

The Utility of Immune Function Profiling in Rheumatoid Arthritis Therapeutic Efficacy Monitoring

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A thesis submitted in partial fulfilment of the requirements of
Oxford Brookes University for the degree of Doctor of
Philosophy

June 2018

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Abstract

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune disease, primarily affecting the small joints of the hands, wrists and feet. Disease onset is insidious with classical symptoms including pain, morning stiffness, and persistent synovitis; accompanied by

fatigue, malaise and weight loss. RA patients also suffer from a number of extra-articular manifestations and additional comorbidities, including rheumatoid nodules and scleritis; as well as an increased risk of cardiovascular disease (CVD), lymphoma and infection. Ultimately, RA is a devastatingly progressive disease, if a diagnosis and an effective therapeutic regimen is not established quickly.

Identifying when a therapy is proving ineffective can be difficult, however, and is reliant upon accurate formal joint counts, CRP measurements and patient global assessments. These are potentially insensitive means of assessing remission and may result in delayed changes to therapeutic regimens. The identification of new biomarkers to improve the predictive value of these evaluation models would therefore greatly benefit patient care. Based upon current evidence, it has been hypothesized that RA patients who fail to make a clinical improvement following the initiation of csDMARD and bDMARD therapeutic regimens are identifiable by their profile of the following CD4⁺ T-cell populations: follicular helper, memory helper, antigen-specific and naturally-occurring regulatory CD4⁺ T-cells.

As such, flow cytometric assays have developed for the identification and enumeration of these respective CD4⁺ T-cell populations; the relative and absolute counts of which were investigated within a cohort of healthy controls, with normal distributions established. Subsequently, we have identified distinct alterations to the CD4⁺ T-cell compartments of newly-diagnosed, treatment-naïve and longstanding RA patients, with clear associations with patient clinical outcomes observed. Of notable significance, the relative distribution of CCR4⁻ Th2-like follicular helper CD4⁺ T-cells within newly-diagnosed, treatment-naïve RA patients at the time of diagnosis, has proven highly predictive of therapeutic efficacy; with changes in the relative distribution of Th17-like follicular helper and Th17 memory helper CD4⁺ T-cells also associated with changes to disease activity over time.

Furthermore, consistent with previous studies, the CMV status of newly-diagnosed, treatment-naïve and longstanding RA patients, is associated with an aggravated clinical course; with changes in CMV-specific CCR4⁺ Th17 cell responses related to changes in disease activity 12-months after the initiation of a csDMARD therapeutic regimen. No such relationships could, however, be discerned within the naturally-occurring regulatory CD4⁺ T-cell compartment, limiting the utility of employing naturally-occurring regulatory CD4⁺ T-cell measurements in therapeutic efficacy monitoring. Nevertheless, these preliminary results indicate that the addition of follicular helper, memory helper and CMV-specific CD4⁺ T-cell responses to current remission assessment practices, could improve the identification of RA therapy non-responders, enabling the swift adjustment of RA patient therapeutic regimens and, consequently, improving disease management.

List of Publications and Presentations

Publications

Dhalla [F](#), [Fox H](#), [Davenport EE](#), [Sadler R](#), [Anzilotti C](#), [van Schouwenburg PA](#), [Ferry B](#), [Chapel H](#), [Knight JC](#), [Patel SY](#). Chronic mucocutaneous candidiasis: characterization of

a family with STAT-1 gain-of function and development of an ex-vivo assay for Th17 deficiency of diagnostic utility. Clin Exp Immunol. 2016 May; 184(2):216-27.

Presentations

H. Fox. Immune Function Profiles in Rheumatoid Arthritis. Oral presentation given at 2015 Research Student Symposium (2015).

H. Fox. Immune Function Profiling in Rheumatoid Arthritis. Poster presentation at 2016 Research Student Symposium (2016).

H. Fox, R. Sadler, L. Ayers, E. Bateman, R. Jain, S. Misbah, S. Patel, B. Ferry. Follicular helper T-cell subpopulation profiling in Common Variable Immunodeficiency Disorders. Poster presentation at 17th Biennial Meeting of the European Society for Immunodeficiencies (2016).

Acknowledgements

Firstly, I would like to express my gratitude to my supervisors Professor Berne Ferry, Professor Susan Brooks, Dr Ross Sadler and Dr Lisa Ayers for their advice and support during my PhD studies. I would particularly like to thank Dr Ross Sadler and Dr Lisa Ayers for their time, knowledge and review of this thesis during its write-up; who

consistently allowed this work to be my own, but steered me in the right direction when needed.

I would also like to thank the many clinicians and research nurses, based at the Nuffield Orthopaedic Centre, who were involved in patient recruitment and sample collection. In particular, I would like to thank Karen Doig and Helen Bunting, for their untiring dedication to the IMPRA study; without which this work would not have been possible. My thanks must also go to all the patients and healthy controls who kindly donated blood samples to this study.

Finally, I would like to thank all my family, friends and colleagues for their unfailing support and continuous encouragement throughout my years of study. Above all, special thanks must go to my parents, who had no doubt that I would succeed in finishing my studies, even when I did, on many occasions!! This accomplishment would not have been possible without them. Thank you.

Hannah

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Chapter 4

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Table 4.5 *Relative distribution of CD25⁺CD134⁺ activated CD4⁺ T-cells and their subpopulations, following stimulation with HSV within newly-diagnosed, treatment-naïve RA patients, longstanding RA patients, as well as disease and healthy controls.*

Table 4.6 *Relative distribution of CD25⁺CD134⁺ activated CD4⁺ T-cells and their subpopulations, following stimulation with Aspergillus within newly-diagnosed, treatment-naïve RA patients, longstanding RA patients, as well as disease and healthy controls.*

Chapter 5

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List of Abbreviations

ABT	Abatacept
ADA	Adalizumab
ADCC	Antibody-dependent cell mediated cytotoxicity
AICD	Activation-induced cell death
APCs	Antigen-presenting cells
aTcs	CD25+CD134+ activated CD4+ T-cell responses
BCL-6	B-cell lymphoma 6
bDMARD	Biological disease-modifying anti-rheumatic drugs
CCP	Cyclic citrullinated proteins
CCR4	C-C Chemokine receptor 4
CCR6	C-C Chemokine receptor 6
CCR7	C-C Chemokine receptor 7
CD40L	CD40 ligand
CD62L	L-selectin
CDC	Complement dependent cytotoxicity
cDCs	Conventional dendritic cells

cFth	Circulating blood-derived follicular helper CD4 ⁺ T-cells
CMV	Cytomegalovirus
CRP	C-reactive protein
csDMARD	Conventional synthetic disease-modifying anti-rheumatic drugs
cTECs	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
CVD	Cardiovascular disease
CXCR3	C-X-C Chemokine receptor 3
CXCR5	C-X-C Chemokine receptor 5
CZP	Certolizumab pegol
DAS28	28 joint disease activity score
DCG	Disease control group
DHFR	Dihydrofolate reductase
DMARD	Disease-modifying anti-rheumatic drugs
DP	Double positive
EAM	Extra-articular manifestations
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-Linked ImmunoSpot
ELS	Ectopic lymphoid structures
ERA	Newly-diagnosed, treatment-naïve RA patients
ESR	Erythrocyte sedimentation rate
ETA	Etanercept
EULAR	European league against rheumatism
FOXP3	Forkhead box P3
FRS	Framingham risk score
GATA-3	GATA Binding Protein 3
GCs	Germinal Centres
GITR	Glucocorticoid-induced TNF receptor family-related protein
GOL	Golimumab
HC	Healthy controls
HCQ	Hydroxychloroquine

HDL	High density lypoprotein
HSV	Herpes simplex virus
ICOS	Inducible costimulator
IFN- γ	Interferon gamma
IFX	Infliximab
IL-10	Interleukin-10
IL-10R	Interleukin 10 receptor
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-15	Interleukin-15
IL-17	Interleukin-17
IL-17A	Interleukin-17A
IL-17F	Interleukin-17F
IL-2	Interleukin-2
IL-21	Interleukin-21
IL-22	Interleukin-22
IL-27	Interleukin-27
IL-2R α	α -chain of the IL-2 receptor
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
INN	Tofacitinib
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
JAK	Janus kinase
LEF	Leflunomide
MHC	Major histocompatibility complex
mTECs	Medullary thymic epithelial cells
mTh	Memory helper CD4+ T-cells
MTX	Methotrexate
nTregs	Naturally-occurring regulatory CD4+ T-cells

OCSD	Other conventional synthetic disease-modifying anti-rheumatic drug
PAD4	Peptidyl arginine deiminase type IV
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PHA	Phytohaemagglutinin
RA	Rheumatoid Arthritis
RA (patient group)	Longstanding RA patients
RF	Rheumatoid factor
ROR γ t	Retinoic acid receptor-related orphan nuclear receptor <i>gamma t</i>
RTX	Rituximab
SAP	SLAM-associated protein
SCORE	Systematic COronary Risk Evaluation
SP	Single positive
SSZ	Sulfasalazine
STAT-3	Signal transducer and activator of transcription 3
T-bet	T-box transcription factor TBX21
TCR	T-cell receptor
TGF- β 1	Transforming Growth Factor β 1
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNF- α	Tumour necrosis factor alpha
TOC	Tocilizumab
VCA	viral capsid antigen
XLP	X-linked lymphoproliferation syndrome

Chapter 1: An Introduction to Rheumatoid Arthritis

1.1. Introduction

1.1.a. *Clinical Presentation*

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune disease, primarily affecting the small joints of the hands, wrists and feet. Affecting approximately 0.5 – 1.0% of adults, and with an annual incidence of 5 – 50 cases per 100,000 within industrialized countries, rheumatoid arthritis is a debilitating disease in which chronic inflammation ultimately results in joint destruction and subsequent deformity (Klareskog, Catrina and Paget, 2009; Scott, Wolfe and Huizinga, 2010). Largely a disease of the elderly, but capable of occurring at any age, the peak age of onset is between 40 and 70 years of age, with consistently higher rates in females. As such, the lifetime risk of developing RA in adults is 3.6% in women and 1.7% in men (Crowson et al., 2011; Lee and Weinblatt, 2001).

Disease onset is usually insidious with classical symptoms including pain, morning stiffness and persistent synovitis; accompanied by fatigue, malaise and weight loss (Hashizume and Mihara, 2011; Klareskog, Catrina and Paget, 2009). Morning stiffness is a common feature of active RA, with symptoms that last more than one hour reflecting a high level of inflammation and disease activity that rarely occurs outside of RA (Boers et al., 2015; Gibofsky, 2012). Synovitis of the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints of the fingers, the interphalangeal joints of the thumbs, the wrists, and the metatarsophalangeal (MTP) joints of the toes often occurs early in disease; a pattern of joint involvement that in general does not occur in other forms of arthritis (Figure 1.1) (Aletaha et al., 2010; Gibofsky, 2012; Kay and Upchurch, 2012).

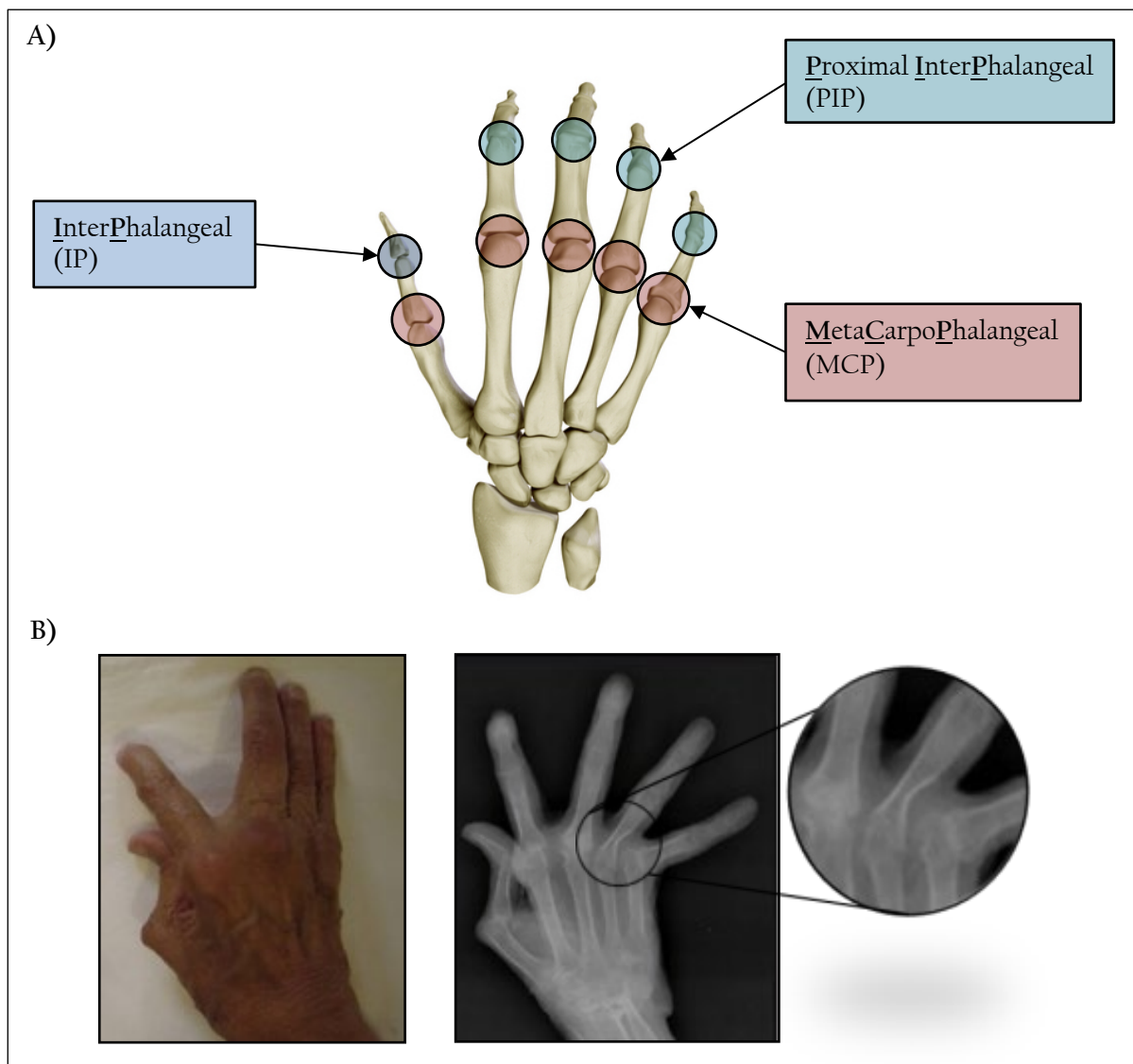


Figure 1.1:- A) Schematic displaying the proximal interphalangeal (PIP), interphalangeal (IP) and metacarpophalangeal (MCP) joints, of the hands, typically affected in Rheumatoid Arthritis (RA); B) Inflammation of the small joints of the hands may result in permanent hand deformities and joint damage, if diagnosis is delayed or immunomodulatory therapies prove ineffective. Images downloaded and adapted from <https://cgtrader.com/3d-models/character> and <http://images.rheumatology.org/bp/#!/folder/75692/5231176>, respectively in September 2017.

In addition to these classical symptoms of RA, approximately 18.0 – 41.0% of patients also suffer from a number of extra-articular manifestations (EAM), such as rheumatoid nodules, scleritis, amyloidosis and sjögren's syndrome; with 1.5 – 21.5% of patients presenting with the most severe forms of EAM (Table 1) (Gibofsky, 2012; Prete et al., 2011; Turesson et al., 2006). Incidence rates of EAM are highly varied amongst study groups and according to geographical location suggesting that, as with the overall pathogenesis of RA, genetic and environmental factors play a pivotal role in EAM development. Of the environmental factors more commonly investigated, a strong association between the

occurrence of extra-articular manifestations and a history of smoking is of particular importance; with smoking also a strong predictor of radiographic progression in newly diagnosed RA (Saevarsdottir et al., 2015; Saevarsdottir et al., 2011). Interestingly, RA patients with extra-articular manifestations are also more likely to possess the serological component rheumatoid factor (RF) (94.0% of patients with EAM vs 71.0% of patients without EAM ($p=0.006$)), as well as antibodies directed against cyclic citrullinated proteins (CCP) (77.0% of patients with EAM vs. 56.0% of patients without EAM ($p=0.03$)), with levels of these two components also tending to be higher in patients with extra-articular manifestations than those without (Turesson et al., 2007). As the presence of these serological components may in fact be demonstrated up to 15 years before disease onset, the measurement of RF and anti-CCP antibodies are often used diagnostically, as well as a marker of disease severity (Aletaha et al., 2010; Kay and Upchurch, 2012; Law et al., 2012; Mouterde et al., 2014).

Over the years it has been demonstrated that RA patients suffer from a number of additional comorbidities including an increased risk of infections, malignancies, typically lymphomas, and cardiovascular disease (CVD) (Table 1) (Aaltonen et al., 2015; Klareskog, Catrina and Paget, 2009). As the leading cause of premature death in RA, especially in patients with longstanding disease, it has been estimated that patients with RA have an increased cardiovascular mortality ratio that ranges between 1.6 and 1.7, as compared to the general population (Gkaliagkousi et al., 2012). This increased risk also appears to be independent of traditional cardiovascular disease risk factors, such as smoking, hypertension and dyslipidaemia, and as a consequence it has been demonstrated that the standard coronary risk evaluation models, such as the Framingham Risk Score (FRS) and the Systematic COronary Risk Evaluation (SCORE), used to assess the 10-year coronary heart disease risk (based upon age, total cholesterol, high density lipoprotein (HDL) cholesterol, smoking history and systolic blood pressure) can significantly underestimate the risk of cardiovascular disease in RA patients (Artigao-Rodenas et al., 2013; Gomez-

Vaquero et al., 2012; Liao and Solomon, 2013; Primdahl, Clausen and Horslev-Petersen, 2013). To date the most typical cardiovascular complications to occur within the setting of RA include pericarditis, cardiomyopathy and ischaemic heart disease (Table 1.1) (Guedes et al., 2001; Turiel et al., 2010).

Alongside this increased risk of cardiovascular disease, RA patients are also increasingly susceptible to infections, particularly bacterial infections of the skin, joints and lungs (Doran et al., 2002; Smitten et al., 2008). Incidence rates for RA patient hospitalizations suggest that RA patients are twice as likely to be hospitalized due to a serious infection, such as pneumonia, compared to healthy individuals; with age, extra-articular manifestations, leukopaenia, as well as the use of corticosteroids all identified as predictors of infection (Doran et al., 2002; Doran et al., 2002; Smitten et al., 2008). The use of immunomodulatory agents, in any disease setting, undoubtedly predisposes patients to an increased risk of infection, as by design both widespread and targeted therapies are intended to limit and suppress inflammation. However, the susceptibility of RA patients to infection has been largely deemed independent of the immunosuppression resulting from therapeutic interventions, and is in fact thought to be a result of immune dysregulation, a state which may not only be involved in the inflammatory processes occurring within these patients, but may also contribute to their increased risk of cardiovascular disease (Aaltonen et al., 2015; Doran et al., 2002; Listing, Gerhold and Zink, 2013). The relationship between rheumatoid arthritis and numerous infectious agents has long been an area of intense investigation, as is the case with a number of other autoimmune diseases. This line of enquiry is not only due to the increased infection burden in these patients, but also for their perceived involvement in RA pathogenesis; with the viral agents CMV, EBV and HSV, along with the periodontal disease causing *porphyromonas gingivalis* of particular interest (Davis et al., 2012; Magnusson et al., 2010; Pierer et al., 2012; Sandal et al., 2016).

System Affected	Extra-articular Manifestation /Co-morbidity	Prevalence (%)	Symptoms
Cardiovascular	Pericarditis Cardiomyopathies Ischaemic heart disease Hypertension	30.0 – 50.0 (Turiel et al., 2010) (symptomatic in 1.0 – 4.0) (Prete et al., 2011) 3.0 – 30.0 (Voskuyl, 2006) 5.4 (Lopez-Longo et al., 2009) 33.0 (Naranjo et al., 2008)	Inflammation of the pericardium, resulting in chest pains and echocardiographic changes (Khandaker et al., 2010). Conduction system abnormalities, developing secondary to focal, diffuse, necrotising or granulomatous myocarditis, or the use of disease-modifying anti-rheumatic drugs (Turiel et al., 2010). A group of diseases that includes myocardial infarction, stroke and sudden cardiac death (Turiel et al., 2010). A long term medical condition in which the blood pressure in the arteries is persistently elevated greater than 140/90 mmHg (Gomez-Vaquero et al., 2012).
Cutaneous	Rheumatoid nodules Raynaud's phenomenon	30.0 (90.0 in RF+ patients) (Prete et al., 2011; Turesson et al., 2006) 5.0 – 17.0 (Prete et al., 2011)	Subcutaneous nodules commonly occurring on the forearms, fingers and heels (Prete et al., 2011). Reduced blood flow to the extremities, most notably the fingers and toes, due to cold or emotional stress (Prete et al., 2011).
Haematological	Felty's syndrome	1.1 (Turesson et al., 2007)	A rare complication of RA presenting with the triad of chronic polyarthritis (resulting in severe joint destruction), neutropaenia and splenomegaly (Balint and Balint, 2004).
Immunological (Infection)	CMV EBV HSV-1/HSV-2 Porphyromonas gingivalis	48.0 CMV IgG+ (Davis et al., 2012) 95.0 EBV IgG+ (Davis et al., 2012) 33.0 HSV DNA in PBMCs (Burgos et al., 2015) 67.0 p. gingivalis IgG (Mikuls et al., 2009)	Viral infections belonging to the family of human herpes viruses. Latent infection is ubiquitous in individuals worldwide. Thought to exacerbate RA disease severity (Davis et al., 2012; Pierer et al., 2012). Gram-negative bacteria associated with periodontal disease (Mikuls et al., 2009).
Nephritic	Glomerulonephritis Secondary Amyloidosis	0.2 (Prete et al., 2011) 5.0 – 16.0 (Gómez-Casanovas et al., 2001) (Clinical and sub-clinical)	A collective term used to describe inflammation of the glomeruli of the kidneys, often resulting in proteinuria and haematuria (Karie et al., 2008). Deposition of insoluble amyloid A fibres in the kidney, liver and rheumatoid synovium, ultimately resulting in tissue damage and organ failure (Gómez-Casanovas et al., 2001).
Ocular	Scleritis Episcleritis Keratoconjunctivitis sicca Secondary sjögren's syndrome	2.0 (Vignesh and Srinivasan, 2015) 3.0 (Vignesh and Srinivasan, 2015) 45.0 (Murray and Rauz, 2016) 5.0 – 20.0 (Romanowska-Próchnicka et al., 2016) (Clinical and sub-clinical)	Painful inflammation of the sclera (Murray and Rauz, 2016). Recurrent inflammation of the episclera, or the layer superficial to the sclera (Murray and Rauz, 2016). Dry eye syndrome, resulting in discomfort and visual disturbances (Murray and Rauz, 2016). Characterized by lymphocytic infiltration of the exocrine glands, resulting in dry gritty eyes, dry mouth and swollen parotid glands (Romanowska-Próchnicka et al., 2016).
Oncological	All malignancies Hogdkin's lymphoma Lymphoma Lung cancer	3.3 (Abásolo et al., 2008) 0.5 (Abásolo et al., 2008) 0.2 (Abásolo et al., 2008) 2.7 (Abásolo et al., 2008)	Highly varied depending on the location, size of disease burden and cellular origin.
Pulmonary	Pulmonary nodules Interstitial lung disease	78.6 (Yuksekkaya et al., 2013) (Clinical and sub-clinical) 8.0 – 15.0 (Zamora-Legoff et al., 2016)	Identical to those found subcutaneously, approximately 0.5–5.0 cm in diameter, usually located at the peripheral zones of the upper and middle lung regions. May undergo cavitation, calcification, or an increase in size, or they may resolve spontaneously (Yuksekkaya et al., 2013). Not always symptomatic, but typical symptoms include dyspnea and cough (Zamora-Legoff et al., 2016).

Table 1.1:- Extra-articular manifestations and co-morbidities typically associated with RA.

The increased incidence of malignancy, as compared to the general population, also represents a significant source of morbidity and mortality within RA patients; with the overall relative risk of malignancy in rheumatoid arthritis increased, as well as the individual risk of lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma and lung cancer also documented (Simon et al., 2015; Smitten et al., 2008). The well recognized causal links between smoking and lung cancer, and independently smoking with RA pathogenesis and disease severity would certainly imply an indirect association between rheumatoid arthritis and lung cancer development. However, RA has also been shown to affect the lungs, with mortality due to pulmonary diseases, such as interstitial lung disease, shown to be twice as likely to occur in RA as compared to the general population (Prete et al., 2011; Simon et al., 2015; Smitten et al., 2008). As such, various modes of action have been postulated to account for the increased risk of malignancy in RA; the most well described models the effects of chronic inflammation. In such a scenario, chronic stimulation, either by an infectious agent, environmental factor, such as the components of cigarette smoke, or RA-associated pulmonary disease, result in the development of a chronic inflammatory state that increases the likelihood of malignant transformation. Concomitant reductions in regulatory T-cell and NK cell activity, both of which would potentially limit malignant cell growth, are also proposed (Simon et al., 2015; Smitten et al., 2008). Interestingly, the RA comorbidities described above appear to be more prominent in RF and anti-CCP antibody positive patients. As such, in addition to their previously described diagnostic utility and use as EAM indicators, the presence of these biomarkers is also associated with an increased risk of overall morbidity and mortality (Arnab et al., 2013; Klareskog, Catrina and Paget, 2009). Ultimately, with the combined effects of joint destruction, extra-articular manifestations and comorbidities, RA is a devastatingly progressive disease, particularly when a diagnosis and an effective therapeutic regimen are not established quickly.

1.1.b. Diagnostic Criteria - 1987 vs. 2010 Classification Criteria

Until recently the diagnosis of RA had been based heavily upon the presence of end-stage disease factors, such as radiographic changes, including evidence of bony erosions or decalcifications, as well as non-articular features, such as rheumatoid nodules and scleritis (Figure 1.1 and Table 1.1) (Gibofsky, 2012; Kay and Upchurch, 2012; Scott, Wolfe and Huizinga, 2010). However, it has now been recognized that achieving a diagnosis at this stage of disease is highly detrimental to patient quality of life, with a huge amount of largely irreversible damage having already occurred. As such in 2010, the American College of Rheumatology (ACR) and the European League against Rheumatism (EULAR) formed a collaboration, designed to review the classification criteria used in the diagnosis of RA; with the primary focus to develop a set of classification criteria that would enable clinicians to establish an earlier diagnosis, thereby permitting anti-inflammatory and disease modifying therapies to be initiated before permanent joint damage, extra-articular manifestations and co-morbidities have occurred (Figure 1.2) (Aletaha et al., 2010; Gibofsky, 2012; Kay and Upchurch, 2012).

With this aim in mind the 2010 classification criteria are therefore based much more heavily upon the assessment of the number of joints involved, particularly the small joints and irrespective of any radiographic changes; as well as the duration of symptoms (≥ 6 weeks), in combination with the presence of serological markers, such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), RF and anti-CCP antibodies, designed to serve as surrogate markers of active inflammation and disease activity (Table 1.2). The evaluation of each of these criteria allows the clinician to generate a score upon patient assessment, with a score $\geq 6/10$ indicative of rheumatoid arthritis (Aletaha et al., 2010; Gibofsky, 2012; Kay and Upchurch, 2012).

	1987 Criteria		2010 Criteria	
Criteria	Description	Score	Description	Score
Morning Stiffness	In and around joints, for at least 1 hour.	1	Clinical synovitis/swelling in ≥1 joint not explained by another disease.	N/A
Joint Involvement	Physician observed soft tissue swelling or fluid in 3 of 14 possible joints.	1	1 large joint 2 – 10 large joints 1 – 3 small joints (with or without large joints) 4 – 10 small joints (with or without large joints) ≥10 joints (at least 1 small)	0 1 2 3 5
Arthritis of hand joints	At least 1 swollen hand or wrist area.	1	N/A	N/A
Symmetric Arthritis	Simultaneous bilateral involvement.	1	N/A	N/A
Rheumatoid Nodules	Subcutaneous nodules over bony prominences, extensor surfaces, or in juxtaarticular regions observed by physician.	1	N/A	N/A
Serology	Positive RF serum test.	1	Negative RF and ACPA Low Positive RF or ACPA High Positive RF or ACPA	0 2 3
Radiographic Changes	Erosions or unequivocal bony decalcification in or adjacent to the involved joints, but not consistent with osteoarthritis.	1	N/A	N/A
Acute Phase Reactants	CRP and ESR	N/A	Normal CRP and ESR Abnormal CRP or ESR	0 1
Duration of Symptoms	First 4 criteria must be present for ≥6 weeks	N/A	<6 weeks ≥6 weeks	0 1
Criteria score required		≥4/7		≥6/10

Table 1.2:- Comparison of ACR/EULAR 1987 and 2010 RA Classification Criteria. Adapted from Gibofsky (2012).

1.1.c. Therapeutic Strategies - Conventional Synthetic vs Biologic Therapies

The therapeutic regimens employed in the treatment of RA have also been extensively reviewed in recent years, with the primary goal to reduce disease activity and/or achieve disease remission before permanent joint damage can be incurred. As such, in an additional change to the 2010 classification criteria, as well as EULAR's recommendations for RA management, it is now also highly recommended that once identified, RA patients immediately begin a thorough therapeutic regimen, involving the use of either and/or both synthetic and biological disease-modifying anti-rheumatic drugs (DMARDs) (Figure 2) (Smolen et al., 2014).

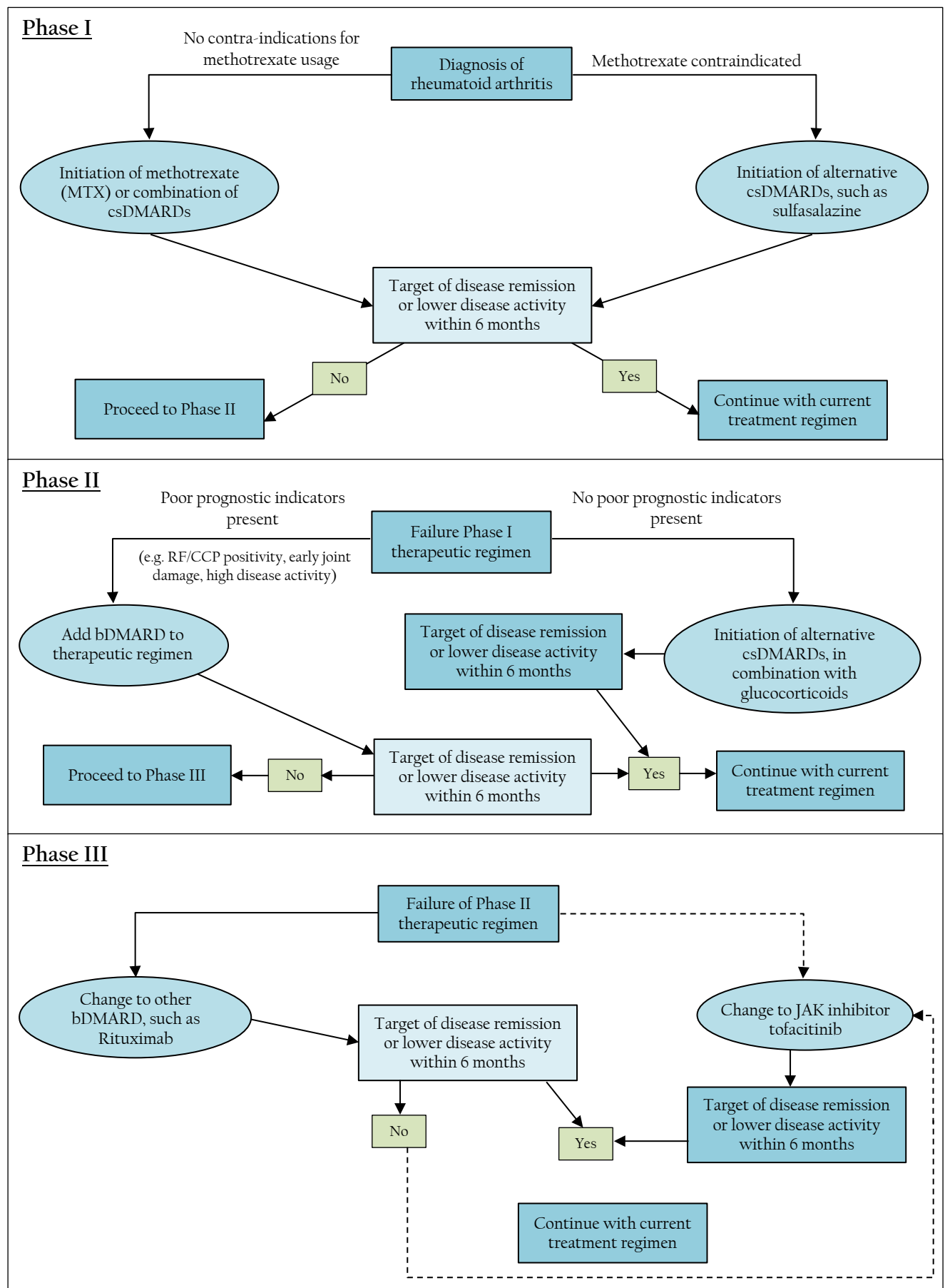


Figure 1.2:- EULAR therapeutic algorithm based on the 2013 recommendations for the management of RA. MTX = Methotrexate; csDMARD = conventional synthetic disease modifying anti-rheumatic drug; bDMARD = biologic disease modifying anti-rheumatic drug; JAK = janus kinase inhibitor. Dotted line denotes treatment strategies in which therapeutic efficacy is yet to be ascertained. Adapted from Smolen et al (2014).

The specific modes of action of conventional synthetic and biological DMARDs are highly varied, with conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs) generally demonstrating widespread immunomodulatory effects. This is also the case with the gold standard of RA therapy, methotrexate (MTX), which has multiple modulatory effects, one of which being to disrupt the metabolism of folic acid (Baggott et al., 1993; Cutolo et al., 2001). Although, likely not its primary mode of action in the treatment of rheumatoid arthritis, it is well documented that MTX acts as a structural analogue for folic acid, directly competing with folic acid for binding to the enzyme dihydrofolate reductase (DHFR). This competitive binding ultimately results in reduced folic acid metabolism, hindering DNA synthesis, and subsequently compromising cellular integrity, which along with the desired immunosuppressive effects, also has far reaching consequences for other bodily functions (Table 1.3) (Baggott and Morgan, 2009; Baggott et al., 1993). In the treatment of rheumatoid arthritis many of these undesirable effects are counterbalanced by the supplementation of MTX-based therapeutic strategies with folate, which is used to mitigate the toxic effects of DHFR inhibition instigated by MTX (Baggott et al., 1993; Cutolo et al., 2001).

The widespread actions of csDMARDs, such as MTX, are in direct comparison to the biological disease-modifying anti-rheumatic drugs (bDMARDs) which generally display far more targeted immunomodulatory effects; such as the B-cell depleting monoclonal antibody, rituximab. The chimeric monoclonal antibody rituximab has been specifically designed to target CD20 expressing B-cells, as well as short-lived plasma cells, for antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC), thereby decreasing autoantibody production, as well as B-cell mediated antigen presentation (Table 1.3) (Tchetina et al., 2016). Given the considerable selection of csDMARDs and bDMARDs, that are now widely available for use in the regulation of rheumatological diseases; taking into consideration their relative modes of action, accompanying side effects, as well as their potential financial burdens to healthcare

establishments; EULAR have recently published a set of recommendations for the management of rheumatoid arthritis, based upon five extensive systematic literature reviews performed by a panel of experts in the field (Figure 1.2) (Gaujoux-Viala et al., 2010; Gorter et al., 2010; Knevel et al., 2010; Nam et al., 2010; Schoels et al., 2010; Smolen et al., 2016; Smolen et al., 2014).

Despite recent advances in the development of monoclonal antibody therapies, the use of the conventional synthetic DMARD methotrexate (MTX) is still considered the mainstay of RA therapy (Aletaha et al., 2010; Smolen et al., 2014). Due to its widespread immunosuppressive activity, MTX is able to induce low disease activity rates or 70.0% improvement rates, as defined by the ACR, in about 25.0 – 50.0% of early RA patients (Smolen et al., 2014). In cases with MTX contraindications or intolerance, such as pregnancy, liver damage, cytopenias and severe infection, the DMARDs leflunomide and sulfasalazine may instead be used, and have also shown good clinical efficacy (Salliot and van der Heijde, 2009). These conventional synthetic disease-modifying drugs may be given singularly or in combination with glucocorticoids for the initial phase of treatment, to provide immediate pain relief and reduce inflammation, whilst other csDMARD doses are gradually increased; with glucocorticoids ideally tapered within 6 months, to avoid unwanted side-effects (Figure 1.2) (Caplan et al., 2017; Czock et al., 2005; Smolen et al., 2014). EULAR recommendations further stipulate that during active disease and the initiation phase of therapy, frequent monitoring should occur, ideally every 1 – 3 months. If no improvement in disease activity has been observed within 3 months, or the target of remission has not been reached by 6 months, it is recommended that the therapeutic regimen be adjusted, as 30.0 – 40.0% of patients will prove resistant to therapy, with persistent synovitis and rapid erosion progression evident (Figure 1.2) (Picchianti Diamanti et al., 2014; Pitzalis, Kelly and Humby, 2013; Smolen et al., 2014).

Therapeutic Agent		Proposed modes of action
Conventional synthetic DMARDs:-		
Methotrexate	MTX	a) MTX acts as a structural analogue for folic acid, directly competing with folic acid for binding to the enzyme DHFR; thereby reducing folic acid metabolism and hindering DNA synthesis (Baggott and Morgan, 2009; Cutolo et al., 2001). b) The inhibition of DHFR also inhibits the formation of tetrahydrofolate, required for the synthesis of methionine, an essential amino acid, which acts as a methyl group donor in a number of cellular reactions, including the formation of polyamines. Increased polyamine levels have been demonstrated in synovial fluid of RA patients, where their metabolism by accumulating monocytes may result in the formation of the harmful NH_3 and H_2O_2 (Cronstein, 2005). c) Finally, MTX increases extracellular adenosine release, via the inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase; thereby blocking de novo purine synthesis (Tian and Cronstein, 2007).
Leflunomide	LEF	a) At low, therapeutic levels, LEF and its active metabolite, A77 1726, reversibly inhibit the mitochondrial enzyme dihydro-orotate dehydrogenase, which plays a key role in the de novo synthesis of the pyrimidine uridine monophosphate (rUMP), fundamentally required for DNA and RNA synthesis (Cutolo et al., 2003). b) At higher doses, LEF inhibits the expression of several tyrosine kinases, inducing the downregulation of cell signalling in the G_0/G_1 phase of the cell cycle (Cutolo et al., 2003). c) A77 1726 is also able to suppress the activation of the transcription factor complex nuclear factor κB (NF-κB), a potent mediator of inflammation (Cutolo et al., 2006).
Sulfasalazine	SSZ	a) After oral administration SSZ is partially cleaved by intestinal bacteria, resulting in the formation of 5'-aminosalicylic acid (5'ASA) and sulfapyridine (SP). SSZ and its derivatives are thought to primarily exert their immuno-modulatory effects via the inhibition of NF-κB, thereby resulting in the inhibition of inflammatory cell chemotaxis, cytokine expression in mononuclear cells, lymphocyte proliferation and activation, and angiogenesis (Rodenburg et al., 2000). b) SSZ has also been shown to induce apoptosis, via a mechanism that is yet to be fully elucidated, but seems to involve the activation of caspases 8 and 3, the activity of which further inhibits the NF-κB activation pathway (Rodenburg et al., 2000).
Hydroxychloroquine	HCQ	a) HCQ blocks a signalling pathway common to TNF-β and IL-1β, which depends on the activation of endosomal NADPH oxidase 2 (NOX2). The major effect of HCQ is the prevention of agonist-induced gp91phox translocation into the endosome, which abrogates the function of NOX2 and the production of reactive oxygen species (Muller-Calleja et al., 2016). b) HCQ decreases the release of prostaglandin E_2 (PGE_2), a principle mediator of inflammation in RA, via the upregulation of the inhibitory enzyme 15-hydroxyprostaglandin dehydrogenase (HPGD) (Kim et al., 2015). c) HCQ blocks the binding of endosomal toll-like receptors (TLR) 3, 8 and 9 to their ligands dsRNA, ssRNA and unmethylated ssDNA, respectively, thereby reducing the activation of plasmacytoid dendritic cells (pDCs) and the production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) that are responsible for disease development and progression (Kuznik et al., 2011).

Table 1.3:- Conventional synthetic (cs) and biological (b) disease-modifying anti-rheumatic drugs (DMARDs) used in the treatment of RA therapies.

Glucocorticoids	GCs	a) GCs, such as prednisone, prednisolone and methylprednisolone, exert their effects by binding to the glucocorticoid receptor (GR). Upon binding, the activated GR complex, translocates to the nucleus where it upregulates the transcription of anti-inflammatory cytokines, such as interleukin-10 (IL-10) (transactivation), whilst simultaneously inhibiting the activities of the pro-inflammatory transcription factor NF- κ B (transrepression) (Caplan et al., 2017; Czoek et al., 2005).
Biological DMARDs:-		
Infliximab	IFX	a) IFX is a human-mouse chimeric IgG₁ monoclonal antibody, consisting of murine-derived immunoglobulin heavy and light chain variable regions coupled to human IgG₁ immunoglobulin heavy and light chain constant regions. Capable of binding with high affinity to both soluble and membrane-bound forms of TNF-α, IFX is able to neutralize TNF-α, preventing its binding to the receptors, tumour necrosis factor receptor 1 (TNFR1) and tumour necrosis factor receptor 2 (TNFR2); thereby inhibiting the activation of NF-κB and the subsequent production of further pro-inflammatory cytokines (Liang et al., 2013). b) Due to its possession of a human IgG₁ immunoglobulin constant region, IFX also has the ability to induce antibody-dependent cell mediated cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), as well as the apoptosis of pro-inflammatory cells bearing membrane-bound TNF-α (Nesbitt et al., 2007).
Adalimumab	ADA	a) ADA is a fully humanized IgG₁ monoclonal antibody, capable of binding, with high affinity, to TNF-α. Due to its intact and humanized immunoglobulin constant regions, ADA is able to induce ADCC, CDC and the apoptosis of cells bearing membrane-bound TNF-α (Haraoui et al., 2011; Nesbitt et al., 2007). b) As with IFX, ADA is also able to neutralize all forms of TNF-α: soluble, transmembrane and receptor-bound, reducing pro-inflammatory signalling via NF-κB, downstream of TNFR1 and TNFR2 (Nesbitt et al., 2007).
Golimumab	GOL	a) GOL is a fully humanized IgG ₁ monoclonal antibody, capable of binding to and inhibiting the activity of soluble and transmembrane forms of TNF- α . A highly stable monoclonal antibody, with low immunogenicity, but possessing a high affinity for TNF- α , GOL has the capacity to neutralize TNF- α , as well as induce ADCC, CDC, or apoptosis of pro-inflammatory cells bearing membrane-bound TNF- α (Kay and Rahman, 2010; Shealy et al., 2010).
Certolizumab pegol	CZP	a) CZP is a PEGylated Fab' fragment of a humanized monoclonal antibody that binds to, and neutralizes, TNF- α . The attachment of the polyethylene glycol (PEG) moiety to the monoclonal antibody Fab' fragment substantially improves the half-life of CZP to that of an intact monoclonal antibody, without increasing its immunogenicity. As CZP lacks the Fc domain of an intact immunoglobulin molecule, binding of CZP to TNF- α does not induce ADCC, CDC, or apoptosis of pro-inflammatory cells bearing membrane-bound TNF- α , but instead more likely results in the neutralization of soluble TNF- α , thereby limiting its downstream pro-inflammatory effects (Nesbitt et al., 2007).
Etanercept	ETA	a) Unlike the other anti-TNF-α agents used in the treatment of rheumatoid arthritis, ETA is a fusion protein, composed of a human IgG₁ immunoglobulin constant region fragment (Fc), coupled to two copies of the p75 subunit, the extracellular portion of the human TNFR2 receptor (Kaymakcalan et al., 2009). The unique structure of ETA confers the ability to neutralize soluble and membrane-bound TNF-α, although the efficacy of ETA to bind membrane-bound TNF-α is generally agreed to be less than that of IFX (Nesbitt et al., 2007). b) Unlike IFX, ADA, GOL and CZP, ETA is also capable of neutralizing lymphotoxin, also known as tumour necrosis factor β (TNF-β); although the relative contribution of lymphotoxin to RA pathogenesis is uncertain (Nesbitt et al., 2007). c) Finally, ETA is also able to induce CDC and ADCC, although to a lesser extent than IFX and ADA (Nesbitt et al., 2007).

Table 1.3 continued:- Conventional synthetic (cs) and biological (b) disease-modifying anti-rheumatic drugs (DMARDs) used in the treatment of RA therapies.

Abatacept	ABT	a) ABT is a dimeric fusion protein composed of the human cytotoxic T lymphocyte antigen 4 (CTLA-4) extracellular domain, coupled to a human Fc portion of IgG₁. This fusion creates a soluble receptor that is able to bind with high affinity to the co-stimulatory molecules CD80 and CD86, which in turn blocks the activation of otherwise pro-inflammatory T-cells (Picchianti Diamanti et al., 2014). b) ABT is also able to reduce the adherence and transendothelial migration of monocytes, to endothelial cells, rendering pro-inflammatory monocytes incapable of penetrating the synovial tissue where they would otherwise contribute to the overall pathology of RA, including joint damage and destruction (Picchianti Diamanti et al., 2014).
Rituximab	RTX	a) RTX is a chimeric monoclonal antibody specifically designed to deplete CD20 expressing B-cells, as well as short-lived plasma cells. Its precise mode of action is yet to be fully elucidated, but likely involves the targeting of CD20 expressing cells for destruction via ADCC, CDC and apoptosis (Dass et al., 2008).
Tocilizumab	TOC	a) TOC is a humanized monoclonal antibody specifically designed to abrogate the actions of the pro-inflammatory cytokine interleukin 6 (IL-6); a cytokine whose pro-inflammatory actions are pivotal in the pathogenesis of RA; by blocking the binding of IL-6 to the membrane bound and soluble forms of the interleukin-6 receptor (IL-6R) (Burmester et al., 2016).
Tofacitinib	INN	a) INN is an oral janus kinase (JAK) inhibitor that preferentially impedes receptor signalling through JAK1 and JAK3, by directly interfering with JAK3- and JAK1-dependent signal transducer and activator of transcription (STAT) activation. As such, signalling via the γc cytokines and their receptors, including interleukin-2 (IL-2), interleukin-4 (IL-4) and interleukin-9 (IL-9), may be interrupted. In the setting of RA tofacitinib is thought to impair the production of inflammatory Th17 cells, that are otherwise generated in response to IL-1β, IL-6 and interleukin-23 (IL-23), whilst enhancing the production of IL-17A from anti-inflammatory Th17 cells generated in the presence of IL-6 and transforming growth factor (TGF)-β (Ghoreschi et al., 2011).

Table 1.3 continued:- Conventional synthetic (cs) and biological (b) disease-modifying anti-rheumatic drugs (DMARDs) used in the treatment of RA therapies.

In the event of therapeutic failure or intolerance, the use of a biological DMARD may be considered, usually in combination with a conventional synthetic DMARD, designed to reduce the likelihood of patients developing autoantibodies against their monoclonal therapy, which can substantially reduce bDMARD therapeutic efficacy (Emery, Sebba and Huizinga, 2013; Wolbink et al., 2006). The introduction of the tumour-necrosis factor (TNF) inhibitors, infliximab, adalimumab, etanercept, golimumab and certolizumab pegol are often considered at this stage; whilst the IL-6 receptor blocker tocilizumab and the CTLA-4 fusion protein abatacept may also be considered in the event of TNF inhibitor inefficacy (Smolen et al., 2014). Patients should again be monitored closely at this stage, with the aim of achieving remission within a further 6 months. If, however, the therapeutic target is not reached within this time frame, a change in TNF inhibitors or the introduction of a different bDMARD, such as the anti-CD20 antibody rituximab, should again be considered (Harrold et al., 2015; Picchianti Diamanti et al., 2014; Smolen et al., 2014). Other biologic agents, such as the JAK inhibitor Tofacitinib may also be utilized, particularly when other therapeutic agents have failed to be efficacious (Ghoreschi et al., 2011; Smolen et al., 2014). Any indicators of increased disease activity, such as early joint damage, or the presence of anti-CCP antibodies and rheumatoid factor, should result in the further tailoring of the prescribed therapeutic regimen, in order to appropriately manage patients presenting with increased disease activity (Espinoza, Fabre and Pers, 2016). Altogether, using this algorithm patient disease activity can be closely monitored and therapy swiftly altered in the event of intolerance or inefficacy, thereby reducing the risk of progressive joint damage.

1.1.d. Current Remission Monitoring Practices

Identifying when a therapy is proving ineffective can be extremely challenging and, in general rheumatology practice, is reliant upon accurate formal joint counts, such as those used to generate the 28 joint disease activity score (DAS28), as well as the ACR-EULAR criteria for remission. These criteria comprise of tender and swollen joint counts, CRP

and erythrocyte sedimentation rate (ESR) measurements, as well as patient global assessments, such as the health assessment questionnaire (HAQ); all of which are designed to assess a patient's current inflammatory state (Felson et al., 2011). This is a potentially insensitive means of assessing remission, in part due to the often disassociated levels of the inflammatory markers CRP and ESR with a patient's clinical disease activity index (CDAI); as assessed by formal joint counts in combination with patient and physician global assessments; combined with the relative subjectivity of questionnaires (Kay et al., 2014). As the clinical approach to RA therapy is now based heavily upon the concept of 'treating to target' (T2T), the relative insensitivity of these remission monitoring practices may potentially hinder timely alterations to therapeutic regimens, ultimately compromising patient care and disease prognosis (Smolen et al., 2016).

Phase III clinical trials are also met with similar challenges, particularly when the efficacy of a new therapeutic regimen is to be assessed. Both ACR and EULAR response criteria are typically adopted as measures of clinical response, with ACR20, ACR50 and ACR70 response criteria arguably the most widely used (Criteria, 2007; Ward, Guthrie and Alba, 2014). However, these response criteria are also dependent upon the conventional disease activity measures named above, with 20.0%, 50.0% or 70.0% improvement rates in swollen and tender joint counts a prerequisite; accompanied by 20.0%, 50.0% or 70.0% improvement in 3 out of 5 other clinical measures, including the clinician's assessment of disease activity, the patient's assessment of disease activity, the patient's assessment of pain, the patients assessment of physical function and the levels of the inflammatory markers, CRP and ESR (Criteria, 2007; Ward, Guthrie and Alba, 2014). As such the identification of new biomarkers to improve the predictive value of these remission evaluation models would greatly benefit patient care and has therefore been a major focus (Alzabin et al., 2012; Ponchel et al., 2013).

1.1.e. Disease Pathogenesis – Key Modulators of Disease

1.1.e.i. T-cell development - In recent years, several studies have demonstrated the utility of measuring CD4⁺ T-cell subsets, in order to predict the achievement of clinical remission in RA patients. Generated in the thymus, T-lymphocytes (T-cells) develop from common lymphoid progenitors (CLPs); lymphocyte precursors derived from haematopoietic stem cells produced in the bone marrow, with the capacity to develop into T-, B- and NK-cells given the appropriate developmental signals (Figure 1.3) (Jin et al., 2012; Rothenberg, Moore and Yui, 2008; Yui and Rothenberg, 2014). During naïve T-cell development, CLPs migrate to the thymus, entering and populating the subcapsular region of the thymic cortex, where they receive signals from thymic-resident stromal cells, enforcing their commitment to the T-cell lineage (Hu et al., 2015; Klein et al., 2014). At this developmental checkpoint, T-lineage specific genes are activated, via Notch-1 signalling, inducing the expression of CD2, CD5 and CD127, the α -chain of the interleukin-7 receptor (IL-7R); all of which are required for continued T-cell commitment and survival (Figure 1.3) (Rothenberg, Moore and Yui, 2008; Spits, 2002; Wiekmeijer et al., 2016; Yui and Rothenberg, 2014).

With the upregulation of these early T-cell molecules, the rearrangement of T-cell receptor (TCR) genes and the generation of a functional TCR is initiated within these immature thymocytes. It is during this phase of development that the maturation of juvenile T-cells is further committed to one of two pathways; that of $\alpha\beta$ or $\gamma\delta$ T-cells, depending upon their successful rearrangement of the α - and β -chain genes or the γ - and δ -chain genes of the TCR, respectively, in a process known as V(D)J recombination (Yui and Rothenberg, 2014). In healthy individuals, approximately 95.0% of immature thymocytes enter the circulation as naïve $\alpha\beta$ T-cells, the fundamental constituent of T-cell immunity in healthy individuals; with the remaining 5.0% consisting of $\gamma\delta$ T-cells (Figure 1.3) (Davis and Bjorkman, 1988; Spits, 2002; Takahama, 2006).

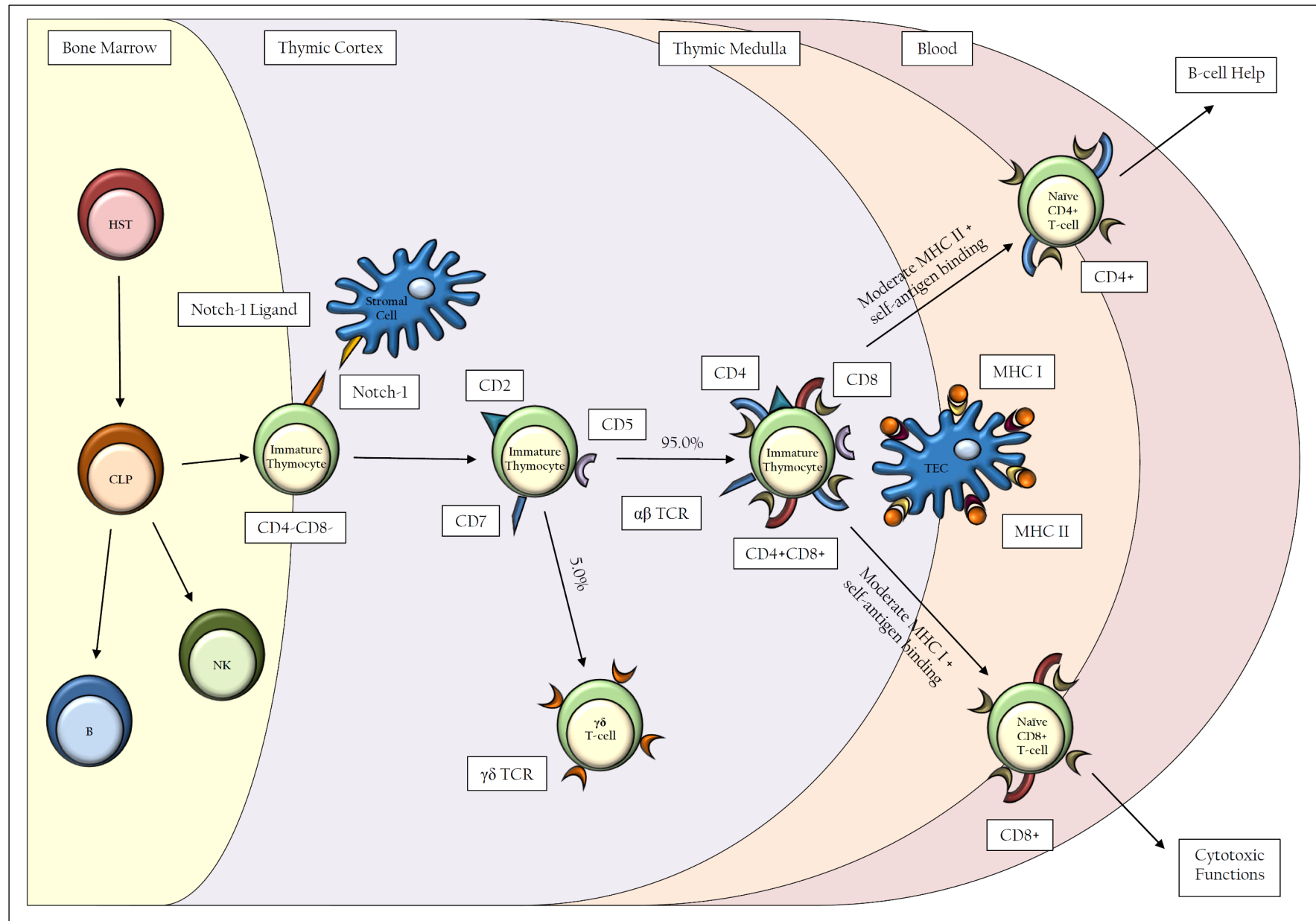


Figure 1.3:- Proposed mechanism for CD4⁺ and CD8⁺ naïve T-cell development, within the cortical and medullary regions of the thymus. Adapted from Spits (2002).

Following V(D)J recombination, and the successful expression of a pre-TCR on the cell surface, these immature thymocytes undergo an intense period of proliferation, after which mature TCR:CD3 complexes, together with co-expression of CD4 and CD8 signalling molecules, are presented upon the cell surface (Yui and Rothenberg, 2014). At this stage of development, juvenile $\alpha\beta$ T-cells must successfully navigate through two further developmental checkpoints; ensuring their ability to respond to foreign antigens, whilst demonstrating “tolerance” to self-antigens (Klein et al., 2014; Moran and Hogquist, 2012; Persaud et al., 2014). During these positive and negative selection processes, immature $\alpha\beta$ T-cells are inspected for self-antigen:major histocompatibility complex (MHC) binding ability and affinity, the successful completion of which pledges immature $\alpha\beta$ T-cells and their continued development to one of two pathways; that of CD4+ or CD8+ $\alpha\beta$ T-cells, depending upon their recognition of, and strength of binding to, self-antigen:MHC class I or class II complexes presented on the surface of cortical (cTECs) and medullary thymic epithelial cells (mTECs), respectively (Figure 1.3) (Chen et al., 2017; Hu et al., 2015; Klein et al., 2014; Lee et al., 2012).

With these developmental checkpoints successfully navigated, naïve CD4+ and CD8+ T-cells migrate through the thymic cortex into the medullary region of the thymus, before entering the circulation as mature, “self-tolerant” CD4+ and CD8+ T-cells (Hu et al., 2015; Jin et al., 2012; Persaud et al., 2014). These naïve CD4+ and CD8+ T-cells migrate to secondary lymphoid tissues, cycling between the circulation and the lymphatics, until such a time as they come into contact with their cognate antigen, in association with the appropriate co-stimulatory signals; presented by professional antigen-presenting cells (APCs) (Chen et al., 2017; Jin et al., 2012). Upon activation, both CD4+ and CD8+ T-cells acquire further helper and cytotoxic functions, respectively, depending upon the cytokine milieu and their interactions with other cellular components, including professional APCs and B-cells (Figure 1.3) (Chen et al., 2017; Kim et al., 2013).

Broadly speaking, CD4⁺ and CD8⁺ T-cells bear contrasting functional capabilities; with CD4⁺ T-cells considered critical in the provision of B-cell help to undergo somatic hypermutation, affinity maturation and class-switch recombination, for the production of immunoglobulins of the IgG, IgA and IgE isotypes (Chevalier et al., 2011; Ma et al., 2012; Morita et al., 2011). In addition, peripheral tolerance to self-antigens is maintained via the suppressive actions of naturally-occurring regulatory T-cells, which develop in the thymus from immature CD4⁺ thymocytes (Lee et al., 2012; Nunes-Cabaco et al., 2011). In contrast, CD8⁺ T-cells possess cytotoxic functions, producing granzyme B and perforin, packaged into cytolytic granules; effector molecules that are pivotal for immune defence against intracellular pathogens, including viral and mycobacterial infections. Moreover, the cytotoxic functions of CD8⁺ T-cells may also contribute to anti-tumour immunity (Figure 1.3) (Chang, Wherry and Goldrath, 2014; Wolint et al., 2004).

Further to their principal roles, CD8⁺ and, in particular, CD4⁺ T-cells may be further subdivided into functionally distinct helper T-cell subpopulations, based upon their transcription factor, cytokine and chemokine receptor expression profiles. Presently, Th1, Th2, Th17, Th1/17, Th9, Th22 and follicular helper T-cells have all been described, with each subpopulation thought to differentially contribute to the formation of adaptive immune responses (Acosta-Rodriguez et al., 2007; Kara et al., 2013; Morita et al., 2011; Okada et al., 2015; Plank et al., 2017; Sallusto et al., 1998). With such contrasting, and intricate roles to play in adaptive immunity, it is conceivable that defects in any number of, or a combination of, these T-cell subpopulations could contribute to a loss in “self-tolerance” and the development of autoimmune conditions, including rheumatoid arthritis. As such, the characterization, and measurement, of CD4⁺ T-cell subsets has become a growing area of interest.

1.1.e.ii. General principles of RA development - RA has a complicated and highly heterogeneous pathophysiology, which appears to involve a break in tolerance to self-antigens, such as

vimentin, fibrinogen and aggrecan, following their modification by the enzyme peptidyl arginine deiminase type IV (PAD4) (Cordova et al., 2013; Shin et al., 2013; Snir et al., 2011; von Delwig et al., 2010). Such post-translational modifications may occur following episodes of bronchial stress or periodontal disease, for example, conditions in which the expression of PAD4 enzymes is increased. This increased enzymatic activity results in the introduction of citrulline residues into the primary structure of proteins such as fibrinogen, thereby increasing their affinity for the shared epitope QKRAA, a common amino acid motif found in the HLA-DRB1 molecule of genetically susceptible individuals (McInnes and Schett, 2011; Shin et al., 2013). The increased affinity of these citrullinated peptides for HLA-DRB1 results in the uptake and subsequent expression of these peptides by APCs, such as dendritic cells and macrophages, that may consequently migrate to secondary lymphoid organs, where they are able to activate self-reactive CD4⁺ helper T-cells (Figure 1.4) (James et al., 2010; McInnes and Schett, 2011).

The activation of APCs, and subsequently self-reactive CD4⁺ helper T-cells, has a number of downstream effects, including the increased production of pro-inflammatory cytokines, such as interleukin 6 (IL-6), interleukin 1 (IL-1), interleukin 17 (IL-17) and tumour necrosis factor- α (TNF- α). These pro-inflammatory cytokines perpetuate local and systemic inflammation, by promoting the activation of endothelial cells, increasing the expression of adhesion molecules, as well as cytokine and chemokine receptors, whilst also promoting local angiogenesis (McInnes and Schett, 2011). The augmented production of IL-6, IL-1 and TNF- α also supports the development of osteoclasts, whose bone resorption and remodelling activities are heavily implicated in the development of RA radiographic changes and end stage joint destruction (Figure 1.4). The production of IL-17, as well as the expression of receptor activator of NF- κ B ligand (RANK-L) by self-reactive CD4⁺ helper T-cells has also been implicated in this process (Sato et al., 2006). In addition to their involvement in osteoclastogenesis, self-reactive T-cells are also capable of providing co-stimulation to self-reactive B-cells; promoting affinity maturation, class switch

recombination and their maturation into antibody secreting plasma cells, giving rise to the production of IgG autoantibodies (Figure 1.4) (McInnes and Schett, 2011; Sato et al., 2006; Snir et al., 2011).

The evidence supporting the role of T-cells in RA pathophysiology is therefore three-fold. Firstly, antibodies directed against cyclic citrullinated peptides may be detected in RA patients up to 15 years before disease onset, supporting the theory of a loss of tolerance to self-antigens; a state which is usually tightly regulated by T-cell selection processes within the thymus, as well as by naturally-occurring regulatory T-cells out in the periphery (Cooles, Isaacs and Anderson, 2013; James et al., 2010; Law et al., 2012). Secondly, although not considered pathogenic, anti-CCP antibodies are of the IgG isotype, indicating that auto-reactive B-cells producing antibodies against cyclic citrullinated proteins have received T-cell help to undergo class switch recombination (Snir et al., 2011). Finally, self-reactive CD4⁺ helper T-cells are capable of inducing osteoclastogenesis via their production of IL-17 and the induction of RANK-L on synovial fibroblasts, upregulating osteoclast activity (Sato et al., 2006). Based on this evidence the T-cell subsets of particular interest have been follicular and antigen-specific helper T-cell subpopulations, as well as naturally occurring regulatory T-cells, namely for their roles in providing B-cell help, controlling peripheral tolerance to self-antigens, and the induction of osteoclastogenesis, respectively (Cooles, Isaacs and Anderson, 2013; Morita et al., 2011; Sato et al., 2006).

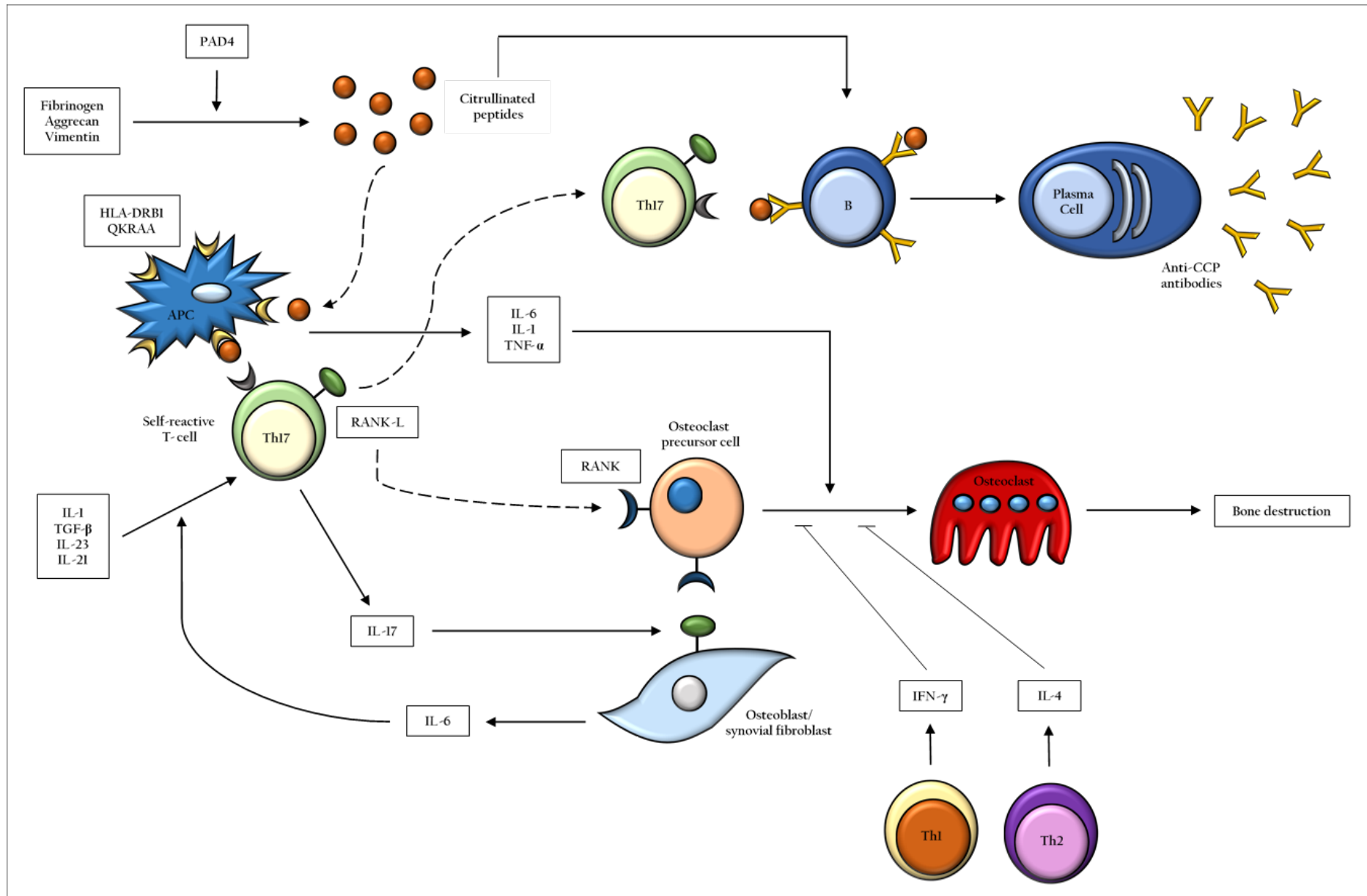


Figure 1.4:- Proposed pathogenic mechanism of rheumatoid arthritis. Adapted from Sato et al (2006).

1.1.e.iii. *Follicular helper CD4⁺ T-cells* - Specialized for providing B-cell help within the germinal centres of secondary lymphoid tissue, follicular helper T-cells are a subset of memory CD4⁺ helper T-cells expressing the chemokine receptor CXCR5; allowing their migration into B-cell follicles in response to the ligand CXCL13 (Craft, 2012; Morita et al., 2011). Further distinguished by their expression of CD40-ligand (CD40L), programmed cell death 1 (PD-1) and inducible costimulator (ICOS), as well as their ability to secrete the cytokines interleukin 4 (IL-4), interleukin 10 (IL-10) and interleukin 21 (IL-21), the primary function of follicular helper T-cells is to promote the proliferation, differentiation, class switch recombination and affinity maturation of activated B-cells in response to antigenic challenge (Li et al., 2012; Ma et al., 2012; Morita et al., 2011; Tangye et al., 2013).

Historically, the most accurate means of assessing follicular helper T-cells, within health and disease, is to acquire lymph node samples. However, this can prove challenging, and as such it has become widely accepted that the measurement of peripheral blood-derived CD4⁺ helper T-cells bearing CXCR5 and CD45RO, an isoform of CD45 only found on circulating memory cells, may serve as a surrogate measure of lymph node-derived follicular helper T-cells (Chevalier et al., 2011; Li et al., 2012). There is also mounting evidence to show that this specialized group of memory CD4⁺ helper T-cells is highly heterogeneous, with distinct subsets of follicular helper T-cells possessing highly diverse cytokine secretion profiles (Fazilleau et al., 2009; Morita et al., 2011; Reinhardt, Liang and Locksley, 2009). Indeed, in 2011, Morita et al demonstrated the ability to subdivide blood-derived follicular helper T-cells into Th1-, Th2- and Th17-type cell subsets, based upon their expression of the chemokine receptors CCR6 and CXCR3; with the Th17-type cell subset, defined as CCR6⁺CXCR3⁻, the most efficient at helping B-cells to make immunoglobulins of the IgG, IgA and IgM isotypes. In contrast, the Th2-type subset, defined as CCR6⁻CXCR3⁻, was the only blood-derived follicular helper T-cell subpopulation capable of inducing the production of IgE (Ma et al., 2015; Morita et al., 2011). This group then went on to further show that the skewed distribution of follicular

helper T-cell subpopulations towards Th2/Th17-type cell subsets correlates with disease activity in patients with juvenile dermatomyositis (Morita et al., 2011). Since then further studies have demonstrated an increase in Th17 cells in RA patients, as compared to healthy controls; with sustained numbers of circulating Th17 cells present following an incomplete response to TNF- α blockade, further cementing the potential role of follicular helper T-cells in RA pathogenesis (Alzabin et al., 2012; Chen et al., 2011).

1.1.c.iv. Antigen-specific CD4⁺ T-cells - The role of antigen-specific CD4⁺ T-cells, and their composite subpopulations, has also become an area of interest over recent years. Infectious agents have long been considered to be potential triggers of RA development, and although previous studies have failed to identify a single infectious agent with a direct pathophysiological role, viral agents, such as cytomegalovirus (CMV), herpes simplex virus (HSV) and epstein barr virus (EBV) have been of particular interest (Magnusson et al., 2010; McInnes and Schett, 2011). This is in part due to the isolation of CMV from the synovial fluid of RA patients, as well as an observed increase in EBV-DNA, EBV-specific antibodies and EBV-infected cells within RA patients, as compared to healthy controls. In addition, it has also been documented that latent infection with CMV may in fact aggravate the clinical course of RA, resulting in advanced joint destruction, and consequently an increase incidence of joint-related surgical procedures (Davis et al., 2012; Magnusson et al., 2010; Pierer et al., 2012).

