# The role of regulatory T cells in the pathophysiology of aplastic anaemia and their potential use as a treatment

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# The role of regulatory T cells in the pathophysiology of aplastic anaemia and their potential use as a treatment

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*"Considerate la vostra semenza:*
*fatti non foste a viver come bruti,*
*ma per seguir virtute e canoscenza".*

*"Consider well the seed that gave you birth:*
*You were not made to live as brutes,*
*But to follow virtue and knowledge".*

[Dante Alighieri
La Divina Commedia
Inferno
Canto XXVI, vv.118-12]

## Declaration

I hereby declare that I alone composed this thesis and that the work is my own, except where stated otherwise (APPENDIX 5: CONTRIBUTIONS).

# ABSTRACT

Aplastic anaemia is an immune mediated bone marrow failure syndrome characterized by reduced and dysfunctional regulatory T cells. Immunosuppressive treatment with anti-thymocyte globulins and ciclosporin A or allogeneic stem cell transplantation are the treatment of choice above and below forty years of age respectively. Not many options are available for elderly patients not fit for immunosuppressive treatment or relapsing after that.

The aim of this Ph.D. project was to investigate in more depth the role of regulatory T cells in the pathogenesis of aplastic anaemia and to explore if *ex vivo* expansion of Tregs could be an alternative treatment for refractory/relapsed aplastic anaemia in patients without a suitable human leukocyte antigen-matched donor.

During the first part of the project, we were able to identify, through mass cytometry, a specific immune signature - based on Treg subsets named A and B - able to predict response to treatment. Non-responders to immunosuppressive treatment seemed more likely to have higher Treg A number compared with non-responders, whereas responders had higher Treg B.

The following part of the Ph.D. has been aimed to assess whether Treg A and B are also functionally different, and we were able to show that Treg A and B are not only immunophenotypically different, but they also show a distinct functional profile, ontogeny, gene signature, and apoptosis propensity. The next aspect we wanted to explore, is weather Tregs are expandable to be used, *in vitro* first and *in vivo* afterwards, as a cellular therapy for aplastic anaemia. All our data show that aplastic anaemia Tregs are expandable, have a Treg-like immunophenotype, are functional,

stable, cannot be induced to secrete interleukin-17A, and increase phosphorylated B cell lymphoma-2 expression.

Having assessed their "safety" *in vitro*, we investigated their use *in vivo* and preliminary data show that, in a graft *versus* host disease mouse model, they are able to mitigate autoimmunity and prolong overall survival.

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# ABBREVIATIONS

| | |
|---|---|
| $A_{2A}R$ | Adenosine receptor 2A |
| ACC1 | Acetyl-CoA carboxylase 1 |
| AHR | Aryl hydrocarbon receptor |
| AIRE | Autoimmune regulator |
| AML | Acute myeloid leukaemia |
| AMPK | AMP-activated protein kinase |
| AP-1 | Activator protein 1 |
| APAF | Apoptotic protease activating factor 1 |
| APCs | Antigen presenting cells |
| APECED | Autoimmune polyendocrinopathy candidiasis ectodermal dysplasia |
| ASXL1 | Additional sex combs like 1 |
| ATG | Anti-thymocyte globulin |
| ATRA | All-trans retinoic acid |
| BATF | B cell-activating transcription factor |
| BCA | Bicinchoninic acid |
| Bcl-2 | B cell lymphoma 2 |
| Bcl-6 | B cell lymphoma 6 |
| BH3 | Bcl-2 homology domain 3 |
| BID | BH3-interacting death domain |
| Bim | Bcl-2 interacting mediator of cell death |
| BM | Bone marrow |
| BMF | Bone marrow failure |
| bp | Base pair |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CCL19 | Chemokine (C-C motif) ligand 19 |
| CCL21 | Chemokine (C-C motif) ligand 21 |
| CCR | CC chemokine receptor |
| CCR7 | C-C chemokine receptor-7 |
| CD | Cluster of differentiation |
| CD40L | CD40 ligand |
| cDNA | Complementary deoxyribonucleid acid |
| CDR1 | Complementarity determining region 1 |
| CDR2 | Complementarity determining region 2 |
| CDR3 | Complementarity determining region 3 |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CMV | Cytomegalovirus |
| CNIs | Calcineurin inhibitors |
| CsA | Ciclosporin A |
| CSM | Cell staining medium |
| CTLA-4 | Cytotoxic T lymphocyte associated protein 4 |
| CTLs | Cytotoxic T lymphocytes |
| CXCR3 | CXC chemokine receptor 3 |
| CyTOF | Time of flight mass cytometry |
| D | Diversity |
| DAPI | Diamidino-phenylindole |
| DC | Dyskeratosis congenita |
| DCs | Dendritic cells |
| DLI | Donor lymphocyte infusion |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DNMT3A | DNA methyltransferase |
| $ddH_2O$ | Double-distilled water |
| dNTPs | Deoxyribonucleotide triphosphates |

| | |
|---|---|
| DP | Double positive |
| EBMT | European bone marrow transplantation |
| EBV | Epstein Barr virus |
| EDTA | Ethylene diamine tetra-acetic acid |
| EMA | European medicines agency |
| EOMES | Eomesodermin |
| ER | Endoplasmic reticulum |
| ETC | Electron transport chain |
| FA | Fanconi anaemia |
| FACS | Fluorescence-activated cell sorting |
| FADD | FAS associated death domain protein |
| FCS | Foetal calf serum |
| FDR | False discovery rate |
| FLAER | Fluorescent aerolysin |
| FLOCK | |
| FOXO | Forkhead box O |
| Foxp3 | Forkhead box protein 3 |
| GALT | Gut-associated lymphoid tissue |
| G-CSF | Granulocyte-colony stimulating factor |
| gDNA | Genomic deoxyribonucleid acid |
| GEP | Gene expression profile |
| GFI1 | Growth-factor independent 1 |
| GITR | Glucocorticoid-induced tumour necrosis factor-related receptor |
| GLUT1 | Glucose transporter 1 |
| GPI | Glycerophosphatidylinositol |
| GVHD | Graft *versus* host disease |
| GVL | Graft *versus* leukaemia |
| HAT | Histone acetyltransferase |
| Hb | Haemoglobin |
| HbF | Foetal haemoglobin |
| HBSS | Hanks' balanced salt solution |
| HCL | Hairy cell leukaemia |
| HDAC | Histone deacetylase |
| H&E | Haematoxylin and eosin |
| HIF1 | Hypoxia-inducible factor 1 |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| HRP | Horseradish peroxidase |
| HSCT | Haematopoietic stem cell transplantation |
| HSPCs | Haematopoietic stem progenitor cells |
| IBD | Inflammatory bowel disease |
| IBMF | Inherited bone marrow failure |
| ICOS | Inducible co-stimulator |
| IDO | Indoelamine 2,3-dioxygenase |
| IFI | Invasive fungal infection |
| IFN-α | Interferon-α |
| IFN-β | Interferon-β |
| IFN-γ | Interferon-γ |
| Ig | Immunoglobulin |
| IKK | Inhibitor of nuclear factor-κB kinase |
| IL-2R | IL-2 receptor |
| IL-12 | Interleukin-12 |
| IPA | Ingenuity Pathway Analysis |
| IPEX | Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome |
| IRF4 | Interferon regulatory factor 4 |
| IST | Immunosuppressive therapy |
| ITAMs | Immuno-receptor tyrosine-based activation motifs |

| | |
|---|---|
| iTregs | Induced Tregs |
| J | Joining |
| JAK | Janus kinase |
| KLF-2 | Krüppel-like factor 2 |
| LAG | Lymphocyte activation gene 3 |
| LAP | Latency-associated peptide |
| LCK | Lymphocyte-specific protein tyrosine kinase |
| LKB1 | Liver kinase B1 |
| MACS | Magnetic-activated cell sorting |
| MAF | Macrophage-activating factor |
| MAPK | Mitogen-activated protein kinase |
| MDS | Myelodysplastic syndrome |
| MHC | Major histocompatibility complex |
| MHC-I | Major histocompatibility complex class I |
| MHC-II | Major histocompatibility complex class II |
| MHC-III | Major histocompatibility complex class III |
| MMF | Mycophenolate mofetil |
| MOMP | Mitochondrial outer membrane permeabilization |
| MSD | Matched sibling donor |
| mTOR | Mechanistic target of rapamycin |
| mTORC | mTOR complex |
| MUD | Matched unrelated donor |
| NCBI | National Center for Biotechnology Information |
| NFAT | Nuclear factor of activated T cells |
| NF-κB | Nuclear factor-κB |
| NGS | Next generation sequencing |
| NKs | Natural killers |
| NKTs | Natural killer T cells |
| NR4A | Nuclear receptor 4A |
| NRM | Non-relapse mortality |
| NSAA | Non-severe aplastic anaemia |
| nTregs | Naturally derived Tregs |
| OS | Overall survival |
| OXPHOS | Oxidative phosphorylation |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PB | Peripheral blood |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD-1 | Programmed cell death 1 |
| PDK | Phosphoinositide-dependent protein kinase |
| PD-L | Programmed cell death ligand |
| P/S | Penicillin/Streptomycin |
| PE | Phycoerythrin |
| PFA | Paraformaldehyde |
| PIGA | Phosphatidylinositol glycan anchor biosynthesis A |
| PIP2 | Phosphatidylinositol-4,5-biphosphate |
| PIP3 | Phosphatidylinositol-3,4,5-trisphosphate |
| PKM2 | Pyruvate kinase M2 |
| PLT | Platelets |
| PMA | Phorbol myristate acetate |
| PMN | Polymorphonucleated |
| PNH | Paroxysmal nocturnal haemoglobinuria |
| PTEN | Phosphatase and tensin homologue |
| RBC | Red blood cells |
| RNA | Ribonucleic acid |
| RAR | Retinoic acid receptor |
| RORγt | Retinoid-related orphan receptor γt |

| | |
|---|---|
| RPMI | Roswell Park Memorial Institute |
| RUNX3 | Runt-related transcription factor 3 |
| SAA | Severe aplastic anaemia |
| SHM | Somatic hypermutation |
| SOCS3 | Suppressor of cytokine signalling 3 |
| SP | Single positive |
| SPADE | Spanning-tree progression analysis of density-normalized events |
| SPI6 | Serine protease inhibitor 6 |
| SPIA | Single primer isothermal amplification |
| STAT | Signal transducer and activator of transcription |
| TAP | Transporter associated with antigen processing |
| TCA | Tricarboxylic acid |
| TCF1 | T cell factor 1 |
| Tconv | Conventional T cells |
| TCR | T cell receptor |
| Teff | Effector T cell |
| Tfh | Follicular helper T cells |
| TGF-β | Transforming growth factor-β |
| Th | T helper |
| Th1 | T helper 1 |
| Th2 | T helper 2 |
| Th17 | T helper 17 |
| TNF | Tumour necrosis factor |
| Tpo | Thrombopoietin |
| TRAIL | TNF related apoptosis-inducing ligand |
| TRAIL-DR5 | TNF related apoptosis inducing ligand-death receptor 5 |
| TRAILR | TNF related apoptosis-inducing ligand receptor |
| Tregs | Regulatory T cells |
| TSDR | Treg-specific demethylated region |
| t-SNE | t-distributed stochastic non-linear embedding |
| UCB | Umbilical cord blood |
| V | Variable |
| Vβ | Variable gene segment β |
| VCT | Violet cell trace |
| VHL | Von Hippel-Lindau |
| viSNE | visual t-distributed stochastic neighbour embedding |
| VSAA | Very severe aplastic anaemia |
| WB | Western blot |
| XIAP | X-linked inhibitor of apoptosis protein |

# 1 INTRODUCTION

## 1.1 The immune system

The word immunity derives from the Latin word *immunitas*, generally referred to the protection from legal prosecution offered to Roman senators while they were in charge. Traditionally, immunity refers to the protection from disease, more specifically infectious disease. The apparatus of cells and molecules responsible for immunity is the immune system, and their coordinated response to foreign substances is defined as immune response.[1]

The physiologic function of the immune system is defence against infectious microbes; nevertheless, even non-infectious foreign substances can elicit immune responses. Defence against microbes is mediated by the early reactions of innate immunity and the later responses of adaptive immunity (Figure 1.1).[1]

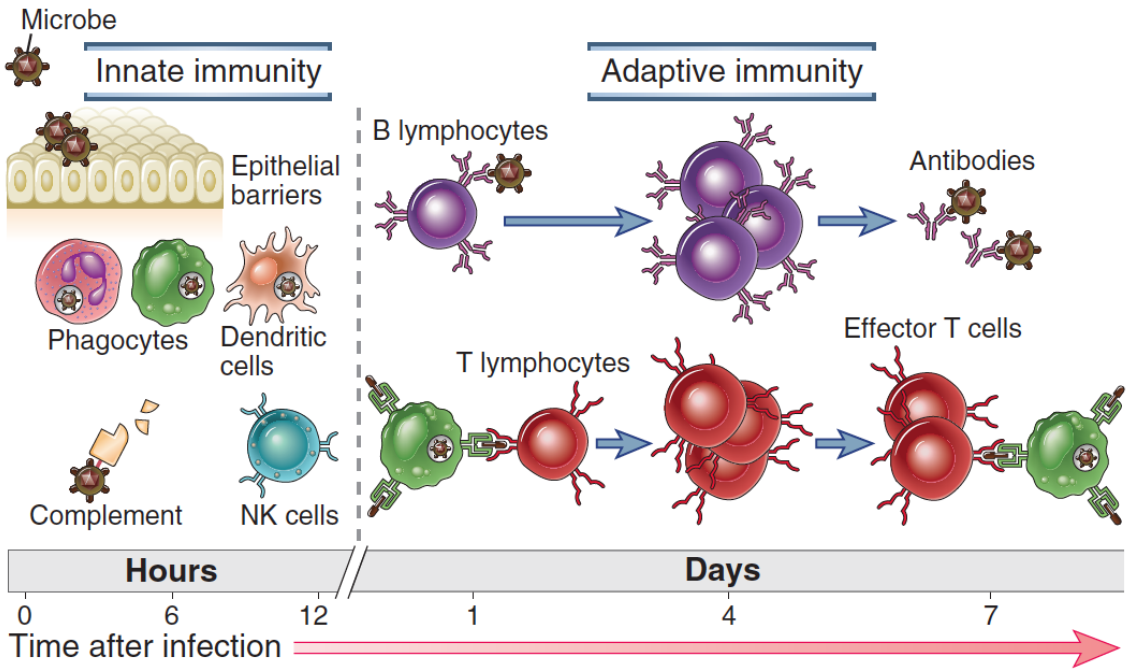



**Figure 1.1 Innate and adaptive immunity**

The initial defence is provided by the mechanisms of innate immunity while adaptive immune responses develop later and consist of activation of lymphocytes.[1]

### 1.1.1 Innate immunity

As the focus of this project was mainly on T cells, only a brief background on innate immunity will follow.

Innate immunity is the first line of defence against infections. The cells and soluble molecules of innate immunity either exist in a fully functional state before encounter with microbes or are rapidly activated by microbes, faster than the development of adaptive immune responses.[1]

Innate immunity has three main functions:[1]

1. It is the initial response to microbes that prevents, controls, or eliminates infection of the host by many microbes.
2. Its mechanisms recognize the products of damaged and dead host cells and serve to eliminate these cells and to initiate the process of tissue repair.
3. Innate immunity to microbes stimulates adaptive immune responses and can influence the nature of the adaptive responses to make them optimally effective against different types of microbes.

The two major types of responses of the innate immune system that protect against microbes are inflammation and antiviral defence.[1]

The cellular components of the innate immune system are:[1, 2]

- epithelial barriers;
- phagocytes;
- dendritic cells;
- natural killer cells;
- T and B lymphocytes with limited antigen receptors specificities;
- mast cells.

### 1.1.1.1 Epithelial barriers

Intact epithelial surfaces form physical barriers between microbes in the external environment and host tissue, and epithelial cells produce antimicrobial chemicals that further impede the entry of microbes (Figure 1.2). Epithelial cells as well as some leukocytes produce peptides that have antimicrobial properties. Barrier epithelia contain certain types of lymphocytes, including intraepithelial T lymphocytes, that recognize and respond to commonly encountered microbes.[1, 2]

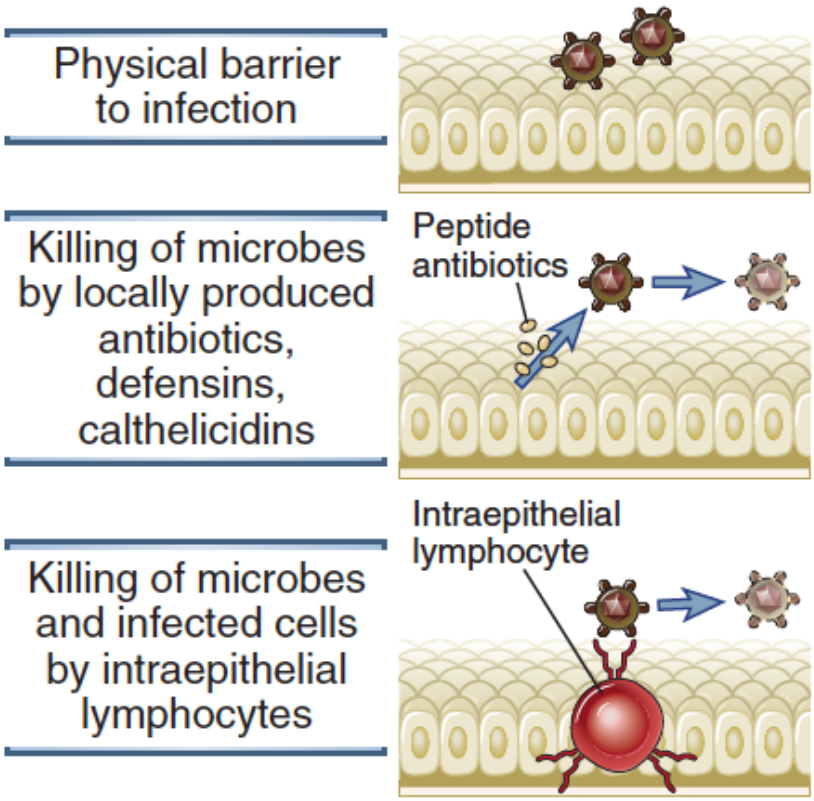



**Figure 1.2 Epithelial barriers**

Epithelia provide a physical barrier, produce antimicrobial molecules, and harbour intraepithelial lymphocytes.[1]

### 1.1.1.2 Phagocytes

Phagocytes are macrophages or neutrophils, which have specialised phagocytic functions and are the first line of defence against microbes, which successfully penetrate epithelial barriers. They have two main functions. First, they are able to internalize and kill microbes. Second, phagocytes respond to microbes by producing

various cytokines that promote inflammation and also enhance the antimicrobial function of host cells at the site of infection.[1]

#### 1.1.1.3 Dendritic cells

Dendritic cells have essential recognition and effector roles in innate immunity. This heterogeneous family of bone marrow-derived cells with long dendrite-like cytoplasmic processes, are constitutively present in most tissues of the body, including epithelia. Dendritic cells are uniquely capable of triggering and directing adaptive T cell-mediated immune responses, and this is dependent on their innate immune responses to microbes. This capability reflects the ability of dendritic cells to take up microbial protein antigens, to transport them to lymph nodes where naïve T cells home, and to alter and display the protein antigens in a way that the T cells can recognize. Depending on the nature of the microbe that induces the innate response, a dendritic cell will direct naïve T cell differentiation into distinct types of effector cells, such as interferon (IFN)-γ producing T helper 1 (Th1) cells or IL-17-producing Th17 cells.[1, 2]

#### 1.1.1.4 Natural killers

Natural killers (NKs) are lymphocytes distinct from T and B cells that play important roles in innate immune responses mainly against intracellular viruses and bacteria. NKs do not express highly diverse, clonally distributed antigen receptors typical of B and T cells. Rather, they use deoxyribonucleic acid (DNA)-encoded receptors to distinguish pathogen-infected from healthy cells. NKs distinguish infected and stressed cells from healthy cells, and NKs activation is regulated by a balance between signals that are generated from activating receptors and inhibitory receptors.

Most NKs express inhibitory receptors that recognize major histocompatibility (MHC)-I molecules, which are cell surface proteins normally expressed on almost all healthy cells in the body. The effector functions of NKs are to kill infected cells and to activate macrophages to destroy phagocytosed microbes (Figure 1.3).[1, 2]

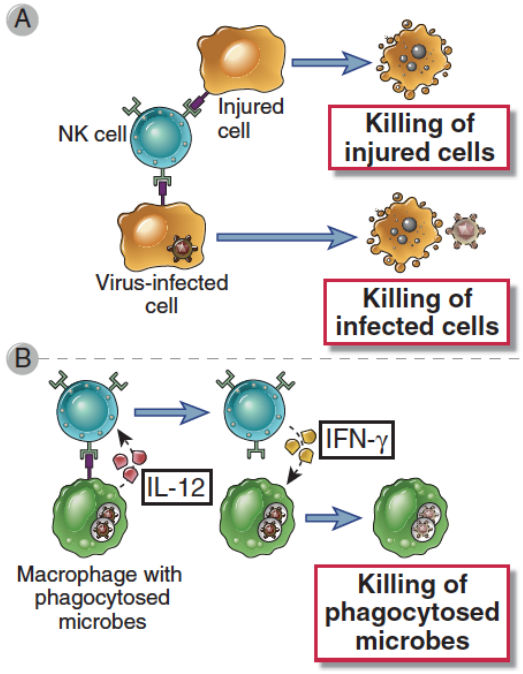



**Figure 1.3 Functions of natural killers**

A. NKs recognize ligands on infected cells and are able to kill them to eliminate reservoirs of infection as well as dysfunctional cells. B. NKs respond to macrophages-secreted IL-12 and produce IFN-γ, which activates the macrophages to kill phagocytosed microbes.[1]

### 1.1.1.5 T and B cells with limited antigen receptor specificities

Most T and B lymphocytes are components of the adaptive immune system and are characterized by a highly diverse repertoire of specificities for different antigens. However, certain subsets of T and B lymphocytes have very little diversity because the same antigen receptor gene DNA segments are recombined in each clone and there is little or no modification of junctional sequences. These T and B cell subsets recognize structures expressed by many different or commonly encountered microbial species. Although these T and B cells perform similar effector functions as do their more clonally diverse counterparts, the nature of their specificities places

them in a special category of lymphocytes that is akin more to effector cells of innate immunity than to cells of adaptive immunity.[1]

#### 1.1.1.6 Mast cells

Mast cells are present in the skin and mucosal epithelium and rapidly secrete proinflammatory cytokines and lipid mediators in response to infections and other stimuli (Figure 1.4). These cells contain abundant cytoplasmic granules containing various inflammatory mediators that are released when the cells are activated. The granule contents include histamine, that cause vasodilation and increased capillary permeability, and proteolytic enzymes that can kill bacteria or inactivate microbial toxins. Mast cells also synthesize and secrete prostaglandins and tumour necrosis factor (TNF).[1]

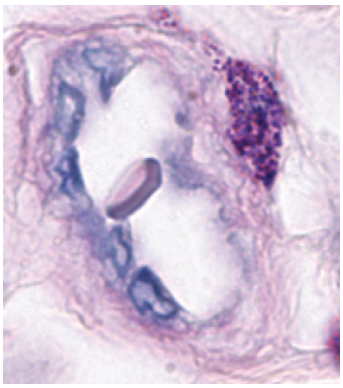


**Figure 1.4 Mast cell**

In the upper right corner a mast cell is visible.[1]

### 1.1.2 Adaptive immunity

In contrast to innate immunity, there are other immune responses that are stimulated by exposure to infectious agents and increase in magnitude and defensive capabilities with each successive exposure to a particular microbe. Because this form of immunity develops as a response to infection and adapts to the infection, it is called adaptive immunity. The defining characteristics of adaptive immunity are exquisite specificity

for distinct molecules and an ability to "remember" and respond more vigorously to repeated exposures to the same microbe.[1, 3]

There are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity, that are mediated by different components of the immune system, and function to eliminate different types of microbes (Figure 1.5).[1, 3]

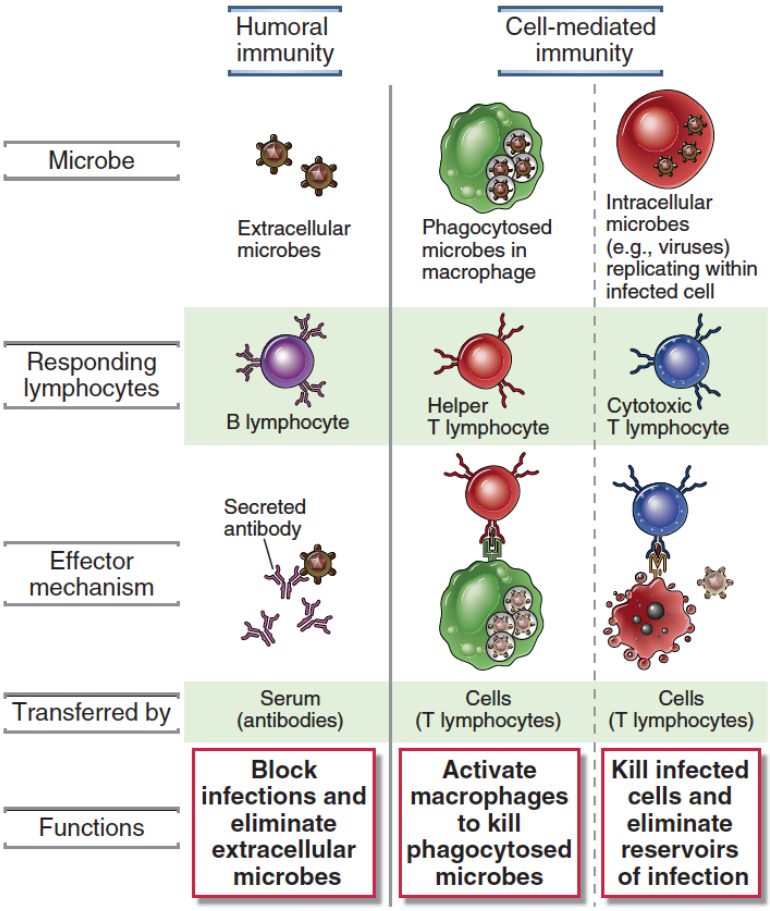



**Figure 1.5 Types of adaptive immunity**

In humoral immunity, B cells secrete antibodies that prevent infections by and eliminate extracellular microbes. In cell-mediated immunity, Th activate macrophages to kill phagocytosed microbes or cytotoxic T lymphocytes (CTLs) directly destroy infected cells.[1]

The cardinal features of adaptive immunity are:[1]

- specificity;
- diversity;
- memory;
- clonal expansion;

- specialization;
- contraction and homeostasis;
- non-reactivity to self.

These features are summarised in Table 1.1 and Figure 1.6.

**Table 1.1 Cardinal features of adaptive immune responses**

| Feature | Functional significance |
|---|---|
| Specificity | Ensures that the immune response to a microbe (or non-microbial antigen) is targeted to that microbe (or antigen) |
| Diversity | Enables immune system to respond to a large variety of antigens |
| Memory | Increases ability to combat repeat infections by the same microbe |
| Clonal expansion | Increases ability to combat repeat infections by the same microbe |
| Specialisation | Generates responses that are optimal for defence against different types of microbes |
| Contraction and homeostasis | Allows immune system to recover from one response so that it can effectively respond to newly encountered antigens |
| Non-reactivity to self | Prevents injury to host during responses to foreign antigens |

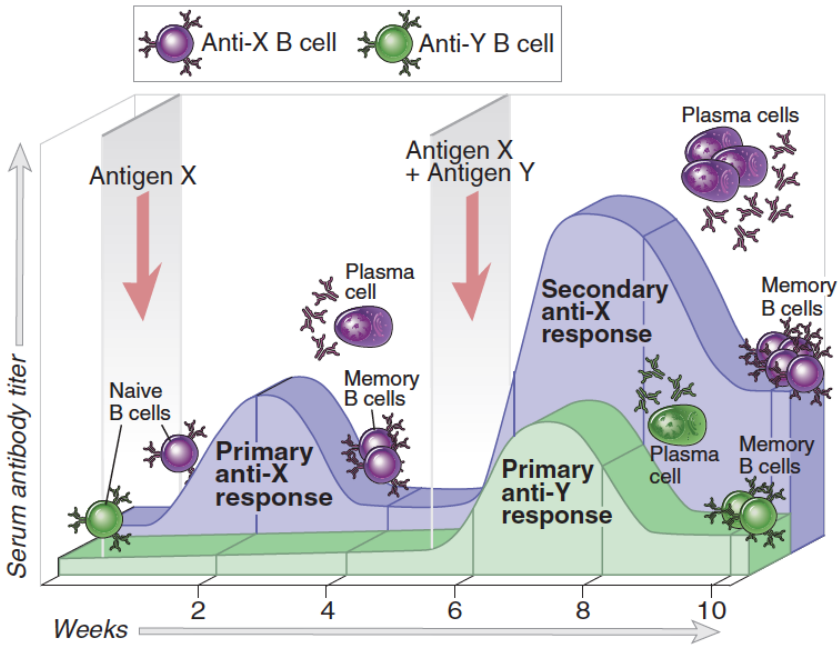



**Figure 1.6 Specificity, memory and contraction of adaptive immune responses**

Antigens X and Y induce the production of different antibodies (specificity). The secondary response to antigen X is more rapid and larger than the primary response (memory). Antibody levels decline with time after each immunization (contraction, the process that maintains homeostasis). The same features are seen in cell-mediated immune responses.[1]

The cellular components of the adaptive immune system are B and T lymphocytes [subdivided in Th, cytotoxic T lymphocytes (CTLs), and regulatory T cells (Tregs)] (Figure 1.7).[1, 3]

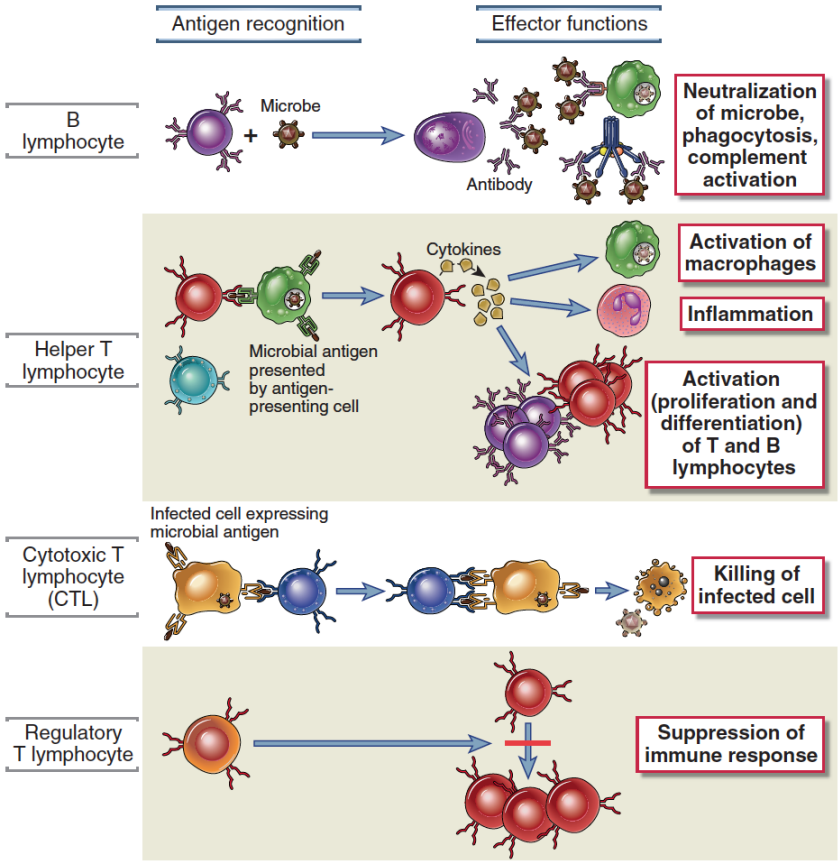



**Figure 1.7 Classes of lymphocytes**

B lymphocytes recognize soluble antigens and become antibody-secreting cells. Th recognize antigens on the surfaces of antigen presenting cells (APCs) and secrete cytokines, which trigger different mechanisms of immunity and inflammation. CTLs recognize antigens on infected cells and kill them. Tregs suppress and prevent immune response to self-antigens.[1]

B cells origin, function and development will not be discussed as the focus of this project was mainly on T cells, specifically Tregs.

## 1.1.3 T cell development and differentiation

### 1.1.3.1 Early development

All lymphocytes derive from pluripotent haematopoietic stem cell precursors in the bone marrow. T cells predominantly develop in the thymus, whereas B cells and natural killers T cells (NKTs) generate in the BM.[1]

Based on T cell receptor (TCR) expression, mature T cells can be subdivided into two lineages: [4, 5]

- TCR  cells, which recognise antigens associated with self-MHC molecules;
- TCR  cells, which recognise antigens not associated with self MHC molecules.

Immature lymphocytes commit to the TCR first, and then become either $CD4^+$ or $CD8^+$ cells. Mature $CD4^+$ and $CD8^+$ cells recognise antigenic peptides complexed with MCH-II or MCH-I molecules, respectively.[1] The differentiation of naïve T cells into effector cells requires interactions with dendritic cells, follicular dendritic cells, and antigen receptor signaling.[1] The cytokine milieu is also crucial for this differentiation process. All these complex interactions take place in secondary lymphoid organs such as the spleen, lymph nodes, and Peyer's patches (Figure 1.8).[1]

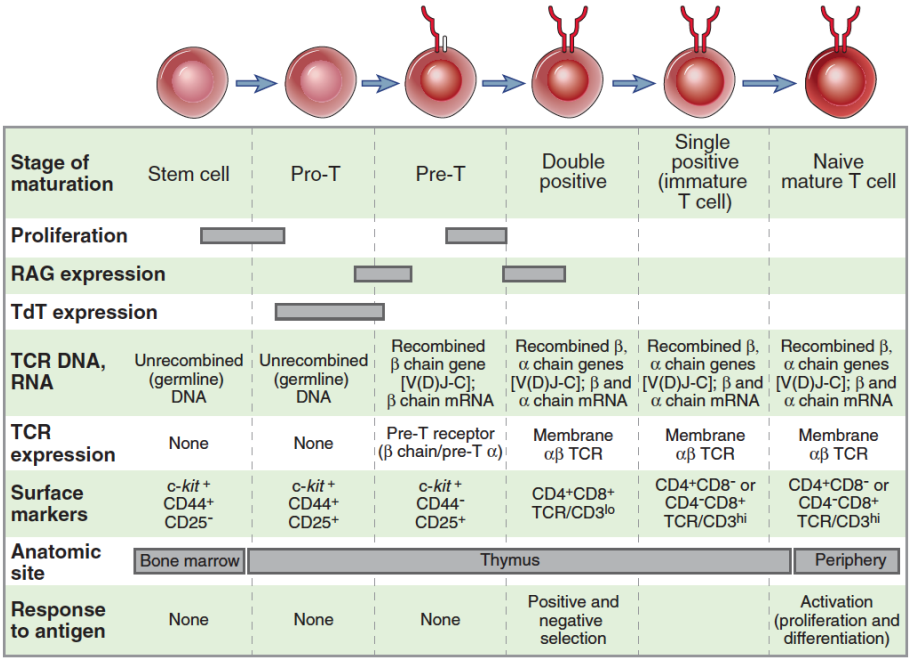



**Figure 1.8 Stages of T cell maturation**

Different stages of maturation in the thymus and export to the periphery are illustrated. Thymocytes in early stages of development do not express any important cell surface molecules. The final thymocytes are "single-positive" $CD4^+\alpha\beta^+$, $CD8^+\alpha\beta^+$, and $CD4^-CD8^-\gamma\delta^+$ cells.[1]

TCR is composed of a α and a β chain in most T cells, and it intervenes in T cell recognition of MCH-peptide antigen complexes. TCR contains variable and constant regions: for TCR Vβ, the variable region is encoded by V, D, and J gene segments, and can be divided in TCR Vα and TCR Vβ families (Figure 1.9).[5, 6]

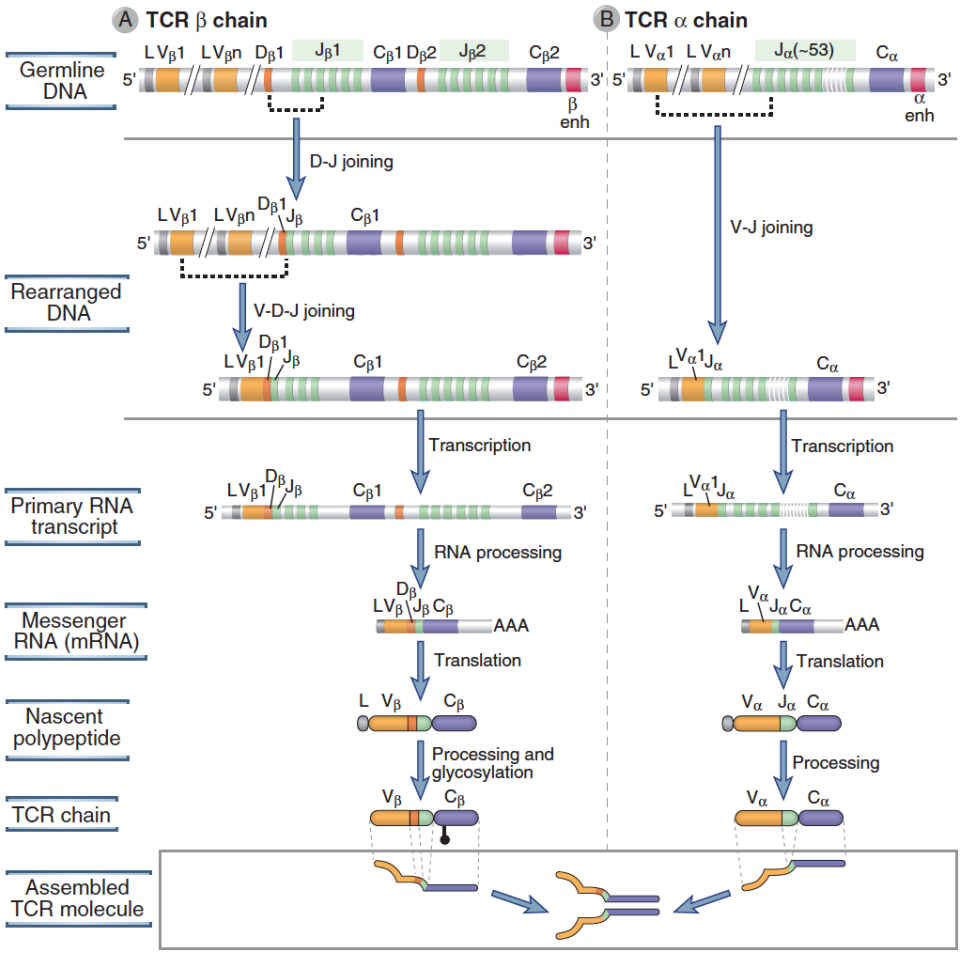



**Figure 1.9 TCR α and β chain gene recombination and expression**

Sequential rearrangements of TCR gene consist of three main steps: D to J, V to D-J rearrangements and transcription. This complex and random selection of V, D, J and C forms an mRNA that is finally translated into a TCR protein chain.[1]

Complementarity determining region 1 (CDR1), complementarity determining region 2 (CDR2), and complementarity determining region 3 (CDR3) define TCR families: CDR3 defines the T cell antigen/MHC specificity and is composed of the distal portion of the V, the proximal J region, and for Vβ, the D region. During T cell ontogeny, V, D, and J gene segments are not only rearranged, but also truncated or

elongated in order to get different sequences and lengths in CDR3.[5] Most of the sequence variability in TCR is concentrated in the CDR3 regions due to this complex re-arrangement.[1, 7]

The main function of CDR1 and CDR2 is to stabilise the ligated CDR3, while CDR3 mainly recognise and ligate the human leukocyte antigen (HLA) presented antigenic peptides.[8, 9] Upon binding with peptide-MHC complexes, the TCR complex initiate signals that activate T cells effector functions.[1] The TCR complex consists of TCR (with α and β chain), CD3 and γε chain (Figure 1.10).

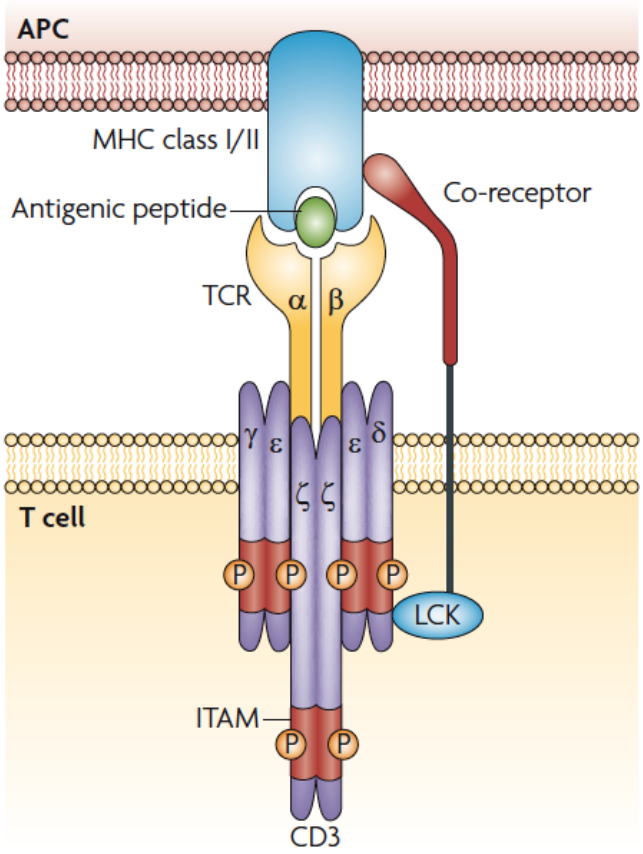



**Figure 1.10 TCR complex**

The TCR complex of MHC-restricted T cells consists of the αβ TCR non-covalently linked to the CD3 and γε proteins. The association of these proteins with one another is mediated by charged residues in their transmembrane regions.[10]

In a mature T cell, the TCR complex is assembled in the endoplasmic reticulum (ER) and transported to the membrane. After antigen recognition by T cells, SRC family

kinases such as lymphocyte-specific protein tyrosine kinase (LCK) and FYN phosphorylate tyrosine residues within the immuno-receptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of CD3 and γε proteins. LCK associates with the cytoplasmic tail of CD4 and CD8 (co-receptors), and FYN is physically linked to CD3. The phosphor-tyrosines in the ITAMs become docking sites for a tyrosine kinase with tandem SRC homology 2(SH2) domains. This kinase, called ZAP-70, is recruited and triggers signal transduction pathways that ultimately lead to changes in T cell gene expression.[1, 7]

### 1.1.3.2 Antigen recognition by T cells: major histocompatibility complex restriction

An important role in antigen processing and presentation of peptides to T cells is played by MHC.[1] T cells are able to recognise foreign peptide antigens/self MHC complexes as integral components of the ligands that T cells recognise (Figure 1.11).[1]

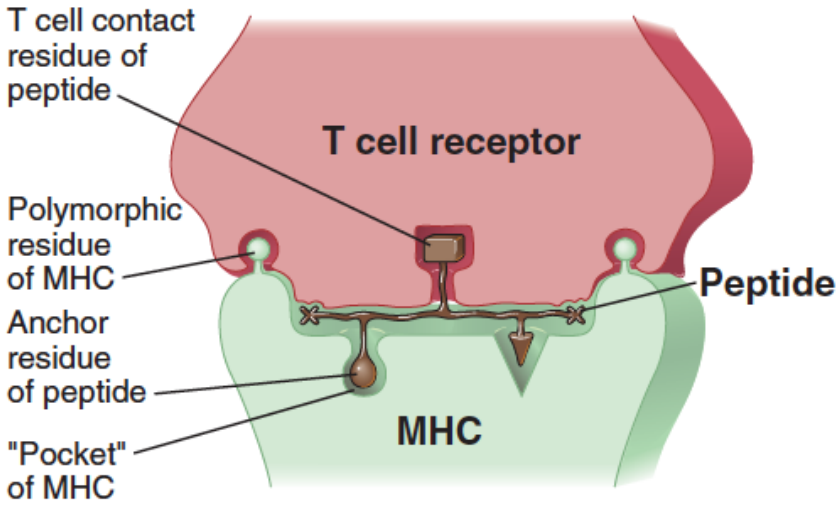



**Figure 1.11 T cell recognition of a peptide/major histocompatibility complex**

Two polymorphic residues of MHC molecule and one peptide residue connect to T cell receptor. One or two other peptide's residues are also connecting to MHC molecule in a groove called "pocket".[1]

MHC molecules are present in all vertebrates with a similar pattern of inheritance and transcription of the genome. In humans these molecules are called HLA. There

are two types of polymorphic MHC genes, which encode two groups of proteins, class I and class II. MHC-I molecules present peptides to and are recognised by $CD8^+$, whereas MHC-II molecules present peptides to $CD4^+$.[1, 11]

MHC genes are highly polymorphic, with more than 250 alleles for some of these genes in human.[1] The set of MHC alleles on each chromosome is called "MHC haplotype", and each HLA allele has a numerical designation.[1]

Figure 1.12 shows a schematic map of human and mouse MHC *loci*.

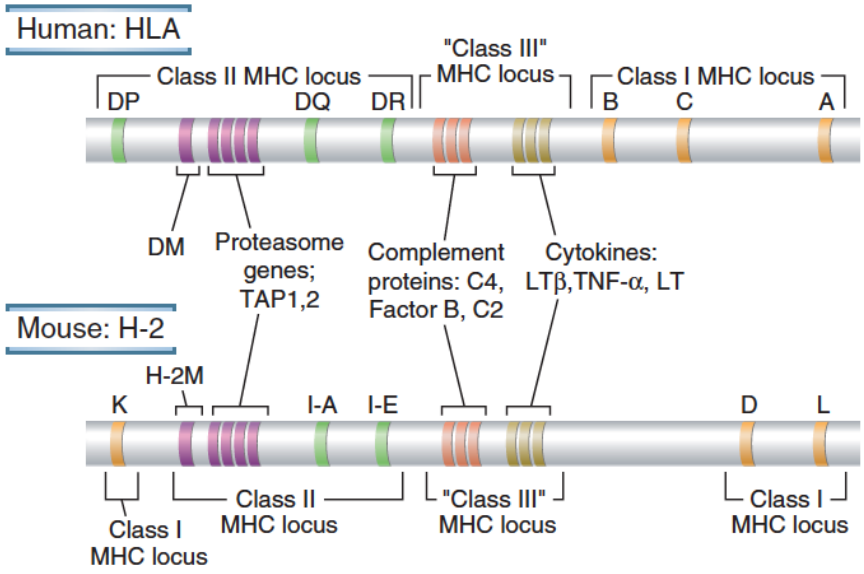



**Figure 1.12 Human and mouse major histocompatibility complex *loci* map**

The MHC locus organisation is very similar in human and mouse. Although the class II *loci* are shown as single blocks, each locus consists of several genes. The MHC-III *locus*, consists of genes that encode non peptide-display molecules and this term is not commonly used.[1]

There are three main components to each MHC molecule:

- an extracellular peptide-binding cleft (groove);
- an immunoglobulin (Ig)-like domain;
- a transmembrane and cytoplasmic domain.

All these polypeptides are MHC-coded, except from $\beta_2$-microglobulin on class I MHC.[1] The peptide-binding cleft of MHC-I is made by $\alpha_1$ and $\alpha_2$ segments of $\alpha$ chain, while in MHC-II is formed by $\alpha_1$ and $\beta_1$ segments (Figure 1.13).

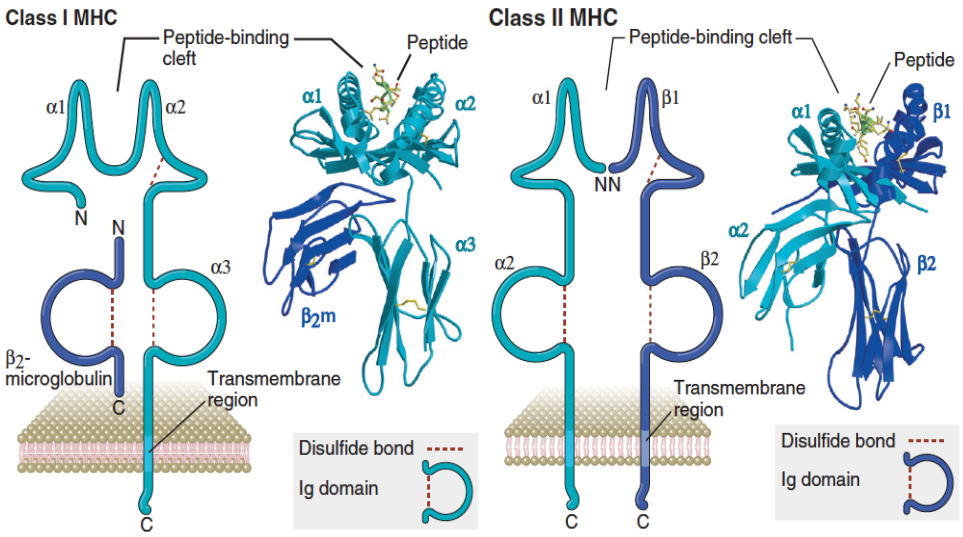



**Figure 1.13 Polymorphic residues of major histocompatibility complex molecules**

The peptide-binding cleft of MHC-I is formed by the polymorphic residue of $\alpha_1$ and $\alpha_2$. In MHC-II, this cleft is formed by $\alpha_1$ and $\beta_1$ residue. Binding sites for CD8 and CD4 are located on $\alpha_3$ and $\beta_2$ chain respectively.[1]

The size of the cleft in MHC-I is large enough to bind peptides of eight to eleven amino acids, while the MHC-II can accommodate peptides with the length of ten to thirty amino acids or more. The optimal length, however, is twelve to sixteen residues.[1, 11]

The $\beta_2$ segment of MHC-II contains the binding site for CD4, whereas the binding site for CD8 is located in the $\alpha_3$ segment of MHC-I.[1]

Class I molecules are virtually expressed on all nucleated cells, and the effector function of class I-restricted $CD8^+$ is to kill cells infected with intracellular microbes such as viruses.[1] It has been shown that the expression of MHC molecules is increased by cytokines produced during both innate and adaptive immune response: interferon (IFN)-α, interferon (IFN)-β and IFN-γ are shown to increase the expression level MHC-I. IFN-γ has the similar effect on MHC-II.[1]

### 1.1.3.3 Antigen processing and presentation to T cells

T cells are only able to recognise antigen when presented in an MHC context. T cells expressing antigen receptors specific for peptides bound to self MHC are

selected to survive, whereas the cells that do not recognise self MHC are allowed to die.[1] However, the processing and presentation of an antigen to T cells is significantly different between $CD4^+$ and $CD8^+$. $CD4^+$ class II restricted T cells recognise peptides derived mainly from extracellular proteins internalised into the vesicles of antigen presenting cells (APCs), whereas $CD8^+$ recognise peptides derived from cytosolic, usually endogenously synthesised, proteins (Figure 1.14).[1]

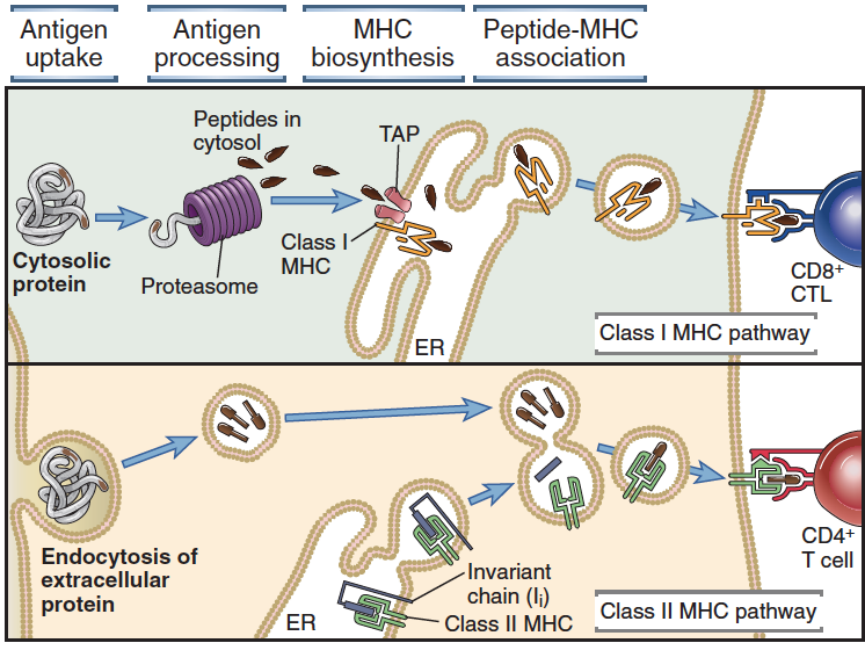



**Figure 1.14 Antigen processing and presentation**

In the MHC-II pathway, an extracellular protein is endocytosed and processed. The resultant peptide will be bound and be presented to $CD4^+$ through MHC-II. In the class I MHC, cytosolic proteins are processed by proteasomes and peptides are transported into the ER to be bind to the MHC-I and presented to $CD8^+$.[1]

Almost all nucleated cells are expressing MHC-II and therefore capable of presenting peptides to CTLs. However, professional APCs (mainly dendritic cells - DCs) can ingest and process virally infected or tumoral cells and present their surface antigen(s) to CTLs. This process is called cross-presentation, to indicate that one cell type (DCs) can present antigens from another cell and prime or activate antigen specific T cells.[1] Macrophages, dendritic cells and B lymphocytes express MHC-II molecules and co-stimulators, and are capable of activating $CD4^+$. While the main role of DCs

is to activate naïve T cells, macrophages and B cells activate effector T cells. These pathways of MHC restricted antigen presentation make T cells capable of screening most of the body cells for the presence of foreign antigens.[1]

### 1.1.3.4 Education and self-tolerance (central tolerance)

A key aspect of T cell development is their "education" to avoid self-antigen recognition. This process takes place in the thymus and the thymic environment provides *stimuli* that are required for the proliferation and maturation of thymocytes. Two types of molecules are particularly important in the process of maturation and education of thymocytes: MHC molecules and cytokines.[1] MHC molecules are an important part of positive and negative selection of thymocytes, a process during which thymocytes will be selected or deleted based on their affinity for self MHC-peptide complex. $CD4^+CD8^+$ thymocytes are mainly in the cortex, and those recognising self MHC-peptide with low avidity are stimulated to survive (positive selection). $CD4^+CD8^+$ in the thymic medulla go through negative selection, mediated by medullary epithelial cells. As the pre-mature T cells are $CCR7^+$, they are attracted to CCR7 specific chemokines, chemokine (C-C motif) ligand 21 (CCL21) and chemokine (C-C motif) ligand 19 (CCL19), in the medulla. Thymic medullary epithelial cells express a nuclear protein called autoimmune regulator (AIRE) that induces the expression of several tissue-specific genes in the thymus and provide an array of tissue specific peptides to be presented to the T cells. T cells with high avidity recognition of self-peptide-MHC complex will be induced to undergo apoptosis. This process is called negative selection (or central tolerance) and leads to clonal deletion of auto-reactive T cells (Figure 1.15).[12]

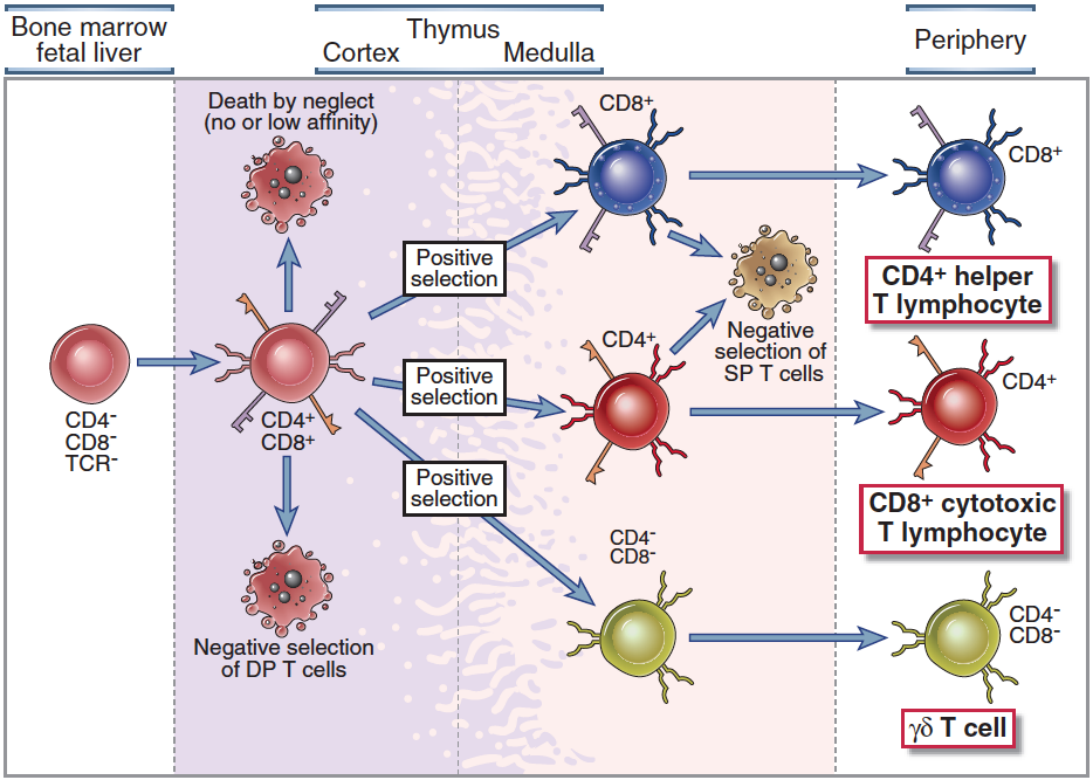



**Figure 1.15 Maturation of T cells in the thymus**

Precursors of T cells travel from the bone marrow through the blood to the thymus. In the thymic cortex, progenitors of αβT cells express TCRs and CD4 and CD8 co-receptors. Selection processes eliminate self-reactive T cells in the cortex at the double positive stage and also single positive medullary thymocytes. They promote survival of thymocytes whose TCRs bind self-MHC molecules with low affinity. Functional and phenotypic differentiation into $CD4^+CD8^-$ or $CD8^+CD4^-$ T cells occurs in the medulla, and mature T cells are released into the circulation.[1]

Interestingly, some of the T cells with the capability of self-peptide-MHC recognition differentiate into Tregs, which migrate to the periphery and prevent response to the self-antigen (peripheral tolerance). This mechanism is explained in more detail in the sections below.

There are similar mechanisms for the B cells, however they usually will not die but change the specificity of their receptor with a process called receptor editing.[1]

### 1.1.3.5 T cell differentiation of $CD4^+$ and $CD8^+$

T cells are extensively plastic in response to antigens and can differentiate along different lineages. The main distinction between different T cells is the different panels of cytokines produced[1, 13]

After recognising an antigen, T cells start secreting cytokines and subsequently expand clonally and differentiate into effector or memory T cells (Figure 1.16). Although the response phases are relatively similar in both $CD4^+$ and $CD8^+$ T-cells, the $CD4^+$ response to antigen is mainly cytokine production and induction of an "active" environment, which finally activates macrophages and B lymphocytes, whereas $CD8^+$ CTLs respond mainly by killing the antigen positive cells.[1, 14]

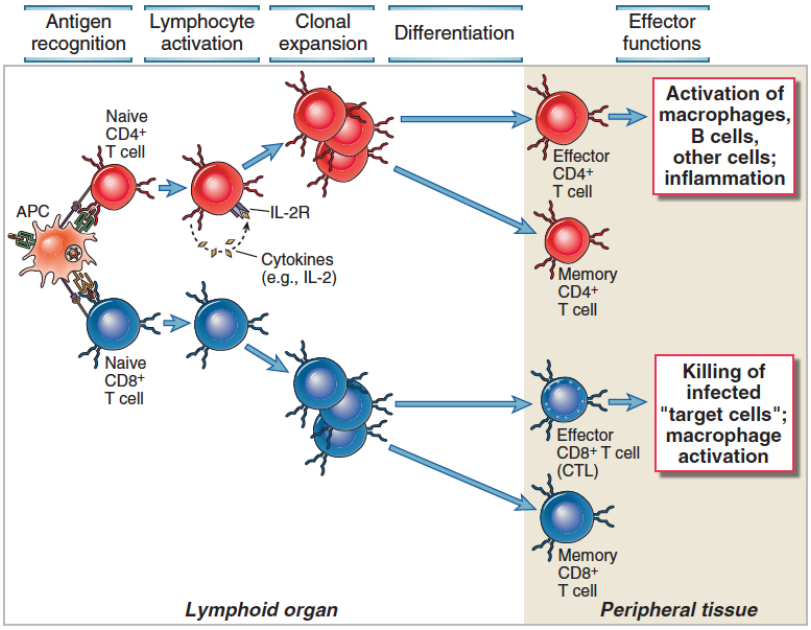



**Figure 1.16 Phases of T cell responses**

Antigen recognition by T cells induces cytokine secretion, particularly in $CD4^+$, clonal expansion as a result of cell proliferation, and differentiation of the T cells into effector cells or memory cells. In the effector phase of the response, the effector $CD4^+$ respond to antigen by producing cytokines that have several actions, such as the recruitment and activation of leukocytes and activation of B lymphocytes, and $CD8^+$ CTLs respond by killing other cells.[1]

The differentiation of $CD8^+$ depends upon recognition of antigens through professional APCs and additional co stimulators (CD80, CD86) in the presence of cytokines secreted by APCs and/or $CD4^+$. The presences of co-stimulatory signals are critical in the process of $CD8^+$ response (Figure 1.17): antigen presentation in the absence of co-stimulation (by resting APC or non-professional antigen presenting cells) usually leads to anergy.[1]

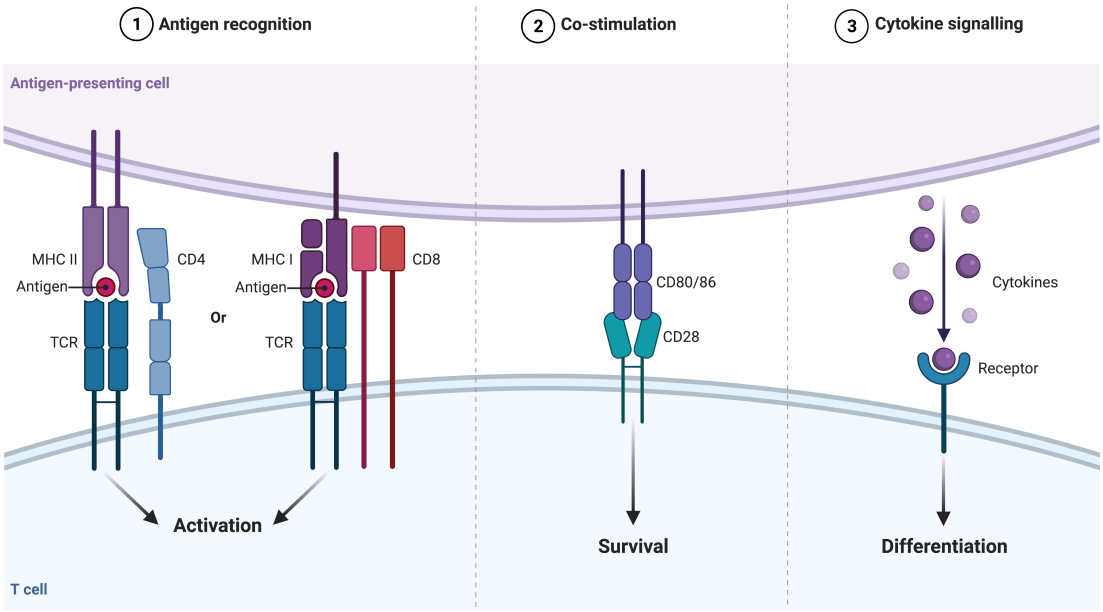



**Figure 1.17 Three signals of T cells activation**

1. Antigen recognition occurs when the APC presents an antigen to the TCR. 2. Co-stimulation involves the binding of molecules on the APC such as CD80/86 to the CD28 receptor on the T cell, this activity aids T cell activation. 3. Cytokine signalling induces the differentiation of the T cell into either effector or regulatory phenotypes.

The ability to secrete cytokine defines different subsets of $CD8^+$: Tc1 (producing IFN-γ), Tc2 (producing IL-4) and Tc17 (producing IL-17).[15] Despite the different cytokine profiles, the function of $CD8^+$ is highly dependent on their homing receptor and their anatomical distribution.[16]

$CD4^+$ T cell lineages were traditionally considered to be Th1 and Th2, producing IFN-γ and IL-4 respectively, however there has been a major advance in the understanding the function and cytokines profiles of these cells and newer lineages were identified within $CD4^+$.[1]

Although the profile of cytokines produced by different $CD8^+$ T cell subsets is different, their main function remains the killing of antigen positive cells. On the other hand, $CD4^+$ T cells are not only different in their cytokine profile but also in their function, ranging from IFN-γ or IL-4-secreting cells (pro-inflammatory) to different immune regulatory T cells.[1]

The main subtypes of $CD4^+$ are Th1 (producing IFN-γ and TNF-α), Th2 (producing IL-4 and IL-13) and Th17 (producing IL-17), and they have different transcription factors as their lineage master regulator (Figure 1.18).[1] Two more lineages, not belonging to the Th subfamily, are identified within $CD4^+$ T cells:

- Tregs [(identified by the production of inhibitory cytokines like transforming growth factor (TGF-β) and IL-10 and expression of the transcription factor forkhead box protein 3 (Foxp3)];
- Follicular helper T cells (Tfh) (providing helper functions to B cells).[17]

Another feature of different T cell subtype is the production of different cytokines, expression of specific cytokine receptors, response to different priming and autocrine cytokines, signal transducer and activator of transcription (STAT) regulators and lineage-specific transcriptional regulators.

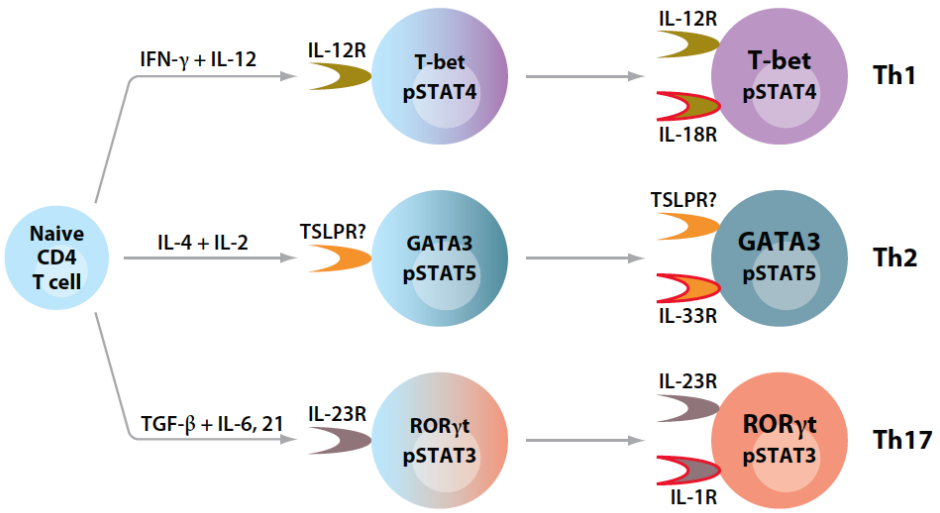



**Figure 1.18 Subtypes of T helper cells**

Cytokines play critical roles in differentiation and effector functions of Th1, Th2, and Th17 cells. Upon TCR activation triggered by antigen-presenting cells, naive $CD4^+$ T cells differentiate into distinct Th lineages in the context of combinations of cytokines. The differentiation processes involve up-regulation of master transcriptional regulators and activation of STAT proteins.[18]

There have been major advances in the understanding the function and the importance of different $CD4^+$ subsets, leading to further insights into the pathogenesis of cancer initiation, progression and immune escape.[19]

As the main focus of this study is the role of Tregs in the pathogenesis of AA, this $CD4^+$ subset and its role in central and peripheral tolerance will be reviewed in more detail.

### 1.1.4 Immunologic tolerance

Immunologic tolerance is characterized by unresponsiveness to an antigen induced by previous exposure to the same antigen. A lymphocyte which encounters an antigen, has two different options:

- it may be activated, leading to immune responses;
- it may be inactivated or eliminated, leading to tolerance.[1]

The same antigen may induce an immune response or tolerance, depending on the conditions in which it is displayed to specific lymphocytes (e.g., in the presence or absence, respectively, of inflammation and innate immune responses). Antigens able to induce tolerance are called tolerogens, while those able to generate immunity are defined as immunogens.[1, 20]

There are several characteristics of tolerance that both T and B cells have in common and is important to list them before describing T cell tolerance in more detail (Figure 1.19):[1]

- in the absence of autoimmunity, there is tolerance of self-antigens because the lymphocytes that recognize them are either killed or inactivated;
- tolerance is the result of the recognition of antigens by specific lymphocytes;

- self-tolerance can either be central (induced in immature self-reactive lymphocytes in the primary lymphoid organs) or peripheral (induced in mature lymphocytes in the periphery);
- central tolerance occurs during the maturation of lymphocytes in the primary lymphoid organs, where the encounter with self-antigens may lead to cell death or replacement of a self-reactive antigen receptor with a new one;
- peripheral tolerance occurs when, because of recognizing self-antigens, mature lymphocytes either become incapable of responding to that antigen, or are induced to die by apoptosis, or are actively suppressed Tregs.

