KLRG1 and its role in Rheumatoid Arthritis

A thesis submitted in candidature for the degree of Doctor of Medicine

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January 2019

Infection and Immunity

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SHORT SUMMARY

Introduction: Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic joint inflammation. A homeostatic disequilibrium of the immune system is implicated in the failure of systemic tolerance. A part of this disequilibrium may be the rise of $CD3^+CD8^+CD28^-$ T cells in RA. Their role in the pathogenesis of RA remains unknown.

Methods: Analysis of peripheral blood samples from RA patients using flow cytometry were compared to healthy controls (RA patients n=50, healthy controls n=25).

Results: $CD3^+CD8^+CD28^-$ cells are higher in RA. Further subdivision of this group revealed that $CD3^+CD8^+CD28^-$ cells are higher in Early RA as well as Established RA. This subset of $CD3^+CD8^+CD28^-$ cells also correlated with disease duration in Early RA patients. The cell surface receptor KLRG1, is expressed by a high percentage of $CD3^+CD8^+CD28^-$ cells.

As well as being associated with clinical and serological markers in RA, $CD3^+CD8^+CD28^-KLRG1^+$ cells are higher in the Early RA patients who had a poor response to therapy at six months. $CD3^+CD8^+CD28^-KLRG1^+$ cells were found to produce more IL-10 than $KLRG1^-$ cells.

The percentage of $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^-KLRG1^+$ cells is higher in CMV positive than CMV negative RA patients but CMV distribution is similar across the responders and non-responders to treatment in Early RA. CMV positive $CD3^+CD8^+CD28^-KLRG1^+$ cells produce more IL-10 than $KLRG1^-$ cells.

Conclusion: The higher percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells is associated clinical and serological markers as well as poor response to treatment in Early RA patients. This reinforces the importance of this subtype of T regs in the course of RA. However, the precise reason is unclear and requires further investigation.

DECLARATION

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For you Dad

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my supervisors; to Professor Ernest Choy for this unique opportunity and for your enthusiasm and vast knowledge; to Professor Anwen Williams for her extensive experience, immense knowledge and support and to Dr Gareth Jones for your infective passion, patience and dedication, that really kept me going when times were tough. Guidance from you throughout has been invaluable.

The work carried out in this thesis would not have been possible without the help, support and guidance of numerous people in the Tenovus building. I owe a great deal to all the patients that have taken the time to be part of these studies: thank you.

To my work colleagues and, more importantly, friends: Ruth, Jess, Katie, Gareth, Lauren, Cath, Justine, Vicky, Javier, Rav, Anne, Saydul, Selinda, Aisling, Dave, Anna, Jason, Alicia, Tommy, Ben. Your advice, chats and giggles have been invaluable.

To Mum and Mark, for listening to my highs and lows with equal attention and such patience. Thank you for helping me throughout and for believing in me. To my younger but hugely impressive brother, you are always there for me, a strong arm to lean on and someone to make me laugh at myself again. Thank you to my ladies, Amber, Jen, George, Emily and Alice for such emotional support.

My Dad. This is for you. I finished this with the strength you gave me. Your never-ending belief in me stoked the fire to help me get over the finish line. Infact you built the fire. I cannot count the number of times I so wished I could discuss this with you, to hear your balanced, logical and always insightful view on a subject. If I listen hard, I still hear your voice.

Lastly to my men, Mike and Leonardo. You have both given and sacrificed so much in order to help me do this, your help and support has been immeasurable. You are the light at the end of the tunnel. Thank you.

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ABBREVIATIONS

ACR – American College of Rheumatology

ACR50 –Improvement in the number of tender and swollen joints, and 50% improvement in ≥3 of: the patient's global assessment of disease status; the patient's assessment of pain; the patient's assessment of function; the physician's global assessment of disease status; serum CRP levels.

ACPA – Anti Citrullinated Protein Antibody

APCs – Antigen presenting cells

BNF – British National Formulary

DAS28 – Disease Activity Score 28

CRP – C-reactive protein

DMARDs – Disease Modifying Anti-Rheumatic Drugs

dH2O – distilled water

EDTA - Ethylenediaminetetraacetic

ef450 - eFluor450

ESR – Erythrocyte sedimentation rate

EULAR – European League against Rheumatism

FITC – Fluorescein isothiocyanate

HLA – Human Leucocyte antigen

IL – Interleukin

MCTD – Mixed Connective Tissue Disease

MHC – major histocompatibility antigen

NICE – National Institute for Health and Care Excellence

NK cells – Natural Killer

NSAIDs – Non-steroidal anti-inflammatory drugs

PCP - Peridinin-chlorophyll proteins

PIP – Phosphatidylinositol-bisphophate (cell membrane phospholipid)

PE - R-phycoerythrin

RA – Rheumatoid Arthritis

SLE – Systemic Lupus Erythematosus

T1DM – Type 1 Diabetes Mellitus

TNF – Tumour necrosis factor

Th – T helper

1 Chapter 1: General Introduction

1.1 Rheumatoid Arthritis

1.1.1 Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease that causes chronic joint inflammation. The pathogenesis is multifactorial and complex. RA is characterised by synovial inflammation caused by the infiltration of inflammatory cells into the joint. This leads to synoviocyte proliferation and hyperplasia (called pannus). Pannus invades and erodes cartilage and destroys bone[1]. Persistant inflammation causes joint damage so treating RA-associated inflammation as early as possible and keeping disease activity low, is the target for any successful therapy[2]. The loss of function associated with an arthritic joint may be irreversible, therefore prevention of joint damage is the preferred outcome for the patient.

RA is perhaps the best example of a condition where research into disease pathogenesis has led to the development of targeted therapy. Cytokine inhibitors have benefited millions of patients. Indeed, tumour necrosis factor (TNF) antagonists, have revolutionised the treatment and prognosis of RA[3]. However, despite the growing numbers of medication options available for RA therapy, still 6% of patients do not respond to even the third line biologic treatment[4]. This is because their disease is refractory to such therapy or contra-indicated due to co-morbidities (e.g. chronic infection, cardiac failure).

The prognosis of RA can vary from non-erosive to severe destructive disease over 10 years. There is a widely accepted therapeutic window of opportunity to prevent joint damage[5,6]. The National Institute for Health and Clinical Excellence advocates aggressive treatment ideally within three months of onset of symptoms with a combination of disease modifying anti-rheumatic drugs (DMARDs) and corticosteroids. This approach can expose patients with benign disease to potential toxicity from unnecessary treatment. Ideally, biomarkers of poor prognosis can be identified so that RA patients can be selected for the more aggressive treatment. These biomarkers of prognosis could

also potentially provide insight into the pathogenesis of RA and provide the opportunity to develop novel targets for treatment.

1.1.2 Clinical Features of RA

RA classically presents as a symmetrical polyarthritis, predominantly with swelling, stiffness and pain. Symptoms are normally worse in the mornings, then improve throughout the day. The symptoms do, however, typically return after episodes of inactivity[7].

RA onset can range from an insidious start over weeks to months to an acute onset. The number of joints affected is variable but RA normally affects five or more joints. The pattern of joints affected is also important. The most commonly affected joints are the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints of the hand, the wrist, and metatarsophalangeal (MTP) joints of the feet[8].

Fatigue, fever, weight loss and malaise are often non-specific clinical signs associated with RA. Although the articular structures are predominantly affected by RA, extra-articular manifestations can include secondary Sjögren's syndrome, pulmonary fibrosis, renal amyloidosis, rheumatoid nodules, sensory neuropathy and cardiovascular disease[9,10].

1.1.3 Diagnostic Criteria

In 2010, revised diagnostic criteria from the American College of Rheumatology was developed for the diagnosis of RA (**Table 1-1**)[11]. The new criteria included the novel serological Anti-Citrullinated Peptide Antibody (ACPA) test as well as excluding previous criteria used, for example subcutaneous nodules[12]. This modification of criteria was introduced because of the need to identify patients with early disease.

A) Joint Involvement (0-5)	
1 large joint	0
2-10 large joints	1
1-3 small joints including wrists (with or without involvement of large joints)	2
4-10 small joints including wrists (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B) Serology (0-3)	
Negative RF and negative ACPA	0
Low-positive RF or low positive ACPA	2
High positive RF or high positive ACPA	3
C) Acute phase reactant (0-1)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D) Duration of symptoms (0-1)	
<6 weeks	0
>6 weeks	1

Table 1-1: 2010 American College of Rheumatology and European League against Rheumatism classification criteria for RA. These classification criteria are aimed at newly presenting patients. Patients who are tested should have at least one joint with definite clinical synovitis, which is also not explained by another disease. Classification criteria for RA is a score of ≥6 out of 10.

1.1.4 Epidemiology of RA

The typical age of onset of RA is between 30 and 60 years and is 2-4 times more likely in females[13]. RA has a highly variable annual incidence (12-1200 per 100,000 population)[14]. Interestingly the disease prevalence is about 1% in Caucasians but varies between 0.1% (in rural Africans) and 5% (in North American indigenous peoples)[15,16].

1.1.5 Risk Factors for Developing RA

Environmental and genetic factors are thought to contribute to RA development. Genetic twin studies in RA have a concordance rate of 15-30% the strongest of which is associated with HLA-DR[17]. The PTPN22 gene has also been implicated in RA. The normal function of this gene is a negative regulation of T cell activation. A missense single-nucleotide polymorphism within the gene has been shown to have a demonstrable effect associated with dysregulation of T cell activation[18].

Genome-wide association studies (GWAS) have recently been used to great effect to identify genetic susceptibility at over 100 loci for RA[19]. Since 2010, more loci specific to ethnic groups have been identified but no single gene has been identified and targeted as the leading cause of RA. Attention has turned to whether these loci can help predict disease response to medication[20].

Along with genetic predisposition, environmental factors such as smoking in particular, are also thought to play a role. Although smoking has long been identified as a risk factor for RA, emerging evidence suggests a new association between genetic predisposition, smoking and the generation of ACPA[21,22].

Källberg et al (2010) estimated that smoking was responsible for 35% of ACPA positive cases. The amount of smoking in pack years, as well as the number of HLA-DR genes carried, both increased the risk of ACPA positivity[23]. In individuals carrying two copies of the HLA-DR1 gene, 55% of ACPA-positive RA was attributable to smoking. It also appears that the interaction between the HLA-DRB1 and PTPN22 genes, increase the risk of ACPA positive RA [24]. Interestingly the HLA-DRB1 gene in which the linkage to the HLA region is observed in RA families, is absent in ACPA negative families[25].

Increased amounts of citrullinated proteins have been found in the lungs[26]. These proteins may provide a substrate for immune activation. Citrullinated proteins (for example vimentin, histone and fibrinogen) were not only found in the lungs but also the inflamed RA joints[27]. This suggests that auto-immunity to citrullinated self-molecules, may be triggered in the lungs and then affect joints elsewhere.

Porphyromonas gingivalis is a pathogenic gram-negative bacterium that causes periodonitis, and has been linked to the development of RA. It releases an enzyme, peptidyl arginine deiminase (PAD) that can cause the conversion of arginine to citrulline[28]. These bacteria and its enzyme can migrate into the bloodstream and cause remote citrullination. Citrullination of peptides predispose to autoimmunity in RA through the development of ACPA.

Smoking is also a risk factor for periodontitis[29]. A hypothesis that smoking increases the risk of RA through promoting periodontal disease was tested using 103 pre-RA cases. Antibody levels to PAD peptides were higher in smokers but were not associated with risk of RA, or development of ACPA[30]. There appears to be a significant positive correlation between the years a patient has smoked for and the levels of Rheumatoid Factor (RF)[31]. However, the mechanism for this remains unclear.

Some viral infections such as Epstein-Barr virus (EBV), Cytomegalovirus (CMV) and Parvovirus B19 are candidates for the trigger of autoimmunity but no clear link has yet been shown. CMV causes an increase in the number of T cells and possibly ages the immune system[32,33]. This aging or T cell senescence and exhaustion includes immunosuppressive T cells. This could result in the ineffective homeostatic controls by suppressive T cells and thereby contribute to the pathogenesis of autoimmune arthritis.

There are high levels of EBV positivity in RA patients and evidence of EBV and parvovirus within synovial tissues[34,35,36]. An EBV viral protein gp110 contains a sequence of amino acids (QKRAA). This corresponds to a region of HLA-DRB1 alleles associated with RA risk[37]. T cells positively selected in the thymus by low affinity interactions with self-major

histocompatibility complex (MHC) peptides may later be triggered when exposed to these EBV foreign peptides similar enough to cross-react and precipitate an autoimmune reaction[38].

Hormones may be another factor. Broadly speaking, oestrogen has inflammatory properties and androgens are anti-inflammatory[39]. Accordingly, the ratio of oestrogens to androgens is significantly higher in the synovial fluid of RA joints. This is the case in both men and women[40]. The female predisposition to developing RA is well documented, and it is well established that the physiological changes in pregnancy can affect the course of RA, with symptomatic relief in the first trimester and recurrence postpartum[41,42]. The postpartum increased risk of developing RA may be due to rapid progesterone withdrawal or increased levels of prolactin (given that oestrogen levels are reduced)[43,44]. This demonstrates the complexity of the hormonal association with RA.

1.1.6 Role of autoantibodies: RF and ACPA

RF is an autoantibody against the Fc portion of IgG and ACPA occurs naturally during inflammation, apoptosis and keratinisation[45]. Significantly higher numbers of these peptides are found in RA synovium in comparison to Osteoarthritis (OA)[46].

The presence of RF and ACPA in RA, is associated with more erosive disease and poorer response to treatment[47,48]. This implicates these disease-specific autoantibodies in the pathogenesis and perpetuation of RA[49]. However, van Steenbergen et al (2015) did not find that RF gives an additive effect on bone erosion in ACPA-positive patients, making the association not as clear[50].

ACPA has been found to directly induce bone loss. ACPA directed against citrullinated vimentin induces osteoclastogenesis and activated bone resorption[51]. A study in 2016 showed that mice receiving ACPA from RA patients, developed arthralgia and systemic bone loss before signs of joint inflammation appeared[52]. Similar observations are seen clinically in ACPA-positive disease, with structural bone damage before clinical onset[53].

ACPA also promotes the release of autoantigens from neutrophils. ACPA bind to Fc receptors on myeloid cells and activate the complement system[54]. Activation of macrophages by ACPA induces production of TNF-α, one of the key inflammatory cytokines in RA[55]. This activation of macrophages appears to be further amplified by RF, causing an increase in the secretion of Interleukin 1 (IL), IL-8 and IL-6, which activate synoviocytes[56].

Neutrophil extracellular traps (NETs) cause cell death via the extrusion of intracellular material. ACPA was shown to potently induce NET formation in RA[57]. NETs also increase the activity of synovial fibroblasts which are responsible for active invasion into articular cartilage, stimulation of pro-inflammatory mediators and angiogenesis. NET formation in turn is a further source of citrullinated antigens for ACPA generation[58]. ACPA formation of NETs may amplify autoimmunity and perpetuate the inflammation in RA.

In terms of prediction of development of RA in healthy individuals with relatives with RA, the positive predictive values of positivity for ACPA or RF was 64%[59]. Individuals with non-specific musculoskeletal symptoms and ACPA positivity demonstrated rates of development of RA from ~40 to 60% over 2–5 years of follow-up[60]. So strong is the correlation with ACPA that there is emerging evidence that ACPA positive and ACPA negative RA may behave very differently and may even be two distinct diseases.

1.1.7 Immunopathogenesis of RA

Both the adaptive and innate immune responses are implicated in the destructive rheumatoid process. It is thought that genetically predisposed individuals have repeated activation of the innate and adaptive immune system which leads to the breakdown of self-tolerance, leading to auto-antigen presentation and antigen specific T and B cell activation[61]. A mixture of cells is thought to be involved including, monocytes, antigen presenting cells[62,63] (**Figure 1-1**).

Initiation of the inflammatory and immune response is mediated by Toll-like receptors (TLR). These act as a key part of the innate immune system and activate the adaptive immune system. TLRs recognise Pathogen Associated Molecular Patterns (PAMPs)

expressed on microbial pathogens or Danger Associated Molecular Patterns (DAMPs) that can be expressed by cells under stress for example adenosine triphosphate (ATP) or heat shock proteins. A few microbial PAMPs in RA include LPS on gram-negative bacteria, peptidoglycan on gram-positive bacteria and envelope protein on viruses[64]. Particular TLRs like TLR2 and TLR4 expression is increased in synovial tissue in RA[65,66]. However, endosomal TLR3 and TLR7 are raised in early as well as established RA[67]. It is not yet clear whether TLRs play a role in the pathogenesis as well as the perpetuation and progression of RA.

Activated T and B cells produce cytokines and chemokines. Aberrant production and regulation of both pro-inflammatory and anti-inflammatory cytokines and cytokine pathways are found in RA. Experimental models suggest that synovial macrophages and fibroblasts may become autonomous in the presence of a pro-inflammatory cytokine network leading to chronic inflammation[68].

B cells also have a role in the pathologic process and may serve as antigen-presenting cells which are required for T-cell activation and T cell help[69,70]. This was confirmed in B cell depletion studies[71]. The lineage of B cells (plasma cells) also secrete cytokines and produce numerous autoantibodies, for example RF[72]. These antibodies form immune complexes and can activate the complement cascade causing tissue damage.

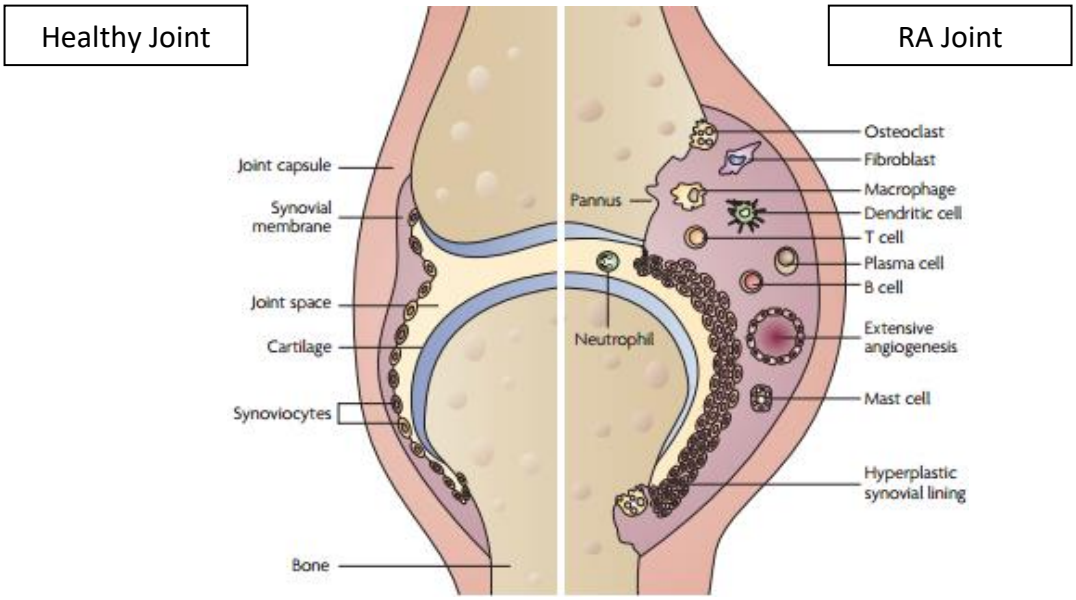


Figure 1-1: Cell types involved in the pathogenesis of RA. Inflammatory cells invade the normally acellular synovium and surrounding area. This leads to the formation of hyperplastic invasive tissue leads called 'Pannus'. Synovial fibroblasts and osteoclasts are involved in the destruction of cartilage and bone. The immune effector cells initiate and maintain the inflammation. Figure from Ademowo et al, 2013[73].

1.1.8 Cytokines in RA

Cytokines are the chemical messengers that regulate a large range of the inflammatory processes in RA. The increase in auto-immune activity in RA shows that the balance between pro- and anti- inflammatory cytokine signalling is disrupted, favouring pro-inflammatory cytokine activity and thereby joint damage **(Table 1-2**). Some cytokines such as TNF, IL-6 and IL-1 promote inflammatory responses and others such as IL-10 and IL-4 function mainly as an anti-inflammatory cytokine in the RA context[74]. However, no cytokine is inherently pro- or anti-inflammatory. These properties are dependent on their environment. This thesis will be mainly identifying the role of cytokines in the context of RA.

Three key pro-inflammatory cytokines implicated in RA are TNF, IL-6 and IL-1. Successful blockade of these particular cytokines has created efficacious treatment for RA (**Section 1.1.9**). Murine models suggested TNF induced arthritis and was an inflammatory mediator for IL-1, the crucial cytokine causing cartilage destruction[75, 76].

TNF is known to be responsible for endothelial cell activation, induction of metalloproteinases and adhesion molecules, angiogenesis, bone erosion and activation of fibroblasts, keratinocytes and enterocytes. TNF inhibition reduces expression of adhesion molecules and cellularity in RA synovium. This shows that the anti-inflammatory effects could be partly explained by the down-regulation of cytokine-inducible vascular adhesion molecules in synovium, with a consequent reduction of cell traffic into joints[77]. Circulating levels of IL-1 and IL-6 are also decreased after treatment. Upon TNF blockade, angiogenesis is also significantly reduced and lymphangiogenesis increased.

IL-6 is a complex cytokine due to its ability to promote osteoclastogenesis and degenerative processes within the inflamed joint, and its capacity to activate lymphocytes. IL-6 also appears to also have homeostatic roles. This is dependent on the pathway of signalling. Homeostatic properties and regenerative activities of IL-6 are mediated by classic signalling whereas the pro-inflammatory responses of IL-6 are rather mediated by trans-signalling[78]. In RA, IL-6 primarily promotes synovitis and joint

destruction in its pro-inflammatory role as evident by benefit of IL-6 inhibitors which are approved treatment for RA **(Section 1.1.9).**

IL-1 is a key mediator in bone resorption and cartilage destruction by inducing prostaglandin E_2 and proteolytic enzymes. A gene transfer study in rabbits, which introduced IL-1β into the joint induced clinical and histological features of RA[79].

IL-17 is produced mainly by T helper cell population (Th17) but also $CD8^+$ T cells. It is a highly pro-inflammatory cytokine. Reports indicate that high levels of IL-17 in RA, can promote joint degradation[80,81]. A combination of IL-17 and TNFα was found to be predictive for poor outcome in RA[82]. In 2015 Secukinumab, an anti-IL-17 treatment, was licensed for psoriasis, and subsequently psoriatic arthritis and ankylosing spondylitis[83]. However, it has failed to show convincing efficacy in RA[84].

Interferon α (IFNα) is linked to the progression of arthritis and its gene expression was identified in a subgroup of ACPA positive patients with destructive RA[85,86]. Although it has not been clearly categorised as either pro- or anti-inflammatory, it has been found at the site of inflammation in RA[87,88,89].

Immunomodulatory cytokines, IL-10 and IL-4 regulate production of IL-1 and TNF in the synovium of RA[90,91]. These anti-inflammatory cytokines can inhibit T cell activity by suppressing IFN and can also inhibit macrophage activity. IL-4 gene therapy reduced IL-17 expression in the synovium and prevented bone erosion. IL-4 did this by suppressing osteoprotegerin ligand expression, which reduced osteoclast numbers[92].

Cytokine	Pro- or Anti-Inflammatory	Source	Action
IL-1	Pro	Macrophages	T cell activation and stimulates pro-inflammatory cytokine production. Osteoclast activation.
TNF α	Pro	Macrophages	Stimulates pro-inflammatory cytokine production, activates fibroblasts, T cell activation, pannus formation, destruction of cartilage and bone.
IL-17	Pro	Th17 cells	Up regulation of pro-inflammatory cytokines, osteoclastogenesis.
IL-10	Anti	Monocytes, macrophages, mast cells, NK cells, Neutrophils, Eosinophils, Lymphocytes.	Down-regulates cell surface levels of MHC II, adhesion and co-stimulatory molecules. Inhibits cytokine production and CD4+ proliferation. Protect against cartilage damage.
IL-4	Anti	Th2 Cells, basophils[93].	Decreases Th1 lymphocyte production, stimulates B Cell antibody production and fibroblast development, inhibit cytokine production.
IL-6	Both	Monocytes, Fibroblasts, Endothelial Cells, Keratinocytes, Mesangial cells and T and B lymphocytes.	Stimulate final stages of B cells maturation, antibody production, activation of osteoclasts and T cell growth and differentiation. OR Inhibit pro-inflammatory cytokines.
IFNα	Both	T cells and NK Cells	Promote Th1 polarisation, boost antigen processing and facilitate cytotoxicity.

Table 1-2: Basic actions and origins of some pro- and anti- inflammatory cytokines in RA

1.1.9 Treatment of RA

Medications for RA target the reduction of inflammation thereby improving symptoms and preventing joint damage. There are three main groups of medications in RA: non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and disease-modifying anti-rheumatic drugs (DMARDs). Several studies have provided evidence that early treatment with DMARDs results in superior clinical and radiological outcomes[94,95,96].

DMARDs are divided into categories: synthetic, target synthetic and biologic. Synthetic DMARDS, e.g. methotrexate (MTX), hydroxychloroquine (HCQ), leflunomide (LFM) and sulphasalazine (SSZ) are cheaper and are normally used as a first-line according to UK NICE, EULAR and ACR guidelines **(Figure 1-3)**[97,98,99]. Progression through the guidelines in the UK is based on DAS28 score (**Table 1-3**)[100].

Targeted synthetic DMARDs (tsDMARDs) are orally administered janus kinase inhibitors (JAK inhibitors). JAKs transduce signals from cytokine receptors and phosphorylate Signal Transducers and Activators of Transcription (STAT). Upon phosphorylation, STATs translocate to the nucleus where they bind DNA, and regulate gene expression (**Figure 1-2)**. JAK inhibitors prevent the phosphorylation of the STATs. Two examples are tofacitinib and baricitinib, which have been approved in Europe for the treatment of RA. Both have shown equivalence to anti-TNF therapy in patients who have not responded to methotrexate[101,102].

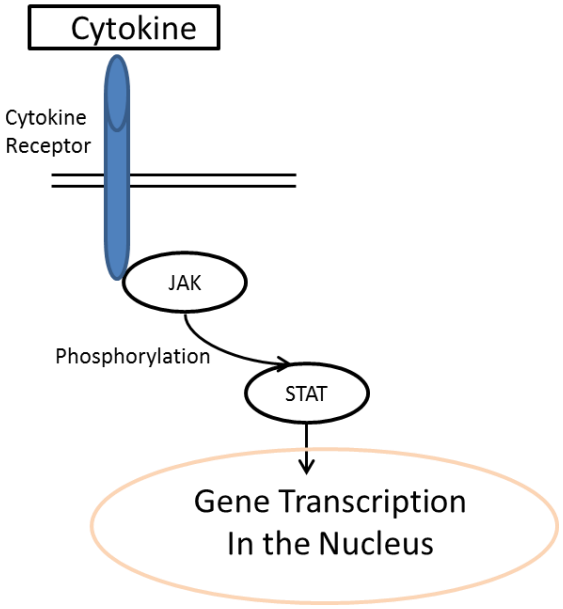


Figure 1-2: The cytokine receptor JAK-STAT signalling cascade. JAK inhibition prevents phosphorylation substrates such as STATs, and therefore prevents the cytokine dependent gene regulation.

Biologic DMARDs are often used when synthetic DMARDs fail to control RA. Development of biologic DMARDs resulted from identifying "therapeutic targets" that may be responsible for driving inflammation. Monoclonal antibodies or immunoconstructs can then be developed for these targets. The first successful target was the cytokine, TNF. Sir Marc Feldmann and Sir Ravinder Maini formulated their hypothesis implicating TNF in the pathogenesis of RA in the 1980s[103]. Subsequent clinical trials using infliximab, a chimeric anti-TNF monoclonal antibody, and the immunoconstruct etanercept, confirmed the importance of TNF in RA and led to the licence approval of these biologic DMARDs for RA. Etanercept was licenced for RA in 1998 and is a soluble p75 TNF receptor fusion protein that is made up of two TNF receptors. These are bound to the Fc portion of IgG, therefore bivalent binding two TNF molecules per etanercept molecule. Since then, five different anti-TNF agents have been developed and approved for use: adalimumab and golimumab, two fully human anti-TNF antibodies and the polyethylene-glycolated Fab' fragment with anti-TNF reactivity, certolizumab pegol[104].

An alternative blockade was sought after. Anakinra is an IL-1 receptor antagonist, licenced for the treatment of RA in 2006. However, it has been shown to be less potent

than the TNF inhibitors in most patients and as a result, is now used less frequently in RA treatment[105,106]. Anti IL-1β (Canakinumab) is now used in the treatment of gout. Monosodium urate crystals stimulate the release of IL-1 by activating the NLRP3-inflammasome via monocytes or macrophages[107]. The first pilot study in 2007 looked at ten patients, who had failed NSAIDs. The study showed that anakinra was an effective therapy for acute gout[108].

Abatacept, developed in 2007, is a T cell inhibitor (**Figure 1-7**). A meta-analysis showed that patients on abatacept were significantly more likely than patients on a non-biologic DMARD, to achieve an ACR50 response at one year and significant inhibition of radiographic progression[109]. Physical function improvement and reduced disease activity and pain were also significant. However, there were a significantly increased number of serious infections at a year[110]. A number of studies have also documented the efficacy of abatacept in certain important subsets of patients with RA[111,112,113,114].

A fourth biologic, rituximab, is a chimeric anti-CD20 monoclonal antibody that depletes B cells. It does this through one or more of the antibody-dependent mechanisms; Fc receptor gamma-mediated antibody-dependent cytotoxicity, complement-mediated cell lysis, growth arrest and B-cell apoptosis[115]. Rituximab is mostly used in the treatment of RA in patients who have failed TNF inhibitors. Seropositive patients are more likely to respond to rituximab which may reflect the importance of B cells in driving autoantibody generation and autoantibody-mediated RA[116].

Tocilizumab, an anti-IL-6 receptor (IL-6R) monoclonal antibody, was developed in 2009[117]. Another anti-IL-6R monoclonal antibody sarilumab, was approved in 2017. Although a very effective treatment for RA, several side effects have been reported including increased infection rates, increased serum cholesterol levels, abnormalities in liver function tests and increased risk of gastrointestinal perforation if the patient has pre-existing diverticulitis[118,119]. IL-6 is needed for the expression of C-Reactive Protein (CRP), therefore IL-6 inhibition causes normalisation of CRP[120]. This means that CRP cannot be relied upon as an acute phase reactant during treatment with IL-6 inhibitors.

Treatment options for RA have dramatically transformed in the last 20 years with more target specific therapy. Unfortunately, this revolution of discovery comes at a cost. Increasing therapeutic options and growing populations makes competition fierce and attention needs to be turned to health as well as a cost-effective solution for the future. Biologics and tsDMARDs are expensive. Drug-free remission is uncommon. For most patients, sustained treatment is needed to control disease. Promoting immunoregulation has been a key research strategy to achieve drug-free remission.

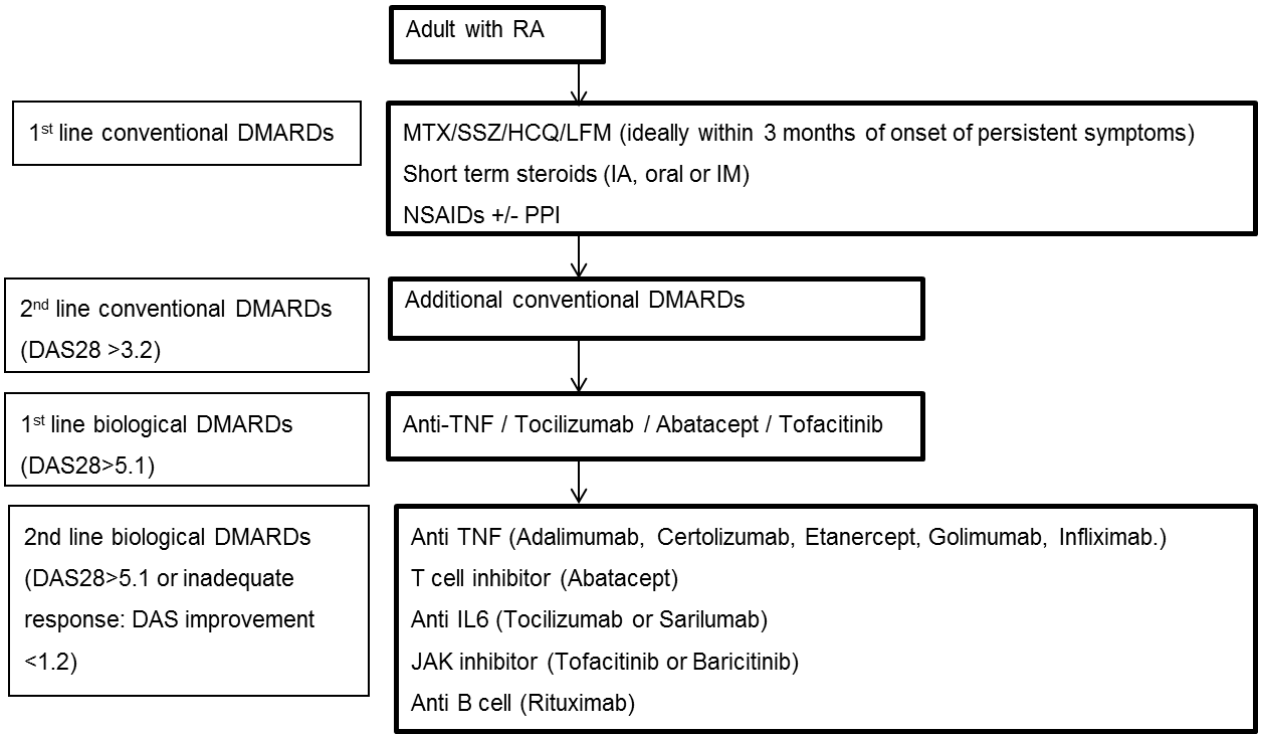


Figure 1-3: Basic treatment guideline for patients diagnosed with RA in the UK. This is based on the UK NICE, EULAR and ACR guidelines. One main difference is that the ACR guidelines promote the use of biological DMARDs earlier in the course of disease. Progression through the guideline is based upon DAS28 scores **(Table 1-3)** but also the measurement of prognostically unfavourable factors such as RF, ACPA and the presence of radiographic joint erosion.

DAS28 score	DAS28 group
<2.6	Remission
2.6-3.2	Mild
3.2-5.1	Moderate
>5.1	Severe

Table 1-3: Stratification of disease activity by DAS28 score. This score is calculated using 28 tender and 28 swollen joint counts, ESR and a subjective global health assessment of disease activity by the patient during the preceding 7 days on a scale between 0-100 ('0': no activity, '100': highest activity possible). *Formula: DAS28(4) = 0.56*sqrt(t28) + 0.28*sqrt(sw28) + 0.70*Ln(ESR) + 0.014*GH*

1.2 The Role of T Lymphocytes in the Pathogenesis of RA

Both the adaptive and innate immune responses are implicated in the destructive rheumatoid process. A combination of T and B cells, monocytes, antigen presenting cells and cytokines are shown to be involved[121].

Optimal activation of T cells is completed with two signals. The first signal is antigen specific stimulation delivered through the TCR on naïve T cells and MHC molecules. The second signal is designed to de-select any T cells which react to self. This is antigen independent and non-specific. This activation signal is delivered through the co-stimulatory molecule present on antigen presenting cells (APCs) and is required for sustained T cell proliferation and effector or memory cell generation. If the co-stimulatory signal is absent, the cell will undergo clonal anergy or deletion.

1.2.1 $CD4^+$ T cells in RA

Dysregulation of $CD4^+$ T cell balance has been substantiated as a cause of rheumatic inflammation. Subsets of $CD4^+$ can be distinguished by their cytokine production and divided into the six subsets below**.** $CD4^+$ T cells activate plasma cells which release autoantibodies and immune complexes causing joint injury. Macrophages activated by

$CD4^+$ cells, release cytokines which stimulate fibroblasts, chondrocytes and synovial cells. These lead to pannus formation and joint damage.

Synovial inflammation was reduced after administration of anti-CD4 monoclonal antibody in patients with early RA, leading to local depletion of $CD4^+$ T cells in RA[122]. When activated by antigen presenting cells, $CD4^+$ T helper cells produce IL-2 and IFNγ cytokines[123]. These pro-inflammatory cytokines promote T cell proliferation to sustain effector T cell responses, activate macrophages and other cell populations, including synovial fibroblasts.

Earlier data on T helper 1 (Th1) cells strongly implied a predominant role in the pathogenesis of RA as they were found to be associated with inflammatory responses and cellular immunity. The Th1 cytokine signature is IFNγ but they also secrete IL-2, IL-3 and TNF[124]. However, the Th1 phenotype does not entirely explain all the mechanisms of inflammation.

Th2 cells are involved with allergic reactions and modulation of eosinophils. They secrete IL-4, IL-5 and IL-13 which work on B cell activation and antibody-based immunity.Th2 cells normally counteract Th1 cells[125]. In RA, Th2 cells are barely detectable in RA.

Th17 cells are increased in RA[126]. Th17 cells produce IL-17 and they require IL-23 for their long-term survival and pathogenic function[127]. In mice, they develop from naïve T cells stimulated with transforming growth factor-β1 and IL-6. It is likely that Th17 cells are driving the inflammatory force behind many autoimmune models. IL-17 increases IL-6 production, collagen destruction and collagen synthesis. Th17 cells are also pro-osteoclastogenic and therefore contribute to bone destruction[128].

T follicular cells (Tfh) are a part of the body's humoral immune response. They produce IL-21 to support B cell proliferation and plasma cell differentiation. IL-21 is raised in RA and correlates with disease activity[129,130]. The main transcription factor for Tfh cells is B-cell lymphoma 6 protein (Bcl-6). Deletion of Bcl-6 blocked Tfh differentiation resulting in a reduction of autoantibody formation and thereby inhibition of arthritis[131].

Th9 cells are a type of $CD4^+$ T cells that generate IL-9, IL-10 and IL-21. Functionally they appear to have a role in inflammatory disease including allergic inflammation, anti-tumour immunity and autoimmune inflammation. A complex milieu of cytokines (mostly IL-4 and TGFβ) and transcription factors including STAT6, drive the differentiation of these cells[132]. In patient studies, IL-9 is strongly correlated with Immunoglobulin E (IgE) and asthma but moreover, there are conflicting reports as to the role of Th9 cells in autoimmune disease[133]. In some reports, blocking IL-9 resulted in amelioration of autoimmune encephalitis, but other reports found that IL-9 may promote regulatory T cell function and stop experimental autoimmune uveitis[134],[135]. Immune responses that are strictly dependent on Th9 cells have not yet been established.

A key gene implicated in RA is HLA-DRB1. This encodes the MHC II chain molecule. As $CD4^+$ helper T cells (Th) become activated when they bind to MHC class II complexes, this further reinforces that $CD4^+$ T cells are key players in the pathogenesis of RA. $CD4^+$ cells are dominant in the inflammatory infiltrates of the RA synovium[136,137,138]. They orchestrate local inflammation and cellular infiltration and initiate Collagen-Induced Arthritis (CIA) in murine models[139]. Finally, the clinical success of the biologic abatacept (Cytotoxic T-Lymphocyte-associated antigen 4 (CTLA-4) Immunoglobulin) for RA, also indicates significant role of $CD4^+$ T cells.

1.2.2 $CD4^+$ regulatory T cells (T regs) in RA

In view of autoimmunity, some self-reactive T cells escape the primary mechanism of deletion in the thymus. This breakdown of self-tolerance results in autoimmune diseases[140]. Regulatory mechanisms in the peripheral immune system are required to protect against the generation of self-directed immune responses. Overwhelming evidence suggests that $CD4^+$ T cells with a regulatory capacity (T regs) are responsible for this[140,141]. Normally T regs can suppress proliferation of effector T cells, but in RA their ability to do this is defective[142,143].

There is no definite surface marker for T regs but a generally considered characteristic feature is the expression of CD25. However, CD25 is also expressed by activated T and B

cells, macrophages and some dendritic cells. Other surface markers for T regs have been reported including CTLA-4, CD62 ligand, glucocorticoid-induced tumour necrosis factor receptor (GITR), membrane bound TGF-β, CD95, programmed cell death-ligand 1 (PD-L1) and $\alpha_4\beta_7/\alpha_4\beta_1$ integrin[144].

$CD4^+CD25^+$ T regs have been found in higher numbers in synovial fluid compared to peripheral blood in RA patients[145]. The synovial fluid $CD4^+CD25^+$ T regs also demonstrated an enhanced suppressive activity compared to peripheral blood which was related to their activation status. However, activated responder T cells in the synovial fluid had a reduced susceptibility to these $CD4^+CD25^+$ T regs.

Depletion of $CD4^+CD25^+$ T regs was associated with enhanced induction and severe disease in the murine CIA model[146]. Furthermore, the transfer of $CD4^+CD25^+$ T regs effectively treats experimental inflammatory arthritis by reducing osteoclast formation and systemic inflammation[147].

As well as high CD25 expression, the low expression of CD127 has also been associated with T regs. This subset of $CD4^+CD25^+CD127^{low}$ cells were found to be significantly lower in the peripheral blood of RA patients with active disease, compared to low/moderate disease activity[148].

Forkhead box P3 (FOXP3) is the most specific intracellular transcription factor identifying T regs. This is needed in order to maintain the suppressive properties of T regs[149]. TNF causes FOXP3 to lose its suppressive capacity by dephosphorylation in RA[150]. This could partially explain why these cells are unable to arrest breakdown in self-tolerance. It also appears that in RA patients who respond to TNF inhibitor therapy, $CD4^+CD25^+FoxP3^+$ T regs regain some function[151].

1.2.3 $CD8^+$ T cells in RA

$CD8^+$ T cells are known as cytotoxic cells and are key players in the body's defense against viral infections and cancer. In comparison to the wealth of literature available

on $CD4^+$ cells involvement in autoimmunity, the role of $CD8^+$ T cells is less defined, particularly in regard to their involvement in the progression of RA.

Like $CD4^+$ cells, $CD8^+$ cells have subsets with different immunological features. They are divided into pro-inflammatory and regulatory cells. This is evident from the array of cytokines they produce including IL-6 and TNF, IFN, IL-17 and IL-10[152,153]. There is growing evidence that $CD8^+$ T cells play an important role in autoimmune disease, particularly at the target sites of inflammation.

A key indication that $CD8^+$ T cells play a role in certain autoimmune diseases, is the evidence that HLA class I molecules have a strong positive association with the susceptibility to autoimmune disease. This is especially the case for Ankylosing Spondylitis with HLA B27 and Psoriatic Arthritis with HLA-C[154,155]. In Type 1 Diabetes, $CD8^+$ T cells are thought to be involved in the both the initiation of the disease and the destruction of beta cells[156,157].

In relation to RA, evidence shows that the number of $CD8^+$ cells is raised in the peripheral blood in comparison with healthy controls[158]. This is similarly the case in early RA (<1 year) where absolute numbers of $CD8^+$ T cells in peripheral blood are higher than healthy controls and are also expanded in the synovial fluid of patients with RA[159,160]. The increased pro-inflammatory cytokine production observed in peripheral blood $CD8^+$ effector during RA also normalises when patients enter disease remission[161].

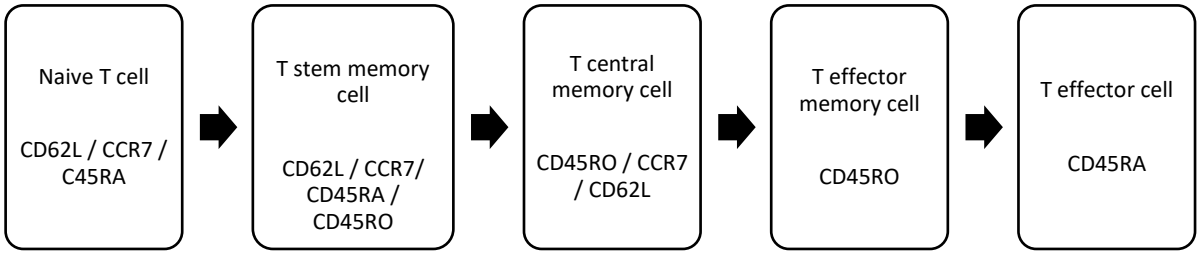


Figure 1-4: The differentiation of $CD3^+CD8^+$ inflammatory T cells. The different subsets can be defined by the surface protein expression. The effector function is increased upon $CD3^+CD8^+$ differentiation, while memory function and proliferation are decreased.

When activated, $CD8^+$ T cells develop into memory or effector cells **(Figure 1-4**)[162]. $CD8^+$ effector T cells produce IFNγ which activates macrophages and induces expression of MHC class II. Aberrant expression is associated with autoimmune and auto inflammatory diseases.

Memory T cells enable lifelong immunity but some subsets of memory $CD8^+$ T cells up regulate negative co-stimulatory markers and either develop an exhausted phenotype or display a tissue-instructed differentiation. TIM3 is a $CD8^+$ T cell inhibitory receptor. Interestingly, TIM3 expression is increased in the peripheral blood of patients with RA and is even higher in the synovial fluid[163]. Other $CD8^+$ T cell inhibitory receptors include PD1 and CTLA4. These induce a co-inhibitory signal in activated T cells to promote T cell anergy. Overexpression of these is also a feature of 'exhausted cells,' well described in chronic viral infections and cancer[164].

RA patients may have increased levels of these inhibitory receptors in attempt to re-establish control in inflammatory environments after recurrent stimulation. However, the signal may be ineffective or insufficient to control the inflammation. . . The up-regulation of inhibitory receptors may be a compensatory mechanism. This hypothesis is based on similar phenomenon demonstrated in $CD4^+$ T cells in synovial fluid[165].

1.2.4 $CD8^+$ T cells linking viral infection with development of RA

There is a suggestion that RA could be triggered by viruses such as EBV and CMV. A combined effect of HLA genotype and EBV exposure has been reported to increase the

risk of developing RA and furthermore, several studies have detected CMV and EBV in the RA synovial joint at higher rates than in other joint diseases ($p<0.05$) [166,167, 168]. CMV up-regulates an inhibitory receptor on T cells called Leukocyte immunoglobulin-like receptor 1 (LIR-1). The $CD8^+$ T cell receptor LIR-1 was found at increased frequencies in CMV-positive RA patients compared to CMV-positive healthy controls. The numbers of $LIR\text{-}1^+CD8^+$ T cells increased with disease activity, suggesting their involvement in the pathogenesis of RA[169].

Cytotoxic ($CD8^+$) T cells are triggered by dendritic cells that have taken up viral antigens, and exhibit these on their surface with MHC class I proteins. Binding to the antigen alone does not cause activation of the cytotoxic T cell. For this, IL-2 secretion from adjacent activated helper T cells is also needed[170].

Macrophages phagocytose free extracellular viruses and then process and present antigen in association with class II MHC proteins, to the helper T cells. The macrophages produce a co-stimulus and IL-2 to stimulate proliferation of the helper T cell. IL-2 also stimulates the cytotoxic T cells bound to the surface of the virus infected cell, to proliferate.

Activated cytotoxic T cells exocytose vesicles containing perforin into the extracellular space between the T cell and virus infected cell. Perforin inserts into the target cells' membrane and forms channels through the membrane. The target cells become leaky and die. When the virus is released into the extracellular fluid, it can be directly neutralized by circulating antibody. Some cytotoxic T cells generated during the proliferation following an initial antigenic stimulation do not complete their full activation at this time but remain as memory cells.

1.2.5 $CD8^+$ T reg cells

In health, T regs cells classically maintain self-tolerance by suppressing pathological immune responses against auto antigens. Although $CD8^+$ cells have been thought to have immunoregulatory function, their phenotypic markers are less well established.

