Investigation into the development of thymopoiesis, mechanisms of action and assessment of quality of life in a cohort of paediatric patients with acute graft-versus-host disease treated with extracorporeal photopheresis

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April 2018

Abstract

The primary immunological aim following allogeneic haematopoietic stem cell transplantation (HSCT) is to achieve complete, sustained immune reconstitution and requires a functioning thymus for naïve T-lymphocyte neogenesis. Acute graft-versus-host disease (aGVHD) remains a major obstacle and targets the thymus, with damage to the thymic microenvironment impairing thymopoiesis, further compounded by corticosteroid treatment. Extracorporeal photopheresis (ECP) is an alternative therapy for aGVHD, possibly acting in an immunomodulatory fashion, although the true *in vivo* effects are not established.

21 paediatric patients were recruited; 8 ECP patients, 6 patients with no aGVHD (control group 1) and 7 patients with aGVHD not requiring ECP (control group 2). Thymic recovery was quantitatively measured by enumeration of $CD3^+CD4^+CD45RA^+CD31^+$ naïve T-lymphocytes by flow cytometry and TRECs using PCR, and qualitatively by TCR DNA spectratyping. Investigations of mechanisms of action of ECP included examination of dendritic cell (DC) subset patterns and regulatory T-lymphocytes (Tregs) using flow cytometry, and Th1 and Th2 cytokine profiles using ELISA. Quality of life was assessed using the PedsQL Generic Core Scales at the beginning and end of ECP treatment, and at 4 months post-HSCT for the control groups.

Four patients completed ECP therapy and demonstrated qualitative and quantitative improvement in thymic output. Faster thymic recovery was evident in those who started ECP earlier. Two patients remain on prolonged ECP treatment but with negligible thymic output, suggesting irreversible thymic damage. Comparison with the control groups demonstrated superior thymic recovery in the ECP group compared to control group 2. An increase in cDC and pDC populations was observed with a decline in the cDC/pDC ratio. There was reduced DC co-stimulatory marker expression in the responding ECP patients. Variable patterns in Tregs, and a decline in Th1 and incline in Th2 cytokines in the responding ECP patients with treatment progression were seen. QOL of life was lowest in the ECP group pre ECP, and post ECP there was an improvement in QOL parent-proxy scores.

These data demonstrate that ECP facilitates thymopoiesis in some, but not all, patients. Early initiation may promote faster thymic recovery. Patients with aGVHD not treated

with ECP appeared to have slower thymic recovery. Exploration of the mechanisms supported promotion of a tolerogenic environment in responding patients. Although additional data are needed, ECP appears to promote immune tolerance and, by reducing aGVHD and immunosuppression, facilitates thymic recovery and complete immune reconstitution post-HSCT.

Declaration

The material contained in this thesis is entirely the work of this author, unless otherwise stated, and has not been submitted for a degree previous to this or any other university.

Dr Aisling Flinn

April 2018

Acknowledgements

I would like to thank my supervisors Dr Andrew Gennery and Dr Xiao Nong Wang for their incredible support, guidance and friendship, and for playing a huge role in the development of my enthusiasm and passion for research and questioning. I have truly loved every minute.

The amazing ECP team, their friendship and support – Julie Guest, Jennifer Lawrence, Claire Welton and Carol Louden - who I am much indebted to!

Dr Emily Mavin and Dr Charlotte Alston, for their valuable support and advice with flow cytometry and spectratyping analyses.

My MRes students Catherine Roberts and Anna Ehrlich, and Dr Tom Altmann (Academic Foundation Programme trainee) for their enthusiasm, hard work and contribution to the study.

Everyone in the lab for their kindness and support, who were always willing to help in any way possible including Jean Norden, Dr Rachel Crossland, Liz Douglas, Dr Amy Publicover, Dr Venetia Bigley, Dr Urszula Cytlak-Chaudhuri, Sarah Pagan and all the Flow Cytometry staff.

Dr Stuart Adams for his help in establishing the TREC assay, and Drs Alessandra Sottini and Luisa Imberti for kindly supplying the TREC/TRAC plasmid.

The Bubble Foundation and Mallinckrodt Pharmaceuticals for their financial support.

Ciaran, for his patience and support, listening to me practice presentations and talk enthusiastically about the thymus.

To all the patients and families involved, without whom this study would not have been possible.

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Tables and Figures

Abbreviations

8-MOP	8-methoxypsoralen
aGVHD	Acute graft-versus-host disease
Allo-HSCT	Allogeneic haematopoietic stem cell transplantation
APC	Antigen presenting cell
ATG	Anti-thymocyte globulin
AHA	Autoimmune haemolytic anaemia
AIRE	Autoimmune Regulator
BO	Bronchiolitis obliterans
cGVHD	Chronic graft-versus-host disease
CSA	Ciclosporin
CDR3	Complementarity determining region 3
CR	Complete response
CT	Computed tomography
CHS	Contact hypersensitivity
cDC	Conventional DC
cTEC	Cortical thymic epithelial cell
CTCL	Cutaneous T-cell lymphoma
CTLA4	Cytotoxic T-lymphocyte antigen
DC	Dendritic cell
DP	Double positive
ECP	Extracorporeal photopheresis
FC	Facilitating cells
Flt3	Fms-like tyrosine kinase 3
FOXP3	Forkhead box p3
GIT	Gastrointestinal tract
GILZ	Glucocorticoid-induced leucine zipper
GITR	Glucocorticoid-induced tumour necrosis factor receptor-related protein
GARP	Glycoprotein A repetitions predominant
GVL	Graft-versus-leukaemia
HSCT	Haematopoietic stem cell transplantation
HPE	Homeostatic peripheral expansion
HLA	Human leukocyte antigen
IR	Immune reconstitution
ITP	Immune thrombocytopaenia
IDO	Indoleamine-2,3-deoxygenase
ICOS-L	Inducible co-stimulator ligand
iTreg	Inducible Treg
ILC	Innate lymphoid cell
KGF	Keratinocyte growth factor
LAP	Latency-associated peptide
LTBP	Latent-TGF-β-binding protein
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MFI	Median fluorescent intensity
mTEC	Medullary thymic epithelial cell
moDC	Monocyte-derived DC
MMF	Mycophenolate mofetil

NK	Natural killer
nTreg	Natural Treg
Nrp-1	Neuropilin-1
NR	No response
PR	Partial response
PedsQL	Pediatric Quality of Life
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid DC
PCR	Polymerase chain reaction
PID	Primary immune deficiency
PFTs	Pulmonary function tests
QOL	Quality of life
RTE	Recent thymic emigrant
Treg	Regulatory T-lymphocyte
SP	Single positive
Th	T helper
TCR	T-cell receptor
TREC	T-cell receptor excision circle
TEC	Thymic epithelial cell
TSDL	Thymic stromal-derived lymphopoietin
TRA	Tissue-restricted self-antigen
tol-DC	Tolerogenic DC
TRM	Transplant-related mortality
TN	Triple negative
UVA	Ultraviolet A

Thesis Structure

This thesis is divided into 6 chapters as follows:

1. General introduction and overall aims
2. Clinical progression with ECP treatment
3. Thymopoiesis with progression of ECP therapy
4. Mechanisms of action underlying ECP
5. Quality of life of patients receiving ECP treatment
6. Conclusions

Chapter 1 introduces the background of the project, includes a brief summary of the subsequent parts and states the overall aims of the project.

Chapter 2 contains information on patient recruitment and characteristics, sample collection and outlines results regarding clinical response to treatment and outcomes.

Chapters 3 - 5 contain a more detailed review of the literature relevant to each chapter, and the aims, methods, results, discussion and conclusion relevant for each part are described.

Part 6 is the overall discussion and conclusions bringing all the preceding chapters together.

The objective for this structure is to present the background information and data in the most logical relevant fashion to facilitate reading and interpretation.

Chapter 1 . General Introduction and Overall Aims

1.1 Background

1.1.1 Haematopoietic stem cell transplantation

Allogeneic haematopoietic stem cell transplantation (HSCT) is a potentially curative treatment option for a wide variety of disorders, including primary immune deficiency (PID) and metabolic disorders, haematopoietic malignancies, and bone marrow failure syndromes. Huge advances have been made since the first transplant in 1957[1], including improved human leukocyte antigen (HLA)-typing techniques, less toxic conditioning regimens and better supportive care, resulting in improved clinical outcomes and enabling more patients to avail of this therapeutic option. In the treatment of malignancies, disease eradication is achieved by a combination of pre-HSCT conditioning and the allogeneic graft-versus-leukaemia (GVL) effect. In other indications for HSCT including primary immune deficiencies, the defective or deficient component of the immune system is replaced by donor-derived stem cells, and in this situation, there is no benefit from the GVL effect. The primary immunological aim following HSCT is to achieve complete and durable immune reconstitution with a diverse and self-restricted T-cell receptor (TCR) repertoire capable of recognising a broad range of pathogens. Effective sustained adaptive T-lymphocyte immunity is essential to protect against infection, disease relapse and development of secondary malignancies.

1.1.2 Graft-versus-host-disease

Despite advances made in the management of HSCT, acute graft-versus-host disease (aGVHD) remains a leading cause of morbidity and mortality[2], and limits both the success and more widespread application of this therapy. The incidence of grade 2–4 aGVHD in children ranges from 14%-40%[3,4], depending on factors such as the degree of histocompatibility mismatch, age of the recipient, the underlying condition and the conditioning regimen used[5]. Higher grades have been associated with worse transplant-related mortality (TRM) and lower overall survival rates[6].

Acute GVHD is mediated by mature donor stem cell-derived, donor thymus-tolerised T-lymphocytes that recognise and attack disparate host antigens resulting in a harmful inflammatory response. The most important targets are the HLAs, encoded by the major histocompatibility complex (MHC) located on the short arm of chromosome 6, which

play a key role in tissue histocompatibility and T-lymphocyte recognition[7]. The degree of MHC mismatch between donor and recipient is the most important determinant of aGVHD, most importantly at the HLA-A, -B, -C and DRB1 loci[2]. However, even in the setting of a HLA identical sibling HSCT, alloreactivity can still occur due to mismatch between minor histocompatibility antigens[8].

1.1.3 Pathophysiology of aGVHD

The Billingham criteria identified the three requirements necessary for the development of aGVHD[9]:

(1) The graft must contain immunocompetent cells

(2) There must be a disparity between host antigens and those in the graft

(3) The host must be unable to launch an immune response against this process

Elucidation of aGVHD pathophysiology is based on experimental models[10]:

- Damage to host tissue by conditioning regimens, underlying disease, and/or infections leads to release of pro-inflammatory cytokines such as IFNγ, TNFα and IL-1 resulting in an inflammatory environment leading to the activation and maturation of host antigen presenting cells (APCs), and upregulation of adhesion and costimulatory molecules. This cultivates an environment that promotes the recruitment of donor alloreactive T-lymphocytes.
- Donor T-lymphocytes recognise disparate alloantigens on activated host APCs and become activated, proliferate, differentiate, produce further inflammatory cytokines and migrate to target organs directed by chemokines, selectins and integrins.
- Effector cells, primarily cytotoxic T-lymphocytes and natural killer (NK) cells, and soluble effectors cause apoptosis of target cells mediated by perforin/granzyme and Fas/Fas ligand pathways[11].

1.1.4 Clinical features

Historically, aGVHD was defined as occurring within the first 100 days following HSCT, and chronic GVHD (cGVHD) as after 100 days. However, with the development of new

strategies such as reduced intensive conditioning, this definition is less clear and a more recent re-classification now includes both late aGVHD occurring after 100 days and overlap syndrome with features of both (Table 1.1)[12].

Acute GVHD principally involves the skin, gastrointestinal tract (GIT) and liver, with skin manifestations occurring most commonly and usually the earliest following engraftment[2,10,13,14]. Patients typically develop a pruritic maculopapular rash, initially around the neck and shoulders, often involving the palms and soles but sparing the scalp. In severe cases, blistering and ulceration can occur. Gastrointestinal aGVHD usually involves diarrhoea but may also manifest as vomiting, nausea, anorexia, abdominal pain and bleeding. Liver involvement typically manifests as cholestasis due to damage to the bile canaliculi, with elevated alkaline phosphatase and serum bilirubin (Table 1.2). The thymus has also been shown to be a target in aGVHD, with a detrimental impact to both the thymic stroma and developing thymocytes. Whilst accepted as part of the spectrum of cGVHD, pulmonary involvement in aGVHD is more controversial. Although well documented, supported by compelling experimental evidence, knowledge in pulmonary aGVHD is more deficient due to limited clinical data and difficulty in histopathological and clinical diagnosis due to overlap with other aetiologies[15,16].

Acute GVHD is staged according to the extent of involvement of the skin, GIT and liver (Table 1.3)[14]. Increasing grades of aGVHD are associated with increasing TRM[6]. Severe aGVHD is associated with a poor prognosis with a 5% long-term survival for grade 4, and 25% for grade 3 disease[10].

Type		Definition
Acute	Classic acute GVHD	Onset ≤100 days post HSCT/DLI, features of acute GVHD
	Persistent/recurrent/late-onset acute GVHD	Onset >100 days post HSCT/DLI, features of acute GVHD
Chronic	Classic chronic GVHD	Onset at any time post HSCT/DLI, features of chronic GVHD
	Overlap syndrome	Onset at any time post HSCT/DLI, features of both acute and chronic GVHD

Table 1.1 Classification of GVHD as per the National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report[12].

	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4
Skin	No rash	Rash <25% of BSA	25-50% BSA	>50% Generalized erythroderma	Plus desquamation and bullae
Gut	Diarrhoea <10mls/kg/d	10-19.9mls/kg/d	20-30mls/kg/d	30mls/kg/d	Severe abdominal pain +/- ileus, frank blood or melena
Upper GI	-	Severe nausea/vomiting	-	-	-
Liver	Bilirubin ≤2mg/dL	2.1-3mg/dL	3.1-6 mg/dL	6.1 – 15 mg/dL	>15mg/dL

Table 1.2 Acute GVHD staging of individual organ involvement using the modified Seattle Glucksberg criteria[17,18].

Overall aGVHD grade	Skin stage	Liver stage	GIT stage	Upper GI stage
Grade 1	1-2	0	0	0
Grade 2	3	1	1	1
Grade 3	-	2-3	2-4	-
Grade 4	4	4	-	-

Table 1.3 Overall aGVHD grading using the modified Seattle Glucksberg grading system[17,18].

1.1.5 Thymopoiesis and T-lymphocyte reconstitution post-HSCT

The thymus gland is the primary site of T-lymphocyte development. Following recruitment of progenitor stem cells from the bone marrow and a sequential developmental process within the specialised microenvironment of the thymic stroma, naïve T-lymphocytes are exported into the circulation. Crucially, developing thymocytes undergo an education process termed positive and negative selection before their exit to the periphery. This process, called central tolerance, enables T-lymphocytes to only recognise antigen in the context of self-MHC and prevents reactivity to self-antigen. Maintenance of the thymic microenvironment is essential for the normal thymopoiesis process, and, conversely, normal thymic development depends on input from the developing thymocytes. The thymus is also responsible for the production of natural regulatory T-lymphocytes (nTregs), another essential tool in the maintenance of tolerance.

Following HSCT, complete and sustained reconstitution of the T-lymphocyte compartment requires donor progenitor cells to enter the thymus and undergo the normal developmental process, resulting in the production of naïve self-tolerant T-lymphocytes with a broad repertoire of TCRs enabling an appropriate immune response to a vast range of antigens. Damage to the thymic microenvironment as a result of chemotherapy, radiotherapy and/or aGVHD can disrupt this process, delaying successful restoration of adaptive T-lymphocyte immunity.

1.1.6 Acute GVHD and the thymus

It is now well established that the thymus is also a sensitive target in aGVHD, affecting both the stromal compartment and the developing thymocytes[19-21] with negative consequences on both the quality and quantity of thymic output, reducing the number of naïve T-lymphocytes and causing a distorted TCR repertoire. First line treatment of aGVHD is corticosteroids, a potent systemic immunosuppressive and anti-inflammatory agent, but which also impairs thymopoiesis, further compounding the disturbance of thymic function, subjecting the patient to increased risk of infections and other complications.

1.1.7 Extracorporeal photopheresis

Extracorporeal photopheresis (ECP) involves the collection of peripheral blood mononuclear cells (PBMCs) by apheresis, exposure to the photoactive drug 8-methoxypsoralen (8-MOP) and ultraviolet A (UVA) radiation, followed by re-infusion of the photoactivated cells back into the patient[22]. It was initially advocated for the treatment of cutaneous T-cell lymphoma (CTCL)[23], but has since been shown to also be effective in a wide variety of clinical settings, including GVHD[24]. The clinical efficacy of ECP in the treatment of aGVHD in patients has been demonstrated in several studies, as well as the corticosteroid-sparing effects, safety and tolerability[24-29]. A systematic analysis of prospective studies examining ECP treatment outcomes in corticosteroid refractory/dependent/intolerant aGVHD in both adults and children found an overall response rate of almost 70% in all organs, which is an encouraging result compared to other second line treatments used[30]. Based on the evidence available, the UK Expert Photopheresis Group guidelines state that ECP should be considered as second line therapy for patients with aGVHD grades 2-4 who are steroid refractory/dependant/intolerant[31].

ECP is given as one cycle weekly (2 treatments over 2 consecutive days) and is recommended for a minimum of 8 weeks[31]. Three treatments per week for the first 4 weeks may be considered for those with grade 3 or 4 disease. The adverse side effects of ECP treatment are minimal and predominantly related to central venous access. Children can develop hypovolaemia although this issue is less problematic with erythrocyte priming of the ECP circuit. Barriers to ECP treatment include access as it is carried out in a limited number of institutions, cost and the need for long-term central venous access.

The mechanisms behind ECP have not been fully elucidated but, in the setting of aGVHD, it is believed to act in an immunomodulatory fashion with evidence suggesting a key role played by dendritic cells (DCs) and regulatory T-lymphocytes (Tregs)[32,33]. It is novel in the fact that it has shown potential to treat pathophysiologically very different diseases, with mechanisms that appear to be both immune-stimulatory to treat CTCL, and immune-tolerating as observed in aGVHD, although the pathways which result in these opposing outcomes is not clear.

The major, and unique, advantage of ECP as a therapeutic modality is the lack of global immunosuppression with preservation of adaptive immune responses to novel and

recall antigens, therefore not increasing the risk of serious infections or disease relapse, or interfering with the GVL effect[34,35]. By promoting immune tolerance and resolving aGVHD, and by reducing the burden of immunosuppressive medications such as corticosteroids with their associated unwanted effects, damage to the thymus is mitigated, potentially allowing regeneration of the thymic microenvironment and restoration of thymopoiesis, paving the way for robust adaptive immunity, improving the ability to fight infection and reduce the risk of secondary malignancy or relapse.

1.1.8 Quality of life

Health related quality of life (QOL) assessment is a key component in the evaluation of a treatment outcome, providing essential patient information that can help to optimise delivery of care. ECP, whilst safe with few reported adverse side effects, is invasive and time consuming for patients and families, and there is currently no information available regarding QOL in paediatric patients requiring ECP for aGVHD. It is imperative to understand that despite the negative aspects of ECP, the benefits it entails in terms of both clinical outcome and patient and family satisfaction, present it as a feasible and accepted therapeutic modality.

1.2 Overall aims

The overall aims of the study were to:

1. Evaluate if ECP allows restoration of thymopoiesis in paediatric patients with aGVHD
2. Investigate the underlying mechanisms of ECP
3. Assess whether ECP treatment for aGVHD improves quality of life

Chapter 2 . Clinical Progression with ECP Treatment

2.1 Background

ECP has been shown to be an effective therapeutic modality in the treatment of corticosteroid-refractory or corticosteroid-dependent aGVHD[29,30,36,37]. Superior response rates are reported in cutaneous involvement compared to disease affecting the GIT or liver[29,30], although a prospective study involving 72 paediatric patients did not demonstrate a difference in complete response rates in organ-specific disease (78%, 76% and 84% in skin, GIT and liver aGVHD respectively)[36]. Early initiation of ECP treatment is associated with better clinical outcomes[29], and the potential in GVHD prophylaxis has also recently been demonstrated[38]. A key advantage of ECP over alternative second line interventions in aGVHD is the lack of generalised immunosuppression, and several studies have demonstrated the corticosteroid-sparing benefits[29,39].

Although the aim of this study was not to assess clinical outcomes following ECP treatment, this information is important in the interpretation of results in the subsequent chapters.

2.2 Materials and methods

2.2.1 Study participants

The ECP patient cohort was composed of paediatric patients who attended the Great North Children's Hospital for ECP treatment of aGVHD following allo-HSCT for either underlying PID or malignancy. In addition, there were two control groups in this study; (1) paediatric patients who received an allo-HSCT and did not develop aGVHD, and (2) paediatric patients who received an allo-HSCT and developed aGVHD but did not require treatment with ECP. Decision to treat with ECP was a physician-led clinical judgement. Both control groups attended the Great North Children's Hospital. Treatment of patients was not affected by this study. Ethical approval was granted by the South Eastern Scotland Research Ethics Committee (16/SS/0019/AM03). Informed consent was obtained from all patients or their legal guardians.

Hospital records of each patient were reviewed and clinical details recorded included demographics, underlying diagnosis, details regarding the HSCT and conditioning regimen, immunosuppressive agents used and dosages, type of GVHD and stage,

response to ECP and overall outcome. The conditioning regimen was described as myeloablative (MA) if cyclophosphamide was used in combination with busulfan or with total body and cranial irradiation, reduced toxicity MA if cyclophosphamide was used in combination with fludarabine, and reduced intensity conditioning (RIC) if fludarabine and treosulfan were used, with or without additional thiotepa. Overall grade of aGVHD was determined using the modified Glucksberg criteria. Clinical response to ECP was defined as complete (CR) if there was complete resolution of GVHD, partial (PR) if there was some improvement in GVHD or no response (NR).

2.2.2 Administration of ECP

ECP was administered on 2 consecutive days every week for the first 9 weeks, every 2 weeks for the next 7 weeks, every 3 weeks for the following 4 weeks and monthly thereafter until it was decided to cease treatment, although some patients deviated from this protocol based on clinical judgement. ECP was delivered using the CELLEX® System (Mallinckrodt Pharmaceuticals, NJ, USA) apheresis machine as previously described[40]. All patients <40kg were blood-primed prior to the procedure with packed erythrocytes. One ECP treatment was defined as two ECP procedures over two consecutive days.

2.2.3 Sample collection

For the ECP patient cohort, blood samples were taken from a central venous line before ECP treatment on day 1 and again on day 2 of each cycle. Blood samples included 15mls of whole blood in a universal tube containing heparin and 5mls in a gold top BD Vacutainer Blood Collection Tube (BD Biosciences) containing clot activator and separation gel.

For the control patients, initial blood samples were taken at 4 months post-HSCT at the same time blood was taken for routine clinical purposes either peripherally or from a central venous line. Blood samples included up to 5mls of whole blood in a universal tube containing heparin and up to 5mls in a gold top BD Vacutainer Blood Collection Tube (BD Biosciences) containing clot activator and separation gel. Subsequent blood samples were taken at 8 and 12 months post-HSCT. The frequency of activated T-lymphocytes ($CD3^+CD4^+HLA\text{-}DR^+$ T-lymphocytes) was measured as part of the Trucount® panel described in section 3.4.2. Of note, data collection at 8 and 12 months

post-HSCT is ongoing and the number of samples available for analysis at each time point is shown in Table 2.1. Statistical analysis was performed using Prism v5.00 (GraphPad Software, Inc.), with a significant p value defined as <0.05.

	4 months	8 months	12 months
ECP group	n = 8	n = 8	n = 5
Control group 1	n = 6	n = 4	n = 4
Control group 2	n = 7	n = 5	n = 3

Table 2.1 Numbers of prospective blood samples from each group available for each time point. Data collection is ongoing at 8 and 12 months.

2.3 Results

2.3.1 Prospective patient recruitment

Twenty-one patients in total were recruited from June 2016 - January 2018, including 8 ECP patients. At the time of data analysis, 4 ECP patients successfully completed therapy, 2 are still receiving ECP treatment and 2 patients were withdrawn from treatment. Six control patients with no aGVHD (group 1) were recruited and 7 control patients with aGVHD not requiring ECP (group 2) were recruited. One patient from group 2 had relapse of leukaemia 10 months post-HSCT and was withdrawn from the study.

2.3.2 Patient characteristics

Three ECP patients had an underlying diagnosis of PID and 5 had underlying malignancy (two patients with relapsed acute myeloid leukaemia (AML), two with high-risk AML and one patient with relapsed acute lymphoblastic leukaemia (ALL)). Median age at time of HSCT was 5.8 years (range 0.8-13 years) in the ECP group, 7.3 years in control group 1 with no aGVHD (range 0.6-18.5 years) and 6.6 years in control group 2 with aGVHD (range 1.1-15 years). A summary of the clinical characteristics of the ECP patients and control patients is provided in Table 2.2. Median weight of patients at the time of ECP commencement was 16.6kg (range 11.5-27.6kg). Median time from HSCT to commencement of ECP was 48 days (range 35-173 days).

Details of aGVHD diagnoses and management are shown in Table 2.3. All patients in control group 2 had skin aGVHD, with a maximum grade of 2. Two patients received treatment with topical corticosteroids only, along with continuation of aGVHD prophylaxis. All ECP patients had a maximum grade 2-3 aGVHD and received multiple other immunosuppressive agents prior to ECP, including continuation of aGVHD prophylaxis. Seven ECP patients received infliximab prior to ECP. ECP was well tolerated by all patients with no procedure-related adverse events.

Patient 1, who remains on prolonged ECP treatment, was initially diagnosed with skin aGVHD at day +12 post-HSCT which responded to systemic corticosteroids and topical treatment. This was followed by an episode of pneumonitis of unclear cause on day +43, which improved with systemic corticosteroids and infliximab, alongside antimicrobial and antifungal therapies, although respiratory symptoms persisted. Subsequent worsening of symptoms precipitated a chest computed tomography (CT) on day +151 suggestive of bronchiolitis obliterans (BO), leading to a diagnosis of lung GVHD. No infectious cause was identified although lung biopsy was not performed due to poor clinical condition. Pulmonary function tests (PFTs) demonstrated a low FEV1 (32.5%) and FEV1/FVC ratio (63.7%) and high dose corticosteroids and ECP were started. There was initial overall improvement in respiratory symptoms, repeat chest CT after cycle 15 of ECP therapy showed no progression and a corticosteroid dose nadir of 0.1mg/kg was reached at cycle 30 (week 57 of ECP, 80 weeks post-HSCT) without deterioration in respiratory symptoms. At this point (on four weekly ECP), patient 1 developed liver aGVHD, confirmed on biopsy (maximum bilirubin 5.6mg/dL), requiring re-initiation of high dose corticosteroids, commencement of sirolimus, and resumption of weekly ECP. Liver dysfunction subsequently improved with normalisation of bilirubin, respiratory symptoms remained stable (dyspnoea on exertion) and most recent PFTs demonstrated an FEV1 29.6% and FEV1/FVC ratio 57.8. No other features suggesting cGVHD were present.

Patient 8 developed respiratory symptoms on day +39 post-HSCT (cough and dyspnoea with exertion). PFTs demonstrated a restrictive pattern (FEV1 35% and FEV1/FVC 91%). Corticosteroids 2mg/kg and inhaled budesonide were commenced with some initial improvement. Symptoms worsened upon weaning of corticosteroids, chest CT demonstrated progressive diffuse pulmonary nodularity with no evidence of infection or BO, and due to steroid-dependency ECP was commenced on day +103, leading to improvement in PFTs (FEV1 68.2%, FEV1/FVC ratio 95.3%). At cycle 11, following

reduction in ECP frequency to two weekly, alongside reduction in corticosteroids to 0.4mg/kg, there was worsening in repeat PFTs (FEV1 61.2%, FEV1/FVC ratio 87.9%). Lung biopsy at this stage showed active mild $CD3^+$ lymphocytic bronchiolitis, consistent with partially treated aGVHD and no infectious causes were identified. Corticosteroids were increased again with a good response with a plan to wean more slowly. Patient 8 had no other aGVHD organ involvement, and no other features of cGVHD.

	ECP Group N=8	Control Group 1 N=6	Control Group 2 N=7
Age at HSCT (years)			
Median	5.8	7.3	6.6
Range	0.8 - 13	0.6 – 18.5	1.1 - 15
Gender			
Male	6 (75%)	3 (50%)	4 (57.1%)
Female	2 (25%)	3 (50%)	3 (42.1%)
Underlying diagnosis			
Immune deficiency	3 (37.5%)	3 (50%)	4 (57.1%)
Malignancy/Haematological	5 (62.5%)	3 (50%)	3 (42.9%)
HSCT Source			
BM	6 (75%)	5 (83.3%)	3 (42.9%)
PBSC	2 (25%)	1 (16.7%)	4 (57.1%)
HSCT Donor			
Sibling/MFD	4 (50%)	4 (66.7%)	0
MUD	4 (50%)	2 (33.3%)	6 (85.7%)
Haploidentical	0	0	1 (14.3%)
HLA Matching			
10/10	7 (87.5%)	6 (100%)	4 (57.1%)
<10/10	1 (12.5%)	0	3 (42.9%)
Conditioning			
MA	5 (62.5%)	0	3 (42.9%)
Reduced toxicity MA	0	2 (33.3%)	0
RIC	3 (37.5%)	4 (66.7%)	4 (57.1%)
Serotherapy			
Yes	5 (62.5%)	5 (83.3%)	7 (100%)
No	3 (37.5%)	1 (16.7%)	0
GVHD prophylaxis			
CSA/MMF	3 (37.5%)	4 (66.7%)	3 (42.9%)
CSA alone	5 (62.5%)	2 (33.3%)	3 (42.9%)
None	0	0	1 (14.3%)

Table 2.2 Summary of ECP patient and control patient characteristics.

BM, bone marrow; PBSC, peripheral blood stem cells; MFD, matched family donor; MUD, matched unrelated donor; MA, myeloablative; RIC, reduced intensity conditioning; CSA, ciclosporin; MMF, mycophenolate mofetil.

	aGVHD organ involvement (max stage)	Maximum grade	Therapies (excluding ECP)	Reason for ECP
ECP group				
Patient 1	Skin (3), lung, liver (2)	3	CS, *CSA, MMF*, IFX, BUD	CS refractory
Patient 2	GIT (3), skin (2)	3	CS, *CSA*, IFX	CS refractory
Patient 3	Skin (3)	2	CS, *CSA*, MMF*, IFX	CS refractory
Patient 4	GIT (3), skin (2)	3	CS, *CSA*, IFX	CS refractory
Patient 5	GIT (3)	3	CS, *CSA*, MMF*, IFX	CS refractory
Patient 6	Skin (3)	2	CS, *CSA*, MMF*, IFX	CS refractory, CMV viraemia
Patient 7	Skin (3)	2	CS, *CSA*, IFX	CS dependency
Patient 8	Lung	?	CS, *CSA*, BUD	CS dependency
Control Group 2				
Patient 1	Skin (1)	1	Topical CS, *CSA, MMF*	N/A
Patient 2	Skin (3)	2	Topical + systemic CS, *CSA*	N/A
Patient 3	Skin (3)	2	Topical + systemic CS	N/A
Patient 4	Skin (1)	1	Topical CS, *CSA*	N/A
Patient 5	Skin (3)	2	Topical + systemic CS, *CSA, MMF*	N/A
Patient 6	Skin (3)	2	Topical + systemic CS, *CSA*	N/A
Patient 7	Skin (3)	1	Topical + systemic CS, *CSA, MMF*	N/A

Table 2.3 Details of GVHD of the ECP patients and patients from control group 2.

CS, corticosteroids; CSA, ciclosporin; IFX, infliximab; MMF, mycophenolate mofetil; BUD, budesonide (inhaled); *CSA - CSA later changed to tacrolimus. Medications in italics denote continued aGVHD prophylaxis.

2.3.3 Clinical outcomes

Among the patients who completed ECP, all had complete resolution of aGVHD (patients 2, 4, 5 and 6). Patient 3 was withdrawn after cycle 16 of ECP. Corticosteroids had been weaned to 0.4mg/kg by cycle 11, skin aGVHD had resolved and there was early evidence of restored thymic output. Clinical deterioration started at cycle 13, was subsequently diagnosed with Progressive Multifocal Leukoencephalopathy of unclear cause despite extensive investigation, and he died 17 months post-HSCT. Clinical consensus was that this patient had an unknown dormant neurotrophic virus, and weaning of immune suppression alongside immune reconstitution, led to an attempt to eradicate the virus resulting in widespread and destructive brain inflammation. Patient 7, who was high-risk with minimal residual disease at time of transplantation, was withdrawn after cycle 17 due to relapse of acute myeloid leukaemia (AML), despite evidence of early T-lymphocyte recovery and complete resolution of aGVHD. Patients 1 and 8 had a partial response with some improvement in clinical condition, but with ongoing chronic respiratory symptoms and persisting abnormalities on chest CT.

2.3.4 Frequency of activated T-lymphocytes

Elevated frequency of activated $CD4^+$ HLA-DR^+ T-lymphocytes was seen in all patients before starting ECP, with an overall decline in the frequency with progression of ECP (Figure 2.1). For patient 3, following an initial decline, there was an increase in frequency following clinical deterioration. Similarly, patient 1 had a transient increase correlating with the development of liver aGVHD, followed by a second decline. For patients 1 and 8, the overall decline in frequency of activated T-lymphocytes occurred in parallel with stabilisation of respiratory status, but not resolution of disease, unlike the patients who successfully completed treatment.

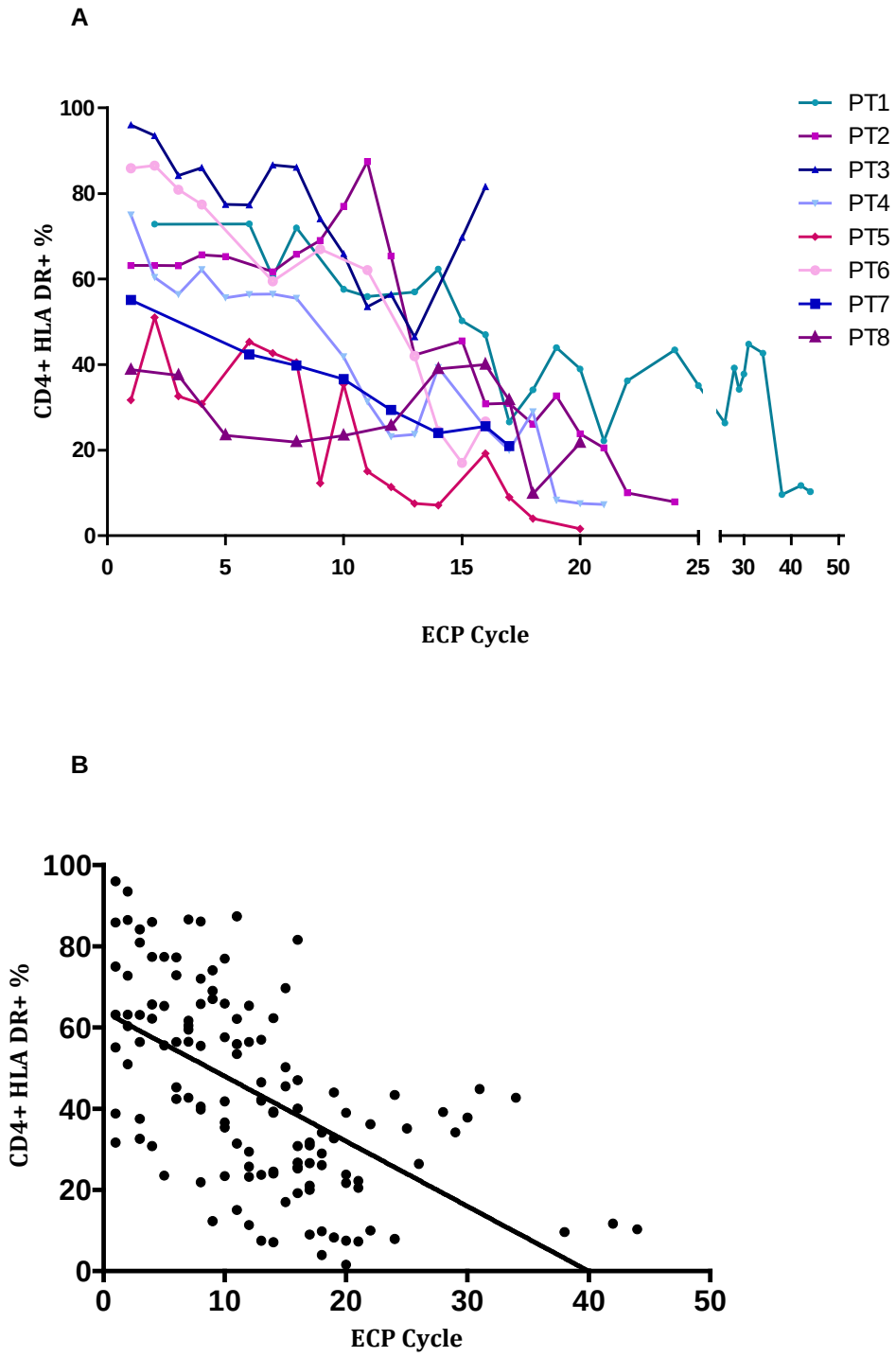


Figure 2.1 (A) An overall decline in the frequency of activated $CD4^{+}HLA\text{-}DR^{+}$ T-lymphocytes with progression of ECP was observed, with linear regression analysis (B) demonstrating a significant reduction ($p<0.0001$). An increase was evident in patient 3 prior to ECP cessation following deterioration and a transient increase, followed by a decrease, in patient 1, corresponding to the development of liver aGVHD.

2.3.5 Weaning of immune suppression

Immune suppression was successfully weaned in all patients. All patients were discontinued from further infliximab therapy (n=7). Five patients (Figure 2.2) had a gradual reduction in corticosteroid dose. Patient 1 required repeated high dose corticosteroid treatment following development of liver aGVHD at cycle 30, but prior to this immune suppression was successfully weaned. Patient 8 was weaned to 0.5mg/kg prednisolone, but, following repeat PFTs demonstrating deterioration from previous, this was increased with a view to wean more slowly. Patient 3 had been weaned from corticosteroids and mycophenolate mofetil (MMF), but restarted high dose corticosteroids due to deterioration in his clinical condition which was unrelated to aGVHD.

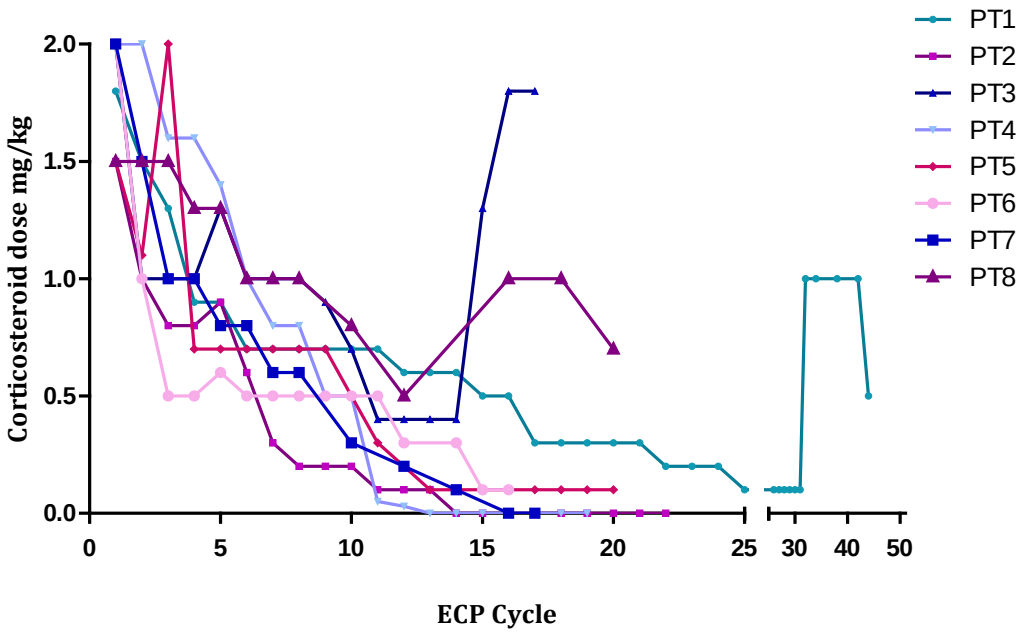


Figure 2.2 Five patients were successfully weaned from corticosteroids. Two patients (patient 1 and 8) required re-initiation of high dose corticosteroids during ECP but at a lower dose relative to the pre-ECP dose. Patient 3 was restarted on high dose corticosteroids for the management of a complication unrelated to aGVHD.

2.3.6 Outcome of control groups

In control group 1, one patient, with a previous underlying diagnosis of severe aplastic anaemia, developed immune thrombocytopaenia (ITP) 10 months post-HSCT which responded to a course of corticosteroids. In control group 2, two patients developed autoimmune haemolytic anaemia (AHA) post-HSCT, at 6 months and 7 months respectively. One patient responded to corticosteroids, and the second patient required treatment with corticosteroids, sirolimus and rituximab. One patient from group 2 was diagnosed with relapse of leukaemia at 10 months post-HSCT.

2.4 Discussion

Those who completed ECP therapy did well, with resolution of aGVHD and successful weaning of immune suppression. These patients commenced ECP early (median 47.5 days) post-HSCT and had disease limited to skin and/or GIT. The two patients that were withdrawn also started ECP early (both 35 days post-HSCT) for skin aGVHD. Their complications highlight important points related to immune reconstitution; patient 3 was thought to have developed a known rare complication of central nervous system immune reconstitution inflammatory syndrome (IRIS)[41], and patient 7 developed relapse of his disease despite showing evidence of early T-lymphocyte recovery. Both situations highlight that even when immune reconstitution is in progress, these patients remain vulnerable to devastating complications, either related to immune recovery or ongoing immune deficiency. ECP was well tolerated by all patients, reinforcing the positive safety profile of ECP including for patients with a low body weight.

Neither patient 1 nor 8 had a straightforward diagnosis. Patient 1 appears to have overlap syndrome, with features consistent with aGVHD involvement (skin and liver) and cGVHD (BO). Patient 8 does not fulfil diagnostic criteria for lung aGVHD[42] due to lack of other features of aGVHD, although these criteria are not specific for paediatric patients. In addition, there are no other cGVHD manifestations and a restrictive pattern of involvement is less typical of cGVHD[43]. Pulmonary disease in both appeared to have a degree of corticosteroid-responsiveness, with some improvement in respiratory status.

Autoimmunity is a recognised complication following allo-HSCT and is strongly associated with cGVHD[44]. Failure in normal thymic negative selection is implicated to be part of the underlying pathogenesis, along with failure of normal Treg formation, cytokine imbalance and autoreactive B-lymphocytes[20]. Mouse models have shown that thymic injury leads to impaired negative selection resulting in self-reactive T-lymphocytes and autoimmune manifestations[45]. The development of AHA in two patients from control group 2 supports the concept that thymic damage and consequent impaired thymopoiesis is an integral pathophysiological component behind the development of post-HSCT autoimmune disorders, although it is not known if the escaped potentially self-reactive T-lymphocytes can respond to self-antigens and initiate autoimmunity such as AHA. It also remains unclear why certain isolated autoimmune disorders develop, in certain patients, even without the presence of cGVHD. It is intriguing that a patient in control group 1 also developed an autoimmune

haematological disorder (ITP), raising the question of whether, even in the absence of overt clinical aGVHD, unknown thymic damage may be present.

2.5 Conclusion

Following allo-HSCT, patients are subjected to a period of vulnerability, susceptible to infections, GVHD and other complications. Exposure to prolonged immunosuppressive therapies and GVHD extends this vulnerable period further, and damage to the thymic microenvironment from these same insults, and others, can lead to further complications related to inadequate immune reconstitution. Efforts need to be made on minimising this period of vulnerability, by developing targeted therapies without systemic immunosuppression such as ECP, and by protecting the thymus, thereby supporting complete, fast and robust immune reconstitution to allow return to a normal life.

Chapter 3 . Thymopoiesis with Progression of ECP Treatment

3.1 Background

3.1.1 Thymic structure and normal thymopoiesis

The thymus is the primary lymphoid organ responsible for the continuous and life-long production of a functional pool of T-lymphocytes exhibiting a widely diverse TCR repertoire, capable of reacting with harmful foreign antigens, but that also recognise and tolerate self-antigens. Thymic structural development and T-lymphocyte production are processes which have been evolutionary conserved across species and over time[46], reinforcing the importance of this organ, although underestimated by many over the years[47]. It is divided into the subscapular region, the cortex, the cortico-medullary junction and the medulla. The major cellular components of the thymic stroma include epithelial cells, DCs, reticular fibroblasts and macrophages together forming a specialised 3-dimensional microenvironment responsible for the recruitment of T-lymphocyte precursors followed by an orderly sequential process of T-lymphocyte development and maturation[48]. Thymic epithelial cells (TECs) are the major component of the thymic stromal scaffold, divided into two main compartments – the cortical (c) and medullary (m) TECs which exhibit distinct functional properties. A correct and undisrupted thymic microenvironment is essential for normal T-lymphocyte development and, conversely, normal thymic development is also dependent on input from the developing thymocytes, so called 'thymic crosstalk'[49,50].

Because the thymus does not contain haematopoietic stem cells, progenitor cells are recruited from the bone marrow and enter the thymus at the cortico-medullary junction, with P-selectin and platelet P-selectin glycoprotein ligand on the progenitor cells playing an important role in this homing process[51] (Figure 3.1). Commitment to the T-lymphocyte lineage occurs following interaction between Notch1 receptor on the precursor cells and delta-like 4 ligand expressed by the cTECs[52]. At this stage, thymocytes express a 'triple negative' (TN) phenotype, devoid of CD3, CD4 and CD8 surface markers. Following expansion of the TN thymocytes, controlled by signals such as IL-7 and fms-like tyrosine kinase 3 (Flt3) ligand, both CD4 and CD8 are gained to acquire a 'double positive' (DP) phenotype with a heterodimeric TCRαβ complex[53,54]. TCR diversity is generated by random genetic rearrangements of the TCR loci and is estimated to be in the region of 10^{20} α-β chain combinations[48]. Because these genetic combinations have the potential to generate self-reactive TCRs which carry the risk of

autoimmunity, thymocytes are subjected to a rigorous two-stage selection process to identify and remove these potentially damaging self-reactive T-lymphocytes[48,55]. The first stage (positive selection) takes place in the cortex where DP thymocytes are exposed to a peptide/MHC complex presented by cTECs. Thymocytes that recognise this complex with intermediate affinity proceed to the next stage of development, ensuring recognition of antigen in association with self-MHC molecules. If the TCR does not recognise the complex or recognises with high affinity, the T-lymphocyte will undergo apoptosis or 'death by neglect'.

Following positive selection, the surviving thymocytes migrate to the medulla, predominantly regulated by chemokine receptor 7 and the corresponding ligands CCL19 and CCL21 expressed by mTECs, and subsequently lose either CD4 or CD8, dependent on which MHC class they associated with during positive selection, to become 'single positive' (SP) cells[54]. The second stage of TCR selection, designated negative selection, occurs in the medulla where SP thymocytes are exposed to a self-peptide/MHC complex presented by mTECs and DCs. Medullary TECs possess the unique ability of ectopic expression of a wide range of peripheral tissue-restricted self-antigens (TRAs). This so called 'promiscuous gene expression' is partly controlled by the Autoimmune Regulator (AIRE) transcription factor, as well as the more recently described Fezf2 transcription factor[56], to form a 'molecular mirror of peripheral self'[57,58]. TCRs that react with high affinity to the TRA/MHC complexes are deleted as these harbour the potential to elicit autoimmunity. Transfer of peptides between mTECs and intrathymic DCs is also thought to increase the likelihood of autoreactive T-lymphocytes encountering rare peptides[59]. Negative selection is an indispensable part of central tolerance, a key process that renders T-lymphocytes tolerant of self. Disruption to the *AIRE* gene leads to impaired negative selection and multi-organ autoimmunity, manifesting in humans as the rare condition autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED)[58]. Similarly, loss of Fezf2 in mouse models leads to autoantibody production and evidence of autoimmune disease[56].

The time interval required for intrathymic development and maturation is approximately 4 months[60]. The surviving mature self-tolerant, MHC-restricted thymocytes, termed recent thymic emigrants (RTEs), are exported into the circulation and expand in response to exposure to antigen or signals indicating lymphocytopaenia (homeostatic peripheral expansion, HPE). In order to maintain flexibility in diversity, thymic negative selection is not absolute, and ultimately some self-reactive T-

lymphocytes also enter the periphery. Other additional mechanisms are therefore in place to counteract this abscondment, one of these being the production of regulatory T-lymphocytes (Tregs) which play an essential role in down-regulating peripheral immune responses and limiting inflammation that may be harmful to the host but also in the maintenance of self-tolerance[61].

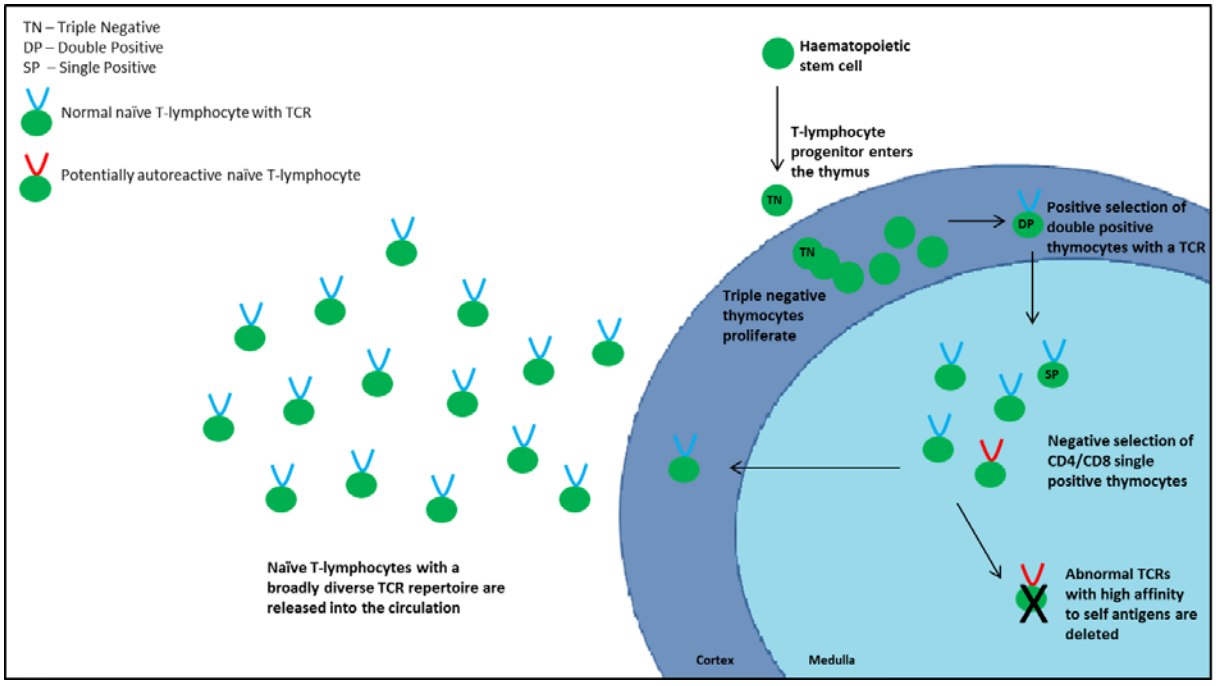


Figure 3.1 Normal thymopoiesis resulting in export of self-tolerant naïve T-lymphocytes with a diverse TCR repertoire into the circulation[62]**.**

3.1.2 Assessment of thymic output

The peripheral naïve T-lymphocyte pool is maintained by a combination of proliferation of the circulating naïve T-lymphocytes in response to exposure to antigen or homeostatic signals and thymic export of naïve T-lymphocytes balanced with apoptosis or differentiation into effector or memory T-lymphocytes. The size of the T-lymphocyte pool is predominantly determined by HPE, but the production of new T-lymphocytes is essential to maintain diversity of the TCR repertoire. Measuring thymic output provides an indicator of functional T-lymphocyte immunity and post-HSCT it indicates *de novo* production of T-lymphocytes from haematopoietic stem cells. Early effective reconstitution of the T-lymphocyte compartment has shown to be associated with

improved long-term T-lymphocyte reconstitution and overall outcome, emphasising the importance of monitoring in the early days following HSCT[63,64].

