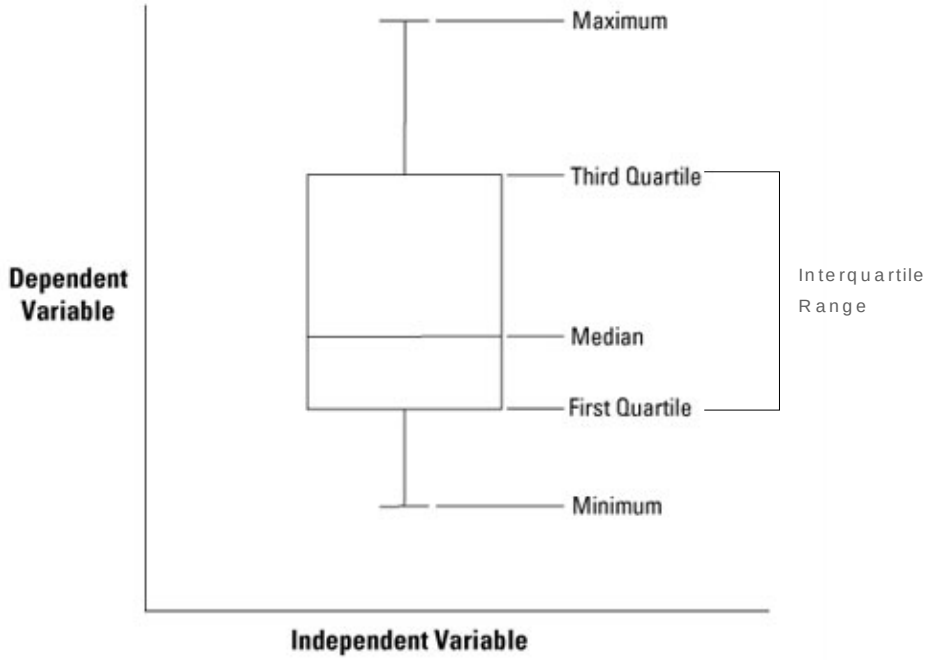
First quartile, Q_1 – is the central point between the smallest score and the median

Median, Q_2 - is the central point between all values in the data set

Third quartile, Q_3 – is the central point between the highest score and the median

Maximum value – is the highest value from the data set

Interquartile range is a measure of the variability around the median and is the difference between the 75^{th} and 25^{th} quartiles i.e. $Q_3 - Q_1$



When there are large discrepancies in the data values between different groups, it was not convenient to use the linear (i.e. absolute values) scale. In such cases, the logarithmic scale was used.

3.2.7.2 Hypothesis Testing

Normality tests were also important in order to determine which tests to conduct in hypothesis testing. If the data was normally distributed parametric tests were chosen while for non-normally distributed data non-parametric tests were used.

Table 3.13 helps one to choose which statistical test is most appropriate for hypothesis testing based on two things: a) whether the data is normal or non-normally distributed and b) whether comparison is between two groups or more.

Throughout the study, it was of interest to compare pairwise all of the groups and not just compare each group with a control group. The one-way ANOVA compares the

means of the two or more groups and is therefore suitable for normally distributed data while the Kruskal-Wallis non-parametric test compares the distribution rather than the mean across the groups and is more suitable for non-normally distributed data (Marusteri & Bacarea, 2010).

	Normal Distribution (parametric tests)	**Non-Normal Distribution (non-parametric tests)**
Comparison between two groups	Student t-test	Mann-Whitney U Test
Comparison between more than 2 groups	**Analysis of Variance (ANOVA)**	**Kruskal-Wallis Test**

Table 3.13: Choice of statistical tests based on distribution of data and whether comparison is between two or more groups. Tests used in the study are given in bold.

If the *p* value generated from the statistics software was more than 0.05, then the Null Hypothesis was accepted meaning that differences, if any, between samples was attributed due to chance. If the *p* value was less than or equal to 0.05, then the Null Hypothesis was rejected and the Alternative Hypothesis was accepted.

Accepting the Null Hypothesis, H_0 meant that:

1. Treatment had no effect on the phenotype of Treg (for phenotype assays)
2. Treatment had no effect on the expression of specific markers (for phenotype assays)
3. Treatment had no effect on the proliferation of Tresp or Treg (for proliferation assays)
4. Treatment had no effect on the suppressive capabilities of Treg on Tresp (for proliferation assays)

5. Treatment had no effect on the cytokine production in the cell supernatants (for cytokine analysis).

Rejecting the Null Hypothesis and accepting the Alternative Hypothesis meant that:

1. Treatment had an effect on the phenotype of Treg (for phenotype assays)
2. Treatment had an effect on the expression of specific markers (for phenotype assays)
3. Treatment had an effect on the proliferation of Tresp or Treg (for proliferation assays)
4. Treatment had an effect on the suppressive capabilities of Treg on Tresp (for proliferation assays)
5. Treatment had an effect on the cytokine production in the cell supernatants (for cytokine analysis).

When using one-way ANOVA or the Kruskal-Wallis test to test differences between three or more groups, a single *p* value is generated which indicates whether the groups are statistically significantly different from each other, however it does not identify which of the groups are statistically significantly different from each other. For this reason, post-hoc tests were done to determine where the statistical significant differences lied.

For the one-way ANOVA, the post-hoc test used was either the Tukey's test or the Dunnett's T3 test. Tukey's Honestly Significant Difference Test (HSD) test is used to do a single-step multiple comparisons to find means which are statistically significant from one another (Nayak & Hazra, 2011). Tukey's test compares the means of every treatment to

the means of every other treatment and identifies any difference between two means that is greater than the expected standard error.

Assumptions of Tukey's HSD test:

1. The observations being tested are independent within and among the groups.
2. The groups associated with each mean in the test are normally distributed.
3. There is equal within-group variance across the groups associated with each mean in the test (homogeneity of variance). i.e. equal sample size

To check whether there is equal within-group variance the Levene's test was carried out. This test was used to assess whether the variance between two or more groups is equal. If the *p* value is greater than 0.05 then the groups being compared have equal population variances while if the *p* value is smaller than 0.05 then the groups do not have equal variances and therefore rule number 3 for the Tukey's test is violated and the test cannot be used. In the case were the groups have unequal distribution of variance, the Dunnett's T3 test was used.

For the Kruskal-Wallis non-parametric test, the Dunn's post-hoc test was used to highlight which groups were statistically significant from each other (Nayak & Hazra, 2011). In this case, the Kruskal Wallis test is not affected by unequal distribution of variance (Neuhauser, 2002).

3.2.7.3 Representing Statistical Significance

When two groups were statistically significant, these were marked with numbers on the box and whisker plots. Matching numbers mean that the two groups are statistically significant from each other. The number was followed by an Asterix/is as follows:

* indicates a statistical difference of $p \leq 0.05$

** indicates a statistical difference of $p \leq 0.005$

*** indicates a statistical difference of $p \leq 0.0005$

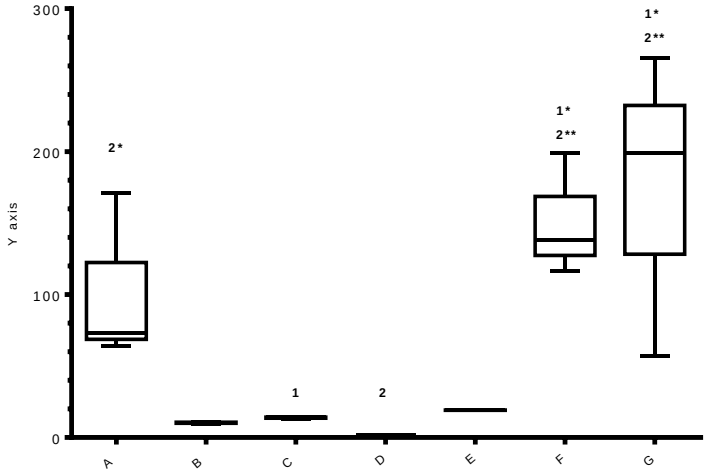


Figure 3.7: Representing Statistical Significance. In this example, Group C is statistically significant from groups F and G ($p \leq 0.05$ in both) while group D is statistically significant from group A ($p \leq 0.05$) and from groups F and G ($p \leq 0.005$ in both).

4 RESULTS

4.1 Analysing the Effects of Interferon-γ

4.1.1 Phenotype Analysis

To investigate the effect of IFN-γ on the phenotype of Tregs, monocultures of Treg cells were activated using anti- CD3/CD28. On the same day, two different concentrations of IFN-γ (25ng/ml and 50ng/ml) were tested and added to the cells while one sample was left untreated. The cells were cultured for a total of 6 days and phenotype changes detected by flow cytometry using both extra- and intracellular antigen markers and the results of the untreated and treated cells were compared. FOXP3, CD25, T-bet, CD183, CCR4, CCR6 and CRTH2 were the panel of antibodies that were used. Data acquisition was carried out on FACS Aria III (BD Biosciences) and analysis was carried out using FlowJo software.

A double positive phenotype was used to characterise different CD4+ T cells types. A FOXP3+CD25+ phenotype typically characterizes Treg, a T-bet+CD183+ phenotype typically characterises Th1, a CCR4+CRTH2+ phenotype typically characterises Th2 whilst the FOXP3-CCR4+CCR6+ phenotype typically characterises Th17. For the latter, it was essential to gate on the FOXP3- cells as CCR4 and CCR6 are also expressed in Treg but FOXP3 is not expressed in Th17. The dot plots were gated on single cells (singlets) using the FSC-A versus FSC-H strategy described in the methods. To omit non-specific binding of the antibody, which might over-represent positive expression, the histograms for the antibody-stained sample were overlain on top of their respective isotype-stained sample. Range gates were then drawn to delineate the non-overlapping histogram area to the right of the isotype which represents specific binding. For the same biological replicate, the same instrument settings were used

throughout analysis and the same gates were used across all samples throughout analysis. Once gated on the singlet population and specific binding was identified, gated dot plots were constructed and the percentage of cells that were positive for both markers in each phenotype was derived from FlowJo software. The results achieved are discussed below.

4.1.1.1 IFN-γ Reduces the Percentage of Treg cells with FOXP3+CD25+ Phenotype

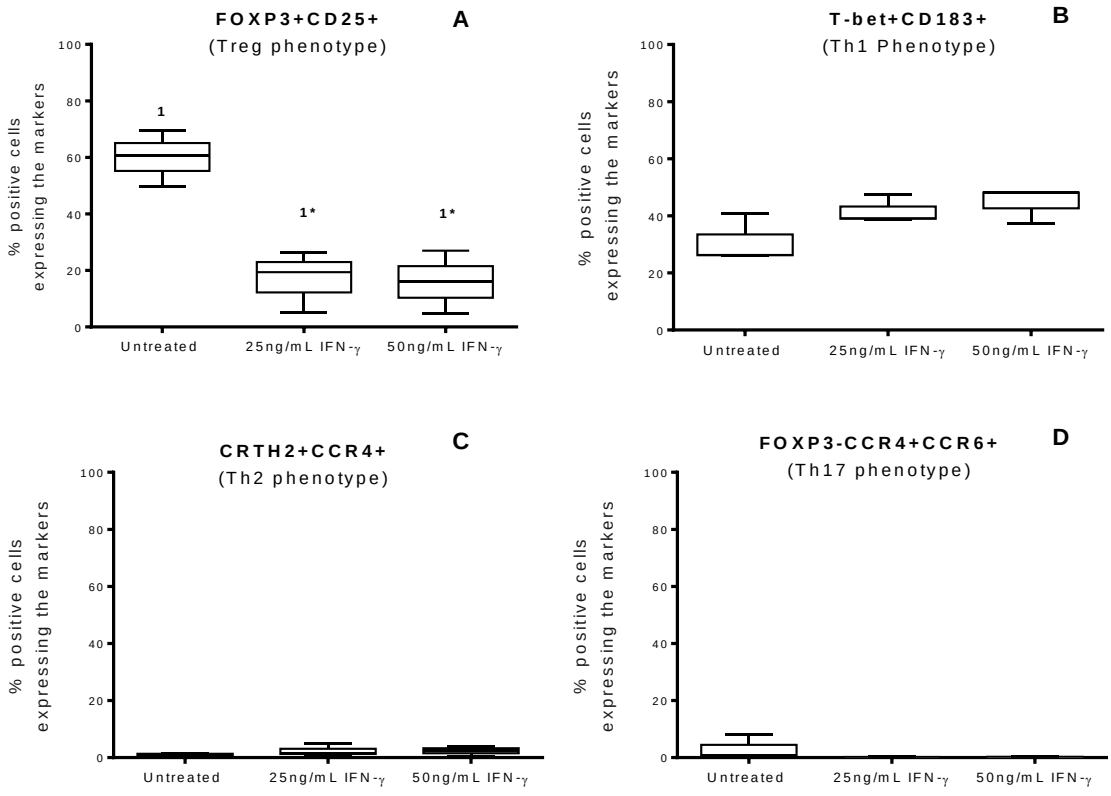


Figure 4.1: Box and whisker plots showing the effects of IFN-γ on the phenotype of Treg monocultures. Data collected 6 days post-activation and treatment. Data is presented as median percentage positive cells with 75th and 25th quartiles (n = 3 from different blood donors). Differences between treatments for (A), (B) and (C) were tested using one-way ANOVA with Tukey's test while difference in (D) was tested using the Kruskal-Wallis non-parametric test with pairwise comparison. No statistical significant differences were observed for (B), (C) and (D). * $p \leq 0.05$.

Figure 4.1 (plot A) shows that there was a statistically significant decrease in the percentage of cells that were FOXP3+CD25+ in the IFN-γ treated cultures relative to the untreated one (p = 0.006 in both cases). Therefore, treating Treg cells with IFN-γ seemed to cause a reduction in the percentage of cells with a typical Treg phenotype. Plot B, on the other hand, shows that a higher percentage of cells were CD183+T-bet+ in the treated cultures relative to the untreated ones although the increase was not statistically significant ($p > 0.05$). Therefore, upon treatment with IFN-γ, a higher percentage of cells attained a phenotype that typically characterises Th1. A moderate percentage of CD183+T-bet+ cells was also observed in the untreated sample and when this was investigated, it was found out that cells in the untreated culture expressed high levels of CD183 (but only minimal amounts of T-bet). Although CD183 is associated with Th1 CD4+ T cells, expression of the marker in CD25+FOXP3+ CD4+ Tregs has been reported in other studies and can sometimes also be expressed in up to 30–40% of human Tregs (Hoerning *et al.,* 2011).

Plots C and D show that there was no evident change in the percentage of CRTH2+CCR4+ and FOXP3-CCR4+CCR6+ cells between the untreated and the IFN-γ-treated Treg ($p > 0.05$) meaning that treatment with IFN-γ does not favour a shift towards a Th2 or Th17 phenotype respectively.

4.1.1.2 IFN-γ Reduces Expression of FOXP3 and Upregulates Expression of T-bet

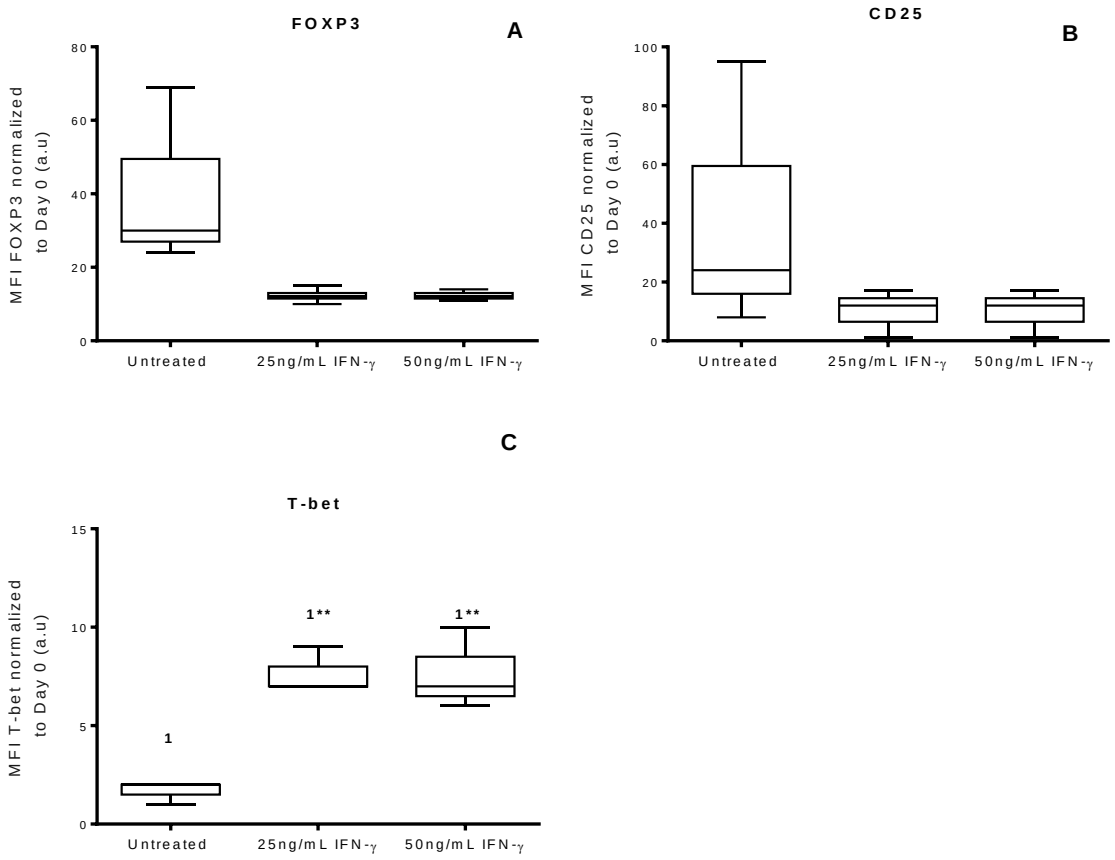


Figure 4.2: Box and Whisker plots showing Median Fluorescent Intensity (MFI) of (A) FOXP3; (B) CD25; (C)T-bet expression in the untreated and IFN-γ treated Treg. Data was collected 6 days post-activation and treatment. Units are arbitrary and have been normalized to the MFI of Day 0. Data is presented as median MFI with 75^{th} and 25^{th} percentiles (n = 3 from different blood donors). Difference in (A) and (B) MFIs between treatments was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons and difference in (C) MFI between treatments was tested using one-way ANOVA with Tukey's test . No statistical significant differences were observed for (A) and (B) * $p \leq 0.05$, ** $p \leq 0.005$

Figure 4.2 shows the median cellular level of expression for FOXP3, CD25 and T-bet separately rather than percentage of cells expressing the markers. This is represented as the median fluorescent intensity (MFI) at day 6 post-activation and treatment normalized to the MFI of Day 0 before activation. Upon activation (i.e. relative to Day 0) the expression of markers FOXP3 and CD25 increased in all of the activated samples, yet the expression of both

markers in the IFN-γ-treated samples did not increase as much as they did in the untreated samples (Plots A and B). These differences in expression were, however, not statistically significant ($p > 0.05$). Only when FOXP3 and CD25 were considered together (and the percentage of cells positive for both markers derived) were the differences statistically significant as shown in (plot A).

Figure 4.2 (Plot C) shows that the increase in T-bet MFI, and therefore expression of the marker, relative to Day 0 in Treg cells treated with IFN-γ increased more than untreated Treg and indeed the differences were significant ($p = 0.005$ in both cases). This shows that the increase in the percentage of cells that were CD183+T-bet+ in the treated samples (Figure 4.1, plot B) was not due to an increase in CD183 expression but rather due to an increase in T-bet expression. CD183 expression was already significant in the untreated samples and remained more or less the same when the Tregs were treated with IFN-γ.

Phenotype analysis therefore indicates that IFN-γ seems to upregulate expression of T-bet while downregulating that of FOXP3 and CD25. This means that the cytokine might be promoting Th1 differentiation of Treg cells by increasing the expression of the transcription factor responsible for Th1 differentiation and decreasing expression of Treg markers.

To check whether the addition of IFN-γ had an effect on the Treg's ability in suppressing Tresp, immunosuppression analysis were performed and is discussed in the next section.

4.1.2 Proliferation Assays

To investigate the effect of IFN-γ on the immunosuppressive capabilities of Treg on Tresp, isolated populations of Tresp and Treg cells (from same blood donor) were stained with

CFSE and eFluor™ 670 proliferation dyes respectively and activated separately using anti-CD3/CD28. IFN-γ was added to Treg on the same day. On day 2 post-activation, co-cultures of 1:1 Tresp to Treg cells were prepared. An untreated co-culture of Tresp and Treg (i.e. no IFN-γ addition) and monocultures of Treg and Tresp were also prepared as controls. Additionally, monocultures of Tresp (in the absence of Treg) treated with IFN-γ were prepared to check whether the IFN-γ had a direct effect on Tresp alone and thus be able to determine whether changes in immunosuppression in the co-culture were due to an effect of the IFN-γ on Treg or, alternatively, directly on the Tresp. A positive control for immunosuppression (where suppression of Tresp proliferation is expected) was also prepared by culturing Tresp in the presence of the immunosuppressant Rapamycin at a concentration of 300ng/ml.

After adding the Treg to the Tresp, the cells were cultured for a further 5 days (for a total of 7 days post-activation) and flow cytometric analysis was analysed by monitoring the dilution of the proliferation dyes.

The dot plots were gated on the singlet cells using the FSC-A versus FSC-H strategy described in the method. For the same biological replicate, the same instrument settings were used throughout analysis and the same gates were used across all samples throughout analysis.

The fold dilution of the dye was calculated by dividing the median fluorescent intensity (MFI) of inactivated cells i.e. cells not activated with anti-CD3/CD28, by the MFI of the activated untreated or treated cells in all the samples. The greater the dilution of the dye in the activated samples from that of the inactivated sample, the greater the number of cell divisions and hence the higher the proliferation.

4.1.2.1 IFN-γ Reduces Treg-Mediated Suppression of Responder T cells Not Due to a Reduction in Treg Proliferation

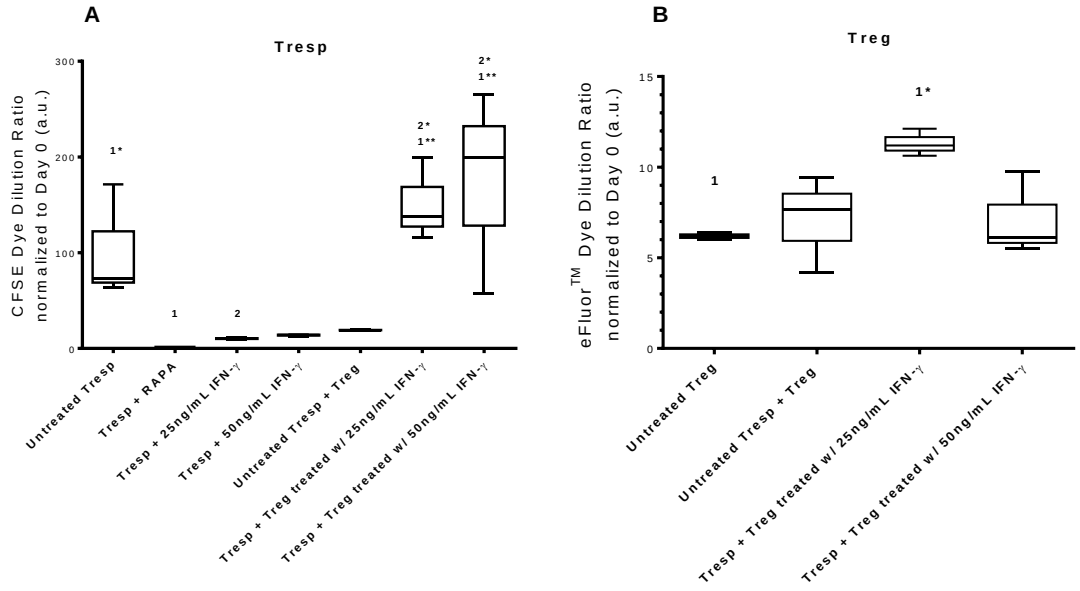


Figure 4.3: Cell proliferation of (A) Tresp and (B) Treg by monitoring the dilution of CFSE and eFluor™ respectively under different IFN-γ treatment conditions. Units are arbitrary and is the ratio of the MFI of inactivated Tresp/Treg cells to the MFI of the activated Tresp/Treg in the mono/co-cultures 7 days post-activation and treatment (and 5 days in co-culture). Data is presented as median CFSE/eFluor™ dye dilution as box plots with 75th and 25th percentiles (n = 3 from different blood donors). Difference between treatments for (A) was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons while difference between treatments for (B) was tested using one-way ANOVA with Dunnett's T3 test. * $p \leq 0.05$, ** $p \leq 0.005$

Figure 4.3 (plot A) above shows that amongst the Tresp and Treg co-cultures, CFSE dilution was higher in the IFN-γ-treated co-cultures, and the effect seems to increase proportionally to the increase in the concentration of IFN-γ added. This suggests that proliferation of Tresp was higher when the cocultures were treated with IFN-γ. The differences are not statistically significant from those of the untreated co-culture ($p > 0.05$), but might have reached significance if a higher number of biological replicates were used.

Interestingly, CFSE dilution in the IFN-γ-treated co-cultures is even higher than that of the Tresp monocultures in the absence of Treg and IFN-γ, meaning that the addition of IFN-γ significantly overcomes the effects of the Tresp suppression by Treg and facilitates their proliferation profoundly. These differences were however again not significant ($p > 0.05$).

This effect on Tresp proliferation exerted by IFN-γ is somehow only observed when the Tresp are cultured with Treg as plot A also shows that culturing Tresp alone in the presence of IFN-γ, seems to severely reduce Tresp proliferation rather than promote it. Indeed, the dye dilution was minimal and comparable to that of the positive control for immunosuppression (rapamycin-treated Tresp). The CFSE dye dilution in the Tresp monocultures treated with 25ng/ml IFN-γ was significantly different from that of the co-cultures treated with 25ng/ml ($p = 0.014$) and 50ng/ml IFN-γ ($p = 0.011$).

In summary, the data in Plot A is showing that the proliferation of Tresp is inhibited in the presence of Rapamycin and slowed down considerably to similar levels in the presence of Treg in the untreated co-culture (with the Treg suppressing the Tresp). IFN-γ acts directly on Tresp by inhibiting their proliferation (exerting a strong suppression as much as Treg). However, when IFN-γ-treated Treg are added to Tresp, the suppressive capacity of Treg is decreased and the Tresp are able to proliferate.

Plot B shows that the extent of Treg proliferation remained roughly the same under all conditions except for the co-culture treated with 25ng/ml IFN-γ. In fact, there was a statistical significant difference in the dye dilution between the latter and the Treg monocultured in the absence of IFN-γ ($p = 0.015$). Such results therefore indicate that IFN-γ does not slow down Treg proliferation but on the contrary treating the co-cultures with 25ng/ml IFN-γ seemed to enhance Treg. This also indicates that the enhanced Tresp proliferation observed in the

Tresp:Treg co-cultures treated with IFN-γ was not because the cytokine was causing a reduction in Treg proliferation. Therefore, either the Tresp proliferated more in response to the treated Treg or IFN-γ was acting directly on Treg, reducing their suppressive capabilities but not by reducing their numbers. The reduction in Treg's ability to suppress Tresp might be linked to the decrease in the expressions of FOXP3 or CD25 observed in IFN-γ-treated Treg and possibly also due to an increase in the expression of the Th1 transcription factor T-bet. For instance, CD25 is the receptor for IL-2 which is needed by both Tresp and Treg to proliferate so a reduction in CD25 reduces the ability of Treg to sequester IL-2 from Tresp.

Since the conventional method of dye dilution takes into consideration the Median Fluorescent intensity, this method only gives the extent to which the dye has been diluted relative to Day 0, and although it provides an idea of how treatments affected proliferation, the kinetics of how the treatment affected proliferation might be overlooked.

Sometimes a more informative way to analyse proliferation data is to use flow cytometry analysis software programs that offer a proliferation modelling platform. These software platforms generate a number of indices that give more information regarding the proliferation patterns under different treatments. Two of these indices include the Proliferation Index and Division Index. As described in the method section, the proliferation index is defined as the average number of cell divisions that dividing cells in the population have undergone whilst the division index is the average number of divisions the cells in the entire population has undergone. These two indices therefore give different information since the proliferation index only takes into consideration dividing cells (and therefore how responding cells are responding to the treatment), while the division index takes into account

the whole population (i.e. dividing and non-dividing cells), avoids bias associated with the proliferation index and reflects what the entire system is doing.

To generate these proliferation indices, the .fcs files were loaded onto FlowJo Version 10.6.1. and the Proliferation Modelling platform offered by this version was used to derive proliferation and division indices for both Tresp and Treg. The same model adjustments were used to compare samples belonging to the same biological replicate so that they were consistent across the proliferation analysis.

4.1.2.2 IFN-γ Increases Tresp Proliferation and Division Indices In Tresp and Treg Co-Cultures

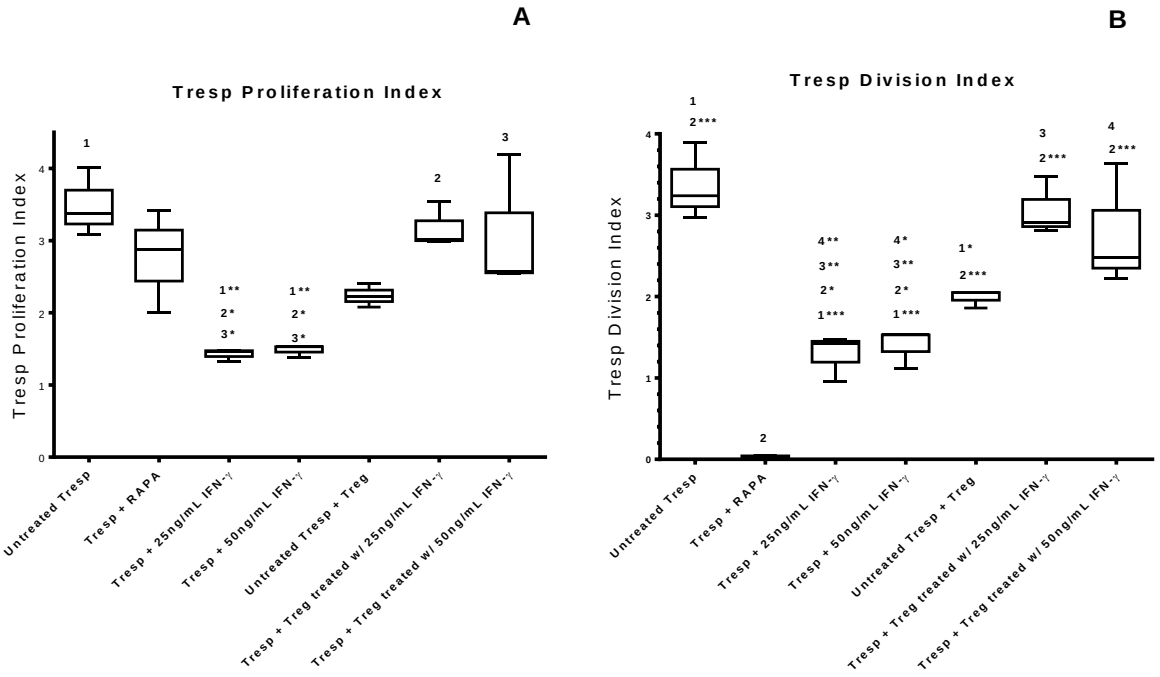


Figure 4.4: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of Tresp in the absence and presence of Treg under different IFN-γ treatment conditions. Data was collected 7 days post-activation and treatment (5 days in co-culture). Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data are presented as median Proliferation/Division Index with 75th and 25th percentiles (n = 3 from different blood donors). Difference between groups was tested using the one way ANOVA with Tukey's test. * p≤ 0.05, ** p≤ 0.005 *** p ≤ 0.0005

Figure 4.4, Box plot A shows that the highest proliferation indices where recorded in the Tresp cultured in the absence of Treg and IFN-γ, and in the Tresp cultured in the presence of Treg treated with IFN-γ. Although the latter two co-cultures showed no significant differences with the untreated co-culture (p > 0.05), the presence of IFN-γ, despite the presence of Treg, caused an increase in the average number of cell divisions that proliferating Tresp cells were undergoing. This was approximately by one more division (an increase from two to three average cell divisions). The high proliferation index value for Tresp treated with

rapamycin at first might seems odd given that in this sample Tresp proliferation was severely suppressed. One must bear in mind however that the proliferation index only takes into account proliferating cells. Most of the cells in this sample were therefore not considered (since they did not undergo any cell division at all). The proliferation index therefore only considered the very small number of cells that were immune to the effects of rapamycin and underwent some cell division. In fact, when one looks at the division index (plot B), which considers the entire cell population including the non-dividing cells, the index was very low (practically zero).

For the division indices, the same pattern as for the proliferation indices was observed: the highest indices recorded where recorded in the Tresp monocultures in the absence of IFN-γ and in Tresp co-cultured with Treg treated with IFN-γ.

Taken together, this means that treating Tresp directly with IFN-γ seems to be reducing the number of cell divisions proliferating cells undergo (proliferation index) as well as the number of divisions of the entire Tresp population (division index). On the other hand, adding IFN-γ-treated Treg to Tresp seems to enhance the number of cell divisions in proliferating Tresp cells and in the entire Tresp population.

4.1.2.3 IFN-γ at a Concentration of 25ng/ml Increases Treg Division Index

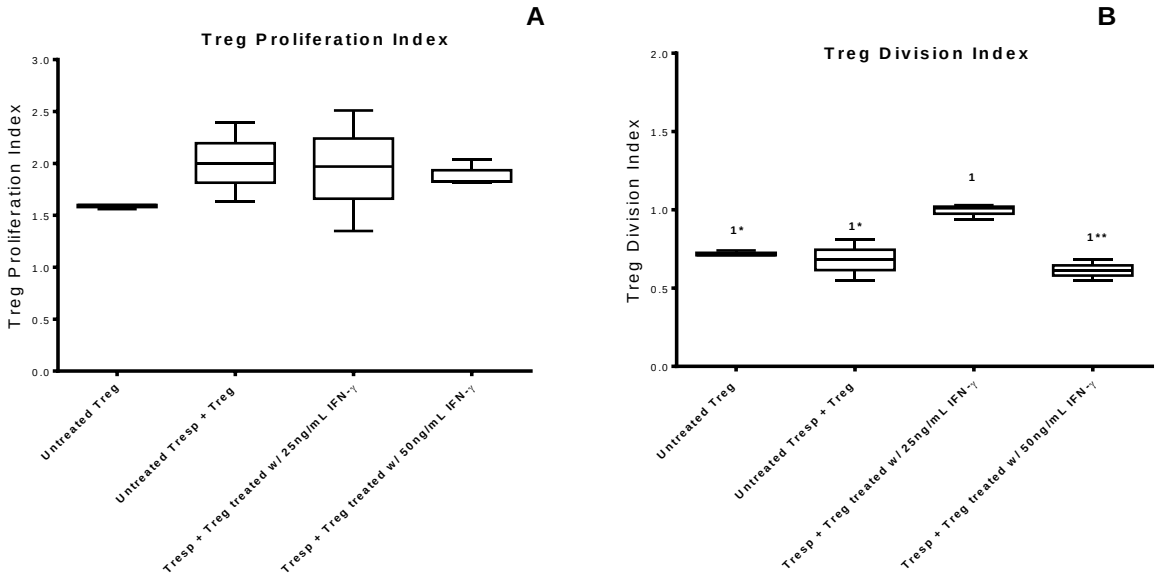


Figure 4.5: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of Treg in monocultures and in Tresp:Treg co-cultures under different IFN-γ treatment conditions. Data was collected 7 days post-activation (5 days post-coculturing). Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data are presented as median in box plots with 75th and 25th quartiles (n = 3 from different blood donors). Difference between groups was tested using one-way ANOVA with Tukey's test. * p≤ 0.05, ** p≤ 0.005

Figure 4.5 (Plot A) shows that the proliferation indices generated for Treg do not change significantly across different treatment conditions meaning that the average number of cell divisions undergone by the proliferating Treg cells was not affected by the addition of IFN-γ.

The number of dividing cells increased slightly (though not significant) when the Treg were co-cultured with the Tresp probably because the presence of the latter stimulated further the expansion of Treg.

However, plot B shows that the average number of cell divisions that the entire population of cells underwent increased when the Treg were treated with 25ng/ml IFN-γ and

co-cultured with Tresp, meaning that more cells in the entire population were undergoing cell division. The division index in this co-culture was significantly higher from those of the rest of the mono/co-cultures (Tresp + Treg treated w/ 25ng/ml IFN-γ vs Untreated Tresp + Treg p = 0.005; Tresp + Treg treated w/ 25ng/ml IFN-γ vs Tresp + Treg treated w/ 50ng/ml IFN-γ p = 0.001; Tresp + Treg treated w/ 25ng/ml IFN-γ vs Untreated Treg p = 0.010).

Proliferation and division indices derived for Treg therefore suggest, in agreement with the dye dilution method, that IFN-γ exerts no effect, or perhaps slightly enhances the proliferation of Treg at a concentration of 25ng/ml.

4.1.3 Cytokine Analysis

Cytokines produced by T cells, in addition to controlling T cell abundance, also drive the phenotypic and functional specialization of the cells. An analysis of the cytokines present in the cell culture supernatants was therefore carried out using a custom-designed multi-analyte flow assay kit by Biolegend®. The presence of a number of differentiation and effector cytokines for Treg, Th1, Th2 and Th17 were tested.

4.1.3.1 IFN-γ Enhances the Production of IL-10 in Tresp and Treg Co-Cultures

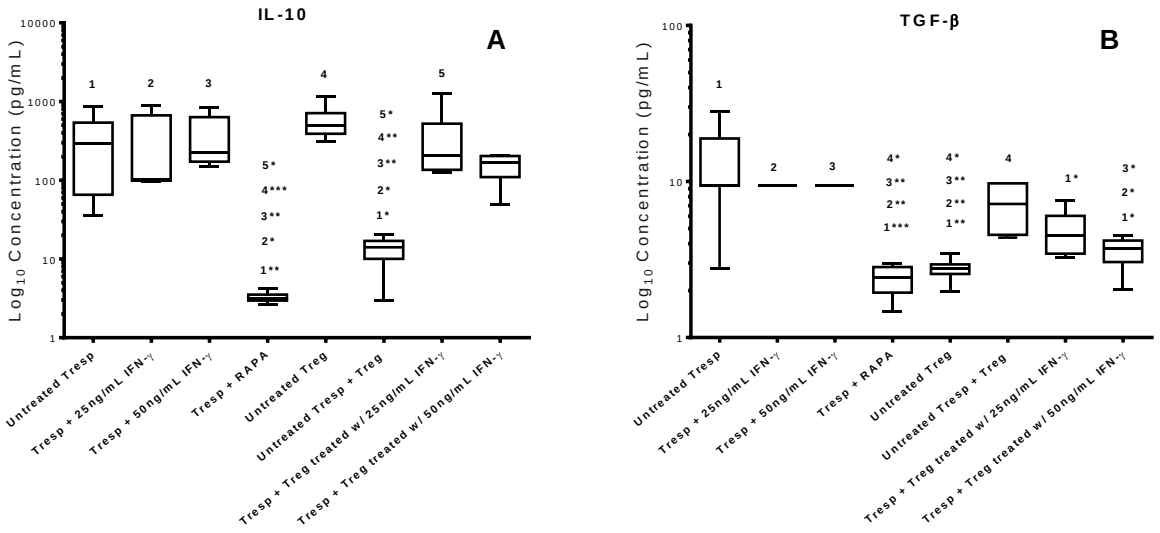


Figure 4.6: Box and Whisker plots showing, in logarithmic scale, the concentrations in pg/ml of anti-inflammatory cytokines (A) IL-10 and (B) free active TGF-β1 obtained from cell supernatants exposed to different IFN-γ treatment conditions. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th an 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p≤0.05, ** p≤0.005, *** p≤0.0005

IL-10 and TGF-β are anti-inflammatory cytokines and have been described in literature as being the effector cytokines for Treg. These cytokines are inhibitory cytokines on effector cells, are involved in the suppressive function of regulatory T cells and play critical roles in maintaining immune homeostasis, minimizing tissue damage and preventing autoimmunity.

However, Figure 4.6, (plot A), shows that, IL-10 was not only produced by the Treg monocultures, but also by Tresp cells both in the absence and presence of IFN-γ. This therefore indicates that IL-10 production is not only restricted to Treg but is also produced by other non-Treg cells. Th2 cells, for instance, are known to also produce IL-10. Although Treg monocultures produced appreciable amounts of IL-10, surprisingly, the amount of IL-10 detected in the untreated co-cultures of Treg and Tresp went down despite the fact that in

this co-culture Tresp proliferation was being suppressed (as shown in the functional assays). Indeed, there was a significant decrease in the cytokine production from the Treg monoculture to the untreated Treg and Tresp co-culture (p = 0.001) . This therefore indicates that IL-10 production was not the main mechanism by which the Treg were suppressing the Tresp in the untreated co-culture. The concentration of IL-10 started to increase again in the co-cultures once the Treg were treated with IFN-γ especially at a concentration of 25ng/ml with the latter being significantly different from untreated co-culture control (p = 0.015). In the untreated co-culture, Tresp proliferation was being slowed down by the Treg and therefore the total cytokine production of IL-10 might have been lowered due to a reduction in the amount released by Tresp such as Th2. However, the increase in this anti-inflammatory cytokine in the treated co-cultures might also be an opposing response to the proliferating Tresp in the co-cultures where the Treg had been treated with IFN-γ. Indeed, IL-10 is known to act directly on CD4+ T cells, inhibiting proliferation and production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α which are required by CD4+ cells to proliferate. Therefore, treating Treg with IFN-γ might have reduced Treg-mediated suppression of Tresp which in turn might have induced the production of suppressing cytokines such as IL-10 by the Tresp or the Treg to control the uninhibited Tresp proliferation.

Plot B shows that the quantity of TGF-β detected in all samples was very low. TGF-β exists in two forms: latent TGF-β and free-active TGF-β. The former cannot be detected by standard kits and if the sample is not activated, one will only be able to detect the free-active TGF-β and the quantities are usually very low and beyond the detection limit. However, the levels of free-active TGF-β can be increased naturally when an activation signal is provided because the latent form will convert into the active form. Therefore, by activating the cells (in this case using anti-CD3/CD28) one would be able to detect both forms of TGF-β. Nonetheless,

despite the fact that the samples were activated, the levels of the cytokine were still low under all treatment conditions. Although in low quantities, free active TGF-β was detected even in untreated and IFN-γ-treated Tresp monocultures. Again, this shows that Tresp also produce this suppressing cytokine, and that it's production is not only restricted to Treg. Indeed, significant differences were observed in the TGF-β concentrations produced by the Tresp monocultures (both untreated and treated with IFN-γ) and Tresp and Treg co-cultures (treated with IFN-γ) with the former being higher than the latter ($p < 0.05$ in all cases) . The reduction in this cytokine in the IFN-γ-treated co-cultures might be one mechanism by which the Tresp are overcoming suppression by Treg. However these concentration differences were very minimal and a better understanding would require further investigation.

4.1.3.2 IFN-γ Enhances the Production of Granzymes A and B in Tresp and Treg Co-Cultures

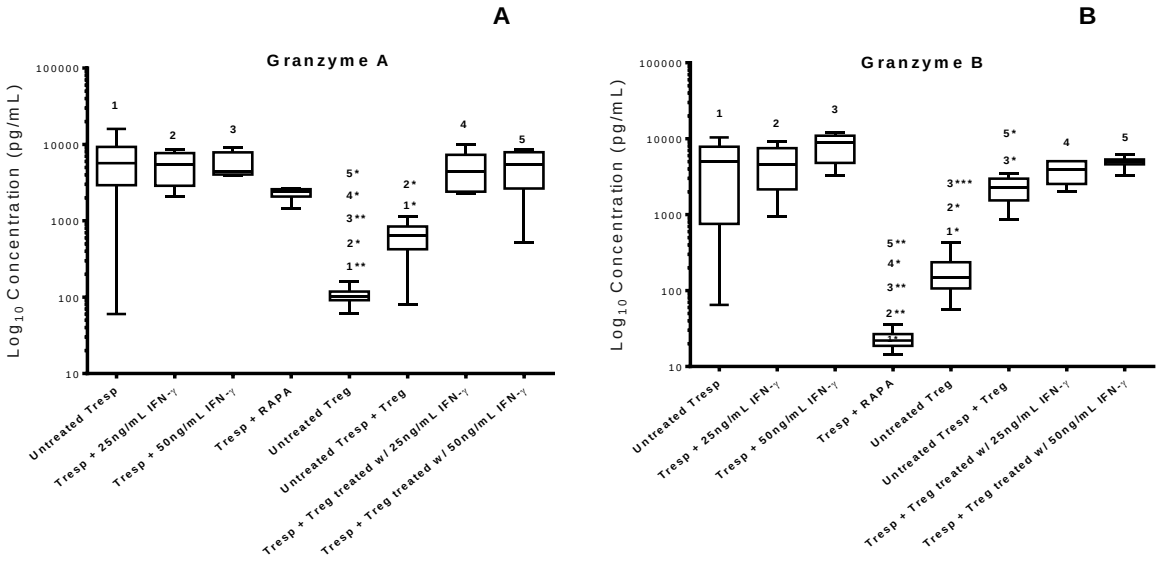


Figure 4.7: Box and Whisker plots showing, in logarithmic scale, concentrations in pg/ml of (A) Granzyme A and (B) Granzyme B obtained from cell supernatants exposed to different IFN-γ treatment conditions. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$

Granzyme A and B are serine protease released by cytoplasmic granules, and induce cell apoptosis. These enzymes are the main mechanism by which cytotoxic T cells and natural killer cells induce apoptosis of pathogen-infected cells and tumour cells. However, granzymes have also been proposed as being another mechanism by which Treg mediate suppression of Tresp cells (Karreci *et al.*, 2017).

The results in Figure 4.7 (plot A), however indicate that Granzyme A is also produced by CD4+ Tresp monocultures and only minimally by Treg when cultured alone. Indeed, there was a significant difference in the concentrations produced by Tresp and Treg monocultures

(Untreated Tresp vs untreated Treg p = 0.005; Untreated Tresp + 25ng/ml IFN-γ vs untreated Treg p = 0.010; Untreated Tresp + 50ng/ml IFN-γ vs untreated Treg p = 0.002). Addition of IFN-γ did not significantly affect the production of this protease enzyme in the Tresp monoculture. The co-culture of Tresp and Treg in the absence of IFN-γ produced lower amounts of Granzyme A and Granzyme B when compared to the IFN-γ- treated co-cultures. This was probably also due to the fact that the reduction in Tresp proliferation in the untreated co-cultures resulted in a lowered production of the enzyme. On the other hand, in the IFN-γ-treated co-cultures, the Granzyme A concentrations increased again proportional to the increase in Tresp proliferation (as shown from the proliferation studies) due to production by the proliferating Tresp themselves or by the Treg in an attempt to suppress Tresp proliferation.