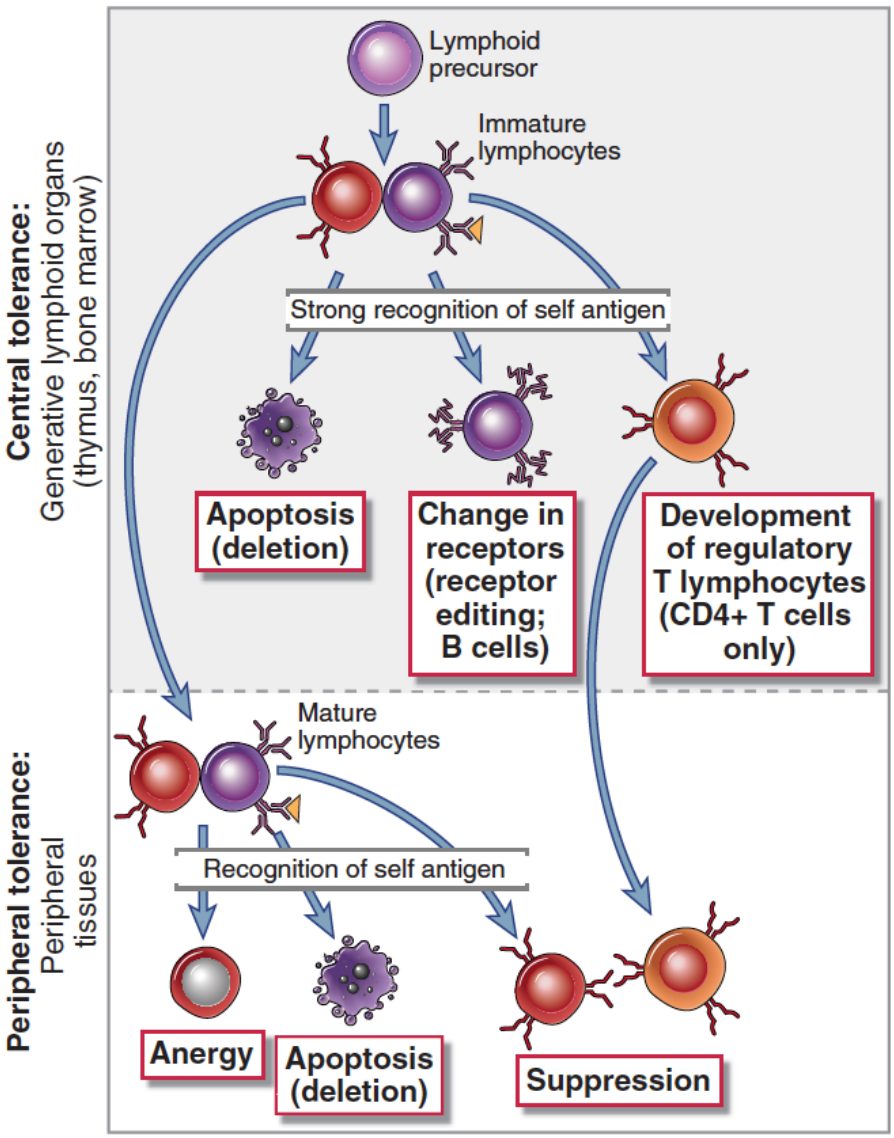



**Figure 1.19 Central and peripheral tolerance to self-antigens**

Immature lymphocytes specific for self-antigens may encounter these antigens in the generative lymphoid organs and either are deleted or develop into regulatory lymphocytes (central tolerance). Some self-reactive lymphocytes may mature and enter peripheral tissues and may be inactivated or deleted by encounter with self-antigens in these tissues or be suppressed by the regulatory T cells (peripheral tolerance).[1]

### 1.1.4.1 Central tolerance in T cells

During their maturation in the thymus, many immature T cells able to recognize antigens with high avidity are deleted, and some of the surviving $CD4^+$ develop into Tregs (Figure 1.20).

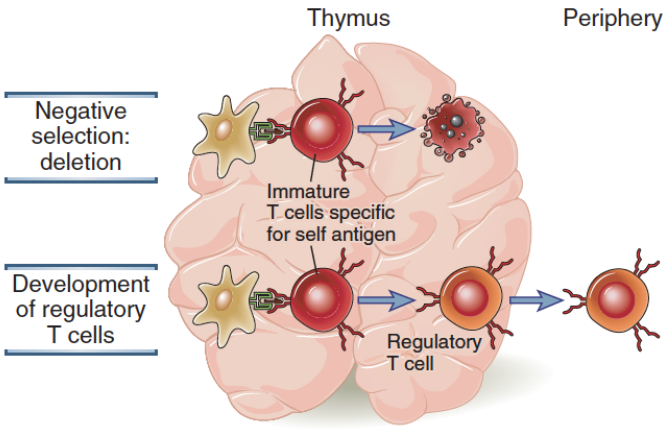



**Figure 1.20 Central T cell tolerance**

Self-antigens recognition by immature T cells in the thymus may lead to either death of the cells (negative selection) or the development of Tregs that enter the periphery.[1]

This process affects both class I and class II MHC-restricted T cells and is therefore important for tolerance in both $CD8^+$ and $CD4^+$. The end result of negative selection of thymocytes is that the repertoire of mature T cells that leave the thymus and populate peripheral lymphoid tissues is unresponsive to the self-antigens.[1, 21] The two main factors responsible for the negative selection of self-reactive thymocytes are:

- the presence of that antigen in the thymus;
- the affinity of the thymocyte TCRs that recognize the antigen.[1]

Thus, relevant aspects to negative selection are:

- which self-antigens are present in the thymus;
- how the immature T cells that recognize these antigens are killed.[1]

Self-proteins in the thymus are processed and presented in association with MHC molecules on APCs. The antigens present in the thymus include both circulating and cell-associated proteins with a wide distribution in tissues. The thymus has a specific mechanism for expressing antigens that are typically present only in certain peripheral tissues, allowing immature autoreactive T cells deletion from the developing T cell repertoire. Some of these antigens are expressed in thymic

medullary epithelial cells under the control of the AIRE protein, which functions as a transcription factor able to promote the expression of selected tissue antigens in the thymus. AIRE is a component of a multi-protein complex involved in transcriptional elongation and chromatin remodelling. There is also evidence for AIRE-independent mechanisms of deletion in the thymus.[1, 22]

If an immature thymocyte has a high-affinity receptor for self-antigens, dies by apoptosis in the thymus. Negative selection can occur in both double-positive T cells in the thymic cortex or newly generated single-positive cells in the medulla. Some self-reactive $CD4^+$ T cells that see self-antigens in the thymus are not deleted but instead differentiate into antigen specific Tregs.[1]

### 1.1.4.2 Historical perspective on the discovery of regulatory T cells

Treg cells were identified as $CD4^+$ T cells expressing high levels of CD25 or low levels of CD45RB, and able to protect from autoimmune pathology induced by neonatal thymectomy or by reconstitution of lymphopenic rodents.[23-28] The lack of specificity of these markers, shared with activated T cells, and the controversial nature of the experimental settings, initially led to scepticism as to their true relevance to immune tolerance.[29] This scepticism was enhanced by the "embarrassing" collapse of the "suppressor cells" theory in the early 1980s.[30] The breakthrough that led to general acceptance of Treg cells as a distinct phenotype/lineage was the identification of the transcription factor FoxP3[31-33] and of its unique expression in Treg cells.[34-36] Deficiencies in Foxp3 were shown to cause lymphoproliferation and multiorgan autoimmunity in scurfy mutant mice and human IPEX.[37] This pathology could be rescued by transfer of Treg cells,[34] and was reproduced by inactivation of FoxP3 uniquely in T cells,[35] proving the T-cell

autonomous role of FoxP3. Aside from providing a unique molecular identifier, these results established that Tregs were not solely constructions from "experimental systems" in mice, but played a non-redundant role in immune tolerance and homeostasis.[29]

### 1.1.4.3 Thymic selection of regulatory T cells: underlying concepts

The first clue that Tregs are generated in the thymus originated from the neonatal thymectomy experiment, in which the removal of the thymus on day three, but not day seven, after birth results in the spontaneous development of a variety of autoimmune pathologies.[38] This observation became one of the foundations of the Tregs field, as it demonstrated that thymus-derived Tregs that migrate to the periphery after day 3 are essential for self-tolerance.[39, 40]

There are two models for thymic development of Tregs: the instructive model and the stochastic model.[41] According to the instructive model, cell fate determination is based on the strength of T cell receptor stimulation: intermediate levels of TCR stimulation induce forkhead box P3 expression, whereas higher levels induce negative selection. Low-level TCR signalling allows the cells to mature and emigrate as conventional naive T cells. According to the stochastic or selective model, the induction of FOXP3 expression is attributable to a TCR-independent signal, perhaps at the early stage of $CD4^{-}CD8^{-}$ double-negative thymic progenitors. Compared with $FOXP3^{-}$ thymocytes, $FOXP3^{+}$ cells are relatively resistant to negative selection, which is induced by a high level of self-reactivity of the TCR. Thus, these self-reactive $FOXP3^{+}$ thymocytes survive to generate the Treg subset. Although once controversial, the preponderance of available data supports the hypothesis that self-reactivity is the primary determinant that directs developing thymocytes to undergo

thymic Treg differentiation. The self-antigens for many Tregs appear to be uncommon, rather than ubiquitous, based on the observation that thymocytes with the same TCR specificity undergo intraclonal competition for a small Treg developmental "niche".[41] The dependence of thymic Tregs selection on self-reactivity is remarkable on many levels. It limits the export of self-reactive conventional $CD4^+$ T cells to the periphery. It creates a T cell subset that, at the population level, can distinguish self. Whereas self-reactive T cells would be biased towards natural Tregs, T cells recognizing foreign antigens would not have that bias, resulting in a form of self/non-self discrimination. Finally, the principle of clonal expansion is likely to be as crucial for Tregs function as it is for conventional $CD4^+$ T cells, allowing Tregs to dynamically respond to changes in self-antigen presentation due to trauma or homeostatic cell death processes.[41]

#### 1.1.4.4 Differences between thymic and induced regulatory T cells: knock-out models' lessons

There are several types of regulatory T cells but only the naturally occurring $CD4^+CD25^{high}Foxp3^+$ thymic Treg (tTreg) differentiate in the thymus as part of T cell ontogeny.[42-45] Induced Treg (iTreg) can be generated from $CD4^+CD25^-$ cells *in vitro* in both mouse and human in the presence of TGF-β; however, human iTreg are not suppressive, suggesting that mouse and human Tregs have different differentiation requirements.[46] Foxp3 is a master transcriptional regulator that controls the tTreg differentiation program, is essential for tTreg function, and its constitutive expression distinguishes tTreg from other cells, such as activated Teff, that transiently express Foxp3.[47] A subset of Treg with a comparable phenotype ($CD4^+CD25^{high}Foxp3^+$) has also been demonstrated in the mouse to differentiate in

peripheral tissues and is referred to as peripheral Treg (pTreg).[47] Whether pTreg are also present in humans is uncertain since their identification is difficult because definitive markers that distinguish between the two subsets are presently missing.[48]

Immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome is a rare but highly instructive example of a monogenic autoimmune disease of tTreg differentiation or function.[49] The analysis of a naturally occurring Foxp3 mutation in the scurfy mouse enabled several groups of investigators to identify IPEX to be the consequence of mutations in FOXP3, which is localized on the X chromosome.[50] The clinical phenotype of male scurfy mice matched the clinical presentation of 19 related males patients initially reported in 1982 by Powell and colleagues.[51] Subsequent studies in transgenic and genetically engineered mice established the link between FOXP3 mutations, a lack of tTreg, $CD4^+$ T cell hyperproliferation, and tissue infiltration and destruction.[52] Correlations between IPEX genotype and phenotype have not been consistent, and the reasons for this discrepancy remain so far unknown.[53] Unlike Foxp3 knock-out mice, most patients are not null for FOXP3. Instead, expression is either decreased or mutant isoforms are generated that retain some biological activity. The incomplete loss of FOXP3 function probably explains the later onset of symptoms in patients compared to that of mice.[47]

The AIRE gene and pathogenic mutations were identified by positional cloning of the gene responsible for APS1, which was originally called autoimmune polyendocrinopathy candidiasis ectodermal dysplasia (APECED).[54, 55] The classical manifestations of autoimmune polyglandular syndrome type 1 (APS1) were a triad of chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency, but the clinical phenotype is expanding with improved detection of

AIRE mutations.[47] This is analogous to the situation with IPEX where broader availability of genetic testing for FOXP3 mutations has led to an expanded range of clinical manifestations and disease progression than predicted with the classical definition. An interesting difference observed between APS1 and the engineered mouse model of AIRE deficiency is the observation that patients present with decreased peripheral numbers of tTregs, which is not seen in the $AIRE^-$ mice, despite their tTreg developmental defect. In addition to the altered TCR repertoire of tTregs due to intrathymic defects in selection, the tTregs from APS1 patients have intrinsic defects of decreased FOXP3 expression and function.[56] In athymic nude mice transplanted with both a normal and AIRE-deficient thymus, autoimmunity still developed despite the presumed normal generation of Tregs by one of the thymi.[57] It is not known if the relative tTregs output of the mouse thymus is the same as human, nor is it known if transplanted thymus is equally as efficient as a native organ.[47] Nevertheless, if these murine results were also true of APS1 patients, they would have significant therapeutic implications. First, if there were a therapy that allowed normal tTregs generation,[58] then thymectomy to remove the native AIRE-deficient thymus would be necessary to prevent further production of autoreactive Teff cells. Second, the efficacy of the newly produced tTregs would likely be enhanced if ablative lymphocytotoxic therapy was first administered to eliminate autoreactive T cells that had already been made.[47]

#### 1.1.4.5 Peripheral tolerance in T cells

Despite the central tolerance mechanisms, some autoreactive T cells can escape from this crucial control and react against autoantigens. To prevent any damage, there is a further check point in the immune system that control and "regulate" these cells in

the periphery: the peripheral tolerance.[1] Central tolerance acts *via* three mechanisms (Figure 1.21):[1]

- anergy;
- deletion of T cells by apoptosis;
- suppression by Tregs.

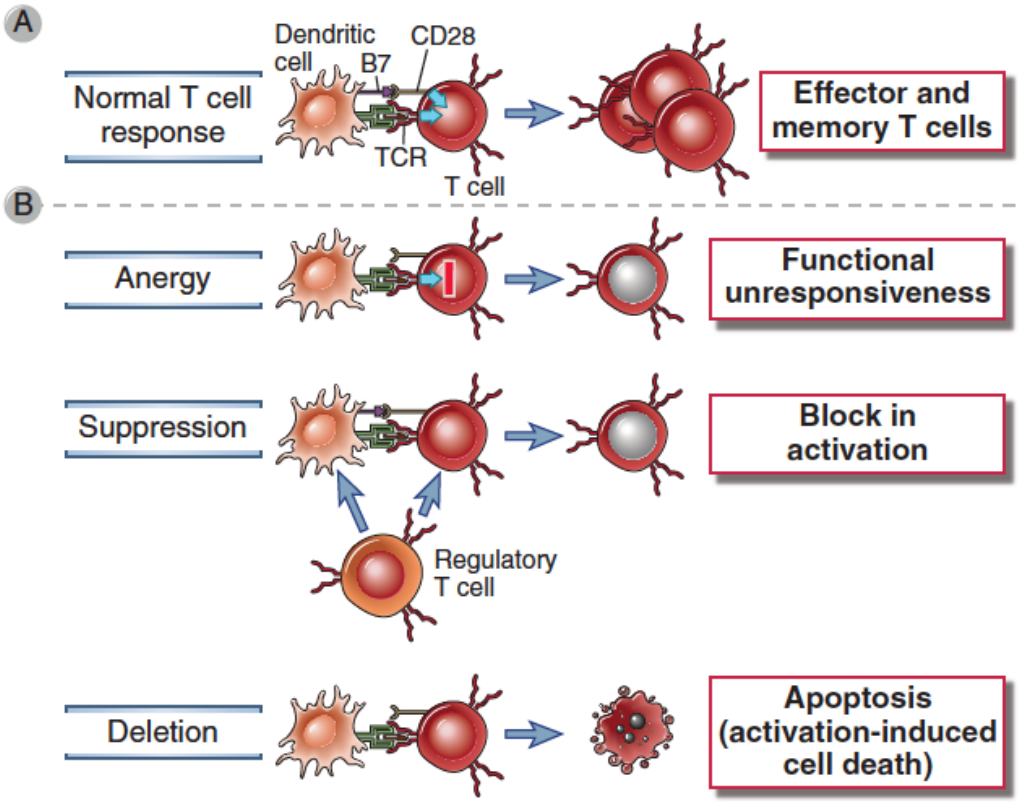



**Figure 1.21 Mechanisms of peripheral T cell tolerance**

The signals involved in a normal immune response (A) and the three major mechanisms of peripheral T cell tolerance (B) are illustrated.[1]

### *1.1.4.5.1 Anergy*

When a mature $CD4^+$ T cell is exposed to an antigen in the absence of co-stimulation, it may make the cells incapable of responding to that antigen. In this process, the self-reactive cells do not die but become unresponsive to the antigen (Figure 1.22).[1, 59]

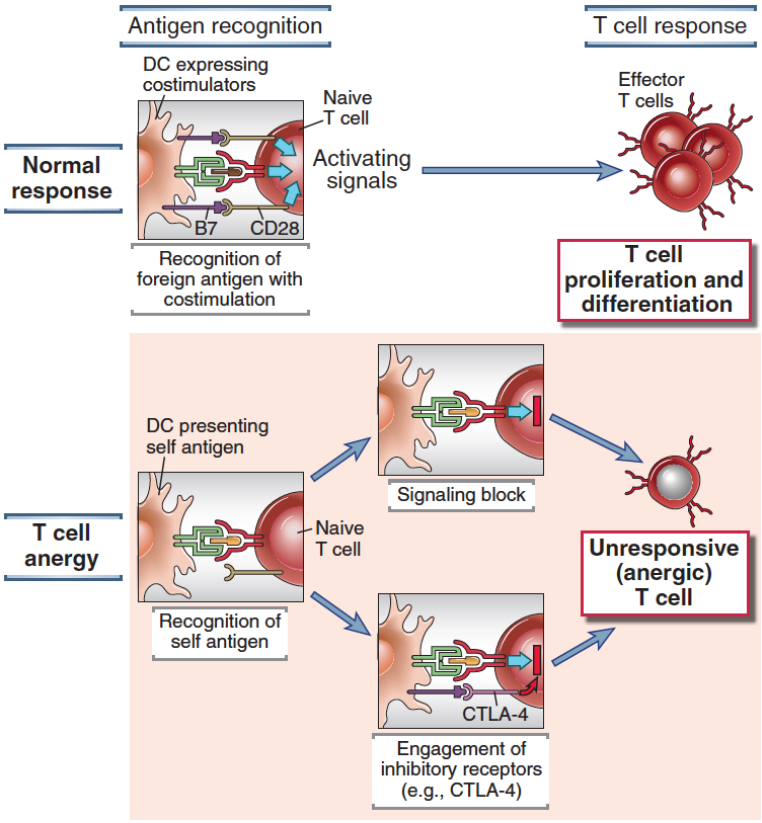



**Figure 1.22 Mechanisms of T cell anergy**

T cell responses are induced when the cells recognize an antigen presented by a professional APC and activating receptors on the T cells recognize co-stimulators on the APCs. If the T cell recognizes a self-antigen without co-stimulation, the T cell becomes unresponsive to the antigen because of a block in signalling from the TCR complex or engagement of inhibitory receptors.[1]

Several biochemical and genetic alterations reduce the ability of lymphocytes to respond to self-antigens (Figure 1.22):

- block in TCR-induced signal transduction (can be attributed to reduced TCR expression);[1]
- self-antigen recognition may activate cellular ubiquitin ligases, responsible for the ubiquitination of TCR-associated proteins and their targeting for proteolytic degradation in proteasomes or lysosomes;[1]
- self-antigens recognition by T cells may trigger the engagement of inhibitory receptors of the CD28 family, whose function is to terminate T cell responses. Cytotoxic T lymphocyte associated protein 4 (CTLA-4) and programmed death-1 (PD-1) are two receptors involved in self-tolerance. CTLA-4

competes with CD28 for B7 co-stimulators and excludes CD28 from the site of T cell recognition,[1] whereas engagement of PD-1 by its ligands (PD-L1 or PD-L2) transduces a signal that inhibits T cell proliferation, cytokine production, and cytolytic function;[60]

- tissue DCs are normally in an immature state and do not express co-stimulatory molecules. Presentation of an antigen by these immature DCs may induce tolerance in T cells and make them unresponsive.[1]

### *1.1.4.5.2 Deletion of T cells by apoptosis*

The ability to recognize self-antigens without inflammation or a repeated stimulation by antigens is able to induce T cell death by apoptosis through two major pathways: the mitochondrial pathway and the death receptor pathway (Figure 1.23).[1]

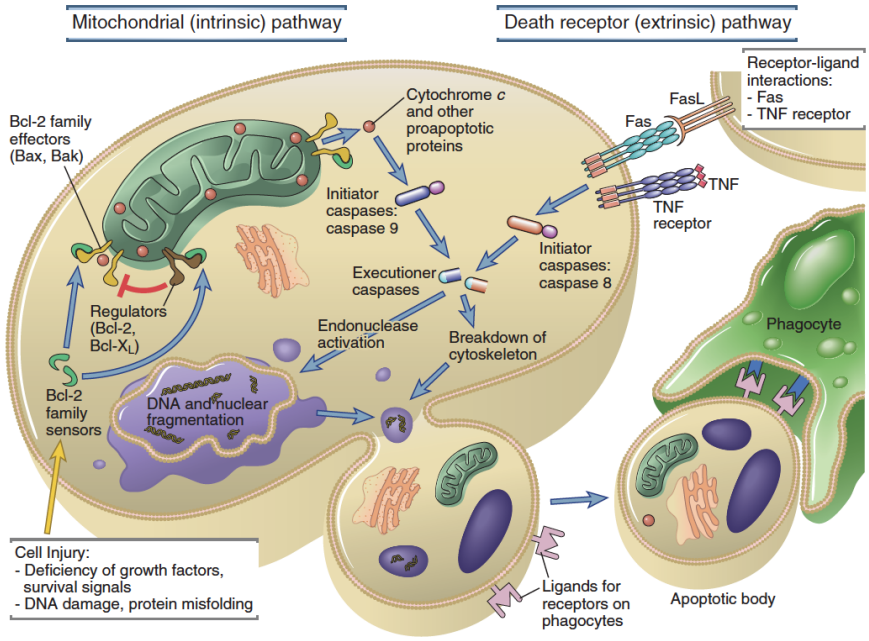



**Figure 1.23 Pathways of apoptosis**

Apoptosis is induced by the mitochondrial and death receptor pathways, which culminate in fragmentation of the dead cell and phagocytosis of apoptotic bodies.[1]

The mitochondrial (or intrinsic) pathway is mediated by the anti-apoptotic family of proteins Bcl-2. In normal circumstances, signals from the TCR, co-stimulators and growth factors stimulate the expression of anti-apoptotic proteins of Bcl-2 family and

these proteins promote cell survival and proliferation. When T cells are stimulated in the absence of proper co-stimulation and/or inflammation, a pro-apoptotic protein called Bcl-2 interacting mediator of cell death (Bim) becomes activated, resulting in T cell apoptosis.[1]

In the death receptor (or extrinsic) pathway, the TNF-homologous cell surface receptors are engaged by their ligands. As a result of these receptors oligomerization, cytoplasmic adaptor proteins are activated, and caspase 8 is subsequently assembled and cleaved. The active caspase 8 then cleaves a series of other caspases, again resulting in apoptosis.[1]

#### *1.1.4.5.3 Suppression by regulatory T cells*

The ability of some T cells to suppress other T cells' function was firstly described in the early 1970s, supported by a number of studies.[61-63] Nevertheless, the knowledge on this field has had a stop, mainly because initial attempts to define populations of suppressor cells and their mechanisms of action were largely unsuccessful.[1] Twenty-five years later, the concept of "suppressive T cells" has had a remarkable rebirth, due to better approaches to define, purify, and analyse these subpopulations of T cells.[1, 24, 64]

Regulatory T lymphocytes, a $CD4^+$ T cells subset, are responsible to suppress immune responses and maintain self-tolerance (Figure 1.24).

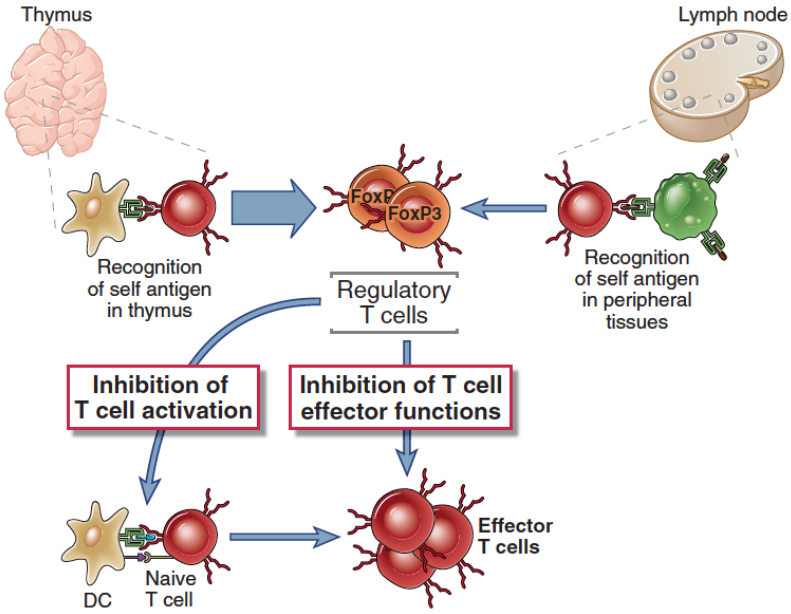



**Figure 1.24 Regulatory T cells**

Regulatory T cells are generated by self-antigen recognition in the thymus and by antigen recognition in peripheral lymphoid organs. The development and survival of these cells require IL-2 and the transcription factor Foxp3. In peripheral tissues, regulatory T cells suppress the activation and effector functions of other, self-reactive and potentially pathogenic lymphocytes.[1]

The majority of Tregs express high levels of the IL-2 receptor α chain (CD25) but no other markers of T cell activation. The transcription factor Foxp3, a member of the forkhead family of transcription factors, plays a critical in the development and function of the majority of Tregs.[1]

### *1.1.4.5.3.1Phenotypic markers of regulatory T cells*

Tregs have a distinct phenotype from other lymphocyte populations (Table 1.2 Characteristics of CD4+ subsets).[1]

**Table 1.2 Characteristics of $CD4^+$ subsets**

| | Tregs | Naïve T cells | Effector and memory cells |
|---|---|---|---|
| Surface markers | $CD25^{high}$<br>CTLA-4<br>GITR<br>$CD127^{low}$ | CD25<br>$CD127^{high}$ | $CD25^{medium/high}$<br>$CD127^{low}$ (effectors)<br>$CD127^{high}$ (memory) |
| Cytokines produced upon activation | TGF-β<br>IL-10 | IL-2 | IFN-γ<br>IL-4<br>IL-5<br>IL-17 |
| Chemokines receptors | CCR6 | CCR7 | CXCR3 |
| Growth factor requirement | IL-2 | IL-7 | IL-2 (effectors)<br>IL-4 (effectors)<br>IL-7 (memory) |

| | | | |
|---|---|---|---|
| Major transcription factor expressed | Foxp3<br>STAT5 | KLF-2 | T-bet (effectors)<br>GATA-3 (effectors)<br>RORγt (effectors)<br>Various STATs (effectors)<br>BLIMP-1 (memory) |

Although numerous T cell subsets may have suppressive activity, the cell type whose regulatory role is best established is $CD4^+Foxp3^+CD25^{high}$. Both Foxp3 and CD25 are essential for the generation, maintenance, and function of Tregs. These cells typically express low levels of IL-7 receptor (CD127), and they use IL-2 but not IL-7 as their growth and survival factor. Tregs also typically express high levels of CTLA-4, which is required for their function.[1]

#### *1.1.4.5.3.2 Differences between murine and human Tregs*

In the early 1990s, expression of CD25 was used to identify human Tregs on the total circulating $CD4^+$ T cells with values oscillating between 10 and 30%.[65] High expression of CD25 in human $CD4^+$ T cells has been used to define Tregs, representing approximately 1–2% of the total $CD4^+$ subset in peripheral blood and exhibiting the greatest suppressive function.[66] However, the definition of "high" mean fluorescence intensity can be inaccurate as it depends, among others, on the specific configuration of the flow cytometers and on the characteristics of the antibodies; moreover, CD25 is expressed heterogeneously on T cells.[67]

The crucial role of FoxP3 as a master regulator in the development and function of Tregs was demonstrated by a frameshift mutation in the *FOXP3* gene that leads to its impaired expression and to the development of a syndrome known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in humans and scurfy in mice, resulting in a variety of autoimmune disorders that could be incompatible with life.[68] In humans, the percentage of $CD4^+FoxP3^+$ cells is

variable, representing approximately 1–10% of the $CD4^+$ population in peripheral blood.[64] In the early 2000s, the addition of FoxP3 to CD25 efficiently improved the identification of Tregs.[64] However, such a combination of CD25 and FoxP3 is not perfect, as the expression of these markers can change depending on the inflammatory status.[69] July 2006, two groups introduced a new marker for the identification of Tregs; they found that low or lack of expression of CD127 in CD251 T cells identify human Tregs with a potent suppressive function.[70]

In mice, the identification of Tregs as $CD4^+CD25^+$ T cells has been used widely, representing approximately 5–10% of $CD4^+$ T cells in the periphery.[64] As well as in humans, the co-expression of CD25 and FoxP3 for the characterization of Tregs in rodents is accepted widely. Analyses using both markers have shown a decreased frequency of this subpopulation when compared to expression of total FoxP3 alone. This could be explained by the transient loss of CD25 expression in $CD4^+FoxP3^+$ T cells after chronic activation by internalization of the IL-2/IL-2R complex.[71] The use of CD127 to identify mice Tregs has also been suggested, because their expression levels are lower than in $CD4^+CD25^-$ cells, and the depletion of Tregs through anti-CD25 antibodies leads to a 90% reduction of $CD4^+CD25^{high}CD127^{low}$ cells.[72] Similar to what occurs in humans, the use of this marker could also be controversial, as there is a subpopulation of activated $CD4^+FoxP3^+$ Tregs with high expression of CD127.[73]

In humans, several authors have reported that $CD4^+CD25^{high}$ T cells express intracellular CTLA-4 constitutively, which correlates with a high suppressive activity while in mice, the expression of CTLA-4 by Treg cells can vary depending on the tissue evaluated.[69]

In healthy individuals, approximately 4% of human $CD4^+FoxP3^+$ Tregs express PD-1 in peripheral blood. However, under certain conditions, such as chronic viral infection, its expression could be upregulated. Conversely, compartmentalization of PD-1 expression has been reported in mice, as approximately 90% of the $CD4^+FoxP3^+$ in healthy mice express PD-1 intracellularly in lymph nodes and spleen.[67]

#### *1.1.4.5.3.3Generation and maintenance of regulatory T cells*

Tregs are mainly generated by self-antigen recognition in the thymus and by recognition of self and foreign antigens in peripheral lymphoid organs. In the thymus, one of the fates of a CD4-committed lymphocyte recognising self-antigens, could be its development in a Treg; Tregs developed in the thymus are called nTregs (Figure 1.25). In peripheral lymphoid organs, antigen recognition in the absence of strong innate immune responses promotes the generation of Tregs from naive $CD4^+$; these are called iTregs (Figure 1.25). Due to their different ontogeny, nTregs are specific for self-antigens because these are the antigens mainly encountered in the thymus; on the other hand, iTregs may be specific for self or foreign antigens. It is not clear yet if Treg subsets contribute equally to the maintenance of self-tolerance.[1]

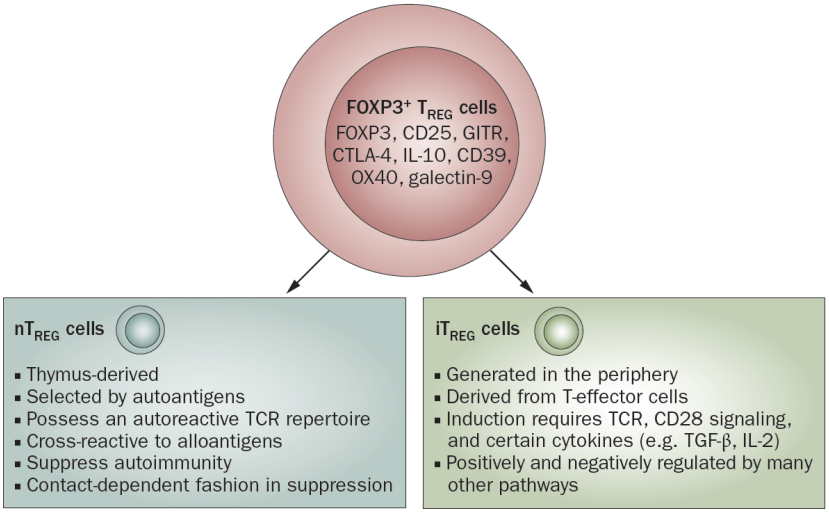



**Figure 1.25 Key features and differences between nTregs and iTregs**

Both subsets express the transcription factor FOXP3 and a variety of other cell surface factors, but they are strikingly different in origin, induction, TCR repertoire and in antigen specificity.[74]

*In vivo* animal data suggest that stimulation of mouse T effector by $CD103^+$ DCs in the presence of TGF-β and retinoic acid induces the generation of $Foxp3^+$ T cells in the gut-associated lymphoid tissue (GALT).[75-79] Moreover, Tregs can be induced in the periphery after exposure to αVβ8-integrin-expressing DCs[80] or suppressor of cytokine signalling 3 (SOCS3)-deficient DCs.[81] In addition to its role in generating iTregs, TGF-β may also have an important role in helping to maintain Foxp3 expression by nTregs.[82]

In human, Foxp3 induction by TCR stimulation in the presence of TGF-β does not always confer a regulatory to T cells.[46]

Human iTregs ($CD4^+CD45RA^+$ T cells stimulated with CD3 and CD46 specific antibodies) exert their suppressive function through the secretion suppressive cytokines and through the expression of granzyme B and to kill target cells in a perforin-dependent manner.[83] In contrast to naturally occurring Tregs, induced Tregs often have a restricted specificity for particular cell types, like tumours or foreign antigens.[84] Data from *in vivo* experiments are challenging to interpret as it is

not easy to assess the contribution of nTregs *versus* iTregs in the secretion of inhibitory molecules, such as IL-10 or TGF-β.[85]

IL-2 promotes differentiation of T cells into Tregs and is also required for the survival and maintenance of this cell population. IL-2 activates the transcription factor STAT5, which may enhance expression of Foxp3 as well as other genes known to be involved in the function of regulatory T cells.[1].

### *1.1.4.5.3.4 Mechanisms of suppression*

Tregs have the ability to inhibit T effectors through four basic modes of action (Figure 1.26):

- suppression by inhibitory cytokines;[85]
- suppression by cytolysis;[85]
- suppression by metabolic disruption;[85]
- suppression by DCs modulation.[85]

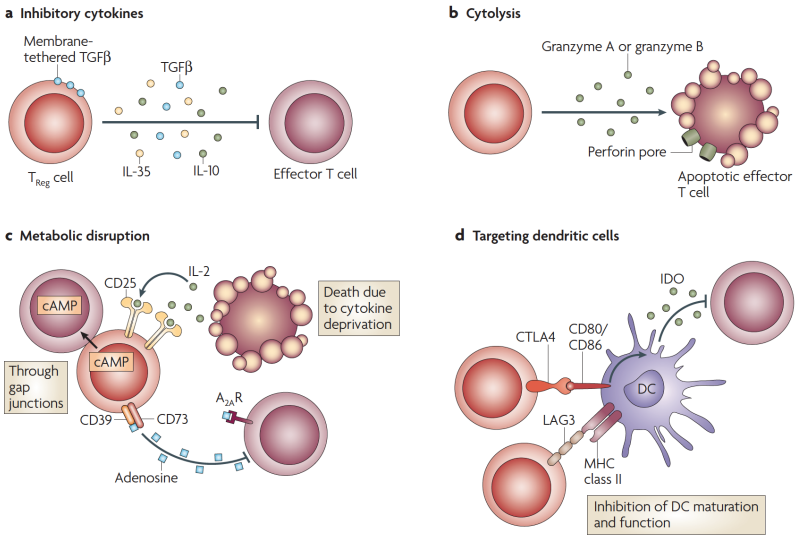



**Figure 1.26 Suggested mechanisms of Tregs function**

(A) Inhibitory cytokines include IL-10, IL-35 and TGF-β. (B) Cytolysis includes granzyme A and granzyme B-dependent and perforin-dependent killing mechanisms. (C) Metabolic disruption includes high-affinity CD25-dependent cytokine deprivation-mediated apoptosis, cyclic adenosine monophosphate (cAMP)-mediated inhibition, and CD39 and/or CD73-generated, adenosine receptor 2a ($A_{2A}R$)-mediated immunosuppression. (D) Targeting DCs includes mechanisms that modulate DC maturation and/or function such as LAG3-MHC II-mediated suppression of DC maturation, and CTLA4-CD80/CD86-mediated induction of indoelamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs.[85]

#### 1.1.4.5.3.4.1 Suppression by inhibitory cytokines

Despite some controversy in this matter, the cytokines traditionally considered "regulatory" are IL-10 and TGF-β.[85] IL-10 secretion by Tregs has been shown to be effective in prevention of inflammatory bowel disease (IBD) in mice.[86] Moreover, tumour microenvironment could promote the generation Tregs whose function is IL-10 dependent and cell contact independent.[87] Nevertheless, IL-10 is not exclusively secreted by Tregs and in specific circumstances t effectors could secrete IL-10.[88]

While TGF-β plays a crucial role in the development and maintenance of iTregs, its relevance for nTregs function is a controversial topic.[85] However, some studies suggest a suppressive effect on T effector by Tregs-secreted TGF-β (in a mouse model of IBD).

An inhibitory role for IL-35 has emerged in recent years. This cytokine is preferentially secreted by Tregs and is required for their maximal suppressive activity.[89] IL-35 is a member of the IL-12 family and is produced by Tregs, but not resting or activated T effector.[89] Remarkably, IL-35 was sufficient for suppressive activity, as ectopic expression of IL-35 conferred regulatory activity on naïve T cells and recombinant IL-35 suppressed T cell proliferation *in vitro*.[85, 89]

Although IL-10, IL-35 and TGF-β have been shown to be key mediators of Tregs suppressive abilities, their distinct role in different pathogenic and homeostatic settings suggests a non-overlapping function.[85]

#### 1.1.4.5.3.4.2 Suppression by cytolysis

Granzymes-mediated cytolysis is a well-established method of cell killing by NK cells and $CD8^+$ cytotoxic lymphocytes, but also many human $CD4^+$ have been demonstrated to have a similar cytotoxic activity,[90, 91] such as activated human

nTregs which have been shown to express granzyme A. Furthermore, it has been shown that Tregs mediated target-cell killing is mediated by granzyme A and perforine.[91] Tregs were also shown to suppress NK cells and CTLs anti-tumour activity by killing these cells in a granzyme B and perforine-dependent manner.[92] The proof of Tregs granzyme B-mediated cell killing is that, effector T cells overexpressing the granzyme B specific inhibitor serine protease inhibitor 6 (SPI6) are resistant to Treg mediated suppression.[85]

Some apoptosis-inducing factors have recently been identified on Tregs: galectin-1 and TNF-related apoptosis-inducing ligand (TRAIL)-DR5 are among the up-regulated factors, which are up-regulated and can induce apoptosis on T effector.[93, 94]

#### 1.1.4.5.3.4.3 Suppression by metabolic disruption

Due to Tregs' high expression of CD25, they consume a significant amount of IL-2 in the environment. This IL-2 consumption may lead to a local deficit of IL-2 and therefore actively starve T effector which are in cell cycle.[85] There is evidence that Tregs-induced cytokine deprivation may cause T effector apoptosis.[95] However, it seems that IL-2 depletion alone is not enough for Tregs to suppress T effector, therefore more work needs to be done to clarify this aspect.[96]

IL-2 deprivation is not the only mechanism through which Tregs cause metabolic disruption: concordant expression of CD39 and CD73 has been shown to generate peri-cellular adenosine, which suppresses T effector function through adenosine receptor 2A ($A_{2A}R$) activation.[97-99] This mechanism is also responsible for the enhanced generation of iTregs through the inhibition of IL-6 expression while promoting TGF-β secretion.[85, 100] Tregs are also able to suppress T effectors function

by direct transfer of cyclic adenosine monophosphate (cAMP) into T effector themselves, through membrane gap junctions.[101]

#### 1.1.4.5.3.4.4 Suppression by targeting dendritic cells

It is not yet clear how much of the regulatory potency of Tregs is directed towards DCs *versus* T effector, despite several studies have demonstrated immunomodulatory effects of Tregs on DC maturation and function[85, 102-105]. The main candidate molecule to DCs modulation is CTLA-4. More specifically: in the absence of functional CTLA-4 (using CTLA-4 specific blocking antibodies as well as in CTLA-4 deficient Tregs), Treg-mediated suppression of T effector via DCs was reduced.[106, 107] Tregs have also the ability to induce DCs to express indoelamine 2,3-dioxygenase (IDO). This is a potent regulatory molecule, known to induce the production of pro-apoptotic metabolites from the catabolism of tryptophan. This results in the suppression of T effector through a mechanism dependent on interactions between CTLA-4 and CD80/CD86.[108, 109]

## 1.1.5 $CD4^+$ plasticity

Each T cell subset can be characterized by its ability to sense different inductive cytokines, programme the expression of distinct transcription factors and function by producing selected cytokines and chemokine receptors to best control specific pathogens or prevent immune pathology (Figure 1.27).[110]

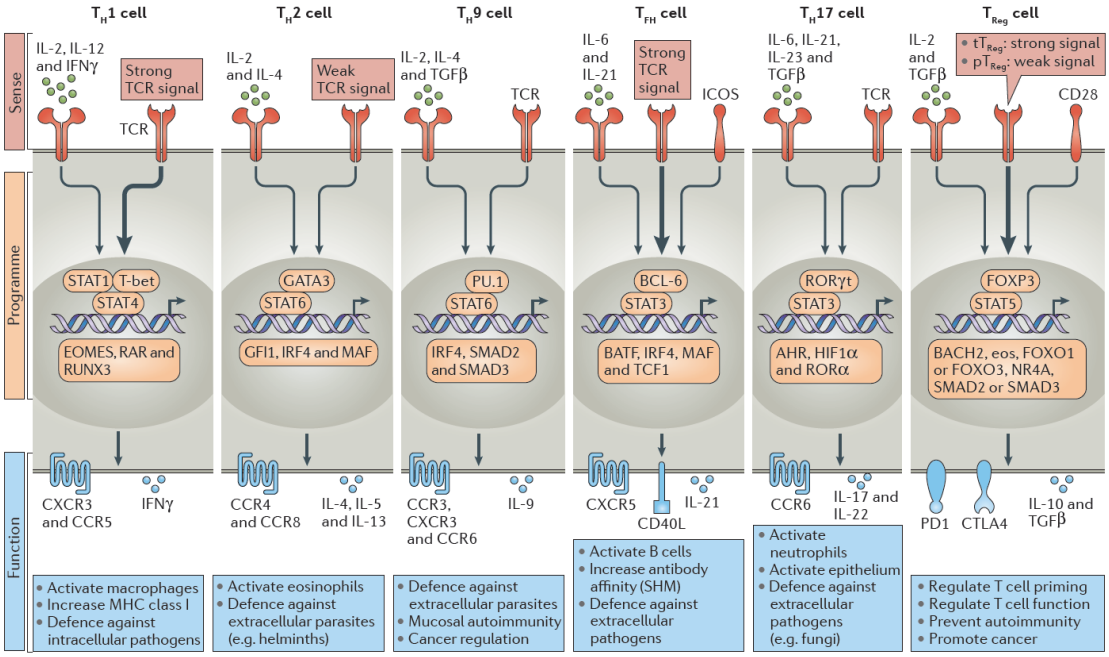



**Figure 1.27 Polarised CD4⁺ T cell subsets**

Each CD4⁺ T cell subset can be defined by their distinct abilities to sense (red), programme (orange) and function (blue) in the control of specific pathogens or immune pathologies. The inductive cytokines, polarizing transcription factors and cytokines or chemokine receptors that are characteristic of each subset are shown, along with their association with specific forms of immune defence.[110]

However, new evidence is available, and it shows the capacity of polarised T cells to change their phenotype and repolarize towards mixed or alternative fates, fuelling the hypothesis that CD4⁺ T cells are adaptable and can exhibit phenotypic plasticity in response to changing contexts.[111-114]

CD4+ plasticity can be regulated at three levels (Figure 1.28):[110]

- extracellular;
- cytosolic signalling;
- gene regulation.

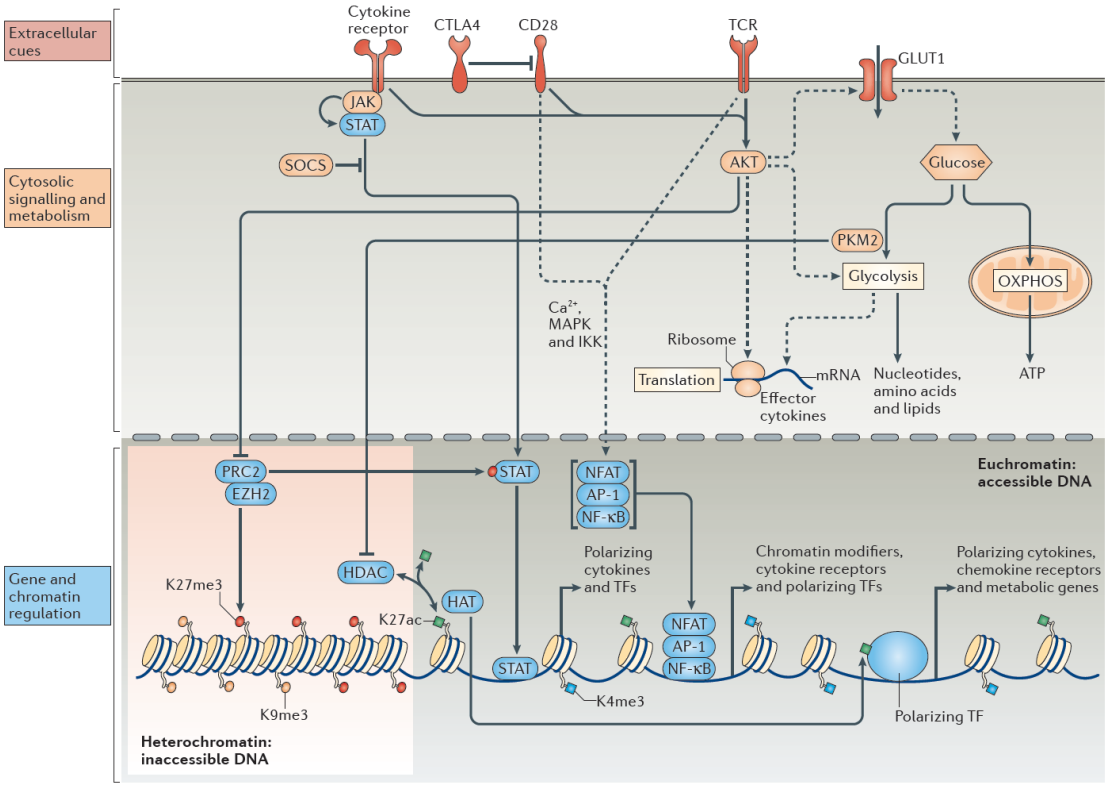



**Figure 1.28 The integration of signals at many levels in T cells regulates plasticity**

The regulation of T cell plasticity is depicted at three levels in the cell: extracellular cues (red), cytosolic signalling and metabolic programmes (orange) and transcription factor (TF)- or chromatin-mediated gene regulation (blue). These pathways are integrated by mechanisms linking these levels of regulation. Direct protein-protein interactions are indicated by solid lines, whereas indirect links between proteins are denoted with dashed lines.[110]

### 1.1.5.1 Regulation of plasticity at the extracellular level

#### *1.1.5.1.1 Cytokines*

Specific cytokines have a clear and dominant role in driving the plasticity between $CD4^+$ T cell subsets (Figure 1.29). This is due in part to the capacity of key inductive cytokines to provide a simple and direct conduit between the environment and gene regulation.[115] The majority of these polarizing cytokines function by engaging their receptors and inducing a phosphorylation cascade of receptor-associated Janus kinase (JAK) and signal transducer and STAT proteins, leading to the nuclear localization of the STAT proteins where they act as transcription factors.[110] The cytokine environment can even influence plasticity between inflammatory and regulatory programmes. TGF-β is crucial for the conversion of Th17 cells towards a regulatory

phenotype through the promotion of Foxp3 or IL-10 expression.[116-118] Conversely, the treatment of Treg cells from mice or humans with the Th17 cell-inducing cytokines IL-6, IL-1 and IL-23 can destabilize Foxp3 expression and induce IL-17 production in vitro.[119, 120]

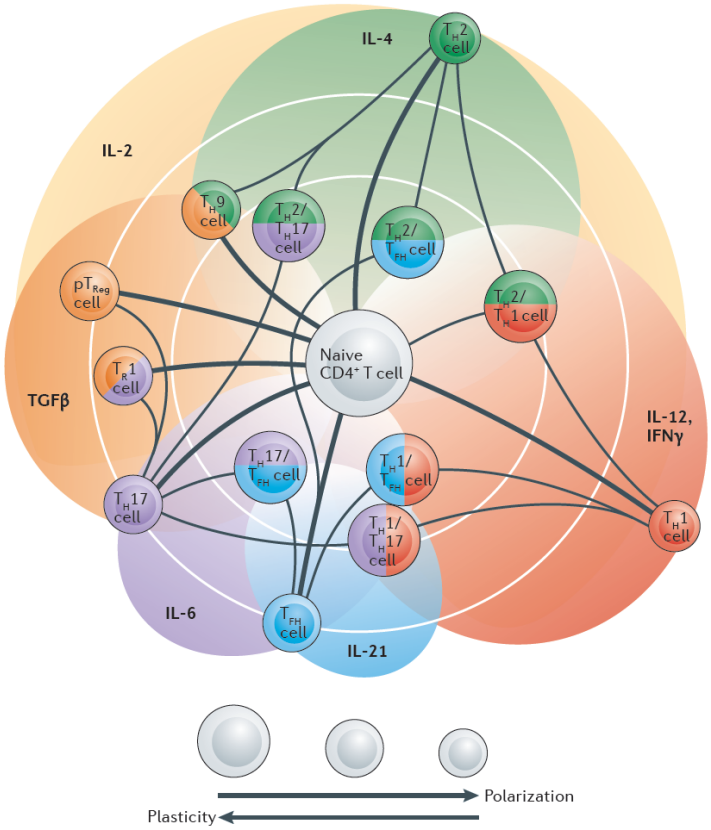



**Figure 1.29 Cytokine-driven T cell plasticity**

The key inductive cytokines IL-4, IFN-γ, IL-12, IL-21, IL-6, TGF-β and IL-2, alone or in concert, can polarize naive $CD4^+$ T cells towards different functions. This pinwheel depiction is intended to reveal the interconnectedness of these different programmes based on the capacity of these inductive cytokines to promote polarization or plasticity between subsets. Polarization, and hence more restricted cellular function, is depicted both by the distance from the central naive T cell and by decreasing cell size. Grey lines linking the subsets depict known transitions. Evidence suggests that reprogramming between subsets may occur by transitioning through intermediate stages in which cells exhibit phenotypes of many subsets.[110]

### *1.1.5.1.2 TCR and co-stimulatory signal strength*

The affinity of a TCR for its cognate antigen on MHC molecules, combined with co-stimulatory receptor-ligand interactions at the cell surface, generates variable intensities of cytosolic signals that flux through the cell, driving the activities of transcription factors such as AP-1, nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB) (Figure 1.28). Variations in signalling intensities can alter

the differentiation of $CD4^+$ cell subsets by tuning the receptiveness of a cell to different cytokines, by inducing the expression of specific cytokine receptors or by impinging directly on the activation of specific STATs.[121, 122]

Weak TCR signalling, by transient or low-affinity TCR interactions, favours the induction of Foxp3 expression.[123, 124] Importantly, TCR signal strength drives the first divergence of inflammatory *versus* regulatory subsets during T cell development in the thymus.

An increased strength of signal, which is imparted by TCRs that recognize self-antigen, imprints a distinct epigenetic state and induces Foxp3 expression to generate the Treg cell lineage.[41, 125]

### 1.1.5.2 Regulation of plasticity by cytosolic signalling

#### *1.1.5.2.1 PI3-AKT-mechanistic target of rapamycin signalling*

Activated by numerous cues in T cells, PI3K activates AKT by generating the phospholipid PIP3, which acts as a docking and activation site for the kinase.

AKT can then phosphorylate many substrates, including regulators of the canonical mechanistic target of rapamycin (mTOR) complex 1 leading to its activation. Importantly, PI3K is opposed by the activity of phosphatase and tensin homologue (PTEN), which converts PIP3 to PIP2.[110]

The PI3K-AKT-mTOR pathway is a crucial bifurcation point for inflammatory *versus* Treg programmes, as the activation of this pathway is required for the polarization and function of most T helper cells but is largely repressed in Treg.[126, 127] AKT function is blunted in Tregs by the activity of PTEN; removal of this regulation in Tregs with PTEN deficiency results in severely compromised Tregs stability and in their conversion into inflammatory TH1 and TH17 cells.[128, 129]

#### *1.1.5.2.2 Cellular energetics and metabolism*

Following antigenic stimulation, T cells rapidly mould the acquisition and use of metabolites to meet their energetic and biosynthetic needs, and the metabolic programmes that are engaged can directly affect T cell function.[110] CD28 signalling directly controls the metabolic switch to glycolysis during T cell activation by up-regulating the expression of glucose transporter 1 (GLUT1) in a PI3K–AKT-dependent manner, thereby increasing the import of glucose into the cell.[130, 131] Importantly, Tregs do not use glycolysis after stimulation, largely owing to their selective blockade of PI3K-AKT activation, preventing GLUT1 up-regulation. Instead, Tregs heavily rely on fatty acid oxidation to feed the tricarboxylic acid cycle and generate energy through oxidative phosphorylation.[132-134] Thus, PTEN deficiency in Tregs may drive loss of Foxp3 expression and effector cytokine production by enforcing glycolytic metabolism.[128, 129] In addition to glucose or fatty acids, glutamine is an important biosynthetic precursor that tips the balance between Th1 and Treg polarization. Glutamine metabolism generates α-ketoglutarate, which is required for Th1 cells but blocks Tregs differentiation in an mTORC1-dependent manner.[135]

### 1.1.5.3 Regulation of plasticity by gene regulation

#### *1.1.5.3.1 Transcription factors*

STATs drive Th cell polarization or plasticity in direct response to the binding of cytokines to receptors.[110] The so-called "master regulators" or "lineage-defining" transcription factors T-bet, GATA3, retinoid-related orphan receptor γt (RORγt), B cell lymphoma-6 (BCL-6) and Foxp3 have a substantial, but often incomplete, role

in setting the transcriptional programmes of Th1, Th2, Th17, TFH and Tregs, respectively (Figure 1.30).[125, 136-138]

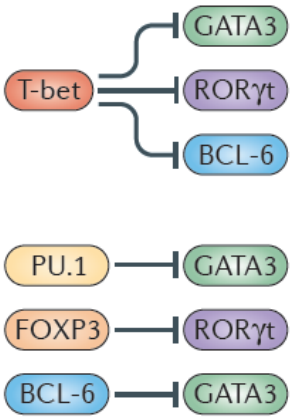



**Figure 1.30 Mechanisms of gene regulation in T cell plasticity (I)**

Direct interactions between transcription factors can antagonize the function of opposing transcription factors.[110]

These so-called master transcription factors behave less like lineage-definers and more like executors, expressed in response to environmental cues to carry out the induction of a defined set of gene effectors.

The expression of specific STATs and master transcription factors is not sufficient for polarization or plasticity of T helper cell subsets; rather, a collection of additional transcription factors is required.[139, 140]

### *1.1.5.3.2 DNA accessibility by modification of DNA and histones*

In the nucleus, a delicate balance is struck between the logistics of packing DNA into this confined space (in heterochromatin) and providing access to DNA for transcription (in euchromatin).

Post-transcriptional modifications of histone proteins, which make up nucleosomes, control how tightly nucleosomes are packed, and this has been adopted by the cell to regulate transcription in a semi-stable manner, along with the direct modification of DNA by methylation.

These forms of gene regulation are termed "epigenetic" because they promote the heritable transmission of distinct transcriptional programmes despite identical DNA sequences.[110]

### *1.1.5.3.3 Generating accessible DNA*

Regulation of T cell polarization by chromatin structure and DNA accessibility was firstly hypothesised with the discovery that cytokine expression after TCR stimulation required a discrete number of cellular divisions.[141-144] This is largely due to the requirement for chromatin to be reorganized to create access to key differentiation *loci*. The opening of chromatin is aided by the recruitment of histone acetyltransferases, chromatin remodelling enzymes and histone methyltransferases, as well as the recently discovered histone lysine demethylases and ten-eleven translocation proteins that initiate DNA demethylation (Figure 1.31).[145-151]

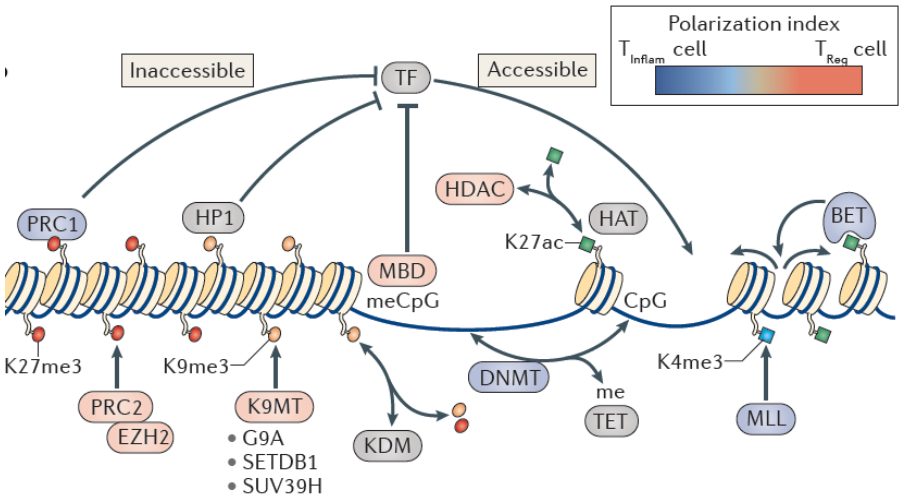



**Figure 1.31 Mechanisms of gene regulation in T cell plasticity (II)**

DNA accessibility controls gene expression. Histone modifications and DNA methylation can drive changes in chromatin structure, altering the accessibility of DNA to transcription factors. These epigenetic changes act to stabilize gene expression programmes during T cell responses in which driving or opposing transcription factors may be transiently lost or gained, respectively. A polarization index indicates proteins or processes that favour inflammatory (blue) or regulatory (red) T cell programmes.[110]

Polarizing transcription factors, such as STATs, may participate in opening or stabilizing the local chromatin structure by recruiting these enzymes in a site-specific manner during Th polarization.[147, 150-153]

#### *1.1.5.3.4 Inhibiting DNA accessibility by DNA methylation*

Equally important to opening *loci* for transcription factors to bind, is the ability to block accessibility to genes that would oppose the directed polarization of effector functions.[110] Cytosine methylation directly blocks the binding of transcription factors to DNA and, through binding methyl-CpG-binding domain proteins, recruits additional chromatin modifiers, such as histone deacetylases or repressive histone methyltransferases, to generate heterochromatin.[111, 146] Ultimately, the effect on gene expression can vary depending on whether enhancer or repressor elements are methylated.[110]

### 1.1.5.4 Phenotypic plasticity in inflammatory and regulatory T cell lineages

Tregs originating in the thymus provide the best example of a distinct lineage of $CD4^+$ T cells. They differ extensively from the naïve precursors of Th that also originate in the thymus. Similar to the separation of the $CD8^+$ and $CD4^+$ T cell lineages, Tregs diverge from naïve $CD4^+$ T during T cell development in the thymus, recognize a distinct set of antigens,[41] have different epigenetic landscapes,[125] and uniquely express a defining transcription factor: Foxp3 (Figure 1.32).[110]

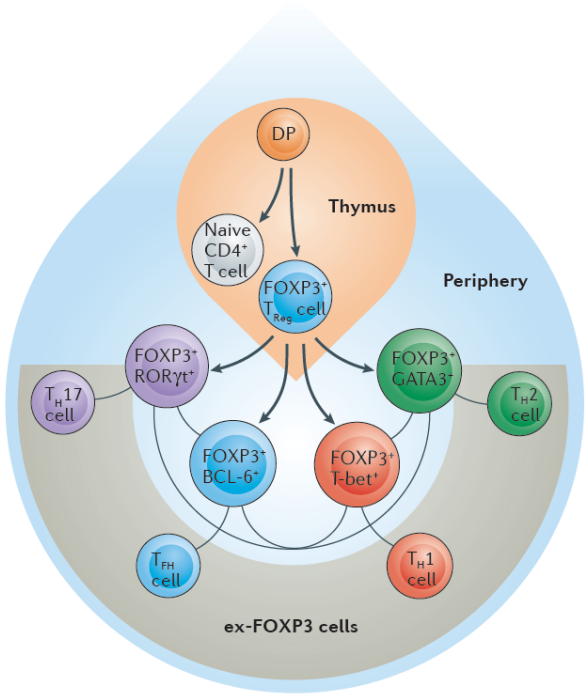



**Figure 1.32 Phenotypic plasticity in inflammatory and regulatory T cell lineages**

The figure schematically represents the extreme plasticity of T cells, both in inflammatory lineages and in Tregs.[110]

The finding that Foxp3$^+$ Tregs can co-express each of the so-called master transcription factors of each Th subset is important to our understanding of T cell plasticity.[154] Although the acquisition of properties defining each Th subset is hypothesized to allow Tregs to mirror, and thus better regulate, specific types of immune responses,[154, 155] co-expression of these transcription factors with Foxp3 supports the notion that the expression of these transcription factors is not lineage-defining but is responsive to environmental cues.[110] Furthermore, although often a topic of controversy, the stability of Foxp3 expression in thymic Tregs is pronounced, especially when compared to the plasticity with which the other master transcription factors can be induced or repressed.[110] Nonetheless, the loss of Foxp3 expression and acquisition of inflammatory cytokine production does occur in several settings, most prominently during lymphopenia or massive inflammation,[156-159] and is augmented by disrupting specific genes and pathways in Tregs.[128, 160, 161] It is these

transitions, between inflammatory and regulatory programmes, that are likely to be of greatest importance in immunological disease as well as for therapeutic manipulation to treat disease.[110]

## 1.2 Aplastic anaemia

### 1.2.1 Definition

Aplastic anaemia is a rare and heterogeneous type of bone marrow failure syndrome, idiopathic in 70-80% of the cases.[162] It is defined as one or peripheral blood (PB) pancytopenias with a hypocellular bone marrow in the absence of an abnormal infiltrate or marrow fibrosis.[163]

The incidence is 2-3 per million per year in Europe, but higher in East Asia.[164] There distribution is biphasic, with peaks at 10-25 years and over 60 years.[163]

### 1.2.2 Perspective on different causes of aplastic anaemia

#### 1.2.2.1 Haematopoietic stem and progenitor cells

Immune-mediated destruction of bone marrow haematopoietic stem and progenitor cells is a well-known mechanism of AA pathophysiology, but recent studies have demonstrated that haematopoietic stem progenitor cells (HSPCs) in subgroups of patients with AA carry intrinsic defects,[165] including telomere shortening and presence of myelodysplastic syndrome (MDS) related somatic mutations.

Significant telomere shortening has been reported in subpopulations of patients with AA, especially in non-responders to immunosuppressive treatment (IST).[166, 167] Mutations in telomerase complex genes on progenitor cells lead to defects in telomere length maintenance, leading to deficient haematopoietic survival and proliferative capacity, and to a reduced haematopoietic stem cell pool.[168-170]

Accelerated telomere shortening in subsets of untreated or refractory AA patients has been linked to an increased HSPCs proliferation.[166, 171] Therefore, in subpopulations of patients with AA, restricted clonal haematopoiesis and "regenerative stress" leading to chromosomal instability in HSPCs, may be the cause of telomere shortening.[172, 173]

There is a correlation between telomere length and persistent cytopenias after IST: shortened telomeres are associated with a higher risk of relapse, clonal evolution and monosomy 7 as well as inferior Overall survival (OS).[173, 174] Short and dysfunctional telomeres affect the proliferation of normal HSPCs, induce chromosomal instability and increase the risk of malignant transformation. Therapeutic up regulation of telomerase by androgen or other pharmacological agent might reduce the predisposition to clonal evolution.[173]

The potential association and co-operation between mutations in epigenetic regulators and immune-mediated bone marrow failure (BMF) may be explained by the fact that the majority of mutated genes are involved in epigenetic regulation of DNA transcription.[167]

#### 1.2.2.2 Genetic contribution

The most frequent and difficult differential diagnosis of AA is hypocellular MDS. Morphologic differences between the two conditions may be very difficult to see, and often the cellularity of the sample is too low to make a thorough morphologic characterization.[167] The lack of established diagnostic criteria to distinguish these two entities is a major reason for this inconsistency.[175] However, the significance of abnormal cytogenetic clones, detected in 10-15% of patients with AA, is controversial and may not indicate a diagnosis of MDS.[176] Some abnormal clones, like +8[177] and

del13q,[178] are associated with a good response to IST. Moreover, detection of single nucleotide polymorphism array karyotype abnormalities may identify those AA patients who are at risk of clonal evolution.[179] Kulasekararaj *et al.* have identified clonal hematopoiesis in a fifth of a cohort of AA patients and specific gene mutations were associated with transformation to MDS.[167] The majority of the mutated genes are involved in epigenetic regulation of DNA transcription. Mutations of additional sex combs like 1 (ASXL1), DNMTA, BCOR, and TET2 in this cohort, together with published expression data, suggests a role for the potential association between mutations of epigenetic regulators and immune-mediated BMF.[167]

Yoshizato *et al.* performed next-generation sequencing and array-based karyotyping on blood samples obtained from 439 AA patients, and analysed serial samples obtained from 82 patients. One third of the patients were found to have somatic mutations in myeloid cancer candidate genes. Forty-seven per cent of the patients developed clonal hematopoiesis, mainly as acquired mutations. The mutations seemed to have an age-related signature, as their prevalence increased with age. While DNA methyltransferase (DNMT3A) and ASXL1-mutated clones showed a trend to increase in size over time, the size of BCOR, BCORL1-mutated, and phosphatidylinositol glycan anchor biosynthesis A (PIGA)-mutated clones decreased or remained stable. A better response to IST with a longer and a higher rate of overall and progression-free survival correlated with mutations in PIGA, BCOR, and BCORL1; mutations of DNMT3A, and ASXL1 were associated with a worse outcome. Nevertheless, the high variability of clonal dynamics did not necessarily predict the response to therapy and long-term survival among individual patients.[180]

To summarise, the presence of mutated clones could be a hallmark of clonal haematopoiesis (like in MDS) or of a selective adaptation in the context of immune surveillance/subversion secondary to AA.[167]

### 1.2.2.3 Regulatory T cells

Aplastic anaemia exact aetiology is still unknown, but previous studies have showed that it is an immune-mediated disease in which autologous T cells induce haematopoietic cells apoptosis.[162, 181] Previous data have shown inadequate numbers of PB Tregs in patients with AA.[182, 183] Furthermore, the number of Tregs in AA patients could improve substantially after IST.[184]

Considering the role of Tregs in AA, the decreasing dose of Ciclosporin a (CsA) seems to allow a better clinical effect.[184] Kordasti *et al.* have shown the reduction in absolute number and frequency of Tregs, which also correlates with disease severity, and sorted Tregs from AA patients were unable to suppress cytokine secretion (both IL-2 and IFN-γ) by autologous conventional T cells.[185] In the same study, it was shown that Tregs from AA patients secrete pro-inflammatory cytokines.[185]

Another study also reported the decreased frequencies of PB and bone marrow (BM) Tregs, reversed ratio of Treg frequencies of BM *versus* PB, the abnormality of migratory potential, and defective immunosuppression on conventional T cells in vitro.[186] A recent paper has also shown how the composition of Treg subsets is able to predict response to IST.[187]

### 1.2.2.4 Rationale for and brief history of immunosuppressive treatment

The pathophysiology of aplastic anaemia has not been fully elucidated and the best evidence for an autoimmune pathogenesis is found in the response to

immunosuppressive treatment.[188] As a substantial proportion of patients are non-responders, the question remains as to whether these are patients with inadequately treated autoimmune marrow failure, who may be rescued by an improvement of immunosuppressive or immunomodulating treatment, or whether these patients may have a different pathophysiology and will, therefore, never respond to immunosuppressants, whatever the intensity or specificity. Such patients may include individuals with undiagnosed hereditary marrow failure syndromes, hypoplastic myelodysplastic syndrome and possibly also true quantitative stem cell failure.[189]

Immunosuppression to treat severe aplastic anaemia was initially pioneered by Georges Mathé and Bruno Speck among others in the late 1970s. It is of interest that the first patients received haploidentical marrow along with anti-thymocyte globulin (ATG) and it took a while to discover that the response was due to ATG and not to the marrow infusion.[190] ATG and CsA has been the standard treatment over the last 30 years with continued improvement of results, probably more due to improvement in supportive care and increasing knowledge about best use of the established therapeutic tools than due to changes of the immunosuppressive treatment strategy.[189] A more comprehensive overview of all the treatment options will be given in 1.2.6.

### 1.2.3 Disease severity

To diagnose aplastic anaemia there must be at least two of the following:[191]

- haemoglobin (Hb) < 100 g/l;
- platelets (PLT) < 50 x $10^9$/l;
- polymorphonucleated (PMN) < 1.5 x $10^9$/l.

The modified Camitta criteria are used to assess severity:[191, 192]

- very severe AA:
  - marrow cellularity < 25% (or 25–50% with <30% residual haematopoietic cells), plus at least two of:
  - PMN < 0.2 x $10^9$/l;
  - PLT < 20 x $10^9$/l;
  - reticulocyte count < 20 x $10^9$/l;
- severe AA:
  - as for very severe aplastic anaemia (VSAA) but neutrophils < 0.5 x $10^9$/l;
- non-severe AA:
  - AA not fulfilling the criteria for severe aplastic anaemia (SAA) or VSAA.