With a potential viral aetiology, coupled with the widely accepted understanding that CD4⁺ and CD8⁺ T-cell responses are fundamentally required to orchestrate an effective and efficient anti-viral response, a recent study sought to profile immune responses to the herpesviruses CMV and EBV, within RA patients, so as to further investigate their role in radiographic joint damage. This group were able to show a significant increase in IFN- γ and interleukin-13 (IL-13) production, within RA patients with a high degree of joint damage, as compared to healthy controls and RA patients with a lower degree of joint

damage; thereby implicating T-cell subset anti-viral responses in RA joint destruction. Thus, based upon such results, a thorough investigation of antigen-specific T-cell responses in rheumatoid arthritis patients would seem warranted (Davis et al., 2012).

Until recently, however, the investigation of antigen-specific responses was limited to two lines of inquiry; namely detection of the pathogen directly, or the measurement of the immune responses to those pathogens, either via the detection of immunoglobulin or cytokine responses (Sadler et al., 2014; Zaunders et al., 2009). Recent publications have since described a flow cytometric technique, utilizing a whole blood stimulation method to viral, fungal and bacterial lysates, in which antigen-specific CD4⁺ T-cell responses can be detected and quantified, by monitoring for the upregulation of the alpha subunit of the IL-2 receptor (IL-2R α), CD25, and the costimulatory molecule OX40 (also known as CD134), both of which are required for activated T-cell survival (Hsu et al., 2012; Sadler et al., 2014; Zaunders et al., 2009). A method such as this would allow the direct investigation of antigen-specific CD4⁺ T-cell responses to agents, such as CMV, within RA patients; whilst the expansion of this flow cytometric assay to incorporate the T helper lineage-specific chemokine receptors CCR6 and CXCR3 would also facilitate a more thorough examination of their composite memory CD4⁺ T-cell subpopulations, and accordingly their role in RA pathogenesis (Ma et al., 2015; Morita et al., 2011). Moreover, given the proposed relationship between infectious agents and RA development, this method could also conceivably provide a further measure of disease activity (Davis et al., 2012).

1.1.e.v. Naturally-occurring regulatory CD4⁺ T-cells - The role of naturally-occurring regulatory T-cells in RA pathophysiology and as a consequence disease activity monitoring has also been extensively investigated. Constituting approximately 5.0 – 10.0% of CD4⁺ T-cells, naturally occurring regulatory T-cells originate in the thymus as a specialized subset of helper T-cells, designed to suppress and limit T cell responses in the periphery (Byng-

Maddick and Ehrenstein, 2015; Wing et al., 2014). Expressing high levels of CD25, cytotoxic T lymphocyte-induced antigen 4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related protein (GITR), naturally-occurring regulatory T cells also produce high levels of the anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor beta (TGF- β) (Blache et al., 2011; Byng-Maddick and Ehrenstein, 2015; Picchianti Diamanti et al., 2014). Their exact mode of action is not fully understood, but it is thought that this particular form of regulatory T-cells exerts their suppressive function, via their secretion of IL-10 and TGF- β , as well as via direct cell-to-cell contact through CTLA-4 (Blache et al., 2011; Cribbs et al., 2014). Due to their highly suppressive capabilities naturally-occurring regulatory T-cells play a critical role in limiting the actions of auto-reactive T-cells, that have failed to be removed during positive and negative selection processes in the thymus, and have escaped into the periphery; and as a consequence, their role in preventing autoimmunity is pivotal (Blache et al., 2011; Byng-Maddick and Ehrenstein, 2015).

The impact of regulatory T-cells on RA disease course and therapy efficacy has become even more evident following a recent publication by Nie et al who demonstrated the effect of TNF- α on the activity of naturally-occurring regulatory T-cells (Nie et al., 2013). Naturally-occurring regulatory T-cell function and homeostasis is highly dependent upon the stable expression of the regulatory T-cell-specific transcription factor FoxP3, a protein which undergoes a number of post-translational modifications, including methylation and phosphorylation, to reach its active form. Numerous disturbances in FoxP3 post-translational modifications have now been documented in patients with active RA, with reductions in regulatory T-cell suppressive capabilities correlating with the presence of these disturbances (Kennedy et al., 2014; Nie et al., 2013). Interestingly, it has recently been shown that one such disturbance, namely the dephosphorylation of FoxP3, occurs in the presence of TNF- α , a cytokine that is abundantly present in the joints of patients with active RA (Nie et al., 2013). This dephosphorylation reduces the suppressive capabilities

of regulatory T-cells in these patients, thereby contributing to their continuing active inflammatory state. Of even more importance is the subsequent demonstration of regulatory T-cell function restoration following the successful treatment of these patients with anti-TNF- α agents (Gao et al., 2015; Nie et al., 2013). This work not only sheds light on the probable role of defective regulatory T-cells in RA pathogenesis, but also highlights the potential benefit of monitoring regulatory T-cell function in the detection of therapeutic efficacy and the establishment of clinical remission.

1.2. Aims of the Study

1.2.a. *Study Hypothesis*

Based on the current evidence, the working hypotheses of this study are therefore that RA patients who fail to make a clinical improvement following the initiation of csDMARD and bDMARD therapeutic regimens are identifiable by their profile of the following CD4⁺ T-cell populations: follicular helper CD4⁺ T-cells, memory helper CD4⁺ T-cells, antigen-specific CD25⁺CD134⁺ activated CD4⁺ T-cells and naturally-occurring regulatory CD4⁺ T-cells. Therefore, the addition of these parameters to current clinical remission assessment practices, will improve the identification of RA therapy non-responders, enabling better disease management. This work is to be conducted as part of a much wider study, which is funded by a CSO NIHR Healthcare Science Research Fellowship.

1.2.b. *Aims and Objectives*

1. To investigate the relative distribution and absolute counts of follicular and memory helper CD4⁺ T-cells and their composite subpopulations, in longstanding RA patients, disease and healthy control groups.
2. To investigate the relative distribution of antigen-specific CD25⁺CD134⁺ activated CD4⁺ T-cell recall responses to PHA, CMV, HSV and aspergillus, as well as their composite subpopulations, in longstanding RA patients, disease and healthy control groups.
3. To investigate the relative numbers of naturally-occurring regulatory CD4⁺ T-cells and their suppressive capabilities, following antigenic challenge, within longstanding RA patients, disease and healthy control groups, via the study of FoxP3 expression and its phosphorylation status.

4. To assess follicular helper, memory helper, antigen-specific CD25+CD134+ activated and naturally-occurring regulatory CD4+ T-cell subsets, and as a result the immune function profiles of newly-diagnosed, treatment-naïve RA patients.
5. To conduct the longitudinal follow-up of newly-diagnosed, treatment-naïve RA patients at 3-, 6- and 12-monthly intervals, to determine the clinical utility of introducing T-cell subpopulation profiling in therapy efficacy and clinical remission monitoring.

Chapter 2: Materials and Methods

2.1. Study Design

2.1.a. Study Participants

Sample size calculations were performed using the online calculator found at powerandsamplesize.com/Calculators. Using a 2-sample, 2-sided equality calculator, pilot data gathered from follicular helper CD4⁺ T-cell, antigen-specific CD4⁺ T-cell and naturally-occurring regulatory CD4⁺ T-cell assays was analysed (Table 2.1). From this provisional data, it was established that in order to detect a significant difference between patient and control groups, data from a minimum of 21 longstanding RA patients and 47 healthy controls would be required; assuming a type I error rate of 5.0% with a 90.0% power of rejecting the null hypothesis (Figure 2.1).

Power Calculation Parameters						
Assay	Assay Parameter	Group A Mean	Group B Mean	Group B SD	Sampling Ratio	Sample Size
Follicular helper CD4 ⁺ T-cells	Follicular Helper T-cell (CD45RO ⁺ CXCR5 ⁺) (%)	11.1	7.9	3.8	0.46	$\frac{A = 21}{B = 47}$
Antigen-specific CD4 ⁺ T-cells	CD25 ⁺ CD134 ⁺ activated CD4 ⁺ T-cell (% to PHA)	50.7	67.4	8.9	0.46	$\frac{A = 5}{B = 10}$
Naturally-occurring regulatory CD4 ⁺ T-cells	FoxP3 (of CD25 ⁺ CD127 ^{lo} CD4 ⁺ T-cells (% to PHA)	21.8	34.6	9.1	1.86	$\frac{A = 17}{B = 9}$

Table 2.1:- Power calculations performed on pilot data obtained from follicular helper, antigen-specific and naturally-occurring regulatory CD4⁺ T-cell assays. Group A = longstanding RA patients, Group B = healthy controls, SD = Standard Deviation, Sampling Ratio = Number of Group A/Number of Group B, Sample Size = Number of Group A and Group B participants required to find a statistically significance difference between Group A and B with a type I error rate of 5% and a power of 90%.

With the stratification of longstanding RA patients and disease controls, according to their use of csDMARD and bDMARD therapeutic regimens also to be investigated, the results of these sample size calculations suggest that the recruitment of longstanding RA patients undertaking csDMARD and bDMARD therapeutic regimens to a sampling ratio of 0.46 would facilitate the detection of statistically significant differences between

patient and control groups undertaking csDMARD therapeutic regimens, as compared to those receiving bDMARD-based therapeutics; assuming a type I error rate of 5.0%, with a 90.0% power of rejecting the null hypothesis.

Based upon the above calculations, fifty-six longstanding RA (RA) patients, with a diagnosis of >3 years; 20 newly-diagnosed, treatment-naïve rheumatoid arthritis (ERA) patients, with a diagnosis of <3 years; 32 age- and sex-matched disease controls (DCG), as well as 32 age- and sex-matched healthy controls (HC) were recruited onto the study (Table 2.2).

Study Participants					
Participant Demographics			Therapeutic Regimen		
Study Group	Median Age	M:F Ratio	No therapy	csDMARDs only	bDMARDs
RA (n = 56)	66.0 (29.0 – 89.0)	24:32 (0.75)	4	37	15
ERA (n = 20)	57.0 (35.0 – 84.0)	11:9 (1.22)	20	0	0
DCG (n = 32)	57.0 (35.0 – 74.0)	14:18 (0.78)	0	21	11
HC (n = 32)	55.0 (30.0 – 75.0)	14:18 (0.78)	32	0	0
Total (n = 140)	60.0 (29.0 – 89.0)	63:77 (0.82)	57	60	26

Table 2.2:- Study demographics, including longstanding RA (RA); newly diagnosed, treatment naïve RA (ERA); disease controls (DCG) and healthy control (HC) participants.

All RA and ERA patients were diagnosed according the 2010 ACR/EULAR Classification Criteria for Rheumatoid Arthritis. DCG consisted of 17 psoriatic arthritis, 5 ankylosing spondylitis, 3 granulomatosis with polyangiitis (GPA), 2 systemic lupus erythematosus (SLE), 2 giant cell arteritis (GCA), 1 dermatomyositis, 1 polymyositis, 1 mixed connective tissue disease (MCTD), and 1 IgG4-related disease patient; all of which were undertaking therapeutic regimens consisting of csDMARD and bDMARDs, identical to those employed in the treatment of RA (Table 2.3). All DCG had been diagnosed according to their respective ACR and EULAR diagnostic criteria.

DMARD Combination Therapeutic Strategy	Study Group	
csDMARD Therapeutic Strategy:-	RA No. (%Total)	DCG No. (% Total)
MTX		
MTX Only	6	9
MTX + HCQ	10	1
MTX + HCQ + OCSD	4	0
MTX + OCSD	1	4
MTX + Steroids	2	2
MTX + HCQ + Steroids	1	2
MTX Total	24 (46.2%)	18 (56.3%)
OCSD		
OCSD Only	3	2
OCSD + HCQ	5	0
OCSD + Steroids	1	0
OCSD Total	9 (17.3%)	2 (6.2%)
HCQ		
HCQ Only	3	1
HCQ + Steroids	1	0
HCQ Total	4 (7.7%)	1 (3.1%)
csDMARD Total	37 (71.2%)	21 (65.6%)
bDMARDs Therapeutic Strategy:-	RA No. (%Total)	DCG No. (% Total)
ADA		
ADA Only	0	3
ADA + MTX	2	0
ADA + MTX + HCQ	1	0
ADA + MTX + OCSD	0	1
ADA Total	3 (5.7%)	4 (12.5%)
ETA		
ETA Only	0	2
ETA + MTX	1	1
ETA + MTX + HCQ	1	0
ETA + OCSD	1	1
ETA + Steroids	1	0
ETA + HCQ	1	0
ETA + MTX + OCSD	2	1
ETA Total	7 (13.5%)	5 (15.6%)
IFN		
IFN Only	0	0
INF + MTX	1	0
IFN Total	1 (1.9%)	0 (0.0%)
RTX		
RTX Only	1	0
RTX + Steroids	2	2
RTX + Steroids + Steroid Sparing Agents	1	0
RTX Total	4 (7.7%)	2 (6.3%)
bDMARD Total	15 (28.8%)	11 (34.4%)
DMARD Total	52	32

Table 2.3:- Therapeutic regimens employed in the treatment of longstanding RA patients (RA) and disease controls (DCG) at the time of recruitment; with absolute and relative numbers displayed. csDMARD = conventional synthetic disease modifying anti-rheumatic drug; bDMARD = biological disease modifying anti-rheumatic drug; MTX = methotrexate; HCQ = hydroxychloroquine; OCSD = other conventional synthetic disease modifying anti-rheumatic drug; ADA = adalimumab; ETA = etanercept; IFN = infliximab; RTX = rituximab.

All RA and ERA patients, as well as DCG were recruited from rheumatology outpatient clinics held at the Nuffield Orthopaedic Centre (NOC), Oxford University Hospitals NHS Foundation Trust, Oxford; with 14mls of blood acquired from all RA and ERA patients, as well as DCG and HC. DAS28 scores, CRP and ESR were recorded for all RA and ERA

patients, at the time of participant recruitment, as measures of disease activity. An ESR and CRP measurement of $<20.0\text{mm/hr}$ and $<5.0\text{mg/l}$, respectively were considered to represent low disease activity. The following disease activity targets were used to assess DAS28 scores:- <2.6 = Remission; $2.6 - 3.2$ = Low Disease Activity; $>3.2 - 5.1$ = Moderate Disease Activity; >5.1 = High Disease Activity.

Informed written consent was obtained from all participants. Ethical approval was granted by NRES Committee South Central - Oxford A (REC Reference Number:- 14/SC/0025).

2.1.b. Longitudinal follow-up of newly-diagnosed, treatment-naïve RA patients

Newly-diagnosed, treatment-naïve RA patients were longitudinally monitored for one year after the time of diagnosis; with ERA participant follow-ups occurring at 3-, 6- and 12-monthly intervals, in accordance with ACR-EULAR criteria for remission and ‘Treating to Target’ (T2T) therapeutic regimens. Average ERA follow-up times and participant retention rates were 3.4 months (75.0%), 7.0 months (60.0%) and 11.4 months (70.0%), respectively. Upon diagnosis all ERA patients were commenced on a csDMARD therapeutic regimen, consisting of methotrexate, in combination with hydroxychloroquine and folic acid. All changes to ERA therapeutic regimens were recorded at the participants’ scheduled rheumatology clinic follow-up appointments. DAS28 scores, CRP, ESR and FBC results were re-recorded at the time of each scheduled follow-up appointment.

2.2. Methods

2.2.a. *Follicular and Memory CD4⁺ Helper T-cell Measurements*

Follicular and memory helper T-cells were identified and enumerated via cell surface staining of 100µl EDTA whole blood using 5µl of the following monoclonal antibodies:- CD3 PerCP, CD4 APC-H7, CD45RO PE, CXCR5 Alexa Fluor 488, CCR6 Alexa Fluor 647, CXCR3 PE-Cy7 and CCR4 BV421 (BD Biosciences). Samples were incubated for 15 minutes at room temperature, in the dark, after which 2ml of FACSlyse (BD Biosciences) solution was added for 6 minutes. Samples were subsequently washed with PBS (Oxoid) and then fixed with 400µl 1% formaldehyde ready for acquisition on a BD FACSCanto II. All follicular and memory T-cell subpopulations were expressed as a percentage of CD3⁺CD4⁺CD45RO⁺CXCR5⁺ and CD3⁺CD4⁺CD45RO⁺CXCR5⁻ T-cells, respectively.

2.2.b. *Lymphocyte Subset Enumeration*

T⁺, B⁺ and NK-cell enumeration (TBNK) was performed using EDTA whole blood, BD Multitest™ CD3/CD8/CD45/CD4 and CD3/CD16+CD56/CD45/CD19 antibody cocktails, in combination with BD Trucount™ tubes (BD Biosciences); from which the absolute counts of all follicular, memory and naïve helper T-cell subpopulations were calculated.

All samples for follicular helper T-cell, memory helper T-cell and lymphocyte subset enumeration were acquired on a BD FACSCanto II and analysed using FACSDiva and FACSCanto software, respectively (BD Biosciences).

2.2.c. *Peripheral Blood Mononuclear Cell (PMBCs) Separation*

Lithium heparin blood was diluted 1:2 with RPMI 1640 culture medium (Sigma-Aldrich), supplemented with L-glutamine and Penicillin/Streptomycin (Sigma-Aldrich). Diluted heparin blood was layered over 4ml of lymphoprep (Alere) and centrifuged at 1000g for 22 minutes. The mononuclear cell layer was subsequently removed and washed twice in 10ml

of supplemented RPMI 1640, after which PBMCs were resuspended in 1ml supplemented RPMI 1640 in preparation for counting on a haemocytometer.

To count, all PBMC preparations were diluted 1:5 with trypan blue (Sigma-Aldrich), with 10µl of diluted cell suspension placed onto a disposable counting slide (Immune Systems Limited). Cell counting was performed using a Nikon Eclipse TS100 inverted light microscope (Nikon Instruments Europe B.V.); with a final cell concentration of 2.5×10^7 cells/ml prepared for intracellular transcription factor staining.

2.2.d. Intracellular Transcription Factor Staining - T-bet, ROR γ t, GATA-3

Follicular and memory helper T-cells were identified via cell surface staining of PBMCs, prepared from lithium heparin blood, obtained from seven healthy controls. PBMCs were stained using the following monoclonal antibodies:- CD3 PerCP, CD4 APC-H7, CD45RO PE, CXCR5 Alexa Fluor 488, CCR6 Alexa Fluor 647, CXCR3 PE-Cy7 and CCR4 BV510 (BD Biosciences). Samples were incubated for 15 minutes at room temperature, in the dark, after which samples were washed with PBS (Oxoid) and then permeabilized according to manufacturer's instructions, using the Transcription Factor Buffer Set (BD Biosciences). Once permeabilized, samples were stained intracellularly with either 20µl T-bet BV421 or 20µl ROR γ t BV421 overnight, or 20µl GATA-3 BV421 for 40mins. All samples were subsequently fixed with 1% formaldehyde in preparation for sample acquisition.

All samples for transcription factor expression analysis were acquired on a BD FACs Aria, and analysed using FACs Diva software (BD Biosciences).

2.2.e. Antigen-specific CD4⁺ T-cell Measurements

Heparin blood was diluted 1:1 in ISCOVES modified DMEM (Sigma-Aldrich), with 500µl diluted blood transferred to sterile, capped 5ml flow cytometry tubes (BD Biosciences).

To each relevant tube, the following quantities of whole viral/fungal lysates were added:- 442ng/ml of CMV (Advanced Biotechnologies Inc.), 2.0µg/ml of HSV (Advanced Biotechnologies Inc.) or 20µg/ml of aspergillus fumigatus (Microgen Bioproducts). Positive and negative control tubes were also included for each sample, consisting of 15µg/ml of PHA (Sigma Aldrich) or media alone.

Following incubation for 42 - 44 hours at 37°C with 5% CO₂, samples were vortexed and 200µl stimulated blood transferred to new 5ml flow cytometry tubes (BD Biosciences). Samples were then stained with the following monoclonal antibodies:- CD4 APC-H7, CD14 PerCP, Viaprobe Cell Viability Solution (7-AAD), CD25 FITC, CD134 (OX40) PE, CCR6 Alexa Fluor 647, CXCR3 PE-Cy7 and CCR4 BV421 (BD Biosciences). Samples were incubated for 20 minutes at room temperature, in the dark, after which 2ml FACSlyse (BD Biosciences) solution was added for 20 minutes. Samples were subsequently washed with PBS (Oxoid) and then fixed with 400µl 1% formaldehyde in preparation for acquisition on a BD FACSCanto II.

All antigen-specific T-cell samples were acquired on a BD FACSCanto II, with 30,000 CD4+ T-cell events acquired for each antigenic stimulation. All results were analysed with FACSDiva software (BD Biosciences), with antigen-specific T-cell subpopulations expressed as a percentage of live CD4+CD14-CD25+CD134+ T-cells. Antigen-specific T-cell responses <0.3%, and with less than 50 activated T-cells, to CMV, HSV and Aspergillus were not analysed further for chemokine receptor expression profiles.

2.2.f. CMV Immunoglobulin G Serological Assessments

CMV IgG serological analysis was performed by the Microbiology Laboratory, Oxford University Hospital NHS Foundation Trust, Oxford via the use of the ARCHITECT CMV IgG chemiluminescent microparticle immunoassay (Abbott Diagnostics). The following

manufacturer's reference ranges were used in result interpretation:- <6.0 AU/ml = Nonreactive; 6.0 – 15.0 AU/ml = Equivocal; ≥15.0 AU/ml = Reactive.

2.2.g. Naturally-occurring Regulatory T-cell Measurements

Heparin blood was diluted 1:1 in ISCOVES modified DMEM (Sigma-Aldrich) with 500µl diluted blood transferred to sterile, capped 5ml flow cytometry tubes (BD Bioscience). To each relevant tube, the following quantities of antigen were added:- 20µg/ml of aspergillus fumigatus (Microgen Bioproducts) or 15µg/ml of phytohaemagglutinin (PHA) (Sigma-Aldrich). A negative control tube was included for each sample, consisting of media alone.

Following incubation for 42 - 44 hours at 37°C with 5% CO₂, samples were vortexed and 100µl stimulated blood transferred to new 5ml flow cytometry tubes (BD Biosciences). Samples were then stained with the following monoclonal antibodies:- CD4 APC-H7, CD14 PerCP, Viaprobe Cell Viability Solution (7-AAD), CD25 FITC, and CD127 PE-Cy7 (BD Biosciences). Samples were incubated for 20 minutes at room temperature, in the dark, after which 2ml FACSlyse (BD Biosciences) solution was added for 20 minutes. Samples were subsequently washed with PBS (Oxoid) and then permeabilized using the Transcription Factor Buffer Set (BD Biosciences), according to manufacturer's instructions. Once permeabilized, samples were stained intracellularly with either 5µl FoxP3 PE (BD Biosciences), or 1µl phospho-FoxP3 (S418) antibody (Abgent) with 5µl anti-rabbit IgG PE (R&D Systems). Samples were subsequently fixed with 1% paraformaldehyde in preparation for acquisition on a BD FACSCanto II.

Samples were acquired on a BD FACSCanto II, with 50,000 CD4⁺ T-cell events acquired for each antigenic stimulation. All results were analysed with FACSDiva software (BD Biosciences), with CD25⁺CD127^{lo} naturally-occurring regulatory T-cells expressed as a percentage of live CD4⁺ T-cells, and total FoxP3⁻ and phospho-FoxP3 (S418)-expressing

regulatory T-cell populations expressed as a percentage of live CD4⁺CD14⁻CD25⁺CD127^{lo} T-cells.

2.2.h. Statistics

All statistical analyses were performed using Microsoft Excel and GraphPad PRISM 5 software (GraphPad PRISM, San Diego, CA, USA). Normal ranges for both relative and absolute counts, of follicular and memory T-cell subpopulations, antigen-specific T-cell subpopulations and naturally-occurring regulatory T-cell populations were established by determining the 5th and 95th percentiles of our adult, age- and sex-matched, healthy control cohort, whilst the median and inter-quartile ranges (IQR) were used to further describe the distribution of T-cell subpopulations. Kruskal-Wallis one-way analysis of variance tests were used to compare subpopulation distributions patient and control groups, as well as the stratification of RA patients and DCG according to therapeutic interventions. Dunn's Multiple Comparison Test was used post hoc to correct for multiple comparison analysis. Mann Whitney U tests were used to further compare T-cell subpopulation distributions between individual disease and control groups, as well as to investigate the effects of sex on subpopulation distribution within HC. Spearman's Rank Correlations were used to assess associations of age, as well as the relationship between current disease activity measures and T-cell subpopulations; with r -values $\geq \pm 0.50$ considered to represent an association between two variables. The Wilcoxon matched pairs signed-ranks test was used to assess the longitudinal effects of therapeutic interventions on ERA patients. The delta changes in patient clinical outcomes and T-cell subpopulations, in combination with Spearman's Rank Correlations, were used to assess therapeutic efficacy in ERA patients. For all analyses, a p value of ≤ 0.05 was considered statistically significant.

Chapter 3: The differential expression of CCR4 delineates a subpopulation of Th17-like follicular helper T-cells which are expanded within rheumatoid arthritis following the initiation of anti-TNF- α therapeutic interventions

3.1. Introduction

3.1.a. *Role of Follicular Helper T-cells in the Healthy Immune System*

The fundamental role of follicular helper T-cells in B-cell maturational processes has been extensively investigated over recent years. This specialized subset of memory CD4⁺ T-cells, bearing the lymph node homing C-X-C chemokine receptor 5 (CXCR5) and CD45RO, an isoform of CD45 exclusively found on memory cells, are uniquely capable of migrating to the T-B cell regions of secondary lymphoid organs, in response to the ligand CXCL13, following antigenic stimulation and activation (Ma et al., 2012; Mazerolles et al., 2013; Morita et al., 2011). Further distinguished by their high surface expression of CD40-ligand (CD40L), programmed cell death 1 (PD-1), inducible costimulator (ICOS) and SLAM-associated protein (SAP), as well as their ability to secrete high levels of the cytokines interleukin-4 (IL-4), interleukin-10 (IL-10) and interleukin-21 (IL-21), follicular helper T-cells are highly proficient at promoting the proliferation, class switch recombination, somatic hypermutation and affinity maturation of activated B-cells (Chevalier et al., 2011; Ma et al., 2012). Altogether, the precise orchestration of these B-cell maturational processes, by follicular helper T-cells (Fth), leads to the rapid formation of germinal centres (GCs), along with the generation of a circulating reservoir of class-switched memory B-cells and long-lived plasma cells, capable of excreting high affinity immunoglobulins of the IgG, IgA and IgE isotypes (Figure 3.1) (Chevalier et al., 2011; Morita et al., 2011).

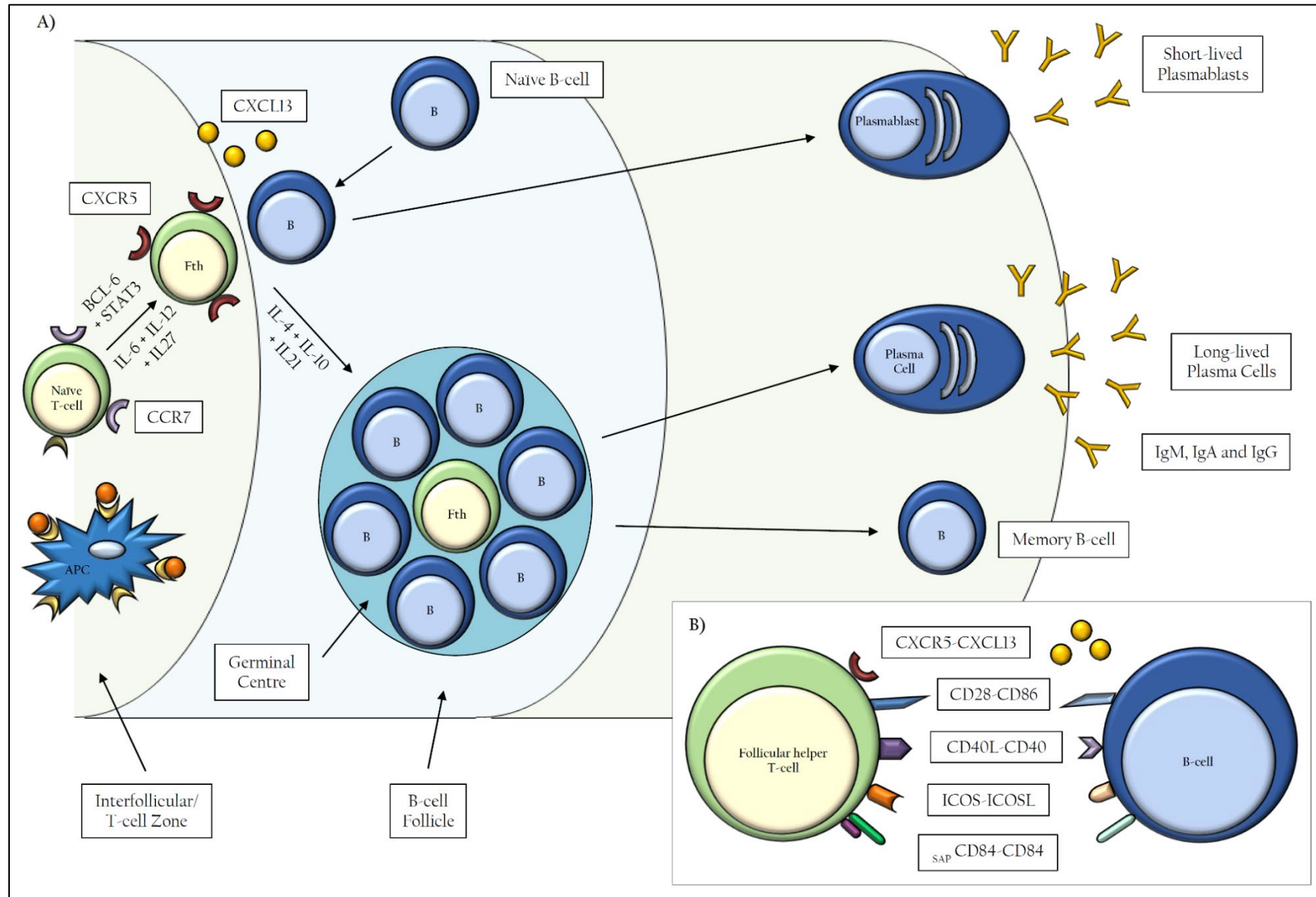


Figure 3.1:- A) Proposed mechanism and localization of follicular helper T-cell and B-cell interactions, within secondary lymphoid structures, during the generation of a humoral immune response. B) Molecular interactions required for follicular helper T-cell development and B-cell maturation. Adapted from Ma et al (2012).

The interactions between Fth and B-cells of several maturational stages is highly symbiotic, with direct cell-cell contact and reciprocal cytokine signalling both required to maintain the Fth and B-cell reservoirs, respectively (Table 3.1). The impact of Fth on B-cell maturation and immunoglobulin production is well established, with the frequency of circulating Fth shown to correlate with the efficacy of humoral immune responses after infection and vaccination (Bentebibel et al., 2013; Locci et al., 2013; Ma et al., 2016). The reliance of Fth on these interactions is also now much more understood, with their interdependency evidenced by the lack of Fth found in patients suffering from a number of primary humoral immunodeficiency disorders, including X-linked agammaglobulinaemia (XLA), hyper IgM syndrome (CD40L deficiency) and ICOS deficiency; immunodeficiency disorders in which B-cell developmental and/or maturational pathways are defective (Chevalier et al., 2011; Ma et al., 2015).

Molecule		Mode of Action	Associated Immunodeficiency
Cell Surface Signalling:-			
C-X-C chemokine receptor type 5	CXCR5	Facilitates the migration of follicular helper T-cells to the T-B cell regions of secondary lymphoid organs, in response to the chemokine CXCL13 (Mazerolles et al., 2013; Morita et al., 2011).	No associated immunodeficiencies currently described.
CD40 Ligand	CD40L	In combination with IL-4 secretion, CD40-CD40L signalling induces the expression of activation-induced cytidine deaminase (AID) in naïve B-cells, an enzyme required for class switch recombination and affinity maturation (Weinstein et al., 2016).	Hyper IgM Syndrome (Ma et al., 2015).
Programmed Cell Death 1	PD-1	Regulates follicular helper T-cell activity, by constraining proliferation and increasing apoptosis, thereby limiting B-cell access to follicular helper T-cell help. Regulatory function prevents the development of autoimmunity (Good-Jacobson et al., 2010).	No associated immunodeficiencies currently described.
Inducible Costimulator	ICOS	Costimulatory signalling through ICOS-ICOS-L is fundamentally required for the maintenance of the follicular helper T-cell phenotype, as well as the development and maintenance of germinal centres and humoral immune responses (Bossaller et al., 2006).	ICOS Deficiency (Bossaller et al., 2006).
SLAM-associated Protein	SAP	Required for follicular helper T-cells to form stable contacts with cognate B-cells to promote their activation, differentiation and subsequent germinal centre formation (Cannons et al., 2010).	X-linked lymphoproliferative disease (XLP) (Cannons et al., 2010).
Tumour necrosis factor receptor superfamily, member 4 (TNFRSF4)	CD134 OX40	Promotes follicular helper T-cell differentiation by inducing the expression of follicular helper T-cell associated genes, including CXCR5, BCL6, IL-21 and CXCL13 (Jacquemin et al., 2015).	Autosomal recessive OX40 Deficiency (Byun et al., 2013).
Cytokines:-			
Interleukin-4	IL-4	Produced by follicular helper T-cells. In combination with CD40-CD40L signalling, IL-4 induces the expression of AID in naïve B-cells, an enzyme required for class switch recombination and affinity maturation (Weinstein et al., 2016).	No associated immunodeficiencies currently described.
Interleukin-6	IL-6	Acts synergistically with IL-21 to induce BCL-6 expression and follicular helper T-cell development, via a STAT3 dependent pathway (Choi et al., 2013).	No associated immunodeficiencies currently described.
Interleukin-10	IL-10	Produced by follicular helper T-cells. Enhances DNA synthesis and expansion of activated B-cells, supporting the differentiation of long-lived plasma cells. Regulatory functions limit B-cell access to follicular helper T-cell help, via the formation of a negative feedback loop (Cai et al., 2012).	IL-10 and IL-10R Deficiency (Engelhardt et al., 2013).
Interleukin-12	IL-12	Promotes follicular helper T-cell development by inducing the expression of follicular helper T-cell associated genes, including IL-21, CXCR5, ICOS, Bcl-6 and c-Maf, via a STAT4 dependent pathway. Fundamentally required for germinal centre formation (Schmitt et al., 2013).	Mendelian susceptibility to mycobacterial infections (MSMD) (Prando et al., 2013).

Table 3.1:- Membrane-bound receptor, cytokine and transcription factor signalling involved in the maintenance and function of follicular helper T-cells, as evidenced by known primary immunodeficiencies.

Molecule		Mode of Action	Associated Immunodeficiency
Cytokines continued:-			
Interleukin-21	IL-21	Produced by follicular helper T-cells. A key regulator of the germinal centre response, required for affinity maturation and the generation of long-lived plasma cells (Weinstein et al., 2016).	IL-21 Deficiency (Salzer et al., 2014).
Interleukin-27	IL-27	Promotes follicular helper T-cell development by inducing the expression of follicular helper T-cell associated genes, including IL-21, CXCR5, PD-1 and ICOS, in a STAT3 dependent manner. Fundamentally required for germinal centre formation (Batten et al., 2010).	No associated immunodeficiencies currently described.
Transcription Factors:-			
B-cell lymphoma 6	BCL-6	The archetypal follicular helper T-cell transcription factor, required for the activation of genes associated with follicular helper T-cell migration and T-B cell interactions, including CXCR5, CD40L and SAP, respectively (Kroenke et al., 2012).	No associated immunodeficiencies currently described.
Signal transducer and activator of transcription 3	STAT3	Fundamental in the generation of follicular helper T-cells, due to its involvement in the downstream signalling of interleukin-6 (IL-6), interleukin-21 (IL-21) and interleukin 27 (IL-27) (Ma et al., 2012).	Autosomal dominant hyper IgE syndrome (Mazerolles et al., 2013).

Table 3.1 continued:- Membrane-bound receptor, cytokine and transcription factor signalling involved in the maintenance and function of follicular helper T-cells, as evidenced by known primary immunodeficiencies.

Interestingly, the functional properties of Fth have also proven to be highly heterogeneous, with distinct subsets of Fth possessing highly diverse cytokine secretion and co-stimulatory molecule expression profiles, as well as distinct migratory patterns, which vary considerably depending on their developmental stage, as well as their respective involvement in primary or secondary humoral immune responses. Indeed, during the development of a primary immune response, the migration of activated Fth appears to be largely restricted to the germinal centre (GC) of origin, with the germinal centres of primary immune responses thus considered 'closed structures', in which naïve B-cell access to T-cell help is restricted, thereby ensuring low-affinity, self-reactive B-cells are not selected for propagation (Fazilleau et al., 2009; Reinhardt, Liang and Locksley, 2009; Suan et al., 2015). In subsequent secondary humoral immune responses, follicular helper T-cells, having acquired their 'classical' memory helper phenotype, expressing CD45RO, gain the ability to cycle between their germinal centre of origin, neighbouring germinal centres and the follicular mantle of secondary lymphoid structures, permitting the rapid generation of secondary immune responses to recall antigens. As such, Fth are a dynamic T-cell population, whose close interactions with the B-cells of secondary lymphoid structures not only perpetuates their own maturation, but also tightly regulates B-cell development, ensuring the appropriate generation of primary and secondary humoral immune responses (Figure 3.1) (Fazilleau et al., 2009; Reinhardt, Liang and Locksley, 2009; Suan et al., 2015).

Historically, the most accurate means of assessing Fth is to acquire lymph node samples. However, this can prove challenging, and as such it has become widely accepted that the measurement of peripheral blood-derived CD4⁺ helper T-cells, bearing CXCR5 and CD45RO, may serve as a surrogate measure of mature lymph node-derived Fth (Chevalier et al., 2011; Mazerolles et al., 2013; Morita et al., 2011). Indeed, several studies have indicated that circulating peripheral blood-derived follicular helper T-cells (cFth) may in fact represent a circulating pool of putative lymph node-derived Fth, evidenced by their

shared functional properties, as well as the diminished levels of this T-cell subpopulation found in patients with hyper IgM syndrome and ICOS deficiency; humoral immunodeficiency disorders in which lymph node-derived Fth are also absent (Ma et al., 2012; Ma et al., 2015; Morita et al., 2011). Lymph node- and blood-derived Fth are not, however, without their differences. Compared to lymph node-derived Fth, cFth express lower levels of ICOS, IL-21, OX40 and BCL-6, the archetypical transcription factor of Fth. The activation molecule CD69 is also absent, suggesting that cFth remain in a quiescent state, unlike their lymph node-derived counterparts. In contrast, C-C chemokine receptor 7 (CCR7) and the adhesion molecule L-selectin (CD62L), both of which are required for entry into secondary lymphoid organs via high endothelial venules (HEVs), are highly expressed on cFth, but remain absent on lymph node-derived Fth throughout their migration between the follicular mantle zones and germinal centres of the secondary lymphoid organs (Fazilleau et al., 2009; Morita et al., 2011). As such, cFth possess reduced B-cell stimulatory functions, compared to the functions of putative lymph node-derived Fth, but retain their capacity to enter secondary lymphoid structures upon activation, implying a role for cFth in immune surveillance (Chevalier et al., 2011; Fazilleau et al., 2009; Mazerolles et al., 2013).

To add further complexity to this paradigm, several groups have now demonstrated the ability to further subdivide cFth into Th1-, Th2- and Th17-like subpopulations, based upon the expression of the chemokine receptors C-C chemokine receptor 6 (CCR6) and C-X-C chemokine receptor 3 (CXCR3); the expression of which is largely restricted to Th17 and Th1 cells, respectively (Dhalla et al., 2016; Morita et al., 2011). These cFth subpopulations have been shown to possess distinct transcription factor expression profiles and to provide varying degrees of B-cell help; with the Th17-like subpopulation, defined as CCR6⁺CXCR3⁻, the most efficient at helping B-cells to make immunoglobulins of the IgG, IgA and IgM isotypes. In contrast, the Th2-like subpopulation, defined as CCR6⁻CXCR3⁻

, was the only cFth subpopulation capable of inducing the production of IgE (Ma et al., 2015; Morita et al., 2011).

Given the influential role of individual cFth subpopulations on B-cell maturation and immunoglobulin production, it seems reasonable to hypothesize that defects in, or alterations to the distribution of, cFth subpopulations, in particular the Th17-like cFth compartment, may contribute to the development of autoantibodies, and by supposition autoimmune disorders; with some of these defects perhaps even representing the primary immunological defect. Indeed, previous studies have implicated cFth subpopulations in the development of a number of autoimmune disorders, including systemic lupus erythematosus (SLE), sjögren's syndrome and juvenile dermatomyositis (JDM) (Li et al., 2012; Morita et al., 2011; Simpson et al., 2010; Verstappen et al., 2017). One such study, investigating the pathogenesis of JDM, was able to demonstrate the skewed distribution of cFth subpopulations towards a Th2/Th17-like phenotype, within patients suffering from JDM. This group then went on to further demonstrate that the skewed distribution of cFth subpopulations correlates with disease activity, as well as the frequency of plasmablasts, found in JDM patients (Morita et al., 2011). Rheumatoid arthritis is another such chronic autoimmune disease, in which autoantibodies against numerous self-antigens are detectable. As such, the detection and characterization of cFth within this disease setting has also offered new avenues of investigation.

3.1.b. Follicular Helper T-cells and Rheumatoid Arthritis

Although not considered pathogenic, the presence of rheumatoid factor and anti-CCP antibodies, in 79.0% and 63.0% of RA patients, respectively advocates follicular helper T-cell involvement in disease pathogenesis, due to their fundamental role in T-dependent immune responses and B-cell maturation (Suan et al., 2015; Turesson et al., 2007). This hypothesis is further supported by the previously documented presence of ectopic lymphoid structures (ELS) found within the synovial tissues of 40.0 – 50.0% of RA

patients; with highly organized structures closely resembling the germinal centres of secondary lymphoid organs found in approximately 25.0% of RA patients (Croia et al., 2013; Humby et al., 2009; Weyand and Goronzy, 2003). As with the germinal centres of secondary lymphoid organs, these ELS consist of a network of follicular dendritic cells, B-cells and follicular helper T-cells, which have proven capable of supporting the maturation of class-switched, long-lived plasma cells, as well as the ongoing production of anti-CCP antibodies. In addition, the presence of these ELS has also been shown to correlate with measures of inflammation, including ESR, CRP and white blood cell counts (WBC), cementing their role, as well as their cellular constituents, in RA pathogenesis (Bombardieri, Lewis and Pitzalis, 2017; Humby et al., 2009; Thurlings et al., 2008).

As a major component of secondary lymphoid organs and ELS, numerous studies have sought to characterize follicular helper T-cell involvement in RA development, with an increase in the proportion of cFth in newly-diagnosed, treatment-naïve RA patients, compared to healthy controls, the most frequent finding reported (Ma et al., 2012; Wang et al., 2013). Interestingly, this observation has not, however, been replicated in other studies investigating cFth in longstanding RA patients, receiving a variety of conventional synthetic and biological DMARDs, suggesting that the initiation of DMARD therapeutic regimens is able to alter the cFth compartment. As such, their continued measurement may be beneficial in disease activity monitoring (Chakera et al., 2012).

With Th17-like cFth of particular relevance to autoantibody production, further studies have also investigated the relative distribution of cFth subpopulations within RA patients, with one such study able to demonstrate the skewed distribution of cFth towards a Th17/Th2-like phenotype, in newly-diagnosed, treatment naïve RA patients (Pandya et al., 2016). These results, coupled with the sustained numbers of circulating Th17 cells found in RA patients following an incomplete response to TNF- α blockade, would support the further characterization and measurement of individual cFth subpopulations in disease

activity and therapy efficacy assessments (Alzabin et al., 2012; Chen et al., 2011). A thorough search of the literature, however, suggests that no such studies have sought to compare cFth subpopulations within newly-diagnosed, treatment-naïve RA patients with those of longstanding RA patients; whilst limited data is also available from longitudinal follow-up studies, regarding the long term immunological profile of newly-diagnosed RA patients following the initiation of immunomodulatory therapies.

As such, a thorough investigation of follicular and memory helper CD4⁺ T-cell subpopulations within newly-diagnosed, treatment-naïve and longstanding RA patients seems warranted; with the longitudinal follow-up of newly-diagnosed RA patients at 3-, 6- and 12-months after the time of diagnosis also considered of benefit. In addition to the previously published characterization of cFth subpopulations, according to the expression of CCR6 and CXCR3 alone, the introduction of the skin-homing and Th2-specific C-C chemokine receptor 4 (CCR4) permits the identification of eight follicular and memory helper CD4⁺ T-cell subpopulations. Furthermore, a strategy such as this also facilitates the further characterization of the CCR6-CXCR3⁻ cFth subpopulation previously designated as Th2-like follicular helper T-cells (Hu et al., 2015; Ma et al., 2015; Morita et al., 2011).

3.2. Aims and Objectives

Based upon the literature presented above, the working hypothesis of this chapter is therefore that newly-diagnosed, treatment-naïve RA patients, as well as longstanding RA patients who have failed to make a clinical improvement following the initiation of a conventional synthetic or biologic DMARD therapeutic regimen are identifiable by the profile of their follicular and memory helper T-cell subpopulations. As such, the monitoring of these parameters will improve the identification of RA therapy non-responders, enabling better disease management. The aims of this chapter are therefore:-

1. To identify and fully characterize eight follicular and memory helper CD4⁺ T-cell subpopulations, utilizing the chemokine receptors CCR6, CXCR3 and CCR4, according to their expression of the Th1-, Th2- and Th17-polarising transcription factors T-bet, GATA-3 and ROR γ t.
2. To establish the normal distribution, for relative and absolute counts, of follicular and memory helper CD4⁺ T-cell subpopulations, in a cohort of adult, age and sex-matched healthy controls and to determine the presence of any age and sex associations.
3. To compare the relative and absolute counts of follicular and memory helper CD4⁺ T-cell subpopulations in longstanding and newly diagnosed, treatment-naïve RA patients with those of age- and sex-matched disease and healthy controls; and to determine associations with disease activity measures.
4. To stratify longstanding RA patients according to their therapeutic regimens, in order to further investigate the effects of immunosuppressive therapy on follicular and memory helper T-cell subpopulation distribution.

5. To longitudinally monitor follicular and memory helper T-cell subpopulations in newly diagnosed, treatment-naïve RA patients, at 3-, 6- and 12-monthly intervals, following the initiation of immunosuppressive therapy; to further investigate the effect of RA therapeutic regimens on follicular and memory helper T-cell subpopulation distribution.

3.3. Results

3.3.a. Follicular helper and memory helper CD4⁺ T-cells may be subdivided into eight distinct subpopulations based upon their expression of the chemokine receptors CCR6, CXCR3 and CCR4

Previous studies have extensively characterized subpopulations of cFth, and to a lesser extent memory helper CD4⁺ T-cells (mTh), based upon the expression of CCR6 and CXCR3 alone (Ma et al., 2015; Morita et al., 2011). To our knowledge, however, no such investigations have sought to additionally assess the expression of the Th2-specific and skin-homing chemokine receptor CCR4, within both of these CD4⁺ T-cell compartments. As such a flow cytometric assay was designed to facilitate the identification of cFth, within CD4⁺ T-cells, based upon their dual expression of CD45RO and the chemokine receptor CXCR5; with mTh discriminated by their expression of CD45RO in the absence of CXCR5. Within each of these CD4⁺ helper T-cell compartments, eight distinct subpopulations were subsequently distinguishable, based upon their differential expression of the chemokine receptors CCR6, CXCR3 and CCR4 (Figure 3.2).

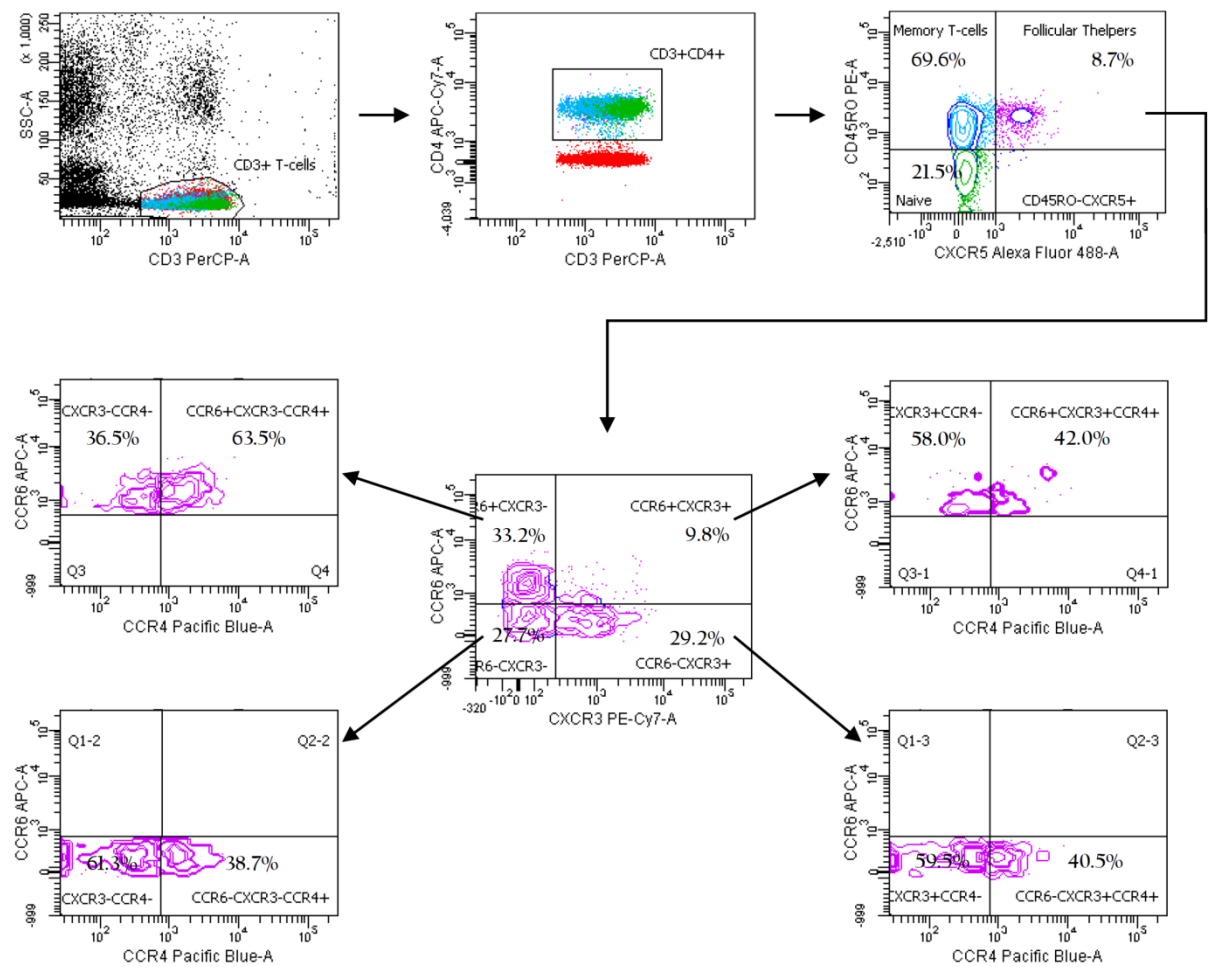
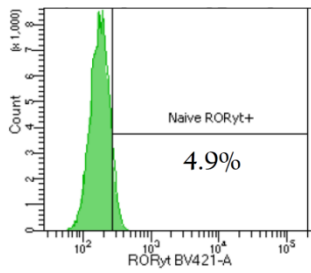


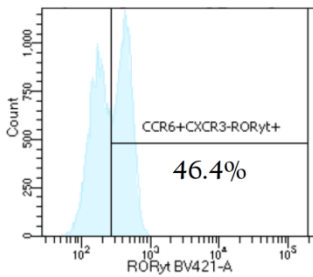
Figure 3.2:- Representative FACS plots depicting gating strategy employed for the identification of follicular (cFth) and memory (mTh) helper CD4⁺ T-cell subpopulations. Samples were gated so as to acquire 30,000 CD4⁺ T-cell events. CD45RO⁺CXCR5⁺ cFth and CD45RO⁺CXCR5⁻ mTh were then gated and assessed for their expression of the chemokine receptors CCR6 and CXCR3, after which cFth and mTh subpopulations were further analysed for their expression of the chemokine receptor CCR4.

Following the addition of CCR4, to the previously published protocol, it was considered necessary to corroborate the transcription factor expression profiles of the cFth and mTh subpopulations identified, respectively. Expression of the follicular transcription factor B-cell lymphoma 6 (BCL-6) has been previously investigated by other groups; with comparable levels of BCL-6 demonstrated in all follicular helper T-cell subpopulations investigated. As such, transcription factor expression profiling of cFth and mTh was limited to the Th1⁻, Th17⁻ and Th2-polarising transcription factors T-bet, ROR γ t and GATA-3, respectively (Figure 3.3) (Ma et al., 2015; Morita et al., 2011).

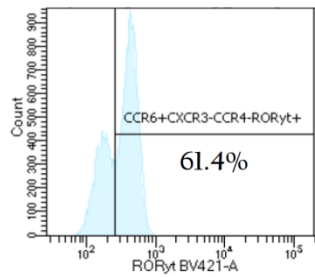
A) Naïve T-cells



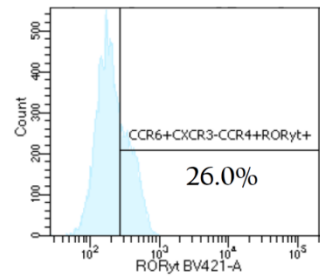
B) CCR6+CXCR3-



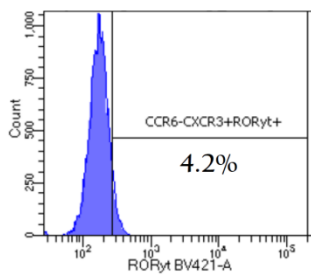
C) CCR6+CXCR3-CCR4-



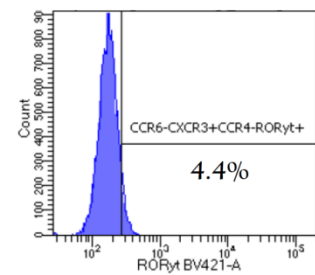
D) CCR6+CXCR3-CCR4+



E) CCR6-CXCR3+



F) CCR6-CXCR3+CCR4-



G) CCR6-CXCR3+CCR4+

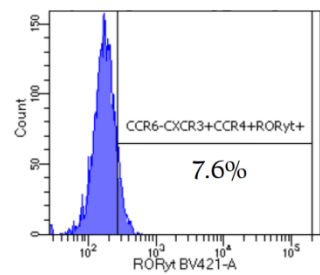


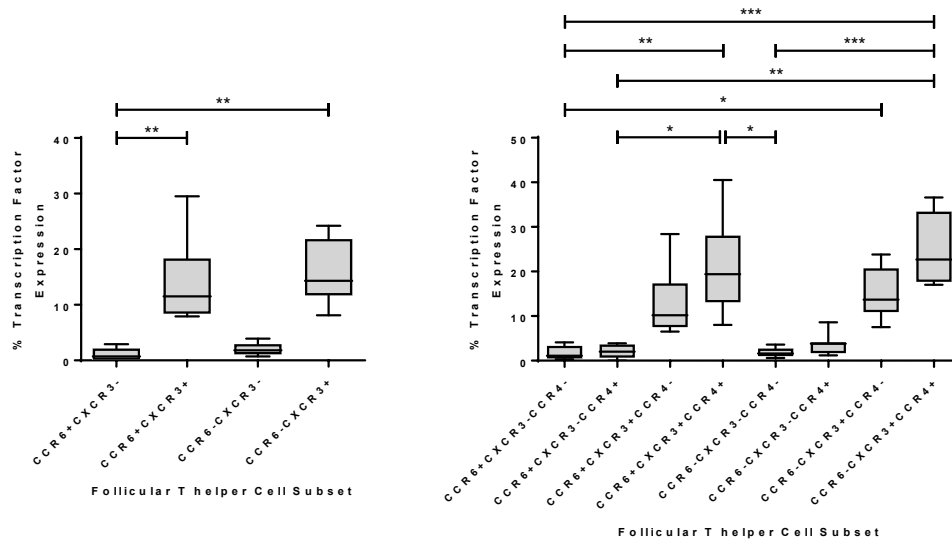
Figure 3.3:- Representative FACS plots depicting intracellular staining for the transcription factor ROR γ t, within A) Naïve T-cells; B) CCR6+CXCR3-; C) CCR6+CXCR3-CCR4-; D) CCR6+CXCR3-CCR4+; E) CCR6-CXCR3+; F) CCR6-CXCR3+CCR4- and G) CCR6-CXCR3+CCR4+ memory helper CD4+ T-cells. Samples were gated so as to acquire 1,000,000 lymphocytes events. CD45RO+CXCR5- memory T-cells were then identified and assessed for their expression of the chemokine receptors CCR6, CXCR3 and CCR4 as previously described. Naïve CD4+ T-cells were assessed for transcription factor expression as a measure of non-specific background.

3.3.a.i. *Follicular helper CD4+ T-cells* - As expected, intracellular staining for T-bet, ROR γ t and GATA-3, performed on PMBCs prepared from seven healthy controls, demonstrated the presence of distinct transcription factor expression profiles within cFth subpopulations. Based upon the expression of CCR6 and CXCR3 alone, significantly higher levels of T-bet were present within CXCR3-expressing cFth subpopulations ($p < 0.05$); with significantly increased expression of ROR γ t associated with CCR6 ($p < 0.05$)

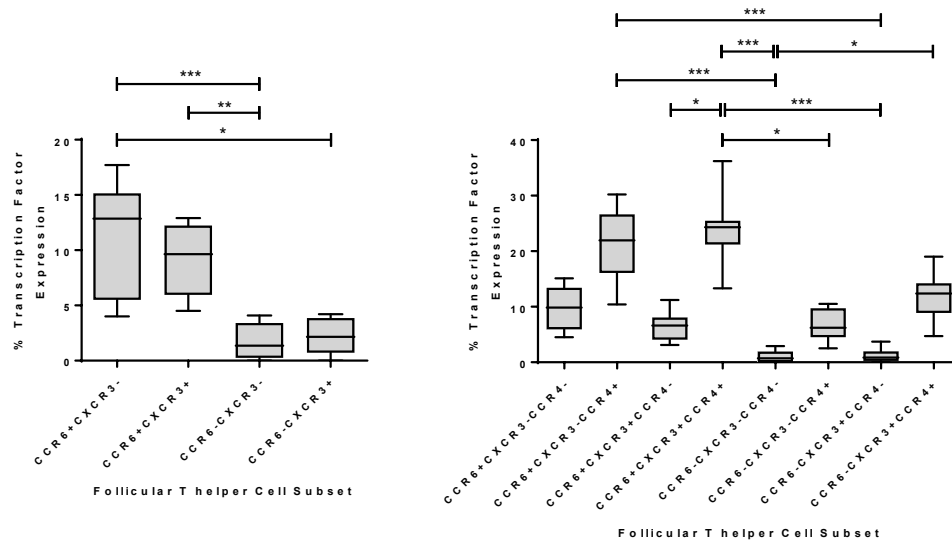
(Figure 3.4A - B). GATA-3 was universally expressed across all cFth subsets. However, consistent with previous studies, significantly lower amounts of GATA-3 were detected in CXCR3-expressing cFth subpopulations ($p<0.05$); a finding in keeping with the well-documented antagonistic behaviour of T-bet and GATA-3 (Figure 3.4C) (Jenner et al., 2009; Kanhere et al., 2012).

When analysed further according to the expression of CCR4, the relative expression levels of T-bet, ROR γ t and GATA-3 appeared to vary considerably, with the highest levels of T-bet and ROR γ t occurring in CXCR3+CCR4+ and CCR6+CCR4+ subpopulations, respectively; although these observations failed to reach statistical significance, when compared to their CCR4- counterparts (Figure 3.4A - B). GATA-3 expression was comparable across all eight cFth subpopulations, excluding CCR6+CXCR3+CCR4+ cFth, in which the relative expression levels of GATA-3 were significantly increased ($p<0.01$); and CCR6-CXCR3+CCR4- cFth, wherein GATA-3 expression levels were significantly reduced ($p<0.05$) (Figure 3.4C).

A) T-bet



B) ROR γ t



C) GATA-3

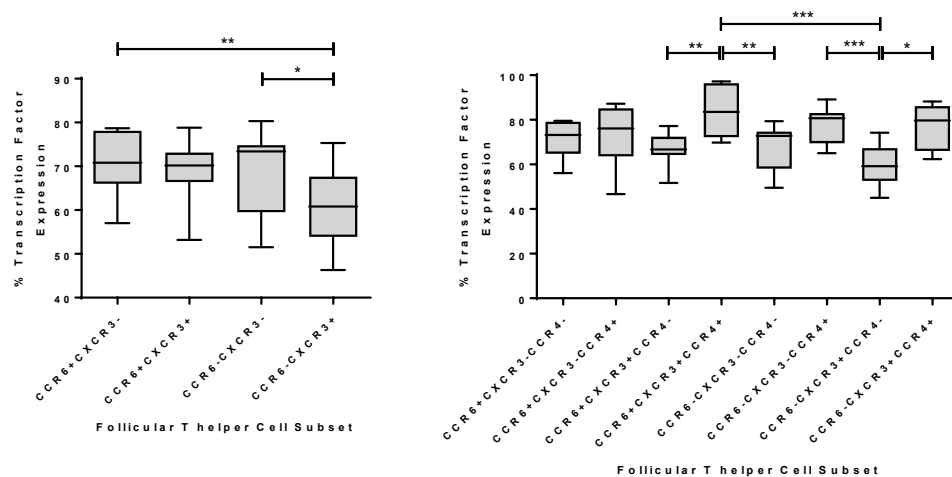
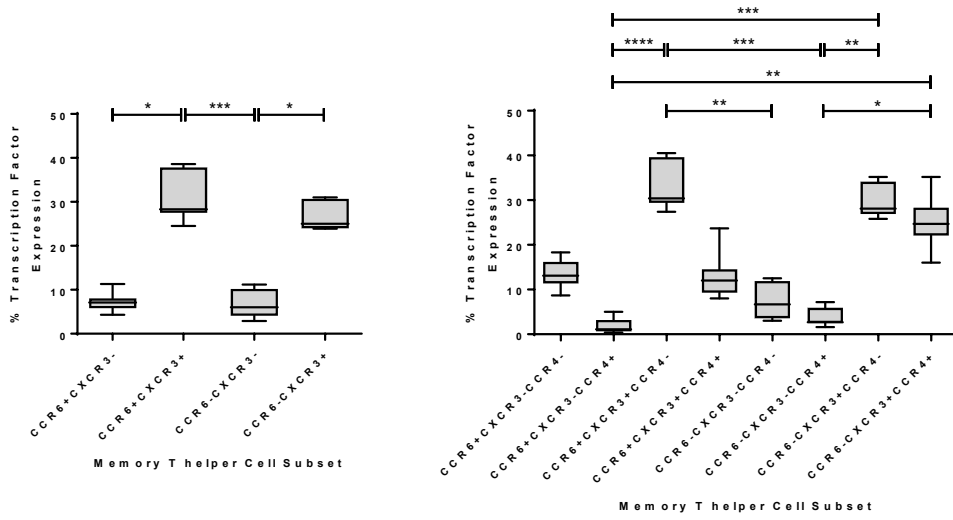


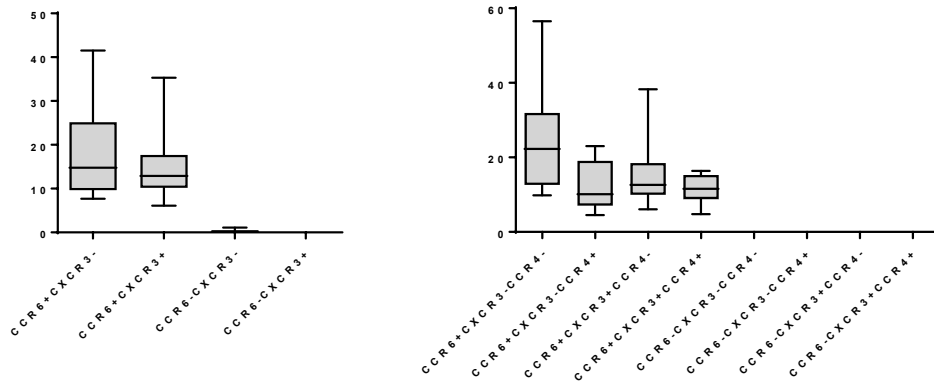
Figure 3.4:- Transcription factor expression profiles of follicular helper T-cell subpopulations, characterized by the expression of CCR6, CXCR3 and CCR4, following intracellular staining of PBMCs extracted from seven healthy controls (HC). Box plots show the median, interquartile and minimum and maximum values of expression for the transcription factors A) T-bet, B) ROR γ t and C) GATA-3, following the subtraction of non-specific staining assessed within naïve CD4⁺ T-cells. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.3.a.ii. *Memory helper CD4⁺ T-cells* - Transcription factor expression profiles also appeared to vary substantially within the memory helper CD4⁺ T-cell compartment. Consistent with the transcription factor expression profiles of cFth, when analysed according to the expression of CCR6 and CXCR3 alone, significantly higher levels of T-bet and ROR γ t were detected within CXCR3⁻ ($p<0.05$) and CCR6-expressing mTh, respectively ($p<0.01$); with GATA-3 universally expressed across all mTh subpopulations (Figure 3.5A - C). With the addition of CCR4, however, it became clear that the transcription factor expression profiles of mTh subpopulations differ considerably to those of their cFth counterparts; with CCR4 shown to enhance the expression of T-bet and ROR γ t within the cFth compartment, but not within the mTh compartment. As such, compared to cFth subpopulations, T-bet expression levels were significantly higher in CCR6⁺CXCR3⁺CCR4⁻ mTh ($p<0.01$), followed closely by CCR6⁻CXCR3⁺CCR4⁻ ($p<0.01$) and CCR6⁻CXCR3⁺CCR4⁺ ($p<0.05$) mTh, respectively (Figure 3.5A). The relative expression levels of ROR γ t also varied considerably, with the highest levels of ROR γ t expressed within CCR6⁺CXCR3⁻CCR4⁻ mTh ($p<0.01$), followed closely by the remaining three CCR6⁺ mTh subpopulations (Figure 3.5B). Consistent with results obtained in cFth subpopulations GATA-3 was evenly expressed across all eight mTh subpopulations, with no significant differences in expression observed (Figure 3.5C).

A) T-bet



B) ROR γ t



C) GATA-3

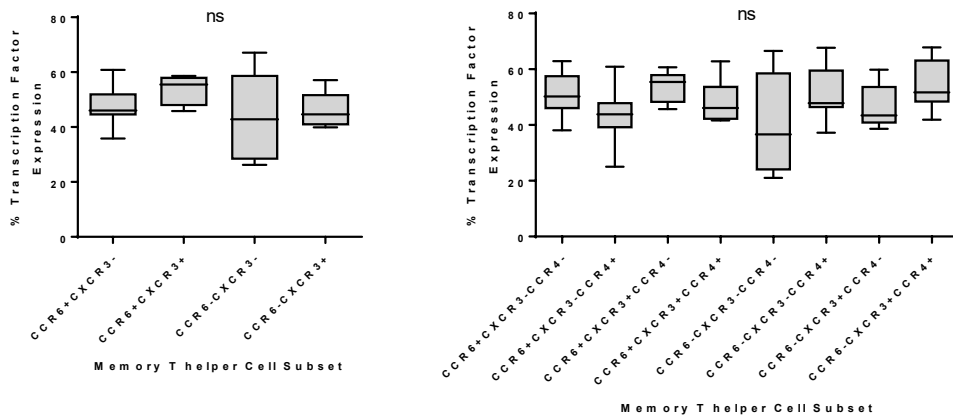


Figure 3.5:- Transcription factor expression profiles of memory helper CD4⁺ T-cell subpopulations, characterized by the expression of CCR6, CXCR3 and CCR4, following intracellular staining of PBMCs extracted from seven healthy controls (HC). Box plots show the median, interquartile and minimum and maximum values of expression for the transcription factors A) T-bet, B) ROR γ t and C) GATA-3, following the subtraction of non-specific staining assessed within naïve CD4⁺ T-cells. ns = non-significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

With T-bet and ROR γ t expression associated with the presence of CCR6 and CXCR3, respectively within cFth and mTh, albeit to varying degrees with the addition of CCR4; combined with the universal expression of GATA-3 across all cFth and mTh subpopulations, the following arbitrary designations have been assigned to cFth and mTh subpopulations alike and will be used hereafter: CCR6+CXCR3⁻ (CCR4+/-) Th17-like/Th17, CCR6+CXCR3⁺ (CCR4+/-) Th1/17-like/Th1/17, CCR6-CXCR3⁻ (CCR4-/+) Th2-like/Th2 and CCR6-CXCR3⁺ (CCR4+/-) Th1-like/Th1 cFth and mTh (Table 3.2).

Helper CD4 ⁺ T-cell Subpopulation	Transcription Factor Expression			Subpopulation Designation
	T-bet	ROR γ t	GATA-3	
CCR6+CXCR3 ⁻	-	+	+	Th17
CCR6+CXCR3 ⁺	+	+	+	Th1/17
CCR6-CXCR3 ⁻	-	-	+	Th2
CCR6-CXCR3 ⁺	+	-	+	Th1

Table 3.2:- Follicular and memory helper CD4⁺ T-cell subpopulation designations, based upon the relative expression levels of T-bet, ROR γ t and GATA-3.

3.3.b. Follicular helper and memory helper CD4⁺ T-cell subpopulations are differentially expressed in healthy controls

3.3.b.i. *Follicular helper CD4⁺ T-cells* - With follicular and memory helper CD4⁺ T-cell subpopulations clearly defined according to their transcription factor expression profiles, the relative and absolute counts, of each compartment were assessed within a cohort of 32 adult healthy controls. Within healthy controls, cFth made up 7.8% (5.7 – 14.5%) (median; inter-quartile range (IQR)) and 72.9 x 10⁶/l (47.8 – 96.0 x 10⁶/l) of CD4⁺ T-cells, respectively. A more detailed analysis of cFth chemokine receptor expression revealed a distinct subpopulation expression profile, with Th1-like cFth relative and absolute counts significantly increased, as compared to Th17-like (p<0.001 and ns, respectively), and Th1/17-like (p<0.0001, respectively) cFth. This increase in Th1-like cFth was followed closely by a significant increase in the relative and absolute counts of Th2-like cFth, as compared to Th17-like (p<0.05 and ns, respectively) and Th1/17-like cFth (p<0.0001, respectively) (Figure 3.6A and 3.6C). Further analysis for CCR4 expression revealed the presence of substantial CCR4⁻ Th1-like and CCR4⁻ Th2-like cFth subpopulations, the

percentages of which were significantly elevated as compared to all other subpopulations ($p < 0.0001$ and $p < 0.05$, respectively) (Figure 3.6B and 3.6D).