Figure 4.7 (plot B) shows that Tresp monocultures release Granzyme B but Treg monocultures do so in very small quantities when cultured alone (similarly to Granzyme A). Again, the differences between Tresp and Treg monocultures are statistically significant Untreated Tresp vs untreated Treg p = 0.028; Untreated Tresp + 25ng/ml IFN-γ vs untreated Treg p = 0.036; Untreated Tresp + 50ng/ml IFN-γ vs untreated Treg p = 0.002). Similar to Granzyme A, the concentrations detected in the untreated co-culture were observed to be low but levels rise again when the Treg in the co-cultures are treated with IFN-γ.

Collectively, these results indicate that CD4+ Tresp also produce suppressor cytokines and granzymes and that in this case Treg are suppressing Tresp by additional mechanisms apart from the production of IL-10 and granzymes.

These suppressor molecules might have been upregulated in the treated Treg and Tresp co-cultures (either by the Tresp themselves or by the suppressive Treg) as a responding mechanism to control the enhanced proliferation of Tresp.

4.1.3.3 IFN-γ Augments the Secretion of TNF-α in Treated Tresp:Treg co-cultures

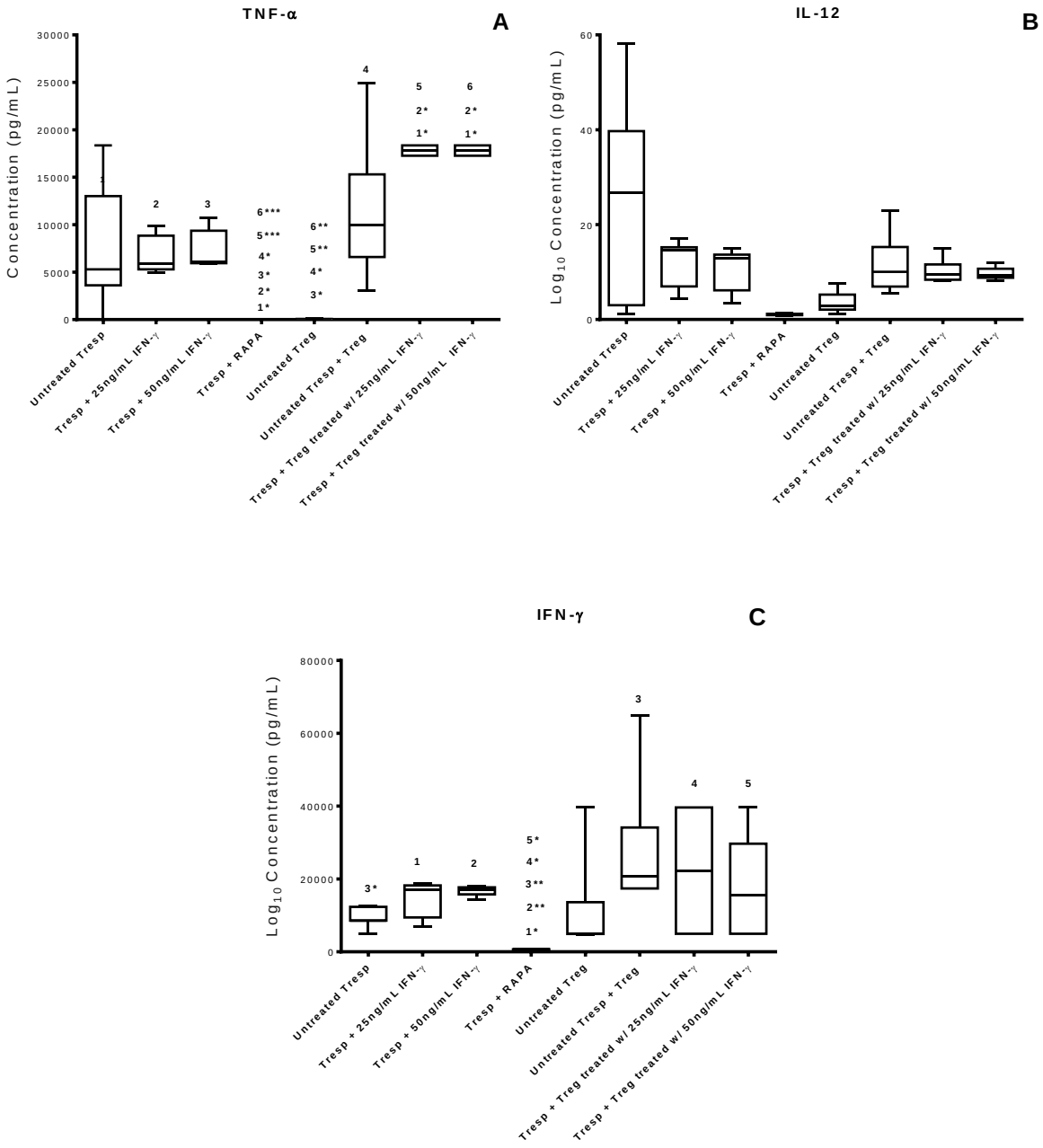


Figure 4.8 : Box and Whisker plots showing, in linear scale, the concentrations in pg/ml of pro-inflammatory cytokines (A) TNF-α, (B) IL-12 and (C) IFN-γ obtained from cell supernatants exposed to different IFN-γ treatment conditions. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles ($n \geq 3$). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. No statistical significant differences were found in (B) * p≤ 0.05, ** p≤0.005, *** p≤0.0005

TNF-α and IL-12 are described as pro-inflammatory cytokines that induce the differentiation of naïve CD4+ T cells to Th1. IL-12 is also a strong inducer of IFN-γ and causes the differentiation of naïve CD4+ cells to Th1. IFN-γ is the effector cytokine for Th1.

As expected, Figure 4.8 (plot A) shows that Tresp monocultures produced TNF-α (Th1 portion) and treatment of the Tresp monocultures with IFN-γ did not significantly alter production of this cytokine. As also expected, untreated Treg monocultures did not produce the cytokine. The levels of the untreated Treg and Tresp co-cultures were comparable to those of the untreated and treated Tresp monoculture. However, treating the Treg in the co-cultures with IFN-γ increased profoundly the production levels of TNF-α. Although the differences were not significant from that of the untreated co-culture ($p > 0.05$), they were significantly higher from those of the Tresp monocultures untreated ($p = 0.31$ in both cases) or treated with 25ng/ml IFN-γ ($p = 0.37$ in both cases). Therefore, this indicates that IFN-γ upregulated the production of TNF-α in Tresp and Treg co-cultures. It might be possible that the IFN-γ-treated Treg in the co-cultures acquired Th1 characteristics and the ability to produce that "extra" TNF-α in the treated co-cultures. This might possibly be linked to the increase in T-bet expression observed in the Treg treated with IFN-γ (as shown in Figure 4.8, plot C). The increase in TNF-α in turn might have prompted the Tresp to proliferate even in the presence of Treg. Alternatively, the increase in TNF-α could have been due release by the highly proliferating Tresp cells (Th1 fraction) in the IFN-γ-treated co-cultures.

Figure 4.8, Plot B shows that no statistical significant differences were observed in the production of IL-12 between the different treatment conditions ($p > 0.05$) and levels detected were extremely low. This shows that IFN-γ does not affect IL-12 production in Tresp monocultures as well as in co-cultures of both Tresp and Treg.

Box and whisker plot C shows that IFN-γ was produced by both Tresp and Treg monocultures in high and similar amounts. Treg that produce IFN-γ during stimulation have been described in other studies (Volker, Wang, Sadeghi, & Opelz, 2014) and this is essential for their protective function against autoimmunity and graft versus host disease (Koenecke *et al.*, 2012; Daniel, Trojan, Adamek *et al.*, 2015). The levels recorded in the untreated co-culture were significantly higher than in the Tresp monocultures (p = 0.16), probably due to a dual release of the cytokine by both Tresp and Treg.

4.1.3.4 IFN- γ Might be Enhancing IL-4 Production in Tresp Monocultures and IL-4 and IL-17 in Tresp and Treg co-cultures

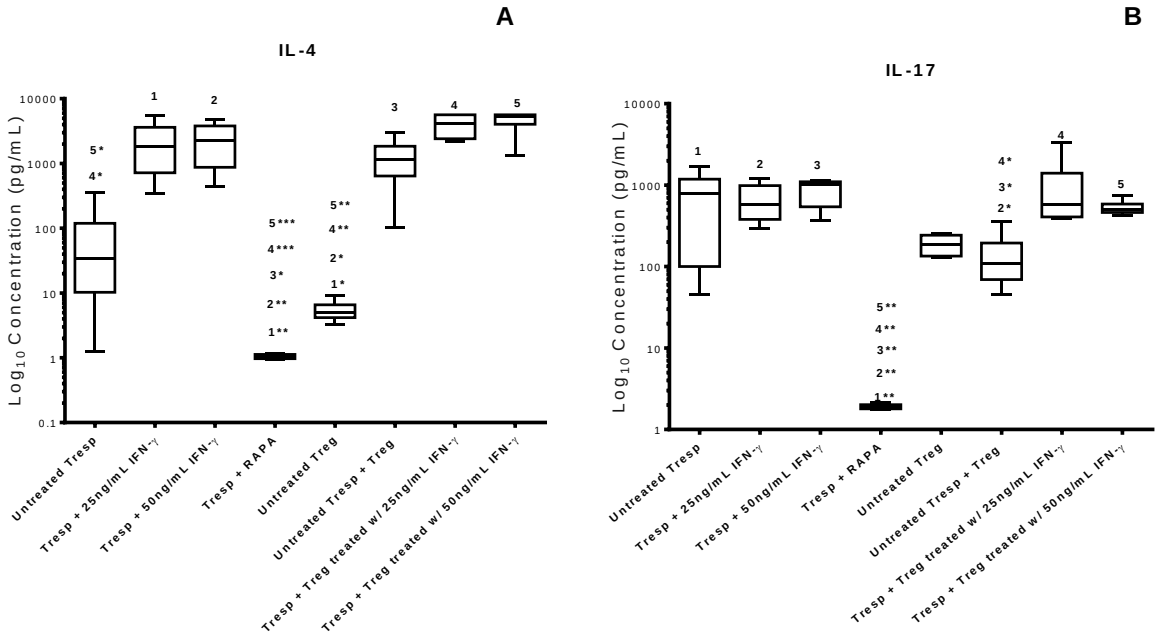


Figure 4.9 : Box and Whisker plots showing, in logarithmic scale, the concentrations in pg/ml of the differentiation cytokine for Th2 (A) IL-4 and effector cytokine for Th17 (B) IL-17 obtained from cell supernatants exposed to different IFN-γ treatment conditions. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles ($n \geq 3$). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$

IL-4 is the differentiation cytokine for Th2 and IL-17 is the effector cytokine for Th17. Figure 4.9 (plot A) show that untreated Tresp monocultures produced very low levels of IL-4 meaning only a small population of Tresp cells were expected to differentiate into Th2. Also, as expected, very little IL-4 was detected in the Treg monocultures. However interestingly, adding IFN-γ increases IL-4 production relative to their untreated corresponding control in both Tresp monocultures and Treg and Tresp co-cultures. Although no significant differences were observed ($p > 0.05$), the increase is evident and would have probably reached significance if higher number of replicates were considered. IL-4 is known to promote Th2 differentiation and stability but it also inhibits Th1-cell differentiation and is antagonistic for many of the activities of IFN-γ. Therefore, it is possible that IL-4 production might have increased in response to addition of the Th1 effector cytokine IFN-γ in an attempt to inhibit Th1 responses. Therefore, it might be probable that a proportion of proliferating cells were in fact producing IL-4 to limit Th1 responses.

Plot B shows that treating Tresp monocultures with IFN-γ does not alter IL-17 production in Tresp monocultures. As expected, lower levels of the cytokine were recorded in the Treg monoculture as well as the untreated Tresp and Treg co-cultures. The levels of the cytokine were observed however to increase in co-cultures where Treg had been treated with IFN-γ. Some studies have reported that Treg under certain inflammatory conditions acquire Th17-like characteristics. Therefore, it could be possible that a portion of treated Treg acquired these characteristics and started producing IL-17. In fact the cytokine concentrations produced by the co-culture treated with 25ng/ml IFN-γ was significantly higher than that of the untreated co-culture ($p = 0.044$).

4.2 Analysing the Effects of Endogenous TLR7/8 Ligand - Human RNA

4.2.1 Phenotype Analysis

To investigate the immunostimulatory effects of human endogenous RNA on the phenotype of Treg, Treg cells were activated using anti- CD3/CD28 in the presence of IL-2. On the same day of activation, two different concentrations of RNA were tested (1µg/ml and 10µg/ml) and the Treg cells were transfected with the RNA using polyethylenimine (PEI) as a transfection agent as described in the method. Transfection using a suitable transfection agent was important in order to ensure uptake of the 'self' RNA and prevent its degradation by nucleases before they can make it to the endosomes where the TLR7 and 8 are located.

The cells were cultured for a further 6 days and phenotype analysis using both extra- and intracellular markers was carried out by flow cytometry to compare phenotypic changes in the untreated and treated samples. For the same biological replicate, the same instrument settings on the FACS Aria III were used throughout analysis. The .fcs files were analysed using FlowJo software, the cells were gated on the singlet population, specific binding was identified using an isotype control and the same gates were used across all samples throughout analysis for the same biological replicate.

4.2.1.1 Endogenous RNA Decreases the Percentage of FOXP3+CD25+ but Increases the Percentage of CRTH2+CCR4+ Cells

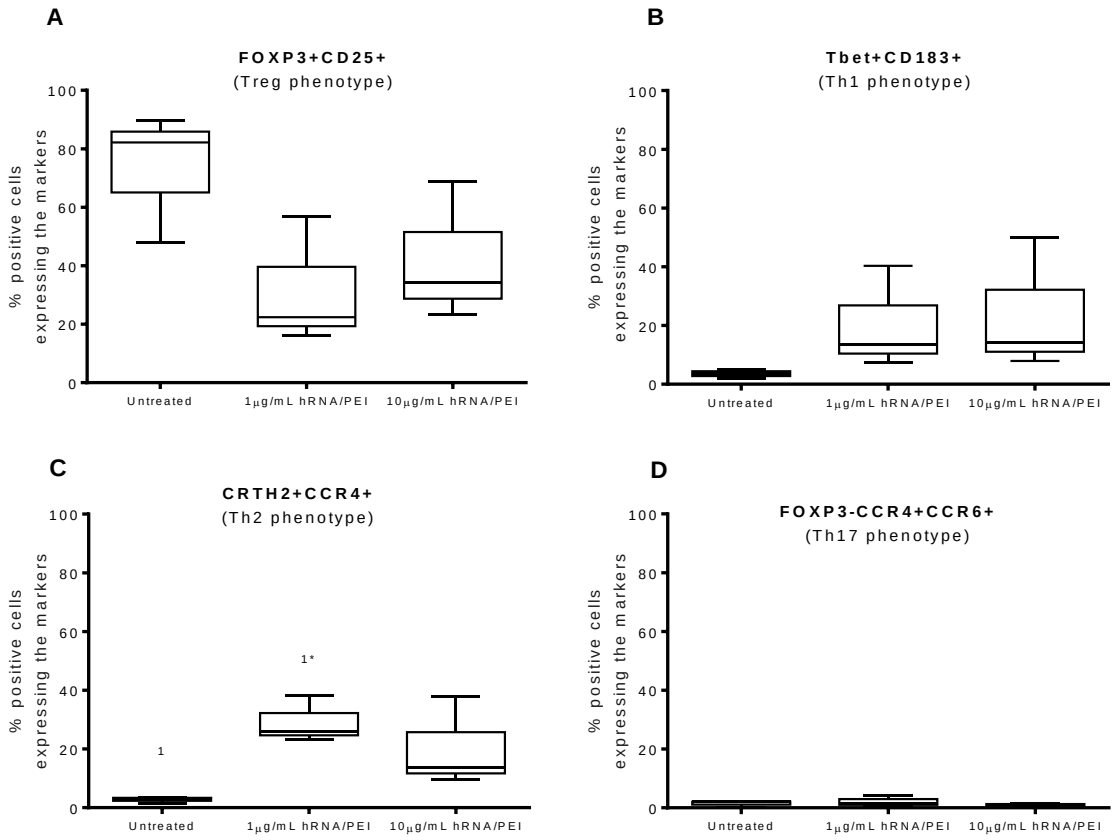


Figure 4.10: Graph showing the phenotypic effects of transfecting Tregs with endogenous RNA 6 days post-activation and transfection. Data is presented as median percentage of cells with 75th and 25th quartiles (n =3 from different blood donors). Difference in percentage of cells that were (A) FOXP3+CD25+ and (C) CRTH2+CCR4+ and (D) FOXP3-CCR4+CCR6+ between treatments was tested using one-way ANOVA with Tukey's test. Difference in percentage of cells that were (B) Tbet+CD183+ between treatments was tested using Kruskal Wallis non-parametric test with pairwise comparisons. No statistical significant differences were observed for (A), (B) and (D) * $p \leq 0.05$

Figure 4.10, plot A, shows that treating Treg with human RNA complexed to PEI decreased the percentage of cells that are FOXP3+CD25+ thus disfavouring a Treg phenotype, though the differences were not significantly different from those of the untreated Treg ($p > 0.05$). Moreover, plot B shows a higher percentage of cells that are T-

bet+CD183+ in the treated Treg possibly favouring a Th1 phenotype. The differences in the percentage of cells that were FOXP3+CD25+ and T-bet+CD183+ between untreated and treated Treg were however not statistically significant ($p > 0.05$).

Interestingly, the percentage number of CRTH2+CCR4+ cells in the treated Treg also increased relative to the untreated. This is a shift which was not observed with any of the other TLR ligands discussed further on. The percentage difference between the untreated Treg and those treated with 1µg/mL RNA was significant ($p = 0.40$). This means that transfecting Treg with 1µg/mL RNA derived from 'self' cells, might possibly be favouring a Th2 phenotype. No changes in the percentage of cells that were FOXP3-CCR4+CCR6+ was observed.

The increase in the percentage of positive cells is usually a result of higher upregulation in the expression of one of the markers. When all the markers were analysed separately by looking at the median fluorescent intensity given off, shifts in the MFI were observed for markers CD25, CD183 and CRTH2 as below shows.

4.2.1.2 Endogenous RNA Reduces CD25 Expression While Upregulating CRTH2 Expression in FOXP3+ Cells

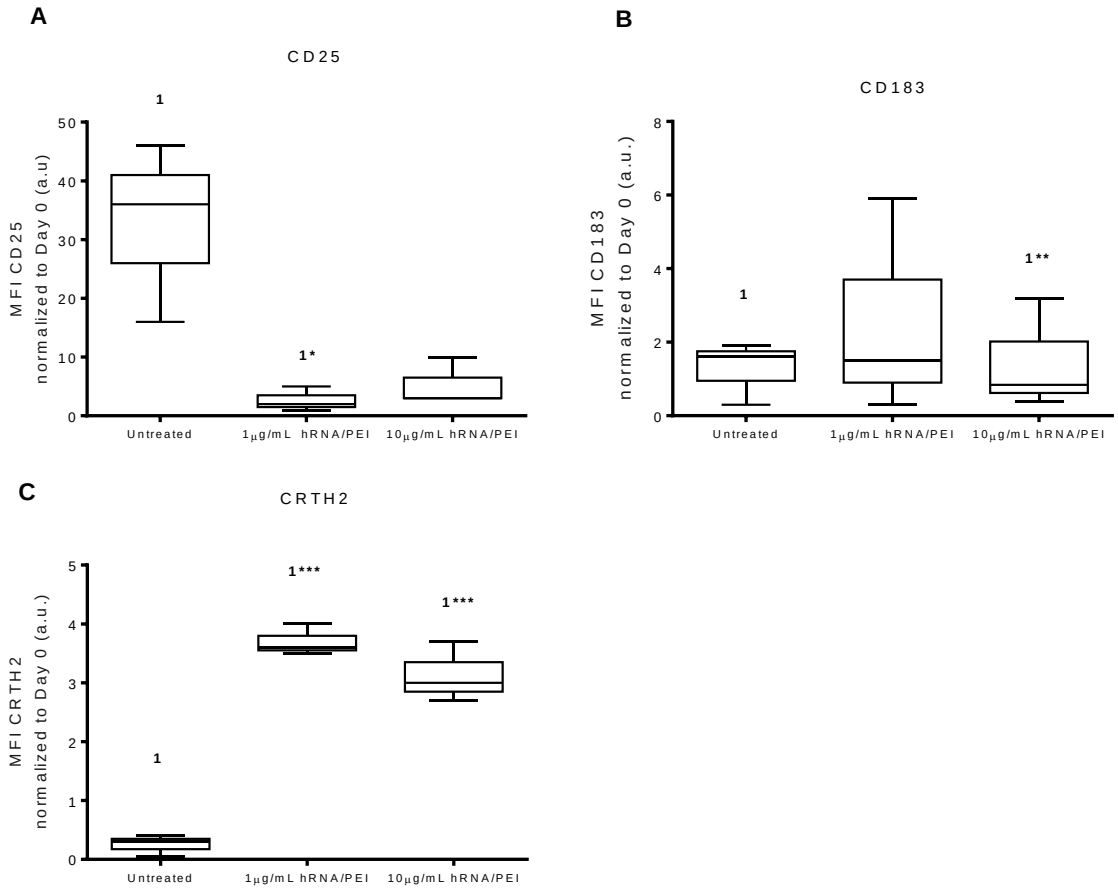


Figure 4.11: Box and Whisker plots showing Median Fluorescent Intensity (MFI) of (A) CD25 (B) CD183; (C) CRTH2 fluorescene in the untreated Treg and Treg transfected with endogenous RNA. Units are arbitrary and have been normalized by dividing the MFI on 6 days post-treatment by that of Day 0. Data are presented as median MFI with 75th and 25th percentiles (n = 3 from different blood donors). Difference in MFI between treatments for (A) and (B) was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons while for (C) they were tested using one way ANOVA with Tukey's test. * p≤ 0.05, ** p≤ 0.005, *** p ≤ 0.0005

Figure 4.11 (plot A) shows that treating Treg with 1µg/ml RNA caused a significant decrease in the MFI for CD25 relative to the untreated (p = 0.05). This shows that the decrease in FOXP3+CD25+ percentage of cells was due to a decrease in the expression of CD25. In fact the expression of FOXP3 remained relatively unchanged (data not shown).

Plots B and C show, on the other hand, that the MFIs for CD183 and CRTH2 respectively increased in the treated samples. When other TLR8 ligands such as CL097 were used (results discussed further on), the percentage of CD183+T-bet+ cells was due to an increase in T-bet+ which is not the case with endogenous RNA as it was the expression of CD183 that was observed to increase. Since CD183 is also expressed in some Treg, one cannot really claim that RNA is favouring a Th1 phenotype (Akimova *et al.*, 2017). In fact, Figure 4.12 plot A below shows that cells expressing high levels of CD183 in the treated samples still retained high expression of FOXP3 and thus were expected to remain suppressive cells.

However, the fact that RNA increases the expression of CRTH2 is very interesting. CRTH2 is not expressed in Treg and has been described as the most reliable marker for the detection of human Th2 cells in health and disease (Cosmi *et al.*, 2000). The differences in expression of the marker in the untreated Treg and both cultures of Treg transfected with endogenous RNA were significant ($p \leq 0.0005$ in both cases). Therefore, it might be possible that treating Treg with human RNA stimulates TLR 7 and/or 8 and favours a flip towards a Th2, rather than a Th1, phenotype. Cells in the treated samples that expressed high levels of CRTH2 were still found to be positive for FOXP3 as shown in plot B, possibly representing an intermediate flipping stage between Treg and Th2. Differences in percentage of FOXP3+CRTH2+ cells between untreated and treated Treg were also statistically significant (Untreated Treg vs Treg treated with 1µg/ml hRNA p = 0.11; Untreated Treg vs Treg treated with 10µg/ml hRNA p = 0.018).

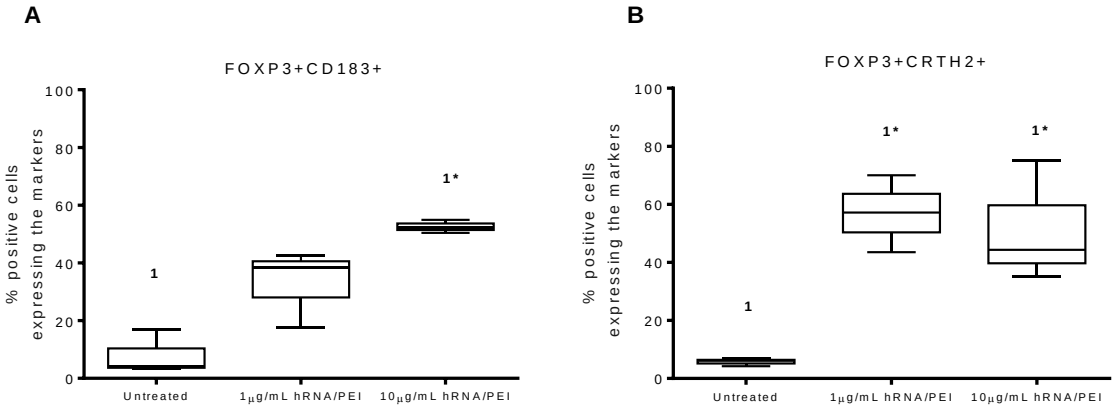


Figure 4.12: Box and whisker plots showing the percentage of (A) FOXP3+CD183+ and (B) FOXP3+CRTH2+ cells in untreated Treg and Treg transfected with endogenous RNA. Data are presented as median with 75th and 25th quartiles (n = 3 from different blood donors). Differences between treatments were tested using one way ANOVA with Tukey's test. * $p \leq 0.05$

4.2.2 Proliferation Assays

4.2.2.1 PEI Does Not Induce Toxic Effects on the Proliferation of Responder T Cells

A preliminary test was done to check whether the presence of PEI itself (without being complexed to nucleic acids) induces toxicity and whether it effects T cell proliferation. The figure below shows the Tresp proliferation pattern obtained for activated Tresp: Treg coculture in activating medium only (RPMI-1640 + anti-CD3/CD28 + IL-2) and that obtained for the same co-culture in activating medium plus PEI not complexed to nucleic acids.

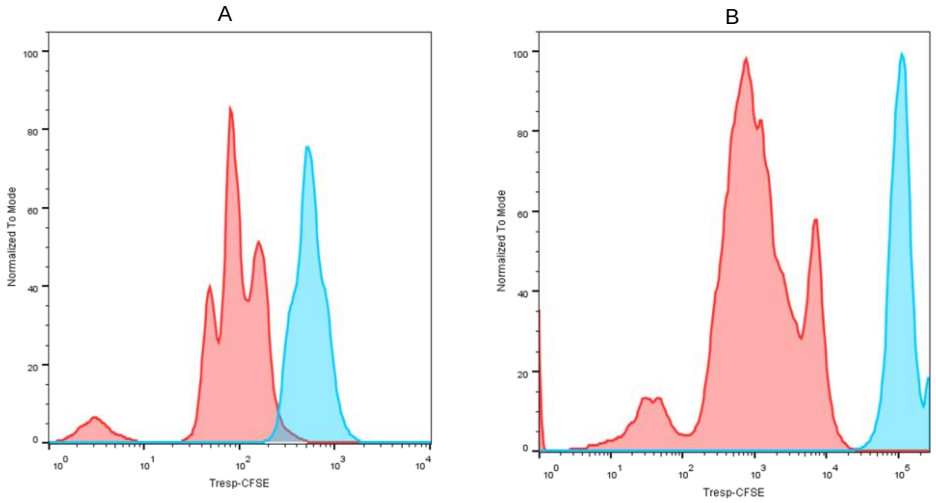


Figure 4.13: Testing the effect of PEI alone on T cell proliferation. (A) Histogram showing Tresp proliferation in the absence of PEI (B) Histogram showing Tresp proliferation in the presence of 40µl PEI not complexed to nucleic acids (40 µl is the maximum volume of PEI used in the study). Blue peak – Day 0 (day of activation), red peaks – 96 hours post-activation.

As Figure 4.13 shows, PEI (containing no nucleic acids complexed with it) does not induce toxicity and Tresp proliferate in the same way as to the control where no PEI was added. Therefore, in order to save the reagent, PEI was omitted from the negative controls in subsequent analysis.

To investigate the effect of transfecting Treg with endogenous RNA on the immunosuppressive capabilities of Treg on Tresp, Tresp and Treg cells were stained with CFSE and eFluor™ 670 proliferation dyes respectively and activated separately using anti-CD3/CD28 and in the presence of IL-2. Following 2 days after transfecting the Treg and washing (so as to remove any residual RNA which had not entered the Treg), the Treg were added to the Tresp in a 1:1 ratio. Untreated monocultures of Treg and Tresp were also prepared as well as monocultures of Tresp treated with rapamycin or transfected

with RNA (using PEI) at the same two concentrations. The latter cultures were set up to investigate the effect of RNA on Tresp alone. After preparing the co-cultures, the cells were cultured for a further 5 days and flow cytometric analysis of cell proliferation was carried out by monitoring the dilution of the proliferation dyes. The greater the dilution of the dye, the greater the number of cell divisions and hence the higher the cell proliferation.

For the same biological replicate, the same instrument settings on the FACS Aria III were used throughout analysis. The .fcs files were analysed using FlowJo, the cells were gated on the singlet population and the same gates were used across all samples throughout analysis for the same biological replicate.

4.2.2.2 Endogenous RNA has No Effect on Treg Suppressor Function

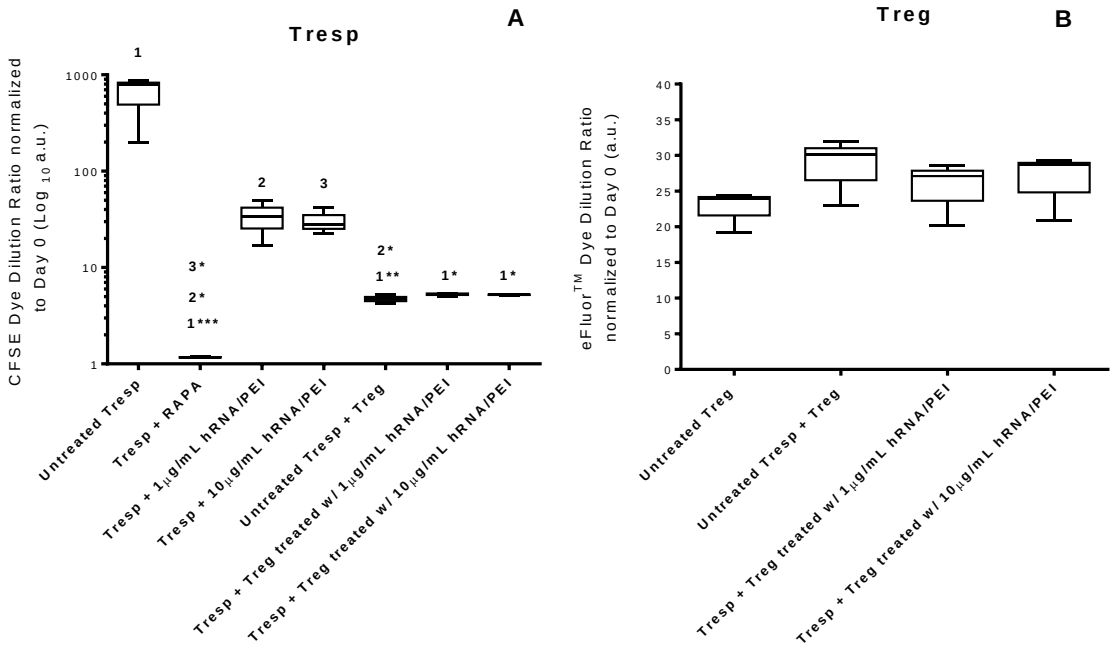


Figure 4.14: Cell proliferation of (A) Tresp and (B) Treg by monitoring the dilution of CFSE and eFluor™ respectively under different treatment conditions of endogenous RNA . Units are arbitrary and is the ratio of the MFI of inactivated Tresp/Treg cells to the MFI of the activated Tresp/Treg in the mono/co-cultures 7 days post-activation and transfection (and 5 days in co-culture). Data is presented as median CFSE dye dilution with 75th and 25th percentiles (n = 3 from different blood donors). Difference between treatments for (A) was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons while difference between treatments for (B) was tested using one-way ANOVA with Tukey's test. No statistical significant differences were observed in (B). * $p \leq 0.05$, ** $p \leq 0.005$ *** $p \leq 0.0005$.

Figure 4.14 plot A shows that the highest CFSE dilution, and therefore the highest Tresp proliferation, was in the untreated Tresp monoculture. The dilution of the dye was still high (but lower than the untreated) in the transfected Tresp monocultures and not significantly different from the untreated Tresp monoculture ($p > 0.05$ in both cases).

However, the CFSE is significantly less diluted in the Tresp and Treg co-cultures and the dilution is more or less equal in all of the three co-cultures. The differences were

significantly lower than those of the Tresp monocultures ($0.005 \leq p < 0.05$). This suggests that endogenous RNA does not affect the Treg's suppressive function, and that the lower CFSE dilutions in the co-cultures relative to the Tresp monocultures was due to the typical presence of Treg suppressing Tresp proliferation. This also means that the decrease in CD25 expression observed in the treated Treg (Figure 4.11, plot A) did not reduce the immunosuppressive power of the Treg probably due to the fact that FOXP3 expression was retained.

Plot B shows that transfecting Treg in co-cultures with endogenous RNA does not affect Treg proliferation either as the dilution of eFluor® dye did not change significantly between the different treatments ($p > 0.05$ in all cases).

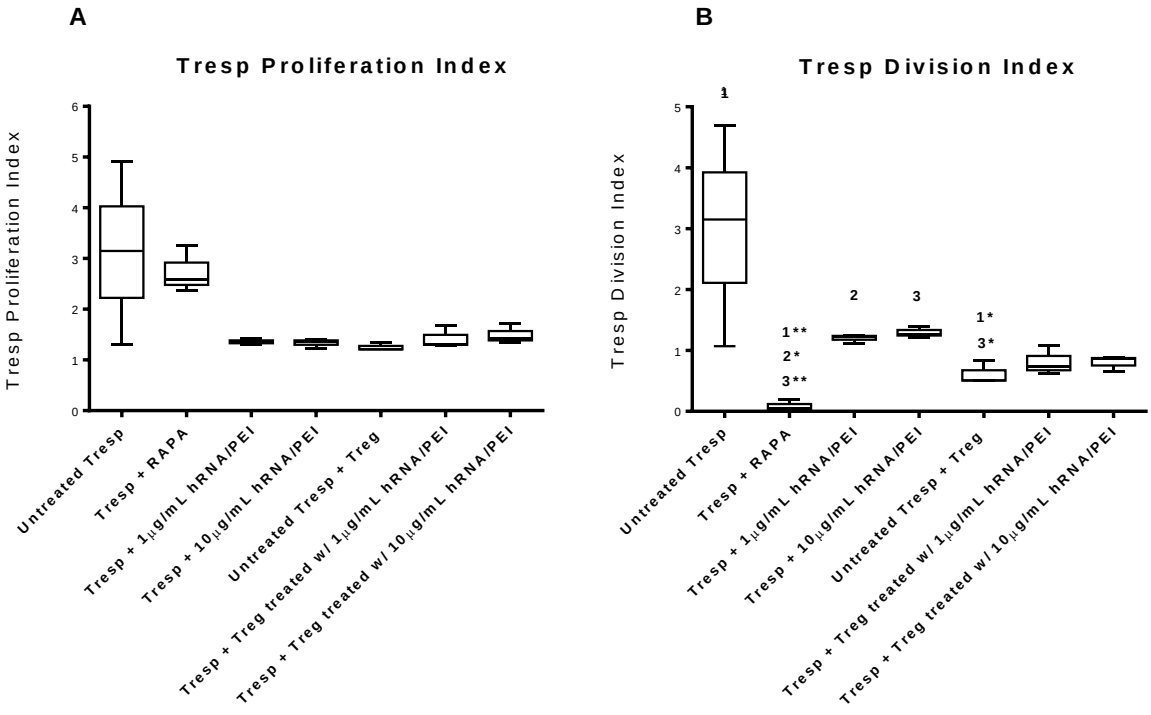


Figure 4.15: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of Tresp under different treatment conditions of endogenous RNA. Data was collected 7 days post-activation and transfection (and 5 days in co-culture). Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data is presented as median Proliferation/Division Index with 75th and 25th percentiles (n = 3 from different blood donors). Difference between treatments was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons. No statistical significant differences were observed for (A) . * p≤ 0.05, ** p≤ 0.005

Figure 4.15 plot A shows that there were no statistical significant differences observed in the proliferation indices of Tresp between the different treatments (p > 0.05 in all cases). This shows that for cells that have undergone at least one cell division, there was no difference in the average number of cell divisions that they underwent. This must mean that what is different (and what is causing differences in the CFSE dye dilution) is the average number of cell divisions that the cells in the entire population undergo i.e. the division index. In fact, plot B shows that the co-cultures had lower division indices than the Tresp monocultures with the untreated co-culture being significantly different

from the untreated Tresp monoculture (p = 0.021) and from the Tresp monoculture treated with 10µg/ml endogenous RNA (p = 0.025). Therefore culturing the Tresp in the presence of Treg reduces the number of cells that undergo any cell division at all.

4.2.2.3 Endogenous RNA Might Be Mildly Slowing Down Treg Proliferation

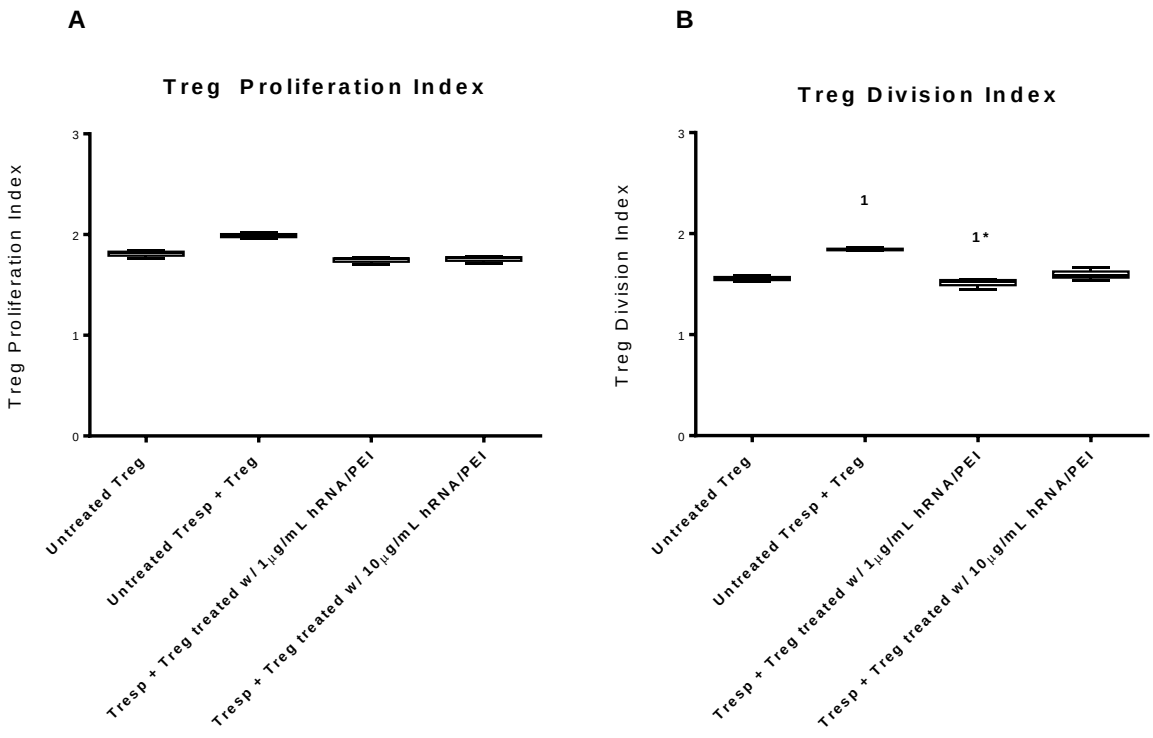


Figure 4.16: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of Treg under different treatment conditions of endogenous RNA. Data was collected 7 days post-activation and transfection (a total of 5 days in co-culture). Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data is presented as median Proliferation/Division Index with 75th and 25th percentiles (n = 3 from different blood donors). Difference between treatments was tested using the one-way ANOVA with Tukey's test. No statistical significant differences were observed for (A) .
* p< 0.05, ** p< 0.005

Figure 4.16 plot A shows that there were no significant differences in the proliferation indices of Treg under the different conditions (p > 0.05) meaning that the

average number of cell divisions that dividing Treg underwent did not vary. However, looking at plot B, it can be observed that there is a significant difference between the Treg division index of the untreated co-culture and the one where the Treg were treated with 1µg/mL with RNA (p = 0.038). Though a mild effect, transfecting Treg with endogenous RNA might be slowing down Treg proliferation by reducing the number of Treg that undergo any cell division at all.

4.2.3 Cytokine Analysis

Cytokine analysis was carried out to investigate cytokine environment induced under different treatment conditions.

4.2.3.1 Treating the Co-Cultures with Endogenous RNA Reduces IL-10 Production

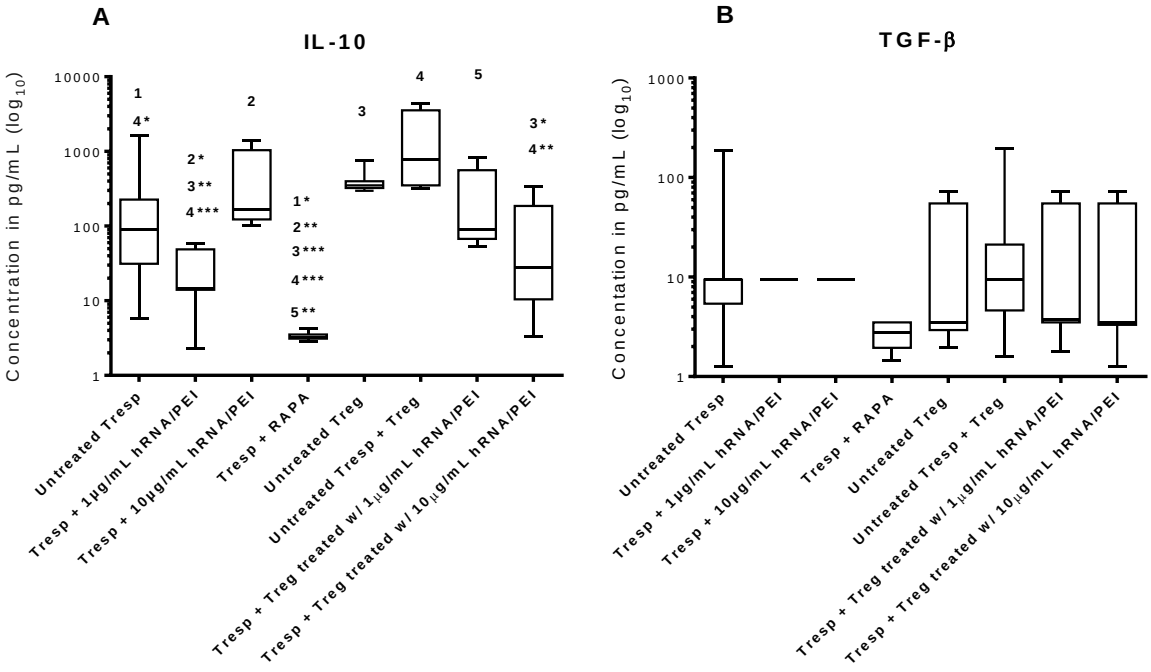


Figure 4.17: Box and Whisker plots showing, in logarithmic scale, concentrations in pg/mL of anti-inflammatory cytokines (A) IL-10 and (B) TGF-β obtained from cell supernatants exposed to different treatment conditions of endogenous RNA. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles (n ≥ 3). Difference between treatments was tested using the non-parametric Kruskal Wallis test with pairwise comparison. No statistical significant differences were observed for (B). * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005

Figure 4.17 plot A shows that IL-10 is produced by both Tresp and Treg monocultures. The highest levels of the anti-inflammatory cytokine were recorded in the untreated co-culture indicating that secretion of IL-10 might be one of the predominating mechanism by which Treg were exerting suppression of Tresp. Transfecting Treg in co-cultures with RNA seems to reduce the production of IL-10 and this effect seems to be dose-dependent, decreasing as the concentration of RNA is increased. A significant difference is observed between the untreated co-culture and that where the Treg are treated with 10µg/mL hRNA (p = 0.003). Nonetheless, alternative mechanisms of

immunosuppression were in operation other than IL-10 production given that Treg-mediated suppression was maintained in the treated co-cultures despite IL-10 levels went down.

Plot B shows no significant differences in TGF-β production among the different cultures ($p > 0.05$) and the concentration of free-active TGF-β detected, as observed in the other experiments, was rather low.

4.2.3.2 Granzyme B Production is Reduced When Tresp and Treg Co-cultures Are Treated With Endogenous RNA

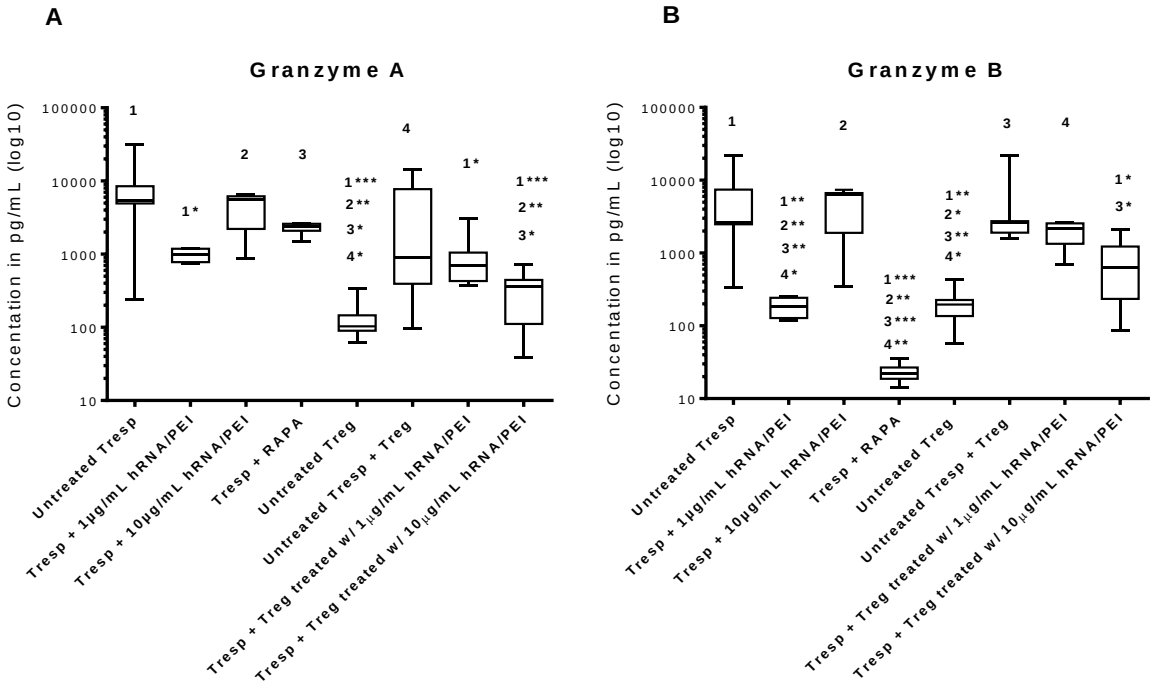


Figure 4.18: Box and Whisker plots showing in logarithmic scale, concentrations in pg/mL of Granzymes (A) Granzyme A and (B) Granzyme B obtained from cell supernatants exposed to different treatment conditions of endogenous RNA. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles (n ≥ 3). Difference between treatments was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005

Figure 4.18 shows that the production of granzyme B was reduced in the treated co-cultures (plot B) while no significant differences were observed in the concentration of Granzyme A other than between the monocultures and co-cultures (plot A). The decrease in the concentration of Granzyme B seem to be dose dependent and a statistically significant difference in the concentration of the enzyme was observed between the untreated co-culture and that were Treg were transfected with 10µg/mL of endogenous RNA (p = 0.046).