### 1.2.4 Clinical presentation

The most common symptoms at diagnosis are those related to anaemia and thrombocytopenia, whereas serious infections are not a frequent early symptom in the course of the disease.[163] If there is a history of jaundice, this may suggest a post-hepatitis aplastic anaemia.[163] Even if almost two thirds of the cases are idiopathic, a careful drug, occupational exposure and family history should always be obtained.[163] Any drug possibly responsible should be discontinued and the patient should not be given again the same medication.[163] Hepatomegaly, splenomegaly or lymphadenopathy, are generally absent (except in case of infection).[163] In young adults with short stature, abnormally pigmented skin and skeletal abnormalities, particularly affecting the thumb, Fanconi Anaemia should be ruled out.[193] The presence of nail dystrophy, reticular skin pigmentation, and oral leucoplakia is

characteristic of DC.[193] The presence of peripheral lymphoedema may indicate a diagnosis of Emberger syndrome due to germline GATA2 mutation.[163]

### 1.2.5 Investigations required for the diagnosis

There is no single test that reliably diagnoses idiopathic acquired AA, therefore it is a diagnosis of exclusion.[163] Consequently, alternative aetiologies of BMF must be excluded in the diagnostic assessment.[163] An "empty" marrow on histology is a distinguishing feature and a prerequisite for the diagnosis.[163] There is increasing evidence that prevalence of inherited bone marrow failure (IBMF) syndromes is much higher than previously thought and may as well present in adulthood. The diagnostic investigations are required to confirm the diagnosis, and:

- exclude other causes of pancytopenia and a hypocellular bone marrow;[163]
- exclude IBMF syndromes;[163]
- screen for other underlying cause;[163]
- document co-existence of abnormal cytogenetic and paroxysmal nocturnal haemoglobinuria (PNH) clones.[163]

The list of investigations to be performed routinely in a suspect of aplastic anaemia is described below:[163]

- full blood count:[163]

  usually the Hb, PMN and PLT are uniformly decreased. In the early stages, isolated cytopenia, particularly thrombocytopenia, may occur. Lymphocyte counts are usually preserved. Presence of monocytopenia needs further investigation to exclude hairy cell leukaemia (HCL) or inherited bone marrow failure due to GATA2 mutation;[163]

- reticulocyte count:[163]

automated reticulocyte counting generally over-estimates the count compared to the levels of the Camitta criteria[191], as those were defined on manual counts. This criterion has now been changed from manual percentages to absolute reticulocyte levels < 60 x $10^9$/l as assessed by automated technologies;[194]

- blood film examination:[163]

  macrocytosis and anisopoikilocytosis are frequent, PMN may show toxic granulation, PLT are mainly small; exclude presence of dysplasia, abnormal platelets, blasts and other abnormal cells;[163]

- foetal haemoglobin (HbF)%:[163]

  to be measured pre-transfusion in children, its level is often elevated in constitutional syndromes;[163]

- peripheral blood chromosomal breakage analysis with diepoxybutane if patient aged < 50 years to exclude Fanconi anaemia (FA);[163]

- flow cytometry for glycophosphatidylinositol (GPI)-anchored proteins to detect PNH clone [six colours including fluorescent aerolysin (FLAER];[163]

- vitamin $B_{12}$ and folate:[163]

  a diagnosis of AA should be confirmed after proper correction of vitamin $B_{12}$ or folate deficiency;[163]

- liver function tests:[163]

  liver functions test should always be performed to exclude ongoing hepatitis;[163]

- hepatitis A, hepatitis B, hepatitis C, Epstein Barr virus (EBV), Cytomegalovirus (CMV), human immunodeficiency virus (HIV), parvovirus B19:[163]

  post-hepatitis AA is rare and it generally occurs 2-3 months after an acute episode of hepatitis;[195] CMV should be assessed if SCT is being considered; HIV is a more common cause of isolated cytopenias but is a very rare cause of AA;[196, 197] parvovirus B19 is more usually associated with pure red aplasia;[198]
- anti-nuclear antibody and anti-double stranded DNA:[163]

  very rarely systemic lupus erythematosus is associated with pancytopenia due to hypocellular marrow;[163]
- chest X-ray and other radiology:[163]

  useful at diagnosis to exclude infection; X-rays of the hands, forearms and feet may be indicated if an IBMF syndrome is suspected;[163]
- abdominal ultrasound scan and echocardiogram:[163]

  hepatomegaly, splenomegaly or enlarged lymph nodes may suggest another haematological malignancy as the underlying cause for the pancytopenia.[163]

There are also emerging diagnostic tests, which are not yet part of the routine but will most likely be soon:

- peripheral blood leucocyte telomere length;[199]
- next generation sequencing gene panels for telomere gene complexes mutations, other IBMF syndromes, acquired somatic mutations to help discriminate AA from hypoplastic MDS;[167]
- single nucleotide polymorphism array karyotyping to detect unbalanced chromosomal defects.[179]

## 1.2.6 Treatment

Treatment algorithms for acquired SAA and refractory SAA are shown in Figure 1.33[200] and in Figure 1.34.[201]

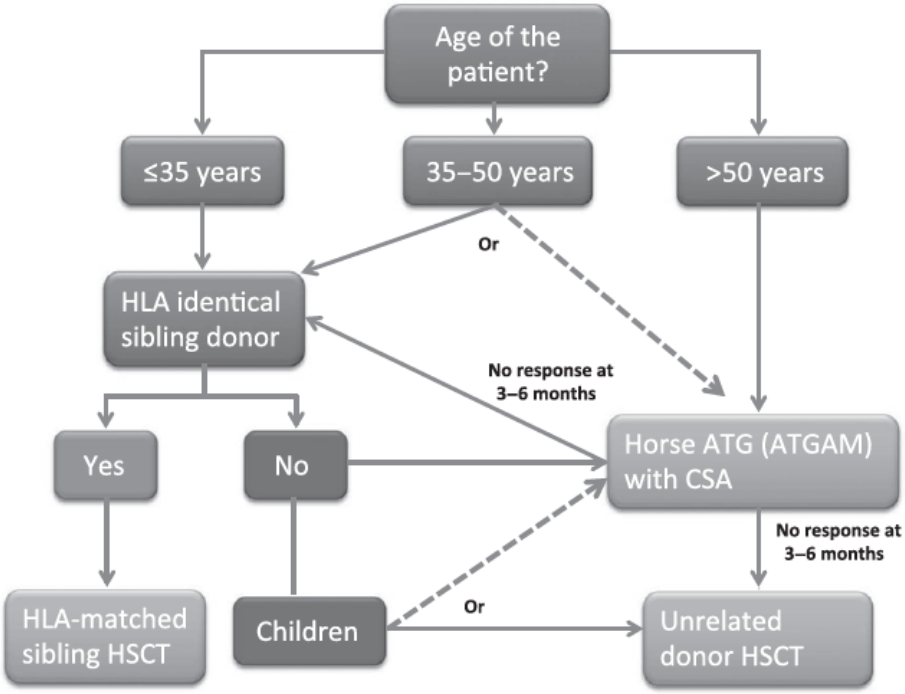



Figure 1.33 Treatment of acquired SAA

Haematopoietic stem cell transplantation (HSCT) may be considered for patients aged 35-50 or > 50 years who fail to respond to first line IST.[200]

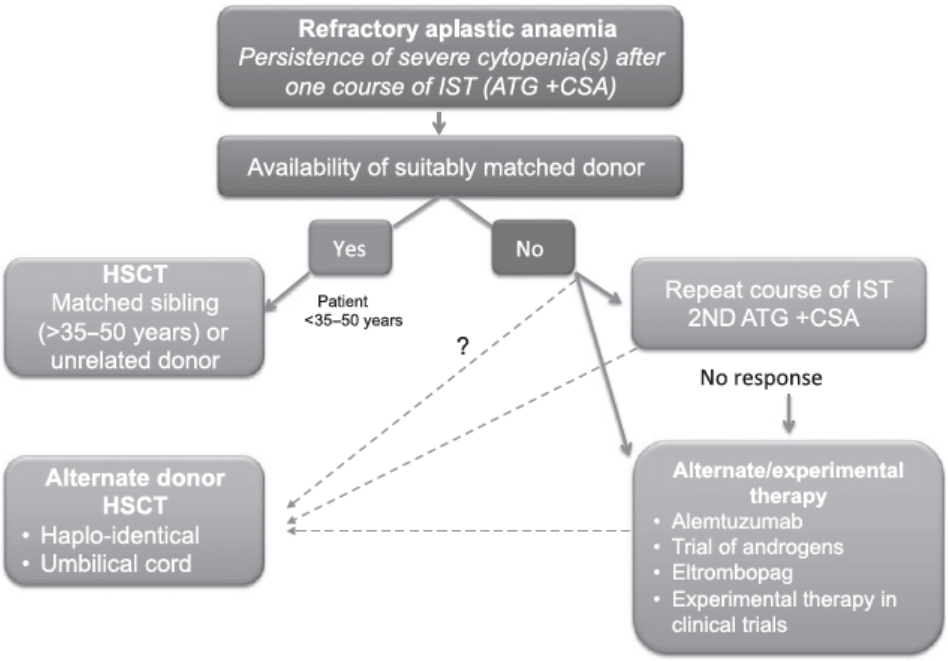



Figure 1.34 Treatment of adult refractory SAA

### 1.2.6.1 Supportive care

#### *1.2.6.1.1 Blood product support*

Transfusion with red blood cells (RBC) is often essential to maintain a safe blood count, relieve symptoms of anaemia and improve quality of life. The decision to transfuse RBC should be based on signs of anaemia, also considering the patient's age and cardiac, pulmonary or vascular co-morbidities.[163] The main aim of RBC transfusion is to maintain quality of life and avoid symptoms, therefore the Hb level to trigger the transfusion should be set according to the patient's age, comorbidities and severity of symptoms. Alloimmunization and iron overload are the commonest risks in transfusion dependent patients.[163]

There is no specific evidence regarding PLT transfusions in AA, all the data are taken from studies addressing the need for PLT transfusion support in patients with reversible thrombocytopenia.[202-204] Prophylactic PLT transfusions should be given to AA patients on active treatment if the PLT count < 10 x $10^9$/l. In case of sepsis,

PLT should be kept higher than 20 x $10^9$/l. During treatment with anti-thymocyte globulin (ATG), worsening thrombocytopenia can be observed. Despite the lack of evidence, most clinicians use a threshold of 20 x $10^9$/l.[205, 206]

The transfusion of irradiated granulocytes should only be taken into consideration in patients with life-threatening infections.[207] The actual effectiveness of granulocyte concentrates is not very well known and its use can cause adverse events, such as transfusion-related acute lung injury, alloimmunization and febrile reactions.[163]

All AA patients treated with ATG, alemtuzumab and/or candidate to have a HSCT must be transfused with irradiated blood products.[208] Since in the United Kingdom all the blood components are leucodepleted, the use of CMV-negative blood products is no longer recommended (with the only exception being pregnancy).[163]

#### *1.2.6.1.2 Iron chelation*

RBC transfusion-dependent patients will almost certainly develop tissue iron overload. For patients who will be treated with HSCT with a myeloablative regimen, a raised serum ferritin is an adverse predictor of outcome.[209] Serum ferritin still remains the most widely used parameter for assessment of iron overload. Magnetic resonance imaging (T2* or R2) can be used to quantify cardiac and liver iron, although its utility in AA has not been established.[163]

The first line treatment for iron chelation is desferrioxamine, if it is inadequate or contra-indicated, deferasirox should be used, although renal function should be strictly monitored in patients assuming CsA. Deferiprone is efficacious but not recommended in neutropenic patients.[210]

For IST responders, or after a successful HSCT, venesection is recommended for iron overload.

#### *1.2.6.1.3 Infection: prevention and treatment*

Infections are the major cause of death in AA.[201] The prolonged and persistent neutropenia of these patients results in a higher incidence of invasive fungal infections (IFI) and severe bacterial sepsis.[163]

Prophylactic antibiotics with either two non-absorbable (e.g., colistin and neomycin) or quinolone should be given, although the preference should be according to local policy. An aspergillus-active azole (itraconazole or posaconazole) should be used as prophylaxis. Antiviral prophylaxis with aciclovir or valaciclovir should be used during and after ATG therapy.[163]

Clinicians should follow their local protocols and guidelines for the management of febrile neutropenia, including the assessment and management of fungal infections.[211] Empirical anti-fungal therapy, as per local guidelines, should be initiated promptly for patients with clinically suspected IFIs.[163]

### 1.2.6.2 Immunosuppressive treatment

Standard first line IST is the combination of horse ATG (ATGAM®; Pfizer, New York, NY, USA) and CsA with no indication for routine use of granulocyte-colony stimulating factor (G-CSF) with ATG + CsA.[212] Prednisolone is used with ATG to prevent side effects of ATG.[163]

The combination of ATG and CsA is the first line treatment for:

- Non-severe aplastic anaemia (NSAA) patients who are transfusion dependent, bleeding, encountering infections or for lifestyle (activities);
- SAA/VSAA patients in the absence of an HLA-matched sibling;
- SAA/VSAA patients older than 35-50 years of age (Figure 1.33).

Although there is no upper age limit for ATG administration, an increased mortality in patients older than 60 years treated with ATG has been shown.[212, 213] In refractory/relapsing patients, or if the patient is ineligible for HSCT from an unrelated donor, a second course of ATG could be given (Figure 1.34).[162, 206, 214] A second course of horse ATG may be associated with more immediate and late (serum sickness) side effects.[215]

ATG is a powerful immunosuppressive agent, therefore it should only be used as in-patients in centres that are familiar with it and with its side effects. Before starting ATG:

- the patient should be clinically stable and afebrile;[163]
- PLT refractoriness should be investigated with PLT count increment studies;[163]
- prophylactic antiviral, antibiotic and antifungal drugs should be administered according to local guidelines;[163]
- for patients older than 60 years, careful assessment of co-morbidities is necessary to determine medical fitness, as there is an increased mortality from infection and bleeding after ATG in this setting.[163]

The dose of horse ATG is 40 mg/kg/d for four days. It should be given as a 12-18 hour intravenous infusion through a double lumen central venous catheter. Due to high risk of anaphylaxis, a test-dose must be given prior the full dose. Intravenous methylprednisolone 1 mg/kg, chlorphenamine, and PLT (aiming to keep the count > 20-30 x $10^9$/l) should be given before each dose of ATG.[162, 206] Irrespective of the neutrophil count, in case of fever, broad-spectrum intravenous antibiotics should be given. As fluid retention is common during ATG treatment especially in older

patients, therefore careful attention to fluid balance is important. Prednisolone (1 mg/kg/d) should be started the day after ATG is completed and should be given for two weeks, followed by rapid tapering over the two weeks. Side effects of ATG are:

- early reactions (including fever, rash, rigors, hypotension, hypertension, fluid retention, rarely acute pulmonary oedema, adult respiratory distress syndrome and anaphylaxis);
- later reactions (serum sickness occurring days 7-14 from the start of ATG, most commonly with arthralgia, myalgia, rash and fever).

CsA (5 mg/kg/d) should be started as the prednisolone dose is tapered, to achieve blood levels of 100-200 μg/l. CsA should be continued whilst the blood count continues to rise. A slow tapering of the drug (25 mg every 2-3 months) can be started after at least a further twelve months of therapy, to reduce the risk of later relapse.[216] Response criteria are shown in Table 1.3.[162]

**Table 1.3 Criteria for response to immunosuppressive therapy in aplastic anaemia**

| Response criteria to IST in SAA | |
|---|---|
| None | Severe disease criteria still fulfilled |
| Partial | Transfusion independent<br>No longer meet criteria for severe disease |
| Complete | Haemoglobin concentration normal for age and gender<br>Neutrophil count >1.5 x $10^9$/l<br>Platelet count >150 x $10^9$/l |
| **Response criteria to IST in NSAA** | |
| None | Blood counts are worse, or do not meet criteria below |
| Partial | Transfusion independence (if previously dependent)<br>Doubling or normalization of at least one cell line<br>Increase of baseline<br>Haemoglobin concentration of > 30 g/l (if initially < 60)<br>Neutrophils of > 0.5 x $10^9$/l (if initially < 0.5)<br>Platelets of > 20 x $10^9$/l (if initially < 20) |
| Complete | Same criteria as for severe disease |

Response to ATG is generally 3-4 months delayed. Six months after the first course of ATG, the response rate is around 70%. Five-year OS is age-dependent: 100% for

age < 20 years, 92% for 20-40 years, 71% for 40-60 years and 56% for > 60 years.[212] For NSAA, the response rate to ATG and CsA is 74%.[217] Relapse after ATG occurs in up to 35% of patients; the risk of later clonal evolution to MDS or acute myeloid leukaemia (AML) is 15%, and PNH 10%.[206, 218] Response to a second course of ATG is around 35% for refractory AA and 55-60% for relapsed AA.[162, 206, 214]

There is not enough evidence to support the use of mycophenolate mofetil, sirolimus, corticosteroids and cyclophosphamide for treatment of AA.[163] There are *in vivo* data, which support the use of rapamycin for BMF syndromes. Feng *et al.* have shown that rapamycin was an effective therapy in mouse models of immune-mediated bone marrow failure, acting through different mechanisms to cyclosporine. Its specific expansion of Tregs and elimination of clonogenic $CD8^+$ effectors support its potential clinical utility in the treatment of aplastic anemia.[219]

### 1.2.6.3 Eltrombopag

Approximately fifteen years ago, agonists of the thrombopoietin (Tpo) receptor, which stimulated megakaryocytes to produce platelets, were approved for immune thrombocytopenia.[220] These agents led to platelet count recovery in the majority of refractory cases of immune thrombocytopenia.[221] Apart from erythropoietin and granulocyte-colony stimulating factor, Tpo has distinct properties that could be effective in stimulating haematopoietic stem cells. This hormone, firstly cloned in 1994, was initially associated with megakaryocyte stimulation and platelet production.[222-224] However, *in vitro* and experimental data implicated that Tpo also had an important role in stem cell proliferation and maintenance.[220] Firstly, unlike erythropoietin and granulocyte-colony stimulating factor, the Tpo receptor is expressed in stem cells.[225] Secondly, in Tpo receptor knock-out models, aside from

megakaryocytopenia and thrombocytopenia, significant reductions in haematopoietic stem cells were observed.[226-229] Thirdly, Tpo is commonly used along with other growth factors and interleukins in culture to stimulate stem cells in vitro.[225] Fourthly, in a rare form of bone marrow failure, amegakaryocytic thrombocytopenia, mutations in the Tpo receptor resulted in multilineage cytopenias and significant haematopoietic stem cell deficit in humans.[230-232] Thus, a Tpo mimetic could in theory be active in ameliorating marrow function in aplastic anaemia and associated disorders. The very elevated endogenous Tpo serum levels in severe aplastic anaemia patients raised concerns of the ineffectiveness of this approach.[233, 234] Notwithstanding, a prospective dose escalation study was developed with eltrombopag in severe aplastic anaemia patients with an insufficient response to initial immunosuppression. Later, due to the single-agent activity observed in these earlier studies, eltrombopag was combined with immunosuppressive treatment in front line.[220] Eltrombopag represents an important addition to the armamentarium in AA that, for several decades, lacked approval of new therapies. Eltrombopag is currently approved as a single agent in patients with an insufficient response to initial immunosuppression. The immune and non-immune properties of eltrombopag may be complementary or synergize with those of immunosuppression, contributing to a more rapid and robust recovery of blood counts when given in combination in severe aplastic anaemia.[220] Thus, combining therapies with different modes of action in improving hematopoiesis is proving to be beneficial in this setting.[220] The addition of androgens and possibly other growth factors may further improve bone marrow function and blood count recovery, and these possibilities are likely to be investigated

in clinical research protocols. In coming years, real word evidence will further solidify the role of the Tpo receptor agonists in bone marrow failure syndromes.[220]

### 1.2.6.4 Haematopoietic stem cell transplantation

Up-front HSCT from matched sibling donor (MSD) is indicated for SAA in young and adult patients who have a sibling donor. However, due to the different outcome between young and adult, a full co-morbidities assessment should be carried out for patients aged 35-50 in order to determine if IST could be a safer option.[200] Matched unrelated donor (MUD) HSCT is indicated for SAA after failure to respond to one course of IST. There is no formal upper age limit, but this should be carefully evaluated on an individual patient basis.[163]

The donor should be 10/10 or 9/10 matched based on HLA high resolution typing for class I (HLA-A, B, C) and class II (HLA-DRB1, DQB1) antigens. Alternative donor HSCT using either cord blood, a haploidentical family donor or a 9/10-matched unrelated donor might be considered after failure to respond to IST and in the absence of a MSD.[235, 236] If there is a syngeneic donor available, HSCT should be considered in all patients regardless of age as long term OS is 90%.[201]

The choice of conditioning regimens to use depends on:

- patient age;[163]
- type of donor;[163]
- centre preference for choice of T cell depletion (ATG[237, 238] or alemtuzumab[239]).

A recent European Bone Marrow Transplantation (EBMT) analysis has shown that unrelated donor HSCT is a viable option as the outcome of MUD HSCT is no longer inferior to MSD HSCT.[240, 241]

### 1.2.6.5 Treatment in the elderly

The treatment of patients older 60 years with AA is more complex and the outcome is worse due to inferior tolerability of the treatment. Therefore patients should be individually assessed for co-morbidities and their specific wishes should be respected, as the quality of life is as important as the clinical outcome.[163] It is also important to rule out hypoplastic MDS, as MDS is a far more common BMF syndrome in this age group.[163]

Immunosuppression is still considered the treatment of choice in this setting. There is no indication for allogeneic HSCT as first line treatment in patients aged > 60 years, although HSCT can be considered in selected patients. Ideally, the least toxic treatment should be given. However, another consideration is how quickly a response is required, such that those with a more severe disease should be treated more intensely than those with less severe disease.[163]

Despite being more effective than CsA alone, treatment with ATG and CsA requires hospitalization and has a higher risk of acute and delayed toxicity than younger patients.[217] Patients must be assessed carefully for their risk of infection, bleeding, heart failure and arrhythmias, as with ATG these complications are more frequent. Moreover, older patients have an inferior OS after ATG and CsA compared to a younger cohort of patients.[213]

Alternative treatments are CsA alone, oxymetholone (or danazol) or alemtuzumab. CsA alone can be given in an outpatient setting but nephrotoxicity and hypertension must be carefully monitored. After a very careful assessment in older patients, alemtuzumab may be used as a single agent in refractory/relapsed AA.[242]

Oxymetholone or danazol are an option in patients intolerant or unresponsive to CsA. As danazol has fewer masculinizing side effects than oxymetholone it is the drug of choice women.[243, 244] Oxymetholone can cause nephrotoxicity, hepatic tumours, mood changes, cardiac failure, prostate enlargement, and raised blood lipids. Finally, patients who are intolerant, or who decline IST should be offered best supportive care.[163]

Eltrombopag is the thrombopoietin receptor agonist. It has been used in 43 AA patients with an overall response rate of 40% and a good tolerance. The main concern was the clonal evolution, especially in monosomy 7 patients.[245] In August 2015, it has been licensed by the European Medicine Agency (EMA) for IST-refractory SAA or heavily pre-treated patients unsuitable for HSCT. It should be used with careful long-term monitoring for clonal evolution. Abnormal cytogenetic clone typical of MDS (particularly monosomy 7) should be excluded prior to starting treatment.[163]

## 1.3 *Ex vivo* expanded regulatory T cells as a therapy

### 1.3.1 Regulatory T cells as a therapy

There is an increasing body of evidence that the adoptive transfer of Tregs in multiple disease settings, including type 1 diabetes, results in disease prevention and, in many cases, disease remission.[246] It has been demonstrated that Tregs are defective in several autoimmune diseases.[247] These defects consist in loss of Treg number in inflamed tissues, reduced signalling through the IL-2 receptor, and variability of the suppressive activities of the cells *in vitro* and *in vivo*.[248] These findings have introduced the concept of Tregs as an immunotherapy, especially if the abnormalities observed *in vivo* can be corrected through *ex vivo* Tregs expansion.

### 1.3.1.1 Regulatory T cells therapy in graft *versus* host disease prevention

Rejection, graft versus host disease and infections are major complications of allogeneic hematopoietic stem cell transplantation and remain the main causes of transplant related morbidity and mortality.[249] Tregs are ideal candidates for tolerance induction in the context of HSCT and may represent an ideal tool for an adoptive cellular therapy for inducing tolerance, preventing graft *versus* host disease (GVHD) and for improving the quality of immune reconstitution without compromising the beneficial graft *versus* leukaemia (GVL) effect.[249] Table 1.4 shows a list of trials with adoptive Tregs therapy.[249]

**Table 1.4 Adoptive cellular therapy with Tregs**

| Author | Setting | Total patients | Intervention and Treg doses | Main results |
|---|---|---|---|---|
| Brunstein *et al.*[250] | Prevention of GVHD after double UCB transplantation | 23 | Infusion immediately after transplantation of *ex vivo* expanded UCB-derived nTregs, average 64% Foxp3$^+$ after expansion. | Reduced incidence of acute GVHD compared to historical controls. Similar incidence of opportunistic infections or relapse. |
| Edinger *et al.*[251] | Patients with high risk of AML relapse after HSCT | 9 | Infusion of freshly isolated donor Treg. Up to 5 x $10^6$ cells per kg (> 50% Foxp3$^+$). After an observation of 8 weeks, additional Tconv were administered at the same dose to promote GVL. | No Treg transfusion-related adverse events were observed despite the absence of pharmacologic immunosuppression. Neither GVHD nor opportunistic infections or early disease relapses occurred after Treg transfusion. |
| Di Ianni *et al.*[252] and Martelli *et al.*[253] | Improving the quality of immune reconstitution after haploidentical *ex vivo* T cell depleted transplantation | 43 | Infusion of donor CD4$^+$CD25$^{++}$ Tregs, followed by an inoculum of Tconv and positively selected CD34$^+$. Patients did not receive any prophylactic immunosuppression. | Effective not only in improving the immune reconstitution, but it was also associated with a low incidence of AML and GVHD prevention. Non-relapse mortality (NRM) remained significantly high. |
| Trzonkowski *et al.*[254] | Patients with chronic GVHD and resistant acute GVHD | 2 | Infusion of *in vitro* expanded donor Treg (90% Foxp3$^+$, dose of 1 x $10^5$/kg for patient with chronic GVHD and 3 x $10^6$/kg for patient with resistant acute GVHD). | Contributed to amelioration of chronic GVHD and allowed to reduce immunosuppressive drugs. For resistant acute GVHD no benefit was observed. |

| | | | | |
|---|---|---|---|---|
| Brunstein *et al.*[255] | Prevention of GVHD after double-UCB transplantation | 11 | Treg doses from 3 to 100 x $10^6$/kg. | Tregs were safe and resulted in lower rates of acute and chronic GVHD. |

#### *1.3.1.1.1 Cord blood derived regulatory T cells*

In the first in human clinical trial performed in 23 patients immediately after double-umbilical cord blood transplantation, the infusion of umbilical cord blood (UCB)-derived Tregs was associated with a reduced incidence of acute GVHD compared to historical control subjects. Moreover, there wasn't an increased incidence of opportunistic infections or relapse, suggesting that Tregs could provide an immediate, albeit possibly transient, immune suppression to control GVHD without long-term deleterious effects.[250] In a newer study, 11 patients were treated with Tregs doses from 3 up to 100 x $10^6$/kg. Clinical outcomes were compared with contemporary controls receiving the same conditioning regimen and immunosuppression. The incidence of grade II-IV acute GVHD at 100 days was 9% *versus* 45% in controls ($p = 0.05$) and chronic GVHD at 1 year was 0% in patients treated with Tregs and 14% in controls. Moreover, no increase in relapse, infection, or toxicity was observed between the two groups of patients, confirming the safety of Tregs infusion.[255]

#### *1.3.1.1.2 Donor-derived regulatory T cells*

In a phase I study, nine patients with a high risk of post-transplant leukaemia relapse received freshly isolated donor Tregs (as a pre-emptive strategy). Up to 5 x $10^6$/kg cells were administered after stopping any pharmacological GVHD prophylaxis. After an observation period of eight weeks, additional conventional T cells (Tconv) were administered at the same dose to promote GVL. Despite the absence of pharmacological immunosuppression, no Tregs transfusion-related adverse events were observed. Furthermore, neither GVHD nor opportunistic infections or early

disease relapses occurred after Tregs transfusion, suggesting safety and feasibility of this cellular therapy.[251] In a trial of 43 consecutive AML patients undergoing haploidentical using an *ex vivo* T cell depleted graft, authors investigated the infusion of donor Tregs (2 x $10^6$/kg), followed 4 days later by an injection of mature donor T cells (1 x $10^6$/kg) and positively immune selected $CD34^+$ cells. Patients did not receive any prophylactic immunosuppression after transplant. The early, post-transplant adoptive transfer of Tregs resulted effective not only in improving the immune reconstitution, but it was also associated with a low incidence of AML relapse in a group of patients with very high-risk disease.[252, 253]

#### *1.3.1.1.3 Drugs with a direct effect on in vivo expansion of regulatory T cells*

The use of isolated and *ex vivo* expanded Tregs is quite challenging. The difficulties related to low cells numbers and the need of expanding these cells with appropriate GMP procedures, led investigators to search for drugs able to expand Tregs *in vivo*.[249]

Authors from Harvard published two clinical trials (one phase I and one phase II) whose results suggest that low-dose IL-2 may be a reasonable candidate as adjuvant for adoptive Tregs cell therapy.[256, 257] Nonetheless, the impact of the therapeutic administration of IL-2 can be controversial for its role on NKs expansion.[258] This effect could be regarded as positive for its ability to promote a significant GVL protective effect[259] or potentially negative in relation to a possible proinflammatory activity as observed in various autoimmune models and in patients.[260]

Several experimental models suggest the *in vivo* expansion of Tregs when cyclophosphamide is given post-transplant for GVHD prophylaxis and tolerance induction.[261, 262] Despite the potential dominant effect of a deep depletion of

alloreactive cytotoxic T cells, these studies suggest that expansion of Tregs is also contributing to the therapeutic effect of this drug. Tregs resistance to post-transplant cyclophosphamide appears to be related to induction of aldehyde dehydrogenase expression upon allogeneic stimulation, in contrast to a condition of equilibrium in which this enzyme is minimally expressed by human Tregs.[263] Preservation of regulatory T cells by post-transplant cyclophosphamide has been postulated even when this GVHD prophylaxis has been used after myeloablative, HLA-matched transplant in AML and MDS patients.[264]

Another interesting drug to expand Tregs *in vivo* is represented by rapamycin, an inhibitor of the mTOR-AKT pathway that promotes growth and expansion of Tregs.[265, 266] In a first study with GVHD prophylaxis based on rabbit ATG, rituximab and oral rapamycin and mycophenolate, T cell reconstitution was rapid and skewed toward Tregs. Interestingly, the occurrence and severity of GVHD was negatively correlated with Tregs frequency.[267, 268]

Promising results indicate that azacytidine can have an immunomodulatory effect by increasing functional Treg numbers. Azacytidine, in addition to the direct cytotoxic effect, seems to accelerate the reconstitution of Tregs and induce $CD8^+$ T cell response to candidate tumour antigens, enhancing the GVL effect and reducing the risk of GVHD.[269-271] In this context, the potential mechanism of action of azacytidine seems to be related to a delayed effect of the drug leading to demethylation of the Foxp3 promoter and overexpression of Foxp3, driving T cell differentiation toward a regulatory phenotype.[271]

Ruxolitinib is a potent inhibitor of the JAK1 and JAK2 signal transduction pathway and it has been licensed for the treatment of patients with myelofibrosis.[249]

Interestingly, ruxolitinib has been reported to be clinically effective in a cohort of 95 patients with steroid-refractory acute and chronic GVHD with an overall response rate of 81% with a complete response of 46% and a 6 months survival of 79%.[272] Drugs able to induce *in vivo* expansion of Tregs are listed in Table 1.1.

**Table 1.5 Drug induced *in vivo* expansion of Tregs**

| Author | Setting | Total patients | Intervention and Treg doses | Main results |
|---|---|---|---|---|
| Koreth *et al.*[256] | Treatment of steroid-refractory chronic GVHD | 29 | Low-dose s.c. IL-2 (0.3 x $10^6$, 1 x $10^6$ or 3 x $10^6$ IU/$m^2$) for 8 weeks. | The maximum tolerated dose of IL-2 was 1 x $10^6$ IU/$m^2$. Administration was associated with Treg cell expansion *in vivo* and improvement of chronic GVHD in 12 out of 23 evaluable patients. |
| Koreth *et al.*[273] | Treatment of steroid-refractory chronic GVHD | 35 | Daily IL-2 (1 x $10^6$ IU/$m^2$ per day) for 12 weeks. | 20 out of 33 (61%) evaluable patients had clinical responses. Compared with pre-treatment levels, Treg and NKs rose more than fivefold and fourfold, respectively. |
| Kennedy-Nasser *et al.*[274] | GVHD prophylaxis in paediatric patients | 16 | Ultra-low-dose IL-2 injections (100,000-200,000 IU/$m^2$ 3 x per week). | No IL-2 patients developed grade 2-4 acute GVHD, compared with 4 out of 33 (12%) of the control group. Among IL-2 recipients, *in vivo* expansion of Tregs was observed. |
| Peccatori *et al.*[267] | GVHD prophylaxis in haploidentical transplant using PBSC grafts | 121 | Sirolimus based, calcineurin-inhibitor free prophylaxis of GVHD. | T cell reconstitution was rapid and skewed toward Tregs. The occurrence and severity of GVHD was negatively correlated with Tregs frequency. |
| Cieri *et al.*[268] | GVHD prophylaxis in haploidentical transplant using PBSC grafts | 40 | Post-transplant Cy and sirolimus-based GVHD prophylaxis (Sirolimus and post-transplant cyclophosphamide). | Grade II to IV and III-IV acute GVHD were 15% and 7.5%, respectively. The 1-year cumulative incidence of chronic GVHD was 20%. The number of circulating Tregs at day 15 after HSCT was predictive of subsequent GVHD occurrence. |
| Goodyear *et al.*[270] | Azacytidine administration after reduced intensity HSCT for AML | 27 | Monthly courses of azacytidine after reduced intensity HSCT. | Azacytidine after transplantation was well tolerated with a low incidence of GVHD. Azacytidine increased the number of Tregs within the first 3 months. |

| | | | | |
|---|---|---|---|---|
| Schroeder *et al.*[275] | Azacytidine and DLI administration as salvage therapy for relapse after HSCT | 13 | Azacytidine 100 mg/m$^2$/day on days 1-5 or 75 mg/m$^2$/day on days 1-7 every 28 days and DLI after every second azacytidine cycle. | After 4 azacytidine cycles an increase in the absolute number of Tregs was observed, especially in patients relapsing early after HSCT. A relatively low rate and mild presentation of GVHD despite a dose escalating DLI schedule was reported. |
| Choi *et al.*[276, 277] | Prevention of GVHD after HSCT | 50 | Vorinostat (100 mg or 200 mg, twice a day) combined with standard prophylaxis for GVHD. | Grade 2-4 acute GVHD by day 100 was lower than expected. Vorinostat enhances Tregs after HSCT. |

### 1.3.1.2 Regulatory T cells therapy in type 1 diabetes

Bluestone *et al.* conducted a phase I, two-centres, open-label, dose-escalation study conducted at the University of California, San Francisco and Yale University in which participants with recent-onset type 1 diabetes received a single infusion of *ex vivo* expanded autologous $CD4^+CD25^{high}CD127^{low}$ polyclonally expanded Tregs.[278] Primary objectives were safety, laboratory abnormalities, and other signs of toxicity. The secondary endpoint was diabetes-related outcome measures including C-peptide response during mixed meal tolerance tests, insulin use, and haemoglobin A1c.[278]

The tolerance to Treg infusions was good, and the infusions were shown to be safe over a more than 500-fold dose range. Cytokine release, infusion reactions, or infectious complications were not observed. The infused Tregs did not change their phenotype into a pathologic one.[278] Thus, this study demonstrates the successful isolation, expansion, and reinfusion of polyclonally expanded Tregs derived from patients with type 1 diabetes. This clinical trial can be considered a platform for additional studies in this and other autoimmune diseases. Treg-promoting therapies, such as low-dose IL-2,[279] are likely to be complementary to this current adoptive

transfer effort, potentially resulting in a safe and effective combination therapy, which, may induce durable remission and tolerance in this disease setting.[280]

### 1.3.1.3 Regulatory T cells therapy in solid organ transplantation

Polyclonally expanded Tregs have shown a favourable safety profile and efficacy (Table 1.6).[250, 252, 254, 281]

**Table 1.6 Clinical trials using *ex vivo* expanded Tregs**

| Clinical trial number | Setting | Patients recruited | Isolation | Treg doses | Study overview and results |
|---|---|---|---|---|---|
| N/A[254] | Graft *versus* host disease adult | 2 | FACS $CD4^+CD25^+$ $CD127^-$ | 1 x $10^5$/kg → 3 x $10^6$/kg | The first patient had chronic GVHD 2 years post BMT. After receiving 0.1 x $10^6$/kg FACS purified *ex vivo* expanded Tregs from the donor, the patient was successfully withdrawn from immunosuppression without evidence of recurrence. The second patient had acute GVHD at one-month post transplantation, treated with several infusions of expanded donor Tregs. Despite the initial and transitory improvement, the disease progressed and ultimately resulted in the patient's death |
| NCT00602693[250] | Graft *versus* host disease adult | 23 | CliniMACS $CD25^+$ | 0.1, 0.3, 1 and 3 x $10^6$/kg | Tregs were isolated from a third party UCB graft and expanded polyclonally with anti-CD3/CD28 coated beads and recombinant IL-2 for 18 days. Compared with the 108 historical controls, there was a reduced incidence of grades II–IV acute GVHD (from 61–43%), although the overall incidence of GVHD was not significantly different |
| N/A[252] | Graft *versus* host disease adult | 28 | CliniMACS $CD4^+CD25^+$ | 2-4 x $10^6$/kg | Patients received donor Tregs without *ex vivo* expansion and donor effector T cells without any other adjuvant immunosuppression. The dose of 1 x $10^6$/kg Teff with 2 x $10^6$/kg Tregs was reported to be safe. Patients receiving Tregs demonstrated accelerated immune reconstitution, reduced CMV reactivation, and a lower |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | incidence of tumour relapse and GVHD when compared to historical controls.<br>Only 13 out of the 26 patients surviving |
| N/A[281] | Type 1 diabetes children | 12 | FACS $CD4^+CD25^+$ $CD127^-$ | 10-20 x $10^6$/kg | One-year follow-up of 12 children with Type 1 diabetes treated with autologous-expanded *ex vivo* Tregs.<br>The data supported the safety of the infused Tregs, with 8/12 treated patients requiring lower requirements of insulin, with two children completely insulin independent at 1 year |
| NCT01210664[282] | Type 1 diabetes children | 14 | FACS $CD4^+CD25^+$ $CD127^-$ | 5 x $10^6$/kg → 2.6 $10^9$/kg | Infusion of 14 type 1 diabetic patients with *ex vivo* expanded Tregs. Enrolment and infusion is complete |

The last few years have also witnessed the start of two clinical trials of Treg immunotherapy in solid-organ transplantation at King's College London, the ONE Study (NCT02129881) and ThRIL (NCT02166177).[283]

The ONE study is a multicentre phase I/II study, on the safety of and potential efficacy of infusing *ex vivo* expanded Tregs. The immunosuppression (prednisolone, tacrolimus, and mycophenolate mofetil) regimen of the patients receiving expended Tregs will be similar across the centres. The production feasibility of each of the cell products will also be assessed.[283]

The ThRIL study (NCT02166177) is a combined phase I/II clinical trial of Treg immunotherapy in the setting of liver transplantation. Safety, tolerability, and efficacy of polyclonally expanded Tregs will be among the primary endpoints (thymoglobulin and an mTOR-inhibitors will be the immunosuppression regimen). ThRIL trial is currently in the recruitment stage.[283]

Adjunct immunosuppressive regimens, the timing and number of injections, the dose of Tregs with the desired specificity, and the trafficking properties of the infused cells, are all issues which need to be addressed prior to the design of such trials.[283]

#### 1.3.1.4 Association of *ex vivo* expanded regulatory T cells and immunosuppression

The efficacy of Treg therapy requires a "friendly" *in vivo* cytokine milieu, supporting both cell engraftment and the chance of inducing tolerance.[284, 285]

The main question is: how will this microenvironment affect the Tregs *in vivo* following post-expansion transfer?[283] Since Tregs strongly depend on the exogenous supply of IL-2, the use of calcineurin inhibitors may have a negative impact on the survival and suppressive ability of the expanded Tregs.[266, 286] However, CNI may be taken into consideration as a therapy to induce a favourable environment before Treg infusion[287]. The effects of mycophenolate mofetil (MMF) on Tregs have not been fully analysed and the few studies published are not consistent. The effect of this drug on cell division may negatively influence the expansion of antigen specific Tregs and prevent the development of a long-term tolerance.[288] Other studies have shown that MMF has no effect on Tregs and promotes the induction of a more tolerogenic environment.[266] Dexamethasone and prednisolone have been used for a long time as immunosuppressors for the treatment of inflammatory diseases and in the transplant setting. Glucocorticoids have been shown to enhance the IL-2-dependent expansion of Tregs *in vivo*,[289] induce an increased Foxp3 expression in Tregs in asthma patients,[290] and restore the suppressive function of Tregs in patients with relapsing multiple sclerosis.[291] Thus, the combination of a tailored immunosuppressive therapy

along with the administration of *ex vivo* expanded Tregs may potentially maintain tolerance, accomplishing the ultimate aim of Treg immunotherapy.[283]

### 1.3.2 Regulatory T cells manufacture

Treg manufacturing plans with protocols that are compliant with good manufacturing practice GMP are of paramount importance for the implementation of Treg therapy. The clinical Treg selection protocol used by the Immunoregulation Laboratory at King's College London is a combination of depletion of $CD8^+$ cells and positive selection of $CD25^+$ cells using the automated CliniMACS plus system (Miltenyi Biotec). This process doesn't allow the selection of Tregs based on multiple parameters; moreover, the lack of distinction of $CD25^{high}$ cells may lead to a contaminating of Tconv, explaining the lower purity of this protocol for Treg isolation.[292, 293]

In this regard, GMP-compliant fluorescence-activated cell sorting will broaden the possibility of isolating Treg subsets with potent suppressive function, specificity, and epigenetically stable.[283] The most promising marker combinations for Treg isolation are:

- $CD4^+CD25^{high}CD45RA^+$,[294]
- $CD4^+CD25^{high}CD127^{low}$.[295]

The last few years have witnessed significant efforts to obtain the relevant regulatory approvals to integrate the FACS cell sorter into a clinical cell production process.[283]

### 1.3.3 Optimization of culture conditions for polyclonal regulatory T cells expansion

Tregs rarity in the circulation is one of the main limitations in the implementation of protocols for adoptive cell therapy; therefore, it is necessary to expand these cells *ex*

*vivo* to obtain clinically relevant numbers, prior to their re-administration. Tregs can be expanded using anti-CD3/CD28-coated beads and high dose IL-2,[293] nevertheless Tconv have the potential to proliferate vigorously in the same conditions: this may hamper the potential safety and the efficacy of the final product. Because of this, several groups have focused on the optimization of culture conditions to ensure the expansion of enough Tregs with the minimum number of contaminant cells.[283]

### 1.3.3.1 Role of IL-2 for regulatory T cells development and homeostasis

IL-2 was the first cytokine to be molecularly cloned, and based on its function, was called "T cell growth factor".[296] IL-2 is mainly secreted by activated $CD4^+$ T cells and, to a lesser extent, by activated $CD8^+$ T cells, activated dendritic cells, natural killer cells and NKT cells.[297] IL-2 has pleiotropic effects; it supports the development of Tregs in the thymus, although it may not be indispensable for this role.[298] It is a crucial survival factor for Tregs in the periphery, and is needed for the functionality and stability of Tregs.[299, 300]

The molecular mechanism of IL-2-induced Treg cell stability involves, at least partially, epigenetic changes in the Foxp3 *locus*, moreover Tregs are not able to produce IL-2.[125, 160] Other main effects of IL-2 are to support the proliferation of $CD4^+$ T cells, and the terminal differentiation of $CD8^+$ T cells.[301] IL-2 has been demonstrated to promote the development of naive $CD4^+$ T cells into iTregs,[302] Th2,[303] Th9[304] and, in some studies, Th1[305].

On the other hand, IL-2 suppresses the differentiation of $CD4^+$ T cells into Th17[306] and T follicular helper (Tfh) cells[307] by a STAT5-dependent mechanism (Figure 1.35).

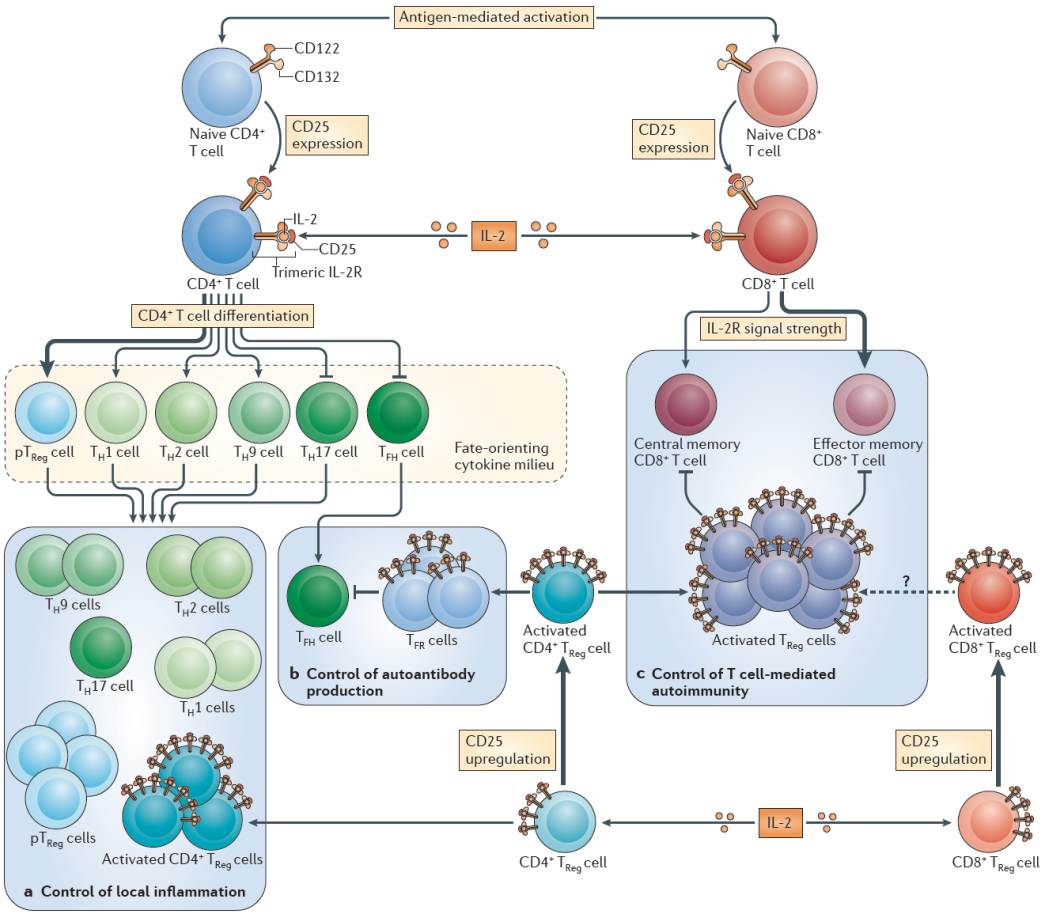



**Figure 1.35 Pleiotropic effects of IL-2 in controlling autoimmunity**

Upon T cell receptor ligation and co-stimulation, naïve CD4⁺ or CD8⁺ T cells transiently express CD25 and respond to IL-2 through the high-affinity IL-2R. The differentiation fate of CD4⁺ T cells depends on the cytokine milieu: IL-12 and IL-18 favour the differentiation of Th1; IL-4 and IL-33 favour Th2 cells; TGF-β and IL-4 favour Th9 cells; TGF-β, IL-1, IL-6 and IL-23 favour Th17 cells; TGF-β favours peripherally iTregs; and IL-6 and IL-21 favour Tfh cells. As part of the fate-orienting milieu, IL-2 favours the differentiation of Th1, Th2, Th9 and iTregs, and inhibits the differentiation of Th17 and Tfh cells. The differentiation fate of CD4⁺ and CD8⁺ T cells is influenced by the strength of IL-2 signalling, with greater signal strength favouring differentiation into iTreg cells and effector memory T cells, respectively. Treg cells constitutively express CD25 and respond vigorously to IL-2 by up-regulating CD25 and becoming more potent (activated) suppressor cells. Some of these cells migrate to lymph node germinal centres where they are known as T follicular regulatory cells. Together, these changes contribute to tipping the immune balance towards regulation rather than inflammation, notably by favouring the differentiation of naïve CD4⁺ T cells into iTregs rather than Th17 cells (part a); helping to control autoantibody production by favouring the differentiation of TFR cells over Tfh cells (part b); and helping to control autoreactive CD8⁺ effector T cells by increasing the number and function of Treg cells (part c). The mechanisms of the suppressive activity of CD8⁺ Tregs *in vivo* remain to be determined.[279]

Tregs have a ten to twenty-fold lower activation threshold for IL-2 than Tconv [assessed by level of phosphorylated STAT5 (pSTAT5)]. Moreover, the activation of numerous genes important for cell function requires an amount of IL-2 that is hundred times lower for Tregs than for Tconv.[308] The characteristic sensitivity of Tregs to IL-2 could be due, along with the higher expression of CD25, to IL-2R

signalling specificity as Tregs may be more dependent on IL-2-STAT5 signalling.[126, 309] IL-2 has been demonstrated to support qualitative aspects of the Treg development, in addition to simply adding a survival advantage, as Tregs lacking IL-2 signalling express low levels of numerous markers associated with the typical Treg phenotype (CTLA-4, CD39, CD73).[310] The paramount role of IL-2 in modulating the size of the peripheral Treg compartment has led to therapeutic IL-2 use in several human clinical trials.[256, 257, 311, 312]

#### 1.3.3.2 The mTOR pathway and its role in regulatory T cells expansion

The mechanistic target of Rapamycin is a key regulator of T cell metabolism that is needed to integrate nutrient sensing pathways with signalling pathways involved in differentiation, growth, survival, and cell proliferation.[313] Conventional $CD4^+$ and $CD8^+$ T cells, upon stimulation, utilise the mTOR pathway to meet the increased metabolic requirements of T cell activation by switching from primarily oxidative phosphorylation, seen in resting T cells, toward a state of enhanced aerobic glycolysis (Figure 1.36).[314, 315]

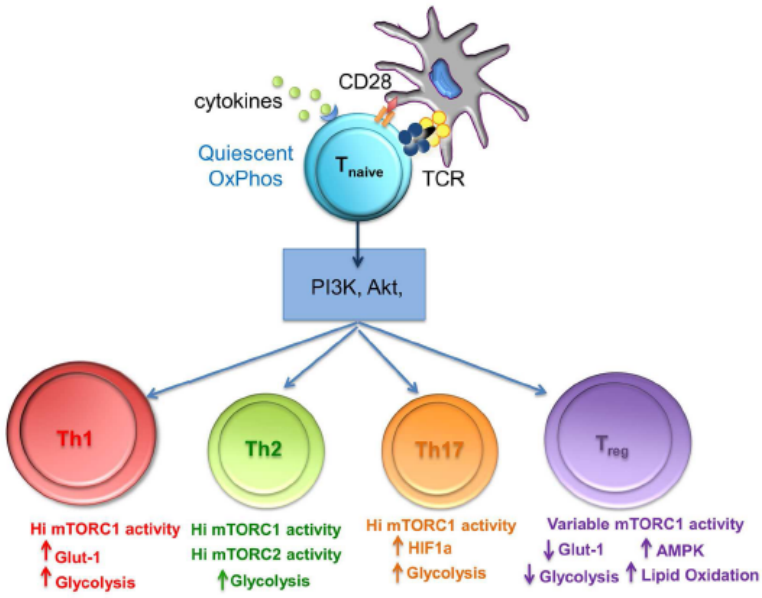



**Figure 1.36 The level of mTORC activity in the differentiation of T cell subsets**

mTORC1 integrates nutrient sensing and signalling pathways to match the energy requirements of activated T cells. Th1, Th2, and Th17 cells require high levels of glycolysis that is mediated by high mTORC1 activity, wh[316]ereas Treg differentiation requires variable mTorc activity, reduced glycolysis, and lipid oxidation.

The importance of this phenomenon in determining T cell fate was first observed using a selective mTOR inhibitor, rapamycin, which prevented the generation of Tconv responses and induced the generation of Tregs.[265, 317-319] Tregs have higher levels of AMP kinase activity and their metabolism preferentially relies on lipid oxidation;[133] the AMP-activated kinase acts as a sensor of the AMP/ATP ratio, which is increased during hypoxia and inhibits mTOR kinase to promote mitochondrial oxidative metabolism rather than glycolysis.[320, 321] While inhibition of mTOR enhances Treg generation during an immune response, mTOR activity is known to be required to maintain their suppressive capabilities.[316] There are two structurally distinct complexes of mTOR (mTORC1 and mTORC2). Both of them are localized within different subcellular compartments and have different functions. As recently shown, the mTORC1 complex seems to have a crucial role for the suppressive activity of Treg; indeed, mTORC1 activity was demonstrated to be

higher in Treg than naïve T cells under steady state conditions.[322, 323] Mechanistically, the mTORC1 pathway in Treg was shown to be necessary to up-regulate the expression of surface CTLA-4 and inducible co-stimulator (ICOS), key intrinsic receptors for Treg-mediated suppression. Moreover, mTORC1 was shown to induce cholesterol and lipid metabolism as well as proliferation in Tregs.[324] Following antigenic stimulation, T cells rapidly mould the acquisition and use of metabolites to meet their energetic and biosynthetic needs, and the metabolic programmes that are engaged can directly affect T cell function. A crucial decision for the T cell is how (and whether) to catabolize glucose. Oxidative phosphorylation breaks down glucose to yield the maximum amount of ATP, whereas glycolysis alone generates less ATP but creates precursors of amino acids, nucleotides and lipids from glucose (Figure 1.37). Aerobic glycolysis, also known as the Warburg effect, allows rapidly proliferating cells to meet their biosynthetic needs by using glycolysis, rather than oxidative phosphorylation, when oxygen is replete.[325] CD28 signalling directly controls the metabolic switch to glycolysis during T cell activation by up-regulating the expression of GLUT1 in a PI3K-AKT dependent manner, thereby increasing the import of glucose into the cell.[130, 131] Importantly, Tregs do not use glycolysis after stimulation, largely owing to their selective blockade of PI3K-AKT activation, preventing GLUT1 up-regulation. Instead, Tregs heavily rely on fatty acid oxidation to feed the tricarboxylic acid cycle and generate energy through oxidative phosphorylation.[132-134]

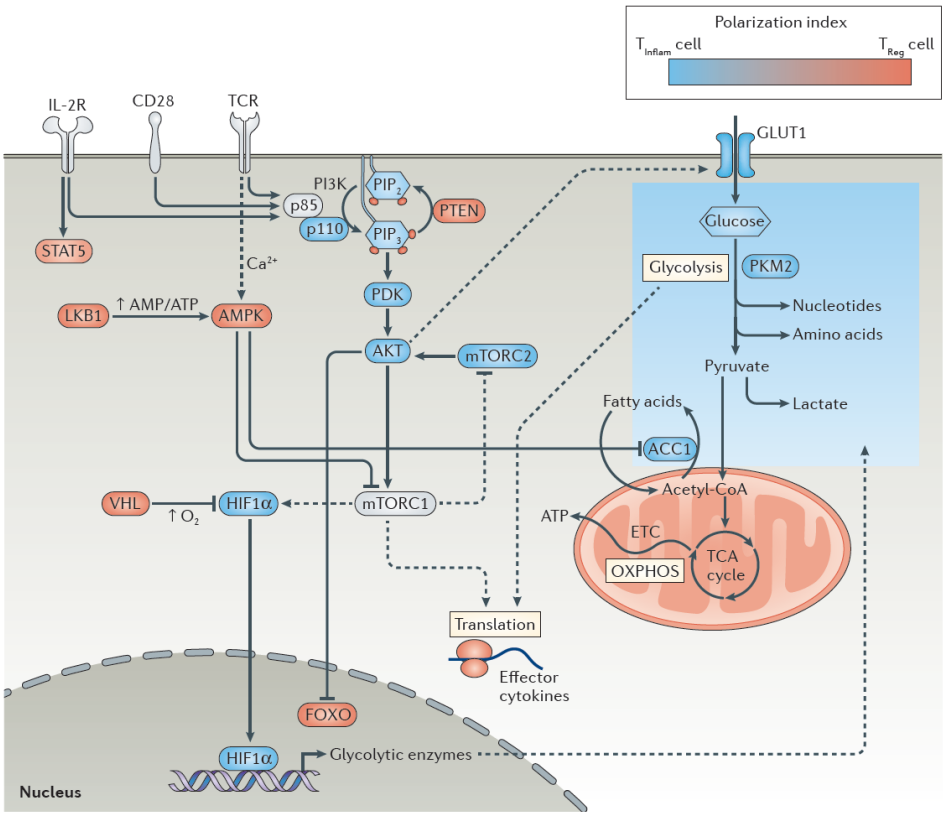



**Figure 1.37 The PI3K-AKT-mTOR pathway and metabolic programmes converge to regulate the plasticity of inflammatory *versus* Tregs**

Links between extracellular cues, PI3K-AKT-mTOR pathway, metabolic programmes and gene regulation are depicted. A polarization index indicates proteins or processes that favour the induction of inflammatory (blue) or Tregs (red) programmes. Solid lines indicate direct protein-protein interactions, whereas indirect links between proteins are denoted with dashed lines.[110]

The PI3K-AKT-mTOR pathway is a crucial bifurcation point for inflammatory versus regulatory T cell programmes, as the activation of this pathway is required for the polarization and function of most T helper cells but is largely repressed in $Foxp3^+$ Tregs.[126, 127]

### 1.3.3.3 All-trans retinoic acid and regulatory T cells function

The primary biologically active metabolite of vitamin A, all-trans retinoic acid, plays vital roles in embryonic development, vision, skin homeostasis and reproduction, and it also a key factor for maintenance of the immune system.[326] DCs' derived all-trans retinoic acid (ATRA) promotes the *de novo* generation of Tregs from naïve $CD4^+CD25^-$ T cell in mice,[76, 77] but also suppresses the de novo differentiation of

naïve CD4⁺ cells into Th17.[327] The RA receptor/retinoid X receptor heterodimer is responsible for the effect of ATRA on Treg and Th17.[79, 328] Through the induction of the expression of CCR9 and α4β7, ATRA seems to promote gut homing of CD4⁺ T cells.[329] RORγt, is involved in the gene transcription of Th17 cells. TGF-β can induce high levels of RORγt and further promotes Th17 cell development in the presence of IL-6. However, the addition of ATRA to cultures containing TGF-β and IL-6 greatly reduces RORγt expression and Th17 cell differentiation.[330] The crucial role played by ATRA in immune tolerance is through the induction of iTreg cells (Figure 1.38). While TGF-β alone is not enough to induce the development of human iTreg cells, the addition of ATRA adds the necessary stimulus for human iTreg cell induction, demonstrating its value in clinical translation.[331]

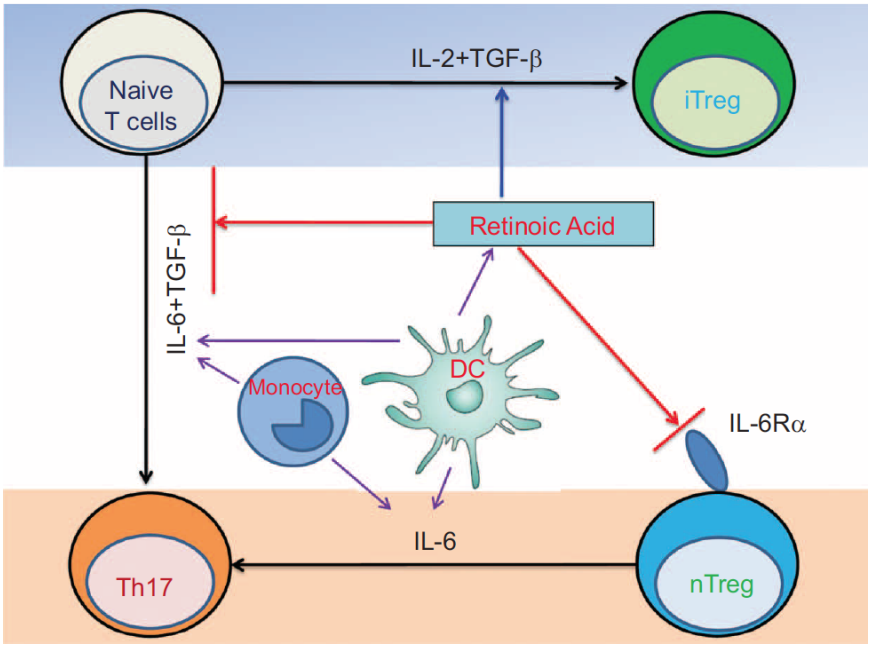



**Figure 1.38 Immunomodulatory effect of ATRA on CD4⁺ T cell subsets**

ATRA maintains immune homeostasis by working with TGF-β to promote Treg cell induction from naïve T cells, while inhibiting Th17 cell induction in the presence of inflammatory cytokines such as IL-6. In addition, ATRA inhibits Th17 differentiation from nTreg cells by reducing their expression of IL-6R .[332]

Foxp3 DNA methylation is responsible for its expression and maintenance by Treg cells,[333] nevertheless ATRA induces the differentiation and stability of iTreg cells in

the absence of any alteration of DNA methylation. Instead, ATRA is able to increase histone methylation and acetylation within the promoter and conserved non-coding DNA sequence elements at the Foxp3 gene locus;[334] however its effect can be dual: it can inhibit Foxp3 methylation in nTreg in a pro-inflammatory environment.[335] ATRA has also been shown to maintain Foxp3 expression during nTreg cell expansion, also in humans.[327, 335] In an inflammatory cytokine milieu, Foxp3 expression and Tregs function stabilising effect of ATRA is superior to rapamycin.[335] ATRA increases the expression of CTLA-4. The B7/CTLA-4 signal is very important for the development and function of iTreg cells.[336] ATRA also induces the expression of surface TGF-β on nTreg cells,[335] through which it may induce nTreg cell stabilization. Moreover, ATRA also down-regulates IL-6R expression and its signalling on nTregs, making nTreg cells resistant to this pro-inflammatory cytokine, which is usually increased in autoimmunity.[337]

## 1.4 Aims of this study

Treatment options for aplastic anaemia include HSCT (for patients younger than 40 years), IST and, more recently, eltrombopag.[187] Unfortunately there is a proportion of patients (lack of bone marrow donor, ineligibility for IST or disease relapse) for which treatment is quite challenging.[187] Additional novel treatments are needed for patients who fail to respond to IST and, along with this, more robust diagnostic tests able to predict response to treatment.[187]

The first part of my Ph.D. was aimed to:

1. Identify an immune signature for AA compared to healthy individuals based on Tregs subsets (chapter 3)

2. Correlate the above-mentioned immune signature with response to immunosuppression (chapter 3)

Once established that responders to IST have a different Treg signature (immunophenotypically) compared to non-responders, I wanted to assess whether this difference correlated also with functional and ontogenetic features such as:

1. Suppressive ability of different Treg subsets (is there any difference of suppressive ability between Treg A and B?) (chapter 4)
2. T cell receptor diversity (do Treg A and B share any T cell receptor sequence? Are they from the same "cell of origin"?) (chapter 4)
3. Genetic signature (investigated with both gene expression profiling and RNA sequencing) (chapter 4)
4. Propensity to apoptosis through the FAS/FAS-L pathway (why is there a reduction of Treg B in AA? Is this Treg subset more prone to go through apoptosis? If so, through which pathway? Can early apoptotic cells be rescued? Is this mechanism IL-2 dependant?) (chapter 4)

Finally, after categorising these two subsets in such depth, the final part of the Ph.D. was aimed to expand Tregs (including subsets A and B) and to characterise them to assess if there is a rationale to consider expanded Tregs as a potential cellular therapy for AA (with IST non-responders or IST non-eligible in mind as a potential target cohort for this treatment). More specifically, I explored Tregs sensitivity to high doses of IL-2 as a starting point before any *ex vivo* expansion (chapter 5). Having established their sensitivity to high doses of IL-2, I studied in more depth expanded Tregs features such as:

1. Phenotype stability (do expanded Tregs remain Tregs? Does the same concept apply to expansion of Treg subsets such as A and B?) (chapter 5)
2. Functionality (do expanded Tregs and their subsets suppress conventional T cells proliferation?) (chapter 5)
3. Stability (is the Treg-specific demethylated region still demethylated after *ex vivo* culture? In other words, does the Treg-friendly expansion *medium* affects their stability) (chapter 5)
4. Clonality (do Treg A and B come from the same "cell of origin"? Do these two different subsets share any T cell receptor sequence? Are expanded Tregs skewed towards any specific Treg sub-clone?) (chapter 5)
5. Plasticity (are expanded Tregs plastic? More specifically, can they be polarised towards a more "inflammatory" T cell subset such as Th17?) (chapter 5)
6. Apoptosis propensity (after expansion, are Treg B still more prone to apoptosis than their "A" counterpart?) (chapter 5)
7. In vivo use (can *ex vivo* expanded Tregs ameliorate bone marrow aplasia in a GVHD mouse model? Does it affect mice overall survival?) (chapter 5)

# 2 MATERIALS AND METHODS

In this chapter the general methods used in this project are discussed. Any specific method or deviation from standard will be discussed at the beginning of each relevant chapter.

## 2.1 Cell isolation

### 2.1.1 Gradient cell separation

Peripheral blood leukapheresis,[338] were obtained from National Health Service Blood and Transplant (NHSBT) as anonymised leukocyte reduction chambers (also named leukocyte cones). Cells were recovered aseptically in a laminar hood (class II microbiological safety cabinets)[339] and PBMC were isolated from the leukapheresis product by Ficoll-Paque density gradient centrifugation. Leukocyte cones were swabbed with 70% ethanol, their contents drained into sterile 50 ml tubes, flushed with 20 ml phosphate buffered saline (PBS), topped up to 35 ml with PBS and gently inverted to mix. In a sterile 50 ml tube, 35 ml diluted leukapheresis product was carefully layered onto 15 ml of Ficoll-Paque gradient (previously warmed to room temperature) and centrifuged at 900 x $g_{max}$ with full acceleration and zero brake for 20 minutes at 25 ˚C. Upon completion of centrifugation cycle, the clearly visible white PBMC layer (Figure 2.1) was collected into a new sterile 50 ml tube, topped up to 50 ml with Hank's balanced salt solution (HBSS) and washed twice at 300 x $g_{max}$ (all the reagents are listed in Table 2.1).

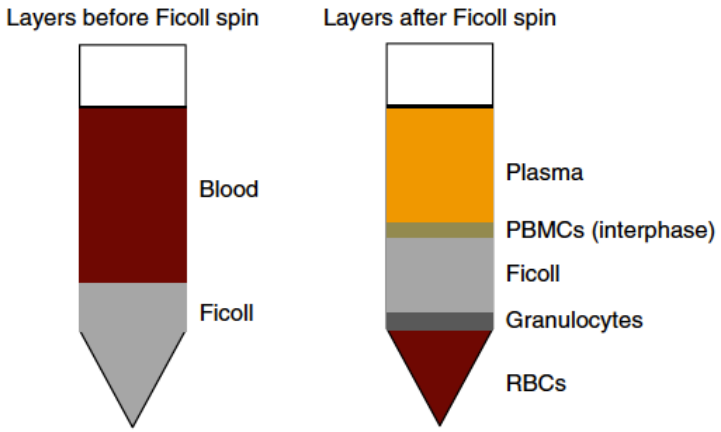



**Figure 2.1 Leukocyte cone pre- and post-centrifugation**

After centrifugation cells are separated based on their density. From the top to the bottom, we find PLT (the least dense compared with the Ficoll), PBMCs (placed in the interface), red blood cells, Ficoll and the other white blood cells.[340]

**Table 2.1 Reagents for gradient cell separation**

| Product | Company | Catalogue # |
|---|---|---|
| PBS | Sigma-Aldrich | D866 |
| HBSS | Sigma-Aldrich | 55037C |
| Ficoll-Paque | GE Healthcare Life Sciences | 17-1440-02 |

## 2.1.2 Trypan blue counting

The number of leukocytes obtained was determined by manual counting, using a hemacytometer in the presence of trypan blue. The method is based on the property of the dye to selectively stain dead cells in blue as the plasma membrane is disrupted. Live cells, which have an intact plasma membrane, are not stained. Ten µl of the cell suspension was transferred into the counting chamber and all the cells in the central large square of the hemacytometer were counted. The total number of cells was calculated (cells/ml) as: cells counted (25 squares grid) x dilution factor x $10^4$ x volume of the cell suspension. Trypan blue was used in a ratio of 1:1 therefore the total calculated cell number was multiplied by two (all the reagents are listed in Table 2.2).

**Table 2.2 Reagents for cell counting**

| Product | Company | Catalogue # |
|---|---|---|
| Hemacytometer | Thomas scientific | CTL-HEMM-GLDR |
| Trypan blue | Sigma-Aldrich | T8154 |

## 2.1.3 Bead separation

These methods were used to enrich different subtypes of T cells for different assays. When kits were used, manufacturer instructions were always followed unless otherwise specified.

### 2.1.3.1 $CD4^+$ isolation

Human $CD4^+$ T cells were negatively selected from peripheral blood mononuclear cells (PBMCs) by depletion of non-$CD4^+$ cells, using the $CD4^+$ T cell negative isolation kit and magnetic-activated cell sorting columns. After collecting the PBMCs by centrifugation at 220 x $g_{max}$ for 10 minutes, the media was removed, and the cell pellet resuspended in MACS buffer [1 x PBS, 2mM ethylene diamine tetra-acetic acid (EDTA), 0.5% BSA} according to the manufacturer instructions. To isolate $CD4^+$ T cells, a cocktail of biotin-conjugated monoclonal antibodies against non-$CD4^+$ T cells was added to the cell pellet and incubated for 10 minutes at 4 °C. Microbeads conjugated to a monoclonal anti-biotin antibody provided with the kit, were then added and incubated for 10 minutes at 4 °C. After washing the cells with 2 ml MACS buffer and centrifugation at 220 x $g_{max}$ for 10 minutes, the pellet was resuspended in 500 µl of MACS buffer and then placed in a MACS column. The column magnetically retains the non-$CD4^+$ cells, while the $CD4^+$ cells in the flow-through were collected (negative selection). Cells were then centrifuged at 220 x $g_{max}$ for 10 minutes and the pellet was resuspended in cell media according the use (all the reagents are listed in Table 2.3).

**Table 2.3 Reagents for $CD4^+$ isolation**

| Product | Company | Catalogue# |
|---|---|---|
| MACS BSA Stock Solution | Miltenyi Biotec | 130-091-376 |
| MACS Separation Buffer | Miltenyi Biotec | 130-091-221 |
| LS columns | Miltenyi Biotec | 130-042-401 |
| CD4+ T Cell Isolation Kit | Miltenyi Biotec | 130-096-533 |

| | | |
|---|---|---|
| MidiMACS Separator | Miltenyi Biotec | 130-042-302 |
| MiniMACS separator | Miltenyi Biotec | 130-042-102 |
| MACS multistand | Miltenyi Biotec | 130-042-303 |

### 2.1.3.2 Tregs isolation

For Tregs enrichment, PBMCs were resuspended in 135 µl of buffer/$10^7$ total cells and incubated in the fridge for 5 minutes with 15 µl of $CD4^+$ T cell biotin-antibody cocktail/$10^7$ total cells. Thirty µl of anti-biotin microbeads/$10^7$ cells were added and the cell suspension was incubated in the fridge for 10 minutes. The first part of the Treg isolation includes a depletion of non-$CD4^+$ T cells in which the cell suspension is passed through a pre-rinsed LD column. The total effluent was collected as the non-labelled, pre-enriched $CD4^+$ cell fraction. The column was rinsed twice. The second part of the Treg isolation is performed through a magnetic labelling of $CD4^+CD25^+$ regulatory T cells. The pre-enriched fraction of $CD4^+$ T cells was resuspended in 135 µl of buffer/$10^7$ cells with 15 µl/$10^7$ cells of CD25 microbeads. The mix was incubated in the fridge for 5 minutes. After this step, cells were washed with 2 ml of buffer (at 300 x $g_{max}$ for 10 minutes) and resuspended in 500 µl of buffer (up to $10^8$ cells). The cell suspension was applied to a pre-rinsed MS column and the column was washed three times. After the washes, the column was removed from the separator and placed on a suitable collection tube, where the enriched Tregs fraction was flushed with a column plunger. This last step was repeated twice to increase Tregs purity (all the reagents are listed in Table 2.4).