A candidate marker for regulatory T cell activity includes the loss of surface CD28 expression by CD8 T cells. In 2005, Davila et al showed that $CD8^+CD28^-CD56^+$ cells could suppress memory T cell responses[171]. This set of T cells was shown to express high levels of FOXP3, IL-10 and TGFβ[172]. The $CD8^+CD28^-$ T reg cells' immunosuppressive role has been established in cancer, transplantation and systemic lupus erythematosus (SLE)[173,174,175].

Synovial fluid in RA joints contains high local concentrations of IL-15 and IL-12. These cytokines down-regulate CD28 and enhance 4-1BB, ICOS and PD-1 expression by $CD8^+$ T cells and increase $CD8^+$ cell survival[176].

HLA-E deficient murine models showed that $CD8^+$ T reg cells can limit the T cell response via the molecule Qa-1 (murine HLA-E). Qa-1 knockout mice appear to develop autoimmune disease due to the absence of $CD8^+$ T regs function[177].

1.2.6 CD28 expression by $CD8^+$ lymphocytes

Activation of the T cell receptor with APCs via MHC-I-bound peptide antigen has low affinity and is therefore insufficient to induce full activation and survival of T cells. A co-stimulatory signal from CD28 cell surface receptor is needed[178]. As the T cell receptor (TCR) recognizes MHC-I-bound peptide antigen, presented by APCs, the CD28 receptor interacts with CD86 or CD80 **(Figure 1-5)**. CD28 receptor on T cells is needed to prolong T cell responses. Since activation of these T regs could be therapeutic in autoimmune diseases and cancer, CD28 was investigated as a therapeutic target. In 2006, an infamous phase 1 clinical trial at Northwick Park Hospital, administered a CD28 superagonist antibody (TGN1412) to six healthy male volunteers. After one infusion the six subjects developed multi-organ failure due to a cytokine storm[179]. Fortunately, they all survived after intensive care. One patient developed dry gangrene and lost the tips of his fingers and toes, but otherwise no long-term sequelae are apparent.

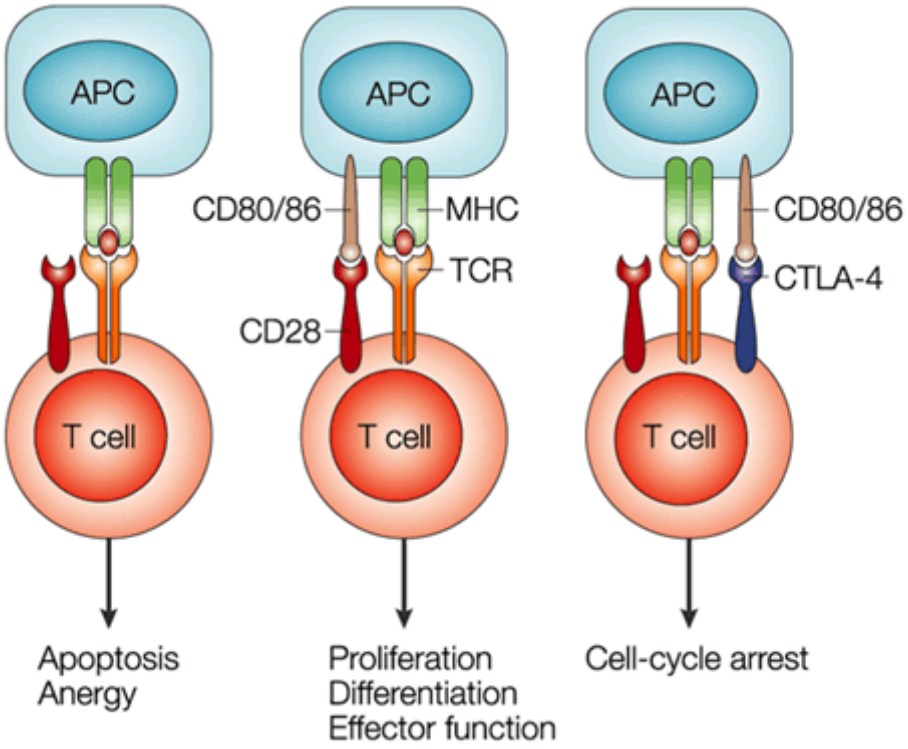


Figure 1-5: T cell activation and co-stimulatory receptors. Simultaneous recognition of a specific major histocompatibility complex (MHC)–peptide complex by the T cell receptor (TCR) and CD80 or CD86 by the co-stimulatory receptor $CD28^-$ results in T-cell activation, cytokine production, proliferation and differentiation. In the absence of CD28 ligation, T cells undergo apoptosis or become anergic. After T cell activation and up regulation of cytotoxic T lymphocyte antigen 4 (CTLA-4), co-ligation of the TCR and CTLA-4 results in cell-cycle arrest and termination of T cell activation. Figure from Alegre et al, 2010[180].

Despite the loss of CD28, Larbi et al (2006) found that $CD8^+$ cells possess a pre-assembled signalosome that might account for their ability to maintain CD28 signaling (via Akt phosphorylation)[181]. The location of CD28 may be as important as the surface expression level.

Persistent T cell stimulation and proliferation appears to down-regulate CD28 expression, with critically shortened telomeres[182,183]. Telomeres are the non-coding nucleotide sequence at the end of a chromosome .Telomeres prevent the chromosomal instability, rearrangement and end-to-end fusion[184]. Shortend telomere changes could

imply immune senescence because they have previously been linked with a higher frequency of infections and incidence of malignancy in older adults[185].

High TNF-α concentrations, as in the case with RA, abrogate CD28 transcription[186]. The loss of CD28 is combined with increased expression of CD57[187]. CD28 loss and CD57 gain is also observed on $CD4^+$ T cells during chronic immune activation in stable HIV patients[188]. The proportion of $CD8^+CD57^+$ T cells expressing was significantly higher in RA patients compared with age-matched controls[189]. Activated $CD8^+CD28^-$ T regs cells produced high levels of immunosuppressive form of IFN-γ, similar to that produced by $CD4^+CD28^-$ T cells[190,191].

Unlike $CD4^+CD25^+$ T regs, $CD8^+CD28^-$ T regs function mainly through the action of IL-10 and transforming growth factor (TGF)-β[192]. Neutralization of TGF-β consistently reduced $CD8^+CD28^-$ T regs suppressor function in vitro[197]. IL-10 is predominantly anti-inflammatory, and therefore has been proposed as protective in the pathogenesis of RA. IL-10 also has a stimulatory effect on B cell autoantibody production in RA, whilst being predominantly regulatory, suppressing IL-6[193,194]. IL-10 based clinical trials showed limited efficacy[195]. A single dose of IL-10 was found to be non-toxic however neutrophilia, monocytosis, and lymphopenia, occurred at serial administration of higher doses[196]. Like $CD4^+CD25^+$ T regs, the $CD8^+CD28^-$ T cell subset also expresses high levels of FOXP3.

TNF inhibition therapy has been shown to partially restore $CD8^+CD28^-$ T reg function in vivo and in vitro[197]. Restoration is particularly in relation to an increase in IL-10 receptor (IL-10R) expression on autologous T cells in RA. This suggests only a temporary incapacitation by TNFα. Notably, expression of IL-10R in individuals with RA remained lower than healthy controls expression.

Although the number of $CD8^+CD28^-$ T regs is raised in RA, the reduced sensitivity of T responder cells and defective T reg function, impact on their regulatory function[158]. This lack of immune regulation could be implicated directly in the pathogenesis of autoimmune diseases.

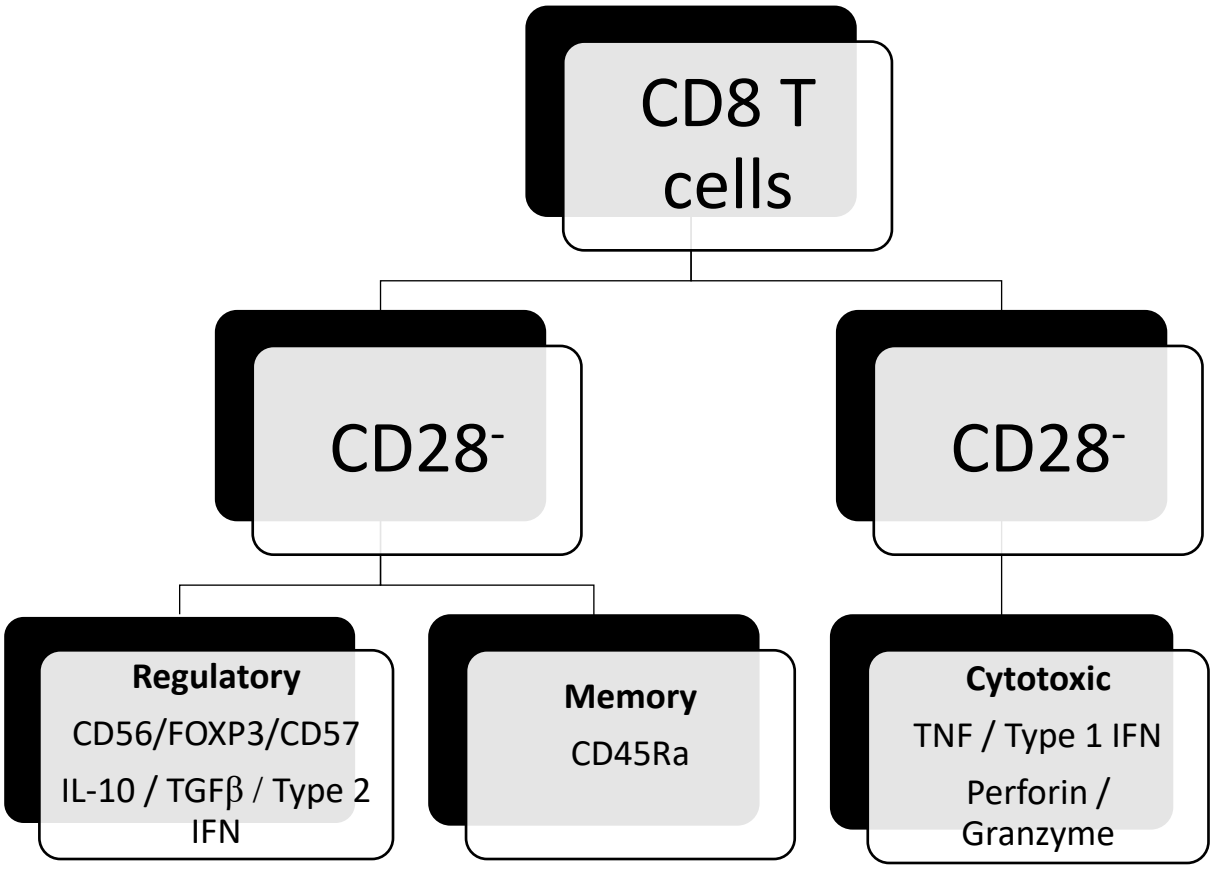


Figure 1-6: Proposed existing subsets of CD8 T cells.

1.3 Immune Checkpoints

Although T regs are regarded as homeostatic controls of the immune system, thereby normally suppressing the immune response, their functions vary. These functions can depend on immune checkpoints which are expressed on the cell surface. Immune checkpoints are key in the role of self-tolerance and autoimmunity.

1.3.1 Cytotoxic T Lymphocyte Antigen 4 (CTLA-4)

Checkpoints are co-stimulatory and inhibitory signals. CTLA-4 is highly expressed on T regs. CTLA-4 is a transmembrane glycoprotein that is a homolog of CD28. CTLA-4 binds to the CD80 and CD86 proteins with a greater affinity, than CD28[198]. $CD8^{+}CD28^{-}$ T regs up-regulate of CTLA4, which arrests the T cell cycle and may contribute to the immunopathology of RA **(Figure 1-5)**[199,200,201]. The blockade of CD28 by CTLA-4 leads to inhibition of T regs[202].

The CD28 co-stimulatory pathway has also been targeted in RA therapy with abatacept. This selective co-stimulation modulator causes the inhibition of T cells and has proven to be a successful biologic therapy. It is a soluble fusion protein comprising CTLA-4 and the Fc portion of IgG1. It prevents CD28 from binding to its counter-receptor CD80/CD86 **(Figure 1-7).**

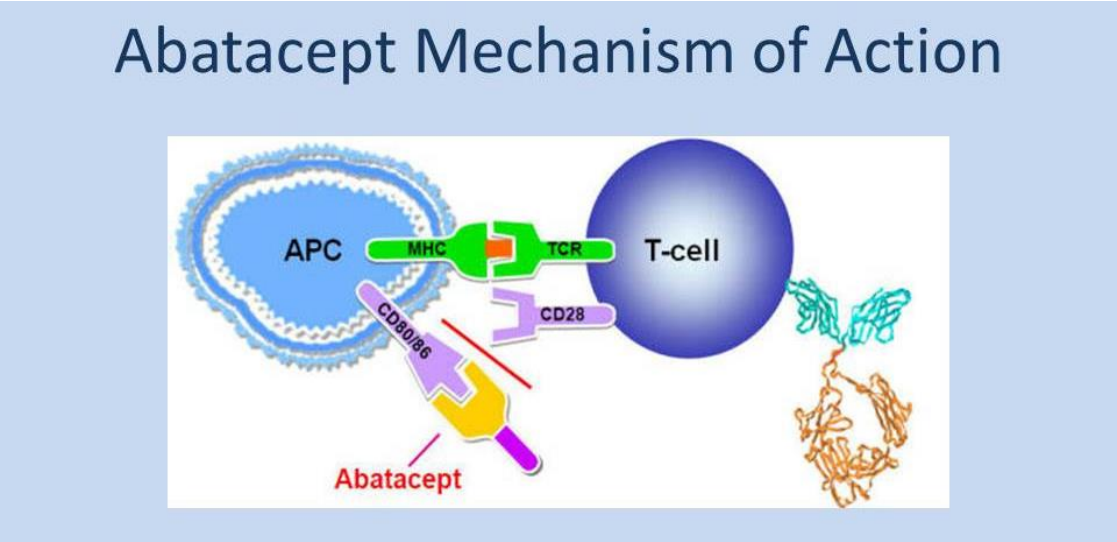


Figure 1-7: Abatacept mechanism of action. Abatacept modulates the immune response by binding to CD80/86, thus preventing the binding of the co-stimulatory CD28. This attenuates T cell activation. Figure from Orencia[203]

1.3.2 Inducible Co-Stimulator (ICOS)

Activated T cells also express other co-stimulatory molecules such as ICOS. Co-stimulation through ICOS, have all been shown to enhance the proliferation and telomerase activity in $CD8^{+}CD28^{-}$ T cells[204]. A subset of $CD8^{+}CD28^{-}$ T regs cells have been found to be increased numerically in RA, but have reduced expression of ICOS and programmed death 1 (PD-1) compared to healthy or disease controls[197].

1.3.3 Programmed Death 1 (PD-1)

PD-1 mainly affects T cell activity within the tissues and tumours as opposed to regulating T cell activation in lymphoid organs. It also binds to CD80 to inhibit T cell proliferation[205].

1.3.4 Lymphocyte Activation Gene 3 (LAG-3)

LAG-3 also dampens T cell immune responses. It inhibits $CD8^{+}$ effector T cell function and can enhance the suppressive activity of T regs[206]. LAG-3 is up-regulated on anergic T cells, the blockade of which resulted in a degree of reversal of anergy[202]. The signalling pathways of LAG-3 are largely unknown.

1.3.5 T cell immunoglobulin mucin 3 (TIM3)

In 2002, TIM-3 was found on $CD8^+$ T cells as a marker of IFN gamma[198]. Galectin is the main ligand for TIM-3. When TIM-3 binds to Galectin-9, $CD8^+$ T cells decrease production of IFN and reduce T cell proliferation[207].

1.3.6 Glucocorticoid-induced TNFR family related gene (GITR)

GITR is also expressed on T regs. Activation of GITR results in T reg attenuation and enhancement of effector T cell responses[208]. Overexpression of GITR is associated with autoimmunity[209].

1.3.7 4-1BB

4-1BB receptor by contrast is an activating checkpoint on $CD8^+$ T cells (as well as other immune cells). Once engaged with its ligand, it promotes survival and pro-inflammatory pathways[210].

1.4 Hypothesis

This MD thesis hypothesises that the lack of appropriate immune responsiveness and reaction, is signposted by the cell surface loss of CD28. $CD8^+CD28^-$ T cellscould be a marker of poor prognosis RA where the overactive nature of the disease causes pathology. CD8+ cells could be further divided into subsets of cells using cells surface markers including CD57, CD45Ra, CD56 and KLRG1.

1.5 Aims

The overall aim of the study was to examine the role of $CD3^+CD8^+CD28^-$ cells in Early and Established RA using human samples.

- To look at whether these $CD3^+CD8^+CD28^-$ cells are raised in early and established RA in the peripheral blood compartment.
- To assess if there is an association with serological and clinical factors regularly used in medical practice.

- To investigate the cytokine production of $CD3^{+}CD8^{+}CD28^{-}$ cells in the peripheral blood compartment.
- To address the need for a positive marker for these cells.

2 Chapter 2: Materials and Methods

2.1 Materials

All plastic-ware was obtained from Greiner Bio-One Ltd, Gloucestershire, GL10 3SX (unless stated otherwise). All general laboratory chemicals and reagents were obtained from Fisher Scientific UK Ltd, Loughborough, LE11 5RG. Flow cytometry analysis tubes were Titertube™ microtest tube and supplied by Bio-Rad Laboratories, Inc. Purple topped 4 ml BD vacutainers™ containing EDTA were used for blood. These were sourced from Becton Dickinson UK Ltd (BD).

2.1.1 Buffers

Dulbecco's Phosphate Buffered Saline (PBS) pH 7.2, free of calcium and magnesium was supplied by Life Technologies Ltd. Fluorescence-activated cell sorting (FACS) buffer consisted of PBS with 0.5% (w/v) bovine serum albumin (Life Technologies Ltd), 5 mM EDTA, 7.5 mM sodium azide. Both buffers were stored at 4˚C.

2.1.2 Flow cytometry reagents

Antibodies used for flow cytometry are listed in **Table 2-1**. Phorbol 12-myristate 13-acetate (PMA) and ionomycin for cell stimulations were supplied by Sigma Aldrich Company Ltd. PMA (1mg) was dissolved in 1ml Dimethyl Sulphoxide (DMSO). Once dissolved, culture media was added to prepare a final concentration of 50 µg/ml PMA; this was kept at -20^{o}C. Ionomycin (1 mg) was dissolved in 2 ml DMSO to yield a stock solution of 0.5 mg/ml, which was stored at -20^{o}C. Monensin (500 mg; eBioscience™) was dissolved in 24 ml ethanol and the 30 mM stock solution was kept at 4^{o}C wrapped in foil.

Intracellular cytokine staining was performed using the BD Cytofix/Cytoperm™ kit (BD Biosciences). Following flow cytometric staining, cells were stored in BD Cellfix diluted 1 in 10 with distilled H_2O, as recommended by the manufacturer (BD

Biosciences). Cells were transferred to Titertube microtest tubes (Bio-Rad) before acquisition using a CyAn ADP flow cytometer (Beckman Coulter UK Ltd).

The cell culture media was RPMI 1640 (Life Technologies) supplemented with 10% Fetal Calf Serum (Thermofisher). Lymphoprep^{TM} density gradient medium (Stemcell Technologies UK Ltd.) was used to isolate the mononuclear cells from the peripheral blood samples.

Antibody	Clone	Company
Cell Surface Staining		
CD8 Pe-Vio770	BW135/80	Miltenyi Biotec Ltd
CD3 APC-Cy7	557943	BD Pharminogen
CD28 eFluor 450	CD28.2	eBioscience
KLRG1 APC		Miltenyi Biotec Ltd
CD56 PeCy5.5	CMSSB	eBioscience
CD57 PE	NK-1	Becton Dickinson
CD45Ra PCP-Cy5	HI100	eBioscience
REA APC (KLRG1 isotype)	130-104-614	Miltenyi Biotec Ltd
Mouse IGG1K (CD56 Isotype) PeCy5.5	P3.6.2.8.1	eBioscience
Mouse IgM (CD57 Isotype) PE	G155-228	Becton Dickinson
Mouse IgG2bK (CD45Ra Isotype) PCP5.50	eBMG2b	eBioscience
Mouse IgG1 (CD28 Isotype) ef450	P3.6.2.8.1	eBioscience
Intracellular Staining		
IL6 PE	MQ2-13A5	eBioscience
IL10 FITC	IC2172F	R&D Systems Inc.
IL4 AF488	MQ2-13A5	eBioscience
IFN PE	4S.B3	eBioscience
IL17A PCP5.5	eBio6DEC17	eBioscience
Mouse IgG1 kappa (IFN Isotype) PE	MOPC-21	Becton Dickinson
Mouse IG1 kappa (IL17 Isotype) PCP5.5	P3.6.2.8.1	eBioscience
Mouse IgG1 kappa (IL4 Isotype) af488	P3.6.2.8.1	eBioscience
Rat IgG1 kappa (IL6 Isotype) PE	eBRG1	eBioscience
Mouse IgG1 (IL10 Isotype) FITC	P3.6.2.8.1	eBioscience

Table 2-1: Antibodies for flow cytometry. Fluorescently conjugated antibodies were used in flow cytometry to identify singular leukocyte populations, to measure receptor expression and to evaluate cytokine responses. Yellow: cell surface antibodies, green: cell surface antibody isotype controls. Grey: intracellular antibodies, blue: intracellular antibody isotype controls. Species and isotype matched antibodies were used as controls.

2.2 Methods

2.2.1 Ethical Approval and Recruitment

Recruitment for this Inflammation and Immune Regulation in Early Inflammatory Arthritis study was granted ethical approval by the South East Wales Research Ethics Committee, Panel B in 2011 (REC reference: 11/WA/0326). Cardiff University was responsible for the governance of the study with reference number 11/CMC/5299. The Cardiff and Vale University Health Board Research & Development Office approved the proposal in 2013.

2.2.2 Selection

Twenty-five patients were recruited to each study arm, according to the inclusion and exclusion criteria (**Figure 2-1**):

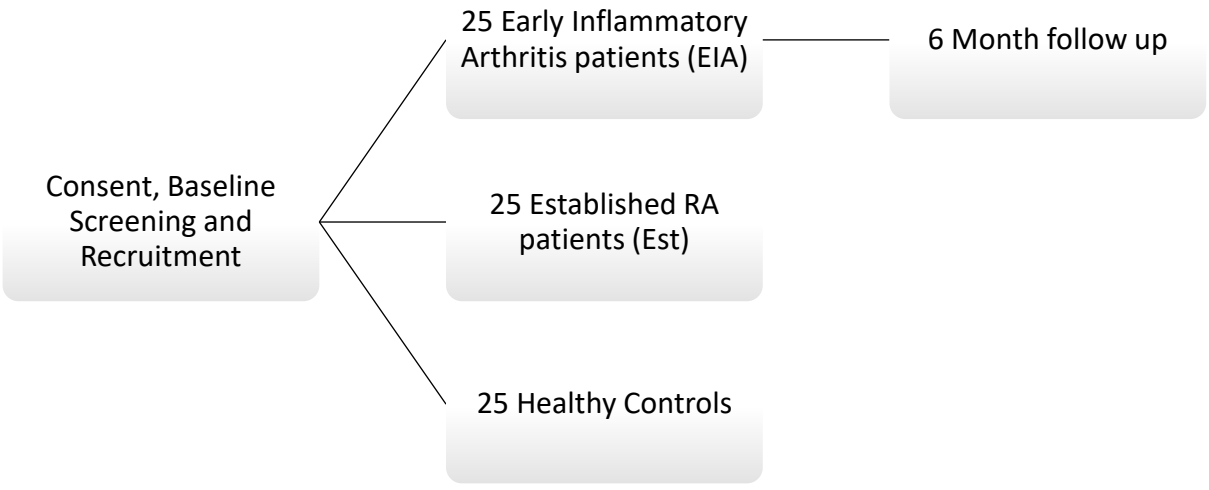


Figure 2-1: Patient recruitment into the three arms of the study.

Patients Inclusion criteria

- Age 18 years or over;
- Able and willing to give written consent and comply with the requirements of the study protocol;
- Synovitis in at least one joint;
- *Duration of persistent symptoms in the EIA group of 4 weeks to 6 months*[211,212]
- *For the Established (Est) RA arm, >2 years since diagnosis*

Patients Exclusion Criteria

- Definite other autoimmune rheumatic disease (e.g. Systemic Lupus Erythematosus, Mixed connective tissue disease, Scleroderma, Polymyositis);
- Heart disease classified as New York Heart Foundation Functional class IV (ACR classification);
- Treatment with intravenous gamma globulin, plasmapheresis or Prosorba™ column within the last 6 months;
- Current other inflammatory joint disease (e.g. gout, reactive arthritis, Lyme disease).

Healthy Controls Inclusion Criteria (Attempted to age match to patients)

- Age18 years or over
- Able and willing to give written consent and comply with the requirelemtns of the study protocol;

Healthy Controls Exclusion Criteria

- Definite autoimmune rheumatic disease (e.g. Rheumatoid Arthritis)

2.2.3 Recruitment

Patients were recruited from the Cardiff University Hospital of Wales (UHW) outpatient clinic. Once identified, the patients were given a patient information leaflet and contacted a minimum of 24 hours after. If they were willing to participate a baseline clinical assessment was scheduled.

Patients (age ≥18 years) with persistent symptoms (4 weeks to 6 months) of synovitis in at least one joint and ability to consent, were recruited. Peripheral blood samples were collected and flow cytometry completed. Patients with early inflammatory arthritis and established RA patients (>2 years of diagnosis) recruited from the University Hospital of Wales Rheumatology clinic were compared with healthy controls recruited from Rheumatology Staff from the clinic. Age, sex, serology status, disease activity (DAS28) and duration of disease were recorded (**Table 3-1**).

2.2.4 Clinical Assessments

During baseline clinical assessments, the study was discussed, and the patients were given the opportunity to ask questions regarding their involvement. After written consent was obtained, the following was data was collected:

- Age
- Sex
- RA disease duration
- Disease activity level using DAS28 score[213]
- Rheumatoid Factor
- Anti CCP
- Erythrocyte Sedimentation Rate (ESR)
- C-Reactive Protein (CRP) level
- CMV status
- Current medications (see **Table 3-2** and **Table 3-3**)

Flow Cytometry

Flow cytometry was used to evaluate the expression of cell surface receptors and intracellular cytokines in $CD8^+$ T Lymphocytes isolated from the peripheral blood of the study subjects[197]. Each blood sample was divided into two aliquots in order that cell surface staining and intracellular staining could be performed in parallel.

2.2.5 Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

Blood (3 ml) was collected from consenting volunteers into two 4 ml BD Vacutainer™ blood collection tubes containing EDTA. One tube was allocated to cell surface staining and the other to intracellular staining. Each tube of blood was diluted with an equal volume of PBS and the resulting 6 ml of diluted blood was carefully layered over 3 ml of Lymphoprep™. To isolate PBMCs, samples were centrifuged (800 g, 15 min) at room temperature with the brake turned off. The separated PBMC fraction was recovered using a 1000 µl pipette taking care not to disturb the mononuclear cell layer (**Figure 2-2**). Recovered cells were then centrifuged (250 g, 10 minutes) and washed twice in PBS to remove contaminating platelets. Finally, purified PBMCs were re-suspended in cell culture media before proceeding with flow cytometry: For each millilitre of whole blood collected, PBMCs were re-suspended in 1 ml (for intracellular) or 0.5 ml (for cell surface staining) of supplemented RPMI 1640 cell media.

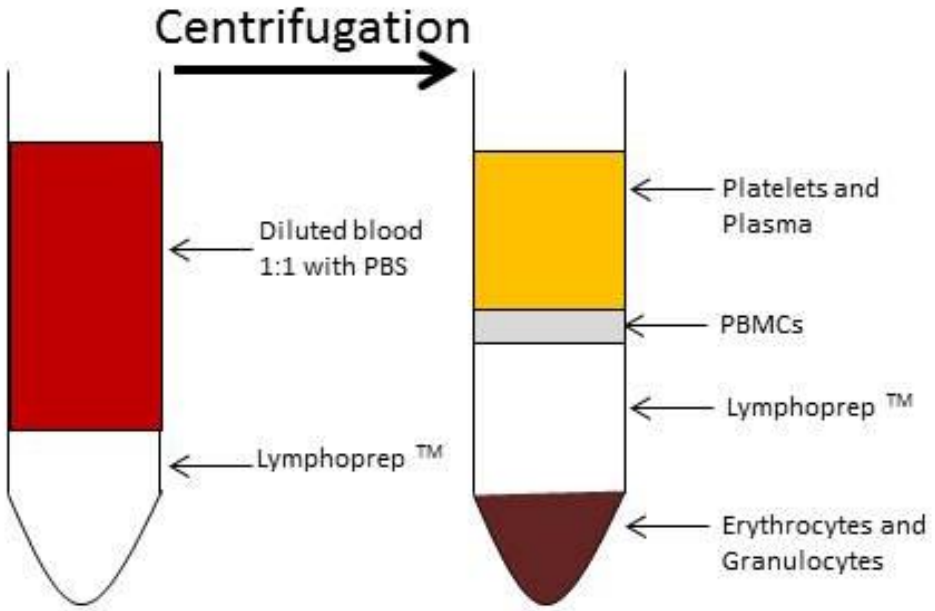


Figure 2-2: Purification of PBMCs. Following centrifugation, diluted blood layered over Lymphoprep™ form distinct layers according to their density. The PBMC layer forms a white cloudy band between the yellow plasma and the clear Lymphoprep media. During centrifugation, the brake must be deactivated to avoid disturbing the distinct layers formed.

2.2.6 Cell Surface Staining

PBMCs (50 µl) were pipetted into wells of a 96-well U-bottom plate, and an equal volume of FACS buffer containing 5% (v/v) rabbit serum (Life Technologies) was added. Cells were then incubated at 4°C for 20 minutes. This blocked non-specific binding of the Fc portion of fluorescently conjugated antibodies during flow cytometry to leukocyte Fc receptors.

A master mix of fluorescently conjugated antibodies specific for cell surface receptors was prepared (**Table 2-1**). The antibody mix (50 µl per well) was then added to the appropriate wells of the 96-well plate so that a final antibody dilution of 1/100 was used. The antibody and cell mixture were then incubated at 4°C for 30 minutes. Following this, cells were centrifuged (500 g, 5 min) and washed in FACS buffer twice,

before finally re-suspending in BD CellFix. Cells were stored in the dark at 4˚C for up to 24 hours, until acquisition.

2.2.7 Intracellular Flow Cytometry

Purified PBMCs (100 µl) were pipetted into wells of a sterile 96-well U-bottom plate (CELLSTAR™ plates from Greiner Bio-One). Cells were stimulated using 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of the Golgi transport inhibitor, monensin (3 µM)[214]. Cells were then stored in the fridge at 4˚C for four hours. Antibody staining of cell surface receptors was performed as described above before proceeding with cell fixation, permeabilisation and intracellular antibody staining. For this, cells were centrifuged (500 g, 3 minutes) and re-suspended in 100 µl of Cytofix/Cytoperm™ and incubated at 4˚C for 20 minutes. The cells were then centrifuged as above, washed twice with BD Perm/Wash, before incubating with antibodies specific to cytokines.

A master mix of cytokine-specific antibodies was prepared in BD Perm/Wash (**Table 2-1**). The cells were re-suspended in 100 µl of the prepared master mix which contained antibodies at a final dilution of 1/50. Cells were incubated at 4˚C for 30 minutes. Following this, the cells were again centrifuged (500 g, 3 min) and washed twice to remove excess antibody. The cells were finally re-suspended in 200 µl of BD Cellfix™ and stored in the dark at 4˚C for up to 24 hours, until acquisition.

2.2.8 Flow Cytometry Analysis

A minimum of 50,000 events were acquired using a CyAn ADP flow cytometer (Beckman Coulter). All acquisitions and analysis were performed using the program Summit (software version 4.3; Beckman Coulter). Firstly, the channel voltage was adjusting using unstained cells to set acquisition parameters. The unstained cells were positioned into the lower left quadrant between 10^0 and 10^1 on the Log fluorescent intensity and acted as a comparator (**Figure 2-3**). This ensured that the fluorochromes were not signalling in error **(Figure 2-3**).

Digital compensation was performed in Summit after sample acquisition and before final analysis. This was achieved using PBMCs stained with single fluorochrome-conjugated antibodies (**Figure 2-3**). Compensation corrects for emission spectra overlap between different fluorochromes in a multicoloured sample. This ensures that the fluorescence measured at any particular detector is being emitted from correct fluorochrome.

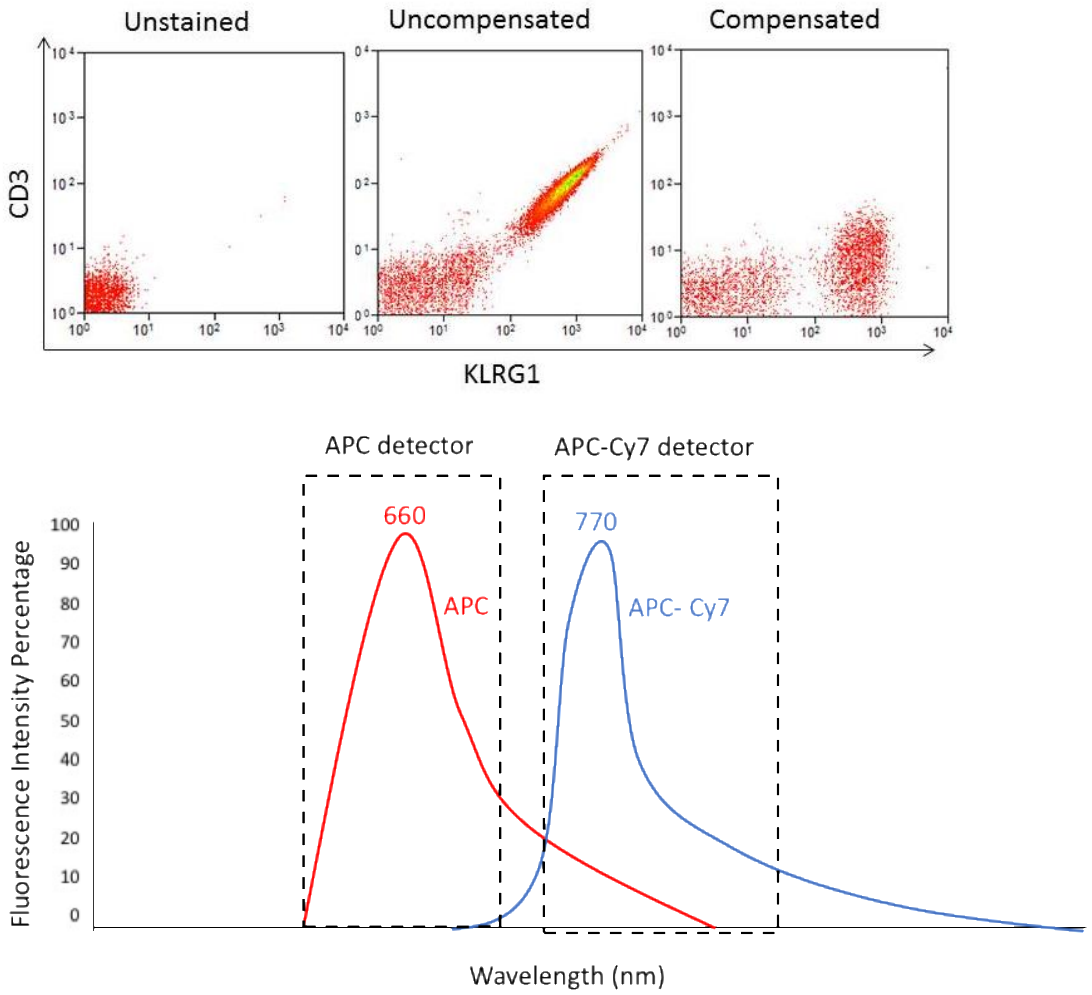


Figure 2-3: Compensation of the spectral overlap between APC and APC-Cy7. APC and APC-Cy7 fluorochromes have overlapping emission spectra. Voltage adjustment ensured that control unstained cells were detected in the lower-left quadrant of the dot plots (**left plot**). In uncompensated data, positive control cells stained with an APC-conjugated antibody are detected in both the APC channel and at the APC-Cy7 detector (**middle plot**). Digital compensation and adjustment of the spillover in to the APC-Cy7 detector, ensured that the fluorescence from APC-conjugated antibodies were only measured at the detector assigned to APC (**right plot**). The spectral emission curve (**lowermost plot**) shows the spillover from APC into APC-Cy7 and from APC-Cy7 into APC. The APC and APC-Cy7 detectors have been compensated to minimise spillover.

2.2.9 Gating Strategy

The gating strategy for the analysis of $CD3^+CD8^+$ T cells can be seen in **Figure 2-4**. Due to the characteristics of the different leucocytes, the event size (FS Linear) and granularity (SS Linear) are used to differentiate cell types and exclude dead cells and debris (**Figure 2-4A**).

Figure 2-4B shows the discrimination of 'doublet' events which were removed from the analysis. Multiple cells passing through the flow cytometer in close proximity were excluded as falsely identified single events and was achieved by comparing forward scatter area against forward scatter linear. $CD3^+CD8^+$ cells were then gated for further analysis (**Figure 2-4C**).

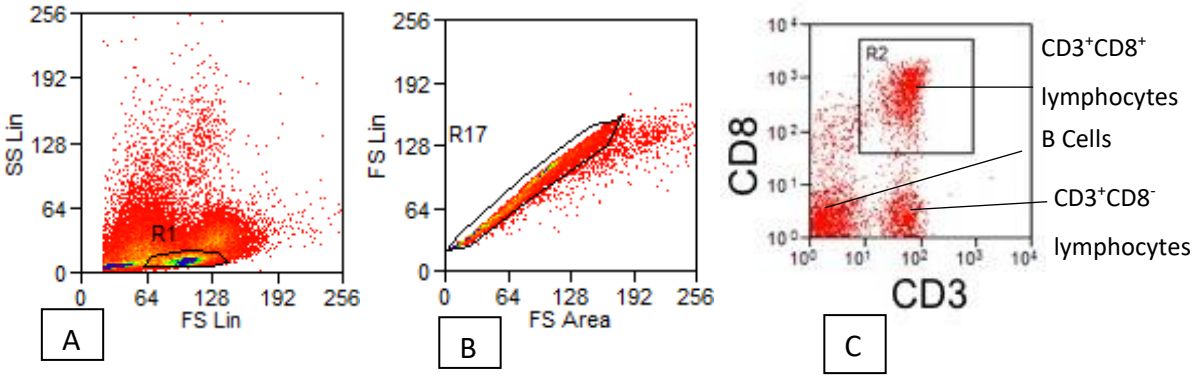


Figure 2-4: Gating strategy for the analysis of peripheral $CD8^+$ T cells. A) Linear Forward Scatter (FS Lin) reflects particle size while Side Scatter (SS Lin) reflects particle granularity, allowing discrimination of leukocyte populations such as lymphocytes (R1). Populations deselected include neutrophils which are relatively small but demonstrate granular morphology and monocytes, which are large and relatively granular cells in comparison to lymphocyte population. B) Gating of single cells using Forward Scatter Area against Forward Scatter Linear allows discrimination of events based on size. Multiple cells detected as a single event result in the Forward Scatter Area being disproportionately high in relation to Forward Scatter Linear and

are excluded from further analysis. C) $CD3^{+}CD8^{+}$ T cells were selected using gate R2 for further analysis.

2.3 Statistics

Statistical analysis was performed using the Statistical Analysis software program Graph Pad Prism v5. When presenting the data, the median was used, if not stated otherwise. The median was chosen rather than the mean, because it is robust against outliers and skewed data distribution.

One outlier in the Controls group was excluded due the absolute number of $CD3^{+}CD8^{+}CD28^{-}$ cells being significantly higher than the other controls ($p<0.05$). This sample was removed from all analyses. On two occasions the flow cytometer broke so analysis was carried out on a different model (CANTO^{TM}). This affected the intracellular staining analysis of eight samples so these sample were removed.

The data did not have a normal distribution when tested with Shapiro-Wilk normality test[215]. Non-parametric statistical tests were therefore applied.

Statistical tests used depended on the analysis being completed. A comparison of the means of two groups was achieved using the non-parametric Mann-Whitney U Test. The Kruskal-Wallis test was used to analyse the statistical difference between the three or more unmatched groups, applying post-hoc Dunn's analysis for relevant pairwise multiple comparisons. Differences that were considered significant were $p<0.05$.

The non-parametric Spearman's rank correlation was used to analyse statistical associations in the data. A line of linear regression was added the graphs to illustrate the relationship.

The power calculation to determine the number of subjects needed in each arm of the study was completed midway through data collection, on the existing data using the statistical analysis software SPSS (IBM). Sample size estimation showed that at least 25 patients were needed in each arm to demonstrate a 2-sample 2-sized

difference of 15% with a SD of 16 for $CD3^{+}CD8^{+}CD28^{-}$ T cells with 90% statistical power.

3 Chapter 3: The role of $CD3^+CD8^+CD28^-$ cells in RA

3.1 Introduction

$CD3^+CD8^+$ T lymphocytes are raised the peripheral blood of RA patients in comparison to healthy controls[216]. These cells are also found in abundance in the synovial fluid of patients with RA[217]. $CD3^+CD8^+$ T cells can be divided into pro-inflammatory and regulatory cells. Increased pro-inflammatory cytokine production by $CD3^+CD8^+$ effector memory T cells in peripheral blood of patients with RA, normalises in the remission phase[216].

CD28 is a pivotal cell surface marker on $CD3^+CD8^+$ T cells. When CD28 is expressed, the T cell plays a predominantly pro-inflammatory role. A loss of CD28 occurs during antigen-driven differentiation toward a terminal phenotype. When CD28 is lost, the T cell has more of an immunosuppressive role. This has been shown in cancer, transplantation and systemic lupus erythematosus (SLE)[218,219,220].

Although the loss of CD28 is associated with immunosuppression, mechanistic studies will benefit from establishing a positive marker for the $CD3^+CD8^+CD28^-$ T reg cells. This chapter aims to establish a robust positive marker for this subset of lymphocytes. Flow cytometry was used to identify the subset of $CD3^+CD8^+CD28^-$ lymphocytes and to correlate these with standard clinical and serological markers that are regularly used in medical practice.

3.1.1 Hypothesis

This MD thesis hypothesises that the lack of appropriate immune responsiveness and reaction, signposted by a rise in $CD3^+CD8^+CD28^-$ T cells, could be associated with disease phenotype, severity and response to treatment in RA.

3.1.2 Aims

- Assess prevalence of $CD3^+CD8^+CD28^-$ cells in the peripheral blood compartment in Early and Established Rheumatoid Arthritis and assess correlation with clinical and serological markers.
- Assess cytokines produced by $CD3^+CD8^+CD28^-$ cells in Early and Established Rheumatoid Arthritis in the peripheral blood compartment.
- Establish positive cell surface marker for $CD3^+CD8^+CD28^-$ cells.

3.2 Subject demographics

RA disease activity (DAS28) in the Established Arthritis groups was higher compared to subjects with Early RA. Seropositivity for ACPA was higher than RF in both Early (79%:68%) and Established RA (63%:56%). These proportions reflect that of the RA patients[221,222]. Differences between the groups did not reach statistical significance.

The proportion of female subjects (3:1) was similar to those in the RA population and this was matched in the Controls[223].

	Healthy Controls	Early Rheumatoid Arthritis	Established Rheumatoid Arthritis
Number of patients	25	25	25
Mean Age	41±12	56±12	62±12
Mean disease duration	N/A	4 Months	10 Years
Rheumatoid Factor Positive %	N/A	68 (17)	56 (14)
ACPA positive %	N/A	79 (19)	63 (15)
Mean DAS28	N/A	4.07±1.3	5.4±1.9
Female %	68(17)	64 (16)	68 (17)
CRP	N/A	14±13	24±30
ESR	N/A	31±26	27±26

Table 3-1: Demographics of Health Controls, Early and Established RA patients recruited. Percentages are shown with number of subjects in brackets. Mean verage and standard deviation are shown.

Early RA							
Patient No.	**NSAIDs**	**Steroids**	**DMARDs**	**Biologic**	**Analgesia**	**CRP**	**ESR**
1	Ibuprofen		Methotrexate			1	6
2			Methotrexate Hydroxychloroquine			14	42
3					Tramadol	17	48
4			Methotrexate Hydroxychloroquine			4	5
5		Prednisolone 5mg			Paracetamol	21	28
6	Naproxen		Methotrexate			3	9
7						0	9
8			Methotrexate Hydroxychloroquine		Paracetamol Tramadol	32	30
9			Sulphasalazine			8	22
10		Prednisolone 10mg	Methotrexate Hydroxychloroquine			3	4
11		Prednisolone 20mg	Methotrexate			2	5
12					Co-codamol	0	5
13			Methotrexate			5	19
14			Methotrexate Hydroxychloroquine			46	94
15		Prednisolone 10mg	Methotrexate		Co-codamol	3	5
16			Methotrexate Hydroxychloroquine		Paracetamol	4	12
17		Prednisolone 10mg	Methotrexate Hydroxychloroquine			5	90
18		Prednisolone 10mg			Paracetamol	15	40
19	Naproxen	Depo-Medrone 120mg				10	27
20	Naproxen					15	24
21		Prednisolone 2mg				33	60
2	Naproxen				Tramadol	20	38
23			Sulphasalazine		Tramadol	42	85
24		Prednisolone 15mg				24	28
25	Naproxen					19	30

Table 3-2: Medication for Early RA patients (n=25). None of the early RA patients were on biologic medication. All doses were standard according to the BNF unless stated. Patient 7 refused all treatment, patient 20 refused DMARDs.

Established RA							
Patient No.	**NSAIDs**	**Steroids**	**DMARDs**	**Biologic**	**Analgesia**	**CRP**	**ESR**
1			Mycophenolate Mofetil			10	32
2		Prednisolone 10mg	Mycophenolate Mofetil			92	51
3		Prednisolone 5mg	Hydroxychloroquine Sulphasalazine		Gabapentin Oxycontin	6	54
4					Co-codamol	109	94
5	Diclofenac		Methotrexate		Paracetamol Co-codamol	<1	8
6			Methotrexate Hydroxychloroquine		Tramadol	35	2
7						33	53
8		Prednisolone 7.5mg			Amitriptyline Buprenorphine	<1	39
9			Leflunomide Hydroxychloroquine Sulphasalazine		Paracetamol	4	43
10	Naproxen		Methotrexate Hydroxychloroquine Sulphasalazine			10	3
11			Methotrexate			8	7
12			Methotrexate Sulphasalazine		Tramadol	8	35
13	Ibuprofen		Methotrexate		Paracetamol	56	32
14		Prednisolone 5mg	Methotrexate		Paracetamol	7	1
15						1	1
16			Methotrexate Sulphasalazine			<1	11
17	Naproxen	Prednisolone 5mg	Methotrexate			23	40
18			Sulphasalazine			7	2
19		Prednisolone 7.5mg	Mycophenolate Mofetil	Adalimumab	Amitriptyline	3	6
20			Methotrexate Hydroxychloroquine	Certolizumab		72	83
21				Adalimumab	Co-codamol	7	3
22			Methotrexate	Certolizumab	Tramadol	2	2
23		Prednisolone 15mg	Methotrexate	Adalimumab		16	28
24	Etoricoxib		Methotrexate Sulphasalazine	Adalimumab		11	16
25			Methotrexate	Etanercept		8	17

Table 3-3: Medication for Established RA patients (n=25). Seven established RA patients were on biologic medication. All doses were standard according to the BNF unless stated.

Optimisation of flow cytometry antibody detection of $CD3^{+}CD8^{+}CD28^{-}$ cells

Optimisation of a robust fluorochrome conjugated antibody for the detection of CD28 was key to this thesis. Using three different conjugates PCP5.5, eFluor450 (ef450) and PE, comparison of the fluorochrome shift was made (**Figure 3-1**). The PE and PCP5.5 fluorochromes resulted in significant background staining. The fluorochrome shift using ef450 on CD28 resulted in a more defined shift from isotype control-stained samples in comparison to PCP5.5 and PE. This antibody was therefore selected to identify the CD28 surface marker.

