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**The pathogenicity and regulation
of CD161⁺ Th17 lineage cells
in rheumatoid arthritis**

2015

Sharee Ann Basdeo

B.A. (Mod.) Molecular Medicine



A thesis submitted to

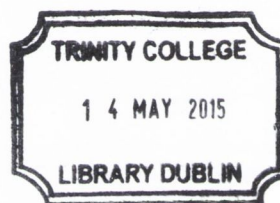
Trinity College Dublin

as completion of the degree of Doctor of Philosophy

Supervisor: Prof. Jean Fletcher

Translational Immunology

Schools of Medicine and Biochemistry/Immunology.



Thesis 10523

Declaration of Authorship

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A handwritten signature in black ink, appearing to be 'Sharee Basdeo', with a long horizontal flourish extending to the right.

Sharee Basdeo.

Dedicated to the memory of my beloved Pappy

who taught me,

amongst many things,

that if at first you don't succeed

try and try again.

Abstract

Pathogenic Th17 cells, which produce their signature cytokine IL-17, play a key role in autoimmune diseases such as rheumatoid arthritis (RA) and therefore, have been the target of recently developed therapeutics. On the other hand, Treg cells act to constrain effector T cell responses and play an important role in maintaining tolerance. The aim of this study was to investigate the regulation of Th17 cells by Treg cells in the context of the RA joint. In recent years it has emerged that both Treg and Th17 cells exhibit significant functional plasticity, particularly under conditions of inflammation. However, the role of this plasticity in autoimmune inflammation is still unclear. Th17 cells originate from CD161⁺ cells and retain expression of CD161 throughout their lifespan, despite exhibiting functional plasticity and becoming IFN- γ -producing cells, or so-called ex-Th17 cells. In this study, the plasticity and pathogenicity of both Th17 and Treg cells extracted from the RA joint was examined. In addition, the ability of Treg cells to suppress different populations of effector T cells was assessed.

The data demonstrated that CD161⁺ Th17 lineage cells were highly enriched in the RA joint and the majority expressed IFN- γ but not IL-17, indicating that these cells were mostly comprised of ex-Th17 type cells rather than bona fide Th17 cells. CD161⁺ Th cells were highly polyfunctional in terms of cytokine production. In addition, CD161⁺ Th cells had significantly longer telomeres and higher expression of anti-apoptotic Bcl2 compared with CD161⁻ Th cells, indicating that CD161⁺ Th cells may have increased capacity for survival and replication. When taken from the hypoxic environment of the RA joint, CD161⁺ Th cells expressed significantly more HIF-1 α compared with CD161⁻ Th cells. Synovial fibroblast cells cultured with CD161⁺ Th cell supernatants had enhanced expression of IL-8, IL-6, RANKL and ICAM-1 compared with CD161⁻ Th cell supernatants, suggesting that CD161⁺ Th cells may significantly contribute to the propagation of fibroblast hyperplasia, autoimmune inflammation and bone degradation.

Treg cells from the RA joint exhibited a functional phenotype but nonetheless, when compared with those from healthy controls, exhibited reduced ability to suppress autologous effector cells. The differential Treg cell mediated suppression of CD161⁺ and CD161⁻ Th cells was assessed and the data showed that effector CD161⁺ Th cell proliferation and cytokine production were significantly less suppressed by Treg cells compared with the suppression of effector CD161⁻ Th cells. When CD4⁺ T cells were sorted into discrete Th1, Th17 and ex-Th17 cells, it was evident that CD161⁺ ex-Th17 cells were inherently resistant to Treg cell mediated suppression whereas bona fide CD161⁺ Th17 cells were adequately suppressed.

These data suggest that CD161⁺ Th cells play a key role in propagating inflammation in RA by secreting multiple pro-inflammatory cytokines which can signal to immune cells in the synovium and to surrounding fibroblast cells. These functionally plastic CD161⁺ Th cells were less susceptible to suppression by Treg cells, suggesting that they may evade regulation in the RA joint. Moreover, CD161⁺ Th cells exhibited characteristics indicative of increased survival capacity in the hypoxic RA joint which may allow them to persist for extended periods during chronic inflammation. Collectively, these data demonstrate that plastic CD161⁺ Th cells, and in particular ex-Th17 cells are highly pathogenic in the context of RA. These data have contributed to better understanding the nature of Th17-lineage cells and, therefore, may help to improve therapeutic strategies for autoimmunity in general.

Acknowledgements

I would like to express the deepest appreciation to Prof. Jean Fletcher, creator of questions and questioner of answers. Thank you for your creativity, leadership and accessibility. It has been an honour to learn from you and this thesis would not have been possible without your guidance, patience and encouragement. From gloving up and helping out, to tirelessly reading over this thesis, you have been with me every step of the way and I really appreciate all the time you have invested in me and this project. I would also like to gratefully acknowledge Science Foundation Ireland for awarding Jean a Starting Investigator's Research Grant, which funded this PhD project.

To the delightful Deborah Cluxton, it has been a pleasure to have you on the team as a scientist and friend. Thanks for all the laughs, shared sorrows and the ideas tennis (not to mention the wonderful cake- an integral fuel for the last lap of thesis writing). To the venerable Barry Moran, FACS guru and friend, thanks for all your help, for the countless sorting hours you devoted to this project and for the chats during hours of acquisition.

A big thank you is due to Prof. Kingston Mills, mentor on the grant for this project and our gracious host for the first 18 months of my PhD. Thanks also to the Mills' lab, past and present, for training, troubleshooting, sharing reagents and for great nights out. With particular thanks to the lovely Lara Dungan for helping me to find my feet in the 1st year of my PhD and for being a great friend beyond the boundaries of science. Also, to Neil Marshall, my SPICE mentor – thanks for your patience in teaching me how to use the software.

None of the clinical work in this project would have been possible without our excellent collaborators in the Department of Rheumatology, Vincent's hospital and, of course, all the patients and controls who gave samples for research. Thanks to Prof. Ursula Fearon for supporting the research pursuits of this thesis and for willingly sharing precious samples. Thanks to the wonderful Jenny McCormick for processing samples, sharing protocols and hunting down patient information, you have been a great support. Thanks also to Mary Connolly for primary RA fibroblast cells and Trudy McGarry for the K4 fibroblast cell line.

Thanks to all the staff in TBSI that support our research on a daily basis; to Mary-Pat O'Sullivan, Liam Cross, Noel Breslin, Quentin Comerford, the lads in stores, the lads in solvent stores/waste disposal and the teaching lab- especially Audrey Carroll for safety training.

To my science friends; Ania S, Sinead, Louise, Graham, Claire, Cheryl, Conor, Sarah, Shane, Chris, Karen, Michelle, Alicja, Ania M, Heather, Jan, Ewa, Mieszko, Ali, Laura and Jessie. This chapter of my life has been greatly enhanced by knowing you, it has been a wonderful journey and when the path got rough, it was really great to have friends to share the burden.

Thanks to my school friends; Amber, Gemma and Suzanne, who kept me sane and social during the final months of this project- you have been a great support.

To the Kelly family, who have been a great support during the writing of this thesis, I really appreciate your love and interest in my scientific endeavours.

To my wonderful Nan, whose stoic belief in me has always surpassed my ability to believe in myself, thank you for the unwavering love and support you have lavished on me all my life. To my Mam, who has loved and encouraged me since before I can remember, thank you for the opportunities you have given me and for being the wind in my sails. To Naomi, for keeping me humble, teaching me to laugh at myself and for your quiet pride in me. Thanks for the phone calls and for always asking how I'm getting on. You have held my hand since I was very small and I really appreciated the metaphoric hand-holding throughout the writing of this thesis. And to my Dad, from whom I inherited a strong "left-brain", without you all of my achievements have felt incomplete. I have missed you every day.

Finally, to the sublime Dr Patrick Kelly, you are the love of my life, and I am forever indebted to science for crossing our paths together. Thank you for encouraging me to apply for Jean's PhD in the first place and then for your endless support, for sharing your ideas and for hiding me away under your arm when the world gets tough.

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Sweeney, C.M., Loneragan, R., **Basdeo, S.A.**, Kinsella, K., Dungan, L.S., Higgins, S.C., Kelly, P.J., Costelloe, L., Tubridy, N., Mills, K.H., Fletcher, J.M., IL-27 mediates the response to IFN- β therapy in multiple sclerosis patients by inhibiting Th17 cells. *Brain Behav Immun*, 2011. 25(6): p. 1170-81

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Cluxton D., **Basdeo S.A.**, Moran B., Mills K.H., Fletcher J.M. Distinct metabolic and functional features of human Th1, Th17 and ex-Th17 cells. (Manuscript in preparation).

List of abbreviations

Ab	Antibody
ACPA	Anti-citrullinated protein antibodies
ADP	Adenosine diphosphate
AF	Alexa fluor
Ag	Antigen
AKT	Protein kinase B (serine/threonine kinase)
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APC	Antigen presenting cell (or allophycocyanin in flow cytometry)
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
Bcl	B cell lymphoma
BCR	B cell receptor
BV	Brilliant violet
°C	Degrees Celsius
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CIA	Collagen-induce arthritis
CO ₂	Carbon dioxide
CNS	Central nervous system
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte antigen-4
CTV	Cell trace violet
CXCR3	Chemokine (C-X-C motif) receptor 3
d	Day
DAMP	Danger associated molecular pattern
DAS28	Disease activity score 28
DC	Dendritic cell
DMARD	Disease modifying anti-rheumatic drug
DMSO	Dimethyl sulfoxide
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme linked immunosorbant assay
ESR	Erythrocyte sedimentation rate
FACS	Fluorescent-activated cell sorting
FCS	Foetal calf serum
FISH	Fluorescent <i>in situ</i> hybridisation
FITC	Fluorescein iosthiocyanate
FoxP3	Forkhead Box P3
FSc	Forward scatter
g	relative centrifugal force
GATA-3	GATA-binding protein 3
GARP	Glycoprotein A repetitions predominant
GITR	Glucocorticoid induced TNFR-related protein
GM-CSF	Granulocyte macrophage colony stimulating factor
Gy	Gray
HBSS	Hank's balanced salt solution
HC	Healthy control
Hi	High intensity fluorescence
HIF-1 α	Hypoxia-inducible factor-1 α
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
IBD	Inflammatory bowel disease
ICAM	Intracellular adhesion molecule
ICOS	Inducible co-stimulator
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
Irr	Irradiated
iTreg	Induced regulatory T
IU	International units
JAK	Janus Kinase
JIA	Juvenile Idiopathic Arthritis

LAG-3	Lymphocyte activation gene-3
LLT1	Lectin-like transcript 1
Lo	Low fluorescence intensity
LPS	Lipopolysaccharide
M	molar
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MFI	Median fluorescence intensity
mg	milligram
MHC	Major histocompatibility complex
ml	millilitre
mM	milimolar
MMP	Matrix metalloprotease
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
µg	microgram
n	experimental number of replicates
NFκB	Nuclear factor kappa B
ng	Nanogram
NK	Natural Killer
NKRP1A	Natural killer cell surface protein P1A
NSAID	non-steroidal anti-inflammatory drug
nTreg	Natural regulatory T
PAMP	Pathogen associated molecular pattern
PB	Peripheral blood
PBMC	Peripheral blood mononucleocytes
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP-Cy5.5	PerCP tandem conjugate with cyanine 5.5 dye
PFA	Paraformaldehyde
pg	picogram
PI-3K	Phosphatidylinositol 3-kinase
PILAR	Proliferation-induced lymphocyte-associated ligand
PMA	Phorbol myristate acetate

PNA	Peptide nucleic acid
PRR	Pathogen recognition receptor
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa B (NFκB) ligand
RF	Rheumatoid factor
ROR-γt	RAR-related orphan receptor gamma t
ROS	Reactive oxygen species
RPMI	Rosewell Park Memorial Institute cell culture medium
SD	Standard deviation
SF	Synovial fluid
SFMC	Synovial fluid mononucleocytes
SLE	Systemic lupus erythematosus
SPICE	Simplified presentation of extremely complex evaluations
SSc	Side scatter
STAT	Signal transducer and activator of transcription
T cell	Thymically-derived CD3-expressing lymphocyte
T-bet	T-box transcription factor TBX21
Tc	Cytotoxic T cell
TCR	T cell receptor
Tfh	T follicular helper cell
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor, formerly TNF-α
Treg	Regulatory T cell
VCAM	Vascular cell adhesion molecule
v/v	Volume per volume

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*“After climbing a great hill, one only finds that there are
many more hills to climb”*

Nelson Mandela

Chapter 1

General Introduction

Chapter 1 Introduction

1.1 The immune system

The immune system provides defence against infection. Multi-cellular organisms are persistently exposed to micro-organisms, many of which are pathogenic; however, incidences of infection remain relatively rare. Co-habiting the world with microbes has placed a strong evolutionary pressure on multi-cellular life and led to the formation of a finely tuned, highly adaptable, specifically sensitive and exquisitely precise immune system.

The immune system is composed of a network of organs, interacting cells and cell products. This system protects the entire body from pathogens and other foreign (non-self) substances, destroys infected or malignant cells and eradicates cellular debris. It can exert its effects in every tissue of the body and thus, is integral to the maintenance of homeostasis. The myriad of small signalling molecules involved in orchestrating the immune response can have both pleiotropic and redundant roles, adding to the complexity of immune signalling. The healthy immune system is maintained in a fine homeostatic balance between pro-inflammatory responses which protect the body from infection and anti-inflammatory responses that prevent inappropriate responses that can cause collateral damage. Dysregulation of the immune system can contribute to either excessive pro-inflammatory responses to self tissues (autoimmunity) or excessive anti-inflammatory responses, allowing cancerous cells to evade detection and preventing clearance of infections (figure 1.1).

The mammalian immune system can be broadly divided into two components; the innate and adaptive immune systems. For simplicity, these two systems are often described as discrete entities, however, in reality these two arms of the immune response are intrinsically linked and co-ordinate each other to orchestrate the appropriate immune response to any given infection. The innate immune system includes barrier functions to hinder entry to the body as well as a cellular response which recognises invading pathogens (Murphy et al., 2012). Barrier mechanisms include skin, anti-bacterial lysozyme in tears to protect the conjunctiva and in the saliva, movement by peristalsis in the gastro-intestinal tract or beating cilia in the respiratory tract to remove pathogens and the secretion of mucus to trap microbes. If these barriers are breached, innate immune cells can detect the presence and nature of the invading pathogen and up-regulate pro-inflammatory responses to aid clearance of the pathogen. The innate immune response also activates and directs the adaptive immune response against the invading pathogen (Murphy et al., 2012).

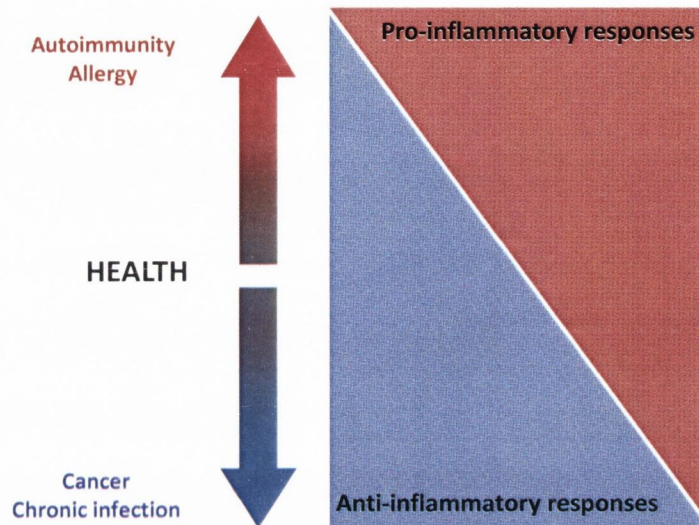


Figure 1.1 **Dysregulation of immune responses in disease.**

The homeostatic immune system is maintained by balancing pro-inflammatory responses with anti-inflammatory, tolerogenic responses. A pro-inflammatory response to pathogens or a tolerogenic response to innocuous signals transiently shifts the system off balance but the system is speedily returned to equilibrium to maintain the healthy state. In the case of autoimmune pathologies, the system is skewed to pro-inflammatory responses and fails to return to a healthy balance. Conversely, during chronic infections or the growth of cancerous cells, the immune system is shifted towards a chronically tolerogenic state.

1.2 The innate immune system

The innate immune system is the first line of defence against invading pathogens. It constitutively employs barrier functions to prevent the entry of pathogens into the body; these include the skin and mucosal membranes as well as specialist barriers such as cilia lining the trachea, anti-bacterial lysozyme in tears and the low pH of the stomach (Murphy et al., 2012). If these barriers are breached, inducible defence mechanisms are utilised to detect the invading pathogen and stymie its replication. Inducible innate responses rely largely on the recognition of invading pathogens and are mediated mainly by phagocytic cells such as macrophages, dendritic cells (DC) and neutrophils. These cells can endocytose the pathogen or infected cell and kill it, recruit other immune cells to the site of infection and are imperative to the initiation of the adaptive immune response (Murphy et al., 2012).

Innate immune cells express pattern recognition receptors (PRR) which include toll-like receptors (TLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR) (Creagh and O'Neill, 2006). These PRRs engage a range of highly conserved pathogen associated molecular patterns (PAMPs) present on the surface of the invading pathogen, or endogenous danger associated molecular patterns (DAMPs) which are released from damaged or infected host cells (Matzinger, 2002). Specific types of PAMPs are recognised by one of the subclasses of PRRs, eliciting an innate response appropriate to the nature of the invader. For example, TLR4 specifically recognises lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria whereas TLR3, 7 and 9 can recognise viral nucleic acids (Boehme and Compton, 2004).

Ligation of a PRR triggers a signalling cascade within the cell, culminating in the translation and secretion of defensive proteins such as chemokines (which recruit specific immune cells to the site of infection along a chemical gradient), cytokines (signalling molecules that direct the immune response and can be directly involved in clearance), and opsonins and complement (which coat the invader, enhancing phagocytosis by innate cells). These early signalling mediators cause inflammation to facilitate the clearance of the invading pathogen (Murphy et al., 2012). Acute inflammation occurs within minutes or hours and resolves when the insult has been removed. It is characterised by five cardinal signs; dolor (pain), calor (heat), rubor (redness), tumor (swelling) and function laesa (loss of function). The heat, redness and swelling are indicative of increased blood flow and permeability, allowing immune cells to traffic into the site of inflammation and kill the pathogen. Pain and loss of function also aid healing by ensuring the site is rested and protected by the host (Murphy et al., 2012).

As well as causing inflammation, innate cells directly activate the adaptive immune response by functioning as antigen presenting cells (APC). In addition to up-regulating the expression of soluble mediators, the ligation of PRRs with cognate PAMPs up-regulates the surface expression of co-stimulatory molecules which enhance the activation of adaptive cells.

The major histocompatibility complex (MHC) present antigens, which are proteins derived from a pathogen that have been processed into short peptides, to adaptive T cells (Murphy et al., 2012). The T cell receptor (TCR) can only recognise antigens when bound to MHC molecules. There are two classes of MHC molecules: MHC-I and MHC-II. MHC-I presents antigen to CD8⁺ T cells and MHC-II presents antigen to CD4⁺ T cells (Murphy et al., 2012). MHC-I is expressed by all nucleated cells and allows for the presentation of endogenous self or pathogen-derived antigens. In this way, viral proteins, intracellular bacteria and tumour antigens can be recognised. However, DC are specialised to process exogenous antigens and load them into MHC-I molecules for presentation to naive CD8⁺ T cells, in a practice known as cross-presentation (Belz et al., 2002).

In contrast, MHC-II expression is restricted to B cells and innate APC and presents antigens from phagocytosed pathogens to prime naive CD4⁺ T cells. It is the presentation of the antigen along with the ligation of co-stimulatory molecules and the cytokine milieu created by the APC that directs the subsequent adaptive immune response (Murphy et al., 2012).

1.3 The adaptive immune system

The adaptive immune system provides long-lived protection against pathogens and bolsters the innate response during protracted infections. The adaptive immune response is characterised by 3 fundamental features; specificity, diversity and memory.

The specific recognition of the invading organism occurs via a vastly diverse array of unique receptors, which are randomly created by the somatic rearrangement of gene segments. Adaptive immunity can be divided into two categories; humoral and cell-mediated. B cells mediate humoral immunity through the production of antibodies against the invading pathogen. T cells are responsible for cell-mediated immunity and secrete cytokines to enhance immune responses as well as directly killing infected cells. Both B and T cells originate from a common lymphoid progenitor, derived from the pluripotent haematopoietic stem cells of the bone marrow.

Activated B and T cells traffic to the site of inflammation during an infection. When the pathogen is eliminated and the inflammation is resolved, the majority of effector cells die by apoptosis, however, a small population of long-lived memory cells remain (Murphy et al., 2012).

1.3.1 Immune memory

The capacity to generate immunological memory is a principal hallmark of the adaptive immune response. When B cells or T cells are initially activated by a specific pathogenic antigen, a small cohort of the effector cells that are clonally expanded to clear the pathogen become memory cells after inflammation has resolved (Murphy et al., 2012). Upon subsequent encounters with the same pathogen, memory lymphocytes can confer immediate protection in the periphery and swiftly induce a recall response to that antigen in the secondary lymphoid organs (Sallusto et al., 2004). It is this valuable feature of the immune response that is manipulated to confer protection in prophylactic vaccinations.

B cell memory consists of two response arms; protective memory and reactive memory. Protective memory is mediated by previously differentiated, long-lived plasma cells which confer immediate protection by secreting antibodies (Slifka et al., 1998). Reactive memory is mediated by memory B cells that proliferate and differentiate into plasma cells in response to subsequent encounters with previously recognised antigens (Ochsenbein et al., 2000). Similarly, T cell protective memory is mediated by effector memory cells that traffic to inflamed peripheral tissues and exert immediate effector function. Central memory cells mediate reactive memory, when activated by a secondary antigenic stimulation, by proliferating and differentiating into effector cells in the secondary lymphoid organs (Sallusto et al., 2004).

1.3.2 B cells

Immature B cells develop in the bone marrow and migrate to the spleen. The antigen specific moiety of the B cell receptor (BCR) is composed of immunoglobulin (Ig) molecules elicited by the random recombination of multiple gene segments at the BCR locus during development. Membrane bound Ig recognises specific epitopes on the surface of pathogens. When a B cell is activated by the presence of pathogenic epitopes, Ig with the same antigen specificity is secreted as antibody. There are 5 classes of Ig in mammals; IgM, IgA, IgD, IgG and IgE, each with a distinct structure and function in humoral immunity. Mature naïve B cells produce IgM and IgD. Upon activation, B cells can undergo class switching to produce IgG, IgA or IgE antibodies, specific to the nature of the pathogen. At this stage, B cells are terminally differentiated antibody secreting plasma cells. Antibodies activate the complement cascade and opsonise pathogens to enhance phagocytosis. B cells also express MHC-II and can therefore present

antigen to T cells. T cells in turn can provide help to B cells by producing cytokines that promote growth and differentiation and can direct Ig class switching (Murphy et al., 2012).

1.3.3 T cells

T cell development

The vast majority of TCR are composed of an α and a β chain. There are two types of T cell which express an $\alpha\beta$ TCR; $CD4^+$ T cells are defined as T helper (Th) cells and $CD8^+$ T cells are cytotoxic T lymphocytes (Tc). $CD8^+$ T cells recognise endogenous antigen loaded on to an MHC class I molecule and are stimulated to kill virally infected or transformed cells. $CD4^+$ T cells recognise exogenous antigen presented by an MHC-II molecule and promote the immune response via several mechanisms, including the activation of B cells, macrophages and $CD8^+$ T cells (Murphy et al., 2012).

The thymus was first identified as the location of T cell development in the early 1960s (Miller, 1963). T cell progenitors migrate from the bone marrow to the thymus. Progenitor cells initially entering the thymus lack the surface molecules characteristic of mature T cells. Stromal cells in the thymus signal to the progenitor, via Notch1, to switch on specific genes that commit the cell to the T cell lineage and induce the TCR genes to undergo rearrangement thus, creating the vast diversity of the TCR repertoire (Murphy et al., 2012). Initially, neither CD4 nor CD8 are expressed so these cells are termed “double-negative” thymocytes. First, expression of CD3, part of the TCR complex, is induced. Most TCRs are composed of the conventional $\alpha\beta$ heterodimer and these cells become CD4 and CD8 “double-positive” cells. A minority lineage is also produced by unconventional $\gamma\delta$ TCR chains. These unconventional T cells are exported to the periphery where they function as early mediators of the inflammatory response. The conventional double positive T cells undergo both a positive and negative selection process; immature T cells capable of binding with antigen complexed to the self-MHC are selected for survival and maturation whilst cells that react too strongly with self antigens are eradicated from the repertoire. The selected cells then lose either CD4 or CD8 expression to become single positive, naive T cells (von Boehmer, 1992). These mature T cells migrate to the peripheral lymphoid compartments where they await priming by activated innate APC.

In order for a naive T cell to be fully activated, it must receive three signals; the ligation of the MHC-bound antigenic peptide with the specific TCR, ligation of co-stimulatory molecules on the surface of the APC with cognate receptors on the T cell and signals from the cytokine milieu produced by innate cells which polarise the T cell into a particular phenotype (Murphy et al.,

2012). If the naive T cell does not receive the necessary co-stimulation in conjunction with TCR engagement, it becomes anergic. Correctly activated T cells clonally expand and exert effector function on the invading pathogen.

T helper cell differentiation

Naive CD4⁺ Th cells are multipotent; when the three signals from innate APC are recognised and integrated by the naive Th cell, a distinct programme of differentiation is selected. Clonal expansion of this specifically activated T cell occurs to generate progeny that have bespoke functions to eliminate the specific pathogen. It is the co-evolution of different classes of pathogens (and symbiotes) with the adaptive immune response that has diversified the functional specificity of the CD4⁺ Th cell subsets. Once human naive Th cells experience antigen and become differentiated into Th subsets, they lose the expression of the naive marker, CD45RA, and gain expression of the memory marker, CD45RO.

The concept of discrete Th cell lineages was first introduced in the mid 1980s when two distinct T cell clones were identified by their cytokine profiles (Mosmann et al., 1986). From this the Th1/Th2 paradigm developed and classification of Th cells subsets according to their cytokine production became dogma. Th1 cells are defined also by their response to intracellular infections, which evolved to surmount the evasive strategies of pathogens that survive and replicate inside cells. In contrast, Th2 cells coordinate the elimination of parasitic helminth infections. More recently, Th17 cells, which orchestrate the immune response against extracellular bacteria and fungi (Harrington et al., 2006); and T follicular help (fh) cells, which trigger antigen specific B cell activation and germinal centre formation (Breitfeld et al., 2000), have also been identified. Th9 and Th22 subsets have also been described, however, their status as truly distinct subsets and their physiological role is uncertain (Annunziato and Romagnani, 2009).

Differentiation of naive CD4⁺ T cells into the distinct Th subsets is dependent upon the cytokine environment in which the T cell receives the TCR signal and co-stimulation (figure 1.2). Th1 cells are induced by the cytokine interleukin 12 (IL-12) which signals through STAT4 to promote Th1 differentiation (Thierfelder et al., 1996). Differentiated Th1 cells express the transcription factor T-bet and produce their signature cytokine, interferon- γ (IFN- γ) (Szabo et al., 2000), along with other cytokines such as TNF and IL-2 (Glimcher and Murphy, 2000). IFN- γ is a pro-inflammatory cytokine that can act in an autocrine manner to up-regulate its own production and signals through the STAT1 to turn on IFN- γ inducible genes (Darnell et al., 1994). IFN- γ can also negatively regulate differentiation into other Th cell subsets. Functionally, IFN- γ activates

macrophages to kill engulfed pathogens and promotes B cell class switching to produce IgG to opsonise intracellularly infected cells (Basu et al., 2013).

IL-4 induces the differentiation of naive Th cells into Th2 cells. IL-4 signals via STAT6 to promote the expression of the transcription factor GATA-3 thereby committing the naive Th cell to the Th2 pathway of differentiation (Ouyang et al., 1998). Activated Th2 cells produce IL-4, IL-5, IL-10 and IL-13. IL-4, like IFN- γ , promotes the preferential development of its own lineage by established positive and negative feed-back loops (Murphy et al., 2012). Th2 cells promote B cell class switching to IgE which activates granule release from mast cells and basophils, enhances mucus production and recruits eosinophils (Basu et al., 2013).

Differentiation of human naive Th cells into Th17 cells is induced by a combination of IL-21 and TGF- β (Yang et al., 2008), IL-6 and TGF- β (Veldhoen et al., 2006), IL-6 and IL-1 β or IL-1 β and IL-23 (Acosta-Rodriguez et al., 2007a). IL-6, IL-21 and IL-23 all activate STAT3, which is a critical factor for Th17 differentiation as it regulates ROR- γ t, the master regulator of Th17 transcription (O'Shea et al., 2009). There is conflicting literature regarding the role of TGF- β in human Th17 differentiation (Acosta-Rodriguez et al., 2007a, Yang et al., 2008) which may be reconciled by evidence suggesting that TGF- β indirectly promotes Th17 cells by suppressing Th1 development (Santarlaschi et al., 2009). Human Th17 cells originate from CD4⁺ CD161⁺ naive T cells found in both umbilical cord blood and the thymus (Cosmi et al., 2008) and are characterised by the expression of the IL-23 receptor, CCR6, CCR4 and CD161 (Annunziato et al., 2012). The master regulator of Th17 transcription is ROR- γ t and these cells can produce IL-17A, IL-17F, IL-22, IL-21, TNF and GM-CSF (Annunziato et al., 2012). IL-21 regulates Th17 differentiation and therefore acts in an autocrine manner to amplify the Th17 response (Annunziato et al., 2012). Th17 cells recruit and activate neutrophils and promote B cell class switching to opsonising IgG antibodies. The IL-17 receptor is expressed on many cell types, enabling IL-17 to exert global pro-inflammatory effects, including induction of pro-inflammatory cytokines, chemokines and adhesion molecules on fibroblasts cells (Yao et al., 1995), epithelial cells (Lee et al., 2008), endothelial cells (Hot et al., 2012) and keratinocytes (Chiricozzi et al., 2011). In addition, Th17 production of IL-21 promotes the differentiation of Tfh cells via STAT3 signalling (Liu et al., 2013). Tfh cells depend on the transcription factor Bcl-6 and are required to produce the full range of B cell antibodies (Annunziato and Romagnani, 2009).

Naive CD4⁺ Th cells may also differentiate into regulatory T (Treg) cells under the influence of TGF- β and IL-2. Treg cells induced in the periphery (iTreg) and thymically derived natural Treg cells (nTreg) express the transcription factor FoxP3 and are phenotypically indistinguishable

from each other. STAT5, downstream of IL-2 signalling, plays a crucial role in nTreg and iTreg cell development (Mahmud et al., 2013). Both iTreg and nTreg cells serve to dampen excessive inflammation through the production of anti-inflammatory cytokines, such as IL-10 and TGF- β and also, through contact-dependant mechanisms.

A summary of Th cell differentiation into polarised, specific subsets is shown in figure 1.2.

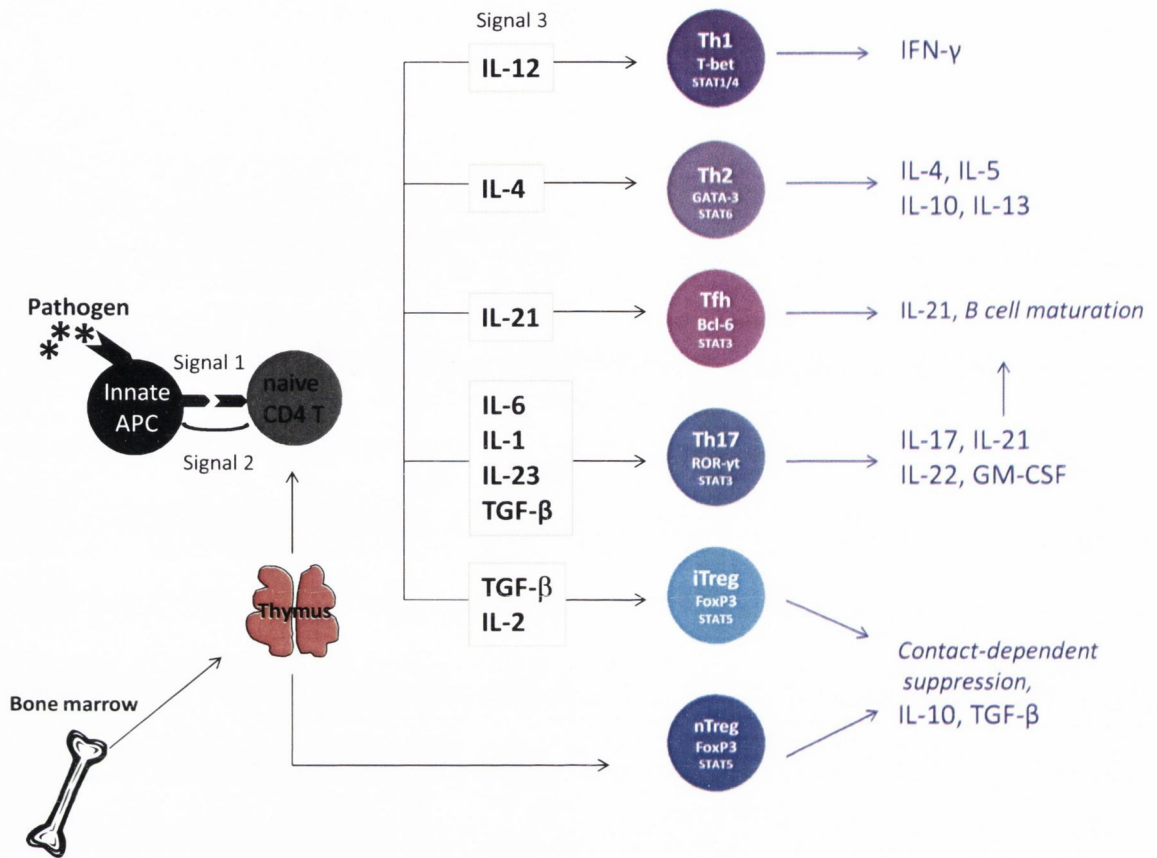


Figure 1.2 Th cell differentiation into “discrete” subsets.

Hematopoietic stem cells in the bone marrow traffic to the thymus where they develop into T cells. CD4⁺ naive T cells leave the thymus expressing a unique T cell receptor. Conserved motifs expressed by pathogens are recognized by pattern recognition receptors on innate cells. This signaling causes the up-regulation of co-stimulatory molecules (signal 2) and the secretion of cytokines (signal 3). The up-taken foreign matter is processed and the antigenic peptide fragment is loaded onto the MHC-II molecule and trafficked to the cell surface. The antigenic peptide encased in the MHC-II binds to the TCR receptor on a specific naive T cell, denoted as signal 1 in the diagram. Co-stimulation is provided by the innate antigen-presenting cell, denoted as signal 2 on the diagram. The T cell must receive a 3rd signal from the cytokine environment in order to differentiate into the appropriate subset. T helper cell subsets produce their own cytokines and have distinct effector functions.

Th cell plasticity

Th cell differentiation gives rise to a diverse range of Th cells with specialised functions. It was originally thought that these differentiated Th cells adhered to their characteristic gene expression profile with high fidelity. Emerging data, however, indicates ever increasing flexibility within these “distinct” lineages (Hirahara et al., 2013). Phenotypic promiscuity has been documented in all pathways of differentiation. For example, Th2 type cells can switch toward the Th1 programme of differentiation by acquiring the expression of Tbet and co-producing IL-4 and IFN- γ (Zhu and Paul, 2010, Peine et al., 2013). Tfh cells are also functionally capable of up-regulating Tbet or GATA-3 (Cannons et al., 2013) and therefore producing IFN- γ or IL-4, which tailors the B cell antibody response to bacterial or parasitic infections, respectively (Reinhardt et al., 2009).

Th17 cells have been the protagonists demonstrating the concept of plasticity in recent years with amassing evidence indicating that Th17 cells can phenotypically switch into “Th1-like” cells (Lexberg et al., 2008, Lee et al., 2009a). In addition, these plastic, switched Th1-like ex-Th17 cells that result from the maturation or differentiation of bona fide Th17 in inflamed tissues are thought to exhibit marked pro-inflammatory function (Sundrud and Trivigno, 2013) and have been identified in the context of both experimental and clinical autoimmunity (Hirota et al., 2011). Published data from both human and mouse models indicate that Th17 cells, as defined by their production of IL-17, may not be an end stage differentiated Th subset. However, Th17 cells, as defined by their expression of CCR6 and CD161 incorporate three discrete subpopulations representing the distinct stages of phenotypic switching; firstly, the bona fide IL-17 producing Th17 cells which lack Th1 associated gene expression, secondly, the hybrid IL-17 and IFN- γ co-producing cells that up-regulate a Th1 gene transcription and thirdly, the IFN- γ producing Th17 cells that no longer express IL-17 (Annunziato et al., 2012). Although this switching is largely regarded to be unidirectional, some evidence indicating that the *il-17* gene locus is epigenetically stable (Cohen et al., 2011) and that IL-17⁺ Th17 cells are poised, awaiting environmental cues to re-activate IL-17 expression (Wan et al., 2011) suggests that plastic Th17 cells may be functionally capable of returning to IL-17 gene expression.

On the other hand, the hypothesis suggesting that Th17 cells are early precursors that linearly differentiate into ex-Th17/Th1-like cells in inflamed tissues is supported by observations showing that ex-Th17 cells constitute the majority of tissue-infiltrating CD161⁺ CD4⁺ T cells in the joints of autoimmune arthritis patients (Nistala et al., 2010, Cosmi et al., 2011), affected gut of Crohn’s disease sufferers (Maggi et al., 2013, Kleinschek et al., 2009) and the cerebrospinal fluid

of MS patients (Kebir et al., 2009). These studies suggest that Th17 cell plasticity may occur locally however, because ex-Th17 cells also exist in the peripheral blood, trafficking of these cells from the periphery into inflamed tissues cannot be ruled out. It has not yet been deciphered if all Th17 cells end up in an ex-Th17 state in non-lymphoid tissues or if the switching pressure is unique to chronic inflammation or autoimmunity. Interestingly, bona fide IL-17 producing Th17 cells are enriched in chronically inflamed skin psoriatic lesions (Zheng et al., 2007, Harper et al., 2009) and in the asthmatic lung (Alcorn et al., 2010) implying that the particular inflammatory milieu directs the plasticity or stability of these cells. Moreover, recent data suggests that Th17 cell plasticity is not limited to the acquisition of a Th1-like phenotype but may also switched towards a Th2-like phenotype as a small subset of CD4⁺ CD161⁺ CCR6⁺ T cells which co-express IL-17 and IL-4 have been identified in the circulation of asthma patients (Cosmi et al., 2010).

In addition to their pathogenic role in autoimmunity, plastic ex-Th17 type cells may mediate protective anti-tumour immunity. Whilst the effects of Th17 cells in cancers are tissue specific, it is largely thought that IL-17 promotes oncogenic signaling and enhances angiogenesis (Numasaki et al., 2003). However, it has been shown that if Th17 cells exhibit a plastic polyfunctional cytokine profile; expressing IFN- γ and TNF, their numbers at the tumour site of ovarian cancer are predictive of improved patient outcome (Kryczek et al., 2009). Similar correlations between Th17 cell plasticity and improved outcome have been demonstrated in many other cancers including lung (Ye et al., 2010) and prostate (Sfanos et al., 2008) cancer. Moreover, plastic IL-17⁺ IFN- γ ⁺ cytotoxic CD8⁺ T cells, which were converted from non-cytotoxic IL 17⁺ T cells, exhibited strong anti-tumour activity in a murine model (Tajima et al., 2011).

The literature indicates that Th17 cells may be, or at least incorporate, a pluripotent memory Th cell population which mediates the pathology in autoimmunity, allergy and cancer (Wilke et al., 2011). Manipulating the plasticity of these cells may serve as a “volume control” to turn down polyfunctional pro-inflammatory responses in the context of autoimmunity or amplify the plasticity to eradicate cancers. Furthermore, these cells may promote beneficial vaccine responses, given their dynamic nature and propensity to generate long-term T cell memory (Kryczek et al., 2011), which will be discussed further below.

A summary of Th17/Th1 cell plasticity is shown in figure 1.3.

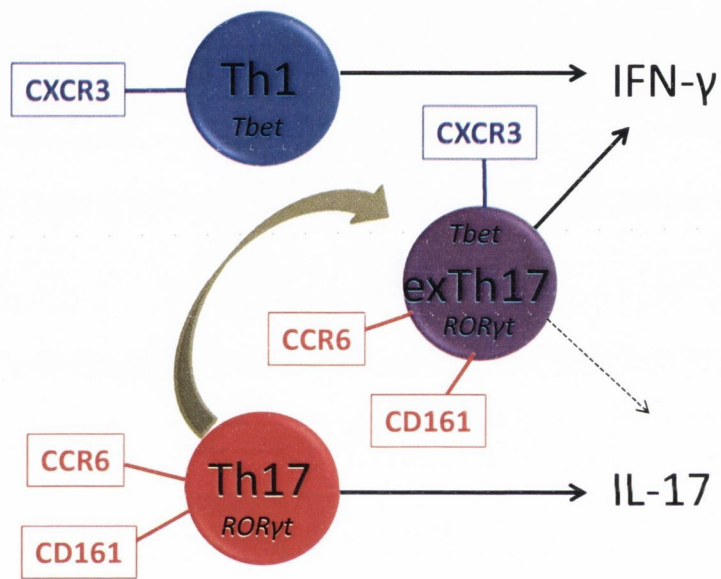


Figure 1.3 Th17 cells can switch into plastic ex-Th17 cells.

Th1 cells (blue) express CXCR3 on their cell surface and produce IFN- γ . Th17 cells (red) express CD161 and CCR6 on their cell surface and can produce IL-17. Under certain inflammatory conditions, Th17 cells are plastic and can switch into IFN- γ producing cells which express CXCR3 but retain the expression of CD161 and CCR6. These plastic ex-Th17 cells (purple) initially co-produce IFN- γ and IL-17, which may represent an intermediate state of plasticity, before becoming cells which produce IFN- γ in the absence of IL-17.

Th17 type cells have stem cell-like features which may enhance their longevity.

Memory T cells are long-lived cells which are primed by an initial encounter with a pathogen and, hence, respond with enhanced capacity upon subsequent encounters with the same pathogen. Circulating memory T cells can be divided into two distinct pools on the basis of effector function and migration potential (Sallusto et al., 1999); central memory cells express CCR7 and are postulated to reside predominantly in lymph nodes, spleen and blood; whereas effector memory cells do not express CCR7 and are predominately found in peripheral non-lymphoid tissue, such as the gut, liver and lungs (Masopust et al., 2001). Effector memory cells are shorter-lived and more prone to apoptosis compared with central memory cells which are long-lived (Sallusto et al., 2004). Human Th17 cells are largely found within the effector memory compartment and exhibit a terminally differentiated phenotype (Kryczek et al., 2011). Given their terminally differentiated phenotype, it might be assumed that Th17 cells have a short half-life and display an exhausted or senescent phenotype but, to the contrary, human Th17 cells express high levels of stem cell associated genes including Bcl2, HIF-1 α , Notch and Nanog (Kryczek et al., 2011). These data indicate that Th17 type cells are long-lived with a high capacity for self-renewal and resistance to apoptosis, characteristics which are likely to enhance their pathogenicity in the context of autoimmunity.

CD161 expression on Th cells

Although it is now established that human Th17 cells are marked by the expression of CD161, CD161 expression is not exclusive to any predefined T cell subset. CD161, or NKR1A was originally identified as the human homologue of rodent NKR1; an NK cell marker expressed on the majority of rodent NK cells but only on a subset of human NK cells (Lanier et al., 1994). In addition, CD161 is not expressed on rodent T cells, however, it is expressed on approximately 15-25% of human T cells, including both CD4 and CD8 T cells, in adult peripheral blood and is preferentially expressed in cells with a memory phenotype (Lanier et al., 1994).

CD161 is expressed on CD4⁺, CD8⁺ and double negative $\alpha\beta$ TCR T cells and is also expressed on a population of $\gamma\delta$ TCR T cells (Maggi et al., 2010). CD161 is significantly up-regulated in Th17 cell clones compared to Th1 and Th2 cells (Cosmi et al., 2008). IL-17 secreting CD4⁺ Th cells are contained within the CD161⁺ population of Th cells and express ROR- γ t, therefore, CD161 expression is now considered a hallmark of Th17 cells. CD161 expression is associated with a memory phenotype in adults and, moreover, the vast majority of CD161^{hi} Th cells reside within the effector memory pool. This suggests that CD161 may be a marker of activation or memory, however, CD161⁻ Th cells do not up-regulate CD161 expression upon anti-CD3 and anti-CD28

stimulation nor in an antigen specific manner upon restimulation (Northfield et al., 2008). Moreover, CD161 is expressed by subsets of naive T cells in the fetal liver and thymus (Lanier et al., 1994, Martin et al., 2009) and in umbilical cord blood (Cosmi et al., 2008). The presence of these naive CD161⁺ T cells suggests that expression is induced early in ontogeny and therefore, is not merely a marker of activation. The fact that the vast majority of CD161⁺ Th cells in adult blood are confined to the memory pool suggests that these cells have all been exposed to activation and may therefore serve as an integral part of host defense.

CD161 plays a role in transendothelial migration even in the absence of chemotactic stimuli (Poggi et al., 1997), which suggests that these cells may preferentially home to specific tissues. This is further supported by evidence indicating that approximately 50% of T cells express CD161 in both the epithelial and lamina propria layers of the healthy human duodenum and colon (O'Keeffe et al., 2004). CD4⁺ CD161⁺ Th cells are selectively recruited to the liver during chronic hepatitis infection and co-express IFN- γ and IL-22 (Kang et al., 2012), which implies that they are plastic, polyfunctional, ex-Th17 type cells. Interestingly, IFN- γ is anti-viral whereas IL-22 is hepatoprotective which, therefore, indicates that CD161⁺ Th cell polyfunctionality is finely tuned to respond to the requirements of the infection. Hence, this plasticity may have evolved to allow the Th cells to raise the ante against pathogens at the risk of increasing the propensity to establish inappropriate chronic inflammation. As such, CD161⁺ T cells have been implicated in the pathogenesis of many autoimmune diseases such as multiple sclerosis (MS) (International Multiple Sclerosis Genetics et al., 2007), psoriasis (Cosmi et al., 2008), Crohn's disease (Cosmi et al., 2008, Kleinschek et al., 2009), rheumatoid arthritis (RA) (Chalan et al., 2013) and juvenile idiopathic arthritis (JIA) (Cosmi et al., 2011). More recently, a subpopulation of CD161⁺ Th cells that co-expressed CD39 have been documented to be increased in the peripheral blood and lamina propria of patients with active Crohn's disease, suggesting that monitoring the frequencies of the subpopulation may serve as a biomarker of progression and response to therapy (Bai 2014JI). Furthermore, the association of CD161 with CD39 resulted in the population being refractory to purinergic signalling and having enhanced acid sphingomyelinase activity, which controls mTOR and STAT3. These data indicate a functional role for CD161 in conjunction with CD39 in controlling Th17 responses.

CD161 currently has two known ligands; lectin-like transcript 1 (LLT1) and proliferation-induced lymphocyte-associated receptor (PILAR). LLT1 is expressed on TLR activated DC and is up-regulated on activated B cells, NK cells and T cells (Germain et al., 2011). Ligation of CD161 with LLT1 inhibits IFN- γ production in NK cells and conversely, enhances the production of IFN- γ

(Aldemir et al., 2005) and IL-17 (Germain et al., 2011) in T cells stimulated with anti-CD3. PILAR is expressed on lymphocytes and is markedly enhanced on CD4⁺ T cells post TCR engagement. In the absence of CD28 mediated co-stimulation, PILAR promotes TCR stimulation and T cell proliferation by increasing anti-apoptotic Bcl-xL and inducing the production of pro-inflammatory cytokines, which suggests that CD161 may act as a receptor for co-stimulation (Huarte et al., 2008).

1.3.3 Immune Regulation

The pro-inflammatory response to harmful stimuli must be tightly regulated to limit collateral damage to surrounding tissues. The system must also be regulated to ensure that an inappropriate response, such as a response to self peptides or harmless environmental stimuli, does not trigger a pro-inflammatory response. "Tolerance" is a central tenet in immunology, describing the ability of the immune system to decipher dangerous from innocuous signals and pro-actively suppress a response to self or harmless non-self. The balance between tolerogenic and pro-inflammatory responses maintains the system in homeostatic equilibrium (figure 1.4A). A breakdown in tolerance causes disequilibrium in the system (figure 1.4B), culminating in allergy (the response to innocuous environmental stimuli) or autoimmunity; an immune response mounted against self antigens resulting in damage to healthy tissues and eventual clinical presentation of tissue, organ-specific or even multi-organ pathology.

There are various tolerance mechanisms which prevent autoimmunity. These include central tolerance, where self-reactive T cells are deleted in the thymus, and peripheral tolerance. Peripheral tolerance mechanisms include induction of anergy in the absence of co-stimulation, threshold antigen concentrations, anti-inflammatory cytokine production and regulatory T cells (Treg) (Mueller, 2010). Treg cells act as the brakes for the immune response; dampening inflammation, encouraging resolution and upholding tolerance.

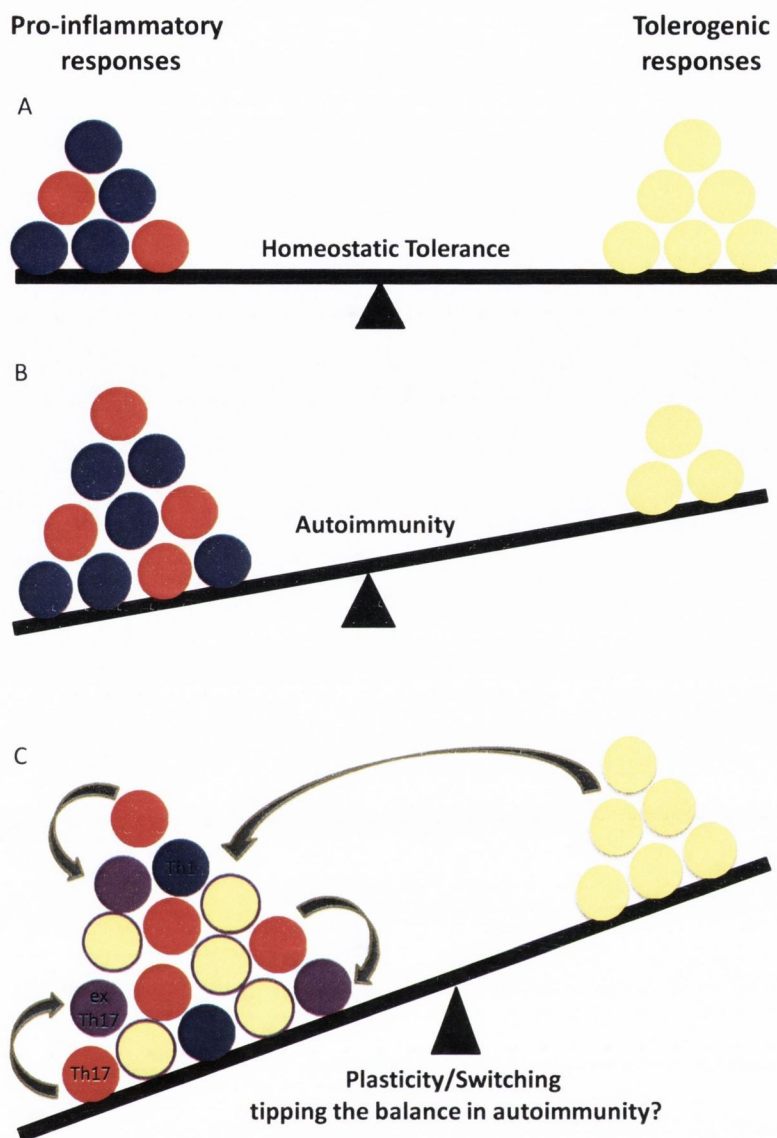


Figure 1.4 The fine balance between effector and regulatory responses.

The immune system is maintained in equilibrium between pro-inflammatory effector responses and tolerogenic, regulatory responses (A). The system is primed to shift either way depending on the nature of the stimulus; during infection, the pro-inflammatory response predominates until the infection is cleared and homeostatic equilibrium is re-established. If self or harmless non-self antigens are presented, tolerance predominates until the signal is quelled and the system returns to homeostasis. In the case of autoimmunity, the whole system is shifted off balance and inappropriate pro-inflammatory effector response to self-antigens chronically predominates (B). This could be due to an increase in numbers on the effector arm with a concomitant decrease on the tolerogenic side or may be caused by a defect in tolerogenic responses or conversely, by pro-inflammatory responses becoming impervious to regulation. It is also plausible to postulate that the autoimmune state may be caused or perpetuated by plasticity between T cell subsets; switching from one effector phenotype to another may propagate inflammation, making the pro-inflammatory responses harder to suppress (C). Treg cells may also succumb to their highly inflammatory environment, converting into effector-type cells, which produce pro-inflammatory cytokines.

Treg cell development

Miller, after describing the role of the thymus in T cell development, recognised its direct association with immune tolerance (Miller and Basten, 1996). The autoreactive T cells are deleted during negative selection in the thymus in a process called central tolerance. Autoreactive T cells that escape the selection check points in the thymus can be constrained by Treg cells in the periphery (peripheral tolerance). nTreg cells develop under the influence of IL-2 in the thymic medulla. Sakaguchi was the first to identify thymically derived nTreg cells as a minor population of CD4⁺T cells that co-express CD25, the alpha-chain of the IL-2 receptor, and exhibit regulatory function in mice (Sakaguchi et al., 1995). The Foxp3 transcription factor was subsequently found to be selectively expressed in the CD4⁺CD25⁺T cell population and required for nTreg development (Hori et al., 2003, Fontenot et al., 2003). The importance of this Treg cell population is demonstrated by the fatal autoimmune syndromes that develop in scurfy mice and humans; genetic Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, which are caused by a mutation in Foxp3, resulting in a Treg cell deficiency (Bennett et al., 2001, Lahl et al., 2007).

Treg selection in the thymus is thought to be based on an intermediate level of TCR affinity with self-peptide in the MHC complex as opposed to strongly autoreactive T cells that are deleted or self non-reactive T cells that are selected to become effectors (Murphy et al., 2012). Treg cell TCRs are more diverse than the effector repertoire and biased towards self-peptides, making them the quintessential guards against autoimmunity (Wing and Sakaguchi, 2011). nTreg cells can suppress all the major elements of the immune response; CD4⁺T cells, CD8⁺T cells, NKT cells, dendritic cells, monocytes/macrophages, B cells, mast cells, basophils and eosinophils and can signal to other non-immune cells such as osteoblasts, epithelia and endothelia (Shevach, 2009).

Naive effector T cells in the periphery can be differentiated into FoxP3 expressing iTreg cells when the cognate antigen is presented in the presence of TGF- β and IL-2 (Chen et al., 2003, Chen et al., 2011). iTreg cells are thought to differ from nTreg cells in several ways including, their cognate antigens (nTreg cell recognise self antigens in the thymus whereas iTreg cells recognise tissue-specific and foreign antigens), their requirements for differentiation (nTreg cells emerge from the thymus fully mature but iTreg cells require further signaling from the microenvironment post differentiation) and their need for co-stimulation via CD28 (required by the nTreg but not by the iTreg cell) (Bluestone and Abbas, 2003, Horwitz et al., 2008). However, the role of both regulatory subsets is to suppress the proliferation and effector function of

activated T cells, and although they are currently phenotypically indistinguishable in humans, much effort has been made to differentiate these two subpopulations of Treg cells. However, the characteristics thought to distinguish nTreg cells from iTreg cells are not universally accepted.

Pioneering work by Rudensky's team uncovered a role for conserved non-coding DNA (CNS) elements in the FoxP3 gene that define the frequency and stability of Treg cells as well as their origin; i.e. thymic or peripheral (Zheng et al., 2010). CNS1 was shown to be responsible for the generation of iTreg cells from naive CD4⁺ T cells in the periphery but had no role in thymic differentiation of nTreg cells. CNS2 maintains the expression of FoxP3 in the progeny of Treg cells. CNS3 is required for the induction of FoxP3 in both the thymus and the periphery. Foxo transcription factors are also thought to play a prominent role in controlling Treg cell development and function as mice with T cell specific deletion of Foxo1 had increased effector memory populations and spontaneous encephalitis (Kerdiles et al., 2010). Additionally, Treg cells from Foxo1 deficient mice, despite having normal FoxP3 expression, had reduced CD62L, CD25 and CTLA-4 expression and thus, reduced Treg cell effector function.

Other markers have been suggested to distinguish peripheral iTreg cells from thymic nTreg cells, however, with some controversy. Helios, an Ikaros transcription factor family member, is thought to differentiate thymic-derived nTreg cells from iTreg cells induced in the periphery (Sugimoto et al., 2006, Thornton et al., 2010). Other reports suggest that Helios may not be a suitable nTreg cell marker as it is induced during T cell activation and expressed by Th2 and Tfh cells (Akimova et al., 2011, Serre et al., 2011) and moreover, has been reported to be expressed in iTreg cells *in vitro* and *in vivo* (Gottschalk et al., 2012). Neuropilin-1 has also been identified as a marker to distinguish between nTreg and iTreg cells (Yadav et al., 2012) by showing that only 6% of peripheral iTreg cells express neuropilin-1 whereas 25% express Helios in a mouse lacking CD4⁺ FoxP3⁺ cells in the thymus. Neuropilin-1 has been suggested to be a superior marker to Helios and improve the fidelity of the Helios marker to nTreg cells when both used together (Lin et al., 2013). However, neuropilin-1 is also expressed on effector T cells (Milpied et al., 2009).

Interestingly, augmentation of the induction of iTreg cells is amongst the pleiotropic immunomodulatory effects of vitamin A, a hormone-like growth factor best known for its role in scotopic and colour vision. Vitamin A is consumed in the diet as all-trans-retinol and enzymatically oxidised to all-trans-retinoic acid (ATRA). ATRA works in synergy with TGF- β to promote the differentiation of iTreg cells and can negate the Th17 skewing influence of IL-6 (Zhou et al., 2010). ATRA has been shown *in vitro* to expand the nTreg cell population and

stabilise the expression of FoxP3 and suppressive activity in the presence of IL-6 (Zhou et al., 2010). In the presence of TGF- β , ATRA has been demonstrated to effectively convert naive CD4⁺ T cells into stable, FoxP3-expressing iTreg cells with potent suppressive ability (Wang et al., 2009).

Th3 cells are iTreg cells induced by orally ingested antigens and TGF- β in the gut mucosa. Th3 cells invoke tolerance by producing anti-inflammatory TGF- β , which promotes Ig class-switching to non-inflammatory IgA, and by inhibiting Th1 and Th2 responses (Jonuleit and Schmitt, 2003). A reciprocal relationship between Th3 cells and Th17 cells in the gut has also been shown, with ATRA acting to promote Th3 induction over Th17 cells (Nistala and Wedderburn, 2009, Mucida et al., 2007).

Naive T cells can also be differentiated in the presence of antigen into a regulatory-like phenotype by IL-10 in the environment. These cells are called T regulatory type 1 cells (Tr1) are FoxP3 negative but capable of potent suppression by producing IL-10 and TGF- β (Fujio et al., 2010, Beissert et al., 2006, Roncarolo et al., 2006, Jonuleit and Schmitt, 2003). Tr1 cells also secrete low levels of IFN- γ and can be maintained by the growth factor IL-15, even in the absence of TCR stimulation (Bacchetta et al., 2002).

Research to delineate the roles of the distinct Treg sub-types has been hampered by the lack of markers to functionally distinguish between them. Helios, a member of the ikaros transcription factor family, was identified as a marker of thymically-derived Treg cells (Thornton et al., 2010). The fidelity of this marker however, is not assured.

Canonical Treg cell markers

Treg cells do not have any unique surface markers therefore a panel of markers including CD4, CD25, CD127 and FoxP3 are used to identify the population as CD4⁺ CD25⁺ CD127^{lo} FoxP3⁺. The expression of the FoxP3 transcription factor is the most reliable marker of Treg cells. However, as FoxP3 is intranuclear, it cannot be used to sort and purify a population of viable Treg cells. Furthermore, FoxP3 can be transiently up-regulated in activated T cells, particularly in humans. CD25 expression can be either high or intermediate on Treg cells however, activated CD4⁺ T effector cells also up-regulate their expression of CD25. CD127 (IL-7 receptor) expression is down-modulated on Treg cells compared with mature T cells and monocytes. Other cell surface molecules associated with enhanced suppressive function can be used to identify activated Treg cells; however, these markers are not restricted to the Treg cell population. The consensus in the literature supports the purification of viable Treg cells on the basis of cell surface CD4⁺ CD25⁺

CD127^{lo} expression (Yu et al., 2012). Populations of CD8⁺ Treg cells (Cone et al., 2009, Hu et al., 2004, Jiang et al., 1998) and CD25⁻ Treg cells (Horwitz, 2010) have also been identified in the literature, further complicating the isolation of the total Treg cell cohort from other T cells.

Resting nTreg cells are CD45RA⁺ (naive) FoxP3^{lo} and when activated become CD45RO⁺ (memory) FoxP3^{hi} (Miyara et al., 2009). Both resting and activated nTreg cells express the IL-2 receptor; CD25, adhesion molecules; CD62L and CD44, cytotoxic T-lymphocyte antigen 4 (CTLA-4), co-stimulatory CD28, chemokine receptors; CCR7, CXCR4, the survival receptor OX40 and glucocorticoid-induced tumour necrosis factor-receptor-related protein (GITR) (Beissert et al., 2006, Lim et al., 2006). Expression of CD39 marks Treg cells with strong suppressor function and can be used as an additional marker to isolate functionally-active Treg cells (Mandapathil et al., 2009).

The nTreg profile is highly conserved in mammals. Non-human primates exhibit the same nTreg proteome and functionality as human nTreg cells but some subtle differences exist between rodents and humans (Roncarolo and Battaglia, 2007). This high extent of conservation across mammalian species indicates that Treg cells are integral to the workings of the immune system and survival. Moreover, it is thought that iTreg cells emerged in placental animals to enforce maternal tolerance to foetal alloantigens (Samstein et al., 2012), further highlighting the importance of Treg cells across many species.

Treg cell mechanisms of action

Treg cells are anergic *in vitro* but continuously proliferate *in vivo*, most likely due to recognition of self-antigens and commensal bacteria (Sakaguchi et al., 2008). The threshold of Treg cell TCR stimulation by antigen is reduced compared with the concentration of antigen required to stimulate the TCR of effector T cells (Sakaguchi et al., 2008). Once activated in an antigen specific manner, the Treg cell exerts its suppressive effects in a non-specific manner. Treg cells can constrain the activation and expansion of naive and effector CD4⁺ Th cells, CD8⁺ Tc cells and NK cells as well as inhibit the maturation and function of innate cells, including APCs. Treg cells exert their effects via cell-contact dependant mechanisms, production of soluble factors, metabolic disruption, targeted cell death and APC dependant mechanisms. Treg cells can suppress the immune response by producing certain cytokines, forcing the balance of the milieu towards tolerogenic regulation, by cytotoxic killing of effector cells through the release of perforin and granzyme, by contact signalling to the DC, blocking co-stimulation and by enzymatically processing pro-inflammatory molecules into anti-inflammatory molecules (Shevach, 2009).

CTLA-4 can interact with CD80/86 on the APC to co-stimulate the Treg cell to exert its suppression and furthermore, may also be able to strip the APC of its co-stimulatory molecules by high avidity binding causing removal of the ligand from the cell surface (Qureshi et al., 2011, Miyara and Sakaguchi, 2007). CTLA-4 can directly mediate suppression by inducing the enzyme indoleamine 2,3-dioxygenase (IDO) in DCs which facilitates the catabolism of tryptophan into immunosuppressive metabolites (Boasso et al., 2005).

Lymphocyte activation gene 3 (LAG-3) is a CD4-associated adhesion molecule expressed transiently on the surface of activated T cells and expressed highly and persistently in activated Treg cells (Huang et al., 2004). LAG-3 is capable of binding MHC-II molecules, with higher affinity than CD4; therefore impairing TCR mediated activation of CD4⁺ T cells (Huard et al., 1995).

GITR is highly expressed on the majority of Treg cells but is also expressed at lower levels on B cells, NK cells and macrophages whereas its cognate ligand is expressed on APC such as DC (Shevach and Stephens, 2006). GITR is thought to negatively modulate the magnitude of suppression as stimulation of GITR on Treg cells with its ligand abrogates suppression (Shimizu et al., 2002).

TCR-inducible co-stimulatory receptor (ICOS) provides co-stimulation for the expansion of activated Treg cells and is thought to be integral for the generation and function of Treg cells in certain experimental disease settings including helminth infection, (Redpath et al., 2013), experimental diabetes (Kornete et al., 2012), experimental airway allergy (Busse et al., 2012) and during EAE (Rottman et al., 2001). Moreover, ICOS regulates the generation and function of human Treg cells (Zheng et al., 2013) and Treg cells expressing ICOS have been identified in human disease settings including cancer (Strauss et al., 2008) and SLE (Liu et al., 2011).

Glycoprotein A repetitions predominant (GARP) is selectively up-regulated on activated Treg cells and is thought to mark Treg cells with enhanced suppressive function (Chen and Oppenheim, 2011). GARP is required for binding latent TGF- β on the surface of Treg cells (Stockis et al., 2009).

HLA-DR is expressed on approximately one third of effector Treg cells in human PBMC (Chen and Oppenheim, 2011) and is associated with highly suppressive FoxP3⁺ populations which function by contact-dependent mechanisms (Baecher-Allan et al., 2006).

CD39 is an ectonucleotidase that cleaves adenosine tri-phosphate (ATP) to adenosine mono-phosphate (AMP) (Kaczmarek et al., 1996). CD39 is expressed on the vast majority of FoxP3⁺

Treg cells in mice however, in the healthy human population; CD39 is expressed by approximately half of FoxP3⁺ Treg cells (Borsellino et al., 2007). Extracellular ATP exerts marked pro-inflammatory effects and its catabolism by CD39 therefore results in a net anti-inflammatory effect (Borsellino et al., 2007). CD73 can remove the final phosphate group to form adenosine which ligates to the A2A receptor expressed on T cells resulting in increased intracellular cAMP and suppression of effector T cell function (Deaglio et al., 2007).

These aforementioned cell surface molecules may be used as markers of activated, suppressive Treg cells in combination with the established Treg markers.

Treg cell production and release of regulatory cytokines, such as IL-10 and TGF- β non-specifically suppress the inflammatory environment independent of cell-to-cell contact (Taylor et al., 2006). These regulatory cytokines can suppress effector T cells directly by inducing anergy or indirectly via modulation of APC function (Taylor et al., 2006). The induction of iTreg cells from naive precursors, under the influence of IL-10 and TGF- β , serves to amplify the regulatory response whilst preventing additional differentiation of naive T cells into pro-inflammatory effector cells. Furthermore, Treg cell reliance on exogenous IL-2 can indirectly suppress effector cells by acting as an IL-2 sink, competitively removing the growth factor from the environment and inducing IL-2 deprivation mediated apoptosis (Vignali, 2012, Pandiyan et al., 2007). Interestingly, Treg cells' ability to sequester IL-2 has been implicated in the enhancement of Th17 cell differentiation as IL-2 has been shown to promote Th1 whilst diminishing Th17 cell differentiation in mice (Kryczek et al., 2007, Laurence et al., 2007, Fujimura et al., 2013), thus, adding to the complexity of Treg/Th17 balance. Additionally, recently published work indicates that exogenous IL-2 controls the balance between Treg, Th1 and Th17 cells *in vitro* from RA patients and healthy controls (Kosmaczewska et al., 2014).

Treg cell subsets and phenotypes are summarised in figure 1.5.

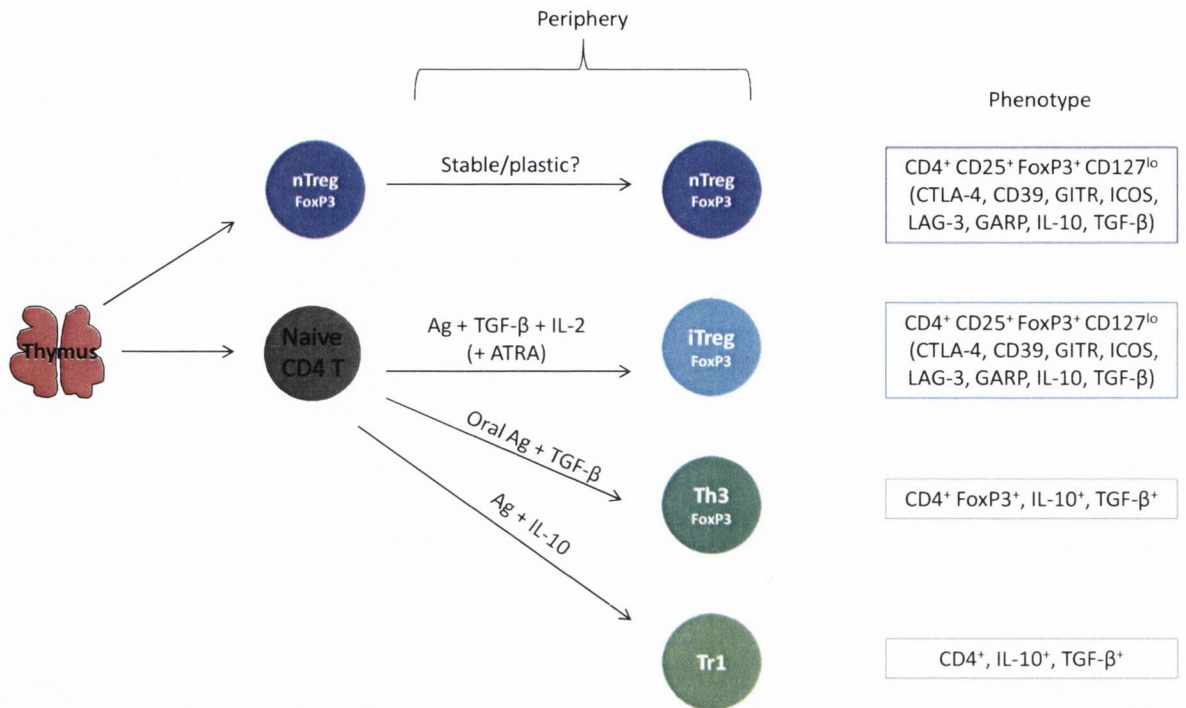


Figure 1.5 **CD4⁺ Regulatory T cell subsets**

nTreg cells develop in the thymus in response to self antigens along with naive effector T cells. nTreg cells are released into the periphery to regulate peripheral immune responses. The stability and life-long suppressive capacity of the nTreg in chronic inflammatory environments is unknown. In the periphery, naive Th cells can be converted into regulatory cells by signaling from the cytokine milieu. iTreg cells are converted from naive Th cells in the presence of antigen (Ag) under the influence of TGF- β and IL-2. The phenotype of nTreg cells and iTreg cells is very similar. Phenotypic markers shown in brackets represent markers that are not universally expressed by the whole population. Th3 cells are differentiated in the gut mucosa under the influence of TGF- β in the presence of low doses of orally ingested Ag. Th3 cells express FoxP3 and produce TGF- β and IL-10. Ag presented in the presence of IL-10 induces naive CD4⁺ T cells into Tr1 cells. These cells do not express FoxP3 but produce high levels of IL-10 and can also produce TGF- β .

Treg cell plasticity

Th cells were originally thought to be terminally differentiated, however, in recent years there has been an increasing number of publications suggesting that this is not the case (Zhou et al., 2009). Treg cells are no exception and a vast literature which documents Treg cell plasticity is rapidly emerging. The iTreg and Th17 axes of differentiation intersect at an early stage of development as they both rely on TGF- β signalling for lineage commitment. Depending on the cytokine milieu, TGF- β promotes an anti-inflammatory response or, in conjunction with IL-6, a pro-inflammatory Th17 response (Kimura and Kishimoto, 2010). These lineages were therefore thought to be mutually exclusive, divergent pathways. However, as evidenced in the literature, Treg and Th17 cells may in fact be dynamic inter-related partners, held in equilibrium by their environment. The discovery of T cells co-expressing FoxP3 and ROR- γ t (Voo et al., 2009, Zhou et al., 2008, Ayyoub et al., 2009) and Treg cells secreting IL-17 (Fletcher et al., 2009) have reinforced this notion. In addition to Treg cells existing in a “hybrid” Treg-effector state, FoxP3⁺ Treg cells have also been shown to differentiate into FoxP3⁻ Th17 (Valmori et al., 2010, Komatsu et al., 2014) and Tfh (Tsuji et al., 2009) cells. This Treg cell plasticity may contribute to their inability to constrain chronic inflammation in autoimmune diseases by tipping the balance towards an effector response (figure 1.4C).

Treg and effector cell plasticity may, however, be a dynamic equilibrium as it has recently been shown that Th1 cells exhibit plasticity and can convert to a Treg cell phenotype (Amarnath et al., 2011). Moreover, CD161 was recently identified on a subset of Treg cells and marks Treg cells that are capable of producing pro-inflammatory cytokines, including IL-17 (Afzali et al., 2013, Pesenacker et al., 2013). This evidence implies that iTreg cells can also be induced from previously differentiated effector Th1 or Th17 cells. A summary of Th1, Th17 and Treg cell plasticity is shown in figure 1.6.

There may be inherent differences in the stability of nTreg cells versus iTreg cells due to epigenetic differences at their *FoxP3* locus (Huehn et al., 2009), however, this research has been hampered by the lack of adequate markers to decipher nTreg cells from iTreg cells generated *in vivo* and therefore, has only examined *in vitro* generated iTreg cells.

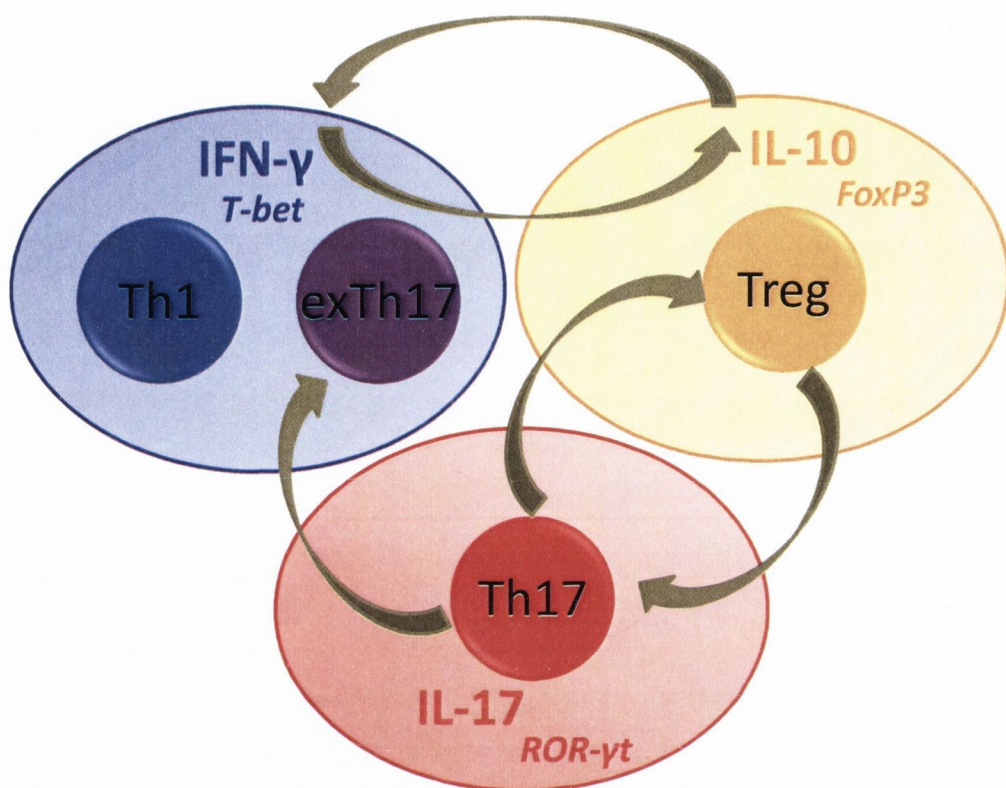


Figure 1.6 Plasticity between Th17, Th1 and Treg cell subsets.

