Developing Regulatory T Cells as a Cellular Therapy for Inflammatory Bowel Disease

Canavan, James Benedict

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E-thesis Declaration

James Benedict Canavan

Developing Regulatory T Cells as a Cellular Therapy for Inflammatory Bowel Disease

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by

James Benedict Canavan

Department of Experimental Immunobiology

Division of Transplant Immunology and Mucosal Immunology

&

Medical Research Council Centre for Transplantation

King's College London

5th Floor, Tower Wing

Guy's Hospital

London SE1 9RT

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1 Declaration

The work presented here is my own and all experiments, except where acknowledged in the text, were performed by myself.

James Benedict Canavan

2 Abstract

Crohn's disease (CD) is a chronic, currently incurable inflammatory bowel disease. There remains an unmet need to develop novel therapies, as currently used medications often fail to induce or maintain long-term remission and may also be complicated by significant side-effects. $CD4^+CD25^{hi}CD127^{lo}FOXP3^+$ regulatory T cells (T_{regs}) modulate immune activation and contribute substantially to peripheral tolerance. Observations from monogenetic disorders in humans and experimental models of colitis in mice suggest that T_{regs} have a role in modulating intestinal inflammation. Recent clinical trials provide proof-of-principal that T_{regs} can be selected and expanded *in vitro* under "good manufacturing practice" conditions while retaining their function and are safe when adoptively transferred to humans. This thesis addresses potential barriers to the development of *in vitro* expanded T_{regs} as a cell based therapy for CD by showing that it is possible to expand T_{regs} from the peripheral blood of CD patients. Extending observations made by other investigators, this thesis shows that FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{reg} subset is likely to be the optimum population from which to expand T_{regs} *in vitro* for potential clinical use, based on *in vitro* suppressive ability, stability of FOXP3 expression and cytokine expression in comparison with T_{regs} expanded from MACS-enriched $CD4^+CD25^{hi}$ or FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ subsets. T_{regs} expanded *in vitro* from the $CD45RA^+$ T_{reg} subset express $\alpha_4\beta_7$ integrin and home to human small bowel in a *Scid* mouse bearing a human small intestinal xenograft. A rapid 7-hour assay of T_{reg} function is validated, in comparison with a "classic" 96-hour CFSE dilution assay, and used to

demonstrate that T_{regs} expanded from the $CD45RA^+$ T_{reg} subset suppress activation of $CD3^+$ lymphocytes obtained from inflamed CD mucosa and mesenteric lymph node. Taken together, these observations suggest that T_{regs} expanded *in vitro* from FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors have many characteristics which make them suitable for consideration as a potential cell-based therapy for CD. These findings pave the way for a further development towards a clinical trial of autologous *in vitro* expanded T_{regs} for CD.

3 Acknowledgements

3.1 Supervision

The research reported in this thesis was supervised by Professor Graham M Lord and Dr Maria P Hernandez-Fuentes at King's College London (KCL), and Professor Thomas T Macdonald at Bart's and the London School of Medicine and Dentistry. Additional guidance was provided by Professor Giovanna Lombardi and Professor Randy Noelle at KCL and Professor Nahum Shpigel at the Hebrew University of Jerusalem (HUJI), Rehovot, Israel. Clinical supervision was provided by Dr Peter Irving and Dr Jeremy Sanderson at Guy's & St Thomas NHS Foundation Trust.

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Service (NHS) Foundation Trust and King's College London. The views expressed are those of the author and not necessarily those of the NHS, the NIHR, or the Department of Health.

3.3 Attribution of experiments

The experiments described in Chapter 10 (page 160) were designed, executed and analysed by myself with the assistance of Dr Behdad Afzali. The *in vitro* expanded T_{reg} lines used in this chapter were cultured by Dr Cristiano Scottà and Dr Henrieta Fazekasova. This chapter was published as:

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The experiments described in Chapter 11 (page 196) were designed, executed and analysed by myself with the assistance of Mr Matthew Elder. Assessment of *FOXP3* demethylation shown in Figure 11.8 (page 225) was performed by Epiontis GmBH (Berlin, Germany). The C.B-17 *scid* human intestinal xenograft model was established by Professor Nahum Shpigel and maintained by Dr Irit Shoval at the Hebrew University of Jerusalem. The immunofluorescence panels shown in Figure 11.9 (page 231) were stained by Dr Ellen Marks (Postdoctoral Research Fellow, KCL) and Dr Irit Shoval (HUJI). Chapter 11 has been submitted for publication.

Professor Toby Prevost (KCL) performed the statistical modelling comparing two designs of a phase I clinical trial, discussed in Section 12.9.5 (page 272).

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7 List of Abbreviations

The following abbreviations have been used to denote regulatory T cell subsets, consistent with nomenclature in original publications and recent expert recommendations (Abbas et al., 2013; Miyara et al., 2009):

T_{reg}	Thymically-derived FOXP3^{+} regulatory T cell. These have also been abbreviated as "tT_{regs}", where the use of "T_{reg}" might cause confusion, e.g. "tT_{regs} *vs.* iT_{regs}".
aT_{reg}	Activated CD4^{+}CD25$^{hi(+++)}$CD45RA^{-}FOXP3^{hi} T_{reg} subset
iT_{reg}	Peripherally (extra-thymically) induced CD4^{+} T_{reg}
rT_{reg}	Resting CD4^{+}CD25$^{hi(++)}$CD45RA^{+}FOXP3^{lo} T_{reg} subset
Th3	TGF-β-expressing peripherally induced regulatory T cell
Tr1	Type 1 regulatory T cell (IL-10-producing peripherally induced regulatory T cell)
iTr35	IL-35-producing peripherally induced regulatory T cell

The following additional abbreviations are used throughout this thesis:

A_{2A}AR	A_{2A} adenosine receptor
ADA	Adalimumab

ADP	Adenosine diphosphate
AE	Attaching-effacing
AHR	Aryl hydrocarbon receptor
AIEC	Adherent invasive *Escherichia coli*
APC	Allophycocyanin
APC	Antigen presenting cell
ARE	AU-rich element
ATG16L1	Autophagy related 16-like 1 (*S. cerevisiae*)
APC	Antigen presenting cell
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
AZA	Azathioprine
BLT	Bone marrow, liver and thymus
B_{reg}	Regulatory B cell
CBA	Cytometric bead array
CCR	Chemokine receptor
CD	Crohn's disease
CD (number)	Cluster of differentiation, specified by the numerical suffix
CDAI	Crohn's disease activity index
CESAME	"Cancers Et Surrisque Associé aux Maladies inflammatoires intestinales En France" registry

CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CpG-ODN	CpG Oligodeoxynucleodide
CRM	(Bayesian) Continual reassessment model
CTLA-4	Cytotoxic T lymphocyte antigen-4
CTV	CellTrace Violet
CTZ	Certolizumab
DC	Dendritic cell
DLT	Dose limiting toxicity
DEREG	Depletion of regulatory T cell mouse model
DN	Dominant negative
DSS	Dextran sodium sulphate
DT	Diphtheria toxin
EAE	Experimental autoimmune encephalomyelitis
Ebi3	Epstein Barr virus induced gene 3
EBV	Epstein-Barr virus
EC-IBD	European Collaborative Study Group on IBD
ELISA	Enzyme Linked Immunosorbent Assay
EPEC	Enteropathogenic *Escherichia coli*
ER	Endoplasmic reticulum
FACS	Fluorescence Activated Cell Sorting

Flt3L	FMS-like tyrosine kinase 3 ligand
FOXP3, *FOXP3*	Forkhead box P3
GETAID	"Groupe d'Etude Thérapeutique des Affections Inflammatoires Digestives"
GITR	Glucocorticoid induced tumour necrosis factor receptor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-monocyte colony stimulating factor
GMP	Good manufacturing practice
GvHD	Graft *vs.* host disease
GWAS	Genome wide association study
HR	Hazard ratio
HSTCL	Hepatosplenic T cell lymphoma
IBSEN	"Inflammatory Bowel South-Eastern Norway" study group
ICOS	Inducible T cell co-stimulator
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IF	Immunofluorescence
IFN-γ, *IFNG*	Interferon-gamma
IFX	Infliximab
Ig	Immunoglobulin, e.g. IgA, IgG, IgM.
IKKγ	IκB kinase-γ

IL-(number)	Interleukin, specified by the numerical suffix
IL-(number)R	Interleukin receptor
ILC	Innate lymphoid cell
IQR	Interquartile range
IRB	Institutional Review Board
IP	Intraperitoneal
IV	Intravenous
LB	Lysogeny broth
LRR	Leucine rich repeat domain
LP	Lamina propria
LPMC	Lamina propria mononuclear cell
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetic cell sorting
MBP	Myelin basic protein
MDP	Muramyl dipeptide
MDR1	Multi-drug resistance 1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MLNMC	Mesenteric lymph node mononuclear cell

MTX	Methotrexate
NK	Natural killer cell
NKT	Natural killer T cell
NHS	National Health Service
NOD2	Nucleotide-binding oligomerisation domain containing 2
OR	Odds ratio
OVA	Ovalbumin
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PE	Phycoerythrin
PML	Progressive multifocal leukoencephalopathy
Poly(I:C)	Polyinosinic-polycytidylic acid
PSA	Polysaccharide A
QC	Quality control
RALDH2	Retinaldehyde dehydrogenase 2
RAG, *RAG2*	Recombinase activating gene (2)
REC	Research Ethics Committee
rh	Recombinant human cytokine, e.g. rhIL-2
ROC	Receiver operating characteristic (curves)

RORγt, *RORC*	RAR-related orphan receptor C
RR	Relative risk
RTE	Recent thymic emigrant
SAA	Serum amyloid A
SAMP1/Yit(Fc)	Senescence accelerated mouse P1/Yit or senescence accelerated mouse P1/YitFc mouse strains
SB	Small bowel
SCFA	Short chain fatty acid
SCID	Severe combined immunodeficiency
SD	Standard deviation
SFB	Segmented filamentous bacteria
SIR	Standardised incidence ratio
SNP	Single nucleotide polymorphism
SPECT	Single Photon Emission Computed Tomography
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
T_{con}	Conventional $CD4^+CD25^{lo/int}$ or $CD4^+CD25^-$ T cells, as specified in the text.
TCR	T cell receptor
TGF-β, *TGFB*	Transforming growth factor - beta
Th	$CD4^+$ T helper subset

TLR	Toll-like receptor
TNF, *TNF*	Tumour necrosis factor
TREAT	"Therapy, Resource, Evaluation and Assessment Tool" CD infliximab treatment registry
TRUC	*TBX21*$^{-/-}$ x *RAG2*$^{-/-}$ Ulcerative Colitis model of experimental colitis
TSDR	T_{reg} specific demethylated region
UC	Ulcerative colitis
XG	Xenograft
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein
WT	Wild type

8 Introduction

8.1 Introduction

$CD4^+CD25^{hi}CD127^{lo}FOXP3^+$ thymus-derived regulatory T cells (T_{regs}) are key mediators of peripheral tolerance (Huibregtse et al., 2007). T_{regs} can modulate the activation of multiple effector cell types, including conventional $CD4^+CD25^{lo/int}$ T lymphocytes (T_{cons}), $CD8^+$ lymphocytes and γδ T cells, natural killer (NK) cells and dendritic cells (DCs). Pre-clinical models of experimental colitis and rare immunodeficiency syndromes in humans provide evidence that T_{regs} have a role in maintaining intestinal mucosal homeostasis, in addition to peripheral tolerance. T_{reg} cell therapy is a realistic possibility, now that T_{regs} can be reliably identified, isolated and expanded *in vitro*, while maintaining suppressive function. These observations prompted an investigation into the feasibility of developing T_{regs} as an autologous cell therapy for Crohn's disease (CD): a currently incurable, chronic inflammatory disease of the intestine.

This introduction reviews the burden of CD on the population and individuals, currently available therapies and treatment paradigms and emphasises the need for the development of novel therapies for this relapsing-remitting chronic disease. The complex interaction between environment, genes, microbiota and the intestinal immune system will be discussed, in health and disease. Observations suggesting that T_{regs} have a role in mucosal immune regulation will be put into context and potential opportunities and barriers to the development of T_{regs} as a cell-based therapy will be reviewed. Finally, the hypotheses generated by this review will be discussed. The experimental

approaches taken to address these hypotheses will be discussed in subsequent chapters.

8.2 Introduction to Crohn's disease

Crohn's disease (CD) is an inflammatory bowel disease that is characterised by chronic, idiopathic, granulomatous segmental inflammation of the gastrointestinal tract (Abraham and Cho, 2009). Wilhelm Fabry (1623), Giovanni Morgagni (1761) and Samuel Wilks (1859) all provided early descriptions of cases consistent with CD (Jones, 1960; Kirsner, 1988). The Glasgow surgeon T Kennedy Dalziel described a series of 13 patients with "Chronic Interstitial Enteritis" in 1913, closely resembling Crohn's later report (Dalziel, 1913). However, the 1932 case series of 14 patients with "Regional Ileitis" by Burrill B Crohn, Leon Ginzburg and Gordon D Oppenheimer caught the medical imagination and gave the disease its eponym (Crohn et al., 1932).

8.3 Epidemiology of Crohn's disease

CD is slightly more common in women than men (female:male ratio 1.26-1.53) (Bernstein et al., 2006). Peak incidence of CD is in the second and third decades of life (Cosnes et al., 2011). As shown in Table 8.1 (page 30) the incidence of CD has increased in developed countries over the last 50 years. The incidence of CD in adults appears to be stabilising in well-characterised longitudinal cohorts, while the incidence of CD in teenagers is still rising in northern Europe (Chouraki et al., 2011; Perminow et al., 2009).

Recent estimates for the incidence of CD, in cohorts that include incident patients from the year 2000 onwards, range from 6.3/100,000 (Chouraki et al., 2011) to 20.2/100,000 (Bernstein et al., 2006). Recent estimates for the prevalence of CD range from 155.2/100,000 (Gearry et al., 2006) to 319/100,000 (Bernstein et al., 2006). Rubin *et al.* examined the prevalence of CD in northern England, in a population-based survey of 24 general practices, comprising of 179,496 patients. The prevalence of CD was 144/100,000 in this cohort (Rubin et al., 2000).

The highest incidences of CD have been reported in northern Europe, North America and New Zealand, with lower incidences in developing countries (Baumgart et al., 2011; Cosnes et al., 2011). A North-South gradient has also been identified within Europe (Shivananda et al., 1996). An East-West gradient has been identified across Canada, with incidence (and prevalence) increasing from 8.8/100,000 (161/100,000) in British Columbia to 20.2/100,000 (319/100,000) in Nova Scotia (Bernstein et al., 2006).

The incidence of CD is lower in developing countries and in "minorities" in developed countries, compared with the general population incidence in developed countries (Cosnes et al., 2011). In a study from the southern Californian Kaiser Permanente health system, the prevalence of CD in adult Hispanic and Asian Americans was 4.1/100,000 and 5.6/100,000, respectively, while the prevalence in African Americans was 29.8/100,000, compared with 43.6/100,000 for whites (Kurata et al., 1992). However, the prevalence of CD in African Americans is likely to increase, as the incidence rate in African American children is increasing to rates comparable with non-Hispanic white children (Ogunbi et al., 1998). The incidence

of CD in Canadian Aboriginal groups is one tenth of that seen in the general population (Blanchard et al., 2001).

Table 8.1: The changing incidence and prevalence of CD in selected, longitudinal cohorts.

Location & Reference	Cohort Period or Date	Incidence per 10^5	Prevalence per 10^5
Olmsted County, Minnesota, USA			
Sedlack *et al.* (1980)	1935-1954	1.9	
	1955-1964	4.0	
	1965-1975	6.6	
	1976		105.7
Loftus *et al.* (1998)	1991		133
Loftus *et al.* (2007)	1940-1949	2.3	
	1950-1959	2.6	
	1960-1969	6.5	
	1970-1979	7.9	
	1980-1989	6.8	
	1990-2000	7.9	
	2000		174
Manitoba, Canada			
Bernstein *et al.* (1999)	1989-1994	14.6	
	1994		198.5
Blanchard *et al.* (2001)	1987-1996	15.6	
Bernstein *et al.* (2006)	1998-2000	15.4	271.4
Copenhagen County, Denmark			
Binder *et al.* (1982)	1970-1978	2.7	
	1978		34
Munkholm *et al.* (1992)	1979-1987	4.1	
	1987		50.4
Vind *et al.* (2006)	2003-2005	8.6	
Stockholm County, Sweden			
Lapidus *et al.* (1997)	1955-1959	1.4	
	1960-1964	2.2	
	1965-1969	3.7	
	1970-1974	4.8	
	1975-1979	4.4	
	1980-1984	4.5	
	1985-1989	4.9	
Lapidus *et al.* (2006)	1990-2001	8.3	213
Northern France (EPIMAD registry)			
Whole cohort (Chouraki et al., 2011)	1988-1990	5.2	
	1997-1999	7.1	
	2006-2007	6.7	
Subgroup analysis of the 10-19 age group (Chouraki et al., 2011)	1988-1990	6.5	
	2006-2007	11.1	
Southeast Norway (Inflammatory Bowel South-Eastern Norway (IBSEN) study group)			
IBSEN study group (Moum et al., 1996)	1990-1993	5.8	
IBSEN-II study group (paediatric CD) (Perminow et al., 2009)	2005-2007	6.8	

8.4 Genetic and environmental risks for Crohn's disease

8.4.1 Familial aggregation and concordance in twin studies

A positive family history is the largest independent risk factor for CD (Baumgart and Sandborn, 2007). Between 2.2% and 16.2% of patients with CD will have a first-degree relative with CD, and 5.2-22.5% of probands with CD will have a first-degree relative with IBD (Russell and Satsangi, 2004). A diagnosis of CD increases the risk of CD to first-degree relatives by 5-to-35-fold, while a diagnosis of CD in a proband increases the risk of CD in a sibling by 30-to-42-fold, compared with the risk in the general population (Russell and Satsangi, 2004). The age-corrected risk of developing CD is also two-fold higher in a Southern Californian Ashkenazi Jewish cohort compared with non-Jewish cohorts (Peeters et al., 1996; Roth et al., 1989; Yang et al., 1993).

Recent studies show that between 35% and 63.6% of monozygotic twins and 0-3.6% of dizygotic twins are concordant for CD (Halfvarson et al., 2003; Jess et al., 2005; Spehlmann et al., 2008). Bengtson *et al.* found a 100% disease concordance in proband-sibling pairs (Bengtson et al., 2010). There is also a high degree of concordance in both CD diagnosis (82-83%) and disease site (76-86%) in families with more than two affected members (Bayless et al., 1996; Colombel et al., 1996).

A number of groups have reported genetic anticipation for CD in the offspring of both Jewish and non-Jewish affected parents, including the prospectively acquired IBSEN study group cohort (Bengtson et al., 2009; Cabré et al., 2014; Heresbach et al., 1998; Peeters et al., 1996). However, Hampe *et al.*

suggest that confounders, such as shorter diagnostic interval in offspring and ascertainment bias (i.e. offspring who may develop CD at an older age in the future have not yet developed the disease at the time of ascertainment), mean that evidence for genetic anticipation in CD should be treated with caution (Hampe et al., 2000).

8.4.2 Susceptibility genes and loci

CD is a complex polygenetic disease, where common polymorphisms in multiple genes are likely to confer modest increases, or decreases, in the absolute risk of developing disease (Risch and Merikangas, 1996). The three major approaches to the identification of CD risk alleles are genetic linkage studies, candidate gene association studies and genome-wide association studies (GWAS) (Liu and Anderson, 2014).

Linkage studies assess the co-segregation of marker alleles in haplotype blocks with disease status in affected families, while candidate gene studies attempt to associate markers within individual genes with disease. These approaches can identify low-frequency variants of moderate effect, although with a high false-positive rate. These studies identified 9 chromosomal areas in linkage disequilibrium with IBD, termed IBD1-9 according to the date of discovery, located on chromosomes 16 (haplotype block includes *NOD2* gene), 12, 6 (*MHC*), 14 (*IL25*), 5 (*OCTN1/2*), 19, 1 (*TGFB2*), 16 and 3 (*CCR5*), respectively (Cho et al., 1998; Duerr et al., 2000; Hampe et al., 1999; Hugot et al., 1996; Paavola-Sakki et al., 2003; Rioux et al., 2000; Satsangi et al., 1996; van Heel et al., 2003; Williams et al., 2002).

While linkage studies typically assess haplotype blocks using approximately 300 marker alleles in family groups, GWAS can assess statistically significant associations between allele frequencies and disease across hundreds of thousands of single nucleotide polymorphisms (SNPs) in a population. GWAS can identify common variants of small effect. Due to linkage disequilibrium, $5x10^6$ "common" SNPs, with a minor allele frequency >5%, can be profiled by assessing approximately $5x10^5$ SNPs (Liu and Anderson, 2014).

The original GWAS studies in CD (Duerr et al., 2006; Franke et al., 2007; Hampe et al., 2007; Libioulle et al., 2007; Parkes et al., 2007; Raelson et al., 2007; Rioux et al., 2007; Wellcome Trust Case Control Consortium, 2007; Yamazaki et al., 2005) were followed by meta-analyses performed under the aegis of the International IBD Genetics Consortium (Barrett et al., 2008; Franke et al., 2010; Jostins et al., 2012).

In the most recent study, Jostins *et al.* performed a meta-analysis (with Immunochip validation) on GWAS data from over 75,000 cases and controls (Jostins et al., 2012). 163 disease risk loci were identified, 30 of which were CD-specific and 110 of which were associated with both CD and ulcerative colitis (UC). SNPs in *NOD2* (Odds Ratio [OR] 3.103) and *IL23R* (OR 2.013) were associated with the highest risk of CD and IBD, respectively. The contribution of all other SNPs to CD or IBD was modest, with odds ratios of <1.3 each. These data highlight the contribution of genes involved in immune pathways to the risk of CD, including autophagy (*ATG16L1*, *NOD2*), innate and adaptive immunity (*CARD9*, *RASGRP1*, *RIPK2*, *TEC* and *TNFSF18*), cytokine expression and signalling (*IFNG*, *IFNGR2*, *IL6ST*, *IL12RB*, *IL23R*, *RORC*, *STAT3*, *STAT5A*, *STAT5B*) and immune regulation

(*IL2RA*, *IL10*, *IL10RB*, *SMAD7*). Genes associated with Mendelian susceptibility to mycobacterial disease were also enriched, suggesting selective pressure in evolving the genetic architecture of IBD. The majority of CD-associated SNPs lie in non-coding regions, suggesting that they are likely to affect regulation of the transcription of genes of interest, rather than producing mutated or truncated gene products. Jostins *et al.* then performed a further *in silico* analysis which identified a network of functionally related genes that are over-expressed in bone marrow-derived macrophages, including IBD risk alleles (*CARD9*, *DOK3*, *HCK*, *IL10*, *LGALS9*, *NOD2*, *SLC11A1* and *VDR*) and non-IBD risk alleles (*ALOX5*, *ARC*, *IL23A*, *LST1*, *NCKAP1L*, *PARVG*, *S100A9* and *TBC1D2*). This approach could be used to develop novel hypotheses on IBD biology.

GWAS data currently explains 13.6% of the variance of CD (Jostins et al., 2012), which falls short of the estimated variance attributable to genetic factors in twin studies: a concept that has been termed "hidden heritability" (Satsangi et al., 2011). The contribution of genes to disease risk could be further characterised by novel techniques that increase the coverage of current GWAS, such as evaluating "uncommon" SNPs (minor allele frequency <5%), evaluating polymorphisms in introns, fine mapping of exons and novel computational techniques (Kim et al., 2011; Satsangi et al., 2011; Stahl et al., 2012).

Characterisation of the immunological role of CD risk alleles can further an understanding of the biology of CD. This has been done successfully for the autophagy related 16-like 1 gene (*ATG16L1*). In humans, both CD patients and healthy controls who carry the *ATG16L1* T300A risk allele exhibit increased Paneth cell endoplasmic reticulum (ER) stress (Deuring et al., 2014). Murine

hypomorphic *Atg16l1* activity (*Atg16l1*^{HM}) is associated with Paneth cell dysfunction (Adolph et al., 2013). ATG16L1-mediated autophagosome formation limits ER stress and NF-κB activation in mice that carry an epithelium-specific deletion of the unfolded protein response (UPR) transcription factor *Xbp1*. However, removal of this protective effect by introducing *Atg16l1*^{HM} to *Xbp1*$^{\Delta IEC}$ mice results in transmural CD-like inflammation (Adolph et al., 2013). Amino acids 296-299 of ATG16L1 also constitute a caspase cleavage motif. The T300A CD risk allele (T316A in mice) increases the susceptibility of ATG16L1 to caspase-3-mediated degradation *in vitro*, which provides a link between starvation-induced autophagy, ER stress and Paneth cell dysfunction in ATG16L1 risk allele carriers (Murthy et al., 2014). Paneth cells are discussed further in Section 8.8.2 (page 66).

The biology resulting from mutations in nucleotide-binding oligomerisation domain containing 2 (NOD2), a cytoplasmic muramyl dipeptide (MDP) sensor, is less well understood. Mutations in the leucine-rich repeat domain (LRR), including R702W, G908R and 1007fs, result in impaired recognition of MDP and impaired MDP-mediated NF-κB activation *in vitro* (Netea et al., 2005). While murine *Nod2* deficient models do not recapitulate CD, wild type (WT) *Nod2* restrains NF-κB activation and Interleukin-1β (IL-1β) expression in macrophages, which is lost in *Nod2*^{2939ic} mutant mice (Maeda et al., 2005). *Nod2* sufficiency also facilitates MDP-mediated protection from dextran sodium sulphate (DSS) colitis, which is lost in *frameshift Nod2* mice (Watanabe et al., 2008b). NOD2 also has roles in autophagy and mucosal barrier function (Strober and Watanabe, 2011). Carriage of *NOD2* risk alleles is associated with Paneth cell dysfunction and a reduction α-defensins: a class of luminal antimicrobial peptides secreted by Paneth cells (Kobayashi et al.,

2005; Wehkamp et al., 2005). *Nod2*$^{-/-}$ mice also have decreased luminal defensin-related cryptidin 4 (homolog of human α defensin) compared with WT, which is further decreased following enteric *Listeria* infection. *Listeria* infection is also associated with increased bacterial translocation from the gut in *Nod2*$^{-/-}$ mice, compared with WT controls (Kobayashi et al., 2005). NOD2 polymorphisms might also affect T_{reg}-mediated immune modulation, as NOD2-mediated NF-κB activation in FOXP3^{+} T_{regs} prevents Fas-mediated apoptosis, and this is lost in T_{regs} obtained from patients with *NOD2* risk alleles (Rahman et al., 2010). Associations between CD risk alleles and microbial dysbiosis, epithelial and immune cell dysfunction are discussed in subsequent sections.

Another challenge in CD genetics is the prediction of disease behaviour from the presence or absence of risk alleles. Cleynen *et al.* correlated genotype with clinical outcome data in 1,528 European CD patients, followed for over 10 years, in order to confirm earlier observations that *NOD2* polymorphisms are associated with ileal disease location, earlier age of onset and more aggressive disease course, and to identify new genotype-phenotype associations (Cleynen et al., 2013). *NOD2* polymorphisms were associated with ileal disease, while both *NOD2* and *TNFSF15* risk alleles were associated with risk of resection. Risk alleles in *NOD2* and *JAK2* were associated with a shorter time to stenosing disease. *PDRM1* and *ATG16L1* risk alleles were associated with a shorter time to fistulating disease. Polymorphisms in *NOD2*, *LOC441108*, *SLC22A23*, *PDRM1* and *TAB2/MAP3K71P2* were associated a shorter time to a combined end-point of stenosis, fistula, perianal disease or surgery. *IL23R* polymorphisms were associated with ileocolonic disease but protective against the development of

fistulating disease. With further research and validation, it may be possible in the future to use genetic data to aid in the prediction of outcome in clinical practice.

8.4.3 Environmental risks for Crohn's disease

GWAS data currently explains 13.6% of the variance of CD (Jostins et al., 2012). Modelling of GWAS data in other common polygenetic diseases suggests that genetics may ultimately explain between 43% and 49% of the variance in coeliac disease (excluding the contribution attributable to the major histocompatibility complex [*MHC*] genes), coronary artery disease and type 2 diabetes (T2DM) (Stahl et al., 2012). These data, and the rapid increase in CD incidence rates in the last 40 years in well-studied populations (discussed in Section 8.3 [page 28]) strongly suggest that environmental factors play a role in the aetiology of CD.

As discussed above (Section 8.3 [page 28]), a north-south gradient exists within Europe and between developed and developing countries, suggesting that environmental factors associated with affluence may have a role in the aetiology of CD (Baumgart et al., 2011; Shivananda et al., 1996). In the United States, the incidence of CD is rising amongst Asian, African-American and Hispanic populations (Hou et al., 2009). The offspring of immigrants to countries with high CD incidence have an increased risk of CD, compared with their parents (Li et al., 2011). Multiple associations with relative affluence have been identified, including educational attainment (Rogers et al., 1971), skilled or managerial occupation (Keighley et al., 1976), an urban-rural gradient (Bernstein et al., 2006), the availability of hot running water (Gent et al., 1994) and a sedentary lifestyle

(Bernstein et al., 2001). The "hygiene hypothesis" has been proposed as a unifying hypothesis to associate improved public health and environmental hygiene with an increased incidence of atopy and allergic disease in developed countries. It hypothesises that a lack of exposure to childhood infections results in an abnormal "education" of the immune system, while a lack of exposure to enteric parasites removes their immune-modulating effects, leading to an increased risk of atopy and allergic disease (Carvalho et al., 2009; Lashner and Loftus, 2006). Hugot *et al*. proposed the "cold chain hypothesis", suggesting that bacteria such *Listeria* and *Yersinia*, that can survive and proliferate at refrigerated temperatures, might be responsible for an association between the availability of refrigeration and the incidence of CD (Hugot et al., 2003). These hypotheses remain unproven with respect to CD.

Many groups have searched for a pathogenic bacteria in association with CD, including *Mycobacterium avium paratuberculosis* and adherent-invasive *Escherichia coli*. CD is pathologically similar to Johne's disease, a granulomatous enteritis of cattle caused by *Mycobacteria avium paratuberculosis*. Fastidious *Mycobacteria* have been cultured from CD lesions and serologic studies associate exposure to *Mycobacterium avium paratuberculosis* with CD (Gitnick et al., 1989; McFadden et al., 1987). Similarly, adherent-invasive *Escherichia coli* are enriched in CD lesions and are associated with defective macrophage killing of intracellular bacteria (Darfeuille-Michaud et al., 1998; 2004; Glasser et al., 2001). However, neither of these organisms are widely accepted as causative in CD. The role of the intestinal microbiome in CD is further discussed in Section 8.8.1 (page 62).

Smoking is a major environmental risk factor for CD, although the chemicals that increase this risk are obscure. A recent meta-analysis calculated that smoking increased the risk (OR) of CD to 1.76 (95% confidence interval [CI] 1.40-2.22) (Mahid et al., 2006). There does not appear to be an association between childhood or adult exposure to passive smoking and CD (Jones et al., 2008). Smokers have a more complicated disease course than non-smokers, and smoking cessation is an effective therapy for CD (Johnson et al., 2005). Oral contraceptives increase the risk of CD (Relative risk [RR] 1.51 [95% CI 1.17-1.96]) and this risk reverts to that of the non-exposed population on cessation (Cornish et al., 2008). Results of studies examining breastfeeding and subsequent risk of CD are heterogeneous, while childhood MMR and BCG vaccinations are not risk factors for CD (Baumgart and Sandborn, 2012).

8.5 Phenotype at diagnosis and disease evolution

CD is classified according to the age of onset, disease distribution and disease behaviour. A uniform disease classification system was introduced at the World Congress of Gastroenterology at Vienna in 1998 (Gasche et al., 2000) and modified at a subsequent congress at Montreal in 2005 (Silverberg et al., 2005).

Population-based studies from Olmsted County, Minnesota (Gollop et al., 1988; Thia et al., 2010), the multicentre European Collaborative Study Group on IBD (EC-IBD) (Wolters et al., 2006), south-eastern Norway (IBSEN) (Henriksen et al., 2007) and Northern France (EPIMAD registry) (Gower-Rousseau et al., 2013) are consistent in showing that approximately one third of CD patients each present with disease involving the ileum, the colon or both the ileum and colon, and 10%

have additional upper gastrointestinal involvement. Elderly onset (>60 years) is associated with colonic disease and inflammatory disease behaviour, while children are more likely to present with ileocolonic disease (Gower-Rousseau et al., 2013). The location of disease remains stable over time, with disease developing at a second site in 13.5% at 5 years in the IBSEN cohort (Henriksen et al., 2007), and 15% at 10 years at a Belgian tertiary referral centre (Louis et al., 2001).

Louis *et al.* characterised the natural history of CD, or disease phenotype at presentation and rate of progression to complications, in a cohort of 297 patients at a Belgian tertiary referral centre (Louis et al., 2001). 73.4% had non-stricturing, non-penetrating (inflammatory) disease at presentation, decreasing to 52% at 5 years and 30.6% at 10 years. 10.8% had stricturing disease at presentation, rising to 21.2% at 5 years and 32.2% at 10 years. 15.5% had penetrating disease at presentation, rising to 26.8% at 5 years and 37.2% at 10 years. The population-based IBSEN cohort, with 221 CD patients, yielded similar results (Henriksen et al., 2007). At presentation, 60.5% had inflammatory disease, 27.5% had stricturing disease and 12% had penetrating disease. These figures changed to 46.5%, 33% and 20.5% respectively at 5 years. In a population-based study from the Mayo Clinic including 306 patients, 81%, 5% and 14% had inflammatory, stricturing and penetrating disease at presentation, respectively (Thia et al., 2010). The risk of developing a stricturing or penetrating complication within the first 90 days of diagnosis was 18.6% in this cohort. The cumulative risk of complicated disease was 22% at 1 year, 33.7% at 5 years and 50.8% at 20 years. Isolated colonic disease is more likely to retain an inflammatory phenotype, while ileal disease is

more likely be complicated by strictures or penetrating disease (Cosnes et al., 2011).

The majority of patients have a relapsing-remitting course, while 10-15% have chronic, unremitting disease (Cosnes et al., 2011). Approximately 55% will be in clinical remission each year (Munkholm et al., 1995). The 10-year cumulative relapse rate is 90% (Solberg et al., 2007).

The goals of treatment in CD include resolution of symptoms by inducing and maintaining steroid-free remission, and the longer-term prevention of disease progression to complicated CD, i.e. stricturing or penetrating disease. The high annual rates of active CD and significant risk of progression to complicated CD described above are reported by specialist CD centres that offer standard-of-care management. This illustrates a significant unmet need in CD therapeutics. As discussed in Section 8.7 (page 42), this could be addressed by changing treatment paradigms (from "Step-up" to "Top-down") or treatment goals ("Treat to target") with currently available medications, or by the development of novel therapeutics for CD.

8.6 Clinical symptoms

Clinical symptoms in CD are heterogeneous but are often characteristic of disease site and activity. For example, diarrhoea with abdominal pain can occur the context of terminal ileitis, while bloody diarrhoea and tenesmus is more likely with distal colonic disease. Up to 20% will have perianal fistulae at presentation (Cosnes et al., 2011). Extraintestinal manifestations such as uveitis, sacroileitis, erythema nodosum and pyoderma gangrenosum can also occur. As discussed

further in Section 8.7.4 (page 51), the presence of symptoms is not predictive of progression of anatomic damage (Cosnes et al., 2011).

8.7 Treatment of Crohn's disease

Treatment of CD is complex and treatment choices depend on disease location, disease activity and patient preference. As CD therapeutics have been extensively reviewed elsewhere and are the subject of clinical practice guidelines (Dignass et al., 2010; Terdiman et al., 2013), the purpose of the following sections is to give a focussed overview of currently available therapies, treatment paradigms and potential novel therapies, in order to highlight currently unmet need in CD therapeutics.

Goals of treatment in CD are the induction and maintenance of steroid-free clinical remission with the intention of resolving symptoms and improving quality of life, while minimizing drug toxicity (Cheifetz, 2013). The prevention of disease progression to "complicated" CD (stricturing or penetrating disease) is a longer-term treatment goal. The medications currently used to treat CD are summarized in Table 8.2 (page 44).

The clinical end-points in the studies described below are defined according to the Crohn's disease activity index (CDAI). The CDAI is a validated, multi-parameter disease activity score used in CD research, although not in routine clinical practice (Best et al., 1976). The absence of disease activity is defined as a CDAI of <150, while mild, moderate and severe disease activity are defined as CDAIs of 150-220, 220-450 and >450, respectively. In the context of clinical trials, a clinical remission is defined as a CDAI <150 at the time of assessment, while a

clinical response is defined as a decrease in CDAI of >100 points. More recently, mucosal healing has been investigated as a potential end-point in CD studies (Baert et al., 2010; Frøslie et al., 2007; Neurath and Travis, 2012; Schnitzler et al., 2009). An endoscopic disease severity score has also been validated recently (Ferrante et al., 2013).

Table 8.2: Medications used in the treatment of Crohn's disease.

Medication	Induction of remission (%)*	Maintenance of remission (%)*	Potential serious adverse events
Corticosteroids			
Prednisolone Methylprednisolone Budesonide	51-64% (Benchimol et al., 2008; Greenberg et al., 1994)	No role.	Steroid side-effects, including "moon face", "buffalo hump", glucose intolerance, myopathy, osteoporosis, *etc*.
Thiopurines			
Azathioprine 6-mercaptopurine	No role. (Chande et al., 2013).	51-71% (Prefontaine et al., 2009)	Bone marrow suppression, pancreatitis, lymphoma (rare)
Anti-metabolites			
Methotrexate	39% (Intramuscular) (Feagan et al., 1995) Not recommended for induction of remission in moderate-to-severe CD. (Terdiman et al., 2013)	70% (Patel et al., 2009)	Pulmonary fibrosis, hepatotoxicity, teratogenic
Anti-TNF			
Infliximab (cA2) Adalimumab (D2E7) Certolizumab (CDP580)†	Remission: 30-40% Response: 60-70% (Hanauer et al., 2006; Sandborn et al., 2007; Targan et al., 1997)	Remission: 35-47% Response: 46-63% (Behm and Bickston, 2008; Hanauer et al., 2002; Rutgeerts et al., 2004)	Infection, infusion reactions, lymphoma (rare)
Anti-α_4 integrin			
Natalizumab	Remission: 26% Response: 48% (Targan et al., 2007)	Remission: 44% Response: 61% (Sandborn et al., 2005)	Progressive multifocal leukoencephalopathy (Van Assche et al., 2005)
Anti-$\alpha_4\beta_7$ heterodimer			
Vedolizumab	Remission: 14.5% Response: 31.4% (Sandborn et al., 2013)	Remission: 36-39% Response: 43-45% (Sandborn et al., 2013)	Infections
Biosimilar Infliximab‡			
"Remsima" (CT-P13) "Inflectra" (CT-P13)	Licensed by the European Medicines Agency in June 2013. A clinical trial designed to determine safety and efficacy *vs.* reference infliximab in CD is recruiting (NCT02096861). (Danese et al., 2013; European Medicines Agency, 2013)		Not yet known in CD

* Clinical trial methodology is heterogeneous. The temporal endpoints for induction and maintenance of remission are 6-12 weeks and 36-52 weeks, respectively.

† Certolizumab (CTZ) is effective at maintaining remission in CD, but not effective in inducing remission.

‡ "Biosimilars" or "similar biological medicinal products" are biotechnology-produced medicinal products that are similar to an already licensed biological medicine, developed to take advantage of the expiry of a period of exclusivity for the reference biologic drug (European Medicines Agency, 2012).

8.7.1 "Step-up" therapy for induction of remission

"Step-up" therapy involves the induction of remission with corticosteroids, followed by the use of more recently introduced biologic therapy, if corticosteroids medications fail to induce remission. As corticosteroids induce remission in 51-64% of patients with CD, between one third and one half may require additional therapy to induce remission (Benchimol et al., 2008; Greenberg et al., 1994). The choice of corticosteroid depends on the site of disease and disease activity. For example, budesonide 9mg per day is currently recommended for mildly active ileocaecal CD, while oral prednisolone is recommended for moderately active ileocaecal CD, and prednisolone or intravenous hydrocortisone is recommended for severe ileocaecal CD (Dignass et al., 2010). Corticosteroids are also the first line therapy for colonic CD (Dignass et al., 2010). Corticosteroids are not effective in maintaining remission in CD and are also associated with a range of side-effects, as detailed in Section 8.7.6 (page 53), below. Consequently, early introduction of an immune modulating agent such as azathioprine (AZA) or methotrexate (MTX) is appropriate to maintain remission following the cessation of corticosteroid therapy.

Nutritional therapy with elemental or polymeric diets are effective at inducing remission in children with CD, although a recent Cochrane systematic review found that corticosteroids were more effective at inducing remission in paediatric CD than nutritional therapy (Zachos et al., 2007). Evidence for the effectiveness of nutritional therapy at inducing remission in adult CD is currently lacking (Dignass et al., 2010).

As shown and referenced in Table 8.2 (page 44), infliximab (IFX), adalimumab (ADA), natalizumab and vedolizumab are superior to placebo at inducing remission in CD in patients who have failed previous therapies. Due to the risk of JC virus-induced progressive multifocal leukoencephalopathy with natalizumab (Van Assche et al., 2005), this medication is likely to be superceded in clinical practice by vedolizumab.

8.7.2 "Top-down" therapy for induction of remission

"Top-down" therapy, involving initial induction of remission with anti-tumour necrosis factor (TNF) agents, is an alternative treatment paradigm. The early introduction of biological therapy has the advantage of minimising corticosteroid therapy. Anti-TNF medications may also be more effective when initiated within 2 years of diagnosis, rather than later in disease course (Dignass et al., 2010). However, biologics are expensive and are associated with their own spectrum of side-effects, detailed in Section 8.7.6 (page 53), below. The majority of patients who require treatment respond to corticosteroids (Benchimol et al., 2008; Greenberg et al., 1994). In patients who undergo ileocolic resection for distal ileal CD, there is a 31-50% chance that they will not have disease of similar severity in the future, i.e. requiring surgery (Kim et al., 1997; Weston et al., 1996). In order to balance these factors, there is interest in prospectively identifying patients who are likely to develop more severe disease and who may benefit from more aggressive therapy *ab initio*. Beaugerie *et al.* followed 1,188 patients over the first 5 years of their diagnosis, excluding patients who required surgery within 3 months of diagnosis (Beaugerie et al., 2006). 85.2% developed "disabling disease" within this time, defined as >2 courses of steroids or steroid-dependent disease,

further hospitalisation, disabling chronic symptoms (such as diarrhoea with urgency), and a need for immunosuppressive therapy or surgery. Risks for disabling disease included an initial requirement for steroids (OR 3.1 [95% CI 2.2-4.4]), age <40 (OR 2.1 [1.3-3.6]) and perianal disease (OR 1.8 [1.2-2.8]). In this study, the positive predictive value of 2 or 3 risk factors in developing disabling disease was 84% and 91%, respectively. This model was validated with a second cohort of 302 patients from the same centre (Beaugerie et al., 2006), and an independent cohort of 90 patients from Olmstead County, Minnesota (Seksik et al., 2007). Loly *et al.* used more restrictive criteria to define disabling disease in a cohort of 361 patients: the development of perianal disease or extensive surgery (colectomy, >2 small bowel [SB] resections or 1 SB resection of >50cm, or definitive stoma formation) (Loly et al., 2008). 59.7% developed disabling disease in the first 5 years after diagnosis. Risks included an initial requirement for steroids, perianal disease, ileal and colonic involvement, stricturing behaviour at diagnosis and weight loss >5kg. Age at diagnosis was not found to be a risk in this study. Consequently, patients presenting with 2 or more of these risk factors could be considered to be at risk of a poor outcome and might be candidates for top-down treatment at diagnosis. Smoking is also a well-established risk factor for aggressive disease course in CD (Louis, 2010).

In the "Step-Up/Top-Down study", D'Haens *et al.* showed that top-down treatment is superior to step-up treatment in treatment-naïve patients with active CD (CDAI >200) (D'Haens et al., 2008). Sixty seven patients were randomised to top-down therapy (IFX and AZA *ab initio*), while 66% were randomised to step-up therapy (induction of remission with corticosteroids, ± AZA, ± IFX). At 26 weeks, 60% of the top-down group were in steroid-free remission without surgery

compared to 35.9% of the step-up group (p=0.0062). At 52 weeks, these figures were 61.5% and 42.2%, respectively (p=0.028). Rates of serious adverse events were similar between the two groups.

In the "SONIC" study, Colombel *et al.* compared induction and maintenance of remission with combination therapy of IFX/AZA (n=169) *vs.* IFX monotherapy (n=169) *vs.* AZA monotherapy (n=170) in treatment naïve patients with moderate-to-severe CD (CDAI>250) (Colombel et al., 2010). Steroid-free remission rates at week 26 were 56.8% for the combination therapy group, 44.4% for the IFX-alone group (p=0.02) and 30% for the AZA-alone group (p<0.001). The combined therapy group had fewer serious adverse events compared with the IFX or AZA monotherapy groups (15.1% *vs.* 23.9% [p=0.04] *vs.* 26.7% [p=0.01]).

Few data are available on combination therapy with IFX and MTX. In a recent clinical trial comparing IFX or combination IFX/MTX to maintain remission over 50 weeks in 126 CD patients, no additional benefit to IFX/MTX combination therapy was seen compared with IFX alone (treatment failure 30.6% *vs.* 29.8%; n=126) (Feagan et al., 2014).

Taken together, these results indicate that top-down therapy is superior to step-up therapy in inducing remission in treatment-naïve patients with moderate-to-severe CD, and that combined therapy with IFX and AZA is the treatment of choice. In order to avoid "over-treating" patients who are likely to have an indolent clinical course and to target patients who are likely to progress to complicated CD, a decision to commence top-down therapy could be based on clinical risk factors for disease progression. In clinical practice, clinicians weigh the risks of progression to complicated disease, treatment side effects (Section

8.7.6 [page 53]), treatment costs and patient preferences to tailor treatment to individual patients (Dignass et al., 2010; Terdiman et al., 2013).

8.7.3 Does therapy have an effect on the natural history of Crohn's disease?

Thiopurines have been available for over 30 years. Retrospective epidemiological cohort studies from Cardiff (Ramadas et al., 2010), Hungary (Lakatos et al., 2012) and Canada (Bernstein et al., 2012) suggest that AZA use is associated with a reduction in rates of hospitalisation and surgery in CD; an association that is apparent in cohorts that were diagnosed prior to the widespread introduction of biologic therapy. In contrast, Cosnes *et al.* did not find an association between immunosuppressant use and reduction in surgical rates in a retrospective cohort study from Paris (Cosnes et al., 2005). However, only 9% of patients who underwent surgery in that study received immunosuppressants for more than 3 months, which suggests that AZA was administered for a sub-optimal treatment duration. This was confirmed by Peyrin-Biroulet *et al.*, who found that a short AZA treatment duration (<1.5 months) was an independent risk factor for CD-related abdominal surgery (Peyrin-Biroulet et al., 2011). In a UK population-based, retrospective cohort study, Chatu *et al.* found that the 5-year cumulative probability of thiopurine use increased from 12% to 18% to 25% in cohorts diagnosed from 1989-1993, 1994-1999 and 2000-2005, respectively (Chatu et al., 2014). This mirrored reductions in the rate of first intestinal resection from 15% to 12% to 9% in the three cohorts. Increased duration of thiopurine therapy was associated with increased reduction in surgical rates: 6 months of therapy reduced the risk of surgery by 44%, while 12 months of therapy reduced the risk

of surgery by 69%. Taken together, these observations suggest that long-term thiopurine use may reduce the risk of surgery in CD.

These data should be balanced against the results of two small studies examining early AZA therapy in newly diagnosed CD patients. Cosnes *et al.* compared the introduction of AZA therapy with conventional therapy in 132 patients with CD of less than 6 months duration, who were at risk of the development of "disabling disease" (Cosnes et al., 2013). While the number of trimesters in remission (the primary outcome in this study) and intestinal surgery rates did not differ between groups at 3 years, patients in the AZA group had less perianal surgery than patients in the conventional therapy group (4% *vs.* 18%; p=0.036). Panés *et al.* randomised patients with CD of less than 8 weeks duration to AZA therapy (n=68) or placebo (n=63) and allowed patients to receive corticosteroids but not biologics for disease exacerbations (Panés et al., 2013). Rates of corticosteroid-free remission at 76 weeks (the primary outcome) were similar between groups (44.1% *vs.* 36.5%; p=0.48). In a *post hoc* analysis, patients in the AZA group had fewer exacerbations than patients in the placebo group, defined as CDAI >220 (11.8% *vs.* 30.2%; p=0.01). One additional study, who's main purpose was to correlate CD risk alleles with clinical outcome, found that AZA use within 3 years of diagnosis led to reduced surgical rates, even though patients who were prescribed AZA had a higher risk of progressing to complicated CD than patients who did not receive AZA (Cleynen et al., 2013). However, these studies are limited by small sample size and short duration of follow-up. Magro *et al*. addressed these limitations by reporting their experience of AZA and anti-TNF use and disease progression in 736 CD patients at risk for disease progression, who were followed for a mean of 12.3 (±8.4) years (Magro et al., 2014b). 87% of

patients had an inflammatory phenotype at diagnosis. Of these, 28.5% progressed to stricturing disease, while 23.5% progressed to penetrating disease. Patients with inflammatory disease who received monotherapy with AZA or combination therapy with AZA/anti-TNF had a reduced rate of disease progression, compared with patients who did not receive treatment (Hazard ratios [HR] for disease progression of 0.15 [p<0.001] and 0.33 [p<0.001], respectively). These data suggest that AZA or a combination of AZA/anti-TNF, given early in the course of inflammatory CD in patients who are at risk of disease progression, may reduce the risk of disease progression.

Anti-TNF agents have been available for approximately 15 years. While it seems plausible that widespread use of anti-TNF agents will further reduce the need for surgery in CD (Colombel et al., 2010; Magro et al., 2014b), the Cardiff study showed that surgical rates were falling prior to the introduction of anti-TNF agents (Ramadas et al., 2010). New data should be interpreted in this context.

8.7.4 Disease progression and "treating to target"

Despite the clinical approaches described above, only 10% of CD patients experience prolonged remission over 10 years (Solberg et al., 2007), while over 50% progress from inflammatory to stricturing or penetrating disease within this time (Magro et al., 2014b; Solberg et al., 2007). There is poor correlation between clinical symptoms and endoscopic appearance. In a recent study, 19% of CD patients with significant endoscopic lesions had no symptoms, while symptoms were present in 41% of CD patients with no endoscopic disease (Bouguen et al., 2014). Consequently, strictures and fistulae can develop in the absence of overt

symptoms. This has led to the a proposal that mucosal healing (the absence of ulcers on endoscopy), rather than clinical symptoms, should be used as a treatment goal in a "treat to target" approach to therapy (Bouguen et al., 2013).

Mucosal healing is achievable with currently available treatments (Colombel et al., 2010). Induction of remission with IFX monotherapy is associated with mucosal healing rates of 29% at week 10 (Rutgeerts et al., 2004) and 30% at week 26 (Colombel et al., 2010). Induction of remission with AZA/IFX combination therapy is associated with mucosal healing rates of 44% at week 26 (Colombel et al., 2010) and 73% at week 104 (D'Haens et al., 2008). One small prospective study suggests that maintenance therapy with MTX is not as effective as AZA or IFX at inducing mucosal healing in patients who were in sustained clinical remission following corticosteroid therapy (MTX 11% *vs.* AZA 50% [p=0.01] *vs.* IFX 60% [p=0.008]; n=51) (Laharie et al., 2011).

In the Step-up/Top-down study, the presence of mucosal healing following induction of remission predicted prolonged steroid-free remission over the subsequent two years (70.8% *vs.* 27.3%; p=0.036) (Baert et al., 2010). Mucosal healing is also associated with reduced risk of surgery (Frøslie et al., 2007; Schnitzler et al., 2009). Bouguen *et al.* retrospectively reviewed outcomes in 67 patients treated to a target of mucosal healing, identified by 6-monthly endoscopies (Bouguen et al., 2014). The authors reported that 71% of endoscopies were associated with a change of management, but the study missed the opportunity to compare this with the clinical decisions that would otherwise have been taken with conventional care, in the absence of frequent endoscopies.

The precise role of treating to a target of mucosal healing has yet to be defined in a large, prospective study.

8.7.5 Surgery

Surgery may be used to treat active isolated distal ileal disease, disease that is unresponsive to appropriate medical therapy and complications of CD, such as strictures, fistulae, toxic megacolon and dysplasia. While surgery has a role in the management of CD, it is not curative. Seven to 12% of people with CD undergo surgery within the first year of diagnosis, rising to 40-60% by the tenth year of diagnosis (Bernstein et al., 2012; Peyrin-Biroulet et al., 2011; Vind et al., 2006). Following surgery, disease almost uniformly reoccurs and a subgroup of patients require multiple operations (Cheifetz, 2013).

As discussed above, there is some evidence that treatment with immunomodulators and anti-TNF agents reduce the risk of surgery in CD patients. It is hoped that novel treatment strategies, such as "treat to target", may reduce this risk further. More aggressive treatment strategies will have to be balanced against the risks of side-effects of therapy.

8.7.6 Side-effects of medications for Crohn's disease

Many medications used in the treatment of CD are associated with important side-effects. This means that efficacy needs to be balanced against side-effect profile and patient preference in treatment decisions.

Systemic corticosteroids are associated with a characteristic fat deposition (moon face, buffalo hump, abdominal obesity), striae, hypertension, impaired

glucose tolerance, cataracts, myopathy and psychiatric side-effects ("steroid psychosis") (Lichtenstein et al., 2006). AZA and 6-mercaptopurine are associated with nausea and diarrhoea, myelosuppression (leukopaenia, anaemia, thrombocytopaenia), pancreatitis, hair loss, and liver-related complications such as hepatitis, veno-occlusive disease, peliosis and nodular regenerative hyperplasia (Lichtenstein et al., 2006). MTX is associated with nausea and diarrhoea, mucositis, hepatic fibrosis and interstitial pneumonitis (Lichtenstein et al., 2006). Anti-TNF agents are associated with risk of infusion reactions (IFX), delayed hypersensitivity-like reactions (IFX), injection site induration (ADA, CTZ), reactivation of latent tuberculosis (TB) and drug-induced lupus (Lichtenstein et al., 2006).