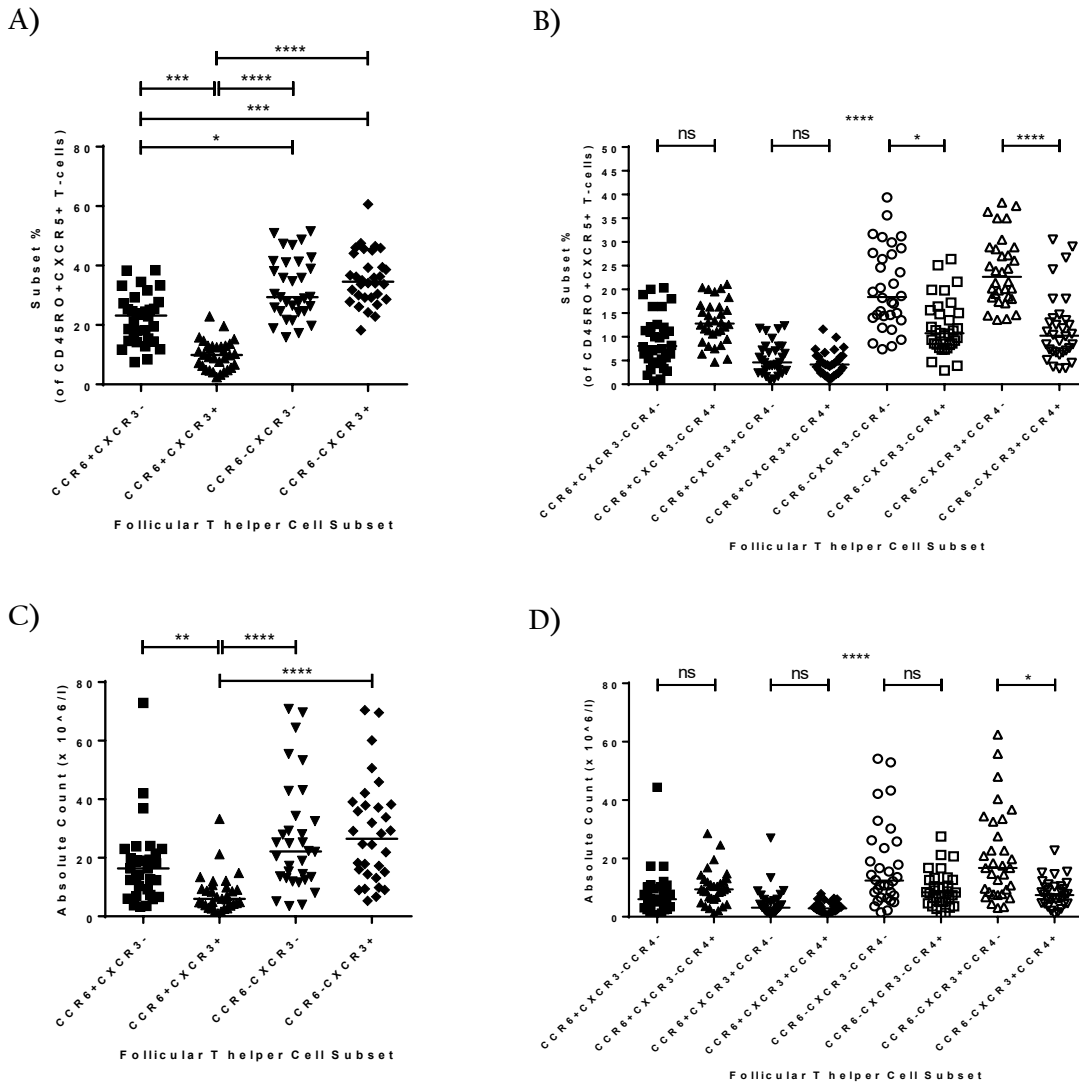


Figure 3.6:- Follicular helper CD4⁺ T-cell subpopulation expression profiles, generated within healthy controls, based upon the expression of the chemokine receptors CCR6, CXCR3 and CCR4. A) Subpopulation percentages, based upon the expression of CCR6 and CXCR3; B) Subpopulation percentages, based upon the expression of CCR6, CXCR3 and CCR4; C) Subpopulation absolute counts (x10⁶/l), based upon the expression of CCR6 and CXCR3; D) Subpopulation absolute counts (x10⁶/l), based upon the expression of CCR6, CXCR3 and CCR4. **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

3.3.b.ii. *Memory helper CD4⁺ T-cells* - Within our healthy control cohort, mTh represented 61.8% (49.0 – 77.4%) of CD4⁺ T-cells; equating to 482.4 x 10⁶/l (336.0 – 738.0 x 10⁶/l) CD4⁺ T-cells, respectively. Consistent with the cFth compartment, the relative and absolute counts of mTh subpopulations were dominated by a Th1 subpopulation, when compared to Th17 ($p < 0.0001$, respectively) and Th1/17 ($p < 0.0001$, respectively) mTh. This increase in Th1 mTh was accompanied by a significant increase in the relative and absolute

counts of Th2 mTh, as compared to Th17 ($p<0.01$ and ns, respectively) and Th1/17 ($p<0.0001$ and $p<0.05$, respectively) mTh (Figure 3.7A and 3.7C). When analysed further for CCR4 expression, the relative and absolute counts of mTh subpopulations were typified by a substantial CCR4⁻ Th1 mTh subpopulation, which was significantly elevated compared to all other mTh subpopulations ($p<0.0001$ and $p<0.01$, respectively). Despite evidence for an increase in Th2 mTh, based upon the expression of CCR6 and CXCR3 alone, no such elevation in CCR4⁻ or CCR4⁺ Th2 mTh percentages or absolute counts was apparent (Figure 3.7B and 3.7D).

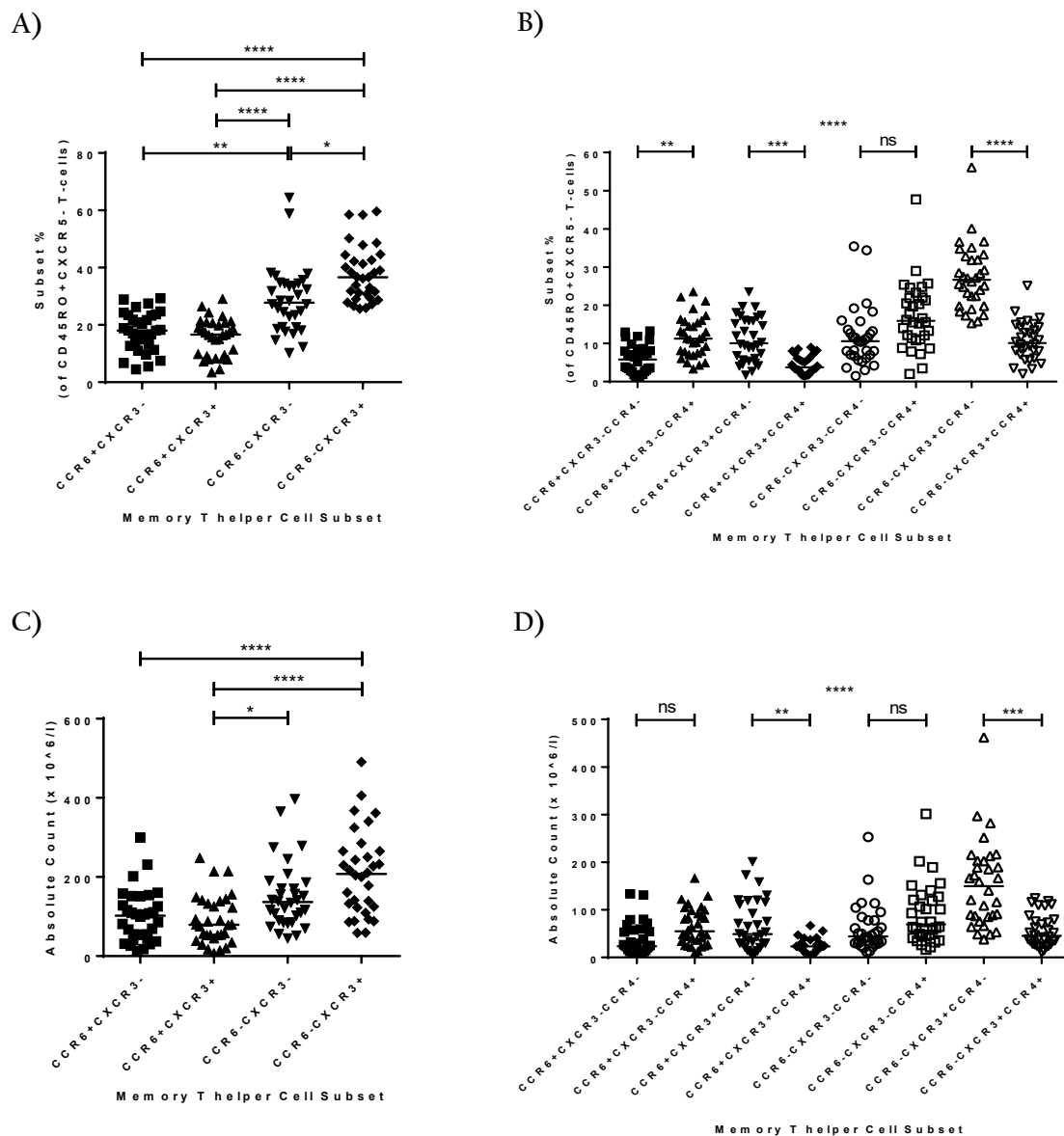


Figure 3.7:- Memory helper CD4⁺ T-cell subpopulation expression profiles, generated within healthy controls, based upon the expression of the chemokine receptors CCR6, CXCR3 and CCR4. A) Subpopulation percentages, based upon the expression of CCR6 and CXCR3; B) Subpopulation percentages, based upon the expression of CCR6, CXCR3 and CCR4; C) Subpopulation absolute counts (x10⁶/l), based upon the expression of CCR6 and CXCR3; D) Subpopulation absolute counts (x10⁶/l), based upon the expression of CCR6, CXCR3 and CCR4. **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

With the relative and absolute counts of cFth, mTh and their subpopulations described, it was considered necessary to ascertain whether any age or sex associations existed within these T-cell parameters prior to their assessment within newly-diagnosed, treatment-naïve and longstanding RA patients. Within the cFth and mTh compartments, no effects of age or sex were noted in the relative or absolute counts of any cFth or mTh subpopulation described. As such, the relative and absolute counts of cFth and mTh

compartments within healthy controls are dominated by a CCR4⁻ Th1-like/Th1 subpopulation, with a CCR4⁻ Th2-like/Th2 subpopulation also contributing significantly to the composition of these T-cell compartments (Table 3.3).

	<u>Follicular Helper T-cells</u> (CD45RO+CXCR5 ⁺)		<u>Memory Helper T-cells</u> (CD45RO+CXCR5 ⁻)	
	%	x 10 ⁶ /l	%	x 10 ⁶ /l
Follicular Helper T-cells (CD45RO+CXCR5 ⁺)	2.5 – 18.1	19.8 – 162.6	–	–
Memory Helper T-cells (CD45RO+CXCR5 ⁻)	–	–	29.5 – 93.3	220.1 – 1003.9
CCR6+CXCR3 ⁻ (Th17)	<u>10.2 – 36.2*</u>	<u>3.7 – 39.2†</u>	6.2 – 28.1	22.5 – 215.1
CCR6+CXCR3 ⁻ CCR4 ⁻	1.5 – 19.4	0.6 – 17.3	1.2 – 12.4	3.7 – 103.1
CCR6+CXCR3 ⁻ CCR4 ⁺	5.9 – 20.3	2.5 – 21.9	4.6 – 21.6	17.7 – 125.3
CCR6+CXCR3 ⁺ (Th1/17)	3.7 – 17.5	1.7 – 17.7	6.0 – 25.3	16.0 – 214.4
CCR6+CXCR3 ⁺ CCR4 ⁻	1.4 – 11.7	0.6 – 10.9	3.5 – 19.6	9.9 – 165.2
CCR6+CXCR3 ⁺ CCR4 ⁺	1.5 – 8.7	1.5 – 8.7	1.6 – 8.4	5.2 – 51.0
CCR6-CXCR3 ⁻ (Th2)	<u>18.1 – 49.7‡</u>	<u>4.6 – 66.8+</u>	<u>13.6 – 47.5‡</u>	<u>54.0 – 317.8‡</u>
CCR6-CXCR3 ⁻ CCR4 ⁻	<u>8.3 – 33.5‡</u>	2.9 – 47.6	3.3 – 26.7	17.0 – 136.6
CCR6-CXCR3 ⁻ CCR4 ⁺	4.3 – 23.2	2.1 – 20.9	5.5 – 27.2	24.0 – 195.1
CCR6-CXCR3 ⁺ (Th1)	<u>23.6 – 47.0†</u>	<u>7.9 – 64.4Δ</u>	<u>26.3 – 58.4‡Σ<</u>	<u>74.1 – 384.8\$/</u>
CCR6-CXCR3 ⁺ CCR4 ⁻	<u>14.2 – 36.9\$</u>	<u>3.9 – 51.5»</u>	<u>16.6 – 38.2Ω</u>	<u>48.6 – 288.8€</u>
CCR6-CXCR3 ⁺ CCR4 ⁺	3.6 – 27.7	1.8 – 15.3	3.4 – 17.5	17.8 – 117.0

Table 3.3:- Relative (%) and absolute counts (x 10⁶/l) of follicular helper (CD45RO+CXCR5⁺) and memory helper (CD45RO+CXCR5⁻) CD4⁺ T-cells, and their subpopulations generated within healthy controls (n = 32). All follicular helper (cFth) and memory helper (mTh) CD4⁺ T-cell subpopulations were expressed as a percentage of CD45RO+CXCR5⁺ and CD45RO+CXCR5⁻ CD4⁺ T-cells, respectively. Underlined figures denote significant results. † = p<0.0001 as compared to Th1/17-like cFth; ‡ = p<0.0001 as compared to Th1/17-like cFth; § = p<0.0001 as compared to Th1/17-like cFth; ¶ = p<0.05 as compared to Th1/17-like cFth; * = p<0.001 as compared to Th1/17-like cFth; \$ = p<0.0001 as compared to all cFth subpopulations; i = p<0.05 as compared to all cFth subpopulation percentages. Δ = p<0.0001 as compared to Th1/17-like cFth; + = p<0.0001 as compared to Th1/17-like cFth; † = p<0.01 as compared to Th1/17-like cFth; » = p<0.05 as compared to CCR4⁺ Th1-like cFth absolute counts. II = p<0.0001 as compared to Th17 mTh; Σ = p<0.0001 as compared to Th1/17 mTh; < = p<0.05 as compared to Th2 mTh; ∂ = p<0.0001 as compared to Th1/17 mTh; ‡ = p<0.01 as compared to Th17 mTh; Ω = p<0.0001 as compared to all mTh subpopulation percentages. \$ = p<0.0001 as compared to Th17 mTh; / = p<0.0001 as compared to Th1/17 mTh; ‡ = p<0.05 as compared to Th1/17 mTh; € = p<0.01 as compared to all mTh subpopulation absolute counts. Figures denote 5th and 95th percentiles.

3.3.c. Follicular and memory helper CD4⁺ T-cell subpopulation distributions are altered in newly-diagnosed and longstanding rheumatoid arthritis patients

3.3.c.i. *Follicular helper CD4⁺ T-cells* - With the relative and absolute counts of follicular and memory helper CD4⁺ T-cells and their subpopulations established within healthy controls, the relative and absolute counts of cFth and mTh were compared between 20 newly-diagnosed, treatment-naïve RA patients (ERA), 56 longstanding RA patients (RA), 32 age- and sex-matched disease controls (DCG) and 32 age- and sex-matched healthy controls (HC). Within the follicular helper T-cell compartment, no significant difference in CD45RO⁺CXCR5⁺ cFth relative or absolute counts was observed between any patient or control group (Table 3.4 and 3.5). When analysed further according to the expression of the chemokine receptors CCR6 and CXCR3 alone, no significant difference in Th17-like cFth percentages or absolute counts was evident between patient and control groups (Figure 3.8A and 3.9A). With the inclusion of CCR4, no significant difference in CCR4⁻ or CCR4⁺ Th17-like cFth percentages or absolute counts was observed between patient and control groups (Figure 3.8B – C; Figure 3.9B – C). Upon further inspection, however, 19/56 (33.9%) RA patients and 10/32 (31.2%) DCG demonstrated CCR4⁺ Th17-like cFth percentages above the 95th percentile of HC (5th – 95th percentile = 5.9 – 20.3%) (median of RA population within 95th percentile of HC = 11.7% [IQR = 9.6 – 13.4%]; median of RA population above 95th percentile of HC = 26.6% [IQR = 23.4 – 35.9%]). Furthermore, 11/56 (19.6%) RA patients and 2/32 (6.3%) DCG also demonstrated CCR4⁺ Th17-like cFth absolute counts above the 95th percentile of HC (5th – 95th percentile = 2.5 – 21.9 x 10⁶/l) (median of RA population within 95th percentile of HC = 7.8 [IQR = 5.0 – 12.2 x 10⁶/l]; median of RA population above 95th percentile of HC = 34.6 [IQR = 26.3 – 36.8 x 10⁶/l]) (Table 3.3 – 3.5; Figure 3.8C and 3.9C).

	<u>ERA</u>	<u>RA</u>	<u>DCG</u>	<u>HC</u>
Follicular Helper T-cells (CD45RO+CXCR5+) (%)	9.8 (6.7 – 14.2)	8.4 (5.2 – 12.5)	7.5 (4.9 – 11.2)	7.8 (5.7 – 14.5)
CCR6+CXCR3- (Th17) (%)	21.5 (14.8 – 26.2)	24.7 (17.4 – 29.0)	22.6 (16.6 – 28.7)	23.2 (14.8 – 27.6)
CCR6+CXCR3-CCR4- (%)	6.7 (5.2 – 10.6)	5.9 (3.4 – 9.1)	5.3 (2.7 – 9.8)	8.1 (4.9 – 12.1)
CCR6+CXCR3-CCR4+ (%)	13.9 (9.6 – 17.1)	13.4 (10.6 – 24.1)	15.0 (10.8 – 22.6)	12.8 (9.8 – 16.3)
CCR6+CXCR3+ (Th1/17) (%)	<u>5.0†Δ</u> (4.0 – 6.4)	8.7 (5.4 – 14.4)	8.7 (4.1 – 13.7)	9.9 (5.8 – 13.0)
CCR6+CXCR3+CCR4- (%)	<u>2.8‡</u> (1.9 – 3.8)	3.1 (2.0 – 5.4)	3.5 (1.6 – 5.8)	4.6 (2.9 – 7.8)
CCR6+CXCR3+CCR4+ (%)	<u>2.4‡¶</u> (1.6 – 3.1)	4.2 (2.2 – 8.7)	3.6 (2.2 – 9.8)	4.2 (2.3 – 6.0)
CCR6-CXCR3- (Th2) (%)	<u>48.3♦Π*</u> (42.8 – 52.2)	36.4 (26.9 – 45.8)	34.3 (25.8 – 49.9)	29.4 (24.6 – 41.2)
CCR6-CXCR3-CCR4- (%)	<u>31.1Σ</u> (23.9 – 35.3)	18.8 (8.0 – 31.2)	18.4 (9.2 – 30.8)	18.4 (14.1 – 27.6)
CCR6-CXCR3-CCR4+ (%)	16.8 (12.2 – 19.7)	15.4 (11.2 – 19.6)	15.5 (11.5 – 22.8)	<u>10.8∂</u> (8.3 – 15.5)
CCR6-CXCR3+ (Th1) (%)	25.0 (21.7 – 31.3)	27.2 (22.9 – 34.1)	30.0 (22.0 – 35.4)	<u>34.6\$‡</u> (29.2 – 42.9)
CCR6-CXCR3+CCR4- (%)	17.9 (13.9 – 21.8)	16.4 (11.2 – 21.5)	16.8 (10.1 – 21.6)	<u>22.7‡«</u> (18.4 – 28.8)
CCR6-CXCR3+CCR4+ (%)	6.5 (5.1 – 11.6)	9.5 (5.4 – 17.5)	9.0 (6.0 – 18.5)	10.3 (6.8 – 13.8)

Table 3.4:- Follicular helper T-cell (CD45RO+CXCR5+) and subpopulation expression profiles, according to percentage (%) of CD3+CD4+ and CD45RO+CXCR5+ T-cells respectively, within early arthritis patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. Underlined figures denote significant results. † = p<0.01 as compared to RA patients and HC; Δ = p<0.05 as compared to DCG; ‡ = p<0.05 as compared to HC; ¶ = p<0.01 as compared to RA patients; ¶ = p<0.05 as compared to DCG; ♦ = p<0.001 as compared to HC; Π = p<0.01 as compared to RA patients; * = p<0.05 as compared to DCG; Σ = p<0.05 as compared to RA patients, DCG and HC; ∂ = p<0.05 as compared to ERA patients and DCG; \$ = p<0.01 as compared to ERA and RA patients; ‡ = p<0.05 as compared to DCG; ‡ = p<0.001 as compared to RA patients; « = p<0.01 as compared to DCG. Figures denote median and inter-quartile ranges (IQR).

	<u>ERA</u>	<u>RA</u>	<u>DCG</u>	<u>HC</u>
Follicular Helper T-cells (CD45RO+CXCR5+) (x10 ⁶ /l)	77.2 (66.0 – 119.4)	73.3 (47.2 – 111.1)	52.0 (30.6 – 108.3)	72.9 (47.8 – 96.0)
CCR6+CXCR3- (Th17) (x10 ⁶ /l)	16.1 (10.8 – 21.2)	16.0 (9.8 – 26.3)	10.8 (8.2 – 25.3)	16.4 (7.8 – 21.0)
CCR6+CXCR3-CCR4- (x10 ⁶ /l)	4.7 (3.6 – 7.6)	4.2 (1.6 – 7.8)	2.4 (1.1 – 8.7)	6.0 (2.7 – 10.0)
CCR6+CXCR3-CCR4+ (x10 ⁶ /l)	10.2 (6.7 – 14.3)	9.7 (6.5 – 18.3)	9.3 (5.8 – 16.3)	9.4 (6.1 – 12.6)
CCR6+CXCR3+ (Th1/17) (x10 ⁶ /l)	4.4 (3.0 – 5.7)	6.0 (3.5 – 11.2)	5.4 (2.1 – 9.4)	5.9 (3.7 – 9.8)
CCR6+CXCR3+CCR4- (x10 ⁶ /l)	2.0 (1.1 – 2.9)	2.7 (1.5 – 4.3)	1.5 (0.8 – 4.0)	3.1 (1.4 – 5.9)
CCR6+CXCR3+CCR4+ (x10 ⁶ /l)	1.9 (1.1 – 3.1)	3.3 (1.7 – 7.2)	2.4 (1.3 – 4.8)	2.9 (1.8 – 4.5)
CCR6-CXCR3- (Th2) (x10 ⁶ /l)	<u>36.9Δ</u> (24.1 – 56.7)	27.4 (11.1 – 46.1)	23.6 (9.7 – 36.2)	22.2 (13.1 – 33.9)
CCR6-CXCR3-CCR4- (x10 ⁶ /l)	<u>23.9II</u> (13.3 – 32.8)	12.5 (3.8 – 25.7)	12.1 (2.6 – 23.9)	12.4 (6.7 – 25.2)
CCR6-CXCR3-CCR4+ (x10 ⁶ /l)	12.1 (9.2 – 15.8)	9.0 (5.5 – 19.5)	9.4 (5.7 – 13.0)	8.4 (5.1 – 12.7)
CCR6-CXCR3+ (Th1) (x10 ⁶ /l)	20.5 (14.0 – 33.6)	19.6 (12.6 – 32.7)	15.9 (8.4 – 32.2)	26.5 (14.6 – 38.1)
CCR6-CXCR3+CCR4- (x10 ⁶ /l)	13.8 (9.0 – 18.2)	12.0 (5.1 – 20.8)	7.7 (3.8 – 22.1)	16.7 (8.0 – 31.3)
CCR6-CXCR3+CCR4+ (x10 ⁶ /l)	5.3 (3.5 – 8.1)	7.0 (3.3 – 10.9)	5.6 (3.6 – 9.9)	7.4 (4.6 – 10.4)

Table 3.5:- Follicular helper T-cell (CD45RO+CXCR5+) and subpopulation expression profiles, according to absolute count (x10⁶/l) of CD45RO+CXCR5+ T-cells respectively, within early arthritis patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. Underlined figures denote significant results. Δ = p<0.05 as compared to DCG; II = p<0.05 as compared to DCG. Figures denote median and inter-quartile ranges (IQR).

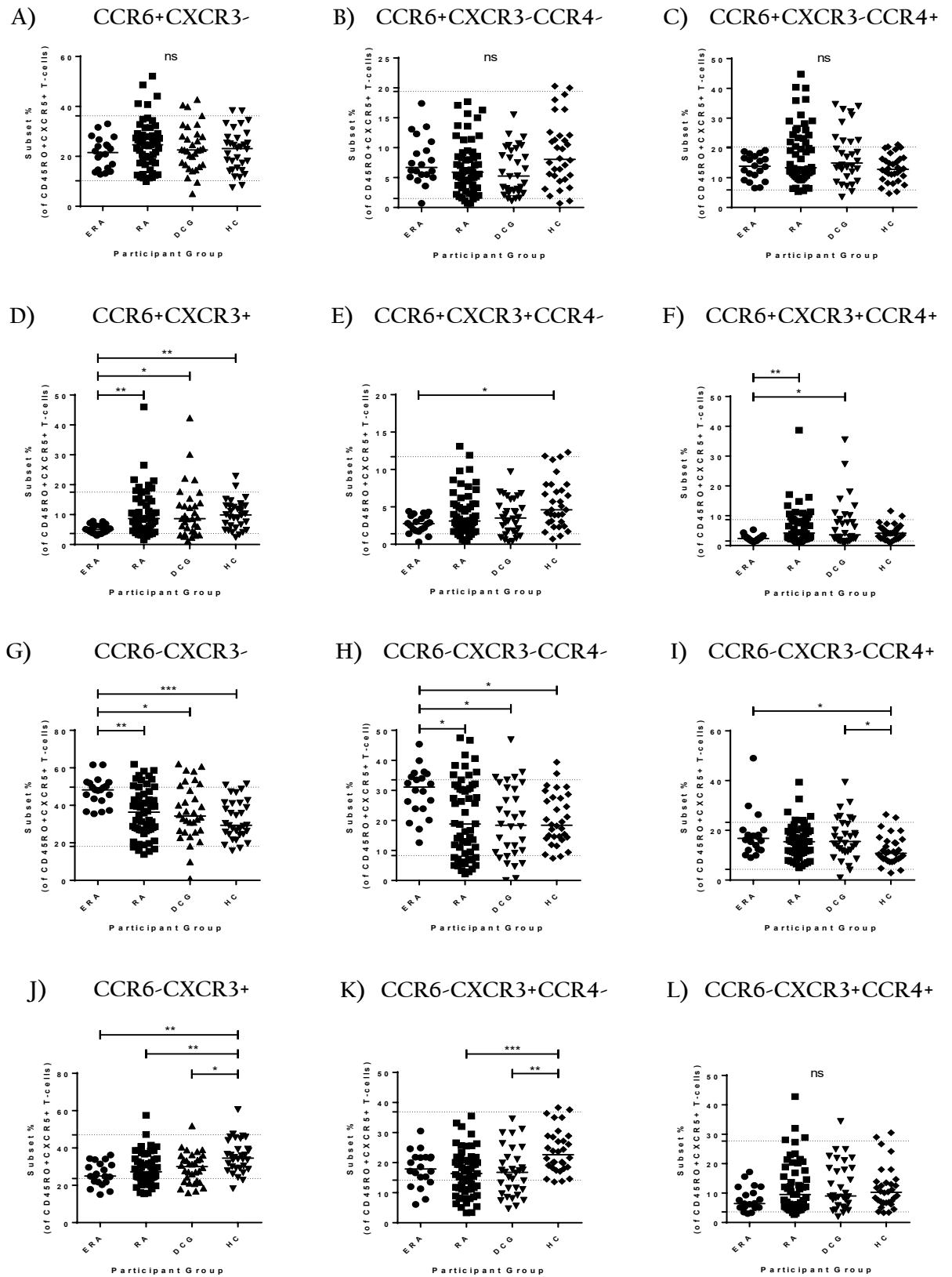


Figure 3.8:- Prevalence (%) of A) CCR6+CXCR3-; B) CCR6+CXCR3-CCR4-; C) CCR6+CXCR3-CCR4+; D) CCR6+CXCR3+; E) CCR6+CXCR3+CCR4-; F) CCR6+CXCR3+CCR4+; G) CCR6-CXCR3-; H) CCR6-CXCR3-CCR4-; I) CCR6-CXCR3-CCR4+; J) CCR6-CXCR3+; K) CCR6-CXCR3+CCR4- and L) CCR6-CXCR3+CCR4+ follicular helper CD4+ T-cell subpopulations in newly-diagnosed treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. ns = non-significant; ----- denotes 5th and 95th percentiles of healthy controls.

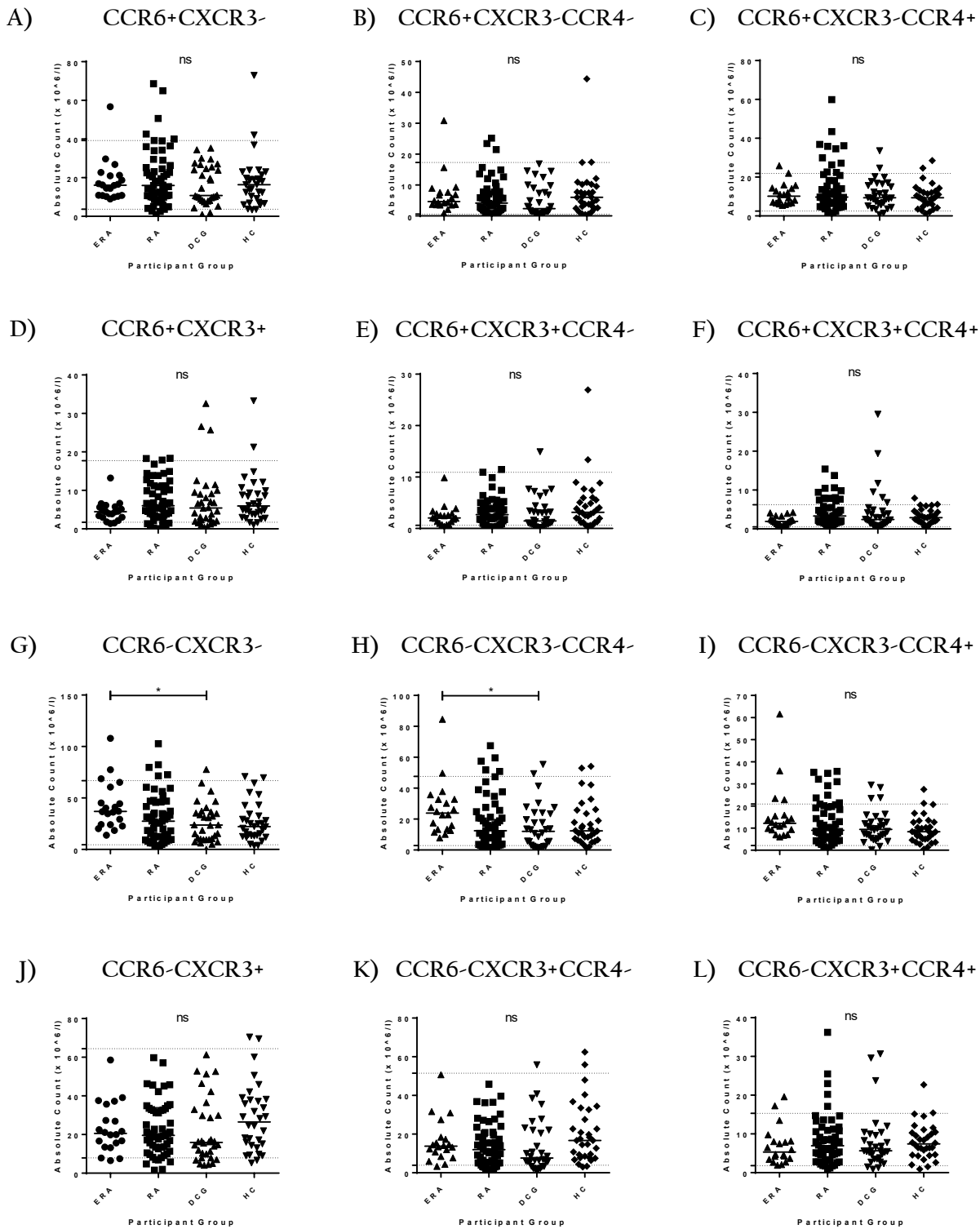


Figure 3.9:- Absolute counts (x 10⁶/l) of A) CCR6+CXCR3-; B) CCR6+CXCR3-CCR4-; C) CCR6+CXCR3-CCR4+; D) CCR6+CXCR3+; E) CCR6+CXCR3+CCR4-; F) CCR6+CXCR3+CCR4+; G) CCR6-CXCR3-; H) CCR6-CXCR3-CCR4-; I) CCR6-CXCR3-CCR4+; J) CCR6-CXCR3+; K) CCR6-CXCR3+CCR4- and L) CCR6-CXCR3+CCR4+ follicular helper CD4⁺ T-cell subpopulations in newly-diagnosed treatment-naive RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. *** = p < 0.001, ** = p < 0.01, * = p < 0.05. ns = non-significant; denotes 5th and 95th percentiles of healthy controls.

Although no significant differences in Th17-like cFth relative or absolute counts were apparent within ERA patients, significant alterations to Th1/17-like, Th2-like and Th1-like

cFth were evident. Th1/17-like cFth percentages were significantly reduced in ERA patients as compared to RA patients ($p<0.01$), DCG ($p<0.05$) and HC ($p<0.01$). This significant reduction was not, however, evident in the absolute counts of Th1/17-like cFth (Table 3.4 and 3.5; Figure 3.8D and 3.9D). With the addition of CCR4, a significant reduction in the percentage of CCR4⁻ Th1/17-like cFth was observed within ERA patients, as compared to HC ($p<0.05$); whilst a significant reduction in CCR4⁺ Th1/17-like cFth percentages was also observed in ERA patients, when compared to RA patients ($p<0.01$) and DCG ($p<0.05$) (Table 3.4; Figure 3.8E - F). No significant reductions in Th1/17-like cFth subpopulations were, however, observed in the absolute counts of these subpopulations (Table 3.5; Figure 3.9E - F).

Alongside these alterations, a significant increase in Th2-like cFth percentages was observed within ERA patients, as compared to RA patients ($p<0.01$), DCG ($p<0.05$) and HC ($p<0.001$); with 8/20 (40.0%) ERA patients also demonstrating Th2-like cFth percentages above the 95th percentile of HC (5th – 95th percentile = 18.1 – 49.7%). Likewise, 12/56 (21.4%) RA patients and 8/32 (25.0%) DCG also demonstrated Th2-like cFth percentages above the 95th percentile of HC (Table 3.4; Figure 3.8G). Accompanying these changes to Th2-like cFth percentages, a significant increase in the absolute counts of Th2-like cFth was observed in ERA patients as compared to HC ($p<0.05$); a finding not reiterated in RA patients (Table 3.5; Figure 3.9G).

When analysed further for CCR4 expression, a significant increase in CCR4⁻ Th2-like cFth percentages was observed within ERA patients, as compared to RA patients ($p<0.05$), DCG ($p<0.05$) and HC ($p<0.05$), which translated into a significant increase in CCR4⁻ Th2-like cFth absolute counts as compared to DCG ($p<0.05$) (Table 3.4 and 3.5; Figure 3.8H and 3.9H). Interestingly, a split population in CCR4⁻ Th2-like percentages was also observed within RA patients, with 26/56 (46.4%) RA patients demonstrating CCR4⁻ Th2-like cFth percentages akin to those observed with ERA patients and 30/56 (53.36%)

RA patients demonstrating CCR4⁻ Th2-like cFth percentages below those observed within ERA patients (median of RA population in keeping with ERA patients = 31.6% [IQR = 27.6 – 36.6%]; median of RA population below those of ERA patients = 9.3% [IQR = 5.5 – 14.2%]) (Figure 3.8H). The relative distribution of CCR4⁺ Th2-like cFth was also significantly increased within ERA patients, and DCG, as compared to HC ($p < 0.05$, respectively) with a visible, yet statistically insignificant increase in CCR4⁺ Th2-like cFth also present in RA patients. No significant alterations in CCR4⁺ Th2-like cFth absolute counts were, however, observed in any patient or control group (Table 3.4 and 3.5; Figure 3.8I and 3.9I).

Finally, a significant reduction in Th1-like cFth percentages was observed within ERA patients ($p < 0.01$), RA patients ($p < 0.01$) and DCG ($p < 0.05$), as compared to HC (Table 3.4; Figure 3.8J). When analysed further for CCR4 expression, a significant reduction in CCR4⁻ Th1-like cFth was observed in RA patients and DCG, as compared to HC ($p < 0.001$ and $p < 0.01$, respectively); with an appreciable, yet non-significant reduction in CCR4⁻ Th1-like cFth also observed in ERA patients (Table 3.4; Figure 3.8K). No such reductions in Th1-like and CCR4⁻ Th1-like cFth absolute counts were observed in ERA patients, RA patient or DCG (Table 3.5; Figure 3.9J – L). Furthermore, no significant difference in CCR4⁺ Th1-like cFth relative and absolute counts were observed in any patient or control group (Table 3.4; Figure 3.8L). In summary, the relative and absolute counts of cFth subpopulations in ERA patients are typified by a significant increase in Th2-like cFth; with concurrent reductions in Th1/17⁻ and Th1-like cFth also observed. In addition to the alterations observed in ERA patients, a proportion of RA patients also displayed an increase in CCR4⁺ Th17-like cFth, above the 95th percentile of HC. Additionally, a split population in the percentage of CCR4⁻ Th2-like cFth was also observed within RA patients; with the relative distribution of CCR4⁻ Th2-like cFth observed, within a proportion of RA patients, akin to those observed in ERA patients.

3.3.c.ii. *Memory helper CD4⁺ T-cells* - Within the memory helper T-cell compartment a significant reduction in CD45RO⁺CXCR5⁻ mTh percentages was observed within ERA patients, as compared to RA patients ($p < 0.05$) and HC ($p < 0.01$). No significant difference in the absolute counts of CD45RO⁺CXCR5⁻ mTh was, however, observed in any patient or control group (Figure 3.10; Table 3.6 and 3.7).

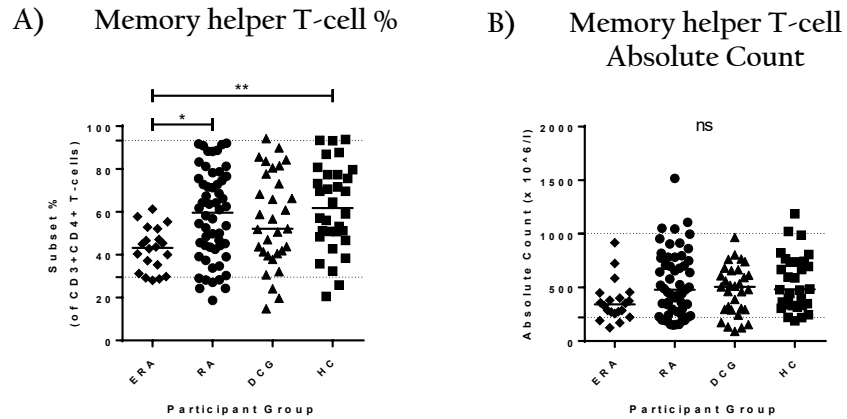


Figure 3.10:- A) Relative distribution (%) and B) absolute counts ($\times 10^6/l$) of memory helper CD4⁺ T-cells in newly-diagnosed, treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. ** = $p < 0.01$, * = $p < 0.05$, ns = non-significant; denotes 5th and 95th percentiles of healthy controls.

	<u>ERA</u>	<u>RA</u>	<u>DCG</u>	<u>HC</u>
Memory Helper T-cells (CD45RO+CXCR5-) (%)	<u>43.2</u> $\Delta^{\dagger}$ (32.2 – 50.9)	59.6 (42.8 – 75.3)	52.1 (40.5 – 77.7)	61.8 (49.0 – 77.4)
CCR6+CXCR3- (Th17) (%)	16.7 (14.5 – 20.4)	19.7 (14.1 – 27.5)	20.5 (15.0 – 26.7)	18.1 (12.8 – 23.4)
CCR6+CXCR3-CCR4- (%)	6.2 (3.8 – 7.6)	4.7 (3.1 – 7.5)	5.1 (2.7 – 8.2)	5.8 (2.8 – 8.5)
CCR6+CXCR3-CCR4+ (%)	11.7 (9.3 – 14.3)	14.1 (9.6 – 2.5)	14.0 (9.8 – 19.4)	11.3 (7.5 – 15.6)
CCR6+CXCR3+ (Th1/17) (%)	<u>8.8</u> Π (7.2 – 12.2)	11.8 (7.7 – 17.0)	13.4 (7.9 – 21.0)	16.6 (9.2 – 20.7)
CCR6+CXCR3+CCR4- (%)	<u>5.9</u> $\$$ (4.5 – 8.0)	7.4 (4.6 – 10.8)	8.3 (4.0 – 13.2)	10.1 (5.8 – 16.1)
CCR6+CXCR3+CCR4+ (%)	3.1 (2.3 – 4.0)	3.8 (2.2 – 6.9)	4.4 (2.6 – 7.1)	3.8 (2.6 – 6.1)
CCR6-CXCR3- (Th2) (%)	<u>41.2</u> $\ddagger$ (33.5 – 46.1)	34.1 (26.6 – 44.5)	30.3 (24.9 – 40.9)	27.8 (19.2 – 34.5)
CCR6-CXCR3-CCR4- (%)	<u>16.3</u> Σ $\P$ (12.9 – 18.6)	10.5 (5.2 – 16.3)	7.7 (5.2 – 11.8)	10.6 (6.8 – 13.5)
CCR6-CXCR3-CCR4+ (%)	<u>26.1</u> δ (16.2 – 30.2)	18.8 (13.3 – 29.0)	20.1 (13.9 – 29.1)	15.9 (11.2 – 22.0)
CCR6-CXCR3+ (Th1) (%)	30.9 (25.5 – 34.4)	<u>30.6</u> $\diamond$ (23.8 – 37.3)	32.9 (27.9 – 40.1)	36.7 (29.8 – 44.0)
CCR6-CXCR3+CCR4- (%)	21.2 (16.5 – 24.0)	<u>18.8</u> $\$$ (13.0 – 27.0)	<u>20.3</u> Ω (13.6 – 27.0)	26.7 (20.5 – 32.6)
CCR6-CXCR3+CCR4+ (%)	9.8 (7.6 – 11.5)	10.3 (6.8 – 13.2)	13.1 (6.7 – 16.0)	10.1 (7.3 – 14.3)

Table 3.6:- Memory helper T-cell (CD45RO+CXCR5-) and subpopulation expression profiles, according to percentage (%) of CD3+CD4+ and CD45RO+CXCR5- T-cells respectively, within early arthritis patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. Underlined figures denote significant results. Δ = $p < 0.01$ as compared to HC; $\dagger$ = $p < 0.05$ as compared to RA patients; Π = $p < 0.01$ as compared to HC; $\$$ = $p < 0.05$ as compared to HC; $\ddagger$ = $p < 0.01$ as compared to HC; $\P$ = $p < 0.05$ as compared to DCG; Σ = $p < 0.001$ as compared to DCG; $\P$ = $p < 0.05$ as compared to RA patients and HC; δ = $p < 0.05$ as compared to HC; $\diamond$ = $p < 0.05$ as compared to HC; $\$$ = $p < 0.01$ as compared to HC; Ω = $p < 0.05$ as compared to HC. Figures denote median and inter-quartile ranges (IQR).

	<u>ERA</u>	<u>RA</u>	<u>DCG</u>	<u>HC</u>
Memory Helper T-cells (CD45RO+CXCR5+) (x10 ⁶ /l)	341.8 (259.4 – 437.1)	478.7 (249.1 – 774.9)	505.8 (293.6 – 656.8)	482.4 (336.0 – 738.0)
CCR6+CXCR3- (Th17) (x10 ⁶ /l)	58.1 (46.8 – 84.8)	71.3 (42.7 – 173.8)	100.6 (46.3 – 130.3)	102.4 (46.5 – 145.5)
CCR6+CXCR3-CCR4- (x10 ⁶ /l)	20.2 (10.7 – 32.4)	17.7 (12.4 – 41.4)	26.3 (8.9 – 36.9)	23.6 (12.2 – 56.8)
CCR6+CXCR3-CCR4+ (x10 ⁶ /l)	37.7 (28.5 – 52.2)	51.6 (26.3 – 125.8)	60.5 (31.9 – 96.6)	55.1 (28.5 – 96.9)
CCR6+CXCR3+ (Th1/17) (x10 ⁶ /l)	<u>29.7Δ</u> (22.8 – 40.4)	63.0 (22.1 – 120.2)	63.0 (15.7 – 120.3)	79.4 (47.1 – 137.1)
CCR6+CXCR3+CCR4- (x10 ⁶ /l)	<u>19.1II</u> (13.5 – 29.2)	34.4 (12.5 – 65.5)	36.1 (10.1 – 77.0)	48.9 (29.6 – 114.8)
CCR6+CXCR3+CCR4+ (x10 ⁶ /l)	<u>9.5Σ</u> (6.4 – 15.4)	17.3 (6.4 – 43.7)	18.7 (7.5 – 44.6)	23.0 (12.0 – 28.9)
CCR6-CXCR3- (Th2) (x10 ⁶ /l)	138.1 (93.4 – 198.8)	145.1 (91.7 – 225.5)	133.7 (80.1 – 194.2)	137.0 (86.6 – 182.4)
CCR6-CXCR3-CCR4- (x10 ⁶ /l)	51.1 (29.6 – 72.5)	32.6 (22.4 – 74.3)	24.8 (15.3 – 63.2)	44.4 (29.6 – 77.8)
CCR6-CXCR3-CCR4+ (x10 ⁶ /l)	87.4 (44.0 – 106.3)	82.9 (52.5 – 142.2)	85.01 (56.8 – 104.2)	69.6 (45.6 – 125.8)
CCR6-CXCR3+ (Th1) (x10 ⁶ /l)	<u>91.6†</u> (70.3 – 147.2)	154.5 (88.3 – 223.3)	141.0 (60.5 – 213.1)	207.5 (123.4 – 265.1)
CCR6-CXCR3+CCR4- (x10 ⁶ /l)	<u>62.1‡</u> (43.1 – 98.5)	87.2 (52.6 – 130.9)	<u>74.4¶</u> (38.0 – 134.5)	149.8 (85.9 – 202.4)
CCR6-CXCR3+CCR4+ (x10 ⁶ /l)	31.7 (18.8 – 44.9)	40.2 (20.8 – 84.5)	43.7 (24.2 – 82.8)	45.2 (27.9 – 78.5)

Table 3.7:- Memory helper T-cell (CD45RO+CXCR5-) and subpopulation expression profiles, according to absolute count (x10⁶/l) of CD45RO+CXCR5- T-cells respectively, within early arthritis patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. Underlined figures denote significant results. Δ = p<0.01 as compared to HC; II = p<0.01 as compared to HC; Σ = p<0.05 as compared to RA patients and HC; † = p<0.01 as compared to HC; ‡ = p<0.01 as compared to HC; ¶ = p<0.05 as compared to HC. Figures denote median and inter-quartile ranges (IQR).

With the addition of CCR6 and CXCR3, no significant difference in Th17 mTh relative or absolute counts was observed between patient and control groups. However, 13/56 (23.2%) and 12/56 (21.4%) RA patients did demonstrate Th17 mTh percentages and absolute counts above the 95th percentile of HC (5th – 95th percentile = 6.2 – 28.1% and 22.5 – 215.1 x 10⁶/l, respectively) (median of RA population within the 95th percentile of HC = 16.6% [IQR = 12.5 – 22.2%] and 55.3 x 10⁶/l [IQR = 34.6 – 109.0 x 10⁶/l], respectively; median of RA population above the 95th percentile of HC = 34.0% [IQR = 29.4 – 37.4%] and 275.9 x 10⁶/l [IQR = 246.2 – 319.6 x 10⁶/l], respectively) (Table 3.6 and 3.7; Figure 3.11A and 3.12A).

When analysed further for CCR4 expression, no significant difference in CCR4- or

CCR4⁺ Th17 mTh percentages or absolute counts was observed. However, 13/56 (23.2%) and 14/56 (25.0%) RA patients displayed CCR4⁺ Th17 mTh percentages and absolute counts above the 95th percentile of HC (5th – 95th percentile = 4.6 – 21.6% and 17.5 – 125.3 x 10⁶/l, respectively) (median of RA population within the 95th percentile of HC = 11.8% [IQR = 8.3 – 15.9%] and 38.6 x 10⁶/l [IQR = 20.9 – 74.9 x 10⁶/l], respectively; median of RA population above the 95th percentile of HC = 24.7% [IQR = 23.2 – 32.5%] and 214.2 x 10⁶/l [IQR = 142.8 – 264.3 x 10⁶/l], respectively) (Figure 3.11B - C; Figure 3.12B - C).

A significant reduction in Th1/17 mTh relative and absolute counts was observed within ERA patients, as compared to HC ($p < 0.01$, respectively) (Table 3.6 and 3.7; Figure 3.11D and 3.12D). When analysed further for CCR4 expression, a significant reduction in the percentage and absolute counts of CCR4⁻ Th1/17 mTh in ERA patients was observed, as compared to HC ($p < 0.05$ and $p < 0.01$, respectively); with a significant reduction in the absolute counts of CCR4⁺ Th1/17 mTh also observed in ERA patients, as compared to RA patients and HC ($p < 0.05$, respectively) (Figure 3.11E - F; Figure 3.12E - F). Accompanying this observed reduction in Th1/17 mTh was a significant increase in Th2 mTh percentages within ERA patients, as compared to DCG and HC ($p < 0.05$ and $p < 0.01$, respectively) (Table 3.6; Figure 3.11G). When analysed further for CCR4 expression, this increase in Th2 mTh translated into a significant increase in the percentage of both CCR4⁻ and CCR4⁺ Th2 mTh within ERA patients, when compared to RA patients ($p < 0.05$ and ns), DCG ($p < 0.001$ and ns) and HC ($p < 0.05$, respectively) (Figure 3.11H and 3.11I). These increases in Th2 mTh subpopulations were not, however, replicated within the absolute counts of CCR6-CXCR3⁻, CCR4⁻ or CCR4⁺ Th2 mTh, respectively (Figure 3.12G - I).

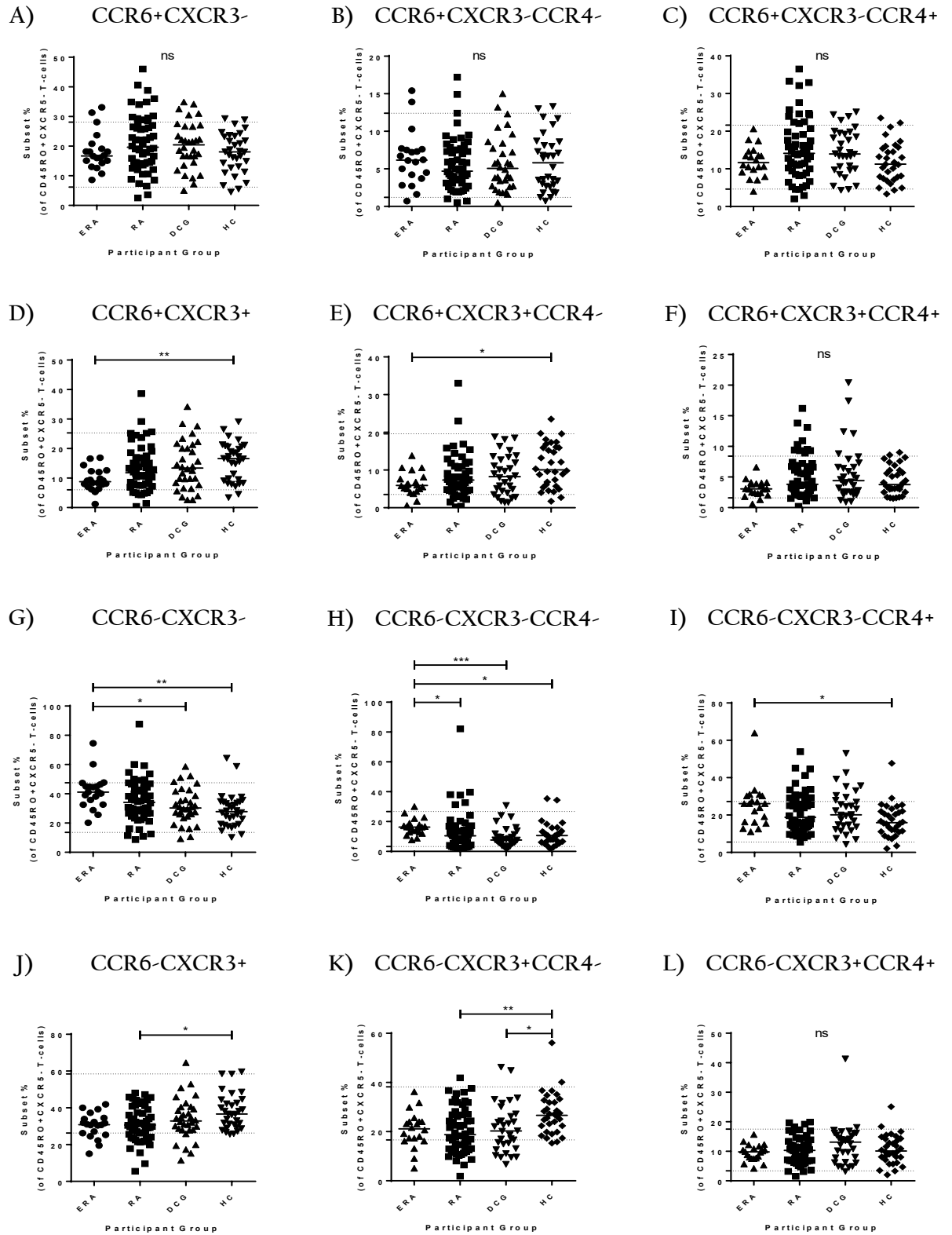


Figure 3.11:- Prevalence (%) of A) CCR6+CXCR3-; B) CCR6+CXCR3-CCR4-; C) CCR6+CXCR3-CCR4+; D) CCR6+CXCR3+; E) CCR6+CXCR3+CCR4-; F) CCR6+CXCR3+CCR4+; G) CCR6-CXCR3-; H) CCR6-CXCR3-CCR4-; I) CCR6-CXCR3-CCR4+; J) CCR6-CXCR3+; K) CCR6-CXCR3+CCR4- and L) CCR6-CXCR3+CCR4+ memory helper CD4+ T-cell subpopulations in newly-diagnosed treatment-naive RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. ns = non-significant; denotes 5th and 95th percentiles of healthy controls.

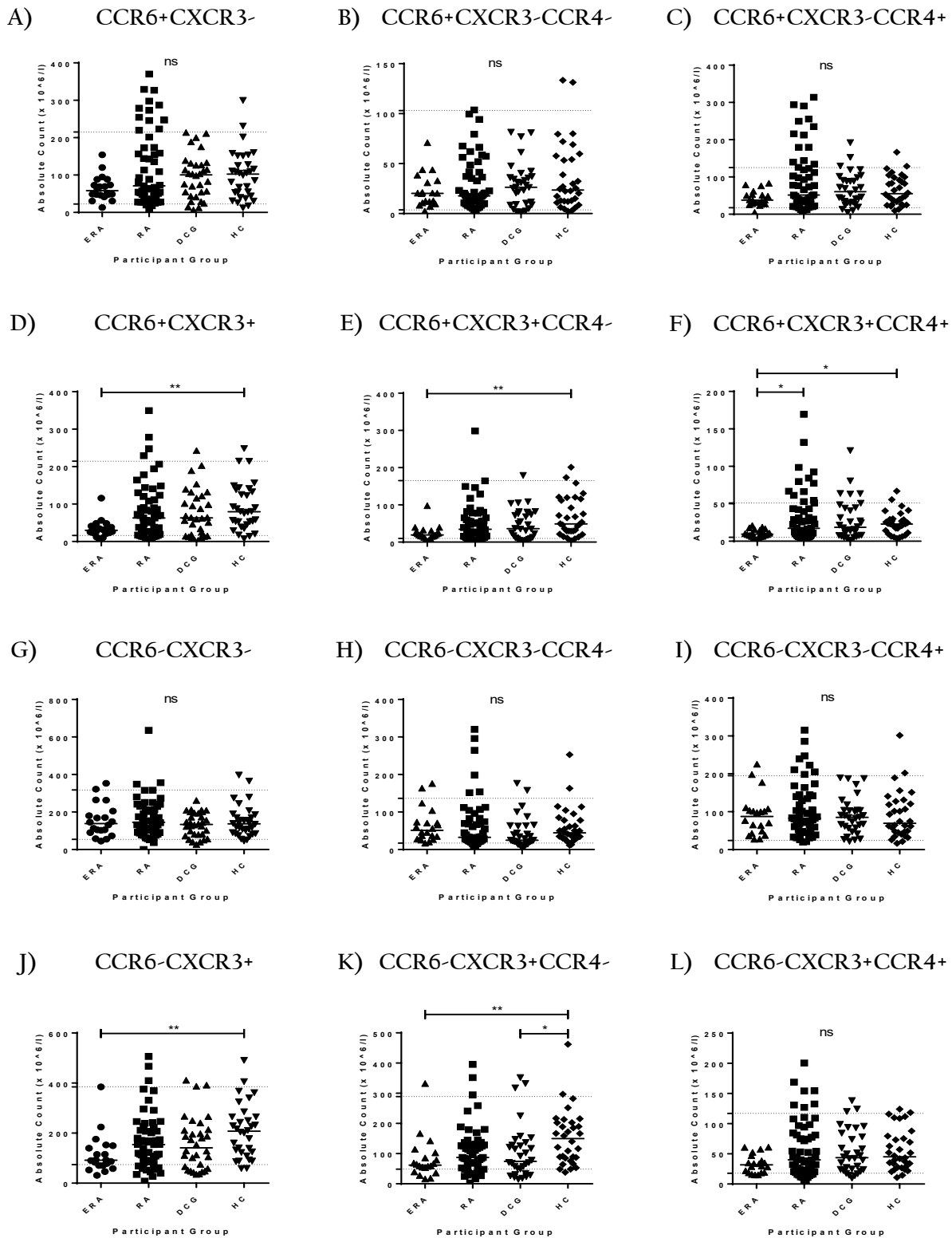


Figure 3.12:- Absolute counts (x 10⁶/l) of A) CCR6+CXCR3⁻; B) CCR6+CXCR3⁻CCR4⁻; C) CCR6+CXCR3⁻CCR4⁺; D) CCR6+CXCR3⁺; E) CCR6+CXCR3⁺CCR4⁻; F) CCR6+CXCR3⁺CCR4⁺; G) CCR6-CXCR3⁻; H) CCR6-CXCR3⁻CCR4⁻; I) CCR6-CXCR3⁻CCR4⁺; J) CCR6-CXCR3⁺; K) CCR6-CXCR3⁺CCR4⁻ and L) CCR6-CXCR3⁺CCR4⁺ memory helper CD4⁺ T-cell subpopulations in newly-diagnosed treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. *** = p<0.001, ** = p<0.01, * = p<0.05. ns = non-significant; ----- denotes 5th and 95th percentiles of healthy controls.

Finally, a significant reduction in Th1 mTh percentages was observed within RA patients, as compared to HC ($p < 0.05$); with a trend towards reduced Th1 mTh percentages within ERA patients and DCG also observed, as compared to HC (Figure 3.11J). Supplementary to the observed changes in Th1 mTh percentages, a significant decrease in Th1 mTh absolute counts was observed within ERA patients, as compared to HC ($p < 0.01$) (Figure 3.12J). Upon further examination for CCR4 expression, a significant reduction in CCR4⁻ Th1 mTh percentages was observed within RA patients and DCG when compared to HC ($p < 0.01$ and $p < 0.05$, respectively); with 23/56 (41.1%) RA patients and 11/32 (34.4%) DCG demonstrating CCR4⁻ Th1 mTh percentages below the 5th percentile of HC (5th – 95th percentile = 16.6 – 38.2%). In addition, a significant reduction in CCR4⁻ Th1 mTh absolute counts was observed in ERA patients and DCG, when compared to HC ($p < 0.01$ and $p < 0.05$, respectively) (Figure 3.11K and 3.12K). In contrast, no significant alterations in CCR4⁺ Th1 mTh relative or absolute counts were observed in any patient or control group (Figure 3.11L and 3.12L). In summary, the relative and absolute counts of mTh subpopulations in ERA patients are characterized by a significant increase in Th2 mTh, with reductions in Th1/17 and Th1 mTh also observed. In addition to the observed alterations in mTh subpopulations within ERA patients, a proportion of RA patients also display an increase in CCR4⁺ Th17 mTh percentages and absolute counts, above the 95th percentile of HC.

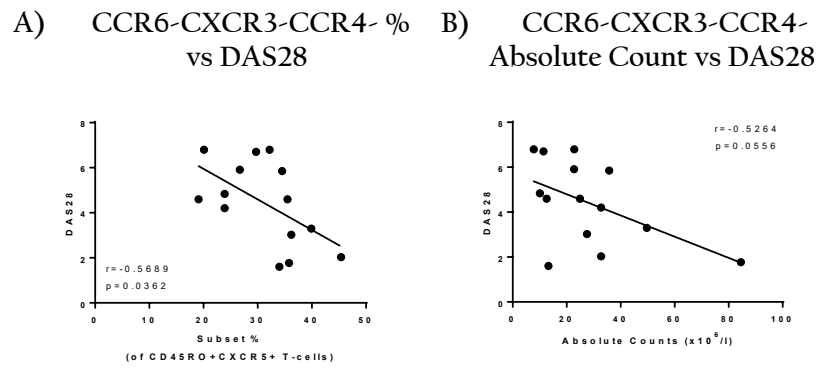
3.3.d. Alterations to the relative and absolute counts of follicular and memory helper CD4⁺ T-cell subpopulations correlate with measures of disease activity in newly-diagnosed, treatment-naïve RA patients

3.3.d.i. *Follicular helper CD4⁺ T-cells* - With the follicular and memory helper CD4⁺ T-cell compartments of ERA patients dominated by an increase in Th2 subpopulations, combined with evidence for concurrent reductions in Th1/17 subpopulations, a thorough investigation for associations between the relative and absolute numbers of these T-cell subpopulations and patient clinical outcomes seemed warranted. In addition, the observed increase in Th17 subpopulations, within a proportion of RA patients, coupled

with the reduced levels of Th1 subpopulations, within the cFth and mTh compartments of RA patients provided another avenue of investigation. As such all cFth and mTh parameters within ERA and RA patients, were compared to the currently available disease activity measures erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and the 28 joint disease activity score (DAS28).

At the time of diagnosis, median ESR, CRP and DAS28 scores within ERA patients were 25.00mm/hr (IQR = 12.75 – 41.25mm/hr), 11.20mg/l (IQR = 3.53 – 37.70mg/l) and 4.40 (IQR = 2.77 – 5.87), respectively. When investigated for associations with cFth subpopulations, no associations between CD45RO+CXCR5+ cFth percentages and absolute counts were noted with ESR, CRP or DAS28 scores. When analysed further according to the expression of the chemokine receptors CCR6, CXCR3 and CCR4, however, a negative association between the relative distribution of CCR4⁻ Th2-like cFth and DAS28 scores was observed within ERA patients ($r=-0.5689$; $p=0.0362$); a relationship which remained evident within the absolute counts of CCR4⁻ Th2-like cFth ($r=0.5264$; $p=0.0556$) (Figure 3.13A - B). No further associations between cFth subpopulation percentages and absolute counts were observed with disease activity measures within ERA patients.

Newly-diagnosed, treatment-naïve RA Patients



Longstanding RA Patients

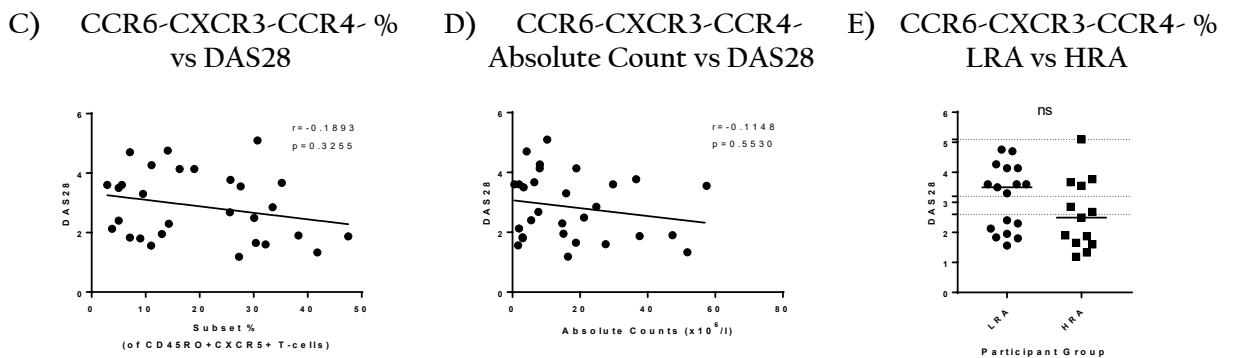


Figure 3.13:- Association of follicular helper CD4⁺ T-cell percentages and absolute counts with DAS28 scores in newly-diagnosed, treatment-naïve and longstanding RA patients. Correlation of A) CCR6-CXCR3-CCR4⁻ Th2-like follicular helper CD4⁺ T-cell percentages with DAS28 score; and B) CCR6-CXCR3-CCR4⁻ Th2-like follicular helper CD4⁺ T-cell absolute counts with DAS28 score, within newly-diagnosed, treatment-naïve RA patients. Association of C) CCR6-CXCR3-CCR4⁻ Th2-like follicular helper CD4⁺ T-cell percentages with DAS28 score; and D) CCR6-CXCR3-CCR4⁻ Th2-like follicular helper CD4⁺ T-cell absolute counts with DAS28 score, within longstanding RA patients. E) DAS28 scores within longstanding RA patients presenting with elevated CCR6-CXCR3-CCR4⁻ Th2-like follicular helper CD4⁺ T-cell percentages (HRA), as compared to longstanding RA patients with reduced CCR6-CXCR3-CCR4⁻ Th2-like follicular helper CD4⁺ T-cell percentages (LRA). Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables, with a p-value <0.05 considered statistically significant.

Within RA patients, limited data was available regarding ESR, CRP and DAS28 scores.

However, of those available the median ESR, CRP and DAS28 scores were 12.00mm/hr (IQR = 7.00 – 18.00mm/hr), 4.35mg/l (IQR = 1.60 – 12.20mg/l) and 2.68 (IQR = 1.89 – 3.64), respectively. When assessed for evidence of associations with cFth subpopulations, no associations between CD45RO+CXCR5⁺ cFth percentages and absolute counts were noted with ESR, CRP or DAS28 scores; whilst no further associations between cFth subpopulation percentages or absolute counts were observed with any disease activity

measure within RA patients (data not shown). This included the previously observed negative association between CCR4⁻ Th2-like cFth percentages, absolute counts and ERA patient DAS28 scores (Figure 3.13C - D). A trend towards a reduction in DAS28 scores was, however, observed within RA patients demonstrating an increase in CCR4⁻ Th2-like cFth percentages, as compared to RA patients demonstrating a reduction in CCR4⁻ Th2-like cFth percentages; although this observation failed to reach statistical significance ($p=0.1938$) (Figure 3.13E). No significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating elevated CCR4⁺ Th17 cFth percentages and absolute counts, as compared to RA patients demonstrating CCR4⁺ Th17 cFth percentages and absolute counts within the 5th – 95th percentile of HC (data not shown). As such, at the time of diagnosis, the relative distribution and absolute counts of CCR4⁻ Th2-like cFth appear to negatively associate with DAS28 scores, indicating a favourable clinical presentation within ERA patients in which CCR4⁻ Th2-like cFth are elevated. This relationship, however, appears to be partially abrogated in RA patients in which a csDMARD or bDMARD therapeutic regimen has been previously established.

3.3.d.ii. *Memory helper CD4⁺ T-cells* – Further to the lack of associations between CD45RO⁺CXCR5⁺ cFth percentages, absolute counts and current disease activity measures, no associations between the relative or absolute counts of CD45RO⁺CXCR5⁻ mTh and any current disease activity measure was observed within ERA patients (data not shown). Within the mTh compartment, however, a trend towards a negative relationship between the relative distribution of Th17 mTh and DAS28 scores was apparent ($r=-0.4774$; $p=0.0856$); an association which reached significance between Th17 mTh absolute counts and DAS28 scores ($r=-0.7159$; $p=0.0040$) (Figure 3.14A - B). When analysed further for CCR4 expression, this relationship translated into a negative association between CCR4⁺ Th17 mTh absolute counts and DAS28 scores ($r=-0.5308$; $p=0.0508$), although no such relationship was observed for CCR4⁺ Th17 mTh percentages (Figure 3.14C and data not shown). In addition to this observed negative relationship, a

positive relationship between the relative distribution of CCR4⁺ Th1 mTh and DAS28 score was observed within ERA patients ($r=0.6351$; $p=0.0168$), although this relationship was not replicated within the absolute counts of CCR4⁺ Th1 mTh (Figure 3.14D and data not shown). No further associations between mTh subpopulation percentages, or absolute counts, were observed with any disease activity measure within ERA patients.

Newly-diagnosed, treatment-naïve RA Patients

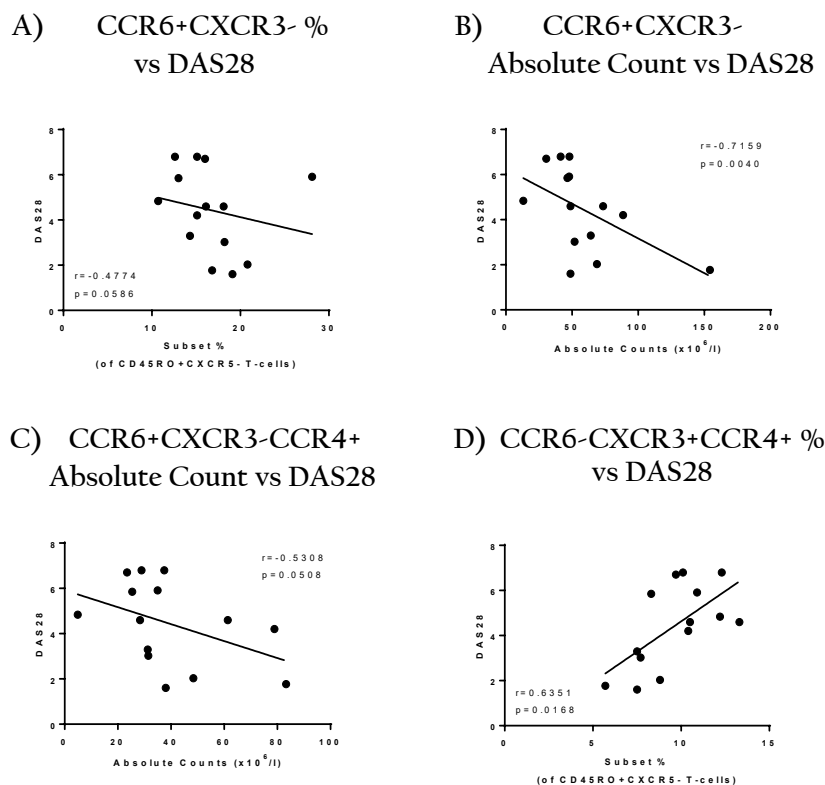


Figure 3.14:- Correlation of A) CCR6+CXCR3⁻ Th17 memory helper CD4⁺ T-cell percentages with DAS28 score; B) CCR6+CXCR3⁻ Th17 memory helper CD4⁺ T-cell absolute counts with DAS28 score; and C) CCR6+CXCR3-CCR4⁺ Th17 memory helper CD4⁺ T-cell absolute counts with DAS28 score, within newly-diagnosed, treatment-naïve RA patients. An R-value ≥ 0.50 was considered to represent an association between two variables, with a p-value < 0.05 considered statistically significant.

In contrast to the observed clinical relationships within ERA patients, no associations between any mTh subpopulation parameter and any current disease activity measure were observed within RA patients (data not shown). Furthermore, irrespective of CCR4 expression, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating elevated Th17 mTh percentages and absolute counts, as compared to RA patients demonstrating Th17 mTh percentages and absolute counts

within the 5th and 95th percentiles of HC (data not shown). As such, the relative and absolute counts of Th17 mTh within ERA patients appear to negatively associate with patient DAS28 scores, indicating a favourable clinical presentation within ERA patients presenting with an increase in Th17 mTh. A positive association between the relative distribution of CCR4⁺ Th1 mTh and DAS28 scores was also apparent within these patients, indicative of an adverse clinical presentation within ERA patients in which Th1 mTh are elevated. However, these relationships appear to be abrogated within RA patients in which a csDMARD or bDMARD therapeutic regimen has been previously established.

3.3.e. The stratification of longstanding RA patients and disease controls according to their therapeutic regimens reveals a distinct increase in Th17-like follicular and Th17 memory helper CD4⁺ T-cells with the use of anti-TNF- α therapeutic agents

3.3.e.i. Follicular helper CD4⁺ T-cells – Despite the lack of associations with patient clinical outcome measures within the follicular and memory helper CD4⁺ T-cell compartments of RA patients, the previously observed increase in Th17-like cFth and Th17 mTh, within a proportion of RA patients and DCG, warranted a more thorough investigation. With a high degree of heterogeneity present in the therapeutic regimens employed within these patients, it was sought to determine if any of the alterations observed within the relative or absolute numbers of cFth and mTh subpopulations, within RA patients and DCG, could be attributed to differences in patient therapeutic regimens. As such, RA patients and DCG were stratified into two distinct therapeutic groups; namely, those undertaking a conventional synthetic disease modifying anti-rheumatic drug (csDMARD) therapeutic regimen and those receiving biological disease modifying anti-rheumatic drug (bDMARD) therapeutics. Following such stratification, no significant difference in the disease activity measures ESR, CRP or DAS28 scores was observed between RA patients receiving csDMARD or bDMARD therapeutic regimens (Figure 3.15A and data not shown).