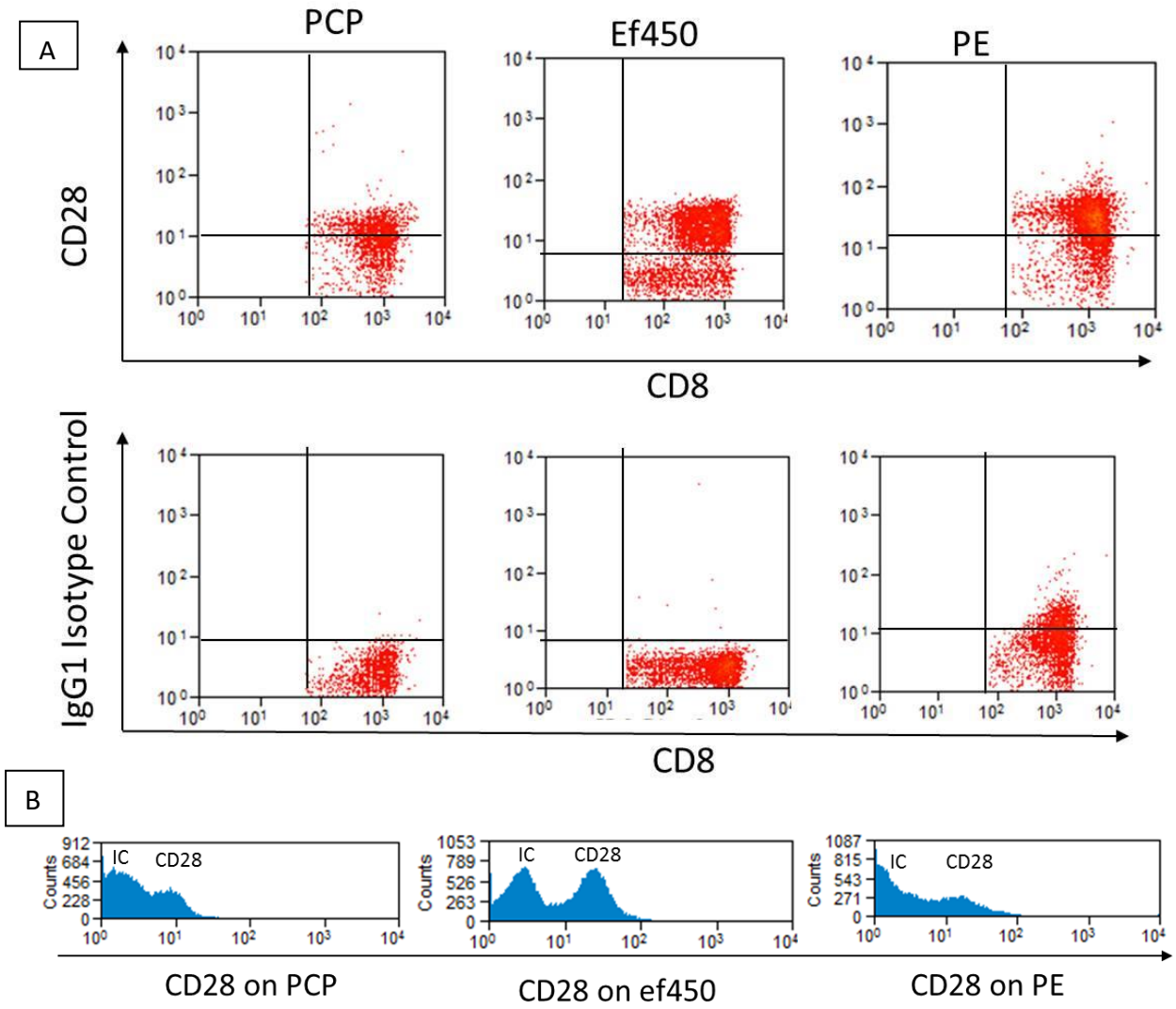


Figure 3-1: Optimisation of CD28 detection by flow cytometry. (A) Flow cytometry plots to compare detection of CD28 on $CD3^{+}CD8^{+}$ T cells using anti-CD28 antibodies conjugated to PCP, ef450 and PE on CD28 with controls directly below. (B) The histograms show the distinctive shift that CD28 had when conjugated to the colour ef450 in comparison to its isotype Control (IC). This distinctive shift is not present when using PCP and PE.

3.4 Proportions of $CD3^+CD8^+CD28^-$ cells using ef450 conjugated CD28 antibody in healthy controls

To ensure experimental data was consistent, the proportion of $CD3^+CD8^+CD28^-$ T cells in six healthy controls (females aged between 25-35) were compared and in keeping with existing literature (**Figure 3-2**). The six controls had between 8-20% $CD3^+CD8^+CD28^-$ T cells. The frequencies of $CD3^+CD8^+CD28^-$ cells were consistent with published literature[197]. The younger average age compared to the RA population could contribute to the low percentages of $CD3^+CD8^+CD28^-$ cells. Given the good reproducibility of the results using the ef450 conjugated antibody to CD28, this was established as the fluorochrome which was used to identify CD28.

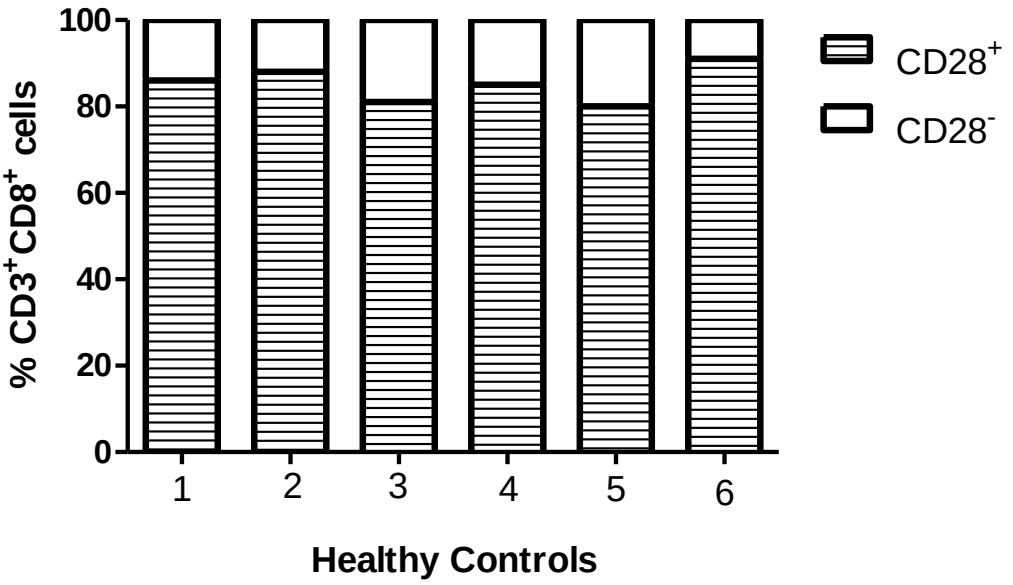


Figure 3-2: Proportions of $CD3^+CD8^+CD28^-$ cells were compared in six healthy controls using the antibody conjugated fluorochrome ef450.

3.5 $CD3^+CD8^+CD28^-$ cells in pooled RA and Controls

The subset of $CD3^+CD8^+CD28^-$ T reg cells was increased in pooled RA patients (Early RA plus Established RA patients) in comparison to the controls (p=0.0114) (**Figure 3-3**). Ceeraz et al (2013) found a similar increase in $CD3^+CD8^+CD28^-$ in RA patients compared to controls ($p<0.0001$)[197]. The absolute number of $CD3^+CD8^+CD28^-$ cells was similar in the controls groups to pooled RA.

The fluorescence-activated cell sorting (FACS) plots displaying $CD3^+CD8^+CD28^-$ cells were gated on lymphocytes, single cells and $CD3^+CD8^+$ cells. The mean percentage of lymphocytes from PBMCs, in the control group (n=24) was 60% and 49% in the pooled RA patients (n=50). The mean percentage of $CD3^+CD8^+$ cells was 14% in healthy controls and 10% in pooled RA patients.

Six patients in the Established RA group were treated with biologic DMARDs and are highlighted in red. The subjects were distributed across the group and the low numbers meant that sub-analysis was not carried out on this group.

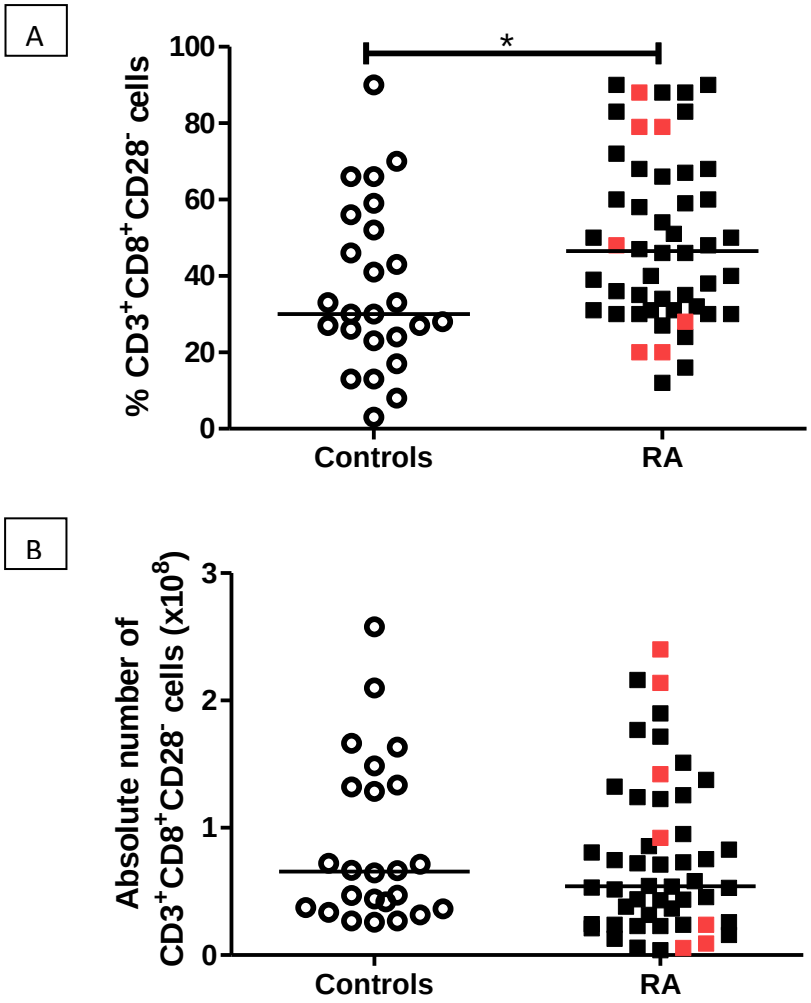


Figure 3-3: The percentage of $CD3^+CD8^+CD28^-$ T Cells is higher in pooled RA patients than Controls. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-$ T Cells shown with median, * $p<0.05$ by Mann-Witney U Test. The six patients on biologic DMARDs are highlighted in red. Controls (n=24), Pooled RA (n=50).

3.6 $CD3^+CD8^+CD28^-$ cells in Early RA, Established RA and Controls

While previous studies have shown an increase of $CD3^+CD8^+CD28^-$ cells in RA, for the first time data presented here revealed that the subdivision of RA patients into Early and Established RA shows a higher percentage of $CD3^+CD8^+CD28^-$ cells in both the Early RA group and Established RA group than controls (**Figure 3-4**).

Although percentage of $CD3^+CD8^+CD28^-$ cells appeared higher in the Established RA group than the Early RA group, this difference was not statistically significant. Increasing the sample size, thereby boosting the statistical the power of the study, would help to determine whether this was a true difference. The absolute number of $CD3^+CD8^+CD28^-$ T cells was similar between controls, Early and Established RA.

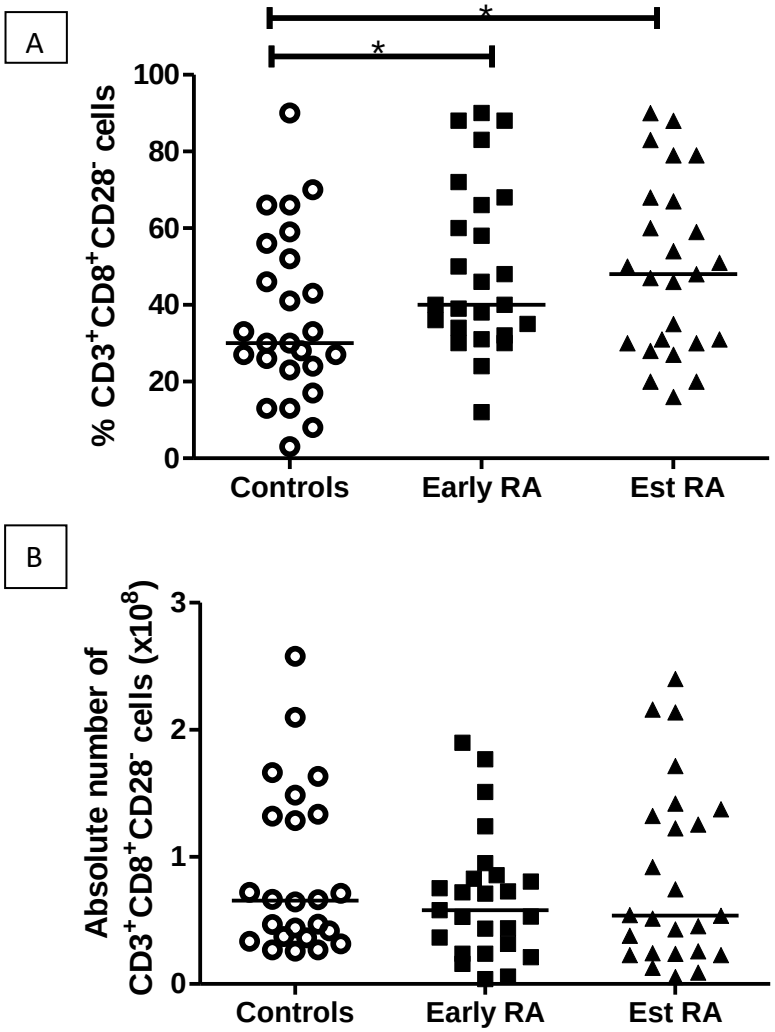


Figure 3-4: The percentage of $CD3^+CD8^+CD28^-$ T cells in Early and Established RA is higher than Controls. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-$ T Cells shown with median, * $p<0.05$ by Kruskal-Wallis ANOVA Test. Controls (n=24), Early RA (n=25), Established RA (n=25).

3.7 There was no correlation between $CD3^+CD8^+CD28^-$ cells and Age

Previous data highlighted a link between $CD3^+CD8^+CD28^-$ T cells and age, telomere length and senescence[224,225]. Ceeraz et al (2013) found significant positive correlation between $CD3^+CD8^+CD28^-$ and age in RA ($r=0.26$, $n=60$, $p=0.042$)[197]. There was no statistically significant correlation between the percentage or absolute number of $CD3^+CD8^+CD28^-$ T cells and age in pooled RA patients (**Figure 3-5**). Thus, there was no relationship between age and expression of CD28 on $CD3^+CD8^+$ T cells.

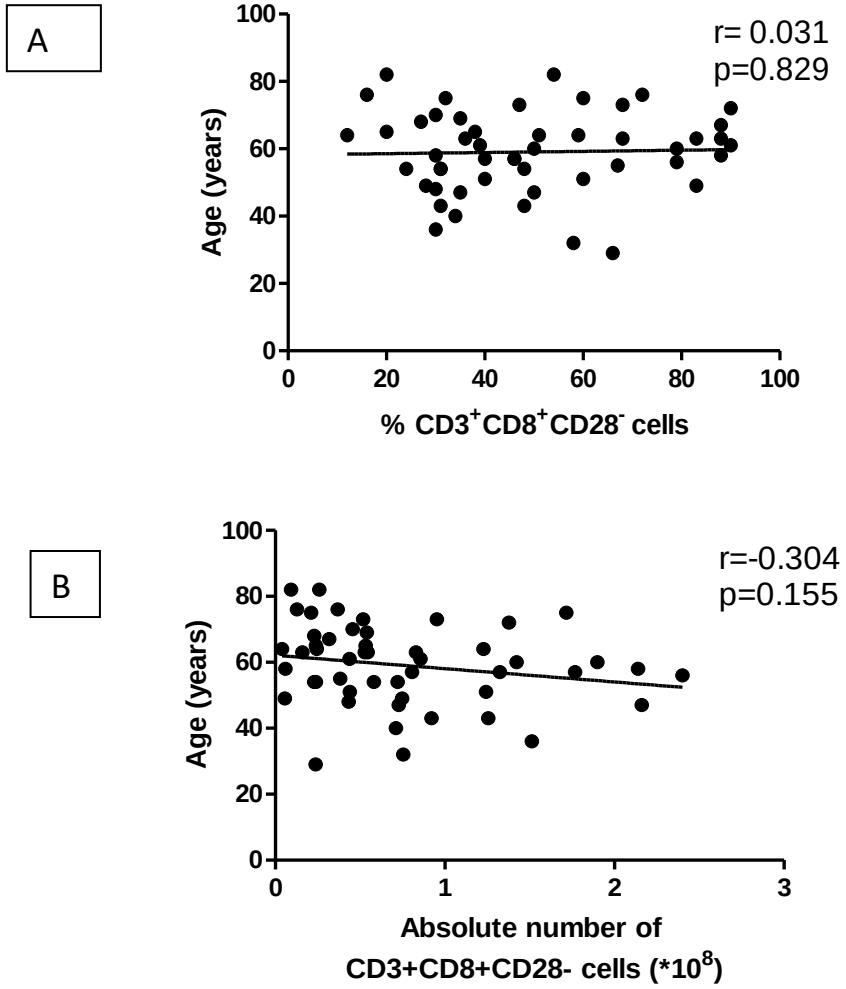


Figure 3-5: Percentage and Absolute number of $CD3^+CD8^+CD28^-$ cells do not correlate with age in pooled RA patients. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-$ T Cells. Correlation was determined using non-parametric Spearman's rank analysis, * $p<0.05$. Pooled RA (n=50).

3.8 The correlation between $CD3^+CD8^+CD28^-$ cells and Sex

Of the 24 controls, 68% were female and of the 50 pooled RA, 66% were female. The pooled RA patients had a higher percentage of $CD3^+CD8^+CD28^-$ cells in the male group compared to the females with RA (**Figure 3-6**, p=0.012). The trend of the percentage of $CD3^+CD8^+CD28^-$ cells was similar in the control group, with higher percentage of $CD3^+CD8^+CD28^-$ cells in the male population, but this difference was not significant. There was no difference between sex and the absolute number of $CD3^+CD8^+CD28^-$ cells in pooled RA patients or controls.

Dudkowska et al (2017) observed higher amounts of $CD8^+CD28^-$ T cells in males than in females between 65-74 in the general population (n=192, p=0.014)[226]. Notably, their results also indicated a higher level of IL-10 in men than women (p=0.018). Correlation between $CD3^+CD8^+CD28^-$ cells and Sex has not been looked at in the RA population previously.

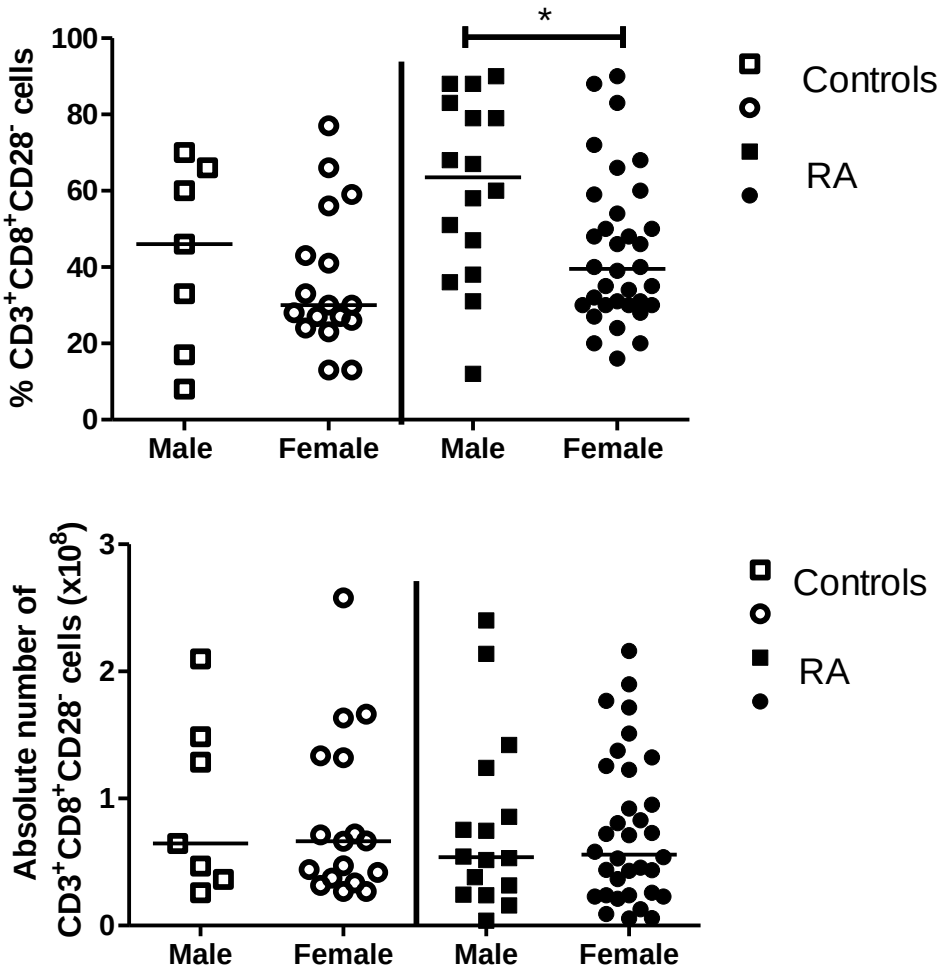


Figure 3-6: The percentage of $CD3^+CD8^+CD28^-$ cells is statistically significantly higher in males compared with females in pooled RA patients. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-$ T Cells. Graphs represent median, * $p<0.05$ using Kruskal-Wallis ANOVA Test. Controls (n=24, Male=7, Female=17), Pooled RA (n=50, Men=16, Women=34).

3.9 Serological status and $CD3^+CD8^+CD28^-$ T cells: RF positivity

Of the pooled RA patients 62% were RF positive. The percentage of $CD3^+CD8^+CD28^-$ cells was significantly higher in RF positive compared to RF negative patients in established RA (p=0.012) (**Figure 3-7C)**. This trend was reflected in the absolute number of $CD3^+CD8^+CD28^-$ cells but the differences were not statistically significant.

In Early RA patients the trend of percentage of $CD3^+CD8^+CD28^-$ cells was higher in the RF negative patients but this difference again was not significant **(Figure 3-7B)**. The differences are summarised in **Table 3-5**. Although the relationship between RF and $CD3^+CD4^+CD28^-$ cells in RA and control patients has previously been investigated, no statistical difference was observed (p=0.062)[227].

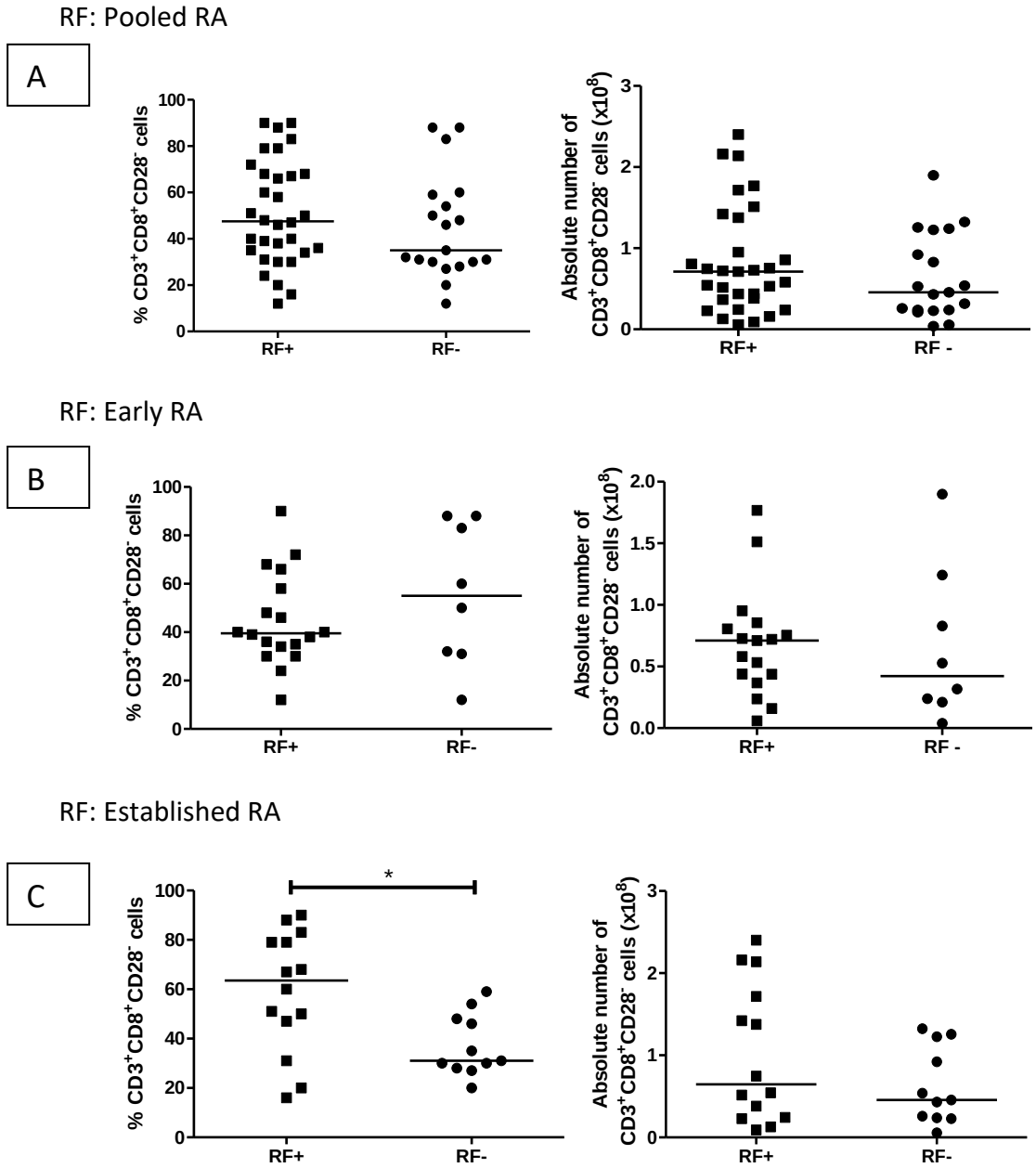


Figure 3-7: Percentage of CD3+CD28$^+$CD28$^-$ cells is higher in RF positive established RA patients. Percentage and absolute number of CD3+CD28$^+$CD28$^-$ cells in (A) pooled RA patients (n=50, RF positive=31, RF negative=19), (B) early RA (n=25, RF positive=17, RF negative=8) and (C) established RA (n=25, RF positive= 14, RF negative=11). Graphs represent median, * $p<0.05$ using Mann-Witney U Test.

3.10 Serological status and $CD3^+CD8^+CD28^-$ T cells: ACPA status

Although the trend appeared to show that the percentage and absolute number of $CD3^+CD8^+CD28^-$ cells is higher in ACPA positive than ACPA negative, this was not statistically significant (**Figure 3-8**). One patient in the Established RA group did not have recorded ACPA serology so was excluded from the analysis. ACPA correlation with $CD3^+CD8^+CD28^-$ cells in RA has not previously been investigated.

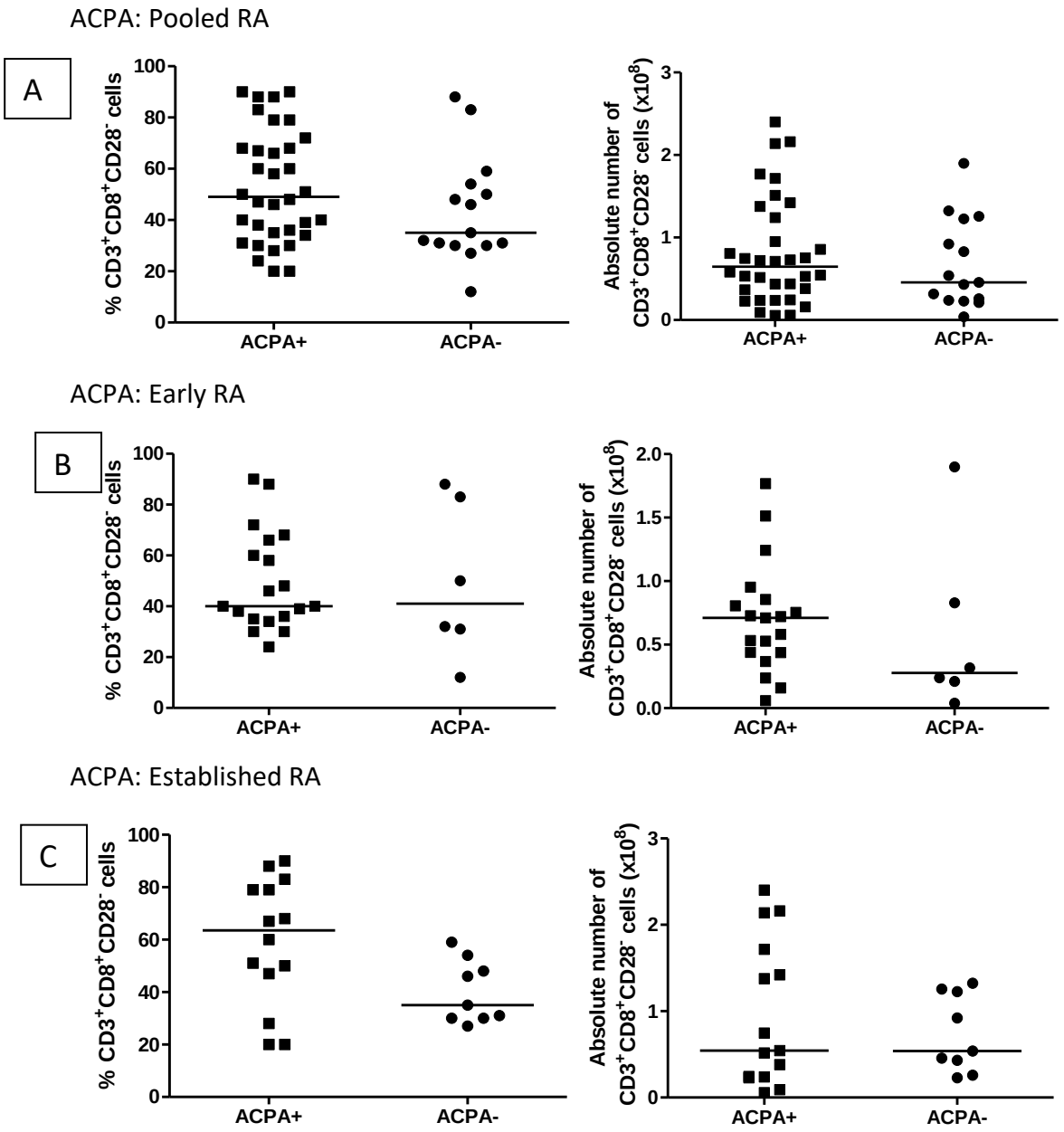


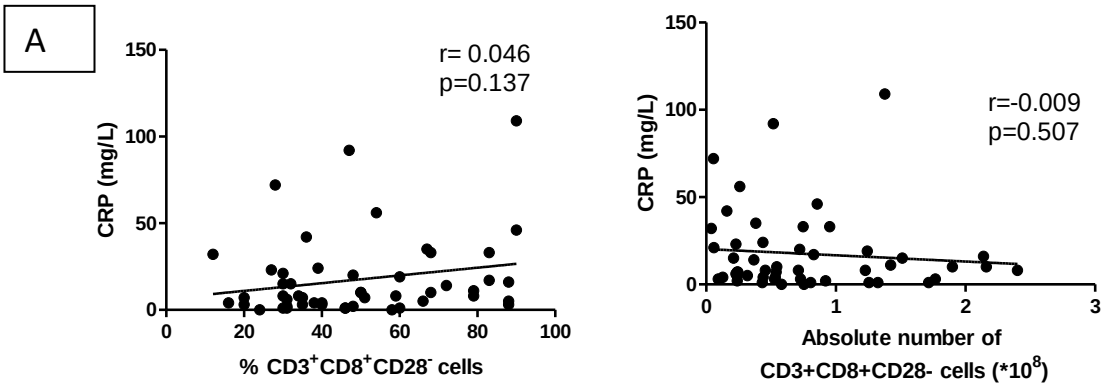
Figure 3-8: Percentage and Absolute number of $CD3^{+}CD28^{+}CD28^{-}$ cells do not correlate with ACPA positivity in RA patients. Percentage and absolute number of $CD3+CD28^{+}CD28^{-}$ cells in (A) pooled RA patients (n=49, ACPA positive=34, ACPA negative=15), (B) early RA (n=25, ACPA positive=19, ACPA negative=6) and (C) established RA (n=24, ACPA positive=15, ACPA negative=9). Graphs represent median, using Mann-Witney U Test. One patient from the Established RA group did not have an ACPA result so was excluded from analysis.

3.11 Laboratory markers and $CD3^+CD8^+CD28^-$ T cells: CRP

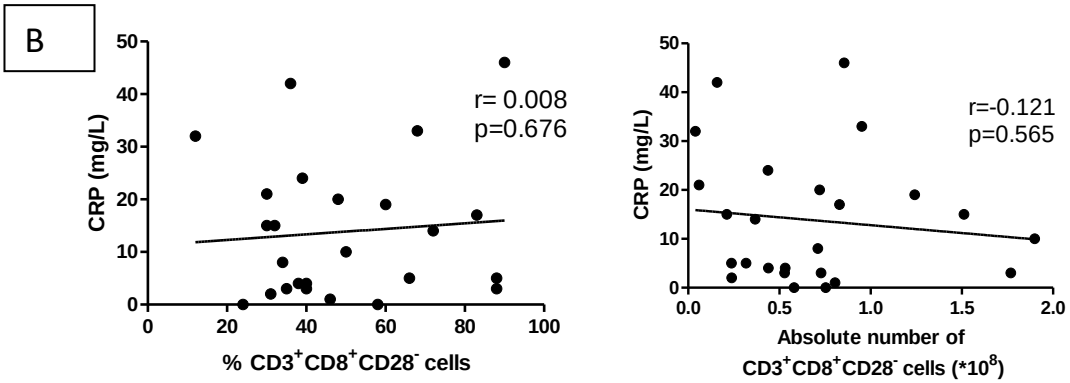
CRP is marker of inflammation used in medical practice to monitor disease activity in RA. When subdivided into Early and Established RA, there was a novel low-level correlation revealed between the percentage of $CD3^+CD8^+CD28^-$ T cells and CRP in Established RA (**Figure 3-9C**).

There was no correlation between the percentage of $CD3^+CD8^+CD28^-$ T cells and CRP in pooled or Early RA. There was no correlation between the absolute number of $CD3^+CD8^+CD28^-$ T cells and CRP (**Table 3-4**). CRP correlation with $CD3^+CD8^+CD28^-$ cells in RA has not previously been investigated.

CRP: Pooled RA



CRP: Early RA



CRP: Established RA

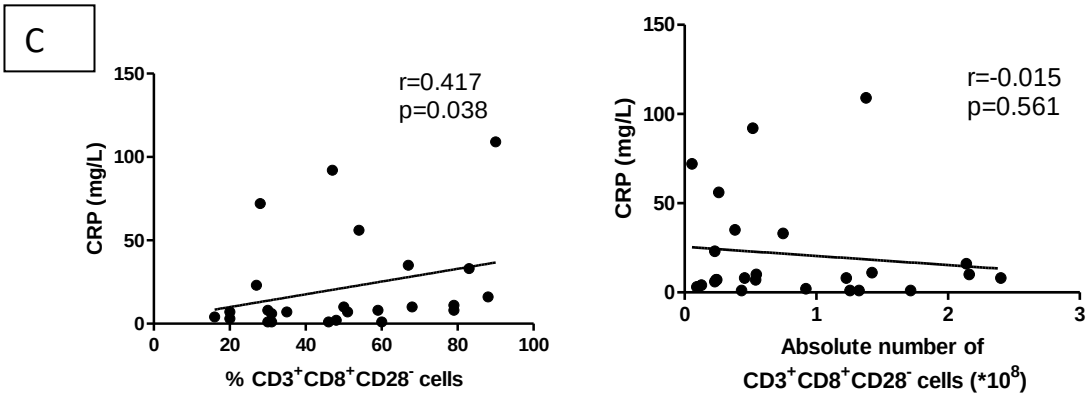


Figure 3-9: There is low level correlation between CRP and the percentage of $CD3^+CD8^+CD28^-$ cells in Established RA. Correlation was determined using non-parametric Spearman's rank analysis, * $p<0.05$. (A) Pooled RA (n=50), (B) early RA (n=25) and (C) established RA (n=25).

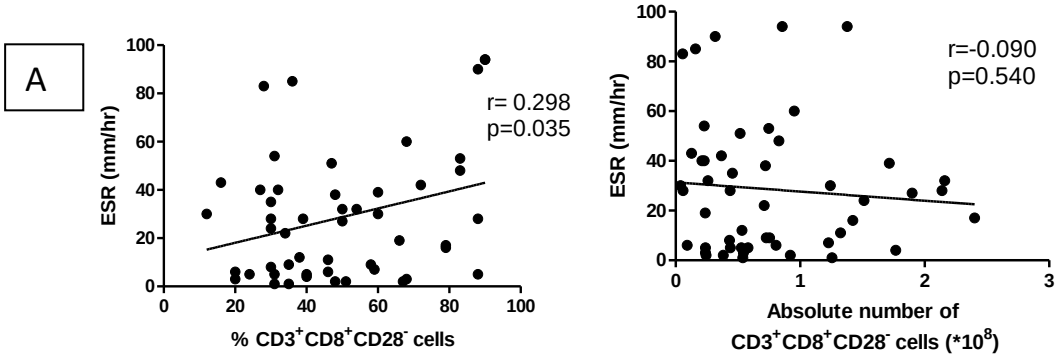
3.12 Laboratory markers and $CD3^+CD8^+CD28^-$ T cells: ESR

There is a low-level correlation between the percentage of $CD3^+CD8^+CD28^-$ T cells and ESR in the pooled RA (**Figure 3-10A**) and Early RA (**Figure 3-10B**).

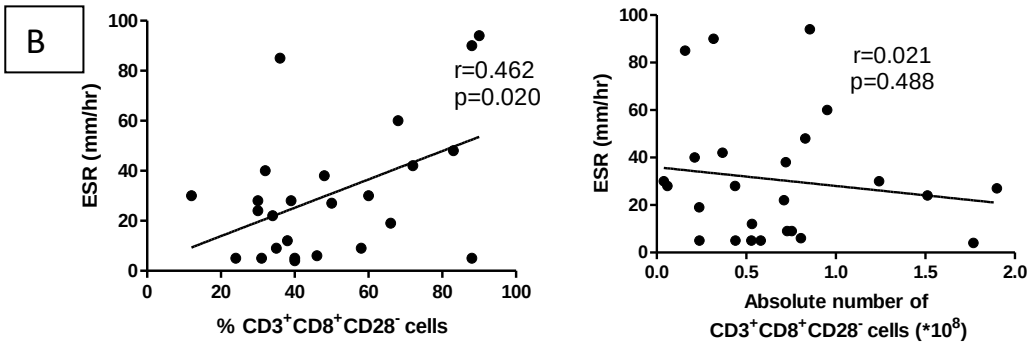
There was no significant correlation between the percentage or absolute number of $CD3^+CD8^+CD28^-$ T cells and ESR in the Established RA patients (**Figure 3-10C**). This was consistent with results by Ceeraz et al (2013) who did not find a significant correlation between $CD3^+CD8^+CD28^-$ T cells and ESR in Established RA[197].

There was no correlation between the absolute number of $CD3^+CD8^+CD28^-$ T cells and ESR in pooled RA, Early RA or Established RA (**Table 3-4**).

ESR: Pooled RA



ESR: Early RA



ESR: Established RA

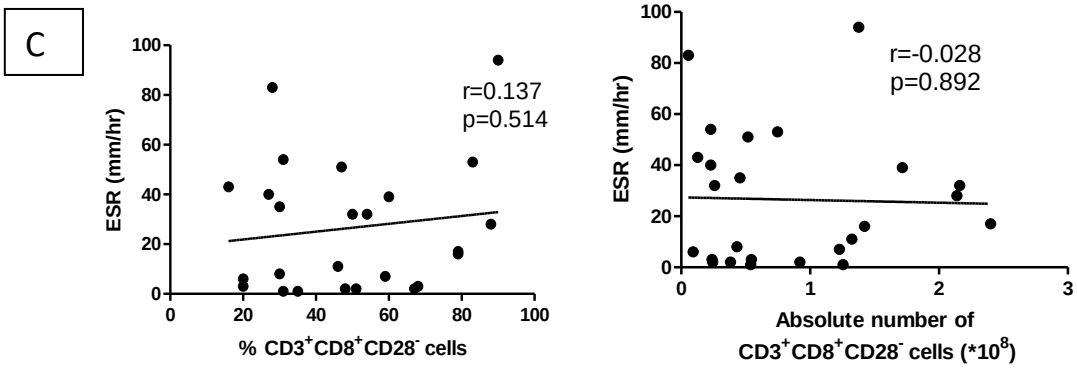


Figure 3-10: There is low-level correlation between the percentage of $CD3^+CD8^+CD28^-$ T cells and ESR in pooled and Early RA. Correlation was determined using non-parametric Spearman's rank analysis, * $p<0.05$. Pooled RA (A, n=50), Early RA (B, n=25), Established RA (C, n=25).

3.13 Clinical markers and $CD3^+CD8^+CD28^-$ cells: DAS28 score and disease duration

There was no statistically significant correlation between the percentage or absolute number of $CD3^+CD8^+CD28^-$ T cells and DAS28 (using ESR) in pooled RA (**Figure 3-11A**). These findings reinforce previous results from the Ceeraz et al (2013) study which did not find a significant correlation between $CD3^+CD8^+CD28^-$ T cells and DAS28[197].

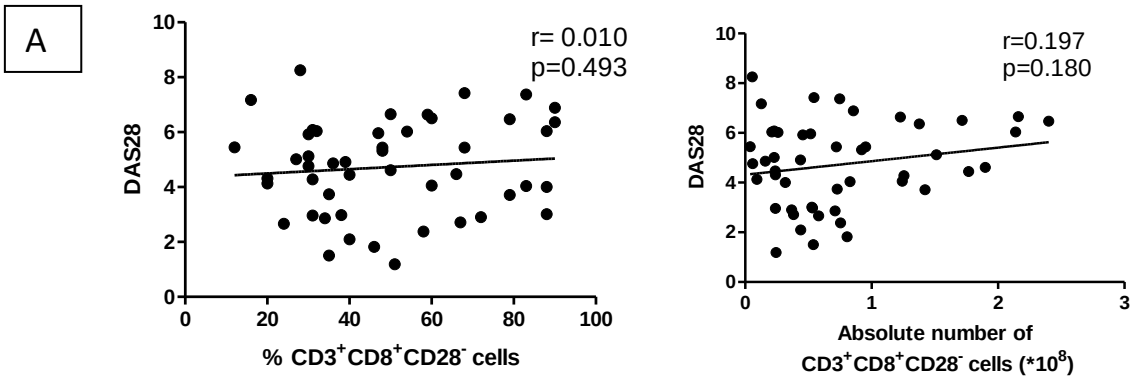
Subdivision into Early and Established RA did not reveal any statistically significant correlation between the percentage or absolute number of $CD3^+CD8^+CD28^-$ T cells and DAS28 (**Figure 3-11B & 3-12C**).

A novel finding that there was a low-level correlation between the percentage of $CD3^+CD8^+CD28^-$ T cells and disease duration in the Early RA group (**Figure 3-12A**).

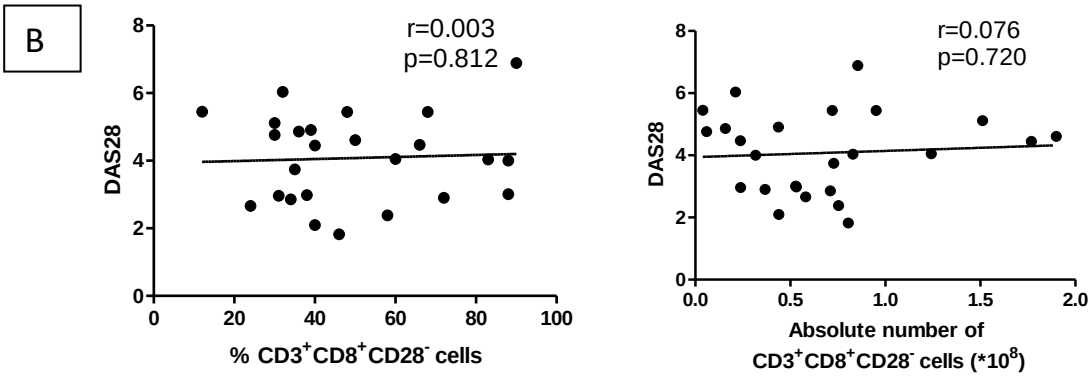
There was no correlation between the percentage of $CD3^+CD8^+CD28^-$ T cells and disease duration in the Established RA group (**Figure 3-12B**). This was also shown by Ceeraz et al (2013) who found no significant correlation with the disease activity score and $CD8^+CD28^-$ cells in Established RA[197].

There was no correlation between the absolute number of $CD3^+CD8^+CD28^-$ T cells and disease duration in either the Early or Established RA (**Table 3-4**). Pawlik et al (2003) also looked at the expansion of $CD4^+CD28^-$ T-cell compartment and disease chronicity in RA, but none was found ($r=0.14$, $n=42$, $p=0.3$)[227].

DAS28: Pooled RA



DAS28: Early RA



DAS28: Established RA

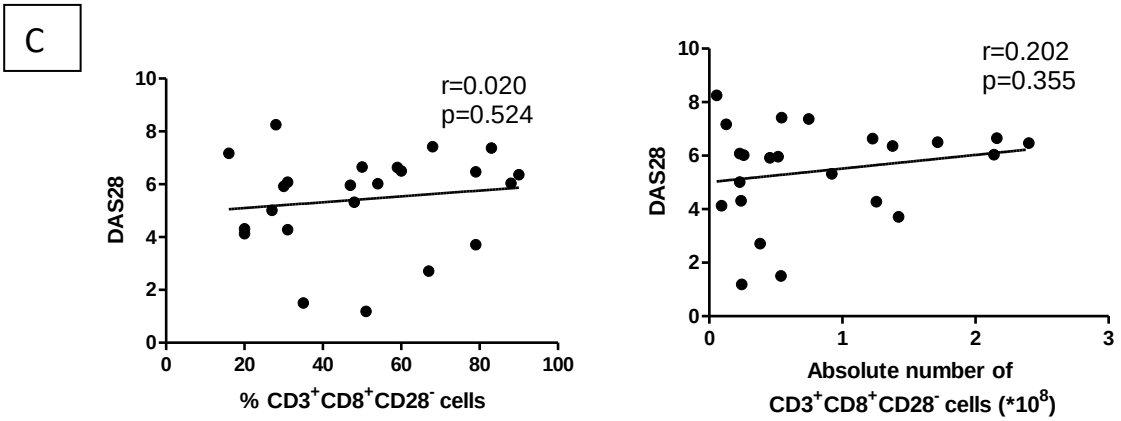
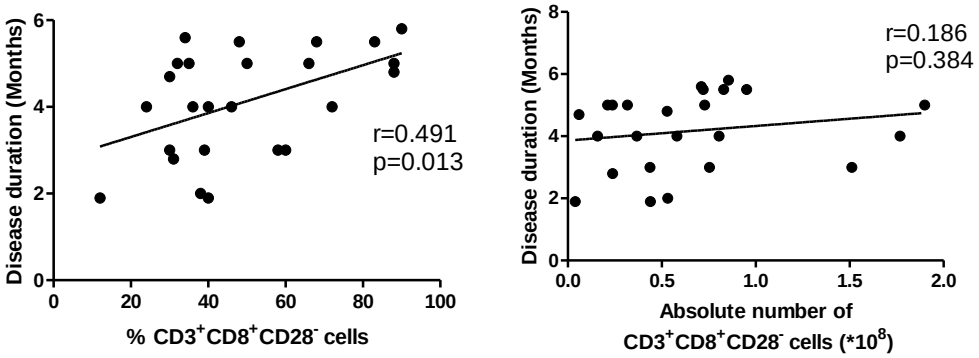


Figure 3-11: Percentage and Absolute number of $CD3^+CD8^+CD28^-$ cells do not correlate with age in pooled RA patients. Two patients in the Established RA group did not have a DAS28 (ESR) recorded at baseline so were excluded (A&C). Correlation was determined using non-parametric Spearman's rank analysis, *p<0.05. Pooled RA (A, n=48), Early RA (B, n=25), Established RA (C, n=23).