3.1.2.1 Quantitative measures of thymic output

T-cell receptor excision circles (TRECs) are stable circular episomal pieces of DNA generated as a by-product following TCR α and β chain formation. Following proliferation of the double negative (DN) thymocytes, the TCRβ chain is formed and generates Dβ-JβTRECs. The TCRα chain is formed at the DP stage of development generating a signal joint (sj) TREC, and approximately 70% of T-lymphocytes migrate from the thymus containing a sjTREC (hereafter referred to as TREC). Two important caveats must be considered in interpretation of TREC values: firstly, because TRECs do not replicate, TREC levels become progressively more dilute with naïve T-lymphocyte proliferation. In addition, persistence of naïve T-lymphocytes in the circulation, with TREC-containing T-lymphocytes detectable months after thymectomy[65], can further complicate interpretation of TRECs as a measure of thymic output. However, TREC quantification using polymerase chain reaction (PCR) does provide a practical and accepted measurement of thymic output by calculation of the frequency of TRECs in a defined population of mononuclear cells or sorted $CD4^+$ or $CD8^+$ T-lymphocytes[66,67], and has been shown to correlate well with recovery of $CD4^+CD45RA^+CD31^+$ T-lymphocyte numbers post-HSCT[66,68]. Calculation of absolute numbers of TRECs per millilitre of blood can overcome the problem that arises with cell division, and has also been shown to correlate with TREC content in a defined number of cells even in the setting of lymphocyte depletion[69]. A more accurate quantification of thymic output is the sjTREC to Dβ-JβTREC ratio (thymic ratio), representing the intrathymic proliferation that occurs between β and α chain rearrangement, the main determinant of thymic cellularity and number of recent thymic emigrants produced[70]. The thymic ratio has the advantage of not being affected by peripheral T-lymphocyte expansion, but use is limited by the fact that it is labour intensive and expensive.

No specific surface markers for RTEs have been identified in humans to date. Naïve T-lymphocyte markers such as CD45RA, CD27 and CD62L are not always reliable as expansion can occur without loss of these markers[71,72]. In addition, $CD45RO^+$ cells can revert back to a $CD45RA^+$ phenotype. With advances in immunophenotyping techniques, the expression of CD31 (platelet endothelial cell adhesion molecule-1) on $CD4^+CD45RA^+$ T-lymphocytes has been used as a marker for RTEs, demonstrated to have a high TREC

content and numbers declined with age[70,73,74]. In healthy children <15 years the frequency of $CD31^+$ expression in $CD4^+CD45RA^+$ T-lymphocytes is approximately 80-90%, decreasing to 60-80% in 20-30 year olds and 40-60% in those >60 years[74]. However, despite RTEs containing a high content of $CD4^+CD45RA^+CD31^+$ T-lymphocytes, $CD4^+CD45RA^+CD31^+$ T-lymphocytes are not exclusive RTE markers as CD31 is not always lost when naïve T-lymphocytes proliferate and CD31 can also be expressed by other cells including endothelial cells, mast cells and NK cells[75]. However, both $CD45RA^+CD27^+$ T-lymphocytes and $CD45RA^+CD31^+$ T-lymphocytes correlate strongly with TRECs in paediatric patients, suggesting either panel can be used in the clinical context for enumerating naïve T-lymphocytes in small blood samples[76].

3.1.2.2 Qualitative measures of thymic output

Quality of the T-lymphocyte compartment is best assessed by measuring diversity of the TCR repertoire and T-lymphocyte function. As the diversity of the TCR repertoire is almost completely reflective of the naïve T-lymphocyte compartment, measurement can provide information regarding thymic output[77,78]. A more diverse TCR repertoire is also associated with higher TREC concentrations[79]. Assessment of TCR diversity can be performed using flow cytometry, spectratyping of the complementarity determining region 3 (CDR3) and nucleotide sequencing. The TCR is composed of α and β chains, each consisting of variable and constant regions. The β chain is encoded by variable (V), diversity (D), junctional (J) and constant (C) gene segments, and the CDR3 region is formed by joining of V-D-J segments followed by the creation of further diversity by addition and removal of nucleotides. This creates a hypervariable region; the part of the TCR that binds to cognate antigen and where the highest level of diversity is found. Spectratyping uses RNA from T-lymphocytes to amplify the cDNA of the CDR3 region providing information about the CDR3 lengths and distribution patterns in each of the Vβ families measured, with normal complexity characterised by a Gaussian distribution. Flow cytometry quantifies the frequency of T-lymphocytes expressing a certain TCR using specific Vβ family subgroup specific monoclonal antibodies but is limited by restricted availability of antibodies. Compared to flow cytometry, spectratyping provides more detailed resolution of TCR diversity, however there is no accepted single standardised method of analysing data at present and this technique gives equal weighting to all Vβ families measured, independent of how many genes they contain[80]. However, analysis of the TCR repertoire using CDR3 spectratyping has shown to

correlate with immune function in HSCT recipients[81]. Nucleotide sequencing of CDR3 regions provides even more in depth analysis, but is expensive and, although evolving, is not widely available at present[82]. T-lymphocyte functional tests involve measuring levels of cytokines following T-lymphocyte stimulation or detecting the presence of antigen-specific T-lymphocytes.

3.1.3 HSCT and T-lymphocyte reconstitution

Conditioning is usually given prior to HSCT to eradicate malignant cells, prevent graft rejection, and to make space in the bone marrow for the incoming graft. As a result, there is an 'aplastic phase', with obliteration of innate and adaptive immune responses, subjecting the patient to a period of increased risk of infection and other complications until the stem cells engraft and reconstitution of the immune system ensues. Recovery of the innate immune system occurs relatively quickly, but effective reconstitution of the adaptive T- and B-lymphocyte compartments is a lengthier and more complex process, and accounts for the prolonged period of immune deficiency and susceptibility to infection following HSCT[83,84].

Swift rebuilding of a competent and normo-cellular T-lymphocyte compartment is an essential prerequisite for a normal life enabling regular development and function. Incomplete or delayed immune reconstitution, particularly of the T-lymphocyte compartment, is associated with increased post-transplant morbidity and mortality[85-87]. Early T-lymphocyte recovery post-HSCT is also predictive of superior long-term T-lymphocyte immune reconstitution[63]. Recovery of $CD3^+$ T-lymphocytes (>1 x 10^9/L) and $CD4^+$ (>0.5 x 10^9/L) takes approximately 6-12 months following HSCT, with slower $CD4^+$ recovery compared to $CD8^+$ T-lymphocytes resulting in an inversed CD4/CD8 ratio, and significant delay is seen (particularly affecting $CD4^+$ T-lymphocytes) when anti-thymocyte globulin (ATG) serotherapy is utilised during conditioning[88].

Restoration of the T-lymphocyte compartment post-HSCT occurs by two parallel pathways[89]. Initially after HSCT, the rise in T-lymphocyte numbers is due to the thymic-independent pathway, with expansion of pre-existing surviving host T-lymphocytes or donor T-lymphocytes transferred with the graft, driven by cytokines IL-15, IL-2 and IL-7. However, TCR diversity is dependent upon the repertoire of the initial donated T-lymphocyte population and expansion results in skewing of the TCR repertoire with time, as well as gradual depletion of T-lymphocytes. This expansion does provide a

degree of immune protection initially in the post-transplant period, particularly the memory host and donor T-lymphocytes against re-exposure to specific antigens such as CMV and EBV[77,90], but is overall limited in its diversity and permanency, with prevailing susceptibility to infections[91,92]. Complete and sustained immune reconstitution following lymphodepletion requires *de novo* regeneration of naïve T-lymphocytes from donor progenitor cells within the thymus, which exhibit a broad TCR repertoire capable of recognising a diverse range of pathogens and tumour antigens (the thymic-dependent pathway)[78,93]. Naïve $CD4^+CD45RA^+$ T-lymphocytes are first detected in the circulation approximately 4 months post-HSCT, reflecting the time interval required for intrathymic development of lymphoid precursors[60]. Low numbers of TRECs are detected 5-6 months post-HSCT, followed by a steady rise correlating with the recovery of naïve T-lymphocytes[73]. Children display a limited TCR repertoire diversity during the first 6 months after HSCT but develop a normal diverse spectratype profile by 12 months[93], and increasing diversity correlates with increasing TREC levels[94].

Long-term recovery of the total $CD4^+$ population is strongly dependent on renewed naïve T-lymphocyte production[78]. This process is dependent on a functioning and structurally intact thymus capable of exporting a regular stream of naïve T-lymphocytes, and can be negatively affected by a number of factors including advanced patient age, T-lymphocyte depletion, myeloablative conditioning and the development of GVHD[91,94,95]. The importance of strategies that preserve and boost thymic function, both before and after HSCT, to optimise immune reconstitution and clinical outcome is thus becoming increasingly recognised.

3.1.4 Effect of aGVHD on the thymus

Although aGVHD principally involves the skin, GIT and liver, the thymus is also a primary target, resulting in disruption of the thymic architecture. Thymic aGVHD has shown to cause loss of demarcation between the cortico-medullary zones, loss of Hassall's corpuscles, alteration of TEC subpopulations and depletion of thymocytes[19-21]. The structural damage to the thymic microenvironment consequently impairs T-lymphocyte formation and export, reflected by lower TREC levels and a distorted TCR repertoire observed in patients, and occurs independent of age[20,67,68,96-98]. Acute GVHD also has detrimental effects on the thymic-independent pathway with reduced expansion of transferred mature donor T-lymphocytes possibly due to loss of peripheral

T-lymphocyte niches[98]. The thymus appears to be particularly sensitive to the effects of aGVHD with thymic output being significantly affected even in grade 1 disease[77]. Subclinical thymic aGVHD may even occur in the absence of overt aGVHD[99], and have an underappreciated adverse effect on reconstitution of adaptive immunity, causing ongoing infections and incomplete immune reconstitution post-HSCT.

Although the precise mechanisms behind how aGVHD causes thymic damage in humans remain incompletely understood, experimental aGVHD models have helped to delineate the cellular and molecular mechanisms underlying thymic injury and the effects on T-lymphocyte development[19]. TECs act as both initiators and targets of thymic aGVHD, capable of directly activating alloreactive donor T-lymphocytes independently of APCs[96]. Activation of alloreactive donor T-lymphocytes leads to IFNγ secretion and stimulation of a STAT1-induced apoptosis pathway resulting in death of TECs[96]. Overall, the result is disruption of the normal architecture and organisation of the thymic microenvironment and it is likely that, as a consequence of this, the immature thymocytes fail to receive the normal necessary signals required to develop leading to thymic atrophy and reduced thymic export.

Murine models have demonstrated that thymocyte damage occurs at two stages of development predominantly resulting in loss of DP thymocytes. The first stage involves failure of normal TN thymocyte proliferation, leading to diminished numbers of DP thymocytes[19,20]. The second stage is increased apoptosis of DP thymocytes[20,100]. Both events contribute to the reduction in thymic lymphoid cellularity, consequent thymic atrophy and reduced thymic export. Patients with aGVHD display a decrease in both βTREC and sjTREC levels, suggestive of an interference at an early developmental stage (prior to Dβ-JβTREC formation) involving either early thymocyte precursors or bone marrow-derived progenitors[67,70].

A distorted TCR repertoire is observed in patients with aGVHD[67]. Normally, all thymic stromal cells of both haematopoietic and epithelial origin which mediate the positive and negative selection processes exhibit the same MHC haplotype. Following HSCT with HLA mismatch, the radio/chemoresistant TECs continue to express recipient MHC molecules while the stromal cells of haematopoietic origin, such as DCs, will be replaced by donor MHC molecules. This MHC disparity is thought to disturb the normal selection process, resulting in thymocytes escaping negative selection, increasing the survival of autoreactive T-lymphocytes, and negatively affecting the TCR repertoire in the

periphery[101-104]. Expression of MHC class II molecules by cTECs in mice, but absent expression by mTECs or DCs, results in increased frequency of autoreactive T-lymphocytes capable of causing autoimmunity[105]. Murine models of aGVHD have demonstrated that damage to mTECs by donor $CD8^+$ T-lymphocytes disrupts normal thymic negative selection with escape of autoreactive $CD4^+$ T-lymphocytes into the circulation[45]. Thus, aGVHD is detrimental to both quantity and quality of T-lymphocyte recovery (Figure 3.2).

It is conceivable that thymic injury from aGVHD resulting in disruption of the normal selection process, as well as thymic Treg development, promotes the escape of potentially autoreactive cells into the circulation, setting the scene for autoimmunity as seen in cGVHD[45]. It is well established that aGVHD predisposes to cGVHD but the mechanistic link between them has been uncertain. Recently, Dertschnig *et al* demonstrated that impaired thymic TRA expression secondary to damaged AIRE-expressing mTECs disrupts negative selection permitting *de novo* production of TRA-specific (autoreactive) T-lymphocytes which escape into the periphery. TRAs most affected were those expressed in tissues known to be targets in cGVHD thus providing a potential link between alloimmunity to the development of autoimmunity[106,107].

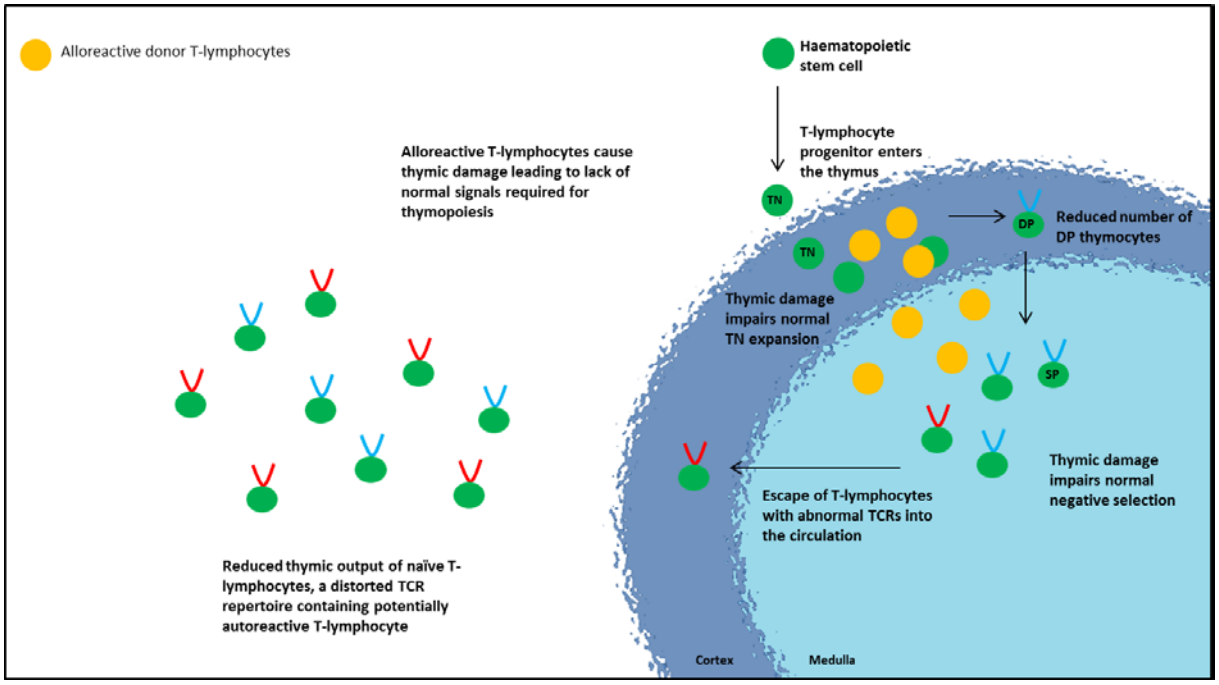


Figure 3.2 Acute GVHD damages the thymic microenvironment and results in reduced output of naïve T-lymphocytes with a distorted TCR repertoire[62].

3.1.5 Effect of immunosuppressive therapies on the thymus

First-line treatment of aGVHD is corticosteroids, which exhibit potent immunosuppressive and anti-inflammatory effects, but also have significant toxic side effects, including increased risk of cardiovascular disease, osteoporosis and insulin resistance[108-110]. Despite their success in some, a complete response is only witnessed in 25-50% of patients[31]. Short intensive corticosteroid courses have shown to induce thymic involution in avian models, causing a profound reduction in naïve T-lymphocyte production, although with complete recovery following cessation of corticosteroid treatment[111]. In mice, corticosteroids cause apoptosis of developing thymocytes in an Apaf-1 and caspase-9 dependent manner, with immature DP thymocytes being particularly sensitive, possibly due to higher expression of specific glucocorticoid receptor promoters[112,113]. Increased levels of endogenous glucocorticoids in humans are associated with transient suppression of thymopoiesis[114]. However, the precise effects in humans and the effects of long-term corticosteroid use on the thymus are unknown. Corticosteroids, in the treatment of aGVHD, further compound the detrimental effect on thymic function and thymopoiesis, and along with the systemic immunosuppressive effects, subject the patient to further increased risk of infection and other complications.

Patients who are refractory to corticosteroid treatment have an unfavourable prognosis with increased TRM[115]. Whilst corticosteroids are well established as first-line therapy for aGVHD, there is no consensus to standard second-line therapy for patients with steroid-refractory disease or steroid-dependency and usually involves intensification of systemic immunosuppression with a plethora of different therapeutic agents such as ciclosporin (CSA), MMF, anti-TNFα antibodies or mammalian target of rapamycin (mTOR) inhibitors[116]. These agents mainly non-selectively target T-lymphocytes resulting in a general immunosuppressive effect, and also likely negatively affect the GVL effect[117], but there is little evidence regarding their effect on thymic function. Experimental studies suggest that while short-term CSA causes reversible thymic damage, long-term treatment (up to 140 days) leads to irreversible changes, with loss of Hassall's corpuscles and reduced MHC II antigen expression[118], but the impact on thymic function, and the effect on humans is not known. Weinberg *et al* reported that prophylactic immunosuppressive agents (CSA/methylprednisolone/ATG) for more than 6 months in the absence of GVHD did not affect TREC levels, but this was postulated based on three patients and a definitive conclusion cannot be made[68]. Fallen *et al* demonstrated no difference in naïve T-lymphocyte numbers or TREC levels between

patients who received CSA for 3 or 12 months following HSCT. This was also based on a small number of predominantly adult patients, and did not take into account other factors such as GVHD or age. In this same study, patients with cGVHD, with or without previous aGVHD, displayed no evidence of thymic recovery, which may be caused by direct GVHD-mediated damage or by exposure to prolonged immunosuppressive therapy used in its management, or both[94], suggesting that thymic injury can have long-lasting effects, at least up to 12 months, although the long-term effect is not known. Further understanding of the effects on the thymus by these medications is needed. Regardless, non-selective immunosuppression increases the risk of opportunistic infections, relapse and the development of secondary malignancies, as well having their associated toxic side effects. Overall, there is a need for more selective thymic-sparing therapies.

3.1.6 Thymic regeneration

The thymus is extremely sensitive to other acute insults including chemotherapy, radiotherapy and infection[119], but, despite its sensitive nature, it has remarkable capacity for endogenous regeneration to restore T-lymphocyte immune competence, although the underlying mechanisms are not fully understood and multiple factors are likely to be involved[120]. Keratinocyte growth factor (KGF), IL-22, IL-7 and Foxn1 have been implicated to play an important role in this process via their positive effects on TEC populations, but much remains to be elucidated[121-123].

Thymic damage associated with aging is a chronic irreversible process, partly attributed to intrinsic thymocyte abnormalities and partly to structural changes that occur resulting in interference with the normal thymocyte-stromal cell signalling network, with contributing factors including reduced expression of Foxn1 and reduced production of IL-7[119]. This leads to decreased thymic output of naïve T-lymphocytes and constriction of the TCR repertoire, resulting in defective adaptive immune responses and increased autoimmunity, further illustrating the importance of optimal thymic function[120]. However, despite this decline with age, functioning persists albeit at a lower rate. Thymic atrophy due to the aging process also impairs thymic regenerative potential following acute insult[124].

Thymic regenerative ability is also impaired by aGVHD through mechanisms which are not fully clear although is thought to be partly due to loss of intrathymic innate

lymphoid cells (ILCs), leading to deficiency of IL-22, an integral cytokine in mediating protection and regeneration of TECs[125]. Indeed, administration of exogenous IL-22 in murine models post-HSCT augmented thymopoiesis[125], but IL-22-deficient mice still do exhibit some thymic tissue regeneration, suggesting involvement of other pathways. Endothelial cells within the thymus have recently shown to be resistant to insults such as chemotherapy and corticosteroids, and contribute towards recovery of thymic function by producing BMP4 which induces TEC expression of Foxn1[126]. KGF has shown to have a protective effect on TECs in an experimental murine model of aGVHD, indirectly promoting thymopoiesis[127]. Another experimental study demonstrated the beneficial effects of KGF on thymic recovery when used in combination with androgen blockade[128]. Research on KGF has been extended into primate HSCT models demonstrating improved thymopoiesis in rhesus macaques, although this was not associated with improved function in terms of protecting against CMV activation[129].

An interesting concept is that thymic involution may be a protective mechanism when continued thymic output in the presence of damage is detrimental to the individual[119]. In the setting of infection, thymic involution resolves after approximately 2 weeks, but diminished export during that time period may protect against the body becoming tolerant to the foreign antigen causing the infection[130]. As part of the aging process, reduced thymic export may be an attempt to protect against autoimmunity as T-lymphocyte development becomes less efficient. Hypothetically, in the context of aGVHD, reduced thymic export could be a mechanism to reduce output of autoreactive abnormal T-lymphocytes until thymic damage is repaired and normal efficient thymopoiesis can be restored. If this hypothesis is true, therapeutic strategies that boost and accelerate endogenous thymic repair would pre-empt the need for this inherent involution process and expedite restoration of functioning thymopoiesis.

3.1.7 Cytokines related to thymic function and regeneration

3.1.7.1 IL-7

IL-7 is a pleiotropic cytokine with a diverse spectrum of effects within the immune system. The thymus is a major source of IL-7 production, predominantly by non-haematopoietic TECs[131]. Other cells which produce IL-7 include smooth muscle cells, endothelial cells and DCs[132]. Intrathymic IL-7 plays an essential multifunctional role in thymopoiesis; absence of IL-7 signalling results in severely impaired immature

thymocyte survival, proliferation and differentiation[133-135]. Early B-lymphocyte development also requires IL-7, but B-lymphocytes become IL-7 independent following successful rearrangement of the Ig heavy chain gene locus[136]. T-lymphocytes, however, maintain a life-long IL-7 dependency and in the periphery, IL-7 is essential for naïve and memory T-lymphocyte homeostasis[137-139]. Consequently, IL-7 plays a critical role in both pathways of T-lymphocyte reconstitution following HSCT[140] and an inverse relationship between IL-7 levels and T-lymphocyte numbers has been described[141], although one adult study did not show a correlation between IL-7 and TRECs[142]. Previous studies have also suggested that high IL-7 levels early after HSCT are predictive of an increased risk of aGVHD, potentially due to IL-7-driven expansion of alloreactive T-lymphocytes within the graft, or occurring indirectly as a consequence of T-lymphocyte activation and subsequent IL7 receptor down-regulation and reduced IL-7 consumption[142-144]. Use of IL-7 therapeutically to promote T-lymphocyte reconstitution post-HSCT has been controversial, with some evidence of improvement in thymocyte proliferation (albeit transient) in murine models, but other studies demonstrated increased T-lymphocyte numbers due to increased peripheral expansion rather than *de novo* thymic production of naïve T-lymphocytes. Importantly, administration of exogenous IL-7 could also increase the risk of aGVHD through promotion of alloreactive T-lymphocyte expansion[145].

IL-7 interacts with the IL-7 heterodimeric receptor complex (IL-7R) composed of the IL-7R α-chain (CD127) and the common γ-chain (CD132). IL-7 binding initiates signal transduction resulting in transphosphorylation and activation of JAK1 and JAK3 kinases followed by downstream activation of STAT5 and PI3 kinase[132]. IL-7R also exists in the circulation as a soluble form (sIL-7R) released from the cell surface and competes with similar affinity for IL-7, resulting in inhibition of IL-7 signalling. The exact mechanisms of IL-7 regulation are not known, but may be due to a negative feedback effect (IL-7 signalling resulting in down regulation of the IL-7R), or dependent on the amount of 'IL-7 consuming cells' (depletion of T-lymphocytes expressing the IL-7R resulting in increased level of IL-7)[146].

3.1.7.2 IL-22

IL-22 is a member of the IL-10 cytokine family, and is predominantly produced by lymphoid cells including helper T-lymphocytes, NKT cells and ILCs, although there are some reports of non-lymphoid IL-22 sources such as neutrophils and macrophages[147]. IL-23 produced by DCs and IL-1β (produced by multiple cell types) are a potent

stimulators of IL-22 secretion[147]. IL-22 receptor is a heterodimeric protein composed of IL-22R1 and IL-10R2, and is not expressed by haematopoietic cells[148]. Consequently, IL-22 does not directly impact immune cells, but acts primarily on epithelial cells and fibroblasts in a diverse spectrum of tissues. IL-22 binding to the receptor leads to activation of the STAT family of transcription factors, particularly STAT3. There is also a soluble form of the IL-22 receptor called IL-22 binding protein (IL-22BP) which has an inhibitory effect on IL-22 and is secreted by various tissues such as the intestine and lymph nodes[149].

IL-22 possesses important protective properties, such as maintaining the integrity of epithelial barriers, tissue regeneration following insult and secretion of antimicrobial peptides[149]. However, there is also evidence that IL-22 has pro-inflammatory effects, for example stimulating production of pro-inflammatory molecules such as IL-1 and IL-6, and has been shown to contribute to systemic inflammatory processes such as rheumatoid arthritis and psoriasis[150,151]. It is thought that the protective versus pathological effects of IL-22 depends on the environment and inflammatory situation in which it is produced, the duration of IL-22 production and synergy with other cytokines secreted simultaneously such as type-1 IFNs[147,152].

In the context of aGVHD, this paradoxical role of IL-22 is evident with both protective and pathogenic effects reported, and may depend on whether the source of IL-22 is recipient or donor-derived. Donor-derived IL-22 resulting in exacerbation of tissue inflammation associated with reduced numbers of Tregs has been demonstrated in experimental models and IL-22-deficient grafts associated with less severe aGVHD[153,154]. In contrast, recipient-derived IL-22 has shown to be tissue protective in the GIT[155].

IL-22 plays an integral role in protecting and maintaining the GI epithelial barrier, and ILC production of IL-22 in the GIT has shown to be protective against GVHD and aid in epithelial recovery following tissue injury. Lindemans *et al* demonstrated that ILC–produced IL-22 increased the growth of murine intestinal organoids, and IL-22 treatment in aGVHD models stimulated intestinal stem cell recovery and epithelial regeneration[156]. In the absence of aGVHD ILCs in the gut can withstand conditioning and secrete IL-22 under the influence of IL-23, but ILC-produced IL-22 is reduced in aGVHD and IL-22 deficiency leads to increased epithelial damage[155]. However, the time frame of this regenerative process is not known, and it is unclear if the same process is duplicated in humans.

IL-22 plays an important role in the process of thymic regeneration and recovery of thymic function following acute damage, driven by intrathymic DC IL-23 production stimulating thymic ILC IL-22 secretion[123,125]. However, the majority of evidence for this derives from animal models, and the exact role of IL-22 in aGVHD in humans and in the context of thymic recovery is not yet known.

3.1.8 Extracorporeal photopheresis – a 'thymic sparing' strategy?

The clinical efficacy and safety of ECP in the treatment of aGVHD is well established, but importantly and uniquely, it does not have a systemic immunosuppressive effect and allows weaning of other concurrent immunosuppressive medications. Thus, ECP can reduce severity of aGVHD without subjecting the patient to increased risk of serious infection or disease relapse, and does not interfere with the GVL effect. By acting in an immunomodulatory fashion, potentially through an effect on DCs and Tregs, and promoting a tolerant environment, ECP could, by reducing aGVHD, and enabling a reduction in concomitant immunosuppression, allow thymic recovery, restoration of normal T-lymphocyte development and complete immune reconstitution with improved clinical outcome. Indeed, Alousi *et al* demonstrated faster recovery of $CD4^+$ and $CD8^+$ T-lymphocytes in those treated with ECP and corticosteroids compared to corticosteroids alone[157]. Beattie *et al* demonstrated evidence of recovery of thymic function in a paediatric patient treated with ECP for corticosteroid-refractory grade IV skin aGVHD, illustrated by a rise in $CD4^+CD45RA^+CD31^+$ naive T-lymphocytes and a reduction in serum IL-7 levels with progression of ECP, along with successful resolution of aGVHD and significant reduction in immunosuppressive medications[158]. Whilst this is promising and suggests that ECP is more favourable compared to other aGVHD therapies in promoting thymic regeneration and reconstitution of the T-lymphocyte compartment, further prospective evidence on a larger number of patients is needed.

3.1.9 Summary

The thymus is an indispensable factory for T-lymphocyte production. Maintaining the delicate microenvironment of this gland, and protecting it from injury, is crucial to allow this process to continue without interruption. The thymus has capacity for regeneration following acute insult, with multiple driving factors involved including cytokines IL-7 and IL-22.

HSCT results in an inevitable period of lymphodepletion, and complete sustained reconstitution of the adaptive T-lymphocyte compartment is dependent upon *de novo* thymic T-lymphocyte production, with a diverse TCR repertoire to defend against a broad range of antigens and protect the patient from infection and relapse. Acute GVHD causes thymic damage, impairing thymopoiesis, and negatively impacts thymic regeneration. Corticosteroids, potent systemic immunosuppressive agents, further compound the detrimental effect on thymopoiesis. Consequently, these vulnerable patients are subject to a high risk of infection and other complications, and overall poorer clinical outcome.

ECP is an alternative immunomodulatory therapeutic strategy for aGVHD. By promoting immune tolerance and simultaneously avoiding systemic immunosuppression, ECP could reduce aGVHD, and enable a reduction in concomitant immunosuppression, allowing thymic recovery, and support restoration of normal T-lymphopoiesis.

3.2 Aims

1. To investigate if thymic output improves both quantitatively and qualitatively over time in paediatric patients receiving ECP for treatment of aGVHD, and how this compares with paediatric patients post-HSCT who do not develop overt aGVHD and those who develop aGVHD but are not treated with ECP, and with a retrospective cohort who previously received ECP.

2. To investigate the trajectory of serum IL-7 levels over the course of ECP treatment and to observe if these levels correlate with thymic output.

3. To investigate the trajectory of serum IL-22 levels over the course of ECP therapy and to observe whether levels correlate with thymic output or disease progression.

3.3 Hypotheses

1. ECP, by reducing the activity of aGVHD, specifically thymic aGVHD, and allowing weaning of concurrent immunosuppressive therapies, facilitates endogenous repair of thymic damage, recovery of thymic function and increased export of recent thymic emigrants.

    a. Thymic function improves both quantitatively as depicted by increased naïve T-lymphocytes and TRECs, and qualitatively with improvement in the diversity of the TCR repertoire as measured by TCR DNA spectratyping.

2. Increasing thymic output is followed by a decline in serum IL-7.

    a. As thymic export increases, serum IL-7 levels decrease either due to a negative feedback effect or increased IL-7 consumption.

3. The process of thymic regeneration and restoration of thymic export in the context of ECP treatment is faster compared to strategies utilising non-specific systemic immunosuppressive agents only.

    a. Thymic export occurs at faster rate in the ECP group compared to the group with aGVHD but not treated with ECP.

4. By facilitating thymic regeneration and restoration of thymic function, IL-22 levels will rise and then fall as the gland repairs itself, and increased IL-22 will precede increased thymic output.

3.4 Methods and materials

3.4.1 Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were prepared by density–gradient centrifugation. Whole blood was diluted 1:1 with phosphate-buffered saline (PBS) and then layered on to Lymphoprep® solution (STEMCELL Technologies). PBMCs were separated by centrifugation at 800g for 15 minutes at 20°C and then collected from the density medium – plasma interface using a sterile Pasteur pipette. Cells were washed in PBS solution, and counted using a Neubauer improved cell counting chamber (Weber Scientific International) in a 1:1 dilution with Trypan blue solution (Sigma-Aldrich). Separated PBMCs were then washed again in PBS and suspended in a freezing solution (consisting of 70% RPMI, 20% foetal calf serum, 10% dimethyl sulfoxide) and stored in 1.8ml Nunc CryoTube® vials (Thermo Fisher Scientific) at -80°C for 24 hours followed by long-term storage at -150°C.

3.4.2 Preparation of Trucount samples

Whole blood was used for true counting by flow cytometry in order to establish absolute cell counts. Samples were collected before ECP on day 1 of the cycle and analysed within 24 hours. 100µL whole blood was stained with an antibody-fluorochrome conjugate panel (Table 3.1) in Trucount® tubes (BD Biosciences) containing a known number of microbeads and incubated for 20 minutes at room temperature in the dark before adding 900µL of red blood cell lysis buffer (sterile water containing 10mM NaOH, 0.155M NH_4Cl, 10mM $KHCO^3$, 0.12mM EDTA). Data was collected using the FortessaX20 (BD Biosciences) 10 minutes after addition of the lysis buffer with a minimum of 50,000 events recorded. Data was analysed using FlowJo® software (BD Biosciences) (gating strategy shown in Figure 3.3 and negative controls in Figure 3.4). Naïve T-lymphocytes were defined as $CD3^+CD4^+CD45RA^+CD31^+$ and true counts were established using the following equation:

$$Cells\ per\ \mu L = \frac{\#\ of\ cell\ events}{\#\ of\ bead\ events} \times \frac{total\ beads\ in\ tube}{volume\ of\ sample}$$

Antibody Target	Fluorochrome	Volume per test	Manufacturer
CD45	APC (630 – 670/14)	5 µL	BD Biosciences™
CD3	PE-Cy7 (561 – 780/60)	2.5 µL	BD Biosciences™
CD4	PE (561 – 584/15)	5 µL	BD Biosciences™
CD45RA	BUV737 (355 – 730/45)	5 µL	BD Biosciences™
CD31	APC Cy7 (640 – 780/60	2 µL	BD Biosciences™
HLA-DR	BUV395 (355 – 379/28)	2.5 µL	BD Biosciences™
CD127	BV421 (405 – 450-50)	2 µL	BD Biosciences™
CD25	FITC (488 – 520/20)	7 µL	BD Biosciences™

Table 3.1 Antibody staining panel for true counting of lymphocyte subsets (naïve T-cells, Tregs, activated T-cells).

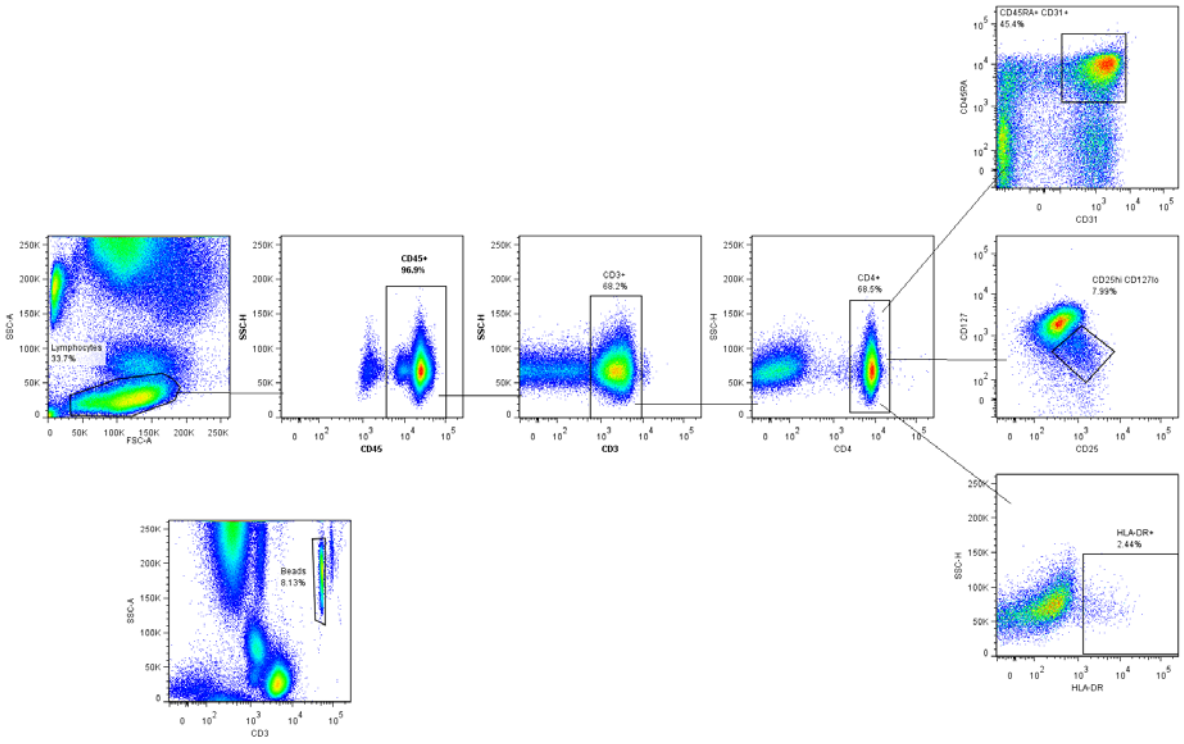


Figure 3.3 Trucount panel gating strategy to measure absolute counts of naïve T-lymphocytes ($CD3^{+}CD4^{+}CD45RA^{+}CD31^{+)}$, Tregs ($CD3^{+}CD4^{+}CD25^{hi}CD127^{lo}$) and activated T-lymphocytes ($CD3^{+}CD4^{+}HLA\text{-}DR^{+}$).

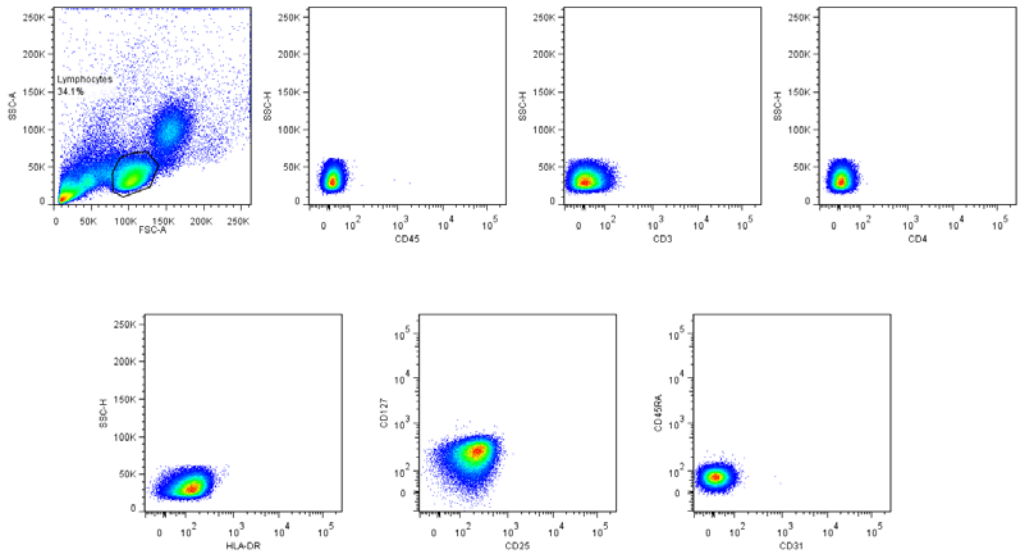


Figure 3.4 Trucount panel negative controls for naïve T-lymphocytes, Tregs and activated T-lymphocytes.

3.4.3 T-cell receptor excision circles

3.4.3.1 Transformation and extraction of plasmid DNA

A triple insert plasmid containing 1µg of TREC/KREC/TRAC DNA was kindly supplied by Drs Alessandra Sottini and Luisa Imberti (Brescia, Italy). Kappa-deleting recombination excision circles (KRECs) are generated during light-chain formation of B-lymphocyte development and are used to assess bone marrow B-lymphocyte output. As the aim of this study was to quantify T-lymphocyte neogenesis, KREC values were not analysed although were part of the protocol for this experiment. The plasmid DNA was transformed using E.coli and TOPO® Cloning Reaction (Thermo Fisher Scientific) performed in Great Ormond Street Hospital supervised by Dr Stuart Adams. The transformed cells were evenly spread on an ampicillin-selective agar plate and incubated at 37°C overnight. Positive colonies were selected and expanded overnight in lysogeny broth. Plasmid DNA was then extracted and purified using the Qiagen Plasmid Purification Kit® according to the manufacturer's instructions and quantified. Serial dilutions of the stock plasmid TREC/KREC/TRAC DNA (2 x 10^6 copies per µl) with 1x TE

buffer containing 50ng/µl tRNA was performed to give final concentrations of 10^6, 10^5, 10^4, 10^3, 10^2 and 10 copies per µl.

3.4.3.2 TREC/TRAC standard curve

The standard curve was set up using the protocol established by Dr Stuart Adams using Real-Time PCR. The following TREC/KREC primers and probes were used (Integrated DNA Technologies): TREC forward primer 5'-CAC ATC CCT TTC AAC CAT GCT-3', TREC reverse primer 5'-TGC AGG TGC CTA TGC ATC A-3', TREC probe 5'-FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT-TAMRA-3', KREC forward primer 5'-TCC CTT AGT GGC ATT ATT TGT ATC ACT-3', KREC reverse primer 5'-AGG AGC CAG CTC TTA CCC TAG AGT-3' and KREC probe 5'-HEX-TCT GCA CGG GCA GCA GGT TGG-TAMRA-3'. For the housekeeper gene TRAC the following primers and probe was used: TRAC forward primer 5' TGG CCT AAC CCT GAT CCT CTT-3', TRAC reverse primer 5'-GGA TTT AGA GCT TCT CAG CTG GTA CAC-3' and TRAC probe 5'-FAM-TCC CAC AGA TAT CCA GAA CCC TGA CCC-TAMRA-3'.

PCR reactions were performed using 96-well MicroAmp plates in triplicates in a final volume of 25µL consisting of 5µL of DNA, 12.5µL 2x TaqMan® Universal PCR master mix containing no AmpEsterase UNG (Applied Biosystems), 4.5µL water and primers and probes for TREC, KREC and TRAC at a final concentration of 45µM and 10µM respectively. Simultaneous detection of TREC, KREC and TRAC copy numbers was done using the 7900HT Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 1 minute (x 40 cycles) The passing standard curve values were:

- Slope: Range -3.1 to -3.6
- Intercept Range: 36-41
- R^2 Range: 0.970 – 1.00

Once the standard curve met the passing values (Figures 3.5 and 3.6), the stock plasmid dilutions 10^6, 10^5, 10^4, 10^3 and 10^2 were aliquoted and stored at -20°C. The standard 10^1 per µl was made fresh for each PCR run.

3.4.3.3 TREC assay using patient samples

DNA was purified from 5 x 10^6 PBMCs using the QIAamp® DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen). Eluted DNA was quantified using the NanoDrop® ND-1000 spectrophotometer (LabTech) and was used immediately or

stored at -80°C. PCR reactions of patient samples were carried out in duplicates alongside no-template controls, and the PCR assay was performed as described above. In each assay run, serial dilutions of the TREC and TRAC plasmid were used to obtain a standard curve for absolute quantification of TREC and TRAC copy numbers and the standard curve values had to meet the passing requirements. Data were analysed using Sequence Detection Systems software version 2.4 (Applied Biosystems). Utilising the TRAC housekeeping gene enables direct DNA quantification as each cell contains two TRAC copies. The number of TRECs per million cells for each patient was established using the following equation:

$$(\text{mean quantity value for TREC})/(\text{mean quantity value for TRAC}/2) \times 10^6$$

Results were recorded as the number of TRECs per 1ml of blood using the following equation, which removes the influence of peripheral proliferation.

$$(\text{mean quantity value for TREC})/(\text{mean quantity value for TRAC}/2) \times (\text{lymphocyte + monocyte count in 1ml of blood})$$

Results of the TREC assay were validated using two patient samples from Great Ormond Street Hospital provided by Dr Adams.

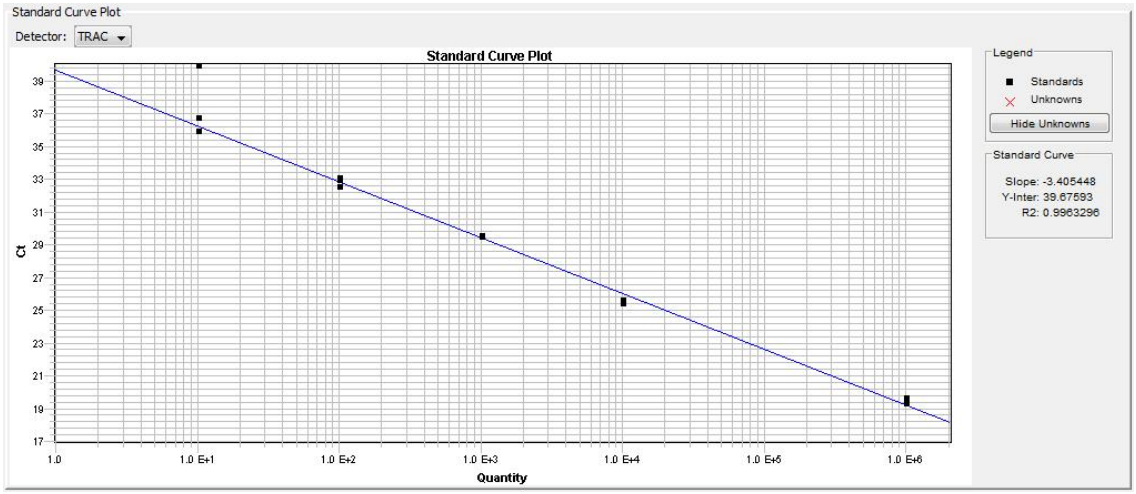


Figure 3.5 The standard curve for TRACs. For each run the passing requirements for the slope, intercept range and R^2 were met for both the TRAC and TREC standard curve.

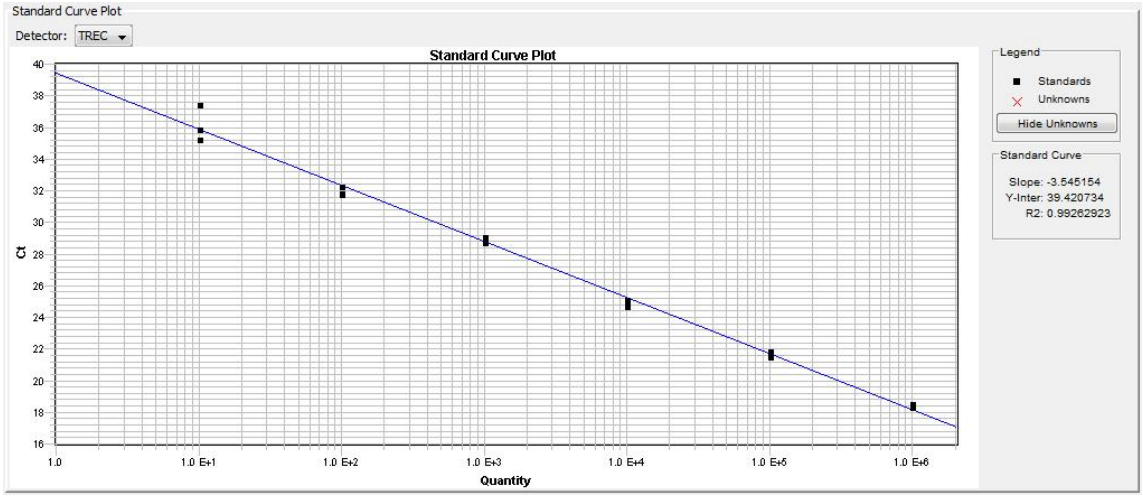


Figure 3.6 The standard curve for TRECs.

3.4.4 T-cell receptor DNA spectratyping

RNA was extracted from PBMCs using the RNeasy® Mini Kit (Qiagen) and quantified using a NanoDrop® ND-1000 spectrophotometer (LabTech). Following isolation, the RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A master mix was prepared which contained 10X Buffer, 25X dNTP mix, 10X random primers, MultiScribe® Reverse Transcriptase (Thermo-Fisher Scientific), RNase inhibitor and nuclease-free water. For each sample the RNA was diluted to a final concentration of 2µg in a total volume of 10µL in RNase-free water. 10µL of the master mix was added to 10µL of RNA in a PCR reaction tube, followed by incubation at 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 15 seconds using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The cDNA was then used immediately or stored at -20°C.

Twenty-three Vβ family-specific RT-PCR reactions were used to determine the complexity of the TCR β-chain repertoire. Vβ primers were reconstituted in TE buffer to make 100µM stock solutions (Table 3.2). Primers Vβ6b1 and Vβ6b2 were mixed in a 1:1 ratio and used as primer Vβ6b. 7.5µl of each of the Vβ primers were combined in pairs (Table 3.3), and added to 885µl of TE buffer to make 12 working primer solutions (0.83pmol/µl). 15µl from each pair of working primers was aliquoted into a 96-well PCR plate (Figure 3.7). 50µl of FAM-labelled Cβ primer was added to 50µl TE buffer to make a working solution of 50pmol/µl.

The TCR DNA spectratyping assay consisted of two PCR reactions followed by CDR3 fragment length analysis by capillary electrophoresis. For the first PCR reaction, 110µl of a master mix containing 10X buffer, MgCl2 (25mM), HotStarTaq® DNA polymerase (Qiagen), dNTPs (25mM) and nuclease-free water was combined with 20µl of cDNA. 10µl from the cDNA/master mix was added to the appropriate wells of the 96-well plate containing the primers, to give a final reaction volume of 25µl, and incubated at 95°C x 10 minutes for one cycle, 40 cycles of 95°C x 25 seconds, 60°C x 45 seconds and 72°C x 45 seconds, and one cycle of 72°C x 5 minutes. 2µl of each PCR product was then combined with 8µl of a master mix (containing 10X buffer, MgCl2 (25mM), HotStarTaq® DNA polymerase (Qiagen), dNTPs (25mM), fluorescent-labelled Cβ primer and nuclease-free water) and pipetted into a new 96-well PCR plate for the run-off labelling PCR reaction as follows: 95°C x 2 minutes for one cycle, 15 cycles of 95°C x 25 seconds, 60°C x 45 seconds and 72°C x 45 seconds, and one cycle of 72°C x 5 minutes.

For the fragment analysis, a 9µl post PCR mix containing Hi-Di Formamide (Thermo Fisher) and GeneScan® 500 ROX Size Standard (Applied Biosystems) was added to 1µl of the PCR product into each well of a new 96-well plate, denatured at 95°C for 3 minutes and then transferred to the 3130 Genetic Analyzer (Thermo Fisher). The electropherograms were analysed using Peak Scanner software version 2.0 (Applied Biosystems). Healthy control samples were first analysed (Figure 3.8) and the following determined:

1. The typical number of peaks per family
2. The number of Gaussian families per spectratype
3. The presence of any large oligoclonal or monoclonal peaks per spectratype

Based on the information from healthy control samples, subsequent electropherograms were then scored as follows:

1. Normal spectratype = ≥22 families having between 5-12 peaks with less than 3 non-Gaussian families and ≤3 large oligoclonal or monoclonal peaks.
2. Abnormal spectratype = all families present with ≥20 having 5-12 peaks, ≥3 but less than 8 families have a non-Gaussian distribution, >3 but ≤6 large oligoclonal or monoclonal peaks.
3. Highly abnormal spectratype = some absent families or <20 families having 5-12 peaks and ≥8 families having a non-Gaussian distribution, >6 large oligoclonal or monoclonal peaks.

In the analysis, the terms Gaussian or non-Gaussian distribution were used as a subjective description of the appearance of each Vβ family to demonstrate the visual change in the TCR repertoire over time, rather than reflecting a statistical description.

Cβ	GGGTGTGGGAGATCTCTGC
Cβ (FAM labelled)	ACACAGCAGCCTCGGGTGGG
Vβ1	CCGCACAACAGTTCCCTGACTTGC
Vβ2	CACAACTATGTTTTGGTATCGTC
Vβ3	CGCTTCTCCCTGATTCTGGAGTCC
Vβ4	TTCCCATCAGCCGCCCAAACCTAA
Vβ5	GATCAAAACGAGAGGACAGC
Vβ6a	GATCCAATTTCAGGTCATACTG
Vβ6b1	CAGGGSCCAGAGTTTCTGAC
Vβb2	CAGGGCTCAGAGGTTCTGAC
Vβ7	CCTGAATGCCCCAACAGCTCT
Vβ8	GGTACAGACAGACCATGATGC
Vβ9	TTCCCTGGAGCTTGGTGACTCTGC
Vβ11	GTCAACAGTCTCCAGAATAAGG
Vβ12	TCCYCCTCACTCTGGAGTC
Vβ13a	GGTATCGACAAGACCCAGGCA
Vβ13b	AGGCTCATCCATTATTCAAATAC
Vβ14	GGGCTGGGCTTAAGGCAGATCTAC
Vβ15	CAGGCACAGGCTAAATTCTCCCTG
Vβ16	GCCTGCAGAACTGGAGGATTCTGG
Vβ17	TCCTCTCACTGTGACATCGGCCCA
Vβ18	CTGCTGAATTTCCCAAAGAGGGCC
Vβ20	TGCCCAGAATCTCTCAGCCTCCA
Vβ21	GGAGTAGACTCCACTCTCAAG
Vβ22	GATCCGGTCCACAAAGCTGG
Vβ23	ATTCTGAACTGAACATGAGCTCCT

Table 3.2 Primers used for TCR DNA spectratyping.