4.2.3.3 Endogenous RNA Enhances IL-12 Secretion in Tresp and Treg Co-Cultures

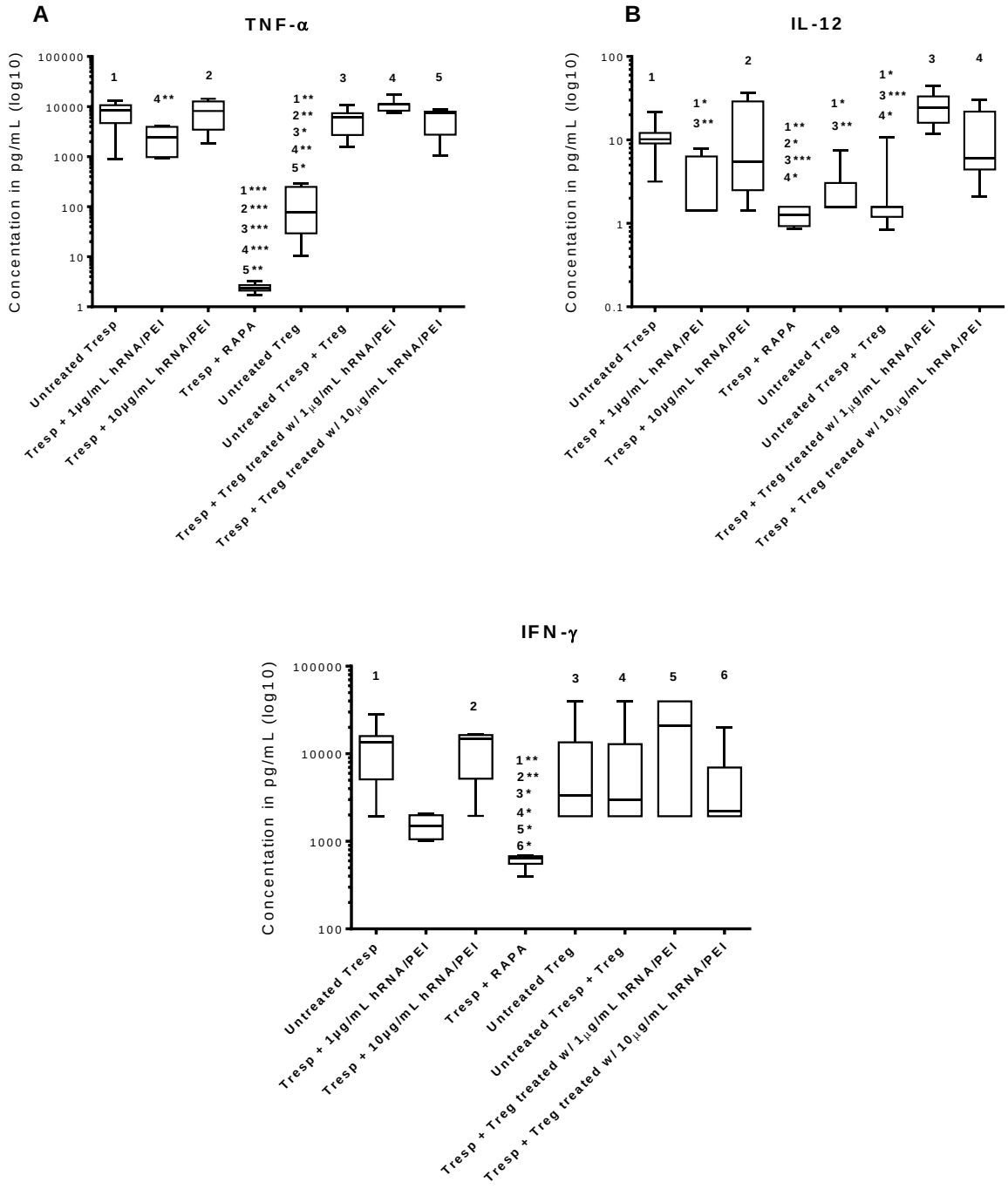


Figure 4.19: Box and Whisker plots showing, in logarithmic scale, concentrations in pg/mL of Th1-associated pro-inflammatory cytokines (A) TNF-α, (B) IL-12 and (C) IFN-γ obtained from cell supernatants exposed to different treatment conditions of endogenous RNA. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles (n ≥ 3). Difference between treatments was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$

As shown in Figure 4.19 box and whisker plots A and C, the TLR8 ligand does not seem to alter production of the Th1-promoting cytokines TNF-α and IFN-γ respectively. The concentrations of these cytokines in the untreated and treated co-cultures remained more or less constant. The levels recorded in the co-cultures were also similar to those of the Tresp monocultures meaning that the Treg did not suppress production of these cytokines.

However, it is of interest to note that plot B shows that the concentrations of IL-12, the differentiation cytokine for Th1, increased significantly in the co-cultures treated with both concentrations of endogenous RNA (Untreated Tresp + Treg vs Tresp + Treg treated with 1µg/ml hRNA/PEI $p \leq 0.0005$; Untreated Tresp + Treg vs Tresp + Treg treated with 10µg/ml hRNA/PEI $p = 0.046$). Transfecting Tresp monoculture with endogenous RNA did not produce this effect.

In the co-cultures, the RNA and transfection medium were added to the Treg separately before these were added to the Tresp, and any RNA which had not entered the Treg was washed off. Therefore, it is probable that the "extra" IL-12 production in the co-cultures was being produced by the transfected Treg and not by the Tresp. Alternatively the Tresp might be responding to some other soluble factor that the treated Treg were releasing by producing IL-12. The later hypothesis is more plausible given that there were no indications that Treg were assuming Th1 characteristics. These results might suggest that treating Treg with endogenous RNA induces cells in the co-culture to promote Th1-polarising environment via the enhanced secretion of IL-12 and this might actually be useful for the purpose of this study.

4.2.3.4 Endogenous RNA Enhances the Production of IL-4 in Tresp and Treg Co-Cultures

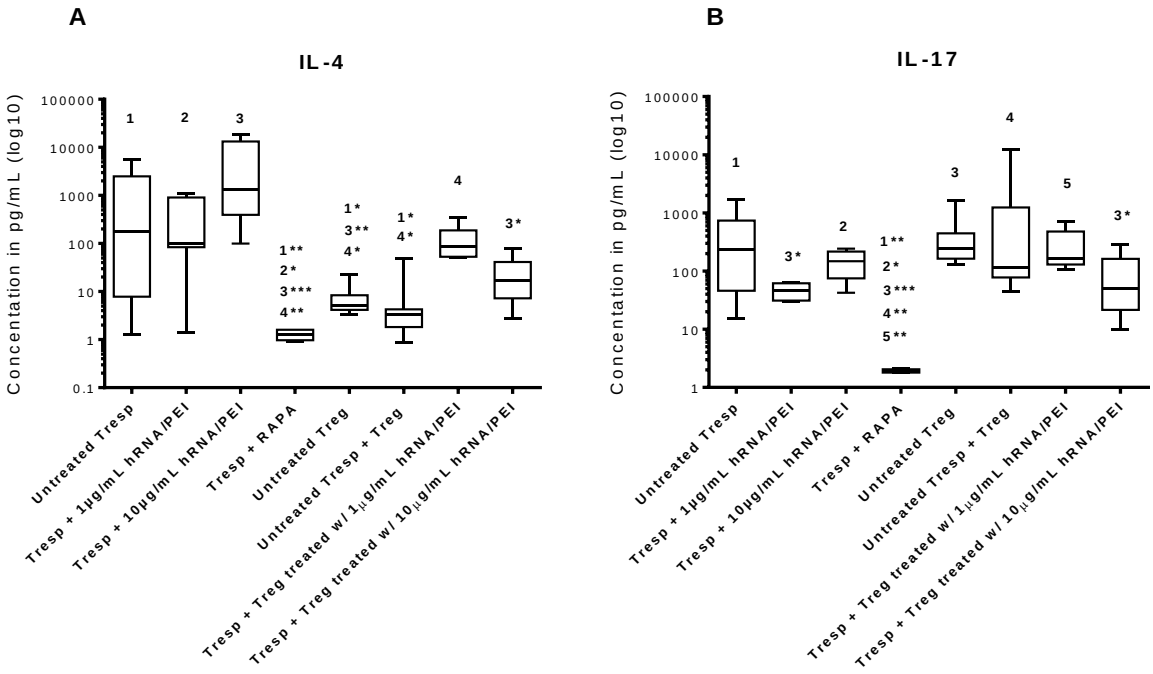


Figure 4.20: Box and Whisker plots showing, in logarithmic scale, concentrations in pg/mL of Th2 differentiation cytokine (A) IL-4 and the effector cytokine for Th17 (B) IL-17 obtained from cell supernatants exposed to different treatment conditions. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles (n ≥ 3). Difference between treatments was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005

An interesting result obtained from these cytokine assays is the increase in IL-4 production in the treated co-cultures as shown in Figure 4.20 (plot A). The concentrations of the cytokine are highest in the Tresp monocultures, as usually observed, and decrease when the Tresp are co-cultured with the Treg (untreated). However, IL-4 production starts to increase again when the Treg in the co-cultures are treated with endogenous RNA with the difference between the untreated co-culture and that treated with 1µg/mL hRNA being statistically significant (p = 0.041). Although it might be that possible that the

Tresp are releasing the IL-4, an alternative hypothesis could that that the transfected Treg are releasing the cytokine. This might be tied to the increase in CRTH2 marker observed in the treated Treg, especially those transfected with 1µg/mL of RNA (plot C) . Indeed, analysing together Figure 4.11 plot C and Figure 4.20 plot A, it is evident that IL-4 production is proportional to the increase in CRTH2 expression. Therefore, it might be that the TLR7/8 ligand is promoting a switch of the Treg to Th2 by upregulating the expression of CRTH2 as well as the production of the Th2 differentiation cytokine IL-4.

Treating the co-cultures with the TLR8 ligand does not seem to particularly affect the production of IL-17 ($p > 0.05$ in most of the cases) and thus differentiation to Th17 (Figure 4.20 plot B).

4.3 Analysing the Effects of Endogenous TLR9 Ligand - Human DNA

4.3.1 Phenotype Analysis

To investigate the effect of cell-free DNA on the phenotype of Treg, Treg cells were activated using anti- CD3/CD28 in the presence of IL-2. On the same day of activation, two different concentrations of cell free DNA were tested (1µg/ml and 10µg/ml) and the Treg cells were transfected with the DNA using PEI as a transfection agent as described in the method. Using a transfection reagent results in a more efficient delivery of DNA into the cell (especially since human genomic DNA is large and therefore unaided uptake would be difficult).

The cells were cultured for a further 6 days and phenotype analysis using both extra- and intracellular markers was carried out by flow cytometry to compare phenotypic changes in the untreated and treated samples. For the same biological replicate, the same instrument settings on the FACS Aria III were used throughout analysis. The .fcs files were analysed using FlowJo, the cells were gated on the singlet population, specific binding was identified and the same gates were used across all samples throughout analysis for the same biological replicate.

4.3.1.1 Endogenous DNA Does Not Induce Phenotypic Changes in Treg cells

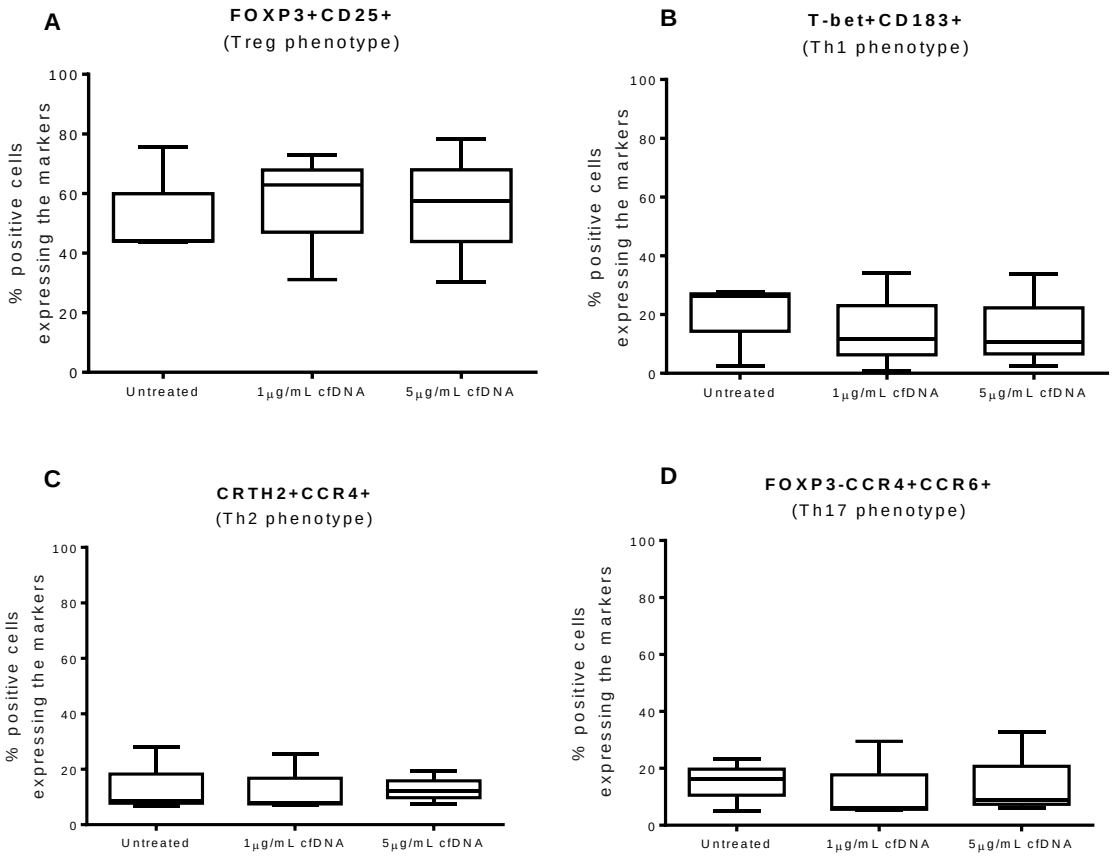


Figure 4.21: Graph showing the effects of transfecting Tregs with endogenous human DNA on their phenotype 6 days post-activation and transfection. Data is presented as median percentage of cells with 75th and 25th quartiles. Difference in percentage of cells that were FOXP3+CD25+ and T-bet+CD183+ between treatments was tested using one-way ANOVA with Tukey's test. Difference in percentage of cells that were CCR4+CRTH2+ and FOXP3-CCR4+CCR6+ between treatments was tested using Kruskal Wallis non-parametric test with pairwise comparisons. No statistical significant differences were observed.

Figure 4.21 shows that there was no apparent change in the phenotype of the Treg when the cells are transfected with endogenous DNA aided by PEI. The majority of the cells in the untreated sample were FOXP3+ and CD25+, which is the phenotype that typically characterises Treg, and remained as such even in the transfected samples.

Therefore, endogenous DNA seems to exert no effect on the phenotype of Treg and this TLR9 ligand does not cause a shift of Treg to other T cell types. No shifts in the MFI, and thus expression was either observed when considering the expression of single markers.

4.3.2 Proliferation Assays

To investigate the effect of endogenous DNA on the immunosuppressive capabilities of Treg on Tresp, Tresp and Treg cells were stained with CFSE and eFluor670 proliferation dyes respectively and activated separately using anti- CD3/CD28 and in the presence of IL-2. Following 2 days after transfecting the Treg with either 1µg/ml or 5 µg/ml DNA (complexed to PEI) and washing (so as to remove any residual DNA which had not entered the Treg), the Treg were added to the Tresp in a 1:1 ratio. Untreated monocultures of Treg and Tresp were also prepared as well as monocultures of Tresp treated with rapamycin and endogenous DNA complexed to PEI at the same two concentrations (the latter to also investigate the its effect on Tresp alone). After preparing the co-cultures, the cells were cultured for a further 5 days and flow cytometric analysis of cell proliferation was analysed by monitoring the dilution of the proliferation dye. The greater the dilution of the dye, the greater the number of cell divisions and hence the higher the cell proliferation.

For the same biological replicate, the same instrument settings on the FACS Aria III were used throughout analysis. The .fcs files were analysed using FlowJo, the cells were gated on the singlet population and the same gates were used across all samples throughout analysis for the same biological replicate.

4.3.2.1 Endogenous DNA Directly Reduces Tresp Proliferation and Enhances Treg Suppressor Function

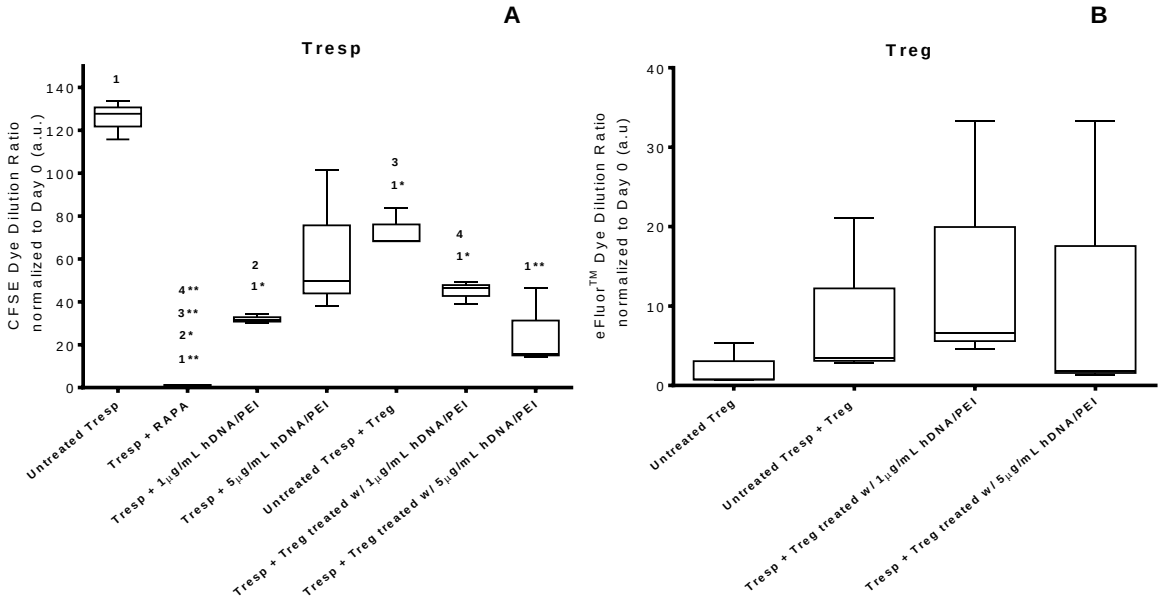


Figure 4.22: Box and whisker plot showing the (A) CFSE dye dilution in Tresp and (B) eFluor™ dye dilution in Treg under different treatment conditions of endogenous DNA. The dye dilution represents the extent of Tresp/Treg proliferation. Data was collected 7 days post-activation and transfection and 5 days after co-culturing. Units are arbitrary and is the ratio of the MFI of inactivated Tresp/Treg cells to the MFI of the activated Tresp/Treg in the mono/co-cultures 7 days post-activation. Data is presented as median CFSE/eFluor™ dye dilution with 75th and 25th quartiles (n = 3 from different blood donors). Difference between groups in (A) was tested using one way ANOVA with Dunnett's T3 test while difference between groups in (B) was tested using Kruskal Wallis non-parametric test with pairwise comparisons. No statistical significant differences were observed in (B). * p≤ 0.05, ** p≤ 0.005

Figure 4.22 plot A shows, as expected, a statistically significant difference in the CFSE dye dilution of Tresp cultured alone and Tresp that were co-cultured in the presence of Treg (untreated and treated with endogenous DNA) showing that the Treg were suppressing Tresp proliferation. The same plot however also shows that the CFSE dye dilution was lower in the DNA/PEI-treated co-cultures than in the untreated co-culture (and proliferation decreases with increasing DNA concentration), although differences

were not statistically significant ($p > 0.05$). It is also interesting to note that even though the co-cultures left untreated and those treated with 1µg/ml DNA/PEI were not significantly from the positive suppressive control (Tresp + rapamycin), this was not the case with the co-culture treated with 5µg/ml DNA/PEI ($p = 0.377$). This shows that the extent of Tresp suppression in this latter co-culture was considerably higher than that in the former two co-cultures and closer to that of the positive suppressive control.

The DNA/PEI also seems to exert a direct negative effect on Tresp proliferation as the CFSE dilution was lower when monocultures of Tresp (in the absence of Treg) were treated with DNA/PEI and in fact the difference between untreated Tresp and those cultured in the presence of 1µg/ml DNA/PEI (both in the absence of Treg) were statistically significant ($p = 0.012$).

The data in general shows that the proliferation of Tresp is slowed down in the presence of DNA/PEI both in the absence and presence of Treg. Since the Treg are transfected with the DNA before adding them to Tresp and the Treg are washed off from the transfection medium after the incubation period, the endogenous DNA in the co-cultures is acting directly on the Treg and somehow increasing their suppressor function. This effect might be dose-dependent. Nonetheless, endogenous DNA also showed a direct inhibitory effect on Tresp in the absence of Treg.

Proliferation of Treg was also monitored to check whether the enhanced suppression of Tresp observed in co-cultures with treated Treg was due to enhanced Treg proliferation. In Figure 4.22 plot B, one can see that the proliferation of Treg is not significantly affected by the addition of DNA/PEI. There was an increase in Treg proliferation in the co-cultures in response to the presence of Tresp but these differences

were not significant ($p > 0.05$). Such results therefore indicate that endogenous DNA does not augment Treg proliferation but it increases their immunosuppressive capacity of the cells.

Proliferation modelling of the data for both Tresp and Treg proliferation was then performed in order to provide more information on the proliferation patterns of the cells under different treatment regimes.

4.3.2.2 Endogenous DNA Decreases Tresp Proliferation and Division Indices in both Tresp Monocultures and When Co-cultured with Treg

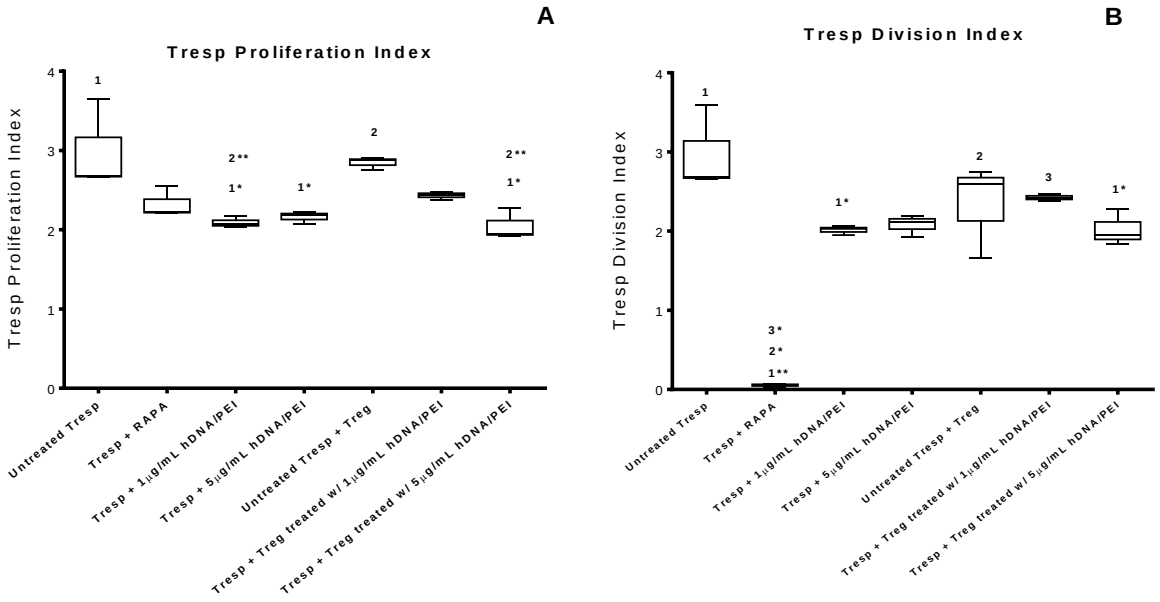


Figure 4.23: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of Tresp in the absence or presence of Treg untreated or transfected with human DNA. Data was collected 7 days post-activation and treatment and 5 days co-culture. Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data are presented as median Proliferation/Division Index with 75th and 25th percentiles (n = 3 from different blood donors). Difference between groups was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons. * p≤0.05, ** p≤ 0.005

The proliferation and division indices in provide further indications that endogenous DNA is indeed suppressing Tresp proliferation both in the absence and presence of Treg. Figure 4.23 plot A shows that the highest proliferation indices where recorded in monocultures and co-cultures that were left untreated with a number of significant differences observed between untreated and treated cultures. Proliferating Tresp in the untreated c-cultures underwent an average of three rounds of cell divisions while treated ones underwent an average of two rounds of cell division. Treatment with endogenous DNA also reduced the average number of cell divisions undergone by the entire population of cells as shown plot B. This was especially evident when one compares the untreated Tresp monocultures and those treated with endogenous DNA where an average of three rounds of cell divisions and two rounds of cell division respectively were observed.

4.3.2.3 Treg Proliferation and Division Indices in Co-Cultures Increase Slightly In the Presence of Endogenous DNA

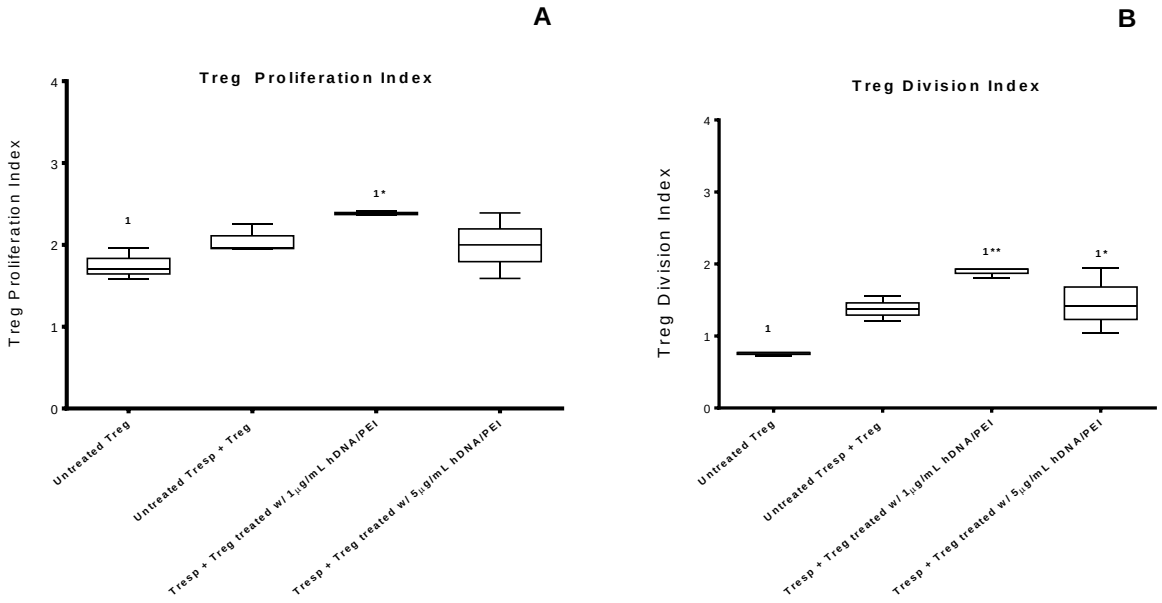


Figure 4.24: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of untreated Treg monocultures and in Tresp:Treg cocultures untreated or transfected with human DNA. Data was collected 7 days post-activation and treatment and 5 days co-culture. Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data are presented as median Proliferation/Division Index with 75th and 25th percentiles (n = 3 from different blood donors). Difference between groups was tested using one-way ANOVA with Tukey's test . * p≤ 0.05, ** p≤ 0.005

Figure 4.24 plot A shows that the highest proliferation indices were recorded in Treg co-cultured with Tresp that had been previously transfected with 1µg/ml endogenous DNA and a significant difference is observed between this co-culture and the untreated Treg monoculture (p = 0.047). The highest division index was also recorded in this co-culture. Division indices in co-culture transfected with both DNA concentrations are significantly different from that of the untreated monoculture of Treg (Untreated Treg vs Tresp + Treg treated w/ 1µg/ml hDNA/PEI p = 0.002; Untreated Treg vs Tresp + Treg treated w/ 10µg/ml hDNA/PEI p = 0.030) . These results show that endogenous DNA

might be, to a certain extent, promoting the number of cell divisions Treg undergo, in contrast to its effect on Tresp which is the opposite. This was unclear when Treg proliferation was analysed using the dye dilution method (Figure 4.22 plot B) and therefore highlights the importance of including proliferation and division indices when analysing cell proliferation data to reveal proliferation patterns that are not immediately apparent when using the dye dilution method.

4.3.3 Cytokine Analysis

Cytokine analysis was carried out on the cell culture supernatants to try and understand which soluble factors were responsible for the enhanced Treg-mediated suppression observed.

4.3.3.1 Low Concentrations of Endogenous DNA Enhance Production of IL-10, TGF-β and Granzyme B

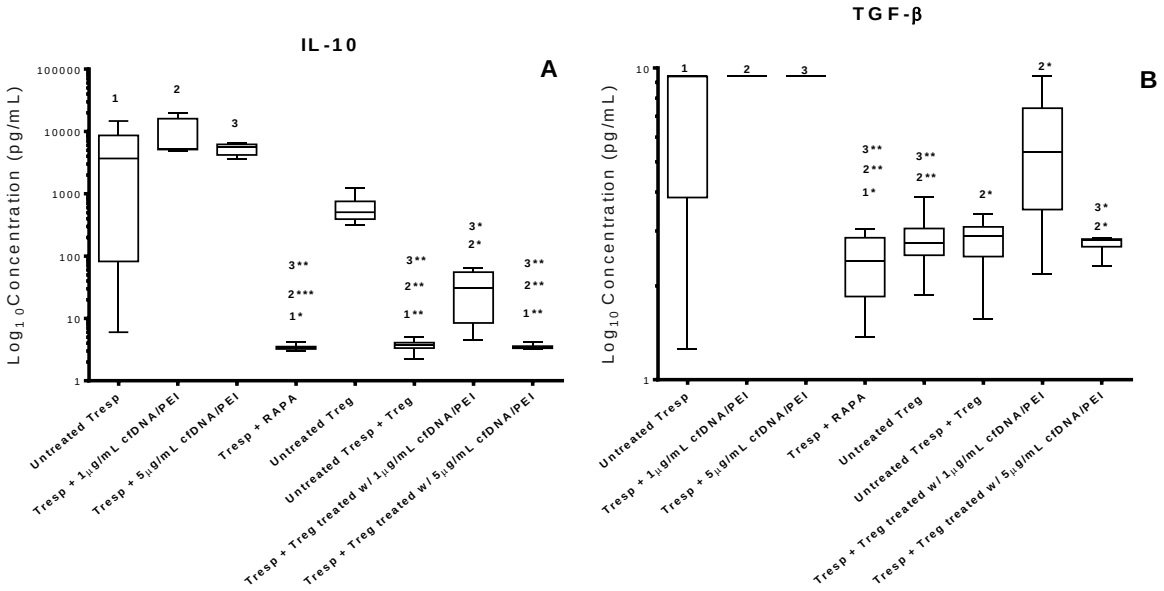


Figure 4.25 : Box and Whisker plots showing, in logarithmic scale, the concentrations in pg/mL of (A) IL-10 and (B) free active TGF-β1 obtained from cell supernatants exposed to different treatment conditions of endogenous DNA. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th an 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005

Figure 4.25 shows that the highest levels of the anti-inflammatory cytokines IL-10 and TGF-β were recorded in the Tresp monoculture in the absence of Treg and the concentrations were statistically significant from the levels observed in some of the Tresp and Treg co-cultures ($0.001 \leq p \leq 0.037$).

Addition of 1μg/ml endogenous DNA complexed to PEI seems to increase production of the two cytokines, yet the difference was not significant from that of the untreated co-culture control ($p > 0.05$). Treating with 5μg/ml seems to restore the cytokines' concentrations back to the levels detected in the untreated samples. This means that Tresp

in this co-culture were being suppressed via some additional mechanism that does not involve increase in the production of these two anti-inflammatory cytokines by the Treg.

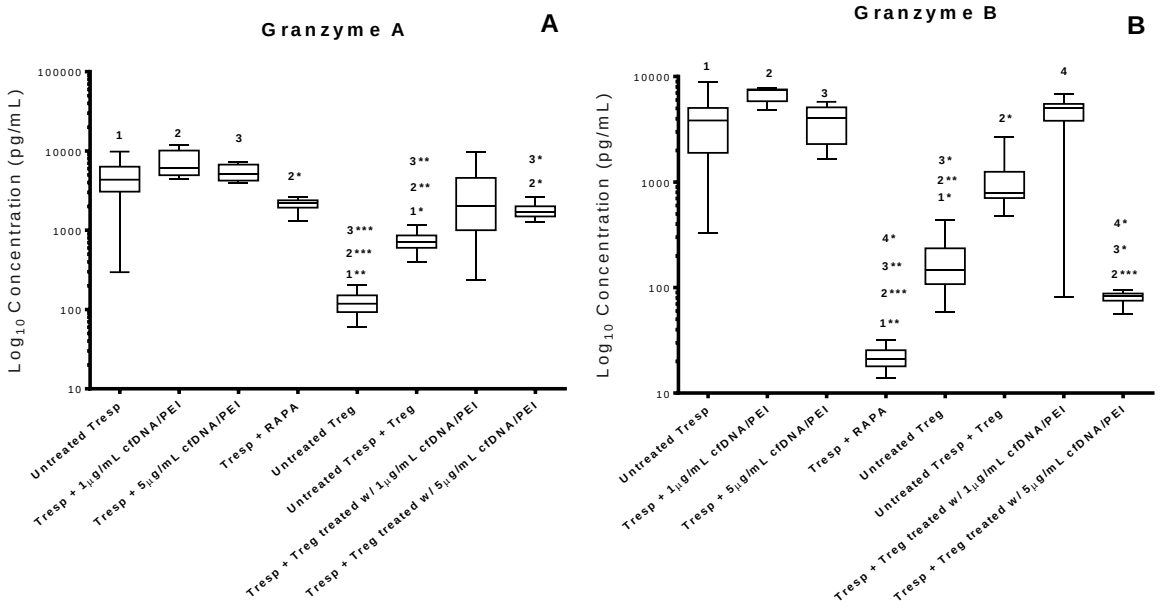


Figure 4.26: Box and Whisker plots showing, in logarithmic scale, concentrations in pg/mL of (A) Granzyme A and (B) Granzyme B; obtained from cell supernatants exposed to different treatment conditions of endogenous DNA. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th an 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005

Similar to the patterns observed with IL-10 and TGF-β production, Figure 4.26 shows that Granzymes A and B were detected in high amounts when Tresp are cultured in the absence of Treg. Treg monocultures produced very low levels of both granzymes and once Tresp and Treg are co-cultured together, Granzyme A and B production is reduced to levels that are significantly lower from those of the Tresp monocultures ($0.001 \leq p < 0.048$ and $p \leq 0047$ respectively). This excludes the co-culture treated with 1µg/ml endogenous DNA where higher levels of especially granzyme B were detected (plot B). Indeed there is a significant difference in granzyme B produced by cells in the co-cultures treated with 1µg/ml endogenous DNA and those treated with 5µg/ml DNA ($p = 0.035$).

4.3.3.2 Low Concentrations of Endogenous DNA Promote the Secretion of Th1-associated Cytokines

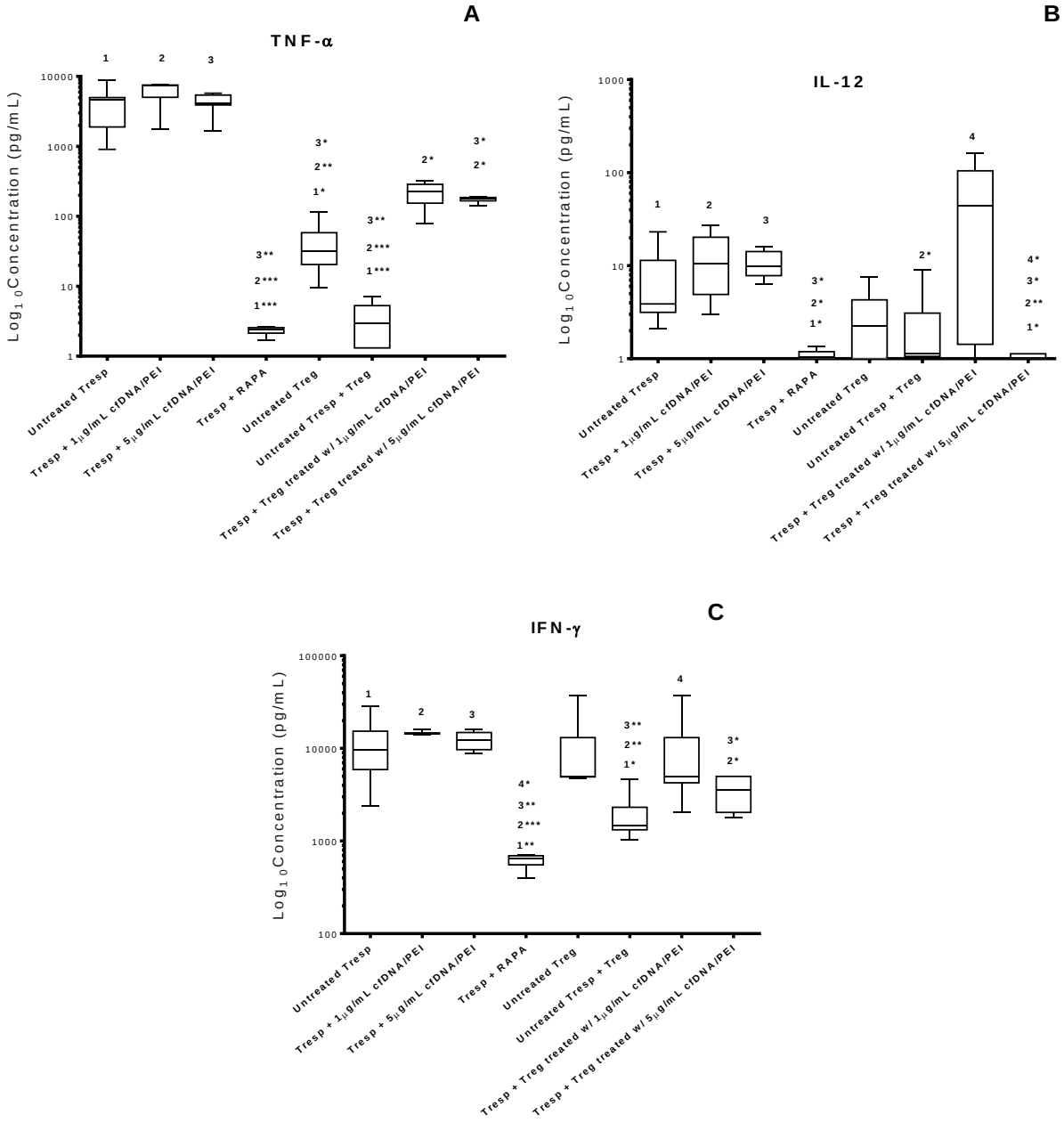


Figure 4.27: Box and Whisker plots showing, in logarithmic scale, concentrations in pg/mL of (A) TNF-α, (B) IL-12 and (C) IFN-γ obtained from cell supernatants exposed to different treatment conditions of endogenous DNA. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th an 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005

Figure 4.27 plot A shows that the levels of TNF-α were the highest when the Tresp were cultured in the absence of Treg and the differences in concentrations were significant from those of the Tresp:Treg co-cultures ($p \leq 0.047$ in such cases). Treating Treg with endogenous DNA in the co-culture however increased the concentration of the cytokine relative to their respective untreated control. These differences are not statistically significant ($p > 0.05$) but might have reached significance is a greater number of replicates were considered. Therefore, it is possible that in the co-cultures, the Tresp were responding to the highly suppressive treated Treg by secreting higher amounts of TNF-α.

Plot B shows that the levels of IL-12 were generally low in all cultures, as was observed in the IFN-γ experiments. Nonetheless, levels were significantly higher in the Tresp monocultures when compared to the Tresp and Treg co-cultures ($0.003 \leq p \leq 0.025$). The only exception was again observed in the co-culture treated with 1µg/ml endogenous DNA. Indeed, a statistically significant difference was observed between the IL-12 concentrations recorded in this co-culture and the one where the Treg in the co-culture had been transfected with 5µg/ml endogenous DNA ($p = 0.033$). Interestingly, the median concentration levels in the coculture treated with 1µg/ml exceeded even those of the Tresp monocultures.

Figure 4.27 Plot B therefore provides indications that when Treg were treated with a concentration of 1µg/ml DNA, Tresp responded back to the treated Treg by upregulating the secretion of the Th1-differentiation cytokine IL-12. This however was not sufficient to counteract the suppressive action of Treg, which clearly, was strengthened with the treatment. In the co-culture treated with 5µg/ml DNA, it is probable that Tresp

were so severely suppressed by the treated Treg that the levels of the IL-12 detected were almost negligible.

Figure 4.27, Box and whisker Plot C shows that IFN-γ was the highest in the Tresp monocultures, Treg monocultures and the co-cultures treated with 1µg/ml of DNA. The untreated co-culture and that treated with 5µg/ml DNA produced lower amounts of the cytokine and the differences were significant from those of the Tresp monocultures ($p \leq 0.037$). Although the co-culture treated with 1µg/ml DNA and the untreated co-cultures were not statistically significant ($p = 0.108$), the cytokine concentrations in the former co-culture were very close and not significantly different from the Tresp monoculture (with $p \geq 0.05$ in all cases).

4.3.3.3 Endogenous DNA Does Not Significantly Alter the Production of IL-4 and IL-17

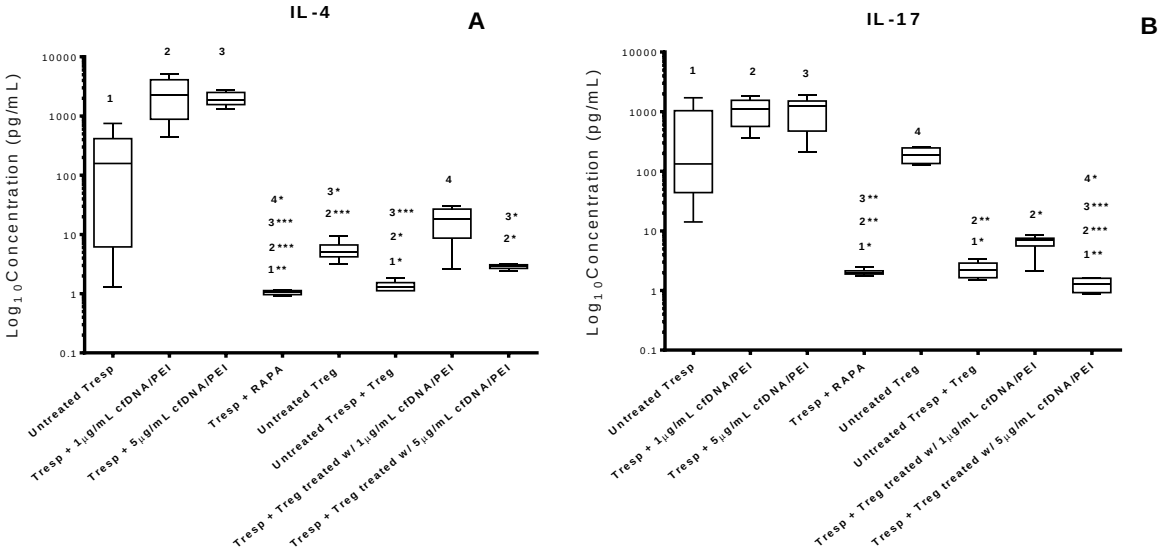


Figure 4.28: Box and Whisker plots showing concentrations in pg/mL of (A) IL-4 and (B) IL-17; obtained from cell supernatants exposed to different treatment conditions of endogenous DNA. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th an 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005

Figure 4.28 plot B shows that the concentrations of the differentiation cytokine for Th2, IL-4, was highest in the Tresp monoculture indicating that a proportion of the proliferating Tresp cells where differentiating into Th2. Adding endogenous DNA to Tresp monocultures seem to slightly enhance production of this cytokine although the differences are not statistically significant ($p > 0.05$). The levels of the IL-4 recorded in the co-cultures was lower than that of the Tresp monocultures, with the untreated co-culture and the one treated with 5µg/ml DNA being significantly lower ($p \leq 0.010$).

Plot B shows that the highest levels of IL-17 were recorded in the Tresp monoculture whilst the levels recorded in the co-cultures were significantly lower. Treg monocultures were also found to produce high levels of the cytokine. IL-17 production was also low in the co-culture where the Treg were treated with 1µg/ml endogenous DNA indicating that endogenous DNA does not affect the production of the effector cytokine for Th17.

4.4 Analysing the Effects of Synthetic TLR7/8 Ligand CL097, a Resiquimod derivative

This section summarises the effects of the first synthetic TLR7/8 ligands on the Treg phenotype, function and cytokine production in Tresp and Treg co-cultures. The results achieved using CL097, a synthetic water-soluble compound of Resiquimod which belongs to the a group of compounds called imidazoquinolines known to have anti-viral and possibly also anti-cancer properties, are presented.

In previous studies, TLR8 ligands have been found to inhibit Treg responses. They have been shown to enhance CD4+ non-Treg cells and induce cytotoxic T cell responses and promote their infiltration in some tumours, as well as augment Th1 proinflammatory cytokine production. These effects are known to be mediated via dendritic cells, however, the direct effects of these ligands on Treg have been poorly investigated.