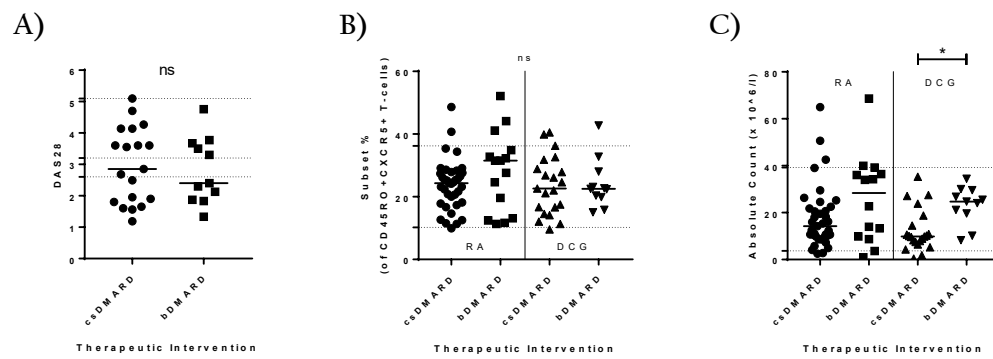


Figure 3.15:- Stratification of long-standing RA patients (RA) and disease controls (DCG) based upon the use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens. A) DAS28 scores within long-standing RA patients undertaking csDMARD therapeutic regimens, as compared to long-standing RA patients receiving bDMARD therapeutics. Dotted lines denote disease activity targets. <2.6 = remission; $2.6 - 3.2$ = low disease activity; $>3.2 - 5.1$ = moderate disease activity; >5.1 = high disease activity. B) Relative distribution of CCR6+CXCR3⁻ Th17-like follicular helper T-cells. C) Absolute counts of CCR6+CXCR3⁻ Th17-like follicular helper T-cells. * = $p < 0.05$, ns = non-significant. Dotted lines denotes the 5th and 95th percentile of healthy controls.

With the further examination of the cFth compartment, no significant difference in the relative or absolute counts of CD45RO+CXCR5⁺ cFth was observed between RA patients undertaking csDMARD or bDMARD therapeutic regimens, respectively (data not shown). Furthermore, when analysed further according to the expression of the chemokine receptors CCR6 and CXCR3 alone, no significant difference in the relative distribution of Th17⁻, Th1/17⁻, Th2⁻ or Th1-like cFth was observed within RA patients or DCG undertaking csDMARD or bDMARD therapeutic regimens. A trend towards an increase in the relative and absolute counts of Th17-like cFth was, however, observed within RA patients receiving bDMARD, as compared to those undertaking csDMARD therapeutic regimens; although these observations failed to reach statistical significance ($p=0.1829$ and $p=0.1894$, respectively) (Figure 3.15B - C).

With the inclusion of CCR4, no significant difference in the relative and absolute counts of CCR4⁺ Th17-like cFth were observed within RA patients undertaking bDMARD therapeutic regimens, as compared to those receiving csDMARDs. Split populations in the relative and absolute counts of CCR4⁺ Th17-like cFth were, however, observed within RA patients receiving bDMARDs, with 8/15 (53.3%) and 5/14 (35.7%) RA patients

receiving bDMARDs demonstrating CCR4⁺ Th17-like cFth above the 95th percentile of HC (Figure 3.16A – B). When investigated further for differences in disease activity measures, however, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating elevated CCR4⁺ Th17-like cFth relative and absolute counts, above the 95th percentile of HC, or those demonstrating CCR4⁺ Th17-like cFth relative and absolute counts below the 95th percentile of HC (Figure 3.16C and data not shown).

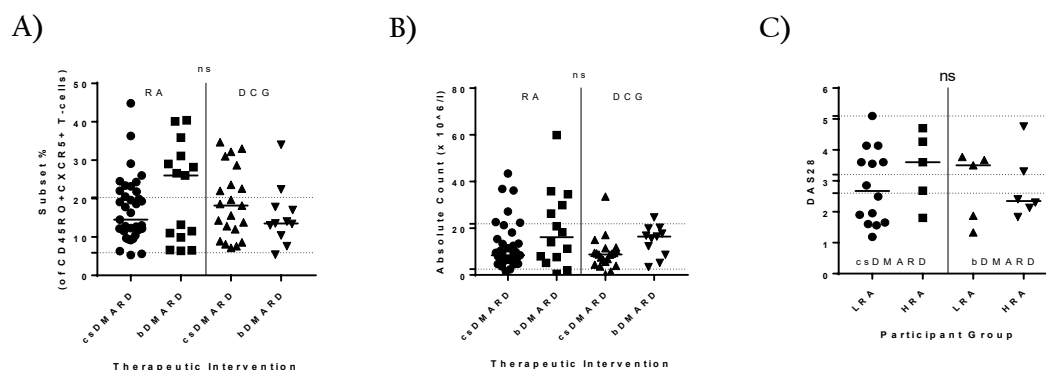


Figure 3.16:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon the use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens. A) Relative distribution of CCR6+CXCR3-CCR4⁺ Th17-like follicular helper CD4⁺ T-cells. B) Absolute counts of CCR6+CXCR3-CCR4⁺ Th17-like follicular helper CD4⁺ T-cells. Dotted lines denotes the 5th and 95th percentile of healthy controls. C) DAS28 scores within longstanding RA patients undertaking csDMARD and bDMARD therapeutic regimens stratified according to the presence (HRA) or absence (LRA) of CCR6+CXCR3-CCR4⁺ Th17-like cFth percentages above the 95th percentile of healthy controls. Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. ns = non-significant.

Further to the observed alterations in the relative and absolute counts of CCR4⁺ Th17-like cFth, no significant difference in the relative distribution of CCR4⁻ Th2-like cFth was observed between RA patients undertaking csDMARD and bDMARD therapeutic regimens. Split populations in the relative distribution of CCR4⁻ Th2-like cFth were, however, observed within RA patients undertaking csDMARD and bDMARD therapeutic regimens, with 17/37 (45.9%) and 6/15 (40.0%) of RA patients receiving csDMARDs and bDMARDs, respectively demonstrating CCR4⁻ Th2-like cFth percentages akin to those previously observed within ERA patients (Figure 3.17A). However, when stratified further according to the relative distribution of CCR4⁻ Th2-like cFth, no significant

difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating CCR4⁻ Th2-like cFth percentages akin to those of ERA patients, and those demonstrating CCR4⁻ Th2-like cFth percentages below those of ERA patients, irrespective of their therapeutic regimen (Figure 3.17B and data not shown). No further alterations to the relative or absolute counts of any cFth subpopulation were observed within RA patients or DCG when stratified according to the use of csDMARD or bDMARD therapeutic regimens.

As such, following the stratification of RA patients according to their use of csDMARD or bDMARD therapeutics, a non-significant increase in the relative and absolute counts of CCR4⁺ Th17-like cFth was observed within a proportion of RA patients undertaking bDMARD therapeutic regimens, as compared to RA patients receiving csDMARD therapeutics. However, of those RA patients undertaking bDMARD therapeutics, no significant difference in the disease activity measures ESR, CRP or DAS28 was observed between RA patients demonstrating elevated CCR4⁺ Th17-like cFth percentages and absolute counts, and those demonstrating CCR4⁺ Th17-like cFth relative and absolute counts within the 95th percentile of HC. In addition to the observed increase in CCR4⁺ Th17-like cFth, split populations in the relative distribution of CCR4⁻ Th2-like cFth were also observed within RA patients undertaking both csDMARD- and bDMARD-based therapeutic regimens. However, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating elevated CCR4⁻ Th2-like cFth percentages, akin to those observed within ERA patients, and RA patients demonstrating CCR4⁻ Th2-like cFth percentages below those of ERA patients, irrespective of their therapeutic regimen.

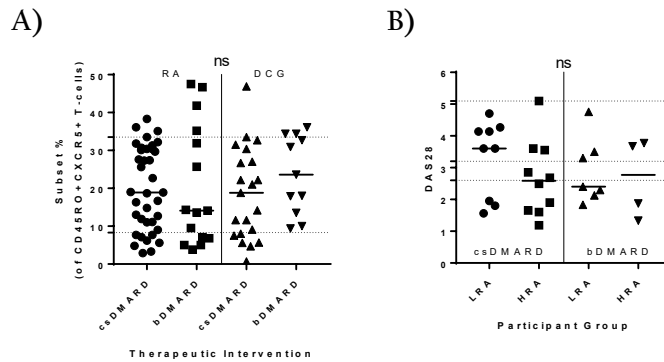


Figure 3.17:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon the use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens. A) Relative distribution of CCR6-CXCR3-CCR4- Th2-like follicular helper CD4+ T-cells. Dotted lines denotes the 5th and 95th percentile of healthy controls. B) DAS28 scores within longstanding RA patients undertaking csDMARD and bDMARD therapeutic regimens stratified according to the presence (HRA) or absence (LRA) of CCR6-CXCR3-CCR4- Th2-like cFth percentages analogous to those observed within newly-diagnosed, treatment-naïve RA patients. Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. ns = non-significant.

With alterations to the relative and absolute counts of CCR4+ Th17-like and CCR4- Th2-like cFth observed within a proportion of RA patients receiving both csDMARD and bDMARD therapeutics, RA patients and DCG were further stratified according to their primary therapeutic agent, in order to investigate the effects of individual csDMARD and bDMARD therapeutic regimens on cFth subpopulations. Following stratification, no significant difference in CD45RO+CXCR5+ cFth percentages was observed in RA patients or DCG, receiving csDMARD-based therapeutics, incorporating methotrexate and methotrexate substitutes, including leflunomide and sulfasalazine; or bDMARD-based therapeutics, such as anti-tumour necrosis factor alpha (TNF- α) and B-cell depleting (rituximab) monoclonal antibodies (data not shown). A significant increase in the absolute counts of CD45RO+CXCR5+ cFth was, however, observed within RA patients receiving anti-TNF- α therapeutics, as compared to those receiving methotrexate, methotrexate substitutes and rituximab ($p < 0.05$, respectively). In contrast, a significant reduction in the absolute counts of CD45RO+CXCR5+ cFth was observed within RA patients receiving rituximab, as compared to those receiving methotrexate ($p < 0.05$), an observation entirely consistent with the symbiotic relationship of circulating B-cells and

cFth. With the use of rituximab proven to result in a global reduction in CD45RO+CXCR5+ cFth, the relative and absolute counts of Th17⁻, Th1/17⁻, Th2⁻ and Th1-like cFth subpopulations within RA patients receiving rituximab were not investigated hereafter (Figure 3.18A).

When analysed further according to the expression of CCR6 and CXCR3, no significant difference in the relative distribution of Th17⁻, Th1/17⁻, Th2⁻ or Th1-like cFth was observed within RA patients or DCG, when stratified according to their primary therapeutic agent. A split population in the relative and absolute counts of Th17-like cFth was, however, evident within RA patients undertaking anti-TNF- α therapeutic regimens; with a significant increase in the absolute counts of Th17-like cFth also observed within RA patients receiving anti-TNF- α monoclonal therapeutics, as compared to those undertaking methotrexate and methotrexate substitutes ($p < 0.05$, respectively) (Figure 3.18B – C). No further alterations to the absolute counts of Th1/17⁻, Th2⁻ or Th1-like cFth were observed within RA patients receiving anti-TNF- α therapeutics.

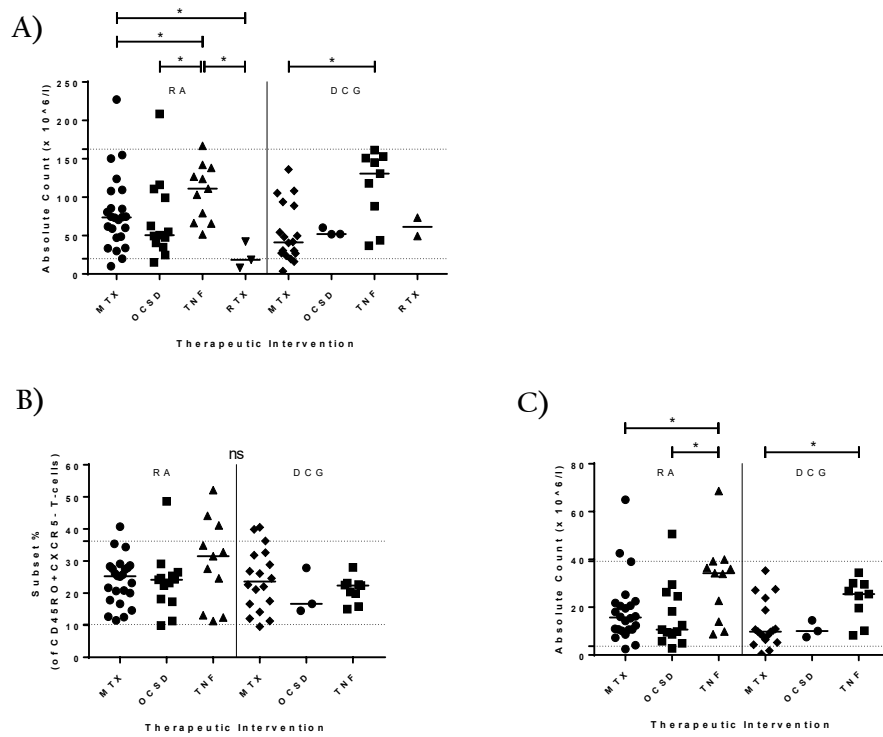


Figure 3.18:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon their use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens. A) Absolute counts of CD45RO+CXCR5+ follicular helper CD4+ T-cells. B) Relative distribution of CCR6+CXCR3- Th17-like follicular helper CD4+ T-cells. C) Absolute counts of CCR6+CXCR3- Th17-like follicular helper CD4+ T-cells. MTX = methotrexate, OCSD = other csDMARD, TNF = tumour necrosis factor alpha, RTX = rituximab. ** = $p < 0.01$, * = $p < 0.05$, ns = non-significant. Dotted lines denotes the 5th and 95th percentile of healthy controls.

With the addition of CCR4, no significant difference in the relative distribution of any cFth subpopulation was observed within RA patients and DCG when stratified according to their primary therapeutic agent (data not shown). A split population in the relative distribution of CCR4+ Th17-like cFth was, however, evident within RA patients undertaking anti-TNF- α therapeutic regimens; with 6/11 (54.5%) RA patients demonstrating CCR4+ Th17-like cFth percentages above the 95th percentile of HC (Figure 3.19A). A significant increase in the absolute counts of CCR4+ Th17-like cFth, above the 95th percentile of HC, was also observed within RA patients undertaking anti-TNF- α therapeutic regimens, as compared to RA patients receiving methotrexate and methotrexate substitutes ($p < 0.05$, respectively). Furthermore, a split population in the absolute counts of CCR4+ Th17-like cFth was also evident within RA patients receiving anti-TNF- α therapeutics; with 5/11 (45.5%) RA patients demonstrating CCR4+ Th17-like

cFth absolute counts above the 95th percentile of HC (Figure 3.19B). However, when investigated further for associations with patient clinical outcomes, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients with elevated CCR4⁺ Th17-like cFth percentages and absolute counts and those demonstrating CCR4⁺ Th17-like cFth percentages and absolute counts within the 95th percentile of HC (Figure 3.19C and data not shown).

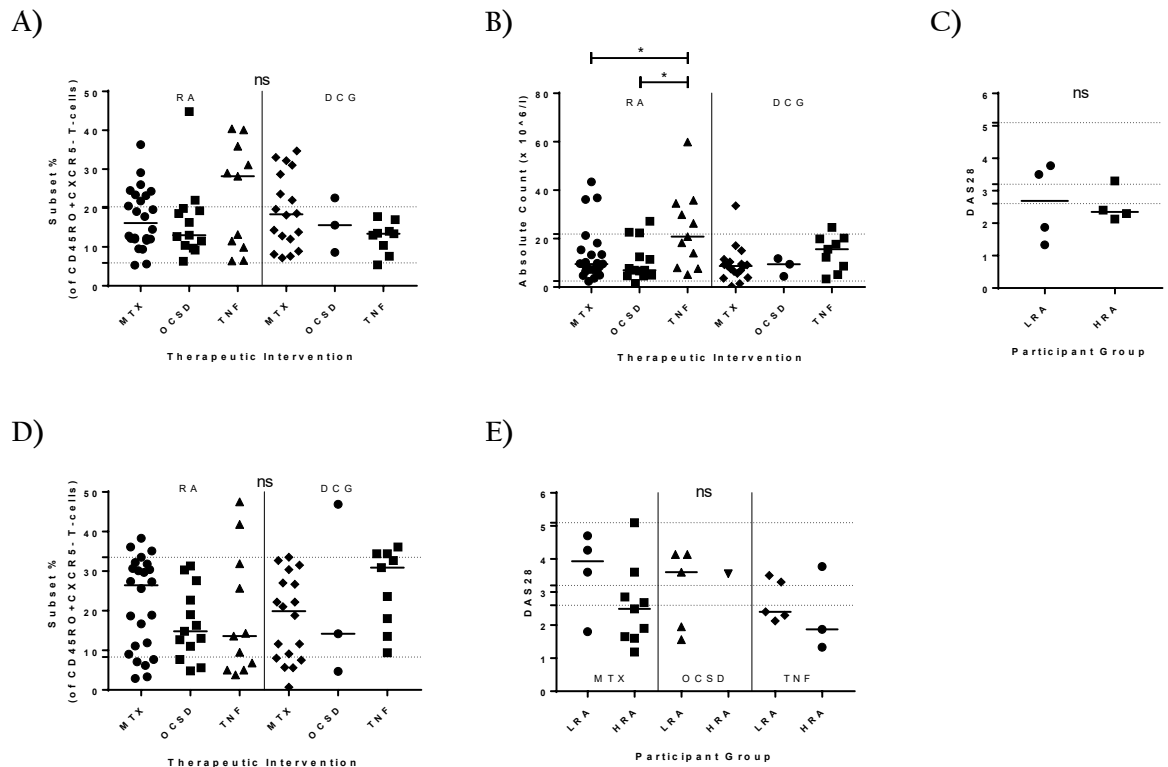


Figure 3.19:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon their use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens. A) Relative distribution of CCR6+CXCR3-CCR4⁺ Th17-like follicular helper CD4⁺ T-cells. B) Absolute counts of CCR6+CXCR3-CCR4⁺ Th17-like follicular helper CD4⁺ T-cells. C) DAS28 scores within longstanding RA patients undertaking anti-TNF- α therapeutic regimens stratified according to the presence (HRA) or absence (LRA) of CCR6+CXCR3-CCR4⁺ Th17-like cFth percentages above the 95th percentile of healthy controls. Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. ns = non-significant. D) Relative distribution of CCR6-CXCR3-CCR4⁺ Th2-like follicular helper CD4⁺ T-cells. E) DAS28 scores within longstanding RA patients undertaking csDMARD and bDMARD therapeutic regimens stratified according to the presence (HRA) or absence (LRA) of elevated CCR6-CXCR3-CCR4⁺ Th2-like cFth percentages akin to those observed with newly-diagnosed, treatment-naïve RA patients. Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. MTX = methotrexate, OCSD = other csDMARD, TNF = tumour necrosis factor alpha. * = $p < 0.05$, ns = non-significant. Dotted lines denotes the 5th and 95th percentile of healthy controls.

Further to the observed increase in CCR4⁺ Th17-like cFth, no significant difference in the relative or absolute counts of CCR4⁻ Th2-like cFth were observed between RA patients undertaking csDMARD and bDMARD therapeutic regimens. Split populations in the relative distribution of CCR4⁻ Th2-like cFth were, however, observed within RA patients receiving methotrexate, methotrexate substitutes and anti-TNF- α monoclonal therapies, with 13/24 (54.2%), 4/13 (30.8%) and 4/11 (36.4%) of RA patients demonstrating CCR4⁻ Th2-like cFth percentages akin to those observed within ERA patients (Figure 3.19D). When investigated further for differences in disease activity measures, however, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating CCR4⁻ Th2-like cFth percentages akin to those observed within ERA patients, as compared to RA patients presenting with CCR4⁻ Th2-like cFth percentages within the 95th percentile of HC, irrespective of their therapeutic regimen (Figure 3.19E and data not shown).

Altogether, these results suggest that the use of csDMARDs and bDMARDs, in particular the use of anti-TNF- α monoclonal antibodies, has a striking effect on the relative and absolute numbers of cFth and their subpopulations; with the previously observed increase in CCR4⁺ Th17-like cFth, within a proportion of RA patients, attributed to the use of anti-TNF- α monoclonal therapeutics. In contrast, however, no single therapeutic agent could be designated responsible for the split populations observed in the CCR4⁻ Th2-like cFth percentages of RA patients. Thus, this observation appears to be independent of the therapeutic interventions utilized within our RA cohort. Accordingly, despite these compelling observations, no significant differences in the disease activity measures ESR, CRP or DAS28 scores were observed in RA patients bearing elevations in CCR4⁺ Th17-like or CCR4⁻ Th2-like cFth, as compared to RA patients demonstrating CCR4⁺ Th17-like and CCR4⁻ Th2-like cFth within the 5th and 95th percentiles of HC, irrespective of the therapeutic regimen utilized. As such, taken together, these results would imply the presence of artefactual changes to CCR4⁺ Th17-like and CCR4⁻ Th2-like cFth, within a

proportion of RA patients, as a direct result of csDMARD and bDMARD therapeutic intervention.

3.3.e.ii. *Memory helper CD4⁺ T-cells* - In contrast to the results obtained within the follicular helper CD4⁺ T-cell compartment, no significant difference in the relative or absolute counts of CD45RO⁺CXCR5⁻ mTh, or indeed their composite subpopulations, when characterized according to the expression of CCR6, CXCR3 and CCR4, was observed within RA patients or DCG undertaking csDMARD or bDMARD therapeutic regimens (data not shown). With the full stratification of RA patients and DCG according to their primary therapeutic agent, no significant difference in the relative and absolute counts of CD45RO⁺CXCR5⁻ mTh was observed within RA patients or DCG receiving csDMARD therapeutic regimens and bDMARD-based therapeutics, including anti-TNF- α monoclonal therapies and rituximab. Accordingly, the use of rituximab in RA patients, therefore, appears to have a far more profound effect on the relative and absolute counts of cFth than their mTh counterparts. As such, the effects of rituximab on mTh subpopulation distributions was therefore included in all further investigations (Figure 3.20A - B).

When analysed further according to the expression of CCR6 and CXCR3, no significant difference in the relative distribution of Th17, Th1/17, Th2 or Th1 mTh was observed within RA patients or DCG when stratified according to their primary therapeutic agent. A trend towards an increase in the percentage of Th17 mTh was observed within RA patients undertaking anti-TNF- α monoclonal therapies, as compared to RA patients receiving methotrexate ($p=0.1498$), methotrexate substitutes ($p=0.0849$) and rituximab ($p=0.0777$), although these observations failed to reach statistical significance (Figure 3.20C). A significant increase in the absolute counts of Th17 mTh was, however, observed within RA patients, receiving anti-TNF- α monoclonal therapies, as compared to RA patients undertaking methotrexate-based therapeutic regimens ($p<0.05$) (Figure 3.20D).

Furthermore, a split population in the relative and absolute counts of Th17 mTh was evident within RA patients receiving anti-TNF- α monoclonal therapeutics, with 5/11 (45.5%) and 4/11 (36.4%) RA patients demonstrating Th17 mTh percentages and absolute counts above the 95th percentile of HC (Figure 3.20C - D).

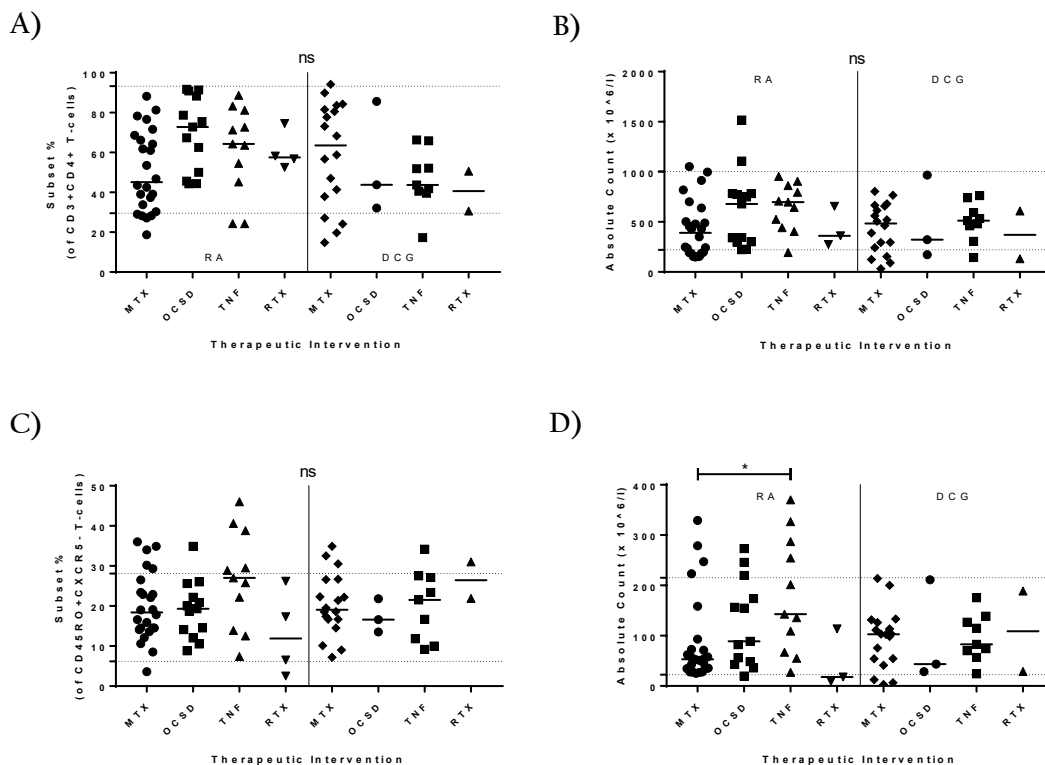


Figure 3.20:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon their use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens. A) Relative distribution of CD45RO+CXCR5⁻ memory helper CD4⁺ T-cells. B) Absolute counts of CD45RO+CXCR5⁻ memory helper CD4⁺ T-cells. C) Relative distribution of CCR6+CXCR3⁻ Th17 memory helper CD4⁺ T-cells. D) Absolute counts of CCR6+CXCR3⁻ Th17 memory helper CD4⁺ T-cells. MTX = methotrexate, OCSD = other csDMARD, TNF = tumour necrosis factor alpha, RTX = rituximab. Dotted lines denotes the 5th and 95th percentile of healthy controls. * = $p < 0.05$, ns = non-significant

With the addition of CCR4, no significant difference in the relative distribution of any mTh subpopulation was observed within RA patients or DCG when stratified according to their primary therapeutic agent. A trend towards an increase in the relative distribution of CCR4⁺ Th17 mTh was observed within RA patients undertaking anti-TNF- α therapeutic regimens, as compared to those receiving methotrexate ($p=0.1548$) and rituximab ($p=0.1040$), although these observations failed to reach statistical significance (Figure 3.21A). Despite the lack of statistical significances observed, 5/11 (45.5%) RA

patients receiving anti-TNF- α monoclonal therapeutics demonstrated CCR4⁺ Th17 mTh percentages above the 95th percentile of HC; a finding reiterated in the absolute counts of CCR4⁺ Th17 mTh observed within RA patients receiving anti-TNF- α therapeutics. Furthermore, a significant increase in the absolute counts of CCR4⁺ Th17 mTh was observed within RA patients receiving anti-TNF- α therapeutics, as compared to RA patients undertaking methotrexate-based therapeutic regimens ($p < 0.05$) (Figure 3.21A - B). However, when investigated further for associations with patient clinical outcomes, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating CCR4⁺ Th17 mTh percentages and absolute counts above the 95th percentile of HC and those demonstrating CCR4⁺ Th17 mTh percentages and absolute counts within the 95th percentile of HC (Figure 3.21C and data not shown). As such, in keeping with results obtained within the cFth compartment, the use of anti-TNF- α therapeutic agents demonstrates the most striking immunological effects on mTh subpopulations, with elevations in CCR4⁺ Th17 mTh, above the 95th percentile of HC, observed within a proportion of RA patients. However, when assessed further for differences in patient clinical outcome measures, no significant difference in ESR, CRP or DAS28 scores was observed in RA patients exhibiting elevations in CCR4⁺ Th17 mTh, as compared to RA patients demonstrating CCR4⁺ Th17 mTh within the 5th and 95th percentiles of HC. Thus, consistent with results obtained in the cFth compartment, elevations in CCR4⁺ Th17 mTh appear to represent a unique response to the utilization of anti-TNF- α monoclonal therapeutics; albeit with these changes seemingly unrelated to disease activity.

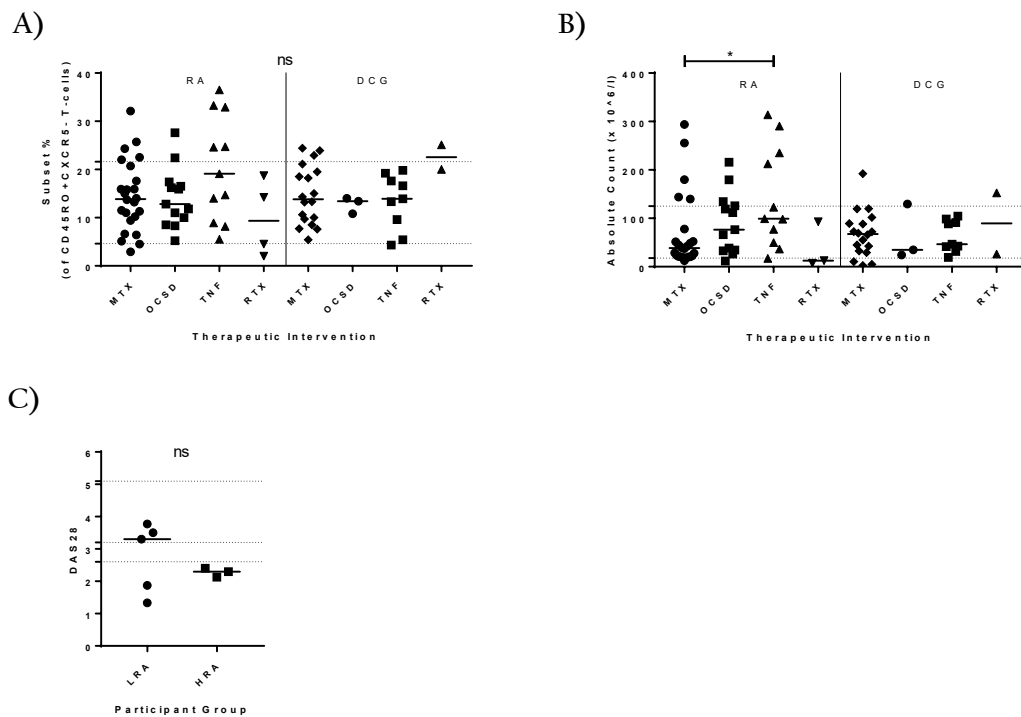


Figure 3.21:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon their use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens. A) Relative distribution of CCR6+CXCR3-CCR4+ Th17 memory helper CD4+ T-cells. B) Absolute counts of CCR6+CXCR3-CCR4+ Th17 memory helper CD4+ T-cells. Dotted lines denotes the 5th and 95th percentile of healthy controls. C) DAS28 scores within longstanding RA patients undertaking anti-TNF- α therapeutic regimens stratified according to the presence (HRA) or absence (LRA) of CCR6+CXCR3-CCR4+ Th17 memory helper CD4+ T-cell percentages above the 95th percentile of healthy controls. Dotted lines denote disease activity targets. <2.6 = remission; $2.6 - 3.2$ = low disease activity; $>3.2 - 5.1$ = moderate disease activity; >5.1 = high disease activity. ns = non-significant. MTX = methotrexate, OCSD = other csDMARD, TNF = tumour necrosis factor alpha, RTX = rituximab. * = $p < 0.05$, ns = non-significant.

3.3.f. The longitudinal monitoring of follicular and memory helper CD4+ T-cell subpopulation percentages may be used to assess therapeutic efficacy in newly-diagnosed RA patients following the initiation of csDMARD therapeutic regimens

3.3.f.i. Follicular helper CD4+ T-cells - With clear associations between patient clinical outcomes and follicular and memory helper CD4+ T-cell subpopulations observed within ERA patients at the time of diagnosis, combined with evidence for alterations to the relative and absolute numbers of cFth and mTh within RA patients following therapeutic stratification, the utility of measuring cFth and mTh parameters to monitor therapeutic efficacy was assessed. As such, the relative and absolute counts of cFth and mTh subpopulations were longitudinally monitored within ERA patients at 3-, 6- and 12-

monthly intervals, following their initiation of csDMARD therapeutic regimens. In conjunction with the monitoring of cFth and mTh subpopulations serial ESR, CRP and DAS28 scores were performed to serve as measures of disease activity. Following 12-months of compliance with a csDMARD therapeutic regimen, consisting of methotrexate, in combination with hydroxychloroquine and folic acid, a significant reduction in CRP and DAS28 scores was observed within ERA patients, as compared to the time of diagnosis ($p<0.01$ and $p<0.05$, respectively) (Figure 3.22A – B). Insufficient ESR measurements were available for assessment 12-months after diagnosis. Therefore, all subsequent comparisons between cFth and mTh subpopulations with the disease activity measure ESR were excluded. Median CRP and DAS28 scores within ERA patients 12-months after the initiation of csDMARD therapeutic regimens were 2.80mg/l (IQR = 4.40 – 42.9mg/l) and 2.40 (IQR = 1.74 – 3.45), respectively.

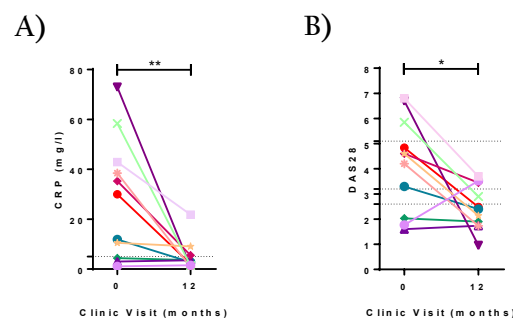


Figure 3.22:- Longitudinal monitoring of C-reactive protein (CRP) and DAS28 scores within newly-diagnosed, treatment-naïve RA (ERA) patients 12-months after the initiation of conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimens. A) CRP measurements (mg/l) at the time of diagnosis (0-months), as compared to 12-months after the initiation of csDMARD therapeutic regimens. Dotted line denotes disease activity target. $<5.0\text{mg/l}$ = remission. B) DAS28 scores at the time of diagnosis (0-months), as compared to 12-months after the initiation of csDMARD therapeutic regimens. Dotted lines denote disease activity targets. <2.6 = remission; $2.6 - 3.2$ = low disease activity; $>3.2 - 5.1$ = moderate disease activity; >5.1 = high disease activity. ** = $p<0.01$, * = $p<0.05$.

Within the follicular helper CD4⁺ T-cell compartment, no significant difference in the relative or absolute counts of CD45RO⁺CXCR5⁺ cFth was observed within ERA patients up to 12-months after the initiation of treatment (data not shown). When analysed further according to the expression of CCR6 and CXCR3 alone, a significant increase in the relative distribution of Th17-like cFth was observed 12-months after the initiation of

csDMARD therapeutic regimens, as compared to the time of diagnosis ($p<0.05$) (Figure 3.23A). These findings were not, however, replicated within the absolute counts of Th17-like cFth (data not shown). No further alterations to the relative or absolute counts of Th1/17-, Th2- or Th1-like cFth were observed up to 12-months post diagnosis (data not shown).

With the addition of CCR4, a significant increase in the relative and absolute counts of CCR4- Th17-like cFth was observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens ($p<0.05$, respectively) (Figure 3.23B and 3.24A). No such difference was, however, observed in the relative or absolute counts of CCR6+ Th17-like cFth. Nevertheless, consistent with the results obtained within longstanding RA patients, a split population in the relative distribution of CCR4+ Th17-like cFth was observed within ERA patients 12-month after diagnosis; a finding reiterated in the absolute counts of CCR4+ Th17-like cFth (Figure 3.23C and 3.24B). When investigated further for differences in patient clinical outcomes, a significant reduction in DAS28 scores was observed within ERA patients demonstrating a higher percentage of CCR4+ Th17-like cFth, as compared to ERA patients demonstrating a lower percentage of CCR4+ Th17-like cFth (median of ERA population demonstrating lower CCR4+ Th17-like cFth percentages = 9.8% [IQR = 9.0 – 10.4%]; median of ERA population demonstrating higher CCR4+ Th17-like cFth percentages = 18.6% [IQR = 16.2 – 20.2%]) ($p<0.05$) (Figure 3.23D). This finding was not replicated within the absolute counts of CCR4+ Th17-like cFth, or indeed, the relative and absolute counts of CCR4- Th17-like cFth (data not shown).

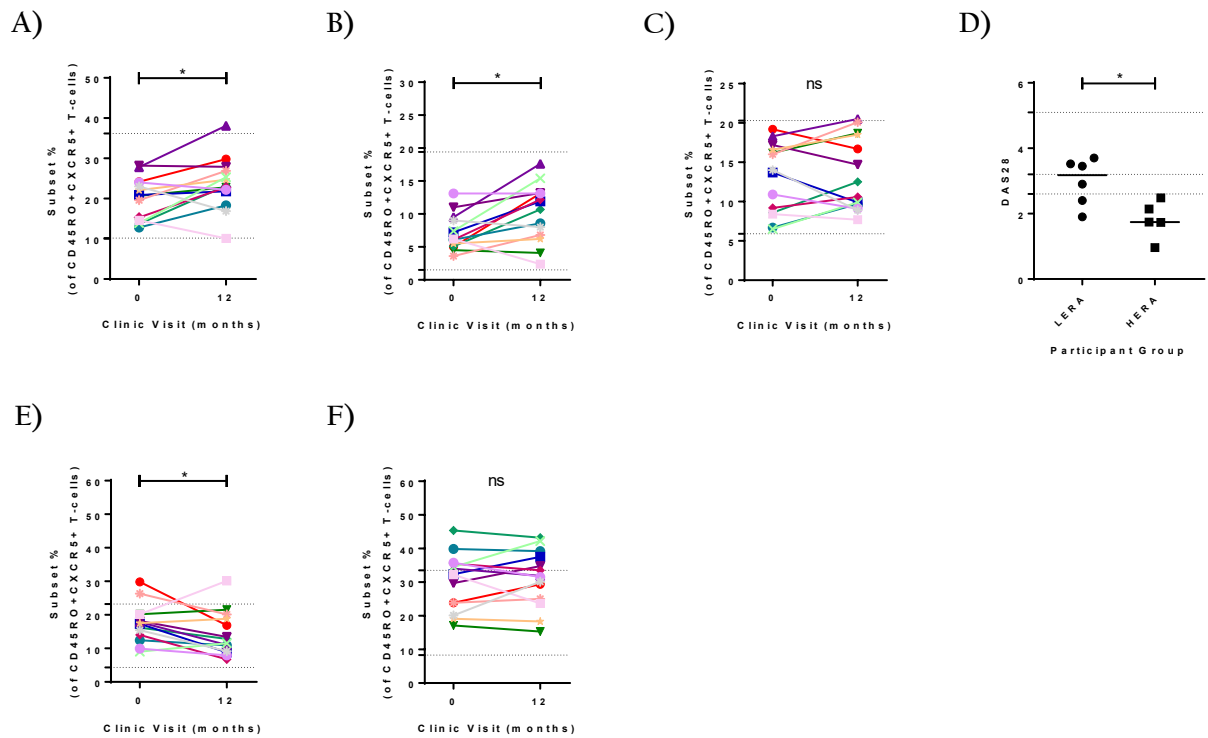


Figure 3.23:- Longitudinal monitoring of follicular helper CD4+ T-cell subpopulations within newly-diagnosed RA patients 12-months after the initiation of conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimens. A) Relative distribution of CCR6+CXCR3- Th17-like follicular helper CD4+ T-cells. B) Relative distribution of CCR6+CXCR3-CCR4- Th17-like follicular helper CD4+ T-cells. C) Relative distribution of CCR6+CXCR3-CCR4+ Th17-like follicular helper CD4+ T-cells. Dotted lines denotes the 5th and 95th percentile of healthy controls. D) DAS28 scores within newly-diagnosed RA patients 12-months after the initiation of csDMARD therapeutic regimens, stratified according to the presence of lower (LERA) or higher (HERA) CCR6+CXCR3-CCR4+ Th17-like follicular helper CD4+ T-cell percentages. Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. E) Relative distribution of CCR6-CXCR3-CCR4+ Th2-like follicular helper CD4+ T-cells. F) Relative distribution of CCR6-CXCR3-CCR4- Th2-like follicular helper CD4+ T-cells. ** = $p < 0.01$, * = $p < 0.05$, ns = non-significant.

Accompanying these alterations to Th17-like cFth subpopulations, a significant reduction in the relative distribution of CCR4+ Th2- like cFth was observed within ERA patients 12-months after the initiation of csDMARD therapeutic interventions, as compared to the time of diagnosis ($p < 0.05$) (Figure 3.23E). This reduction in CCR4+ Th2- cFth was not, however, replicated within the absolute counts of this cFth subpopulation (data not shown). Furthermore, despite evidence for alterations in the relative distribution of CCR4- Th2-like cFth within RA patients, no significant difference in the relative or absolute counts of CCR4- Th2-like cFth was noted within ERA patients up to 12-months after the initiation of csDMARD therapeutic interventions (Figure 3.23F and data not

shown). No further alterations to the relative or absolute counts of any cFth subpopulation were observed up to 12-months after the initiation of csDMARD therapeutic regimens (data not shown).

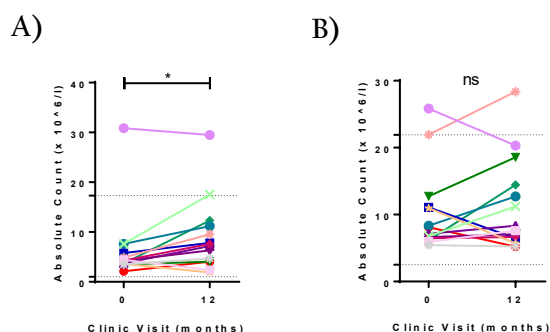


Figure 3.24:- Longitudinal monitoring of follicular helper CD4⁺ T-cell subpopulations within newly-diagnosed RA patients 12-months after the initiation of conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimens. A) Absolute counts of CCR6+CXCR3-CCR4⁻ Th17-like follicular helper CD4⁺ T-cells. B) Absolute counts of CCR6+CXCR3-CCR4⁺ Th17-like follicular helper CD4⁺ T-cells. * = $p < 0.05$, Dotted lines denotes the 5th and 95th percentile of healthy controls.

With evidence for alterations in Th17-like and, to a lesser extent Th2-like, cFth observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens, the relationship between these cFth subpopulations and disease activity was further explored; with the predictability of therapeutic efficacy, as a primary function of cFth characterization at diagnosis of particular interest. In this regard, a negative association between the relative distribution of Th17-like cFth and DAS28 scores was observed within ERA patients 12-months post-diagnosis ($r = -0.6636$; $p = 0.0306$) (Figure 3.25A). With the addition of CCR4, a negative association between the relative distribution of CCR4⁺ Th17-like cFth and DAS28 scores was observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens ($r = -0.7727$; $p = 0.0074$) (Figure 3.25B). When investigated further for changes in disease activity and the relative distribution of Th17-like cFth within ERA patients, following 12-months of therapeutic compliance, a positive association between the delta change in DAS28 scores and the delta change in Th17-like cFth percentages was observed; indicating that the greatest improvement in DAS28 scores is, in fact, associated with a decrease in Th17-like cFth ($r = 0.5273$, $p = 0.1231$) (Figure 3.25C).

This positive association was, however, abrogated with the addition of CCR4 ($r=0.3697$; $p=0.2957$); whilst this relationship remained unreplicable within the absolute counts of Th17-like and CCR4+ Th17-like cFth (data not shown). Furthermore, when the relative and absolute counts of Th17-like and CCR4+ Th17-like cFth within ERA patients at the time of diagnosis were compared to the delta change in disease activity measures 12-months after diagnosis, no relationships with the delta change in CRP or DAS28 scores were demonstrated (data not shown). Thus, although related to the change in disease activity in ERA patients 12-months after diagnosis, the monitoring of Th17-like cFth percentages and absolute counts cannot be used to predict therapeutic efficacy at the time of diagnosis.

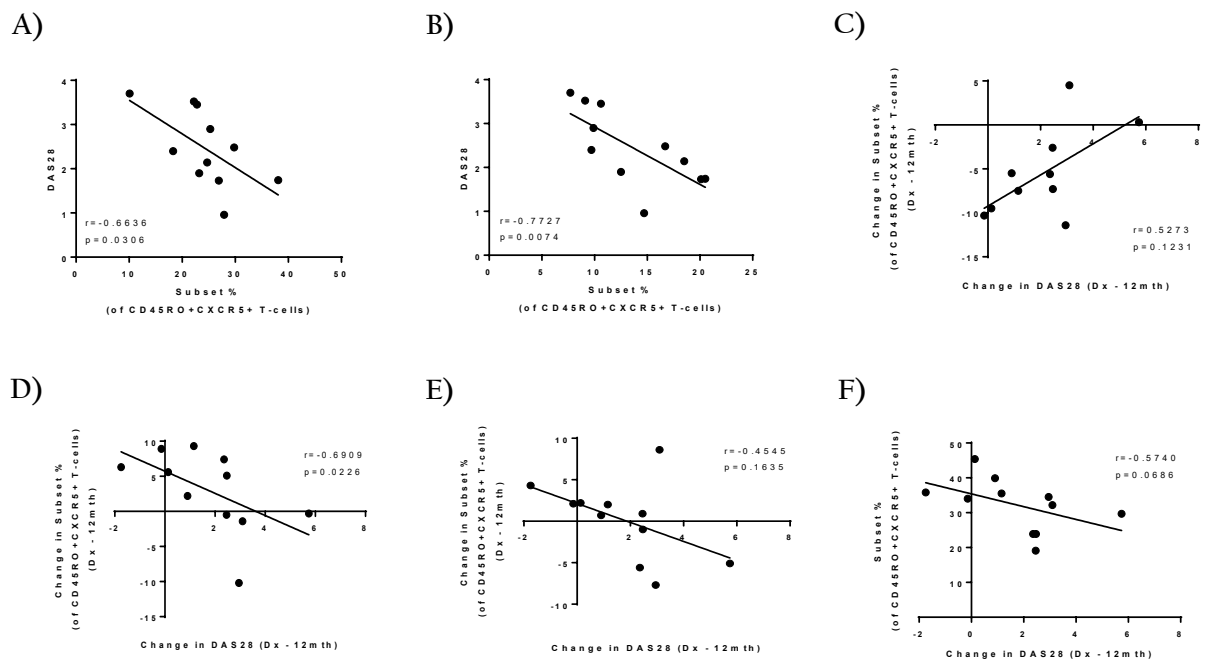


Figure 3.25:- Association of follicular helper CD4+ T-cell subpopulation percentages with DAS28 scores, within newly-diagnosed RA patients 12-months post diagnosis. A) Percentage of CCR6+CXCR3- Th17-like follicular helper CD4+ T-cells compared to DAS28 scores. B) Percentage of CCR6+CXCR3-CCR4+ Th17-like follicular helper CD4+ T-cells compared to DAS28 scores. C) Delta change in DAS28 scores compared to delta change in CCR6+CXCR3- Th17-like follicular helper CD4+ T-cells percentages. D) Delta change in DAS28 scores compared to delta change in CCR6-CXCR3- Th2-like follicular helper CD4+ T-cells percentages. E) Delta change in DAS28 scores compared to delta change in CCR6-CXCR3-CCR4- Th2-like follicular helper CD4+ T-cells percentages. F) Percentage of CCR6-CXCR3-CCR4- Th2-like follicular helper CD4+ T-cells at diagnosis compared to delta change in DAS28 scores. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables, with a p-value < 0.05 considered statistically significant.

No further associations were observed between patient clinical outcomes and the relative or absolute counts of any other cFth subpopulation up to 12-months after the initiation of csDMARD therapeutic regimens; with the previously observed negative association between the relative distribution of Th2-like and CCR4⁻ Th2-like cFth with DAS28 scores apparently abrogated within ERA patients upon the initiation of csDMARD therapeutic regimens. However, when investigated further for changes in disease activity, a negative association between the delta change in DAS28 scores and the delta change in Th2-like cFth percentages was observed ($r=-0.6909$; $p=0.0226$) (Figure 3.25D). This negative relationship was, however, partially abrogated between the delta change in DAS28 scores and the delta change in CCR4⁻ Th2-like cFth ($r=-0.4545$; $p=0.1635$) (Figure 3.25E). Crucially, when the relative distributions of Th2-like and CCR4⁻ Th2-like cFth, within ERA patients at the time of diagnosis, were compared to the delta change in disease activity measures 12-months after the initiation of csDMARD therapeutic regimens, a negative association between the relative distribution of CCR4⁻ Th2-like cFth and the delta change in DAS28 scores was observed ($r=-0.5740$; $p=0.0686$) (Figure 3.25F). As such, following 12-months of compliance with a csDMARD therapeutic regimen, a decrease in the relative distribution of Th17-like cFth is associated with the greatest improvement in ERA patient clinical outcome. In contrast, an increase in the relative distribution of Th2-like cFth was associated with the largest clinical improvement; with the relative distribution of CCR4⁻ Th2-like cFth within ERA patients at the time of diagnosis predictive of therapeutic efficacy.

3.3.f.ii. Memory helper CD4⁺ T-cells - Within the memory helper CD4⁺ T-cell compartment, a significant reduction in the relative and absolute counts of CD45RO⁺CXCR5⁻ mTh was observed within ERA patients 12-months after diagnosis, as compared to the time of diagnosis ($p<0.05$, respectively) (Figure 3.26A and 3.27A). With the addition of CCR6 and CXCR3 alone, no significant difference in the relative or absolute counts of Th17, Th1/17, Th2 or Th1 mTh was observed within ERA patients up to 12-months after the time of

diagnosis (data not shown). A split population in the relative distribution of Th17 mTh was, however, observed within ERA patients after 12-months of compliance with a csDMARD therapeutic regimen (Figure 3.26B). This observation was not, however, replicated within the absolute counts of Th17 mTh (data not shown).

With the addition of CCR4, no significant difference in the relative or absolute counts of CCR4+ Th17 mTh was observed within ERA patients up to 12-months after the initiation of csDMARD therapeutic regimens. A split population in the relative distribution of CCR4+ Th17 mTh was, however, observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens; a finding not echoed within the absolute counts of CCR4+ Th17 mTh (Figure 3.26C and data not shown). When investigated further for differences in patient clinical outcomes, a non-significant reduction in DAS28 scores was observed within ERA patients demonstrating a higher percentage of CCR4+ Th17 mTh, as compared to ERA patients demonstrating a lower percentage of CCR4+ Th17 mTh (median of ERA population demonstrating lower CCR4+ Th17 mTh percentages = 9.8% [IQR = 7.5 – 11.2%]; median of ERA population demonstrating higher CCR4+ Th17 mTh percentages = 17.2% [IQR = 13.9 – 18.8%]) (p=0.0848) (Figure 3.26D).

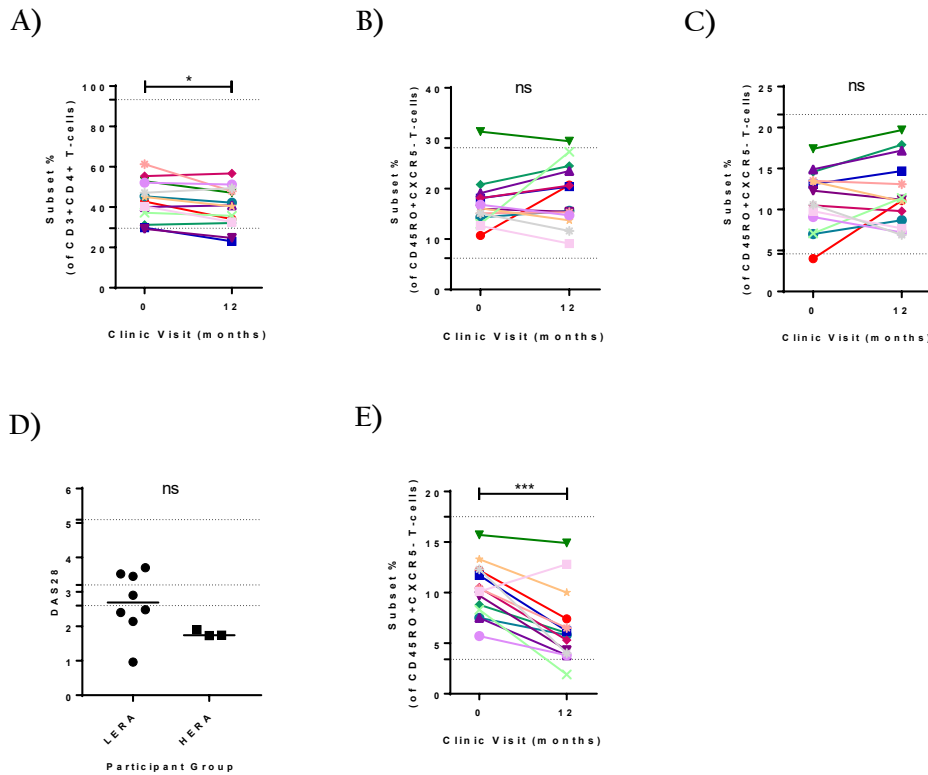


Figure 3.26:- Longitudinal monitoring of memory helper CD4+ T-cell subpopulations within newly-diagnosed RA patients 12-months after the initiation of conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimens. A) Relative distribution of CD45RO+CXCR5- memory helper CD4+ T-cells. B) Relative distribution of CCR6+CXCR3- Th17 memory helper CD4+ T-cells. C) Relative distribution of CCR6+CXCR3-CCR4+ Th17 memory helper CD4+ T-cells. D) DAS28 scores within newly-diagnosed RA patients 12-months after the initiation of csDMARD therapeutic regimens, stratified according to the presence of lower (LERA) or higher (HERA) CCR6+CXCR3-CCR4+ Th17 memory helper CD4+ T-cell percentages. Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. E) Relative distribution of CCR6-CXCR3+CCR4+ Th1 memory helper CD4+ T-cells. *** = $p < 0.001$, * = $p < 0.05$. Dotted lines denotes the 5th and 95th percentile of healthy controls.

Accompanying these alterations in CCR4+ Th17 mTh, a significant reduction in the relative and absolute counts of CCR4+ Th1 mTh was observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens ($p < 0.001$ and $p < 0.01$, respectively), as compared to the time of diagnosis; with 13/14 (92.9%) ERA patients demonstrating a reduction in CCR4+ Th1 mTh relative and absolute counts, signifying a global reduction in CCR4+ Th1 mTh with csDMARD therapeutic usage (Figure 3.26E and 3.27B). No further alterations to the relative or absolute counts of any mTh subpopulation were observed up to 12-months after the initiation of csDMARD therapeutic regimens (data not shown).

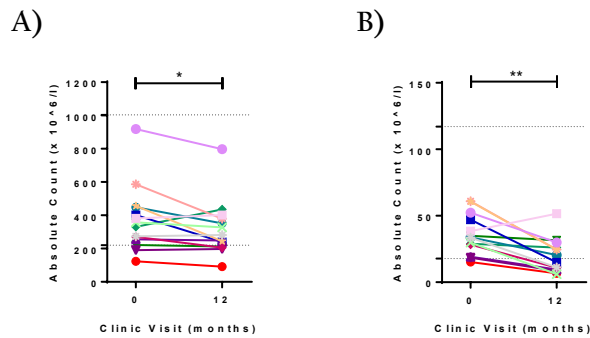


Figure 3.27:- Longitudinal monitoring of memory helper CD4+ T-cell subpopulation absolute counts within newly-diagnosed RA patients 12-months following the initiation of conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimens. A) Absolute counts of CD45RO+CXCR5- memory helper CD4+ T-cells. B) Absolute counts of CCR6-CXCR3+CCR4+ Th1 memory helper CD4+ T-cells. ** = $p < 0.01$, * = $p < 0.05$. Dotted lines denotes the 5th and 95th percentile of healthy controls.

With evidence for alterations in Th17 and Th1 mTh observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens, the relationship between these mTh subpopulations and disease activity was further explored. As such, contrary to the clinical associations observed with cTh subpopulations, no association between the relative distribution of Th17 mTh, CRP and DAS28 scores was observed within ERA patients up to 12-months after diagnosis; irrespective of CCR4 expression (data not shown). Furthermore, when investigated further for changes in disease activity and the relative distribution of Th17 mTh within ERA patients, following 12-months of compliance with a csDMARD therapeutic regimen, no association between the delta change in DAS28 scores and the delta change in Th17 mTh relative and absolute counts was observed, regardless of CCR4 expression (data not shown). Finally, despite evidence for a reduction in CCR4+ Th1 mTh 12-months after the initiation of a csDMARD therapeutic regimen, no associations between the relative distribution of Th1 mTh, CCR4+ Th1 mTh and the disease activity measures CRP or DAS28 scores were observed.

To summarize, 12-months after the time of diagnosis, an increase in the relative distribution of Th17 and CCR4+ Th17 mTh was observed within ERA patients. Accompanying this increase, a non-significant reduction in DAS28 scores was observed within ERA patients demonstrating an increase in CCR4+ Th17 mTh percentages, as

compared to ERA patients presenting with a lower percentage of CCR4+ Th17 mTh. Despite these compelling results, however, no associations between the change in the relative distribution of CCR4+ Th17 mTh and the change in CRP and DAS28 scores were observed. As such, following 12-months of compliance with a csDMARD therapeutic regimen, the relative counts of Th17 mTh are not predictive of therapeutic efficacy within ERA patients.

3.4. Discussion

Rheumatoid arthritis (RA) is a devastatingly progressive and destructive autoimmune disease, in which the actions of T⁺, B⁺ and antigen presenting⁺ (APC) cells have all been implicated in disease pathogenesis (McInnes and Schett, 2011; Sato et al., 2006; Snir et al., 2011). In recent years, a specialized subset of memory CD4⁺ T-cells, bearing the lymph node homing chemokine receptor CXCR5 have been linked to RA development, primarily due to the presence of autoantibodies directed against cyclic citrullinated peptides (CCP) within a proportion of RA patients (Suan et al., 2015; Turesson et al., 2007). The production of these autoantibodies, is dependent upon the proliferation, class switch recombination and affinity maturation of activated B-cells, directed against citrullinated peptides such as fibrinogen, vimentin and aggrecan, all of which are promoted by the activities of follicular helper CD4⁺ T-cells (Fth) (Cordova et al., 2013; Fazilleau et al., 2009; Shin et al., 2013; von Delwig et al., 2010). In 2011, Morita et al demonstrated the ability to subdivide circulating blood-derived follicular helper CD4⁺ T-cells (cFth) into Th1⁺, Th2⁺ and Th17-like subpopulations, based upon the expression of the chemokine receptors CCR6 and CXCR3, the expression of which is restricted to Th17 and Th1 cells, respectively (Dhalla et al., 2016; Morita et al., 2011). These cFth subpopulations have been shown to provide varying degrees of B-cell help, with the Th17-like subpopulation the most efficient at inducing B-cells to produce immunoglobulins of the IgG, IgA and IgM isotypes (Ma et al., 2015; Morita et al., 2011).

With Th17-like cFth the most relevant subpopulation to autoantibody production, a number of studies have demonstrated the skewed distribution of cFth subpopulations towards a Th17/Th2-like phenotype within newly-diagnosed, treatment-naïve RA patients (ERA) (Pandya et al., 2016). However, no such studies have sought to compare cFth, or indeed memory helper CD4⁺ T-cell (mTh) subpopulations within ERA patients to those of longstanding RA patients (RA), in which conventional synthetic and biological disease modifying anti-rheumatic drug (DMARD) therapeutic regimens have been previously

established. Herein, the relative and absolute counts of cFth and mTh within a cohort of 20 ERA patients were compared with those of 56 RA patients, as well as 32 age- and sex-matched disease (DCG) and healthy controls (HC), respectively; with the aim of determining the clinical utility of employing cFth and mTh assessments in therapeutic efficacy monitoring.

Within ERA patients, the cFth and mTh compartments were typified by an expansion of Th2-like/Th2 cFth and mTh; with a significant reduction in Th1/17-like/Th1/17 and Th1-like/Th1 cFth and mTh, also observed, as compared to RA patients, DCG and HC. With the addition of CCR4, a strategy which permits the identification of eight cFth and mTh subpopulations respectively, whilst also facilitating the further characterization of the previously designated Th2-like/Th2 cFth and mTh subpopulations, a significant increase in the relative distribution of both CCR4⁻ and CCR4⁺ Th2-like/Th2 cFth and mTh subpopulations was observed within ERA patients, as compared to RA patients, as well as DCG and HC.

A similar cFth and mTh subpopulation expression profile was also observed within RA patients. However, contrary to the relative and absolute counts of Th17-like/Th17 cFth and mTh observed within ERA patients, a non-significant increase in the relative distribution of CCR4⁺ Th17-like/Th17 cFth and mTh was also observed within 33.9% and 23.2% of RA patients, respectively; with no such alteration observed in their CCR4⁻ counterparts. The precise role of the chemokine receptor CCR4 in this demarcation remains to be fully elucidated. Historically, CCR4 has been considered a skin-homing and Th2-specific chemokine receptor, also permitting the entry of CD4⁺CD8⁺ double positive (DP) thymocytes into the medullary regions of the thymus during negative selection, by responding to the ligands CCL17 and CCL22 (Hu et al., 2015). However, a recent study, investigating the permissiveness of Th17 cells to HIV-1 infection, was able to demonstrate the differential production of interleukin-21 (IL-21) within CCR6⁺CCR4⁻ double negative

(DN) and CCR6⁺CCR4⁺ Th17 memory CD4⁺ T-cells; with CCR6⁺ DN T-cells proven to produce increased levels of IL-21 relative to their CCR4⁺ counterparts, implying increased follicular helper CD4⁺ T-cell function (Wacleche et al., 2016). If this dichotomy in IL-21 expression is also true of cFth subpopulations, it is possible that CCR4⁻ Th17-like cFth may possess greater B-cell helper functions compared to CCR4⁺ Th17-like cFth, and as such an increase in CCR4⁺ Th17-like cFth may disrupt naïve B-cell education, contributing to autoantibody production.

Transcription factor expression profiling performed within our HC cohort would certainly be consistent with the notion of differential subpopulation functional activity amongst cFth and mTh, and between T-cell compartments (Ma et al., 2015; Morita et al., 2011). Indeed, within the cFth compartment, the transcription factors T-bet and ROR γ t were more strongly expressed within CXCR3⁺CCR4⁺ and CCR6⁺CCR4⁺ cFth subpopulations, respectively; whilst within the mTh compartment T-bet and ROR γ t were more prolifically expressed within CCR6⁺CXCR3⁺CCR4⁻ Th1/17 and CCR6⁺CXCR4⁻ CCR4⁻ Th17 mTh subpopulations, respectively. Despite these compelling results, cytokine secretion assays are required to substantiate the cytokine production profiles of these cFth and mTh subpopulations, whilst co-culture experiments, incorporating individual cFth or mTh subpopulations, should be performed with naïve B-cell preparations to confirm the individual effects of cFth and mTh subpopulations on B-cell maturation and class switch recombination. Unfortunately, such experiments were beyond the scope of the present study.

Despite the need for future functional examinations, the distributional changes observed within the cFth and mTh compartments of ERA and RA patients do appear to bear some clinical significance. Within ERA patients a negative association between the relative and absolute counts of CCR4⁻ Th2-like cFth and DAS28 scores was observed, indicating a favourable clinical presentation within ERA in which Th2-like cFth are expanded.

Interestingly, however, this relationship appeared to be completely abrogated within RA patients, in which a therapeutic regimen was already established; a potential effect of DMARD utilization.

Although a similar mTh subpopulation expression profile was evident within ERA and RA patients, differential clinical associations were observed. As such, within the mTh compartment, negative associations between the relative and absolute counts of CCR4⁺ Th17 mTh and DAS28 scores were observed within ERA patients; whilst a positive correlation between the relative distribution of CCR4⁺ Th1 mTh and DAS28 scores was also observed. Altogether, these results would indicate a favourable clinical presentation within ERA patients in which increased Th17 mTh and reduced Th1 mTh are observed. As noted in the cFth compartment, however, this relationship was again disrupted within RA patients, further indicating a potential effect of conventional synthetic (csDMARD) and biological disease-modifying anti-rheumatic drug (bDMARD) therapeutic application.

With this in mind, the further stratification of RA patients according to their therapeutic regimens was performed, so as to assess the differential effects of csDMARD- and bDMARD-based therapeutic regimens on the cFth and mTh compartments, respectively. When stratified according to the utilization of csDMARD or bDMARD therapeutics, no significant difference in CRP, ESR or DAS28 scores was observed between RA patients undertaking csDMARD- or bDMARD-based therapeutic regimens, indicating that the disease activity of RA patients receiving csDMARD and bDMARD-based therapeutics are not intrinsically different once a therapeutic regimen has been successfully established.

Despite the lack of clinical differences, a number of alterations in the relative and absolute counts of cFth and mTh subpopulations were, however, observed. Within the cFth compartment, a trend towards an increase in the relative and absolute counts of CCR4⁺ Th17-like cFth was observed within RA patients undertaking bDMARD-based

therapeutic regimens, as compared to those receiving csDMARD therapeutics. Interestingly, split populations in the relative and absolute counts of CCR4⁺ Th17-like cFth were also observed within RA patients receiving bDMARDs, with 8/15 (53.3%) and 5/14 (35.7%) RA patients receiving bDMARDs demonstrating CCR4⁺ Th17-like cFth percentages and absolute counts above the 95th percentile of HC, respectively. When investigated further for differences in disease activity measures, however, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating elevated CCR4⁺ Th17-like cFth and those demonstrating CCR4⁺ Th17-like cFth within the 95th percentile of HC.

With the stratification of RA patients according to their primary therapeutic agent, a significant increase in the absolute counts of CCR4⁺ Th17-like cFth, above the 95th percentile of HC, was observed within RA patients undertaking anti-TNF- α therapeutic regimens, as compared to RA patients receiving methotrexate and methotrexate substitutes. A split population in the relative distribution of CCR4⁺ Th17-like cFth was also observed within RA patients undertaking anti-TNF- α therapeutic regimens, as compared to RA patients receiving methotrexate and methotrexate substitutes, with 6/11 (54.5%) RA patients receiving anti-TNF- α therapeutics, demonstrating CCR4⁺ Th17-like cFth percentages above the 95th percentile of HC. These results are indicative a unique cFth response to anti-TNF- α therapeutics in a proportion of RA patients. However, despite these dramatic alterations to Th17-like cFth, no significant difference in CRP, ESR or DAS28 scores was observed between RA patients demonstrating elevated CCR4⁺ Th17-like cFth and those demonstrating CCR4⁺ Th17-like cFth within the 95th percentile of HC, indicating that the observed increase in Th17-like cFth within a proportion of RA patients receiving anti-TNF- α therapeutics is, perhaps, an artefactual change, rather than a predictor of therapeutic efficacy.

Further to the alterations observed in CCR4⁺ Th17-like cFth, split populations in the relative distribution of CCR4⁺ Th2-like cFth were also observed within RA patients undertaking csDMARD and bDMARD therapeutic regimens, with 17/37 (45.9%) and 6/15 (40.0%) RA patients receiving csDMARDs and bDMARDs, respectively demonstrating CCR4⁺ Th2-like cFth percentages akin to those previously observed within ERA patients. These split populations remained evident following the further stratification of RA patients according to their primary therapeutic agent, with 13/24 (54.2%), 4/13 (30.8%) and 4/11 (36.4%) RA patients receiving methotrexate, methotrexate substitutes and anti-TNF- α monoclonal therapies, respectively demonstrating CCR4⁺ Th2-like cFth percentages akin to those observed within ERA patients. Despite these apparent alterations, however, when investigated further for differences in disease activity measures no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating CCR4⁺ Th2-like cFth percentages akin to those observed within ERA patients, as compared to RA patients presenting with CCR4⁺ Th2-like cFth percentages within the 95th percentile of HC, irrespective of their therapeutic regimen; reiterating the notion that csDMARD and bDMARD therapeutic regimens are not intrinsically different in their ability to induce a clinical improvement.

Such findings were reiterated in the mTh compartment in which the effects of csDMARD and bDMARD therapeutics appeared far subtler than those generated within the cFth compartment. Thus, no significant difference in the relative or absolute counts of any mTh subpopulation were observed within RA patients when stratified according to the use of csDMARD or bDMARD therapeutic regimens. With the full stratification of RA patients according to their primary therapeutic agent, no significant difference in the relative or absolute counts of CD45RO⁺CXCR5⁺ mTh was observed in RA receiving methotrexate, methotrexate substitutes, anti-TNF- α monoclonal therapeutics or rituximab. In isolation, these results suggest that the use of the B-cell depleting monoclonal agent, rituximab, has a far more profound effect on the cFth compartment than their mTh counterparts. The

literature surrounding the long-term effects of rituximab on the cFth compartment remains, however, somewhat ambiguous, particularly within the setting of RA. As such, these observations remain unconfirmed (Audia et al., 2017; Verstappen et al., 2017).

Despite the lack of changes to the relative and absolute counts of mTh as a whole, the full stratification of RA patients according to their primary therapeutic agent, revealed a trend towards an increase in the relative distribution CCR4⁺ Th17 mTh, above the 95th percentile of HC, within RA patients receiving anti-TNF- α monoclonal therapeutics, as compared to those undertaking methotrexate and rituximab; with a significant increase in CCR4⁺ Th17 mTh absolute counts also observed within RA patients undertaking anti-TNF- α monoclonal therapies, as compared to those receiving methotrexate. However, consistent with results obtained in the cFth compartment, when investigated further for associations with patient clinical outcomes, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating CCR4⁺ Th17 mTh percentages and absolute counts above the 95th percentile of HC and those demonstrating CCR4⁺ Th17 mTh percentages and absolute counts within the 95th percentile of HC.

All things considered, the use of anti-TNF- α monoclonal therapeutics appears to have a profound effect on the relative and absolute counts of cFth, mTh and their subpopulations, in particular Th17-like/Th17 cFth and mTh. The precise relationship between Th17-like cFth, Th17 mTh and anti-TNF- α therapeutics within the setting of RA is an area of intense debate, with TNF- α shown to promote the differentiation of Th17 cells, via the induction of IL-1 β and IL-6 production by monocytes within the synovium of RA (Andersson et al., 2015; Zheng et al., 2014). In turn, the production of IL-17 by activated Th17 cells is thought to promote joint inflammation by acting synergistically with TNF- α and IL-1 β to promote osteoclastogenesis and cartilage destruction (Chen et al., 2011; Zheng et al., 2014). In this regard, the use of anti-TNF- α monoclonal agents is thought to reduce the production of IL-17A, IL-17F and IL-22 by Th17 T-cells via the downregulation of p38-MAPK, ERK-

MAPK and STAT3 phosphorylation; molecules which mediate cytokine production by operating downstream of the T-cell receptor (TCR) and toll-like receptors (TLRs) of mature CD4⁺ T-cells and APCs, respectively (Lin et al., 2017). As such, a sustained increase in circulating Th17 cells has previously been reported in RA patients following an incomplete response to TNF- α blockade; results somewhat at odds with the negative association between DAS28 scores and Th17-like cFth and mTh observed within the ERA patients of the present study (Alzabin et al., 2012; Chen et al., 2011).