Tube 1	Vβ9 and 18 primers
Tube 2	Vβ23 and 6b primers
Tube 3	Vβ16 and 4 primers
Tube 4	Vβ3 and 13a primers
Tube 5	Vβ22 and 11 primers
Tube 6	Vβ21 and 8 primers
Tube 7	Vβ 20 primer
Tube 8	Vβ 15 and 2 primers
Tube 9	Vβ 17 and 13b primers
Tube 10	Vβ 7 and 6a primers
Tube 11	Vβ 12 and 14 primers
Tube 12	Vβ1 and 5 primers

Table 3.3 Working Vβ primer solutions.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Tube 1	Tube 9	Tube 5	Tube 1	Tube 9	Tube 5	Tube 1	Tube 9	Tube 5	Tube 1	Tube 9	Tube 5
B	Tube 2	Tube 10	Tube 6	Tube 2	Tube 10	Tube 6	Tube 2	Tube 10	Tube 6	Tube 2	Tube 10	Tube 6
C	Tube 3	Tube 11	Tube 6	Tube 3	Tube 11	Tube 6	Tube 3	Tube 11	Tube 6	Tube 3	Tube 11	Tube 6
D	Tube 4	Tube 12	Tube 8	Tube 4	Tube 12	Tube 8	Tube 4	Tube 12	Tube 8	Tube 4	Tube 12	Tube 8
E	Tube 5	Tube 1	Tube 9	Tube 5	Tube 1	Tube 9	Tube 5	Tube 1	Tube 9	Tube 5	Tube 1	Tube 9
F	Tube 6	Tube 2	Tube 10	Tube 6	Tube 2	Tube 10	Tube 6	Tube 2	Tube 10	Tube 6	Tube 2	Tube 10
G	Tube 6	Tube 3	Tube 11	Tube 6	Tube 3	Tube 11	Tube 6	Tube 3	Tube 11	Tube 6	Tube 3	Tube 11
H	Tube 8	Tube 4	Tube 12	Tube 8	Tube 4	Tube 12	Tube 8	Tube 4	Tube 12	Tube 8	Tube 4	Tube 12

Figure 3.7 The position of working Vβ primers in 96-well PCR plate.

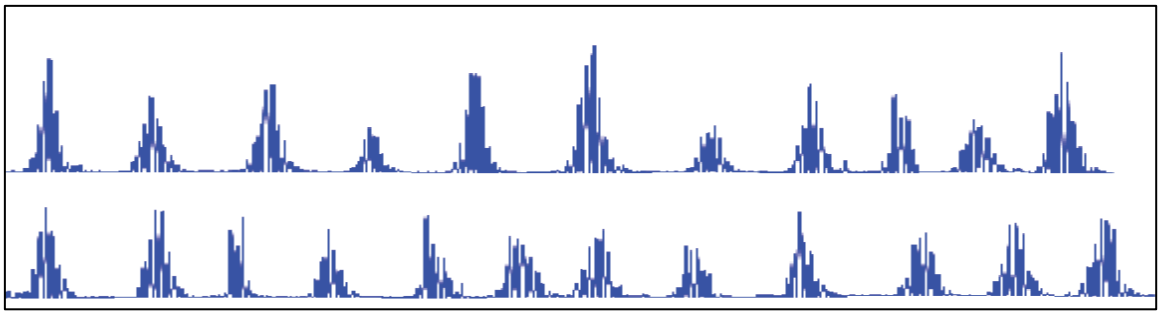


Figure 3.8 Example of a healthy control TCR DNA spectratype.

3.4.5 Serum IL-7 and IL-22 quantification

Serum was obtained by centrifugation of approximately 5mls of blood for 5 minutes ($2.5x10^3$ RPM) followed by transfer of serum to 1.5ml Eppendorf Tubes® (Sigma-Aldrich) and stored at -80°C. Absolute levels of serum IL-7 and IL-22 were quantified using the Quantikine® enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) as per manufacturer's instructions. Previous results indicated that the serum concentration of IL-7 might be higher than the upper limit of the standard curve, therefore serum samples were diluted 1:5 with calibrator diluent. No dilution was performed for IL-22 analysis. All standards were tested in duplicate. Due to restriction in microplate size patient samples were tested in singlet. Analysis of optimal density was performed using a Multiskan Ascent® plate reader (Thermo Scientific) and data were analysed by Ascent® software (Thermo Scientific). For the IL-7 assay the final values were multiplied by 5 to account for the serum dilution. The minimal detectable dose of IL-22 was 2.7pg/ml. Baseline expression of IL-22 in healthy controls is extremely low, with an average of 2pg/ml observed in one study[159] and elevated levels of IL-22 were defined as >2pg/ml. The minimal detectable dose of IL-7 was 0.1pg/ml. Elevated levels of IL-7 were defined as >9.8pg/mL, the maximum of the normal range found in the serum of healthy controls as determined by R&D Systems.

3.4.6 Collection of retrospective data

Details of paediatric patients who received an allogeneic HSCT at the Great North Children's Hospital, Newcastle upon Tyne from June 2010 to April 2016 were collected. Hospital records were reviewed and patients were categorised into four groups: no aGVHD, aGVHD treated topically (corticosteroids +/- tacrolimus), with systemic corticosteroids, or with ECP. These groups were chosen to examine the effect of treatment on thymic output, rather than the severity of GVHD. Excluded were patients with no available data, those with <12 months follow-up, cGVHD, recipients of >1 HSCT or those who received donor lymphocyte infusions. Thymopoietic recovery was measured using historic computer-based laboratory results of $CD4^+$ naïve T-lymphocytes ($CD3^+CD4^+CD45RA^+CD27^+$) measured at 3, 6, 9 and 12 months post-HSCT for all patients. For the ECP group results were additionally recorded at 3, 6, 9 and 12 months during ECP as part of routine monitoring. The latest values of $CD4^+$ naïve T-lymphocytes prior to commencing ECP were also recorded.

3.4.7 Statistical analysis

Group analysis of the prospective ECP patients was performed up to cycle 26, as only one patient remained on ECP beyond this time point. Individual analysis was performed until the end of ECP treatment regardless of duration. Non-linear regression (curve fit) analysis was performed using a second order polynomial (quadratic) model.
Comparison of median values was performed using the Kruskal-Wallis test with post hoc analysis when appropriate using the Dunn Correction test to detect significance between individual groups. Correlation analysis was performed using Spearman non-parametric correlation. P value <0.05 was significant.

3.5 Results

3.5.1 Retrospective results

155 paediatric patients received an allo-HSCT at the Great North Children's Hospital, Newcastle upon Tyne from June 2010 to April 2016. Of 102 eligible patients, 41 (40.2%) had no aGVHD, 23 (22.6%) received topical treatment only (corticosteroids +/- tacrolimus) for grade I-II skin aGVHD, 25 (24.5%) were treated with systemic corticosteroids (one also received infliximab), and 13 (12.7%) had aGVHD and received ECP (Tables 3.4 and 3.5). Naïve $CD4^+CD45RA^+CD27^+$ T-lymphocyte median values (and ranges) following HSCT for the groups at each time point are shown (Figure 3.9 and Table 3.6). There was a significant difference between the rate of thymopoiesis between all four groups at 3, 6, 9 and 12 months post-HSCT ($p=0.002$, $p<0.001$, $p<0.001$, $p=0.001$ for each respective time point). The poorest recovery of thymopoiesis was seen in the ECP group. The median value of naïve T-lymphocytes before ECP was 8 (range 0-47 cells/μL), but thymopoietic recovery, albeit limited, was observed with treatment progression (Figure 3.10)[160].

Patients who received ECP started after failure to respond to systemic corticosteroids and other immunosuppression, with a median duration from HSCT to commencement of ECP of 105 days, range 36-840 days (among the 11 patients with a known date of diagnosis of aGVHD, the median duration from diagnosis of aGVHD to commencement of ECP was 138 days, range 13-900 days). The median number of ECP treatments was 17, with a CR seen in 7/13 (53.8%), 5 (38.5%) had a PR and 1 (7.7%) had NR. Among those who had a PR, four died, three from complications related to immune suppression. All patients who had a CR survived. The non-responding patient was re-transplanted for aGVHD. In 8/13 (61.5%) patients concurrent immunosuppression was reduced whilst receiving ECP, 4/13 (30.8%) ceased immunosuppression, and immunosuppression remained unchanged in the non-responding patient.

	Gender	Median/mean age at HSCT	Diagnosis	Donor	Source
No acute GVHD (n=41)	F = 13 M = 29	0.9/4.2 years	SCID = 14 CID = 17 Neutrophil defects = 6 Autoimmune condition = 2 Haemophagocytic syndrome = 1 HIES = 1	MSD = 8 MUD = 11 MRD = 2 Haplo = 6 MMUD = 6 Unknown = 8	BM = 18 Cord = 3 PBSC = 20
aGVHD + topical treatment (n=23)	F = 8 M = 15	3.6/5.9 years	CID = 12 SCID = 5 Neutrophil defects = 3 Autoimmune condition = 2 Haemophagocytic syndrome = 1	MSD = 1 MUD = 9 MMUD = 2 MMRD = 1 Haplo = 3 Unknown = 7	BM = 6 Cord =2 PBSC = 15
aGVHD + systemic corticosteroids (n=25)	F = 10 M = 15	2.8/4.3 years	CID = 14 SCID = 5 Neutrophil defects = 3 Metabolic = 1 Autoimmune condition = 1 Haemophagocytic syndrome = 1	MSD = 2 MUD = 7 MMUD = 2 Haplo = 3 Unknown = 11	BM = 7 Cord = 3 PBSC = 15
aGVHD + ECP (n=13)	F = 4 M = 9	3.7/4.7 years	CID = 5 SCID = 5 Metabolic = 1 AML = 2	MSD = 3 MUD =9 Haplo = 1	BM = 4 Cord 5 PBSC = 4

Table 3.4 Details of all of the eligible retrospective patients (n=102).

SCID, severe combined immunodeficiency; CID, combined immune deficiency; HIES, Hyper IgE Syndrome; MSD, matched sibling donor; MUD, matched unrelated donor; MRD, matched related donor; MMUD, mismatched unrelated donor; Haplo, haploidentical; BM, bone marrow; PBSC, peripheral blood stem cells; AML, acute myeloid leukaemia

	Underlying diagnosis	Gender	Age at HSCT (mths)	Source of HSC	HLA match (mismatch)	Condit-ioning	Sero-therapy	GVHD prophylaxis	GVHD grade/type	Therapies pre ECP	Reason for ECP	Response	Change in immune suppression	Death
1	SCID (IL2RG)	M	3	MUD CB	9/10, (A)	MA low toxicity	N	CSA, MMF	2, skin, GIT	CS, TAC, IFX	CS dependent	PR	Decrease	Y (Infection)
2	Omenn Syndrome (RAG1)	M	8	Sib BM	10/10	MA low toxicity	N	CSA, MMF	Severe skin, lung	CS, SLM, IFX, ATG, imatinib	CS refractory	PR	Decrease	Y (Pneumonitis)
3	SCID (Artemis)	F	7	MUD CB	10/10	MA low toxicity	Y	CSA, MMF	3, skin	CS, TAC, IFX	CS refractory	NR	Unchanged	N
4	CID (T cell Activation Defect)	M	9	MUD CB	10/10	MA low toxicity	N	CSA, MMF	2, skin	CS, TAC, IFX	CS dependent	CR	Decrease	N
5	SCID (IL2RG))	M	9	MUD CB	10/10	MA low toxicity	N	CSA, MMF	3, skin	CS, CSA	CS refractory	PR	Decrease	N
6	CD4 Lymphopenia	M	13	Haplo PBSC	5/10	MA low toxicity	N	CSA, MMF	2, skin	CS, SLM, IFX	CS dependent	PR	Decrease	Y (Infection)
7	A-T, severe CID	M	44	MUD BM	10/10	MA low toxicity	Y	CSA, MMF	2, skin	CS, TAC, IFX	CS refractory	PR	Decrease	Y (PTLD)
8	DOCK 8 Deficiency	M	53	Sib BM	10/10	MA low toxicity	N	CSA, MMF	2, skin	CS, IFX	CS dependent	CR	Cessation	N
9	NEMO	M	54	MUD PBSC	8/10 (A,C)	MA low toxicity	Y	CSA, MMF	4, skin	CS, TAC, IFX, ATG, MoAb CD52	CS dependent	CR	Decrease	N
10	CD40L Deficiency	M	90	MUD PBSC	9/10, (DQ)	MA low toxicity	Y	None	4, liver, GIT	CS, TAC, SLM, MMF, IFX, ATG	CS refractory	CR	Decrease	N
11	Osteopetrosis	F	168	MUD PBSC	9/10, (A)	MA low toxicity	N	CSA, MMF	2, skin	CS, IFX	CS dependent	CR	Cessation	N
12	AML (high risk)	F	54	MUD double CB	5/6 (DR), 6/8 (DQ)	MA	Y	CSA	3, skin, GIT	CS, IFX, MMF	CS refractory	CR	Cessation	N
13	AML (high risk)	F	221	Sib BM	10/10	MA	N	CSA	4, skin, liver, GIT	CS, IFX	CS refractory	CR	Cessation	N

Table 3.5 Details of the retrospective ECP patients.

CSA, ciclosporin; TAC, tacrolimus; IFX, infliximab; CS, corticosteroids; SLM, sirolimus; ATG, anti-thymocyte globulin; MoAb CD52, alemtuzumab; MMF, mycophenolate mofetil; PTLD, post-transplant lymphoproliferative disorder; CB, cord blood

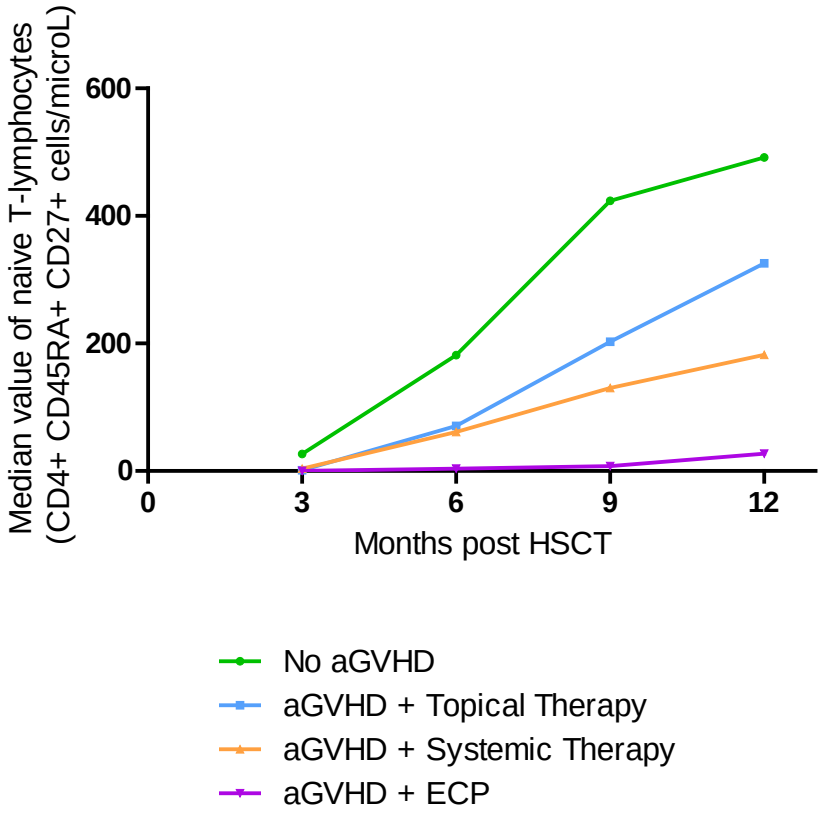
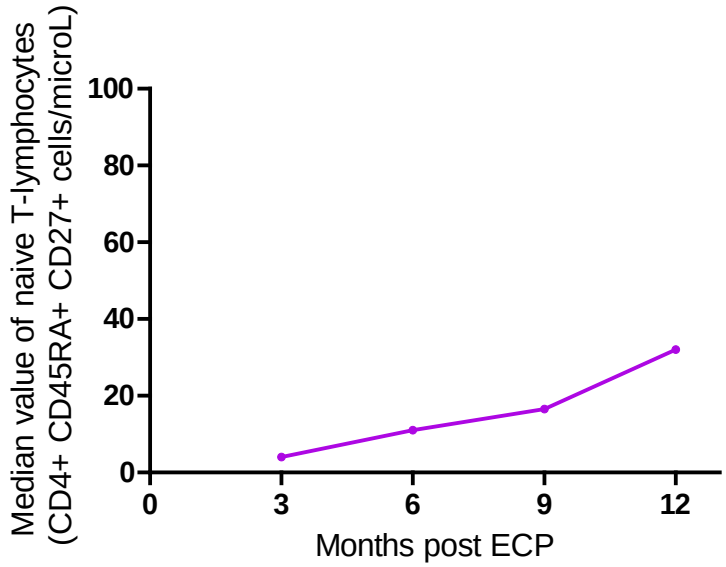


Figure 3.9 Median values of $CD3^+CD4^+CD45RA^+CD27^+$ naïve T-lymphocytes at 3, 6, 9 and 12 months post-HSCT in patients with no aGVHD (green), those with aGVHD treated topically (blue), with conventional systemic immunosuppression (orange) and with ECP (purple)[160]. Patients with no aGVHD demonstrated fastest recovery of naïve T-lymphocytes, with a significant decrease even in those with aGVHD treated with topical therapies. Recovery was most inferior in the ECP group.

	Median value 3 months (range)	Median value 6 months (range)	Median value 9 months (range)	Median value 12 months (range)
No aGVHD	27 (0-384)	182 (1-1788)	424 (6-2804)	492 (10-3081)
aGVHD + Topical Therapy	0 (1-161)	71 (0-1240)	203 (493-1722)	326 (9-1388)
aGVHD + Systemic Therapy	2 (0-103)	67 (0-672)	129 (0-1037)	182 (0-1177)
aGVHD + ECP	0 (0-28)	4 (0-212)	8 (0-95)	27 (7-861)

Table 3.6 Median values and ranges of naïve T-lymphocytes ($CD4^+CD45RA^+CD27^+$ cells/μL) for each group at 3, 6, 9 and 12 months post-HSCT.



Median value 3 months (range)	Median value 6 months (range)	Median value 9 months (range)	Median value 12 months (range)
4 (0-15)	11 (0-488)	16.5 (4-266)	32 (5-862)

Figure 3.10 Patients treated with ECP displayed the lowest number of naïve T-lymphocytes post-HSCT, but did show evidence of limited thymic recovery with progression of ECP[160]. The median values and ranges at 3, 6, 9 and 12 months post ECP are displayed in the table.

3.5.2 Prospective results – Quantitative analyses

3.5.2.1 Quantitative T-lymphocyte recovery

Among the 8 ECP patients, an overall increase in the absolute number and percentage of naïve T-lymphocytes ($CD3^+CD4^+CD45RA^+CD31^+$) was observed with progression of treatment (Figure 3.11). For the purpose of this chapter, further description of 'a responding patient' or 'non-responding patient' refers to response in terms of thymopoietic recovery (rather than clinical response). Individual patient analysis demonstrated that among the ECP patients who successfully completed therapy, 4/4 demonstrated improved thymic output with increased naïve T-lymphocytes and TRECs. These patients also demonstrated high r^2 values using non-linear curve fit analysis (Table 3.7, Figures 3.12 and 3.13) Two ECP patients were withdrawn from therapy (after cycles 16 and 17 respectively) but did show evidence of increasing thymopoiesis prior to cessation of treatment (Figure 3.14). Two patients did not show evidence of increased thymic output of naïve T-lymphocytes and currently remain on ECP therapy (Figure 3.15). Patient 1 demonstrated negligible numbers of naïve T-lymphocytes at 24 months post-HSCT and with prolonged ECP treatment. Patient 8 similarly showed no evidence of improved thymic output at the time of data analysis (cycle 21 of ECP, 54 weeks post-HSCT).

Three patients (patients 2, 6 and 7) had a small naïve T-lymphocyte population (Figure 3.16) initially, which then gradually declined to reach a nadir between cycles 9-13 (weeks 15-23 post-HSCT), followed by a gradual increase in naïve T-lymphocytes, alongside increasing TRECs and improvement in diversity of the TCR repertoire. These patients commenced ECP at days 55, 40 and 35 respectively post–HSCT.

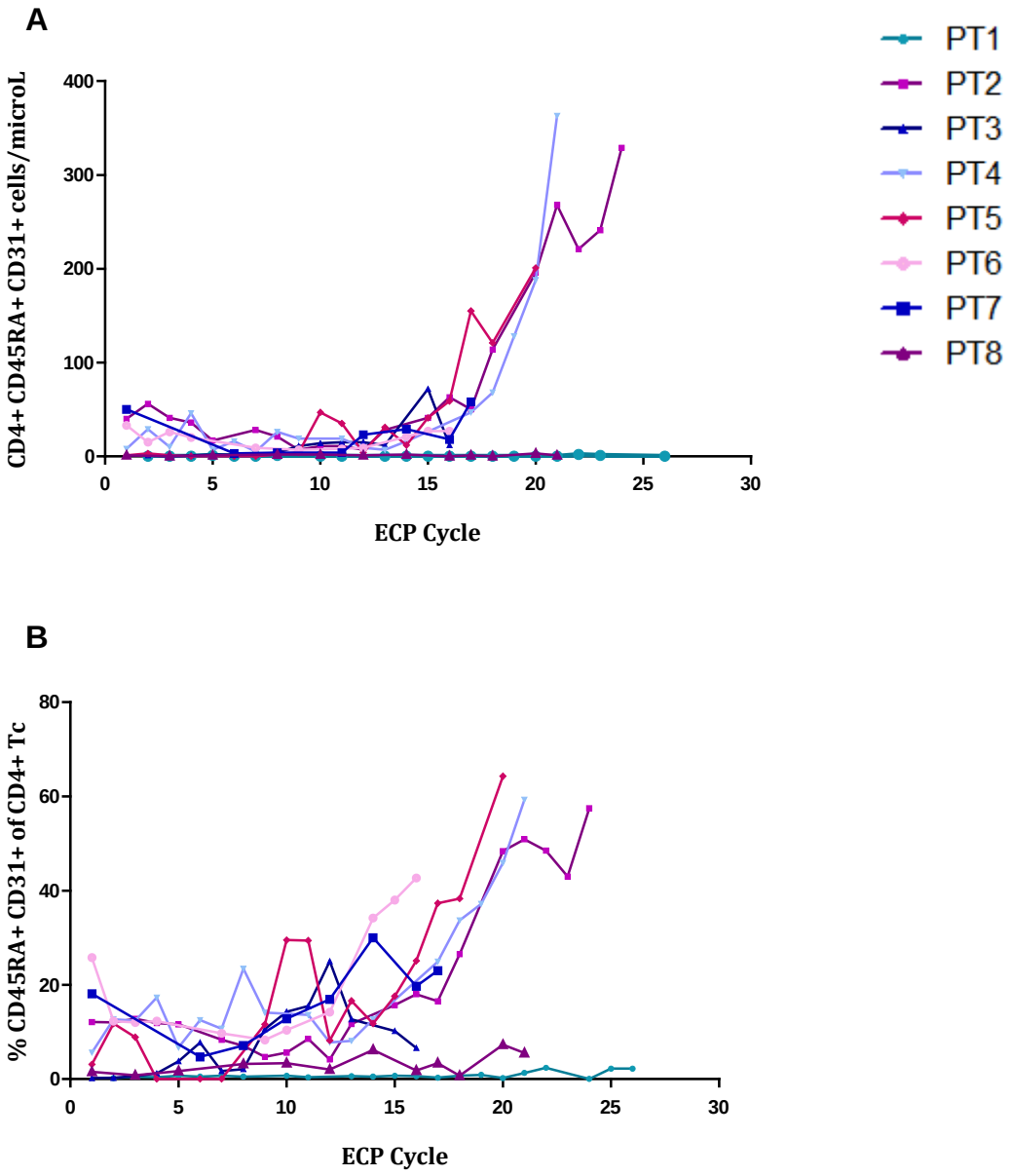


Figure 3.11 Absolute numbers (A) and frequency (B) of naïve T-lymphocytes among all ECP patients with progression of ECP therapy, linear regression analysis demonstrating a statistically significant increase ($p<0.001$). However, patient 1 (green line) demonstrated no evidence of improvement in thymic output despite being 2 years post HSCT and 54 cycles of ECP therapy (results up to cycle 26 shown here). Patient 8 (purple line) has also shown no improvement in thymic output to date.

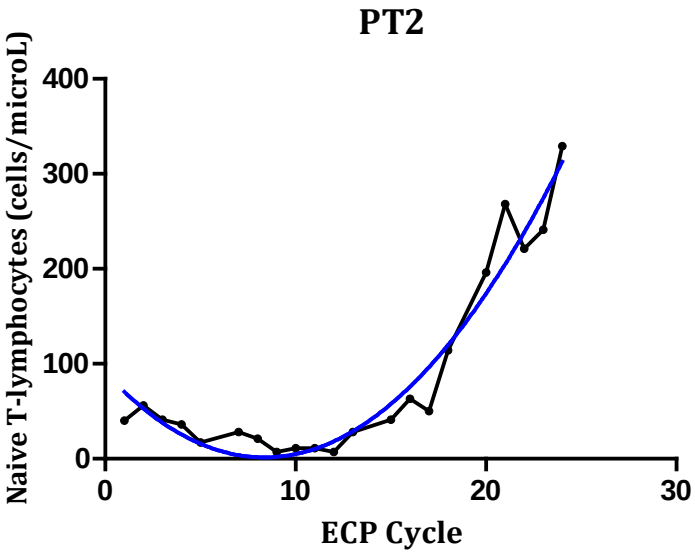


Figure 3.12 Example of non-linear regression (second order polynomial (quadratic) model) in a responding ECP patient, r^2 0.95.

	R^2 value
Patient 1	0.19
Patient 2	0.95
Patient 3	0.47
Patient 4	0.85
Patient 5	0.85
Patient 6	0.98
Patient 7	0.76
Patient 8	0.04

Table 3.7 Results of non-linear regression analyses for all ECP patients, with responding patients demonstrating high r^2 values, and non-responding patients demonstrating low r^2 values.

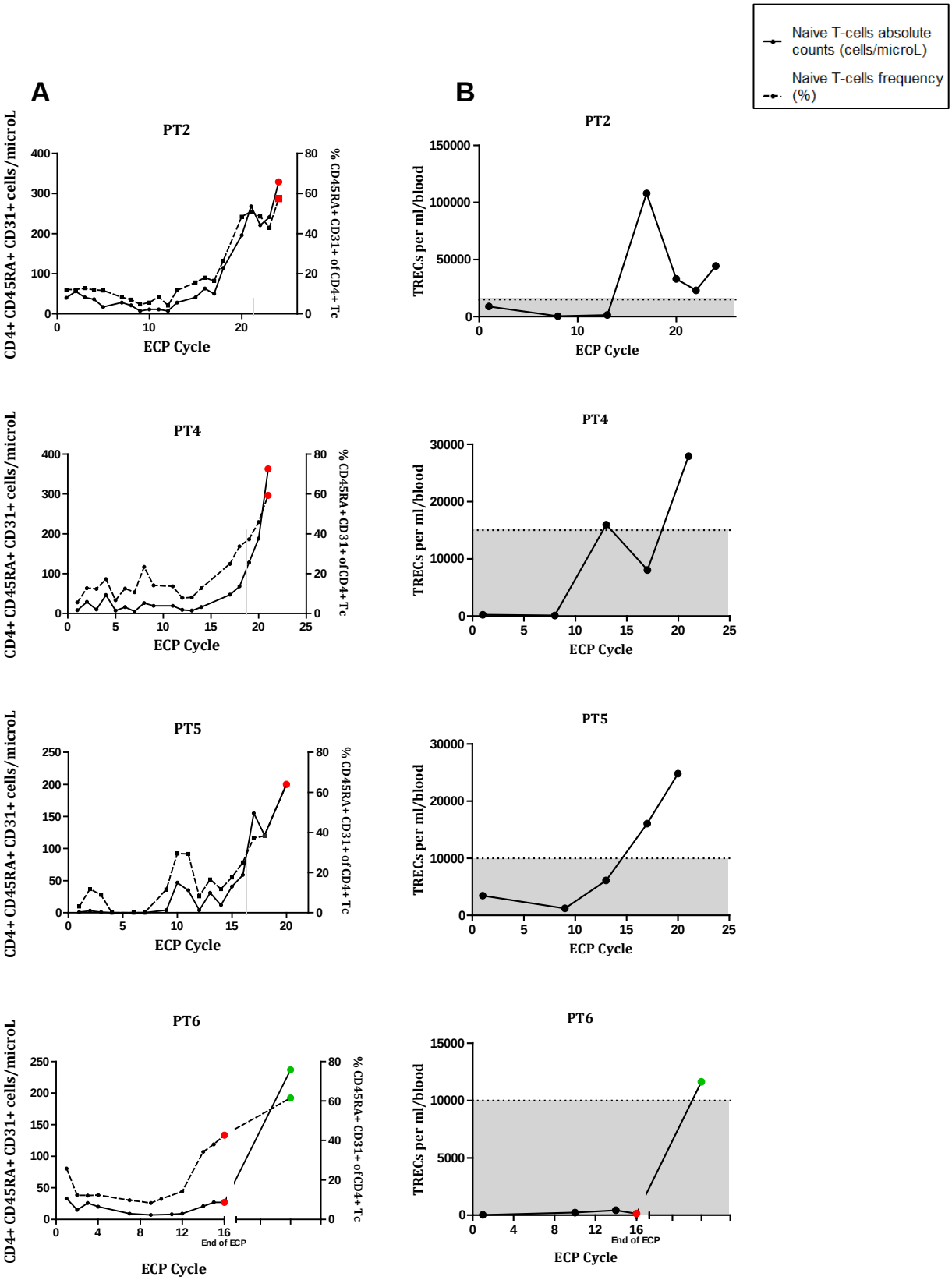


Figure 3.13 Individual quantitative analyses of thymic recovery using (A) absolute numbers (continuous black line) and frequency (dashed line) of naïve T-lymphocytes and (B) number of TRECs per ml/blood showed an improvement in 4 patients who completed ECP therapy. Below the normal age-dependent range for TRECs is demonstrated by the grey shaded area. The red dot denotes the end of ECP, and in patient 6 the green dot denotes follow-up analysis at 8 months post-HSCT.

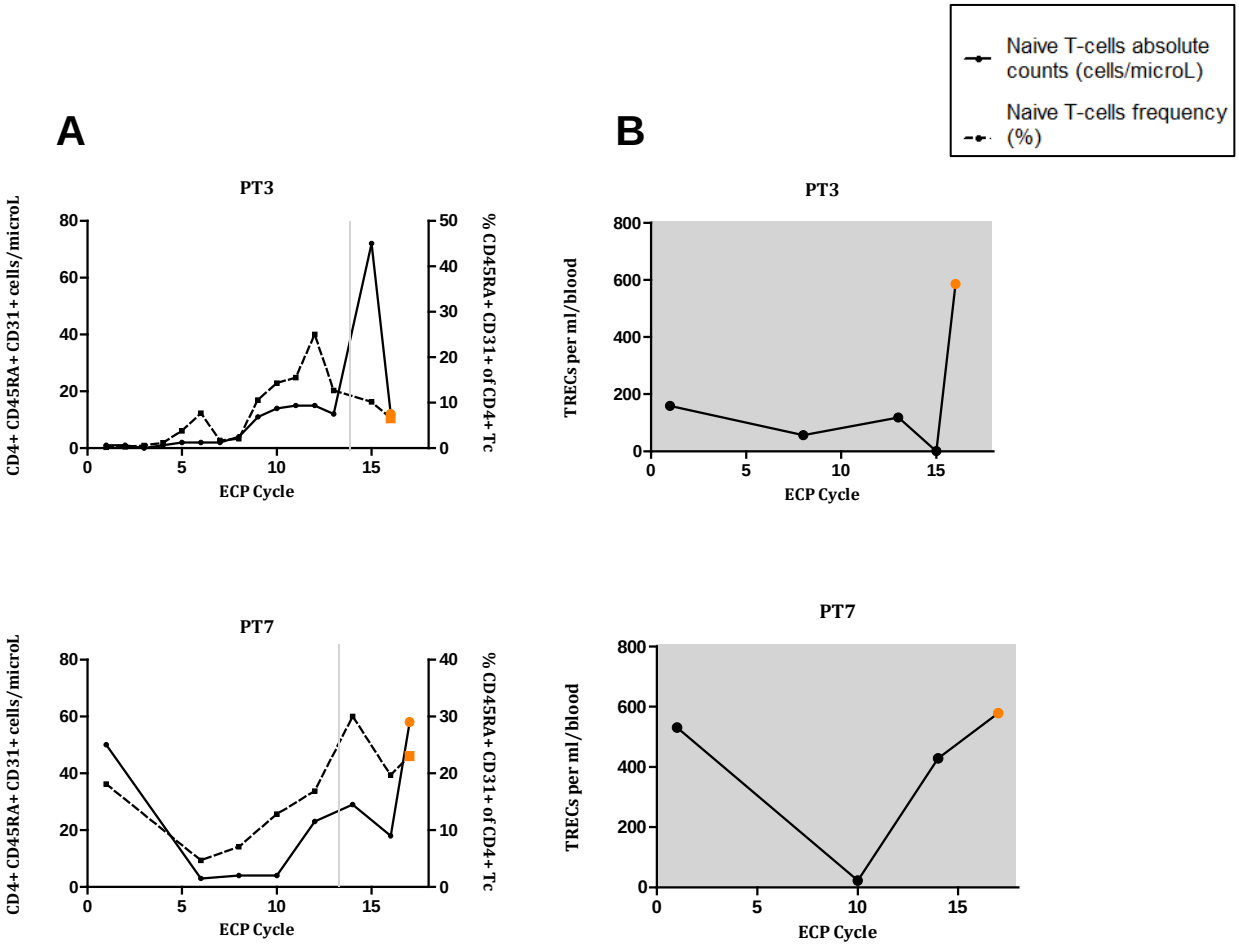


Figure 3.14 Patients 3 and 7 were withdrawn from ECP at cycles 16 and 17 respectively (the orange dot denotes time of withdrawal). Both patients demonstrated evidence of initial thymic recovery prior to withdrawal, with increasing absolute counts (continuous black line) and frequency (dashed line) of naïve T-lymphocytes (A) and TRECs (B) (although still below the normal range of TREC values). Patient 3 had shown an initial increase in absolute numbers of naïve T-lymphocytes at cycle 15, but this decreased following re-introduction of high dose corticosteroids.

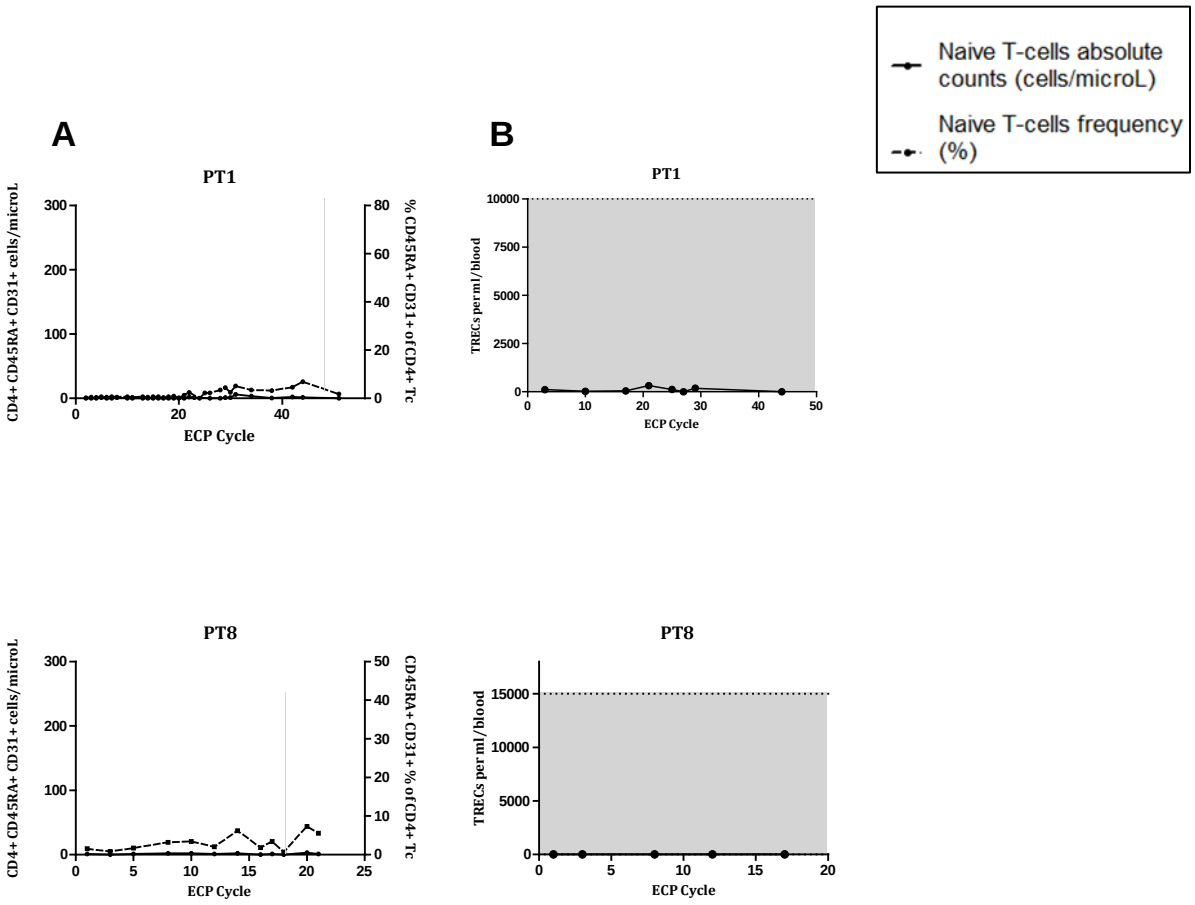


Figure 3.15 No evidence of improvement in thymic output was seen in patients 1 and 8 despite prolonged ECP therapy and weaning of immune suppression. This is illustrated by persisting very low absolute numbers (continuous black line) and frequency (dashed line) of naïve T-lymphocytes (A) and TRECs (B). The grey shaded zone indicates below the age-dependent TREC normal range.

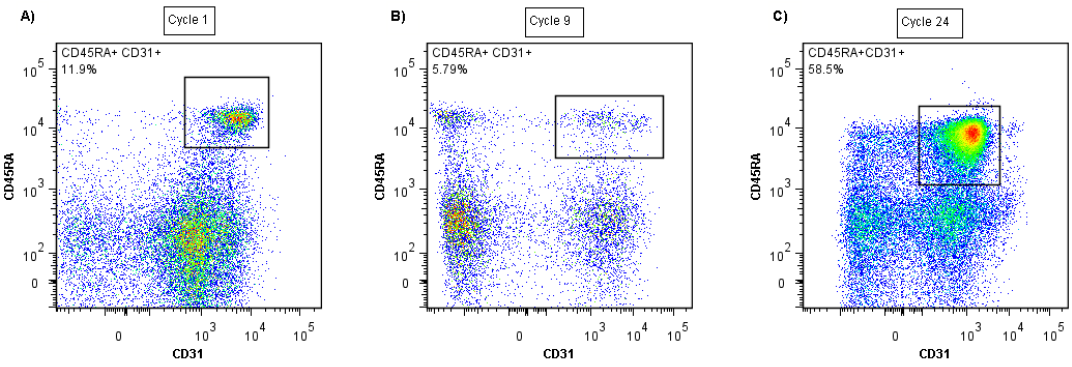


Figure 3.16 An example of an initial naïve T-lymphocyte population evident in patient 2 at (A) the beginning of ECP (55 days post-HSCT), which gradually declined to reach a nadir at cycle 9 (B) and then gradually increased, along with increasing TRECs and improvement in diversity of the TCR repertoire, by the end of ECP (C).

3.5.2.2 Thymic recovery and corticosteroid dose

A significant inverse relationship was observed between the dose of corticosteroids and the absolute numbers of naïve T-lymphocytes (Figure 3.17), using the median dose of corticosteroids (mg/kg) and median absolute number of naïve T-lymphocytes among all of the ECP patients. A rise in naïve T-lymphocytes was observed only when the corticosteroids had been weaned to a low dose, regardless of timing post-HSCT and resolution of clinically overt aGVHD.

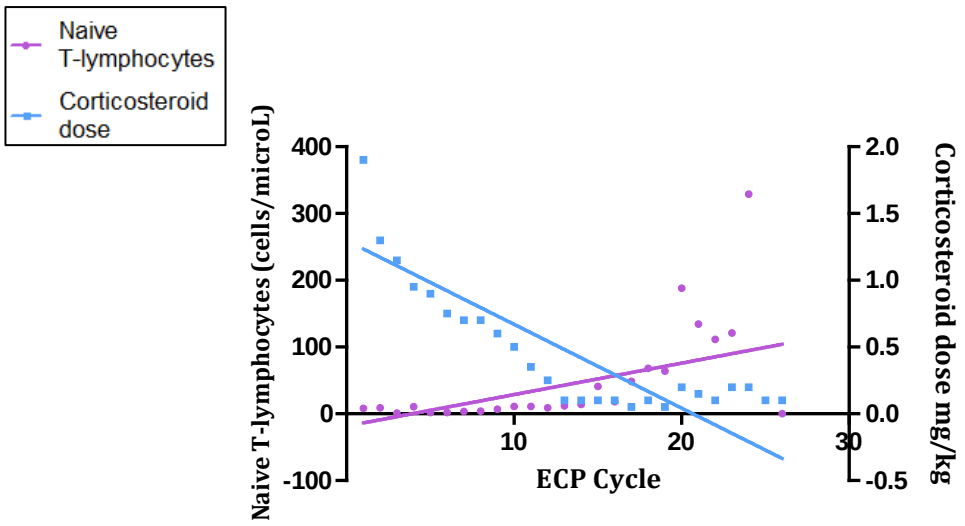


Figure 3.17 An inverse relationship was observed between the median dose of corticosteroids (blue line) and the median number of absolute naïve T-lymphocytes (purple line) at each cycle measured among the ECP patients ($p<0.0001$).

3.5.2.3 Time of ECP initiation and thymopoietic recovery

To ascertain if earlier initiation of ECP resulted in faster naïve T-lymphocyte recovery, the ECP group were divided into those who started ECP<90 days post-HSCT (n=5) and those started >90 days post-HSCT (n=3). The date post HSCT was used at this was a definitive time point for all patients, whereas the date of aGVHD diagnosis, and thus time from aGVHD diagnosis to ECP commencement, was not unequivocally identifiable for all patients. Data demonstrated that those started on ECP earlier had a faster increase in naïve T-lymphocytes compared to those started later (Figure 3.18), although the >90 days group includes the two non-responding patients.

Thymopoietic recovery in the prospective ECP group was compared with the retrospective ECP patients who had a CR to therapy (n=7,) who therefore had more complete follow-up data available (Figure 3.19). The median time from HSCT to commencement of ECP for this CR subgroup was 73 days. Comparison was also made with the prospective ECP subgroup who had a CR (n=4, median time from HSCT to commencement of ECP = 47.5 days). Thymopoietic recovery by 12 months was superior in the prospective ECP groups, with the highest naïve T-lymphocyte counts seen in the prospective CR group. Linear regression analysis demonstrated a statistically significant difference in the slope of the CR retrospective group compared to that of the CR prospective group (p=0.02).

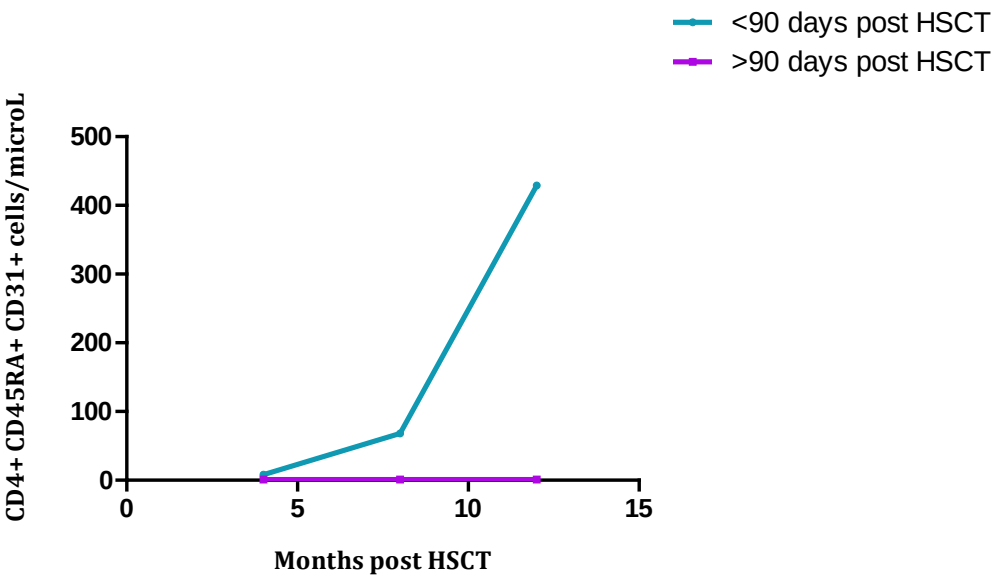


Figure 3.18 Patients started on ECP <90 days following HSCT (n=5) had superior naïve T-lymphocyte recovery (green line) compared to those commenced >90 days (n=3) (purple line), based on the median number of absolute naïve T-lymphocytes of the two groups at 4, 8 and 12 months.

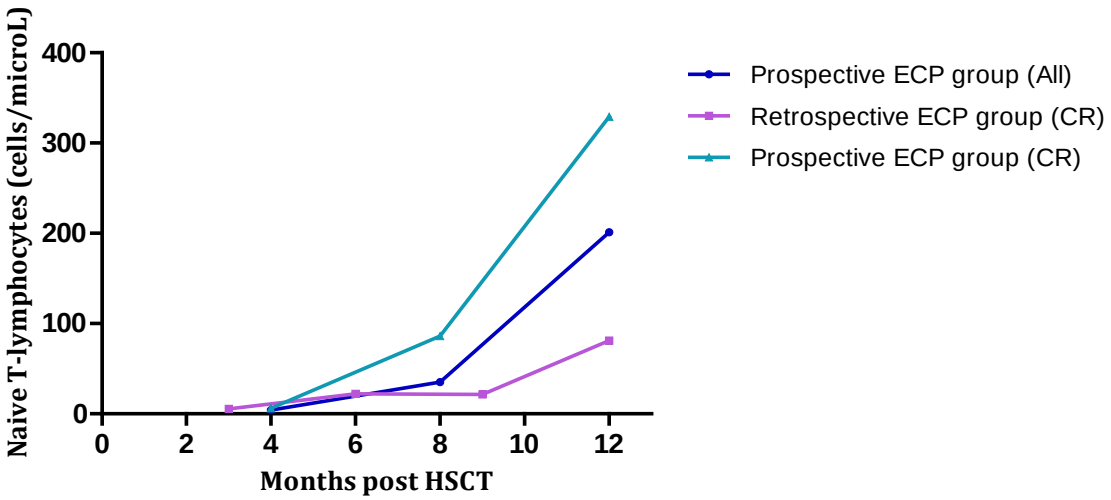


Figure 3.19 Comparison of thymopoietic recovery between the prospective and retrospective ECP groups demonstrated fastest recovery in the prospective group who had a CR to ECP (green line). Of note, naïve T-lymphocytes were defined as $CD4^+CD45RA^+CD27^+$cells in the retrospective group and $CD4^+CD45RA^+CD31^+$ cells in the prospective group, but these populations have shown to correlate well[76].

3.5.2.4 Comparison of thymopoiesis with control groups

To examine whether the naïve T-lymphocyte reconstitution seen in the ECP patients reflected the normal immune reconstitution trajectory of paediatric patients post-HSCT, results were compared with control group 1 (paediatric patients with no aGVHD), and control group 2 (paediatric patients with aGVHD not treated with ECP) at 4, 8 and 12 months following HSCT (Figure 3.20). All groups exhibited similar low median numbers of naïve T-lymphocytes at 4 months post-HSCT, expected at this stage of adaptive immune recovery. At 8 months, control group 1 had the highest median number of naïve T-lymphocytes and this superior upward trend continued at 12 months. At 8 months, the median number of naïve T-lymphocytes was higher in the ECP group compared to control group 2, and a steady incline followed similar to, but at an inferior rate, control group 1. Control group 2 demonstrated the lowest median number of naïve T-lymphocytes at each time point, although data collection is ongoing at the later time points.

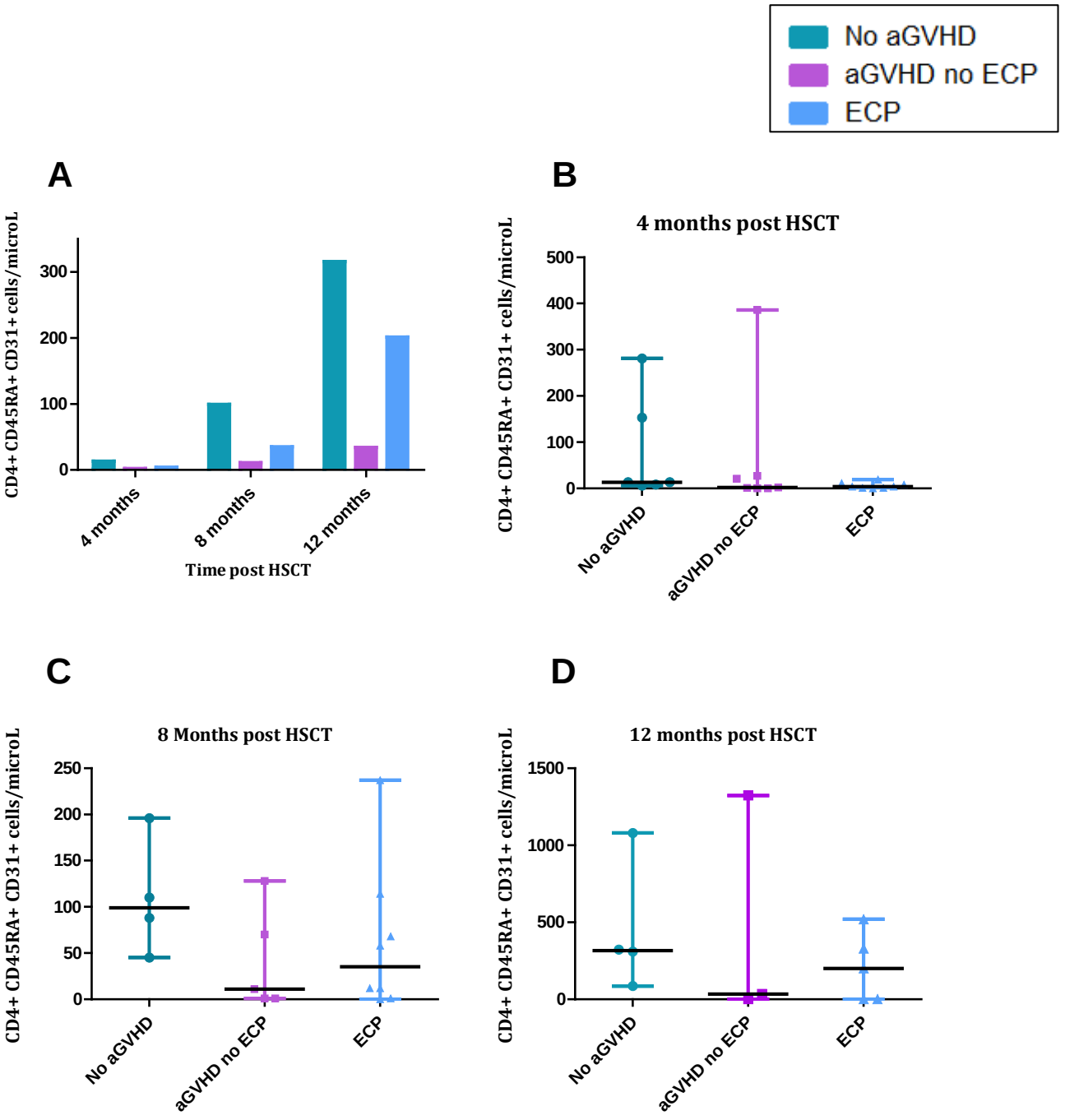


Figure 3.20 (A) Comparison of the naïve T-lymphocyte median values for each group at 4, 8 and 12 months. A steady incline is observed which is fastest in control group 1 with no aGVHD, followed by the ECP group, with the slowest recovery observed in control group 2. (B-D) Scatter plots of each group at 4, 8 and 12 months respectively demonstrate the median values and the ranges of absolute naïve T-lymphocyte numbers within each group. No significant differences in median values were detected at each time point (p=0.16, 0.32 and 0.69 respectively) although data collection at later time points continues.