Disease Duration: Early RA

A



Disease Duration: Established RA

B

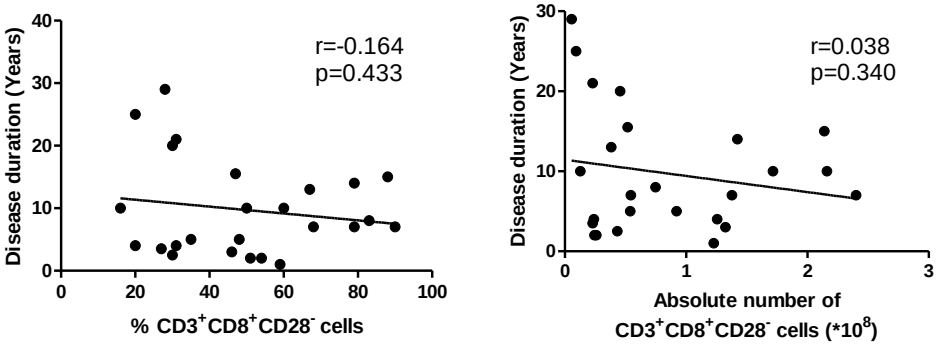


Figure 3-12: There is low level correlation between the percentage of $CD3^+CD8^+CD28^-$ T cells and Disease Duration in Early RA patients. Correlation was determined using non-parametric Spearman's rank analysis, *p<0.05. Disease duration analysis with Early RA (A, n=25), Established RA (B, n=25).

Variable	Patient group	Correlation with $CD3^+CD8^+CD28^-$ % (r value)	p Value	Correlation with $CD3^+CD8^+CD28^-$ Absolute number (r value)	p Value
CRP	Pooled	0.455	ns	0.009	ns
	Early	0.008	ns	0.015	ns
	Est.	0.417	**0.038**	0.015	ns
ESR	Pooled	0.298	**0.035**	-0.090	ns
	Early	0.462	**0.020**	-0.021	ns
	Est.	0.137	ns	-0.028	ns
DAS28	Pooled	0.010	ns	0.197	ns
	Early	0.003	ns	0.076	ns
	Est.	0.020	ns	0.202	ns
Disease Duration	Early	0.491	**0.013**	0.186	ns
	Est.	-0.164	ns	0.038	ns

Table 3-4: Comparison of $CD3^+CD8^+CD28^-$ cells and CRP, ESR, DAS28 and Disease duration. Gaussian approximation Spearman's rank analysis, r value with the significance. P value <0.05 is considered significant.

3.14 $CD3^+CD8^+CD28^-$ cells at baseline do not correlate with six month disease activity (using DAS28)

Disease severity is calculated in medical practice using the DAS28 score which includes the ESR. A positive response is determined as a reduction of DAS28 by >1.2[228]. The Early RA group had their DAS28 score calculated at baseline and 6 months later (n=22). Three patients did not have a follow up DAS28 score recorded. Patients whose DAS28 score reduced by >1.2 were determined as responders.

The trend of percentage of $CD3^+CD8^+CD28^-$ cells was higher in the patients who did not respond to treatment, but this difference was not significant (**Figure 3-13A**). Absolute number of $CD3^+CD8^+CD28^-$ cells was similar in both the responders and non-responders (**Figure 3-13B and Table 3-5**). These results were consistent with Ceeraz et al (2013) who looked at $CD3^+CD8^+CD28^-$ cells and disease duration in Established RA[197].

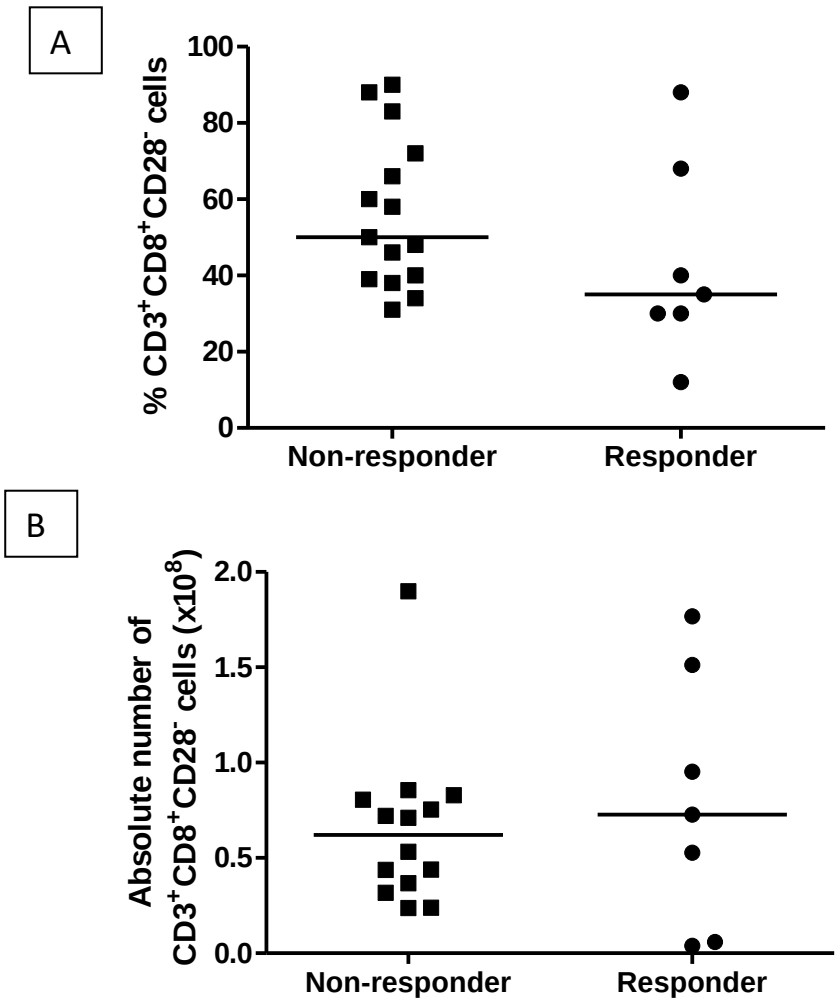


Figure 3-13: $CD3^+CD8^+CD28^-$ T cells do not predict severity of disease at 6 months in Early RA. Three patients were excluded because they did not have a recorded DAS28 at six months. Percentage (A) and absolute number (B) of $CD3^+CD8^+CD28^-$ cells were represented median, using Mann-Witney U Test. Early RA (n=22, Responders=7, Non-responders=15).

Variable	Patient Group	Median % $CD3^+CD8^+CD28^-$ cells		p Value	Median absolute number $CD3^+CD8^+CD28^-$ cells (*10^8)		p Value
		Positive	Negative		Positive	Negative	
RF	Pooled	48	38	ns	0.7	0.4	ns
	Early	39	58	ns	0.7	0.4	ns
	Est	63	30	**0.0199**	0.6	0.4	ns
ACPA	Pooled	58	38	ns	0.6	0.5	ns
	Early	40	40	ns	0.7	0.3	ns
	Est	62	38	ns	0.5	0.5	ns
Responders	Early	46	38	ns	0.6	0.7	ns

Table 3-5: Comparison of $CD3^+CD8^+CD28^-$ cells and RF, ACPA and Responders. Mann-Witney U Test was used to establish significance. P value <0.05 is considered significant.

3.15 Intracellular Cytokines: How active are $CD3^+CD8^+CD28^-$ cells in pooled RA versus Controls?

3.15.1 IFN production

Existing work with $CD4^+CD28^-$ T cells by Teo et al (2013) shows that these cells produce more TNF-α and IFN-γ than $CD4^+CD28^+$ T cells[229,230]. $CD4^+CD28^-$ T cells in synovial fluid from RA patients produce less IFN-γ and TNF-α than peripheral blood $CD4^+CD28^-$ T cells, but they produce more IL-17A[231]. Furthermore, they produce perforin and granzyme B, which can destroy synovial tissue[232]. Notably, reduced responsiveness to $CD4^+CD25^+$ regulatory T cells and resistance to apoptosis further add to their destructive potential[233,234]. This provides rationale for a comprehensive characterisation of the effector characteristics of $CD3^+CD8^+CD28^-$ cells in RA.

In comparison to the research with $CD4^+CD28^-$ T cells, the relationship between $CD3^+CD8^+CD28^-$ T cells, RA progression and effector cytokine production is ill-defined. Comparison of the characteristics of $CD3^+CD8^+CD28^-$ cells revealed very low percentages and absolute number of $CD3^+CD8^+CD28^-$ cells were producing IFN. The amount of $CD3^+CD8^+CD28^-$ cells producing IFN between the controls and pooled RA patients was similar (**Figure 3-14**). There was no difference between $CD3^+CD8^+CD28^-$ cells and $CD3^+CD8^+CD28^+$ cells producing IFN. High concentrations of IFN-γ were produced by stimulated $CD3^+CD8^+CD28^-$ cells in healthy controls as well as RA in one patient study[197].

Subdivision into Early and Established RA showed no difference between the groups for percentage or absolute numbers of $CD3^+CD8^+CD28^-$ or $CD3^+CD8^+CD28^+$ cells producing IFN (**Figure 3-15**).

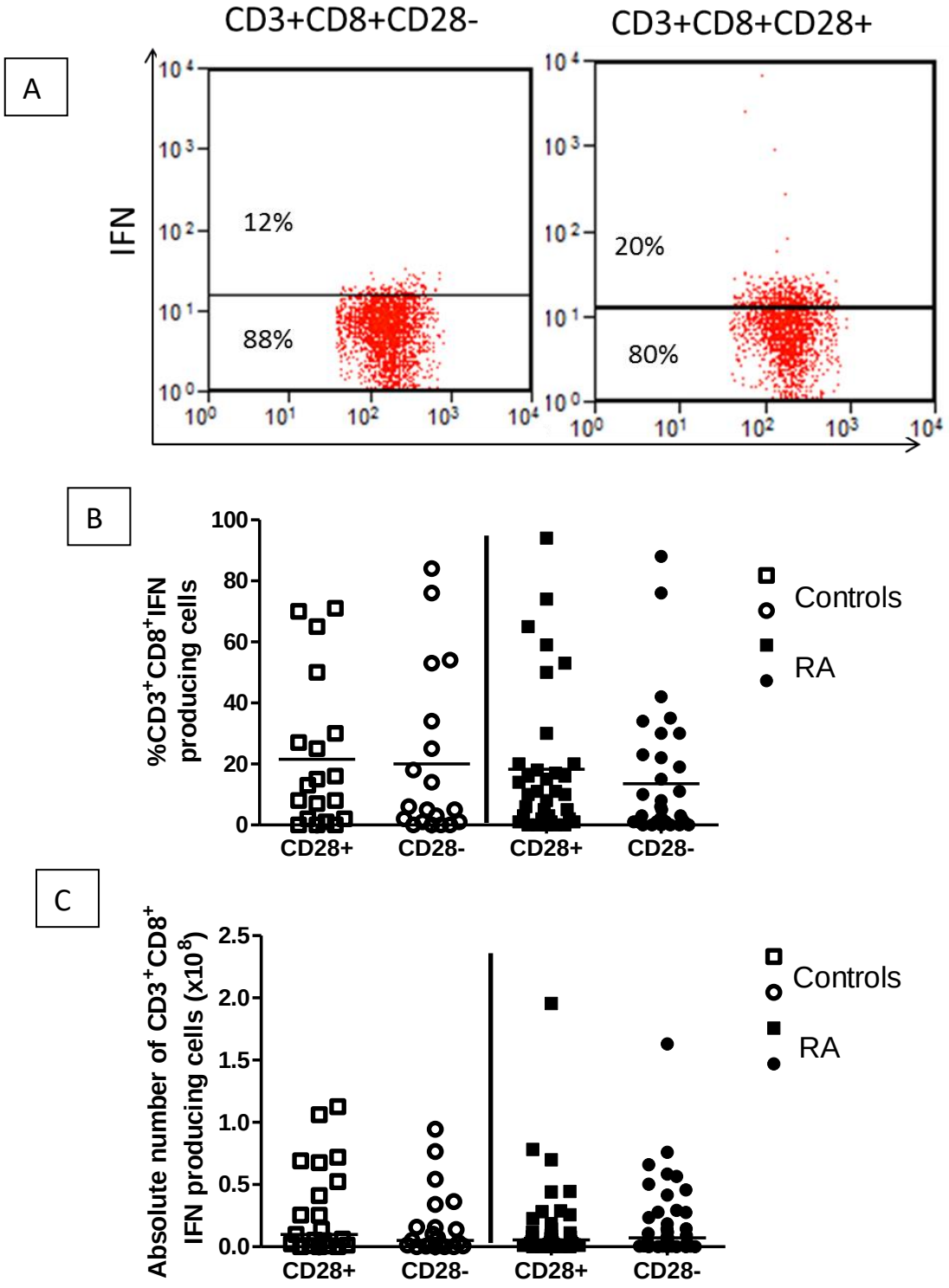


Figure 3-14: $CD3^+CD8^+CD28^-$ T cells produce small amounts of IFN. Summit graphs to illustrate staining for IFN with antibody conjugated on fluorochrome PE (A). The percentage (B) and absolute number (C) of $CD3^+CD8^+CD28^-$ T cells producing IFN in Controls on left and pooled RA patients on the right. Graphs represent median, * $p<0.05$ using Kruskal-Wallis ANOVA test. Controls (n=19), Pooled RA (n=35).

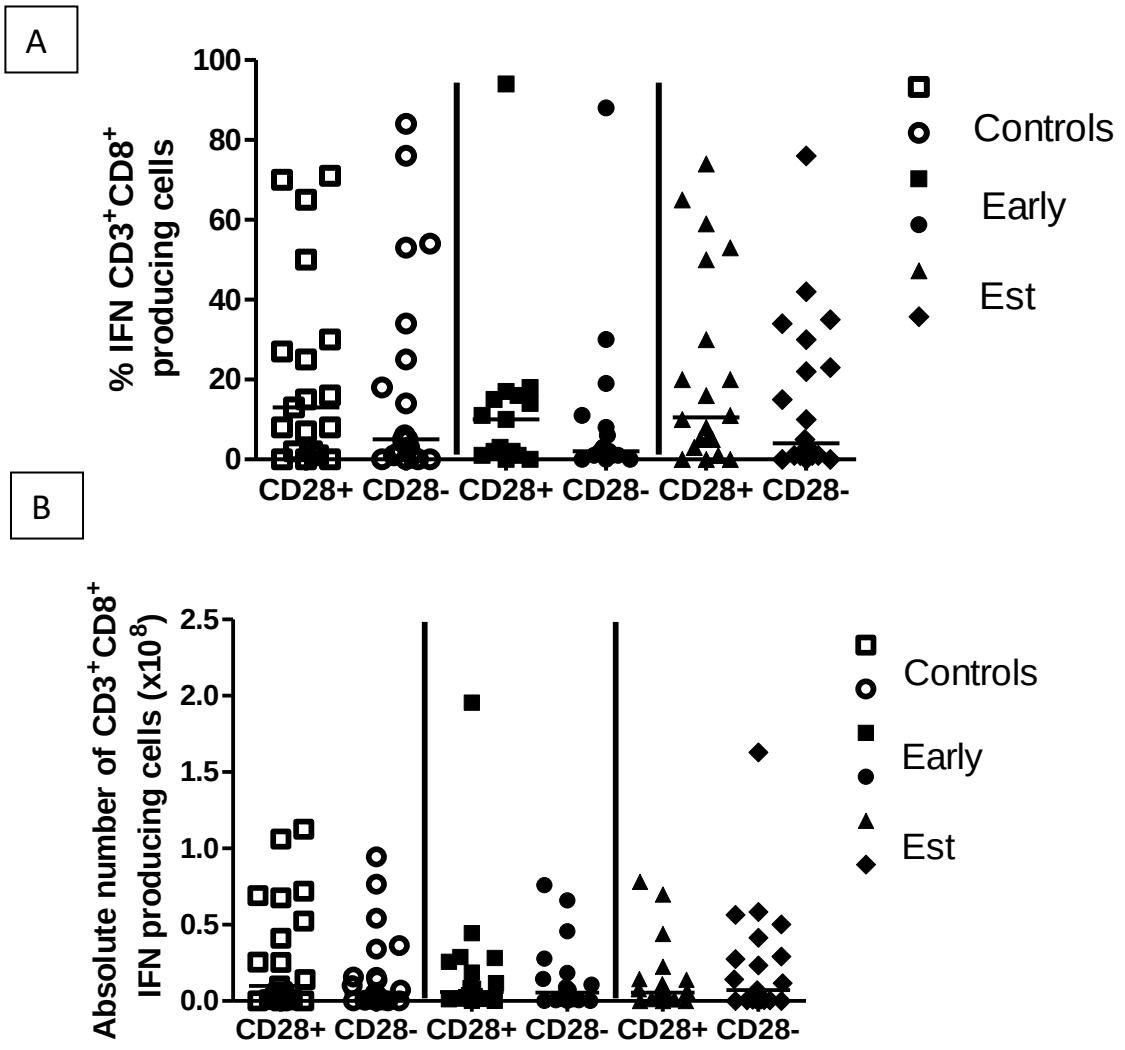


Figure 3-15: Subdivision into Early RA and Est RA groups showed no significant difference in IFN production from $CD3^+CD8^+$ cells. Graphs represent median, *p<0.05 using Kruskal-Wallis ANOVA test for percentage (A) and absolute number (B) of $CD3^+CD8^+CD28^-$/+ cells. Controls (n=19), Early (n=15) and Established (n=20).

IL-10 production

Comparison of the characteristics of $CD3^+CD8^+CD28^-$ cells revealed similar percentages and absolute numbers of $CD3^+CD8^+CD28^-$ cells were producing IL-10. Ceeraz et al (2013) found that RA patients on Methotrexate $CD3^+CD8^+CD28^-$ cell cultures produced more IL-10 following stimulation compared to healthy controls ($p=0.0072$)[197]. There was no difference between the Controls and pooled RA, or between $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^+$ cells, producing IL-10 (**Figure 3-16**).

Novel subdivision into Early and Established RA revealed that the percentage and absolute number of $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^+$ cells producing IL-10 was similar across groups (**Figure 3-17**).

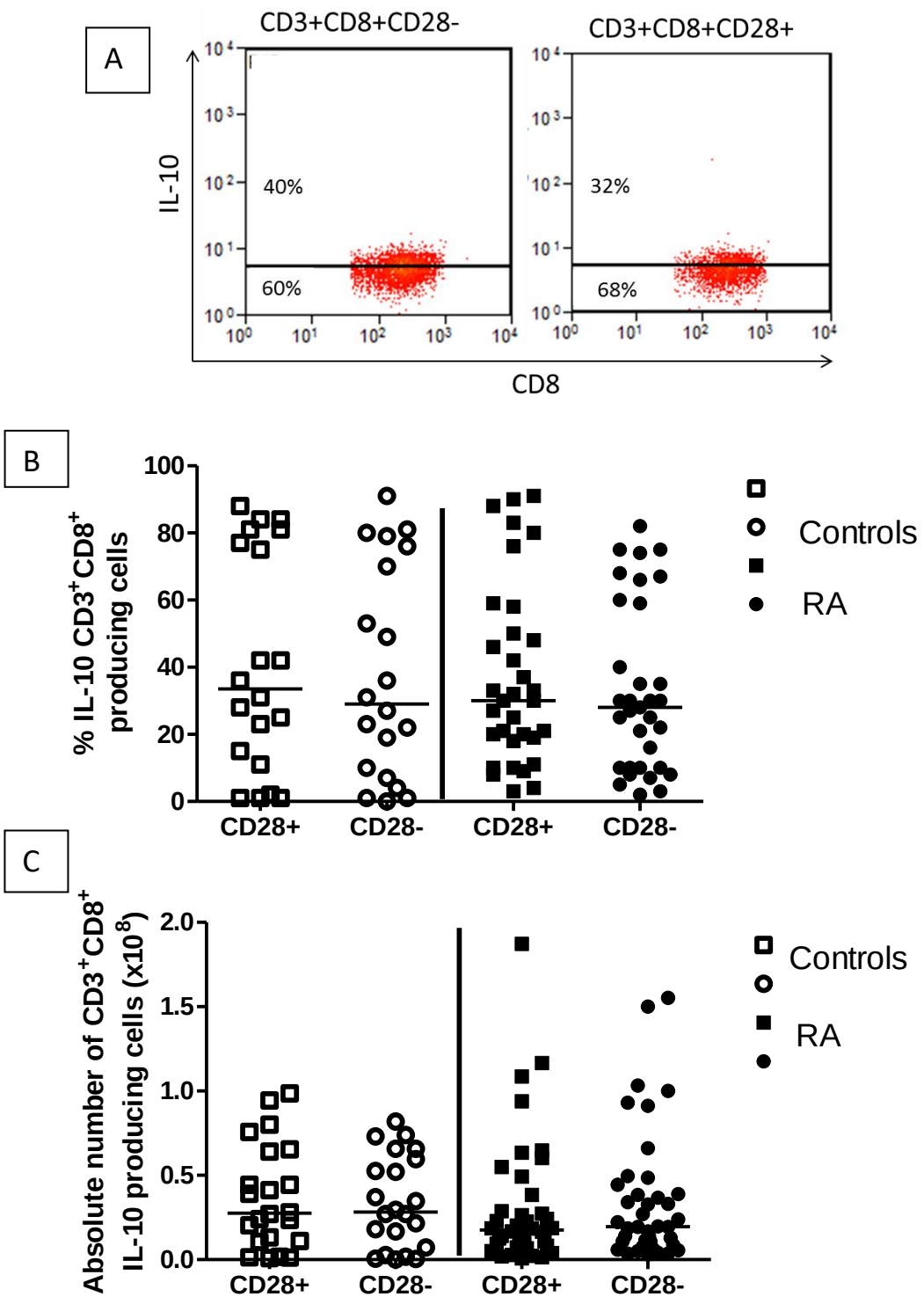


Figure 3-16: $CD3^+CD8^+CD28^-$ cells produce IL-10. Summit graphs to illustrate staining for IL-10 with antibody conjugated on fluorochrome FITC (A). The percentage (B) and absolute number (C) of $CD3^+CD8^+CD28^-$ T cells producing IL-10 in Controls on the left and pooled RA patients on the right. Graphs represent median, Kruskal-Wallis ANOVA test was used. Controls (n=21), Pooled RA (n=37).

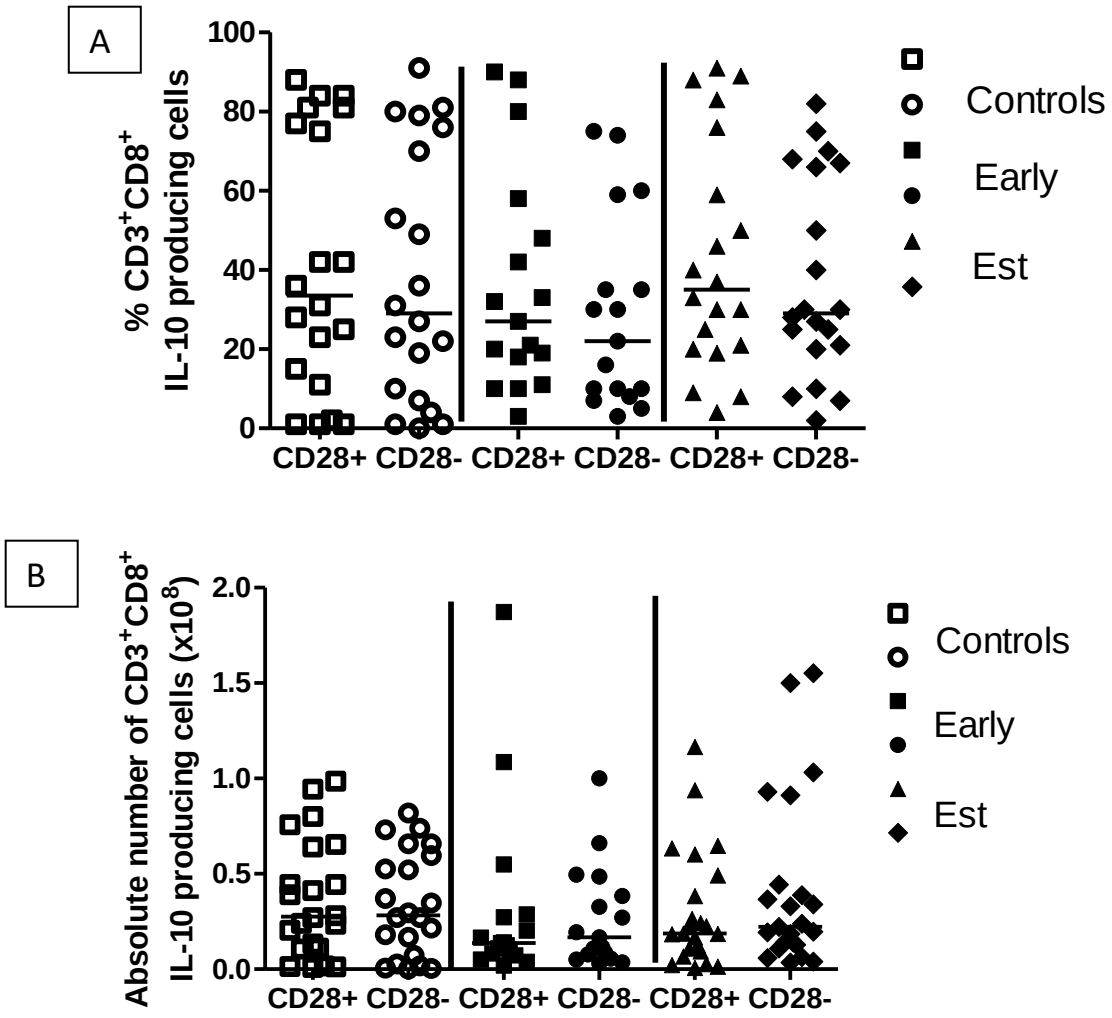


Figure 3-17: Subdivision into Early RA and Established RA groups showed no significant difference in IL-10 production from $CD3^+CD8^+CD28^-$ cells. Graphs represent median, using Kruskal-Wallis ANOVA test for percentage (A) and absolute number (B) of $CD3^+CD8^+CD28^{-/+}$ cells. Controls (n=19), Early (n=17) and Established (n=20).

3.15.3 IL-4 production

Production of IL-4 by $CD3^+CD8^+CD28^-$ cells has not been assessed previously. This study revealed low percentages and absolute numbers of $CD3^+CD8^+CD28^-$ cells were producing IL-4. There was no difference between the controls and pooled RA, or between $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^+$ cells, producing IL-4 (**Figure 3-18**).

Subdivision into Early and Established RA revealed that the percentage and absolute number of $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^+$ cells producing IL-4 was similar across groups (**Figure 3-19**). Thus, these novel investigations reveal that $CD3^+CD8^+CD28^-$ cells are not major producers of IL-4.

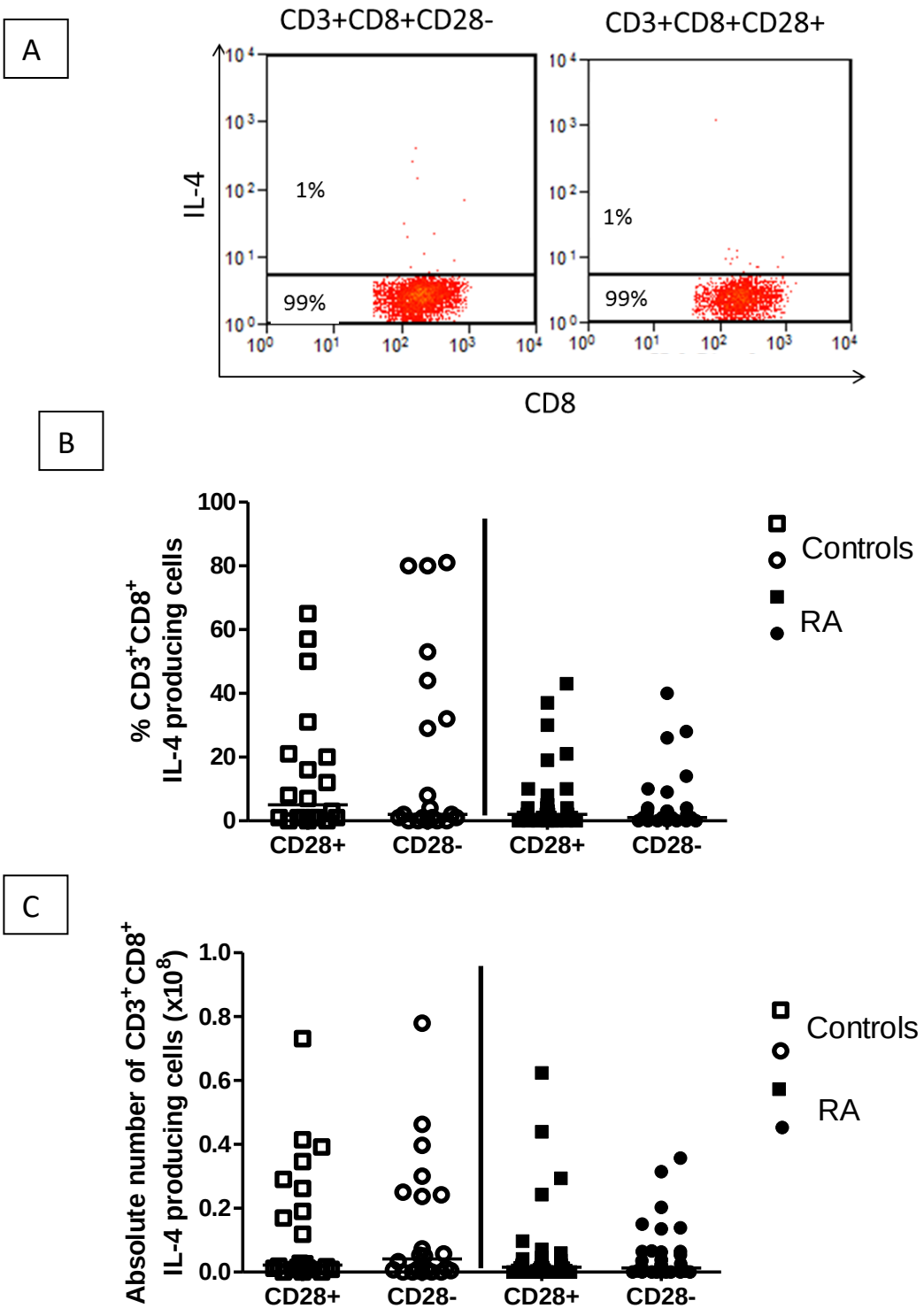


Figure 3-18: Very little IL-4 is produced by $CD3^+CD8^+CD28^-$ T cells. Summit graphs to illustrate staining for IL-4 with antibody conjugated on fluorochrome FITC (A). The percentage (B) and absolute number (C) of $CD3^+CD8^+CD28^{-/+}$ T cells producing IL-4 in controls on the left and pooled RA patients on the right. Graphs represent median, Kruskal-Wallis ANOVA test was used. Controls (n=20), Pooled RA (n=37).

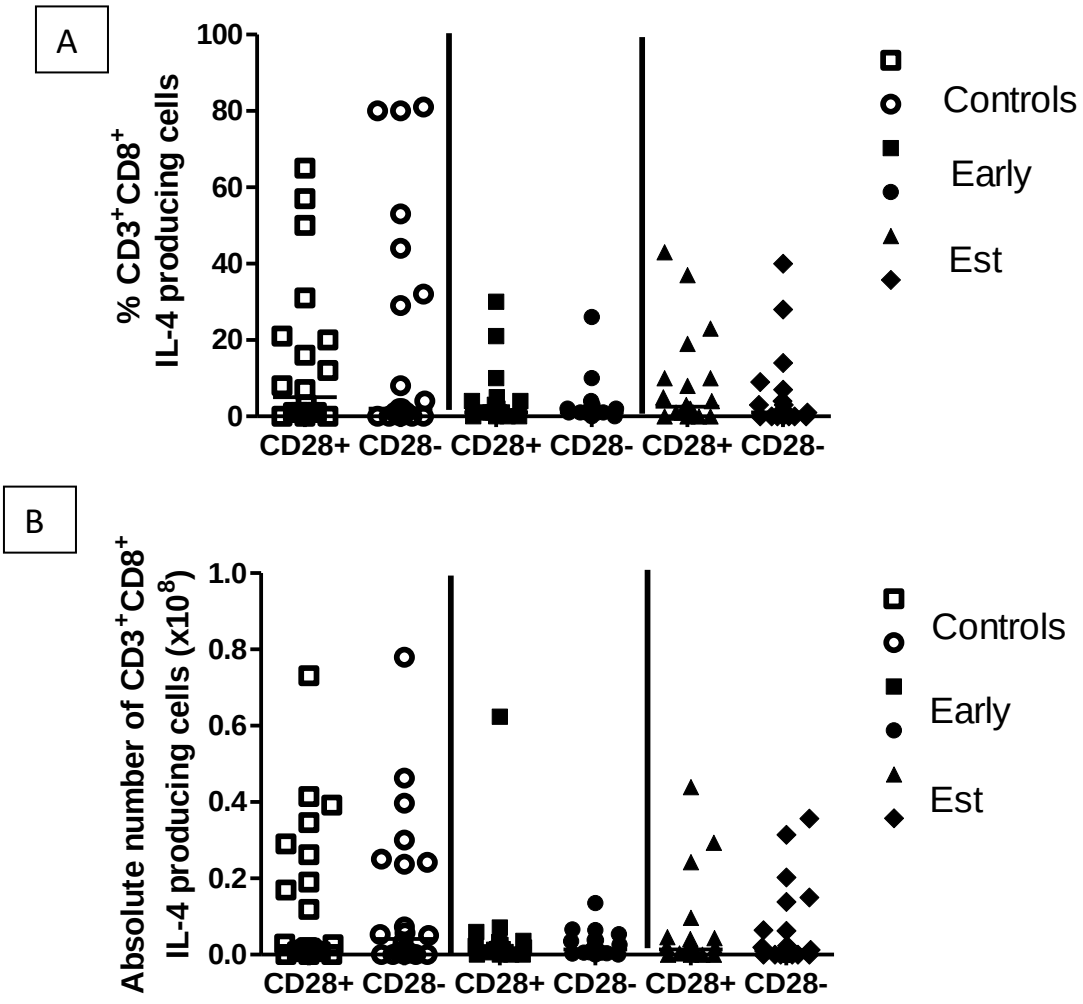


Figure 3-19: Subdivision into Early RA and Est RA groups showed no significant difference in IL-4 production. Graphs represent median, using Kruskal-Wallis ANOVA test for percentage (A) and absolute number (B) of $CD3^{+}CD8^{+}CD28^{-/+}$ cells. Controls (n=19), Early (n=17) and Established (n=20).

3.15.4 IL-6 production

Comparison of the characteristics of $CD3^+CD8^+CD28^-$ cells revealed low percentages and absolute numbers of $CD3^+CD8^+CD28^-$ cells were producing IL-6. The trend of percentage and absolute number of IL-6 producing cells was higher in $CD3^+CD8^+CD28^+$ compared to $CD3^+CD8^+CD28^-$ cells in Controls and also Pooled RA (% only) but this difference was not statistically significant (**Figure 3-20**).

Subdivision into Early and Established RA revealed that the percentage and absolute number of $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^+$ cells producing IL-6 was similar across the groups (**Figure 3-21**). This characterisation of $CD3^+CD8^+CD28^-$ cells is consistent with the established understanding of other T cells populations, where the cells are not potent producers of IL-6[235].

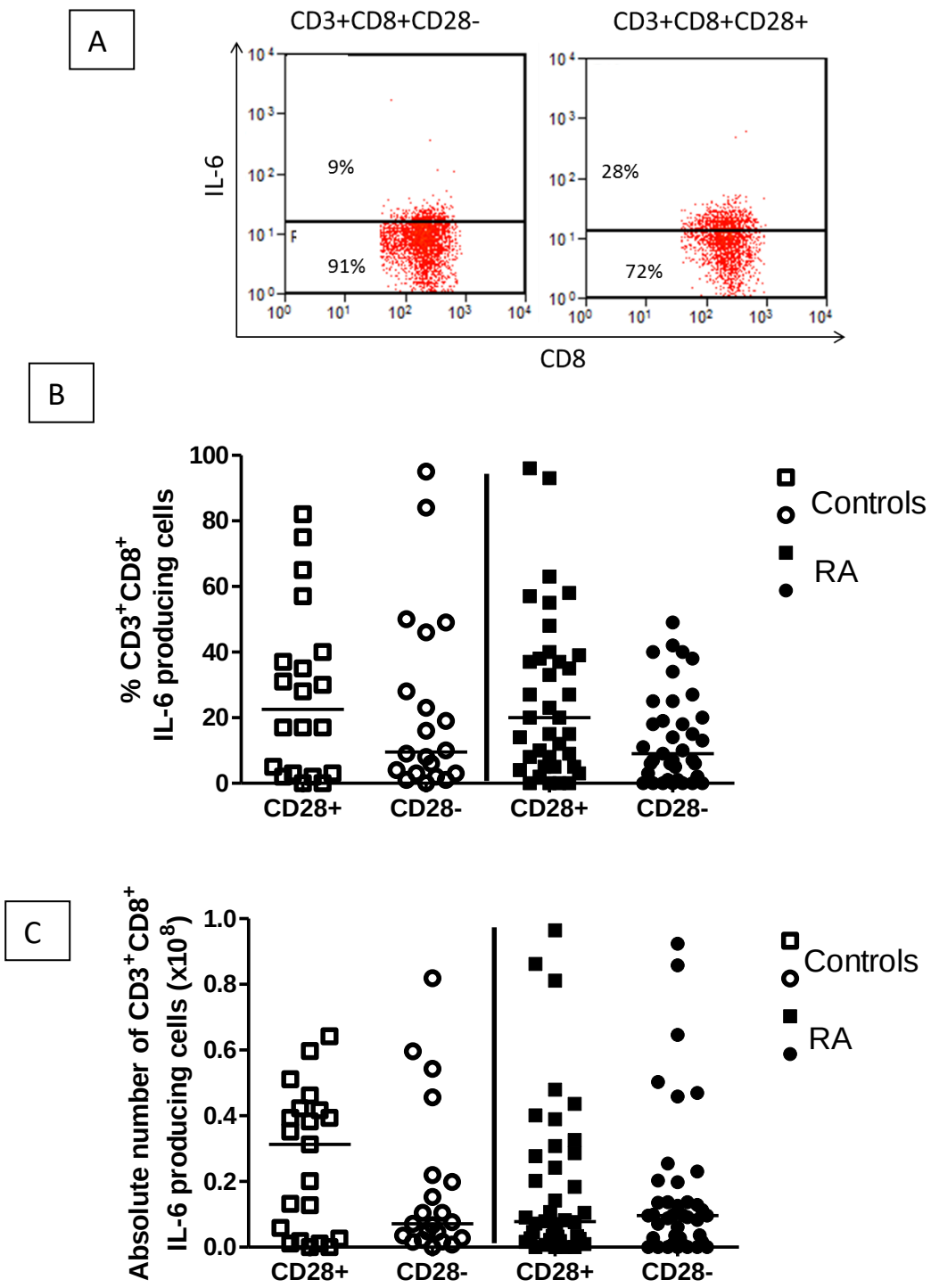


Figure 3-20: $CD3^+CD8^+CD28^-$ T cells produce small amounts of IL-6. Summit graphs to illustrate staining for IL-6 with antibody conjugated on fluorochrome PE (A). The percentage (B) and absolute number (C) of $CD3^+CD8^+CD28^-$ T cells producing IL-6 in Controls on left and pooled RA patients on the right. Graphs represent median, using Kruskal-Wallis ANOVA test. Controls (n=20), Pooled RA (n=30).

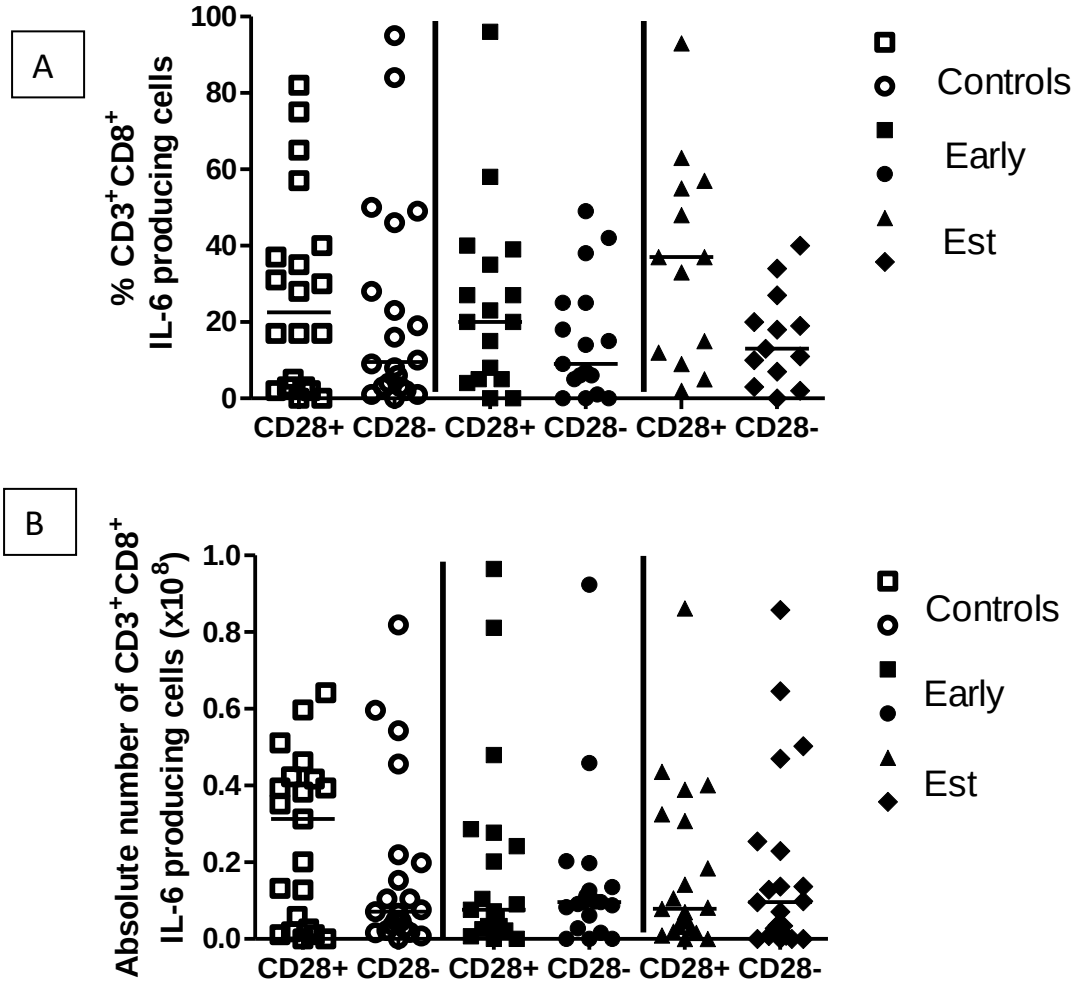


Figure 3-21: Subdivision into Early RA and Established RA groups showed no difference in IL-6 production. Graphs represent median, using Kruskal-Wallis ANOVA test for percentage (A) and absolute number (B) of $CD3^+CD8^+CD28^{-/+}$ cells. Controls (n=20), Early (n=17) and Established (n=13).

3.15.5 IL-17 production

$CD3^+CD8^+CD28^-$ cells do not produce IL-17. There was no difference between the Controls and pooled RA, or between $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^+$ cells, in their ability to produce IL-17 (**Figure 3-22**).

Subdivision into Early and Established RA similarly revealed that the percentage and absolute number of $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^+$ cells producing IL-17 was comparable (**Figure 3-23**).

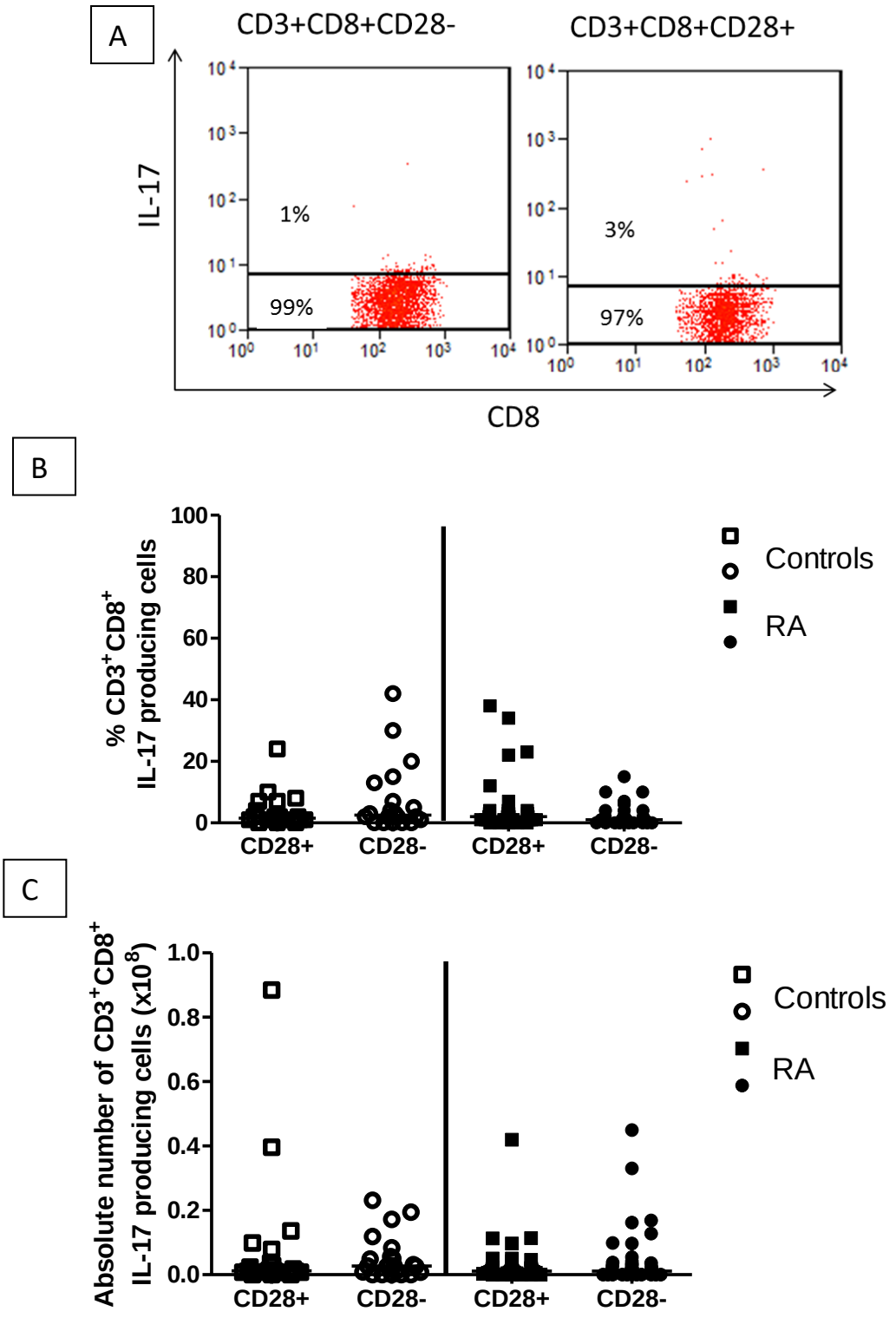


Figure 3-22: $CD3^+CD8^+CD28^-$ T cells do not produce IL-17. Summit graphs to illustrate staining for IL-17 with antibody conjugated on fluorochrome PCP (A). The percentage (B) and absolute number (C) of $CD3^+CD8^+CD28^{-/+}$ T cells producing IL-17 in Controls on left and pooled RA patients on the right. Graphs represent median, using Kruskal-Wallis ANOVA test. Controls (n=20), Pooled RA (n=37).