Treg cells (shown in yellow) can produce pro-inflammatory cytokines such as IL-17 and IFN- γ and switch into ROR- γ t expressing Th17 cells (red) or T-bet expressing Th1 cells (blue). Th17 cells are plastic and can switch into IFN- γ producing, T-bet expressing ex-Th17 cells (purple). In addition, Th1 and Th17 cells can switch into suppressive FoxP3 expressing Treg cells.

Treg cell therapy

Although the therapeutic potential of Treg cells was hypothesised as far back as the 1970s (Gershon, 1975), and despite the exponential growth in Treg literature since then, translating Treg cell therapy from bench to bedside has proven onerous. Administering a drug to enhance Treg cell activity may also enhance effector T cell activity *in vivo*, exacerbating disease. The infamous drug TGN1412, a CD28 superagonist, developed by TeGenero Immuno Therapeutics, was tested in both rodent and non-human primate models and found to preferentially expand Treg cells (Lin and Hunig, 2003) and restore tolerance in the animal model of MS (Beyersdorf et al., 2005). In phase 1 trials, however, the drug caused a cytokine storm in healthy adults (Suntharalingam et al., 2006).

In adoptive cell therapy, the patient donates PBMC, which are sorted *ex vivo* for CD4⁺ CD25⁺ Treg cells, expanded *in vitro* then infused back into the same patient (Riley et al., 2009). This treatment strategy could be hazardous if these expanded Treg cells exhibit any degree of plasticity. It has been shown that the majority of expanded, adoptively transferred, Treg cells retain suppressive activity *in vivo* but a small frequency lose FoxP3 expression and can differentiate into effector T cells (Komatsu et al., 2009). The use of iTreg cells differentiated from naive T cells *in vitro* for adoptive cell therapy has also been a topic of much research; it has been shown that ATRA in combination with IL-2 and TGF- β enhanced the conversion of non-Treg precursor cells into FoxP3⁺ Treg cells and furthermore enhanced suppressive capacity and stability compared with iTreg cells generated with IL-2 and TGF- β alone (Wang et al., 2009, Lu et al., 2010). ATRA induced iTreg cells produced less IFN- γ and IL-2 than iTreg cells induced with IL-2 and TGF- β alone and moreover, retained a protective phenotype in a mouse model of graft-versus-host disease even when treated with pro-inflammatory IL-1 β and IL-6 (Lu et al., 2010). Another study in mice showed that addition of ATRA to nTreg cells abrogated IL-6 mediated Treg cell plasticity to a Th17 phenotype and maintained the expression of FoxP3 (Zhou et al., 2010).

1.4 Autoimmunity

Autoimmunity occurs when tolerance is broken, allowing an inappropriate immune response to self-tissues, which can manifest as clinically apparent diseases such as type-I diabetes, systemic lupus erythematosus (SLE), MS and RA. The precise aetiologies of many autoimmune disorders remain elusive however; associations to certain kinds of infections have been discovered which, along with other environmental triggers (such as nutrient status/smoking) and a genetic predisposition are thought to elicit disease. The most common genetic linkages to autoimmune diseases are found in the MHC-II alleles and specifically, HLA-DR polymorphisms have been

associated with both RA and MS (Nagy et al., 2010, Sadovnick, 2012). Autoimmunity tends to occur at a higher prevalence in females; both RA and MS exhibit a 2:1 ratio of female to male patients (Whitacre, 2001).

Historically, T cell mediated autoimmunity was attributed to pathogenic Th1 cells whilst a Th2 type response was thought to be protective. More recently, the field has shifted focus to Th17 cells, which have been demonstrated to play a key role in pathogenesis of a variety of autoimmune diseases including RA, psoriasis, SLE, inflammatory bowel disease (IBD) and MS (Maddur et al., 2012). The emerging concept implicates the disequilibrium between auto-reactive pathogenic Th1/Th17 responses and protective Treg cell responses in the origin and subsequent pathophysiology of autoimmunity. Intuitively, Treg cells are a promising candidate for adoptive cell therapy, however, due to the dynamic and plastic nature of the immune response, the true efficacy and safety of this approach is yet to be established. In addition, targeting Th17 cells therapeutically is also a rational strategy for ameliorating autoimmune pathologies and this is undergoing investigation with several different approaches including blocking the differentiation of Th17 cells, blocking the cytokines they produce or by inhibiting the transcription factors specific for Th17 cells (Maddur et al., 2012).

1.4.1 Rheumatoid Arthritis

Epidemiological studies of RA indicate an average population prevalence of about 0.5% to 1% globally (Silman and Pearson, 2002). European countries and the USA are included in this bracket, however, China and Japan have a lower prevalence and the disease is nearly undetectable in rural Africa. A significant increase in prevalence is observed in Pima and Chippewa Native Americans with 5% to 7% occurrence in the population. Twin and migrant studies have strongly implicated a genetic association with the disease but other causative factors, such as hormone levels and diet are also associated risks factors. Smoking has also emerged as an environmental factor implicated in the pathogenesis of RA and is now identified as a major preventable risk factor in RA aetiology (Kallberg et al., 2011). RA is a chronic, systemic, relapsing-remitting, autoimmune disease that manifests mainly in synovial joints such as wrists, fingers, knees and ankles. The inflamed synovium undergoes marked hyperplasia and synovial fluid accumulates in the joint causing swelling, severe pain and loss of function. In addition, inflammation triggers the degradation of bone and cartilage leading to destruction of the joint architecture. Pain is clinically assessed using the disease activity score 28 (DAS28) formula which accounts for the number of tender and swollen joints (out of a possible 28), the erythrocyte sedimentation rate (ESR; which marks inflammation) and the patient's own

assessment of their global health. A DAS28 score of more than 3.2 indicates active disease, between 2.6 and 3.2 denotes well controlled disease and below 2.6 is considered remission. The concentration of C-reactive protein (CRP) in the serum of patients is also used as an indicator of active inflammation. As inflammation is systemic, other organs can become damaged; patients often suffer with rheumatoid lung, vasculitis and atherosclerosis which account for the significant mortality and morbidity in these patients.

The immuno-pathology of RA

The RA synovial joint contains a mélange of inflammatory infiltrates including T and B cells, macrophages, DCs, neutrophils, NK cells and mast cells. These lymphoid cells can also form follicular aggregates, germinal center-like structures and granulomas (Cope, 2008). These structures create an environment, at the site of inflammation, capable of supporting auto-antigen encounter, processing and presentation of self-proteins to T cells (Cope, 2008). Dysregulated positive-feedback signalling between leukocytes, synovial fibroblasts, chondrocytes and osteoclasts propagate the pathogenesis of RA.

Autoantibodies have been identified in RA, including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs). A gene-environment interaction that confers a high risk of seropositive (antibody positive) RA has been identified between shared epitope genes in the HLA-DR and smoking (Padyukov et al., 2004). The shared epitope hypothesis is based on the structural differences in MHC-II/HLA molecules which allow more than one antigenic peptide to fit into the groove and be displayed to the T cell (Gregersen et al., 1987). It is now well established that smoking causes the citrullination of proteins in the lung. Citrullinated proteins can fit into shared epitopes of certain HLA molecules to induce an immune response which generates ACPAs (Baka et al., 2009). These ACPAs can remain latent in the lung for several years. It has been postulated that an infection or some form of damage which causes inflammation in the joint, promotes the citrullination of structural proteins such as collagen, vimentin or filaggrin which the ACPAs generated in the lung can recognise (Baka et al., 2009). ACPAs are detected in the synovial fluids of up to 80% of RA patients (Reparon-Schuijt et al., 2001, Raptopoulou et al., 2007) and may play a key role in the pathogenesis, as well as the aetiology, of RA as their presence is correlated with disease severity (Lundberg et al., 2005). ACPAs are now being used in the clinic as serological markers of RA in addition to the longstanding antibody marker and clinical parameter, RF.

Macrophages contribute to synovitis through phagocytosis and APC function as well as the production of cytokines (TNF, IL-1, IL-6, IL-12, IL-15, IL-18 and IL-23), reactive oxygen species

(ROS), nitroxides, icosanoids and matrix-degrading enzymes (McInnes and Schett, 2011). Interaction with T cells, immune complexes and cytokines further drives macrophage activation. Neutrophils produce prostaglandins, ROS and proteases. Mast cells generate vasoactive amines, chemokines, proteases and cytokines. Moreover, mast cells have been reported to be the primary source of IL-17 in the RA synovium, (Hueber et al., 2010, Suurmond et al., 2011) however, these studies used immunohistochemistry and fluorescent microscopy to draw this conclusion but these techniques cannot definitively prove that the mast cell produces IL-17. Alternatively, this data may indicate that mast cells merely bind the IL-17 produced by T cells. Fibroblast-like synoviocytes in the RA joint become hyper-proliferative, anchorage independent, lose contact inhibition and have an altered resistance to apoptosis, similar to transformed cancer cells, and express cytokines, chemokines, adhesion molecules and matrix metalloproteases (MMPs) (Filer et al., 2006). In addition to directly damaging cartilage in its vicinity, synovial fibroblasts promote the survival of T and B cells.

Th cells and their cytokines in RA

CD4⁺ T cells are enriched in the synovial joints of RA patients and play a central role in the pathogenesis of RA through the production of pro-inflammatory cytokines (Astry et al., 2011). A Th1 cytokine profile has been identified in cells taken from the synovial fluid of RA patients (Dolhain et al., 1996) and furthermore, Th1 cells specific for citrulline are significantly enriched in RA patients compared with healthy controls (James et al., 2014). Th17 cells induce the production of MMPs which result in the degradation of cartilage and also induce a highly pro-inflammatory phenotype in fibroblast cells, creating a pro-inflammatory positive feedback loop which may drive chronic inflammation and degradation in RA (van Hamburg et al., 2011). In addition, plastic ex-Th17 type cells have been identified in inflammatory arthritis (Nistala et al., 2010, Cosmi et al., 2011, Chalan et al., 2013). Th1 and Th17-type cells mediate their effects through the production of pro-inflammatory cytokines, including TNF, IFN- γ , IL-17 and GM-CSF.

TNF, produced by Th1 and Th17 type cells, is an important autocrine and paracrine inducer of other pro-inflammatory cytokines and chemokines and propagates inflammation in RA by up-regulating the expression of adhesion molecules on the cells that line the synovial joint (Choy and Panayi, 2001). The importance of TNF in the pathogenesis of RA is indicated by the success of anti-TNF therapies. There is controversy surrounding the role of IFN- γ , the seminal cytokine of Th1 cells, in RA. Some evidence suggests that IFN- γ is rarely detectable in RA synovial fluid and tissue (Firestein and Zvaifler, 1987) and studies in animal models imply a protective role for IFN- γ based on the observation that IFN- γ deficiency exacerbates disease (Hirota et al., 2007).

However, administration of recombinant IFN- γ to RA patients did not significantly improve clinical outcomes (Veys et al., 1997). Other data shows that Th1 cells are enriched in RA joint compared with peripheral blood (Yamada et al., 2008, Suzuki et al., 1999) and moreover, IFN- γ is produced spontaneously from RA synovial Th cells *ex vivo* (Sattler et al., 2009). IFN- γ can act as a pro- or anti- bone resorptive cytokine but overall exerts net stimulatory effects on bone resorption and bone loss by stimulating T cell activation and secretion of RANKL and TNF (Gao et al., 2007). GM-CSF secreted by Th cells plays a key role in propagating inflammation in RA by recruiting and activating innate immune cells in a positive feedback manner. Th17 cells have been identified as a major source of GM-CSF and data from murine models implicate Th17 derived GM-CSF in the pathogenesis of autoimmunity (Codarri et al., 2011, El-Behi et al., 2011). Administration of recombinant GM-CSF to mice primed for a suboptimal collagen induced arthritis (CIA) response increased the disease incidence, accelerated onset time and exacerbated the clinical score and joint swelling of mice (Campbell et al., 1997). As a result, GM-CSF is now being targeted therapeutically in early clinical trials with RA patients; preliminary evidence indicates that the drug is well tolerated and has efficacy in RA, shown by a significantly reduced DAS28 score (Behrens et al., 2014). IL-17, the signature cytokine of Th17 cells, drives the recruitment and activation of neutrophils, stimulates the release of cytokines and chemokines and induces the production of prostaglandins and matrix metalloproteinases (Weaver et al., 2007). In mouse models of arthritis, inhibition of IL-17 suppressed joint inflammation and conversely, over-expression of IL-17 exacerbated joint inflammation and damage (Lubberts et al., 2005). Furthermore, IL-17 has been proven to synergise with TNF to enhance inflammation in the RA joint (Miossec, 2003, Griffin et al., 2012). Unfortunately, the efficacy of anti-IL-17 therapy in phase II trials has been disappointing; however, phase III trials are currently underway and may decipher the true value of IL-17 as a therapeutic target (Kellner, 2013).

Th17/CD161 plasticity and polyfunctionality in RA

Murine studies of autoimmunity heavily implicate Th17 cells in disease pathogenesis. However, functionally plastic IFN- γ producing Th17 cells (or ex-Th17/non-classical Th1 cells) have been observed, calling the stability of the Th17 cell lineage into question (Lee et al., 2009b, O'Shea and Paul, 2010). Tracking the plasticity of Th17 cells by fate mapping has yielded compelling data suggesting that plastic Th17 cells which co-produce IL-17 and IFN- γ are responsible for mediating autoimmune damage in experimental autoimmune encephalomyelitis (EAE), the mouse model of MS (Hirota et al., 2011). Moreover, plastic Th17 cells have been shown to be a feature of chronic inflammation, such as in the case of autoimmunity, in contrast to acute

inflammation as a result of candida albicans infection, which did not elicit IFN- γ from Th17 cells (Hirota et al., 2011).

CD161, a lectin-like receptor, is a marker of human Th17 type cells (Cosmi et al., 2008), as discussed in the above section *CD161 expression on Th cells*. Importantly, CD161 expression is retained on Th17 type cells even after they switch to an ex-Th17 cell that co-produces IL-17 and IFN- γ , or IFN- γ alone (Annunziato et al., 2013). The frequencies of circulating CD161⁺ Th cells have recently been demonstrated to correlate with disease activity in RA (Miao et al., 2013). However, caution must be exercised when examining features of peripheral blood; depending on the proximity of sampling to an inflammatory event, the blood may show inverse results to those observed at the site of autoimmune inflammation in the joint. This phenomenon may be due to cell trafficking, resulting in an apparent deficit in the circulation but enrichment in the joint or vice versa. Recent data indicates that frequencies CD4⁺ T cells expressing CD161 are decreased in the peripheral blood of newly diagnosed RA patients but enriched in the synovial fluid of both newly diagnosed and late-stage RA (Chalan et al., 2013). In addition, the study showed that CD161⁺ Th17 type cells from the synovial fluid of a small cohort of late-stage RA patients had a Th1 cell phenotype, indicating that the CD161⁺ Th17-type cells had switched into plastic IFN- γ producing ex-Th17 cells. More extensive work has been published concerning the role of CD161⁺ Th cells in JIA. CD161⁺ Th1-type cells (CXCR3⁺ CCR6⁻/IFN- γ ⁺ IL-17⁻) and CD161⁺ Th17/Th1 type cells (CXCR3⁺ CCR6⁺/IFN- γ ⁺ IL-17⁺) accumulate in the synovial fluid of patients with JIA but, interestingly, CD161⁺ bona fide Th17-type cells (CXCR3⁻ CCR6⁺/IFN- γ ⁻ IL-17⁺) are not enriched in joint (Cosmi et al., 2011). TCR V β profiling by two independent groups has shown that CD161⁺ cells with a Th1 phenotype (ex-Th17 cells) share a common clonal ancestry with CD161⁺ Th17-type cells indicating that ex-Th17 type cells are derived from bona fide Th17 cells in JIA synovial fluid (Nistala et al., 2010, Cosmi et al., 2011). Furthermore, the highly pro-inflammatory environment of the JIA joint has been shown to be responsible for promoting Th17 cell plasticity via an IL-12 mediated mechanism (Cosmi et al., 2011, Nistala et al., 2010). In addition, the frequency of total CD4⁺ CD161⁺ T cells and CD4⁺ CD161⁺ IFN- γ ⁺ IL-17⁺ positively correlates with ESR and serum levels of CRP in JIA, which strongly suggests that CD161⁺ Th cells and, in particular, plastic ex-Th17 CD161⁺ Th cells are directly linked to the disease activity of arthritis (Cosmi et al., 2011).

Plastic Th17 cells co-producing IL-17 and IFN- γ have recently been shown to be polyfunctional due to the concomitant production of GM-CSF in mice during EAE (Duhon et al., 2013) and, more recently, in the joints of JIA patients (Piper et al., 2014). Synergy between different cytokines

derived from Th17-type cells is likely to be mechanistically important for Th17 mediated pathogenic effector functions in RA, or, indeed autoimmunity in general.

The role of Treg cells in RA

Understanding the altered nature of immuno-regulatory responses during autoimmune inflammation is fundamental to understanding the overall disease. A deficit in overall Treg cell numbers or a defect in Treg cell function are both plausible contributors to the chronic inflammation observed in the RA joint. Several studies examining Treg cell phenotype and function in RA have drawn incongruent conclusions. Conflicting data are largely due to the fact that some studies examined cells taken from the peripheral blood of RA patients whereas others looked at cells from the site of inflammation, in the RA synovial fluid. Researchers examining the peripheral blood of RA patients have reported a decrease in the frequency of Treg cells compared with healthy controls (Sempere-Ortells et al., 2009, Cao et al., 2003, Raghavan et al., 2009), however, studies which extracted lymphocytes from the synovial fluid of RA patients have reported enhanced frequencies of Treg cells (van Amelsfort et al., 2004, Cao et al., 2004, Raghavan et al., 2009). Considering the highly mobile nature of T cells during an immune response, discrepancies between the frequencies of Treg cells in the blood and in the joint may be due to cells trafficking out of the circulation to the site of inflammation.

Inflammation persists in RA despite high frequencies of Treg cells in the joint and an explanation as to why Treg cells fail to constrain or resolve the chronic inflammatory response remains elusive. The frequency of Treg cells in blood has been inversely correlated to disease activity (Sempere-Ortells et al., 2009, Kawashiri et al., 2011). However, there is no association between the frequency of Treg cells in RA patients and clinical parameters such as the presence of rheumatoid factor or bone erosion (Cao et al., 2004). The expression of CTLA-4 on Treg cells in the peripheral blood is contentious; one report has shown increased CTLA-4 expression on RA Treg cells (Sempere-Ortells et al., 2009) whilst another report has found significantly reduced CTLA-4 expression on Treg cells from RA patient peripheral blood (Ehrenstein et al., 2004). Further to the latter group's findings, Treg cells from the peripheral blood of active RA patients were able to suppress effector cell proliferation but not pro-inflammatory cytokine production (Ehrenstein et al., 2004). The data indicated that this defective suppression was caused by an increased CTLA-4 internalisation rate. Driving CTLA-4 to the surface of the Treg cell by treating it with PMA restored the Treg cell's ability to suppress IFN- γ production by effector cells (Flores-Borja et al., 2008). Another report has shown significantly increased frequencies of Treg cells expressing CTLA-4, GITR and OX-40 in RA synovial fluid (van Amelsfort et al., 2007).

Furthermore, that report demonstrated that Treg cells from the peripheral blood of RA patients suppressed the production of TNF and IFN- γ .

Suppression assays carried out by two independent groups illustrated that Treg cells from the synovial fluid of RA patients suppressed effector cell proliferation and reduced the concentrations of TNF and IFN- γ produced in the supernatants (van Amelsfort et al., 2004, Cao et al., 2004), suggesting that Treg cells in the synovial joint are suppressive and yet fail to constrain inflammation.

In JIA, elevated numbers of Treg cells have been reported in the synovial fluid compared with peripheral blood (Haufe et al., 2011). Furthermore, suppression assays were carried out on sorted Treg and effector cells from the peripheral blood of healthy controls or synovial fluid of JIA patients; the data demonstrated a reduced inhibition of JIA synovial fluid derived effector cells by synovial Treg cells. Cross-over studies showed the impaired suppression was due to the nature of synovial effector cells and was not a defect in Treg cells; healthy control Treg cells could not suppress JIA synovial effector cells as effectively as autologous effectors but Treg cells from the JIA synovium were capable of suppressing healthy control effector cells as effectively as healthy control Treg cells (Haufe et al., 2011).

Role of fibroblast cells in propagating autoimmune inflammation in RA

In a healthy joint, the synovium is comprised of fibroblast cells and macrophages, two to three cells thick, which serves as a barrier attaching the bone and joint capsule together (Filer, 2013). In RA, the synovial lining becomes hyperplastic due to the inhibition of apoptotic pathways and results in the lining becoming ten to fifteen cells thick. This layer can invade adjacent cartilage and bone, resulting in degradation and eventual loss of mobility in the joint. In addition, the underlying sub-layer, which is composed of less densely packed fibroblast cells, contains large numbers of infiltrating immune cells. These neighbouring fibroblast and immune cells influence each other through the secretion of soluble factors such as cytokines.

Fibroblast cells mediate much of the structural damage in the RA joint by secreting MMPs and cathepsins, which degrade bone and cartilage (Ritchlin, 2000). Moreover, fibroblast cells express and secrete RANKL to promote osteoclast activation and bone resorption (Shigeyama et al., 2000). Fibroblast cells potentiate the persistence of inflammation by maintaining the recruitment, survival and retention of lymphocytes in the inflamed joint (Buckley, 2011) by constitutively expressing chemokines and modulating endothelial cells (McGettrick et al., 2010). Furthermore, fibroblast cells establish chronic inflammation by inappropriately blocking

apoptosis and secreting cytokines that promote survival of T cells, B cells and neutrophils (Buckley et al., 2001, Filer et al., 2006).

T cells reciprocally affect fibroblast cells via the secretion of cytokines which elicit an enhanced pro-inflammatory phenotype in the fibroblast cells. Interestingly, even unstimulated T cells induce synovial fibroblast cells to produce prostaglandin E₂, IL-6 and IL-8 and this production is synergistically augmented by the addition of recombinant IL-17 (Yamamura et al., 2001). More recently, it has been established that IL-17 synergises with other pro-inflammatory cytokines, such as TNF, to induce a pro-inflammatory phenotype from fibroblast cells (Benedetti and Miossec, 2014, Parsonage et al., 2008). Moreover, fibroblast cells have been shown to up-regulate expression of CD40, ICAM-1 and MHC-II in response to IFN- γ (Kato et al., 2013). Synovial fibroblast cells express the GM-CSF receptor (Postiglione et al., 1998) however, little is known about their response to GM-CSF.

Hypoxia in the RA joint; HIF-1 α in T cells.

Low oxygen tension is a well established feature of RA synovial joints (Lund-Olesen, 1970). Despite the up-regulation of angiogenic factors in the RA joint, tissue oxygen levels fail to recover due to elevated oxygen consumption caused by synovial hyperplasia and increased metabolic demand. Tissue oxygen pressure is inversely correlated with synovitis, indicating that increased inflammation is significantly associated with a more hypoxic environment (Ng et al., 2010). In addition, an inverse relationship between tissue oxygen pressure and T cell infiltration was demonstrated in the joints of RA patients (Ng et al., 2010). In order to survive under hypoxic conditions, cells must switch from oxidative phosphorylation to the anaerobic process of glycolysis to meet their metabolic demands. This switch is orchestrated by the transcription factor hypoxia-inducible factor-1 α (HIF-1 α) (Semenza, 2009). HIF-1 α is expressed during normoxia but the presence of oxygen hydroxylates two proline residues within the HIF-1 α molecule which marks the protein for ubiquitination and degradation. Under hypoxic conditions, HIF-1 α is not tagged for ubiquitination and is therefore stabilised in the cell.

T cells, due to their highly mobile nature, are subjected to a wide range of oxygen tensions; from the relatively hypoxic environments of the bone marrow (Parmar et al., 2007) and thymus (Braun et al., 2001) to circulation in the oxygen-rich blood. Effector memory T cells retained in non-lymphoid tissues and at sites of inflammation may undergo protracted exposure to hypoxia, as evidenced by the expression HIF-1 α in T cells present in inflammatory bowel disease (Higashiyama et al., 2012) and RA (Gaber et al., 2009)

HIF-1 α is required for the differentiation of Th17 cells in mice and additionally, is more highly expressed in Th17 cells compared with other Th cells (Shi et al., 2011). HIF-1 α is thought to preferentially enhance Th17 development and concomitantly down-regulate Treg cell development in mice (Shi et al., 2011, Dang et al., 2011). However, conflicting data suggests that hypoxia and HIF-1 α promote FoxP3 expression and Treg cell function (Ben-Shoshan et al., 2008, Clambey et al., 2012). The definitive role of hypoxia in T cell differentiation remains elusive in human cells, however, one thing is clear; in order for T cells to survive under hypoxic conditions they must stabilise expression of HIF-1 α .

Interestingly, certain subtypes of stem cells exist in hypoxic environments and it has therefore been postulated that hypoxia may be advantageous to stem cell longevity (Davy and Allsopp, 2011). Furthermore, HIF-1 α expression maintains functional levels of telomerase, the enzyme that lengthens telomeres, in murine embryonic stem cells (Davy and Allsopp, 2011).

Therapeutic strategies for RA

Disease modifying anti-rheumatic drugs (DMARD) are the frontline therapy for RA, with treatment commencing at the time of diagnosis (Sizova, 2008). These include methotrexate, hydroxychloroquine, cyclosporine, gold salts, sulfasalazine and non-steroidal anti-inflammatory drugs (NSAID). In cases where these DMARD therapies fail to show efficacy, cyclophosphamide or steroid therapies are utilised to stabilise uncontrolled autoimmune inflammation. Most conventional DMARD therapies do not have a rational basis for treating RA but their efficacy has rather been discovered by serendipity (Suresh, 2010). As a result, these therapies work via “sledgehammer” style mechanisms to inhibit immune responses non-specifically, causing severe adverse effects. Methotrexate, for example is an anti-folate drug used as a cancer chemotherapeutic and for ectopic pregnancies in addition to its use for a range of autoimmune diseases including RA, psoriasis, SLE and Crohn’s disease (Chan and Cronstein, 2013, Cecchino et al., 2014). Methotrexate exerts a variety of pharmacological effects and it is therefore thought to be a combination of these effects that result in its anti-proliferative and globally immunosuppressive effects in RA (Chan and Cronstein, 2013). Because management of RA is largely focused on ameliorating joint symptoms, prophylactic or therapeutic treatments for co-morbidities such as heart disease are not always considered in the therapeutic strategy for the patient. The treatment outcomes for the majority of patients are suboptimal as they fail to reach full remission status (Keystone et al., 2012). The disease, like many other autoimmune disorders, exhibits a significant degree of heterogeneity across the patient population, resulting in varied drug responses.

Research over the past 2 decades has elucidated the pathophysiology of RA and has led to the development of rationally designed novel biological drugs with immunomodulatory effects such as Infliximab (anti-TNF), Abatecept (CTLA-4 Ab), Tocilizumab (anti-IL6) and Rituximab (B cell depleting mAb). Clinicians are improving treatment strategies by using DMARD and biologic combination therapies, yet an approach that adequately produces therapeutic tolerance, sufficient to retain the remission state long-term or to cure the disease outright, has thus far remained unattainable (Suresh, 2010).

The emergence of the biologic era of drugs arose as a consequence of advancement in biochemical techniques, which allowed for the identification of cytokines in tissues derived from the RA joint and a concomitant advancement in protein engineering allowing for the production of highly specific, clinical grade antibodies (Taylor, 2011). TNF was the first cytokine identified as a potential therapeutic target for RA. Anti-TNF therapy was validated in clinical trials for eliciting significant benefit in most patients (Elliott et al., 1994) furthermore, in combination with methotrexate treatment, anti-TNF showed marked efficacy in improving joint swelling as well as halting structural damage (Emery et al., 2009) which had previously been considered an inexorably progressive feature of RA. Despite the success of anti-TNF therapy, many caveats exist with its use and with the use of biological treatments generally, including a high rate of primary non-response in 25-30% of patients and the loss of response over time in initially responsive patients, incomplete pain relief despite reduced inflammation and an insufficient overall response to achieve stable remission. Moreover, targeting cytokines that are critical to host defence may cause a significant increase in the incidence of infection. Notably, anti-TNF therapy is associated with reactivation of latent tuberculosis (Miller and Ernst, 2009).

The effect of anti-TNF therapies on Th1 and Th17 type cells has been assessed *in vitro* and *ex vivo* from RA patients. *In vitro* differentiated Th1 cells were found to be more susceptible to treatment with infliximab than Th17 cells (Kanayama et al., 2011). Another study found that infliximab therapy increased the frequency and absolute number of Th cells producing IL-17 in the peripheral blood of treated RA patient as well as the concentration of IL-17 produced by patient PBMC stimulated *ex vivo* (Alzabin et al., 2012). Moreover, in an animal model of RA, CIA, non-response to anti-TNF is associated with increased concentration of IL-17 in cells stimulated *ex vivo* from peripheral blood and increased frequency of IL-17 producing cells in the joints of mice (Alzabin et al., 2012). These findings may explain why reduction in disease activity in response to anti-TNF is often incomplete but given the efficacy of anti-TNF therapy, may also indicate that the full pathogenicity of Th17 cells requires the presence of TNF. This postulation is

further supported by evidence of synergy between IL-17 and TNF which exacerbates cartilage destruction (Van Bezooijen et al., 2002, Moran et al., 2009). Interestingly, it has recently emerged that JIA patients treated with etanercept (a decoy receptor that binds to TNF) have reduced frequencies of CD161⁺ IFN- γ ⁺ ex-Th17 type cells in their peripheral blood. In addition, recombinant TNF promoted the plasticity of sorted Th17 cells from the peripheral blood of healthy donors into IFN- γ producing ex-Th17 cells and this was reduced in the presence of etanercept (Maggi et al., 2014).

The rationale for targeting IL-17 developed from observations in the CIA mouse model of RA showing markedly attenuated disease in IL-17 deficient mice (Nakae et al., 2003). Furthermore, mice treated with anti-IL-17 after the onset of CIA exhibited reduced joint inflammation and bone and cartilage destruction (Lubberts et al., 2004). Clinical evidence demonstrated that IL-17 concentrations were increased in the serum and synovial fluid of RA patients (Metawi et al., 2011, Moran et al., 2009, Park et al., 2005) and positively correlated with DAS28 scores (Metawi et al., 2011). The pleiotropic effects of IL-17 in the rheumatoid joint include synovial inflammation, cartilage destruction, bone erosion and fibroblast activation which are all consistent with a pathogenic role for IL-17 in RA and provide rationale for targeting IL-17 therapeutically in RA.

As with all immuno-modulatory therapies, a holistic view is required to ensure that the patient does not become severely immunodeficient. Th17 cells play a central role in host defense against extracellular bacteria and fungi at mucosal surfaces. Therefore, targeting IL-17 may increase the risk of infection which needs to be weighed against the potential benefit. People with genetic defects eliciting IL-17 deficiencies develop chronic mucocutaneous candidiasis but do not develop serious or life threatening infections or immunopathologies (Puel et al., 2011). However, given that IL-17 is a family of cytokines (A-F), blocking IL-17A therapeutically for autoimmunity may not be sufficient to increase susceptibility to mucocutaneous infections.

Despite high expectations for the efficacy of anti-IL-17 treatment for RA, results from phase II trials have been disappointing. Secukinumab failed to reach its primary endpoint and although ixekizumab in combination with DMARD therapy attained a statistically significant primary response, this was based on ACR20; indicating only a 20% improvement in disease compared with placebo (Kellner, 2013). Furthermore, brodalumab (anti-IL-17RA), another IL-17 targeting antibody, showed no evidence of significant clinical efficacy. Interestingly, all three of these IL-17 targeted drugs have proven highly efficacious in psoriasis (Leonardi et al., 2012, Papp et al.,

2012, Papp et al., 2013), suggesting that the lack of efficacy in RA is not due to pharmacokinetics but rather due to the role of IL-17 in pathology.

Given that Th17 cells are plastic in the context of inflammation, perhaps targeting IL-17 in established RA is obsolete as the cells may have already switched (or at least received the signal to switch) into ex-Th17 cells that no longer produce IL-17. For this reason, targeting CD161⁺ Th cells may prove to be more efficacious than targeting just IL-17 as it would deplete both bona fide Th17 and ex-Th17 cells as well as their full arsenal of pro-inflammatory mediators, not just IL-17. Furthermore, if plasticity is central to the pathogenicity of autoimmune inflammation, depletion of plastic cells may help to return the skewed autoimmune imbalance back to homeostasis (figure 1.4C). This may prevent further manifestations of autoimmunity in RA patients and may help to prevent secondary inflammatory morbidities. Plastic IL-17⁺ IFN- γ ⁺ T cells have been detected in the artery wall of patients with symptomatic coronary atherosclerosis and induce pro-inflammatory responses in vascular smooth muscle cells (Eid et al., 2009). It may therefore be postulated that RA associated atherosclerosis may be exacerbated by plastic ex-Th17 cells. Targeting these cells may also have a prophylactic impact on secondary inflammation in the vital organs.

1.5 Working hypothesis

Certain subpopulations of Th cells may contribute significantly more than other populations to autoimmune inflammation and damage in the RA joint. In addition, Treg cells may not effectively constrain pathogenic Th cells in the RA joint. Recent advances in the literature suggest that T cells are capable of functional plasticity in certain pro-inflammatory environments, therefore it was hypothesized that Th cells, especially Treg and Th17-lineage cells, from the synovial fluid of RA patients may be functionally plastic and this may contribute to the propagation of autoimmune inflammation. Figure 1.7 outlines the working hypothesis of this study.

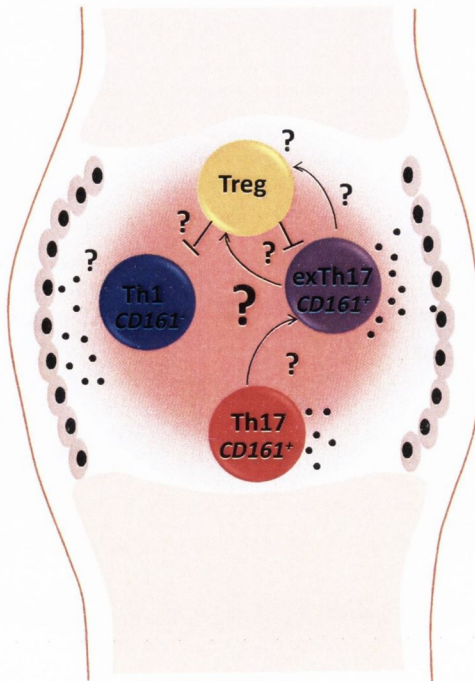


Figure 1.7 Plasticity and aberrant regulation may play a key role in the propagation of autoimmune inflammation in RA.

Fibroblast cells line the synovial compartment and play an active role in contributing to inflammation in RA through the production of pro-inflammatory cytokines and chemokines. T cells play a central role in the propagation of inflammation in the RA joint. The diagram illustrates a working hypothesis which suggests that a certain subpopulation of Th cells may contribute significantly more than other populations to autoimmune inflammation and damage in the RA joint. In addition, Treg cells may not effectively constrain Th cells in the RA joint. Recent advances in the literature suggest T cells are capable of functional plasticity in certain pro-inflammatory environments, therefore it was hypothesized that Th cells, especially Treg and Th17-lineage cells, from the synovial fluid of RA patients may be functionally plastic and this may contribute to the propagation of autoimmune inflammation.

1.6 Aims

- **To assess the phenotype and function of effector Th cells in the RA joint.**

The cytokine profile and cytokine polyfunctionality (the ability to express multiple cytokines concomitantly) in cells extracted from RA synovial fluid, RA peripheral blood and healthy control peripheral blood will be evaluated along with the expression of CD161. Features of CD161⁺ Th cells which may promote persistence in the chronically inflamed RA joint will be examined to gain insight into the pathogenic capacity of these cells. In addition, the differential effect of CD161⁺ and CD161⁻ Th cells on synovial fibroblast cells will be examined.

- **To assess the phenotype and function of Treg cells in the RA joint.**

Analysing the phenotype of Treg cells may demonstrate a defect in the Treg cell compartment which may be causative of Treg cell failure to constrain inappropriate inflammation in RA. Functional analysis of Treg cell mediated suppression of effector cells will illustrate if a defect exists in Th cell regulation in RA. Plasticity of Treg cells in the RA joint will also be assessed to evaluate their contribution to inflammation.

- **To analyse the differential Treg cell mediated regulation of CD161⁺ and CD161⁻ Th cells and moreover, assess the differential regulation of discrete Th1, Th17 and ex-Th17 cells.**

Purified populations of effector and Treg cells were sorted and co-cultured in a suppression assay. Treg cell mediated suppression of proliferation and pro-inflammatory cytokines will be robustly assessed.

Chapter 2

Methods

Chapter 2 Methods

2.1 Isolation of PBMCs from human venous blood

Healthy donor and patient blood samples, obtained with informed consent, were collected in sterile 9ml heparin-coated tubes. The blood was diluted 1 in 2 with sterile PBS and layered over Lymphoprep (Axis-Shield poC AS, Oslo, Norway; up to 30 ml of diluted blood over 20 ml of Lymphoprep).

For large numbers of healthy control PBMC, buffy coat blood packs (the plasma-depleted fraction of anti-coagulated blood) were obtained with consent from the Irish Blood Transfusion Board. Blood was diluted 1 in 2 with sterile PBS and centrifuged at 1250 g for 10 minutes at room temperature. The buffy coat layer containing leukocytes was removed (figure. 2.1A), diluted in 200 ml of sterile PBS and layered over Lymphoprep (20 ml Lymphoprep and 25 ml diluted buffy coat per tube).

PBMC were isolated by density-gradient centrifugation (900 g, 20 minutes, 20°C, with the brake off). The PBMC layer was removed using a Pasteur pipette (figure 2.1B), washed in sterile PBS and centrifuged at 650 g for 10 minutes. The supernatant was completely discarded and the pelleted PBMC were resuspended in sterile PBS and centrifuged at 290 g for 10 minutes. The supernatant was completely discarded and the pelleted PBMC were resuspended in sterile PBS and centrifuged at 290 g for 7 minutes to remove platelets. The supernatant was discarded and PBMC were resuspended in complete RPMI (cRPMI; Biosera; appendix 1) and counted.

2.2 Isolation of SFMC from synovial fluid of RA patients

Synovial fluid samples were obtained with informed consent from patients with RA attending rheumatology clinics at St Vincent's University Hospital. Ethical approval was granted by the St Vincent's Research Ethics Committee. Synovial fluid was aspirated into a syringe and allowed to cool to room temperature. Hyaluronidase (0.5 mg/ml) was added to the fluid and incubated for 1 hour at 37°C. Synovial fluid samples were then diluted 1 in 4 with Hank's balanced salt solution (HBSS; Sigma Aldrich) and 20 ml of diluted fluid was layered over 5 ml of Lymphoprep and centrifuged at 400 g for 25 minutes with the brake off. The cloudy synovial fluid mononuclear cell (SFMC) layer was removed from the interface, washed twice in HBSS and centrifuged at 500 g for 10 minutes. Cells were cryopreserved in FCS containing 10% DMSO at -80°C overnight then transferred to liquid nitrogen. Cells were thawed into warm cRPMI for use, washed, pelleted by centrifugation at 300 g for 5 minutes, resuspended in cRPMI and counted.

2.3 Patient information

Variable	# of patients
Male	17
Female	24
DAS28 > 3.2	21
DAS28 2.6-3.2	5
DAS28 < 2.6	1
methotrexate	5
methotrexate + anti-TNF	11
methotrexate + other biologic	2
anti-TNF	4
anti-TNF + cyclosporin	1
sulfasalazine	2
mycophenolate	1
no treatment	9

Please note that at time of printing the DAS28 scores of 14 patient samples were unknown and the treatment at time of sampling for 6 patients was unknown.

Graphs illustrating individual patient’s ages and DAS28 scores with mean and standard deviation are shown in figure 2.2. For phenotyping studies, age and sex matched healthy control PBMC were used to compare with RA PBMC and SFMC.

2.4 Cell Counting

Cells in cRPMI were diluted in trypan blue (Sigma Aldrich) and viable mononuclear cells were counted on a light microscope (Leica DM500) using a haemocytometer (Improved Neubauer, Hawksley or Hycor Glasstic slide, Kova). The total number of cells was calculated from the formula:

Average cell number (in one quadrant) x 10⁴ x trypan blue dilution factor x volume (ml) = total cell number

2.5 Purification of total CD4⁺, CD4⁺ CD45RA⁺ (naive) and CD4⁺ CD45RO⁺ (memory) T cell by negative selection using magnetic activated cell sorting (MACS).

PBMC were isolated from blood samples as described in section 2.1 and total CD4⁺ T cells, naive CD4⁺ T cells or memory CD4⁺ T cells were isolated using the relevant MACS T cell isolation kit (Miltenyi Biotech) according to the manufacturer's protocol. Cells were washed in MACS buffer and pelleted by centrifugation at 360 g for 5 minutes. The supernatant was completely discarded and the pellet was resuspended in 40 µl of MACS buffer per 10⁷ cells. Biotin conjugated antibody cocktail was added to the cells at 10 µl per 10⁷ cells. The solution was mixed thoroughly, allowed to incubate for 10 minutes at 4°C, washed in MACS buffer and pelleted by centrifugation at 650 g for 10 minutes. The supernatant was completely discarded and the pellet was resuspended in 80 µl of MACS buffer per 10⁷ cells. Anti-biotin micro beads (Miltenyi Biotech) were added to the cells at 20 µl per 10⁷ cells. The cell/bead solution was mixed thoroughly, incubated for 15 minutes at 4°C then washed in MACS buffer and pelleted by centrifugation at 650 g for 10 minutes. The pellet was resuspended in 500 µl (for up to 10⁸ cells, or scaled up appropriately for larger cell numbers) of MACS buffer and passed through a 70 µm cell strainer (BD falcon) to remove clumps. An LS magnetic column (Miltenyi Biotech) was placed in the magnet and washed with MACS buffer (3 ml). The cell sample was then loaded onto the column followed by 3 washes of MACS buffer (3 ml per wash). The desired cell fraction (total CD4⁺, CD4⁺ CD45RA⁺ or CD4⁺ CD45RO⁺, depending on the kit used) was contained in the 12 ml eluate. Purified cells were washed in cRPMI (Biosera), pelleted by centrifugation at 360 g for 5 minutes and counted. The purity of the sorted cells was assessed by flow cytometry and was routinely > 95% (figure. 2.3).

2.6 Cell tracer labelling

Cells for labelling were washed in sterile PBS to remove FCS or glutamine as the presence of amine groups quenches tracer dyes. Tracer dyes were diluted in sterile, warm PBS to avoid precipitation and an equal volume added to cells suspended in warm PBS (2x10⁶ cells / ml) to yield a final concentration of 1 µM for Cell Trace Violet (CTV; appendix 1) or 0.5 µM for carboxyfluorescein succinimidyl ester (CFSE; appendix 1). Cells were incubated at 37°C for 10 minutes. Tracer dye labelling was stopped by the addition of at least 4 times the labelling volume of cRPMI (Biosera). Cells were cooled on ice for 5 minutes, pelleted by centrifugation (360 g for 5 minutes) and resuspended in cRPMI (Biosera). A sample of unlabelled cells, CTV

labelled cells and CFSE labelled cells were fixed with 4% paraformaldehyde (PFA; Santa Cruz Biotech) immediately after staining on day (d) 0 for use as compensation controls on d 4 and d 5.

2.7 Treg cell Suppression Assays

CD4⁺ T cells were purified using MACS (section 2.5) from human healthy control (HC) PBMC, or SFMC from RA patients (St. Vincent's University Hospital, Dublin) with active disease (DAS28 > 3.2). CD4-enriched HC PBMC and RA SFMC were stained in cRPMI for extracellular markers; CD39-FITC, CD25-PE, CD4- PerCP-Cy5.5 and CD127-Alexafluor647, and sorted by fluorescence activated cell sorting (FACS) on a Moflo™ cell sorter (Beckman Coulter). Cells were sorted into responder (CD4⁺ CD25⁻, Treg depleted) and Treg subsets (CD4⁺ CD25⁺ CD127^{lo} CD39⁺ and CD4⁺ CD25⁺ CD127^{lo} CD39⁻). The purity of sorted T cell subsets was assessed by flow cytometry and was routinely greater than 98%. The fidelity of the CD4⁺ CD25⁺ CD127^{lo} sorting panel for Treg cell isolation was assessed by intranuclear FoxP3 staining post sorting. Sorted Treg cells were routinely >90% FoxP3⁺ (figure 2.4). Responder cells were labelled with CTV or CFSE (section 2.6), according to the manufacturers protocol. Labelled cells were washed and counted. Responder cells were cultured at 5x10⁴ / well alone or in co-culture with unlabelled or CTV labelled (section 2.6) Treg cells at 5x10⁴ / well or 2.5x10⁴ / well. Cells were stimulated with plate bound α-CD3 (appendix 1; 1 µg/ml bound in PBS for a minimum of 2 hours at 37°C) in the presence of 3x10⁵ irradiated (60 Gy), allogeneic, unlabelled or CFSE labelled (section 2.6) PBMC to provide co-stimulation (figure 2.5). Depending on the experimental outcome, different cell labelling was chosen for certain populations; responder cells were labelled with CTV and Treg cells were left unlabelled, where possible, as Treg cell numbers are usually limiting and Treg cells are more sensitive to experimental handling (figure 2.5A). When the proliferation of the Treg cell was assessed, responder cells were labelled with CFSE and Treg cells were labelled with CTV (figure 2.5B). Cells were maintained in humidified incubators at 37°C with 5% CO₂. Proliferation of responder cells was checked on d 4 by assessing CTV dilution by flow-cytometry (BD Fortessa™). On d 5, supernatants were harvested and cells were restimulated with PMA and ionomycin in the presence of brefeldin A golgi block (appendix 1) and analysed for cytokine production and proliferation by flow cytometry. Irradiated PBMC were excluded from analysis and cytokine production from the responder cell populations was assessed by gating on tracer label⁺ cells.

To further analyse the suppression of CD4⁺ T cell subpopulations, CD4⁺ memory (CD45RO⁺ CD45RA⁻) T cells were isolated by MACS (section 2.5). A Moflo™ sort was then undertaken to isolate CD161⁺ and CD161⁻ fractions or Th1, Th17 and ex-Th17 subsets. For total CD161⁺ and CD161⁻ subsets, memory CD4⁺ T cells were stained with CD45RA-FITC, CD161-PE, CD4-PerCP-

Cy5.5 and CD25-APC. CD4⁺ CD45RA⁻ CD25⁻ CD161⁺ and CD4⁺ CD45RA⁻ CD25⁻ CD161⁻ responder cells were sorted with routinely >98% purity (figure 2.6), labelled with CTV and co-cultured with Treg cells, as described above. These CD161^{+/+} T cell subsets thus contained only antigen experienced CD4⁺ T cells, excluding Treg cells. A panel of chemokine receptor markers was used together with CD161 to purify Th1, Th17 and ex-Th17 cells (Crome et al., 2010b, Acosta-Rodriguez et al., 2007b, Annunziato et al., 2012). MACS isolated CD4⁺ memory cells (section 2.5) were stained with CCR6-FITC, CXCR3-PE, CD161-PE-Cy7 and CCR4-A647. CD161⁻ CXCR3⁺ CCR4⁻ CCR6⁻ Th1 cells, CD161⁺ CXCR3⁻ CCR4⁺ CCR6⁺ Th17 cells and CD161⁺ CXCR3⁺ CCR6⁺ ex-Th17 cells were sorted with routinely >98% purity according to the gating strategy shown in figure 2.7. Sorted Th subsets were labelled, counted and co-cultured with Treg cells, as described above. Remaining cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours to assess cytokine production from each Th subset in order to ensure that the sorting panel isolated cells with the correct cytokine signature (figure 2.8). If cell numbers yielded from RA patient SFMC sorts for CD161⁺ and CD161⁻ Th cells or HC PBMC sorts for Th1, Th17 and ex-Th17 subsets were lower than 2x10⁴, cells were plated in 384 well plates and cultured with 1x10⁵ irradiated APC.

2.8 *In vitro* Treg cell induction from non-Treg cell precursors

Naive CD4⁺ T cells were purified from HC PBMC as described in section 2.5. Cells were stained in cRPMI for extracellular markers; CD45RA-FITC, CD25-PE and CD4- PerCP-Cy5.5 and sorted by FACS. Sorted CD4⁺ CD45RA⁺ CD25⁻ cells were seeded at 1x10⁶ / well in a 12-well plate with IL-2 (500 IU; appendix 1), TGF- β (10 ng/ml; appendix 1) and All-Trans-Retinoic Acid (ATRA; 100 nM; appendix 1). A Treg expansion kit (Miltenyi Biotech), consisting of beads coated with α -CD3 and α -CD28, was used to activate cells at a ratio of 1:1 beads to cells. Cells were maintained in humidified incubators at 37°C with 5% CO₂. After 5, 7, 10 and 12 days in culture, the induction of iTreg cells was assessed by flow cytometric staining for FoxP3⁺ CD25⁺ CD127^{lo} CD4⁺ T cells.

2.9 iTreg and nTreg function and stability assays

iTreg cells were induced from HC CD4⁺ CD45RA⁺ CD25⁻ naive non-Treg cells *in vitro* (section 2.8). HC PBMCs from the same donor were CD4-enriched by MACS (section 2.5) and total responder cells (CD4⁺ CD25⁻) and Treg cells (CD4⁺ CD25⁺ CD127^{lo}) were sorted by FACS. Sorted responder cells were labelled with CFSE (section 2.6) and cultured alone (50,000 cells / well) or in co-culture with *in vitro* iTreg cells (50,000 cells / well) in a 96 well-plate. Cells were stimulated with anti-CD3 (1 μ g/ml; appendix 1) in the presence of 300,000 irradiated APC (60 Gy) for 5 days. Proliferation of responder cells was checked on d 4 by assessing CFSE dilution on a flow-

cytometer (BD Fortessa™). On d 5, cells were restimulated with PMA and ionomycin in the presence of brefeldin A golgi block (appendix 1) and analysed for cytokine production and proliferation by flow cytometry. Irradiated PBMC were excluded from analysis and cytokine production from the responder cell populations was assessed by gating on CTV⁺ cells.

To assess the stability of different Treg cell subsets in pro-inflammatory environments, *ex vivo* Treg and *in vitro* iTreg cells (50,000 cells / well) were stimulated with anti-CD3 (1 µg/ml; appendix 1) in the presence of 300,000 irradiated APC (60 Gy) and cultured with or without synovial fluid (SF; diluted 1:4 with medium) extracted from the joints of RA patients during active disease. Where applicable, cells were treated with IL-2 (40 IU / ml) and were maintained in humidified incubators at 37°C and 5% CO₂ for 5 days. Cells were analysed for expression of CD4, CD25, CD127 and FoxP3 (section 2.11). Cells were restimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours to assess cytokine production (section 2.11).

2.10 Measurement of cytokine production by ELISA

The concentrations of human IL-17A, IFN-γ, TNF, GM-CSF, IL-6 and IL-8 were quantified by ELISA. 75 µl of capture antibody diluted in coating buffer was applied to high-binding 96-well plates (Greiner Bio-one). Plates were incubated overnight at 4°C, capture antibody was removed and non-specific binding sites were blocked with appropriate blocking solution for 1 hour at room temperature. After blocking, plates were washed in PBS-tween solution (appendix 1), dried and supernatant samples were loaded into wells in triplicates either neat or diluted 1 in 3 with assay diluent. A standard curve of serially diluted recombinant cytokine standard was also loaded onto the plates in triplicate, with the top working standard at 2000 pg/ml. Blank wells, containing assay diluents only, were included on each plate to allow the subtraction of background from each sample. Samples were incubated overnight at 4°C. After washing, biotinylated detection antibody was added to each well and incubated for 2 hours at room temperature. Plates were washed and horseradish-peroxidase (HRP) conjugated to streptavidin was applied to wells for 30 minutes in the dark. Wells were thoroughly washed and the substrate, TMB, was added. The enzyme-mediated colour reaction was protected from light while developing and stopped with the addition of 1M H₂SO₄ (half the volume in the well added; i.e. 1:3 dilution). The optical density of the colour was determined by measuring the absorbance at 450 nm (TMB) using a microtiter plate reader (BioTek, Epoch). Cytokine concentrations were calculated from the standard curve using Gen5[®] software.

2.11 Flow-cytometric staining and analysis

Treg cell staining:

Human HC or RA PBMC and RA SFMC were stained *ex vivo* for Treg analysis. Treg cells were identified using the following standard panel of markers: CD4⁺CD25⁺FoxP3⁺CD127^{lo} in addition to other markers of interest. Cells were first stained for cell surface expression of CD4, CD25 and CD127, followed by intranuclear staining for FoxP3 using a specialised staining kit (eBioscience FoxP3 staining buffer kit). The Fix/Perm solution was diluted 1 in 4 with diluent and 250 µl was applied to cells for 30 minutes in the dark at 4°C. Cells were then washed and pelleted twice in 1 ml of permeabilisation buffer (diluted 1 in 10 with dH₂O) then resuspended in 50 µl of permeabilisation buffer containing fluorochrome conjugated antibodies against intracellular and intranuclear markers and incubated for 30 minutes in the dark at 4°C. Cells were washed and acquired on a FACSCanto™ II or LSRFortessa™ (both BD Biosciences). Compensation beads singly stained with every fluor channel utilised were acquired to adjust for spectral overlap. Fluorescence minus one (FMO) controls were acquired and, where appropriate, Treg depleted populations were stained and used to set negative gates during analysis. An unstained sample was also run as a control. RA SFMC samples often exhibited higher intensity staining compared with PBMC, therefore, the gating strategy for RA SFMC was determined by an SFMC FMO control. Lymphocytes were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively) and live cells were then identified as viability dye^{lo/-} (figure 2.9A and B). In cells stained *ex vivo*, CD4⁺ T cells were directly identified (figure 2.9C).

Intracellular cytokine staining:

Cytokine analysis required stimulation with PMA (Sigma, 50 ng/ml) and ionomycin (Sigma, 500 ng/ml) in the presence of brefeldin A (Sigma, 5 µg/ml) for 5 hours. Every stimulated sample was controlled by an unstimulated sample (brefeldin A only). PMA/ionomycin stimulation down regulates the expression of CD4 on CD4⁺ T cells, therefore, stimulated cells and controls were stained for CD3 and CD8 instead; gating on the CD3⁺CD8⁻ population to identify the CD4⁺ T cells (figure 2.9D). Stimulation also affects CD25 and FoxP3 expression, therefore when analysing cytokine production from Treg cells, cells were stimulated for 4 hours to reduce the effect. Cells were washed in FACS buffer (appendix 1), pelleted by centrifugation at 340 g for 5 minutes and resuspended in 50 µl of FACS buffer containing antibody-conjugated-fluors against extracellular markers of interest. The samples were incubated for 15 minutes in the dark at room temperature, washed in FACS buffer and pelleted by centrifugation. Supernatants were removed and cells were resuspended in 50 µl of fixative solution. For general Th cell intracellular

cytokine staining, the Caltag Fix and Perm (BioSciences) part A, was applied to cells for 10 minutes in the dark at room temperature. Cells were then washed, pelleted by centrifugation (340 g for 5 minutes) and resuspended in the permeabilising buffer (part B) containing the antibody-fluors against the desired cytokines or other intracellular markers. Intracellular stains were incubated for 15 minutes in the dark at room temperature, washed in FACS buffer and acquired on a BD FACS Canto II or LSRFortessa™ (both BD Biosciences) flow cytometer. Analysis and presentation of overlapping subsets of cytokine producing cells was performed using SPICE version 5.3 software, downloaded from <http://exon.niaid.nih.gov> (Roederer et al., 2011).

The gating strategy to assess live CD4⁺ Th cells in samples stained *ex vivo* and in samples stimulated with PMA and ionomycin is shown in figure 2.9.

Assessment of telomere length by flow-FISH:

Thymuses were isolated from 6-8 week old female C57/BL6 mice and passed through a 70 µm sieve. Thymocyte cells in suspension were counted and cryopreserved in FCS containing 10 % DMSO at -80°C. HC PBMC were isolated (section 2.1) and sorted by MACS for CD4⁺ T cells (section 2.5). CD4-enriched cells were stained in cRPMI (Biosera) for CD45RA-FITC, CD161-PE, and CD25-APC and sorted by FACS. Total PBMC, sorted CD45RA⁺ naive Th cells and sorted CD45RA⁻ memory Th cells were assessed as controls for cells of interest; sorted CD4⁺ CD45RA⁻ CD25⁻ CD161⁺ and CD4⁺ CD45RA⁻ CD25⁻ CD161⁻ cells. Sorted cells were split into triplicate tubes and combined with 200,000 CFSE labelled (section 2.6) murine thymocytes to act as an internal control. Cells were fixed with 4% PFA (Santa Cruz Biotech) for 15 minutes at room temperature, washed in PBS and pelleted by centrifugation at 400g for 5 minutes. Supernatants were completely discarded and 1 ml of hybridisation buffer (appendix 1) was added to the pellet while vortexing. Cells were pelleted by centrifugation at 800g for 7 minutes, the supernatants were completely discarded and the residual volume was made up to exactly 100 µl in each tube. The peptide nucleic acid (PNA) Cy5 telomere probe (Cambridge Research Biochemicals) was diluted to 50 µg/ml in dH₂O. The probe was further diluted to 1.5 µg/ml in hybridisation buffer (appendix 1) then added to cells in a 1:1 ratio eliciting a final concentration of 750 ng/ml. Cells were vortexed and incubated in sealed tubes at 82°C for 10 minutes in a water bath then snap cooled in ice-water. The probe was allowed to hybridise for 1 hour, in the dark, at room temperature. The cells were then washed twice in 1 ml of post-hybridisation solution to remove excess unbound probe and pelleted by centrifugation at 800 g for 7 minutes. Cells were then washed twice in PBS, pelleted by centrifugation at 400 g for 5 minutes and immediately acquired on the FACSCanto™ II. The median fluorescence intensity (MFI) of the Cy5 probe was averaged

for all thymocytes (except samples lying outside the standard deviation, which were excluded) and this value was used to normalise the MFI value in each sample.

2.12 Collating multi-parameter flow cytometry using SPICE software.

The co-fluorescence of each of cell with every other fluorescent parameter examined can be deduced by creating Boolean combinations of gates. In mathematics, Boolean algebra is the algebra of truth values; 0 or 1. Creating Boolean combinations therefore elicits a yes or no answer, yielding, in this instance, every possible combination of every cytokine with every other cytokine investigated. The Boolean variables can be put into the software programme Simplified Presentation of Incredibly Complex Evaluations (SPICE) to create a pie-chart style diagram that quantitatively represents all the permutations of each of the parameters with each other (Roederer et al., 2011). The sections within the pie chart represent the frequency of cells producing combinations of cytokines. The legends for the colours within the chart are prohibitively large, therefore, cannot be shown alongside the pie charts but are available for perusal in appendix 2. This legend, however, is made somewhat redundant by the presence of the arcs around the circumference of the pie chart which denote that the proportion of the pie chart lying under the arc is producing the cytokine shown in the arc legend. Where there are multiple arcs over the same section of the pie chart, this signifies that the cells are producing all the cytokines represented by the surrounding arcs within the time period of stimulation.

2.13 Synovial fibroblast cell culture.

Synovial tissue harvested from RA patients during active disease was digested with collagenase type 1 (1 mg/ml) in RPMI for 4 hours in a humidified incubator at 37°C with 5% CO₂. Dissociated cells were grown to confluence in cRPMI and used between passages 4 and 8. The K4 immortalised synoviocyte fibroblast cell line (a kind gift from Dr Ursula Fearon) were also cultured and used between passages 30-40. Primary RA fibroblast cells and K4 fibroblast cells were rendered quiescent by omitting serum for a minimum of 8 hours before stimulating with T cell supernatants or recombinant cytokines.

2.14 Statistical Analysis

Statistical analyses were performed using Prism 5 software; the One-way or Two-way ANOVA with Tukey's multiple comparison and Bonferroni's post-tests, respectively, was used for comparing 3 or more groups. Paired t-tests were used to compare variance between 2 groups within one donor sample. P values of <0.05 were considered significant and denoted with an

asterisk. Normality tests were carried out to ensure that the data was normally distributed for parametric statistical tests.

2.15 Figures

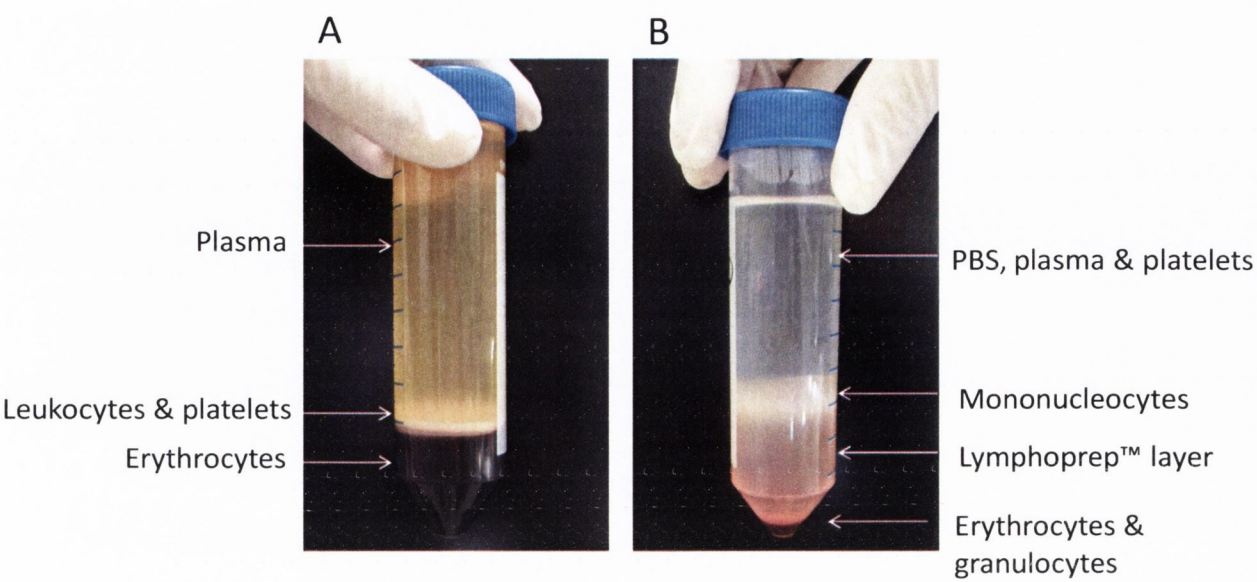


Figure 2.1 Isolation of HC PBMC from buffy coat blood packs.

Blood was diluted 1 in 2 with sterile PBS and centrifuged at 1250 g for 10 minutes at room temperature with the brake off. The blood separates into its constituent parts (A). The buffy coat layer containing leukocytes was removed, diluted in sterile PBS, layered over Lymphoprep and centrifuged at 900 g for 20 minutes at 20°C, with the brake off. The desired mononucleocyte-enriched layer is the cloudy interface between the PBS-containing layer and the Lymphoprep™ layer (B).

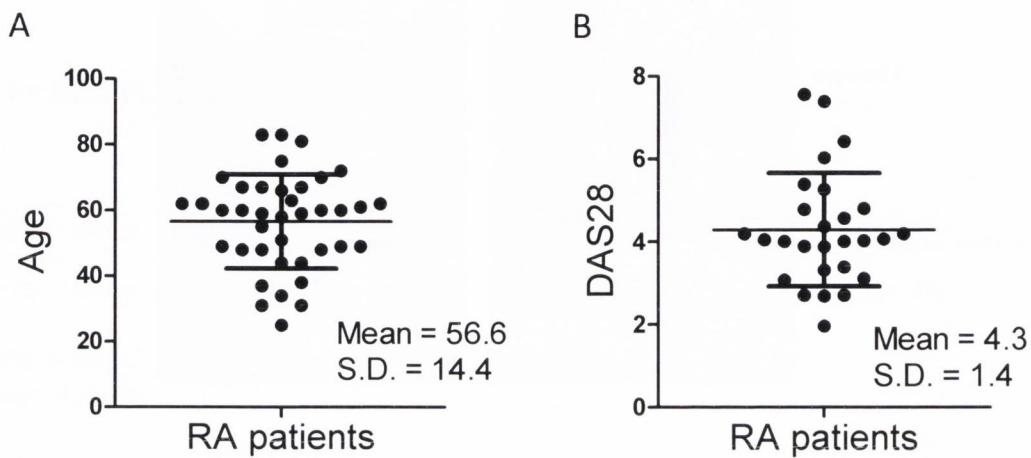


Figure 2.2 RA patient age and DAS28 scores.

RA PBMC and SFMC were obtained from patients with informed consent. Patient age (A) and DAS28 scores (B) were recorded at time of sampling. The data is normally distributed. Mean age is 56.6 ± 14.4 years and mean DAS28 score is 4.3 ± 1.4 , indicating that the majority of patients are experiencing active disease at time of sampling.

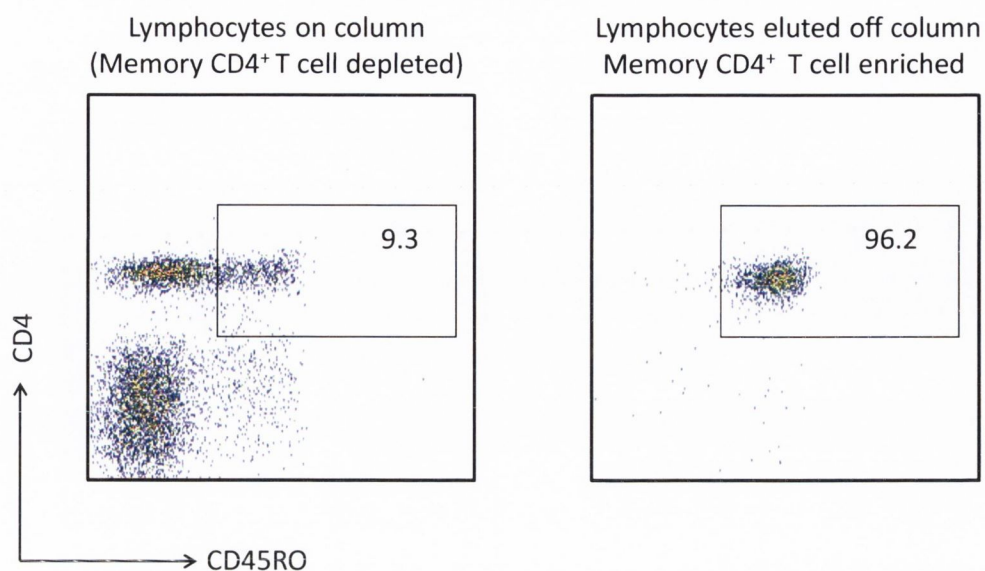


Figure 2.3 Purity of CD4⁺ CD45RO⁺ T cells post negative magnetic sorting from PBMC.

PBMC were isolated from healthy controls and labelled with magnetic beads, according to the manufacturer's protocol. The desired CD4⁺ CD45RO⁺ fraction was eluted off the column (shown on the right hand side). The column was removed from the magnet and remaining cells were flushed off (shown on the left hand side). Cells were stained with fluorochrome-conjugated antibodies against CD4 and CD45RO and analysed by flow cytometry.

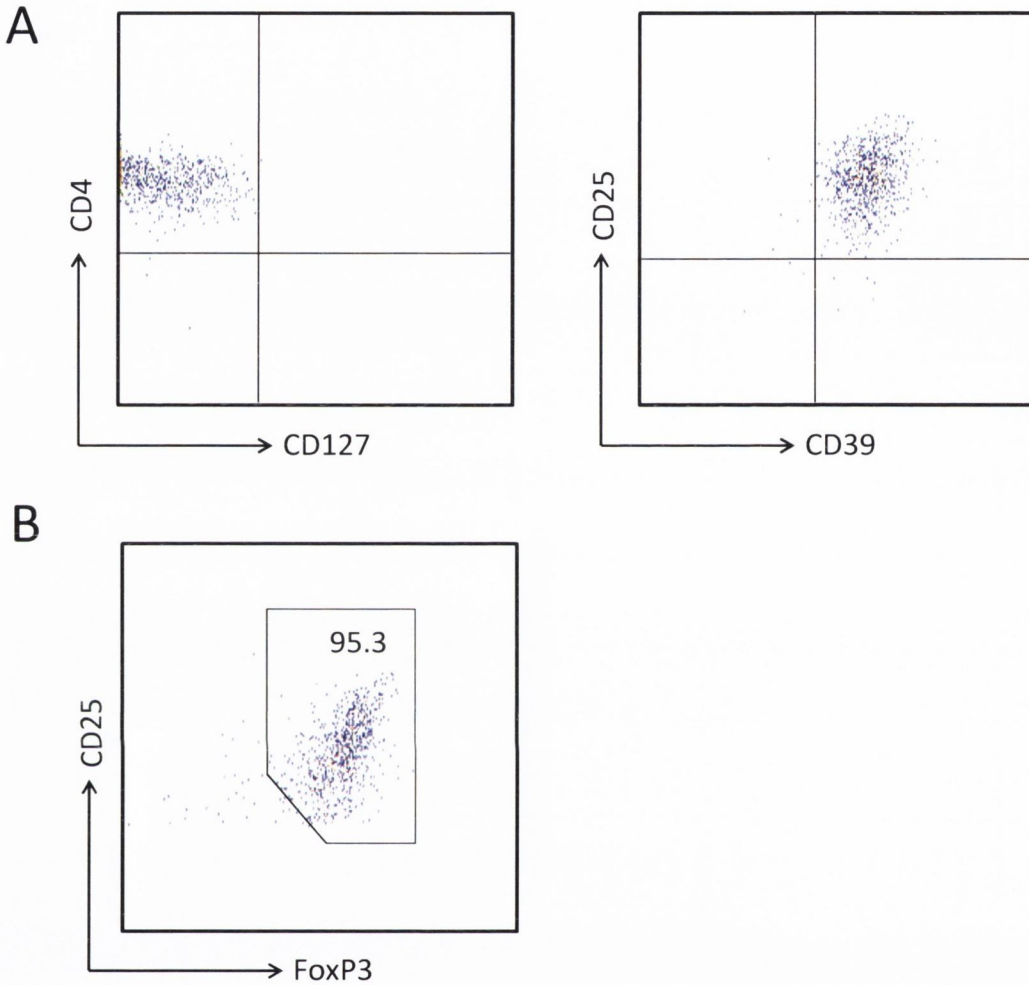


Figure 2.4 Purity of FACS sorted CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells.

CD4⁺ T cells were purified from healthy control PBMC by MACS and stained with fluorochrome-conjugated antibodies specific for CD4, CD127, CD25 and CD39. CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Sorted cell populations were acquired on a flow cytometer to confirm their purity (A). Sorted Treg cells were stained intranuclearly with a fluorochrome-conjugated antibody specific for FoxP3 to ensure the sorted Treg cells were FoxP3⁺ (B).

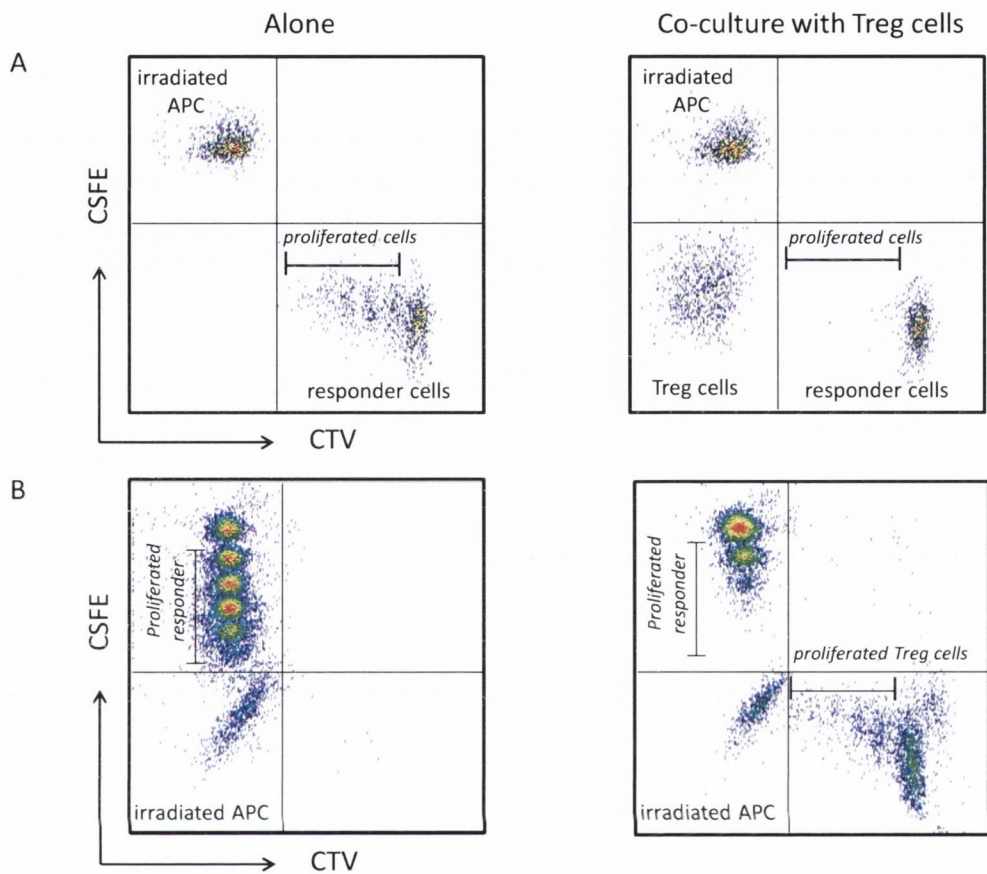


Figure 2.5 Use of cell tracer labels for flow-cytometry based suppression assays.

T cell subsets were sorted by flow-cytometry and labelled with CSFE, CTV or left unlabelled. Cells were cultured with irradiated PBMC and anti-CD3 for 4-5 days. In some experiments, irradiated PBMC were labelled with CSFE (to aid their exclusion from analysis), responder cells were labelled with CTV and Treg cells were unlabelled (A). In experiments analysing Treg cell proliferation, sorted responder cells were labelled with CSFE, Treg cells were labelled with CTV and irradiated PBMC were left unlabelled (B). The proliferation of cells was analysed by measuring the dilution of the cell tracer labels.

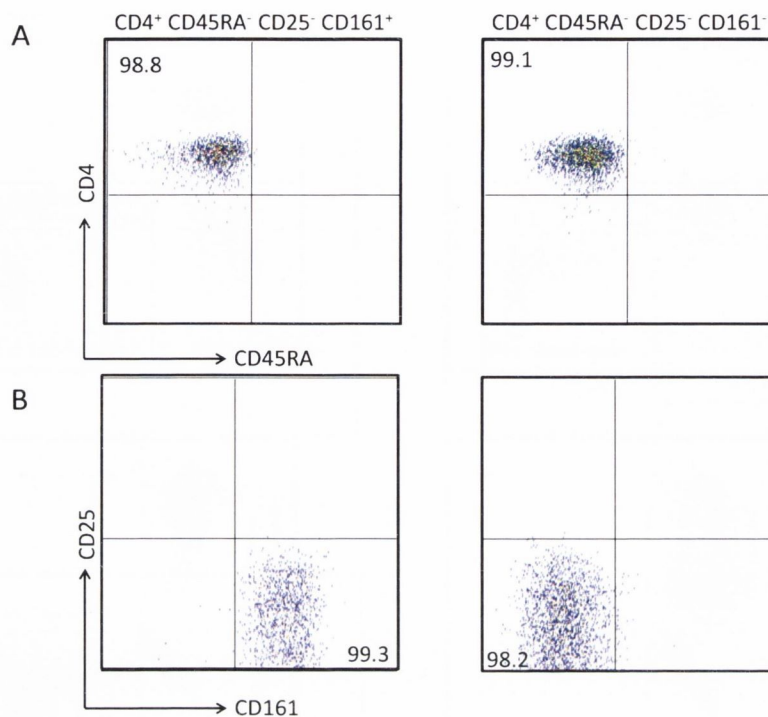


Figure 2.6 Purity of FACS sorted CD4⁺ CD45RA⁻ CD25⁻ CD161^{+/-} responder cells.