3.5.3 Prospective results – Qualitative analyses

3.5.3.1 Qualitative T-lymphocyte recovery

Spectratype analysis of a control patient with no aGVHD at 12 months post-HSCT displayed a normal TCR repertoire (Figure 3.21, Table 3.8). In comparison, a control patient with aGVHD treated with systemic corticosteroids demonstrated a highly abnormal TCR repertoire with frequent monoclonal and oligoclonal peaks at 12 months post HSCT (Figure 3.22, Table 3.8).

All patients in the ECP group exhibited a highly abnormal TCR repertoire prior to commencing ECP therapy. Many Vβ families were missing, did not display a normal Gaussian distribution, or families consisted of a limited number of irregular peaks (Table 3.9). Two patients had a normal spectratype analysis at the end of ECP therapy, correlating with increased RTEs in the periphery (Figure 3.23). One patient demonstrated an improvement with a reduced number of monoclonal peaks and a reduced number of non-Gaussian families. Patient 6, who completed ECP earliest compared to the other patients (cycle 16, 6 months post-HSCT) had a highly abnormal at the end of ECP, but repeat analysis at 8 months post-HSCT demonstrated further improvement, with less frequent monoclonal peaks, but did not yet fulfil criteria to be classified as normal. Patient 1 continued to display a highly abnormal TCR repertoire at cycle 44 of ECP (19 months post-HSCT) (Figure 3.24). The two patients withdrawn from ECP had a highly abnormal TCR spectratype analysis at the time of withdrawal (both at 8 months post-HSCT). Patient 3 exhibited a deterioration compared to the pre-ECP analysis, whereas patient 7 had a degree of improvement evident, with a reduced number of monoclonal peaks and non-Gaussian families. TCR DNA spectratype analyses of the remaining ECP patients are shown in Appendix 1.

Patient	Date	Result	Description
Control group 1 (no aGVHD)	12 months post HSCT	Normal	One family < 5 peaks. All other families present with between 5-12 peaks, 1 family with non-Gaussian distributions, 3 monoclonal peaks.
Control group 2 (aGVHD no ECP)	12 months post HSCT	Highly abnormal	All other families present with between 5-12 peaks, all with Gaussian distributions, 13 monoclonal/oligoclonal peaks.

Table 3.8 TCR spectratype analyses demonstrated a normal TCR repertoire in a patient from control group 1 with no aGVHD and a highly abnormal TCR repertoire in a patient from control group 2 with aGVHD at 12 months post-HSCT. A normal spectratype was defined as ≥22 families having between 5-12 peaks with less than 3 non-Gaussian families and ≤3 large oligoclonal or monoclonal peaks.

Patient	Pre ECP result	Description	Post ECP result	Description
PT1	Highly abnormal	22 families with ≥5 peaks, 17 oligo/monoclonal peaks, 10 non-Gaussian families	ECP ongoing Cycle 44: highly abnormal	One family < 5 peaks. All other families present with between 5-12 peaks, 2 families with non-Gaussian distributions, frequent (x15) monoclonal peaks.
PT2	Highly abnormal	22 families with ≥5 peaks, 15 oligo/monoclonal peaks, 9 non-Gaussian families	Abnormal but improved	22 families with ≥5 peaks, 2 non-Gaussian families, 6 clonal peaks.
PT3	Highly abnormal	21 families with ≥5 peaks, 9 oligo/monoclonal peaks, 13 non-Gaussian families	Withdrawn at C16: highly abnormal	Majority of families non-Gaussian, 6< 5 peaks, 1 absent family, with frequent monoclonal peaks (>10).
PT4	Highly abnormal	23 families with ≥5 peaks, 11 oligo/monoclonal peaks, 3 non-Gaussian families	Normal	All families present with between 5-12 peaks, 2 families with non-Gaussian distributions, 3 monoclonal peaks
PT5	Highly abnormal	22 families with ≥ 5 peaks, 9 oligo/monoclonal peaks 4 non-Gaussian families	Normal	22 families with ≥5 peaks, all Gaussian distributions, 2 monoclonal peaks
PT6	Highly abnormal	23 families with ≥5 peaks, 13 oligo/monoclonal peaks, 12 non-Gaussian families	Highly abnormal	One family < 5 peaks. All other families present with between 5-12 peaks, 7 families with non-Gaussian distributions, frequent (x16) monoclonal peaks
PT7	Highly abnormal	22 families with ≥5 peaks, 9 non-Gaussian families, 10 oligo/monoclonal peaks, 9 non-Gaussian families	Withdrawn at C17: highly abnormal	4 families with <5 peaks, 4 non-Gaussian, 5 monoclonal peaks.
PT8	Highly abnormal	23 families ≥ 5 peaks, 16 oligo/monoclonal peaks, 8 non-Gaussian families	ECP ongoing	*

Table 3.9 Summary of TCR spectratyping results for the ECP patients before and after ECP treatment. Of note, analyses were performed at variable durations post-HSCT).

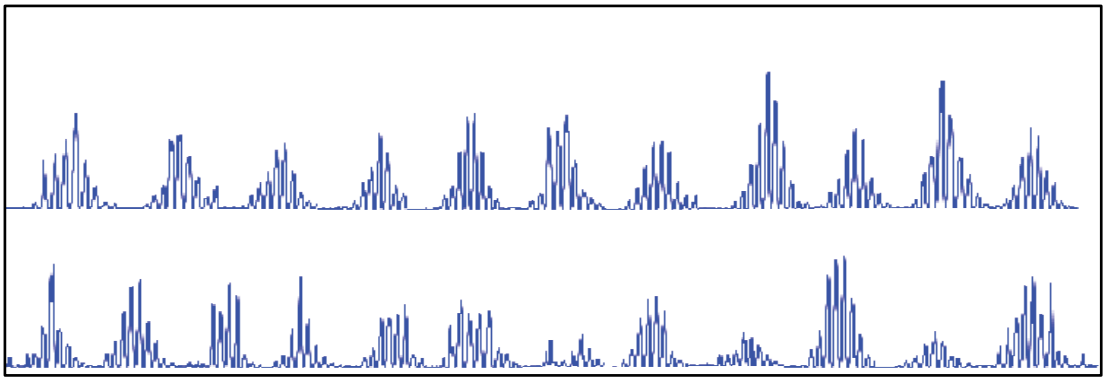


Figure 3.21 Normal TCR spectratype analysis of a paediatric patient at 12 months post-HSCT with no aGVHD. Almost all Vβ families are present with between 5-12 peaks and a normal Gaussian distribution, with infrequent monoclonal peaks.

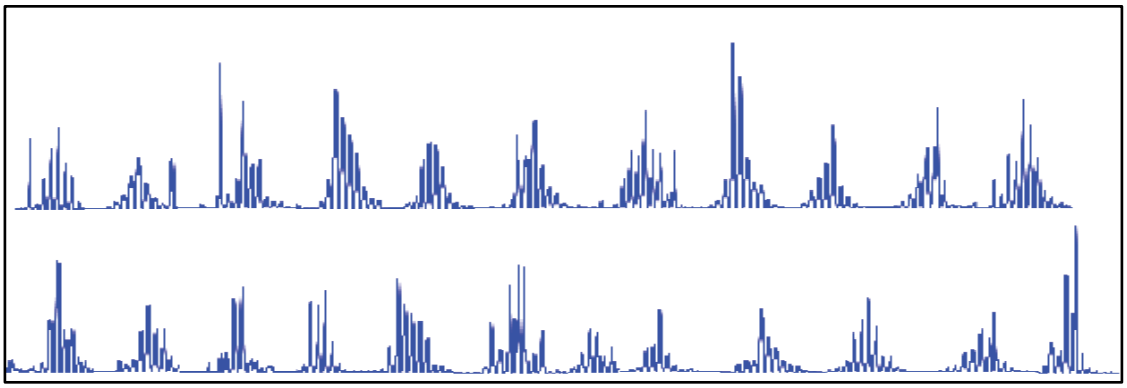
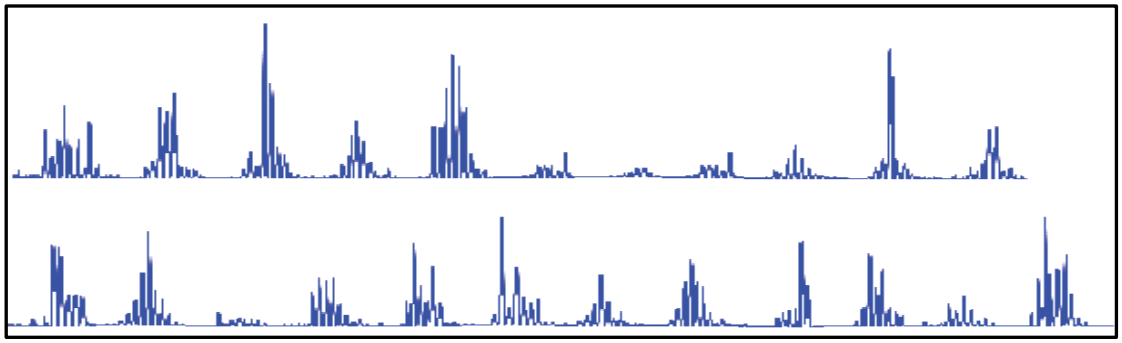


Figure 3.22 A patient from control group 2 who had aGVHD treated with corticosteroids but did not receive ECP exhibited a highly abnormal TCR spectratype analysis at 12 months post-HSCT. All Vβ families are present but there are frequent oligoclonal and monoclonal peaks observed suggesting ongoing abnormality of the TCR repertoire.

(A)



(B)

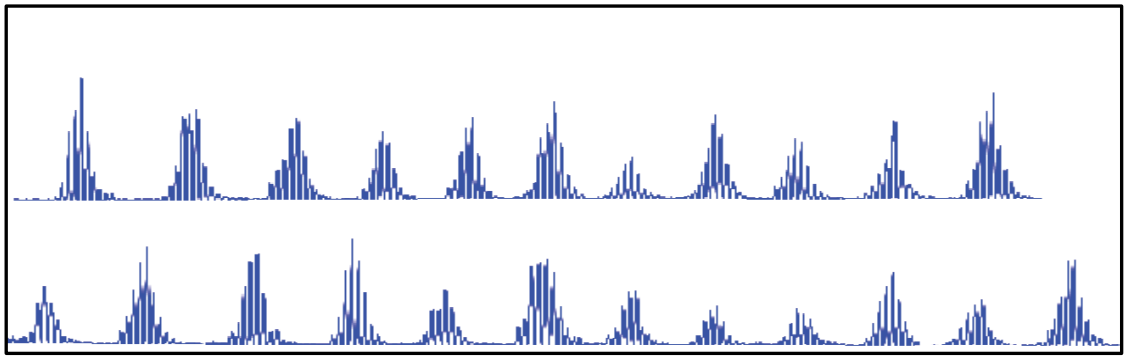
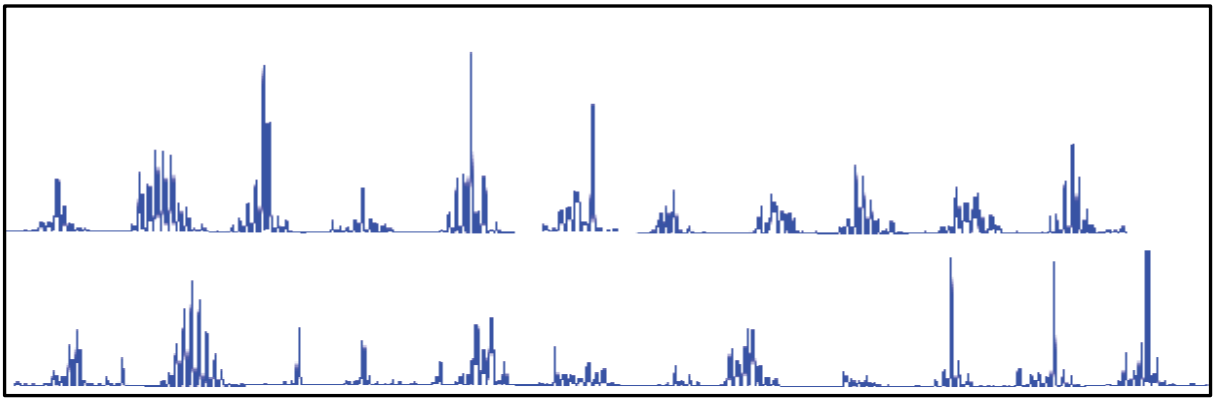


Figure 3.23 (A) TCR DNA spectratype analysis of a responding ECP patient, with a highly abnormal TCR repertoire evident before ECP therapy, with frequent oligoclonal/monoclonal peaks and non-Gaussian distribution of several families. (B) Post ECP analysis (following 20 cycles of ECP and at 12 months post-HSCT) showed marked improvement and normalisation of the TCR repertoire.

(A)



(B)

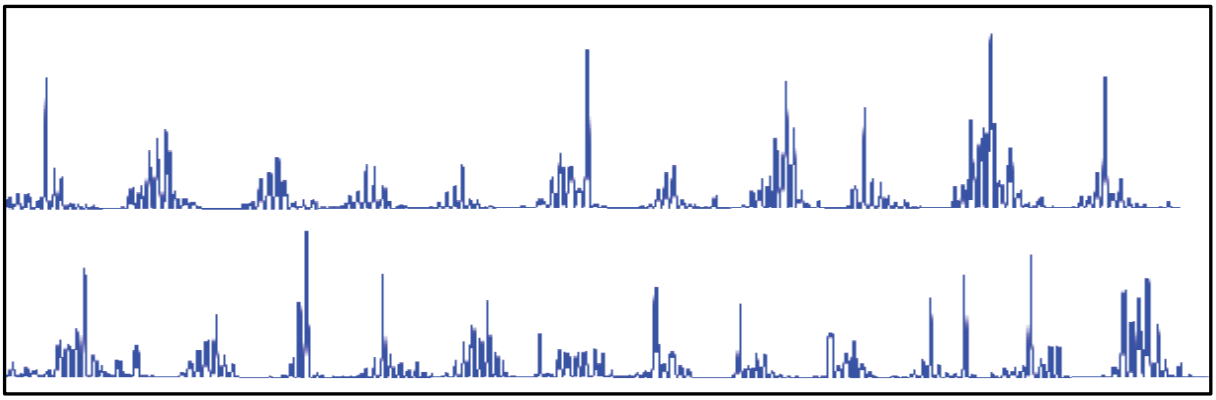


Figure 3.24 TCR DNA spectratype analysis of patient 1 (A) pre ECP demonstrated a highly abnormal repertoire, with frequent oligoclonal/monoclonal peaks. Follow up analysis (B) at cycle 44 of ECP remained highly abnormal.

3.5.4 Prospective results – Cytokines

3.5.4.1 IL-7

There was an overall decline in serum IL-7 levels among all of the ECP patients with progression of treatment (Figure 3.25). Linear regression analysis demonstrated a significant inverse relationship between serum IL-7 levels and cycles of ECP ($p = 0.006$). Individual analysis demonstrated a negative correlation between serum IL-7 and absolute numbers of naïve T-lymphocytes in 5/8 ECP patients (Table 3.10). Levels of elevated IL-7 were variable with peak levels ranging from 16.1 – 99.7pg/mL. IL-7 levels decreased to and then were maintained within or close to the normal range in parallel with increasing naïve T-lymphocyte numbers. In the responding ECP patients who completed therapy (Figure 3.26), 3/4 demonstrated an inverse relationship between IL-7 and naïve T-lymphocytes. Patient 6, who completed treatment earlier than the other patients, did not have later time points available at the time of analysis. The non-responding patients (patients 1 and 8) did not display a negative correlation between IL-7 and naïve T-lymphocytes (Figure 3.27-A). In patient 1, an increase in IL-7 was evident at cycles 20-25 (maximum level of 22.2pg/mL), but with no significant corresponding increase in naïve T-lymphocytes, and otherwise remained just above the normal range throughout treatment (6.6 – 12.7pg/mL, overall median 11.3pg/mL, mean 23.0pg/mL). Patient 8 displayed a raised IL-7 above the normal range, with a gradual incline (maximum level 19.1pg/ml), but, similar to patient 1, did not demonstrate a corresponding rise in naïve T-lymphocytes. The two patients withdrawn from ECP, who both displayed an initial rise in naïve T-lymphocytes prior to withdrawal, had a negative correlation between IL-7 and naïve T-lymphocytes (Figure 3.27-B).

3.5.4.2 Comparison with control groups

Median serum IL-7 levels were highest in control group 1 at 4 months, and steadily declined at 8 and 12 months post-HSCT (Figure 3.28). At 4 months, median IL-7 levels were similar between control group 2 and the ECP group, levels were marginally higher in control group 2 at 8 months, and this gap increased further at 12 months.

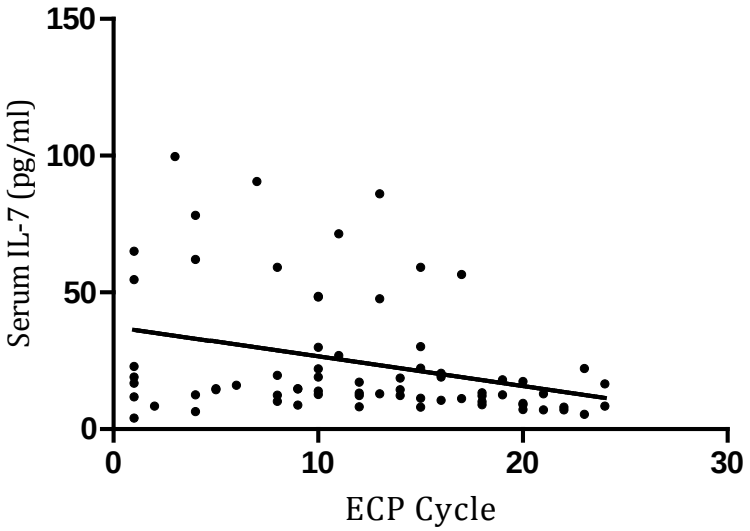


Figure 3.25 Serum IL-7 levels measured in all ECP patients with progression of treatment demonstrated an overall decline (p = 0.006). Normal range of IL-7 was defined as 0.27 – 9.80pg/mL, mean 2.51pg/mL.

Patient 1	Spearman r P value	0.271 0.348
Patient 2	Spearman r P value	-0.883 <0.001*
Patient 3	Spearman r P value	-0.241 0.582
Patient 4	Spearman r P value	-0.762 0.037*
Patient 5	Spearman r P value	-0.694 0.069
Patient 6	Spearman r P value	0.564 0.350
Patient 7	Spearman r P value	-0.800 0.333
Patient 8	Spearman r P value	0.866 0.083

Table 3.10 Spearman rank correlation analysis illustrating the relationship between serum IL-7 levels and naïve T-lymphocyte absolute counts with progression of ECP. An asterisk denotes statistical significance (p value <0.05) and Spearman r denotes the correlation coefficient.

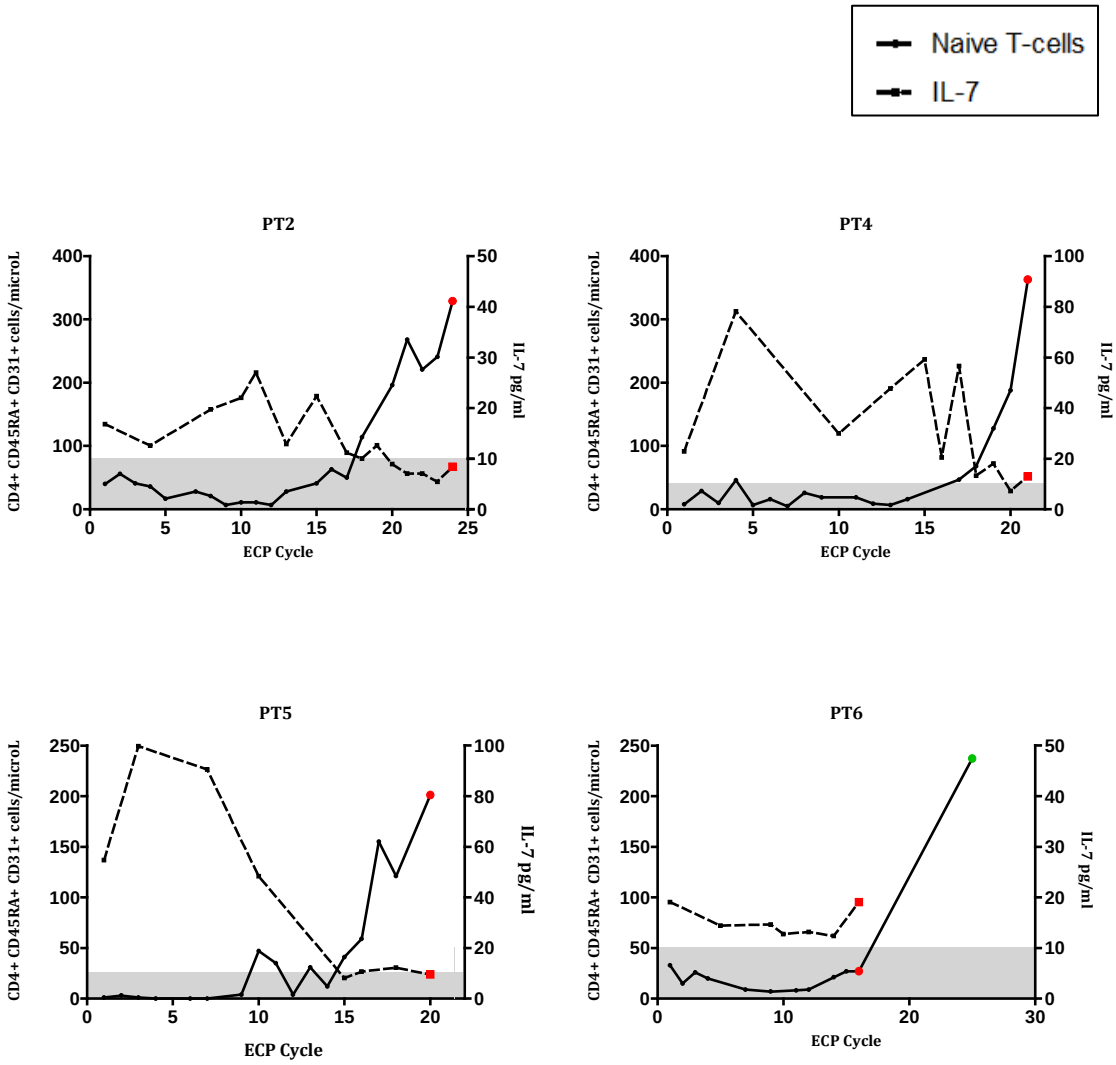


Figure 3.26 Serum IL-7 levels (dashed black line) and the relationship to absolute counts of naïve T-lymphocytes (continuous black line) in the responding patients who completed ECP treatment. The red dots denote the end of ECP treatment. The green dot for patient 6 denotes follow up at 8 months post-HSCT. The grey shaded zone represents the normal range of IL-7. An inverse relationship was demonstrated in patients 2, 4 and 5.

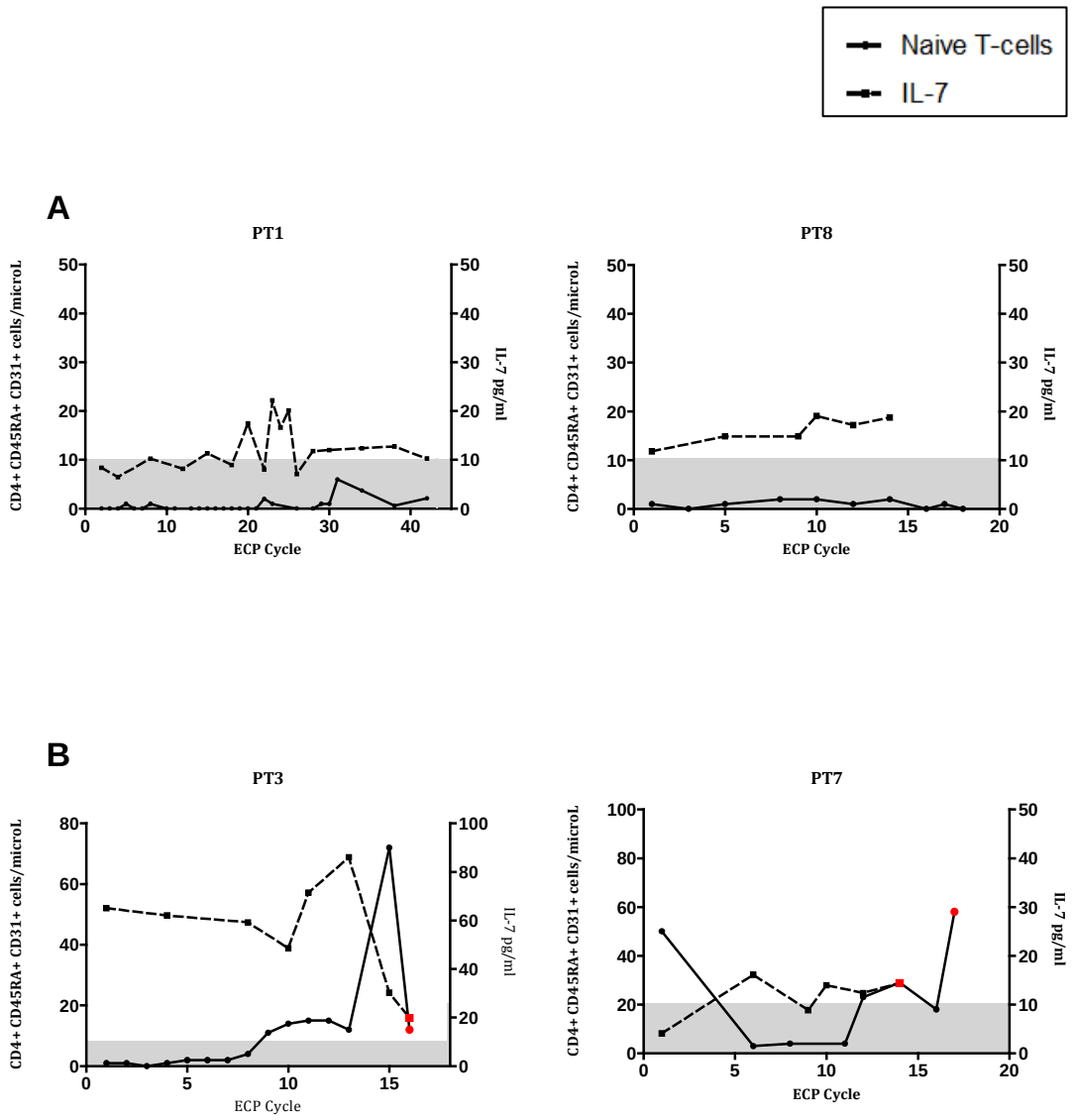


Figure 3.27 Serum IL-7 levels (dashed black line) and the relationship to absolute counts of naïve T-lymphocytes (continuous black line) in (A) the non-responding patients and (B) the patients withdrawn from ECP treatment. The red dots denote the end of ECP treatment. The grey shaded zone represents the normal range of IL-7. Patients 3 and 7 demonstrated evidence of an inverse relationship between IL-7 and naïve T-lymphocytes. Patient 1 had an isolated increase in IL-7 between cycles 20-25 with no corresponding significant increase in naïve T-lymphocytes. Patient 8, despite IL-7 being above the normal range, similarly showed no corresponding increase in naïve T-lymphocytes.

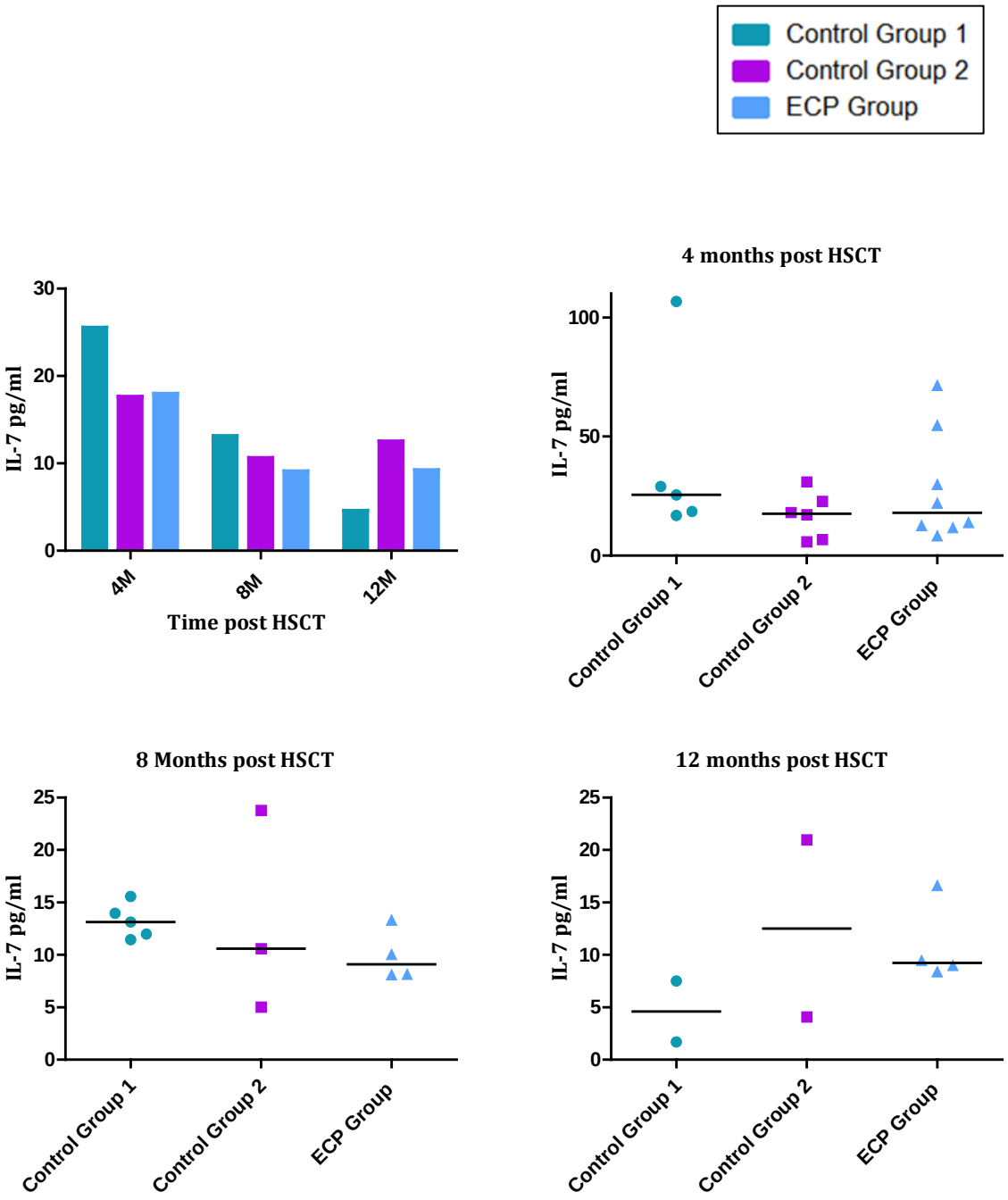


Figure 3.28 Comparison of median serum IL-7 levels between the ECP group and the control groups at 4, 8 and 12 months post-HSCT. No statistically significant differences at 4, 8 or 12 months post-HSCT were detected (p= 0.40, 0.26, 0.24 respectively). At 4 months, median serum IL-7 levels were highest in control group 1, and highest in control group 2 at 8 and 12 months post-HSCT.

3.5.4.3 IL-22

Two patterns of serum IL-22 were observed among the ECP patients. In patients 1, 2, 3 and 6, high levels of IL-22 were detected at the beginning of ECP, either from the onset or very early in treatment, followed by a decline and a later second peak, the timing of which was variable for each patient (ranging from cycle 12 – 23). In contrast, patient 4, 5, 7 and 8 IL-22 levels were low initially, followed by a later incline. In patients 4, 6 and 8, IL-22 levels peaked between cycles 10-16, followed by a decline. For patient 7, IL-22 levels gradually increased, but the final 3 measurements (prior to withdrawal from treatment) were not available at the time of analysis.

The range of IL-22 detected was variable among patients. Patient 5 displayed significantly higher IL-22 levels compared to the other ECP patients, with a median value of 48.6pg/ml (range 2.4 – 118.0pg/ml). There was a gradual increase with low levels initially, with a peak at cycle 14 and persistently very elevated levels between cycles 14 – 18, followed by a drop in IL-22 at the end of ECP. Patient 3 also had high levels of IL-22 (median 49.1pg/mL, range 11.3 – 156.2pg/ml). This patient had high levels at the onset of ECP, followed by a decline, and a later surge to a peak of 156.2pg/ml at cycle 14, coinciding with the timing of his clinical deterioration. Patient 4 had lower IL-22 levels compared to the other ECP patients with a peak value measured of 15.9pg/ml. Four healthy control serum samples were analysed concurrently and IL-22 was non-detectable.

3.5.4.4 IL-22 relationship with naïve T-lymphocytes

A rise in serum IL-22 occurred prior to the increase in naïve T-lymphocytes in patients 3, 4, 5, 6 and 7, with the opposite observed in patient 2 (Figure 3.29). The surge in IL-22 observed in patient 3 at cycle 14 occurred immediately prior to the sharp increase in naïve T-lymphocytes. The increase in IL-22 in patients 1 and 8 was not associated with any corresponding change in naïve T-lymphocytes.

3.5.4.5 IL-22 relationship to IL-7

Among those with two IL-22 peaks (at the beginning of and later in the course of ECP therapy), IL-7 levels peaked before the second peak in patients 2, 3 and 6 (Figure 3.30). Among those with a later rise in IL-22, IL-7 peaked prior to IL-22 in patients 4, 5 and 7.

The peak in IL-22 coincided with the IL-7 peak in patients 1 and 8. For patient 1, both IL-7 and IL-22 increased concurrently then decreased, whereas for patient 8, IL-7 continued to incline, although later time points were not available at the time of analysis. An initial rise in IL-22 was evident for patients 7 and 8, but as ECP was ongoing for these patients at the time of IL-22 analysis, complete analysis was not possible.

3.5.4.6 Comparison of IL-22 with the control groups

Comparison of the ECP group IL-22 levels with those of the control groups at 4, 8 and 12 months post-HSCT demonstrated that at 4 months, the median IL-22 level was highest in control group 2, with similar median levels between control group 1 and the ECP group. At 8 and 12 months post-HSCT, median IL-22 levels were highest in the ECP group, although the number of patients analysed, particularly at 12 months, were low (Figure 3.31). Despite these trends, there was no statistical difference at any of the time points measured ($p=0.63$, 0.23, 0.22 at 4, 8 and 12 months respectively).

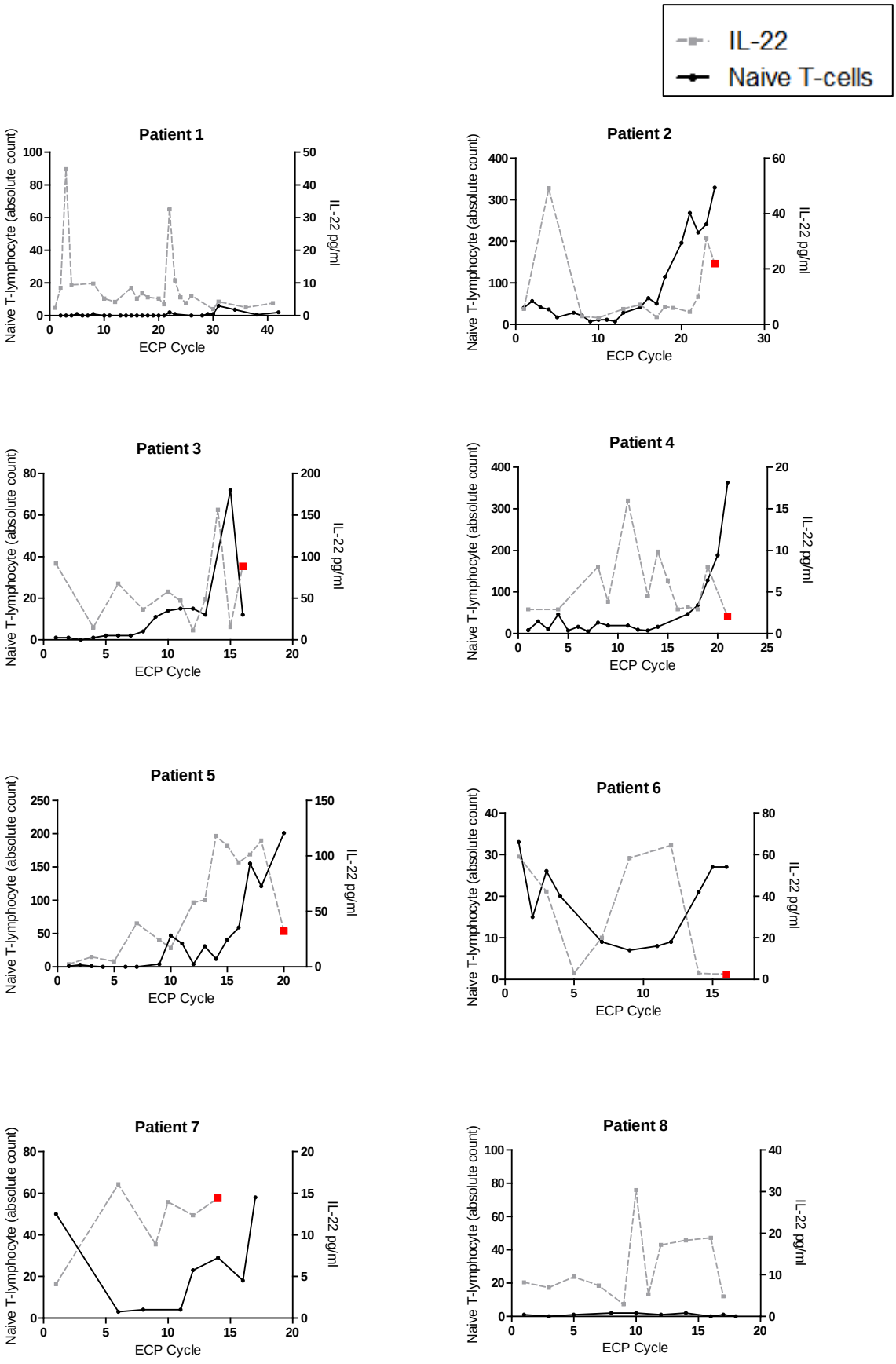


Figure 3.29 Patterns of serum IL-22 (dashed grey line) with progression of ECP, and the relationship with naïve T-lymphocytes (continuous black line). Note that different right *y* axis scales are used as the IL-22 range was variable among patients. The red square indicates completion of ECP therapy. Patients 1, 2, 3 and 6 demonstrated a similar pattern with high levels initially, followed by a decline, and a later second peak.

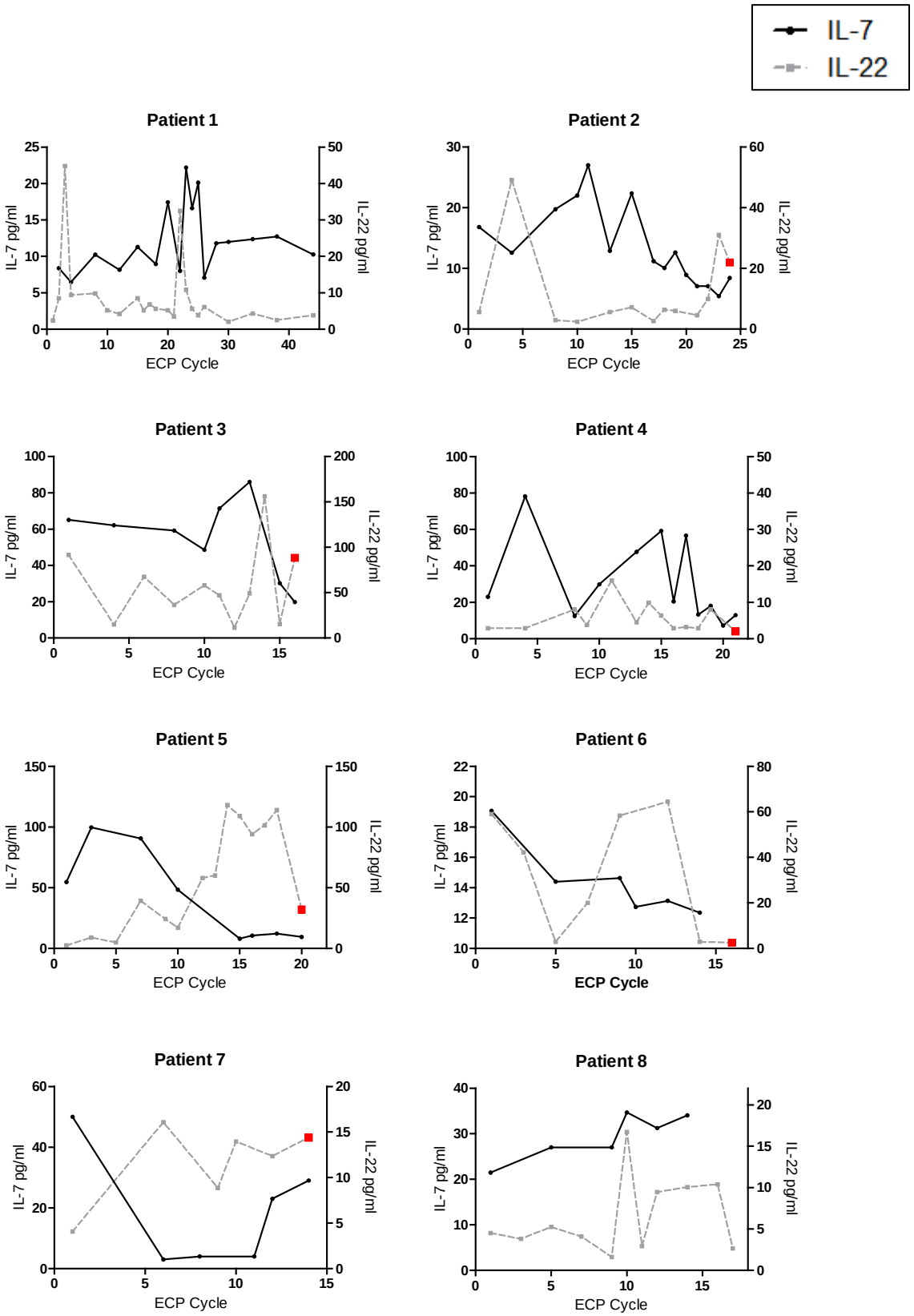


Figure 3.30 Pattern of serum IL-22 (dashed grey line) in the ECP patients with progression of treatment relative to serum IL-7 levels (continuous black line). A peak in IL-7 was observed before the peak in IL-22 in patients 4, 5 and 7, and before the second IL-22 peak in patients 2, 3 and 6.

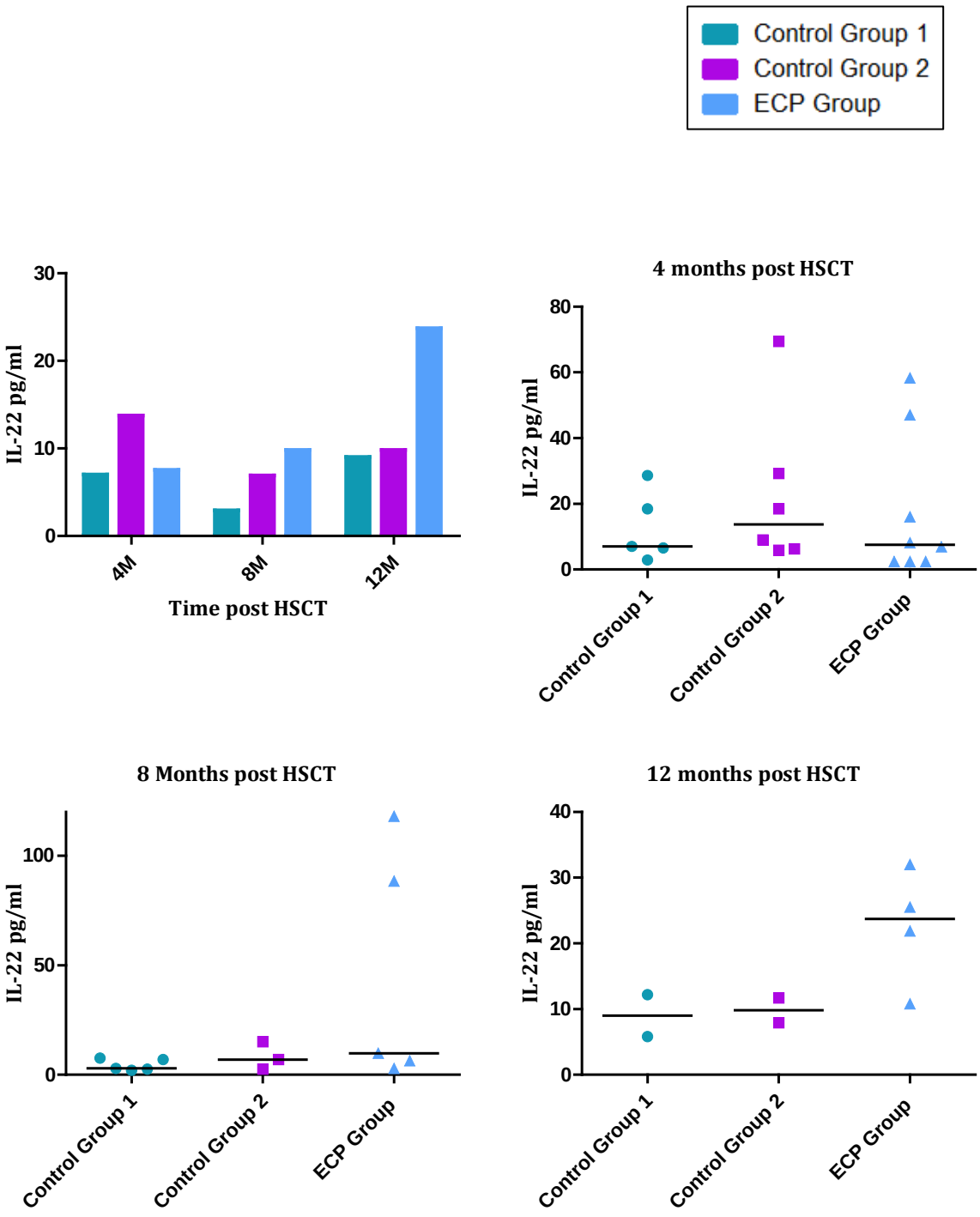


Figure 3.31 Comparison of serum IL-22 levels between the ECP group and the control groups. Control group 2 had the highest median level of IL-22 at 4 months, and the highest median IL-22 levels at 8 and 12 months were seen in the ECP group. No statistically significant differences were found at 4, 8 or 12 months post-HSCT (p=0.63, 0.23 and 0.22 respectively).

3.6 Discussion

3.6.1.1 Retrospective results

The retrospective data highlight the repressive effect of aGVHD and treatment with corticosteroids on thymic function and restoration of naïve T-lymphocytes post-HSCT; a detrimental effect seen even with mild aGVHD treated with topical therapies. Although it is unknown if these effects persisted beyond 12 months, during this lymphopaenic period these patients are subject to a high risk of complications, particularly viral infections. Indeed, the majority of deaths among the ECP-treated patients were related to complications of long-term immunosuppressive therapy. Those treated with ECP had the lowest numbers of naïve T-lymphocytes post-HSCT, most likely reflecting more severe aGVHD within this group, the concurrent prolonged use of multiple immunosuppressive therapies, and the delay in commencing ECP for some patients (median duration 105 days from HSCT). However, patients in this group demonstrated some, albeit very limited, thymic recovery, suggesting that ECP can facilitate thymopoietic recovery by reducing aGVHD and concurrent immunosuppression. Limitations include the retrospective nature of data collection and missing data at some time points. Only quantitative analysis of thymic recovery using naïve T-lymphocyte absolute counts was possible, and other quantitative parameters such as TRECs, or qualitative parameters such as assessment of diversity of the TCR repertoire, were not available for a more complete picture of thymic recovery. Three of the ECP patients died within 12 months of commencing ECP, and this loss of data may bias the results among the survivors. Despite this, the trend of naïve T-lymphocytes among the groups firmly highlights the detrimental effects of aGVHD and corticosteroids on thymic output, supports pre-existing evidence that ECP can facilitate thymic recovery[158], and raises the important question of whether earlier initiation of ECP may allow further improvement by reducing the degree of thymic damage inflicted from aGVHD and prolonged corticosteroid and other systemic immunosuppression exposure.

3.6.1.2 Prospective analysis of thymic recovery

All the prospective ECP patients demonstrated low thymic output and a highly abnormal TCR repertoire at the beginning of ECP treatment, regardless of the timing post-HSCT. Four patients exhibited improved thymopoiesis with progression of ECP therapy, evidenced by the rise in absolute numbers and frequency of $CD4^+CD45RA^+CD31^+$ naïve

T-lymphocytes and TRECs, and indirectly demonstrated by the inverse relationship with serum IL-7 levels, indicating that as thymic output is restored, IL-7 is consumed by IL-7R-expressing T-lymphocytes and serum IL-7 levels fall. Importantly, this was also associated with qualitative improvement, illustrated by the increased diversity of the TCR repertoire, as a widely diverse TCR repertoire is a prerequisite to protect against future potential pathogenic antigens encountered. Two patients should evidence of preliminary thymic recovery, with an increase in naïve T-lymphocytes, but subsequently were withdrawn from treatment.

In contrast, two patients (patients 1 and 8), both with pulmonary involvement, demonstrated no improvement in thymic output with ECP progression. Patient 1 had negligible thymic output 2 years post-HSCT, with a persisting abnormal TCR repertoire, despite 54 cycles of ECP treatment. Contributing factors for this outcome may include; prolonged exposure to high dose corticosteroids prior to ECP, slower weaning of immune suppression compared to other patients, re-initiation of high dose corticosteroids at cycle 30, comparatively late initiation of ECP (173 days post-HSCT), and a complicated clinical course. Patient 8 was also exposed to prolonged high dose corticosteroids, with slow weaning of immunosuppression, started ECP at 4 months post-HSCT and displayed no evidence of thymic recovery at 12 months post-HSCT (and 21 cycles of ECP). An alternative explanation could be that the block in T-lymphocyte production is before the thymus, related to a problem of the lymphoid progenitor population. Further investigation is needed to elucidate this. Whilst it is unknown at this stage whether this is a permanent immune outcome for these patients, the aforementioned factors, coupled with other pre and peri-HSCT factors such as chemotherapy, collectively may have caused unbeknownst thymic damage, raising the clinically relevant question of whether sustained, multiple and/or intense insult to the thymus can result in irreversible damage with an associated bleak clinical outcome.

Three patients had evidence of circulating RTEs at the beginning of ECP, which declined in the first few weeks of treatment. This transiency suggests that these naïve T-lymphocytes originated within the graft, or were residual surviving host naïve T-lymphocytes, rather than *de novo* ontogenesis. This is supported by the lack of correlation with TRECs, the presence of the highly abnormal TCR repertoires at this point, and the fact that these patients were started on ECP within 2 months post-HSCT. Fallen *et al* previously demonstrated that naïve T-lymphocytes may be transferred from the donor, but these will be negligible by approximately 3 months post-transplant[94].

Naïve T-lymphocytes can also be detected in the periphery due to re-expression of naïve surface markers but by corroborating flow cytometric evidence of thymic recovery with TRECs and spectratyping analysis, the inference is strengthened that the increased naïve T-lymphocytes observed are due to *de novo* thymic production, as re-expression of naïve surface markers or proliferation of the naïve T-lymphocyte population would result in dilution of TRECs, and ongoing restriction of the TCR repertoire.

An interval was observed between the onset of ECP treatment and establishment of improved thymic output, with initial evidence of sustained increasing thymic output in responding patients seen at cycles 10-13, corresponding to approximately 3 months of treatment. This pattern of thymic recovery may reflect the normal trajectory of T-lymphocyte reconstitution following HSCT, with thymic output reported to normally occur after approximately 4 months. Against this argument is the fact that despite inconsistencies in the timing from HSCT when ECP was started, the responding patients demonstrated improvement at approximately the same time following initiation of ECP. Patient 5 commenced ECP at 4 months post-HSCT with negligible thymic output at this time point, and showed the same pattern of thymopoietic recovery, with increasing RTEs detected from cycle 10 onwards (7 months post-HSCT). This suggests that the pattern of recovery is more reflective of the time required for the repair of thymic tissue and for the normal process of thymocyte development and export to resume.