4.4.1 Phenotype Analysis

To investigate the effect of CL097 on the phenotype of Treg, flow cytometric analysis was carried out to compare any phenotypic changes that result when Treg are treated with the ligand and compared with the phenotype of untreated Treg.

Treg were activated using anti-CD3/CD28 in the presence of IL-2. On the day of activation, the Treg were treated with two different concentrations of CL097 (Invivogen, USA) that stimulate human TLR7/8 according to the manufacturer's instructions. The Tregs were also treated with CL097 in combination with bacterial LPS which acts as a TLR4 ligand. LPS is known to induce Th1 responses and release of IL-12 via a TLR4-mediated mechanism, however it has also been demonstrated in studies that TLR4 and TLR8 work synergistically together and promote Th1 responses even further (Napolitani et al., 2005).

Another treatment combination that was tested consisted of combining CL097 and the pro-inflammatory and differentiation cytokine for Th1, IL-12, as it has also reported that these two together promote Th1. To check whether these combinations were indeed due a synergistic effect and not due to the sole action of one agent, the Treg were also treated with LPS or IL-12 alone, in the absence of CL097. The purpose of setting up these conditions was to investigate whether creating a Th-1 promoting environment via TLR7/8-signalling induces changes in Treg.

Six days post treatment, the Treg cells were stained with extra- and intracellular markers that characterise different CD4+ cell populations. Analysis to compare phenotypic changes between treated and untreated Treg was carried out using flow cytometry. The cells were gated on the singlet population and the same gates were used throughout analysis for all samples that were derived from the same biological replicate.

4.4.1.1 CL097, LPS and IL-12 Promote Treg Phenotype

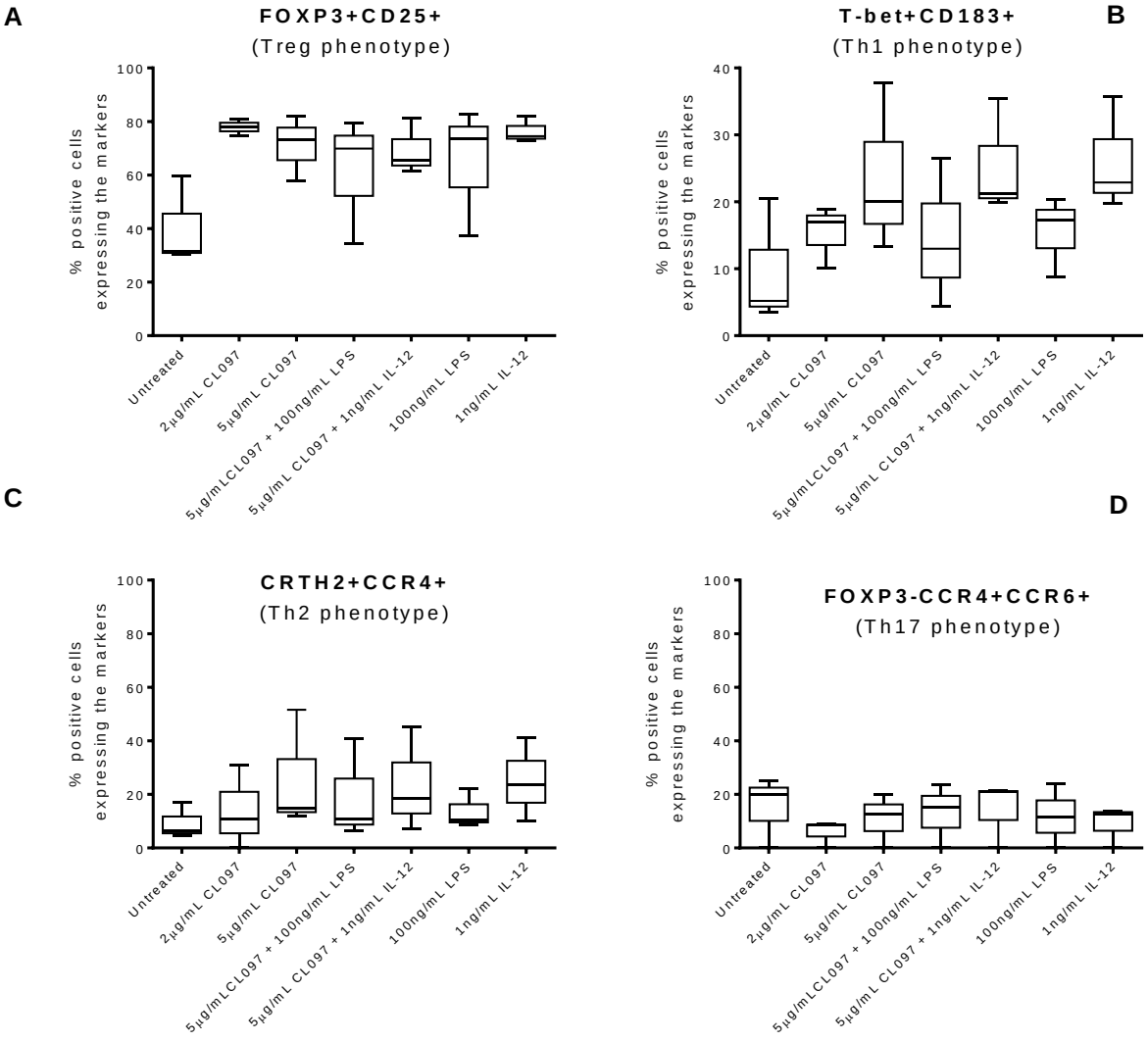


Figure 4.29: Box and whisker plots showing the effects of CL097 on the phenotype of Treg monocultures 6 days post-activation and treatment. Data is represented as median percentage positive cells with 75th and 25th quartiles (*n* = 3 from different blood donors). Differences between treatments for (A), (C) and (D) were tested using Kruskal-Wallis non-parametric test with pairwise comparison while for (B) differences were tested using one-way ANOVA with Tukey's test. No statistical significant differences were observed.

Figure 4.29 (plot A) shows that treating the Treg with CL097 alone or in combination with LPS or IL-12 increased the percentage of cells that were FOXP3+CD25+ indicating that these agents enhanced further a Treg phenotype in the proliferating cells. No synergistic

effect was observed when coupling CL097 with LPS or IL-12. The same effect was achieved when the Treg were treated with either LPS or IL-12. Although the percentage increase doubled relative to the untreated control, these differences did not reach statistical significance ($p > 0.05$).

Interestingly, Figure 4.29 (plot B) shows that the treated samples also showed an increase in the percentage of cells that were T-bet+CD183+ especially when treated with 5µg/ml CL097 and IL-12 alone or combined together. This means that that these two agents, apart from enhancing a Treg phenotype might also be promoting the co-expression of Th1 markers. Again, these differences were not statistically significant relative to the untreated control ($p > 0.05$) and the effect of combining CL097 with IL-12 was not a synergistic or even an additive one.

Figure 4.29 (plots C and D) show that there was no evident change in the percentage of cells that were CRTH2+CCR4+ or FOXP3- CCR4+CCR6+ relative to the untreated control meaning that none of the agents, alone or in combination, favoured phenotypes associated with Th2 or Th17 cells.

4.4.1.2 CL097 and IL-12 Upregulate CD25 and T-bet Expression

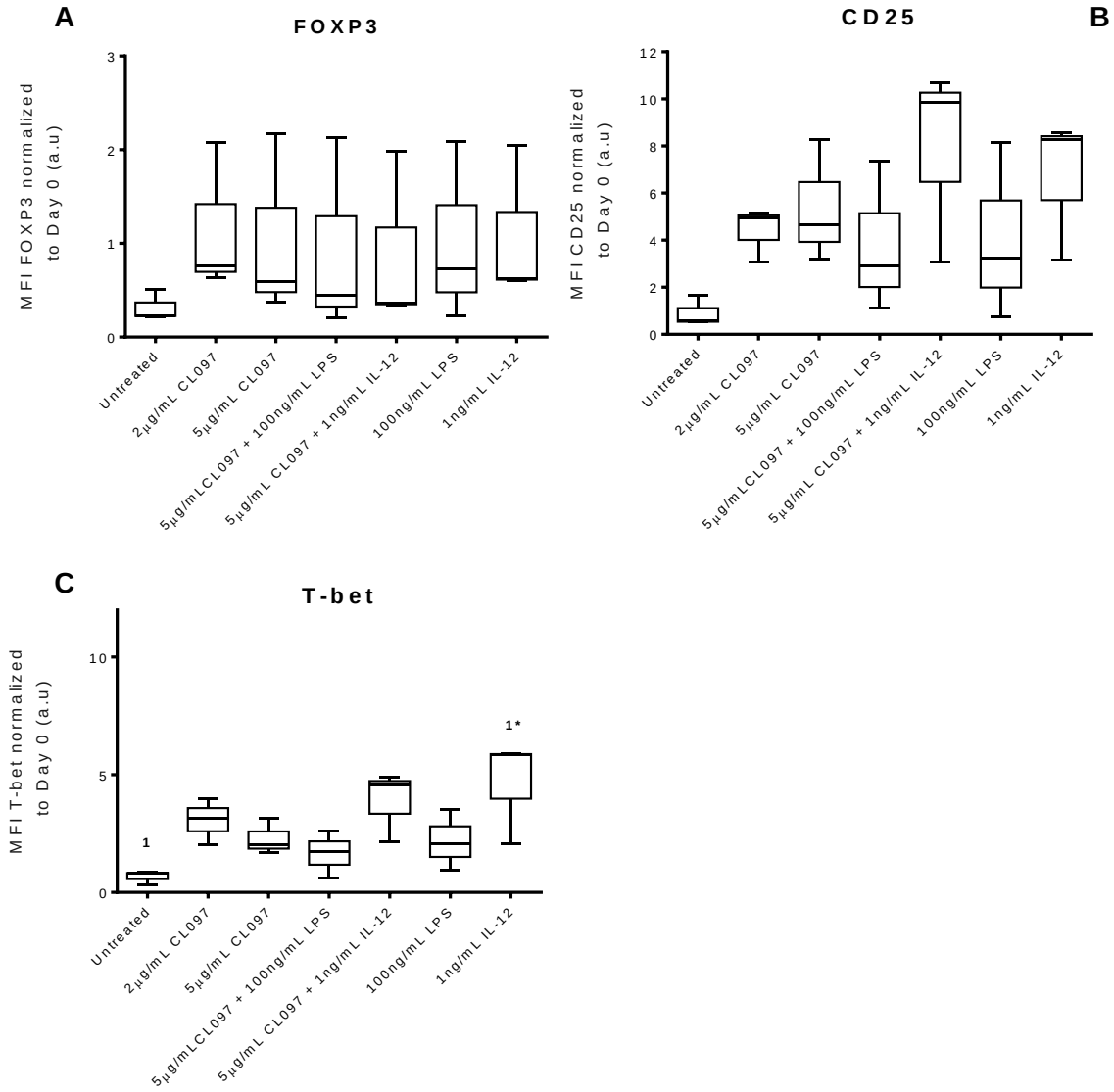


Figure 4.30: Box and Whisker plots showing Median Fluorescent Intensity (MFI) of (A) FOXP3; (B) CD25; (C)T-bet fluorescence in the untreated cultures and cultures treated with CL097 without and in combination with LPS or IL-12. Units are arbitrary and have been normalized by dividing the MFI on 6 days post-treatment by that of Day 0. Data is represented as median MFI with 75th and 25th percentiles. Differences in FOXP3 MFI between treatments was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons whilst differences in CD25 and T-bet MFI between treatments was tested using one-way ANOVA with Tukey's test. No statistical significance was found between groups in (A) and (B) * $p \leq 0.05$, ** $p \leq 0.005$

When the relative FOXP3 and CD25 MFIs in the untreated and treated Tregs were compared, an increase in both markers' MFIs was observed in the treated Treg, meaning that expression of both FOXP3 and CD25 was enhanced upon treatment. These changes are illustrated in Figure 4.30 (plots A and B above respectively). The MFIs for the CD25 marker were markedly increased (whilst not so much for FOXP3) in the treated Tregs, especially those exposed to 5µg/ml CL097 together with 1ng/ml IL-12 whereby it increased by almost tenfold relative to that of the untreated control. These MFI differences were not statistically significant ($p > 0.05$) but would have probably reached significance if a larger number of replicates were used in the analysis.

Plot C, on the other hand, shows that the treated Tregs were observed to have higher MFI values for transcription factor T-bet relative to the untreated Tregs, which might explain why a higher percentage of T-bet+CD183+ cells was recorded in the treated samples. This effect was the greatest in the presence of IL-12. Although most of the differences did not reach statistical significance in comparison to the untreated Treg, the T-bet MFI values of Treg treated with 1ng/ml IL-12 were significantly greater than that of the untreated Treg ($p = 0.025$). This is not surprising given that IL-12 is a Th1 differentiation cytokine which might have in turn upregulated the Th1-transcription factor T-bet. Although Tregs treated with CL097 alone or in combination with IL-12 did cause an increase in T-bet expression relative to untreated Treg, combination of the two agents did not produce a combined effect. In fact T-bet expression was the highest in the Tregs treated with IL-12 alone.

These results are interesting in that they indicate that simulating an inflammatory environment using CL097, LPS and IL-12 alone or in combination maintain and upregulate even more expression of Treg markers, but at the same time also promote the Th1 transcription factor T-bet such that it is uncertain whether these agents are reinforcing a Treg phenotype or perhaps beginning to induce a flip to a Th1 phenotype. To check whether the population of cells that upregulated T-bet also maintained FOXP3 expression or lost it, the percentage of cells that were FOXP3+T-bet+ in the untreated and treated Tregs were compared and this is shown in below.

4.4.1.3 Treated Treg That Upregulated T-bet Retained FOXP3 Expression

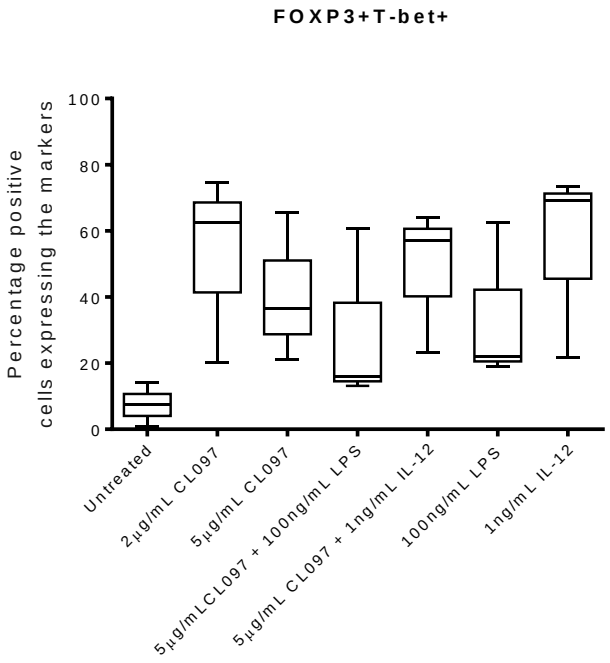


Figure 4.31: Box and whisker plot showing the percentage of FOXP3+T-bet+ cells of untreated Treg or after exposure of Treg to CL097 alone or in combination with LPS or IL-12. Data was collected 6 days post-activation and treatment. Data are presented as median percentage of cells with 75th and 25th quartiles. Differences between treatments were tested using Kruskal-Wallis non-parametric test with pairwise comparison. No statistical significant differences were observed.

Figure 4.31 shows that in the untreated Treg, only a very small percentage of cells were FOXP3+T-bet+ while the percentage of cells with this phenotype increased markedly in the treated cells. The results indicate that treating Tregs with CL097 alone or in combination with IL-12 (and to a lesser extent with LPS) resulted in the upregulation of T-bet expression in FOXP3+ cells. The same effect was achieved when the Treg were exposed to IL-12 and, to a lesser extent LPS, in the absence of CL097. This shows that simulating a pro-inflammatory environment through the addition of CL097, LPS and IL-12, induces the expression of T-bet in FOXP3+ cells. Although this at first seems rather surprising given that T-bet is the transcription factor for Th1 and FOXP3 for Treg, other studies have similarly observed upregulation of T-bet in the FOXP3+ T cells under Th1-inducing environments (these studies are further discussed in the discussion). They also observed however that, rather than rendering them more similar to Th1, cells with such phenotypes were rendered more immunosuppressive towards Th1.

To investigate whether this was also true in this study, immunosuppression assays were carried out to check whether the suppressive capabilities of treated Treg relative to untreated Treg was altered.

4.4.2 Proliferation Assays

To investigate whether the increased expression of FOXP3 and CD25, and the upregulation of T-bet in FOXP3+ cells altered the immunosuppressive capabilities of the treated Tregs, functional analysis was carried out by coculturing Treg and CD4+CD25- Tresp in the presence of the same agents and compared to untreated cocultures.

The Tresp cells and Treg cells were stained with CFSE and eFluor670 proliferation dyes respectively and activated separately using anti-CD3/CD28 in the presence of IL-2. The Treg cells were treated on the same day of activation and 2 days later, co-cultures of 1:1 Tresp to Treg cells were prepared. Untreated monocultures of Treg and Tresp as well as Tresp monocultured in the presence of CL097 were also prepared for comparison purposes. A positive suppressive control of Tresp cultured in the presence of 300ng/ml rapamycin was also prepared. The cells were cultured for a further 7 days post-treatment (and 5 days post-coculturing) and cell proliferation analysis of both Tresp and Treg was carried out by monitoring the dilution of the two proliferation dyes. The greater the dilution of the dye the greater the number of cell divisions and hence proliferation. The same instrument settings during acquisition and the same gates during analysis were used for the same set of biological replicates.

4.4.2.1 CL097 Enhances Treg Function Despite Upregulation of T-bet

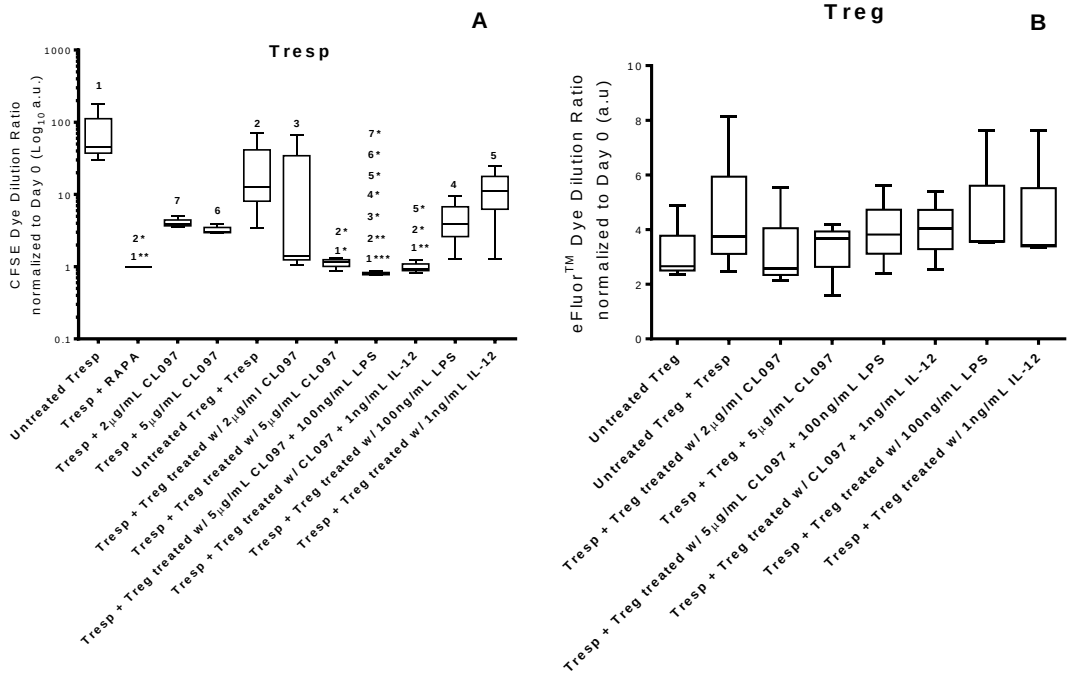


Figure 4.32: Box and whisker plot showing the (A) the CFSE and (B) eFluor™ dye dilution under different treatment conditions of CL097 in the absence or presence of LPS or IL-12. The fold dye dilution represents the extent of Tresp proliferation in (A) and Treg proliferation in (B). Data was collected 7 days post-activation and treatment. Units are arbitrary and represent the ratio of the MFI of inactivated Tresp cells (Day 0) to the MFI of the activated Tresp in the mono/co-cultures 7 days post-activation. Data is presented as median CFSE/eFluor™ dye dilution with 75th and 25th percentiles (n = 3 from different blood donors). Difference between groups was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons. * $p \leq 0.05$, ** $p \leq 0.005$ *** $p \leq 0.0005$

It is evident that none of the treatment caused a reduction in Treg function when Tresp and Treg were co-cultured at a ratio of 1:1. In fact, most treatments involving CL097 actually enhanced suppression of Tresp. Figure 4.32 (plot A) above shows that the CFSE dye dilution, as expected, was low in Tresp treated with the immunosuppressive drug rapamycin, and highest in the Tresp cultured in the absence of Treg as no suppression was being exerted on their proliferation. However, the same graph also shows that the

dye fold dilution was very low and comparable to that of rapamycin-treated Tresp, in the co-culture treated with the higher concentration of CL097 alone or in combination with LPS or IL-12. The differences in the dye dilution between the untreated co-culture and all co-cultures treated with 5µg/ml CL097 were significant ($0.003 \leq p \leq 0.043$). The dye dilution was observed to rise again (to levels comparable to those of the untreated coculture) when treated with LPS or IL-12 in the absence of CL097. Differences between co-cultures treated with CL097 combined to LPS and LPS only were statistically significant ($p = 0.027$) as were those between cocultures treated with CL097 combined with IL-12 and IL-12 only ($p = 0.047$). However, no synergistic effects were observed.

CL097 also seems to act directly on Tresp monocultures by reducing their proliferation, although differences were not significant from those of the untreated Tresp ($p > 0.05$).

These results therefore indicate that proliferation of Tresp cells was significantly reduced and Treg function reinforced in the presence of CL097 at a concentration of 5µg/ml both in the absence and presence of LPS or IL-12. The fact that Tresp suppression when the co-cultures were treated with LPS or IL-12 alone remained unchanged relative to the untreated co-culture, shows that it was the action of CL097 which was somehow enhancing it. These results thus indicate that CL097 might be directly reducing Tresp proliferation but, even more evidently, augmenting further the suppressor function of treated Treg. This observation might be possibly tied to the upregulation of T-bet in FOXP3+ cells which, as described earlier, might have rendered the cells more potently immunosuppressive towards Th1.

To investigate whether CL097 enhanced Treg proliferation, the Tregs were stained with a different proliferation dye, eFluor™, and their proliferation also monitored by flow cytometry. The results are shown in Figure 4.32 (plot B). This plot shows that there were no significant differences in the eFluor™ dye dilution between the different cultures ($p > 0.05$ in all cases). This therefore indicates that CL097 does not particularly affect the proliferation of Treg and thus the reduction in Tresp proliferation observed in CL097-treated co-cultures was not due to a direct increase in Treg cell numbers. It suggests that CL097 enhanced the Treg's suppressor function and the upregulation of FOXP3, CD25 and possibly also that of T-bet, might be amongst the contributing factors. In particular, elevated expression of CD25, which acts as an IL-2 receptor, increases the ability of Treg to sequestrate IL-2 which is also required by Tresp to proliferate. Coupled to this, the upregulation of T-bet in the FOXP3+ cells might be increasing the Treg's ability to suppress the Th1 subset of responder cells, as other studies have previously shown. Nonetheless, the increase in FOXP3 and CD25 expression and the upregulation of T-bet in Tregs was also observed when Tregs were treated with LPS and IL-12 alone, which means that there must be some other additional mechanism by which CL097 at concentrations of 5μg/ml is enhancing Treg-mediated immunosuppression.

4.4.2.2 The Majority of Responder T Cells in CL097-treated Co-Cultures Do Not Undergo Cell Division

Including the division and proliferation indices provides more information on the patterns of proliferation observed under different treatments.

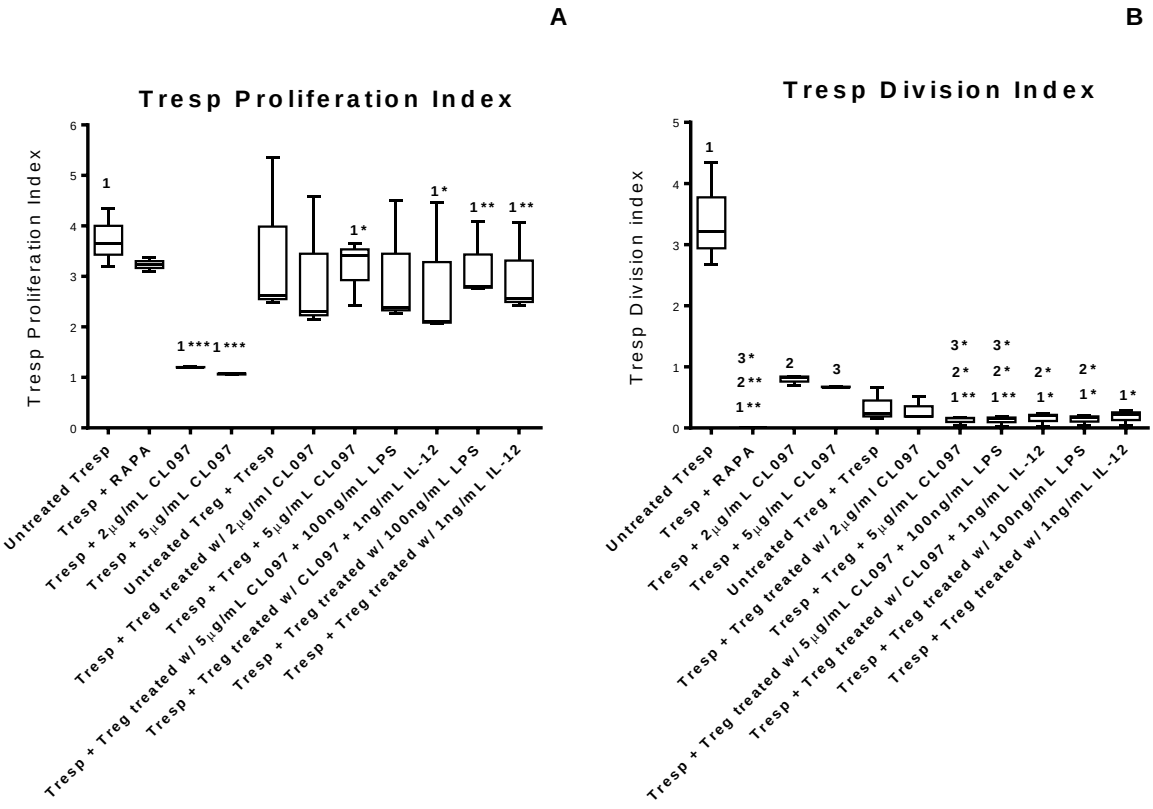


Figure 4.33: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of Tresp in the absence or presence of Treg untreated to exposed to different treatment conditions of CL097 in the absence or presence of LPS or IL-12. Data was collected 7 days post-activation and treatment. Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data is represented as median Proliferation/Division Index with 75th and 25th percentiles (n = 3 from different blood donors). Difference between groups from (A) was tested using one-way ANOVA with Tukey's test while for (B) differences were tested using the Kruskal-Wallis non-parametric test with pairwise comparisons. No statistical significance was observed for (A) * $p \leq 0.05$, ** $p \leq 0.005$

The proliferation index reflects how responding cells (i.e. cells that are dividing) are affected by the different treatments. Figure 4.33 (Plot A) shows that when the Tresp monocultures were treated with CL097 in the absence of Treg, the proliferation index decreased by fourfold relative to the untreated Tresp monoculture showing that CL097 directly reduced the number of cell divisions (from a median of almost 4 to 1 cell divisions) dividing cells underwent. On the other hand, the proliferation index in the co-

cultures, whether treated or untreated, remained more or less the same. Therefore, treatment did not affect the number of cell divisions dividing cells in the co-culture underwent (remaining with a median of approximately 2 cell divisions). This means that once a cell started to divide, treatment did not affect the amounts of cell division (relative to the untreated co-culture).

The division indices (plot B) of the different co-cultures were however extremely low showing that, when considering the entire population of Tresp cells, only a few actually divided. In fact, the average number of cell divisions that the entire population in the co-cultures underwent was less than one meaning that the majority of the Tresp cells did not divide at all. It means that most of the Tresp cells in the co-culture did not even undergo 1 cell division (as shown by the division) whilst the small number of cells that did undergo cell division underwent an average of 2 to 3 divisions (as shown by the proliferation index). The division indices recorded in the co-cultures were significantly lower than those of the Tresp monocultures ($0.004 \leq p \leq 0.041$). Although treating the Tresp monocultures with CL097 reduced the average number of cell divisions by approximately three-fold, the majority of Tresp still managed to undergo one cell division and the degree of suppression was therefore not as profound as in the co-culture.

4.4.2.3 CL097 Has No Effect on Treg Proliferation and Division Indices

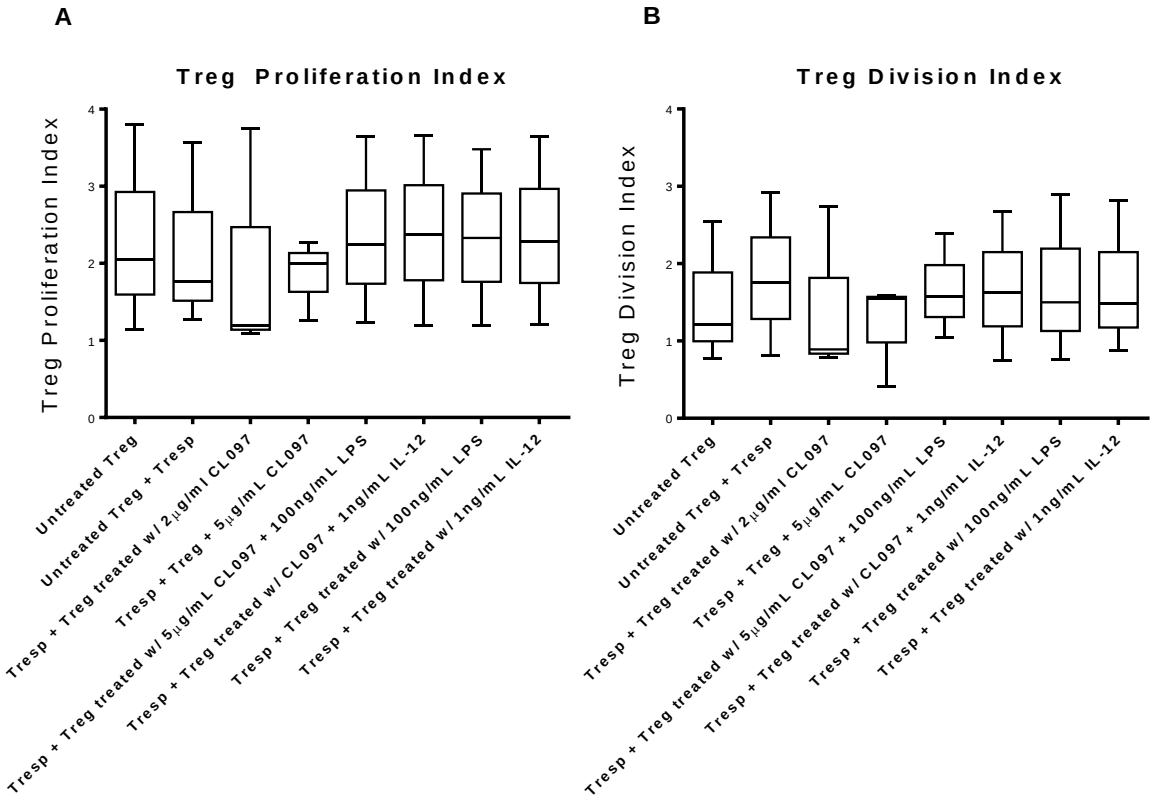


Figure 4.34: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of Treg in the absence and presence of Tresp, untreated or treated with CL097 alone or in combination with LPS or IL-12. Data was collected 7 days post-activation and treatment. Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data is presented as median Proliferation/Division Index with 75th and 25th percentiles (n = 3 from different blood donors). Difference between groups was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons. No statistical significant differences were observed.

Figure 4.34 (Plot A) shows that the median Treg proliferation indices in the co-cultures treated with 5µg/ml CL097 relative to the untreated co-culture rises only slightly and the differences are not statistically significant ($p > 0.05$). This means that the agents do not as such affect the average number of cell divisions dividing Treg are undergoing. The agents do not effect either the number of cell divisions the entire population is

undergoing i.e. the division indices (Plot B). These results are in agreement with the conclusions drawn out when Treg proliferation was analysed using the dye dilution method. Both methods suggest that treating Treg with the CL097, LPS and IL-12 (alone or in combination) do not affect Treg proliferation or the number of cell divisions they undergo.

4.4.3 Cytokine Analysis

In an attempt to try and understand which soluble factors were responsible for the observations made, cytokine analysis was carried out.

4.4.3.1 CL097 with IL-12 Promotes the Production of IL-10 and TGF-β

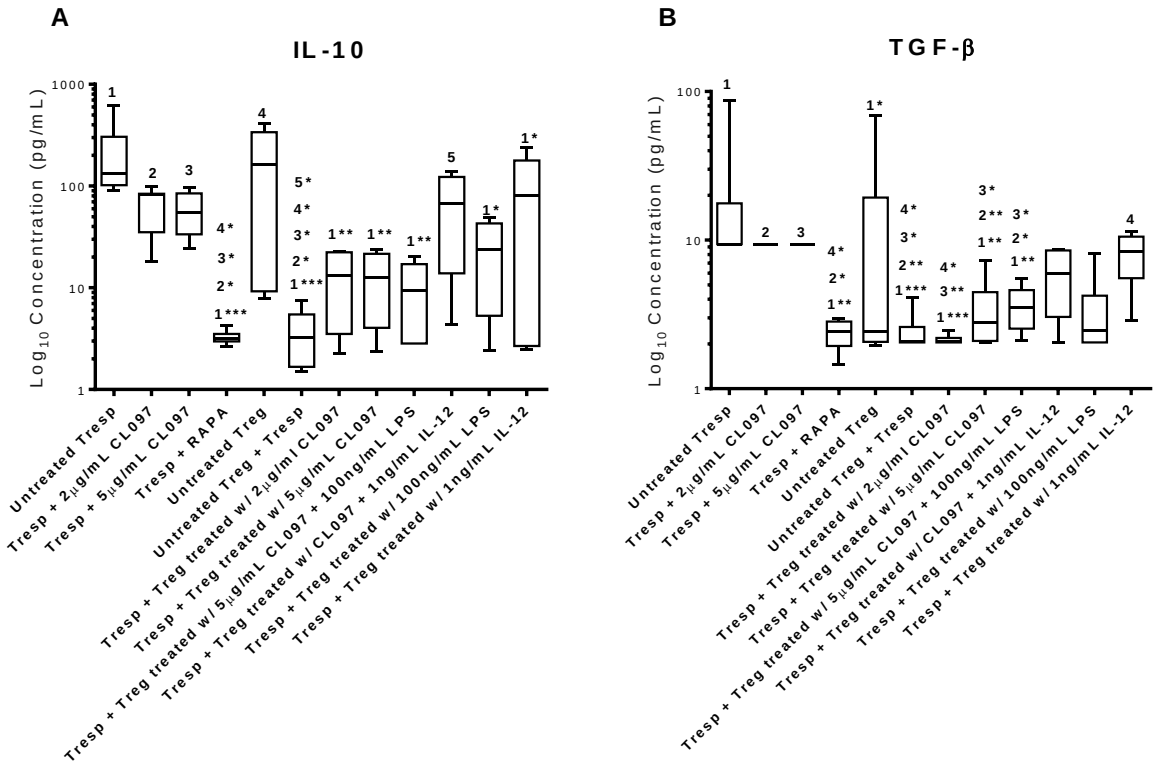


Figure 4.35 : Box and Whisker plots showing, in logarithmic scale, concentrations in pg/ml of (A) IL-10 and (B) TGF-β obtained from cell supernatants exposed to different treatment conditions of CL097 in the absence or presence of LPS or IL-12. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p≤ 0.05, ** p≤ 0.005, *** p≤0.0005

Figure 4.35 (plot A) shows that IL-10 was highly produced by Tresp monocultures and Treg monocultures. Treatment with CL097 does not seem to significantly alter production of this cytokine in Tresp monocultures relative to the untreated monoculture. The levels of IL-10 detected in most of the Tresp and Treg co-cultures were significantly lower than those of the Tresp monocultures ($p \leq 0.043$). The lowest levels of the cytokine were detected in the untreated Tresp and Treg co-culture and the levels of the cytokine

increased only slightly when Treg were treated with CL097 alone or in combination with LPS. However, in the presence of IL-12, IL-10 production increased and this effect was observed both when IL-12 was combined to CL097 and when added on its own. A significant difference was observed between the untreated co-culture and the one treated with 5µg/ml CL097 combined to IL-12 ($p = 0.047$). IL-10 is known to downregulate IL-12 (Ozato, Tsujimura, & Tamura, 2002) and therefore the exogenous addition of IL-12 might have been promoting the release of IL-10 as an opposing mechanism to counteract the inflammatory signal triggered by IL-12.

Similar patterns of cytokine production were observed for TGF-β (plot B) in that Tresp monocultures (both untreated and CL097-treated) produced significantly higher amounts of the cytokine than Treg monoculture and Tresp and Treg co-cultures ($p \leq 0.028$). Treatment with CL097 did not affect production levels of this cytokine in the Tresp monocultures. Lower levels of the cytokine were observed in the untreated Tresp and Treg co-cultures and yet again, upon treatment with IL-12 (alone or combined with CL097), production of the cytokine increased in a similar way as that observed with IL-10. A significant difference was observed in the cytokine levels produced by the co-culture treated with IL-12 alone and the untreated coculture ($p = 0.031$) as well as coculture treated with 2µg/ml CL097 ($p = 0.007$).

These results therefore indicate that CL097 at a concentration of 5µg/ml coupled to IL-12 increases production of IL-10 and TGF-β. Given that this effect was also seen when treatment involved the addition of IL-12 only, it is likely that IL-12 was the main contributor to this effect.

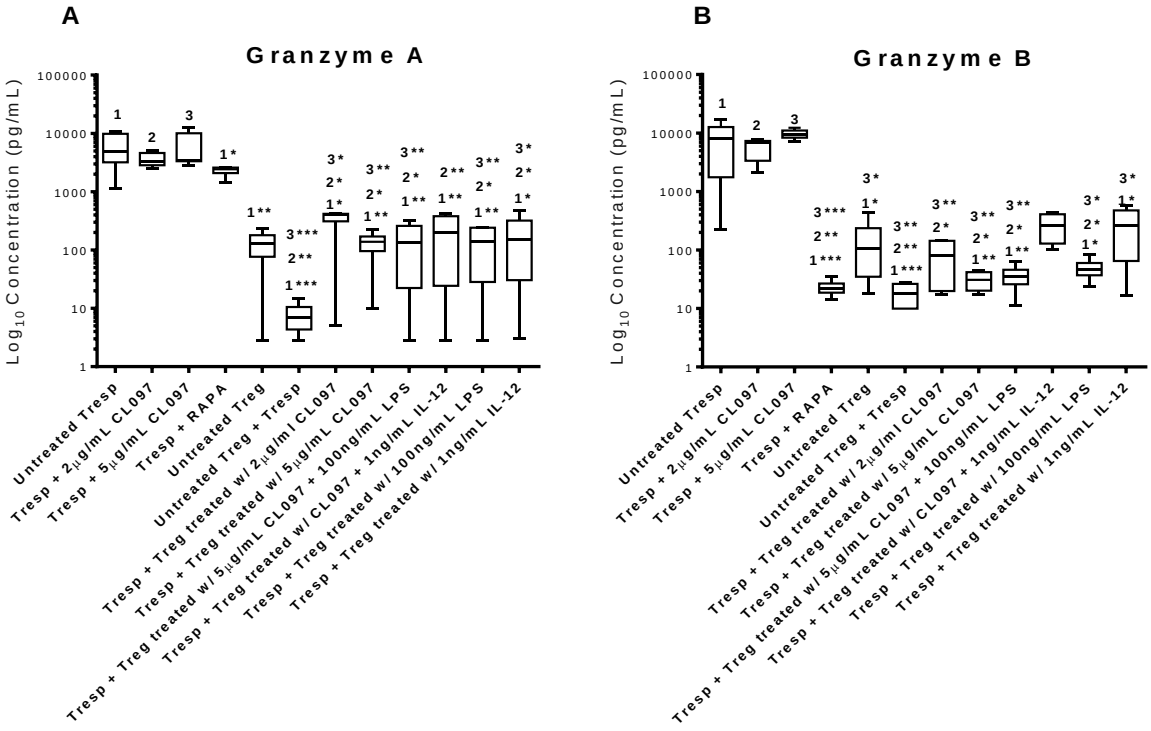


Figure 4.36 : Box and Whisker plots showing, in logarithmic scale, concentrations in pg/ml of (A) Granzyme A and (B) Granzyme B obtained from cell supernatants exposed to different treatment conditions of CL097 in the absence or presence of LPS or IL-12. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p≤ 0.05, ** p ≤0.005, *** p≤0.0005

Same as with IL-10 and TGF-β, Figure 4.36 shows that the highest granzymes production was in the Tresp monoculture and the concentrations were statistically significantly higher than those of the Treg monocultures, as well as the Tresp and Treg co-cultures. In the Tresp monocultures, CL097 did not seem to alter the production of both enzymes relative to their corresponding untreated control. The levels did however rise slightly in the treated co-cultures although the differences are not statistically significant from those of the untreated co-culture ($p > 0.05$).

4.4.3.2 CL097 with IL-12 Promotes the Secretion of TNF-α and IFN-γ

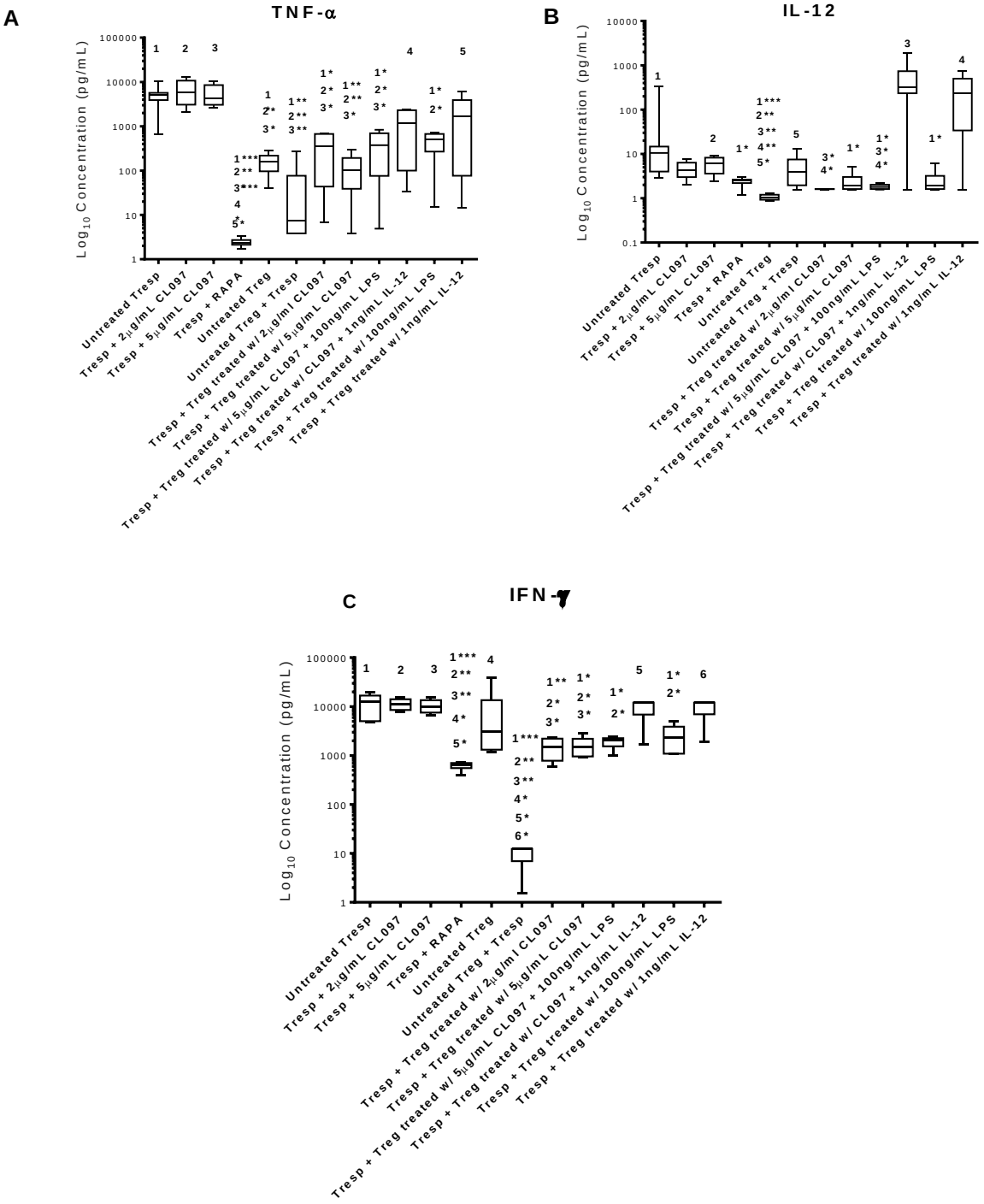


Figure 4.37 : Box and Whisker plots showing, in logarithmic scale, the concentrations in pg/ml of (A) TNF-α, (B) IL-12 and (C) IFN-γ obtained from cell supernatants exposed to different treatment conditions of CL097 in the absence or presence of LPS or IL-12. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p≤ 0.05, ** p≤ 0.005, *** p≤0.0005

Figure 4.37 (plot A) shows that, as expected, Tresp monocultures produce TNF-α in high amounts. Again, addition of CL097 did not seem to affect the production of the cytokine. Interestingly, Treg monocultures also produced TNF-α in the absence of Tresp, despite it being pro-inflammatory (TNF-α- producing Treg have been described in other studies), however levels go back down when the Treg and Tresp are co-cultured together. When CL097 is added to the co-cultures, the TNF-α levels started increasing again, especially in the co-cultures treated with 5µg/ml CL097 and IL-12 as well as when treated with IL-12 alone. Although no significant differences were observed between treated co-cultures and the untreated ones ($p > 0.05$), a significant difference was observed between the co-cultures treated with 5µg/ml CL097 and IL-12 and the positive suppressive control, where TNF-α production by the Tresp was being inhibited by the rapamycin ($p = 0.040$). This control however was not different from the untreated co-culture. The same also holds for the co-culture treated with IL-12 alone in that it was different from the Tresp treated with rapamycin ($p = 0.021$).