In order to further substantiate these findings, whilst also assessing the utility of employing cFth and mTh subpopulation measurements in therapeutic efficacy monitoring, the relative and absolute counts of cFth and mTh subpopulations were longitudinally monitored within ERA patients up to 12-months after the initiation of csDMARD therapeutic regimens. Within the cFth compartment, a significant increase in CCR4⁺ Th17-like cFth percentages was observed in ERA patients 12-months after diagnosis, with no such alteration to the relative or absolute counts of CCR4⁺ Th17-like cFth observed. Interestingly, however, consistent with the results obtained within RA patients, a split population in the relative distribution of CCR6⁺ Th17-like cFth was observed within ERA patients 12-month after diagnosis; a finding reiterated in the absolute counts of CCR4⁺ Th17-like cFth. Nonetheless, when investigated further for differences in patient clinical outcomes, a significant reduction in DAS28 scores was observed within ERA patients demonstrating a higher percentage of CCR4⁺ Th17-like cFth, as compared to ERA patients demonstrating a lower percentage of CCR4⁺ Th17-like cFth, indicating that an increase CCR4⁺ Th17-like cFth is associated with a favourable clinical outcome. In further support of these findings a negative association between the relative distribution of CCR4⁺ Th17-like cFth and DAS28 scores was also observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens.

In direct contradiction with these findings, however, a positive association between the delta change in Th17-like cFth percentages and the delta change in DAS28 scores was also observed within ERA patients 12-months after diagnosis; curiously signifying that the greatest improvement in DAS28 scores over 12-months was, in fact, associated with a decrease in Th17-like cFth. Despite these compelling results, the percentage of Th17-like cFth within ERA at diagnosis proved not to be predictive of the delta change in DAS28 scores 12-months after the initiation of csDMARD therapeutic regimens. Altogether, these results would therefore suggest that the measurement of Th17-like cFth cannot be used to predict therapeutic efficacy, but may still prove beneficial in the longitudinal monitoring of therapeutic response, particularly in cases where routine ESR, CRP and DAS28 scores have not been possible.

Accompanying these changes in Th17-like cFth, a significant reduction in the relative distribution of CCR4⁺ Th2-like cFth was also observed within ERA patients 12-months after diagnosis. This reduction in CCR4⁺ Th2⁻ cFth was not, however, replicated within the absolute counts of this cFth subpopulation. Furthermore, despite evidence for significant alterations in the relative distribution of CCR4⁻ Th2-like cFth within RA patients, no significant difference in the relative or absolute counts of CCR4⁻ Th2-like cFth was noted within ERA patients up to 12-months after the initiation of csDMARD therapeutic interventions. Moreover, the previously observed negative association between the relative distribution of Th2-like and CCR4⁻ Th2-like cFth with DAS28 scores appeared completely abrogated within ERA patients following the initiation of csDMARD therapeutic regimens.

When investigated further for changes in disease activity, however, a negative association between the delta change in DAS28 scores and the delta change in Th2-like cFth percentages was apparent within ERA patients 12-months after diagnosis; although this relationship was partially abrogated between the delta change in DAS28 scores and the

delta change in CCR4⁺ Th2-like cFth. Crucially, however, when the relative distribution of CCR4⁺ Th2-like cFth, within ERA patients at the time of diagnosis, was compared to the delta change in disease activity measures 12-months after the initiation of csDMARD therapeutic regimens, a negative association between the relative distribution of CCR4⁺ Th2-like cFth and the delta change in DAS28 scores was observed, indicating that the greatest improvement in disease activity was observed within ERA patients presenting with a lower percentage of Th2-like cFth at the time of diagnosis. As such, changes to Th2-like cFth percentages appear to not only correlate with changes in disease activity, but their measurement within ERA patients at the time of diagnosis also appear to be predictive of therapeutic efficacy, prior to the initiation of csDMARD therapeutic regimens; a biomarker which could prove invaluable in the selection of patient therapeutic regimens.

Within the mTh compartment, no significant difference in the relative or absolute counts of Th17 or CCR4⁺ Th17 mTh was observed within ERA patients up to 12-months after the initiation of csDMARD therapeutic regimens. In a similar vein to results obtained within the cFth compartment, however, a split population in the relative distribution of CCR4⁺ Th17 mTh was observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens; a finding unsubstantiated by the absolute counts of CCR4⁺ Th17 mTh. Nevertheless, when investigated for differences in patient clinical outcomes, a non-significant reduction in DAS28 scores was observed within ERA patients demonstrating a higher percentage of CCR4⁺ Th17 mTh, as compared to ERA patients demonstrating a lower percentage of CCR4⁺ Th17 mTh, indicating that an increase in CCR4⁺ Th17 mTh is associated with a favourable clinical outcome.

Subsequent investigations for associations with patient clinical outcomes were unable, however to substantiate this notion, with no association between the relative distribution of CCR4⁺ Th17 mTh and DAS28 scores observed within ERA patients 12-months after

diagnosis. Additionally, when investigated further for changes in disease activity and the relative and absolute counts of Th17 mTh within ERA patients, following 12-months of compliance with a csDMARD therapeutic regimen, no association between the delta change in DAS28 scores and the delta change in CCR4+ Th17 mTh relative and absolute counts was observed. Furthermore, akin to results obtained within the cFth compartment, when the relative counts of Th17 and CCR4+ Th17 mTh within ERA patients at the time of diagnosis were compared to the delta change in disease activity measures 12-months after the initiation of csDMARD therapeutic regimens, no relationships with the delta change in CRP or DAS28 scores were demonstrated, signifying that the measurement of Th17 mTh cannot be used as a predictor of therapeutic efficacy.

With a similar subpopulation expression profile observed within both the cFth and mTh compartments of ERA patients, with analogous clinical associations observed within both ERA and RA cohorts, the results presented herein are compelling. With all ERA patients placed on a csDMARD therapeutic regimen consisting of methotrexate, hydroxychloroquine and folic acid, the application of DMARD therapeutic regimens does indeed appear to be responsible for the differential cFth and mTh subpopulation expression profiles observed within RA patients, as compared to ERA patients prior to the initiation of csDMARD therapeutic regimen. In particular, the observed increase in Th17-like cFth and Th17 mTh is seemingly a unique response to the utilization of anti-TNF- α monoclonal therapeutics. Unlike previous studies, however, the data presented herein would suggest that the observed increase in Th17-like cFth and Th17 mTh still represents a favourable clinical response to therapeutic implementation. With limited clinical outcome measures available, however, for our RA cohort, it was unfortunately unfeasible to definitively determine whether this increase in Th17-like cFth and Th17 mTh is indeed associated with a long-term favourable clinical outcome, or is in fact merely a non-specific by-product of anti-TNF- α monoclonal therapeutic utilization. Furthermore, to our

knowledge, the relationship between Th2-like cFth, RA pathogenesis and the longitudinal monitoring of Th2-like cFth in therapeutic efficacy monitoring has not been definitively described elsewhere.

With these questions remaining, the direct comparison of cFth and mTh subpopulation expression profiles with their synovial counterparts is a potential area for future investigations, particularly in the light of the present findings. Indeed, Th17 cells, and to a lesser extent Th2 cells, have been previously implicated in RA pathogenesis, as well as a number of other autoimmune diseases, with raised numbers of Th17 cells previously documented in active sjögren's syndrome, ankylosing spondylitis and rheumatoid arthritis (Li et al., 2012; Pandya et al., 2016; Shen, Goodall and Hill Gaston, 2009). With no such increase in CCR4⁺ Th17-like cFth and Th17 mTh observed within ERA patients at the time of diagnosis, it is plausible for such cells to have been directed to the joints at disease onset, where they contribute to the development of ectopic lymphoid structures (ELS) (Bombardieri, Lewis and Pitzalis, 2017; Humby et al., 2009; Thurlings et al., 2008). In such a scenario, CCR4⁺ Th17-like cFth and Th17 mTh may remain sequestered away within the joints of RA patients during active disease, actively contributing to joint inflammation and destruction, until such a time as an effective csDMARD or bDMARD therapeutic regimen can be established, upon which these cells may be redirected back into the circulation; a favourable response represented in our cohort by a negative association between the relative distribution of CCR4⁺ Th17-like cFth and Th17 mTh with DAS28 scores.

The presence of CCR4⁻ Th2-like cFth within the periphery of ERA and RA patients may be interpreted in a similar fashion. With the greatest improvement in clinical course observed within ERA patients in which an increase in CCR4⁻ Th2-like cFth was observed; it can be postulated that the successful establishment of a csDMARD therapeutic regimen results in the egress of CCR4⁻ Th2-like cFth from the synovium of RA patients back into the periphery. Under such circumstances, the presence of increased CCR4⁻ Th2-like cFth

percentages within ERA patients, following the implementation of csDMARD therapeutic regimens may in fact delineate a group of patients in which a divergent disease pathogenesis is at work; with Th2-like cFth the dominant pathogenic CD4⁺ T-cell; a hypothesis which has been presented by other groups (Pandya et al., 2017; Pandya et al., 2016). As such, the further examination of paired synovial fluid and peripheral blood samples from ERA and RA patients, with active disease, should be considered in future studies, so as to extrapolate when and why these phenomena occur. Such investigations would also determine whether the observed cFth and mTh repertoire within the periphery is truly reflective of those populations within ELS, or merely remnants following pathogenic CD4⁺ T-cell migration to the synovium, further aiding our understanding RA pathogenesis.

In conclusion, consistent with previous studies, the cFth and mTh compartments of ERA patients are indeed skewed towards a Th2-like/Th2 phenotype, with the relative distribution of Th2-like cFth indicative of disease severity (Pandya et al., 2016). With the initiation of csDMARD and bDMARD therapeutic regimens, the relative and absolute counts of cFth and mTh subpopulations are altered, with an increase in Th17-like cFth and Th17 mTh observed, particularly following the utilization of anti-TNF- α monoclonal therapeutics (Alzabin et al., 2012; Chen et al., 2011). Critically, changes in the relative distribution of Th17-like cFth and Th17 mTh are related to changes to disease activity, providing a further means of longitudinally assessing therapeutic efficacy, once a therapeutic regimen has been established. Of yet further importance is the discovery that the relative distribution of Th2-like cFth within ERA patients at the time of diagnosis is highly predictive of therapeutic efficacy. As such, the continued monitoring of Th2-like cFth, as well as Th17-like cFth and Th17 mTh percentages may be highly advantageous, particularly in cases where the standard disease activity measures ESR, CRP and DAS28 scores have either become dissociated with disease activity, or their measurement was

simply not feasible; a conclusion which should be substantiated in future studies (Kay et al., 2014).

Chapter 4: CMV-specific CD25⁺CD134⁺ activated CD4⁺ T-cell responses are predictive of disease severity within rheumatoid arthritis

4.1. Introduction

4.1.a. The Role of Antigen-specific T-cell Responses in the Healthy Immune System

A critical component of any adaptive immune response is the ability to generate immunological memory; a state in which antigen-experienced T- and B- lymphocytes are able to rapidly reactivate and expand upon re-exposure to their predisposing antigenic targets. This pivotal constituent of adaptive immunity ensures the production of antigen-specific secondary immune responses that possess greater efficiency and potency than their primary immunological counterparts, thereby safeguarding against future antigenic challenge (Kallies, 2008). Such cellular processes, underpinning the basis for vaccination technology and infection control, are fashioned during pathogen-induced primary immune responses, with memory T-cells typically generated during the contraction phase of a primary immune response (Bautista et al., 2016; Kallies, 2008; Kim et al., 2013). During this maturation stage, the majority of previously activated effector T-cells are deleted via apoptosis, leaving a small, but stable pool of circulating memory T-cells that contribute to continued immune surveillance against the offending pathogen and may be rapidly reactivated upon pathogen re-exposure (Bautista et al., 2016; Kallies, 2008; Kim et al., 2013).

The generation and maintenance of memory CD4⁺ T-cells is a multi-faceted, yet poorly understood process, in which the repeated exposure of effector CD4⁺ T-cells to their cognate antigen leads to the development of two functionally distinct subsets of memory CD4⁺ T-cells, delineated by their expression of the chemokine receptor C-C chemokine receptor 7 (CCR7) and the adhesion molecule L-selectin (CD62L) (Figure 4.1) (Bautista et

al., 2016; Sallusto et al., 1999). Effector memory CD4⁺ T-cells, identified by their lack of CCR7 expression, in combination with heterogeneous levels of CD62L, are characterized by their rapid production of the pro-inflammatory cytokines interferon- γ (IFN- γ), interleukin-4 (IL-4) and interleukin-5 (IL-5), within a few hours of reactivation. In addition to their release of pro-inflammatory cytokines, effector memory CD4⁺ T-cells also possess the ability to migrate to non-lymphoid sites of inflammation, based upon their expression of tissue-homing chemokine receptors, including CXCR3 and CCR6 (Pepper and Jenkins, 2011; Sallusto et al., 1999). In contrast, central memory CD4⁺ T-cells, identified as CCR7⁺CD62L⁺, produce large amounts of the cytokine interleukin-2 (IL-2), a potent T-cell survival factor, which promotes the widespread proliferation of memory CD4⁺ T-cells following reactivation. Their continued expression of CD62L and CCR7 also ensures their retained ability to migrate between secondary lymphoid organs, whilst they also remain capable of acquiring effector memory T-cell functions at a later stage (Pepper and Jenkins, 2011; Sallusto et al., 1999). As such, these two subsets of memory CD4⁺ T-cells occupy differing immunological niches, with effector memory CD4⁺ T-cells representing terminally-differentiated cells that contribute immediate protection following pathogen re-exposure; whilst their central memory counterparts provide reactive protection, via their activation of antigen-presenting dendritic cells and their provision of cytotoxic CD8⁺ T-cell and B-cell help (Figure 4.1) (Lanzavecchia and Sallusto, 2000; Sallusto, Geginat and Lanzavecchia, 2004).

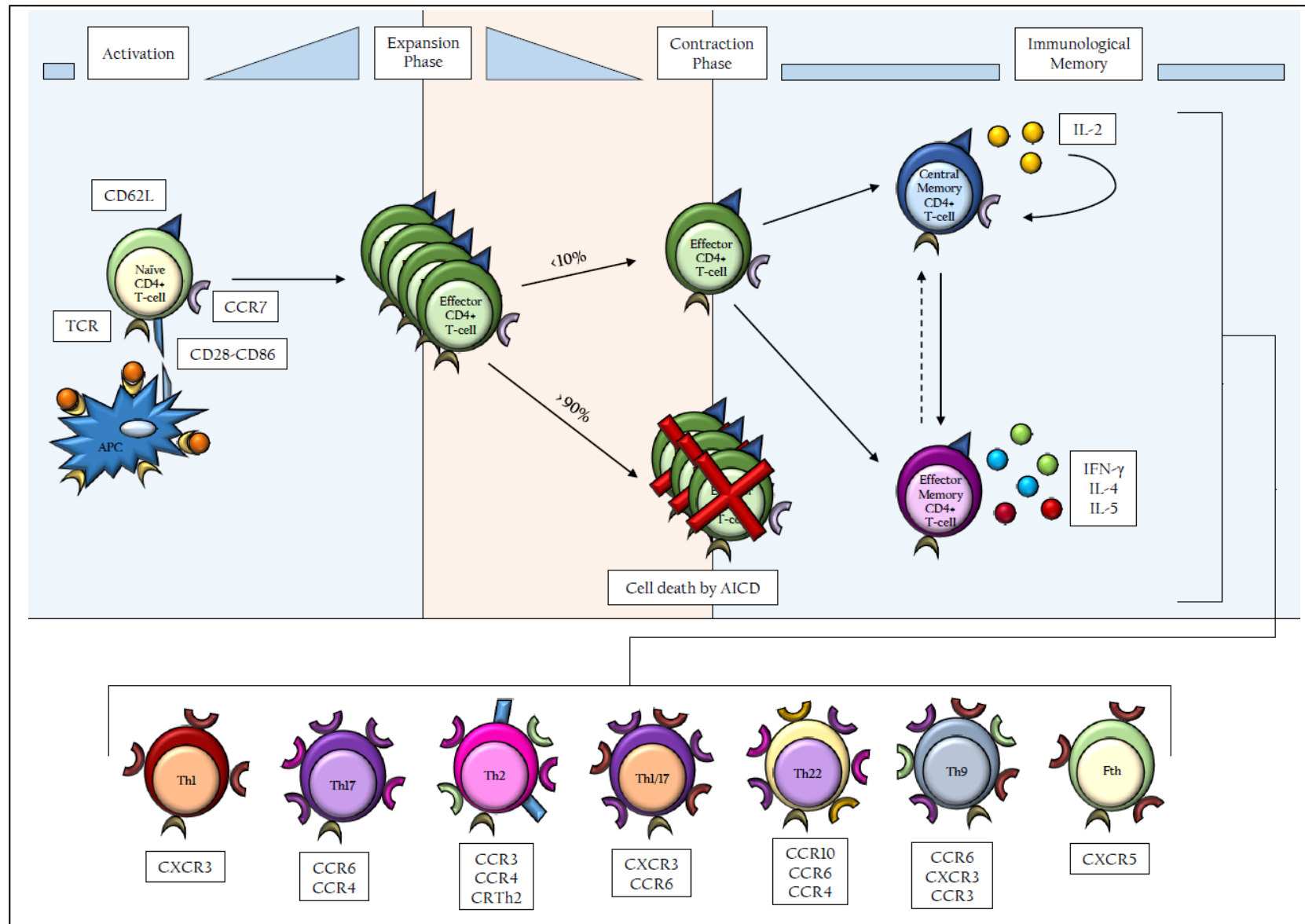


Figure 4.1:- Proposed model for effector memory and central memory CD4⁺ T-cell generation, as well as the memory helper T-cell subpopulations currently characterized. AICD = Activation induced cell death; TCR = T-cell receptor. Adapted from Sallusto et al (2004).

Supplementary to their categorization according to CCR7 and CD62L, effector memory and central memory CD4⁺ T-cells may be further subdivided into functionally distinct helper T-cell subpopulations, based upon their transcription factor, cytokine and chemokine receptor expression profiles. To date, Th1, Th2, Th17, Th22, Th9, Th1/17 and follicular helper T-cells have all been described, with each subpopulation thought to possess unique helper T-cell functions that differentially contribute to adaptive immunity (Table 4.1) (Acosta-Rodriguez et al., 2007; Kara et al., 2013; Morita et al., 2011; Okada et al., 2015; Plank et al., 2017; Sallusto et al., 1998). For example, Th1 memory CD4⁺ T-cells, identified by their expression of the chemokine receptor CXCR3, in the absence of CCR6, are characterized by their expression of the transcription factor T-bet, as well as their production of the pro-inflammatory cytokines IL-2 and IFN- γ . As such, this T-cell subset is typically involved in defence against intracellular pathogens and viral agents, aiding cytotoxic CD8⁺ T-cell and macrophage function (Kanhare et al., 2012; Sallusto et al., 1998). In contrast, Th17 memory CD4⁺ T-cells, distinguished by their expression of CCR6, in the absence of CXCR3, as well as their expression of the transcription factor ROR γ t and the cytokines interleukin-17 (IL-17), interleukin-21 (IL-21) and interleukin-22 (IL-22), are typically involved in anti-fungal immunity and defence against extracellular pathogens (Acosta-Rodriguez et al., 2007; Dhalla et al., 2016). As such, each infectious agent activates a unique profile of memory CD4⁺ T-cells that are ideally suited to contain and limit the spread of the offending pathogen (Table 4.1).

Memory T-cell Subpopulation	Master Transcription Factor	Chemokine Receptors and Cell Surface Molecules	Cytokines	Role in Adaptive Immunity
Th1	T-bet (Kanhare et al., 2012)	CXCR3 (Sallusto et al., 1998)	IL-2 IFN- γ (Ma et al., 2016; Sallusto et al., 1998)	Mediate defence against intracellular pathogens and viral agents, aiding cytotoxic CD8 ⁺ T-cell and macrophage function (Ma et al., 2016; Sallusto et al., 1998).
Th2	GATA-3 (Kanhare et al., 2012)	CCR3 CCR4 CRTh2 (Acosta-Rodriguez et al., 2007)	IL-4 IL-5 IL-13 (Kanhare et al., 2012)	Provide protective immunity against parasitic infections, via the direct actions of IL-4, IL-5 and IL-13, combined with the activation of eosinophils and basophils (Guo et al., 2015; Sallusto et al., 1998).
Th17	ROR γ t (Acosta-Rodriguez et al., 2007)	CCR6 CCR4 (Acosta-Rodriguez et al., 2007; Dhalla et al., 2016)	IL-17A IL-17F IL-21 IL-22 (Plank et al., 2017)	Mediate anti-fungal immunity, against pathogens such candida albicans, and defence against other extracellular pathogens, via the recruitment and activation of neutrophils (Acosta-Rodriguez et al., 2007; Dhalla et al., 2016; Okada et al., 2015).
Th22	BNC-2 FOXO4 (Eyerich et al., 2009)	CCR10 CCR6 CCR4 (Duhén et al., 2009; Eyerich et al., 2009)	IL-22 TNF- α (Eyerich et al., 2009; Plank et al., 2017)	Thought to contribute to immune surveillance of the skin, by inducing the production of the T-cell and NK-cell promoting factors, IL-15 and IL-7, from keratinocytes (Duhén et al., 2009; Eyerich et al., 2009).
Th9	PU.1 (Gerlach et al., 2014)	CCR6 CXCR3 CCR3 (Kara et al., 2013)	IL-9 (Kara et al., 2013)	Precise functional significance is yet to be determined, but it is hypothesized that Th9 cells initiate immunity against parasitic infections; whilst also contributing to anti-tumour immunity (Kaplan, Hufford and Olson, 2015; Kara et al., 2013).
Th1/17	T-bet ROR γ t (Acosta-Rodriguez et al., 2007)	CCR6 CXCR3 (Acosta-Rodriguez et al., 2007; Okada et al., 2015)	IFN- γ IL-17A IL-17F (Okada et al., 2015)	Provide protective immunity against intracellular pathogens, including mycobacterial disease, via their production of IFN- γ and their subsequent enhancement of macrophage function. Also provide anti-fungal immunity, against pathogens such as candida albicans, via their simultaneous production of IL-17A and IL-17F, and their recruitment and activation of neutrophils (Acosta-Rodriguez et al., 2007; Okada et al., 2015).
Follicular helper T-cells	BCL-6 (Ma et al., 2015; Morita et al., 2011)	CXCR5 (Chevalier et al., 2011; Ma et al., 2015)	IL-4 IL-10 IL-21 (Morita et al., 2011)	Promote the proliferation, class switch recombination, somatic hypermutation and affinity maturation of activated B-cells (Chevalier et al., 2011; Morita et al., 2011).

Table 4.1:- Transcription factor, chemokine receptor and cytokine expression profiles of memory helper T-cell subpopulations and their role in adaptive immunity.

4.1.b. Infection and Rheumatoid Arthritis

With a causal link between various pathogenic agents and the development of autoimmunity well documented, infectious agents have long been considered potential triggers of RA pathogenesis; with the gram-negative bacteria *porphyromonas gingivalis* and the viral agents cytomegalovirus (CMV), herpes simplex virus (HSV) and Epstein Barr virus (EBV) of particular interest (Burgos et al., 2015; Croia et al., 2013; Magnusson et al., 2010; Mikuls et al., 2009; Pierer et al., 2012). The relationship between the periodontal disease causing *porphyromonas gingivalis* and rheumatoid arthritis has been comprehensively investigated, with a direct pathophysiological link between the presence of periodontal disease, raised protein arginine deiminase 4 (PAD4) levels and the production of anti-cyclic citrullinated peptide (CCP) antibodies extensively described (Johansson et al., 2016; Mikuls et al., 2009). To date, the direct relationship between RA pathogenesis and the human herpesviruses is less conclusive. Previous studies have been able to isolate CMV from the synovial fluid of RA patients; whilst an increase in EBV-DNA, EBV-specific antibodies and EBV infected cells have all been documented, when compared to disease and healthy controls (Burgos et al., 2015; Davis et al., 2012; Magnusson et al., 2010; Pierer et al., 2012). A recent study also observed the presence of active EBV infections within the ectopic lymphoid structures of RA synovium, in association with the local differentiation of CCP-reactive B-cells (Croia et al., 2013).

The clinical course of rheumatoid arthritis also appears to be affected by the presence of these viral agents, with latent CMV infections shown to aggravate the clinical course of seropositive RA patients, as compared to CMV seronegative RA patients, resulting in radiographic evidence of advanced joint destruction and an increased incidence of joint-related surgical procedures (Davis et al., 2012; Pierer et al., 2012). Despite mounting evidence to support the increased incidence of infectious agents within RA patients, a direct causal link between these infectious agents and RA pathogenesis remains elusive. As CD4⁺ T-cells are intricately involved in adaptive host responses to viral agents, and are

also heavily implicated in the pathogenesis of rheumatoid arthritis, a thorough investigation of antigen-specific T-cell responses seems warranted. With this in mind, a recent study sought to profile immune responses to the herpesviruses CMV and EBV, within RA patients, to further investigate their role in radiographic joint damage. This group were able to show a significant increase in IFN- γ and interleukin 13 (IL-13) production, within RA patients with a high degree of joint damage, as compared to healthy controls and RA patients with a lower degree of joint damage, thereby implicating Th1 and Th2 memory CD4⁺ T-cell subsets in joint destruction (Davis et al., 2012). A subsequent study by Davis et al further demonstrated that the increased production of IFN- γ and IL-4, by peripheral blood mononuclear cells (PBMCs) upon CMV/EBV stimulation, was predictive of an inadequate 28 joint disease activity score (DAS28) response in RA patients, following the initiation of disease modifying anti-rheumatic drug (DMARD) therapeutic regimens (Davis et al., 2013). Altogether, these studies validate the potential utility of employing antigen-specific T-cell immune response monitoring in the modelling of RA patient therapeutic outcomes, with the identification of differential CMV-specific immune responses within RA patients potentially able to discriminate between DMARD responders and non-responders, prior to the initiation of a therapeutic regimen (Davis et al., 2013).

4.1.c. Antigen-specific Immune Responses - Current Detection Methods

Until recently, the investigation of antigen-specific immune responses had been limited to two lines of inquiry; namely the detection of the pathogen directly or the measurement of immune mediators produced in direct response to the offending pathogens. The former incorporates the use of microbial cultures and/or molecular techniques, including quantitative real-time polymerase chain reactions (PCR) for pathogen recognition; whilst the latter integrates the measurement of specific immunoglobulins and pro-inflammatory cytokines to further investigate host immunity (Marchese et al., 2009; Rangaka et al., 2012; Tan et al., 2016). In isolation, these traditional detection methods are primarily used to

confirm the prior exposure of an individual to a known pathogen. For example, enzyme-linked immunosorbent assays (ELISAs) and enzyme-linked ImmunoSpot (ELISpot) assays are frequently used in routine diagnostic practice to quantify the release of IFN- γ from PMBCs, following their stimulation with the mycobacterium tuberculosis-specific antigens ESAT-6 and CPF-10, the detection of which can be used to establish the presence of latent tuberculosis (TB) infections (Rangaka et al., 2012). In the setting of RA, as well as other autoimmune conditions in which the use of tumour necrosis factor alpha (TNF- α) monoclonal therapeutic agents are often utilized, such a detection method is vital; with TNF- α a pivotal cytokine in the suppression of mycobacterial infection reactivation (Olsen et al., 2016).

The combined use of direct pathogen detection methods, in concert with the measurement of specific immune mediators, permits a far more rigorous investigation of immune competence, thereby aiding the diagnosis of suspected primary and secondary immunodeficiency disorders. For example, the presence of chronically high EBV viral loads, as determined by PCR for EBV DNA, in the absence of EBV viral capsid antigen (VCA) and epstein-barr virus nuclear antigen (EBNA) specific immunoglobulins, of the IgG isotype, is a characteristic feature of X-linked lymphoproliferative disease (XLP), an immunodeficiency disorder in which male carriers of SLAM-associated protein (SAP) mutations are incapable of mounting an appropriate adaptive immune response against EBV infection, leading to fulminant infectious mononucleosis, dysgammaglobulinaemia and an increased risk of lymphoproliferative disease (Gaspar et al., 2002; Sakamoto et al., 1982; Seemayer et al., 1995).

Although able to provide vital information in the above lines of inquiry, these conventional antigen-specific detection methods provide limited data regarding the efficacy of the immune response generated. Several publications have, however, since described a flow cytometric technique, utilizing a whole blood stimulation method to viral, fungal and

bacterial lysates, in which antigen-specific CD4⁺ T-cell responses can be detected and quantified. This method not only provides a rapidly inexpensive means of assessing antigen-specific immune responses, but also advances our understanding of memory CD4⁺ T-cell generation, a field that has previously lagged behind our knowledge of CD8⁺ T-cell recall responses (Hsu et al., 2012; Sadler et al., 2014; Zaunders et al., 2009). This novel flow cytometric assay detects and enumerates antigen-specific memory CD4⁺ T-cells via their upregulation of the alpha subunit of the IL-2 receptor (IL-2R α), CD25, in combination with the co-stimulatory molecule OX40 (also known as CD134), a member of the tumour necrosis factor receptor (TNFR) superfamily, both of which are required for activated T-cell survival and whose expression is amplified within 24-48 hours of memory T-cell re-activation (Gramaglia et al., 2000; Sadler et al., 2014).

Conceivably, this assay therefore has a number of potentially valuable applications, with the investigation of aberrant recall responses in relation to primary and secondary immunodeficiencies an appropriate line of inquiry (Sadler et al., 2014). However, a method such as this could also provide valuable information in relation to autoimmune conditions, including RA, for which an infectious aetiology has been proposed. By facilitating the direct investigation of antigen-specific CD4⁺ T-cell responses to infectious agents, including the herpesviruses CMV and HSV, a more thorough investigation of their pathogenic role in RA development is possible. Given the proposed relationship between various infectious agents, RA disease pathogenesis and DMARD therapeutic efficacy, a method such as this would indeed pose an asset, theoretically providing a further measure of disease activity and severity. Moreover, the expansion of this flow cytometric assay to include the chemokine receptors CCR6 (Th17-specific), CXCR3 (Th1-specific) and CCR4 (Th2-specific) would also facilitate the examination of their composite memory CD4⁺ T-cell subpopulations; an approach which has been successfully utilized in the characterization of blood-derived follicular helper T-cells (Ma et al., 2015; Morita et al., 2011). A stratagem such as this may therefore provide valuable insight into the nature of

antigen-specific CD4⁺ T-cell responses against viral, bacterial and fungal stimuli, within the general population, as well as in the setting of rheumatoid arthritis (Acosta-Rodriguez et al., 2007; Dhalla et al., 2016; Kanhere et al., 2012; Sallusto et al., 1998).

4.2. Aims and Objectives

Based upon the current evidence presented above, the working hypothesis of this chapter is that newly-diagnosed, treatment-naïve RA patients, as well as longstanding RA patients who have, or will fail, to make a clinical response following the initiation of a conventional synthetic (cs) or biologic (b) DMARD therapeutic regimen may be identified by their profile of antigen-specific CD4⁺ T-cell responses. As such, the addition of these parameters to current disease management protocols will improve the discrimination between RA therapy responders and non-responders, improving the efficacy and efficiency of patient management. The aims of this chapter are therefore:-

1. To establish the normal distribution, for total PHA, CMV, HSV and aspergillus antigen-specific T-cell responses, as well as their composite subpopulation distributions, in a cohort of adult, age- and sex-matched healthy controls.
2. To compare the total and relative subpopulation distributions of antigen-specific T-cell responses to PHA, CMV, HSV and aspergillus in newly-diagnosed, treatment-naïve and longstanding RA patients with those of age- and sex-matched disease and healthy controls.
3. To investigate the relationship between the CMV-specific T-cell responses and CMV serological responses of newly-diagnosed, treatment-naïve and longstanding RA patients, as well as age- and sex-matched disease and healthy controls.
4. To stratify longstanding RA patients according to their therapeutic regimens, in order to further investigate the effects of conventional synthetic and biologic immunosuppressive therapies on antigen-specific T-cell responses, as well as their relative subpopulation distributions.

5. To longitudinally monitor antigen-specific T-cell responses and their relative subpopulation distributions to PHA and CMV in newly-diagnosed, treatment-naïve rheumatoid arthritis patients, at 3-, 6- and 12-monthly intervals, following the initiation of immunosuppressive therapy; to further investigate the effect of RA therapeutic regimens on antigen-specific T-cell subpopulation distribution.

4.3. Results

4.3.a. Activated CD4⁺ T-cell subpopulations are differentially expressed according to divergent antigenic stimulations

A flow cytometric assay was developed allowing the identification of eight distinct subpopulations of CD25⁺CD134⁺ activated CD4⁺ T-cells (aTc), following antigenic stimulation with PHA, CMV, HSV and aspergillus, respectively (Figure 4.2). The following subpopulation designations have been used throughout: CCR6⁺CXCR3⁻ (CCR4⁺/-) Th17, CCR6⁺CXCR3⁺ (CCR4⁺/-) Th1/17, CCR6⁻CXCR3⁻ (CCR4⁺/-) Th2 and CCR6⁻CXCR3⁺ (CCR4⁻/+) Th1 cells (see Chapter 3).

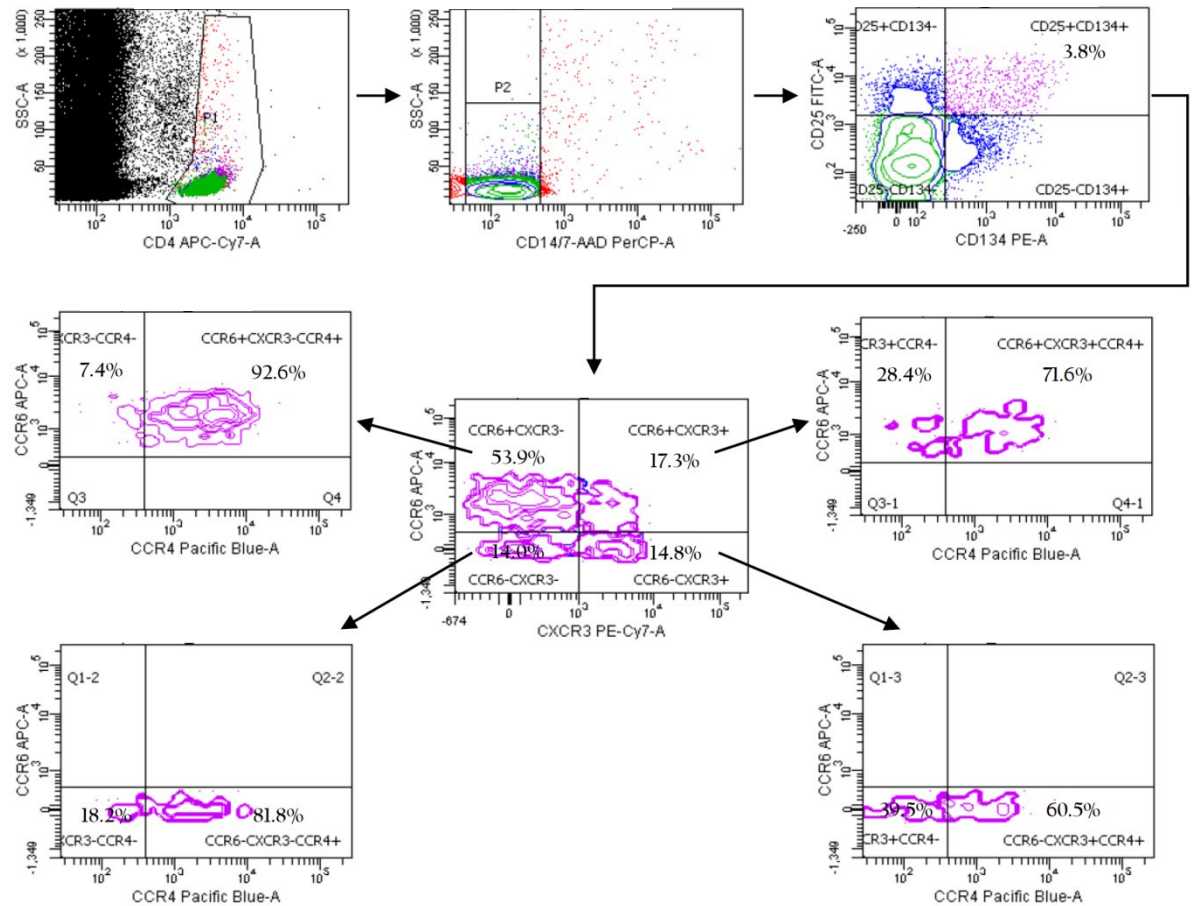


Figure 4.2:- Representative FACS plot depicting gating strategy employed for the identification of antigen-specific T-cells. FACS plots depict sample gating following culture with HSV antigen. Samples were gated so as to acquire 30,000 CD4⁺ events, with subsequent removal of monocytes and/or dead cells stained with CD14 and the viability marker 7-AAD. Live CD25⁺CD134⁺CD4⁺ T-cells were then gated, with sample gating based upon the identification of 0.1% of CD25⁺CD134⁺CD4⁺ T-cells following culture with media alone. CD25⁺CD134⁺CD4⁺ T-cells were then assessed for their expression of the chemokine receptors CCR6 and CXCR3, after which CD25⁺CD134⁺ T-cells were then further analysed for their expression of the additional chemokine receptor CCR4.

Within our healthy control cohort, CD25+CD134+ antigen-specific CD4+ T-cell responses made up 67.6% (62.1 – 71.6%) (median; inter-quartile ranges (IQR)), 5.8% (3.6 – 7.4%), 2.1% (1.2 – 3.3%) and 1.0% (0.7 – 1.5%) of live CD4+ T-cells, following stimulation with PHA, CMV, HSV and aspergillus, respectively (Figure 4.3A). Antigen-specific CD4+ T-cell responses following stimulation with PHA were significantly increased, as compared to aTc responses following stimulation with CMV ($p<0.05$), HSV ($p<0.0001$) and aspergillus ($p<0.0001$), respectively; with a significant increase in CMV-specific CD4+ T-cell responses also observed, as compared to aspergillus-specific CD4+ T-cell responses ($p<0.01$) (Figure 4.3A). When analysed further according to the expression of CCR6 and CXCR3, aTc responses following stimulation with PHA, were typified by a significant increase in Th1 and Th2 cell subpopulations, as compared to Th17 ($p<0.0001$, respectively) and Th1/17 ($p<0.0001$, respectively) cell subpopulations, respectively (Figure 4.3B). In contrast, following stimulation with CMV, HSV and aspergillus, aTc responses were dominated by a Th17 cell subpopulation, as compared to Th1/17 ($p<0.0001$, respectively), Th2 ($p<0.001$, non-significant and $p<0.0001$, respectively) and Th1 ($p<0.0001$, respectively) cell subpopulations, respectively (Figure 4.3C – E).

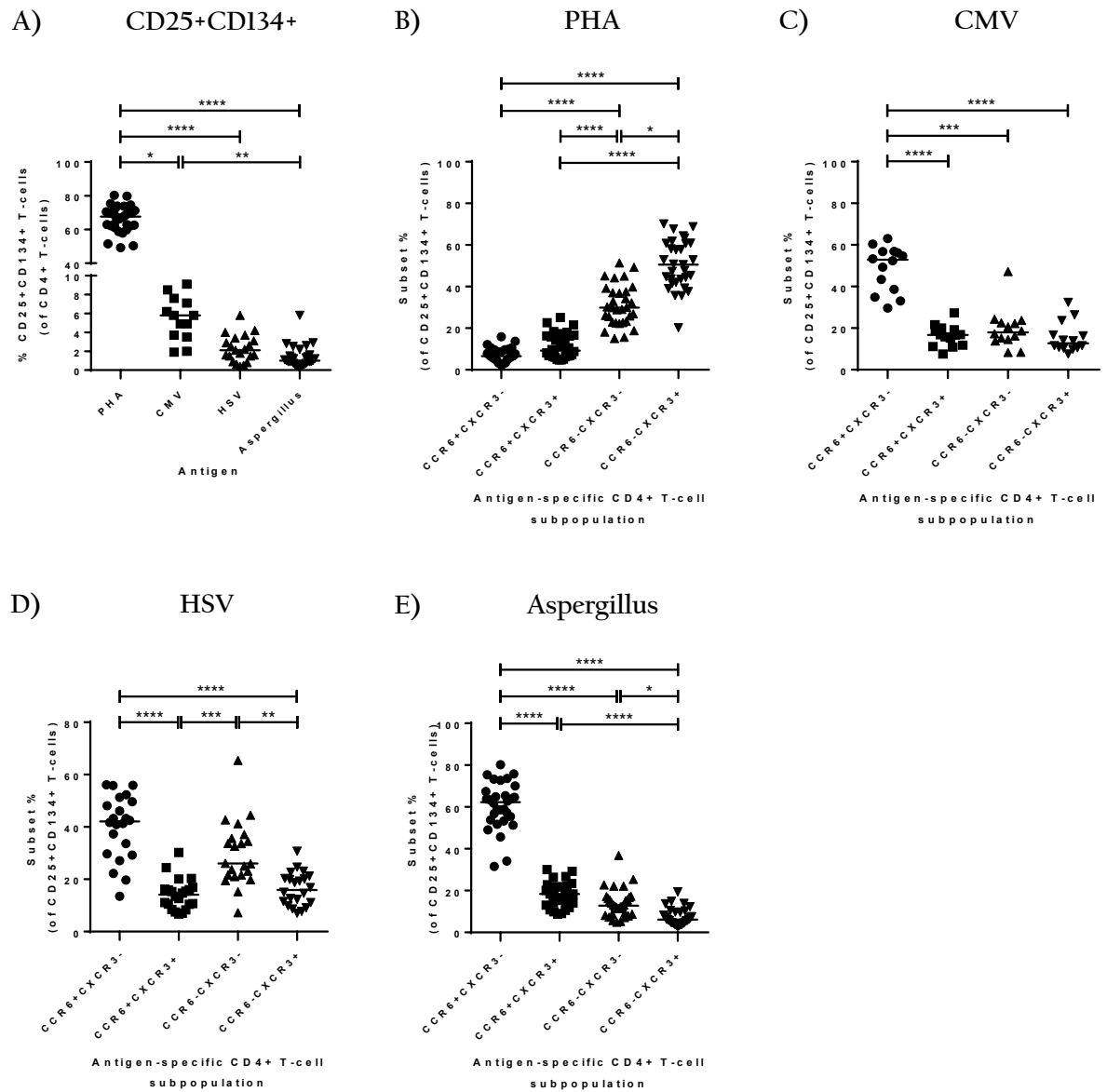


Figure 4.3:- Relative distribution (%) of antigen-specific CD4⁺ T-cells responses and their subpopulations, according to the expression of the chemokine receptors CCR6 and CXCR3 alone, within healthy controls. A) Relative distribution of antigen-specific CD4⁺ T-cell responses, following the exclusion of CD25+CD134⁺ T-cell responses <0.3% with less than 50 activated T-cells. Relative distribution of antigen-specific CD4⁺ T-cell subpopulations following stimulation with B) PHA; C) CMV; D) HSV and E) Aspergillus. **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

With the addition of the Th2-specific chemokine receptor CCR4, a dichotomy between the expression of CCR4⁻ and CCR4⁺ antigen-specific CD4⁺ T-cell subpopulations became apparent; with the relative distribution of CCR4⁺ T-cells consistently and significantly higher than their CCR4⁻ counterparts, following stimulation with PHA, CMV, HSV and aspergillus ($p < 0.01$, respectively) (Figure 4.4A – D). As such, following stimulation with PHA, the dominant subpopulations generated were CCR4⁺ Th1 and CCR4⁺ Th2 cells, respectively (Figure 4.4A). In contrast, the overriding subpopulation

produced following stimulation with CMV, HSV and aspergillus was CCR4⁺ Th17 cells (Figure 4.4B – D).

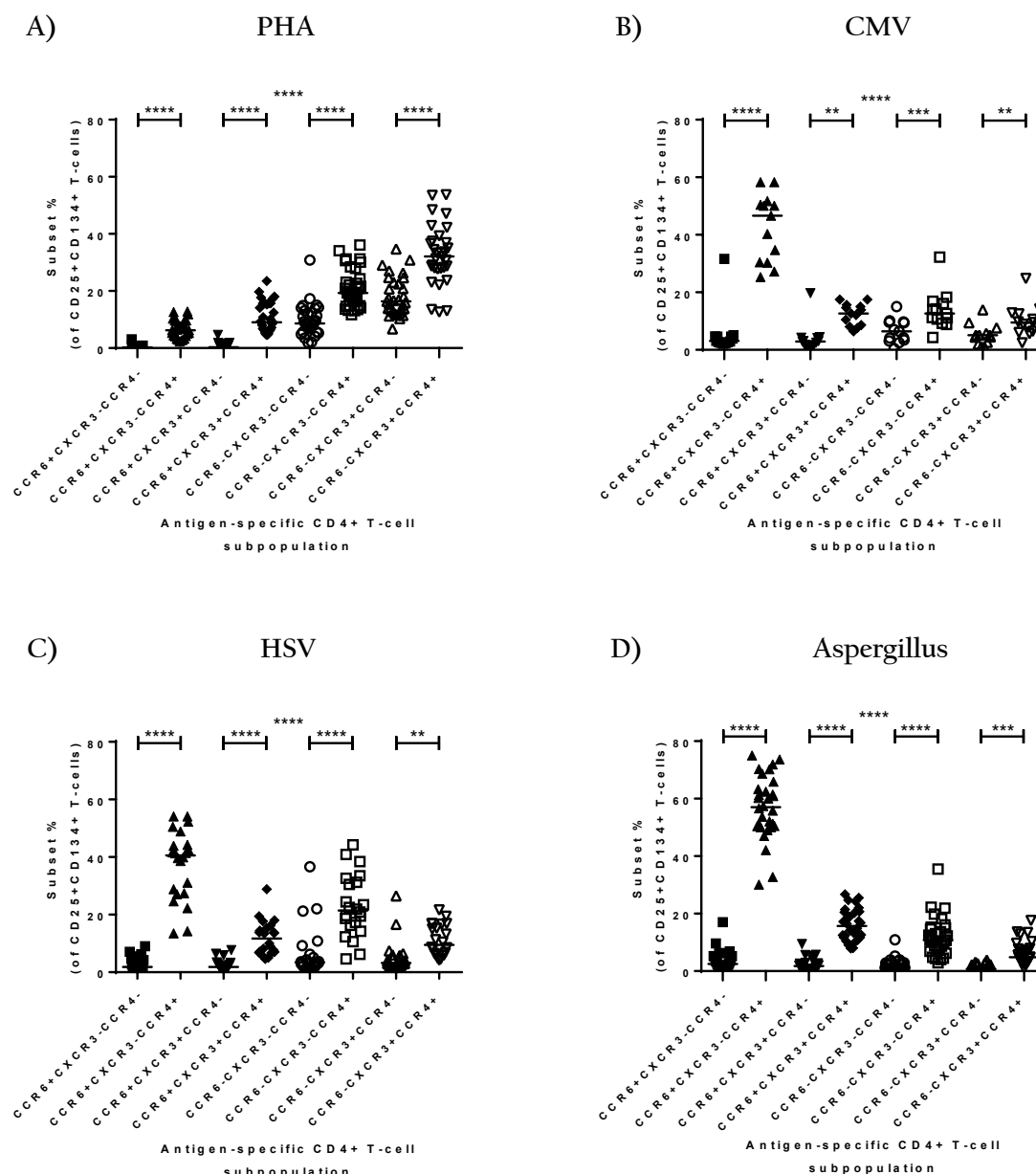


Figure 4.4:- Relative distribution (%) of antigen-specific CD4⁺ T-cell subpopulations, based upon expression of the chemokine receptors CCR6, CXCR3 and CCR4, generated within healthy controls following stimulation with A) PHA; B) CMV; C) HSV and D) Aspergillus. **** = p<0.0001, *** = p<0.001, ** = p<0.01.

Although distinctive subpopulation expression profiles were not evident for each individual antigenic stimulus investigated, excluding PHA, the distribution of antigen-specific CD4⁺ T-cell subpopulations did differ according to the antigenic stimulant utilized (Figure 4.5). For example, a significant increase in the relative distribution of Th17 cells was observed following stimulation with aspergillus, as compared to

stimulation with PHA ($p<0.0001$), HSV ($p<0.01$) and CMV ($p<0.01$) (Figure 4.5A). With the addition of CCR4, a significant increase in CCR4⁺ Th17 cells was observed following stimulation with aspergillus, as compared to stimulation with CMV and HSV ($p<0.01$, respectively), with no such difference in CCR4⁻ Th17 cells observed (Figure 4.5B - C).

In contrast, a significant increase in Th2 cells was observed following stimulation with HSV, as compared to aspergillus ($p<0.0001$) (Figure 4.5G). With the addition of CCR4, a significant increase in CCR4⁺ Th2 cells was observed following stimulation with HSV, as compared to CMV and aspergillus ($p<0.05$ and $p<0.0001$, respectively), whilst a significant increase in CCR4⁻ Th2 cells was also observed following stimulation with HSV, as compared to aspergillus ($p<0.05$) (Figure 4.5I). Despite evidence for differing CD4⁺ T-cell subpopulation contributions to antigen-specific CD4⁺ T-cell responses, following stimulation with PHA, CMV, HSV and aspergillus, no associations between the relative distribution of aTc responses and the relative distribution of CCR4⁻ or CCR4⁺ Th17, Th1/17, Th2 or Th1 cells were observed (data not shown).

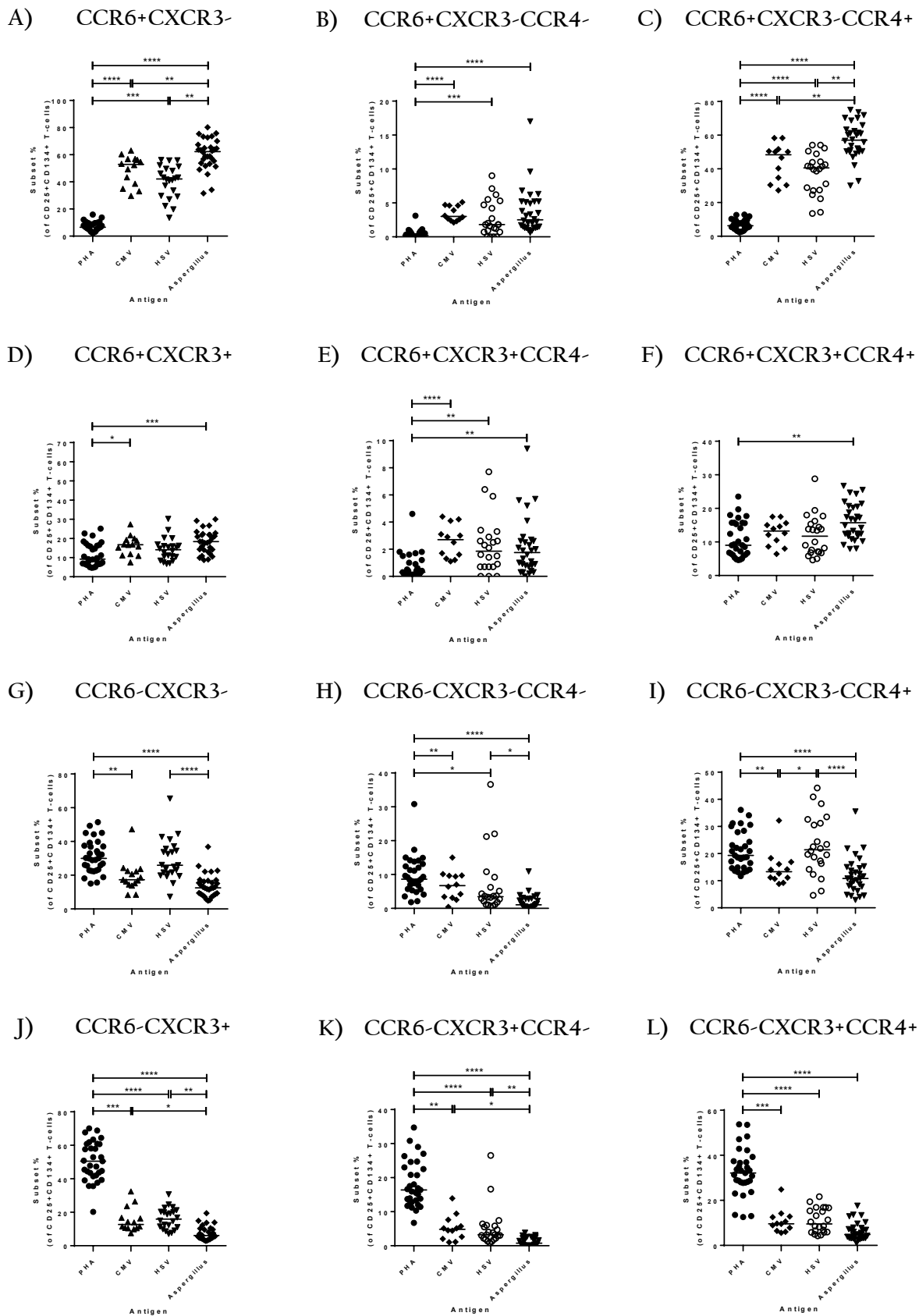


Figure 4.5:- Relative distribution (%) of A) CCR6+CXCR3-; B) CCR6+CXCR3-CCR4-; C) CCR6+CXCR3-CCR4+; D) CCR6+CXCR3+; E) CCR6+CXCR3+CCR4-; F) CCR6+CXCR3+CCR4+; G) CCR6-CXCR3-; H) CCR6-CXCR3-CCR4-; I) CCR6-CXCR3-CCR4+; J) CCR6-CXCR3+; K) CCR6-CXCR3+CCR4- and L) CCR6-CXCR3+CCR4+ activated CD4+ T-cells following stimulation with PHA, CMV, HSV and Aspergillus, within healthy controls. **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

4.3.a.i. *PHA* - With the normal distribution of antigen-specific CD4⁺ T-cell subpopulation expression profiles established, it was felt necessary to corroborate whether any age or sex associations existed within the observed aTc responses or their composite subpopulations; prior to their assessment within newly-diagnosed, treatment-naïve and longstanding RA patients. As such, following stimulation with PHA, no association between the relative distribution of aTc responses and age was observed within our healthy control cohort (data not shown). When analysed further according to the expression of CCR6, CXCR3 and CCR4, no significant relationships between the relative distribution of any antigen-specific CD4⁺ T-cell subpopulation and age were observed (data not known). In addition, no significant effects of sex were observed within aTc responses or their constituent CD4⁺ T-cell subpopulations (data not shown).

4.3.a.ii. *CMV* - Within our healthy control cohort, CMV serological assessments indicated a CMV carriage rate of 11/32 (34.4%). Consistent with serological CMV carriage rates, the relative distribution of aTcs, following stimulation with CMV, indicated prior exposure to CMV (i.e. relative distributions of aTcs >0.3%) in 13/32 (40.6%) of healthy controls; with a positive association between the relative distribution of aTcs and CMV IgG measurements observed ($r=0.7279$; $p<0.0001$) (Figure 4.6). No associations between CMV IgG serological measurements and the relative distribution of Th17, Th1/17, Th2 or Th1 cells irrespective of CCR4 expression were, however, observed (data not shown).

Despite evidence in the literature for an increase in CMV carriage rates with age, no associations between CMV immunoglobulin G (IgG) measurements (AU/ml) or the relative distribution of aTcs were observed with age (data not shown) (Parry et al., 2016; Vyse, Hesketh and Pebody, 2009). When analysed further according to the expression of CCR6, CXCR3 and CCR4, no associations between the relative distribution of any T-cell subpopulation characterized and age were observed (data not shown). Furthermore, no significant effects of sex were observed within aTc responses or their constituent CD4⁺ T-

cell subpopulations (data not shown). As such, following stimulation with CMV, the relative distribution of aTcs and CMV IgG serological measurements are positively associated within healthy controls. No single activated CD4⁺ T-cell subpopulation, however, appears associated with CMV IgG levels.

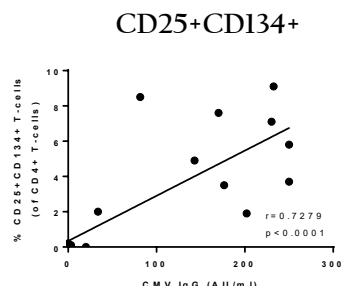


Figure 4.6:- Association of CD25+CD134⁺ activated CD4⁺ T-cell percentages with CMV IgG serological assessments, following stimulation with CMV in healthy controls. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables, with a p-value < 0.05 considered statistically significant.

4.3.a.iii. *HSV* - Following stimulation with HSV, no relationship between the relative distribution of aTc responses and age was observed (data not shown). With the addition of CCR6, CXCR3 and CCR4, no associations with the relative distribution of any activated CD4⁺ T-cell subpopulation and age were observed (data not shown). Additionally, when analysed further for effects of sex, no significant sex associations were observed within aTcs or the relative distribution of any activated CD4⁺ T-cell subpopulation with or without the addition of CCR4.

4.3.a.iv. *Aspergillus* - Following stimulation with aspergillus, no association between the relative distribution of aTcs and age was observed (data not shown). When analysed further according to the expression of CCR6, CXCR3 and CCR4, no associations were observed between the relative distribution of any aspergillus-specific CD4⁺ T-cell subpopulations and age. Furthermore, no effects of sex were noted within any aspergillus-specific CD4⁺ T-cell subpopulation characterized (data not shown). As such, no significant effects of age or sex were apparent within the aTc responses of our healthy control cohort, irrespective of the antigenic stimulus utilized. Therefore, following stimulation with the mitogen PHA, aTc responses are dominated by CCR4⁺ Th1 and CCR4⁺ Th2 cell subpopulations. In contrast, following stimulation with CMV, HSV and

aspergillus, aTc responses are typified by a CCR4⁺ Th17 subpopulation; with CCR4⁺ Th2 cells also contributing significantly to HSV-specific CD4⁺ T-cell responses (Table 4.2).

	<u>PHA</u>	<u>CMV</u>	<u>HSV</u>	<u>Aspergillus</u>
CD25+CD134 ⁺ activated CD4 ⁺ T-cell (%)	<u>51.0 – 77.4</u> ^{†Δ}	<u>2.0 – 8.7</u> ^{II}	0.4 – 4.0	0.4 – 2.9
CCR6+CXCR3 ⁻ (Th17) (%)	<u>2.8 – 12.8</u> ^{¶Σ}	31.6 – 61.4	22.1 – 56.1	<u>35.8 – 75.6</u> [‡]
CCR6+CXCR3 ⁻ CCR4 ⁻ (%)	<u>0.05 – 1.1</u> ^{f\$}	2.2 – 15.7	0.0 – 8.6	1.0 – 8.2
CCR6+CXCR3 ⁻ CCR4 ⁺ (%)	<u>2.8 – 12.2</u> ^Ω	26.4 – 58.3	22.1 – 53.8	<u>33.6 – 72.8</u> [€]
CCR6+CXCR3 ⁺ (Th1/17) (%)	<u>4.9 – 22.0</u> ^{i\$}	9.5 – 23.9	7.1 – 23.8	9.5 – 28.0
CCR6+CXCR3 ⁺ CCR4 ⁻ (%)	<u>0.1 – 1.8</u> ^{io}	1.2 – 10.5	0.0 – 6.3	0.3 – 5.7
CCR6+CXCR3 ⁺ CCR4 ⁺ (%)	<u>4.7 – 18.8</u> ^o	7.1 – 17.5	5.3 – 19.2	8.6 – 25.1
CCR6-CXCR3 ⁻ (Th2) (%)	<u>17.0 – 47.0</u> ^{«»}	11.6 – 33.5	<u>15.8 – 42.6</u> [«]	5.5 – 24.1
CCR6-CXCR3 ⁻ CCR4 ⁻ (%)	<u>2.8 – 16.0</u> ^{×!}	1.6 – 12.1	<u>1.0 – 20.3</u> [»]	0.0 – 4.5
CCR6-CXCR3 ⁻ CCR4 ⁺ (%)	<u>13.2 – 32.5</u> ^{/^}	7.0 – 23.9	<u>6.9 – 38.0</u> ^{#]}	4.2 – 22.1
CCR6-CXCR3 ⁺ (Th1) (%)	<u>35.7 – 68.2</u> ^{[≈}	8.9 – 28.8	8.7 – 30.4	<u>3.2 – 17.2</u> ^{μ{}
CCR6-CXCR3 ⁺ CCR4 ⁻ (%)	<u>10.8 – 29.8</u> ^{*}}	1.1 – 11.2	0.9 – 16.5	<u>0.0 – 3.0</u> ^{&+}
CCR6-CXCR3 ⁺ CCR4 ⁺ (%)	<u>13.3 – 50.7</u> ^{αε}	4.3 – 18.4	4.7 – 23.3	2.4 – 15.6

Table 4.2:- Relative distribution (%) of antigen-specific CD4⁺ T-cell responses and their subpopulations within healthy controls (n = 32), following stimulation with PHA, CMV, HSV and aspergillus, respectively. All antigen-specific CD4⁺ T-cell subpopulations were expressed as a percentage of CD25+CD134⁺ activated CD4⁺ T-cells. Underlined figures denote significant results. † = p<0.0001 as compared to HSV and aspergillus; Δ = p<0.05 as compared to CMV; II = p<0.01 as compared to aspergillus; ¶ = p<0.0001 as compared to CMV and aspergillus; Σ = p<0.001 as compared to HSV; ‡ = p<0.01 as compared to CMV and HSV; f = p<0.0001 as compared to CMV and aspergillus; \$ = p<0.001 as compared to HSV; Ω = p<0.0001 as compared to CMV, HSV and aspergillus; € = p<0.01 as compared to HSV and CMV; i = p<0.001 as compared to aspergillus; \$ = p<0.05 as compared to CMV; i = p<0.0001 as compared to CMV; o = p<0.01 as compared to HSV and aspergillus; o = p<0.01 as compared to aspergillus; « = p<0.0001 as compared to aspergillus; » = p<0.01 as compared to CMV; × = p<0.0001 as compared to aspergillus; ! = p<0.01 as compared to CMV; ? = p<0.05 as compared to HSV; » = p<0.05 as compared to aspergillus; / = p<0.0001 as compared to aspergillus; ^ = p<0.01 as compared to CMV; # = p<0.0001 as compared to aspergillus;] = p<0.05 as compared to CMV; [= p<0.0001 as compared to HSV and aspergillus; ≈ = p<0.001 as compared to CMV; μ = p<0.01 as compared to HSV; { = p<0.05 as compared to CMV; * = p<0.0001 as compared to HSV and aspergillus; } = p<0.001 as compared to CMV; & = p<0.01 as compared to HSV; + = p<0.05 as compared to CMV; α = p<0.0001 as compared to HSV and aspergillus; ε = p<0.001 as compared to CMV. Figures denote 5th and 95th percentiles.

4.3.b. Antigen-specific CD4⁺ T-cells are differentially expressed in newly-diagnosed, treatment-naïve and longstanding RA patients

4.3.b.i. PHA - With the relative distribution of PHA⁻, CMV⁻, HSV and aspergillus-specific CD4⁺ T-cell responses and their subpopulations characterized within healthy controls, the relative distribution of aTcs, and their subpopulations, were compared between 20 newly-diagnosed, treatment-naïve RA patients (ERA), 56 longstanding RA patients (RA), 32 age- and sex-matched disease controls (DCG) and 32 age- and sex-matched healthy controls (HC). Following stimulation with PHA, a significant reduction in the relative distribution of aTcs was observed within ERA ($p < 0.01$) and RA patients ($p < 0.0001$), as well as DCG ($p < 0.001$), as compared to HC; with 8/20 (40.0%) ERA patients, 16/56 (28.6%) RA patients and 9/32 (28.1%) DCG presenting with aTc percentages below the 5th percentile of HC (5th – 95th percentile = 51.0 – 77.4%) (Figure 4.7A; Table 4.3). When analysed further according to the expression of CCR6 and CXCR3 alone, no significant difference in the relative distribution of any activated CD4⁺ T-cell subpopulation was observed between patient and control groups (Table 4.3). A trend towards an increase in the percentage of CCR6-CXCR3⁻ Th2 cells was, however, observed within RA patients, with 10/56 (17.9%) RA patients demonstrating CCR6-CXCR3⁻ Th2 cell percentages above the 95th percentile of HC (5th – 95th percentile = 17.0 – 47.0%) (Figure 4.7B; Table 4.3).

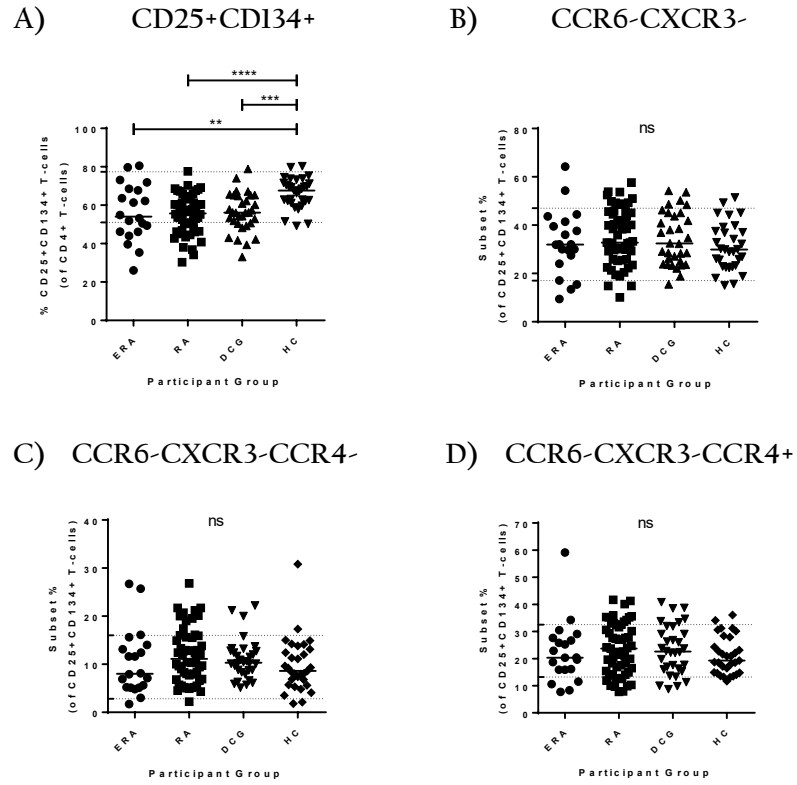


Figure 4.7:- Relative distribution (%) of A) CD25+CD134+; B) CCR6-CXCR3- Th2; C) CCR6-CXCR3-CCR4- Th2 and D) CCR6-CXCR3-CCR4+ Th2 activated CD4+ T-cells following stimulation with PHA, within newly-diagnosed, treatment-naïve RA patients (ERA) and longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, ns = non-significant; denotes 5th and 95th percentiles of healthy controls.

With the addition of CCR4, no significant difference in the relative distribution of any activated CD4+ T-cell subpopulation was observed between patient and control groups (Table 4.3). Upon further inspection, a trend towards an increase in the relative distribution of CCR4- Th2 cells was observed within RA patients, with 11/56 (19.6%) RA patients demonstrating CCR4- Th2 cell percentages above the 95th percentile of HC (5th – 95th percentile = 2.8 – 16.0%) (Figure 4.7C; Table 4.3). A trend towards an increase in the relative distribution of CCR4+ Th2 cells was also observed within RA patients and DCG, with 12/56 (21.4%) RA patients and 6/32 (18.7%) DCG demonstrating CCR4+ Th2 cell percentages above the 95th percentile of healthy controls (5th – 95th percentile = 13.2 – 32.5%) (Figure 4.7C; Table 4.3). As such, following stimulation with PHA, the relative distribution of aTcs is significantly reduced within ERA and RA patients, as well as DCG, as compared to HC; with a proportion of RA patients and DCG also demonstrating an increase in the relative distribution of Th2 activated CD4+ T-cells.

	<u>ERA</u>	<u>RA</u>	<u>DCG</u>	<u>HC</u>
CD25+CD134+ activated CD4+ T-cell (%)	<u>54.0</u> Π (46.1 – 68.3)	<u>55.7</u> $\dagger$ (47.5 – 61.2)	<u>56.1</u> Δ (50.5 – 65.1)	67.6 (62.1 – 71.6)
CCR6+CXCR3- (Th17) (%)	6.5 (4.1 – 10.5)	6.3 (4.2 – 9.8)	6.4 (4.9 – 8.8)	6.5 (4.5 – 9.6)
CCR6+CXCR3-CCR4- (%)	0.3 (0.2 – 0.5)	0.2 (0.1 – 0.4)	0.2 (0.1 – 0.6)	0.2 (0.1 – 0.6)
CCR6+CXCR3-CCR4+ (%)	6.1 (3.7 – 9.4)	6.2 (4.0 – 9.7)	6.0 (4.8 – 8.8)	6.2 (4.2 – 9.4)
CCR6+CXCR3+ (Th1/17) (%)	<u>11.0</u> (7.0 – 15.8)	<u>8.6</u> (5.0 – 12.1)	<u>9.0</u> (6.4 – 15.3)	<u>9.2</u> (6.6 – 16.3)
CCR6+CXCR3+CCR4- (%)	0.3 (0.2 – 0.5)	0.2 (0.1 – 0.4)	0.3 (0.1 – 0.8)	0.3 (0.1 – 1.0)
CCR6+CXCR3+CCR4+ (%)	10.3 (6.5 – 14.5)	8.3 (4.8 – 11.0)	8.6 (6.3 – 14.0)	9.0 (6.2 – 15.47)
CCR6-CXCR3- (Th2) (%)	<u>32.0</u> (24.9 – 40.9)	<u>32.8</u> (25.8 – 45.1)	<u>32.4</u> (24.1 – 44.7)	<u>29.9</u> (22.9 – 37.5)
CCR6-CXCR3-CCR4- (%)	8.0 (5.1 – 13.8)	11.1 (6.9 – 15.6)	10.3 (8.4 – 12.8)	8.6 (5.7 – 13.1)
CCR6-CXCR3-CCR4+ (%)	20.3 (15.9 – 27.4)	23.7 (14.9 – 32.4)	22.6 (16.1 – 31.2)	19.3 (14.8 – 27.9)
CCR6-CXCR3+ (Th1) (%)	<u>43.7</u> (36.5 – 60.6)	<u>45.9</u> (37.7 – 57.8)	<u>47.0</u> (40.0 – 53.7)	<u>50.5</u> (42.0 – 60.8)
CCR6-CXCR3+CCR4- (%)	19.4 (14.1 – 23.5)	15.8 (9.8 – 24.0)	16.6 (12.0 – 21.1)	16.4 (12.8 – 23.0)
CCR6-CXCR3+CCR4+ (%)	26.7 (21.0 – 35.3)	27.3 (22.0 – 36.2)	28.3 (25.6 – 34.8)	32.1 (27.9 – 37.4)

Table 4.3:- Relative distribution (%) of CD25+CD134+ activated CD4+ T-cells and their subpopulations, following stimulation with PHA within newly-diagnosed, treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. Underlined figures denote significant results. $\dagger$ = $p < 0.0001$ as compared to HC; Δ = $p < 0.001$; Π = $p < 0.01$ as compared to HC. Figures denote median and inter-quartile ranges (IQR).

4.3.b.ii. CMV - Serological assessments revealed CMV carriage rates of 10/20 (50.0%) within ERA patients, 20/56 (35.7%) within RA patients and 13/32 (40.6%) within DCG, respectively. No significant difference in CMV serological status or CMV IgG serological quantification was observed between patient and control groups (data not shown). Consistent with serological CMV carriage rates, the relative distribution of aTcs following stimulation with CMV, indicated prior exposure to CMV, in 10/20 (50.0%) ERA patients, 17/56 (30.3%) RA patients and 13/32 (40.6%) DCG respectively. When analysed further for differences in the relative distribution of aTcs, a significant reduction in the relative distribution of aTcs was observed within RA patients, as compared to HC ($p < 0.05$)

(Figure 4.8A; Table 4.4). A trend towards a reduction in the relative distribution of aTcs was also observed within ERA patients and DCG, as compared to HC. However, these observations failed to reach statistical significance ($p=0.1428$ and $p=0.0931$, respectively) (Figure 4.8A; Table 4.4).