The patients started on ECP later had an inferior rate of thymic recovery compared to the patients started within 90 days of HSCT, suggesting that limiting exposure to thymic insults may reduce the degree of damage inflicted, permitting earlier repair and restoration of function. However, this is based on a limited number of patients and does not inform of us of whether patients with less severe aGVHD would also display the same thymopoietic benefit if started on ECP early. Further data are required before conclusions can be made, but this supports previous studies that earlier adoption of ECP may produce better outcomes[161,162] (although the impact on immune reconstitution has not been studied), and that consideration for ECP treatment should be made early following lack of response to corticosteroids.

The key role of IL-7 in thymopoiesis following HSCT is demonstrated by the inverse relationship in those who completed ECP and displayed thymic recovery; IL-7 levels were highest when naïve T-lymphocyte counts were low, and declined with increasing naïve T-lymphocyte numbers. IL-7 is essential in thymocyte development from the DN

stage onwards with a controlled spatiotemporal balance of intra-thymic IL-7 required[163], and absence or disturbance of this delicate environment results in impairment of thymocyte survival and development. Increased IL-7 production supports the presence of a functioning thymic stroma. However, IL-7 levels did not peak until several cycles into ECP treatment for some patients, with lower levels initially despite low levels of RTEs. This time lapse may reflect initial thymic stromal damage with consequent inability to produce IL-7, and, following reduction in aGVHD activity and corticosteroids, thymic stromal renewal allows for restoration of appropriate IL-7 production in response to lymphopaenia.

Patient 6, who successfully discontinued ECP at cycle 16, had elevated IL-7 levels evident throughout ECP, but a decline was not seen, most likely because the steep rise in RTEs occurred after ECP treatment was completed and serum IL-7 at these later time points was not available at the time of analysis. A similar pattern was seen for patient 7 who was withdrawn at cycle 17. In patient 1, the isolated transient elevation of IL-7 does not result in increased thymic output of naïve T-lymphocytes, potentially due to a need for sustained elevated IL-7 to stimulate and maintain thymopoiesis. As TECs are the major source of IL-7, lack of sustained IL-7 in patient 1 reinforces the presence of thymic damage and consequent impairment of IL-7 production. However, patient 8 had steadily elevated IL-7 from cycles 1–16, although not as high as seen in other patients, but with no resulting increased thymic output supporting the presence of functioning IL-7-producing TECs. This suggests involvement of other factors preventing normal thymopoiesis, such as disturbance of the normal intra-thymic IL-7 spatiotemporal balance or lack of other thymopoiesis-supporting factors. Potentially, in the presence of thymic impairment, supra-optimal levels of IL-7 are needed to drive thymopoiesis, and levels seen in patient 8 are inadequate. IL-7 can also be produced by other non-thymic derived cells, thus presence of IL-7 does not rule out the presence of thymic TEC damage.

Variable levels of elevated IL-7 were seen between patients, with peak levels ranging from 16.1 – 99.7pg/ml. This may reflect varying degrees of thymic damage present, or an individual need for a higher driving force to provide adequate thymopoiesis. Higher levels of IL-7 did not appear to result in superior T-lymphocyte reconstitution, although longer-follow up is needed. Variability in results may also be related due to inter-assay variation (CV 7.8–11.8%). It is also important to consider that measuring IL-7 in the serum may not accurately reflect intra-thymic IL-7 levels and production, and further

investigation is needed to determine if there are other causes of suboptimal IL-7 levels in the setting of severe lymphopaenia.

Thymopoietic recovery occurred in parallel with decreased frequency of activated HLA-DR$^+$ T-lymphocytes and improvement in overall clinical condition, reflecting a reduction in aGVHD disease activity, and gradual withdrawal of corticosteroids and other immunosuppressive agents. Both reduction in aGVHD and weaning of immunosuppression are the likely reasons for thymic recovery, rather than a direct ECP effect, although it is not clear which of these detrimental factors has a predominant effect. Thymic recovery was established as the patients reached a low dose of corticosteroids per kilogram bodyweight, suggesting a dominant corticosteroid negative effect. This would support a careful but rapid weaning approach. Clinical aGVHD subsided within weeks of starting ECP in all patients apart from patients 1 and 8; however, even in the absence of overt aGVHD activity, isolated subclinical thymic aGVHD can be present which may account for the delayed thymic recovery. In the non-responding patients 1 and 8, clinical disease persisted but a decrease in HLA-DR+ T-lymphocytes was also observed, and, even with reduction of immune suppression, there was no evidence of thymic output. This suggests persisting active thymic aGVHD may be present or irreparable damage. Both patients 1 and 8 remained exposed to corticosteroids throughout (minimum dose reached was 0.5mg/kg for patient 8 and 0.1mg/kg for patient 1) and required re-initiation of increased corticosteroids, which may also be responsible or at least contribute to the ongoing suppression of RTE production.

Comparison with the control groups provided insight into the interpretation of thymopoiesis relative to the normal trajectory of recovery following HSCT, and the potential benefit of ECP when compared to those with aGVHD treated with corticosteroids only. Low numbers of naïve T-lymphocytes were detected in all groups at 4 months post-HSCT. At 8 months, thymic recovery was fastest in control group 1 with no aGVHD, and continued an upward trend and by 12 months, the median number of naïve T-lymphocytes was 316 (range 85-1079). This superior recovery of thymic output in control group 1 supports the detrimental impact of aGVHD and corticosteroids on thymic output as observed in control group 2 and the ECP group who displayed lower numbers of naïve T-lymphocytes at 8 months post-HSCT. By 12 months, the median number of naïve T-lymphocytes was superior in the ECP group compared to control group 2. However, it is important to highlight that the number of 12 month samples

available was limited at the time of analysis and further sample collection is ongoing. In addition, while this does not include the ECP patients who were withdrawn from treatment, thus subjecting results to potential attrition bias, it does include the non-responding ECP patients.

Confounding variables which affect the rate of T-lymphocyte reconstitution must be considered, in particular the use of serotherapy[164]. All patients in control group 2 received serotherapy, however, 6/7 (85.7%) in control group 1 also received serotherapy, as did the majority (62.5%) of the ECP patients. Age-related thymic involution and the impact of age on thymic recovery is another important variable impacting T-lymphocyte recovery. The median age in control group 1 was the highest (7.3 years), and median ages were similar in control group 2 and the ECP group (6.6 and 5.8 years respectively). Thymic regeneration post-HSCT is slower and the degree of T-lymphocyte recovery is less with increasing age[124], but even older adult patients with the lowest numbers of $CD4^+$ T-lymphocytes experience a modest increase in the frequency of naïve T-lymphocytes and TRECs, and among individuals <25 years of age, thymic damage secondary to aGVHD is suggested to be fully reversible[77], suggesting that paediatric patients should have the capacity for thymic recovery and development of a robust adaptive T-lymphocyte compartment post-HSCT.

IL-22 is integral in protecting and maintaining epithelial tissues, and stimulating recovery following injury. Patient 5 displayed particularly high levels of IL-22. This patient, with stage 3 GIT aGVHD, had low IL-22 levels early in ECP treatment which may reflect loss of IL-22-producing ILCs in the gut, and with treatment progression and improvement of aGVHD, regeneration of these cells and restored IL-22 production may account for the elevated IL-22 levels evident 7 weeks after commencing ECP, promoting GIT epithelial repair. A similar scenario could be reflected in the thymus; the rise in IL-22 also occurs in parallel with increasing RTEs, until the end of treatment when IL-22 levels fall. This could reflect thymic repair due to beneficial thymic ILC production of IL-22, and when repair is complete and thymic output is restored, IL-22 is redundant and levels return to baseline. A similar rise in IL-22 in parallel with increasing RTEs, followed by a decline at the end of ECP, was observed in several patients, further supporting this. However, one of the caveats of measuring cytokines in the serum is that results do not identify what tissue cytokine levels are, or what tissues they are produced from.

IL-7 plays a critical role in promoting expansion of IL-22-producing immune cells[147], and the peak in IL-7 levels prior to the surge in IL-22 seen in the majority of patients supports this, although the functional role IL-7 has in IL-22 production is not known. In patient 1, the second peak in IL-22 occurred in parallel with the isolated peak of IL-7, followed by a decline of both cytokines, suggesting failure to maintain production, potentially due to irreparable thymic damage and poor immune reconstitution of IL-22-producing immune cells.

The pathological or protective impact of IL-22 is thought to be related to whether it is of donor or recipient origin. Although thought to be able to withstand conditioning, recipient IL-22-producing ILCs are eliminated in aGVHD, at least in the GIT[155]. This would imply that elevated IL-22 early in ECP treatment (and thus at the peak of the aGVHD course) is donor-derived and pro-inflammatory, although not all patients with aGVHD exhibit this pattern. The later rise in IL-22 may then represent the restoration of beneficial recipient-derived IL-22.The timing of when, and for how long, donor-derived IL-22 is predominant post-HSCT, and the timing of recovery of recipient IL-22-producing cells, is not known, and it can be argued that recovery of recipient IL-22 producing cells is not possible as they will be replaced by donor cells as long as chimerism is 100% donor. The pathological or protective effect of IL-22 may be more dependent on the inflammatory environment specific to that individual, as well as influenced by levels of other circulating cytokines, and individual patients with different patterns of IL-22 may reflect the diversity of other cytokine patterns present. It is also important to note that the true paediatric normal range of IL-22 is not known, and that while regular time points throughout ECP treatment were measured, IL-22 levels in between these time points is unknown.

If thymic damage is present, one could assume that, once the offending factors are reduced or removed, there is a minimum duration required for the processes of TEC regeneration and normal thymocyte development to occur before new T-lymphocytes enter the circulation. An important question is whether a delay in removing/reducing the offending factor(s) prolongs the aforementioned time interval or impairs the overall outcome, and conversely whether early removal shortens the time interval. Results from patients 1 and 8 support this hypothesis; both have ongoing disease and exposure to corticosteroids and display negligible thymic output. Patients who displayed successful thymic recovery had faster resolution of disease and weaning of immune suppression. The true short- and long-term effects of corticosteroids on human thymic function are

not known. Corticosteroids have a large amount of historic clinical experience, are convenient and accessible, and relatively inexpensive compared to new, more expensive therapies such as ECP, but perhaps the potential long-term detrimental effects of corticosteroids on the immune system and post-HSCT immune reconstitution are underestimated. The data presented here support earlier initiation of ECP therapy following non-response to first line therapy for aGVHD and a careful but aggressive approach to weaning corticosteroids and other immunosuppressive agents with disease resolution.

It is critical to further understand the regenerative potential of the thymus and if multiple compounding malefactors together can inflict overwhelming thymic damage to a point of 'no return', where the degree of damage is so great that the intrinsic ability of the thymus to recover is inhibited, independent of age-related changes, resulting in a thymus that is "burnt out" with persistent loss of thymic function, even in young patients. Although there are tools to measure thymic output, there are currently no tools available to measure the presence and degree of severity of thymic damage. This highlights the need for a biomarker that enables us to diagnose thymic damage, to monitor and assess the response to treatment, with the aim of avoiding irreparable damage. A biomarker would be useful to guide individualised treatment, providing information about when to avoid treatments known to exacerbate thymic damage.

Further therapies that protect and enhance thymic function are needed. Several clinical trials have been established examining the potential of exogenous growth factors, hormones and cytokines in this regard. One phase IIa study (NCT02406651) is examining the effect of recombinant IL-22 in combination with corticosteroids in the treatment of grade II-IV GIT aGVHD, and another clinical trial (NCT01746849) is evaluating if KGF plus luteinizing hormone-releasing hormone (LHRH)-agonist treatment promote immune recovery post allo-HSCT. These are important exciting steps forward, but further studies are required to examine if patients with evidence of profound or irreversible damage will benefit from these exogenous therapies. Patients with severe or apparently irreversible thymic damage may benefit from a combination of such therapies, or development of novel strategies such as thymic transplantation or T-cell progenitor therapy.

3.7 Conclusion

The endogenous capacity of the thymus to regenerate and recovery of thymic function following insult is crucial, although the mechanisms by which this occurs are not fully understood. Protection of the thymus from damage, preservation of thymic function and conservation of this regenerative potential is paramount to achieve the best clinical outcome for patients undergoing HSCT, especially as more HSCTs are performed on older patients with lower residual thymic function. This requires further understanding of the effects current therapies have on the thymus, which may allow us to make better 'thymic-protective' therapeutic decisions and initiation of less 'thymic-toxic' management strategies. Experimental treatments such as IL-22, Flt3 and KGF show promise in terms of promoting thymic regeneration and boosting thymic function and continued development of these strategies is necessary. Further understanding of the mechanisms driving thymic damage and repair in aGVHD, is needed to allow exploitation of these pathways and potential development of new therapeutic strategies.

ECP presents itself as a potential strategy that can indirectly promote both thymic function and protection; restoration of thymic function by specific targeting of alloreactive T-lymphocytes and reducing aGVHD activity and protection from further damage inflicted by other immunosuppressive agents by facilitating their withdrawal. These data demonstrate that ECP can facilitate thymic recovery in some patients. Importantly, thymic recovery was not witnessed for all patients, raising the question of whether there is a "thymic threshold", a limit to the degree of damage that be inflicted before it becomes irreversible and the endogenous capacity to regenerate is lost. Identification of patients before this point is reached is crucial, highlighting the need to identify a biomarker to diagnose thymic damage, monitor for its' development in the transplant setting and assess response to treatment. A biomarker would be of unquestionable benefit to help individualise treatment regimens; earlier awareness of the presence of thymic damage or thymic aGVHD would allow for intervention before damage becomes extensive and irreversible, such as earlier initiation of ECP or perhaps even prophylactic use of ECP in high-risk patients.

The importance of studying T-lymphocyte regeneration in the post-HSCT period is demonstrated in this study. Whilst it is known that the rate of thymic-dependent T-lymphocyte recovery decreases with increasing age, it should not be assumed that in paediatric patients recovery of the T-lymphocyte compartment is unequivocal and

further understanding of the limits of thymic regenerative capacity at different ages is needed. Clearer elucidation of thymic reparative pathways is also needed and would greatly assist in the development of new innovative therapeutic strategies to optimise immune recovery and minimise the vulnerable period of immune depletion where the risk of opportunistic infections and relapse is high. A multifaceted approach utilising a combination of strategies that can protect, restore and maximise thymic function could augment immune recovery, for example, a synergistic combination of ECP with other non-immunosuppressive strategies, such as administration of IL-7 targeting the developing thymocytes or IL-22 targeting the thymic epithelium. Adopting an approach that promotes an immune tolerant environment post-HSCT may be more conducive to robust T-lymphocyte reconstitution compared to an immune suppressed environment and ideally, identification of patients who would benefit most from this approach and tailor their treatment accordingly.

Chapter 4 . Mechanisms of Action underlying ECP

4.1 Background

Although the mechanisms behind ECP have not yet been fully elucidated, it is believed to act in an immunomodulatory fashion with potential to treat pathogenically contrasting diseases; upregulation of the immune-stimulatory response to treat CTCL and promotion of immune tolerance in acute and chronic GVHD. Existing evidence of ECP-induced immune tolerance in the setting of aGVHD suggests involvement of DCs and Treg populations, and alteration of cytokine patterns.

4.1.1 Apoptosis and an antigen-specific response

Exposure of mononuclear cells to 8-MOP/UVA within the ECP circuit results in the formation of covalent bonds with pyrimidine bases and subsequent cross-linking of DNA. This DNA damage induces apoptosis of the exposed cells, with the activated T-lymphocytes affected preferentially[22,165,166]. Apoptosis occurs several hours after ECP and peaks on day 3[167], possibly due to increased Fas-mediated pro-apoptotic signalling[168]. However, only 5-10% of the total lymphocytes are harvested and exposed during the procedure. This is an insufficient number to entirely account for the effects of ECP being mediated through destruction of GVHD-causing T-lymphocytes, particularly as the majority of activated T-lymphocytes reside in the tissues rather than the blood, and it is speculated that the ECP-exposed cells have indirect immunomodulatory actions on other non-exposed immunocompetent cells following reinfusion.

Phagocytosis of the apoptotic cell fragments leads to an immune response specifically directed against the activated T-lymphocytes. Ben-Nun *et al* demonstrated in murine models with experimental autoimmune encephalomyelitis that reinfusion of treated cells elicited an immune response specifically targeting the same untreated pathogenic T-lymphocyte clone, but sparing other non-disease causing clones[169]. This was the first evidence supporting the "Vaccination Theory". In the setting of CTCL, it is thought that phagocytosis of the apoptotic cells provokes a cytotoxic clonal response against the malignant T-lymphocytes. More recently, ECP demonstrated clinical effectiveness in the treatment of autoimmune disorders, transplant rejection and GVHD leading to the idea that ECP was also able to promote immune tolerance. This unveiled the exciting new prospect of a therapy that could be utilised in disorders caused by aberrant T-lymphocytes that was able to discriminate between pathogenic and non-pathogenic clones, thus avoiding the undesirable side effects associated with generalised T-

lymphocyte elimination such as increased frequency of severe infections or disease relapse. What it is that induces activation of the appropriate immune pathway resulting in these opposing outcomes is not clear, but is suspected to involve APCs.

The specificity characteristic of the immune response is thought to be because activated T-lymphocytes are particularly susceptible to the pro-apoptotic effects of ECP, undergo apoptosis faster than other cells, and thus are phagocytosed preferentially by the APCs[170]. In a contact hypersensitivity (CHS) murine model, ECP-treated leukocytes inhibited the CHS immune response in an antigen-specific manner which did not occur if they received untreated leukocytes or if leukocytes were treated with 8-MOP or UVA radiation alone. This inhibition was mediated by $CD4^+CD25^+$ T-lymphocytes, and also depended on the presence of $CD11c^+$ cells, supporting the role of DCs and Tregs as key players in the underlying process[171].

4.1.2 Dendritic cells

4.1.2.1 Origin and classification

DCs form the intricate link between the innate and adaptive immune systems. Termed professional APCs based on their primary ability to internalise, process and present antigen to T-lymphocytes, resulting in activation and directing T-lymphocyte polarisation. DCs also play a key role in maintaining immune homeostasis, with involvement in both central and peripheral tolerance, disruption of which can result in autoimmune and inflammatory disorders or tumour development[172].

Following their initial identification by Ralph Steinman in 1973[173], DCs have been challenging to classify due to their considerable heterogeneity and overlapping characteristics, and different studies have used various nomenclatures complicating interpretation. DCs can originate from bone marrow-derived haematopoietic stem cells or can be generated from monocytes in inflammatory conditions. Guilliams *et al* proposed a classification in 2014 based on ontogeny, and broadly classifies DCs into three main subsets; conventional (or myeloid) DCs (cDCs) 1 and 2 and plasmacytoid DCs (pDCs)[174]. DCs can be identified as the lineage $(CD3/CD19/CD20)^-$ $HLA\text{-}DR^+$ population in mononuclear cells. Conventional DCs typically express CD11c but can be distinguished from monocytes by the lack of CD14 and CD16[175], and are divided into $CD1c^+$ and $CD141^+$ subsets. Conventional DC1s, characterised by high expression of CD141, secrete TNFα, CXCL10 and IFNγ, are efficient at cross presentation of antigen to

$CD8^+$ T-lymphocytes and elicit a Th1 response[175]. The cDC2 subset express CD1c, are more numerous in the circulation compared to cDC1s, produce high levels of IL-12 and are more efficient at presenting antigen to naïve T-lymphocytes, promoting Th1 and Th17 responses[175]. Conventional DC2s have been shown to also be efficient at cross presentation of antigen and priming CD8+ cytotoxic responses[176].

Plasmacytoid DCs, the most numerous DC population in the blood, are characterised by expression of CD123, CD303 and CD304 surface markers, display high levels of the pattern-recognition receptors TLR7 and TLR9 and are potent producers of type 1 interferons, important in viral immunity[175]. Plasmacytoid DCs have a lower capacity to capture antigen and stimulate naïve T-lymphocytes compared to cDCs[177], and can direct a Th1 or Th2 response; contact with invading viruses leads to IFNα production by pDCs, stimulating virus-specific Th1 effector cell activation, whereas absence of IFNα leads to Th2 polarisation and attenuation of an ongoing Th1 immune response[178,179]. The plasticity in pDC function is also likely influenced by both the activation state of the pDC and by factors from the surrounding microenvironment. It is suggested that cDCs play a more prominent role in antigen capture and initiation of effector immune responses, and pDCs are more involved in antiviral immunity and maintaining peripheral tolerance to self-antigens.

In the steady state, DCs mainly reside in the peripheral tissues where they sample the microenvironment for antigens. Uptake of pathogenic antigen triggers DC maturation by upregulation of MHC II, adhesion molecules, co-stimulatory molecules (CD40, CD80, CD83 and CD86) and trafficking receptors such as CCR7, increased secretion of pro-inflammatory cytokines such as IL-12, followed by migration to secondary lymphoid tissues where they activate antigen-specific T-lymphocytes, generating an effector immune response.

4.1.3 Tolerogenic dendritic cells

As well as being potent inducers of immune stimulation, DCs display important regulatory or tolerogenic functions. DCs participate in central tolerance by their involvement in negative selection of developing T-lymphocytes in the thymus[180,181]. As part of maintaining peripheral tolerance in the steady state, DCs regularly sample self-antigens to detect and subsequently delete auto-reactive T-lymphocytes, as well as regulate tolerance to non-pathogenic environmental antigens and commensal

microbiota[182]. Engulfment of autologous apoptotic cells resulting from normal cell turnover by tolerogenic DCs (tol-DCs) is thought to be an important physiological mechanism of self-tolerance and prevention of autoimmunity[183].

The stage of activation and maturation is a critical determinant of the immune stimulatory or immune regulatory capacity of DCs, with regulatory DCs exhibiting a semi-mature state, endowed with high phagocytic ability and reduced MHC II and co-stimulatory molecule expression[184]. Insufficient co-stimulatory molecule help in the immunological synapse can result in deletion or anergy of the corresponding T-lymphocytes. The maturation state of DCs appears to be modulated by the nature of antigen it is exposed to and whether or not it conveys a 'danger signal', the surrounding microenvironment and by particular subsets of cells such as Tregs[185,186]. For example, in the presence of infection, internalisation of microbial components stimulates DC maturation with upregulation of the immune response and subsequent inflammation. In contrast, in the presence of autologous antigens, these signals are not transmitted leading to down-regulation of the immune response or tolerance.

The ability of DCs to adopt a particular state of activation when inducing tolerance compared to when inducing an effector immune response against invading pathogens reflects the innate plasticity and multi-functional capacity characteristic of DCs, with involvement of the NFκB and p38SAPK signalling pathways in regulating the maturation process[187,188]. In essence, depending on the response and degree of maturation of DCs following their interaction with antigen, they can stimulate inflammation or induce tolerance, and up or down regulate the immune response in an antigen-specific manner. However, tol-DCs are not confined to a semi-mature phenotype and maturation can be induced upon exposure to inflammatory signals. This instability presents a barrier to exploit DC tolerogenic properties in terms of therapeutic application. There is also evidence that fully mature DCs can exert regulatory effects leading to suggestions that the functional capacity of DCs may be more dependent on exogenous signals it receives such as TGFβ and IL-10[172].

Tol-DCs display multiple inhibitory pathways to mediate peripheral tolerance including stimulation of Treg differentiation, reduced secretion of pro-inflammatory cytokines, in particular IL-12, increased production of anti-inflammatory cytokines such as IL-10 and TGFβ, expression of membrane receptors that inhibit T-lymphocyte activation such as ILT-4 and PD-L1/2, and stimulation of indoleamine-2,3-deoxygenase (IDO) activity, an

enzyme involved in breakdown of tryptophan, particularly by pDCs[184,189,190]. Depletion of tryptophan and accumulation of its metabolites has been shown to cause T-lymphocyte apoptosis, prevent T-lymphocyte proliferation and stimulate Treg expansion[172,191]. IL-10 is indispensable in immune down-regulation and induction of tolerance, and prevents DC maturation by down-regulation of MHC II and co-stimulatory molecule expression[184]. DCs cultured with IL-10 also exhibit increased expression of inhibitory proteins such as ILT2 and ILT4, and stimulate further DC production of IL-10, creating a positive feedback loop[184].

Plasmacytoid DCs have been identified as a particularly important regulatory subset[178,192], promoting T-lymphocyte anergy and production of IL-10-producing Tr1 Tregs through the expression of inducible co-stimulator ligand (ICOS-L)[193-195].The chemokine receptor CCR9 has been identified as a marker of this tolerogenic immature pDC subset, most populous in secondary lymphoid tissues, with tolerance-inducing effects that include suppression of aGVHD in animal models[192]. Plasmacytoid DCs have also been shown to assume an important role in allo-HSCT, facilitating engraftment and mediating GVL activity and aGVHD tolerance[196]. Precursor pDCs have been identified as the predominant population in $CD8^+/TCR^-$ 'facilitating cells' (FC) which enhance engraftment of allogeneic HSCs and induce tolerance to donor-specific skin allografts[197]. This heterogeneous FC population consists of numerous cell subpopulations, with DCs being the major component, which in turn is composed primarily of pre-pDCs ($CD11c^{dim}/B220^+/CD11b^-$), although the optimal beneficial effect exerted by pre-pDCs is witnessed only when the other FC components are present, suggesting interaction with other cells is necessary. The therapeutic potential of DCs in the context of HSCT and protection against GVHD has become a recent focus of research as the ability of DCs to induce donor T-lymphocyte tolerance to self-antigen is increasingly recognised.

4.1.4 Dendritic cells and HSCT/acute GVHD

Circulating cDCs and pDCs, although detectable as early as 7-14 days post-HSCT, are reduced in number following allo-HSCT but progressively increase, reaching normal levels by a year post-HSCT, although the rate of DC reconstitution in the peripheral lymphoid tissues is unknown. Reported kinetics of cDC and pDC recovery are mixed, with some studies demonstrating faster reconstitution by cDCs[198] and other showing no difference in the early post-HSCT period[199,200]. Recovery has shown to be faster in RIC

compared to MA conditioning[201], but appears to be unaffected by serotherapy[202]. Impaired DC reconstitution is associated with increased TRM and risk of relapse, and poorer overall survival[203].

In the context of aGVHD, host DCs play a pivotal role in priming allo-reactive T-lymphocyte responses, as demonstrated by a murine aGVHD model in which depletion of host APCs prevented aGVHD occurrence[204], although not by depletion of host DCs alone[205]. Acute GVHD impairs DC recovery; patients with aGVHD have lower numbers of cDCs and pDCs (especially of pDCs) post-HSCT compared to patients without aGVHD, although the specific effects on DCs are not known[206,207]. Corticosteroids are known to have a DC depleting effect[208] and down-regulate expression of co-stimulatory molecules[209,210], but patients with aGVHD have reduced numbers of DC subsets prior to the onset of clinical symptoms suggesting an independent adverse impact. Other agents reported to affect DC recovery and/or function include G-CSF, rapamycin and MMF[206]. Pre-clinical and clinical studies have demonstrated a role for pDCs in mediating protection from aGVHD, in terms of both the donor graft pDC content and post-HSCT reconstitution[211,212], and low numbers of pDCs early after HSCT have shown to be predictive for aGVHD severity[200,203,213]. However, while *in vitro* studies have supported the tolerogenic effects of pDCs on the allogeneic T-lymphocyte response, the exact effects *in vivo* are still uncertain[178].

Horvath *et al* found that pDCs and cDCs in patients post-HSCT expressed higher levels of co-stimulatory molecules initially, but normalised to the levels of expression seen in healthy controls by day +100, with no significant difference seen in expression between patients with and without aGVHD[207]. Little is known about DC function post-HSCT, although one study demonstrated reduced *ex vivo* type 1 IFN production by pDCs upon HSV-1 stimulation suggesting dysfunction in anti-viral responses[214].

4.1.5 ECP and the effect on dendritic cells

Monocytes also undergo apoptosis, but more slowly than lymphocytes following ECP[171]. Studies have demonstrated that ECP promotes differentiation of exposed monocytes into functional DCs without the need of added high concentrations of stimulating cytokines or growth factors, and occurs independently of the disease setting[215]. This process is stimulated by the physiological interaction of monocytes with adherent platelets during passage through the ECP chamber[215,216]. Hannani *et al* demonstrated

that although by day 6 post-ECP 80% of monocytes were apoptotic, their functional abilities such as T-lymphocyte stimulation, differentiation into DCs and endocytosis were preserved, but there was impairment of migratory capacities[217]. As the majority of DCs typically reside in the tissues, this monocyte-DC differentiation potentially introduces a much larger number of DCs into the circulation than is normally seen, thus increasing the antigen-processing and presenting capacity. Following ECP, apoptotic cells are localised primarily in the liver and spleen, regions rich with APCs, in particular DCs, but how this migration of apoptotic cells occurs is not clear[218].

Phagocytosis of ECP-exposed apoptotic peptide fragments results in DCs acquiring an immature tolerogenic state, characterised by down-regulation of maturation markers and co-stimulatory molecules such CD40, CD80, CD83 and CD86 and increased secretion of anti-inflammatory cytokines such as TGFβ and IL-10, resulting in enhanced phagocytic activity but a reduced ability to stimulate an effector T-lymphocyte immune response[166,219-224]. Monocyte-derived immature DCs also show upregulated expression of the glucocorticoid-induced leucine zipper (GILZ) gene, a marker of tol-DCs, following ECP exposure[225]. GILZ encodes an intracellular protein that transduces various immunosuppressive signals, including inhibition of NFκB. Upon interaction with T-lymphocytes, tol-DCs can induce anergy or apoptosis, or stimulate the production of Tregs, but the predominant tolerance-inducing mechanism in ECP is unknown. DCs are not confined to this immature state and can respond to inflammatory signals such as lipopolysaccharide (LPS) resulting in full maturation[166]. Binding of apoptotic cells to DCs via TAM receptors has been demonstrated to reduce production of pro-inflammatory cytokines such as TNFα and IL-6[226]. ECP treatment of GVHD (both acute and chronic) has shown to increase the pDC population and the pDC/cDC ratio, whereas the reverse trend was observed in CTCL, which may shift towards a Th2/Treg environment[227-229]. A switch from a predominant cDC to pDC population in cGVHD was also suggested by Gorgun *et al*[224].

The timing of the ECP effect on DC phenotype *in vivo* is unknown and results from *in vitro* studies are variable. Berger *et al* demonstrated that ECP-induced DCs from CTCL, GVHD and normal subjects exhibited a similar maturation phenotype following 18 hours *in vitro* incubation. It may take longer than 18 hours for DCs to process the apoptotic antigens leading to subsequent phenotype alteration; Di Renzo *et al* found *in vitro* reduced DC expression of co-stimulatory molecules on day 9 of ECP treatment[220], and Lamioni *et al* showed evidence of phagocytosis of ECP-treated mononuclear cells after

24 hours of co-culture and demonstrated a tol-DC phenotype post ECP in paediatric patients treated for transplant rejection, although this study did not stipulate at what stage in the course of ECP treatment this occurred[222]. In contrast, Holtick *et al* demonstrated transient and partial maturation of *in vitro* monocyte-derived DCs (mo-DCs) 4 and 8 hours after PUVA exposure, but not after 24 hours[227]. This occurred independent of exposure to apoptotic T-lymphocytes, suggesting that either apoptotic T-lymphocytes do not play a role in modulating the DC phenotype, or that PUVA induces initial partial maturation, but more long-lasting effects on the DC phenotype and the subsequent fate may depend upon additional contact with the apoptotic T-lymphocytes; malignant clonal T-lymphocytes causing CTCL stimulating DC maturation and upregulation of the immune response, or alloreactive T-lymphocytes causing aGVHD stimulating ongoing partial maturation and promotion of tolerance. If the ECP-exposed monocytes undergo apoptosis as suggested by several studies[222,227] they may also influence the phenotype of other non-exposed DCs[221]. Whilst *in vitro* experiments have helped enormously in gaining insight to the potential underlying mechanisms of ECP, their reflection of the true *in vivo* ECP cellular effects remains obscure and little work has been done directly examining the *in vivo* effects of ECP on DCs or the differential expression of the co-stimulatory markers by pDCs and cDCs.

In aGVHD, DCs, as the predominant APC, are culpable for presenting disparate host antigens to the donor T-lymphocytes resulting in activation and propagating the pathway of cellular injury. Inducing a DC tolerogenic state and dampening their ability to activate T-lymphocytes could theoretically lead to assimilation of the trigger of the aGVHD pathophysiological process. Indeed, prophylactic use of ECP before conditioning in murine models demonstrated that an ECP-induced tolerogenic environment improved survival by preventing the initiation of aGVHD. This immune tolerance occurred due to reduced DC activation, as well as increased $CTLA4^{+}$Tregs[230]. Modulation of DCs, in terms of both number and function, appears to be a central process behind the mechanism of ECP, but further investigation of the *in vivo* effects in humans is required.

4.1.6 Regulatory T-lymphocytes

4.1.6.1 Origin and classification

Tregs are another key cellular component of immune tolerance regulation, shown to play an essential role in maintaining immune homeostasis, limiting chronic

inflammation and preventing autoimmunity. Tregs are a subset of $CD4^+$ T-lymphocytes with high expression of IL-2 receptor (CD25) and can be divided into two main types based on their origin; natural and inducible. Natural Tregs (nTregs) are produced within the thymus whereas inducible Tregs (iTregs) develop from naïve T-lymphocyte in the periphery upon antigen exposure, with TGFβ and retinoic acid potentially involved in this process[231]. How Tregs are generated in the thymus is not clearly understood. It is thought that they display a higher than normal signal strength upon interaction with the MHC/self-peptide complex at the negative selection stage, but it is unknown why this leads to subsequent redirection to a distinct Treg lineage and not apoptosis. There is increasing evidence that AIRE expression plays a role in this process, directing differentiation of autoreactive T-lymphocytes into Forkhead box p3 ($FOXP3^+$) Tregs[232]. FOXP3 is a transcription factor that plays a critical role in both Treg development and suppressive function, and is currently the most reliable marker used to detect Tregs in the circulation[231]. Mutations in the *FOXP3* gene in humans cause the rare condition IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), characterised by markedly reduced or absent FOXP3-expressing Tregs[233]. However, FOXP3 expression can also be induced upon T-lymphocyte activation without displaying immunosuppressive properties negating it as a truly specific marker, although expression is transient and at a lower level compared to stable $FOXP3^+$ Tregs[234]. An alternative means to identify circulating Tregs is by selecting the $CD4^+CD25^{hi}CD127^{lo}$ compartment which expresses a high level of FOXP3 and has potent suppressive activity[235].

Discrimination between nTregs and iTregs is essential to understand fully their specific functions in regulating immune homeostasis as well as their role in different disease states. Experimental models suggest that nTregs are sentinels of systemic and tissue-specific autoimmunity, preventing inappropriate immune responses to self antigens, whereas iTregs control inflammation at mucosal surfaces and impact gut microbiota, controlling immune responses to harmless non-self antigens[236]. Expression of Helios (a zinc finger transcription factor and member of the Ikaros transcription factor family) has been used as a marker of nTregs, although this has been challenged following the demonstration of induction of Helios expression both *in vitro* and *in vivo*[237-239]. Furthermore, Helios negative cells can be identified within the naïve $CD45RA^+CD31^+CD62L^+CCR7^+FOXP3^+$ Treg population[240]. Whilst Helios positive and negative subsets of Tregs are recognised, its' specificity for thymic origin is disputed, but

may represent a Treg subset with superior suppressive activity[241]. Neuropilin-1(Nrp-1, CD304) is a surface maker important in Treg interaction with DCs and is also thought to play a role in mediating Treg migration[242]. It has been suggested as a potential nTreg marker from murine studies[243,244] but its' applicability as a marker of origin in humans has been contested[245,246]. At this time, a specific lineage marker for Tregs remains to be identified and results from Treg murine studies appear to not be consistently translatable to humans. Helios is the strongest marker at present as an identifier of thymic origin, but results should be interpreted with caution.

The immune regulating mechanisms of Tregs are multifarious and include effects on DCs (modulation of DCs required for effector T-lymphocyte activation, CTLA4, LAG3), production of inhibitory cytokines (IL10, TGFβ, IL-35), cytolytic effects (secretion of granzymes and perforin, TRAIL-DR5 pathway) and metabolic disruption (IL-2 depletion, release of adenosine nucleosides via CD39 and CD73, inhibition of IL-6)[247]. TGFβ is a key player both in Treg differentiation and function. It is part of a complex composed of latent-TGF-β-binding protein (LTBP), latency-associated peptide (LAP) and TGFβ. LAP regulates TGFβ activity, and is a marker of TGFβ-producing cells, but is also thought to modulate immune responses independently[248]. Glycoprotein A repetitions predominant (GARP) is a cell surface molecule anchoring the TGFβ/LAP latent complex to the Treg surface, and identifies a subset of activated Tregs with potent suppressive function[249]. GARP also has a positive feedback effect on FOXP3 expression and absent GARP expression on $FOXP3^+$ Tregs decreases FOXP3 expression and suppressive capacity. Cytotoxic T-lymphocyte antigen (CTLA4) is expressed on the surface of Tregs and, upon interaction with DCs, can stimulate DC production of IDO which suppresses effector T-lymphocyte function. A reciprocal relationship exists between tol-DCs and Tregs, with tol-DCs driving Treg differentiation and, conversely, Tregs are capable of down-regulating DC maturation and function, maintaining the DC tolerogenic state[250]. In the thymus, DCs have shown to induce Treg production in response to thymic stromal-derived lymphopoietin (TSDL), and pDCs in particular may play an important role in stimulating nTreg production via the CD40-CD40L pathway[251].

4.1.7 Tregs and HSCT/acute GVHD

The normal Treg frequency within the $CD4^+$ T-lymphocyte population ranges from 4-9%, with a slight increase seen with ageing. In a study by Kielsen *et al* involving 29 paediatric patients post-HSCT, the proportion of circulating Tregs was normal by 3 months (median 4.2%), increasing to 7.0% by 6 months, with a higher frequency and

numbers observed in those who developed aGVHD. Treg reconstitution was negatively associated with ATG treatment, and positively associated with higher cell dose and lower age[252]. Corticosteroids have been associated with increased numbers of Tregs[253] although the impact on function is not known. Transfer of Tregs has been performed as a treatment for aGVHD with some success in experimental models. Nguyen *et al* demonstrated that Tregs reduced aGVHD and protected thymic and lymphoid tissue, accelerating T-lymphocyte immune reconstitution with a diverse TCR repertoire and protecting against CMV infection[254]. However, difficulties remain in obtaining a pure population of Tregs due to deficiencies in our knowledge of their characterisation. Their relative scarcity in the peripheral blood means that *ex vivo* expansion is necessary to induce adequate Treg numbers for therapeutic purposes, and there is also a concern regarding maintaining a stable Treg immunoregulatory phenotype in an inflammatory environment and the risk of conversion to effector T-lymphocytes.

4.1.8 ECP and Tregs

The generation of Tregs is thought to be a central immunomodulatory action of ECP. Gatza *et al* demonstrated in aGVHD murine models that ECP-treated splenocytes improved aGVHD and immune reconstitution by reducing the number of non-exposed $CD8^+$ effector lymphocytes, suppressing allogeneic T-lymphocyte proliferation and increasing the number of Tregs. These changes were lost in the presence of anti-CD25 confirming the requirement of Tregs for this process[255]. Although Treg subpopulations were not specifically examined in this study, it was noted that there were an increase in Tregs that expressed $CD62L^+$ and $CD103^+$. Attenuation of aGVHD in murine models by ECP-treated splenocytes was also associated with an increase in Tregs as demonstrated by Capitini *et al*[219]. In the CHS model previously mentioned, tolerance was lost upon depletion of $CD4^+CD25^+$ cells, further supporting a central role played by Tregs[171]. Other studies have suggested a significant role of IL-10-producing Tr1 Tregs in the mechanisms behind ECP[256].

The contribution of Tregs to the mechanisms of ECP has also been investigated in clinical studies. Lamioni *et al* demonstrated increased levels of Tregs with suppressive function in four paediatric patients post heart or lung transplantation[222]. In another study, increased $CD39^+$ Tregs was observed in ECP-treated patients post heart transplantation, but no change in $CD62L^+$, $CD120b^+$ and $CD147^+$ Treg subsets[229]. A study

by Biagi *et al* involving 10 patients (including 4 paediatric patients) with acute and chronic GVHD showed a significant increase in functional Tregs following ECP, which was also accompanied by increased glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR) expression which identifies activated Tregs[257]. A larger study involving 27 patients with acute and chronic GVHD showed a significant increase in Tregs numbers in those who responded to ECP treatment[258]. Schmitt *et al* also demonstrated increased Treg levels post ECP and increased Treg suppressive capacity, but which did not reach the levels seen in healthy controls[259].

4.1.9 Cytokines – Th1 and Th2 subsets

The T-lymphocyte population is divided into $CD4^+$ T helper (Th) lymphocytes and $CD8^+$ cytotoxic T-lymphocytes. $CD4^+$ Th lymphocytes are further divided into subsets depending on the environment they are exposed to following interaction with the corresponding MHC II/APC and upon activation, proliferate and secrete distinct cytokine profiles that will discriminate them as Th1, Th2, Th17 or Th22 lymphocytes eliciting different immune responses.

Th1 and Th2 subsets, their associated cytokine profiles and their different functional properties were first identified by Mosmann *et al* in 1986[260]. Th1 lymphocytes have a signatory pro-inflammatory profile, characterised by production of IL-2, IFNγ and TNF, with an essential protective role in the generation of cell mediated immune responses. However, excessive Th1 responses have been linked to autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis[261] and are implicated in aGVHD. The Th2 subset is characterised by production of IL-4, IL-5, IL-10 and IL-13, plays a key role in the production of IgE and response to parasitic infections, but excessive activity is implicated in allergic diseases. IL-6 is a cytokine produced by a number of different types of cells and has both inflammatory and regenerative effects[262]. IL-6 also plays a role in directing Th polarisation, promoting Th2 and inhibiting Th1 differentiation[263]. Th2 cytokines have been described as anti-inflammatory due to their ability to counteract the Th1 response, but also have been found to be pathogenic in certain autoimmune conditions, potentially by enhancing production of autoantibodies[261,264]. Thus both Th1 and Th2 have vital roles in promoting protection, but excessive response of either pathway can result in detrimental inflammation and tissue damage.

4.1.10 Cytokines and acute GVHD

Chemotherapy and radiation-induced tissue damage during conditioning produces an inflammatory "cytokine storm", characterised by high levels of TNFα, IL-1 and IL-6, further compounded by inflammation associated with co-existing infection or pre-existing organ damage, setting the platform for the initiation of aGVHD. Damage to mucosal barriers, particularly of the GIT, permits translocation of pathogen- and danger -associated molecular patterns, such as LPS, derived from the GI microbiome into the circulation, triggering further TNFα and IL-1 production, intensifying the cytokine storm[265,266] (Figure 4.1).

The cytokine storm of the initial phase is responsible for driving T-lymphocyte differentiation to Th1 predominance in the second phase, with high levels of TNFα and IFNγ associated with earlier onset and more severe disease, particularly of the GIT[266-270]. IL-12 plays a crucial role in driving Th1 polarisation of the second phase. TNFα and IFNγ contribute to tissue damage by upregulation of host MHC molecules, promoting cytotoxic T-lymphocyte maturation and recruitment of monocytes and macrophages[267,271]. Promotion of Th2 activity, particularly IL-10, has been suggested to be protective in the context of aGVHD, with downregulation of Th1 pro-inflammatory cytokines[265,272,273]. However, this is not thought to be a clear-cut paradigm; although Th1 predominance is well established, a complex interface between cytokines appears to exist impacting both the organ involvement and the severity of aGVHD[274], with experimental studies demonstrating Th2 and Th17 involvement in cutaneous and lung pathology, and Th1-mediated GI and hepatic damage[275]. Dysregulation of the Th1/Th2 balance in aGVHD may be more accountable for causing and maintaining tissue damage rather than the effects of specific cytokines.

Therapeutic targeting of the pathogenic cytokine profiles attributed to the damaging effects of aGVHD has achieved some clinical success, but results from experimental studies have not consistently translated into the clinical setting, and their use is limited by the 'off-target' effects, including compromise of the GVL effect, and global immunosuppressive effects[276]. Corticosteroids non-selectively diminish cytokine production, affecting both Th1 and Th2 pathways, although may not affect certain cytokines involved in tissues repair such as TGFβ and platelet-derived growth factor[277]. Corticosteroids impair both cytokine production and actions, and because cytokines exist in a cascading network of positive and negative feedback mechanisms, reduction of a particular cytokine consequently can impact the levels of others.

Further understanding of the disturbed cytokine balance in aGVHD, the role of different Th lymphocyte subsets in different aGVHD-damaged tissues and development of non-immunosuppressive therapies that restore the delicate Th1/Th2 balance would be a beneficial approach to management.

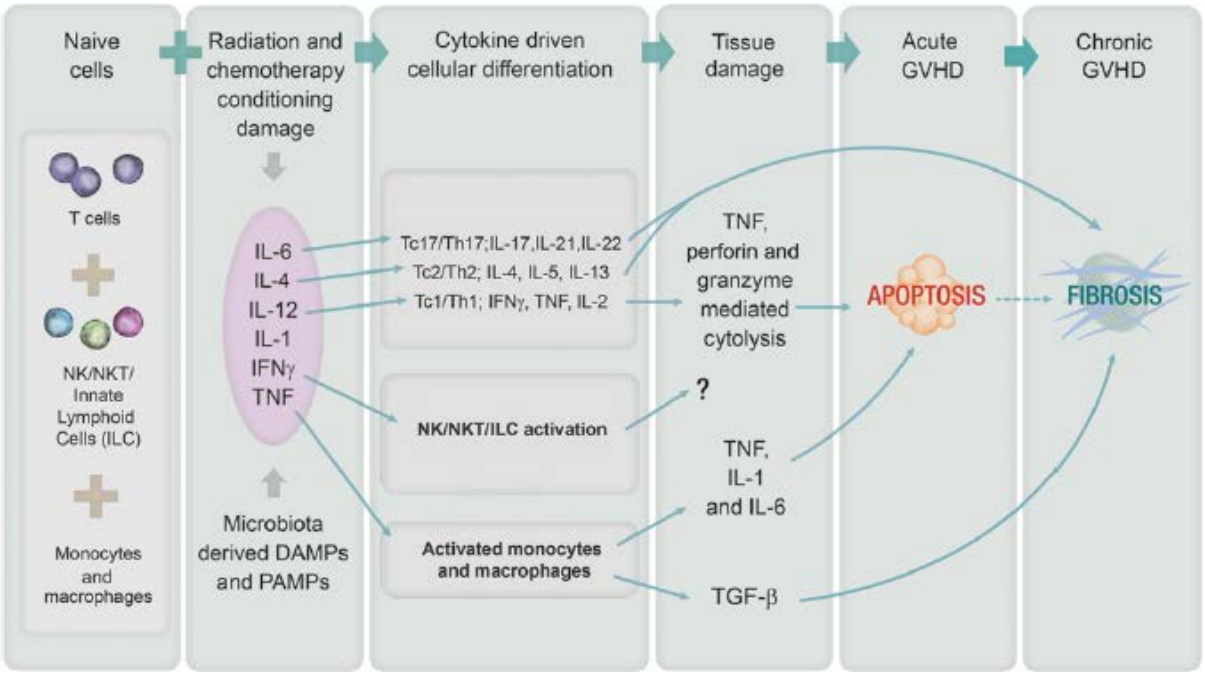


Figure 4.1 The cytokine phases of GVHD, taken from Henden AS *et al* Cytokines in Graft-versus-Host Disease[265].

4.1.11 ECP and the balance of cytokines

Restoration of the Th1/Th2 cytokine balance is suggested to be a key mechanism behind ECP; phagocytosis of ECP-exposed apoptotic cells by APCs leading to increased anti-inflammatory cytokines (IL-10, TGFβ) and decreased pro-inflammatory cytokines (IFNγ, TNFα, IL-1 and IL-12), with necrotic cells having opposing effects on the cytokine response[278-280]. The important role of IL-10 in particular has been observed in several experimental animal aGVHD models investigating the therapeutic effect of ECP[219,230]. An ECP-induced shift from a Th1 to Th2 cytokine profile was supported by *in vitro* and *ex vivo* studies utilising ECP models and patient cells[220,223-225,281,282], possibly related to an effect on arginine metabolism[283]. However, these studies vary in the patient population examined (cGVHD and aGVHD patients, healthy volunteers), and methods, such as measurement of cytokines using different types of ECP-exposed cells. Holtick *et al*, using an *in vitro* ECP-model, demonstrated that the Th1 to Th2 shift is at least partly due to the effects of ECP on mo-DCs, with not only a deviation towards Th2 cytokine polarisation, but also decreased expression of Th1-associated chemokine receptors CCR5/CXCR3 and increased Th2 receptors CCR4/CCR10 when ECP-treated mo-DCs were incubated with allogeneic naïve T-lymphocytes.

The Th1 to Th2 shift has been previously quantified using a ratio of Th1/Th2 cytokines, or, more specifically, using the ratio of IFNγ to IL-4 as a surrogate marker[229,284,285]. While the effects of apoptotic cells on cytokine patterns is well studied, evidence of ECP-induced alterations in cytokine patterns *in vivo* is lacking, particularly in the context of aGVHD, and the validity of the Th1 to Th2 shift remains to be proven.

4.1.12 Conclusion

In summary, although not yet fully deciphered, the mechanisms behind ECP are multifaceted, with effects on different components of the immune system, culminating in an antigen-specific immune tolerogenic response in the setting of aGVHD. Key changes include preferential apoptosis of activated T-lymphocytes, modulation of APCs, in particular DCs, with promotion of a tol-DC phenotype, increased generation of Tregs and alteration of cytokine profiles with increased production of anti-inflammatory cytokines and reduced pro-inflammatory cytokine levels. Many of the studies exploring the potential mechanisms incorporate *in vitro* ECP models or animal models, and much remains to be elucidated regarding the *in vivo* effects of ECP. It is not known whether the effects of ECP are due to a single predominant mechanism, or multiple mechanisms contributing together to culminate in an overall effect.

One of the factors precluding more widespread application of ECP as a therapeutic option for aGVHD, and other conditions, is lack of a full understanding of the underlying mechanisms of action. The development of pathogen-specific therapies is highly desirable, rather than depending on systemic immunosuppressive agents, and further understanding of the antigen-specific tolerance achieved by ECP not only could potentially broaden the therapeutic and prophylactic use of ECP in the HSCT setting, but also introduce novel concepts of how tolerance can be achieved, and extension of this knowledge to other realms of medicine, such as chronic inflammation or malignancy.

4.2 Aims

Dendritic cells:

1. To examine *in vivo* DC subset patterns (absolute numbers of cDC1, cDC2 and pDCs) in paediatric patients with aGVHD receiving ECP over the course of treatment.
2. To compare the DC subset patterns of paediatric patients treated with ECP with paediatric patients post-HSCT with no aGVHD and those with aGVHD not treated with ECP at 4, 8 and 12 months post-HSCT.
3. To examine the phenotype of circulating cDCs and pDCs; specifically expression of CD80, CD86, CD83, and CCR9 over the course of ECP treatment.

Regulatory T-lymphocytes:

1. To examine the pattern of Tregs in paediatric patients receiving ECP treatment for aGVHD (absolute counts and frequencies of $CD25^{hi}CD127^{lo}CD4^{+}$ T-lymphocytes) over the course of treatment.
2. To compare the Treg patterns of paediatric patients treated with ECP with paediatric patients post-HSCT with no aGVHD and those with aGVHD not treated with ECP at 4, 8 and 12 months post-HSCT.
3. To examine the frequency of $FOXP3^{+}$ Tregs in paediatric patients receiving ECP for aGVHD and if expression of Helios changes with treatment progression.

Th1 and Th2 cytokines:

1. To investigate the pattern of Th1 and Th2 cytokines in paediatric patients receiving ECP treatment for aGVHD over the course of treatment.
2. To compare Th1 and Th2 cytokine patterns of paediatric patients treated with ECP with paediatric patients post-HSCT with no aGVHD and those with aGVHD not treated with ECP at 4, 8 and 12 months post-HSCT.

4.3 Hypothesis

1. ECP increases the number of circulating DCs and promotes a predominant tolerogenic phenotype with reduced expression of co-stimulatory molecules.

2. ECP increases the number and frequency of circulating Tregs with progression of treatment.

    a. As thymic function improves, the number of Helios positive Tregs increases reflecting recovery of central tolerance.

3. ECP induces a shift in the cytokine profile over time from a Th1 dominant environment, with high levels of IFNγ, TNF, IL-1 and IL-2, to a Th2 dominant environment with increasing levels of IL-4, IL-5, IL-6 and IL-10.