These results therefore indicate that simulating an inflammatory environment through the addition of CL097, LPS and IL-12 (alone or in combination) upregulates the production of the Th1-associated cytokine TNF-α in Tresp and Treg co-cultures. The greatest effect was seen when IL-12 is included in the treatment, however no synergistic effect was observed when combining with CL097. Indeed, other studies have reported that treating other immune cells (such as macrophages) with imidazoquinolines (the group of compounds to which CL097 belongs) activates TNF-α (Hemmi *et al.*, 2002). This might also be true in CD4+ T cells. It is not clear whether the "extra" TNF-α was being produced by the Treg or by the Tresp. However since treating Tresp monocultures with CL097 did not alter production of the cytokine, it is probable that it was being released by

the treated Treg. Alternatively, it might have been produced by the Tresp themselves in response to the higher suppressor action exerted by the treated Treg in the co-culture. Nonetheless, if the latter hypothesis were the case, the increase in the production of TNF-α by Tresp was not enough to overcome suppression by Treg. Indeed, some studies have suggested that TNF-α released by activated Tresp activates and enhances the suppressive function of Treg cells to help limit the collateral damage caused by excessive immune activation (X. Chen & Oppenheim, 2010; X. Chen et al., 2008)

Figure 4.37 (plot B) show that the levels of IL-12 did not change much in the different co-cultures where IL-12 had not been added exogenously. Treatment with CL097 did not significantly alter the production of the cytokine in the Tresp monocultures either. As expected, low levels of the cytokine were detected in the Treg monocultures and the levels were statistically lower from those of the Tresp monocultures ($p \leq 0.04$ in such cases). CL097 or LPS do not seem to alter production of the cytokine in the Tresp and Treg co-cultures when compared to the untreated co-culture. The co-cultures were IL-12 production was highest was in those to which IL-12 added. The exogenous addition of the cytokine might have further prompted the release of more IL-12 by the cells in the co-culture as other studies have shown that IL-12 upregulates its own production (Hoffman, Benz, Silberstein *et al.*, 2013).

Figure 4.37 (plot C) show that the levels of the Th-1 effector cytokine, IFN-γ were highest in the Tresp monocultures and also in the Treg monocultures. Treg that produce IFN-γ were already observed in the cell-free DNA experiments. Adding CL097 to the Tresp monocultures did not alter the cytokine levels relative to the untreated control. Levels of the cytokine in the untreated co-cultures are significantly lower than those of the Tresp monocultures and although co-cultures treated with CL097 increase their production of

IFN-γ, the concentrations are still statistically lower than those of the Tresp monocultures ($p \leq 0.05$). The only co-cultures where IFN-γ was high and not statistically different from those of the Tresp monocultures were those treated with IL-12 (alone or in combination with CL097) and in fact the concentrations recorded were similar to those observed in the Tresp monocultures. Therefore here one observes again an increase in the production of yet another pro-inflammatory cytokine in co-cultures triggered by CL097 and, further enhanced by the exogenous addition of IL-12.

4.4.3.3 IL-4 and IL-17 Production Are Not Affected By CL097

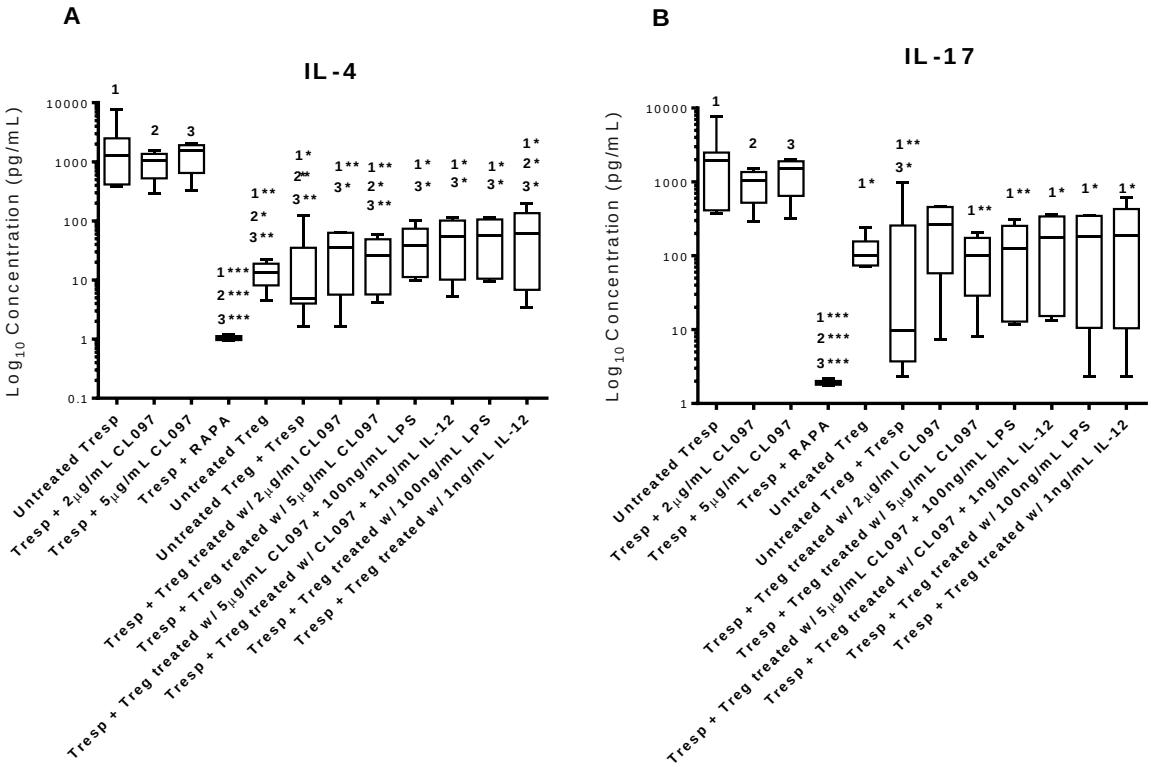


Figure 4.38 : Box and Whisker plots showing, in logarithmic scale, the concentrations in pg/ml of (A) IL-4 and (B) IL-17 obtained from cell supernatants exposed to different treatment conditions of CL097. Data was collected 7 days post-activation and treatment. Data are presented as median concentrations with 75th and 25th quartiles (n ≥ 3). Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * p≤ 0.05, ** p≤ 0.005, *** p≤0.0005

Figure 4.38 plot A in shows that the production of the Th2 differentiation cytokine, IL-4, was highest in the Tresp monocultures and treating these cells with CL097 does not affect production of this cytokine. The concentrations of the cytokine in the co-cultures were significantly lower than those of the Tresp monocultures ($0.001 \leq p \leq 0.046$) and treatment with any of the agents raised the concentration of the cytokine only minimally relative to the untreated and none of the differences were statistically significant (with $p \geq 0.05$ in all cases). Therefore, CL097, LPS or IL-12 do not seem to alter the production of IL-4, and, unlike the effect of endogenous RNA, does not promote Th2 differentiation.

Plot B in Figure 4.38 shows that IL-17, which is an effector cytokine for Th17 was the highest in the Tresp monocultures and the concentrations recorded in the untreated Tresp monoculture were statistically higher from those of most Treg and Tresp co-cultures ($0.03 \leq p \leq 0.045$). Addition of CL097 did not alter cytokine production in the Tresp monoculture or in the Tresp and Treg co-cultures. Treatment with LPS and IL-12 alone did not seem to effect IL-17 production either. Therefore, none of the agents seemed to effect the production of the cytokine associated with Th17-effector responses.

Summing up the results of the cytokine assay, it seems that CL097 supplemented with IL-12 (as well as IL-12 alone) might be promoting the release of anti-inflammatory cytokines such as IL-10 and TGF-β implied in Treg function. These might contribute (coupled to the phenotypic changes also observed) to the enhanced Treg suppressor function in the treated co-cultures. The effects exerted by CL097 alone are weaker than when combined to IL-12.

There are however also some preliminary indications that CL097 co-promotes the production of pro-inflammatory cytokines associated with Th1, primarily TNF-α and IFN-γ. Again, this effect is at its strongest when exogenous IL-12 was added. Therefore, coupling IL-12 to a TLR7/8 ligand might indeed augmenting -Th1 proinflammatory cytokine production. However, functional analysis has also demonstrated that this was not sufficient to abrogate Treg-mediated immunosuppression of the Tresp when cultured in equivalent ratios. None of the agents seem to affect the production of Th2 and Th17-associated i.e. IL-4 and IL-17 respectively.

4.5 Analysing the Effects of Synthetic TLR7/8 Ligand Single Stranded Poly-Uridine

This section of results summarizes the results achieved when the second synthetic TLR7/8 ligand was tested. This ligand is single-stranded Poly-Uridine (ssPolyU) which is a synthetic oligonucleotide containing only uridine nucleosides. The occurrence of many uridine nucleosides also present in viral RNA. The ligand was purchased complexed with the cationic lipid LyoVec^{TM} from InvivoGen in order to protect it from degradation by nucleases and facilitate its uptake by cells.

4.5.1 Phenotype Analysis

The same procedure used for Treg activation and treatment when testing the effects of CL097 was used in that the Treg cells were activated using anti-CD3/CD28 and IL-2 and the agents added on the day of activation. The cells were cultured for a total of 6 days and flow cytometric analysis was carried out to compare phenotypic changes between untreated and treated Tregs using the same combination of markers that characterise different CD4+ T cell populations. The two concentrations of ssPolyU tested were 1µg/ml and 10µg/ml as, according to manufacturer's instructions, stimulation of TLR8 by human cells are achieved within this range of concentrations. It was also of interest to compare results with those achieved for endogenous RNA, hence also why the same concentrations were used. As with CL097, the effect of coupling the TLR8 ligand with LPS and IL-12 was also investigated. The same instrument settings were used during data acquisition and the same gates were used during data analysis for the same set of biological replicates.

4.5.1.1 Single Stranded PolyU Reduces The Percentage of FOXP3+CD25+ Cells Only When Coupled to LPS and IL-12 in One Treatment

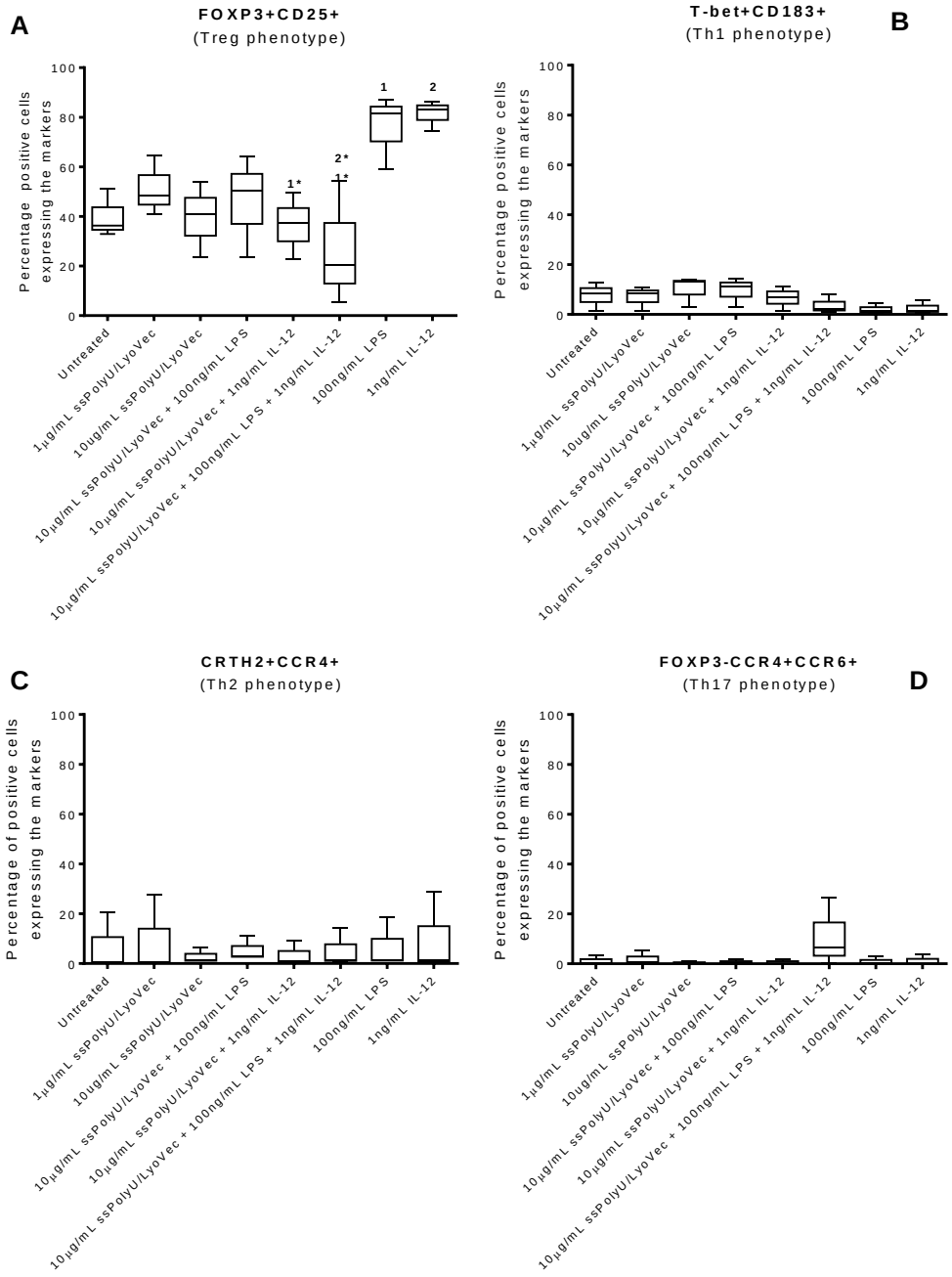


Figure 4.39: Box and whisker plots showing the effects of ssPolyU in the absence or presence of LPS and/or IL-12 on the phenotype of Treg monocultures 6 days post-activation and treatment. Data is represented as median percentage positive cells with 75th and 25th quartiles (*n* = 3 from different blood donors). Differences between treatments was tested using one-way ANOVA with Tukey's test. No statistical significant differences were observed for (B), (C) and (D).

Figure 4.39 (Plot A) shows that treating cells with ssPolyU (alone or in combination with either LPS or/and IL-12) did not significantly alter the percentage of cells that were FOXP3+CD25+ relative to untreated Treg. Same as observed in the CL097 set of experiments, the percentage of cells with this phenotype was raised to twice as much the untreated control when the cells were treated with either LPS or IL-12 alone. This means that Treg phenotype is more enhanced in the sole presence of LPS or IL-12 than when these are combined with ssPolyU. Interestingly, however when combining all three reagents together, a slight opposite effect was observed in that the percentage of cells with this phenotype was lowered to half the amount of that in the untreated co-culture (difference not statistically significant) and to quarter the amounts of that in the co-cultures treated with LPS alone or IL-12 alone. The latter two were statistically different from the co-cultures treated with ssPolyU + IL-12 and co-culture treated with ssPolyU + LPS + IL-12 ($p \leq 0.05$ in such cases). Contrary to endogenous RNA and CL097 (whereby percentage of FOXP3+CD25+ cells are reduced and increased respectively), ssPolyU alone does not induce phenotypic changes in Treg unless combined with LPS and IL-12.

No significant changes in the percentage of cells that were CD183+Tbet+, CRTH2+CCR4+ or FOXP3+CCR4+CCR6 (Plots B, C and D respectively) were observed ($p > 0.05$).

4.5.1.2 The Level of Expression of FOXP3 and CD25 Is Not Altered When ssPolyU is Combined to LPS and IL-12

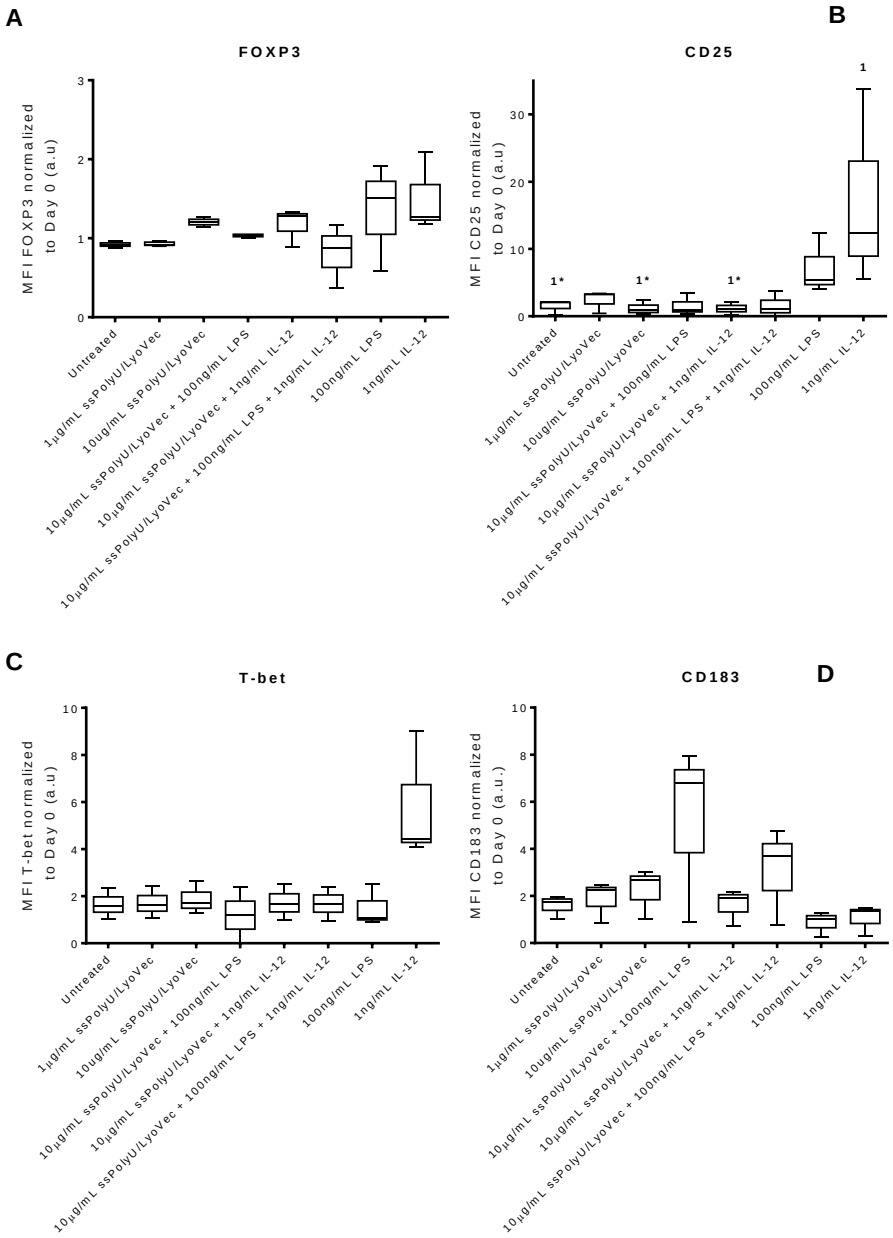


Figure 4.40: Box and Whisker plots showing Median Fluorescent Intensity (MFI) of (A) FOXP3; (B) CD25; (C)T-bet and (D) CD183 cells after treating with ssPolyU in the absence or presence of LPS and/or IL-12. Units are arbitrary and have been normalized by dividing the MFI on 6 days post-treatment by that of Day 0. Data is represented as median MFI with 75th and 25th percentiles. Difference in MFIs between treatments was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons and difference in MFI for (B) between treatments was tested using one-way ANOVA with Tukey's test. No statistical significance was found between groups in (A), (C) and (D) * p≤ 0.05, ** p≤0.005

Figure 4.40 shows that LPS and IL-12 increased the expression of both FOXP3 and CD25 (Plots A and B) in Treg (as similarly observed in the CL097 set of results). Expression of CD25 is statistically higher in these Treg than in those untreated and those treated with 10μg/ml ssPolyU in the absence or presence of IL-12 ($0.045 \leq p \leq 0.050$*).* Therefore, ssPolyU (alone or combination with LPS and/or IL-12) does not alter expression of FOXP3 and CD25 relative to the untreated control. This means that treating Treg with ssPolyU, LPS and IL-12 in one treatment decreases the percentage number of cells with a FOXP3+CD25+ phenotype but the level of expression of the markers (represented by the MFI) in the cells that positively express them remained unchanged.

Unlike CL097, ssPolyU does not alter T-bet expression (plot C) in the treated Treg and the only Tregs that upregulated the Th1-transcription factor were the ones treated with IL-12 alone (as observed in the CL097 set of experiments).

What is worth noting is that the expression of CD183 increased when the ssPolyU was coupled to LPS both in the absence and presence of IL-12 (plot D). This effect was not observed when the Treg were treated with either ssPolyU or with LPS alone which means that it was the combined effect of ssPolyU and LPS that was inducing this upregulation. Despite median levels increased to almost four times as much, the differences were not statistically significant with those of the untreated Treg ($p > 0.05$).

It has already been implied that CD183 is a marker for Th1, but this marker is not exclusive to Th1 and in fact it is also expressed in some Treg. Therefore, it was essential to check whether the T cells that upregulated CD183 expression still maintained FOXP3.

4.5.1.3 Combining ssPolyU with LPS Increase the Percentage of Cells with a FOXP3+CD183+ Phenotype

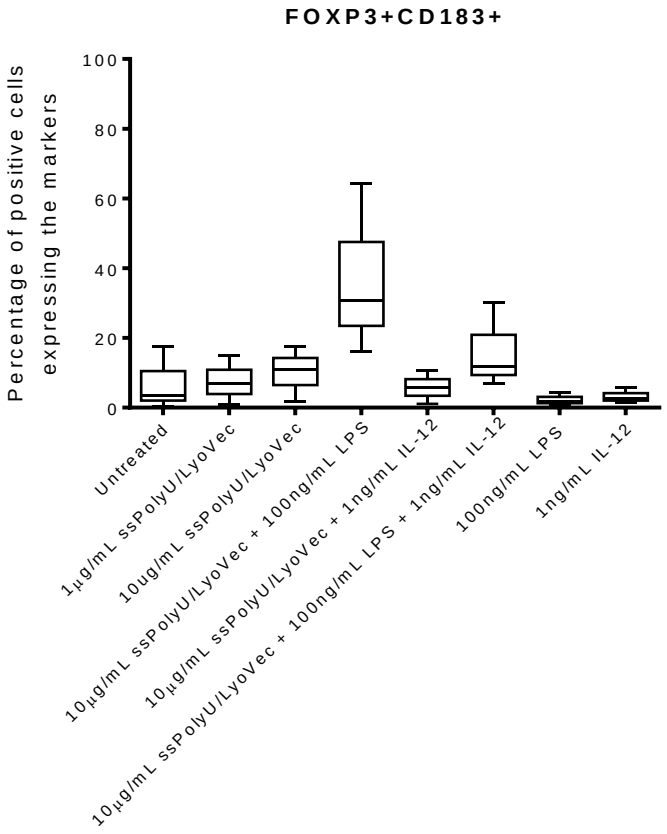


Figure 4.41: Box and whisker plot showing the percentage of cells that were FOXP3+CD183+ under different treatment conditions of ssPolyU in the absence or presence of LPS and/or IL-12. Results were collected 6 days post-activation and treatment. Data are presented as median percentage of cells with 75th and 25th quartiles. Differences between treatments were tested using Kruskal-Wallis non-parametric test with pairwise comparison. No statistical significant differences were observed.

Figure 4.41 shows that the Treg that upregulated CD183 expression (i.e. those treated with ssPolyU + LPS and those treated with ssPolyU + LPS + IL-12) retained FOXP3 expression. This indicates that these cells were expected to remain suppressive in nature.

4.5.2 Proliferation Assays

Despite no change in the phenotype, the effect of ssPolyU on the suppressor function of Treg on Tresp was still investigated. This is because differences in effector function do not always necessarily induce a phenotypic change. Functional assays were conducted by monitoring the proliferation of both Tresp and Treg in their co-cultures.

The same procedure as for CL097 was followed in that the Tresp and Treg were first stained separately with CFSE and eFluor™ respectively, were then activated using anti-CD3/CD28 and IL-2, and treatment agents added on the same day to the Treg. Two days post-activation and treatment, the treated Treg were added to the Tresp. The cells were cultured for a further 5 days and flow cytometric analysis was carried out to compare the proliferation of Tresp as well as that of Treg.

4.5.2.1 Single Stranded PolyU at 1µg/ml but not at 10µg/ml Reduces Treg Numbers and their Suppressor Function

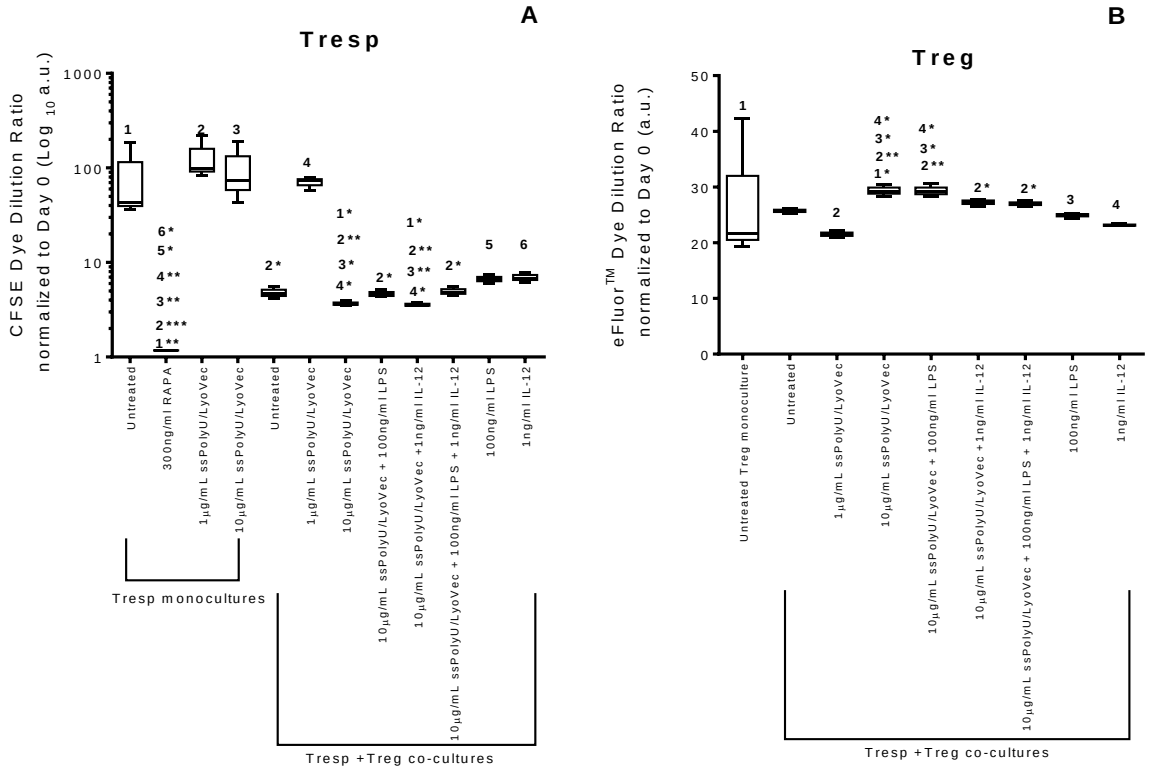


Figure 4.42: Box and whisker plot showing the (A) the CFSE and (B) eFluor™ dye dilution under different treatment conditions of ssPolyU in the absence and presence of LPS and/or IL-12. The dye dilution represents the extent of Tresp proliferation in (A) and Treg proliferation in (B). Data was collected 7 days post-activation and treatment. Units are arbitrary and is the ratio of the MFI of inactivated Tresp cells to the MFI of the activated Tresp in the mono/co-cultures 7 days post-activation. Data is presented as median CFSE/eFluor™ dye dilution with 75th and 25th percentiles (n = 3 from different blood donors). Difference between groups was tested using the Kruskal-Wallis non-parametric test with pairwise comparisons. * $p \leq 0.05$, ** $p \leq 0.005$ *** $p \leq 0.0005$

Figure 4.42 (plot A) shows that treating Tresp monocultures with ssPolyU did not affect proliferation of Tresp. Interestingly, when in turn the Treg were treated with the ligand at a concentration of 1µg/ml and then added to the Tresp two days later, the proliferation of the Tresp was enhanced relative to the untreated co-culture despite the

presence of Treg. This means that treating the Treg at this concentration reduced their suppressive capabilities and the Tresp proliferated unhindered as they did in the Tresp monocultures.

However, treating the Treg with the higher concentration of ssPolyU (both in the absence and presence of LPS and/or IL-12) seems to abolish the effect as Tresp proliferation in co-cultures treated with 10µg/ml ssPolyU was as low as that of the untreated co-culture. Tresp proliferation in the co-cultures treated with 10µg/ml ssPolyU were statistically lower than that of the untreated and treated Tresp monocultures ($0.0005 < p < 0.05$) as well as than the co-culture where the Treg had been treated with 1µg/ml ssPolyU ($p = 0.009$). Treating the co-cultures with LPS or IL-12 and excluding ssPolyU improved only slightly Tresp proliferation relative to the untreated co-culture.

Therefore these results suggest that ssPolyU does not have a direct effect on Tresp proliferation. It is also indicated that at a concentration of 1µg/ml, ssPolyU promotes Tresp proliferation (by directly reducing Treg suppressor function), but at a higher concentration of 10µg/ml, the ligand exerted an inhibitory effect on Tresp by supporting Treg-mediated suppression.

Figure 4.42 (plot B) shows the effect of ssPolyU, LPS and IL-12 on the proliferation of Treg. It can be noted that treating Treg with 1µg/ml ssPolyU (and then adding them to Tresp) reduces Treg proliferation relative to all other cultures. Treg proliferation in co-cultures treated with 1µg/ml ssPolyU were statistically lower than all those treated with 10µg/ml ssPolyU ($0.0005 < p < 0.05$ in these cases). Therefore, Tresp proliferation was not suppressed in co-cultures treated with 1µg/ml possibly because suppressor Treg numbers were being reduced in this co-culture. Treg numbers then started to increase again (even

more than the untreated control) when treated with a concentration of 10µg/ml ssPolyU, restoring suppression of Tresp proliferation.

These results suggest that at low concentrations, ssPolyU promotes Tresp proliferation by suppressing the treated Treg. Treg function is however quickly restored and Tresp are once again suppressed when the Treg are treated with tenfold the concentration, possibly in response to the excessive signalling induced by high doses of the synthetic ligand.

4.5.2.2 1µg/ml Single Stranded PolyU Increases Tresp Proliferation Index

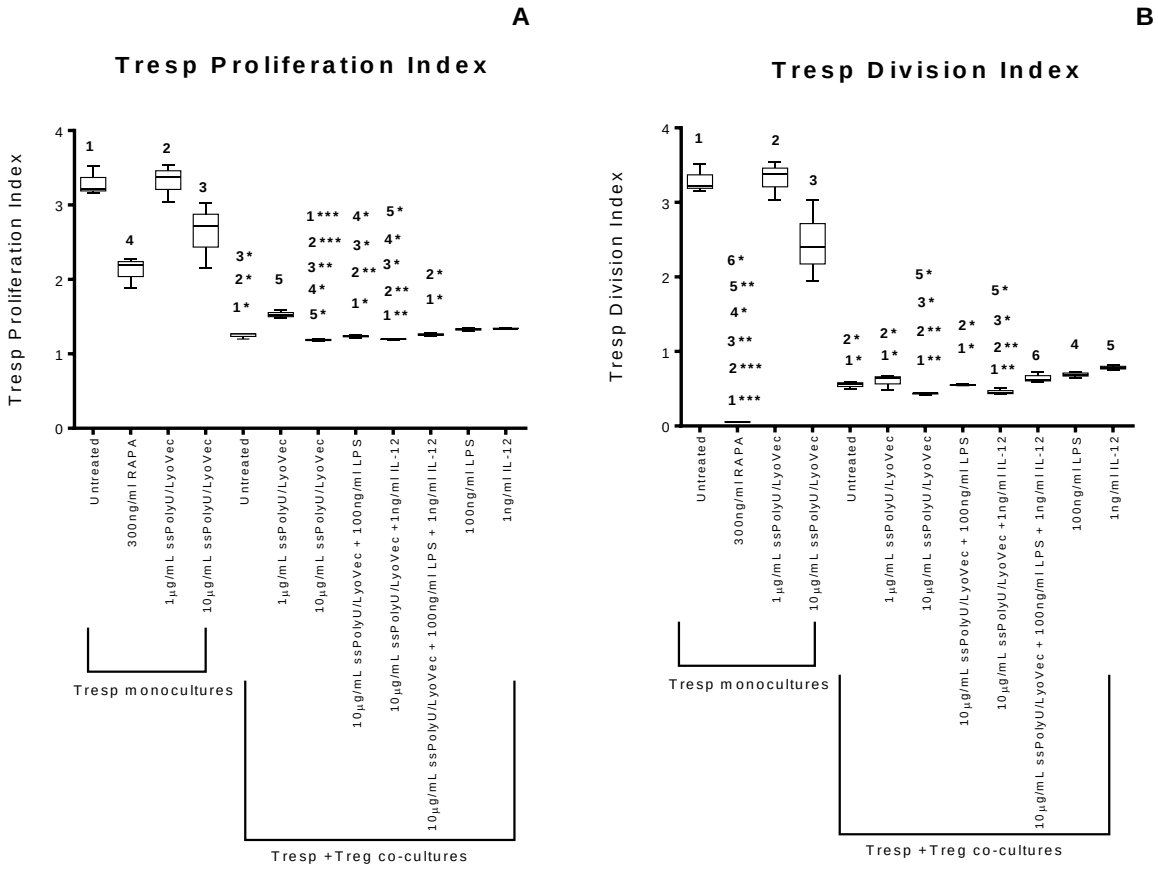


Figure 4.43: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of Tresp in the absence and presence of Treg untreated or treated with ssPolyU in the absence or presence of LPS and/or IL-12. Data was collected 7 days post-activation and treatment and 5 days in co-culture. Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data are presented as median Proliferation/Division Index with 75th and 25th percentiles (n = 3 from different blood donors). Difference between groups was tested using Kruskal Wallis non-parametric test. * p≤ 0.05, ** p≤ 0.005

Figure 4.43 (plot A) indicates that the Treg added to the Tresp are reducing the number of cell divisions (relative to the Tresp monocultures) that proliferating Tresp are undergoing (from a median of 3 to 1 cell divisions). However, treating the Treg in the co-cultures with 1µg/ml ssPolyU improves this number of cell divisions (by an average of 0.5) respective to the untreated co-culture and the co-cultures treated with 10µg/ml ssPolyU.

Significant differences are observed between the co-cultures treated with 1µg/ml ssPolyU and two out of four co-cultures treated with 10µg/ml ssPolyU (p = 0.001 both for Tresp + Treg treated with 10µg/ml ssPolyU as well as Tresp + Treg treated with 10µg/ml ssPolyU + 1ng/ml IL-12).

Figure 4.23 (plot B) on the other hand shows that there was no statistical differences in the division indices between co-cultures treated with 1µg/ml ssPolyU and the untreated co-cultures or co-cultures treated with 10µg/ml (p > 0.05*)*. This means that the low doses of ssPolyU only increased the number of cell divisions in proliferating cells (as shown by the proliferation index) whilst the average number of cell divisions the entire population underwent remained the same (as shown by the division indices).

It is evident that the enhanced Tresp proliferation in the co-culture treated with 1µg/ml ssPolyU (relative to the untreated co-culture) was more pronounced when proliferation data was analysed using the dye dilution method than when the data was analysed using the proliferation modelling available on FlowJo software. This variance can be attributed to the fact that the two techniques analyse the data differently. Both of the above mentioned techniques have their own associated advantages and limitations. One limitation of the dye dilution method is that some loss of fluorescence might not always be due to cell proliferation resulting in the overestimation of cell proliferation. On the other hand, the model used to generate proliferation and division indices might not always fit the acquired data perfectly. These limitations are acknowledged and this is why data was analysed using both methods.

4.5.2.3 1µg/ml ssPolyU Causes a Decrease in Treg Proliferation and Division Indices

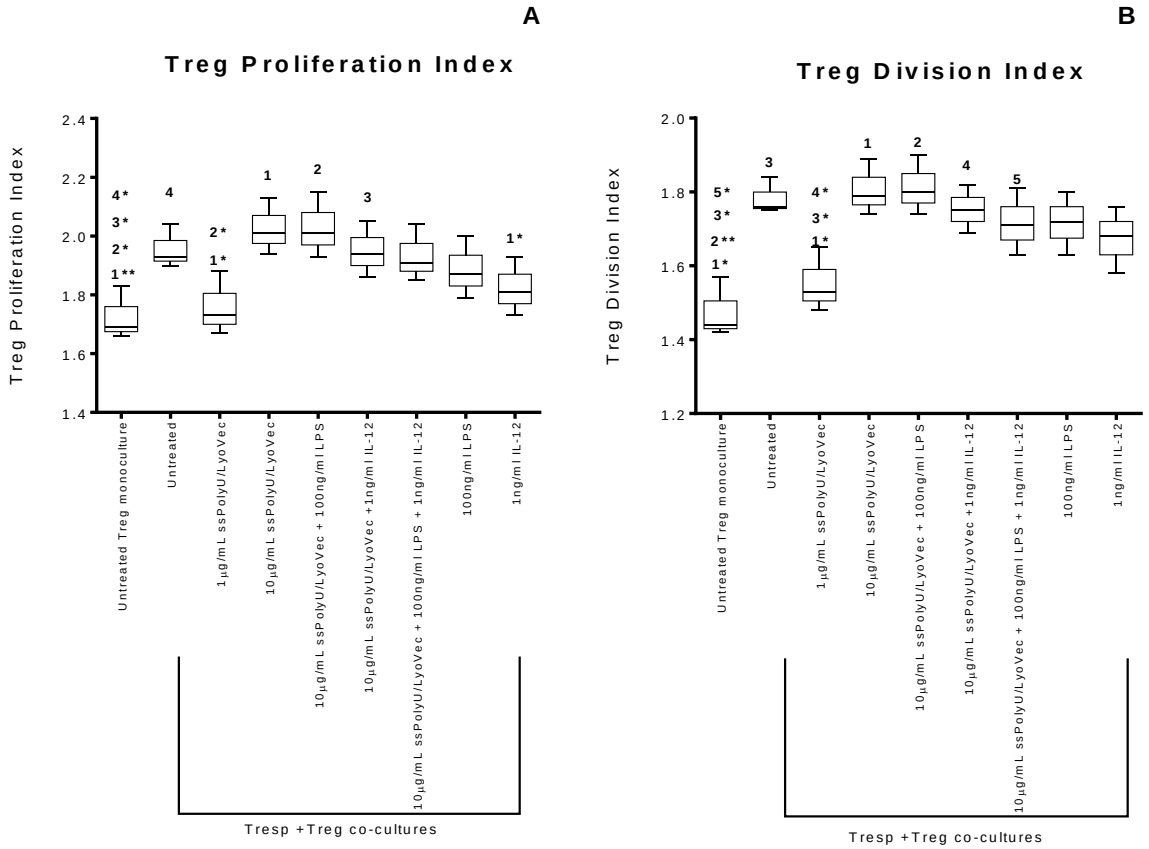


Figure 4.44: Box and whisker plots showing (A) Proliferation Indices and (B) Division Indices of untreated Treg monocultures and of Treg in Tresp:Treg co-cultures untreated or treated with ssPolyU in the absence or presence of LPS and/or IL-12. Data was collected 7 days post-activation (5 days post-treatment). Units are arbitrary and were generated using FlowJo Version 10.6.1 proliferation modelling platform. Data are presented as median Proliferation/Division index with 75th and 25th quartiles (n = 3 from different blood donors). Difference between groups was tested using Kruskal Wallis non-parametric test. * p≤ 0.05, ** p≤ 0.005

Figure 4.44 (plot A) shows that treating Treg in the co-culture with 1µg/ml ssPolyU reduces the number of cell divisions (by an average of 0.3) proliferating Treg undergo. The proliferation index of this co-culture is statistically lower than that of two out of four co-cultures treated with 10µg/ml ssPolyU (p = 0.014 with Tresp + Treg treated with

10µg/ml ssPolyU and $p = 0.018$ with Tresp + Treg treated with 10µg/ml ssPolyU and 100ng/ml LPS), but is similar to the proliferation indices of untreated Treg monoculture.

Plot B shows that treating the co-culture with 1µg/ml ssPolyU also reduces the Treg's division index (i.e. the cell divisions the entire Treg population is undergoing) and follows the same pattern as with the proliferation indices with significant differences observed between this co-culture and three out of four co-cultures untreated ($p = 0.24$) or treated with 10µg/ml ssPolyU ($p = 0.022$ with Tresp + Treg treated with 10ug/m0l ssPolyU*; p* = 0.016 with Tresp + Treg treated with 10µg/ml ssPolyU + 100ng/ml LPS). Therefore, proliferation modelling has further shown that treating Treg with 1µg/ml PolyU reduces their proliferation and this is therefore the likely reason why Tresp proliferation was improved in this co-culture.

4.5.3 Cytokine Analysis

To check whether any soluble factors were responsible for the observed changes, cytokine analysis was carried out on the cell culture supernatants collected after 7 days of activation and treatment.

4.5.3.1 Single Stranded PolyU In Combination with IL-12 Increases IL-10 and Granzyme B Production in Tresp and Treg Co-Cultures

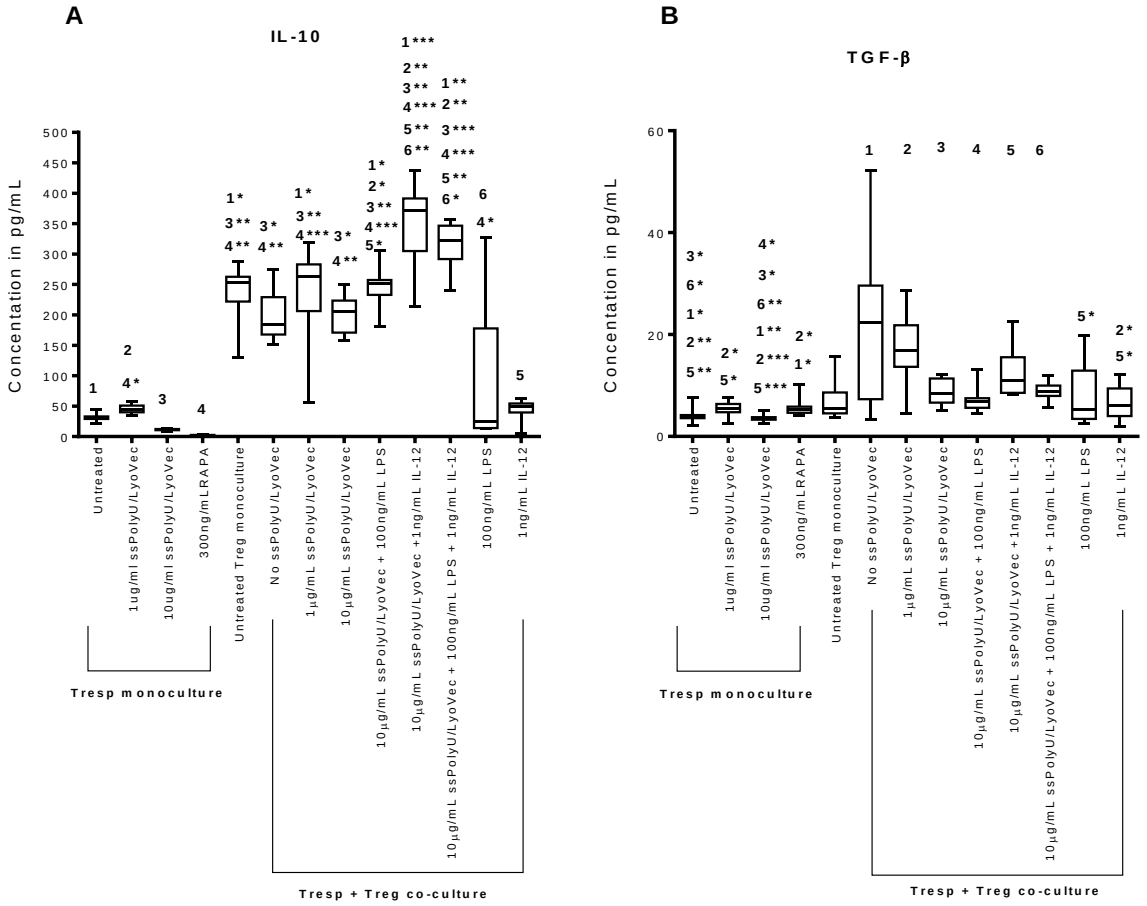


Figure 4.45 : Box and whisker plots showing, in linear scale, the concentrations in pg/mL of (A) IL-10 and (B) free active TGF-β1, obtained from supernatants of Treg and Tresp monocultures as well as Tresp and Treg cocultures left untreated or treated with ssPolyU with or without LPS or/and IL-12. Data was collected 7 days post-treatment. Data are presented as median concentration with 75th and 25th quartiles. Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison.* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$

Figure 4.45 (plot A) demonstrates that significantly higher levels of IL-10 were found in the Tresp:Treg co-culture (and Treg monoculture) than in the Tresp monoculture, most probably owing to release by the Treg which were absent in the Tresp monoculture. The concentration of the cytokine in the Tresp monoculture was extremely low and addition

of ssPolyU did not seem to induce any effect. Addition of ssPolyU to the co-cultures did not alter production levels of IL-10 either and the concentrations recorded were similar to those of the untreated co-culture. However, when ssPolyU was combined to IL-12, the concentrations of the cytokine were observed to increase. This seems to be a combinatory effect of the two agents together as treating with ssPolyU alone did not affect IL-10 concentration whereas treating with IL-12 alone actually resulted in lowered IL-10 concentrations relative to the untreated control. Although none of the differences were significantly different from those of the untreated co-cultures ($p > 0.05$), it is still worth noting that combining the TLR7/8 ligand (ssPolyU) and the Th1-differentiation cytokine (IL-12) resulted in higher productions of the anti-inflammatory cytokine IL-10. It is unknown whether the extra IL-10 was being produced by the treated Treg or by the Tresp reacting to the treated Treg, however a regulatory mechanism to counteract the effects of simulating a Th1-promoting environment might be at play.

The levels of TGF-β recorded (plot B) were also significantly higher in the Tresp:Treg co-cultures than in the Tresp monoculture (also owing to release by Treg in the co-cultures). These differences were however very small considering that the amounts of free-active TGF-β detected in the supernatants was very low in the first place (Figure 7, plot B). Adding ssPolyU alone or in combination with LPS and/or IL-12 did not seem to effect production of the cytokine by the Treg.

Therefore, ssPolyU alone does not seem to promote an increase in IL-10 or TGF-β. This means that, although an increase in Tresp suppression was observed in co-cultures treated with the TLR7/8 ligand, suppression is not promoted through increased release of these anti-inflammatory cytokines.