**Table 2.4 Reagents for Tregs isolation**

| Product | Company | Catalogue # |
|---|---|---|
| MACS BSA Stock Solution | Miltenyi Biotec | 130-091-376 |
| MACS Separation Buffer | Miltenyi Biotec | 130-091-221 |
| Regulatory T cells isolation kit | Miltenyi Biotec | 130-091-301 |
| LD columns | Miltenyi Biotec | 130-042-901 |
| MS columns | Miltenyi Biotec | 130-042-201 |
| MidiMACS Separator | Miltenyi Biotec | 130-042-302 |
| MiniMACS separator | Miltenyi Biotec | 130-042-102 |

| | | |
|---|---|---|
| MACS multistand | Miltenyi Biotec | 130-042-303 |

## 2.2 Cryopreservation and thawing of peripheral blood mononuclear cells

### 2.2.1 Peripheral blood mononuclear cells cryopreservation

Live cells were preserved at cryogenic temperature (below 150 °C) in liquid nitrogen in order to suspend their normal metabolic activity so that they could be thawed and used as needed.[341]

After isolation of PBMCs, 1 ml of PBS was added, and cells were spun at 300 x $g_{max}$ for 10 minutes. Supernatant was removed by aspiration and pellet was resuspended in 1 ml of Cryomaxx at the concentration of 50 x $10^6$ cells/ml (all the reagents are listed in Table 2.5).

**Table 2.5 Reagents for PBMCs cryopreservation**

| Product | Company | Catalogue # |
|---|---|---|
| Cryomaxx | GE Healthcare Life Sciences | J05-012 |
| Prime-XV | Irvine Scientific | 91141 |

### 2.2.2 Peripheral blood mononuclear cells thawing

Frozen cells were thawed at 37 °C in a water bath till the Cryomaxx liquefied. The cell suspension was transferred in a 15 ml tube. Five ml of Prime-XV were added and then span at 300 x $g_{max}$ for 10 minutes (this step was performed twice). Cells were then resuspended in 1 ml of media and counted to assess the post-thaw viability (as already described).

## 2.3 Staining

Before being analysed on a flow cytometer, cells were stained for both surface and intracellular markers. All the staining protocols include viability dye step, described in the surface staining paragraph. The antibody panel is listed in each section of the relevant chapter.

### 2.3.1 Surface staining

Before the viability dye staining, cells were washed twice in PBS without $Ca^{++}/Mg^{++}$ (in the absence of any protein) and resuspended in 1 ml of PBS without $Ca^{++}/Mg^{++}$; for the live/dead discrimination, 1 µl of the dye was added (to give a 1:1000 final dilution) and the cell suspension was left in incubation at room temperature for 30 minutes in the dark. Cells were washed twice (300 x $g_{max}$ for 10 minutes) with 2 ml of FACS washing buffer [PBS with 2.5% foetal calf serum (FCS), and 0.1% $NaN_3$]; the immunoglobulin molecules contained in the serum would block the Fc receptors in monocytes reducing non-specific binding of the fluorochrome-labelled antibodies during surface staining. After the washes, an appropriate volume of cocktail was added and incubated at room temperature for 30 minutes in the dark. Cells were washed twice with 2 ml FACS wash buffer (300 x $g_{max}$ for 10 minutes) (all the reagents are listed in Table 2.6).

**Table 2.6 Reagents for surface staining**

| Product | Company | Catalogue # |
|---|---|---|
| PBS w/o $Ca^{++}/Mg^{++}$ | Sigma Aldrich | D8537 |
| $NaN_3$ | Sigma Aldrich | S8032 |
| Foetal calf serum | ThermoFisher Scientific | 10500-064 |
| L/D eFluor 780 | eBioscience | 65-0865 |

### 2.3.2 Intracellular staining

Unless otherwise specified, the surface staining was always performed before the intracellular staining as already described. For the intracellular staining, cells were fixed/permeabilised first, by the addition of 1 ml of freshly prepared eBioscience fixation/permeabilization solution and incubated in the fridge for 45 minutes.

After this step, cells were washed (300 x $g_{max}$ for 10 minutes) with 2 ml of 1x eBioscience permeabilization buffer directly to tubes. After the supernatant was

decanted, 2 µl of normal rat serum was added to the cell pellet in residual buffer and incubated in the fridge for 15 minutes.

Without washing, the appropriate antibody was added, and the samples were left in incubation in the fridge for 30 minutes. Cells were washed twice (300 x $g_{max}$ for 10 minutes) with 2 ml of 1x eBioscience permeabilization (all the reagents are listed in Table 2.7).

**Table 2.7 Reagents for intracellular staining**

| Product | Company | Catalogue # |
|---|---|---|
| PBS w/o $Ca^{++}/Mg^{++}$ | Sigma Aldrich | D8537 |
| $NaN_3$ | Sigma Aldrich | S8032 |
| Foetal calf serum | ThermoFisher Scientific | 10500-064 |
| Fixation/Permeabilization concentrate | eBioscence | 00512343 |
| Fixation/Permeabilization diluent | eBioscence | 00522356 |
| Permeabilization washing buffer | eBioscence | 00833356 |

### 2.3.3 Proliferation dye staining for proliferation assays

All the proliferation assays were performed in an autologous setting, unless otherwise specified. All the reagents and samples were kept on ice for the duration of the experiment. After the cells count, Tconv were washed with staining buffer (PBS with 1% FCS). The proliferation dye [violet cell trace (VCT) or carboxyfluorescein succinimidyl ester (CFSE)] was diluted 1:5 in dimethyl sulfoxide (DMSO) to have a working solution of 1 mM. Cells were resuspended in sterile staining buffer ($10^6$ cells/ml). Without touching or pre-wetting, the walls of the tube, the cell suspension was placed at the bottom of the tube. Holding the tube nearly horizontal, 1 µl of proliferation dye was pipetted onto the clean and dry wall of the tube (1 µM final concentration). After carefully closing the tube, the cell suspension was shacked and vortexed several times and incubated at room temperature for 5-10 minutes.

After this step, 10 ml of ice-cold Roswell Park Memorial Institute (RPMI) 1640/FCS 10% were added to the tube and the cell suspension was left on ice for 5 minutes.

Cells were span down (300 x $g_{max}$ for 10 minutes) and washed again (300 x $g_{max}$ for 10 minutes) with ice-cold *medium*. Cells were resuspended at 100,000/100 µl in ice-cold PRIME-XV/10% Human Ab serum and plated with Tregs (1:1 ratio, unless otherwise specified) and anti CD3/CD28 beads (cell:bead = 20:1, unless otherwise specified).

After five days of culture, cells were harvested and stained with Fixable Viability Dye eFluor 780, anti-human CD3 VioGreen, and CD4 PerCp Cy5.5. The samples were run on a Canto II (BD Biosciences) and data were analysed with FlowJo, version 7.6 (Tree Star). The same method was used to assess sorted Tregs functionality. All the supernatants from these assays were harvested and kept at -20 ˚C for cytokine measurement. All the reagents are listed in Table 2.8.

**Table 2.8 Reagent list for proliferation dye staining for proliferation/suppression assay**

| Product | Company | Catalogue # |
|---|---|---|
| PBS w/o $Ca^{++}$/$Mg^{++}$ | Sigma Aldrich | D8537 |
| RPMI 1640 | ThermoFisher Scientific | 12633-012 |
| Foetal calf serum | ThermoFisher Scientific | 10500-064 |
| Dynabeads Human T-Activator | Gibco | 11132D |
| CellTrace Violet | Life Technologies | C34557 |
| CellTrace CFSE | Life Technologies | C34554 |
| Fixable Viability Dye eFluor 780 | eBioscience | 65-0865-18 |
| CD3 VioGreen | Miltenyi Biotec | 130-098-164 |
| CD4 PerCp Cy5.5 | BioLegend | 317428 |
| PRIME-XV T Cell Expansion XSFM | Irvine Scientific | 91141 |
| AB human serum | Sigma-Aldrich | H4522 |

## 2.4 Compensation and data analysis

Compensation is required for a flow cytometry experiment because of the physics of fluorescence. A fluorochrome is excited and emits a photon in a range of wavelengths. Some of those photons spill into a second detector, causing single stained samples to appear double positive.

Before running the samples, a compensation adjustment was carried out to counterbalance the spectra overlap, using BD CompBeads (BD Biosciences, catalogue # 552843).

The proper laser voltages were verified by running the setup beads using FACSDiva software (BD Biosciences). These procedures ensured the consistency of laser voltages during the entire project.

The compensation beads with the antibody used in the actual experiment were run on the FACS machine after initial checks and compensation. Compensation beads were acquired and recorded for 20,000 events to identify the target population. Raw files from the recorded experiments were analysed with FACSDiva software (BD Biosciences).

FlowJo software v 7.6 (Tree Star) was used to produce FACS plots for this thesis.

### 2.4.1 Antibodies for mass cytometry

The quality of metal tagged antibodies was assessed by staining healthy donor PBMCs followed by fluorescence conjugated secondary antibody and run on laser-based flow-cytometer as well as using biaxial plots after mass-cytometer run. Collected cells were stained with metal conjugated antibodies with or without a 4-hour stimulation with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A. Intracellular staining for transcription factors and cytokines was performed after fixation and permeabilization according to the manufacturer's instructions (see the intracellular staining paragraph).

The panel of antibodies was designed and optimised based on surface markers, transcription factors and cytokines. Each antibody was tagged with a rare metal isotope and its function has been verified by conventional flow-cytometry prior to

mass-cytometry. CyTOF 2 mass cytometer (Fluidigm) has been used for data acquisition. Acquired data were normalised based on normalisation beads (Ce140, Eu151, Eu153, Ho165 and Lu175) and analysed as already described.[342] In brief, automated clustering was performed on a subset of 800,000 cells sampled from all individuals. The number of cells sampled from each individual was proportional to the total number of cells in that sample.

### 2.4.2 Cell staining for mass cytometry

Cells stained for CyTOF were either PBMCs or expanded Tregs, this will be stated in each section of the results.

Before the staining, half of the cells were stimulated with leukocyte activation cocktail, with BD GolgiPlug for 4 hours. After stimulation, cells were harvested, washed twice (300 x $g_{max}$ for 10 minutes) with PBS without $Ca^{++}/Mg^{++}$, and counted prior to staining. Cells were stained with 500 µl of 1x Rh-intercalator at room temperature for 15 minutes and washed twice (300 x $g_{max}$ for 10 minutes) with cell staining medium (CSM, made with PBS without $Ca^{++}/Mg^{++}$ and 2.5% FCS). The Fc blocking step was done by adding 10 µl (diluted 1:20) human Ig solution (stock solution provided at 100 mg/ml) at room temperature for 10 minutes. Before proceeding to the surface staining, the Ab master mix was span at 5,600 x $g_{max}$ for 5 minutes. After this step, cells were stained at room temperature and washed twice (300 x $g_{max}$ for 10 minutes) with CSM. For the intracellular staining, cells were fixed/permeabilised first, by the addition of 1 ml of freshly prepared eBioscience fixation/permeabilization solution and incubated in the fridge for 45 minutes. After this step, cells were washed (400 x $g_{max}$ for 8 minutes) with 2 ml of 1x eBioscience permeabilization buffer. The intracellular master mix was added, and cells were

incubated in the fridge for 30 minutes and washed (400 x $g_{max}$ for 8 minutes) once with 2 ml of 1x eBioscience permeabilization buffer. Finally, cells were fixed in 500 µl of paraformaldehyde (PFA) 4% and left in the fridge over-night. The following day, before injection, cells were stained in 500 µl 1x Ir-intercalator at room temperature for 20 minutes, washed once with PBS (600 x $g_{max}$ for 7 minutes), counted and washed twice (600 x $g_{max}$ for 7 minutes) with MilliQ water (all the reagents are listed in Table 2.9).

**Table 2.9 Reagents for cell staining for mass cytometry**

| Product | Company | Catalogue # |
|---|---|---|
| Leukocyte Activation Cocktail, with BD GolgiPlug | BD Biosciences | 550583 |
| PBS w/o $Ca^{++}$/$Mg^{++}$ | Sigma Aldrich | D8537 |
| Foetal calf serum | ThermoFisher Scientific | 10500-064 |
| Fc blocking reagent | Immunostep | FCR-2 ml |
| Fixation/Permeabilization concentrate | eBioscence | 00512343 |
| Fixation/Permeabilization diluent | eBioscence | 00522356 |
| Permeabilization washing buffer | eBioscence | 00833356 |

## 2.4.3 ProcartaPlex™ multiplex immunoassay

ProcartaPlex™ immunoassays incorporate magnetic microsphere technology licensed from the Luminex™ Corporation to enable the simultaneous detection and quantitation of multiple protein targets in diverse matrices. The platform allows the simultaneous detection from a single sample of up to 100 protein targets on the Luminex™ 100/200™ and FLEXMAP 3D™ platforms. The reagents are listed in Table 2.10.

**Table 2.10 Reagents list for ProcartaPlex™ multiplex immunoassay**

| Product | Company | Catalogue # |
|---|---|---|
| HS HU IL-17A SIMPLEX | Affymetrix | EPXS010-12017-901 |
| HS HU IL-1β SIMPLEX | Affymetrix | EPXS010-10224-901 |
| HS HU IFN-γ SIMPLEX | Affymetrix | EPXS010-10228-901 |
| HS HU TNF-α SIMPLEX | Affymetrix | EPXS010-10223-901 |
| HS HU IL-2 SIMPLEX | Affymetrix | EPXS010-10221-901 |
| HS HU IL-10 SIMPLEX | Affymetrix | EPXS010-10215-901 |
| HS HU BASIC KIT | Affymetrix | EPXS010-10420-901 |

### 2.4.3.1 Sample preparation

Samples were thawed on ice and mixed very well by vortexing. This step was followed by centrifugation at 10,000 x $g_{max}$ for 5 minutes. Samples were kept on ice until they were used.

### 2.4.3.2 Reagents preparation

Wash buffer concentrate (10x) was brought at room temperature and vortexed for 15 seconds. Twenty ml of the wash buffer concentrate (10x) were mixed with 180 ml of double-distilled water (dd$H_2O$). Ten ml of universal assay buffer (10x) were mixed with 90 ml of dd$H_2O$.

Each different antigen standard set vial was centrifuged at 2,000 x $g_{max}$ for 10 seconds and the lyophilised antigen was reconstituted by adding 50 µl of cell culture medium. Each vial was vortexed for 10 seconds and span down for 10 seconds to collect the content at its bottom.

Vials were put on ice for 10 minutes to ensure complete reconstitution. The content of each vial was pooled together.

Using the PCR 8 tubes strip provided by the kit was, a 4-fold serial dilution was prepared (Figure 2.2).

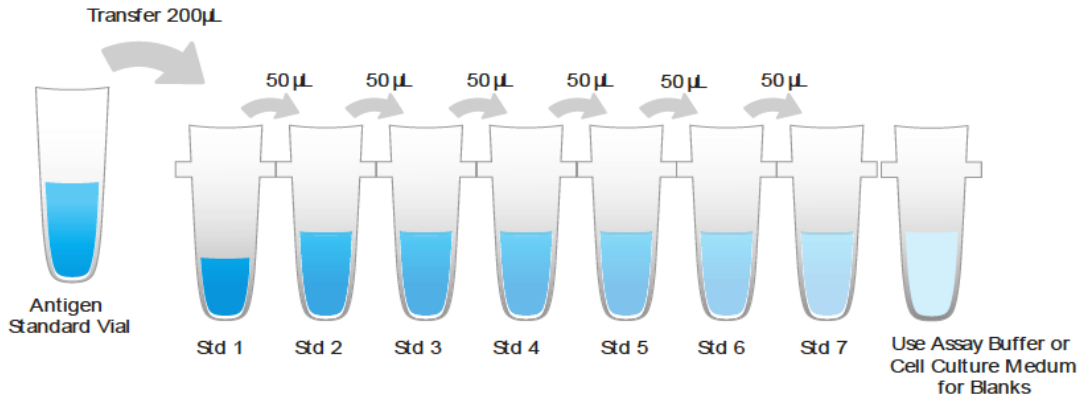



Figure 2.2 Serial dilution for standard preparation

### 2.4.3.3 Assay protocol

The plate map was defined as recommended by the protocol. After vortexing for 30 seconds, 50 µl of magnetic bead solution were added to each well. Beads were washed once with wash buffer (1x) by using a magnetic block. Following the washed, sample-specific buffer, samples, standards and blanks, were added to the plate. Once ready, the plate was covered with a lid and shacked at 500 rpm at room temperature for two hours. After two washes, the detection antibody mixture was added and incubated for 30 minutes. The streptavidin-PE was added after two more washes and incubated for 30 minutes. Before reading the plate, 120 µl f reading buffer were added and the plate was shacked for 5 minutes at room temperature.

### 2.4.3.4 Instrument setup

The instrument was set up according to the manufacturer instructions (Table 2.11).

**Table 2.11 Instrument settings for the cytokine measurement assay**

| Instrument | Sample size | DD gate | Timeout | Bead event/bead region |
|---|---|---|---|---|
| Luminex™ 100/200™ FLEXMAP 3D™ | 50 µl | 5,000-25,000 | 60 seconds | 50-100 |

## 2.5 Treg-specific demethylated region analysis by bisulfite deep amplicon sequencing on the Illumina MiSeq platform

Treg-specific demethylated region (TSDR) is an evolutionary conserved element in the 5' untranslated region of the *foxp3 locus*. It has been shown previously that the CpG motifs in this element are demethylated in thymus derived Tregs and can serve as a specific epigenetic marker for Foxp3$^+$ Tregs with a stable phenotype.[333, 343] In humans the *foxp3* gene [National Center for Biotechnology Information (NCBI) gene ID 50943] is located on the minus strand of chromosome Xp11.23.

The assay described here interrogates 15 CpG motifs within the human TSDR in the following positions (reference GRCh38.p2).

As the *foxp3* gene is located on the X chromosome, female individuals will have two alleles and males one allele. Currently, it is unknown to which extent the *foxp3 locus* in females is subject to X inactivation, which might lead to methylation of CpGs in one copy of the TSDR of stable regulatory T cells. To avoid difficulties in data interpretation it is preferable to run TSDR methylation assays on samples from male donors. Data derived from female donors need to be corrected for the presence of the second allele as follows:

- FAm[♂/♀]: apparent fraction of methylated sites [male/female];
- FAdm[♂/♀]: apparent fraction of demethylated sites [male/female];
- FTm[♂/♀]: true fraction of methylated sites [male/female];
- FTdm[♂/♀]: true fraction of demethylated sites [male/female].

For male samples (only one X chromosome):

- FTm[♂] = FAm[♂]
- FTdm[♂] = FAdm[♂]

For female samples (two X chromosomes with one X chromosome inactivated):

- FTdm[♀] = 2 x FAdm[♀];
- FTm[♀] = 1 – [2 x (1 – FAm[♀])].

In cases where the gender of the donor is unknown (e.g., when working with NHSBT leukocyte cone), the genomic DNA can be used to establish the gender. The laboratory for molecular haematology at the Rayne Institute performs a chimerism assay, part of which is a PCR test on the amelogenin gene.

The result of this PCR will indicate the gender of the donor of the genomic DNA sample.

### 2.5.1 Isolation of genomic DNA

Genomic DNA is isolated from fresh or frozen cell pellets of pure Tregs in PBS. The procedure follows closely the Qiagen protocol "Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol)" for the DNeasy Blood & Tissue kit. The DNA was eluted from DNA from the DNeasy Mini spin columns by adding 40 µl of AE buffer to the column, incubating for 3 minutes at room temperature, spinning. This step was repeating by adding 20 µl of AE buffer only. An aliquot of 1.5-2 µl was then measured for DNA concentration on a NanoDrop spectrophotometer (see the NanoDrop paragraph).

The concentration was then used as a guide for the dilution factor for the precise determination of DNA concentration by the PicoGreen assay (all reagents are listed in Table 2.12).

**Table 2.12 Reagents for genomic DNA extraction**

| Product | Company | Catalogue # |
|---|---|---|
| DNeasy Blood & Tissue Kit | Qiagen | 69504 |
| QIAamp DNA Mini Kit | Qiagen | 51304 |

### 2.5.2 NanoDrop

DNA concentration was determined by NanoDrop spectrophotometry, measuring the absorbance of the DNA at 260 nm. Typically, 1 µl of DNA in TE buffer was placed on the NanoDrop optical fibre and the absorbance was measured against a blank of $dH_2O$. The 260 nm/280 nm ratio of absorbances is a good indicator of protein contamination and a value of 1.5 or higher indicates a reasonable purity of DNA. The Beer-Lambert equation was used to correlate absorbance with

concentration (A = ε x b x c), where A is the measured absorbance, ε is the dependent molar absorbitivity, b is the path length and c is the analyte concentration.[344]

### 2.5.3 Quantification of genomic deoxyribonucleic acid with the PicoGreen assay

The PicoGreen dsDNA assay (Quant-iT™ PicoGreen dsDNA Assay Kit, ThermoFisher Scientific, P11496) was used to establish an accurate concentration of genomic DNA or PCR fragments/libraries, as a precise value was needed for downstream applications like bisulfite conversion or high-throughput sequencing. The assay measures the fluorescence of the PicoGreen reagent, when bound to double-stranded DNA, and compares the value to a standard curve made from lambda (λ) DNA. In contrast to measurements on a spectrophotometer, the PicoGreen method is insensitive to the presence of RNA, single-stranded DNA or nucleotides.

#### 2.5.3.1 Preparation of the DNA standard curve

The PicoGreen dsDNA Assay kit contains a vial of DNA at a concentration of 100 ng/ l. Before the assay, the λ DNA was diluted to a working concentration of 2 ng/ l. further five 1:2 dilutions with TE buffer were made to set up the standard curve, resulting in the following range: 2 ng/ l, 1 ng/ l, 0.5 ng/ l, 0.25 ng/ l, 0.125 ng/ l and 0.0625 ng/ l.

#### 2.5.3.2 Preparation of the double stranded DNA samples

Using the concentration estimate from the NanoDrop, the experimental samples of DNA were diluted to a maximum of 0.25 – 0.5 ng/µl with TE buffer.

Diluted samples can be measured directly but should eventually have a concentration larger than 0.0625 ng/µl to lie within the range of the standard curve.

#### 2.5.3.3 Pipetting the assay

Five µl of DNA (experimental sample or standard curve) were pipetted into two or three separate 0.1 ml Rotor-Gene style tubes. Then 5 µl of a 1:100 dilution of PicoGreen dsDNA reagent (with TE buffer) were added into each tube.

#### 2.5.3.4 Running the assay on the Corbett Rotor-Gene 6000

All samples were measured on the Rotor-Gene 6000 real-time PCR cycler with the programme "Nucleic Acid Quantification". The programme will first heat the samples to 50 °C and then establish an appropriate gain setting for the fluorescence measurement. For this to work correctly, the sample with the highest DNA concentration must be placed in position 1 on the 72-well rotor.

### 2.5.4 Bisulfite conversion of genomic deoxyribonucleic acid

A bisulfite conversion reaction is required to investigate methylation patterns of genomic DNA. The method is based on the property of sodium bisulfite to selectively deaminate cytosine residues to uracil (Figure 2.3).

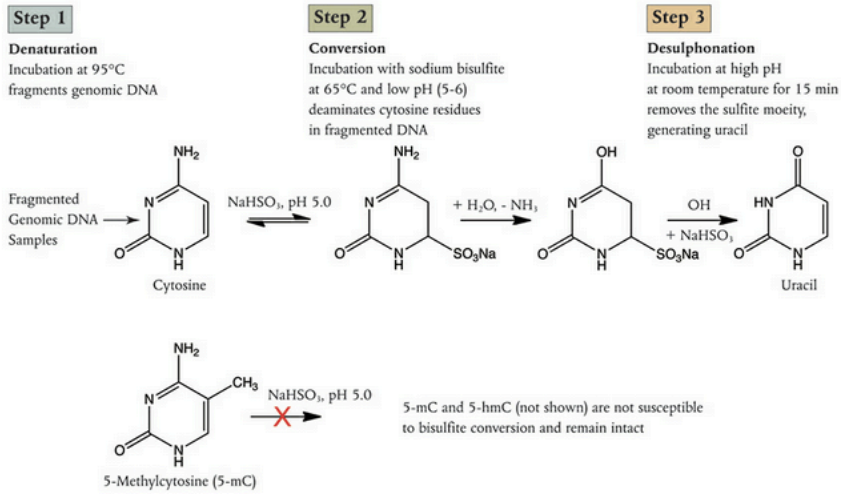



**Figure 2.3 The bisulfite conversion**

Step 1 – Denaturation - involves a reaction between cytosine and bisulfite that leads to deamination (step 2 - Conversion) at acid pH. Therefore, desulphonation at alkaline pH produces uracil (step 3 - Desulphonation). Adapted from www.neb.com.

Since 5-methylcytosine is not converted under these conditions, it is possible to distinguish between methylated and unmethylated cytosines (Figure 2.4).[345]

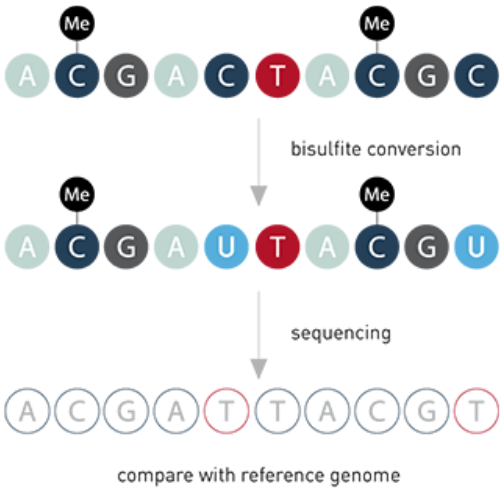



**Figure 2.4 Conversion of cytosine**

Bisulfite treatment converts cytosine residues to uracil but leaves 5-methylcytosine residues unaffected. Adapted from https://www.diagenode.com.

The genomic DNA was bisulfite converted using the EpiTect Plus DNA Bisulfite Kit (Qiagen, 59124). The samples were prepared by adding the bisulfite mix and incubated in a thermocycler using the bisulfite conversion program (Table 2.13).

Table 2.13 Bisulfite conversion thermal cycle condition

| Step | Time | Temperature |
|---|---|---|
| Denaturation | 5 minutes | 95 °C |
| Incubation | 25 minutes | 60 °C |
| Denaturation | 5 minutes | 95 °C |
| Incubation | 85 minutes | 60 °C |
| Denaturation | 5 minutes | 95 °C |
| Incubation | 175 minutes | 60 °C |
| Hold | Indefinite | 20 °C |

Once the conversion was completed, samples were transferred into two separate 1.5 ml microcentrifuge tubes. A total of 310 µl of BL buffer containing 10 µg/ml carrier RNA was added to each sample. Samples were mixed by vortexing for 10 seconds. Two hundred and fifty µl of ethanol (100%) were added to each sample and mixed by vortexing for 15 seconds. The entire mixture from each tube was transferred into two separate MinElute DNA spin columns placed in a collection tube. The two spin columns were centrifuged at maximum speed for 1 minute and the flow-through was discarded. A total of 500 µl BW buffer was added to each spin column and spun for 1 minute at 300 x $g_{max}$ and the flow-through was discarded. A total of 500 µl BD buffer was added to each spin column and left to incubate for 30 minutes at 37 °C. Spin columns were centrifuged at 300 x $g_{max}$ for 1 minute and the flow-through was discarded. A total of 550 µl of BW buffer was added to each spin column and centrifuged for 1 minute at 300 x $g_{max}$ and the flow-through was discarded. A total of 250 µl of ethanol (100%) was added to each sample and then centrifuged for 1 minute at 300 x $g_{max}$. Spin columns were placed into two new clean tubes and centrifuged for 1 minute at 300 x $g_{max}$ to remove any residual liquid. Spin columns were placed into two DNALoBind tubes. The genomic DNA was eluted by adding 20 µl EB buffer, incubated for 1 minutes at room temperature and spun for 1 minute at 400 x $g_{max}$. The genomic DNA was kept at 4° C.

### 2.5.5 PCR amplification of the bisulfite converted TSDR region

After bisulfite conversion of the genomic DNA, the TSDR of the Foxp3 *locus* was amplified in a PCR reaction with a primer pair specific for the region and containing appropriate tags for later sample indexing in a multiplex sequencing setup (all reagents are listed in Table 2.14).

**Table 2.14 Reagents for PCR amplification of the bisulfite converted TSDR region**

| Product | Company | Catalogue # |
|---|---|---|
| EpiMark Hot Start Tag DNA Polymerase | New England Biolabs | M04905 |
| Deoxynucleotide (dNTP) Solution Set | New England Biolabs | N0446S |

The primer pair used is shown in Table 2.15. These two primers are derivatives of the original primers published by Baron *et al.*[343]

**Table 2.15 Primer used in the PCR amplification of the bisulfite converted TSDR region**

| Primer name | Sequence |
|---|---|
| TSDR_3_bst_fwd | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGTTTGGGGGTAGAGGATTT |
| TSDR_3_bst_rev | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATCACCCCACCTAAACCAA |

The mix added to each reaction is detailed in Table 2.16.

**Table 2.16 Reaction mix**

| Component | Volume per 25 l reaction |
|---|---|
| Bisulfite converted DNA | 4 µl (equivalent to 10 ng genomic DNA) |
| RNase-free water | 14.375 µl |
| 5X EpiMark Hot Start Taq Reaction Buffer | 5 µl |
| dNTPs (10 mM each) | 0.5 µl |
| Primer TSDR_3_bst_fwd (10 M) | 0.5 µl |
| Primer TSDR_3_bst_rev (10 M) | 0.5 µl |
| EpiMark Hot Start Taq DNA Polymerase | 0.125 µl |

The reaction tubes were then placed in a thermocycler and run with the conditions shown in Table 2.17.

**Table 2.17 Cycling conditions for PCR amplification of the bisulfite converted TSDR region**

| Step | Time | Temperature |
|---|---|---|
| Initial denaturation | 30 seconds | 95 °C |
| Amplification for 38 cycles | 25 seconds | 95 °C |
| | 45 seconds | 56 °C |
| | 45 seconds | 68 °C |
| Final extension | 5 minutes | 68 °C |
| Hold | Indefinite | 4 °C |

After amplification, the samples were stored in the fridge for short term and frozen if used after an extended period of time.

### 2.5.6 First gel purification of PCR amplicons

This step is needed to clean up the PCR amplification product (all reagents are listed in Table 2.18).

Table 2.18 Reagents for first gel purification of PCR amplicons

| Product | Company | Catalogue # |
|---|---|---|
| QIAquick Gel Extraction Kit | Qiagen | 28704 |
| Agarose | Sigma-Aldrich | A9539 |
| Ethidium bromide | Sigma-Aldrich | E1510 |
| GeneRuler DNA ladder mix, 100-10,000 bp | Thermo Fisher Scientific | 11531605 |

Fifty ml of TAE buffer with 1.8% agarose were poured into a small gel tray. A 15-well comb was used to create sample wells large enough to hold around 25 µl, but small enough to minimise the amount of agarose in the isolation procedure. Twenty µl of the reaction were mixed with loading dye and run at 80 V to 100 V for around 45 minutes until a proper separation of the 400 bp amplicon was achieved. The gel was put a GelDoc station to document the fragment size(s) and purity of the PCR amplification product. The 400 bp fragment was cut from the agarose gel (with a scalpel under UV illumination) and the gel block was placed into a clean pre-weighed 1.5 ml microcentrifuge tube. The gel extraction procedure was done with the Qiagen QIAquick Gel Extraction Kit and follows closely the protocol "QIAquick Gel Extraction Kit Protocol using a microcentrifuge" in the QIAquick Spin Handbook. The gel extracted agarose fragment containing the DNA of interest was mixed with 3 volumes of PBI buffer, then incubated at 50 °C for 10 minutes. Once the agarose slice was dissolved, the solution was applied onto a QIAquick spin column and

centrifuged at 400 x $g_{max}$ for 10 minutes. A total of 750 µl of PE buffer were added to the QIAquick column and then centrifuged 400 x $g_{max}$ for 10 minutes. Twenty µl of EB buffer were applied to the column and incubated for 5 minutes at room temperature. The column was spun at 400 x $g_{max}$ for 1 minute to recover the DNA fragment. DNA was then stored at -20 ˚C in a DNALoBind tube.

### 2.5.7 Sample indexing of PCR amplicons for multiplex sequencing

For next-generation sequencing assays on the MiSeq instrument, several multiplexed samples are typically combined into one library and loaded on the whole onto the sequencing chip (all reagents are listed in Table 2.19).

**Table 2.19 Reagents for sample indexing of PCR amplicons for multiplex sequencing**

| Product | Company | Catalogue # |
|---|---|---|
| Nextera XT Index Kit | Illumina | FC-131-1002 |
| GoTaq Hot Start Colorless Master Mix | Promega | M5132 |

In order to be able to retrieve the sequences belonging to a single original sample the PCR amplicons from separate samples are barcoded before they can be pooled. The Nextera XT Index Kit contains a number of different PCR primers (forward and reverse), which attach to the common tag in the TSDR_3_bst primers and add a unique combination of indices to each sample.

Nextera XT Index primers come in two groups, so called i5 and i7 primers, and using a combination of one i5 and one i7 primer for the PCR amplification of a single sample will attach a unique identifier. Indexing reactions are typically set up in a 96-well plate format, were each well of the 8 rows has the same i5 primer and each well of the 12 columns has the same i7 primer. Before indexing, the total number of samples pooled into the final library and loaded onto the sequencing chip needs to be established, so that each sample can receive a unique identifier.

A low-cycle PCR amplification reaction was set up (see Table 2.20), where the TSDR amplicon from bisulfite converted DNA was used as template. Individual amounts from different samples were normalised, so that each indexing reaction received a similar amount of template.

**Table 2.20 Amplification reaction for sample indexing of PCR amplicons for multiplex sequencing**

| Component | Volume per 15 l reaction |
|---|---|
| TSDR PCR amplicon (gel-purified DNA) | 0.5 µl-1.0 µl (from a total of 40 l DNA) |
| RNase-free water | 4.0-4.5 µl (adjust template to 5 µl in total) |
| GoTaq Mix | 7.5 µl |
| Index Primer 1 (i5) | 1.25 µl |
| Index Primer 2 (i7) | 1.25 µl |

The reactions tubes are then placed in a thermocycler and run with the conditions showed in Table 2.21.

**Table 2.21 Cycling conditions for amplification reaction for sample indexing of PCR amplicons for multiplex sequencing**

| Step | Time | Temperature |
|---|---|---|
| Initial denaturation | 3 minutes | 95 °C |
| Amplification for 8 cycles | 20 seconds | 95 °C |
| | 30 seconds | 55 °C |
| | 30 seconds | 72 °C |
| Final extension | 5 minutes | 72 °C |
| Hold | Indefinite | 4 °C |

After amplification the sample was stored in the fridge for short term and frozen for an extended period of time.

## 2.5.8 Second gel purification of PCR amplicons

The methodology used has already been described above. After the gel purification of indexed PCR amplicons, the DNA was pooled. To have a similar sequencing read depth for all samples loaded, all fragments should be present in equimolar amounts.

## 2.5.9 Quantification of PCR amplicons with the PicoGreen assay

The methodology used has already been described above (page 129).

### 2.5.10 Next-generation sequencing of PCR amplicons on the Illumina MiSeq platform

All the reagents used for this step are listed in Table 2.22.

**Table 2.22 Reagents for next-generation sequencing of PCR amplicons on the Illumina MiSeq platform**

| Product | Company | Catalogue # |
|---|---|---|
| MiSeq Reagent Nano Kit v2 | Illumina | MS-103-1003 |
| MiSeq Reagent Kit v3 | Illumina | MS-102-3003 |
| Agencourt AMPure XP | Beckman Coulter | A63880 |

#### 2.5.10.1 Pooling the samples into a single library

At this stage, the final PCR purification with the Agencourt AMPure XP beads was done on individual samples or on the pooled library, according to each experiment. Using the accurate molar DNA concentrations for all samples to be pooled, correct volumes for equimolar amounts were calculated and combined.

#### 2.5.10.2 Clean-up of the sample

To purify PCR products, 1.8 µl of AMPure XP were added for 1.0 µl of sample. The DNA fragments the bind to paramagnetic beads. The following step consisted in separating of beads and DNA fragments from contaminants. After separation, beads and DNA fragments were washed twice with ethanol (70%) to remove contaminants. The final part included the elution purified DNA fragments from beads (the workflow for purification is represented in Figure 2.5).

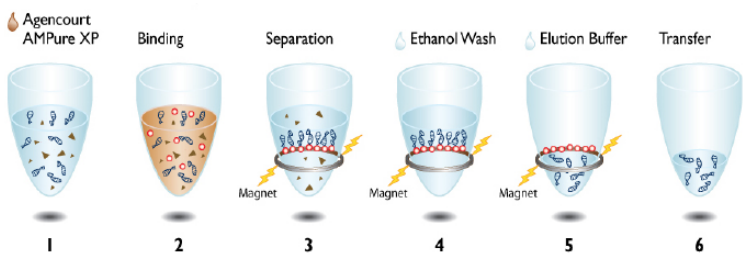



**Figure 2.5 Workflow for PCR purification**

The figure represents the sequence of steps leading to the sample clean-up (adapted from Agencourt AMPure XP instructions for use).

#### 2.5.10.3 Agarose gel and PicoGreen assay on the pooled library

Once the library had been pooled and bead purified, an aliquot was loaded onto an agarose gel to establish purity to make sure that there are no low-molecular weight fragments or primer dimers present, as these would dominate the sequencing reaction. The DNA concentration of the pooled library was established with the PicoGreen assay (see above) and the mass concentration was converted into a molar concentration, depending on the size distribution.

#### 2.5.10.4 Denature and dilute Library

The subsequent steps follow closely the Illumina protocol "Preparing Libraries for Sequencing on the MiSeq". Depending on the reagent version used for sequencing, different staring DNA concentrations were used. The commonly used concentration was 4 nM, so in a first step the pooled DNA library was diluted to a final concentration of 4 nM with water. The 4 nM library was then denatured with 0.2 M NaOH and neutralised with HT1 buffer resulting in a 20 pM library.

#### 2.5.10.5 Prepare PhiX control

A library of phage PhiX DNA was spiked into the sequencing reaction. This is especially important for low-complexity libraries, such as PCR amplicon sequencing libraries. Once the control was spiked into the sequencing sample, the sample was loaded into the MiSeq sequencing reagent cartridge.

#### 2.5.10.6 Sequencing run

The set-up of the MiSeq instrument for sequencing was done according to the Illumina protocols supplied with the individual kits used. For kits with v2 reagent

there is “MiSeq Reagent Kit v2 Reagent Preparation Guide”. A short description will follow.

The hybridization buffer and the reagent cartridge were removed from the freezer and thawed to room temperature. Once thawed, the reagent cartridge was placed in a water bath containing enough room temperature deionized water to submerge the base of the reagent cartridge up to the water line printed on the reagent cartridge.

The reagent cartridge was allowed to thaw at room temperature water bath for approximately one hour and them thoroughly dried. Finally, the cartridge was inspected to make sure it was fully mixed, free of precipitates and with no air bubbles.

In the next generation technology, DNA polymerase catalyses the incorporation of fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) into a DNA template strand during sequential cycles of DNA synthesis. During each cycle, at the point of incorporation, the nucleotides are identified by fluorophore excitation. The critical part is that next generation sequencing extends this process across millions of fragments in a massively parallel fashion.

Illumina next generation sequencing (NGS) workflows include four basic steps:

1. library preparation [the sequencing library is prepared by random fragmentation of the DNA or complementary DNA sample, followed by 5’ and 3’ adapter ligation (Figure 2.6 A)];
2. cluster generation [for cluster generation, the library is loaded into a flow cell where fragments are captured on a lawn of surface-bound oligos complementary to the library adapters; each fragment is then amplified into distinct, clonal clusters through bridge amplification (Figure 2.6 B)];

3. sequencing [Illumina technology uses a reversible terminator-based method that detects single bases as they are incorporated into DNA template strands Figure 2.6 C)];
4. data analysis [during data analysis and alignment, the newly identified sequence reads are aligned to a reference genome (Figure 2.6 D)].

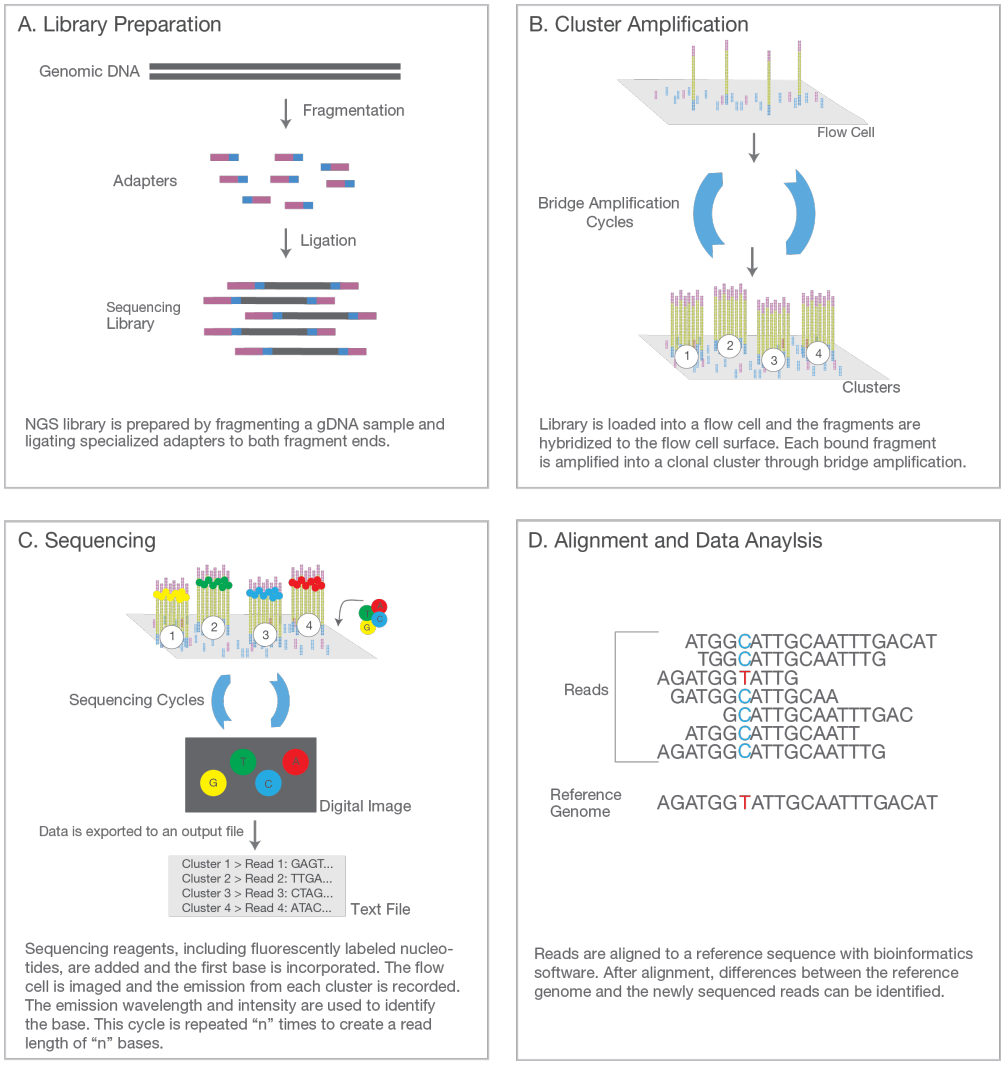



**Figure 2.6 Next generation sequencing chemistry overview**

Illumina next generation sequencing includes four steps: (A) library preparation, (B) cluster generation, (C) sequencing, and (D) alignment and data analysis (adapted from "An introduction to next generation sequencing technology").

The main features of this technology are:

- automation (after setting up the run, no additional hands-on time is required);

- prefilled reagent cartridge (a specially designed single-use prefilled reagent cartridge provides reagents for cluster generation and sequencing, including paired-end sequencing reagents and indexing reagents);
- interface controls (the MiSeq control software interface provides controls to configure the instrument, set up and monitor runs, and perform maintenance procedures);
- convenient flow cell loading (a clamping mechanism auto-positions the flow cell as it is loaded onto the instrument);
- innovative fluidics architecture (the MiSeq fluidics system enables unmatched efficiency in chemistry cycle time during sequencing);
- real-time analysis (integrated analysis software performs real-time on instrument data analysis during the sequencing run, which includes image analysis and base calling);
- MiSeq reporter (integrated secondary analysis software processes data from analysis to perform alignment and provide information about each sample analysed).

## 2.6 T cell receptor sequencing

In order to assess the clonality of expanded T cells, we have sent samples to Adaptive Biotechnology to sequence the T cell receptor. A brief overview of the assay is described below. The immunoSEQ hsTCRB kit combines highly optimized multiplex PCR primers, a set of built-in controls consisting of synthetic immune receptor sequence analogs, and advanced bioinformatics. The assay specifically targets the CDR3 of human TCRB gene sequences following imperfect rearrangement of the V, D and J gene segments, which includes non-templated

nucleotide insertions and deletions at the gene segment junctions. All possible recombined receptor sequences in a sample are amplified using a proprietary mix of multiplexed V- and J-gene primers in a first round of PCR amplification (Figure 2.7 A).

High-throughput sequencing and unique identification of each library are made possible through the addition of universal adapter sequences and DNA barcodes unique to each PCR replicate in a second round of PCR (Figure 2.7 B). Following the second PCR amplification, sequencer-ready libraries (Figure 2.7 C) are pooled for sequencing on an Illumina MiSeq.

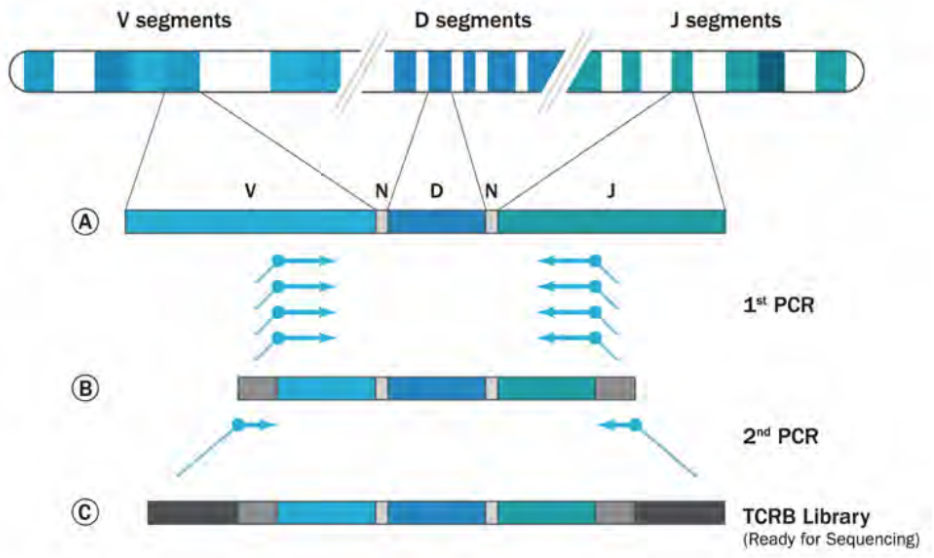



**Figure 2.7 immunoSEQ assay overview**

V, D and J segments are recombined to form the CDR3 of a rearranged TCRB gene. (A) Multiplex PCR to amplify the highly variable CDR3 region. (B) PCR to add barcodes and adaptor sequences for sequencing via the Illumina MiSeq. (C) Sequencer-ready libraries (adapted from hsTCRB immunoSEQ kit manual, Adaptive Biotechnologies).

After sequencing, raw sequence output from the MiSeq is transferred to Adaptive Biotechnologies using the immunoSEQ data assistant. Raw data were processed to report the normalised and annotated TCRB repertoire profile for each sample.

Data quality also was checked to identify issues with cross-contamination, low coverage and missing or unexpected samples.

These results, along with several data analysis and visualization tools were made available through our immunoSEQ analyser account.

## 2.7 Gene expression profiling

### 2.7.1 RNA extraction by TRIzol/RNeasy hybrid protocol

Cells were homogenised using an appropriate volume of TRIzol and stored at room temperature for 5 minutes. Chloroform was added to the homogenate (0.2 ml chloroform/1 ml of TRIzol used), mixed vigorously for 15 seconds, and incubated for 2-3 minutes at room temperature. The mixture was centrifuged at 1,200 x $g_{max}$ for 15 minutes at 4 ˚C, the aqueous phase (top) was removed and mixed with an equal volume of ethanol (70%). Sample was then loaded into RNeasy column seated in a collection tube and centrifuged for 30 seconds at 8,000 x $g_{max}$. The column was washed twice with guanidinium thiocyanate (10%) plus ethanol (10%) in RPE buffer. The RNA was eluted into microcentrifuge tube using 30-50 µl of RNase-free water. RNA concentration was measured by NanoDrop (all the reagents are listed in Table 2.23).

Table 2.23 Reagents for RNA extraction by TRIzol/RNeasy hybrid protocol

| Product | Company | Catalogue # |
|---|---|---|
| Trizol | ThermoFisher Scientific | 15596-026 |
| Chloroform | Sigma-Aldrich | C2432 |
| Ethanol | Sigma-Aldrich | 652261 |
| Guanidinium thiocyanate | Sigma-Aldrich | 3563461 |
| RNeasy MinElute clean-up | Qiagen | 74204 |

### 2.7.2 Complementary deoxyribonucleic acid preparation from total RNA

Samples for gene expression analysis were sent to the Genomics Centre in Waterloo campus. All the experiments (as described below) were performed there by the Genomics Centre technicians.

Total RNA was amplified, and biotin labelled using the Ovation Pico WTA V2 and Encore Biotin Module kits (NuGEN, 3302). The first strand complementary deoxyribonucleic acid (cDNA) is prepared from total RNA using a unique first strand DNA/RNA chimeric primer mix and reverse transcriptase. The primer mix contains a unique mixture of random and oligo dT primers such that priming occurs across the whole transcript. RT extends the 3' DNA end of each primer generating first strand cDNA.

The resulting cDNA/mRNA hybrid molecule contains a unique RNA tag sequence (single primer isothermal amplification - SPIA tag) at the 5' end of the cDNA strand, which will be used as a priming site for the SPIA process. Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second cDNA strand, which includes DNA complementary to the 5' SPIA tag sequence from the first strand chimeric primers. The result is a double-stranded cDNA with a DNA/RNA heteroduplex corresponding to the SPIA tag at one end. SPIA is a rapid, simple and sensitive strand-displacement amplification process developed by NuGEN. It uses a DNA/RNA chimeric primer (SPIA primer), DNA polymerase and RNase H in an isothermal assay. RNase H removes the RNA portion of the heteroduplex SPIA tag sequence, revealing a site for binding the SPIA primer.

DNA polymerase synthesizes cDNA starting at the 3' end of the primer, displacing the existing forward strand. Priming with the chimeric SPIA primer recapitulates the heteroduplex SPIA tag, creating a new substrate for RNase H and the initiation of the next round of cDNA synthesis.

The process of SPIA DNA/RNA primer binding, DNA replication, strand displacement and RNA cleavage, is repeated in a highly processive manner, resulting in rapid accumulation of micrograms of amplified cDNA from pg of total RNA.

### 2.7.3 Array processing and data analysis

Biotinylated cDNA was applied and hybridised to Human Gene 2.0 ST arrays (Affymetrix, 902459) following NuGEN's guidelines. Arrays were processed using the Hybridisation Wash Stain kit and scanned following manufacturer's guidelines (Affymetrix, 900720).

CEL files were processed in Expression Console to generate normalised, summarised and background-corrected data (CHP files), and further analysed for differential gene expression using gene set enrichment analysis (GSEA).[346, 347]

Publically available free software for gene set enrichment analysis was used for enrichment and leading-edge analysis: http://software.broadinstitute.org/gsea/msigdb/search.jsp.

## 2.8 RNA sequencing

RNA sequencing is a powerful method for analysing global gene expression levels and providing in-depth analysis of the transcriptome.

RNA sequencing can be used to accurately analyse low expression genes and transcripts often not detected by other methods, discover novel genes and isoforms, and assemble transcriptomes not previously studied.

RNA extraction, RNA library preparations, sequencing reactions, and initial bioinformatics analysis were conducted at GENEWIZ, LLC (South Plainfield, NJ, USA).

### 2.8.1 Total RNA extraction and sample quality control

Total RNA was extracted following the TRIzol reagent user guide (Thermo Fisher Scientific). One µl of glycogen (10 mg/ml) was added to the supernatant to increase RNA recovery. RNA was quantified using Nanodrop 2000 (Thermo Scientific), Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked with TapeStation (Agilent Technologies, Palo Alto, CA, USA) if the concentration met the requirements.

### 2.8.2 Single cell, low-input RNA library preparation and multiplexing

SMART-Seq v4 ultra low input kit for sequencing was used for full-length cDNA synthesis and amplification (Clontech, Mountain View, CA), and Illumina Nextera XT library was used for sequencing library preparation. Briefly, cDNA was fragmented, and adaptor was added using transposase, followed by limited-cycle PCR to enrich and add index to the cDNA fragments. The final library was assessed with Qubit 2.0 fluorometer and Agilent TapeStation.

### 2.8.3 Sequencing 2 x 150 bp pair-end

The sequencing libraries were multiplexed and clustered on two lanes of a flowcell. After clustering, the flowcells were loaded on the Illumina HiSeq instrument according to manufacturer's instructions. The samples were sequenced using a 2 x 150 paired end configuration. Image analysis, and base calling were conducted by the HiSeq control software on the HiSeq instrument. Raw sequence data (.bcl files) generated from Illumina HiSeq were converted into fastq files and de-multiplexed using Illumina bcl2fastq v. 2.17 program. One mismatch was allowed for index sequence identification.

### 2.8.4 Data analysis

Reads were trimmed for adapters using cutadapt and aligned to hg19 genome using STAR.[348] Quantification in expected counts from genes and isoforms were computed by RSEM[349] using genecode annotation v.26 [https://www.gencodegenes.org/]. We used TMM method to estimate scale factors between samples followed by the voom function in limma to convert into log2counts per million (logCPM) and quantile normalisation. Finally, differential expression between groups were evaluated by LIMMA bioconductor package.[350] Genes with a fold change of 1.5 and moderated p-value < 0.05 were considered as significantly different.

The next step of data analysis was done using Ingenuity Pathway Analysis (QIAGEN). Ingenuity path analysis (IPA) is a web-based software able to reveal significant molecules, biological pathways, and networks underlying complex 'omics data. It is used for the analysis, integration, and interpretation of data derived from gene expression experiments including RNA-Seq, microRNA and SNP microarrays, metabolomics, proteomics, and small-scale experiments that generate gene and chemical lists. Downstream Effects Analysis predicts cellular functions, disease processes, and other phenotypes impacted by patterns in an analysed dataset. Upstream Regulator Analysis identifies regulators (transcription factors, cytokines, kinases, etc.) directly linked to the targets in analysed data and whose activation or inhibition may account for observed changes. The Causal Network Analysis feature of IPA Advanced Analytics uses powerful algorithms to generate multi-levelled regulatory networks that may explain the gene expression changes exhibited in a dataset.

The core Upstream Regulator Analysis of IPA identifies upstream molecules that are directly connected to the targets exhibiting changes in a dataset. Causal Network Analysis enables the discovery of novel regulatory mechanisms by expanding upstream analysis to include regulators that are not directly connected to targets in the dataset [IPA features, from QIAGEN website: https://www.qiagenbioinformatics.com/products/features/].

## 2.9 Cell lysis and bicinchoninic acid assay for protein quantification

### 2.9.1 Lysis of non-adherent cells

Cells were harvested and span down at 500 x $g_{max}$ for 5 minutes and washed in ice-cold PBS. Ice-cold 1x Milliplex MAP lysis buffer and freshly prepared protease inhibitors were added to cells (1 ml/$10^7$ cells). The addition of a protease inhibitor cocktail or protease inhibitor ensures the integrity of proteins for downstream analysis and further characterization of samples. The lysate was rocked for 15 minutes at 4 ˚C. After lysis, the sample was filtered and diluted 1:4 for bicinchoninic acid assay (BCA) assay (see below). All the reagents are listed in Table 2.24.

**Table 2.24 Reagents list for lysis of non-adherent cells**

| Product | Company | Catalogue # |
|---|---|---|
| PBS | Sigma-Aldrich | D866 |
| Protease Inhibitor Cocktail Set III | Merk | 535140 |
| Milliplex MAP Lysis Buffer | Millipore | 43-040 |

### 2.9.2 Bicinchoninic acid protein assay

The BCA protein assay is based on the reduction of $Cu^{++}$ to $Cu^{+}$ by protein in an alkaline solution, and a concentration-dependent detection of the monovalent copper ions produced. Bicinchoninic acid is a chromogenic reagent that chelates with

the reduced copper, producing a purple reaction complex with strong absorbance at 562 nm.[351]

### 2.9.2.1 Preparation of BSA standards and working reagents

BSA standards were prepared as shown in Table 2.25 and the working reagents as shown in Table 2.26. The kit used is from Novagen, 71285-3.

**Table 2.25 Preparation of BSA standards**

| Dilutions for standard assay | | | |
|---|---|---|---|
| **Tube** | **Volume of BSA** | **Volume of diluent** | **Final BSA concentration** |
| 1 | 250 µl from 2 mg/ml solution | 250 | 1,000 g/ml |
| 2 | 250 µl from tube 1 | 250 | 500 g/ml |
| 3 | 250 µl from tube 2 | 250 | 250 g/ml |
| 4 | 250 µl from tube 3 | 300 | 125 g/ml |
| 5 | 250 µl from tube 4 | 400 | 25 g/ml |
| 6 | 0 | 400 | 0 g/ml |
| **Dilutions for enhanced assay** | | | |
| **Tube** | **Volume of BSA** | **Volume of diluent** | **Final BSA concentration** |
| 1 | 100 µl from 2 mg/ml solution | 700 | 250 g/ml |
| 2 | 400 µl from tube 1 | 400 | 125 g/ml |
| 3 | 300 µl from tube 2 | 450 | 50 g/ml |
| 4 | 200 µl from tube 3 | 200 | 25 g/ml |
| 5 | 100 µl from tube 4 | 400 | 5 g/ml |
| 6 | 0 | 400 | 0 g/ml |

**Table 2.26 Preparation of BSA working reagents**

| | For each sample | X 20 |
|---|---|---|
| Test tube assay | | |
| BCA solution | 1 ml | 20 ml |
| 4% cupric sulfate | 20 µl | 400 l |
| Micro-scale assay | | |
| BCA solution | 200 µl | 4 ml |
| 4% cupric sulfate | 4 µl | 80 l |

### 2.9.2.2 Pipetting the assay

The following procedures describe two assay protocols. The test tube assay requires a larger volume of protein sample (50 µl).

However, the effect of interfering substances is minimized because the ratio of BCA working reagent to protein sample is 20:1.

The micro-scale assay requires less of the protein sample (25 µl) and offers the convenience of a 96-well plate.

However, because the ratio of BCA working reagent to protein sample is 8:1 the effect of interfering substances will be greater.

#### *2.9.2.2.1 Test tube assay*

Fifty µl of each standard or protein sample replicates were pipetted into labelled test tubes with the addition of 1.0 ml BCA working reagent and mixed by gentle vortexing. Tubes were incubated at 37 °C for 30 minutes and then allowed to cool to room temperature. Then, 1 ml water was added to a clean cuvette and the absorbance reading was adjusted at 562 nm to zero. The mix was transferred to clean cuvettes and the absorbance (A562) of all reactions was measured and recorded within 10 minutes. The corrected absorbances, were obtained by subtracting the absorbance of the blank standard from the absorbance measurement of all other standard and protein samples. To generate the standard curve, the corrected absorbances were plotted *versus* the known mass of the BSA standards. Using the standard curve, the recorded corrected absorbance reading for the samples assayed, falling within the linear range of the standard curve, were interpolated. The final amount of protein present in the original sample was calculated by correcting for the dilution and sample volume.

#### *2.9.2.2.2 Micro-scale assay*

Twenty-five µl of each standard or protein sample replicates were pipetted into individual wells of a 96 well plate with the addition of 200 µl BCA working reagent and mixed by gentle vortexing. Plate was incubated at 37 °C for 30 minutes and then allowed to cool to room temperature.

The absorbance reading was adjusted at 562 nm to zero. The corrected absorbances, were obtained by subtracting the absorbance of the blank standard from the absorbance measurement of all other standard and protein samples. To generate the standard curve, the corrected absorbances were plotted *versus* the known mass of the BSA standards.

Using the standard curve, the recorded corrected absorbance reading for the samples assayed, falling within the linear range of the standard curve, were interpolated. The final amount of protein present in the original sample was calculated by correcting for the dilution and sample volume.

## 2.10 Early and late apoptosis protein detection

### 2.10.1 Preparation of reagents for the immunoassay

The following description refers to the user manual of Millipore, 48-669MAG (early apoptosis kit) and Millipore, 48-670MAG (late apoptosis kit).

#### 2.10.1.1 Preparation of magnetic beads

Milliplex MAP magnetic beads were provided as a 20x stock solution. Before use, they were sonicated for 15 seconds and vortexed for 30 seconds. Beads were diluted 1x by combining 0.150 ml beads with 2.85 ml of Milliplex MAP Assay Buffer 2, vortexed for 15 seconds and transferred with a pipette into a reservoir before use.

#### 2.10.1.2 Preparation of biotin-labelled detection antibody and streptavidin-PE

Milliplex MAP detection antibody was provided as a 20x stock solution. Before use, it was vortexed for 10 seconds and centrifuged briefly after vortexing for complete recovery of contents. Detection Antibody was diluted to 1x by combining 0.150 ml

of Detection Antibody with 2.85 ml of Milliplex MAP Assay Buffer 2. The Milliplex MAP streptavidin-PE solution was vortexed for 10 seconds and diluted by combining 0.120 ml of streptavidin-PE with 2.88 ml of Milliplex MAP cell signalling assay buffer 2. The 1x biotinylated detection antibody and streptavidin-PE were transferred with a pipette to separate reservoirs before use.

#### 2.10.1.3 Preparation of lyophilised Milliplex MAP cell lysates

Milliplex MAP HeLa cell lysate: lambda phosphatase was provided as a lyophilized stock of cell lysate prepared from HeLa cells treated with lambda phosphatase and was used as an unstimulated control. Jurkat cell lysate: anisomycin was provided as a lyophilized stock of cell lysate prepared from Jurkat cells treated with 25 µM of anisomycin (4 hours). A549 cell lysate: camptothecin was provided as a lyophilized stock of cell lysate prepared from A549 cells stimulated with 5 µM of camptothecin (overnight). Jurkat cell lysate: paclitaxel was provided as a lyophilized stock of cell lysate prepared from Jurkat cells treated with 5 µM of paclitaxel (overnight). Each of the cell lysates were prepared in Milliplex MAP lysis buffer containing protease inhibitors and lyophilized for stability. All the reagents are listed in Table 2.27.

**Table 2.27 Reagents list for preparation of lyophilised Milliplex MAP cell lysates**

| Product | Company | Catalogue # |
|---|---|---|
| HeLa cell lysate: lambda phosphatase | Millipore | 47-229 |
| Jurkat cell lysate: anisomycin | Millipore | 47-207 |
| A549 cell lysate: camptothecin | Millipore | 47-218 |
| Jurkat cell lysate: paclitaxel | Millipore | 47-220 |

Each of the lyophilized cell lysates was reconstituted in 100 µl of ultrapure water (giving a total protein concentration of 2 mg/ml). The reconstituted lysates were vortexed and incubated for 5 minutes at room temperature (store on ice). After

incubation, 150 µl of Milliplex MAP assay buffer 2 were added to each cell lysate vial and vortexed. If aliquots were made, they were stored at -80 ˚C.

### 2.10.2 Immunoassay

Filtered lysates were diluted 1:1 in Milliplex MAP assay buffer. The suggested working range of protein concentration for the assay was 1 to 25 µg of total protein/well (25 l/well at 40 to 1,000 g/ml). Each well of the plate was filled with 50 µl of Assay Buffer. The plate was covered and put on a plate shaker (600-800 rpm) for 10 minutes at room temperature. The assay buffer was removed by inverting the plate and tapping it onto absorbent towels several times. The beads suspension was vortexed for 10 seconds before use, and 25 µl of 1x bead suspension were added to each well. Twenty-five µl of assay buffer were added to blank wells, reconstituted control cell lysates and sample lysates and the plate was put in incubation overnight (16 hours) at 4 ˚C on a plate shaker (600-800 rpm) protected from light. The following day, the plate was shacked for 10 minutes at room temperature. Samples and controls were decanted after the plate has been sitting on the handheld magnetic separation block for one minute. The plate was removed from the magnetic separation block and washed twice with 100 µl of assay buffer per well. After adding 25 µl/well of 1x Milliplex MAP detection antibody, the plated was sealed, covered with lid and incubated with agitation on a plate shaker for one hour at room temperature. Samples and controls were decanted after the plate has been sitting on the handheld magnetic separation block for one minute and 25 µl of 1x Millipore MAP streptavidin-PE were added. The plated was sealed, covered with lid and incubated with agitation on a plate shaker for 15 minutes at room temperature. Without removing streptavidin-PE, 25 µl of Milliplex MAP amplification buffer were added to each well. The plated was

sealed, covered with lid and incubated with agitation on a plate shaker for 15 minutes at room temperature. Streptavidin-PE and amplification buffer were decanted after the plate has been sitting on the handheld magnetic separation block for one minute. Before analysis on the Luminex system, beads were resuspended in 150 µl of Milliplex MAP assay buffer (per well) and mixed on plate shaker for 5 minutes.

## 2.11 Animal model

### 2.11.1 Lentiviral vector and lentivirus production

Lentiviral vector containing GFP Luciferase vectors was produced from transient calcium phosphate transfected 293Tx cells. Nine x $10^6$ 293Tx cells were seeded in a 25 cm culture dish using 15 ml of DMEM (1x) + GlutaMax media [Gibco, plus 10% FBS and 1% penicillin/streptomycin (P/S)] and incubated overnight at 37 ºC in a humidified incubator with a 5% $CO_2$ concentration. The following day, 9 µg pMD2.G (encoding VSV-G), 20 µg pCMV8.9 (for Gag/Pol) and 32 µg of GFP Luciferase vector plasmid were mixed and diluted to a total volume of 1,125 µl with $dH_2O$. Then 125 µl of $CaCL_2$ was added to the mixture and the mixture was incubated at room temperature for five minutes on a roller. Then 1.25 ml of 2x HEBS buffered saline 2X (sigma #51558) was added slowly while vortexing. Then the mixture was added slowly onto the cells and gently mixed for even distribution, followed by 16 hours incubation at 37 ºC in a humidified incubator with 5% $CO_2$. The medium was replaced with 15 ml fresh DMEM (1x) + GlutaMax and incubated for additional 24 hours. Cell culture supernatant containing the vector were collected at 48 hours post-transfection and left at 4 ºC overnight. Fifteen ml of fresh media were added to the cells and incubated overnight at 37 ºC in a humidified incubator with 5% $CO_2$. Next day Cell culture supernatant containing the vector were

collected and mixed up with the collection from the previous day. Vector supernatant was concentrated by centrifugation at 22,000 rpm for 2 hours at 4 ºC. Media was removed, and the pellet was resuspended in up to 0.2 ml of StemSpan (Stem Cell Technologies) and stored at -80 ºC.

### 2.11.2Lentiviral infection of regulatory T cells

One x $10^5$ Tregs were used for the production of GFP Luciferase transduced T regulatory cells. Prior transduction, Tregs were pre-activated by using CD3/CD28 beads in a 1:1 ratio in a media containing rapamycin and ATRA in a 24 well plate (for media composition, please refer to "Regulatory T cells expansion"). Cells were incubated overnight at 37 ºC in a humidified incubator with 5% $CO_2$. Next day, activation beads were removed by mixing the cells with a pipette and then incubating the cells on a magnet, followed by removing the supernatant that contained the Tregs. Following on, Tregs were infected once with lentiviral supernatant (multiplicity of infection of 1:30). After 24 hours, cells were washed with PBS (with 2% FCS and P/S) and fresh media (same solution of rapamycin and ATRA) was added (1 x $10^5$ cells/ml). Cells were incubated for up to 7 days (media replaced every 2 days). Luciferase-transduced Tregs were isolated based on their GFP expression using by using BD Aria. Non-transduced Tregs were used as a control from day 1.

### 2.11.3 Bioluminescence imaging

Animals anesthetized with isofluorane were imaged using the Xenogen IVIS imaging system for up to 55 min following D-luciferin (Caliper Life Sciences, Cambridge, UK) injections. D-luciferin was injected via the intra-peritoneal (150 mg/kg) route. Bioluminescence images were taken from dorsal side of the mice. The photons

emitted from luciferase expressing Tregs, expressed as Flux (photons/second/$cm^2$/steradian) were quantified and analysed using the "Living image" software (Caliper Life Science).

### 2.11.4Immunofluorescence of mouse bones

Harvested bones and other soft tissues from mice were recovered and fixed overnight in 10% neutral buffered formalin. Bones were then decalcified with Osteosoft (Millipore) for 7 days. All the other tissues were processed, paraffin embedded and then sectioned (5 μm) for histological studies. Following on, haematoxylin and eosin staining was performed to assess quality of the sections and to analyse the tissue sections under the microscope.

For immunofluorescence studies, heat antigen retrieval was performed. Primary unconjugated antibodies employed were specific for the following proteins: human CD45 and endomucin. Secondary fluorescent antibodies used in this assay were obtained from Invitrogen. Tissue images were taken using Zeiss Axio Scan.Z1 slice scanner using Zen blue edition software. Data from images was obtained using Fiji software equipped with both grids overlay and Cell Counter plugins.