4.4 Methods and materials

4.4.1 Trucount analysis

For DC absolute count analysis, 200µL whole blood was stained with an antibody-fluorochrome conjugate panel (Table 4.1) in Trucount® tubes (BD Biosciences) containing a known number of microbeads and incubated for 40 minutes at room temperature in the dark before adding 900µL of red blood cell lysis buffer. Data were collected using the Fortessa X20 flow cytometer (BD Bioscience), with a minimum of 50,000 events recorded and analysed using FlowJo® software (BD Biosciences) (gating strategy shown in Figure 4.2). Conventional DCs were identified as $CD3^{-}CD19^{-}CD20^{-}CD34^{-}CD14^{-}CD16^{-}DR^{+}CD11c^{+}$, and divided into the cDC1 subset ($CD141^{+}$) and cDC2 subset ($CD1c^{+}$). Plasmacytoid DCs were defined as $CD3^{-}CD19^{-}CD20^{-}CD34^{-}CD14^{-}CD16^{-}DR^{+}CD11c^{-}CD123^{+}$. Tregs ($CD3^{+}CD4^{+}CD25^{hi}CD127^{lo}$) were included in the panel described previously in section 3.4.2, with absolute counts and frequency within the $CD4^{+}$ T-lymphocyte population reported. Normal values of cDCs and pDCs were referenced from Heinze *et al*[286].

4.4.2 Tolerogenic DC staining

PBMCs were prepared as described previously in section 3.4.1. 1 x 10^{6} PBMCs were added to polystyrene round-bottom BD Falcon® tubes (BD Biosciences), washed with FACS buffer (98% PBS, 2% FCS, 1mM endotoxin-free EDTA) and re-suspended in the remaining 50µL buffer. The cells were stained with an antibody-fluorochrome conjugate panel as shown in Table 4.2 and incubated in the dark at 4°C for 20 minutes. Cells were washed again with FACS buffer and data were collected using the BD FACSymphony flow cytometer (BD Biosciences). Analysis was performed using FlowJo software (BD Biosciences) (gating strategy shown in Figure 4.3). Median fluorescent intensity (MFI) was used to analyse CD80, CD86, CD83 and CCR9 expression by cDC2 and pDC populations. Samples were analysed at serial time points; pre-ECP, mid-ECP and end of ECP (if completed treatment) or late ECP (if treatment is ongoing).

4.4.3 Intracellular FOXP3 Treg staining

1 x 10^{6} PBMCs were added to polystyrene round-bottom BD Falcon® tubes (BD Biosciences), washed with FACS buffer and re-suspended in the remaining 50µL buffer. The cells were stained with the extracellular antibodies (Table 4.3) and incubated for 20

minutes at 4°C. After washing with FACS buffer, cells were fixed with Fixation/Permeabilisation Buffer (eBioscience). Normal mouse serum 2µL was added to each sample to block non-specific binding. Intracellular antibodies were added (Table 4.3) and incubated for 20 minutes at 4°C. The cells were washed with 2mls of Permeabilisation Buffer and resuspended in 300µL FACS buffer. Data was collected using the BD FACSymphony flow cytometer (BD Biosciences) and analysis was performed using FlowJo software (BD Biosciences) (gating strategy shown in Figure 4.4). Samples were analysed at serial time points; pre-ECP, mid-ECP and end of ECP (if completed treatment) or late ECP (if treatment is ongoing). This work was partially done by Masters of Research student Anna Ehrlich under my supervision.

4.4.4 Th1 and Th2 cytokine analysis

Serum was obtained by centrifugation of approximately 5mls of blood for 5 minutes ($2.5x10^3$ RPM) followed by transfer of serum to 1.5ml Eppendorf Tubes® (Sigma-Aldrich) and stored at -80°C. Absolute levels of serum Th1 (IL-1β, IL-2, IFN-γ, TNF-α) and Th2 (IL-4, IL-5, IL-6, IL-10) cytokines were quantified using the Enhanced Sensitivity Cytokine Bead Assay (BD Biosciences) performed as per the manufacturer's instructions. Briefly, 50µL of standard or patient sample was added to a mixture of 20µL of each of the capture beads and detection reagent A, followed by incubation at room temperature for 2 hours. The samples were washed and incubated with 100µL detection reagent B at room temperature for 1 hour. A final wash was performed to remove any unbound detector reagent followed by flow cytometric analysis (FACS Canto II, BD Biosciences) to quantify the amount of each cytokine. Prism v5.00 (GraphPad Software, Inc.) was used to formulate a standard curve and cytokine concentrations were determined from these standard curves. If a cytokine level was not detected, the sample result was omitted from the analysis. This was because it could not be known with certainty whether this was due to true undetectable circulating levels or due to an error in the assay. This approach was adopted to maintain consistency throughout, and to not underestimate cytokine levels. IL-1 was unavailable from the supplier for the final assay, therefore IL-1 analysis was included with results available from previous assays and completion of IL-1 analysis will be performed at a later stage when available. This work was partially done by Masters of Research student Catherine Roberts under my supervision.

4.4.5 Statistical analysis

Group analysis of the ECP patients was performed up to cycle 30, as only one patient remained on ECP beyond this time point. Individual analysis was performed until the end of ECP treatment regardless of duration. Comparison of median values was performed using the Kruskal-Wallis test with post hoc analysis when appropriate using the Dunn Correction test to detect significance between individual groups. P value <0.05 was significant.

Antibody Target	Fluorochrome (Laser/bandpass filter)	Volume per test	Manufacturer
CD3	FITC (488-520/20)	3.5µL	BD Biosciences
CD4	PE (561-584/15)	5µL	BD Biosciences
CD16	PE-Dazzle (561-610/20)	5µL	BD Biosciences
CD123	PerCP-Cy5.5 (561-710/50)	3.5µL	BD Biosciences
CD141	APC (635-670/14)	3.5µL	BD Biosciences
CD45	AF700 (635-730/45)	1µL	Biolegend
CD34	APC Cy7 (635-780/60)	3.5µL	BD Biosciences
CD8	APC Cy7 (635-780/60)	3.5µL	BD Biosciences
CD11c	BV421 (405-450/50)	3.5µL	BD Biosciences
HLA-DR	V500 (405-525/50)	5µL	BD Biosciences
CD14	BV650 (405670/30)	3.5µL	BD Biosciences
CD1c	PeCy7 (561-780/60)	3.5µL	BD Biosciences

Table 4.1 Antibody panel for counting DC subset absolute numbers.

Antibody Target	Fluorochrome (Laser/bandpass filter)	Volume per test	Manufacturer
CD1c	PeCy7 (561-780/60)	4µL	BD Biosciences
Lin 3 19 20	AF700 (635-730/45)	3µL	BD Biosciences
CD141	BV510 (405-525/50)	3µL	BD Biosciences
CD14	BV650 (405670/30)	3µL	BD Biosciences
CD11c	BV711 (405-710/50)	4µL	BD Biosciences
HLA-DR	BUV395 (355-379/28)	3µL	BD Biosciences
CD16	PE-CF594 (561-610/20)	4µL	BD Biosciences
CD123	BV785 (405-780/60)	4µL	Biolegend
CD80	PE (561-584/15)	7.5µL	BD Biosciences
CD83	BV421 (405-450/50)	4µL	BD Biosciences
CD86	FITC (488-520/20)	10µL	BD Biosciences
CCR9	AF647 (635-670/14)	3µL	BD Biosciences
DAPI	(355-450/50)	1µg/ml	BD Biosciences

Table 4.2 Antibody panel for tolerogenic DCs.

Antibody Target	Fluorochrome (Laser/bandpass filter)	Volume per test	Manufacturer
Extracellular antibodies			
CD3	PE-Cy7 (561-780/60)	2.5µL	BD Biosciences
CD4	PE (561-584/15)	10µL	BD Biosciences
CD25	Alexa Fluor 700 (635-730/45)	2.5µL	BD Biosciences
Zombie Aqua	(405-525/50)	1 µL (1/500 dilution)	Biolegend
Intracellular antibodies			
FOXP3	Alexa Fluor 647 (635-670/14)	2.5µL	BD Biosciences
Helios	Alexa 488 (488-520/20)	3µL (1/10 dilution)	BD Biosciences

Table 4.3 Antibody panel for FOXP3$^+$ Tregs.

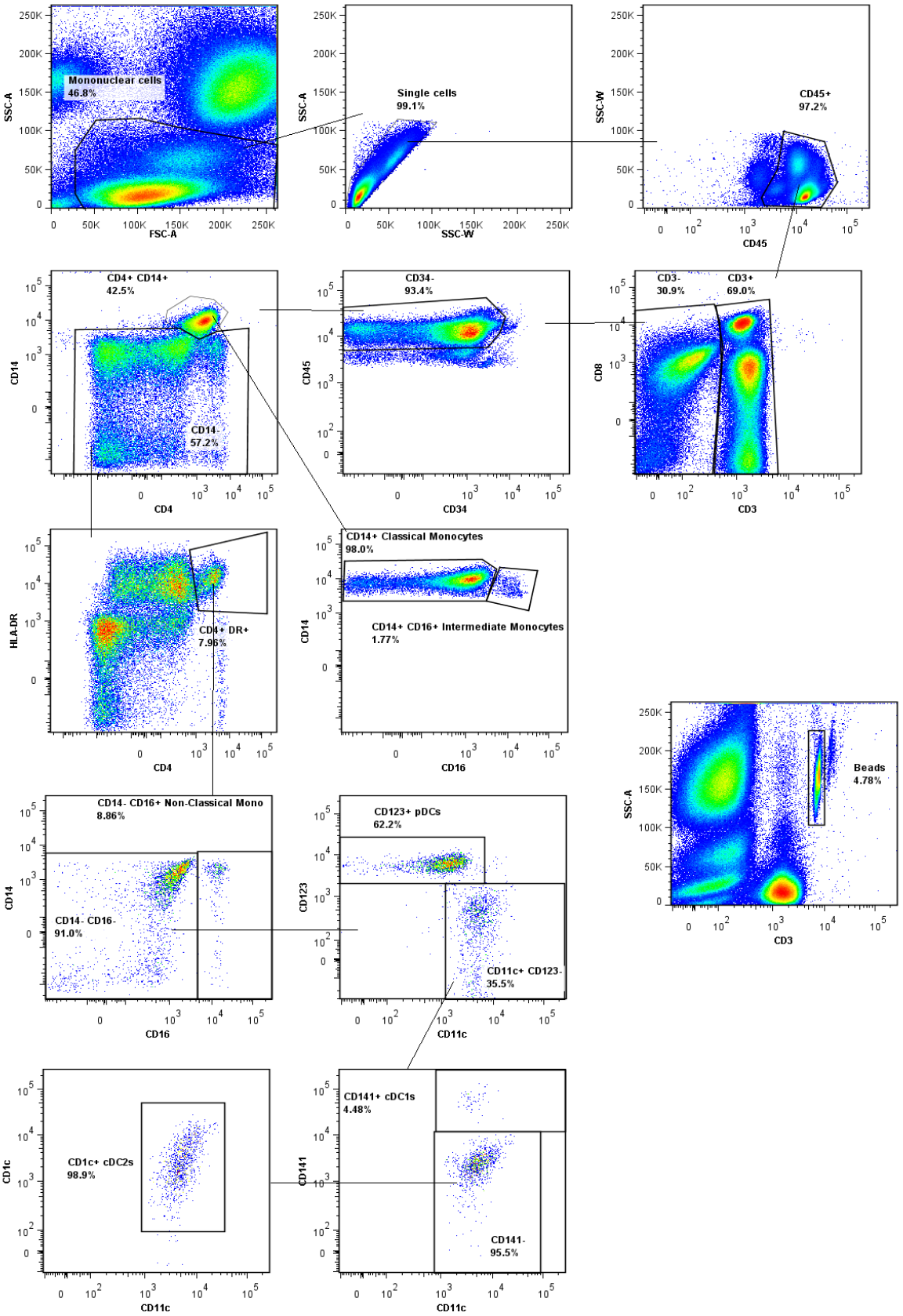


Figure 4.2 Gating strategy for DC Trucount analysis.

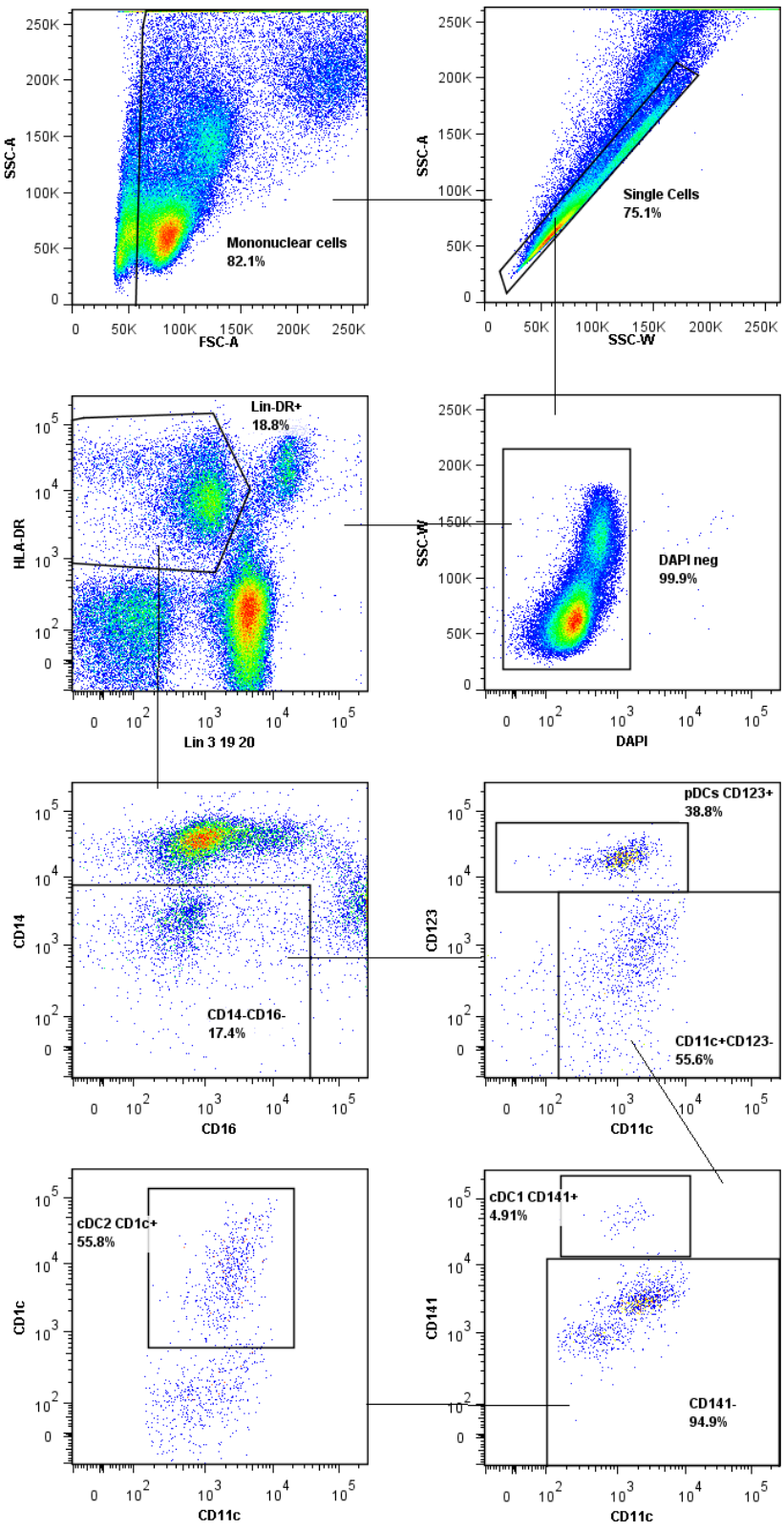


Figure 4.3 (A) Gating strategy to analyse tolerogenic DCs.

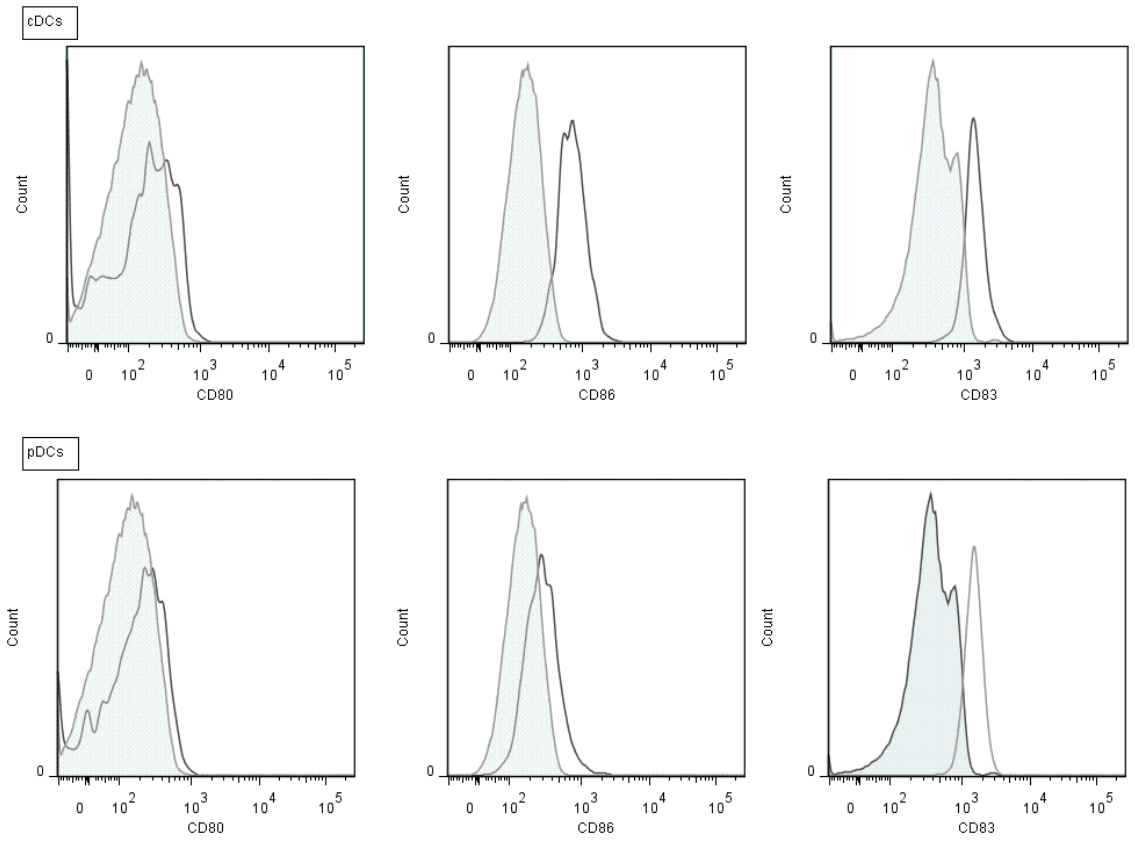


Figure 4.3 (B) Healthy control expression of CD86, CD80 and CD83 by cDC2s and pDCs with isotype controls (shaded).

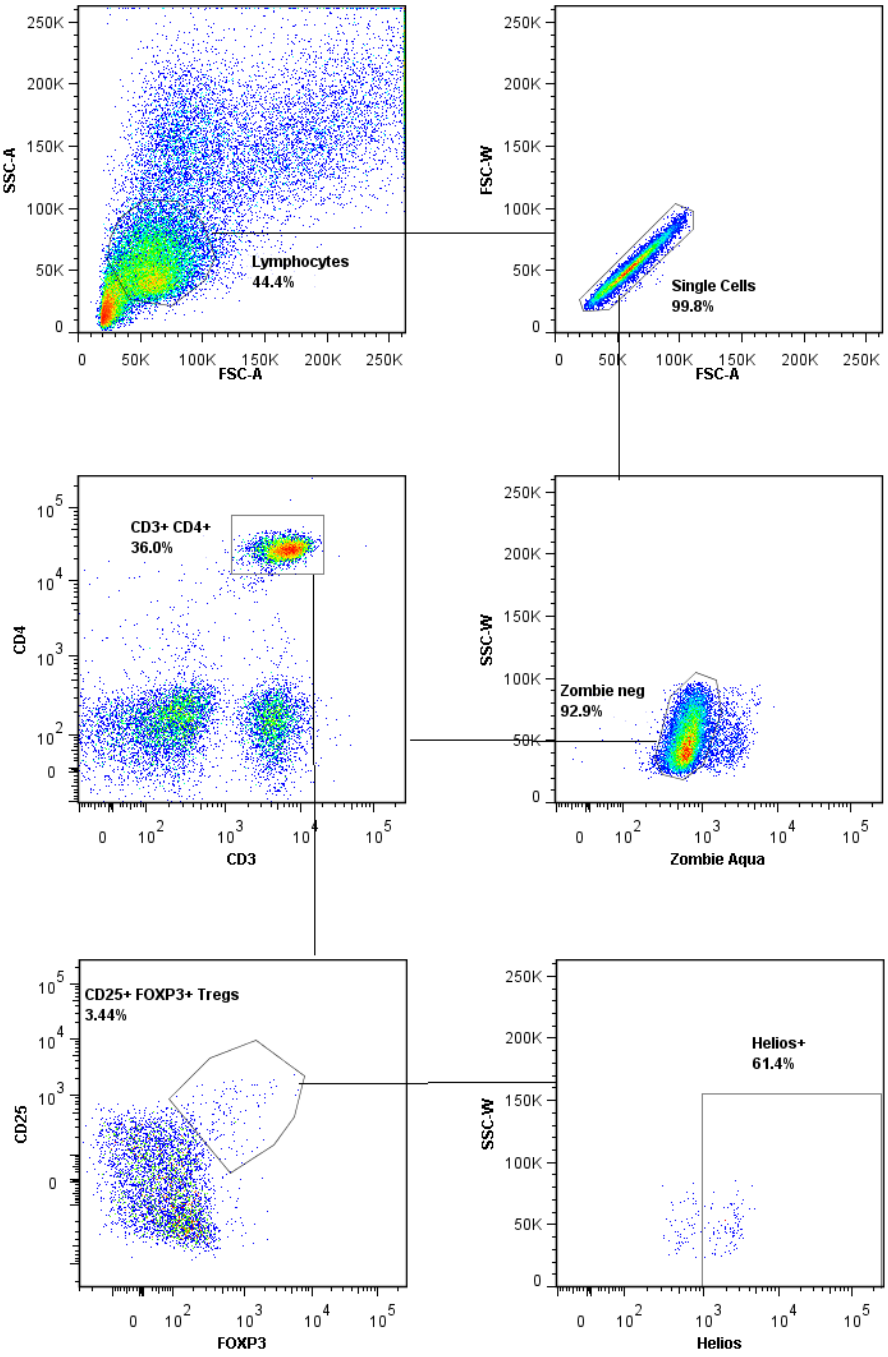


Figure 4.4 Gating strategy to analyse FOXP3⁺ Tregs and Helios expression.

4.5 Results

4.5.1 Dendritic cell subset absolute numbers

Analysis of absolute numbers of DCs demonstrated consistently very low numbers of the cDC1 population in all patients (absolute count range 0 – 1.4 cells/μL) therefore the total cDC CD11c+ population was used (CD1c+/CD141+CD11c+CD14-CD16-DR+Lin- cells).

4.5.1.1 Dendritic cell subset changes within the ECP group

Within the whole ECP group, there was an overall increase in the pDC and cDC absolute counts with progression of ECP treatment, with a concurrent decline in the cDC/pDC ratio (Figure 4.5). However, by 12 months post-HSCT, at which stage the majority of patients had completed ECP therapy, the cDC/pDC ratio increased again, comparable to pre-ECP levels, although remained lower compared to the control groups (Figure 4.6-C).

Individual analyses of DC subsets in the ECP patients (Figure 4.7) demonstrated very low absolute numbers of both pDCs and cDCs in patients 1, 3 and 8. Patients 2, 4, and 6, who all successfully completed ECP, demonstrated a similar pattern; cDC counts were higher at the early stages of therapy, paralleled with a peak in the cDC/pDC ratio, followed by a decline in cDCs (and consequently the cDC/pDC ratio). Towards the end of ECP treatment, there was an increase in both cDC and pDC populations. Plasmacytoid DC counts remained low in patient 6, although this may be related to the earlier stage post-HSCT compared to patients 2 and 4. Patient 5, who also successfully completed ECP, demonstrated a more erratic cDC pattern, but despite this, similarities to patients 2, 4 and 6 were seen, although occurred later in the course of treatment; cDCs and the cDC/pDC ratio peaked initially (between cycles 10-13), followed by a decline, and an increase in pDCs and cDCs was observed at the end of treatment. Patient 7 (withdrawn from ECP at cycle 17) had a peak in the cDC/pDC ratio in the early stages of ECP, followed by a decline. In parallel with this, an increase in cDCs and pDC was seen, although at the time of diagnosis of AML relapse and withdrawal from ECP, both cDCs and pDCs decreased.

4.5.1.2 Comparison of DCs with control groups

The cDC population rose steadily in control group 1, and was within the normal range by 12 months post-HSCT (Figure 4.6-A). A steady rise was similarly seen in the ECP group, reaching the lower limit of the normal range by 12 months, although remained lower compared to control group 1. In contrast, the median cDC absolute count in control group 2 remained static below the lower limit of the normal range at 4, 8 and 12 months.

No significant differences were detected in the median cDC counts between the 3 groups at any of the time points measured (p=0.09, 0.25 and 0.21 at 4, 8 and 12 months respectively).

Plasmacytoid DC reconstitution was slower compared to cDC reconstitution, with absolute numbers at 12 months post-HSCT remaining below the lower limit of normal in all 3 groups (Figure 4.6-B). A steady incline in pDCs was observed in control group 1. A similar incline was seen in the ECP group, although at an inferior rate compared to control group 1. A different pattern was observed in control group 2, with the pDC median absolute count highest at 4 months post-HSCT, followed by a decline at 8 months, and remained static at 12 months. A significant difference was detected in the median pDC counts at 4 months (p=0.02), but not at 8 or 12 months (p=0.48 and 0.37 respectively).

A similar pattern in the cDC/pDC ratio was evident in control groups 1 and 2, with a peak at 8 months, highest in control group 2, followed by a decline at 12 months (Figure 4.6-C). The cDC/pDC ratio was lower in the ECP group at 8 months post-HSCT compared to the other two groups (p=0.43). By 12 months post-HSCT (at which stage the majority of patients had completed ECP treatment), the cDC/pDC ratio was comparable between the 3 groups (p=0.45).

4.5.2 Dendritic cells and corticosteroids

Among the ECP patients who completed ECP therapy and had successful weaning of corticosteroids (patients 2, 4, 5 and 6), all had a statistically significant inverse relationship between the corticosteroid dose and absolute numbers of pDCs (p=0.0005, p<0.0001, p<0.0001, p=0.015 respectively) and two patients had a significant inverse relationship between the corticosteroid dose and absolute numbers of cDCs (p=0.66, p=0.02, p=0.04 and p=0.10 respectively). The three ECP patients who had high dose corticosteroids re-started during treatment (patients 1, 3 and 8) had baseline very low numbers of both pDCs and cDCs at all doses of corticosteroids.

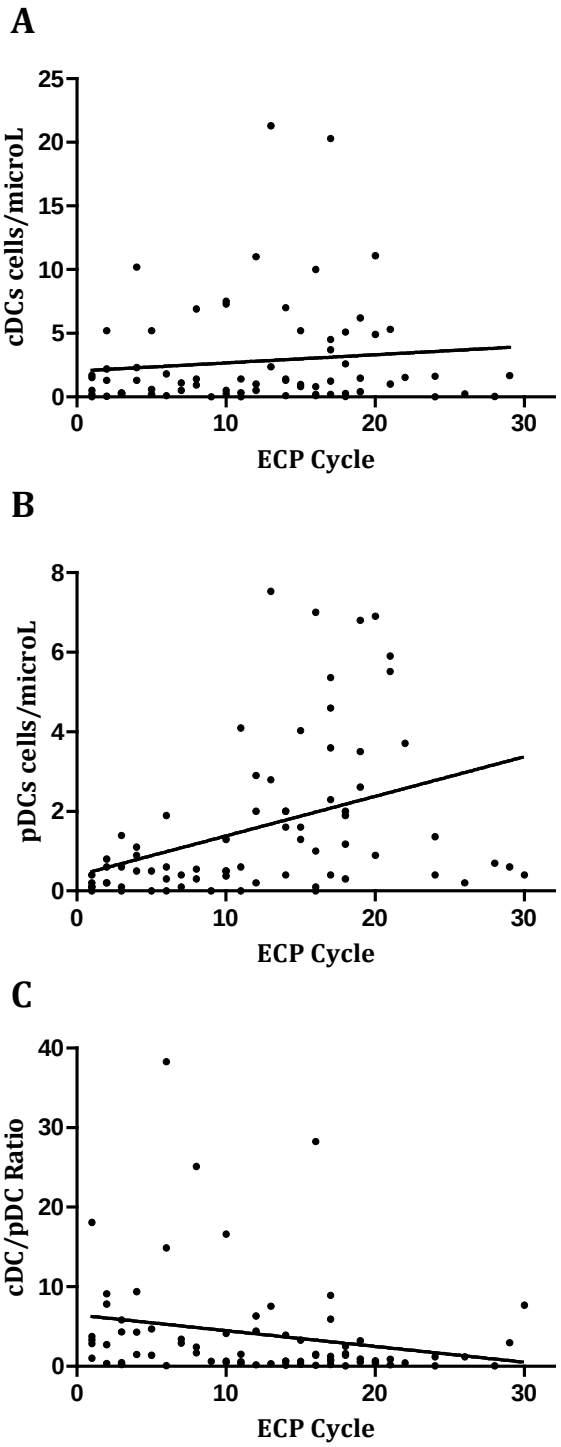


Figure 4.5 Overall trend of pDC and cDC absolute counts and the cDC/pDC ratio during ECP therapy demonstrated; (A) an increase in cDCs, although not statistically significant using linear regression analysis (p=0.34), (B) a statistically significant increase in pDCs (p<0.0007), and (C) a decline in the cDC/pDC ratio, although not statistically significant (p=0.05).

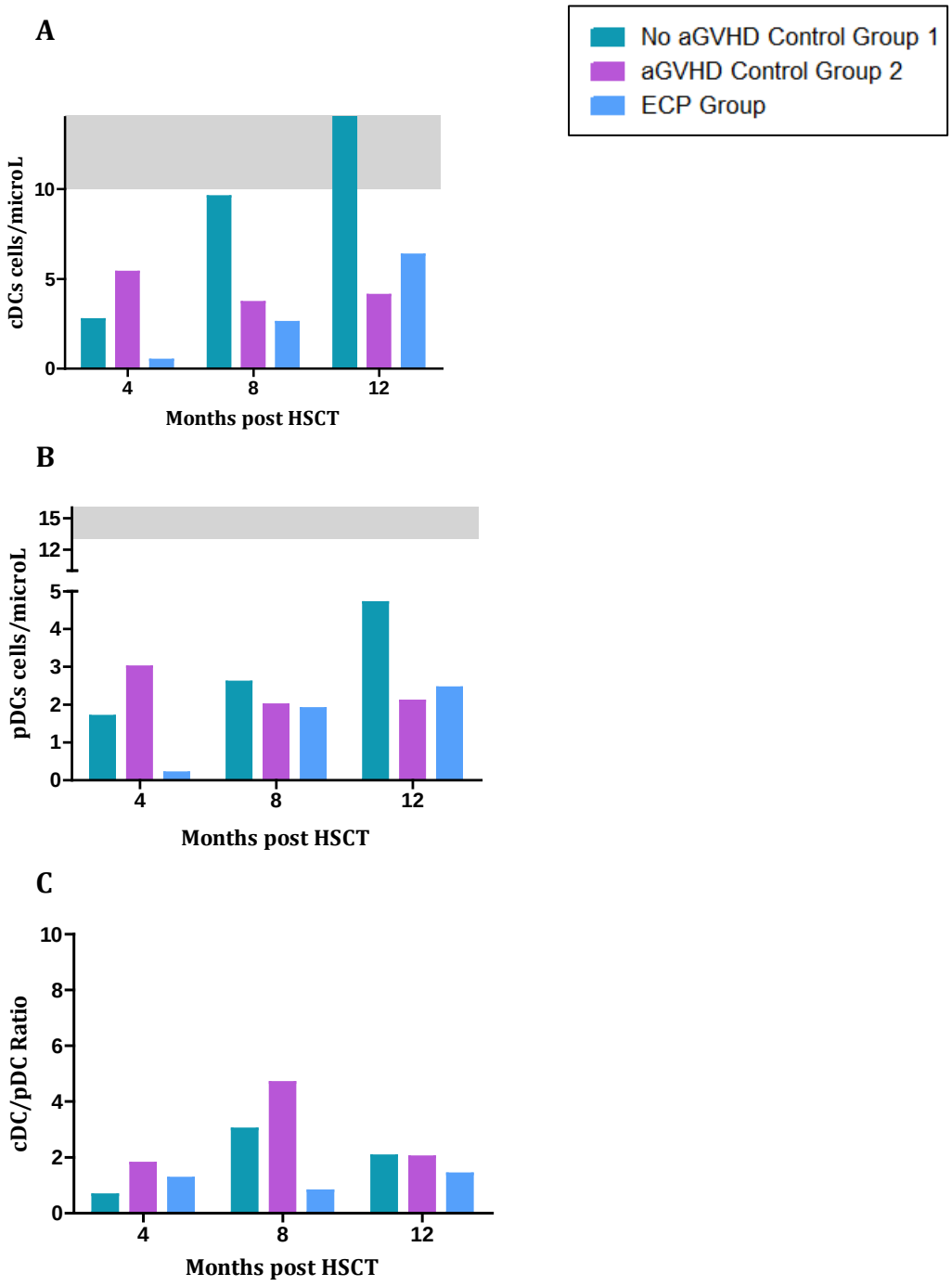


Figure 4.6 Comparison of the median pDC and cDC absolute counts (A and B) and the median cDC/pDC ratios (C) between the ECP group and control groups 1 and 2 at 4, 8 and 12 months post-HSCT. No significant differences were detected in the median cDC counts or cDC/pDC ratios at any of the time points. A significant difference was detected in the median pDC counts at 4 months (p=0.02), with Dunn's Post Test analysis identifying a significant difference between control group 2 and the ECP group but no significant difference at 8 or 12 months (p=0.48 and 0.37 respectively). The grey shaded area depicts the normal range[286].

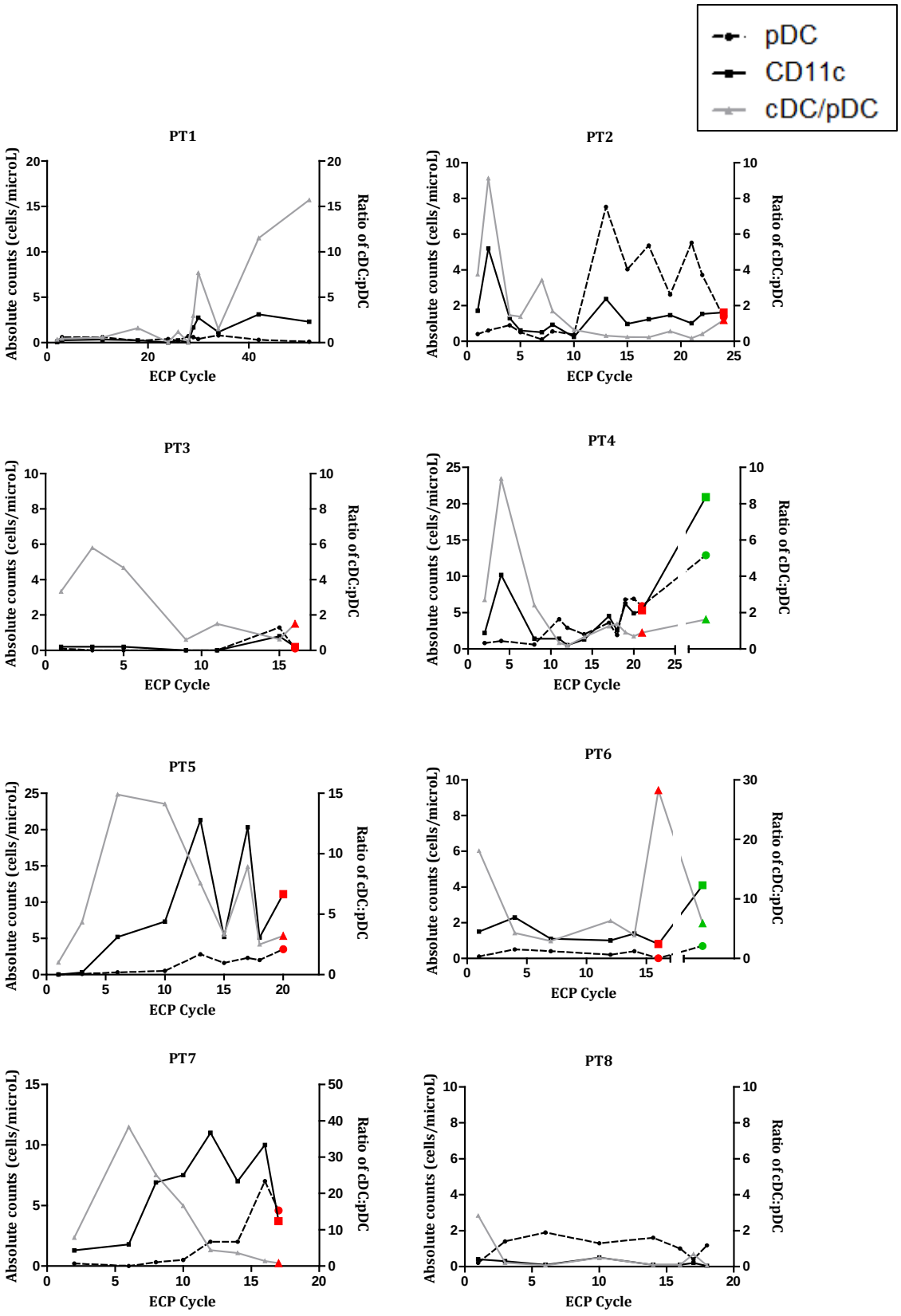


Figure 4.7 Analyses of individual ECP patient pDC (black dotted line) and cDC (black continuous line) absolute counts and cDC/pDC ratios (grey line). The red dots denote end of ECP treatment and the green dot denotes follow-up samples at 12 months post-HSCT (patient 4) and 8 months post-HSCT (patient 6). Low absolute numbers of pDCs and cDCs were observed in patients 1, 3 and 8.

4.5.3 Dendritic cell phenotype

As the cDC1 populations were very small for all of the patients, only the phenotype of cDC2s and pDCs were examined. Conventional DC2 and pDC expression of the surface co-stimulatory molecules CD80, CD86 and CD83 was examined in the following control patients; a normal adult healthy control, 2 patients from control group 1 at 4 months post-HSCT and 2 patients from control group 2 (patient 1 who was on a low dose of corticosteroids at 4 and 8 months post-HSCT, and patient 2 who was on a physiological dose of corticosteroids at 4 months, but no corticosteroids at 8 months post-HSCT). Plasmacytoid expression of CCR9 was also examined.

No significant differences in cDC2 or pDC expression of the co-stimulatory markers between the control patients were observed (Figure 4.8). There was no evidence of significantly reduced expression in those who were on low-dose corticosteroids at the time points measured. Patient 2 from control group 2 demonstrated lower expression of CD83 by pDCs and CD80 by cDC2s, but otherwise results were similar.

Figure 4.9 demonstrates co-stimulatory marker expression by cDC2s and pDCs in the ECP patients 2, 4, 5 and 6 at several progressive time points over the course of treatment. These patients were 'responders' to ECP treatment; they completed therapy with successful resolution of aGVHD, weaning of concurrent immunosuppression and had evidence of thymic recovery. Expression was reduced compared to that seen in the control patients also shown, particularly within the pDC population. There was a pattern of higher expression before ECP treatment was started followed by a reduction, although this was not universal. There was also a pattern of increasing expression over the course of ECP treatment. This was particularly evident in patient 4; high expression of all co-stimulatory molecules before ECP treatment, followed by a reduction, and then increased expression at the end of treatment (although not to pre-treatment levels). Figure 4.10 demonstrates co-stimulatory marker expression by cDC2s and pDCs in the ECP patients 1 and 8 (treatment non-responders). The pDC population for patient 8 at cycle 17 was too small to measure co-stimulatory marker expression, therefore only cycle 3 was included. Although some time points display lower MFI values compared to the controls, overall the trend is of higher expression and a more erratic pattern compared to the 'responding patients', with occasional high peaks of MFI. Patient 1, who remains on prolonged ECP treatment, displayed increased expression at cycle 38, coinciding with the development of liver aGVHD and re-initiation of high dose corticosteroids.

CCR9 expression by pDCs was also examined in all patients; no consistent patterns were observed, with MFI values of the ECP patients similar to that seen in the control patients.

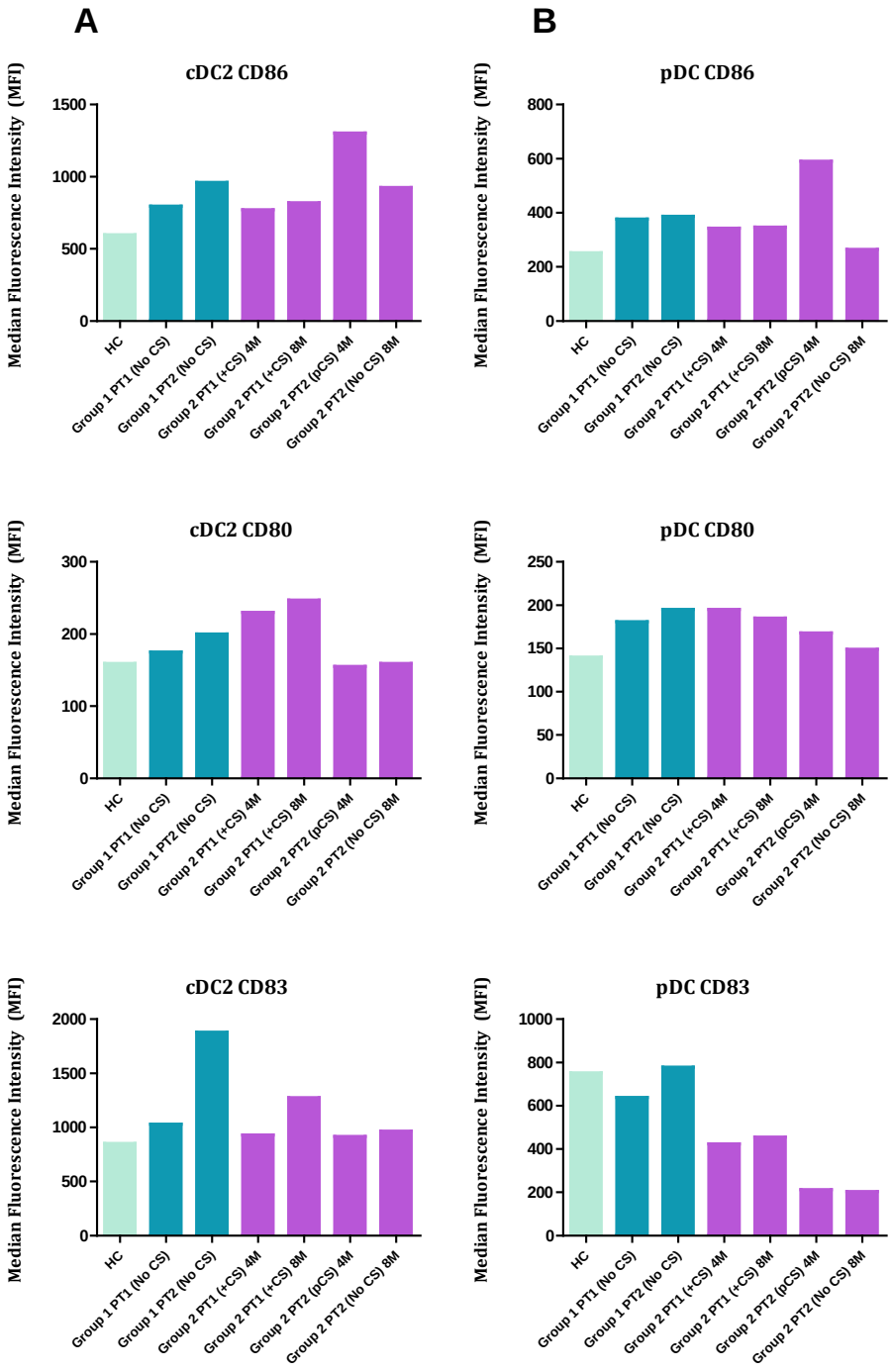


Figure 4.8 Expression of CD86, CD80 and CD83 by cDC2s (A) and pDCs (B)was measured in the following controls; healthy adult control (light green), two patients from control group 1 with no aGVHD (dark green) and two patients from control group 2 with aGVHD who did not receive ECP (pink). Patient 1 from group 2 was on low dose corticosteroids at both 4 and 8 months post-HSCT. Patient 2 from group 2 was on a physiological dose of corticosteroids (pCS) at 4 months but no corticosteroids at 8 months post-HSCT. No significant differences in co-stimulatory marker expression were observed between the control patients.

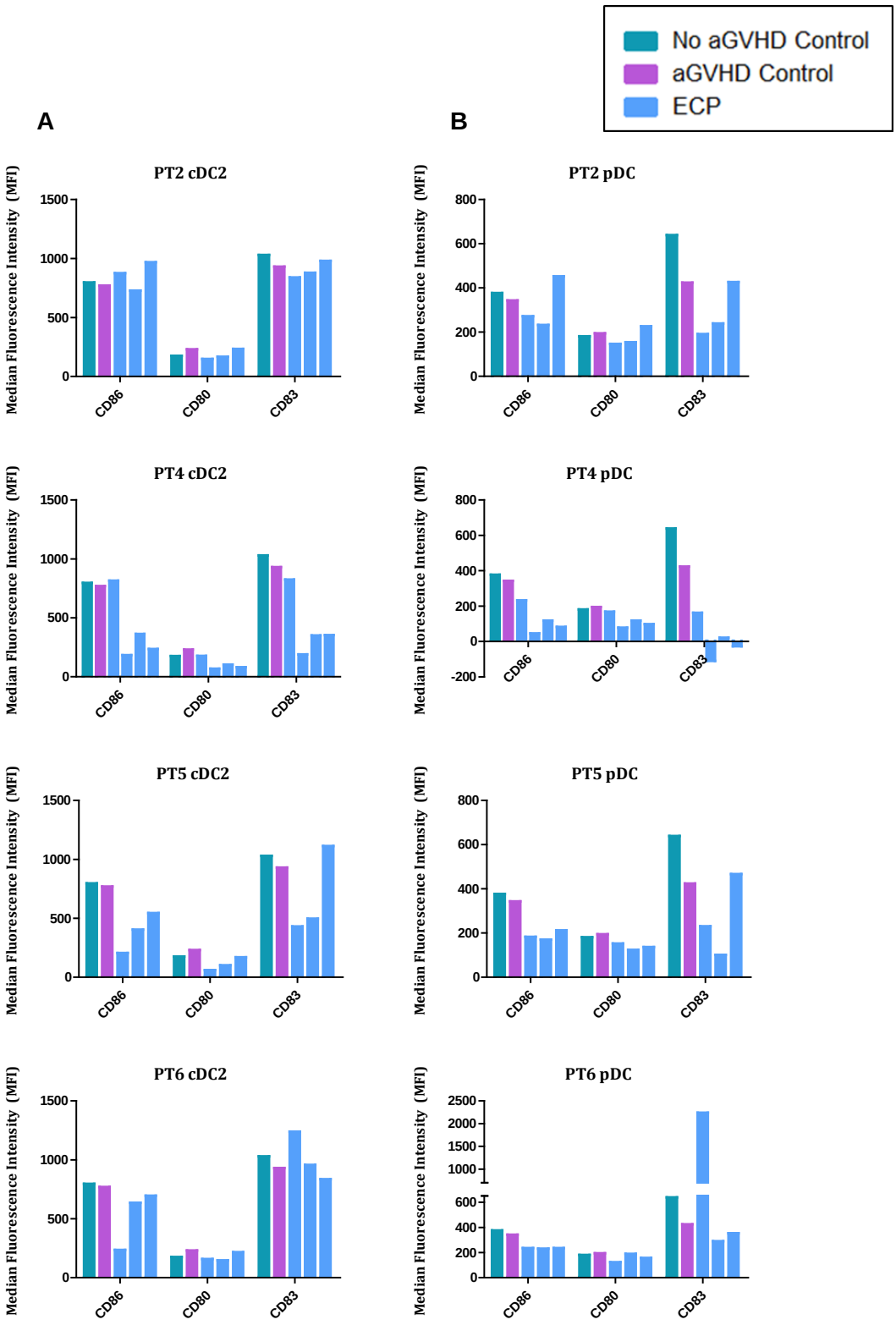


Figure 4.9 Expression of CD80, CD86 and CD83 by cDC2s (A) and pDCs (B) in the responding ECP patients 2, 4, 5 and 6 (blue). The expression of CD86, CD80 and CD83 was measured at serial time points during the course of ECP. The bars to the left represent the pre-ECP values, with subsequent courses represented to the right. For context, patients from control group 1 (green) and 2 (pink) are included. Overall, a trend of reduced co-stimulatory marker expression compared to controls was observed, particularly in the pDC population.

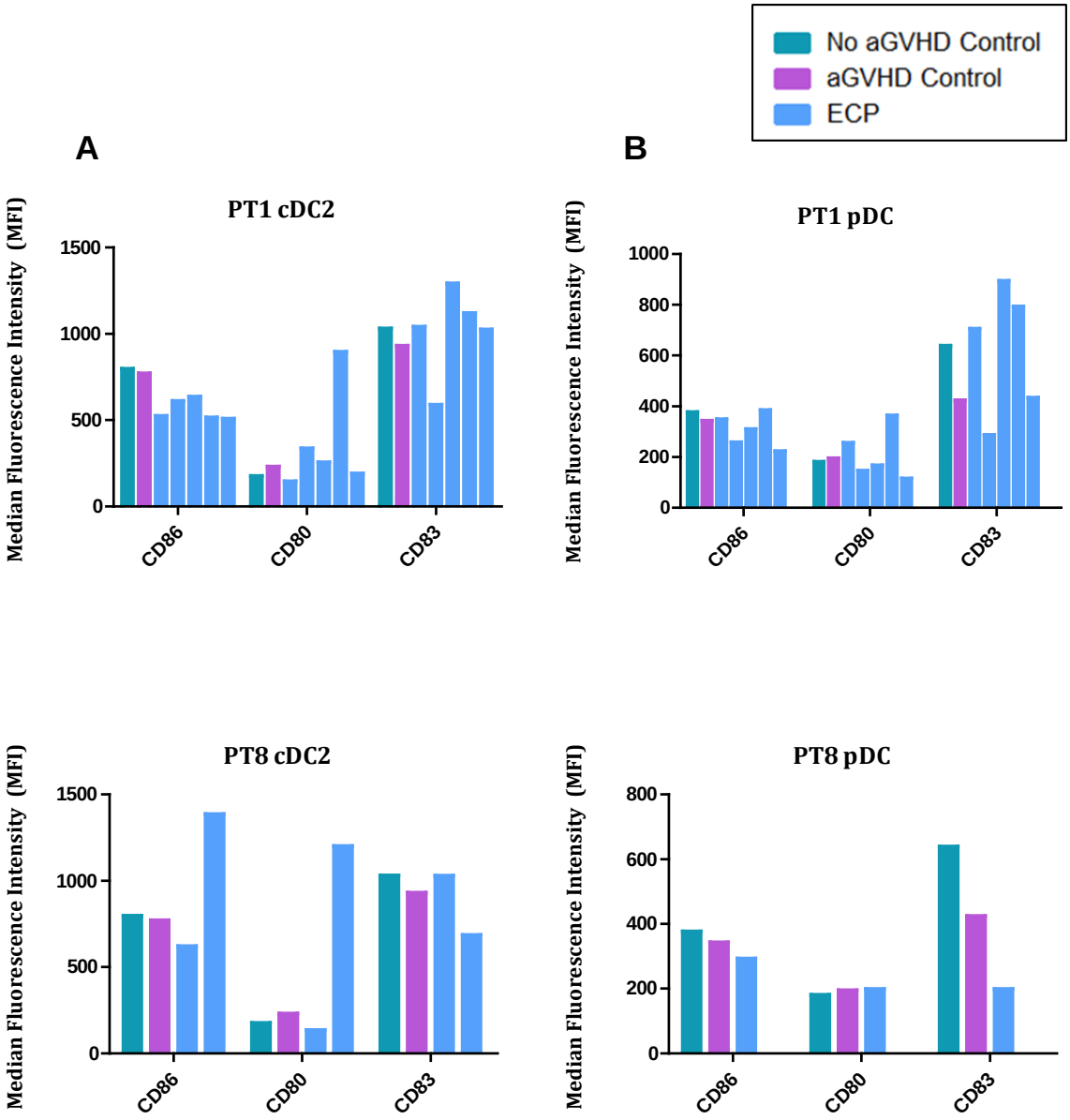


Figure 3.10 Expression of CD80, CD86 and CD83 by cDC2s (A) and pDCs (B) in the ECP 'non-responding' patients 1 and 8. Samples were taken pre-ECP, mid-ECP and late ECP. The bars to the left represent the pre-ECP values, with subsequent courses represented to the right. For context, patients from control group 1 (green) and 2 (pink) are included. Overall, with some exceptions, co-stimulatory marker expression appeared higher, or similar to the controls, with occasional peaks in MFI values.