8.7.7 Medication use and risk of lymphoid malignancy in Crohn's disease

CD is not intrinsically associated with an increased risk of lymphoproliferative disease (Beaugerie, 2011). However, thiopurine use is associated with an increased risk of lymphoma in CD patients, although large datasets are required to evaluate this risk. The "Cancers Et Surrisque Associé aux Maladies inflammatoires intestinales En France" (CESAME) registry recently reported the risk of lymphoproliferative disease in their cohort of 19,486 IBD patients (60.3% CD), who were followed for a median of 35 months (Beaugerie et al., 2009). The incidence of lymphoproliferative disease was 0.9/1,000 (95% CI 0.50-1.49) patient-years in IBD patients receiving thiopurines, 0.2/1,000 (95% CI 0.02-0.72) patient-years in patients who had discontinued thiopurines and 0.26/1,000 (95% CI 0.10-0.57) patient-years in patients who had never received thiopurines (HR: 5.28 [95% CI 2.01-13.9; p=0.0007]) (Beaugerie et al., 2009).

Prior exposure to thiopurines in CD patients was also associated with an increased risk of myeloid leukaemia and myelodysplasia (Standardised incidence ratio [SIR]: 6.98 [95% CI 1.44-20.36]) (Lopez et al., 2014).

Most cases of CD thiopurine-associated lymphoma in the CESAME registry were Epstein-Barr virus (EBV)-associated B cell lymphomas, which are more typically seen in immunosuppressed patients with EBV reactivation following solid organ transplantation (Beaugerie et al., 2009; Magro et al., 2014a).

Fatal lymphoproliferation following primary EBV infection can also occur in the context of transplant immunosuppression (Posthuma et al., 1995) and in patients with X-linked mutations in *SH2D1A* (or *SAP*) or *XIAP* (Pachlopnik Schmid et al., 2011; Rigaud et al., 2006). Fatal lymphoproliferation following primary EBV infection was described in a 19 year-old man receiving azathioprine and prednisolone for CD (Posthuma et al., 1995). Two young men (<35 years old) in the CESAME registry also developed fatal thiopurine–associated post-mononucleosis lymphoproliferative disorder (Beaugerie et al., 2009). This suggests that thiopurines should be prescribed cautiously to young men with CD who are EBV seronegative.

Approximately 40 cases of thiopurine-associated hepatosplenic T cell lymphoma (HSTCL) have been described in patients with IBD, following a median duration of thiopurine exposure of 6 years (Magro et al., 2014a). The majority of HSTCL cases (>90%) have been described in young men (<35 years old) and 50% have been associated with thiopurine/anti-TNF combination therapy. While the absolute risk of HSTCL is small, it is uniformly fatal in the absence of haematopoietic stem cell transplantation (HSCT). Current recommendations are

to avoid exposure to thiopurine/anti-TNF combination therapy for greater than 2 years in young men (Magro et al., 2014a).

Pooled data from anti-TNF registration clinical trials (Lichtenstein et al., 2012b), data from the Crohn's "Therapy, Resource, Evaluation, and Assessment Tool" (TREAT) registry, which includes 6,273 IFX-receiving CD patients, who have been followed for a mean of 5.2 years (Lichtenstein et al., 2014), and a systematic review of the paediatric CD literature (Dulai et al., 2014) suggest that anti-TNF therapy has not yet been associated with an increased risk of lymphoma in CD. However, larger cohorts with longer follow-up are needed to answer this question more fully.

CD patients receiving immunosuppressants are at increased risk of non-melanoma skin cancer, while patients receiving anti-TNF agents are at increased risk of melanoma, suggesting that CD patients should avoid sun exposure (Magro et al., 2014a).

8.7.8 Medication use and risk of infection in Crohn's disease

Treatment with anti-TNF agents is associated with a risk of opportunistic infections, including TB and atypical mycobacterial and fungal infections, *Pneumocystis jiroveci* pneumonia and *Varicella zoster* infections (Lawrance et al., 2010; Lichtenstein et al., 2012a). In a recent pooled analysis of 5 pivotal placebo-controlled trials of IFX ± thiopurine/MTX use in IBD, 49.1% of IFX-treated patients experienced at least 1 infection (*vs.* 45.3% for placebo; p=0.40) (Lichtenstein et al., 2012b). The proportion of patients with "serious infections" requiring hospital admission was also similar between groups (4.5% *vs.* 5.6%; p=0.55). The use of

immunomodulators (*vs.* no immunomodulators) did not increase the risk of infection in CD, but increased this risk slightly in UC.

The TREAT registry offers a longer period of follow-up than pivotal clinical trials (n=6,273; mean follow-up 5.2 years) (Lichtenstein et al., 2012a). The unadjusted rate of serious infections in patients who had received IFX in the preceding 3 months was 2.06/100 patient-years, compared with 1.42/100 patient-years in patients receiving other treatments (RR 1.45 [95% CI 1.10-1.91]). Multivariate logistic regression was then performed in order to identify independent risk factors for serious infection. Independent risk factors for serious infection were moderate-to-severe disease activity (HR 2.24 [1.57-3.19]), use of narcotic analgesics (HR 1.98 [1.44-2.73]), prednisone therapy (HR 1.57 [1.17-2.10]) and IFX treatment in the preceding 3 months (HR 1.43 [95% CI 1.11-1.84]). This suggests that patients who developed serious infections were sicker than other patients, with worse disease activity and a requirement for more aggressive CD therapy. Treatment with corticosteroids or opiate analgesics and increasing age, but not recent IFX therapy, were independent risk factors for mortality in this cohort.

In a recent pooled analysis of the paediatric literature, the rate of serious infections was similar in patients receiving immunomodulators or anti-TNF agents (3.33/100 patient-years and 3.52/100 patient-years, respectively) (Dulai et al., 2014). Both of these were lower than the expected rate of serious infections in patients receiving corticosteroids (7.30/100 patient-years).

There is some evidence that current thiopurine or anti-TNF agents may increase the risk of infectious complications following IBD surgery (Myrelid et al.,

2009; Syed et al., 2013). However, there is a paucity of data on the risk of infection with methotrexate use in CD (Terdiman et al., 2013).

8.7.9 Natalizumab and risk of progressive multifocal leukoencephalopathy

Natalizumab is a humanised monoclonal antibody that prevents $\alpha_4\beta_7$ integrin-mediated leukocyte adhesion and intestinal migration mediated by binding α_4 integrin. While natalizumab failed to make its primary end-point of induction of clinical response *vs.* placebo in the ENACT-1 trial, on-going maintenance natalizumab therapy in responders (the ENACT-2 trial) resulted in higher rates of sustained response (61% *vs*. 28%; $p<0.001$) and sustained remission (44% *vs.* 26%; $p=0.003$) than placebo (Sandborn et al., 2005). A subsequent study showed that natalizumab was effective at inducing remission in moderate-to-severe CD (the ENCORE study) (Targan et al., 2007).

Natalizumab also interferes with $\alpha_4\beta_1$ integrin-mediated leukocyte migration to the central nervous system, which forms the basis of its biological activity in multiple sclerosis. In 2005, three cases of progressive multifocal leukoencephalopathy (PML) were reported in natalizumab-treated patients (2 with MS, 1 with CD), due to CNS reactivation of the human polyoma JC virus (Van Assche et al., 2005). In addition, 1 patient in the ENACT studies developed PML (Sandborn et al., 2005). Following this discovery, Natalizumab was withdrawn and subsequently re-introduced on the condition that recipients participate in a registry. As vedolizumab targets the $\alpha_4\beta_7$ heterodimer and does not interfere with $\alpha_4\beta_1$-mediated leukocyte homing to the CNS, it is likely that vedolizumab will supersede natalizumab in clinical practice.

8.7.10 New and emerging therapies for Crohn's disease

As described above, a significant proportion of CD patients will have a sub-optimal response to currently available therapies, or be at risk for the development of side-effects, including infection and lymphoma. These reflect a currently unmet need in CD therapeutics. Therapies currently under investigation for CD are summarised in Table 8.3 (page 60), while Table 8.4 (page 61) summarises investigational therapies that failed to meet their primary end-point in recent clinical trials in CD. "Inflectra" and "Remsima" (CT-P13), two biosimilar IFXs, have been licensed for the treatment of CD in Europe (Danese et al., 2013; European Medicines Agency, 2013), although a clinical trial designed to show non-inferiority with reference IFX in CD has not yet been completed. These are included in Table 8.2 (page 44). The large number of therapies currently under investigation for CD highlights current interest in the development of new treatments to address unmet therapeutic needs.

Table 8.3: Investigational therapies for Crohn's disease.

Target	Name	Class	Comments
α4 integrin	AJM300	Small molecule	Efficacy in phase II study in CD. Published in abstract form only. (Takazoe et al., 2009b)
HGH	Somatotropin	Recombinant hormone	Efficacy in adult phase II study (Slonim et al., 2000). No additional efficacy when given with steroids in paediatric CD (Denson et al., 2010).
IL-6R	Tocilizumab (Atlizumab/MRA)	Humanised mAb	Efficacy in phase II study (Ito et al., 2004).
IL-10	rhuIL-10	Recombinant cytokine	Phase IIa clinical trials, testing SC and bacterially-delivered IL-10. (Braat et al., 2006; Fedorak et al., 2000; Schreiber et al., 2000). Other companies have IL-10-based molecules in their pipelines.
IL-12p40	Ustekinumab	Humanised mAb	Efficacy in phase II study (Sandborn et al., 2012b).
Janus kinase	Tofacitinib	Small molecule	Not superior to placebo in recent phase II study, but very high placebo response rate (Sandborn et al., 2014).
MAdCAM-1	PF-00547659	Orally active compound	Phase II study in CD recruiting ("OPERA study"; NCT01276509).
Multiple (DC and Th1 responses)	Laquinimod	Small molecule	Efficacy in experimental autoimmune encephalitis and multiple sclerosis (phase III). Phase II study in CD completed (NCT00737932).
TNF	Golimumab	Fully human mAb	Efficacy shown in phase III study in UC (Löwenberg and D'Haens, 2013).
TNF	Thalidomide	IMiD	Positive results in phase II clinical trials (Ehrenpreis et al., 1999; Vasiliauskas et al., 1999).
TNF	Semapimod	MAPK inhibitor	Phase I (Hommes et al., 2002).
Microbiome	Rifaximin	Antibiotic	Phase II (Prantera et al., 2012).

Table 8.4: Investigational therapies for Crohn's disease that have failed in clinical trials.

Target	Name	Class	Comments
CCR9	Vercirnon (CCX-282B)	Small molecule	Successful phase II trial (Keshav et al., 2013). Phase III terminated early.
CD3	Visilizumab	Humanised mAb	Cytokine release syndrome and liver injury in phase IIa studies (Baumgart et al., 2009).
CTLA-4	Abatacept	Humanised mAb	Phase II (Sandborn et al., 2012a).
ICAM-1	Alicaforsen (ISIS 2302)	Antisense oligonucleotide	IV administration failed to induce remission in moderate-to-severe CD (Yacyshyn et al., 2007). Topical administration shows some promise in UC.
IFN-γ	Fontolizumab	Humanised mAb	Failed to meet primary endpoint of efficacy at day 29 in phase II trial. Decreased CRP in treatment group (Reinisch et al., 2010).
IL-11	Oprelvekin	Recombinant cytokine	Superior to placebo in 1 phase II study (Sands et al., 2002), but inferior to prednisolone in a second phase II study (Herrlinger et al., 2006).
IL-12p40	Briakinumab (ABT 874)	Humanised mAb	Phase II (Löwenberg and D'Haens, 2013).
IL-12/IL-23	Apilimod mesylate (STA-5326)	Small molecule	Phase II (Sands et al., 2010).
IL-17A	Secukinumab	Humanised mAb	Phase III (Hueber et al., 2012).
GM-CSF	Sargramostim	Recombinant cytokine	Phase II (Korzenik et al., 2005; Takazoe et al., 2009a).
TNF	CDP517	Humanised mAb	Phase III. Sub-group analysis suggested efficacy in subjects with CRP>10mg/L (Sandborn et al., 2004).
TNF	Lenalidomide	IMiD	Phase II; moderate CD (Mansfield et al., 2007).
TNF	Doramapimod (BIRB 793)	MAPK inhibitor	Phase II (Schreiber et al., 2006).
TNF p55	Onercept	Soluble p55R	Phase III (Rutgeerts et al., 2006).
TNF p75	Etanercept	Soluble p75R fusion protein	Phase II (Sandborn et al., 2001).
Dietary supplement	Fructo-oligosaccharides		Phase II (Benjamin et al., 2011).
Dietary supplement	Omega-3 fatty acids		For prevention of relapse. Initial positive phase II study (Belluzzi et al., 1996). Subsequent negative definitive studies (Feagan et al., 2008; Lorenz-Meyer et al., 1996).
Studies performed in moderate-to-severe CD, unless otherwise stated.			

8.8 The mucosal immune system and Crohn's disease

The mucosal immune system is separated from the highly immunogenic environment of the intestinal lumen by a single layer of epithelial cells, overlaid by mucus. The following section discusses the inter-relationship between the intestinal microbiota, the epithelium and intra-epithelial lymphocytes, and cells of the mucosal immune system. Under "steady state" conditions, commensal bacteria, intestinal epithelial cells (IECs) and immune cells interact to prevent inappropriate mucosal inflammation to commensal bacteria and luminal antigens. Disruption of this balance may initiate or potentiate the chronic intestinal inflammation seen in CD.

8.8.1 Intestinal microbiota

Commensal intestinal bacteria interact with the mucosal epithelium and are required for the normal development of the mucosal immune system. Germ-free mice have reduced secretory Immunoglobulin A (IgA), reduced lamina propria mononuclear cells and absent mesenteric lymph nodes (MLN). Germ-free mice also succumb to "low dose" 1% DSS colitis, in contrast to conventionally housed mice (Kitajima et al., 2001). There are a numerous specific examples of bacteria modulating the mucosal immune system. Segmented filamentous bacteria (SFB) adhere to terminal ileal epithelium in mice and induce a transcriptional program in the terminal ileal mucosa (Ivanov et al., 2009). SFB-induced mucosal expression of serum amyloid A (SAA) induces IL-6 and IL-23 expression in lamina propria (LP) DCs, which allows LP DCs to induce T helper 17 (Th17) polarisation *in vitro*, consistent with Th17 $CD4^+$ T cells (expressing IL-17, IL-22 and RORγt in this

paper) seen in SFB colonised mice (Ivanov et al., 2009). SFB colonisation is also associated with a mucosal transcriptional program of antimicrobial peptides, including RegIIIγ, and protection from *Citrobacter rodenium*-induced colitis. There is currently considerable interest in determining if a Th17-inducing equivalent to SFB exists for humans.

Commensal bacteria can also modulate the mucosal immune system. *Bacteroides fragilis* induces peripheral conversion of $CD4^+$ T cells to $FOXP3^+$ $IL\text{-}10^+$ peripherally induced regulatory T cells (iT_{regs}), that can also express TGF-β (Round and Mazmanian, 2010), and protect against experimental colitis. iT_{regs} are induced by bacterial Polysaccharide A (PSA) and this induction is Toll Like Receptor (TLR) 2-dependent. *Clostridium spp.* can induce iT_{regs} (Atarashi et al., 2013) and $IL\text{-}10^+$ macrophages (Hayashi et al., 2013) in murine LP and protect against experimental colitis. Commensal bacteria, including *Clostridium spp.*, can ferment dietary fibre to short chain fatty acids (SCFA), which are a major energy source for intestinal epithelium, can induce iT_{regs} in both the small and large intestines and can prevent experimental colitis (Arpaia et al., 2013; Atarashi et al., 2013; Furusawa et al., 2013; Smith et al., 2013). Incubation of CD mucosal explants with *Lactobacillus casei* significantly decreases pro-inflammatory cytokine expression in culture supernatants, including TNF, Interferon-γ (IFN-γ), IL-2, IL-6, IL-8 and CXCL1 (Llopis et al., 2009).

The intestinal microbiome is required for the development of colitis in some mouse models (Sellon et al., 1998). It is likely that the intestinal microbiome contributes to the pathogenesis of CD in humans. Faecal stream diversion can improve CD in distal bowel segments (Rutgeerts et al., 1991; Winslet et al., 1994).

Rifaximin showed promise in a phase II clinical trial in moderately active CD (Prantera et al., 2012). Pouchitis (following ileorectal anastomosis in UC) is amenable to probiotic or antibiotic therapy (Sartor, 2004).

The intestinal microbiome consists of four major bacterial phyla: Firmicutes, Proteobacteria, Actinobacteria and Bacteroides, in addition to many uncharacterised bacteria. Mucosal inflammation in CD is associated with both decreased diversity of the intestinal microbiome and a change in relative composition. Microbial dysbiosis, meaning reduced microbial complexity and altered microbial composition, has been identified in active CD, in comparison with healthy intestinal mucosa, and in inflamed *vs.* non-inflamed areas within affected individuals (Sepehri et al., 2007). Dysbiosis is also seen in unaffected first-degree relatives and siblings of CD patients (Hedin et al., 2014; Joossens et al., 2011). A number of independent groups have identified dysbiosis of SCFA/butyrate-producing bacteria in CD, including reduced complexity within the butyrate-producing Firmicutes phylum (Manichanh et al., 2006) and relative decreases in butyrate-producing *Clostridium spp.*, *B. fragilis*, *B. vulgatus*, *Eubacterium rectale*, *Ruminococcus spp.* and *Faecalibacterium prausnitzii* (Andoh et al., 2009; Gevers et al., 2014; Kang et al., 2010). In some studies, a relative loss of SCFA/butyrate-producing bacteria correlates with clinical outcome. Absent mucosal *F. prausnitzii* has been associated with an increased risk of endoscopic recurrence of CD following ileal resection (Sokol et al., 2008). Low rates of the Firmicutes *Bacteroides*, *Clostridium coccoides* and *F. prausnitzii* have also recently been associated with a more rapid time to relapse following cessation of IFX in a microbiome sub-study of the Groupe d'Etude Thérapeutique des Affections Inflammatoires Digestives (GETAID) "STORI" study (Louis et al., 2012; Rajca et al.,

2014). Relative loss of SCFA-producing bacteria has been proposed as a potential mechanism by which the negative immune-modulating effect of the intestinal microbiome may be lost in active CD (Louis and Flint, 2009). Multiple pathogenic and conditionally pathogenic bacteria have been shown to be relatively increased in active CD, including *Enterococcus*, *Shigella flexneri*, *Listeria*, *Fusobacterium*, *Veillonella* and *Haemophilus* (Gevers et al., 2014; Kang et al., 2010). These changes are primarily seen in the mucosa-associated microbiome, rather than the luminal microbiome (Gevers et al., 2014). Based on data from 447 treatment-naïve paediatric CD patients and 221 controls, Gevers *et al.* recently proposed that the dysbiosis seen in biopsies obtained from the terminal ileum and rectum was able to identify CD with good discrimination (AUC 0.85 and 0.78, respectively), and could be developed as a diagnostic instrument in the future (Gevers et al., 2014). In the same cohort, a predictive model formed from a combination of mucosal oxidative stress gene expression and microbiome composition was able to predict 6-month steroid-free remission more accurately than clinical factors alone (Gevers et al., 2014; Haberman et al., 2014).

Adherent-invasive *Escherichia coli* (AIEC) has been specifically associated with inflamed mucosa in CD and a CD-like ileitis in boxer dogs. AIEC are enriched in inflamed CD mucosa and adhere tightly to the mucosal epithelium, which they can invade. AIEC replicate within mucosal macrophages *in vitro*, which then over-express TNF, a cytokine associated with CD pathogenesis (Darfeuille-Michaud et al., 1998; 2004; Glasser et al., 2001). AIEC have also been identified within macrophages in CD mucosal biopsies. *Fusobacterium* is a similarly adherent-invasive bacterium that is associated with UC but has not been reproducibly identified in CD (Mangin et al., 2004).

Interestingly, both *Nod2*$^{-/-}$ in mice and carriage of *NOD2* CD risk alleles in humans are associated with distinct patterns of microbial clustering in non-inflamed ileal mucosa in mice and humans, in addition to a decrease in *F. prausnitzii* in humans (Rehman et al., 2011). In another study, *NOD2* and *ATG16L1* risk alleles were associated with different patterns of mucosal dysbiosis in surgically resected CD specimens (Frank et al., 2011).

Taken together, these observations suggest that the intestinal microbiome plays a role in the development and modulation of the mucosal immune system, and that alterations in the intestinal microbiome that are associated with CD can alter the balance between anti-inflammatory and pro-inflammatory effects of luminal bacteria on the mucosal immune system.

8.8.2 The epithelium, Paneth cells and intraepithelial lymphocytes

8.8.2.1 Barrier function of the epithelium and mucus layer

A single layer of polarised epithelium, held together by tight junctions and overlaid by mucus, forms a physical barrier between the intestinal microbiota and other luminal antigens and the mucosal immune system. Chimeric transgenic mice that carry a dominant negative (DN) mutation in N-cadherin in IECs develop crypt and intestinal inflammation that is limited to areas of chimeric epithelium, suggesting that focally increased epithelial permeability is sufficient to induce mucosal inflammation (Hermiston and Gordon, 1995). Increased epithelial permeability precedes the onset of inflammation in the senescence accelerated mouse P1/YitFc (SAMP1/YitFc) model of spontaneous transmural ileitis and caecitis, which is a close phenocopy of human CD (further discussed in Section

8.13.3 [page 109]; Matsumoto et al., 1998). Keratins provide mechanical support to epithelial cells. Keratin-8 mutations cause epithelial hyperplasia and colonic inflammation in mice (Baribault et al., 1994) and have been identified in patients with both CD and UC (Owens et al., 2004). Finally, epithelial disruption by DSS or oxazolone results in Th1 or Th2-mediated colitis, respectively; although these are considered to model UC more closely than CD (Wirtz et al., 2007).

Intestinal permeability can be measured by intestinal uptake of polyethylene glycol 400 or lactulose, rhamnose and mannitol. Increased intestinal permeability has been reported in patients with CD and in first-degree relatives carrying *NOD2* 3020insC or compound 3020insC and R702W mutations, but not in WT non-carriers (Buhner et al., 2006; Hollander et al., 1986; Katz et al., 1989; Munkholm et al., 1994; Teahon et al., 1992). A genetic association with intestinal permeability in first-degree relatives could explain the heterogeneity of results seen in these studies. Normal intestinal permeability is also associated with relative protection from disease relapse in CD. Arnott *et al.* reported that CD patients in remission with normal intestinal permeability were unlikely to relapse within a year of assessment (n=1/31), while those with increased intestinal permeability were more likely to relapse (n=8/18, $p<0.0001$) (Arnott et al., 2000).

Intestinal mucus, secreted by goblet cells, limits the interaction between commensal bacteria and the epithelium. Deletion or mutations in the mucus *MUC2* gene are associated with a superficial colitis that resembles UC in mice (Van der Sluis et al., 2006). While impaired mucus production could conceivably alter interactions between luminal bacteria and the epithelium, missense mutations in

MUC2 cause inflammation due to ER stress from accumulation of non-glycosylated MUC2 precursor in goblet cells (Heazlewood et al., 2008).

8.8.2.2 Paneth cells and intestinal epithelial cells

Paneth cells are specialised epithelial cells that are found in highest numbers in the terminal ileum. They synthesise and release antimicrobial factors into the gut lumen, including α-defensins, RegIIIγ and secretory phospholipase A. Paneth cell secretion of antimicrobial factors is induced by luminal bacteria in a MyD88-dependent manner (Menendez et al., 2013), and by bacterially-derived TLR agonists, including lipopolysaccharide (LPS, TLR4), flagellin (TLR5), polyinosinic-polycytidylic acid (Poly[I:C], TLR3) and CpG-oligodeoxynucleotide (CpG-ODN, TLR9) (Rumio et al., 2012). Paneth cell degranulation can also be induced by immune cell-derived IFN-γ (Farin et al., 2014). As highly secretory cells, Paneth cells are susceptible to ER stress or the "unfolded protein response" (UPR), which is initiated by misfolded proteins in the ER, results in NF-κB activation and leads to apoptosis, if unchecked (Kaser and Blumberg, 2011). As discussed in Section 8.4.2 (page 32), there is an interaction between risk alleles for CD and cellular pathways of bacterial sensing, autophagy and ER stress. Carriage of CD risk alleles in *NOD2* (Kobayashi et al., 2005; Wehkamp et al., 2005) and *ATG16L1* (Adolph et al., 2013; Deuring et al., 2014) are associated with a defective UPR, ER stress, Paneth cell dysfunction and decreased secretion of α-defensins in both mice and humans. CD patients with quiescent disease carrying the *ATG16L1* T300A allele have overexpression of ER stress markers GRP78 and pEIF2α in Paneth cells (Deuring et al., 2014). Adolph *et al.* recently showed that epithelium-

specific deletion of the UPR gene *Xbp1* in *Atg16l1*^{HM} mice resulted in a spontaneous CD-like transmural ileitis (Adolph et al., 2013). The *ATG16L1* risk alleles T300A and T316A also increase the susceptibility of ATG16L1 to caspase-3-mediated processing, suggesting that the effects of ATG16L1 mutations on Paneth cell dysfunction can be worsened by increased degradation of ATG16L1 by inflammation-induced autophagy in risk allele carriers (Murthy et al., 2014). As Paneth cells are a major source of antimicrobial factors, Paneth cell dysfunction may be associated with microbial dysbiosis. Indeed, carriage of both *NOD2* and *ATG16L1* risk alleles has been associated with distinct mucosal microbial clustering in the ileum, compared with non-carriers (Frank et al., 2011; Rehman et al., 2011).

IECs restrain luminal bacteria by secreting β defensins, and by transcytosis of IgA produced by plasma cells located in sub-epithelial lymphoid follicles. IECs also express IL-10 and TGF-β, contributing to the prevention of inappropriate mucosal immune activation (Colgan et al., 1999; Jarry et al., 2008), and thymic stromal lymphopoietin (TSLP), which induces tolerogenic DCs. Pattern recognition receptor expression in IECs is anatomically oriented with IEC polarisation to prevent inappropriate immune activation in response to luminal microbiota. TLR5 is exclusively expressed on the basolateral surface of IECs, limiting NF-κB activation in the absence of mucosal damage (Gewirtz et al., 2001). Basolateral TLR9 stimulation leads to NF-κB activation, while repeated apical TLR9 stimulation leads to an attenuated response (Lee et al., 2006). It is likely that increased epithelial permeability and epithelial disruption seen in active CD allows luminal bacteria to translocate to the sub-epithelial space, where TLR5 and -9 can

be activated. IEC TLR4 expression is also up-regulated in active CD (and in the non-inflamed mucosa of *NOD2*$^{-/-}$ mice), which can contribute to NF-κB activation and inflammation in active CD (Cario and Podolsky, 2000). It is possible Paneth cell dysfunction and reduced luminal antimicrobial peptides contributes to microbial dysbiosis, and dysbiosis then combines with increased intestinal permeability allowing bacterial translocation across the epithelium to the mucosa. These may be early or initiating events in CD.

As discussed above, the epithelium employs multiple strategies to prevent inappropriate NF-κB activation by commensal bacteria. However, NF-κB signalling within the epithelium is also required to prevent intestinal inflammation. The NEMO$^{IEC\text{-}KO}$ mouse has IEC-specific ablation of IκB kinase-γ (IKKγ) which is essential for NF-κB activation, and develops spontaneous transmural colitis starting at 1-2 weeks of age(Nenci et al., 2007; Zaph et al., 2007). By 2 weeks, the colonic inflammatory infiltrate is comprised of DCs and granulocytes, with mucosal expression of *Il1b*, *Il6*, *Tnf* and *Ccl2* mRNA. Lymphoid follicles appear by 12 weeks of age. Mucosal inflammation is associated with decreased IEC production of β-defensin-3 and TNF-induced IEC apoptosis. Inflammation in this model is dependent on TLR signalling via Myd88 in IECs and intact TNF signalling (Nenci et al., 2007). This phenotype can be recapitulated with an IEC-specific deletion of IKKα or IKKβ. *Ikkb*$^{\Delta IEC}$ IECs have significantly reduced TSLP secretion, impaired Th2 responses to *Trichuris* infection, increased production of TNF and IL-12p40 by mucosal DCs, and increased expression of IFN-γ and IL-17 in CD4^{+} lymphocytes (Zaph et al., 2007). These observations suggest that tightly controlled NF-κB signalling within the epithelium is required to maintain the epithelial barrier and

restrain inappropriate mucosal inflammation, and that consequently, novel agents that target NF-κB signalling should be used with caution in CD.

Under non-inflammatory conditions, IECs suppress $CD4^+$ activation (Cruickshank et al., 2004), but there is evidence to suggest that IECs can present antigen in the context of mucosal inflammation. IECs from both healthy controls and CD patients take up orally administered ovalubumin (OVA) *in vivo*, where it is targeted to late endosomes (Büning et al., 2006). MHC-II is stored in late endosomes in professional APCs. MHC-II expression is seen in late endosomes and the basolateral membrane in IELs obtained from patients with inflamed CD, but not from patients with CD in remission or healthy controls (Büning et al., 2006). IECs can also express co-stimulatory molecules, including CD40, CD80 and CD86 (Cruickshank et al., 2004; Nakazawa et al., 2004) and molecules that can directly activate $CD4^+$ T cells, including lectins and carboxylated glycans (Hokama et al., 2004; Srikrishna et al., 2005). Consequently, it is possible that atypical antigen presentation and immune cell activation by IECs can contribute to mucosal inflammation in CD.

8.8.2.3 Intraepithelial lymphocytes

The epithelial layer contains a population of T cells, termed intraepithelial lymphocytes (IELs), which include thymically-derived $TCR\gamma\delta^+$ or $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ T cells. IELs can also be induced in the periphery from $CD4^+$ or $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ lymphocytes that encounter their cognate antigen (Cheroutre et al., 2011). IELs exist in a "poised" state, as they express genes and cell surface markers of activation, such as CD44, CD69 and NK cell markers, but also inhibitory molecules

such as CD8αα (Denning et al., 2007; Leishman et al., 2001; Shires et al., 2001). IEL migration to the small bowel is mediated by $\alpha_E\beta_7$ integrin and CCR9, which interact with epithelial E-cadherin and CCL25, respectively (Andrew et al., 1996). TCRγδ$^+$ and CD8αα$^+$ TCRαβ$^+$ have numerous protective functions in murine models, including limiting immune cell activation by secreting of TGF-β and IL-10, helping IgA production, promoting IEC repair and proliferation, limiting epithelial damage in bacterial or chemically-induced colitis in mice, and cytotoxic effects against cells infected with intracellular parasites or viruses (Cheroutre et al., 2011). However, TCRγδ$^+$ cell dysfunction is associated with mucosal inflammation in murine models, such as *TCRα* mutant, *CD5*$^{-/-}$*β7*$^{-/-}$, and *CD4*^{Cre} *x Pdk1*^{Flox} mice (Kawaguchi-Miyashita et al., 2001; Mizoguchi et al., 2003; Park et al., 2010). Active CD is associated with increased TCRγδ$^+$ cells in the peripheral blood (PB) (Giacomelli et al., 1994) and IELs in ileal, colonic and rectal mucosa (Caballero et al., 1995; Kanazawa et al., 2001; Meresse et al., 2001). There is also evidence of increased lymphocyte migration to the epithelium in active CD (Meresse et al., 2001). IELs express IFN-γ and can be induced to express IL-17 by macrophage and DC-derived IL-23 in active CD (Liu et al., 2011). In an experimental autoimmune encephalomyelitis (EAE) model, IL-23$^+$ TCRγδ$^+$ cells prevent the peripheral induction of FOXP3$^+$ iT$_{regs}$ *in vitro* and *in vivo*, and impair T$_{reg}$-mediated suppression of CD4$^+$ proliferation *in vitro* (Petermann et al., 2010). Consequently, while IELs help maintain epithelial integrity and prevent mucosal inflammation in response to commensal bacteria and luminal antigens, it is also possible that IELs can contribute to mucosal inflammation in CD.

8.8.3 Dendritic cells and macrophages in the lamina propria

8.8.3.1 Mucosal CX_3CR1^+ and $CD103^+$ dendritic cells

The two subsets of LP myeloid cells that are classically considered to be mucosal DCs can be distinguished on the basis of CX_3CR1 and CD103 (α_E integrin) expression (Schulz et al., 2009; Varol et al., 2009). Expression of CX_3CR1 and CD103 is mutually exclusive between these DC subsets.

CX_3CR1^+ cells arise from $Ly6C^{hi}$ monocytes under the control of granulocyte-monocyte colony stimulating factor (GM-CSF) (Varol et al., 2009) and are reduced in the LP of germ-free mice (Niess and Adler, 2010). Under non-inflammatory conditions, mucosal antigen presenting cells (APCs) sample luminal antigens that are translocated across the epithelium by specialised epithelial microfold, or M cells at lymphoid follicles or Peyer's patches. $CX3CR1^+$ DCs also sample luminal antigens by passing dendrites through epithelial tight junctions into the intestinal lumen (Niess et al., 2005; Rescigno et al., 2001). Dendrite formation in $CX3CR1^+$ DCs is stimulated by interaction between the luminal microbiota and IECs and requires MyD88 sufficiency in IECs (Chieppa et al., 2006). CX_3CR1^+ DCs are pro-inflammatory: on stimulation with fractalkine/CX_3CL1, these cells express IL-6 and TNF (Niess and Adler, 2010). TNF production by CX_3CR1^+ DCs exacerbates DSS colitis (Varol et al., 2009). CX_3CR1^+ DCs induce Th1 and Th17 differentiation *in vitro* (Niess and Adler, 2010). Mixed Th1/Th17 inflammation seen in $CD45RB^{hi}$ adoptive transfer colitis is ameliorated when this model is recapitulated by adoptively transferring WT T_{cons} to *Cx3cr1*$^{-/-}$ recipients (Niess and Adler, 2010).

There is on-going controversy as to whether CX_3CR1 DCs can truly be considered to be DCs, or whether they are a population of tissue-resident macrophages (Geissmann et al., 2010). $CX3CR1^+$ DCs were originally characterised as $CX3CR1^+CD11b^+CD11c^+$ cells (Niess et al., 2005), but mucosal CX_3CR1^+ DCs also express monocyte-macrophage markers such as $F4/80^+$ and CD14 (Medina-Contreras et al., 2011; Varol et al., 2009). CX_3CR1 DCs do not proliferate *in vivo* in response to FMS-like tyrosine kinase 3 ligand (Flt3L) or GM-CSF (Schulz et al., 2009). In addition, CX_3CR1 DCs do not migrate to MLNs *in vivo* and are less efficient at generating all-trans retinoic acid (ATRA) than $CD103^+$ DCs (Schulz et al., 2009). These observations suggest that mucosal CX_3CR1 DCs may be a population of tissue-resident macrophages that induce mucosal inflammation when activated.

$CD103^+$ DCs arise from Flt3L-induced macrophage-DC precursors *via* pre-DCs (Varol et al., 2009). CD103 is the ligand for E-cadherin and this allows $CD103^+$ DCs to closely interact with IECs. $CD103^+$ DCs turn over rapidly *in vivo* and are responsive to both Flt3L and GM-CSF (Schulz et al., 2009). In contrast to CX_3CR1^+ DCs, $CD103^+$ DCs migrate to MLNs (Schulz et al., 2009), fulfilling a classic antigen-presenting DC role *in vivo*. $CD103^+$ DCs have two other distinctive roles: inducing T_{regs} in the periphery and imprinting expression of gut homing receptors on lymphocytes.

$CD103^+$ DCs induce T_{cons} to become $FOXP3^+$ iT_{regs} in an ATRA and TGF-β-dependent manner (Coombes et al., 2007; Sun et al., 2007). $CD103^+$ DC frequency increases in a gradient from the duodenum to the colon and this correlates with the frequency of mucosal $FOXP3^+$ T_{regs} (Denning et al., 2011). As discussed in

Section 8.15.2 (page 114), TGF-β-mediated induction of T_{regs} from T_{cons} requires sub-immunogenic doses of antigen with sub-optimal T cell receptor (TCR) ligation or co-stimulation. High levels of antigen or high levels of co-stimulation impair TGF-β-mediated iT_{reg} induction (Benson et al., 2007; Kretschmer et al., 2005). However, ATRA synergises with TGF-β to allow iT_{reg} induction even in the face of high levels of co-stimulation (Benson et al., 2007). This suggests that $CD103^+$ DC-mediated iT_{reg} induction might be preserved in the context of inflammation. ATRA also induces FOXP3 expression *via* Smad3 and inhibits expression of IL-6Rα, IRF-4 and IL-23R, thus facilitating iT_{reg} induction and inhibiting IL-6 and IL-23-driven T_{con} polarisation to Th17 (Elias et al., 2008; Mucida et al., 2007; Xiao et al., 2008). ATRA also inhibits expression of other lineage-skewing cytokines by memory T_{cons}; specifically IFN-γ, IL-4 and IL-21 (Hill et al., 2008). iT_{reg} induction is also influenced by the intestinal microbiota. $CD103^+$ DCs from SFB-colonised mice are less efficient at inducing iT_{regs} and more efficient at inducing IL-17^+ T_{cons} when compared with $CD103^+$ DCs from SFB-negative mice (Denning et al., 2011). This shows that environmental Th17 cytokines impair iT_{reg} induction in the steady-state, and suggests that iT_{reg} induction might also be reduced by Th17-mediated mucosal inflammation.

$CD103^+$ DC-mediated induction of $FOXP3^+$ iT_{regs} from T_{cons} is dependent on $\alpha_V\beta_8$ integrin expression by $CD103^+$ DCs (Paidassi et al., 2011). TGF-β is secreted as an inactive precursor and requires activation by $\alpha_V\beta_8$ integrin to become biologically active. Mucosal and MLN $CD103^+$ DCs generate more iT_{regs} *in vitro* than $CD103^-$ DCs, when supplemented with latent TGF-β (Paidassi et al., 2011). Selective depletion of $\alpha_V\beta_8$ on DCs results in colitis, in addition to wasting (Travis

et al., 2007). These mice also have a 50% reduction in colonic T_{reg} numbers, while T_{reg} numbers at extra-intestinal sites are preserved, suggesting that $CD103^+$ DC-mediated iT_{reg} induction substantially contributes to the mucosal T_{reg} population.

Peyer's patch and MLN $CD103^+$ DCs also induce ATRA-dependent $\alpha_4\beta_7$ integrin and CCR9 expression on lymphocytes, which confers gut-homing phenotype and allows lymphocytes to recirculate to the mucosa (Annacker et al., 2005; Iwata et al., 2004; Johansson-Lindbom et al., 2005; Mora et al., 2003). This is supported by ATRA production by MLN stromal cells (Hammerschmidt et al., 2008). Interaction with $CD103^+$ DCs also induces CD103 expression on both thymically-derived T_{regs} (tT_{regs}) and iT_{regs}, allowing them to interact with E-cadherin on the basolateral surface of IECs and become tissue-resident T_{regs} (Siewert et al., 2008). However, it is not currently possible to distinguish between tT_{regs} and iT_{regs} in the mucosa in humans, making characterisation of the relative contribution of these cells to CD difficult. Peripheral induction of iT_{regs} by TGF-β (in the absence of ATRA) is discussed in Section 8.15.2 (page 114).

8.8.3.2 Mucosal macrophages

Under non-inflammatory conditions, PB monocytes are attracted to the LP by IL-8 and TGF-β, where they become tissue-resident macrophages and, mediated by TGF-β, down-regulate expression of CD14, CD40, CD80, CD86, CD89 (IgA receptor), reduce responsiveness to stimulation through TLRs 3-9, and secrete IL-10 following TLR ligation (Monteleone et al., 2008; Smith et al., 2001; Smythies et al., 2006; 2010). LP macrophages (variously characterised as $CD14^+CD209^+$ or $F4/80^+$) express retinaldehyde dehydrogenase 2 (RALDH2), can synthesise ATRA

and can also induce iT_{regs} from T_{cons} *in vitro* (Denning et al., 2011; Kamada et al., 2009). $CD14^+CD209^+$ LP macrophages also present antigens to mucosal T cells and macrophage-induced Th17 polarisation is increased in the presence of commensal bacterial antigens in samples obtained from CD LP, but not in samples obtained from healthy controls (Kamada et al., 2009). Mucosal DCs also have an IL-10-dependent hyporesponsiveness to bacterial TLR ligands, despite higher expression of TLR2, 3, 4 and 9 than splenic or MLN DCs (Monteleone et al., 2008). IL-10 signalling in myeloid cells is required to restrain mucosal inflammation. Mice with a myeloid-specific deletion in *Stat3* develop chronic enterocolitis with increased TNF, IFN-γ, IL-1 and IL-6 expression and Th1 polarisation (Takeda et al., 1999). Enterocolitis in this model is mediated by TLR4 and a Th1 response, as double knock-outs with *Tlr4*$^{-/-}$ or *Il12p40*$^{-/-}$ *were* not associated with disease, while a double knock-out with *Tnf* did not attenuate colitis in this model (Kobayashi et al., 2003). In contrast to tissue-resident LP macrophages, the inflammatory $CD14^+$ monocyte/macrophage population seen inflamed IBD mucosa probably derives from infiltrating PB monocytes (Rugtveit et al., 1997).

NOD2 is expressed in mucosal macrophages and, as discussed in Section 8.4.2 (page 32), polymorphisms in *NOD2* are the commonest monogenetic risk for CD. NOD2 senses bacterial MDP and the MDP:NOD2 interaction results in NF-κB activation (Girardin et al., 2003; Hugot et al., 2001; Netea et al., 2005). Identifying how NOD2 risk alleles might alter monocyte/macrophage biology to increase the risk of mucosal inflammation has proven challenging, as NOD2 is also expressed in IECs and Paneth cells. *Nod2*$^{-/-}$ mice have abnormalities in intestinal permeability, bacterial translocation and anti-microbial peptide secretion that could be attributed to the effects of NOD2 deletion in those tissues (Kobayashi et al., 2005).

In addition, *Nod2*$^{-/-}$ mice and mice with NOD2 mutations that recapitulate human risk alleles (*Nod2*^{2939ic} and *frameshift Nod2* mice) do not develop spontaneous intestinal inflammation (Kobayashi et al., 2005; Maeda et al., 2005; Watanabe et al., 2008b).

Treatment of human PB monocytes with MDP activates NF-κB and induces TNF, IL-1β and IL-8 expression. PB monocytes from humans with NOD2 risk alleles have decreased NF-κB activation in response to MDP or LPS, compared with PB monocytes from non-carriers (Bonen et al., 2003). These cells also have decreased MDP-induced expression of TNF, IL-1β and IL-8, compared with non-carriers (Beynon et al., 2008; Li et al., 2004; van Heel et al., 2005). However, MDP treatment also induces expression of inhibitors of the NOD2-NF-κB pathway, such as IRAK-M, an inhibitor of IRAK-1 (Hedl et al., 2007). PB monocytes from non-carriers that have been pre-treated with MDP have decreased pro-inflammatory cytokine expression on re-treatment with MDP or LPS, while this attenuated response to MDP or LPS re-treatment is not seen in monocytes with NOD2 risk alleles (Hedl et al., 2007). Intestinal macrophages are chronically exposed to bacterial products *in vivo*. Freshly isolated intestinal macrophages from healthy controls do not express TNF in response to MDP, despite intact phagocytosis (Hedl et al., 2007). These observations suggest that chronic exposure to MDP and TLR ligands restrains NF-κB activation through the induction of regulatory molecules and that this mechanism is lost in patients with NOD2 risk alleles. It is possible that loss of an attenuated NF-κB response due to chronic MDP exposure might increase the risk of mucosal inflammation in humans with NOD2 risk alleles.

PB monocytes from humans with NOD2 risk alleles also have increased basal expression of IL-12p40 (Beynon et al., 2008). Stimulation of murine macrophages with MDP reduces IL-12p40 expression and both *Nod2*$^{-/-}$ macrophages and macrophages from human carriers have increased IL-12p40 expression and NF-κB activation in response to peptidoglycan stimulation (Watanabe et al., 2004). This suggests that NOD2 risk alleles might also increase the risk of Th1 mediated inflammation in carriers.

Polymorphisms in *ATG16L1* are associated with increased risk of CD (Hampe et al., 2007; Rioux et al., 2007). *ATG16L1* risk alleles are associated with ER stress and Paneth cell dysfunction in mice and humans (Adolph et al., 2013; Deuring et al., 2014). *ATL16L1* risk alleles T300A and T316A also result in increased caspase-3-mediated degradation of ATG16L1 and defective autophagy (Murthy et al., 2014). This is associated with decreased clearance of *Yersinia enterocolitica* and increased expression of TNF, IL-1β and IL-6 in infected macrophages (Murthy et al., 2014). Adherent-invasive *E. coli* can also persist and replicate within macrophages, resulting in increased TNF and IL-6 expression by infected cells (Glasser et al., 2001; Lapaquette et al., 2012). MDP can activate autophagy *via* NOD2 and disruption of *NOD2* can interact with *ATG16L1* mutations to impair autophagy, resulting in intracellular persistence of AIEC and increased TNF and IL-6 expression in response to AIEC infection (Homer et al., 2010; Lapaquette et al., 2012). An exaggerated inflammatory response by macrophages that fail to kill intracellular bacteria provides another link between macrophages, *ATG16L1* and *NOD2* risk alleles, and CD.

8.8.4 Th1 and Th17 CD4+ lymphocytes in the lamina propria

8.8.4.1 Mucosal Th1 lymphocytes

Current conceptualisation of CD pathogenesis suggests that microbial dysbiosis, increased epithelial permeability, Paneth cell dysfunction and activation of the innate immune system are likely to be the initiating events in mucosal inflammation. However, activation of the adaptive immune system, particularly T_{cons}, is likely to perpetuate mucosal inflammation once this is established. The importance of Th1 and Th17 responses in the intestinal mucosa is highlighted by recent GWAS results, which show that SNPs in *IFNG*, *IFNGR2*, *IL12B*, *IL21*, *IL23R*, *RORC*, *STAT1* and *STAT4* are risk alleles for CD (Franke et al., 2010; Jostins et al., 2012).

Th1 $CD4^+$ lymphocytes are characterised by IFN-γ, IL-12, T-bet and CXCR3 expression (Mosmann et al., 1986; Powell et al., 2012; Szabo et al., 2000). T-bet expression is induced in $CD4^+$ lymphocytes by IFN-γ *via* signal transducer and activator of transcription (STAT) 1, and stabilised and potentiated by IL-12 *via* STAT4 (Afkarian et al., 2002; Mullen et al., 2001; Szabo et al., 2000), both of which are produced by activated APCs and lymphocytes in the LP (Neurath, 2014). A prominent mucosal Th1 response is seen in human CD and multiple animal models of intestinal inflammation, including $CD45RB^{hi}$ adoptive transfer colitis, TNBS colitis, the BM→Tgε26 model, spontaneous colitis in *Il10*$^{-/-}$ mice and the transmural colitis seen in *Stat4* transgenic mice (Neurath et al., 2002; Simpson et al., 1998; Wirtz et al., 1999). In the $CD45RB^{hi}$ adoptive transfer model, anti-IFN-γ antibody or adoptive transfer with *Stat4*$^{-/-}$ or *Tbx21*$^{-/-}$ T_{cons} prevents induction of

colitis, while T_{cons} with T-bet over-expression get worse disease (Neurath et al., 2002; Powrie et al., 1996; Simpson et al., 1998). IL-21 blockade also reduces IFN-γ, T-bet and phosphorylated STAT4 expression in CD LPMCs (Monteleone et al., 2005b). Glucocorticoids inhibit Th1 polarisation by direct interaction between the glucocorticoid receptor and T-bet (Liberman et al., 2007). Fontolizumab, an anti-IFN-γ monoclonal antibody, demonstrated initial safety in CD (Hommes et al., 2006; Reinisch, 2005). A phase II clinical trial did not demonstrate a significant difference in clinical response rates *vs.* placebo at day 29, although recipients had a significant reduction in C-reactive protein (CRP) levels in recipients, suggesting that fontolizumab has a biological effect in patients with CD (Reinisch et al., 2010).

8.8.4.2 Mucosal Th17 lymphocytes

IL-17 was first cloned in 1993 and originally described as a $CD4^+$ T cell product (Park et al., 2005; Rouvier et al., 1993; Yao et al., 1995; 1996), although $TCR\gamma\delta^+$ lymphocytes are also a major source of IL-17 (Lockhart et al., 2006). Th17 lymphocytes are characterised by IL-17, IL-22, IL-23, RORγt (RORC in humans) and CCR6 expression. Under steady-state conditions, the intestinal microbiota (including SFB in mice) induces mucosal DCs to express IL-6 and IL-23, which polarise T_{cons} to Th17 (Becker et al., 2003; Ivanov et al., 2009; 2008). In CD and murine models of mucosal inflammation, Th17 cells can be induced by macrophage or DC expression of IL-6, IL-21, IL-23 or TL1A (Kamada et al., 2010; Yen et al., 2006). Th17 polarisation can be augmented by macrophage-derived IL-2, IL-15 and IL-18 (Hoeve et al., 2006). IL-1β can also drive Th17 polarisation by inducing the migration of IL-6 and IL-23-expressing innate immune cells to the

mucosa, or by a direct stimulation of IL-1βR-sufficient T_{cons} (Coccia et al., 2012; Shaw et al., 2012).

Th17 cells have a protective effect at environmental interfaces, *via* IL-6 and IL-23-mediated expression of IL-22 (Zheng et al., 2007). IL-22 activates STAT3 in IECs, which induces β-defensin, RegIIIβ and RegIIIγ expression and increases IEC proliferation and migration (Brand et al., 2006; Wolk et al., 2004). IL-22-induced anti-microbial peptide production can be augmented by IL-17A and IL-17F (Liang et al., 2006). IL-22 can also be expressed by innate lymphoid cells, $TCR\gamma\delta^+$ lymphocytes, $NKp46^+$ NK cells and protects from adherent-invasive bacteria, particularly *Citrobacter rodentium* in murine models (Basu et al., 2012; Mielke et al., 2013; Ota et al., 2011; Satoh-Takayama et al., 2008; Sonnenberg et al., 2011; Zheng et al., 2008). In contrast to other Th17 cytokines, induction of IL-22 expression is inhibited by TGF-β (Leung et al., 2014; Zheng et al., 2007). Mucosal IL-22 expression in active CD is either increased or equivalent to healthy controls (Brand et al., 2006; Leung et al., 2014).

Th17 cells also have multiple pro-inflammatory effects, including induction of TNF, IL-1β, IL-6 and IL-8 in innate immune cells, neutrophil recruitment and activation of fibroblasts (Neurath, 2014). The identification of the contribution of various Th17 cytokines to mucosal inflammation has been a source of controversy. Adoptive transfer of *Rorc*$^{-/-}$ T_{cons} in the $CD45RB^{hi}$ adoptive transfer model did not induce colitis, but subsequent treatment of these mice with IL-17A induced colitis, suggesting that IL-17A has a colitogenic role (Leppkes et al., 2009). However, adoptive transfer of *Il17a*$^{-/-}$ T_{cons} did not result in a reduction in colitis, but rather an exaggerated Th1 response with worse colitis and wasting, suggesting that IL-

17^+ T_{cons} cells can restrain Th1 differentiation (Leppkes et al., 2009; O'Connor et al., 2009). *RAG1*$^{-/-}$ mice injected with anti-CD40 antibody develop colitis and wasting. Colitis in this model is dependent on IL-23p19 expression by mucosal DCs and IL-17 expression within the intestine, while wasting is dependent on IL-12p40, IFN-γ and TNF (Uhlig et al., 2006b). IL-12 and IL-23 share the IL-12p40 subunit and their receptors share the IL-12Rβ1 chain. It is now clear that IL-12R and IL-23R also have discrete patterns of expression on $CD4^+$ lymphocytes, which correlates with Th1 or Th17 phenotypes, respectively (Chognard et al., 2014). IL-23R and IL-23 expression is required for pathogenicity in murine Th17 cells, and pathogenic and non-pathogenic Th17 cells have a distinct gene expression signatures (Ghoreschi et al., 2010; Lee et al., 2012). IL-23 also prevents mucosal induction of iT_{regs} (Izcue et al., 2008). IL-23 has also been implicated as a pathogenic cytokine in murine models of psoriasis, multiple sclerosis, arthritis and in the pulmonary immune response to *Mycobacteria tuberculosis* infection (Chan et al., 2006; Cua et al., 2003; Ghoreschi et al., 2010; Lockhart et al., 2006; Lubberts et al., 2005; Zheng et al., 2007).

These observations on the heterogeneity of Th17 cells might explain apparently conflicting results in two recent clinical trials. Ustekinumab, a monoclonal antibody against the common IL-12p40/IL-23p40 subunit, was superior to placebo in both induction and maintenance of remission in a phase III clinical trial (Sandborn et al., 2012b). However, blockade of IL-17 with secukinumab was ineffective in CD and resulted in a higher rate of adverse events compared with placebo, particularly in the group with elevated inflammatory markers (CRP ≥10 mg/L or faecal calprotectin ≥200 ng/ml; Hueber et al., 2012). This is consistent with the idea that IL-23 is a pathogenic cytokine in CD, while IL-

17, or IL-17$^+$ expressing cells, have some protective effects. Ramesh *et al.* recently discovered that *IL23R* expression and IL-23 responsiveness is restricted to a subset of human Th17 cells, characterised by CCR6$^+$CXCR3^{hi}CCR4^{lo}CCR10$^-$CD161$^+$ expression (Ramesh et al., 2014). This subset was enriched in inflamed CD mucosa. This subset also expressed multi-drug resistance type 1 (MDR1) and were resistant to glucocorticoid-mediated suppression *in vitro*. This suggests that pathogenic human Th17 cells in CD mucosa are IL-23-responsive and can contribute to steroid-resistant disease. Further studies to associate the presence of Th17 subsets in CD mucosa with responsiveness to steroid therapy might allow early identification of patients who are unlikely to respond to glucocorticoids and early institution of an anti-TNF agent to induce remission.