### 2.11.5 Xenotransplantation

NOD/SCID/IL2rγ$^{-/-}$/IL-3/GM/SF (NSG-S) mice were a kind gift of Dr Leonard Shultz (The Jackson Laboratory). All animal experiments were performed in accordance with Home Office and CRICK guidelines. Up to 5 x $10^6$ PBMCs (with or without Tregs; 1:1 ratio) from healthy controls were injected via the intravenous route. Mice that were injected with PBMCs with Tregs received additional injections of Tregs after every two weeks until sacrifice. Human cell engraftment was

assessed at sacrifice (12-18 weeks) or at the time of death. Live cells were stained (hCD45, mCD45, hCD3, hCD19) and analyzed using Fortessa (BD Biosciences, Oxford, UK). All the animal experiments have been carried out by Dr Syed Mian.

# 3 DEEP PHENOTYPING OF TREG SUBSETS IN AA

## 3.1 Introduction

It has already been shown that in AA there is a reduction in the number and function of Tregs.[182, 185, 186, 352] Tregs from these patients also secrete pro-inflammatory cytokines.[185]

The correlation between this reduction in number and function and response to IST has not been studied, and still it is not clear if Tregs from AA patients are genuine Tregs or come from a "more Tconv-like" cell.[187]

IST with ATG and CsA is a very toxic treatment, for which not all patients are eligible, especially among the elderly.[206, 245, 353] Therefore, an immune signature able to predict the response to IST would be a very useful tool to select patients with the highest likelihood to respond to IST.[187]

In this chapter the differences between HDs and AA immune signature are described, as well as the immunological features (based on Tregs subsets and Tconv) able to predict the response to IST.[187]

## 3.2 Materials and methods

### 3.2.1 Patients and controls

PBMCs from 16 patients (12 IST responders and 4 non-responders) were used for initial CyTOF analysis; samples from another 15 patients (11 IST responders and 4 non-responders) were used as validation cohort.

Response to IST was evaluated 6 months post therapy. Median age was 45 years (range 20-72 years). IST-eligible AA patients were randomly invited to participate

in this study before commencement of therapy. All patients younger than 35 years of age were screened for Fanconi anemia.[187]

### 3.2.2 Mononuclear cell separation

Mononuclear cells were separated from peripheral blood by density gradient sedimentation as already described (page 114).

### 3.2.3 Surface and intracellular staining for mass cytometry analysis

AA and HDs PBMCs were stained according to the protocol already described in material and methods chapter (page 123). The antibodies used are listed in Table 3.1 and Table 3.2.

**Table 3.1 Antibody panel for mass cytometry staining (without any stimulation)**

| Channel | Target | Clone | Company | Catalogue # | Panel |
|---|---|---|---|---|---|
| 141 | CD3 | UCHT1 | BioLegend | 300443 | I |
| 142 | CD19 | HIB19 | Fluidigm | 3142001B | I |
| 143 | CD56 | HCD56 | BioLegend | 318345 | I |
| 144 | CD11B | ICRF44 | Fluidigm | 3144001B | I |
| 145 | CD4 | RPA-T4 | Fluidigm | 3145001B | I |
| 146 | CD196 (CCR6) | G034E3 | BioLegend | 353427 | I |
| 147 | CD20 | 2H7 | Fluidigm | 3147001B | I |
| 148 | CD16 | 3G8 | Fluidigm | 3148004B | I |
| 149 | CD194 (CCR4) | 205410 | Fluidigm | 3149003A | I |
| 150 | CD62L | DREG-56 | BioLegend | 304835 | I |
| 151 | CD123 | 6H6 | Fluidigm | 3151001B | I |
| 152 | CD103 | Ber-ACT8 | BioLegend | 350202 | I |
| 153 | CD45RA | HI100 | Fluidigm | 3153001B | I |
| 154 | CD45 | HI30 | Fluidigm | 3154001B | I |
| 156 | CD95 | DX2 | BioLegend | 305631 | I |
| 158 | CD161 | HP-3G10 | BioLegend | 339919 | I |
| 159 | CD154 | 24-31 | BioLegend | 310835 | I |
| 160 | CD28 | CD28.2 | Fluidigm | 3160003B | I |
| 162 | CD69 | FN50 | Fluidigm | 3162001B | I |
| 164 | CD45RO | UCHL1 | Fluidigm | 3164007B | I |
| 165 | CD152 | L3D10 | BioLegend | 349902 | I |
| 166 | CD33 | WM53 | BioLegend | 303419 | I |
| 166 | CD34 | 581 | Fluidigm | 3166012B | I |
| 166 | CD15 | W6D3 | BioLegend | 323035 | I |
| 167 | CD27 | O323 | Fluidigm | 3167002B | I |
| 168 | CD8 | SK1 | Fluidigm | 3168002B | I |
| 169 | CD25 | 2A3 | BioLegend | 3169003B | I |
| 169 | CD25 | M-A251 | BioLegend | 356102 | I |
| 170 | CD7 | CD7-6B7 | BioLegend | 343111 | I |

| | | | | | |
|---|---|---|---|---|---|
| 171 | Foxp3 | 150D | BioLegend | 320002 | I |
| 171 | Foxp3 | 259D | BioLegend | 320202 | I |
| 171 | Foxp3 | PCH101 | eBioscience | 14-4776-82 | I |
| 172 | CD183 (CXCR3) | G025H7 | BioLegend | 353733 | I |
| 174 | HLA-DR | L243 | Fluidigm | 3174001B | I |
| 175 | CD279 | EH12.2H7 | Fluidigm | 3175008B | I |
| 176 | CD127 | A019D5 | Fluidigm | 3176004B | I |

Table 3.2 Antibody panel for mass cytometry staining (staining after 4 hours stimulation with PMA/ionomycin)

| Channel | Target | Clone | Company | Catalogue # | Panel |
|---|---|---|---|---|---|
| 139 | CD39 | A1 | BioLegend | 328221 | II |
| 141 | CD3 | UCHT1 | BioLegend | 300443 | II |
| 142 | CD19 | HIB19 | Fluidigm | 3142001B | II |
| 143 | CD56 | HCD56 | BioLegend | 318345 | II |
| 144 | CD11b | ICRF44 | Fluidigm | 3144001B | II |
| 145 | CD4 | RPA-T4 | Fluidigm | 3145001B | II |
| 147 | CD20 | 2H7 | Fluidigm | 3147001B | II |
| 148 | IL-4 | MP4-25D2 | BioLegend | 500829 | II |
| 149 | CD194 (CCR4) | 205410 | Fluidigm | 3149003A | II |
| 150 | CD62L | DREG-56 | BioLegend | 304835 | II |
| 151 | CD123 | 6H6 | Fluidigm | 3151001B | II |
| 152 | TNF-α | MAb11 | Fluidigm | 3152002B | II |
| 153 | CD45RA | HI100 | Fluidigm | 3153001B | II |
| 154 | CD45 | HI30 | Fluidigm | 3154001B | II |
| 156 | IL-6 | MQ2-13A5 | Fluidigm | 3156011B | II |
| 158 | IL-2 | MQ1-17H12 | Fluidigm | 3158007B | II |
| 159 | CD154 | 24-31 | BioLegend | 310835 | II |
| 160 | Tbet | 4B10 | Fluidigm | 3160010B | II |
| 162 | CD69 | FN50 | Fluidigm | 3162001B | II |
| 164 | IL-17A | N49-653 | Fluidigm | 3164002B | II |
| 165 | IFN-γ | B27 | Fluidigm | 3165002B | II |
| 166 | CD33 | WM53 | BioLegend | 303419 | II |
| 166 | CD34 | 581 | Fluidigm | 3166012B | II |
| 166 | CD15 | W6D3 | BioLegend | 323035 | II |
| 167 | GATA3 | TWAJ | Fluidigm | 3167007A | II |
| 168 | CD8 | SK1 | Fluidigm | 3168002B | II |
| 169 | CD25 | 2A3 | BioLegend | 3169003B | II |
| 169 | CD25 | M-A251 | BioLegend | 356102 | II |
| 170 | IL-10 | JES3-9D7 | BioLegend | 501423 | II |
| 171 | Foxp3 | 150D | BioLegend | 320002 | II |
| 171 | Foxp3 | 259D | BioLegend | 320202 | II |
| 171 | Foxp3 | PCH101 | eBioscience | 14-4776-82 | II |
| 172 | CD38 | HIT2 | Fluidigm | 3172007B | II |
| 174 | HLA-DR | L243 | Fluidigm | 3174001B | II |
| 175 | CD279 | EH12.2H7 | Fluidigm | 3175008B | II |
| 176 | CD127 | A019D5 | Fluidigm | 3176004B | II |

### 3.2.4 Surface and intracellular staining for conventional flow cytometry (validating cohort)

AA and HDs PBMCs were stained according to the protocol already described in material and methods chapter (page 118). The antibodies used for this panel are listed in Table 3.3.

**Table 3.3 Antibody panel for flow cytometry validating cohort**

| Product | Company | Catalogue # |
|---|---|---|
| L/D eFluor 780 | eBioscience | 65-0865 |
| CD4 PerCP Cy5.5 | BioLegend | 3000530 |
| CD25 PE | BioLegend | 356104 |
| CD127 | eBioscience | 25-1278-42 |
| CD45RA | FITC | 304106 |
| CD95 BV 421 | BioLegend | 305624 |
| CCR4 BV510 | BioLegend | 359416 |
| Foxp3 Pe Cy5 | eBioscience | 15-5773-82 |

## 3.3 Results

### 3.3.1 Identification of an immune signature for aplastic anaemia based on regulatory T cell subsets

Thirty-one AA patients at diagnosis and five HDs were analysed. The first 16 patients were part of an initial test cohort, and the other 15 were part of a validation cohort.

Metal-tagged antibodies against surface and intracellular markers were used in two separate panels (with or without stimulation with PMA/ionomycin) to stain T cells, including known markers for Tregs (CD25, CD127, Foxp3), naïve/memory subsets, homing/trafficking receptors, and differentiation/activation markers (the antibody panels are summarised in Table 3.1 and in Table 3.2).

$CD4^+$ and $CD8^+$ T cells and B cells were clustered using a "whole panel" clustering approach to minimize bias. $Foxp3^+$ cells were also clustered within $CD4^+$ T cells (Figure 3.1 and Figure 3.2).

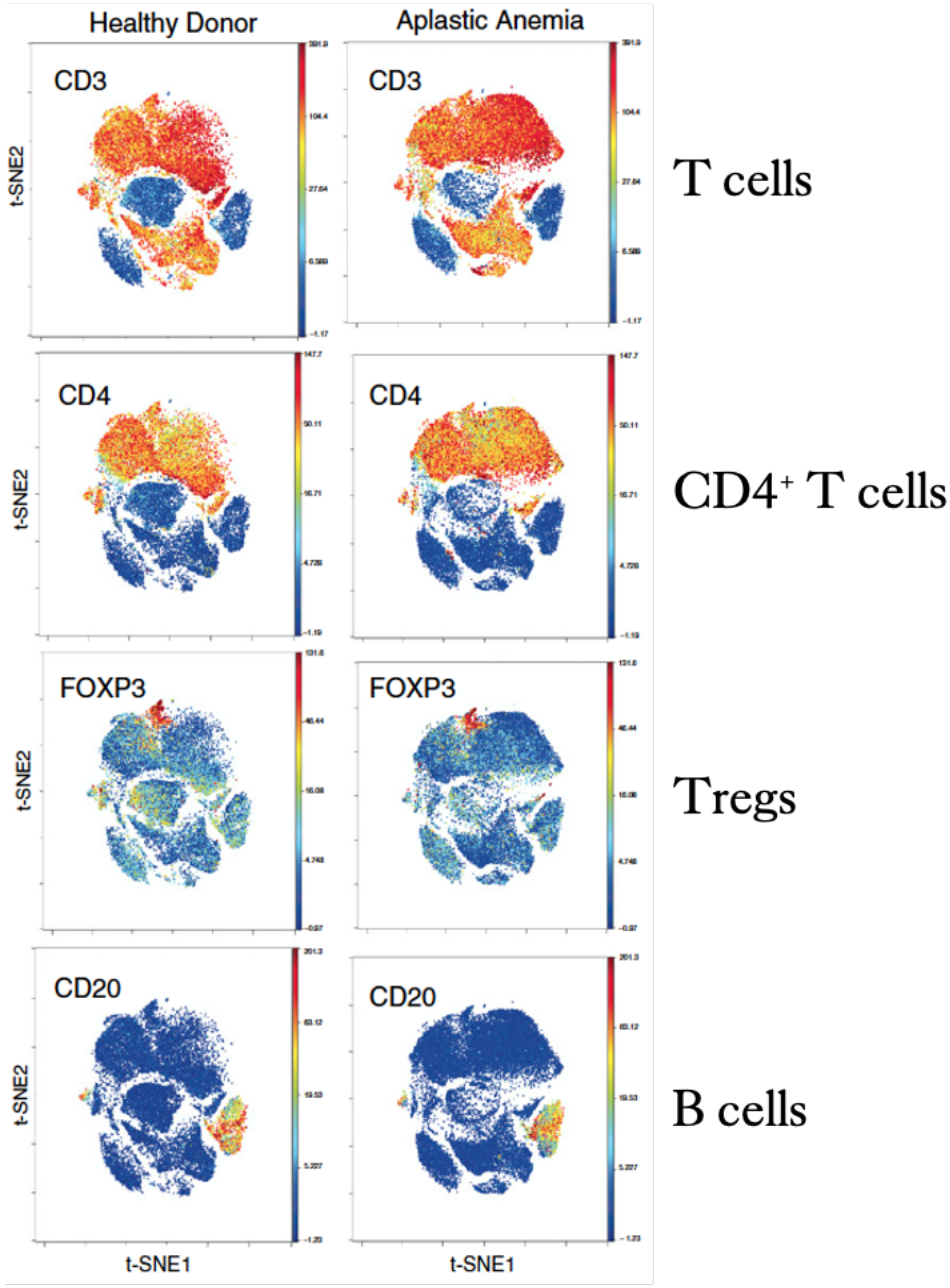



**Figure 3.1 PBMCs staining and clustering (I)**

PBMCs from aplastic anaemia and healthy donors were clustered using 34 surface and intracellular markers according to panel I (Table 3.1). Intact cells were gated based on Iridium-191 and event length, followed by Iridium-191 and Iridium-193 gating. Viable cells were selected based on CD45 expression and negativity for Rhodium. All FCS files were first normalized using control beads and analysed using Cytobank web-based software. $CD4^+$ and $CD8^+$ T cells and B cells clustered together in both aplastic anaemiaand healthy donors. Figure is representative of 16 aplastic anaemia and 5 healthy donors.[187]

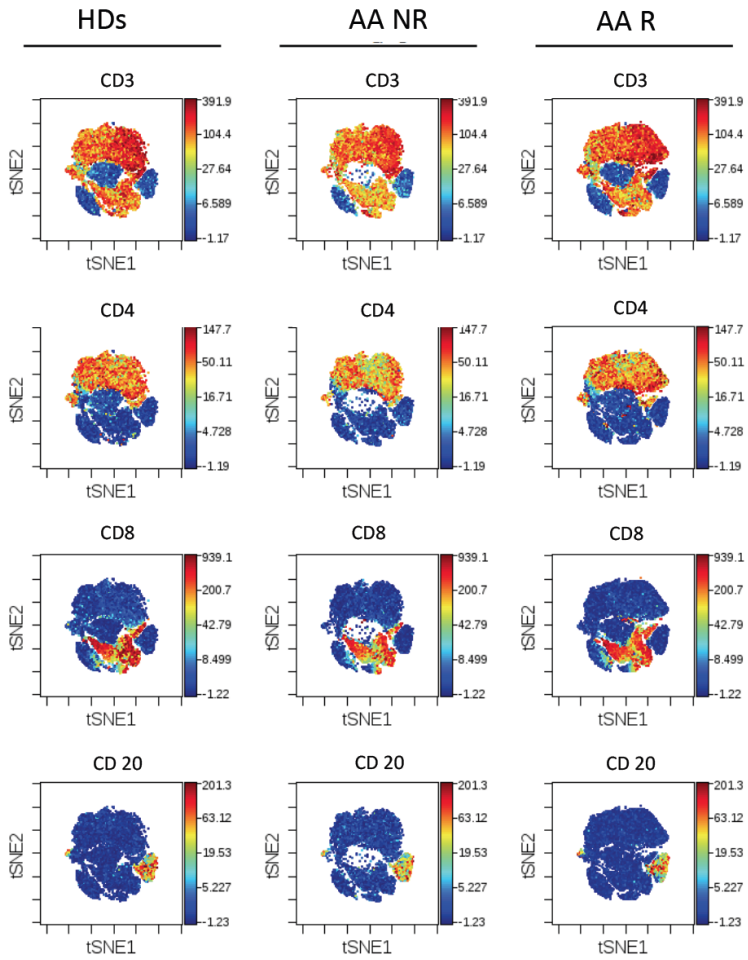



**Figure 3.2 PBMCs staining and clustering (II)**

PBMCs from aplastic anaemia and healthy donors were stained with panel I (Table 3.1) and data acquired using CyTOF 2. Acquired data were normalised based on normalisation beads (Ce-140, Eu-151, Eu-153, Ho-165 and Lu-175). Both $CD4^+$ and $CD8^+$ T cells and B cells were clustered distinctively in aplastic anaemia and healthy donors.[187]

After gating for $CD3^+$, $CD4^+$, and $CD8^-$, and merging all samples, viSNE was performed, and cells were clustered based on 13 markers that most clearly identified Tregs (Figure 3.3).

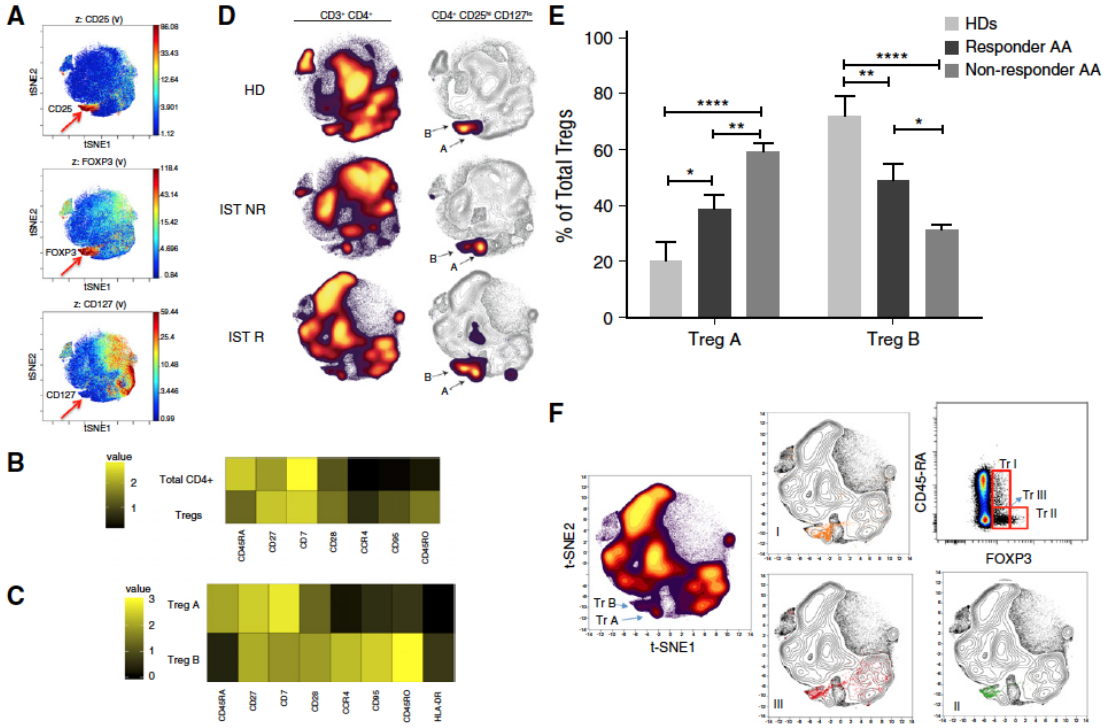



**Figure 3.3 Identification of Treg subsets by automated clustering**

(A) After initial gating for $CD3^+$, $CD4^+$, and $CD8^-$ T cells, the gated cells were clustered using viSNE. Treg populations were identified based on high expression of CD25 and Foxp3 and low expression of CD127. (B) Median expression of the seven most discriminative parameters between total $CD4^+$ cells and Tregs as identified by the automated clustering algorithm FLOCK on a subset of 700,000 cells proportionally selected from all samples. Tregs were defined as clusters whose median expression was simultaneously higher than the 90% quantile of Foxp3 expression, higher than the 90% quantile of CD25 expression, and lower than the 50% quantile of CD127 expression across all $CD3^+CD8^-$ cells. Heat map plot is based on 19 AA samples (pre- and post-IST) and 5 HDs. (C) Median expression of the eight most discriminative parameters between the two Treg subpopulations identified by the automated clustering algorithm FLOCK. Expression values were transformed using the asinh function in a cofactor of 5. Heat map plot is based on 19 AA samples (pre- and post-IST) and 5 HDs. (D) The density plot of viSNE plots revealed 2 subpopulations within Tregs, designated as Treg A and B (arrows). The frequencies of Treg A and B were different between HDs and AA patients. Patients who did not respond to IST had a higher number of Treg A at the time of diagnosis compared with responder patients and HDs. The viSNE plots (right) are an overlay of Tregs' contour plots coloured by density and $CD4^+$ T cells uncoloured contour plots. (E) At the time of diagnosis and before treatment, Treg A frequency was higher in responders as well as non-responders compared with HDs (38.8% ± 5% and 63.5% ± 4.5% vs 20.3% ± 6.6%, $p = 0.05$, $p = 0.0001$), whereas the frequency of Treg B was lower in both responders and non-responders AA patients at the time of diagnosis compared with HDs (48.8% ± 6.1% and 28.9% ± 2.7% *versus* 72.2% ± 6.7%, $p = 0.005$, $p = 0.0001$). The non-responders, however, had significantly higher Treg A and lower Treg B compared with responders (63.5% ± 4.5% *versus* 38.8% ± 5.0, $p = 0.005$ for Treg A; 28.9% ± 2.7% *versus* 48.8% ± 6.1%, $p = 0.05$ for Treg B). Error bars are standard error of mean. Kruskal-Wallis 1-way analysis of variance test was used for statistical analysis. **** $p = 0.0001$, *** $p = .001$, ** $p = 0.01$, * $p = 0.05$. (F) The overlap between the Treg subpopulations identified using viSNE and manually gated Treg populations based on CD45RA and Foxp3 expression. Although subpopulations A and B mainly overlap with subpopulations I ($CD45RA^{high}Foxp3^{low}$) and II ($CD45RA^{low}Foxp3^{high}$) respectively, subpopulation III ($CD45RA^{low}Foxp3^{low}$) was spread over population B as well as outside the Treg area. Figures are overlays of manually gated Tregs on viSNE plots of total $CD4^+$ T cells from an IST responder.[187]

Treg subpopulations were clustered and identified by high expression of CD25 and Foxp3 and low expression of CD127 (Figure 3.3 A). Identified Tregs expressed $CD27^{high}$, $CD45RA^{low}$, $CD45RO^{high}$, $CD95^{high}$, $CD7^{low}$, $CD28^{high}$, and $CCR4^{high}$

compared with the total $CD4^+$ T cell subpopulation (Figure 3.3). The frequency of total Tregs was significantly lower in AA patients compared with HDs (2.7% *versus* 5.7% of $CD4^+$ T cells, $p = 0.01$), confirming our previously published findings.[185] The number of Tregs was not significantly different between patients with SAA, VSAA, and NSAA.

Although viSNE clustered the Treg population in one area, density plots revealed a heterogeneous distribution of cells. Two subpopulations within Tregs with a different frequency between AA and HDs were identified and designated as Treg A and Treg B (Figure 3.3 C-D).

To confirm the presence of these two subpopulations and eliminate any bias, dimensionality reduction and automated unsupervised clustering methodology was applied independently by a bioinformatician; this confirmed the presence of two subpopulations within Tregs (Figure 3.3 C). These two subpopulations showed distinct markers in AA Tregs (16 patients, 12 IST responders and 4 non-responders) as well as 5 HDs.

Although both subpopulations were $CD25^{high}$, $Foxp3^{high}$, and $CD127^{low}$ compared with total $CD4^+$ T cells, subpopulation B was additionally characterized by a lower expression of CD45RA ($p = 0.0001$), CD7 ($p = 0.001$), and CD27 ($p = 0.05$) and a higher expression of CCR4 ($p = 0.0001$), CCR6 ($p = 0.0001$), CD25 ($p = 0.0001$), CD28 ($p = 0.01$), CD45RO ($p = 0.0001$), CD95 ($p = 0.0001$), CXCR3 ($p = 0.05$), Foxp3 ($p = 0.0001$), and HLA-DR ($p = 0.0001$) compared with subpopulation A (Kruskal-Wallis 1-way analysis of variance by ranks).

In addition to total Tregs, the percentage of Tregs B was significantly lower in AA patients compared with HDs (40.8% ± 13.7% *versus* 72.2% ± 15%, $p = 0.008$).[187]

### 3.3.2 Regulatory T cells composition at diagnosis predicts response to immunosuppressive therapy

When patients were stratified into IST responders (n = 12) and non-responders (n = 4), although the overall frequency of Treg B was lower in both responders and non-responders at the time of diagnosis compared with HDs (n = 5) (48.8% ± 6.1% and 28.9% ± 2.7% *versus* 72.2% ± 6.7%, $p = 0.005$, $p = 0.0001$), non-responders had significantly higher Treg A and lower Treg B compared with responders (63.5% ± 4.5 *versus* 38.8% ± 5.0%, $p = 0.005$ for Treg A, 28.9% ± 2.7 *versus* 48.8% ± 6.1, $p = 0.05$ for Treg B) (Figure 3.3 E and Table 3.4).[187]

**Table 3.4 Treg A and B markers and summary of changes in AA**

| Treg subset | Markers | HDs | IST-responder pre-treatment | IST-non-responder pre-treatment | IST-responder post-treatment | IST-non-responder post-treatment |
|---|---|---|---|---|---|---|
| Treg A | CD45RA↑, CD7↑, CD27↑, CCR4↓, CCR6↓, CD25↓*, CD28↓, CD45RO↓, CD95↓, CXCR3↓, Foxp3↓, HLA-DR↓ | Minor population 20.3% ± 6.6 | 38.8% ± 5.0 | 63.5% ± 4.5 | 19.26% ± 2.43 | NSC 73.9% ± 6.8 |
| Treg A (TNF-$\alpha^+$) | TNF-α↑ IL-10↓, CD279↓, HLA-DR↓, CD38↓ | R 1.2% ± 0.3 | NSC 0.75% ± 0.14 | NSC 0.79% ± 0.13 | 0.32% ± 0.12 | NSC 1.81% ± 0.19 |
| Treg B | CD45RA↓, CD7↓, CD27↓, CCR4↑, CCR6↑, CD25↑**, CD28↑, CD45RO↑, CD95↑, CXCR3↑, Foxp3↑, HLA-DR↑ | Major population 72.2% ± 6.7 | 48.8% ± 6.1 | 28.9% ± 2.7 | NSC 59.98% ± 3.18 | NSC 21.8% ± 4.3 |

NSC: no significant change, R: reference value, * lower expression compared to Treg B, ** higher expression compared to Treg A – The comparison is pre- *versus* post-treatment

To investigate the overlap between our Treg subsets with the ones identified by Miyara *et al.*,[354] Tregs were gated based on CD45RA and Foxp3 expression. Although subpopulations A and B mainly overlapped with subpopulations I ($CD45RA^{high}Foxp3^{low}$), and II ($CD45RA^{low}Foxp3^{high}$) respectively, subpopulation

III ($CD45RA^{low}$, $Foxp3^{low}$) was spread over both population A and B, and some cells clustered outside the Treg area (Figure 3.3).[187]

Following IST response, the frequency of population A was significantly reduced in responders (from 38.8% ± 5.0% to 19.2% ± 2.4, *p = 0.01*), but not in non-responders. Treg B frequency was significantly higher in responders compared with non-responders (59.9% ± 3.4% *versus* 21.8% ± 4.3, *p = 0.0001*) and closer to HDs (Table 3.4). Treg A or B were not significantly different between patients with SAA/VSAA (n = 11) and NSAA (n = 5).[187]

The cytokine profile of Tregs following stimulation with PMA/ ionomycin, and brefeldin A was investigated. Treg subpopulations A and B were identified within Tregs (Figure 3.4).

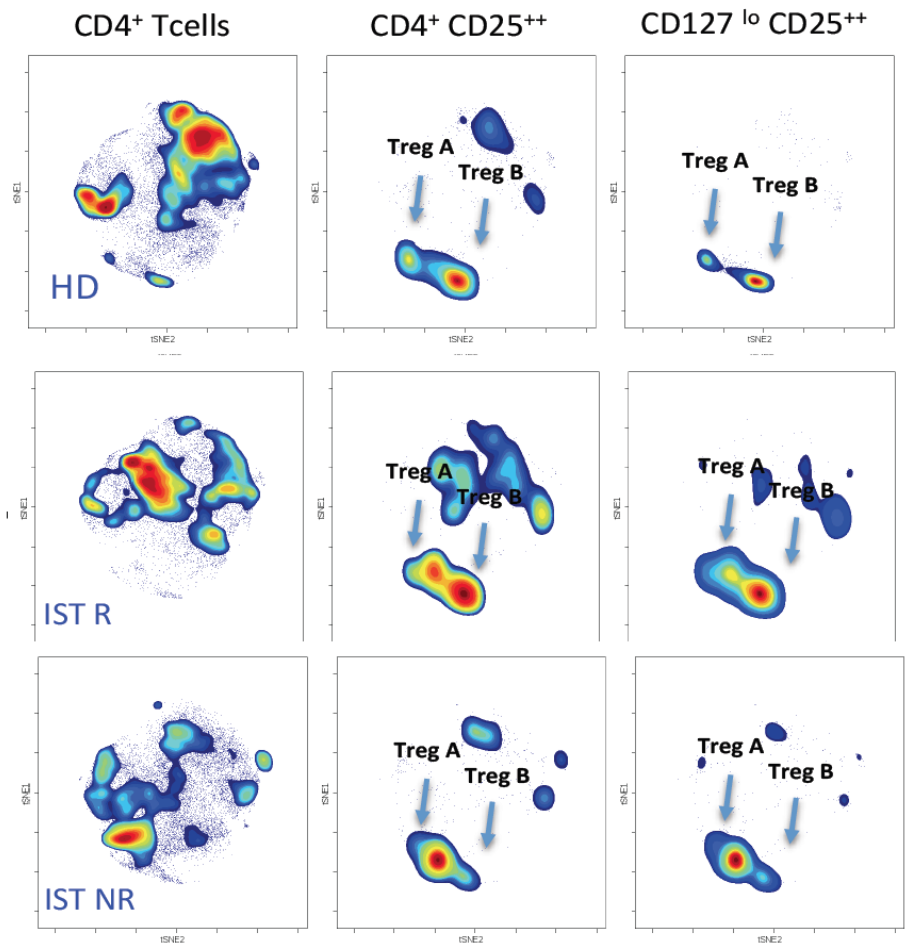



**Figure 3.4 viSNE analysis of PMA/ionomycin stimulated CD4⁺ cells**

This analysis revealed similar subsets of Tregs (A and B) as unstimulated CD4⁺ T cells in both aplastic anaemia patients and healthy donors. To make the two populations more visible, a "gating filter" was applied based on high expression of CD25 (middle plots) and low expression of CD127 (right plots).[187]

Stimulated Treg clusters expressed higher CD25 ($p = 0.001$) and Foxp3 ($p = 0.001$) and lower CD127 ($p = 0.001$) compared with total CD4⁺ T cells. The majority of CD4⁺ T cells with pro-inflammatory cytokine properties clustered outside the "Treg area"; thus, Treg clusters expressed negligible amounts of pro-inflammatory cytokines IFN-γ, and IL-17 (Figure 3.5 A). Tregs expressed significantly higher IL-10 compared with total CD4⁺ T cells ($p = 0.002$); however, the IL-10 expression was not significantly different between the two Treg subsets. Although TNF-α-expressing cells were clustered within non-Treg subpopulations, a cluster of CD4⁺ TNF-α T cells clustered within the Treg A subpopulation (Figure 3.5 B).[187]

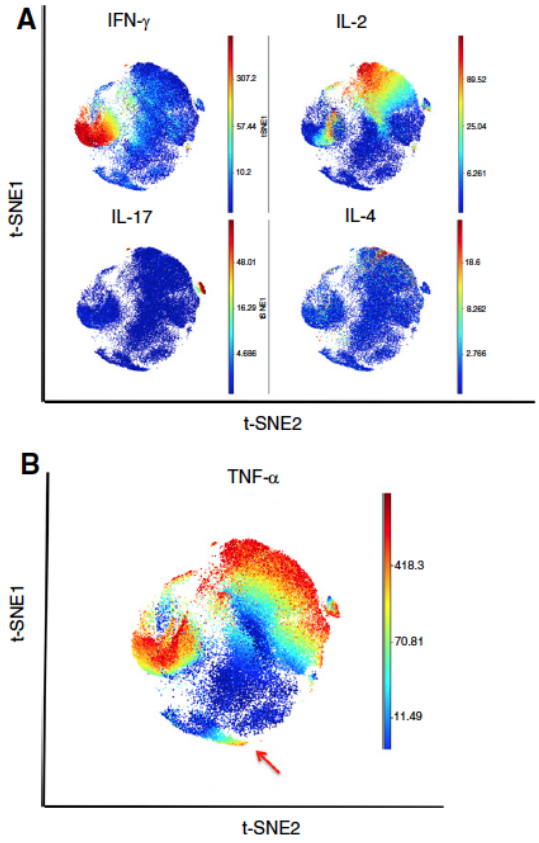



**Figure 3.5 PMA/ionomycin-stimulated Treg subsets (I)**

(A) Following four hours stimulation with PMA/ionomycin in the presence of brefeldin A, PBMCs were stained for surface and intracellular markers (Table 3.2) followed by mass cytometry and viSNE on $CD4^+$ T cells. Cytokine-secreting $CD4^+$ T cells, including IFN-γ, IL-$2^+$, IL $17^+$, and IL-$4^+$, localized distinctly with minimal overlap and outside the "Tregs area." Both Treg A and Treg B show higher expression of IL-10 compared with "non-Tregs" and TNF-α-secreting Tregs, but there was no significant difference between Treg A and B in terms of IL-10 expression. (B) Unlike the rest of cytokine-secreting $CD4^+$ T cells, TNF-α-secreting cells were spread over several areas, including the Treg A subpopulation (red arrow).[187]

These TNF-$\alpha^+$ cells expressed significantly higher CD127 ($p = 0.001$), and lower IL-10 ($p = 0.001$), CD279 ($p = 0.001$), HLA-DR ($p = 0.003$), CD38 ($p = 0.001$), CD25 ($p = 0.001$), and Foxp3 ($p = 0.001$) compared with total Tregs. Although at the time of diagnosis the frequency of TNF-$\alpha^+$ cells was not significantly different between IST responders and non-responders, IST responders had significantly lower TNF-$\alpha^+$ cells compared with non-responders following response to IST (0.32% *versus* 1.81%, $p = 0.008$) (Figure 3.6).[187]

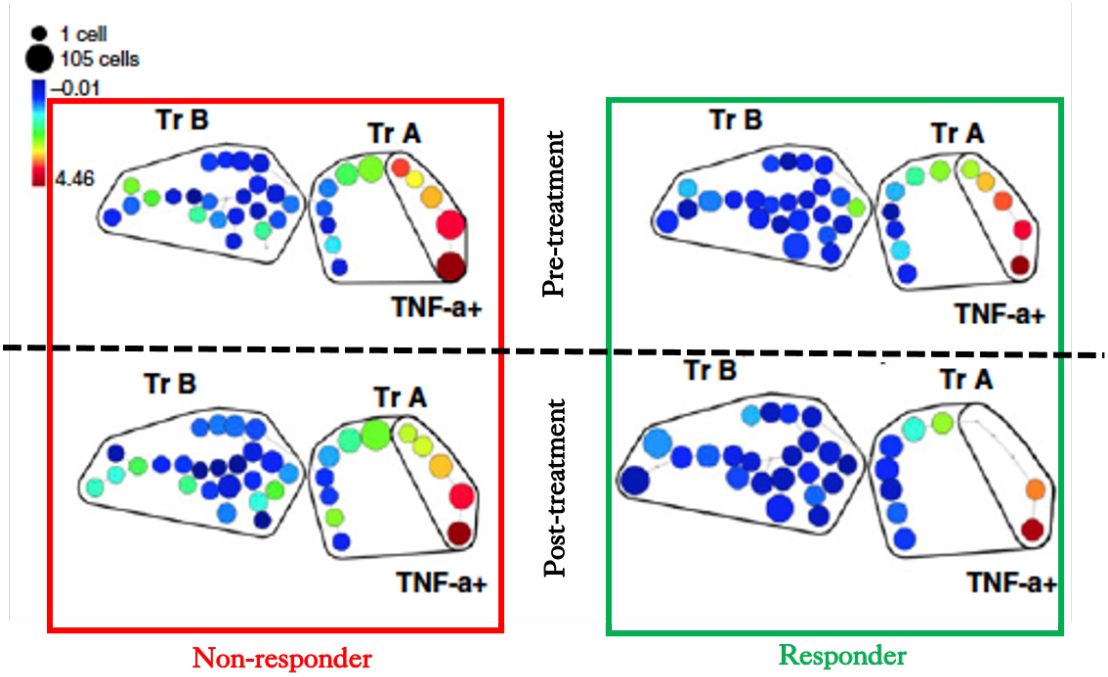



**Figure 3.6 PMA/ionomycin-stimulated Treg subsets in aplastic anaemia patients (II)**

SPADE analysis of Treg A and B following four hours stimulation with PMA/ionomycin and intracellular staining (Table 3.2). Although the TNF-α-secreting cells within the Treg A subpopulation reduce after immunosuppressive treatment in responder aplastic anaemia patients, there is no similar reduction in non-responders.[187]

### 3.3.3 Validation by conventional flow cytometry

Conventional flow cytometry was performed on peripheral blood from a separate validation cohort of 15 AA patients (11 IST responders and 4 non-responders) at the time of diagnosis to confirm that the identified markers were sufficient to detect Treg subpopulations and whether the CyTOF-identified combination of markers could still be predictive for IST response. PBMCs were stained with anti-CD4, CD25, CD127, Foxp3, CD95, CCR4, and CD45RA and, similar to the initial cohort, Treg A were significantly higher in non-responders compared with responders (37.82% ± 5.11 *versus* 18.33 ± 2.31, $p = 0.006$), whereas Treg B were significantly higher in responders (69.97% ± 3.02 *versus* 49.34% ± 4.92, $p = 0.01$) (Figure 3.7).

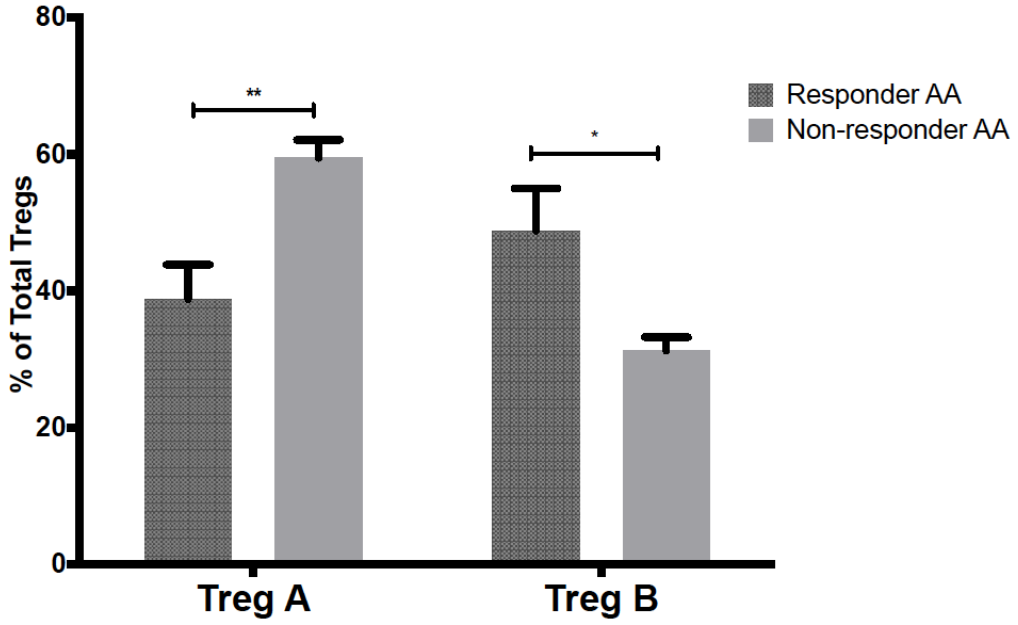



**Figure 3.7 Differences in Tregs subsets (A and B) between responders and non-responders in the validation cohort evaluated by conventional flow cytometry**

Significant difference between responders to immunosuppressive treatment (n = 11) and non-responder to immunosuppressive treatment (n = 4) patients (validation cohort) regarding Treg population A and B, using conventional flow cytometry. PBMCs from 15 aplastic anaemia patients were stained with anti-CD4, CD25, CD127, Foxp3, CD95, CCR4 and CD45RA and analysed by polychromatic flow cytometry. viSNE plot of the acquired data revealed similar Treg subpopulations A and B. Similar to the initial cohort, Treg A were significantly higher in non-responders compared to responders (37.82% ± 5.11 *versus* 18.33 ± 2.31, $p = 0.006$) whereas Treg B were significantly higher in responders (69.97% ± 3.02 *versus* 49.34% ± 4.92, $p = 0.01$). Error bars are standard error of mean. Kruskal-Wallis 1-way analysis of variance test was used for statistical analysis.[187]

## 3.3.4 Conventional CD4⁺ T cells

Our marker panels also identified and clustered Tconv. The Treg population was first gated out on a viSNE plot. Then a SPADE clustering based on CD45RA, CD45RO, CD27, and CD62L was performed. Tconv subpopulations were defined as naïve ($CD45RA^{+}CD45RO^{-}CD27^{high}$), memory ($CD45RA^{-}CD45RO^{+}CD27^{low}$), central memory ($CD45RA^{-}CD45RO^{+}CD27^{low}CD62L^{high}$), effector memory ($CD45RA^{-}CD45RO^{+}CD27^{low}CD62L^{low}$), effector ($CD45RA^{-}CD45RO^{+}$ $CD27^{low}$), and terminal effectors ($CD45RA^{+}CD45RO^{-}CD27^{low}$). Subset frequencies were not significantly different between IST responders (n = 12) and non-responders (n = 4) at the time of diagnosis. However, effector $CD4^{+}$ T cells expressed a significantly

higher CD161 level in non-responders compared with responders ($p = 0.01$) (Figure 3.8).[187]

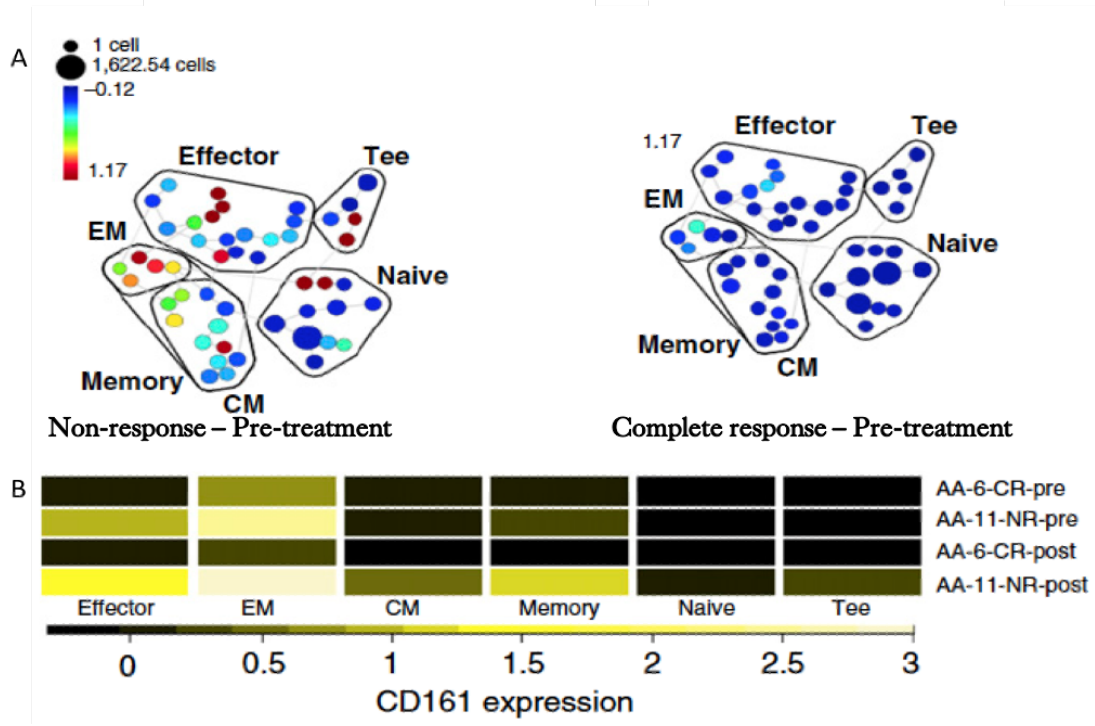



**Figure 3.8 PMA/ionomycin Tconv subpopulations in aplastic anaemia patients**

SPADE clustering based on CD45RA, CD45RO, CD27, and CD62L. The Tconv subpopulations were defined as naïve ($CD45RA^+CD45RO^-CD27^{high}$), memory ($CD45RA^-CD45RO^+CD27^{low}$), central memory ($CD45RA^-CD45RO^+CD27^{low}CD62L^{high}$), effector memory ($CD45RA^-CD45RO^+CD27^{low}CD62L^{low}$), effector ($CD45RA^-CD45RO^+CD27^{low}$), and terminal effectors ($CD45RA^+CD45RO^-CD27^{low}$). Tconv with effector phenotype expresses higher CD161 in non-responder patients at the time of diagnosis compared with responder aplastic anaemia patients. The frequencies of these subpopulations were not significantly different between IST responder and IST non-responder patients at the time of diagnosis. The naïve Tconv from IST non-responder patients were expressing slightly higher CCR4 compared with responder patients.[187]

## 3.4 Summary of data

Although it is already known that Tregs play an important role in the pathophysiology of autoimmune diseases, the definition and significance of Treg subsets is less clear. Their identification is challenging in autoimmune diseases, as generally the number of Tregs is usually low and they may express aberrant markers; in addition, gating strategies for Treg subpopulations are often subjective. Biomarkers able to distinguish AA patients from HDs and able to identify, at the

time of diagnosis, patients less likely to respond to IST, have not been identified yet.[187]

With a relatively new technology (CyTOF), it is now possible to extensively characterize rare, heterogeneous populations of cells with minimal bias.[342, 355] CyTOF allows to measure the expression level of more than forty parameters at a single cell level.[355-357] The complexity of Treg subsets in HDs has been previously demonstrated by mass cytometry;[358] however, their biological importance has not been investigated.

In the current project, by using multidimensional phenotyping and unbiased approach, two distinct Treg subpopulations were characterized in HDs and AA patients and the changes in these subsets were able to predict response to IST.[187]

The analytical strategy used, eliminated the unavoidable subjectivity of Treg subpopulation definition based on two-dimensional gating. Within the $CD25^{high}CD127^{low}Foxp3^{high}$ Tregs, AA Tregs expressed $CD27^{high}CD45RA^{low}CD45RO^{high}CD95^{high}CD7^{low}CD28^{high}CCR4^{high}$ compared with the total $CD4^{+}$ T cell. It was possible to identify two well-defined subpopulations within this Treg population (Treg A and B). Although total Treg numbers were reduced in AA, Treg A was significantly higher in AA patients compared with HDs. In contrast, the number of Treg B subpopulation was significantly lower in AA patients compared with HDs (Table 3.4). Subpopulation B was characterized by a lower expression of CD45RA, CD7, and CD27 and a higher expression of CCR4, CCR6, CD25, CD28, CD45RO, CD95, CXCR3, Foxp3, and HLA-DR. The most significantly different markers were CD95, CCR4, and CD45RO.[263, 359, 360] The identified Treg subpopulations were compared with

established Treg subpopulation definitions.[354] Although Tregs A and B overlap with Treg subpopulations I and II respectively, this approach is able to highlight those Treg III closer to Tregs, and to combine them with subset A or B based on their phenotype. This eliminates cells that are closer to Tconv, therefore less likely to be regulatory.[187]

There is an unmet need for more robust predictive factors for response to IST.[187] Known predictive factors for response to IST include less severe disease, young age, and absolute reticulocyte and lymphocyte counts of ≥ 25 and ≥ 1.0 x $10^9$/l, respectively.[361] Short telomeres in children, but not in adults, also predict response to IST.[174, 362] The presence of a PIGA mutation is predictive for response to IST, as it is a somatic BCOR/BCORL mutation. In contrast, somatic mutation of DNMT3A or ASXL1 is associated with worse outcomes following IST.[180] This immune signature identified by our group can predict response to IST at time of AA diagnosis. Non-responders to IST seem more likely to have higher Treg A number compared with non-responders, whereas responders had higher Treg B number compared with HDs (Figure 3.9). Treg B are characterized by a more "activated/memory" phenotype.[187]

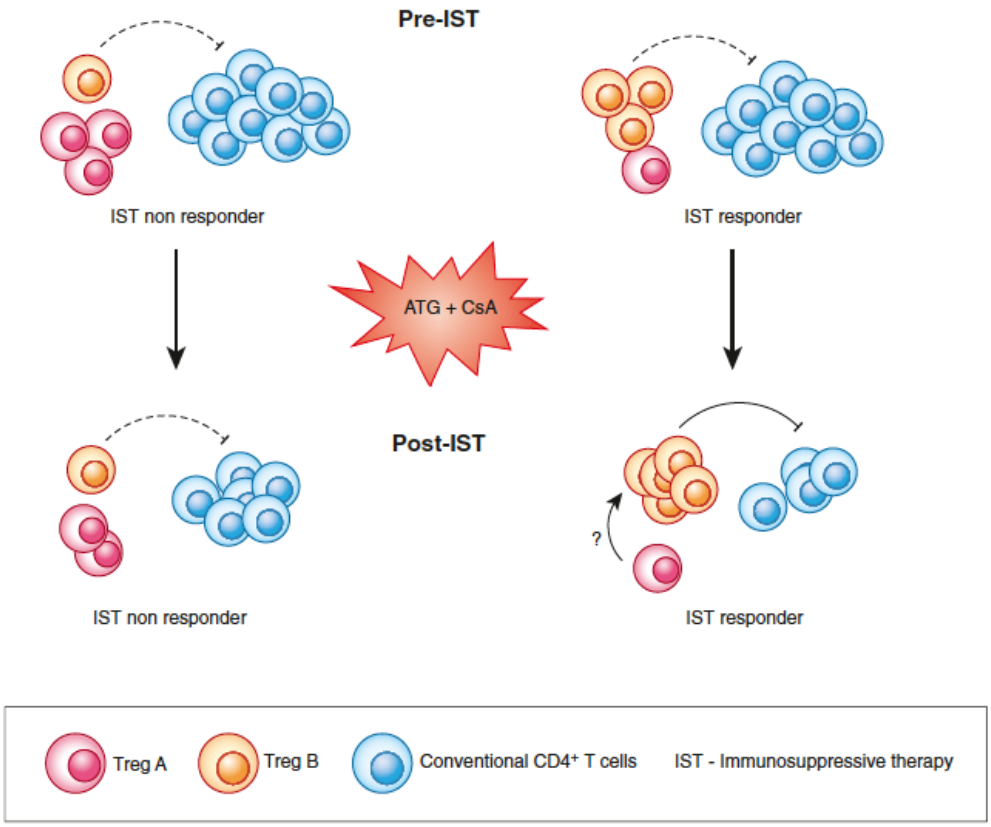



**Figure 3.9 Hypothesised mechanism of aplastic anaemia pathophysiology based on Treg subsets: visual summary**

Aplastic anaemia patients with higher number of Treg B before immunosuppressive treatment, are more likely to respond to therapy. Following response to immunosuppressive treatment, responder patients have a higher number of Treg B cells compared with non-responders. Treg B are enriched with cell cycle-related proteins and are, probably, more likely to enter the cell cycle compared to Treg A.[187]

This specific immune signature, identified by high-resolution mass cytometry, could also be determined by the use of conventional flow cytometry, and confirmed the predictive value for response to IST.[187] Considering the ability of the Treg subsets composition to predict response to IST, and the role of Tregs in AA pathophysiology, we wanted to explore the potential therapeutic application of expanded Tregs for AA. This will be discussed in the next chapter.

# 4 FUNCTION, ONTOGENY AND APOPTOSIS PROPENSITY OF REGULATORY T CELL SUBSETS

## 4.1 Introduction

Treatment options for idiopathic AA include allogeneic hematopoietic stem cell transplantation or IST, and recently eltrombopag for refractory SAA.[206, 245, 353] Some patients are ineligible for hematopoietic stem cell transplantation because of older age or lack of a suitable donor. IST itself could be a very toxic treatment, especially among the elderly, and after IST one-third of patients fail to respond, and 35% relapse after responding. Up to 20% of patients transform to myelodysplastic syndrome or acute myeloid leukaemia after IST.[167, 206, 214, 215, 363] Therefore, additional novel therapeutic approaches are needed for AA patients who fail to respond (or relapse) to IST.[187]

As already mentioned, Tregs have been proven to play an important role in AA pathophysiology.[185, 187, 364] It has already been shown in a mouse model, that aberrant FAS and FAS-L expression on lymphocytes is able to attenuate induced BMF syndrome.[365] Our hypothesis is that Tregs may be lower in number (especially Treg B) because they have already gone through apoptosis at the moment of disease presentation. We also hypothesise that, due to the pro inflammatory bone marrow environment, there is a relative lack of IL-2, which contributes to Tregs death. Moreover, IL-2 has been shown to act as an anti-apoptotic cytokine by inducing the expression of Bcl-2 and Bcl-XL (Figure 4.1).[366]

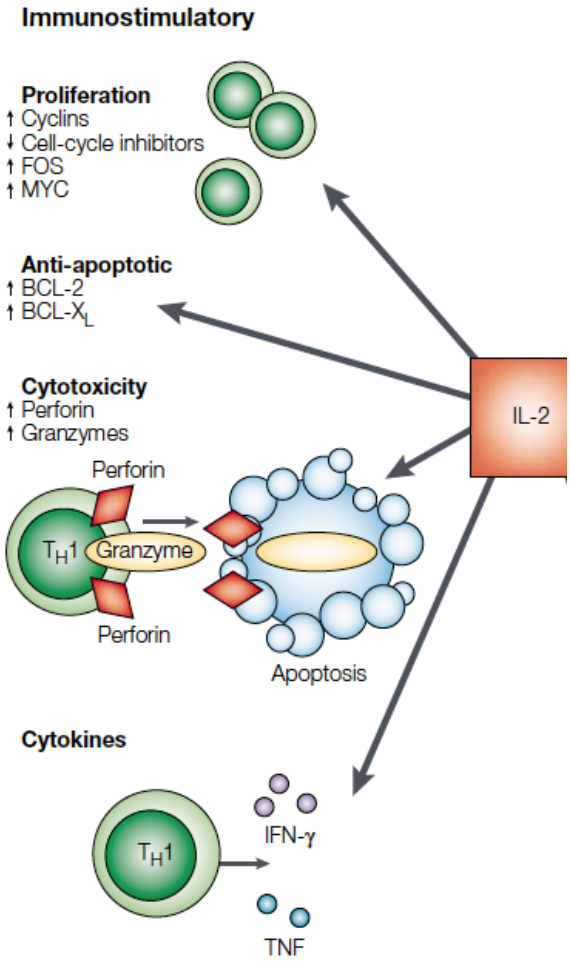



**Figure 4.1 Immunostimulatory effects of IL-2**

IL-2 is well known for its ability to promote proliferation, to inhibit apoptosis and to induce cytokines. Its main, non-redundant *in vivo* function is to limit lymphoid expansion and promote peripheral tolerance.[366]

Considering all these aspects, we wanted to investigate Tregs function, ontogeny and apoptosis propensity in order to test their expandability in "Treg-friendly" conditions with high dose IL-2, rapamycin and ATRA (described in chapter 5).[367] Tregs function, ontogeny and apoptosis propensity are described in this chapter.

## 4.2 Materials and methods

### 4.2.1 Pro-inflammatory cytokine measurement

Cytokines were measured from cells culture supernatant harvested as described before (page 120). The ProcartaPlex immunoassay has been performed as already described (page 124).

## 4.2.2 Treg A and B sorting

Peripheral blood mononuclear cells were enriched for $CD4^+$ T cells as described (page 116). The $CD4^+$ enriched fraction was stained for CD4, CD25, CD127, CD45RA, CD95 and CCR4 and sorted on a FACS Aria 1 at the BRC Flow Core Facility at Guy's Hospital.

Sorting efficiency was always > 95% and purity (calculated only if cell number > 100,000) of the sorted fractions was always > 98%. All the reagents are listed in Table 4.1.

**Table 4.1 Reagents list for Treg A and B sorting**

| Product | Company | Catalog # |
|---|---|---|
| MACS BSA Stock Solution | Miltenyi Biotec | 130-091-376 |
| MACS Separation Buffer | Miltenyi Biotec | 130-091-221 |
| CD4+ T Cell Isolation Kit | Miltenyi Biotec | 130-096-533 |
| PBS w/o $Ca^{++}/Mg^{++}$ | Sigma Aldrich | D8537 |
| RPMI 1640 | ThermoFisher Scientific | 12633-012 |
| Foetal calf serum | ThermoFisher Scientific | 10500-064 |
| DAPI | BioLegend | 422801 |
| CD4 PerCp Cy5.5 | BioLegend | 317428 |
| CD25 PE | BioLegend | 356104 |
| CD127 Pe Cy7 | eBioscience | 25-1278-42 |
| CD45RA FITC | BioLegend | 304106 |
| CD95 APC | BioLegend | 305612 |
| CCR4 BV510 | BioLegend | 359416 |

## 4.2.3 T cell receptor sequencing

To evaluate the clonality of Treg A and B we have sent genomic DNA to Adaptive Biotechnologies (Seattle, U.S.). The procedure has already been described in the material and methods section (page 141).

## 4.2.4 Gene expression profile

For the gene expression profile data, RNA was extracted following a "hybrid" method (page 143). The RNA was processed, and the data analysed as already described in the general materials and methods section (pages 143-145).

### 4.2.5 Mapping protein interaction networks

We have further analysed the gene expression profile (GEP) data from Treg A and B and mapped the protein interaction networks of the proteins encoded by the mRNA that are enriched in the Treg B subpopulation. We mapped Treg B proteins onto high-confidence human soluble complexes[368] which were verified by a combination of cutting-edge biochemical fraction mass spectrometric profiling and several computational analyses such as Bayesian probabilistic functional network[369] and conservative clustering algorithm.[370] Firstly, proteins directly interacting with Treg B are identified, and secondly the complexes involving Treg B proteins were classified. In studies of human primary lymphocytes and $CD34^+$ cells, it had already been shown that quiescent (G0) cells do not contain many proteins required for cell proliferation (e.g. DNA synthesis and mitosis) or molecules that regulate the cell cycle.[371-373] Each of these proteins is synthesised *de novo* as cells progress through G1 for the first time.[374] Entry into the cell cycle from quiescence requires cells to progress through the G0 → G1 commitment point, and many of these proteins are synthesized post commitment.[372]

### 4.2.6 Apoptosis induction and annexin V staining

As Treg B express higher levels of cell cycle genes, and are defined as $CD95^+$ (FAS), we investigated the apoptosis propensity of these Treg subsets (due to low cells numbers, apoptosis was induced on $CD4^+$ T cells, and Treg A and B discrimination was evaluated afterwards by conventional flow cytometry). $CD4^+$ T cells were enriched as already described (page 116). The enriched $CD4^+$ fraction was resuspended at 0.4 x $10^6$/200 µl and cultured with and without anti-human FAS (5 µg/ml) for five hours. Then cells were harvested and washed once with FACS buffer

(PBS with 2.5% FCS and 0.1% $NaN_3$). Cells were stained according to the manufacturer instructions. To summarise: the surface staining was performed first, followed by the Fixable Viability Dye (as describe at page 119). After the live/dead staining, cells were resuspended in 1x binding buffer and annexin V antibody was added. After 15 minutes at room temperature, samples were washed with 1x binding buffer and analysed on a Canto II (BD Biosciences). All the reagents are listed in Table 4.2.

**Table 4.2 Reagents list for apoptosis induction and annexin V staining**

| Product | Company | Catalogue # |
|---|---|---|
| PBS w/o $Ca^{++}/Mg^{++}$ | Sigma Aldrich | D8537 |
| Foetal calf serum | ThermoFisher Scientific | 10500-064 |
| CD4+ T Cell Isolation Kit, human | Miltenyi Biotec | 130-096-533 |
| MACS BSA Stock Solution | Miltenyi Biotec | 130-091-376 |
| MACS Separation Buffer | Miltenyi Biotec | 130-091-221 |
| LS columns | Miltenyi Biotec | 130-042-401 |
| L/D eFluor 780 | eBioscience | 65-0865 |
| PRIME-XV T Cell Expansion XSFM | Irvine Scientific | 91141 |
| AB human serum | Sigma-Aldrich | H4522 |
| CD4 PerCP Cy5.5 | BioLegend | 300530 |
| CD25 PE | BioLegend | 356104 |
| CD127 Pe Cy7 | eBioscience | 25-1278-42 |
| CD45RA FITC | BioLegend | 304106 |
| CD95 BV421 | Biolegend | 305624 |
| CCR4 BV510 | BioLegend | 359416 |
| Annexin V Apoptosis Detection Kit APC | eBioscience | 88-8007-74 |
| Anti-Fas (human, activating) | Millipore | 05-201 |

### 4.2.7 Apoptosis prevention with low-dose IL-2

Treg were magnetically enriched from two HDs as already described (page 117). Once enriched, Tregs were cultured with anti-human FAS, with or without low-dose IL-2 (10 IU/ml) for five hours. Tregs were then stained as described in "Apoptosis induction and annexin V staining" (page 179), using the same antibody panel. Cells were analysed on a Fortessa (BD Biosciences) and the data were analysed with FlowJo (Tree Star).

### 4.2.8 RNA sequencing

RNA sequencing was conducted at GENEWIZ, LLC (South Plainfield, NJ, USA) as already described (page 145) and data were analysed as reported in 2.8.4.

## 4.3 Results

### 4.3.1 Functionality of regulatory T cell subsets

To assess the function of the Treg subsets, HDs $CD4^+$ Tregs were sorted based on CD25, CD127, and CD95 expression, which showed the highest differences between subpopulations A and B on viSNE clusters, excluding intracellular markers to avoid fixation and permeabilization of the cells. Sorted cells were $CD4^+CD25^{high}CD127^{low}CD95^{low}CD45RA^{high}CCR4^{low}$ (subpopulation A) and $CD4^+CD25^{high}CD127^{low}CD95^{high}CD45RA^{low}CCR4^{high}$ (subpopulation B). To confirm these markers were enough to identify Treg subpopulations, viSNE runs were performed based on these markers and both subpopulations were identified (Figure 4.2).

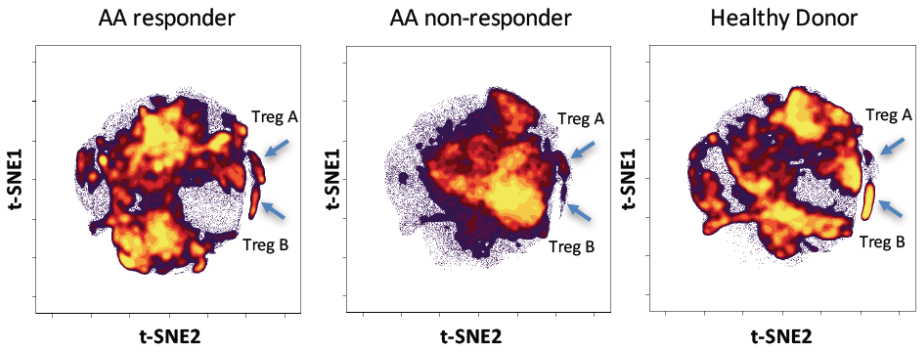



**Figure 4.2 viSNE plots from aplastic anaemia and healthy donors**

These viSNE plots are based on CD4, CD25, CD127, CD95, CD45RA, CCR4. The minimum 6 markers were enough to reasonably distinguish Tregs populations in healthy donors as well as AA patients. viSNE is a visualization tool for high-dimensional single-cell data based on the t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm. viSNE finds a 2D representation of single cell data that best preserves its local and global geometry. The resulting viSNE map provides a visual representation of the single-cell data that is like a biaxial plot, with the positions of cells reflecting their proximity in high-dimensional space.[187]

Treg B cells were significantly more functional compared with those of subpopulation A in suppression of both IFN-γ and TNF-α secretion by Tconv ($p = 0.05$) (Figure 4.3).