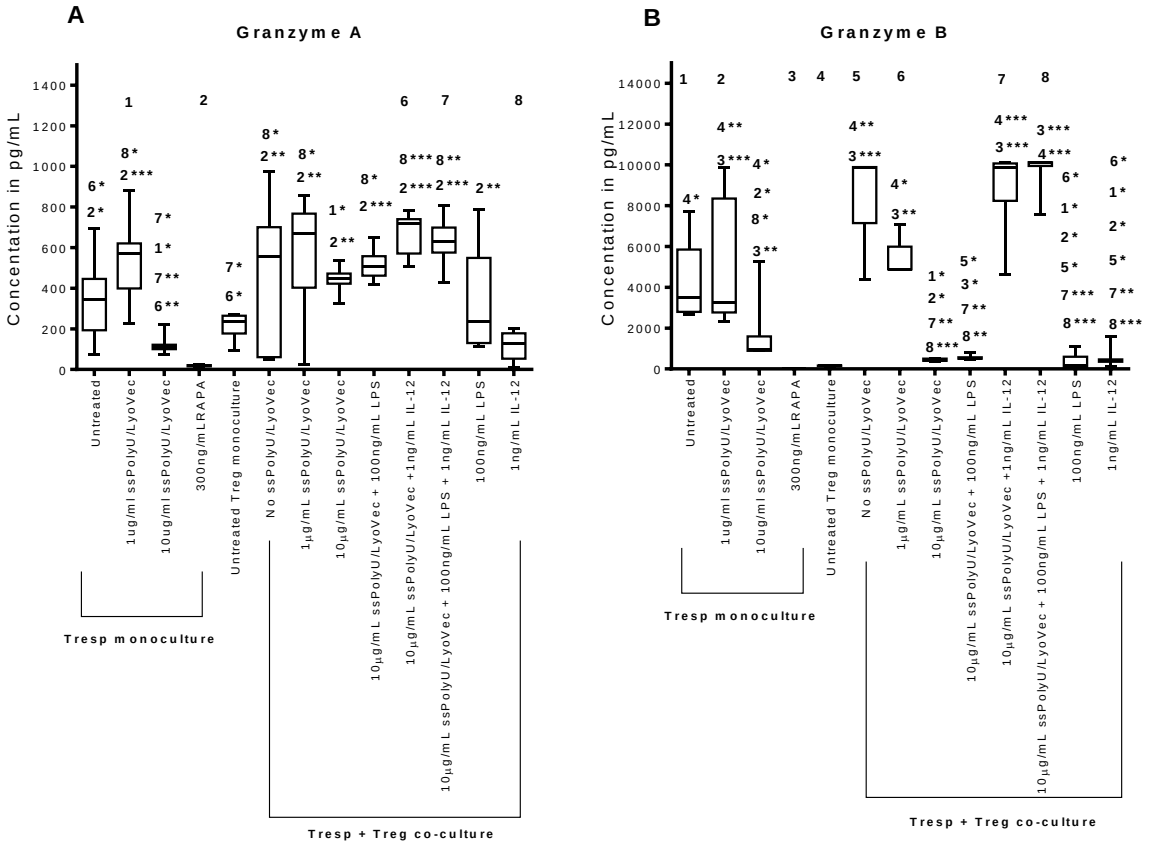


Figure 4.46 : Box and whisker plots showing, in linear scale, the concentrations in pg/mL of (A) Granzyme A and (B) Granzyme B, obtained from supernatants of Treg and Tresp monocultures as well as Tresp and Treg cocultures left untreated or treated with ssPolyU with or without LPS or/and IL-12. Data was collected 7 days post-treatment. Data are presented as median concentration with 75th and 25th quartiles. Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison.* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$

Figure 4.46 plot A shows that treating the Tresp monoculture with 1µg/ml ssPolyU seemed to slightly induce higher production of Granzyme A relative to the untreated monoculture (difference not statistically significant), yet treating with a higher concentration seemed to lower production levels. Indeed, the difference in the concentration levels detected in the two monocultures treated with different concentrations of ssPolyU were statistically significant (p = 0.011). The same effect was seen when the co-cultures were first treated with 1µg/ml ssPolyU (levels higher than in

the untreated co-culture) and when then treated with 10µg/ml (levels lower than the untreated co-culture). This shows that low concentrations of the TLR7/8 ligand promoted the production of the granzyme but higher concentrations tended to cause an opposite effect. Combining ssPolyU with IL-12 seemed to re-promote the production of Granzyme A, despite the fact that the higher concentration of ssPolyU was used. This seemed to be also a case of a combined effect since treating the co-culture with IL-12 alone actually caused a reduction (to statistically significant levels) in Granzyme A concentrations.

A similar kind of pattern was observed with Granzyme B (plot B) in that a reduction in the granzyme levels were observed in the monocultures as well as co-cultures treated with 10µg/ml ssPolyU, and that production levels started to increase again when the co-cultures were treated with 10µg/ml ssPolyU in combination with IL-12.

Therefore, ssPolyU alone does not cause an increase in the production of the suppressor molecules discussed, which would be beneficial in a tumour micro-environment. However, combining ssPolyU with IL-12 might on the other hand prove disadvantageous in such a scenario as an increase in IL-10 and granzymes production levels was observed under these treatment conditions. An increase in these suppressor molecules would impede anti-tumour immunity since these suppressor molecules are employed by Treg to suppress Tresp.

4.5.3.2 Single Stranded PolyU In Combination with IL-12 Promotes the Release of TNF-α and Further Enhances IL-12 Production in Tresp and Treg Co-Cultures

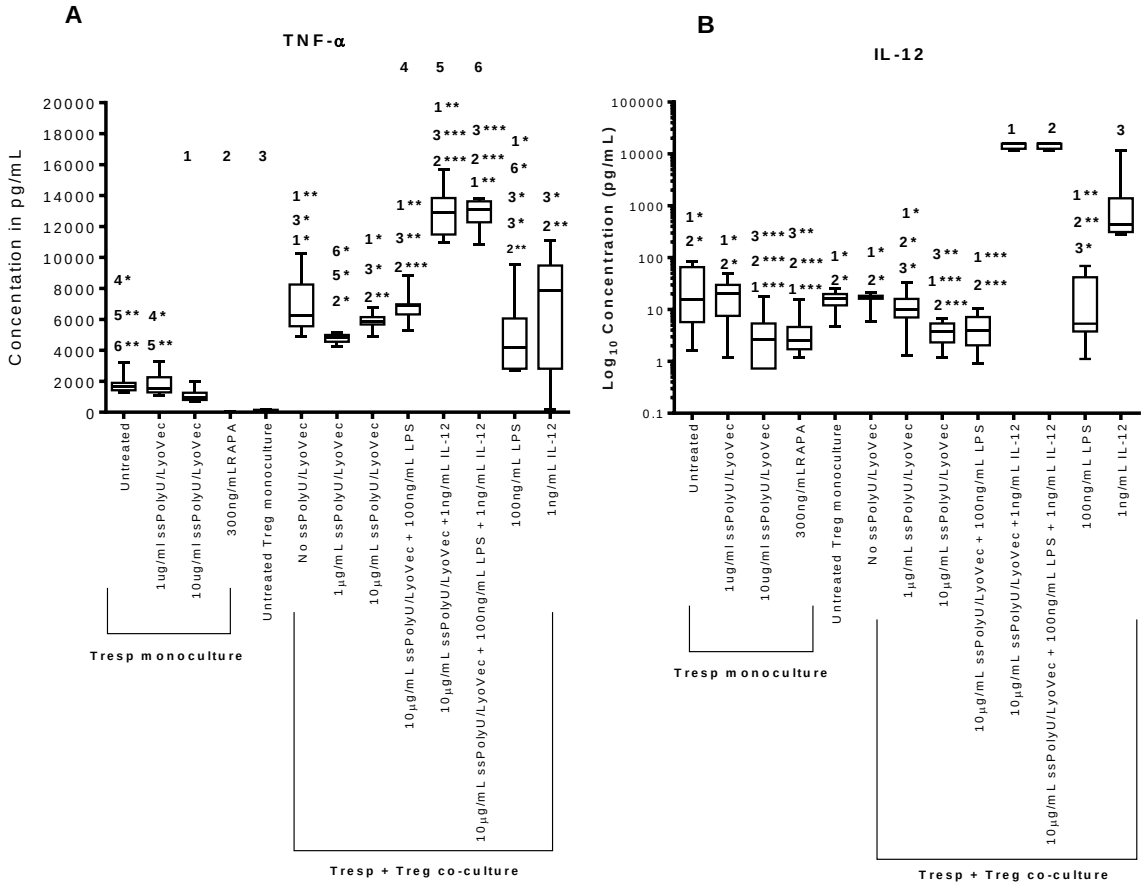


Figure 4.47 : Box and whisker plots showing, in linear or logarithmic scale, the concentrations in pg/mL of (A) TNF-α and (B) IL-12 obtained from supernatants of Treg and Tresp monocultures as well as Tresp and Treg cocultures left untreated or treated with ssPolyU with or without LPS or/and IL-12. Data was collected 7 days post-treatment. Data are presented as median concentration with 75th and 25th quartiles. Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison.* $p \leq 0.05$, ** $p \leq 0.05$, *** $p \leq 0.005$

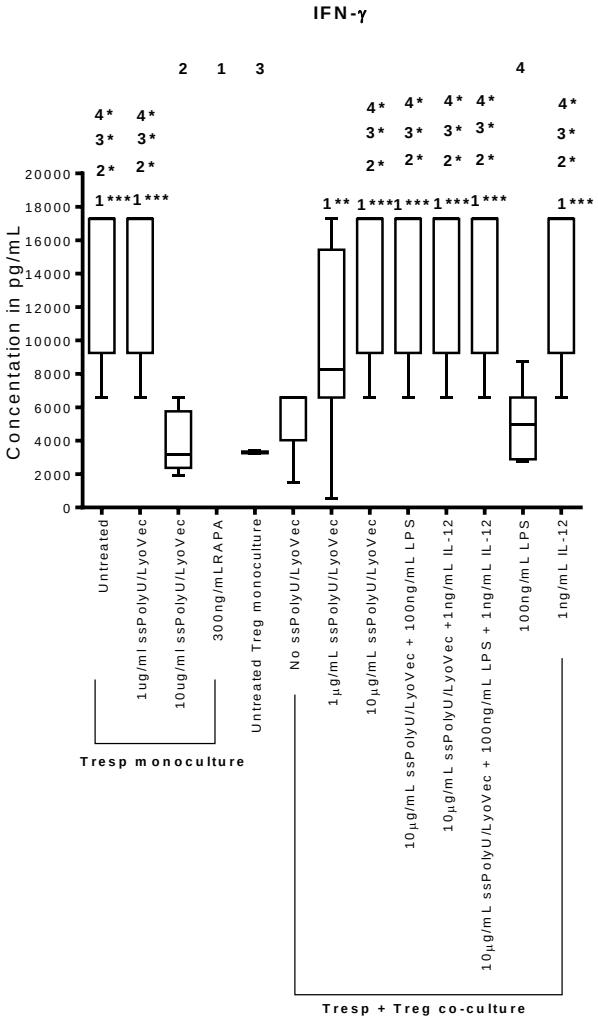


Figure 4.48 : Box and whisker plots showing, in linear scale, the concentrations in pg/mL of IFN-γ, obtained from supernatants of Treg and Tresp monocultures as well as Tresp and Treg cocultures left untreated or treated with ssPolyU with or without LPS or/and IL-12. Data was collected 7 days post-treatment. Data are presented as median concentration with 75th and 25th quartiles. Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison.* $p \leq 0.05$, ** $p \leq 0.05$, *** $p \leq 0.005$

TLR7 or TLR8-activation by ssPolyU has been reported to induce the release of pro-inflammatory cytokine when DCs are present including TNF-α, IL-12 and IFN-γ. Figure 4.47(Plot A) shows that treating Tresp monocultures with ssPolyU (in the absence of DCs) does not seem to particularly effect the production of the pro-inflammatory cytokine TNF-α. Treating the Tresp:Treg co-cultures with ssPolyU alone also seemed to exhibit no

effect either. However, when combined to IL-12, levels of the cytokine increase dramatically with the concentration differences being significant to those of the co-culture treated with 1µg/ml ssPolyU ($p < 0.05$). Interestingly, the TNF-α concentrations in the co-culture treated with ssPolyU and IL-12 were also even significantly higher than those of the Tresp monocultures ($p < 0.05$). Same as with IL-10, this effect is not due to the action of IL-12 alone, as treating the co-cultures with it alone retains the TNF-α concentration similar to that of the untreated co-culture. This means that ssPolyU on its own is not sufficient to induce the release of TNF-α by CD4+ T cells, and that it requires the addition of the Th1-differentiation cytokine IL-12. In other studies where DCs where present, it is likely that ssPolyU triggers the release of IL-12 from DCs which in turn stimulates the production of TNF-α (and other pro-inflammatory cytokines) by CD4+ T cells. With DCs being absent in this study, the action of ssPolyU was only possible when IL-12 was added exogenously. The increase in TNF-α under these conditions could also explain why an increase in IL-10 was observed in the same co-cultures. The increase in TNF-α might have prompted the upregulation of IL-10 to counteract the inflammatory responses that would have been induced by TNF-α (and IL-12).

Single stranded PolyU alone also does not promote IL-12 production in Tresp monocultures or Tresp:Treg co-culture (Figure 4.47 plot B). This is probably because the main source of IL-12 are dendritic cells which were absent in the mono- and co-cultures. IL-12 concentration only increased when IL-12 was added, but increased by tenfold to the concentration originally added probably because the presence of IL-12 promoted its own secretion. In an *in vivo* tumour micro-environment where DCs are present, ssPolyU might promote the release of IL-12 by DCs which in turn would produce this same effect as seen when IL-12 was added exogenously.

Figure 4.48 shows that low levels of IFN-γ were detected in the untreated co-culture, yet treating the co-culture with ssPolyU (alone or in the presence of LPS and/or IL-12) restores the concentrations of the cytokine to the same levels that are produced by the Tresp monocultures where no Treg are present. Although these differences were not statistically significant, the difference is still evident (p = 0.68*).* Therefore, ssPolyU restores IFN-γ concentrations that would otherwise be reduced in the presence of Treg.

4.5.3.3 Single Stranded PolyU with IL-12 Weakly Induces IL-4 While 1µg/ml But Not 10µg/ml ssPolyU Promotes IL-17

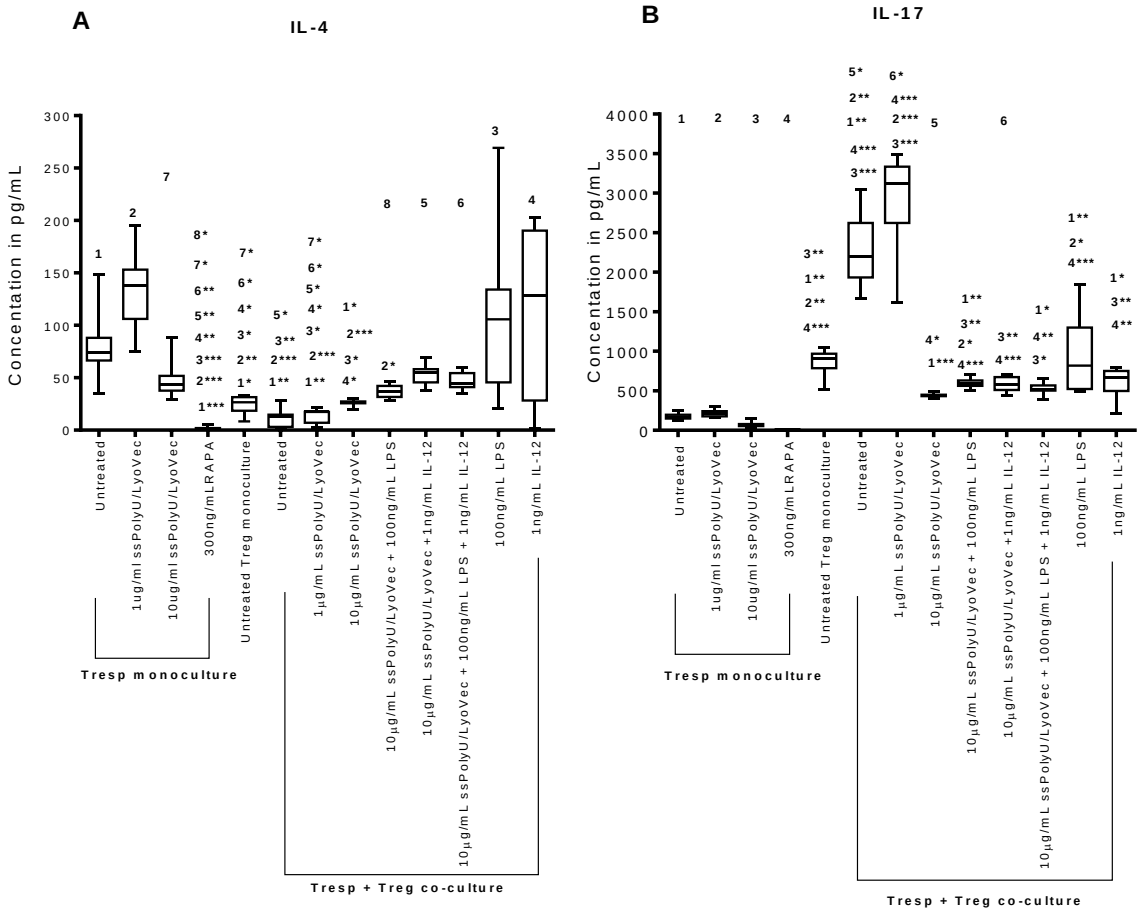


Figure 4.49 : Box and whisker plots showing concentrations in pg/mL of (A) IL-4 and (B) IL-17, obtained from supernatants of Treg and Tresp monocultures as well as Tresp and Treg cocultures left untreated or treated with ssPolyU with or without LPS or/and IL-12. Data was collected 7 days post-treatment. Data are presented as median concentration with 75th and 25th quartiles. Difference between groups was tested using the non-parametric Kruskal Wallis test with pairwise comparison. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$

Single stranded PolyU alone also does not seem to effect the cytokine concentration of the Th2-differentation cytokine IL-4 in the co-cultures as the levels detected were similar to those of the untreated co-culture (Figure 4.49 plot A). The highest levels of the cytokine were detected in the Tresp monocultures and the co-cultures treated either with

LPS or IL-12 alone. This means that in these co-cultures, some Tresp were differentiating into Th2. Interestingly, however, combining ssPolyU with IL-12 promotes a slight increase in the concentration of IL-4 and the differences were significant from those of the untreated co-culture (p = 0.009) and the co-cultures treated with ssPolyU on its own (p = 0.009 when treated with 1µg/ml ssPolyU and p = 0.011 when treated with 10µg/ml ssPolyU). Similarly to IL-10, IL-4 suppresses Th1 cells so creating a Th1-polarizing environment (ssPolyU in conjunction with IL-12) might also have induced the co-production of yet another Th1-inhibtory cytokine to regulate Th1 responses.

The concentrations of IL-17 detected in the Tresp monocultures were very low indicating that only a small amount of these CD4+ cells were Th17 (Figure 4.49, plot B). Interestingly, high levels of IL-17 were detected were in the untreated co-cultures and levels were even higher when the Treg in the co-cultures had been treated with 1µg/ml ssPolyU. This might indicate that ssPolyU at this concentration promotes Th17. At higher concentrations of ssPolyU, IL-17 concentrations are significantly lowered even when combining them with LPS and/or IL-12. Therefore, high concentrations of ssPolyU do not enhance the production of the Th17 effector cytokine.

5 DISCUSSION

5.1 Evaluating the Suitability of IFN-γ In the Modulation of Treg for Cancer Immunotherapy

The role of IFN-γ on CD4+CD25+ Treg remains controversial. Wei *et al.*, 2007 demonstrated that the addition of the Th1 effector cytokine to cultures of naïve $CD4^+$ T cells resulted in a substantial reduction in the percentage of FOXP3 expression. Chang, Kim, Han, & Kang, 2009 have demonstrated, in *in vitro* differentiation studies, that conversion of naïve T cells into $FOXP3^+$ Treg was significantly inhibited by IFN-γ. IFN-γ inhibiting Treg generation during activation *in vitro* was also seen in studies by Caretto, Katzman, Villarino, Gallo, & Abul, 2010 and this inhibition was independent of T-bet expression.

On the other hand, Wang *et al.*, 2006 have shown that IFN-γ, both in the presence and the absence of TCR co-stimulation, leads to the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ Treg and increases FOXP3 expression in both mouse and human experimental systems.

The results of this present study coincided with those of Wei *et al.*, 2007 where a reduction in FOXP3 expression was observed when Treg were treated with IFN-γ. The decrease was the same irrespective of whether the Treg were treated with 25ng/ml or with 50ng/ml IFN-γ.

FOXP3 is a critical regulator of CD4+CD25+ Treg function. Fontenot *et al.*, 2005 observed that deletion of the FOXP3 gene in mice resulted in severe autoimmunity where the mice succumbed to an aggressive lymphoproliferative syndrome. When FOXP3 was transduced into CD4+CD25- T cells, these cells acquired suppressive function and the mice

survived. Therefore, stringent regulation of FOXP3 expression is required to maintain homeostasis of T-cell-mediated immune responses.

Whilst this immunosuppression is essential for self-tolerance, it can be highjacked in the case of tumours to prevent a curative immune response. Thus in oncology, reducing Treg function is a potential useful target. In this study, exposure to IFN-γ reduced FOXP3 expression in Treg which might explain why the treated Treg were less suppressive towards Tresp in the proliferation assays. IFN-γ did not slow down the expansion of Treg in these proliferation assays, meaning that the reduced Treg-mediated immunosuppression in IFN-γ-treated co-cultures was probably due to a change in the Treg phenotype and not Treg numbers.

The mechanisms by which FOXP3 endows Treg with a suppressive function remains unclear but Fontenot *et al.*, 2005 hypothesized that FOXP3 might be inducing the expression of IL-10, an anti-inflammatory cytokine implicated in Treg effector function.

This study also demonstrated that IFN-γ-treated Treg upregulated the expression of T-bet which is the master transcription factor of Th1 cells. T-bet is known to induce and upregulate IFN-γ production however the reverse induction has also been observed. Therefore, IFN-γ regulates the transcription factor that promotes its' own production in a positive feedback loop (Lighvani *et al.*, 2001). Although this has been observed in Th1 cells, the same effect might also be true to Treg induced by IFN-γ to take on Th1 inflammatory characteristics, thus suggesting a possible mechanism of plasticity of Treg under inflammatory environments.

FOXP3+ Treg that express T-bet have been reported in studies and in particular they have been implicated in inflammatory conditions such as colitis. It has been reported that under Th1 inflammatory conditions induced by IFN-γ, Tregs upregulate Th1-related

transcription factor T-bet and express the pro-inflammatory cytokine IFN-γ. This suggests that at a certain point, possibly onto the conversion from acute to chronic inflammation, Tregs might contribute to the inflammatory process, rather than suppress it (Giovangiulio *et al.*, 2019).

In another study, it was shown that Th1-like Treg cells are found in higher numbers in subjects suffering from relapsing remitting multiple sclerosis (RRMS) in comparison to healthy subjects. These Th1-like Treg cells induced by the inflammatory conditions associated with RRMS secrete IFN-γ and these Treg showed reduced suppressive activity *in vitro,* which was partially reversed by IFN-γ–specific antibodies (or removal of IL-12) (Dominguez-Villar, Baecher-Allan, & Hafler, 2013).

Similarly, in this study, IFN-γ seems to reverse the suppressive activity of the Treg and render them more Th1-like. The addition of exogenous IFN-γ might be promoting T-bet expression in Treg (while reducing FOXP3 expression) inducing them to be pro-inflammatory rather than anti-inflammatory and immune-suppressive. Although this is undesirable in autoimmune diseases such as colitis and multiple sclerosis, it might have important applications in the cancer therapy to boost the anti-tumour response.

Adding IFN-γ to CD4+CD25- directly to non-Treg cells in this present study seems to cause the opposite effect to those seen by Wang *et al.,* 2006. These cells already produce substantial amounts of IFN-γ and the exogenous addition of more IFN-γ might have triggered a negative feedback loop that suppresses proliferation in order to reduce the chances of autoreactivity. Indeed, in several studies, it has been shown that after the release of IFN-γ from Th1 during acute inflammation, high concentrations of the cytokine then promote activation-induced cell death (AICD) in effector T cells (Tresp) as a mechanism to prevent

excess tissue damage (Dalton, Haynes, Chu, Swain, & Wittmer, 2000; Fulda & Debatin, 2002; Refaeli, Parijs, Alexander, & Abbas, 2002). In fact, in this study, adding IFN-γ directly to CD4+CD25- T cells (Tresp) monocultures severely reduced their proliferation which could be possibly accounted for by AICD.

The self-regulatory mechanism by proliferating Tresp is also evident when one looks at the cytokine production of IL-10 under the different treatments. Although IFN-γ is considered as the signature cytokine for Th1, other cytokines may also be produced by Th1, namely pro-inflammatory cytokines IL-2 and TNF-α as well as the anti-inflammatory cytokine IL-10 (Ma, Fan & Ribas, 2014). The latter is also produced by Th2 (Rosloniec, Latham, & Guedez, 2002). Some studies have suggested that Th1 effector cell responses are autoregulated through a negative feedback loop via the co-induction and expression of IL-10. The relative amounts of IFN-γ and IL-10 produced by such double positive cytokine-secreting Th1 cells subsets and their ability of "cytokine switching" might define the inflammatory response within the tumour environment (Ma, Fan & Ribas,2014). IL-10 is one of the effector cytokines of Treg and was therefore expected to be high, at least, in the untreated co-culture where Tresp suppression was evident. This however was not the case and it was in fact found to be higher in the IFN-γ-treated co-cultures where Tresp proliferation was high. IL-10 is not the only mechanism by which Treg exert immunosuppression, and in this case Tresp suppression by Treg in the untreated sample might have been primarily mediated by some other mechanism. IL-10 is also produced by effector Th1 cells to help limit the damage caused by inflammation induced by these types of cells themselves (Trinchieri, 2007). This might explain the higher levels of IL-10 found in the Tresp monoculture and the co-cultures treated with IFN-γ where proliferation of Tresp was significant. Being pro-inflammatory, IFN-γ might have induced the proliferation of Tresp (in particular Th1) but this sudden increase in Tresp

numbers might have in turn triggered the production of IL-10 by the proliferating Th1 themselves (rather than by the Treg) as a self-regulatory mechanism.

Another cytokine produced in higher amounts in IFN-γ-treated samples was IL-4. Although this cytokine is considered as pro-inflammatory and is the differentiation cytokine for Th2, it also functions in suppressing Th1 (Silva-Filho, Caruso-Neves, & Pinheiro, 2014). Indeed, IL-4 production increased when both Tresp monocultures and Treg in Tresp and Treg co-cultures had been treated with IFN-γ. The increase in IL-4, probably by the Tresp (since Treg do not usually produce IL-4), might also be acting as a Th1-controlling mechanism induced by the Th1 effector cytokine IFN-γ. Indeed, a study has shown that IL-4 was found to enhance IL-10 production in Th1 cells (Mitchell *et al.*, 2017). In another study by Wurtz & Baje, 2004, IL-4 blocked Th1 differentiation and redirected it towards the Th2 lineage, whereas in already differentiated effector Th1 cells, IL-4 seemed to transiently and reversibly reduce the transcription of the IFN-γ gene. Therefore the Th1 responses induced by the addition of IFN-γ might have prompted increased production of IL-4 in treated co-cultures in order to counteract Th1-induced inflammation. This might be advantageous in the tumour microenvironment as at first a "sudden" acute Th1 inflammatory response is generated (which is required to kill off tumour cells) but the effect is self-controlled in order to prevent auto-reactivity and the consequences of excessive Th1 responses.

IFN-γ also induced the production of the Th1 pro-inflammatory cytokine TNF-α in the Tresp and Treg co-cultures. Sol *et al.*, 2008 observed that TNF-α production is induced by IFN-γ treatment in a murine macrophage cell line and, since TNF-α, is also produced by Th1, the same effect might be observed in Tresp and Treg co-cultures. It is however unclear whether this "extra" TNF-α production in the IFN-γ-treated co-cultures is by the Tresp (Th1 population) or the IFN-γ-treated Treg, or both. Some studies have shown that although Treg cells secrete

anti-inflammatory cytokines they can also acquire the ability to produce pro-inflammatory cytokines such as TNF-α under inflammatory conditions (Jung, Lee, Kwak, & Shin, 2019). Interestingly, these TNF-producing Treg cells exhibit Th17-like features in acute hepatitis (Wohlfert & Belkaid, 2010; Jung, Lee, Kwak, & Shin, 2019). Indeed an increase in IL-17 (effector cytokine for Th17) was observed in the co-culture treated with 25ng/ml IFN-γ which could suggest this. However phenotype analysis proved otherwise since the percentage of cells that were FOXP3-CCR4+CCR6+ (characteristic of a Th17 phenotype) remained extremely low in the treated Treg.

The enhanced production of TNF-α with regards to cancer has a dual effect. On one hand, TNF-α could have undesirable effects as it acts as an endogenous tumour promoter and stimulates cancer cells' growth, proliferation, invasion and metastasis, as well as tumour angiogenesis. On the other hand, however, TNF-α could also act as a cancer killer, as its name implies (Wang & Lin, 2008). Therefore, the upregulation of TNF-α secretion by IFN-γ-exposed lymphocytes could actually result in a good outcome for cancer therapy. Secretion by the lymphocytes in the tumour microenvironment is helpful as it reduces the toxicity of systematic TNF-α administration and risk of autoimmune responses.

Therefore, treating Treg present within the tumour micro-environment with IFN-γ might offer potential in cancer immunotherapy as it has been shown to downregulate FOXP3 which endows Treg with their suppressive power. Additionally, it induces the production of Th1-associated cytokines that promote anti-tumour immunity, whilst at the same time it also stimulates the co-production of other cytokines that regulate Th1- inflammation thus preventing tissue damaged induced by an over-reactive Th1 response. It is however important that the responses are retained within the tumour micro-environment as systematic

administration will result in Treg with a reduced ability to prevent the development of systematic autoimmune disease.

5.2 Evaluating The Immunomodulating Effects Of Endogenous Nucleic Acid TLR Ligands

It is well known from several studies that natural viral and bacterial nucleic acids can act as modulators of the immune system. For instance, viral double stranded RNA is recognized by TLR3 while viral single stranded RNA is recognized by TLR7 and TLR8. On the other hand, TLR9 is the only known receptor for DNA primarily for bacterial DNA containing unmethylated CpG motifs. The denotation 'CpG' is shorthand for the occurrence of a cytosine linked, through a phosphate bond, to a guanine.

However, it has also been shown that, despite the immune system having evolved adaptations to recognize self from non-self-nucleic acids, TLR ligands can sometimes also respond to endogenous nucleic acids released from the host's apoptotic and dead cells.

Single stranded RNA has been reported to act as an endogenous ligand when it is released during chronic inflammation or tissue damage. It has been implicated in the pathogenesis of systematic autoimmune diseases such as systematic lupus erythematosus by launching a potent inflammatory response while inhibiting the function of Treg (Wu, Tang, & Zuo, 2015).

TLR9 was originally thought to bind and respond only to unmethylated (CpG) DNA. This exclusivity was thought to be due to the fact that mammalian DNA has a low frequency of CpG dinucleotides, which if present, are methylated (Hemmi *et al.*, 2000; Rich, 2007). However, it is now clear that TLR9 also recognizes host DNA and has also been implicated in the pathogenesis of certain autoimmune diseases including Systematic

Lupus Erythematosus (SLE) and psoriasis (Barrat *et al.*, 2005; Yasuda *et al.*, 2009). Indeed, Barrat *et al.*, 2005 have shown that in SLE, mammalian DNA and RNA form antibody-nucleic acids complexes, which are endocytosed and delivered to endosomal compartments where they act as ligands to TLR9 and TLR7 respectively and induce IFN-α production in dendritic cells resulting in autoimmunity (Means *et al.*, 2005). These antibody-nucleic acid complexes are thought to form due to a reduced ability by macrophages to clear off apoptotic cells in SLE patients (Barrat *et al.*, 2005).

Nonetheless, stimulation by endogenous nucleic acids is still a rare event because it is difficult for endogenous nucleic acids to enter cells. The mammalian immune system has evolved mechanisms to ensure that degradation of extracellular nucleic acids takes place. Nucleases including DNases (that degrade DNA) and RNases (that degrade RNA) that exist in serum degrade extracellular nucleic acids (Hornung, Roers, Carus, & Universita, 2016). These nucleic acids released from necrotic or apoptotic cells are also normally degraded by macrophages before they can access the intracellular TLR compartments (Barton, Kagan, & Medzhitov, 2006; Notley, Jordan, Mcgovern, Brown, & Ehrenstein, 2017; Gordon & Pluddemann, 2018).

Sequence specificity is another mechanism which discriminates between self and non-self-nucleic acids. For instance, methylated CpG single-stranded DNA and double-stranded DNA found in mammalian cells display much lower affinities to TLR9, and as such, they also have a lower tendency to induce TLR9 dimerization which is required for signalling (Hornung *et al.*, 2016). However, although the self-recognition can be reduced by specificity to a particular nucleic acid sequence, this mechanism is clearly not enough to avoid autoimmunity. Indeed, unmethylated CpG motifs are present in mammalian genomic DNA, albeit at a much lower frequency than in viral and bacterial genomes

(Barton *et al.*, 2006). Additionally, Yasuda *et al.*, 2009 have shown that although no activation is induced by DNA fragments lacking CpG dinucleotides, this CpG-free DNA can induce DC activation if internalized by aided (liposomal) transfection. This shows that if a transfecting agent is used and if successfully delivered inside the endosomes, endogenous nucleic acids might activate immune cells regardless of the nucleic acid pattern (Hornung *et al.*, 2016).

An additional mechanism that further allows discrimination of self from non-self-nucleic acids is the location of the TLRs (Barrat *et al.*, 2005). DNA- and RNA-specific TLRs are localised inside endosomal compartments and not on the cell surface. These compartments are usually devoid of host nucleic acids because extracellular nucleic acids present in serum are degraded before they can enter the cells or are found below the immunostimulatory threshold. However, nucleic acids associated with microbial pathogens are protected on their way to these compartments and stabilized to avoid to avoid extracellular digestion (Anz *et al.*, 2010) .

Breaking down these barriers, however, by delivering nucleic acid ligands to these compartments using suitable agents (e.g. PEI or lipofectamine) can trigger immune responses (Hornung *et al.*, 2016). Delivery vehicles such as PEI facilitate endocytosis of nucleic acids into the cell, release of the nucleic acids into the endosomal system and provide protection against nucleases (Wegmann *et al.*, 2012).

Lamphier, Sirois, Verma, Golenbock, & Latz, 2006 have demonstrated that TLR9 itself binds rather indiscriminately to a broad range of DNA sequences and when introduced using delivery vehicles, many non-stimulatory DNAs become stimulatory, suggesting that selectivity is in part determined by uptake efficiency.

Barton *et al.*, 2006 describe the creation of a chimeric TLR9 receptor that was localized to the cell surface (rather than on endosomes). This relocated TLR9 receptor responded normally to synthetic TLR9 ligands but not to viral nucleic acids and was able to recognize self-DNA, which usually does not stimulate wild-type TLR9. Thus, they demonstrated that intracellular localization of wild-type TLR9 prevents activation by self-DNA (and is important for activation by viral nucleic acids), whereas a chimeric TLR9 receptor re-localised to the cell surface could respond to mammalian DNA. Therefore, it is not surprising that host nucleic acid ligands are able to modulate the immune response if their delivery to the endosomal compartments is facilitated.

Although chronic TLR signalling by endogenous TLR ligands has been implicated in the pathogenesis of autoimmune diseases as well as tumourigenesis, activation of acute inflammation induced by endogenous TLR ligands (instead of microbial components) is potentially interesting in cancer therapy. In such instances, nucleic acids are released from dying cells and damaged tissues and a transient and controlled TLR-induced inflammation might actually promote anti-tumour responses in the tumour micro-environment. For this reason, endosomal delivery of nucleic acids (whether self or foreign) can be exploited as an adjuvant for vaccination and immunotherapy. Despite this, the effect of TLR signalling on Treg using endogenous TLR ligands (in a non-autoimmune pathological environment) has been poorly investigated. TLRs 7, 8 and 9 are expressed in Treg (Guangwei Liu & Zhao, 2007) therefore in this study, the immunomodulatory effects of introducing endogenous RNA and DNA (aided by PEI) into these cells was investigated and are discussed next.

5.2.1 Evaluating the Effects of Endogenous RNA

Heil, Hemmi, & Hochrein, 2004 have proposed that TLR7 or TLR8 can recognize GU-rich single-stranded RNA sequences present in self-messenger RNA (mRNA) or ribosomal RNA (rRNA). In this study, directly transfecting Treg (using PEI as a transfecting agent) with RNA isolated from human cells, seemed to reduce the percentage of cells with a Treg phenotype and interestingly, to increase the percentage of cells that express a Th2-like phenotype i.e. a CRTH2+CCR4+ phenotype. It has also been observed to reduce the expression of CD25 in Treg and upregulate the expression of a unique marker for Th2, CRTH2. The latter observation is interesting and has not been reported elsewhere in published literature, to our knowledge. The mechanisms by which TLR8 reduces the suppressive activity of Treg in other studies has been poorly described, but one possible explanation demonstrated in this study might be the downregulation of CD25. This CD marker is the receptor for IL-2, which is required by Treg to proliferate, and Treg need the receptor to competitively sequester the cytokine from Tresp (hence why Treg usually have higher expression of CD25).

Upon further investigation, it was also observed that FOXP3 expression was retained in these Treg and that the percentage of cells that upregulated CRTH2 were also positive for FOXP3 suggesting that the majority of treated Treg cells attained a FOXP3+CRTH2+ phenotype. As mentioned earlier throughout the text, FOXP3 is required for the suppressive function of Treg so its retention raised the question as to whether the treated Treg had indeed lost their ability to suppress Tresp. Proliferation studies revealed that this was not the case, so that despite loss of CD25, these cells still suppressed responder cells.

Cells with FOXP3+CRTH2+ have been reported elsewhere in other studies which did not involve TLRs and their ligands. Chantveerawong, Jindarat, Fuengthong *et al.*, 2018 have observed that cells with such phenotypes were found circulating in the blood of asthmatic patients and these cells were found to have a reduced regulatory function (despite FOXP3 expression). Schmidl, Andreesen, Hoffmann, & Rehli, 2011, on the other hand, found that Treg with a memory cell phenotype (characterised as $CD45RA^-$) strongly upregulated Th2 genes including GATA-3, IL-4, IL-5, and IL-13 (presence of CRTH2 was not tested) but lost FOXP3 expression. Similarly, Wang, Souabni, Flavell, & Wan, 2010 showed that reduced levels of FOXP3 expression (but not complete loss) led to the conversion of natural Treg into Th2 cells that could produce IL-4. Halim *et al.*, 2017 observed that a high number of Th2-like Treg were found in malignant tissues of patients with melanoma and colorectal cancer. These cells were found to exhibit the highest migratory capacity in response to chemokines enriched at tumour sites. They revealed that these cells may contribute to a tumourigenic environment as a result of increased malignant cell survival and their ability to suppress Th-1 responder cells.

Therefore this interplay between Treg and Treg attaining Th2-like characteristics has been reported numerous times but never in the context of TLR8-signalling using endogenous RNA. Contrary to Schmidl, Andreesen, Hoffmann, & Rehli, 2011 and Wang, Souabni, Flavell, & Wan, 2020, this study revealed that expression of FOXP3 in CRTH2-expressing Treg was still maintained (only CD25 was downregulated). Contrary to Chantveerawong, Jindarat, Fuengthong *et al.*, 2018, proliferation studies performed here revealed that the FOXP3+CRTH2+ cells generated did not result in a significant reduction in suppressive functions relative to the untreated control. It is unknown how TLR8 signalling modulates the Treg suppressive function, however one explanation could be

the upregulation or downregulation of FOXP3 after stimulation by the TLR ligands (Sutmuller *et al.*, 2006). Since FOXP3 expression was maintained in the treated co-culture in this present study, the suppressive abilities of the Treg were not affected. Then again, this would be contradictory to what was observed by Chantveerawong, Jindarat, Fuengthong *et al.*, 2018 who reported that FOXP3+CRTH2+ had a reduced suppressor function despite retaining FOXP3 expression. Moreover, both Treg and Th2 suppress Th1 and by attaining Th2-like characteristics, the treated Treg cells in the co-culture might be just using a different mechanism to suppress proliferation of Th1-differentiated Tresp. Additionally, the ratios of Treg to Tresp used in other studies wherein TLR 8 ligands were shown to reverse Treg function were lower (1:10 in Peng *et al.*, 2005) than the ratio used in this study (1:1). The higher amounts of Treg used in this present study might have rendered the Treg cells refractory to the pro-inflammatory effects of the TLR8 ligand.

Proliferation modelling did reveal, however that, transfecting Treg with 1μg/ml endogenous RNA might slightly reduce the proliferation of Treg although it was only a minimal effect, possibly explaining why the Treg were still suppressive (coupled the fact that they retained FOXP3 expression). Nonetheless, this slight reduction in Treg proliferation might be tied to the reduced expression of CD25. Being the receptor for IL-2, CD25 is critical for the development and peripheral expansion of Treg (Nelson, 2004). Therefore, a reduction in the expression of the IL-2 receptor may have resulted in a reduced ability by the Treg to use IL-2 for their expansion.

In treating Treg with endogenous RNA, some putative desirable results were also achieved. Despite maintenance of FOXP3 expression, a reduction in the production of suppressor molecules implicated in Treg-mediated immunosuppression was observed in the treated co-cultures. These include IL-10 and Granzyme B (Schmidt *et al.*, 2012). IL-10

usually inhibits the synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3 and TNF-α as well as maintain FOXP3 expression in Treg (Wang *et al.*, 2016). Granzyme B is highly upregulated in human Treg and induces apoptosis in target cells (Karreci *et al.*, 2017). In the untreated co-culture, it was possible that one of the mechanisms by which the Treg were suppressing Tresp was via the release of IL-10 and Granzyme B. Although IL-10 is also known to be produced by Th2 cells (Ozato *et al.*, 2002), interestingly, there was a decrease in IL-10 production in the treated co-cultures despite the Treg were being observed to attain a Th2-like phenotype. The reduction in IL-10 and Granzyme B production observed in the co-culture when the Treg were treated with endogenous RNA would therefore be valuable in a tumour micro-environment as it reduces suppression of Tresp mediated by these molecules.

Also very interesting is that transfection of the Treg with endogenous RNA increased the expression of cytokine IL-12 required for Th1 differentiation. This result was rather unexpected at first given that the transfected Treg attained Th2-like characteristics (rather than Th1). It is unclear whether the cytokine was being released by the Treg or the Tresp, however this result is very promising given that CD4+ T cells do not usually produce IL-12 (main source are usually DCs). One hypothesis could be that IL-12 was being released to promote Th1 that will in turn inhibit Th2 induced by endogenous RNA treatment. Additionally, IL-12 might have been released to counteract the effects of the Th2-differentiation cytokine IL-4 which was also found to be released in higher quantities in the treated co-cultures relative to the untreated. Indeed, if one compares the production profiles of IL-4 and IL-12, one observes a similar pattern: the increase in the production of IL-12 was directly proportional to the increase in IL-4. Indeed, IL-4 has been shown to upregulate IL-12 expression (in DCs and macrophages but perhaps also in CD4+

cells) to counteract IL-4's powerful induction of the Th2 response (Tak and Saunders, 2005). The production of elevated levels of IL-12 to promote Th1 differentiation coupled with a decrease in the production of IL-10, which neutralizes the anti-tumour effect of IL-12 (Tugues *et al.*, 2015), might be advantageous in the tumour micro-environment.

When considering together all the results described above, overall it is suggested that treating Treg with self-RNA does not elicit an immune response that favours anti-tumour immunity because Treg suppressor function is retained. It has already been mentioned earlier that Th2 suppress Th1 and therefore since the latter are the Th subset that are the most anti-tumourigenic, conversion to Th2-like cells is undesirable in a tumour micro-environment. Nonetheless, the study provided further insights on the plasticity of Treg and demonstrated their ability of not only being able attain Th1 characteristics, but also to Th2. This effect is dependent not only on the TLR itself but also on the nature of the ligand. Contrary to viral RNA, endogenous RNA might be in fact eliciting an immunosuppressive response rather than an immunogenic one (Anz *et al.,* 2010) possibly to prevent self-reactivity and autoimmunity.

5.2.2 Evaluating the Effects of Endogenous DNA

LaRosa *et al.*, 2007 have shown that TLR9 is directly stimulated by synthetic CpG DNA and it has been shown that treating Treg with such ligands abrogates their suppressive function directly and this effect was independent of APCs. Additionally they demonstrated that CpG DNA also works directly on CD4+CD25- responder T cells and increases their ability to escape Treg-mediated immunosuppression and this effect was dependent on the expression of Myeloid differentiation primary response 88 (MYD88)

protein in CD4+CD25- T cells. They also observed that loss of Treg function was not due to downregulation of FOXP3 as no change in the expression of the transcription factor was observed in their studies.

LaRosa *et al.* conducted their studies on mice and while CpG-rich oligodeoxynucleotides (CpG-ODNs) are potent immune activators in mice, their stimulatory effects are often less dramatic in humans, and this discrepancy between rodents and humans has been attributed to differences in TLR9 expression in the different species (Mutwiri, van Drunen Littel-van den Hurk, & Babiuk, 2009).

In this study, treating Treg with endogenous human DNA (rather than synthetic bacterial DNA analogues) and aiding its uptake into the cells by PEI, produced an opposite effect in that it enhanced rather than abrogated Tresp suppression by Treg, and this effect seemed to be dose-dependent. No change in FOXP3 expression (or any of the markers tested) was observed in the endogenous DNA-transfected Treg. These contrasting results might be attributed to the nature of the stimulatory DNA sequence used in the two studies. The genomic DNA used in this study was of mammalian origin which, contrary to the DNA used by LaRosa *et al.*, has a low frequency of CpG motifs, which if present, are methylated. Methylation of the CpG motif strongly reduces the affinity of TLR9 and reduces pro-inflammatory responses. Moreover, double stranded DNA (as used in this study) is less likely to bind to TLR9 than single stranded DNA and conversion of one form to another is achieved by DNAses (Agrawal and Gait, 2019) however the action of the enzyme in this study was prevented by complexing the nucleic acid to PEI.