8.8.4.3 Introduction to mucosal regulatory T cell populations

FOXP3$^+$ T_{regs}, FOXP3$^+$ iT_{regs}, FOXP3$^-$ IL-10$^+$ type 1 regulatory T cells (Tr1s), TGF-β$^+$ peripherally induced regulatory T cells (Th3) and IL-35$^+$ peripherally induced regulatory T cells (iTr35 cells) have all been implicated in the prevention of mucosal inflammation. Active mucosal inflammation results in a relative increase of T_{cons} compared with T_{regs}. A cell-intrinsic defect in LP T_{con} responsiveness to T_{reg}-mediated suppression has also been identified in inflamed CD mucosa (Fantini et al., 2009). NOD2-mediated NF-κB activation in FOXP3$^+$ T_{regs} prevents Fas-mediated apoptosis, and this is lost in T_{regs} obtained from patients with *NOD2* risk alleles (Rahman et al., 2010). There is also controversy as to whether mucosal T_{regs} can be subverted to a Th1 or Th17 phenotype and contribute to mucosal inflammation in CD, or whether T_{regs} simply co-opt the

cellular machinery associated with Th1 (Koch et al., 2009; Oldenhove et al., 2009) and Th17 (Chaudhry et al., 2009) polarisation to home to inflamed sites and suppress Th1, Th2 or Th17-mediated inflammation, respectively. These points are discussed in detail below, while T_{reg}-Th17 plasticity is discussed in Chapter 11 (page 196).

8.8.5 Tumour necrosis factor in Crohn's disease

TNF is produced by activated mucosal macrophages, DCs and lymphocytes and has multiple pro-inflammatory effects, including inducing activation and cytokine expression in immune cells, conferring resistance to apoptosis in T_{cons}, inducing apoptosis of epithelial cells and fibroblast activation (Neurath, 2014). TNF also has a direct effect on the suppressive ability of T_{regs}. TNF acts through TNFRII to reduce expression of *FOXP3* mRNA and FOXP3 protein in T_{regs} obtained from patients with rheumatoid arthritis. These T_{regs} have defective *in vitro* suppression of autologous T_{con} proliferation, which is reversed following IFX therapy (Valencia et al., 2006).

TNF also plays a central role in a number of mouse models of intestinal inflammation. A20 is a cytoplasmic zinc finger protein that inhibits TNF-induced NF-κB activation and *A20*$^{-/-}$ cells fail to terminate TNF-induced NF-κB activation (Lee et al., 2000; Wertz et al., 2004). *A20*$^{-/-}$ mice develop multisystem inflammation, including chronic colitis with crypt abscesses and crypt distortion. This phenotype also occurs in *RAG1*$^{-/-}$*A20*$^{-/-}$ mice, suggesting that innate immune cells and non-immune cells are sufficient to induce pathology in this model. While this model demonstrates that tight control of TNF-induced NF-κB activation is

required for mucosal homeostasis, it is not a tractable model for studying CD due to high and early mortality. Severe multi-organ inflammation is also not a feature of CD in humans.

Mice with mutations in the AU-rich elements (ARE) of *TNF* have abnormal regulation of *Tnf* mRNA and increased constitutive and inducible TNF expression by both immune and non-immune cells (Kontoyiannis et al., 1999). This results in a transmural CD-like ileitis with granuloma and arthritis. The ileitis is dependent on lymphocytes and TNF signalling, as *RAG1*$^{-/-}$ *x TNF*$^{\Delta ARE}$ and *TNFRII*$^{-/-}$ *x TNF*$^{\Delta ARE}$ mice are protected. Ileitis in *TNF*$^{\Delta ARE}$ mice is also dependent on activated CD8^{+} lymphocytes, IL-12 and IFN-γ (Kontoyiannis et al., 2002). CCL19 and CCL21 are expressed in both inflamed CD mucosa and in ectopic lymphoid tissue in *TNF*$^{\Delta ARE}$ inflamed ileum (McNamee et al., 2013). CCL19 and CCL21 mediate CCR7-dependent lymphocyte homing. In the *TNF*$^{\Delta ARE}$ mouse, anti-CCR7 treatment decreases lymphocyte trafficking from the mucosa to MLN, causing lymphocyte retention in inflamed ileum. If this observation holds true for humans, this suggests that anti-CCR7 antibody treatment may prevent the egress of CCR7^{+} effector-memory T_{cons} from inflamed CD mucosa and that anti-CCR7 antibodies may be inappropriate in CD. In addition to illustrating a central role for TNF in CD pathogenesis, this shows that the *TNF*$^{\Delta ARE}$ mouse model can also be used to assess potential therapies for CD.

The mechanism of action of anti-TNF agents in CD has been a source of controversy. As summarised in Table 8.2 (page 44) and Table 8.4 (page 61), infliximab, adalimumab and certolizumab pegol are effective in CD. However, anti-TNF agents such as etanercept, which have proven successful in other diseases,

have failed in clinical trials in CD. Both IFX and etanercept neutralise soluble TNF, but only IFX binds to transmembrane TNF on activated PB lymphocytes and LP lymphocytes from CD patients, or Jurkat T cells transfected with transmembrane TNF (Mitoma et al., 2005; Van den Brande et al., 2003). IFX activates caspase-3 and induces lymphocyte apoptosis on binding to transmembrane TNF, while etanercept does not. Atreya *et al.* identified a second mechanism by which select anti-TNF agents induce apoptosis in lymphocytes (Atreya et al., 2011). Transmembrane TNF is expressed more highly on mucosal $CD14^+$ cells obtained from CD patients, than on mucosal $CD4^+$ cells. Both IFX and ADA block the interaction between transmembrane TNF on $CD14^+$ cells and TNFRII on mucosal $CD4^+$ T_{cons}. Blockage of this interaction induces T_{con} apoptosis on co-culture. In this study, IFX-induced T_{con} apoptosis was prevented by IL-6, suggesting that the activation of other inflammatory pathways could potentially cause primary treatment failure or loss of response to anti-TNF agents.

8.9 Regulatory T cells

8.9.1 Identification of T_{regs} in mice and humans

Central tolerance is a mechanism to prevent autoimmunity by deleting auto-reactive T cells within the thymus. This is a two-stage process, involving positive selection of thymocytes that have the capacity to recognize MHC molecules on thymic epithelial cells, followed by clonal deletion or induction of anergy in thymocytes that strongly recognize self-antigens. In contrast, peripheral tolerance refers to the prevention of autoimmunity by immune modulating cells in the periphery.

Key experiments from the 1960's to the 1990's implicated thymically-derived $CD4^+CD25^+$ T cells as mediators of peripheral tolerance. Mice that are thymectomied on post-natal day 3 develop multi-system autoimmunity, which can be avoided by delaying thymectomy until after day 7 and prevented by infusion of thymocytes (Kojima et al., 1976; Nishizuka and Sakakura, 1969; Sakaguchi et al., 1982; Taguchi and Nishizuka, 1981). It was subsequently found that adoptive transfer of $CD4^+$ lymphocytes depleted of $CD5^{hi}$ or $CD45RB^{lo}$ fractions to nude mice caused multi-system autoimmunity or colitis, which could be prevented by co-transfer of the depleted fraction (Morrissey et al., 1993; Powrie and Mason, 1990; Powrie et al., 1993; Sakaguchi et al., 1985). Hall *et al.* identified CD25 (IL-2Rα) as a cell surface marker of $CD4^+$ lymphocytes that prevented cardiac allograft rejection in rats (Hall et al., 1990). Subsequent adoptive transfer experiments demonstrated that $CD4^+CD25^+$ lymphocytes were responsible for prevented adoptive transfer colitis in mice (Sakaguchi et al., 1995; 1996). *In vitro* suppressive $CD4^+CD25^{hi}$ T_{regs}

were identified in humans in 2001 (Baecher-Allan et al., 2001; Dieckmann et al., 2001; Jonuleit et al., 2001; Levings et al., 2001a; Ng et al., 2001; Taams et al., 2002).

8.9.2 FOXP3 as a T_{reg} "marker"

The X-linked forkhead/winged-helix family transcription factor *FOXP3* is the master regulator of T_{reg} differentiation and function. The phenotype of the Scurfy mouse, where hemizygous males develop rapidly fatal multi-system autoimmunity with uncontrolled lymphoproliferation and defective T_{regs}, maps to *Foxp3* (Brunkow et al., 2001). *Foxp3*$^{-/-}$ mice develop a similar fatal autoimmunity which can be rescued by $CD4^+CD25^+$ T_{regs} (Fontenot et al., 2003; Hori et al., 2003). Ectopic *Foxp3* expression in $CD4^+CD25^-$ lymphocytes confers a suppressor function (Fontenot et al., 2003; Hori et al., 2003). In humans, *FOXP3* mutations are associated with multi-system autoimmunity and defective T_{regs} in the Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome, in which gastrointestinal inflammation may be a feature (Bennett et al., 2001; Wildin et al., 2000). However, *FOXP3* expression in humans is not restricted to T_{regs} and can be induced, albeit at lower levels, in T_{cons} upon activation. Key to the stable expression of *FOXP3* in humans and maintenance of a stable, suppressive T_{reg} phenotype is demethylation of the *FOXP3* T_{reg} Specific Demethylation Region (TSDR), which is completely demethylated in T_{regs} but not in T_{cons} (Baron et al., 2007; Floess et al., 2007; Polansky et al., 2008). Barzaghi *et al.* recently described a cohort of patients with "IPEX-like syndrome", a syndrome of severe multisystem autoimmunity in the absence of detectable *FOXP3* mutations, which was associated with incomplete TSDR demethylation despite a normal

proportion of circulating T_{regs} (Barzaghi et al., 2012). This suggests that complete TSDR demethylation is a marker of functional T_{regs} *in vivo* in humans.

8.9.3 Cell surface markers to delineate T_{reg} subsets

Naïve $CD4^+CD25^{lo}$ T_{cons} express the heterodimeric low-affinity IL-2 receptor (IL-2R), comprised of CD122 [IL-2Rβ] and CD132 [the common gamma chain, γ_c]). CD25 (IL-2Rα) is a non-signalling molecule that binds IL-2 and interacts with the heterodimeric IL-2R to form the trimeric high affinity IL-2R. This increases the affinity of the IL-2R for its ligand by 10-100 fold (Boyman and Sprent, 2012). Even though both T_{regs} and activated T_{cons} express the trimeric IL-2R, it is expressed at a higher density per cell in T_{regs}. This allows T_{regs} to selectively respond to low concentrations of IL-2. T_{regs} require IL-2 and intact IL-2 signalling for thymic development, peripheral homeostasis and effector action, as discussed in Section 8.11.3 (page 96), below. In humans, there is an overlap in CD25 expression between $CD4^+CD25^{int}$ activated T_{cons} and $CD4^+CD25^{hi}$ T_{regs}, meaning that it is not possible to delineate T_{regs} on the basis of CD25 expression alone. In contrast, mice have a bimodal distribution of CD25 expression, meaning that murine T_{regs} can be discriminated from T_{cons} by CD25 expression alone.

In addition to CD25, numerous cell surface molecules have been proposed as specific T_{reg} markers in humans, including cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Wing et al., 2008), glucocorticoid-induced TNF receptor family related protein (GITR) (Shimizu et al., 2002), inducible co-stimulator (ICOS) (Ito et al., 2008) and HLA-DR (Baecher-Allan et al., 2006). However, these markers are also expressed by activated T_{cons}, which complicates the delineation of functional T_{regs}

in humans. This "activated" phenotype is consistent with a requirement for TCR:MHC-II interaction and co-stimulation during thymic T_{reg} differentiation (Povoleri et al., 2013).

CD127 (IL-7Rα) is differentially expressed between non-suppressive $CD4^+CD25^{lo/int}CD127^{hi}$ T_{cons} and suppressive $CD4^+CD25^{hi}CD127^{lo}$ T_{regs}, and inversely correlates with FOXP3 expression, facilitating identification and isolation of T_{regs} in humans (Liu, 2006; Seddiki, 2006), which is relevant to a successful program of cell therapy.

Human T_{regs} are heterogeneous. $CD4^+CD25^{hi}CD127^{lo}$ cells can be phenotypically subdivided into three subsets on the basis of CD25 and CD45RA expression:

(i) $CD4^+CD25^{hi(++)}CD45RA^+FOXP3^{lo}$ resting T_{regs} (rT_{regs}; Fr I),

(ii) $CD4^+CD25^{hi(+++)}CD45RA^-FOXP3^{hi}$ activated T_{regs} (aT_{regs}; Fr II) and

(iii) $CD4^+CD25^{hi(++)}CD45RA^-FOXP3^{lo}$ non-suppressive cells (Fr III) (Miyara et al., 2009).

Freshly isolated PB rT_{regs} have low Ki-67 expression. These cells have low FOXP3 expression with a demethylated *FOXP3* TSDR and do not express significant amounts of effector cytokines following stimulation with PMA/ionomycin. Following TCR ligation *in vitro*, they proliferate and are highly suppressive. They also express $CD31^+$, a marker of recent thymic emigrants (RTEs). Adoptive transfer experiments and longitudinal analysis of T_{reg} clones from a healthy individual indicate that rT_{regs} up-regulate FOXP3 expression and give rise to aT_{regs} (Miyara et al., 2009). Freshly isolated aT_{regs} express Ki-67 (50%), CTLA-4 and

HLA-DR. They retain a demethylated *FOXP3* TSDR and do not express cytokines on stimulation. Following TCR ligation *in vitro*, they avidly suppress but are susceptible to apoptosis, which may have been interpreted as anergy in earlier studies (Sakaguchi et al., 2010). The third population of non-suppressive cells has an incompletely demethylated TSDR and expresses effector cytokines on stimulation, suggesting that these are activated T_{cons}. These data contribute to observations from other groups that suggest that T_{regs} may be most efficiently expanded from $CD45RA^+$ precursors, as discussed in Section 8.16 (page 119), below.

8.10 T_{regs} and control of mucosal inflammation

Two strands of evidence suggest that T_{regs} may have a role in modulating intestinal inflammation in mice and humans:

(i) The phenotype of murine or human diseases that are associated with genetic deletions or mutations in molecules required for T_{reg} function support a role for T_{regs} in preventing inappropriate intestinal inflammation. Rare genetic mutations support a role for T_{regs} in preventing mucosal inflammation in humans

(ii) T_{regs} are effective at preventing or curing colitis in experimental models of intestinal inflammation.

8.11 Rare mutations are associated with T_{reg} dysfunction and mucosal inflammation in humans

Multiple mechanisms for T_{reg}-mediated immune modulation have been proposed, including secretion of cytokines such as TGF-β (Maloy et al., 2003; Powrie et al., 1996), IL-10 (Asseman et al., 1999; 2003; Belkaid et al., 2002; Maloy et al., 2003), and IL-35 (Collison et al., 2007); environmental depravation of IL-2 (Pandiyan et al., 2007) or inhibition of T_{con} IL-2 synthesis (Thornton and Shevach, 1998); cell contact-dependent inhibition (Ng et al., 2001; Takahashi et al., 1998; 2000; Thornton and Shevach, 1998); granzyme B and perforin-mediated cytolysis of target cells (Cao et al., 2007; Gondek et al., 2005); inhibition of APC function (DiPaolo et al., 2007); and metabolic disruption (Deaglio et al., 2007). This section will discuss observations made in humans with mutations or deletions in genes, who's products are thought to contribute to T_{reg} function and use these examples to illustrate the relative importance of these mechanisms for T_{reg}-mediated modulation of immune activation in the gut. Specifically, mutations in *FOXP3*, *IL10RA*, *IL10RB*, *IL2RA* (CD25), *STAT5B* and *WAS* are associated with T_{reg} dysfunction and intestinal inflammation in humans.

8.11.1 Mutations in *FOXP3* reveal a role for T_{regs} in preventing autoimmunity

FOXP3 mutations cause the *Scurfy* mouse and IPEX in humans, discussed in Section 8.9 (page 88). *Scurfy* is associated with *Foxp3* mutations in mice and can be rescued by a *Foxp3* transgene (Brunkow et al., 2001). Lahl *et al.* developed a transgenic mouse with expression of a diphtheria toxin (DT) receptor-green fluorescent protein fusion protein under the control of the *Foxp3* promoter,

termed the DEREG ("Depletion of Regulatory T cell") mouse model (Lahl et al., 2007). DT-mediated deletion of FOXP3$^+$ T cells recapitulates the *Scurfy* phenotype, confirming that loss of peripheral tolerance in the absence of FOXP3$^+$ T_{regs} caused *Scurfy* in mice. These observations provide a "classic" genotype-phenotype correlation between defective T_{regs} and multisystem autoimmunity.

However, *Scurfy* mice do not have prominent colitis (Boehm et al., 2012; Lahl et al., 2007). Mucosal inflammation in IPEX is characterised by increased IELs and villous blunting in the small intestine (De Benedetti et al., 2006; Wildin et al., 2002). Aside from mucosal oedema and superficial erosions in the colon, this appearance is more consistent with coeliac disease, rather than IBD. Two recent publications employed the DEREG model and *Scurfy* T_{regs} to show that homing of FOXP3$^+$ T_{regs} to the intestinal LP is required for oral tolerance to OVA or MOG_{35-55} peptide (Cassani et al., 2011; Hadis et al., 2011). While depletion of FOXP3$^+$ T_{regs} may cause a loss of oral tolerance to mucosal antigens and enteropathy, more severe mucosal disease may be restrained by FOXP3$^-$ regulatory cell populations or non-T_{reg} sources of immune modulating cytokines, such as IL-10 (Cheroutre et al., 2011; Hadis et al., 2011; Jarry et al., 2008).

8.11.2 Mutations in *IL10*, *IL10RA* and *IL10RB* suggest a role for T_{reg}-derived and non-T_{reg}-derived IL-10 in preventing mucosal inflammation

Global IL-10 mutations are associated with intestinal inflammation. *Il10*$^{-/-}$ mice develop spontaneous enterocolitis (Kühn et al., 1993) and administration of an anti-IL10R antibody to a WT mouse is sufficient to induce colitis (Asseman et al., 2003), demonstrating that IL-10 has an important role in modulating the

mucosal immune system. In humans, mutations in *IL10RA* and *IL10RB* are associated with "Very Early Onset" IBD (before 2 years of age) (Glocker et al., 2009; Moran et al., 2013). Even though IL-10 is widely expressed by non-T_{reg} innate and adaptive immune cells, including non-T_{reg} $CD4^+$ and $CD8^+$ lymphocytes, IL-10^+ Tr1 cells, B cells, monocyte/macrophages, DCs, IELs and IECs, it is likely that IL-10 expression by T_{regs} contributes to mucosal immune homeostasis (Saraiva and O'Garra, 2010). In an murine IL-10/FOXP3 dual reporter mouse, IL-10^+FOXP3^+ $CD4^+$ regulatory cells are enriched in the colon, while IL-10^+FOXP3^- $CD4^+$ cells are enriched in the small bowel, and extra-intestinal FOXP3^+ T_{regs} do not express IL-10 (Maynard et al., 2007). The contribution of T_{reg} IL-10 to mucosal homeostasis is discussed below, while Tr1s are discussed in Section 8.15.1 (page 113).

T_{regs} contribute to IL-10-mediated prevention of mucosal inflammation. In the CD45RB^{hi} adoptive transfer model of colitis, IL-$10^{-/-}$ T_{regs} are unable to prevent colitis induced by adoptive transfer of CD45RB^{hi} naïve T_{cons} (Asseman et al., 1999), and cure of colitis by WT Tregs is abrogated by anti-IL-10R (Uhlig et al., 2006a). However, another group reported that adoptively transferred IL-$10^{-/-}$ T_{regs} are able to prevent CD45RB^{hi} colitis but that recipient IL-10 sufficiency is required for T_{reg}-mediated prevention of colitis in this model (Murai et al., 2009). Adoptively transferred T_{reg}-derived IL-10 inhibited *Helicobacter hepaticus*-mediated intestinal inflammation in a RAG$^{-/-}$ mouse (Maloy et al., 2003). Deletion of *Il10* under the control of the *Foxp3* promoter (*Il10$^{flox/flox}$* x *Foxp3$^{YFP\text{-}Cre}$*) deletes IL-10 in FOXP3^+ T_{regs} (Rubtsov et al., 2008). In this system, mice develop spontaneous colitis, immune mediated lung hypersensitivity and dermatitis, suggesting that IL-10 in T_{regs} has a non-redundant role in restricting inflammation at mucosal surfaces and the skin (Rubtsov et al., 2008). Consistent with this hypothesis are recent

observations that IL-10$^+$ T_{regs} control Th17 responses *in vitro* and reduce LP IL-17$^+$ T_{cons} in established colitis (Huber et al., 2011) and ablation of *Il10r* in murine T_{regs} impairs STAT3-dependent suppression of Th17-mediated colitis (Chaudhry et al., 2009).

In various murine models, mucosal inflammation can be abrogated by exogenous IL-10 (Powrie et al., 1994), forced IL-10 over-expression in otherwise pathogenic T_{cons} (Hagenbaugh et al., 1997), or mucosal delivery of IL-10 by genetically modified *Lactobacillus lactis* in mice and humans (Braat et al., 2006; Steidler et al., 2000). This indicates that the source of IL-10 may be less important than the presence of IL-10 in the mucosa. Taken together, these observations suggest that IL-10 plays a non-redundant role in restraining the mucosal immune system and that T_{regs} are likely to contribute to this function in mice and humans.

8.11.3 Mutations in *IL2*, *IL2RA* and *STAT5A/B* suggest a role for the IL-2 pathway in T_{reg}-mediated prevention of autoimmunity and mucosal inflammation

The low-affinity IL-2R is composed of a heterodimer of CD122 (IL-2Rβ) and CD132 (the γ_c chain). CD25 (IL-2Rα) is a non-signalling molecule that binds IL-2 and interacts with the heterodimeric IL-2R to form a trimeric receptor complex and increase the affinity of IL-2R for IL-2 by 10-100 fold (Boyman and Sprent, 2012). As discussed in Section 8.9.3 (page 90), both CD4$^+$CD25^{int} activated T_{cons} and CD4$^+$CD25^{hi} T_{regs} express CD25, meaning that it is difficult to delineate between activated T_{cons} and T_{regs} on the basis of CD25 expression alone.

In T_{cons}, IL-2-mediated signalling *via* STAT5 contributes to Th1 polarisation, *via* STAT5A and STAT5B binding to the *Tbx21* and *Il12rb* loci, augmenting T-bet and IL-12Rβ2 expression (Liao et al., 2011). IL-2 also has a role in Th2 polarisation. STAT5 binds to chromatin and stabilises the accessibility of the *Il4* gene (Cote-Sierra et al., 2004). STAT5 also binds to the *Il4ra* locus and is required for optimum IL-4Rα and IL-4 expression (Liao et al., 2008). IL-2 also restrains Th17 polarisation in T_{cons}, *via* STAT5 binding to the *Il17a* promoter and STAT5-mediated reduction in IL-6Rα and gp130 expression (Kryczek et al., 2007; Laurence et al., 2007; Liao et al., 2011).

The IL-2 pathway is also important in thymic development and peripheral maintenance of T_{regs}. Deletion of *Il2*, components of the trimeric IL-2R or its transcriptional target, *Stat5*, required for the maintenance of FOXP3 expression, result in substantially decreased T_{reg} numbers and multi-system autoimmunity (Burchill et al., 2003; Fontenot et al., 2005; Malek et al., 2002; Papiernik et al., 1998; Snow et al., 2003; Yao et al., 2007). Autoimmunity can be prevented in $Il2^{-/-}$ mice by administration of IL-2 or reconstitution, as a bone marrow chimera, with IL-2-sufficient cells (Almeida et al., 2002). Similarly, autoimmunity can be prevented in $Il2rb^{-/-}$ mice by adoptive transfer of WT $CD4^+CD25^+$ T_{regs} or by thymic expression of IL-2Rβ (Malek et al., 2002). Adoptive transfer of WT T_{regs} to $Stat5^{-/-}$ mice also rescues autoimmunity, while *Stat5* activation is sufficient to increase $CD4^+CD25^+$ T_{regs} in the absence of IL-2 (Antov et al., 2003). *CD25* and *STAT5B* mutations have been described in humans with multi-system autoimmunity and intestinal inflammation (Torgerson and Ochs, 2007), supporting the importance of the IL-2 axis in peripheral tolerance in humans.

Binding of IL-2 to the IL-2R causes the IL-2:IL-2R complex to be internalised within the T cell, followed by recirculation of CD25 to the cell surface (Boyman and Sprent, 2012). T_{reg}-mediated cytokine depravation-induced apoptosis of T_{cons} has been proposed as a mechanism of T_{reg}-mediated immune modulation (Pandiyan et al., 2007). T_{regs} can also inhibit IL-2 mRNA expression in T_{cons} *via* a cell contact-dependent mechanism (Thornton and Shevach, 1998). It has also been proposed that IL-2 uptake by the trimeric high-affinity IL-2R on T_{regs} can cause T_{regs} to act as "IL-2 sinks", leading to environmental depletion of IL-2 and cytokine depravation-mediated apoptosis of local T_{cons} (Pandiyan et al., 2007).

8.11.4 Mutations in *WAS* support a role for T_{regs} in preventing autoimmunity and mucosal inflammation

T_{reg} defects are also seen in Wiskott-Aldrich syndrome (WAS). This is an X-linked primary human immunodeficiency caused by mutations in Wiskott-Aldrich syndrome protein (WASP), a protein that links TCR ligation to the actin cytoskeleton. It is characterized by recurrent infections, thrombocytopenia, eczema, autoimmunity, in addition to the development of colitis in both mice and humans (Maillard et al., 2007). Defective expression of WASP results in decreased T_{reg} numbers, decreased IL-10 and TGF-β expression and decreased T_{reg} activation in response to TCR/CD28 ligation (Humblet-Baron et al., 2007; Maillard et al., 2007; Marangoni et al., 2007). WASP deficiency is also associated with impaired amelioration of $CD45RB^{hi}$ colitis in mice, and defective *in vitro* suppression of T_{cons} in both mice and humans. Interestingly, WASP deficiency in the innate immune system also impairs the ability of WT T_{regs} to suppress $CD45RB^{hi}$ colitis, although this can be overcome by an increased T_{reg} dose (Nguyen et al., 2012).

These observations support a role for T_{regs} in preventing inappropriate intestinal inflammation and autoimmunity in humans.

8.12 Additional proposed mechanisms of T_{reg}-mediated suppression

The preceding section discussed rare mutations affecting molecules and pathways that have been implicated in T_{reg} function and can lead to mucosal inflammation. This section discusses additional molecules that have been shown to be important for T_{reg} function in mice. As discussed below, the relative contribution of each pathway to suppression of mucosal inflammation often depends on the model studied.

8.12.1 TGF-β

TGF-β has distinct roles: as a T_{reg} effector cytokine, which is discussed below, and in inducing Th3 cells, which is discussed in Section 8.15.3 (page 117). The role of TGF-β in T_{reg}-Th17 plasticity is discussed in more detail in Chapter 11 (page 196).

TGF-β is expressed by almost every cell type. Mice with a global deletion of *Tgfb1* are well for an initial 2-3 week *post natal* period but then develop wasting and multisystem autoimmunity, including gastritis and colitis, to which they succumb within 1-2 weeks (Kulkarni et al., 1993; Shull et al., 1992). Depletion of $CD4^+$ or $CD8^+$ lymphocytes improves systemic inflammation in this model (Kobayashi et al., 1999; Letterio et al., 1996). This phenotype is recapitulated by specific deletion of TGF-βRII in $CD4^+$ cells ($Tgfbr2^{flox/flox}$ x $Cd4^{Cre}$; Marie et al., 2006). Blocking TGF-β responsiveness in $CD4^+$ cells by expression of a DN TGF-βRII under

the control of the *Cd4* promoter also leads to multisystem autoimmunity and colitis, although this begins at 3-4 months of age (Gorelik and Flavell, 2000). Expression of a DN TGF-βRII under the control of the human *CD2* promoter in $CD8^+$ lymphocytes results in peripheral lymphoproliferation but with little inflammation (Lucas et al., 2000). These observation suggest that TGF-β responsiveness in $CD4^+$ cells is required to prevent autoimmunity and colitis, although TGF-β responsiveness in other cell types also contributes to the prevention of autoimmunity.

TGF-β is probably not critical for thymic development of T_{regs}, as T_{reg} development is normal in *Tgfb1*$^{-/-}$ mice (Li et al., 2006; Marie et al., 2005) and the reduction in T_{reg} numbers seen in 3-5 day-old mice with a $CD4^+$ TGF-βRI conditional knock-out is quickly corrected by IL-2-mediated T_{reg} proliferation (Liu et al., 2008). However, TGF-β appears to be critical for survival and maintenance of T_{regs} in the periphery. Peripheral T_{reg} numbers are significantly reduced by 8-10 days of age in *Tgfb1*$^{-/-}$ mice, which might account for the timing of autoimmunity and colitis in these animals (Marie et al., 2005). WT T_{regs} also have a selective advantage over *Tgfbr2*$^{-/-}$ T_{regs} in maintaining peripheral T_{reg} numbers in a bone marrow chimera (Li et al., 2006).

TGF-β contributes to mucosal homeostasis in other experimental models. In the $CD45RB^{hi}$ adoptive transfer model, T_{reg}-mediated protection from colitis can be abolished by treatment with an anti-TGF-β antibody (Powrie et al., 1996). WT T_{regs} are unable to prevent colitis induced by adoptive transfer of $CD45RB^{hi}$ T cells expressing DN TGF-βRII, indicating that T_{cons} require TGF-β responsiveness for T_{reg}-mediated prevention of colitis in this model (Fahlén et al., 2005). Fahlén *et al.*

found that DO11.10 *Tgfb1*$^{-/-}$ T_{regs} suppressed adoptive transfer colitis induced by CD45RB^{hi} colitis to an equivalent degree as DO11.10 *Tgfb*$^{+/+}$ T_{regs} and that the protective effect of DO11.10 *Tgfb1*$^{-/-}$ T_{regs} was abolished by treatment with anti-TGF-β (Fahlén et al., 2005). This suggests that T_{reg}-derived TGF-β may not be required to suppress colitis. However, Li *et al.* performed co-transfer experiments with *Tgfb1*$^{flox/flox}$ *x Cd4*^{Cre} mice and found a conflicting result that *Tgfb1*$^{flox/flox}$ T_{regs} were unable to prevent WT CD45RB^{hi} Th1-mediated colitis, suggesting that T_{reg}-derived TGF-β contributed to prevention of colitis (Li et al., 2007a). Co-transfer of *Tgfb1*$^{flox/flox}$ T_{regs} and *Tgfb1*$^{flox/flox}$ CD45RB^{hi} T cells produced a more severe phenotype than co-transfer of *Tgfb1*$^{flox/flox}$ T_{regs} and WT CD45RB^{hi} T cells, suggesting that T_{con}-derived TGF-β modulated inflammation to a minor degree in this model (Li et al., 2007a). Consequently, the precise contribution of T_{reg}-derived TGF-β to mucosal homeostasis is unclear.

Subdivision of human T_{regs} on the basis of ICOS expression yields two functionally distinct subsets: ICOS^{+} T_{regs} that express surface-bound TGF-β and soluble IL-10, and ICOS^{-} T_{regs} that express surface-bound TGF-β alone (Ito et al., 2008). This finding highlight that individual T_{regs} may utilize multiple distinct mechanisms of suppression and this theme will be discussed further in subsequent sections.

8.12.2 CTLA-4

Cytotoxic T lymphocyte antigen (CTLA)-4 is constitutively expressed by both murine CD4^{+}CD25^{+} T_{regs} (Read et al., 2000; Takahashi et al., 2000) and human CD4^{+}CD25^{hi} T_{regs} (Jago et al., 2004; Miyara et al., 2009). *Ctla4* is a target for FOXP3

and FOXP3 amplifies and stabilises CTLA-4 expression (Gavin et al., 2007). Both global *Ctla4*$^{-/-}$ deletion (Khattri et al., 1999; Read et al., 2006; Tang et al., 2004) and specific *Ctla4*$^{-/-}$ deletion under the control of the *Foxp3* promoter in T_{regs} (*Ctla4*$^{flox/flox}$ x *Foxp3*^{Cre}) (Wing et al., 2008) are associated with normal thymic development, proliferation and survival of T_{regs}. However, T_{reg} accumulation in the colon is impaired in *Ctla4*$^{-/-}$ mice (Barnes et al., 2013) Anti-CTLA-4 antibody treatment induces multisystem autoimmunity in WT mice (Takahashi et al., 2000) and abrogates T_{reg}-mediated prevention of CD45RB^{hi} colitis (Read et al., 2000), but not accumulation of T_{regs} in the colon (Read et al., 2006). This effect is due to CTLA-4 blockade on T_{regs}, rather than T_{cons} (Read et al., 2006; Takahashi et al., 2000; Tang et al., 2004). Anti-CTLA-4 treatment also prevents adoptively transferred T_{regs} from modulating *Helicobacter hepaticus*-induced colitis in *RAG2*$^{-/-}$ mice, even though in this model FOXP3^{+} T_{regs} are detectable in the colon following adoptive transfer (Watanabe et al., 2008a). In addition, specific *Ctla4* deletion in T_{regs} results in lymphoproliferation, fatal multisystem autoimmunity and impaired *in vitro* suppression of T_{con} proliferation (Wing et al., 2008).

While these observations support a role for CTLA-4 in T_{reg}-mediated prevention of autoimmunity and mucosal inflammation, discordant results exist. For example, WT T_{regs} require CTLA-4 to suppress T_{con} proliferation *in* vitro, while T_{regs} from *Ctla4*$^{-/-}$ mice can suppress T_{con} proliferation *in vitro* (Tang et al., 2004). T_{regs} from *Ctla4*$^{-/-}$ mice over-express TGF-β and IL-10, in comparison with WT T_{regs}, and their *in vitro* suppressive ability is abrogated by anti-TGF-β (Tang et al., 2004). This suggests that *Ctla4*$^{-/-}$ T_{regs} can use other mechanisms to maintain T_{reg} function *in vitro* and *in vivo*. T_{regs} from *Ctla4*$^{-/-}$ mice can also prevent CD45RB^{hi} adoptive transfer colitis, but adoptive transfer of *Ctla4*$^{-/-}$ T_{regs} to *Ctla4*$^{-/-}$ mice results in

colitis, suggesting that CTLA-4-mediated modulation of T_{con} activation may also have a role in preventing intestinal inflammation (Read et al., 2006). Indeed, selective engagement of CTLA-4 on DCs by T_{cons} results in the induction of antigen-specific $CD25^{+}FOXP3^{+}$ iT_{regs} and $CD25^{-}TGF\text{-}\beta^{+}$ iT_{regs} both *in vitro* and *in vivo* (Li et al., 2007b). CTLA-4 is also required for TGF-β-mediated induction of $CD4^{+}CD25^{+}FOXP3^{+}$ T cells from $CD4^{+}CD25^{-}$ T cells *in vitro* (Zheng et al., 2006). These observations suggest that iT_{reg} generation is likely to be impaired in *Ctla4*$^{-/-}$ mice, providing an explanation for the requirement of CTLA-4 in the recipient to modulate $CD45RB^{hi}$ colitis.

CTLA-4-mediated down-regulation of CD80 and CD86 on APCs is a key mechanism of CTLA-4-mediated immune modulation by T_{regs}. CLTA-4-mediated prevention of $CD45RB^{hi}$ colitis by adoptively transferred T_{regs} or T_{reg}-mediated protection from diabetes in pre-diabetic non-obese diabetic (NOD) mice is dependent on interaction between CD28 and CD80 (B7) (Liu et al., 2001; Salomon et al., 2000). WT T_{regs} down-regulate CD80 and CD86 expression on DCs, while T_{regs} with T_{reg}-specific *Ctla4* deletion do not (Wing et al., 2008). Qureshi *et al.* showed that $CTLA\text{-}4^{+}$ cells capture CD80 and CD86 from the cell surface of target APCs, which is then degraded within $CTLA\text{-}4^{+}$ cells (Qureshi et al., 2011). This limits the ability of APCs to provide co-stimulation to T cells *via* CD28:B7 interaction.

8.12.3 CD39/CD73

CD39 and CD73 are expressed on T_{regs} in mice (Borsellino et al., 2007; Deaglio et al., 2007; Kobie et al., 2006) and activated T_{regs} in humans (Afzali et al.,

2013a; Borsellino et al., 2007; Deaglio et al., 2007; Dwyer et al., 2010). FOXP3 also amplifies and stabilises CD39 and CD73 expression (Gavin et al., 2007). CD39 is an ectonuclease triphosphase diphosphohydrolase and CD73 is a 5'-ectonucoeosidase, both of which sequentially hydrolyse extracellular adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and adenosine (Kaczmarek et al., 1996). T_{regs} have been shown to hydrolyse extracellular ADP to adenosine *in vitro* (Deaglio et al., 2007). Pericellular adenosine has an anti-inflammatory effect on T_{cons} via the A_{2A} adenosine receptor ($A_{2A}AR$) (Deaglio et al., 2007; Naganuma et al., 2006), inhibiting IL-2 and TNF expression by T_{cons} and IL-2-mediated T_{con} proliferation *in vitro* (Erdmann et al., 2005). ATL-146e, a selective $A_{2A}AR$ agonist, attenuates both acute and chronic/relapsing formalin-immune complex colitis in rabbits, CD-like chronic ileitis in SAMP1/YitFc mice, and ileitis in *Scid* mice adoptively transferred with $CD4^+$ lymphocytes obtained from SAMP1/YitFc mouse MLNs (Odashima et al., 2005). $A_{2A}AR$ sufficiency is required in both T_{cons} and T_{regs} for T_{reg}-mediated prevention of $CD45RB^{hi}$ adoptive transfer colitis (Naganuma et al., 2006). *Cd39*$^{-/-}$ T_{regs} have impaired *in vitro* suppression of WT T_{con} proliferation and impaired skin allograft survival, in comparison with WT T_{regs} (Deaglio et al., 2007). While both $CD39^+$ and $CD39^-$ T_{regs} suppress IFNγ expression by T_{cons} *in vitro*, only $CD39^+$ T_{regs} suppress T_{con} IL-17 expression (Fletcher et al., 2009). These observations suggest that an intact CD39/CD73-pericellular adenosine-$A_{2A}AR$ pathway is required for T_{reg}-mediated immune modulation in the gut in mice, and that this pathway has a role in T_{reg}-mediated mucosal immune homeostasis in humans.

The CD39/CD73-pericellular adenosine-$A_{2A}AR$ pathway has also been proposed as one mechanism by which methotrexate prevents joint inflammation

in rheumatoid arthritis. MTX increases extracellular adenosine and CD73 and A_{2A}AR are required for the action of methotrexate in reducing inflammation in experimental models of acute inflammation (Montesinos et al., 2003; 2007).

8.12.4 IL-35 and iTr35 cells

IL-35 is a heterodimeric cytokine comprised of IL-27β, encoded by the Epstein-Barr-virus-induced gene (*Ebi3*), and IL-12α, and is highly expressed by FOXP3$^+$ T_{regs} (Collison et al., 2007). *Ebi3* and *Il12a* mRNA and IL-35 protein expression are up-regulated in T_{regs} on co-culture with T_{cons} (Collison et al., 2007; 2009). *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ T_{regs} have a diminished ability to both suppress T_{con} proliferation *in vitro* and cure CD45RB^{hi} adoptive transfer colitis *in vivo* (Collison et al., 2007). Immune regulation by IL-35$^+$ T_{regs} is likely to be a two-step process. Initial *in vitro* suppression of T_{con} proliferation by IL-35$^+$ T_{regs} requires both cell-cell contact and T_{reg} activation, and is augmented by activated T_{cons}. Once activated, IL-35$^+$ T_{regs} can suppress T_{con} proliferation across a transwell membrane in an IL-35 and IL-10-dependent manner (Collison et al., 2009). This is likely to be due to IL-35-mediated peripheral induction of a population of anergic IL-35$^+$ regulatory cells (iTr35) from FOXP3$^-$ T_{cons}, which has been demonstrated *in vitro* in both humans and mice (Collison et al., 2010). iTr35 cells do not express FOXP3 and have a gene expression profile more similar to T_{cons} than to T_{regs}. Adoptive transfer of iTr35 cells prevents the *Scurfy* phenotype in *Foxp3*$^{-/-}$ *mice* and also prevents the development of EAE in susceptible mice. iTr35 cells can also be induced *in vivo* in the small intestine and colon by *Trichuris muris* infection, and in a tumour microenvironment (Collison et al., 2010). IL-35-mediated iTr35

induction is likely to be one of the mechanisms by which T_{regs} can induce infectious tolerance *in vivo*.

8.12.5 Granzyme A, granzyme B and perforin

Cell contact-dependent induction of apoptosis in target cells has also been proposed as a mechanism of action of T_{regs}. Both human and murine T_{regs} express granzyme A on activation and can induce perforin-dependent apoptosis in a range of target immune cells, including activated T_{cons}, $CD8^+$ lymphocytes, $CD14^+$ monocytes and both immature and mature DCs (Grossman et al., 2004). Killing in this system is independent of Fas-FasL interaction, but dependent on both perforin and interaction with CD18 (Grossman et al., 2004). T_{regs} can also induce contact-dependent apoptosis of target T_{cons} in a granzyme B-dependent, perforin-independent manner (Gondek et al., 2005). Granzyme B is also up-regulated in T_{regs} in the tumour microenvironment and T_{regs} isolated from experimental tumours can kill cytotoxic $CD8^+$ lymphocytes and NK cells in a granzyme B and perforin-dependent manner (Cao et al., 2007).

8.12.6 LAG-3

LAG-3 is a CD4 homolog that binds to MHC-II and is expressed by activated T_{regs}. LAG-3 is expressed by both tT_{regs} and iT_{regs}, and CD49b/LAG-3 co-expression was recently proposed as a marker forTr1 cells (Gagliani et al., 2013; Huang et al., 2004). *Lag3*$^{-/-}$ T_{regs} have impaired *in vitro* suppression of T_{con} proliferation and this is recapitulated by treatment of co-cultures with anti-LAG-3 antibodies in both murine and human cells (Huang et al., 2004). Anti-LAG-3 also impairs iT_{reg}-

mediated resolution of a pneumonitis model *in vivo*. A regulatory phenotype can also be conferred on T_{cons} by over-expression of LAG-3.

8.13 T_{regs} in experimental colitis

A number of mouse models of intestinal inflammation have already been discussed above, including DN N-cadherin TG mice, keratin-8 deficient mice, $NEMO^{IEC\text{-}KO}$ mice, STAT3 deficiency in myeloid cells, *Il10*$^{-/-}$ mice, *A20*$^{-/-}$ mice, *TNF*$^{\Delta ARE}$ mice, and the $CD45RB^{hi}$ adoptive transfer model of colitis. This section focuses on mouse models in which T_{regs} have been shown to have a significant role in preventing mucosal inflammation or are able to modulate inflammation following adoptive transfer.

8.13.1 $CD45RB^{hi}$ adoptive transfer colitis

Scid or *RAG1/2*$^{-/-}$ mice reconstituted with $CD4^+CD45RB^{hi}$ lymphocytes develop a Th1 or mixed Th1/Th17-mediated colitis with IFN-γ-mediated wasting (Denning et al., 2005; Morrissey et al., 1993; Powrie et al., 1993). This model can be recapitulated on an immunodeficient TGε26 background to yield effector $CD4^+$ cells that are responsive to caecal bacterial antigens (Veltkamp et al., 2006). Co-transfer of $CD4^+CD45RB^{lo}$ lymphocytes at a 1:1 ratio prevents the development of wasting and colitis. $CD4^+CD25^+$ T_{regs} within the $CD4^+CD45RB^{lo}$ fraction were shown to be responsible for protection from colitis in this model. As discussed above, T_{reg}-mediated protection from colitis can be abrogated by anti-CTLA-4, anti-IL-10, anti-IL-10R or anti-TGF-β mAbs. Adoptive transfer of $CD4^+CD25^+$ T_{regs} also cures established colitis (Mottet et al., 2003). While intestinal lymphocyte homing

is required for the initiation of colitis, intestinal T_{reg} homing is not required to prevent colitis in this model (Denning et al., 2005). Co-transferred β_7 integrin-deficient T_{regs} efficiently proliferate in spleen and lymph nodes and prevent accumulation of effector lymphocytes in the colonic mucosa, even though intestinal T_{reg} accumulation is impaired and regulatory activity cannot be recovered from the colons of mice co-transferred with $\beta_7^{-/-}$ T_{regs} (Denning et al., 2005).

8.13.2 The TRUC mouse model

The TRUC mouse (*Tbx21*$^{-/-}$ x *RAG2*$^{-/-}$ UC) is a model of microbially-driven colonic inflammation mediated by the innate immune system (Garrett et al., 2007; Powell et al., 2012). T-bet functions as a negative regulator of *Tnf* in myeloid cells. Early TRUC colitis is mediated by TNF overproduction in colonic DCs and is amenable to anti-TNF treatment (Garrett et al., 2009). T-bet is also a transcriptional repressor of *Il7ra.* Consequently, *Tbx21*$^{-/-}$ may affect the homeostasis of IL-7Rα-expressing innate lymphoid cells (ILCs), in addition to promoting IL-17A expression in ILCs, in the absence of efficient IFN-γ production. ILCs drive inflammation in chronic TRUC colitis by overexpressing IL-17A and IL-22 in response to TNF and IL-23, and chronic TRUC colitis is responsive to anti-IL-17A and ILC-depleting anti-CD90 mAbs (Powell et al., 2012). *Helicobacter typhlonius* is a causative organism in TRUC colitis. Adoptively transferred $CD4^+CD25^+$ T_{regs} co-localize with mucosal DCs and can cure colitis in this model. Even though the TRUC mouse model more closely resembles UC than CD, this model shows that T_{regs} can modulate TNF production by innate immune cells *in vivo*.

8.13.3 SAMP1/YitFc mice

The SAMP1/Yit strain was developed at the Yakult Central Institute for Microbiological Research, Tokyo, by mating and back-crossing brother-sister pairs of SAMP1 mice that developed skin ulceration, which correlated with the development of spontaneous ileitis (Matsumoto et al., 1998). The SAMP1/YitFc strain was developed at the University of Virginia by mating two mice from the original SAMP1/Yit colony and back-crossing the offspring (Rivera Nieves et al., 2003). Both the SAMP1/Yit and SAMP1/YitFC strains develop commensal bacteria-dependent spontaneous transmural ileitis, associated with T cell, neutrophil and macrophage infiltration. In addition to ileitis, a proportion of SAMP1/YitFc mice also develop fistulating perianal disease. The spontaneous disease seen in these strains is a close phenocopy of human CD.

The SAMP1/YitFc phenotype has been linked with a strain-specific absence of PPAR-γ expression in mucosal crypts, suggesting that this phenotype is mediated by metabolic stress in crypt epithelium (Sugawara et al., 2005). Increased epithelial permeability and IFN-γ expression in ileal mucosa precedes the onset of ileitis (Rivera Nieves et al., 2003). SAMP1/YitFc mouse bone marrow-derived macrophages also have decreased expression of IL-6, IL-10 and TNF in response to MDP stimulation, suggesting dysregulated NOD signalling (Corridoni et al., 2013). A slight female preponderance is seen in CD. Ileitis in female SAMP1/YitFc mice occurs earlier and is more severe than ileitis in male littermates. This sex difference can be explained by differing responses to oestrogen between male and female mice (Goodman et al., 2014). Treatment of male mice with oestrogen results in T_{reg} proliferation and attenuation of ileitis.

However, female mice are resistant to oestrogen-mediated immune modulation. Depletion of T_{regs} with an anti-CD25 antibody also worsens ileitis, showing that T_{regs} restrain mucosal inflammation in this model (Ishikawa et al., 2013). Adoptive transfer of SAMP1/YitFc MLN cells to a *Scid* mouse causes colitis in the recipient, which is worsened by T_{reg} depletion with anti-CD25 antibodies. A second adoptive transfer of T_{regs} from control AKR mice ameliorated colitis, while adoptive transfer of SAMP1/YitFc T_{regs} did not, (Ishikawa et al., 2013). These observations indicate that T_{regs} have a role in modulating ileal inflammation in SAMP1/YitFc mice may have defective T_{reg}–mediated suppression of inflammation *in* vivo. Were these results to hold true across species, this suggests that freshly isolated, autologous T_{regs} may not be an effective therapy for CD in humans and that T_{regs} might need to be manipulated *in vitro* in order to augment their ability to regulate inflammation following adoptive transfer.

8.13.4 Additional models in which T_{regs} show a beneficial effect on mucosal inflammation

In a murine model of infectious colitis that more closely resembles UC than CD, *RAG2*$^{-/-}$ mice infected with the murine pathogen *Helicobacter hepaticus* develop colonic inflammation, which is worsened by adoptive transfer of $CD4^{+}CD45RB^{hi}$ lymphocytes at the time of infection (Maloy et al., 2003; Watanabe et al., 2008a). *H. hepaticus*-mediated intestinal pathology can be prevented by T_{reg} transfer prior to infection. This protective effect is abrogated by blockade of IL-10R, TGF-β or CTLA-4. Interestingly, adoptively transferred T_{regs} are also able to suppress colitis induced by 2.25% DSS ingestion in $RAG2^{-/-}$ mice, although this protective effect is

lost at higher doses (Huber et al., 2004). These observations suggest that T_{regs} are effective at modulating intestinal inflammation in selected pre-clinical models.

8.14 T_{regs} in the blood and mucosa of patients with active Crohn's disease

$CD4^+CD25^{hi}CD127^{lo}$ T_{regs} comprise 5-10% of human PB $CD4^+$ lymphocytes and 10-15% of LP $CD4^+$ lymphocytes in health. Active Crohn's disease is associated with a decrease in the proportion of PB T_{regs} and an increase in the proportion of T_{regs} in the LP and MLN, compared to healthy controls (Makita et al., 2004; Maul et al., 2005; Reikvam et al., 2011; Saruta et al., 2007). In resections obtained from patients with active CD, $FOXP3^+$ T cells in accumulate in the LP at areas of T cell infiltration and non-caseating granulomas, and at T cell-rich areas in associated MLNs (Saruta et al., 2007). FOXP3 is also more highly expressed by polymerase chain reaction (PCR) (Maul et al., 2005) and immunofluorescence (IF) (Reikvam et al., 2011) in inflamed Crohn's mucosa, in comparison with healthy controls. These data are consistent with a hypothesis that T_{regs} migrate to inflamed mucosa or are induced *de no*vo in the setting of inflammation. However, interpretation of these data is difficult, as activated mucosal T_{cons} may also express the T_{reg} markers interrogated in these studies: FOXP3, CD25 and CTLA-4.

T_{regs} obtained from inflamed Crohn's MLNs suppress proliferation of autologous PB T_{cons} (Saruta et al., 2007). Similarly, T_{regs} obtained from inflamed Crohn's LP suppress proliferation of autologous PB T_{cons} (Kelsen et al., 2005; Makita et al., 2004; Saruta et al., 2007), and suppress T_{con} production of IFN-γ and TNF in co-culture supernatants (Kelsen et al., 2005). However, LP T_{regs} do not suppress proliferation of autologous T_{cons} obtained from inflamed Crohn's mucosa

(Fantini et al., 2009). This impaired response to T_{reg}-mediated suppression of proliferation is caused by overexpression of Smad7, an inhibitor of TGF-β signalling at the TGF-β receptor, in T_{cons} obtained from inflamed Crohn's mucosa (Fantini et al., 2009). Responsiveness to T_{reg}-mediated suppression can be regained by treatment with an anti-sense oligonucleotide to Smad7 (Fantini et al., 2009).

Activated T_{cons} express an effector-memory phenotype. PB T_{regs} suppress proliferation and IL-2 production less well from antigen-experienced $CD4^+CD45RA^-$ T_{cons}, compared with naïve $CD4^+CD45RA^+$ T_{cons} (Afzali et al., 2011).

These observations suggest that enhancement of T_{reg} function may be required to overcome T_{con} resistance to T_{reg}-mediated suppression seen in active Crohn's mucosa.

8.15 Other cell types with regulatory potential

As discussed above, multiple immune cell types can modulate immune activation in the mucosa, including tT_{regs}, ATRA/TGF-β-induced iT_{regs} , IL-35^+ T_{regs} and iTr35 cells, TCR$\gamma\delta^+$ and CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ IELs, $CD103^+$ DCs, and tissue-resident macrophages. T_{cons} can acquire regulatory activity (± FOXP3 expression) in the periphery on stimulation with IL-10 (Tr1 cells) or TGF-β in the absence of ATRA (iT_{regs} and Th3 cells). $CD8^+CD28^-$ T cells, NK cells, natural killer T (NKT) cells, and regulatory B cells have all been shown to have immune regulating properties *in vitro* or *in vivo*. The following section summarises non-T_{reg} immune modulating cells and illustrates inter-related themes in both generation and effector function between immune modulating cell types.

8.15.1 Regulatory $CD4^+$ cells induced by IL-10 in the periphery

Tr1 cells are $FOPX3^-$ T cells that are peripherally induced to express IL-10. Tr1 cells can be induced from naïve $CD4^+$ T cells by repeated antigenic stimulation in the presence of IL-10 (Groux et al., 1997) or IL-10 with IFN-α (Levings et al., 2001b); by immature DCs *in vitro* or *in vivo* (Dhodapkar et al., 2001; Jonuleit et al., 2000) or $IL\text{-}10^+$ tolerogenic DCs (Gregori et al., 2010), or by stimulation of the complement receptor CD46 (Cardone et al., 2010; Kemper et al., 2003). As T cell stimulation with CD46 sequentially induces a Th1, then Tr1 phenotype, it is possible that this subset of Tr1 cells may represent quiescent or "tired" former effector T cells (Cardone et al., 2010). In addition, Maynard *et al.* used a IL-10/FOXP3 dual reporter mouse system to determine that $IL\text{-}10^+$ regulatory cells can develop *in vivo* from both $FOXP3^+$ and $FOXP3^-$ precursors, and that this development is dependent on TGF-β but not IL-10 (Maynard et al., 2007). In this model, $IL\text{-}10^+FOXP3^+$ T_{regs} were enriched in the colon, while $IL\text{-}10^+FOXP3^-$ Tr1s were enriched in the small bowel. The mucosal environment probably influences peripheral induction of Tr1s from naïve $CD4^+$ T cells, as ATRA produced by mucosal DCs suppresses TGF-β-mediated induction of Tr1s, while DC activation *via* TLR9 induces Tr1s (Maynard et al., 2009). Lentiviral transduction of *IL10* induces a Tr1 phenotype in human $CD4^+$ lymphocytes (Andolfi et al., 2012). Until recently, characterisation of Tr1 cells was limited by a lack of specific markers. However, co-expression of CD49b and LAG-3 has recently been proposed as a Tr1-specific marker in both mice and humans (Gagliani et al., 2013).

Tr1 cells are anergic and proliferate poorly in response to antigenic stimulation (Groux et al., 1997). As with T_{regs}, Tr1s are likely to suppress immune

responses *via* multiple mechanisms, including IL-10 and TGF-β (Akdis et al., 2004; Huber et al., 2011), CTLA-4 and PD-1 (Meiler et al., 2008), metabolic disruption via CD39 and CD73 (Mandapathil et al., 2010) and granzyme B and perforin-dependent lysis of target cells (Magnani et al., 2011). Tr1 cells can modulate IL-17-mediated colitis in mice (Huber et al., 2011).