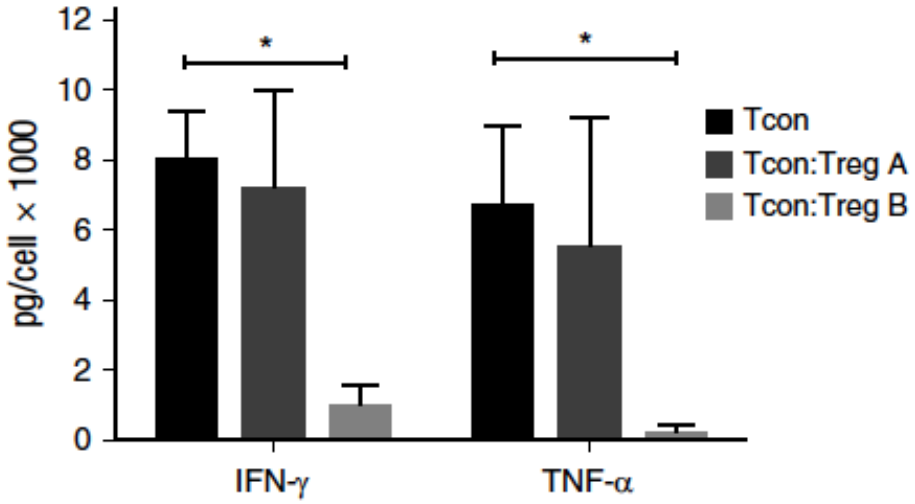



**Figure 4.3 Functionality of Tregs subsets: inhibition of pro-inflammatory cytokine secretion By Tregs subsets (A and B)**

Suppression of Tconv cytokine secretion by Treg subpopulations: $CD4^{+}CD25^{high}CD127^{low}CD452RA^{high}CD95^{low}CCR4^{low}$ (subpopulation A), $CD4^{+}CD25^{high}CD127^{low}CD45RA^{low}CD95^{high}CCR4^{high}$ (subpopulation B) and $CD4^{+}CD25^{low}$ Tconv were sorted (Facs Aria) and cultured for five days with anti CD3/CD28 beads (Tconv:Treg:bead = 20:20:1). After five days of culture, the supernatant was analysed with ProcartaPlex (eBioscience) according to the manufacturer's instructions. The cytokine concentrations were corrected for the cell number. Treg B cells were able to significantly reduce both IFN-γ and TNF-α secretion by Tconv ($p = 0.05$). Average of three replicates, Student t test, * $p = 0.05$. Error bars are standard error of mean.[187]

## 4.3.2 Clonality of Treg A and B

Genomic DNA from sorted Treg A and B was used for TCR Vβ chain CDR3 high-throughput sequencing.[375] On average, subpopulations A and B had 33,773 and 26,090 unique TCR sequences, respectively, and 233 sequences were common to both ($r = 0.008$) (Figure 4.4).[187]

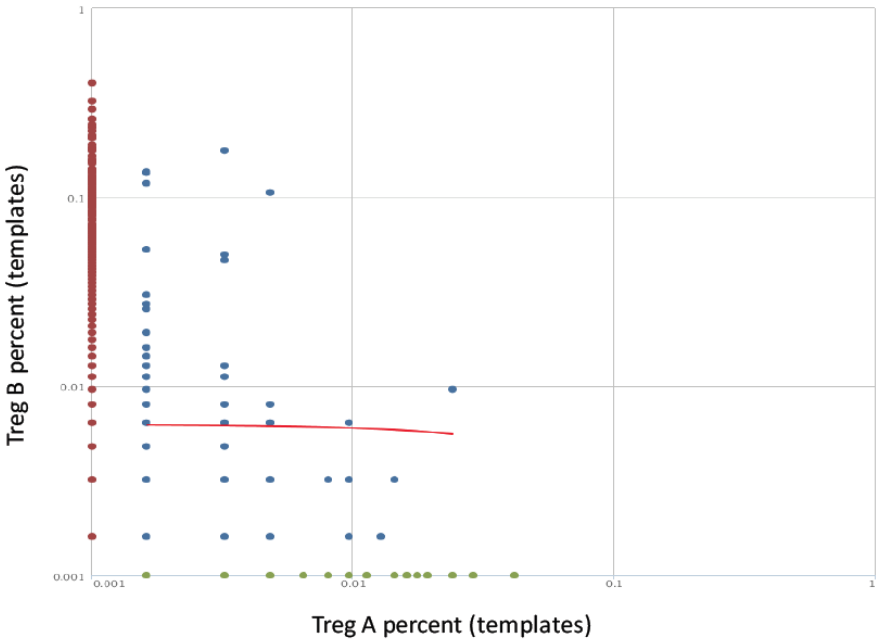



**Figure 4.4 Overlap of TCR Vβ sequences between Treg A and B**

Genomic DNA from sorted Treg A and B was used for TCR Vβ chain CDR3 high-throughput sequencing. On average, subpopulation A and B had 33,773 and 26,090 unique TCR sequences respectively and 233 sequences were common to both (*r = 0.008*). Figure is generated using immunoSEQ online software.[187]

Tconv also shared 137 and 383 sequences with subpopulations A and B, respectively (*r = 0.154* and *r = 0.092*, respectively). In assessing a naturally, highly diverse TCR subpopulations, under-sampling could introduce bias.[376, 377] We therefore used PG index for pairwise comparison of TCR repertoire overlap.[376] Overlap between Treg A, B, and Tconv was small, suggesting these subpopulations were distinct (Figure 4.5).[187]

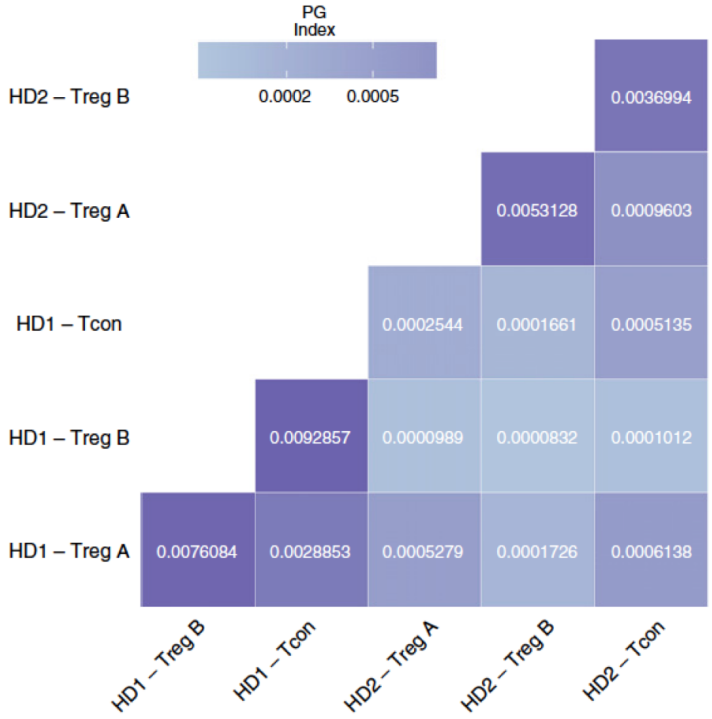



**Figure 4.5 Clonality of Treg A and B: T cell receptor sequences overlap between Treg subsets**

Pairwise comparison of TCR repertoire overlaps. The colour shading reflects the numerical value of the PG indices. The TCR sequences shared between Tconv, Treg A, and Treg B were very low with a PG index < 0.001 in all comparisons. The PG index (power-geometric) is a general geometric index parametrized by two nonnegative parameters, and therefore able to put weight on rare (or abundant) receptors in a more flexible way.[187]

### 4.3.3 Gene expression profile analysis of Treg subpopulations and protein interaction

Whole GEP data showed both Treg A and Treg B had different GEP compared with Tconv.[378, 379] Nevertheless, when principal component analysis was performed, Treg B and Tconv showed the most striking differences, whereas the Treg A subpopulation showed a transcriptional profile in between Treg B and Tconv (Figure 4.6).[187]

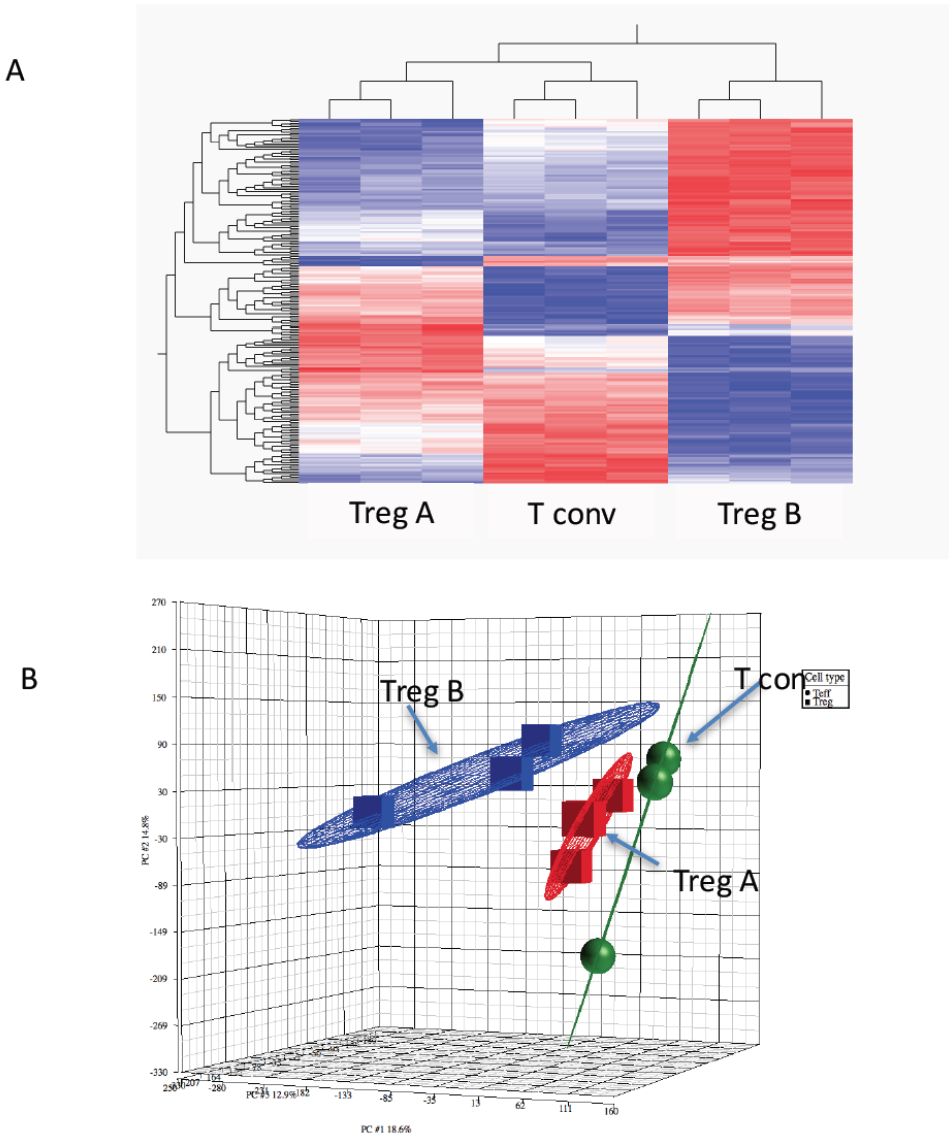



**Figure 4.6 Gene expression profile analysis of regulatory and conventional T cells**

We have compared the global GEP of Treg A, B and Tconv to identify gene signatures related to these populations in comparison to Tconv. When we compered the unbiased whole GEP data, all three populations showed different gene expression signatures (A). However, principal component analysis showed that Treg B and Tconv are the most distant populations and Treg A subpopulation is placed between the other two (B). Median expression of 3 HDs sorted Treg A, Treg B and Tconv.[187]

The comparison of Treg subsets and Tconv GEP, using the human Treg gene signature,[380] showed that both Treg A and B subpopulations were significantly enriched in genes up-regulated in human Tregs, including *IL-2RA*, *FOXP3*, *IKZF2*, *TIGIT*, and *CTLA4* [false discovery rate (FDR) < *0.0001*) for both Treg subpopulations compared with Tconv. Treg B were enriched with Treg-related

memory/activation genes compared with Treg A, including *JAKMIP1*, *CCR8*, *TRIB1*, and *GZMK* (*FDR < 0.0001*) (Figure 4.7).[187]

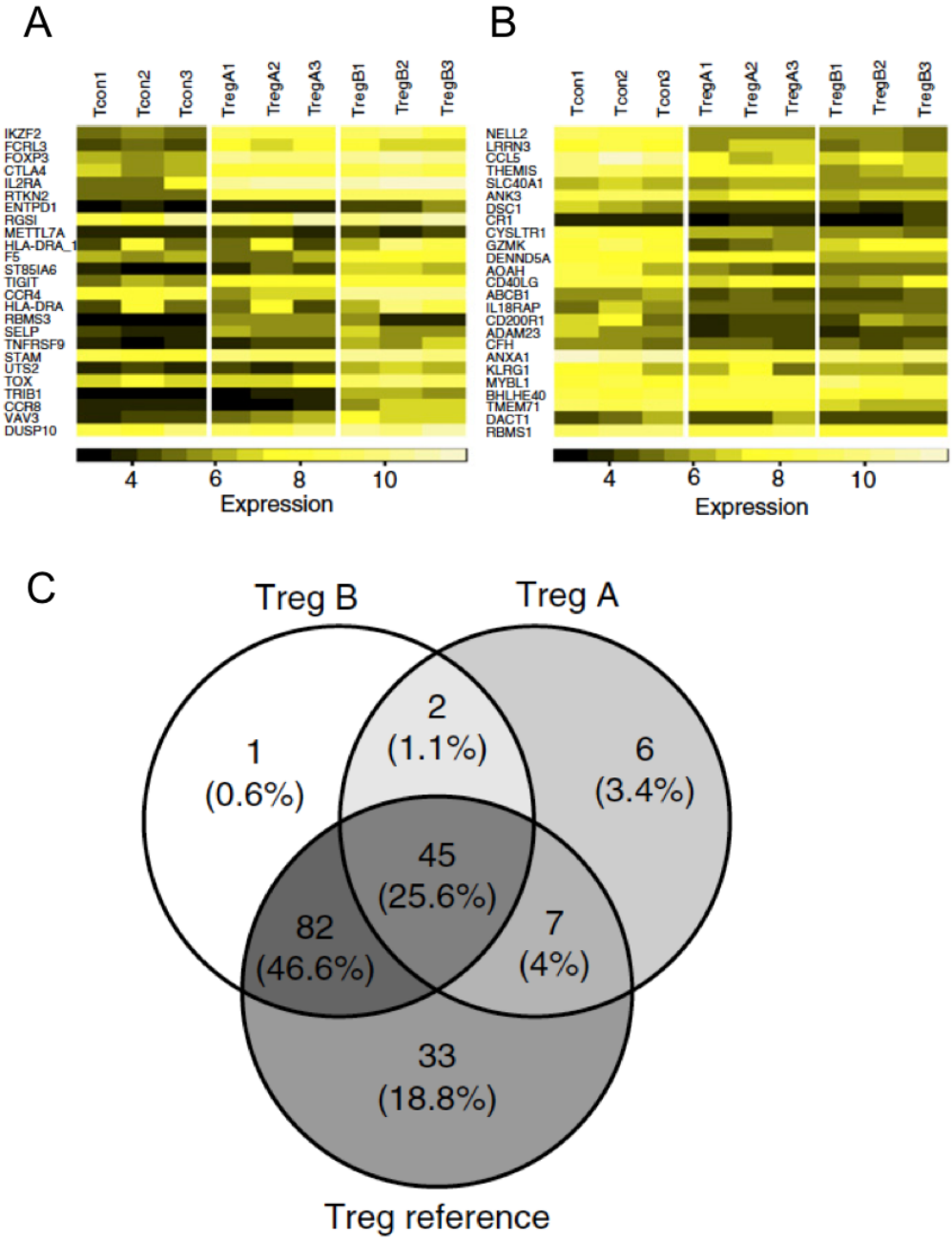



**Figure 4.7 Gene expression profile analysis of regulatory T cell subsets and conventional T cells**

For the gene expression profile of Treg A, B and Tconv, all three T cell populations were compared with the Ferraro list.[380] (A-B) Both Treg A and Treg B were enriched with Treg-related genes, in particular *IKZF2*, *FCRL3*, *FOXP3*, *CTLA4*, and *IL-2RA*. However, the genes that are expressed at a lower level in human Tregs were enriched in Tconv, but in none of the Treg subpopulations. (C) The frequency of common genes between Treg A, Treg B, and referenced published genes[380] are demonstrated, and includes genes that are highly expressed by human Tregs.[187]

Thus, although both Treg subpopulations were enriched with Treg-associated genes, Treg B were characterized by an activation gene signature, in agreement with mass cytometry findings.[187]

Gene set enrichment functional analysis[346] highlighted several gene sets as significantly overexpressed in Treg B, including G2M checkpoint (*FDR < 0.0001*), mitosis (*FDR = 0.015*), M phase of mitotic cell cycle (*FDR = 0.018*), IL-2-STAT5 signalling (*FDR = 0.023*), and immune response genes (*FDR = 0.032*) (Table 4.3).[187]

**Table 4.3 Genes that are up regulated in Treg B compared with Treg A**

| Genes | FDR q value | Normalised enrichment score | Significant genes |
|---|---|---|---|
| G2M checkpoint | < 0.0001 | 2.0188198 | *CASC5, NUSAP1, CENPE, TPX2, TOP2A, KIF11, BARD1, EZH2, SLC7A5, HN1, GINS2, CKS2, BUB1, SMC4, STIL, BRCA2, CHEK1, SAP30, E2F3, MKI67, CDKN2C, NEK2, KIF15, KPNA2, KIF23, ZAK, POLQ, WHSC1, TFDP1, UCK2, E2F2, CDKN3, CDC7, E2F1, AURKA, CDC20, EFNA5, KIF4A, EXO1, CDC25A, PRC1, KIF5B* |
| Mitosis | 0.014934987 | 2.020 | *NCAPH, NUSAP1, NDC80, CENPE, BUB1B, TPX2, KIF11, TTN, BUB1, NEK2, SMC4, PCBP4, PAM* |
| M phase of mitotic cell cycle | 0.01755665 | 2.0006573 | *NCAPH, NUSAP1, NDC80, CENPE, BUB1B, TPX2, KIF11, TTN, BUB1, NEK2, SMC4, PCBP4, PAM* |
| IL-2-STAT5 signalling | 0.02301426 | 1.5519865 | *SYT11, CCR4, TNFRSF9, CST7, ADAM19, IL1R2, ANXA4, PHLDA1, SLC1A5, IL18R1, FGL2, TNFRSF18, TNFRSF4, GALM, CXCL10, BATF, SPP1, TNFSF10, PHTF2, CD86, AHNAK, IL10, LIF, TLR7, F2RL2, CD79B, CTLA4, FURIN, TNFRSF1B, CAPG, ALCAM, CSF1, CASP3, CSF2, MUC1, MYO1E RORA, ITGAV, PRNP, ICOS, UCK2, CYFIP1, SOCS1* |
| Immune response genes | 0.031976275 | 1.9183398 | *IL7, CCR2, CCR8, CCR4, IL1R2, CCR6, CST7, CCL20, GZMA, CADM1, AIM2, CIITA, CCR5, CTSC, IL12A, NCF4, IL4, CCR9, GEM, IL32, TNFRSF4, LAX1, DEFB4A, FCGR3B, TLR7, CD74, APOA4, CCL5, APOBEC3G, CD79B, CTLA4* |

Protein interaction networks of proteins encoded by messenger RNA that were enriched in Treg B were also mapped (Figure 4.8).[187]

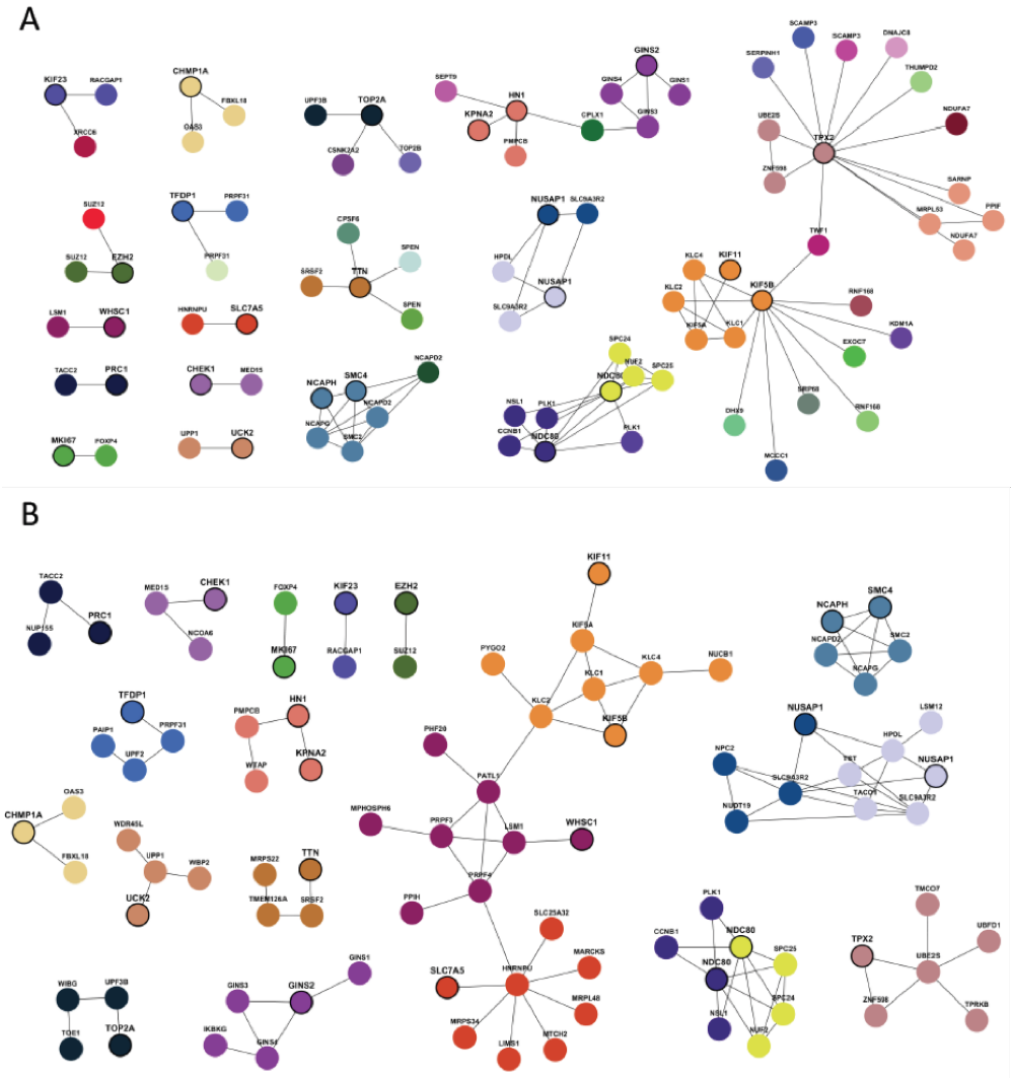



**Figure 4.8 Complexes of Treg B genes among the Human Soluble Protein Complex**

Treg B genes are mapped to Human Soluble Complexes[368] using Cytoscape[381] and 24 proteins of the genes are in the dataset. Proteins are nodes or circles, and their interactions are edges. The nodes are coloured by the complexes that are co-fractionated human soluble protein complexes.[368] (A) Proteins of Treg B genes and their direct interacting proteins [Treg B genes (black border nodes) and their 1st neighbours in Human Soluble Protein complexes]. (B) Protein complexes involving proteins of Treg B genes. [187]

Ontology analyses of the functions of these protein complexes (Table 4.4 and Figure 4.9) showed they are involved in mitotic functions, DNA replication, and cell cycle-dependent transcription.

MKI67 mRNA (encoding nuclear Ki67 protein) was enriched in Treg B subpopulation cells.[187]

This could be important as the Ki67 protein is often used as a marker for cell proliferation.[382, 383]

Table 4.4 Functional enrichment analysis of Treg B genes

| Enriched function | Group | Group genes |
|---|---|---|
| Mitotic spindle organisation | Group 0 | *AURKA, BRCA2, BUB1, BUB1B, CDC20, CENPE, CHEK1, CHMP1A, EFNA5, KIF11, KIF15, KIF20B, KIF23, KIF4A, NDC80, NEK2, NUSAP1, PRC1, STIL, TPX2* |
| Nuclear division | Group 1 | *AURKA, BRCA2, BUB1, BUB1B, CASC5, CDC20, CDC25A, CDK3, CENPE, CHEK1, CHMP1A, CKS2, KIF11, KIF15, KIF20B, KIF23, KIF4A, MKI67, NCAPH, NDC80, NEK2, NUSAP1, SMC4, STIL, TOP2A, TPX2, TTN* |
| Microtubule cytoskeleton organisation involved in mitosis | Group 2 | *AURKA, BRCA2, CENPE, CHMP1A, KIF11, KIF15, KIF20B, KIF23, KIF4A, NDC80, NEK2, NUSAP1, PRC1, STIL, TPX2* |
| Nuclear chromosome segregation | Group 3 | *AURKA, BRCA2, BUB1, BUB1B, CASC5, CDC20, CDC7, CENPE, CHEK1, CHMP1A, KIF11, KIF20B, KIF23, KIF4A, NCAPH, NDC80, NEK2, NUSAP1, PRC1, SMC4, STIL, TOP2A, TTN* |
| Double strand break repair via homologous recombination | Group 4 | *BARD1, BRCA2, CDC7, CHEK1, EXO1, GINS2, WHSC1* |
| Mitotic nuclear division | Group 5 | *AURKA, BRCA2, BUB1, BUB1B, CASC5, CDC20, CDC25A, CDK3, CENPE, CHEK1, CHMP1A, KIF11, KIF15, KIF20B, KIF23, KIF4A, NCAPH, NDC80, NEK2, NUSAP1, SMC4, STIL, TPX2, TTN* |
| Meiotic cell cycle | Group 6 | *AURKA, BRCA2, CDC20, CKS2, EXO1, MKI67, NEK2, SMC4, TOP2A* |
| Negative regulation of fat cell proliferation | Group 7 | *E2F1, E2F3, TFDP1* |
| Chromosome segregation | Group 8 | *AURKA, BRCA2, BUB1, BUB1B, CASC5, CDC20, CDC25A, CDK3, CENPE, CHEK1, CHMP1A, EFNA5, KIF11, KIF15, KIF20B, KIF23, KIF4A, NCAPH, NDC80, NEK2, NUSAP1, SMC4, STIL, TOP2A, TPX2, TTN* |
| Spindle organization | Group 9 | *AURKA, BRCA2, CDC7, CHMP1A, KIF11, KIF20B, KIF23, KIF4A, NDC80, NEK2, NUSAP1, PRC1, STIL, TPX2* |
| Regulation of DNA recombination | None 0 | *CHEK1, KPNA2, WHSC1* |
| Regulation of cyclin-dependent protein serine/threonine kinase activity | None 1 | *CDC25A, CDKN2C, CKS2, STIL* |

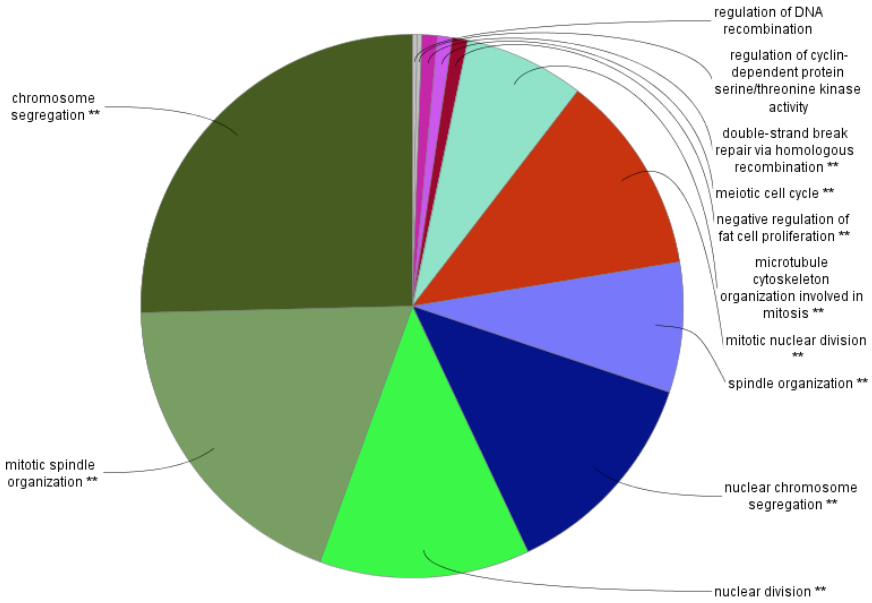



**Figure 4.9 ClueGO biological process enrichment**

Representative functional terms from functional enrichment analysis of Treg B genes using ClueGO.[384] The pie chart shows enriched functional terms listed in the Table 4.4 and the ratio of the enrichment based on the enrichment test from the original paper method.[187, 384]

## 4.3.4 RNA sequencing

RNA sequencing of three AA samples has shown that, in a Treg B *versus* Treg A comparison, Treg B have more activated cell death pathways. The top-10 pathway list is shown in **Errore. L'origine riferimento non è stata trovata.**.

The same analysis has been repeated after expansion (chapter 5), and even after four weeks in culture, expanded AA Treg B have an increased activation of CD28 signalling and death receptor signalling compared to expanded Treg B from HDs. Post-expansion AA Treg A and B do not present any difference in pathway activation.

**Table 4.5 Top-10 list of RNA sequencing analysis. IPA results of the comparison Treg B *versus* Treg A**

| Ingenuity Canonical Pathways | -log(p-value) | Ratio | Molecules |
|---|---|---|---|
| Th1 and Th2 Activation Pathway | 5.87 | 0.133 | PTGDR2, TNFRSF4, GRB2, TGFBR3, IL6R, MAF, BMPR2, IL12RB2, HLA-DQB1, TGFBR2, ITGB2, NFIL3, IL17RB, HLA-DRB1, CCR4, |

| | | | |
|---|---|---|---|
| | | | CD80, CD40, PIK3R6, CD86, NFATC2, CD274, STAT1, GATA3, TIMD4 |
| Osteoarthritis Pathway | 5.85 | 0.126 | CTNNA1, VEGFB, BMPR2, CASP4, SDC4, SMAD5, SLC39A8, HMGB1, IL1R2, VEGFA, TGFBR2, PRG4, ANXA5, NAMPT, CASP8, ADAMTS4, CASP10, ITGA4, ITGB1, SMAD7, ANXA2, IL1R1, TCF3, RBPJ, LEF1, LRP1 |
| Molecular Mechanisms of Cancer | 5.82 | 0.101 | PRKACB, CDKN2A, WNT10B, CTNNA1, HRAS, BMPR2, CDKN2C, ARHGEF1, SMAD5, CDKN2B, RHOH, FAS, TGFBR2, PAK1, NFKBIA, BBC3, CCND3, RHOU, AKT3, CASP8, PRKD3, E2F2, ITGA4, CASP10, ITGB1, ARHGEF12, GRB2, ADCY3, SMAD7, MAPK9, BAX, TCF3, RASGRF2, NFKBID, E2F1, PIK3R6, LEF1, RBPJ, LRP1 |
| Th2 Pathway | 5.18 | 0.137 | PTGDR2, TNFRSF4, GRB2, TGFBR3, MAF, BMPR2, IL12RB2, HLA-DQB1, TGFBR2, ITGB2, IL17RB, HLA-DRB1, CCR4, CD80, CD40, PIK3R6, CD86, NFATC2, GATA3, TIMD4 |
| B Cell Development | 4.59 | 0.276 | PTPRC, HLA-DRB1, CD40, CD80, CD86, IGHM, HLA-DQB1, IL7 |
| T Helper Cell Differentiation | 4.36 | 0.174 | TGFBR2, HLA-DRB1, CD40, CD80, IL6R, FCER1G, CD86, IL12RB2, HLA-DQB1, RORC, STAT1, GATA3 |
| Colorectal Cancer Metastasis Signaling | 4.36 | 0.104 | PRKACB, WNT10B, GRB2, GRK3, RACK1, IL6R, ADCY3, HRAS, MAPK9, VEGFB, BAX, TCF3, BIRC5, RHOH, MLH1, TGFBR2, VEGFA, TLR1, RHOU, PIK3R6, MSH6, AKT3, LEF1, PTGER2, STAT1, LRP1 |
| iCOS-iCOSL Signaling in T Helper Cells | 4.21 | 0.136 | GRB2, CSK, TRAT1, PLCG1, HLA-DQB1, PTPRC, NFKBID, NFKBIA, HLA-DRB1, CD80, CD40, PIK3R6, FCER1G, AKT3, NFATC2, PLEKHA1 |
| Myc Mediated Apoptosis Signaling | 3.94 | 0.158 | CDKN2A, YWHAQ, IGF1, GRB2, PIK3R6, IGF1R, MAPK9, HRAS, AKT3, BAX, CASP8, FAS |
| CD28 Signaling in T Helper Cells | 3.83 | 0.126 | GRB2, CSK, MAPK9, PLCG1, HLA-DQB1, PTPRC, NFKBID, PAK1, NFKBIA, HLA-DRB1, CD80, PIK3R6, FCER1G, CD86, AKT3, NFATC2 |

### 4.3.5 FAS-L-induced apoptosis leads to a reduction of Treg B and this effect can be reduced by low-dose IL-2

Given the role of FAS-L positive T cells in the pathophysiology of AA pathophysiology and the fact that CD95 (FAS) is one of the significant non-redundant markers that define Treg B, we tested the FAS-L sensitivity of these cells (magnetically isolated $CD4^+$, see page 116). After a five hour culture with FAS-L (5

μg/ml), the number of apoptotic cells was significantly higher in Treg B compared to Treg A (3 replicates, *p = 0.019*) (Figure 4.10).

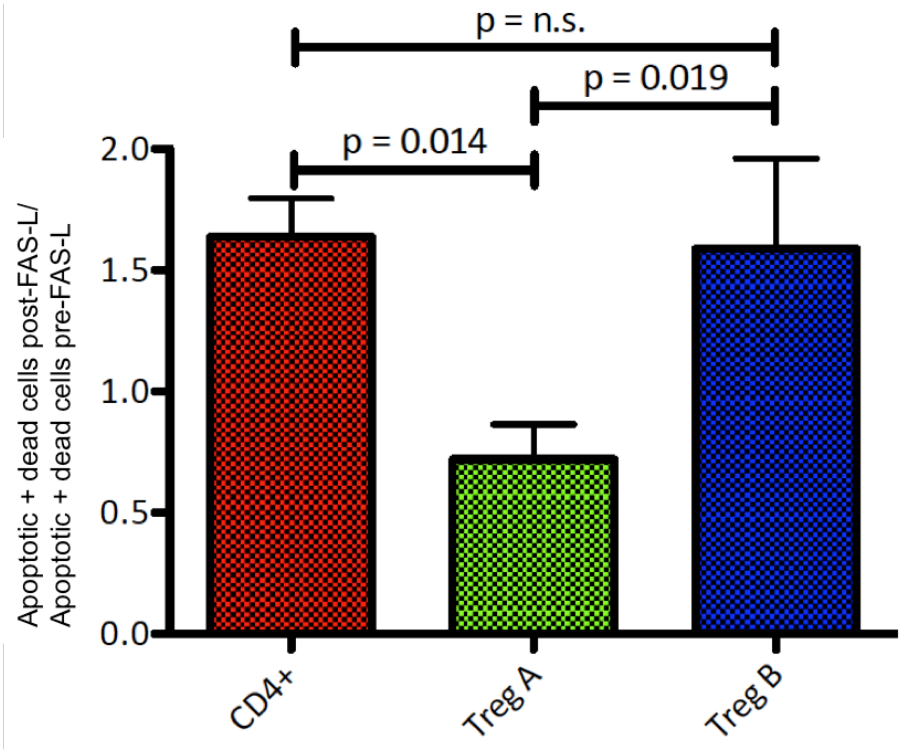



**Figure 4.10 Apoptosis induction on CD4⁺: differences between Treg A and B**

After adding FAS-L (5 μg/ml) for 5 hours, Treg B (and CD4⁺) have a higher rate of apoptotic and dead cells than Treg A. Each bar represents the ratio between apoptotic and dead cells after FAS-L addition and apoptotic and dead cells before FAS-L addition. Error bars represent mean ± standard deviation.Groups were compared by analysis of variance (ANOVA).

This effect could be prevented by the addition of low-dose IL-2 (5 IU/ml) to the culture (Figure 4.11).

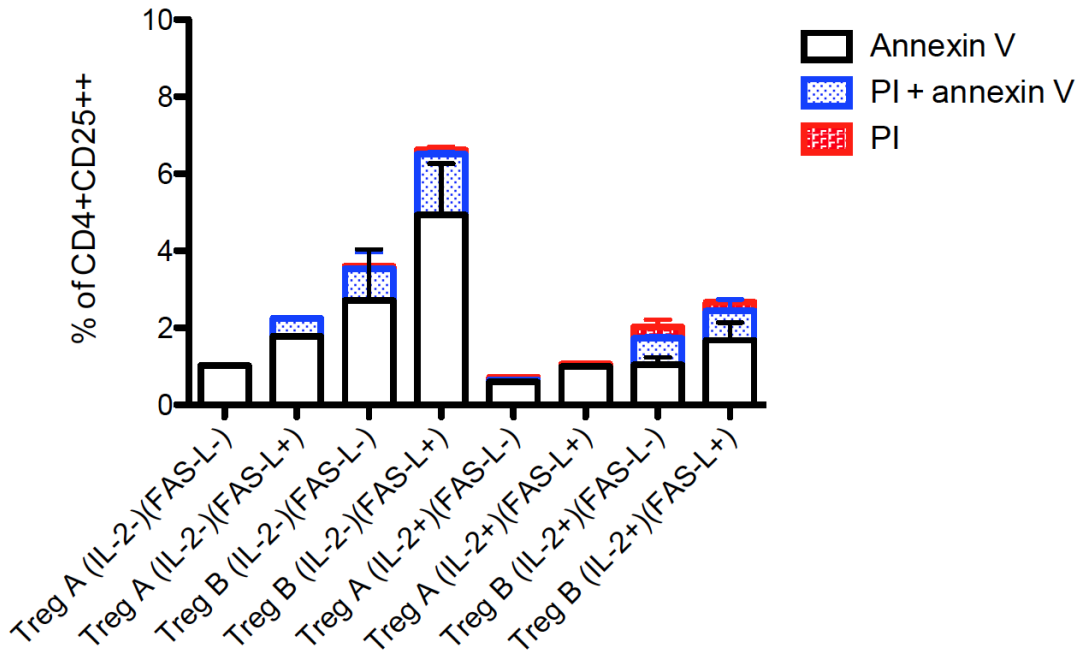



**Figure 4.11 Apoptosis prevention by addition of IL-2**

The figure shows the combination of two experiments. By adding low-dose IL-2 (5 IU/ml) after inducing apoptosis, Treg B can be rescued but not Treg A. Error bars represent mean ± standard deviation.

## 4.4 Summary of data

We have previously shown that, with a novel strategy for multidimensional deep phenotyping, we can reliably identify an immune signature for AA based on Treg subpopulations. This approach also identified an immune signature that predicts for response to IST at time of diagnosis.[187]

We then wanted to assess whether these two Treg subsets are functionally and ontogenetically different to establish if they could be potentially used, once expanded, as a potential treatment for AA.

The first finding was that freshly sorted Treg A and B (as described before) showed a different functional profile: in fact, only Treg B were able to suppress the pro inflammatory cytokine secretion by autologous Tconv. This demonstrated that Treg A and B are not only phenotypically, but also functionally, two distinct Treg subpopulations, confirming the mass cytometry data from AA samples.[187] To have a

more complete characterisation of these two different subsets, we investigated their clonality and were able to prove that they are polyclonal but do not share almost any TCR sequence, suggesting a possible distinct developmental origin.[187]

Considering that Treg A and B were proven to be immunophenotypically, functionally and ontogenetically different, the next step was to try to identify a gene signature able to differentiate them. The comparison of Treg subsets and Tconv GEP, using the human Treg gene signature,[380] showed that both Treg A and B subpopulations were significantly enriched in genes up-regulated in human Tregs, including *IL-2RA*, *FOXP3*, *IKZF2*, *TIGIT*, and *CTLA4* (*FDR < 0.0001*). Treg B were enriched with Treg-related memory/activation genes compared with Treg A, including *JAKMIP1*, *CCR8*, *TRIB1*, and *GZMK* (*FDR < 0.0001*) (Figure 4.7).[187] Although both Treg subpopulations were enriched with Treg-associated genes, Treg B showed an activation gene signature, in agreement with our mass cytometry findings.[187] Gene set enrichment functional analysis[346] highlighted several gene sets as significantly overexpressed in Treg B, including G2M checkpoint, mitosis, M phase of mitotic cell cycle, IL-2-STAT5 signalling, and immune response genes.[187] Ontology analyses of the functions of these protein complexes showed they are involved in mitotic functions, DNA replication, and cell cycle-dependent transcription. RNA sequencing of patient' samples has shown an increase in death cell pathway activation in Treg B *versus* Treg A.

CD95 (FAS) is one of the defining, non-redundant markers able to differentiate Treg A and B. FAS, after binding with FAS-L, mediates apoptosis through the activation of caspase 8.[385] Moreover, its aberrant expression on lymphocytes is likely to play a role in BMF pathophysiology in an animal mode.l[365] Therefore, we investigated the

different “apoptotic propensity” between Treg A and B and the latter have shown to be more prone to FAS-FAS-L-mediated apoptosis.

IL-2, in addition to being crucial for Tregs development and survival, it also has immunostimulatory effects, including an increased expression of the anti-apoptotic Bcl-2 and Bcl-XL.[366] The following step was to test whether these IL-2 “anti-apoptotic” properties could be exploited to prevent Treg B from going to apoptosis: we were able to show that low-dose IL-2 can prevent FAS-FAS-L-mediated apoptosis.

To summarise: we have shown that Treg A and B are not only immunophenotypically different, but they also show a distinct functional profile, ontogeny, gene signature, and apoptosis propensity.

In the next chapter Treg sensitivity to IL-2, expandability, and *in vivo* use will be described.

# 5 POTENTIAL USE OF *EX VIVO* EXPANDED REGULATORY T CELLS AS A CELLULAR THERAPY FOR AA

## 5.1 Introduction

Aplastic anaemia has a dual distribution around twenty and sixty years of age.[163] Younger patients are generally fitter, with lower comorbidities, therefore almost always eligible for HSCT (if an MSD is available) or immunosuppressive treatment (IST) with ATG and CsA. On the contrary, elderly patients are often unfit due to the high number of comorbidities. Patients' poor performance status often prevents clinicians from offering standard IST, due to its toxicity profile.[163] Moreover, one third of patients who are given ATG and CsA relapse or do not respond; in this setting the efficacy of a second course of IST is still a matter of debate. Therefore, clinicians do not have many options for elderly patients unfit for treatment or relapsed patients with SAA and VSAA.[163]

Adoptive transfer of autologous Tregs represents an exciting immunotherapeutic strategy.[386, 387] Generally, protocols for adoptive transfer include Treg isolation from the host, enrichment, expansion, and re-infusion (Figure 5.1).[387] Advantages of an *ex vivo* expansion strategy include the ability to perform careful cellular phenotyping and control the dose of administered cells.[388]

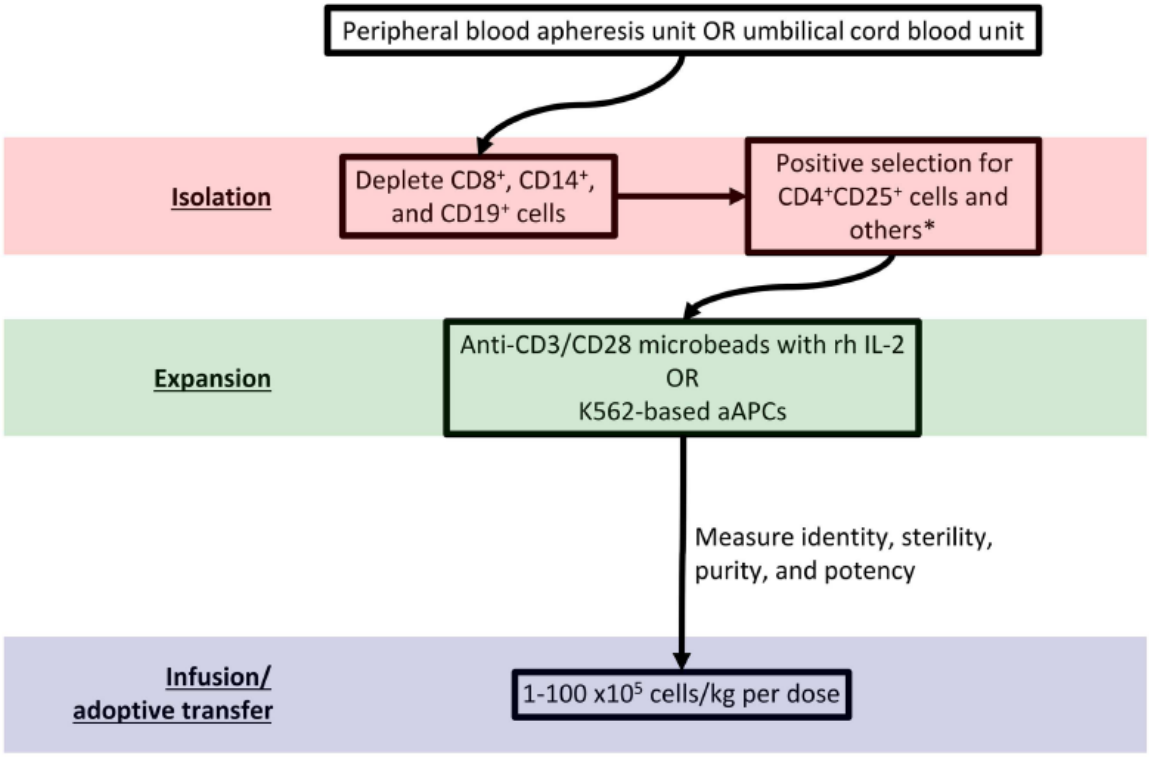



**Figure 5.1 Schematic representation of the strategy to isolate, expand and infuse Tregs**

Generally, protocols for adoptive transfer include Treg isolation from the host, enrichment, expansion, and re-infusion.[387]

Successful isolation requires labelling cell surface markers with a tagged antibody and sorting *via* FACS or magnetic bead separation.

Unfortunately, no cell surface markers uniquely identify Tregs. Although Foxp3 expression specifies the Treg lineage in mice,[35] T cells promiscuously express Foxp3 in humans.[389] Regardless, Foxp3 detection requires cell permeabilization, which makes cells unusable for adoptive transfer.[387]

As activated $CD4^+$ conventional T cells may also transiently express CD25, patterns of CD127 (the IL-7 receptor  chain),[390] CD49b (the integrin VLA-4  4 1  chain),[391] lymphocyte activation gene 3 (LAG-3),[391] CD45RA, CD45RO, and latency associated peptide (LAP)[392] can identify Tregs and facilitate their isolation. Although Tregs express CTLA-4, glucocorticoid-induced tumour necrosis factor-related

receptor (GITR),[393] CD69,[394] andCD44,[395] activated non-Tregs may also express these markers.[387]

A list of relevant Treg markers for immunotherapy is reported in Table 5.1.[387]

**Table 5.1 Treg markers relevant to their use as immunotherapy**

| Marker | Function | Relevance to Treg immunotherapy |
|---|---|---|
| FoxP3 | Transcription factor, master regulator of Tregs development and function | Identifies Treg lineage in mice, expressed in human $CD4^+$ Tregs[36] |
| CTLA-4 | Transmits inhibitory signals to APC | Important mechanism of Tregs suppressive function[396] |
| LAP | Component of TGF-β latent complex | Identifies Treg subset with TGF-β-mediated function[392] |
| GITR | Cell signalling | Important mechanism of Tregs suppressive function[65] |
| ICOS | Co stimulator on T cells | Involved in Treg expansion and IL-10 production, particularly during Th2 inflammation[397] |
| LAG-3 | CD homolog with MHC II binding properties | Expressed on Tregs[391] |
| CD3 | TCR signal transduction | Stimulation required for Treg expansion |
| CD4 | Interacts with MHC II molecules on APCs and amplifies TCR signals | Identifies $CD4^+$ lymphocytes subset |
| CD25 | IL-2 receptor component | Expressed by $CD4^+$ $Foxp3^+$ Tregs but also by other T cells[24] |
| CD28 | Co stimulator required for T cell activation | Stimulation required for Treg expansion[398] |
| CD44 | Hyaluronic acid receptor | Marker of activated Tregs[395] |
| CD45RO | Protein tyrosine phosphatase, receptor type, C | Positive Treg marker, also identifies memory T cells |
| CD45RA | Protein tyrosine phosphatase, receptor type, C | Minor Treg marker, also identifies naïve T cells |
| CD49b | Cell adhesion and signalling | Expressed on Tregs[391] |
| CD62L | Lymphocyte cell adhesion molecule | May be marker of effective disease modulating Treg subset[399] |
| CD69 | Cell signalling | Marker of activated Tregs that suppress via membrane-bound TGF-β1[394] |
| CD127 | IL-7 receptor | Negative Treg marker[390] |

Tregs have already been used in clinical settings such as allograft tolerance,[250-252, 254, 400]and autoimmunity (diabetes mellitus type I).[281, 401, 402]

The adoptive transfer of autologous, *ex vivo* expanded Tregs has never been investigated in AA.

In this chapter Treg expandability and stability, clonality, plasticity of expanded Tregs will be described.

The animal model for the *in vivo* use of these cells will also be discussed.

## 5.2 Materials and methods

### 5.2.1 Suppression assay

To test expanded Tregs function, autologous Tconv were stained with VCT and Tregs with CFSE (described at page 120) and cultured for five days (Tconv:Treg:bead = 20:20:1). After five days cells were harvested, stained for live/dead and surface CD3/CD4. Samples were acquired on a Canto II (BD) (see page 120 for more details).

### 5.2.2 IL-2 sensitivity (STAT5 phosphorylation)

PBMCs from 2 HDs and 6 AA patients were stimulated with IL-2 at different concentrations (0.1-1,000 IU/ml) for 15 and 30 minutes. Cells were than fixed with 1 ml of BD fix/lyse buffer for 10 minutes and washed twice with ice-cold wash buffer (PBS with 0.2% BSA). The permeabilization was done by adding ice-cold methanol 100% 1 ml for 20 minutes. After permeabilization, cells were washed (800 x $g_{max}$ for 5 minutes) three times with ice-cold wash buffer and stained with the antibody master mix (in the fridge for 1 hour). After staining, cells were washed twice with ice-cold wash buffer and run on Fortessa (BD Biosciences). All the reagents are listed in Table 5.2.

Table 5.2 Reagents list for STAT5 phosphorylation

| Product | Company | Catalogue # |
|---|---|---|
| PBS | Sigma-Aldrich | D866 |
| BSA, Albumin, Bovine Fraction V solution 7.5% | Sigma-Aldrich | A8412 |
| Methanol 100% | AnaIR | BDH 1015866B |
| BD Lyse/Fix Buffer | BD Biosciences | 558049 |
| Proleukin | Novartis | CLB-P-476-750-14002_GB |

| | | |
|---|---|---|
| CD45RA PE Cy7 | BD Biosciences | 337186 |
| pSTAT5 FITC | BD Biosciences | 612598 |
| CD25 APC | BD Biosciences | 555434 |
| CD25 APC | BD Biosciences | 340907 |
| CD4 Alexa-700 | BioLegend | 300526 |
| Foxp3 PE | BioLegend | 320208 |

### 5.2.3 Phosphorylated STAT5 expression with Western blot

Protein samples were separated by Polyacrylamide Gel Electrophoresis using the NuPAGE system (polyacrylamide gel electrophoresis). Protein lysates of between 1 x $10^5$ and 3 x $10^5$ cells were loaded per lane of a 4-12% (w/v) bis-tris polyacrylamide gel. Either NuPAGE Multimark pre-stained ladders were used as protein 86 standards to determine the size of any bands detected. Gels were assembled in a NuPAGE gel apparatus and run for 1 hour in 1X MES SDS Running Buffer (50 mM MES, 50 mM Tris, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.3) at 200 V. After electrophoresis, the separated proteins were transferred to a nitrocellulose-coated nylon membrane using the NuPAGE semi-dry gel blotting module according to the manufacturer instructions. Transfer was for 1.5 hours in NuPAGE Transfer Buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.2) with 20% (v/v) methanol at 25 V. For very large proteins, transfer was for 20 hours at 10 V in a 40 °C cold room. After transfer the membrane was removed from the apparatus and blocked for 30 minutes in 10% (w/v) non-fat dried milk in PBS/0.05% Tween-20. Blocking reagent was removed by three brief washes in PBS/0.05% Tween-20 before detection of proteins.

Primary antibodies were made up to working dilutions in PBS/0.05% (v/v) Tween-20 with 3% (w/v) BSA. For most of the antibodies, the dilution was 1 in 1000, giving a range of 0.1 μg to 1 μg. Blots were incubated with the antibody solution for either 1 hour at room temperature or overnight at 40 °C. After incubation the blots were

washed 3 times for 5 minutes in PBS/0.05% (v/v) Tween-20. Horseradish peroxidase conjugated secondary antibodies (Dako) appropriate to the species in which the primary was raised were diluted in PBS/0.05% (v/v) Tween-20 with 10% (w/v) non-fat dried milk. The working concentration of secondary antibodies was 0.5 μg/ml. Blots were incubated for 30 minutes to 1 hour at room temperature, then washed 3 times for 5 minutes in PBS/0.05% (v/v) Tween-20. Bands were visualised using a chemiluminescent detection system (ECL or ECL plus, GE Healthcare), in which horseradish peroxidase (HRP) cleaves the luminol reagent to produce light. Protein bands were detected by Azure Imager for various times ranging from 1 second to 5 minutes. All the reagents are listed in Table 5.3.

**Table 5.3 Reagent list for phosphorylated STAT5 Western blot**

| Product | Company | Catalogue # |
|---|---|---|
| Bolt™ Welcome Pack, 4-12%, 12-well | ThermoFisher Scientific | NW0412C |
| NuPAGE™ MES SDS Running Buffer (20X) | ThermoFisher Scientific | NP0002 |
| Hybond-C Extra (30 cm x 3 m) | GE Healthcare | RPN303E |
| NuPAGE™ Transfer Buffer (20X) | ThermoFisher Scientific | NP0006 |
| Tween™ 20 Surfact-Amps™ Detergent Solution | ThermoFisher Scientific | 85113 |
| Rabbit Anti-Mouse Immunoglobulins/HRP | Dako | P026002-2 |
| STAT5 Antibody | Cell Signaling | 9363 |
| Phospho-Stat5 (Tyr694) (D47E7) XP® Rabbit mAb | Cell Signaling | 4322 |
| Pierce™ ECL Plus Western Blotting Substrate | ThermoFisher Scientific | 32132X3 |

## 5.2.4 Regulatory T cells expansion

For unfractionated Treg expansion, cells were magnetically sorted as described at page 117. For Treg subsets expansion, cells were sorted as described at page 178.

Freshly isolated live Tregs were stimulated with anti-CD3/CD28 beads (1:1 ratio) and high-dose (1,000 IU/ml) IL-2 for four weeks with ATRA 2 μM and rapamycin

100 nM. Culture medium (XV Prime, Irvine Scientific, supplemented with AB serum 10%) and beads were replenished every week. After four weeks of expansion, cells were rested with decreasing doses of IL-2 for five days.[367] All the reagents are listed in Table 5.4.

**Table 5.4 Reagents list for Treg expansion**

| Product | Company | Catalogue # |
|---|---|---|
| Dynabeads Human T-Activator | Gibco | 11132D |
| PRIME-XV T Cell Expansion XSFM | Irvine Scientific | 91141 |
| AB human serum | Sigma-Aldrich | H4522 |
| Rapamycin | LC Laboratories | R-5000 |
| ATRA | Sigma | R2625-100 mg |
| Proleukin | Novartis | CLB-P-476-750-14002_GB |

### 5.2.5 Treg function (proliferation assay and cytokine measurement)

Treg function was assessed as already described (page 120). Supernatant was collected and stored at -20 °C and then used later for cytokine measurement using xMAP technology.

### 5.2.6 TSDR methylation status of expanded Regulatory T cells

Tregs stability was assessed by the methylation status of the TSDR as described at page 126.

### 5.2.7 T cell receptor sequencing

To evaluate the clonality of Treg A and B we have sent genomic DNA to Adaptive Biotechnologies (Seattle, U.S.). The procedure has already been described (page 141).

### 5.2.8 Regulatory T cells polarisation toward IL-17A secretion

To test Treg plasticity after expansion, cells were rested for three days after expansion with decreasing doses of IL-2. After three days of rest, cells were resuspended up to 3 x $10^6$/ml and cultured for five days in Th17 polarising conditions

with IL-1β (10 ng/ml), IL-6 (25 ng/ml) and IL-2 (10 IU/ml). After five days of culture, cells were harvested and stained to measure IL-17 secretion. All the reagents are listed in Table 5.5.

**Table 5.5 Reagents list for polarisation towards IL-17A secretion**

| Product | Company | Catalogue # |
|---|---|---|
| PRIME-XV T Cell Expansion XSFM | Irvine Scientific | 91141 |
| AB human serum | Sigma-Aldrich | H4522 |
| Dynabeads Human T-Activator | Gibco | 11132D |
| Proleukin | Novartis | CLB-P-476-750-14002_GB |
| Fixable Viability Dye eFluor 780 | eBioscience | 65-0865-18 |
| CD4 PerCp Cy5.5 | BioLegend | 317428 |
| CD25 V710 | BioLegend | 302636 |
| CD45RA FITC | BioLegend | 304106 |
| CD95 BV421 | BioLegend | 305624 |
| CCR4 BV510 | BioLegend | 359416 |
| Foxp3 PE | eBioscience | 12-4776-42 |
| IL-17A APC | BioLegend | 512334 |
| Human IL-1β, premium grade | Miltenyi Biotec | 130-093-897 |
| Human IL-6, premium grade | Miltenyi Biotec | 130-095-352 |

### 5.2.9 B cell lymphoma-2 Western blot

Whole cell lysates were prepared using 1x Radio-Immunoprecipitation Assay (RIPA) Lysis Buffer. 15-20 μg of lysates were separated on NuPAGE Novex 4-12% Bis-Tris Protein Gels, transferred to a PVDF membrane and blocked with 5% non-fat dried milk in 0.1% Tween 20/PBS buffer for an hour. Membranes were then probed with primary antibodies overnight at 4 °C. After washing, membranes were subsequently probed with secondary antibodies for an hour at room temperature. Membranes were then incubated in Pierce ECL Western Blotting Substrate for 5 minutes and proteins were exposed to CL-Xposure Clear Blue X-ray Film (Thermo Fisher Scientific) for the preferable time using Photon Imaging Systems SRX-101A. The reagents are listed in Table 5.6.

**Table 5.6 Reagents list for Bcl-2 WB**

| Product | Company | Catalogue # |
|---|---|---|
| RIPA Lysis Buffer System | Santa Cruz Biotechnology | sc-24948A |

| | | |
|---|---|---|
| NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm | ThermoFisher Scientific | NP0321BOX |
| PVDF Pre-cut Blotting Membranes, 0.2 µm pore size | ThermoFisher Scientific | LC2002 |
| Tween™ 20 Surfact-Amps™ Detergent Solution | ThermoFisher Scientific | 85113 |
| Pierce™ ECL Plus Western Blotting Substrate | ThermoFisher Scientific | 32132X3 |
| CL-XPosure™ Film, 5 x 7 in. (13 x 18 cm) | ThermoFisher Scientific | 34090 |
| Konica SRX-101A | Konica Minolta | SRX-101A |

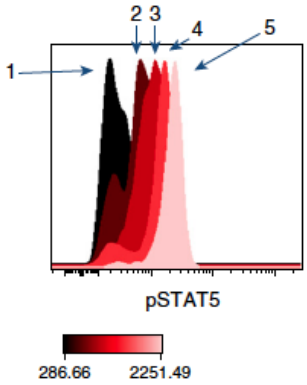
### 5.2.10 Animal model

Al the methods used for this section are described in full details at page 154.

## 5.3 Results

### 5.3.1 Regulatory T cells response to IL-2

One of the aims of this study was to investigate the potential expandability of Tregs in aplastic anaemia. As Tregs are exogenously dependant on IL-2 to survive, proliferate and function, we firstly tested the IL-2 sensitivity of AA Tregs based on STAT5 phosphorylation, the main mean to assess Tregs proliferation upon IL-2 binding to its receptor.[403] Freshly isolated PBMCs from 2 HDs and 6 AA patients (3 IST responders and 3 non-responders, at diagnosis) were cultured in the presence of IL-2 at escalating concentrations (from 0.1 to 1000 IU/ml). Treg pSTAT5 expression, assessed by flow cytometry, significantly increased after fifteen minutes culture with IL-2 (0.5 IU/ml) (median fluorescence intensities, 92.6 ± 35.1 pre-IL-2 *versus* 787 ± 109.6 post-IL-2, $p = 0001$), confirming their responsiveness to IL-2. There was no difference between HDs and AA Tregs in response to IL-2 (Figure 5.2).




| Condition | Cells | IL-2 | Time |
|---|---|---|---|
| 1 | Tregs | 0 | 0 |
| 2 | HD Tregs | 0.5 U/mL | 15 min |
| 3 | AA Tregs | 0.5 U/mL | 15 min |
| 4 | HD Tregs | 1 U/mL | 30 min |
| 5 | AA Tregs | 1 U/mL | 30 min |

**Figure 5.2 STAT5 phosphorylation in response to IL-2**

Freshly isolated PBMCs from two healthy donors and six aplastic anaemia patients (3 IST responders and 3 non-responders, at diagnosis) were cultured in the presence of IL-2 at different concentrations (from 0.1 to 1000 IU/ml). Treg pSTAT5 expression significantly increased after 15 minutes culture with IL-2 (0.5 IU/ml) (median fluorescence intensities, 92.6 ± 35.1 pre-IL-2 versus 787 ± 109.6 post-IL-2, *p = 0001,* one way analysis of variance), confirming their responsiveness to IL-2.

As a confirmation, the Western blot of the phosphorylated STAT5 shows that Treg B have a higher expression of the protein both before and after addition of low dose (1 IU/ml) IL-2 (Figure 5.3).

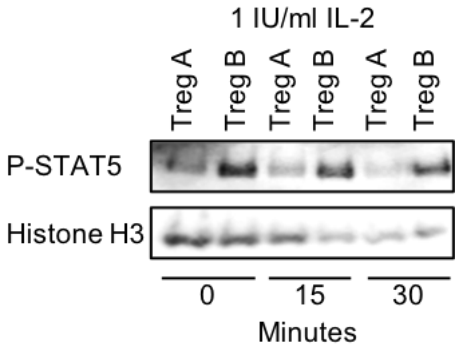



**Figure 5.3 Phosphorylated STAT5 in Tregs before and after addition of low-dose IL-2**

Treg A and B from 3 HDs were sorted as described in page 178. Isolated Treg A and B were cultured in low dose IL-2. 15-20 µg of lysates were separated on NuPAGE Novex 4-12% Bis-Tris Protein Gels, transferred to a PVDF membrane and blocked with 5% non-fat dried milk in 0.1% Tween 20/PBS buffer for an hour. Membranes were then probed with primary antibodies overnight at 4 °C. After washing, membranes were subsequently probed with secondary antibodies for an hour at room temperature. Membranes were then incubated in Pierce ECL Western Blotting Substrate for five minutes and proteins were exposed to CL-Xposure Clear Blue X-ray Film (Thermo Fisher Scientific) for the preferable time using Photon Imaging Systems SRX-101A.

## 5.3.2 Tregs expandability in healthy donors and aplastic anaemia and their phenotype

To test their *ex vivo* expandability, Tregs were obtained from 6 AA patients (3 IST responders and 3 non-responders, at diagnosis) and 8 HDs and were cultured and

stimulated with anti-CD3/CD28 beads (1:1 ratio) and high-dose IL-2 (1000 IU/ml) for four weeks with added ATRA 2 μM and rapamycin 100 nM.[367] AA Tregs expanded in a comparable rate to HDs, with a median of 33 fold increase (range 29-149) compared with a 21 fold increase (range 8-36) in HD (Figure 5.4).[187]

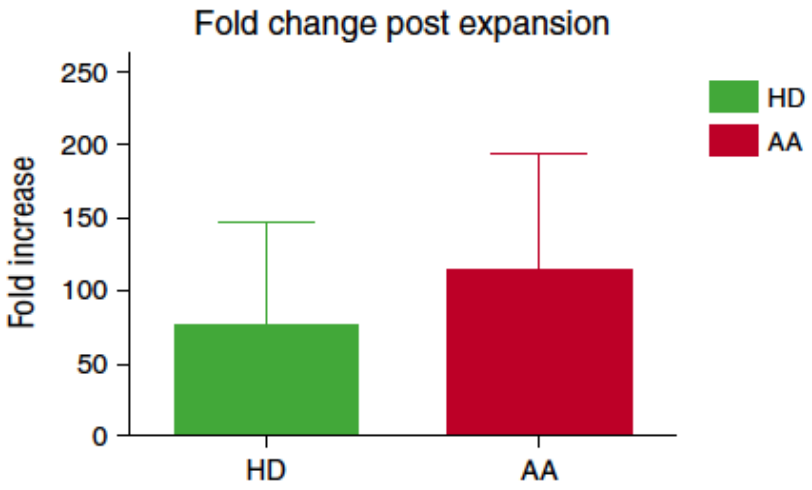



**Figure 5.4 Tregs expandability in healthy donors and aplastic anaemia patients**

Freshly isolated live Tregs were stimulated with anti-CD3/CD28 beads (1:1 ratio) and high-dose (1,000 IU/ml) IL-2 for four weeks with ATRA 2 μM and rapamycin 100 nM. Culture medium (XV Prime, Irvine Scientific, supplemented with AB serum 10%) and beads were replenished every week. After four weeks of expansion, cells were rested with decreasing doses of IL-2 for five days. There was no significant difference between AA and HD Tregs in terms of fold-change increase after four weeks expansion (represents median-fold increase of three healthy donors and three aplastic anaemia patients). Error bars are standard error of mean.[187]

Expanded Tregs demonstrated more than 90% Foxp3$^+$ expression in both AA and HDs (Figure 5.5), confirming their "Treg phenotype" after *ex vivo* expansion.[187]

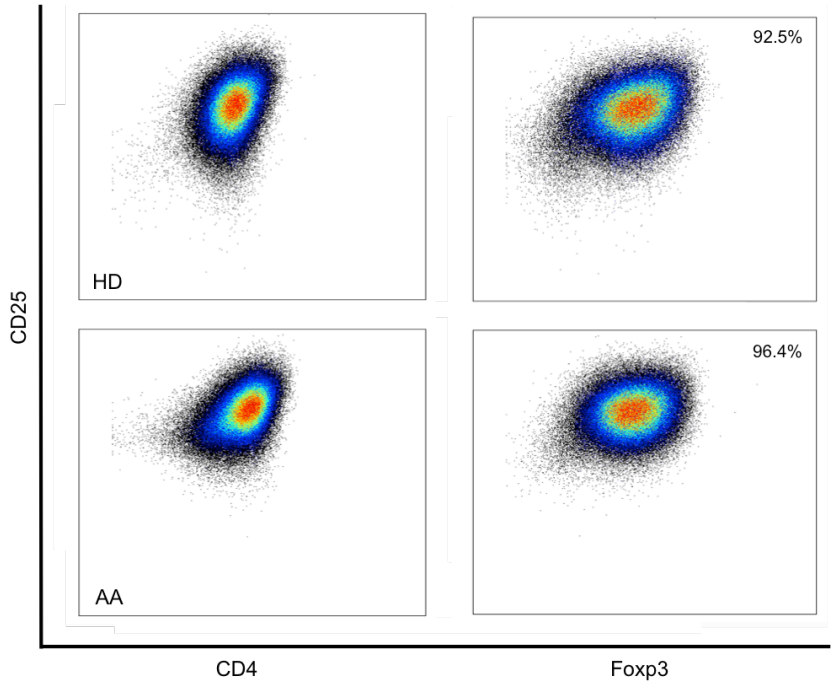



**Figure 5.5 Phenotype of expanded Tregs (healthy donors *versus* aplastic anaemia patients)**

Expanded Tregs were > 90% Foxp3$^{+}$ following expansion, confirming their "Treg-phenotype". This is representative of one HD and one AA patient. Tregs were defined as CD4$^{+}$CD25$^{high}$CD127$^{low}$Foxp3$^{+}$.[187]

The heterogeneity of *in vitro* expanded Tregs with Treg A and Treg B was investigated next. As the sorted Tregs were stimulated and treated in a Treg skewing environment, the expression level of some markers, such as CD25 or Foxp3, were high in expanded Tregs, making it difficult to cluster expanded Tregs with Treg A or B for comparison. To overcome this technical issue, we used an alternative analysis approach based on distance calculation and relative expression of markers. The subpopulations of Tregs expanded *in vitro* were assessed using the Euclidian distance between the mean expression for each parameter in Treg A and B (Figure 5.6 and Figure 5.7).[187] Using a 1-tail Welch's 2-sample *t* test, the null hypothesis that the distance between Tregs A and expanded Tregs is lower than in Tregs B ($p < 2.2 \times 10\text{-}16$) was rejected, suggesting that expanded Tregs are more similar to subpopulation B than A.[187]

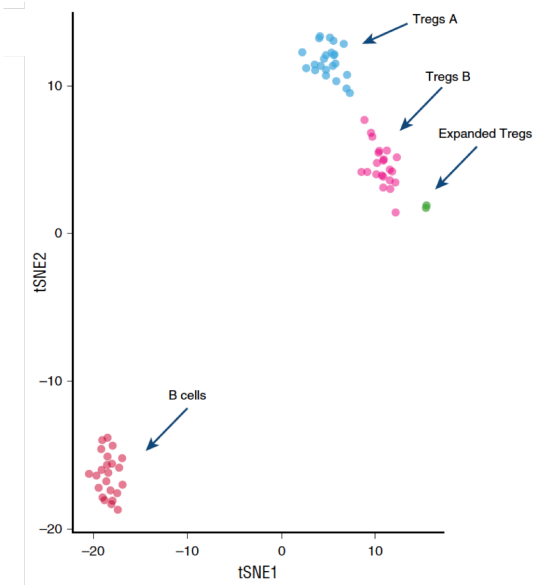



**Figure 5.6 Heterogeneity of expanded regulatory T cells**

The composition of *in vitro* expanded Tregs was assessed by calculating the Euclidian distance between the mean expression for each parameter in Treg A and B, calculated in 24 Treg A and B (5 HD and 19 AA samples) and 5 (2 AA and 3 HDs) *in vitro* expanded Tregs. B cells were used as an "irrelevant" control. The following parameters were used for the Euclidian distance calculation: Foxp3, CD25, CD127, CD45RA, HLA-DR, CCR6, CCR4, CD69, CD27, CXCR3, CD45RO, CD4, CD20, CD95, CD161, CD28, CD152, CD7, CD279, and CD19. t-SNE1 and t-SNE2 were used for distance calculation. viSNE plot of expression centroids for all Treg cell subpopulations, B cells, and expanded Tregs across all samples. Treg A and Treg B were automatically gated from 24 individual samples (19 samples from AA patients, 5 HDs) using the automated clustering algorithm FLOCK on a subset of 700,000 cells proportionally selected from all samples. B cells were gated from the same 24 samples in Cytobank. Expanded Tregs were from 3 HDs and 2 AA patients. Expression centroids were computed for each cell population and used as input for the dimensionality algorithm t-SNE as implemented in the tool cyt. Each dot in the plot represents one cell population in a particular sample. Expression values were transformed using the asinh function in a cofactor of 5. Using a 1-tailed Welch's 2-sample *t test*, we can reject the null hypothesis that the distance between Treg A and expanded Tregs is lower than in Treg B ($p < 2.2 \times 10^{-16}$), which suggests that expanded Tregs are more like subpopulation B than A.[187]

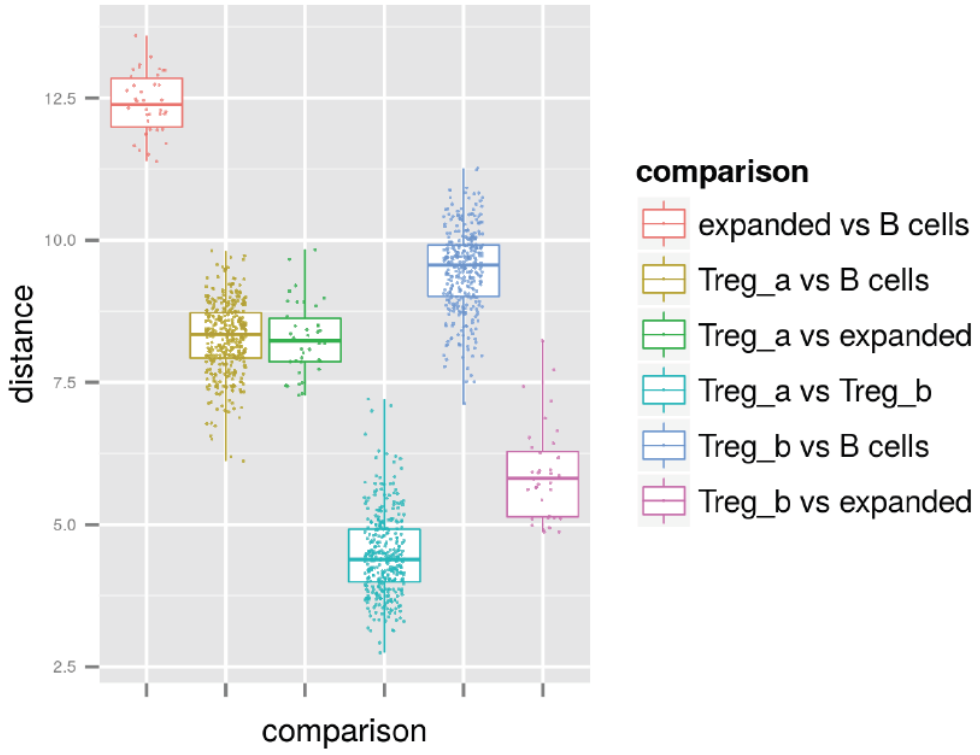



**Figure 5.7 Boxplots of pairwise Euclidian distances between cell population expression centroids across samples**

Treg A and Treg B were automatically gated from 24 individuals (19 aplastic anaemia patients and 5 healthy donors) using the automated clustering algorithm FLOCK on a subset of 700,000 cells proportionally selected from all samples. B cells were manually gated from the same 24 samples in Cytobank. Expanded Tregs were from 3 HDs and 2 AA patients. Boxplots depict the minimum, 1st quartile, median, 3rd quartile and maximum of the distribution of pairwise distances for the corresponding comparison. Expression values were asinh-transformed using a cofactor of 5.[187]

As Treg B have shown a gene signature skewed to cell cycle and proliferation genes, we wanted to assess Treg subsets expandability to see if this would translate in a higher proliferation and expansion rate. Treg A and B were sorted from 6 HDs and 4 AA patients, and then expanded for 4 weeks. Treg A and B expanded equally in HDs (Treg A 45 fold increase, and B 33 fold increase, $p = n.s.$), whereas Treg A expanded at a higher rate compared to Treg B in AA (Treg A 2,120 fold increase, and B 90 fold increase, $p = 0.03$) (Figure 5.8).[187]

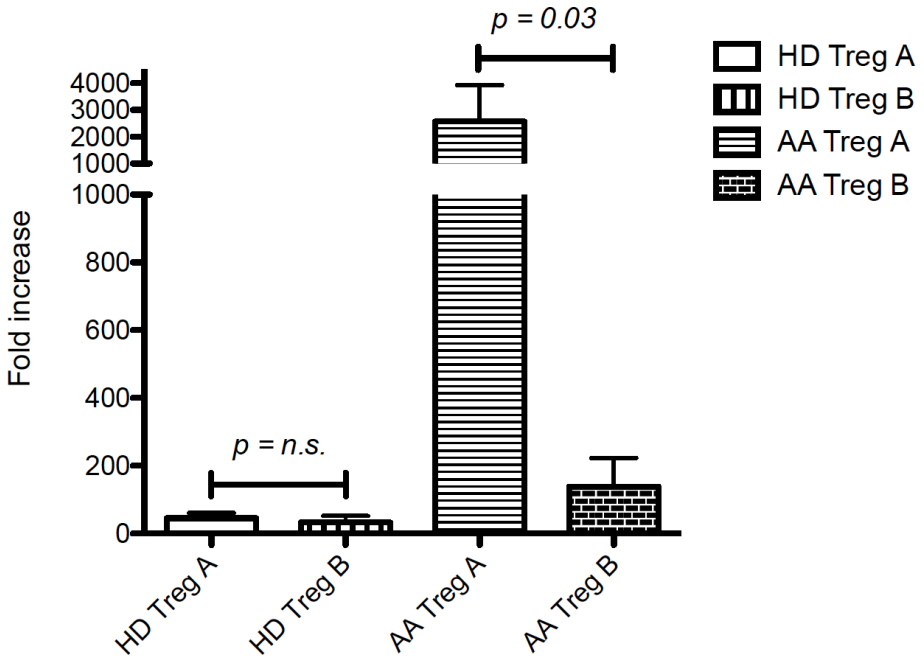



**Figure 5.8 Treg subsets expandability in healthy donors and aplastic anaemia**

Freshly isolated live Tregs were stimulated with anti-CD3/CD28 beads (1:1 ratio) and high-dose (1,000 IU/ml) IL-2 for four weeks with ATRA 2 μM and rapamycin 100 nM. Culture medium (XV Prime, Irvine Scientific, supplemented with AB serum 10%) and beads were replenished every week. After four weeks of expansion, cells were rested with decreasing doses of IL-2 for five days. There was no significant difference between Treg subsets expandability in helathy donors, while in aplastic anaemia Tregs A expanded significantly more than Treg B (represents median-fold increase of 6 HDs and 4 AA). Error bars are standard error of mean. One-way analysis of variance.