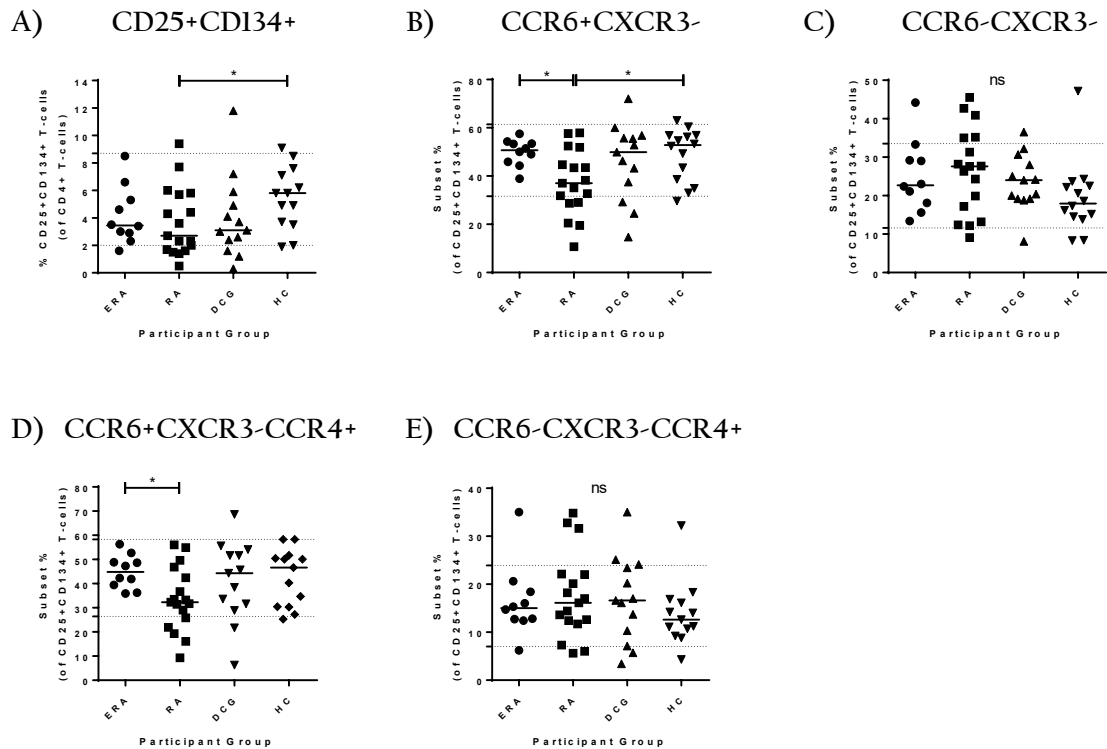


Figure 4.8:- Relative distribution (%) of A) CD25+CD134+; B) CCR6+CXCR3-; C) CCR6-CXCR3; D) CCR6+CXCR3-CCR4+ and E) CCR6-CXCR3-CCR4+ activated CD4+ T-cells following stimulation with CMV, within newly-diagnosed, treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. * = $p<0.05$, ns = non-significant; denotes 5th and 95th percentiles of healthy controls.

When analysed further according to the expression of CCR6 and CXCR3 alone, a significant reduction in the relative distribution of Th17 cells was observed within RA patients, as compared to ERA patients and HC ($p<0.05$, respectively) (Figure 4.8B; Table 4.4). No significant difference in the relative distribution of Th1/17, Th2 or Th1 cells was, however, observed between any patient or control group (Table 4.4). A trend towards an increase in the relative distribution of Th2 cells was observed within RA patients, as compared to HC. However, this observation failed to reach statistical significance ($p=0.0721$) (Figure 4.8C; Table 4.4).

	<u>ERA</u>	<u>RA</u>	<u>DCG</u>	<u>HC</u>
CD25+CD134+ activated CD4+ T-cell (%)	3.4 (2.7 – 5.6)	<u>2.7†</u> (1.6 – 5.7)	3.1 (2.0 – 5.4)	5.8 (3.6 – 7.3)
CCR6+CXCR3- (Th17) (%)	50.7 (45.5 – 53.6)	<u>37.0Δ</u> (28.8 – 48.3)	49.9 (33.3 – 56.2)	52.8 (37.7 – 56.8)
CCR6+CXCR3-CCR4- (%)	4.1 (2.5 – 8.1)	2.8 (1.1 – 4.5)	3.9 (1.7 – 4.6)	3.1 (2.5 – 4.6)
CCR6+CXCR3-CCR4+ (%)	44.8 (38.6 – 49.8)	<u>32.3II</u> (23.8 – 44.6)	44.3 (30.2 – 52.8)	46.6 (30.3 – 51.0)
CCR6+CXCR3+ (Th1/17) (%)	11.4 (9.5 – 15.9)	12.4 (8.9 – 15.5)	13.2 (9.0 – 14.8)	16.7 (11.6 – 19.4)
CCR6+CXCR3+CCR4- (%)	1.9 (0.9 – 2.7)	1.1 (0.4 – 2.7)	2.5 (1.0 – 3.7)	2.9 (1.4 – 4.1)
CCR6+CXCR3+CCR4+ (%)	9.1 (8.1 – 13.1)	8.9 (6.9 – 14.2)	10.5 (6.7 – 11.7)	12.6 (8.3 – 15.3)
CCR6-CXCR3- (Th2) (%)	22.7 (17.5 – 30.2)	27.6 (15.2 – 35.1)	24.0 (19.2 – 29.4)	18.0 (14.3 – 22.8)
CCR6-CXCR3-CCR4- (%)	3.9 (2.3 – 8.0)	7.4 (4.8 – 13.1)	7.9 (4.2 – 12.1)	6.4 (3.2 – 9.6)
CCR6-CXCR3-CCR4+ (%)	15.0 (12.6 – 18.9)	16.1 (12.0 – 22.0)	16.6 (8.7 – 23.7)	12.6 (9.9 – 16.5)
CCR6-CXCR3+ (Th1) (%)	11.8 (9.1 – 15.6)	17.4 (10.6 – 22.1)	13.8 (10.3 – 25.2)	12.7 (10.5 – 18.4)
CCR6-CXCR3+CCR4- (%)	4.4 (3.0 – 7.0)	3.7 (1.9 – 6.9)	3.2 (2.3 – 6.9)	5.1 (2.3 – 6.6)
CCR6-CXCR3+CCR4+ (%)	10.1 (7.4 – 11.4)	7.4 (5.1 – 10.2)	12.3 (5.9 – 19.3)	9.5 (6.2 – 12.6)

Table 4.4:- Relative distribution (%) of CD25+CD134+ activated CD4+ T-cells and their subpopulations, following stimulation with CMV within newly-diagnosed, treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. Underlined figures denote significant results. † = $p < 0.05$ as compared to HC; Δ = $p < 0.05$ as compared to ERA and HC; II = $p < 0.05$ as compared to ERA. Figures denote median and inter-quartile ranges (IQR).

With the addition of CCR4, a significant reduction in the relative distribution of CCR4+ Th17 cells was observed within RA patients, as compared to ERA patients ($p < 0.05$); with a trend towards a reduction in CCR4+ Th17 cells also observed in RA patients, as compared to HC ($p = 0.0922$) (Figure 4.8D; Table 4.4). No significant difference in the relative distribution of any further activated CD4+ T-cell subpopulation characterized was observed between patient and control groups (Table 4.4). A trend towards an increase in the relative distribution of CCR4+ Th2 cells was observed within RA patients, as compared to HC, although this observation failed to reach statistical significance ($p = 0.1798$) (Figure 4.8E; Table 4.4). In summary, following stimulation with CMV, the

relative distribution of aTcs appears reduced within ERA and RA patients, as well as DCG, as compared to HC; with a significant reduction in Th17 cells and a concomitant increase in Th2 cells observed within RA patients, as compared to ERA patients and HC. In contrast, no significant differences in the relative distribution of any activated CD4+ T-cell subpopulation were observed within ERA patients.

4.3.b.iii. HSV - Following stimulation with HSV, the relative distribution of aTc responses indicated prior exposure to HSV in 12/20 (60.0%) ERA patients, 42/56 (75.0%) RA patients and 22/32 (68.8%) DCG, respectively. A significant increase in the relative distribution of aTcs was observed within ERA patients, as compared to RA patients, DCG and HC ($p < 0.05$, respectively) (Figure 4.9; Table 4.5). When analysed further according to the expression of CCR6, CXCR3 and CCR4, however, no significant difference in the relative distribution of any activated CD4+ T-cell subpopulation characterized was observed between patient and control groups (Table 4.5). As such, following stimulation with HSV, a significant increase in the relative distribution of aTcs was observed within ERA patients, as compared to RA patients, as well as DCG and HC. However, the relative distribution of the composite T-cell subpopulations remained unaltered. With no significant difference in the relative distribution of aTc subpopulations, observed beyond those observed following stimulation with PHA or CMV, the effects of HSV stimulation will not be investigated further from hereafter.

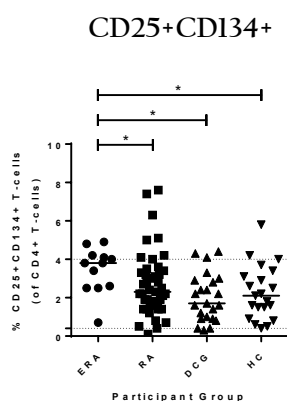


Figure 4.9:- Relative distribution of CD25+CD134+ activated CD4+ T-cells following stimulation with HSV, within newly-diagnosed, treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. * = $p < 0.05$, ns = non-significant; denotes 5th and 95th percentiles of healthy controls.

	<u>ERA</u>	<u>RA</u>	<u>DCG</u>	<u>HC</u>
CD25+CD134+ activated CD4+ T-cell (%)	<u>3.8†</u> (2.5 – 4.2)	2.3 (1.6 – 3.4)	1.7 (0.9 – 2.9)	2.1 (1.2 – 3.2)
CCR6+CXCR3- (Th17) (%)	46.7 (35.9 – 55.1)	45.8 (33.7 – 53.6)	43.2 (38.6 – 53.4)	42.5 (31.6 – 50.4)
CCR6+CXCR3-CCR4- (%)	2.0 (1.6 – 6.9)	1.9 (1.0 – 3.3)	2.4 (1.0 – 6.8)	1.8 (0.8 – 5.0)
CCR6+CXCR3-CCR4+ (%)	44.1 (31.1 – 49.9)	43.0 (32.3 – 48.5)	39.6 (33.8 – 46.8)	41.3 (28.1 – 46.5)
CCR6+CXCR3+ (Th1/17) (%)	13.4 (11.0 – 17.5)	12.9 (10.7 – 16.4)	13.4 (10.8 – 19.8)	14.7 (9.4 – 16.5)
CCR6+CXCR3+CCR4- (%)	2.3 (1.3 – 2.7)	1.5 (0.9 – 2.3)	1.9 (0.7 – 5.3)	1.6 (0.7 – 2.7)
CCR6+CXCR3+CCR4+ (%)	11.3 (8.5 – 13.4)	11.2 (8.3 – 14.2)	12.2 (7.8 – 14.7)	13.4 (7.0 – 15.7)
CCR6-CXCR3- (Th2) (%)	24.6 (19.3 – 30.0)	25.4 (19.0 – 32.2)	25.3 (19.3 – 31.8)	25.9 (21.3 – 35.0)
CCR6-CXCR3-CCR4- (%)	3.8 (2.8 – 7.7)	3.1 (1.5 – 5.5)	3.9 (1.2 – 6.2)	3.3 (1.4 – 5.6)
CCR6-CXCR3-CCR4+ (%)	18.1 (15.6 – 25.7)	21.4 (16.7 – 28.8)	20.5 (12.5 – 30.4)	20.9 (15.2 – 30.8)
CCR6-CXCR3+ (Th1) (%)	13.7 (7.1 – 16.3)	15.2 (10.0 – 18.9)	12.8 (10.8 – 15.9)	16.8 (11.3 – 20.7)
CCR6-CXCR3+CCR4- (%)	3.2 (1.8 – 9.2)	3.7 (1.9 – 5.4)	4.6 (2.1 – 7.1)	3.2 (2.2 – 5.9)
CCR6-CXCR3+CCR4+ (%)	6.4 (5.4 – 10.5)	10.2 (5.9 – 13.1)	8.9 (3.6 – 12.5)	10.4 (6.2 – 16.8)

Table 4.5:- Relative distribution (%) of CD25+CD134+ activated CD4+ T-cells and their subpopulations, following stimulation with HSV within newly-diagnosed, treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. Underlined figures denote significant results. † = $p < 0.05$ as compared to RA patients, DCG and HC. Figures denote median and inter-quartile ranges (IQR).

4.3.b.iv. *Aspergillus* - Following stimulation with *Aspergillus*, no significant difference in the relative distribution of aTcs was observed between patient and control groups (Table 4.6). Upon further analysis for expression of the chemokine receptors CCR6 and CXCR3 alone, no significant difference in the relative distribution of Th17 or Th1 cells was observed between patient and control groups (Table 4.6). A significant reduction in the relative distribution of Th1/17 cells was, however, observed within ERA and RA patients, as well as DCG, as compared to HC ($p < 0.05$, respectively) (Figure 4.10A; Table 4.6). This reduction in Th1/17 cells was accompanied by a significant increase in the relative distribution of Th2 cells within ERA and RA patients, as compared to HC ($p < 0.01$ and $p < 0.05$, respectively). Furthermore, 10/56 (17.8%) RA patients demonstrated Th2 cells

percentages above the 95th percentile of healthy controls (5th – 95th percentile = 5.5 – 24.1%) (Figure 4.10B; Table 4.6).

	<u>ERA</u>	<u>RA</u>	<u>DCG</u>	<u>HC</u>
CD25+CD134+ activated CD4+ T-cell (%)	0.8 (0.5 – 1.4)	0.8 (0.4 – 1.5)	1.1 (0.6 – 2.5)	1.0 (0.7 – 1.5)
CCR6+CXCR3- (Th17) (%)	59.9 (53.1 – 63.4)	61.4 (51.6 – 69.9)	63.2 (54.9 – 67.2)	62.3 (53.7 – 68.0)
CCR6+CXCR3-CCR4- (%)	1.9 (0.6 – 6.0)	1.2 (0.8 – 3.3)	1.3 (0.5 – 3.4)	2.5 (1.4 – 5.2)
CCR6+CXCR3-CCR4+ (%)	55.7 (48.1 – 60.4)	57.4 (48.6 – 69.1)	59.6 (51.5 – 62.9)	57.0 (50.3 – 63.9)
CCR6+CXCR3+ (Th1/17) (%)	<u>12.6†</u> (11.2 – 19.8)	<u>12.5Δ</u> (9.7 – 16.7)	<u>12.6Π</u> (9.0 – 19.4)	18.3 (13.7 – 22.0)
CCR6+CXCR3+CCR4- (%)	1.3 (0.0 – 1.7)	<u>0.5‡</u> (0.0 – 1.2)	0.9 (0.0 – 2.9)	1.7 (0.8 – 2.6)
CCR6+CXCR3+CCR4+ (%)	<u>11.3¶</u> (9.4 – 19.2)	<u>12.0Σ</u> (8.9 – 15.8)	<u>11.9ð</u> (8.8 – 15.8)	15.7 (11.7 – 20.5)
CCR6-CXCR3- (Th2) (%)	<u>19.6‡</u> (14.9 – 22.1)	<u>16.4Ω</u> (12.7 – 23.1)	14.5 (9.2 – 23.6)	12.7 (8.5 – 16.8)
CCR6-CXCR3-CCR4- (%)	1.7 (1.2 – 4.8)	1.1 (0.7 – 2.4)	1.1 (0.2 – 2.4)	1.0 (0.4 – 2.8)
CCR6-CXCR3-CCR4+ (%)	<u>15.1i</u> (12.4 – 20.1)	<u>15.3\$</u> (9.8 – 21.5)	14.5 (6.8 – 21.6)	10.8 (7.6 – 15.1)
CCR6-CXCR3+ (Th1) (%)	5.4 (4.1 – 9.8)	5.7 (3.7 – 9.8)	5.2 (3.6 – 8.9)	6.1 (4.4 – 9.8)
CCR6-CXCR3+CCR4- (%)	0.9 (0.0 – 2.4)	0.4 (0.0 – 1.2)	0.6 (0.2 – 1.2)	0.7 (0.0 – 2.2)
CCR6-CXCR3+CCR4+ (%)	4.2 (2.9 – 6.6)	4.7 (3.4 – 9.3)	4.1 (1.9 – 7.8)	4.8 (3.2 – 7.7)

Table 4.6:- Relative distribution (%) of CD25+CD134+ activated CD4+ T-cells and their subpopulations, following stimulation with Aspergillus within newly-diagnosed, treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. Underlined figures denote significant results. † = p<0.05 as compared to HC; Δ = p<0.05 as compared to HC; ‡ = p<0.01 as compared to HC; Π = p<0.05 as compared to HC; ¶ = p<0.05 as compared to HC; Σ = p<0.01 as compared to HC; ð = p<0.05 as compared to HC; ‡ = p<0.01 as compared to HC; Ω = p<0.05 as compared to HC; i = p<0.05 as compared to HC; \$ = p<0.05 as compared to HC. Figures denote median and inter-quartile ranges (IQR).

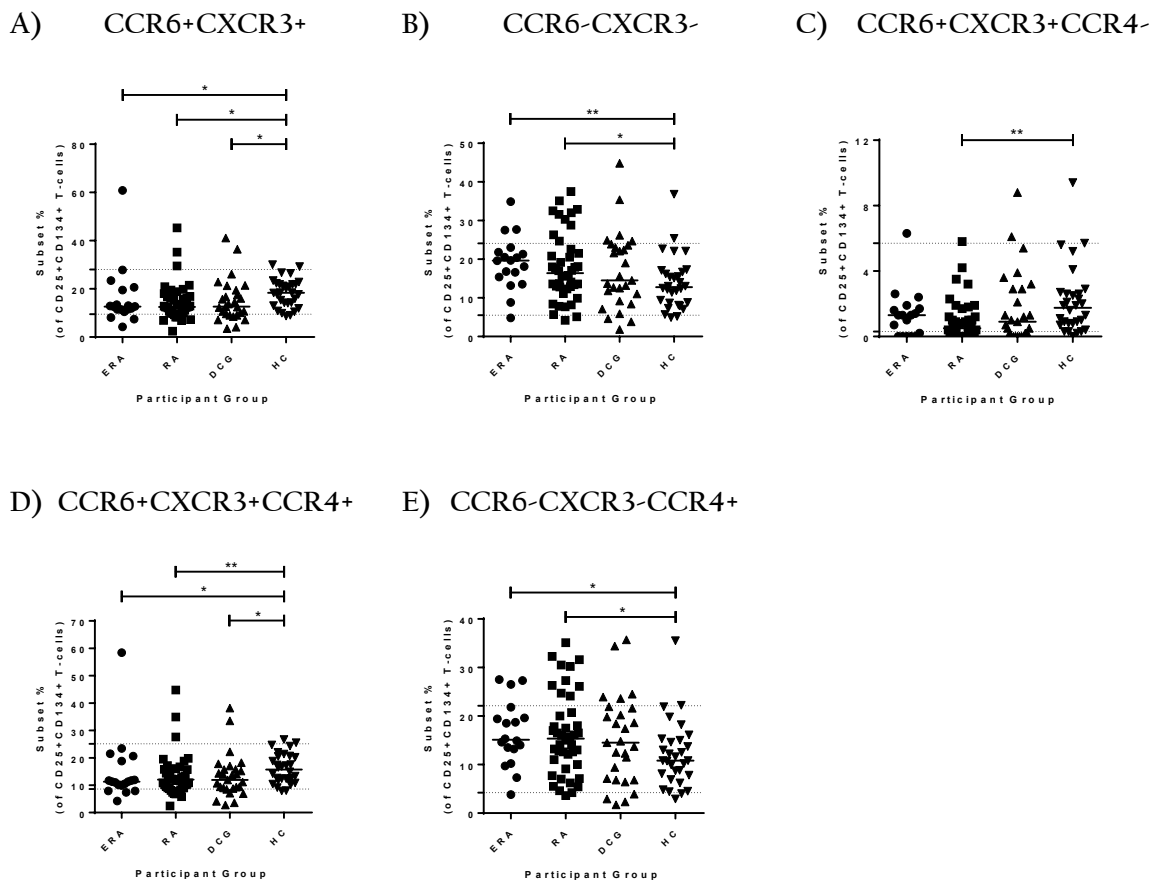


Figure 4.10:- Relative distribution (%) of A) CCR6+CXCR3+; B) CCR6-CXCR3-; C) CCR6+CXCR3+CCR4-; D) CCR6+CXCR3+CCR4+ and E) CCR6-CXCR3-CCR4+ activated CD4+ T-cells following stimulation with aspergillus, within newly-diagnosed, treatment-naïve RA patients (ERA), longstanding RA patients (RA), as well as disease (DCG) and healthy (HC) controls. ** = $p < 0.01$, * = $p < 0.05$, ns = non-significant; denotes 5th and 95th percentiles of healthy controls.

With the addition of CCR4, a significant reduction in the relative distribution of CCR4- Th1/17 cells was observed within RA patients, as compared to HC ($p < 0.01$) (Figure 4.10C). A significant reduction in the relative distribution of CCR4+ Th1/17 cells was also observed within ERA and RA patients ($p < 0.05$ and $p < 0.01$, respectively), as well as DCG ($p < 0.01$), as compared to HC (Figure 4.10D; Table 4.6). Accompanying these reductions in Th1/Th17 cells, a significant increase in the relative distribution of CCR4+ Th2 cells was observed within ERA and RA patients, as compared to HC ($p < 0.05$, respectively), with 10/56 (17.8%) RA patients also demonstrating CCR4+ Th2 activated CD4+ T-cells percentages above the 95th percentile of HC (5th – 95th percentile = 4.2 – 22.1%) (Figure 4.10E). As such, following stimulation with aspergillus, a significant reduction in the

relative distribution of Th1/17 cells, with a concurrent increase in the relative distribution of Th2 cells, was observed within ERA and RA patients, as compared to HC.

4.3.c. The relative distribution of activated CD4+ T-cell subpopulations are associated with measures of clinical outcome within newly-diagnosed, treatment-naïve and longstanding RA patients in an antigen-specific manner

4.3.c.i. PHA - With the antigen-specific CD4+ T-cell responses of ERA and RA patients dominated by an increase in Th2 cells, following stimulation with PHA, CMV and aspergillus, combined with evidence for concurrent reductions in Th1/17 and Th17 cells documented, following stimulation with aspergillus and CMV, respectively, a thorough investigation for associations between the relative distribution of activated CD4+ T-cell responses and patient clinical outcomes seemed warranted. As such, despite evidence for a reduction in the relative distribution of aTcs within ERA and RA patients, no associations between the relative distribution of aTcs and the current disease activity measures erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and DAS28 scores were observed within ERA or RA patients, following stimulation with PHA (data not shown). Furthermore, no significant difference in ESR, CRP or DAS28 scores was observed between ERA and RA patients demonstrating aTc percentages below the 5th percentile of HC, and ERA and RA patients demonstrating aTc percentages within the 5th and 95th percentile of HC (Figure 4.11 and data not shown).

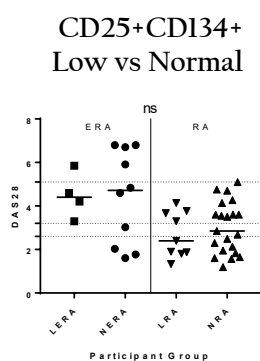


Figure 4.11:- DAS28 scores within newly-diagnosed, treatment-naïve RA (ERA) and longstanding RA (RA) patients presenting with CD25+CD134+ activated CD4+ T-cell percentages below the 5th percentile of healthy controls (LERA and LRA, respectively), as compared to newly-diagnosed, treatment-naïve RA and longstanding RA patients presenting with CD25+CD134+ activated CD4+ T-cell percentages within the 5th and 95th percentiles of healthy controls (NERA and NRA, respectively), following stimulation with PHA. Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. A p-value <0.05 considered statistically significant.

When analysed further according to the expression of CCR6, CXCR3 and CCR4, no associations between the relative distribution of any PHA-specific activated CD4⁺ T-cell subpopulation characterized and ESR, CRP and DAS28 scores were observed (data not shown). Moreover, despite evidence for a non-significant increase in the relative distribution of Th2 cells, above the 95th percentile of HC, within a proportion of RA patients, no significant difference in ESR, CRP or DAS28 scores was observed within RA patients demonstrating CCR4⁻ Th2 or CCR4⁺ Th2 cells above the 95th percentile of HC, as compared to RA patients demonstrating CCR4⁻ Th2 and CCR4⁺ Th2 cells within the 95th percentile of HC (data not shown). As such, following stimulation with PHA, no significant associations between patient clinical outcomes, aTcs and any activated CD4⁺ T-cell subpopulation characterized were observed within ERA or RA patients.

4.3.c.ii. CMV - No associations between CMV IgG serological measurements and ESR, CRP or DAS28 scores were observed within ERA or RA patients (data not shown). Furthermore, following stimulation with CMV, no association between the relative distribution of aTcs and ESR, CRP or DAS28 scores was observed within ERA or RA patients (data not shown). A trend towards an increase in DAS28 scores was, however, observed within CMV positive RA patients, as compared to CMV negative RA patients, according to aTc responses. However, this observation failed to reach statistical significance ($p=0.0886$). No such relationship was observed within ERA patients ($p=0.6114$) (Figure 4.12A). With the exclusion of CMV negative patients, no associations between the relative distribution of aTc responses and ESR, CRP or DAS28 scores was observed within ERA patients (data not shown). In contrast, a positive association between the relative distribution of aTcs and CRP ($r=0.6710$; $p=0.0044$) was observed within RA patients (Figure 4.12B). No further associations with ESR or DAS28 scores were observed.

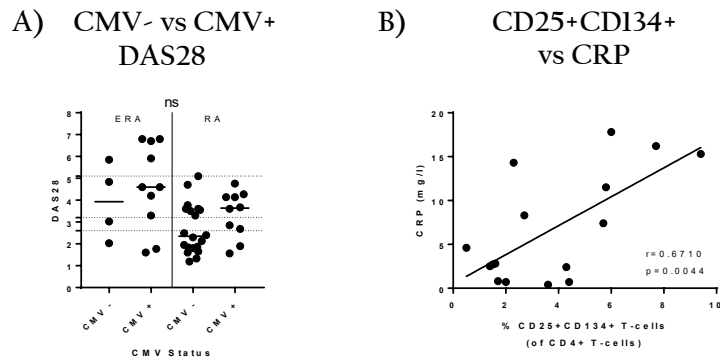


Figure 4.12:- Association of activated CD4+ T-cell subpopulation percentages with ESR, CRP and DAS28 scores, within newly-diagnosed, treatment-naïve RA and longstanding RA patients, following stimulation with CMV. A) DAS28 scores within CMV- and CMV+ newly-diagnosed, treatment-naïve RA (ERA) and longstanding RA (RA) patients, according to CD25+CD134+ activated CD4+ T-cell responses. Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. B) Percentage of CD25+CD134+ activated T-cells vs CRP within longstanding RA patients. A CD25+CD134+ activated CD4+ T-cell response $\geq 0.3\%$ was considered to represent a positive response to CMV stimulation. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables. A p-value <0.05 was considered statistically significant.

With the addition of CCR6 and CXCR3, a negative association between the relative distribution of Th17 cells and DAS28 score was observed within ERA patients ($r = -0.5000$; $p = 0.1411$); with the relative distribution of CCR4+ Th17 cells seemingly contributing more to this relationship than their CCR4- counterparts ($r = -0.5183$; $p = 0.1248$ and data not shown) (Figure 4.13A - B). No such relationship was observed within RA patients. Moreover, despite evidence for a reduction in Th17 and CCR4+ Th17 cells within RA patients, as compared to ERA patients and DCG, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating Th17 or CCR4+ Th17 cell percentages below the 5th percentile of HC, as compared to RA patients demonstrating Th17 cell percentages within the 5th and 95th percentile of HC (data not shown).

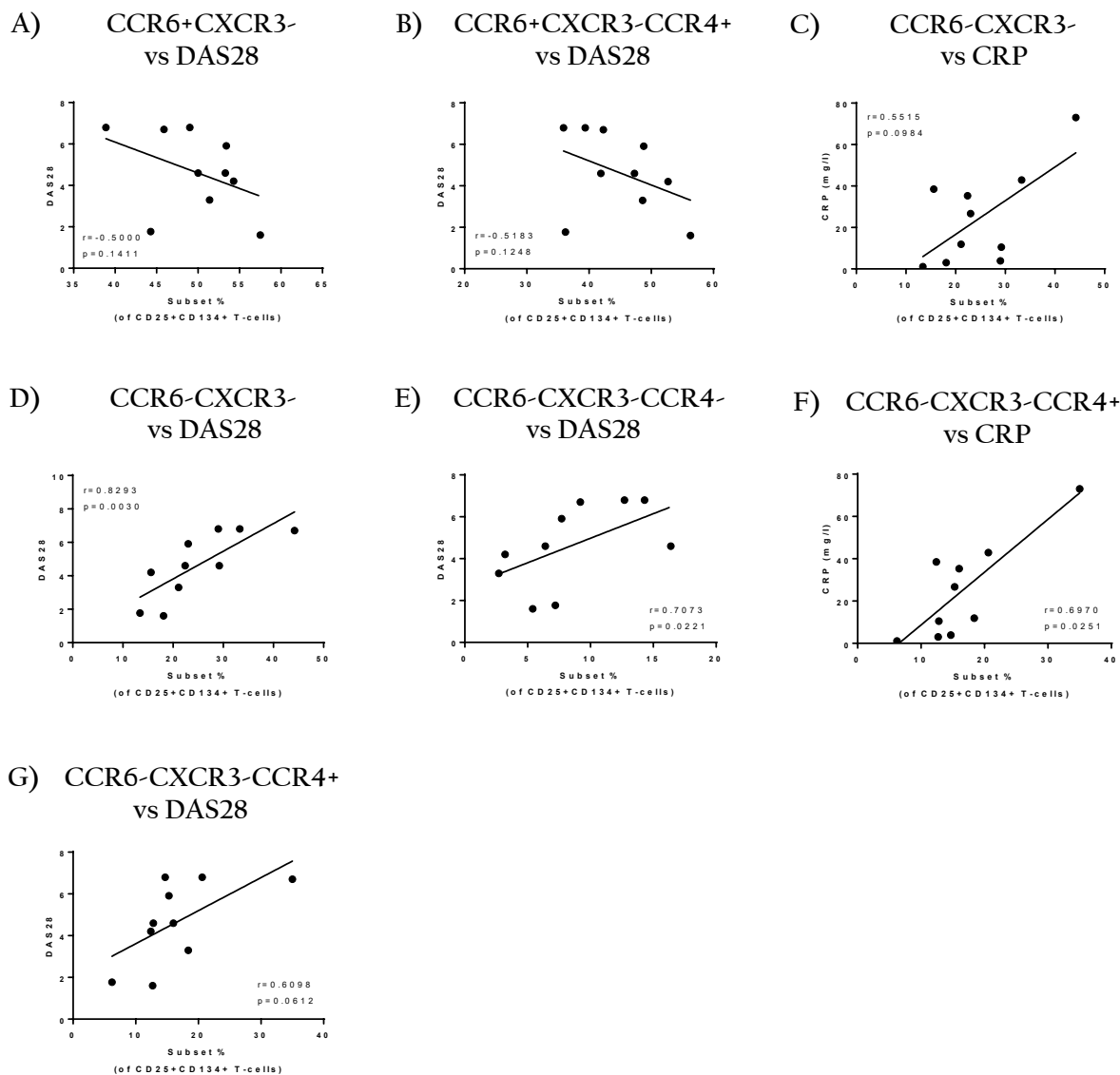


Figure 4.13:- Association of activated CD4⁺ T-cell subpopulation percentages with CRP and DAS28 scores, within newly-diagnosed, treatment-naïve RA patients, following stimulation with CMV. A) Percentage of CCR6+CXCR3⁻ Th17 activated T-cells vs DAS28; B) Percentage of CCR6+CXCR3-CCR4⁺ Th17 activated T-cells vs DAS28. C - D) Percentage of CCR6-CXCR3⁻ Th2 activated T-cells vs CRP and DAS28, respectively. E) Percentage of CCR6-CXCR3-CCR4⁻ Th2 activated T-cells vs DAS28. F) Percentage of CCR6-CXCR3-CCR4⁺ activated T-cells vs CRP. G) Percentage of CCR6-CXCR3-CCR4⁺ activated T-cells vs DAS28. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables. A p-value < 0.05 was considered statistically significant.

Accompanying these associations with CCR4⁺ Th17 cells, positive associations between the relative distribution of Th2 cells, CRP ($r = 0.5515$; $p = 0.0984$) and DAS28 scores ($r = 0.8293$; $p = 0.0030$) were observed within ERA patients (Figure 4.13C – D). With the addition of CCR4, a positive association between the relative distribution of CCR4⁻ Th2 cells and DAS28 scores was observed within ERA patients ($r = 0.7073$; $p = 0.0221$); with positive associations between the relative distribution of CCR4⁺ Th2 cells, CRP ($r = 0.6970$;

p=0.0251) and DAS28 scores ($r=0.6098$; $p=0.0612$) also observed (Figure 4.13E-G). In contrast to the relationships observed within ERA patients, no associations between the relative distribution of Th2 cells, or indeed their CCR4⁻ and CCR4⁺ subpopulations, and ESR, CRP or DAS28 scores were observed within RA patients (data not shown). Furthermore, despite evidence for a non-significant increase in Th2 cells within RA patients, as compared to HC, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating Th2 cell percentages above the 95th percentile of HC and those demonstrating Th2 cell percentages within the 95th percentile of HC (data not shown). In summary, following stimulation with CMV, an increase in the relative distribution of CCR4⁺ Th17 cells appears to be associated with a favourable clinical outcome within ERA patients; whilst an increase in both CCR4⁻ and CCR4⁺ Th2 cells appears to correlate with an adverse clinical presentation in ERA patients. These relationships, however, are completely abrogated within RA patients in which csDMARD therapeutic regimens have been previously established.

4.3.c.iii. *Aspergillus* - Following stimulation with aspergillus, a positive association between the relative distribution of aTcs and DAS28 scores was observed within ERA patients ($r=0.6402$; $p=0.0282$) (Figure 4.14A). No such relationship was, however, observed within RA patients (data not shown). Despite a lack of evidence for a significant difference in the relative distribution of Th17 cells within ERA and RA patients, as compared to DCG and HC, with the addition of CCR6 and CXCR3, a positive association between the relative distribution of Th17 cells and DAS28 scores was observed within ERA patients ($r=0.7193$; $p=0.0084$); a result in direct juxtaposition to those observed following stimulation with CMV (Figure 4.14B). With the addition of CCR4, a positive association between the relative distribution of CCR4⁺ Th17 cells and DAS28 scores was observed within ERA patients ($r=0.7715$; $p=0.0033$) (Figure 4.14C). No such relationship was, however, observed within RA patients.

No further associations between ESR, CRP and DAS28 scores were observed with the relative distribution of Th1/17, Th2 or Th1 cells, irrespective of CCR4 expression, within ERA and RA patients (data not shown). Moreover, despite evidence for split populations in the relative distribution of Th2 and CCR4+ Th2 cells of RA patients, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating Th2 and CCR4+ Th2 cell percentages above the 95th percentile of HC, and those demonstrating CCR4+ Th2 cell percentages below the 95th percentile of HC (data not shown). As such, following stimulation with aspergillus, an increase in the relative distribution of aTcs and CCR4+ Th17 cells is associated with an adverse clinical presentation within ERA patients; with no such relationships observed with RA patients.

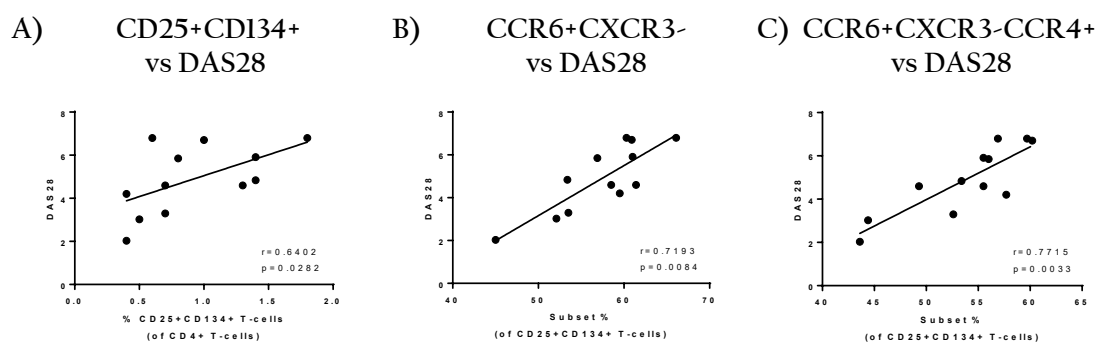


Figure 4.14:- Association of activated CD4⁺ T-cell subpopulation percentages with DAS28 scores, within newly-diagnosed, treatment-naïve RA patients, following stimulation with Aspergillus. A) Percentage of CD25+CD134⁺ activated CD4⁺ T-cells vs DAS28 scores. B) Percentage of CCR6+CXCR3⁻ Th17 activated CD4⁺ T-cells vs DAS28 scores. C) Percentage of CCR6+CXCR3⁻CCR4⁺ Th17 activated CD4⁺ T-cells vs DAS28 scores. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables. A p-value < 0.05 was considered statistically significant.

4.3.d. *The relative distribution of CD25+CD134⁺ activated CD4⁺ T-cells and their subpopulations are altered by the use of biological disease-modifying anti-rheumatic drugs in longstanding RA patients and disease controls*

4.3.d.i. PHA - With alterations to the relative distribution of aTcs and their subpopulations observed within ERA and RA patients, coupled with evidence for antigen-dependent associations with patient clinical outcomes, a more detailed investigation for the effects of therapeutic interventions on aTc responses was deemed necessary. As such, during initial investigations, RA patients and DCG were stratified into two distinct

therapeutic groups, i.e. those undertaking a csDMARD therapeutic regimen and those receiving bDMARD therapeutics. Following stimulation with PHA, a significant reduction in the relative distribution of aTcs was observed within RA patients receiving bDMARD-based therapeutic regimens, as compared to those receiving csDMARD therapeutic interventions ($p<0.05$) (Figure 4.15A). When investigated further for differences in disease activity measures, however, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating aTc percentages below the 5th percentile of HC, as compared to RA patients presenting with aTc percentages within the 5th and 95th percentiles of HC, irrespective of their therapeutic regimen; signifying the existence of a bDMARD-specific effect on aTc responses, following stimulation with PHA, independent of disease activity (Figure 4.15B).

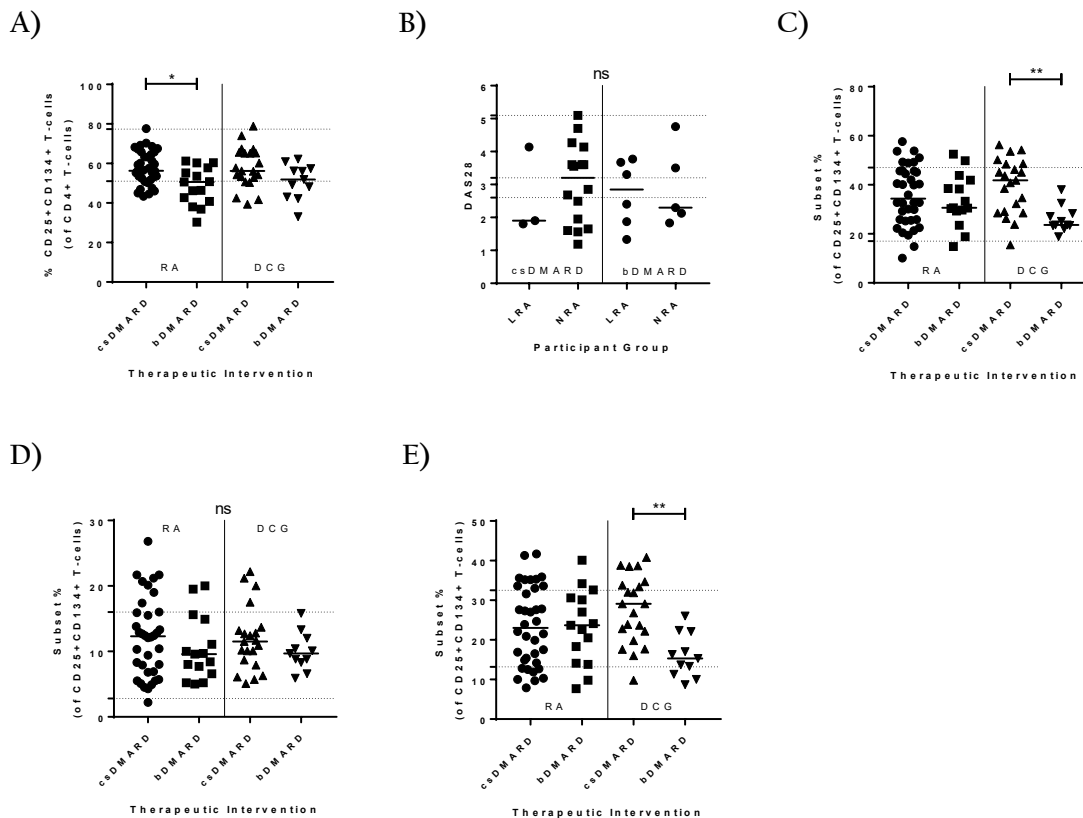


Figure 4.15:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon the use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens, following stimulation with PHA. A) Relative distribution of CD25+CD134+ activated CD4+ T-cells within longstanding RA patients and disease controls. Dotted lines denotes the 5th and 95th percentile of healthy controls. B) DAS28 scores within longstanding RA patients presenting with CD25+CD134+ activated CD4+ T-cell percentages below the 5th percentile of healthy controls (LRA), as compared to longstanding RA patients presenting with CD25+CD134+ activated CD4+ T-cell percentages within the 5th and 95th percentiles of healthy controls (NRA), stratified according to their use of csDMARD- or bDMARD-therapeutic regimens, respectively. Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. C) Relative distribution of CCR6-CXCR3- Th2 cells within longstanding RA patients and disease controls. D) Relative distribution of CCR6-CXCR3-CCR4- Th2 cells within longstanding RA patients and disease controls. E) Relative distribution of CCR6-CXCR3-CCR4+ Th2 cells within longstanding RA patients and disease controls. ** = $p < 0.01$; * = $p < 0.05$. Dotted lines denotes the 5th and 95th percentile of healthy controls.

When analysed further according to the expression of CCR6 and CXCR3 alone, no significant difference in the relative distribution of any activated CD4+ T-cell subpopulation was observed within RA patients or DCG receiving bDMARD- or csDMARD-based therapeutic interventions. However, in keeping with evidence for an increase in Th2 cells within a proportion of RA patients, 7/36 (19.4%) RA patients undertaking csDMARD therapeutic regimens demonstrated Th2 percentages above the

95th percentile of HC (Figure 4.15C). With the addition of CCR4, no significant difference in the relative distribution of any activated CD4⁺ T-cell subpopulation was observed between RA and DCG receiving csDMARD and bDMARD therapeutic regimens. However, 8/36 (22.2%) and 10/36 (27.8%) RA patients undertaking csDMARD therapeutic regimens demonstrated CCR4⁻ and CCR4⁺ Th2 cell percentages above the 95th percentile of HC, respectively (Figure 4.15D - E). When investigated further for differences in disease activity measures, however, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating CCR4⁻ and CCR4⁺ T-cell percentages above the 95th percentile of HC, as compared to RA patients presenting with CCR4⁻ and CCR4⁺ T-cell percentages within the 5th and 95th percentiles of HC, irrespective of their therapeutic regimen (data not shown).

With evidence for a significant reduction in the relative distribution of aTcs within RA patients undertaking bDMARD-based therapeutic regimens, coupled with a non-significant increase in the relative distribution of CCR4⁻ and CCR4⁺ Th2 cells within a proportion of RA patients receiving csDMARDs, RA patients and DCG were stratified further according to their primary therapeutic agents; to facilitate a more thorough inspection for the effects of individual csDMARD and bDMARD therapeutic agents on aTc responses and their composite subpopulations. Following stratification, a significant reduction in the relative distribution of aTcs was observed within RA patients receiving anti-TNF- α therapeutics, as compared to RA patients receiving methotrexate ($p < 0.05$). Furthermore, 7/11 (63.6%) RA patients receiving anti-TNF- α therapeutics demonstrated aTc percentages below the 5th percentile of HC (Figure 4.16A). No significant difference in ESR, CRP or DAS28 scores was, however, observed between RA patients receiving anti-TNF- α therapeutics, demonstrating aTc percentages below the 5th percentile of HC and those demonstrating aTc percentages within the 5th and 95th percentiles of HC (Figure 4.16B).

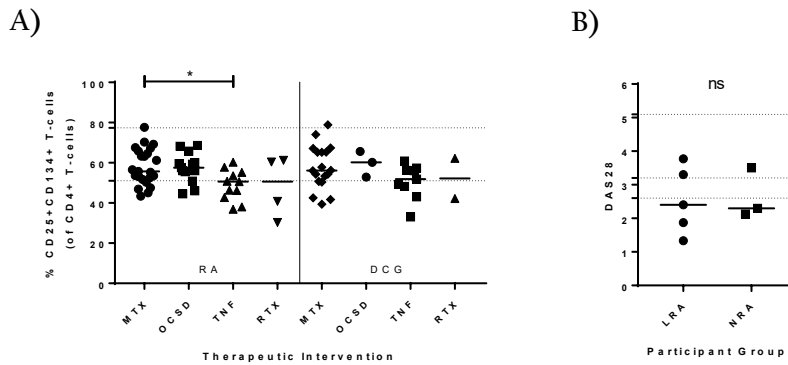


Figure 4.16:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon the use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens, following stimulation with PHA. A) Relative distribution of CD25+CD134+ activated CD4+ T-cells within longstanding RA patients and disease controls undertaking methotrexate-, methotrexate substitute-, anti-TNF- α monoclonal- and rituximab-based therapeutic regimens. MTX= methotrexate, OCSD = other csDMARD, TNF = tumour necrosis factor alpha, RTX = rituximab. Dotted lines denotes the 5th and 95th percentile of healthy controls. B) DAS28 scores within longstanding RA patients, undertaking anti-TNF- α therapeutic regimens, presenting with CD25+CD134+ activated CD4+ T-cell percentages below the 5th percentile of healthy controls (LRA), as compared to longstanding RA patients presenting with CD25+CD134+ activated CD4+ T-cell percentages within the 5th and 95th percentiles of healthy controls (NRA). Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. * = $p < 0.05$, ns = non-significant.

When analysed further according to the expression of the chemokine receptors CCR6, CXCR3 and CCR4, no significant difference in the relative distribution of any activated CD4+ T-cell subpopulation was observed within RA patients or DCG receiving methotrexate-, methotrexate substitute-, anti-TNF- α or rituximab-based therapeutic regimens; with neither methotrexate or methotrexate substitutes seemingly contributing more to the observed increase in CCR4- and CCR4+ Th2 cells (data not shown). As such following stimulation with PHA, the relative distribution of CD25+CD134+ activated CD4+ T-cells is significantly reduced within RA patients undertaking bDMARD-based therapeutic regimens, consisting of anti-TNF- α monoclonal agents, as compared to RA patients and DCG receiving methotrexate-based therapies. When assessed further for associations with current disease activity measures, however, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients receiving anti-TNF- α therapeutics, demonstrating aTc responses below the 5th percentile of HC, and RA patients demonstrating aTc percentages with the 5th and 95th percentile of HC. Taken

together, these results indicate that the reduction in aTc responses observed within RA patients undertaking bDMARD therapeutic regimens, consisting of anti-TNF- α monoclonals, is an artefactual phenomenon in response to the utilization of anti-TNF- α monoclonal agents, independent of disease activity. Supplementary to these anti-TNF- α -specific effects, a non-significant increase in Th2 cell percentages was also observed within RA patients undertaking csDMARD therapeutic regimens. However, no singular therapeutic agent could be deemed responsible for the increase in this activated CD4⁺ T-cell subpopulation.

4.3.d.ii. CMV - No significant difference in CMV IgG serological measurements were observed between RA patients and DCG undertaking csDMARD or bDMARD therapeutic regimens (data not shown). Furthermore, following stimulation with CMV, no significant difference in the relative distribution of aTcs was observed within RA patients and DCG receiving csDMARD- or bDMARD-based immunosuppressive therapies, with/without the exclusion of CMV negative RA patients and DCG (data not shown). When analysed further according to the expression of CCR6 and CXCR3 alone, no significant difference in the relative distribution of Th17, Th1/17, Th2 or Th1 cells was observed within RA patients or DCG undertaking csDMARD and bDMARD therapeutic interventions (data not shown). A trend towards a reduction in Th17 cells was observed within RA patients undertaking bDMARD-based therapeutic regimens, as compared to those receiving csDMARD therapeutic regimens. However, this observation failed to reach statistical significance ($p=0.1464$) (Figure 4.17A). With the addition of CCR4, however, a significant reduction in the relative distribution of CCR4⁺ Th17 cells was observed within RA patients undertaking bDMARD therapeutic regimens, as compared to those receiving csDMARD-based therapeutics ($p<0.05$) (Figure 4.17B).

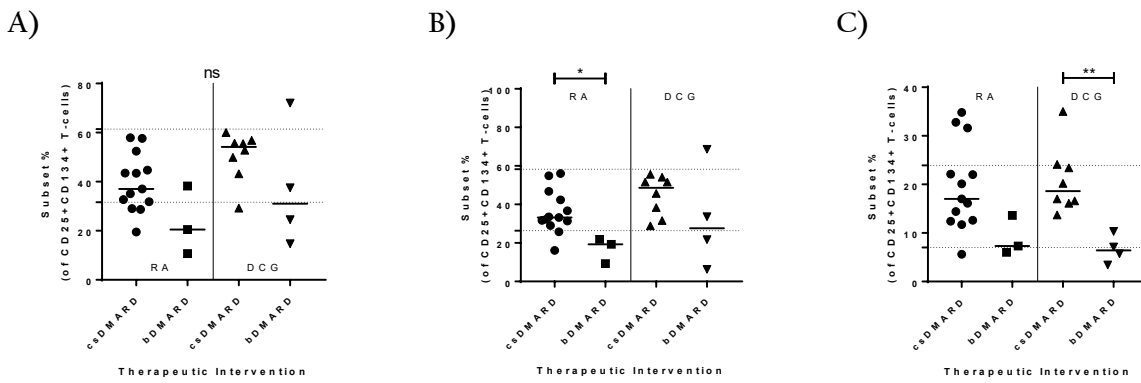


Figure 4.17:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon the use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens, following stimulation with CMV. A) Relative distribution of CCR6+CXCR3-
Th17 activated CD4+ T-cells within longstanding RA patients and disease controls undertaking csDMARD and bDMARD therapeutic regimens. B) Relative distribution of CCR6+CXCR3-CCR4+ Th17 activated CD4+ T-cells within longstanding RA patients and disease controls undertaking csDMARD and bDMARD therapeutic regimens. C) Relative distribution of CCR6-CXCR3-CCR4+ Th2 activated CD4+ T-cells, following stimulation with CMV, within longstanding RA patients and disease controls undertaking csDMARD and bDMARD therapeutic regimens. ** = $p < 0.01$, * = $p < 0.05$, ns = non-significant. Dotted lines denotes the 5th and 95th percentile of healthy controls.

Accompanying this reduction in CCR4+ Th17 cells, a trend towards a reduction in the relative distribution of CCR4+ Th2 cells was observed within RA patients receiving bDMARD-based therapeutics, as compared to RA patients undertaking csDMARD therapeutic regimens, although this observation failed to reach statistical significance ($p=0.0821$). A significant reduction in the relative distribution of CCR4+ Th2 cells was, however, observed within DCG undertaking bDMARD-based therapeutic regimens, as compared to those receiving csDMARDs ($p < 0.01$) (Figure 4.17C). No further alterations to the relative distribution of any other activated CD4+ T-cell subpopulation were observed within RA patients or DCG receiving bDMARD- or csDMARD-based therapeutics (data not shown).

With the full stratification of RA patients and DCG according to their primary therapeutic agent, low participant numbers were experienced; with no CMV positive individuals shown to be undergoing a bDMARD therapeutic regimen, consisting of anti-TNF- α monoclonal agents. Accordingly, due to a lack of statistical power, no significant difference in the relative distribution of aTcs, or their composite subpopulations, was

observed within RA patients or DCG undertaking methotrexate-, methotrexate substitute- or rituximab-based therapeutic regimens (data not shown). Moreover, no conclusions regarding the disease activity of RA patients demonstrating reductions in CCR4⁺ Th17 and CCR4⁺ Th2 cells could be drawn, in relation to their individual therapeutic regimens. To summarize, following stimulation with CMV, a reduction in the relative distribution of CCR4⁺ Th17 and CCR4⁺ Th2 cells was observed within RA patients undertaking bDMARD therapeutic regimens, as compared to those receiving csDMARD-based therapeutics. However, due to a lack of statistical power, these changes could not be fully investigated, nor attributed to the effects of individual therapeutic agents.

4.3.d.iii. Aspergillus - Following stimulation with aspergillus, no significant difference in the relative distribution of aTcs was observed within RA patients and DCG undertaking csDMARD or bDMARD therapeutic regimens (data not shown). With the addition of CCR6 and CXCR3, no significant difference in the relative distribution of any activated CD4⁺ T-cell subpopulation was observed within RA patients and DCG receiving csDMARD and bDMARD therapeutics (data not shown). A trend towards an increase in the relative distribution of Th2 cells was observed within RA patients receiving csDMARD-based therapeutics, as compared to those undertaking bDMARD therapeutic regimens. However, this observation failed to reach statistical significance ($p=0.1609$). Despite the lack of statistical significance, 9/27 (33.3%) RA patients undertaking csDMARD therapeutic regimens demonstrated Th2 cell percentages above the 95th percentile of HC; a trend not observed within RA patients receiving bDMARDs (Figure 4.18A).

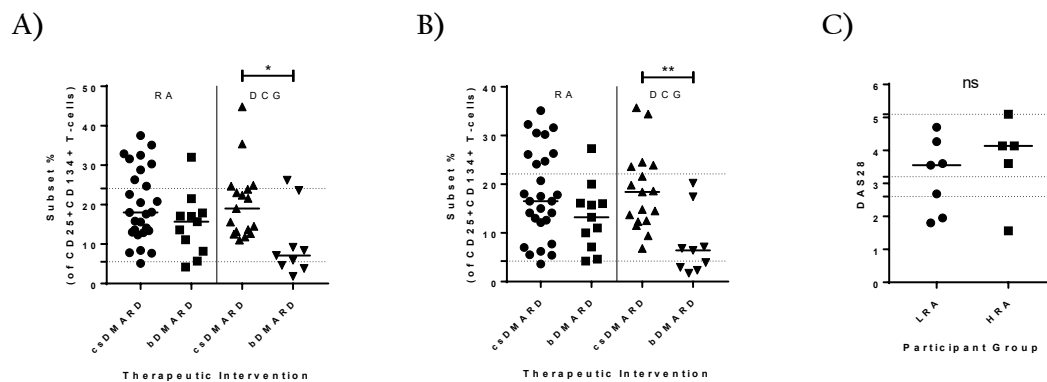


Figure 4.18:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon the use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens, following stimulation with Aspergillus. A) Relative distribution of CCR6-CXCR3- Th2 activated CD4+ T-cells within longstanding RA patients and disease controls undertaking csDMARD and bDMARD therapeutic regimens. B) Relative distribution of CCR6-CXCR3-CCR4+ Th2 activated CD4+ T-cells within longstanding RA patients and disease controls undertaking csDMARD and bDMARD therapeutic regimens. Dotted lines denotes the 5th and 95th percentile of healthy controls. C) DAS28 scores within longstanding RA patients, undertaking csDMARD therapeutic regimens, presenting with CCR6-CXCR3-CCR4+ Th2 activated CD4+ T-cell percentages within the 5th and 95th percentile of healthy controls (LRA), as compared to longstanding RA patients presenting with CCR6-CXCR3-CCR4+ Th2 activated CD4+ T-cell percentages above the 95th percentiles of healthy controls (HRA). Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. ** = $p < 0.01$, * = $p < 0.05$, ns = non-significant.

With the addition of CCR4, a trend towards an increase in the relative distribution of CCR4+ Th2 cells was also observed within RA patients undertaking csDMARD therapeutic regimens, as compared to those receiving bDMARD-based therapeutics. However, this observation failed to reach statistical significance ($p=0.1472$). Nevertheless, 9/27 (33.3%) RA patients undertaking csDMARD therapeutic regimens demonstrated CCR4+ Th2 cell percentages above the 95th percentile of HC (Figure 4.18B). When investigated further for differences in disease activity measures, however, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients undertaking csDMARD therapeutic regimens, demonstrating CCR4+ Th2 cell percentages above the 95th percentile of HC and those demonstrating CCR4+ Th2 cell percentages within the 5th and 95th percentile of HC (Figure 4.18C). No further alterations to any activated CD4+ T-cell subpopulations were observed within RA patients or DCG undertaking csDMARD or bDMARD therapeutic regimens (data not shown).

With the full stratification of RA patients and DCG according to their primary therapeutic agent, no significant difference in the relative distributions of aTcs, or their composite subpopulations, was observed within RA patients or DCG undertaking methotrexate-, methotrexate substitute-, anti-TNF- α - or rituximab-based therapeutic regimens; with no single therapeutic agent seemingly responsible for the non-significant increase in CCR4+ Th2 cells previously observed within a proportion of RA patients (data not shown). As such, following stimulation with aspergillus, the relative distribution of Th2 activated CD4+ T-cells appears increased within a proportion of RA patients undertaking csDMARD-based therapeutic regimens. However, no single therapeutic agent appears responsible for this observation. Moreover, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating Th2 cell percentages above the 95th percentile of HC, and RA patient demonstrating Th2 cell percentages within the 5th and 95th percentile of HC. As such, the observed increase in CCR4+ Th2 cells appears to be an artefact of csDMARD therapeutic usage; irrespective of disease activity.

4.3.e. The longitudinal monitoring of activated CD4+ T-cell subpopulations may be used to assess therapeutic efficacy within newly-diagnosed RA patients, in an antigen-specific manner

4.3.e.i. PHA - With clear associations between patient clinical outcomes and activated CD4+ T-cell subpopulations observed within ERA patients at the time of diagnosis, coupled with evidence for alterations to the relative distribution of activated CD4+ T-cell subpopulations within RA patients, following therapeutic stratification, the utility of measuring antigen-specific CD4+ T-cell parameters to monitor therapeutic efficacy was assessed. As such, the relative distribution of aTcs, and their composite CD4+ T-cell subpopulations, following stimulation with PHA, CMV and aspergillus, were longitudinally monitored within ERA patients at 3-, 6- and 12-monthly intervals, following their initiation of csDMARD therapeutic regimens. Following stimulation with PHA, no

significant difference in the relative distribution of aTcs was observed within ERA patients up to 12-months after diagnosis. A trend towards an increase in the relative distribution of aTcs was observed within ERA patients 12-month post-diagnosis, as compared to those at the time of diagnosis. However, this relationship failed to reach statistical significance ($p=0.0681$) (Figure 4.19A). When analysed further according to the expression of CCR6, CXCR3 and CCR4, no significant difference in the relative distribution of any activated CD4⁺ T-cell subpopulation was observed within ERA patients up to 12-months after the initiation of a csDMARD therapeutic regimen (data not shown).

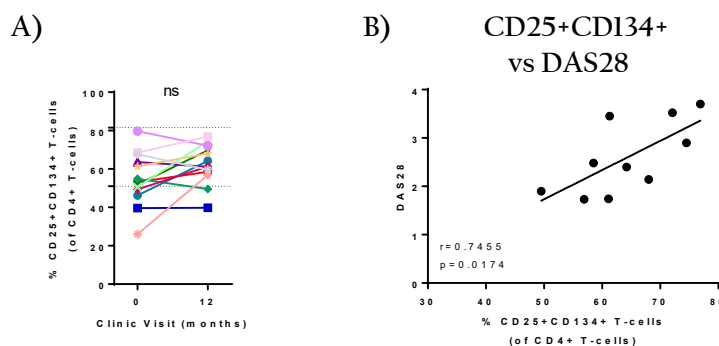


Figure 4.19:- Longitudinal monitoring and clinical outcome associations of CD25+CD134+ activated CD4⁺ T-cells within newly-diagnosed RA patients 12-months after the initiation of conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimens, following stimulation with PHA. A) Relative distribution of CD25+CD134+ activated CD4⁺ T-cells 12-months after diagnosis, as compared to the time of diagnosis. B) Relative distribution of CD25+CD134+ activated CD4⁺ T-cells compared to DAS28 scores, within newly-diagnosed RA patients 12-months post diagnosis. Dotted lines denotes the 5th and 95th percentile of healthy controls. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables. A p-value < 0.05 was considered statistically significant. ns = non-significant

With evidence for alterations in PHA-specific aTc responses observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens, the relationship between this CD4⁺ T-cell subpopulation and disease activity was further explored; with the predictability of therapeutic efficacy, as a primary function of activated CD4⁺ T-cell response monitoring at diagnosis of particular interest. Insufficient ESR measurements were available for assessment 12-months after diagnosis. Therefore, all subsequent comparisons between activated CD4⁺ T-cell subpopulations with the disease activity measure ESR were excluded. In this regard, when analysed further for

associations with the patient clinical outcome measures CRP and DAS28 scores, a positive association between the relative distribution of aTcs and DAS28 scores was observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens ($r=0.7455$; $p=0.0174$) (Figure 4.19B). When investigated further for changes in disease activity and the relative distribution of aTcs over time, however, no association was observed between the delta change in CRP and DAS28 scores and the delta change in aTc responses (data not shown). Furthermore, no association between the relative distribution of aTcs at the time of diagnosis and the delta change in CRP or DAS28 scores was observed within ERA patients 12-months after the initiation of a csDMARD therapeutic regimens. As such, following stimulation with PHA, an increase in the relative distribution of aTcs, 12-months after the time of diagnosis, appears to correspond with an adverse clinical response within ERA patients. However, neither the relative distribution of aTcs at the time of diagnosis, nor the change in aTc percentages, following 12-months of compliance with a csDMARD therapeutic regimen, are predictive of patient disease activity. Therefore, the monitoring of PHA-specific CD4⁺ T-cell responses may not be used to predict or monitor therapeutic efficacy.

4.3.e.ii. *CMV* - Based upon aTc responses, following stimulation with CMV, no evidence of CMV conversion was evident within ERA patients up to 12-months after diagnosis. Furthermore, no significant difference in the relative distribution of aTcs was observed with ERA patients up to 12-months after the initiation of csDMARD therapeutic regimens. With the exclusion of CMV negative ERA patients, a trend towards a reduction in the relative distribution of aTcs was observed 12-months after the initiation of csDMARD therapeutic regimens. However, this observation failed to reach statistical significance ($p=0.0781$) (Figure 4.20A). With the addition of CCR6 and CXCR3 alone, no significant difference in the relative distribution of any activated CD4⁺ T-cell subpopulation was observed within ERA patients up to 12-months after the initiation of csDMARD therapeutic regimens. A trend towards an increase in the relative distribution

of Th2 cells was observed within ERA patients at 12-month post diagnosis; with 5/8 (62.5%) ERA patients demonstrating Th2 cell percentages above the 95th percentile of HC. However, this observation failed to reach statistical significance ($p=0.0781$) (Figure 4.20B). Furthermore, no significant difference in CRP or DAS28 scores was observed between ERA patients demonstrating Th2 cell percentages above the 95th percentile of HC, and those demonstrating Th2 percentages within the 5th and 95th percentile of HC (data not shown).

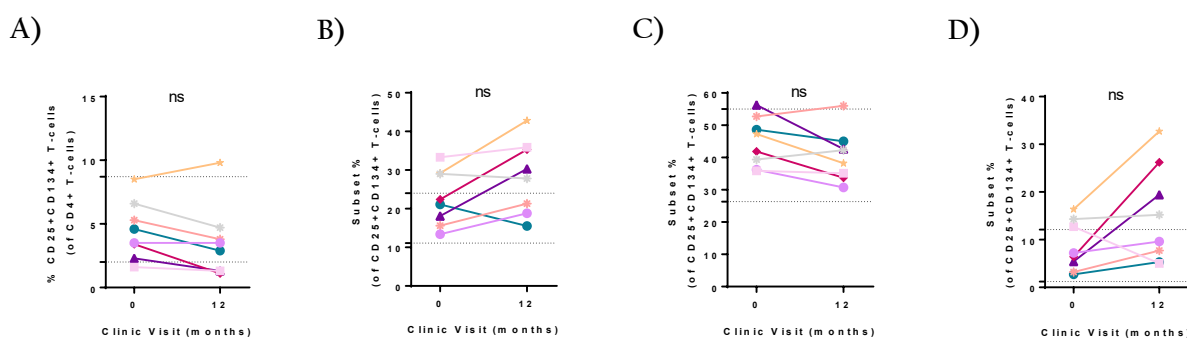


Figure 4.20:- Longitudinal monitoring of CD25+CD134+ activated CD4+ T-cells and their subpopulations within newly-diagnosed RA patients 12-months following the initiation of conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimens, following stimulation with CMV. A) Relative distribution of CD25+CD134+ activated CD4+ T-cells, of CD4+ T-cells. B) Relative distribution of CCR6-CXCR3- Th2 activated CD4+ T-cells. C) Relative distribution of CCR6+CXCR3-CCR4+ Th17 activated CD4+ T-cells. D) Relative distribution of CCR6-CXCR3-CCR4- Th2 activated CD4+ T-cells. Dotted lines denotes the 5th and 95th percentile of healthy controls. ns = non-significant.

With the addition of CCR4, no significant difference in the relative distribution of any activated CD4+ T-cell subpopulation was observed within ERA patients up to 12-months after diagnosis. A trend towards a reduction in the relative distribution of CCR4+ Th17 cells was, however, observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens, as compared to the time of diagnosis; although this observation failed to reach statistical significance ($p=0.0781$) (Figure 4.20C). Additionally, consistent with previous observations in RA patients, a trend towards an increase in the relative distribution of CCR4- Th2 cells was observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens, as compared to the time of diagnosis; with 4/8 (50.0%) ERA patients demonstrating CCR4- Th2 cell percentages

above the 95th percentile of HC. However, this observation failed to reach statistical significance ($p=0.0781$) (Figure 4.20D). Consistent with previous observations, no significant difference in CRP or DAS28 scores was observed between ERA patients demonstrating CCR4⁺ Th2 cell percentages above the 95th percentile of HC and ERA patients demonstrating CCR4⁺ Th2 cell percentages within the 5th and 95th percentile of HC (data not shown).

With clear differences in CMV-specific activated CD4⁺ T-cell subpopulations evident within ERA patients 12-months after the initiation of a csDMARD therapeutic regimen, the relationships between these CD4⁺ T-cell subpopulations and patient clinical outcomes were further investigated. In this regard, no association between the relative distribution of aTcs and the patient clinical outcomes CRP or DAS28 scores was observed within ERA patients up to 12-months after diagnosis (data not shown). When analysed further according to the expression of CCR6 and CXCR3, however, a negative association between the relative distribution of Th17 cells and DAS28 scores was observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens ($r=-0.7143$; $p=0.0881$) (Figure 4.21A). With the addition of CCR4, a negative association between the relative distribution of CCR4⁺ Th17 cells and DAS28 scores was observed within ERA patients 12-months after diagnosis ($r=-0.7857$; $p=0.0480$) (Figure 4.21B). When investigated further for changes in disease activity and the relative distribution of CCR4⁺ Th17 cells over time, a negative association between the delta change in DAS28 scores and the delta change in CCR4⁺ Th17 cell percentages was observed ($r=-0.5357$; $p=0.2357$); indicating that the greatest improvement in disease activity is associated with an increase in the relative distribution of CCR4⁺ Th17 cell (Figure 4.21C). Despite these compelling results, no association between the relative distribution of CCR4⁺ Th17 cells at the time of diagnosis and the delta change in CRP and DAS28 scores was observed; indicating that the monitoring of CCR4⁺ Th17 cells may not be used as a predictor of therapeutic efficacy, but may still prove beneficial in the monitoring of patient disease activity.

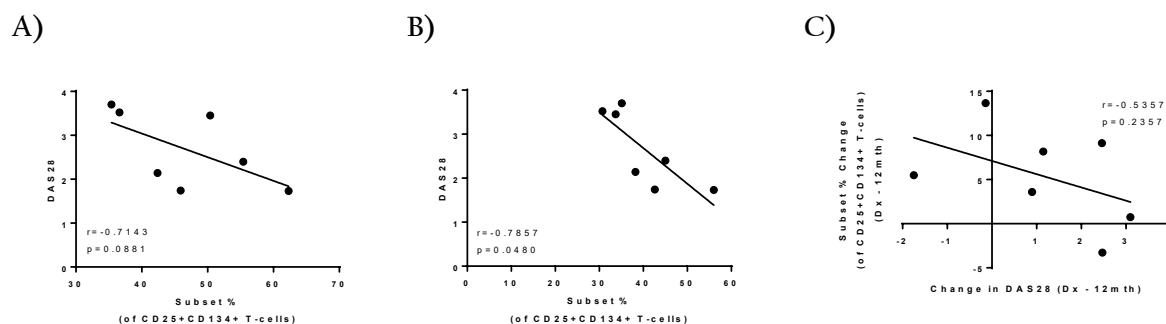


Figure 4.21:- Association of CD25+CD134+ activated CD4+ T-cells and their composite subpopulations, within newly-diagnosed RA patients following stimulation with CMV, with DAS28 scores. A) Relative distribution of CCR6+CXCR3⁻ Th17 activated CD4+ T-cells compared to DAS28 scores. B) Relative distribution of CCR6+CXCR3-CCR4⁺ Th17 activated CD4+ T-cells compared to DAS28 scores. C) Delta change in the relative distribution of CCR6+CXCR3-CCR4⁺ Th17 cells compared to the delta change in DAS28 scores. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables. A p-value < 0.05 considered statistically significant.

Finally, despite evidence for an increase in the relative distribution of Th2 and CCR4⁺ Th2 cells within ERA patients, 12-months after the initiation of a csDMARD therapeutic regimen, no associations with the disease activity measures CRP and DAS28 scores were observed (data not shown). Furthermore, no associations between the delta change in CRP and DAS28 scores were observed with the delta change in Th2 cell percentages; indicating that the monitoring of CMV-specific Th2 cells cannot be used to monitor therapeutic efficacy (data not shown). In conclusion, following stimulation with CMV, 12-months after the initiation of csDMARD therapeutic regimens, an increase in the relative distribution of Th17 cells appears to be indicative of a favourable clinical outcome; with the greatest improvement in disease activity 12-months after diagnosis associated with an increase in the relative distribution of CCR4⁺ Th17 cells.

4.3.e.iii. *Aspergillus* - Following stimulation with aspergillus, no significant difference in the relative distribution of aTcs, or indeed their composite subpopulations, was observed within ERA patients up to 12-months after the initiation of csDMARD therapeutic regimens (data not shown). Furthermore, no relationships were observed between any activated CD4+ T-cell subpopulation and the clinical outcome measures CRP and DAS28 scores (data not shown). With no significant difference in the relative distribution of

aspergillus-specific aTcs up to 12 months after the time of diagnosis, combined with a lack of associations with patient clinical outcome measures, the longitudinal monitoring of aspergillus-specific aTcs provides no benefit in therapeutic efficacy monitoring within newly-diagnosed RA patients.

4.4. Discussion

An infectious aetiology has long been considered a contributing factor to RA pathogenesis, with the gram-negative bacteria *porphyromonas gingivalis*, and the viral agents CMV, HSV and EBV of particular interest (Burgos et al., 2015; Magnusson et al., 2010; Mikuls et al., 2009; Pierer et al., 2012). The causal relationship between the periodontal disease causing *porphyromonas gingivalis* and RA has been comprehensively investigated, with a direct link between periodontal disease, raised PAD4 enzyme levels and the production of anti-CCP antibodies extensively described (Johansson et al., 2016; Mikuls et al., 2009). The direct relationship between RA pathogenesis and the human herpesviruses is, however, less conclusive, despite the isolation of CMV and EBV from the synovium of RA patients, and an aggravated clinical course described in the presence of latent CMV infection (Davis et al., 2012; Magnusson et al., 2010; Pierer et al., 2012). However, with conventional antigen-specific detection methods providing limited data regarding the efficacy of a generated immune response, the precise demarcation of these infectious agents in RA pathogenesis has remained elusive.