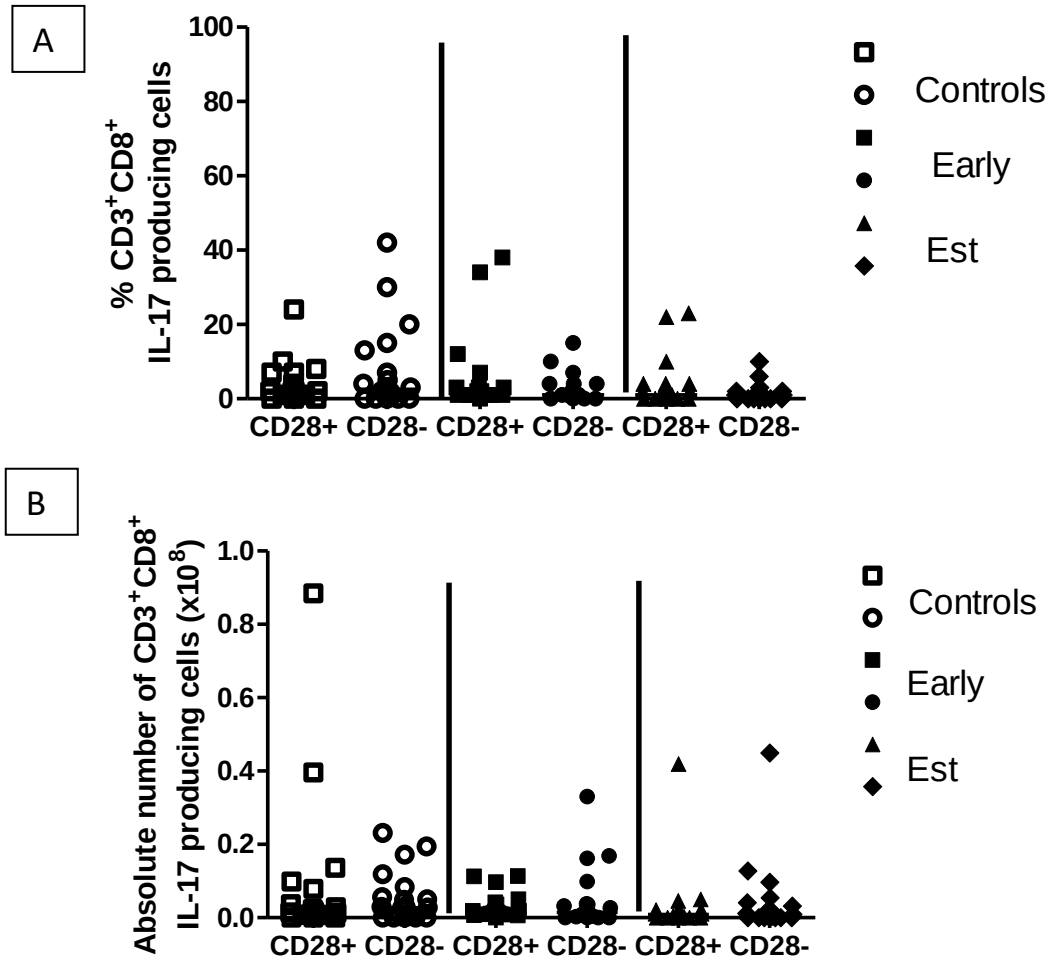


Figure 3-23: Subdivision into Early RA and Est RA groups showed no significant difference in IL-17 production. Graphs represent median, using Kruskal-Wallis ANOVA test for percentage (A) and absolute number (B) of $CD3^+CD8^+CD28^{-/+}$ cells. Controls (n=20), Early (n=17) and Established (n=20).

3.16 Cell surface markers expressed by $CD3^+CD8^+CD28^-$ T Cells

Although the loss of CD28 is associated with immunosuppression, mechanistic studies would benefit from establishing a positive marker for the $CD3^+CD8^+CD28^-$ T reg cells. This section investigates previously proposed markers for $CD3^+CD8^+CD28^-$ and directly compares these to the marker Killer Cell Lectin-like receptor subfamily G member 1 (KLRG1).

3.16.1 CD57

The CD57 antigen is a 100-115 kD terminally sulphated carbohydrate epitope that was originally found on human natural killer (NK) cells[236]. CD57 acquisition is seen on $CD16^+CD56^-$ cytotoxic and inflammatory NK cells, signalling the differentiation. Notably, it is not observed on regulatory $CD16^+CD56^+$ NK cells, even during chronic infections[237].

More recently CD57 has also been found to be expressed on $CD4^+$ and $CD8^+$ lymphocytes after repeated antigen stimulation[238]. Furthermore, the proportion of $CD8^+CD57^+$ T cells was significantly higher in RA patients compared with age-matched controls[239]. CD57 is now used to identify terminally differentiated 'senescent' cells with reduced proliferative capacity and altered functional properties. Although $CD8^+CD57^+$ T cells can be defined as senescent, they are not functionally exhausted. Senescence and exhaustion mechanisms of a cell are independently regulated. Exhaustion is characterised by the progressive loss of T cell function due to a chronically high antigen load[240]. IL-2 production and proliferative capacity are the first functions to be lost by exhausted T cells, followed later by loss of TNF-α production and cytotoxic activity.

$CD57^+$ T cells are presumed to lack proliferative capacity and are known to increase in frequency with chronic immune activation as well as during normal aging[241,242,243,244]. In $CD8^+$ T lymphocytes, CD57 identifies a reduced proliferative response to T cell receptor triggering in the presence of IL-2, IL-7 or IL-15, as well as increased susceptibility to antigen-induced apoptosis[245].

Senescent, late-differentiated $CD57^+$ T cells are generally $CD45Ro^-$ and have a reduced expression of the co-stimulatory molecules CD27/$CD28^-$ and chemokine receptor CCR7. They gain the re-expression of CD45Ra (**Figure 1-4**).

3.16.2 CD45Ra

CD45, a protein tyrosine phosphatase regulating Src-family kinases, is expressed on all hematopoietic cells. CD45Ra is expressed on naïve T cells and effector cells in both $CD4^+$ and $CD8^+$. Naïve T cells express CD45Ra along with CCR7 and CD62L; CD45Ra is expressed on effector T cells which lack CCR7 and CD62L expression. A 2006 paper demonstrated that CD45Ra expression indicates time since the latest stage of antigenic stimulation[246]. $CD45Ra^+CCR7^-CD8^+$ T cells are resting memory cells that on stimulation, initially lose their CD45Ra and gain CCR7. If the $CD45Ra^-CCR7^+CD8^+$ T cells are not stimulated further, lose the CCR7 and regain CD45Ra.

Effector $CD8^+CD28^-$ ($CD8^+CD57^+$) T cells tend to express CD45Ra rather than CD45Ro[247,248]. T cells are effector cells that re-express CD45Ra, and are late stage memory T cells[249,250,251]. This T cell subset of effector T cells that re-express the naive-cell marker CD45Ra (TEMRA) cells, classified as $CD45Ra^+CCR7^-CD95^+CD28^-$ can rapidly express cytokines and cytotoxic molecules such as granzymes and perforin after antigen stimulation[252].

3.16.3 CD56

CD56, also known as neural cell adhesion molecule (NCAM), is mostly associated with natural killer (NK) cells[253]. However, CD56 has also been detected on dendritic cells as well as other lymphoid cells, including gamma delta (γδ) T cells and activated $CD8^+$ T cells.

$CD8^+CD56^+$ T cells represent 1–11% of the peripheral T cell pool. One study clearly indicated that $CD56^+$ cells, which are generally $CD45Ra^+$, better demarcate a subset with the properties of effector lymphocytes[254]. These $CD8^+CD56^+$ cells recognise HLA

class I molecules and as well as having an effector memory phenotype, have high tumour cytotoxic capacity and cytokine production[255]. Both CMV infection and ageing are also associated with increased expression of CD56 on T cells in humans[256].

Senescent terminally differentiated $CD8^+CD45Ra^+$ T (TEMRA) effector T cells with complete competence, develop features of NK cells. This comprises of the up-regulation of NK cell receptors, including CD56. TEMRA cells use their acquired NK cell machinery to maintain rapid effector functions throughout life, tackling the increased burden of tumours and infections in the elderly[257].

In 2005, Davila et al showed that $CD8^+CD28^-CD56^+$ cells could suppress memory T cell responses and were therefore anti-inflammatory[258]. Ceeraz et al (2013) looked at CD56 as a marker of $CD8^+CD28^-$ suppressor function **(Figure 3-24**)[197]. $CD56^+$ T cells were shown to tolerise APCs, stopping the priming of $CD4^+$ T cells and suppressing the memory responses, a display of strong anti-inflammatory activity. Down-regulation of the co-stimulatory ligands CD80 and CD86 on synovial fibroblasts was identified as one mechanism of immunosuppression.

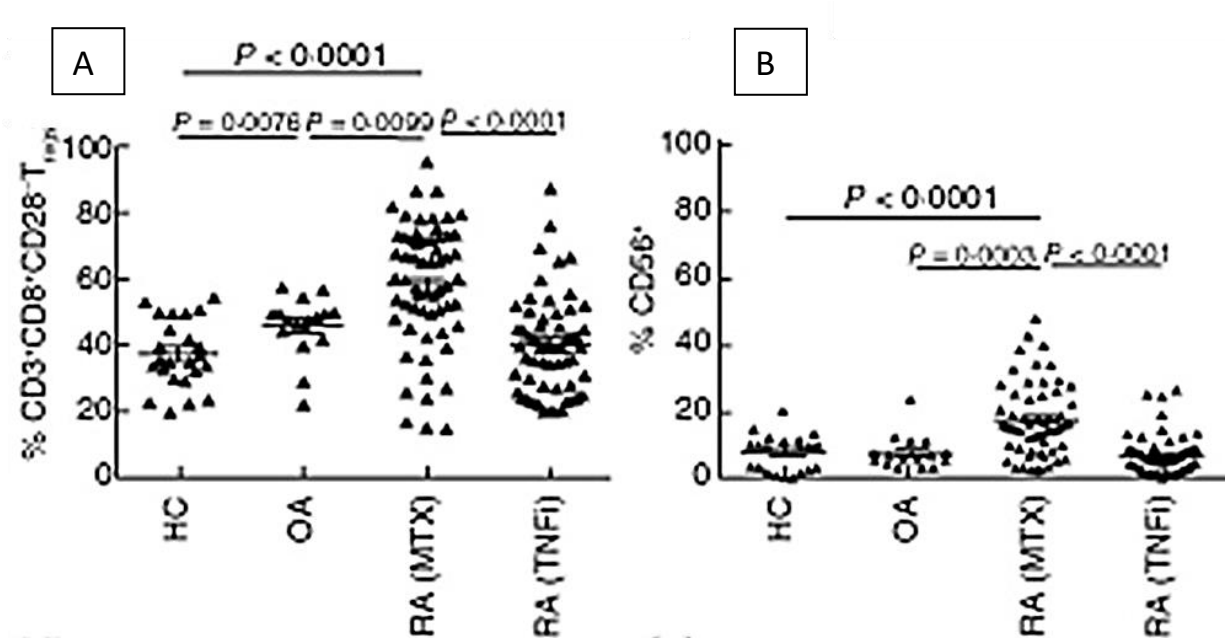


Figure 3-24: Percentage of $CD3^+CD8^+CD28^-$ T cells in RA patients on methotrexate are raised in comparison to healthy controls (HC). $CD3^+CD8^+CD28^-$ cell percentage is significantly raised in RA patients on methotrexate in comparison to Osteoarthritis (OA) and RA patients on TNF, as well as healthy controls (A). CD56 (B) is also significantly higher in RA patients in comparison to the same groups. $CD28^-$ and $CD56^+$ are both markers of senescent effector $CD8^+$ T cells, which indicate repeated antigen stimulation. Image from Ceeraz et al, 2013[197].

3.16.4 Killer Cell Lectin-like receptor subfamily G member 1 (KLRG1)

KLRG1 is an inhibitory transmembrane protein preferentially expressed on T cells. Increased KLRG1 expression signifies persistent antigen stimulation, gives rise to highly differentiated $CD8^{+}CD28^{-}KLRG1^{+}$ T cells and could distinguish the differentiation stage of the cell[259]. As $CD8^{+}$ T cells lose their $CD28^{-}$, it appears they gain CD57 as well as KLRG1.

KLRG1 is a transmembrane protein preferentially expressed on T cells and CD56 NK cells. KLRG1 is a cadherin receptor: recognising E-, N- and R- Cadherin. E-cadherin is expressed on epithelial cells, peripheral blood and Langerhans cells whilst N- and R-cadherin are expressed by the nervous system. Cadherins are a class of type 1 transmembrane Ca2+ dependent glycoprotein with a role in cell adhesion.

KLRG1 acts mainly through E–Cadherin. Increased expression of E-Cadherin observed a negative regulatory effect on fibroblast proliferation. Dermal fibroblast proliferation is inhibited by E-Cadherin expression[260]. The use of KLRG1-transgenic mice showed that antigen-stimulated T cells in the presence of KLRG1's ligand, E-Cadherin, inhibited the proliferative capacity of $CD8^{+}$ T cells[261]. High levels of E-Cadherin were needed to show a modest degree of inhibition.

Blocking KLRG1 signaling during TCR activation (antibodies against E-Cadherin), enhances proliferative activity that was linked directly to an Akt (a key signaling kinase). Akt-mediated synthesis of cyclin D and E was increased and there was a decrease in the cyclin inhibitor p27[262]. KLRG1 mediated inhibition of NK cell function revealed that KLRG1/ligand interactions inhibit the cytolytic activity of polyclonal human NK cells by interfering with both degranulation and IFNγ release.

KLRG1 acts through SHIP-1 and SHP-2 to degrade PIP3 to PIP2, preventing phosphorylation of Akt (**Figure 3-25**)[263]. KLRG1 also possesses an immune receptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain, suggesting that it may play a functional role in the immune system[264]. Blocking KLRG1 causes the

conversion of PIP2 to PIP3 and restores the Akt signalling pathway and therefore proliferation in otherwise dysfunctional cells[262].

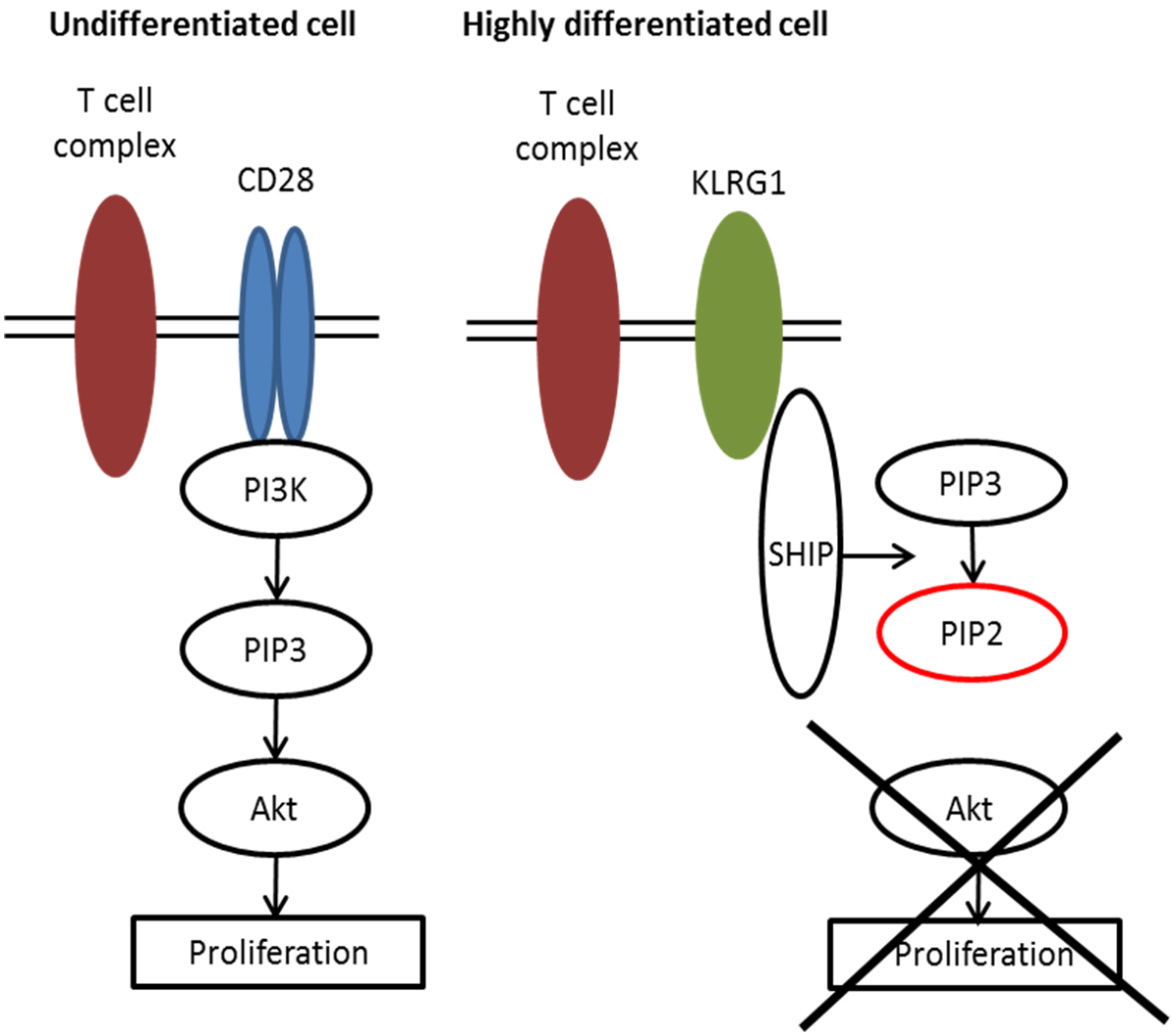


Figure 3-25: Changes in Akt signalling with differentiation of $CD3^+CD8^+$ T cells and co-inhibitory receptor KLRG1. Undifferentiated cells signal through $CD28^-$, initiating the Akt signalling pathway. This induces a broad range of cell functions including proliferation. Highly differentiated cells lose their $CD28^-$ and gain the inhibitory molecule KLRG1. KLRG1 acts through SHIP-1 and SHP-2 to degrade PIP3 to PIP2 and prevents the Akt phosphorylation. (PI3K is lipid kinase involved in cellular signal transduction systems. SHIP-1 and SHP-2 are phosphatases that regulate cell responses in lymphocytes and myeloid cells. PIP is a membrane phospholipid.)

3.16.5 KLRG1 as a signal of differentiation or ageing

KLRG1 signifies persistent antigen stimulation and short telomeres which may lead to decreased responsiveness[265]. Expression of KLRG1 rises dramatically with age, with greater than 90% expression of KLRG1 being seen on $CD8^+$ T cells in individuals over 65 years of age[266,267,262]. KLRG1 is higher with age as well as differentiation, the highest percentage of expression on memory and highly differentiated end stage cells[268,269]. Degree of immune response decreases with increasing age, so there is a theory that inhibiting KLRG1 may enhance vaccine efficacy.

However, age aside, KLRG1 positivity is highest on $CD28^-$ cells in healthy individuals. It is unclear whether CD28 loss is due to cell senescence or in response to increased stimulation. High expression of KLRG1 on $CD8^+CD28^-$ T cells may be partially responsible for the cells' apparently decreased proliferative response to stimulation. This occurs in both the young (<35 years) and old (>65 years[262]. Highly differentiated T cells expressing KLRG1, decrease the efficiency of the immune system.

Persistent antigen stimulation gives rise to highly differentiated $CD8^+CD28^-KLRG1^+$ T cells with the shortest telomeres and a decline in the immune system responsiveness[270,271]. These changes imply immune senescence and are linked with a higher frequency of infections and incidence of malignancy in older adults[272]. Over 92% of $CD8^+$ cells specific for latent Epstein Barr Virus (EBV) or Cytomegalovirus (CMV) expressed KLRG1. Lower percentages of KLRG1 expression were observed for Influenza and HIV. As well as being highly expressed on cells infected with EBV and CMV, KLRG1 expression is up-regulated on healthy NK cells chronically infected with Hepatitis C Virus[273]. This could indicate that KLRG1 has immunosuppressive properties that enable cells to persistently exist, despite infection.

3.17 Establishing a positive marker for CD28 negativity: comparison of KLRG1, CD57, CD56 and CD45Ra

Given that CD57, CD45Ra and CD56 are existing markers of CD28 negativity, these were used as a comparison against the marker KLRG1. CD57 appears to be the most established and sensitive marker of CD28 negativity in $CD8^+$ cells at present[274,275].

Of the $CD3^+CD8^+CD28^-$ cells in controls and pooled RA patients, the percentage of KLRG1, CD57 and CD45Ra were similar, with mean percentages ranging between 74-84%. The mean percentage of $CD3^+CD8^+CD28^-CD56^+$ cells was lower (**Figure 3-26A**, 22%, $p<0.05$).

This is illustrated by the FACS plot analysis of flow cytometry in **Figure 3-26B.** CD57, KLRG1 and CD45Ra show a clear shift and greater proportion of positivity in $CD3^+CD8^+CD28^-$ cells. The proportion of CD56 positivity was less than the other three markers. This data suggests that CD56 is not as strong a marker of CD28 negative cells as CD57, CD45Ra and KLRG1.

Although CD57, CD45Ra and KLRG1 were good markers of CD28 negativity, it is important to look at whether they are also positive on other cells, resulting in non-specific detection and analysis of non-target cells. When looking at $CD3^+CD8^+CD28^+$ cells, a large proportion of them were also CD45Ra positive (**Figure 3-26C,** 66%). The mean averages of $CD3^+CD8^+CD28^+$ cells that were positive for the other markers were lower: KLRG1 (23%), CD57 (8%) and CD56 (3%) (not shown below). This suggests that KLRG1 and CD57 are more sensitive markers of CD28 negative cells than CD45Ra. Overall, KLRG1 and CD57 were the best markers for the positive selection of CD28 negative cells and the exclusion of CD28 positive cells.

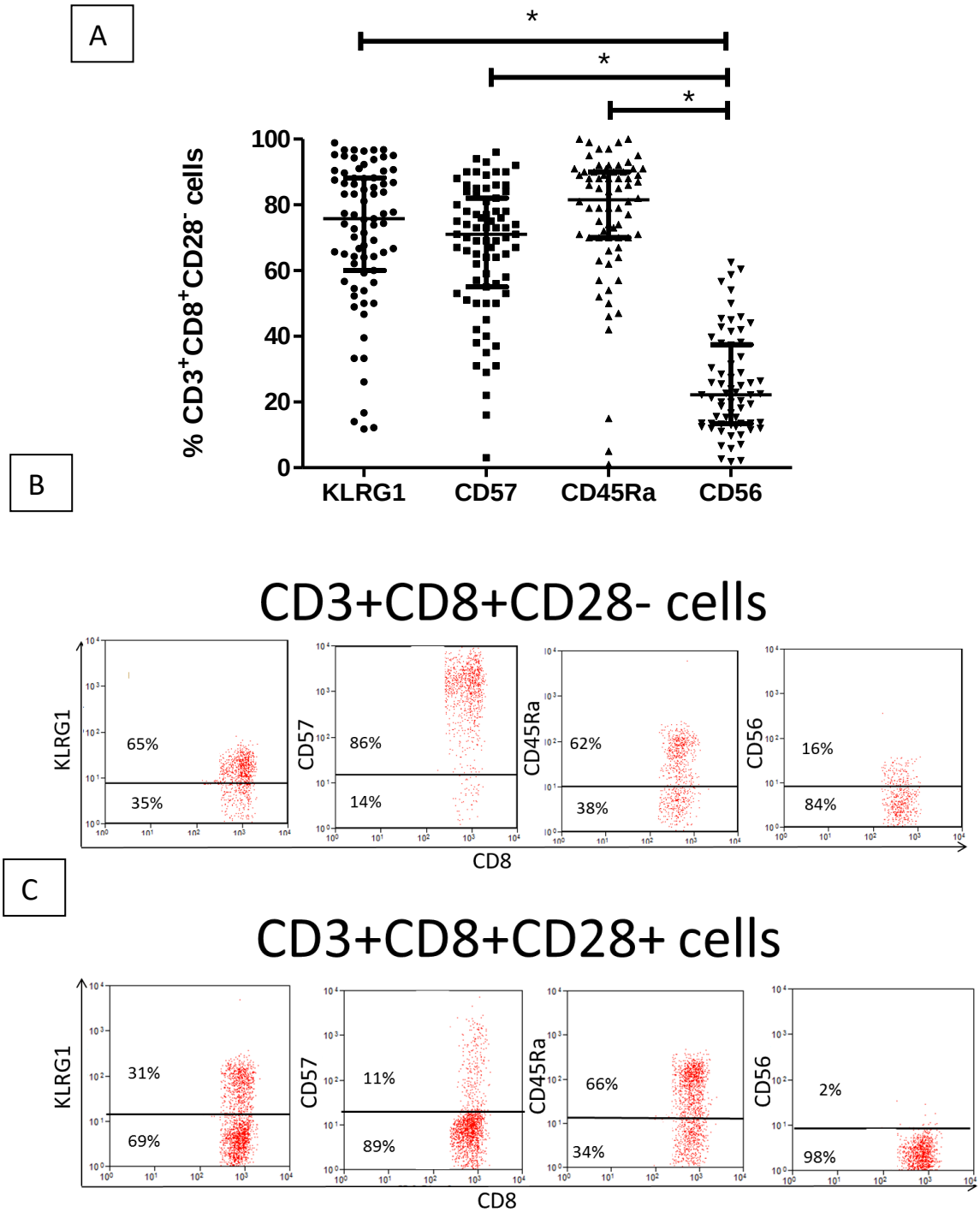


Figure 3-26: KLRG1, CD57 and CD45Ra are highly expressed on $CD3^+CD8^+CD28^-$ cells. KLRG1, CD57 and CD45Ra were expressed more than CD56 ($p<0.05$) on $CD3^+CD8^+CD28^-$ cells (A). Summit plots of $CD3^+CD8^+CD28^-$ cells show higher percentages of $CD3^+CD8^+CD28^-$ cells express KLRG1, CD57 or CD45Ra than CD56 (B). $CD3^+CD8^+CD28^+$ cells express less KLRG1, CD57, CD45Ra and CD56 (C). KLRG1 (N=75, Median = 75.8), CD57 (N=75, Median = 70.7), CD45Ra (N=70, Median= 81.4), CD56

(N=66, Median = 22.2). Median and interquartile range shown, *p<0.05 using Kruskal-Wallis ANOVA test.

3.18 $CD3^+CD8^+CD28^-$ cells are positive for both KLRG1 and CD57

Although CD57 and KLRG1 appear to be the most robust markers of $CD3^+CD8^+CD28^-$ T cells, analysis was completed to establish which of these markers were the most effective in detecting the cells of interest, and whether these markers were selecting the same, or potentially different cells. Co-expression of CD57, CD45Ra, CD56 by $CD3^+CD8^+CD28^-$ cells with KLRG1 was therefore assessed (**Figure 3-27**).

CD57 and KLRG1 identified the most $CD3^+CD8^+CD28^-$ cells (mean=56, SD=22). Comparison with CD45Ra showed the majority of $CD3^+CD8^+CD28^-$ cells were both positive for KLRG1 as well as CD45Ra (mean=56, SD=22). Although a similar proportion of $CD3^+CD8^+CD28^-$ cells were positive for both KLRG1 and CD57 as KLRG1 and CD45RA, the proportion of $CD3^+CD8^+CD28^-$ cells that were positive for KLRG1 as well as CD45Ra was less distinctive on the FACS plots than CD57 or KLRG1 (**Figure 3-27**). When compared with CD56, a smaller proportion of $CD3^+CD8^+CD28^-$ cells were both positive for KLRG1 as well as CD56 (mean=19, SD=17). The difference between CD57 and CD45Ra against CD56 was statistically significant (**Figure 3-28A**).

Analysis of $CD3^+CD8^+CD28^+$ cells, showed that CD45Ra has the highest mean percentage of $KLRG1^+$ cells (24%) in comparison to CD57 (13%) and CD56 (4%) (**Figure 3-27B**). This showed that CD45Ra was less discriminatory than CD57 and CD56 because it is positive on CD28 positive cells as well as CD28 negative cells. The percentage of CD57, CD45Ra and CD56 positivity is higher on $CD3^+CD8^+CD28^-KLRG1^+$ cells than $CD3^+CD8^+CD28^+KLRG1^+$ cells (**Figure 3-28B**).

Further evaluation of whether KLRG1 or CD57 would be best if used as a single marker of $CD3^+CD8^+CD28^-$ cells only, was completed. KLRG1 and CD57 were selected because CD45Ra and CD56 did not discriminate the $CD3^+CD8^+CD28^-$ cells to the same extent. In this regard, KLRG1 worked marginally better than CD57 by detecting 71% of $CD3^+CD8^+CD28^-$ cells whereas CD57 effectively marked 66% of these cells. While 56% of the $CD3^+CD8^+CD28^-$ cells were positive for both CD57 and KLRG1 (**Figure 3-29**).

This suggests that KLRG1 is more highly expressed by $CD3^+CD8^+CD28^-$ T cells than CD57 in RA.

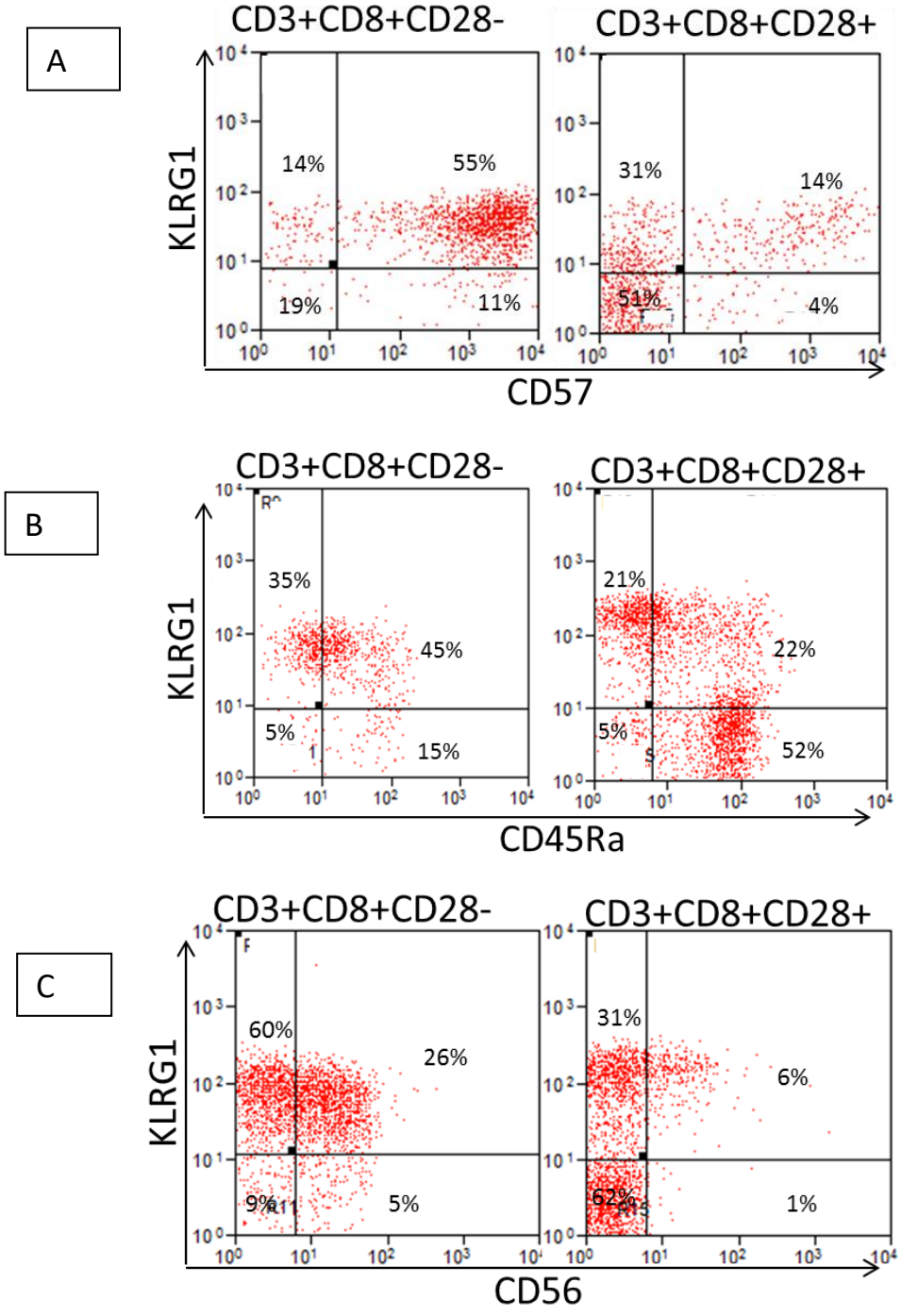
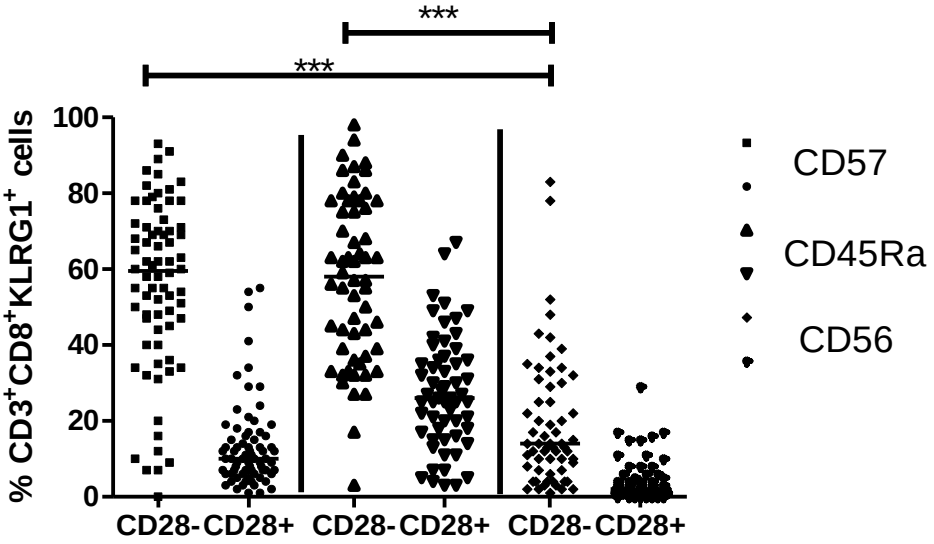


Figure 3-27: The majority of $CD3^+CD8^+CD28^-CD57^+$ cells are also $KLRG1^+$. Summit plot (A) displays the majority of $CD3^+CD8^+CD28^-$ cells are positive for both KLRG1 and CD57 (55%) in Controls and RA patients (n=72). In plot (B) 45% of $CD45Ra^+$ cells are $KLRG1^+$ (n=58). The overlap is the least for $CD56^+$ and $KLRG1^+$ cells in plot (C) (n=66). KLRG1 also marks a proportion of $CD3^+CD8^+CD28^-$ cells

A



B

	$CD3^+CD8^+CD28^-$ $KLRG1^+$	$CD3^+CD8^+CD28^+$ $KLRG1^+$
$CD57^+$	56	13
CD45Ra+	57	24
CD56+	19	4

Figure 3-28: The majority of $CD3^+CD8^+CD28^-KLRG1^+$ cells are $CD57^+$ and $CD45Ra^+$ but not $CD56^+$. The mean percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells is higher for $CD57^+$, $CD45Ra^+$ and $CD56^+$ than $CD3^+CD8^+CD28^+KLRG1^+$ cells (B). Graph (A) represents the median, ***p<0.001 using Kruskal-Wallis ANOVA test. CD57 (n=72), CD45Ra (n=58) and CD56 (n=66).

Surface Marker	KLRG1^{+}57^{-}	KLRG1^{+}57^{+}	KLRG1^{-}57^{-}	KLRG1^{-}57^{+}
Percentage	15	56	19	10

Table 3-6:KLRG1 and CD57 in 56% of CD3^{+}CD8^{+}CD28^{-} cells. The mean percentage of CD3^{+}CD8^{+}CD28^{-} cells is shown (n=72).

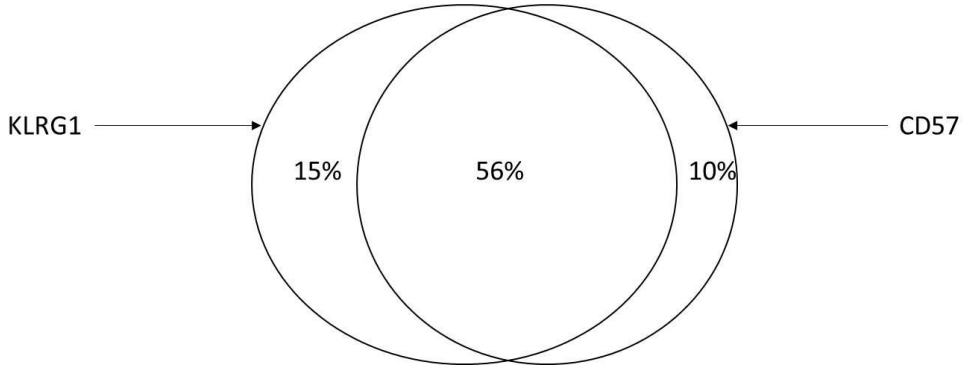


Figure 3-29: KLRG1 is a better single marker for CD3^{+}CD8^{+}CD28^{-} cells than CD57. More CD3^{+}CD8^{+}CD28^{-} cells are KLRG1^{+}CD57^{-} (15%) than are CD57^{+}KLRG1^{-} (10%).71% of the CD3^{+}CD8^{+}CD28^{-} cell pool was KLRG1^{+} (56%+15%), in comparison to 66% being CD57^{+} (56%+10%)(n=72).

3.19 Discussion

In establishing the methodology for this results chapter, previous studies have confirmed that $CD3^+CD8^+CD28^-$ cells are higher in RA, but also provided novel evidence that this is also the case in Early RA[197]. $CD3^+CD8^+CD28^-$ cells also correlate with disease duration in Early but not Established RA, thereby suggesting a possible role early in the disease process. The expansion of this cell subset could be driving the immune disturbance in RA. Alternatively, this could be due to the autoimmune process in early disease. This is supported by the association of $CD3^+CD8^+CD28^-$ cells with RF, CRP and ESR. Multiple studies have linked RF and ACPA seropositivity and inflammatory changes in the pre-RA stages[276,277]. Thus, expansion of $CD3^+CD8^+CD28^-$ cells might occur prior to clinical disease.

The two groups of patients were on different treatments at the time of their baseline assessments. This is highlighted in **Table 3-2** and **Table 3-3**. Particular attention needs to be brought to the six patients in the Established RA group that were on biologic DMARDs. These patients would normally be expected affect the proportions of different T cells, however **Figure 3-3** highlights that these patients were equally distributed throughout the data.

Consistently there are three poor prognostic markers for RA: high disease activity, serology (RF and/or ACPA) and early erosions[278]. However, DAS28 did not correlate with $CD3^+CD8^+CD28^-$ cells. DAS28 is a measure of inflammation at a point in time used regularly in clinical practice. It is not however, reflective of the immune process.

Previous studies in non-RA individuals found increased $CD3^+CD8^+CD28^-$ cells with age[279]. The lack of association in patients with the RA disease process may possibly be due to expansion of $CD3^+CD8^+CD28^-$ cells being driven by immune activation in these patients rather than repeated infections.

A 2010 systematic review looked at eleven studies regarding the effect of sex on remission of disease activity. Male sex was shown to be an independent predictor of

remission in five studies[280]. Hyrich et al (2006) found that women were significantly less likely to achieve remission compared with men[281]. However, a systematic review found that six studies concluded that sex was not an independent predictor of remission when adjusted for other variables. A novel finding in this chapter was that there was a higher percentage of $CD3^{+}CD8^{+}CD28^{-}$ cells in male RA patients. The percentage of male patients was 36% in the pooled RA. Despite being representative of the RA population, the number of patients were small, so this result will need to be confirmed by studies of larger sample size. This finding could be a type I statistical error; results that incorrectly reject the null hypothesis.

There was no correlation between $CD3^{+}CD8^{+}CD28^{-}$ cells and Early RA responders. However, the numbers of follow-up patients at six months was small (n=22), therefore there was insufficient power to establish a firm conclusion. Furthermore the cut off of DAS28 improvement ≥1.2 could have been arbitrary, and therefore there may have been underestimating the number of patients that improved at six months (but less than a DAS28 score of 1.2) The proportion of responders (32%) was lower than the average population. One study found 64% patients still taking methotrexate with significant improvement at 5 years[282]. Looking more closely at the Early RA patients investigated, 12/22 (55%) patients were on DMARDs and 8/22 (36%) were on steroids. Bearing in mind the Treat to Target guidelines of early and effective treatment for RA, the proportions of Early RA patients on active treatment were low[283]. Reasons for this could include patient choice, contraindications to medications and that patients were recruited from a range of consultants. Furthermore, the majority of patients at the time of the baseline visit did not meet the criteria of RA, therefore only warranting corticosteroids as treatment at the time.

There was no correlation between $CD3^{+}CD8^{+}CD28^{-}$ cells and the cytokine profile they produce. This could also be attributed to the diversity of production of cytokines these cells produce or possibly to the lower number of subjects included in the analysis[284]. Another consideration could be the cytokine stimulant. PMA is considered a good stimulant of IFN and IL-4 but not IL-10 or IL-6. Instead, stimulating with lipopolysaccharide may have been more effective[285].

KLRG1 is marker of a subset of $CD3^+CD8^+CD28^-$ cells. There was a majority of overlap with cells signposted with the $CD57^+$ surface marker. The high levels of double positivity of KLRG1 and CD57 mean that it is worth considering whether the double positive with CD57 could be used to identify $CD3^+CD8^+CD28^-$ cells in the future.

3.20 Conclusions

- The percentage of $CD3^+CD8^+CD28^-$ cells was higher and also associated with disease duration in Early RA.
- The percentage of $CD3^+CD8^+CD28^-$ cells was higher in male patients with RA.
- The percentage of $CD3^+CD8^+CD28^-$ cells in RA patients was associated with RF positivity, CRP and ESR.
- KLRG1 is expressed by a high percentage of $CD3^+CD8^+CD28^-$ cells.

4 Chapter 4: The role of $CD3^+CD8^+CD28^-KLRG1^+$ cells in RA

4.1 Introduction: KLRG1 in arthritis

KLRG1 may be associated with the function observed in highly differentiated T cells. A 2013 study looking at $KLRG1^+$ T cells in synovitis has shown the KLRG1 levels are higher in RA and spondyloarthropathy (SpA) patients in comparison to crystal induced arthritides. This suggests that $KLRG1^+$ T cells may have a significant role in the pathogenesis of autoimmune arthritis[286]. In particular, $KLRG1^+$ T cells were found in higher numbers in the synovial fluid of arthritic joints, than peripheral blood of SpA and RA patients.

The T cell compartment in RA has been found to have an immunosenescent phenotype[287,288]. However, $KLRG1^+$ cells are not exhausted[289]. For example, they are capable of demonstrating cytokine responses[290,291,]. In this regard, Melis et al (2013) found that $CD8^+KLRG1^+$ T cells were more functionally active than $KLRG1^-$ T cells and could secrete TNF-α after stimulation with CMV in vitro[286].

Another role observed is that KLRG1 can also inhibit NK and CD8 T cell function[292,293]. Membrane-bound E-cadherin is the main ligand for the KLRG1 receptor. Recently, it has been also been shown that the soluble E-cadherin (sE-cadherin) ligand can influence inhibitory KLRG1 signalling[294]. SE-cadherin levels were higher in synovial fluid versus peripheral blood of RA and SpA patients[286]. This would indicate that KLRG1 in inflammatory arthritis is inhibiting cell functions.

Functional studies have shown that the inhibitory function of KLRG1 was only observed when both the T cell receptor and KLRG1 signals were delivered from the same site[295]. An anti-KLRG1 monoclonal antibody that blocks the inhibitory effects of KLRG1 was found to increase cytolytic activity of $CD8^+$ cells[292].

Further implicating KLRG1 in inflammatory arthritis is the finding that membrane-bound E-cadherin is expressed on synovial tissue and leukocytes with the highest levels found on myeloid dendritic cells[296]. Inflammatory matrix metalloproteinases (MMPs) cleave membrane-bound E-cadherin. It is likely that sE-Cadherin is made locally in the joint, as these MMPs are expressed in highly in arthritic joints.

KLRG1 is likely to be key to the autoimmune response in RA because it contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain[297]. ITIM is a part of the immune cycle and can provide negative or positive regulatory capacity for the immune system.

4.1.1 Hypothesis

Chapter 3 found that KLRG1 is highly expressed by $CD3^{+}CD8^{+}CD28^{-}$ cells. As $CD3^{+}CD8^{+}CD28^{-}$ cells are raised in RA, this suggests that KLRG1 could also be raised in RA. This MD thesis hypothesises that $CD3^{+}CD8^{+}CD28^{-}$ cells, signposted by the cell surface marker KLRG1, could be associated with disease phenotype, severity and response to treatment in RA.

4.1.2 Aims

The aim of Chapter 4 was to assess association of $CD3^{+}CD8^{+}CD28^{-}KLRG1^{+}$ cells in Early and Established Rheumatoid Arthritis.

- Assess prevalence of $CD3^{+}CD8^{+}CD28^{-}KLRG1^{+}$ cells in the peripheral blood compartment in Early and Established Rheumatoid Arthritis patients and assess correlation with clinical and serological markers.
- Assess activity of $CD3^{+}CD8^{+}CD28^{-}KLRG1^{+}$ cells in the peripheral blood compartment in Early and Established Rheumatoid Arthritis by looking at cytokine production.

4.2 Evaluation of $CD3^+CD8^+CD28^-KLRG1^+$ cells in RA patients and Healthy Controls

Chapter 3 showed that $CD3^+CD8^+CD28^-$ cells were raised in RA and that KLRG1 was highly expressed on these cells. Therefore, comparison of $CD3^+CD8^+CD28^-KLRG1^+$ cells between healthy controls and RA was made.

The trend showed that RA patients (comprising of both Early and Established RA) had a higher percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells than the Control group (**Figure 4-1A**, p=0.052). The absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ T cells was similar between controls and Pooled RA. Melis et al (2013) did not find a difference between levels of KLRG1 in the peripheral blood of inflammatory arthritis and healthy controls[286].