### 5.3.3 Expanded regulatory T cells function

To assess the suppressive activity of expanded Tregs, Tconv were stained with a fluorescent proliferation dye and co-cultured with autologous *ex vivo* expanded Tregs (1:1 ratio) for five days in the presence of anti-CD3/CD28 beads (Tconv:Treg:beads = 20:20:1).

Expanded aplastic anaemia Tregs suppressed proliferation of $CD4^+$ Tconv (an average reduction from 43% to 5%, $p = 0.009$) in both autologous and allogeneic conditions (Figure 5.9).

The suppressive activity of AA expanded Tregs was not significantly different from HD expanded Tregs.[187]

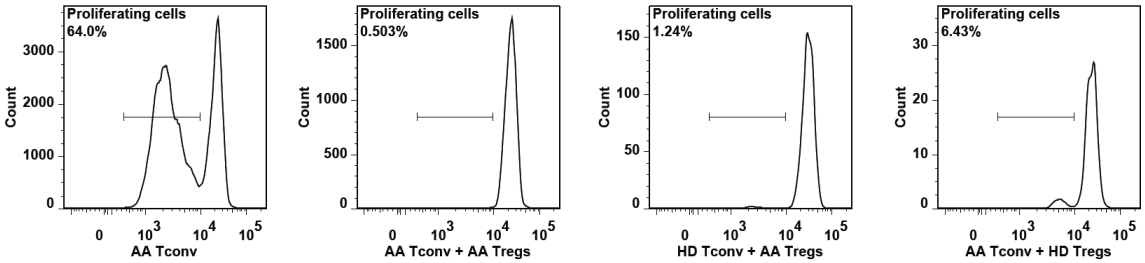



**Figure 5.9 Suppressive capacity of healthy donord and aplastic anaemia expanded regulatory T cells: criss-cross assay**

Tconv were stained with a fluorescent proliferation dye and co-cultured with autologous expanded Tregs (1:1 ratio) for five days in the presence of anti-CD3/CD28 beads (Tconv:Treg:beads = 20:20:1). Expanded AA Tregs suppressed proliferation of $CD4^+$ Tconv (an average reduction from 43% to 5%, $p = 0.009$, one way analysis of variance) in both autologous and allogeneic conditions. The suppressive activity of AA expanded Tregs was not significantly different from HD expanded Tregs.[187]

As *in vivo* physiologic Tconv:Tregs ratio is not very well understood, we have tested Tconv:Treg ratios from 1:1 up to 8:1 (Figure 5.10 and Figure 5.11) and we have observed that there was no difference between HDs and AA. The suppressive ability decreased along with the increase of Tconv:Treg ratio (from Tconv:Treg = 8:1 to 1:1, proliferating cells were 48.5% *versus* 43% *versus* 29.5% *versus* 13.5% for HDs and 56% *versus* 54% *versus* 34% *versus* 15% for AA , respectively, $p = 0.001$). These experiments were also repeated with HDs and AA expanded Treg A and B with similar results (Figure 5.12).

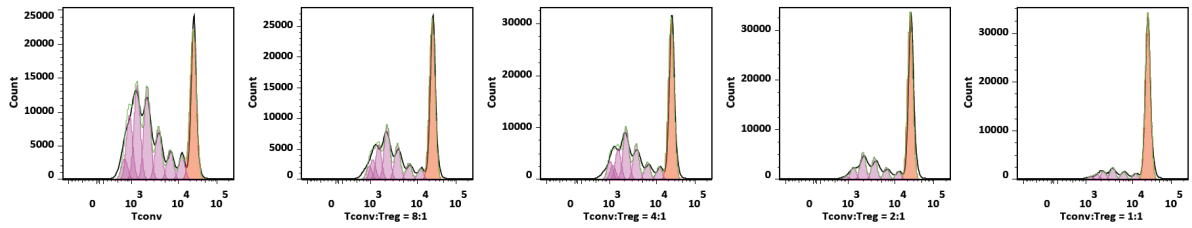



**Figure 5.10 Suppression assay with escalating (from 8:1 to 1:1) Tconv:Treg ratios**

Tconv were stained with a fluorescent proliferation dye and co-cultured with autologous expanded Tregs (1:1 ratio) for five days in the presence of anti-CD3/CD28 beads (Tconv:Treg:beads = 20:20:1). Expanded Tregs suppressed proliferation of $CD4^+$ Tconv. In this figure one experiment is shown.

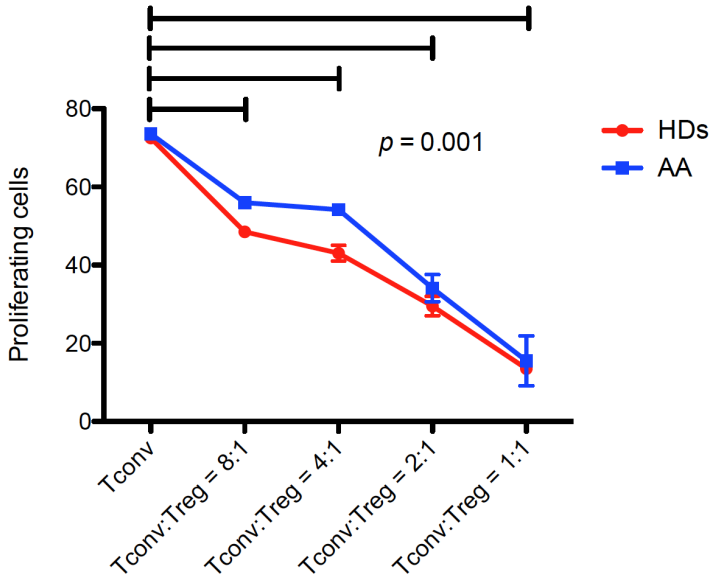



**Figure 5.11 Suppression assay with expanded "total" Tregs at de-escalating ratios (healthy donors and aplastic anaemia patients)**

Tconv were stained with a fluorescent proliferation dye and co-cultured with autologous expanded Tregs (1:1 ratio) for five days in the presence of anti-CD3/CD28 beads (Tconv:Treg:beads = 20:20:1). Expanded Tregs suppressed proliferation of $CD4^+$ Tconv (from Tconv:Treg = 8:1 to 1:1, proliferating cells were 48.5% *versus* 43% *versus* 29.5% *versus* 13.5% for HDs and 56% *versus* 54% *versus* 34% *versus* 15% for AA, respectively, $p = 0.001$, one way analysis of variance, LSD *post hoc* test). The suppressive activity of AA expanded Tregs was not significantly different from HD expanded Tregs. The experiment was repeated twice (two healthy donors and two aplastic anaemia patients).

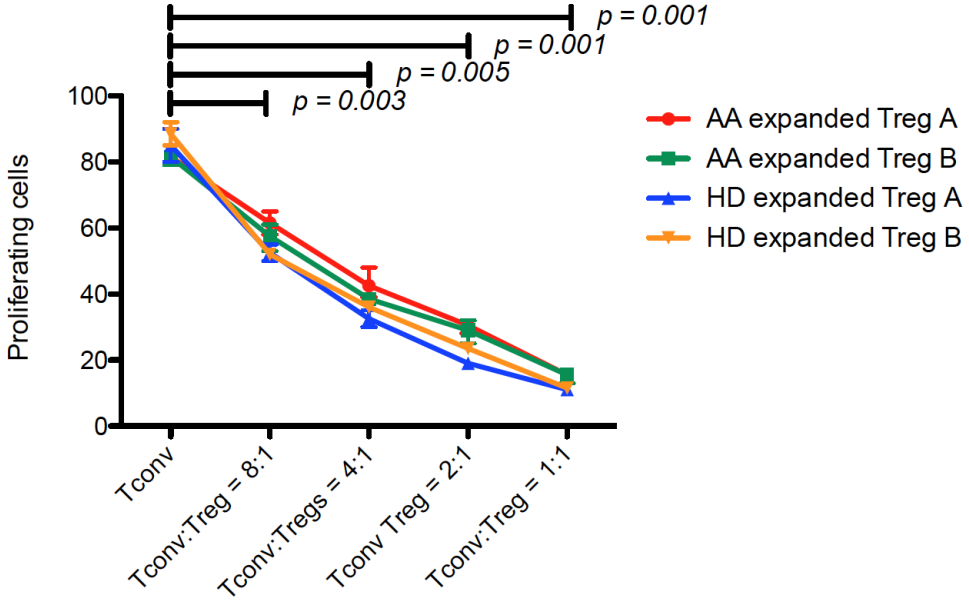



**Figure 5.12 Suppression assay with expanded Treg A and B (healthy donors and aplastic anaemia patients)**

Tconv were stained with a fluorescent proliferation dye and co-cultured with autologous expanded Tregs (1:1 ratio) for five days in the presence of anti-CD3/CD28 beads (Tconv:Treg:beads = 20:20:1). Expanded Tregs (A and B) suppressed proliferation of $CD4^+$ Tconv (from Tconv:Treg = 8:1 to 1:1, proliferating cells were 52% *versus* 32% *versus* 19% *versus* 11% for HDs Treg A, 52% *versus* 36% *versus* 23% *versus* 11% for HDs Treg B, 61% *versus* 43% *versus* 30% *versus* 15% for AA Treg A, 57% *versus* 38% *versus* 29% *versus* 11% for AA Treg B respectively, $p = 0.003$, $p = 0.005$, $p = 0.001$, $p = 0.001$, one-way analysis of variance, LSD *post hoc* test). The suppressive activity of AA expanded Tregs was not significantly different from HD expanded Tregs. The experiment was repeated six times (two HDs and four AA).

The ability of expanded Treg A and B to suppress the secretion of pro-inflammatory cytokines (IFN-γ and TNF-α) was equal (*p = n.s.*). Quite notably, only expanded Treg B were able to suppress IL-17A secretion (*p < 0.05*) (Figure 5.13).

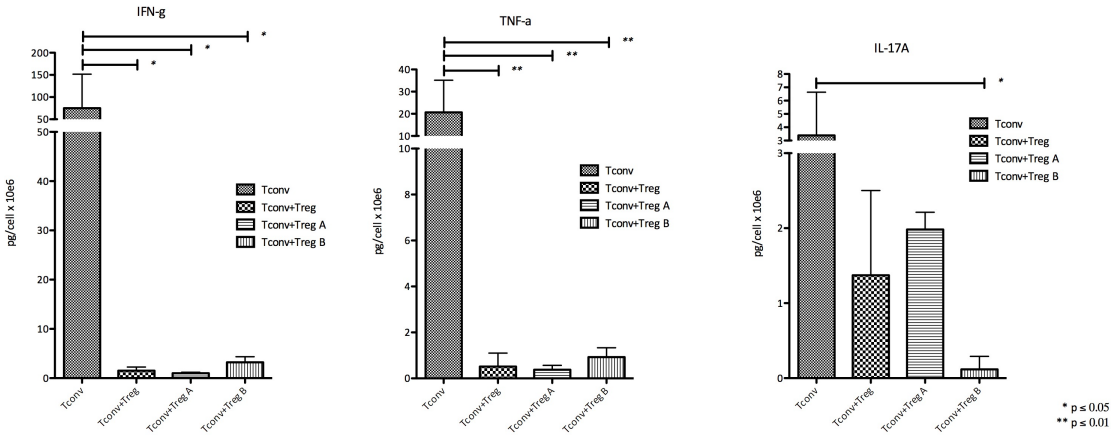



**Figure 5.13 Pro-inflammatory cytokines inhibition by expanded Treg A and B**

Total Treg, Treg A and Treg B equally suppress the secretion of IFN-γ and TNF-α; nevertheless, IL-17A secretion is selectively suppressed by expanded Treg B only. The concentration of each cytokine is expressed as pg/cell x $10^6$. The cytokine concentration was determined using xMAP technology (Luminex$^{TM}$) on the cell culture supernatant according to the manufacturer instructions (one-way analysis of variance, LSD *post hoc* test).

## 5.3.4 Expanded regulatory T cells stability

To assess the stability of expanded Tregs, we investigated the methylation status of 15 cytosine guanine dinucleotide sites within the Foxp3 TSDR[404] by ampilcon sequencing of bisulfite treated DNA on an Illumina MiSeq sequencing platform. TSDR cytosine guanine dinucleotide sites in expanded HDs and AA Tregs were more than 98% unmethylated, confirming the stability of expanded Tregs (Figure 5.14).[187]

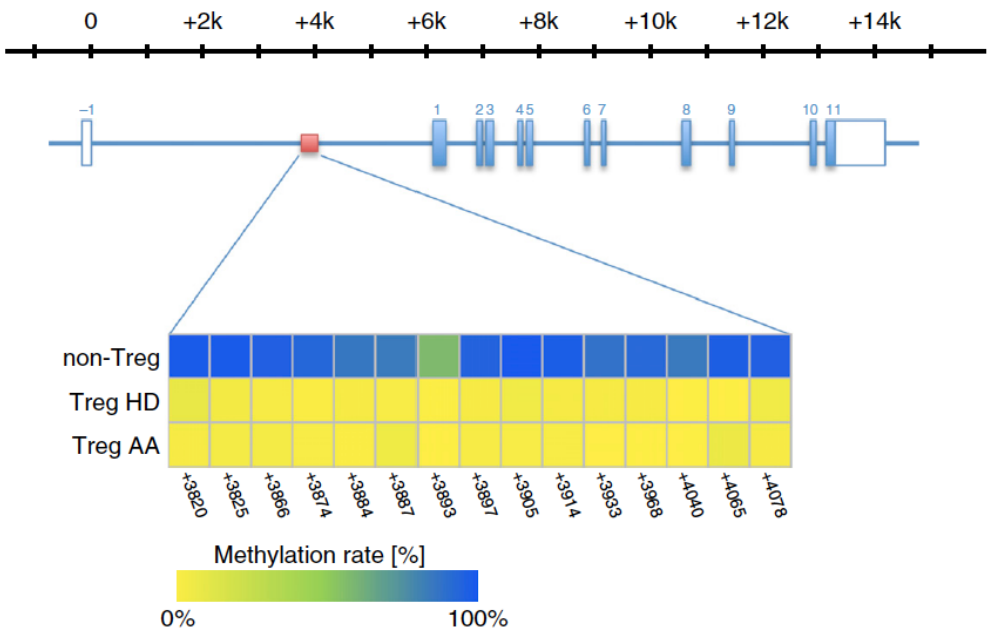



**Figure 5.14 Human *FOXP3* gene *locus* on chromosome Xp11.23 (reverse strand)**

Schematic representation of the human *FOXP3* gene *locus*. The exon/intron structures are in blue, and the TSDR region in red (square). The lower panel shows the methylation statues of TSDR in healthy donors and aplastic anaemia expanded Tregs (yellow squares) compared with non-Tregs (blue and green squares on the upper row).[187]

Treg subsets stability was assessed as well: TSDR CpG sites in the expanded Treg A and Treg B populations from HD and AA patients are 2% and 13% methylated respectively, confirming their stability (Figure 5.15).

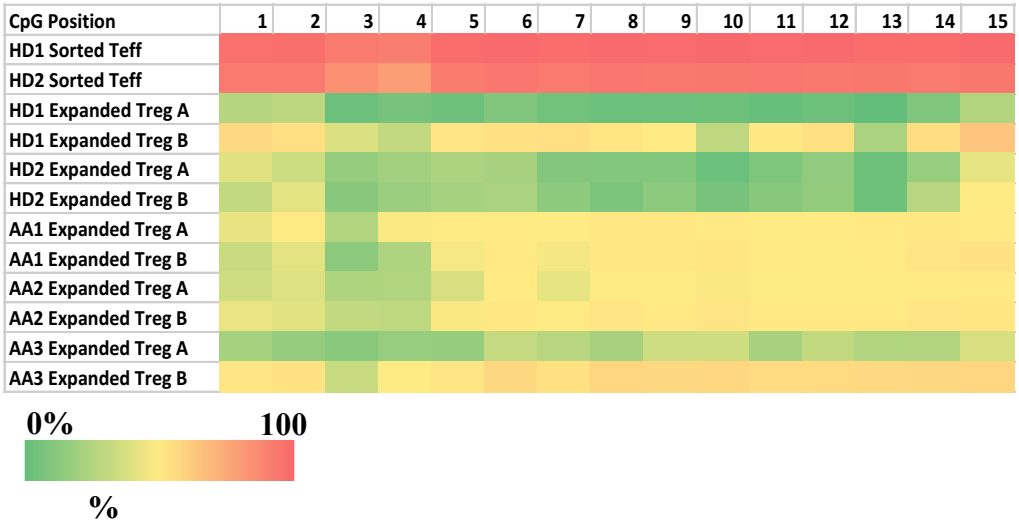



**Figure 5.15 Treg-specific demethylated region of expanded Treg A and B (healthy donors and aplastic anaemia patients)**

FoxP3 Treg-specific demethylated region analysis was performed by deep amplicon bisulphite sequencing. The lower panel (shades of yellow/orange and green) shows the methylation statues of TSDR in healthy donors and aplastic anaemia patients (as indicated in the lefy column) expanded Treg subsets compared with non-Tregs (first and second row, in shades of red).

## 5.3.5 Expanded regulatory T cells clonality

TCR Vβ CDR3 high-throughput sequencing of expanded Tregs was used to investigate their clonality. The normalized Shannon entropy of expanded Treg repertoire was used to calculate the degree of clonality that, on average, was 0.12 (4 expanded Tregs, 1 being "monoclonal" and 0 being "polyclonal"). Both AA and HDs expanded Tregs showed a comparable level of TCR Vβ CDR3 diversity, as defined in Figure 5.16.[187]

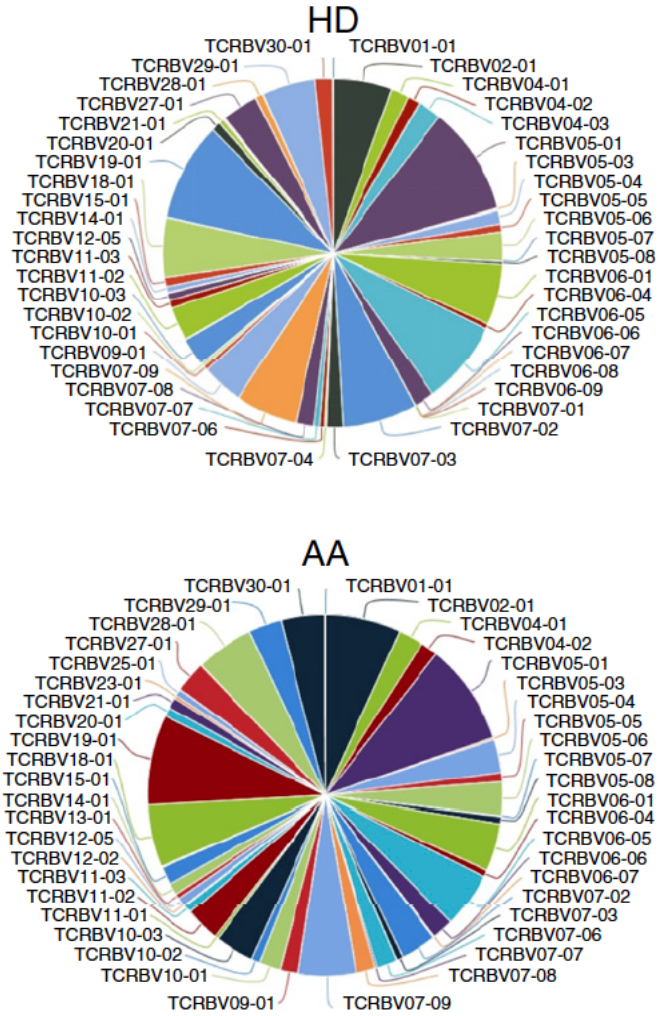



**Figure 5.16 Expanded Tregs clonality by T cell receptor complementarity determining region 3 sequencing**

Amplification and sequencing of T cell receptor B complementarity determining region 3 was performed using immunoSEQ platform (Adaptive Biotechnology), Seattle, WA). A power geometric (PG) index with Horvitz-Thompson type was used as correction for under sampling and Good-Turing coverage adjustment. Each pie chart represents the TCRBV families in expanded "total" Tregs (healthy donor and aplastic anaemia respectively). Each slice of the pie chart represents a TCRVB clone.

Expanded Tregs were polyclonal in both AA and HDs. The normalized Shannon entropy of expanded Treg repertoire was used to calculate the degree of clonality, which, on average, was 0.12.[187]

Expanded Treg A and B were also found to be polyclonal as shown in Figure 5.17.

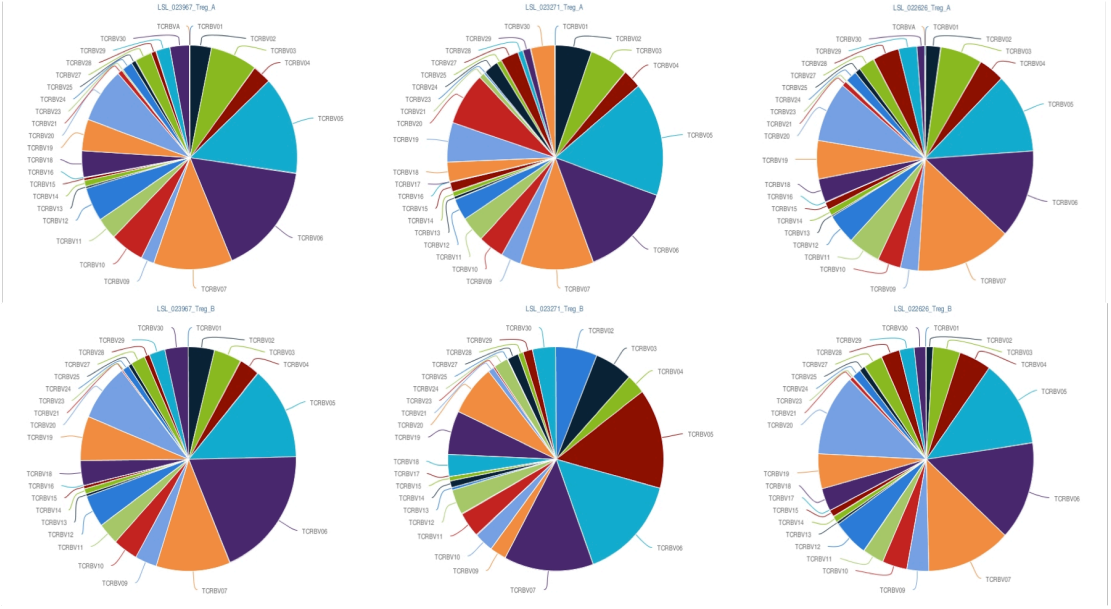


**Figure 5.17 Expanded Treg A and B clonality by T cell receptor complementarity determining region 3 sequencing**

Amplification and sequencing of T cell receptor B complementarity determining region 3 was performed using immunoSEQ platform (Adaptive Biotechnology), Seattle, WA). A power geometric (PG) index with Horvitz-Thompson type was used as correction for under sampling and Good-Turing coverage adjustment. Each pie chart represents the compositions of TCRBV families in each expanded Treg subsets (the top row shows Treg A from three AA patients and the bottom row shows Treg B from the same patients).

Moreover, these Treg subsets seem to originate from different clones, as they do not share the same TCR repertoire (Figure 5.18).

| | LSL_023271_Treg_A | LSL_022626_Treg_A | LSL_023967_Treg_A | LSL_023967_Treg_B | LSL_022626_Treg_B | LSL_023271_Treg_B |
|---|---|---|---|---|---|---|
| LSL_023271_Treg_A | | 0.009 | 0.007 | 0.003 | 0.003 | 0.003 |
| LSL_022626_Treg_A | 0.009 | | 0.002 | 0.006 | 0.005 | 0.001 |
| LSL_023967_Treg_A | 0.007 | 0.002 | | 0.004 | 0.003 | 0.001 |
| LSL_023967_Treg_B | 0.003 | 0.006 | 0.004 | | 0.002 | 0.000 |
| LSL_022626_Treg_B | 0.003 | 0.005 | 0.003 | 0.002 | | 0.000 |
| LSL_023271_Treg_B | 0.003 | 0.001 | 0.001 | 0.000 | 0.000 | |

**Figure 5.18 TCR overlap between expanded Treg subsets**

The figure represents TCR overlap between expanded Treg A and B from three AA patients. It shows an overlap metric value for each possible pair-wise percent sharing between all pairs of samples. This is computed by averaging across the two ratios of shared reads over total reads for each sample. A power geometric (PG) index with Horvitz-Thompson type was used as correction for under sampling and Good-Turing coverage adjustment

## 5.3.6 Expanded regulatory T cells plasticity

To investigate their plasticity, "total" Tregs, Treg A, and Treg B were cultured for five days in a Th17 polarizing culture (containing IL-1β, IL-6, IL-2). After five days of culture, only total Tregs started secreting IL-17A ($p = 0.013$), but neither Treg A, nor Treg B did (Figure 5.19). Due to the low cell numbers and the rarity of the disease, these experiments were performed on HDs only.

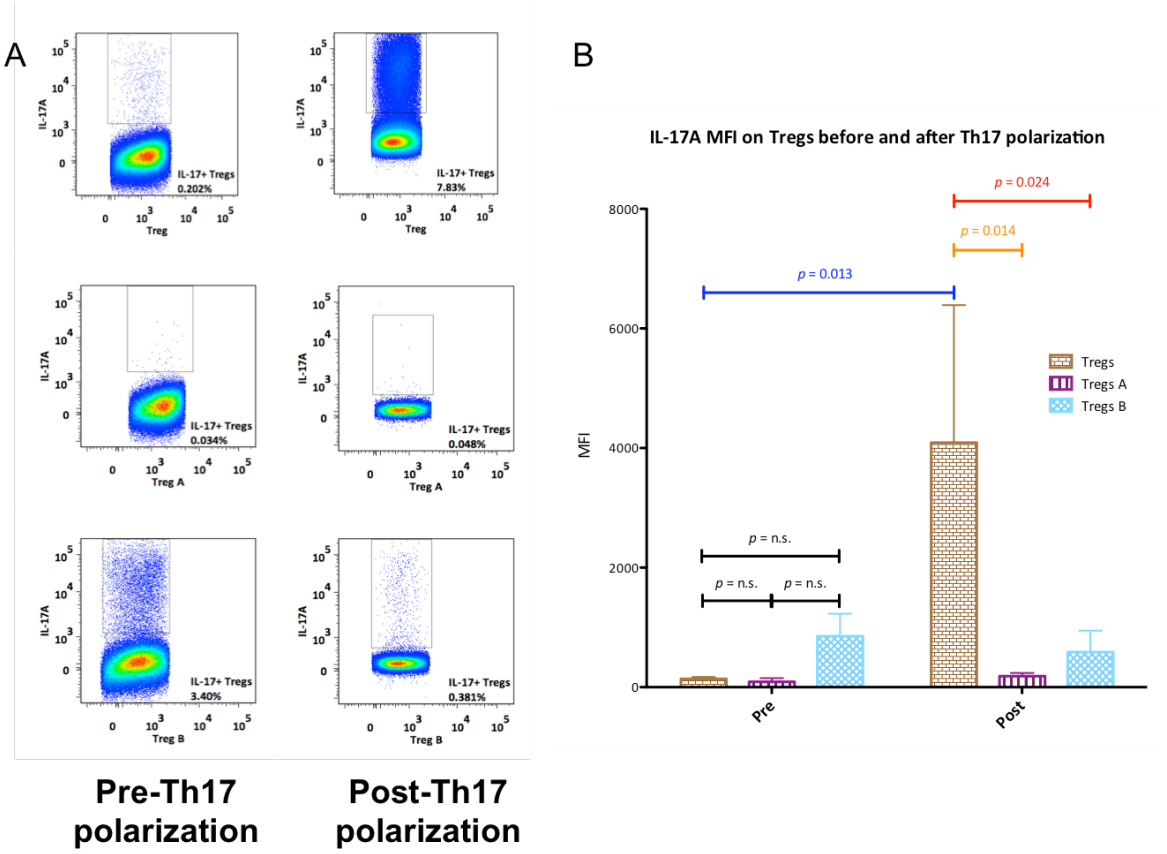



**Figure 5.19 Expanded Tregs and their subsets plasticity**

"Total" Tregs, Treg A, and Treg B were cultured for five days in a Th17 polarizing culture (containing IL-1β, IL-6, IL-2 as described at page 202). After five days of culture, only total Tregs started secreting IL-17A ($p = 0.013$, unpaired Student t test), but neither Treg A, nor Treg B did. (A) One representative experiment. (B) Combination of three experiments (healthy donor only).

Despite IL-17A secretion, Tregs were still able to suppress autologous Tconv proliferation (n = 3, $p = 0.001$, one was analysis of variance), as shown inFigure 5.20.

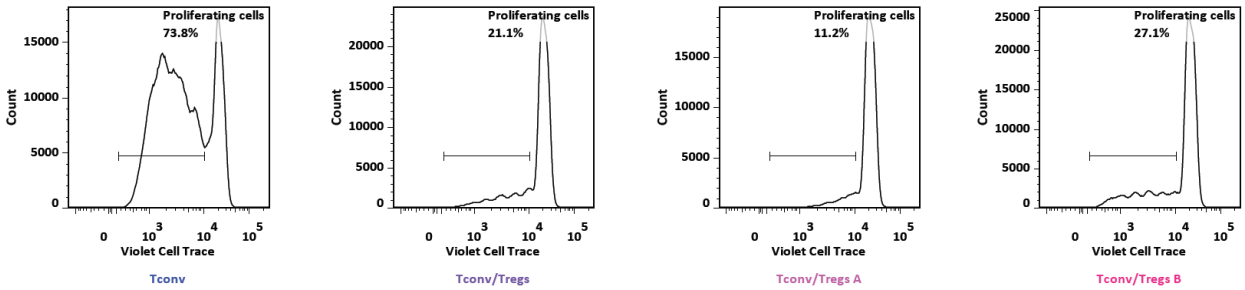



**Figure 5.20 Suppressive function of Tregs and their subsets after culture in a Th17 polarising environment**

After five days of culture in IL-1β, IL-6, IL-2, autologous Tconv were stained with VCT (as described at page 120) and co-cultured with Tregs ("total Tregs, Treg A and Treg B in a Tconv:Treg:bead = 20:20:1) for five days. Cells were harvested, stained for live/dead and surface CD3/CD4 and analysed on a Canto II (BD).

### 5.3.7 Expanded regulatory T cells become apoptosis resistant through phosphorylated-B cell lymphoma-2

Bcl-2, encoded in humans by the BCL2 gene, is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis).[405, 406] BCL-2 is localized to the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins.[407] Considering Bcl-2 antiapoptotic role, we decided to use it as an anti-apoptotic marker to assess apoptosis sensitivity after expansion. As shown in Figure 5.21, the phosphorylated form of Bcl-2 is significantly more expressed in expanded Treg A and B, more evidently in AA patients, hence demonstrating that *ex vivo* culture of Tregs may increase their resistance to apoptosis.

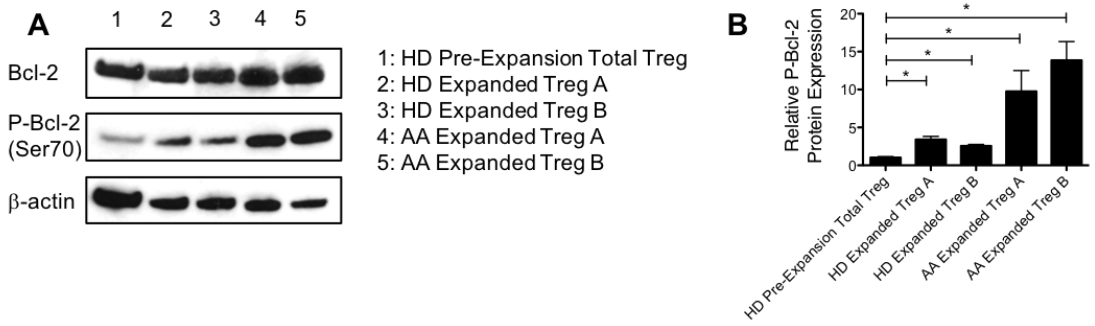



**Figure 5.21 Bcl-2 expression in Tregs subsets pre- and post-expansion**

Western blot was performed in total Tregs (pre-expansion) and Treg A and B (post-expansion) (A). Relative expression of Bcl-2 in higher in expanded Treg (both subsets A and B when compared to pre-expansion (B). * $p < 0.01$, one way analysis of variance.

### 5.3.8 Injected regulatory T cells longevity in mice

As previously described (page 154), Tregs were transduced with lentivirus and injected in mice as shown in Figure 5.22. After injection with D-luciferin, serial images were taken at 4, 24, 48 hours and then weekly. As shown in the figure, transduced Tregs were detectable in mice organs up to 14 days after injection.

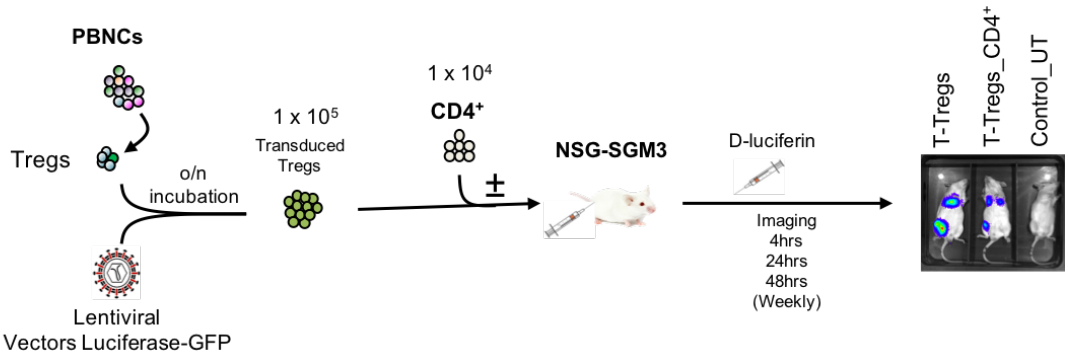



**Figure 5.22 Lentivirus-transduced Tregs longevity in mice**

1 x $10^5$ Tregs were used to produce GFP Luciferase transduced T regulatory cells. Prior transduction, Tregs were pre-activated by using CD3/CD28 beads in a 1:1 ratio in a media containing rapamycin and ATRA in a 24 well plate (for media composition, please refer to "Regulatory T cells expansion"). Cells were incubated overnight at 37 ºC in a humidified incubator with 5% $CO_2$. Next day, activation beads were removed by mixing the cells with a pipette and then incubating the cells on a magnet, followed by removing the supernatant that contained the Tregs. Following on, Tregs were infected once with lentiviral supernatant (multiplicity of infection of 1:30). After 24 hours, cells were washed with PBS (with 2% FCS and P/S) and fresh media (same solution of rapamycin and ATRA) was added (1 x $10^5$ cells/ml). Cells were incubated for up to 7 days. Luciferase-transduced Tregs were isolated based on their GFP expression using by using BD Aria. Non-transduced Tregs were used as a control from day 1.

## 5.3.9 *In vivo* use of expanded regulatory T cells

To evaluate the suppressive ability of the expanded Tregs in *in vivo*, we used NOD/SCID/IL2rγ−/−/IL-3/GM/SF (NSG-SGM3) humanized mice (Figure 5.23).

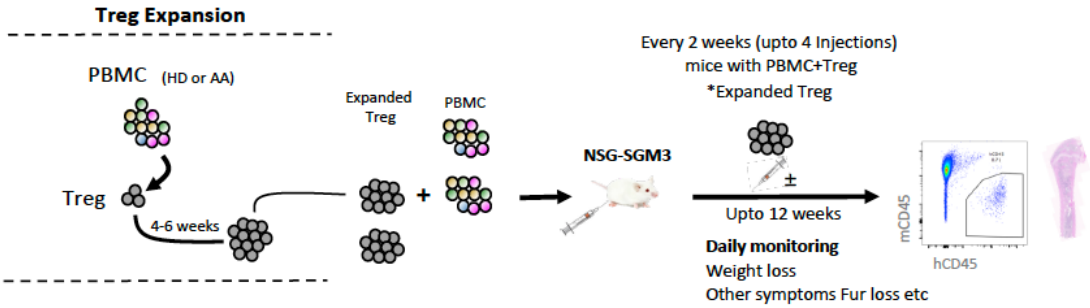



**Figure 5.23 *In vivo* use of Tregs: schematic representation of the animal work protocol**

Schematic representation of isolation of Tregs, followed by xenotransplantation and downstream analysis. Human healthy donors' peripheral blood mononuclear cells were injected into NSG-SGM3 mice with or without expanded Tregs (1:1 ratio; 10 x $10^6$). Following on, mice that were injected with PBMC + Tregs also received additional doses of Tregs every two weeks post-injection. Two weekly Treg injection doses were chosen based on earlier experiment showing the exhaustion of Tregs after five weeks in mice.

NSG-SGM3 mouse model has been previously shown to efficiently support the development and maintenance of human Tregs.[408] Firstly, to study the kinetics of human Tregs *in vivo*, HD Tregs were transduced with a bi-cistronic vector co-

expressing GFP and luciferase, and then co-injected with or without human healthy donors $CD3^+$ cells in NSG-SGM3 mice (Figure 5.24).

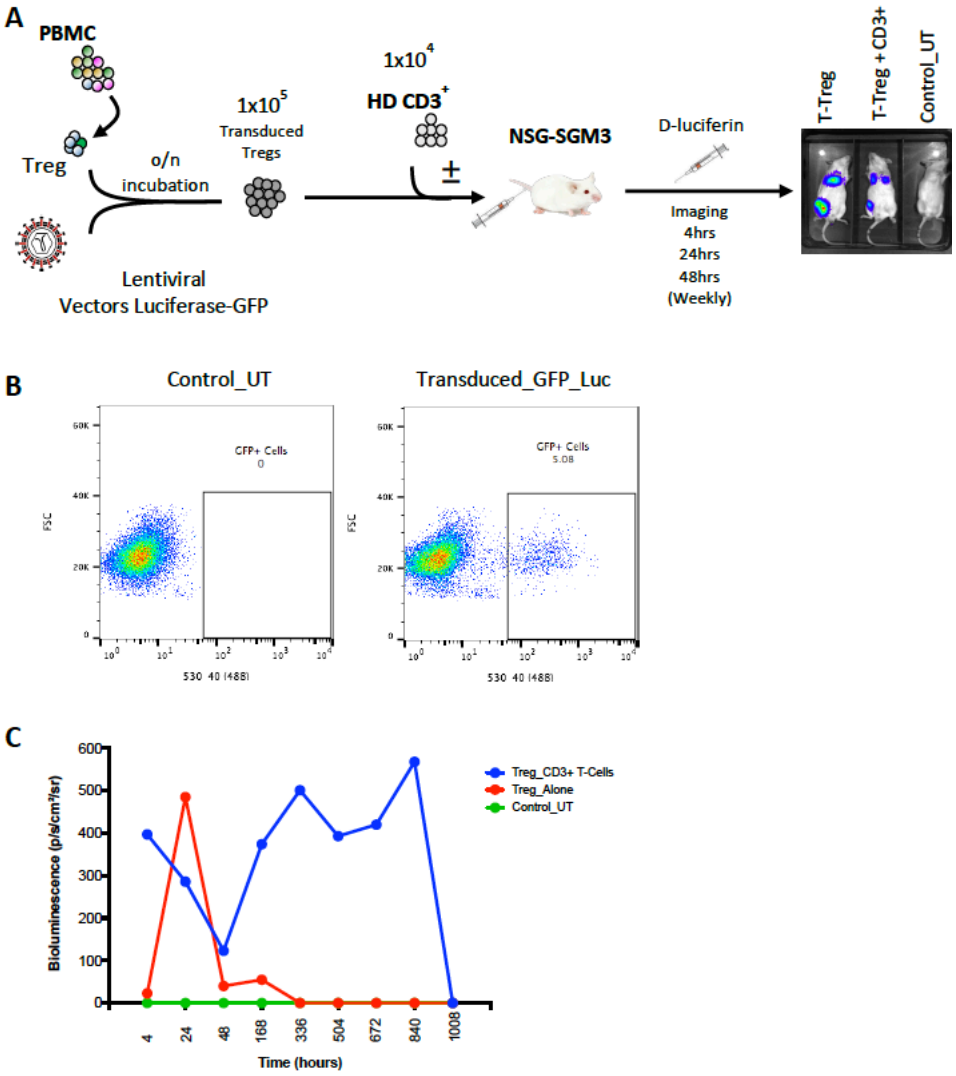



**Figure 5.24 Kinetics of healthy donors' Tregs**

(A) Schematic representation of isolation of Tregs, lentiviral transduction of Tregs with GFP-Luciferase vector, followed by xenotransplantation and *in vivo* imaging. (B) Transduction efficiency of Tregs as shown by GFP-positive cells on the right dot plot. (C) Bioluminescence (expressed in p/s/cm$^2$/sr) of mice recipient injected with $CD3^+$ alone (n=1 – blue line), $CD3^+$ T cells with transduced Tregs (n=1 – red line) and un-transduced Tregs alone (n=1 – green line). UT: Untreated cells.

Using whole body bioluminescence imaging, we were able to detect Tregs for up to 5 weeks in mice that were co-injected with $CD3^+$ T cells (Figure 5.24).

Next, human HD PBMCs were injected into NSG-SGM3 mice with or without expanded Tregs (1:1 ratio; 10 x $10^6$). Following on, mice that were injected with

PBMC + Tregs also received additional doses of Tregs every two weeks post-injection (Figure 5.24 C).

This two weekly Treg injection doses were chosen based on our earlier experiment showing the exhaustion of Tregs after 5 weeks in mice (Figure 5.24 C). Tregs or PBMC + Tregs recipient mice showed no, or little clinical disease symptoms associated with xeno-GVHD. Tregs or PBMC + Tregs recipient mice had less body weight lost as compared to mice injected with PBMC alone (Figure 5.25).

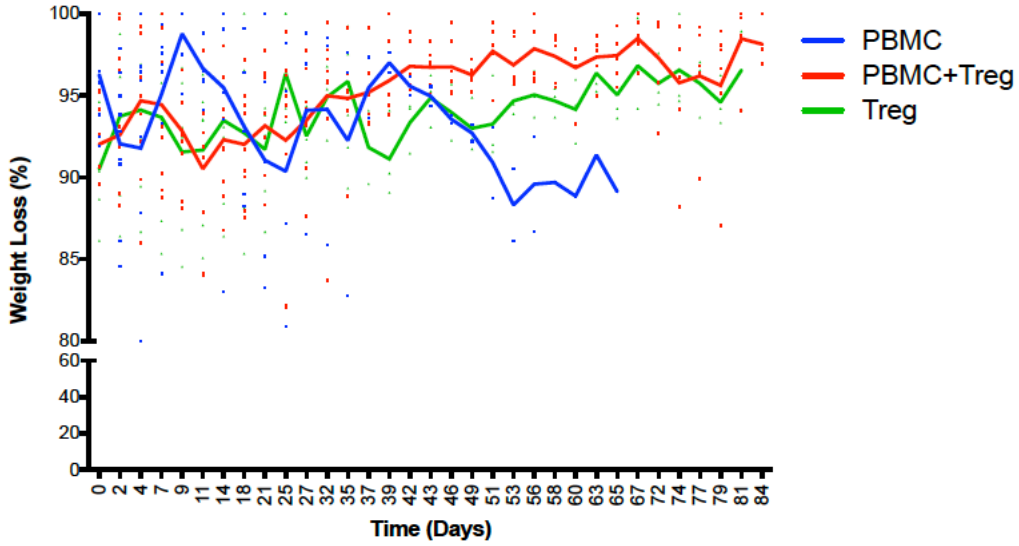



**Figure 5.25 Measurement of body weight loss in our animal model**

Measurement of body weight loss (representing GVHD disease clinical feature) in recipient mice that were injected with healthy donors' derived peripheral blood mononuclear cells without (n=14 – blue line) or with Tregs (autologous or allogenic – red line) (n=9) or Tregs alone (n=3 – green line).

Notably, mice injected with either Tregs or PBMC + Tregs had a significantly better overall survival compared to the mice injected with PBMCs alone (Figure 5.26).

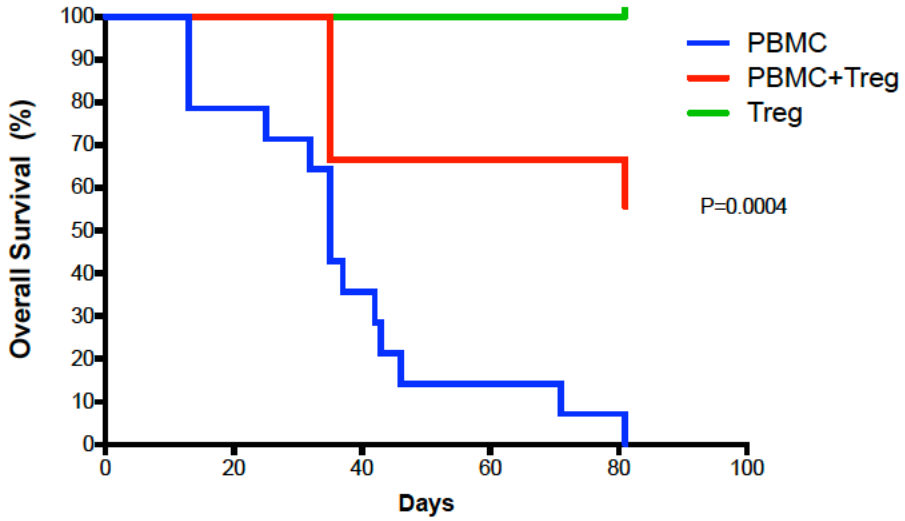



**Figure 5.26 Effect of *ex vivo* expanded Tregs injection: mice overall survival**

Overall survival of mice that were injected with healthy donors derived peripheral blood mononuclear cells without (n=14 – blue line) or with Tregs (autologous or allogenic – red line) (n=9) or Tregs alone (n=3 – green line). Cumulative survival calculated according to Kaplan-Meier method.

Following the xenotransplantation, male recipient mice had a better survival than female counterparts (data not shown). Histological assessment of mouse femurs and spleen at the time of death illustrated that tissue from PBMCs recipient mice had acellular bone marrow and severe disruption of tissue architecture in the bone marrow as well as spleen (Figure 5.27).

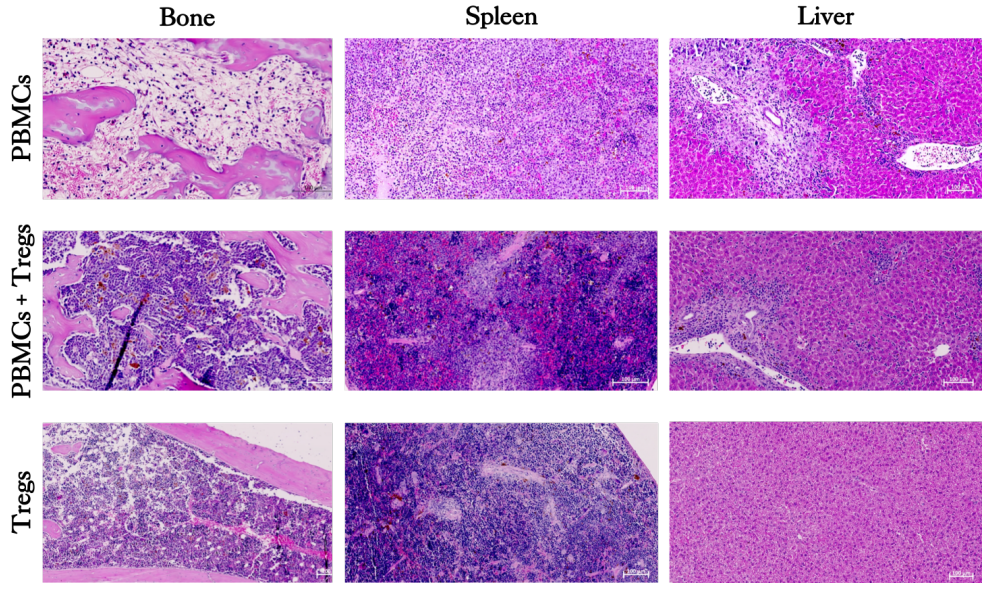



**Figure 5.27 Haematoxylin and eosin (H&E) staining of bone, spleen, and liver**

H&E staining shows an increased cellularity in mice injected with Tregs. Spleen and liver slides show a reduced lymphocyte infiltrate, as to suggest an attenuation in organ specific GVHD.

Morphological assessment of the BM sections demonstrates atypical haematopoiesis with prominent fibrotic tissue in mice injected with PBMC alone. However, mice injected with Tregs with or without PBMC have normocellular marrow with no or little evidence of marrow fibrosis (Figure 5.27).

We next tested the expanded Tregs from AA patients in NSG-SGM3 mice. Similar to HD Tregs, AA expanded Tregs (1:1 ratio; up to 4.5 x $10^6$ cells) provided the protective effect from xeno-GVHD symptoms, and mice with either Tregs or PBMC + Tregs survived significantly longer compared to the mice injected with PBMC alone (Figure 5.28).

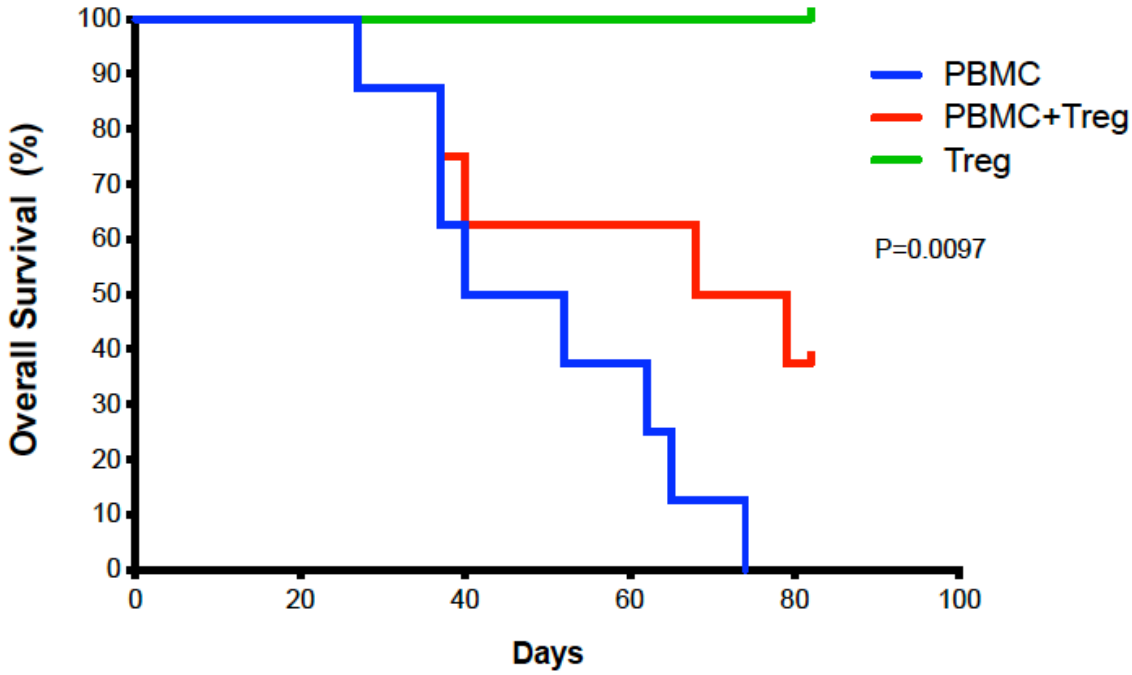



**Figure 5.28 Injection of *ex vivo* expanded aplastic anaemia Tregs: effect on mice overall survival**

Overall survival of mice that were injected with aplastic anaemia patient-derived peripheral blood mononuclear cells without (n=5 – blue line) or with aplastic anaemia Tregs (autologous or allogenic red line) (n=8) or AA Tregs alone (n=2 – green line). Cumulative survival calculated according to Kaplan-Meier method.

It is noteworthy that the GVHD suppressive ability of healthy donors Tregs was better compared to aplastic anaemia Tregs, even though recipient mice with HD cells received higher doses of PBMCs (AA: 1.75 - 4.5 x $10^6$ *versus* HD: 10 x $10^6$).

## 5.4 Summary of data

As mentioned previously, therapeutic options for refractory or relapsed AA are limited, especially in the elderly patients setting or when a matched related donor is not available.[163] This is the reason why we have chosen to focus on Treg expansion, to possibly develop another therapeutic tool for AA. On the other hand, expanding Tregs and "safely" injecting them to AA patients also presents its risks.

Regulatory T cell functional stability represents a challenge for using Tregs for immunotherapy as a minor population of $Foxp3^+$ cells loses Foxp3 expression overtime; these "ex-$Foxp3^+$" cells may display an activated conventional T cell phenotype and become pathogenic *in vivo*.[156] Loss of Foxp3 expression has been associated with a pro-inflammatory microenvironment and switching to an effector T cell phenotype characterized by IL-17 and IFN-γ secretion.[158, 409, 410] While Tregs delivered to a normal host tend to retain their suppressive function, a proportion of Tregs adoptively transferred into a lymphopenic environment may differentiate into pathogenic T cells.[159, 411] Despite the fact that some co-stimulatory pathways differentially affect conventional T cells *versus* Tregs, no single pathway completely selects for a specific T cell subset.[387, 412] Therefore, administration of pharmaceuticals that stimulate Tregs may also activate conventional T cells (off-cell effect).[387] In addition to that, Tregs are low in number in AA[185] and it is very difficult to expand them *in vivo* with conventional IST.[187]

Therefore, before testing their efficacy *in vivo*, we have proved their sensitivity to IL-2, their expandability, function, stability, and scarce plasticity in a pro-inflammatory environment. Then we have tested their function in an *in vivo* model.

Freshly isolated PBMCs (2 HDs and 6 AA) were cultured with low doses of IL-2 and STAT5 phosphorylation (investigated by flow cytometry) increased equally, with no differences between HDs and patients. After having established that AA Tregs are sensitive to IL-2, we were also able to prove that, if cultured in a "Treg-friendly" environment, Tregs can also be expanded *in vitro*. Interestingly, both HDs and AA Tregs expand *in vitro*, with a post-expansion purity > 90% and a Treg B-like immunophenotype. As a Foxp3 positivity is not enough to define human Tregs, we have also proved their ability to suppress autologous Tconv proliferation (in an *in vitro* suppression assay with Tconv:Treg ratios ranging from 1:1 to 8:1) and pro-inflammatory cytokines secretion.

Typically, AA is preceded by a viral infection, often clinically silent. Therefore, we may "miss" the pathogenetic evolution and the patient comes to the clinician's attention when the blood count is already very low. If we will be successful in taking this project to a clinical trial, we must consider that we will have to deal with very low cell numbers, especially with low Treg count (with Treg B being even lower in non-responders). Therefore, we have assessed Treg subsets expandability and, interestingly, we have found that Treg A and B grow equally in HDs but in AA Treg A expand more than Treg B. This finding was quite surprising, as we would expect Tregs B to proliferate more, considering they are enriched in cell cycle genes. One possible explanation, although just a speculation, is that Tregs A need more IL-2 to enter the cell cycle and a potential "local" IL-2 deprivation prevents this subset to proliferate. Once expanded *ex vivo* and exposed to very high (non-physiologic) IL-2 concentration, they enter the cell cycle and proliferate vigorously. Additionally, as

Tregs B are more prone to proliferate, they may be more "exhausted" from a cell cycle pint of view as they have been to more cycles compared to Tregs A.

Expanded Treg subsets can suppress the proliferation of autologous Tconv with no difference, but only expanded Treg B are able to suppress the secretion of IL-17A.

As already stated, growing Tregs *in vitro* is only the first aspect. The second one was to prove their stability through high-throughput sequencing of their TSDR: Tregs and their subsets have shown a low methylation of the TSDR, confirming their stability. Having assessed that we were able to expand functional and stable Tregs, we moved on to investigate their clonality and TCR sequencing data have shown that both "total" Tregs and their subsets (A and B) are polyclonal and almost don't share any TCR sequence.

$CD4^+$ T cells are known to be "plastic", able to switch from one phenotype to another unless terminally differentiated.[110] Treg plasticity, after injection to AA patients, where the microenvironment is probably pro-inflammatory, could be detrimental and counterproductive. Therefore, we tested the *in vitro* plasticity of "our" expanded Tregs and we have shown that only the expanded total Tregs could be induced to secrete IL-17A, but neither Treg A, nor Treg B. Our hypothesis is that Treg III (which is included in total Tregs, but almost absent from A and B), the cytokine secreting Tregs, "contaminate" the starting cells population and preferentially expand despite the presence of rapamycin. In addition to their stability, expanded Tregs (both A and B from HDs and AA patients) show an increased resistance to apoptosis, as demonstrated by the increase in phosphorylated Bcl-2 protein.

We can conclude that AA Tregs are expandable, have a Treg-like immunophenotype, are functional, stable, cannot be induced to secrete IL-17A, and increase phosphorylated Bcl-2 expression.

Having assessed their "safety" *in vitro*, we investigated their use *in vivo*. Although the subtypes of AA patients involved in this study were heterogenous, we hereby demonstrated that *in vitro* expanded Tregs from AA are functional and stable with minimal plasticity, giving the potential of using these expanded Tregs as an additional strategy to restore the Treg reduction in AA patients which may improve the clinical outcome of the standard treatment using IST. Indeed, by using NSG-SGM3 mouse model we have shown the efficacy of *in vitro* expanded Tregs in protecting mice against GVHD. While AA and HD Tregs were able to similarly suppress the proliferation of Tconv *in vitro*, the efficacy of AA expanded Tregs in suppressing GVHD *in vivo* was found slightly lower than the HD expanded Tregs. These differences in the *in vivo* immunosuppressive ability of AA and HD Tregs might be due to the pre-activation of T effector cells from AA patients which were co-injected with Tregs and was not the case in HD T effector cells.[185,413] In other words, AA expanded Tregs were inhibiting less "suppressible" T effector cells compared to HD counterparts. This could be a less problematic issue in a clinical setting where AA patients would receive IST first, that would eliminate the activated T effector cells.

# 6 GENERAL DISCUSSION AND FUTURE DIRECTIONS

## 6.1 Background and research questions

Aplastic anaemia is a rare form of bone marrow failure and, without appropriate treatment, carries high mortality and morbidity rates. There is a geographical variation in the incidence, with two to three cases per million per year in the Western population but it is three-fold higher in Asians. AA shows two peaks of incidence, typically in the first three decades of life, with a median age of approximately 20 years, while a second peak occurs around the age of 60.[164, 414-416]

Immune dysregulation is a key component in the pathogenesis of AA. In the idiopathic form of AA, that may follow a preceding viral infection, T cell mediated autoimmunity against autologous bone marrow stem cell progenitors leads to defective or absent haematopoiesis. It is hypothesised that unidentified antigenic exposure leads to a polyclonal expansion of $CD4^+$ T cell dysregulation and an overproduction of pro-inflammatory cytokines such as IFN-γ and TNF-α. In addition, oligoclonal expansion of a dysregulated cytotoxic $CD8^+$ T cell population has also been demonstrated in *ex vivo* bone marrow models of AA patients.[417-420]

Increases in Th17 cells are also demonstrable in the peripheral blood and bone marrow of AA patients.[421] The increase in Th17 cell elevation seems to correlate with the amount of IFN-γ-producing cells and overall disease severity; furthermore Th17 cell numbers correlate negatively with Treg populations.[421] The concept of aberrant, disordered T cell populations initiating and propagating the pathology of AA is also supported by the finding that that T cell directed immunosuppressive therapy such

as the combination of ATG and CsA are able to induce response in up to 80% of patients with severe aplastic anaemia.[422]

$CD8^+CD57^+$ cells, a subset of T cells known as effector memory cells that activate as a result of antigen affinity and stimulation, are shown to be expanded in the peripheral blood of AA patients and demonstrate features of oligoclonality.[423] Given the role of $CD8^+CD57^+$ effector memory cells in immune surveillance, their expansion may precipitate the abnormal oligoclonal expansion seen in AA.[423]

Regulatory T cells (Tregs) also play an important role in the pathogenesis of AA. A marked quantitative and qualitative deficit in Tregs, which normally suppress autoreactivity of other T cell populations to normal tissue including cells in the bone marrow microenvironment and HSCs, has been shown in AA.[182, 186]

However, the definition of human Tregs and their subtypes could be challenging and controversial.

Earlier studies of HLA expression in AA have also supported their role in provoking the aberrant T cell homeostasis based on the finding of a higher frequency of both HLA-DR2 and HLA-DR15 presence in AA patients.[424, 425] The overrepresentation of these specific HLA molecules in AA patient may effectively augment the otherwise physiologic and constitutive expression of HLA class II molecules on antigen-presenting cells to interact with $CD4^+$ T cells and ultimately present a HSC-derived antigen resulting in subsequent HSC immune-mediated destruction.[424] In addition, cells expressing the HLA-DR2 antigen have been previously shown to augment the release of TNF-$\alpha$.[425]

The main therapies for AA are HSCT and IST. For patients who do not have a sibling donor for HSCT, the standard first line IST is the combination of horse ATG

(ATGAM®; Pfizer, New York, NY, USA) and CsA with no indication for routine use of G-CSF with ATG + CsA.[212] Unfortunately, not all the elderly patients (especially if with severe comorbidities) are eligible for this treatment, as it has several toxicities and a poor tolerance, often requiring long hospitalisation. Moreover, relapsed or refractory patients are another challenging cohort of patients to manage.[426] Until recently, there have been only limited data for the management of patients with refractory or relapsed AA after IST with horse ATG and CsA. A multicentre study in Italy for the second course of rabbit ATG and CsA showed an overall response rate of 77%, including 30% complete response rate, in patients refractory to one course of horse ATG and CsA.[427] NIH also analysed the outcomes of rabbit ATG and CsA for patients who were refractory or relapsed after one course of horse ATG and CsA. This study showed an overall response rate of 30 % in refractory patients and 65% in relapsed patients.[428] A third course of ATG-containing IST showed benefit only in previous responders, but not in patients who were refractory to previous IST.[429] On the other hand, cyclophosphamide or alemtuzumab can be used as alternative IST regimen in these patients.[242, 430-434] Despite the results of IST have been improved in the past two decades, treatment failures such as unresponsiveness, relapse, and clonal evolution remain a major problem and many studies, which conducted intensified IST by adding other immunosuppressants, failed to show noteworthy results to improve response and survival.[426]

Another challenging aspect of treatment with IST, is the lack of reliable biomarkers to predict response to therapy.

With this in mind, this PhD project was aimed to try to address the following research questions:

1. Is there a specific immune signature that predicts the response to IST in AA?
2. Are Tregs from AA suppressive?
3. What is the mechanism of Treg B reduction in AA?
4. Why the other subset of Tregs fail to proliferate and compensate the lack of Treg B population?
5. Are AA Tregs expandable *in-vitro*? If so, can we use them as a potential therapy in AA?

## 6.2 Summary of Findings

### 6.2.1 Immunosuppressive treatment responders and non-responders have different regulatory T cell signatures

It is already established that Tregs play an important role in the pathophysiology of autoimmune diseases, less clear is the significance of Treg subsets. Tregs identification is challenging, as generally the numbers are low, and they may express aberrant markers. By using CyTOF technology, it is now possible to extensively characterize rare, heterogeneous populations of cells with minimal bias.[342, 355] By using multidimensional phenotyping and unbiased approach, I was able to characterise two distinct Treg subpopulations both in HDs and AA patients and the changes in these subsets were able to predict response to IST.[187] It was possible to identify two well-defined subpopulations (Treg A and B). Although total Treg numbers were reduced in AA, Treg A were significantly higher in AA patients compared with HDs. In contrast, the number of Treg B subpopulation was significantly lower in AA patients compared with HDs (Table 3.4). Subpopulation B

was characterized by a lower expression of CD45RA, CD7, and CD27 and a higher expression of CCR4, CCR6, CD25, CD28, CD45RO, CD95, CXCR3, Foxp3, and HLA-DR. The most significantly different markers were CD95, CCR4, and CD45RO.[263, 359, 360] The identified Treg subpopulations were compared with established Treg subpopulation definitions by the Sakaguchi group.[354]

Although Tregs A and B overlap with Treg subpopulations I and II respectively, this approach can highlight those Treg III closer to Tregs, and to combine them with subset A or B based on their phenotype. This eliminates cells that are closer to Tconv, therefore less likely to be regulatory (the so-called "cytokine-secreting Tregs).[187]

As explained above, there is an unmet need for more robust predictive factors for response to IST.[187] ATG and CsA is a treatment with potentially serious side effects and a documented endothelial toxicity, therefore not every patient is suitable to receive such treatment. This is more evident in elderly patients, who often suffer from cardiovascular diseases. Therefore, for elderly patients not eligible for IST, therapeutic options are scarce, hence the importance of having response predictors to treatment.

This specific immune signature identified by our group demonstrated to predict response to IST at time of AA diagnosis. Non-responders to IST seem more likely to have higher Treg A number compared with non-responders, whereas responders had higher Treg B number compared with HDs (Figure 3.9). CyTOF technology allowed to investigate and use around thirty markers (both surface and intracellular) to differentiate Tregs A and B. Nevertheless, this technology has not been

implemented in the day-to-day clinical practice, therefore I validated CyTOF results with conventional flow cytometry, routinely used for clinical purposes.

In aplastic anaemia, Treg B are characterized by a more "activated/memory" phenotype, as suggested by the fact that they are $CD45RO^{+}$.[187] The cytokine profile upon PMA/ionomycin and brefeldin A stimulation showed that T cells with pro-inflammatory properties (such as secretion of IFN-γ and TNF-α) clustered outside the "Treg area". Despite the used panel was specifically designed for Tregs, it allowed also to study Tconv and to identify only one difference: CD161 expression is higher in non-responders. This needs further investigation as the clinical significance of its increased expression is not very well understood yet.

### 6.2.2 Different regulatory T cell subsets are not equally functional and have different ontogeny and propensity to FAS/FAS-L mediated apoptosis

After identifying an immune signature for aplastic anaemia based on Treg subpopulations, [187] I assessed whether these two Treg subsets were functionally and ontogenetically different to establish if they could be potentially used, once expanded, as a "cellular therapy" for AA. Functionally, only freshly isolated Treg B were able to suppress the pro inflammatory cytokine secretion by autologous Tconv (IFN-γ mainly). The different functional profiles suggests that Treg A and B are two distinct Treg subpopulations, confirming the mass cytometry data from AA samples.[187] The next step was to establish if Treg A and B are "just" different phenotypically and functionally, or if they proliferate from a different "cell of origin" altogether. The TCR was used as a hallmark of "clonality" and it emerged that both

subsets are polyclonal and share very little TCR sequences, suggesting a possible distinct developmental origin.[187]

Considering that subset A and B show clear distinguishing features, I wanted to investigate if they also have a distinct gene signature or if their differences are only "environmentally induced and inducible". GEP analysis demonstrated that both Treg A and B subpopulations have a Treg gene signature, but Treg B were enriched with Treg-related memory/activation genes compared with Treg A, including *JAKMIP1*, *CCR8*, *TRIB1*, and *GZMK* (Figure 4.7).[187] Moreover, Treg B showed an activation gene signature, in agreement with our mass cytometry findings.[187] Gene set enrichment functional analysis[346] highlighted several gene sets as significantly overexpressed in Treg B, including G2M checkpoint, mitosis, M phase of mitotic cell cycle, IL-2-STAT5 signalling, and immune response genes.[187] Ontology analyses of the functions of these protein complexes showed they are involved in mitotic functions, DNA replication, and cell cycle-dependent transcription. Therefore Tregs B have a specific gene signature which makes them more prone to proliferate and enter the cell cycle.

However, it was not clear as why Treg B are reduced in aplastic anaemia and why Treg A do not "over-proliferate" to compensate this relative reduction in Tregs. In other words, the mechanistic reason of this Treg B "loss" was not clear.

In principle, a cell subset could be reduced in number either because that specific subset proliferate less (and this does not seem to be the case as Treg B are enriched in cell cycle genes), or because that specific subsets goes through apoptosis more. Considering all the previous findings, I have hypothesised that the latter could be the mechanistic aspect to be investigated in more depth. The "proof of principle" is that

Treg B could be more sensitive to FAS-L, considering the highest expression of CD95 (FAS). The gene expression data have indeed suggested that this is the case and Tregs from AA patients have indeed show higher expression of FAS-FAS-L mediated apoptosis.

Nevertheless, this does not explain why Treg A cannot expand and provide necessary protection against self-reactive T cells in response to increased apoptosis of their B counterpart.

My hypothesis was that Treg A cannot efficiently phosphorylate STAT-5 in response to low dose IL-2 (the "physiologic", *in vivo* concentration). The STAT-5 experiment showed that it was the case and when Treg A and B were cultured in the presence of low dose IL-2, Treg A showed lower/delayed level of STAT-5 phosphorylation. The reason for this "delayed", weaker response to IL-2 is not clear yet, but it could be due to a lower expression of IL-2 receptor (CD25) by these cells compared to Treg B.

To summarise: we have shown that Treg A and B are not only immunophenotypically different, but they also show a distinct functional profile, ontogeny, gene signature, and apoptosis propensity, hence suggesting a role as prognosticator and player in AA pathogenesis.

### 6.2.3 Autologous, *ex vivo*, expanded regulatory T cells may have a therapeutic potential in AA

As mentioned above, therapeutic options for refractory or relapsed AA are limited, especially in the elderly patients setting.[163] Considering that aplastic I have focussed on Treg expansion, to possibly investigate if they could be used as a cellular therapy

for AA as opposed to the traditional pharmacological approach with immunosuppressive treatment.