Human Tr1 clones can be generated and expanded *in vitro* (Brun et al., 2009). Desreumaux *et al.* recently conducted a Phase Ib/IIa clinical trial of OVA-specific Tr1 clones in 20 patients with CD, administering 10^6-10^9 cells per patient with an acceptable safety profile and 8/20 (40%) clinical response by week 8 (Desreumaux et al., 2012).

8.15.2 Regulatory $CD4^+$ cells induced by TGF-β^+ (without ATRA) in the periphery

FOXP3 expression and regulatory phenotype can be induced in murine $CD4^+CD25^-$ T_{cons} by TGF-β alone, or a combination of TGF-β, IL-2, and sub-immunogenic or mucosal antigen exposure and co-stimulation from immature DCs (Apostolou and Boehmer, 2004; Chen et al., 2003; Coombes et al., 2007; Kretschmer et al., 2005; Oliveira et al., 2011; Park et al., 2004; Selvaraj and Geiger, 2007), or by adoptive transfer of naïve T_{cons} to a lymphopaenic host (Curotto de Lafaille et al., 2004; Sun et al., 2007).

FOXP3 induction in peripherally induced $FOXP3^+$ T_{regs} depends on sub-maximal TCR signalling. This is illustrated by reduction in iT_{reg} generation associated with increased antigen dose (Kretschmer et al., 2005), increased stimulation *via* CD3 or CD28 (Benson et al., 2007) and increased TCR signalling

conferred by deletion of *Cblb*, an inhibitory E3 ubiquitin ligase (Wohlfert et al., 2006). Indeed, either antigen-specific or anti-CD3-mediated sub-maximal TCR signalling in the absence of TGF-β is sufficient to induce iT_{regs} *in vitro* (Oliveira et al., 2011). Premature termination of TCR signalling by inhibition of PI3K, Akt or mTOR, or mutations that reduce mTOR signalling following TCR ligation results in increased iT_{reg} induction (Sauer et al., 2008; Strainic et al., 2013).

TGF-β acts *via* Smad3, NFAT and TIEG1 to induce FOXP3 expression (Tone et al., 2007; Venuprasad et al., 2008) and TGF-β is sufficient to induce FOXP3 expression in murine T_{cons} (Benson et al., 2007). Withdrawal of TGF-β following *in vitro* iT_{reg} generation results in loss of FOXP3 expression within approximately 4 days (Selvaraj and Geiger, 2007). FOXP3 expression is required for the suppressive effect of iT_{regs}. However, the gene expression profile induced by TGF-β in iT_{regs} is distinct from the FOXP3-mediated gene expression signature seen in tT_{regs}. This TGF-β -induced iT_{reg} gene expression signature is independent of FOXP3, as it can be induced by TGF-β treatment in *Foxp3*$^{-/-}$ *Scurfy* T_{cons} (Hill et al., 2007). It is likely that the requirements for iT_{reg} generation in humans differ from those in mice, as TGF-β is not sufficient to induce stable FOXP3 expression or a suppressive phenotype *in vitro* in human $CD4^+$ T_{cons} (Tran et al., 2007). However, other groups suggest that suppressive iT_{regs} can be generated *in vitro* from human PB $CD4^+CD25^-$ cells by 24 hour stimulation with anti-CD3/anti-CD28 antibodies followed by a 9-day culture without further TCR ligation (Walker et al., 2005; 2003); from $CD4^+CD25^-CD45RA^+$ cells by TCR ligation with anti-CD3/anti-CD28-coated beads, IL-2 ± rapamycin (Hippen et al., 2011a); or from $CD4^+CD31^+$ RTEs with anti-CD3 antibody and TGF-β (Paiva et al., 2013).

The ontogeny of iT_{regs} is controversial in mice and humans. Zelany *et al.* noted intra-thymic differentiation of antigen-specific iT_{regs} following footpad injection with myelin basic protein (MBP) peptide and adjuvant in MBP-TCR transgenic *RAG1*$^{-/-}$ mice: a mouse strain that does not constitutively express $CD4^+CD25^+FOXP3^+$ T_{regs} (Zelenay et al., 2010). Paiva *et al.* showed that $CD4^+CD8^-FOXP3^-$ thymocytes and $CD4^+CD25^-$ RTEs are sensitive to TCR ligation/TGF-β-induced iT_{reg} generation *in vitro* and iT_{reg} generation on adoptive transfer to a lymphopaenic host, while $CD4^+CD31^+$ RTEs are more susceptible to *in vitro* iT_{reg} generation than $CD4^+CD31^-$ T_{cons}. (Paiva et al., 2013). Consequently, it is possible that the ability to convert to an iT_{reg} is restricted to thymocytes and RTEs, and progressively lost with maturation in the periphery.

iT_{regs} share multiple mechanisms to modulate immune responses with tT_{regs}, including contact-dependent suppression *via* CTLA-4 and contact-independent suppression *via* IL-10 (Park et al., 2004). iT_{regs} are as effective as tT_{regs} in suppressing mucosal inflammation in experimental models (Curotto de Lafaille et al., 2008; Haribhai et al., 2009; Kretschmer et al., 2005). It was recently proposed that tT_{regs} and iT_{regs} could be distinguished by expression of Neuropilin-1 (encoded by the *Nrp1* gene) (Weiss et al., 2012; Yadav et al., 2012) or Helios (Thornton et al., 2010). However, both Neuropilin-1 and Helios are induced by cell activation in humans (Himmel et al., 2013; Milpied et al., 2009), meaning that it is not currently possible to determine if the increase in LP T_{regs} seen in active CD is due to migration of tT_{regs} to inflamed mucosa or *de novo* generation of iT_{regs} in the mucosa. In mice, *Nrp1*$^{+/+}$ and *Nrp1*$^{-/-}$ $CD4^+CD25^+$ T_{regs} have similar TCR specificities and home to the same anatomical niches when adoptively transferred with naïve T_{cons} to a *Scid* mouse (Huang et al., 2014). When co-transferred to a lymphopaenic

animal, *Nrp1*$^{+/+}$ T_{regs} out-compete *Nrp1*$^{-/-}$ T_{regs}, suggesting that tT_{regs} are "fitter" than iT_{regs} *in vivo* and, consequently, may play a greater role in maintaining peripheral tolerance (Huang et al., 2014). However, abolition of thymic T_{reg} generation by the *king* mutation to the *Carma1* gene results in a near-complete depletion of T_{regs} in the thymus, spleen, peripheral LN and mesenteric LN, with a relative preservation of T_{regs} in the colonic LP (22.5% of LP $CD4^+$ cells in C57BL/6 vs. 8.4% in *Carma1*$^{k/k}$; Barnes et al., 2009). Small intestinal T_{regs} were not examined in this study. Both TGF-β/IL-2-mediated *in vitro* generation of iT_{regs} and *in vivo* proliferation of iT_{regs} in response to mouse cytomegalovirus infection were preserved in *Carma1*$^{k/k}$ mice (Barnes et al., 2009). This suggests that iT_{regs} are preferentially induced in the intestinal LP *in vivo* and that iT_{reg} induction is independent of thymic tT_{regs}

8.15.3 T helper 3 cells

T helper 3 (Th3) cells were originally described as IL-4^+ IL-10^+ TGF-β^+ $CD4^+$ clones that were induced in MLNs following oral challenge with MBP (Chen et al., 1994). Th3 generation was facilitated by "low-dose" (1mg x 5) but not "high-dose" (25mg x 1) oral MBP (Chen et al., 1996). MBP antigen-specific Th3s prevented both MBP-driven and alloantigen proteolipid protein-induced EAE (Chen et al., 1994; 1996; Inobe et al., 1998). Cross-linking of CTLA-4 induced a TGF-β^+ regulatory phenotype in $CD4^+$ T_{cons} and inhibited their expression of IL-4 and IFN-γ (Chen et al., 1998; Li et al., 2007b). Th3 induction was enhanced by culture with TGF-β, IL-4, IL-10 and anti-IL-12; and inhibited by IFN-γ (Inobe et al., 1998; Seder et al., 1998). Both *in vitro* suppression of T_{con} proliferation and *in vivo* protection

from EAE were abolished by anti-TGF-β treatment (Chen et al., 1998; 1994). Based on these observations, it is possible that Th3 cells contribute to oral tolerance. However, it is not possible to distinguish between Th3 cells and iT_{regs}, which limits their further characterisation.

8.15.4 iTr35 cells

Induction of iTr35 cells from $FOXP3^-$ T_{cons} and their role *in vitro* and *in vivo* is discussed above in Section 8.12.4 (page 105).

8.15.5 Other immune regulating cell types

$CD8^+CD28^-$ T cells are antigen-specific $IL\text{-}10^+$ cells that can be induced by CD40L-activated plasmacytoid DCs or antigen-specific culture *in vitro*. $CD8^+CD28^-$ T cells suppress T cell proliferation and APC activation by expressing IL-10 and inhibiting co-stimulation by down-regulating CD40 expression on T cells, and CD80/CD86 expression on DCs, including inhibition of CD86 ligation-induced NF-κB transcription (Gilliet and Liu, 2002; Jiang et al., 1998; Li et al., 1999).

In addition to providing cell-mediated immunity against virally-infected or malignant cells, NK cells can down-regulate immune responses through multiple mechanisms, including killing mature DCs and inducing cell cycle arrest in proliferating T cells (Chiesa et al., 2003; Trivedi et al., 2005; Zhang et al., 2006). NKT-mediated up-regulation of PD-1 in lymphocytes induces IL-10 expression in T_{regs} and reduces IFN-γ expression in T_{cons} (Hongo et al., 2012).

$CD19^+CD24^{hi}CD38^{hi}$ regulatory B cells (B_{regs}) are induced by CD40 stimulation and inhibit T cell proliferation *via* IL-10 and a CD80/CD86-dependent

mechanism (Blair et al., 2010; Lemoine et al., 2011). B_{regs} also induce FOXP3 expression in co-cultured T cells (Lemoine et al., 2011). Renal transplant tolerance, seen in a cohort with stable creatinine and no immunosuppression, is associated with a PB B cell-related gene expression signature, increased PB naïve and transitional B cells (the B cell population in which B_{regs} are enriched) and increased *CD20* mRNA in urine sediment cells, suggesting that B_{regs} may also play a role in restraining an allogeneic immune response in renal transplantation (Newell et al., 2010).

8.16 T_{regs} as a cellular therapy for CD

Data from pre-clinical models of intestinal inflammation and rare immunodeficiency syndromes in humans suggest that T_{regs} play a role in modulating the mucosal immune response. Adoptively transferred *in vitro* expanded human T_{regs} prevent the rejection of human skin transplants following adoptive transfer of allogeneic PBMCs (Issa et al., 2010; Sagoo et al., 2011) and prevent arteriosclerosis in xeno-transplanted internal mammary artery grafts (Nadig et al., 2010). These cells also prevent GvHD in humanized mouse models (Ermann et al., 2005; Scottà et al., 2013). In these models, in addition to solid organ transplantation and HSCT in humans, uncontrolled allo-reactive immunity is modulated by adoptively transferred T_{regs}. However, potentially large cell numbers may be required for T_{reg}-mediated immune modulation. For example, T_{reg} doses at a 1:1 ratio with adoptively transferred effector cells are required to suppress GvHD in murine models (Hoffmann et al., 2002). PB T_{regs} are approximately 5% of PB $CD4^+$ lymphocytes, or 1% of all PBMCs. Consequently, multiple groups have focussed on developing techniques to expand T_{regs} either *in*

vivo following infusion, or *in vitro* for subsequent infusion in order to obtain infused T_{reg}:PBMC ratios close to 1:1.

8.16.1 Therapeutic use of unexpanded, freshly isolated or stored T_{regs} in humans

One approach is to infuse fresh or stored T_{regs} to a lymphopaenic recipient and rely on subsequent homeostatic expansion *in vivo*. Di Ianni *et al.* administered 2-4 x 10^6/kg unexpanded, third party haploidentical $CD4^+CD25^{hi}$ T_{regs} to 28 subjects, 4 days prior to HSCT with $CD34^+$ stem cells and T_{cons} (Di Ianni et al., 2011). T_{reg} infusion was associated with prevention of GvHD in 26/28 recipients. T_{reg} infusion did not impair post-transplant immune recovery, NK cell reconstitution or response to influenza vaccination. One year following HSCT, disease-free survival was 46%. Consequently, infusion of freshly isolated or stored T_{regs} are a promising approach for prevention of GvHD following HSCT.

In contrast to patients undergoing HSCT, CD patients are not lymphopaenic. Indeed, conditioning therapy for the induction of lymphopaenia is highly risky in CD. A recent clinical trial of autologous HSCT in CD was terminated early due to adverse events (ASTIC study, ClinicalTrials.gov identifier: NCT00297193; Hawkey and Trialists, 2014). Most other categories of patients are not lymphopaenic and consequently will require *in vitro* T_{reg} expansion in order to obtain the T_{reg} numbers that are proposed to be required to induce a biological effect.

8.16.2 Approaches to *in vitro* expansion of T_{regs} and therapeutic use of *in vitro* expanded T_{regs} in humans

Many groups have shown that human T_{regs} can be expanded *in vitro* while maintaining stable *FOXP3* expression and a suppressive phenotype *in vitro* (Battaglia et al., 2006; Brunstein et al., 2011; Golovina et al., 2011; Hippen et al., 2011b; Hoffmann et al., 2006; 2004; Marek-Trzonkowska et al., 2012; Putnam et al., 2009; Scottà et al., 2013; Strauss et al., 2009; 2007b; Tresoldi et al., 2011; Trzonkowski et al., 2009) A protocol for Good Manufacturing Practice (GMP)-grade *in vitro* T_{reg} expansion that will be applied in a sub-study of the forthcoming "ONE study", a clinical trial to determine if antigen-specific *in vitro* expanded T_{regs} reduce the requirement for immunosuppression following renal transplantation, was recently published (Putnam et al., 2013).

Published techniques for *in vitro* T_{reg} expansion utilise polyclonal stimulation and "high dose" recombinant human (rh) IL-2 (300-1000 IU/ml replenished every 48-96 hours). T_{regs} that are repetitively stimulated in this milieu lose FOXP3 expression and their *in vitro* suppressive ability unless cultured with supplemental rapamycin (Battaglia et al., 2006; Golovina et al., 2011; Hoffmann et al., 2004; Putnam et al., 2009; Scottà et al., 2013; Strauss et al., 2007b; Tresoldi et al., 2011). Pim 2 is a serine/threonine kinase that is induced by FOXP3 and is constitutively expressed in T_{regs} but not T_{cons}. Rapamycin prevents out-growth of contaminating T_{cons} in T_{reg} cultures, while pim 2 expression confers resistance to rapamycin-mediated inhibition of T_{reg} proliferation *in vitro* (Basu et al., 2008). Rapamycin also limits potential for Th17 plasticity in *in vitro* expanded T_{regs} (Scottà et al., 2013; Tresoldi et al., 2011). However, in the three reported clinical

trials, T_{regs} were expanded *in vitro* in the absence of rapamycin, although the immunosuppressive regimen used in Brunstein *et al.* included rapamycin (Brunstein et al., 2011; Marek-Trzonkowska et al., 2012; Trzonkowski et al., 2009). T_{regs} were safely infused in each of these studies. Rapamycin is also not used in the planned T_{reg} expansion protocol for the ONE study (Putnam et al., 2013).

There are a number of theoretical advantages to expanding T_{regs} from CD patients in the presence of rapamycin. Firstly, human PB and lymphoid tissue contains a population of FOXP3$^+$ T_{regs} that express IL-17 on activation (Koenen et al., 2008; Voo et al., 2009). IL-17$^+$ T_{regs} can also be identified in inflamed Crohn's mucosa (Hovhannisyan et al., 2011). IFN-γ can induce Th1 plasticity in tT_{regs}, with T-bet and IFN-γ expression (Koch et al., 2009; Oldenhove et al., 2009). *In vitro* expanded T_{regs} cultured without rapamycin retain the potential to express IFN-γ and IL-17 (Battaglia et al., 2006; Scottà et al., 2013; Strauss et al., 2007b; Tresoldi et al., 2011). Both Th1 and Th17 cytokines have been implicated in the pathogenesis of CD, but the contribution of T_{reg}-derived effector cytokines to disease pathogenesis is unclear. Nevertheless, adoptive transfer of *in vitro* expanded T_{regs} with the potential to express effector cytokines and contribute to disease pathogenesis may be of concern in CD. Secondly, LP T_{cons} obtained from inflamed CD mucosa are resistant to T_{reg}-mediated suppression (Fantini et al., 2009). LP T_{cons} from inflamed CD mucosa over-express Smad7, an inhibitor of TGF-β signalling, which confers a cell-intrinsic resistance to TGF-β-mediated suppression by autologous T_{regs}. This defect is restricted to LP T_{cons}. LP T_{regs} suppress proliferation of autologous PB T_{regs} normally. T_{regs} from patients with autoimmune diseases or end-stage renal failure that are expanded *in vitro* with

supplemental rapamycin are more potently suppressive than freshly isolated T_{regs} (Afzali et al., 2013a; Cao et al., 2010). This may allow *in vitro* expanded T_{regs} to overcome T_{con} resistance to T_{reg}-mediated suppression seen in inflamed Crohn's mucosa (Fantini et al., 2009). Consequently, rapamycin may be useful in the generation of T_{regs} from CD patients.

The optimum starting population from which to isolate T_{regs} for subsequent expansion from CD patients is currently unknown. There are a number of theoretical advantages to expanding PB T_{regs} from the naïve $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{reg} subset. Firstly, initial enrichment on the basis of $CD45RA^+$ excludes the population of non-regulatory $CD25^{hi(++)}CD45RA^-FOXP3^{lo}$ T cells with an effector phenotype that lie within the $CD4^+CD25^{hi}CD127^{lo}$ gate (Miyara et al., 2009). Naïve $CD45RA^+$ T_{regs} can be highly expanded to yield a homogenous population of suppressive $FOXP3^+$ cells (Hoffmann et al., 2004). Secondly, *in vitro* expanded $CD4^+CD25^{hi}CD45RA^+$ cells from healthy donors retain CD62L and CCR7 expression following expansion, which is associated with therapeutic efficacy in pre-clinical models (Ermann et al., 2005; Hoffmann et al., 2004; Issa et al., 2010). Thirdly, freshly isolated $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} (Miyara et al., 2009) and T_{regs} expanded *in vitro* from healthy donor $CD45RA^+$ T_{regs} (Hoffmann et al., 2004) have reduced or absent expression of pro-inflammatory cytokines, in contrast to other freshly isolated T_{reg} populations, or T_{regs} expanded *in vitro* from $CD45RA^-$ precursors. This suggests that this strategy will minimise the potential for T_{reg} plasticity following expansion. Consistent with this finding are the observations that both freshly isolated $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} and T_{regs} expanded from $CD45RA^+$ precursors have an epigenetically stable *FOXP3* locus with extensive TSDR demethylation, which confers stable FOXP3 expression

(Baron et al., 2007; Hoffmann et al., 2009; Miyara et al., 2009). T_{regs} expanded *in vitro* from $CD45RA^+$ precursors retain stable FOXP3 expression even in the absence of supplemental rapamycin (Hoffmann et al., 2009). Consequently, naïve $CD45RA^+$ T_{regs} may be an appropriate population from which to expand T_{regs} in CD.

The requirements for *in vitro* expanded T_{reg} homing for a cure of CD in humans is unknown. Adoptively transferred $\beta_7^{-/-}$ T_{regs} prevent $CD45RB^{hi}$ colitis despite being unable to traffic to the LP (Denning et al., 2005). T_{reg} culture with rapamycin promotes expression of CD62L and CCR7 (Strauss et al., 2009), which are associated with homing to lymphoid tissue and T_{reg}-mediated transplant survival and prevention of GvHD (Ermann et al., 2005; Issa et al., 2010). $\alpha_4\beta_7$ integrin expression can be induced by supplementation of T_{reg} cultures with ATRA, at a cost of reduction in suppressive ability and increased IL-17 expression (Golovina et al., 2011; Scottà et al., 2013).

In support of this approach, there are three reports of *in vitro* expanded T_{reg} products being used safely in humans. The technical aspects of T_{reg} isolation, expansion and dosing in these studies are summarised in Table 8.5 (page 126).

Trzonkowski *et al.* treated one patient with chronic GvHD and one patient with acute GvHD with T_{regs} expanded from allogeneic $CD4^+CD25^{hi}CD127^{lo}$ precursors (Trzonkowski et al., 2009). The chronic GvHD patient experienced a significant improvement. PB T_{regs}, as a proportion of $CD4^+$ cells, doubled from 2.5% to 5% six months later. T_{reg} infusion was associated with an improvement in bronchiolitis obliterans, cessation of mycophenolate mofetil and a reduction in prednisolone from 60mg per day to 5mg per day. The acute GvHD patient showed

a temporary improvement in symptoms but subsequently deteriorated and died. No adverse events were noted.

Brunstein *et al.* treated 23 patients with T_{regs} expanded *in vitro* from umbilical cord blood (UCB) after double UCB transplantation (Brunstein et al., 2011). 74% received the target dose. Infusion-related toxicities included hypertension (n=2) and neurologic changes that were attributed to pre-existing medications (n=2). Adoptively transferred T_{regs} were detectable in PB for up to 14 days following infusion. Engraftment, disease-free survival and opportunistic infections were comparable to historic controls, while the incidence of grade II-IV GvHD in UCB T_{reg} recipients was 41%, compared with 61% for historical controls (p=0.05).

Marek-Trzonkowska *et al.* treated 10 patients with type 1 diabetes mellitus (T1DM) diagnosed in the preceding 2 months with T_{regs} expanded *in vitro* from autologous $CD4^{+}CD25^{hi}CD127^{lo}$ T_{regs} (Marek-Trzonkowska et al., 2012). One patient was diagnosed with influenza the day after infusion. No other infusion-related toxicities were reported. Signs of potential efficacy were noted. 4-5 months after T_{reg} infusion, plasma C peptide levels were significantly higher in recipients, compared with an untreated control group, indicating preservation of insulin secretion. 8/10 recipients were in clinical remission at 4-5 months, compared with 4/10 in the control group.

The lack infusion-related toxicities and absence of T_{reg}-mediated excess immunosuppression (e.g. excess infections) seen in these studies is promising safety data for *in vitro* expanded T_{regs} as a therapeutic agent, and supports the further development of *in vitro* expanded T_{regs} as a cell-based therapy. The current

ClinicalTrials.gov listings for planned or on-going studies utilising *in vitro* expanded T_{regs} is summarised in Table 8.6 (page 127).

Table 8.5: Published clinical trials of *in vitro* expanded T_{regs}.

Study	Context	Enrichment Protocol	Expansion Protocol	Dose
Trzonkowski *et al.* (2009)	Treatment of acute and chronic GvHD n=2	T_{regs} from allogeneic buffy coat. $CD4^+$ negative bead selection, followed by FACS-based sorting of $CD4^+CD25^{hi}CD127^{lo}$ cells.	RPMI 1640 with 10% autologous plasma. IL-2 (1000 IU/ml) Anti-CD3/anti-CD28 beads (1:1). 3 weeks.	Acute GvHD: 1×10^5/kg Chronic GvHD: 3×10^6/kg
Brunstein *et al.* (2011)	Prevention of GvHD following UCB transplantation n=23	$CD25^+$ bead positive selection	X-VIVO15 with 10% human AB serum. IL-2 (300 IU/ml) Anti-CD3/anti-CD28 beads (1:2). 18 ± 1 days.	0.1-30 $x10^5$/kg after double UCB transplantation
Marek-Trzonkowska *et al.* (2012)	Safety of autologous *in* vitro expanded Tregs in paediatric type 1 DM T1DM n=10	FACS-based sorting of $CD3^+CD4^+$ $CD25^{hi}CD127^{lo}$ cells	CellGro medium with 10% autologous plasma. IL-2 (1000 IU/ml) Anti-CD3/anti-CD28 beads (1:1). Up to 2 weeks.	4 patients: $10x10^6$/kg 6 patients: 20×10^6/kg

Table 8.6: Current ClinicalTrials.gov listing of planned or recruiting clinical trials utilising *in vitro* expanded T_{regs}.

Study name/PI	Leading Sites	ClinicalTrials.gov identifier	Context
Baron/Beguin	University Hospital of Liege	NCT01903473	Treatment of chronic GvHD.
Bluestone/Feng	University of California, San Francisco	NCT02188719	Antigen-specific expanded T_{regs}. Reduction in acute or chronic rejection following liver transplantation.
Bluestone/Herold	University of California, San Francisco	NCT01210664	Polyclonally expanded T_{regs}. Safety in paediatric T1DM.
Brunstein/Blazar	U Minnesota Masonic Cancer Centre, Minneapolis	NCT02118311	Phase II clinical trial of polyclonally expanded UCB T_{regs}. Prevention of acute GvHD with double UCB HSCT.
Bykovskaia/Kaabak	Russian State Medical University, Moscow	NCT01446484	Polyclonally expanded T_{regs}. Prevention of renal transplant rejection in children.
The ONE Study (ONETreg1)	King's College London Oxford University	NCT02129881	Polyclonally expanded T_{regs}. Prevention of renal transplant rejection.
The "ThRIL" study Sanchez-Fueyo	King's College London	NCT02166177	Antigen-specific expanded T_{regs}. Reduction in immunosuppressive burden following liver transplantation.
Pidala	Lee Moffitt Cancer Centre, Tampa, Florida	NCT01795573	Polyclonally expanded T_{regs}. Prevention in acute GvHD following UCB transplantation.

Finally, the dominant antigens in human CD are unknown. Consequently, protocols to expand T_{regs} *in vivo* will involve polyclonal stimulation. Antigen-specific T_{regs} are more potent inhibitors of immune responses than polyclonal T_{regs} , and adoptively transferred T_{regs} induce transplant tolerance in an antigen-specific manner (Hall et al., 1990). However, the TCR repertoire of T_{regs} is largely auto-reactive (Hsieh et al., 2006; Wong et al., 2007), so it is likely that polyclonal expansion of autologous T_{regs} will result in a population that will activate following recognition of self-antigens following adoptive transfer. The classical view of TCR specificity is that each TCR allows a high affinity interaction between a single peptide-MHC complex. However, the poor shape complementarity at the TCR-peptide-MHC interface allows individual TCRs to recognise multiple peptides at low affinity, including peptides without a strong sequence homology, and the cross-reactivity of the TCR from a single cell clone expands on T cell activation (Amrani et al., 2001; Anderson et al., 1988; Fujinami and Oldstone, 1985; Garboczi et al., 1996; Garcia et al., 1996; Mason, 1998; Nanda et al., 1995; Wraith et al., 1992; Wucherpfennig and Strominger, 1995). Antigen-specific T_{reg} activation also allows T_{regs} to suppress inflammation mediated by other antigens, a phenomenon termed "bystander suppression" (Chen et al., 1994; Elinav et al., 2008; Thornton and Shevach, 2000).

"Infectious tolerance" is another overarching mechanism by which T_{regs} can induce transplantation tolerance in experimental systems, by which T_{regs} induce an immune regulating phenotype in non-T_{regs} (Qin et al., 1993). As discussed above, T_{regs} can employ multiple mechanisms to induce immune regulating cells, including IL-10, TGF-β and IL-35, in addition to CD40:CD40L-mediated B_{reg} activation, modulation of DC activation and granzyme-mediated killing of activated APCs.

Characterisation of the potential mechanisms of action of an *in vitro* expanded T_{reg} cell product would be sensible prior to clinical use.

8.16.3 Non-T_{reg} cellular therapy for CD

At the time of writing, multiple cell products are in development for the treatment of CD, including autologous HSCT (ASTIC study, ClinicalTrials.gov identifier: NCT00297193; Hawkey and Trialists, 2014), allogeneic bone marrow-derived mesenchymal stem cells (MSC) ("Prochymal", Mesoblast/Osiris; ClinicalTrials.gov identifier: NCT00482092), allogeneic adipose tissue-derived MSC ("Cx601", TiGenix; ClinicalTrials.gov identifier: NCT01541579) and OVA-specific IL-10^+ $CD4^+$ lymphocytes (Desreumaux et al., 2012).

8.17 Aims and Hypotheses

The aim of this work is to determine if T_{regs} can be developed as an autologous cell therapy for CD. To this end, the individual aims and hypotheses are as follows:

(i) **To validate a novel, rapid assay of T_{reg} function for *in vitro* expanded T_{regs}, thus improving the safety of T_{reg} clinical trials and developing a novel measure of T_{reg} function for laboratory-based practice.** This tests the null hypothesis that *in vitro* expanded T_{reg}-mediated suppression of CD69 and CD154 on T_{cons} does not correlate with *in vitro* expanded T_{reg}-mediated suppression of T_{con} proliferation at 96h.

(ii) **To determine if T_{regs} can be isolated from the blood of Crohn's patients and expanded *in vitro* in the presence of supplemental rapamycin to yield phenotypically stable cells.** This tests the null hypothesis that T_{regs} expanded from $CD45RA^+$ and $CD45RA^-$ precursors have equivalent potential for Th17 conversion and equivalent *FOXP3* TSDR demethylation.

(iii) **To determine if T_{regs} can be isolated from the blood of Crohn's patients and expanded *in vitro* in the presence of supplemental rapamycin to yield cells with the capacity to suppress mucosal inflammation.** This tests the null hypothesis that T_{regs} expanded from Crohn's blood are not able to overcome the *in vitro* resistance to T_{reg}-mediated suppression previously described in T_{cons} isolated from diseased mucosa. The assay validated in aim (i), above will be employed here.

(iv) **To determine if T_{regs} can be isolated from the blood of Crohn's patients and expanded *in vitro* in the presence of supplemental rapamycin to yield cells with the capacity to home to human bowel *in vivo*.** This tests the null hypothesis that T_{regs} expanded from Crohn's blood do not express intestinal and lymphoid homing receptors *in vitro* and are unable to home to human small bowel *in vivo*.

9 Materials and Methods

9.1 Samples from healthy donors

Healthy donor peripheral blood mononuclear cells (PBMCs) were obtained from anonymized human leukocyte cones supplied by the NHS National Blood Service (Tooting, London, UK), following research ethics committee (REC) approval (Hammersmith, Queen Charlotte's & Chelsea REC; reference 09/H0707/86).

9.2 Samples from patients with Crohn's disease

A new REC approval was required prior to obtaining samples from CD patients. This was obtained from South East London REC 2 (reference 10/H0804/65). This application was created on 19th May 2010, followed by initial submission on 13th July, resubmission on 30th September and approval on 20th December 2010. NHS R&D approval was obtained from Guy's & St Thomas' NHS Foundation Trust (reference RJ110/N321; approved 4th January 2011). This study was also adopted onto the NIHR Clinical Research Network (CRN) registry (NIHR CRN reference 9929), which provided a *per capita* reimbursement for each accrued subject, which could then be used to purchase research staff time.

In order to allow patients sufficient time to consider providing samples for research, patients were contacted at home by telephone prior to attending clinic or presenting for admission for elective surgery. CD patients attending the IBD clinic at Guy's & St Thomas' NHS Foundation Trust were invited to donate blood for T_{reg} expansion. In addition, CD patients undergoing intestinal resection were invited to

donate blood, mesenteric lymph node (MLN) and mucosal samples. Patients were contacted by telephone prior to their scheduled clinic visit or elective surgery so that they would have sufficient time to consider participation. Approximately 50% of patients approached this way agreed to donate samples for the studies reported here.

9.3 Cell culture media and buffers

"Complete X-VIVO-15" medium was used to expand T_{regs} *in vitro*. This consisted of X-VIVO-15 (Lonza, Walkersville, MD), supplemented with 5% human AB serum (Biosera, Uckfield, UK) and rapamycin (100 nM, Rapamune, Pfizer, Sandwich, UK).

All other experiments were performed using "complete RPMI" medium, consisting of RPMI 1640 medium (PAA Laboratories, Pasching, Austria) supplemented with HEPES (10 mM, Thermo Fisher Scientific, Loughborough, UK), L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 g/ml), sodium pyruvate (1 mM), MEM nonessential amino acids (0.1 mM), and 10% foetal calf serum (all PAA).

MACS buffer consisted of phosphate buffered saline pH 7.2 (PBS) without Ca^{2+} and Mg^{2+} (PAA) supplemented with 0.5% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) and 2mM EDTA (Sigma-Aldrich).

9.4 PBMC isolation

For PBMC isolation from leukocyte-enriched buffy coats, leucocytes were diluted up to 30ml with sterile PBS and layered over 20ml Lymphocyte Separation

Medium 1077 (LSM 1077; PAA) followed by centrifugation at 2,000 RPM for 30 minutes at 20°C with slow acceleration and no brake. The interface was harvested and washed twice with PBS at 1,800 RPM for 10 minutes at 4°C with normal acceleration and deceleration. Cell count and viability were confirmed with trypan blue staining (Sigma-Aldrich).

For PBMC isolation from PB, blood was diluted 2:1 with sterile PBS and layered over LSM 1077, followed by centrifugation and treatment, as described above.

9.5 MACS-based enrichment for $CD4^+$ lymphocytes by negative selection

The MACS $CD4^+$ T Cell Isolation Kit II (Miltenyi, Bergisch Gladbach, Germany) was used to enrich PBMCs for $CD4^+$ lymphocytes by negative selection, as per manufacturer's instructions. Briefly, PBMCs were incubated with Biotin-Antibody Cocktail 10 μl per 10^7 cells in a final volume of 50μl/10^7 cells for 10 minutes at 4°C, followed by the addition of anti-biotin MicroBeads 20μl/10^7 cells in a final volume of 100μl/10^7 cells for a further 15 minutes. After washing, the labelled PBMCs were applied to a MACS LS column (Miltenyi) to yield an effluent containing >95% "untouched" $CD4^+$ T cells. Next, the effluent was incubated with CD25 MicroBeads II (Miltenyi) 10 μl per 10^7 cells in a final volume of 100μl/10^7 cells for 15 minutes at 4°C, followed by application to a MACS LS column once washed. A representative example is illustrated in Figure 11.2 (page 205).

9.6 MACS-based enrichment for $CD4^+$ lymphocytes by positive selection

The CliniMACS device (Miltenyi) is designed for GMP cell enrichment. In order to duplicate a GMP workflow using non-GMP reagents for the experiments described in Chapter 11 (page 196), $CD4^+$ lymphocytes were enriched to >95% from PBMCs by positive MACS selection (Miltenyi). Briefly, PBMCs were incubated with anti-$CD4^+$ MicroBeads 20 μl per 10^7 cells in a final volume of 100μl/10^7 cells for 15 minutes at 4°C, followed by positive selection on a MACS LS column, to yield >95% $CD4^+$ cells. A representative example is illustrated in Figure 11.3 (page 207).

9.7 Cell sorting

$CD4^+$ cells were stained with a Human Regulatory T Cell Sorting Kit (BD Biosciences, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, enriched $CD4^+$ cells were labelled with mouse anti-human CD4–PerCP-Cy5.5 (clone L200), anti-CD25–phycoerythrin (PE) (clone 2A3), anti-CD127–AlexaFluor 647 (clone 40131.111) ± anti-CD45RA–FITC (clone HI100). Cells were then sorted to $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} or $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ (Population I) and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ (Population II & III) T_{reg} subsets, on a FACSAria (BD Biosciences). $CD4^+CD25^-$ T_{cons} were contemporaneously sorted for later use as autologous responder cells. Post-sort viability was confirmed by microscopy with trypan blue stain. Post-sort purity is summarized in Table 9.1 and 9.2 (page 136). Representative examples of these sort strategies are illustrated in Figure 10.1 (page 165) and Figure 11.4 (page 208).

Table 9.1: Post-sort purity of $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} using the sort strategy illustrated in Figure 10.1 (page 165).

Population (n=10)	Pre-sort (%)	Post-sort (%)
$CD4^+CD25^-$ T_{cons}	55.7 (34.5 – 61.2)	98.3 (94.4 – 98.9)
$CD4^+CD25^{hi}$ T_{regs}	6.7 (5.3 – 8.9)	94.1 (89.5 – 96.9)
$CD4^+CD25^{hi}CD127^{lo}$ T_{regs}	4.8 (3.5 – 6.5)	94.1 (89.6 – 96.5)
n=10. Data presented as median (IQR).		

Table 9.2: Post-sort purity of $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ (population I) and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ (population II & III) T_{reg} subsets using the sort strategy illustrated in Figure 11.4 (page 208).

Population (n=13)	Pre-sort (%)	Post-sort (%)
$CD4^+CD25^-$ T_{cons}	67.0 (61.1 – 71.8)	98.0 (97.6 – 98.7)
$CD4^+CD25^{hi}$ T_{regs}	7.4 (8.1 – 5.2)	95.9 (94.3 – 97.6)
$CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs}	2.4 (1.7 – 3.1)	86.5 (80.8 – 91.6)
$CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ T_{regs}	3.8 (3.1 – 5.9)	92.7 (87.8 – 94.9)
n=13. Data presented as median (IQR).		

9.8 *In vitro* T_{reg} expansion

T_{regs} were expanded *in vitro* as described previously (Canavan et al., 2012; Sagoo et al., 2011; Scottà et al., 2013). FACS-sorted T_{reg} populations were plated at 10^6/mL in complete X-VIVO-15 medium and activated with anti-CD3/anti-CD28–coated beads (Dynabeads®, Invitrogen, Paisley, UK) at a 1:1 bead:cell ratio. rhIL-2 (Proleukin®, Novartis, Camberley, UK) 1,000 IU/ml was added at the start of the culture and replenished every second day. The optimum re-stimulation frequency for *in vitro* expansion of T_{regs} from the PB of CD patients was initially investigated by daily assessment of proliferation by microscopy and daily assessment of Ki-67 expression by FACS. Assessment of Ki-67 expression is illustrated in Figure 11.5 (page 212). A head-to-head comparison of candidate re-stimulation frequencies was then performed, as illustrated in Figure 11.6 (page 214). Every 10-12 days, beads were removed by magnetic adherence and cells were re-stimulated with new anti-CD3/anti-CD28 beads after 10-12 days of culture, with the addition of fresh rapamycin and IL-2. The phenotype and suppressive ability of *in vitro* expanded T_{reg} lines was assessed after 28 days of culture.

9.9 Assessment of CD69 and CD154 expression in lymphocytes

The BD FastImmune Human Regulatory T Cell Function Kit (BD Biosciences) was used according to the manufacturer's instructions to determine the cell surface expression of CD69 and CD154 on FACS-sorted $CD4^+CD25^-$ T_{cons}, and T_{reg}–mediated suppression of CD69 and CD154 expression on T_{cons}.

$5x10^4$ T_{cons} per well were cultured either alone or in co-culture with sorted $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} at T_{con}:T_{reg} ratios titrated from 1:1 to 32:1 (constant T_{con}

numbers) in 220 μL complete RPMI in 96-well U-bottomed plates (Nunc, Roskilde, Denmark). T_{cons} were stimulated with anti-CD3/anti-CD28–coated beads (Dynabeads) at a bead:T_{con} ratio of 0.2. A "2X" control condition was included in each assay, containing 10^5 T_{cons} per well, to determine the level of non-specific inhibition of CD69 or CD154 expression seen in this system, due to increased cell density or limited access to beads at higher T_{con}:T_{reg} ratios. Anti-CD154–Allophycocyanin (APC) (clone 89-76) antibody was added at time 0 to capture transient cell surface expression of CD154 during T_{con} activation (Chattopadhyay et al., 2005). Control conditions included an unstained, unstimulated T_{con} condition, a stained, unstimulated T_{con} condition, and a stained, stimulated T_{con} condition. After incubation at 37°C/5% CO_2 for 7 hours, cells were stained as required with a titrated antibody cocktail of anti–CD3–PerCP–Cy5.5 (clone SK7), CD4–FITC (SK3), CD25–PE (2A3), and CD69–PE–Cy7 (L78) and acquired on an LSRII cytometer (BD Biosciences) running FACSDiva 6.1.3 software.

This assay was initially optimised using $CD4^+CD25^-$ T_{cons} as responder cells. Optimisation of the activation conditions for this assay is discussed in Section 10.4 (page 166) and illustrated in Figure 10.2 (page 167). Validation of the gating strategy using T_{cons} as responder cells is discussed in Section 10.5 (page 168) and illustrated in Figure 10.3 (page 169).

In all cases, percentage suppression (S) of marker frequency was calculated using the formula:

$$S = 100 - \left(\left[\frac{a}{b}\right] x\ 100\right)$$

where *a* is percentage positive in the presence of T_{regs} and *b* is percentage positive in the absence of T_{regs}.

9.10 Assessment of lymphocyte proliferation

T_{cons} were labelled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) or CellTrace Violet (CTV) according to standard protocols. Briefly, cells were washed in warm (37°C) phosphate buffered saline (PBS, Gibco, Paisley, UK) to remove excess protein. Resuspended cells were then incubated with 1 mL warm 1 μM CFSE working solution in the dark at room temperature for 3 minutes, or 1 mL warm 1 μM CTV working solution in the dark at 37°C for 20 minutes. The fluorescent dye was then quenched by the addition of 9ml warm PBS, followed by resuspension in complete medium.

$5x10^4$ T_{cons} were then cultured alone or in co-culture with T_{regs} at T_{con}:T_{reg} ratios titrated from 1:1 to 32:1 (constant T_{con} numbers), in 200 μL complete RPMI in 96-well U-bottomed plates. A "2X" control condition was included in each assay, containing 10^5 T_{cons} per well, to determine the level of non-specific inhibition of proliferation seen in this system due to increased cell density or limited access to beads at higher T_{con}:T_{reg} ratios.

For experiments in which $CD4^+CD25^-$ T_{cons} were used as responder cells, cells were activated by anti-CD3/anti-CD28 coated microbeads at a bead:T_{con} ratio of 0.02.

For experiments in which MLN or LP mononuclear cells were used as responder cells, cells were activated by plate-bound anti-CD3 and anti-CD28 antibodies (R&D Systems, Abingdon, UK). Briefly, 100 μL PBS containing 2 μL/ml

of anti-CD3 antibody and 2 μL/ml of anti-CD28 antibody was added to relevant wells of a 96 well U-bottomed plate. Plates were incubated overnight at 4°C. The following day, plates were washed twice with PBS prior to use.

Assessment of proliferation by flow cytometry was performed at 96 hours. Cells were acquired in a total volume of 300 μL containing 30 μL counting beads (CountBright, Invitrogen), where indicated. Acquisition was limited by gating on counting beads, collecting at least 5,000 events per sample. Dead cells were excluded with propidium iodide (Invitrogen) or Live/Dead Yellow (Invitrogen) according to the manufacturer's instructions. The numbers of non-proliferating cells (events in the first peak) and precursor cells generating each cell division were calculated using standard formulas (Wells et al., 1997).

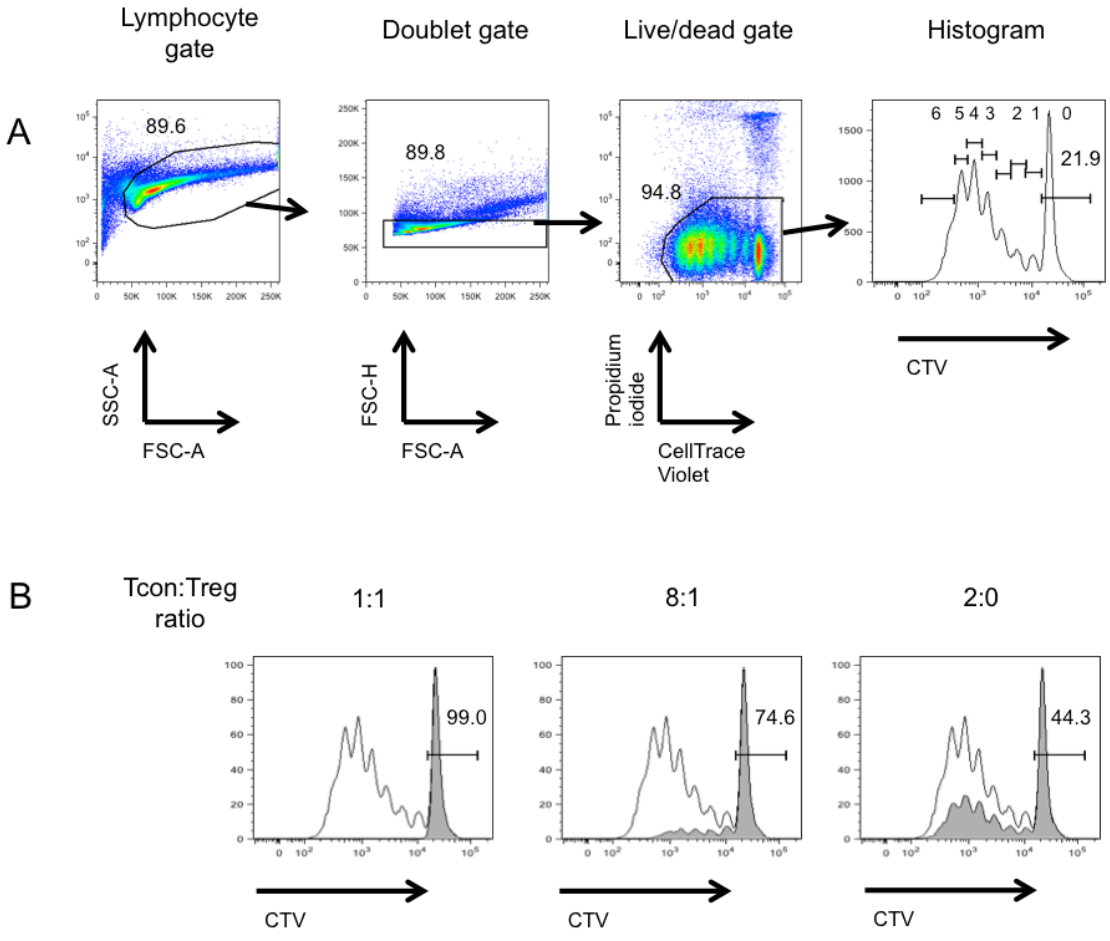
Percentage suppression (S) of proliferation was calculated using the formula:

$$S = 100 - \left(\left[\frac{c}{d}\right] x\ 100\right)$$

where c is percentage proliferating precursors in the presence of T_{regs} and d is percentage proliferating precursors in the absence of T_{regs}. The gating strategy used to assess proliferation in this system is illustrated in Figure 9.1 (page 141).

Figure 9.1: Gating strategy to assess proliferation in the CFSE (or CTV) dilution assay.

(A & B) Representative FACS plots illustrating the gating strategy to analyse proliferation of T_{cons} labelled with a fluorescent dye. **(A)** T_{cons} (cultured alone in this example) were gated on lymphocytes with subsequent exclusion of doublets, dead cells and unlabelled live cells. The histogram (far right panel) illustrates the undivided peak (division 0, proportion of events in this gate is also shown) and peaks representing subsequent divisions (1-6). From these data, the number of precursors giving rise to each cell division was calculated. **(B)** Proliferation in T_{cons} co-cultured with T_{regs} at a 1:1 ratio (left panel) and an 8:1 ratio (middle panel), showing a dose-response in T_{reg}-mediated suppression of proliferation. Non-specific inhibition of proliferation due to cell density was assessed by including a 2:0 T_{con}:T_{reg} condition (right panel), demonstrating that the suppression seen in the 8:1 condition (and above, in this assay) was due to T_{regs}. Study condition (shaded); 1:0 condition (clear line).



9.11 Flow cytometric evaluation of T_{reg} subsets.

An 11-colour (14 parameter) flow cytometric panel was optimized for the identification of T_{reg} subsets and assessment of T_{reg} maturation, differentiation and transcription factor expression. This panel allowed the identification of PB and LP $CD4^{+}CD25^{hi}CD127^{lo}$ T_{regs} and T_{reg} subsets (Miyara et al., 2009), and assessment of the purity and phenotype of *in vitro* expanded T_{reg} lines. Reagents used are listed in Table 9.3 (page 143). Representative FACS plots are illustrated in Figure 9.2 (page 144).

Briefly, surface-staining antibodies including Live/Dead Blue were added for 30 minutes at 4°C. Cells were then washed in PBS, followed by fixation and permeabilisation with a FOXP3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA). Intracellular staining was performed for 30 minutes at 4°C. Samples were acquired on a LSRII or Fortessa flow cytometer (BD Biosciences) with application settings, to ensure comparability of longitudinally acquired data.

Gating was based on natural populations, requiring the addition of a fully stained PBMC condition when assessing *in vitro* expanded T_{reg} lines. A "Fluorochrome Minus One" (FMO) control condition is a fully stained sample except for a single fluorochrome of interest and is used to define a "negative" population, taking into account the auto-fluorescence of the cells of interest and signal spillover from other fluorochromes in the panel. They are most useful in defining true positive gating for epitopes that are not bimodally expressed, hence without natural "positive" and "negative" populations. FMO controls were used to assist in accurate gating of $CD45RA^{+}$ and $CD45RO^{+}$ populations.

Table 9.3: Antibodies used in the flow cytometric evaluation of T_{reg} subsets.

Marker	Fluorochrome	Clone
CD3	APC-H7	SK7
CD4	eFluor® 450	RPA-T4
CD8	V500	RPA-T4
CD25	PE	2A3
CD127	PerCP-Cy5.5	eBioRDR5
CD45RA	AlexaFluor® 700	HI100
CD45RO	PE-Cy7	UCHL1
CD161	Brilliant Violet™ 605	HP3G10
Foxp3	FITC	PCH101
Helios	APC	22F6
Live/Dead Blue	DAPI (UV 450/50)	-

Figure 9.2: Gating strategy used in the flow cytometric evaluation of T_{reg} subsets.

(A) Cells (PBMCs in this example) were gated on lymphocytes with subsequent exclusion of doublets, dead cells and positive identification of $CD3^+CD4^+$ events. **(B)** Sub-division of live $CD4^+$ events on the basis of CD25 expression in $CD4^+$ lymphocytes yielded three phenotypically distinct $CD4^+$ subsets. $CD4^+CD25^{lo}$ events (naïve and un-activated $CD4^+$ lymphocytes) were identified by comparing CD25 expression in $CD4^+$ and $CD4^-$ events (to the left of the dotted line in the left panel). The titrated dose of anti-CD25-PE (2A3) allowed reproducibly clear discrimination between $CD4^+CD25^{hi}$ events (T_{regs}) and $CD4^+CD25^{int}$ events (activated $CD4^+$ lymphocytes).

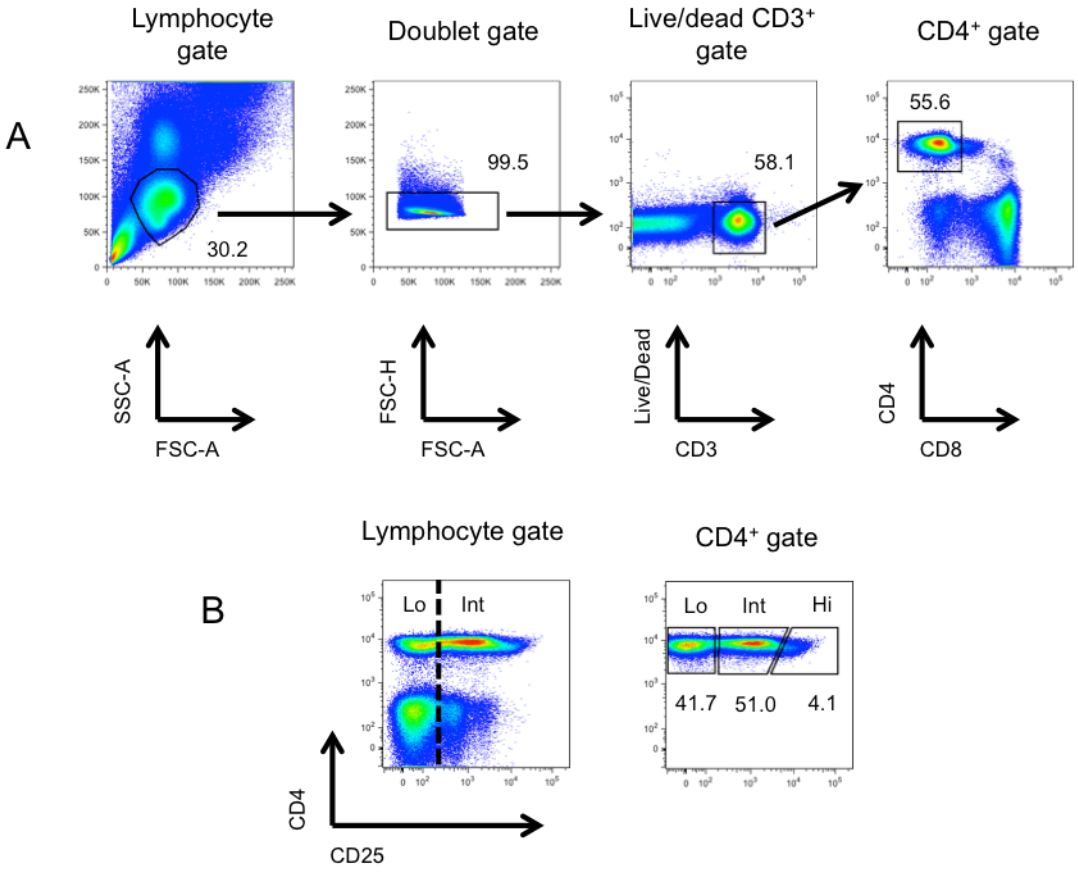
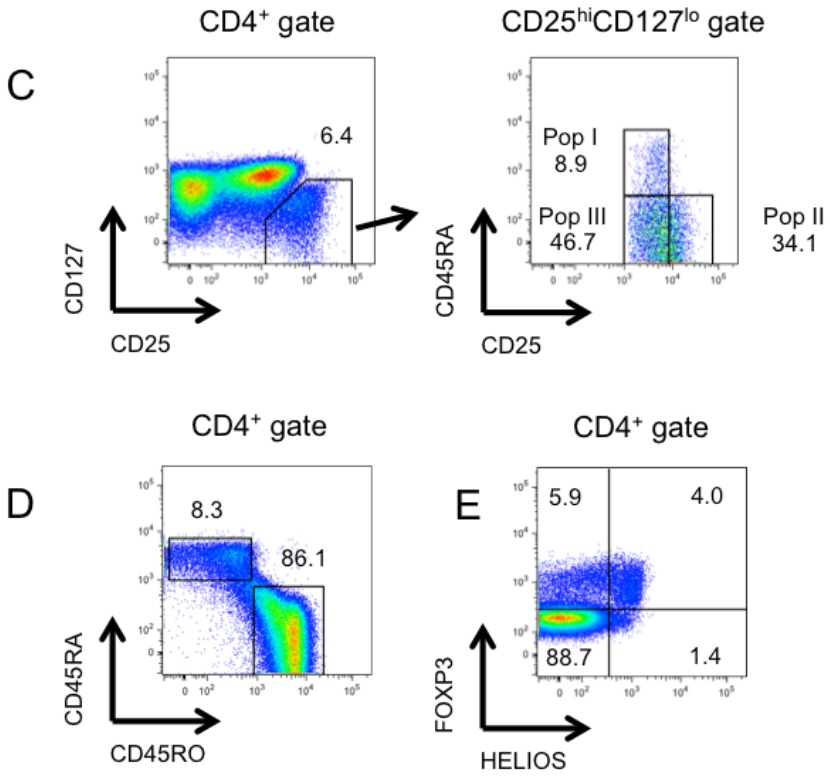


Figure 9.2 (continued): Gating strategy used in the flow cytometric evaluation of T_{reg} subsets. **(C)** T_{reg} subsets were identified by gating on $CD4^+CD25^{hi}CD127^{lo}$ events. **(D & E)** The panel described in Table 9.3 (page 143) also allowed easy evaluation of CD45RA, CD45RO and FOXP3 expression in live $CD4^+$ events.