Subdivision into Early and Established RA revealed no differences between the groups of percentages or absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells (**Figure 4-2**).

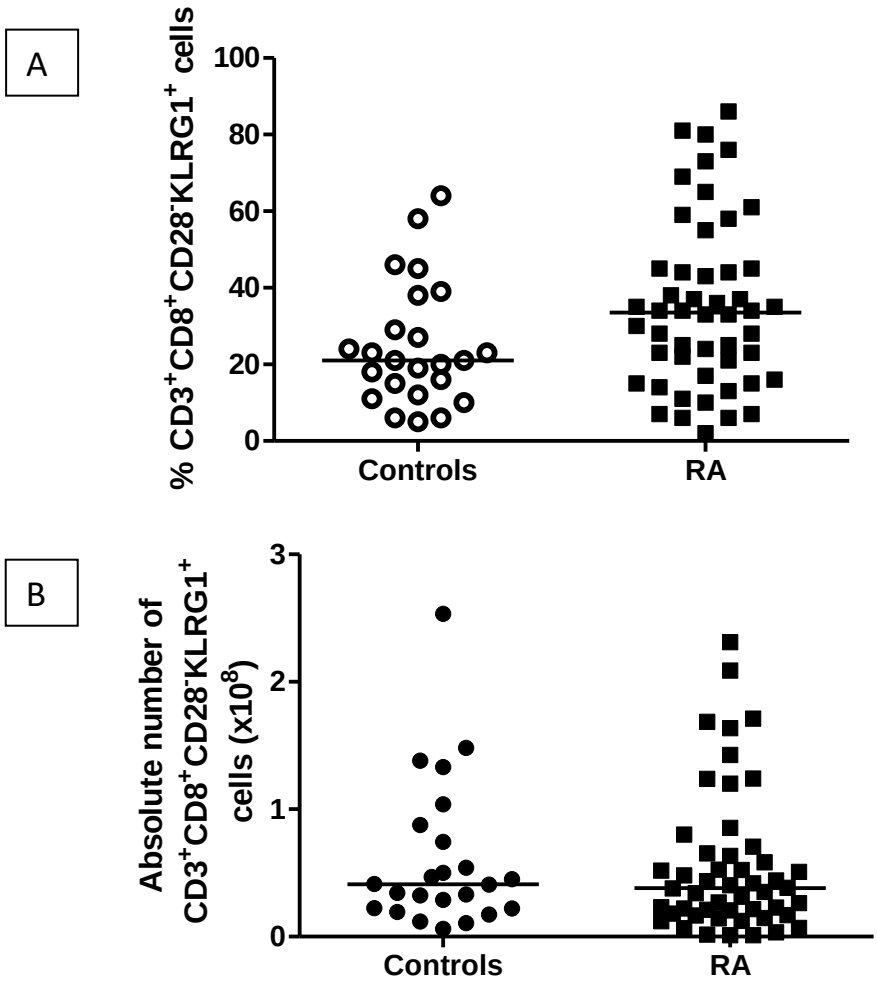


Figure 4-1: The percentage of $CD3^+CD8^+CD28^-KLRG1^+$ T Cells is comparable in pooled RA patients compared to Controls. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-KLRG1^+$ T Cells shown with median. Statistical analysis performed using Mann-Witney U Test. Controls (n=24), Pooled RA (n=50)

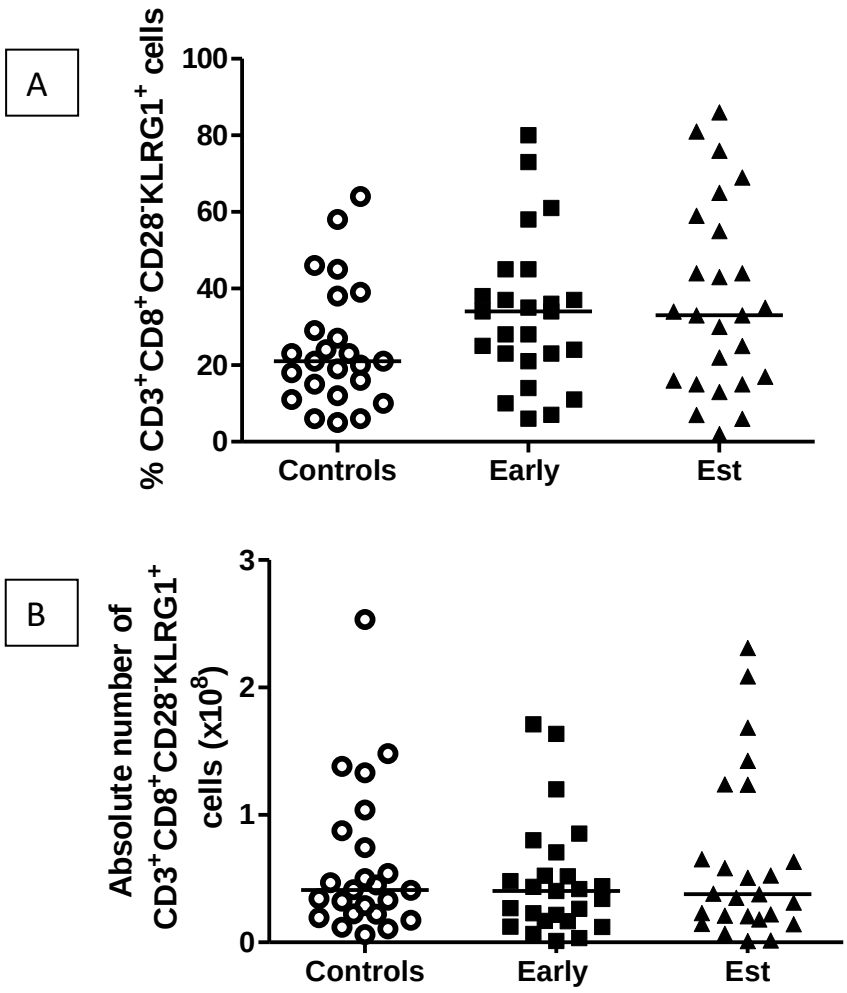


Figure 4-2: Percentage and absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells are similar in Early and Established RA compared to Controls. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-KLRG1^+$ T Cells shown with median, the statistical test used was Kruskal-Wallis ANOVA Test. Controls (n=24), Early RA (n=25), Established RA (n=25).

4.3 The correlation between $CD3^+CD8^+CD28^-KLRG1^+$ cells and Age

Multiple studies have found an association between KLRG1 and age[298]. However, further investigation has revealed KLRG1 as a marker of T cell senescence following persistent antigen stimulation[296]. KLRG1 has not previously been investigated in the context of age and RA. There was no correlation between the percentage or absolute number of $CD3^+CD8^+CD28^-$ $KLRG1^+$ cells and age in pooled RA patients (**Figure 4-3A & 4-3B**).

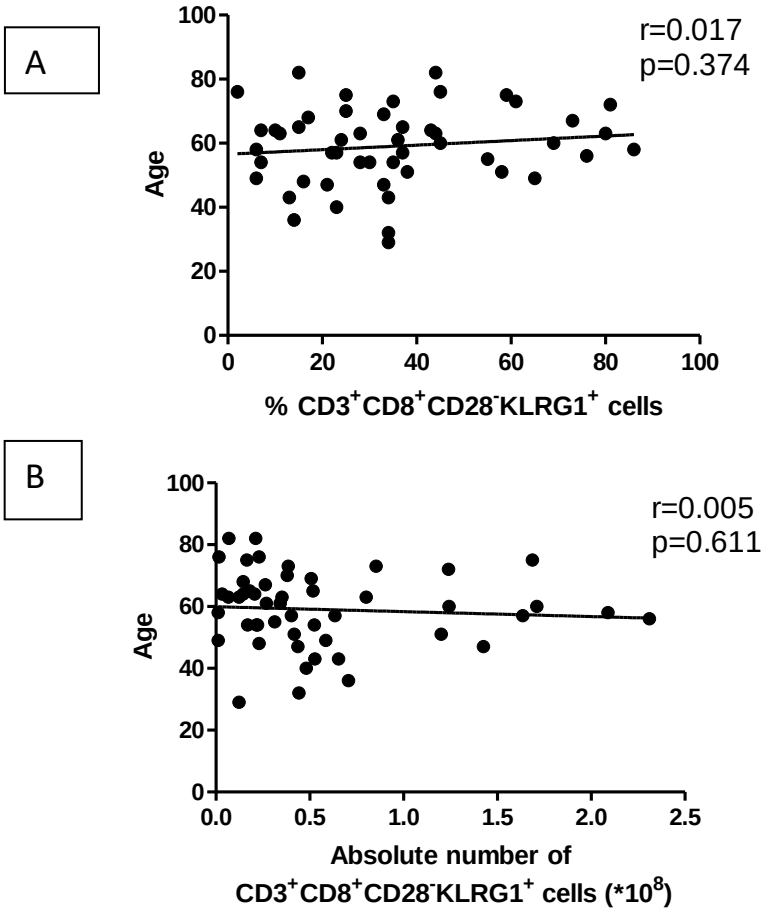


Figure 4-3: Percentage and Absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells do not correlate with age in pooled RA patients. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-KLRG1^+$ T Cells. Correlation was determined using non-parametric Spearman's rank analysis. Pooled RA (n=50).

4.4 The correlation between $CD3^+CD8^+CD28^-KLRG1^+$ cells and Sex

In Chapter 3 there was an observation whereby $CD3^+CD8^+CD28^-$ cells were raised in male RA patients in comparison to female patients. Further investigation into whether this difference was echoed by $CD3^+CD8^+CD28^-KLRG1^+$ cells also showed that pooled RA patients had a higher percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells in the male RA patients compared to females RA patients ($p<0.05$) (**Figure 4-4A**). Sex with respect to $CD3^+CD8^+CD28^-KLRG1^+$ cells in RA has not previously been investigated.

This trend was not echoed in the control group where percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells was similar between male and female patients. When looking at sex in healthy controls there was no difference in the absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells (**Figure 4-4B**). 68% of the Controls were female and of the 50 pooled RA, 66% were female. There was no difference between the $CD3^+CD8^+CD28^-KLRG1^-$ cells and sex (data not shown).

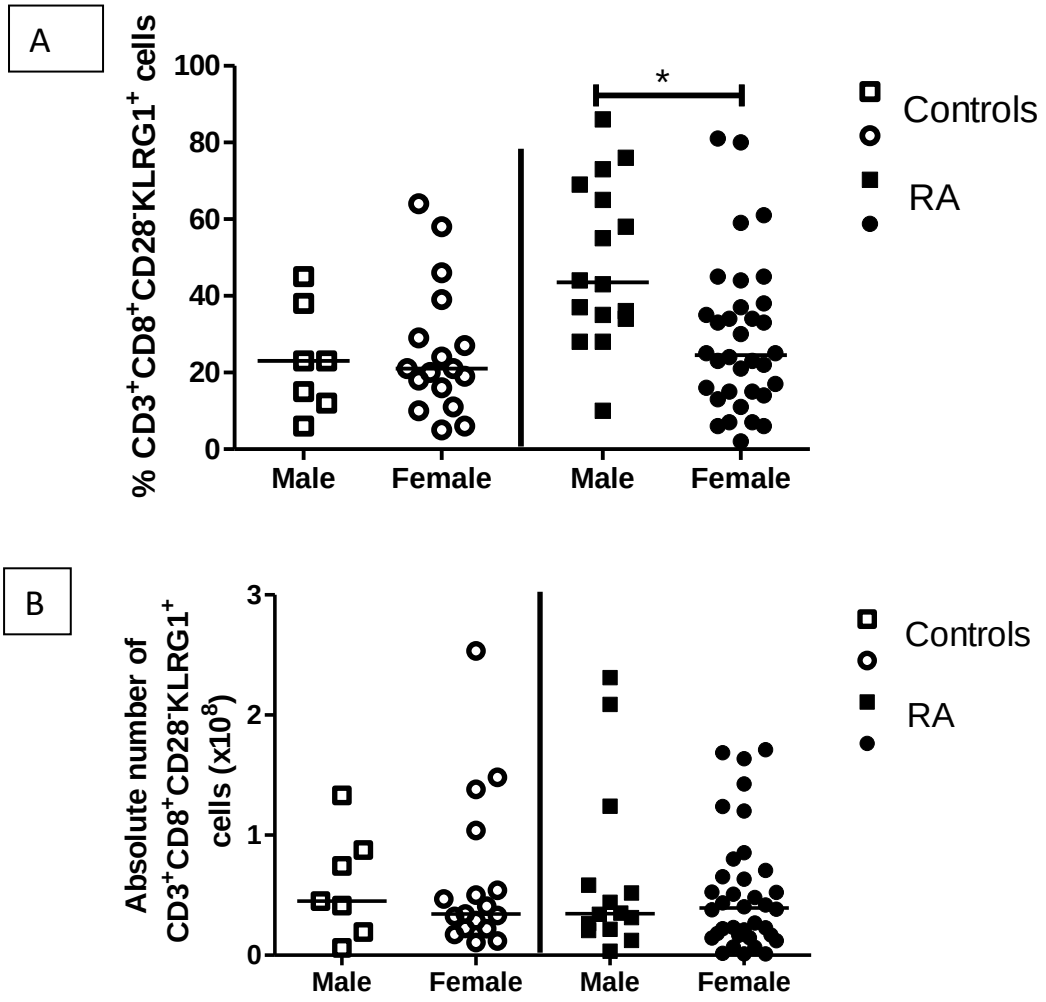


Figure 4-4: The percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells is higher in males than females with RA. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-KLRG1^+$ T Cells. Graphs represent median, *p<0.05 using Kruskal-Wallis ANOVA statistical test. Controls (n=24, Male=7, Female=17), Pooled RA (n=50, Men=16, Women=34).

4.5 Serological status and $CD3^+CD8^+CD28^-KLRG1^+$ T cells: RF positivity

$CD3^+CD8^+CD28^-$ cells in Chapter 3 were higher in RF positive Established RA patients compared to RF negative. This finding was reaffirmed because the percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells was also higher in RF positive Established RA patients compared to RF negative (p=0.0062) **(Figure 4-5C)**. This has not previously been investigated.

This trend was reflected in the absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells but the differences were not significant. In Early RA patients, a trend suggesting an increased percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells was also observed in RF negative patients (**Figure 4-5B**). However, this trend was also not statistically significant. A larger patient cohort would confirm whether this was indeed a reliable finding. There was no difference found between the groups of RF positive and negative in pooled RA (**Figure 4-5A**)

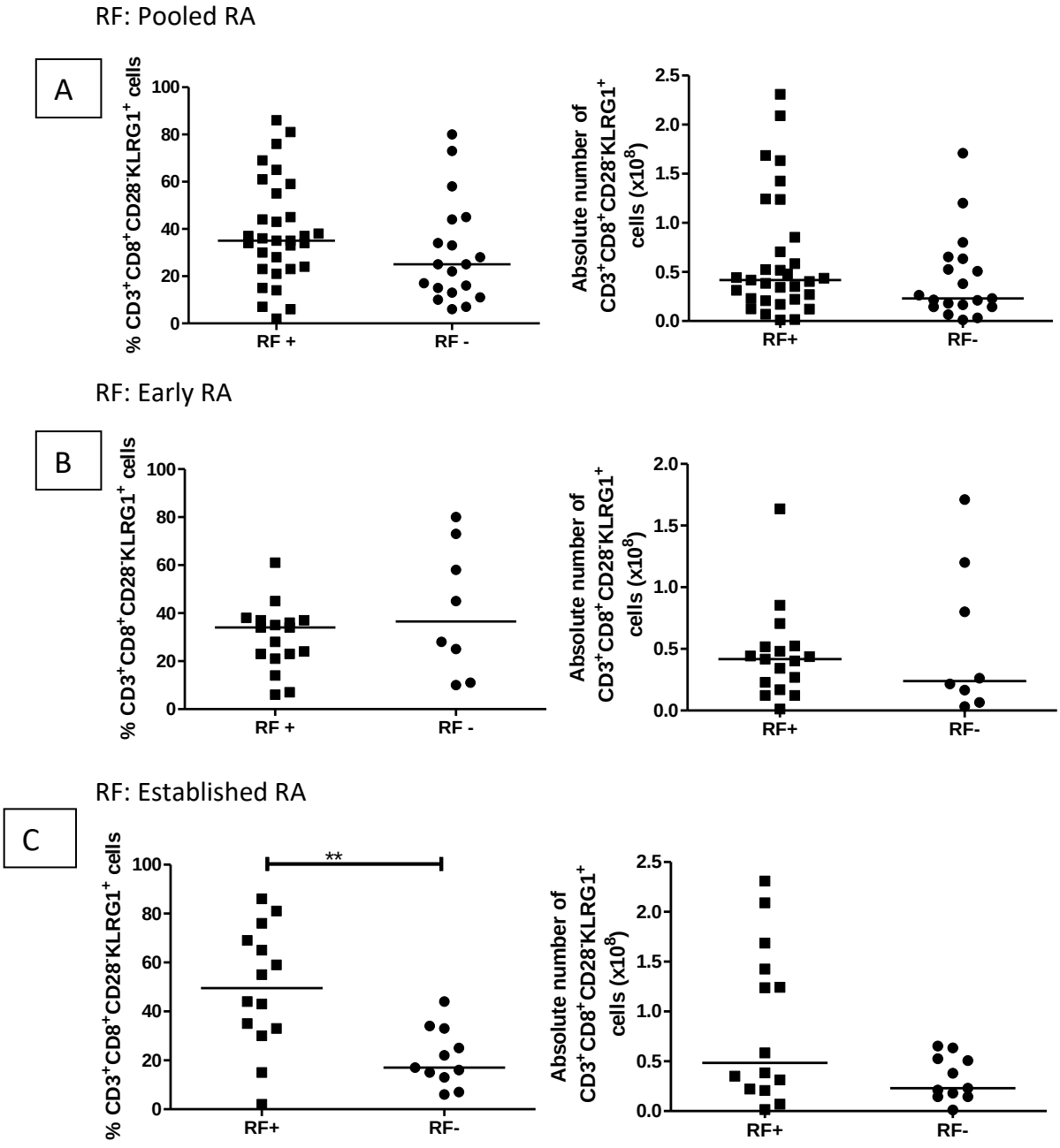


Figure 4-5: Percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells is higher in RF positive established RA patients. Percentage and absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells in (A) pooled RA patients (n=50), (B) Early RA (n=25), (C) Established RA (n=25). Graphs represent median, **p<0.01 using Mann-Witney U Test.

4.6 Serological status and $CD3^+CD8^+CD28^-KLRG1^+$ T cells: ACPA positivity

Given the increase of $CD3^+CD8^+CD28^-KLRG1^+$ cells observed for the RF autoantibody status of the RA patients, it was also determined if there was a similar increase for autoantibodies against Citrullinated proteins.

The percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells were found to be higher in ACPA positive Established RA patients (p=0.0317) (**Figure 4-6C)**. This was a novel finding. The absolute numbers of $CD3^+CD8^+CD28^-KLRG1^+$ cells were similar in ACPA positive and negative disease. There was no difference between the ACPA positive and negative groups and $CD3^+CD8^+CD28^-KLRG1^+$ cells in Early and Pooled RA patients (**Figure 4-6A & 4-6B)**.

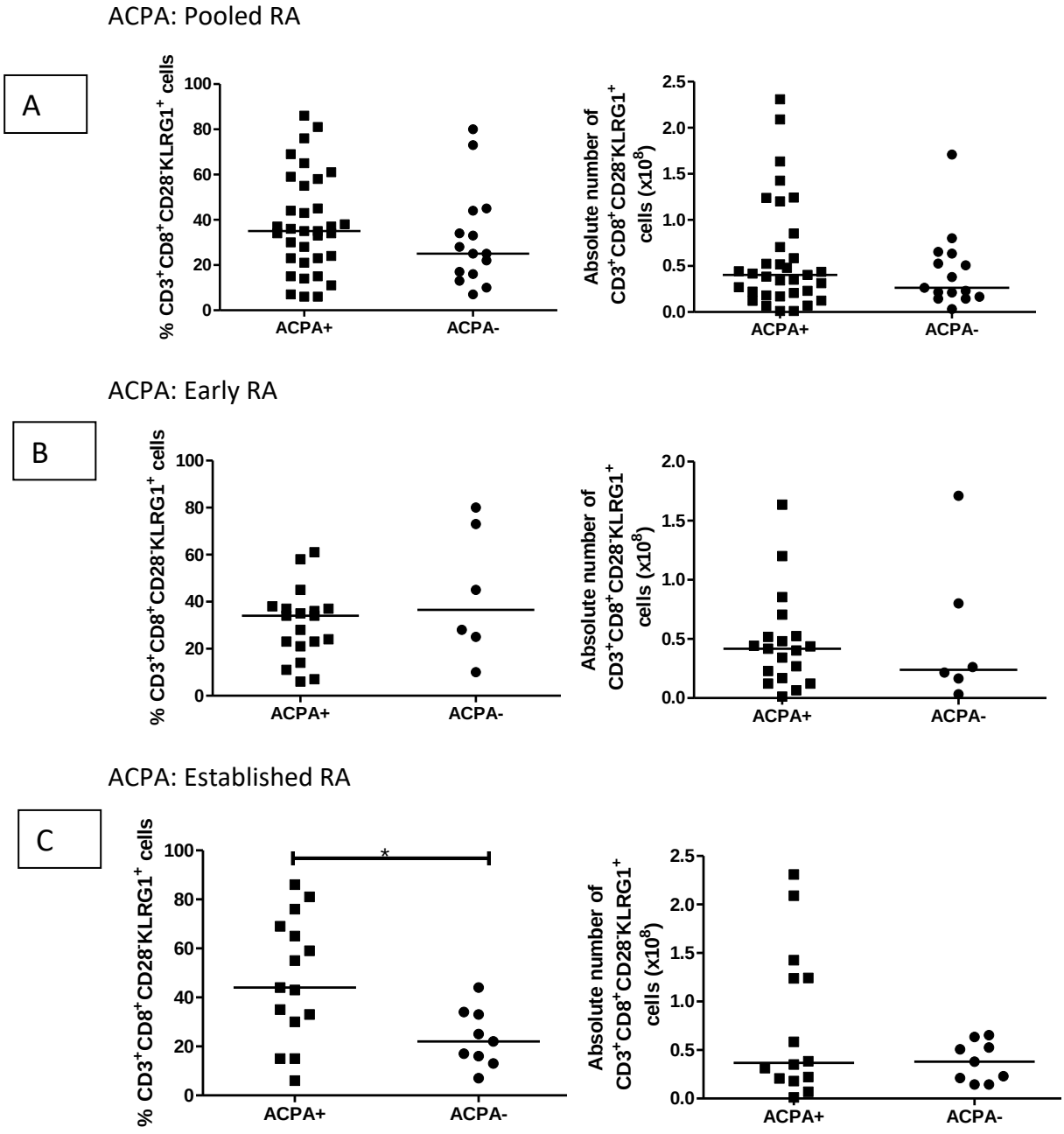


Figure 4-6: Percentage $CD3^+CD8^+CD28^-KLRG1^+$cells are higher in ACPA positive established RA patients. Percentage and absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells in (A) pooled RA patients (n=50), (B) Early RA (n=25), (C) Established RA (n=25). Graphs represent median, **p<0.01 using Mann-Witney U Test. One patient from the Established RA group did not have an ACPA result so was excluded from analysis.

4.7 Laboratory markers and $CD3^+CD8^+CD28^-KLRG1^+$ T cells: CRP and ESR

Chapter 3 found that $CD3^+CD8^+CD28^-$ T cells correlated with the ESR and CRP as systematic markers of chronic disease inflammation. However, there was no statistically significant correlation between the percentage or absolute numbers of $CD3^+CD8^+CD28^-KLRG1^+$ T cells and CRP (**Figure 4-7A, 4-7B & 4-7C).**

In keeping with Chapter 3, there was found to be a low level correlation between the percentage of $CD3^+CD8^+CD28^-KLRG1^+$ T cells and ESR in the pooled RA (**Figure 4-8A**) and Early RA (**Figure 4-8B**). This was a novel finding. There was no significant correlation between the absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ T cells and ESR in RA patients (**Figure 4-8A, 4-8B & 4-8C**).

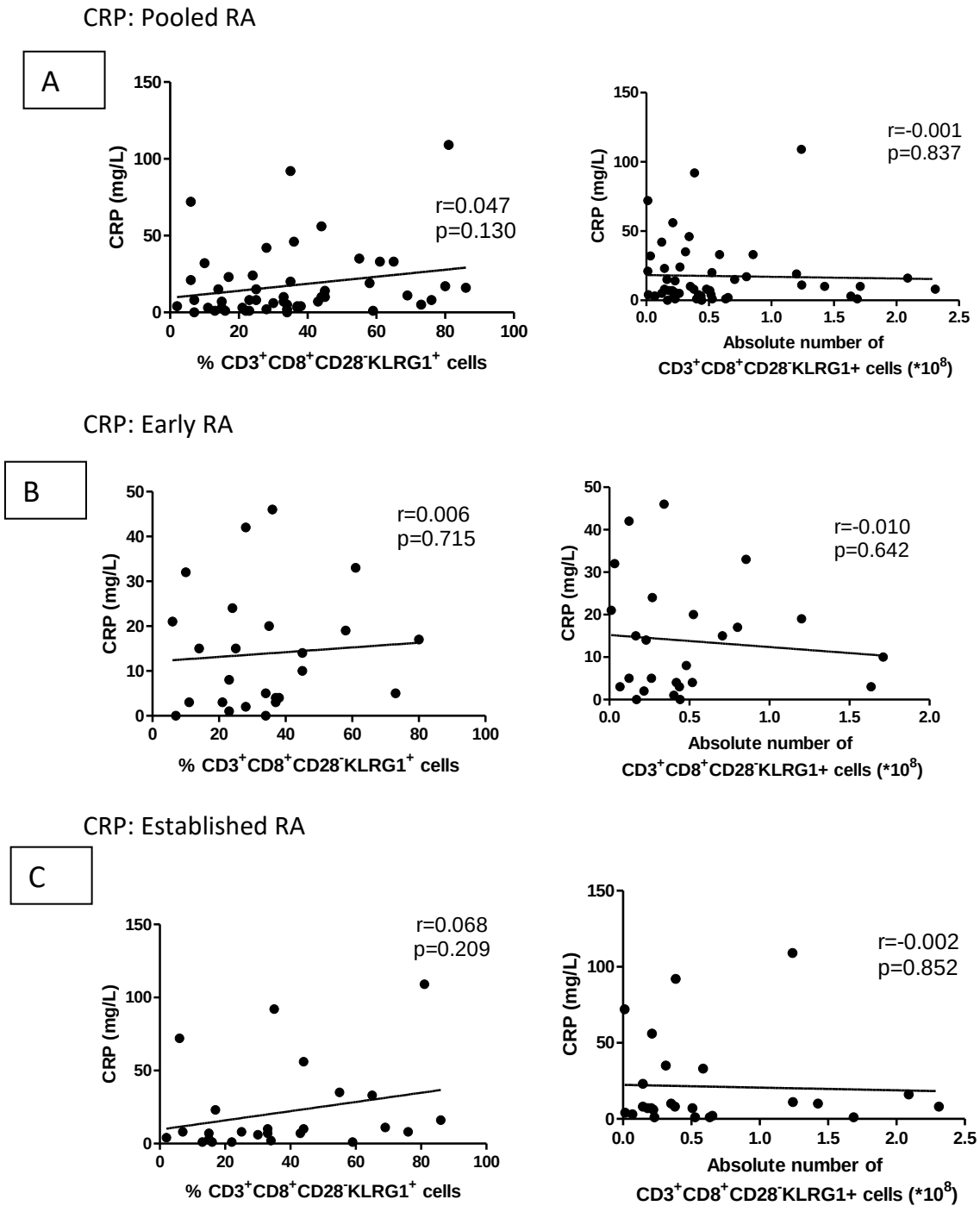


Figure 4-7: There was no correlation between CRP and $CD3^+CD8^+CD28^-KLRG1^+$ T cells. Correlation was determined using non-parametric Spearman's rank analysis. Pooled RA (A, n=50), Early RA (B, n=25), Established RA (C, n=25).

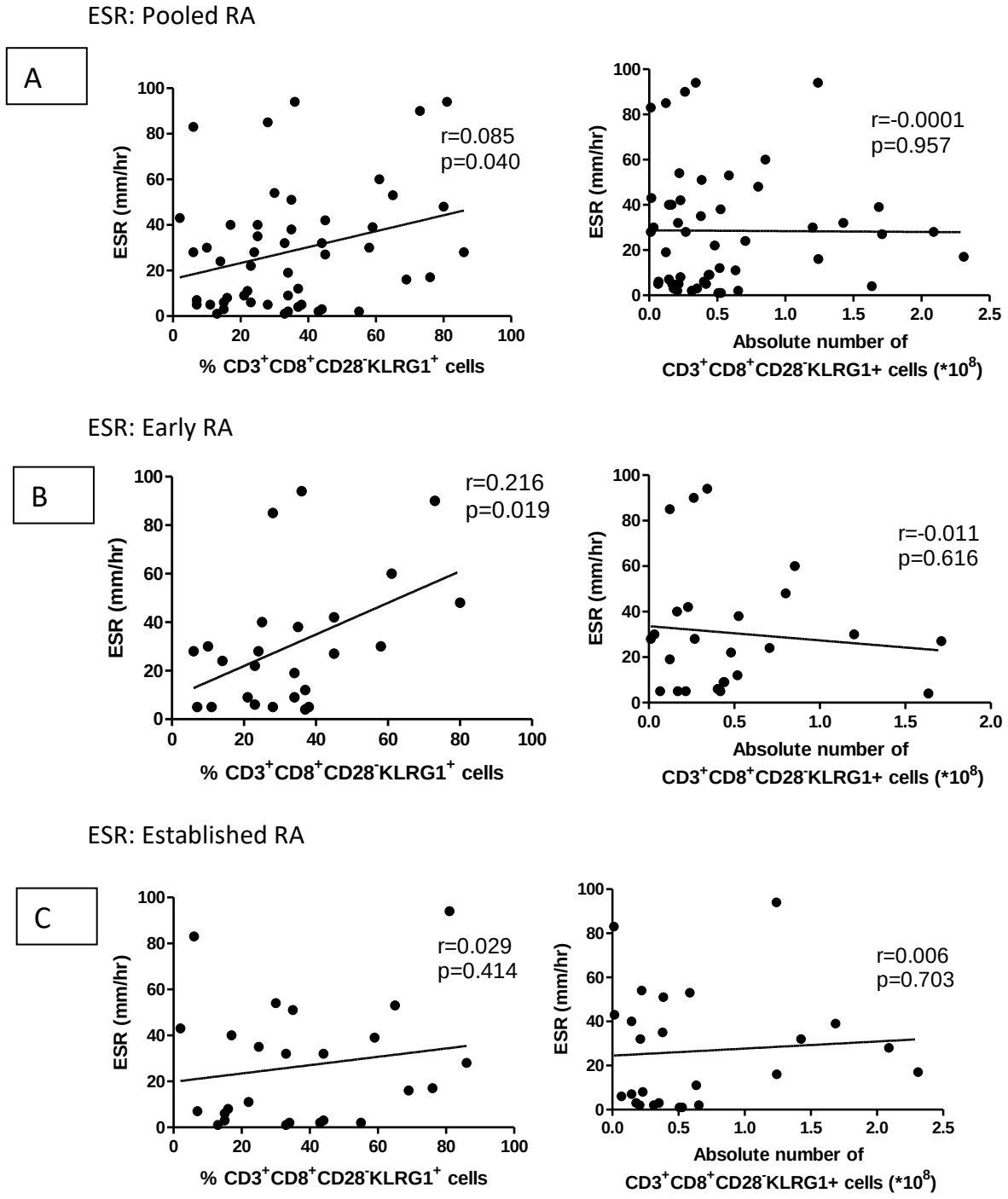


Figure 4-8: There is low-level correlation between the percentage of $CD3^+CD8^+CD28^-KLRG1^+$ T cells and ESR in pooled and Early RA. Correlation was determined using non-parametric Spearman's rank analysis, * $p<0.05$. Pooled RA (A, n=50), Early RA (B, n=25), Established RA (C, n=25).

4.8 Clinical status and $CD3^+CD8^+CD28^-KLRG1^+$ T cells: DAS28

There was no correlation between the percentage or absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ T cells and DAS28 in pooled, Early or Established RA (**Figure 4-9A, 4-9B & 4-9C**).

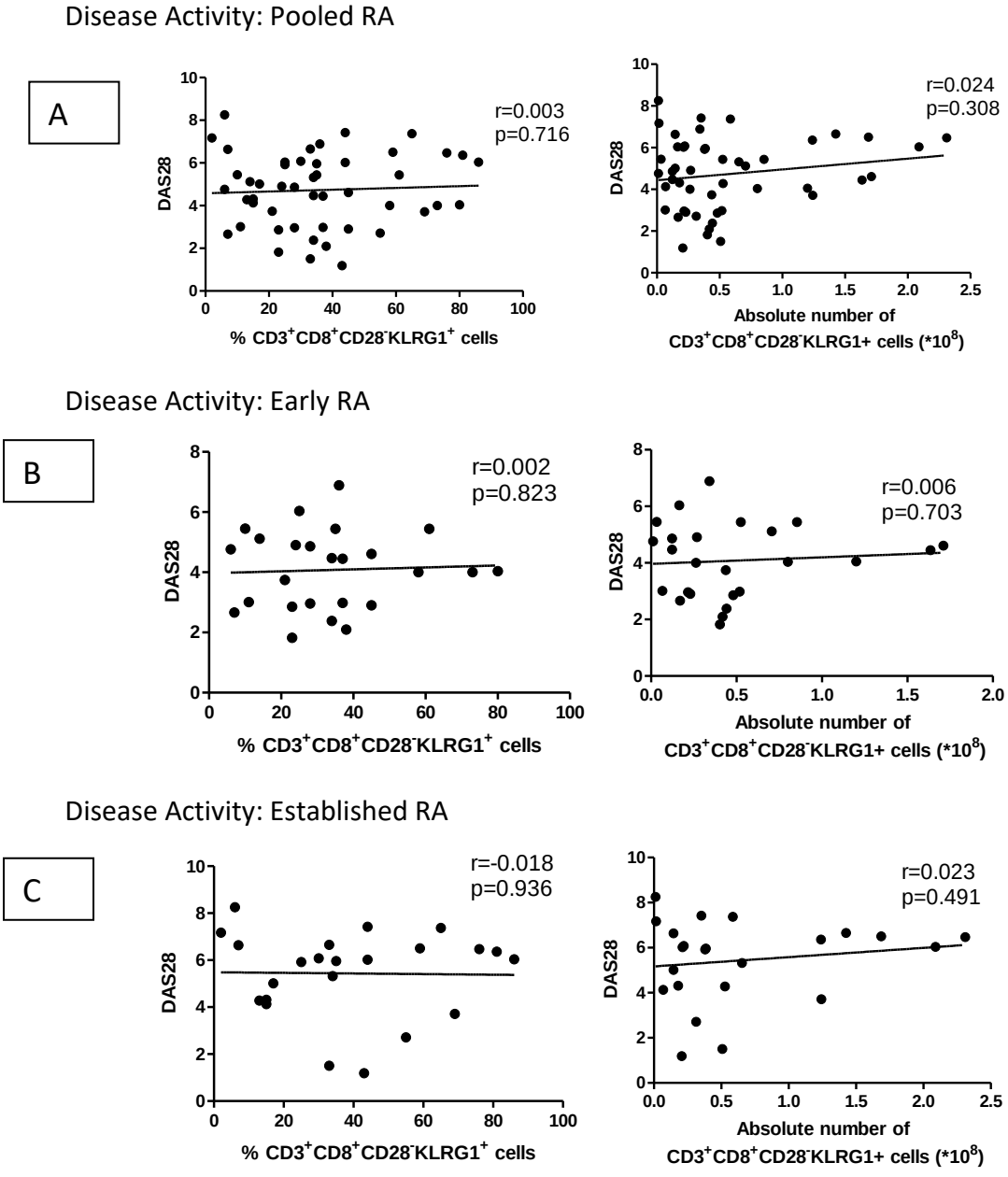
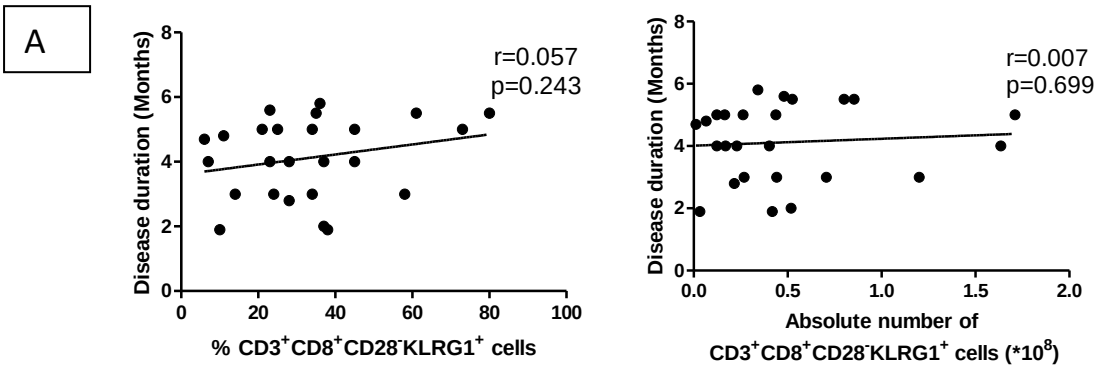


Figure 4-9: There is no correlation between the DAS28 and $CD3^+CD8^+CD28^-KLRG1^+$ T cells in RA. Correlation was determined using non-parametric Spearman's rank analysis. Pooled RA (A, n=48), Early RA (B, n=25), Established RA (C, n= 23). Two Established RA patients did not have a recorded DAS28 score.

4.9 Clinical status and $CD3^+CD8^+CD28^-KLRG1^+$ T cells: Disease duration

Subdivision into Early and Established RA showed no correlation between the percentage or absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ T cells and disease duration in Early or Established RA (**Figure 4-10A & 4-10B**).

Disease Duration: Early RA



Disease Duration: Established RA

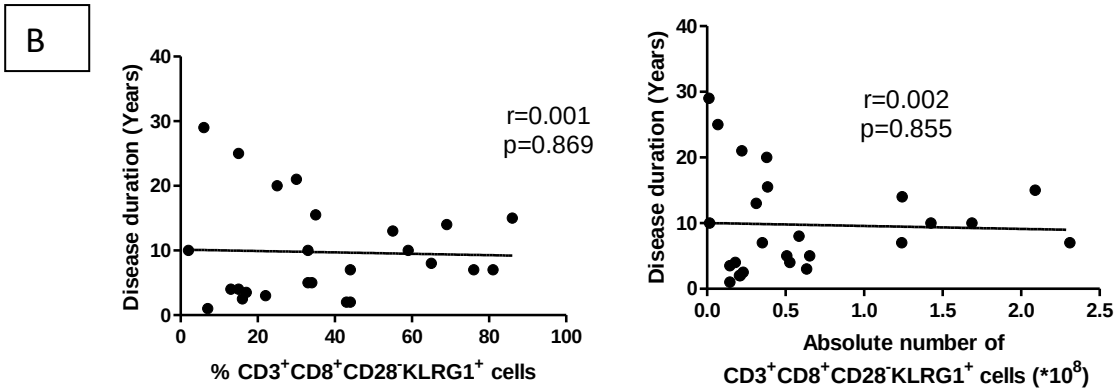


Figure 4-10: Percentage and Absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ T cells do not correlate with Disease Duration in RA. Correlation was determined using non-parametric Spearman's rank analysis. Early RA (A, n=25), Established RA (B, n= 25).

4.10 $CD3^+CD8^+CD28^-KLRG1^+$ cells at baseline correlate with 6-month disease activity (using DAS28)

$CD3^+CD8^+CD28^-$ cells did not predict severity of disease at 6 months in Early RA patients. However, using the same measure of response rate as in Chapter 3 (DAS28 reduction of >1.2), revealed for the first time that the percentage of $CD3^+CD8^+CD28^-KLRG1^+$ T Cells is higher in Early RA non-responders than responders (**Figure 4-11A**, p=0.031)[299]. The absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells was similar in the two groups of responders and non-responders **(Figure 4-11B)**. Three patients did not have a 6 month follow up DAS28 score recorded so were excluded from analysis.

The value of this analysis is that it reveals that KLRG1 as marker of a subtype of $CD3^+CD8^+CD28^-$ cells for RA that may be less responsive to treatment. KLRG1, as a marker of more refractory disease, could potentially warrant a higher degree of intense treatment with DMARDs. This type of research has the potential to identify new markers that could inform clinicians with regard to the likely response to therapy.

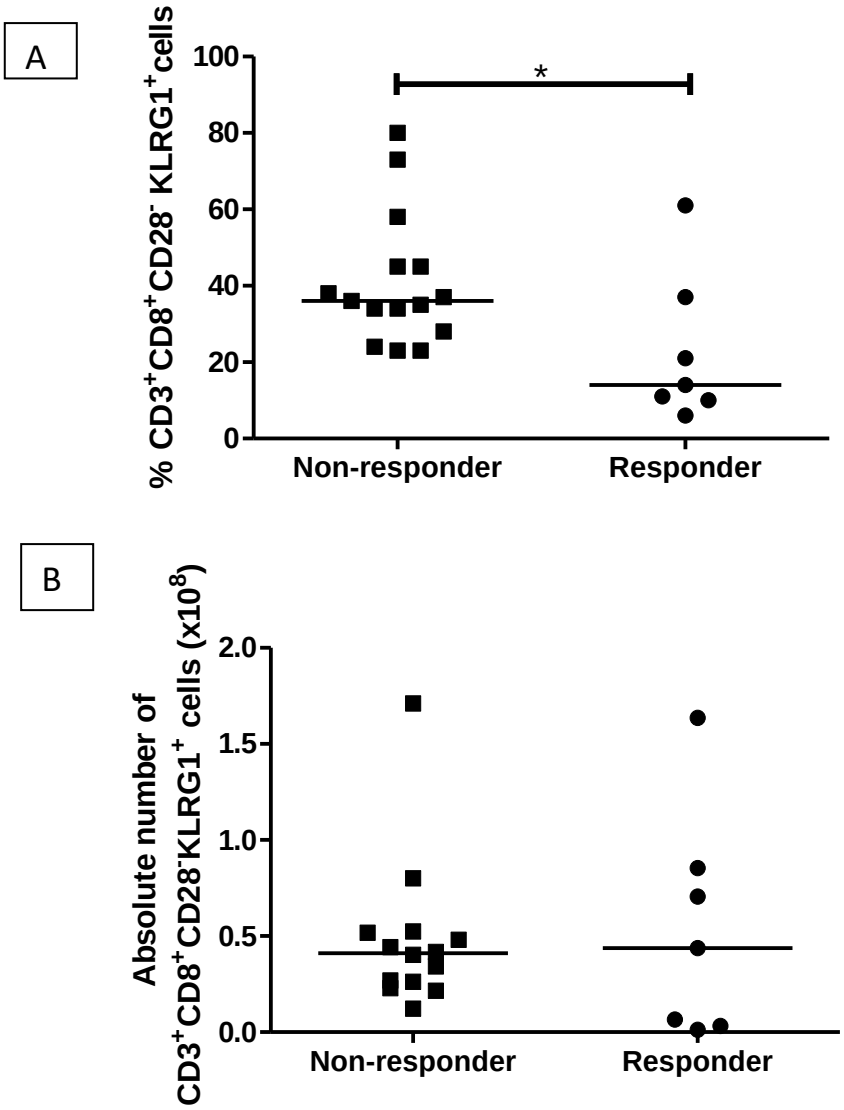


Figure 4-11: Percentage of $CD3^+CD8^+CD28^-KLRG1^+$ T cells predicts the response to therapy at 6 months in Early RA. Percentage (A) and absolute number (B) of $CD3^+CD8^+CD28^-KLRG1^+$ cells were represented median, * $p<0.05$ using Mann-Witney U Test. Three patients were excluded because they did not have a recorded DAS28 at 6 months. Early RA (n=22, Responders=7, Non-responders=15).

4.11 Intracellular cytokines: the activity of CD3+CD8+CD28-KLRG1+ cells

This Results Chapter has found that $CD3^+CD8^+CD28^-KLRG1^+$ cells are higher in non-responders to treatment at 6 months in Early RA. The cellular components of the immune system are modulated by cytokine signals; therefore, it was investigated whether these cells were producing particular cytokines.

Melis et al (2014) found when looking at $CD4^+$ and $CD8^+$ $KLRG1^+$ cells; that IFN production was not different in $KLRG1^+$ compared to $KLRG1^-$ in inflammatory arthritis compared to crystal arthritides[286].

The selection of cytokines IFN, IL-10, IL-4, IL-6 and IL-17 from Chapter 3 were analysed using the same process and gating as in the Chapter 3. It was observed that there were no differences between $CD3^+CD8^+CD28^-$ in controls and RA for IFN, IL-4, IL-6 and IL-17 however, below is an interesting observation in relation to IL-10 secretion by $CD3^+CD8^+CD28^-KLRG1^+$ cells. The other cytokine analysis is presented in **Appendix 7.1**.

4.11.1 Measuring IL-10 production

Comparison of the characteristics of $CD3^+CD8^+CD28^-KLRG1^+$ cells revealed that similar percentages $CD3^+CD8^+CD28^-KLRG1^+$ cells were producing IL-10 between Controls and RA (**Figure 4-12A**). However, the absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells producing IL-10 in pooled RA was higher than $CD3^+CD8^+CD28^-KLRG1^-$ cells (**Figure 4-12B,** $p<0.01$).

This was a novel finding. Increased production of IL-10 from this subset of cells suggests that not only are these cells not exhausted, but instead that $CD3^+CD8^+CD28^-KLRG1^+$ cells are producing immunoregulatory IL-10 in order to control the inflammation or in response to inflammation in RA.

Subdivision into Early and Established RA showed no difference in the percentage and absolute number of $CD3^+CD8^+CD28^-$ cells producing IL-10.