CD4⁺ CD45RO⁺ T cells were purified from healthy control PBMC by MACS and stained with fluorochrome-conjugated antibodies specific for CD4, CD45RA, CD25 and CD161. CD4⁺ CD45RA⁻ CD25⁻ CD161⁺ and CD4⁺ CD45RA⁻ CD25⁻ CD161⁻ cells were sorted by FACS. Sorted cell populations were acquired on a flow cytometer to confirm their purity. Cells were gated to select the CD4⁺ CD45RA⁻ memory T helper cell population (A). Selected cells were further gated to exclude activated cells or Treg cells expressing CD25 and finally sorted according to their expression of CD161 (B).

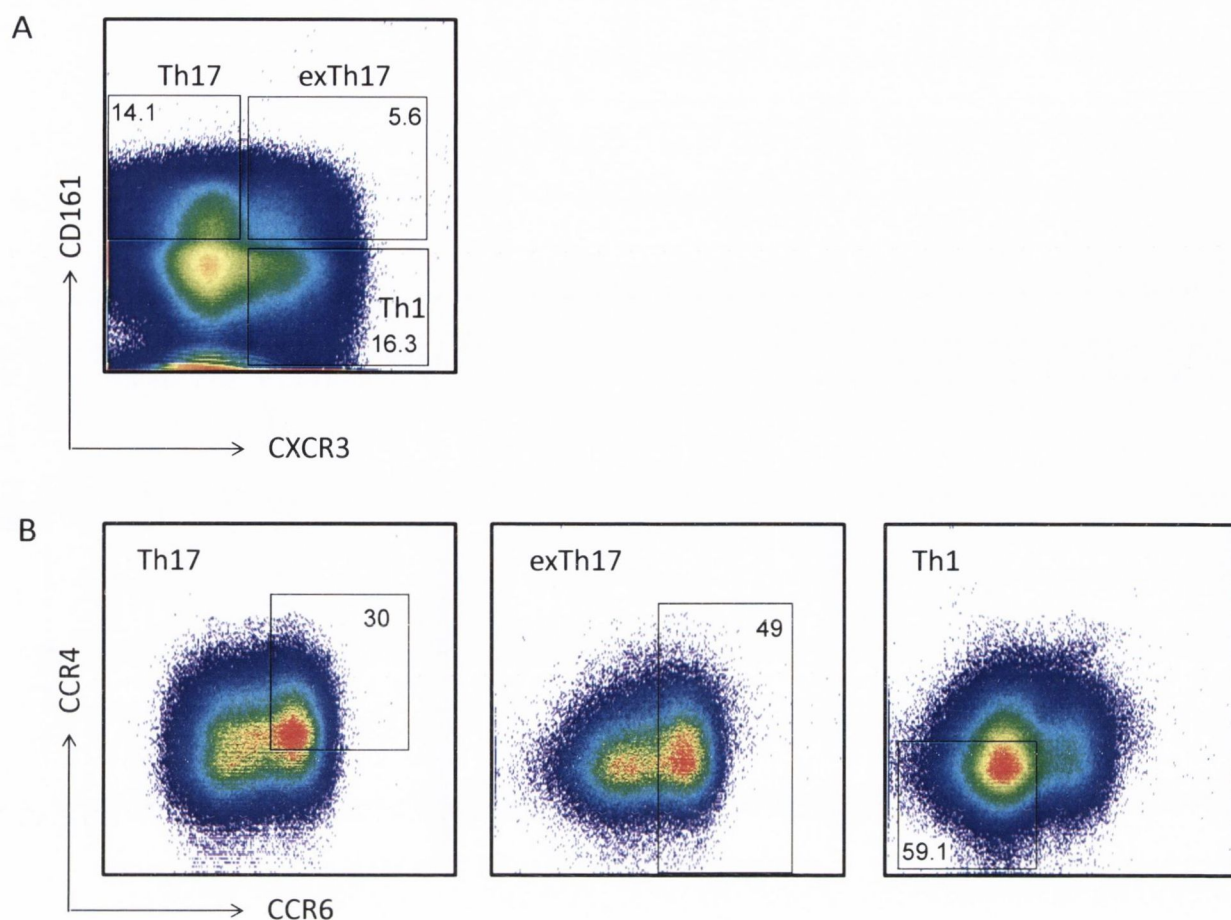


Figure 2.7 Gating strategy for sorting discrete Th1, Th17 and ex-Th17 cells.

CD4⁺ CD45RO⁺ T cells were purified from healthy control PBMC by MACS and stained with fluorochrome-conjugated antibodies specific for CXCR3, CD161, CCR4 and CCR6. Th1, Th17 and exTh17 cell subsets were sorted by FACS. Initially, cells were gated according to their expression of CD161 and CXCR3 (A). These populations were then further gated according to their expression of CCR4 and CCR6 (B) to yield CD161⁻ CXCR3⁺ CCR4⁻ CCR6⁻ Th1 cells, CD161⁺ CXCR3⁻ CCR4⁺ CCR6⁺ Th17 cells and CD161⁺ CXCR3⁺ CCR6⁺ exTh17 cells.

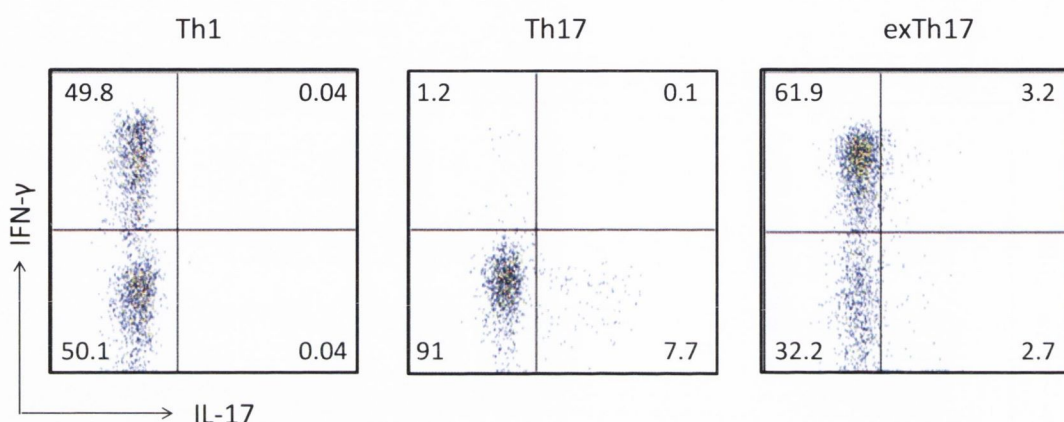


Figure 2.8 Day 0 cytokine production from sorted Th1, Th17 and ex-Th17 cells.

CD4⁺ CD45RO⁺ T cells were magnetically sorted from healthy control PBMC and stained with fluorochrome-conjugated antibodies specific for CXCR3, CD161, CCR4 and CCR6. Cells were sorted using a MoFlo[™] high performance cell sorter. Sorted cell subsets were stimulated with PMA and ionomycin for 5 hours in the presence of brefeldin A. Cells were stained for intracellular IL-17 and IFN-γ.

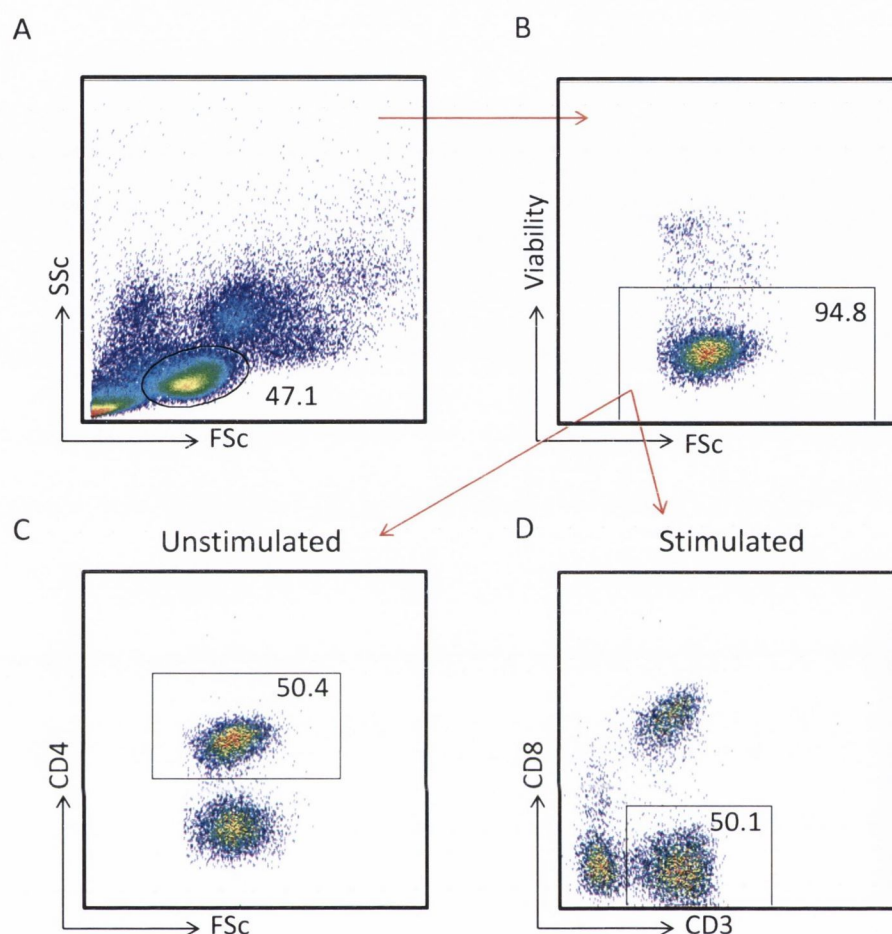


Figure 2.9 Flow cytometry gating strategy to identify live CD4⁺ T cells.

PBMC were isolated from healthy donors, stained with cell viability dye and fluorochrome-conjugated antibodies specific for CD3, CD4 and CD8 and analysed by flow cytometry. The lymphocyte population was identified on the basis of size (FSc) and granularity (SSc) (A). Dead cells stain positive for the viability dye, therefore, live cells are viability^{lo/-} (B). CD4⁺ cells were gated directly from the live lymphocyte population in unstimulated samples (C), however, when cells were stimulated with PMA and ionomycin, surface expression of CD4 was compromised thus, the CD4⁺ T cell population was identified indirectly by gating on the CD3⁺ CD8⁻ population (D).

Chapter 3

CD161⁺ Th cell pathogenicity in RA

Chapter 3 CD161⁺ Th cell pathogenicity in RA

3.1.1 Introduction

Th1 and Th7 type CD4⁺ T cells are abundant in the synovial joints of RA patients and play a central role in the pathogenesis of RA through the production of pro-inflammatory cytokines including; TNF, IFN- γ , GM-CSF and IL-17 (Astry et al., 2011).

TNF, produced by Th1 and Th17 type cells, amplifies inflammation in an autocrine and paracrine manner by inducing production of other pro-inflammatory cytokines and chemokines and up-regulating the expression of adhesion molecules to promote trafficking to the site of inflammation (Choy and Panayi, 2001). TNF was the first cytokine identified as a major contributor to autoimmune inflammation in RA and subsequently anti-TNF therapy was validated in clinical trials for eliciting significant benefit in the majority of treated patients (Elliott et al., 1994). Furthermore, in combination with methotrexate treatment, anti-TNF showed marked efficacy in improving joint swelling as well as halting structural damage (Emery et al., 2009). Unfortunately however, there is a high rate of primary non-response (25-30% of patients) to anti-TNF therapy and loss of response over time in initially responsive patients (Miller and Ernst, 2009).

During an immune response, IFN- γ activates macrophages (Reed, 1988), enhances NK cell activity (Carnaud et al., 1999) and regulates B cell function (Jurado et al., 1989). In addition, IFN- γ promotes bone resorption and bone loss by stimulating T cell activation and secretion of RANKL and TNF (Gao et al., 2007). This cytokine is therefore thought to be a contributing factor to pathology in RA.

GM-CSF secreted by Th cells plays a key role in propagating inflammation in RA by activating macrophages, increasing DC maturation and APC function and by recruiting circulating neutrophils, monocytes and lymphocytes to the site of inflammation (Shi et al., 2006). GM-CSF has recently become a therapeutic target and is currently in early clinical trials with RA patients; preliminary evidence indicates that the drug is well tolerated and has efficacy in RA, shown by a significantly reduced DAS28 score (Behrens et al., 2014).

IL-17 drives the recruitment and activation of neutrophils, stimulates the release of cytokines and chemokines and induces production of prostaglandins and MMPs (Weaver et al., 2007). The receptor for IL-17 is widely expressed on many cell types including non-haematopoietic cells, such as fibroblast and epithelial cells, as well as innate immune cells such as neutrophils and macrophages (Yao et al., 1995, Gaffen, 2009). In mouse models of arthritis, inhibition of IL-17

suppressed joint inflammation and conversely, over-expression of IL-17 exacerbated joint inflammation and damage (Lubberts et al., 2005). Unfortunately, the efficacy of anti-IL-17 therapy in phase II trials has been disappointing; however, phase III trials are currently underway and may clarify the true value of IL-17 as a therapeutic target (Kellner, 2013).

In addition to their singular affects which promote the propagation of inflammation in different ways, these pro-inflammatory cytokines can have combinatorial and synergistic effects. For example, IL-17 and TNF synergistically enhance the production of pro-inflammatory mediators from synovial fibroblast cells and endothelial cells (Miossec, 2003, Griffin et al., 2012). Polyfunctional cells, capable of producing several cytokines concomitantly or in close succession may therefore, contribute more than monofunctional cells to the propagation of autoimmune inflammation. Plastic Th17 cells co-producing IL-17 and IFN- γ have recently been shown to be polyfunctional due to the concomitant production of GM-CSF in mice during EAE (Duhon et al., 2013) and, more recently, in the joints of JIA patients (Piper et al., 2014). Synergy between different cytokines derived from Th17-type cells is likely to be mechanistically important for Th17 mediated pathogenic effector functions in RA, or, indeed autoimmunity in general.

Interestingly, Th17 cells have been shown to exhibit stem cell-like features, which indicate that Th17 type cells are long-lived with a high capacity for self-renewal and resistance to apoptosis (Kryczek et al., 2011). These characteristics suggest that Th17-type cells may persist for extended periods of time during chronic autoimmunity.

T cells, like all other somatic cells, have a finite proliferative lifespan determined by the length of their telomeres, which are stretches of DNA that cap the ends of chromosomes. Every time a cell replicates, part of the telomere is eroded away until a minimum length is reached and the cell is induced into replicative senescence. Given that Th17 type cells have stem cell-like features, one could postulate that they must have longer telomeres than other Th cells; however, this has not yet been shown in the literature.

In order for T cells to survive under hypoxic conditions of the RA joint, they must up-regulate HIF-1 α , which acts as an oxygen sensor and coordinates the switch to anaerobic glycolysis. Interestingly, HIF-1 α is required for the differentiation of Th17 cells in mice and additionally, is more highly expressed in murine Th17 cells compared with other Th cells (Shi et al., 2011). However, the expression of HIF-1 α in human Th cells in the healthy state or during disease induced hypoxia is under studied.

CD161, a lectin-like receptor, is a marker of human Th17 type cells (Cosmi et al., 2008), which is retained even after they switch to an ex-Th17 cell that co-produces IL-17 and IFN- γ , or IFN- γ alone (Annunziato et al., 2013). This marker can therefore be used to track Th17 plasticity and identify Th17 lineage cells.

3.1.2 Aims

The aim of the experiments in this chapter was to examine the phenotype and function of Th cells extracted from the site of autoimmune inflammation, in the RA joint. In particular, this study sought to identify highly pro-inflammatory subsets within the CD4⁺ T cell compartment, which may be a cornerstone of chronic autoimmune inflammation and may be potential therapeutic targets. Specifically, this chapter aimed to:

- Examine the phenotype and function of CD4⁺ effector T cells in RA SFMC
 - Establish the cytokine profile of effector Th cells in the RA synovial fluid and to examine, for the first time, Th cell polyfunctionality in the RA joint.
- Examine the plasticity and pathogenicity of CD161⁺ Th cells in RA SFMC
 - Assess the expression of CD161 on CD4⁺ T cells in RA synovial fluid and, for the first time, examine differential cytokine expression in CD161⁺ versus CD161⁻ Th cells.
 - Evaluate the novel differences in the longevity of CD161⁺ versus CD161⁻ Th cells and therefore, assess their likelihoods of persistence in the RA joint.
- Examine the phenotype and function of CD161⁺ Th cells in HC PBMC
 - Ascertain if CD161⁺ Th cells in healthy donors are inherently more polyfunctional, in terms of pro-inflammatory cytokine production, and therefore likely to play a prominent role in several Th cell mediated autoimmune diseases.
- Examine the effects of CD161⁺ and CD161⁻ Th cells on synovial fibroblast cells

3.2 Results

3.2.1 The phenotype and function of CD4⁺ T effector cells from the RA joint compared with peripheral blood.

Pro-inflammatory cytokine production by CD4⁺ T cells plays an important role in promoting and propagating joint inflammation and destruction in RA. Therefore, the phenotype and function of CD4⁺ effector T cells found within the synovial fluid (SF) of the inflamed joint were examined compared with CD4⁺ effector T cells in RA patient peripheral blood (PB) and healthy control PB. 10-colour flow cytometry was utilised to allow for the simultaneous analysis of multiple parameters in one sample, enabling the identification of cells that co-express multiple cytokines and markers.

3.2.1a CD4⁺ Th cells within RA SFMC are proliferating, effector memory cells with a differentiated phenotype.

PBMC and SFMC were isolated from RA patients with active disease and compared with age and sex matched HC PBMC. Lymphocytes were identified by their size and granularity, determined by the forward scattering (FSc) and the side scattering (SSc) of light, respectively. All samples were pre-stained with a viability exclusion dye which irreversibly stains dead cells, allowing for their exclusion from analysis. The viability dye was analysed against FSc and viability^{lo/-} cells were gated for further analysis (gating strategy shown in methods, figure 2.9). Samples were stained for cell surface expression of CD4 and intranuclear expression of the Treg cell marker, FoxP3, and proliferation marker, Ki67. The frequencies of CD4⁺ T cells were assessed in RA SFMC compared with RA and HC PBMC. No significant difference in the frequency of CD4⁺ T cells was found between the groups (figure 3.1A, left graph). However, the frequency of non-Treg cell effector FoxP3⁻ CD4⁺ T cells actively undergoing cell cycling, as marked by the expression of Ki67, was significantly increased in the RA SFMC compared with both HC and RA PBMC, suggesting that the CD4⁺ T cells at the site of inflammation in the RA joint were more proliferative than CD4⁺ T cells in the periphery (figure 3.1A, right and figure 3.1B).

In order to identify the memory and differentiation status of Th helper cells, samples were stained with CD4, CD45RO, CCR7, CD27 and CD28. CD45RO identifies antigen experienced (memory) cells, whereas the expression of CCR7 that is present on central memory cells is absent in effector memory cells. Upon further differentiation expression of CD27 and then CD28 is progressively lost (Fritsch et al., 2005, Sallusto et al., 1999, Okada et al., 2008). On average, 80% of CD45RO⁺ memory CD4⁺ T cells in the RA joint displayed an effector memory phenotype (CCR7⁻) compared with an average of approximately 50% in both HC and RA PBMC; figure 3.2A).

Furthermore, the effector memory CD4⁺ cells in RA SFMC were highly differentiated (shown by a significant loss of CD27 and CD28 co-expression) compared with effector memory cells present in HC blood (figure 3.2B). Representative FACS plots show that all CD4⁺ T cells in the RA joint exhibited a memory phenotype, as expected, whereas CD4⁺ T cells in HC and RA PBMC contain a population of CD45RO⁻ naive cells (figure 3.2C). Moreover, these representative FACS plots illustrate the enrichment of effector memory cells in RA SFMC compared with HC and RA PBMC (figure 3.2C). RA SFMC effector memory CD4⁺ T cells exhibited a reduced frequency of CD27⁺ CD28⁺ double positive cells compared with HC and RA PBMC, indicating that effector memory cells in the RA joint are more highly differentiated compared with peripheral blood (figure 3.2D). Taken together these data indicate that although Th cells in the RA joint are not enriched compared with blood, they are highly proliferative and have been driven to become highly differentiated effector memory cells.

3.2.1b Cytokine production from CD4⁺ Th cells in RA SFMC compared with HC and RA PBMC.

In order to examine cytokine production, RA SFMC and RA or HC PBMC were stimulated *ex vivo* with PMA and ionomycin (which activate protein kinase C to mimic signaling via the TCR) in the presence of brefeldin A (which blocks the golgi apparatus and therefore prevents the exocytosis of produced cytokines). The PMA stimulation negatively affects the cell surface expression of CD4, therefore, when cells were stimulated, the CD4⁺ population was identified indirectly by gating on CD3⁺ CD8⁻ cells (see gating strategy in methods, figure 2.9). Cells were pre-stained with a viability dye before cell surface staining for CD3 and CD8 followed by intracellular staining for cytokines; TNF, GM-CSF, IFN- γ and IL-17.

CD4⁺ effector T cells from the RA SFMC exhibited significantly increased production of pro-inflammatory GM-CSF compared with both HC and RA PBMC (figure 3.3A, left). The frequency of TNF-producing CD4⁺ T cells in RA SFMC was similar to that produced by HC PBMC, however, there appeared to be a trend toward reduced TNF production in RA PBMC compared with RA SFMC (figure 3.3A, right). Interestingly, representative FACS plots demonstrate that GM-CSF was produced exclusively by cells that also make TNF (figure 3.3B; GM-CSF expression is evident only in the upper right-hand quadrant, indicating that it is co-expressed with TNF). Furthermore, the frequency of TNF and GM-CSF co-producing CD4⁺ T cells was markedly increased in RA SFMC compared with HC and RA PBMC. In addition, CD4⁺ effector T cells from RA SFMC exhibited significantly increased production of pro-inflammatory IFN- γ compared with both HC and RA PBMC (figure 3.4A, left). The frequency of CD4⁺ T cells producing IL-17 was also significantly increased compared with RA PBMC, although the percentage was relatively low compared with

other cytokines (figure 3.4A, right). Representative FACS plots show IFN- γ expression versus IL-17 expression (figure 3.4B); the frequency of total IFN- γ or IL-17, shown in the graphs, consists of the combined frequencies of the upper left and right quadrants or the lower and upper right quadrants, respectively. It is evident from these plots that the majority of IL-17 expressed by CD4⁺ T cells in the RA SF was co-expressed with IFN- γ , indicating that Th17-type cells in the RA SF were ex-Th17 type cells.

It is evident from the FACS data that these pro-inflammatory cytokines were not only enhanced in CD4⁺ T cells from the RA joint, but seemingly produced by the same polyfunctional cells, concomitantly. To more extensively evaluate the polyfunctional potential of RA SFMCs, a panel of cytokines was assessed by 10-colour flow cytometry and analysed using SPICE software (section 2.9). In this case, the diagram represents 5 cytokines produced in every possible combination with each other in the CD4⁺ T cells averaged and overlaid from 4 HC PB samples, 4 RA PB samples and 4 RA SF samples (figure 3.5). The colours within the pie chart represent different populations producing different combinations of cytokines and the relative size of each segment correlates to the frequency of the population. The abridged legend shown indicates the percentage of the population producing different combinations of cytokines. A full legend for the colours within the pie chart is available in appendix 2 (figure app. 2.1). The arcs surrounding the pie charts represent the particular cytokines being produced (see arc legend). All the area lying under the circumference of the arc represents the frequency of cells producing that cytokine. Areas of the pie surrounded by multiple arcs are polyfunctional cells, producing all the cytokines denoted by the arc under which they lie. The strikingly increased yellow pie slice in the RA SFMC represents cells producing GM-CSF (red arc), IFN- γ (blue arc) and TNF (purple arc) concomitantly (figure 3.5). The dark green pie slice lying under both blue (IFN- γ) and purple (TNF) arcs is also significantly increased in RA SFMC compared with HC and RA PBMC. In addition, the grey pie slice with no arcs overlying it, indicating the inactive cells not producing any of the cytokines examined is significantly reduced in RA SFMC compared with HC and RA PBMC. IL-10 production (the section of the pie chart over-arc'd in green) is very low in all groups, most likely due to the difficulties associated with identifying IL-10 production by flow cytometry. IL-17 producing cells (lying under the magenta arc) are very highly polyfunctional. This is a significant finding, despite their low frequency, because it suggests that the IL-17 producing Th17-type cells in the RA joint may be in a highly dynamic state.

In summary, the frequencies of Th cells producing a range of pro-inflammatory cytokines were enriched in RA SF and furthermore, the cells producing cytokines were significantly more polyfunctional compared with those from peripheral blood.

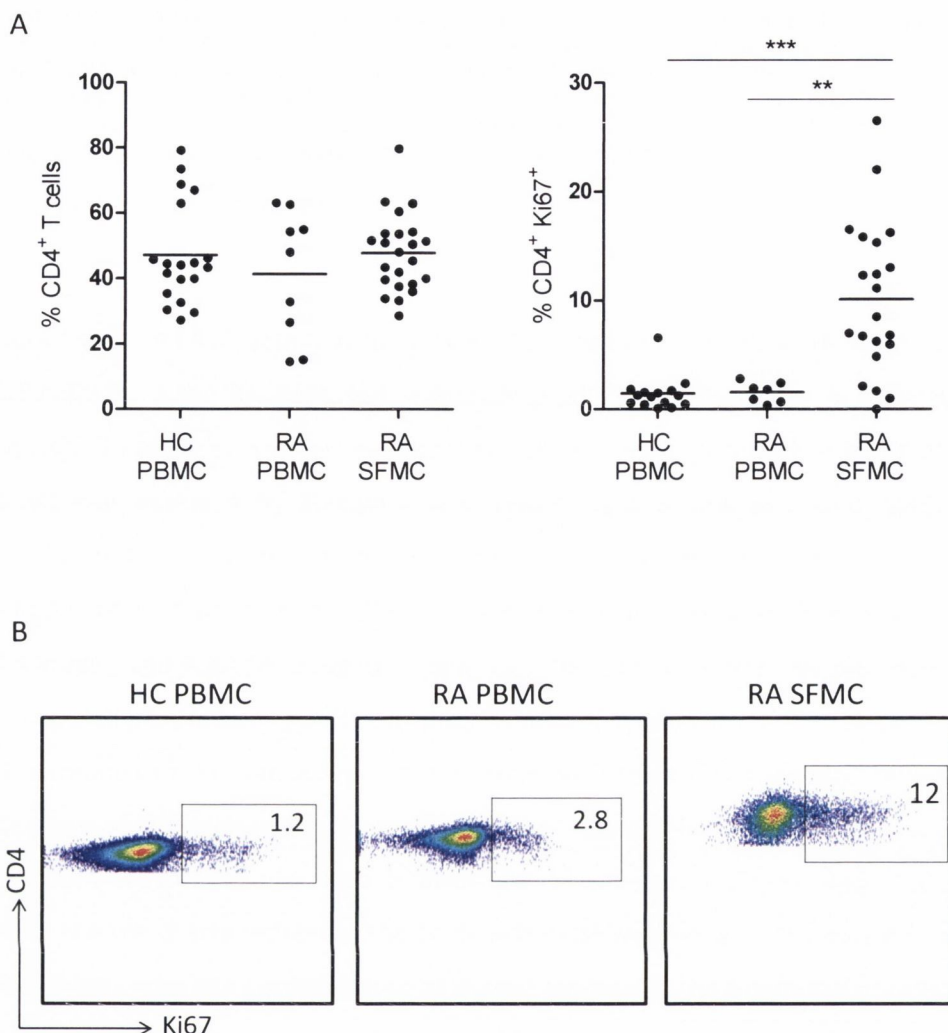


Figure 3.1 The frequency of proliferating CD4⁺ T cells is significantly enhanced in RA SFMC compared with RA and HC PBMC.

PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, FoxP3 and Ki67, which marks active cell cycling, and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD4⁺ cells were gated. The graphs represent the frequency of total CD4⁺ cells (A), and the frequency of FoxP3⁻ effector CD4⁺ T cells expressing Ki67 (B), in each group. Representative FACS plots show the expression of Ki67 in the effector CD4⁺ T cell population (C). Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test; ** $p < 0.01$ *** $p < 0.001$. CD4 stain: HC PBMC $n = 19$, RA PBMC $n = 9$, RA SFMC $n = 23$. Ki67 stain: HC PBMC $n = 15$, RA PBMC $n = 7$, RA SFMC $n = 21$.

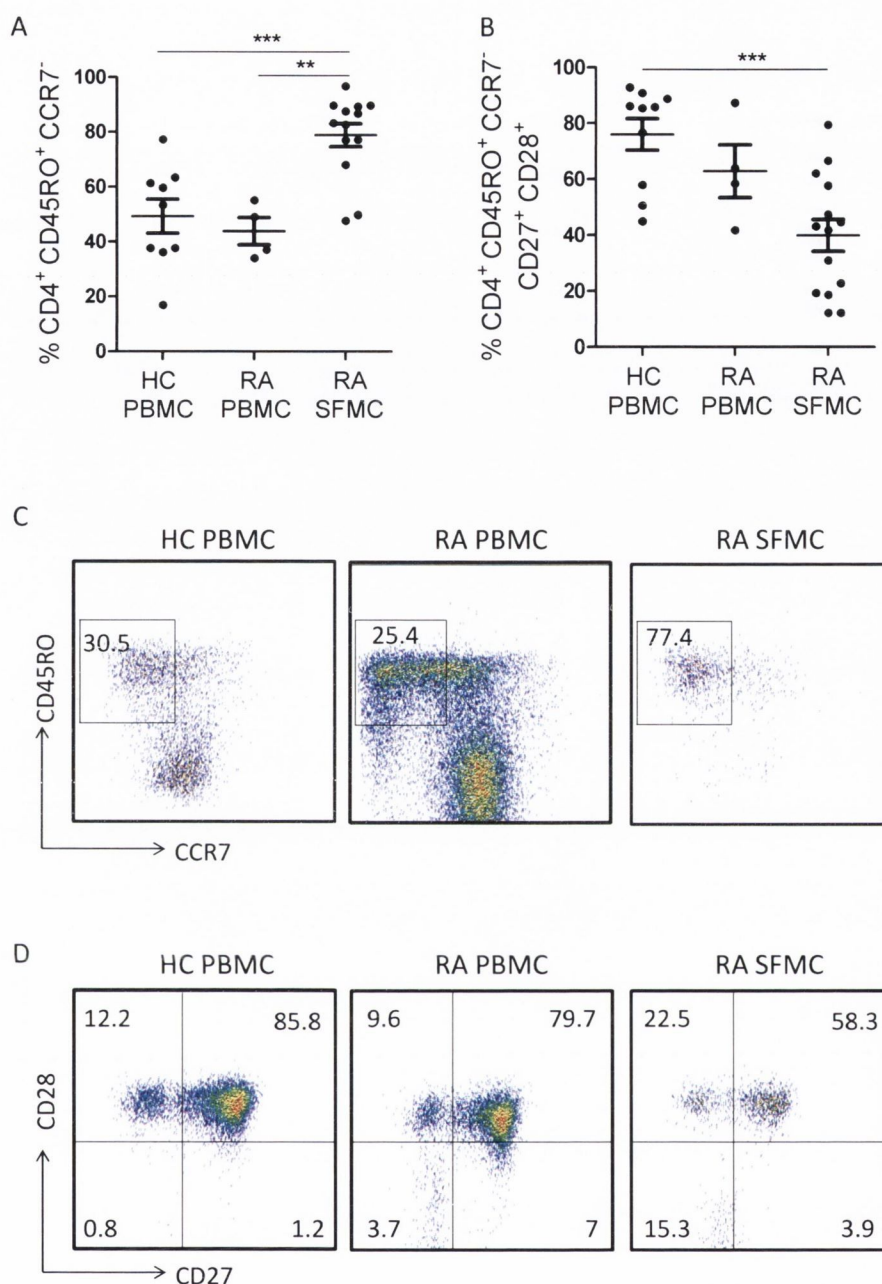


Figure 3.2 The majority of CD4⁺ T cells in RA SFMC are effector memory cells and are significantly more differentiated compared with effector memory cells from HC PBMC.

SFMC and PBMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, CD45RO, CCR7, CD27 and CD28 and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD4⁺ CD45RO⁺ cells were gated. The graphs represent the frequency of effector memory cells within the CD4⁺ memory population (CD4⁺ CD45RO⁺ CCR7⁻) (A) and the frequency of effector memory cells co-expressing CD27 and CD28 (B). Representative FACS plots show the frequency of effector memory cells within the total CD4⁺ population (C). Representative FACS plots show the frequency of CD4⁺ effector memory cells co-expressing CD27 and CD28 (D). Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison post-tests; **p<0.01, *** p<0.001. (A) HC PBMC n=9, RA PBMC n=4, RA SFMC n= 13. (B) HC PBMC n=10, RA PBMC n=4, RA SFMC n=14.

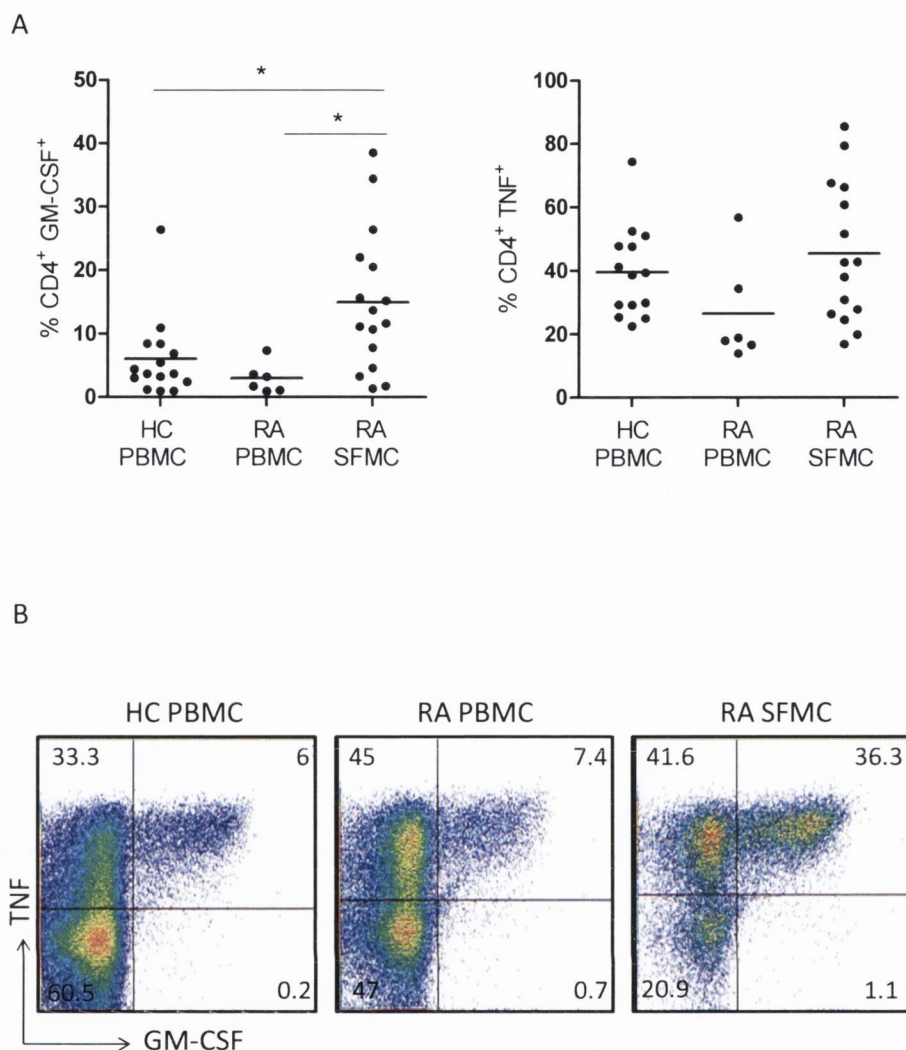


Figure 3.3 The frequency of CD4⁺ T cells producing GM-CSF is significantly increased in RA SFMC compared with HC PBMC and RA PBMC.

PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A, stained with fluorochrome-conjugated antibodies specific for CD3, CD8, TNF and GM-CSF and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD3⁺ CD8⁻ cells were gated to identify CD4⁺ Th cells. The graphs represent the frequency of CD4⁺ T cells expressing cytokine in each group (A). Representative FACS plots show the expression and co-expression of TNF and GM-CSF in the total CD4⁺ T cell population (B). Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test; * $p < 0.05$. GM-CSF stain: HC PBMC $n = 15$, RA PBMC $n = 6$, RA SFMC $n = 16$. TNF stain: HC PBMC $n = 14$, RA PBMC $n = 6$, RA SFMC $n = 15$.

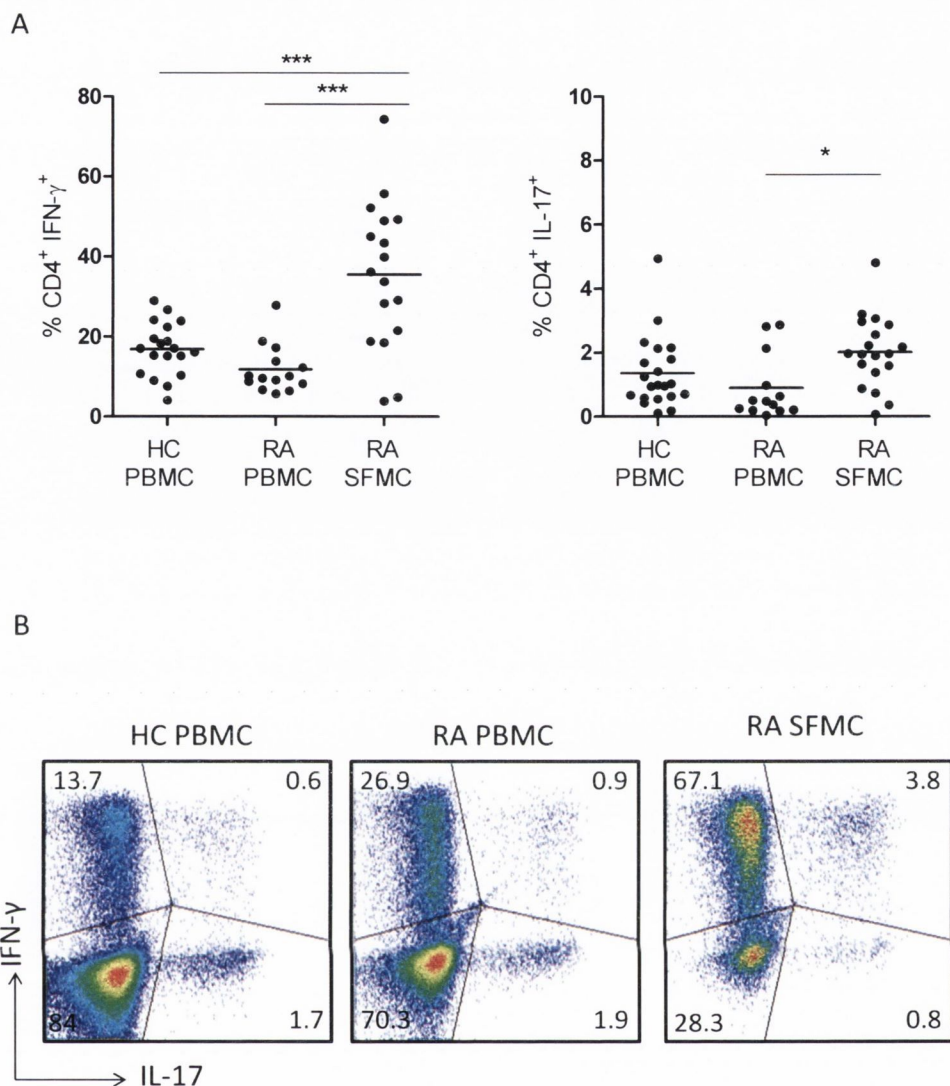


Figure 3.4 The frequency of CD4⁺ T cells producing IFN-γ or IL-17 is significantly increased in RA SFMC compared with HC PBMC and RA PBMC.

PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A, stained with fluorochrome-conjugated antibodies specific for CD3, CD8, IFN-γ and IL-17 and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD3⁺ CD8⁻ cells were gated to identify CD4⁺ Th cells. The graphs represent the frequency of CD4⁺ T cells expressing IFN-γ and IL-17 in each group (A). Representative FACS plots show the expression and co-expression of IFN-γ and IL-17 in the total CD4⁺ T cell population (B). Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test; *P<0.05, *** p<0.001. IFN-γ stain: HC PBMC n=19, RA PBMC n=14, RA SFMC n=17. IL-17 stain: HC PBMC n=21, RA PBMC n=13, RA SFMC n=19.

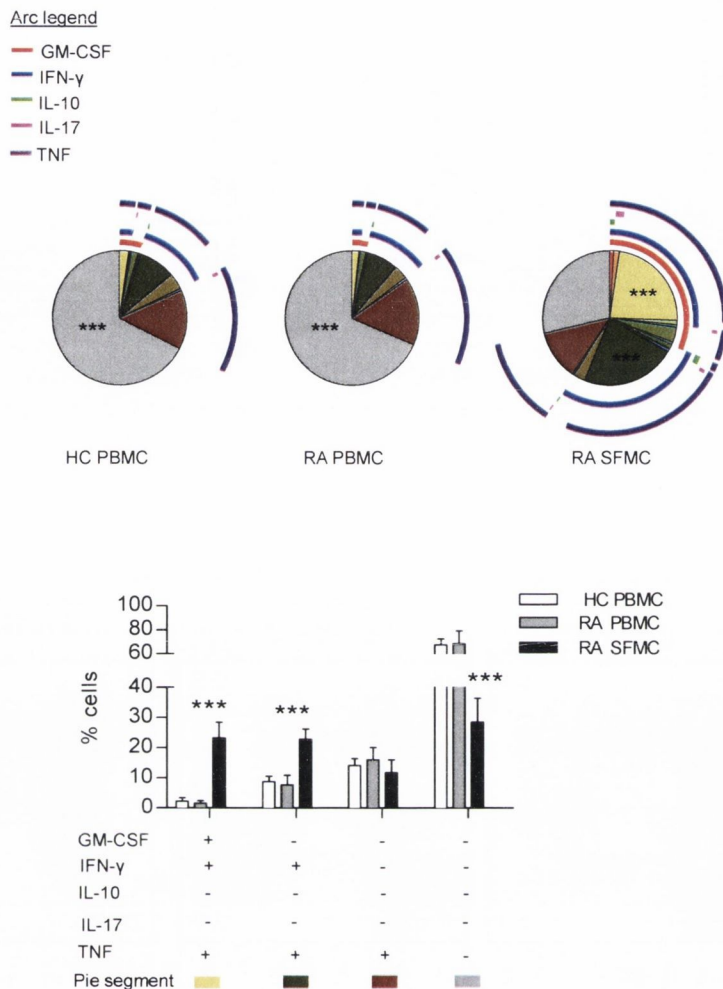


Figure 3.5 The production of cytokines by CD4⁺ T cells in RA SFMC is significantly more polyfunctional compared with HC and RA PBMC.

PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A, stained with fluorochrome-conjugated antibodies specific for CD3, CD8, GM-CSF, IFN- γ , IL-17, IL-10 and TNF and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD3⁺ CD8⁻ cells were gated to identify CD4⁺ Th cells before gating on cytokine⁺ cells. The pie charts represent the frequencies of cells producing every possible combination of the 5 cytokines analysed. The different colours of each segment within the pie chart denote populations producing different combinations of cytokines (see bar graph; abridged legend showing major populations). The relative size of the pie segment correlates to the frequency of the particular population. The arcs around the circumference of the pie chart indicate that a particular cytokine (see pie chart arc legend) is produced by all the cells in the pie chart that lie under the arc. Parts of the pie chart surrounded by multiple arcs are, therefore, polyfunctional cells. Statistical differences between groups were determined by two-way ANOVA with Bonferroni post-tests; ***p<0.001. Asterisks mark segments that are significantly increased compared with both HC and RA PBMC. For full pie chart legend, see appendix 2.

3.2.2 Plasticity and pathogenicity of CD161⁺ Th17-type cells in RA joint compared with peripheral blood.

The increased frequency of CD4⁺ T cells in the inflamed joint that co-produced IL-17 and IFN- γ (figure 3.4B) suggested that these CD4⁺ T cells might be functionally plastic. Evidence in the literature indicates that Th17 cells are capable of switching to “Th1-like” cells, which produce IFN- γ via an intermediate state in which IFN- γ and IL-17 are co-produced (Lee et al., 2009b). CD161 is a cell surface marker present on precursor Th17 cells and bona fide Th17 cells and is retained on Th17 cells that have switched to Th1-like cells (Cosmi et al., 2008). These switched Th17-type cells are referred to as “ex-Th17” or “non-classical Th-1” because they produce IFN- γ , the Th1 signature cytokine. However, it remains unclear as to whether these “non-classical Th1” switched Th17 cells merely behave like bona fide Th1-type cells, or if they exhibit a distinct phenotype. This question will be addressed in more detail in chapter 5. This study sought to examine the differences between CD161⁺ Th cells, which encompass both bona fide Th17 and ex-Th17 type cells, and CD161⁻ Th cells, which broadly include Th1 and Th2 type cells, in the context of the inflamed RA joint.

3.2.2a CD161⁺ Th cells are enriched in the RA joint and express significantly more IFN- γ compared with peripheral blood.

SFMC and PBMC were isolated from RA patients during active disease and were compared with age and sex matched HC PBMC. Firstly, the frequency of Th cells expressing CD161 was analysed then expression of CD161 was examined in conjunction with cytokine production (from PMA/ionomycin stimulated cells) in order to track the fate of Th17 cells in the RA joint. CD4⁺ T cells from the RA SF exhibited significantly enhanced expression of CD161 compared with both HC and RA PBMC (figure 3.6A, left). Furthermore, CD4⁺ CD161⁺ T cells in the RA SF produced significantly more IFN- γ compared with RA PBMC (figure 3.6A, centre) but the frequency of IL-17 production was similar between groups (figure 3.6A, right). Representative FACS plots show that the majority of IL-17 was produced by CD161⁺ Th cells (figure 3.6B, lower panel) although some IL-17 appeared to come from CD161^{lo} cells. On average, approximately 50% of CD161⁺ Th cells in the RA joint expressed IFN- γ (figure 3.6A, middle) and could, therefore, be classified as ex-Th17 type cells.

3.2.2b Significantly higher frequencies of CD161⁺ Th cells produce pro-inflammatory cytokines compared with CD161⁻ Th cells from RA SFMC

Next, the frequencies of CD161⁺ versus CD161⁻ Th cells producing pro-inflammatory cytokines were assessed in order to ascertain if these CD161⁺ Th cells may be more pathogenic, in the

context of autoimmune inflammation, than CD161⁺ Th cells. To this end, SFMC were isolated from RA patients during active disease. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A and stained for CD3, CD8, CD161, IFN- γ , TNF, GM-CSF and IL-17. Comparable frequencies of CD161⁺ and CD161⁺ Th from RA SF produced IFN- γ , however; significantly higher frequencies of CD161⁺ Th cells produced TNF, GM-CSF and IL-17 (figure 3.7A). Representative dot plots gated on CD4⁺ CD161⁺ and CD4⁺ CD161⁺ cells from RA SFMC illustrate that higher frequencies of CD161⁺ Th cells produce pro-inflammatory cytokines than CD161⁺ Th cells (figure 3.7B). This data indicates that CD161⁺ Th cells are a richer source of pro-inflammatory cytokines than CD161⁺ Th cells in the RA joint.

3.2.2c CD161⁺ Th cells exhibit enhanced pro-survival characteristics compared with CD161⁺ Th cells.

Th17 cells have been reported to have stem cell-like characteristics which are likely to enhance their pathogenicity and longevity in the context of autoimmunity (Kryczek et al., 2011), therefore, the longevity and survival characteristics of both HC PBMC and RA SFMC CD161⁺ versus CD161⁺ Th cells were assessed. The expression of Bcl2, an important anti-apoptotic protein, was assessed by intranuclear staining and flow cytometry in CD161⁺ and CD161⁺ Th cells from HC PBMC and RA SFMC (figure 3.8A; collated data top panel, representative histograms below). The data demonstrated that CD161⁺ Th cells expressed significantly more Bcl2 protein than their CD161⁺ Th cell counterparts in both HC PBMC and RA SFMC. It is well established that the RA joint is a hypoxic environment; therefore, cells need to up-regulate HIF-1 α and other shock proteins in order to survive. Evidence from murine research suggests that HIF-1 α is required for Th17 differentiation by promoting ROR- γ t and moreover, can promote Th17 cells in hypoxic environments (Shi et al., 2011). The expression of HIF-1 α in CD161⁺ versus CD161⁺ Th cells was examined in HC PBMC and RA SFMC. Interestingly, under conditions of normoxia as found in HC PBMC, no significant differences were observed between CD161⁺ and CD161⁺ Th cells (figure 3.8B, left). However, when cells extracted from the hypoxic RA joint were examined, CD161⁺ Th cells exhibited significantly higher expression of HIF-1 α compared with CD161⁺ Th cells (figure 3.8B, right). Finally, telomere length was assessed by flow-FISH, to ascertain if there were differences in replicative potential between the CD161⁺ and CD161⁺ Th cell subsets. Both HC PBMC and RA SFMC derived CD161⁺ Th cells displayed significantly longer telomeres compared with their CD161⁺ Th cell counterparts (figure 3.8C).

In summary, RA SF CD161⁺ Th cells exhibited characteristics which suggest that they may have an increased capacity for survival and persistence within the inflamed, hypoxic RA joint (higher

Bcl2 and HIF-1 α expression and longer telomeres) compared with their CD161⁻ Th cell counterparts.

Taken together, these data showed that CD161⁺ Th cells were enriched in the RA joint and produce mostly IFN- γ , indicating that the CD161⁺ Th cell subset consisted mainly of ex-Th17 type cells rather than bona fide IL-17 producing Th17 cells. In addition, significantly higher frequencies of CD161⁺ Th cells produced pro-inflammatory cytokines compared with CD161⁻ Th cells from the RA SF. Cumulatively, this data strongly suggests that CD161⁺ Th cells are pathogenic in the context of the RA joint and may, therefore, be a plausible therapeutic target.

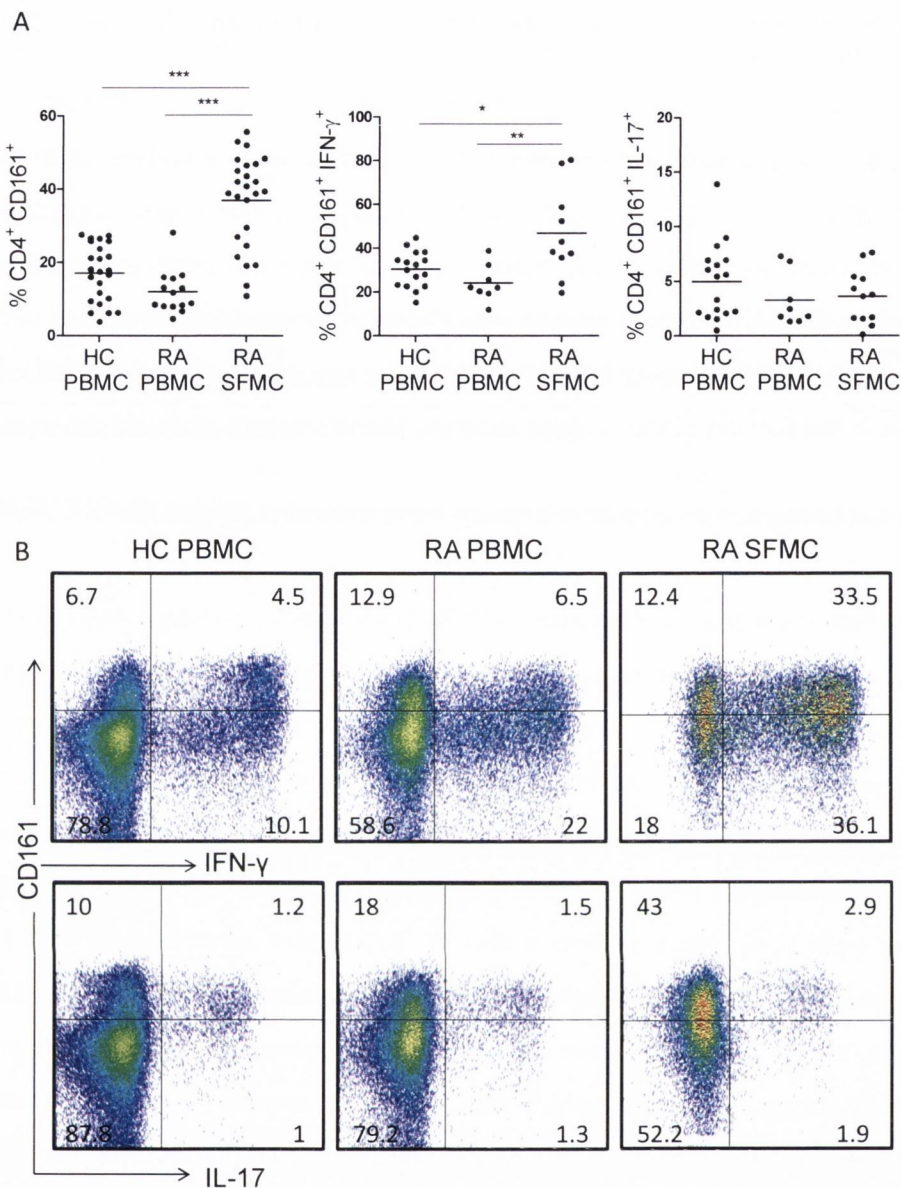


Figure 3.6 Expression of CD161 is significantly enhanced in RA SFMC CD4⁺ T cells and a significantly greater frequency of CD4⁺ CD161⁺ T cells produce IFN-γ in RA SFMC compared with PBMC.

PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stimulated for 5 hours with PMA, ionomycin and brefeldin A, stained for IL-17, IFN-γ, CD3, CD8 and CD161 and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD3⁺ CD8⁻ cells were gated to identify CD4⁺ Th cells before gating on CD161⁺ cells and cytokines. The graphs illustrate the frequency of CD4⁺ T cells expressing CD161 (A, left) the frequency of CD4⁺ CD161⁺ cells producing IFN-γ (A, middle); and CD4⁺ CD161⁺ cells producing IL-17 (A, right)). Representative FACS plots show stimulated CD4⁺ CD161⁺ cells producing cytokines (B). Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test; * $p < 0.05$, *** $p < 0.001$. CD161 stain (A, left) HC PBMC $n = 24$, RA PBMC $n = 14$, RA SFMC $n = 25$. CD161⁺ IFN-γ⁺ stain (A, middle) HC PBMC $n = 16$, RA PBMC $n = 8$, RA SFMC $n = 10$. CD161⁺ IL-17⁺ (A, right) HC PBMC $n = 17$, RA PBMC $n = 8$, RA SFMC $n = 12$.

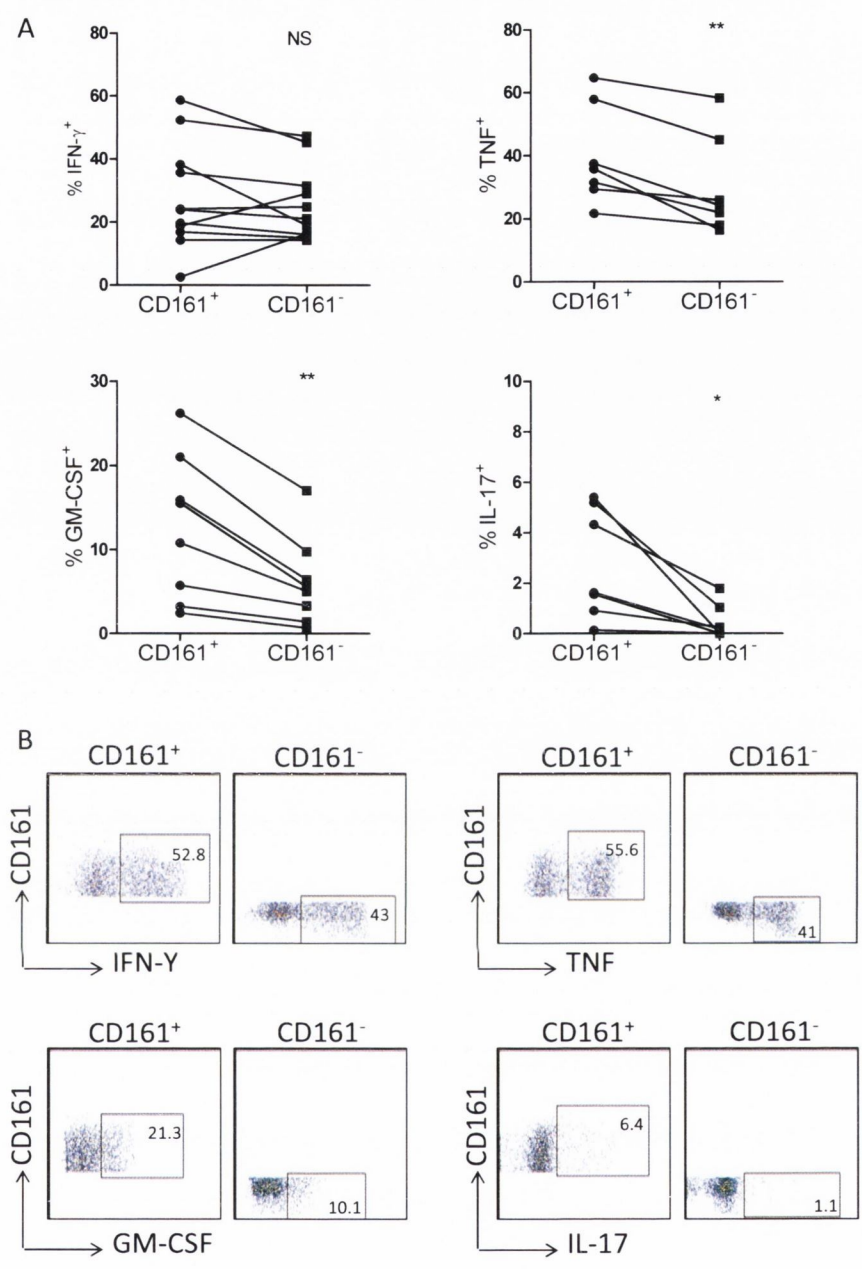


Figure 3.7 Significantly higher frequencies of CD161⁺ Th cells produce pro-inflammatory cytokines compared with CD161⁻ Th cells in the RA joint.

SFMC were isolated from RA patients with active disease. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A, stained with fluorochrome-conjugated antibodies specific for CD3, CD8, CD161, IFN- γ , TNF, GM-CSF and IL-17 and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD3⁺ CD8⁻ cells were gated to identify CD4⁺ Th cells before gating on CD161⁺ and CD161⁻ cells. (A) Each pair of data points represents the cytokine production from CD161⁺ and CD161⁻ Th cells within one patient sample. (B) Dot plots show the frequency of cells producing cytokines in the CD161⁺ and CD161⁻ subpopulations. Statistical differences were determined by paired T-tests; * $p < 0.05$, ** $p < 0.01$. IFN- γ stain $n = 11$, TNF stain $n = 7$, GM-CSF stain $n = 8$ and IL-17 stain $n = 7$.

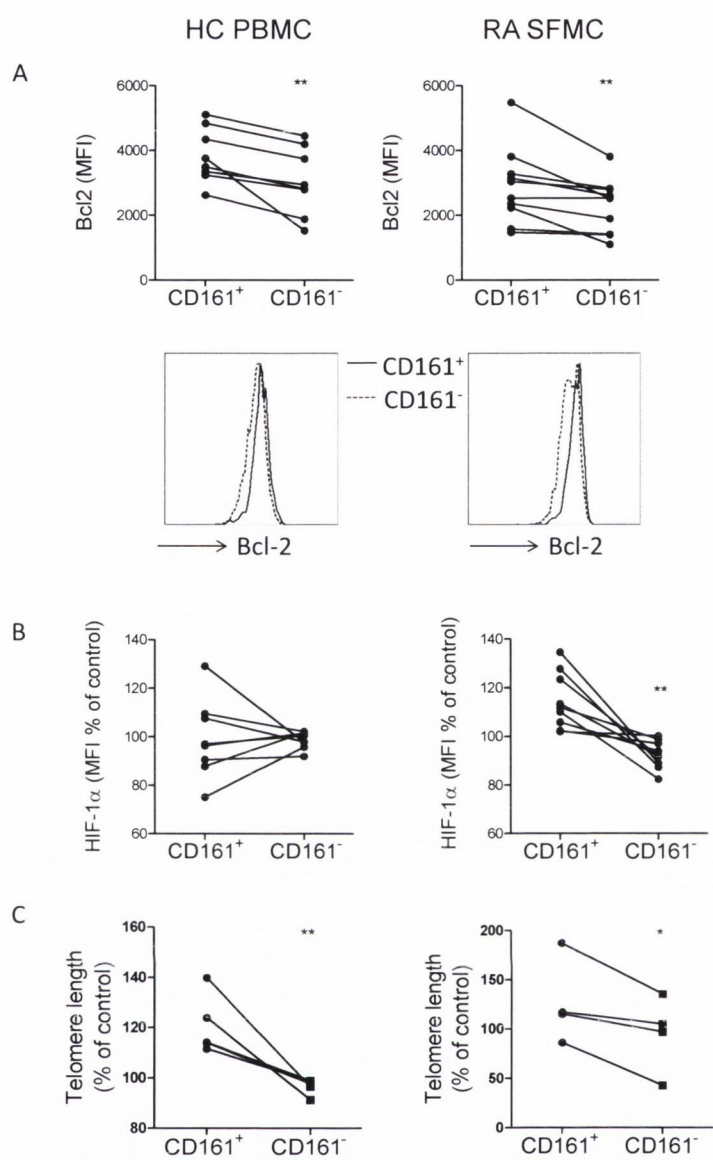


Figure 3.8 CD161⁺ Th cells from the RA joint exhibit enhanced expression of Bcl2 and HIF-1α and longer telomeres compared with CD161⁻ Th cells.

SFMC were isolated from RA patients with active disease. PBMC were isolated from healthy donors. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, CD45RO, CD161, Bcl2 and HIF-1α and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD3⁺ CD8⁻ CD45RO⁺ cells were gated to identify memory CD4⁺ Th cells before gating on CD161⁺ and CD161⁻ cells. (A) The graphs show the expression of Bcl2 (top) with representative histograms (below) and HIF-1α (B; expressed as percentage of total CD4⁺ CD45RO⁺ HIF-1α MFI) in CD161⁺ and CD161⁻ memory Th cells from healthy controls (left) and RA SFMC (right). Each pair of data points represents a single donor (HC PBMC n=8, RA SFMC n=10). Total CD4⁺CD45RO⁺ and CD4⁺CD45RO⁺CD25⁻CD161⁺ and CD4⁺CD45RO⁺CD25⁻CD161⁻ effector T cell subsets were sorted from HC PBMC and RA SFMC by FACS. Telomere length was assessed by flow-FISH and expressed as a percentage of total CD4⁺CD45RO⁺ from the same donor (C). Each pair of data points represents a single donor (HC n=5, RA n=4). Statistical differences were determined by paired T-tests with two-tailed p-values; *p<0.05, **p<0.01.

3.2.3 CD161 marks Th17 cells and plastic, polyfunctional Th17 type cells in HC PBMC.

In the previous section, the data established that CD161⁺ Th cells were enriched in the joint and were a rich source of pro-inflammatory cytokines. Furthermore, CD161⁺ Th cells have the capacity to survive for longer periods and replicate more than CD161⁻ Th cells. In order to further the hypothesis that CD161⁺ Th cells are highly pathogenic in RA and may therefore be a potential therapeutic target, this study sought to ensure the fidelity of the CD161 marker to Th17-type cells and assess the differences in the polyfunctional nature of CD161⁺ and CD161⁻ Th subsets. Moreover, this study aimed to clarify the grey area surrounding IL-17 production from seemingly CD161⁻ Th cells.

3.2.3a ROR- γ t⁺ cells express CD161 and CD161⁺ Th cells produce significantly more IL-17 but similar concentrations of IFN- γ and TNF compared to CD161⁻ Th cells.

Firstly, the co-expression of CD161 and ROR- γ t (the transcription factor that directs Th17 cell function) in CD4⁺ T cells was assessed in HC PBMC *ex vivo* by flow cytometry (figure 3.9A). Cells were stained for the cell surface expression of CD4 and CD161, followed by intranuclear staining for ROR- γ t. The histogram shows CD161 expression on total CD4⁺ T cells (red) and on CD4⁺ ROR- γ t⁺ cells (blue), clearly demonstrating that CD161 marks ROR- γ t⁺ cells. FACS sorted Treg-depleted memory CD161⁺ and CD161⁻ Th cells were stimulated with PMA and ionomycin, in the presence of brefeldin A, to assess cytokine production by flow cytometry (figure 3.9B). The representative FACS plots illustrate that similar frequencies of CD161⁺ and CD161⁻ Th cells expressed IFN- γ whereas IL-17 was predominantly expressed by CD161⁺ Th cells. Secondly, the concentrations of IL-17, IFN- γ and TNF in the supernatants of sorted Treg-depleted memory CD161⁺ and CD161⁻ Th cells, stimulated for 5 days with anti-CD3 and irradiated APC, were assessed by ELISA. CD161⁺ Th cells produced significantly more IL-17 than CD161⁻ Th cells but notably, CD161⁻ Th cells produced quantifiable, albeit very low, concentrations of IL-17 (figure 3.10, top). Interestingly, both CD161⁺ and CD161⁻ Th cells produced comparable concentrations of IFN- γ and TNF (figure 3.10, middle and bottom). In summary, ROR- γ t⁺ Th17 type cells are marked by the expression of CD161 and CD161⁺ CD4⁺ T cells produced IL-17, IFN- γ and TNF.

3.2.3b CD161⁺ Th cells have a polyfunctional cytokine profile whereas CD161⁻ Th cells have a mono- or bi-functional cytokine profile.

Since IL-17 producing cells in RA SFMC were so highly polyfunctional (figure 3.5), this raised the question of whether polyfunctionality might be a feature of CD161⁺ CD4⁺ T cells. Using the SPICE platform, differential polyfunctionality was assessed in the CD161⁺ and CD161⁻ memory

Th cells from 20 healthy donors on day 3 post anti-CD3 stimulation. The pie charts represent the 6 cytokines produced in every possible combination with each other in CD161⁺ CD4⁺ CD45RO⁺ T cells (left) compared with CD161⁻ CD4⁺ CD45RO⁺ T cells (right), averaged and overlaid from 20 HC PBMC samples (figure 3.11). For parity, all inactive cells (cells not producing any of the cytokines examined) have been excluded. The colours within the pie chart are heat map styled to represent increasing polyfunctionality (from grey to dark red) and the relative size of each segment correlates to the frequency of the population (figure 3.11A). The arcs surrounding the pie charts represent the particular cytokines being produced (see arc legend). All the area lying under the circumference of the arc represents the frequency of cells producing that cytokine. Areas of the pie surrounded by multiple arcs are polyfunctional cells, producing all the cytokines denoted by the arc under which they lie. The orange and red inner pie segments, which indicate cells producing 3 or more cytokines, are noticeably larger in the CD161⁺ Th helper cells compared with the CD161⁻ Th cells, whereas the grey (1 cytokine) and yellow segments (2 cytokines) were relatively increased in the CD161⁻ Th cells. In particular, the proportion of cells producing GM-CSF, IFN- γ , IL-2 and TNF (dominant red pie segment over arced by pink, green, blue and purple) was relatively increased in the CD161⁺ Th cells. Figure 3.11B shows the percentage of cells that produced 1, 2, 3, 4, 5 or 6 cytokines. There were significantly more CD161⁻ Th cells that produced only one or 2 cytokines, whereas the proportion of polyfunctional cells that produced 3, 4, 5 or 6 cytokines was significantly increased in the CD161⁺ Th cell population. The abridged legend showing the frequencies of subpopulations over 1% producing different combinations of cytokines, indicates that significantly more CD161⁺ cells are producing multiple cytokines compared with CD161⁻ Th cells which are more mono- and bi-functional and specifies the cytokine combinations being produced (figure 3.11C).

These data demonstrate that in healthy donors, CD161⁺ memory Th cell subset are significantly more polyfunctional than their CD161⁻ memory Th cell counterparts. Observation of such a phenomenon in a healthy population suggests that this feature is intrinsic to the nature of Th cells marked by CD161, and not an aberration induced by the autoimmune environment.

3.2.3c CD161 expression is not induced in CD161⁻ Th cells upon stimulation.

In order to ensure that the highly active cytokine polyfunctionality observed in stimulated PBMC was not an artefact of stimulation falsely inducing CD161 expression, a simple experiment was designed to trace the expression of CD161 on FACS sorted Treg-depleted memory CD161⁺ and CD161⁻ Th cells. Sorted cells were labelled with CTV and grown for 5 days in the presence of anti-CD3 and irradiated APC. Expression of CD161 was examined on both CD161⁺ and CD161⁻ sorted

subsets (figure 3.12). Expression of CD161 was not induced on CD161⁻ sorted cells (figure 3.12, right). Interestingly, previously CD161⁺ sorted cells appeared to lose CD161 expression in later generations of cell division (i.e. as CTV labelling becomes more diluted; figure 3.12, left). This may have been due to asymmetric division of the cells as evidence in the literature suggests that activated T cells can divide asymmetrically (Chang et al., 2007) i.e. disproportionate amounts of the contents of the cell as well as the proteins embedded in the cell membrane are inherited by daughter cells. In this way, parent CD161⁺ cells may give rise to one CD161⁺ and one CD161^{lo/-} daughter cell as a result of an asymmetric mitotic cell division. This may, at least in part, explain the production of IL-17 observed in the CD161^{lo} population (figure 3.7B, lower panel). It is also plausible that CD161 was not induced on activated CD161⁻ Th cells because the assay lacked a 3rd signal, such as addition of instructive cytokines. Thus, *in vivo*, CD161 expression may be induced but, importantly, it is not induced by activation in the experimental system used here.

In summary, the expression of CD161 marks a population of CD4⁺ T cells in healthy controls which incorporates the vast majority of IL-17 producing cells but also contains Th17 cells that have switched from producing IL-17 to producing IFN- γ . CD161⁺ Th cells were significantly more polyfunctional, in terms of their cytokine production, compared with CD161⁻ Th cells. CD161 was not induced on CD161⁻ cells upon stimulation; however, expression of CD161, in some individuals, may be reduced on cells that have undergone several rounds of division.

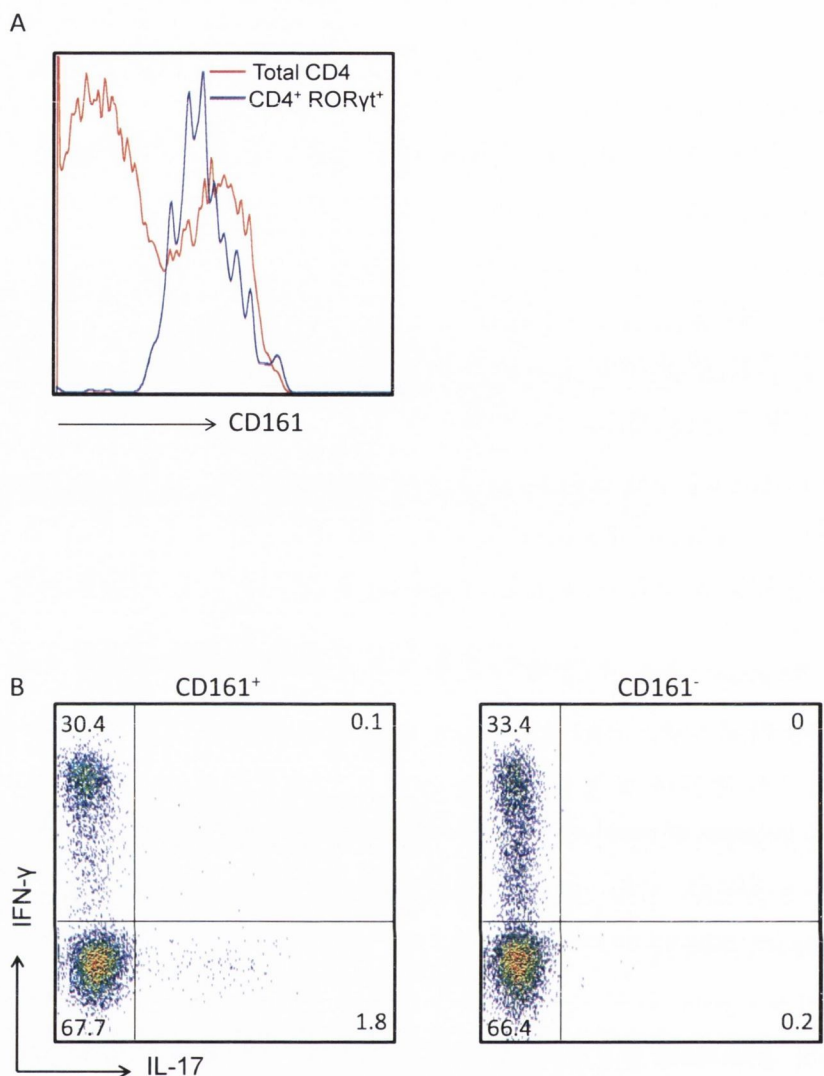


Figure 3.9 In healthy control blood, CD161 is expressed on ROR- γ ⁺ Th17-type cells and CD161⁺ Th cells produce IL-17 and IFN- γ .

PBMC were isolated from healthy donors, stained with fluorochrome-conjugated antibodies specific for CD4, CD161 and ROR- γ t and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD4⁺ cells were gated. The expression of ROR- γ t in total CD4⁺ cells (red) and CD161⁺ CD4⁺ cells (blue) is shown in the histogram (A). CD4⁺ memory cells were purified from HC PBMC by MACS. CD4⁺CD45RO⁺CD25⁻CD161⁺ and CD4⁺CD45RO⁺CD25⁻CD161⁻ effector T cells were sorted by FACS. Representative FACS plots show cells stimulated, directly post sorting, with PMA and ionomycin in the presence of brefeldin A, stained for IL-17 and IFN- γ and analysed by flow cytometry (B).

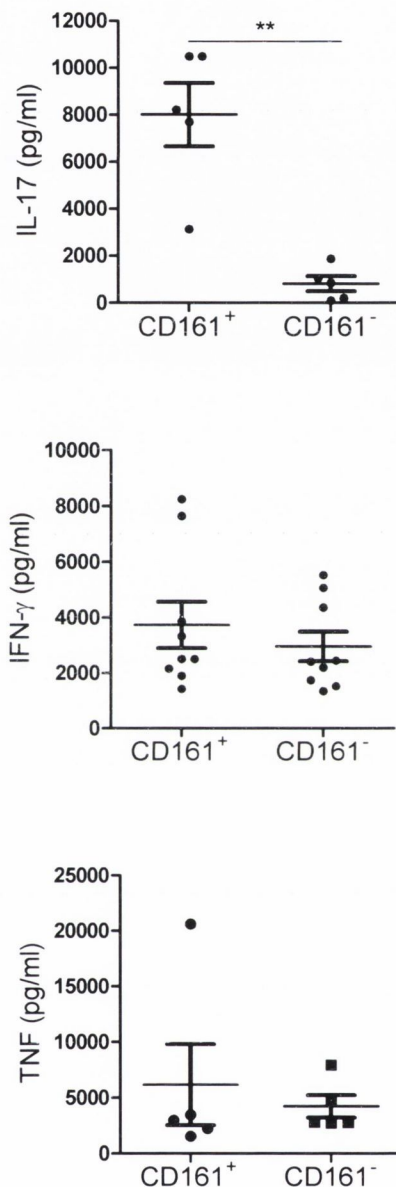


Figure 3.10 The concentrations of IL-17, IFN-γ and TNF produced by CD161⁺ and CD161⁻ Th cells.

PBMC were isolated from healthy donors. CD4⁺ memory T cells were purified from PBMC by MACS. CD4⁺CD45RO⁺CD25⁻CD161⁺ and CD4⁺CD45RO⁺CD25⁻CD161⁻ effector T cells were sorted by FACS. Effector cells were and cultured for 5 days in the presence of anti-CD3 and allogeneic irradiated APC. The concentrations of IL-17, IFN-γ and TNF in the supernatants were assessed by ELISA. Each data point is representative of an averaged triplicate from an independent experiment; IL-17 and TNF n=5, IFN-γ n=9. Statistical differences in concentrations were determined by paired T-tests; **p<0.01.