9.12 Flow cytometric evaluation of cytokine and transcription factor expression in T_{regs}

A 13-colour (16 parameter) flow cytometric panel was optimized for the detection of intracellular cytokine and transcription factor expression. Reagents used are listed in Table 9.4 (page 147). Cytokine and transcription factor expression was assessed after stimulation with 50 ng/ml PMA, 1 μg/ml Ionomycin and 3 μM Monensin (all Sigma-Aldrich) in complete RPMI medium for 4 hours in a in a 37°C/5% CO_2 incubator. Cells were stained and acquired as described above. The gating strategy is illustrated in Figure 9.3 (page 148).

Table 9.4: Antibodies used in the flow cytometric evaluation of cytokine and transcription factor expression in T_{regs}.

Marker	Fluorochrome	Clone
CD3	APC-H7	SK7
CD4	V500	RPA-T4
CD8	PE-Cy7	RPA-T8
CD25	PE	2A3
CD127	Brilliant Violet™ 650	A019D5
CD45RA	AlexaFluor® 700	HI100
T-bet	PerCP-Cy5.5	O4-46
Foxp3	FITC	PCH101
RORγt	APC	AFKJS-9
IFN-γ	Brilliant Violet™ 570	4S.B3
IL-17	Pacific Blue	N49-653
CD161	Brilliant Violet™ 605	HP-3G10
Live/Dead Blue	DAPI (UV 450/50)	-

Figure 9.3: Gating strategy used in the flow cytometric evaluation of cytokine and transcription factor expression in T_{regs}.

(A) $CD4^{+}CD25^{hi}CD127^{lo}$ T_{regs} were identified from PBMCs by gating on lymphocytes with exclusion of doublets, dead cells and positive identification of $CD3^{+}CD4^{+}$ events.

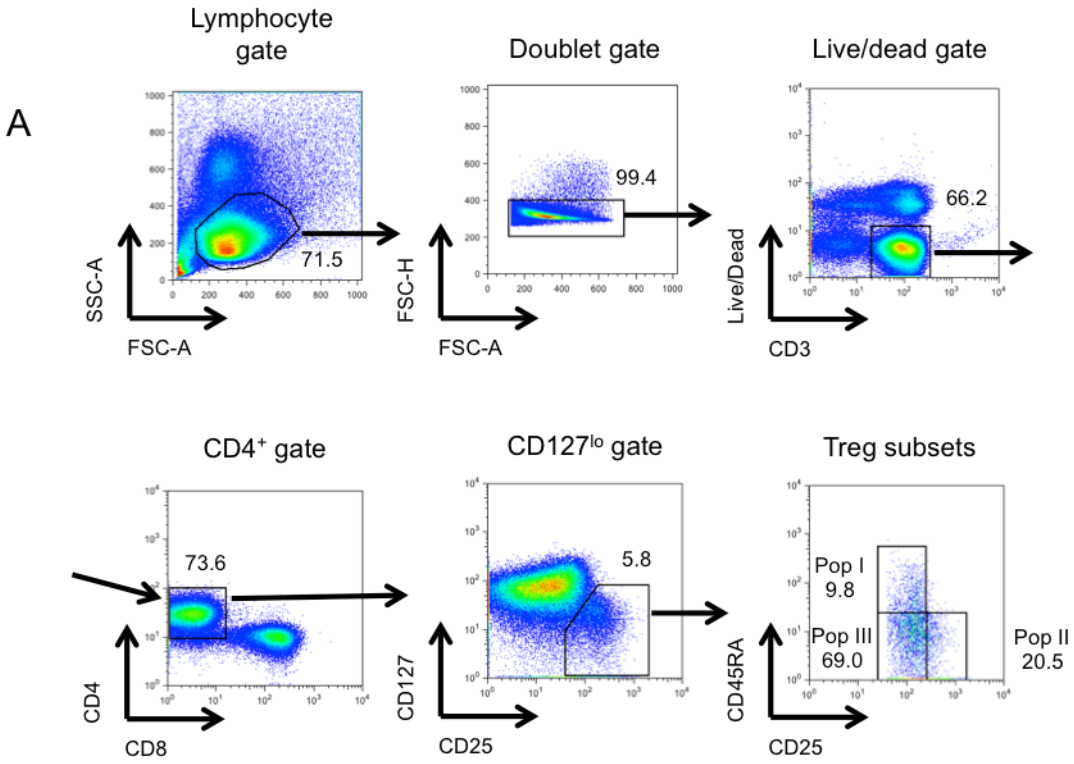
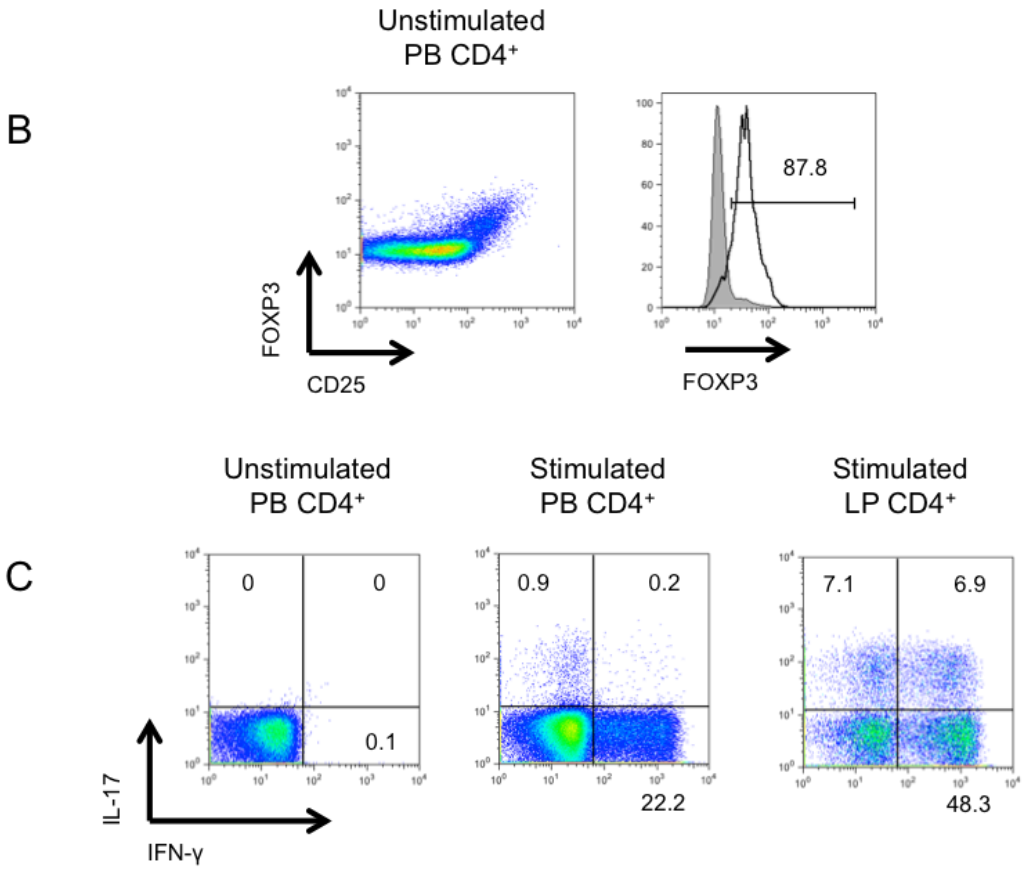


Figure 9.3 (continued): Gating strategy used in the flow cytometric evaluation of cytokine and transcription factor expression in T_{regs}.

(B) FOXP3 expression in unstimulated $CD4^+$ cells was predominantly seen within the $CD4^+CD25^{hi}$ fraction (left panel). The right panel illustrates FOXP3 expression in $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} (clear line), compared with expression in the whole $CD4^+$ population (shaded line). **(C)** Panels gated on live $CD4^+$ events illustrating positive staining for IL-17 and IFN-γ in PB and LP $CD4^+$ cells.



9.13 Flow cytometric evaluation of homing receptor expression in T_{regs}

Two 14-colour (17 parameter) flow cytometric panels were optimized for the detection of cell surface proteins important for lymphocyte homing to immune and intestinal tissue. Reagents used are listed in Table 9.5 (page 151). Cells were stained and acquired as described above.

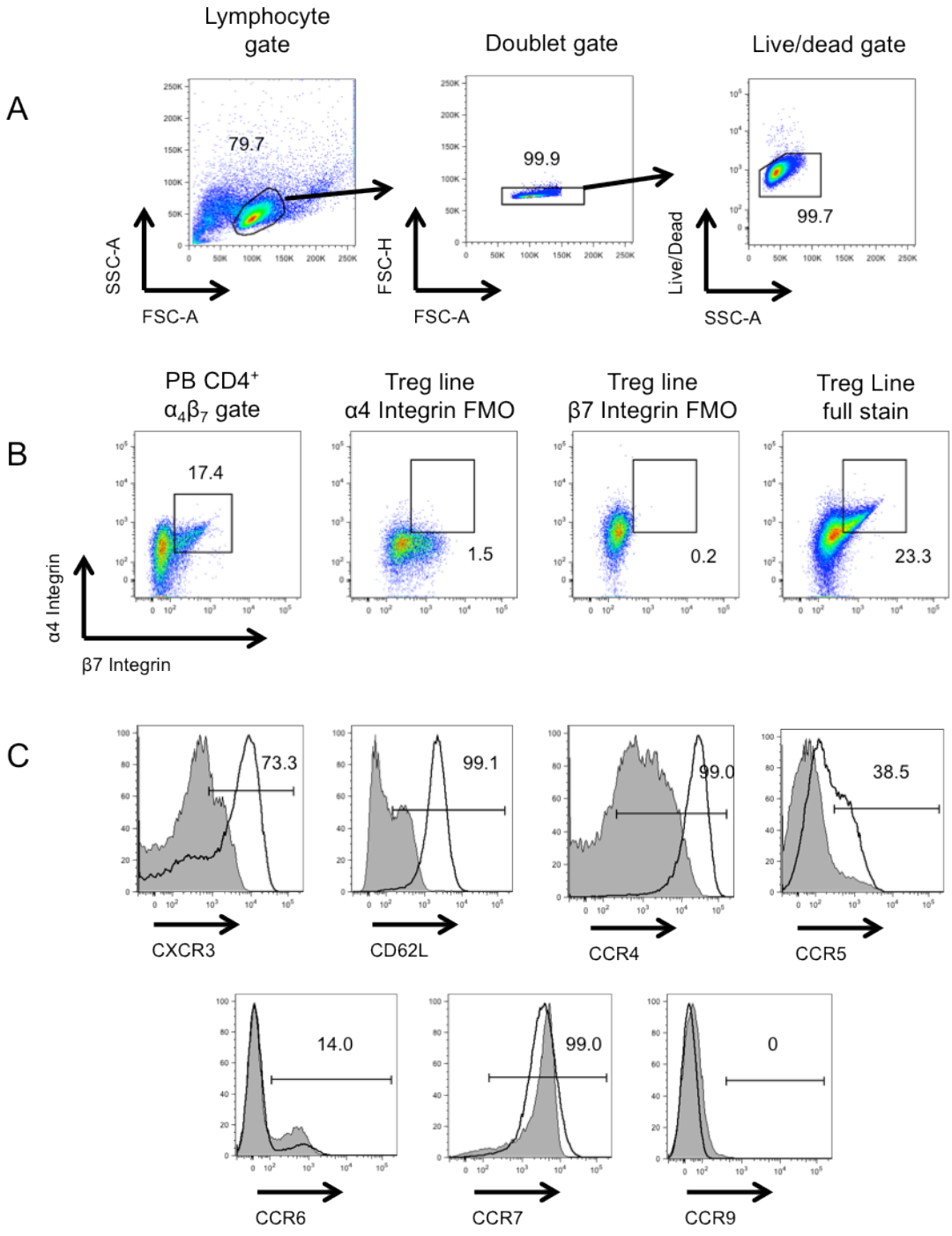
For the assessment of homing receptor expression in *in vitro* expanded T_{reg} lines, both a fully stained PBMC control and FMO controls for each homing receptor were obtained to define positive gates. The gating strategy is illustrated in Figure 9.4 (page 152).

Table 9.5: Flow cytometric evaluation of homing receptor expression in T_{regs}.

Core Panel		
Specificity	**Fluorochrome**	**Clone**
CD3*	APC-H7	SK7
CD4*	V500	RPA-T4
CD25*	PE	2A3
CD127*	Brilliant Violet™ 650	A019D5
CD45RA*	AlexaFluor® 700	HI100
CD45RO*	Brilliant Violet™ 570	UCHL1
CD49d (α_4 integrin)	PerCP-Cy5.5	9F10
β_7 integrin	FITC	FIB504
CD62L	Pacific Blue	DREG-56
CD161	Brilliant Violet™ 605	HP3G10
Live/Dead Blue	DAPI (UV 450/50)	
Additional Antibodies for Panel 1		
Specificity	**Fluorochrome**	**Clone**
CCR7 (CD197)	PE-CF594	150503
CCR6 (CD196)	APC	G034E3
CXCR3 (CD183)	PE-Cy7	IC6/CXCR3
Additional Antibodies for Panel 2		
Specificity	**Fluorochrome**	**Clone**
CCR5 (CD195)	PE-CF594	2D7/CCR5
CCR9 (CD199)	APC	BL/CCR9
CCR4 (CD194)	PE-Cy7	TG6/CCR4

Figure 9.4: Flow cytometric evaluation of homing receptor expression in T_{regs}.

(A) In this representative example, T_{regs} from an *in vitro* expanded T_{reg} line were identified by forward- and side-scatter properties with the exclusion of doublets and dead cells. **(B)** $\alpha_4\beta_7$ integrin expression in PB $CD4^+$ from a healthy donor (first panel). FMOs from T_{reg} lines were required to ensure accurate gating (second-to-fourth panels). **(C)** Representative examples of homing receptor expression in *in vitro* expanded T_{regs}. Expression in PB $CD4^+$ (shaded). Expression in T_{reg} line (clear). Gates drawn on basis of FMO controls.



9.14 Estimation of cytokine concentrations

Cytokine concentrations were estimated in culture supernatants using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences) or sandwich ELISAs, as indicated. CBAs were performed by incubating 50 μL of culture supernatant with cytokine capture beads and PE detection reagent for 3 hours at room temperature in the dark, before washing and acquisition on a Fortessa cytometer (BD Biosciences). Sandwich ELISAs (R&D Systems, Abingdon, UK) for human IL-17 were performed in duplicate according to manufacturer's instructions, measuring optical density at 450nm on a Bio-Tek EL800 automatic plate reader (Wolf Laboratories, York, UK). For both techniques, cytokine concentrations were interpolated from contemporaneously acquired standard curves.

9.15 rtPCR

Following total RNA extraction from Trisure (Bioline, London, UK), cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit and multiplex rtPCR performed in duplicate using the Maxima Probe/ROX qPCR Master Mix (both Thermo Fischer Scientific) on a BioRad C1000 Thermal Cycler. The following FAM-conjugated probes were used: AHR (Hs00169233), CCR9 (Hs01890924), IL-17A (Hs00174383) and RORC (Hs01076112). GAPDH-VIC (Applied Biosystems, Paisley, UK) was used as an endogenous housekeeping gene and relative expression calculated by $2^{-\Delta Ct}$.

9.16 Assessment of IL-17 production under pro-inflammatory conditions

In vitro generated T_{regs} were activated with anti-CD3/anti-CD28 coated beads at a 1:1 bead:T_{reg} ratio and cultured at 10^6 cells/ml in complete RPMI for 5 days at 37°C/5% CO_2, supplemented with the following cytokine cocktails, as previously described (Koenen et al., 2008; Scottà et al., 2013; Tresoldi et al., 2011): (a) IL-2 (10 IU/ml, Proleukin). (b) IL-2, IL-1 (10 ng/ml), IL-6 (4 ng/ml) and TGF-β (5 ng/ml). (c) IL- 2, IL-21 (25 ng/ml), IL-23 (25 ng/ml) and TGF-β (all R&D Systems). Supernatant IL-17 concentrations were measured by ELISA.

9.17 Assessment of *FOXP3* TSDR demethylation

Genomic DNA was isolated from T_{regs} using the DNeasy kit (Qiagen, Manchester, UK). Bisulfite conversion and assessment of the methylation status of the *FOXP3* T_{reg} Specific Demethylated Region (TSDR) was performed by Epiontis GmbH (Berlin, Germany), as described previously (Baron et al., 2007; Polansky et al., 2008; Sehouli et al., 2011). The genomic locations of *FOXP3* and *GAPDH* CpG-rich regions interrogated have been reported previously (see Sehouli et al., 2011).

9.18 Isolation of lamina propria mononuclear cells and mesenteric lymph node cells

Partial thickness samples were obtained from intestinal resection specimens under the supervision of a consultant histopathologist and washed with PBS. The mucosal layer was dissected and cut into <5 mm sections. The following steps were then performed using pre-warmed media (37°C) in a 37°C/5% CO_2 incubator with constant agitation provided by a portable magnetic stirrer. The

epithelial layer was removed by incubation with Hank's balanced salt solution (HBSS, Gibco) supplemented with EDTA (1 mM; Sigma-Aldrich, Gillingham, UK) and gentamycin (30 μg/ml; PAA) for 30 minutes. The epithelial suspension was decanted. The crude lamina propria mixture was then digested in complete RPMI supplemented with gentamycin, collagenase D (25 μg/ml; Roche, Welwyn Garden City, UK) and DNase I (10 μg/ml; Roche) for 60 minutes. The cell digest was then filtered through a 100 μm mesh (BD Biosciences) and collagenase activity quenched with a cold PBS wash. Lamina propria mononuclear cells (LPMCs) were then enriched by density gradient centrifugation. LPMCs were harvested from the interface and cell count and viability confirmed with trypan blue staining.

Mesenteric lymph nodes (MLN) were mechanically disrupted between mesh, then washed with cold RPMI, to yield a single-cell MLN suspension.

9.19 C.B-17 SCID mouse human intestinal xeno-transplant model

This model was established by Professor Nahum Shpigel at the Koret School of Veterinary Medicine, Hebrew University of Jerusalem, Rehovot, Israel. IRB approval was prospectively obtained (Ethics Committee for Animal Experimentation, Hebrew University of Jerusalem; MD-11-12692-4 and the Helsinki Committee of the Hadassah University Hospital; 81-23/04/04). Women undergoing legal terminations of pregnancy gave written, informed consent for use of foetal tissue in this study.

C.B-17 SCID mice were purchased from Harlan, Israel and housed under specified pathogen free conditions in filter-topped cages, receiving autoclaved food and water *ad libitum*.

Human foetal small bowel up to 16 weeks gestational age was implanted subcutaneously on the dorsum of the mouse, as described previously (Golan et al., 2010; 2009; Howie et al., 1998). Tissues were transported on ice, then washed in cold RPMI and cleaned of mesentery prior to cutting into 3-4 cm sections. Following anaesthesia, small incisions were made in the suprascapular region and flank of the C.B-17 SCID mouse. Blunt dissection was used to create a subcutaneous tunnel, through which a forceps could be passed. The forceps was then used to thread a foetal intestinal segment into the subcutaneous space. The intestinal segment was trimmed as required and the skin closed with a tissue adhesive. Grafts developed for 12-16 weeks prior to manipulation. Each mouse could accommodate up to two intestinal xeno-transplants. This model is illustrated in Figure 11.9 (page 231).

9.20 Induction of graft inflammation with enteropathogenic *Escherichia coli*

Green fluorescent protein (GFP)-expressing WT enteropathogenic *Escherichia coli* (EPEC) were grown in Lysogeny broth (LB) medium (Sigma-Aldrich) overnight at 27°C without agitation, as described previously (Golan et al., 2010). Xenograft inflammation was induced by intraluminal injection of up to 10^8 bacteria in 100 µl PBS via a 23G needle, 6-8 hours prior to assessment. As mice could accommodate a single human intestinal xenograft on each side, the contralateral graft was injected with 100 µl of PBS to act as a non-inflamed control in the same animal.

9.21 Adoptive transfer of *in vitro* expanded T_{regs}

$CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} were expanded *in vitro* from the blood of patients with Crohn's disease, as described above (Section 9.8, page 137), and frozen in human AB serum (Biosera) supplemented with 10% DMSO (Sigma-Aldrich), then transported to the Hebrew University of Jerusalem on dry ice. On receipt, cells were thawed and cultured for 7-10 days before use.

Because mice were on a SCID background, mice received 1 g rabbit anti-mouse anti-Asialo GM1 antibody (Cedarlane, Burlington, ON, Canada) by intraperitoneal (IP) injection, 24 hours prior to adoptive transfer. Mice were also treated IP with IL-2 ($2x10^4$ IU, Proleukin) directly prior to T_{reg} administration (Tresoldi et al., 2011). Between $20x10^6$ and $30x10^6$ *in vitro* expanded T_{regs} were injected into the lateral tail vein of study mice, using a standard sterile technique. T_{reg} homing was assessed 24 hours later.

9.22 Detection of adoptively transferred T_{regs} by FACS

The spleen was mechanically disrupted with a pestle between two sheets of 100 μm mesh to yield a single cell suspension. Disrupted tissue was then filtered through 100 μm mesh and washed with cold RPMI, followed by red cell lysis. Cell viability was determined by trypan blue staining.

The intestinal xenograft was retrieved from the dorsum of the animal and opened longitudinally. Inspissated mucus was removed and a single cell suspension prepared, as described in Section 9.18 (page 154).

Single cell suspensions were stained with anti-mouse CD45-PerCP-Cy5.5 (clone 30-F11), anti-human CD45-eFluor®450 (HI30), anti-human CD4-FITC (RPA-T4) (all eBioscience), anti-human CD3-APC-H7 (SK7, BD Pharmingen) and Live/Dead Blue (Invitrogen). Cell suspensions were passed through a 40 μm filter prior to acquisition on a LSRII cytometer.

9.23 Detection of adoptively transferred T_{regs} by immunofluorescence

Tissue for immunofluorescence was snap-frozen in OCT (Tissue-Tek, Sakura, Torrance, CA, USA). Cryostat sections of 7 μm were fixed in 50% acetone, then 100% acetone, then air-dried. After washing and blocking with 20% horse serum (PAA), sections were stained with anti-human CD45-FITC (2D1) and anti-human CD3-biotin (OKT3, both eBioscience), followed by streptavidin-AlexaFluor 594 (Invitrogen). To visualize EPEC, sections were stained with phalloidin-rhodamine (Sigma). Nuclei were stained with DAPI (1 g/mL, Invitrogen). Negative controls were stained with isotype-matched antibodies. Images were acquired on an Olympus BX51 microscope using Micro-Manager software (Vale Lab, UCSF, San Francisco, CA, USA).

9.24 Statistical analysis

Flow cytometric data were analysed with FlowJo (TreeStar, Ashland, OR, USA) or FACSDiva (BD Biosciences). Statistical analysis was performed using GraphPad Prism 5.0d for Mac OSX (GraphPad Software, La Jolla, CA, USA).

Statistical analysis of the performance of CD69 and CD154 suppression at 7 hours as a "diagnostic test" for suppression of proliferation at 96 hours was

performed using XLSTAT (Addinsoft, Paris, France) with Microsoft Excel 2011 (Microsoft Corporation, Seattle, WA, USA).

Continuous data are presented as mean ± standard deviation (SD) or median (interquartile range [IQR]) for parametric and nonparametric data, respectively. Comparison of central tendency was performed using paired parametric and nonparametric tests as appropriate (t test or Wilcoxon signed rank test, respectively). Similarly, multiple means (or ranks) were compared by 1-way ANOVA or Kruskal-Wallis test, as appropriate. Linear variables were compared using correlation or linear regression, yielding a correlation coefficient (r) or regression coefficient (R^2) and p value. Linear variables were compared with nonlinear variables (e.g., 2-fold dilutions of T_{reg} concentrations in co-culture with T_{cons}) using semilogarithmic regression, yielding a regression coefficient (R^2) alone.

The performance of CD69 and CD154 suppression at 7 hours as a "diagnostic test" for critical values of suppression of proliferation at 96 hours was examined using Bland-Altman plots (Bland and Altman, 1986) and receiver operating characteristic (ROC) curves. The Youden index (sensitivity+specificity-1) (Youden, 1950) was used as a continuous variable to determine the range over which suppression of CD69 and CD154 performed optimally as a test to identify suppression of proliferation. The proportion of true-positive, false-positive, true-negative, and false-negative results was calculated and tabulated for critical values of CD69 and CD154 suppression in identifying critical values of suppression of proliferation.

A p value of less than 0.05 was considered statistically significant throughout.

10 Results (1): A rapid diagnostic test for human regulatory T cell function that may enable regulatory T cell therapy

10.1 Introduction

Immunologic tolerance to self-components is critically dependent on a balance between $CD4^{+}CD25^{hi}CD127^{lo}FOXP3^{+}$ regulatory T cells (T_{regs}) and inflammatory T helper (Th) 1 and Th17 lineages, which are associated with autoimmune diseases and transplant rejection (Afzali et al., 2007; Atalar et al., 2009; Baecher-Allan and Hafler, 2006). This balance is suggested by abnormalities of T_{reg} number or function in human autoimmune diseases (Afzali et al., 2007; Chatila et al., 2000; Lindley et al., 2005; Sugiyama et al., 2005; Valencia et al., 2007; Viglietta, 2004) and by the induction of autoimmune diseases in animal models through excess Th1 and/or Th17 in the absence of defective T_{regs} (Ferber et al., 1996; Langrish et al., 2005; Liblau et al., 1995; Lubberts et al., 2002). Broad-spectrum immunosuppressive medicines non-specifically target both Th1 and Th17 cells and have unacceptable long-term side effects. Consequently, cell-based therapy with autologous T_{regs} may be an attractive alternative.

Adoptive cell therapy with autologous T_{regs} for the treatment of autoimmune disease or the induction of tolerance to transplanted tissues is a realistic possibility. *In vitro* expansion of T_{regs} using R&D-grade reagents has been demonstrated by many groups (Battaglia et al., 2006; Golovina et al., 2011; Hippen et al., 2011b; Hoffmann et al., 2006; 2004; Scottà et al., 2013; Strauss et al., 2007b; 2009; Tresoldi et al., 2011). Putnam *et al.* recently published a protocol for GMP-grade *in vitro* expansion of antigen-specific T_{regs} that will be used in a forthcoming

clinical trial (Putnam et al., 2013). *In vitro* expanded T_{regs} have recently been safely infused to patients in phase I clinical trials with the intention of treating or preventing GvHD (Brunstein et al., 2011; Trzonkowski et al., 2009) or improving glycaemic control in paediatric type 1 diabetes (T1DM) (Marek-Trzonkowska et al., 2012).

A successful program of cell therapy relies critically on reproducible and safe expansion of a non-contaminated cell product. The quality of the cell product is determined by passing pre-specified "lot release criteria". For example, the lot release criteria in Brunstein *et al.*'s study included ">70% viability, $CD4^+CD25^{hi}$ purity >60%, <10% $CD8^+$ cells, anti-CD3/anti-CD28 bead count <100 per $3x10^6$ cells, Gram stain with "no organisms", and endotoxin <5 EU/kg" (Brunstein et al., 2011). The current gold standard method for assessing T_{reg} function *in vitro* involves the determination of T_{reg}–mediated suppression of proliferation or cytokine expression by $CD4^+CD25^-$ T_{cons}. This assay has been used in recent clinical trials on *in vitro* expanded T_{reg} populations "for information" only, meaning that the release of a cell product lot has not been dependent on the result. However, as the relationship between *in vitro* suppressive ability and biological activity becomes clearer with greater clinical trial experience, it may become expedient to accurately assess the suppressive capability of T_{regs} immediately before administration into patients. However, the 4-to-5 day period required to perform a standard *in vitro* suppression assay is a distinct kinetic disadvantage for this technique, because T_{regs} intended for therapy will be 4 to 5 days older by the time results are available. As *in vitro* expanded T_{regs} have finite life spans, maintaining suppressive function for a maximum of 4 to 6 weeks *in vitro* (our observations), successful functional assessment prior to adoptive transfer may require a more

rapid test of suppressive efficacy: one available within 12 hours of setting up the assay.

T cell activation is followed within hours by increased expression of surface markers, such as CD25, CD69, CD71, and CD154. CD69, a C-type lectin, is an early activation marker that can be reliably detected by flow cytometry (Mardiney et al., 1996). CD154 (CD40L) is a co-stimulatory molecule expressed on activated T cells that engages CD40 on antigen-presenting cells (APC), resulting in APC activation and T cell help to B cells (Kawabe et al., 1994; Van Kooten and Banchereau, 2000). However, because CD154 is only transiently expressed on the cell surface (Armitage et al., 1992; Yellin et al., 1994), detection of CD154 expression in short-term cultures by flow cytometry can only be achieved by addition of fluorochrome-conjugated anti-CD154 antibody to the cell culture, as CD154-bound antibody remains cell associated even if CD154 is internalized (Chattopadhyay et al., 2005; Frentsch et al., 2005). In cultures with *Staphylococcus* enterotoxin B superantigen, CD154 is maximally expressed by antigen-responsive TCR-Vβ12 $CD4^+$ T cells within 4 to 12 hours, and high expression is maintained up to 24 hours (Chattopadhyay et al., 2005; Frentsch et al., 2005). CD154 expression identifies activated $CD4^+$ cells as evidenced by both subsequent cytokine production and cell proliferation. In this model, CD69 is also highly expressed on CD154-expressing cells at 6 hours. Consequently, staining for both CD154 and CD69 allows estimation of T_{con} activation in short-term T cell cultures, and inhibition of this activation by T_{regs} can act as an early readout for T_{reg}-mediated suppression.

This chapter investigates the role of a rapid, 7-hour assay in the functional assessment of both freshly isolated PB $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} from healthy

donors and T_{regs} expanded *in vitro* from PB $CD4^{+}CD25^{hi}CD127^{lo}$ precursors from healthy donors, in comparison with the current gold standard: a 96-hour assessment of T_{reg}-mediated inhibition of T_{con} proliferation. This 7-hour flow-based assay takes advantage of T_{reg}-mediated inhibition of expression of CD154 and CD69 on T_{cons} stimulated with anti-CD3/anti-CD28 beads. Suppression of T_{con} surface marker expression at 7 hours by both freshly isolated $CD4^{+}CD25^{hi}CD127^{lo}$ T_{regs} and *in vitro* expanded T_{regs} was compared with inhibition of CFSE dilution and cytokine production at 4 days, demonstrating significant correlation between the novel and gold standard assays of T_{reg} function. These data were then used to develop critical values for the 7-hour assay as a "diagnostic test" of freshly isolated and *in vitro* expanded T_{reg} function.

This 7-hour assay could be incorporated into the protocols of forthcoming clinical trials of T_{regs} in blood, renal and liver transplantation, and autoimmune disease, in order to gain additional phenotypic information by correlating T_{reg}-mediated inhibition of T_{con} activation *in vitro* with biological function *in vivo*. Were *in vitro* functional testing to be incorporated into "lot release" criteria, a 7-hour assay would dramatically shorten the time required for functional testing of cellular products, compared with the current gold standard assay, enabling functional assessment and infusion of GMP-T_{regs} on the same day.

10.2 Description and nomenclature of the fresh T_{reg} population used in this study

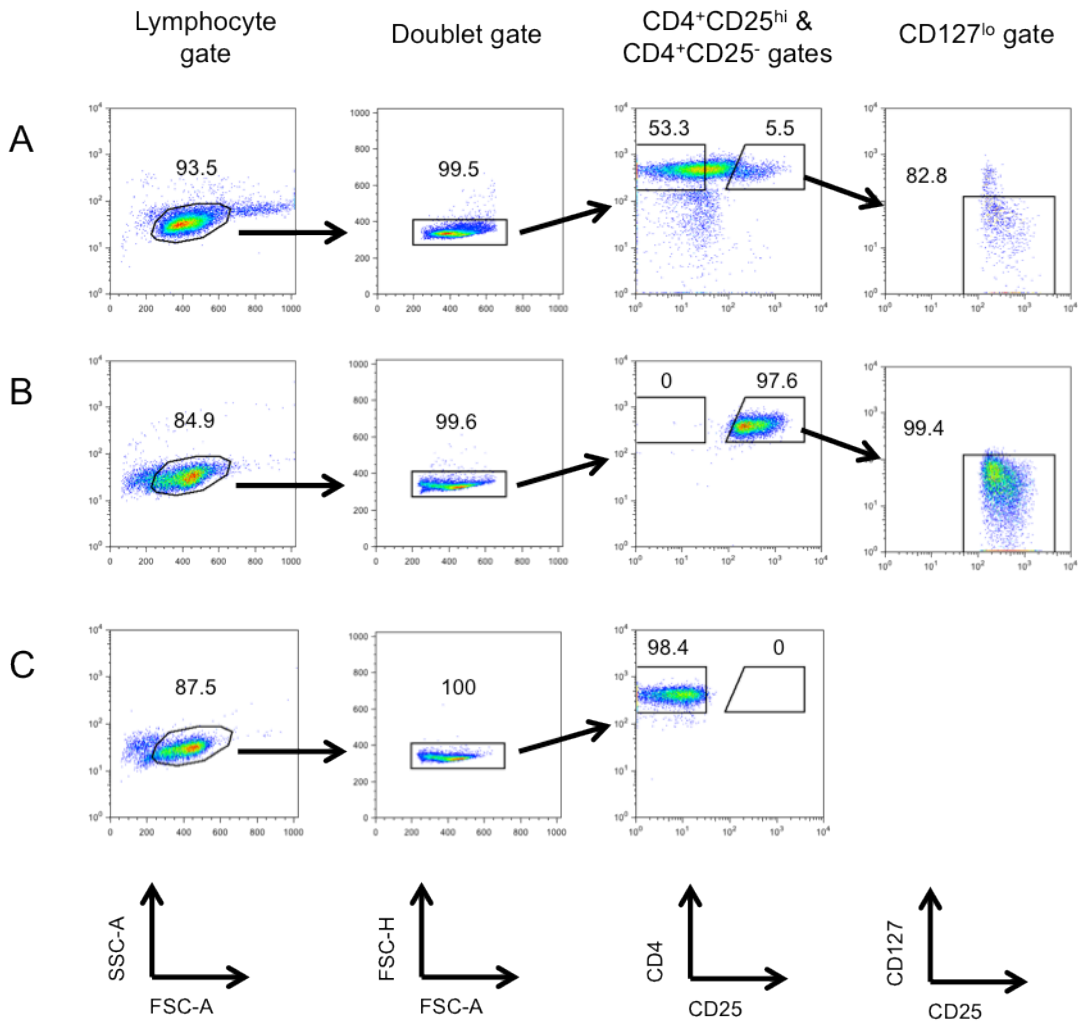
PBMCs were obtained from 10 healthy donor buffy coats (leukocyte collars, NHS Blood) and enriched for $CD4^+$ by MACS negative selection, followed by flow cytometric sorting to $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} (median purity 94.1% [IQR 89.6%-96.5%]; n=10) and autologous $CD4^+CD25^-$ T_{cons} (purity 98.0% [97.6%-98.7%]; n=10) (Figure 10.1 [page 165]). These freshly sorted cell populations were used in experiments involving "freshly isolated" or "fresh" T_{regs}.

10.3 Description and nomenclature of the *in vitro* expanded T_{reg} population used in this study

PBMCs were obtained from 4 healthy donor buffy coats and enriched for $CD4^+$ by MACS negative selection, followed by MACS positive selection for $CD25^+$. The resultant $CD4^+CD25^+$ precursor population was then cultured in X-VIVO15 supplemented with 5% human AB serum, 1000 IU/ml IL-2, 100nM rapamycin and anti-CD3/anti-CD28 Dynabeads at a 1:1 bead:cell ratio (as described in Section 9.8 [page 137]) and as previously published Afzali et al., 2013a; Canavan et al., 2012; Scottà et al., 2013). Additional IL-2 was added every other day and cells were re-stimulated with anti-CD3/anti-CD28 Dynabeads every 10 days. Cells were obtained for the experiments involving *in vitro* expanded T_{regs} after 28 days of culture. Autologous $CD4^+CD25^-$ T_{cons} were obtained at day 0 as described above and stored at -80°C until use. As each T_{reg} population used in this study was quickly shown to suppress activation or proliferation of autologous T_{cons}, the term "putative T_{regs}" is not used in this chapter.

Figure 10.1: Sort strategy to obtain $CD4^+CD25^{hi}CD127^{lo}$ T_{regs}.

(A-C) Representative FACS plots illustrating the sorting strategy to obtain $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} and autologous $CD4^+CD25^-$ T_{cons}. **(A)** PBMCs were enriched for $CD4^+$ lymphocytes by negative selection and stained with the Human Regulatory T Cell Sorting Kit (BD). Gating on lymphocytes and exclusion of doublets allowed identification of $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ populations. The $CD4^+CD25^{hi}$ population was further sorted on the basis of $CD127^{lo}$ expression. **(B)** Post-sort $CD4^+CD25^{hi}CD127^{lo}$ specimen showing >97% purity. **(C)** Post-sort $CD4^+CD25^-$ specimen illustrating 98.4% purity.



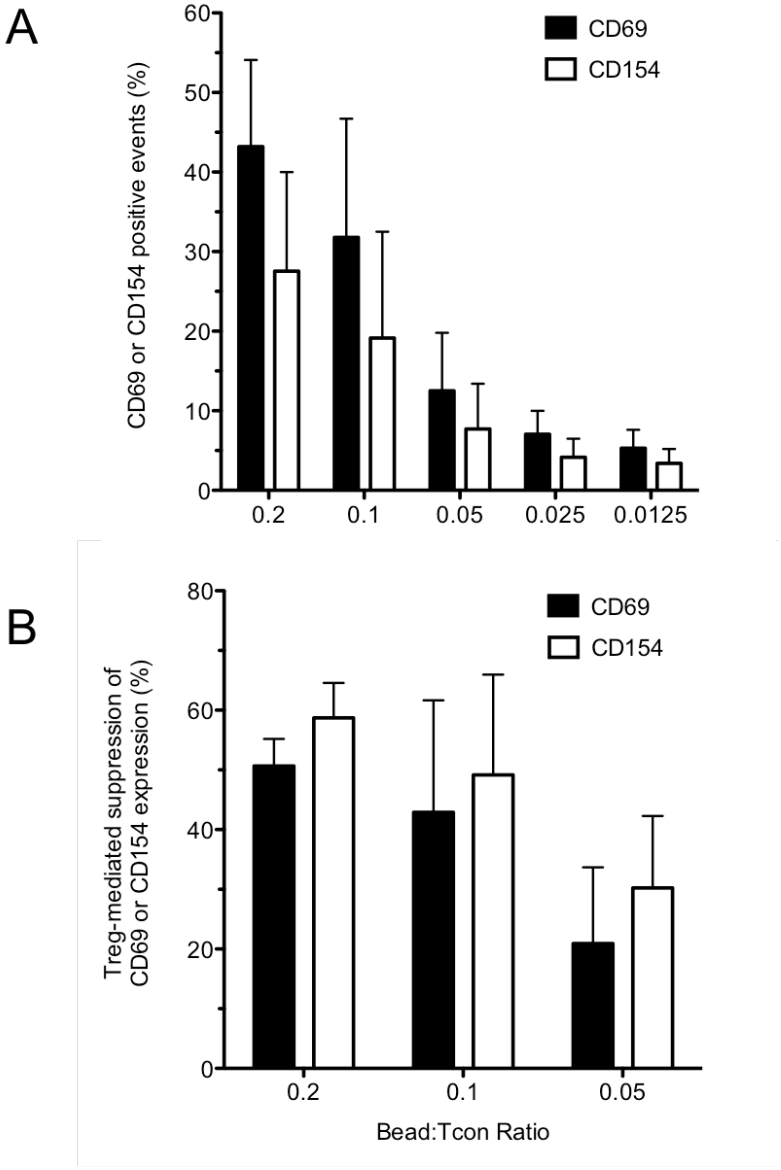
10.4 Optimisation of the 7-hour assay for use with autologous T_{cons} as responder cells: Activation conditions

The BD FastImmune Human Regulatory T Cell Function Kit (BD Biosciences) was optimised by the manufacturer for use with PBMCs as responder cells. This introduced two sources of variability into the assay: (i) the proportion of $CD4^+$ lymphocytes in the PBMCs was *a priori* undefined, and (ii) $CD4^+CD25^{hi}$ T_{regs} were still present in the PBMC population, which may have an effect on the outcome of a co-culture experiment. To remove PBMCs as a potential source of variability, the assay was optimised with autologous $CD4^+CD25^-$ T_{cons} as responder cells.

In order to determine optimum activation conditions using T_{cons} as responder cells, T_{cons} were stimulated with anti-CD3/anti-CD28 coated microbeads at bead:T_{con} ratios ranging from 0.1 (1:2.5) to 0.0125 (1:80) and CD69 and CD154 expression determined at 7 hours (Figure 10.2 A [page 167]). Significant cell death occurred at the 0.1 bead:T_{con} ratio, which did not occur at the other bead:T_{con} ratios tested. Consequently, this condition is not illustrated in the figure. CD69 and CD154 expression fell by 60% between the 0.2 and 0.05 0.1 bead:T_{con} ratios. Next, sorted $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} were co-cultured with autologous T_{cons} at a 1:1 T_{con}:T_{reg} ratio and T_{reg}–mediated suppression of T_{con} CD69 and CD154 expression assessed at 7 hours (Figure 10.2 B. Gating strategy illustrated in Figure 10.3 A-D [page 169]). Maximum T_{reg}–mediated suppression of T_{con} CD69 and CD154 expression was seen at the 0.2 bead:Tcon ratio. Based on these data, all experiments were carried out at a bead:Tcon ratio of 0.2.

Figure 10.2: Optimisation of bead:T_{con} ratio to enable the detection of CD69 and CD154 expression in T_{cons} after 7 hours of activation.

(A) Bar chart showing CD69 and CD154 expression in sorted $CD4^+CD25^-$ T_{cons} cultured for 7 hours, following activation by anti-CD3/anti-CD28 beads at the bead:T_{con} ratios indicated. **(B)** Autologous $CD4^+CD25^{hi}CD127^{lo}$ T_{reg}-mediated suppression of CD69 and CD154 expression in T_{cons} co-cultured for 7 hours with Tregs, following activation by anti-CD3/anti-CD28 beads at the bead:T_{con} ratios indicated. The gating strategy for calculation of T_{reg}-mediated suppression is illustrated in Figure 10.3 A-D (page 169). Graphs summarise the results of two independent experiments using cells from healthy donors. Bar = mean ± SEM.



10.5 Optimisation of the 7-hour assay for use with autologous T_{cons} as responder cells: Gating strategy for analysis

The 7-hour assay depends on an accurate and standardised assessment of CD69 and CD154 expression on T_{cons}. However, T_{regs} may also express CD69 and CD154 on activation. The manufacturer previously developed a gating strategy for the analysis of this assay using PBMCs as responder cells, as described in Section 9.9 (page 137). In this schema, co-cultured T_{regs} were excluded from the analysis by gating on $CD4^+CD25^-$ events, illustrated in Figure 10.3 A-D (page 169), using T_{cons} as responder cells.

In order to validate this gating strategy using T_{cons} as responder cells and to determine if co-cultured T_{regs} needed to be differentially labelled in this assay to facilitate subsequent exclusion from analysis, T_{regs} were labelled with a cell tracking dye (CellTrace Violet, CTV) and co-cultured for 7 hours with autologous T_{cons} at T_{con}:T_{reg} ratios ranging from 1:1 to 32:1. Expression of CD69 and CD154 on T_{cons} was identified using two different gating strategies: (i) Excluding T_{regs} by gating on CTV^- T_{cons}, (Figure 10.3 E, top panel) or (ii) Excluding T_{regs} by gating on the whole lymphocyte population, including CTV^+ T_{regs} and CTV^+ T_{cons} , then gating on $CD25^-$ events (Figure 10.3 E, bottom panel). T_{reg}-mediated suppression of CD69 and CD154 expression was then calculated at each T_{con}:T_{reg} ratio. Analysis of T_{reg}-mediated suppression of CD69 and CD154 expression in both populations yielded similar results (Figure 10.3 F). These data suggest that gating on $CD25^-$ events is sufficient to exclude co-cultured T_{regs} from the analysis and that this strategy yields similar results to an analysis based on CD69 and CD154 expression in the whole T_{con} population, obviating the need to routinely label cell populations in this assay.

Figure 10.3: Gating strategy to assess T_{reg}-mediated suppression of CD69 and CD154 expression in T_{cons}.

(A-D) Illustration of the gating strategy to analyse CD69 and CD154 expression in T_{cons} .

(A) Unstimulated, unstained $CD4^+CD25^-$ T_{cons} were used to identify a CD25 negative gate to exclude $CD25^+$ T_{regs} from further analysis. **(B)** This gate was then applied to unstimulated T_{cons} that were stained with anti-CD25, anti-CD69 and anti-CD154-conjugated antibodies, to identify the threshold for $CD69^+$ and $CD154^+$ events. Stimulated T_{cons} cultured alone **(C)** or at a 1:1 T_{con}:T_{reg} ratio **(D)** were then gated as above to identify T_{reg}-mediated change in CD69 and CD154 expression.

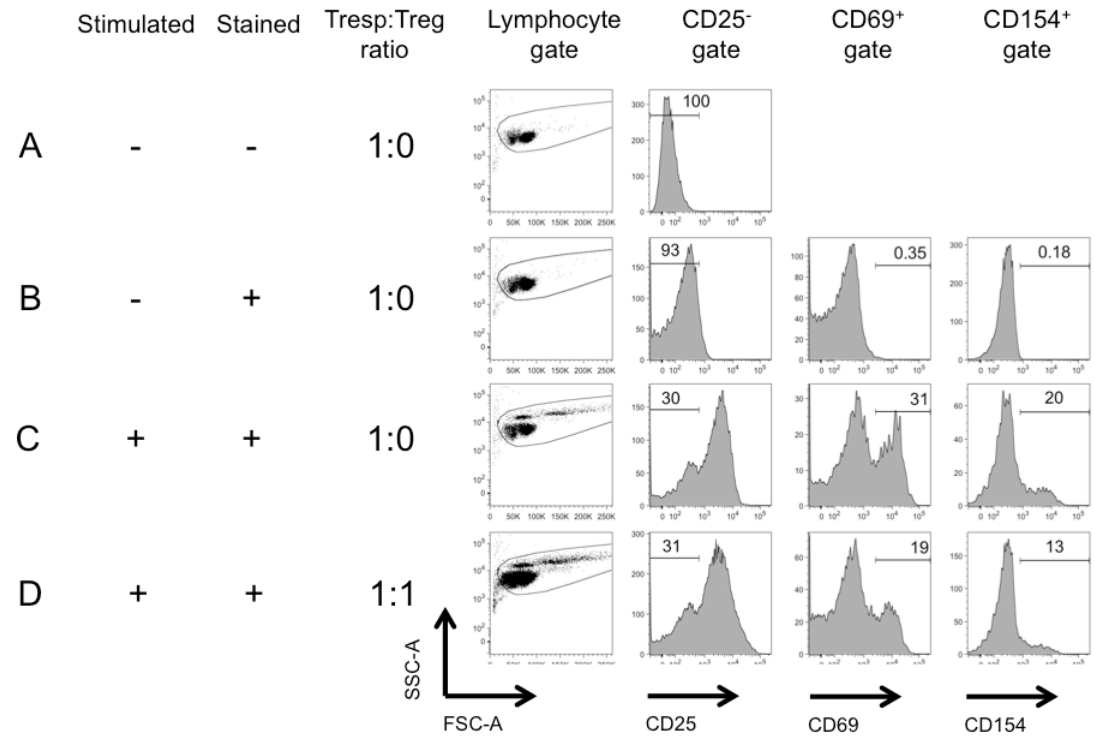
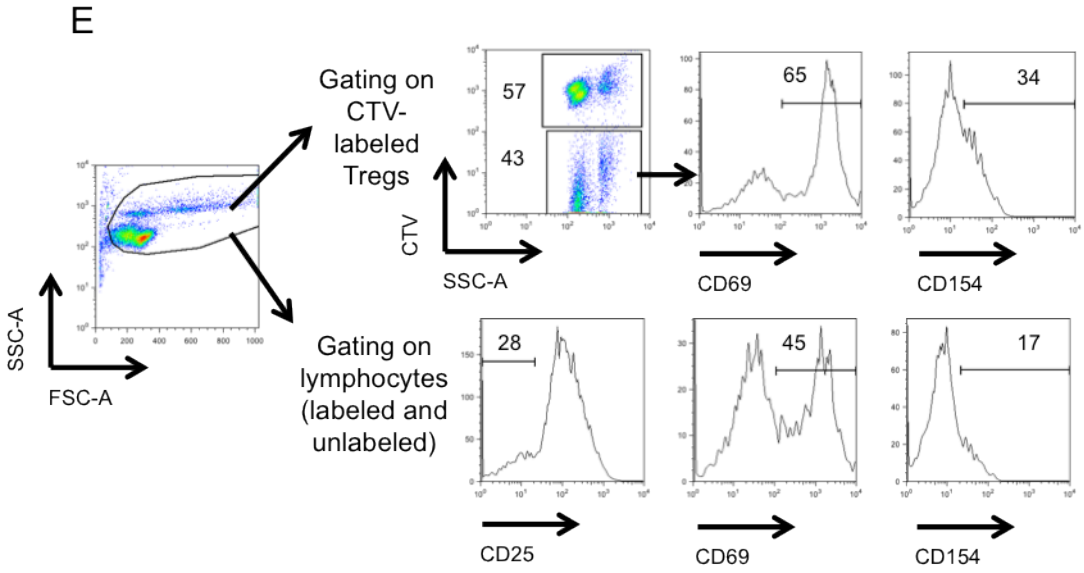
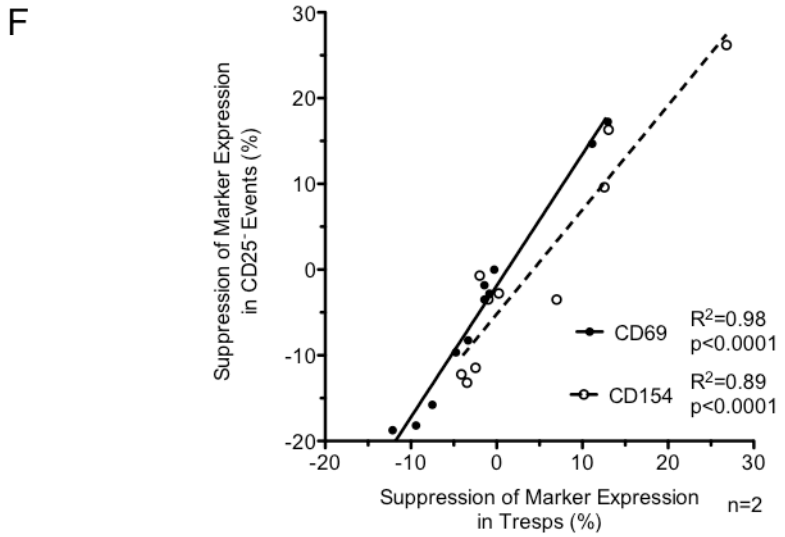


Figure 10.3 (continued): Gating strategy to assess T_{reg}-mediated suppression of CD69 and CD154 expression in T_{cons}.

(E & F) To determine if the exclusion of activated $CD4^+CD25^{int}$ T_{cons} affected the results of the assay, T_{regs} were labelled with CTV and the assay repeated. After 7h, T_{reg}-mediated suppression of CD69 and CD154 was analysed in all (CTV-negative) T_{cons} **(E top panels)** and $CD25^-$ T_{cons} **(E bottom panels)**. CTV labelling reduced the suppressive activity of T_{regs}. **(F)** Even so, excellent correlation was seen between T_{reg}-mediated suppression of CD69 and CD154 expression in T_{cons} gating on $CD25^-$ events in the lymphocyte gate and gating on the whole T_{cons} population: unlabelled T_{cons} in the lymphocyte gate. Pooled data from 2 independent experiments.