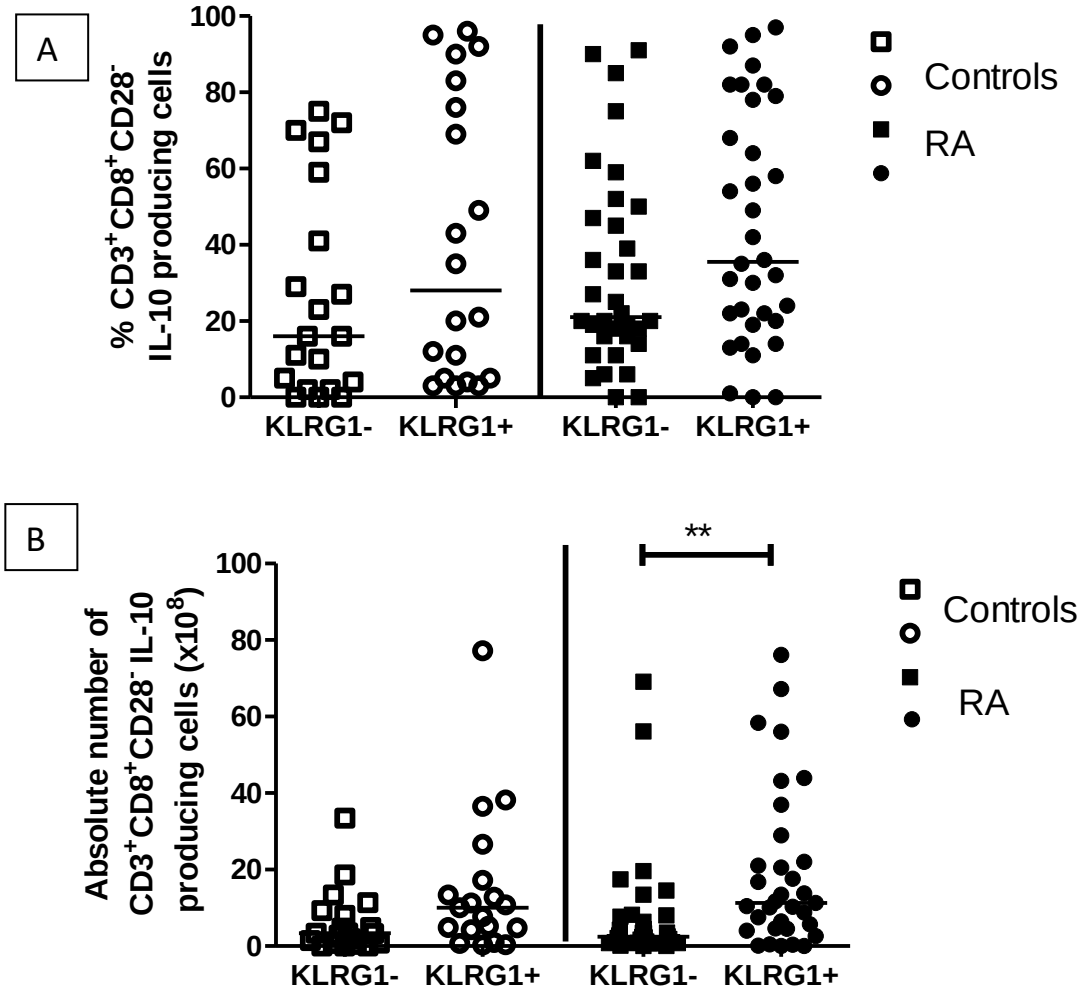


Figure 4-12: Absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ T cells producing IL-10 are higher than $CD3^+CD8^+CD28^-KLRG1^-$ in pooled RA. Percentage (A) and absolute number (B) of $CD3^+CD8^+CD28^-KLRG1^+$ cells were represented median, ** $p<0.01$ statistical analysis was done using Kruskal-Wallis ANOVA test. Controls (n=19), Pooled RA (n=33).

4.12 Discussion

The $CD3^+CD8^+CD28^-KLRG1^+$ cell phenotype can be used to identify a subset of T cells that are associated with increased IL-10 secretion than $CD3^+CD8^+CD28^-KLRG1^-$ cells. This is in contrast to the cytokine profile for $CD3^+CD8^+CD28^-$ cells being similar between $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^+$ cells in Controls and RA in Chapter 3. Banh et al (2009) completed a murine study looking at mature NK and T cells expressing KLRG1. The study found that KLRG1 and cadherin interaction enhanced IL-10 release[300].

As in Chapter 3, with $CD3^+CD8^+CD28^-$ cells being higher in male than female RA patients, $CD3^+CD8^+CD28^-KLRG1^+$ cells were higher in male RA patients than female. Dudkowska et al (2017) looked at the ages 65+ in the normal population and found a higher amount of $CD8^+CD28^-$ T cells in males than in females and also a higher level of IL-10 in men than women ($p=0.018$)[301]. It is unclear whether the increase in IL-10 was associated with aging or sex but testosterone does increase IL-10 synthesis[302]. There are also studies that have found higher IL-10 in women than men[303]. With respect to RA, men have a favourable prognosis[304]. The higher percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells in RA men, conflicts with the concept that $KLRG1^+$ cells are driving RA. This finding could be potentially be explained by other factors increasing these cells in men such as viral infections, hormones or genes on sex chromosomes[305]. $CD3^+CD8^+CD28^-KLRG1^+$ cells may have a role related to hormones, particularly as $KLRG1^-$ cells did not correlate with sex.

$CD3^+CD8^+CD28^-KLRG1^+$ T cells were significantly higher in patients that have not responded after six months of treatment. The increase could indicate a greater immune activity, but it is not clear whether they are causing the inflammation or trying to control it. This increase was perhaps insufficient to control the increase in autoimmunity and inflammation. Other cytokines including IFN, IL-6, IL-4 and IL-17 were investigated but the difference was not significant.

$CD3^+CD8^+CD28^-KLRG1^+$ cells are not raised in RA compared to healthy controls (p=0.052). However, this difference should be taken with caution because it could change if the sample size of this study was increased. This is supported by the study from Melis et al (2013), which found higher levels of $CD3^+CD8^+CD28^-KLRG1^+$ cells in inflammatory arthritis in comparison to crystal arthritides and healthy controls[286].

When considering serological and clinical parameters, there was no correlation between $CD3^+CD8^+CD28^-KLRG1^+$ cells with age, CRP, DAS28 or disease duration. CRP is used in RA to assess disease activity but the systemic inflammatory marker used for the disease activity scoring is normally the ESR. The DAS28 score was originally developed and validated using the ESR[306]. Subsequent comparison with CRP has shown lower DAS28-CRP scores in comparison with DAS-ESR[307,308]. Potentially this could indicate that CRP is not as sensitive a marker as ESR in RA, or perhaps that ESR is a measure of chronic inflammation while CRP reflects acute inflammation.

RF, ACPA and ESR correlated with the percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells. This is in keeping with the hypothesis that these cells indicate a poor prognosis where RF, ACPA and ESR are indeed associated with a poor prognosis in RA[304]. RF and ACPA may contribute to the repeated immune stimulation and development of $CD3^+CD8^+CD28^-KLRG1^+$ cells. These autoantibodies can directly stimulate macrophages and activate complement[309,310,311]. The increase in inflammation would, in turn, increase the ESR.

4.13 Conclusions

- The percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells was higher in male RA patients than female RA patients.
- The percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells in patients with established RA was associated seropositive disease, either RF or ACPA.
- The percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells correlated with ESR especially in patients with early RA
- The percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells are higher in Early RA patients who were conventional DMARD non-responders at six months.

- $CD3^+CD8^+CD28^-KLRG1^+$ cells produce more IL-10 than $CD3^+CD8^+CD28^-KLRG1^-$ cells.

5 Chapter 5: The role of Cytomegalovirus and $CD3^+CD8^+CD28^-$ T cells in RA

5.1 Introduction

Human Cytomegalovirus (CMV) belongs to the herpes virus family (**Table 5-1**). CMV is present in approximately 50% of the adult population and 90% of the elderly[312]. After primary infection, it persists in the host in a latent state. In healthy individuals, CMV and the host exist in a symbiotic equilibrium, so that infectious symptoms are barely experienced. However, for the immunocompromised or the developing foetus, CMV infection can lead to a number of complications (**Section 5.1.3**)

Name	Synonym	Disease
HHV-1	Herpes Simplex Virus-1	Oral Herpes
HHV-2	Herpes Simplex Virus-2	Genital Herpes
HHV-3	Varicella Zoster Virus	Chickenpox and Shingles
HHV-4	Epstein Barr Virus	Infectious Mononucleosis, Burkitt's Lymphoma, HIV associated Hairy Leucoplakia
HHV-5	Cytomegalovirus	Infectious Mononucleosis like syndrome, Retinitis.
HHV-6A and 6B	Roseolovirus and Herpes Lymphotropic Virus	Sixth disease, Roseola Infantum
HHV-7	Roseolovirus	Pytiriasis Rosea, Roseola Infantum
HHV-8	Kaposi's Sarcoma Associated Herpesvirus	Kaposi's Sarcoma

Table 5-1: Family of Herpes Viruses and diseases caused.

5.1.1 Epidemiology:

CMV prevalence generally correlated inversely with socioeconomic development, with the highest rates being in Africa and Asia[313]. A study in 1973 found that CMV seropositivity varied throughout the world from twenty-seven different countries between 40% (France / Germany) and 100 % (Fiji / Uganda) of the population aged 20-40[314].

The prevalence of CMV increases with age. A study in England and Wales found rates of positivity of 15% in those aged 1–4 years to 80% in those aged >65 years[315]. In the UK there was no association with sex or geographic region[315]. Studies in America and Finland reflect similar trends[316,317]. Other factors positively associated with CMV seropositivity included female sex, low household income, household crowding, and low household education[318,319].

5.1.2 Transmission

Transmission can occur via multiple routes: sexual exposure, saliva, urine, blood, breastmilk, transfusion of blood products, transplantation of organs and finally perinatal exposure. The foetus may become infected with CMV in utero or during the neonatal period via exposure to secretions in the birth canal.

The largest concern with CMV infection is the vertical transmission from mother to baby during pregnancy. CMV seropositivity rates in American women of childbearing age (18–45 years) was 51.7%, however it is CMV infection during the pregnancy that creates the biggest risk to the fetus[319]. Congenital CMV infection can cause significant complications and the mortality rate among symptomatic newborns is approximately 5%; with 50-60% of survivors develop serious long-term neurologic morbidity[320,321].

The rate of acquisition and prevalence of CMV infection is higher in the very young, particularly during nursery school years[322]. The higher rates of transmission are propagated by the close person-to-person contact common in this less hygienically aware population. Subsequent transmission from child to previously seronegative

parent plays a significant part in the acquisition of CMV infection in adult life, suggesting that adults with young children are most at risk[323].

5.1.3 Symptoms

More common symptoms that normally resolve within three weeks of primary infection:

- Temperatures of ≥38°C
- Generalised myalgia
- Fatigue
- Nausea
- Sore throat
- Lymphadenopathy
- Rash

Less common complications include:

- Gastro-intestinal: colitis, oesophagitis, gastritis, ileitis, appendicitis
- Respiratory: pneumonia
- Hepatic: hepatitis, portal vein thrombosis
- Ophthalmological: retinitis, anterior uveitis
- Neurological: encephalitis, Guillain-Barré syndrome, focal defects
- Cardiac: atherosclerosis, pericarditis, myocarditis

5.1.4 Treatment

Healthy patients do not normally need treatment when infected with CMV. NSAIDs can help relieve mild symptoms. Severe CMV symptoms are treated with anti-viral medications including ganciclovir, valganciclovir or foscarnet. These are mainly reserved for immunosuppressed patients.

5.1.5 $CD8^+$ T cells in CMV

$CD8^+$ T cells are believed to be most important in controlling CMV infection. Acutely, viral infection induces a massive expansion of $CD8^+$ cells. During the first stage of

infection these cells express CD45Ro, CD38 and HLA-DR, CD28, and CD27, whereas later in infection CD28 and CD27 may become down-regulated[324]. Along with CD28 loss, $CD8^+$ T cell have shortened telomeres[325]. T cells restrict the CMV replication and prevent disease but do not eliminate or stop transmission. Repeated rounds of antigen-driven proliferation by CMV continue to cause the clonal expansion of CMV-specific $CD8^+$ T cells, which therefore accumulate with age.

The primary infection of CMV has a relatively reduced viral and long replication period. This may give time to induce recruitment of CMV-specific T cells into the memory compartment and explain the high proportion of CMV-specific T cells. Approximately 10% of both the $CD4^+$ and $CD8^+$ memory compartments in peripheral blood are made up of CMV specific T cells[326]. The frequencies of CMV specific T cells in healthy subjects are much higher than those observed for other human viruses.

A major subset of CMV specific T cells is defined as $CD8^+CD28^-CD57^+CCR7^-$. One study showed that this phenotype can constitute up to one-quarter of the total CD8 T cell population[327]. Another study involving a cohort of 220 healthy children demonstrated that $CD8^+CD45RA^+CD27^-$ T cells correlated with prior CMV infection ($p<0.0001$). Interestingly, the number of this $CD8^+$ T cell subset remained stable for three years in 40 of the children[328].

5.1.6 $CD4^+$ T cells and CMV

Alongside $CD8^+$ T cells, $CD4^+$ T cells also play a key role in the defence against CMV infection. CMV infection increases the number of $CD4^+CD28^-$ T cells[329]. CMV specific $CD4^+$ T cells precede the appearance of CMV specific $CD8^+$ T cells during primary infection, but are delayed in symptomatic patients[330,331].

Several studies have reported significant differences between RA patients and healthy controls with respect to the frequency of $CD4^+CD28^-$ T cells[332,333,334]. However, few have accounted for CMV seropositivity. RA patients have significant expansions of the $CD4^+CD28^-$ T cells in CMV positive compared to CMV negative and this is associated with more severe joint destruction[335].

5.1.7 T cells, aging and CMV

As discussed in Chapter 1, T cells that are chronically stimulated lose their co-stimulatory CD28 receptor. As a result, their proliferation rate decreases, known as T cell immunosenescence[336]. Aging, but also chronic infections such as CMV can cause increased stimulation and therefore loss of CD28.

Increased mortality and morbidity occur in people with antigenic overload. Swedish longitudinal studies examined 86-92 year olds and defined predictors of mortality as increased $CD8^+$ cells, low $CD4^+$ cells, poor T cell proliferation and low IL-2 production[337,338]. These parameters were significantly associated with CMV seropositivity[339].

CMV seropositivity is associated with an increased number of $CD4^+CD28^-$ and $CD8^+CD28^-$ cells. Although this phenotype has been associated with age, it could be argued that this may in fact be primarily associated with CMV status because CMV seropositivity increases with age.

5.1.8 CMV and Cytokines

IL-10:

It has been found that frequencies of $CD8^+$ T cells stimulated with CMV antigen produce low levels of IL-10. The frequency of $CD8^+$ T cells was higher in the group of younger subjects than the group of older subjects ($P=0.001$)[340].

A viral homolog of IL-10, expressed during latent CMV infection, has been shown to reduce expression of MHC class I and II molecules and inhibit proliferation of PBMCs and the production of inflammatory cytokines[341]. This suppression of lymphocytes could reduce the number of effective T regs. This reduction could contribute to aberration in homeostatic control of the overactive immune system in RA.

IFN:

After in vitro stimulation with CMV peptide antigen, the CMV specific $CD8^+$ T cells from elderly individuals are characterized by a decreased frequency of cells releasing IFN-γ compared to the cells from young subjects ($p<0.001$)[340].

Older patients were shown to have a higher ratio of IL-10 to IFN-γ producing cells in response to CMV, than young people (3.8 versus 9.6). This indicates a trend towards a more anti-inflammatory immune response against CMV in the older patients, however the number of subjects studied was small (n=22)[340]. Repeated stimulation by CMV may lead to initially an expansion of IFN-γ producing cells, which then stimulate the homeostatic control response of increasing the IL-10 producing T cells.

5.1.9 KLRG1 and CMV

KLRG1 has been investigated as a response regulator for anti-viral $CD8^+$ T cells. KLRG1 is expressed on 50% of $CD8^+$ cells in young patients, whereas in the elderly the percentage is 80%. Furthermore, about 90% of CMV positive $CD8^+$ T cells in the elderly are also positive for KLRG1 compared to 70% in the young. Thus, persistent infection with CMV may lead to a gradual increase in the already high number of $CD8^+KLRG1^+$ cells during ageing[342]. Increase in KLRG1 expression is not unique in the elderly and CMV seropositive subjects but also found in Hepatitis C viral infection[343,344]. KLRG1 could be signposting anergic anti-inflammatory cells that are enabling persistent and indolent viral infection in the subject without clearance.

Latent CMV infection and RA share several phenotypical features in the T cell lymphocytes. $CD8^+CD28^-$ T cells are expanded in healthy individuals chronically infected with CMV, and even more so in CMV infected RA patients[345,346]. CMV positivity has been associated with more severe joint destruction in RA[347]. CMV chronic infection phenotype represents a population of T cells that can still efficiently control latent infection, while certain levels of effector function are diminished to prevent overwhelming immunopathologic changes due to collateral auto reactivity[348].

5.1.10 Hypothesis

In RA, CMV infection is associated with an expansion in $CD3^+CD8^+CD28^-$ cells, which have phenotypic and cytokine prolife associated with $CD3^+CD8^+CD28^-$ T reg cells.

CMV and $CD3^+CD8^+CD28^-$ cells could be associated with disease phenotype, severity and response to treatment in RA.

5.1.11 Aims

- Assess association between $CD3^+CD8^+CD28^-$ cells and CMV in patients with RA (both early and established RA) in the peripheral blood compartment.
- Assess association between $CD3^+CD8^+CD28^-$ cells and CMV in the peripheral blood compartment in patients with RA in relation to clinical and serological markers.
- Assess association between $CD3^+CD8^+CD28^-KLRG1^+$ cells and CMV in the peripheral blood compartment in patients with RA.
- Assess association between responders to DMARDs and CMV in patients with RA.

5.2 RA patients and demographics

In this study, 37 patients with RA (25 patients with early RA and 12 patients with established RA) were included in the analysis. The mean age of the Early RA CMV negative group (52±15) was less than the other groups but this difference was not significant. The mean duration of disease was similar for the CMV positive and negative groups **(Table 5-2)**.

The percentage of seropositivity for RF was highest in the CMV positive Established RA group (80%, n=5). ACPA positivity was highest in the CMV negative Early RA group (83%, n=12).

Patients with Established RA had a higher mean DAS28 score in both CMV positive (5.3±1.0, n=5) and negative groups (4.8±2.3, n=7) in comparison to the Early RA group (CMV positive, 4.2±1.6, n=13), (CMV negative, 4.0±1.1, n=12).

The female percentage was similar across the groups (58%-80%). The distribution of CMV seropositivity was similar across the pooled and Early RA groups (Pooled RA CMV positive 51% and Early RA CMV positive 52%.)

Patient and disease characteristics were analysed using the non-parametric Kruskal-Wallis ANOVA test, and none of the differences was statistically significant.

	Early RA		Established RA		Pooled RA	
	CMV negative	**CMV positive**	**CMV negative**	**CMV positive**	**CMV negative**	**CMV positive**
Number of patients	12	13	7	5	19	18
Mean duration of disease	3.7 months	4.1 months	8.1 years	11.8 years	N/A	N/A
Mean Age	52±15	61±8	65±10	66±11	56±14	56±9
RF positive %	75%(9)	54%(7)	43%(3)	80%(4)	63%(12)	61%(11)
ACPA positive %	83%(10)	69%(9)	43%(3)	60%(3)	68%(13)	67%(12)
Mean DAS28	4.0±1.1	4.2±1.6	5.3±1.0	4.8±2.3	4.4±1.2	4.3±1.8
Female %	58%(7)	77%(10)	71%(5)	80%(4)	68%(13)	78%(14)

Table 5-2: Demographics of the patients tested for CMV positivity at baseline. Percentages are shown with number of subjects in brackets.

5.3 $CD3^+CD8^+CD28^-$ cells and CMV status in pooled RA and Early RA patients

The percentage of the $CD3^+CD8^+CD28^-$ T cells was increased in CMV positive pooled and Early RA patients in comparison to CMV negative patients (Pooled RA: p=0.040) (**Figure 5-1A**). The absolute number of cells for pooled RA was similar for CMV positive and negative.

The percentage and absolute number of $CD3^+CD8^+CD28^-$ T cells in Early RA was also increased in CMV positive (**Figure 5-1B**).

$CD3^+CD8^+CD28^-$ T cells have been found to be significantly increased in CMV positive compared to CMV negative in other autoimmune diseases[349]. Although in RA patients the number of EBV-specific $CD8^+$ T-cells correlates positively with the viral load, the $CD8^+$ T-cell responses to CMV antigens do not[350].

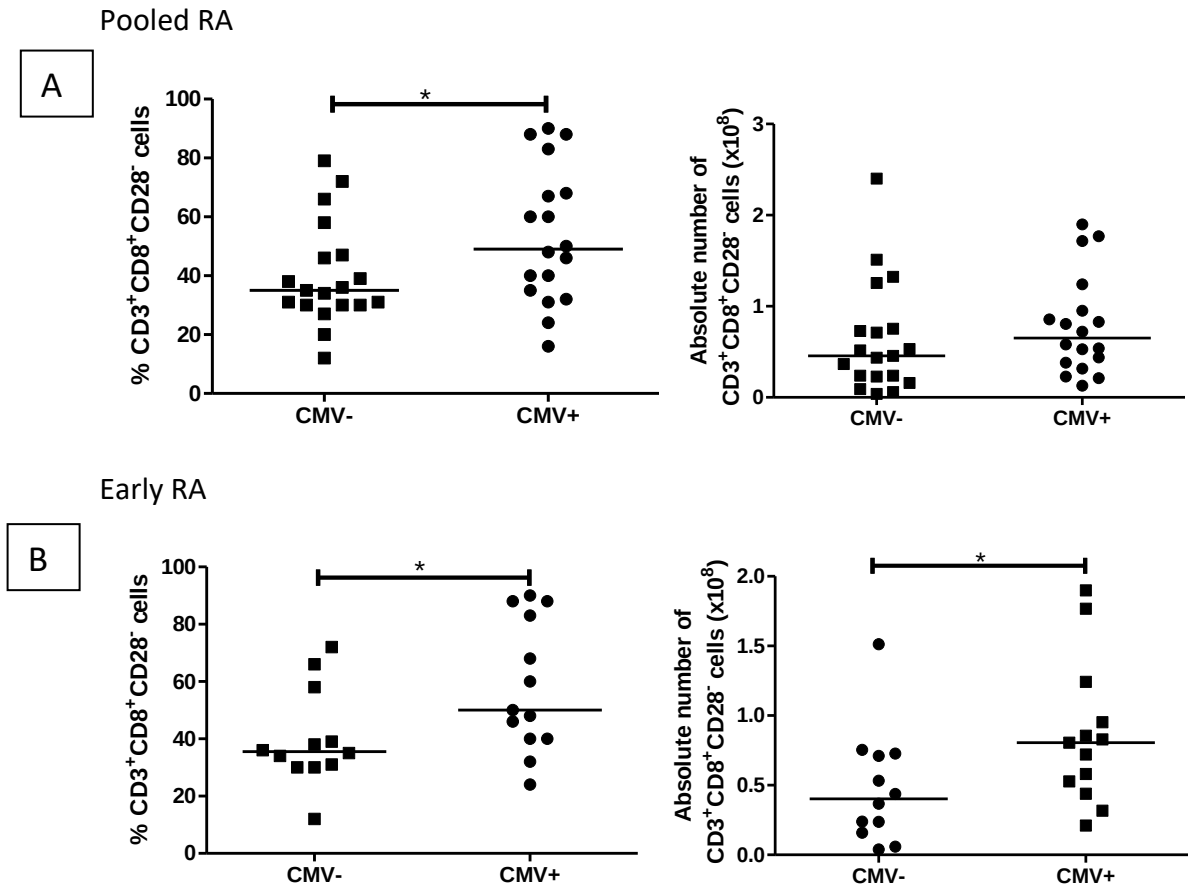


Figure 5-1: Peripheral blood $CD3^+CD8^+CD28^-$ T cells are increased in CMV positive RA patients. Number and percentage of CD8CD28- cells in (A) pooled RA (n=37, CMV positive=19, CMV negative=18), and (B) Early RA (n=25, CMV positive=13, CMV negative=12). Graphs represent median, * $p<0.05$ using Mann-Witney U Test.

5.4 Association of $CD3^+CD8^+CD28^-$ T cells and CMV status with Sex

Of the CMV positive Pooled RA (n=37), 68% were female and of the CMV negative, 78% were female. The percentage of $CD3^+CD8^+CD28^-$ cells was higher in male subjects compared to females in the CMV positive pooled RA patients (**Figure 5-2A**). This has not previously been investigated within an RA patient cohort.

There was no significant difference between sex and CMV status for absolute number of $CD3^+CD8^+CD28^-$ cells in pooled RA patients (**Figure 5-2B & Table 5-3**).

Pooled RA

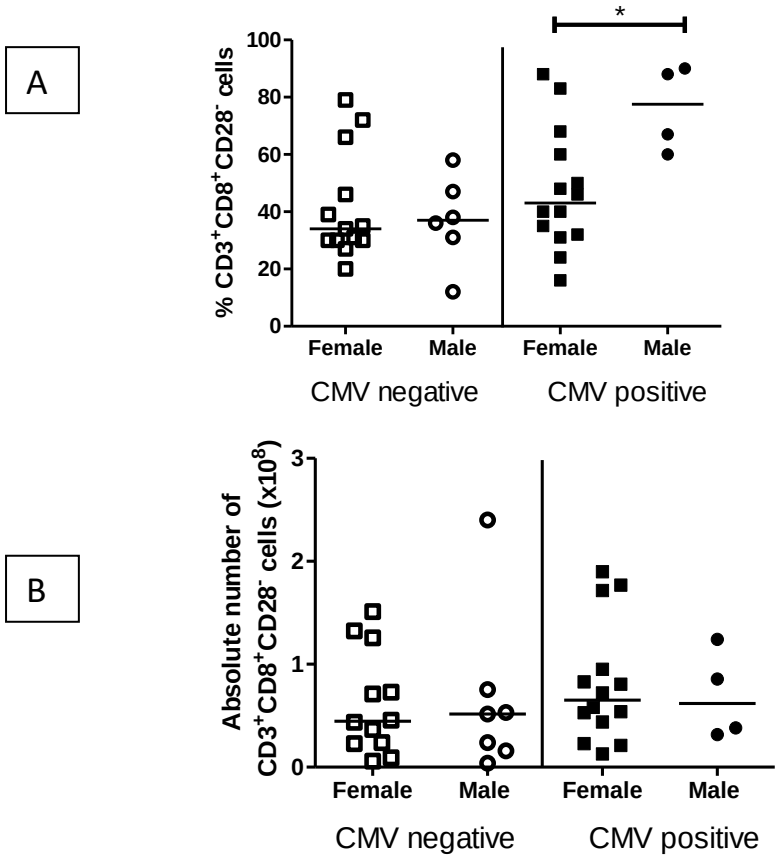


Figure 5-2: $CD3^+CD8^+CD28^-$ cells are similar according to CMV status and sex in pooled RA patients. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-$ T Cells. Graphs represent median, * $p<0.05$ using Kruskal-Wallis ANOVA Test. Pooled RA (n=37).

5.5 Association of $CD3^+CD8^+CD28^-$ T cells and CMV status with RF status in pooled RA

For RF negative patients, the percentage of $CD3^+CD8^+CD28^-$ cells was higher in CMV positive pooled RA than CMV negative ($p<0.05$) (**Figure 5-3A**). This is a novel finding. There was no difference in the absolute number of $CD3^+CD8^+CD28^-$ cells for RF or CMV seropositivity (**Figure 5-3B & Table 5-3**).

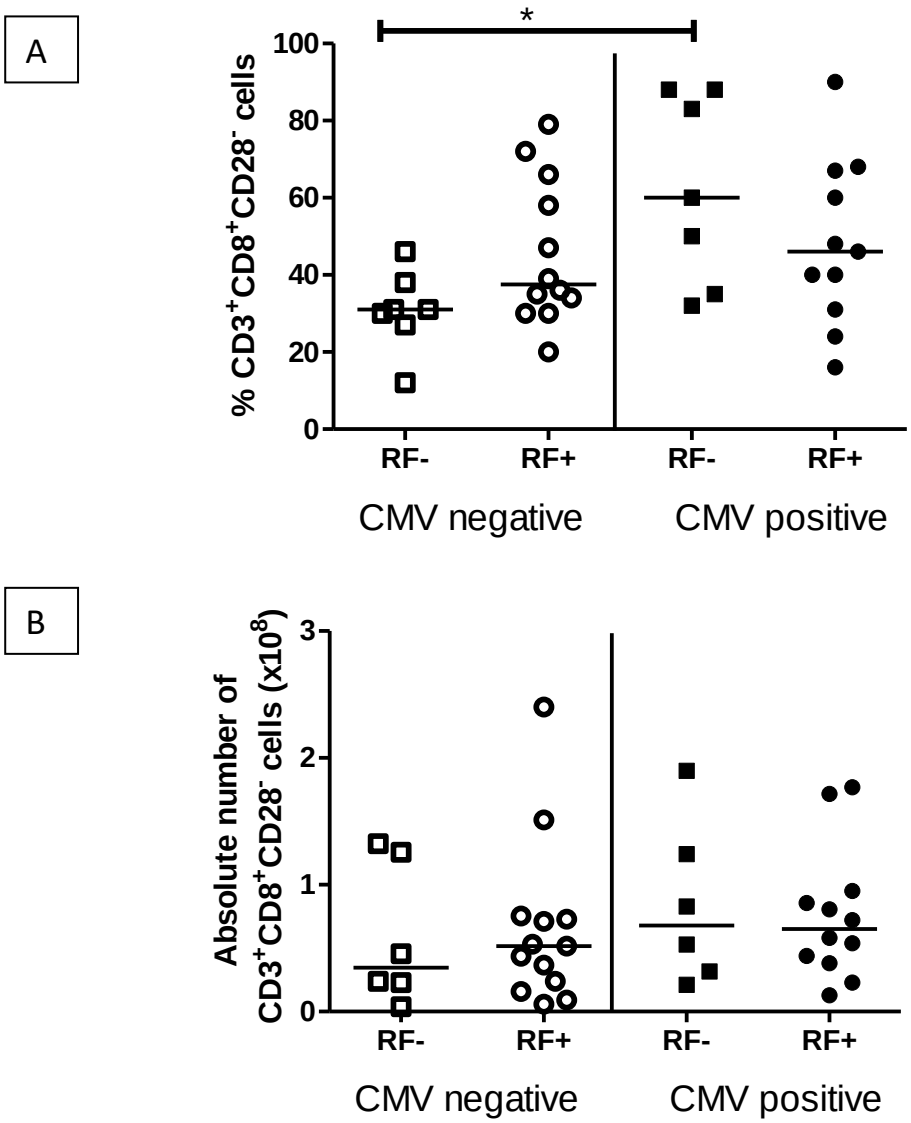


Figure 5-3: Percentage of $CD3^+CD8^+CD28^-$ cells is higher in CMV positive, RF negative RA patients. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-$ T Cells. Graphs represent median, * $p<0.05$ using Kruskal-Wallis ANOVA Test. Pooled RA (n=37).

5.6 Association of $CD3^+CD8^+CD28^-$ T cells and CMV status with ACPA status

For ACPA negative and ACPA positive patients, a similar trend was observed to that seen for RF autoantibody positivity. However, the increased percentage of $CD3^+CD8^+CD28^-$ cells in CMV positive compared CMV negative did not reach statistical significance (**Figure 5-4A**).

There was no difference in the absolute number of $CD3^+CD8^+CD28^-$ cells for ACPA or CMV seropositivity (**Figure 5-4B & Table 5-3**).

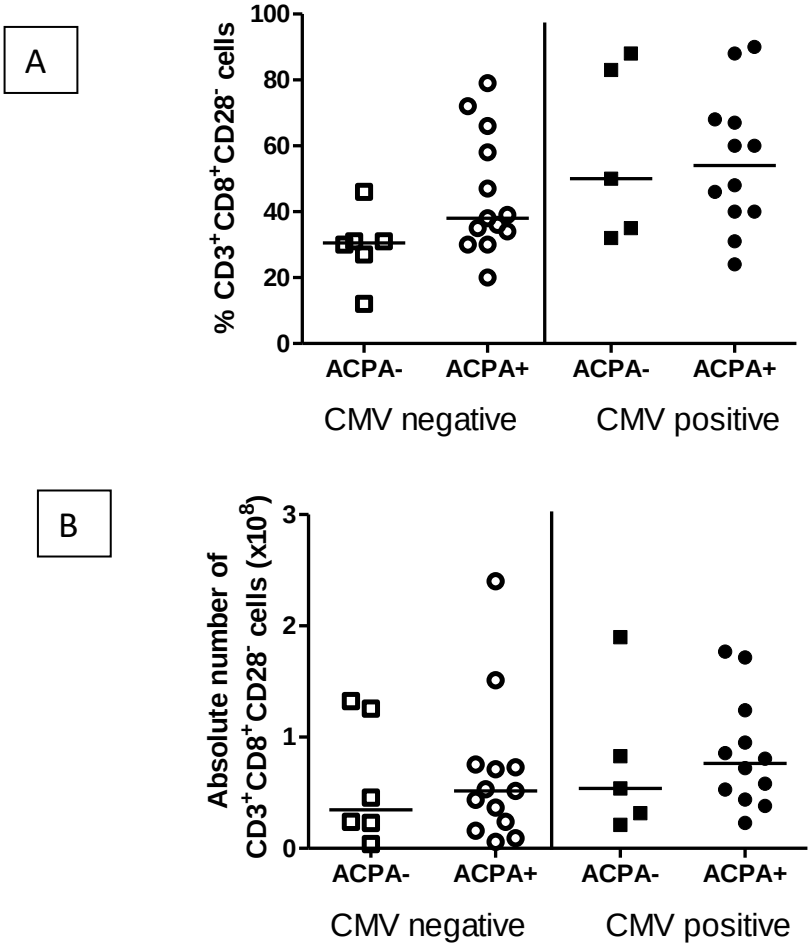


Figure 5-4: Percentage of $CD3^+CD8^+CD28^-$ cells is higher in CMV positive ACPA positive RA than CMV negative, ACPA negative RA patients. Percentage (A) and Absolute Number (B) of $CD3^+CD8^+CD28^-$ T Cells. Graphs represent median, * $p<0.05$ using Kruskal-Wallis ANOVA Test. Pooled RA (n=37).

		Female	Male	RF+	RF-	ACPA+	ACPA-
CMV-	**Number of Patients**	12	6	12	7	13	6
	Mean % $CD28^-$ cells	38±19	37±16	46±19	31±10	45±18	30±11
CMV+	**Number of Patients**	15	4	11	7	12	5
	Mean % $CD28^-$ cells	47±21	76±15	48±22	62±24	55±21	58±26

Table 5-3: The distribution of sex and seropositivity (RF and ACPA) was similar across CMV positive and negative pooled RA patients. Mean is shown with Standard Deviation.

5.7 $CD3^+CD8^+CD28^-$ and CMV seropositivity distribution with Age

The pooled RA patients (n=37) were separated into age tertiles. The distribution of CMV seropositivity is equal across the three age tertiles.

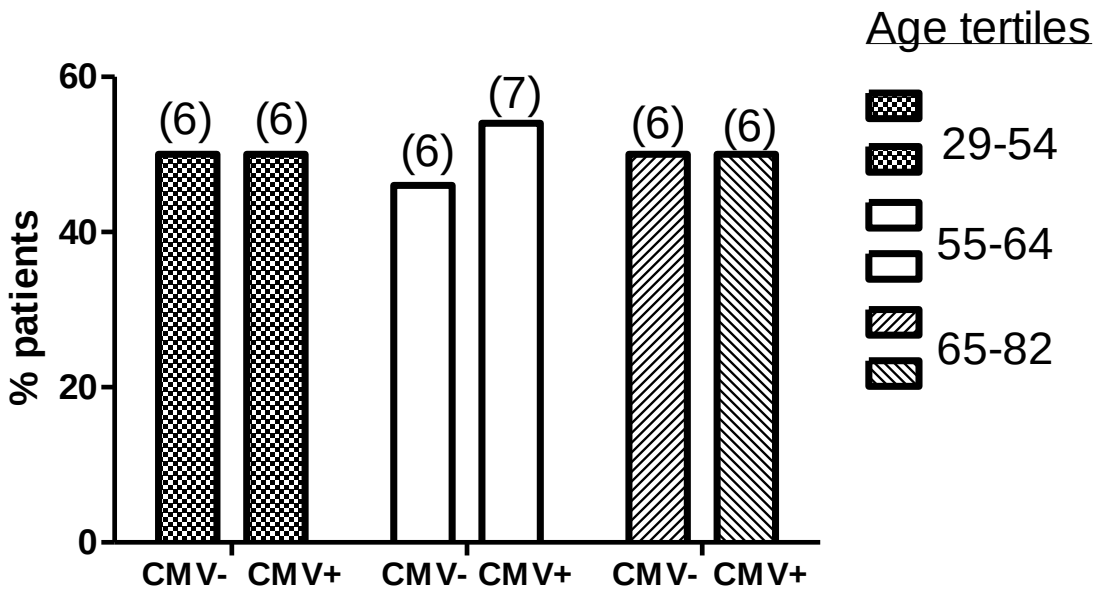


Figure 5-5: The distribution of CMV positivity is even across the three age groups. There were no significant differences between the groups using the Kruskal-Wallis ANOVA test. Pooled RA (n=37) (Age 29-54 n=12, Age 55-64 n=13, Age 65-82 n=12).

5.8 $CD3^+CD8^+CD28^-$ cells and CMV status correlation with Disease Activity (using DAS28)

There was no correlation between the percentage or absolute number of $CD3^+CD8^+CD28^-$ T cells and DAS28 in pooled RA patients (**Figure 5-6A & 5-6B**).

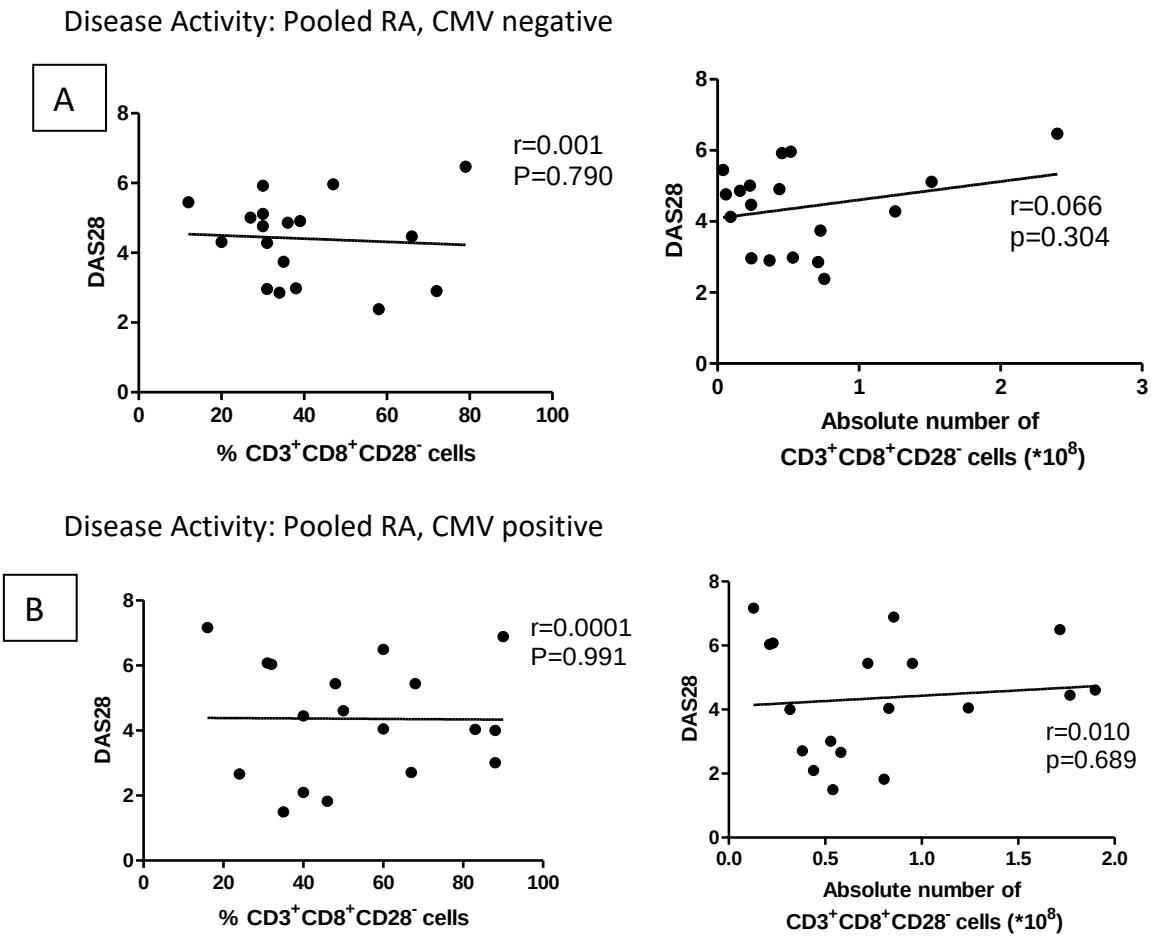


Figure 5-6: There is no correlation between $CD3^+CD8^+CD28^-$ cells, DAS28 score and CMV status. Correlation was determined using non-parametric Spearman's rank analysis, * $p<0.05$. Pooled RA (n=36) (A is CMV negative, n=18) (B is CMV positive, n=18). One patient did not have a baseline DAS28 so was excluded.

5.9 $CD3^+CD8^+CD28^-$ cells and CMV status correlation with CRP

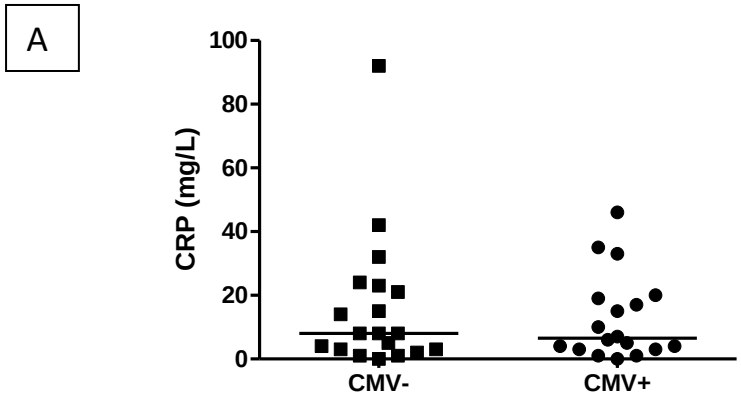
CRP levels were similar in CMV positive and negative patients (**Figure 5-7A**)**.**

Correlation between CRP and percentage of $CD3^+CD8^+CD28^-$ T cells was assessed in CMV negative (**Figure 5-7B**) and CMV positive patients (**Figure 5-7C**). There is a weak but statistically significant correlation between the percentage of $CD3^+CD8^+CD28^-$ cells and CRP in CMV positive pooled RA patients (**Figure 5-7C**).

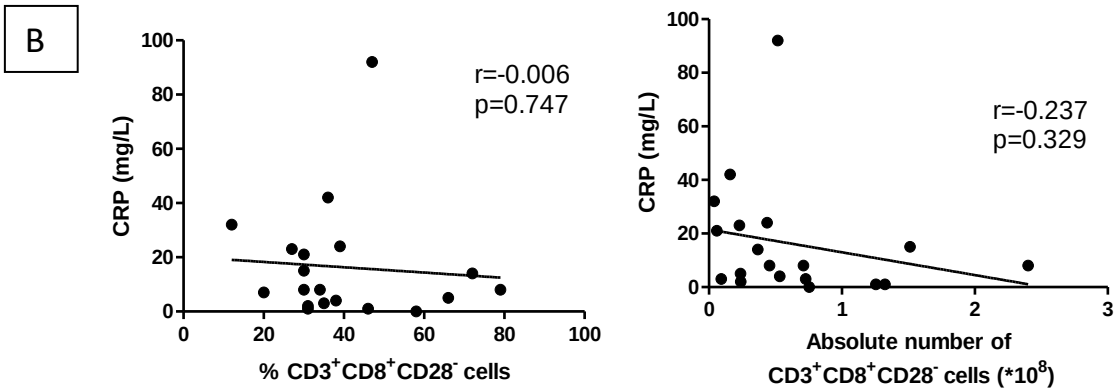
There was no correlation between the absolute number of $CD3^+CD8^+CD28^-$ T cells and CRP in pooled RA (**Figure 5-7B & 5-7C**).

CRP has not previously been found to be a critical factor for $CD3^+CD8^+CD28^-$ cell accumulation. Pera et al (2018) found that the difference in $CD4^+CD28^-$ and $CD8^+CD28^-$ T-cells frequencies between CMV positive individuals and CRP levels was not statistically significant[351].

CRP: Pooled RA



CRP: Pooled RA, CMV negative



CRP: Pooled RA, CMV positive

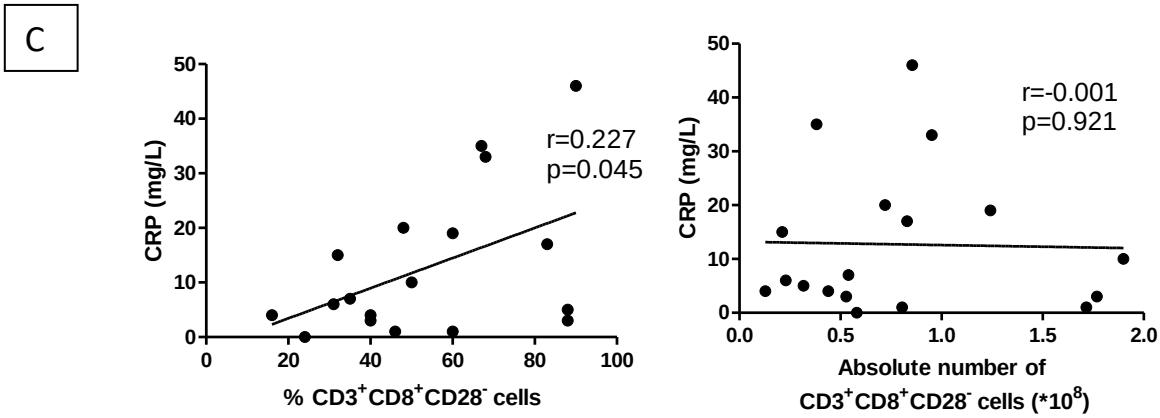


Figure 5-7: There is a weak but statistically significant correlation between the percentage of $CD3^+CD8^+CD28^-$ cells and CRP in CMV positive pooled RA patients. Correlation was determined using non-parametric Spearman's rank analysis, *p<0.05. Pooled RA (A, n=37), CMV negative (B, n=19) CMV positive (C, n=18)

5.10 $CD3^+CD8^+CD28^-$ cells and CMV status correlation with ESR

Correlation between ESR and CMV status was assessed by dividing the pooled RA (n=37) into CMV negative (**Figure 5-8B**) and CMV positive groups (**Figure 5-8C**). There was no correlation between the percentage or absolute number of $CD3^+CD8^+CD28^-$ T cells and ESR in CMV positive or CMV negative pooled RA patients (**Figure 5-8A & 5-8B**).