4.5.4 Regulatory T-lymphocytes

4.5.4.1 $CD25^{hi}CD127^{lo}$ Treg absolute counts

Patient 1 displayed a different pattern of Treg frequency compared to the other ECP patients (Figure 4.11 A and B). Overall, the proportion of Tregs within the $CD4^+$ T-lymphocyte population was above the normal range throughout, with a very high frequency (45.9%) at the beginning of ECP, and a decline in frequency with progression of ECP therapy (10.8% at cycle 44). Patient 1 also had relatively higher absolute Treg numbers at the beginning of ECP followed by a decline, with a peak observed at cycle 31 correlating with re-initiation of high dose corticosteroids. The remaining ECP patients displayed a fluctuating pattern of intermittent increased Treg frequency above the normal range at some stage of ECP therapy, primarily seen at variable points between cycles 6 – 13, but without a consistent upward or downward trend evident.

When comparing the median Treg frequency between the ECP and control groups at 4, 8 and 12 months post-HSCT, control group 2 (aGVHD not treated with ECP) had a higher frequency at 4 and 8 months compared to the other two groups, but by 12 months the frequency between all three groups was similar (Figure 4.12). Absolute counts steadily increased in the ECP group and control group 1, but control group 2 exhibited a peak at 8 months followed by a decline at 12 months, with numbers inferior to the other two groups.

$FOXP3^+$ Tregs were analysed at 3 time points in the course of ECP treatment (Figure 4.13); pre-ECP, mid-ECP and late ECP (if ongoing treatment) or end of ECP (if treatment ceased). A similar frequency to $CD25^{hi}CD127^{lo}$ Tregs was observed; patient 1 displayed a high frequency of $FOXP3^+$ Tregs initially (34.7%) which gradually declined, and the other ECP patients displayed frequencies between 3.2 – 10.2%. The pattern of $FOXP3^+$ Tregs with ECP progression was variable, with 3/5 patients' exhibiting an overall decline, with an incline observed for patient 6. Patient 8 displayed similar frequencies of FOXP3+ Tregs at each time point measured. The frequency of $Helios^+$ $FOXP3^+$ Tregs was consistently high, predominantly between 80-90%, which was higher than that seen in the healthy adult control (median 74.3%) and patients from control group 1 (median 76.9%) but similar to that seen in patients from control group 2 (89.9%).

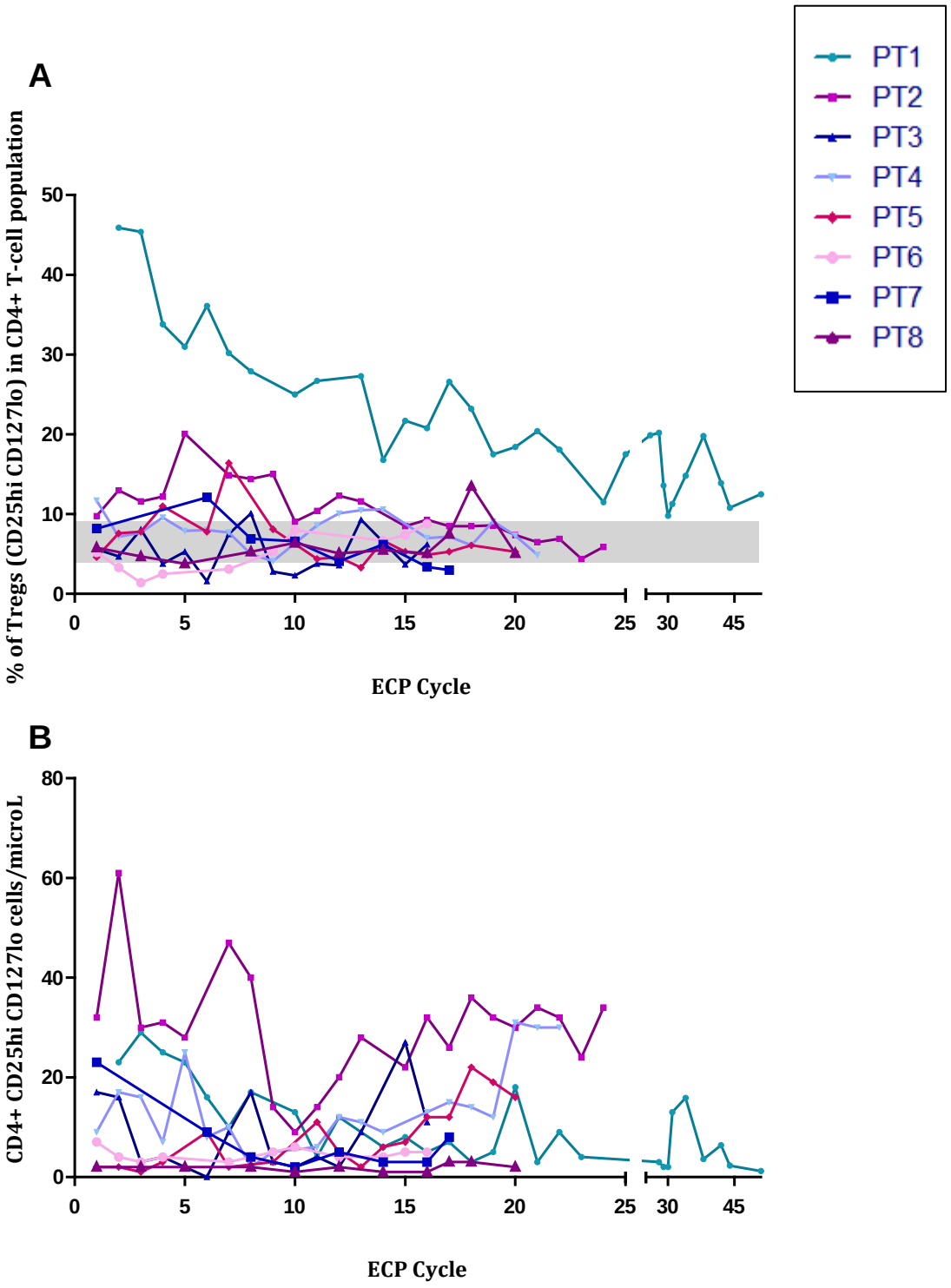
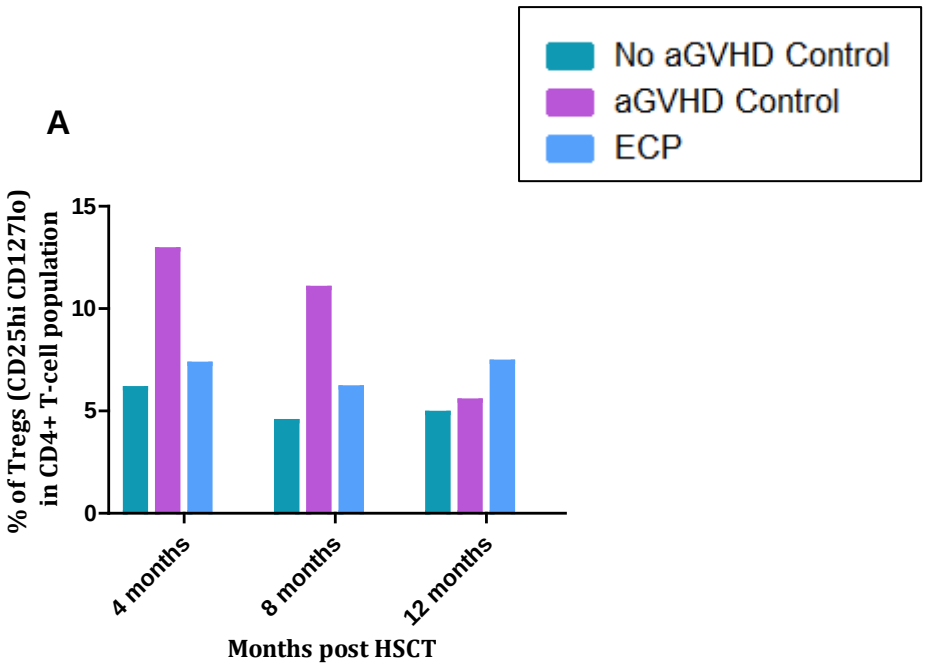
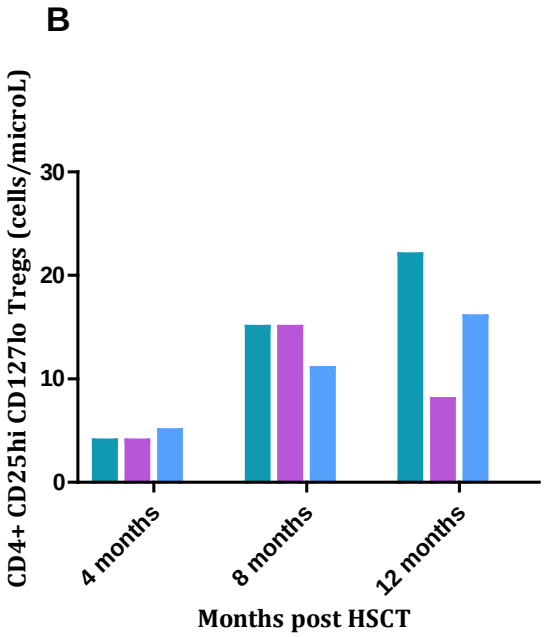


Figure 4.11 Frequency of $CD25^{hi}CD127^{lo}$ Tregs within the CD4+ T-lymphocyte population (A) and absolute numbers (B) of Tregs for each ECP patient with progression of ECP treatment. Patient 1 demonstrated a different pattern compared to the other ECP patients with a high frequency initially followed by a decline. The grey shaded area in (A) represents the normal Treg frequency range.





Range of Treg %	No aGVHD Control	aGVHD Control
4 months	3.9 – 8.6%	3.4 – 15.9%
8 months	4.4 – 5.5%	3.8 – 12.9%
12 months	4.7 – 5.7%	5.0 – 12.5%
Range of Treg absolute counts	**No aGVHD Control**	**aGVHD Control**
4 months	3.4 – 46.3	0.9 – 91.5
8 months	4.8 – 25.0	8.2 – 24.3
12 months	10.8 – 91.3	7.0 – 16.7

Figure 4.12 Median frequency (A) and absolute counts (B) of $CD4^{+}CD25^{hi}CD127^{lo}$ Tregs in each group at 4, 8 and 12 months post-HSCT. A gradual incline in Treg absolute numbers was seen in control group 1 and the ECP group. No significant differences in the median values of the frequency or absolute numbers was observed using Kruskal-Wallis analysis (p= 0.06 and 0.36 respectively).

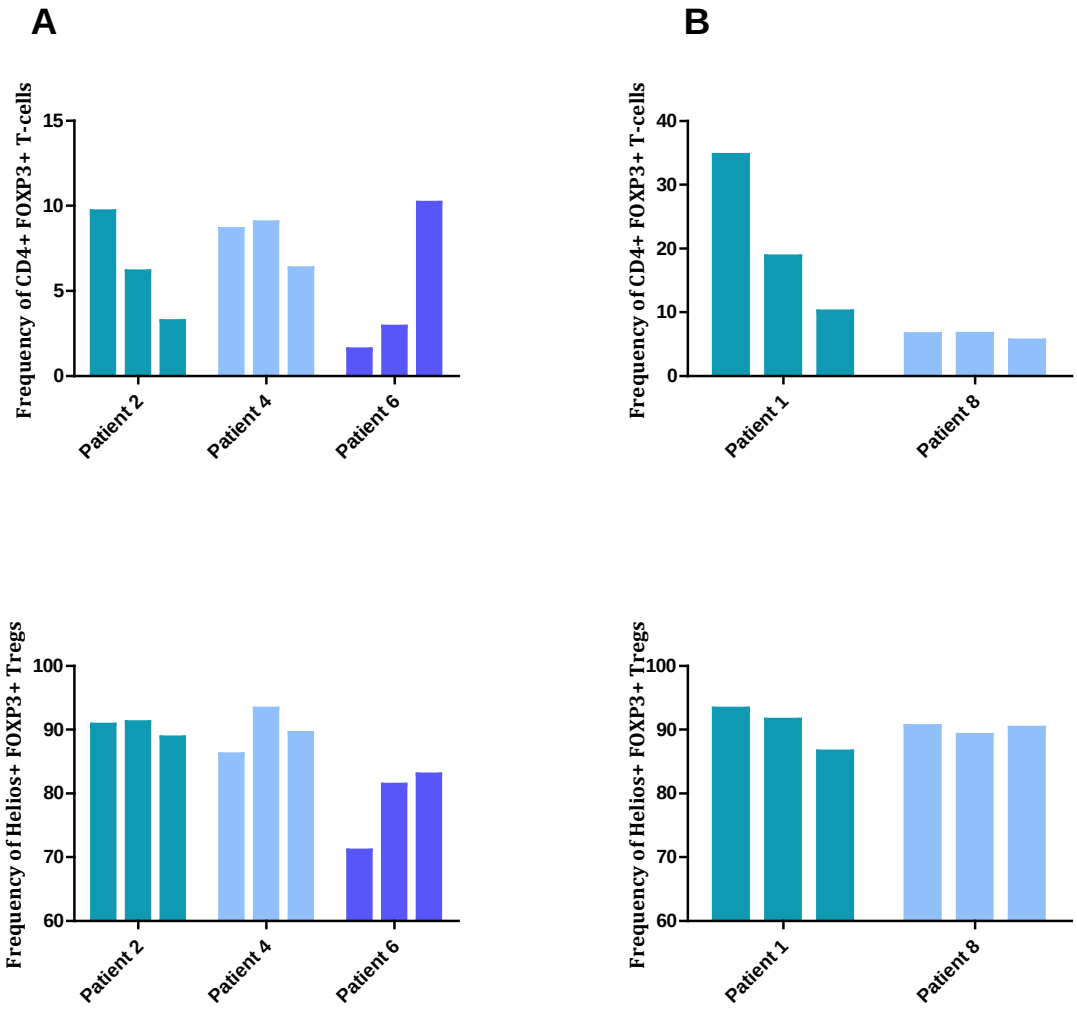


Figure 4.13 Frequency of CD4⁺ FOXP3⁺ Tregs and frequency of Helios⁺ within the FOXP3⁺ Treg population in responding ECP patients 2, 4 and 6 (A) and ECP patients 1 and 8 (B). Samples were taken (displayed from left to right) pre-ECP, mid-ECP and late/end of ECP. The pattern of FOXP3+ Tregs over the course of ECP treatment was variable, with patients 1, 2 and 4 demonstrating a decline in frequency, an incline was observed in patient 6 and a static pattern seen in patient 8. Overall, the frequency of Helios⁺FOXP3⁺ Tregs was high, predominantly between 80-90%. In comparison, the range of Helios⁺FOXP3⁺ Tregs in healthy control samples was 65.1 – 77.2%, median 74.3%.

4.5.5 Th1 and Th2 cytokine analysis

Results were divided into the responding patients who completed ECP therapy (patients 2, 4, 5 and 6) who were analysed together, the non-responding patients (1 and 8) who remain on ECP treatment, and the two patients withdrawn from treatment were analysed individually.

There was a wide variation in the serum cytokine concentrations between patients and within individual patients as shown in Table 4.4. Patient 5, in particular, had consistently higher levels of all cytokines compared to the other 3 responding patients. For this reason, each cytokine is represented graphically twice; firstly as the raw data with the line of best fit to demonstrate the overall trend, and secondly with a split *y* axis to demonstrate more clearly the distribution of individual results. An overall decline in serum IL-2, IFNγ and TNF (Figure 4.14), and an incline in IL-4, IL-5, IL-6 and IL-10 (Figure 4.15) were observed in the responding patients with progression of ECP. Analysis of IL-1 in 3 patients demonstrated a non-significant incline with treatment progression, although this data was incomplete (Figure 4.14). There was no change in the IFNγ:IL-4 ratio with progression of treatment (Figure 4.16).

To investigate if a different Th1/Th2 cytokine profile was present between responding and non-responding ECP patients, results were analysed separately. Because patient 1 was on ECP treatment for a considerably longer duration compared to patient 8, analysis of these patients was performed individually. For both patients 1 and 8, an overall increasing trend in all cytokine concentrations was seen with ECP progression, with the exception of serum IL-4 in patient 8 in which there was a downward trend (Figures 4.17 and 4.18). Both patients displayed a significant increase in TNF levels. Patient 1 displayed an isolated surge in IFNγ from cycles 23-27 which correlated with an intercurrent influenza A infection. Patient 8 also exhibited an isolated surge in IFNγ at cycle 17 which correlated with Human Herpes Virus 6 (HHV6) positivity in the blood.

Patients 3 and 7, who were withdrawn from ECP therapy, were also analysed individually. Patient 3 displayed low levels of serum IFNγ, IL-2, IL-1 and IL-4 until the last sample measurement prior to withdrawal when a surge in IL-1, IL-2 and IL-4 occurred, in parallel with a drop in IL-10. There was also an isolated surge in IFNγ and IL-6 at cycle 11 which correlated with an intercurrent influenza A infection. TNF and IL-5 levels exhibited a downward trend. Patient 7 demonstrated a universal decline in all cytokines. Cytokine results for patients 3 and 7 are shown in Appendix 2.

To explore the pattern of Th1 and Th2 cytokines in the first 12 months post-HSCT, the median values of each cytokine measured from patients from control groups 1 and 2 at 4, 8 and 12 months post-HSCT, were compared with the median values of the ECP patients at the same time points (Figure 4.19). At 12 months post-HSCT, the median values of all cytokines, except IL-2, were higher in the ECP group compared to both control groups. At 4 months, median values of IFNγ, TNF, IL-1, IL-2 and IL-4 were all higher in control group 1 compared to the ECP group (n=4 and 8 respectively regarding the number of samples measured at this time point). At 8 months, median values of IL-2, IL-1, IL-4 and IL-5 were similar between control group 1 and the ECP group, with higher TNF, IL-6 and IL-10 levels but lower IFNγ seen in control group 1. The median value of the cytokines in control group 2 were predominantly lower compared to the other two groups but the number of samples measured was lower (n=3, 2 and 3 at 4, 8 and 12 months respectively).

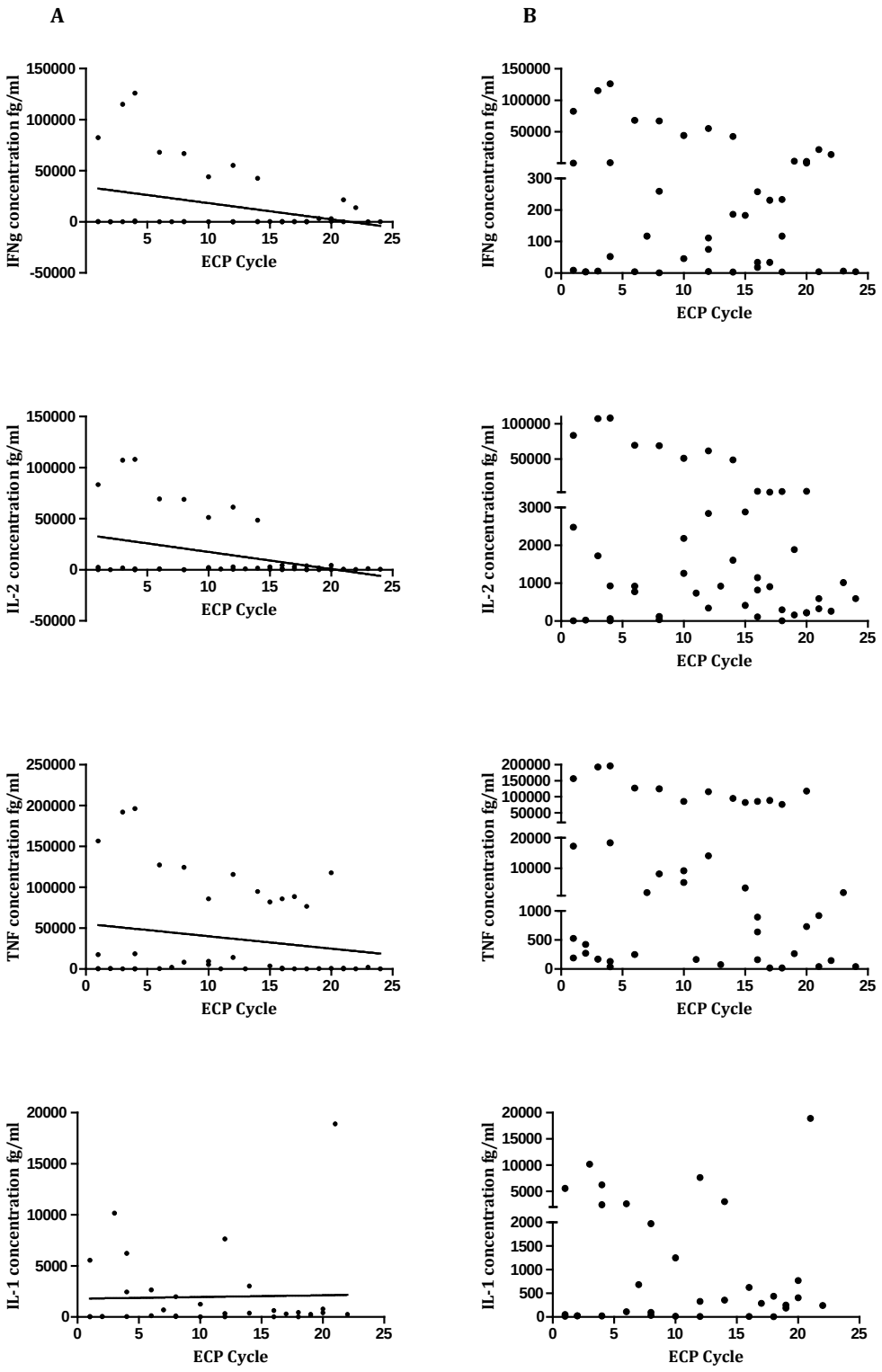


Figure 4.14 Th1 cytokines in the responding ECP patients: linear regression analyses demonstrated a decline in serum IFNγ ($p<0.01$), IL-2 ($p=0.02$) and TNF ($p=0.23$) concentrations in patients 2, 4, 5 and 6. IL-1, analysed in 3 patients demonstrated a non-significant incline ($p=0.87$). Figures in column (A) represent the raw data and (B) represent the same data but with a split *y* axis to highlight the wide variability in individual values.

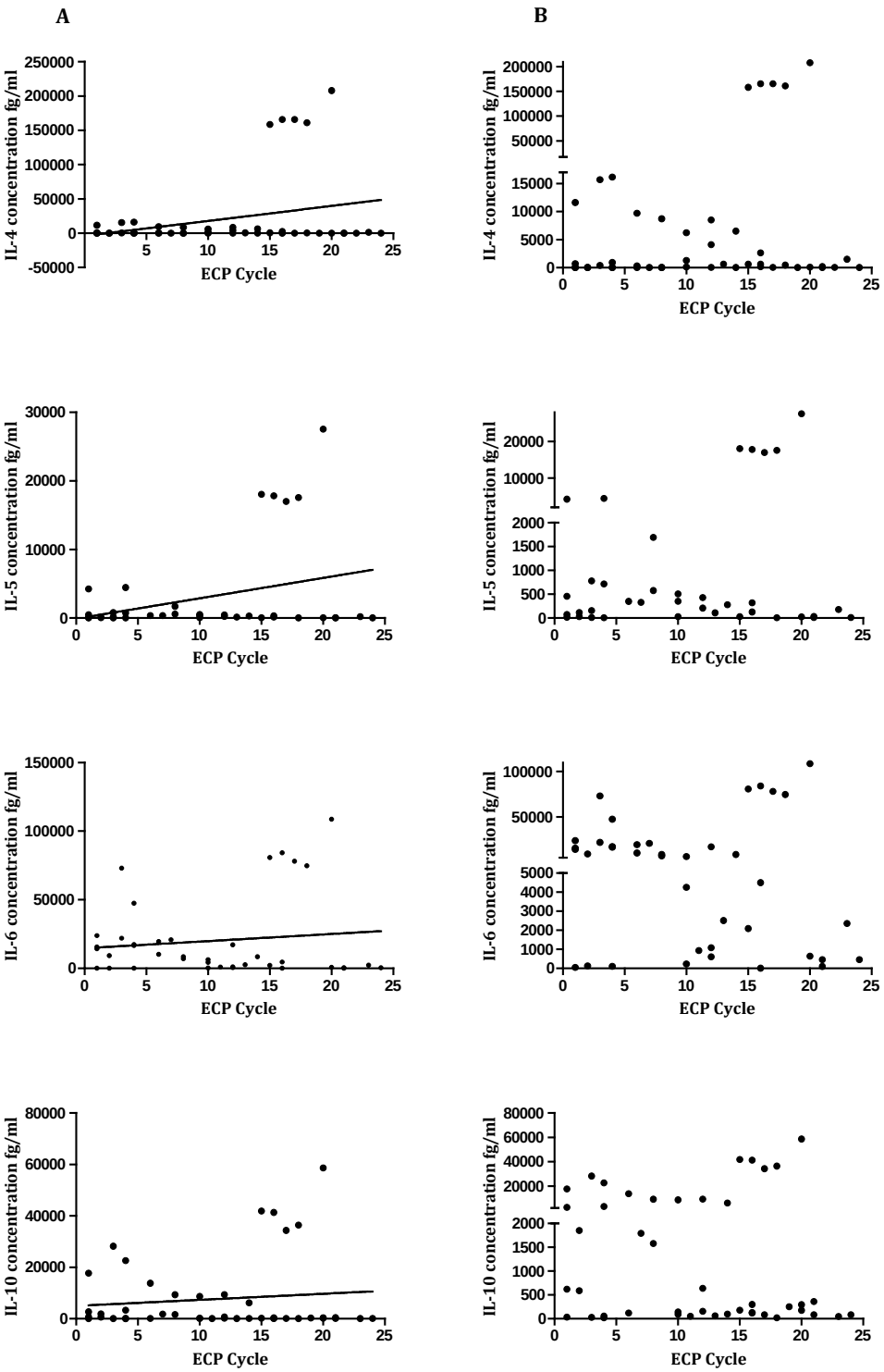


Figure 4.15 Th2 cytokines in the responding ECP patients: linear regression analyses demonstrated an incline in serum IL-4 (p=0.06), IL-5 (p=0.06), IL-6 (p=0.45) and IL-10 (p=0.445) concentrations in patients 2, 4, 5 and 6. Figures in column (A) represent the raw data and (B) represent the same data but with a split *y* axis to highlight the wide variability in individual values.

	Patient 2	Patient 4	Patient 5	Patient 6
IFNγ	3.3 - 21629	1 - 2142	183 - 42609	3.2 – 6.3
IL-2	5.4 - 2845	7 – 1889	4311 - 108056	412 – 1722
TNF	16 - 18370	41 - 527	76499 - 196093	74 – 5351
IL-6	3 – 20759	40 – 1079	6277 – 108740	934 – 73124
IL-4	3.7 – 4095	2 – 179	6248 – 208167	97 - 909
IL-5	4 - 4437	8 - 112	208 – 27534	4 – 320
IL-10	15 – 3271	53 – 618	6179 – 58546	15 - 179

Table 4.4 A wide range of concentration values for each cytokine was observed both between the responding patients and within individual patient results. All numbers are in fg/ml.

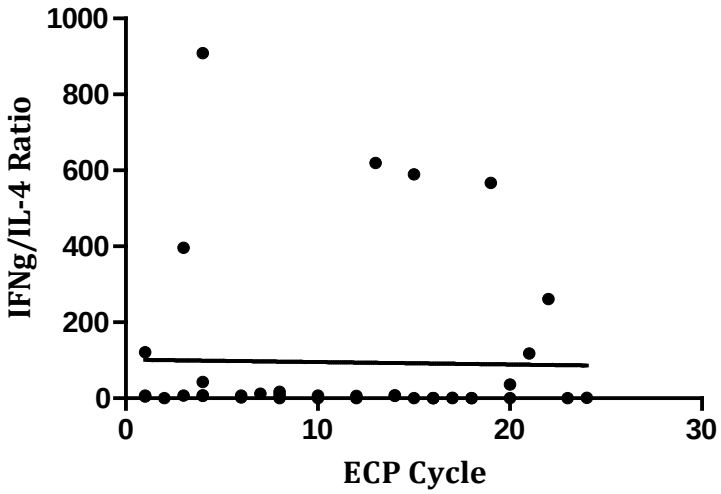


Figure 4.16 IFNγ:IL-4 ratio in the responding patients with progression of ECP demonstrated no overall change (p=0.90).

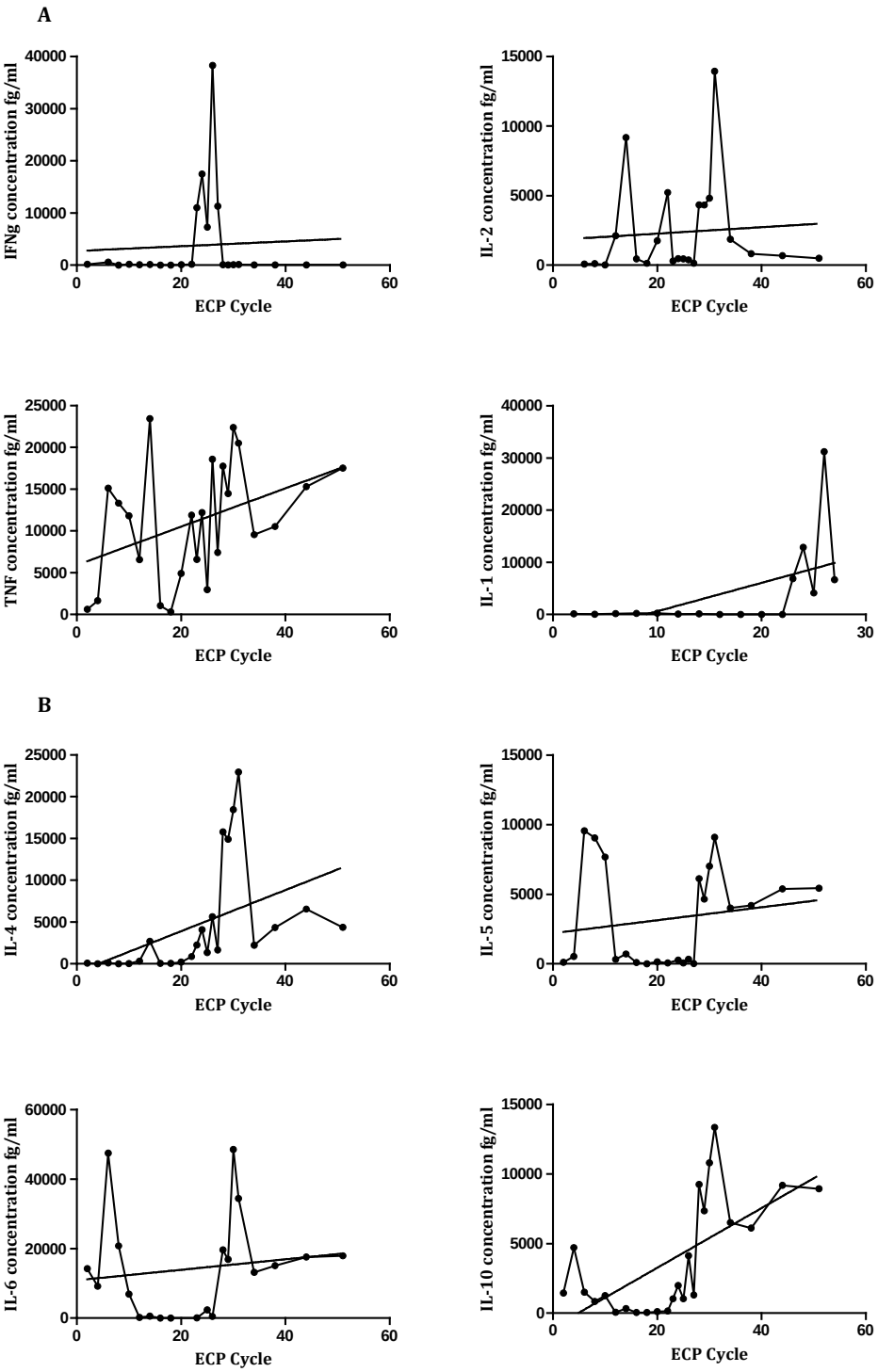


Figure 4.17 Cytokine profiling with progression of ECP therapy in patient 1 demonstrated (A) an increase in Th1 serum cytokines IL-2 (p=0.74), TNF (p=0.04) and IL-1 (p=0.03), with an isolated high peak of IFNγ between cycles 23-27 which correlated with intercurrent influenza A infection. (B) An overall increase in Th2 cytokines IL-4 (p=0.02), IL-5 (p=0.44), IL-6 (p=0.56) and IL-10 (p=0.0006) was also observed.

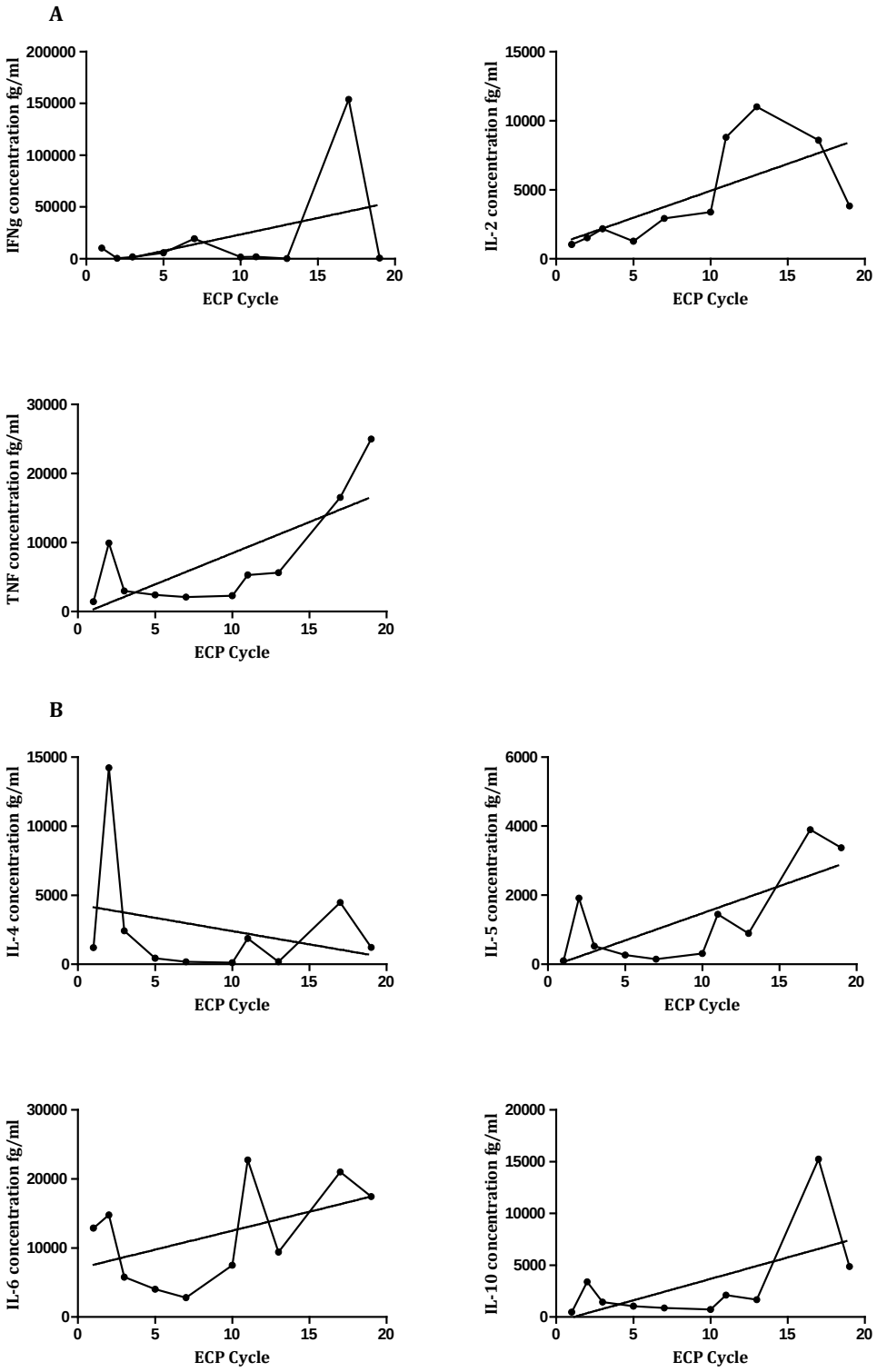


Figure 4.18 Cytokine profiling with progression of ECP therapy in patient 8 demonstrated (A) increasing Th1 serum cytokines IFNγ (p=0.23), IL-2 (p=0.03), TNF (p=0.02), with a high peak of IFNγ at cycle 17 which correlated with HHV6 positivity. IL-1 analysis was not performed on this patient. (B) Analysis of Th2 cytokines demonstrated a decrease in IL-4 (p=0.43), and an increase in IL-5 (p=0.02), IL-6 (p=0.15) and IL-10 (p=0.08).

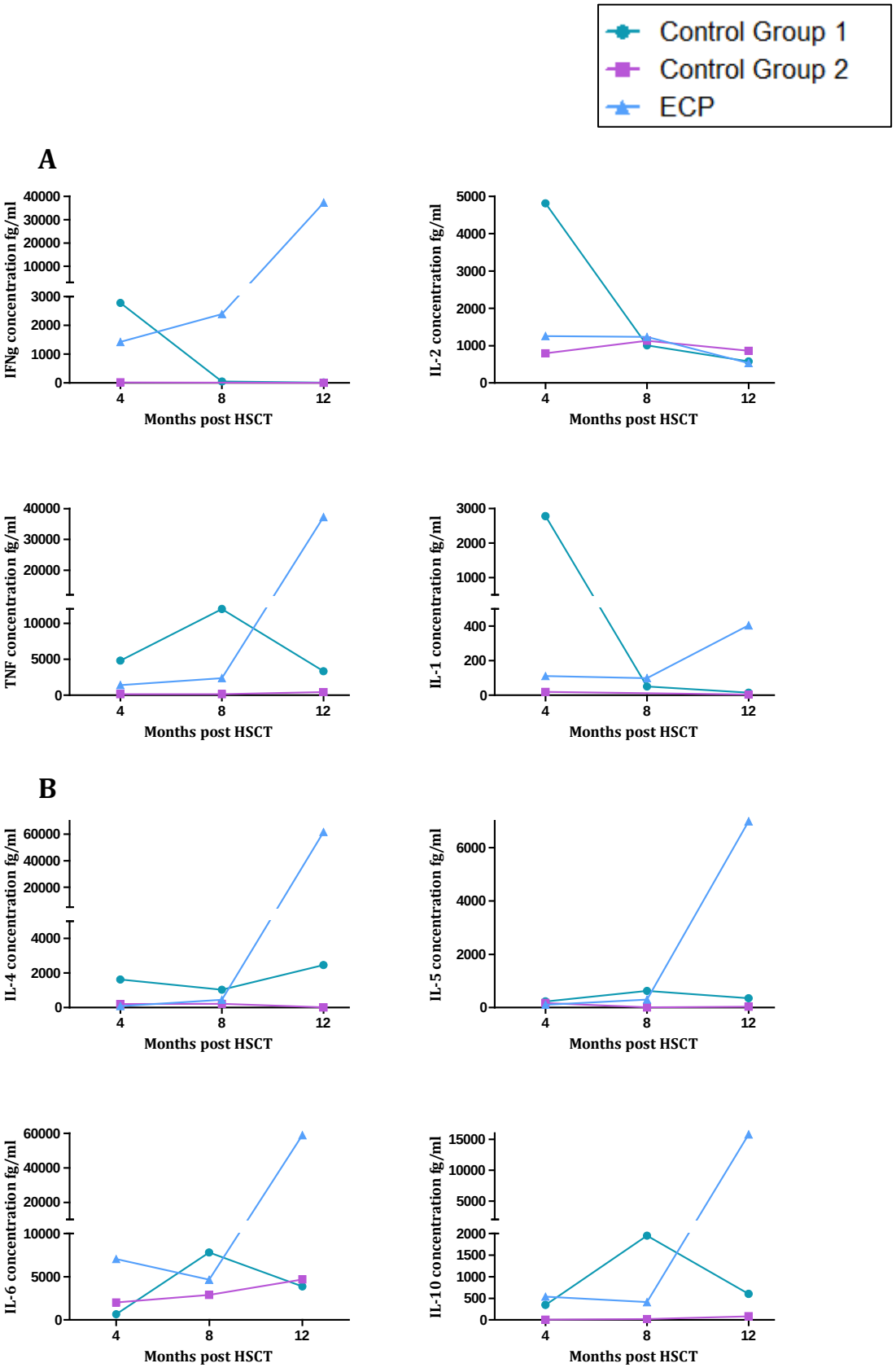


Figure 4.19 Median values of (A) Th1 cytokines and (B) Th2 cytokines for control groups 1 (green) and 2 (pink) and the ECP group (blue) at 4, 8 and 12 months post-HSCT.

4.6 Discussion

In vitro studies have demonstrated that ECP induces DC differentiation from monocytes, and, following phagocytosis of apoptosed T-lymphocytes, promotes development of a tol-DC phenotype. The data shown here tentatively supports an *in vivo* effect of ECP on DC populations, but the co-existence of multiple variables that also impact the rate of DC recovery and DC phenotype and function embroils the ability to clearly answer the question of whether the effect of ECP on DCs is facilitation of normal haematopoiesis and DC reconstitution post-HSCT and/or if ECP actively influences DC numbers, phenotype and function, accounting for, or at least contributing towards, the therapeutic effect. A repeated pattern observed was that in the recovery of absolute cell counts (DC subsets, Tregs and naive T-lymphocytes) the ECP group followed a similar trend to that seen in control group 1, although at an inferior rate. In contrast, control group 2 did not exhibit the same pattern for any of the cell populations studied.

In previous studies, corticosteroids have shown to have a DC depleting effect. An inverse relationship between the corticosteroid dose and the absolute numbers of DCs was observed in the responding ECP patients. However, patients 1, 3 and 8 displayed very low numbers of both pDCs and cDCs at all doses of corticosteroids, with no increase observed even at the lowest doses achieved, suggesting involvement of other factors in suppressing DC recovery. The type of graft has also been shown to impact DC populations. Arpinati *et al* demonstrated that PBSC grafts contain higher numbers of pDCs compared to cDCs potentially due to Th2 polarisation induced by G-CSF, with the opposite found in BM grafts. The only clinical outcome impacted by the graft DC composition was cGVHD, with higher pDC numbers being associated with increased risk[179]. Supporting DC recovery post-HSCT, for example by reducing corticosteroids, is important as faster recovery of DCs, and in particular pDCs, is associated with lower risk of relapse and non-relapse mortality and overall better survival[213,287,288].

To help decipher how the pattern of DC recovery in the ECP patients reflected the normal course of DC reconstitution post-HSCT, and to aid in the evaluation of the impact of other variables, in particular the use of corticosteroids, the DC absolute counts and the cDC/pDC ratios were compared with the control groups at 4, 8 and 12 months post-HSCT. The cDC population rose steadily in control group 1 with no aGVHD, reaching normal levels by 12 months. Plasmacytoid DC reconstitution was slower compared to reconstitution of the cDC population, consistent with previous studies, with absolute

numbers at 12 months remaining below the lower limit of normal. Within the ECP group, an overall increase in both pDC and cDC populations was seen with treatment progression. At 4 months post-HSCT, the absolute numbers of both DC subsets in the ECP group was lower compared to both control groups, but increased at 8 and 12 months, and overall post-HSCT recovery was similar to control group 1, although occurred at an inferior rate. Acute GVHD and immunosuppressive medications can hinder haematopoietic recovery and this combination likely contributed towards the low cDC and pDC numbers at 4 months, and towards the persistently low numbers in patients 1 and 8 who also displayed no evidence of thymopoietic recovery. Other potential contributing factors for the strikingly lower circulating DC numbers in these patients include homing of DCs to inflamed tissues and removal by activated donor T-lymphocytes.

Interestingly, control group 2 (aGVHD not treated with ECP) displayed the highest median counts of both cDCs and pDCs at 4 months post-HSCT, followed by a decline in both subsets at 8 and 12 months. At 4 months, no patient in control group 2 remained on high dose corticosteroids; 2 had been weaned completely, 2 remained on a physiological dose and 2 patients were on a low dose (0.1 - 0.2mg/kg). It is unclear why this initial higher number of DCs was observed, as although high DC counts in the early period post-HSCT have been seen in children who later develop aGVHD[200], more often aGVHD is associated with lower DC counts by 2 months post-HSCT. After 4 months, the decline in absolute DC counts in control group 2 could reflect ongoing subclinical aGVHD with corticosteroid weaning. Importantly, two patients from control group 2 were later re-started on corticosteroids for treatment of autoimmune haemolytic anaemia which may have impacted results at the later time points. Furthermore, this analysis is of a small number of patients and additional data collection is required.

The increase in DC subsets in the ECP group was associated with a concurrent decline in the cDC/pDC ratio indicating that a relatively higher increase occurred in the pDC population during this period. The cDC/pDC ratio in the ECP group displayed a different post-HSCT pattern compared to both control groups. A notable jump in the ECP group median pDC counts was observed between 4 and 8 months, resulting in the cDC/pDC ratio nadir at 8 months (when all patients were on ECP treatment, with the exception of patient 6 who completed ECP at 6 months post-HSCT), whereas a peak in the ratio was observed at the same time point in both control groups. By 12 months post-HSCT (at which stage the majority of patients had completed ECP), the cDC/pDC ratio was

comparable between the 3 groups. A decline in the cDC/pDC ratio in the treatment of aGVHD with ECP was described previously by Shiue *et al*, with the opposite occurring in the context of CTCL treatment[228], although how this shift to encourage pDC development occurs is not yet known. There was no evidence of increased pDC CCR9 expression in the ECP group, although this may be because $CCR9^+$ pDCs are predominantly found in lymphoid tissues, at least in murine models[192].

The similar, although inferior, increasing trend in DC subsets compared to control group 1, but the different pattern in the ratio of subsets suggests that ECP may support both DC reconstitution and impact the balance of circulating subsets. The different pattern of DCs in control group 2 suggests factors other than corticosteroids impact DC recovery, as a steady increase was not observed with corticosteroid cessation, although interpretation is complicated by two patients re-starting corticosteroid treatment. Two other important points to highlight include the fact that the time frame of ECP does not correlate with the post-HSCT time frame, with patients commenced on ECP at different time points in their post-HSCT course, and secondly for ECP patients 1, 3 and 8, the absolute numbers of DCs were extremely low, thus the cDC/pDC ratio should be interpreted with caution, as small changes in the absolute numbers can have a disproportionate impact on the ratio. These results were included in the analysis and this limitation should be noted.

Plasmacytoid DCs are a subset known to have tolerogenic properties and have shown to assume an important role in allo-HSCT, facilitating engraftment and mediating GVL activity and aGVHD tolerance[196]. However, high pDC counts have been reported to be associated with higher risk of relapse[213]. It is interesting to consider that excessive pDCs could be detrimental by promoting an 'overly-tolerant' environment, particularly to those whose indication for HSCT was high-risk malignancy. Patient 7, who despite displaying early evidence of thymic recovery, suffered from relapse at 7 months post-HSCT, following 17 cycles of ECP. At this time, the cDC/pDC ratio was 0.8, indicating a predominant circulating pDC population. Whilst this does not indicate that ECP increases the risk of relapse, it suggests the need for further monitoring of cellular populations during treatment to see if such patterns are replicated and how they concur with the clinical situation.

Functional DC immaturity has been described to be protective against aGVHD[289]. Corticosteroids down-regulate expression of co-stimulatory molecules and impair DC

immunostimulatory capacity, at least in *in vitro* generated mo-DCs using high dose dexamethasone[290]. The true *in vivo* effects and effects at different pharmacological doses is not known. Patient 1 from control group 2 was on low dose corticosteroids at both time points measured, and both cDC2 and pDC CD80, CD86 and CD83 expression was similar compared to the patients in control group 1 with no aGVHD or corticosteroids. This suggests that low dose corticosteroids do not significantly impact expression of these markers, but does not inform us regarding the impact of higher doses. In some of the responding ECP patients there was a gradual incline in expression observed with ECP treatment progression, which could be induced upon corticosteroid weaning. However, several patients demonstrated high MFI values at the onset of ECP when they would be receiving the highest dose of corticosteroids. Furthermore, results from patient 1 support the involvement of other factors involved in reducing expression, as higher expression was observed upon re-initiation of high dose corticosteroids for management of liver aGVHD, although this could also be related to increased disease activity.

The results demonstrate that DC expression of co-stimulatory molecules CD80, CD86 and CD83 was lower in the responding ECP patients which may contribute to a reduction in aGVHD activity due to lack of adequate costimulatory help. Although there are some inconsistencies, there was a pattern in the responding patients of higher expression at the onset of ECP treatment, which may reflect the active state of aGVHD, followed by a reduction in expression following initiation of ECP treatment. In some cases expression continues to decline or remains stable, and for some expression gradually increases again by the end of ECP. Lack of a consistent pattern between the patients, and between specific molecules, likely is related to the heterogeneity of the patients and the presence of individual factors impacting the DC environment, such as intercurrent viral infections or reactivation and concurrent medications and doses specific to each patient.

Reduced expression early in ECP treatment followed by an increase by the end treatment was the pattern most frequently observed in the responding ECP patients. Early in ECP treatment, aGVHD disease is active, as depicted by the high frequency of circulating activated donor-derived and donor thymic-educated T-lymphocytes. These activated T-lymphocytes undergo preferential apoptosis during ECP, are phagocytosed by DCs and this is thought to generate a tolerogenic phenotype. Later in the course of ECP, activated T-lymphocytes are reduced or have returned to normal levels. Resolution

of aGVHD has allowed thymic function and the normal process of central tolerance to return, with the export of donor stem cell-derived but now recipient thymic-educated self-tolerant T-lymphocytes into the circulation, precluding resurgence of aGVHD. If the frequency of activated T-lymphocytes has reduced, reduced ECP-induced apoptosis leads to reduced uptake of apoptotic alloreactive T-lymphocytes and less tol-DCs. Curtailment of ECP apoptosis of activated T-lymphocytes, in the setting of resolved aGVHD and systemic inflammation, means that tol-DCs are redundant. Until this point, in the absence of thymic function, ECP may play the role of a stop-gap tolerising machine, increasing peripheral mechanisms of achieving tolerance. The return of thymic function and reinstatement of central tolerance mechanisms allows normal DC function and phenotype to resume, as suggested by the increasing co-stimulatory marker expression with treatment progression, in parallel with increasing thymic function and resolution of aGVHD. The tol-DC environment is beneficial, but importantly, homeostasis is returned, as it would be undesirable for a predominant tolerogenic environment to persist beyond necessary, or indeed permanently. However, if this hypothesis is true, patient 1, with continued absent thymic function and thus absent functional central tolerance, has decreasing Treg frequency and does not demonstrate firm evidence of a predominant tol-DC environment even in the presence of a high frequency of activated T-lymphocytes. The results are complicated to interpret due to the presence of a complicated clinical course and the possible presence of irreversible thymic and pulmonary damage, but lack of upregulation of peripheral tolerance with ECP treatment potentially could be a contributing factor towards the lack of clinical response and the development of liver aGVHD while on treatment. Patient 8, although limited data were available at the time of analysis, demonstrated reduced expression of CD83 but otherwise did not show convincing evidence of a tol-DC phenotype, and apart from one isolated peak in Tregs at cycle 18, the Treg frequency remained within the normal parameters. Investigation of further responding and non-responding patients is required to elucidate this further.

While this theory may explain the transition in DC phenotype in the setting of aGVHD, it introduces an interesting segue of questions regarding other clinical situations. For example, in CMV infection, activated anti-CMV cytotoxic lymphocytes could undergo apoptosis during ECP leading to tolerance to CMV antigens. In the treatment of CTCL, a cytotoxic immune response is generated against the tumour antigens rather than tolerance. It remains unclear how the deviation in the DC phenotype occurs, how specific

apoptosed antigens trigger immune tolerance or immune upregulation. While a stable tol-DC phenotype directed towards the pathogenic target is desirable, it is also important that an appropriate immune-stimulatory response can be generated against non-allograft stimuli such as intercurrent infections.