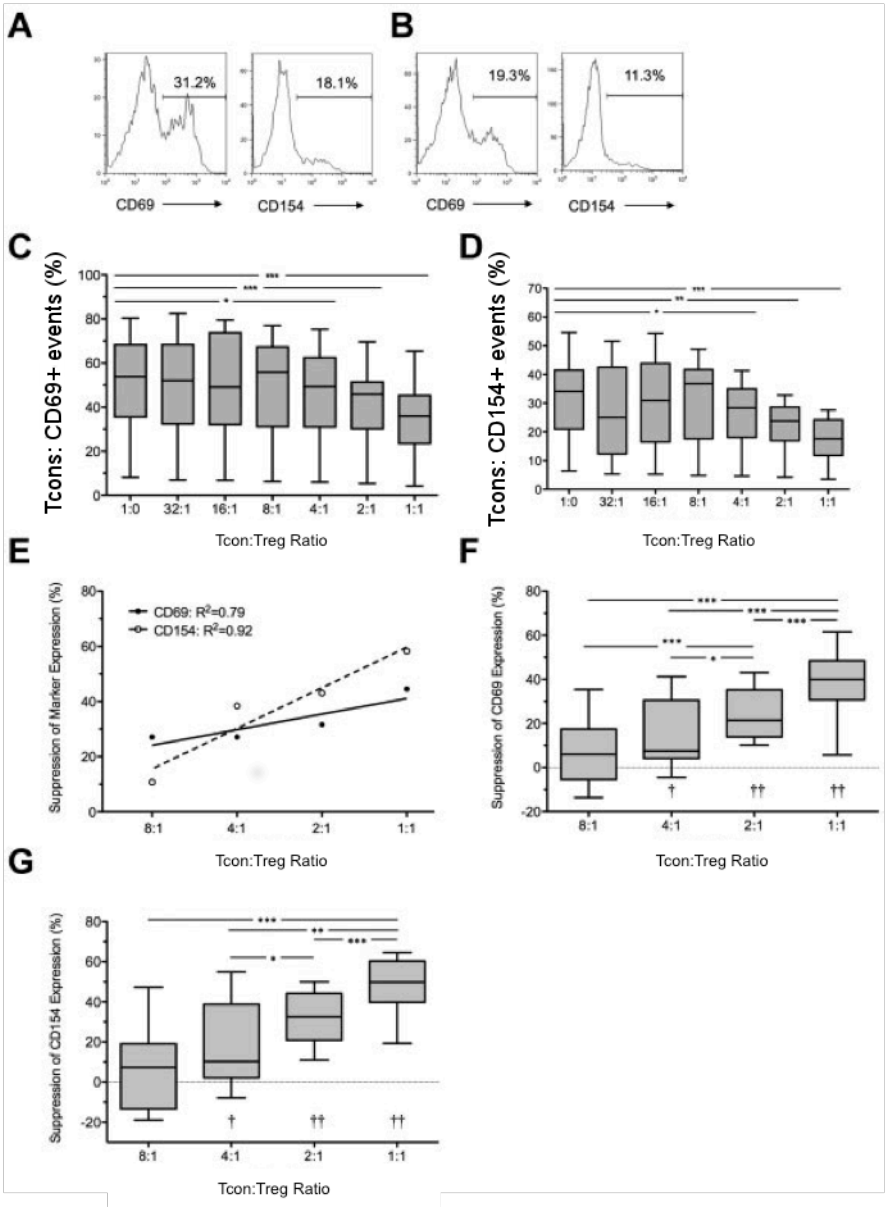
10.6 *In vitro* suppressive effect of freshly isolated T_{regs} at 7 hours

Having optimised the conditions for T_{con} culture and detection of the early activation markers CD69 and CD154 on T_{cons} (see Section 9.9 [page 137]), the ability of freshly isolated T_{regs} to suppress cell surface expression of CD69 and CD154 on T_{cons} after 7 hours of co-culture was assessed. $CD4^+CD25^-$ T_{cons} were cultured both alone and together with autologous freshly isolated $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} at T_{con}:T_{reg} ratios ranging from 1:1 to 32:1 in the presence of anti-CD3/anti-CD28–coated microbeads.

At 7 hours, the median frequency of CD69 and CD154 expression in unstimulated T_{cons} was 2.0% (IQR 1.5%-2.6%, n=10 independent experiments) and 1.8% (0.8%-2.0%, n=10), respectively and in stimulated T_{cons} 54.1% (34.5%-64.5%, n=10) and 34.7% (19%-40%, n=10), respectively (Figure 10.4 A-D [page 172]). CD69 expression in stimulated T_{cons} was consistently higher than CD154 expression ($p<0.001$**;** Figure 10.4 C-D). The addition of autologous T_{regs} at a 1:1 ratio reduced median frequencies of $CD69^+$ and $CD154^+$ events in T_{cons} to 35.9% (23.5%-45.3%, n=10) and 17.6% (11.8%-24.2%, n=10) respectively, median suppression of 40% and 50%, respectively ($p<0.001$ for both comparisons; Figure 10.4 B, E-G). Percentage suppression correlated with the T_{con}:T_{reg} ratio. Statistically significant suppression could only be seen at a ratio of 4:1 (median 7.5% [IQR 4.1%-39.5%]; and 10.2% [2.1%-38.8%] for CD69 and CD154, respectively, n=10), 2:1 (21.4% [13.9%-35.2%] and 32.5% [20.9%-44.2%] for CD69 and CD154, respectively, n=10), and 1:1. At higher dilutions, freshly isolated T_{regs} did not suppress the percentage of $CD69^+$ or $CD154^+$ T_{cons} (Figure 10.4 E-G).

Figure 10.4: Suppression of CD69 and CD154 expression on T_{cons} by autologous, freshly isolated T_{regs}.

(A-B) Representative examples of CD69 and CD154 expression on T_{cons} either cultured alone **(A)** or co-cultured with T_{regs} (**B**, here at a 1:1 T_{con}:T_{reg} ratio). **(C-D)** Cumulative data showing CD69 **(C)** or CD154 expression **(D)** in T_{cons} cultured alone or co-cultured with T_{regs}. Pooled data from 10 independent experiments. **(E-G)** Percentage suppression of CD69 and CD154 on T_{cons} by T_{regs} showing a representative experiment (**E**) and box-and-whisker plots (**F-G**) of pooled data from 10 independent experiments. *p<0.05, **p<0.01, ***p<0.001. †p<0.05, ††p<0.001 with respect to "T_{con} alone" cultures (T_{con}:T_{reg} ratio of 1:0).



Under all conditions, a significant linear relationship was seen between both expression of CD69 and CD154 (R^2=0.85; p<0.0001) and percentage suppression of these markers by T_{regs} (R^2=0.94; p<0.0001).

10.7 Freshly isolated T_{reg}-mediated suppression of T_{con} CD69 and CD154 expression at 7 hours predicts suppression of proliferation at 96 hours

Freshly isolated T_{regs} were cultured with autologous (unlabelled and CFSE-labelled) T_{cons} at T_{con}:T_{reg} ratios ranging from 1:1 to 32:1 and stimulated with anti-CD3/anti-CD28 beads. T_{reg}-mediated inhibition of T_{con} proliferation at 96 hours was compared with T_{reg}-mediated inhibition of CD69 and CD154 expression at 7 hours. The addition of T_{regs} at a 1:1 ratio was associated with a median of 28.2% (IQR 16.3%-42.5%; n=10) suppression of T_{con} proliferation (Figure 10.5 A-C [page 175]). Significant suppression of proliferation was not seen at T_{con}:T_{reg} ratios more dilute than 4:1.

Because suppression of marker expression was only seen at a T_{con}:T_{reg} ratios of more than or equal to 4:1, further comparisons were limited to these experimental conditions. Bland-Altman plots (Bland and Altman, 1986) suggested that suppression of marker expression at 7 hours tended to overestimate suppression of proliferation at 96 hours by (mean±SD) 6.1%±13.7% for CD69 and 11.9%±19.7% for CD154 (Figure 10.5 D & E). A statistically significant linear relationship was found between suppression of marker expression at 7 hours and proliferation at 96 hours for both CD69 (R^2=0.38, p<0.001; Figure 10.5 F) and CD154 (R^2=0.41, p<0.001; Figure 10.5 G).

Next, to determine the usefulness of marker suppression at 7 hours as a "diagnostic test" for suppression of proliferation at 96 hours, quartiles of suppression of proliferation (median=15.8%, upper and lower quartiles of 7.4% and 26.4%) were used as critical values to generate receiver operating characteristic (ROC) curves (Figure 10.5 H-I). CD69 showed good discrimination with an area under the curve (AUC) of 0.93 (95% CI, 0.86-0.99; $p<0.0001$) for suppression of proliferation more than or equal to 7.4%, AUC of 0.77 (0.61-0.93; $p<0.005$) for suppression of proliferation more than or equal to 15.8%, and AUC of 0.78 (0.64-0.93; $p<0.0001$) for suppression of proliferation more than or equal to 26.4% (Figure 10.5 H). CD154 showed similarly good discrimination with AUC for suppression of proliferation more than or equal to 7.4% of 0.89 (0.79-1.00; $p<0.0001$), AUC of 0.77 (0.62-0.93; $p<0.005$) for suppression of proliferation more than or equal to 15.8%, and AUC of 0.82 (0.68-0.96; $p<0.0001$) for suppression of proliferation more than or equal to 26.4% (Figure 10.5 I).

The Youden index (Youden, 1950) was then calculated for each critical value of CD69 and CD154 suppression and graphed to illustrate the range of each assay maximizing discrimination: approximately 33% to 39% suppression for CD69 and 38% to 46% suppression for CD154 (Figure 10.5 J-K). An arbitrary cost was then assigned to each critical value to severely penalize the assay for false positives as false positive results will be the most deleterious to programs of cell therapy. Threshold values of 37.4% and 43% suppression for CD69 and CD154, respectively, minimize the arbitrary cost of the assay and effectively exclude false positives (Figure 10.5 J-K). The diagnostic characteristics of threshold values of CD69 and CD154 suppression at 7 hours in correctly identifying median suppression of proliferation at 96 hours are shown in Table 10.1 (page 178).

Figure 10.5: Suppression at 7 hours predicts suppression of proliferation at 96 hours.

(A) Representative example of a 96-hour CFSE dilution assay, gated on live T_{cons}, cultured alone (left) or at a 1:1 T_{con}:T_{reg} ratio (right). Gates illustrating each division are shown. The number of non-proliferated precursors (as a percent of precursors of all divided cells) is highlighted.

(B-C) Suppression of proliferation at 96 hours at increasing T_{con}:T_{reg} ratios from a representative experiment (**B**) and pooled data from 10 independent experiments (**C**). **(D-E)** Agreement between suppression of CD69 and CD154 expression at 7h and inhibition of proliferation at 96h. Bland-Altman plots showing the limits of agreement (mean and 95% confidence interval) between paired values for freshly isolated T_{reg}-mediated suppression of T_{con} proliferation at 96h ("gold standard") and T_{reg}-mediated suppression of CD69 (**D**) or CD154 (**E**) expression ("comparator" tests) at 7h. **(F-G)** Comparison of suppression of CD69 (**F**) and CD154 (**G**) expression at 7 hours with paired suppression of autologous T_{con} proliferation at 96 hours, with regression lines. (**D-G**) Pooled data from T_{con}:T_{reg} ratios 4:1 to 1:1 from 10 independent experiments. *p<0.05, **p<0.01, ***p<0.001. #p<0.005, ##p<0.0001 with respect to "T_{con} alone" cultures (T_{con}:T_{reg} ratio of 1:0).

Panels A-G are on the following page.

Figure 10.5 (continued): Suppression at 7 hours predicts suppression of proliferation at 96 hours. Panels A-G (legend on previous page).

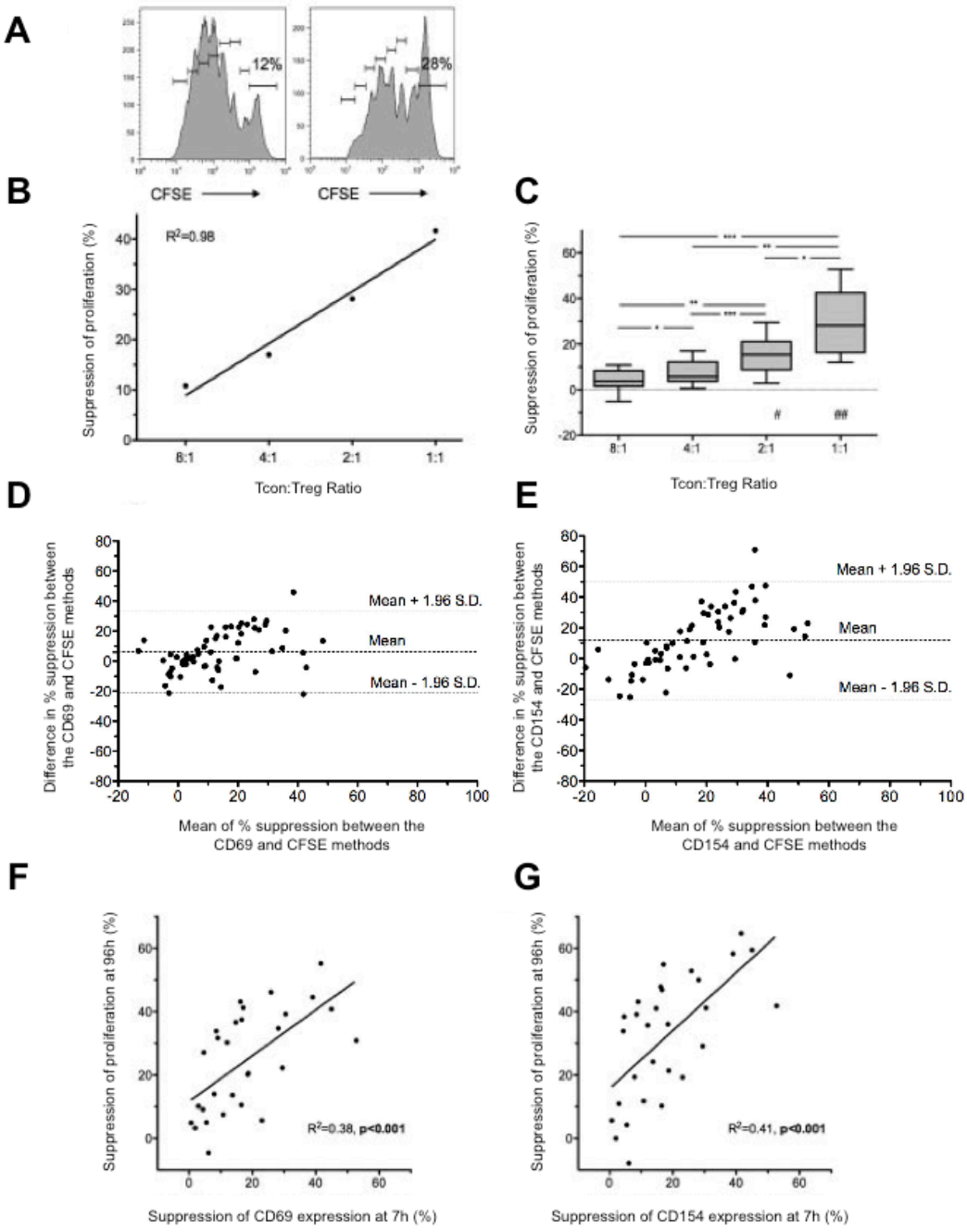


Figure 10.5 (continued): Suppression at 7 hours predicts suppression of proliferation at 96 hours.

(H-I) ROC curves illustrating the performance of CD69 suppression (**H**) and CD154 suppression (**I**) at correctly identifying suppression of T_{con} proliferation at 96 hours, for 3 critical values of the CFSE dilution assay. **(J-K)** Performance of CD69 (**J**) and CD154 suppression (**K**) at correctly identifying median suppression of proliferation. Median and distribution statistics for each marker are also given. The left and right y-axes illustrate the Youden index for threshold values and arbitrary cost of threshold values, penalizing for false positives, respectively. (**H-K**) Pooled data from T_{con}:T_{reg} ratios 4:1 to 1:1 from 10 independent experiments. †p<0.005, ††p<0.0001. AUC indicates Area Under the Curve. UQ indicates upper quartile; and LQ, lower quartile.

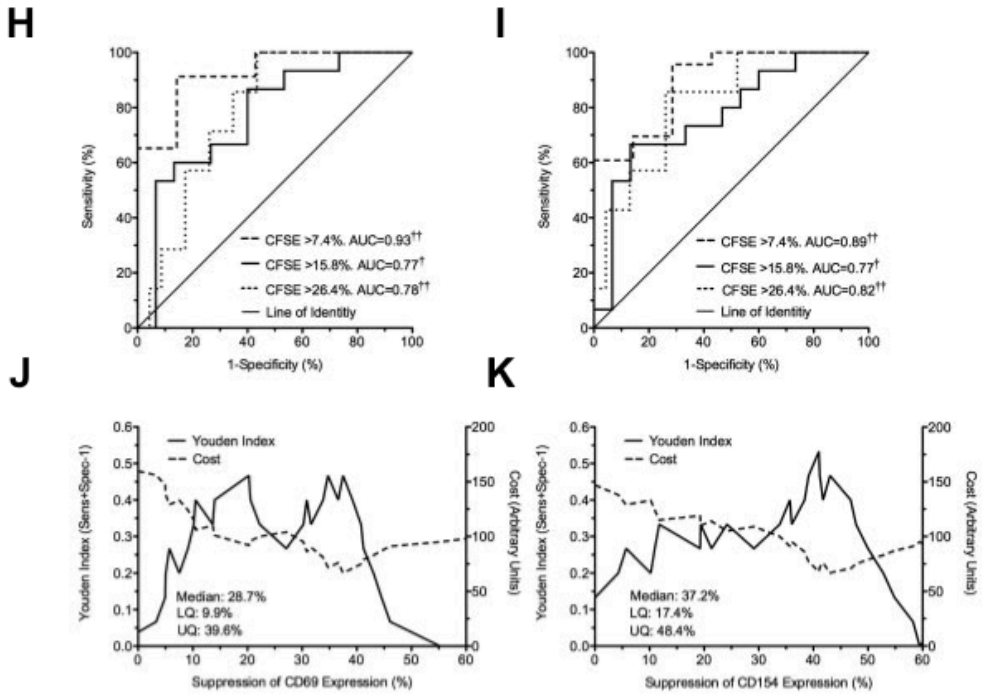


Table 10.1: Critical values of CD69 and CD154 suppression by freshly isolated T_{regs} as a "diagnostic test" for inhibition of proliferation at 96 hours.

Marker and Critical Value	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	LR+	LR-
CD69						
Lower quartile: >9.9%	93% (68-100%)	40% (20-64%)	61%	86%	1.6	0.2
Median: >28.6%	67% (42-85%)	67% (42-85%)	67%	67%	2.0	0.5
Upper quartile: >39.6%	40% (20-64%)	93% (68-100%)	86%	61%	6.0	0.6
Minimum cost: 37.4%	53% (30-75%)	93% (68-100%)	89%	67%	8.0	0.5
CD154						
Lower quartile: >17.4%	86% (61-97%)	40% (20-64%)	59%	75%	1.4	0.3
Median: >37.2%	67% (42-85%)	73% (48-89%)	71%	69%	2.5	0.5
Upper quartile: >48.4%	33% (15-59%)	93% (68-100%)	83%	58%	5.0	0.7
Minimum cost: 43%	53% (30-75%)	93% (68-100%)	89%	67%	8.0	0.5

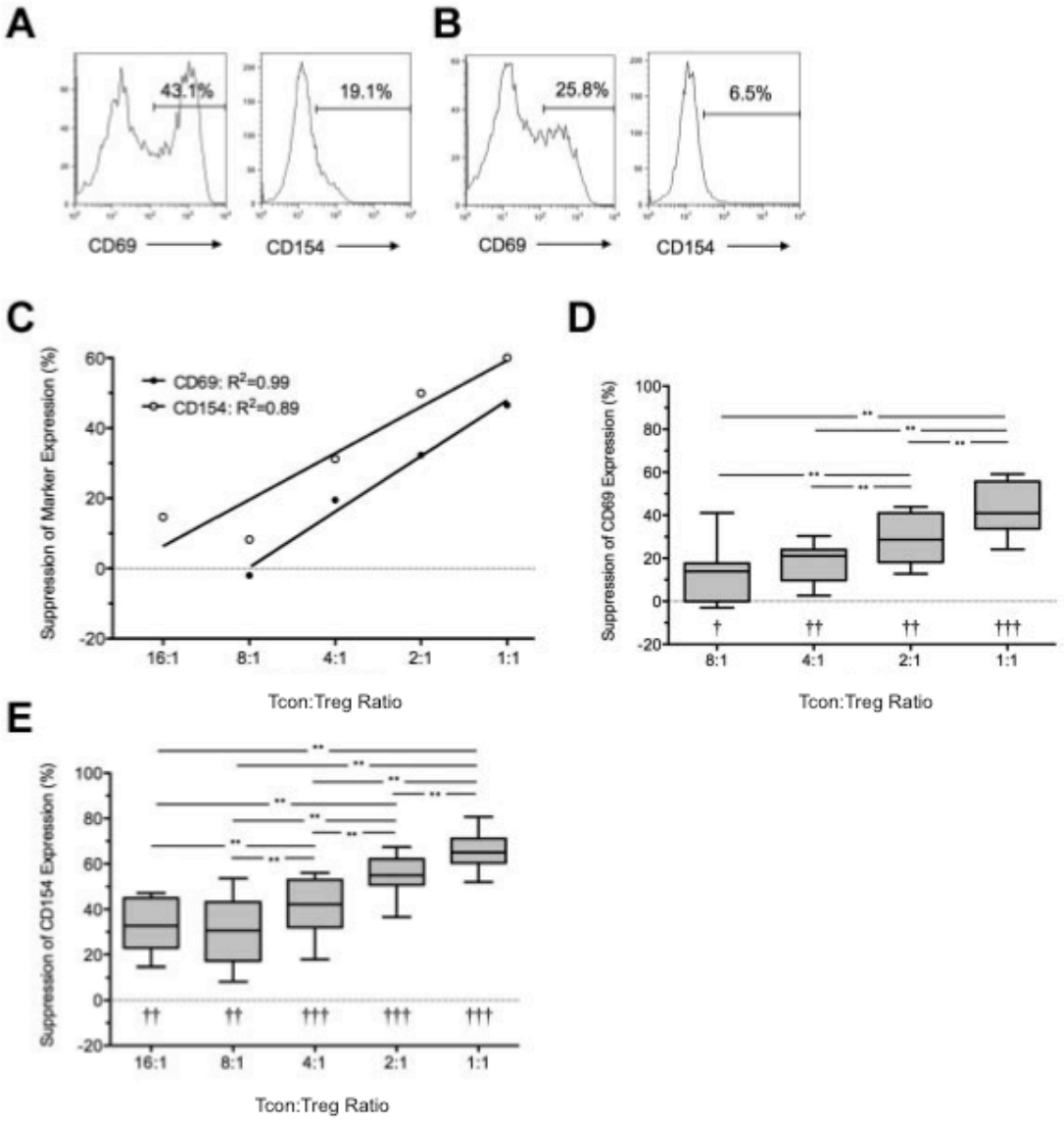
PPV = positive predictive value; NPV = negative predictive value;
LR+ = likelihood ratio of a positive result;
LR- = Likelihood ratio of a negative result; CI = Confidence interval.

10.8 *In vitro* expanded T_{reg}-mediated suppression of T_{con} CD69 and CD154 expression at 7 hours predicts suppression of proliferation at 96 hours

To determine whether suppression of CD69 and CD154 can be used as a test of the suppressive function of *in vitro* expanded T_{regs}, PB $CD4^+CD25^+$ precursor cells were expanded from healthy donors and then used in co-culture experiments with autologous T_{cons}. *In vitro* expanded T_{regs} suppressed both CD69 and CD154 at 7 hours (Figure 10.6 [page 180]) and proliferation at 96 hours (Figure 10.7 [page 183]).

Co-culture of T_{cons} with *in vitro* expanded T_{regs} at a 1:1 ratio resulted in median suppressions of 41% and 65% for CD69 and CD154 expression, respectively ($p<0.01$ compared with T_{cons} alone for both comparisons; Figure 10.6 A-C). As before, percentage suppression correlated with the T_{con}:T_{reg} ratio, although suppression of CD69 was not seen at T_{con}:T_{reg} dilutions less than 8:1 (Figure 10.6 D), in contrast to suppression of CD154, which was observed down to a dilution of 16:1 (Figure 10.6 E).

Figure 10.6: Suppression of CD69 and CD154 expression on T_{cons} by *in vitro* expanded T_{regs}. **(A-B)** Representative examples of CD69 and CD154 expression on T_{cons} either cultured alone (**A**) or co-cultured with *in vitro* expanded T_{regs} (**B**, here at a 1:1 T_{con}:T_{reg} ratio). **(C-E)** Percentage suppression of CD69 and CD154 expression on T_{cons} by T_{regs}, showing a representative experiment (**C**) and box-and-whisker plots of pooled data from 8 independent experiments (**D-E**). Please note that the regression line for CD69 extends only from the 8:1 to 1:1 conditions as suppression was barely visible below the 4:1 ratio. **p<0.01. †p<0.05, ††p<0.001, †††p<0.0001 with respect to "T_{con} alone" cultures (T_{con}:T_{reg} ratio of 1:0).



In vitro expanded T_{regs} potently suppressed T_{con} proliferation (median suppression of 77.9% [IQR 56.3%-91.1%; n=8] at 1:1 ratio; Figure 10.7 A-C; [page 183]), with significant suppression of proliferation observed at all T_{con}:T_{reg} dilutions down to 16:1 (Figure 10.7 B-C), agreeing with previous observations that *in vitro* expanded T_{regs} are stronger suppressors than freshly isolated T_{regs} (Afzali et al., 2013a; Cao et al., 2010).

Bland-Altman plots suggested that suppression of CD69 expression at 7 hours tended to underestimate regulation of proliferation at 96 hours by (mean±SD) 17.5%±27.2% for CD69 (Figure 10.7 D). However, better agreement was seen between suppression of CD154 and suppression of proliferation (0.6%±21.9%; Figure 10.7 E). Because inhibition of marker expression was not seen at T_{con}:T_{reg} ratios less than 8:1 for CD69 and less than 16:1 for CD154, further comparisons were limited to the 8:1 to 1:1 and 16:1 to 1:1 conditions for these markers, respectively. A statistically significant linear relationship was found between suppression of CD69 expression at 7 hours and proliferation at 96 hours (R^2=0.24, p<0.01; Figure 10.7 F). However, the linear relationship between suppression of CD154 expression and suppression of proliferation was stronger (R^2=0.46, p<0.0001; Figure 10.7 G).

To determine the usefulness of marker suppression at 7 hours as a "diagnostic test" for suppression of proliferation at 96 hours, quartiles of suppression of proliferation (median=56.9%, IQR=30.9%-78.4% for CD69 tests; and median=40.2%, IQR=17.7%-68.7% for CD154) were used as critical values to generate ROC curves (Figure 10.7 H-I). CD69 showed poor discrimination with an AUC of 0.68 (95% CI, 0.44-0.92; p=not significant) for suppression of proliferation

more than or equal to 30.9%, AUC of 0.72 (0.54-0.89; $p<0.05$) for suppression of proliferation more than or equal to 56.9%, and AUC of 0.78 (0.64-0.92; $p<0.0001$) for suppression of proliferation more than or equal to 78.4%. In contrast, CD154 showed good discrimination with AUC for suppression of proliferation more than or equal to 17.7% of 0.81 (0.69-0.93; $p<0.0001$), AUC of 0.82 (0.71-0.93; $p<0.001$) for suppression of proliferation more than or equal to 40.2%, and AUC of 0.87 (0.78-0.96; $p<0.0001$) for suppression of proliferation more than or equal to 68.7%.

The Youden index was also calculated for each critical value of CD69 and CD154 suppression to illustrate the range of each assay maximizing discrimination, which turned out to be approximately 15% to 20% suppression for CD69 and 37% to 57% suppression for CD154 (Figure 10.7 J-K). A threshold value of 54% for suppression of CD154 minimized the arbitrary cost of the assay and effectively excluded false positives. In contrast, suppression of CD69 was poor at excluding false positives, only doing so at a threshold of 43.8%. The characteristics of threshold values of CD69 and CD154 suppression at 7 hours in correctly identifying median suppression of proliferation at 96 hours are shown in Table 10.2 (page 186).

Figure 10.7: Suppression at 7 hours by *in vitro* expanded T_{regs} predicts suppression of proliferation at 96 hours.

(A) Representative example of a CFSE dilution assay showing T_{cons} cultured alone (left) or in co-culture with T_{regs} at 1:1 T_{con}:T_{reg} ratio (right). The number of non-proliferated precursors (as a percent of precursors of all divided cells) is highlighted. **(B-C)** Suppression of proliferation at 96 hours at increasing T_{con}:T_{reg} ratios, showing a representative experiment (**B**) and pooled data from 8 independent experiments (**C**). **(D-E)** Bland-Altman plots showing the limits of agreement (mean and 95% confidence interval) between paired values for suppression of proliferation and suppression of CD69 (**D**) or CD154 (**E**) expression using *in vitro* expanded T_{regs}. **(F-G)** Comparison of suppression of CD69 (**F**) and CD154 (**G**) expression at 7 hours with paired suppression of T_{con} proliferation at 96 hours, with regression lines. (**C-G**) Pooled data from 8 independent experiments from T_{con}:T_{reg} ratios 8:1 to 1:1 for CD69 and 16:1 to 1:1 for CD154. *p<0.05. #p<0.05, ##p<0.01, ###p<0.0001 with respect to "T_{con} alone" cultures (T_{con}:T_{reg} ratio of 1:0).

Panels A-G are on the following page.

Figure 10.7 (continued): Suppression at 7 hours by *in vitro* expanded T_{regs} predicts suppression of proliferation at 96 hours. Panels A-G (legend on previous page).

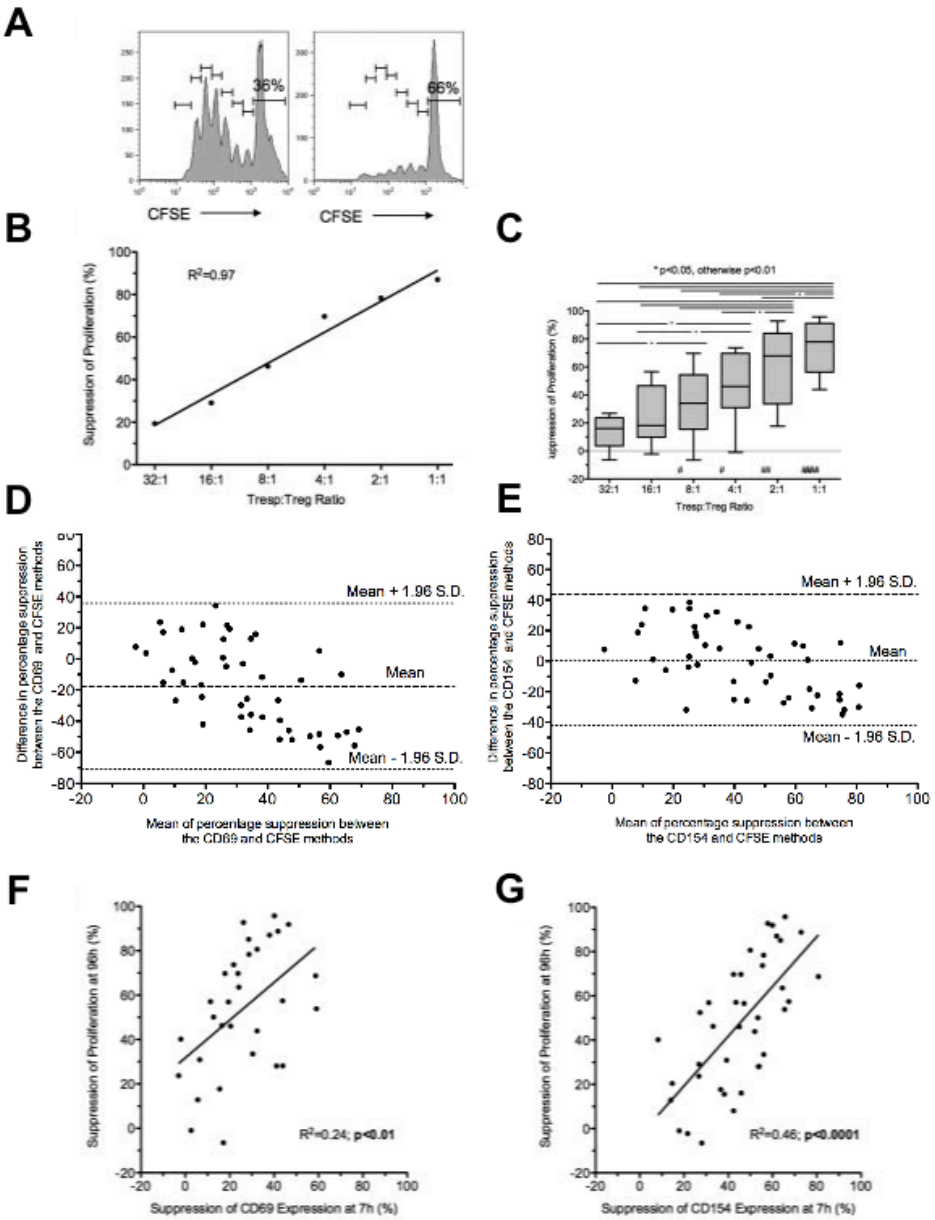


Figure 10.7 (continued): Suppression at 7 hours by *in vitro* expanded T_{regs} predicts suppression of proliferation at 96 hours.

(H-I) ROC curves illustrating performance of CD69 (**H**) and CD154 suppression (**I**) at correctly identifying suppression of T_{con} proliferation at 96 hours, for 3 critical values of the CFSE dilution assay. **(J-K)** Graphs illustrating the performance of CD69 (**J**) and CD154 suppression (**K**) at correctly identifying median suppression of proliferation. Median and distribution statistics for each marker are also given. The left and right y-axes illustrate the Youden index for threshold values and arbitrary cost of threshold values, penalizing for false positives, respectively.

(C-I) Pooled data from 8 independent experiments from T_{con}:T_{reg} ratios 8:1 to 1:1 for CD69 and 16:1 to 1:1 for CD154. *p<0.05, ††p<0.0001.

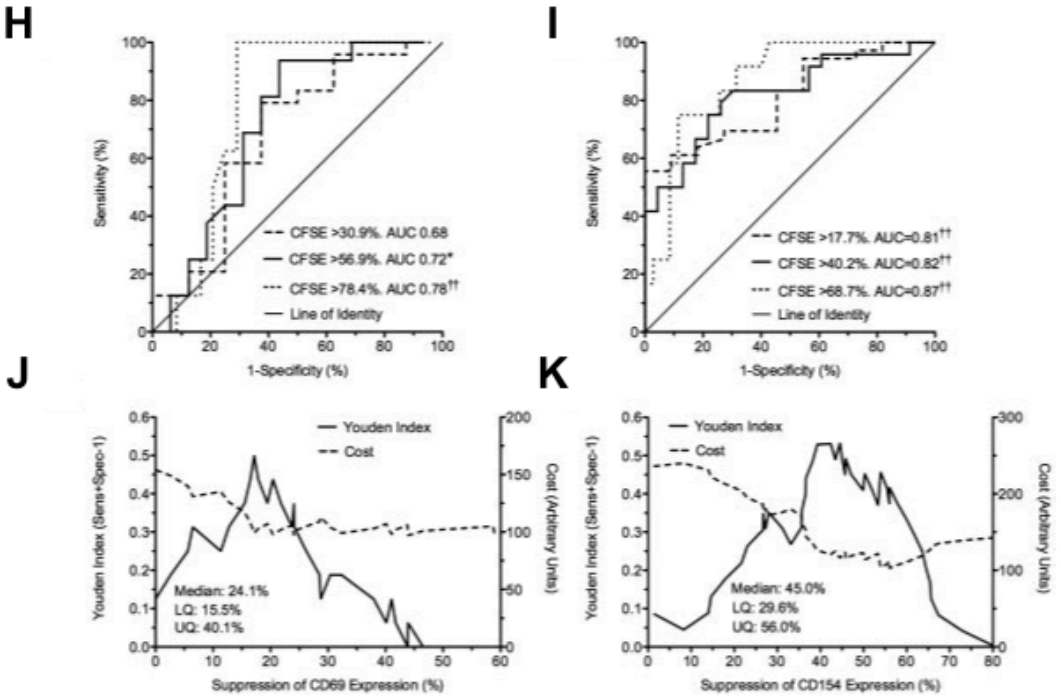


Table 10.2: Critical values of CD69 and CD154 suppression by *in vitro* expanded T_{regs} as a "diagnostic test" for inhibition of proliferation at 96 hours.

Marker and Critical Value	Sensitivity (95% CI)	Specificity (%, 95% CI)	PPV	NPV	LR+	LR-
CD69						
Lower quartile: >15.5%	94% (69-100%)	44% (23-67%)	63%	88%	1.7	0.1
Median: >24.1%	63% (39-82%)	69% (44-86%)	6%7	65%	2.0	0.5
Upper quartile: >40.1%	25% (10-50%)	81% (56-94%)	57%	52%	1.3	0.8
Minimum cost: 43.8%	13% (3-38%)	94% (69-100%)	67%	52%	2.0	0.9
CD154						
Lower quartile: >29.6%	92% (73-98%)	44% (26-63%)	63%	83%	1.6	0.2
Median: >45.0%	71% (51-85)	78% (58-91%)	77%	72%	3.2	0.4
Upper quartile: >56.0%	41% (32-69%)	96% (77-100%)	91%	61%	9.5	0.6
Minimum cost: 54%	50% (32-69%)	96% (77-100%)	92%	65%	11.5	0.5

PPV = positive predictive value; NPV = negative predictive value;
LR+ = likelihood ratio of a positive result;
LR- = Likelihood ratio of a negative result; CI = Confidence interval.

10.9 T_{reg}-mediated suppression of CD69 and CD154 measured at 7 hours predicts T_{reg}-mediated suppression of cytokine expression by T_{cons} at 96 hours.

Because suppression of T_{con} cytokine production may be as important as suppression of proliferation, the effectiveness of the 7-hour assay to predict inhibition of cytokine production at 96 hours was next determined. Both freshly isolated $CD4^{+}CD25^{hi}CD127^{lo}$ T_{regs} and *in vitro* expanded T_{reg} lines were co-cultured with autologous T_{cons} and T_{reg}–mediated suppression of T_{con} CD69 and CD154 expression was compared with T_{reg}–mediated suppression of cytokine expression in co-culture supernatants at 96 hours. Concentrations of IL-2, IFN-γ, IL-10, and IL-17 were measured in 96 hour co-culture supernatants.

Freshly isolated T_{regs} inhibited production of IFN-γ and IL-2 (Figure 10.8 A-B, left panels [page 189]), but not IL-10 nor IL-17. Suppression of both CD69 and CD154 expression on T_{cons} at 7 hours correlated with regulation of IL-2 (R^2=0.42, $p<0.05$ and R^2=0.64, $p<0.001$, respectively) and IFN-γ (R^2=0.47, $p<0.01$ and R^2=0.64, $p<0.05$, respectively) by fresh T_{regs} (Figure 10.8 C left panels).

In vitro expanded T_{regs} potently suppressed IL-2 but increased IFN-γ on activation *in vitro* (Figure 10.8 A-B, right panels). Scottà *et al.* recently showed that *in vitro* expanded T_{regs} can produce IFN-γ both *in vitro* and *in vivo* (Scottà et al., 2013). Consequently, absence of T_{reg}-mediated suppression of IFN-γ may be due to expression of IFN-γ by activated *in vitro* expanded T_{regs}. *In vitro* expanded T_{reg}-mediated inhibition of both CD69 and CD154 at 7 hours correlated with suppression of IL-2 (R^2=0.33, $p<0.001$ and R^2=0.49, $p<0.0001$, respectively). However, an inverse relationship was observed between suppression of CD69 and

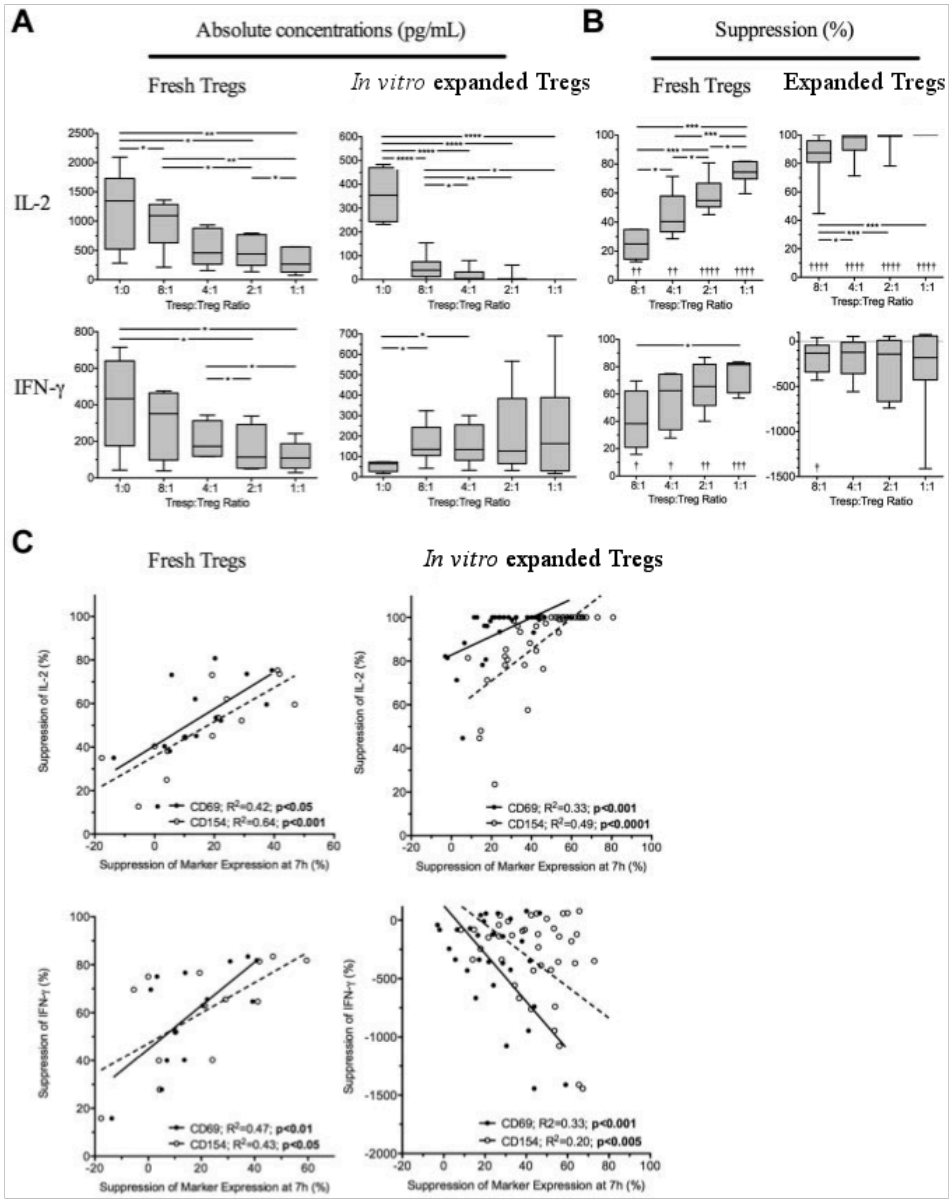
CD154 on T_{cons} and IFN-γ concentrations in supernatants of cultures containing *in vitro* expanded T_{regs} (R^2=0.33, p<0.001 and R^2=0.20, p<0.005, respectively; Figure 10.8 C right panels), supporting the notion that IFN-γ production may not impair *in vitro* expanded T_{reg}-mediated suppression of target cells.

ROC curves constructed to explore the usefulness of marker suppression at 7 hours as a "diagnostic test" for median values of IL-2 suppression at 96 hours showed excellent discrimination for both freshly isolated T_{regs} (AUC of 0.92, 95% CI, 0.87-0.97, p<0.0001; and AUC of 0.98, 0.85-1.0; p<0.0.0001 for CD69 and CD154 suppression, respectively) and *in vitro* expanded T_{regs} (AUC of 0.79, 95% CI, 0.64-0.95; p<0.0001; and AUC of 0.97, 0.84-0.99; p<0.0001; for CD69 and CD154 suppression, respectively). ROC curve analysis also determined that freshly isolated T_{reg}-mediated suppression of CD69 and CD154 on T_{cons} was not useful in discriminating median values of IFN-γ suppression at 96 hours (AUC of 0.74, 95% CI, 0.46-1; p=not significant; and AUC of 0.71, 0.44-0.99; p=not significant; for CD69 and CD154 suppression, respectively). Because *in vitro* expanded T_{regs} did not suppress IFN-γ expression in co-culture supernatants, this analysis was not performed for *in vitro* expanded T_{regs}.

Taken together, these data suggest that suppression of IL-2 at 96 hours can be predicted by suppression of CD69 and CD154 at 7 hours for both freshly isolated and *in vitro* expanded T_{regs}.

Figure 10.8: Suppression of cytokine production at 96 hours is predicable at 7 hours.

(A) Absolute concentrations of IL-2 and IFN-γ in 96-hour culture supernatants from T_{cons} cultured alone or together with freshly isolated T_{regs}, or GMP-T_{regs} at various ratios. **(B)** Suppression of IL-2 and IFN-γ in supernatants by T_{regs}. **(C)** Relationship between suppression of CD69 and CD154 expression at 7 hours and inhibition of IL-2 and IFN-γ at 96 hours. **(A-C)** Cumulative data from 4 independent experiments with each type of T_{reg} showing freshly isolated T_{regs} (left panels) and GMP-T_{regs} (right panels). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. †p<0.05, ††p<0.01, †††p<0.001, and ††††p<0.001 with respect to "T_{con} alone" cultures (T_{con}:T_{reg} ratio of 1:0).



10.10 Discussion.

T_{regs} are promising agents to reduce the requirement for immunosuppression following solid organ transplantation and for the prevention or treatment of a number of inflammatory diseases in humans. This position is supported not only by murine data demonstrating these predicates *in vivo* but also recent studies showing that human T_{regs} can be expanded in large numbers *in vitro* while retaining their suppressive and phenotypic characteristics (Battaglia et al., 2006; Golovina et al., 2011; Hippen et al., 2011b; Hoffmann et al., 2006; 2004; Putnam et al., 2009; Scottà et al., 2013; Strauss et al., 2007b; 2009; Tresoldi et al., 2011). A protocol for GMP-grade expansion of antigen-specific T_{regs} for use in a forthcoming clinical trial has recently been published (Putnam et al., 2013). Furthermore, a recent phase I clinical trial of *in vitro* expanded T_{regs} for the prophylaxis of GvHD (Brunstein et al., 2011), a case series of T_{reg} therapy for the treatment of acute and chronic human GvHD (Trzonkowski et al., 2009) and a phase I clinical trial in paediatric T1DM (Marek-Trzonkowska et al., 2012) suggest that large-scale T_{reg} therapy is likely to be safe in humans. A number of groups demonstrated in humanized mouse models that *in vitro* expanded T_{regs} can prolong survival of human skin transplants (Issa et al., 2010; Sagoo et al., 2011) and prevent transplant arteriosclerosis (Nadig et al., 2010), suggesting that T_{reg} cell therapy may be a successful strategy in solid organ transplantation with the intention of reducing a requirement for short- or long-term immunosuppression, or chronic rejection.

Quantification of T_{reg}–mediated inhibition of T_{con} proliferation, assessed by CFSE dilution or thymidine incorporation, is the current gold standard assay for

the *in vitro* assessment of T_{reg} function. Lack of availability of a simple and rapid assay to assess T_{reg} function before infusion into humans has been a disadvantage in the planning of trials of cell therapy because freshly isolated or *in vitro* expanded T_{regs} are 4 to 5 days older by the time results from traditional assays of suppression are available, with no guarantee that their suppressive function and phenotype is the same as it was 5 days earlier.

In the present work, the performance of a commercially available flow cytometry-based assay for the determination of T_{reg}-mediated suppression of T_{con} activation was compared with contemporaneous gold standard 4-day tests of T_{reg}-mediated suppression of T_{con} proliferation and IL-2 production, set up in parallel. Correlation analysis, Bland-Altman plots and ROC curve analysis all demonstrate that T_{reg}-mediated inhibition of CD69 and CD154 expression in T_{cons} at 7 hours predicts T_{reg}-mediated suppression of proliferation and cytokine production in T_{cons} at 96 hours . Because the translation of quantitated *in vitro* suppression to qualitative clinical outcomes is unknown, 3 critical values for suppression of proliferation were arbitrarily defined, based on statistical criteria (the median, lower and upper quartiles of pooled data from multiple experiments) to assess the performance of the CD69 and CD154 assays. ROC curve analysis based on these criteria showed excellent performance of the 7-hour assay relative to proliferation, although CD69 performed less well as a predictive test for GMP-T_{regs} compared with their freshly isolated counterparts. Because clinical therapy is probably most deleterious in the face of false-positive tests of suppression, determination of critical values was weighted to effectively exclude false-positive results. For freshly isolated T_{regs}, the critical values of CD69 and CD154 suppression to minimize false-positive results were 37.4% and 43%, respectively. These values

for GMP-T_{regs} were 43.8% and 54%, respectively. The short assay was highly predictive of gold standard assays of suppression and performed well in comparison.

Publication of this work (Canavan et al., 2012) was narrowly preceded by a publication from the manufacturer (Ruitenberg et al., 2011). Ruitenberg *et al.* expanded sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} from healthy donors *in vitro* for 14 days and then cultured these *in vitro* expanded T_{regs} for 7 hours with autologous PBMCs. In agreement with the present work, Ruitenberg *et al.* showed that *in vitro* expanded T_{regs} reduced CD69 and CD154 expression in $CD3^+CD4^+$ events at 7 hours. Treatment of the co-culture with CpG, a synthetic TLR9 agonist, impaired *in vitro* expanded T_{reg}–mediated suppression of T_{con} CD69 and CD154 expression in $CD3^+CD4^+$ events. Ruitenberg *et al.* also report that *in vitro* expanded T_{reg}–mediated suppression of CD69 in $CD3^+CD4^-$ events (i.e. $CD8^+$ lymphocytes, as PBMCs were stimulated with anti-CD3/anti-CD28 beads) did not correlate with *in vitro* expanded T_{reg}–mediated suppression of $CD3^+CD4^-$ proliferation in a 5-day co-culture assay. However, the results of these assays for $CD3^+CD4^+$ events were not reported. The present work extends this report by robustly showing that the results of the 7-hour assay correlate with T_{reg}–mediated suppression of $CD4^+CD25^-$ T_{con} proliferation and co-culture supernatant IL-2 concentrations at 96 hours, for both freshly isolated and *in vitro* expanded T_{regs}. These novel data suggest that the 7-hour assay can be used as an alternative to the gold standard assay of *in vitro* T_{reg} function.

At present, *in vitro* expanded T_{reg} cell products must meet pre-specified "lot release criteria" prior to potential infusion. Published lot release criteria for *in*

vitro expanded T_{regs} have focussed on the technical results of the manufacturing process, including cell purity, viability and the absence of microbial contamination (Brunstein et al., 2011). CFSE dilution assays have been used in recent clinical trials of *in vitro* expanded T_{regs} "for information" only, meaning that the release of a cell product lot has not been dependent on the result. As the relationship between *in vitro* suppressive ability and *in vivo* biological activity following infusion becomes clearer with greater clinical trial experience, it may become worthwhile to assess the suppressive capability of T_{regs} immediately before administration into patients. A 7-hour assay might be ideal for this purpose, as it would facilitate assessment and infusion of cells on the same working day. With this in mind, critical values of the 7-hour assay for predicting T_{reg}-mediated suppression of T_{con} proliferation at 96 hours were generated to show, in principal, how the 7-hour assay might be used to assess *in vitro* expanded T_{regs}. The "cut-off" values were deliberately conservative to exclude false-positive results, as "making the cut" at 7 hours might lead to T_{reg} infusion the same day, while failing to "make the cut" might require completion of the 96-hour CFSE dilution assay, before a clinical decision on cell infusion. Because protocols to expand and assess GMP-T_{regs} may vary from institution to institution, individual centres may have to determine the optimum critical values for their own T_{reg} cellular products. However, these data can be quickly obtained by incorporating the 7-hour assay into functional assessment protocols during in-house development of GMP-T_{regs}, and such an approach may also strengthen QC and safety data for the registration of subsequent clinical trials.

In vitro expanded T_{regs} were clearly stronger suppressors than their freshly isolated counterparts, both in the 7-hour and 96-hour assays. *In vitro* expanded

T_{regs} also expressed IFN-γ in culture. Far from compromising T_{reg} function, IFN-γ production by T_{regs} has been shown to be both necessary for their function(Sawitzki et al., 2005) and for their ability to suppress Th1 responses *in vivo* in mice (Koch et al., 2009; Oldenhove et al., 2009). Thus, the relationship observed here between higher IFN-γ concentrations in supernatant of *in vitro* expanded T_{regs} and greater suppression of CD69 and CD154 on T_{cons} is particularly interesting. Although a small number of T_{cons} expressed IL-17, *in vitro* expanded T_{regs} did not suppress IL-17 expression in co-culture supernatants. This is consistent with a previous observation that freshly isolated human T_{regs} do not suppress IL-17 expression by $CD4^+$ T_{cons} *in vitro* (Evans et al., 2007).

Assessment of the demethylation status of *in vitro* expanded T_{regs}' *FOXP3* TSDR might be a complementary approach to a rapid assay of *in vitro* expanded T_{reg} function. *FOXP3* TSDR demethylation correlates with stable FOXP3 expression and a regulatory phenotype in T_{regs} obtained from healthy donor PB (Baron et al., 2007; Floess et al., 2007; Polansky et al., 2008) . *FOXP3* TSDR demethylation also correlates with stable FOXP3 expression in *in vitro* expanded T_{regs} (Baron et al., 2007; Hoffmann et al., 2009), although the degree of demethylation maintained in this region following expansion may depend on the precursor population (Ukena et al., 2011). Golovina *et al.* reported that the *FOXP3* demethylation status of T_{reg} lines expanded under various conditions from two healthy donors correlated with the *in vitro* suppressive ability of those lines (Golovina et al., 2011). This assay could also be performed in-house on a same-day basis. However, as with the 7-hour T_{reg} functional assay, a correlation between *FOXP3* TSDR demethylation status and *in vivo* biological activity will have to be determined. This will be particularly interesting, as TSDR demethylation status is inversely proportional to

cytokine expression in freshly isolated PB T_{regs} from healthy donors, whereas evidence from murine models suggests that T_{regs} co-opt the cellular machinery associated with Th1 (Koch et al., 2009; Oldenhove et al., 2009), Th2 (Zheng et al., 2009) and Th17 (Chaudhry et al., 2009) polarisation to home to inflamed sites and suppress Th1, Th2 or Th17-mediated inflammation, respectively.

In conclusion, this 7-hour assay evaluating T_{reg}-mediated suppression of CD69 and CD154 expression in co-cultured T_{cons} was predictive of T_{reg}-mediated suppression of T_{con} proliferation at 96 hours, for both freshly isolated and *in vitro* expanded T_{regs} from healthy donors. The 7-hour assay has a significant kinetic advantage over a 96-hour assay of T_{reg} function. Functional assessment of *in vitro* expanded T_{reg} lots should be included for information in forthcoming clinical trials of T_{reg} cell therapy, as correlation between *in vitro* suppressive capacity and *in vivo* biological activity may provide insights into the biology of adoptively transferred *in vitro* expanded T_{regs} in a human system. Furthermore, as additional data become available, a role for this assay in the formal assessment of *in vitro* expanded T_{reg} lots prior to infusion to humans may become apparent.