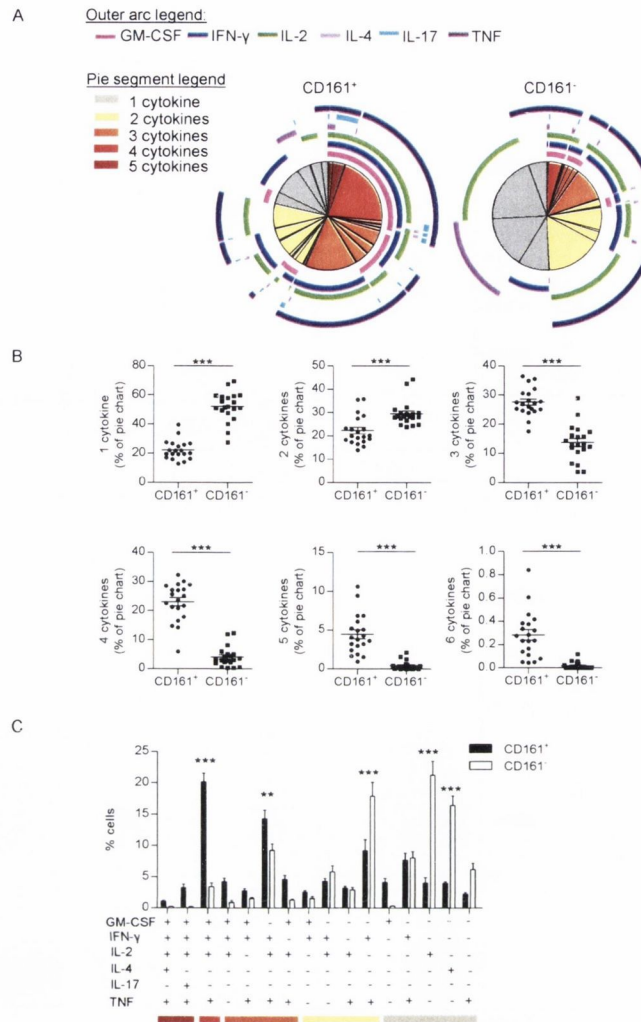


Figure 3.11 CD161⁺ Th cells are more polyfunctional, in terms of cytokine production, than CD161⁻ Th cells.

PBMC were isolated from healthy donors and cultured for 3 days with anti-CD3 before re-stimulation for cytokine production. Cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD8, CD45RO, CD161, GM-CSF, IFN- γ , IL-2, IL-4, IL-17 and TNF, analysed by flow cytometry and results were analysed using SPICE software. (A) The pie charts represent the average frequencies of active cytokine-producing cells producing every possible combination of the 6 cytokines analysed across 20 donors. The segments within the pie chart denote populations producing different combinations of cytokines and are heat-map coded (pie legend: grey-red) to indicate increasing polyfunctional cytokine production. The size of the pie segment correlates to the frequency of the particular population. The arcs around the circumference indicate the particular cytokine (see arc legend) produced by the proportion of cells that lie under the arc. Parts of the pie surrounded by multiple arcs represent polyfunctional cells. The frequency of cells within the CD161⁺ and CD161⁻ Th cell populations producing between 1-6 cytokines is represented graphically in (B; n=20). (C) The abridged bar graph shows frequencies (over 1%) of combinations of cytokines produced by CD161⁺ (black bars) versus CD161⁻ Th cells (white bars). Statistical differences were determined by paired T-Tests (B) or by two-way ANOVA (C); ***p<0.001.

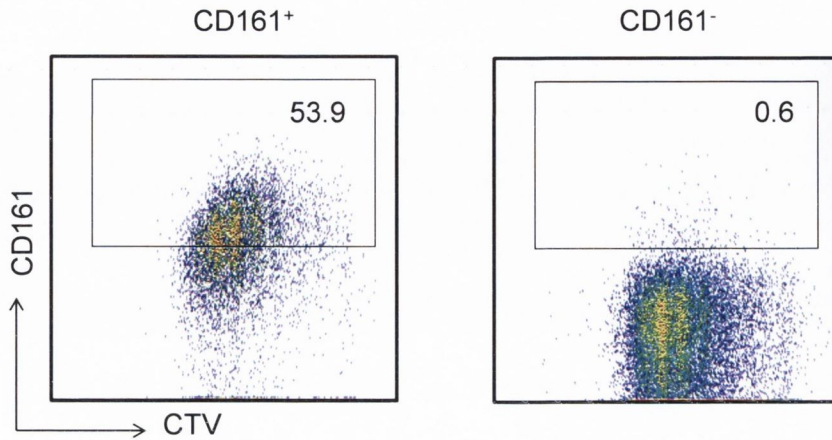


Figure 3.12 CD161 expression is not induced upon stimulation of CD161⁻ Th cells but may be reduced in CD161⁺ Th cells after several rounds of cell division.

PBMC were isolated from healthy donors. CD4⁺ memory cells were purified from PBMC by MACS. CD4⁺CD45RO⁺CD25⁻CD161⁺ and CD4⁺CD45RO⁺CD25⁻CD161⁻ effector T cells were sorted by FACS. Effector cells were labelled with CTV and cultured for 5 days in the presence of anti-CD3 and CFSE labelled allogeneic irradiated APC. Cells were stained with a fluorochrome-conjugated antibody specific for CD161 and analysed by flow cytometry. Dead cells and doublets were excluded, CTV⁺ CFSE⁻ cells were gated. Proliferation was quantified by CTV dilution and CD161 expression was analysed. This data is representative of 3 independent experiments.

3.2.4 CD161⁺ Th cells mediate inflammation via effects on synovial fibroblast cells.

Fibroblast cells line the entire synovial membrane of synovial joints and, although they are not classically considered to be immune cells, they play an active role in immune-mediated damage by expressing cytokines, chemokines, cathepsins, MMPs, adhesion molecules and osteoclastic factors. On the basis of the working hypothesis which suggests that CD161⁺ Th cells may be more pathogenic than CD161⁻ Th cells, the differential effects of these Th subpopulations on fibroblast cells were examined to decipher if HC CD161⁺ Th cells were capable of inducing a more pro-inflammatory phenotype in fibroblast cells compared with HC CD161⁻ Th cells. The effects of recombinant cytokines on fibroblast cells were also assessed in an attempt to pinpoint the factors responsible for inducing the pro-inflammatory phenotype in fibroblast cells.

Initially, experiments were carried out on a K4-immortalised synoviocyte fibroblast cell line and then repeated in primary fibroblast cells derived from 3 RA patients.

3.2.4a CD161⁺ Th cell supernatants induce a significantly more pro-inflammatory phenotype in K4 fibroblast cells compared with CD161⁻ Th cell supernatants.

Supernatants were harvested from FACS sorted Treg-depleted memory CD161⁺ and CD161⁻ Th cells grown in culture for 5 days in the presence of anti-CD3 and irradiated APC. K4 fibroblast cells were grown to 90% confluence, serum starved for 8 hours and treated with T cell supernatants (diluted 1 in 4) for 24 hours. The concentrations of IL-8, a chemokine which recruits neutrophils to the site of inflammation, and IL-6, a key pyrogenic cytokine which promotes antibody production and Th17-type responses, were assessed by ELISA. Cells were trypsinised, stained for expression of ICAM-1 (an adhesion molecule responsible for the trafficking of lymphocytes into the joint) and membrane-bound RANKL (which activates osteoclasts to promote bone resorption) and analysed by flow cytometry. The data illustrates that CD161⁺ Th cell supernatants (from 4 independent experiments; each paired data points represents the average of triplicates) induced significantly more IL-8 and IL-6 from K4 fibroblasts compared with CD161⁻ Th cell supernatants (figure 3.13A). Moreover, representative histograms show that CD161⁺ Th cells (blue line) induced higher expression of ICAM-1 and RANKL compared with CD161⁻ Th cells (red line; figure 3.13B). This data indicated that CD161⁺ Th cells evoke a more highly pro-inflammatory phenotype in K4 fibroblast cells.

3.2.4b Induction of IL-8 and IL-6 in K4 fibroblast cells by recombinant cytokines and combinations of recombinant cytokines.

K4 fibroblast cells were grown to 90% confluence, serum starved for 8 hours and treated with recombinant cytokines; IFN- γ , IL-17, GM-CSF and TNF, serially diluted 1 in 10 from 100 ng/ml to

0.1 ng/ml. Induction of IL-8 and IL-6 in K4 fibroblasts by serially diluted pro-inflammatory cytokines was assessed by ELISA (figure 3.14). Notably, only TNF was able to dose-dependently induce the production of IL-8 (figure 3.14A). Low concentrations of IL-6 were induced by IFN- γ (at all concentrations) and IL-17 (at 100 ng/ml), however, these concentrations were minute (approximately 0.5 ng/ml) compared with the dose-dependent production of IL-6 elicited by TNF (starting at approximately 1.5 ng/ml induced by 1 ng/ml of TNF up to approximately 20 ng/ml, induced by a concentration of 100 ng/ml of TNF; figure 3.14B). This study has previously shown that HC CD161⁺ and CD161⁻ Th cells contained comparable frequencies of cells producing TNF when stimulated *ex vivo* (figure 3.11, upper graphs) and furthermore, exhibited comparable concentrations of TNF (figure 3.10, bottom graph). Therefore, the effect of treating K4 fibroblasts with multiple combinations of pro-inflammatory cytokines was examined next (figure 3.15). As established in the literature, TNF and IL-17 synergise to induce pro-inflammatory cytokines from primary fibroblast cells (Katz et al., 2001). This phenomenon was also observed here in the K4 fibroblast cell line (figure 3.15). The addition of IFN- γ or GM-CSF, or both, to fibroblast cells treated with both IL-17 and TNF did not significantly alter the production of IL-8 (figure 3.15, left). Conversely, no synergistic induction of IL-6 was observed between IL-17 and TNF in K4 fibroblast cells (figure 3.15, right). However, cells treated with TNF, IL-17 and IFN- γ produced significantly more IL-6 compared with cells treated with TNF alone. Moreover, the concentration of IL-6 was further increased by the addition of GM-CSF to cells treated with TNF, IL-17 and IFN- γ . These data suggest that combinations of particular cytokines have synergistic pro-inflammatory effects on K4 fibroblast cells.

3.2.4c CD161⁺ Th cell supernatants induce a significantly more pro-inflammatory phenotype in primary fibroblast cells from RA patients compared with CD161⁻ Th cell supernatants.

In order to assess if CD161⁺ Th cells might induce a pro-inflammatory phenotype in surrounding synovial fibroblast cells during autoimmune inflammation in RA, primary fibroblast cells derived from RA patients during active disease were used. Primary fibroblast cells were used between passages 3-5, grown to 90% confluence, serum starved for 8 hours and treated with supernatants from FACS sorted Treg-depleted memory CD161⁺ and CD161⁻ Th cells which were grown in culture for 5 days in the presence of anti-CD3 and irradiated APC. The addition of CD161⁺ and CD161⁻ Th cells supernatants to primary RA fibroblast cells elicited a similar trend to that observed with the K4 fibroblast cell line (figure 3.16). Each data point was obtained by averaging triplicate wells from each independent experiment. The addition of CD161⁺ Th cell supernatant from 1 HC donor to RA fibroblasts from 3 donors induced more IL-8 compared with CD161⁻ Th cell supernatants, from the same donor, in all 3 RA fibroblast cell lines (figure 3.16A,

left). This trend did not reach statistical significance. CD161⁺ Th cell supernatants induced more IL-6 in 2 out of 3 RA fibroblast donors compared with CD161⁻ Th cell supernatants from the HC same donor and no difference was observed in the concentration of IL-6 in the remaining RA fibroblast donor (figure 3.16A, right). The addition of CD161⁺ Th cell supernatant from 3 HC donors to RA fibroblast cells from 1 donor induced significantly more IL-8 compared with CD161⁻ Th cell supernatants and the same trend was observed for the production of IL-6 (figure 3.16B). Primary fibroblast cells from 3 RA patients, treated with CD161⁺ and CD161⁻ Th cell supernatants from 1 HC donor, were trypsinised, stained with fluorochrome conjugated antibodies specific for ICAM-1 and RANKL and analysed by flow cytometry (figure 3.17). The graphs illustrate that the expression of ICAM-1 and membrane bound RANKL were significantly increased in primary fibroblasts from all 3 RA donors upon addition of CD161⁺ Th supernatants compared with supernatants from CD161⁻ Th cells from the same donor (figure 3.17A). Representative histograms qualitatively demonstrate this increase with CD161⁺ Th cell supernatant (blue line) compared with CD161⁻ Th cell supernatant (red line; figure 3.17B). In summary, these data indicated that soluble factors produced by CD161⁺ Th cells induced a more highly pro-inflammatory phenotype in primary RA fibroblast cells compared with CD161⁻ Th cells.

3.2.4d Induction of IL-8 and IL-6 in primary fibroblast cells from RA patients by recombinant cytokines and combinations of recombinant cytokines.

Primary RA fibroblast cells, derived from RA patients during active disease, were used between passages 3-5, grown to 90% confluence, serum starved for 8 hours and treated with recombinant cytokines; IFN- γ , IL-17, GM-CSF and TNF, serially diluted 1 in 10 from 100 ng/ml to 0.1 ng/ml. The induction of IL-8 and IL-6 in primary RA fibroblast cells, with serially diluted pro-inflammatory cytokines was assessed by ELISA (figure 3.18). Interestingly, IL-17 (at 100 ng/ml) significantly induced IL-8 production (approximately 1 ng/ml) from RA patient fibroblast cells, however, TNF (at 10 and 100 ng/ml) induced significantly higher concentrations of IL-8 (approximately 6 and 10 ng/ml, respectively) than that elicited by IL-17. IFN- γ and GM-CSF, at any concentration, did not induce IL-8 from primary RA fibroblast cells (figure 3.18A). IL-17 (at 10 and 100 ng/ml) and TNF (at 1, 10 and 100 ng/ml) induced IL-6 production in primary RA fibroblast cells (figure 3.18B). However, IFN- γ and GM-CSF did not induce the production of IL-6 from primary RA fibroblast cells at any concentration.

The effects of treating primary RA fibroblast cells with multiple combinations of pro-inflammatory cytokines were examined (figure 3. 19). Treating primary RA fibroblast cells with TNF and IL-17 synergistically increased the concentration of IL-8 and IL-6 produced by the RA

fibroblast cells. Intriguingly, addition of IFN- γ or GM-CSF, or both, to cells treated with IL-17 and TNF significantly reduced the concentration of IL-8 (figure 3.19, left). IFN- γ elicited a significantly greater reduction in IL-8 concentration compared with GM-CSF. The addition of IFN- γ and GM-CSF to cells treated with TNF and IL-17 bore no effect on the production of IL-6 (figure 3.19, right).

These results demonstrated that IL-17 and TNF induced the production of IL-8 and IL-6 in primary RA fibroblast cells. Moreover, IL-17 synergistically enhanced the ability of TNF to induce IL-8 production from RA fibroblast cells whilst concomitant treatment with IFN- γ or GM-CSF, or both, significantly reduced the additive effects of IL-17 and TNF. This effect was specific to IL-8 production as IL-6 was not similarly affected. These data suggested that combinations of cytokines may differentially modulate the immune response during RA.

3.2.4e The expression of ICAM-1 induced in K4 and primary fibroblast cells from RA patients by combinations of recombinant cytokines.

In a similar manner, K4 and primary RA fibroblast cells were treated with combinations of recombinant cytokines and the induction of ICAM-1 was assessed by flow cytometry (figure 3.20). Fibroblast cells were grown to 90% confluence, serum starved for 8 hours and treated recombinant cytokines; IFN- γ , IL-17 and TNF, alone and in combinations. Representative histograms show that TNF in combination with IFN- γ induced higher expression of ICAM-1 in both K4 and primary RA fibroblasts cells compared with TNF alone or in combination with IL-17 (figure 3.20A). Data collated from primary RA fibroblast cells (n=3) illustrates the MFI of ICAM-1 expression on primary RA fibroblast cells treated with TNF, IL-17 and IFN- γ , alone and in combination (figure 3.20B). TNF in combination with IFN- γ induced significantly higher staining intensity for ICAM-1 compared with untreated, TNF alone, IFN- γ alone, IL-17 alone or TNF in combination with IL-17. These data indicate that TNF synergised with IFN- γ , but not IL-17, to significantly enhance the expression of ICAM-1 on K4 fibroblast cells and on primary RA fibroblast cells (n=3).

In summary, soluble factors produced from CD161⁺ Th cells induced a significantly enhanced pro-inflammatory phenotype in K4 fibroblast cells and in primary RA fibroblast cells compared with soluble factors produced by CD161⁻ Th cells. Moreover, recombinant IL-17 synergised with TNF to induce enhanced concentrations of IL-8 but in primary RA fibroblast cells, the presence of IFN- γ reduced this effect. IFN- γ synergised with TNF to induce ICAM-1 expression on fibroblast cells. These data suggest that Th17 cell plasticity from IL-17 producing cells into IFN- γ producing cells may modulate the immunogenic phenotype of fibroblast cells in the RA joint; initially IL-17

production can induce IL-8, which recruits neutrophils. As plasticity towards an ex-Th17 phenotype occurs in Th17 cells, the IFN- γ signalling may disrupt the synergy between IL-17 and TNF, reducing the concentration of IL-8 produced by fibroblast cells and potentially down-modulating the recruitment of neutrophils. IFN- γ synergises with TNF to up-regulate ICAM-1 expression on fibroblast cells, which aids the recruitment of lymphocytes into the joint.

Collectively, these data indicate that plastic CD161⁺ Th cells are likely to be highly pathogenic in the context of autoimmune RA.

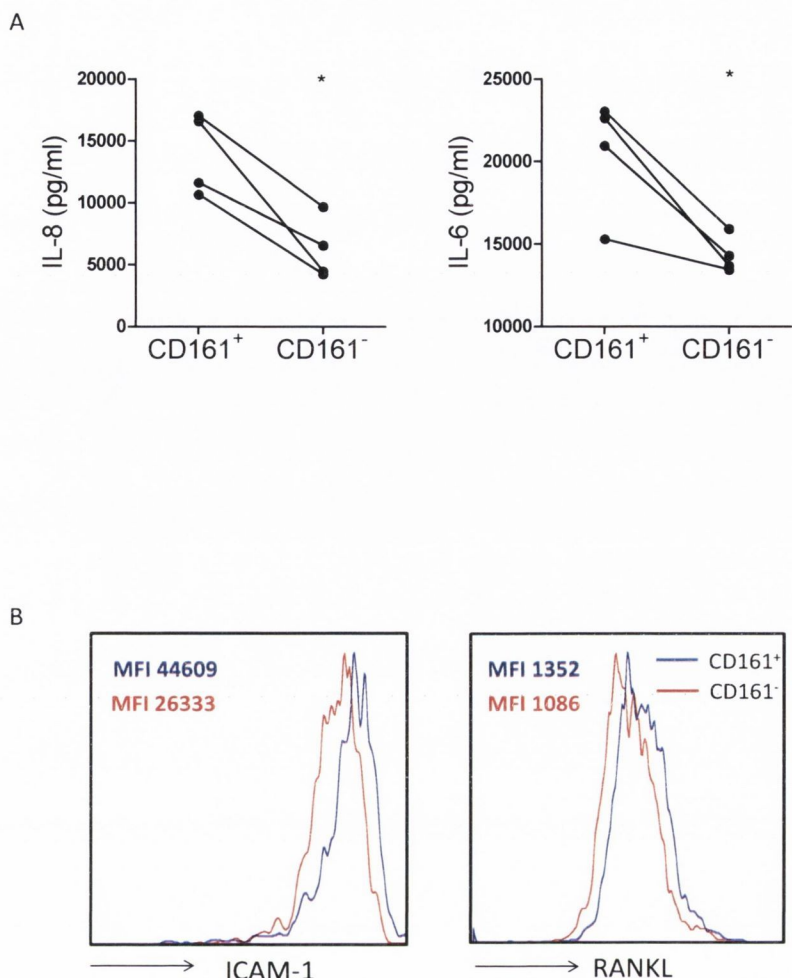


Figure 3.13 CD161⁺ Th cells induce an enhanced pro-inflammatory phenotype in K4 fibroblast cells compared with CD161⁻ Th cells.

PBMC were isolated from healthy donors. CD4⁺ memory T cells were purified from PBMC by MACS. CD4⁺CD45RO⁺CD25⁻CD161⁺ and CD4⁺CD45RO⁺CD25⁻CD161⁻ effector T cells were further sorted by FACS. Effector T cells were cultured 5 days in the presence of anti-CD3 and irradiated, allogeneic APC and supernatants were harvested. K4 fibroblast cells were seeded in 48-well plates, grown to 90% confluence and serum starved for 8 hours. CD161⁺ and CD161⁻ Th cell supernatants were applied to fibroblast cells for 24 hours and the concentrations of IL-8 and IL-6 were assessed by ELISA (A). Each pair of data points represents the average of triplicate wells from a single, independent experiment (n=4). Fibroblast cells were stained with fluorochrome-conjugated antibodies specific for ICAM-1 and RANKL and analysed by flow cytometry. (B) Representative histograms (n=3) show the differential induction of ICAM-1 and RANKL and their median fluorescence intensity (MFI) on K4 fibroblast cells treated with CD161⁺ (blue) and CD161⁻ (red). Statistical differences were determined by paired T-tests with two-tailed p values; *P<0.05.

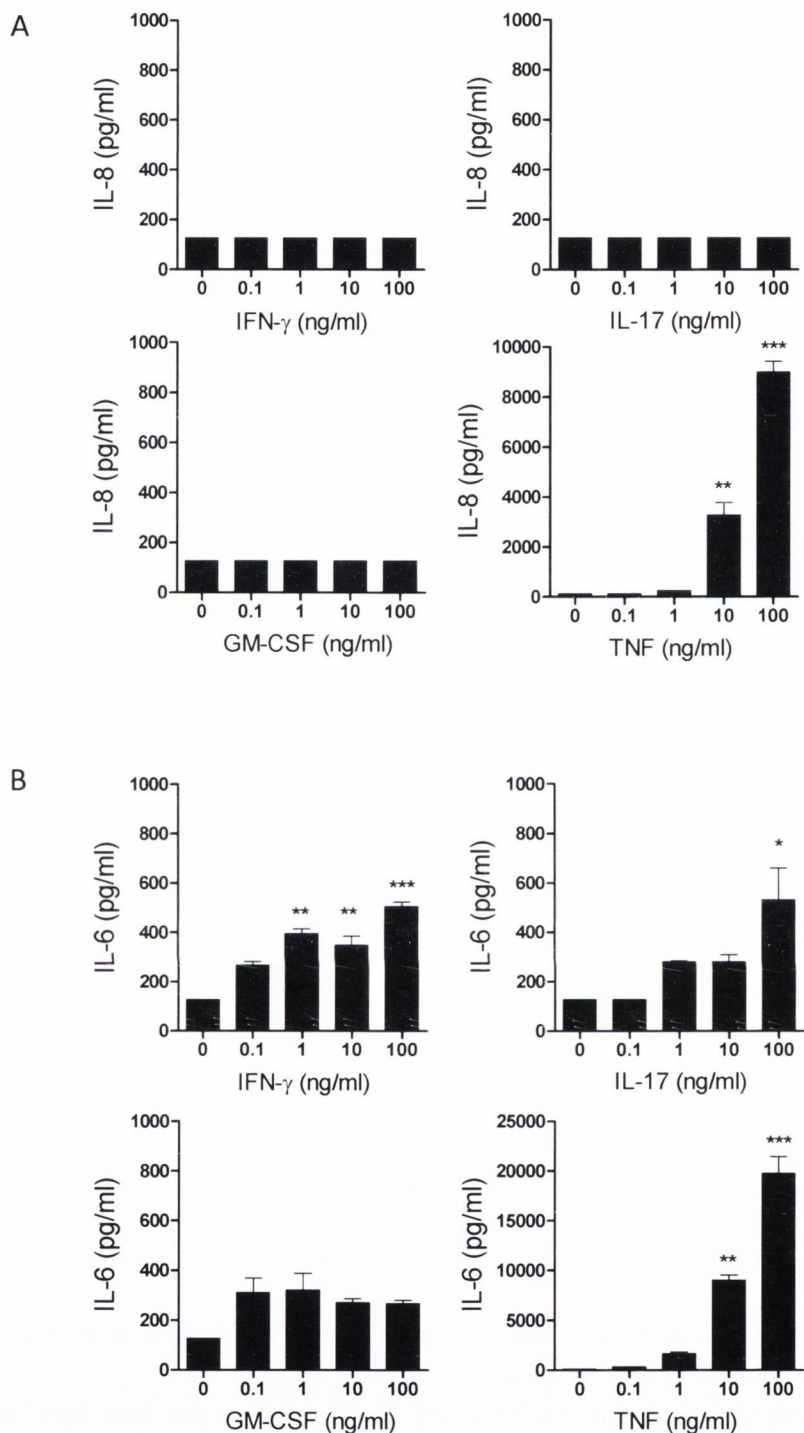


Figure 3.14 Induction of IL-8 and IL-6 in K4 fibroblast cells by recombinant pro-inflammatory cytokines.

K4 fibroblast cells were seeded in 48-well plates, grown to 90% confluence and serum starved for 8 hours. Recombinant IFN- γ , IL-17A, GM-CSF and TNF were applied to cells (at a concentration of 0.1, 1, 10 and 100 ng/ml) for 24 hours. Concentrations of IL-8 (A) and IL-6 (B) in the supernatants were assessed by ELISA. Statistical differences between cytokine treated and untreated cells were determined by one-way ANOVA with Tukey's Multiple Comparisons post-tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. This data is representative of 3 independent experiments.

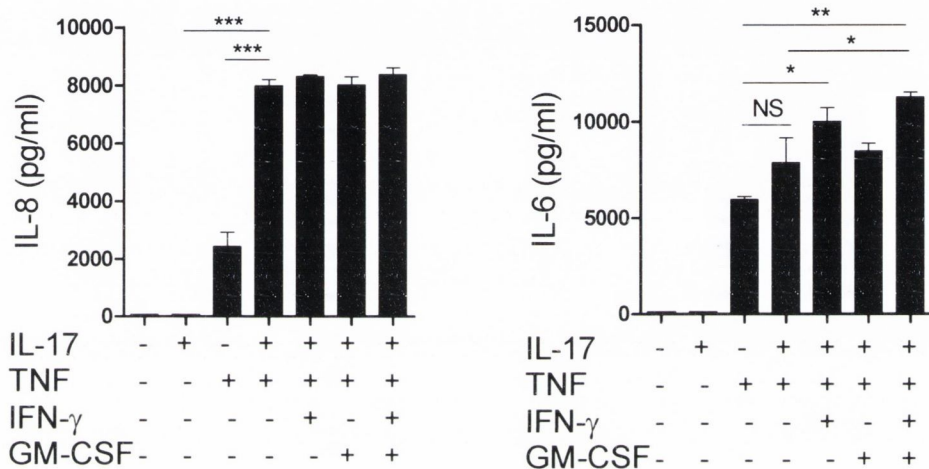


Figure 3.15 Induction of IL-8 and IL-6 in K4 fibroblast cells with combinations of recombinant pro-inflammatory cytokines.

K4 fibroblast cells were seeded in 48-well plates, grown to 90% confluence and serum starved for 8 hours. Recombinant IFN- γ , IL-17A, GM-CSF and TNF were applied to cells (alone and in combination, at 10 ng/ml) for 24 hours. Concentrations of IL-8 (A) and IL-6 (B) in the supernatants were assessed by ELISA. Statistical differences were determined by one-way ANOVA with Tukey's Multiple Comparisons post-tests; *p<0.05, **p<0.01, ***p<0.001. This data is representative of 3 independent experiments.

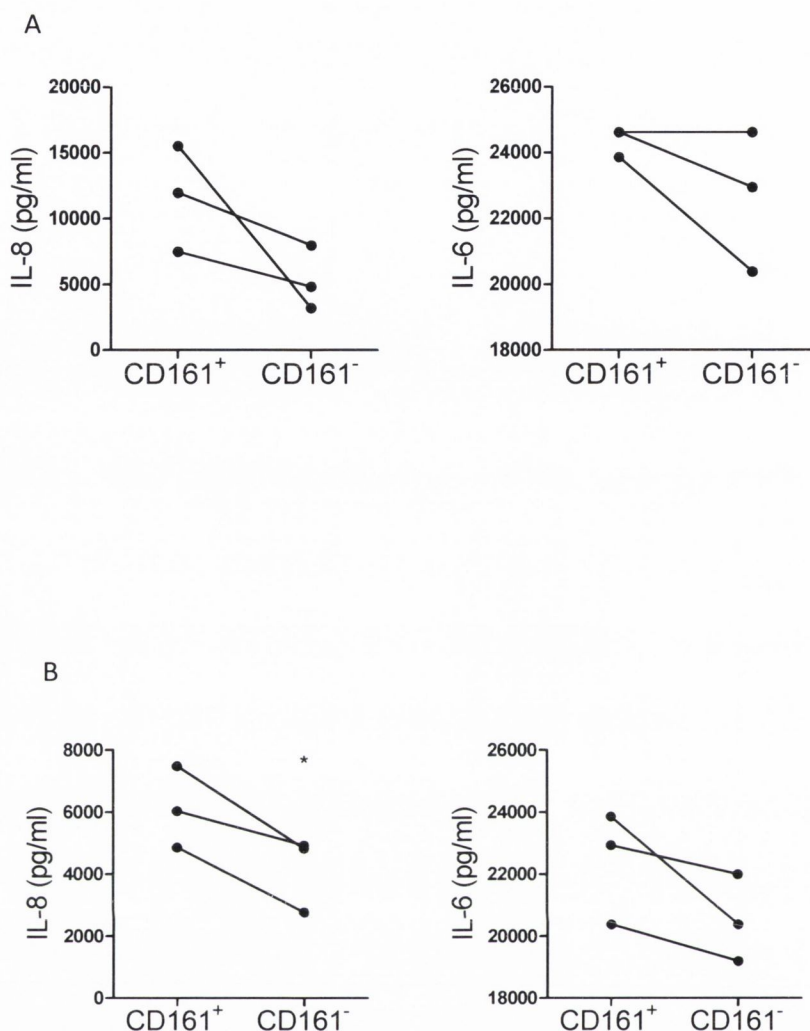


Figure 3.16 CD161⁺ Th cells induce an enhanced pro-inflammatory phenotype in primary RA fibroblast cells compared with CD161⁻ Th cells.

PBMC were isolated from healthy donors. CD4⁺ memory T cells were purified from PBMC by MACS. CD4⁺CD45RO⁺CD25⁻CD161⁺ and CD4⁺CD45RO⁺CD25⁻CD161⁻ effector T cells were further sorted by FACS. Effector cells were cultured 5 days in the presence of anti-CD3 and irradiated, allogeneic APC and supernatants were harvested. Primary fibroblast cells were isolated from RA patients during active disease. Fibroblast cells were seeded in 96-well plates, grown to 90% confluence and serum starved for 8 hours. CD161⁺ and CD161⁻ Th cell supernatants were applied to fibroblast cells for 24 hours and the concentrations of IL-8 and IL-6 were assessed by ELISA. The graphs show the concentrations of IL-8 (left) and IL-6 (right) induced by 1 HC donor CD161⁺ and CD161⁻ Th supernatants on primary fibroblast cells from 3 RA donors (A). The graphs show the concentrations of IL-8 (left) and IL-6 (right) induced by 3 HC donor CD161⁺ and CD161⁻ Th cell supernatants on primary fibroblast cells from 1 RA donor. Statistical differences were determined by paired T-tests with two-tailed p values; *p<0.05.

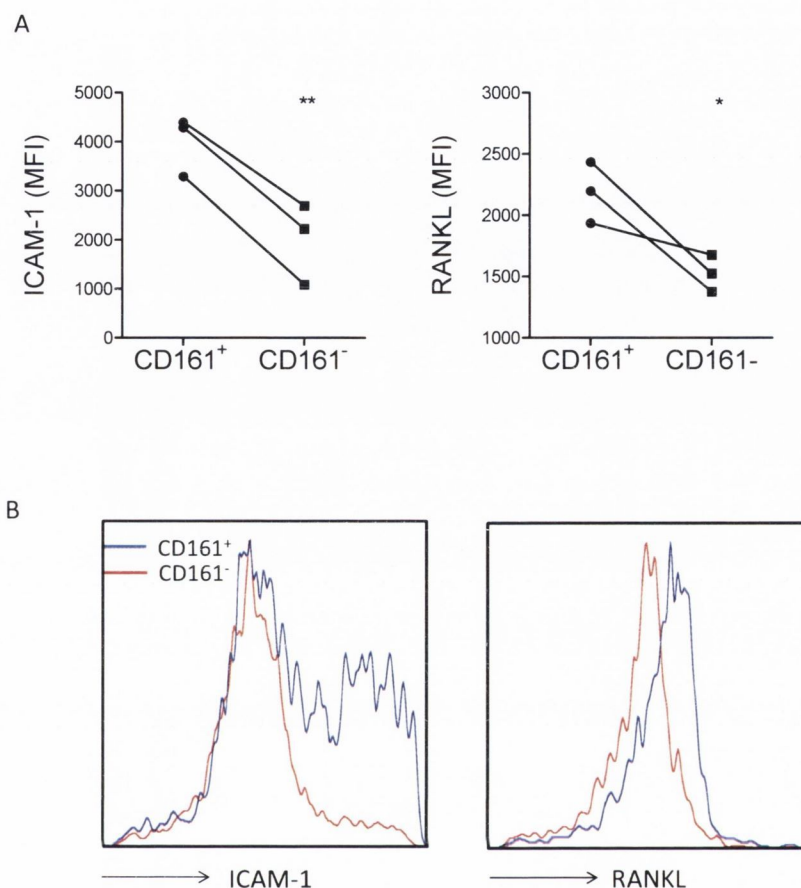


Figure 3.17 Differential induction of primary RA fibroblast cell surface expression of ICAM-1 and RANKL by CD161⁺ and CD161⁻ Th cell supernatants.

PBMC were isolated from a healthy donor and CD4⁺ memory T cells were purified by MACS. CD4⁺CD45RO⁺CD25⁻CD161⁺ and CD4⁺CD45RO⁺CD25⁻CD161⁻ effector T cells were sorted by FACS. Effector cells were cultured 5 days in the presence of anti-CD3 and irradiated, allogeneic APC and RA SFMC and RA or HC PBMC supernatants were harvested. Primary fibroblast cells were isolated from RA patients (n=3) during active disease. Fibroblast cells were seeded in 96-well plates, grown to 90% confluence and serum starved for 8 hours. CD161⁺ and CD161⁻ Th cell supernatants were applied to fibroblast cells for 24 hours. Fibroblast cells were stained with fluorochrome-conjugated antibodies specific for ICAM-1 and RANKL and analysed by flow cytometry. The graphs show the expression of ICAM-1 and RANKL (MFI) induced by CD161⁺ and CD161⁻ cell supernatants (A). Each pair of data points represents an RA fibroblast donor (n=3). Representative FACS plots show the expression of ICAM-1 (right) and RANKL (left) induced by CD161⁺ Th cell supernatants (blue) and CD161⁻ Th cell supernatants (red). Statistical differences were determined by paired T-tests with two-tailed p values; *p<0.05, **p<0.01.

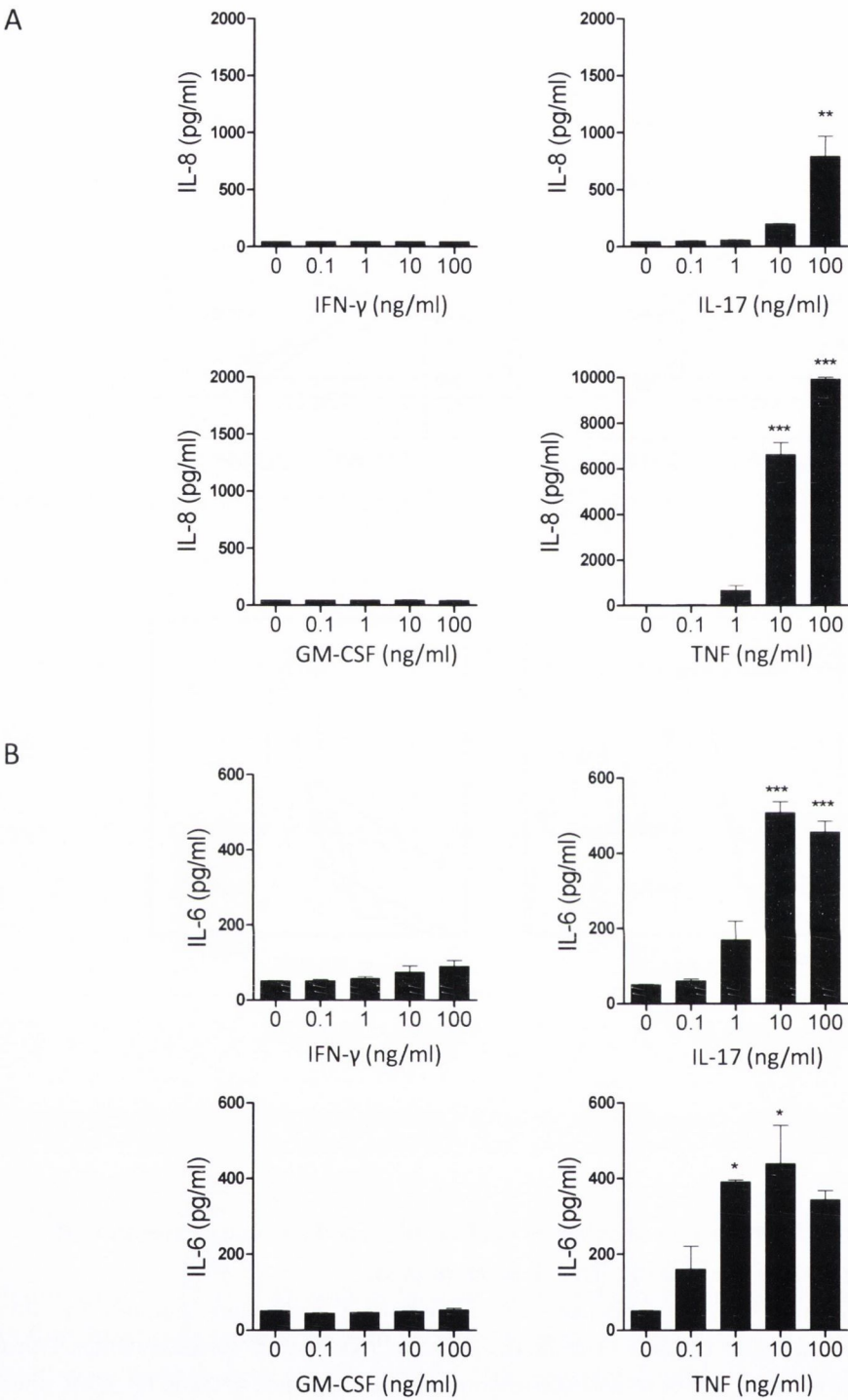


Figure 3.18 Induction of IL-8 and IL-6 in primary RA fibroblast cells by recombinant pro-inflammatory cytokines.

Primary RA fibroblast cells were isolated from patients during active disease. Cells were seeded in 96-well plates, grown to 90% confluence and serum starved for 8 hours. Recombinant IFN- γ , IL-17A, GM-CSF and TNF were applied to cells (at a concentration of 0.1, 1, 10 and 100 ng/ml) for 24 hours. Concentrations of IL-8 (A) and IL-6 (B) in the supernatants were assessed by ELISA. Statistical differences between cytokine treated and untreated cells were determined by one-way ANOVA with Tukey's Multiple Comparisons post-tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. This data is representative of 3 independent experiments.

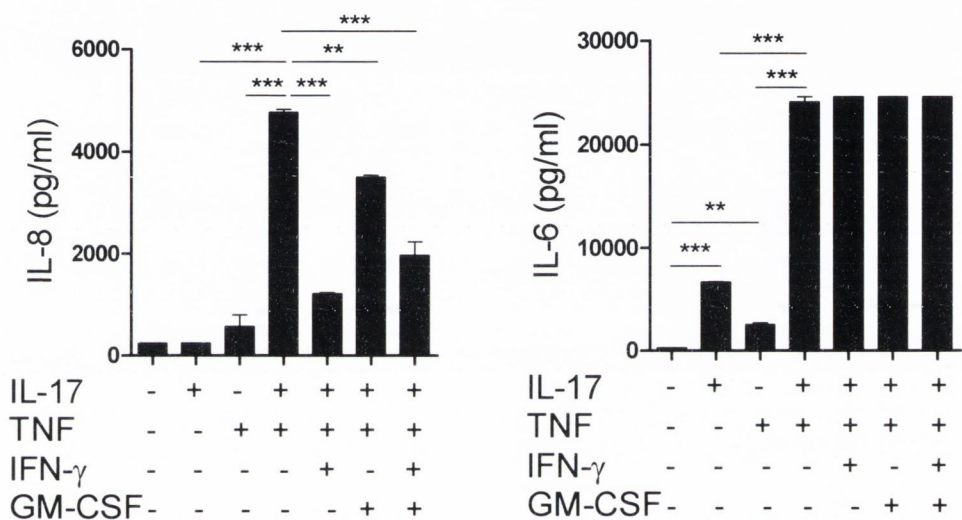


Figure 3.19 Induction of IL-8 and IL-6 in primary RA fibroblast cells with combinations of recombinant pro-inflammatory cytokines.

Primary RA fibroblast cells were seeded in 96-well plates, grown to 90% confluence and serum starved for 8 hours. Recombinant IFN- γ , IL-17A, GM-CSF and TNF were applied to cells (alone and in combination, at 10 ng/ml) for 24 hours. Concentrations of IL-8 (A) and IL-6 (B) in the supernatants were assessed by ELISA. Statistical differences were determined by one-way ANOVA with Tukey's Multiple Comparisons post-tests; **p<0.01, ***p<0.001. This data is representative of 3 independent experiments.

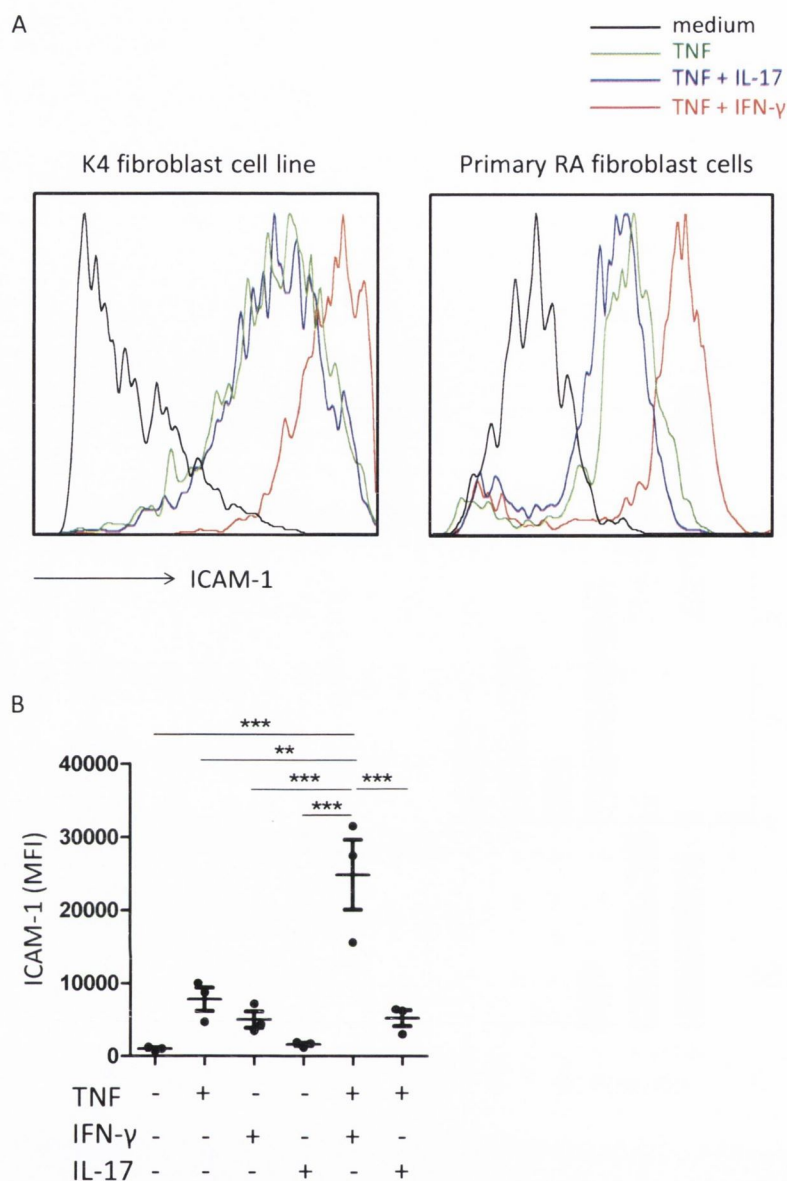


Figure 3.20 TNF synergises with IFN- γ to induce the expression of ICAM-1 on fibroblast cells.

RA fibroblast cells were isolated from RA patients during active disease. K4 fibroblast cells and primary RA fibroblast cells were seeded in 96-well plates, grown to 90% confluence and serum starved for 8 hours. Recombinant IFN- γ , IL-17A and TNF were applied to cells (alone and in combination, at 10 ng/ml) for 24 hours. Cells were trypsinised, stained with a fluorochrome-conjugated antibody specific for ICAM-1 and analysed by flow cytometry. (A) Representative histograms show the intensity of ICAM-1 staining in K4 fibroblast cells (left) and primary RA fibroblast cells (right). (B) The graph shows ICAM-1 (MFI) expression in 3 RA donors. Statistical differences were determined by one-way ANOVA with Tukey's Multiple Comparisons post-tests; ** $p < 0.01$, *** $p < 0.001$.

3.3 Discussion

This chapter has uncovered some novel data suggesting that CD161⁺ Th cells are highly pathogenic in the context of RA. In addition, it has confirmed the findings of previously published work. The present study has shown that CD4⁺ T cells were proliferating in the joints of RA patients and displayed a differentiated effector memory phenotype. In addition, significantly higher frequencies of CD4⁺ T cells in RA SFMC were producing GM-CSF, IFN- γ and IL-17, compared with CD4⁺ T cells from PBMC. Moreover, pro-inflammatory cytokines produced by CD4⁺ T cells in RA SFMC were produced concomitantly with each other or TNF, and were therefore, defined as polyfunctional cells. CD161 expression was markedly increased on CD4⁺ T cells in the RA joint. High frequencies of these CD161⁺ Th cells produced IFN- γ , suggesting that these Th17-lineage cells were ex-Th17 type cells. CD161⁺ Th cells from RA SFMC exhibited a phenotype that may be considered pathogenic in the context of autoimmunity; increased production of pro-inflammatory cytokines, increased expression of pro-survival HIF-1 α and Bcl2 and elongated telomeres. Finally, in order to demonstrate the pathogenic potential of CD161⁺ Th cells in the RA joint, fibroblast cells were treated with supernatants from sorted CD161⁺ and CD161⁻ Th cells. CD161⁺ Th cell supernatants induced significantly more IL-6 and IL-8 production from fibroblast cells and up-regulated their expression of ICAM-1 and RANKL. Cumulatively, these data strongly suggest that CD4⁺ CD161⁺ T cells are pathogenic in the RA joint and are, thus, a rational target for therapy.

This study found no significant difference between the frequencies of CD4⁺ T cells within the lymphocyte compartment of HC PBMC, RA PBMC and RA SFMC. However, this finding is significant because it shows that CD4⁺ T cells exist in the RA joint at a similar frequency to which they circulate in the blood, indicating that the T cells are actively trafficking into the joint. Moreover, the CD4⁺ T cells in the RA joint were more actively proliferating than CD4⁺ T cells in the peripheral blood and displayed a highly differentiated effector memory phenotype, consistent with the literature (Schmidt et al., 1996), which indicates that these cells have experienced an activating signal.

Cytokines produced by CD4⁺ T cells are thought to play a key role in the pathogenesis of RA, and therefore a panel of cytokines was assessed to examine in detail the overlapping populations of cytokine producing cells in the RA joint. This study found that the frequencies of CD4⁺ T cells producing IFN- γ were significantly increased in RA SFMC compared with HC and RA PBMC. This is consistent with published work that showed IFN- γ was significantly increased in Th cells from RA SFMC compared with PBMC (Yamada et al., 2008). IFN- γ has been shown to promote bone loss

and promote immune cell trafficking (Gao et al., 2007, Griffin et al., 2012) indicating that the increased expression of IFN- γ observed in RA SF is likely to have pathogenic effects in the context of the RA joint. GM-CSF secreted by Th cells plays a key role in propagating inflammation in RA by recruiting and activating innate immune cells in a positive feedback manner. This study examined the production of GM-CSF from CD4⁺ T cells in the RA joint, which had hitherto not been robustly assessed in the literature. The frequencies of CD4⁺ T cells producing GM-CSF were significantly increased in RA SFMC compared with HC and RA PBMC. Other work has shown that GM-CSF is expressed in RA SFMC, however, these papers have not shown GM-CSF production directly from CD4⁺ T cells nor have they illustrated increased frequencies of GM-CSF compared with PBMC (Alvaro-Gracia et al., 1991, Haworth et al., 1991). In agreement with the present study in RA, an increase in the frequency of SF derived Th cells producing GM-CSF compared with PBMC has been recently documented in JIA (Piper et al., 2014).

TNF plays a central role in autoimmune inflammation in the RA joint as evidenced by the use of anti-TNF therapy in the clinic. Surprisingly however, in the present study TNF production by Th cells was not significantly increased in RA SFMC compared with PBMC. This may be due to the fact that most memory Th cells can produce TNF when stimulated, therefore, Th cells in the peripheral blood when fully activated with PMA and ionomycin, may produce TNF at maximum capacity. The rationale for targeting TNF came from studies showing that TNF was present in the synovial tissues and cartilage pannus (Chu et al., 1991, Brennan et al., 1989) but focused largely on innate cells, such as macrophages, as the source of TNF in the joint (Kinne et al., 2000). The data from this study suggest that Th cells are also a rich source of TNF in the RA joint.

IL-17 is considered to be an important cytokine in the context of RA as it drives the recruitment and activation of neutrophils, stimulates the release of cytokines and chemokines and the induces production of prostaglandins and MMPs (Weaver et al., 2007). In this study, the overall frequency of IL-17 detected in CD4⁺ T cells was low, although IL-17 production was significantly increased in RA SFMC compared with RA PBMC. Furthermore, the majority of the IL-17 produced was co-expressed with IFN- γ , TNF or GM-CSF indicating that the Th17 cells in RA SFMC were functionally plastic and polyfunctional. This finding is consistent with previously published work showing that IL-17⁺ Th cells in RA SFMC co-express TNF (Church et al., 2010) and, in JIA, co-express IFN- γ (Cosmi et al., 2011). However to date, there has been no other forthcoming evidence illustrating quadruple cytokine polyfunctionality in RA SFMC Th cells.

The data presented here shows for the first time that CD4⁺ T cells from the inflamed RA joint are highly polyfunctional in terms of their cytokine production. In general, activated Th cells produce TNF, however, in the RA joint there was a significant increase in the proportion of polyfunctional Th cells producing GM-CSF, IFN- γ and TNF concomitantly. Moreover, the IL-17 producing cells, despite being low in frequency, were highly polyfunctional. This polyfunctional cytokine production suggests that they may be pathogenic in the context of the inflamed joint. These data demonstrate cytokine polyfunctionality for the first time in RA and are supported by recent findings in JIA which show Th17-type cell polyfunctionality by illustrating that GM-CSF was produced concomitantly with IFN- γ in CD161⁺ Th cells (Piper et al., 2014). The data also highlights the inaccuracy of defining discrete Th subsets based solely on the production of a cytokine and indicates that a high level of functional plasticity exists between the Th subsets under certain inflammatory conditions, such as the pro-inflammatory milieu of the RA joint.

Consistent with evidence in JIA and recent findings in RA (Cosmi et al., 2011, Nistala et al., 2010, Chalan et al., 2013), this study demonstrated that CD161⁺ Th cells were enriched in RA SFMC compared with HC and RA PBMC. The data exhibited a significant spread which distributed broadly into 2 clusters; 8 out of 25 samples displayed frequencies of CD4⁺ CD161⁺ cells similar to HC PBMC whereas the remaining 17 samples had significantly enriched frequencies. This may represent different sub-types of disease in a cohort of the patients examined or may be the result of a particular treatment. The patient cohort examined here varied according to treatment and other clinical parameters. However no segregation of CD161 expression was observed according to treatment type or disease severity. The enrichment of CD161⁺ Th cells failed to significantly correlate with patient DAS28 score (data not shown) despite recently published evidence suggesting that CD161⁺ Th17 cells in the peripheral blood correlate with DAS28, ESR and CRP (Miao et al., 2013). In addition, the paper also showed that CD161⁺ “Th-1 like” cells are negatively correlated with disease activity in the peripheral blood. Their finding is at odds with data from JIA studies of CD161 in SFMC, showing significant correlations between the frequencies of total CD161⁺ Th cells and IL-17⁺ IFN- γ ⁺ plastic CD161⁺ Th cells with ESR and CRP (Cosmi et al., 2011). This discrepancy in the literature further highlights the need for caution when interpreting results from peripheral blood and the requirement to examine cells from the site of autoimmune inflammation, in the synovial fluid.

CD161⁺ Th cells in RA SFMC produced significantly more IFN- γ compared with CD161⁺ Th cells in the peripheral blood. Interestingly, only a small fraction of these CD161⁺ Th cells produced IL-17 in response to stimulation *ex vivo* whereas high proportions expressed IFN- γ . This indicates that

CD161⁺ cells, which are thought to be of a Th17 cell lineage, are functionally plastic and switch their cytokine profile to a “Th1-like” phenotype. The idea that the inflammatory joint environment promotes Th17 plasticity is consistent with studies in JIA showing that CD161⁺ Th17 cells switch to become CD161⁺ ex-Th17 (“Th1-like”) cells (Nistala et al., 2010, Cosmi et al., 2011). The present study demonstrated that these plastic CD161⁺ Th cells produced equally high frequencies of IFN- γ as the bona fide CD161⁻ Th1 type cells in the RA joint. However, CD161⁺ Th cells from RA SFMC produced significantly more TNF, GM-CSF and IL-17 compared with CD161⁻ Th cells, further indicating that these plastic, polyfunctional CD161⁺ Th cells are more likely to be pathogenic in the RA joint.

Although IL-17 producing cells were in the minority in the RA joint, one could speculate that since the majority of polyfunctional effector cells are derived from CD161⁺ Th17 cells, targeting CD161⁺ Th cells would still be an effective therapeutic strategy. IL-17 is now considered an important therapeutic target for autoimmune disease. A recent phase II trial with anti-IL-17 antibodies in RA revealed similar efficacy to TNF inhibitors (Kellner, 2013), however, the efficacy in RA was pale in comparison with the results elicited in psoriasis patients (Papp et al., 2012, Leonardi et al., 2012). This study shows that IL-17 production in the RA joint is highly dynamic and in the patients examined, the majority of CD161⁺ Th17 type cells had switched to IFN- γ producing ex-Th17 cells. Hence, the CD161⁺ IL-17⁻ plastic, polyfunctional cells would not be subject to inhibition by anti-IL-17. Thus, it is possible that the relative lack of efficacy of anti-IL-17 therapy in RA may be due in part to the plasticity of Th17 cells.

It has been reported that Th17 cells have unique stem cell-like characteristics allowing them to persist longer *in vivo* (Kryczek et al., 2011). This “stemness” of Th17 cells was suggested to enhance the anti-tumour capacity of Th17 cells while promoting their capacity for perpetuating autoimmune inflammation. The question of whether or not these plastic ex-Th17 type cells retain their stem cell-like features has hitherto been unexplored. This study demonstrated for the first time that CD161⁺ Th cells from RA SFMC, which are comprised mostly of IFN- γ producing plastic ex-Th17 type cells, express significantly more Bcl2 than their CD161⁻ Th cell counterparts. This implies that CD161⁺ Th cells are more resistant to apoptosis and therefore, may persist longer in the RA joint. In addition, CD161⁺ Th cells extracted from the hypoxic environment of the RA joint expressed significantly more HIF-1 α compared with CD161⁻ Th cells, conferring a further survival advantage on CD161⁺ Th cell. Interestingly, HC CD161⁺ Th cells from the peripheral blood do not exhibit an increased expression of HIF- α but they do exhibit an increased variance (i.e. that data is more spread) compared with CD161⁻ Th cells. HIF-1 α is

constantly produced by all cells and rapidly degraded in normoxia, therefore, significant differences between HIF-1 α expression may only become apparent under conditions of hypoxia. However, murine Th17 cells exhibited higher mRNA levels of HIF-1 α compared with Th1, Th2 and Treg cells (Shi et al., 2011). Interestingly, it has been shown that human Th17 cells have increased mRNA transcripts of HIF-1 α compared with Th1 and Th2 cells and when HIF-1 α expression is knocked down with shRNA in Th17 cells, the relative expression of Bcl2 is consequently reduced (Kryczek et al., 2011). This suggests that increased HIF-1 α in Th17 cells may be responsible for other pro-survival features of Th17 lineage cells, including anti-apoptotic Bcl2.

It has been documented that telomeres shorten as differentiation increases (Fletcher et al., 2005) however, T cells can up-regulate telomerase activity when activated, a mechanism that helps to mitigate the loss of telomeres after multiple cell divisions (Akbar and Vukmanovic-Stejic, 2007). The present study showed that CD161⁺ Th cells in both HC PBMC and RA SFMC had significantly longer telomeres than CD161⁻ Th cells. This suggests that, despite their highly differentiated effector memory phenotype, CD161⁺ Th cells can overcome replicative exhaustion. Moreover, this is the first time that relatively longer telomeres have been demonstrated on a particular subset of Th cells in RA. Interestingly, HIF-1 α has been shown to up-regulate telomerase in human placental cells (Nishi et al., 2004), raising the possibility that increased HIF-1 α in the hypoxic RA joint may provide a mechanism for T cells to escape or delay replicative senescence via upregulation of telomerase. The evidence in this study supports published data indicating that Th17 cells have a stem cell-like phenotype and gives further impetus to the working hypothesis of this study by showing that CD161⁺ Th cells have the necessary attributes to persist for a protracted time in the chronically inflamed environment of the RA joint. In the context of RA, the majority of previous telomere studies showed overall reduced telomere length and a premature ageing phenotype in a gross population of leukocytes (Steer et al., 2007) which was particularly associated with the HLA-DRB1*04 gene that confers risk for developing RA (Schonland et al., 2003). Unfortunately, in the present study the HLA type of patients was not available.

One caveat of this study is the lack of a highly appropriate control; in a healthy joint, there is no build up of synovial fluid that can be drained off, nor is there an enrichment of immune cells trafficking into the joint. Indeed, the frequencies of T cells residing in healthy synovial joints are largely unknown. Synovial fluids drained from the joints of patients suffering with other types of arthritis are not effective controls because, by its very nature, all types of arthritis are

characterised by an immune cell infiltration and inflammation. Osteoarthritis, for example, was historically considered to be a non-inflammatory disease but is now recognised as having an inflammatory, T cell mediated component (Sakkas and Platsoucas, 2007).

This study also delineated, for the first time, the phenotype and function of CD161⁺ Th cells in a healthy population; showing that *ex vivo* CD161⁺ Th cells have an intrinsically increased survival capacity (via increased Bcl2 expression and long telomeres) and activated CD161⁺ Th cells are more polyfunctional than CD161⁻ Th cells. Furthermore, this study suggests that an intrinsic difference exists between bona fide Th1 cells and plastic ex-Th17 type cells that have switched into “non-classical Th1 cells”; plastic IFN- γ producing CD161⁺ Th cells are more polyfunctional than IFN- γ producing CD161⁻ Th cells and, because these cells are longer-lived, it is plausible to postulate that these cells, despite being “Th1-like”, may have enhanced effector features which add to their pathogenicity in the context of autoimmunity. Production of IFN- γ marks the transition of the bona fide Th17 cell phenotype to the ex-Th17 cell phenotype. IFN- γ producing ex-Th17 type cells are thought to be highly pathogenic in JIA (Maggi et al., 2012) and in RA, as demonstrated by the current study, and therefore, distinct from the more mildly pro-inflammatory IFN- γ producing Th1 cells. Therefore, production of IFN- γ by ex-Th17 type cells may not cause the highly pathogenic phenotype but may rather mark the transition to cytokine polyfunctionality as demonstrated by this body of work in conjunction with recent data showing that ex-Th17 cells can express GM-CSF in addition to IFN- γ (Duhon et al., 2013, Piper et al., 2014). There has previously been no forthcoming evidence in the literature proposing that plastic ex-Th17/ “non-classical Th1” type cells are inherently different to bona fide Th1 cells, therefore, functional and phenotypic differences between bona fide Th1, bona fide Th17 and plastic ex-Th17/non-classical Th1 type cells will be explored in a subsequent chapter. Much of the focus in the field of autoimmunity has been on the role of single cytokines such as IL-17 or IFN- γ in autoimmune inflammation. The data presented here suggests that a more relevant focus might be on the characteristics of the particular cell types and the combinations of cytokines that they produce.

This study confirmed that CD161 is a good marker of ROR- γ t⁺ Th17-type cells but also showed that CD161^{lo/-} Th cells can produce small amounts of IL-17. This is consistent with published work showing that IL-17 can be produced from CCR4⁺ CCR6⁺ CXCR3⁻ CD161⁻ Th cells, albeit at lower frequencies than the production from CCR4⁺ CCR6⁺ CXCR3⁻ CD161⁺ Th cells (Crome et al., 2010a). Moreover, the published study showed that mRNA levels of RORC were significantly increased in those CD161⁻ Th17 cells compared with Th1 cells but significantly reduced compared with

Chapter 4

Treg cells in RA

Chapter 4 Treg cells in RA

4.1.1 Introduction

Understanding the altered nature of immuno-regulatory responses during autoimmune inflammation is fundamental to understanding the overall disease. There are conflicting data regarding Treg cell frequency and function in RA, however, some studies examined cells taken from the peripheral blood of RA patients whereas others examined cells from the site of inflammation, in the RA synovial fluid, which may explain the variability in the literature. Researchers examining the peripheral blood of RA patients have reported a decrease in the frequency of Treg cells compared with healthy controls (Sempere-Ortells et al., 2009, Cao et al., 2003, Raghavan et al., 2009), whereas studies which extracted lymphocytes from the synovial fluid of RA patients have reported enhanced frequencies of Treg cells (van Amelsfort et al., 2004, Cao et al., 2004, Raghavan et al., 2009). This literature demonstrates that the source of patient Treg cells being examined is of utmost importance as conclusions drawn from peripheral blood samples may differ vastly to those from synovial fluid. Moreover, when examining peripheral blood, the timing of sampling relative to an inflammatory flare in the joint is important because, depending on the timing, cells may have trafficked into the joint causing an apparent deficit in the circulation. Thus, cells extracted from RA synovial fluid are more pertinent to understanding the pathology.

Inflammation persists in RA despite high frequencies of Treg cells in the joint, hence, the phenotype and function of Treg cells from RA synovial fluid have been the subject of much investigation. Published findings indicate that RA synovial fluid Treg cells have significantly increased frequencies of CTLA-4, GITR and OX-40 compared with peripheral blood (van Amelsfort et al., 2007). Furthermore, RA synovial fluid derived Treg cells have been shown to be functionally suppressive *ex vivo* despite being unable to restrain inflammation during RA (van Amelsfort et al., 2004, Cao et al., 2004). An explanation as to why Treg cells fail to constrain or resolve the chronic inflammatory response in RA remains elusive. A possible explanation of this phenomenon has emerged in a seminal study of JIA, which showed that Treg cells from the synovial fluid cannot successfully constrain autologous effector cells due to the effector cell exhibiting an aggressive phenotype that results in the evasion of Treg cell mediated suppression (Haufe et al., 2011).

Plasticity of Treg cells toward a Th17 cell phenotype creates a plausible pathogenic role for Treg cells at sites of chronic inflammation. These hybrid “Treg-effector” cells have very recently been shown to express CD161 and are enriched in the synovial fluid of JIA patients (Pesenacker et al.,

2013) and RA patients (Wang et al., 2014). Both studies showed that CD161⁺ Treg cells exhibited phenotypic characteristics of both Th17 and Treg cells and were functionally suppressive when taken from the blood of JIA and RA patients or healthy controls. The addition of recombinant IL-6 alone or in conjunction with IL-23 to Treg cells sorted from RA patients and healthy controls elicited a significant increase in the frequency of FoxP3⁺ Treg cells producing IL-17 (Wang et al., 2014). These data suggest that FoxP3⁺ Treg cells can be converted into inflammatory cytokine producing cells as a result of pro-inflammatory cytokines that are present in the RA microenvironment. Furthermore, these plastic “Treg-effector” hybrid cells are restricted to the CD161⁺ subset of Treg cells. These data therefore indicate that Treg cells can potentially become pro-inflammatory and this has implications for the adoptive transfer of Treg cells as a therapy for autoimmunity.

Adoptive Treg cell therapy may be a plausible method by which the chronic inflammation in RA could be effectively constrained. However, this therapeutic approach could prove disastrous if the transferred Treg cells are capable of functional plasticity and transition into hybrid “Treg-effector” cells which produce pro-inflammatory cytokines. Treating Treg cells with ATRA *in vitro* is thought to enhance their stability (Wang et al., 2009, Lu et al., 2010). Furthermore, ATRA treated nTreg cells improved joint swelling when transferred therapeutically into mice with established collagen induced arthritis (Zhou et al., 2010). However, the stability of *in vitro* differentiated iTreg cells for therapeutic adoptive transfer has been understudied in the context of RA. Synovial fluid drained from RA patients during active disease can be used to mimic the pro-inflammatory environment of the joint *in vitro*, thus, providing the opportunity to investigate the stability of Treg cells *in vitro* but under the stress of a physiologically relevant pro-inflammatory environment.

4.1.2 Aims

The aim of the experiments in this chapter was to characterise the phenotype and function of Treg cells present in the RA SFMC compared with HC and RA PBMC Treg cells. Specifically, this study aimed to:

- Analyse the frequencies and phenotype of Treg cells present in RA SFMC.
 - Assess CD39 and Ki67 expression on Treg cells, which have hitherto gone unstudied and CTLA-4 expression, which is controversial in the literature.
- Examine hybrid “Treg-effector” cells in RA SFMC.
 - Confirm the presence of CD161 on a subset of Treg cells in RA SFMC
 - Assess differential cytokine production from CD161⁺ versus CD161⁻ Treg cells.
- Investigate the propensity of Treg cells to survive in the RA joint.
 - Determine, for the first time, HIF-1 α expression in Treg cells from RA SFMC to deduce their likelihood of surviving in the hypoxic RA joint.
 - Examine the expression of anti-apoptotic Bcl2 on Treg cells from RA SFMC to determine if RA Treg cells are more or less prone to death by apoptosis compared with HC PBMC Treg cells and autologous responder cells.
- Measure Treg cell mediated suppression of responder cells from RA SFMC compared with HC PBMC.
 - Evaluate Treg cell mediated suppression of proliferation, IFN- γ and TNF production by responder cells and, for the first time, robustly assess the Treg cell mediated suppression of IL-17 and GM-CSF.
- Determine the stability of Treg cells cultured with synovial fluids from RA patients.

4.2 Results

4.2.1 The frequency and phenotype of Treg cells present in the RA joint SF compared with peripheral blood.

Having demonstrated increased cytokine production and polyfunctionality in CD4⁺ effector T cells within the RA joint (section 3.2.1), the next question addressed was whether these cells were subject to regulation by Treg cells in the RA joint. This study sought to characterise the Treg cells present in the highly inflammatory, hypoxic environment of the joint in order to assess if the regulatory T cell compartment was defective in terms of frequency, phenotype or function. Lymphocytes were isolated from the SF of RA patients during active disease, as well as from the blood of RA patients and age and sex-matched healthy controls. Cells were stained *ex vivo* for Treg cell markers and analysed by flow cytometry. Many previously published studies used only the expression of CD25 and CD4 as markers of Treg cells. However, CD25, or the IL-2 receptor, is an inadequate marker of Treg cells because its expression is also up-regulated on activated effector cells. Therefore, this study used a combination of surface CD4, CD25, CD127 and intranuclear FOXP3 staining, since no single marker can accurately identify human Treg cells. Live CD4⁺ lymphocytes were identified according to the gating strategy discussed in section 2.11 (figure 2.9). FOXP3⁺ CD127^{lo} cells were gated (figure 4.1A, left) and expression of CD25 was assessed (figure 4.1A, right), thus, Treg cells were identified as being CD4⁺CD25⁺CD127^{lo}FOXP3⁺. For simplicity, Treg cells are often illustrated by FOXP3 expression versus CD25 expression (figure 4.1B).

4.2.1a Treg cells are enriched in RA SFMC compared with RA and HC PBMC.

RA SFMC and PBMC were isolated from RA patients during active disease and compared with HC PBMC. Cells were stained *ex vivo* for Treg cell markers, as described above, and analysed by flow cytometry. Consistent with many studies carried out on SF-derived cells, a significant increase in the frequency of CD4⁺ CD25⁺CD127^{lo} FOXP3⁺ Treg cells was observed in CD4⁺ T cell populations from RA SFMC compared with HC and RA PBMC (figure 4.2). On average, Treg cells constituted approximately 5% of the total CD4⁺ T cell population in the peripheral blood whereas in RA SFMC, the average frequency significantly increased to approximately 12% (figure 4.2A). In addition, a higher staining intensity of CD25 and FOXP3 co-expression was observed in RA SF Treg cells compared with peripheral blood (figure 4.2B); representative histograms show the marked increase in FoxP3 expression in Treg cells RA SFMC compared with Treg cells from matched PB with collated data illustrating that this trend is statistically significant (figure 4.2C).

No significant differences were observed between the frequencies of Treg cells present in RA PBMC compared with HC PBMC (figure 4.2A).

4.2.1b Treg cells exhibit a functional phenotype in RA SFMC.

Next, the phenotype of Treg cells was examined. CD39 is a cell surface ectonuclease that cleaves ATP (an extracellular pro-inflammatory signal) into AMP which, in the presence of CD73, can be further dephosphorylated to form the anti-inflammatory molecule, adenosine. Treg cells utilise CD39 as a mechanism to suppress effector cells (Borsellino et al., 2007) and furthermore, Treg cells expressing CD39 have been shown to have enhanced suppressive function compared with CD39⁻ Treg cells (Deaglio et al., 2007). Therefore, the expression of CD39 was examined on Treg cells from RA SFMC compared with RA and HC PBMC. Cells were isolated from HC and RA patients during active disease and stained *ex vivo* for Treg cell markers and cell surface CD39. Treg cells were identified by the gating strategy outlined above (section 4.2.1).

Interestingly, the frequency of RA SFMC derived Treg cells expressing CD39 was significantly increased, with an average of approximately 80%, compared with approximately 50% expression in Treg cells in RA or HC PBMC (figure 4.3). Treg cells can also suppress via CTLA4, which blocks co-stimulation of effector T cells. Expression of CTLA4 was examined and found to be significantly increased on Treg cells from RA SFMC compared with RA and HC PBMC, with no significant differences observed between RA and HC PBMC (figure 4.4). The expression of Ki67, a marker exclusively expressed in the nucleus of proliferating cells during all stages of cell cycle, was assessed on Treg cells from RA SFMC compared with RA and HC PBMC. Significantly increased frequencies of Treg cells in RA SFMC were undergoing active cell cycling (an average of approximately 21%) compared with RA PBMC (approximately 12%) and HC PBMC (approximately 6%; figure 4.5). The difference in Treg cell Ki67 expression between HC and RA PBMC did not reach statistical significance. Interestingly, the average frequency of proliferation was noticeably higher in Treg cells than in effector cells (see section 3.2.1a, figure 3.1) in both PBMC and SFMC (an average of 1.5% of effector cells compared with 6% of Treg cell expressed Ki67 in HC PBMC, 1.8% of effector cells compared with 12% of Treg cells expressed Ki67 in RA PBMC and 10% of effector cells compared with 21% of Treg cells expressed Ki67 in RA SFMC).

In summary, Treg cells were highly enriched in the RA joint and exhibited increased expression of FoxP3 and increased frequencies of suppressive molecules CD39 and CTLA4 compared with Treg cells in the PB. In addition, Treg cells present in RA SFMC were highly proliferative, as indicated by increased expression of Ki67. These data suggest that there may not be any

apparent defect in the Treg cell compartment in the RA joint in terms of frequency or phenotype. RA SFMC Treg cells would therefore be predicted to be functionally suppressive.

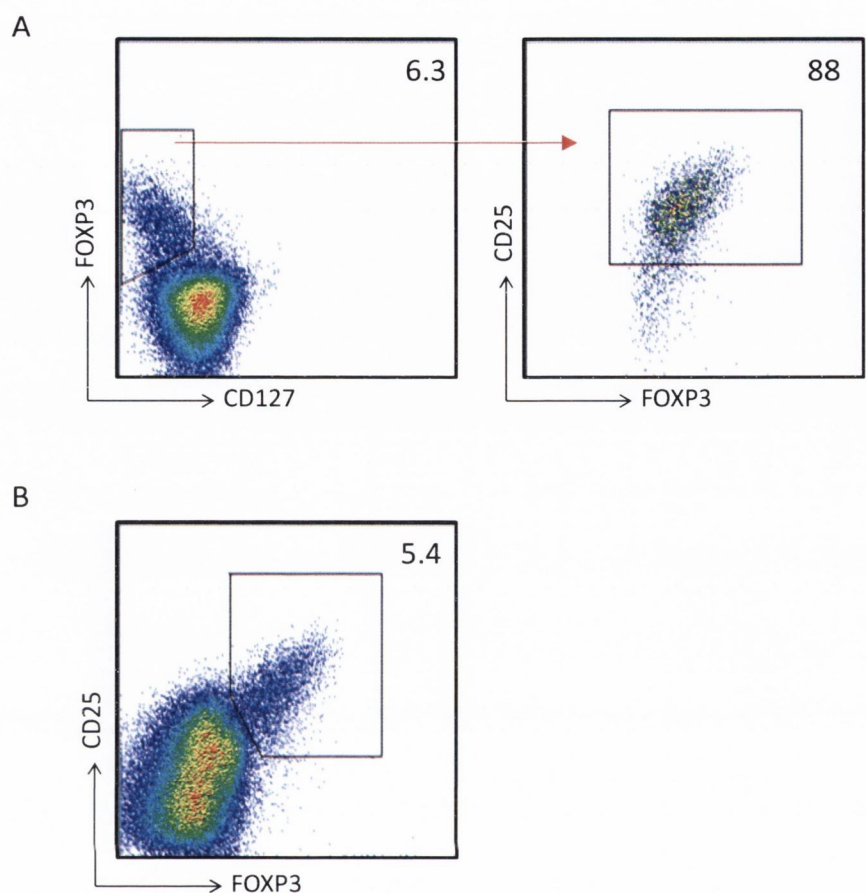


Figure 4.1 The flow cytometry gating strategy for identifying Treg cells.

SFMC or PBMC were stained with fluorochrome-conjugated antibodies specific for CD4 and Treg cell markers; FOXP3, CD25 and CD127, and analysed by flow cytometry. Representative FACS plots show the gating strategy for identifying the Treg cell population. The lymphocyte population was identified on the basis FSc and SSc (data not shown). CD4⁺ T cells within the lymphocyte gate were gated on the basis of CD4 versus forward scatter. Expression of FOXP3 and CD127 was examined in the CD4⁺ population. CD4⁺ FOXP3⁺CD127^{lo} cells were gated (A, left) and then assessed for expression of CD25 (A, right). Thus, Treg cells were identified as being CD4⁺ CD25⁺ CD127^{lo} FOXP3⁺, and their frequency was calculated as a percentage of CD4⁺ T cells i.e. 88% of 6.31 = 5.55%. For simplicity, Treg cells may also be demonstrated as CD25⁺ FOXP3⁺ within the CD4⁺ gate (B).