Despite the lack of unmethylated CpG DNA, the endogenous DNA ligand used still seemed to modulate Treg function, albeit not as desired since Treg function was enhanced. Lawless *et al.*, 2018 have demonstrated that methylated (but not unmethylated) CpG DNA had an anti-inflammatory effect. They demonstrated that DNA derived from calf thymus cells suppressed *in vitro* proliferation of lymphocytes. They hypothesized that this induced suppression was due to the generation of Treg cells which they demonstrated by the enhanced FOXP3 expression in treated cells. The precise mechanism by which methylated CpG DNA induced FOXP3 expression was not shown. However, one hypothesis they came up with was its effect on the methylation status of the Treg gene. Treg-specific DNA hypomethylation is required for Treg development (Morikawa & Sakaguchi, 2014) and a methylated CpG motif can presumably result in the induction of FOXP3 expression by hypomethylation of the FOXP3-related genes. A second possibility is that methylated CpG nucleic acids result in Treg proliferation (independent of FOXP3- intensified expression). The latter hypothesis coincides with the observations reported in this study since, it was observed, that treating the Treg with DNA/PEI complex, particularly in low doses of 1µg/ml, promotes slightly more the proliferation of Treg relative to the untreated control.

This is not the only study which demonstrated that TLR9 stimulation may result in immunosuppressive and tolerogenic effects. In another study, systematic administration of high doses of CpG DNA stimulated Treg in mouse spleen to acquire potent suppressor activity and was mediated by plasmacytoid dendritic cells that express the immunosuppressive enzyme IDO (which in turn activates Treg) after TLR9 ligation (Baban *et al.,* 2009). TLR9-mediated IDO induction of immunosuppressive properties depended on the type of TLR9 ligand used as well as the dose and route of administration (Nicoli *et*

al., 2018), which shows that the nature and structure of the ligand can elicit different responses even though they bind to the same receptor. In this study, the enhanced Treg function must have been mediated by some other mechanism independent of IDO given that DCs were absent in the co-culture.

Although no increase in FOXP3 expression was observed in this study, treating the Treg (which already expressed FOXP3) with endogenous DNA (that contained primarily methylated CpG DNA) maintains expression of the transcription factor. Therefore, these results indicate that mammalian methylated CpG moieties, in contrast to bacterial unmethylated equivalent, are non-inflammatory and rather immunosuppressive. DNA methylation status might therefore govern the type (whether pro- or anti-) of inflammatory response. These disparate responses would in general be beneficial: when unmethylated DNA (bacterial origin) is detected, a pro-inflammatory response is generated to fight off the infection whereas when methylated DNA (host origin) is detected, an anti-inflammatory response is generated in order to prevent autoimmunity.

In fact, Notley, Jordan, Mcgovern, Brown, & Ehrenstein, 2017) observed that apoptotic cell DNA and mammalian double-stranded DNA from healthy individuals is methylated and was found to be immunosuppressive by activating Tregs and promoting the release of TGF-β. In contrast, double stranded DNA from patients with autoimmune diseases such as rheumatoid arthritis or SLE was found to be demethylated, to promote inflammation and to favour the production of IL-6 which acts antagonistically to Treg. Methylation of DNA therefore inhibits inflammation and, if unmethylated, it triggers autoimmunity.

In this study, the response elicited by the addition of PEI/DNA complexes was more an anti-inflammatory one given that it acted directly on Treg, enhancing their function. Yet the study also shows that adding these PEI/DNA complexes to Tresp monoculture also had a direct inhibitory effect on the Tresp proliferation without the intervention of Treg. Again, this observation is contrary to the effect observed by LaRosa *et al.*, 2007 who used unmethylated (rather than methylated as in this study) CpG DNA.

The mechanism by which Treg function was enhanced in this study is not completely understood, however an increase in the two anti-inflammatory cytokines IL-10 and TGF-β was observed in the co-culture where the Treg had been treated with 1µg/ml DNA complexed to PEI. Surprisingly, the increase in the production of the two cytokines was not PEI/DNA complex-dose dependent and in fact lower levels of the cytokines were found in the co-culture where the Treg had been treated with 5µg/ml DNA/PEI complex (the higher amount). Production of these two cytokines was not affected when the Tresp monoculture was treated with DNA/PEI which could indicate that the "extra" cytokine production in the co-cultures was either being released by the Treg or by the Tresp in response to the treated Treg (which were absent in the monoculture). Moreover, an increase in the production of the two granzymes A and B was also observed in the co-cultures treated with 1µg/ml relative to the control and the production profile in the different cultures follows the same pattern as with IL-10 and TGF-β (i.e. production at 5µg/ml DNA/PEI complex is lower than production at 1µg/ml DNA/PEI complex and treating Tresp only with DNA/PEI does not affect the production levels of the granzymes). This indicates that endogenous DNA/PEI complexes at a concentration of 1µg/ml enhances the production of suppressor molecules but not when treated with 5µg/ml DNA/PEI. This could be due to the fact that in the co-culture treated

with 1µg/ml Treg proliferation was slightly higher and therefore there was a greater number of cells secreting these suppressor molecules.

Interestingly, treating Treg with 1µg/ml endogenous DNA complexed to PEI and then adding to Tresp also enhanced the production of certain pro-inflammatory molecules, including TNF-α (also at 5µg/ml) and IL-12. Again, treating Tresp monocultures with the DNA/PEI complex did not affect production of these cytokines. Therefore it is possible that the increased pro-inflammatory cytokine production in the Tresp:Treg co-cultures (where the Treg had been treated) was due to Tresp attempting to counter-act the intensified suppression by the treated Treg.

Since mammalian DNA has a low frequency of CpG DNA (which when present is methylated), it is unknown whether the results observed in this study were being mediated via TLR9. Stetson & Medzhitov, 2006 used DNA that lacks CpG motifs to look at DNA-induced responses independently of TLR9 recognition. The DNA used induced type I IFN production in TLR9-deficient cells in a sequence-independent manner and only required an intact sugar phosphate backbone. The authors also found that chloroquine treatment (which inhibits endosomal acidification and prevents TLR9 activation) of macrophages abrogated CpG DNA-induced type I IFN production but had no effect on type I IFN production stimulated by the DNA that lacked CpG motifs. Taken together, their results provide evidence of a yet unravelled novel pathway for recognition of DNA independent of TLR9. Therefore, it is possible that the results observed in in this study may have been mediated via this unravelled mechanism which might also be present in Treg. Therefore, future studies should aim at trying to identify whether these responses in Treg are mediated via TLR9 or, if not, identify the DNA sensor responsible.

To conclude, treating Treg with endogenous DNA, as observed with endogenous RNA, does not elicit an immune response that favours anti-tumour immunity since the suppressive function of Treg was enhanced. Nonetheless, the study has shown that the origin of stimulatory DNA influences the outcome of the immune response. It has been shown that endogenous DNA results in enhanced immunosuppression, and although it is undesirable in cancer, it might offer a therapeutic potential in the management of autoimmune diseases.

5.3 Evaluating the Immunomodulatory Effects of Synthetic TLR 7/8 Ligands

The two synthetic ligands investigated in this study, CL097 and ssPolyU are well recognized ligands for TLR7 and TLR8. CL097 is a highly water-soluble derivative of R848 also known as Resiquimod. Its chemical formula is $C_{13}H_{14}N_4O$. These belong to a class of compounds called imidazoquinolines. Like R848, CL097 is a ligand for TLR7 in murine cells and a ligand for both TLR7 and TLR8 in human cells. Although sequence specificities of TLR7 and TLR8 have not been conclusively elucidated yet, CL097 mostly activates TLR7 and to a lesser extent TLR8 (Schön & Schön, 2008). CL097 was selected since it activates both TLR7 and 8 and is therefore expected to elicit a stronger immune response than other imidazoquinoline compounds such as imiquimod or its derivative, which only activates TLR7 (Yang, 2009). Moreover, CL097 is highly soluble and easily usable in *in vitro* assays (Caron et al., 2005).

The effects of CL097 and other imidazoquinoline compounds are mainly the secretion of pro-inflammatory cytokines and they have been implicated in producing a Th1-weighted anti-tumoral cellular immune response through the secretion of Th1-

associated pro-inflammatory cytokines (Reiter *et al.*, 1994; Stanley, 2002 as described by Schön & Schön, 2008). They also act by suppressing the humoral response and inhibit the production of Th2 cytokines such as IL-4 (Chosidow & Dummer, 2003). These effects have been described to be primarily mediated via DCs.

Synthetic single stranded ssPolyU is a synthetic single stranded RNA made up of only uridine nucleosides. The chemical formula of uridine is $C_9H_{12}N_2O_6$. These synthetic strands are able to activate immune cells by mimicking the nucleic acid motifs present in viral RNA. Apart from ssPolyU, TLR7 also senses synthetic RNA oligonucleotides derived from HIV, influenza virus, Newcastle diseases viruses and vesicular stomatitis virus while TLR8 recognizes mainly RNA rich in guanosine and uridine such as those found in coxsackie B virus and human parechovirus (Lund *et al.*, 2004; Meås *et al.*, 2020; Melchjorsen *et al.*, 2005; Triantafilou *et al.*, 2005). Although it is not excluded that TLR7 has a preference for a particular RNA motif, the fact that it mediates responses through PolyU, suggests that synthetic nucleotides with simple non-viral motifs can be used as adjuvant therapies. Non-viral synthetic RNA oligonucleotides are always safer to use than natural viral RNA. These are usually complexed to cationic lipids (complexed to lipid LyoVec in this present study) to mimic viral particles, and these cationic lipids promote uptake of RNA as well as protection from degradation by nucleases.

Guanosine and uridine-rich RNA nucleotides ligands are usually known to induce IFN-α production in plasmacytoid dendritic cells (pDCs) in a TLR7-mediated way, whilst adenosine and uridine-rich RNA nucleotides induce the release of TNF-α and IL-12 pro-inflammatory cytokines in a TLR8 dependent-manner (Gorden *et al.*, 2005; Ishii & Akira, 2008). The presence of uridine in synthetic RNA was found to be a prerequisite for secretion of IL-6, TNF-a, and ILI2p4, the latter being an essential inducer of Th1 cells

development (Heil, Hemmi, & Hochrein, 2004). Based on this, uridine is the most powerful nucleoside in activating both TLR7 and TLR8. Therefore, this is the reason why PolyU was used in this study rather than other synthetic RNA nucleotides such as PolyA or PolyG.

Although the primary responsive cell type for synthetic TLR7/8 ligands have always been reported in many studies as being dendritic cells, TLR7 and TLR8 are also expressed on Treg. Peng *et al.*, 2005 have shown that Treg express high levels, especially of TLR8 (when compared to other non-Treg CD4+ T cells) and therefore should be able to respond also to synthetic ligands as well. Therefore, this study was novel in that it investigated the direct effects of these two synthetic ligands on Treg cells and Treg:Tresp co-cultures in the absence of DCs. The results of this study might provide new insights useful for therapies that target Treg in cancer and autoimmune disease.

5.3.1 Evaluating the Effects of CL097

In most experimental *in vitro* studies imiquimod (also an imidazoquinoline compound) in concentrations of up to 5µg/mL elicited a robust proinflammatory response by dendritic cells. The same concentration and a lower concentration (2µg/mL) of CL097 was used in this study. Resiquimod (from which CL097 is derived) induces more pronounced cytokine secretion, macrophage activation and enhancement of cellular immunity as compared to related compound imiquimod (Wagner *et al.*, 1997, 1999; Imbertson *et al.*, 1998; Bernstein *et al.*, 2000; Burns *et al.*, 2000 as described by Schön & Schön, 2008).

However, there are a number of contrasting studies on the effect of imidazoquinolines on Treg. Nishii, Tachinami, Kondo, & Xia, 2018 observed that low doses of Resiquimod in a squamous cell carcinoma cell line derived from C3H/HeN mice (in which a high percentage of tumour infiltrating lymphocytes are CD4+FOXP3+ Tregs) caused a reduction in Treg. On the other hand, Van *et al.*, 2011 observed that Treg counts increased in the lung of Resiquimod-treated mice and mediate suppression of asthma symptoms when experimental asthma had been induced in the mice. Forward, Furlong, Yang, Lin, & Hoskin, 2010 observed that imiquimod and gardiquimod (another imidazoquinoline compound) enhanced the suppressor function of Treg isolated from mice.

In this study, an increase in CD25 expression was observed in all of the treated Treg. Resiquimod derivatives have been reported in several studies to upregulate CD25 expression in T cells. Zhang *et al.*, 2014 showed that CL097, at concentrations as low as 1µg/ml, enhanced CD25 expression in activated γδ T cells from mice. Li *et al.*, 2019 similarly observed significantly enhanced CD25 expression in CD8 cells activated with anti-CD3 in the presence of 10 µg/mL R848. Our studies show that the Resiquimod derivative CL097 has the same effect on Treg cells.

The strongest upregulation of CD25 was observed when CL097 was combined with IL-12, and although the effect was greater than when treating the Treg with CL097 and IL-12 on their own, the effect was not a synergistic one. Many studies have reported that IL-12 stimulates CD25 expression in immune cells to much higher levels than are reached in response to just TCR stimulation and co-stimulation with IL-12. Nguyen, Wang, & Russell, 2000 have demonstrated that IL-12 induces the up-regulation of CD25 surface expression in T cells which is dependent upon p38 mitogen-activated protein (MAP) kinase activity.

IL-12 does not only promote strong CD25 expression in T cells, but also in NK cells (Duggan *et al.*, 2018) and CD8 cells (Valenzuela, Schmidt, & Mescher, 2002). Nguyen *et al.*, 2000 demonstrated that a high concentration of IL-2 alone did not induce CD25 upregulation as much as when combining IL-2 and IL-12. This was also the case in this study since treating Treg with IL-2 and IL-12 upregulated CD25 to eight times as much as in the untreated control (treated with IL-2 only). Combining IL-12 with CL097 produced an even stronger ten-fold CD25 increase relative to the untreated control. During infection, it makes sense that IL-12 upregulates the expression of CD25 in non-Treg T cells in order for the cells to be able to bind more IL-2 enabling them to proliferate. This study however shows that IL-12 makes no discrimination between Treg and non-Treg cells and IL-12 is also well capable of upregulating CD25 expression in Tregs as well. Therefore, combining the TLR7/8 ligand with IL-12 strongly promotes activation markers in Treg, much in the same way as with non-Treg T cells.

CD25 expression was also weakly upregulated when CL097 was combined with the TLR4 ligand LPS or when the Treg were treated with LPS alone. Both ligands have been reported in order studies to upregulate CD25 in immune cells. A study conducted by Monguió-Tortajada, Franquesa, Sarrias, & Borràs, 2018 showed that monocytes (which also express both TLR4 and TLR7/8) treated seperately with R848 (from which CL097 is derived) and LPS, induced CD25 in both treatments although the effect of combining them together was not investigated. In this study, it was demonstrated that both the R848 derivative and LPS upregulated CD25 expression in Treg cells, although combining both TLR ligands did not induce a stronger upregulation. In fact, treating Treg with CL097 alone produced a greater effect. Caramalho *et al.*, 2003 demosntrated that exposure of CD4+ CD25+ cells to LPS induced up-regulation of several activation markers that

enhanced their survival/proliferation. CD25 is one of the activation markers required by Treg to proliferate since it is the receptor for IL-2. Therefore, given these observations, it is not suprising that LPS upregulates CD25 and that it promotes Treg.

Forward *et al.*, 2010 also observed that TLR7 signalling using imiquimod leads to an increase in the expression of CD25 (the receptor for IL-2). They hypothesized that IL-2, which is produced by the Tresp, then acts on Treg cells via CD25 to upregulate FOXP3 expression which in turn increases the suppressor activity of Treg cells. Indeed, in their study, FOXP3 expression in Treg cells increased significantly when treated with imiquimod and IL-2. Similarly, in this present study, treating Treg with CL097 (and IL-2) also increased (although slightly) FOXP3 expression relative to the untreated Treg. This increased expression was also observed when the Treg were treated with IL-12 and LPS alone (where CD25 was also upregulated).

Using the same hypothesis by Forward *et al.*, an increase in CD25 expression upon treating Treg with the three agents CL097, LPS and IL-12 (alone or combination) might have contributed to the increase in FOXP3 expression. This slight increase in FOXP3 expression might in turn have rendered the Treg more suppressive, as was in fact observed in this study. In contrast, TLR7/8 ligands of endogenous origin i.e. endogenous RNA did not induce CD25 in Treg (on the contrary, they reduced it). This might also explain why with this ligand of self origin, FOXP3 levels remaining unchanged, since no increase in CD25 was observed.

An interesting observation made also in this study is that Treg treated with CL097 also upregulated their expression of T-bet. This was especially evident when the Treg were treated with CL097 alone or in combination with IL-12. Expression of T-bet was not

checked in these other studies mentioned above involving Treg. However Li *et al.*, 2019 observed that, apart from upregulation of CD25, R848 also increased the expression of the transcription factors T-bet in CD8+ T cells. This study has shown for the first time that this is also the case in Treg, especially when the ligand is coupled to the differentiation cytokine for Th1, IL-12. Zhao, Zhao, & Perlman, 2012 have also reported that T-bet expression is increased in Foxp3+ Tregs after IL-12 treatment.

Despite upregulation of T-bet+, the highest percentage of treated cells in this study were FOXP3+T-bet+. For this reason, since FOXP3+ expression was maintained, the treated Treg retained a suppressor function.

Treg have been shown to transiently or permanently express T-bet while maintaining FOXP3 expression (Giovangiulio *et al.*, 2019). During inflammation (including release of IFN-γ), FOXP3 induces T-bet expression in Treg to recruit them at the sites of Th1 inflammation and control the inflammation (Koch *et al.*, 2009). These T-bet+ Treg were observed to be suppressive particularly of Th1 (Levine *et al.*, 2017). This means that these T-bet+ Treg cells generated suppress Th1 responses and do not acquire pro-inflammatory effector functions (Koch *et al.*, 2012).

Here, one might argue why, in the present study, no suppression in proliferation was observed when Tresp were co-cultured with IFN-γ-treated Treg. Here, upregulation of T-bet expression was also observed (induced by the IFN-γ added exogenously). However, FOXP3 expression was reduced with IFN-γ, which was not the case with CL097, LPS and IL-12-treated Treg. This continues to highlight how critical FOXP3 expression remains for maintenance of Treg suppression. Indeed, in the study by Levine *et al.*, 2017, it was observed that $FOXP3_{high}$T-bet+ Treg were immunosuppressive towards Th1

whereas FOXP3$_{low}$T-bet+ Treg lost their suppressive capacity. Although in this present study, treating Treg with TLR7/8 ligand (with or without LPS or IL-12) did upregulate the expression for the Th1 transcription factor (and therefore at first seem to favour a shift towards Th1), since FOXP3 expression was still maintained it was debatable whether the suppressive function of Treg had been reversed.

In fact, proliferation studies showed that the Treg suppressive power towards Tresp was increased in the presence of the TLR7/8 ligand. This increase in Tresp suppression by Treg was observed with CL097 since treating the Treg with LPS and IL-12 alone showed no further suppression of Tresp than that of the untreated co-culture control. Surprisingly, treating the Tresp monoculture with CL097 also suppressed them to levels that were comparable to those of the untreated co-culture. Therefore, CL097 was exerting an inhibitory effect on Tresp in the monoculture in the same way as untreated Treg were in a co-culture. Unlike this present study, Forward *et al.*, 2010, did not observe any effect of imiqumod alone on activated Tresp. They also observed that imiquimod did not have an effect on Treg proliferation. Likewise, in this study, CL097 did not affect Treg proliferation, meaning that the increased suppression observed in the Treg:Tresp co-cultures was not due to an increase in Treg numbers but rather due to an increase in the ability of the Treg to suppress Tresp proliferation. The increased ability might be attributed to the enhanced sequestration of IL-2 by Treg due to the increase in CD25, which might have also prompted an increase in FOXP3, as well as the upregulation of T-bet which made them more suppressive towards Th1 cells (Levine *et al.*, 2017).

CL097 and IL-12 treatment also increased the production of anti-inflammatory molecules associated with Treg function including IL-10 and TGF-β. These might also be contributing to the increased suppressive function of the Treg in the treated co-cultures.

Enhanced production of IL-10 in CD4+ T cells after treatment with resiquimod (R848) has been reported in other studies (Caron et al., 2005); although in another study, IL-10 levels dropped when treated with resiquimod (Van *et al.*, 2011). Levels of these suppressor molecules did not change much when the Tresp monocultures were treated with CL097 which means that the treated Treg were raising their concentrations. However, Lu *et al.*, 2010 found 40-fold increases in IL-10 levels in the serum of mice treated with imiquimod and they observed that, rather than being released from FOXP3+ Treg cells, the IL-10 was being released by non-Treg CD4+ cells. Their data suggests that the excessive inflammation induced by TLR agonists may result in a self-regulatory mechanism in CD4+ effector T cells and thus induction of IL-10. Therefore, it is also possible that, in this study, IL-10 was being released by the non-Treg CD4+ cells responding to the treated Treg. IL-10 production seemed to be particularly enhanced in those co-cultures treated with IL-12 (both in the absence and presence of CL097). The additional inflammatory signal induced through the exogenous addition of IL-12 might have stimulated the greatest induction of IL-10. Moreover, given that IL-10 downregulates IL-12 (Rahim, Khan, Boddupalli, Hasnain, & Mukhopadhyay, 2005), the upregulation of IL-10 is likely to be a counteracting response to the exogenous addition of IL-12.

However, treating the Treg with the CL097 and IL-12 also triggered the secretion of pro-inflammatory cytokines namely TNF-α and IFN-γ, the latter of which was also observed to be upregulated when CD4+ T cells were treated directly with R848 independent of antigen-presenting cells (Caron et al., 2005)

Production of these two cytokines in Tresp monoculture was not altered when they were treated with CL097 meaning that it is the Treg that are most probably releasing these cytokines in the co-culture or, alternatively, the Tresp are doing so only in the

presence of the treated Treg. IL-12 production, however, did not change much in co-cultures were exogenous IL-12 was not added, meaning that CL097 and LPS (alone or in combination together) do not affect the cytokine's production by CD4+ cells.

Imiquimod and resiquimod are known to stimulate DCs and macrophages to produce IL-12 (as well as IFN-α, IL-6 and IL-8) which consequently, result in induction of Th1 cell-mediated immunity via the release of TNF-α and IFN-γ (Brugnolo *et al.*, 2003). It is possible that the increase in the production levels of IL-12 was absent (unless exogenously added) in this present study because DCs and macrophages were absent in the Tresp:Treg co-cultures that were used. Nonetheless, this present study has shown that CL097 induces TNF-α and IFN-γ production in T cells independent of DCs or macrophages, but for a stronger induction one would require to add IL-12 (if DCs are absent).

Production levels of IL-4 and IL-17 in this present study did not change much in the treated cultures meaning that the CL097, LPS or IL-12 do not promote Th2 or Th17 respectively. Therefore, whilst endogenous RNA seems to favour Th2 (upregulation of CRTH2 and IL-4 production), this is not the case with the ligand for the same TLRs CL097, highlighting further that the nature of the ligand itself (and not just the toll like receptor) will determine the outcome of the immune response.

Therefore, this study suggests that treating Treg with CL097 produces a dual effect: on one part it promotes Treg suppressive function by upregulating CD25 (and to a lesser extent FOXP3) and promotes the production of Treg-associated suppressor molecules, whilst at the same time it promotes the production of Th1 pro-inflammatory cytokines. Therefore, its use in cancer therapy to modulate Treg remains controversial.

5.3.2 Evaluating the Effects of Single Stranded Poly-Uridine

There are a number of conflicting results when it comes to studies of the effects of synthetic RNA oligonucleotides on Treg. Anz *et al.*, 2010 showed that synthetic RNA oligonucleotides potently inhibited Treg cell-induced suppression through TLR7 in a sequence dependent manner. Synthetic Poly(A) RNA containing only adenosine monophosphates had no effect and only synthetic RNAs that contained guanosine and uridine were able to bring about these changes. Immunostimulatory RNA upregulated the expression of CD69 in DCs and induced the production of IL-6 by APCs. IL-6 provides protection against Treg-induced suppression and acts as a co-stimulatory factor to enhance proliferation and cell survival by counteracting activation-induced cell death (Meås *et al.*, 2020). Therefore, they concluded that inhibition of Treg cell function by immunostimulatory RNA was not due to direct targeting of Treg but was being mediated via DCs.

In contrast, Forward, Furlong, Yang, Lin, & Hoskin, 2010 observed that ssPolyU enhanced Treg-cell mediated suppression via TLR7 and the MyD88 signalling pathway. By knocking down MyD88 in Tresp cells and by activating them in the presence of anti-CD3/anti-CD28 monoclonal antibody-coated beads, the same study also revealed that this is a direct effect by TLR7 agonists present on Treg cells rather than an indirect effect mediated through other immune cells. Therefore, these observations appear to contradict those of by Anz *et al.*,2010 who proposed that TLR-7 signalling is solely mediated via DCs.

In this present study, ssPolyU did not exert much effect on the phenotype of Treg which contrasted to those achieved using CL097. For the latter, an increase in FOXP3, CD25 and T-bet expression was observed. This means that although both CL097 and ssPolyU are

TLR7/8 ligands, the nature of the ligand itself determines the effects on the Treg phenotype. Indeed, in the study by Forward, Furlong, Yang, & Lin, 2010, only imiquimod, which belongs to the same class of compounds as CL097, was reported to have caused increase in CD25 expression in Treg but ssPolyU, which was also tested in the study, is not mentioned to have exerted such an effect.

Using a concentration of 1µg/ml ssPolyU, in this present study, reduced Treg-mediated suppression and enhanced Tresp proliferation as shown in the Tresp cell proliferation assays, despite the lack of an obvious change in the phenotypic markers tested. Incidentally, Anz *et al.*, 2010 used twice this concentration (2µg/ml) of synthetic RNA to abrogate Treg-mediated immunosuppression. They did not use concentrations higher than 2µg/ml. This present study, however, also gives indications that at a higher concentration, ssPolyU might exert an opposite effect. Indeed, at a concentration of 10µg/ml, ssPolyU seemed to sustain Treg-mediated immunosuppression, although Forward *et al.*, 2010 observed 80% suppression even at concentrations as low as 1µg/ml.

The opposite effect is seen on Treg proliferation: 1µg/ml ssPolyU reduced Treg proliferation while 10µg/ml enhanced proliferation relative to untreated Treg. Therefore, Tresp proliferation might have been enhanced when Treg were treated with low concentrations of ssPolyU because the agent was causing a direct reduction in Treg cell numbers. Therefore, it is possible that ssPolyU can activate a self-regulatory feedback where at low concentrations it enhances Tresp proliferation to induce an anti-viral response, but at high concentrations, Treg are induced to suppress Tresp and thus lessen the chances of tissue damage as a result of excessive “viral” inflammation.

Forward *et al*, 2010 hypothesized that the ssPolyU-induced enhancement of Treg suppressor function might be the result of an evolutionary adaptation by single stranded RNA viruses to escape the anti-viral immune response. During infection, TLR7 Treg bind to viral (or synthetic) nucleic acids and enhance Treg cell suppressor function to potentially limit immune-mediated damage to virus-infected tissues. Alternatively, excessive activation of Treg cells through TLR7 signalling in response to viral nucleic acids might represent a potential immune evasion strategy by single stranded RNA viruses.

Although high concentrations of both CL097 and ssPolyU seem to enhance Treg-mediated immunosuppression, the effect is weaker when using ssPolyU. This difference could be attributed to weaker TLR7/8 signalling when using ssPolyU due to its higher instability when compared to CL097. Single stranded PolyU also, did not seem to effect the proliferation of Tresp in monocultures (contrary to CL097) which, coupled to the higher instability of ssPolyU, could also be attributed to the lower expression of TLR7/8 in Tresp when compared to Treg.

Anz *et al.*, 2010 did not exclude that viral RNA could also directly target Treg, given that they express TLR7 and TLR8. They debated however that delivery of nucleic acids inside the endosomal compartment is difficult in T cells including Treg. In their studies, they used cationic lipid Dioleoyl-3-trimethylammonium propane (DOTAP) for its ability to deliver RNA inside the endosomal compartment (when compared to the more commonly used lipofectamine which is more suitable to deliver RNA inside the cytoplasm). Nonetheless, they reported that no FITC-labelled RNA uptake was seen in T cells even with DOTAP whilst DCs were efficiently transfected with both DOTAP or lipofectamine. They therefore proposed that the absence of a direct effect on Treg could have been the result of poor RNA delivery inside Treg cells.

In this present study, the ssPolyU was purchased already complexed to LyoVec^{TM}. This proprietary cationic-lipid based agent is designed to facilitate the cellular entry of RNA or DNA oligonucleotides that act as endosomal TLR7/8 ligands (Katashiba *et al.*, 2011). It is evident in this study, that uptake of ssPolyU/LyoVec by the Treg did occur given that there was a change in Treg-mediated suppression of Tresp proliferation (especially when using 1µg/ml). What cannot be conclusive is that whether the ssPolyU was successfully delivered inside the cytosol or inside the endosomal compartment where the TLR7 and TLR8 are localised. Non-TLR pattern recognition receptors that recognize molecules found in pathogens (PAMPs) or released by damaged cells (DAMPs) are also found present in the cytosol. One group of these receptors are the retinoic-inducible gene (RIG-1)-like receptors (Amarante-Mendes *et al.*, 2018). Engagement of these pattern recognition receptors induces co-stimulatory signals for T cells.

Anz *et al.,* 2010 observed that Treg function was lost when treated with stimulatory RNA but this effect was independent of TLR-signalling. They discovered that both Treg and Tresp expressed retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated protein 5 (MDA-5) both of which are retinoic-inducible gene (RIG-1)-like receptors. They suggested that activation of these RNA-sensing receptors in Treg cells may directly block their suppressive function because when MDA-5 was deficient in Treg cells, their suppressive function was not lost when infected with viral RNA. Therefore, it is possible that the results achieved in this present study might have been mediated by these cytosolic RNA receptors rather than TLRs in the endosome compartment, or it might have also been mediated by both.

Whatever the mechanism, at a concentration of 1µgml ssPolyU, the results of these studies are in agreement with those of Anz *et al.*, 2010 in that Tresp proliferation was

enhanced and, additionally, it has also shown that Treg proliferation was decreased. When a stronger inflammatory stimulus was induced using a higher concentrations of ssPolyU, Treg might have been re-induced to exert suppression and control the proliferation of Tresp. Therefore the intensity of receptor signalling might result in different outcomes promoting either Tresp or Treg. Activation of TLRs has been reported to either increase or reduce Treg function with opposite consequences depending on factors such as the specific TLRs involved but also on the concentration of the TLR ligands (Nyirenda, O'Brien, Sanvito, Constantinescu, & Gran, 2009). High doses of TLR ligands can result in overactivation of TLRs which disrupts the immune homeostasis leading to autoimmunity and inflammatory diseases (El-Zayat, Sibaii, & Mannaa, 2019). Indeed, overactivation of TLR3, TLR7, and TLR9 is causative for inflammatory disease in mice (McAlpine *et al.*, 2018). Since uncontrolled or prolonged activation of TLRs can lead to inflammatory diseases, TLR activation is tightly controlled to prevent sustained activation of the receptors and enhanced production of pro-inflammatory cytokines. These include signal-specific regulators that inhibit TLR signalling and gene-specific regulators that suppress TLR target gene transcription. These regulators render TLR ligands insensitive or hyporesponsive to subsequent ligand stimulation. This is known as TLR tolerance which was initially characterised using LPS where repeated activation of TLR4 led to refractoriness towards further stimulation (Geisel *et al.*, 2007). Later, it was discovered that TLR tolerance also occurs in TLRs 2, 5 and 7 (Broad, Kirby, & Jones, 2007; Geisel *et al.*, 2007; Hayashi *et al.*, 2009).

TLR signalling is negatively regulated by several negative regulators but they function via sequestration of signalling molecules, blockade of signalling molecules recruitment, degradation of target proteins or inhibition of transcription (Lai & Gallo, 2008). A detailed

review of the negative regulators involved in TLR tolerance can be found in Lai & Gallo, 2008.

However, Souza-Fonseca-Guimaraes, Parlato, Fitting, Cavaillon, & Adib-Conquy, 2012 also discovered that Treg also play an active role in TLR tolerance. After exposing NK cells to TLR2 (Pam3CysSK4), 4 (LPS) and 9 (CpG DNA) ligands, they observed that the cells became tolerant to signalling after repeated exposure to these TLRs. Interestingly, they found that this tolerance was induced by Treg and was mainly mediated by TGF-β.

Therefore, it is possible that in this present study excessive TLR7/8 signalling in Treg triggered by high doses of ssPoly enhanced their proliferation and suppressor function to oppose excessive TLR signalling.

This might also possibly explain why certain TLRs (including TLR7 and TLR8) are more highly expressed in Treg when compared to non-CD4+ Treg cells (Caramalho *et al.*, 2003; Kabelitz, 2007; Komai-Koma, Jones, Ogg, Xu, & Liew, 2004). Competitive binding and sequestration of the ligand by the Treg will reduce the chances of the ligand from binding to effector T cells and thus preventing excessive inflammatory responses.

It is unlikely that the functional modulation of Treg by ssPolyU was being mediated via soluble molecules present in the cell culture supernatants. This is because the concentration of anti-inflammatory molecules, granzymes and pro-inflammatory molecules did not change much in the co-cultures treated with 1µg/ml as well as 10µg/ml of ssPolyU when compared to the untreated co-cultures (except IFN-γ). Significant changes in cytokine productions were only observed when treatment involved coupling ssPolyU with IL-12. Under this treatment, the concentration of IL-10, granzyme B, TNF-α, IFN-γ and IL-4 increased. This means that this combinatory treatment resulted in the

induction of both pro-inflammatory (TNF-α and IFN-γ) and anti-inflammatory molecules (IL-10, granzyme B and IL-4). Granzyme B and IL-4 are described here as anti-inflammatory because granzyme B can be released by Treg to suppress Tresp whilst IL-4 can be released by Th2 to inhibit Th1 responses. Therefore, ssPolyU alone is not sufficient to induce changes in the production of these molecules and IL-12 is also required. This requirement was also important when using CL097. The main source of IL-12 are dendritic cells. Since these immune cells were absent in the Treg:Tresp co-cultures, ssPolyU only became active when the IL-12 was sourced and added exogenously. This would however be different in the tumour where dendritic cells are present (Fu & Jiang, 2018). Oth, Vanderlocht, Elssen, Bos, & Germeraad, 2016 observed a positive correlation between the amount of IL-12 produced by monocytic-derived DC and the differentiation of naïve CD4+ T cells to Th1. They highlighted that IL-12 production is a requirement for this, and failure of DC to produce enough IL-12 might be one of the factors that limits the effects of certain anti-cancer therapies.

Future work should focus on trying to understand the mechanisms and which receptors synthetic RNA uses to act directly on Treg. Low doses of single stranded PolyU and other synthetic immunostimulatory RNA bound to a suitable transfection agent might actually offer potential in Treg-targeting anti-cancer therapy provided that the right dosage is administered and provided that all the requirements for its action are present. Since high doses of the agent will re-activate Treg suppressor function, the modulatory effects of ssPolyU on Treg can be self-controlled and therefore the risks of tissue damage *in vivo* due to excessive inflammatory signals will be minimised.

5.4 Limitations

One of the first practical issues noted was blood donor to donor variability. Treg counts and the percentage of Treg from CD4+ was found to vary from donor to donor. Their proliferative abilities and phenotype were also found to vary. Although the donor's general health was verified during the process of blood donation, factors such as age, sex, genetic constitution, environmental factors and lifestyle affect the frequencies, subset distribution and functional competence of lymphocytes, including Treg. For instance, there is evidence of age-related loss of Treg function. Data on these influencing factors was missing, since due to ethical issues, no information on the donors was provided whilst collecting the buffy coats from NBTS. Pooling of lymphocytes from different blood donors to reduce donor to donor variability was also not possible as this would have produced a mixed lymphocyte reaction.

A major limitation in the study was the inherent differences between *in vitro* conditions and the actual conditions present in an *in vivo tumour* environment. One of the weaknesses associated with *in vitro* experiments is that many times, they fail to replicate the complex environment that would normally be present *in vivo* making it difficult to extrapolate the results and therefore predict behaviour in the intact organism or in the tumour microenvironment.

In the present study, the Treg were only co-cultured in the presence of other CD4+ cells from the same donor. These Treg might have behaved differently in the presence of other cells present in the complex tumour micro-environment. Tumour microenvironments are extremely heterogenous and this complexity is all dependent on the release of soluble factors by the various types of cells that would be present. For instance, treating the Treg with IFN-γ suggested that the Treg might have acquired Th1-like characteristics, but their

interaction with CTLs and whether the latter's cytotoxic function is enhanced (which is ultimately what would result in tumour elimination) was not tested.

Moreover, *in vitro* experiments do not address problems that may arise from systematic modulation of Treg which might lead to autoreactivity if numbers/reactivity are changed outside of the tumour micro-environment. Therefore an adequate administration route would need to be developed for any modifying factors, which could involve either direct injection of the agent in the tumour or modifying the Treg's cells *in vitro* and then inject them into tumour .

Finally, another limitation was that the actual mechanism through which the ligands were mediating their effects was not determined. Although PEI has been used in a number of studies to deliver nucleic acids to the endosomal compartments, it could not be ascertained whether the nucleic acids were indeed successfully delivered to the TLRs localised on the endosomes. During the method optimization phase, flow cytometry using fluorescent oligonucleotides was carried out to confirm that PEI successfully delivered the nucleic acids inside the cells. However, their localisation to the endosomal compartment would need to be confirmed using other techniques such as fluorescent microscopy, with specific markers targeting the cell compartment. Ascertaining whether the effects were being mediated via TLR and not through some other alternative mechanism would require gene knockout/siRNA experiments on mice or in cellular co-cultures.

6 CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

Treating Treg with IFN-γ was shown to reduce the expression of FOXP3 and CD25 markers typically highly expressed in Treg, whilst upregulating the expression of the Th1-master transcription factor T-bet.

IFN-γ also abrogated Treg-mediated immunosuppression as Tresp cells co-cultured in the presence of the treated Treg were observed to proliferate uninhibited. The decrease in FOXP3 expression seems to be the most plausible reason for the decrease in suppressor function of Treg since treatment with IFN-γ did not affect their proliferation. Treating Tresp directly with IFN-γ negatively impacted their proliferation which means that therapeutic attempts using IFN-γ to boost anti-tumour responses should focus on treating the Treg only.

IFN-γ also promoted the release of the Th1 effector cytokines TNF-α which could act as a double edged sword in that it can be an anti-tumour as well as pro-tumour agent. IFN-γ was also found to promote the co-secretion of other cytokines that inhibit Th1 including IL-10 and IL-4 probably in response to counter regulate the Th1-promoting environment induced by IFN-γ.

Early studies have already revealed the extensive anti-tumour potential of IFN-γ since it augments cytotoxic function of NK cells and CTLs. Additionally, this study has also shown that IFN-γ can be used to modulate Treg since it downregulates FOXP3 expression, thus Treg function, and therefore preventing inhibition of NKs and CTLs. Moreover, it was also shown to have the ability to activate self-controlling mechanisms that prevent detrimental overactivation of the immune response.

This study has also shown that nucleic acids of endogenous origins are also immunomodulatory. In this study, RNA and DNA isolated from human cells were used a putative ligands for TLR7/8 and TLR9 respectively. Their uptake by cells into the endosomal compartment was facilitated by complexing with PEI.

Transfecting Treg with endogenous RNA decreased the expression of CD25 and upregulated the Th2 marker CRTH2. The upregulation of the latter whilst still maintaining FOXP3 suppression suggests that the Treg attained a mix of Treg and Th2 characteristics, and therefore retained a suppressor function towards Th1. Indeed, the treated Treg still suppressed Tresp proliferation. The downregulation of CD25 might explain the slight reduction in Treg proliferation when these were treated with endogenous RNA.

The endogenous ligand still generated some desirable results including the decrease in suppressor molecules IL-10 and Granzyme B and an increase in the Th1-differentiation cytokine IL-12. The increased production in the latter cytokine might have been triggered in response to counteract the induction of the Th2 response by IL-4, which was also observed to increase in the treated co-cultures. Overall, endogenous RNA is not be a suitable TLR ligand candidate for cancer therapy since it seems as though it favours an immunosuppressive response rather than an anti-tumourigenic one.

Transfecting Treg with human DNA did not induce any changes in Treg phenotype or shifts in the expression of the markers tested. The Treg still maintained expression of FOXP3 and CD25. Treg suppressor function on Tresp was nonetheless enhanced relative to the untreated co-culture which shows that functional modulations are not always accompanied by phenotypic changes.

Treating Treg with 1µg/ml endogenous DNA slightly enhanced Treg proliferation. This might also explain the higher concentrations of Treg-associated suppressor molecules such as

IL-10, TGF-β and Granzyme B detected in co-cultures where Treg had been treated with this concentration of DNA. However, the increase in these suppressor molecules did not seem to be the main mechanism by which the Tresp were being suppressed. Therefore, the mechanism by which endogenous DNA boosted Treg suppressor function remains unresolved.

Additionally, the co-culture where Treg had been transfected with 1μg/ml DNA produced higher levels of TNF-α, IFN-γ, and, as with endogenous RNA, IL-12. Nevertheless, the increase in these Th1 pro-inflammatory molecules was not enough to counteract the intensified suppressor action of treated Treg.

Therefore, endogenous DNA also does not seem to be a suitable TLR ligand candidate for cancer treatment. It is suggested that the nature and methylation status of the DNA determines what kind of inflammatory response is generated. Methylated DNA of mammalian origin seems to elicit primarily an anti-inflammatory responses and might therefore be more appropriate in the treatment of autoimmune diseases where Treg function needs to be enhanced.

This present study has shown that the synthetic TLR7/8 ligand derivative of Resiquimod, CL097, initially thought to suppress Treg, generated some rather unexpected results. It was observed that treating Treg with CL097, especially in combination with IL-12, but also with LPS, mildly upregulated the expression of FOXP3 but highly upregulated the expression of CD25 in a similar way as it does in non-Treg T cells. Therefore, CL097, LPS and IL-12 are unable to discriminate between Treg and non-Treg T cells and will also enhance CD25 expression in Treg.

CL097 combined with IL-12 (as well as IL-12 alone) also upregulated the expression of T-bet which rather than rendering the Treg more Th1-like, endowed them with a greater suppressor function. It was hypothesized that this observation was due to the fact that the

Treg cells that upregulated T-bet retained FOXP3 expression and therefore attained a FOXP3+T-bet+ phenotype. Cells with such phenotypes have been found to be more suppressive towards Th1 than FOXP3+ cells that do not express T-bet.

CL097 (alone or in combination with LPS or IL-12) did not enhance Treg expansion which further supports the hypothesis that the enhanced Treg suppressor function was not due to a boost in Treg numbers, but rather due to the upregulation of the Treg markers FOXP3 and CD25, and the co-upregulation of T-bet.

CL097 alone does not particularly affect cytokine secretion unless combined to IL-12. Once combined with IL-12, treatment enhanced the production of IL-10 and TGF-β implicated in Treg function.

Moreover, this present study has shown that CL097 and IL-12 upregulated the production of TNF-α and IFN-γ by T cells, but the strongest effect was observed when the two were combined together. Therefore, this shows that the robust pro-inflammatory responses that CL097 generated were partly dependent on the presence IL-12 which is usually sourced by DCs.

Unlike the endogenous nucleic acids used, CL097 alone was incapable of inducing IL-12 production in T cells and therefore, *in vivo,* the presence of IL-12-secreting DCs would be required. The upregulation of these pro-inflammatory cytokines was insufficient to overcome the enhanced suppression mediated by treated Treg.

Therefore, the use of imidazoquinoline compounds such as CL097 in cancer treatment remains controversial. Although other studies have shown that such compounds induce Th1 and CTLs and therefore boost the anti-tumourigenic properties of the immune system,

CL097 also targets directly and enhances Tregs which oppose the action of the former, especially if present in substantial amounts within the tumour.

Contrary to CL097, synthetic single stranded polyuridine, also a ligand for TLR 7 and 8, did not induce any phenotypic changes in treated Treg and expression of FOXP3 and CD25 remained unchanged.

However, at a concentration of 1µg/ml ssPolyU, Tresp proliferation in the presence of the treated Treg was improved. At this concentration, Treg proliferation was slightly reduced making this the most plausible reason why Tresp proliferation was enhanced. However, at a high concentration of 10µg/ml, Treg suppressor function was restored and it was hypothesized that this occurred in order to regulate excessive TLR signalling and prevent inflammatory diseases and autoimmunity.

Single stranded PolyU alone (at a concentration of 1µg/ml but not 10µg/ml) only seems to induce increases in IL-17. Treatment with the TLR7/8 ligand alone did not seem to significantly alter cytokine secretion relative to the control. For it to exert any effect, the presence of IL-12 was a requirement. In fact when combined with IL-12, an increase in IL-10, granzyme B, TNF-α, IFN-γ and IL-4 was observed. Same as with CL097, synthetic ssPolyU alone was unable to induce IL-12 production. Therefore, as with CL097, ssPolyU induces changes in cytokine production only in the presence of IL-12, which in a tumour microenvironment would be supplied by APCs.

Low doses of single stranded PolyU were therefore found to be effective in modulating Treg function for the benefit of cancer immunotherapy. Future attempts should focus on determining the most appropriate delivery vehicle for efficient uptake by cells as well as develop an appropriate treatment regime (e.g. frequent low doses) which is important in

order to prevent excessive TLR signalling which would render the treatment ineffective and would re-promote Treg function.

6.2 Future Work

The next phase of *in vitro* studies for the future should be try and understand better the dose-dependent effects of ssPolyU to determine the point up to which its effect on Treg is reversed. Moreover, since it was not tested, it would be interesting to check what effects will 1μg/ml ssPolyU have on Treg if combined with IL-12.

IFN-γ was found to reduce FOXP3 expression, enhance T-bet expression and reduce Treg-mediated suppression of Tresp, whilst low doses of ssPolyU were found to reduce Treg numbers. Therefore, it would be worthwhile testing a combinational treatment using the two agents.

This study has shown that "normal" mammalian methylated nucleic acids elicit an anti-inflammatory response and maintain (or enhance) Treg function. It would also be interesting to test the effect of abnormal nucleic acids extracted from tumour cells to check whether they will induce the same changes in Treg as "normal" nucleic acids did. DNA methylation patterns becomes variable in tumour cells and it is possible that this might elicit different responses. This would be important to notice whether the body recognises damage to tumour tissue as different from damage to other normal tissue by excessive inflammatory reactions.

A very important next step would be to co-culture *in vitro* the Treg treated with IFN-γ and/or low doses of ssPolyU, not only with autologous CD4+ T cells, but also with CTLs and tumour cells (all derived from a tumour patient or at least animal model) to check whether

the cytotoxic activity of CTLs on tumour cells is enhanced in the presence of the treated Treg. This set up would be important to check whether the IFN-γ-treated Treg that supposedly acquired Th1 characteristics did indeed promote CTL activity. This would also be important to check whether the direct reduction in Treg numbers after treatment with 1µg/ml ssPolyU would result in improved CTL activity.

The ultimate phase would then be to move on to *in vivo* studies using mouse models to check the efficacy of the treatment in different types of complex tumour microenvironments. This is also important in order to determine the right treatment doses and the best route of administration in order to prevent extensive modulation of Treg in the periphery.