11 Results (2): The peripheral blood $CD4^+CD25^{hi}CD127^{lo}$ $CD45RA^+$ T cell subset may be an appropriate population for *in vitro* expansion for future use as a T_{reg}-based autologous cell therapy for Crohn's disease

11.1 Introduction.

Thymically-derived $FOXP3^+$ regulatory T cells are key mediators of peripheral tolerance and are likely to have a role in preventing inappropriate mucosal inflammation in response to bacterial and other luminal antigens (Huibregtse et al., 2007). In mice, depletion of $CD4^+CD25^+$ T_{regs} disrupts oral tolerance (Veltkamp et al., 2006). Adoptively transferred T_{regs} can prevent the onset of colitis or treat established colitis in a number of murine models (Garrett et al., 2007; Maloy et al., 2003; Mottet et al., 2003; Powrie et al., 1993). Disruption of *FOXP3* leads to multisystem autoimmunity in both mice (Brunkow et al., 2001) and humans (Wildin et al., 2000), in which enteropathy is a feature. Disruption of other molecules implicated in T_{reg} function, such as TGF-β, CTLA-4, IL-10R, IL-2 or its receptor subunits, can also be associated with autoimmunity and intestinal inflammation (Sakaguchi et al., 2009).

Many groups have shown that human T_{regs} can be expanded *in vitro* from PB or UCB (Battaglia et al., 2006; Brunstein et al., 2011; Golovina et al., 2011; Hippen et al., 2011b; Hoffmann et al., 2006; 2004; Marek-Trzonkowska et al., 2012; Putnam et al., 2013; Scottà et al., 2013; Strauss et al., 2007b; 2009; Tresoldi et al., 2011; Trzonkowski et al., 2009), through TCR stimulation in the presence of IL-2. *In vitro* expanded human T_{regs} have been shown to promote transplant tolerance

(Issa et al., 2010; Sagoo et al., 2011), prevent transplant arteriosclerosis (Nadig et al., 2010) and graft *vs*. host disease (GvHD) (Ermann et al., 2005; Scottà et al., 2013) in humanized immunodeficient mice reconstituted with PBMCs.

Brunstein *et al.* recently showed that *in vitro* expanded UCB T_{regs} were safe and well tolerated in preventing GvHD following UCB transplantation (Brunstein et al., 2011). This study also provided a signal of efficacy in comparison with historical controls. Trzonkowski *et al*. recently treated two patients with allogeneic *in vitro* expanded PB T_{regs}: one with acute and one with chronic GvHD (Trzonkowski et al., 2009). T_{reg} therapy was well tolerated by both recipients. In addition, autologous *in vitro* expanded T_{regs} therapy was well tolerated in a recent phase I clinical trial of 10 children with T1DM (Marek-Trzonkowska et al., 2012). Phase I clinical trials of autologous T_{reg} cell therapy with the intention of reducing the burden of immunosuppression are currently planned for renal transplantation (The ONE Study) and liver transplantation (ThRIL study) (Leslie, 2011; Putnam et al., 2013).

LP T_{regs} are increased in the mucosa of patients with active CD and decreased in PB, compared to healthy controls (Maul et al., 2005; Reikvam et al., 2011; Saruta et al., 2007). LP T_{regs} taken from inflamed CD mucosa suppress proliferation of T_{cons} obtained from blood but not LP T_{cons} (Fantini et al., 2009), suggesting that at least a component of impaired mucosal T_{reg} function in active CD may be a cell-extrinsic defect due to LP T_{con} resistance to suppression. Activated T_{cons} express an effector-memory phenotype, which confers relative resistance to T_{reg}-mediated suppression (Afzali et al., 2011). LP T_{cons} from CD mucosa also overexpress Smad7, an inhibitor of TGF-β signalling, which confers resistance to

T_{reg}-mediated suppression (Fantini et al., 2009). However, T_{regs} expanded *in vitro* in the presence of rapamycin from the PB of patients with end-stage renal failure (ESRF), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS) and asthma suppress *in vitro* T_{con} proliferation more potently than freshly isolated PB T_{regs} from the same donors (Afzali et al., 2013a; Cao et al., 2010). Cao *et al.* attempted to show that this was also true for PB T_{regs} obtained from CD and UC patients, but their analysis was limited by the number of assays performed on freshly isolated T_{regs} (CD n=1; UC n=2) (Cao et al., 2010). If it can be demonstrated that T_{regs} expanded *in vitro* from CD patient's PB can suppress activation or proliferation of T_{cons} obtained from inflamed CD mucosa or mesenteric lymph node (MLN), thus overcoming previously reported mucosal T_{con} resistance to T_{reg}–mediated suppression, autologous T_{reg}-based cell therapy might become an attractive option to induce remission in active CD.

IL-17 contributes to mucosal homeostasis but has also been implicated in the pathogenesis of CD (MacDonald et al., 2011). Human T_{regs} isolated from healthy donor PB or tonsils can be induced to express IL-17 and the Th17 transcription factor RORC when activated *in vitro* in the presence of IL-1 IL-2, IL-21 and IL-23 (Afzali et al., 2013b; Bettelli et al., 2006; Koenen et al., 2008; Voo et al., 2009). Although major sources of IL-17 in the gut include T_{cons} and TCR$\gamma\delta^+$ T cells, a proportion of LP T_{regs} obtained from inflamed CD mucosa express IL-17 (Hovhannisyan et al., 2011). Th1 T_{reg} plasticity has also been described *in vitro* and *in vivo*, as murine T_{regs} can be induced to express IFN-γ and the Th1 transcription factor T-bet in response to a Th1-polarised inflammatory environment *in vivo* (Koch et al., 2009; Oldenhove et al., 2009). In humans, phenotypically distinct T_{reg} populations can be delineated on the basis of CD45RA expression (Miyara et al.,

2009). $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} (population I: $FOXP3^{lo}CD45RA^+$) are resistant to induction of IL-17 and IFN-γ *in vitro* (Miyara et al., 2009). In contrast, $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ T_{regs} (both population II [$FOXP3^{hi}CD45RA^-$] and population III [$FOXP3^{lo}CD45RA^-$]) can be induced to express IL-17 and IFN-γ *in vitro* (Miyara et al., 2009). A physiological role for T_{reg} plasticity in humans is currently unclear. However, a number of groups have shown that murine T_{regs} utilise the cellular machinery associated with Th1 (IFN-γ, T-bet, CXCR3; Koch et al., 2009; Oldenhove et al., 2009), Th2 (IRF-4; Zheng et al., 2009) or Th17 (STAT3; Chaudhry et al., 2009) polarisation to home to inflamed sites and suppress Th1, Th2 or Th17-mediated inflammation, respectively. In humans, $IL\text{-}17^+$ T_{regs} obtained from healthy donors and patients with end-stage renal failure or CD retain their suppressive ability *in vitro* (Afzali et al., 2013b; Hovhannisyan et al., 2011; Koenen et al., 2008; Voo et al., 2009). However, the potential for adoptively transferred T_{regs} to exacerbate inflammation in CD lesions through the production of pro-inflammatory cytokines is of concern.

This chapter addresses a number of perceived barriers to T_{reg}-based cell therapy in CD. Prior to the development of a cell therapy programme, it will be necessary to define a precursor population from which to expand a putative T_{reg} cell population, to show that this precursor population can be isolated from the PB of patients with CD and to show that these cells can then be expanded *in vitro* using technologies amenable to subsequent transfer to a GMP platform. Furthermore, it is desirable that these expanded putative T_{regs} retain a suppressive phenotype following expansion (assessed by *in vitro* suppression of T_{con} proliferation), in addition to epigenetically stable *FOXP3* expression. It is also desirable to

demonstrate that these cells have the capacity to home to target organs. Finally, it is germane to show that these expanded T_{regs} have the capacity to suppress the *in vitro* activation or proliferation of inflammatory cells obtained from the immune compartments in which the T_{regs} will be expected to exert a suppressive effect *in vivo*, in order to produce a clinical response.

Hoffman *et al.* previously demonstrated that initial T_{reg} enrichment on the basis of $CD45RA^+$ expression was required to expand homogenous and stable T_{reg} cell lines from healthy donors in the absence of rapamycin (Hoffmann et al., 2006). A number of groups subsequently showed that the addition of rapamycin to *in vitro* expansion protocols facilitates the selective expansion of T_{regs}, while inhibiting expansion of contaminating T_{cons} (Battaglia et al., 2006; Golovina et al., 2011; Scottà et al., 2013; Strauss et al., 2007b; 2009; Tresoldi et al., 2011). Using cell enrichment strategies achievable with currently available GMP technologies (although R&D grade reagents were used throughout), I wished to determine if initial enrichment on the basis of $CD45RA^+$ expression was required to generate a homogenous and epigenetically stable T_{reg} population following expansion, in the presence of rapamycin, from the PB of CD patients. The characteristics of T_{regs} expanded from an initial MACS-enriched ($CD8^-$ depleted and $CD25^+$ enriched) cell population were compared with T_{regs} expanded from initial FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ (population I) or $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ (combined populations II and III) populations (Miyara et al., 2009). This study provides data suggesting CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors may be the most appropriate cell population from which to expand T_{regs} *in vitro* for forthcoming clinical trials of autologous T_{reg}-based cell therapy for CD.

11.2 Precursor populations used for *in vitro* T_{reg} expansion

11.2.1 Overview of enrichment strategies

Two general enrichment strategies were pursued to generate cell populations from CD PB, for subsequent *in vitro* expansion:

(i) An exclusively MACS-based strategy involving initial depletion of $CD8^+$ cells, followed by enrichment of $CD25^+$ cells, or;

(ii) A strategy involving initial MACS-based enrichment of $CD4^+$ cells, followed by flow cytometric sorting to either $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ (population I) or $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ (combined populations II & III) Treg populations.

These strategies were designed to be achievable with currently available GMP-grade technologies and reagents, illustrated in Figure 11.1 (page 203). It is important to note, however, that all the experiments described in this chapter were performed with R&D-grade reagents.

11.2.2 Nomenclature used for *in vitro* expanded T_{reg} lines.

For brevity, the following nomenclature for putative T_{regs} expanded from each of these precursor populations will be used:

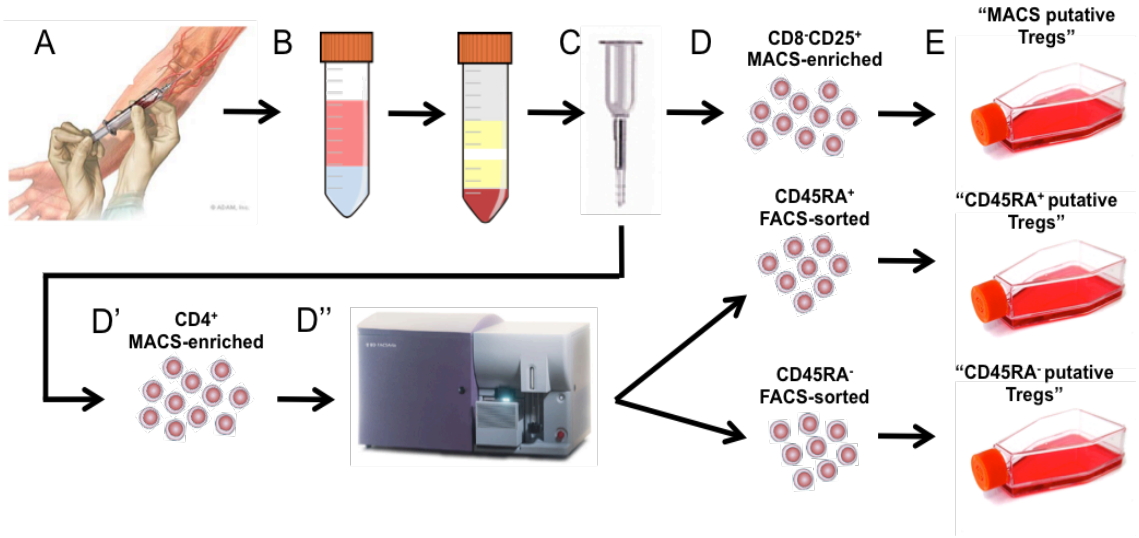
- $CD8^-CD25^+$ MACS-enriched precursors: "MACS putative T_{regs}".
- FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors: "$CD45RA^+$ putative T_{regs}".

- FACS-sorted $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{-}$ precursors: "$CD45RA^{-}$ putative T_{regs}".

Once these expanded cell populations are shown to be "regulatory" by suppressing autologous T_{con} proliferation *in vitro*, the word "putative" will be removed from their shorthand nomenclature.

Figure 11.1: Strategy to determine the optimum enrichment method for *in vitro* expansion of tT_{regs} from PB of CD patients.

(A-E) Cartoon illustrating the strategy used to enrich starting populations for T_{reg} expansion. **(A)** PB was obtained from CD patients and **(B)** PBMCs enriched by density gradient centrifugation. **(C)** Cells for culture were then enriched with MACS technology using two strategies achievable with currently available reagents for the Miltenyi "CliniMACS" system. **(D)** PBMCs were enriched in a two-step selection involving $CD8^+$ depletion, followed by $CD25^+$ enrichment. **(D')** Alternatively, PBMCs were positively selected for $CD4^+$, then stained for CD4, CD25, CD127 and CD45RA, **(D")** followed by sorting on a BD FACSAria II into $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ subsets. **(E)** Each enriched population was stimulated with anti-CD3/anti-CD28 coated Dynabeads and cultured for 20-24 days in X-VIVO15 supplemented with 5% human AB serum, IL-2 and rapamycin. IL-2 was replenished every other day. Cells were re-stimulated and placed in fresh medium at day 12.



11.2.3 Two-step MACS enrichment of a $CD8^{-}CD25^{+}$ precursor population

Professor Lombardi's group recently developed a two-step MACS-based method to enrich a precursor population for subsequent T_{reg} expansion. This method is compatible with currently available reagents from Miltenyi Biotec's GMP-compatible CliniMACS system. A limitation of the CliniMACS system is that a cell population can be labelled for selection or depletion on the basis of cell surface antigen expression only once: i.e. cells that are labelled for positive selection on the basis of $CD4^{+}$ cannot be further enriched by positive selection on the basis of $CD25^{+}$. Consequently, an initial $CD8^{+}$ negative selection was incorporated to remove $CD3^{+}CD8^{+}$ cells, as $CD8^{+}$ lymphocytes can proliferate in the presence of IL-2 and may contaminate an expanded T_{reg} population (Boyman and Sprent, 2012). A subsequent $CD25^{+}$ positive selection was then used to enrich the resulting cell population for CD25 expressing cells, many of which will be $CD4^{+}CD25^{hi}$ T_{regs}. This technique is described in more detail in Section 9.5 (page 134).

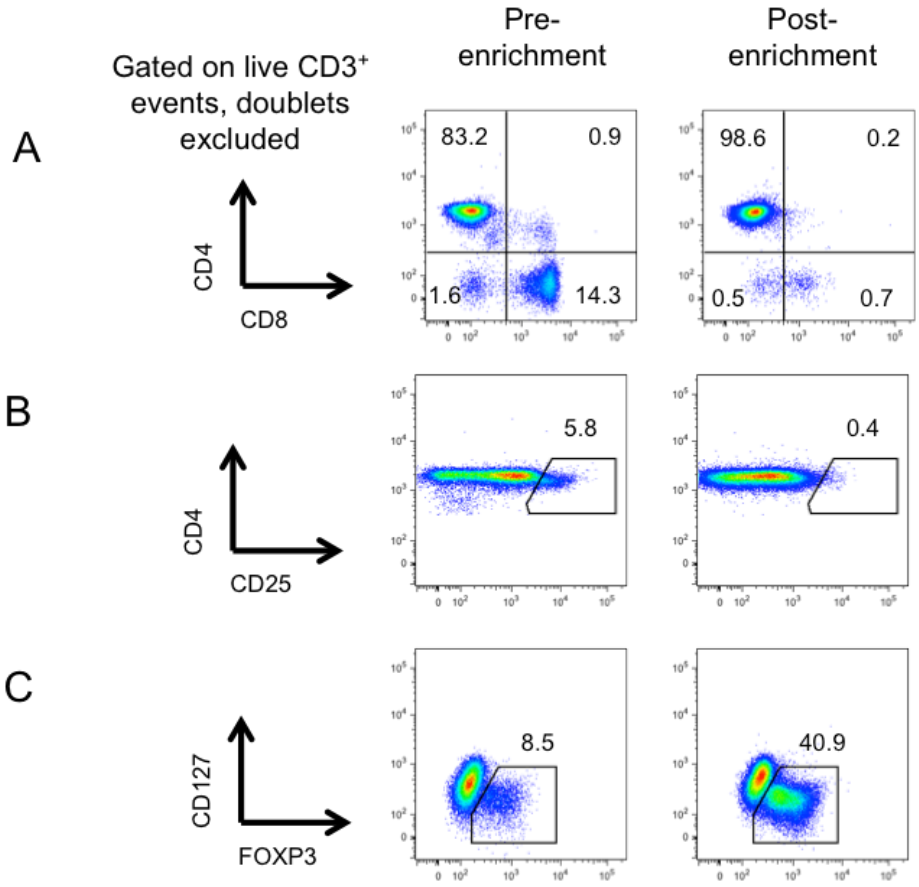
Ten CD patients donated a median of 100ml PB per person (IQR 92.5–100ml). Demographic details are shown in Table 11.1 (page 209). PB was depleted for $CD8^{+}$, followed by $CD25^{+}$ enrichment, as described above. These samples yielded a median of 25,350 live cells/ml (IQR 12,980–41,910 $CD8^{-}CD25^{+}$ cells/ml; trypan blue stain).

$CD8^{-}CD25^{+}$ enriched cells were then phenotyped by FACS. Representative panels are shown in Figure 11.2 (page 205). Following two-step enrichment, live cells consisted of 83.6% $CD3^{+}$ (IQR 72.35–85.25%) and 16.2% $CD3^{-}$ events (IQR 14.4–26.05%). Of the live $CD3^{+}$ events, 90.3% were $CD4^{+}$ (IQR 79.8–94.25%),

50.1% were $CD4^+CD127^{lo}FOXP3^+$ (IQR 41.85–59.15%), and 2.33% were $CD8^+$ (IQR 1.17–4.18%).

Figure 11.2: Two-step MACS enrichment of a $CD8^-CD25^+$ precursor population for subsequent T_{reg} expansion.

(A-C) Representative examples of FACS analyses on PBMCs obtained from CD patients' blood before (left panels) and after (right panels) two-step MACS enrichment for $CD8^-CD25^+$ cells, as described in Section 11.2.3 (page 204). **(A)** Two-step MACS enrichment was associated with enrichment of $CD3^+CD4^+$ events and depletion of $CD3^+CD8^+$ events. **(B)** Anti-CD25 microbeads occasionally interfered with subsequent detection of CD25 on the cell surface by FACS, here using anti-CD25-PE (clone 2A3). **(C)** Consequently, initial T_{reg} purity in these samples was be estimated from $CD127^{lo}FOXP3^+$ events.



11.2.4 Flow cytometric enrichment of $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ precursor populations

In order to determine if initial enrichment on the basis of $CD45RA^+$ expression was required to generate a homogenous and epigenetically stable T_{reg} population following expansion, a two-step process was used to enrich PBMCs to $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ precursor populations for subsequent expansion, as illustrated in Figure 11.1 (page 203). In order to enrich for $CD4^+$ lymphocytes using a method compatible with currently available reagents from Miltenyi Biotec's GMP-compatible CliniMACS system, PBMCs were enriched for $CD4^+$ cells by MACS positive selection. $CD4^+$ cells were then stained for CD4, CD25, CD127 and CD45RA expression and sorted to $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ (population I) or $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ (combined populations II and III) populations (Miyara et al., 2009). This technique is described in more detail in Section 9.7 (page 135).

Thirteen CD patients donated a median of 116ml PB per person (IQR 87.5–150ml). Demographic details are shown in Table 11.1 (page 209). MACS $CD4^+$ positive selection yielded a median of $1.74x10^5$ $CD4^+$/ml (IQR $1.49x10^5$–$4.19x10^5$ $CD4^+$/ml), routinely with >95% purity. A representative panel is shown in Figure 11.3 (page 207).

Subsequent FACS for $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors yielded a median of 1,700 cells/ml (IQR 718–3,487 cells/ml). Post-sort purity was 86.5% (IQR 80.8–91.6%). Subsequent FACS for $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ precursors yielded a median of 3,620 cells/ml (IQR 1,686–6,400 cells/ml). Post-sort purity

was 92.7% (IQR 87.8–94.9%). Representative panels illustrating FACS enrichment are shown in Figure 11.4 (page 208).

Figure 11.3: One-step MACS enrichment of a $CD4^+$ population for subsequent FACS sorting to $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ or $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ populations.

(A) Representative example of FACS analyses on PBMCs obtained from CD patients' blood before (left panel) and after (right panel) one-step MACS positive selection for $CD4^+$ cells, as described in Section 11.2.4 (page 206).

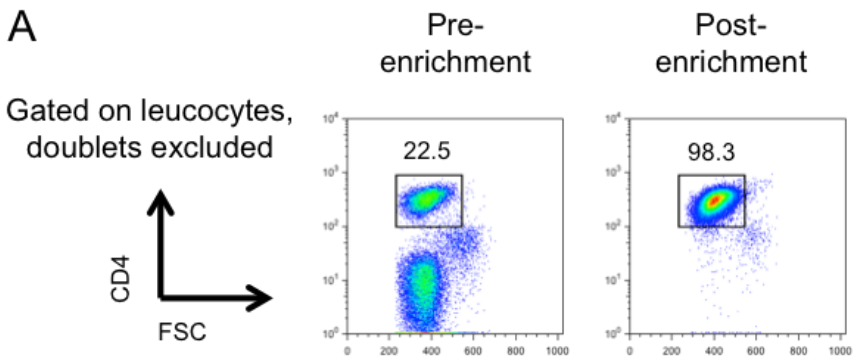
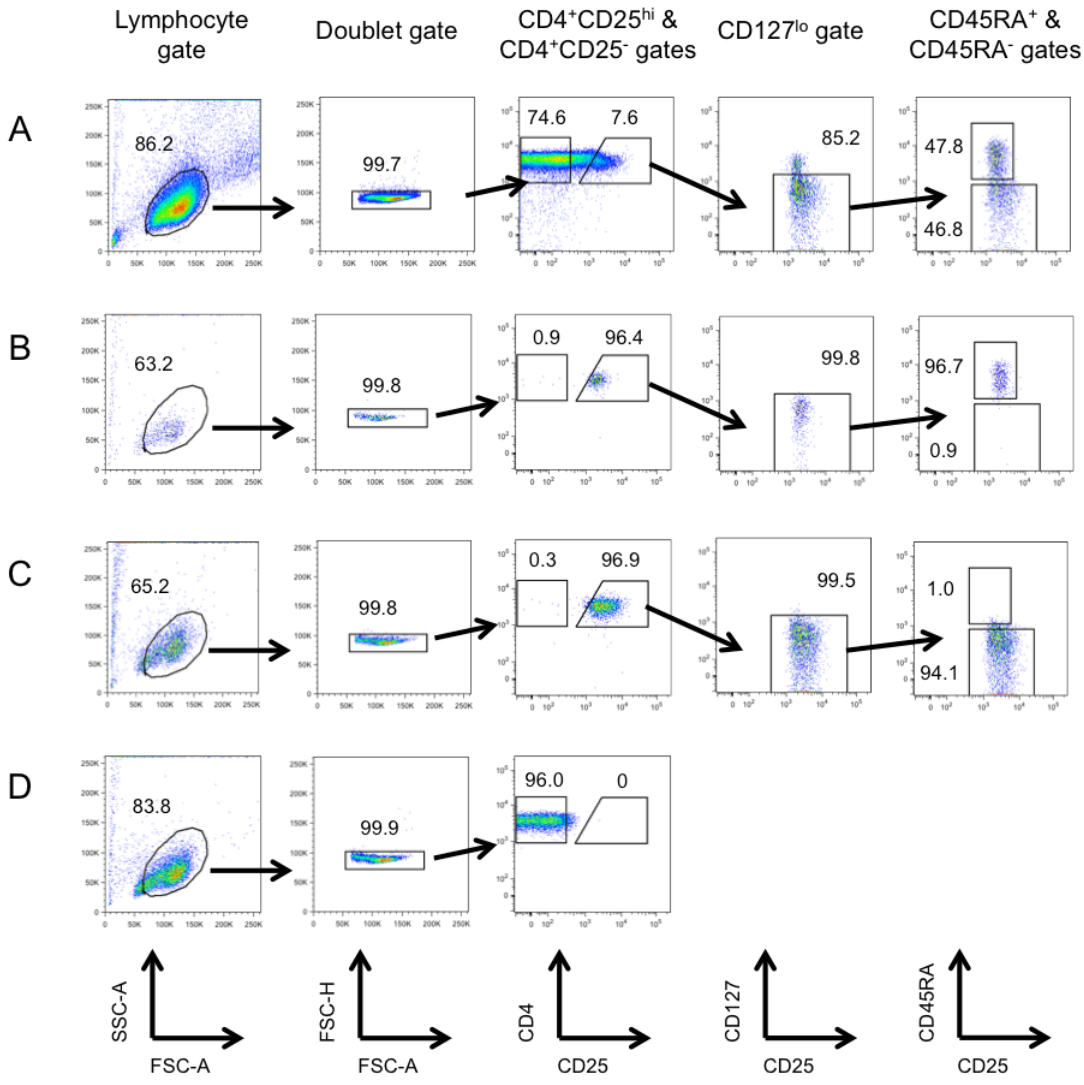


Figure 11.4: FACS sorting strategy to obtain $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ T_{reg} populations for subsequent expansion.

(A-C) Representative FACS plots illustrating the sorting strategy used to obtain $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ populations (and autologous $CD4^+CD25^{lo}$ T_{cons}) for subsequent T_{reg} expansion. **(A)** PBMC were enriched for $CD4^+$ lymphocytes and stained for CD4, CD25, CD127 and CD45RA. Populations for sorting were identified by gating on lymphocytes and excluding doublets, followed by the identification of $CD4^+CD25^{lo}$ T_{cons} and $CD4^+CD25^{hi}$ putative T_{regs}. Putative T_{regs} were then further sorted on the basis of $CD127^{lo}$ and $CD45RA^+$ expression to yield pure $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ populations. **(B)** Sample following sorting for $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ events showing >93% purity. **(C)** Sample following sorting for $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ events, showing >90% purity. **(D)** Sample following sorting for $CD4^+CD25^{lo}$ T_{cons}, showing 96% purity.



11.2.5 Demographic details of patients who donated blood for precursor enrichment and T_{reg} expansion

Ten patients donated blood for T_{reg} expansion following two-step MACS-based enrichment of a $CD8^{-}CD25^{+}$ precursor population. Thirteen patients donated blood for T_{reg} expansion following MACS-based $CD4^{+}$ enrichment and subsequent flow cytometric sorting. Due to limited cell numbers, each donor contributed cells for only one enrichment strategy. As shown in Table 11.1 below, differences between these two groups did not reach statistical significance.

Table 11.1: Demographic details of CD blood donors for T_{reg} expansion.

	MACS alone (n=10)		MACS + FACS (n=13)		p
Female sex	5	(50%)	6	(46.1%)	0.85
Age [years] (median, IQR)	40.9	(30.3-48.6)	40.7	(38.1-49.0)	0.56
Age at diagnosis [years] (median, IQR)	28.2	(16.9-37.9)	20.7	(17.6-36.8)	0.88
Disease duration [years] (median, IQR)	8.5	(4.9-15.6)	17.2	(4.9-21.7)	0.44
Age at Diagnosis*					
Diagnosis <16 y.o. (A1)	2	(20%)	2	(15.4%)	
Diagnosis 17-40 y.o. (A2)	6	(60%)	9	(69.2%)	0.10
Diagnosis >40 y.o. (A3)	2	(20%)	2	(15.4%)	
Location*					
Ileal-only (L1)	2	(20%)	1	(7.7%)	
Colonic only (L2)	2	(20%)	4	(30.8%)	0.63
Ileo-colonic (L3)	6	(60%)	8	(61.5%)	
Concomitant upper GI disease (L4)	0	0	2	(15.4%)	
Perianal disease (p)	2	(20%)	2	(15.4%)	
Disease Behaviour*					
Inflammatory (B1)	4	(40%)	9	(69.2%)	
Stricturing (B2)	2	(20%)	2	(15.4%)	0.32
Penetrating (B3)	4	(40%)	2	(15.4%)	
Disease Activity					
Harvey Bradshaw Index (median, range)	0.5	(0-6)	0	(0-7)	0.66
Active disease HBI >=5	1	(10%)	4	(30.8%)	0.31
Medications and Previous Surgery					
Thiopurines	5	(50%)	7	(53.8%)	1
Anti-TNF agents	3	(30%)	4	(30.8%)	1
Other CD medications	5	(50%)	3	(23.1%)	0.38
Previous Surgery	4	(40%)	8	(61.5%)	0.41

* Montreal classification. Continuous variables compared with Mann-Whitney U tests. Categorical variables compared with χ^2 tests.

11.3 Determination of the optimum re-stimulation frequency for *in vitro* expansion of putative T_{regs} from precursor populations

Many groups have achieved *in vitro* expansion of T_{regs} to yield a suppressive cell population following expansion (Battaglia et al., 2006; Brunstein et al., 2011; Golovina et al., 2011; Hoffmann et al., 2006; 2004; Marek-Trzonkowska et al., 2012; Putnam et al., 2013; Scottà et al., 2013; Strauss et al., 2007b; 2009; Tresoldi et al., 2011; Trzonkowski et al., 2009). Cell expansion is likely to be needed in order to generate the cell numbers required for potential T_{reg}-based cell therapy for CD. In previous studies from our centre involving *in vitro* expansion of T_{regs} from healthy donors, expanding cells were re-stimulated at intervals of either 7 days (Scottà et al., 2013), 10 days (Canavan et al., 2012) or 10-12 days (Afzali et al., 2013a). The proliferation dynamics of precursor populations of *in vitro* expanded putative T_{regs} from the blood of CD patients is unknown. Consequently, the optimum re-stimulation frequency for expansion of putative T_{regs} from these patients was examined.

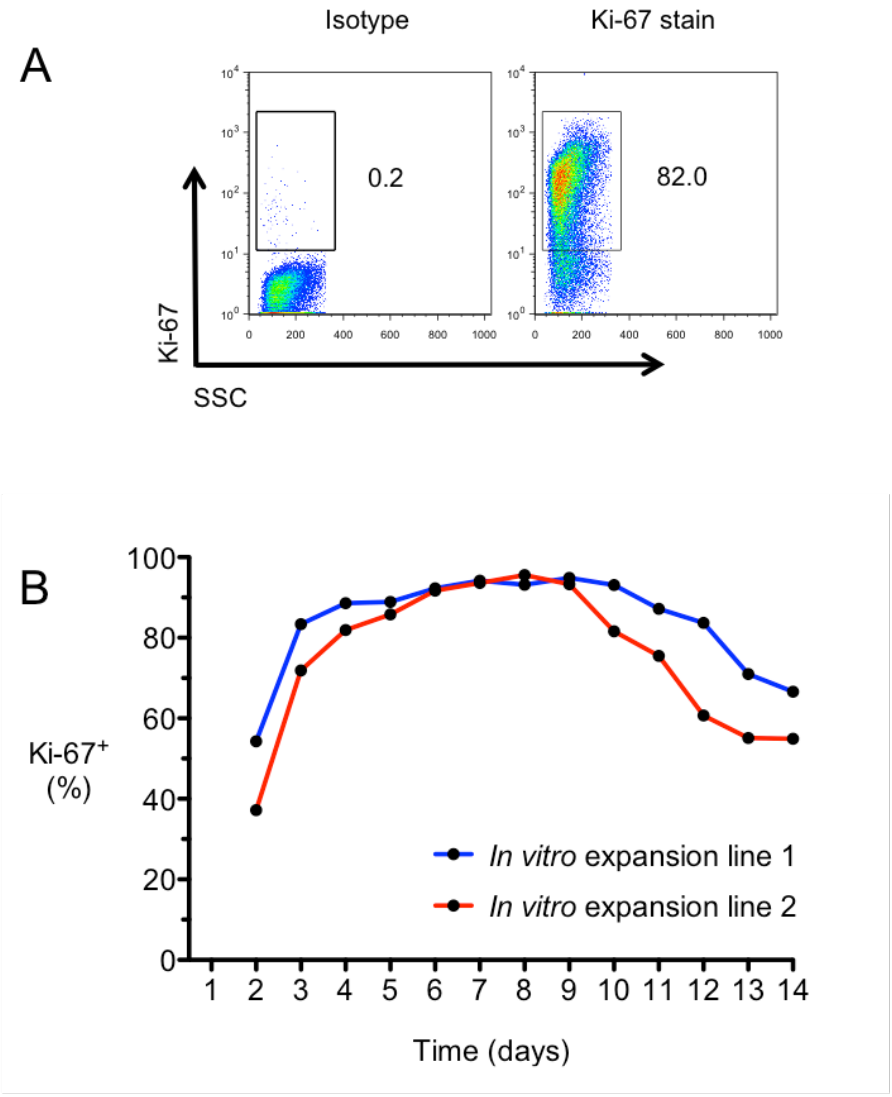
For *in vitro* expansion, freshly isolated $CD4^+$ lymphocytes were enriched by MACS into $CD8^-CD25^+$ precursor populations, as described in Section 11.2.3 (page 204). These cells were then cultured in X-VIVO15 supplemented with 5% human AB serum, 1000 IU/ml IL-2, 100nM rapamycin and anti-CD3/anti-CD28 Dynabeads at a 1:1 bead:cell ratio as described in Section 9.8 (page 137) and previously published(Afzali et al., 2013a; Canavan et al., 2012; Scottà et al., 2013). Additional IL-2 was added every other day.

In order to examine proliferation, $CD8^-CD25^+$ precursors from two healthy donors (leukocyte cones from NHS Blood) were stimulated once with anti-

CD3/anti-CD28 Dynabeads. Proliferation was quantified from days 2-14 of culture by assessment of Ki-67 expression, as shown in Figure 11.5 (page 212). By day 3 following stimulation, clusters of proliferating cells were visible in both lines by light microscopy. Confluent wells were split and re-plated on days 5, 6, 7, 8 and 11; and days 6, 7, 8, 9 and 10, in lines 1 and 2, respectively. By day 11, the cells from both lines had a bland, round appearance by light microscopy and proliferating clusters or activated T cells were no longer seen. This gross phenotype was matched by Ki-67 expression, which rose between days 2-3 to highest expression between days 6-9, thereafter decreasing.

Figure 11.5: Determination of the optimum re-stimulation frequency for *in vitro* expansion of putative T_{regs} from precursor populations.

(A-B) Proportion of proliferating cells assessed by Ki-67 expression in MACS-enriched $CD8^{-}CD25^{+}$ precursor cells during culture. **(A)** Representative FACS plot gated on live MACS-enriched $CD8^{-}CD25^{+}$ cells showing Ki-67 expression (right) with isotype control (left). **(B)** Graph illustrating cumulative FACS data on Ki-67 expression in 2 MACS-enriched $CD8^{-}CD25^{+}$ cell lines from healthy donors.

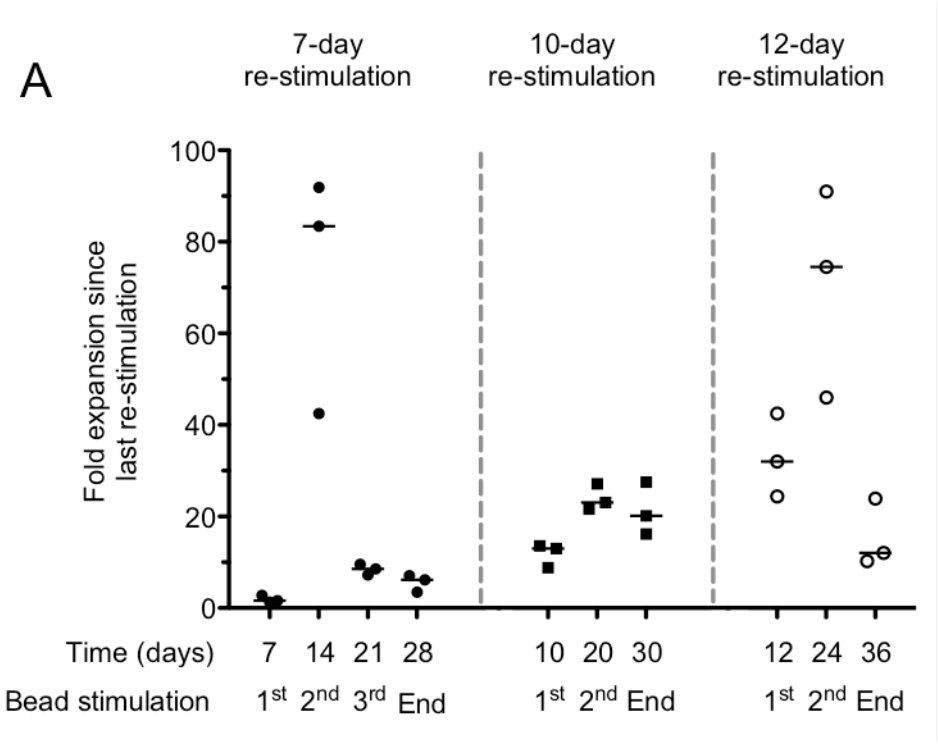


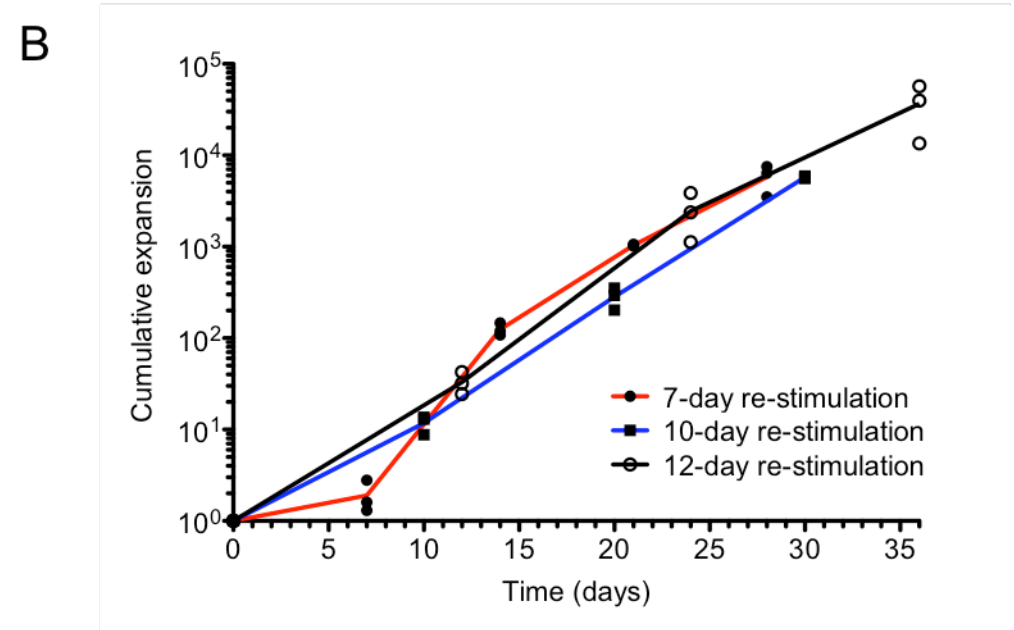
These data suggests that a longer re-stimulation frequency may allow for greater proliferation. In order to examine this, MACS-enriched precursors from three CD donors were cultured as described above and re-stimulated with Dynabeads at 7-, 10- or 12-day intervals. Cell counts were obtained at each re-stimulation. These data are illustrated in Figure 11.6 (page 214).

The lines cultured with a 7-day re-stimulation frequency were notable for little expansion in the first 7 days (median 1.6-fold, range 1.3-2.8), followed by an increased rate of expansion between days 8-14 (median 83.4-fold, range 42.5-91.8), after which the rate of expansion decreased to 8.6-fold between days 15-21 and 6.2-fold between days 22-28 (Figure 11.6 A). The lines cultured with a 10-day re-stimulation frequency expanded a median of 13-, 23.1- and 20-fold between days 0-10, 11-20 and 21-30, respectively. Lines cultured with a 12-day re-stimulation frequency exhibited the consistently highest fold-expansion after the initial stimulation and first re-stimulation, at 32-fold (range 24.4-42.5) and 74.5-fold (range 46-91), respectively, falling to 12-fold (range 10.3-23.9) proliferation after the second re-stimulation. Although limited numbers precluded statistical analysis, cumulative expansion after 24 days in the 12-day re-stimulation frequency group was similar to that seen in the 7-day re-stimulation group, and greater than that seen in the 10-day re-stimulation group (Figure 11.6 B). Consequently, a re-stimulation frequency of 12 days was adopted for *in vitro* expansion of putative T_{regs} from CD patients.

Figure 11.6: Optimisation of anti-CD3/anti-CD28 bead re-stimulation frequencies.

(A-B) Cumulative expansion of MACS-enriched $CD8^{-}CD25^{+}$ precursor cells from 3 CD patient donors, each divided into 3 aliquots and cultured with either 7-, 10- or 12-day re-stimulation frequencies. **(A)** Fold expansion during each stimulation period. **(B)** Graph illustrating cumulative expansion over 28, 30 or 36 days of culture.





11.4 T_{regs} can be expanded from Crohn's disease patient blood using GMP-compatible protocols

Hoffman *et al.* previously showed that initial T_{reg} enrichment on the basis of $CD45RA^+$ expression was required to expand homogenous and stable T_{reg} cell lines from healthy donors in the absence of rapamycin (Hoffmann et al., 2006). A number of groups subsequently showed that the addition of rapamycin to *in vitro* expansion protocols facilitated the selective expansion of T_{regs}, while inhibiting expansion of contaminating T_{cons} (Battaglia et al., 2006; Golovina et al., 2011; Hippen et al., 2011b; Scottà et al., 2013; Strauss et al., 2007b; 2009; Tresoldi et al., 2011). However, the optimum precursor population from which to expand a homogenous, suppressive and epigenetically stable T_{reg} population from CD patients is currently unknown. In order to examine this, blood was obtained from CD donors and the phenotype of putative T_{reg} populations expanded from $CD8^-CD25^+$ MACS-enriched precursors, FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors, or FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ precursors were compared. Cells were expanded as described above for 24 days and re-stimulated with anti-CD3/anti-CD28 beads at day 12.

All MACS and $CD45RA^+$ putative T_{reg} lines proliferated, expanding a median (IQR) of 873-fold (373-1502, n=10) and 175-fold (66-531; n=13p<0.01 *vs.* MACS), respectively, by day 24, (Figure 11.7 A [page 218]). In contrast, 3 of 13 (23%) $CD45RA^-$ T_{reg} lines failed to proliferate and were discontinued. Overall, $CD45RA^-$ putative T_{reg} lines expanded less well than MACS and $CD45RA^+$ putative T_{reg} lines (130-fold; 8-209, n=10), although this difference did not reach statistical significance (Figure 11.7 A).

MACS-enriched precursors were initially contaminated with a median (IQR) of 2.3% (1.2%-4.2%) live $CD3^+CD8^+$ events (Figure 11.7 B). By day 24 of expansion, MACS putative T_{regs} were contaminated by 8.7% (1.3%-20.3%) live $CD3^+CD8^+$ events ($p<0.05$) (Figure 11.7 C).

For the FACS-sorted precursor populations, initial post-sort purity was 86.5% (80.8%–91.6%; n=10) for $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors and 92.7% (87.8%–94.9%) for $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ precursors. In contrast to expanded MACS putative T_{regs}, both $CD45RA^+$ and $CD45RA^-$ putative T_{reg} lines exclusively expressed $CD4^+$ events (Figure 11.7 B).

Next, the ability of day 24 expanded putative T_{reg} lines to suppress the proliferation of autologous $CD4^+CD25^-$ T_{cons} in a 96 hour co-culture assay was examined (Figure 11.7 D-E). CTV-labelled T_{cons} were cultured at $5x10^4$ cells/well (constant T_{con} numbers) with the addition of T_{regs} a various ratios, and stimulated with anti-CD3/anti-CD28 beads at a 1:40 bead:T_{con} ratio. A "2X" condition with 10^5 T_{cons} stimulated with beads at a 1:80 bead:T_{con} ratio was used to attempt to replicate non-specific reduction in T_{con} proliferation that may occur at the 1:1 T_{con}:T_{reg} condition (also 10^5 cells/well at same bead number), due to cell density, physical access to beads, or other factors. T_{reg}–mediated suppression above the reduction in proliferation seen in the "2X" condition can be termed "specific suppression". Specific suppression of T_{con} proliferation was seen for both $CD45RA^+$ and $CD45RA^-$ putative T_{reg} lines above an 8:1 T_{con}:T_{reg} ratio (Figure 11.7 E). $CD45RA^+$ and $CD45RA^-$ putative T_{regs} suppressed T_{con} proliferation to an equivalent degree ($\log_{10}[IC_{50}]$ $CD45RA^+$ *vs.* $CD45RA^-$ putative T_{regs}, $p=0.46$). In contrast, specific suppression was only seen for MACS putative T_{regs} above a 4:1

T_{con}:T_{reg} ratio. The *in vitro* suppressive ability of MACS putative T_{regs} was inferior to that seen for both CD45RA$^+$ and CD45RA$^-$ putative T_{regs} ($\log_{10}$[IC_{50}] CD45RA$^+$ *vs.* MACS and CD45RA$^-$ *vs.* MACS, $p<0.0001$ for both comparisons).

In addition, *in vitro* suppression by both CD45RA$^+$ and CD45RA$^-$ T_{reg} lines was associated with a reduction in IL-2 in co-culture supernatants ($p<0.0001$ and $p<0.001$, respectively; Figure 11.7 F). *In vitro* suppression by CD45RA$^+$ T_{reg} lines was also associated with a reduction in IFN-γ in co-culture supernatants ($p<0.0001$; Figure 11.7 F). However, this was not seen for CD45RA$^-$ T_{reg} lines ($p=0.89$; Figure 11.7 F), suggesting that CD45RA$^-$ T_{reg} lines either do not suppress IFN-γ expression in T_{cons} or that CD45RA$^-$ T_{reg} lines express IFN-γ.

Figure 11.7: Expansion, phenotype and *in vitro* suppressive ability of *in vitro* expanded putative T_{reg} lines.

(A) Cumulative fold-expansion of *in vitro* expanded MACS, $CD45RA^+$ or $CD45RA^-$ putative T_{reg} lines at days 12 and 24 of culture (bar at median). **(B)** Representative FACS plots obtained from MACS, $CD45RA^+$ or $CD45RA^-$ putative T_{reg} lines after 24d of *in vitro* expansion illustrating $CD4^+$ purity and $CD8^+$ contamination. Gated on live $CD3^+$ events. **(C)** Cumulative data from MACS putative T_{reg} lines showing live $CD3^+CD8^+$ events following initial enrichment from peripheral blood (day 0) and after 24 days of *in vitro* expansion. *p<0.05, **p<0.01.

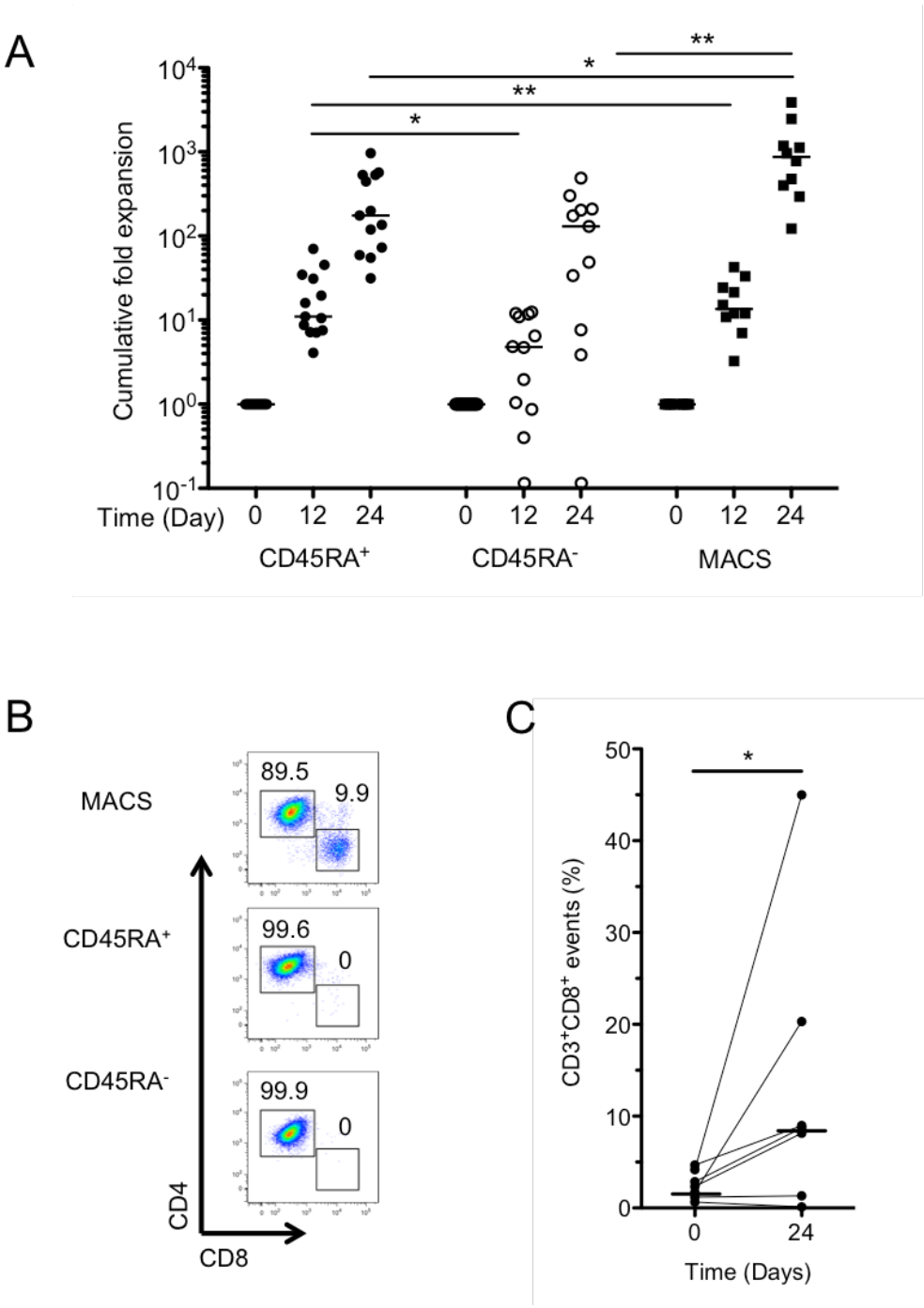
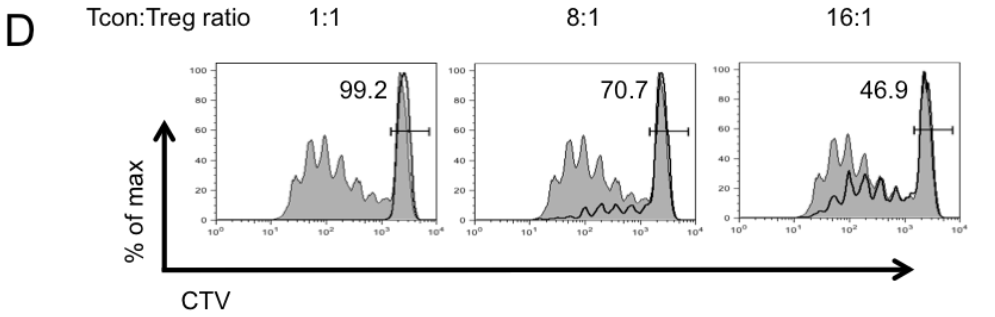
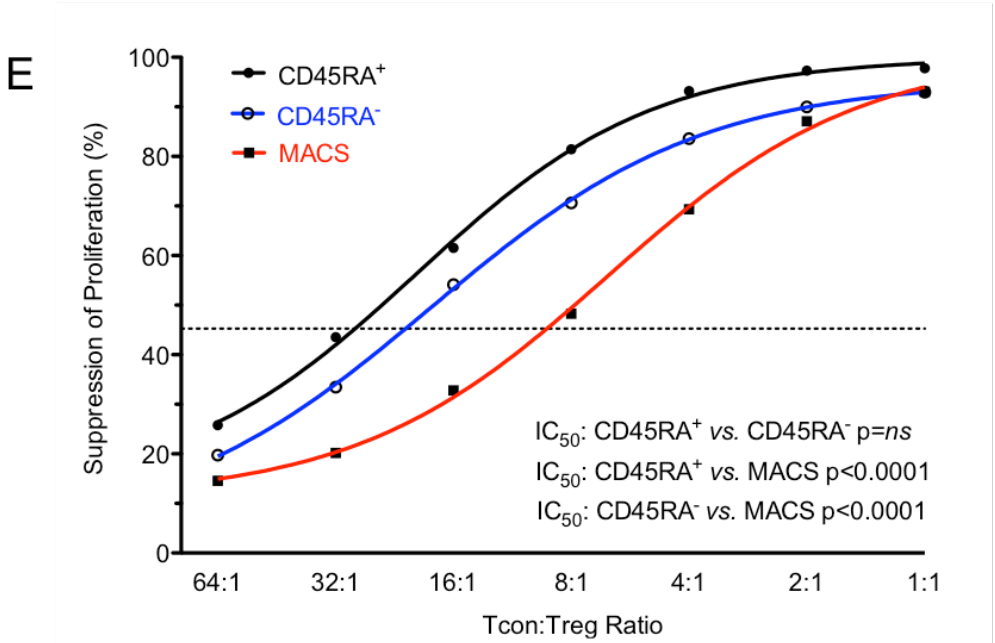


Figure 11.7 (continued): Expansion, phenotype and *in vitro* suppressive ability of *in vitro* expanded putative T_{reg} lines.

(D) Representative plots from an *in vitro* suppression assay, illustrating dose-dependent suppression of autologous $CD4^+CD25^-$ T_{con} proliferation by day 24 $CD45RA^+$ putative T_{regs} after 96h co-culture. Proliferation of CTV-labelled T_{cons} alone (filled) or T_{cons} co-cultured with T_{regs} at various T_{con}:T_{reg} ratios (bold line) is shown. The gate shows the proportion of un-proliferated T_{cons} in each condition. **(E)** Cumulative data from *in vitro* suppression assays, as described above. Mean suppression of autologous T_{con} proliferation by day 24 MACS, $CD45RA^+$ and $CD45RA^-$ putative T_{reg} lines is shown, including a dose-response curve for each condition. Pooled data from 29 T_{reg} lines. Comparisons between suppression seen in study conditions and mean non-specific suppression seen in "2X" control condition (dotted line) are also shown. *p<0.05, ***p<0.001, ****p<0.0001.