A rapid, flow cytometric technique, utilizing a whole blood stimulation method to viral, fungal and bacterial lysates, has however now been described, in which antigen-specific CD4⁺ T-cell responses can be detected and quantified, based upon their expression of the activation markers CD25 and CD134 (Sadler et al., 2014; Zaunders et al., 2009). Such a method provides a viable alternative to the labour-intensive and often expensive tetramer-based examinations that are traditionally used to investigate antigen-specific CD4⁺ T-cell responses; facilitating a more robust investigation of the infectious agents linked to RA pathogenesis (Sadler et al., 2014; Zaunders et al., 2009). With Th17 and Th2 cells previously demonstrated to represent the most relevant T-cell subpopulations to RA pathogenesis, the expansion of this flow cytometric assay to include the chemokine receptors CCR6 (Th17-specific), CXCR3 (Th1-specific) and CCR4 (Th2-specific); a strategy which has been successfully utilized in the characterization of blood-derived

circulating follicular helper CD4⁺ T-cells; a thorough examination of the composite memory CD4⁺ T-cell subpopulations within antigen-specific CD4⁺ T-cell responses is also possible, providing a potential means with which to monitor disease activity and severity within newly-diagnosed, treatment-naïve RA patients (ERA), and longstanding RA patients (RA), in which a therapeutic stratagem has been employed (Ma et al., 2016; Morita et al., 2011).

In order to fully utilize such a method, the normal distribution of CD25⁺CD134⁺ antigen-specific CD4⁺ T-cell (aTc) responses and their subpopulations were fully characterized within healthy controls (HC), following stimulation with PHA, CMV, HSV and aspergillus. Consistent with the mitogenic nature of PHA, aTc responses following stimulation with PHA were significantly increased, as compared to stimulation with CMV, HSV and aspergillus. A significant increase in aTc responses was also observed following stimulation with CMV, as compared to HSV and aspergillus. With the addition of the chemokine receptors CCR6, CXCR3 and CCR4, it was anticipated that each antigenic stimulus would generate a distinct subpopulation expression profile, with viral antigens thought to support the generation of CXCR3⁺ Th1 cell responses and fungal antigens thought to activate CCR6⁺ Th17 cells (Acosta-Rodriguez et al., 2007; Dhalla et al., 2016). However, upon further analysis, it became clear that aTcs generated following stimulation with CMV, HSV and aspergillus were all, in fact, dominated by a Th17 cell subpopulation, with CCR4⁺ Th17 cells more strongly represented than their CCR4⁻ counterparts. The dominance of this Th17 subpopulation did, however, differ according to the antigenic stimulus utilized, with a significant increase in CCR4⁺ Th17 cells observed following stimulation with aspergillus, as compared to CMV, HSV and PHA.

Despite a lack of evidence for distinct subpopulation expression profiles, following stimulation with physiological antigens, the subpopulation expression profile generated following stimulation with the PHA was highly unique, with a significant increase in Th1

cells observed, as compared to stimulation with CMV, HSV and aspergillus. With the addition of CCR4, a significant increase in both CCR4⁻ and CCR4⁺ Th1 cells was observed following stimulation with PHA, as compared to stimulation with CMV, HSV and aspergillus. This increase in Th1 cells was accompanied by a significant increase in Th2 cells, as compared to stimulation with CMV and aspergillus; with a significant increase in CCR4⁺ Th2 cells observed, as compared to stimulation with CMV and aspergillus.

The aTc response generated following stimulation with PHA was not entirely unexpected, with PHA known to bypass the conventional T-cell receptor (TCR) recognition pathways, resulting in widespread T-cell activation (Marshall et al., 2005; Movafagh et al., 2011). However, the subpopulation expression profile observed herein would also suggest that PHA is, in fact, a poor activator of Th17 cells; with far greater Th17 cell percentages generated following stimulation with CMV, HSV and aspergillus, respectively. As such, the use of fungal antigens, such as aspergillus, are perhaps a more appropriate antigenic stimulant to be employed within Th17 cell studies (Acosta-Rodriguez et al., 2007). Future work is, however, required to confirm whether Th17 cell functionality is hampered to the same extent as Th17 cell activation, by PHA stimulation, with cytokine secretion experiments a potential avenue of inquiry. Unfortunately, such investigations were beyond the scope of the present study. In isolation, therefore, these results highlight the importance of utilizing an appropriate antigenic stimulus in the phenotypic study of activated CD4⁺ T-cell subpopulations; with diverse stimuli potentially capable of generating dramatically differing results.

With the normal distribution of aTc responses and their subpopulations established within HC, the effects of age and sex were investigated, prior to their assessment in ERA and RA patients. Within our HC, and universally observed across all antigenic stimulants investigated, no effects of age or sex were observed with the relative distribution of aTcs

or their composite subpopulations. Perhaps, the most surprising finding, therefore, was the lack of age associations with CMV immunoglobulin G (IgG) measurements and the relative distribution of aTcs, following stimulation with CMV. Given that approximately 80% of individuals in the UK ≥ 65 years of age are thought to be infected with cytomegalovirus, our overall CMV detection rate of 53/140 (37.9%) for all participants was unexpected (Vyse, Hesketh and Pebody, 2009). With CMV serological examinations and aTc percentages positively associated and in concordance for CMV positivity in 11/13 (84.6%) HC, however, these results appear to accurately reflect CMV carriage rates within our participants. As such, the reduced carriage rates observed within our study may be a result of differences in the CMV detection methods utilized in this and previous studies (Parry et al., 2016; Vyse, Hesketh and Pebody, 2009). With no significant difference in CMV serological status observed between ERA and RA patients, as well as age- and sex-matched disease (DCG) and healthy (HC) controls, the reduced carriage rates observed within this study were, however, appropriately controlled.

Following the thorough investigation of aTc responses within HC, the relative distribution of aTcs generated within ERA and RA patients were compared to those observed within DCG and HC; with the utility of measuring aTc responses in disease severity and therapeutic efficacy monitoring of particular interest. Following stimulation with PHA, a significant reduction in aTcs was observed within ERA and RA patients, as compared to HC. With a significant reduction in aTcs also observed within DCG, as compared to HC, this reduction in aTc responses is not an RA-specific phenomenon. A reduction in aTcs is perhaps to be expected within RA patients and DCG, given the known immunosuppressive effects of conventional synthetic (cs) and biological (b) disease-modifying anti-rheumatic drugs (DMARDs) (Baggott and Morgan, 2009; Liang et al., 2013). The observed reduction in aTcs within ERA patients is, however, unexpected and implies that CD4⁺ T-cell activation is already impaired within RA patients, prior to the implementation of therapeutic regimens. With a heightened state of disease activity

present within ERA patients at the time of diagnosis, it could be postulated that CD4⁺ T-cells in ERA, and RA, patients in which an effective therapeutic regimen remains to be established, are already present in an activated state and as such are rendered anergic to further exogenous stimulation. However, with no associations between the relative distribution of aTcs and the disease activity measures ESR, CRP and DAS28 scores observed within ERA patients, or indeed RA patients, this hypothesis remains unsubstantiated.

With the addition of CCR6, CXCR3 and CCR4, no significant alterations to any T-cell subpopulation were observed within ERA or RA patients, following stimulation with PHA. A trend towards an increase in the relative distribution of CCR4⁻ and CCR4⁺ Th2 cells was observed within RA patients; with 19.6% and 21.4% of RA patients demonstrating CCR4⁻ and CCR4⁺ Th2 cell percentages above the 95th percentile of HC. No such increase was, however, observed within ERA patients; signifying the presence of a non-significant response to therapeutic intervention, within a proportion of RA patients. Nevertheless, when investigated further for differences in disease activity measures, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating Th2 cell percentages above the 95th percentile of HC and RA patients demonstrating Th2 cell percentages within the 95th percentile of HC, irrespective of CCR4 expression. On a surface level, therefore, the observed increase in Th2 cells appears to be an artefactual change as a result of therapeutic intervention.

Far more compelling results were obtained following stimulation with CMV, with a significant reduction in aTcs observed within RA patients, as compared to HC; with a trend towards a reduction in aTcs also observed within ERA patients and DCG, as compared to HC. Upon first inspection, these results appear entirely consistent with the results obtained following stimulation with PHA. However, when investigated further for changes in disease activity measures, a trend towards an increase in DAS28 scores was

observed within CMV positive RA patients, as compared to CMV negative RA patients, according to aTc responses. Furthermore, with the exclusion of CMV negative RA patients, a positive association between the relative distribution of aTcs and CRP was also observed with RA patients; altogether suggesting that an increase in CMV-specific CD4⁺ T-cell responses is associated with an adverse clinical presentation within RA patients; a result entirely consistent with previous literature (Davis et al., 2012; Pierer et al., 2012).

With the addition of CCR6, CXCR3 and CCR4, a significant reduction in the relative distribution of CCR4⁺ Th17 cells was observed within RA patients, as compared to ERA patients; with a trend towards a reduction in CCR4⁺ Th17 cells also observed as compared to HC. A non-significant increase in the relative distribution of CCR4⁺ Th2 cells was also observed within RA patients, as compared to HC; a trend consistent with the results obtained following stimulation with PHA. No such subpopulation alterations were, however, observed with ERA patients. When investigated further for evidence of associations with patient clinical outcomes, a disparity between ERA and RA patients was clearly present. Within ERA patients, a negative association between the relative distribution of CCR4⁺ Th17 cells and DAS28 scores was observed; with a positive association between the relative distribution of CCR4⁻ Th2 and CCR4⁺ Th2 cells also evident with CRP and DAS28 scores, respectively. Altogether, these results would suggest that an increase in CMV-specific Th17 cells is associated with a favourable clinical presentation in ERA patients; with an increase in CMV-specific Th2 cells associated with an adverse clinical presentation. No such relationships were, however, observed in RA patients; a potential impact of therapeutic intervention and results still in keeping with those obtained following stimulation with PHA.

In contrast to the results obtained following stimulation with PHA and CMV, no significant difference in the relative distribution of aTcs was observed within ERA or RA patients following stimulation with aspergillus. With the addition of CCR6, CXCR3 and

CCR4, however, a significant reduction in the relative distribution of CCR4⁻ and CCR4⁺ Th1/17 cells was observed within RA patients, and, to a lesser extent, ERA patients, as compared to HC. Furthermore, consistent with results obtained following stimulation with PHA and CMV, a significant increase in the relative distribution of CCR4⁺ Th2 cells was also observed with ERA and RA patients, as compared to HC, following stimulation with aspergillus; with 17.8% of RA patients also demonstrating CCR4⁺ Th2 cell percentages above the 95th percentile of HC.

When investigated further for associations with patient clinical outcomes, a positive association between the relative distribution of aTcs and DAS28 scores was observed within ERA patients, despite a previous lack of evidence for alterations in the relative distribution of aTcs, following stimulation with aspergillus. With the addition of CCR6, CXCR3 and CCR4, a positive association between the relative distribution of CCR4⁺ Th17 cells and DAS28 scores was observed within ERA patients; results in direct juxtaposition to those obtained following stimulation with CMV. Altogether these results would indicate that an increase in the relative distribution of aTcs, specifically CCR4⁺ Th17 cells, following stimulation with aspergillus, is associated with an adverse clinical presentation within ERA patients. With aspergillus a potent stimulator of Th17 cells, much more so than any other antigenic stimulant investigated in the present study, it can be posited that the stimulation of CD4⁺ T-cells with aspergillus, is capable of revealing, otherwise occult, pathogenic Th17 cells within ERA patients, perhaps as a result of direct or bystander activation secondary to pro-inflammatory cytokine secretion (Acosta-Rodriguez et al., 2007; Arleevskaya et al., 2016; Zheng et al., 2014). As such, within the setting of RA, an expansion of Th17 cells, following stimulation with aspergillus, appears to carry a deleterious effect. Despite these compelling results, however, and consistent with results obtained following stimulation with PHA and CMV, no such relationship was observed within RA patients, again signifying that the use of csDMARD and

bDMARD therapeutics disrupts the association of aTc responses with conventional measures of disease activity.

With this in mind, the effects of conventional synthetic (csDMARD) and biological disease-modifying anti-rheumatic drug (bDMARD) therapeutic regimens on aTc responses were investigated further within RA patients and DCG. Following stimulation with PHA, a significant reduction in the relative distribution of aTcs was observed within RA patients receiving bDMARD therapeutics, as compared to RA patients undertaking csDMARD-based therapeutic regimens; with 9/15 (60.0%) RA patients undertaking bDMARD-based therapeutic regimens demonstrating aTc percentages below the 5th percentile of HC. When investigated further for differences in disease activity measures, however, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating aTc percentages below the 5th percentile of HC and those demonstrating aTc percentages within the 5th percentile of HC.

With the addition of CCR6, CXCR3 and CCR4, no significant difference in any activated CD4⁺ T-cell subpopulation was observed, following stimulation with PHA. Split populations in the relative distribution of CCR4⁻ and CCR4⁺ Th2 cells were, however, observed within RA patients undertaking csDMARD therapeutic regimens; with 8/36 (22.2%) and 10/36 (27.8%) RA patients undertaking csDMARD therapeutic regimens demonstrating CCR4⁻ and CCR4⁺ Th2 cell percentages above the 95th of HC, respectively. When investigated further for differences in disease activity measures, however, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients demonstrating CCR4⁻ and CCR4⁺ Th2 percentages above the 95th percentile of HC, as compared to RA patients presenting with CCR4⁻ and CCR4⁺ Th2 cell percentages within the 5th and 95th percentile of HC.

With the full stratification of RA patients according to their primary therapeutic agent, a significant reduction in the relative distribution of aTcs was observed within RA patients receiving anti-TNF- α monoclonal therapeutics, as compared to RA patients receiving methotrexate. As a major pro-inflammatory cytokine, the reduction in aTcs is perhaps to be expected. Indeed, TNF- α is one of the earliest cytokines to be produced following T-cell activation (Tsai et al., 1996). With its broadly pro-inflammatory properties, TNF- α activates the NF- κ B pathway, inducing the synthesis of further pro-inflammatory cytokines; whilst simultaneously upregulating anti-apoptotic proteins, which ultimately lead to prolonged cell survival and inflammation (Tsai et al., 1996; Zheng et al., 2014). Within the setting of RA, the introduction of an anti-TNF- α monoclonal therapeutic is purposefully designed to disarm these pro-inflammatory processes, ultimately alleviating inflammation and joint destruction. However, as evidenced by the results presented herein, such effects must also be taken into account when interpreting diagnostic laboratory findings; with the reduction in aTc responses observed within RA patients receiving anti-TNF- α therapeutics, following stimulation with PHA, clearly an artefactual change, as opposed to an indicator of diminished immunity due to RA pathology.

Differences in activated CD4⁺ T-cell subpopulation percentages were also evident following stimulation with CMV; with a significant reduction in the relative distribution of CCR4⁺ Th17 cells observed within RA patients undertaking bDMARD therapeutic regimens, as compared to RA patients receiving csDMARD therapeutics. A trend towards an increase in the relative distribution of CCR4⁺ Th2 cells was also observed within RA patients receiving csDMARD therapeutics, as compared to RA patients undertaking bDMARD therapeutic regimens. As such, the decrease in CCR4⁺ Th17 cells and the concurrent increase in CCR4⁺ Th2 cells previously observed within RA patients, as compared to ERA and HC, respectively appear in fact to be a result of two differing therapeutic regimens; with the utilization of csDMARD therapeutic regimens resulting in an increase in CCR4⁺ Th2 cells, and the implementation of a bDMARD therapeutic

regimen resulting in a decrease in CCR4⁺ Th17 cells; results not entirely inconsistent with those observed following stimulation with PHA.

Unfortunately, with the full stratification of RA patients according to their primary therapeutic agent, low participant numbers became a confounding factor; with no CMV positive individuals shown to be receiving anti-TNF- α monoclonal agents. With low study numbers available, no significant difference in the relative distribution of aTcs, or their composite subpopulations, was observed within RA patients undertaking methotrexate-, methotrexate substitute- or rituximab-based therapeutic regimens. As a result, no firm conclusions could be drawn regarding the effect of individual therapeutic agents on CMV-specific CD4⁺ T-cell responses; albeit the observed reduction in CCR4⁺ Th17 cells within RA patients receiving bDMARD therapeutics, as compared to RA patients undertaking csDMARD therapeutic regimens, can be solely attributed to the use of rituximab. As such, future studies should seek to recruit further CMV positive RA patients in order to substantiate these findings, whilst also investigating the effects of anti-TNF- α monoclonal therapeutics on CMV-specific CD4⁺ T-cells responses.

Supplementary to the changes observed following stimulation with PHA and CMV, no significant difference in the relative distribution of aTcs, or their composite subpopulations, was observed within RA patients undertaking csDMARD and bDMARD therapeutic regimens, following stimulation with aspergillus. Consistent with previous observations, however, a trend towards an increase in the relative distribution of CCR4⁺ Th2 cells was observed within RA patients undertaking csDMARD therapeutic regimens, as compared to RA patients receiving bDMARD-based therapeutics; with 9/27 (33.3%) RA patients undertaking csDMARD therapeutic regimens demonstrating CCR4⁺ Th2 cell percentages above the 95th percentile of HC. Nevertheless, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients undertaking csDMARD therapeutic regimens demonstrating CCR4⁺ Th2 cell percentages above the 95th

percentile of HC and those demonstrating CCR4+ Th2 cell percentages within the 5th and 95th percentile of HC.

All things considered, the relative distribution of aTc subpopulations, appear to be similarly affected by the utilization of csDMARD therapeutics, irrespective of the antigenic stimulant employed; with a non-significant increase in CCR4+ Th2 cells consistently observed within a proportion of RA patients, following stimulation with PHA, CMV and aspergillus. Despite this constant alteration to CCR4+ Th2 cell percentages, however, no significant difference in the disease activity measures ESR, CRP or DAS28 scores could be discerned between RA patients demonstrating elevated CCR4+ Th2 cell percentages and those demonstrating CCR4+ Th2 cell percentages within the 5th and 95th percentile of HC, again irrespective of the antigenic stimulant utilized. Furthermore, with the full stratification of RA patients according to their primary therapeutic agent, no singular therapeutic regimen could be attributed to this increase in CCR4+ Th2 cells. As such, despite evidence for negative associations between the relative distribution of Th2 cells, DAS28 scores and CRP observed within ERA patients, following stimulation with CMV at the time of diagnosis, the stratification of RA patients according to their therapeutic regimen, suggests limited utility is to be found in the longitudinally monitoring of Th2 cells, following the initiation of a csDMARD therapeutic regimen, regardless of the antigenic stimulant in use.

With the efficacy of monitoring aTc responses, following stimulation with PHA, CMV, and aspergillus, still unsubstantiated following therapeutic stratifications, the relative distribution of aTcs, and their composite subpopulations, were longitudinally monitored within ERA patients at 3-, 6- and 12-monthly intervals after the initiation of csDMARD therapeutic regimens; in order to establish whether aTc response measurements in therapeutic efficacy monitoring are truly of benefit. As such, following stimulation with PHA, no significant difference in the relative distribution of aTcs, or their composite

subpopulations, was observed within ERA patients up to 12-months after diagnosis. A trend towards an increase in the relative distribution of aTcs was observed with ERA patients 12-months post diagnosis, as compared to the time of diagnosis, although this observation failed to reach statistical significance. When investigated further for associations with patient clinical outcomes, however, a positive association between the relative distribution of aTcs and DAS28 scores was observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens; indicating that that an increase in the relative distribution of aTcs 12-months after diagnosis is associated with an adverse clinical response. This is not a relationship that was observed within ERA at the time of diagnosis, or indeed within RA patients on pre-established therapeutic regimens. With a diverse array of therapeutic regimens employed in our RA cohort, it is plausible for such a relationship to have been obscured within RA patients, particularly given the inhibitory effect exerted on aTc responses by the use of anti-TNF- α monoclonal therapeutics (Zheng et al., 2014). As such, the continued monitoring of aTc responses within ERA patients, beyond 12-months of compliance with a csDMARD therapeutic regimen should be considered in future studies; with the simultaneous stratification of ERA patients based upon modifications to their therapeutic regimen also of merit; in order to comprehensively investigate the effects of therapeutic regimens on the relationship between aTc responses and conventional disease activity measures. Unfortunately, such investigations were not possible in the present study.

Despite the necessity for future studies, when the change in disease activity and the change in the relative distribution of aTcs was investigated over time, no association between the delta change in aTc responses and the delta change in CRP and DAS28 scores was observed. Furthermore, no association between the relative distribution of aTcs at the time of diagnosis and the delta change in CRP and DAS28 scores was observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens. As such, 12-months after the time of diagnosis, an increase in the relative distribution of aTc

responses appears to correspond with an adverse clinical response within ERA patients. However, following stimulation with PHA, neither the relative distribution of aTc responses at the time of diagnosis, nor the delta change in aTc responses following 12-months of compliance with a csDMARD therapeutic regimen are predictive of patient disease activity. As a consequence, the results presented herein suggest that the monitoring of PHA-specific aTC responses may provide limited benefit in predicting and longitudinally monitoring therapeutic efficacy.

The results obtained following stimulation with CMV, however, were far more encouraging; with a trend towards a reduction in the relative distribution of aTcs observed within ERA patients 12-months after the initiation of a csDMARD therapeutic regimen. With the addition of CCR6, CXCR3 and CCR4, a trend towards a reduction in the relative distribution of CCR4⁺ Th17 cells was also observed within ERA patients 12-months after diagnosis. A trend towards an increase in the relative distribution of CCR4⁺ Th2 cells was also observed within ERA patients 12-months after the initiation of csDMARD therapeutic regimens; results entirely in keeping with those previously observed in RA patients. Furthermore, after 12-months of compliance with a csDMARD therapeutic regimen 4/8 (50.0%) ERA patients also demonstrated CCR4⁺ Th2 cell percentages above the 95th percentile of HC. Consistent with previous observations, however, when investigated further for differences in disease activity measures, no significant difference in CRP or DAS28 scores was observed between ERA patients demonstrating CCR4⁺ Th2 cell percentages above the 95th percentile of HC and ERA patients demonstrating CCR4⁺ Th2 cell percentages within the 95th percentile of HC.

With clear differences in CMV-specific CCR4⁺ Th17 and CCR4⁺ Th2 cells observed within ERA patients 12-months after diagnosis, the utility of employing CMV-specific aTc measurements in therapeutic efficacy monitoring was further assessed. In this regard, a negative association between the relative distribution of CCR4⁺ Th17 cells and DAS28

scores was observed within ERA patients 12-months after diagnosis. When investigated further for changes in disease activity and the relative distribution of CCR4⁺ Th17 cells over time, a negative association between the delta change in DAS28 scores and the delta change in CCR4⁺ Th17 cells was observed; indicating that the greatest improvement in disease activity is associated with an increase in the relative distribution of CCR4⁺ Th17 cells. Despite these compelling results, the percentage of CMV-specific Th17 cells within ERA patients at the time of diagnosis proved not to be predictive of the delta change in DAS28 scores 12-months after the initiation of csDMARD therapeutic regimens. Altogether, therefore, these results would suggest that the measurement of Th17 cells cannot be used to predict therapeutic efficacy, but may still prove beneficial in the longitudinal monitoring of therapeutic responses. The same cannot, however, be said for the measurement of CMV-specific CCR4⁻ Th2 cells, with no association between the relative distribution of CCR4⁻ Th2 cells and CRP and DAS28 scores observed with ERA patients 12-months after diagnosis. Furthermore, no associations between the delta change in CRP and DAS28 scores were observed with the delta change in Th2 cell percentages; indicating that the monitoring of CMV-specific Th2 cells cannot be used to monitor therapeutic efficacy.

Finally, despite evidence for associations with disease severity at the time of diagnosis, no significant difference in the relative distribution of aTcs, or their composite subpopulations, was observed within ERA patients 12-months after diagnosis. Furthermore, when evaluated for associations with patient clinical outcomes, no relationships were observed between any activated CD4⁺ T-cell subpopulations and the clinical outcome measures CRP and DAS28 scores; with no associations between the delta change in aspergillus-specific aTc responses and the delta change in CRP and DAS28 scores observed. As such, the longitudinal monitoring of aspergillus-specific aTcs provides no benefit in therapeutic efficacy monitoring within ERA patients.

In conclusion, consistent with previous studies, aTc responses are indeed altered within ERA and RA patients, with CMV status non-significantly associated with an aggravated clinical course in RA patients (Davis et al., 2012; Pierer et al., 2012). With the initiation of csDMARD and bDMARD therapeutic regimens, the relative distribution of aTC responses are also altered, with an increase in CCR4⁺ Th2 cells universally observed in a proportion of RA patients undertaking csDMARD therapeutic regimens, following stimulation with PHA, CMV and aspergillus. Following stimulation with CMV, a decrease in CCR4⁺ Th17 cells was also observed within RA patients undertaking bDMARD-based therapeutic regimens, consisting of rituximab. Critically, changes in CMV-specific Th17 cell responses are related to changes in disease activity, providing a further means of longitudinally monitoring therapeutic efficacy once a therapeutic regimen has been established. As such, these results advocate for establishing CMV aTcs status at the time of diagnosis; whilst the subsequent longitudinal monitoring of CMV-specific Th17 cell percentages may prove highly advantageous in therapeutic efficacy monitoring in CMV positive patients.

Chapter 5: The phosphorylation status of FoxP3 within naturally-occurring regulatory T-cells may be used as a measure of therapeutic efficacy in rheumatoid arthritis

5.1. Introduction

5.1.a. The Role of naturally-occurring regulatory T-cells in the Healthy Immune System

Constituting approximately 5 - 10% of circulating CD4⁺ T-cells, naturally-occurring regulatory T-cells are a specialized subset of CD4⁺ helper T-cells designed to suppress and limit pathogen-driven cellular responses in the periphery, whilst also maintaining tolerance to self-antigens, thereby preventing the development of autoimmune and allergic disorders (Byng-Maddick and Ehrenstein, 2015; Wing et al., 2014). Originating in the thymus, naturally-occurring regulatory T-cells differentiate from CD4⁺ immature thymocytes, during the course of thymic education; a process in which the developing T-cell receptor (TCR) of immature T-cells is inspected for self-antigen:major histocompatibility complex (MHC) binding ability and affinity (Figure 5.1) (Lee et al., 2012; Nunes-Cabaco et al., 2011; Wing and Sakaguchi, 2010). During naïve T-cell development, CD4⁺CD8⁺ double positive (DP) thymocytes, migrating through the cortical regions of the thymus, are challenged with ectopically expressed self-antigen:MHC class I and class II complexes presented on the surface of cortical thymic epithelial cells (cTECs) (Chen et al., 2017; Hu et al., 2015; Klein et al., 2014). If successfully able to recognize a self-antigen:MHC I or self-antigen:MHC II complex, the developing thymocyte receives survival signals through its T-cell receptor; a process termed “positive selection”, in which the continued survival and differentiation of immature T-cells into CD4⁺CD8⁺ or CD4⁺CD8⁻ single positive (SP) thymocytes is permitted. Those evolving thymocytes that are unsuccessful in traversing this developmental checkpoint are

subsequently deleted from the developing T-cell repertoire via activation-induced cell death (AICD), leaving behind a pool of immature SP T-cells which are reactive against self-antigen:MHC I or II complexes, respectively (Figure 5.1) (Hu et al., 2015; Klein et al., 2014; Nunes-Cabaco et al., 2011).

Following passage through the inner and outer cortex of the thymus, CD8⁺ and CD4⁺ SP thymocytes migrate into the medullary regions of the thymus, where they are again inspected for self-antigen:MHC I and self-antigen:MHC II complex binding ability. During this process of “negative selection”, CD8⁺ and CD4⁺ SP thymocytes capable of binding with high affinity to self-antigen:MHC I or self-antigen:MHC II complexes, expressed on the surface of medullary thymic epithelial cells (mTECs) and conventional dendritic cells (cDCs), are eliminated from the developing T-cell repertoire via AICD (Feng et al., 2015; Guerri et al., 2013; Hu et al., 2015). In contrast, the survival of SP thymocytes binding to self-antigen:MHC I or self-antigen:MHC II complexes with only moderate affinity is perpetuated. As such, the combined effects of positive and negative selection, collectively known as central tolerance, ensure that highly self-reactive immature T-cells are prevented from entering the periphery, where they might otherwise instigate the development of autoimmunity. In contrast, moderately self-reactive CD8⁺ and CD4⁺ SP immature T-cells, which are theoretically capable of responding to “non-self” by cross-reacting with pathogen-specific antigens, are released into the periphery where they may contribute to adaptive immunity (Figure 5.1) (Klein et al., 2014; Moran and Hogquist, 2012; Persaud et al., 2014).

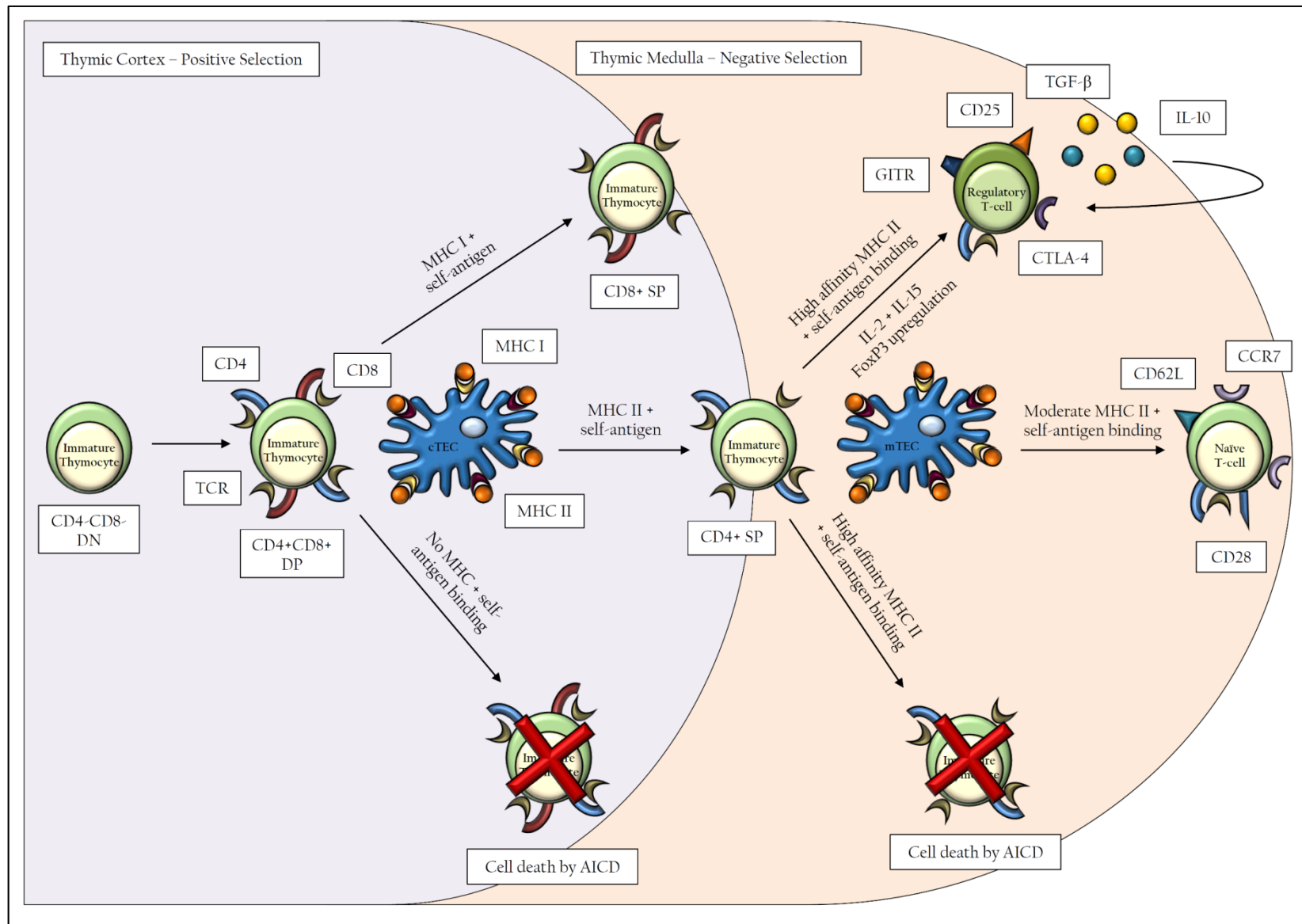


Figure 5.1:- Proposed mechanism for naturally-occurring regulatory T-cell development during thymic education and central tolerance, within the cortical and medullary regions of the thymus. Adapted from Moran et al (2012).

It is during the course of negative selection that naturally-occurring regulatory T-cells develop; with the strength of TCR and self-antigen:MHC complex interactions, in concert with local cytokine signalling, thought to be essential for regulatory T-cell differentiation (Caramalho et al., 2015; Moran and Hogquist, 2012). Whilst chiefly deleted from the evolving T-cell repertoire, highly self-reactive immature CD4⁺ SP T-cells may be rescued from AICD via the induction of the putative regulatory T-cell transcription factor FoxP3; the expression of which is fundamental to confer regulatory T-cell suppressive capabilities (Fontenot, Gavin and Rudensky, 2003; Hinterberger, Wirnsberger and Klein, 2011; Hori, Nomura and Sakaguchi, 2003; Williams and Rudensky, 2007). The precise mechanism by which this reprogramming occurs is yet to be fully elucidated. However, the production of interleukin-2 (IL-2) by developing thymocytes, in combination with the provision of interleukin-15 (IL-15) by thymic-resident antigen-presenting cells (APCs) and thymic epithelial cells, is thought to significantly contribute to this developmental reconditioning (Figure 5.1) (Caramalho et al., 2015; Marshall et al., 2014). Ultimately the “recovery” of self-reactive immature CD4⁺ T-cells, via the induction of this regulatory T-cell developmental pathway, results in the survival and differentiation of a reservoir of self-reactive CD4⁺ naturally-occurring regulatory T-cells (nTregs) that subsequently form the foundation of peripheral tolerance (Nunes-Cabaco et al., 2011; Sakaguchi et al., 2010; Wing et al., 2014).

5.1.b. The Role of Naturally-occurring Regulatory T-cells in Peripheral Tolerance

Expressing high levels of CD25, the α -chain of the IL-2 receptor, in combination with the modulatory receptor cytotoxic T lymphocyte-induced antigen 4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related protein (GITR), nTregs are a fundamental cellular constituent of peripheral tolerance; a process that in itself forms an essential component of adaptive immunity (Table 5.1) (Byng-Maddick and Ehrenstein, 2015; Caramalho et al., 2015; Sakaguchi et al., 2010). The necessity of this operation is due, in part, to the inevitable fragility of central tolerance; a mechanism that, despite the

vigilant and systematic education of naïve T-cells within the thymus, is ultimately beset by the escape of some autoreactive T-cells into the periphery. As such, in order to maintain a balanced and self “tolerant” adaptive immune system, peripheral tolerance is also required to contain and limit the damage that could be generated by the actions of these errant T-cells (Guerri et al., 2013; Klein et al., 2014). Equally, this intricate framework remains on hand to limit the proliferation and expansion of pathogen-specific conventional T-, B- and NK-cells, whilst also restricting the actions of dendritic cells and macrophages during adaptive and innate immune responses, respectively (Heath et al., 2000; Sakaguchi et al., 2010; Wang et al., 2016).

Molecule		Mode of Action
Cell Surface Signalling:-		
Interleukin-2 receptor α chain	CD25	Upregulated on activated CD4 ⁺ and CD8 ⁺ T-cells, whilst constitutively expressed on naturally-occurring regulatory T-cells. Functions to increase the affinity of the IL-2 receptor for its ligand IL-2 by 10 – 100 fold. Amplifies naturally-occurring regulatory T-cell suppressive capabilities by maintaining expression of the putative naturally-occurring regulatory transcription factor FoxP3 (Barron et al., 2010; Boyman and Sprent, 2012)
Interleukin-7 receptor α chain **reduced expression on naturally-occurring regulatory T-cells	CD127	Constitutively expressed on conventional CD4 ⁺ T-cells, with consistently lower levels expressed on human naturally-occurring regulatory T-cells. The binding of FoxP3 to the CD127 promotor region has been speculated to inhibit the expression of the interleukin-7 receptor α chain, signifying that interleukin-7 signalling may not be required to maintain naturally-occurring regulatory T-cell populations (Klein et al., 2010). The combined detection of CD25 expression in concert with the reduced expression of CD127 has therefore been postulated to represent a reliable means of identifying naturally-occurring regulatory T-cells within flow cytometric assays (Klein et al., 2010; Yu et al., 2012).
Cytotoxic T-lymphocyte-associated protein 4	CTLA-4	Constitutively expressed on naturally-occurring regulatory T-cells (Cribbs et al., 2014; Klocke, Holmdahl and Wing, 2017). An essential negative regulator of T-cell immune responses which is thought to function in a non-autonomous manner, via the capture and trans-endocytosis of its cognate ligands, CD80 and CD86, from the surface of antigen-presenting cells (Qureshi et al., 2011; Wing et al., 2014). The removal of these ligands results in the impaired activation of conventional CD4 ⁺ and CD8 ⁺ T-cells (Klocke, Holmdahl and Wing, 2017; Qureshi et al., 2011).
Glucocorticoid-induced TNFR-related protein	GITR	Expressed constitutively on naturally-occurring regulatory T-cells. The precise role of GITR in naturally-occurring regulatory T-cell activity remains to be fully elucidated. However, it is believed that binding of GITR to its soluble ligand GITR-L, expressed on plasmacytoid dendritic cells, macrophages and endothelial cells, enhances the proliferation of naturally-occurring regulatory T-cells. In addition, the ligation of GITR to GITR-L is also thought to be required for optimal naturally-occurring regulatory T-cell suppressive capabilities (Liao et al., 2010; Liao et al., 2014).
Cytokines:-		
Transforming Growth Factor β 1	TGF- β 1	Exerts its regulatory effects via the suppression of IL-2 production, through the direct inhibition of IL-2 promoter activity, inhibiting the proliferation of activated T-, B- and NK-cells, whilst also suppressing the differentiation of activated naïve T-cells into functional CD4 ⁺ and CD8 ⁺ effector T-cells (Heath et al., 2000; Kehrl et al., 1986; Wan and Flavell, 2008). TGF- β 1 is also capable of inducing the expression of FoxP3 within conventional CD4 ⁺ T-cells, conferring regulatory T-cell suppressor functions on conventional T-cell subsets (Fantini et al., 2004; Mayer et al., 2011; Wan and Flavell, 2008).
Interleukin-10	IL-10	Inhibits the synthesis of the pro-inflammatory cytokines interferon- γ (IFN- γ), interleukin-3 (IL-3), tumour necrosis factor- α (TNF- α) and IL-2, produced by antigen-presenting cells (APCs), during the development of innate and adaptive immune responses (Peñaloza et al., 2015; Wang et al., 2016). Maintains the expression and function of FoxP3, synergizing with TGF- β 1 to enhance naturally-occurring regulatory T-cell numbers and their suppressive capabilities (Murai et al., 2009; Wang et al., 2016).
Transcription Factor:-		
Forkhead box P3	FoxP3	The putative transcription factor of naturally-occurring regulatory T-cells. The continued expression of FoxP3 is fundamentally required to confer and maintain the suppressive capabilities of naturally-occurring regulatory T-cells, including the production of IL-10 and the expression of CTLA-4 (Fontenot, Gavin and Rudensky, 2003; Sakaguchi et al., 2010; Williams and Rudensky, 2007).

Table 5.1:- Cytokine, transcription factor and cell surface receptor expression involved in the development, function and identification of naturally-occurring regulatory T-cells.

The fundamental role of nTregs in peripheral tolerance is no more evident than in Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, a devastating condition in which mutations in the gene encoding the regulatory T-cell transcription factor FoxP3 result in impaired/absent nTreg suppressive functions and numbers in affected males (Torgerson and Ochs, 2007; Verbsky and Chatila, 2013). The absence of functional FoxP3 in these patients results in a clinical presentation of multi-organ autoimmunity, typically consisting of a triad of severe diarrhoea due to enteropathy, chronic eczematous dermatitis and polyendocrinopathy, encompassing type 1 diabetes mellitus and hypothyroidism. Infant fatalities are not uncommon, with a life expectancy of just 2 years in such patients, should an effective immunosuppressive therapy and ultimately haematopoietic stem cell transplantation not be performed (Bennett and Ochs, 2001; Torgerson and Ochs, 2007; Verbsky and Chatila, 2013).

Despite the clearly pivotal role of nTregs in the maintenance of peripheral tolerance their exact mode of action remains unclear, although both cytokine secretion and direct cell-to-cell signalling pathways are thought to be involved (Cribbs et al., 2014; Wang et al., 2016; Wing et al., 2014). In keeping with this hypothesis, nTregs are known to produce high levels of the anti-inflammatory cytokines transforming growth factor $\beta 1$ (TGF- $\beta 1$) and interleukin-10 (IL-10), both of which possess broadly immunosuppressive functions that may contribute to the prevention of autoimmunity and allergy, whilst also limiting pathogen-induced adaptive immune responses, following the cessation of infectious disease (Table 5.1) (Blache et al., 2011; Byng-Maddick and Ehrenstein, 2015; Picchianti Diamanti et al., 2014). The first of these cytokines, TGF- $\beta 1$, is thought to chiefly exert its regulatory effects via the suppression of IL-2 production, inhibiting the proliferation of activated T-, B- and NK-cells, whilst also suppressing the differentiation of activated naïve T-cells into functional CD4⁺ and CD8⁺ effector T-cells (Table 5.1) (Heath et al., 2000; Kehrl et al., 1986; Wan and Flavell, 2008). Interestingly, TGF- $\beta 1$ also appears capable of inducing the expression of FoxP3 within conventional CD4⁺ T-cells, and to a lesser extent

CD8⁺ T-cells, thereby conferring regulatory T-cell suppressor functions on conventional T-cell subsets and subsequently perpetuating the actions of the above mentioned regulatory feedback loop (Fantini et al., 2004; Mayer et al., 2011; Wan and Flavell, 2008). Similarly, IL-10 has also been demonstrated to possess broad negative regulatory effects, inhibiting the synthesis of the pro-inflammatory cytokines interferon- γ (IFN- γ), interleukin-3 (IL-3), tumour necrosis factor- α (TNF- α) and IL-2, produced by APCs, including macrophages, during the development of innate and adaptive immune responses (Peñaloza et al., 2015; Wang et al., 2016). Moreover, IL-10 is also capable of influencing nTreg activity, via the maintenance of FoxP3 expression and function, thereby synergizing with TGF- β 1 to enhance nTreg numbers and their suppressive capabilities (Murai et al., 2009; Wang et al., 2016). Of yet further significance is the confirmed ability of IL-10 to specifically curtail Th17 cell effector functions, via the activation of signal transducer and activator of transcription 3 (STAT3), a transcription factor that acts downstream of the IL-10 receptor (IL-10R), and is in itself essential for nTreg function (Chaudhry et al., 2009; Chaudhry et al., 2011).

In addition to their cytokine-driven suppressive activities, nTregs further exert their regulatory functions via direct cell-to-cell contact, through the actions of CTLA-4 (Cribbs et al., 2014; Qureshi et al., 2011; Wing et al., 2014). Whilst only upregulated on conventional T-cells following activation, CTLA-4, a co-inhibitory member of the CD28 family, is constitutively expressed on nTregs (Cribbs et al., 2014; Klocke, Holmdahl and Wing, 2017). An essential negative regulator of T-cell immune responses, this modulatory receptor is thought to function in a non-autonomous manner, via the capture of its ligands, CD80 and CD86, from the surface of APCs, by a process of trans-endocytosis (Qureshi et al., 2011; Wing et al., 2014). The removal of these ligands, which also function as cognate ligands for the costimulatory receptor CD28, results in the impaired activation of conventional CD4⁺ and CD8⁺ T-cells; a mechanism which may serve to limit the expansion of pathogen-induced and auto-reactive T-cell immune responses. As such,

CTLA-4 serves as is a vital constituent of peripheral tolerance and an essential cell surface component of nTregs, a function which has now been utilized in the monoclonal therapy abatacept (ABT) (Klocke, Holmdahl and Wing, 2017; Picchianti Diamanti et al., 2014; Qureshi et al., 2011). This biologic disease modifying anti-rheumatic drug (bDMARD), a dimeric fusion protein composed of the human CTLA-4 extracellular domain coupled to a human Fc portion of immunoglobulin IgG₁, is able to bind with high affinity to CD80 and CD86, blocking costimulatory signals, and as a consequence, inhibiting the activation of pro-inflammatory conventional T-cells (Klocke, Holmdahl and Wing, 2017; Picchianti Diamanti et al., 2014; Qureshi et al., 2011). Altogether the combined regulatory activities of IL-10, TGF- β 1 and CTLA-4 bestow highly immunosuppressive capabilities upon nTregs, securing a critical role in the control of autoreactive T-cells which have escaped into the periphery. As a result, the contribution of nTregs to the prevention of autoimmunity and the development of autoimmune conditions, such as rheumatoid arthritis, is pivotal (Chaudhry et al., 2011; Qureshi et al., 2011; Sakaguchi et al., 2010).

5.1.c. Naturally-occurring regulatory T-cells and Rheumatoid Arthritis

As a major component of peripheral tolerance, the potential impact of nTregs on RA disease activity, severity and therapeutic efficacy has been an area of intense investigation for a number of years; with alterations to nTreg number and function documented. To date, reductions in nTreg numbers, within the peripheral blood, of newly-diagnosed RA patients, as compared to healthy controls, has been the most widely described numerical abnormality (Lawson et al., 2006; Morita et al., 2016). Interestingly, increased numbers of nTregs have conversely been described within the synovium of affected RA patient joints, when compared to the nTreg numbers found within the joint synovium of osteoarthritic patients; intimating the recruitment of nTregs to sites of active immune-mediated inflammation (Lawson et al., 2006; Moradi et al., 2014; Morita et al., 2016; Penatti et al., 2017). Supplementary to these quantitative alterations, modifications to nTreg suppressive functions have also been described, with reductions to CTLA-4 expression

and subsequently the diminished inhibition of conventional T-cell proliferation reported (Cribbs et al., 2014; Klocke, Holmdahl and Wing, 2017). Altogether, these results signify that a state of immune dysregulation, accompanied by defective mechanisms of nTreg induced peripheral tolerance, exists within RA patients, as compared to healthy individuals and patients with non-autoimmune related arthritides (Moradi et al., 2014; Penatti et al., 2017).

Despite these compelling observations, a high degree of ambiguity has remained as to the overall involvement of nTregs in RA pathogenesis, primarily due to inconsistencies in the immunophenotypic characterization of nTregs within the literature (Cribbs et al., 2014; Lawson et al., 2006; Morita et al., 2016). More recently, nTregs have been identified by their high surface expression of CD25, in concert with the low expression of CD127, the α -chain of the interleukin-7 (IL-7) receptor; the expression of which is downregulated on nTregs, as compared to conventional CD4⁺ T-cells (Klein et al., 2010; Sakaguchi et al., 1995; Yu et al., 2012). However, with advances in the detection of the putative nTreg transcription factor FoxP3, as well as recognition of their additional expression of CTLA-4, a myriad of immunophenotypic classifications have been described in the literature, including CD4⁺CD25⁺, CD4⁺CD25⁺CD127^{lo}, CD4⁺CD25⁺FoxP3⁺, CD4⁺CD25⁺CD127^{lo}FoxP3⁺ and CD4⁺CD25⁺CD127^{lo}CTLA⁺ nTregs (Klein et al., 2010; Sakaguchi et al., 1995; Yu et al., 2012). Inevitably, these phenotypic inconsistencies have led to a succession of conflicting results, creating an unreliable representation as to the overall contribution of nTregs to RA pathogenesis and as such investigations regarding therapeutic efficacy have, until recently, remained largely inconclusive (Morita et al., 2016; Yu et al., 2012).

The importance of nTregs in RA pathogenesis has, however, become more evident following a publication by Nie et al, in which the negative effects of TNF- α on nTreg activity was demonstrated, with a direct mechanistic rationale for the use of bDMARDs

directed against TNF- α also proven (Nie et al., 2013). As previously highlighted, nTreg function and homeostasis is highly dependent upon the stable expression of the transcription factor FoxP3, a protein which undergoes a number of post-translational modifications, including methylation, acetylation and phosphorylation, to reach its active form (Gao et al., 2015; Kennedy et al., 2014; Nie et al., 2013; van Loosdregt et al., 2010). Numerous disturbances in FoxP3 post-translational modifications have now been documented in patients with active RA, with reductions in nTreg suppressive capabilities correlating with the presence of these disturbances (Kennedy et al., 2014; Nie et al., 2013). Interestingly, one such disturbance, namely the dephosphorylation of FoxP3 was shown by Nie et al to occur in the presence of TNF- α , a cytokine that is abundantly found in the joints of patients with active rheumatoid arthritis (Gao et al., 2015; Nie et al., 2013). This dephosphorylation was demonstrated via the detection of the phosphorylated form of FoxP3, phospho-FoxP3 (S418), by way of immunoprecipitation and western blot. Using this method Nie et al were able to show that the dephosphorylation of FoxP3 reduces the suppressive capabilities of nTregs within RA patients, thereby perpetuating their active inflammatory state, whilst also providing a rationale for the observed accumulation of nTregs within the synovium of active RA patients joints, with seemingly no beneficial anti-inflammatory effect (Moradi et al., 2014; Morita et al., 2016; Nie et al., 2013). Of even further importance is the subsequent demonstration of nTreg function restoration following the successful treatment of these patients with the anti-TNF- α agent infliximab (Nie et al., 2013).

In isolation, this work not only sheds light on a credible role for defective nTregs in RA pathogenesis, but also highlights the potential benefit of monitoring nTreg function within newly-diagnosed RA patients following the initiation of disease-modifying anti-rheumatic drugs (DMARDs) (Nie et al., 2013). Indeed, a quantitative assay, such as that provided by a flow cytometric platform could prove highly advantageous. With the capacity to measure multiple nTreg parameters simultaneously, the detection of defective

FoxP3 phosphorylation by such a method could potentially provide another means of identifying those patients most likely to benefit from the commencement of an anti-TNF- α therapeutic agent, rather than the continued use of a conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimen (Nie et al., 2013).

5.2. Aims and Objectives

In light of these recent discoveries, the working hypothesis of this chapter is therefore that newly-diagnosed, treatment-naïve RA patients, as well as longstanding RA patients who have failed, or will fail, to make a clinical response following the initiation of a therapeutic regimen may be identified by their nTreg FoxP3 phosphorylation status. As such, the addition of this parameter to current disease management protocols will improve the identification of RA therapy non-responders, permitting the effectual modification of patient therapeutic regimens. The aims of this chapter are therefore:-

1. To establish the normal distribution of CD25⁺CD127^{low}, total FoxP3⁺ and phosphorylated FoxP3-expressing nTreg responses, following culture with media alone, as well as stimulation with PHA and aspergillus respectively, in a cohort of age- and sex-matched healthy controls, following the development of a flow cytometric assay for the detection of phospho-FoxP3 (S418) expression.
2. To compare the total FoxP3⁺ and phosphorylated FoxP3-expressing nTreg responses generated in newly-diagnosed, treatment-naïve and longstanding RA patients with those of age- and sex-matched disease and healthy controls.
3. To stratify longstanding RA patients according to their therapeutic regimens, in order to investigate the effects of immunosuppressive therapy on nTreg FoxP3 phosphorylation status.
4. To longitudinally monitor total FoxP3⁺ and phosphorylated FoxP3-expressing nTreg responses in newly-diagnosed, treatment-naïve rheumatoid arthritis patients, at 3-, 6- and 12-monthly intervals, following the initiation of immunosuppressive therapy; to further investigate the effect of RA therapeutic regimens on nTreg FoxP3 phosphorylation status.

5.3. Results

5.3.a. Naturally-occurring regulatory T-cell number and function is altered in healthy controls following mitogenic stimulation

A flow cytometric assay was designed to assess the number and function of naturally-occurring regulatory CD4⁺ T-cells; with live CD4⁺ regulatory T-cells identified based upon their surface expression of CD25, in combination with their low expression of CD127. Intracellular staining was performed to allow for the detection of the transcription factor FoxP3 within CD25⁺CD127^{lo} nTregs. nTreg function was assessed via intracellular staining for the phosphorylated form of FoxP3, phospho-FoxP3 (S418) (Figure 5.2). The percentage of phosphorylated FoxP3 was calculated using the following formulae:-

$$\frac{(\text{phospho-FoxP3 (S418)} - \text{non-specific background})}{(\text{FoxP3} - \text{non-specific background})} \times 100$$

5.3.a.i. Negative Control - Within our healthy control cohort (n = 16), CD25⁺CD127^{lo} nTregs made up 5.5% (4.3 – 5.9%) of CD4⁺ T-cells (median; inter-quartile range (IQR)), following culture with media alone (Figure 5.3A). Of CD25⁺CD127^{lo} nTregs, 44.0% (40.7 – 51.5%) expressed the regulatory transcription factor FoxP3, with undetectable levels of phospho-FoxP3 (S418) found in 12/16 (75.0%) healthy controls. Within the remaining 4/16 (25.0%) healthy controls, the relative distribution of phospho-FoxP3 (S418) expression was 3.6% (2.3 – 5.6%) of CD25⁺CD127^{lo} nTregs (Figure 5.3B - C). As such, following culture with media alone, phosphorylated FoxP3, and by supposition activated nTregs, were undetectable in 12/16 (75.0%) of healthy controls; with 4/16 (25.0%) healthy controls demonstrating phosphorylated FoxP3 expression levels, and by extension nTreg activation, in 7.9% (4.3 – 14.6%) of CD25⁺CD127^{lo} nTregs (Figure 5.3D).

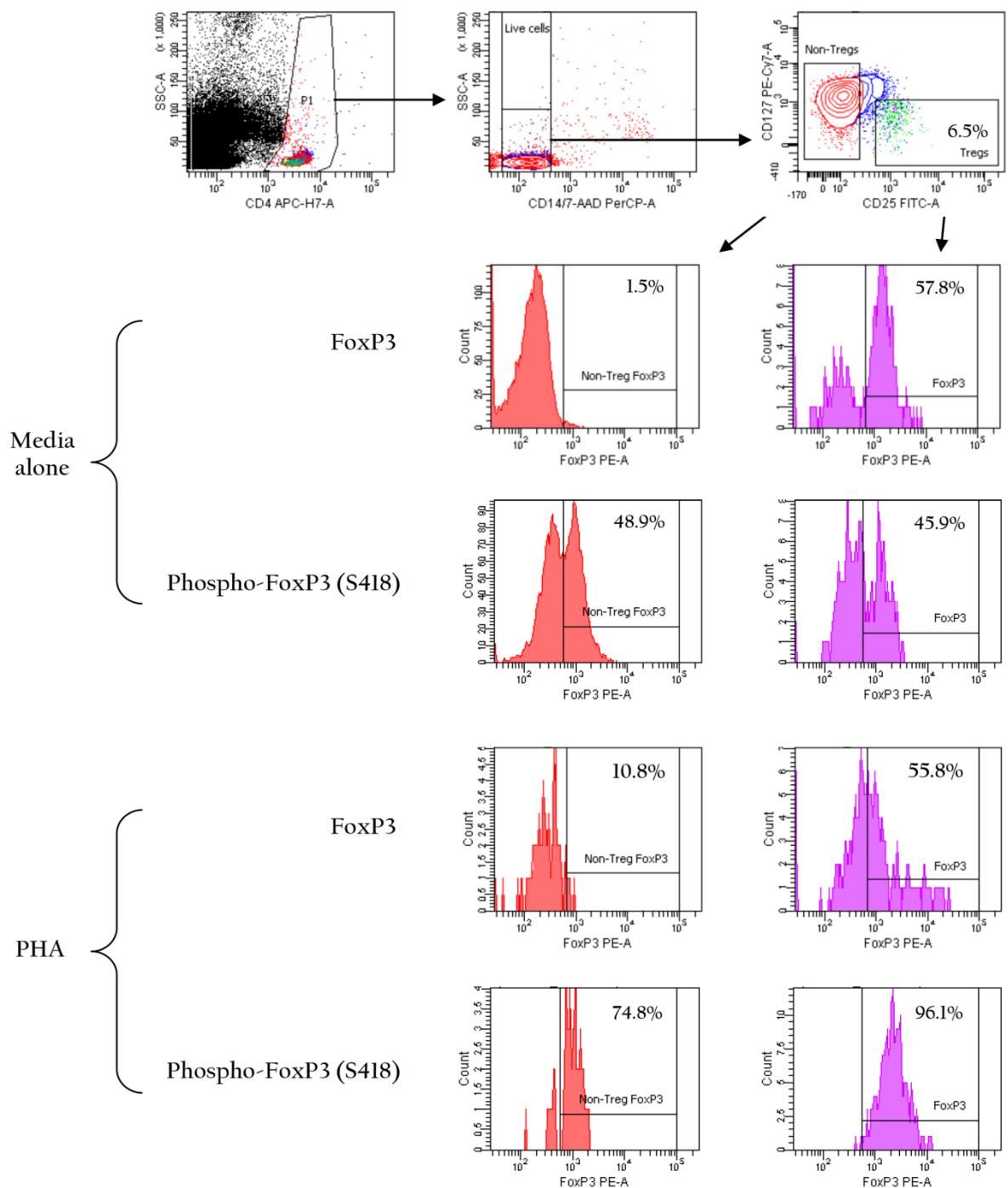


Figure 5.2:- Representative FACS plots depicting gating strategy employed for the identification of FoxP3 and phospho-FoxP3 (S418) expression within live CD4⁺CD25⁺CD127^{lo} naturally-occurring regulatory T-cells. FACS plots depict sample gating following culture with media alone or with PHA. Samples were gated to acquire 50,000 CD4⁺ T-cell events, with removal of monocytes and/or dead cells stained with CD14 and the viability marker 7-AAD. Live CD4⁺CD25⁺CD127^{lo} T-cells were assessed for FoxP3 and phospho-FoxP3 (S418) expression. Live CD4⁺CD25⁻ conventional T-cells were analysed for FoxP3 and phospho-FoxP3 (S418) expression, as a measure of non-specific background. Non-specific background was deducted from regulatory T-cell staining to give a true representation of FoxP3 and phospho-FoxP3 (S418) expression. The percentage of phosphorylated FoxP3 was calculated via the following equation:- $\frac{(\text{phospho-FoxP3 (S418)} - \text{non-specific background})}{(\text{FoxP3} - \text{non-specific background})} \times 100$.

5.3.a.ii. PHA - Following stimulation with PHA a significant increase in CD25+CD127^{lo} nTregs was observed (27.3%; 22.1 – 36.5%) ($p<0.001$), with a significant reduction in the expression of FoxP3 also detected within CD25+CD127^{lo} nTregs (23.8%; 18.5 – 28.8%), as compared to culture with media alone ($p<0.001$) (Figure 5.3A - B). This decrease in FoxP3 expression was, however, accompanied by a significant increase in the relative expression levels of phospho-FoxP3 (S418) within CD25+CD127^{lo} nTregs (13.1%; 9.7 – 16.9%) ($p<0.001$); resulting in the overall increased expression of phosphorylated FoxP3 within CD25+CD127^{lo} nTregs (53.6%; 43.4 – 69.3%), as compared to culture with media alone ($p<0.001$) (Figure 5.3C - D). As such, following stimulation with PHA, the relative distribution of CD25+CD127^{lo} nTregs appears increased, albeit with a concomitant reduction in FoxP3 expression observed. Despite this reduction in FoxP3 expression, the relative expression levels of phospho-FoxP3 (S418) and, by inference, phosphorylated FoxP3 were increased, signifying the presence of increased activated nTreg levels following mitogenic stimulation.

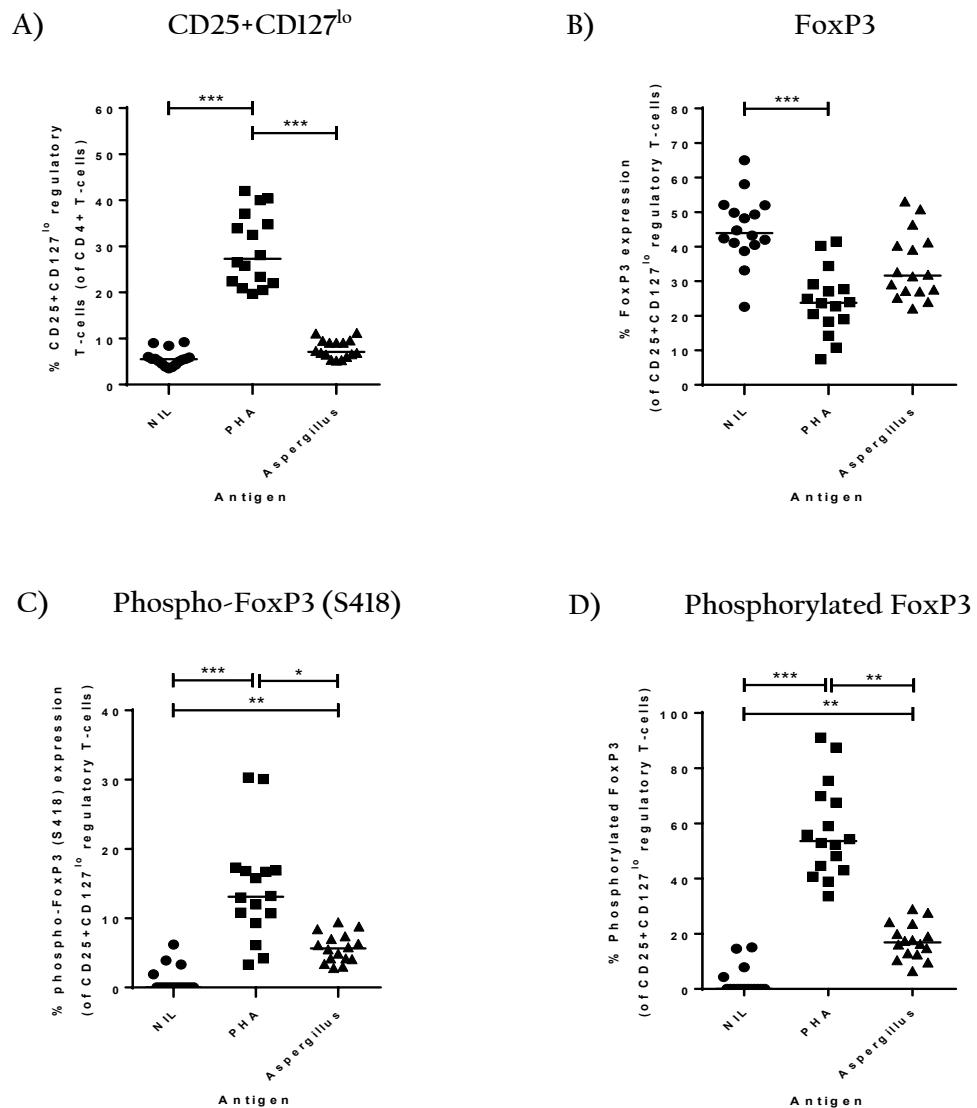


Figure 5.3:- Naturally-occurring regulatory T-cell numbers observed in healthy controls following stimulation with PHA, *Aspergillus* or media alone (NIL). A) Percentage (%) of CD25+CD127^{lo} regulatory T-cells observed, within CD4+ T-cells. B) Percentage of FoxP3 expression, within CD25+CD127^{lo} regulatory T-cells. C) Percentage of phospho-FoxP3 (S418) expression, within CD25+CD127^{lo} regulatory T-cells. D) Percentage of phosphorylated FoxP3, within CD25+CD127^{lo} regulatory T-cells. Phosphorylated FoxP3 percentages were calculated via the following equation:- (phospho-FoxP3 (S418) FoxP3 % - non-specific background)/(FoxP3 % - non-specific background) x 100. *** = p<0.001, ** = p<0.01, * = p<0.05.

5.3.a.iii. *Aspergillus* - Encountered from an early age, the fungal microorganism *aspergillus fumigatus* represents a pathogen against which all immunocompetent individuals are capable of mounting an immune response. The detection of *aspergillus*-specific nTreg responses was therefore incorporated into our flow cytometric assay, in order to represent a more physiological antigenic response than that generated against the mitogen PHA. Following stimulation with *aspergillus*, no significant difference in the relative distribution of CD25+CD127^{lo} nTregs (7.1%; 6.1 – 9.4%), or indeed the relative expression

levels of FoxP3, within CD25+CD127^{lo} nTregs (31.7%; 27.1 – 41.0%), was observed when compared to culture with media alone (Figure 5.3A - B). A significant increase in the relative expression levels of phospho-FoxP3 (S418) (5.7%; 4.1 – 7.3%) and phosphorylated FoxP3 (16.8%; 12.6 – 22.7%) ($p < 0.01$, respectively) were, however, observed as compared to culture with media alone; although the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3 were in fact significantly reduced, as compared to stimulation with PHA ($p < 0.05$ and $p < 0.01$, respectively) (Figure 5.3C - D). As such, following stimulation with aspergillus the relative distribution of CD25+CD127^{lo} nTregs appears unaffected. However, consistent with observations following stimulation with PHA, the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3 were increased, signifying that antigenic stimulation induces the activation of nTregs, without significantly altering their relative distributions (Table 5.2).

	<u>NIL</u>	<u>PHA</u>	<u>Aspergillus</u>
<u>nTreg:-</u>			
CD25+CD127 ^{lo} (%)	3.7 – 9.0	<u>20.3 – 40.8†</u>	5.3 – 11.1
FoxP3 (%)	30.5 – 59.8	<u>10.0 – 40.5Δ</u>	23.5 – 51.4
Phospho-FoxP3 (S418) (%)	0.0 – 4.5	<u>4.0 – 30.2Π</u>	<u>3.0 – 9.0‡Ω</u>
Phosphorylated FoxP3 (%)	0.0 – 14.7	<u>37.6 – 88.4‡</u>	<u>8.9 – 27.9¶Σ</u>

Table 5.2:- Relative distribution (%) of naturally-occurring regulatory T-cells (nTregs) within healthy controls (n = 16), following stimulation with PHA and aspergillus or culture with media alone (NIL). All naturally-occurring regulatory T-cell parameters were expressed as a percentage of CD25+CD127^{lo} CD4⁺ T-cells, respectively. Underlined figures denote significant results. † = $p < 0.001$ as compared to culture with media alone and stimulation with aspergillus; Δ = $p < 0.001$ as compared to culture with media alone; Π = $p < 0.001$ as compared to culture with media alone; ‡ = $p < 0.01$ as compared to culture with media alone; Ω = $p < 0.05$ as compared to stimulation with PHA; ¶ = $p < 0.001$ as compared to culture with media alone; Σ = $p < 0.01$ as compared to stimulation with PHA. Figures denote 5th and 95th percentiles.

With the normal distribution of all measured nTreg parameters established, it was felt necessary to determine whether any age or sex associations were present within our healthy control cohort, prior to the assessment of nTreg number and function within

newly-diagnosed, treatment-naïve and longstanding RA patients. Following such analyses, no significant effects of sex were observed following stimulation with PHA and aspergillus, or indeed following culture with media alone (data not shown). Furthermore, no significant effects of age were observed for any nTreg parameter measured following culture with media alone, or stimulation with aspergillus (data not shown). In contrast, negative associations between age and the relative distribution of CD25⁺CD127^{lo} nTregs ($r=-0.6780$; $p<0.01$), FoxP3 expression levels ($r=-0.8272$; $p<0.0001$) and phospho-FoxP3 (S418) expression levels ($r=-0.7504$; $p<0.0001$) were observed, following stimulation with PHA. A trend towards a negative association between the relative expression levels of phosphorylated FoxP3 and age was also observed, following stimulation with PHA. However, this relationship failed to reach statistical significance ($r=-0.4106$; $p=0.1141$) (Figure 5.4A – D). With no such relationships observed following stimulation with aspergillus, these results would appear to indicate the existence of a PHA-specific reduction in nTreg responses with age, as opposed to a universal age-related reduction in regulatory T-cell parameters. With no significant difference to the relative expression levels of aspergillus-specific nTreg parameters measured, beyond those observed following culture with media alone or stimulation with PHA, the effects of aspergillus stimulation were not investigated further from hereafter.

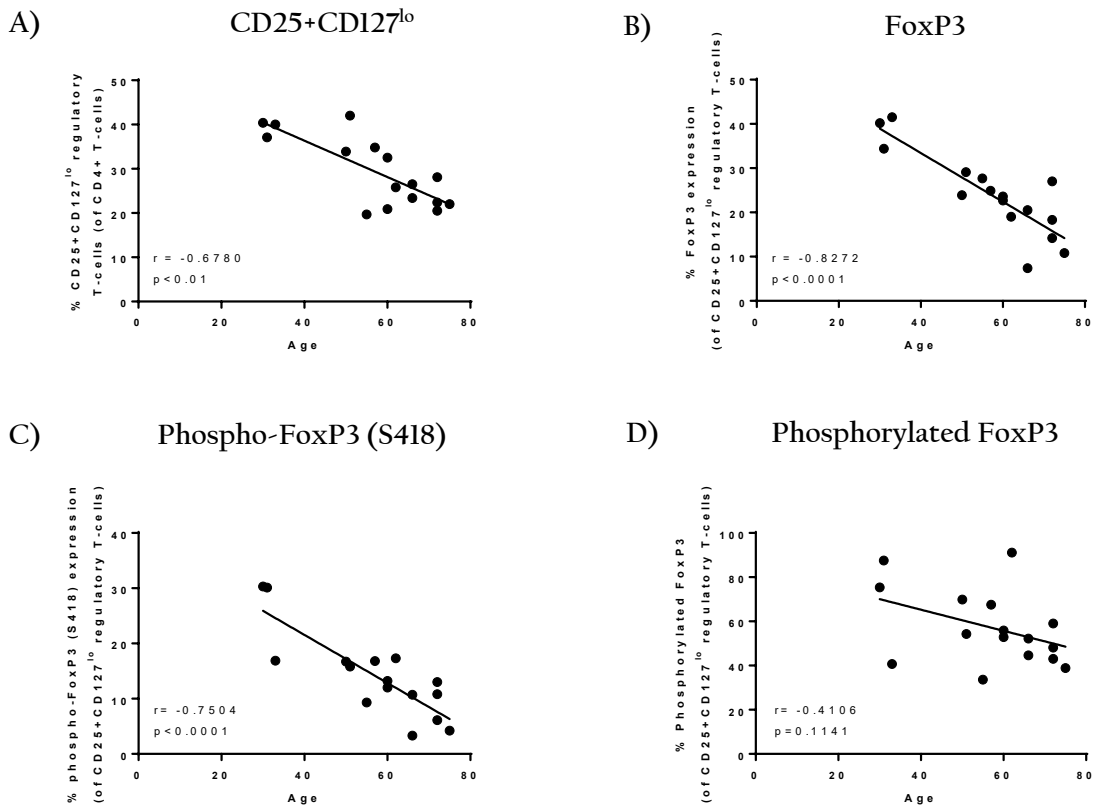


Figure 5.4:- Age and sex associations of naturally-occurring regulatory T-cell numbers and function with age in healthy controls (n = 16), following stimulation with PHA. Percentage of A) CD25+CD127^{lo} naturally-occurring regulatory T-cells observed within CD4+ T-cells; B) FoxP3 expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells; C) Phospho-FoxP3 (S418) expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells and D) Phosphorylated FoxP3, within CD25+CD127^{lo} naturally-occurring regulatory T-cells, compared to age. Phosphorylated FoxP3 percentages were calculated via the following equation:- (phospho-FoxP3 (S418) FoxP3 % - non-specific background)/(FoxP3 % - non-specific background) x 100. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables; with a p-value < 0.05 considered statistically significant.

5.3.b. Naturally-occurring regulatory T-cell number and function are altered in newly-diagnosed treatment naïve RA and longstanding RA patients

5.3.b.i. *Negative Control*:- Following the examination of naturally-occurring regulatory T-cell parameters within healthy controls, the number and function of nTregs was compared between 20 newly-diagnosed, treatment-naïve RA (ERA) and 20 longstanding RA (RA) patients, as well as 18 age- and sex-matched disease controls (DCG) and 16 age- and sex-matched healthy controls (HC). Following culture with media alone, a significant increase in the relative distribution of CD25+CD127^{lo} nTregs was observed within ERA patients, as compared to HC ($p < 0.05$); with no significant difference in CD25+CD127^{lo} nTregs observed within RA patients or DCG (Figure 5.5A; Table 5.3). The relative

expression levels of FoxP3, within CD25⁺CD127^{lo} nTregs, were also altered within ERA patients, with a reduction in the relative expression levels of FoxP3 evident, although this finding failed to reach statistical significance ($p=0.07$) (Figure 5.5C; Table 5.3).