One potential caveat of this approach could be safety, as Tregs functional stability represents a challenge in using Tregs for immunotherapy. Once injected *in vivo*, Tregs may lose their "anti-inflammatory" profile, turn into conventional T cell and become pathogenic *in vivo*, with a detrimental effect.[156] In addition to that, Tregs are reduced in number in AA[185] and it is very difficult to expand them *in vivo* with conventional IST.[187]

Another obstacle to Tregs autologous expansion is that Treg B are even lower in non-responder, and this is the subset one may want to expand as a treatment. In addition to that, Treg A are less responsive to low dose IL-2, but I have shown that they respond well to higher dose IL-2 and phosphorylate STAT-5 accordingly, similarly to Treg B.

Therefore, I have investigated the *ex vivo* expandability of AA Tregs: interestingly, the main expanding subset is Treg A. More specifically, when cultured in a Treg-friendly environment, AA Treg A and total Tregs can be expanded *in vitro* at higher rate compared to healthy donors. The expanded AA Tregs were not only functional (they suppress autologous and allogeneic Tconv, as showed by the criss-cross assay), but also stable as confirmed by culturing in a Th17 polarising environment, therefore showing very scarce plasticity and very low "ability" to differentiate to a Th17-like T cells. Their Treg phenotype is stable as confirmed by the sequencing of TSDR, which was demethylated in expanded Tregs.

Another potential issue could be the longevity of these *ex vivo* expanded cell, as the very "strong" culturing conditions may make them prone to die. On the contrary,

expanded Tregs are resistant to FAS-L mediated apoptosis despite the fact that they are phenotypically closer to Treg B subpopulation (with a higher CD95 expression). Considering the good *ex vivo* expandability of aplastic anaemia Tregs, we have tested the *in vivo* functionality and stability of expanded Tregs, using a GVHD xenograft mouse model: the injected Tregs are able to attenuate GVHD manifestations and symptoms at a tissue level and increase mice overall survival.

Histological assessment of mouse femurs and spleen at the time of death clearly illustrated that tissue from PBMC-only recipient mice had acellular bone marrow and severe disruption of tissue architecture in the BM as well as spleen (which could resemble the histological features of aplastic anaemia). Morphological assessment of the bone marrow sections demonstrates atypical haematopoiesis with prominent fibrotic tissue in mice injected with PBMC alone. However, mice injected with Tregs with or without PBMC have normocellular marrow with no or little evidence of marrow fibrosis. Similarly, AA expanded Tregs (1:1 ratio; up to 4.5 x $10^6$ cells) provided the protective effect from xeno-GVHD symptoms, and mice injectd with either Tregs or PBMC + Tregs survived significantly longer compared to the mice injected with PBMC alone.

It is noteworthy that the GVHD suppressive ability of healthy Tregs was better compared to AA Tregs, even though recipient mice with HD cells received higher doses of PBMCs (AA:1.75 - 4.5 x $10^6$ versus HD:10 x $10^6$). The phenotype of Tregs also remained stable after six weeks.

These *in vivo* data suggest that AA Tregs can be expanded *ex vivo* and that these Tregs become functional, stable, and resistant to FAS-L mediated apoptosis,

therefore, can be considered as a potential therapy for AA as their plasticity towards Th17-like T cells is minimal.

## 6.3 Conclusions and future directions

While the role of Tregs in the pathophysiology of immune aplastic anaemia is well established, this project provides an immune signature which predicts response to IST as well as an in depth understanding of the mechanism that leads to reduction of specific Treg subpopulation and potential target(s) and tools for therapy. The expandability of AA Tregs and their functionality and stability after expansion is another novel aspect of this study (Figure 6.1). I have confirmed that Tregs from patients can be isolated and expanded *ex vivo* and are able to attenuate the immune response in a GVHD mouse model. Indeed, the data I have produced need to be confirmed with GMP compatible reagents to guarantee their reproducibility in a GMP facility.

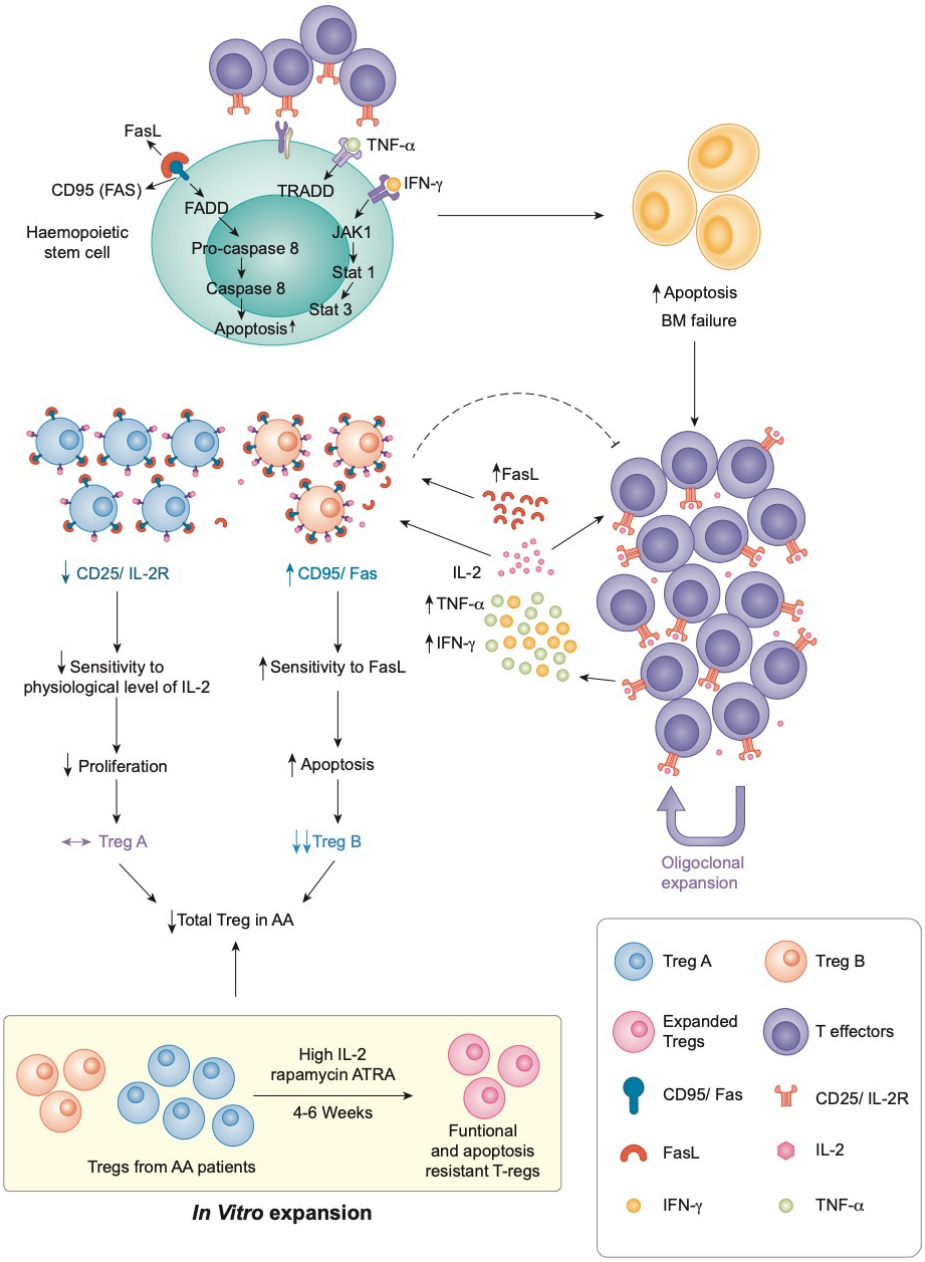



**Figure 6.1 "Visual summary" of the *in-vitro* experiments.**

Aberrant immune responses in AA include the expansion of T effector cells ($CD4^+$ and $CD8^+$) and the increased production of pro-inflammatory cytokines such as IFN-γ and TNF-α, resulting in cell apoptosis through Fas/FasL-dependent pathway. Increased production of IL-2 leads to further expansion of Teff. Treg number and function in term of suppressing Teff are reduced in AA patients as compared to healthy individuals. Treg B, which expressed higher CD95/Fas, are more sensitive to FasL-mediated apoptosis as compared to Treg A, thus resulting in reduced number of Treg B. Although Treg A are less sensitive to FasL, they express lower CD25/IL-2R, do not respond to physiological level of IL-2, and cannot expand in AA. Nevertheless, this does not compensate for the decrease in Treg B, resulting in reduced number of total Treg in AA. In short, the combination of IL-2 deprivation in Treg A and sensitivity to FasL-mediated apoptosis in Treg B results in total Treg reduction in AA. However, Treg A and B isolated from AA patients are expandable *in vitro* with high concentration of IL-2, rapamycin and ATRA for 4 to 6 weeks. These expanded Tregs are functional in suppressing Teff, stable with minimum phenotypic plasticity, and have Treg B-like phenotypes. *In vitro* expanded Tregs are also expressing high level of pro-survival protein, p-BCL-2, representing the potential clinical use of expanded autologous Tregs to improve AA patient clinical outcome in addition or as a replacement to the standard IST.

In addition, these findings suggest a potential role for therapy with low dose and/or low affinity IL-2 in AA as well as the potential clinical use of expanded autologous Tregs to improve patient clinical outcome in addition or as a replacement to standard IST.

However, IST is the standard of care in severe AA and designing a trial based on severe cases may not be feasible at first. Nevertheless, there is no widely accepted approach for the treatment of non-severe cases and these cases might be a better population for phase I/II trial of cell therapy with Tregs (as a first line).

Despite the exciting and promising findings of this study, there are a few aspects which need to be investigated in further details as explained below.

In this study, Tregs were successfully expanded, with no major "failure", and the rate of expansion has always been very encouraging. Nevertheless, the numbers achieved were enough for *in vitro* and *in vivo* studies (in mice) but are negligible if we consider that they will have to be injected "per kilo" in patients, hence the need for a very high expansion yield. Therefore, a combination therapy with low dose IL-2 or allogeneic Tregs might be necessary as tried before.[435] Although "off the shelf" allogeneic Tregs could be used to control the acute phase of AA, Autologous Tregs are shown to survive longer compared to allogeneic Tregs,[436] therefore, may provide a long lived control of immune dysregulation in AA. Importantly, all the expansion experiments will have to be repeated and validated with GMP grade reagents (as stated above), in order to speed up the transition " from the bench to the bedside".

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# APPENDIX 1: TECHNOLOGIES USED IN THIS PROJECT

## A 1.1 Flow cytometry

Flow cytometry is a laser-based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. A flow cytometer is composed of three main subsystems: fluidics, optics, and electronics. The three subsystems work together to simultaneously measure multiple physical characteristics of single particles as they move in a fluid stream through a beam of light.

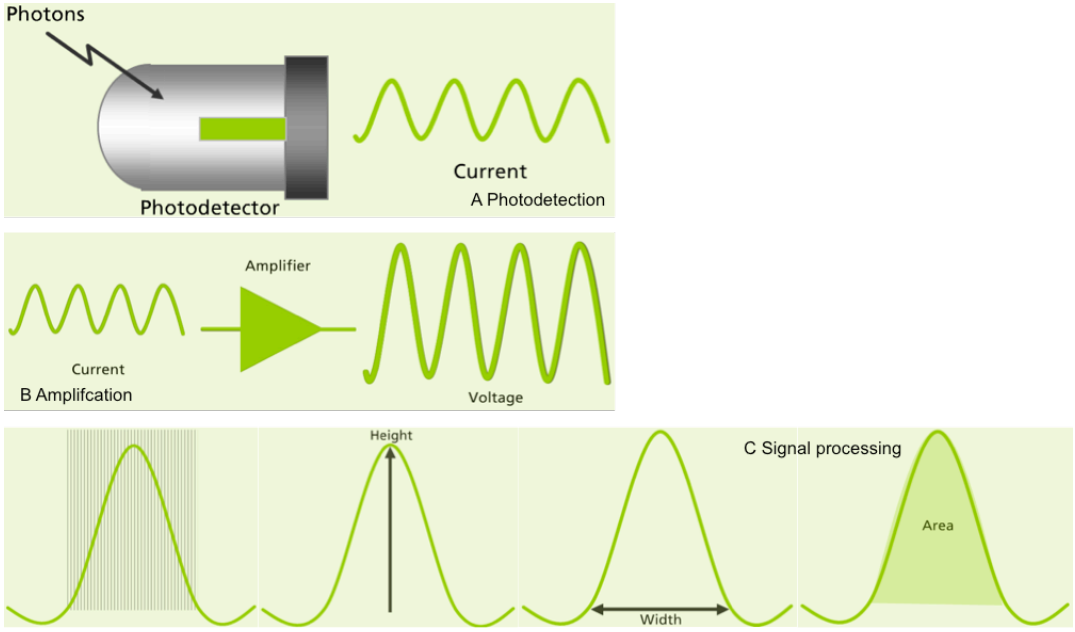
The fluidic system brings the particles of interest to the interrogation point, where they interact with the excitation source (hydrodynamic focusing). With hydrodynamic focusing, the sample core can be maintained within the centre of the sheath fluid.

The optic system provides the excitation source and the components to collect light signals and direct them to the appropriate detectors.

The electronic system converts light signals to equivalent electronic signals as follows:

- photodetectors are light sensors that can detect photons of light; incoming photons cause photodetectors to produce electrical current;
- amplifiers convert electrical current from photodetector into al voltage; the resulting voltages are larger in magnitude than the incoming current;

- signal processors quantify voltage pulses, providing numerical values for pulse height, width, and area.




## A 1.2 Fluorescence activated cell sorting

Cell sorting is the ability to separate cells according to their intracellular (DNA, RNA and protein molecule interaction) or extracellular (size, shape, and surface protein expression) properties. In order to sort a heterogeneous mixture of cells, fluorescence activated cell sorting utilizes flow cytometry to provide a quick, objective and quantitative measurement of intra- and extracellular properties. In FACS, the cell suspension flows in the centre of a narrow, fast flowing stream of liquid. The laminar flow allows a cell separation according to their size. A vibration causes the stream of cells to break into individual droplets containing one cell each. When the stream is about to break into droplets, the flow passes through a fluorescence-measuring tool where the fluorescent property of each cell is measured. An electrical charging tool is placed where the stream breaks into droplets.

A charge is placed on the ring based on the immediately prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge.

In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off.

## A 1.3 Single cell mass cytometry

Mass cytometry offers the possibility of a unique combination of time-of-flight mass spectrometry with Maxpar metal-labelling technology to enable breakthrough discovery and comprehensive functional profiling applications. Cellular targets are labelled with metal-tagged antibodies and detected and quantified by time-of-flight mass spectrometry (www.fluidigm.com). The high purity and choice of metal isotopes ensure minimal background noise from signal overlap or endogenous cellular components. The 135 available detection channels ensure an on-going ability to add more parameters, enabling to fully study the functional complexity of biological systems at a single-cell level. The idea is to tag antibodies with rare isotopes of elements not normally present in cells, stain cells with those tagged antibodies and then pass those cells - one by one - rapidly through a 7,500 K argon plasma. The ions of what were once the individual cells pass into a time-of-flight (TOF) mass spectrometer tuned to the elemental weight range of the isotopes used to tag the antibodies bound to the cells. Every molecule within each individual cell is completely atomized and ionized. Then those ions, falling within a specific mass range, were quantified by time-of-flight mass cytometry (CyTOF). High

dimensional mass cytometry measurements are single cell, quantitative, and well-suited to unsupervised computational analysis.

### A 1.3.1 Data processing, scale transformation, and automatization for mass cytometry

Data were initially processed and analysed using Cytobank. The standalone freeware for clustering and visual t-distributed stochastic neighbour embedding (viSNE) analysis was used as well.

A few methods are available to analyse mass-cytometry complex data by dimensionality reduction and cluster formation into subtypes based on their similarities.[437] One of the most widely used methods is based on t-distributed stochastic non-linear embedding (t-SNE) to visually identify cell populations and delineate subpopulations. Diggins *et al.* have sequentially used a combination of viSNE, spanning-tree progression analysis of density-normalised events (SPADE)[438] and heatmaps to characterize malignant and healthy tissue samples.[342] A similar approach was used to distinguish $CD4^+$ T cell populations and in particular Tregs (viSNE), revealing Treg subsets and characterise the identified subpopulations (heatmaps).

Automated clustering was performed on a subset of 800,000 unstimulated cells sampled from all individuals. The number of cells sampled from each individual was proportional to the total number of cells in that sample. Clustering was performed using flow cytometry clustering without k (FLOCK), a grid density-based unsupervised clustering algorithm, on all cells from all samples.[439] The number of bins used to define the grid and the density threshold used to label a region as dense, were automatically determined by the algorithm while the maximum number of

populations was set to the default value.[382] The following thirteen parameters were chosen for the analysis: Foxp3, CD25, CD127, CD45RA, HLA-DR, CCR6, CCR4, CD62L, CD69, CD27, CXCR3, CD154 and CD45RO. Treg clusters were defined as clusters whose median expression was simultaneously higher than the 90% quantile of Foxp3 expression, higher than the 90% quantile of CD25 expression and lower than the 50% quantile of CD127 expression across all cells.[187]

## A 1.4 Luminex xMAP technology

The xMAP technology can be applied to magnetic and polystyrene beads. The first one is a superparamagnetic 6.5 μm microsphere with a magnetic core and polystyrene surface. The second one is a 5.6 μm polystyrene-only microsphere. Both beads are internally dyed with unique proportions of red and infrared fluorochromes. The different proportions of the red and infrared fluorochromes create 100 unique spectral signatures, which are identified by the Luminex xMAP detection systems. The unique monoclonal antibody-bead conjugation allows the multiple-analytes analysis in a single well. The polystyrene bead requires expensive, and user-unfriendly filter plates. This bead type also makes automating the assay more difficult. The magnetic bead has significant advantages over the polystyrene bead because it is easier and more flexible to use. A flat-bottom plate with either a hand-held magnetic plate washer or an automated magnetic plate washer can be used with magnetic beads. Moreover, a filter plate/vacuum manifold system, commonly used with polystyrene beads, can also be used with magnetic beads. The Luminex System is based on the principles of quantitative fluorescent microscopy or fluorescent flow cytometry. The multiplex system allows the simultaneous measurement of up to 80 different analytes in a single well, using very small sample volumes.

The Luminex 200 and FLEXMAP 3D reader combines two lasers, fluidics, and real-time digital signal processing to distinguish up to 80 different sets of color-coded magnetic microsphere beads, each bearing a different assay. The Luminex reader is an essential tool that performs the key functions of this multiplex technology. The reader detects individual beads by flow cytometry. The fluidics system of the reader aligns the beads into single file as they enter a stream of sheath fluid and then enter a flow cell. Once the beads are in single file within the flow cell, each bead is individually interrogated for bead colour (analyte) and assay signal strength [phycoerythrin (PE) fluorescence intensity)]. The reader uses a 532 nm green laser ("assay" laser) to excite the PE dye of the assay (streptavidin-PE). The 635 nm solid state laser (red "classify" laser) is used to excite the dyes inside the beads to determine their "colour" or "region" and is also used for doublet discrimination by light scatter. The reader has four detectors, one for each of the optical paths. Detectors are used to measure the fluorescence of the assay, to make bead determination (1-100) and to discriminate between single and aggregate beads.

# APPENDIX 2: PUBLICATIONS

## Publications arising directly from this project

blood® Regular Article

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MYELOID NEOPLASIA

# Treg sensitivity to FasL and relative IL-2 deprivation drive idiopathic aplastic anemia immune dysfunction

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**KEY POINTS**

- **FasL-mediated apoptosis plays an important role in Treg depletion and subpopulation imbalance in AA, leading to immune dysregulation.**
- **Remaining AA Tregs become FasL resistant in response to high concentration of IL-2 and are functional in an inflammatory environment.**

**Idiopathic aplastic anemia (AA) has 2 key characteristics: an autoimmune response against hematopoietic stem/progenitor cells and regulatory T-cells (Tregs) deficiency. We have previously demonstrated reduction in a specific subpopulation of Treg in AA, which predicts response to immunosuppression. The aims of the present study were to define mechanisms of Treg subpopulation imbalance and identify potential for therapeutic intervention. We have identified 2 mechanisms that lead to skewed Treg composition in AA: first, FasL-mediated apoptosis on ligand interaction; and, second, relative interleukin-2 (IL-2) deprivation. We have shown that IL-2 augmentation can overcome these mechanisms. Interestingly, when high concentrations of IL-2 were used for in vitro Treg expansion cultures, AA Tregs were able to expand. The expanded populations expressed a high level of *p*-BCL-2, which makes them resistant to apoptosis. Using a xenograft mouse model, the function and stability of expanded AA Tregs were tested. We have shown that these Tregs were able to suppress the macroscopic clinical features and tissue manifestations of T-cell–mediated graft-versus-host disease. These Tregs maintained their suppressive properties as well as their phenotype in a highly inflammatory environment. Our findings provide an insight into the mechanisms of Treg reduction in AA. We have identified novel targets with potential for therapeutic interventions. Supplementation of ex vivo expansion cultures of Tregs with high concentrations of IL-2 or delivery of IL-2 directly to patients could improve clinical outcomes in addition to standard immunosuppressive therapy. (*Blood.* 2020;136(7):885-897)**

## Introduction

Severe aplastic anemia (AA) is a rare and potentially fatal form of bone marrow (BM) failure syndrome, characterized by peripheral blood cytopenia and hypocellular BM. Most cases of acquired AA are associated with autoimmunity.[1-3] In AA, the expansion of T-effector cells ($CD4^+$ and $CD8^+$ T cells), along with the increased levels of proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α, results in the depletion of hematopoietic stem/progenitor cells (HSPCs).[4,5] Immune destruction of HSPCs is also associated with Fas/FasL-dependent apoptotic pathway.[6,7]

Current first-line treatment options for AA patients include hematopoietic stem cell transplantation for younger patients with HLA-matched sibling donors, as well as immunosuppressive therapy (IST) for older patients and patients without a matched sibling donor.[8,9] In hematopoietic stem cell transplant, acute and chronic graft-versus-host disease (GVHD) remain an issue, although occur less frequently using alemtuzumab-based conditioning.[10] Standard IST regimen using antithymocyte globulin and cyclosporine A has shown a response rate of 60% to 70% in AA patients. Nevertheless, 35% of patients relapse after responding, and up to 15% of patients undergo clonal evolution to myelodysplastic syndrome and acute myeloid leukemia following IST.[3,8,9,11-13] More recently, the addition of thrombopoietin receptor agonist (Eltrombopag) to the standard IST has shown a high rate of complete response among patients with

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# Deep phenotyping of Tregs identifies an immune signature for idiopathic aplastic anemia and predicts response to treatment

Shahram Kordasti, Benedetta Costantini, Thomas Seidl, Pilar Perez Abellan, Marc Martinez Llordella, Donal McLornan, Kirsten E. Diggins, Austin Kulasekararaj, Cinzia Benfatto, Xingmin Feng, Alexander Smith, Syed A. Mian, Rossella Melchiotti, Emanuele de Rinaldis, Richard Ellis, Nedyalko Petrov, Giovanni A. M. Povoleri, Sun Sook Chung, N. Shaun B. Thomas, Farzin Farzaneh, Jonathan M. Irish, Susanne Heck, Neal S. Young, Judith C. W. Marsh and Ghulam J. Mufti



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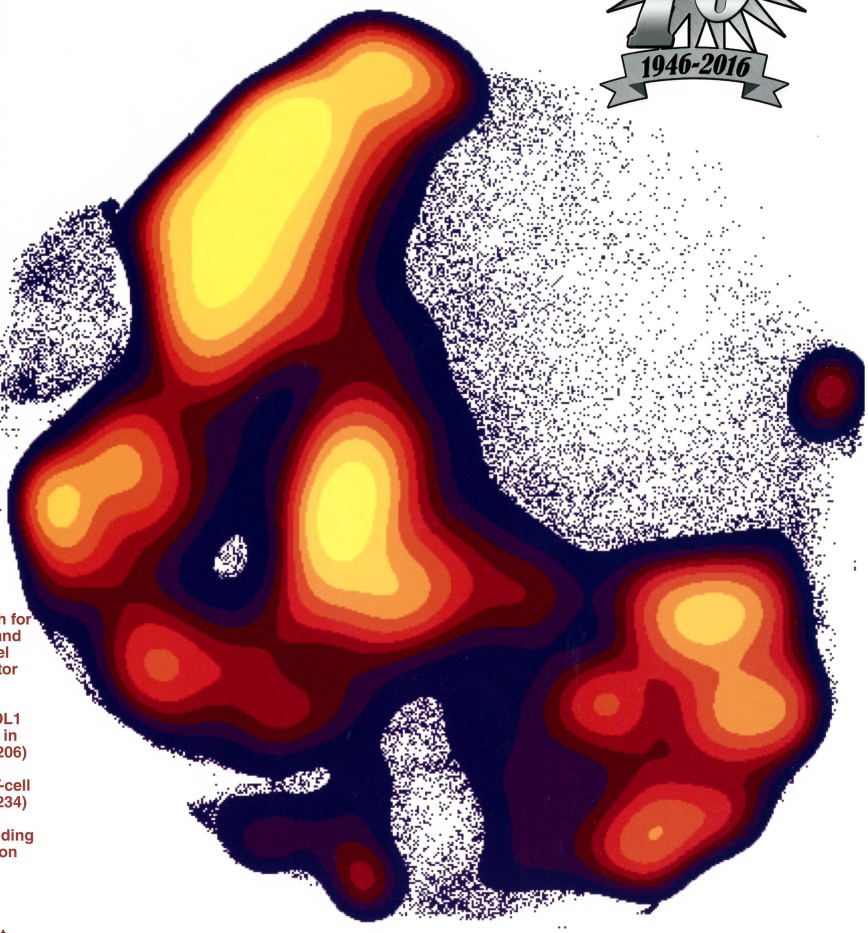

Novel imaging approach for individual stem cells and identification of novel stem cell survival factor (p 1157, p 1181)

Characterization of PDL1 gene rearrangements in lymphoma (p 1159, p 1206)

Novel gene fusions in T-cell lymphoma (p 1161, p 1234)

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Importance of integrin-regulating molecules in platelet function (p 1165, p 1282)

High incidence of *CRBN* and Ras pathway genes in myeloma (p 1226)

*Cover:*
Identification of a clinically relevant population of Tregs in aplastic anemia (p 1158, p 1193)

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# Publications arising from collaborations during this project

OncoImmunology

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## Expansion of myeloid derived suppressor cells correlates with number of T regulatory cells and disease progression in myelodysplastic syndrome

Astrid Olsnes Kittang, Shahram Kordasti, Kristoffer Evebø Sand, Benedetta Costantini, Anne Marijn Kramer, Pilar Perezabellan, Thomas Seidl, Kristin Paulsen Rye, Karen Marie Hagen, Austin Kulasekararaj, Øystein Bruserud & Ghulam J. Mufti

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# Immunomodulatory Effects of Tyrosine Kinase Inhibitor In Vitro and In Vivo Study

Elena Marinelli Busilacchi [1,2,†], Andrea Costantini [1,3,†], Nadia Viola [3], Benedetta Costantini [4], Jacopo Olivieri [5], Luca Butini [3], Giorgia Mancini [2], Ilaria Scortechini [2], Martina Chiarucci [2], Monica Poiani [1,2], Antonella Poloni [1,2], Pietro Leoni [1,2], Attilio Olivieri [1,2,*]

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Tyrosine kinase inhibitors (TKIs)
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T regulatory cells
Cytokine production

A B S T R A C T

Pathogenesis of chronic graft-versus-host disease (cGVHD) is incompletely defined, involving donor-derived CD4 and CD8-positive T lymphocytes as well as B cells. Standard treatment is lacking for steroid-dependent/refractory cases; therefore, the potential usefulness of tyrosine kinase inhibitors (TKIs) has been suggested, based on their potent antifibrotic effect. However, TKIs seem to have pleiotropic activity. We sought to evaluate the in vitro and in vivo impact of different TKIs on lymphocyte phenotype and function. Peripheral blood mononuclear cells (PBMCs) from healthy donors were cultured in the presence of increasing concentrations of nilotinib, imatinib, dasatinib, and ponatinib; in parallel, 44 PBMC samples from 15 patients with steroid-dependent/refractory cGVHD treated with nilotinib in the setting of a phase I/II trial were analyzed at baseline, after 90, and after 180 days of therapy. Flow cytometry was performed after labeling lymphocytes with a panel of monoclonal antibodies (CD3, CD4, CD16, CD56, CD25, CD19, CD45RA, FoxP3, CD127, and 7-amino actinomycin D). Cytokine production was assessed in supernatants of purified $CD3^+$ T cells and in plasma samples from nilotinib-treated patients. Main T lymphocyte subpopulations were not significantly affected by therapeutic concentrations of TKIs in vitro, whereas proinflammatory cytokine (in particular, IL-2, IFN-γ, tumor necrosis factor-α, and IL-10) and IL-17 production showed a sharp decline. Frequency of T regulatory, B, and natural killer (NK) cells decreased progressively in presence of therapeutic concentrations of all TKIs tested in vitro, except for nilotinib, which showed little effect on these subsets. Of note, naive T regulatory cell (Treg) subset accumulated after exposure to TKIs. Results obtained in vivo on nilotinib-treated patients were largely comparable, both on lymphocyte subset kinetics and on cytokine production by CD3-positive cells. This study underlines the anti-inflammatory and immunomodulatory effects of TKIs and supports their potential usefulness as treatment for patients with steroid-dependent/refractory cGVHD. In addition, both in vitro and in vivo data point out that compared with other TKIs, nilotinib could better preserve the integrity of some important regulatory subsets, such as Treg and NK cells.



## INTRODUCTION

Chronic graft-versus-host disease (cGVHD) is a major complication of allogeneic stem cell transplantation (allo-SCT) and is characterized by multiorgan involvement resembling autoimmune diseases. The pathogenesis of cGVHD has not been fully elucidated [1]; however, recent insights suggest that several players and different pathways are involved, including imbalance of T and B cells and exaggerated collagen production. These phenomena are associated with typical alterations of the cytokine network, such as increased levels of transforming growth factor (TGF)-β and inflammatory cytokines.

Similarly to that observed in systemic autoimmune diseases, a variety of autoantibodies has been reported in patients affected by cGVHD [2,3], and many of them develop multiorgan disease with fibrotic features, resembling systemic scleroderma. Typical biologic data are characterized by

*Financial disclosure:* See Acknowledgments on page 274.
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† Elena Marinelli Busilacchi and Andrea Costantini contributed equally to this work.

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ARTICLES

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# Human retinoic acid–regulated CD161+ regulatory T cells support wound repair in intestinal mucosa

Giovanni A. M. Povoleri[1,2], Estefania Nova-Lamperti[1,2], Cristiano Scottà[1,2], Giorgia Fanelli[1,2], Yun-Ching Chen[3], Pablo D. Becker[1,2], Dominic Boardman[1,2], Benedetta Costantini[4], Marco Romano[1,2], Polychronis Pavlidis[1,2], Reuben McGregor[1,2], Eirini Pantazi[1,2], Daniel Chauss[5], Hong-Wei Sun[6], Han-Yu Shih[7], David J. Cousins[8], Nichola Cooper[9], Nick Powell[1,2], Claudia Kemper[10], Mehdi Pirooznia[3], Arian Laurence[11], Shahram Kordasti[4], Majid Kazemian[12], Giovanna Lombardi[1,2,14] and Behdad Afzali[1,5,13,14]*

**Repair of tissue damaged during inflammatory processes is key to the return of local homeostasis and restoration of epithelial integrity. Here we describe CD161+ regulatory T ($T_{reg}$) cells as a distinct, highly suppressive population of $T_{reg}$ cells that mediate wound healing. These $T_{reg}$ cells were enriched in intestinal lamina propria, particularly in Crohn's disease. CD161+ $T_{reg}$ cells had an all-*trans* retinoic acid (ATRA)-regulated gene signature, and CD161 expression on $T_{reg}$ cells was induced by ATRA, which directly regulated the *CD161* gene. CD161 was co-stimulatory, and ligation with the T cell antigen receptor induced cytokines that accelerated the wound healing of intestinal epithelial cells. We identified a transcription-factor network, including BACH2, RORγt, FOSL2, AP-1 and RUNX1, that controlled expression of the wound-healing program, and found a CD161+ $T_{reg}$ cell signature in Crohn's disease mucosa associated with reduced inflammation. These findings identify CD161+ $T_{reg}$ cells as a population involved in controlling the balance between inflammation and epithelial barrier healing in the gut.**

Regulatory T ($T_{reg}$) cells are a non-redundant, suppressive subset of CD4+ helper T cells that are critical for preventing autoimmunity and ideal for cell-based immunotherapy of autoimmunity and prevention of transplant rejection[1]. $T_{reg}$ cells express the master transcription factor FOXP3, the interleukin-2 (IL-2) receptor component CD25 and the inhibitory co-receptor CTLA4[2], and depend on the transcription factor BACH2[3]. $T_{reg}$ cells are derived thymically and peripherally and can also be induced in vitro. There are no universally accepted ways to distinguish these populations, although expression of Helios and neuropilin and methylation status of the $T_{reg}$ cell–specific demethylation region (TSDR) have been proposed[4–8].

Conventional T ($T_{conv}$) cells express their own master transcription factors; these include T-BET+ type 1 helper T ($T_H1$) cells, GATA3+ $T_H2$ cells and RORγt+ $T_H17$ cells[9]. These transcription factors are considered to antagonize $T_{reg}$ cell development: in mice, induction of high T-bet expression in $T_{reg}$ cells within inflamed bowel drives $T_{reg}$ cells into a pro-inflammatory phenotype reminiscent of $T_H1$ cells[10]. This view has been challenged by specific deletions of these factors specifically within Foxp3+ cells of mice[11–14]. For example, T-bet expression within Foxp3+ $T_{reg}$ cells is required for trafficking to and suppression of $T_H1$ cell–mediated inflammation[13], and Gata3 is required for full $T_{reg}$ cell function in the gut[14]. These findings support a 'compartmentalized' view of $T_{reg}$ cells, suggesting multiple subpopulations defined by expression of transcription factors associated with $T_{conv}$ cell lineages and by specialized functions. Indeed, the transcription factor circuitry of $T_{reg}$ cells is complex, with significant interplay between Foxp3 and other lineage-associated transcription factors[15].

In humans, heterogeneous populations of $T_{reg}$ cells have been reported, although typically defined by surface markers (for example, CD39, HLA-DR and CD45RA[5]) rather than transcription factors. Whether these subpopulations have the ability to suppress specific parts of the human immune system has yet to be fully elucidated. Conventional methods for delineating $T_{reg}$ cell subsets are limited by numbers of markers that can be concurrently used and by biased approaches to data analysis (gating of $T_{reg}$ cell subsets)[16]. This has led to conflicting results, with memory $T_{reg}$ cells being reported as both non-suppressive[17] and highly suppressive[18]. By contrast, unbiased multidimensional analysis can delineate the most suppressive $T_{reg}$ cell subpopulations, identify new ones and exclude those less likely to be regulatory[16].

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# APPENDIX 3: CONSENT FORM FOR SAMPLES' TISSUE BANKING

NRES Reference 08/H0906/94+5

KING'S College LONDON

**PARTICIPANT INFORMATION SHEET**

**King's College London Haemato-Oncology Tissue Bank: The Collection and Storage of Blood and Tissue for Use in Future Studies into the Causes, Diagnosis and Treatment of Haematological Disorders.**

You are being invited to make a voluntary contribution of tissue to a Tissue Bank that will be used for research into blood disorders. Before you decide it is important for you to understand why the Tissue Bank is needed and what research will be carried out using tissue from the Tissue Bank. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Please ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

**What is the purpose of the Tissue Bank and the Research it will be used for?**

Medical research depends upon a steady supply of tissue from patients. The purpose of a Tissue Bank is to conserve tissue so that samples in sufficient numbers are ready when they are needed. Our Tissue Bank will supply scientists who are undertaking research to gain a better understanding of the causes of blood disorders such as aplastic anaemia, lymphomas, leukaemia, myelodysplastic syndromes (MDS) and myeloma. As our understanding in this area is advancing rapidly, we would like your permission to store some of your bone marrow, blood cells, serum and any tissue surplus to that required to confirm a diagnosis.

**Do I have to take part?**

You are not under any obligation to take part. Your participation is entirely voluntary and there will be no payment for entering into the study. If you decide to take part you will be asked to sign a consent form and you will be given this information sheet to keep. Staff will explain all the risks, benefits and alternatives before they ask you to sign the consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive. Should you decide to withdraw your consent for continued retention of your tissue, any tissue remaining in the Tissue Bank at that time will be destroyed according to your wishes, as far as possible, and any data relating to you and your tissues, other than that needed to record the receipt and fate of your tissue, will be deleted. Please note that if you do withdraw your consent after donating tissues it may not be possible for researchers to delete data already obtained from research using your tissue.

**What will happen if I take part?**

All these samples will be collected during normal routine blood or bone marrow collections and there will be no additional procedures that you will have to undergo and no additional discomfort.

At diagnosis, you may be invited to provide up to 60ml (4 tablespoons) of blood and 20ml (1½ tablespoons) of bone marrow. We may also invite you to provide the collected cells if you need to have a procedure called leukapharesis (the cells collected from a machine if your white blood cells are high in the blood - what is involved in this and the consent for this procedure will be explained and taken separately)

If a biopsy is performed, we may invite you to consent for the storage of any excess tissue which would otherwise be destroyed after the diagnosis is confirmed.

We may also invite you to provide a sample of buccal cells from your mouth. This involves rinsing your mouth with a solution of salt and collecting the liquid or using a cheek swab. Both methods are painless.

We would like to collect material at regular intervals during the course of your treatment. During and after treatment, you may be invited to provide additional samples when you have your routine blood and bone marrow collections. We will not ask you to donate for this purpose any more than 60ml of blood or 5ml of bone marrow in any 28 day period.

Samples will be stored within our Tissue Bank, which has been licensed by the Human Tissue Authority.

Tissues will subsequently be used by scientists carrying out research into the causes, diagnosis and treatment of Haematological Disorders. In some cases we may need to send a portion of your tissue to our collaborators in academic institutions, or occasionally in commercial organisations, in other parts of the UK or abroad. Some of the research may involve the use of animals, but only when this is strictly necessary. If you choose to donate your tissue to the Tissue Bank but do not wish those tissues to be used in studies involving animals, please indicate this on the consent form and we will respect your wishes. Samples will not be used for any form of reproductive research. In all cases we will treat your tissue with respect and all experiments will have been approved by independent reviewers.

**What are the side effects?**

Blood sampling, bone marrow examination and leukapharesis are routine examinations performed in the haematology department. Each of the procedures

will be explained in full and consent taken. No additional discomfort would be expected from the procedures for this study.

**What is the potential benefit of participation?**

Samples may be tested for genetic abnormalities which may be responsible for blood disorders, therefore leading to a better understanding of the causes of the condition and better treatment. The information could also predict how patients will respond to treatment and be used to monitor how effective the treatment has been.

You may not benefit directly from our work, but if we do discover information that could help your treatment then you and your doctor will be informed.

Results from this study will be published in scientific journals, but no information will be included which will allow participants to be identified.

**What if something goes wrong?**

If you wish to make a complaint about any aspect of the Tissue Bank, please contact the Director of Research Management and Director of Administration (Health Schools), King's College London, Strand, London, WC2R 2LS

**Will my taking part in this study be kept confidential?**

Your contribution to the Tissue Bank will be kept strictly confidential. All your sample(s) and medical information will be stored securely and will be anonymised on receipt by the Tissue Bank so that researchers will not be able to identify you.

**Who is organising and funding the research?**

Professor Stephen Devereux and Ms Rajani Chelliah are the Director and Manager of the Tissue Bank, respectively.

Leukaemia & Lymphoma Research (formerly the Leukaemia Research Fund) provided funding to set up the Tissue Bank. The project has also been reviewed by a committee of the UK National Research Ethics Service.

**Contact for further information**

If you require any further information about participating in the KCL Haemato-Oncology Tissue Bank please contact Ms Rajani Chelliah (Tissue Bank Manager) or Professor Stephen Devereux (Director), Department of Haematological Medicine, King's College London, Rayne Institute, 123 Coldharbour Lane, London, SE5 9NU, telephone 020 7848 5815.

If you do decide to donate to the Tissue Bank you will be given a copy of this information sheet and a copy of the consent form to keep.

**Thank you for your interest in donating samples to our tissue bank**

## CONSENT FORM

**King's College London Haemato-Oncology Tissue Bank: The Collection and Storage of Blood and Tissue for Use in Future Studies into the Causes, Diagnosis and Treatment of Haematological Disorders**

Professor Stephen Devereux, Ms Rajani Chelliah

| | | *Please initial* |
|---|---|---|
| 1 | I confirm that I have read and understood the information sheet version 4.2 dated 1st May 2017 for the above Tissue Bank. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. | |
| 2 | I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected. | |
| 3 | I understand that the following tissues will be collected:<br>*(person taking consent: please delete those below that do not apply)*<br>Blood / Bone Marrow / Buccal Cells (Mouth Wash)<br>Lymph Node / Skin / Other (specify) | |
| 4 | I understand that my tissues may be used in research involving genetic analysis. | |
| 5 | I understand that my tissues may be used in research that involves animal experimentation *(please do not initial this part of the form if you do not wish your samples to be used in this way).* | |
| 6 | I understand that my tissues may be passed to researchers outside of King's College London. | |
| 7 | I understand that relevant sections of my medical notes and data collected during the study may be looked at by researchers from the study, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. | |
| 8 | I agree to take part in the above Tissue Bank. | |

| Patient Details | | | |
|---|---|---|---|
| Surname | | Forename(s) | |
| Date of Birth | Hospital Number | NHS Number | Hospital |
| Signature | | | Date |

| Person Taking Consent | |
|---|---|
| Name (please print) | Signature & Date |

| For Laboratory Use | | | | | |
|---|---|---|---|---|---|
| Research Case Code | Sample Number(s) | | | | |
| RC - - | LSL/ | LSL/ | LSL/ | LSL/ | LSL/ |

## CONSENT FORM

**King's College London Haemato-Oncology Tissue Bank: The Collection and Storage of Blood and Tissue for Use in Future Studies into the Causes, Diagnosis and Treatment of Haematological Disorders**

Professor Stephen Devereux, Ms Rajani Chelliah

| | | *Please initial* |
|---|---|---|
| 1 | I confirm that I have read and understood the information sheet version 4.2 dated 1st May 2017 for the above Tissue Bank. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. | |
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| 3 | I understand that the following tissues will be collected:<br>*(person taking consent: please delete those below that do not apply)*<br>Blood / Bone Marrow / Buccal Cells (Mouth Wash)<br>Lymph Node / Skin / Other (specify) | |
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| 8 | I agree to take part in the above Tissue Bank. | |

| Patient Details | | | |
|---|---|---|---|
| Surname | | Forename(s) | |
| Date of Birth | Hospital Number | NHS Number | Hospital |
| Signature | | | Date |

| Person Taking Consent | |
|---|---|
| Name (please print) | Signature & Date |

| For Laboratory Use | | | | | | |
|---|---|---|---|---|---|---|
| Research Case Code | Sample Number(s) | | | | | |
| RC - - | LSL/ | LSL/ | LSL/ | LSL/ | LSL/ | |

*Patient copy* □ *Notes copy* □ *Tissue Bank copy (original must accompany sample(s))* ☑

# APPENDIX 4: ATG PROTOCOL FOR APLASTIC ANAEMIA AT KING'S COLLEGE HOSPITAL

King's College Hospital NHS
NHS Foundation Trust

**Department of Haematology**

## PROTOCOL FOR THE TREATMENT OF APLASTIC ANAEMIA WITH HORSE ANTI-THYMOCYTE GLOBULIN (hATG, ATGAM®)

**CONTENTS**

## 1. INTRODUCTION and CAUTIONS

This document is based on our current practice of using horse anti-thymocyte globulin (ATG) for aplastic anaemia (AA) at King's College Hospital.

**Reasons for change in the version of protocol**

Recent guidance from the European Blood and Marrow transplant (EBMT) Severe Aplastic Anaemia Working Party and the British Committee for Standards in Haematology (BCSH) advises that horse ATG (ATGAM®) with ciclosporin is currently the preferred first choice of immunosuppressive therapy for patients with AA.

Because of the withdrawal of horse ATG (Lymphoglobuline®) from the market, there have been two prospective studies comparing the use of rabbit ATG with horse ATG as first line of treatment for AA, and surprisingly a significantly worse response was seen with rabbit ATG (35-37% at 6 months, compared to around 75% with horse ATG). Furthermore, survival outcomes were significantly inferior after rabbit ATG (67%) compared to horse ATG (78%), and inferior transplant free survival of 37% for rabbit ATG compared to 70% for horse ATG). In the EBMT study there were a higher number of deaths from infection after rabbit ATG.

There is another preparation of horse ATG which is made and was until very recently used almost exclusively in the USA (ATGAM®). This preparation was used in the prospective study from NIH confirming significantly better response and survival outcomes compared to rabbit ATG. Horse ATG (ATGAM®, Pfizer) is now available in the UK as a named patient product from Pfizer.

Before considering using ATG at a particular hospital it is important that haematologists are aware of the following points relating to the administration of this drug:

- It is of paramount importance that ATG is only used by physicians who are familiar with administering ATG and that the medical and nursing teams are aware of the side effects and how to treat these promptly and appropriately.

- ATG is highly immunosuppressive. It should only be used in centres with at least level 2 facilities. Patients should be nursed in a single or double isolation room. ATG must never be given as an out-patient.

- Patients over the age of 60 years, in particular, should be carefully assessed medically beforehand to determine whether they are fit enough to tolerate ATG treatment. (see BCSH guidelines for the diagnosis and management of aplastic anaemia )

- It is recommended that the haematologist responsible for the patient should contact a centre/specialist with expertise in AA beforehand to discuss the management plan (and possibly review the bone marrow slides).

 Because AA is a rare disease, it is important that, whenever possible, patients are entered into ongoing National or European (EBMT) prospective clinical trials.

 For further written information relating to diagnosis and management of AA the 2009 revised BCSH guidelines are recommended (available on BCSH website: bcshguidelines.com).

 Doses of drugs other than ATG discussed in this document relate to adults. Appropriate dose reductions are necessary for children.

**CONTACT DETAILS**

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## 2. INDICATIONS

2.1. Non-severe acquired AA and transfusion dependent.

2.2. Acquired severe or very severe AA age, with no HLA identical sibling donor. For children, first line matched unrelated donor HSCT instead of ATG may be considered if a fully matched donor is readily available.

2.3. Acquired AA age > 35-50* years with a matched sibling donor

Patients over the age of 60 years have the same response rate to ATG as younger patients, but have increased risk of death from infection and bleeding after ATG. It is therefore important that before considering ATG treatment in such patients, that they are very carefully assessed beforehand to determine whether they are fit enough to tolerate a course of ATG.

* Between the ages of 35-50 years, the decision for first line ATG or HLA matched sibling donor HSCT depends on co-morbidities of patient (BCSH adult aplastic anaemia revised 2015 edition, under final BSH review).

## 3. PREPARATION

Horse ATG (ATGAM®, Pfizer) (each 5ml ampoule contains 250 mg of horse gammaglobulin)

## 4. DOSAGE

The dose of ATGAM® is **40mg/kg/day for 4 days**.

For obese patients, the dose must be calculated according to ideal body weight.

## 5. ASSESSMENT OF THE PATIENT BEFORE STARTING ATG

5.1 Investigations to confirm a diagnosis of AA (see BCSH guidelines on diagnosis and management of acquired AA, (www.bcshguidelines.com)

5.2 Medical assessment prior to starting ATG
- Exclude active infection
- Chest XRay
- ECG (for patients > 60 years, also perform ECHO)

5.3 Assessment of platelet and red cell transfusional requirements

It is important to ensure an adequate platelet increment occurs after platelet transfusions, because ATG results in a drop in the platelet count and can precipitate bleeding. If refractory to random donor platelets, postpone ATG treatment until further investigations. If HLA antibodies are detected, arrange adequate supply of HLA matched platelets to cover course of ATG. Ensure a DAT is sent pre-ATG.

Irradiated platelet transfusions are now recommended for patients receiving ATG treatment (see BCSH guidelines on diagnosis and management of acquired AA, www.bcshguidelines.com). No

recommendation is possible on the duration of irradiated blood products after ATG; one option may be to continue until the lymphocyte count recovers to > 1.0 x $10^9$/l, or indefinitely.

5.4 Obtain informed written consent. A patient information sheet is available and should be given to the patient to read beforehand and this should be documented in the patient notes.

**6. MEDICATIONS TO USE WITH ATG (Ciclosporin and prophylactic medications)**

6.1 Start oral ciclosporin on Day 1 of ATG (section 16 & appendix 1)

6.2 Oral antibiotic prophylaxis: oral ciprofloxacin 250mg 12 hourly

6.3 Chlorhexidine 0.2% mouthwash 10ml 6 hourly

6.4 Antifungal prophylaxis: oral posaconazole suspension 200mg TDS

NB. when using posaconazole in combination with ciclosporin consider reducing the dose of ciclosporin by 25% and titrate according to ciclosporin levels because posaconazole increase ciclosporin blood levels

6.5 Antiviral prophylaxis: oral aciclovir 400mg 12 hourly

6.6 Pneumocystis prophylaxis is probably unnecessary after ATG treatment as we have not seen a case of pneumocystis after ATG treatment given for AA

6.7 Duration of prophylaxis: for all patients, prophylaxis should continue for a minimum of 4 weeks, and longer if very severe AA with neutrophil count < 0.2 x $10^9$/l

6.8 Omeprazole 20mg daily

6.9 Norethisterone oral 5mg 8 hourly for pre-menopausal females

**7. GENERAL NURSING POINTS**

The patient should ideally be nursed in a single or double room, preferably with positive pressure ventilation or laminar air flow. Barrier nursing must be employed, and a 'clean diet' provided for the patient when neutropenic.

**8. ADMINISTRATION**

8.1. Patients must be admitted as an in-patient for ATG treatment. **ATG must not be given as an out-patient.** The average length of stay required is around 2-3 weeks providing there are no complications, such as infections, which may necessitate a longer in-patient stay.

8.2 In general, patients should remain an in-patient for around 2-3 weeks so they can be monitored closely and treated promptly for serum sickness and other possible complications. Admission for just the 5 days of ATG treatment may be considered if there is immediate access to inpatient or day care facilities, for treatment of late complications such as serum sickness, infection or bleeding

(BCSH 2009). However, this should be discussed on an individual patient basis and dependent on the clinical condition of the patient. If the patient is stable, then this should be discussed with the referring hospital beforehand. It is recommended that all older patients, at least > 60yr old, should remain in hospital for the full duration.

8.3. Always give ATG through a central line, or a PICC line in the antecubital fossa line with its distal end in a central vein. Severe thrombophlebitis may occur if ATG is administered via a peripheral vein.

8.4. Dilute ATGAM® in 1000 ml sodium chloride 0.9% (or as appropriate such that the concentration should not exceed 4mg/ml) and infuse via a 0.2 micron in-line filter. A test dose must always be given on day 1 of ATG before the first full dose*. To do this, run the infusion slowly at 5ml/hr for the first hour of the infusion. The test dose should be preceded by methylprednisolone and chlorphenamine, as detailed in section 8.7 below. The test dose must be supervised by a doctor with epinephrine (see anaphylaxis protocol), chlorphenamine (10mg IV) and hydrocortisone (100mg IV) drawn up beforehand

***(* Historically a skin test was used as the test dose for horse ATG (ATGAM), but because of a high incidence of false positives and false negatives, current practice in Europe is to use an intravenous infusion test dose as for Lymphoglobuline and Thymoglobuline, (recent survey of EBMT Severe Aplastic Anaemia Working Party, May 2012).***

8.5. A severe systemic reaction or anaphylaxis to the test dose is an absolute contraindication to proceeding with ATG treatment.

8.6. Infuse the remainder of the infusion over 12-18 hours via a 0.2 micron in-line filter. It should be administered within 24 hours of being made up. Infuse the first full dose over 18 hours. If tolerated, subsequent doses can be given over 12 hours.

8.7. Precede each daily dose of ATG with:
 Platelets (one random donor pack or one apheresis pack or one HLA matched pack), aiming to keep platelet > 30 x $10^9$/l. Do not give platelet transfusions during the ATG infusion, because of the anti-platelet activity of ATG.
 Methylprednisolone 1mg/kg IV as a 30 minute infusion, 30minutes before each does of ATG.
 Chlorphenamine 10 mg IV. (See section 11 if reactions still occur during ATG.). It is no longer necessary to give hydrocortisone as methylprednisolone is given instead.

8.8. If possible, avoid giving more than two units of blood each day of the 4 days of ATG, to (a) help reduce the risk of fluid overload and (b) help ensure that the administration of ATG each day starts in the morning

## 9. SIDE EFFECTS

### 9.1 Immediate side effects (during administration of ATG):

 Lymphopenia, neutropenia and thrombocytopenia.
 Fevers and rigors (tend to be worse on the first day and diminish with subsequent doses of ATG).
 Rash, pruritis, urticaria.
 Fluid retention occurs commonly. Acute pulmonary oedema and cardiac failure can develop rapidly if left untreated. Fluid retention needs very close monitoring (see section 12) and early

treatment with furosemide. It is usually multi-factorial in origin, for example, N Saline diluent, blood and platelet transfusions, corticosteroids, chronic anaemia.
- Hypotension or hypertension.
- Elevation of serum transaminases occur commonly
- Cardiac arrhythmias: bradycardia or tachycardias
- Chest pain, loin pain, back pain occasionally
- Nausea, vomiting, diarrhoea may sometimes occur
- Positive direct antiglobulin test and difficulty with cross matching blood due to the presence of anti-red cell antibodies in ATG
- Phlebitis can occur when administered through a peripheral vein
- Anaphylaxis
- Other rare reported side effects are acute haemolysis, massive pulmonary haemorrhage and adult respiratory distress syndrome, acute renal failure and renal impairment.

### 9.2 Late side effects after administration of ATG due to serum sickness

The onset of serum sickness is typically 7-14 days after starting ATG. If a second course of ATG is given, serum sickness may occur earlier.

The manifestations of serum sickness are:

- Fever, rash (variable manifestations: maculopapular or urticarial or sometimes erythema multiforme, starting on trunk or extremities). Serpiginous palmar-plantar distribution is classical. Rash may become purpuric due to platelet consumption during the time of serum sickness.
- Arthralgia, myalgia, nausea, vomiting, proteinuria (usually mild), rarely splenomegaly and lymphadenopathy.
- Increased platelet transfusion requirements due to platelet consumption.
- Glycosuria and/or hyperglycaemia due to corticosteroids.

### 9.3 Other late side effects of ATG

- Rarely, worsening of autoimmune thyroid disorders and fibrosing alveolitis, and precipitation of Guillan Barre syndrome.
- AA patients treated with ATG are at increased risk of later clonal disorders such as MDS, AML and PNH, and to a lesser degree, solid tumours.

## 10. PREVENTION OF SERUM SICKNESS

Day 1-4: Methylprednisolone 1mg/kg/day IV as 30 min infusion, start 30min before each dose of ATG.
Day 5 onwards: Oral prednisolone 0.5mg/kg/day from day 5 and subsequently aim to half the dose every 5 days.

## 11. MONITORING THE PATIENT

Carefully monitor patient clinically for evidence of bleeding, infection, fluid retention and hypo- or hypertension:

 weigh patient twice daily
 keep strict fluid balance chart daily
 4 hourly temperature, pulse, BP, oxygen saturations and respiratory rate
 daily urine test for glucose
 daily FBC, U&Es and LFTs
 there is no indication for routing monitoring of CMV, EBV viral load. CMV or EBV viral reactivation is very common during ATG treatment; EBV reactivation occurs in 90% patients receiving horse ATG, and to date in the world literature there have been only 3 cases reported with EBV LPD, one of which was not biopsy proven (Nakanishi et al. Int. J. Clin. Exp. Pathol. 2014;7(4): 1748).

## 12. TREATMENT OF SIDE EFFECTS

12.1 Immediate allergic side effects usually respond to a dose of hydrocortisone and chlorphenamine. If persistent, give pethidine 25mg IV. Pyrexia during ATG may also be due to infection, so broad spectrum IV antibiotics (as per departmental protocol for neutropenic patients) must be commenced after obtaining blood cultures.

12.2 Treat fluid retention promptly with furosemide and review fluid balance later the same day. If the patient gains more than one kg in weight, or if the amount in is one litre more than the amount out in 24 hours, then give a dose of furosemide. However, assess clinically first, because if febrile, and increased insensible loss, furosemide may not be appropriate.

12.3 If serum sickness occurs despite prednisolone prophylaxis, give IV hydrocortisone 100mg QDS 6 hourly. Serum sickness is self-limiting and usually resolves within 24-72hours. Do NOT increase the dose of oral prednisolone – this is unnecessary and exposes the patient to higher risk of bacterial and fungal sepsis, as well as the longer term side effects of corticosteroids (such as AVN, diabetes etc.).

12.3 If patient is hypertensive, treat any fluid retention if present, and use appropriate anti-hypertensive.

12.4 For anaphylaxis, discontinue ATG immediately and treat anaphylaxis appropriately.

12.5 If bleeding occurs during ATG, stop the ATG infusion and give additional platelets. Resume ATG when bleeding has resolved. Also, check the coagulation screen if bleeding persists despite adequate platelet increment.

## 13. CONTRAINDICATIONS

13.1 Severe systemic reaction to the test dose.
13.2 ATG may exacerbate viral and parasitic infections, so do not give ATG in the presence of active infection.

## 14. TIME TO RESPONSE

Response to ATG does not usually begin to occur before 3-4 months, so red cell and platelet transfusions will need to be continued as needed until the peripheral blood counts start to improve. Continue oral prophylactic antibiotics and antifungals while the patient is severely neutropenic.

## 15. REPEAT COURSES OF ATG

A second course of horse ATG preparation (ATGAM®) may be considered but the risks of side effects and anaphylaxis are likely to be increased, and the onset of serum sickness can occur earlier than after a first course. Always give a test dose before the second course of ATG.

## 16. THE USE OF CICLOSPORIN (CSA) IN THE TREATMENT OF APLASTIC ANAEMIA

The current standard immunosuppressive regimen for the treatment of AA is the combination of ATG and ciclosporin (CSA), and this applies to very severe, severe and non-severe AA.

The dose of oral CSA is 5mg/kg/day aiming to keep whole blood trough drug level between 100 and 200microgram/l. The dose must be reduced if using posaconazole with CSA.

**Oral CSA is usually commenced on the first day of ATG.**

See Appendix 1 for further details on the use of CSA in the treatment of aplastic anaemia.

## 17. THE USE OF G-CSF WITH ATG

The routine use of daily G-CSF with ATG and CSA is no longer recommended. Recent prospective randomized studies have shown no benefit in giving G-CSF daily after ATG and CSA, as there is no difference in incidence of serious infections, trilineage haematological response or survival when G-CSF is used.

## 18. APPENDIX 1: Use of CSA in the treatment of Aplastic Anaemia

### 18.1. Dose

Initial dose of oral ciclosporin (CSA) is 2.5 mg/kg twice daily. Since CSA is lipid soluble, dose is based on actual body weight. For elderly patients, start with lower dose e.g. 1.25 mg/kg twice daily if > 60 y old and adjust according to renal function, blood pressure and CSA levels.

### 18.2. Clinical formulation

Oral CSA (Neoral®) is available as capsules (10mg, 25 mg, 50mg and 100 mg) or in an oily yellow solution (100 mg/ml). Intravenous CSA is given as an IV infusion in 100ml N/Saline or 5% glucose over 2 hrs.

### 18.3. Side effects

 Nephrotoxicity:

Increases in serum creatinine are dose and plasma level related. Additive nephrotoxicity occurs particularly with aminoglycosides, vancomycin and amphotericin B (also with ACE inhibitors, NSAID, quinolones and trimethoprim). Hyperkaemia may occur with long term use of CSA. There is an Increased risk of renal impairment with drugs such as ACE inhibitors and K+ sparing diuretics e.g. amiloride. Avoid high dietary potassium intake. CSA can aggravate hypomagnesaemia in the setting of BMT as in renal transplants.

 Hypertension:

Often associated with fluid retention, and potentiated by methylprednisolone

 Neurological:

Grand mal fits, usually occur in patients with fluid retention, uncontrolled hypertension and high CSA blood levels, often during combined CSA and methylprednisolone therapy.
Tremor - suggests overdose
Muscle cramps, paraesthesiae of hands and feet.

 Gastrointestinal Tract:

Anorexia, nausea, vomiting
Gingival hypertrophy

 Hepatotoxicity:

Associated with hyperbilirubinaemia, which can be potentiated by erythromycin, voriconazole, norethisterone, oxymetholone. Monitor LFTs carefully while on itraconazole.

 Anaphylaxis:

Reactions are due to the drug or emulsifying agent in the IV preparation.

 Hypertrichosis

**1. Assessment of patient prior to commencing CSA**

Check blood pressure (BP), serum electrolytes, urea, creatinine (U&Es) liver function tests (LFTs). Review current medication patient is taking, and ask about herbal remedies.

**2. Monitoring of patient on CSA**

Aim to keep trough whole blood CSA level 100 and 200microgram/l

**Frequency of monitoring**

Whole blood CSA levels should be measured from day + 2 and then twice weekly on inpatients. For outpatients, weekly CSA levels until stable. Levels can then be checked every 2-3 weeks. If renal and hepatic function is abnormal, levels should be checked more frequently.

**Sample Required**

12 hour trough whole blood levels are measured i.e. before the morning dose of CSA.
Send 3 ml blood in EDTA with special request form to the Analytical Unit.
Always take sample from a peripheral vein.
Never take blood for CSA level from the CSA infusion line of the Hickman catheter, even after thorough flushing of the line, otherwise falsely high levels will be obtained.

**Renal function and other electrolytes**

The most frequent dose limiting toxicity is renal impairment.
Monitor renal function daily and liver function 3 times weekly whilst patient is an in-patient.
Monitor serum $Ca^{2+}$ and $Mg^{2+}$ weekly.
A slow rise in serum creatinine to 120-130 µmol/l is common in the first few weeks of therapy.
If the creatinine is > 130 µmol/l a dose adjustment should be made.
If a rapid rise in creatinine occurs, stop CSA for 1-2 doses, monitor renal function and CSA level, make appropriate dose adjustment.

**Blood Pressure**

As CSA can cause hypertension, monitor BP regularly.
Antihypertensive therapy may be necessary (use atenolol or amlodipine)

**3. Timing of CSA after ATG**

CSA is usually now commenced on the first day of ATG.

**4. Duration of treatment**

For the treatment of AA, CSA is given for a minimum of 12 months and usually much longer. If a response occurs, CSA is continued at full dose until the blood count has stopped rising and has plateaued. It is then continued for a further 12 months, followed by a slow taper of around 25mg every 2-3 months. Too rapid dose reduction is associated with a high incidence of relapse of aplastic anaemia. Some patients, however, are CSA dependent and will need a low dose for a long

period of time. In these patients it may be impossible to stop the CSA completely.

**5. CSA drug interactions**

Listed below are drugs which are often used with CSA in BMT patients, together with information on the potential significance of the interaction and what action should be taken. It is not an exhaustive list. If a drug which you wish to use is not listed please contact a pharmacist for further information.
Problems may occur on discontinuation of drugs as well as on starting them e.g. dangerous CSA toxicity will occur when drugs such as rifampicin are discontinued, unless the dose of CSA is reduced.

**(a) Anticonvulsants**
 **Phenytoin, phenobarbital, and carbamazepine**: CSA levels may fall dramatically on starting these drugs (and will rise on discontinuation).
ACTION: Monitor CSA levels. Dose may need to be increased 2-4 fold (2-3 fold reduction on stopping anticonvulsants).

**(b) Antiemetics**
 **Metoclopramide**: increases CSA absorption.
ACTION: Monitor levels and reduce CSA dose if necessary.

**c) Antihypertensives**
 **Nifedipine**: oedema may worsen. It further increases the risk of gingival hyperplasia.

 **Potassium sparing diuretics**: may increase risk of hyperkalemia.

**(d) Antibacterials**
 **Aminoglycosides (gentamicin, amikacin), co-trimoxazole, trimethoprim, vancomycin and quinolones (Ciprofloxacin)**: may increase nephrotoxicity of CSA.
ACTION: Monitor CSA levels and renal function. Reduce antibiotic dose accordingly.

 **Macrolides (erythromycin and clarithromycin)**: causes a marked increase in CSA levels (4-5 fold) on starting the drug and a similar decrease on stopping.
ACTION: Use an alternative e.g. azithromycin if appropriate otherwise reduce CSA dose and monitor levels closely. Increase CSA dose once Erythromycin stopped.

 **Rifampicin**: CSA levels are markedly reduced. Conversely an overshoot of CSA levels will occur when Rifampicin is stopped (see above).
ACTION: Increase CSA dose according to levels. Dose may need to be increased 3-5 fold. Reduce dose when stopping Rifampicin.

 **Imipenen/cilastatin**: isolated reports of increased CSA levels also disorientation, confusion, motor aphasia and tremor.
ACTION: Monitor renal function.

**(e) Antifungals**
 **Amphotericin B:** increases nephrotoxicity of CSA (not with Ambisome or Abelcet).
ACTION: Monitor renal function and CSA levels closely.

 **Itraconazole and posaconazole**: increase CSA levels through inhibition of metabolism (up to 3-4 times).
ACTION: Monitor CSA levels and adjust dose accordingly when introducing/discontinuing itraconazole or posaconazole.

 **Fluconazole**: possible increase in CSA levels of up to 2-3 fold after 2-3 days through inhibition of clearance. Perhaps more likely at higher doses of fluconazole.

ACTION: Monitor CSA levels and adjust dose accordingly particularly when Introducing or discontinuing doses of fluconazole above 100 mg.

 **Voriconazole:** increased ciclosporin levels. Dose of ciclosporin should be halved when voriconazole commenced

**(f) Antivirals**

 **Aciclovir:** isolated reports of increases in serum creatinine and ATN – may increase nephrotoxicity of CSA.
ACTION: Monitor renal function and adjust aciclovir dose if necessary, particularly with IV aciclovir.

 **Foscarnet:** increased risk of nephrotoxicity.

**(g) Anti-ulcer agents**

 **H2 antagonists (cimetidine, ranitidine)**: these do not appear to affect serum CSA levels. Deterioration in renal function may occur.

ACTION: Monitor renal function.

 **Omeprazole**: isolated case reports of increased CSA levels.

ACTION: Monitor CSA levels.

**(h) Steroids**

 **Methylprednisolone**: convulsions, associated with fluid retention and hypertension, have been reported in BMT patients on CSA and Methylprednisolone 5-20 mg/kg/day.

ACTION: Monitor CSA levels.

 **Norethisterone**: isolated reports that withdrawal of norethisterone has reduced CSA levels.

In addition, exacerbation of fluid retention and hypertension may occur.
ACTION: Monitor CSA levels when introducing/discontinuing norethisterone.

**(i) Miscellaneous**

 **Methotrexate:** previous or concurrent treatment with CSA may increase the risk of liver toxicity.

ACTION: Monitor LFTs.

 **Oxymetholone:** risk of hepatoxicity

ACTION: Avoid the combination of CSA and oxymetholone.

 **Grapefruit juice:** can increase CSA levels.

ACTION: Advise patients not to drink grapefruit juice whilst on CSA.

- **Chinese herbal remedies:** can interfere with CSA levels.

**ACTION: Advise patients not to take whilst on CSA.**

## 19. Appendix 2: Selected References and Guidelines

1. Marsh et al, **Br J Haematol. 2009 Oct; 147(1):43-70.**
**Guidelines for the diagnosis and management of aplastic anaemia.**
This British for Standards in Haematology (BCSH) guideline summarises the evidence for treatment of aplastic anaemia, including the use of ATG for patients who are ineligible for haemopoietic stem cell transplantation (HSCT)

2. EBMT SAAWP, **Lancet. 2011 Nov 26; 378(9806):1831-3**
**Rabbit ATG for aplastic anaemia treatment: a backward step?**
The EBMT Severe Aplastic Anaemia Working Party (SAAWP) has recently published guidance concerning the use and availability of ATG with the recommendation to use horse ATG and ciclosporin.

3. Scheinberg et al, **N Engl J Med. 2011 Aug 4;365(5):430-8**
**Horse versus rabbit antithymocyte globulin in acquired aplastic anemia.**
This is the prospective randomised study from NIH, USA of 120 patients, who received either horse ATG (ATGAM) with CSA or rabbit ATG (Thymoglobuline) with CSA. The 6 month response to horse ATG was 68% compared to only 37% with rabbit ATG. Overall survival (OS) at 3 years with horse ATG was 96% (compared to 76% for rabbit ATG).

4. Marsh et al, **Blood** 2012; 119: 5391-5396. **Prospective study of rabbit antithymocyte globulin and ciclosporin for aplastic anemia from the EBMT Severe Aplastic Anemia Working Party.**
This paper summarises the European (EBMT) study showing worse survival and more deaths after rabbit ATG (Thymoglobuline)compared to horse ATG (Lymphoglobuline).

# APPENDIX 5: CONTRIBUTIONS AND COPYRIGHT

Dr Shahram Kordasti performed mass cytometry data analysis (3.3.1, 3.3.2, 3.3.4, 4.3.1, 5.3.2) and RNA sequencing data analysis (4.3.4).

Dr Giovanni A. M. Povoleri performed GEP data analysis (4.3.3).

Dr Sun Sook Chung performed data analysis for protein function analysis with Cytoscale (4.3.3).

Dr Shok Ping Lim performed apoptosis prevention by IL-2 addition experiment (4.3.5) and Bcl-2 WB (5.3.7).

Dr Steven Orr performed phosphorylated STAT5 WB (5.3.1).

Mr Thomas Seidl designed and performed TSDR sequencing and data analysis (5.3.4).

Dr Syed Mian designed and performed all the animal work experiments (5.3.8).

Subash Balakrishnan, on behalf of Elsevier Permissions Granting Team, granted permission to use all the figures taken from "Cellular and Molecular Immunology", 7th edition[1] (Figure 1.1 Innate and adaptive immunity Figure 1.2 Epithelial barriers Figure 1.3 Functions of natural killers Figure 1.4 Mast cell Figure 1.5 Types of adaptive immunity Figure 1.6 Specificity, memory and contraction of adaptive immune responses Figure 1.7, Figure 1.8, Figure 1.9, Figure 1.10, Figure 1.11, Figure 1.12, Figure 1.13, Figure 1.14, Figure 1.15, Figure 1.16, Figure 1.17, Figure 1.19, Figure 1.20, Figure 1.21, Figure 1.22, Figure 1.23, Figure 1.24). Reference: "Book (1172836) [210603-014848]".