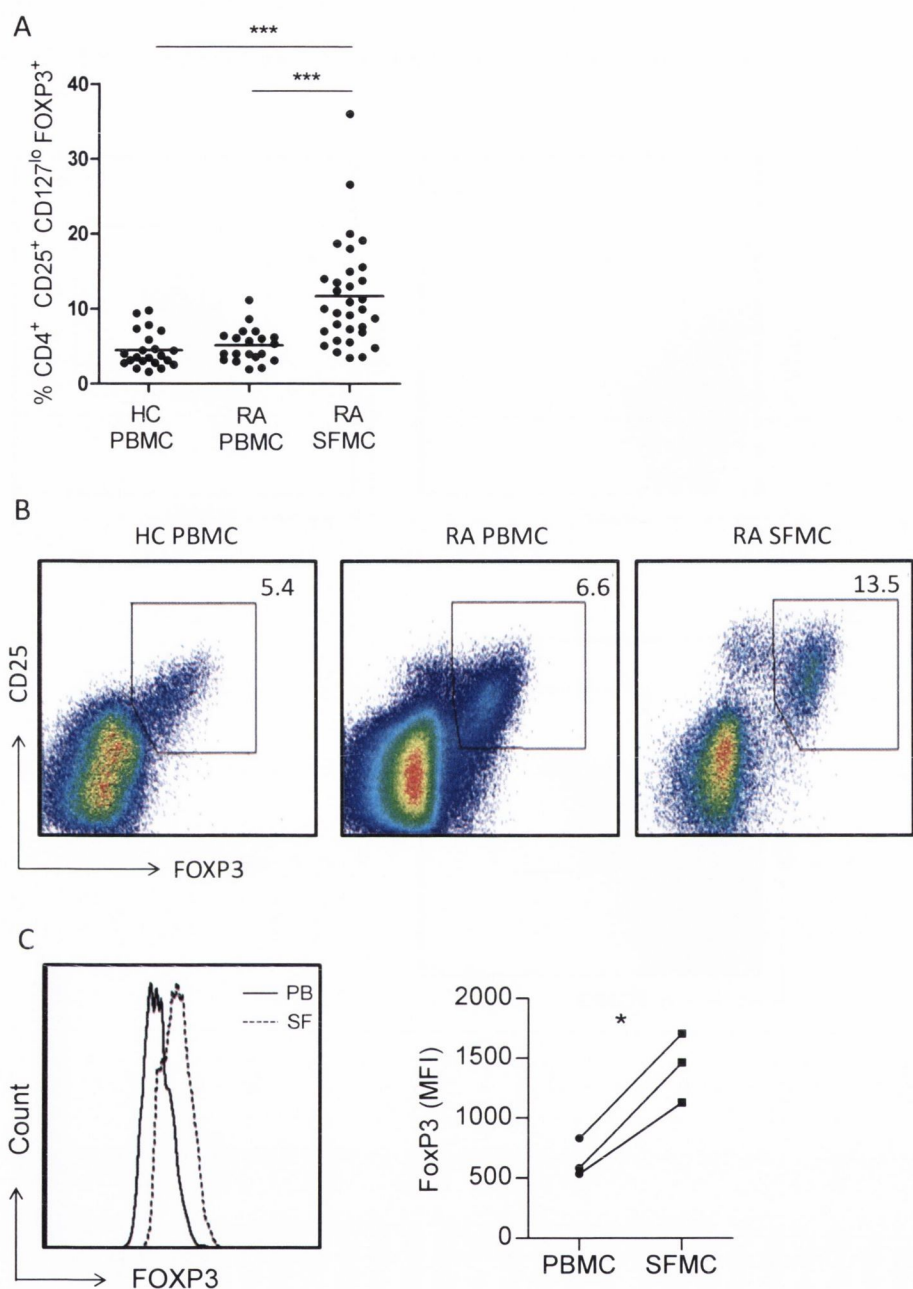


Figure 4.2 The frequency of CD4⁺Treg cells in RA SFMC is significantly increased compared with HC PBMC and RA PBMC and Treg cells from RA SFMC have significantly increased fluorescence intensity for FoxP3 compared with matched PBMC.

RA PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, FOXP3, CD25 and CD127, and analysed by flow cytometry. The graph represents the frequency of Treg cells as a percentage of the total CD4⁺ population in each group (A). Representative FACS plots show the Treg cell populations, gated on CD25⁺ FoxP3⁺ cells (B). Overlaid histograms illustrate the shift in median fluorescence intensity (MFI) for FoxP3 in PB (solid line) compared with SF (dashed line; C). The graph illustrates the expression of FoxP3 (MFI) in PBMC from 3 RA patients compared with matched SFMC. Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test; *** $p < 0.001$. HC $n = 22$, RA PBMC $n = 20$, RA SFMC $n = 32$ (A) or by Student's paired T-test; * $p < 0.05$ (C).

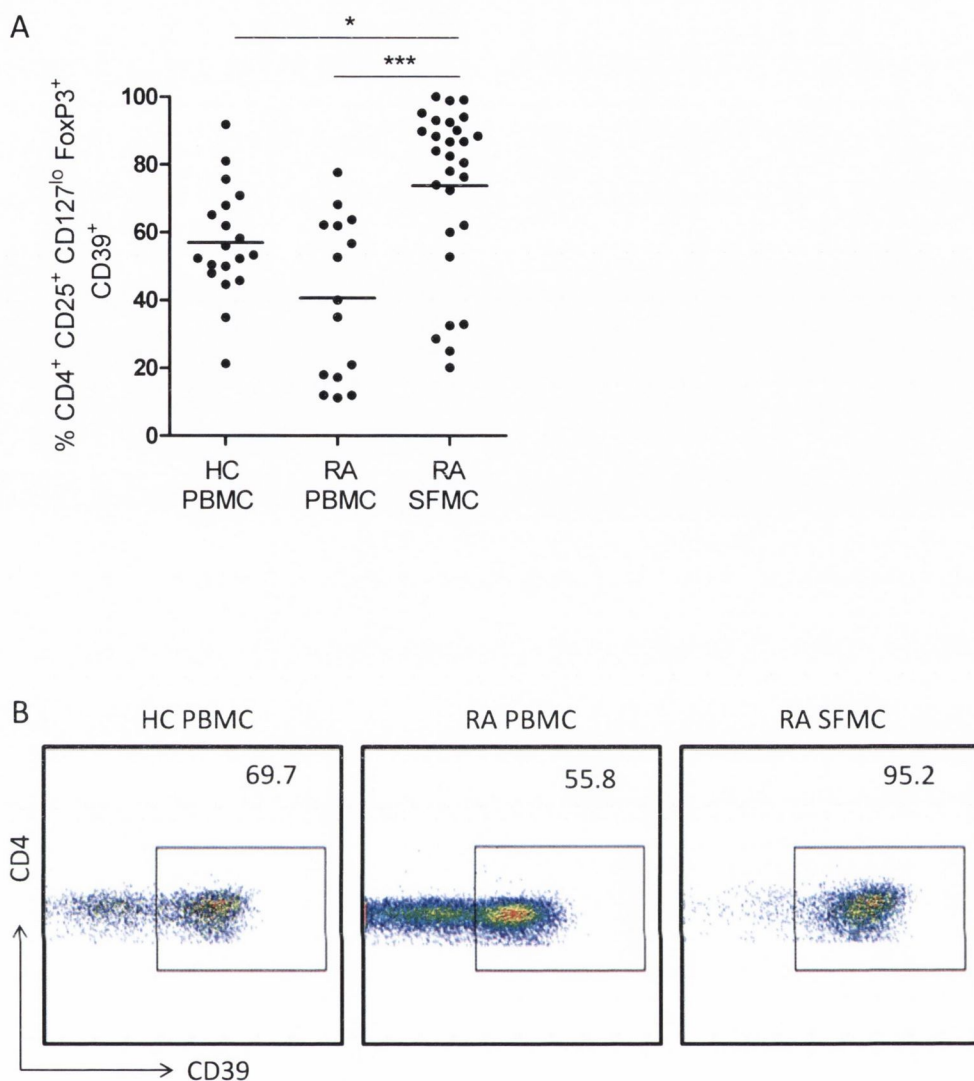


Figure 4.3 The frequency of CD39⁺ Treg cells in RA SFMC is significantly increased compared with HC PBMC and RA PBMC.

RA PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, FOXP3, CD25, CD127 and CD39 and analysed by flow cytometry. The graph represents the frequency of Treg cells expressing CD39 in each group (A). Representative FACS plots show the expression of CD39 in the Treg population (B). Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test; * p < 0.05 *** p < 0.001. HC n=19, RA PBMC n=15, RA SFMC n=28.

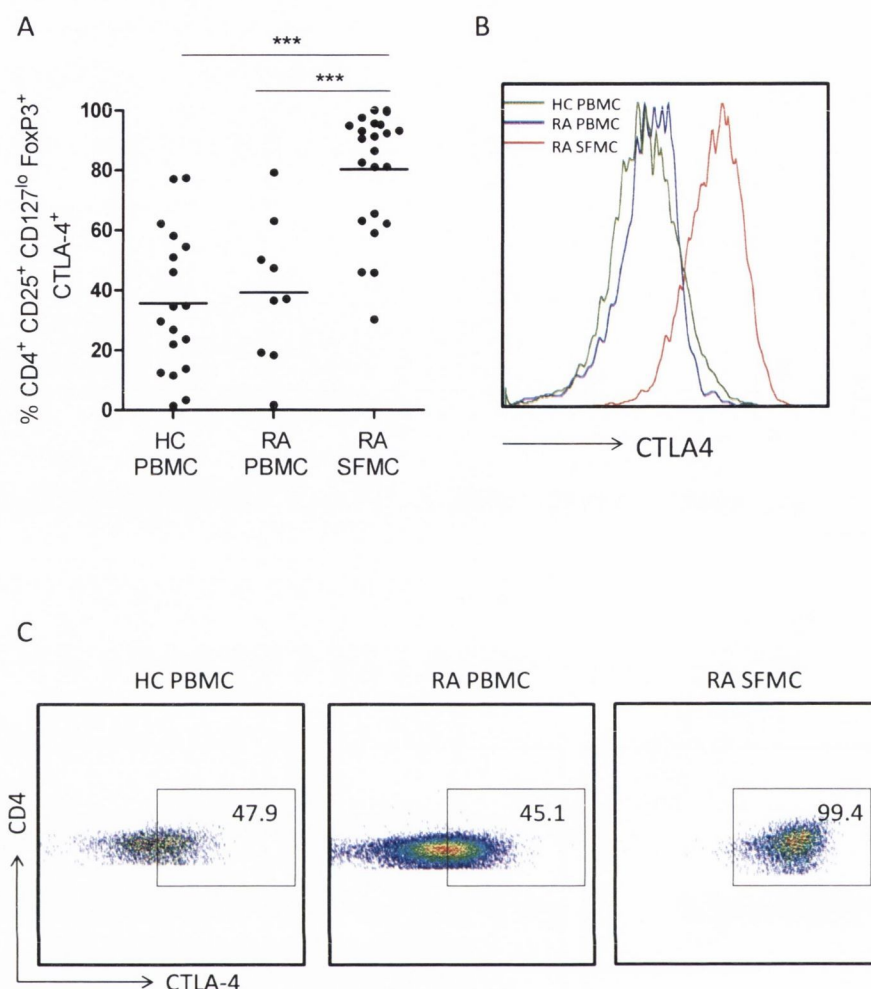


Figure 4.4 The frequency of CTLA-4⁺ Treg cells in RA SFMC is significantly increased compared with HC PBMC and RA PBMC.

RA PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, FOXP3, CD25, CD127 and CTLA-4 and analysed by flow cytometry. The graph represents the frequency of Treg cells expressing CTLA-4 in each group (A). Overlaid histograms compare CTLA-4 expression in each group (B). Representative FACS plots show the expression of CTLA-4 in the Treg population (C). Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test; ** $p < 0.01$. HC $n = 18$, RA PBMC $n = 9$, RA SFMC $n = 23$.

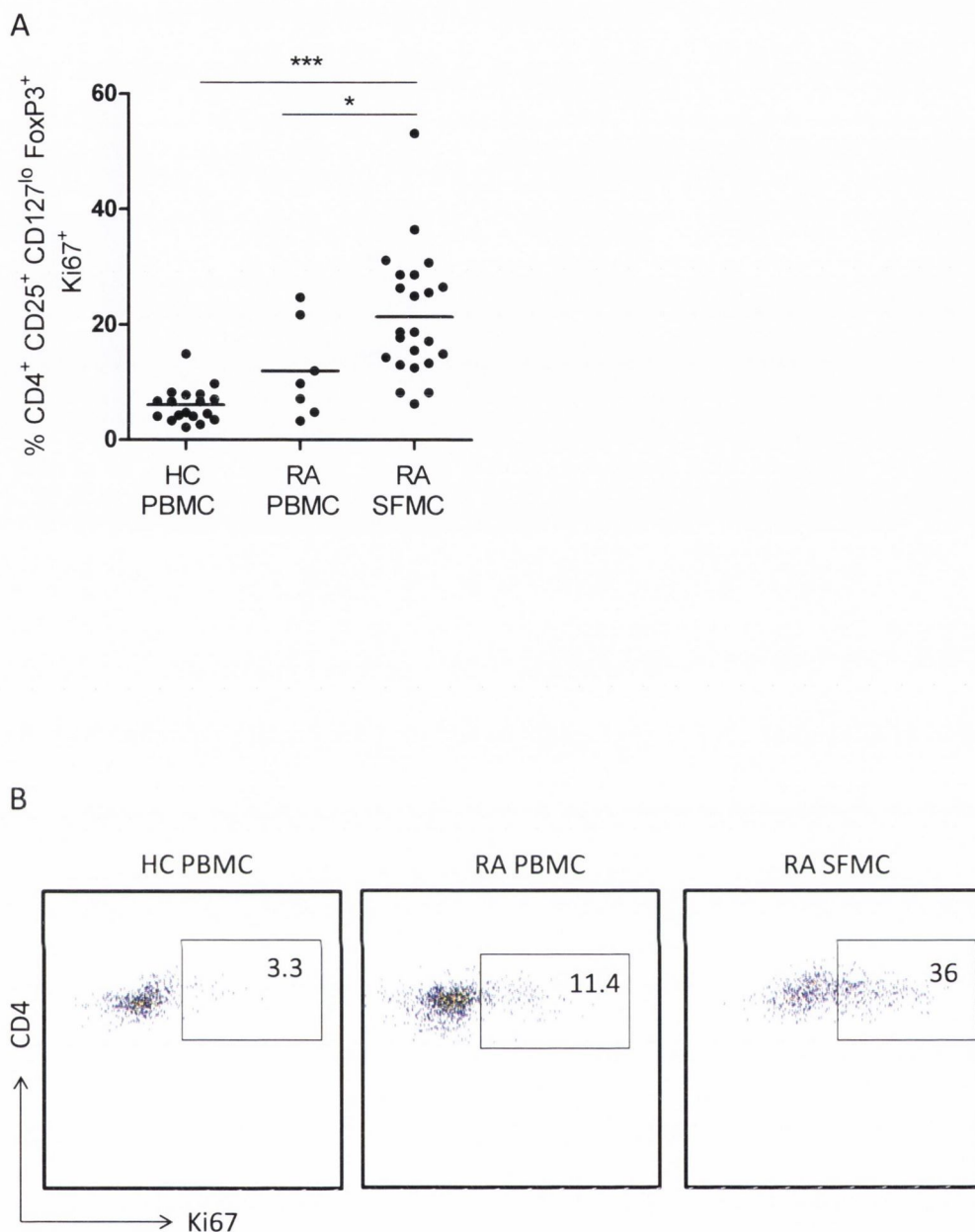


Figure 4.5 The frequency of proliferating Treg cells in RA SFMC is significantly increased compared with HC PBMC and RA PBMC.

RA PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, CD25, CD127, FoxP3 and Ki67 and analysed by flow cytometry. The graph represents the frequency of Treg cells expressing Ki67 in each group (A). Representative FACS plots show the expression of Ki67 in the Treg cell population (B). Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test; * $p < 0.05$ *** $p < 0.001$. HC $n = 18$, RA PBMC $n = 7$, RA SFMC $n = 23$.

4.2.2 Hybrid “Treg-effector” cell function and phenotype in the RA joint.

Traditionally, Treg cells were thought to be anergic cells incapable of producing inflammatory cytokines. However, it has recently emerged that particular subsets of Treg cells are capable of producing pro-inflammatory cytokines in certain microenvironments. Since CD4⁺ effector T cells in RA joint were found to be plastic and polyfunctional (figure 3.6 and 3.7), the possibility that Treg cells in the same microenvironment might also be plastic was considered and investigated.

4.2.2a CD161⁺ Treg cells from RA SFMC are capable of producing pro-inflammatory cytokines.

In order to investigate Treg cell plasticity, RA SFMC, RA PBMC and HC PBMC were stimulated with PMA and ionomycin in the presence of brefeldin A, stained for Treg cell markers, CD161, IFN- γ , IL-2 and IL-17 and analysed by flow cytometry. The stimulation time was reduced, compared to standard cytokine stimulation, to minimize possible non-specific increases in expression of CD25 and FoxP3 caused by PMA stimulation. When Treg cells from RA SF were stimulated, significantly increased frequencies of Treg cells produced IFN- γ compared with Treg cells in RA PBMC (figure 4.6A, left). A trend toward increased IL-17 production from RA SF Treg cells was also observed, however, this was not statistically significant when compared with RA and HC PBMC (figure 4.6A, right). Representative FACS plots illustrate that the majority of Treg cells that produced IL-17 from RA SF were functionally plastic due to presence of an IL-17⁺ IFN- γ ⁺ double positive population (figure 4.6B, right). The frequency of Treg cells expressing CD161 was significantly increased in RA SFMC compared with HC PBMC (figure 4.7A). Expression of CD161 in Treg cells from RA PBMC was slightly elevated compared with HC PBMC; however, this trend was not statistically significant. SPICE analysis demonstrated that CD161⁺ Treg cells from RA SFMC were significantly more active, in terms of cytokine production, than their CD161⁻ Treg cell counterparts- as illustrated by a significantly reduced proportion of grey area within the pie chart (figure 4.7B). Interestingly, IL-17 producing Treg cells, although low in frequency, were highly polyfunctional; producing IL-17 concomitantly with IFN- γ and IL-2, as illustrated by the red pie slice over-arc'd by red, blue and green arcs. In summary, these data demonstrated that Treg cells from RA SFMC were functionally plastic and produced pro-inflammatory cytokines. Additionally, these hybrid pro-inflammatory cytokine producing “Treg-effector” cells were restricted to Treg cells that expressed CD161.

4.2.2b HIF-1 α expression is significantly increased in RA SFMC Treg cells compared with HC Treg cells and CD161⁺ Treg cells exhibit significantly greater HIF-1 α expression compared with autologous CD161⁻ Treg cells.

In order to survive under hypoxic conditions, cells must stabilise the expression of HIF-1 α . Thus in order to ascertain the relative capacity of Treg cells to survive during hypoxia, the expression of HIF-1 α in RA SFMC Treg cells was compared with HC PBMC. Cells were isolated from RA patients during active disease and age and sex matched HC. Cells were stained *ex vivo* for Treg cell markers; CD4, CD25, CD127 and FoxP3, as described in section 4.2.1. Additionally, cells were stained for CD45RO, CD161 and intranuclear HIF-1 α . Memory CD45RO⁺ Th cells were assessed to ensure that HC PBMC results were comparable to RA SFMC, as the vast majority of Th cells in the RA joint are CD45RO⁺ memory cells. The data showed that expression of HIF-1 α was significantly increased on Treg cells in RA SFMC compared with HC PBMC (figure 4.8A). Furthermore, when differential expression of HIF-1 α was assessed on CD161⁺ and CD161⁻ Treg cells, FOXP3⁺ cells co-expressing CD161 expressed significantly more HIF-1 α in both HC PBMC and RA SFMC compared with FOXP3⁺ CD161⁻ cells (figure 4.8B). These data demonstrated that Treg cells in the hypoxic RA joint stabilised their expression of HIF-1 α and therefore would be expected to have a survival advantage in hypoxic conditions. Moreover, CD161⁺ Treg cells exhibited significantly more HIF-1 α expression compared with CD161⁻ Treg cells in both RA SFMC and HC PBMC.

4.2.2c RA SFMC Treg cells are not more prone to apoptosis than HC PBMC Treg cells or autologous RA SFMC responder cells.

Treg cells have previously been shown to be prone to apoptosis (Taams et al., 2001). Given that Treg cells were highly proliferative in the RA joint (figure 4.5), it is plausible that their increased proliferation may be balanced by increased apoptosis in order to maintain homeostasis. To examine this, the expression of Bcl2, an anti-apoptotic protein, was assessed in Treg and responder cells. Cells were isolated from HC and RA patients during active disease and stained for Treg cell markers, CD45RO, CD161 and intranuclear Bcl2. Memory CD45RO⁺ Th cells were assessed to ensure that HC PBMC results were comparable to RA SFMC, as the vast majority of Th cells in the RA joint are CD45RO⁺ memory cells. In HC PBMC, Treg cells exhibited significantly reduced Bcl2 expression compared with responder cells however, no significant difference was observed in the expression of Bcl2 between responder and Treg cells from RA SFMC or in Treg cells from HC and RA SFMCs (figure 4.9A). This was accounted for by the lower expression of Bcl2 in the responder cells from the RA joint compared with the HC. Representative histograms illustrate the increased Bcl2 expression in responder cells compared with Treg cells in HC PBMC

(left) and no difference in the expression of Bcl2 in responder and Treg cells from RA SFMC (right; figure 4.9B). These data suggest that, contrary to the case in healthy control blood, RA SFMC Treg cells are not more prone to apoptosis than their responder cell counterparts. In addition, differential expression of Bcl2 in CD161⁺ and CD161⁻ Treg cells was assessed in HC PBMC and RA SFMC. Interestingly, CD161⁺ Treg cells from RA SFMC expressed significantly more Bcl2 than their CD161⁻ Treg cell counterparts; however, in the HC the difference in Bcl2 expression in CD161⁺ and CD161⁻ Treg cells was not significantly different (figure 4.9C). In summary, these data showed that Treg cells in RA SFMC are not more prone to apoptosis compared with HC PBMC Treg cells and RA SFMC responder cells. Furthermore, CD161⁺ Treg cells in RA SFMC expressed significantly more Bcl2 than their CD161⁻ Treg cell counterparts, which suggests that CD161⁺ hybrid “Treg-effector” cells may have a survival advantage over anti-inflammatory CD161⁻ Treg cells.

Taken together these data indicate that CD161⁺ Treg cells, which are enriched in the RA joint, mirror their CD161⁺ Th effector counterparts in terms of enhanced cytokine polyfunctionality and expression of Bcl2 and HIF-1 α , which will likely provide a survival advantage in the hypoxic RA joint.

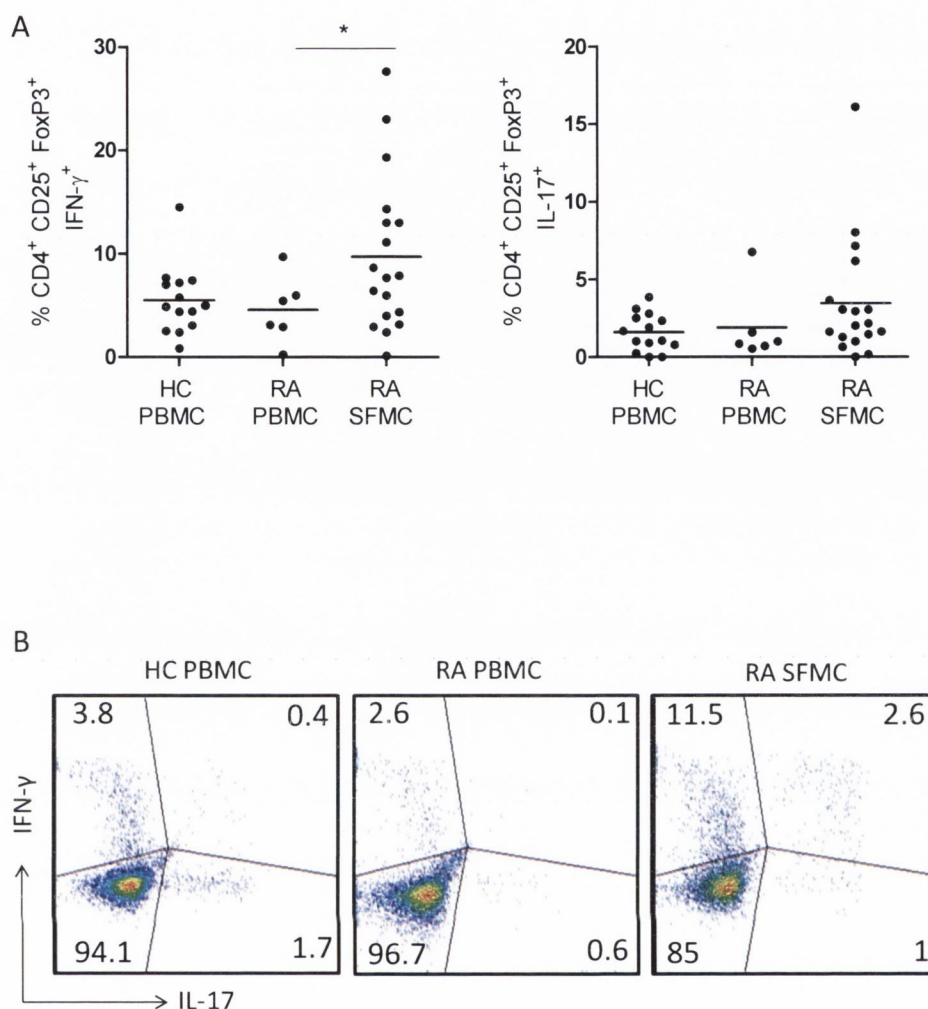


Figure 4.6 The frequency of Treg cells producing IFN-γ is significantly increased in RA SFMC compared with HC PBMC and RA PBMC.

RA PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stimulated with PMA and ionomycin, in the presence of brefeldin A, stained with fluorochrome-conjugated antibodies specific for CD3, CD8, FoxP3, CD25, IFN-γ and IL-17 and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD3⁺ CD8⁻ cells were gated to identify the CD4⁺ Th cell population. Treg cells were then gated as previously shown (figure 4.1). The graphs represent the frequency of Treg cells expressing cytokine in each group (A). Representative FACS plots show the expression and co-expression of IFN-γ and IL-17 in the Treg population (B). Statistical differences in mean frequency between the groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test; * p<0.05. (A) HC n=14, RA PBMC n=6, RA SFMC n=18.

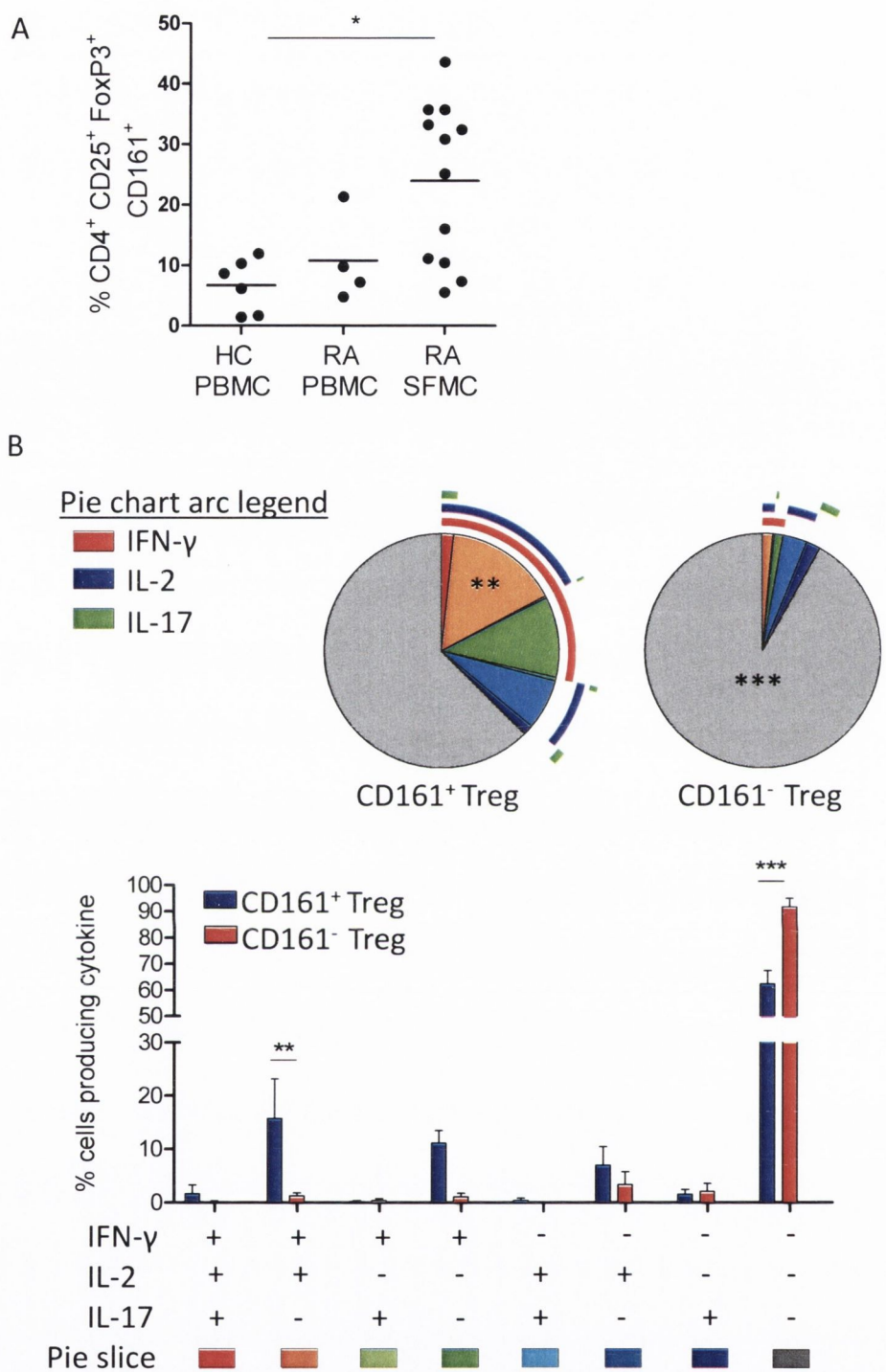


Figure 4.7 The frequency of Treg cells expressing CD161 are increased in RA SFMC and have significantly increased cytokine expression compared with CD161⁻ Treg cells.

PBMC and SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A, stained with fluorochrome-conjugated antibodies specific for CD3, CD8, CD45RO, CD161, FOXP3, IFN- γ , IL-2 and IL-17 and analysed by flow cytometry. (A) CD161 expression in CD4⁺ CD25⁺ FOXP3⁺ Treg cells from HC PBMC (n=6), RA PBMC (n=4) and RA SFMC (n=12). (B) SPICE analysis shows the production of cytokines from CD161⁺ and CD161⁻ Treg cells from RA SFMC (n=3). The bar graph shows statistical differences in areas of the pie slice. Statistical differences were determined by one way ANOVA with Tukey's multiple comparison post tests (A); *p<0.05, or by a two-way ANOVA with a Bonferroni post test (B); **p<0.01, ***p<0.001.

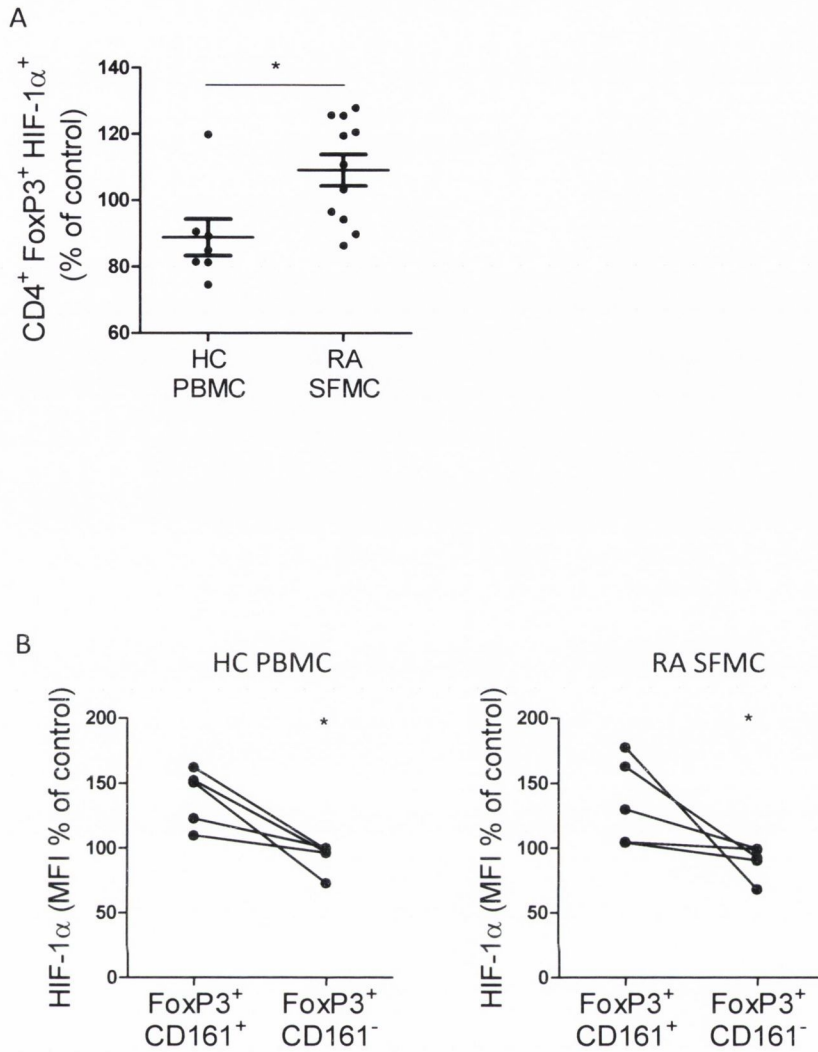


Figure 4.8 The frequencies of Treg cells expressing HIF-1α are increased in RA SFMC and CD161⁺ Treg cells have significantly increased expression of HIF-1α compared with CD161⁻ Treg cells.

SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, CD45RO, CD161, FOXP3 and HIF-1α and analysed by flow cytometry. (A) HIF-1α expression in CD4⁺ FOXP3⁺ T cells from HC PBMC (n=7) and RA SFMC (n=11). (B) HIF-1α expression in CD4⁺ FOXP3⁺ CD161⁺ T cells compared with CD4⁺ FOXP3⁺ CD161⁻ T cells from HC PBMC (left, n=5) and RA SFMC (right, n=5). Values are expressed as percentage of control (i.e. HIF-1α MFI value of the total CD4⁺ for A and CD4⁺ FOXP3⁺ gate for B). Statistical differences were determined by unpaired T-tests; *p<0.05 .

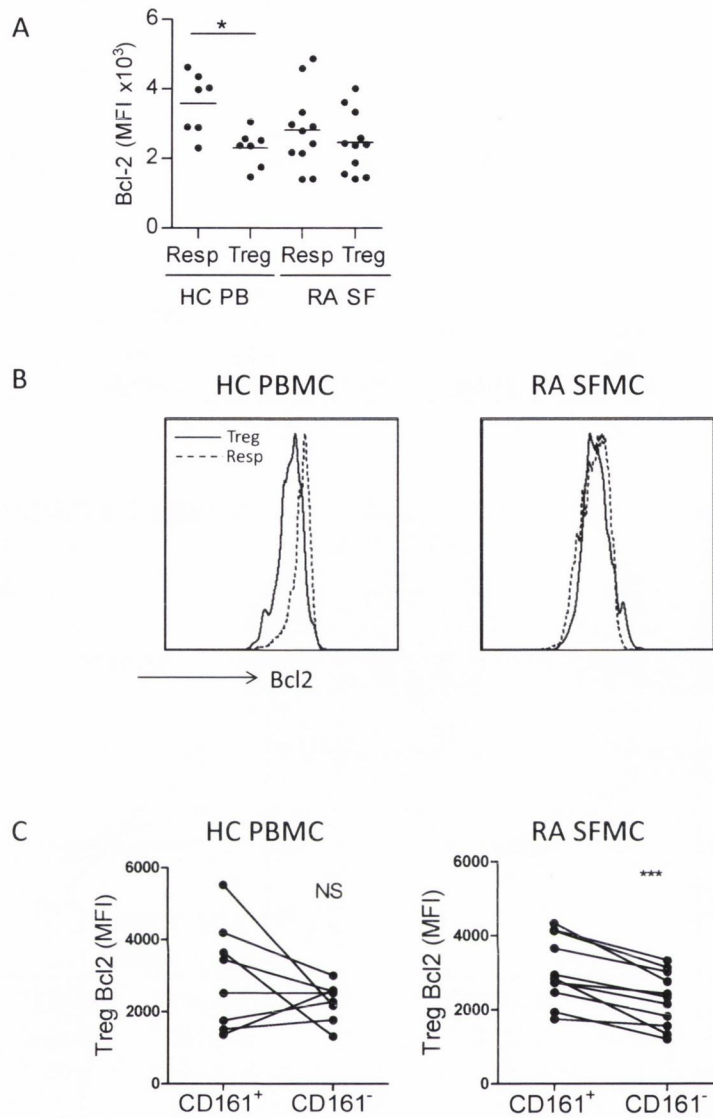


Figure 4.9 The frequency of memory Treg cells expressing Bcl2 in RA SFMC is not significantly different to autologous responder cells or Treg cells from HC PBMC.

SFMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, CD45RO, FOXP3 and Bcl2 and analysed by flow cytometry. Lymphocytes were gated on the basis of forward and side scatter. Dead cells and doublets were excluded and CD45RO⁺ CD4⁺ cells were gated before gating on FoxP3⁺ and FoxP3⁻ cells to analyse Bcl2 expression. (A) Bcl2 expression (MFI) in CD4⁺ CD45RO⁺ FOXP3⁺ Treg cells and CD4⁺ CD45RO⁺ FOXP3⁻ responder cells from HC PBMC (n=7) and RA SFMC (n=11). (B) Representative histograms illustrate Bcl2 expression in responder and Treg cells from HC PBMC (left) and RA SFMC (right). (C) Bcl2 expression (MFI) in CD161⁺ and CD161⁻ Treg cells from HC PBMC (n=8) and RA SFMC (n=11). Statistical differences were determined by paired T-tests with two-tailed p-values; *p<0.05, ***p<0.001, NS= not significant.

4.2.3 Treg cells from RA SFMC are functionally suppressive but significantly less suppressive than Treg cells from HC PBMC.

Treg cells are enriched in the RA joint and display a highly active and suppressive phenotype (section 4.2.1). Despite this, inflammation persists in the RA joint. This raised the question of whether or not the Treg cells are functionally impaired or if the nature of the effector cells in the joint renders them less susceptible to Treg cell mediated suppression. The next experiments were designed to assess the ability of Treg cells from RA SFMC and HC PBMC to suppress autologous responder cells (the Treg-depleted fraction of CD4⁺ T cells).

4.2.3a The flow cytometry-based suppression assay

The suppression of responder cell proliferation and pro-inflammatory cytokine production was assessed using a flow cytometry based suppression assay (see chapter 2, section 2.7). Obtaining a population of highly purified Treg cells is challenging because FOXP3, the most reliable marker of Treg cells, is intranuclear, therefore, it cannot be used to sort live cells. Instead, a panel of extracellular markers, including CD4, CD25 and CD127, was used. Sorting CD4⁺ CD25⁺ CD127^{lo} cells consistently enriches Treg cells, however, when this population is stained for the presence of FOXP3, the percentages vary between 60-90% FOXP3⁺ cells (data not shown). CD39 expression was significantly increased in RA SF Treg cells, so, in order to make the HC and RA suppression assays directly comparable, CD39 was added to the Treg cell sorting panel. Interestingly, the addition of CD39 greatly improved the fidelity of the Treg cell sorting panel to the expression of FOXP3 (figure 4.10A). The representative FACS plots show that the Treg-depleted responder population contained no appreciable CD25⁺ FOXP3⁺ Treg cells (figure 4.10A, left). Treg cells sorted by CD4⁺ CD25⁺ CD127^{lo} CD39⁺ were over 95% FOXP3⁺ (figure 4.10A, middle) whereas Treg cells sorted by CD4⁺ CD25⁺ CD127^{lo} CD39⁻ have inconsistent frequencies of FOXP3 expression; in this example only 33% of sorted cells expressed FOXP3 (figure 4.10A, right). FACS sorted responder cells were labelled with CTV and cultured alone or co-cultured with equal numbers of unlabelled responder cells (as a control) or equal numbers of Treg cells, in the presence of anti-CD3 and irradiated APC (figure 4.10B). Responder cells co-cultured with unlabelled responder cells displayed no noteworthy suppression of proliferation. CD39⁺ Treg cells effectively suppressed responder cell proliferation from 84.9% to 35.4% (overall suppression of 58.3%) compared with CD39⁻ Treg cells which, despite being less pure, suppressed responder cell proliferation from 84.9% to 64.1% (an overall suppression of 24.5%). On the basis of these findings, all further suppression assays shown in this study were carried out with CD39⁺ Treg cells. Suppression could be attributed directly to the suppressive effects of Treg cells, not by indirect competition for cell culture resources, as shown by the responder cells

co-cultured with unlabelled responder cells. In summary, the flow cytometry based suppression assay described above was found to be an effective method of assessing Treg cell function and allowed for the measurement of both effector cell proliferation and cytokine production. Importantly, it could distinguish between responder cell and Treg cell cytokine production.

4.2.3b RA SFMC Treg cells suppress responder cell proliferation and pro-inflammatory cytokine production but are significantly less suppressive than HC PBMC Treg cells.

In order to examine the suppressive function of Treg cells *in vitro*, cryopreserved RA SFMC and HC PBMC CD39⁺ Treg cells and Treg-depleted responder cells (CD4⁺ CD25⁻) were sorted by FACS. Responder cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated, allogeneic APC. Cells were analysed by flow cytometry and proliferation was measured by CTV dilution. The data indicates that both RA SFMC Treg cells and HC PBMC Treg cells were capable of suppressing autologous responder cell proliferation (figure 4.11). However, the extent of this suppression varied between HC and RA donors; representative histograms show that 93.5% of HC responder cells had proliferated and this was reduced to 30.1% in the presence of the HC Treg. This was an overall suppression of 67.8% (figure 4.11A). 81.6% of RA responder cells had proliferated and this was reduced to 50.9% in the presence of the RA Treg, yielding an overall suppression of 37.6% (figure 4.11B). When the data was collated from 11 HC PBMC donors and 8 RA SFMC donors, it was evident that both HC and RA Treg cells significantly reduced the proliferation of autologous responder cells (figure 4.11C and D). Notably, when the percent suppression was calculated, the data demonstrated that the extent of Treg cell mediated suppression of responder cells was significantly reduced in RA SFMC compared with HC PBMC (figure 4.11E).

Next, the suppression of pro-inflammatory cytokines (IFN- γ , TNF, GM-CSF and IL-17) was examined by flow cytometry and confirmed by ELISA. After 5 days in culture, cells were restimulated with PMA and ionomycin in the presence of brefeldin A to determine the suppression of cytokine production. Cells were intracellularly stained with fluorochrome-conjugated antibodies specific for IFN- γ , TNF, GM-CSF and IL-17. Cytokine production was examined in proliferated (CTV^{lo}) cells, while cytokine induced by PMA stimulation of undivided cells was excluded from the analysis. 34.1% of HC responder cells had proliferated and produced IFN- γ (figure 4.12A, upper panel). This frequency was effectively suppressed in the presence of the HC Treg cells to 2.4% (93% suppression compared with control). 65.3% of RA responder cells had proliferated and produced IFN- γ and, in the presence of the Treg cell, this was effectively suppressed to 31.6% (51.6% suppression compared with control; figure 4.12A, lower panel). The

frequencies of IFN- γ ⁺CTV^{lo} responder cells cultured alone and co-cultured with Treg cells were collated from 8 healthy donors and 5 RA patients (figure 4.12B and C, respectively). Both HC and RA Treg cells significantly suppressed autologous responder cells, however, when the percent suppression was calculated, it was evident that HC Treg cells were significantly superior suppressors of responder cell IFN- γ compared with RA Treg cells (figure 4.12D). The concentration of IFN- γ , accumulated in the supernatants over the 5 day culture, was assessed by ELISA (figure 4.12E). The representative graph shows that the production of IFN- γ by the responder cell was significantly suppressed by Treg cells in both HC and RA donors. However, the concentration of IFN- γ was significantly increased in the supernatant of RA responder cells co-cultured with Treg cells compared with the concentration of IFN- γ in the supernatant of HC responder cells co-cultured with Treg cells, confirming the flow cytometry data suggesting that RA Treg cells are less capable of suppressing autologous IFN- γ production than HC Treg cells.

TNF production was examined in the same way as IFN- γ (figure 4.13). 67.9% of HC responder cells were proliferating and produced TNF (figure 4.13A, upper panel). This frequency was effectively suppressed to 5.2% in the presence of the HC Treg cells (92.3% suppression compared with control). 64.9% of RA responder cells had proliferated and produced TNF and this was effectively suppressed to 30.1% in the presence of the Treg cell (53.6% suppression compared with control; figure 4.13A, lower panel). The frequencies of TNF⁺CTV^{lo} responder cells cultured alone and co-cultured with Treg cells were collated from 7 healthy donors and 4 RA patients (figure 4.13B and C, respectively). HC cells significantly suppressed autologous responder cells and RA Treg cell exhibited a trend of suppression. When the percent suppression was calculated, it was evident that HC Treg cells were significantly superior suppressors of responder cell TNF production compared with RA Treg cells (figure 4.13D). The concentration of TNF, accumulated over the 5 day culture, was assessed by ELISA (figure 4.13E). The graph shows that the concentration of TNF was significantly suppressed in both HC and RA donors. The concentration of TNF was increased in the supernatant of RA responder cells co-cultured with Treg cells compared with HC; however, this was not statistically significant.

The suppression of GM-CSF was analysed in a similar manner (figure 4.14). 65% of HC responder cells had proliferated and produced GM-CSF (figure 4.14A, upper panel). This frequency was effectively suppressed to 1.6% in the presence of the HC Treg cells (97.5% suppression compared with control). 51.9% of RA responder cells had proliferated and produced GM-CSF and this was effectively suppressed to 24.1% in the presence of the Treg cell (53.6% suppression compared with control; figure 4.14A, lower panel). The frequencies of GM-CSF⁺CTV^{lo} responder

cells cultured alone and co-cultured with Treg cells were collated from 9 healthy donors and 5 RA patients (figure 4.14B and C, respectively). HC cells significantly suppressed autologous responder cells while RA Treg cells exhibited a trend of suppression. When the percent suppression was calculated, it demonstrated that HC Treg cells were superior suppressors of responder cell GM-CSF compared with RA Treg cells; however, this trend did reach statistical significance due to a high degree of variability (figure 4.14D). The concentration of GM-CSF, accumulated over the 5 day culture, was assessed by ELISA (figure 4.14E). The representative graph shows that the concentration of GM-CSF was significantly suppressed in HC and RA donors. The concentration of GM-CSF was increased in the supernatant of RA responder cells co-cultured with Treg cells (approximately 520 pg/ml) compared with HC responder cells co-cultured with Treg cells (approximately 350 pg/ml); however, this was not statistically significant.

Finally, the suppression of IL-17 production was analysed (figure 4.15). 2% of HC responder cells had proliferated and produced IL-17 (figure 4.15A, upper panel). This frequency was effectively suppressed to 0.4% in the presence of the HC Treg cells (80% suppression compared with control). 4.6% of RA responder cells had proliferated and produced IL-17 and this was effectively suppressed to 1.5% in the presence of the Treg cell (67.4% suppression compared with control; figure 4.15A, lower panel). The frequencies of IL-17⁺ CTV^{lo} responder cells cultured alone and co-cultured with Treg cells were collated from 7 healthy donors and 4 RA patients (figure 4.15B and C, respectively). Both HC and RA Treg cells significantly suppressed autologous responder cell production of IL-17. When the percent suppression of IL-17 was calculated, no difference was observed between HC and RA donor. Interestingly, the ability of HC cells to suppress the production of IL-17 was highly variable (figure 4.15D). The concentration of IL-17, accumulated over the 5 day culture, was assessed by ELISA (figure 4.15E). The representative graph shows that the concentration of IL-17 was significantly suppressed in HC responder cells but not in RA responder cells. However, the overall concentrations of IL-17 were low in the RA donor.

In summary, RA SFMC Treg cells significantly suppressed proliferation, IFN- γ and IL-17 production by autologous responder cells and exhibited a trend toward suppression of TNF and GM-CSF. However, when the extent of RA SFMC Treg cell mediated suppression was compared with HC PBMC Treg cell mediated suppression, the data showed that RA SFMC Treg cells were significantly less suppressive than HC Treg cells in terms of their ability to suppress proliferation, IFN- γ and TNF. A trend towards reduced suppression of GM-CSF was observed in RA SFMC Treg

cells compared with HC. Interestingly, the suppression of IL-17 was not reduced in RA SFMC Treg cells compared with HC Treg cells.

4.2.3c Indirect suppression assays indicate that Treg cells in RA SFMC are capable of suppressing IFN- γ and TNF production at physiological frequencies.

Having established that Treg cells from RA SFMC were capable of suppressing autologous responder cells but exhibited reduced suppressive capacity compared with HC Treg cells, the question remained as to whether Treg cell mediated suppression was being exerted in the RA joint where the Treg cells are present at lower frequencies in comparison to the *in vitro* set-up. In order to evaluate this, an indirect suppression assay was established. This assay compared FACS sorted Treg-depleted responder cells to total CD4⁺ T cells, which contained the population of Treg cells at their physiological frequency. Cells were stimulated *in vitro* with anti-CD3 for 5 days in the presence of irradiated APC and the concentrations of pro-inflammatory cytokines in the supernatants were assessed by ELISA (figure 4.16).

IFN- γ production was significantly reduced in the total CD4⁺ T cell population in RA SFMC compared with Treg-depleted responder cells (figure 4.16, top graph). The concentrations of TNF produced were reduced in total CD4⁺ T cells compared with Treg-depleted responder cells; however, this trend did not reach statistical significance (figure 4.16, middle graph). The Treg cells present in the total CD4⁺ T cell population in RA SFMC did not suppress IL-17 production compared with the Treg-depleted fraction (figure 4.16, bottom graph). In fact, the concentrations of IL-17 produced were slightly increased in the total CD4⁺ T cell population. This suggests Treg cells are unable to constrain IL-17 production in the RA joint or that Treg cells from RA SFMC may be producing IL-17 and contributing to the concentrations found in the supernatants. However, it is important to note that the overall concentrations of IL-17 detected in the supernatants of RA SFMC responder and total CD4 cells were at the lower limit of detection in 3 out of 4 experiments shown, therefore, differences between total CD4⁺ T cells and Treg-depleted responder cells may not be real.

In summary, the indirect suppression assay indicated that Treg cells from the RA joint were functionally suppressive at physiological frequencies, as shown by reduced IFN- γ and TNF production in total CD4 T cell population compared with Treg-depleted responder cells. IL-17 production, however, was not reduced which suggests that Treg cells in this system may have produced IL-17 and masked its suppression.

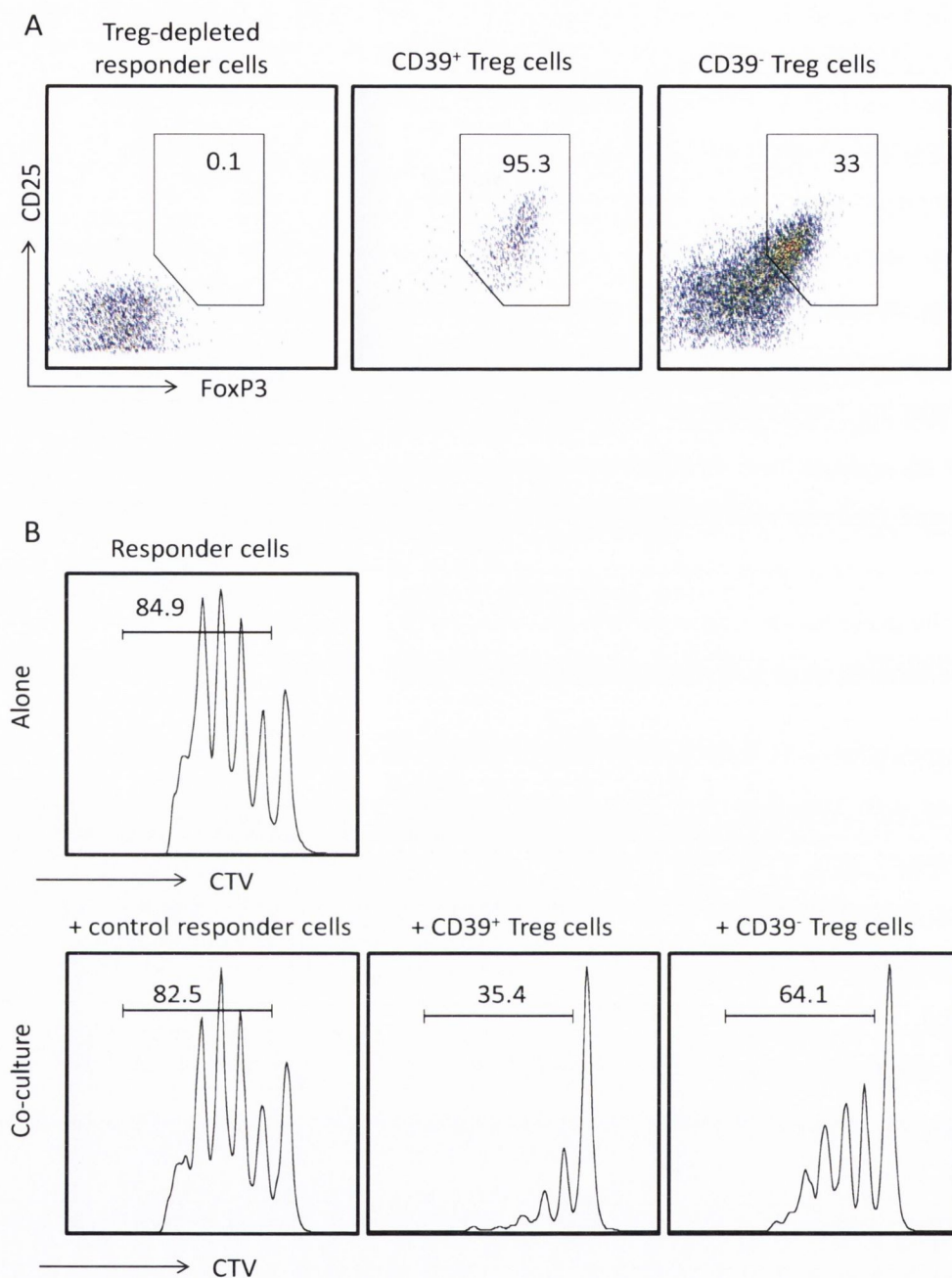


Figure 4.10 Optimisation of the flow cytometry based suppression assay.

PBMC were isolated from healthy donors. CD4⁺ T cells were purified from PBMC by MACS. CD4⁺ CD25⁻ (Treg depleted) responder cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ and CD4⁺ CD25⁺ CD127^{lo} CD39⁻ Treg cells were sorted by FACS. Purified populations were stained with fluorochrome-conjugated antibodies specific for CD25 and FOXP3 and analysed by flow cytometry (A). Responder cells were labelled with CFSE and cultured alone, co-cultured with equal numbers of Treg cells or co-cultured with equal numbers of unlabelled responder cells (as a control) for 5 days in the presence of anti-CD3 and irradiated, allogeneic APC (B). Cells were analysed by flow cytometry; CTV⁺ cells were gated and proliferation was quantified by CTV dilution.

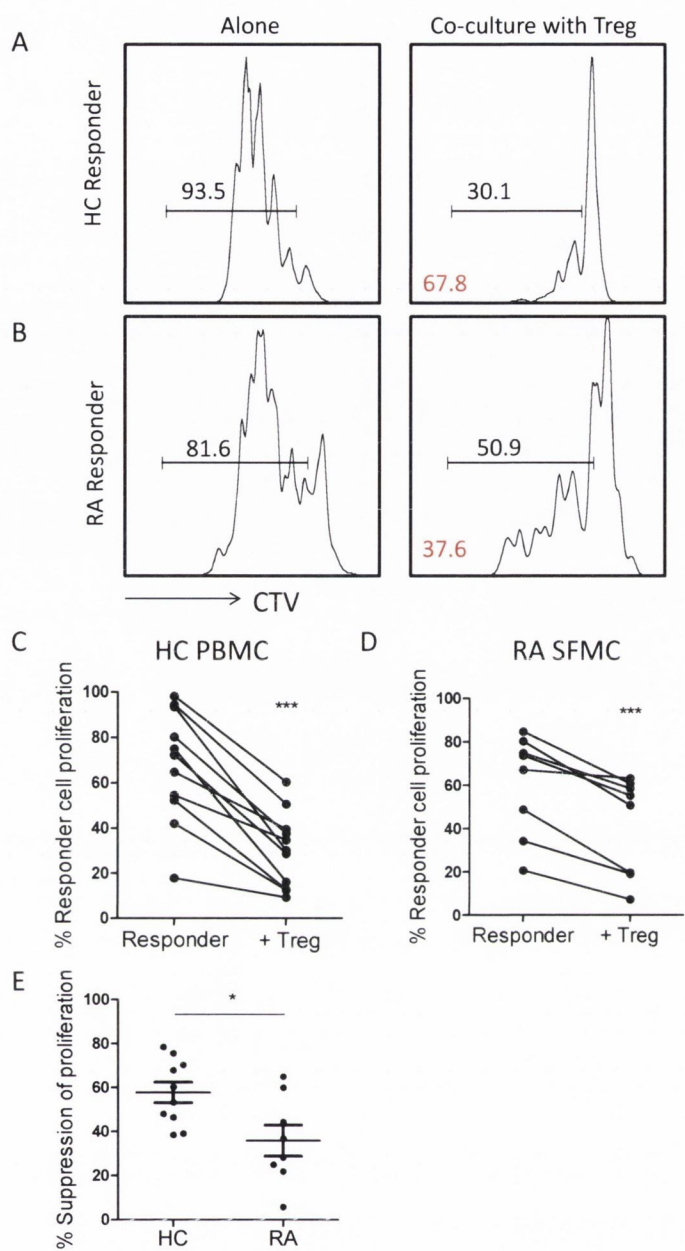


Figure 4.11 RA SFMC derived Treg cells effectively suppress autologous responder cells but this suppression is significantly reduced compared with HC Treg cell suppression of autologous responder cells.

SFMC were isolated from RA patients with active disease and PBMC were isolated from healthy donors. $CD4^+ CD25^-$ (Treg depleted) responder cells and $CD4^+ CD25^+ CD127^{lo} CD39^+$ Treg cells were sorted by FACS. Responder cells were labelled with CTV and cultured alone or co-cultured with equal numbers of autologous Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated, allogenic APC. Cells were analysed by flow cytometry, $CTV^+ CFSE^-$ cells were gated and proliferation was quantified by CTV dilution. Representative histograms from HC PBMC (A) and RA SFMC (B) showing the frequency of cell proliferation (black) and the percent suppression (red) compared with control cells cultured alone. The percent proliferation of HC (C) and RA (D) responder cells cultured alone and co-cultured with Treg cells. Each pair of data points represents a single independent experiment (HC n=11, RA n=8). Statistical significance was determined using a paired T-test with two-tailed p-values; ***p<0.001. (E) Percent Treg cell mediated suppression of responder cell proliferation in HC and RA cells. Statistical significance was determined using an unpaired T-test with two-tailed p-values; *p<0.05.

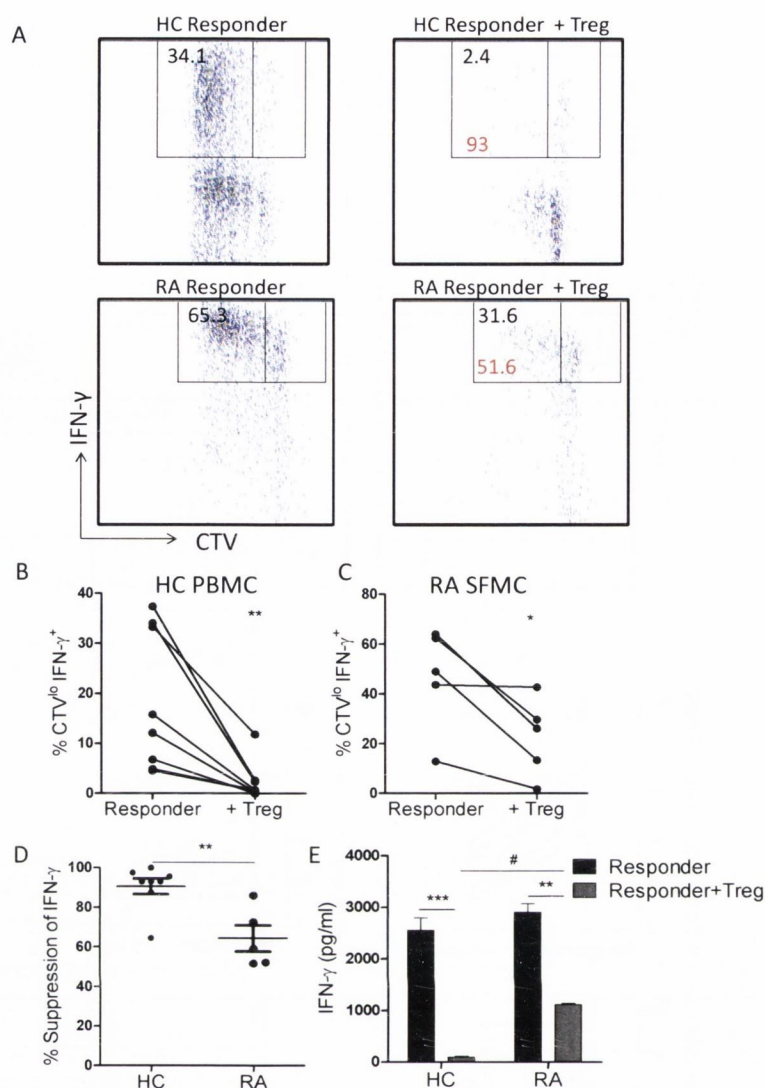


Figure 4.12 RA SFMC derived Treg cells effectively suppress IFN- γ produced by autologous responder cells although their suppressive capacity is significantly reduced compared with HC Treg cells.

SFMC were isolated from RA patients with active disease and PBMC were isolated from healthy donors. CD4⁺ CD25⁻ responder cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Responder cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated, allogenic APC. Supernatants were harvested and cells were restimulated with PMA/ionomycin in the presence of brefeldin A, intracellularly stained for IFN- γ and analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated and expression of IFN- γ was assessed. (A) Representative FACS plots showing the frequency of IFN- γ produced from proliferating cell population (number shown in black) and the percent suppression (red) compared with control cells cultured alone. The frequency of IFN- γ produced by HC (B) and RA (C) responder cells cultured alone and co-cultured with Treg cells. Each pair of data points represents a single independent experiment (HC n=8, RA n=5). (D) Percent Treg cell mediated suppression of IFN- γ produced by HC and RA responder cells. (E) The concentrations of IFN- γ present in the supernatants were measured by ELISA. Statistical significance was determined using a paired T-test with two-tailed p-values (B and C); *p<0.05, **p<0.01, an unpaired T-test with two-tailed p-values (D); **p<0.01 and a two-way ANOVA with Bonferroni post-tests (E); #p<0.05, **p<0.01, ***p<0.001.

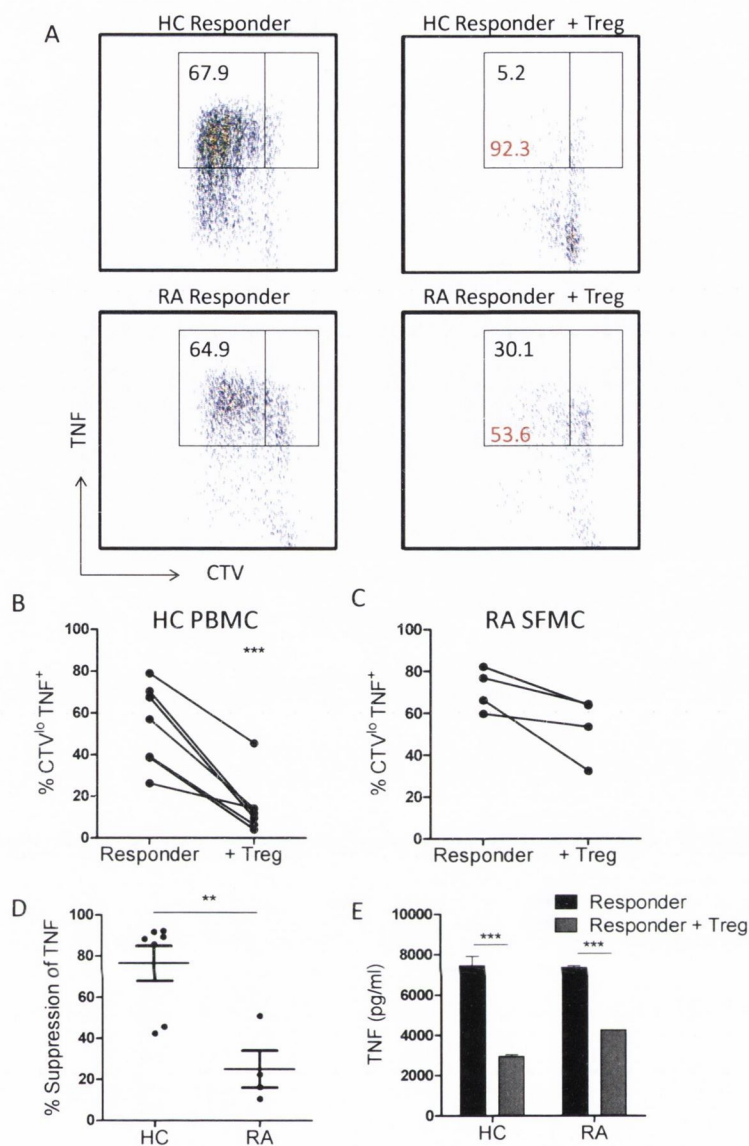


Figure 4.13 RA SFMC derived Treg cells effectively suppress TNF produced by autologous responder cells although their suppressive capacity is significantly reduced compared with HC Treg cells.

SFMC were isolated from RA patients with active disease and PBMC were isolated from healthy donors. CD4⁺ CD25⁺ responder cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Responder cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated, allogenic APC. Supernatants were harvested and cells were restimulated with PMA/ionomycin, intracellularly stained for TNF and analysed by flow cytometry; CTV⁺ CFSE⁺ cells were gated and expression of TNF was assessed. (A) Representative FACS plots showing the frequency of TNF produced from the proliferating cell population (number shown in black) and the percent suppression (red) compared with control cells cultured alone. The frequency of TNF produced by HC (B) and RA (C) responder cells cultured alone and co-cultured with Treg cells. Each pair of data points represents a single independent experiment (HC n=7, RA n=4). (D) Percent Treg cell mediated suppression of TNF produced by HC and RA responder cells. (E) The concentrations of TNF present in the supernatants were measured by ELISA. Statistical significance was determined using a paired T-test with two-tailed p-values (B and C); ***p<0.001, an unpaired T-test with two-tailed p-values (D); **p<0.01 and a two-way ANOVA with Bonferroni post-tests (E); ***p<0.001.

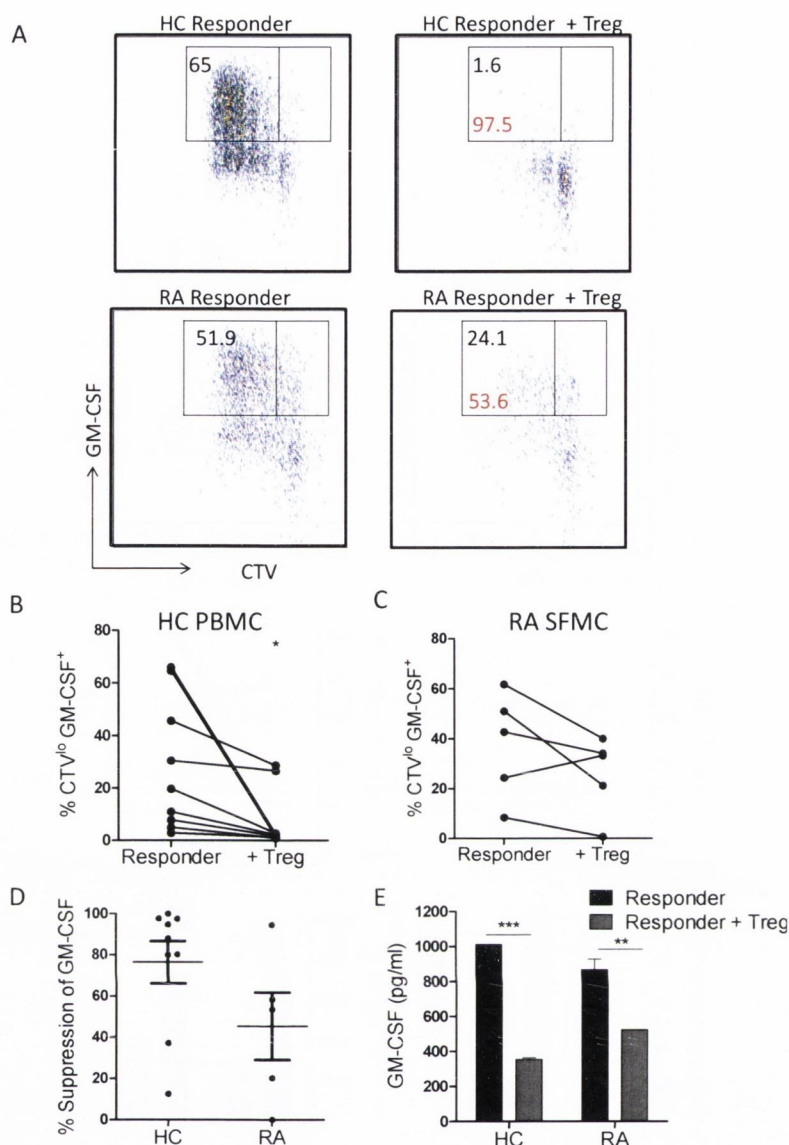


Figure 4.14 RA SFMC derived Treg cells effectively suppress GM-CSF produced by autologous responder cells although their suppressive capacity is reduced compared with HC Treg cells.

SFMC were isolated from RA patients with active disease and PBMC were isolated from healthy donors. CD4⁺ CD25⁻ responder cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Responder cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated, allogenic APC. Supernatants were harvested and cells were restimulated with PMA/ionomycin in the presence of brefeldin A, intracellularly stained for GM-CSF and analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated and expression of GM-CSF was assessed. (A) Representative FACS plots showing the frequency of GM-CSF produced from the proliferating cell population (number shown in black) and the percent suppression (red) compared with control cells cultured alone. The frequency of GM-CSF produced by HC (B) and RA (C) responder cells cultured alone and co-cultured with Treg cells. Each pair of data points represents a single independent experiment (HC n=9, RA n=5). (D) Percent Treg cell mediated suppression of GM-CSF produced by HC and RA responder cells. (E) The concentrations of GM-CSF present in the supernatants were measured by ELISA. Statistical significance was determined using a paired T-test with two-tailed p-values (B and C); *p<0.05, an unpaired T-test with two-tailed p-values (D); however, the data was not statistically significant, and a two-way ANOVA with Bonferroni post-tests (E); **p<0.01, ***p<0.001.

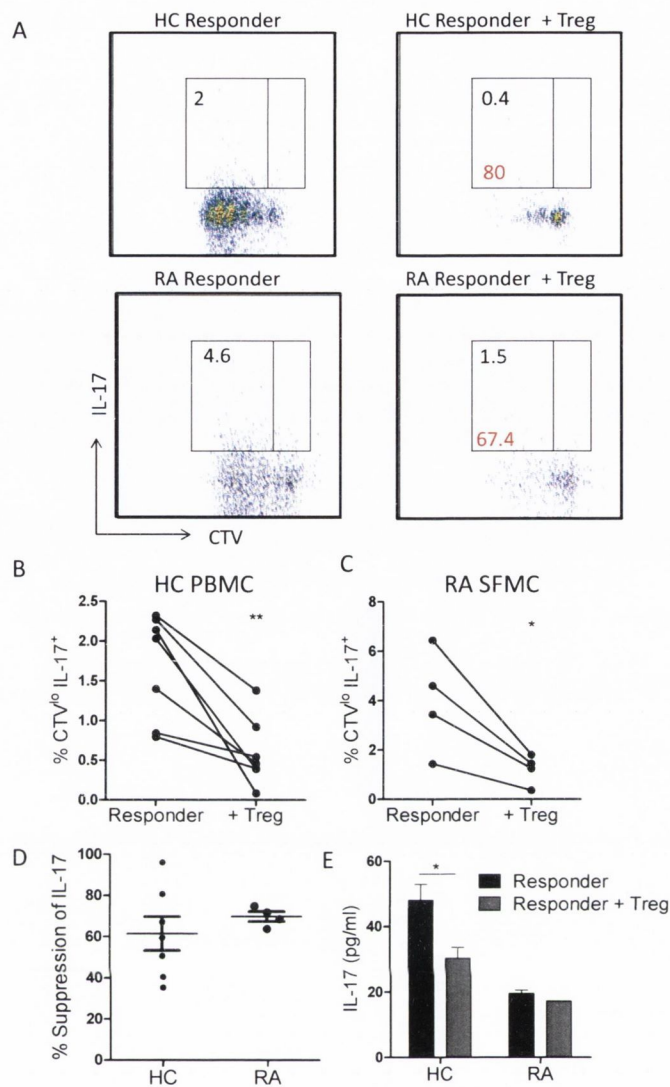


Figure 4.15 RA SFMC derived Treg cells effectively suppress IL-17 produced by autologous responder cells.

SFMC were isolated from RA patients with active disease and PBMC were isolated from healthy donors. CD4⁺ CD25⁻ responder cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Responder cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated, allogenic APC. Supernatants were harvested and cells were restimulated with PMA/ionomycin in the presence of brefeldin A, intracellularly stained for IL-17 and analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated and expression of IL-17 was assessed. (A) Representative FACS plots showing the frequency of IL-17 produced from the proliferating cell population (number shown in black) and the percent suppression (red) compared with control cells cultured alone. The frequency of IL-17 produced by HC (B) and RA (C) responder cells cultured alone and co-cultured with Treg cells. Each pair of data points represents a single independent experiment (HC n=7, RA n=4). (D) Percent Treg cell mediated suppression of IL-17 produced by HC and RA responder cells. (E) The concentrations of IL-17 present in the supernatants were measured by ELISA. Statistical significance was determined using a paired T-test with two-tailed p-values (B and C); *p<0.05, **p<0.01, an unpaired T-test with two-tailed p-values (D); however, the data was not statistically significant, and a two-way ANOVA with Bonferroni post-tests (E); *p<0.05.

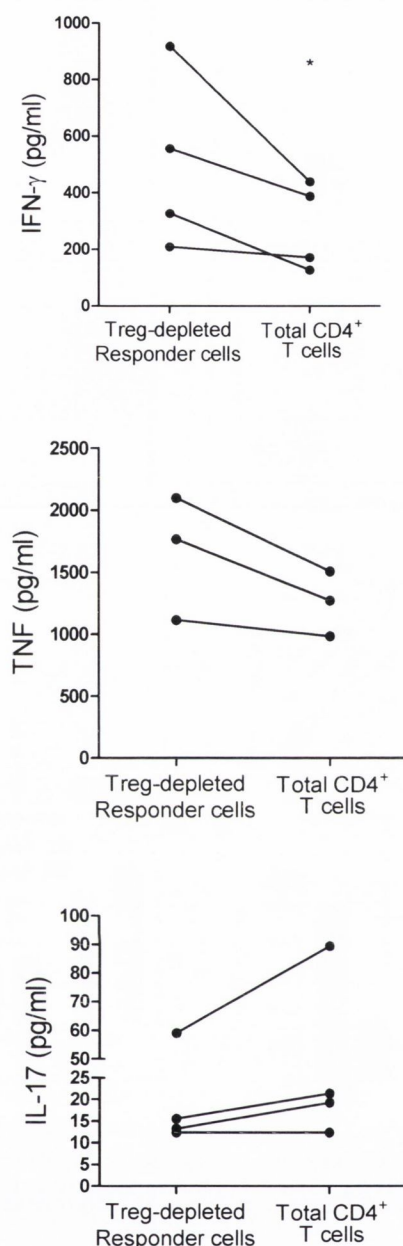


Figure 4.16 RA SF Treg cells present at physiological frequencies mediate suppression of IFN- γ and TNF.