When assessed further for the expression of phospho-FoxP3 (S418), a significant increase in the relative expression levels of phospho-FoxP3 (S418) was observed within RA patients and DCG, as compared to ERA patients ($p<0.05$ and $p<0.01$, respectively) and HC ($p<0.05$, respectively). Furthermore, 9/20 (45.0%) RA patients demonstrated phospho-FoxP3 (S418) expression levels above the 95th percentile of HC (5th – 95th percentile = 0.0 – 4.5%) (Figure 5.5E; Table 5.3). This increased expression in phospho-FoxP3 (S418) was accompanied by a significant increase in the relative expression levels of phosphorylated FoxP3 within RA patients and DCG, as compared to ERA patients ($p<0.05$ and $p<0.001$, respectively) and HC (ns and $p<0.01$, respectively); with 6/20 (30.0%) RA patients demonstrating phosphorylated FoxP3 expression levels above the 95th percentile of HC (5th – 95th percentile = 0.0 – 14.7%) (Figure 5.5G; Table 5.3). Altogether, these results signify that the relative number of CD25⁺CD127^{lo} nTregs is increased within ERA patients following culture with media alone, albeit with a concurrent reduction in FoxP3 expression evident. In contrast, the relative distribution of CD25⁺CD127^{lo} nTregs within RA patients and DCG appear to be numerically unaltered, but functionally active without the prior need for mitogenic or antigenic stimulation (Figure 5.5; Table 5.3).

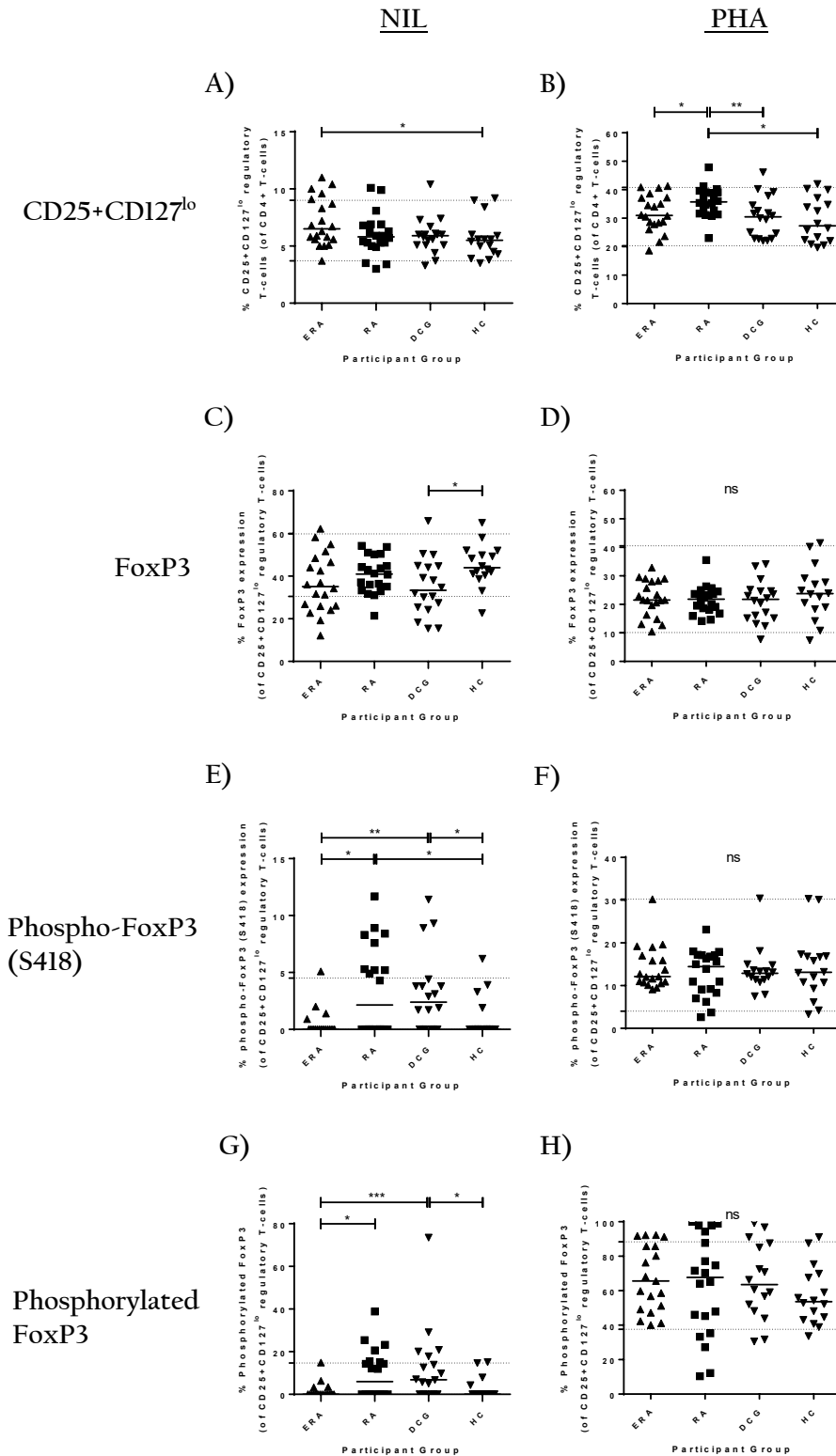


Figure 5.5:- Relative distribution (%) of naturally-occurring regulatory T-cells observed in healthy controls (HC), longstanding RA patients (RA), newly-diagnosed treatment naïve RA patients (ERA) and disease controls (DCG) following culture in media alone or stimulation with PHA, respectively. A - B) Percentage (%) of CD25+CD127^{lo} naturally-occurring regulatory T-cells, of CD4+ T-cells. C - D) Percentage of FoxP3 expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells. E - F) Percentage of phospho-FoxP3 (S418) expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells. G - H) Percentage of phosphorylated FoxP3, within CD25+CD127^{lo} regulatory T-cells. Phosphorylated FoxP3 percentages were calculated via the following equation:- (phospho-FoxP3 (S418) FoxP3 % - non-specific background)/(FoxP3 % - non-specific background) x 100. *** = p<0.001, ** = p<0.01, * = p<0.05, ns = non-significant. Dotted lines denotes the 5th and 95th percentile of healthy controls.

	<u>ERA</u>	<u>RA</u>	<u>DCG</u>	<u>HC</u>
NIL:-				
CD25+CD127 ^{lo} (%)	<u>6.5</u> [†] (5.6 – 9.0)	5.8 (5.0 – 6.8)	5.9 (5.1 – 6.2)	5.5 (4.3 – 5.9)
FoxP3 (%)	35.1 (26.0 – 47.9)	41.0 (33.2 – 50.3)	<u>33.4</u> ^Δ (25.1 – 44.8)	44.0 (40.7 – 51.5)
Phospho-FoxP3 (S418) (%)	0.0 N/A	<u>2.0</u> ^Π (0.0 – 5.9)	<u>2.4</u> ^{‡Ω} (0.0 – 4.0)	0.0 (0.0 – 1.4)
Phosphorylated FoxP3 (%)	0.0 N/A	<u>5.4</u> [‡] (0.0 – 15.2)	<u>6.9</u> ^{¶Σ} (0.0 – 18.4)	0.0 (0.0 – 3.2)
PHA:-				
CD25+CD127 ^{lo} (%)	30.9 (28.0 – 37.2)	<u>35.7</u> ^{§i} (31.5 – 39.0)	30.4 (22.8 – 35.3)	27.3 (22.1 – 36.5)
FoxP3 (%)	21.5 (17.2 – 28.2)	21.8 (17.9 – 24.0)	21.8 (15.2 – 24.8)	23.8 (18.5 – 28.8)
Phospho-FoxP3 (S418) (%)	12.4 (10.8 – 18.5)	14.5 (8.0 – 17.1)	12.4 (10.1 – 13.9)	13.1 (9.7 – 16.9)
Phosphorylated FoxP3 (%)	66.9 (49.6 – 90.0)	67.7 (34.8 – 95.2)	59.9 (40.9 – 85.6)	53.6 (43.4 – 69.3)

Table 5.3:- Relative distribution (%) of naturally-occurring regulatory T-cells observed in healthy controls (HC), longstanding RA patients (RA), newly-diagnosed, treatment-naïve RA patients (ERA) and disease controls (DCG) following stimulation with PHA or culture with media alone (NIL). Underlined figures denote significant results. † = p<0.05 as compared to HC; Δ = p<0.05 as compared to HC; Π = p<0.05 as compared to ERA patients and HC; ‡ = p<0.01 as compared to ERA patients; Ω = p<0.05 as compared to HC; ¶ = p<0.05 as compared to ERA patients; ¶ = p<0.001 as compared to ERA patients; Σ = p<0.05 as compared to HC; § = p<0.01 as compared to DCG; i as compared to ERA patients and HC. Figures denote median and inter-quartile ranges (IQR).

5.3.b.ii. **PHA:-** In contrast to the results obtained following culture with media alone, a significant increase in the relative distribution of CD25+CD127^{lo} nTregs was observed within RA patients, as compared to ERA patients, DCG and HC (p<0.05, p<0.01 and p<0.05, respectively), following stimulation with PHA. No such increase was, however, observed within ERA patients; a result in direct opposition to that observed following culture with media alone (Figure 5.5B; Table 5.3). With the further examination of FoxP3, phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels, no significant difference in the relative expression levels of FoxP3, phospho-FoxP3 (S418) or phosphorylated FoxP3 levels were observed with ERA or RA patients, as compared to DCG and HC (Figure 5.5; Table 5.3). A split population did, however, appear evident in the relative expression levels of phospho-FoxP3 (S418) (median of higher population = 17.1% [IQR = 15.7 – 17.9%]; median

of lower population = 8.3% [IQR = 5.0 – 10.1%]) and phosphorylated FoxP3 within RA patients (median of higher population = 82.4% [IQR = 70.7 – 97.9%]; median of lower population = 34.3% [IQR = 16.0 – 45.8%]); a phenomenon not observed within ERA patients or DCG and HC (Figure 5.5; Table 5.3). Altogether, the mitogenic stimulation of CD25+CD127^{lo} nTregs appears to abrogate the previously observed increase to nTreg numbers within ERA patients; with no significant difference in FoxP3, phospho-FoxP3 (S418) or phosphorylated FoxP3 expression levels observed within this patient group. Within RA patients, however, phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels were highly variable, with split populations in the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3 observed, indicating that a high degree of variability in nTreg functional activity exists within RA patients undertaking csDMARD and bDMARD therapeutic regimens.

5.3.c. Alterations to naturally-occurring regulatory T-cell parameters, within longstanding RA patients, correlate with measures of disease activity

5.3.c.i. *Negative Control:-* With alterations to the relative distribution and functional activities of nTregs evident within ERA and RA patients, a thorough investigation of patient clinical outcomes seemed warranted, to corroborate whether any direct associations between nTreg parameters and patient disease activity measures exist. As such, the relative distributions and functional activity of nTregs, within ERA and RA patients, following stimulation with PHA, or culture with media alone, were compared to the current disease activity measures erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and the 28 joint disease activity score (DAS28). Despite evidence for an increase in the relative distribution of CD25+CD127^{lo} nTregs within ERA patients, following culture with media alone, no associations with ESR, CRP or DAS28 score were observed for any nTreg parameter measured (data not shown). On a superficial level, the same appeared true within our RA patient cohort; with no associations between nTreg numbers or function observed with ESR, CRP or DAS28 score (data not shown). With

the stratification of RA patients according to their relative expression levels of phospho-FoxP3 (S418), no significant difference in DAS28 scores, ESR or CRP was observed between RA patients with detectable phospho-FoxP3 (S418) expression levels and those with undetectable phospho-FoxP3 (S418) expression levels (Figure 5.6A and data not shown). With the exclusive analysis of RA patients with detectable phospho-FoxP3 (S418) expression levels, however, a positive association between the relative expression levels of phospho-FoxP3 (S418) and DAS28 scores was observed ($r=0.9000$; $p=0.0833$). This positive association with DAS28 scores was replicated within RA patients with detectable phosphorylated FoxP3 expression levels ($r=0.8000$; $p=0.1333$) (Figure 5.6B - C). No significant difference in ESR, CRP or DAS28 scores was, however, observed between RA patients with detectable phosphorylated FoxP3 expression levels and those with undetectable phosphorylated FoxP3 expression levels (data not shown). As such, despite the limited availability of disease activity measurements within our RA cohort, these results suggest that two subgroups of longstanding RA patients exist; those with detectable phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels, and those in which phospho-FoxP3 (S418) and phosphorylated FoxP3 expression is absent, prior to antigenic stimulation. Moreover, although no significant difference in ESR, CRP or DAS28 scores was observed between RA patients with/without detectable phospho-FoxP3 (S418) and phosphorylated FoxP3, the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels did bear clinical significance. Thus, within RA patients with detectable phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels, prior to antigenic stimulation, an increase in the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3, is indicative of increased levels of disease activity.

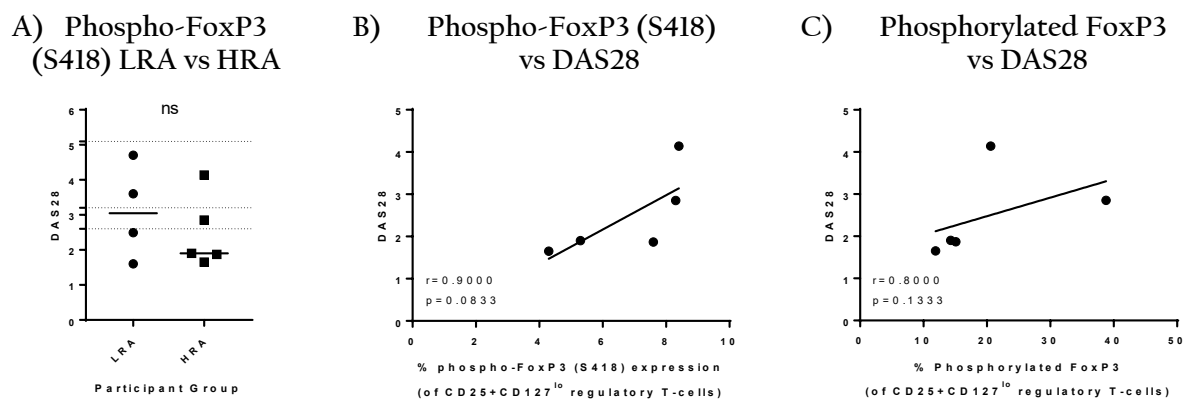


Figure 5.6:- Association of phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels, within CD25+CD127^{lo} naturally-occurring regulatory T-cells, with DAS28 scores in longstanding RA patients, following culture with media alone. A) DAS28 scores within RA patients with undetectable phospho-FoxP3 (S418) expression levels (LRA) vs RA patients with detectable phospho-FoxP3 (S418) expression levels (HRA). Dotted lines denote disease activity targets. <2.6 = remission; 2.6 – 3.2 = low disease activity; >3.2 – 5.1 = moderate disease activity; >5.1 = high disease activity. B) Relative expression levels of phospho-FoxP3 (S418), within CD25+CD127^{lo} naturally-occurring regulatory T-cells, compared to DAS28 scores. C) Relative expression levels of phosphorylated FoxP3, within CD25+CD127^{lo} naturally-occurring regulatory T-cells, compared to DAS28 scores. Phosphorylated FoxP3 percentages were calculated via the following equation:- (phospho-FoxP3 (S418) FoxP3 % – non-specific background)/(FoxP3 % – non-specific background) x 100. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables; with a p-value <0.05 considered statistically significant.

5.3.c.ii. PHA:- Consistent with the lack of alterations to the relative distribution of nTregs within ERA patients following stimulation with PHA, no association with ESR, CRP or DAS28 score was observed between any nTreg parameter measured. Perhaps most striking of all, therefore, were the lack of age associations observed with any nTreg parameter assessed; results also mirrored within our RA cohort. These results are in direct juxtaposition to the negative associations with age previously observed in HC, and as such suggest that the relationship between age and PHA-specific nTreg responses are uncoupled within the setting of rheumatoid arthritis (Figure 5.4 and 5.7). These results would therefore posit the necessity for the introduction of age-related nTreg normal ranges. However, with the stratification of PHA-specific nTreg parameters within HC, ERA and RA patients, according to age, no significant difference in the relative distribution of any nTreg parameter measured was observed, negating the need for age-adjusted ranges in further analyses (Figure 5.8 and data not shown).

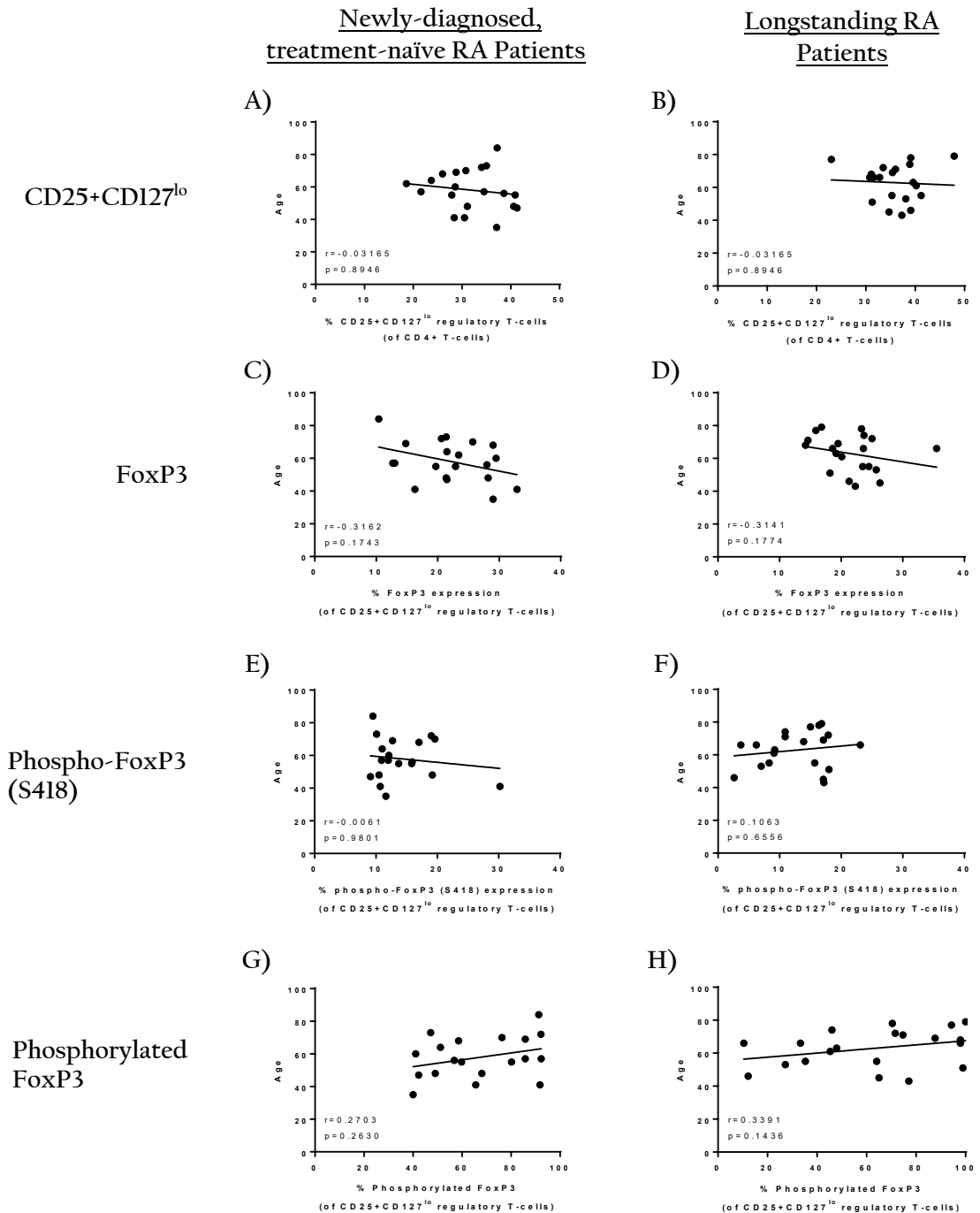


Figure 5.7:- Association of naturally-occurring regulatory T-cell numbers and function with age in newly-diagnosed treatment naïve RA patients (n = 20) and longstanding RA patients (n = 20), following stimulation with PHA. A - B) Percentage of CD25+CD127^{lo} naturally-occurring regulatory T-cells compared to age. C - D) Percentage of FoxP3 expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells compared to age. E - F) Percentage of phospho-FoxP3 (S418) expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells compared to age. G - H) Percentage of phosphorylated FoxP3, within CD25+CD127^{lo} naturally-occurring regulatory T-cells, compared to age. Phosphorylated FoxP3 percentages were calculated via the following equation:- (phospho-FoxP3 (S418) FoxP3 % - non-specific background)/(FoxP3 % - non-specific background) x 100. An R-value ≥ 0.50 was considered to represent a strong correlation. A p-value < 0.05 was considered statistically significant.

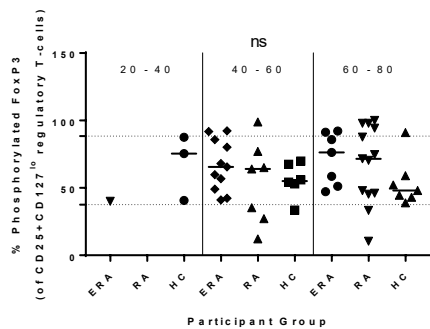
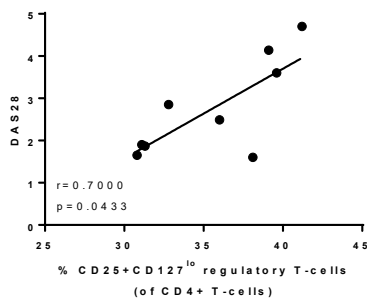


Figure 5.8:- Stratification of healthy controls (HC), newly-diagnosed, treatment-naïve RA (ERA) patients and longstanding RA (RA) patients, according to age (20 – 40 years, 40 – 60 years and 60 – 80 years). Relative distribution (%) of phosphorylated FoxP3 expression levels within CD25+CD127^{lo} naturally-occurring regulatory T-cells of, following stimulation with PHA. A p-value <0.05 was considered statistically significant. Dotted line denotes 5th and 95th percentile of healthy controls.

In keeping with the observed increase to the relative distribution of CD25+CD127^{lo} nTregs within RA patients, a positive association between the relative distribution of CD25+CD127^{lo} nTregs and DAS28 scores was observed ($r=0.7000$, $p=0.0433$) (Figure 5.9A). No further associations with ESR, CRP or DAS28 scores were observed between the relative expression levels of FoxP3, phospho-FoxP3 (S418) or phosphorylated FoxP3, respectively (data not shown).

A) CD25+CD127^{lo} vs DAS28



B) Phosphorylated FoxP3 vs DAS28

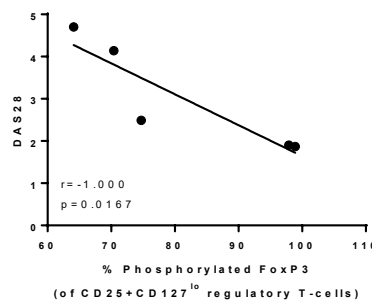


Figure 5.9:- Association of CD25+CD127^{lo} naturally-occurring regulatory T-cells, phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels within CD25+CD127^{lo} naturally-occurring regulatory T-cells with DAS28 scores in longstanding RA patients, following stimulation with PHA. A) Percentage of CD25+CD127^{lo} naturally-occurring regulatory T-cells compared to DAS28 scores. B) Percentage of phosphorylated FoxP3 expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells compared to DAS28 scores, in longstanding RA patients demonstrating higher phosphorylated FoxP3 expression levels. Phosphorylated FoxP3 percentages were calculated via the following equation:- (phospho-FoxP3 (S418) FoxP3 % - non-specific background)/(FoxP3 % - non-specific background) x 100. An R-value $\geq \pm 0.50$ was considered to represent an association between two variables, with a p-value <0.05 considered statistically significant.

Given the split populations previously observed within the relative expression levels of phospho-FoxP3 (S418), and phosphorylated FoxP3, of RA patients following stimulation with PHA, a more thorough investigation of their clinical associations seemed warranted. As such RA patients were further stratified according to their relative expression levels of

phospho-FoxP3 (S418) and phosphorylated FoxP3, respectively and subsequently re-examined for associations with disease activity measures. Following such analyses, no significant difference in ESR, CRP or DAS28 scores was observed between RA patients presenting with lower relative phospho-FoxP3 (S418) expression levels and higher phospho-FoxP3 (S418) expression levels (data not shown). Furthermore, no associations between ESR, CRP, DAS28 scores or age were observed within RA patients presenting with lower relative phospho-FoxP3 (S418) expression levels or, indeed, RA patients presenting with higher relative phospho-FoxP3 (S418) expression levels (data not shown).

A dichotomy in the relative expression levels of phosphorylated FoxP3 did, however, exist within RA patients, following stimulation with PHA. No significant difference in ESR, CRP or DAS28 scores was observed between RA patients presenting with lower relative phosphorylated FoxP3 expression levels and higher relative phosphorylated FoxP3 expression levels (data not shown). Furthermore, no associations between ESR, CRP, or DAS28 scores was observed in RA patients demonstrating lower levels of phosphorylated FoxP3 (data not shown). In contrast, however, a negative association between DAS28 scores and the relative expression levels of phosphorylated FoxP3 was observed within RA patients demonstrating higher levels of phosphorylated FoxP3 (Figure 5.9B). As such, following stimulation with PHA, an increase in the relative distribution of CD25⁺CD127^{lo} nTregs is associated with an adverse clinical response in RA patients. In contrast, an increase in the relative expression levels of phosphorylated FoxP3, within CD25⁺CD127^{lo} nTregs, above 60.0%, is associated with a favourable clinical outcome in RA patients, in which a csDMARD or bDMARD therapeutic regimen has been previously established.

5.3.d. Naturally-occurring regulatory T-cell parameters are not significantly altered within longstanding RA patients when stratified according to csDMARD and bDMARD therapeutic regimens

5.3.d.i. *Negative Control:-* With clear associations between patient clinical outcomes and nTreg parameters evident, within RA patients, the utility of employing nTreg measurements in patient therapeutic efficacy monitoring was further assessed. As such, RA patients and DCG, were stratified according to their therapeutic regimens. In the first instance, RA patients and DCG were categorized into two distinct therapeutic groups; namely those undertaking a therapeutic regimen founded upon the use of a conventional synthetic disease modifying anti-rheumatic drug (csDMARD), or those undertaking a therapeutic regimen based upon the use of a biologic disease modifying anti-rheumatic drug (bDMARD).

Following culture with media alone, no significant difference in the relative distribution of CD25⁺CD127^{lo} nTregs, FoxP3, phospho-FoxP3 (S418) or phosphorylated FoxP3 expression levels were observed within RA patients or DCG when stratified according to their use of csDMARDs or bDMARDs, respectively (Figure 5.10). With the exclusion of RA patients and DCG presenting with undetectable levels of phospho-FoxP3 (S418) and phosphorylated FoxP3, no significant difference in the relative expression levels of phospho-FoxP3 (S418) or phosphorylated FoxP3 were observed between RA patients and DCG receiving csDMARD therapeutic regimens, as compared to those undertaking bDMARD therapeutic regimens (data not shown).

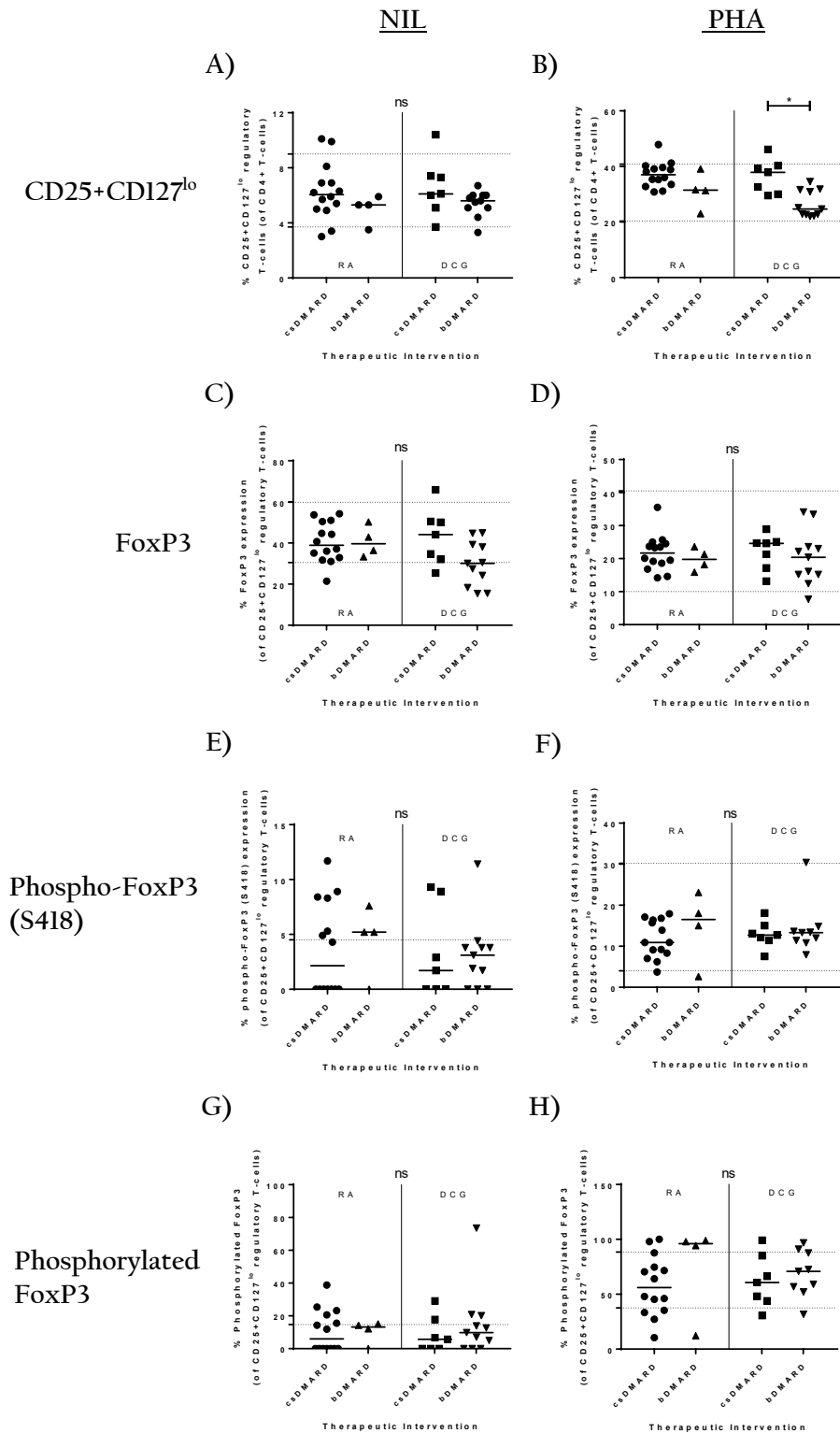


Figure 5.10:- Stratification of longstanding RA patients (RA) and disease controls (DCG) based upon the use of conventional synthetic disease modifying anti-rheumatic drug (csDMARD) or biological disease modifying anti-rheumatic drug (bDMARD) therapeutic regimens, following culture in media alone, or stimulation with PHA, respectively. A - B) Percentage of CD25+CD127^{lo} naturally-occurring regulatory T-cells, of CD4+ T-cells. C - D) Percentage of FoxP3 expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells. E - F) Percentage of phospho-FoxP3 (S418) expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells. G - H) Percentage of phosphorylated FoxP3, within CD25+CD127^{lo} regulatory T-cells. Phosphorylated FoxP3 percentages were calculated via the following equation:- (phospho-FoxP3 (S418) FoxP3 % - non-specific background)/(FoxP3 % - non-specific background) x 100. ** = p<0.01, * = p<0.05, ns = non-significant. Dotted lines denotes the 5th and 95th percentile of healthy controls.

Despite a lack of evidence for differences in nTreg parameters, in RA patients and DCG when stratified according to their use of csDMARDs or bDMARDs alone, RA patients and DCG were stratified further according to their primary therapeutic agent, in order to investigate the effects of individual therapeutic agents on nTreg number and function. Following this stratification, no significant difference in the relative distribution of CD25+CD127^{lo} nTregs, FoxP3, phospho-FoxP3 (S418) or phosphorylated FoxP3 expression levels were observed within RA patients receiving csDMARD therapeutic regimens, incorporating methotrexate and methotrexate substitutes, or RA patients receiving bDMARD therapeutic regimens integrating anti-tumour necrosis factor alpha- (TNF- α) and rituximab-based therapies (data not shown). As such, despite evidence for a positive association between DAS28 scores and the relative expression levels of phospho-FoxP3 (S418), and phosphorylated FoxP3, within RA patients following culture with media alone, no significant difference in any nTreg parameter measured was observed following the stratification of RA patients according to their csDMARD and bDMARD therapeutic regimens. Furthermore, due to limited data regarding measures of clinical outcome within our RA cohort, no significant difference in ESR, CRP, or DAS28 scores was observed between RA patients undertaking csDMARD therapeutic regimens, as compared to RA patients receiving bDMARD (data not shown).

5.3.d.ii. PHA:- Following stimulation with PHA, no significant difference in the relative distribution of FoxP3, phospho-FoxP3 (S418) or phosphorylated FoxP3 were observed within RA patients undertaking csDMARD- or bDMARD-based therapeutic regimens (Figure 5.10). A trend towards a reduction in the relative distribution of CD25+CD127^{lo} nTregs was observed in RA patients undertaking bDMARD therapeutic regimens, as compared to RA patients receiving csDMARD, although this observation failed to reach statistical significance ($p=0.1314$) (Figure 5.10B). With no significant difference in nTreg number or function observed in RA patients and DCG when stratified according to their use of csDMARDs or bDMARDs alone, the full stratification of RA patients and DCG

according to their primary therapeutic agent was performed, to facilitate the investigation of individual therapeutic effects. Following stratification, no significant difference in the relative distribution of CD25+CD127^{lo} nTregs, FoxP3, phospho-FoxP3 (S418) or phosphorylated FoxP3 expression levels was observed within RA patients or DCG (data not shown). As such, following stimulation with PHA, the relative distribution of CD25+CD127^{lo} nTregs appears to be non-significantly reduced within RA patients undertaking bDMARD therapeutic regimens, as compared to RA patients receiving csDMARD-based therapeutics. However, due to limited data regarding patient clinical outcomes within our RA cohort, no significant difference in ESR, CRP, or DAS28 scores was apparent according to therapeutic stratification.

5.3.e. The longitudinal monitoring of naturally-occurring regulatory T-cell parameters may be used to assess therapeutic efficacy in newly-diagnosed RA patients following the initiation of therapeutic regimens

5.3.e.i. *Negative Control*:- Despite limited evidence for alterations to nTreg parameters based upon therapeutic stratification, the previously observed associations between patient clinical outcomes and nTreg parameters within RA patients warranted further investigation within ERA patients. As such, the relative distribution of CD25+CD127^{lo} nTregs, FoxP3, phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels were longitudinally monitored within ERA patients at 3-, 6- and 12-monthly intervals, following their initiation of csDMARD therapeutic regimens; in order to further assess the utility of employing nTreg parameter measurements in therapeutic efficacy monitoring. Following culture with media alone, a significant reduction in the relative distribution of CD25+CD127^{lo} nTregs was observed 12-months post-diagnosis, as compared to the time of diagnosis ($p<0.05$); with a significant reduction in the relative expression levels of FoxP3 also observed 12-months post-diagnosis ($p<0.05$) (Figure 5.11A - B). The relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3 were also altered within ERA patients following the initiation of csDMARD therapeutic regimens, with a

significant increase in the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3 observed 12-months post-diagnosis ($p < 0.05$, respectively) (Figure 5.11C - D).

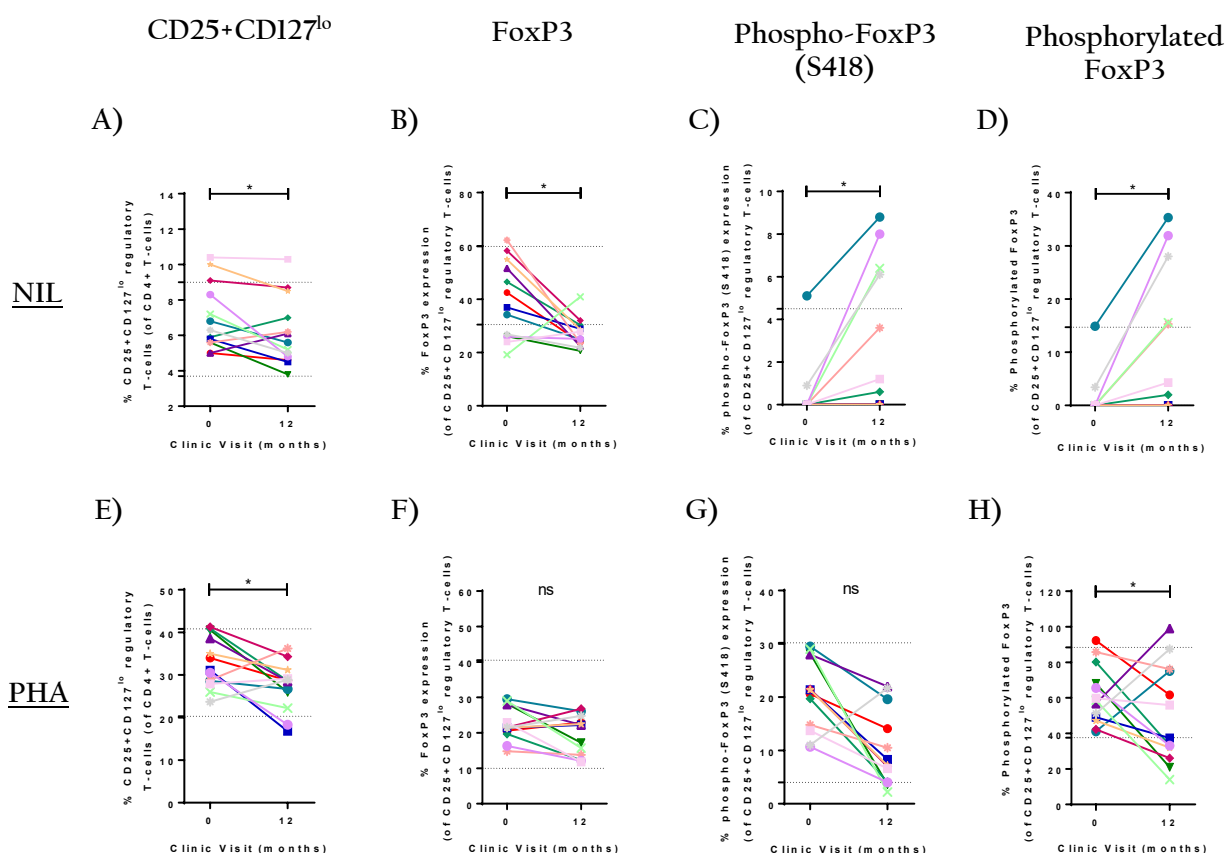


Figure 5.11:- Longitudinal monitoring of naturally-occurring regulatory T-cell parameters within newly-diagnosed RA patients 12-months following the initiation of conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimens, following culture with media alone or stimulation with PHA. A and E) Percentage of CD25+CD127^{lo} naturally-occurring regulatory T-cells, of CD4+ T-cells. B and F) Percentage of FoxP3 expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells. C and G) Percentage of phospho-FoxP3 (S418) expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells. D and H) Percentage of phosphorylated FoxP3, within CD25+CD127^{lo} regulatory T-cells. Phosphorylated FoxP3 percentages were calculated via the following equation:- (phospho-FoxP3 (S418) FoxP3 % - non-specific background)/(FoxP3 % - non-specific background) x 100. * = $p < 0.05$, ns = non-significant. Dotted lines denotes the 5th and 95th percentile of healthy controls.

When analysed further for evidence of clinical associations, no associations with ESR, CRP or DAS28 scores was observed with any nTreg parameter measured 12-months after the initiation of csDMARD therapeutic regimens (data not shown). Moreover, no association between the delta change in ESR, CRP, or DAS28 scores was observed with any nTreg parameter measured 12-months after diagnosis; whilst no significant difference

in ESR, CRP or DAS28 scores was observed when ERA patients were stratified according to the delta change in nTreg parameters (data not shown). With the further exclusion of ERA patients with undetectable levels of phospho-FoxP3 (S418), and phosphorylated FoxP3, no significant associations with ESR, CRP or DAS28 scores, or the delta change in these traditional disease activity measures, were observed up to 12-months after the initiation of csDMARD therapeutic regimens. As such, in keeping with results obtained within RA patients, 12-months after diagnosis and the initiation of csDMARD therapeutic regimens, alterations to the relative distribution of CD25+CD127^{lo} nTregs, FoxP3, phospho-FoxP3 (S418) and phosphorylated FoxP3 are observed; with significant increases in the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3 observed. However, when investigated further for associations with the current disease activity measures ESR, CRP and DAS28, the delta change to nTreg parameters is not significantly associated with fluctuations in patient clinical outcomes; indicating that the monitoring of nTreg parameters, following culture with media alone, cannot be used to predict therapeutic efficacy.

5.3.e.ii. PHA:- Following stimulation with PHA, a significant reduction in the relative distribution of CD25+CD127^{lo} nTregs was observed, within ERA patients 12-months after diagnosis, as compared to the time of diagnosis ($p < 0.05$) (Figure 5.11E). No significant difference in the relative expression levels of FoxP3 and phospho-FoxP3 (S418) were, however, observed up 12-months after the initiation of csDMARD therapeutic regimens. A trend towards a reduction in the relative expression levels of phospho-FoxP3 (S418) was observed 12-months after diagnosis, as compared to the time of diagnosis. However, this observation failed to reach statistical significance ($p = 0.1621$) (Figure 5.11G). A significant reduction in the relative expression levels of phosphorylated FoxP3 was observed within ERA patients 12-months after the time of diagnosis. Furthermore, a split population in the relative expression levels of phosphorylated FoxP3, akin to that previously observed within our RA patient cohort, was observed within ERA patients 12-

months after the time of diagnosis (median of lower population = 32.0% [IQR = 20.9 – 33.3%]; median of higher population = 75.6 [IQR = 60.2 – 90.4%]) (Figure 5.11H).

When analysed further for evidence of associations with clinical outcome measures, no associations with ESR, CRP or DAS28 scores were observed between the relative distribution of CD25+CD127^{lo} nTregs, or the relative expression levels of FoxP3, within ERA patients up to 12-months after the initiation of csDMARD therapeutic regimens (data not shown). Furthermore, no significant difference in ESR, CRP or DAS28 scores was observed when ERA patients were stratified according to the delta change in nTreg parameters, following stimulation with PHA (data not shown). Negative associations between the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3 were, however, observed with DAS28 scores 12-months after the time of diagnosis ($r=-0.5394$, $p=0.1139$ and $r=-0.5515$, $p=0.1049$, respectively); relationships entirely consistent with those previously observed within RA patients (Figure 5.12A - B).

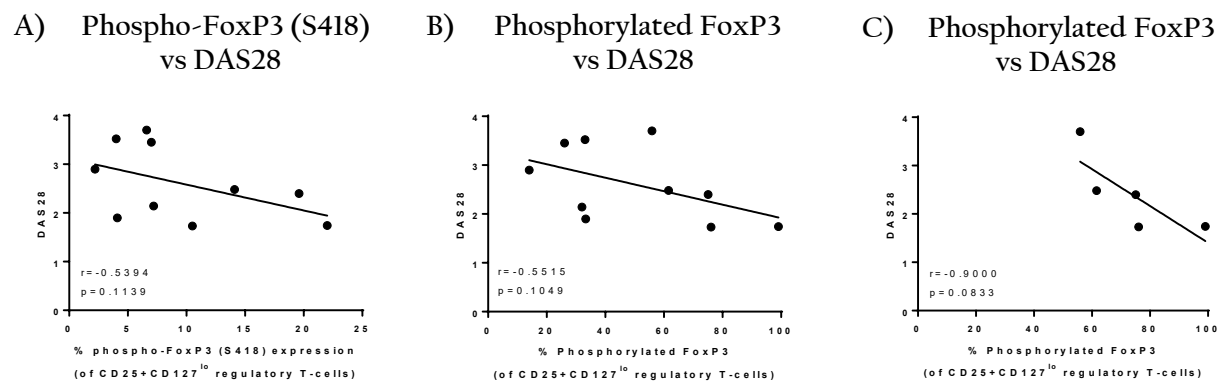


Figure 5.12:- Association of phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels with DAS28 scores in newly-diagnosed RA patients 12-months post diagnosis, following stimulation with PHA. A) Percentage of phospho-FoxP3 (S418) expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells, compared to DAS28 scores at 12-months post diagnosis. B) Percentage of phosphorylated FoxP3 expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells compared to DAS28 scores at 12-months post diagnosis. C) Percentage of phosphorylated FoxP3 expression, within CD25+CD127^{lo} naturally-occurring regulatory T-cells compared to DAS28 scores at 12-months post diagnosis, within newly-diagnosed RA patients demonstrating higher levels of phosphorylated FoxP3 expression. An R-value ≥ 0.50 was considered to represent an association between two variables, with a p-value < 0.05 considered statistically significant.

With the further stratification of ERA patients according to the split populations observed 12-months post-diagnosis, a negative association between the relative distribution of phosphorylated FoxP3 and DAS28 scores was observed within ERA patients presenting with higher levels of phosphorylated FoxP3 ($r=-0.9000$, $p=0.0833$) (Figure 5.12C). No such association was observed within ERA patients presenting with lower levels of phosphorylated FoxP3 12-months after diagnosis. Despite this compelling association, the delta change in the relative expression levels of phosphorylated FoxP3 and DAS28 scores 12-months after the initiation of csDMARD therapeutic regimens were, however, unrelated. As such, within ERA patients, an increase in the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3, following stimulation with PHA, appear to be associated with a favourable clinical outcome, 12-months after the initiation of csDMARD therapeutic regimens. However, the delta change in the relative distribution of nTreg parameters, 12-months after diagnosis, does not appear predictive of therapeutic efficacy.

5.4. Discussion

As a fundamental component of peripheral tolerance, alterations to the number and function of naturally-occurring regulatory T-cells (nTregs) have been extensively investigated in the setting of autoimmunity, with the field of rheumatoid arthritis (RA) no exception. However, due to inconsistencies in the immunophenotypic characterization of nTregs throughout these studies, their role in the pathogenesis of RA, and other such autoimmune conditions, has largely remained ambiguous. The importance of nTregs in RA pathogenesis has, however, become far more evident following a publication by Nie et al, in which the detrimental effects of the pro-inflammatory cytokine TNF- α on nTreg functional activity was extensively investigated (Nie et al., 2013). Within this study, the suppressive capabilities of nTregs was proven to correlate with the phosphorylation status of FoxP3; with the dephosphorylation of FoxP3, and thus a reduction in the functional activities of nTregs, demonstrated to occur in the presence of TNF- α ; a cytokine found in abundance within the joints of RA patients with active disease (Gao et al., 2015; Nie et al., 2013).

This group were therefore able to demonstrate, for the first time, the utility of monitoring FoxP3 phosphorylation status in the assessment of RA disease activity, providing a conceivable means by which therapeutic efficacy can be monitored. Undesirably, however, FoxP3 phosphorylation, within this study, was detected via immunoprecipitation and western blot analyses (Nie et al., 2013). Such methods are labour-intensive, time-consuming and impractical for use within busy diagnostic laboratories; providing limited quantitative parameters by which a response to therapeutic interventions can be monitored. Herein, a quantitative flow cytometric assay was successfully developed, facilitating the detection and characterization of CD25⁺CD127^{lo}-, FoxP3⁻ and phospho-FoxP3 (S418)-expressing nTregs, following culture with media alone, as well as stimulation with PHA.

The importance of employing an intelligible nTreg immunophenotypic characterization was clearly evident from the results obtained within our healthy control cohort, with a supposed increase in the relative distribution of CD25⁺CD127^{lo} nTregs observed, following mitogenic stimulation with PHA. Based upon surface immunophenotyping alone an increase in the relative distribution of nTregs, following antigenic stimulation, would be surmised. However, when considered within the setting of reduced FoxP3 expression levels, this phenotypic increase in nTregs could, in fact, be attributed to the expansion of activated conventional CD4⁺ T-cells; a T-cell subset known to upregulate CD25, as well as downregulate CD127, upon antigenic stimulation (Boyman and Sprent, 2012; Simonetta et al., 2010). Under such circumstances, the stable expression of FoxP3 is perhaps, therefore, a more accurate representation of putative nTreg numbers (Klein et al., 2010; Morita et al., 2016).

Critically, the phosphorylation status of FoxP3 is also altered following antigenic stimulation, with an upregulation in phospho-FoxP3 (S418), and phosphorylated FoxP3, expression observed. Under resting conditions, phospho-FoxP3 (S418) and phosphorylated FoxP3 are minimally expressed within healthy individuals, with negligible nTreg functional capabilities required during times of immunological inactivity. However, following antigenic stimulation, the functional activities of nTregs are fundamentally required to suppress and limit the actions of conventional CD4⁺ and CD8⁺ T-cells, which could otherwise exert potentially damaging pro-inflammatory effects (Kehrl et al., 1986; Wing et al., 2014). Under such conditions, a sharp increase in phospho-FoxP3 (S418) and phosphorylated FoxP3 expression is observed, clearly signifying the activation of nTreg suppressive capabilities. With this in mind, deviations from such phosphorylation expression patterns would indicate a disturbance in nTreg functionality, providing a measureable immunological parameter by which therapeutic efficacy could be monitored.

Within the setting of RA, an increase in the relative distribution of CD25⁺CD127^{lo} nTregs was the only significant alteration to any nTreg parameter observed within newly-diagnosed, treatment-naïve RA (ERA) patients, following culture with media alone. With a non-significant reduction in the relative expression levels of FoxP3 also evident, however, it is tempting to speculate that this increase in CD25⁺CD127^{lo} nTregs, in fact, corresponds to an expansion in activated conventional CD4⁺ T-cells, rather than an increase in nTregs, as previously believed (Boyman and Sprent, 2012; Simonetta et al., 2010; Yu et al., 2012). A result such as this would be entirely consistent with the heightened state of inflammation often detectable within ERA patients, prior to their initiation of disease-modifying anti-rheumatic drug (DMARD) therapeutic regimens (Felson et al., 2011). However, within our ERA patient cohort, no significant associations between any nTreg parameter and the patient clinical outcome measures CRP, ESR or DAS28 scores were observed, following culture with media alone, leaving this hypothesis unsubstantiated.

In addition to the above observations, no significant difference in any nTreg parameter measured was observed within ERA patients, following stimulation with PHA, as compared to disease and healthy controls; with the previously observed increase in CD25⁺CD127^{lo} nTregs apparently abrogated following mitogenic stimulation. Furthermore, no significant associations between CRP, ESR or DAS28 scores were observed with any nTreg parameter measured, following stimulation with PHA. Indeed, the only major alteration observed within ERA patients, and additionally longstanding RA (RA) patients, following stimulation with PHA, was the lack of age associations observed with all nTreg parameters; with negative associations previously observed within our healthy control cohort. However, when stratified according to age, no significant difference in the relative distribution of any nTreg parameter measured was observed, negating the need for age-adjusted ranges in this study. Based upon current data, therefore, the utility of employing nTreg parameter measurements to predetermine

therapeutic efficacy within ERA patients, prior to the initiation of csDMARD therapeutic regimens, remains inconclusive.

Despite the potential lack of clinical utility within ERA patients, the results obtained within RA patients suggest a promising line of enquiry. Indeed, following culture with media alone, a significant increase in the relative expression levels of phospho-FoxP3 (S418), and phosphorylated FoxP3, was observed within our RA cohort. Furthermore, within RA patients with detectable levels of phospho-FoxP3 (S418), and phosphorylated FoxP3, positive associations between the relative expression levels of phospho-FoxP3 (S418), phosphorylated FoxP3 and DAS28 scores were observed. Taken together, these results would suggest that RA patients with detectable levels of phospho-FoxP3 (S418) and phosphorylated FoxP3, following culture with media alone, are subject to increased levels of disease activity, irrespective of their therapeutic regimen.

Interestingly, the relative distribution of nTreg parameters within RA patients was also altered following stimulation with PHA; with a significant increase in CD25+CD127^{lo} nTregs observed. With no significant difference in the relative expression levels of FoxP3, however, detected this result would again be consistent with an increase in activated conventional CD4⁺ T-cells following mitogenic stimulation. Indeed, the observed positive association between the relative distribution of CD25+CD127^{lo} nTregs and DAS28 scores, within RA patients, would support this notion. Despite no further significant alterations to nTreg parameters being evident within RA patients, a high degree of variability in phosphorylated FoxP3 expression levels was apparent, following stimulation with PHA; suggesting that differential groups of RA patients may possess normal nTreg functional capabilities, whilst others may be dysregulated. In this regard, RA patients demonstrating higher levels of phosphorylated FoxP3 expression exhibited a negative association with DAS28 scores, indicating a favourable clinical response; whilst RA patients demonstrating lower levels of phosphorylated FoxP3 displayed no such relationship.

As such, a dichotomy in patient clinical outcomes appears to exist between these two groups of RA patients, based upon the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3, respectively. As such patients have undertaken therapeutic interventions, consisting of a variety of csDMARDs and bDMARDs, the differing effects of these therapeutic interventions were assessed, via the stratification of RA patients according to their therapeutic regimens. However, no significant difference in the relative expression levels of phospho-FoxP3 (S418), or phosphorylated FoxP3, was observed within RA patients receiving csDMARD or bDMARD therapeutics. Furthermore, due to a lack of statistical power, no single therapeutic agent could be attributed to these alterations in phospho-FoxP3 (S418) expression; a line of enquiry which should be explored further in future studies.

With no individual therapeutic effects confirmed, these results presently suggest that the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3 may solely be used as indicators of disease severity, irrespective of the therapeutic intervention employed. The longitudinal monitoring of ERA patients would certainly support this notion; with alterations to all nTreg parameters observed, following culture with media alone and stimulation with PHA, at 12-months post-diagnosis. Critically, the results generated within ERA patients 12-months after the initiation of csDMARD therapeutic regimens are entirely consistent with those previously observed within RA patients. Following stimulation with PHA, a negative association between the relative expression levels of phospho-FoxP3 (S418), phosphorylated FoxP3 and DAS28 scores was observed within ERA patients 12-months after diagnosis; with the most profound negative association observed within ERA patients presenting with higher levels of phosphorylated FoxP3 12-months after the initiation of csDMARD therapeutic regimens. With the results of ERA longitudinal monitoring and RA phenotypic characterization taken together, a rise in phospho-FoxP3 (S418) and phosphorylated FoxP3 expression levels, following culture with media alone, would appear to represent an increase in nTreg functional activity

without prior antigenic activation; a state which corresponds with an adverse clinical response. In contrast, an increase in the relative expression levels of phospho-FoxP3 (S418) and phosphorylated FoxP3, following stimulation with PHA, signifies an appropriate response to antigenic stimulation and as such indicates a favourable clinical response.

The precise mechanism by which disturbances in this particular post-translational modification result in the development of RA is yet to be determined. With genetic mutations in the gene encoding the regulatory T-cell transcription factor FoxP3 proven to result in impaired/absent nTreg numbers and function in the primary immunodeficiency Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, deviations to the post-translational modifications made to FoxP3 during its manufacture seem to induce a comparatively subtle pathology within affected individuals (Kennedy et al., 2014; Torgerson and Ochs, 2007; Verbsky and Chatila, 2013). Based upon the current literature it therefore seems reasonable to hypothesize that the stable expression of FoxP3, irrespective of post-translational modifications, is fundamentally required to maintain a reservoir of nTregs; with the absence of FoxP3 expression resulting in the fulminant loss of nTregs and a predisposition towards the development of IPEX syndrome (Morawski et al., 2013; Torgerson and Ochs, 2007; Verbsky and Chatila, 2013). In contrast, with the stable expression of FoxP3 established, the functional activity of nTregs is dependent upon the appropriate post-translational modification of FoxP3; with irregularities in FoxP3 predisposing towards autoimmunity rather than a fulminant immunodeficiency (Kennedy et al., 2014; Morawski et al., 2013; Nie et al., 2013). With multiple post-translational modifications now documented, including the phosphorylation, acetylation and methylation of FoxP3, it is conceivable that individual modifications might be required to confer specific suppressive capabilities, differentially contributing to the establishment of cytokine secretory and cell-cell signalling suppressive activities (Gao et al., 2015; Kennedy et al., 2014; Morawski et al., 2013; Nie et al., 2013).

Despite convincing evidence for alterations in nTreg number and function within ERA and RA patients, this study was not without its limitations. With participant recruitment restricted, a lack of statistical power is a distinct possibility. However, with the above presented data obtained from appropriately age- and sex-matched patient and control groups, and with clear statistical significances observed, the data generated provides some promising insights into nTreg functionality within RA during the course of immunosuppressive interventions. As such, further investigations are warranted incorporating a larger sample size. Such a study would also benefit from the longitudinal follow-up of ERA patients over a longer period, so as to bridge the gap between the immediate effects of csDMARD therapeutic interventions, on nTreg parameters within ERA patients, and the long-term ramifications of chronic csDMARD and bDMARD usage on nTregs within RA patients.

The use of polyclonal primary and secondary antibodies in the staining of phospho-FoxP3 (S418) was also problematic, introducing the potential for inconsistencies in staining intensity between participants; with the high degree of variability in phospho-FoxP3 (S418) expression observed within all participant groups likely a consequence. However, with promising results demonstrated, future studies should seek to incorporate the use of a fluorochrome-conjugated monoclonal antibody directed against phospho-FoxP3 (S418), or if unavailable, a polyclonal antibody directly conjugated to a suitable fluorochrome would be a viable alternative. Future work should also incorporate the use of functional suppressive assays, to further corroborate the present findings. These studies should seek to evaluate the suppressive capabilities of nTregs within RA patients, presenting with alterations to FoxP3 phosphorylation; an avenue of investigation that was unfortunately beyond the scope of the present study (McMurchy and Levings, 2012; Nie et al., 2013).

At present, the measurement of nTreg parameters, appears to offer limited clinical benefit in the establishment of therapeutic regimens within ERA patients. However, with the

appropriate phenotypic characterization of nTregs, within RA patients who have already commenced on a csDMARD or bDMARD therapeutic regimen, the monitoring of nTreg phosphorylation status may be utilized to identify those RA patients who have, or are, likely to fail their therapeutic regimen. The utility of employing such a strategy in monitoring individual csDMARD and bDMARD therapeutic effects in RA patients, however, remains to be substantiated. As such, with further research, the introduction of this monitoring system into routine clinical operations may prove beneficial to current remission monitoring practices in the future, facilitating the swift adjustment of RA patient therapeutic regimens; improving patient morbidity and mortality.

Chapter 6: General Discussion

Rheumatoid arthritis (RA) is a devastatingly progressive and destructive disease in the event that a swift diagnosis and effective therapeutic regimen cannot be successfully established. Identifying when a therapy is proving ineffective can, however, prove extremely challenging, with current remission monitoring practices heavily reliant upon accurate formal joint counts, acute phase protein measurements, and patient global assessments, such as those recorded by the health assessment questionnaire (HAQ) (Felson et al., 2011). These are potentially insensitive means of assessing patient remission, with the levels of conventional inflammatory markers such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) often dissociated from patient clinical disease activity indices (CDAI), combined with the subjectivity of questionnaires (Felson et al., 2011; Kay et al., 2014).

Throughout this study, it has been our experience that disease severity indicators, such as ESR, CRP and the 28 joint disease activity score (DAS28), are inconsistently utilized in routine clinical practice. This is perhaps due in part to the time pressures experienced in busy rheumatology clinics; coupled conceivably by a lack of confidence in the unreliable and subjective nature of these conventional remission monitoring practices. All in all, the relative insensitivity of these remission monitoring protocols, combined with their sporadic use, may hinder the timely alteration of therapeutic regimens, ultimately compromising patient care and prognosis (Smolen et al., 2016). Thus, the identification of new biomarkers to improve the predictive value of remission evaluation models would be of great benefit.

Adding further complexity to the conundrum is the multifaceted nature of RA pathogenesis, with naturally-occurring regulatory CD4⁺ T-cells (nTregs), circulating blood-derived follicular helper CD4⁺ T-cells (cFth), autoantibody-producing B-cells and CD25⁺CD134⁺ activated CD4⁺ T-cell responses (aTcs) all implicated in disease pathology; causal relationships supported by the present study (Davis et al., 2012; McInnes and Schett, 2011; Nie et al., 2013; Pandya et al., 2016). With such varied cellular constituents involved in disease pathogenesis, however, RA should conceivably be considered a heterogeneous group, or spectrum of disorders, rather than as the single entity it is presently considered. With multiple and contrasting genetic variants previously described in association with RA pathogenesis; likely predisposing to complex and highly variable cellular defects; a decidedly individual patient pathology is entirely plausible (McInnes and Schett, 2011; Scott, Wolfe and Huizinga, 2010). Yet, with RA diagnosis, based upon the 2010 RA classification criteria, focussed heavily upon joint involvement, symptom duration and the presence of serological markers, such as rheumatoid factor (RF) and anti-CCP antibodies, the current diagnostic strategy is an ineffective and incomplete means of characterizing individual patient pathology (Aletaha et al., 2010; Gibofsky, 2012; Smolen et al., 2016).

In isolation, a diagnostic approach such as this seems a rational strategy, designed to establish an early diagnosis and permit the implementation of an effective therapeutic regimen before permanent joint damage, extra-articular manifestations (EAM) and co-morbidities can occur (Aletaha et al., 2010; Kay and Upchurch, 2012; Smolen et al., 2016). However, coupled with the knowledge that 70.0% improvement rates, as defined by the American College of Rheumatology (ACR), are only achieved in approximately 25.0 – 50.0% of early RA patients placed onto conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) therapeutic regimens, consisting of methotrexate (MTX), folic acid and hydroxychloroquine (HCQ), a different approach to RA diagnosis should, perhaps, be considered (Smolen et al., 2014). In this regard, the pathotyping of

prospective RA patients, prior to the implementation of therapeutic regimens, could be highly beneficial and lead to a personalized medicine approach for the monitoring of RA patients. Thus, based upon the present study, we propose that prospective RA patients should not only be inspected for serological markers during diagnosis, but their cellular immune function should also be investigated, with cFth, memory helper CD4⁺ T-cells (mTh), aTcs and nTregs all viable routes for consideration. A strategy such as this would facilitate the identification of RA predisposing cellular defects within newly-diagnosed, treatment-naïve RA (ERA) patients; results which could be used to succinctly identify the causal genetic defect.

Whilst furthering our understanding of RA pathogenesis, the pathotyping of RA patients may also provide a legitimate means by which to predict and monitor therapeutic efficacy. As evidenced in the present study, the measurement of CCR4⁻ Th2-like cFth percentages at the time of diagnosis proved highly predictive of csDMARD therapeutic efficacy, within ERA patients up to 12-months after the time of diagnosis; with the relative distribution of CCR4⁻ Th2-like cFth associated with the delta change in DAS28 scores. Furthermore, the delta changes in CCR4⁺ Th17-like cFth and CCR4⁺ Th17 mTh percentages, within ERA patients after 12-months of compliance with a csDMARD therapeutic regimen, were also associated with the delta change in DAS28 scores; providing a further surrogate means by which therapeutic efficacy can be monitored, particularly in the event that conventional disease activity measures, such as DAS28 scores, are unavailable. Consequently, although further longitudinal studies are required to corroborate these findings, such assays could prove highly advantageous, enabling the tailored implementation of a csDMARD, or where appropriate a bDMARD therapeutic regimen, that specifically targets the patients cellular and pathological defect, ensuring the most efficient containment of disease activity, whilst also minimising the potentially harmful side-effects associated with the use of disease-modifying anti-rheumatic drugs (DMARDs) (Caplan et al., 2017; Salliot and van der Heijde, 2009; Smolen et al., 2014).

Indeed, the identification of deleterious side-effects requires careful patient monitoring, with cytopenias, infection, hepatic disease and hypersensitivity pneumonitis all adverse effects of long-term methotrexate usage alone (Salliot and van der Heijde, 2009; Smolen et al., 2014). Consequently, preventing such damaging side-effects can be a difficult and costly exercise, with the financial constraints associated with an aging population creating further difficulties. Therefore, with the risk of csDMARD and bDMARD toxicity also to be considered, a tailored therapeutic approach could not only reduce the likelihood of disease progression and the development of extra-articular features, but could also prevent the development of deleterious side-effects; a scenario that would greatly improve patient quality of life, whilst also provide huge economic benefits for the national health service (NHS) and society as a whole.

The data presented herein not only advocates for the immune function profiling of prospective RA patients for therapeutic efficacy monitoring, but also posits the necessity of establishing patient infection burden. With RA patients twice as likely to be hospitalized due to serious infections, as compared to healthy individuals, infections are considered a significant cause of morbidity within RA patients; with this increased risk largely thought to be independent of immunosuppressive effects (Doran et al., 2002; Smitten et al., 2008). Alongside this morbidity risk, however, the pathogenesis of RA has long been considered to possess an infectious aetiology, with the herpesviruses CMV, HSV and EBV all implicated, evidenced by the presence of CMV in the synovial fluid of RA patients, as well as an increase in EBV-DNA, EBV-specific antibodies and EBV infected cells documented within RA patients, as compared to disease and healthy controls (Magnusson et al., 2010; Pierer et al., 2012).

The presence of such herpesviruses are also thought to directly contribute to disease severity; with latent CMV infection shown to be associated with an aggravated clinical course in RA patients (Davis et al., 2012; Pierer et al., 2012). The results generated in the

present study would certainly support this notion, with a trend towards increased DAS28 scores observed within CMV positive RA patients, as compared to CMV negative patients, based upon CMV-specific aTc responses. Furthermore, the monitoring of the delta change in the percentage of CMV-specific CCR4⁺ Th17 cells, within ERA patients over 12-months of compliance with a csDMARD therapeutic regimen, also proved highly correlative with the delta change in DAS28 scores; providing an alternative strategy for monitoring therapeutic efficacy within CMV positive RA patients. Based upon the results presented herein, and in other studies, therefore it could be argued that the determination of CMV infection burden at the time of diagnosis should be considered; with this line of enquiry not only identifying another means of monitoring disease activity, but also potentially highlighting another therapeutic avenue (Davis et al., 2013; Sahebjam et al., 2016). With CMV status associated with disease activity, it stands to reason that the use of anti-virals, particular those directed against CMV, would be of clinical benefit. To our knowledge, however, no such strategy has been previously proposed in the setting of RA, a surprising finding given its success in other autoimmune settings (Sahebjam et al., 2016).

With the results presented herein a compelling advocate for immune function profiling, it must be said that the concepts of pathotyping and personalized medicine are not new (Davis et al., 2013; You et al., 2018; Zhang et al., 2018). However, to our knowledge no definitive immune function profile for the characterization of prospective RA patients has, to date, been proposed. Based upon the data presented herein, however, we suggest that the investigation of CCR4⁻ Th2-like cFth, CCR4⁺Th17-like cFth, CCR4⁺ Th17 mTh and CMV-specific Th17 cells should be considered. Furthermore, although a relatively small cohort of ERA and RA patients, were available for analysis, the addition of nTreg phosphorylation status should also be considered. Indeed, consistent with previous studies, the phosphorylation status of FoxP3 within nTregs, following stimulation with PHA, proved to be negatively associated with DAS28 scores, within ERA patients 12-months after the initiation of a csDMARD therapeutic regimen; a relationship also evident

within established RA patients. As such, although the measurement of FoxP3 phosphorylation, at the time of diagnosis, does not appear to be predictive of therapeutic efficacy, the measurement of nTreg parameters may still prove beneficial in the process of therapeutic efficacy monitoring, particularly if these findings can be corroborated in a wider study.

Despite these persuasive arguments, the present study was not without its limitations. Indeed, the evaluation of ERA and RA patient clinical outcomes, within this study, was highly reliant upon the conventional disease severity indicators ESR, CRP and DAS28 scores; the collection of which was, unfortunately, not guaranteed during participant recruitment. Thus, with limited clinical outcome measurements available for RA patients, coupled with the potential for dissociation between CRP, ESR measurements and clinical disease activity, future studies should perhaps consider the assessment of tender and swollen joint counts; disease activity measures which are independent of conventional inflammatory markers, and may also prove more expedient to gather in a busy clinical setting (Kay et al., 2014).

Such studies should also seek to recruit a wider cohort of ERA and RA patients, particularly those with a confirmed positive CMV serological status. An increase in ERA and RA participant numbers would enable a more thorough examination of CMV-specific aTc responses, with the effects of individual csDMARD and bDMARD therapeutic regimens on the relative distribution of CCR4⁺ Th17 and CCR4⁺ Th2 cells of particular interest. A wider ERA and RA cohort would also allow the present nTreg findings to be further substantiated, whilst also facilitating a more detailed examination of the relationship between nTreg parameters and RA disease activity measures; the inspection of which was somewhat limited in the present study by small participant numbers.

Perhaps a further shortcoming of the present study was the heterogeneous nature of the disease control group (DCG) utilized. Although tightly matched with our RA cohort according to their therapeutic regimens, the disease state of our DCG was highly diverse, introducing a confounding factor which may have obscured the expression profiles of our T-cell subpopulations within differing disease states. With the measurement of cFth, mTh, aTcs and nTregs of potential relevance to therapeutic efficacy monitoring in other autoimmune conditions, future studies should consider focussing on a single disease state, such as psoriatic arthritis, an arthritic autoimmune condition in which patients are treated with csDMARDs and bDMARDs akin to those utilized in the treatment of RA (Kay and Rahman, 2010).

Despite these limitations, the present study does herald a potentially interesting line of enquiry should the validity of the present findings be proven by subsequent studies. In such a circumstance, the longitudinal follow-up of cFth, mTh, aTcs and nTregs within ERA patients over an extended time period would also be of benefit, in order to establish whether the relationships observed between our T-cell subpopulations and patient clinical outcomes hold firm beyond that of 12-months, particularly following the adaptation of patient therapeutic regimens to incorporate bDMARD therapeutic agents. These investigations would also lend themselves to more fundamental immunological inquiries, such as the co-culture of differing cFth subpopulations with naïve B-cells, with the further introduction of therapeutic doses of individual csDMARDs and bDMARDs, to examine their effect on B-cell maturation and autoantibody production of particular interest. Moreover, the culture of mTh, aTcs and nTregs, from ERA and RA patients, as well as DCG and HC, in the presence of individual csDMARDs and bDMARDs would facilitate the examination of T-cell immunity under immunosuppressive conditions, advancing our understanding of csDMARD and bDMARD pharmacodynamics in the setting of RA.

In conclusion, the work presented here not only supports the present literature, relating to the potential pathogenesis of rheumatoid arthritis, but also sheds light on the budding merit of using cellular laboratory assays to monitor therapeutic efficacy. Truly, the assays described within, may not only prove highly informative regarding RA pathogenesis, but could also conceivably provide a convenient and cost-effective means of improving patient care and treatment. Furthermore, the assays described herein may not only be applicable to RA, but may also be pertinent to a number of other autoimmune conditions, in which cFth, mTh, aTcs and nTregs are involved in their development, and csDMARDs and bDMARDs are to be utilized (Li et al., 2012; Morita et al., 2011; Verbsky and Chatila, 2013). As such, the potential utility of these three flow cytometric assays has been demonstrated, paving the way for the development of additional flow cytometric assays for the pathotyping of RA, and other autoimmune conditions; with the exploration of further FoxP3 post-translational modifications but one possibility (Kennedy et al., 2014). With the expansive repertoire of such assays, the accurate immune function profiling and therapeutic efficacy monitoring, of patients commencing on csDMARD and bDMARD therapeutic regimens, may help to make the prospect of personalized medicine a reality.

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Appendix

Appendix I

Dhalla [F](#), Fox [H](#), Davenport [EE](#), Sadler [R](#), Anzilotti [C](#), van Schouwenburg [PA](#), Ferry [B](#), Chapel [H](#), Knight [JC](#), Patel [SY](#). Chronic mucocutaneous candidiasis: characterization of a family with STAT-1 gain-of function and development of an ex-vivo assay for Th17 deficiency of diagnostic utility. Clin Exp Immunol. 2016 May; 184(2):216-27.