There are important caveats to consider when interpreting these results. Firstly, the majority of DCs reside within the tissues, with a smaller proportion present in the circulation, therefore sampling this population may not provide a true reflection of the developmental status of tissue-resident DCs or be an accurate representation of the entire DC population. Furthermore, there is a multitude of other confounding variables to consider that may modulate DC phenotype and function, such as the conditioning given pre-HSCT (RIC is associated with reduced co-stimulatory molecule expression and allostimulatory capacity in the first 3-6 months post-HSCT but MA conditioning is not[206]), medications[291] and even vitamin D levels[292]. Intermittent snapshots in the treatment course are examined here, and the DC phenotype in between these time points is not known. More detailed sequential examination of the DC phenotype earlier in the ECP treatment course may yield important information regarding how quickly changes are induced, and continued follow-up examination of the DC phenotype after completion of ECP treatment may reveal if/when a normal stable DC phenotype is restored. While these results support a potential *in vivo* effect of ECP on the DC phenotype, gaps remain in our knowledge regarding the function of these tol-DCs, in the context of ECP including the mode of their regulatory action (or if a combination of multiple inhibitory pathways is involved). Understanding the predominant mechanism, or several simultaneous mechanisms, in the context of aGVHD would also help in the development of novel therapies for inflammatory diseases, or to counter-regulate for the development of cancer immunotherapies.

A bidirectional relationship exists between tol-DCs and Tregs, with tol-DCs promoting Treg generation, and Tregs conversely promote a DC tolerogenic phenotype. While the role of Tregs appears to be important in ECP, it is not clear how and where these Tregs are generated, whether they are dependent on DCs or whether they can be induced independently, or whether it is a combination of both. Results from this study did not demonstrate convincing evidence of increased $CD25^{hi}CD127^{lo}$ Treg populations during the course of ECP treatment, although the frequency was intermittently elevated at certain time points for all patients. Analysis of $FOXP3^{+}$ Tregs similarly displayed frequencies which were largely within the normal range, and although the time points

measured were more limited, apart from patient 6, a decline in FOXP3$^+$ Treg frequency was observed. There was an increase in the absolute Treg counts observed in responding patients towards the end of treatment, but without a corresponding increase in frequency, suggesting this incline was more attributable to the immune recovery also observed in this group. Interestingly, patient 1 demonstrated a different pattern with elevated Treg frequency at the beginning of ECP, with a decline observed over the ensuing ECP cycles. This may be related to exposure to prolonged high dose corticosteroids prior to ECP, although re-initiation of high dose corticosteroids later in the treatment course did not reach the high frequencies observed previously. A high frequency of Helios$^+$ was seen within the FOXP3$^+$ Treg population in the range of 80-90%, with no significant change between the beginning and end of ECP treatment, thus not demonstrating a correlation with increasing thymic output. In patients 1 and 8, the Helios$^+$ frequency was similarly high. As both these patients demonstrated negligible thymic output, this casts further doubt over the reliability of Helios as marker of thymic origin. Several recent studies have also reinforced the claim that Helios is not a marker of nTregs, and represents a stable FOXP3$^+$ population with enhanced immunosuppressive capacity[293].

Whilst these results are not convincing regarding an increase in Tregs as a result of ECP, one explanation for this could be that although the frequency of circulating Tregs remained largely within the normal range, activated Tregs may also have migrated to the sites of tissue inflammation. Importantly it is also unknown what the functional capacity of the Tregs identified is, as improvement in suppressive capacity could be induced by ECP, and this requires further investigation. The relatively high level of Helios expression by Tregs may imply enhanced suppressive capacity. The analysis was also limited to CD4$^+$ Tregs, and a CD8$^+$FOXP3$^+$ Treg population has also been identified with suppressive capacity and attenuating effects on aGVHD murine models[294] although their role in humans is not well studied.

The responding patients demonstrated a decline in Th1 cytokines and an incline in Th2 cytokines with ECP progression, supporting the theory that following the infusion of apoptotic cells, there is a deviation in the Th1/Th2 cytokine balance with ECP treatment. Th2 cytokines inhibit the production of Th1 cytokines, contributing to resolution of tissue damage caused by aGVHD. However, this does not prove that this cytokine shift is an ECP-induced effect, as an alternative explanation could be that this pattern is an indirect consequence of associated improvement in aGVHD[270]. Although a similar

change in cytokine profiles was not observed in control group 2, which could support an ECP-induced effect, this was based on a limited number of samples and further investigation of a larger patient cohort is needed.

In contrast to the responding patients, the non-responding ECP patients exhibited increasing Th1 and Th2 cytokines, particularly of TNF. It is interesting that even with increased Th2 cytokine levels there was no evidence of Th1 cytokines being 'switched off'. Bronchiolitis obliterans syndrome is associated with high levels of pro-inflammatory cytokines including TNF[295]. Prevailing tissue damage with Th1 cytokine production could contribute to the ongoing cytokine dysregulation. Understanding the cytokine patterns in these patients is important to identify avenues for further investigation as to why certain patients do not respond to ECP and to potentially introduce new therapeutic targets that could be used synergistically, for example the addition of an anti-TNF agent.

It is important to highlight the limitations to the cytokine results. Th1 and Th2 cytokine profiling revealed a wide variation in inter-individual serum cytokine levels, particularly patient 5 who displayed a higher range in all cytokines measured. This marked degree of individual variation, potentially due to underlying single nucleotide polymorphisms in cytokine genes, must be considered in the interpretation of results. The cytokine trajectories observed in the responding group of patients demonstrate heteroscedasticity which can impact regression analyses, therefore the inferences from statistical results should be interpreted with caution. Serum cytokine levels may not reflect the concentrations of cytokines in specific tissues of interest, such as in secondary lymphoid tissues where interaction with naïve T-lymphocytes and subsequent polarisation occurs, or provide information on the specific cellular source of each cytokine (for example, if the rise in IL-10 derives from the pDC population). There are also several confounding factors that impact the levels of the cytokines studied, many of which the patients were exposed to, but to different degrees and for varying durations complicating interpretation of results. Infliximab, an anti-TNF monoclonal antibody, was administered to all but one of the ECP patients, which may have contributed to the lower TNF levels observed compared to control group 1 at 4 months post-HSCT. Ciclosporin, which inhibits IL-2 transcription, was given to all of the ECP patients and all but one patient from each control group, but was weaned more quickly in control group 1 and was weaned at variable rates in the patients with aGVHD. Corticosteroids are known to broadly reduce both Th1 and Th2 cytokines, however the

dose at which this occurs *in vivo* and the impact of varying doses of corticosteroids on cytokine levels is not known. Intercurrent infections also impact cytokine levels, as demonstrated by the isolated peaks in IFNγ observed with concurrent viral infections in patients 1, 3 and 8. This highlights the pleiotropic nature of the highly complex cytokine network; high levels of pro-inflammatory cytokines early after HSCT may be contributory to aGVHD, but a later increase may be contributing to protection from an invading microbe, even in the absence of overt clinical infection. The effects of cytokines can vary depending on the tissue or the level of exposure. IFNγ can be damaging in the GIT but protective in pulmonary parenchyma, and 'high-dose' IL-2 stimulates T-lymphocyte proliferation, but 'low dose' has been shown to support Treg development[265].

Despite the aforementioned pitfalls, and with these taken into consideration, this is the first time the *in vivo* Th1 and Th2 cytokine patterns with ECP progression have been examined in detail, and while the results support previous *in vitro* studies, examination of further patients is necessary to determine if the results are consistent and how it compares with a larger control cohort. Rather than a simplistic perception of a Th1/Th2 imbalance with a Th1 predominance in aGVHD, it is likely to be more complex, with the pathogenic or protective effects mediated by the cytokine in question dependent upon the type of cell that produces it, the type of tissue it encounters, and the interaction with other cytokines.

It is possible that different tolerance-inducing mechanisms develop in different patients with different organ-specific damage. In the setting of aGVHD affecting the GIT the cytokine milieu and predominant inflammatory cellular infiltrate is different to inflammation involving the skin or lung[275]. The microenvironment is also affected by co-existing or pre-existing pathologies. Consequently, achieving tolerance in the gut mucosa may involve different pathways to those in the skin or lungs. If APCs present the apoptosed antigens and interact with cognate T-lymphocytes at the site of inflammation, the subsequent steps taken to achieve tolerance may be influenced by the tissue-specific environment. Thus the heterogeneity of the patients may be one reason why a consistent pattern was not seen. In addition, despite this study involving regular blood sampling and more detailed analysis of ECP progression than previous studies, results still reflect only a snapshot at that point. Nonetheless, interesting trends were observed, that support *in vitro* work, and should encourage further investigation on a larger patient cohort.

4.7 Conclusion

The aim after allo-HSCT is to achieve a balance between effector and regulatory T-lymphocytes. Peripheral tolerance mechanisms are essential to protect the body from misguided or excessive immune responses. These protective mechanisms include the generation of tol-DCs and Tregs. If central tolerance mechanisms are deficient, as in the setting of thymic dysfunction, it is interesting to consider the possibility that peripheral tolerance mechanisms can be temporarily increased to counteract this imbalance, and that ECP could be used as a tool in this regard.

The data presented here support an ECP-induced impact on the balance of DC subsets and phenotype, and a shift from a Th1 to Th2 cytokine profile. However, the difficulties in interpreting the results and limitations in the analyses have been outlined and should be considered before drawing firm conclusions. The small number of patients studied means that the analysis is predominantly descriptive in nature. Further work would include more in depth examination of the DC phenotype, such as detailed analysis during a week of ECP treatment, to gather further information with regard to the timing of potential ECP-induced changes, and extension of the analysis to include other markers such as ILT3, IL4 and IDO. Helios did not appear to be a reliable marker of nTregs in this study. However, the high frequency of Helios$^+$ may represent a highly suppressive Treg population, and functional analysis is needed to investigate this further, if adequate cell numbers can be isolated. Further examination of the Treg phenotype would also be valuable, for example measuring CTLA4 and LAP expression over time.

Understanding the different mechanisms underlying immune tolerance at play, perhaps of different mechanisms specific to the site of tissue damage, would help in the endeavour to remove non-specific globally immunosuppressive therapies from our therapeutic repertoire, and replace them with tolerance-inducing, tissue-specific therapies which will undoubtedly improve patient outcomes.

Chapter 5 . Assessment of Health Related Quality of Life in Paediatric Patients treated with ECP

5.1 Background

5.1.1 Introduction

Health-related quality of life (QOL) assessment refers to an individual's self-evaluation of his/her wellbeing and functioning, and the influence that his/her health and experience of the health care system have. Information harnessed from health-related QOL assessment as part of health care research has important applications: it is essential in the evaluation of treatment outcomes and improves our understanding of the overall risk/benefit profile of therapies, provision of information to help improve delivery of services and therapies, and aid in clinical decision-making, so that patient/family-centred care is always at the forefront. The importance and benefits of health-related QOL measurement have been increasingly recognized, and consequently, integrated into health outcome evaluation in clinical trials and other health care service research. Health-related QOL does not replace quantitative measures of clinical efficacy, but provides a more comprehensive evaluation of services from the patient and family perspective by assessment in tandem.

5.1.2 Paediatric QOL assessment

Paediatric health-related QOL assessment must be delivered with appropriate consideration given to the age and cognitive development of the child and should, when possible, include a separate child and parent report to allow perspective from both. Assessment of health-related QOL in children, including those undergoing HSCT, has increased over the last 15 years due to greater availability of improved standardised tools for evaluation of health outcomes[296].

The Pediatric Quality of Life Inventory® 4.0 (PedsQL) Generic Core Scales were designed to measure the core health dimensions as defined by the World Health Organisation; physical, emotional, social and school functioning. It consists of parallel child self-report and parent proxy-report formats. For child self-report, age-specific versions are available: ages 5–7 (young child), 8–12 (child), and 13–18 (adolescent). Separate parent proxy-reports, designed to assess the parent's perceptions of the child's health-related QOL, are formatted for ages 2–4 (toddler), 5–7 (young child), 8–12 (child), and 13–18 (adolescent). A total score of all 23 items is given. Scores are scaled from 0 – 100 with a

higher score reflecting a higher QOL. The PedsQL Infant Scales measures five domains (physical functioning, physical symptoms, emotional functioning, social functioning and cognitive functioning) and consists of a parent-proxy report for infants 1-24 months old. The scoring methods used for the Infant Scales are identical to those used for the Generic Core Scales. Normal values for healthy UK children have been published[297].

5.1.3 Factors impacting paediatric health-related QOL

Paediatric health-related QOL can be affected by demographic factors. Age has been frequently assessed as potential factor impacting health-related QOL, although results have been mixed. In children undergoing curative treatment for cancer, some studies have demonstrated higher QOL in adolescents, but the opposite has also been reported[298]. Female gender and non-white children have also been associated with lower health-related QOL[299,300]. Family-related factors have also been investigated extensively with better family cohesion and resources, higher parental emotional functioning and parental involvement in care associated with higher QOL scores[298,301]. Mothers have been found to report a lower QOL for their child compare to the child themselves or the fathers[302] but other studies have found a good correlation between parent and child reports[303]. In children with cancer, the underlying cancer diagnosis, stage of and type of treatment have all shown to impact health-related QOL[298].

Although PedsQL has been shown to be a feasible, reliable and valid tool to evaluate paediatric health outcomes[304,305], discrepancies in child and parent reporting can arise due to information variance (when the child and parent form different conclusions of QOL based on different sources of information) and criterion variance (different conclusions reached by the child and parent because of comparing functioning to different standards, for example comparison with previous functioning ability or future expected functioning ability) and under rating by parents of children's QOL has been well reported[296]. Discrepancy between parent and child can be more pronounced in the setting of clinical complications such as aGVHD[296].

5.1.4 QOL and the impact of HSCT and aGVHD

The HCST process has a significant effect on both the physical and emotional health of recipients[306]. Patient are subject to intense conditioning regimens with the associated adverse side effects and vulnerability to infection, prolonged hospitalisation in

protective isolation, and risk of complications such as graft rejection and aGVHD. This is in addition to the impact of the underlying condition and the gravity of the diagnosis. Following discharge, patients remain at risk of complications and in protective isolation for several months. However, few studies have been performed that characterise the relationship between clinical outcomes of HSCT and parent/child ratings of paediatric QOL. There are also conflicting reports on whether health-related QOL improves with time following HSCT[302,307]. Those who develop aGVHD have a further decline in QOL compared to those who do not[308]. This is likely related not only to the direct effects of aGVHD, but also the more frequent and longer hospital admissions, immune suppression with their associated toxicities, and development of infectious complications that are more likely to occur. The development of severe complications including acute and chronic GVHD is associated with greater divergence in parent-proxy and child scores, with parents reporting lower QOL, particularly in physical functioning[296,309].

Difficulties in assessment of HSCT-related QOL arise due to heterogeneity in the patient population, such as demographic variability, different underlying diagnoses and different preparative regimens used. Lack of robust information regarding the impact of specific HSCT-related complications and outcomes is likely due to the relative infrequency of these events and the overall small numbers of patients available for evaluation. However, in order to enhance QOL for HSCT recipients, it is essential to better understand the short and long-term difficulties experienced by patients.

5.1.5 QOL and ECP

Despite positive reports regarding the efficacy and advantageous safety profile of ECP, there are risks and barriers to this therapeutic option that should be considered: it is an invasive therapy that requires long term central venous access which carries with it restrictions and risks of complications, it is only provided at certain specialist centres, which often means that patients and families have to travel long distances and the time, expense and disruption to family life that this imposes, and it is a time consuming treatment, with the procedure itself taking several hours. It is also an expensive therapy and requires specialist medical and nursing staff to provide the service, limiting the potential for more widespread application.

With these factors in mind, it is essential to evaluate if ECP improves the QOL of patients. However, the majority of research to date is related to cGVHD and performed on adults;

there is a scarcity of data on the effect of ECP on QOL in aGVHD and in children. Among 38 adults with cGVHD, 17/18 (94%) demonstrated an improvement in QOL after 6 months of ECP treatment, but 11/38 (29%) patients did not complete 6 months of treatment, reflecting the high mortality rate in steroid-refractory disease[310]. Remaining patients did not return the questionnaires, raising the possibility of selection bias, with those completing the questionnaires being the individuals more likely to have noticed an improvement in QOL. An improvement in QOL in adults with cGVHD treated with ECP was also reported by Flowers *et al*[311]. Other studies have used performance status as a substitute marker for QOL. Messina *et al* demonstrated that the median Lansky/Karnofsky performance score improved significantly from 60% before ECP to 100% (range 60–100%) after completion of ECP among paediatric patients with aGVHD, and a similar improvement was witnessed in those with cGVHD[27]. There is currently no data available regarding children with aGVHD treated with ECP using validated health-related QOL questionnaires and understanding QOL outcomes would allow for a more complete risk and benefit analysis of ECP treatment.

5.2 Aim

The aim was to assess the effect of ECP on health-related QOL using a validated questionnaire based assessment at the beginning of ECP therapy and upon completion of ECP therapy. Health-related QOL would be compared to QOL in the general population and to the two control groups at 4 months post-HSCT.

5.3 Methods

The PedsQL Generic Core Scales were administered to a total of 18 parents and 9 children. Parents and patients (when appropriate) were asked to complete one questionnaire to assess QOL at the start of ECP treatment and again upon completion of treatment. Control groups were asked to complete a questionnaire at 4 months post-HSCT. An interpreter was used for 2 patients. Responses to each question were scored 0 – 100. Total scores in each of the individual domains were calculated (Physical, Emotional, Social and School). A summary Psychosocial score was also calculated (a total of the individual Emotional, Social and School domain scores) and a Total score (a total of all domain scores). If the child was too unwell or did not want to participate, only the parent completed the questionnaire.

5.4 Results

Prior to ECP, 8 parents (2 fathers, 6 mothers) completed the questionnaire at baseline and, to date, 4 parents have completed the questionnaire at the end of ECP treatment. Two patients completed the questionnaire at baseline and, to date, 1 patient after completion of ECP treatment. To date, 4 parents and 2 patients from control group 1 (with no aGVHD), and 6 parents and 5 patients from control group 2 (aGVHD not requiring ECP) completed the questionnaire at 4 months post-HSCT (Table 5.1).

The mean total PedsQL scores for the ECP patients, as depicted by the parents, improved from 36.2 before ECP to 61.6 after ECP with all 4 parents reporting an improved QOL (median increase from 30.7 to 53.8). The mean total PedsQL parent-proxy score at 4 months post-HSCT for control group 1 was 67.4 and for control group 2 was 55.1. The mean total PedsQL patient score for control group 1 was 63.1 and for control group 2 was 58.6. All of the mean parent pre-ECP QOL scores were lower compared to the mean scores in both control groups. In the mean child pre-ECP QOL scores, the mean social score was higher compared to both control groups, and the mean emotional score was

the same as control group 1. However, these results are from a limited number of patients, particularly from the ECP group (n=2) and control group 1 (n=2).

In the parent-proxy QOL ratings, the mean psychosocial score (a combination of the emotional, social and school results) and the physical score was highest in control group 1, followed by control group 2, with the lowest scores reported in the pre-ECP group. However, the parent post-ECP psychosocial mean results were superior to control group 2 and similar to that reported in control group 1. In the child assessment, the mean pre-ECP psychosocial and physical scores were lower than both control groups, but were superior to both control groups post-ECP, although this only involved 1 ECP patient.

In the parent-proxy assessment, mean QOL results in all domains were lower in all of the groups compared to the mean UK normal values. In the child assessment, the mean total, physical and psychosocial scores were lower in all groups compared to the mean UK normal values. The post-ECP child report in the emotional domain and the control group 1 child report in the school domain were both higher compared to the UK mean values.

	UK normal values	Pre ECP	Post ECP	Control group 1	Control group 2
Parent Report					
Total	84.6	36.2	61.6	67.4	55.1
Psychosocial	82.2	37.3	64.5	64.3	58.1
Physical	89.1	27.4	62.6	73.7	47.9
Emotional	78.3	46.1	60.1	59.0	70.9
Social	86.8	46.7	66.3	68.8	59.2
School	81.5	32.3	57.5	64.6	44.4
Child Report					
Total	83.9	50.0	69.7	63.1	58.6
Psychosocial	81.8	44.5	71.65	67.3	59.4
Physical	88.5	45.3	75.0	71.9	56.9
Emotional	78.5	65.0	82.5	65.0	66.0
Social	87.7	75.0	60.0	60.0	70.0
School	78.9	37.5	72.5	83.4	41.0

Table 5.1 Overall mean results of the PedsQL questionnaires administered to parents and patients, and normal mean UK values. Overall, the majority of the QOL results from both parents and patients were lower pre-ECP compared to UK normal values and both control groups.

5.5 Discussion

Overall the results demonstrate that low QOL scores were reported pre-ECP by both parents and children, but lower scores were reported by parents compared to those by the children. The child pre-ECP ratings were particularly higher in the emotional and social domains, with the mean social scores actually higher compared to those in both control groups. Higher scores reported by adolescents compared to their caregivers, particularly in the less visible domains of psychosocial health have been previously reported[304] and this reinforces the importance of obtaining self-reports from children and adolescents, particularly in these less visible domains, as caregivers may not be fully aware. However, this represents a small number of patient reports; not all patients were included in the pre-ECP QOL assessment due to poor clinical condition at the time of assessment. Consequently, the child mean scores are biased towards the children who were not as unwell, thus falsely elevating the results. Regardless, the majority of the results are lower compared to the normal mean UK values and both control groups, reinforcing the significant impact aGVHD has on patient morbidity, particularly on those who are refractory to first-line treatment.

An improvement in parent-proxy QOL scores was observed post-ECP, demonstrating that those who successfully completed ECP treatment had an improved QOL. Although a similar trend was seen in the child QOL scores post-ECP, this is based on only one patient, limiting interpretation of this result. These results were subject to attrition bias, as the two patients who were withdrawn due to the development of complications were not included. In addition, two patients remain on ECP, with potentially irreversible tissue damage and chronic symptoms. QOL follow-up in these patients has not yet been performed, but is unlikely to mirror the improved ratings reported by those who responded. This highlights the benefit that would be obtained by identifying those who are likely to respond to ECP treatment, and those to who are unlikely to respond and would benefit from additional or alternative treatment. In addition, incorporating QOL assessment as a routine measure before, during and after ECP treatment for all patients and parents would provide a more comprehensive picture of QOL, providing valuable information from both responding and non-responding patients.

When comparing the post-ECP results with those of the control groups, it is encouraging to see the comparability with control group 1. We can only deduce from this that health-related QOL post-ECP is similar to that reported in patients who have an uncomplicated aGVHD-free course at 4 months post-HSCT. Importantly, the time frames are different

between these two groups, with the ECP patients being farther out from the HSCT date (between 6-12 months post-HSCT). Health-related QOL scores were lower in control group 2 compared to control group 1 at 4 months post-HSCT, likely reflecting the short-term morbidity endured as a result of aGVHD and follow-up would be valuable to assess the long-term impact on QOL. Longer follow-up is also required to discern if the QOL in the 3 patient groups reaches par with the normal mean UK values.

Limitations included the small patient/parent number evaluated, particularly of child self-reports and the results are from a single institution, limiting generalisability. The population studied was heterogeneous with different diagnoses, ages, different preparative regimens, different complications and toxicities incurred over the peri-HSCT period, all of which may impact individual QOL. It is difficult to exclude placebo effect as these scenarios were obviously conducted in a non-blinded manner. In addition, this assessment, while beneficial in obtaining an overall general QOL impression, did not include any feedback specific to the ECP treatment process itself which would be valuable in evaluation of the service and identification of treatment-specific factors that impact QOL either positively or negatively.

5.6 Conclusion

Despite the limitations, the results yield important information that ECP does result in improvement in QOL in patients in those who respond successfully to treatment, at least from a parental perspective. More data is required from a child's perspective, and every effort should be made to include them in this aspect of service evaluation as understanding of risk and protective factors that influence mental health and well-being are imperative to allow optimisation of service delivery. QOL evaluation during treatment would also be important to assess QOL in those who are not responding, or who later develop complications. The results are encouraging but further data collection, with longer follow-up, is essential to build a more complete picture, and to identify specific QOL factors that contribute to the lower QOL in the ECP patients and in the patients from control group 2 which will help us to identify patients who are at risk and tailor management plans and focus resource allocation appropriately.

Chapter 6 . Conclusion

Immune depletion is an anticipated inevitable consequence of HSCT and restoration of the immune system is vital to achieve treatment success. Following engraftment and recovery of the innate immune system, recovery of adaptive immunity is the next critical step, of which a crucial component is thymic production of new antigen-inexperienced self-tolerant T-lymphocytes, able to recognise and appropriately respond to a hugely diverse range of foreign antigens including infectious pathogens and tumour antigens, protecting the patient from these noxious invaders. Normal thymopoiesis requires an intact and undisrupted thymic microenvironment, which is exquisitely sensitive to acute insults such as infections, conditioning therapies, and aGVHD, and undergoes chronic involution as part of the ageing process. However, despite this vulnerability, the thymus is capable of regeneration following injury, allowing restoration of function. Protection of thymic function, or supporting regeneration, is essential for restoration of strong and long-lasting adaptive immunity. As the rates of HSCT survival continue to climb and patients live into adulthood, new factors to consider which optimise long-term outcome and quality of life take increasing importance. Included in this is optimisation of T-lymphocyte reconstitution, both short-term and long-term, by developing therapies that protect the thymus and allow the process of adaptive immune recovery to occur in an undisturbed environment as much as is possible.

This study demonstrated the negative impact that aGVHD has on T-lymphocyte immune reconstitution in paediatric patients following allo-HSCT, with inferior rates of naive T-lymphocyte recovery observed in both the retrospective and prospective groups with aGVHD compared to patients with no aGVHD. This effect may also extend to recovery of DCs and Tregs, as a similar pattern was also observed when examining these populations in the prospective groups. Importantly, ECP therapy appeared to facilitate T-lymphocyte recovery, particularly when started early, with patients who responded successfully to treatment demonstrating good naïve T-lymphocyte recovery both quantitatively and qualitatively.

The results raised many important and thought provoking questions which require further exploration. Firstly, not all patients demonstrated thymic recovery (at least to the point of follow-up), raising the question of whether there is a limit to the degree of damage that can inflicted on the thymus. Preventing this ‘point of no return’ is essential,

as otherwise patients are faced with a bleak clinical outcome. This highlights the need for improved understanding of the limits of thymic regenerative capacity and of the regenerative process, pursuit of thymic 'protective' management strategies and further development of therapies that can promote regeneration, as well as a biomarker that can monitor for and diagnose thymic damage. Secondly, control group 2, who had aGVHD not requiring ECP and therefore had clinically more 'mild' disease appeared, in this small cohort, to potentially have inferior naïve T-lymphocyte immune reconstitution compared to the ECP group who had more severe disease and required more intense immune suppression initially. Complete data collection, further examination of other cell populations such as B-lymphocytes and expansion of patient numbers is essential, as this subgroup may consequently be at higher risk of complications and require a different therapeutic approach. Thirdly, although the responding ECP group demonstrated good immune recovery, this remained inferior to control group 1 at 8 and 12 months post-HSCT. Strategies to narrow this gap further should be explored, as well as longer follow-up to examine if this gap resolves with time.

In the context of allo-HSCT, control is needed of the circulating donor-derived, donor thymic-educated T-lymphocytes. Normally, non-specific suppressive therapies such as CSA, MMF and corticosteroids are utilised to quell the potential damaging effects of these cells, but which also mitigate their beneficial effects such as anti-virus memory T-lymphocytes and GVL activity, until such time that donor-derived, recipient thymic-educated T-lymphocytes have entered the circulation when thymic function and central tolerance are restored. In aGVHD, during this period, patients are suspended in a precarious balance between controlling disease activity, and the risk of complications associated with generalised immune suppression as well as their other toxic side effects. However, the time frame is transient (assuming thymic recovery occurs), and perhaps, as suggested here, promoting a more tolerant environment specific to the pathogenic T-lymphocytes during this period may be more conducive to faster and more robust immune recovery by sparing the thymus and allowing undisturbed thymopoiesis, whilst continuing to avail of the effects from beneficial T-lymphocytes. Patients from control group 1 demonstrated steady naïve T-lymphocyte recovery, but perhaps this could be improved further by replacement of aGVHD prophylaxis with immune modulating therapies, or minimising immune suppression by using it in combination with immune modulation, and regulation of pathogenic T-lymphocytes causing subclinical thymic damage.

Modern therapies are often developed initially by identifying a potential molecular target and demonstrating proof of concept using an experimental model. Knowledge of the target and the mechanism of action lead to development of a new drug, later followed by demonstration of safety and efficacy in humans. ECP has followed the opposite path, with safety and efficacy demonstrated with years of clinical experience, but little known with certainty with regard to the underlying mechanisms. Unravelling the *in vivo* effect of ECP in this backwards manner is complicated by the multitude of confounding variables and heterogeneous nature of the patients who receive ECP. Significant work has been made in *in vitro* and *ex vivo* to explore the mechanisms at play, and the data here, although tainted by the limitations previously outlined, identified differences at cellular level between patients receiving ECP compared to the control patients, and between responding and non-responding ECP patients (in terms of clinical response and thymic recovery), which support some of the concepts that have been suggested, including promotion of pDC predominant environment, a tol-DC phenotype with down-regulation of co-stimulatory molecule expression, and a Th1 to Th2 cytokine shift. Further exploration may explain if the differences observed are a reason for, or a consequence of, lack of a response to ECP treatment and may help to dictate treatment regimens. In addition, understanding of the mechanisms of the specific tolerance induced in ECP may also us to harness these concepts and allow development of new tolerance-promoting therapeutic targets/cellular therapies that could be used in the setting of aGVHD, but also potentially extend application of these therapies to other areas of medicine such as solid organ transplantation.

The patients studied here represent a vulnerable group of individuals, either with a chronic underlying PID or a high-risk malignancy prior to transplant. Successful cure and return to a normal life for these children is our overriding aim. Assessment of quality of life before, during and after the treatment process is essential to ensure that we are reaching this target, and to identify areas for improvement or particularly vulnerable groups. ECP has many positive aspects as a therapeutic modality, but is not without its' disadvantages including the requirement for a central venous line, access to treatment, time and cost. It is reassuring that an improvement in quality of life was shown in the parental assessment of the responding ECP patients, but data obtained from the children was limited making it difficult to draw the same conclusion and one cannot assume the same outcome would be reported by the non-responding or withdrawn patients. While taking into account difficulties with age, development and

clinical condition, more effort is needed to assess the child's perception as this is likely to provide valuable information and unidentified aspects that could be improved. Regular quality of life assessment during ECP treatment of all patients and parents is essential to provide a more complete perspective. In addition, specific feedback related to the ECP treatment (as opposed to the general quality of life assessed here) would be important to further improve the treatment experience.

The aim post-HSCT is to achieve specific and long-lasting immune tolerance to the graft and complete robust immune reconstitution. Immune tolerance is achieved both centrally and peripherally. Ultimately, central tolerance mediated in the thymus is essential to generate tolerant, donor-derived recipient-educated T-lymphocytes. Thymic-mediated central tolerance is out of commission when output is absent or markedly reduced, thus recovery of thymic function after HSCT is imperative, as without it complete and durable T-lymphocyte reconstitution fails and central tolerance does not resume. Management therefore has two aims; minimising the time that the thymus is not functioning optimally and, in the interim, controlling the pathogenic T-lymphocytes but sparing the beneficial T-lymphocytes by utilising other means of peripheral tolerance. ECP appears to be capable of both, supported by the data presented here. However, additional data are needed, and, unsurprisingly, investigation of this exciting area of research has led to more questions to be answered and identification of more avenues to be explored. Specifically, further exploration of thymopoietic recovery should include additional patient numbers, longer follow up and inclusion of adult patients. Improvement in our understanding of the thymic regenerative capacity and the development of a biomarker that could be used to diagnose and monitor for thymic damage would be indispensable in individualising patient care. Further exploration of the mechanisms behind ECP should involve inclusion of a larger patient cohort and additional time points during ECP therapy. A more detailed analysis of the DC and Treg phenotype, ideally using single cell analysis would be desirable, with examination of DC expression of additional co-stimulatory and inhibitory molecules, and analysis of different Treg subpopulations as well as their functional capacity. A more complete assessment of QOL should include longer follow-up, additional data particularly from the child's perspective, evaluation of QOL during the ECP treatment process, and provision of feedback specifically regarding ECP therapy. This additional work will aid in the ongoing development of innovative ways of achieving strong immune reconstitution, essential to achieve a successful cure and return to a normal life.

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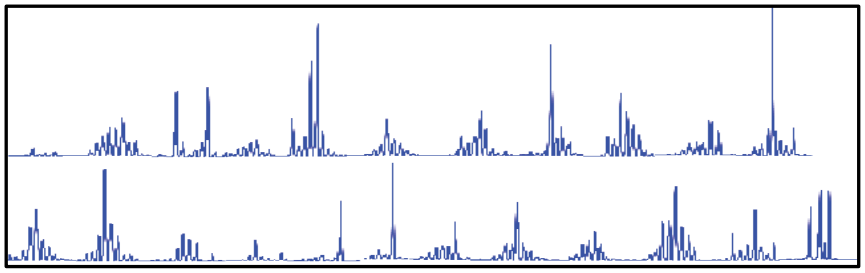
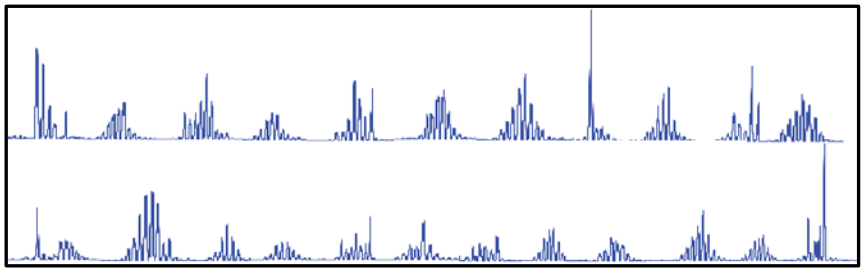
Appendix 1 TCR Spectratyping Results

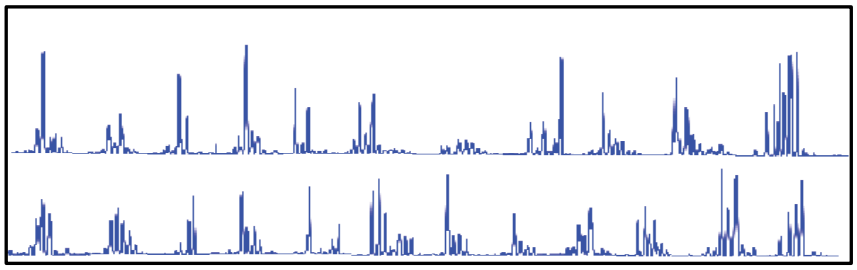
Patient 2 - TCR spectratype analysis pre ECP



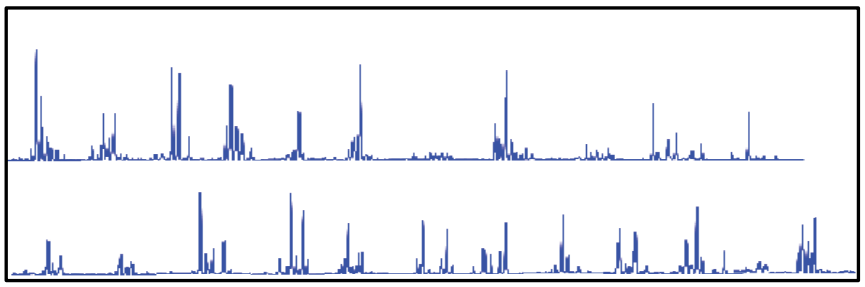
Patient 2 - TCR spectratype analysis post ECP



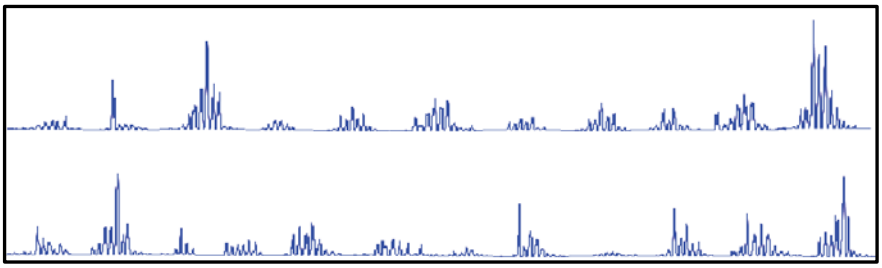
Patient 3 - TCR spectratype analysis pre ECP



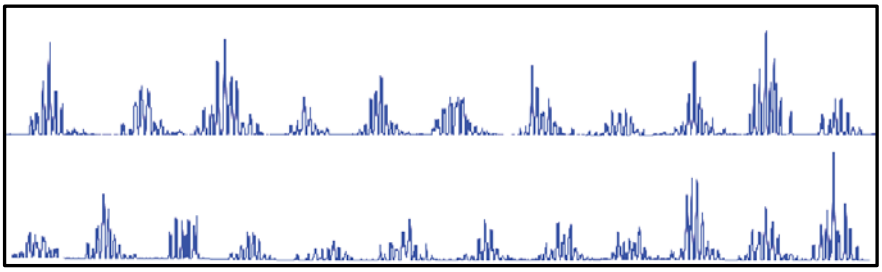
Patient 3 - TCR spectratype analysis at cycle 16 (when withdrawn from ECP treatment)



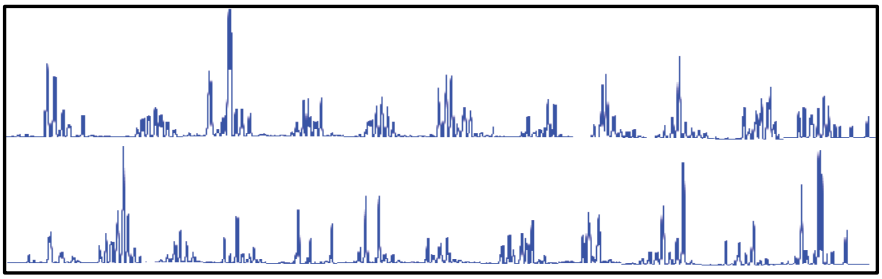
Patient 4 - TCR spectratype analysis pre ECP



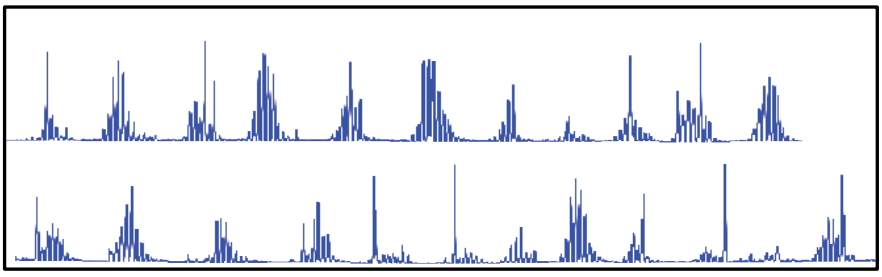
Patient 4 - TCR spectratype analysis post ECP



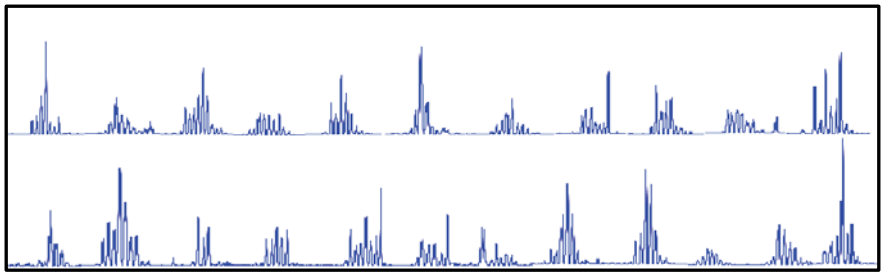
Patient 6 – TCR spectratype analysis pre ECP



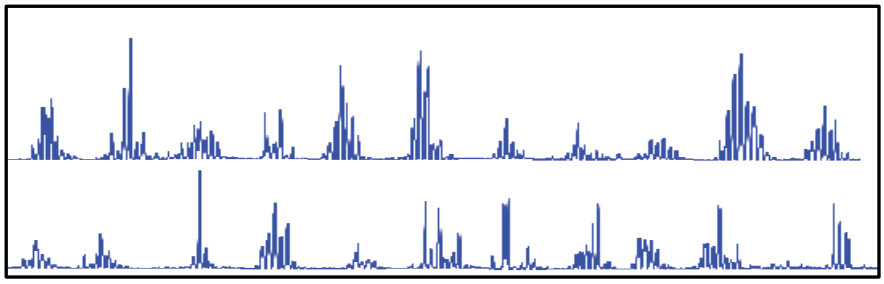
Patient 6 – TCR spectratype analysis at 8 months post-HSCT, remains abnormal but improved from previous analysis



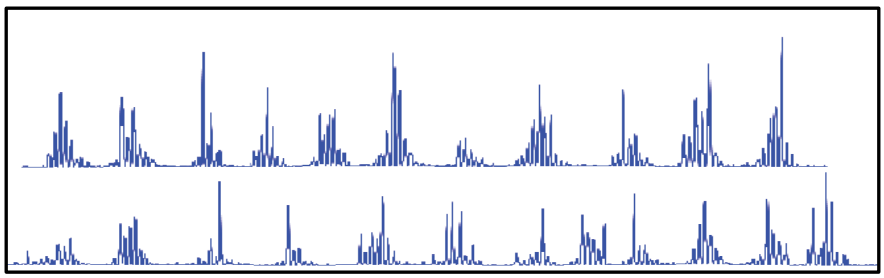
Patient 7 - TCR spectratype analysis pre ECP



Patient 7 – TCR spectratype analysis at cycle 17 (when withdrawn from ECP treatment)



Patient 8 - TCR spectratype analysis pre ECP



Appendix 2 Cytokine results

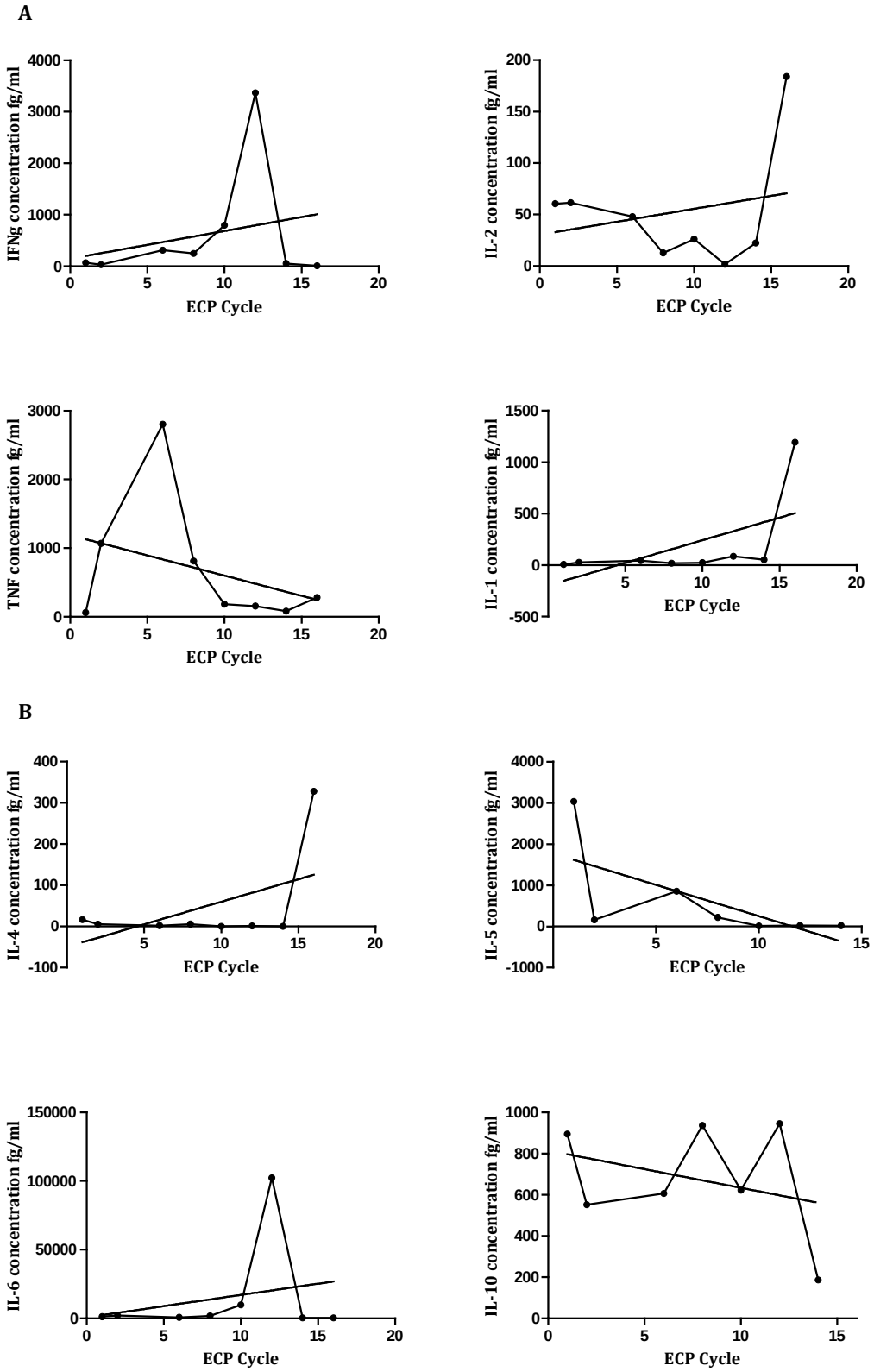


Figure 8.1. Th1 and Th2 cytokine patterns in patient 3 during ECP therapy before withdrawal after cycle 16.

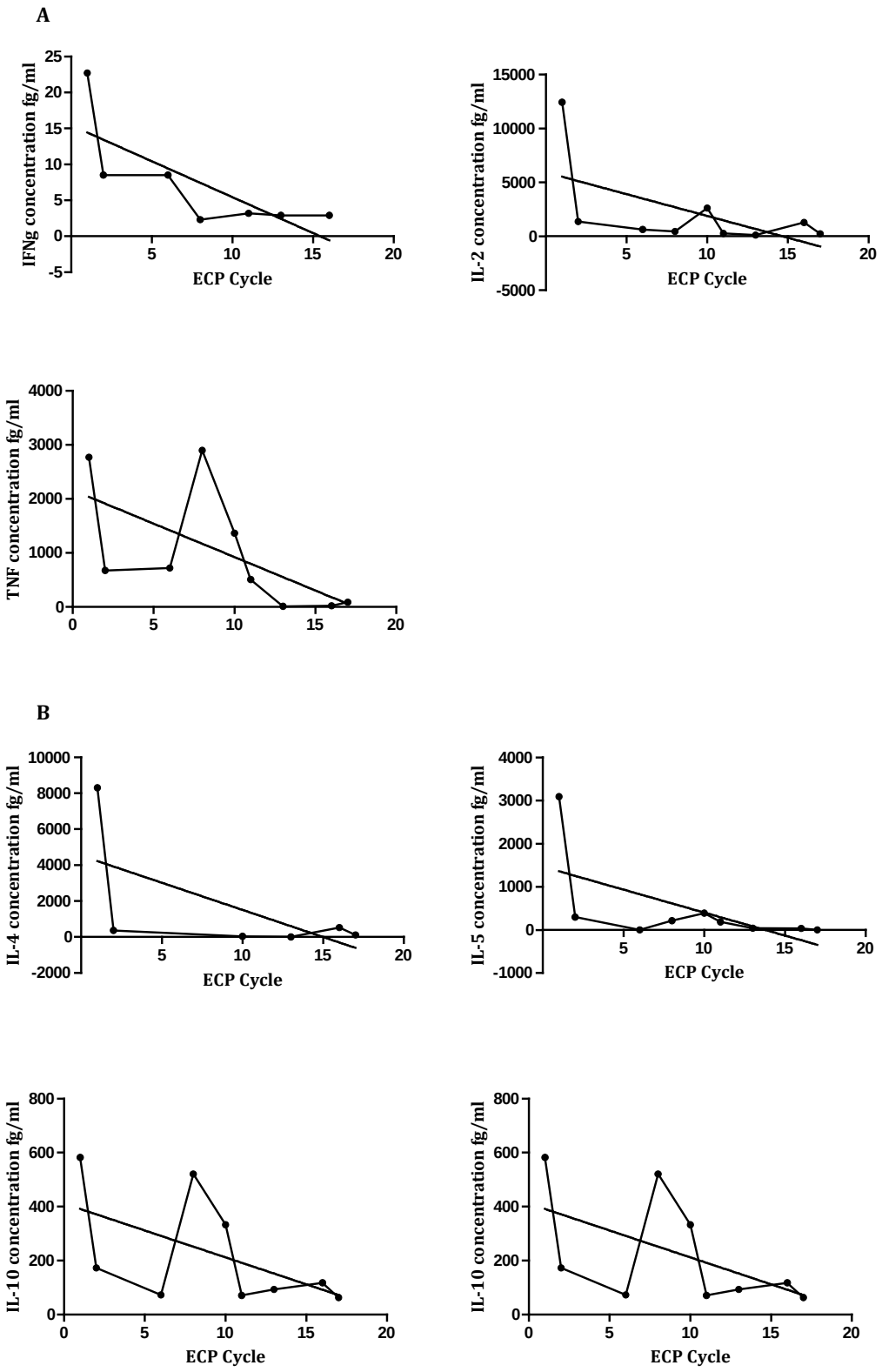


Figure 8.2. Th1 and Th2 cytokine patterns in patient 7 during ECP therapy before withdrawal after cycle 17.

Appendix 3 Manuscripts and Presentations

Publications

Thymopoiesis following HSCT; a retrospective review comparing interventions for aGVHD in a paediatric cohort. Flinn AM, Roberts C, Slatter MA, Skinner R, Robson H, Lawrence J, Guest J, Gennery AR. (2018) Clin Immunol. pii: S1521-6616(17)30780-5. doi: 10.1016/j.clim.2018.01.006. [Epub ahead of print].

Treatment of Pediatric Acute Graft-versus-Host Disease – Lessons from Primary Immunodeficiency? Flinn AM and Gennery AR. Front. Immunol. 8:328. (doi: 10.3389fimmu.2017.00328).

Extracoporeal photopheresis treatment of acute graft-versus-host disease following allogeneic haematopoietic stem cell transplantation. Flinn AM and Gennery AR. F1000Research 2016, 5(F1000 Faculty Rev):1510 (doi: 10.12688/f1000research.8118.1).

Oral presentations

'Thymopoiesis after HSCT, the effect of acute GvHD and how ECP might help'. Presented at the National UK Photopheresis Meeting 2017, London, UK.

'Effects of extracorporeal photopheresis on thymopoiesis in a cohort of paediatric patients with acute graft-versus-host disease'.Institute of Cellular Medicine Director's Day 2017. Awarded prize for best oral presentation. Oral poster presentation at the European Society for Immunodeficiencies meeting, 2017, Edinburgh, UK.

'*Investigation into the mechanism of action, development of thymopoiesis and assessment of quality of life in a cohort of paediatric patients with acute GvHD treated with ECP'.* UK Photopheresis Society Annual Meeting 2016, London, UK.

Published abstracts and poster presentations

Recovery of thymopoiesis in a paediatric cohort with acute GVHD – Can ECP help? Flinn AM, Roberts CF, Wang XN, Gennery AR. European Society for Blood and Marrow Transplantation (EBMT) Annual Meeting 2018, Lisbon, Portugal.

In vivo patterns of dendritic cell and monocyte populations in paediatric patients with acute GVHD treated with ECP. Flinn AM, Altmann TJ, Bigley V, Wang XN, Gennery AR. EBMT Annual Meeting 2018, Lisbon, Portugal.

Thymopoiesis following HCT; a retrospective review comparing interventions for aGvHD in a paediatric cohort. Roberts C, Flinn AM, Slatter MA, Skinner R, Robson H, Cooper J, Guest J, Gennery AR. Published abstract and poster presentation for EBMT 2017, Marseilles.

Extracorporeal Photopheresis affects Dendritic Cells by reducing total numbers and blunting cytokine production in patients with Graft versus Host Disease. Altmann TJ, Bickerton M, Flinn AM, Cytlak U, Milne P, Pagan S, Bigley V, Gennery AR. Published abstract and accepted as poster for EBMT 2017, Marseilles.

A Retrospective Review of a Cohort of Paediatric Patients Treated with ECP for the Management of GVHD. Flinn AM, Slatter MA, Skinner R, Robson H, Cooper J, Guest J, Gennery AR. EBMT Annual Meeting 2016, Valencia, Spain. Bone Marrow Transplantation. Volume 51, Issue S1 (March 2016).