SFMC were isolated from RA patients with active disease. CD4⁺ CD25⁻ Treg-depleted responder cells and total CD4⁺ cells (containing the Treg population at its physiological frequency) were sorted by FACS. Responder cells and total CD4⁺ cells were cultured for 5 days in the presence of anti-CD3 and irradiated, allogenic APC. The concentrations of IFN- γ , TNF and IL-17 present in the supernatants were assessed by ELISA. Each pair of data points represents the average of triplicate wells from a single independent experiment (IFN- γ and IL-17 n=4, TNF n=3). Statistical differences were determined by paired T-tests with one-tailed p values; *p<0.05.

4.2.4 Treg cells proliferate when co-cultured with responder cells.

Treg cells are generally believed to be anergic *in vitro*. However, as established in section 4.2.1 (figure 4.5), a significantly higher proportion of Treg cells from RA SFMC expressed Ki67 and therefore, were undergoing active cell cycling which may lead to replicative exhaustion. Treg cells from RA SFMC exhibited a reduced capacity for suppression compared with HC Treg cells. Therefore, in order to assess if Treg cells from the RA joint may have become exhausted due to their increased replication, the following study was designed to examine the ability of Treg cells from RA SF and HC PB proliferate *in vitro* in response to responder cells. In order to assess the proliferative capacity of Treg cells *in vitro*, the flow cytometry based suppression assay was adapted to allow for the assessment of both responder and Treg cell proliferation. HC PBMC and RA SFMC were sorted by FACS into responder cells (CD4⁺ CD25⁻) and Treg cells (CD4⁺ CD25⁺ CD127^{lo} CD39⁺). Responder cells were labelled with CFSE and Treg cells were labelled with CTV, as illustrated in figure 4.17A (top panel). Cells were stimulated for 5 days with anti-CD3 in the presence of irradiated APC. Proliferation of Treg cells was measured by dilution of the CTV label (figure 4.17A HC= middle panel, RA= lower panel). HC PB and RA SF Treg cells proliferated when cultured alone (22.9% and 20.8% proliferation, respectively) and this proliferation was increased when Treg cells were co-cultured with responder cells (up to 52.1 % in the HC and 27.4% in the RA patient). The proliferation of HC Treg cells was consistently and significantly increased when Treg cells were co-cultured with responder cells from 6 healthy donors (figure 4.17B). The already high rate of proliferation of RA SF Treg cells, as previously demonstrated by Ki67 staining, was somewhat further increased when Treg cells were stimulated and co-cultured with responder cells (figure 4.17C). The extent to which RA Treg cells increased their proliferation in the presence of the responder cells varied amongst the 3 donors examined; hence, this data did not reach statistical significance.

In summary, proliferation of Treg cells was increased in the presence of responder cells but RA Treg cells may have a reduced ability to proliferate in response to co-culture with responder cells. This may be due to elevated basal levels of proliferation in the RA joint, as observed by ki67 staining, which render Treg cells unable to respond fully respond *in vitro* to further proliferate. Alternatively, this may be indicative of a defect in Treg cells from the RA joint.

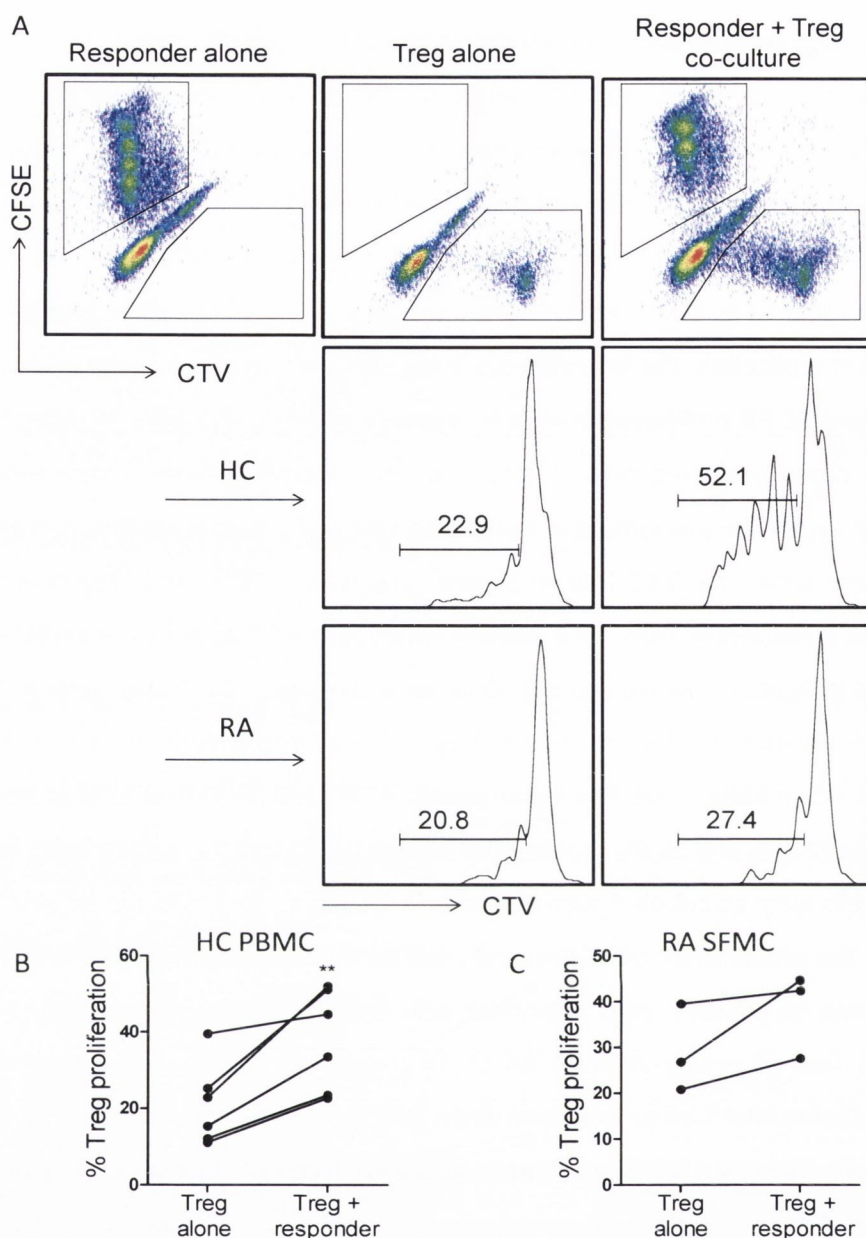


Figure 4.17 HC Treg cells, but not RA Treg cells, exhibit significantly increased proliferation in response to co-culture with autologous responder cells.

SFMC were isolated from RA patients with active disease. PBMC were isolated from healthy donors and $CD4^+$ T cells were purified from PBMC by MACS. $CD4^+$ $CD25^-$ (Treg depleted) responder cells and $CD4^+$ $CD25^+$ $CD127^{lo}$ $CD39^+$ Treg cells were sorted from HC PBMC and RA SFMC by FACS. Treg cells were labelled with CTV and cultured alone or co-cultured with equal numbers of CFSE labelled responder cells for 5 days in the presence of anti-CD3 and irradiated, allogeneic APC (A, top panel). Cells were analysed by flow cytometry; CTV^+ $CFSE^-$ cells were gated and proliferation of Treg cells was quantified by CTV dilution. Representative histograms from HC PBMC (A, second panel) and RA SFMC (A, third panel) showing the frequency of Treg cell proliferation when cultured alone (left) or in co-culture with responder cells (right). The percent proliferation of HC (B) and RA (C) Treg cells cultured alone and co-cultured with responder cells. Each pair of data points represents a single independent experiment (HC n=6, RA n=3). Statistical significance was determined using a paired T-test with two-tailed p-values; **p,0.01.

4.2.5 iTreg cells induced *in vitro* under the influence of TGF- β and ATRA are functionally suppressive and are stable when cultured in RA SF.

The previous data has shown that Treg cells in the RA joint are plastic and responsive to their inflammatory environment (figure 4.6 and figure 4.7). Since the adoptive transfer of Treg cells is being considered as a therapeutic option to combat inflammation, it is important to understand the suppressive capacity and stability of different types of Treg cells under inflammatory conditions, such as the cytokine-rich milieu of the synovial fluid. The aim of the following experiments was:

- to determine the differential phenotype and suppressive functions of *in vitro* induced iTreg cells polarised with and without ATRA.
- to assess the stability of FOXP3 expression in *ex vivo* Treg cells versus iTreg cells induced with and without ATRA in the presence of synovial fluid from the joints of RA patients during active disease.
- to examine the plasticity towards a hybrid “Treg-effector” phenotype of *ex vivo* Treg cells compared with *in vitro* induced iTreg cells polarised with and without ATRA, when cultured with synovial fluid from RA patients during active disease.

4.2.5a The phenotype and cytokine production of iTreg cells treated with ATRA.

iTreg cells were induced from Treg-depleted CD25⁻ CD4⁺ T cells (figure 4.18A) *in vitro* by treatment with high doses of IL-2, TGF- β and ATRA in addition to intense stimulation with anti-CD3 and anti-CD28 coated beads. A CD25⁺ FOXP3⁺ Treg cell population began to emerge *in vitro* after 5 days and gradually increased up to 12 days. On day 7, cells treated with IL-2 alone exhibited a 36.8% frequency of Treg cells compared with 80% of cells treated with IL-2 and TGF- β and 88.3% of cells treated with IL-2, TGF- β and ATRA (figure 4.18B). Next, the phenotype of these iTreg cells was assessed. iTreg cells induced by IL-2 alone and IL-2 and TGF- β expressed CD39 at a frequency of 35.2% and 37.3%, respectively, compared with 42.3% of iTreg cells induced under the influence of IL-2, TGF- β and ATRA (figure 4.18C). CTLA4 expression was also increased in iTreg cells induced with IL-2 and TGF- β or IL-2, TGF- β and ATRA (an MFI of approximately 7,000 and 6,000, respectively) compared with iTreg cells induced by IL-2 alone (exhibiting an MFI of approximately 4,000; figure 4.18D). After 7 days in culture, with combinations of IL-2, TGF- β and ATRA, the concentrations of IFN- γ and TNF were measured by ELISA (figure 4.19). Cells treated with IL-2 alone produced over 20 ng/ml of IFN- γ compared with undetectable amounts in cells treated with IL-2 and TGF- β or IL-2, TGF- β and ATRA (figure 4.19, left graph). Cells treated with IL-2 alone produced TNF and this was significantly decreased in

cells treated with IL-2 and TGF- β . Moreover, TNF was undetectable in cells treated with IL-2, TGF- β and ATRA (figure 4.19, right graph). These data indicated that iTreg cells generated by IL-2, TGF- β and ATRA exhibited a typical Treg cell phenotype and did not produce significant amounts of pro-inflammatory cytokines.

4.2.5b iTreg cells suppress responder cell proliferation and IFN- γ production.

Having demonstrated the successful induction of iTreg with a typical Treg phenotype, suppressive capacity of these iTreg cells was assessed next. On day 7 of iTreg induction, PBMC were isolated from the same healthy donor. Responder cells (CD4⁺ CD25⁻) were sorted by FACS. Responder cells were labelled with CFSE and cultured alone or co-cultured with iTreg cells previously elicited by treatment with IL-2 and TGF- β or IL-2, TGF- β and ATRA. Proliferation was measured by CFSE dilution (figure 4.20A). 85.5% of responder cells proliferated over 5 days in culture with anti-CD3 and irradiated APC. This frequency of proliferation was reduced to 43.7% when responder cells were co-cultured with iTreg cells elicited with IL-2 and TGF- β and further reduced to 30.5% when co-cultured with iTreg cells elicited with IL-2, TGF- β and ATRA. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A and analysed by flow cytometry for production of IFN- γ (figure 4.20B). 36.3% of responder cell produced IFN- γ and this was effectively suppressed to 7.7% in responder cells co-cultured with iTreg cells elicited by IL-2 and TGF- β or 1.5% in responder cells co-cultured with iTreg cells elicited by IL-2, TGF- β and ATRA. These data indicated that iTreg cells were effective suppressors of responder cell proliferation and cytokine production.

4.2.5c The stability of iTreg cells cultured with synovial fluid harvested from RA patients during active disease.

Next, the effect of RA synovial fluid on the stability of *in vitro* converted iTreg cells was compared with *ex vivo* sorted Treg cells. On day 7 of iTreg induction, PBMC were isolated from the same healthy donor and Treg cells (CD4⁺ CD25⁺ CD127^{lo}) were sorted by FACS. Treg cells and autologous iTreg cells were cultured with low doses of IL-2, anti-CD3 and irradiated APC in the presence or absence of SF drained from 9 RA patients during active disease. The stability of the Treg phenotype was assessed as a percentage of control i.e. Treg cells cultured without SF (figure 4.21A). iTreg cells induced with IL-2, TGF- β and ATRA maintained the Treg phenotype significantly more than iTreg cells induced without ATRA. A high degree of variance was observed in the *ex vivo* Treg cell group, which indicated that they may be more sensitive to certain environmental cues compared with iTreg cells generated *in vitro*. Representative plots show the frequencies of CD25⁺ FoxP3⁺ Treg cells in control versus SF treated Treg cells (figure

4.21B). The CD25⁺ FoxP3⁺ Treg cell population in *ex vivo* Treg cells is reduced by 35% from 40.3% under control conditions to 26.1% in the presence of SF. iTreg cells induced without ATRA exhibited 57.5% reduction in the CD25⁺ FoxP3⁺ population in cells treated with SF (18%) compared with control (42.2%). The CD25⁺ FoxP3⁺ population in iTreg cells induced with ATRA was reduced from 39.3% in the control to 30.5% in the presence of SF (a reduction of 22.4%). Interestingly, the control cells exhibited a marked reduction in co-expression of CD25 and FoxP3 (from about 80%, shown in figure 4.18, to around 40%). This may be due to the reduction in IL-2 or the lack of TGF- β in the subsequent assay post induction of iTreg cells. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A to assess pro-inflammatory cytokine production by flow cytometry. iTreg cells induced with ATRA produced less IFN- γ compared with iTreg cells induced without ATRA and *ex vivo* Treg; however, this trend was not significant (figure 4.21B, upper left graph). Both iTreg cells induced by TGF- β only or in combination with ATRA produced significantly less IL-17 compared with *ex vivo* Treg cells (figure 4.21B, upper right graph). ATRA-induced iTreg cells produced significantly less GM-CSF compared with *ex vivo* Treg cells (figure 4.21B, lower left graph). *Ex vivo* Treg cells and ATRA-induced iTreg cells produce significantly less TNF compared with TGF- β only induced iTreg cells (figure 4.21B, lower right graph).

Overall, these data indicated that iTreg cells exhibit a similar phenotype to *ex vivo* Treg cells and are functionally suppressive. In addition, ATRA enhanced the suppressive capacity and stability of iTreg cells in the presence of RA SF and reduced their capacity to produce pro-inflammatory cytokines. Thus, under inflammatory conditions, *in vitro* induced iTreg cells may exhibit superior stability over *ex vivo* isolated Treg cells, which may have important implications for Treg cell-based therapeutic strategies.

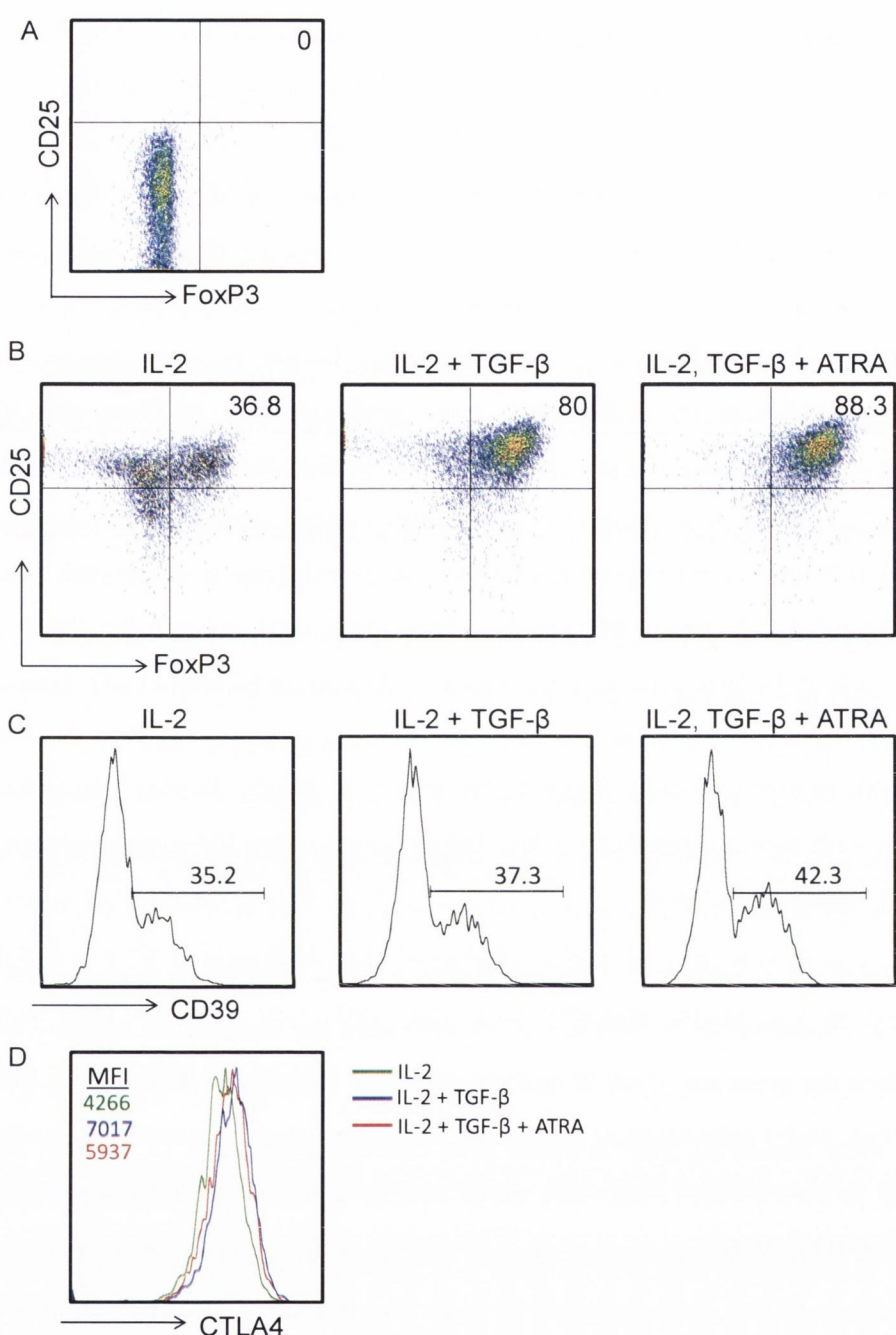


Figure 4.18 Conversion of CD4⁺CD25⁻ (Treg depleted) responder cells into iTreg cells. PBMC were isolated from healthy donors and CD4⁺T cells were purified from PBMC by MACS. CD4⁺ CD25⁻ (Treg depleted) responder cells were sorted by FACS and cultured with beads containing anti-CD3 and anti-CD28 in medium supplemented with IL-2, IL-2 and TGF-β or IL-2, TGF-β and ATRA. (A) The purified population was stained on day 0 with fluorochrome-conjugated antibodies specific for CD25 and FOXP3 to ensure the absence of Treg cells from the culture. (B) On day 7 post-sort, cells were stained with fluorochrome-conjugated antibodies specific for CD25 and FOXP3 and analysed by flow cytometry. (C) On day 7 post-sort, cells were stained with fluorochrome-conjugated antibodies specific for CD25, FOXP3 and CD39 and analysed by flow cytometry. Histograms show the frequency of CD25⁺ FoxP3⁺ Treg cells expressing CD39. (D) On day 7 post-sort, cells were stained with fluorochrome-conjugated antibodies specific for CD25, FOXP3 and CTLA4 and analysed by flow cytometry. Overlaid histograms show the staining intensity of CTLA-4 in CD25⁺ FoxP⁺ Treg cells. This data is representative of 3 independent experiments.

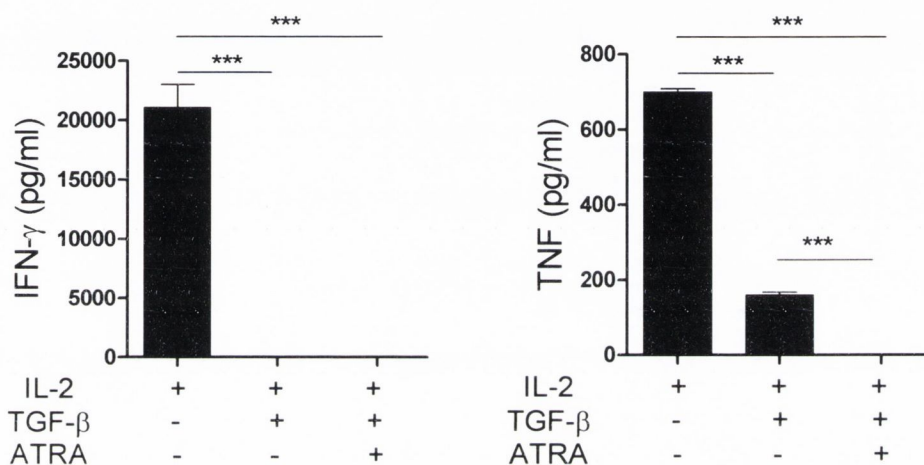


Figure 4.19 The concentrations IFN- γ and TNF are significantly reduced in iTreg cells post conversion with IL-2 and TGF- β or IL-2, TGF- β and ATRA compared with control cells treated with IL-2 alone.

PBMC were isolated from healthy donors and CD4⁺T cells were purified from PBMC by MACS. CD4⁺ CD25⁻ (Treg depleted) responder cells were sorted by FACS and cultured with beads containing anti-CD3 and anti-CD28 in medium supplemented with IL-2, IL-2 and TGF- β or IL-2, TGF- β and ATRA. On day 7, supernatants were harvested and the concentrations of IFN- γ and TNF were quantified by ELISA. Statistical differences were determined by 1-way ANOVA with Tukey's multiple comparison test; *** p<0.001.

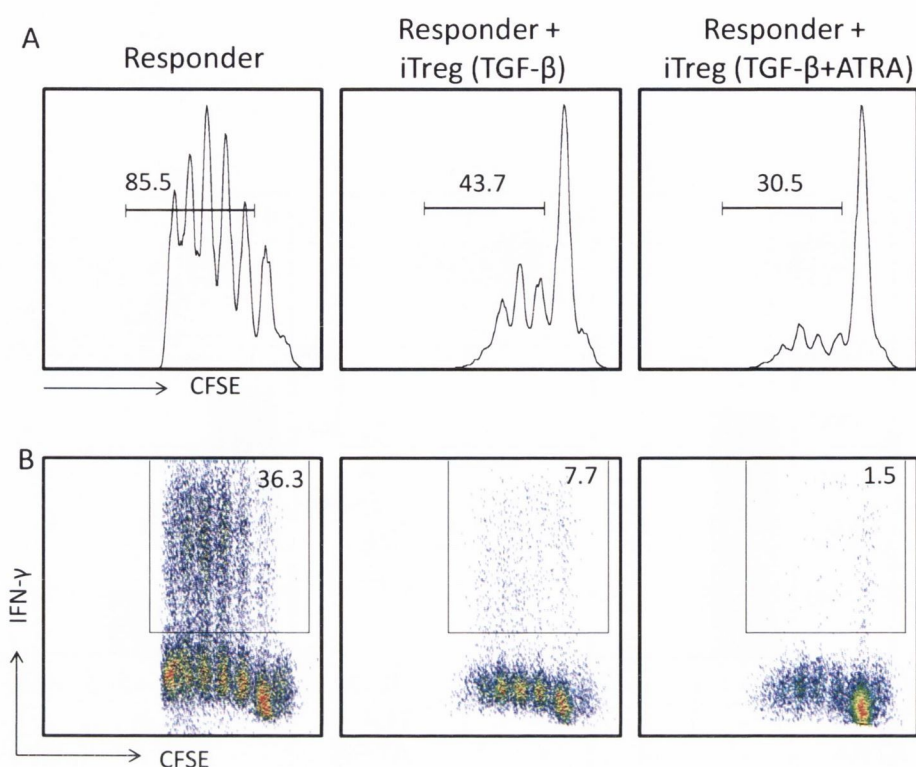


Figure 4.20 iTreg cells generated *in vitro* are functionally suppressive.

PBMC were isolated from healthy donors and CD4⁺ T cells were purified from PBMC by MACS. CD4⁺ CD25⁻ (Treg depleted) responder cells were sorted by FACS and cultured with beads containing anti-CD3 and anti-CD28 in medium supplemented with IL-2, IL-2 and TGF- β or IL-2, TGF- β and ATRA for 7 days. On day 7 of iTreg conversion, PBMC were isolated from the same healthy donor and CD4⁺ T cells were purified from PBMC by MACS. CD4⁺ CD25⁻ (Treg depleted) responder cells were sorted by FACS. Responder cells were labeled with CFSE and cultured alone or co-cultured with equal numbers of iTreg cells (generated with IL-2 and TGF- β or IL-2, TGF- β and ATRA) for 5 days in the presence of anti-CD3 and irradiated, allogeneic APC. Cells were analysed by flow cytometry; CFSE⁺ cells were gated and proliferation was quantified by CFSE dilution (A). Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for IFN- γ and analysed by flow cytometry (B). This data is representative of 2 independent experiments.

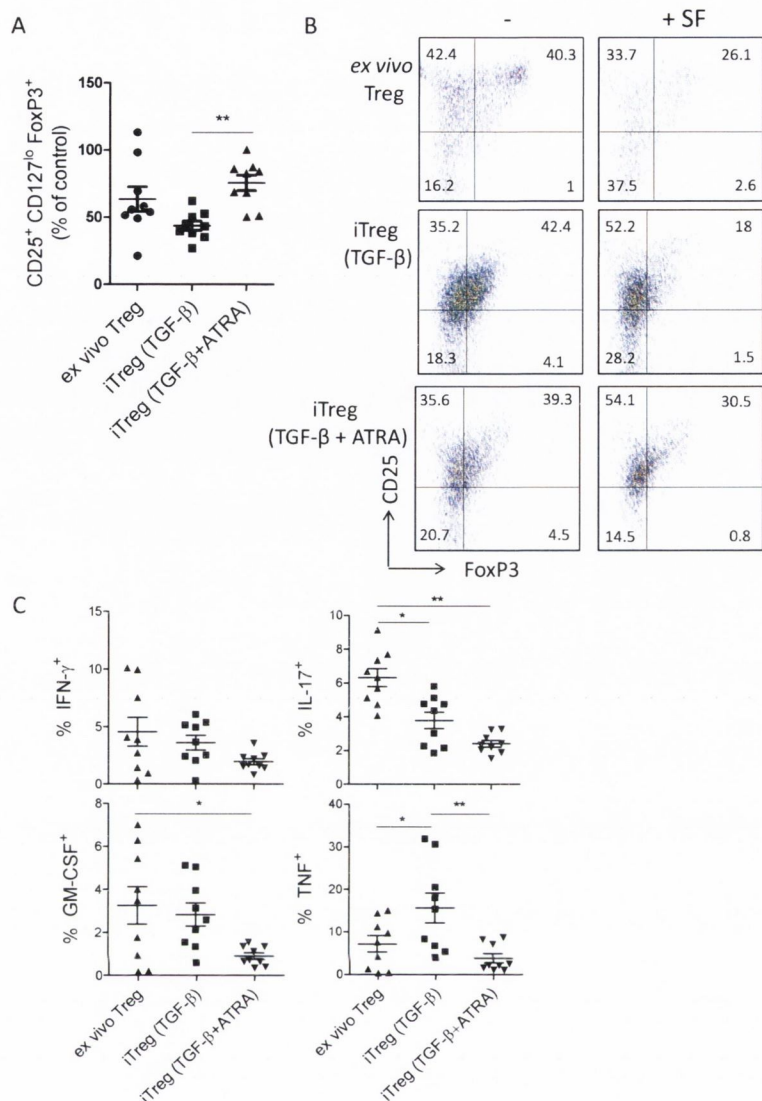


Figure 4.21 iTreg cells generated *in vitro* with IL-2, TGF-β and ATRA exhibit enhanced stability when cultured with RA synovial fluid compared with *ex vivo* Treg cells and iTreg cells generated with IL-2 and TGF-β only.

PBMC were isolated from healthy donors and CD4⁺ T cells were purified from PBMC by MACS. CD4⁺ CD25⁻ (Treg depleted) responder cells were sorted by FACS and cultured with beads containing anti-CD3 and anti-CD28 in medium supplemented with IL-2 and TGF-β or IL-2, TGF-β and ATRA for 7 days. On day 7 of iTreg conversion, PBMC were isolated from the same healthy donor and CD4⁺ T cells were purified from PBMC by MACS. CD4⁺ CD25⁺ CD127^{lo} Treg cells were sorted by FACS. iTreg cells generated *in vitro* and *ex vivo* sorted Treg cells were cultured with or without SF from 9 RA patients in the presence of anti-CD3, IL-2 and irradiated, allogeneic APC. On day 4, cells were stained with fluorochrome-conjugated antibodies specific for CD25, FOXP3 and CD127 and analysed by flow cytometry. The graph shows the retention of the Treg phenotype (CD25⁺ CD127^{lo} FOXP3⁺) as a percentage of the control (i.e. Treg cells cultured without SF; A). Representative dot plots show the frequency of Treg cells present in control conditions versus treatment with SF (B). Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for IFN-γ, IL-17, TNF and GM-CSF and analysed by flow cytometry (C). The graphs show the frequency of cells producing pro-inflammatory cytokines. Statistical differences were determined using a 1-way ANOVA with Tukey's multiple comparison test; *p<0.05, ** p<0.01. This data is representative of 2 independent experiments.

4.3 Discussion

The results from this chapter demonstrated that Treg cells were enriched in the joints of RA patients and exhibited a functional phenotype, with enhanced expression of CD39, CTLA-4 and Ki67, suggesting that these Treg cells were actively proliferating and should be capable of suppression. In addition, these data have shown that Treg cells from RA SFMC produce pro-inflammatory cytokines, and moreover, the production of pro-inflammatory cytokines was restricted to Treg cells that expressed CD161. Suppression assays showed that RA SFMC Treg cells were capable of effectively constraining responder cell proliferation and cytokine production, however, the extent of suppression mediated by RA SFMC Treg cells was markedly reduced compared with HC PBMC Treg cells.

The data illustrating an enrichment of Treg cells in RA SFMC is consistent with published data (van Amelsfort et al., 2004, Cao et al., 2004, Raghavan et al., 2009). However, the present study found no significant difference between the frequencies of Treg cells in HC and RA PBMC. This finding is at odds with reports of decreased frequencies of Treg cells in the peripheral blood of RA patients (Sempere-Ortells et al., 2009, Cao et al., 2003, Raghavan et al., 2009). Another report found significantly increased frequencies of Treg cells in the peripheral blood of RA patients compared with HC, however, this study identified Treg cells as CD4⁺ CD25⁺ and may, therefore, have included activated effector T cells (van Amelsfort et al., 2004). In addition to the differences caused by the use of different combinations of Treg cell markers, these disparities in the literature may be due to differences in the proximity of sampling to an inflammatory flare, an effect of different therapies or may reflect differences in the time from onset; patients sampled close to their time of diagnosis may show different frequencies of peripheral Treg cells compared with patients that have long established disease.

The current study has demonstrated, for the first time that CD39 is enriched on Treg cells in the RA joint. Hitherto, there has been no evidence in the literature suggesting that CD39⁺ Treg cells are increased at sites of autoimmune inflammation. The frequency of CD39⁺ Treg cells was shown to be reduced in the peripheral blood of MS patients (Fletcher et al., 2009) and in keeping with this finding, the results from RA peripheral blood exhibit a trend towards decreased CD39 expression on Treg cells compared with HC. This suggests that CD39⁺ Treg cells may preferentially be recruited to the site of autoimmune inflammation, causing an apparent deficit in the periphery.

CTLA-4 expression was found to be significantly increased in Treg cells from RA SFMC, consistent with published work (van Amelsfort et al., 2007). Whilst increased expression of CTLA-4 implies

that Treg cells may be functionally suppressive, the CTLA-4 molecule may not be fully functional as abnormal CTLA-4 function has been documented in RA and SLE (Flores-Borja et al., 2008, Jury et al., 2010).

In the present study, Treg cells from RA SFMC exhibited increased expression of Ki67 compared with HC and RA PBMC, which indicated that Treg cells in the RA joint are actively undergoing cell proliferation. This finding is novel but in keeping with data that indicates that Treg cells proliferate under homeostatic conditions *in vivo* and that this proliferation is up-regulated during an immune response (Walker, 2004). In agreement with the literature (Vukmanovic-Stejić et al., 2008), analysis of Ki67 staining in HC PBMC demonstrated that low frequencies (an average of 1.4%) of non-Treg CD4⁺ T cells are in active cell cycle (figure 3.1) compared with higher frequencies (an average of 6%) of Treg cells (figure 4.5), during homeostasis in HC PBMC. This simple observation indicates that a small pool of Treg cells may be continuously active, likely due to the recognition of self-antigens, to maintain homeostatic tolerance. Having established that RA SFMC Treg cells are highly proliferative, this suggested that they might be more prone to apoptosis compared with HC PBMC. On the contrary however, the data showed no significant difference in the expression of anti-apoptotic Bcl2 in Treg cells from RA SFMC compared with Treg cells from HC PBMC. In accordance with published literature (Taams et al., 2001), this study found Treg cells from HC PBMC have reduced Bcl2 compared with autologous responder cells. There was, however, no significant difference in Bcl2 expression between RA SFMC Treg and responder cells due to reduced Bcl2 expression in the RA SFMC responder cells. Thus, since Treg cells in the RA joint were actively proliferating and not concomitantly more prone to cell death, this suggests that Treg cells may possibly be switching into effector-like cells.

Plastic, hybrid “Treg-effector” cells capable of producing IL-17 and IFN- γ were evident in RA SFMC and furthermore, were contained largely with a subset of Treg cells expressing CD161. This finding has been supported by recently published work that shows CD161⁺ Treg cells produce pro-inflammatory cytokines in JIA (Pesenacker et al., 2013) and RA patients (Wang et al., 2014). The present study showed that Treg cells expressing CD161 were increased in the joint and, like their effector cell counterparts, CD161⁺ Treg cells were polyfunctional (i.e. capable of making several cytokines concomitantly), as shown by SPICE analysis. This is in keeping with published data indicating that Treg cells can functionally mirror the effector cells in their environment (Duhén et al., 2012). Given that the majority of CD161⁺ effector cells in the RA joint produced IFN- γ , it was fitting that these CD161⁺ Treg cells predominantly produced IFN- γ .

and exhibited low frequencies of IL-17 production. Moreover, CD161⁺ Treg cells exhibited a higher intensity of staining for anti-apoptotic Bcl2 and enhanced expression of HIF-1 α , both of which may promote cell survival in the hypoxic RA joint. These data suggest that the microenvironment of the joint affects Treg cells by inducing pro-inflammatory cytokine production. Or alternatively, that the microenvironment of the RA joint promotes the induction of iTreg cells from non-Treg cell precursors, which may retain certain effector cell characteristics. Since Treg cells expressing CD161 are increased in the joint and exhibit the same pro-survival characteristics as effector CD161⁺ Th cells, this suggests that these Treg cells may be induced from CD161⁺ effector cells. Whilst the consensus in the literature suggests that iTreg cells are induced primarily from naive Th cell precursors, some evidence also indicates that Treg cells may be induced from memory CD4⁺ T cells (Vukmanovic-Stejic et al., 2006). Therefore, it is plausible to postulate that memory CD161⁺ Th cells in RA joint may be converted to iTreg cells, which retain some of the phenotypic features of CD161⁺ effector memory Th cells.

This study found elevated HIF-1 α expression in total Treg cells in RA SFMC compared with HC PBMC. This finding is significant as the effect of HIF-1 α on FoxP3 gene expression is controversial; HIF-1 α is thought to preferentially enhance Th17 cell development and concomitantly down-regulate Treg cell development in mice (Shi et al., 2011, Dang et al., 2011). However, conflicting data suggests that hypoxia and HIF-1 α promote FoxP3 expression and Treg cell function (Ben-Shoshan et al., 2008, Clambey et al., 2012). In light of the fact that Treg cells are found in abundance in the hypoxic RA joint and at other hypoxic sites, such as in malignant tumours (Miller et al., 2006, Eikawa et al., 2010), it is reasonable conclude that Treg cells, or a certain sub-type of Treg cells, are able to effectively up-regulate and stabilise HIF-1 α expression during hypoxia. In addition to finding elevated HIF-1 α in Treg cells from RA SFMC, this study also found that Treg cells expressing CD161 contained significantly more HIF-1 α than their CD161⁻ Treg cell counterparts. It has been demonstrated that similar to the case in mice (Shi et al., 2011, Dang et al., 2011), human CD161⁺ Th17 lineage cells express higher HIF-1 α than other effector Th cells and this is associated with increased glycolytic capacity (unpublished work, Deborah Cluxton). Moreover, chapter 3 of the current study has shown that CD161⁺ Th cells in RA SFMC have increased expression of HIF-1 α compared with their CD161⁻ Th cell counterparts. Thus, it appears that CD161⁺ Treg cells mirror their CD161⁺ effector cell counterparts in terms of increased HIF1- α expression and mostly likely glycolytic capacity. This characteristic is in keeping with published data indicating that Treg cells have the ability to phenotypically mirror the effector Th cells they colocalize with (Duhon et al., 2012).

This study also showed that RA Treg cells were functionally suppressive, however, when the data was analysed in juxtaposition with HC PBMC derived Treg cells; it was evident that the overall suppressive capacity of RA Treg cells was impaired. This may be due to Treg cell defects or exhaustion or an enhanced pro-inflammatory, aggressive phenotype in the responder cell component, which renders the responder cells less susceptible to Treg cell mediated suppression. Other studies have shown that Treg cells from RA synovial fluid were functionally suppressive (van Amelsfort et al., 2004, Cao et al., 2004) however, the suppressive capacity of these Treg cells compared with HC were not robustly assessed. One group showed a trend towards increased capacity of RA SFMC Treg cells to suppress proliferation compared with HC PBMC Treg cells; however, this group isolated Treg cells on the basis of CD4⁺ CD25⁺ expression and failed to show purities for FoxP3 expression within the sorted subset (van Amelsfort et al., 2004). It is likely, therefore, that HC CD4⁺ CD25⁺ Treg cells are less pure than RA SFMC Treg cells as Treg cells in the RA joint are a more homogenous population of CD39⁺ cells, which this study has shown increased the fidelity of CD4⁺ CD25⁺ sorted T cells to FoxP3 expression, compared with peripheral blood. Moreover, tritiated thymidine was used to measure proliferation and therefore the proliferation of the sorted Treg subset cannot be distinguished from the CD25⁻ responder subset in co-culture. Importantly, the published study also showed that responder T cells from RA SFMC were less susceptible to autologous Treg cell mediated suppression compared with PBMC responder cells from the same donor (van Amelsfort et al., 2004). The decreased susceptibility to suppression was observed when Treg cells were derived from either SFMC or PBMC. This published work in conjunction with the data from this chapter, suggests that the reduced Treg cell mediated suppression in RA SFMC compared with HC PBMC is due to the nature of the RA SFMC responder cell, and not a defect in Treg cell activity. Given that chapter 3 identified that CD161⁺ Th cells were enriched in the RA joint, exhibited significantly higher frequencies of pro-inflammatory cytokines and pro-survival factors compared with CD161⁻ Th cells, it is therefore plausible that CD161⁺ Th may be refractory to Treg cell mediated suppression. Since the population of CD161⁺ Th cells is enriched in within responder cells from RA SFMC, their presence may be causing the reduced susceptibility to regulation. This will be further explored in chapter 5.

The question of whether RA SFMC derived Treg cells were defective in terms of their ability to proliferate in co-culture with responder cells was assessed. The majority of published studies on Treg cell function have used thymidine incorporation assays to assess proliferation and ELISA to measure cytokines. The flow cytometry based suppression assay developed in the present study offers the significant advantages of being able to separately track proliferation and cytokine

production by responder cells versus Treg cells. This is particularly important given the significant plasticity of Treg cells which has emerged in recent years. In agreement with published studies (Taams et al., 2001), Treg cells cultured alone were found to be comparatively anergic cells, hence, only a low frequency (approximately 20%) of Treg cells proliferated when stimulated with anti-CD3 and APC *in vitro*. However, when Treg cells were stimulated in the presence of responder cells, Treg cell anergy was overcome as evidenced by a significant increase in Treg cell proliferation. Treg cells from RA SFMC however, did not exhibit such a marked increase in proliferation. One out of three RA patients exhibited an increase in proliferation comparable to HC; from approximately 25% percent to 45%. Treg cells from the second RA patient had a very high frequency of background proliferation (approximately 40% when cultured alone with anti-CD3); as a result, there was no marked increase in proliferation in the presence of the responder cell. Treg cells from the third RA patient showed a small increase in proliferation, from approximately 20% to 27%. The trend towards reduced ability to proliferate in response to *in vitro* co-culture with responder cells may be as a result of the pre-activated state of the RA Treg cell in the joint; Treg cells expressed increased Ki67 and were, therefore, stimulated and proliferating in the joint in the presence of effector cells, perhaps *in vitro* re-stimulation is unable to promote further proliferation of Treg cells. Overall, the number of patients analysed is too small to definitively prove that RA Treg cells do not respond and proliferate effectively when cultured with responder cells. Despite being unable to draw finite conclusions about the proliferative capacity of Treg cells in RA SFMC, this data significantly impacts Treg cell research in general as it showed that human Treg cells proliferate in response to responder cells *in vitro*. This data suggests that Treg cells, although classically thought to be anergic, are in fact able to proliferate in response to inflammation in the same way as effector cells.

Finally, this study determined the differential stability of *ex vivo* Treg cells compared with iTreg cells differentiated *in vitro* with or without ATRA, in the context of the inflammatory RA joint. Cells were converted into iTreg cells with IL-2 and TGF- β in the presence or absence of ATRA. In concordance with published work (Wang et al., 2009), ATRA enhanced the suppressive effects of iTreg cells. In order to investigate the stability of iTreg cells versus *ex vivo* Treg cells, the inflammatory environment of the RA joint was mimicked *in vitro* by the addition of synovial fluids extracted from 9 RA patients during active disease. Studies of this nature have not yet been documented in the literature. It is important to note that iTreg cells, despite exhibiting very high expression of CD25 and FoxP3, were not clean populations of Treg cells. Furthermore, the extent of iTreg cell induction was slightly enhanced in the presence of ATRA. These

disparities were controlled for by analysing the FoxP3 stability of expression data as percentage of control; the control was the Treg cell subset in question, treated with medium only. The data clearly indicates that the function and stability of iTreg cells induced *in vitro* was significantly improved by the addition of ATRA. Interestingly, ATRA significantly reduced the plasticity of iTreg cells into pro-inflammatory cytokine producing “hybrid” Treg-effector cells. This effect was observed most strongly in IL-17 and GM-CSF production by *ex vivo* Treg cells versus *in vitro* iTreg cells differentiated with ATRA, in keeping with the literature that indicates ATRA down regulates Th17-type responses in favour of Treg cell responses (Xiao et al., 2008, Elias et al., 2008). These data indicate that ATRA may be suppressing the induction of Treg cells from CD161⁺ Th cell precursors or may be inhibiting their transition to the “hybrid” Treg-effector phenotype. Unfortunately, the current study did not examine differential expression of CD161 in the iTreg cell populations. However, given the fact that the present study has shown that only CD161⁺ Treg cells produce pro-inflammatory cytokines, the significantly reduced frequencies of pro-inflammatory cytokines produced by iTreg cells differentiated with ATRA indicates that treatment with ATRA may modulate the stability CD161⁺ Treg cells. It is established in the literature that ATRA inhibits Th17 cell polarisation, enhances FoxP3 expression (Elias et al., 2008, Xiao et al., 2008) and shifts the autoinflammatory Th17/Treg imbalance towards regulation (Bai et al., 2009). It is, therefore, rational to postulate that ATRA may be able to down-regulate the “hybrid” Treg-effector nature of CD161⁺ Treg cells.

The data presented here suggests that adoptive Treg cell therapy may be safest with the use of iTreg cells differentiated from naive CD4⁺ CD161⁻ T cells *in vitro* in the presence of ATRA, rather than expanded *ex vivo* Treg cells, which are currently the main focus of adoptive Treg cell therapy (Riley et al., 2009). Depleting the population of CD161⁺ T cells may negate the requirement for ATRA to stabilise the phenotype of iTreg cells, however, this will necessitate further investigation.

In conclusion, this study contributed to the overall understanding of the altered immune-regulatory response in RA by showing that Treg cells are enriched in the RA synovial fluid, exhibit increased expression of important suppressive artillery (CD39 and CTLA-4) and are functionally suppressive *in vitro*. This study also demonstrated that a subset of Treg cells express CD161 and produce pro-inflammatory cytokines which may contribute to autoimmune inflammation in the RA joint. Although RA Treg cells are functionally suppressive *in vitro*, they exhibit a reduced degree of suppression compared with HC Treg cells, which may be due to the differential nature of HC and RA SFMC derived effector cells, rather than a defect in RA Treg cells.

Chapter 5 Treg cell mediated regulation of Th cell subpopulations

5.1.1 Introduction

Differential Treg cell mediated regulation of T helper cell subsets.

There is amassing evidence in the literature implicating the dysregulation of Th17 cells in the pathogenesis of many chronic inflammatory conditions, such as RA, IBD and psoriasis (Tesmer et al., 2008, Crome et al., 2010b). Concomitantly, intense interest in therapeutic strategies designed to block Th17 cells is mounting. However, relatively little is known about the regulation of Th17 cell functions in humans. Treg cells are responsible for constraining proliferation and pro-inflammatory cytokine production by effector T cells during inflammation and furthermore, control autoimmune inflammation. To date, there is no evidence in the literature regarding the differential regulation of CD161⁺ memory Th cells (which incorporate both bona fide Th17 cells and IFN- γ producing ex-Th17 cells) and CD161⁻ memory Th cells (which incorporate Th1 and Th2 cells). Whilst Treg cells have been shown to effectively suppress Th1 cell proliferation and IFN- γ production *in vitro*, the suppression of Th17 cells has been controversial; with some studies indicating that Th17 cells are ineffectively suppressed by Treg cells (Annunziato et al., 2007, Evans et al., 2007) and other data showing Th17 cells are suppressed by Treg cells (Fletcher et al., 2009, Crome et al., 2010a).

Understanding the suppression of human Th17 cells has been thwarted by the lack of a commonly accepted method to reliably differentiate Th17 cells *in vitro* resulting in disparate phenotypes of Th17 cells which cannot be compared across different studies. In addition the plasticity of Th17 cells has complicated the identification of bona fide Th17 cells. The method used to assess suppression is also a key determinant in being able to observe sensitivity to suppression. Established methods, which largely relied on tritiated thymidine incorporation or assessment of cytokine concentration by ELISA, are hampered by the fact that co-cultured responder and Treg cells cannot be differentiated from one another and therefore, only cumulative effects are examined. This can make results difficult to decipher given the plasticity of Treg cell function that has emerged. This can be overcome using a flow cytometry based assay which allows for each subset in co-culture to be labelled and assessed independently of each other (as described in section 2.7).

Classification of Th1, Th17 and plastic ex-Th17 cells.

Identifying a panel of surface markers to reliably isolate discrete Th subsets has been a topic of much investigation (Acosta-Rodriguez et al., 2007b, Maggi et al., 2012). A consensus in the

literature suggests that bona fide Th17 cells can be sorted based on their co-expression of CD161, CCR6 and CCR4 in the absence of CXCR3 expression (Annunziato et al., 2008, Sallusto et al., 2012). Th1 cells can be sorted by CXCR3 and CCR5 expression in the absence of CD161, CCR6 and CCR4 (Annunziato et al., 2008, Sallusto et al., 2012). Plastic Th17 cells which have switched into producing IFN- γ maintain the expression of CD161 and CCR6 and furthermore, gain the concomitant expression of CXCR3 (Annunziato et al., 2008, Sallusto et al., 2012). These plastic ex-Th17 cells which switch into IFN- γ producing “Th-1 like” cells have been referred to, for this reason, in the literature as “non-classical Th1” cells. Only one study to date has investigated the differences between bona fide Th1 cells and plastic ex-Th17 “non-classical Th1” cells in terms of their expression of IFN- γ and IL-17, transcription factors and chemokine receptors (Maggi et al., 2012). This paper showed that “non-classical Th1” cells can be distinguished from bona fide Th1 cells using a combination of surface markers but did not robustly investigate functional differences between the classical and “non-classical Th1-type cells”.

Th17 plasticity, although first established in humans (Annunziato et al., 2007), has been identified as a prominent pathogenic factor in the chronicity of autoimmune inflammation in animal models (Hirota et al., 2011). However, no data has been published to date analysing the pathogenicity of bona fide Th1 cells compared to plastic ex-Th17 “non-classical Th1” cells.

5.1.2 Aims

The experiments in this chapter aimed to determine if specific Th cell subsets are differentially regulated by Treg cells. The specific aims were to:

- Assess differences in Treg cell mediated suppression of CD161⁺ and CD161⁻ Th cells from HC PBMC.
 - Using a flow cytometry based suppression assay to enable the analysis of discrete populations within the co-culture system.
 - Confirm results using an ELISA based suppression assay to evaluate the total concentrations of cytokines present in the cell culture supernatants.
- Examine differential regulation of CD161⁺ and CD161⁻ Th cells from RA SFMC.
- Determine the differential phenotype, function and regulation of Th1, Th17 and ex-Th17 cells.
 - Definitively establish if ex-Th17 cells are increased in the RA joint.
 - Assess cytokine polyfunctionality of the different populations.
 - Examine differential Treg cell mediated regulation of the subpopulations.
 - Hence, deduce the differences between Th1 and ex-Th17 “non-classical Th1” cells.

5.2 Treg cell mediated regulation of Th cell subpopulations

5.2.1 Differential Treg cell mediated suppression of CD161⁺ and CD161⁻ memory Th cells.

Chapter 3 established that CD161⁺ Th cells, which incorporate both bona fide Th17 cells and ex-Th17 type cells, were enriched in the RA joint and produced multiple pro-inflammatory cytokines (figure 3.6 and 3.7). In addition, CD161⁺ Th cells in the RA joint exhibited significantly higher Bcl2 and HIF-1 α expression and had significantly longer telomeres than their CD161⁻ Th cell counterparts (figure 3.8). Moreover, HC CD161⁺ Th cells were highly polyfunctional in terms of cytokine production (figure 3.11) and induced a pro-inflammatory response in fibroblast cells (figure 3.13, 3.16 and 3.17). Chapter 4 demonstrated that Treg cells from the RA joint functionally suppressed autologous responder cells; however, the extent of suppression was significantly reduced compared with HC Treg cell mediated suppression of responder cells (figure 4.11, 4.12 and 4.13). The aim of the following section was to determine whether CD161⁺ and CD161⁻ Th cells might be differentially regulated by Treg cells. Given that CD161⁺ Th cells were enriched in the joint and Treg cells, although functionally suppressive, failed to constrain autoimmune inflammation, the question remained as to whether the lack of regulation was due to the nature of the effector cells as opposed to the effectiveness of the Treg cells. In order to address this question, flow cytometry-based suppression assays were carried out on sorted CD45RO⁺ CD161⁺ and CD45RO⁺ CD161⁻ Th cell populations from healthy controls. The data was further supported by ELISA-based suppression assays.

5.2.1a CD161⁺ Th cells are significantly less susceptible to Treg cell mediated suppression of proliferation and pro-inflammatory cytokine production compared with CD161⁻ Th cells.

HC memory CD4⁺ Treg-depleted CD161⁺ and CD161⁻ effector T cells were sorted by FACS along with CD39⁺ Treg cells. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated APC. Proliferation of effector cells was then measured by CTV dilution and cells were restimulated with PMA and ionomycin in the presence of brefeldin A to assess cytokine production. The results showed that CD161⁻ Th cells were significantly more susceptible to Treg cell mediated suppression of proliferation compared with CD161⁺ cells (figure 5.1A). Representative histograms illustrate that CD161⁺ and CD161⁻ Th cells proliferated to similar extents in culture (figure 5.1B, upper panel; 94.7 and 96.3% proliferation, respectively). In the presence of Treg cells, CD161⁺ Th cell proliferation was reduced to 82.5% (a suppression of 12.9%) whereas CD161⁻ Th cell proliferation was reduced to 58.1% (a suppression of 39.7%;

figure 5.1B, lower panel). Hence, the proliferation of CD161⁺ Th cells was more readily suppressed by Treg cells than CD161⁺ Th cells.

Next, the ability of Treg cells to suppress cytokine production by CD161⁺ or CD161⁺ Th cells was assessed. IFN- γ production was assessed in CTV^{lo} proliferated cells (figure 5.2A), and suppression of IFN- γ was significantly lower in CD161⁺ Th cells compared with CD161⁺ Th cells. Representative FACS plots showed that both CD161⁺ and CD161⁺ Th cells produced IFN- γ (figure 5.2B, upper panel). However, the Treg cell mediated suppression of IFN- γ was vastly different between the two subsets; 40.6% of proliferating CD161⁺ Th cells produced IFN- γ in the presence of the Treg cell (a suppression of 13.2% compared to control) in comparison with 4.2% of proliferating CD161⁺ Th cells (a suppression of 87.7% compared to control cells cultured alone; figure 5.2B, lower panel).

Similarly, suppression of TNF was assessed and found to be significantly more effective in CD161⁺ Th cells compared with CD161⁺ Th cells (figure 5.3A). Representative FACS plots showed that proliferated CD161⁺ and CD161⁺ Th cells produced comparable frequencies of TNF (74.7% and 70.7%, respectively, figure 5.3B, upper panel). When CD161⁺ Th cells were co-cultured with Treg cells, the frequency of proliferating cells producing TNF was reduced to 65.1% (yielding an overall suppression of 12.9% compared with control; figure 5.3, lower panel, left). In contrast, when CD161⁺ Th cells were co-cultured with Treg cells, the frequency of proliferating cells producing TNF is reduced to 10.9% (84.6% suppression; figure 5.3, lower panel, right).

CD161⁺ Th cells exhibited significantly reduced Treg cell mediated suppression of GM-CSF with CD161⁺ Th cells (figure 5.4A). Representative FACS plots showed the frequency of GM-CSF production by proliferating CD161⁺ Th cells (45.2%) and CD161⁺ Th cells (62%; figure 5.4B, upper panel). In this example, when CD161⁺ Th cells were co-cultured with Treg cells, no suppression of GM-CSF was observed i.e. over 45% of proliferating cells were still producing GM-CSF (figure 5.4B, lower panel, left). However, in CD161⁺ Th cells co-cultured with Treg cells, only 4.1% of proliferating cells produced GM-CSF (a suppression of 93.4% compared with control; figure 5.4, lower panel, right). In this example, higher frequencies of CD161⁺ Th cells produced GM-CSF compared with CD161⁺ Th cells yet Treg cell mediated suppression was still more effective in CD161⁺ Th cells.

The production of IL-2 by CD161⁺ Th cells was significantly less well suppressed by Treg cells compared with CD161⁺ Th cells (figure 5.5A). Representative FACS plots showed that the frequency of IL-2 produced by proliferated CD161⁺ Th cells and CD161⁺ Th cells was 49.5% and

76.1%, respectively (figure 5.5B, upper panel). When CD161⁺ Th cells were co-cultured with Treg cells, the production of IL-2 was reduced to 37.5% (24.2% suppression) compared with 26.4% of CD161⁻ Th cells that produced IL-2 in the presence of Treg cells (65.3% suppression; figure 5.4B, lower panel). In this example, higher frequencies of CD161⁻ Th cells produced IL-2 compared with CD161⁺ Th cells, yet the Treg cell mediated suppression was still more effective in CD161⁻ Th cells.

Finally, differential suppression of IL-17 was assessed. Because Th17 type cells are thought to originate exclusively from CD161⁺ precursor cells, CD161⁻ Th cells should not produce IL-17. However, during the course of this study, low frequencies of CD161⁻ Th cells producing IL-17 were observed in certain donors (figure 3.6B, 3.10 and 3.11). This observation has also been noted in published work by other groups (Crome et al., 2010a). In certain instances, CD161⁻ Th cells exhibited only background frequencies of IL-17 staining (<0.5%) and therefore, did not show suppression because the background was the same in cells cultured alone or co-cultured with Treg cells. As a result, the data is less clear than for other cytokines; there was no significant difference between the suppression of IL-17 from CD161⁺ compared with CD161⁻ Th cells (figure 5.6A). Representative FACS plots illustrate that 10.8% of CD161⁺ Th cells produced IL-17 and, in this example, low but appreciable frequencies of CD161⁻ Th cells expressed IL-17 (2.8%; figure 5.6B, upper panel). In co-culture with Treg cells, the frequency of IL-17 production by CD161⁺ Th cells was reduced to 9.3% (13.9% suppression) whereas the frequency of CD161⁻ Th cells that produced IL-17 was reduced to 0.5% (82% suppression; figure 5.6B, lower panel).

In summary, the flow cytometry based suppression assays demonstrated that Treg-depleted memory CD4⁺ CD161⁺ T cells were strikingly less susceptible to Treg cell mediated suppression of proliferation and pro-inflammatory cytokines compared with Treg-depleted memory CD4⁺ CD161⁻ Th cells.

5.2.1b The concentrations of accumulated pro-inflammatory cytokines indicate that CD161⁺ Th cells are less susceptible to Treg cell mediated suppression compared with CD161⁻ Th cells.

To further support the evidence from the flow cytometry-based suppression assay, the concentrations of pro-inflammatory cytokines present in the supernatants of sorted Treg-depleted memory CD4⁺ CD161⁺ and Treg-depleted memory CD4⁺ CD161⁻ T cells after 5 days in culture alone or in co-culture with Treg cells were assessed by ELISA (figure 5.7). Representative ELISA graphs show the concentrations of pro-inflammatory cytokines present in the supernatants of CD161⁺ and CD161⁻ effector cells cultured alone (black bars) compared with effector cells co-cultured with Treg cells (grey bars; figure 5.7A). The production of IFN- γ by

CD161⁺ effector cells co-cultured with Treg cells was not significantly reduced compared with the production of IFN- γ by CD161⁺ effector cells cultured alone. In contrast, the concentration of IFN- γ produced by CD161⁻ effector cells co-cultured with Treg cells was significantly suppressed compared with the concentration present in the supernatant of CD161⁻ effector cells cultured alone (figure 5.7A, upper left graph). The overall extent of suppression (expressed as percent suppression based on the average of triplicate or duplicate wells; shown in red) was calculated and showed that Treg cells reduced the production of IFN- γ in CD161⁺ effector cells by 26.4% compared with 93.5% in CD161⁻ effector cells. When the extent of suppression of IFN- γ production was collated from 5 independent experiments, CD161⁻ effector cells exhibited a significantly higher degree of Treg cell mediated suppression compared with CD161⁺ effector cells (figure 5.7B, upper left graph).

The same trend was observed for the concentrations of TNF in the supernatants; Treg cells did not significantly suppress the production of TNF by CD161⁺ effector cells but significantly suppressed TNF production by CD161⁻ effector cells (figure 5.7A, upper right graph). When the extent of Treg cell mediated suppression of the production of TNF was collated from 4 independent experiments, CD161⁻ effector cells were significantly more susceptible to Treg cell mediated suppression compared with CD161⁺ effector cells (figure 5.7B, upper right graph).

CD161⁺ effector cells cultured alone produced significantly more IL-17 than CD161⁻ effector cells (figure 5.7A, lower left graph). In the presence of Treg cells, the concentration of IL-17 produced by CD161⁺ effector cells was significantly reduced. However, the overall extent of suppression (expressed as percent suppression based on the average of triplicate wells; shown in red) was comparable between CD161⁺ and CD161⁻ effector cells co-cultured with Treg cells. When the extent of Treg cell mediated suppression of the production of IL-17 was collated from 4 independent experiments, no significant differences were found (figure 5.7B, lower left graph).

The production of GM-CSF was significantly suppressed in both CD161⁺ and CD161⁻ effector cells co-cultured with Treg cells (figure 5.7A, lower right graph), however, the extent of suppression differed between the two subsets; the production of GM-CSF was suppressed by 45.9% in CD161⁺ effector cells co-cultured with Treg cells compared with 100% suppression in CD161⁻ effector cells co-cultured with Treg cells. CD161⁺ effector cells cultured alone produced significantly more GM-CSF compared with CD161⁻ effector cells cultured alone. When the extent of Treg cell mediated suppression of the concentration of GM-CSF was collated from 3 independent experiments, CD161⁻ effector cells exhibited higher susceptibility to Treg cell

mediated suppression compared with CD161⁺ effector cells, however, this data did not reach statistical significance (figure 5.7B, lower right graph).

In conclusion, Treg-depleted memory CD4⁺ CD161⁺ and Treg-depleted memory CD4⁺ CD161⁻ T cell subsets were differentially regulated by Treg cells. The CD161⁺ T cell subset was on balance significantly less susceptible to Treg cell mediated suppression of proliferation and pro-inflammatory cytokine production compared with the CD161⁻ T cell subset.

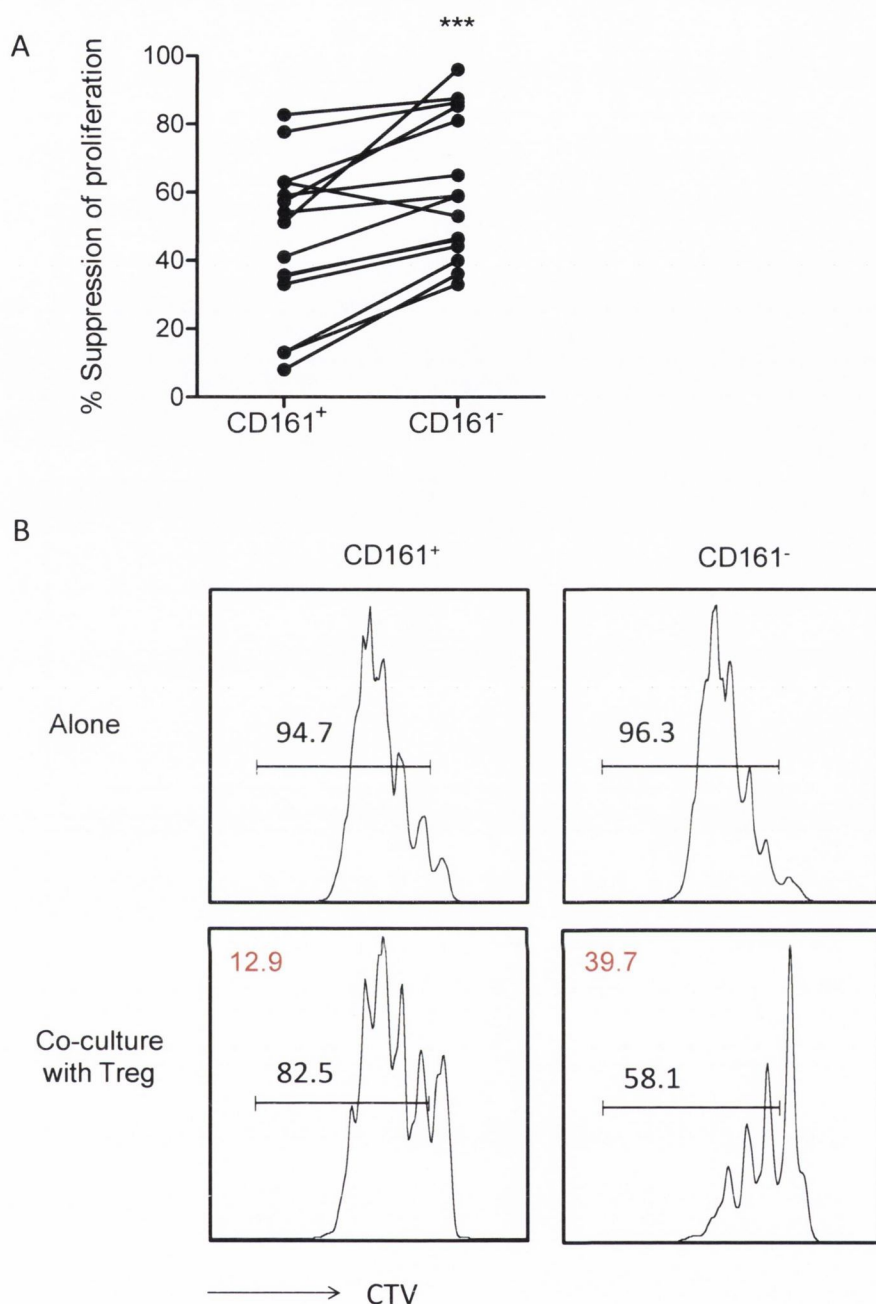


Figure 5.1 CD161⁺ Th cells are less susceptible to Treg cell mediated suppression of proliferation than CD161⁻ Th cells.

PBMC were isolated from healthy donors. CD4⁺ memory cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD25⁻ CD161⁺ and CD4⁺ CD45RO⁺ CD25⁻ CD161⁻ effector T cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated, allogeneic APC. Cells were analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated and proliferation was quantified by CTV dilution. (A) Percent Treg cell mediated suppression of CD161⁺ and CD161⁻ Th cell proliferation. Each pair of data points represents a single independent experiment (n=15). (B) Representative histograms showing the frequency of cell proliferation (black) and the percent suppression (red) compared with control cells cultured alone. Statistical significance was determined using a paired T-test with two-tailed p-values; *** p=0.0005.

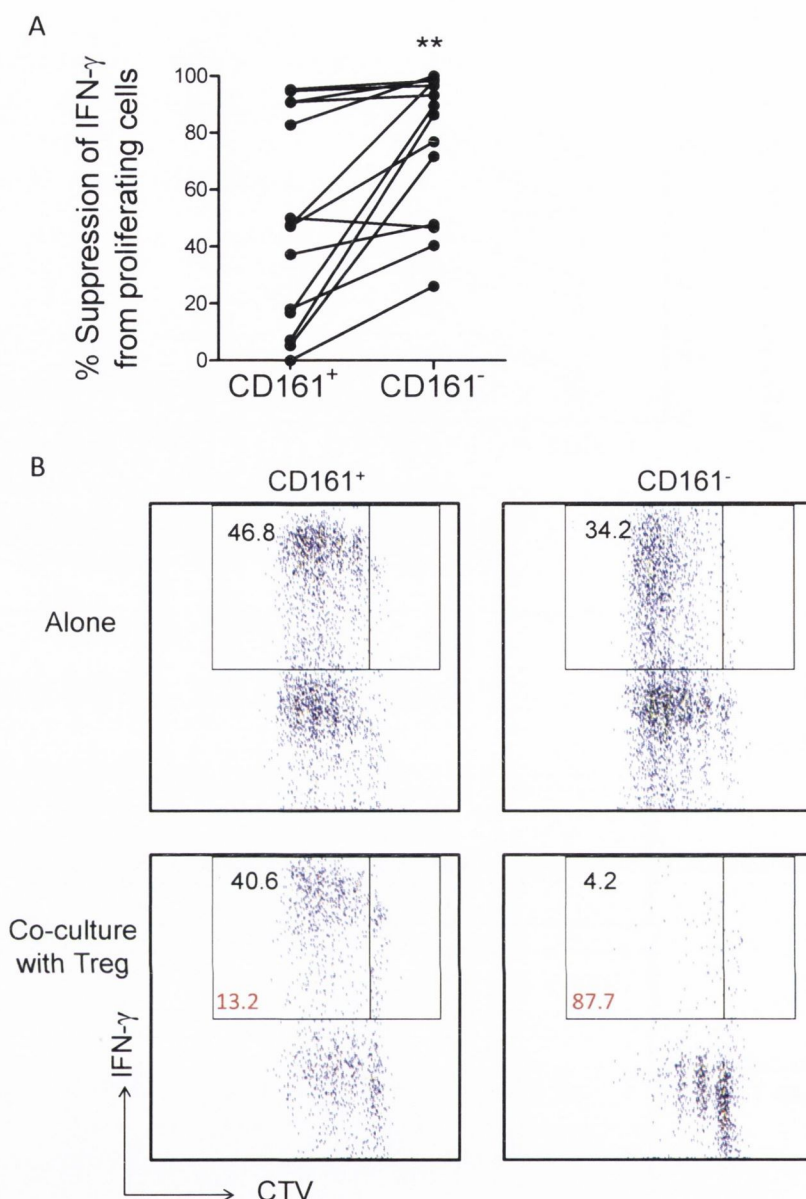


Figure 5.2 CD161⁺ Th cells are less susceptible to Treg cell mediated suppression of IFN- γ production than CD161⁻ Th cells.

PBMC were isolated from healthy donors. CD4⁺ memory cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD25⁻ CD161⁺ and CD4⁺ CD45RO⁺ CD25⁻ CD161⁻ effector T cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled allogeneic irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for IFN- γ and analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated before assessing IFN- γ production. (A) Percent Treg cell mediated suppression of proliferated CD161⁺ and CD161⁻ Th cell IFN- γ production. Each pair of data points represents a single independent experiment (n=14). (B) Representative FACS plots showing the frequency of IFN- γ produced by the proliferating cell population (number shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of IFN- γ were determined by paired T-tests with two-tailed p-values; ** p<0.01.

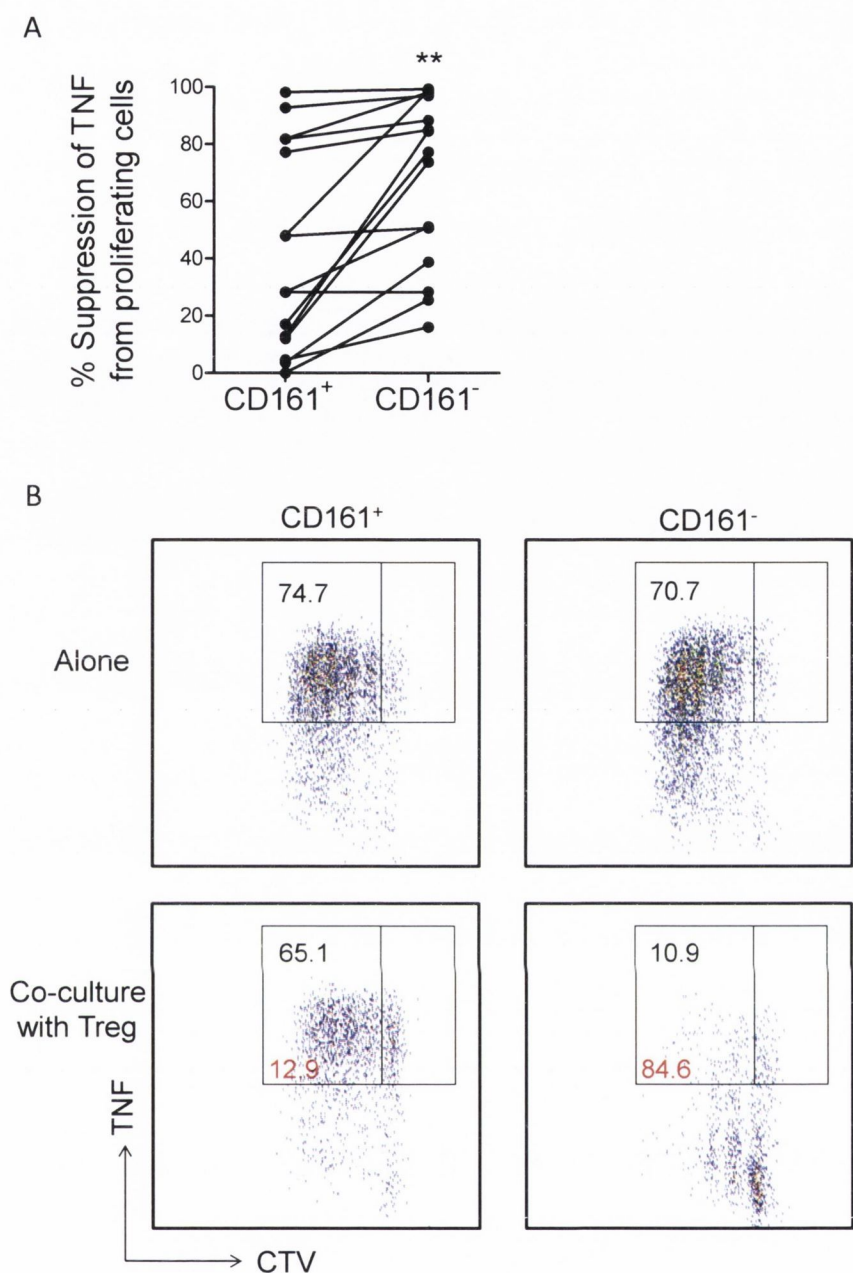


Figure 5.3 **CD161⁺ Th cells are less susceptible to Treg cell mediated suppression of TNF production than CD161⁻ Th cells.**

PBMC were isolated from healthy donors. CD4⁺ memory cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD25⁻ CD161⁺ and CD4⁺ CD45RO⁺ CD25⁻ CD161⁻ effector T cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled allogeneic irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for TNF and analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated before assessing TNF production. (A) Percent Treg cell mediated suppression of proliferated CD161⁺ and CD161⁻ Th cell TNF production. Each pair of data points represents a single independent experiment (n=15). (B) Representative FACS plots showing the frequency of TNF produced by the proliferating cell population (number shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of TNF were determined by paired T-tests with two-tailed p-values; *p<0.05, ** p<0.01.

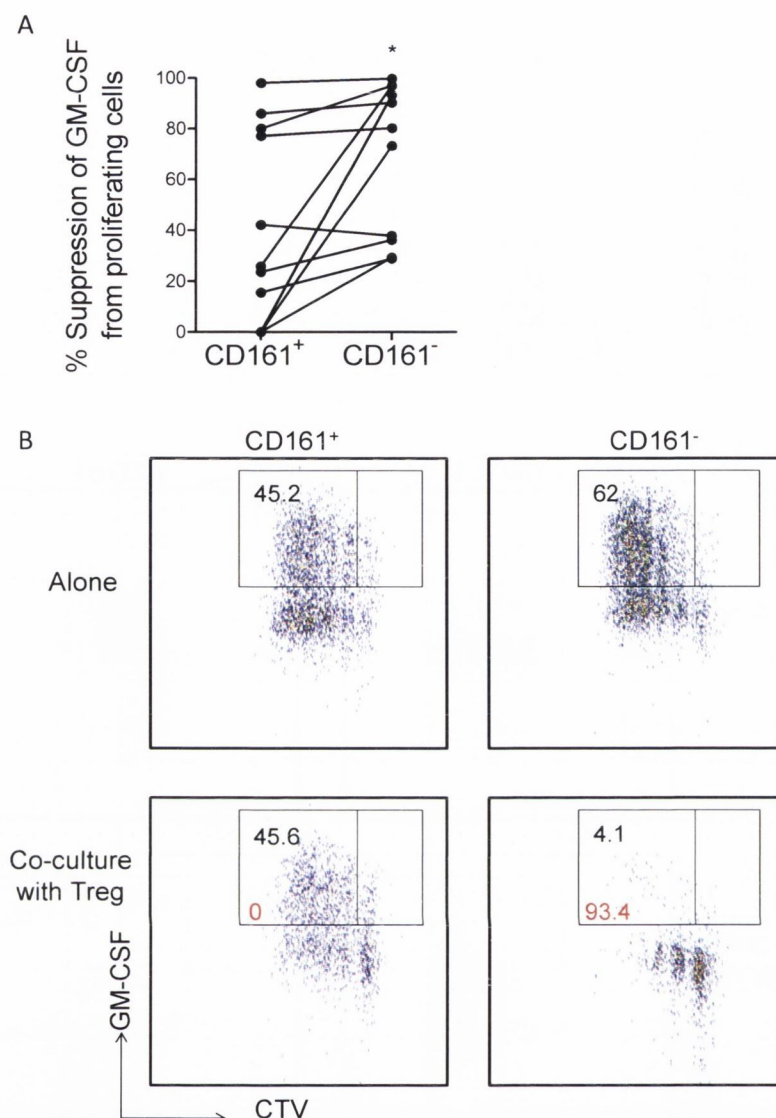


Figure 5.4 CD161⁺ Th cells are less susceptible to Treg cell mediated suppression of GM-CSF production than CD161⁻ Th cells.