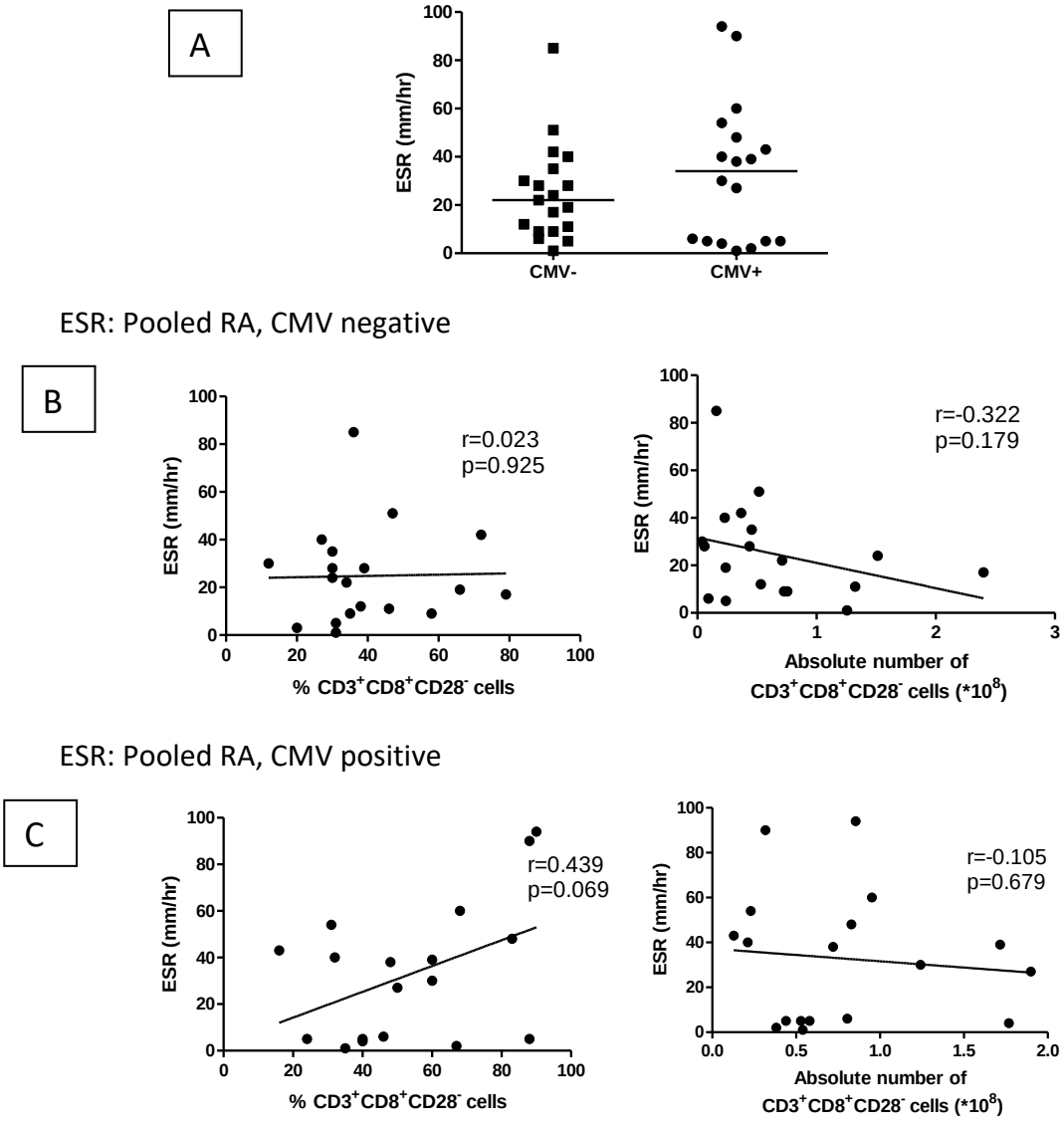


Figure 5-8: There is no correlation between $CD3^+CD8^+CD28^-$ cells and ESR in CMV positive or CMV negative patients. Correlation was determined using non-parametric Spearman's rank analysis, * $p<0.05$. Pooled RA (A, n=37), CMV negative (B, n=19) CMV positive (C, n=18).

CD28 and IL-10 with CMV seropositivity

Percentage of IL-10 producing $CD3^+CD8^+CD28^+$ and $CD3^+CD8^+CD28^-$ T cells in CMV positive and negative patients is shown in **Figure 5-9A**. Although the trend was that a higher percentage of cells produced IL-10 if they were CMV positive rather than CMV negative, this difference was not statistically significant. There was no correlation between the absolute number of cells producing IL-10 and CMV seropositivity (**Figure 5-9B**).

$CD28^-$ and IL-10: Pooled RA

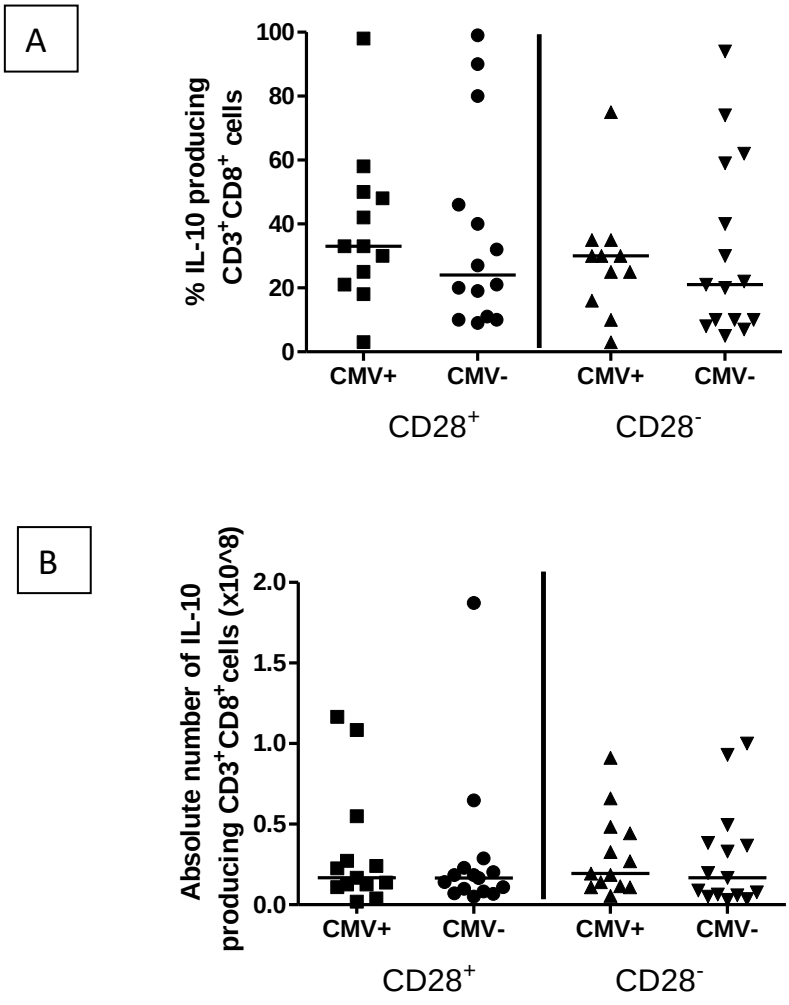


Figure 5-9: There is no difference between CMV positive or negative $CD3^+CD8^+$ cells producing IL-10. Graphs represent median, * $p<0.05$ using Kruskal-Wallis ANOVA test. Pooled RA (n=26, CMV positive= 11, CMV negative =15

5.12 KLRG1 and IL-10 with CMV seropositivity

The percentage of IL-10 producing $CD3^+CD8^+CD28^-KLRG1^+$ and $CD3^+CD8^+CD28^-KLRG1^-$ T cells in CMV positive and negative patients was shown in **Figure 5-10**. In the CMV positive population, there was a higher percentage and absolute number of cells producing IL-10 if they were $KLRG1^+$ rather than $KLRG1^-$ (**Figure 5-10A & 5-10B)**.

IL-10 provides time for CMV to replicate and contributes the virus' persistence within the host. Although human CMV encodes its own unique IL-10 which restricts $CD8^+$ T cell-mediated immune pathology[352],[353]. However, the lack of correlation between CMV and $CD3^+CD8^+CD28^-$ IL-10 producing cells suggests that the control of IL-10 has more to do with KLRG1 than CMV status.

KLRG1 and IL-10: Pooled RA

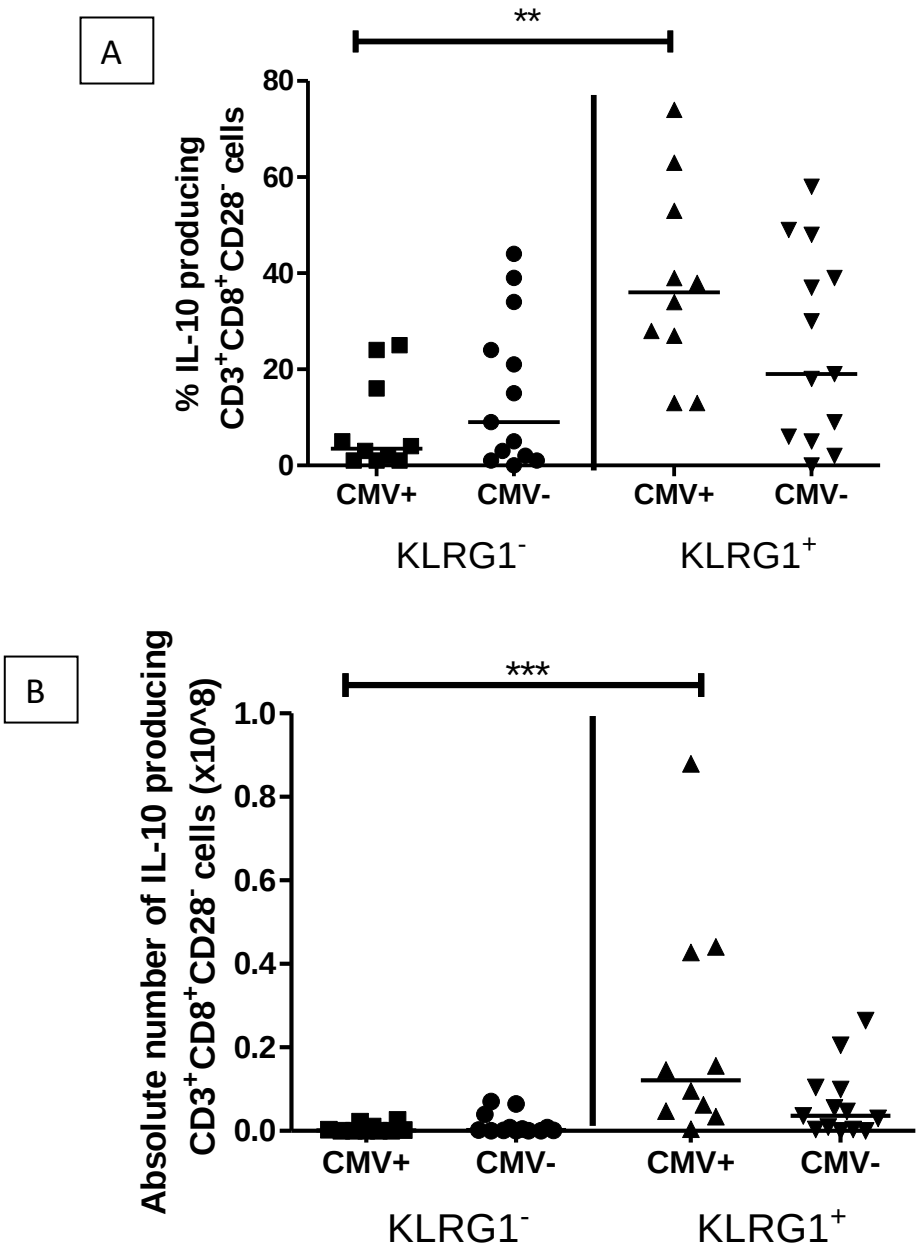


Figure 5-10: The percentage and absolute number of $CD3^+CD8^+CD28^-KLRG1^+$ cells producing IL-10 is higher than $KLRG1^-$, in CMV positive patients Graphs represent median, **p<0.001, ***p<0.0001 using Kruskal-Wallis ANOVA test. Pooled RA (n=23, CMV positive=10, CMV negative=13).

5.13 $CD3^+CD8^+CD28^-KLRG1^+$ cells and CMV status in RA patients

The percentage of $CD3^+CD8^+CD28^-KLRG1^+$ T cells was increased in CMV positive pooled RA patients in comparison to CMV negative patients (**Figure 5-11A**). The absolute number of cells for pooled RA was similar for CMV positive and negative.

The trend of the percentage of $CD3^+CD8^+CD28^-KLRG1^+$ T cells in Early RA was also increased in CMV positive patients but this was not statistically significant (**Figure 5-11B**, p=0.052). The absolute number of cells for Early RA was similar for CMV positive and negative.

Persistent viral infections like CMV, are known to increase highly differentiated $KLRG1^+$ T cells in human peripheral blood[354,355]. However, this has not been looked at in the context of RA.

In addition, the percentage and absolute number of $CD3^+CD8^+CD28^-$ cells with $CD57^+$, $CD45Ra^+$ and $CD56^+$ cells are higher in CMV positive pooled RA patients compared to CMV negative. These findings are presented in the **Appendix 7.2**.

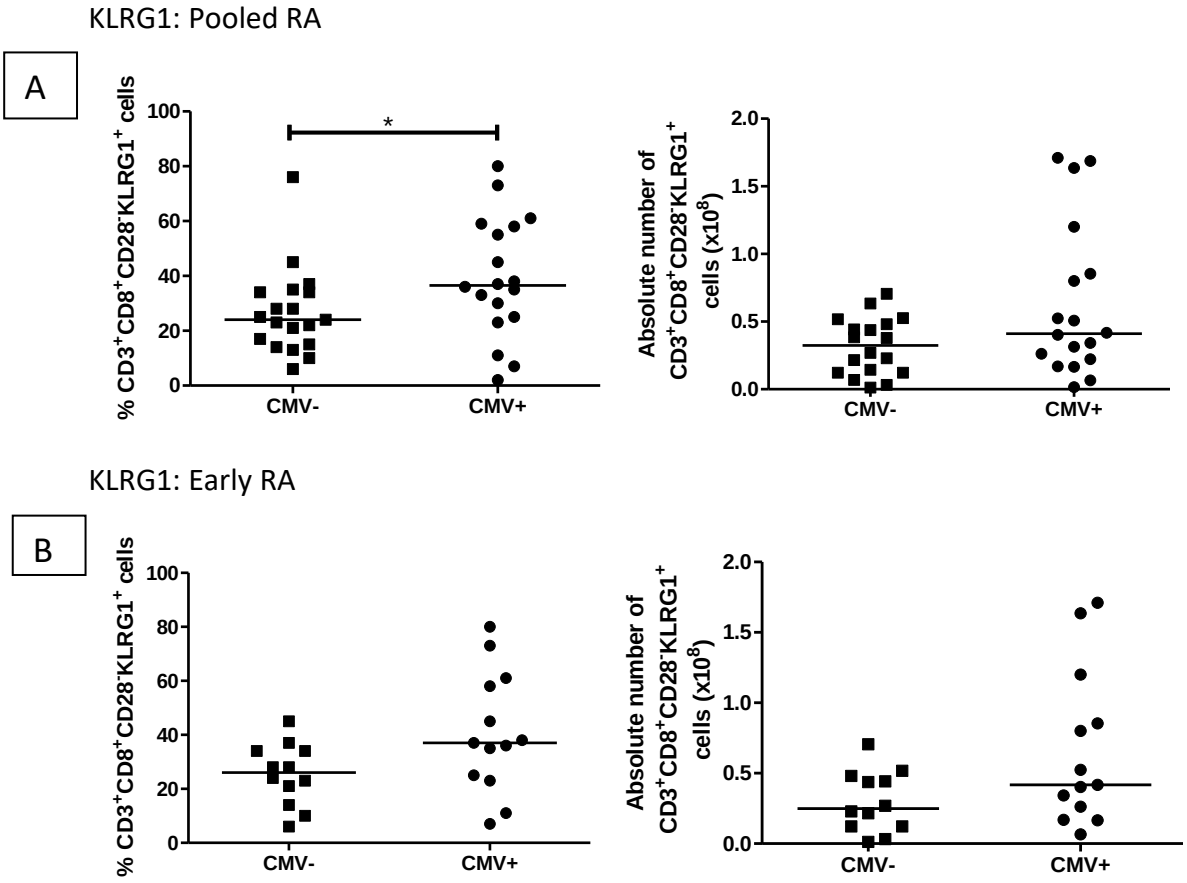


Figure 5-11: Percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells are higher in CMV positive pooled RA patients. Graphs represent median, * $p<0.05$ using Mann-Witney U Test. Pooled RA (A, n=37), Early RA (B, n=25).

5.14 Early RA patient treatment response rate at 6 months and CMV status (using DAS28)

To establish whether the increase in $CD3^+CD8^+CD28^-KLRG1^+$ cells in the non-responder group in Chapter 4 (**Figure 4-11**), was indicative of cell phenotype or CMV status, CMV seropositivity was looked at across the responder groups (**Figure 5-12**). Studies have found that EBV positivity correlated to a favourable treatment response in established RA patients but a correlation between CMV and Early RA has not been looked at previously[356].

As before, the Early RA group was separated into responders and non-responders to treatment according to their DAS28 score which was taken a baseline and 6 months after DMARD treatment (n=22)[357]. Responders are defined as those patients whose DAS28 reduced by >1.2. CMV seropositivity was not found to be higher in the non-responder group. Indeed, CMV seropositivity was similar within the responders (4/7) and non-responders (8/15).

Responders: Early RA

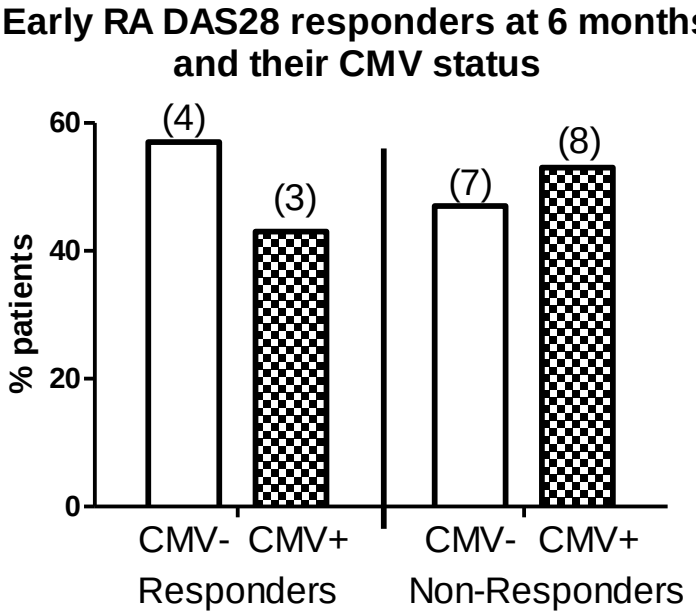


Figure 5-12: CMV seropositivity is equally distributed across the groups of responders and non-responders in Early RA. Statistical differences between the

groups was established using Kruskal-Wallis ANOVA Test. Early RA (n=22). The number of patients in each column is in brackets. Three patients did not have a follow up DAS28 score recorded so were excluded from the analysis.

5.15 Discussion

Chapter 3 showed that that $CD3^+CD8^+CD28^-$ cells are raised in RA, including the novel pattern observed in early RA. In this Chapter, the subdivision into Early RA further revealed a novel finding, whereby both an increased percentage and absolute number of $CD3^+CD8^+CD28^-$ cells were observed in CMV positive patients. The rise could be directly due to the CMV infection because CMV infection is known to increase the absolute number of lymphocytes[358]. The absolute number of $CD3^+CD8^+CD28^-$ cells is higher in Early RA but this is not the case in pooled RA, which suggests that they are involved early on in the RA disease process.

Results in Chapter 4 suggests that $CD3^+CD8^+CD28^-KLRG1^+$ cells were higher in the group of Early RA non-responders to treatment. Given that $CD3^+CD8^+CD28^-KLRG1^+$ cells are also higher in CMV positive patients, CMV could have been the factor responsible for the higher levels of KLRG1 in the non-responders. However, re-examining the groups of responders and non-responders and analysing the CMV seropositivity across these groups, showed that CMV was not higher in the non-responder group compared to the responder group. This would indicate that CMV is not responsible for the higher levels of KLRG1 in the non-responders.

This chapter has shown that CMV positive RA patients have increased levels of $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^-KLRG1^+$ cells than CMV negative patients. Both of these subsets of cells were shown to correlate with clinical and serological factors in Chapter 3 and 4. The finding that CMV correlates with CRP is in keeping with concept that CRP increases in inflammation, infection and tissue injury. However, the release of CRP from hepatocytes is stimulated by IL-6, IL-1 and TNFα, cytokines that are increased in RA but not normally raised in CMV infection (unlike IFNγ)[359,360,361]. This leads me to believe that RA was the primary factor correlated with CRP, rather than CMV. Accordingly, it is notable that CMV did not correlate with RA disease duration, DAS28, ESR, ACPA or RF positivity. Instead CMV correlated with $CD3^+CD8^+CD28^-$ cells in RF negative patients. This shows that when RF was present, this was a greater influence on $CD3^+CD8^+CD28^-$ cells than CMV.

Chapter 4 found that $CD3^+CD8^+CD28^-KLRG1^+$ cells were increased in Early RA patients that did not respond to treatment at six months. The finding that CMV seropositivity was equally distributed across the responder and non-responder groups disproved the hypothesis that CMV was related to treatment response in RA. Although CMV is related to $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^-KLRG1^+$ cells, KLRG1 positivity appears to have a greater influence on treatment response than CMV.

$CD3^+CD8^+$ cells producing IL-10 do not correlate with CMV or CD28 positivity. However, of the CMV positive patients, $CD3^+CD8^+CD28^-KLRG1^+$ cells produce more IL-10 than $KLRG1^-$ cells. This supports findings in existing literature that KLRG1 cells are not anergic but in fact active[362]. If these cells are producing more IL-10, they could be immunoregulatory. Indeed, KLRG1 may be a positive cell surface marker of $CD3^+CD8^+$ T reg cells. The expansion of $CD3^+CD8^+CD28^-KLRG1^+$ T regs may be secondary to persistent immune activation.

The demographics of the RA patient cohort reflect the RA population. The mean age of the Early RA CMV negative group was less than the other groups. This is in keeping with the evidence that CMV positivity correlates with increase of age[315]. An unexpected finding was that CMV distribution across the age tertiles was similar. However, only RA patients were included in this analysis. Given that some studies have implicated CMV in the pathogenesis of disease, there could be a higher prevalence of CMV than average in the younger patients of the group than the average for this age bracket[329,363]. The youngest patient in the cohort was 29 years old, which when considering that 15% of 1-4 year olds are CMV positive rising to 80% in >65 year olds, it is foreseeable that 50% of the patients are CMV positive in the 29-54 age group[315].

A limitation of this Chapter was that the number of Established RA patients' cohort was low (12/37). Therefore, subdivision into Established RA would not create sufficient power for sub-analysis into this group. Increasing the sample size,

particularly the Established RA group, would increase the power of the study and allow for further sub-group analysis.

5.16 Conclusions

- The percentage of $CD3^+CD8^+CD28^-$ and $CD3^+CD8^+CD28^-KLRG1^+$ cells is higher in CMV positive than CMV negative Early and Pooled RA patients.
- $CD3^+CD8^+CD28^-$ cells are higher in male CMV positive pooled RA patients than female.
- In RF negative RA patients, the percentage of $CD3^+CD8^+CD28^-$ cells is higher in CMV positive than CMV negative patients.
- There is weak positive correlation of $CD3^+CD8^+CD28^-$ cells with CRP.
- CMV distribution across age and responders/ non-responders in RA is similar.
- The percentage of $CD3^+CD8^+CD28^-CD57^+$/ $CD45Ra^+$ and $CD56^+$ cells is higher in CMV positive than CMV negative in Pooled RA patients.
- CMV positive $CD3^+CD8^+CD28^-KLRG1^+$ cells produce more IL-10 than $KLRG1^-$ cells.

6 General Discussion

6.1 $CD28^-$ biology – identification of a new subset

This MD thesis has shown for the first time that $CD3^+CD8^+CD28^-$ cells are increased in Early RA as well as Established RA[364]. Furthermore, $CD3^+CD8^+CD28^-$ cells correlate with disease duration in Early RA, which suggests that expansion is related to Early RA disease pathogenesis. Whether $CD3^+CD8^+CD28^-$ cells are driving disease pathogenesis or a result of immune activation is unclear given that $CD3^+CD8^+CD28^-$ are normally associated with immunosuppression or immunosenescence.

Compared to $CD4^+$ T cells, $CD8^+$ T cells are more susceptible to lose CD28. This is due to $CD8^+$ T cells containing a single β-bound protein complex, making it more likely to lose nuclear proteins bound to the β motif of the CD28 promoter and result in CD28 down-regulation[365]. $CD4^+CD28^-$ T cells from RA patients have been shown to support synoviocyte proliferation better than conventional $CD4^+CD28^+$ T lymphocytes[366]. This could suggest that $CD8^+$ cells are more likely to become $CD28^-$ and could support synoviocyte proliferation.

6.2 KLRG1 as a cell surface marker of CD8 T reg

In Chapter 3 of this thesis, I found that a high percentage of $CD3^+CD8^+CD28^-$ T cells that express CD57, also express KLRG1. CD57 is found on many $CD3^+CD8^+CD28^-$ cells[367,368]. $CD3^+CD8^+CD28^-$ cells can be immunosuppressive but whether CD57 is expressed by $CD3^+CD8^+CD28^-$ immunosuppressive cells is inconclusive. Vlad et al (2011) suggested that $CD3^+CD8^+CD28^-$ suppressor cells do not express CD57 and other studies have shown that the majority of $CD8^+CD28^-CD57^+$ cells are actually cytotoxic, expressing granzymes, perforin, IFNγ and TNFα[369,370,371]. $CD8^+CD28^-CD57^+$ cells are raised in autoimmune arthritis and show a highly cytotoxic phenotype associated with more severe disease[372]. As is so frequently the case in the immune system, the environment is fundamental to the functional role. For example, in different disease states, $CD8^+CD28^-CD57^+$ take on an alternative role: for example $CD8^+CD28^-CD57^+$ display an immunosuppressive phenotype in CMV infection, SLE

and myeloma[373,374,375]. The place for $CD8^+CD28^-CD57^+$ cells within autoimmunity is further complicated by the finding that $CD8^+CD28^-CD57^+$ cells also produce IL-5, which is increased in autoimmune asthma patients and drives differentiation of eosinophils[376]. Thus, the development of KLRG1 as amarker for a subset of $CD3^+CD8^+CD28^-$ cells could be crucial to our understanding of the pathogenesis of RA.

IL-10 is an immunosuppressive cytokine previously associated with $CD8^+CD28^-$ T cells. However, not all $CD3^+CD8^+CD28^-$ cells express IL-10. In this thesis, $CD3^+CD8^+CD28^-KLRG1^+$ cells were found to be a distinctive subtype of $CD3^+CD8^+CD28^-$ cells which produce more IL-10 than $CD3^+CD8^+CD28^-KLRG1^-$ cells. This suggests that KLRG1 may be a cell surface marker of $CD8^+$ T regs. The identification of this subset of $CD3^+CD8^+CD28^-KLRG1^+$ cells could be a potential target for the purpose of treatment or patient stratification, however further research into particularly the functional properties of this cell type would be indicated.

In RA, Ceeraz et al (2013) found $CD3^+CD8^+CD28^-$ cells in healthy controls suppressed autologous lymphocyte proliferation but in patients with RA, they were dysfunctional[197]. One explanation was that the IL-10 receptor expression on $CD3^+$ cells in RA was significantly lower than healthy controls[364]. So even though there were high levels of IL-10, $CD3^+$ T cells in RA might not be suppressed. Chronic TNF-α stimulation was implicated since post anti-TNF-α therapy, responder $CD3^+$ T cells increase their IL-10 receptor expression and the $CD3^+CD8^+CD28^-$ cells partially restored their ability to suppress lymphocyte proliferation. Roberts et al (2017) have somewhat reinforced this concept by showing that TNF inhibitors act directly on $CD4^+$ T cells and promote their IL-10 expression[377].

Melis et al (2013) took adult patients with knee synovitis and compared peripheral blood and synovial fluid samples. They found that $CD8^+KLRG1^+$ cells produced more TNF-α than $KLRG1^-$ cells[378]. As well as the numbers being small (n=21), the cohort included a combination of RA (n=10) and different types of seronegative spondyloarthropathy (n=11). This leads me to question the validity of the finding given that they are considerably different arthritides. The study does however,

reinforce that KLRG1 cells are not exhausted which is also backed up further by Reiley et al (2010) who found that $CD4^+KLRG1^+$ T cells make high levels of IFNγ[379].

$CD3^+CD8^+CD28^-KLRG1^+$ cells were associated with treatment response in patients with Early RA at six months. In contrast, $CD3^+CD8^+CD28^-$ cells were not associated with treatment response in patients with Early RA at six months. This indicates that the unique $KLRG1^+$ subtype of $CD3^+CD8^+CD28^-$ cells are active in RA, and as a result could be targeted for clinical utilisation.

6.3 $KLRG1^+$ cells in RA

A higher percentage of $CD3^+CD8^+CD28^-KLRG1^+$ cells was associated with RF and ACPA as well as ESR and to a lesser extent CRP, especially in patients with Early RA. Given that these are established markers of poor prognosis in RA, it reinforces the importance of this subtype of T regs in the course of RA. This is further supported by the association with poor response to therapy at six months. However, the precise reason is unclear and requires further investigation.

6.4 CMV and IL-10 in RA

CMV infection increases $CD3^+CD8^+CD28^-KLRG1^+$ cells, which produce more IL-10. IL-10 limits virus-induced weight loss, pro-inflammatory cytokine production and apoptosis[380]. These effects cumulatively encourage latency of the virus within the host. To reduce the viral load, IL-10 receptor blockade or TNF receptor agonist antibody can be used[381]. This was associated with an increased accumulation of $CD4^+$ T cells expressing IFNγ (**Figure 6-1**). Increases in peripheral IL-10 producing cells suppress antiviral immunity enabling CMV to exploit the immune pathway to promote chronicity[382].

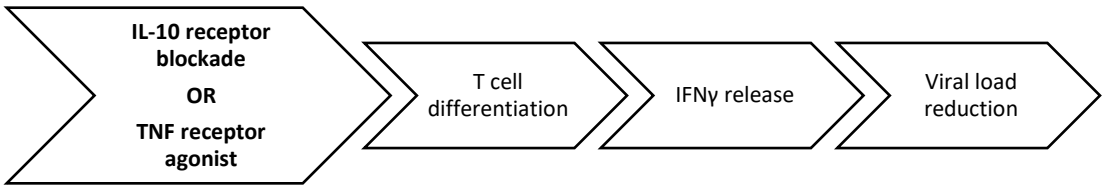


Figure 6-1: CMV immune cascade leading to viral load reduction

The percentage of $CD3^+CD8^+CD28^-$ cells was higher in CMV positive males compared to CMV positive females. This could be an explanation of the unexpected increase in $CD3^+CD8^+CD28^-$ cells and $CD3^+CD8^+CD28^-KLRG1^+$ cells in the male RA subjects compared to female in Chapters 3 and 4. The connection between the sex and CMV seropositivity could be the reason for the significant increase in $CD3^+CD8^+CD28^-$ cells. Indeed, Villacres et al (2004) also showed that sex affects the T cells response to CMV; with men having higher TNF secretion and women predominantly secreting IFNγ[383].

6.5 KLRG1 is associated with poor prognosis in RA but not CMV

CMV infection is associated with an increase in $CD3^+CD8^+CD28^-$ T cells[384]. The T cell immune response to CMV may be associated with non-responders to initial treatment in early RA[385]. In conjunction with the finding that CMV seropositivity is associated with radiographic joint erosions, this could suggest that there is an association between CMV and negative long-term outcomes in RA. CMV patients should arguably be targeted with more comprehensive therapy such as biologic DMARDs[386].

In this thesis, a statistically significant correlation was found between the percentage of $CD3^+CD8^+CD28^-$ cells and CRP in CMV positive RA patients. RF negative RA patients have a higher percentage of $CD3^+CD8^+CD28^-$ cells if they are CMV positive than CMV negative ($p<0.05$). In spite of this, CMV seropositivity was similar across the responders and non-responders. This brings into question the association of CMV and its role in response to therapy and suggests this may be secondary to expansion of $CD3^+CD8^+CD28^-KLRG1^+$ T cells.

CMV up-regulates $CD3^+CD8^+CD28^-KLRG1^+$ T cells and enables these T regs to secrete CMV viral specific IL-10 in order to maintain latency in its host. As T regs, these cells would normally be expected to suppress inflammation. However, in the highly inflammatory environment of early RA, the TNFα rich milieu appears to cause dysregulation of $CD3^+CD8^+CD28^-KLRG1^+$ T cells. This affects the ability of the T regs

to successfully suppress and control the inflammation, leading to full blown RA. The hypothesis of this study is summarised in (**Figure 6-2**).

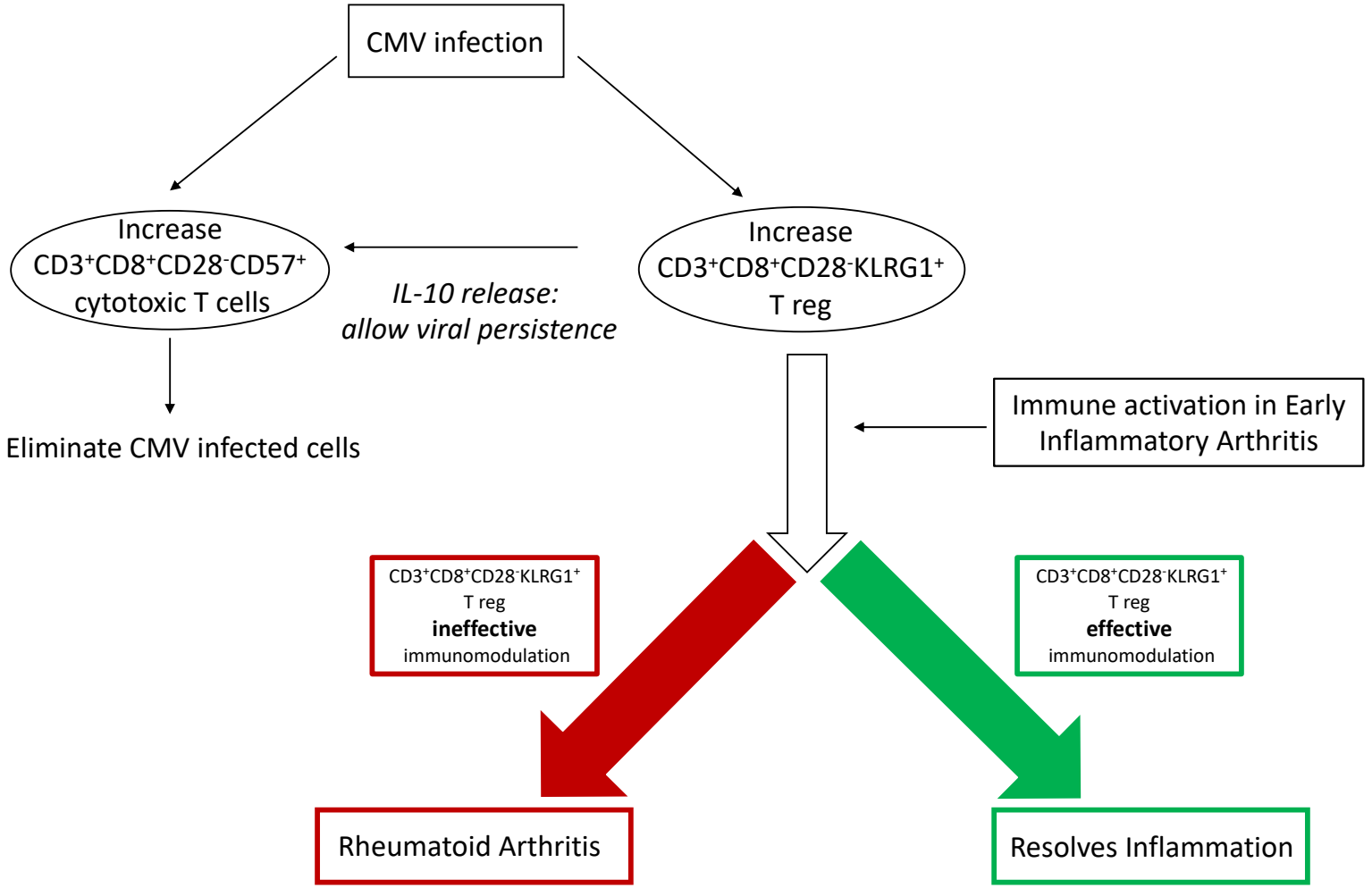


Figure 6-2: Proposed relationship between CMV, T cell subsets and Rheumatoid Arthritis.

6.6 Limitations

There are limitations to the methods of this study. RA is a heterogeneous disease, and this was a non-controlled observational cohort. The patients were treated with different DMARDs, some with and some without corticosteroids or NSAIDs.

Concurrent comorbidities could be influencing six month responder rates. Subjective pain scores, an integral part of the DAS28 disease activity score, could be influenced by pain syndromes that co-exist with RA like Fibromyalgia. Pain assessment questionnaires and factoring in comorbidities would have helped distinguish these impacts on the patient outcomes.

Despite the power calculation to estimate the sample size needed to gain meaningful conclusions, increasing sample size could benefit the study. A larger cohort of patients would make the findings more reliable because the results would be less swayed by individual patients. To do this, an option would be to extend the study to different sites. Consideration would have to be given to challenges such as different ACPA and RF pathology testing in different laboratories and characteristics[387]. There may be considerable national or international variation.

The patient cohort reflected the demographics seen in the general RA population in the UK, with regards to sex, age and levels of disease activity[388]. However, the healthy controls average age was younger than the RA patients. Bearing in mind that age has also previously been found to affect $CD3^{+}CD8^{+}CD28^{-}$ cells, closer age matching could improve the validity of the findings from the data.

Baseline and six month analyses were completed for the Early RA patients. This study would benefit from longer term follow up until a year or even five years to establish the RA burden.

Multiple comparisons have been carried out on the data. This number of comparisons could by chance show a difference with statistical significance and therefore must be acknowledged.

6.7 Directions for future work

To further explore the pathobiologic relevance of $CD3^+CD8^+CD28^-KLRG1^+$ cells, I would like to use immunohistochemistry to analyse RA synovia. Ultrasound guided biopsy samples of affected joints paired with peripheral blood could reveal where $CD3^+CD8^+CD28^-KLRG1^+$ cells predominate and therefore further establish their role in the disease process.

Ultrasound is increasingly used in clinical practice to assess joint involvement in early RA. As well as being used in the diagnosis of RA, ultrasound is used in the monitoring of joint disease. Comparing ultrasound scoring to $CD3^+CD8^+CD28^-KLRG1^+$ cells could reveal a more accurate analysis of progression of disease in contrast to the current standard DAS28 scoring[389,390].

CMV correlates with erosive disease in RA. Correlation of $CD3^+CD8^+CD28^-KLRG1^+$ with erosive disease would be of added value for this thesis. Further comparison with other viruses would be of value for investigation.

Health and wellbeing assessments are subjective measurements of patient outcome. The gold standard for measuring functional status is the Health Assessment Questionnaire (HAQ)[391]. Comparison of $CD3^+CD8^+CD28^-KLRG1^+$ cells could be compared with HAQ outcomes to incorporate a more holistic approach to this analysis.

A functional study to show the role of KLRG1 in immunoregulation in both animal studies and ex vivo studies would be another area of research. In addition, transcriptome profiling would enable researchers to characterise gene expression and activity of $CD3^+CD8^+CD28^-KLRG1^+$ cells.

$CD8^+$ T cells are higher in RA but also appear to play a role in psoriatic arthritis. Menon et al (2014) found that IL-17^+CD8^+ cells are enriched in the Psoriatic Arthritis (PsA) joint but not the RA joint[392]. In addition, the PsA association with HLA class I would indicate a role of $CD8^+$ T cells in the pathogenesis[393]. For that reason, it would be interesting to analyse the role of $CD3^+CD8^+CD28^-KLRG1^+$ cells other inflammatory arthritides, particularly PsA.

Finally, a recent study has shown that CMV is associated with increased risk of cardiovascular (CV) death by over 20%[394]. RA is also an independent risk factor for CV disease[395]. There is increasing evidence that CV risk is associated with $CD4^+CD28^-$ cells and atherosclerosis. Because $CD4^+CD28^-$ and $CD8^+CD28^-$ cells have many similarities, there is a need to consider whether there is also an association between $CD8^+CD28^-$ and atherosclerosis[396,397,398]. There is a significant association of CMV infection with elevated IL-10 in the patients with coronary artery disease. Further establishment of the role of $CD3^+CD8^+CD28^-KLRG1^+$ between CMV, RA and CV disease would be appealing **(Figure 6-3)**[399].

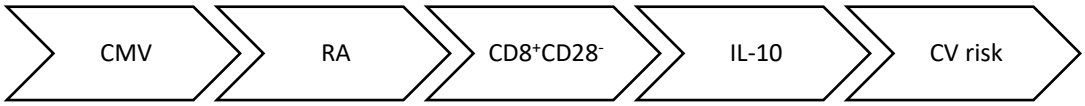


Figure 6-3: Proposed association between CMV and Cardiovascular risk.

6.8 Conclusions

$CD3^+CD8^+CD28^-$ T cells have been found to be increased in several inflammatory disorders and have an effector function of producing inflammatory cytokines and cytotoxicity. Their role in the pathogenesis of autoimmune diseases and particularly RA remains unknown. $CD3^+CD8^+CD28^-KLRG1^+$ cells produce IL-10 and are associated with Early RA patients who do not respond to treatment, suggesting that this subset of T cells have an important role in the immunopathogenesis of RA.

7 Appendix

7.1 Intracellular cytokines production of $CD3^+CD8^+CD28^-KLRG1^+$ cells in pooled RA versus Controls

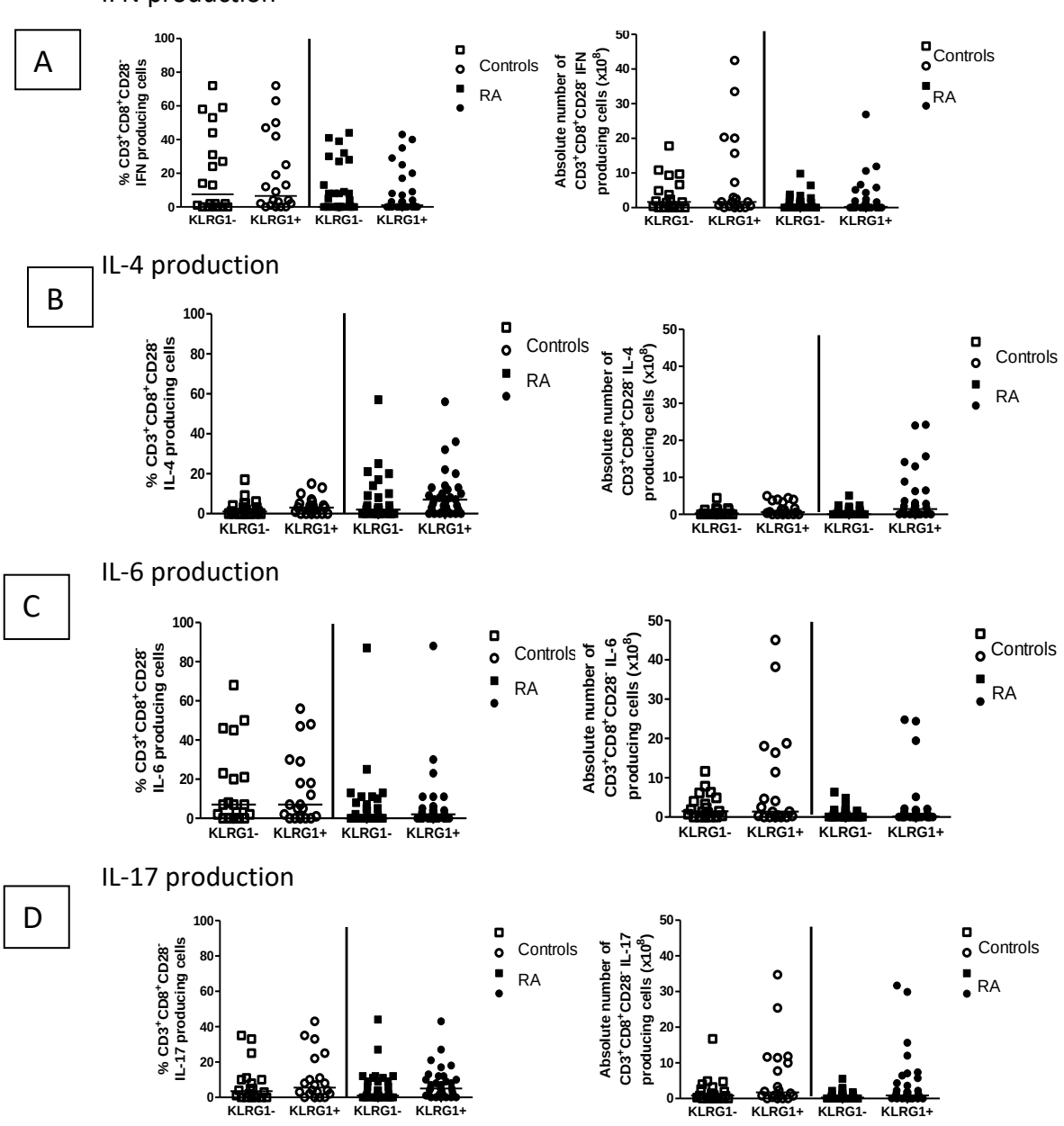


Figure 7-1: $CD3^+CD8^+CD28^-KLRG1^{+/-}$ T cells produce very little IFN, IL-4, IL-6 and IL-17. The percentage and absolute number of $CD3^+CD8^+CD28^-KLRG1^{+/-}$ T cells are presented with controls on left and pooled RA patients on the right. Graphs represent median, with statistical analysis completed using the Kruskal-Wallis ANOVA test. Controls (n=19), Pooled RA (n=31).

7.2 CMV correlation with other $CD3^+CD8^+CD28^-$ cells markers (CD57, CD45Ra and CD56)

The percentage and absolute number of $CD3^+CD8^+CD28^-$ cells which are $CD57^+$, $CD45Ra^+$ and $CD56^+$ are higher in CMV positive than CMV negative pooled RA patients (**Figure 7-2A & 7-2B**). These markers have previously been found to be higher in CMV positive subjects, but have not been looked at in an RA cohort[327,328,400].

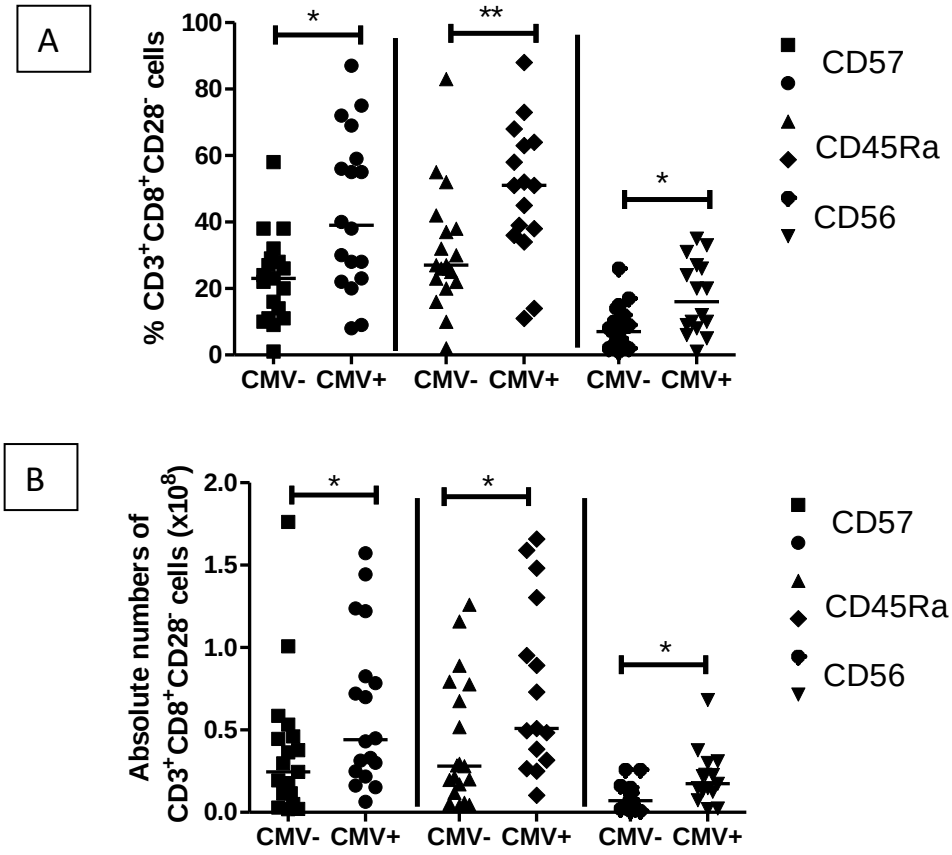


Figure 7-2: The percentage and absolute number of $CD3^+CD8^+CD28^-$ $CD57^+/CD45Ra^+/CD56^+$ cells are higher in CMV positive RA patients. Graphs represent median, * $p<0.05$, ** $p<0.01$ using Mann-Witney U Test. CD57 (n=37, CD45Ra (n=35), CD56 (n=35).

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