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8 APPENDICES

Appendix I: Ethical Consent Approval to Obtain Blood Bags from NBTS

L-Università ta' Malta

Faculty of
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University of Malta
Msida MSD 2080, Malta

Tel: +356 2340 1879/1891/1167
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Ref No: **FRECMDS_1718_073**

Monday 10th December 2018

Ms. Oriana Mazzitelli
Room 404, Centre of Molecular Medicine
and Biobanking

Dear Ms. Mazzitelli,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

Modulating Regulatory T cells for the Effective Application of Cancer Immunotheraphy

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Professor Pierre Mallia
Chairman
Research Ethics Committee

Appendix II: Information Sheet and Consent Form for Blood Donor (English)

Participant Information Sheet And Consent form - Cancer Immunotherapy and Stem Cell Research (University of Malta)

Information.

Dear Blood Donor, whilst I am sure you know you are very appreciated for the role you play in helping patients with various diseases through your donation, it is not always the whole amount of the blood you give that is used, and when this is the case, we would like to give you the opportunity to allow us to use the remaining parts of your blood to conduct research into treating cancer or making stem cells to help many different degenerative diseases (like ALS).

Your white cells are usually removed from the donated blood which is given to patients (as they may harm them) and these can be used for this research. In other times, these white cells are needed and will be kept to help patients with diseases. We will only consider using those which the blood bank does not need.

It is essential that you understand that by taking part in this research, nothing new or different will be done to you or your blood donation. The blood donation procedure with questionnaire, donation, etc. will proceed just as usual. It is just that in those cases where parts of the blood will not be used, it will be used for research at the University of Malta. We need to get your permission below for this. There is no obligation to give us permission and refusal to participate will involve no penalty; however, your participation would be much appreciated.

You or your blood sample will not be in any way identified (no genetic testing is done and we cannot tell and nor do we want to know whom the blood used belonged to). However, when the researcher starts using the blood, consent cannot be withdrawn since the blood sample is no longer linked to the consent form and the sample will then be anonymous. There is no risk involved in your participation, nor will you receive any information about your blood. There may be future benefits to patients with cancer and degenerative disease but not directly to you.

Consent

I, undersigned, hereby declare that I have understood the Patient Information Sheet provided, have had any queries clarified and agree to allow unused parts of my donated blood to be used in this research project.
I am also aware that the participation is voluntary, and that no data regarding me will be collected as blood used will be not identifiable as mine.

_____ _____
Participant's Signature **Date**

_____
Participant's Name. (IN BLOCKS)

The name will only be kept as part of the database in the blood bank/University of Malta so that samples which may be used (as opposed to being discarded) will be matched against this database - no identification of the individual sample will be performed.

Oriana Mazzitelli
Email: oriana.mazzitelli.07@um.edu.mt
Mob 79250456

Simona Maria Pagano
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79361644

Supervisor. (To whom any questions may be directed)

Prof Pierre Scembri Wismayer.
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mob. 99859159, tel. 23402797

Appendix III: Information Sheet and Consent Form for Blood Donor (Maltese)

Fuljett ta 'Informazzjoni tal-Parteċipant u Formola ta' Kunsens – Cancer Immunotherapy and Stem Cells Research (Università ta' Malta)

Informazzjoni

Għażiż Donatur tad-Demm, għalkemm jiena ċert li taf li int apprezzat ħafna għar-rwol li inti tilgħab biex tgħin lil pazjenti b'diversi mard permezz tad-donazzjoni tiegħek, mhuwiex dejjem l-ammont kollu tad-demm li tagħti, jista 'jintuża, u meta dan huwa l-każ, nixtiequ nagħtuk l-opportunità li tippermettulna li jintuża l-bqija tad-demm tiegħek biex twettaq riċerka dwar il-kura tal-kanċer jew li noħolqu *stem cells* li jgħinu ħafna mard deġenerattiv differenti (bħal ALS).

Iċ-ċelloli bojod tiegħek ġeneralment jitneħħew mid-demm li jingħata lill-pazjenti (minħabba li jistgħu jagħmlu ħsara lilhom) u dawn jistgħu jintużaw għal din ir-riċerka. Fi drabi oħra, dawn iċ-ċelloli bojod huma meħtieġa u se jinżammu biex jgħinu lill-pazjenti bil-mard. Aħna ser nikkunsidraw biss l-użu ta 'dawk li l-bank tad-demm m'għandux bżonn.

Huwa essenzjali li inti tifhem li billi tieħu sehem f'din ir-riċerka, xejn ġdid jew differenti jsir lilek jew lid-donazzjoni tad-demm tiegħek. Il-proċedura tad-donazzjoni tad-demm bi kwestjonarju, donazzjoni, eċċ se tipproċedi bħas-soltu. Huwa biss li f'dawk il-każijiet fejn partijiet tad-demm mhux se jintużaw, li se jintużaw għar-riċerka fl-Università ta 'Malta. Għandna bżonn nikseb il-permess tiegħek hawn taħt għal dan. M'hemm l-ebda obbligu li tagħtina l-permess u r-rifjut li tipparteċipa ma tinvolvi l-ebda piena iżda hija apprezzata ħafna.

Int jew il-kampjun tad-demm tiegħek bl-ebda mod ma jiġi identifikat (ma jsir l-ebda test ġenetiku u ma nistgħux ngħidu u lanqas ma rridu nkunu nafu ta' min kien jappartjeni d-demm). Madankollu, meta r-riċerkatur jibda juża d-demm, il-kunsens ma jistax jiġi rtirat peress li l-kampjun tad-demm m'għadux marbut mal-formola tal-kunsens u l-kampjun imbagħad ikun anonimu. M'hemm l-ebda riskju involut fil-parteċipazzjoni tiegħek, u lanqas ser tirċievi xi informazzjoni dwar id-demm tiegħek. Jista 'jkun hemm benefiċċji futuri għal pazjenti b'kanċer u mard deġenerattiv iżda mhux direttament lilek.

Kunsens
Jiena, hawn taħt iffirmat, b'dan niddikjara li qrajt il-Fuljett ta 'Informazzjoni tal-Pazjent ipprovdut, kelli xi mistoqsijiet miġbuha u aċċettajt li l-partijiet mhux użati tad-demm donat tiegħi jintużaw f'dan il-proġett ta' riċerka.
Jiena konxja wkoll li l-parteċipazzjoni hija volontarja, u li l-ebda dejta li tirrigwarda lili ma tinġabar peress li d-demm użat ma jkunx identifikabbli li tiegħi.

_____ _____
Firma tal-Parteċipant **Data**

_____
Isem tal-Parteċipant (FI BLOKKIJIET)

L-isem jinżamm biss bħala parti mid-database fil-bank tad-demm / Università ta 'Malta sabiex il-kampjuni li jistgħu jintużaw (minflok jintremew) ikunu mqabbla ma' din id-database - ma ssir l-ebda identifikazzjoni tal-kampjun individwali.

Riċerkaturi

_____
Oriana Mazzitelli
Email: oriana.mazzitelli.07@um.edu.mt
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_____
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79361644

Superviżur. (Lil min tista issaqsi jekk hemm xi mistoqsijiet)

_____
Prof Pierre Scembri Wismayer.
email: pierre.schembri-wismayer@um.edu.mt.
mob. 99859159, tel. 23402797

Appendix IV: Recipes of Solutions and Buffers

Erythrocyte Lysis Buffer (1000ml)

1. Add 7.49g Ammonium Chloride (NH_4Cl)
2. Add 0.745g Potassium Chloride (KCl)
3. Add 0.04g EDTA
4. Add 0.79 Ammonium bicarbonate (NH_4HCO_3)
5. Dissolve in 1000ml distilled H_2O
6. Autoclave

Isolation Buffer (1000ml)

1. Dissolve 1 sachet PBS (Sigma) in distilled H_2O
2. Add 0.09g EDTA (equivalent to 1mM)
3. Autoclave
4. Add 20ml of sterile FBS (equivalent to 2%)

Blocking Agent (1000ml)

1. Dissolve 1 sachet PBS (Sigma) in distilled H_2O
2. Add 0.18g EDTA (equivalent to 2mM)
3. Autoclave
4. Add 100ml sterile FBS (equivalent to 10%)

Staining Buffer (1000ml)

1. Dissolve 1 sachet PBS (Sigma) in distilled H_2O
2. Add 0.09g EDTA (equivalent to 1mM)
3. Autoclave
4. Add 20ml of sterile FBS (equivalent to 2%)

PEI diluent (1000ml)

1. Add 8.77g NaCl (equivalent to 150mM)
2. Dissolve in 1000ml distilled H_2O
3. Autoclave

Appendix V: Protocol for the Cryopreservation and Thawing of Lymphocytes

Freezing Cells

For long term storage, the cells were frozen at -80^{o}C. In order to prevent the intracellular formation of ice crystals and damage the cells, the freezing medium must contain a cryopreserving such as DMSO. Moreover, intracellular ice formation is also prevented if the cooling process is slow.

The freezing medium which was used when freezing cells was composed of 50% RPMI-1640, 40% FBS and 10% DMSO. This quantity of DMSO was used as higher concentrations of DMSO is known to be toxic to the cells. The freezing medium was kept at a temperature of 4^{o}C before adding to the cells and was prepared fresh each time freezing was required.

The cells were first counted and then centrifuged at 250 × g for 5 minutes. A suitable volume of freezing medium was then added dropwise to the cells such that the final concentration of cells was equal to 1-5 × 10^{7} cells/ml. The cells in freezing medium were then dispensed in cryovials, placed in a Jablo box (to delay cooling) and placed in a -80^{o}C freezer. For longer storage, the cells were placed in liquid nitrogen.

Thawing cells

To minimize damage to the cells and the time the cells were kept in DMSO, fast thawing was performed. The cryovials were removed from the -80^{o}C freezer/liquid nitrogen and thawed in a 37^{o}C water bath. The cells were then rapidly diluted in pre-warmed complete medium (RPMI-1640 with 10% FBS, 1% Pen-strep and 1% Amphotericin B). The cells were then centrifuged at 250 × g for 5 minutes and resuspended in a suitable amount of complete medium. Cell count and viability was then measured. Thawed cells were left incubated at 37^{o}C and 5% CO_2 for at least 2 days after

thawing and before further manipulation, in order to minimize extensive stress to the cells and to allow them rest.

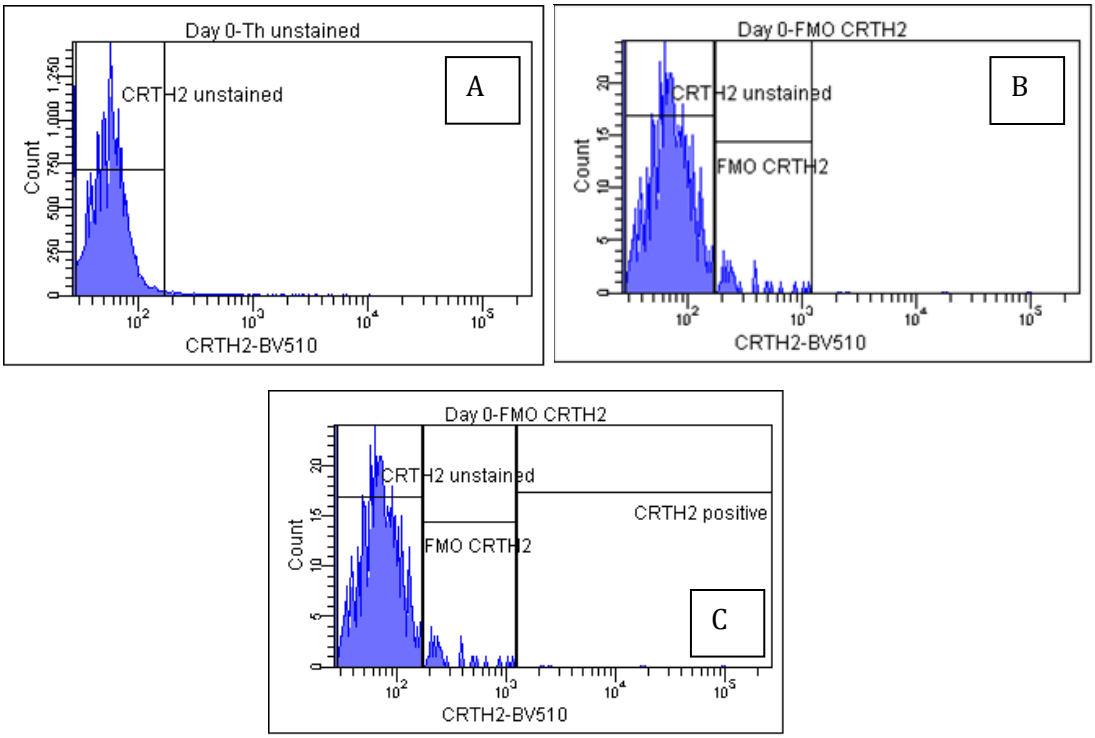
Appendix VI: Determining Positive Expression using Fluorescent Minus One (FMO) Controls

In multicolour analysis, there is substantial overlap between spectra which could result into a false-positive result during flow cytometric analysis due to spread of multiple fluorochromes in a given channel. In order to subtract this spectral overlap and identify the actual positive population, fluorescent minus one (FMO) controls were set before analysing any samples on the BD FACS Aria™ III.

An FMO control contains all the fluorochromes in a panel, except for the one that is being measured. For example, if one is measuring the spread of multiple fluorochromes in the PE channel, the FMO would include all the antibodies with the other fluorochromes but not the antibody conjugated to PE.

The FMO for every fluorochrome was set up. The cells were fixed, stained with extracellular markers, permeabilized and stained with intracellular marker following the procedure described in section 2.14.1 and 2.14.2. An unstained control was also prepared. Histogram plots of cell count versus fluorescent intensity for every fluorochrome were plotted on the BD FACSDIVA™.

The unstained cells were first loaded into the BD FACS Aria III analyser, and any fluorescence detected was regarded as autofluorescence by the cells. Next, the FMO for the different fluorochromes were loaded in series and fluorescence due to spread of other fluorophores was gated by setting interval gates. This was important in order to identify the actual positive population in subsequent analysis. For future analysis, only the fluorescence that was above the fluorescence detected by individual FMO was regarded as positive. Figure 6 shows how the FMOs were set up and how the gating was done.



Histogram A shows the autofluorescence detected by the unstained sample. Histogram B shows the fluorescence detected by the FMO for the BV510 fluorochrome which was prepared by staining the cells with all the different fluorophores in the antibody panel except for the antibody conjugated to BV510. Therefore, fluorescence in the FMO of BV510 that is not due to autofluorescence is due to spread from other fluorophores. Histogram C shows the interval gating at which fluorescence from cells must fall in order for the result to be considered as positive.

Appendix VII: Setting up Compensation

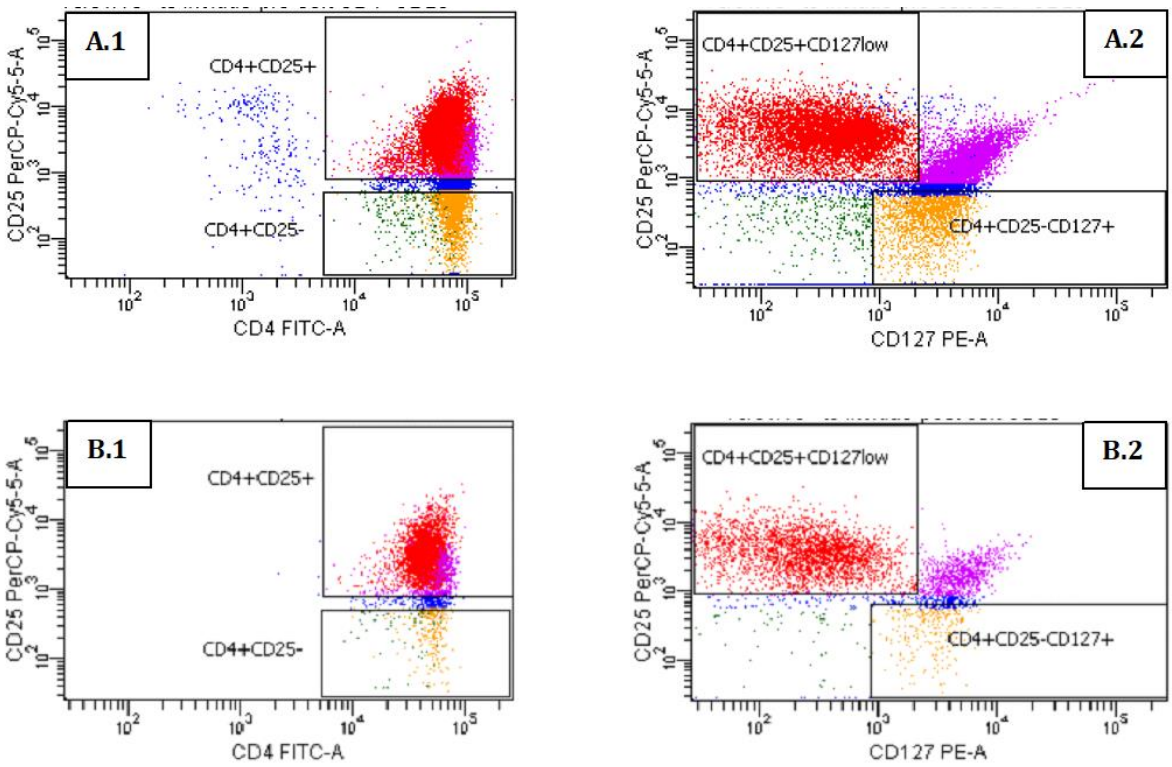
Compensation on the FACS Aria III (BD Biosciences) was set up using BD CompBeads (BD Biosciences) which consisted of Anti-Mouse Ig κ microparticle beads that bind any mouse κ light chain-bearing immunoglobulin, and the Negative Control beads which have no binding capacity.

Eight FACS tube (for every fluorophore) were prepared. To each tube, 1 drop of Anti-Mouse Ig κ beads was added and diluted with a few drops of sheath fluid (BD Biosciences). 1µl of the antibody conjugated to different fluorophores (AF-488/AF-647/APC-Cy7/BV421/PE/PerCP-Cy5.5/BV510) was added separately to each FACS tube. Then, 3 drops of negative control beads were added to each tube. The individual stains were recorded as follows. Using the FACS DIVA™ software, a worksheet for compensation controls was created by selecting *Experiment > Compensation > Create compensation controls*. At this point, a new experiment with compensation controls was created with different tabs for each dye e.g. AF488, APC-Cy7, PE etc. Each tab contained a dot-plot of FSC-A versus SSC-A plot and an adjacent histogram of count versus fluorophore fluorescence.

Each tab was selected and the tube containing the respective fluorophore loaded onto the machine. A low flow rate was used. The bead population was gated on the FSC-A vs SSC-A plot. On the adjacent histogram, two peaks were evident: one representing the negative bead population while the other representing the positively-stained anti-Mouse Ig κ beads. Interval gates were created to represent the negative and positive populations. This procedure was repeated for every fluorophore.

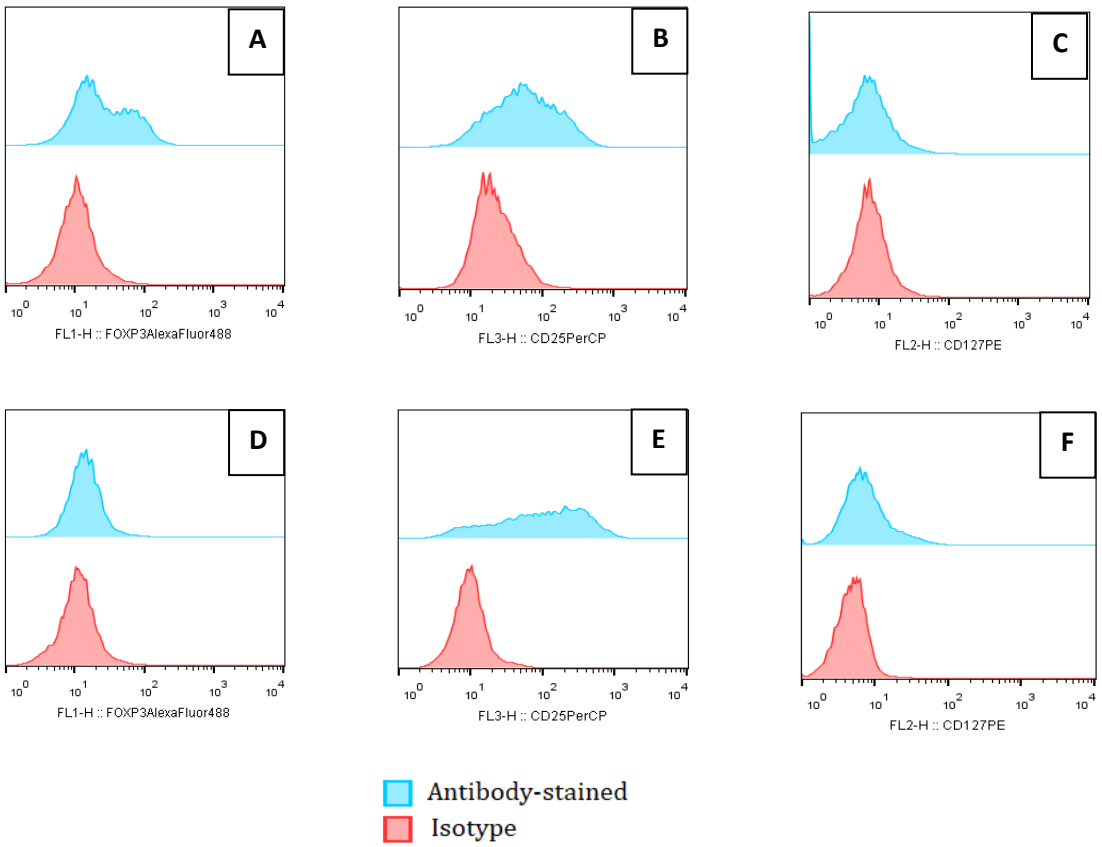
Once all the eight FACS tubes were loaded, the *Experiment > Compensation set up > Calculate compensation* option was selected and the software automatically calculated the spectral overlap. The values for spectral overlap were then copied and used in every subsequent analysis.

Appendix VIII: Confirming Purity of FACS-Isolated CD4+CD25-CD127+ and CD4+CD25+C127-



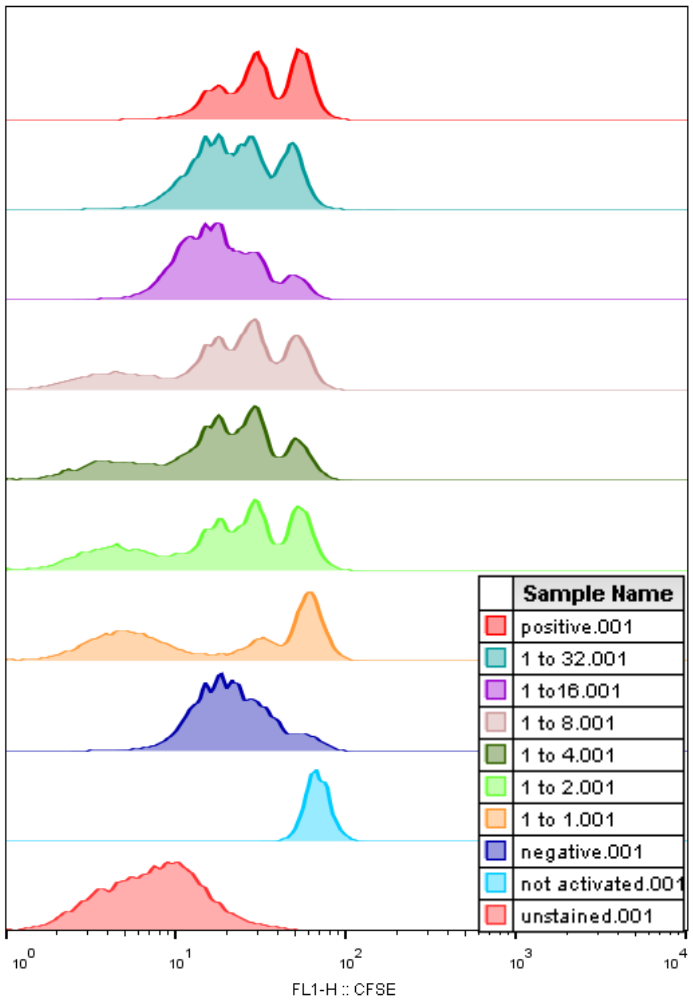
Dot plots showing expression of CD4, CD25 and CD127 Treg. Dot plots A.1 and A.2 show expression of the markers following isolation of Treg using magnetic beads only. There is still some contamination from CD4+CD25-CD127+ cells. Dot plots B.1, B.2 show expression of the same markers after FACS. There is now little contamination from CD4+CD25-CD127+. Cells were sorted on the BD FACS Aria III and analysis carried out using FACS DIVA™ software.

Appendix IX: Confirming Expression of FOXP3 In Isolated Treg



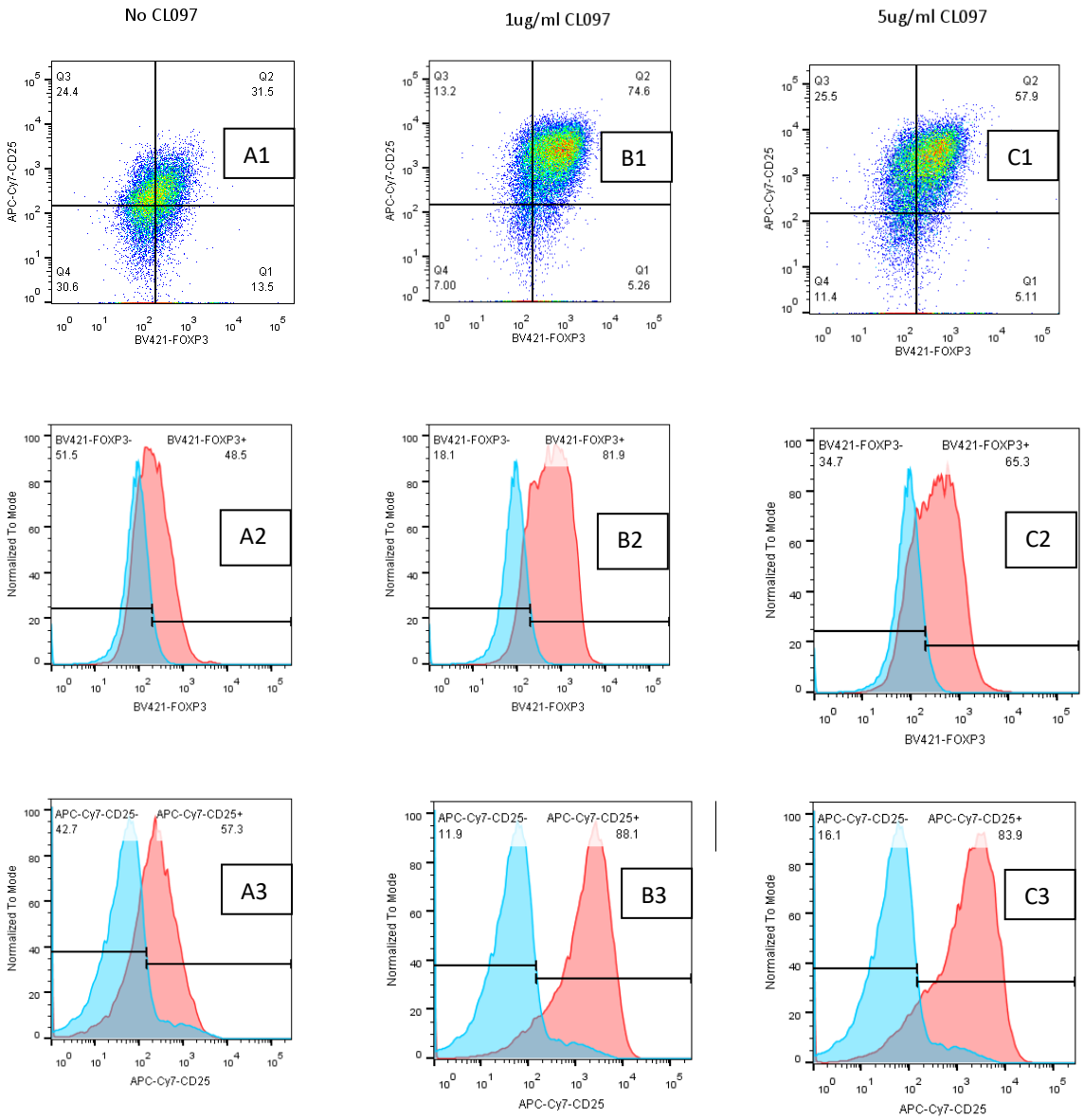
Histogram showing percentage of total gated events versus fluorescent intensities of FOXP3, CD25 and CD127 on day 6 post-expansion. The results of only one replicate are shown. Top three histograms (A-C) show data from the expanded CD4+CD25+ fraction (which were isolated from PBMC using magnetic beads followed by FACS) while the bottom three histograms (D-F) show data from the expanded CD4+CD25- fraction (which was isolated from PBMC using magnetic beads only). Antibody stained sample (represented in blue) are the samples stained with the three antibodies for the markers while the isotype samples (represented in pink) represents fluorescence arising from immunoglobulin non-specific binding. Both fractions were loaded and run using the same instrument settings and the data collected using FACS Diva™. The histograms shown were then generated using FlowJo™. The total number of events were gated on the singlet lymphocyte population as identified from the FSC vs. SSC dot plots.

Appendix X: Confirming Suppressive Function of Isolated Treg



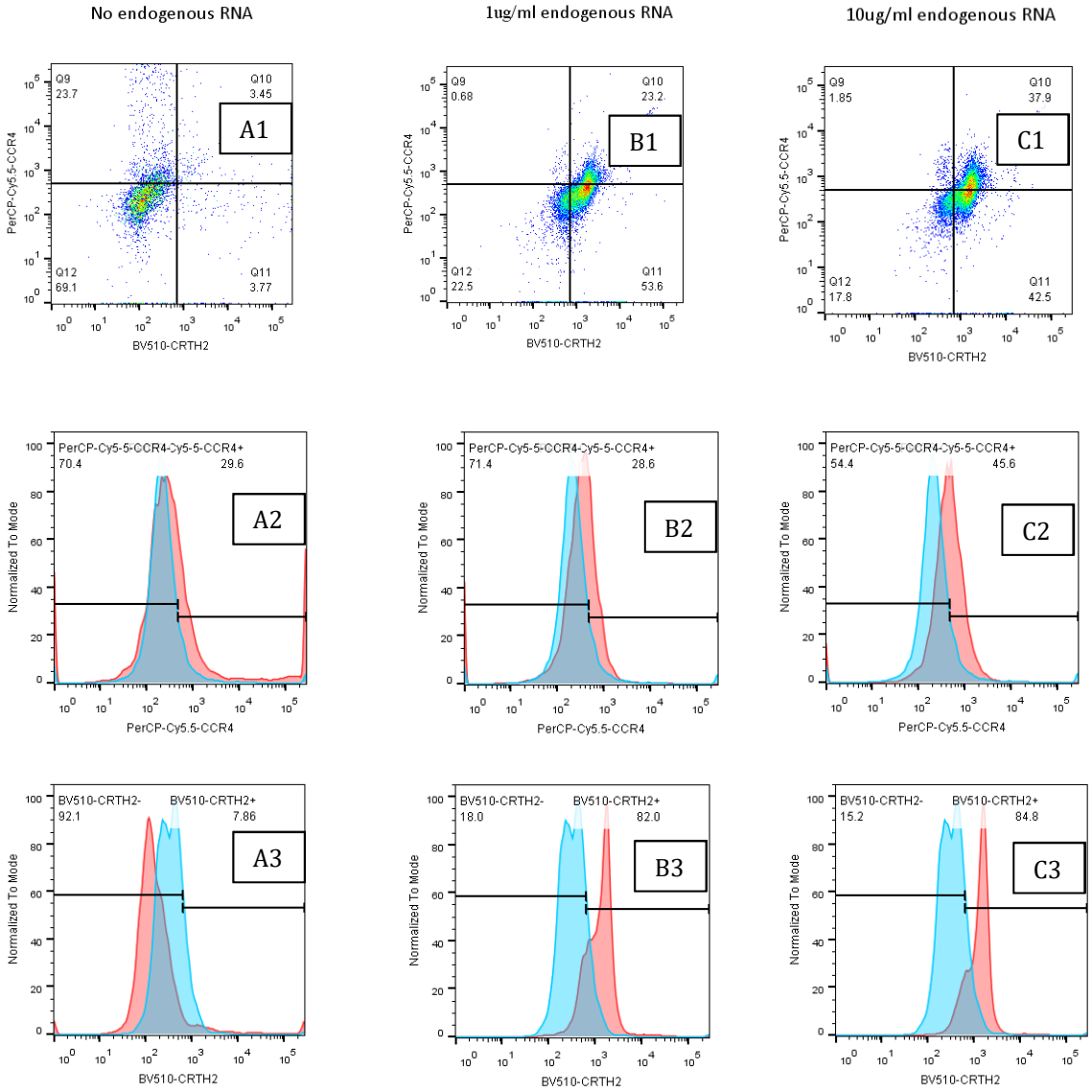
Offset histograms showing proliferation of CFSE-stained Tresp cells activated with anti-CD3/CD28 and cultured alone or in the presence of Treg at different ratios. Readings were taken at 96 hours post-activation. Negative control = Tresp only in the absence of Treg. Positive control – Tresp treated with 300ng/ml Rapamycin. Fluorescence in the unstained sample is due to autofluorescence by cells. The greatest suppression was observed when the Tresp were cultured with the Treg in a 1:1 ratio (orange peak).

Appendix XI: Sample Example of Phenotype Analysis By Flow Cytometry



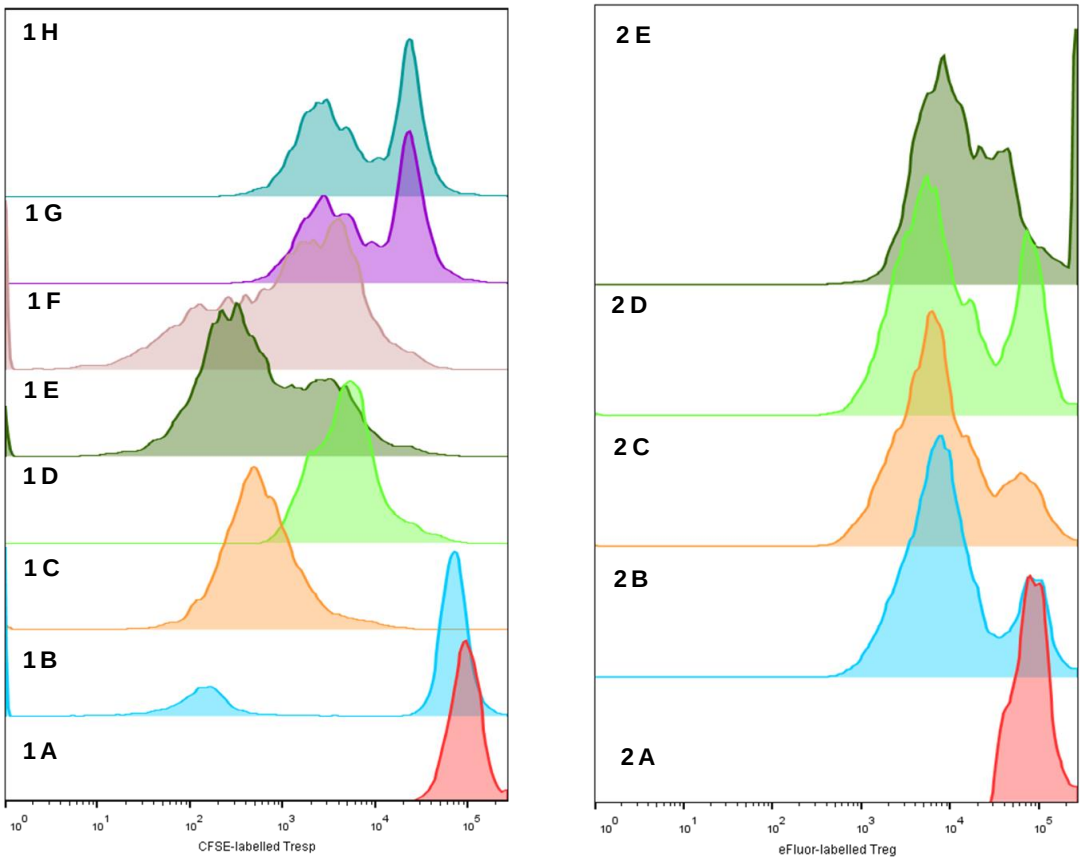
Raw Data For Phenotype Analysis. Represented here is the phenotype results obtained when Treg were treated with CL097. A1 – A3: Untreated; B1 – B3: treated with 2µg/ml CL097; C1-C3: treated with 5µg/ml CL097. For the histograms, the red peak represents the antibody-stained sample while the blue peak represents the isotype sample.

Appendix XII: Sample Example II of Phenotype Analysis By Flow Cytometry



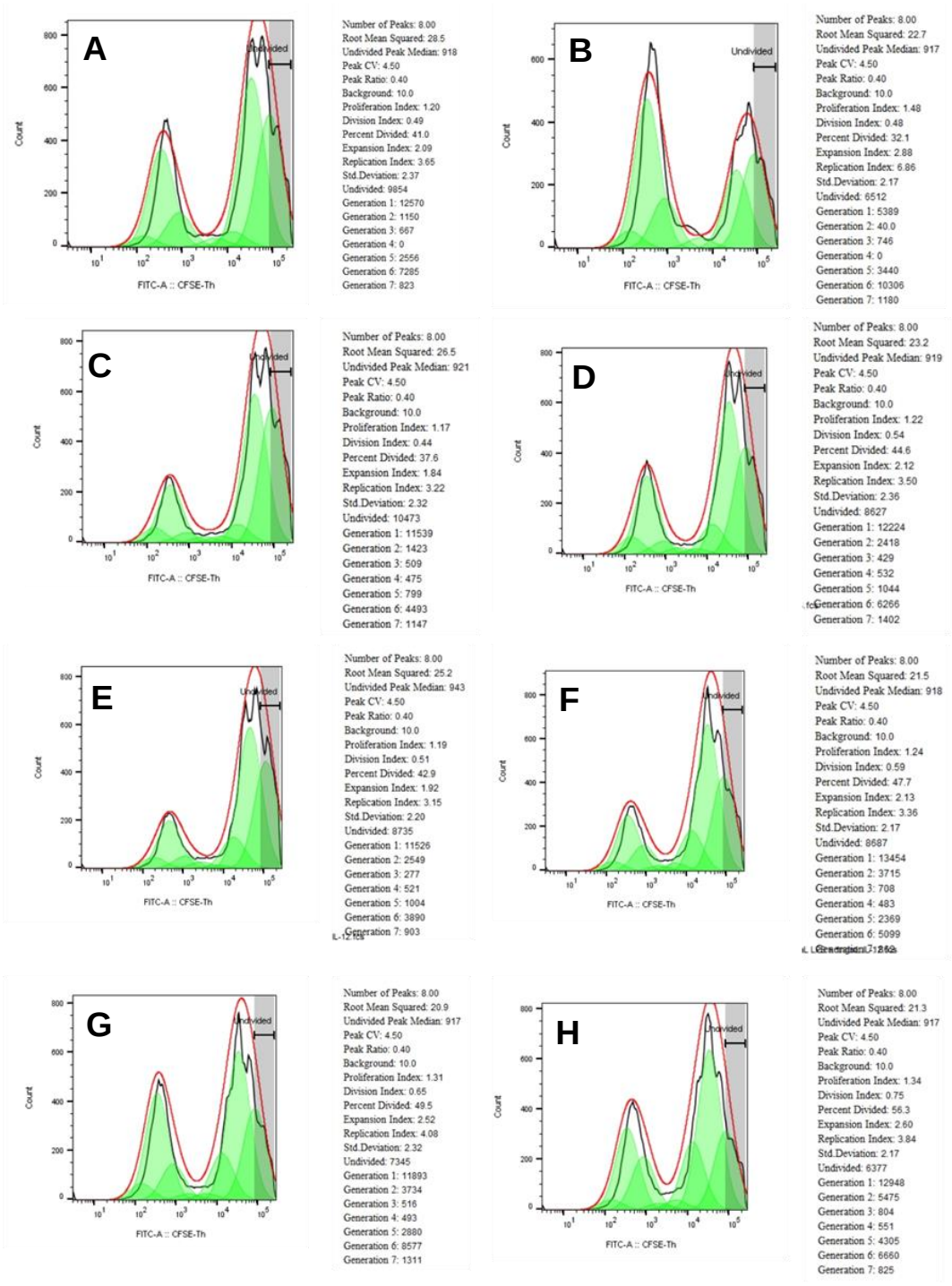
Raw Data For Phenotype Analysis. Represented here is the phenotype results obtained when Treg were treated with endogenous RNA. A1 – A3: Untreated; B1 – B3: treated with 1µg/ml endogenous RNA; C1-C3: treated with 10µg/ml endogenous RNA. For the histograms, the red peak represents the antibody-stained sample while the blue peak represents the isotype sample.

Appendix XIII: Sample Example of Proliferation Analysis by Flow Cytometry



Offset histograms showing proliferation of (1) CFSE-stained Tresp cells and (2) eFluor™-stained Treg cells activated with anti-CD3/CD28 cultured alone or in co-culture. Represented here is the proliferation data obtained when treating with IFN-γ. 1A: Not Activated Tresp (Day 0); 1B: Tresp + 300ng/ml Rapamycin; 1C: Untreated Tresp; 1D: Untreated Tresp + Treg; 1E: Tresp + Treg treated with 25ng/ml IFN-γ; 1F: Tresp + Treg treated with 50ng/ml IFN-γ; 1G: Tresp + 25ng/ml IFN-γ; 1H: Tresp + 50ng/ml IFN-γ. 2A: Not Activated Treg (Day 0); 2B: Untreated Tresp + Treg; 2C: Tresp + Treg treated with 25ng/ml IFN-γ; 2D: Tresp + Treg treated with 50ng/ml IFN-γ; 2E: Untreated Treg. Dye dilution was calculated by dividing the Median Fluorescent intensity of the sample not activated (Day) by the activated (untreated or treated) sample.

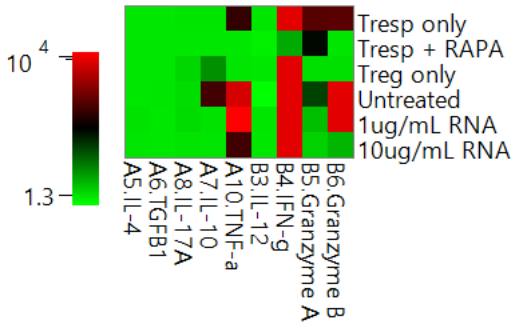
Appendix XIV: Sample Example of Data Proliferation Modelling



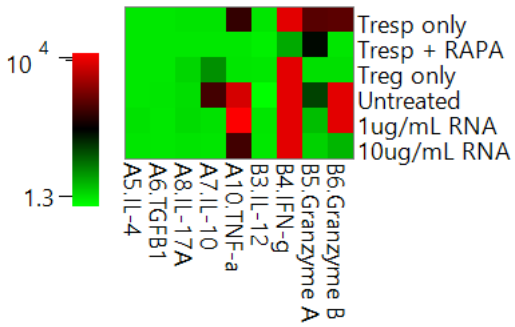
Proliferation Modelling of CFSE-stained Tresp cells using Flow Jo FlowJo Version 10.6.1. Represented here is the data obtained for samples treated with ssPolyU. Statistics including PI and DI shown on the right of each histogram. A: Untreated Tresp + Treg; B: Tresp + Treg + 1µg/ml ssPolyU; C: Tresp + Treg + 1µg/ml ssPolyU; D: Tresp + Treg + 1µg/ml ssPolyU + 100ng/ml LPS; E: Tresp + Treg + 1µg/ml ssPolyU + 1ng/ml IL-12; F: Tresp + Treg + 1µg/ml ssPolyU + 100ng/ml LPS + 1ng/ml IL-12; G: Tresp + Treg + 100ng/ml LPS: H: Tresp + Treg + 1ng/ml IL-12.

Appendix XV: Cytokine Heat Maps

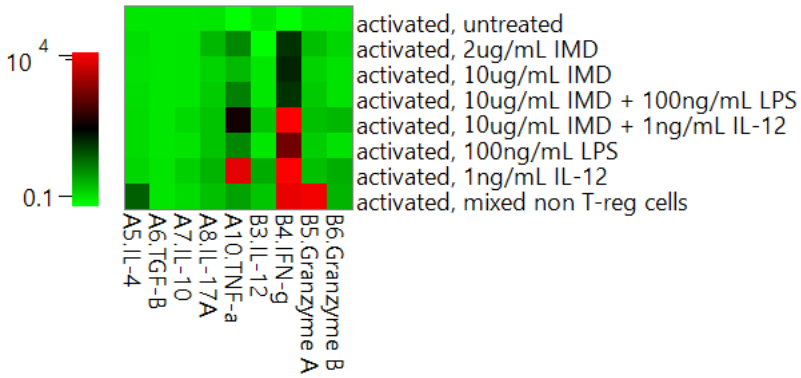
Heat map of cytokine concentration present in supernatants collected from untreated or IFN-γ-treated cultures. Heat map representative of one replicate.



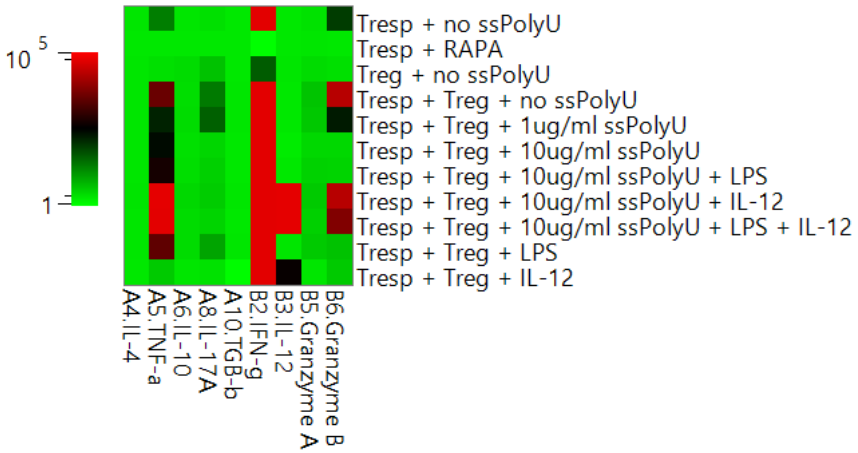
Heat map of cytokine concentration present in supernatants collected from untreated or endogenous RNA-treated cultures. Heat map representative of one replicate.



Heat map of cytokine concentration present in supernatants collected from untreated or endogenous DNA-treated cultures. Heat map representative of one replicate.



Heat map of cytokine concentration present in supernatants collected from untreated or CL097/LPS/IL-12-treated cultures. Heat map representative of one replicate. IMD refers to CL097.



Heat map of cytokine concentration present in supernatants collected from untreated or ssPolyU/LPS/IL-12-treated cultures. Heat map representative of one replicate.