PBMC were isolated from healthy donors. CD4⁺ memory cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD25⁻ CD161⁺ and CD4⁺ CD45RO⁺ CD25⁻ CD161⁻ effector T cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled allogeneic irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for GM-CSF and analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated before assessing GM-CSF production. (A) Percent Treg cell mediated suppression of proliferated CD161⁺ and CD161⁻ Th cell GM-CSF production. Each pair of data points represents a single independent experiment (n=11). (B) Representative FACS plots showing the frequency of GM-CSF produced by the proliferating cell population (number shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of GM-CSF were determined by paired T-tests with two-tailed p-values; * p<0.05.

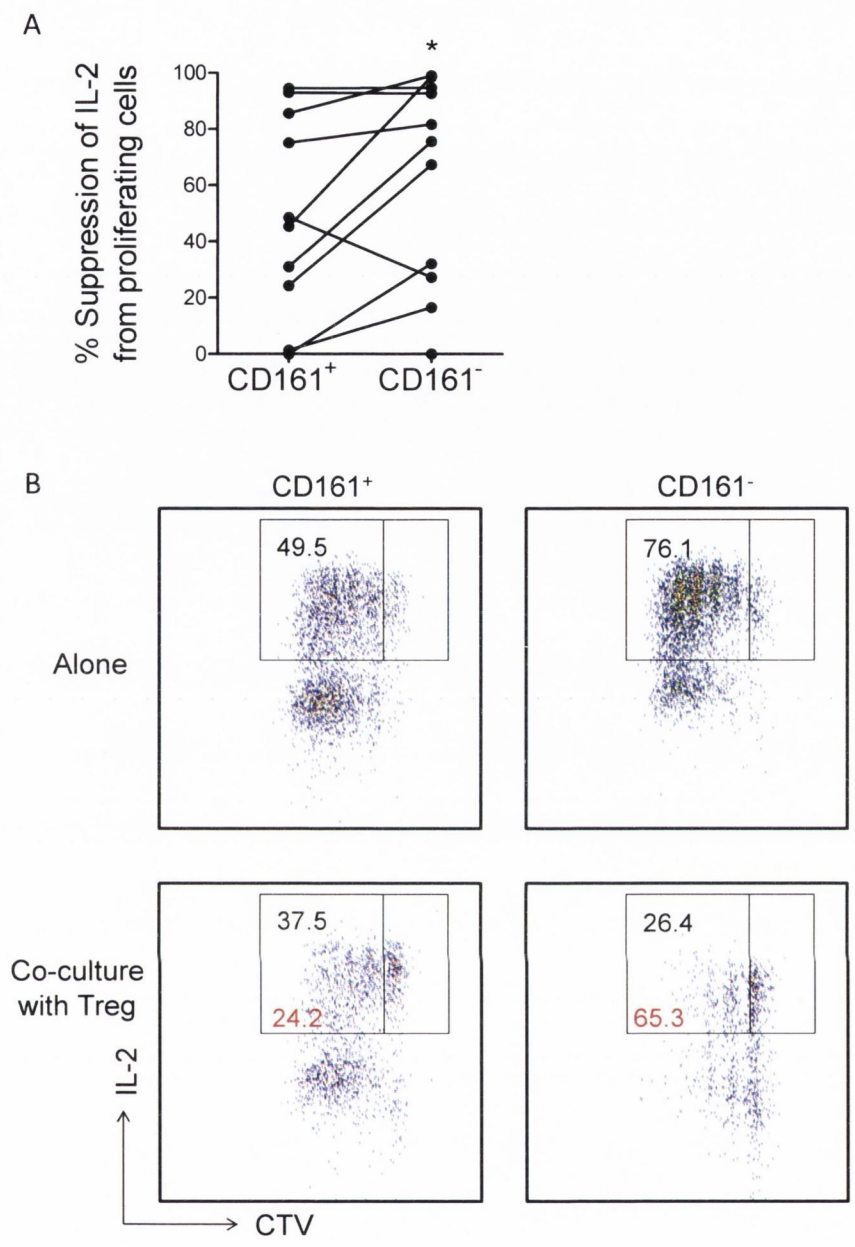


Figure 5.5 **CD161⁺ Th cells are less susceptible to Treg cell mediated suppression of IL-2 production than CD161⁻ Th cells.**

PBMC were isolated from healthy donors. CD4⁺ memory cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD25⁻ CD161⁺ and CD4⁺ CD45RO⁺ CD25⁻ CD161⁻ effector T cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled allogeneic irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for IL-2 and analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated before assessing IL-2 production. (A) Percent Treg cell mediated suppression of proliferated CD161⁺ and CD161⁻ Th cell IL-2 production. Each pair of data points represents a single independent experiment (n=11). (B) Representative FACS plots showing the frequency of IL-2 produced by the proliferating cell population (number shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of IL-2 were determined by paired T-tests with two-tailed p-values; * p<0.05.

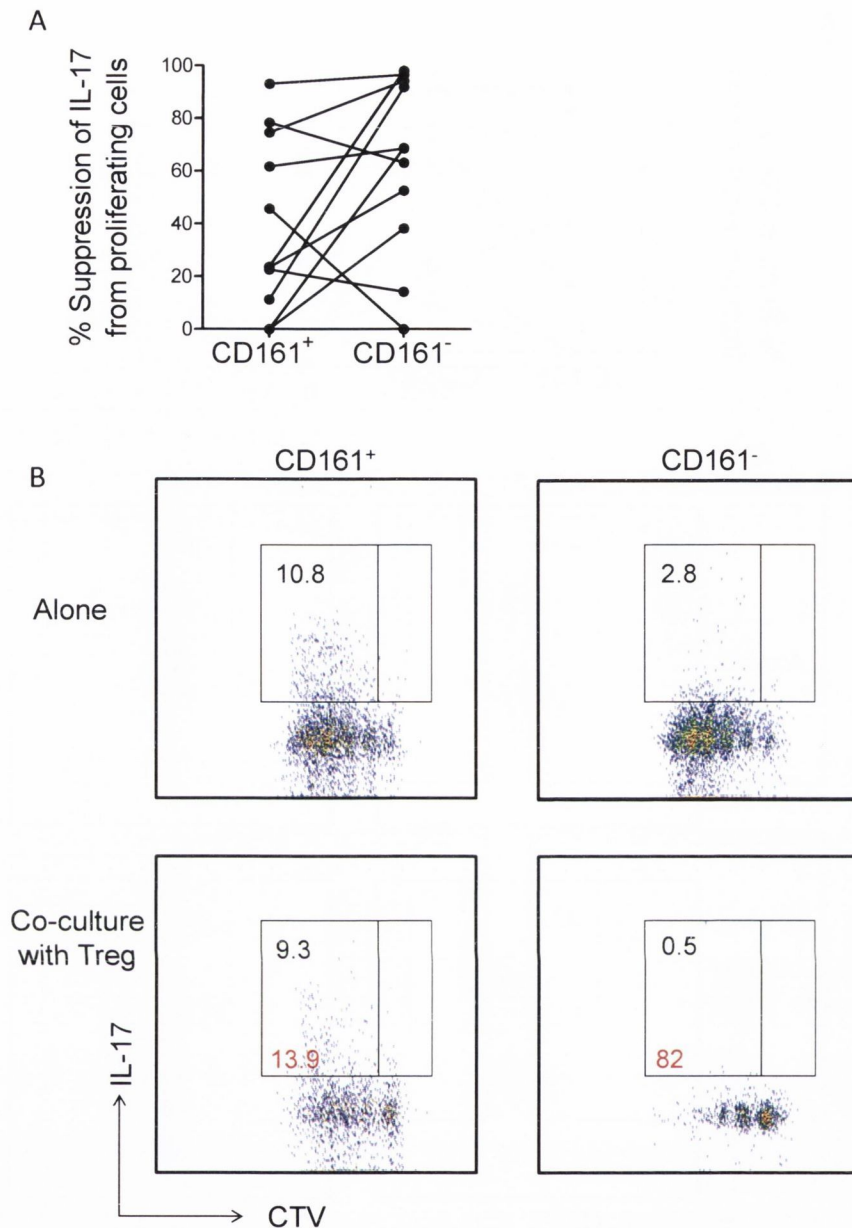


Figure 5.6 No significant difference between Treg cell mediated suppression of IL-17 in CD161⁺ CD161⁻ Th cells.

PBMC were isolated from healthy donors. CD4⁺ memory cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD25⁻ CD161⁺ and CD4⁺ CD45RO⁺ CD25⁻ CD161⁻ effector T cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled allogeneic irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for IL-17 and analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated before assessing IL-17 production. (A) Percent Treg cell mediated suppression of proliferated CD161⁺ and CD161⁻ Th cell IL-17 production. Each pair of data points represents a single independent experiment (n=12). (B) Representative FACS plots showing the frequency of IL-17 produced by the proliferating cell population (number shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of IL-17 were determined by paired T-tests with two-tailed p-values; however, the data did not reach statistical significance.

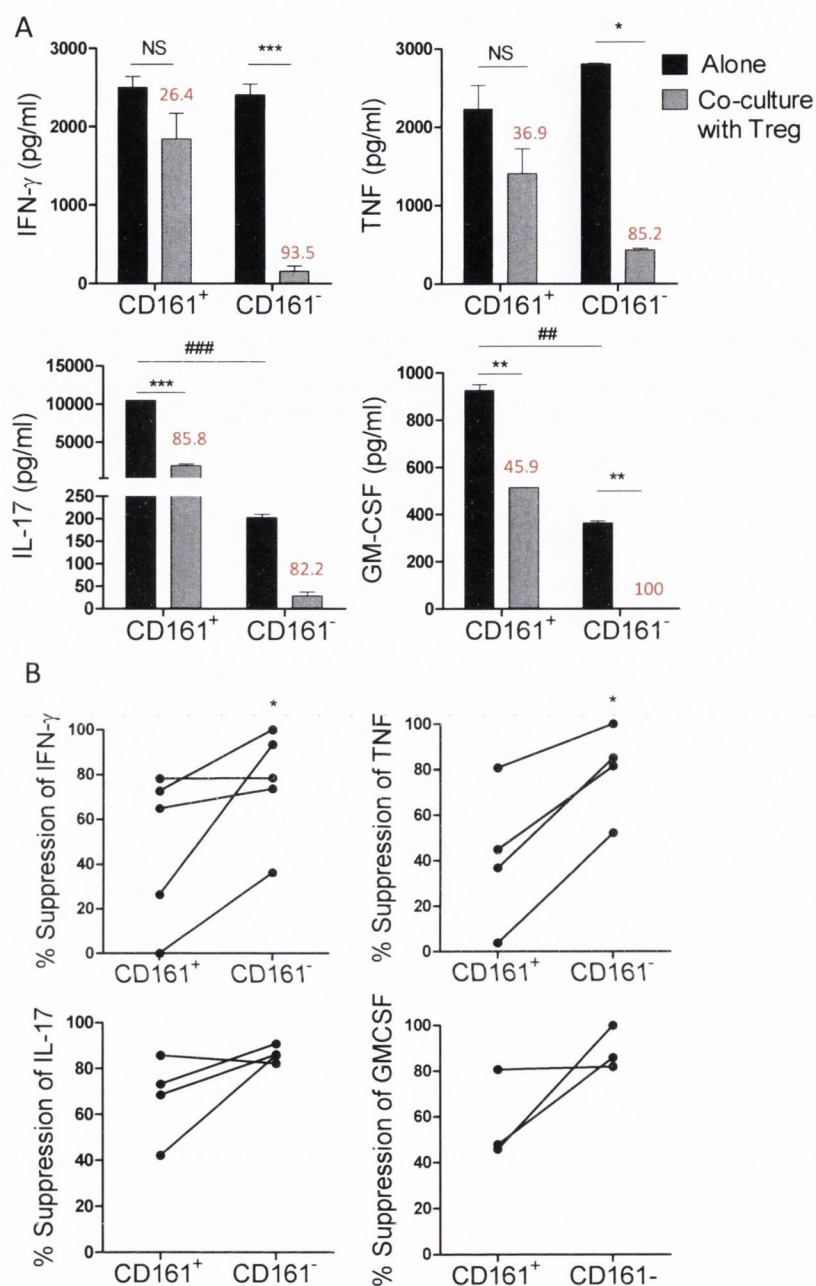


Figure 5.7 CD161⁺ Th cells are less susceptible to Treg cell mediated suppression of pro-inflammatory cytokine production than CD161⁻ Th cells.

PBMC were isolated from healthy donors. CD4⁺ memory cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD25⁻ CD161⁺ and CD4⁺ CD45RO⁺ CD25⁻ CD161⁻ effector T cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and allogeneic irradiated APC. The concentrations of IFN- γ , TNF, IL-17A and GM-CSF in the supernatants were assessed by ELISA. (A) Representative graphs illustrating the concentration of pro-inflammatory cytokines in the supernatants of effector cells cultured alone (black bars) or co-cultured with Treg cells (grey bars). The percent Treg cell mediated suppression of cytokine production is shown in red. (B) Percent Treg cell mediated suppression of pro-inflammatory cytokines. Each pair of data points represents a single independent experiment; IFN- γ (n=5), TNF (n=4), IL-17 (n=4) and GM-CSF (n=3). Statistical differences in cytokine production were determined by a 2-way ANOVA with a Bonferroni post-test; * p<0.05, ** p<0.01, *** p<0.001/ ## p<0.01, ### p<0.001 (A) or determined by a paired T-test; * p<0.05 (B).

5.2.2 Differential regulation of CD161⁺ and CD161⁻ Th cells in RA SFMC.

Having established that CD161⁺ Th cells were enriched in RA SFMC and that HC CD161⁺ Th cells were inherently less susceptible to Treg cell mediated suppression, it may be inferred that CD161⁺ Th cells would also be less well regulated in the context of the RA joint. Indeed, resistance to regulation of the highly enriched CD161⁺ Th compartment could account for the reduced suppression of RA SFMC effector cells by Treg cells that was observed in Chapter 4. In order to directly assess if the regulation of CD161⁺ Th cells was similar in the RA joint as in HC blood, suppression assays were carried out with Treg cells and Treg-depleted memory CD161⁺ and CD161⁻ Th cells sorted from the SFMC of RA patients during active disease. CD161⁺ and CD161⁻ Th cells were labelled with CTV in order to assess proliferation on day 5 post cell sorting. The data demonstrated that CD161⁺ Th cells from RA SFMC were more resistant to Treg cell mediated suppression of proliferation than their CD161⁻ Th cell counterparts in the 4 RA patients assessed; however, this trend did not reach statistical significance (figure 5.8A, top left graph). Cells were restimulated with PMA and ionomycin in the presence of brefeldin A to assess the suppression of cytokine production. A trend towards increased suppression of IFN- γ produced by CD161⁻ Th cells was observed in all 4 donors; however, the effect was very small in 2 donors hence, this data did not reach statistical significance (figure 5.8A, top right graph). The Treg cell mediated suppression of TNF was increased in CD161⁻ Th cells compared with CD161⁺ Th cells in 2 out of 4 RA patient donors whereas no difference was observed between the suppression of the Th cell subsets in the other 2 donors (figure 5.8A, bottom left graph). Treg cell mediated suppression of GM-CSF was increased in CD161⁻ Th cells compared with CD161⁺ Th cells in all 4 RA patient samples; however, this trend failed to reach statistical significance (figure 5.8A, bottom right graph). To assess the contribution of CD161⁺ Th cells to the reduced susceptibility to Treg cell mediated suppression observed in responder cells in the RA joint, CD161⁺ Th cells were next depleted from responder cells and compared with total responder cells from RA SFMC (figure 5.8B and C). Using the flow cytometry based suppression assay and labelling total responder cells and CD161-depleted responder cells with CTV and culturing alone or in co-culture with Treg cells, it was evident that depleting CD161⁺ Th cells from the responder cell population increased their susceptibility to Treg cell mediated suppression. Representative histograms illustrate that the proliferation of total responder cells from RA SFMC was suppressed by 36.7% compared with 63.5% suppression when CD161⁺ Th cells are depleted (figure 5.8B, left). Cells were restimulated with PMA and ionomycin to assess cytokine production; representative dot plots show that responder cell production of IFN- γ was suppressed by 59.7% and this increased to 73.7% when CD161⁺ Th cells were depleted from the

population (figure 5.8B, right). The results from 3 RA patients showed that the suppression of proliferation was significantly increased when CD161⁺ Th cells were depleted from the responder cell population (figure 4.8C). Additionally, the suppression of IFN- γ was also increased when CD161⁺ Th cells were depleted from RA SFMC responder cells.

The suppression of cytokines was also assessed by ELISA (figure 5.9). The data showed that CD161⁺ and CD161⁻ Th cells from RA SFMC generated comparable concentrations of IFN- γ , TNF and GM-CSF; however, the concentration of IL-17 in the supernatant, although increased in CD161⁺ Th cells, was overall very low or negligible. The concentration of IFN- γ was reduced in CD161⁺ Th cells co-cultured with Treg cells but was not significantly lower than CD161⁺ Th cells cultured alone whereas the concentration of IFN- γ produced by CD161⁻ Th cells co-cultured with Treg cells was significantly reduced compared with CD161⁻ Th cells cultured alone (figure 5.9, top left graph). The concentrations of TNF were reduced to similar extents in both Th cell subsets co-cultured with Treg cells; however, this trend was not significant (figure 5.9, top left graph). The concentration of IL-17 produced by CD161⁺ Th cells, albeit very low, was significantly reduced when CD161⁺ Th cells were co-cultured with Treg cells (figure 5.9, bottom left). The concentration of IL-17 produced by CD161⁻ Th cells was at background levels and therefore, no significant decrease was observed when CD161⁻ Th cells were co-cultured with Treg cells. The concentration of GM-CSF was reduced in CD161⁺ Th cells co-cultured with Treg cells; however, this trend was not significant (figure 5.9, bottom right graph). Conversely, Treg cells significantly reduced the concentration of GM-CSF produced by CD161⁻ Th cells.

In summary, the data elicited from suppression assays carried out on RA SFMC derived cells was insufficiently powered to yield significant results in all sets of analysis. Studies such as these are often hampered by the difficulties of working with patient samples, including low cell numbers and low cell viability. However, the trends in the data suggest that, similar to the case for CD161⁺ Th cells from healthy donors, CD161⁺ Th cells from RA SFMC may be less effectively regulated by autologous Treg cells than CD161⁻ Th cells. This data was strengthened by evidence showing that depletion of CD161⁺ Th cells from the responder cell population in RA SFMC increased the extent of Treg cell mediated suppression compared with total responder cells.

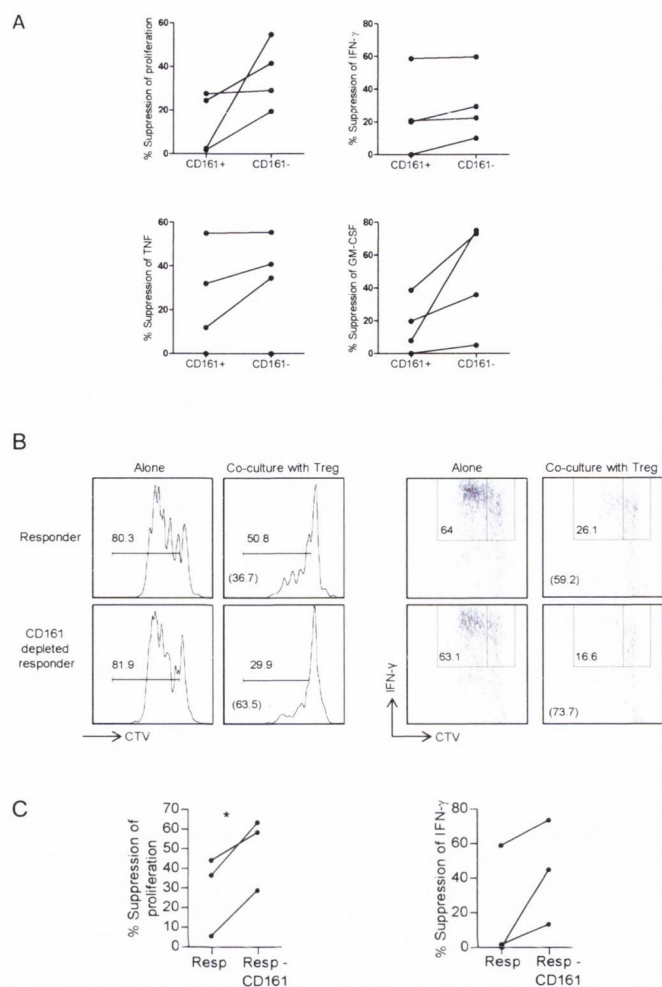


Figure 5.8 Trend towards reduced susceptibility of CD161⁺ Th cells to Treg cell mediated suppression whereas depletion of CD161⁺ Th cells increases responder cell susceptibility to suppression in cells from RA SFMC.

RA SFMC were isolated from patients during active disease. CD4⁺ CD45RO⁺ CD25⁻ (total responder cells), CD4⁺ CD45RO⁺ CD25⁻ CD161⁺ and CD4⁺ CD45RO⁺ CD25⁻ CD161⁻ effector T cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated, allogeneic APC. Cells were analysed by flow cytometry; proliferation was quantified by CTV dilution and cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for IFN- γ , TNF and GM-CSF. (A) Each pair of data points represents a single independent experiment (n=4). (B) Histograms show the frequency of total responder cell (top) and CD161-depleted responder cell (bottom) proliferation (by CTV dilution) in cells cultured alone (left) and co-cultured with Treg cells (right). The percent suppression of proliferation in the presence of the Treg cells is shown in parentheses. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, for cytokine production. Dot plots show the frequency of proliferated cells (CTV^{lo}) producing IFN- γ ; the percent suppression of IFN- γ production is shown in parentheses. (C) Graphs show the percent suppression of proliferation and IFN- γ production in responder cells versus CD161-depleted responder cells in 3 RA patient donors. *p<0.05.

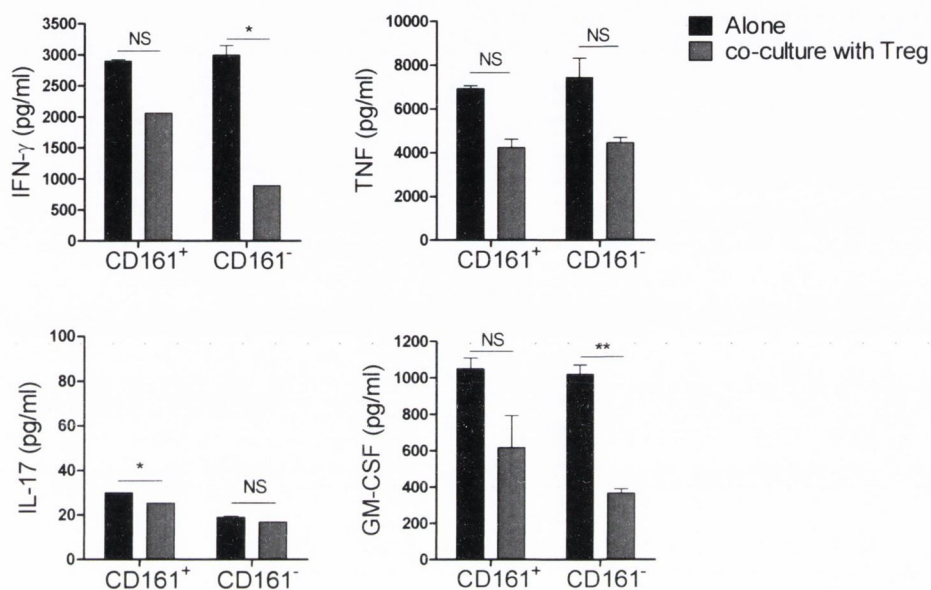


Figure 5.9 Differential Treg cell mediated suppression of RA SF CD161⁺ and CD161⁻ Th cells.

RA SFMC were isolated from RA patients during active disease. CD4⁺ CD45RO⁺ CD25⁻ CD161⁺ and CD4⁺ CD45RO⁺ CD25⁻ CD161⁻ effector T cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and allogeneic irradiated APC. The concentrations of IFN-γ, TNF, IL-17A and GM-CSF in the supernatants were assessed by ELISA. Representative graphs illustrating the concentration of pro-inflammatory cytokines in the supernatants of effector cells cultured alone (black bars) or co-cultured with Treg cells (grey bars). Statistical differences in cytokine production were determined by a 2-way ANOVA with a Bonferroni post-test; * p<0.05, **p<0.01. This data is representative of 2 independent experiments.

5.2.3 ex-Th17 type cells are enriched in the RA joint SF.

In chapter 3, the data demonstrated that CD161⁺ Th cells were enriched in the RA joint (figure 3.6A, left graph) and furthermore, increased frequencies of CD161⁺ Th cells from RA SFMC produced IFN- γ compared with RA and HC PBMC (figure 3.6B). This evidence inferred that the CD161⁺ Th17-type cells in the RA joint were mostly Th17 cells that had switched to become ex-Th17 type cells. In order to directly assess the frequency of Th1, Th17 and ex-Th17 cells in the RA joint, RA SFMC, RA PBMC and HC PBMC were stained *ex vivo* for CD4, CD45RO, CD161, CXCR3, CCR4 and CCR6.

FACS plots show the overall gating strategy; firstly expression of CD161 versus CXCR3 was examined to broadly categorize Th1, Th17 and ex-Th17 cells (figure 5.10A, upper plot). The data indicated that there was no difference between the frequency of CD161⁻ CXCR3⁺ (broadly Th1-type) memory cells in HC PBMC and RA SFMC, however, RA PBMC exhibited a significantly increased frequency of CD161⁻ CXCR3⁺ Th cells compared with HC PBMC (figure 5.10A, lower panel, left graph). The frequency of CD161⁺ CXCR3⁻ (broadly Th17-type) memory cells were slightly elevated in RA SFMC compared with HC and RA PBMC, however, this trend was not significant (figure 5.10A, lower panel middle graph). The frequency of memory cells co-expressing CD161 and CXCR3 (broadly ex-Th17 type cells) were significantly increased in RA SFMC compared with HC PBMC (figure 5.10A, lower panel, right graph). In addition, RA PBMC exhibited a trend toward elevated CD161⁺ CXCR3⁺ co-expression compared with HC PBMC (figure 5.10A, lower panel, right graph).

Secondly, CCR4 and CCR6 expression was used to further define the Th subsets into specific Th1, Th17 and ex-Th17 populations (figure 5.10B, upper panel). Th1 (CD161⁻ CXCR3⁺ CCR4⁻ CCR6⁻) cells were significantly increased in RA PBMC compared with HC PBMC (figure 5.10B, lower panel, left graph). *Bona fide* Th1 (CD161⁻ CXCR3⁺ CCR4⁻ CCR6⁻) and Th17 (CD161⁺ CXCR3⁻ CCR4⁺ CCR6⁺) cells were increased in RA SFMC; however, this trend was not significant (figure 5.10B, lower panel, left and middle graphs). Interestingly, the expression of ex-Th17 type cell markers (CD161⁺ CXCR3⁺ CCR6⁺) were significantly increased in RA SFMC compared with HC PBMC (figure 5.10B, lower panel, right graph). Furthermore, RA PBMC exhibited a trend towards increased frequencies of ex-Th17 cells compared with HC PBMC (figure 5.10B, lower panel, right graph).

In summary, this data indicates that ex-Th17 type cells were significantly enriched during autoimmune inflammation in the RA joint.

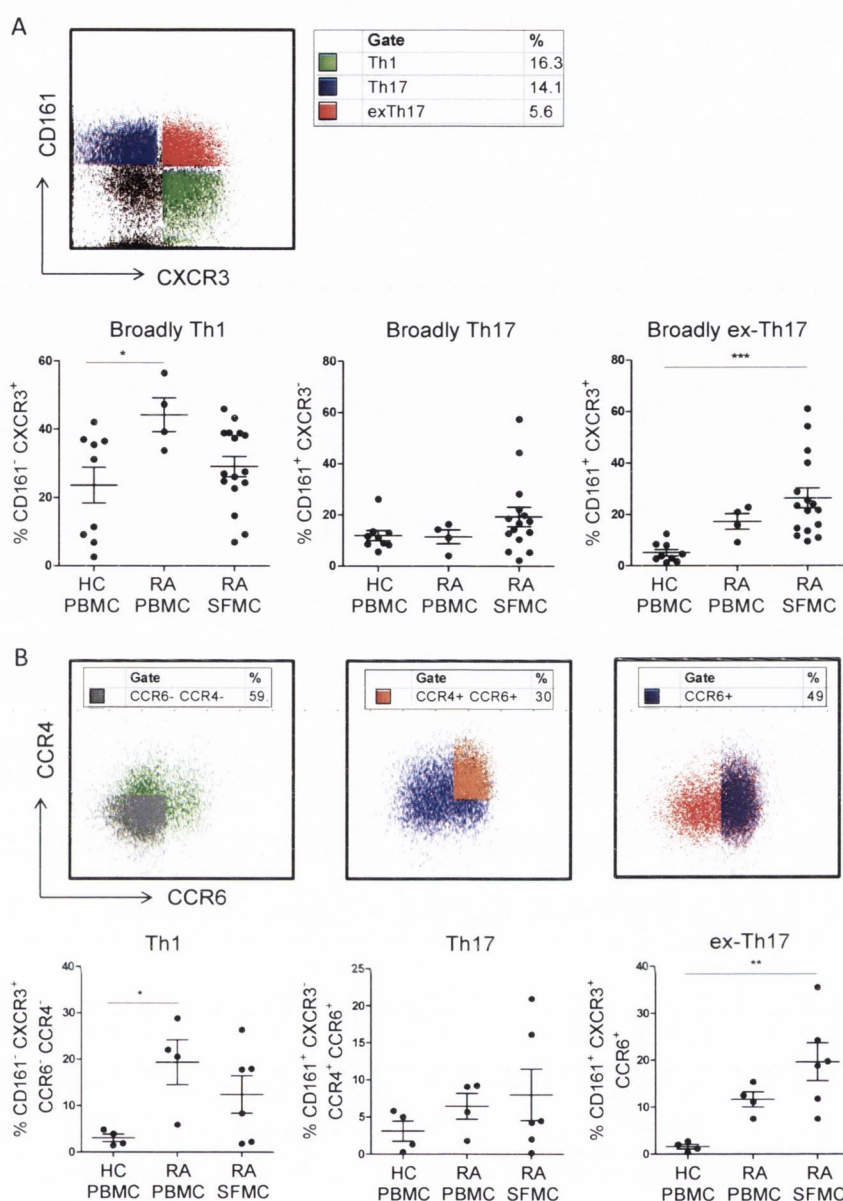


Figure 5.10 Ex-Th17 cells are significantly enriched in RA SFMC compared with HC PBMC.

RA SFMC and PBMC were isolated from RA patients with active disease and compared with HC PBMC. Cells were stained with fluorochrome-conjugated antibodies specific for CD4, CD45RO, CD161, CXCR3, CCR4 and CCR6 and analysed by flow cytometry. (A) FACS plots show the gating strategy for Th1, Th17 and ex-Th17 cells on the basis of CD161 and CXCR3 expression (top plot). The graphs illustrate the frequency of Th17 type cells ($CD161^+ CXCR3^-$), Th1-type cells ($CD161^- CXCR3^+$) and ex-Th17 type cells ($CD161^+ CXCR3^+$) in RA SFMC ($n=16$) compared with HC PBMC ($n=9$) and RA PBMC ($n=4$). (B) Representative FACS plots show CCR expression within the broad CD161/CXCR3 gates; Th1 cells are identified as $CD161^- CXCR3^+$ cells (green) which do not express CCR6 or CCR4 (grey). Th17 cells are $CD161^+ CXCR3^-$ (blue) and express both CCR4 and CCR6 (orange). Ex-Th17 cells are $CD161^+ CXCR3^+$ (red) and express CCR6 (purple). The graphs illustrate the frequency of Th1, Th17 and ex-17 (based on CD161, CXCR3 and CCR expression) in the memory CD4 population of RA SFMC ($n=6$), RA PBMC ($n=4$) and HC PBMC ($n=4$). Statistical differences were analysed by one-way ANOVA with Tukey's multiple comparison post tests; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.2.4 Characteristics of Th1, Th17 and ex-Th17 cells.

Having established that CD161⁺ and CD161⁻ Th cells were differentially regulated and that it is the ex-Th17 component of CD161⁺ Th cells that were particularly enriched in RA SFMC, the next question to be addressed was if this evasion of Treg cell mediated suppression could be attributed to either Th17 or ex-Th17 cells within the gross CD161⁺ Th population. Initially, the characteristics of Th1, Th17 and ex-Th17 cells were compared to deduce the discrete differences between bona fide Th17 cells and ex-Th17 type cells as well as to examine the differences between bona fide Th1 cells and ex-Th17 type cells, which are referred to in the literature as “non-classical Th1” cells due to their production of IFN- γ . A fuller understanding of the characteristics of these cells will be important in deciphering their pathogenic role in autoimmunity.

5.2.4a Ex-Th17 cells have a distinct and significantly more polyfunctional cytokine profile compared with Th1 and Th17 cells.

Differential cytokine expression was examined in Th1, Th17 and ex-Th17 cells. Th cell subsets were sorted by FACS according to the methods described in section 2.7 and the gating strategy shown in figure 2.7. Sorted cells were cultured for 5 days in the presence of anti-CD3 and irradiated, CFSE labelled APC, then restimulated with PMA and ionomycin in the presence of brefeldin A, stained for intracellular cytokines and analysed by flow cytometry. Boolean combinations from multi-parameter flow cytometry were collated using SPICE software. The diagrams represent 5 cytokines produced in every possible combination with each other in the Th subsets averaged and over-laid from 3 independent experiments (figure 5.11). For parity, all inactive cells (cells not producing any of the cytokines examined) have been excluded. The colours within the pie chart represent different populations producing different combinations of cytokines and the relative size of each segment correlates to the frequency of the population. The abridged legend for the pie chart segments shows population greater than 1%. A full legend for the colours within the pie chart is available in appendix 2 (figure app. 2.3). The arcs surrounding the pie charts represent the particular cytokines being produced (see arc legend). All the area lying under the circumference of the arc represents the frequency of cells producing that cytokine. Areas of the pie chart surrounded by multiple arcs are polyfunctional cells, producing all the cytokines denoted by the arc under which they lie. The prominent orange slice in ex-Th17 cells denoted cells that were producing GM-CSF (red arc), IFN- γ (blue arc), IL-2 (green arc) and TNF (purple arc) concomitantly. This proportion of cells was significantly larger in ex-Th17 cells compared with Th17 and Th1 cells. In addition, the yellow slice, signifying the proportion of ex-Th17 cells that produced GM-CSF (red arc), IFN- γ (blue arc) and TNF (purple)

concomitantly, was significantly increased compared with Th1 and Th17 cells. The frequency of bi-functional Th1 and Th17 cells co-producing IL-2 (green arc) and TNF (purple arc) were significantly increased compared with ex-Th17 cells.

In summary, the SPICE data indicates that the cytokine profile of ex-Th17 cells was significantly different to Th1 and Th17 cells. In addition, ex-Th17 cells were significantly more polyfunctional than Th1 and Th17 cells as illustrated by a higher proportion of the circumference of the pie chart being surrounded by 3 or more arcs.

5.2.4b Ex-Th17 cells are mainly effector memory cells and proliferate *in vivo* to a similar extent as Th1 cells but have increased proliferation *in vitro* in response to anti-CD3.

The proliferation of Th1, Th17 and ex-Th17 subsets *in vivo* was assessed by Ki67 staining *ex vivo*. Th subsets were identified from total HC PBMC on the basis of CD161, CXCR3, CCR4 and CCR6 expression (figure 2.7) and the frequencies of these subsets expressing Ki67 were analysed (figure 5.12A). The frequency of Th17 cells actively partaking in the cell cycle were significantly reduced compared with Th1 cells. Th1 and ex-Th17 cells exhibited similar frequencies of Ki67 expression. In order to determine differential proliferative responses to TCR stimulation, proliferation was assessed by CTV dilution after anti-CD3 stimulation *in vitro*. Th1, Th17 and ex-Th17 cells were sorted by FACS, labelled with CTV and cultured for 5 days in the presence of anti-CD3 and irradiated APC. Proliferation was assessed by CTV dilution; the representative histograms showed that 63.5% of Th1 cells had proliferated compared with only 45.3% of Th17 cells and 87.8% of ex-Th17 cells (figure 5.12B). These data suggested that ex-Th17 cells had a greater capacity for proliferation in response to TCR stimulation compared with Th1 and Th17 cells, while Th17 cells had the lowest capacity for proliferation.

In order to ascertain if differences existed between the frequencies of central or effector memory cells in Th1, Th17 and ex-Th17 cell subsets within the total PBMC population, cells were stained for CD4, CD45RO, CCR7, CD161, CXCR3, CCR4 and CCR6 and assessed by flow cytometry. Th1 cells contained significantly higher frequencies of central memory cells compared with Th17 and ex-Th17 cells (figure 5.12C, left). Conversely, both Th17 and ex-Th17 cells contained significantly higher frequencies of effector memory cells compared with Th1 cells (figure 5.12C, right).

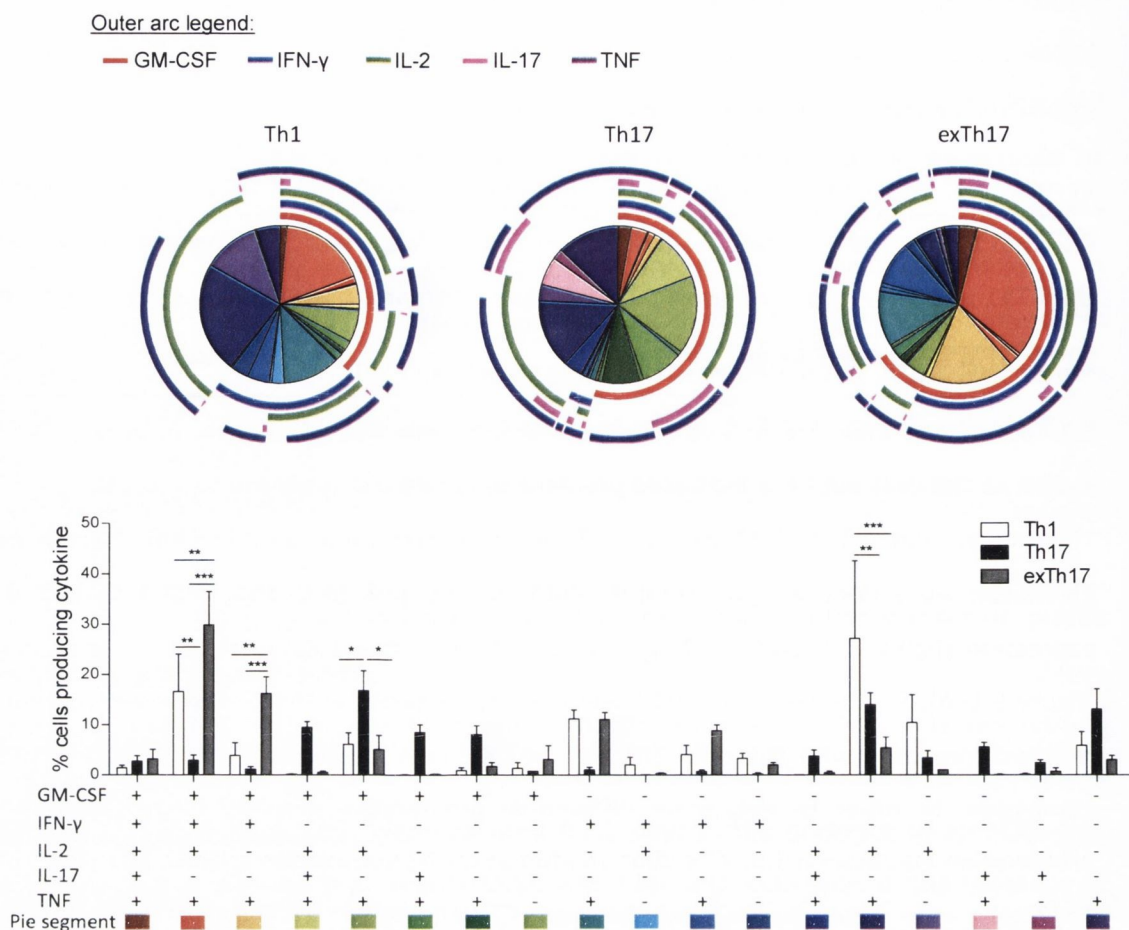


Figure 5.11 SPICE analysis illustrates that ex-Th17 type cells have a distinct cytokine profile and are more polyfunctional compared with Th1 and Th17 cells.

PBMC were isolated from healthy donors. CD4⁺ memory T cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD161⁻ CXCR3⁺ CCR4⁻ CCR6⁻ Th1, CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁻ CCR4⁺ CCR6⁺ Th17 and CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁺ CCR6⁺ ex-Th17 effector cells were sorted by FACS. Cells were cultured for 5 days in the presence of anti-CD3 and CFSE labelled, irradiated APC. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A, stained with fluorochrome-conjugated antibodies specific for GM-CSF, IFN- γ , IL-2, IL-17 and TNF and analysed by flow cytometry; dead cells and doublets were excluded and CFSE⁻ cells were gated to assess cytokine production in the sorted subsets. The pie charts represent the frequencies of active cytokine-producing cells that produce every possible combination of the 5 cytokines analysed. The different colours of each segment within the pie chart denote populations producing different combinations of cytokines. The relative sizes of the pie segment correlates to the frequency of the particular population (see bar graph legend; abridged to show only populations greater than 1%). The arcs around the circumference of the pie charts indicate that a particular cytokine (see pie chart arc legend) is produced by the proportion of cells in the pie chart that lie under the arc. Parts of the pie chart surrounded by multiple arcs are, therefore, polyfunctional cells. Statistical differences between subsets were determined by two-way ANOVA with Bonferroni post-tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. For full pie chart legend, see appendix 2 (figure app. 2.3). SPICE charts represent data averaged and overlaid from 3 independent experiments.

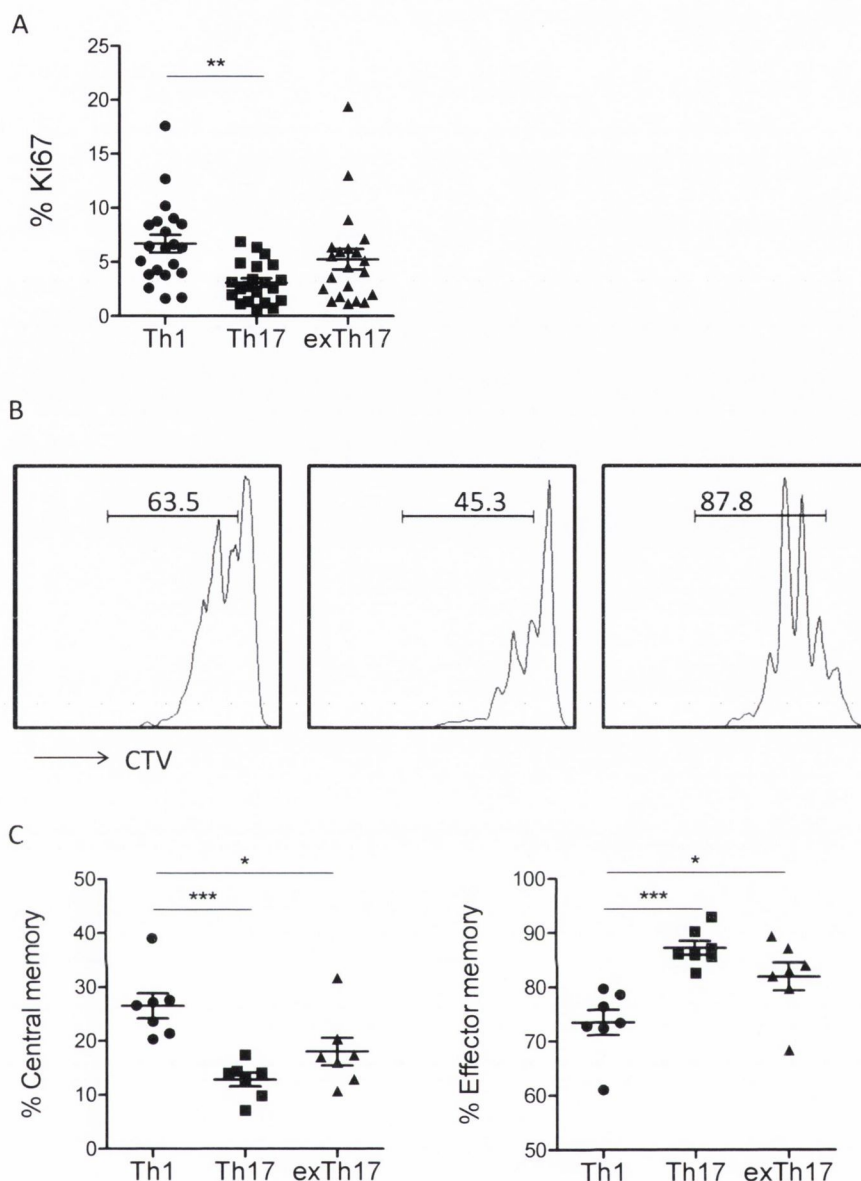


Figure 5.12 Phenotype of Th1, Th17 and ex-Th17 cells.

PBMC were isolated from healthy donors, stained with fluorochrome-conjugated antibodies specific for CD4, CD45RO, CD161, CXCR3, CCR4, CCR6, Ki67 and CCR7 and analysed by flow cytometry. Lymphocytes were identified on the basis of forward and side scatter. Dead cells and doublets were excluded. $CD4^+ CD45RO^+$ cells were gated followed by subset gating before analysing Ki67 or CCR7 expression. The graph shows the frequency on Ki67 expression within each subset (A). $CD4^+$ memory cells were purified from HC PBMC by MACS. $CD4^+ CD45RO^+ CD161^- CXCR3^+ CCR4^- CCR6^-$ Th1, $CD4^+ CD45RO^+ CD161^+ CXCR3^- CCR4^+ CCR6^-$ Th17 and $CD4^+ CD45RO^+ CD161^+ CXCR3^+ CCR6^+$ ex-Th17 effector cells were sorted by FACS. Effector cells were labelled with CTV and cultured for 5 days in the presence of anti-CD3 and CFSE labelled irradiated APC. $CTV^+ CFSE^-$ cells were gated and proliferation was measured by CTV dilution (B). This data is representative of 3 independent experiments. (C) The graphs show the frequency of Th1, Th17 and ex-Th17 cells which are central memory (left) and effector memory (right). Statistical differences were determined using a one-way ANOVA with Tukey's post-tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2.4 Th17 cells switch to producing IFN- γ *in vitro*.

In order to assess the stability of sorted Th17 cells *in vitro*, an experiment was designed to track the induction of IFN- γ production in Th17 cells over time. Given that much of the literature regarding Treg cell mediated suppression of Th17 cells is ambiguous, it was necessary to ascertain if these cells switched their phenotype *in vitro* and the timepoints at which they can still be deemed bona fide Th17 cells. It was hypothesized that bona fide Th17 and ex-Th17 cells may be differentially regulated; therefore the Th17 cell populations that were expanded *in vitro* in the literature may be including an ex-Th17 cell population in some studies and causing disparate conclusions regarding the suppression of Th17 cells.

Th1 and Th17 cells were FACS sorted to high purity on the basis of CD161, CXCR3, CCR4 and CCR6 expression, as previously described. Cells were cultured for 18 days in the presence of IL-2, anti-CD3 and CFSE labelled irradiated APC. Supernatants were harvested on day 5, 12 and 18 to measure the concentrations of pro-inflammatory cytokines produced by Th1 and Th17 cells (figure 5.13A). Th17 cells (white bars) produced consistently high concentrations of IL-17 at the 3 time points assessed. Th1 cells (black bars) did not produce IL-17, as expected. On day 5, Th1 cells produced a high concentration of IFN- γ , which was significantly greater than that produced by Th17 cells. However, by day 12, the concentration of IFN- γ produced by Th1 and Th17 cells was comparable; a significant increase was observed in the concentration of IFN- γ produced by Th17 cells on day 12 compared with day 5. By day 18, Th17 cells produced significantly more IFN- γ than Th1 cells. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A on day 0, 7 and 14 and frequencies of cells producing IFN- γ and IL-17 were analysed by flow cytometry (figure 5.13B). Cells were analysed on day 0, directly post sorting; 62% of sorted Th1 cells produced IFN- γ and 14.7% of Th17 cells produced IL-17. By day 7 in culture, 68.4% of Th1 cells produced IFN- γ and 17.7% of Th17 cells produced IFN- γ only. 7.5% of Th17 cells were co-producing IFN- γ and IL-17 and 17.2% were producing IL-17 only. On day 14, 81.1% of Th1 cells and 38% of Th17 cells produced IFN- γ only. 12.1% of Th17 cells co-produced IFN- γ and IL-17 and 13.7% produced IL-17 only. Notably, no appreciable frequencies of Th1 cells produced IL-17 at any stage during the experiment.

In summary, this data indicates that Th17 cells can spontaneously switch to IFN- γ producing ex-Th17 type cells *in vitro* when stimulated with anti-CD3 in the presence of irradiated APC. Despite the up-regulated production of IFN- γ protein, the production of IL-17 remained high consistently throughout the course of the experiment as it was produced by both bona fide, Th17 cells and ex-Th17 cells that co-produced IL-17 and IFN- γ .

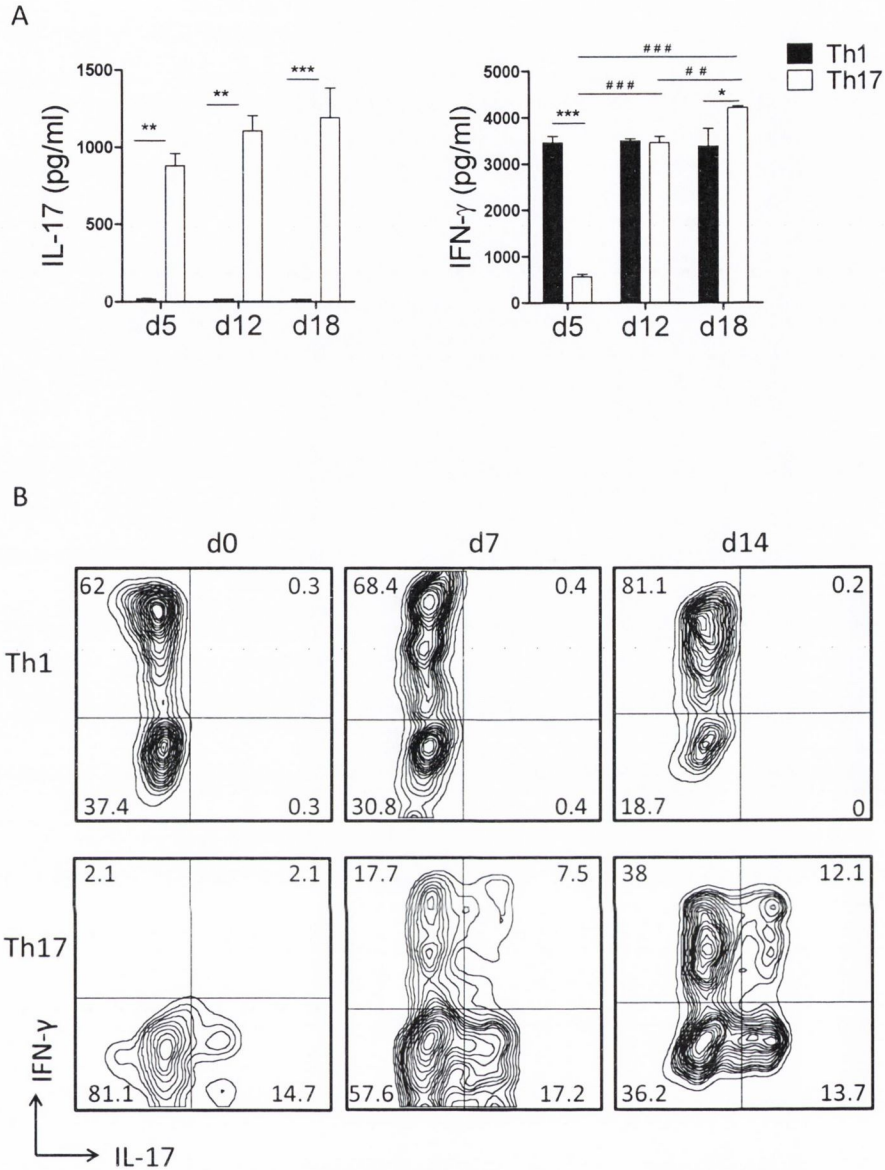


Figure 5.13 The cytokine profile of Th17 cells shifts towards IFN-γ production after *in vitro* stimulation.

CD4⁺ memory cells were purified from HC PBMC by MACS. CD4⁺CD45RO⁺CD161⁻CXCR3⁺CCR4⁻CCR6⁻ Th1, CD4⁺CD45RO⁺CD161⁺CXCR3⁻CCR4⁺CCR6⁺ Th17 and CD4⁺CD45RO⁺CD161⁺CXCR3⁺CCR6⁺ ex-Th17 effector cells were sorted by FACS. Cells were cultured for 18 days in the presence of anti-CD3 and CFSE labelled, irradiated APC. Cells were fed every 5 days with IL-2 and restimulated with fresh anti-CD3 and irradiated APC on day 8. Supernatants were harvested on day 5, day 12 and day 18 to assess the concentration of IFN-γ, IL-17 and TNF produced from Th1 and Th17 cells (A). On day 0, 7 and 14 post-sorting, cells were stimulated with PMA and ionomycin in the presence of brefeldin A, stained with fluorochrome-conjugated antibodies specific for IFN-γ and IL-17 and analysed by flow cytometry (B). Statistical differences between Th1 and Th17 cells were determined by two-way ANOVA with Bonferroni post-tests; *p<0.05, **p<0.01, ***p<0.001. Statistical differences within a subset over time were determined by two-way ANOVA with Bonferroni post-tests; #p<0.05, ###p<0.001. This data is representative of 3 independent experiments.

5.2.5 Differential Treg cell mediated suppression of Th1, Th17 and ex-Th17 cells.

This work has thus far established that CD161⁺ memory Th cells:

- were enriched in the RA joint
- exhibited enhanced expression of pro-survival proteins (Bcl2 and HIF-1 α)
- have relatively longer telomeres but proliferate to the same extent as CD161⁺ memory Th cells
- exhibit highly polyfunctional cytokine expression
- induce an enhanced pro-inflammatory phenotype from fibroblast cells
- were relatively resistant to Treg cell mediated suppression

The CD161⁺ memory Th cell subset comprised bona fide Th17 cells and ex-Th17 type cells. The above study has demonstrated that ex-Th17 type cells were more polyfunctional, in terms of cytokine production, and actively proliferated more than bona fide Th17 cells. In addition, ex-Th17 type cells were significantly enriched in RA SFMC compared with HC PBMC. Because these cells were enriched in RA SFMC, the next question to be addressed was if the resistance to Treg cell mediated suppression observed in the RA joint and in HC CD161⁺ Th cells could be attributed to a particular subset within the gross CD161⁺ population; namely, ex-Th17 type cells. Evidence in the literature suggests that Th1 and Th17 cells are both regulated by Treg cells, however, IL-17 producing Th17 cells exhibit reduced sensitivity to regulation compared with IFN- γ producing Th1 cells (Fletcher et al., 2009). There is no robust evidence, to date, on differential Treg cell mediated suppression of Th1, Th17 and ex-Th17 cells.

5.2.5a Ex-Th17 cells are significantly less susceptible to Treg cell mediated suppression of proliferation and pro-inflammatory cytokine production compared with Th1 and Th17 cells.

The flow cytometry based suppression assay was utilised to examine differential Treg cell mediated suppression of Th1, Th17 and ex-Th17 cells. Th1, Th17 and ex-Th17 cells were sorted by FACS (as described in section 2.7, figure 2.7). Th subsets were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of CFSE labelled, irradiated APC. Responder cell proliferation was measured by CTV dilution. The Treg cell mediated suppression of proliferation was significantly reduced in ex-Th-17 cells, in 11 independent experiments, compared with both Th1 and Th17 cells (figure 5.14A). Representative histograms show that Th1 and ex-Th17 cells proliferated to a similar extent whereas Th17 cells had a somewhat reduced frequency of proliferating cells (figure 5.14B, upper panel). When Th1 cells were co-cultured with Treg cells, proliferation was reduced from 92.7% to 55.1% (a suppression of 40.6%; figure 5.14B lower panel). Similarly, when Th17 cells were co-

cultured with Treg cells, proliferation was reduced from 83.2% to 45.2% (45.7% suppression). However, when ex-Th17 cells were co-cultured with Treg cells, proliferation was reduced from 95% to 86.8% (only 8.6% suppression).

Cells were restimulated with PMA and ionomycin in the presence of brefeldin A to assess pro-inflammatory cytokine production. The suppression of IFN- γ was examined in 9 independent experiments; Treg cell mediated suppression of IFN- γ produced by ex-Th17 cells was decreased compared with the suppression of IFN- γ from Th1 and Th17 cells (figure 5.15A). Interestingly, Treg cell mediated suppression of IFN- γ was significantly higher in Th17 cells compared with ex-Th17 cells. Low frequencies of Th17 cells expressed IFN- γ on day 5, suggesting that they were transitioning *in vitro* to become ex-Th17 type cells which may be effectively suppressed by Treg cells early in their transition.

Next, the Treg cell mediated suppression of TNF was analysed in Th1, Th17 and ex-Th17 cells from 8 independent experiments. Ex-Th17 cells exhibited significantly reduced Treg cell mediated suppression of TNF compared with both Th1 and Th17 cells (figure 5.16A). Representative pseudocolour plots illustrate that 46.7% of Th1 cells were proliferating and producing TNF and in the presence of Treg cells, this was effectively reduced to 25.6% (45.2% suppression; figure 5.16B, left). 51.7% of Th17 cells were proliferating and producing TNF, this frequency was reduced to 22.2% in the presence of the Treg cell (57% suppression; figure 5.16B, middle). 68.1% of ex-Th17 cells were proliferating and producing TNF and this frequency was unaffected by the presence of Treg cells (0% suppression; figure 5.16B, right).

The suppression of GM-CSF was examined in 9 independent experiments and was significantly reduced in ex-Th17 cells compared with Th1 cells (figure 5.17A). The suppression of GM-CSF in Th17 cells was higher compared with ex-Th17 but lower than Th1 cells, however, this trend was not statistically significant. Representative pseudocolour plots illustrate that 40.9% of Th1 cells were proliferating and producing GM-CSF and in the presence of Treg cells, this was effectively reduced to 14.4% (64.8% suppression; figure 5.17B, left). 35% of Th17 cells were proliferating and producing GM-CSF, this frequency was reduced to 11.7% in the presence of the Treg cell (66.6% suppression; figure 5.17B, middle). 51.1% of ex-Th17 cells were proliferating and producing GM-CSF and this frequency is in fact, increased to 59% in the presence of the Treg cell (0% suppression; figure 5.17B, right).

The same trend was observed for the suppression of IL-2; on average, Th1 cells exhibited the largest degree of suppression, followed by Th17 cells with ex-Th17 cells exhibiting the lowest

degree of Treg cell mediated suppression (figure 5.18A). However, this trend was not statistically significant due to variance caused by several data points at zero on the axis; 2/8 in Th1 cells, 4/8 in Th17 cells and 6/8 in ex-Th17 cells. Representative pseudocolour plots illustrate that 42.8% of Th1 cells were proliferating and producing IL-2 and in the presence of Treg cells, this was effectively reduced to 25.8% (39.7% suppression; figure 5.18B, left). 26% of Th17 cells were proliferating and producing IL-2, this frequency was reduced to 10.4% in the presence of the Treg cell (60% suppression; figure 5.18B, middle). 44.5% of ex-Th17 cells were proliferating and producing IL-2 and this frequency was in fact, increased to 54.1% in the presence of the Treg cell (0% suppression; figure 5.18B, right).

The suppression of IL-17 produced by Th17 and ex-Th17 cells was examined in 11 independent experiments and was significantly reduced in ex-Th17 cells compared with Th17 cells (figure 5.19A). Representative pseudocolour plots illustrate that only a background frequency of Th1 cells were proliferating and producing IL-17 (figure 5.19B, left). 20.7% of Th17 cells were proliferating and producing IL-17, this frequency was reduced to 6.5% in the presence of the Treg cell (68.6% suppression; figure 5.19B, middle). 7.4% of ex-Th17 cells were proliferating and producing IL-17 and this frequency was reduced to 5% in the presence of the Treg cell (32.4% suppression; figure 5.19B, right).

In summary, the data from these flow cytometry based suppression assays demonstrated that ex-Th17 cells were significantly less susceptible to Treg cell mediated suppression of proliferation and pro-inflammatory cytokines when compared with Th1 and Th17 cells.

5.2.5b The concentrations of accumulated pro-inflammatory cytokines indicate that ex-Th17 cells are less susceptible to Treg cell mediated suppression compared with Th1 and Th17 cells.

The concentrations of pro-inflammatory cytokines in the supernatants of sorted Th1, Th17 and ex-Th17 cultured alone or co-cultured with Treg cells were quantified by ELISA (figure 5.20). A representative graph shows the concentration of IFN- γ produced by Th1 cells was significantly reduced when cells were co-cultured with Treg cells (figure 5.20A, upper left graph; black versus grey bars). Furthermore, this data demonstrates that IFN- γ produced by ex-Th17 cells was not suppressed in the presence of Treg cells. When the percent suppression of the concentration of IFN- γ was calculated in 3 independent experiments, the suppression of ex-Th17 cells was reduced compared with Th17 cells and significantly reduced compared with Th1 cells (figure 5.20B, upper left graph).

The concentrations of TNF produced by Th1, Th17 and ex-Th17 cells were significantly reduced in the presence of the Treg cell in this example, however, ex-Th17 cells were producing significantly more TNF compared with Th1 and Th17 cells and the concentration of TNF observed in the supernatants of ex-Th17 cells co-cultured with Treg cells was comparable to the concentrations elicited by Th1 and Th17 cultured alone (figure 5.20A, upper right graph). When the percent suppression of the concentration of TNF was calculated in 4 independent experiments, the suppression of ex-Th17 cells was reduced compared with Th17 and Th1 cells; however, this data was not significant (figure 5.20B, upper right graph).

The concentrations of GM-CSF produced by Th1, Th17 and ex-Th17 cells were significantly reduced in all subsets in the presence of Treg cells, however, the extent of suppression differed; Th1 cells were 100% suppressed, Th17 were suppressed by 72% and ex-Th17 cells were suppressed by 40% (figure 5.20A, lower left graph). When the percent suppression of the concentration of GM-CSF was calculated in 2 independent experiments, the suppression of Th17 cells was reduced compared with Th1 cells and the suppression of ex-Th17 cells was further reduced compared with Th17 cells; however, this data was not significant (figure 5.20B, lower left graph).

Only Th17 cells produced a significant concentration of IL-17, which was effectively suppressed by Treg cells (59%; figure 5.20A lower right graph). When the percent suppression of the concentration of IL-17 was calculated in 4 independent experiments, the suppression of ex-Th17 cells was reduced compared with Th17 cells; however, this data was not significant (figure 5.20B, lower right graph).

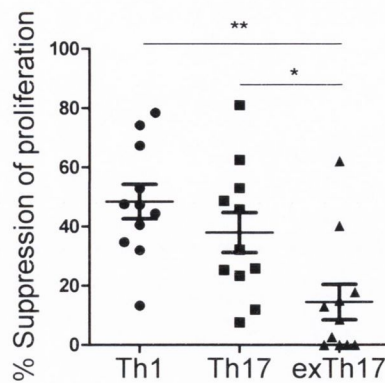
In summary, flow cytometry and ELISA data both indicate that ex-Th17 cells are significantly less susceptible to Treg cell mediated suppression of proliferation and pro-inflammatory cytokine production. These data suggest that the differential suppression observed between CD161⁺ and CD161⁻ Th populations was likely due to the subpopulation of CD161⁺ cells that are classified as ex-Th17 cells. This has important implications in RA, as this study has shown that these ex-Th17 cells were enriched in the RA joint.

5.2.5c Depletion of the ex-Th17 cell population from total CD161⁺ Th cells restores susceptibility to Treg cell mediated suppression.

One final proof-of-principle suppression assay was carried out to demonstrate that the ex-Th17 cells within the CD161⁺ Th cell subset were responsible for the resistance to Treg cell mediated suppression. Total CD4⁺ CD25⁻ CD45RO⁺ CD161⁺, total CD4⁺ CD25⁻ CD45RO⁺ CD161⁻, and ex-Th17

depleted CD4⁺CD25⁻CD45RO⁺CD161⁺CXCR3⁻ effector T cell subsets were sorted by FACS along with CD39⁺ Treg cells. Responder cells were CTV labelled and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled irradiated APC. Proliferation was measured by CTV dilution and cells were restimulated with PMA and ionomycin in the presence of brefeldin A to assess cytokine production. Percent suppression of proliferation, IFN- γ , TNF and GM-CSF were compared in CD161⁻ Th cells, total CD161⁺ Th cells and ex-Th17-depleted CD161⁺ Th cells (figure 5.21). This preliminary data representing two independent experiments shows that Treg cell mediated suppression of CD161⁺ Th cells was restored to a level comparable to CD161⁻ Th cells in CD161⁺ cells depleted of ex-Th17 type cells. In summary, these preliminary findings support the idea that the ex-Th17 pool that exists within the total CD161⁺ Th cell population is responsible for imparting the resistance to Treg cell mediated suppression.

A



B

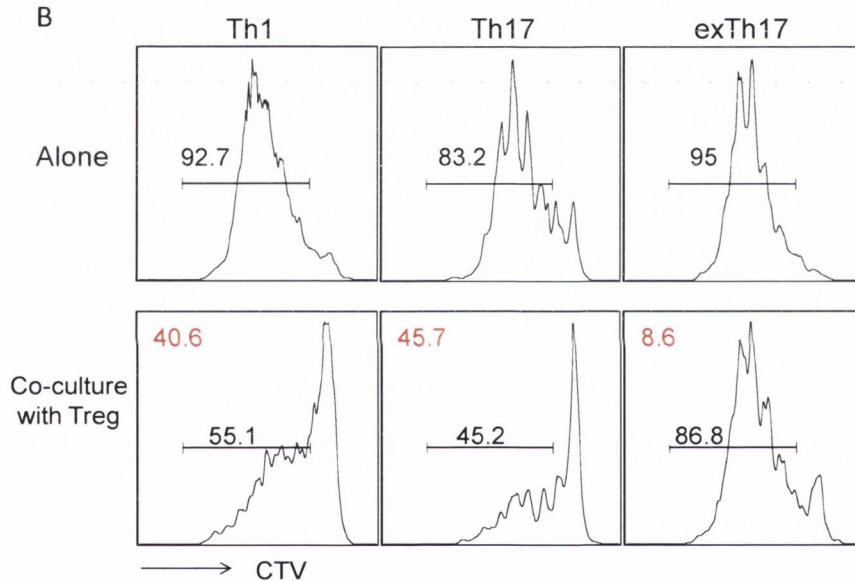


Figure 5.14 Treg-mediated suppression of Th1, Th17 and ex-Th17 cell proliferation.

PBMC were isolated from healthy donors. CD4⁺ memory T cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD161⁻ CXCR3⁺ CCR4⁻ CCR6⁻ Th1, CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁻ CCR4⁺ CCR6⁺ Th17 and CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁺ CCR6⁺ exTh17 effector cells and CD4⁺CD25⁺CD127^{lo}CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled irradiated APC. Cells were analysed by flow cytometry; CTV⁺ CFSE⁻ cells were gated and proliferation was quantified by CTV dilution. (A) Percent Treg cell mediated suppression of Th1, Th17 and exTh17 cell proliferation. Each data point represents a single independent experiment (n=11). (B) Representative histograms showing the frequency of cell proliferation (black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of proliferation were determined by 1-way ANOVA with Tukey's multiple comparison test; * p< 0.05, ** p<0.01.

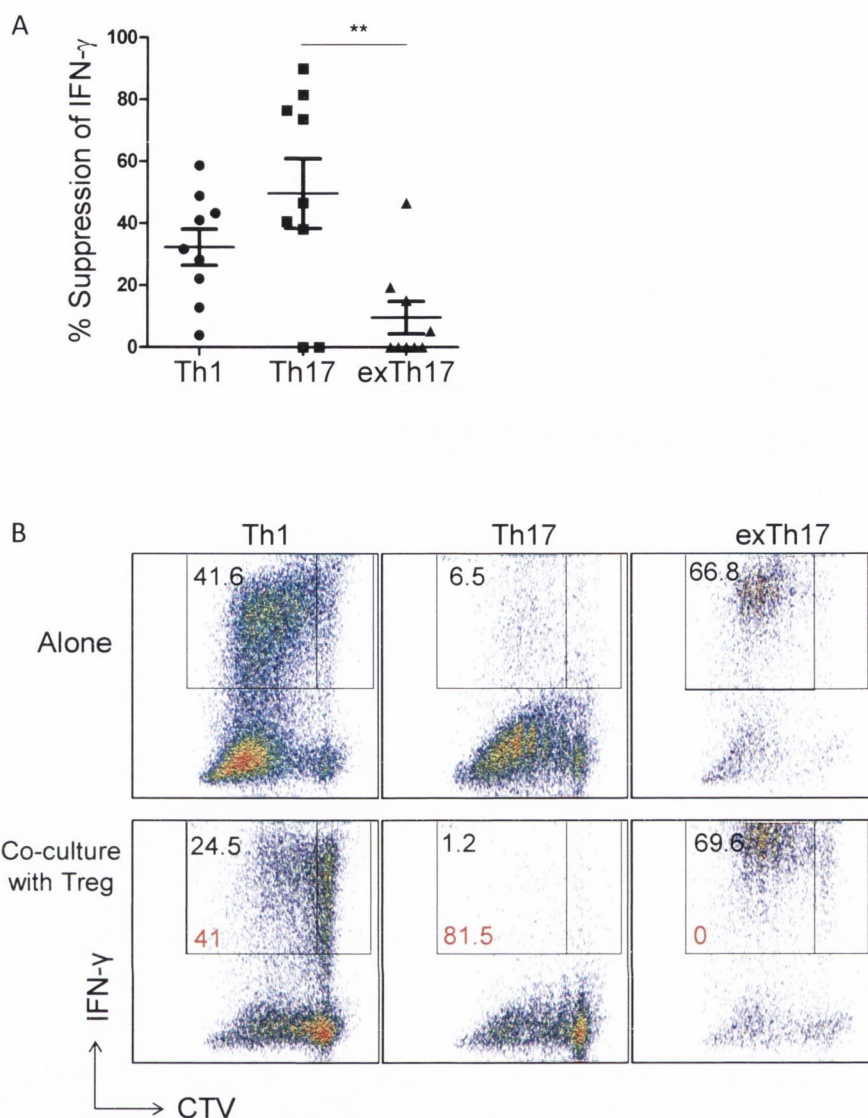


Figure 5.15 Treg cell mediated suppression of Th1, Th17 and ex-Th17 cell production of IFN- γ .

PBMC were isolated from healthy donors. CD4⁺ memory T cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD161⁻ CXCR3⁺ CCR4⁻ CCR6⁻ Th1, CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁻ CCR4⁺ CCR6⁺ Th17 and CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁺ CCR6⁺ exTh17 effector cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for IFN- γ and analysed by flow cytometry. CTV⁺ CFSE⁻ cells were gated before assessing expression of IFN- γ . (A) Percent Treg cell mediated suppression of IFN- γ produced by proliferating Th1, Th17 and exTh17 cells. Each data point represents a single independent experiment (n=9). (B) Representative FACS plots showing the frequency of IFN- γ produced from the proliferating cell population (shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of IFN- γ were determined by 1-way ANOVA with Tukey's multiple comparison test; *p<0.05, ** p<0.01.

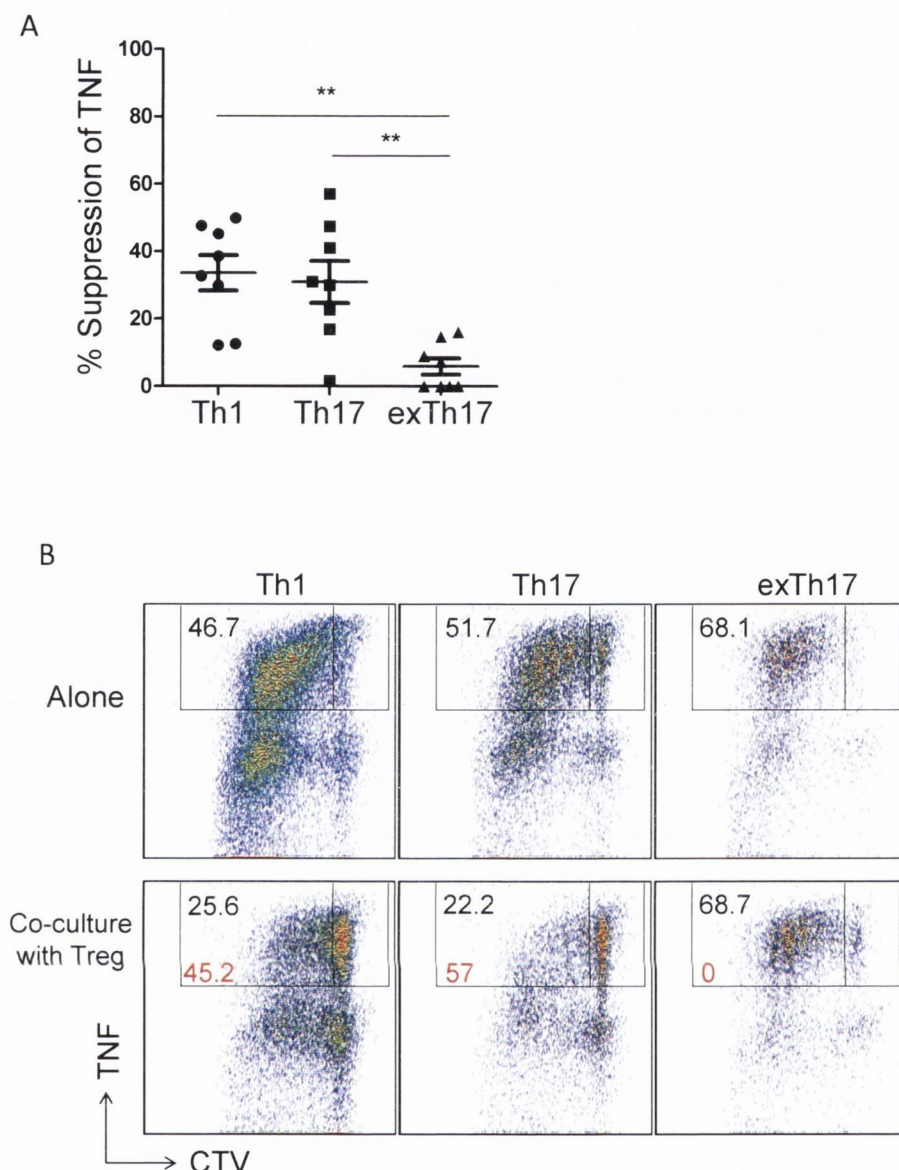


Figure 5.16 Treg cell mediated suppression of Th1, Th17 and ex-Th17 cell production of TNF. PBMC were isolated from healthy donors. CD4⁺ T memory cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD161⁻ CXCR3⁺ CCR4⁻ CCR6⁻ Th1, CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁻ CCR4⁺ CCR6⁺ Th17 and CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁺ CCR6⁺ exTh17 effector cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for TNF and analysed by flow cytometry. CTV⁺ CFSE⁻ cells were gated before assessing expression of TNF. (A) Percent Treg cell mediated suppression of TNF produced by proliferating Th1, Th17 and exTh17 cells. Each data point represents a single independent experiment (n=8). (B) Representative FACS plots showing the frequency of TNF produced from the proliferating cell population (shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of TNF were determined by 1-way ANOVA with Tukey's multiple comparison test; ** p<0.01.

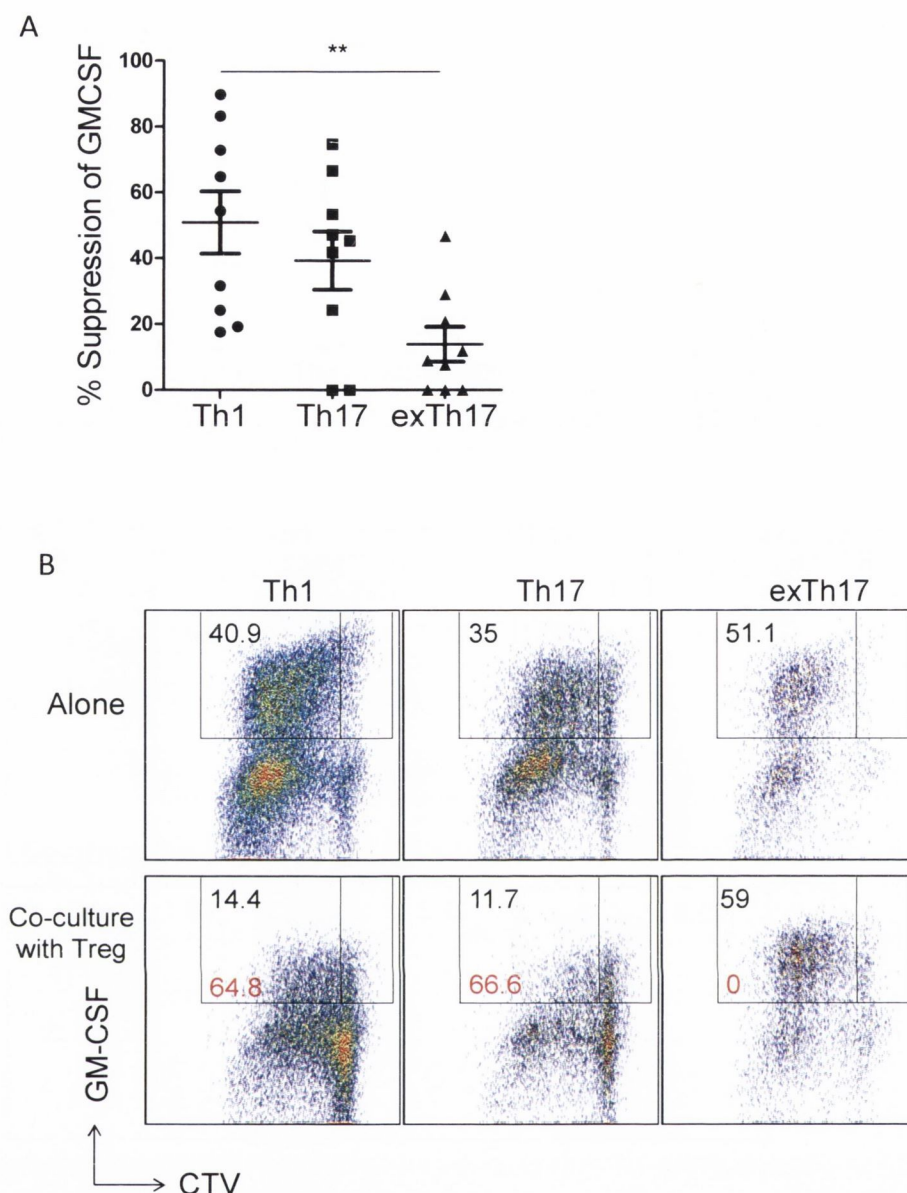


Figure 5.17 Treg cell mediated suppression of Th1, Th17 and ex-Th17 cell production of GM-CSF.

PBMC were isolated from healthy donors. $CD4^+$ memory cells were purified from PBMC by MACS. $CD4^+ CD45RO^+ CD161^- CXCR3^+ CCR4^- CCR6^-$ Th1, $CD4^+ CD45RO^+ CD161^+ CXCR3^- CCR4^+ CCR6^+$ Th17 and $CD4^+ CD45RO^+ CD161^+ CXCR3^+ CCR6^+$ exTh17 effector cells and $CD4^+ CD25^+ CD127^{lo} CD39^+$ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for GM-CSF and analysed by flow cytometry. $CTV^+ CFSE^-$ cells were gated before assessing expression of GM-CSF. (A) Percent Treg cell mediated suppression of GM-CSF produced by proliferating Th1, Th17 and exTh17 cells. Each data point represents a single independent experiment ($n=9$). (B) Representative FACS plots showing the frequency of GM-CSF produced the proliferating cell population (shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of GM-CSF were determined by 1-way ANOVA with Tukey's multiple comparison test; ** $p<0.01$.

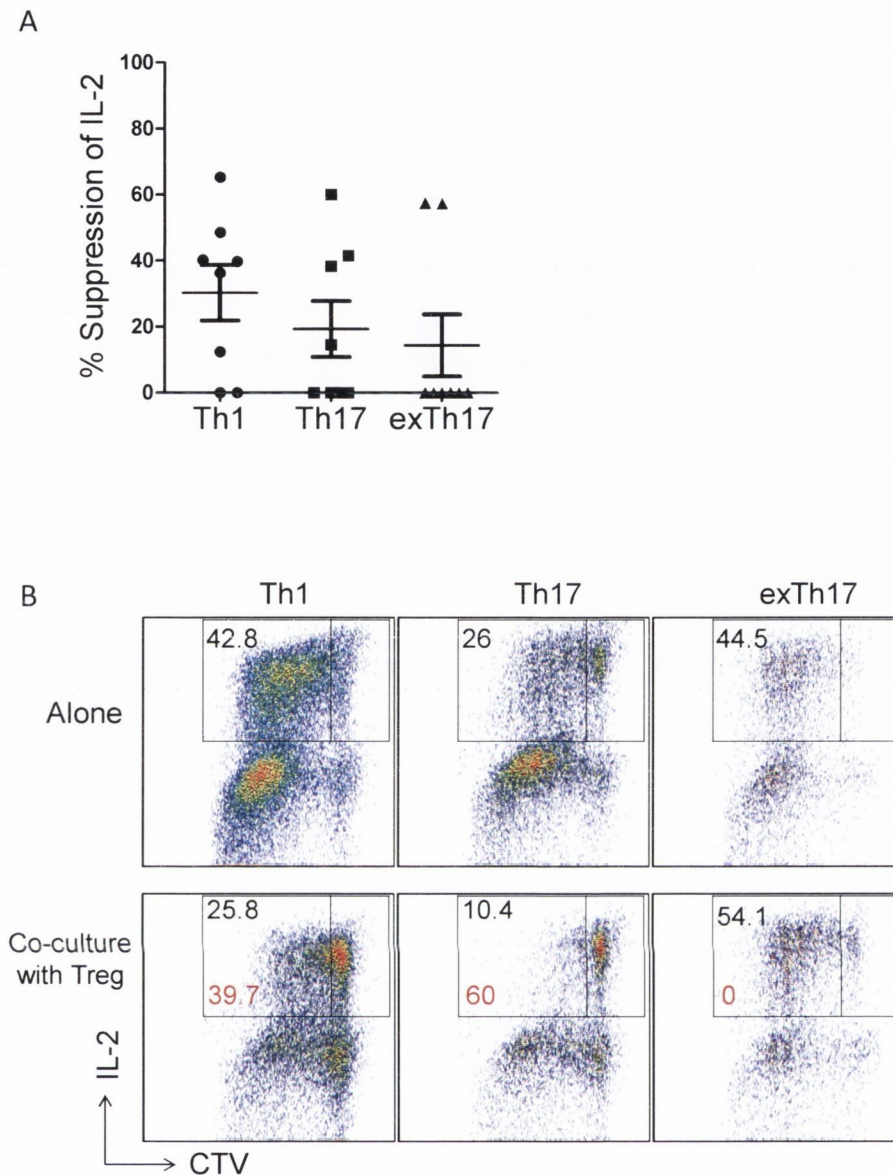


Figure 5.18 Treg cell mediated suppression of Th1, Th17 and ex-Th17 cell production of IL-2. PBMC were isolated from healthy donors. CD4⁺ memory T cells were purified from PBMC by MACS. CD4⁺ CD45RO⁺ CD161⁻ CXCR3⁺ CCR4⁻ CCR6⁻ Th1, CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁻ CCR4⁺ CCR6⁺ Th17 and CD4⁺ CD45RO⁺ CD161⁺ CXCR3⁺ CCR6⁺ exTh17 effector cells and CD4⁺ CD25⁺ CD127^{lo} CD39⁺ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for IL-2 and analysed by flow cytometry. CTV⁺ CFSE⁻ cells were gated before assessing expression of IL-2. (A) Percent Treg cell mediated suppression of IL-2 produced by proliferating Th1, Th17 and exTh17 cells. Each data point represents a single independent experiment (n=8). (B) Representative FACS plots showing the frequency of IL-2 produced from the proliferating cell population (shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of IL-2 were determined by 1-way ANOVA with Tukey's multiple comparison test, however, the data did not reach statistical significance.

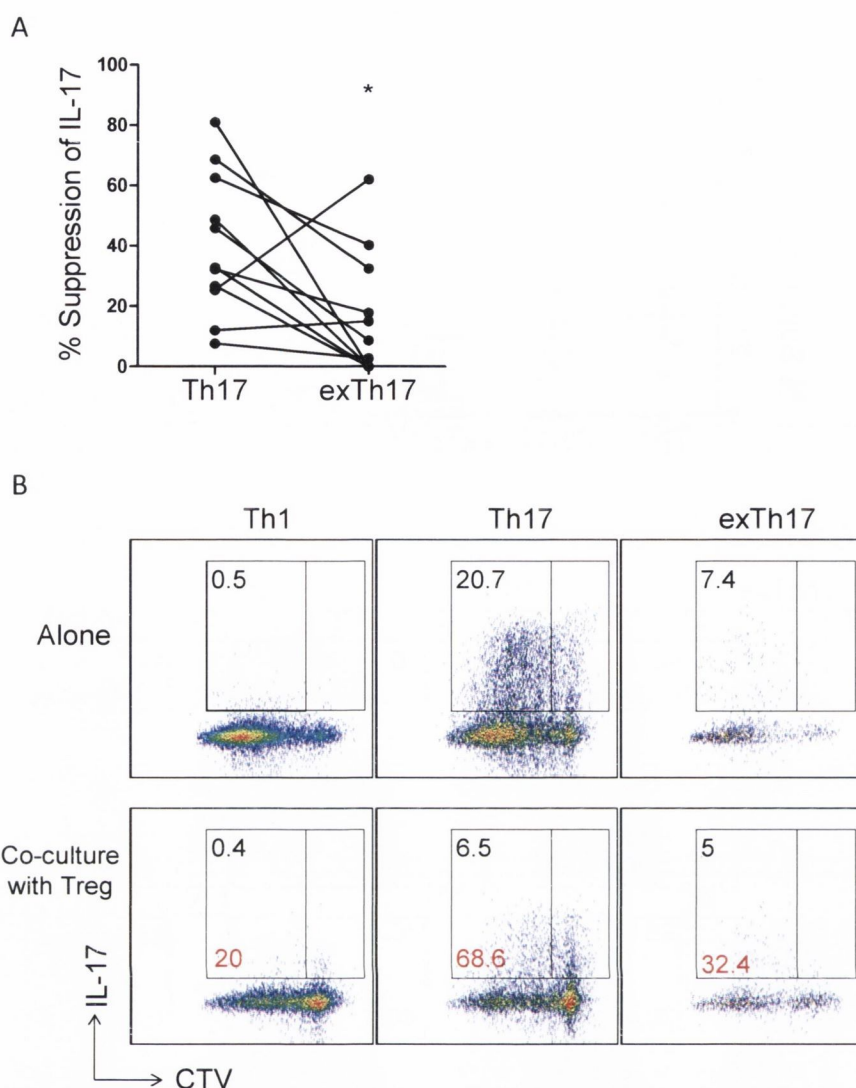


Figure 5.19 Treg cell mediated suppression of Th1, Th17 and ex-Th17 cell production of IL-17.

PBMC were isolated from healthy donors. $CD4^+$ memory T cells were purified from PBMC by MACS. $CD4^+$ $CD45RO^+$ $CD161^-$ $CXCR3^+$ $CCR4^-$ $CCR6^-$ Th1, $CD4^+$ $CD45RO^+$ $CD161^+$ $CXCR3^-$ $CCR4^+$ $CCR6^+$ Th17 and $CD4^+$ $CD45RO^+$ $CD161^+$ $CXCR3^+$ $CCR6^+$ exTh17 effector cells and $CD4^+$ $CD25^+$ $CD127^{lo}$ $CD39^+$ Treg cells were sorted by FACS. Effector cells were labelled with CTV and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled irradiated APC. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A, intracellularly stained for IL-17 and analysed by flow cytometry. CTV^+ $CFSE^-$ cells were gated before assessing expression of IL-17. (A) Percent Treg cell mediated suppression of IL-17 produced by proliferating Th1, Th17 and exTh17 cells. Each pair of data points represents a single independent experiment ($n=11$). (B) Representative FACS plots showing the frequency of IL-17 produced from the proliferating cell population (shown in black) and the percent suppression (red) compared with control cells cultured alone. Statistical differences in suppression of IL-17 were determined by paired T-tests with two-tailed p-values; * $p<0.05$.

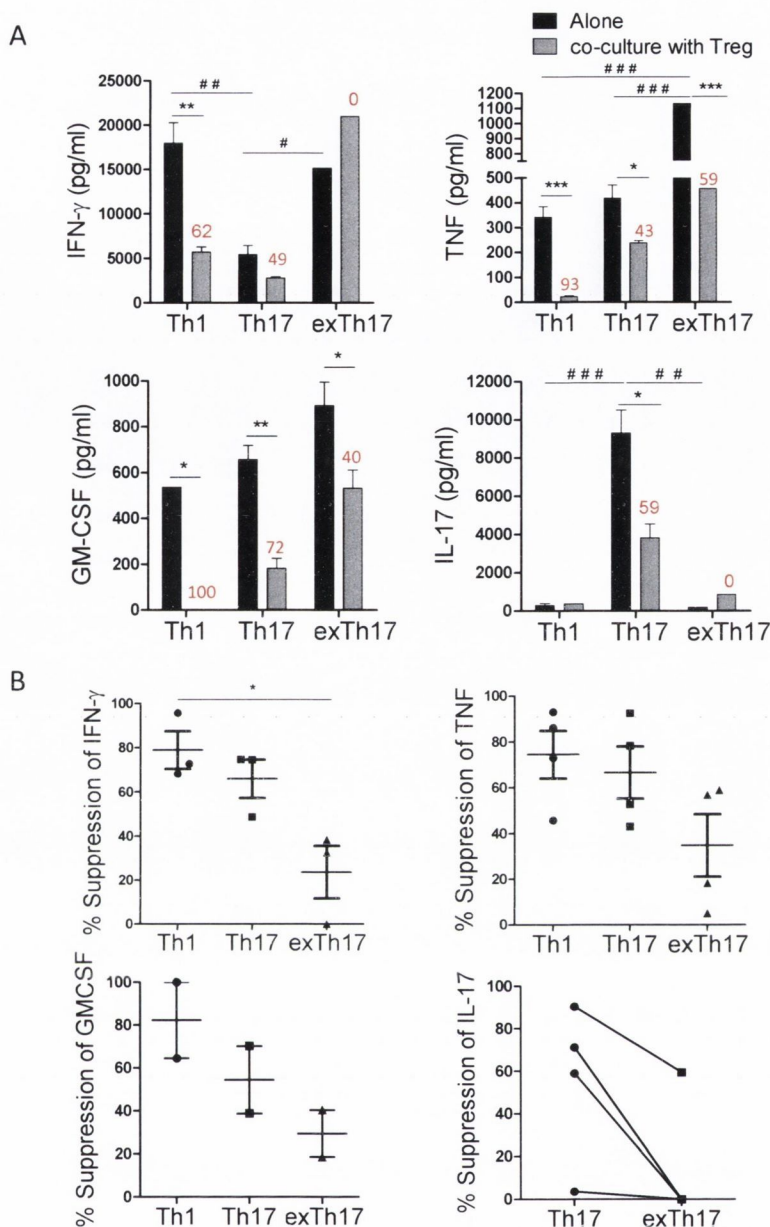


Figure 5.20 Treg cell mediated suppression of Th1, Th17 and ex-Th17 cell pro-inflammatory cytokine production.

PBMC were isolated from healthy donors. CD4⁺ memory T cells were purified from PBMC by MACS. Th1, Th17 and exTh17 effector cells and Treg cells were sorted by FACS, as previously described. Effector cells were cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and allogeneic irradiated APC. The concentrations of IFN- γ , TNF, IL-17A and GM-CSF in the supernatants were assessed by ELISA. (A) Representative graphs illustrating the concentration of pro-inflammatory cytokines in the supernatants of effector cells cultured alone (black bars) or co-cultured with Treg cells (grey bars). The percent Treg cell mediated suppression of cytokine is shown in red. (B) Percent Treg cell mediated suppression of IFN- γ , TNF, GM-CSF and IL-17. Each data point represents a single independent experiment (IFN- γ n=3, TNF n=4, GM-CSF n=2, IL-17 n=4). (A) Statistical differences in cytokine production between effector cells cultured alone or co-cultured with Treg cells were determined by 2-way ANOVA with a Bonferroni post-test; * p<0.05, ** p<0.01, *** p<0.001. Statistical differences in cytokine production between Th1, Th17 and exTh17 cell subsets were determined 2-way ANOVA with a Bonferroni post-test; # p<0.05, ## p<0.01, ###p<0.001. (B) Statistical differences in suppression were determined by 2-way ANOVA with a Bonferroni post-test; * p<0.05.

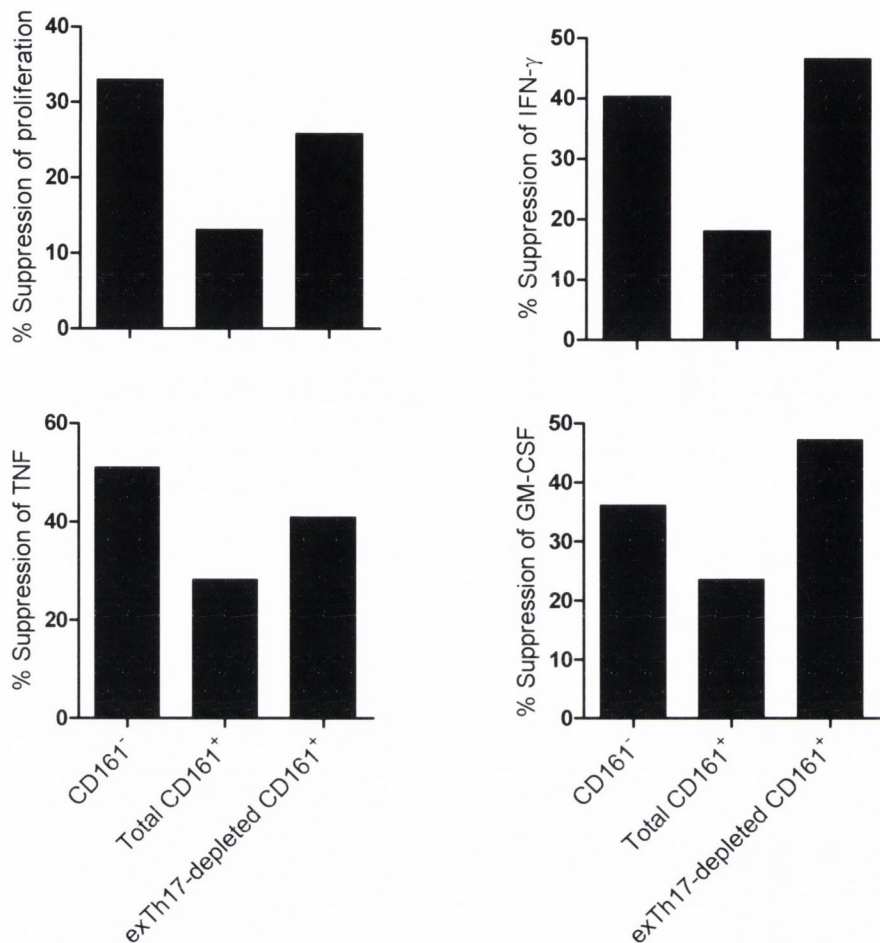


Figure 5.21 Treg cell mediated suppression is restored in CD161⁺ Th cells depleted of ex-Th17 cells.

PBMC were isolated from healthy donors. CD4⁺ memory T cells were purified from PBMC by MACS. Total CD4⁺ CD25⁻ CD45RO⁺ CD161⁺, total CD4⁺ CD25⁻ CD45RO⁺ CD161⁻, and ex-Th17 depleted CD4⁺ CD25⁻ CD45RO⁺ CD161⁺ CXCR3⁻ effector cells were sorted by FACS along with CD39⁺ Treg cells. Effector cells were CTV labelled and cultured alone or co-cultured with equal numbers of Treg cells for 5 days in the presence of anti-CD3 and CFSE labelled allogeneic irradiated APC. CTV⁺ CFSE⁻ cells were gated and proliferation was measured by CTV dilution. Cells were restimulated with PMA and ionomycin in the presence of brefeldin A to assess the suppression of pro-inflammatory cytokine production. Percent suppression was calculated based on the difference between effector cells cultured alone and co-cultured with Treg cells. This data is representative of two independent experiments.

5.3 Discussion

This study demonstrated the novel finding that CD161⁺ Th cells were less susceptible to Treg cell mediated suppression of proliferation and pro-inflammatory cytokine production. Moreover, this study went on to prove that it was the ex-Th17 subpopulation within CD161⁺ Th cells that were responsible for this resistance to suppression. Chapter 3 of this work illustrated that CD161⁺ Th cells were enriched in the joints of RA patients and high frequencies of these cells produce IFN- γ , suggesting that they are ex-Th17 type cells. The current chapter definitively showed that ex-Th17 cells are increased in the RA joint on the basis of cell surface markers and chemokine receptor expression. Importantly, these data showed that ex-Th17 cells were significantly more polyfunctional than bona fide Th17 cells, indicating that Th17 cell plasticity is inherently pathogenic in the context of autoimmunity. This study also showed that purified populations of bona fide Th17 cells switch into IFN- γ producing cells *in vitro*, which has implications for the study of Th17 cell function.

This study is the first to show that CD161⁺ and CD161⁻ Th cells are differentially regulated by Treg cells. Both the flow cytometry and the ELISA based suppression assays showed that memory CD161⁺ Th cells were significantly less susceptible to Treg cell mediated suppression of proliferation and pro-inflammatory cytokine production. Interestingly, a large degree of variance was observed in the extent of Treg cell mediated suppression of proliferation and cytokine production in the healthy donors. This may reflect differences in any given individual's Treg cell function, or may perhaps be due to the nature of the individual's effector cells. Because CD161⁺ Th cells incorporate both bona fide Th17 cells and plastic ex-Th17 cells, the ratio of each type of Th17 cells in any given individual may affect Treg cell mediated suppression. Although the regulation of CD161⁺ cells compared with CD161⁻ cells has hitherto not been assessed, these data are in keeping with other studies suggesting that Th17 lineage cells may not be partial to Treg cell mediated suppression (Annunziato et al., 2007). Given that Th17 lineage cells originate from CD161⁺ Th cells (Cosmi et al., 2008) and Th17 cells require STAT3 for their development (Harris et al., 2007, Yang et al., 2007), these data are consistent with evidence that implicates STAT3 in the mediation of resistance to Treg cell suppression (Goodman et al., 2011).

The differential suppression of IL-17 was difficult to assess by flow cytometry because CD161⁻ Th cells produce very low or background frequencies of IL-17, and IL-17 flow cytometry staining in human cells is technically challenging. For this reason, the ELISA data is invaluable to assess the suppression of IL-17 by examining the concentration accumulated in the supernatant over the 5 day culture period. The ELISA data shows that although CD161⁺ Th cells were producing

significantly higher concentrations of IL-17 than CD161⁻ Th cells, both were suppressed to the same extent. In addition, when the ELISA data was collated from 4 independent experiments, there was no significant difference in the suppression of IL-17; however, IFN- γ , TNF and GM-CSF produced by CD161⁺ Th cells were all significantly less well suppressed compared with CD161⁻ Th cells. This data implies that bona fide IL-17 producing Th17 cells may be subject to a similar degree of suppression as CD161⁻ Th cells whereas plastic, polyfunctional IFN- γ producing ex-Th17 type cells may be the offending component causing the Treg cell insensitivity in total CD161⁺ Th cells. Therefore, the Treg cell mediated suppression of proliferation and pro-inflammatory cytokine production was subsequently examined in discrete Th1, Th17 and ex-Th17 cells as it had thus far gone largely unstudied.

The differential suppression of CD161⁺ and CD161⁻ Th cells taken from SFMC of RA patients during active disease was assessed to ensure that the pattern held true in the context of chronic autoimmune inflammation. The reduced susceptibility of RA SFMC responder cells to Treg cell mediated suppression observed in chapter 4 could plausibly be due the increased frequency of CD161⁺ Th cells in RA SFMC as other studies of JIA have concluded that reduced suppressive function is due to the nature of the responder cells rather than defective Treg cells (Haufe et al., 2011, Wehrens et al., 2011). Although CD161⁺ Th cells from RA SFMC displayed the same trend towards reduced susceptibility to regulation, this did not reach statistical significance. This research was hampered by low cell numbers, poor viability and cell sensitivity as cells from the joint are activated *in vivo* and often die upon reactivation *in vitro*. For this reason, the n numbers for these experiments are low as only approximately 1 in 4 assays sufficiently stimulated the sorted Th cells without inducing death. These results were further confounded by the different treatment regimes of patients. It is also plausible that, if CD161⁺ Th cells are capable of dividing asymmetrically, the chronicity of the inflammation in the RA joint and their enhanced features of longevity may result in an accumulation of CD161^{lo/-} Th cells from a CD161⁺ lineage. If these CD161^{lo} cells were incorporated in the sorted CD161⁻ Th cell subset, then the marked differences between the regulation of CD161⁺ and CD161⁻ Th cells observed in the HC may be somewhat masked in the RA patients. The n numbers in this experiment need to be increased in order to provide sufficient power for the investigation and to decipher the results accurately. However, as a proof-of-principle, the trend of increased susceptibility to Treg cell mediated suppression upon depletion of CD161⁺ Th cells from RA responder cells served to demonstrate that the CD161⁺ Th cell subpopulation, which is enriched in the RA joint, plausibly causes the reduced overall susceptibility to suppression of the total responder cell population observed in comparison with HC in chapter 4.

Having established that CD161⁺ Th cells were less susceptible to Treg cell mediated regulation than their CD161⁻ Th cell counterparts; the next question to address was whether this phenotype was due to the bona fide Th17 cell component of CD161⁺ Th cells or the ex-Th17 cell component. Firstly, the frequency and phenotype of Th1, Th17 and ex-Th17 cells were examined. Thus far, the data collected from RA SFMC had shown that CD161⁺ Th cells were enriched in the RA joint and a high proportion of those CD161⁺ Th cells expressed IFN- γ , suggesting that the CD161⁺ Th cells in the RA joint are plastic ex-Th17 type cells. In order to directly demonstrate the enrichment of ex-Th17 cells in RA SFMC, the panel of chemokines receptors used to sort discrete subsets was used to decipher the frequency of Th1, Th17 and ex-Th17 cell in the RA joint. On the basis of CD161 and CXCR3 expression alone, there was a significant increase in the frequency of ex-Th17 cells in the RA joint compared with HC peripheral blood. Moreover, RA PBMC exhibited a trend towards increased frequencies of broadly ex-Th17 type cells which suggests that plasticity was occurring in the periphery. This data is further supported by data generated using the full panel, which includes CCR4 and CCR6. Additionally, the frequencies of Th1 cells were significantly increased in RA PBMC compared with HC PBMC which implies that inflammation is systemic in these RA patients. This is consistent with recently published data showing that CD161⁺ Th17 type cells are enriched in RA SFMC and have a Th1 cell phenotype, suggesting that these cells are ex-Th17 cells (Chalan et al., 2013). Furthermore, it has been demonstrated in JIA that CD161⁺ Th1-type cells (CXCR3⁺ CCR6⁻/IFN- γ ⁺ IL-17⁻) and CD161⁺ Th17/Th1 type cells (CXCR3⁺ CCR6⁺/IFN- γ ⁺ IL-17⁺) accumulate in the synovial fluid of patients with JIA but CD161⁺ bona fide Th17-type cells (CXCR3⁻ CCR6⁺/IFN- γ ⁻ IL-17⁺) are not enriched in joint (Cosmi et al., 2011).

Further phenotyping indicated that ex-Th17 type cells undergo increased proliferation in response to TCR stimulus *in vitro* compared with Th1 and Th17 cells. Ki67 staining indicated that ex-Th17 cells proliferate *in vivo* to a similar extent as Th1 cells whereas bona fide Th17 have reduced Ki67 expression *ex vivo*. This is in keeping with published work showing, by thymidine incorporation, that Th17 cells have reduced capacity to proliferate in response to TCR stimulus compared with Th1 cells due to their reduced capacity to produce and respond IL-2 (Santarlaschi et al., 2012). Further work by the same group showed that end-stage IFN- γ ⁺ IL-17⁻ ex-Th17 “non-classical Th1” cells have increased proliferation *in vitro* compared with bona fide Th17 cells but reduced proliferation compared with classical Th1 cells (Maggi et al., 2012). Another publication that examined the proliferative response of Th17 cells versus Th17/1 cells (early ex-Th17 cells that co-express IL-17 and IFN- γ) found no difference in their mitogenic index but observed reduced proliferation of the ex-Th17 cells when cells were stimulated in the presence of IL-15

(Annunziato et al., 2007). These studies used clean populations of Th17 and ex-Th17 clones which were expanded *in vitro* prior to use, so, it is plausible that re-stimulation of these expanded cells to check their proliferative capacity may have altered their mitogenic index. The disparity observed may also be due to the use of anti-CD3 with anti-CD28 co-stimulation rather than using the more robust irradiated APC stimulation, as in the present study. Other published work has suggested that anti-CD28 co-stimulation negatively affects the development of Th17-type cells (Bouguerrouh et al., 2009) and moreover, it has been shown that CD161⁺ Th cells do not effectively proliferate when co-stimulated with anti-CD28 but proliferate in the presence of APC (Crome et al., 2010a). This is in keeping with the highly differentiated phenotype of Th17 type cells as indicated by the loss of CD28 expression. Similar to bona fide Th17 cells, the majority of ex-Th17 cells were contained within the effector memory pool whereas Th1 cells contained significantly more central memory cells. This is consistent with data showing that Th17-type cells have an effector memory phenotype (Kryczek et al., 2011). These data indicate that ex-Th17 cells have features of both bona fide Th1 and Th17 cells and moreover, are significantly more polyfunctional in terms of cytokine production than Th1 or Th17 cells. Cumulatively, these data suggest that ex-Th17 cells may be highly pathogenic in the context of autoimmunity as they are capable of producing multiple pro-inflammatory cytokines concomitantly and have increased proliferative capacity compared with their progenitor bona fide Th17 cells. This finding is highly significant given the fact that ex-Th17 cells are enriched in the RA SFMC.

Bona fide Th17 cells stimulated via the TCR seemingly spontaneously shift *in vitro* to producing IFN- γ over a culture time of 1-2 weeks. This finding is supported by published work in mice showing that IL-17⁺ cells shift to becoming IL-17⁺ IFN- γ ⁺ and IL-17⁻ IFN- γ ⁺ cells when stimulated *in vitro* (Lexberg et al., 2008). However, it is also plausible that the addition of exogenous IL-2 to cell culture conditions may play a role in promoting the production of IFN- γ in differentiated memory Th17 cells as murine studies have shown that IL-2 promotes Th1 but inhibits Th17 differentiation from naive Th cells (Laurence et al., 2007, Fujimura et al., 2013) although there was no evidence of a decrease in the production of IL-17 in these data. This shift toward IFN- γ production is somewhat of a confounding factor for assessing the suppression of Th cell subsets over a 5 day period. On day 5, the ELISA data showed only low concentrations of IFN- γ in the supernatant of Th17 cells, however, by day 7, the flow cytometry data indicated that Th17 cells were capable of producing high frequencies of IFN- γ . To assess differential suppression between Th1, bona fide Th17 and ex-Th17 cells, cells were examined either on day 4 or day 5 (i.e. as early

as possible) in order to prevent the *in vitro* switch to ex-Th17 cells from affecting the regulation data.

Finally, the differential Treg cell mediated regulation of Th1, bona fide Th17 and ex-Th17 cells was assessed using the flow cytometry based suppression assay and supported with ELISA data. The data clearly demonstrates that proliferation of Th1 and bona fide Th17 cells are well suppressed by Treg cells but ex-Th17 cells are not subject to the same the degree of Treg cell mediated regulation. This finding is supported by published literature showing that ex-Th17 type cells have reduced suppression of proliferation compared with Th1 cells but is contradictory to data showing that bona fide Th17 cells also have reduced suppression of proliferation compared with Th1 cells (Annunziato et al., 2007). Importantly, this published study used thymidine incorporation in expanded clones to examine the regulation of Th1, Th17 and ex-Th17 type cells. Because Th17 cells *in vitro* are plastic over time, the expanded Th17 clones may not have been bona fide Th17 cells, but rather ex-Th17 cells, by the time they were put into the suppression assay. This may account for the similar behavior of the supposedly Th17 cells to the ex-Th17 cells which exhibited reduced susceptibility to suppression of proliferation. The current body of work has overcome this issue by placing sorted subsets directly into suppression assays rather than expanding clones first. Moreover, the examination of the total cell population, rather than clones, provides more robust insight into the behavior of these subpopulations as a whole.

The suppression of IFN- γ was significantly greater in bona fide Th17 cells, (although only low frequencies produce IFN- γ) compared with ex-Th17 cells. This suggests that nascent ex-Th17 cells may remain subject to Treg cell mediated suppression until some critical threshold is reached in their plastic phenotype. Or conversely, if the frequency of ex-Th17 cells remains low within a bona fide Th17 cell population, resistance to Treg cell mediated suppression may not be apparent. The suppression of TNF was significantly reduced in ex-Th17 cells compared with both Th1 and Th17 cells whereas the suppression of GM-CSF was significantly reduced in ex-Th17 compared with Th1 cells only. This may be due to the early *in vitro* induction of Th17 plasticity which causes a spread in the data; two of the data points for the suppression of GM-CSF in Th17 cells lie on zero, indicating that GM-CSF was not at all suppressed in these samples. Interestingly, there was no significant difference in the Treg cell mediated suppression of IL-2 between the Th subsets. Although the representative FACS plots showed that the suppression of IL-2 followed the same pattern as the suppression of other cytokines, this pattern was not maintained across all donors; 2 independent donors showed no suppression of IL-2 in any subset whereas 2 other donors exhibited increased suppression of IL-2 in ex-Th17 cells compared with

Chapter 6 General Discussion and Future Work

6.1 General Discussion

Introduction

This study has provided new insight into the effector functions and regulation of Th17 lineage cells in health and in the aberrant context of autoimmune RA. As outlined in chapter 1, the balance between Th17 and Treg cells is of utmost importance to the maintenance of homeostasis and an imbalance in these subsets is thought to contribute to autoimmune pathology. Th17 cells have been documented to play a key role in inflammation and autoimmunity, thus, targeting Th17 cells and related cytokines has become a prominent therapeutic strategy. Th17 cells are also a topic of intense investigation for promoting long-term immunity in vaccination. However, the role of Th17-type cells in cancer remains unclear.

Murine models have played a significant role in elucidating the contributions of Th17 cells to autoimmune pathology; however, in order to fully translate these findings into a clinical setting, it is important to have a better understanding of Th17 biology in humans. Human studies, however, are often stymied by an inability to sample cells from pertinent tissues and low cell numbers or poor viability from fluid and biopsy samples. Examining peripheral blood from patients is an accessible and ethical approach; however, lymphocytes in the peripheral blood are not always representative of tissue specific events. In the present study, the availability of synovial fluid samples from RA patients provided an excellent opportunity to study both the Treg and effector cell components of the CD4⁺ T cell response at the site of autoimmune inflammation. Using a combination of *ex vivo* and *in vitro* analysis, this study addressed the hypothesis that Th17 lineage cells may contribute to autoimmune pathology significantly more than other Th cell populations and that regulation by Treg cells may be altered in the context of RA. Moreover, plasticity between Th cell subsets may contribute to the propagation of autoimmune inflammation.

The data herein, showed that CD161⁺ Th17 lineage cells were enriched in the RA joint and exhibited a plastic phenotype; including production of IFN- γ and increased expression of other pro-inflammatory cytokines. These data demonstrated, for the first time, that these CD161⁺ Th cells had increased pro-survival features, which may allow them to persist for protracted periods of time in the RA joint. Furthermore, CD161⁺ Th cell supernatants induced a significantly more pro-inflammatory response from synovial fibroblast cells compared with those from CD161⁻ Th cells. Importantly, these CD161⁺ Th cells were refractory to Treg cell mediated suppression and

this was shown to be due to the presence of ex-Th17 type cells within the total CD161⁺ Th cell population not the bona fide Th17 cells. Treg cells from the RA joint were functionally suppressive but exhibited reduced suppressive capacity overall compared with Treg cells from HC PBMC. This was most likely due to that fact that CD161⁺ Th cells were enriched in the RA joint and, because they were less susceptible to Treg cell mediated suppression, the overall suppressive function of RA SFMC derived Treg cells was reduced. A subpopulation of Treg cells within the RA joint expressed CD161 and produced pro-inflammatory cytokines. In addition, this study was the first to demonstrate that these CD161⁺ functionally plastic Treg cells displayed many of the pro-survival features of CD161⁺ effector Th cells.

These data cumulatively demonstrated that CD4⁺ T cell plasticity is pathogenic in the context of RA, suggesting that therapeutic targeting of CD161⁺ Th cells could be a rational strategy for the amelioration of inflammation in RA. Given that the majority of CD161⁺ Th cells in the RA joint are ex-Th17-type cells, depleting this cell population may have improved efficacy over targeting IL-17 alone.

Implications for autoimmunity in general

These data indicated that Th17 lineage cells have inherently pathogenic characteristics in addition to their ability to produce IL-17. However, the pathogenicity of IL-17 itself has been the major focus of Th17 cell mediated pathology in the field. Th17 cells are capable of functional plasticity and can develop into ex-Th17 cells which have been shown here to retain the pathogenic characteristics of Th17 cells despite the cessation of IL-17 production. Additionally, ex-Th17 type cells have augmented pathogenic potential due to their increased replication and insensitivity to Treg cell mediated suppression.

The ongoing debate over the relative importance of IL-17 versus IFN- γ , or Th17 versus Th1 cells, in the arena of autoimmune mouse models may be reconciled, at least in part, by the plastic nature of Th17 cells which appear to be “Th1-like” due to their production of IFN- γ . In EAE, the mouse model of MS, the deletion of genes encoding cytokines required for the differentiation of Th17 cells are essential for the development of the disease (Cua et al., 2003), however, blocking IL-17 exerted only partial amelioration of disease (Komiyama et al., 2006, Rohn et al., 2006, Hofstetter et al., 2005). This is consistent with the idea that characteristics of the Th17 lineage cells themselves, rather than individual cytokines, are critical for the development of autoimmunity. Given the redundant and pleiotropic effects of cytokines, it seems unlikely that blocking one cytokine will totally abrogate complex autoimmunity. However, the impressive efficacy of anti-IL-17 therapy for psoriasis (Leonardi et al., 2012, Papp et al., 2012, Papp et al.,

2013), which suggests that IL-17 producing cells are not switching into IFN- γ producing cells in inflamed skin, illustrates the need to robustly understand the immunopathologies of specific diseases in order to rationally design therapies tailored to the tissue-specific aberrant immune responses. In keeping with the idea that T cell plasticity is context dependant, it has been found that Th17 lineage cells did not exhibit plasticity in colorectal tumours (unpublished observations, Margaret Dunne). Since IL-17 has been documented to have pro-tumour effects (He et al., 2012), promoting plasticity in this subset may be beneficial for immune mediated anti-tumour therapy.

Murine Th17 cells have been shown to exhibit enhanced trafficking potential into the CNS and other sites of inflammation (Kim, 2009, Rothhammer et al., 2011) but were capable then of switching at the site into ex-Th17 type cells which exhibited polyfunctional cytokine production and mediated autoimmune inflammation (Hirota et al., 2011). GM-CSF has become a prominent cytokine implicated in the pathogenicity of Th17 type cells during EAE (El-Behi et al., 2011, Codarri et al., 2011). The present study has shown that GM-CSF was predominantly produced by stimulated CD161⁺ Th cells and, in particular, the ex-Th17 subcomponent of CD161⁺ Th cells. Interestingly, when examining the multi-parameter flow cytometry data using SPICE software, it became evident that cells producing GM-CSF were highly polyfunctional as they co-produced GM-CSF with IL-17, IFN- γ , IL-2 and TNF, in triple, quadruple and quintuple combinations.

Given the prominent role Th17-type cells have been demonstrated to play in many models of autoimmunity and the identification of CD161⁺ Th cells in human disease settings, the findings herein may be applicable to many types of autoimmune pathologies. In addition these findings may have broader relevance for other diseases such as cancer and chronic infection through the understanding of Th17-type cell behavior in humans.

Cytokine analysis indicated that Th cells generally had the ability to produce more than one cytokine at any given time, with certain subsets exhibiting higher degrees of polyfunctionality than others. These results, in juxtaposition with evidence of plasticity in the cytokine profile of Th17 and Treg cells, strongly suggest that the current dogma of defining subsets based on the expression of one signature cytokine is inadequate. In addition, differential cytokine expression in CD161⁺ and CD161⁻ Th cells from both HC subjects and RA patients indicated that although CD161⁺ Th cells were capable of producing similar frequencies and concentrations of IFN- γ compared with CD161⁻ Th cells, CD161⁺ Th cells contained significantly higher frequencies of GM-CSF, TNF and IL-17 producing cells. Moreover, CD161⁺ IFN- γ producing cells were significantly more polyfunctional than CD161⁻ IFN- γ producing cells. Additionally, ex-Th17 cells

displayed enhanced concomitant production of multiple pro-inflammatory cytokines and retained the stem cell-like features of bona fide Th17 cells. Furthermore, ex-Th17 cells were significantly less susceptible to Treg cell mediated suppression compared with Th1 cells. These data indicated that regarding these plastic ex-Th17 type cells as “non-classical Th1” cells in the literature might be a misnomer as their only functional similarity to Th1 cells appears to be the expression of IFN- γ .

Indeed the role of IFN- γ in the enhanced pathogenicity of plastic Th17 cells is poorly understood. Since IFN- γ can exert both pathogenic and regulatory roles in autoimmunity, controversy over its part in autoimmune inflammation has arisen (Zhang, 2007). The current study suggests that while IFN- γ marks the transition of Th17 cells into the plastic state it is not the feature that increases the pathogenicity of ex-Th17 type cells in the context of autoimmunity; CD161⁺ and CD161⁻ Th cells were both equally capable of producing IFN- γ but it was the CD161⁺ Th cells that exhibited enhanced pathogenicity in terms of their pro-survival features, enhanced cytokine polyfunctionality and reduced susceptibility to regulation. Although the production of IFN- γ by the plastic Th17-type cells is not necessarily the component that adds to the pathogenic potential of Th17 cells, it has become the standard read-out of Th17 plasticity in the literature. Since ex-Th17 cells producing GM-CSF were highly polyfunctional, and given the key role of GM-CSF in models of autoimmune inflammation, increased ability to produce GM-CSF may be a cornerstone of the increased pathogenicity of ex-Th17 cells compared with Th17 cells. It is more likely, however, to be a cumulative effect of several orchestrated changes within the cells as they undergo transition into the ex-Th17 cell phenotype. An understanding of the molecular cues that promote phenotypic and functional plasticity will be key to manipulating T cell responses in autoimmunity, chronic infection, cancer and vaccination.

Implications for therapy

Results from phase II trials indicated that anti-IL-17 therapy had relatively poor efficacy in RA (Kellner, 2013). Since the present study has shown that Th17 cells are plastic in the RA joint, it is plausible that targeting IL-17 in established RA is, at least in part, obsolete as only a low frequency of Th cells appear to be producing IL-17 whereas the vast majority of Th17-lineage cells have switched into ex-Th17 type cells. It is plausible that IL-17 may be produced by other cells; it has been suggested that mast cells produce IL-17 in the RA joint, however, this paper illustrated this by immunohistochemistry and, therefore, cannot differentiate between the production of IL-17 within the cell and cell surface bound IL-17 that may be up-taken from the environment (Hueber et al., 2010). $\gamma\delta$ T cells are also thought to be a major source of IL-17 in

mouse models of autoimmunity, including CIA, but do not produce IL-17 in the RA joint (observed during this study, data not shown) (Ito et al., 2009). Currently, the data indicates that Th17 cells may be the main source of IL-17 in RA, however, IL-17 production from other cells yet to be identified cannot be ruled out. IL-17 is likely to play an important role in immune mediated damage in RA as evidenced by its synergistic effects with TNF on fibroblast cells, its propensity to encourage the recruitment of neutrophils and promote bone degradation. However, given the dynamic nature of plastic Th cell responses, it is plausible that IL-17 production in the RA joint bears effect only at a certain stage in disease, presumably early on (Benedetti and Miossec, 2014). For this reason, targeting the cell type rather than just one cytokine would have added benefit as it would reduce the IL-17 in the tissue along with the plethora of other pathogenic factors associated with Th17-lineage cells. Targeting CD161⁺ Th17-lineage cells would abrogate the effects of both bona fide Th17 and pathogenic ex-Th17 cells. Although one could rationally postulate that this strategy may have enhanced efficacy over neutralising IL-17 alone, the question of eliciting extensive immunodeficiency would need to be addressed. However, specifically depleting CD4⁺ CD161⁺ T cells would be expected to elicit less immunodeficiency compared with severe strategies that target total CD3⁺ or CD4⁺ T cells. Targeting CD161 in general would also deplete CD161 expressing NK and NKT cells, which in addition to causing immunodeficiency, may exacerbate autoimmunity as NK cells have been documented to have protective (as well as detrimental) roles in RA pathology (Shegarfi et al., 2012). Therefore, depleting antibodies with steric ability to target both CD161 and CD4 concomitantly would need to be developed. This strategy would also target CD161⁺ Treg cells which may be either beneficial given their ability to produce pro-inflammatory cytokines or deleterious as they can suppress T cells (Pesenacker et al., 2013) and may also play an active role in dampening pro-inflammatory responses from other cells. Ex-Th17 type cells were found within HC PBMC as well as in the inflamed RA joint, albeit at a much lower frequency. Interestingly they exhibited a similar phenotype regardless of origin, suggesting that their pathogenic pro-inflammatory features are intrinsic to the nature of these cells. These cells may therefore have a specialised role in host defense, suggesting that systemic depletion of CD161⁺ Th cells may cause increased occurrences of infection. Further problems surround the use of CD161 to deplete Th17-lineage cells as there is no mouse homologue that marks Th17 lineage cells and therefore, the required *in vivo* study of CD161⁺ Th17-type cell-depletion would be challenging.

Treg cell mediated therapy

The importance of the imbalance between Th17 and Treg cells in autoimmunity has been outlined by several publications (Eisenstein and Williams, 2009, Niu et al., 2012, Kleczynska et

al., 2011, Jamshidian et al., 2013). A unique relationship exists between Th17 and Treg cells as their differentiation both rely on TGF- β signalling. Thus, iTreg and Th17 cells were thought to be divergent, mutually exclusive cell fates. However, significant plasticity between these two subsets has emerged in the literature (Kleinewietfeld and Hafler, 2013). The present study showed that a subpopulation of Treg cells expressed CD161 and produced pro-inflammatory cytokines in the RA joint. In addition, CD161⁺ Treg cells exhibited significantly increased expression of Bcl2 and HIF-1 α compared with CD161⁻ Treg cells from the RA joint. Because CD161⁺ Treg cells share this phenotype with CD161⁺ effector cells, these data suggest that CD161⁺ effector cells may contain a subset of cells that are capable of functionally switching into Treg cells. This “positive” plasticity in the context of autoimmunity is a subject that is understudied. It is highly plausible, that in a very pro-inflammatory environment, the induction of Treg cells is encouraged, similar to the activation of a small cohort of Treg cells during infection to constrain effector cells and limit collateral damage; analogous to an automatic brake being applied when the accelerator is working at maximum capacity. This may account for the enrichment of Treg cells in RA SFMC, as documented by this study and many others, and may also account for the existence of CD161⁺ Treg cells that are phenotypically and functionally similar to CD161⁺ effector Th cells. It is also plausible, however, that these CD161⁺ Treg cells are thymically derived nTreg cells which have undergone plasticity in the hyper-inflammatory environment of the RA joint and are exhibiting effector phenotypes. If this is the case, it indicates that the inherent ability of CD4⁺ T cells to be plastic is deleterious in the context of chronic inflammation and if the mechanism by which these cells gain the ability to switch can be understood, it may be manipulated to prevent plasticity from adding to autoimmune inflammation.

This evidence of Treg cell plasticity and the documented ability of Treg cells to mirror the effector cells in their environment (Duhon et al., 2012) may have widespread implications for Treg cell therapy in autoimmunity and transplantation settings. Adoptively transferring Treg cells to sites where immune suppression is required may be ineffective or even exacerbate inflammation if the transferred Treg cells were capable of functionally switching into hybrid “Treg-effector” type cells. The data from this study indicated that *ex vivo* Treg cells and *in vitro* induced iTreg cells were functionally plastic and produced pro-inflammatory cytokines when cultured with synovial fluid from RA patients. Moreover, this study showed that inducing Treg cells *in vitro* in the presence of ATRA decreased the propensity of the iTreg cells to switch into pro-inflammatory cytokine producing hybrid “Treg-effector” cells. These data suggest that due caution be exercised when adoptively transferring Treg cells for therapy and indicated that

addition of ATRA to cell culture expansion or induction of Treg cells *in vitro* may reduce the plasticity of the adoptively transferred cells *in vivo*. Furthermore, CD161⁺ Treg cells, which are capable of producing pro-inflammatory cytokines, should be depleted from the Treg cell population to enhance the safety of this therapeutic approach.

Conclusion

This study hypothesized that Th17 lineage cells may contribute to autoimmune pathology significantly more than other Th cell populations and that regulation by Treg cells may be altered in the context of RA. Moreover, plasticity between Th cell subsets was considered likely to contribute to the propagation of autoimmune inflammation. Collectively, this body of work has demonstrated that CD161⁺ ex-Th17 type cells are highly pathogenic in RA and refractory to Treg cell mediated suppression. Plasticity in both Th17-lineage cells and Treg cells contributes to the propagation of inflammation via the production of pro-inflammatory cytokines in RA. This work has provided insights into human Th17 cell biology in general and provided rationale for the therapeutic targeting of Th17-lineage cells in RA. Although the main aims of this project have been achieved, the data herein have opened up a number of additional avenues of investigation, which are discussed in the next section. A summary of the main findings of this thesis are shown in figure 6.1.

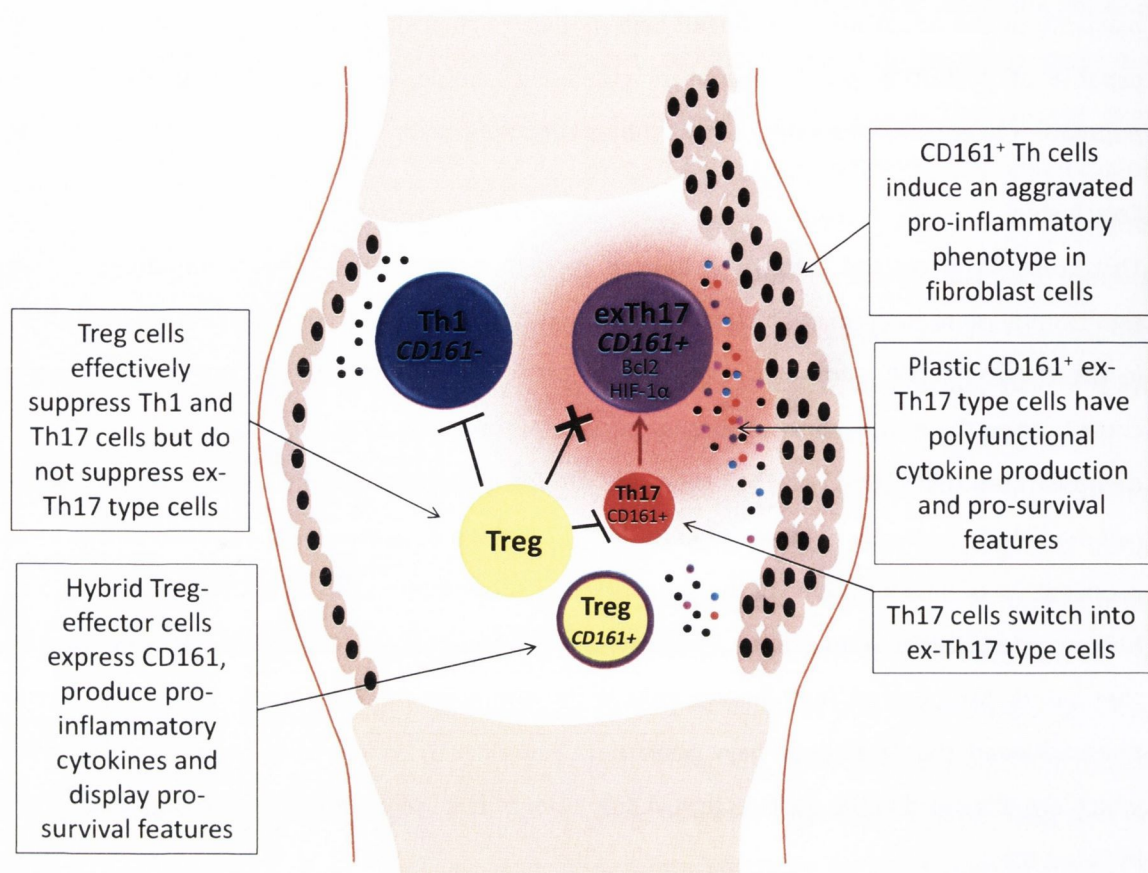


Figure 6.1 Plastic CD161⁺ Th cells play a key role in propagating autoimmune inflammation in RA.

Fibroblast cells line the synovial compartment and become hyperplastic during autoimmune inflammation. These cells invade surrounding cartilage and bone causing structural damage to the joint. CD161⁺ Th cells promote the fibroblast cell mediated destruction of bone by up-regulating the expression of RANKL and furthermore promote the fibroblast cell mediated propagation of inflammation by inducing IL-6, IL-8 and ICAM. T cells play a central role in the propagation of inflammation in the RA joint by expressing pro-inflammatory cytokines. CD161⁺ Th17 cells (shown in red) are plastic and switch into CD161⁺ ex-Th17 type cells (purple) which are enriched in the RA joint and have highly polyfunctional cytokine production. In addition, these CD161⁺ Th cells have increased expression of anti-apoptotic Bcl2 and increased stabilised HIF-1 α which allows the cell to survive under the hypoxic conditions of the RA joint. The frequencies of Treg cells are enriched in the RA joint and exhibit a highly functional phenotype, including high expression of CD39, CTLA4 and Ki67. Treg cells effectively suppress Th1 and Th17 cells but do not adequately constrain ex-Th17 type cells. Expression of CD161 is increased on Treg cells in the RA joint compared with Treg cells in the periphery. These CD161⁺ Treg-effector cells express pro-inflammatory cytokines which may contribute to inflammation in the RA joint.

6.2 Future experiments

As with most scientific studies, this project has unearthed a number of interesting questions which could not be addressed in the time available. Avenues open to investigation on the basis of this project are outlined below.

Investigating common ancestry in Th cells from the RA joint.

Profiling the V β chains of TCRs of sorted subpopulations to identify regions of overlap would ascertain whether plastic ex-Th17 type cells had trafficked into the joint or if they were derived *in situ* from the bona fide Th17 cells in the joint. If the V β profile of ex-Th17 cells' TCRs matched that of Th17 cells' profile, this would indicate that the joint environment itself promotes plasticity. Furthermore, this approach could be used to assess if CD161⁺ Treg cells are derived from CD161⁺ effector cells in the joint. Additionally, the V β profile could be used to further the theory that CD161⁺ Th cells divide asymmetrically, if a proportion of CD161^{lo/-} Th cells shared a clonal ancestry with CD161⁺ Th cells.

Investigating the mechanism behind Th17 cell switching *in vivo*.

It remains unclear as to whether or not the entire Th17 pool is multipotent and capable of functional plasticity, or if a particular subset is more inclined to switch. In order to address this question, the epigenetic status of cloned Th17 cells could be assessed using bisulphate sequencing for methylation patterns.

Since ex-Th17 type cells are evident in the PB of HC donors, this suggests that exposure to certain infections may promote Th17 cell plasticity. Recall experiments could be carried out to identify the common antigens that ex-Th17 type cells have specificity for and furthermore, to identify an infectious agent that may promote Th17 cell plasticity.

Experiments using recombinant cytokines and depleting antibodies in culture with sorted Th17 cells may help to delineate signals promoting Th17 cell plasticity *in vivo*. Because this study illustrated that cultured Th17 cells from healthy donors "spontaneously" shift to producing IFN- γ , further work is required to assess if IFN- γ production represents the end stage of Th17 cell differentiation or if these cells have received a particular signal *in vivo* or even if this might be an *in vitro* effect and therefore an artefact of experimentation.

Given that two ligands have currently been identified for CD161, investigating a functional role for CD161 would be of merit. This could be achieved by blocking CD161 or triggering signaling with the addition of its known ligands and looking for alterations in the phenotype and *in vitro* function of CD161⁺ Th cells. Because ligation of CD161 has been shown to co-stimulate the cell

(via the ligand PILAR) or increase the production of IFN- γ (via the ligand LLT1), this could potentially be contributing to the cells' inherent resistance to regulation or tendency towards plasticity.

Further investigation into the effects of CD161⁺ Th cells in RA SFMC.

The investigation of Treg cell mediated suppression of CD161⁺ and CD161⁻ effector Th cells from RA SFMC was hampered largely by low cell numbers and poor viability. For these reasons, the total number of successful suppression assays carried out on RA SFMC CD161^{+/+} effector cells was low (n=4). Ideally, this should be increased to at least 10, and should preferably be stratified by treatment. Furthermore, cross-over studies with healthy control Treg cells could help to ascertain if the nature of the effector cells in RA SFMC is inherently different to HC PBMC.

This study has shown that CD161⁺ Th cells have longer telomeres than their CD161⁻ Th cell counterparts. Given that these cells are both sorted from memory cells and proliferate to the same extent *in vitro*, this suggests that elongation of the telomere may be occurring in CD161⁺ Th cells. This could easily be assessed using a telomerase assay to show if the enzyme is preferentially up-regulated in CD161⁺ Th cells.

Investigate the effect of ATRA on CD161⁺ Treg cells.

This study found that iTreg cells differentiated in the presence of ATRA had enhanced stability and do not produce pro-inflammatory cytokines when cultured with synovial fluid from RA patients. Because this study, as well as others, has also found that it is the CD161⁺ Treg cells that produce pro-inflammatory cytokines, this data indicates that ATRA may be suppressing the induction of Treg cells from naive CD161⁺ Th cell precursors or may be inhibiting their transition to the "hybrid" Treg-effector phenotype. In light of the fact that published literature suggests that ATRA down-regulates Th17 cells and preferentially expands Treg cells, it would be of interest to assess the effect of ATRA on CD161⁺ Treg cells and the frequency of CD161 expression on iTreg cells induced in the presence or absence of ATRA. It would also be worth comparing the stability of iTreg cells induced from CD161⁻ naive precursor cells in the absence of ATRA compared with the stability of iTreg cells induced from total naive precursor cells to ascertain if depleting CD161⁺ cells from the population negates the need for ATRA to enhance the stability of iTreg cells.

Investigate if plastic ex-Th17 cells in RA have a role in secondary atherosclerosis.

Plastic IL-17⁺ IFN- γ ⁺ T cells have been detected in the artery wall of patients with symptomatic coronary atherosclerosis and induce pro-inflammatory responses in vascular smooth muscle

cells (Eid et al., 2009). Systemically targeting these cells to alleviate the symptoms of RA may also have a prophylactic impact on secondary inflammation in the vital organs. For this reason, it would be of interest to investigate the frequency of ex-Th17 type cells in the peripheral blood of RA patients with atherosclerosis compared with RA patients that do not show signs of secondary damage to vital organs.

Finding a mechanism for reduced susceptibility to Treg cell mediated suppression.

Having established that plastic ex-Th17 type cells are pathogenic in the context of autoimmunity and furthermore, are less susceptible to Treg cell mediated suppression, the next avenue open to investigation is the pursuit of a causal molecular mechanism that elicits the highly aggressive phenotype. The metabolomic profile of these cells may lend insight into differences that might cause the observed phenotype.

Recently, it has emerged that Th17 cells are uniquely sensitive to fluctuations in metabolic intermediates. Consistent with their glycolytic nature, Th17 cells also express elevated HIF-1 α and mTOR (Shi et al., 2011). mTORC1 is activated by PI-3K and AKT and is essential for the development of Th17 cells (Nagai et al., 2013). Signalling through AKT has been reported to be fundamental for the production of cytokines by Th17 cells (Wan et al., 2011). The function of mTORC1 and AKT in controlling Th17 cell effector function and plasticity in human autoimmunity has not yet been examined. Metabolic pathways may therefore hold therapeutic promise for the regulation of autoimmune Th17 cell function. A study examining the Treg cell mediated suppression of effector cells from the joint of patients with JIA showed that suppression is impaired in the synovial fluid compared with the peripheral blood due to resistance of the effector cells rather than a deficit in the function of the Treg cells (Wehrens et al., 2011). Moreover, an AKT inhibitor restored effector cell susceptibility to Treg cell mediated suppression. TNF and IL-6 together were found to induce AKT activation, therefore, causing resistance to suppression (Wehrens et al., 2011).

These published observations indicate that the expression levels of AKT or mTOR may be increased in CD161⁺ Th cells and could be responsible for the phenotype observed. However, given that Th17 cells exhibit enhanced glycolytic capacity, further investigation will be needed to demonstrate a metabolic difference between bona fide Th17 and plastic ex-Th17 cells and thus, find a candidate metabolic intermediate that may be mechanistically responsible for evasion of Treg cell mediated suppression.

Appendix 1 MATERIALS

Cell Culture Medium

Roswell Park Memorial Institute 1640 (RPMI; BioSera) was supplemented with heat-inactivated (56°C for 30 minutes) foetal calf serum (FCS; 10%, Sigma Aldrich), 100mM L-Glutamine (Sigma Aldrich) and 100 µg/ml penicillin/streptomycin (Sigma Aldrich).

MACS Buffer

Sterile Dulbecco’s Phosphate Buffered Saline (PBS; Sigma Aldrich) supplemented with FCS (2%) and EDTA (2 mM, Sigma Alrich), degassed and maintained at 4°C.

FACS Buffer

PBS (Sigma) supplemented with FCS (2%) and Sodium Azide (0.02%)

Flow-FISH REAGENTS

Hybridisation buffer

70% (v/v) formamide (Sigma Aldrich), 25% (v/v) bovine serum albumin (Sigma Aldrich; 4%, dissolved in sterile, distilled water), 2% (v/v) Tris.HCl (Sigma Aldrich; 1 M) and 3% (v/v) NaCl (Sigma Aldrich; 5M).

Post-hybridisation buffer

70% (v/v) formamide (Sigma Aldrich), 2.5% (v/v) bovine serum albumin (Sigma Aldrich; 4% solution in dissolved in sterile, distilled water), 1% (v/v) Tris.HCl (Sigma Aldrich; 1 M), 3% (v/v) NaCl (Sigma Aldrich; 5M), 1% (v/v) Tween20 (Sigma Aldrich; 10% solution dissolved in sterile, distilled water) and 22.5% (v/v) sterile, distilled water.

Flow cytometry software

Cells were acquired on LSR Fortessa (BD) or FACS Canto II (BD) using FACS Diva software (BD). Analysis was carried out with FlowJo 9.4.10.

Fluorochrome-labelled Antibodies for Flow Cytometry

Antibody	Fluor	Reactivity	Supplier	vol.(μ l) /50 μ l test
Bcl2	FITC	Human	eBioscience	1.25
CD3	V500	Human	BD Horizon	1
CD4	PerCP-Cy5.5	Human	eBioscience	1.25
	V500		BD Horizon	1
	PE-CF594		BD Bioscience	1
CD8	A780	Human	eBioscience	1.25
CD25	PE	Human	eBioscience	1.25
	PE-Cy7		eBioscience	1.5
CD27	FITC	Human	eBioscience	1.25
CD28	APC	Human	eBioscience	1.25
CD39	FITC	Human	eBioscience	5
	PerCP-eFluor710		eBioscience	1.5
CD45RA	FITC	Human	eBioscience	5
	PE		eBioscience	5
CD45RO	APC	Human	eBioscience	5
	APC-Vio770		Miltenyi Biotec	2.5
CD127	A647	Human	eBioscience	5
	A780		eBioscience	1.25
CD161	PE	Human	eBioscience	1.25
	PE-Cy7		eBioscience	1.5
	BV 421		BioLegend	1.5
CCR4	A647	Human	BD Bioscience	1.25
CCR6	FITC	Human	BioLegend	5
CCR7	PE	Human	eBioscience	1.25
CTLA-4 (CD152)	APC	Human	BioLegend	1.25
	PE		eBioscience	5
CXCR3	PE	Human	BioLegend	1.25
	efluor 660		eBioscience	1.25
FoxP3	FITC	Human	eBioscience	1.5
	PE		eBioscience	1.25
	e450		eBioscience	1.5

γδ-TCR	APC	Human	BioLegend	1.25
GM-CSF	PE	Human	BioLegend	1.25
HIF1-α	PE	Human	R&D Systems	5
IFN-γ	APC	Human	BD Pharmigen	0.5
	Pacific Blue		BioLegend	0.5
IL-2	APC	Human	eBioscience	1.5
	PE-CF594		BD Biosciences	1
IL-4	PE-Cy7	Human	eBioscience	1.5
IL-10	APC	Human	BD Biosciences	1
	PE		eBioscience	5
IL-17A	Alexa Fluor-488	Human	eBioscience	1.5
	PE		eBioscience	1.5
	Alexa fluor-700		BD Biosciences	1.5
IL-22	PE-Cy7	Human	eBioscience	1.5
Ki67	FITC	Human	eBioscience	1.5
	BV 421		BioLegend	1.25
PNA Telomere probe	Cy5	Human	Cambridge Research Biochemicals	Final conc. 1.5 µg/ml stained in 100 µl
ROR-γt	PE	Human	eBioscience	2
TNF	PerCP-Cy5.5	Human	eBioscience	1.25
Viability dye	efluor 506	Human	eBioscience	1 µl/ml

ELISA REAGENTS

ELISA Wash Buffer

20X PBS (500ml; 80 g NaCl, 11.6 g Na₂HPO₄, 2 g KH₂PO₄ and 2 g KCl all from Sigma Aldrich)

dH₂O (9.5L), Tween ® 20 (2 ml, Sigma Aldrich)

ELISA Substrate Solution TMB

ELISA Stopping Solution Sulphuric Acid (H₂SO₄ ; Sigma Aldrich 1M)

ELISA Kits

Matched antibody pairs	Reactivity	Supplier
IFN-γ	Human	eBioscience
TNF	Human	eBioscience
IL-17A	Human	eBioscience
GM-CSF	Human	eBioscience
IL-6	Human	eBioscience
IL-8	Human	eBioscience

ELISA software

ELISA spectrophotometric analysis was carried out at a wavelength of 450 nm using the Epoch microplate spectrophotometer (BioTek) and Gen5 software (BioTek).

General reagents

Reagent	Supplier	Concentration used (unless otherwise stated)
All Trans Retinoic Acid (ATRA/vitamin A)	Sigma	100 nM
Brefeldin A	Sigma	5µg/ml
Cell Trace Violet (CTV)	Invivogen	1µM
CSFE	Invivogen	0.5µM
Human anti-CD3 (OKT3 clone)	eBioscience	1µg/ml
Human anti-CD28 (CD28.2 clone)	eBioscience	0.5µg/ml
ionomycin	Sigma	500ng/ml
Memory CD4+ T cell isolation kit, human	Miltenyi biotech	according to protocol
Microbeads CD4, human	Miltenyi biotech	Half recommended amount
Naïve CD4+ T cell isolation kit II, human	Miltenyi biotech	according to protocol
PMA	Sigma	50ng/ml
rhGM-CSF	Miltenyi biotech	10 ng/ml
rhIL-2	BD Pharmingen	500 IU iTreg conversion/ 20 IU for cell feeding
rhIL-17A	Miltenyi biotech	10 ng/ml
rhIFN-γ	Miltenyi biotech	10 ng/ml
rhTGF-β	R&D systems	10 ng/ml
rhTNF	Miltenyi biotech	10 ng/ml
Treg expansion kit, human	Miltenyi biotech	1:1 (beads:cells)

Appendix 2 Unabridged SPICE legends

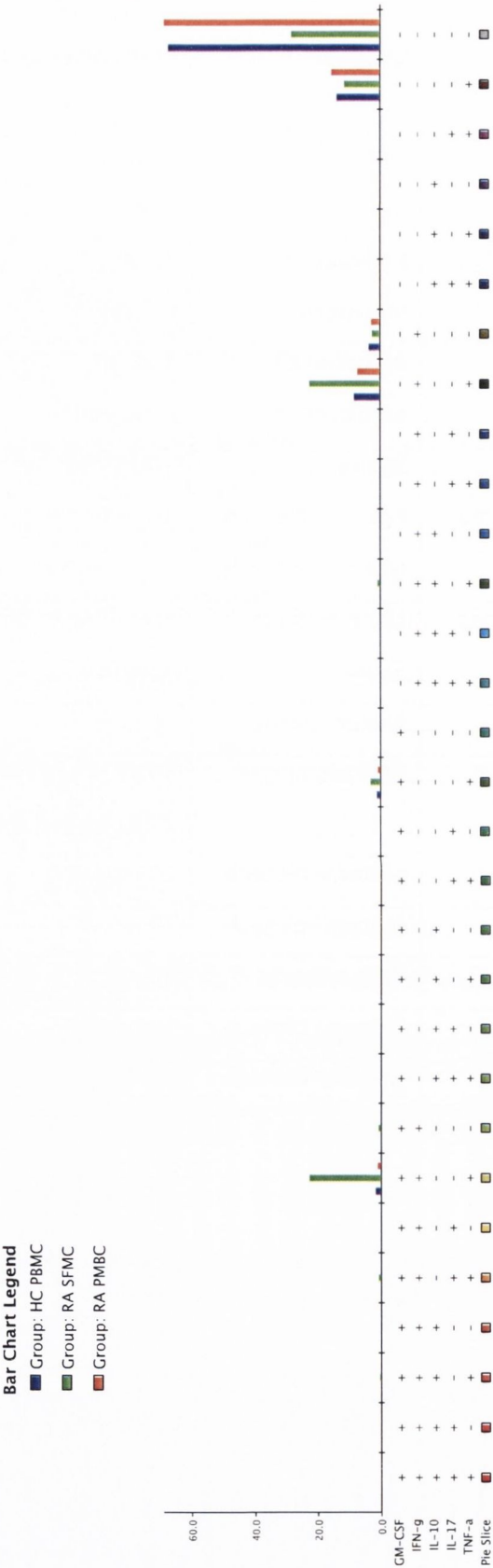


Figure app. 2.1

Bar chart legend for figure 3.5 SPICE diagrams

Bar Chart Legend
 ■ Group: CD161+
 ■ Group: CD161-

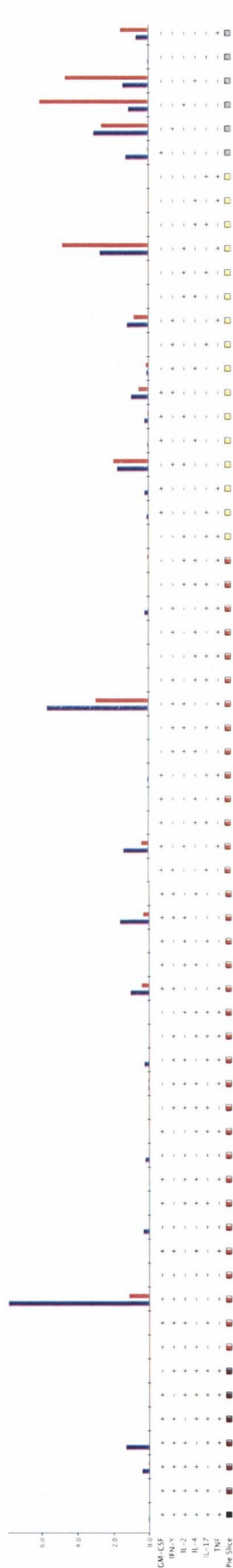
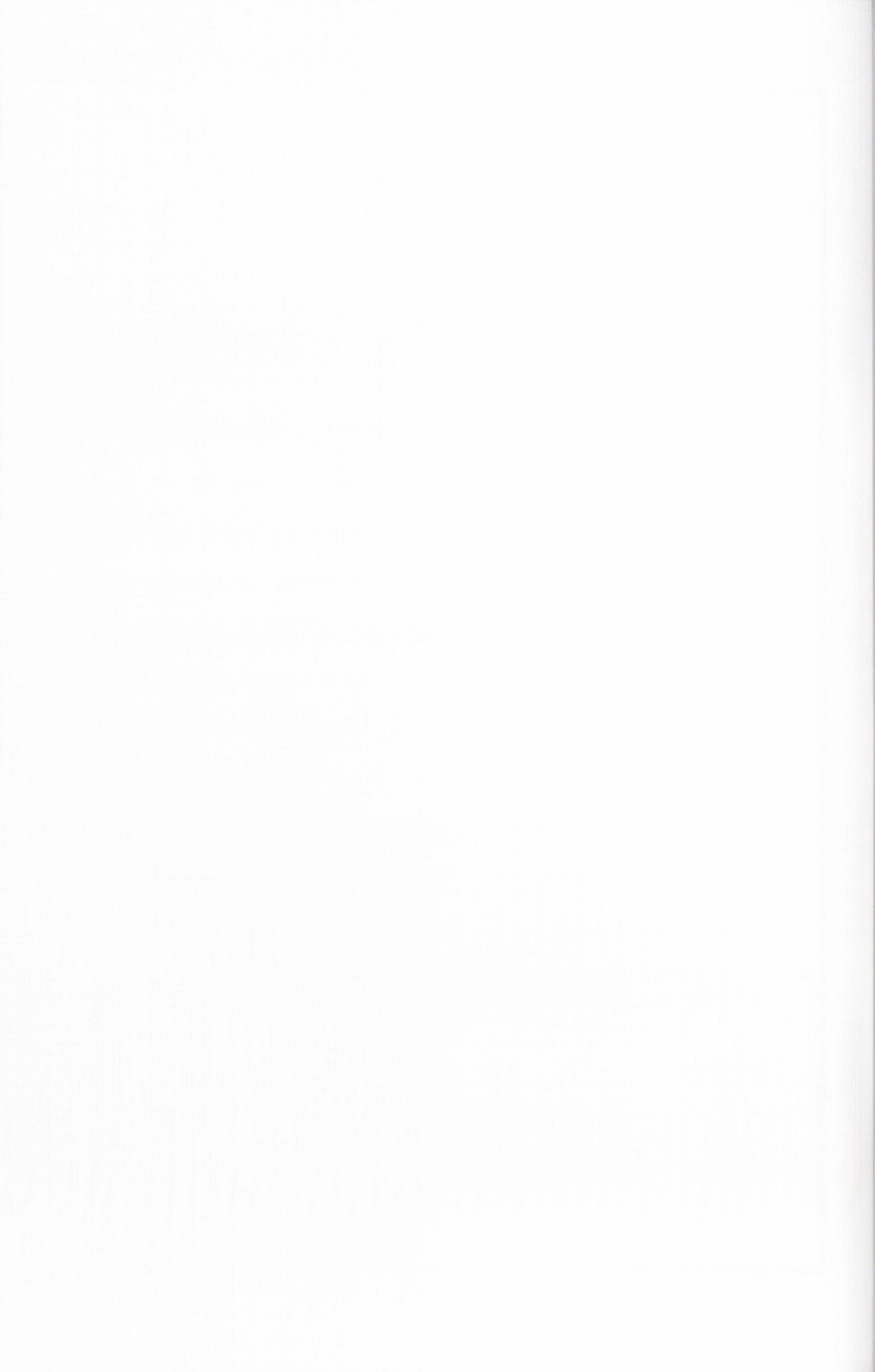


Figure app. 2.2

Bar chart legend for figure 3.11 SPICE diagrams



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