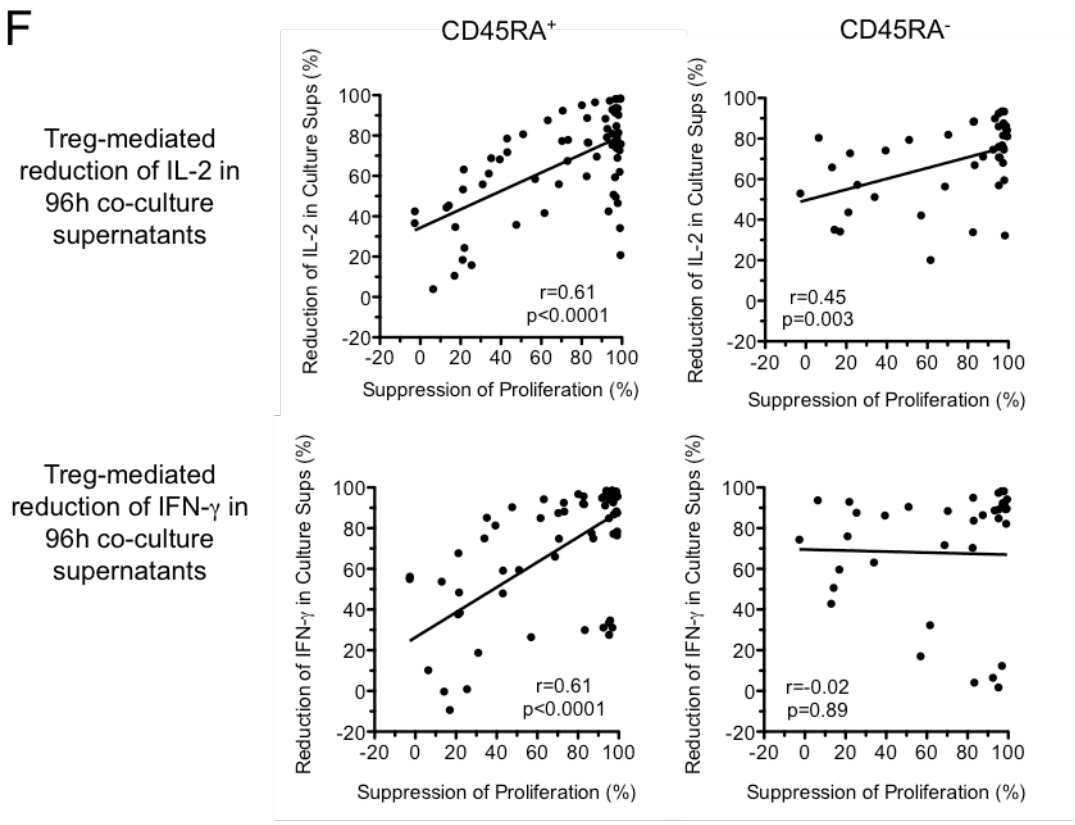




		16:1	8:1	4:1	2:1	1:1
vs. non-specific suppression at each ratio:	$CD45RA^+$	*ns*	***	****	****	****
	$CD45RA^-$	*ns*	*	****	****	****
	MACS	*ns*	*ns*	*	***	***

Figure 11.7 (continued): Expansion, phenotype and *in vitro* suppressive ability of *in vitro* expanded putative T_{reg} lines.

(F) Top panels: Dot plots comparing T_{reg}-mediated suppression of autologous T_{con} proliferation (X axis) and % reduction of IL-2 in co-culture supernatants (Y axis), normalised to supernatant IL-2 concentration in T_{con}-alone conditions. Bottom panels: Dot plots comparing T_{reg}-mediated suppression of autologous T_{con} proliferation (X axis) and % reduction of IFN-γ in co-culture supernatants (Y axis), normalised to supernatant IFN-γ concentration in T_{con}-alone conditions. Data from $CD45RA^+$ and $CD45RA^-$ putative T_{reg} lines are shown. Line-of-best-fit is shown.



11.5 *In vitro* expanded CD45RA$^+$ T_{regs} are resistant to IL-17 induction and stably express FOXP3

LP T_{regs} obtained from inflamed CD mucosa express IL-17 (Hovhannisyan et al., 2011). T_{regs} isolated from healthy donor blood or tonsils can be induced to express IL-17 and the Th17 transcription factor RORC when activated *in vitro* in the presence of IL-1, IL-2, IL-21 and IL-23 (Afzali et al., 2013b; Bettelli et al., 2006; Koenen et al., 2008; Voo et al., 2009). However, the clinical or translational relevance of these observations is unclear. Indeed, human IL-17$^+$ T_{regs} are suppressive *in vitro* (Afzali et al., 2013b; Koenen et al., 2008; Voo et al., 2009). Nevertheless, the potential for adoptively transferred T_{regs} to exacerbate inflammation in CD lesions through the production of pro-inflammatory cytokines is of concern. Consequently, the ability of *in vitro* expanded T_{regs} from CD patients to express pro-inflammatory cytokines was examined.

Day 24 *in vitro* expanded T_{regs} were washed and cultured for a further 24h (in the absence of rapamycin). Cytokine expression in culture supernatants was then assessed (Figure 11.8 A, page 225). IFN-γ expression was similar for CD45RA$^+$ and CD45RA$^-$ T_{regs} (at median 36 pg/ml/10^6 cells [IQR 0-66] and 34 pg/ml [5-503], respectively, p=0.12). However, MACS T_{regs} expressed significantly higher amounts of IFN-γ than either CD45RA$^+$ or CD45RA$^-$ T_{regs} (2,459 pg/ml/10^6 cells [476-3,986], p<0.001 *vs.* CD45RA$^+$, p<0.01 *vs.* CD45RA$^-$). CD45RA$^+$ T_{regs} expressed significantly less IL-4 (1.3 pg/ml/10^6 cells [0-4.6]) than CD45RA$^-$ T_{regs} (44 pg/ml/10^6 cells [3.3-1,186], p<0.01) or MACS T_{regs} (121 pg/ml/10^6 cells [83-657], p<0.01). CD45RA$^+$ T_{regs} also expressed significantly less IL-17 (3.4 pg/ml/10^6 cells [2.3-17]) than CD45RA$^-$ T_{regs} (23 pg/ml/10^6 cells [9.7-32],

$p<0.05$) or MACS T_{regs} (170 pg/ml/10^6 cells [22-264], $p<0.05$). Finally, MACS T_{regs} expressed higher levels of TNF (271 pg/ml/10^6 cells [93-590]) than $CD45RA^+$ T_{regs} (4.1 pg/ml/10^6 cells [0-17], $p<0.05$).

PB T_{regs} can express IL-17 when stimulated with Th17 skewing cytokines *in vitro* (Afzali et al., 2013b; Koenen et al., 2008; Voo et al., 2009). In order to determine if *in vitro* expanded T_{regs} can be induced to express IL-17 on exposure to pro-inflammatory cytokines, D24 *in vitro* expanded T_{regs} were washed and cultured in rapamycin-free medium for a further 5 days in the presence of IL-2 alone, or Th17-inducing cytokine mixes: (i) Mix A: IL-2, IL-1, IL-6 and TGF-β; or (ii) Mix B: IL-2, IL-21, IL-23 and TGF-β (Figure 11.8 B; Koenen et al., 2008; Voo et al., 2009). The cytokine mixes did not induce IL-17 expression in $CD45RA^+$ $T_{regs.}$ In contrast, IL-17 production by both $CD45RA^-$ T_{regs} and MACS T_{regs} was consistently higher than that seen in $CD45RA^+$ T_{regs} in each condition (Figure 11.8 B; $p<0.01$ for each comparison).

Next, the expression of transcription factors thought to be important in Th17 biology was examined by rtPCR in day 24 *in vitro* expanded $CD45RA^+$ and $CD45RA^-$ $T_{regs.}$ MACS T_{regs} were not interrogated, as their mixed populations precluded accurate assessment. *RORC*, *AHR* and *IL-17* were significantly over-expressed in $CD45RA^-$ T_{regs}, in comparison with expression in paired $CD45RA^+$ T_{regs} (Figure 11.8 C; $p<0.05$ for each comparison).

Freshly isolated PB T_{regs} can be characterised by a $CD4^+CD25^{hi}CD127^{lo}$ cell surface phenotype. Expression of this phenotype on day 24 *in vitro* expanded T_{regs} was examined by FACS (Figure 11.8 D). Most $CD45RA^+$ T_{regs} retained a $CD4^+CD25^{hi}CD127^{lo}$ cell surface phenotype following 24 days of *in vitro* expansion

($CD4^+CD25^{hi}CD127^{lo}$ 98.2% [97.1%-98.3%], n=12). In contrast, expression of the $CD4^+CD25^{hi}CD127^{lo}$ cell surface phenotype was significantly reduced in $CD45RA^-$ T_{regs} (93.1% [82.5%-96.9%], $p<0.05$ *vs.* $CD45RA^+$) and MACS T_{regs} (78.3% [71.6%-83.7%], $p<0.0001$ *vs.* $CD45RA^+$).

Demethylation of the *FOXP3* TSDR is associated with stable FOXP3 expression and a regulatory phenotype in humans (Baron et al., 2007; Floess et al., 2007; Miyara et al., 2009). To determine if *in vitro* expanded T_{regs} had epigenetically stable expression of FOXP3, *FOXP3* TSDR demethylation was examined (Figure 11.8 E). The TSDR was completely demethylated in all $CD45RA^+$ T_{reg} lines tested (100%; n=9). In contrast, a median of 90.6% (IQR 36.5%-100%, n=6, $p<0.01$ *vs.* $CD45RA^+$ T_{regs}) TSDR demethylation was found in $CD45RA^-$ T_{regs}, and 4.6% (1.6%-10.5%, n=8, $p<0.001$ *vs.* $CD45RA^+$ T_{regs}) TSDR demethylation was found in MACS T_{regs}. These data suggest that $CD45RA^+$ T_{regs} have epigenetically stable expression of FOXP3 even after 24 days of *in vitro* expansion, while epigenetically stable FOXP3 expression is not seen in MACS T_{regs} expanded *in vitro* from CD patients' blood.

Next, in order to determine if *FOXP3* TSDR demethylation correlated with phenotype (e.g. cell surface T_{reg} phenotype or cytokine expression), correlation plots were constructed to compare TSDR demethylation with cell surface phenotype, IL-17 expression and the *in vitro* suppressive capacity of day 24 *in vitro* expanded T_{regs} (Figure 11.8 F-H). Unfortunately, these plots were not informative for two reasons: (i) *FOXP3* TSDR demethylation was determined in a subset of total lines expanded, limiting the power of this approach, and (ii) due to the

distribution of *FOXP3* TSDR demethylation between groups, CD45RA$^+$ T_{regs} and MACS T_{regs} tended to cluster together in these correlation analyses.

Figure 11.8: *In vitro* expanded T_{reg} plasticity.

(A) IFN-γ, IL-4, IL-17 and TNF secretion by day 24 *in vitro* expanded T_{regs}, detected by cytometric bead array (FACS) on 24h culture supernatants. **(B)** IL-17 detected by ELISA from 5-day culture supernatants of day 24 *in vitro* expanded T_{regs} cultured in the absence of rapamycin but with supplemental IL-2 alone, a cocktail of IL-2, IL-1, IL-6 and TGF-β (Mix A) or a cocktail of IL-2, IL-21, IL-23 and TGF-β (Mix B). Bar at median. *p<0.05, **p<0.01, ***p<0.001.

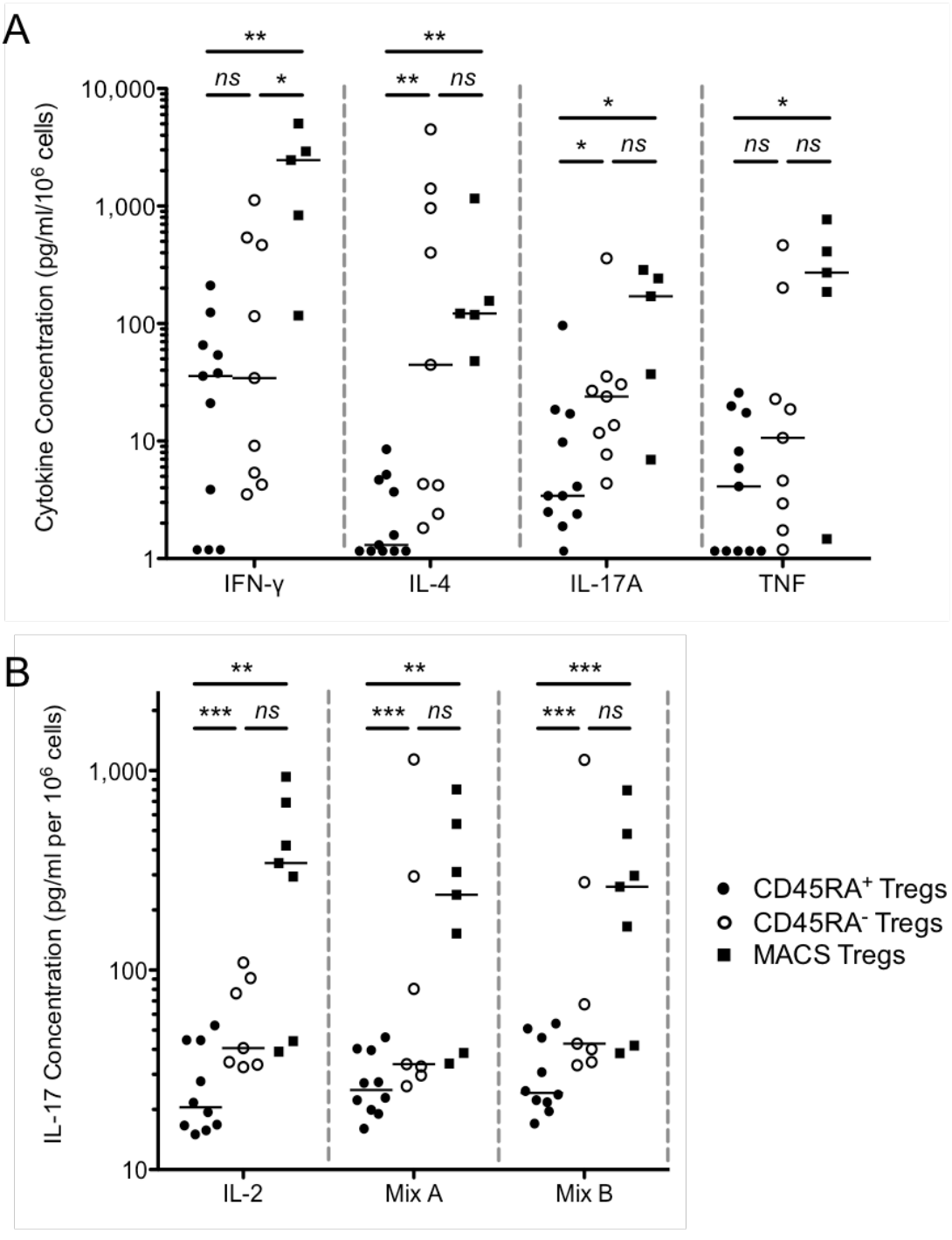


Figure 11.8 (continued): *In vitro* expanded T_{reg} plasticity.

(C) Expression of *RORC*, *AHR* and *IL17A* in day 24 *in vitro* expanded CD45RA$^+$ and CD45RA$^-$ T_{regs}, detected by rtPCR. **(D)** CD4$^+$CD25^{hi}CD127^{lo} cell surface phenotype detected by FACS in day 24 *in vitro* expanded T_{regs}. **(E)** Methylation status of the *FOXP3* TSDR in day 24 *in vitro* expanded T_{regs}. Bar at median. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

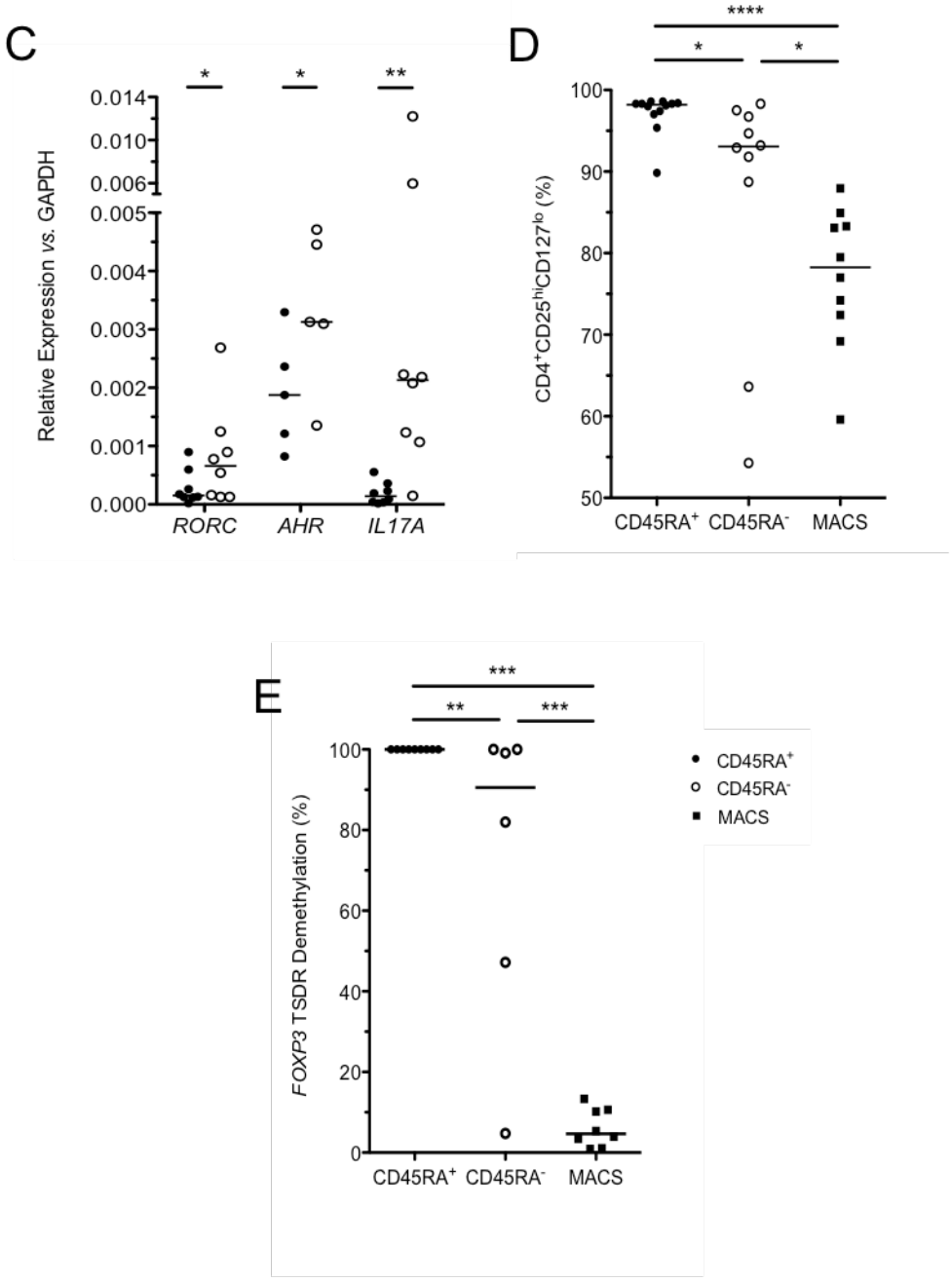
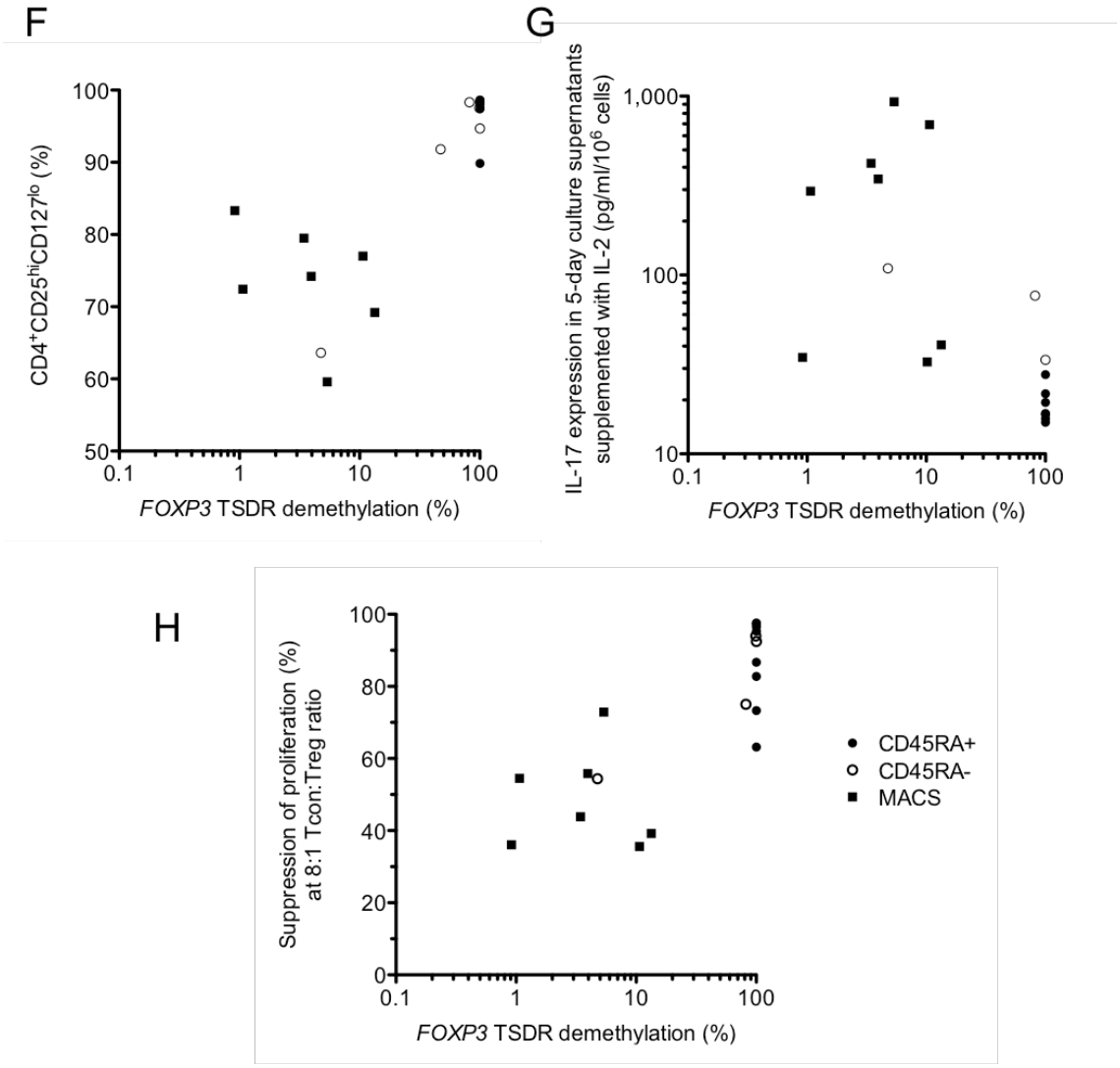


Figure 11.8 (continued): *In vitro* expanded T_{reg} plasticity.

(F-G) Plots exploring the relationship between *FOXP3* TSDR methylation status and day 24 *in vitro* expanded T_{reg} phenotype. **(F)** Cell surface $CD4^{+}CD25^{hi}CD127^{lo}$ phenotype *vs. FOXP3* TSDR demethylation. **(G)** IL-17 expression in 5-day culture supernatants (IL-2 alone condition) *vs. FOXP3* TSDR demethylation. **(H)** Day 24 T_{reg}–mediated suppression of autologous T_{con} proliferation (%) at an 8:1 T_{con}:T_{reg} ratio *vs. FOXP3* TSDR demethylation.



11.6 *In vitro* expanded CD45RA$^+$ T_{regs} express homing receptors for gut and lymphoid tissue

The ability of *in vitro* expanded T_{regs} to home to relevant immune niches, where they may suppress inflammation, may be critical for cell therapy. Consequently, the expression of gut homing receptors on *in vitro* expanded T_{regs} was examined by FACS (Figure 11.9 A-B; page 231). *In vitro* expanded T_{regs} from each precursor population moderately expressed $\alpha_4\beta_7$ integrin (CD45RA$^+$ 21.4% [16.4%-24.0%]; CD45RA$^-$ 22.6% [18.3%-31.2%]; and MACS 18.0% [11.8%-27.1%]) and CCR6 (CD45RA$^+$ 9.7% [5.6%-17.9%]; CD45RA$^-$ 11.2% [6.9%-33.2%]; and MACS 22.8% [14.5%-32.3%]), but did not express CCR9.

The expression of cell surface markers required for lymph node homing was also assessed. CD62L was more highly expressed in *in vitro* expanded CD45RA$^+$ T_{regs} than CD45RA$^-$ T_{regs} (98.2% [69.4%-99.3%] *vs.* 67.2% [40.9%-97.3%], $p<0.05$). Similarly, CCR7 was more highly expressed in *in vitro* expanded CD45RA$^+$ T_{regs} than CD45RA$^-$ or MACS T_{regs} (CD45RA$^+$ 97.0% [90.1%-98.8%] *vs.* CD45RA$^-$ 87.3% [73.5%-95.1%] and MACS 71.2% [64.4%-74.7%] $p<0.05$ for each comparison). These data suggest that *in vitro* expanded CD45RA$^+$ T_{regs} may have the capacity to home to lymphoid tissue *in vivo*. CD45RA$^+$ T_{regs} also moderately expressed CXCR3 (40.2% [21.5%-53.3%]), suggesting that a proportion of these cells may have the capacity to home to sites of Th1-mediated inflammation.

11.7 Adoptively transferred CD45RA$^+$ T_{regs} home to inflamed human small intestine in a C.B-17 SCID human small bowel xeno-transplant model.

In view of the favourable phenotype of *in vitro* expanded CD45RA$^+$ T_{regs} as a candidate cell therapy, the ability of these cells to home to human small bowel *in vivo* was assessed in a murine xeno-transplant model. Human foetal small bowel segments (approximately 2cm in length) were transplanted into a subcutaneous tunnel on the dorsum of a C.B-17 SCID mouse. The xeno-transplanted small bowel segments were then allowed to mature *in situ* for 12-16 weeks. As each animal can accommodate one human small bowel xeno-transplant on each dorsal side, it is possible to manipulate one xeno-transplanted graft in this model, leaving the untouched contralateral graft as a control. Other groups previously characterised this model and showed that xeno-transplanted human small intestinal segments develop into tissue that is morphologically and functionally identical to normal gut, and is capable of peristalsis and nutrient absorption (Golan et al., 2009; 2010; Howie et al., 1998). Furthermore, xenografts possess a chimeric endothelium that expresses human MAdCAM-1(Winter et al., 1991), meaning that $\alpha_4\beta_7$-mediated intestinal homing is feasible in this model. In order to determine if *in vitro* expanded CD45RA$^+$ T_{regs} home to xeno-transplanted human small bowel *in vivo*, in this model system, *in vitro* expanded CD45RA$^+$ T_{regs} were administered intravenously and homing assessed 24h later (Figure 11.9 C-D). Intraluminal injection with enteropathogenic *Escherichia coli* (EPEC) was used to induce mucosal inflammation (Figure 11.9 E). The contralateral xeno-graft was injected with PBS and used as a non-inflamed control.

Following adoptive transfer, human $CD45^+CD3^+CD4^+$ cells were detected in mouse spleen and inflamed human small bowel xeno-graft LP by FACS (Figure 11.9 F-G), indicating that adoptively transferred $CD45RA^+$ T_{regs} homed to inflamed human SB LP in this model. This was confirmed by the detection of human $CD45^+CD3^+$ cells in inflamed human SB LP by immunofluorescence (Figure 11.9 H). Interestingly, human $CD45^+CD3^-$ events were also detected in non-inflamed human small bowel LP in both mice that received IV PBS and IV T_{regs} (Figure 11.9 G), suggesting that a population of long-lived human immune cells was co-transferred with the human small bowel xeno-transplant. Professor MacDonald's group previously showed that human foetal SB contains a population of $CD3^-CD7^+$ cells that persist following xeno-transplantation (Howie et al., 1998).

Figure 11.9: *In vitro* expanded CD45RA$^+$ T_{regs} express gut and lymphoid homing receptors and home to inflamed human LP in a C.B-17 SCID mouse human intestinal xenograft model. **(A)** Representative FACS plots illustrating gut and lymphoid homing receptor expression on D24 CD45RA$^+$ T_{regs} (bold line). Gates were drawn on the basis of fully stained CD4$^+$ lymphocytes (filled) and FMO controls. **(B)** Expression of intestinal and lymphoid homing receptors in D24 T_{regs}. n=17; *p<0.05, **p<0.01.

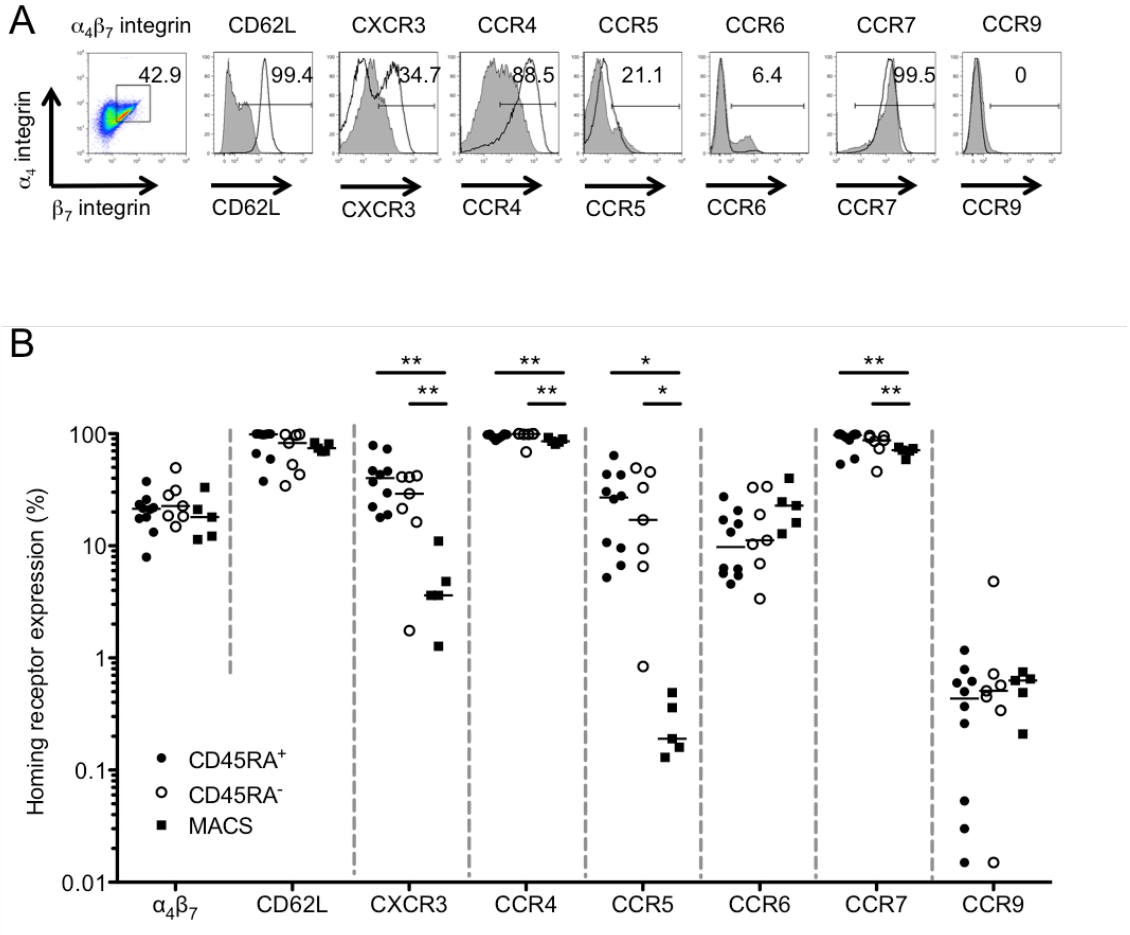
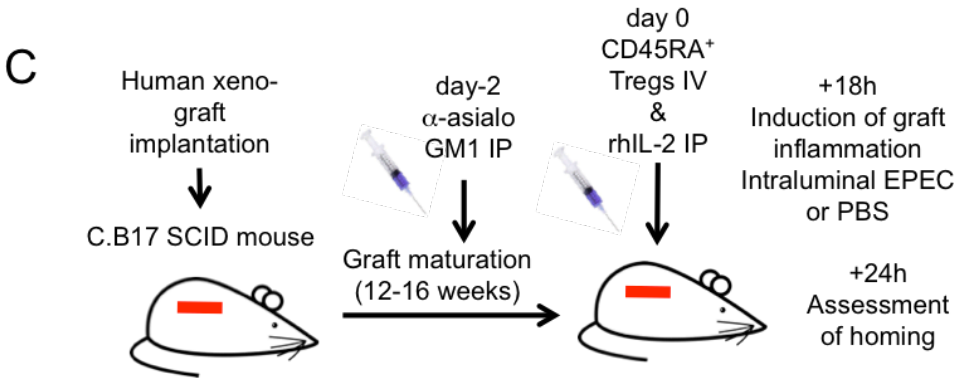


Figure 11.9 (continued): *In vitro* expanded CD45RA$^+$ T_{regs} express gut and lymphoid homing receptors and home to inflamed human LP in a C.B-17 SCID mouse human intestinal xenograft model.

(C) Design of the xenograft mouse experiment. Foetal small intestine was implanted subcutaneously on the dorsum of a C.B-17 SCID mouse and allowed to mature in situ for 12-16 weeks. Following pre-treatment with anti-asialo GM1 and rhIL-2 (Tresoldi et al., 2011), day 24 in vitro expanded CD45RA$^+$ T_{regs} were administered IV. Graft inflammation was induced by intraluminal injection with EPEC. The contralateral graft was injected with PBS and used as a non-inflamed control. Homing was assessed 24h later. **(D)** Left panel: Mature xenografts (circled) are visible subcutaneously on the dorsum of the mouse. Right panel: Dorsal skin has been removed in an anesthetized mouse to reveal the mucus-filled xenograft in situ (right panel). Scale bars=1cm.



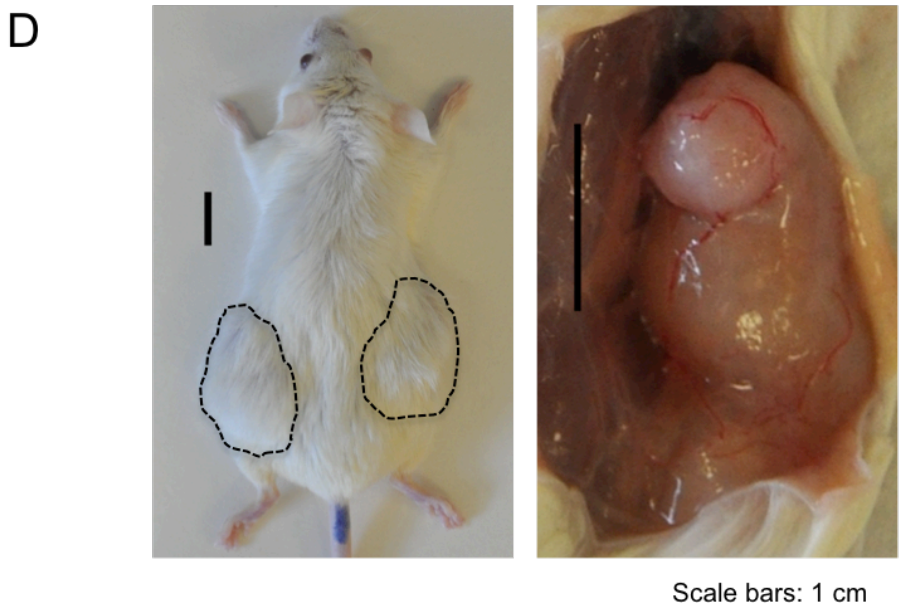
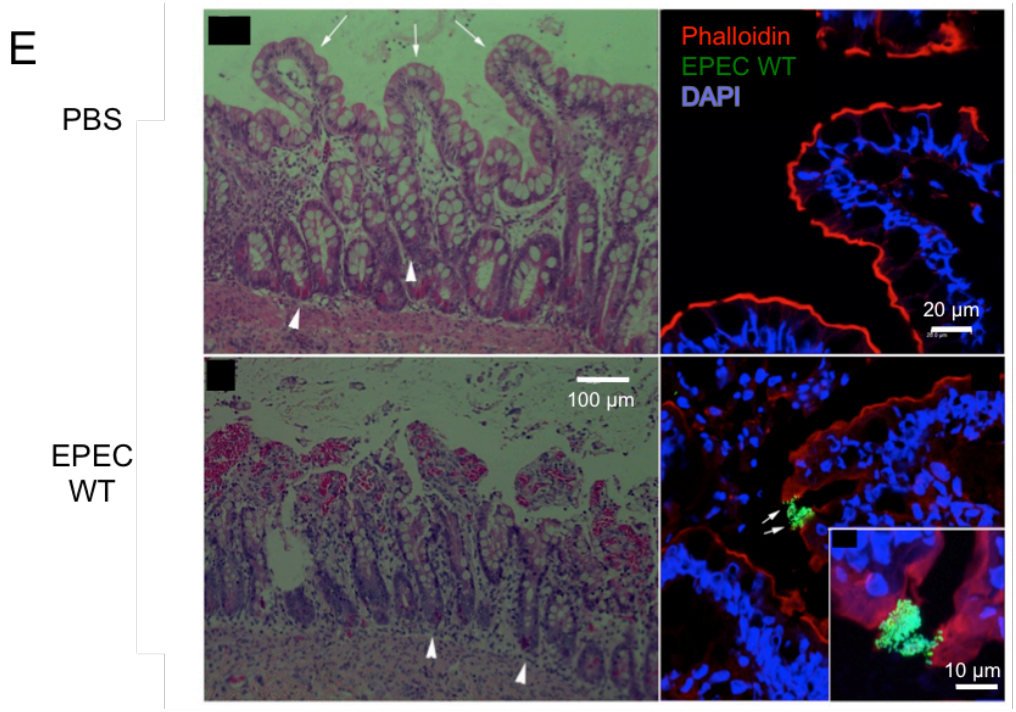


Figure 11.9 (continued): *In vitro* expanded CD45RA$^+$ T_{regs} express gut and lymphoid homing receptors and home to inflamed human LP in a C.B-17 SCID mouse human intestinal xenograft model.

(E) Top left panel: Representative microscopic image of a xenograft 8h after intraluminal injection with PBS, showing non-inflamed small bowel mucosa with normal villi (arrows) and intestinal crypts (arrowheads). H&E, 20X. Bottom left panel: In contrast, intraluminal injection with EPEC caused mucosal inflammation with destruction of villi and cytoplasmic vacuolization. Top right panel: Immunofluorescence staining of PBS-treated xenograft showing a normal small bowel villus. F-Actin is visualized with phalloidin-rhodamine (red). DAPI (blue). Bottom right panel: GFP-expressing EPEC (green, arrows) adhere to mucosal epithelial cells and induce formation of AE lesions. **(F)** FACS plots showing healthy control PBMCs and the gating strategy for identification of live human CD45$^+$CD3$^+$CD4$^+$ events in the C.B17 SCID human intestinal xeno-transplant model.



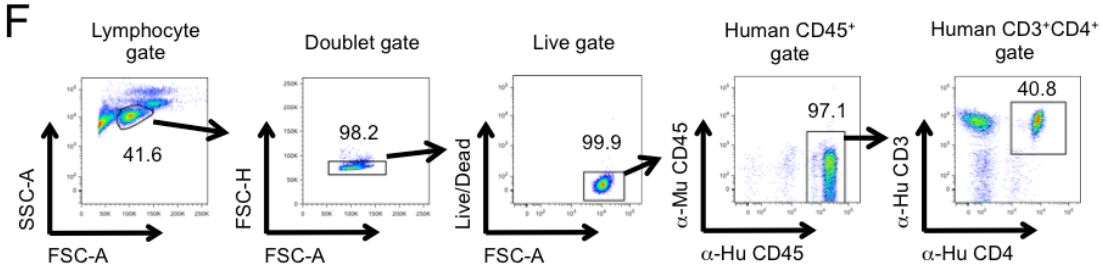
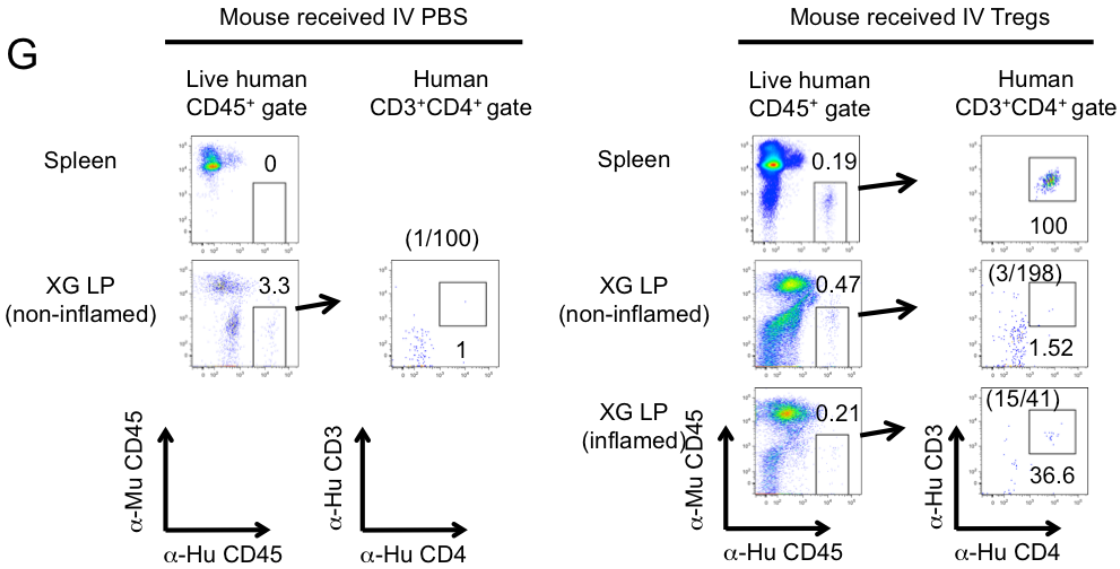
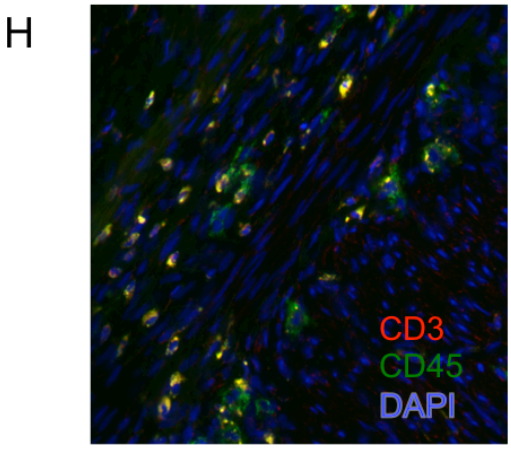


Figure 11.9 (continued): *In vitro* expanded CD45RA$^+$ T_{regs} express gut and lymphoid homing receptors and home to inflamed human LP in a C.B-17 SCID mouse human intestinal xenograft model.

(G) FACS plots showing live human CD45$^+$CD3$^+$CD4$^+$ events in single cell suspensions prepared from murine spleen, non-inflamed and inflamed xenografts (XG), 24h after IV PBS (left panels) or adoptive transfer of T_{regs} (right panels). The gating strategy is illustrated in Figure 11.9 F. The absolute number of CD3$^+$CD4$^+$ events in the xenograft human CD45$^+$ gates are highlighted.

(H) Immunofluorescence staining of xenograft cryosections with anti-human CD3 (red), anti-human CD45 (green) and DAPI (blue). **(F-H)** Representative of 2 independent experiments.





11.8 *In vitro* expanded CD45RA$^+$ T_{regs} suppress proliferation and activation of MLN and LP T cells in active Crohn's disease

In order to be biologically active following adoptive transfer to patients with CD, *in vitro* expanded T_{regs} may have to suppress the activation of effector immune cells in a relevant immune niche, such as the spleen, mesenteric lymph node or intestinal lamina propria. Fantini *et al.* previously found that T_{cons} obtained from inflamed CD mucosa were resistant to T_{reg}-mediated suppression of proliferation *in vitro*, due to over-expression of Smad7, an inhibitor of TGF-β signalling (Fantini et al., 2009). Consequently, it is critical to demonstrate that *in vitro* expanded T_{regs} can overcome potential T_{con} resistance to T_{reg}-mediated suppression and suppress *in vitro* proliferation of T_{cons} obtained from inflamed CD MLN and mucosa.

Unfractionated MLN and LP mononuclear cells (MLNMCs and LPMCs) were obtained from an inflamed ileocaecal CD resection, stimulated with plate-bound anti-CD3/anti-CD28 antibodies and either cultured alone, or co-cultured with day 24 *in vitro* expanded CD45RA$^+$ T_{regs}, at various ratios (Figure 11.10 A-B, [page 237]). Seven hours later, T_{reg}-mediated suppression of MLN and LP CD3$^+$ activation was assessed by determining T_{reg}-mediated suppression of CD154 (CD40L) expression on live MLN and LP CD3$^+$ events (Canavan et al., 2012; 2013). Significant dose-dependent suppression of CD154 expression in live MLN and LP CD3$^+$ events was observed (Figure 11.10 C-D), demonstrating that day 24 *in vitro* expanded CD45RA$^+$ T_{regs} suppress early activation of MLN and LP T_{cons} *in vitro*.

Next, to determine if day 24 *in vitro* expanded CD45RA$^+$ T_{regs} can suppress *in vitro* proliferation of T_{cons} obtained from inflamed CD small bowel, MLNMCs were labelled with CTV and co-cultured with day 24 *in vitro* expanded CD45RA$^+$ T_{regs}. Proliferation of live CD3$^+$ events was assessed at 96h. Dose-dependent T_{reg}-mediated suppression of MLN CD3$^+$ proliferation was seen at each MLNMC:T_{reg} ratio (Figure 11.10 E). It was not possible to determine if day 24 *in vitro* expanded CD45RA$^+$ T_{regs} can suppress *in vitro* proliferation of T_{cons} obtained from inflamed CD LP, as LPMCs obtained from inflamed CD resections died prior to assessment of proliferation at 96h (n=3 independent experiments).

These data suggest that *in vitro* expanded CD45RA$^+$ T_{regs} may be biologically active in the immune niches directly relevant to the pathogenesis of CD.

Figure 11.10: *In vitro* expanded CD45RA$^+$ T_{regs} suppress CD3$^+$ T cell responses in mononuclear cells obtained from inflamed Crohn's MLN and LP.

(A) Fresh ileal resection specimen opened longitudinally to show ileal stricture (marked "S") and proximal inflamed, haemorrhagic mucosa with deep ulceration (arrows). Scale bar: 2cm.

(B) Representative microscopic image from this resection showing mucosal distortion, ulceration (marked "U") and transmural inflammation, including lymphoid aggregates (arrows). 12.5X H&E. Scale bar: 2mm. **(C)** Representative FACS plots gated on live MLN CD3$^+$ events showing CD154 expression on MLN T_{cons} cultured alone (at a 1:0 MLNMC:T_{reg} ratio, top left panel) or with T_{regs} at a 1:1 MLNMC:T_{reg} ratio (bottom left panel). Pooled data showing T_{reg}-mediated suppression of CD154 expression in live MLN CD3$^+$ cells (right panel, n=5). Dotted line shows non-specific suppression from "2X control". Comparisons between observed suppression and non-specific suppression (†p<0.05, ††p<0.01, †††p<0.001, ††††p<0.0001) and observed suppression and no suppression (zero, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001) are shown.

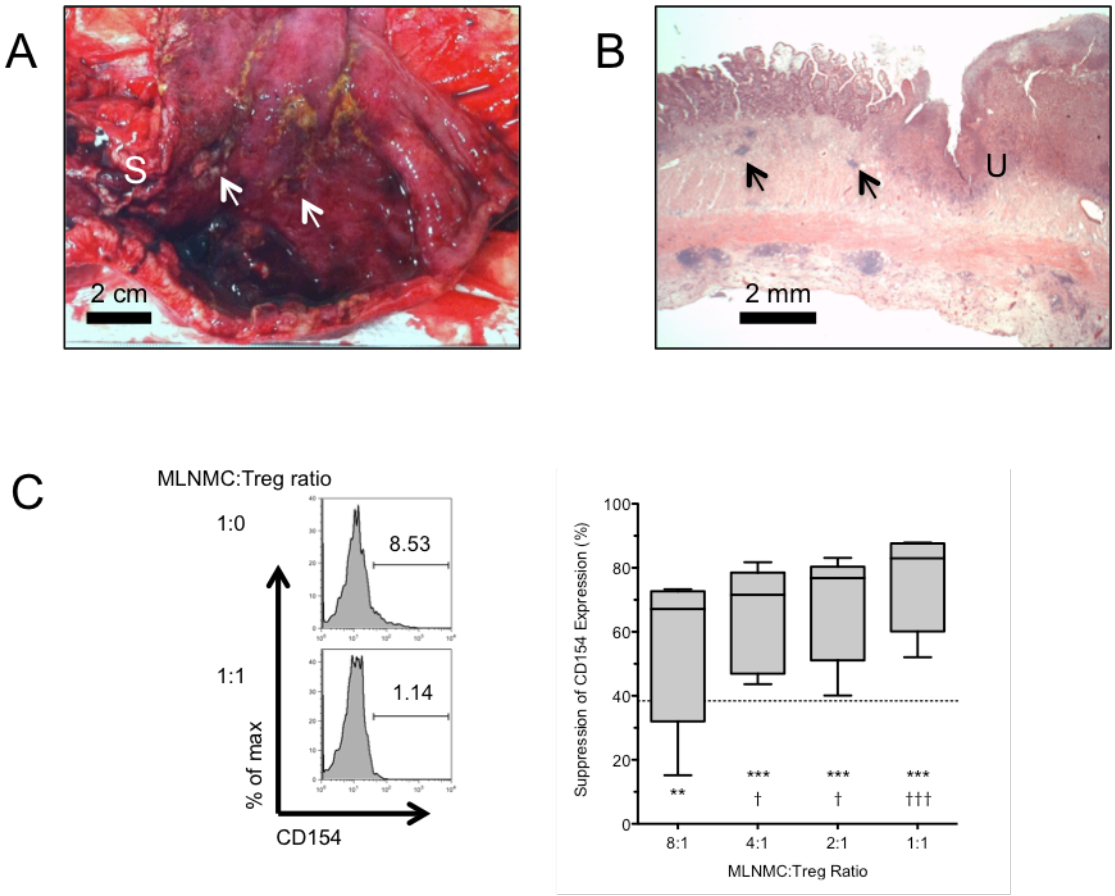
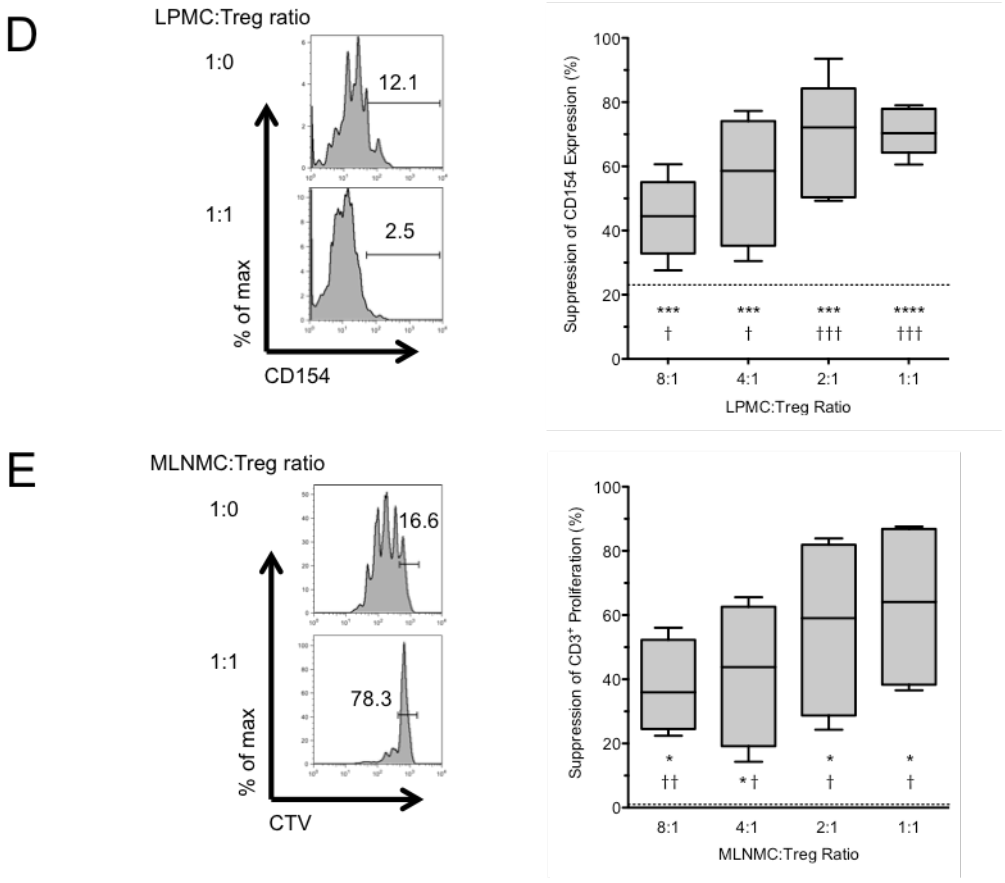


Figure 11.10 (continued): *In vitro* expanded CD45RA$^+$ T_{regs} suppress CD3$^+$ T cell responses in mononuclear cells obtained from inflamed Crohn's MLN and LP.

(D) Representative FACS plots gated on live LP CD3$^+$ events showing CD154 expression on LP T_{cons} cultured alone (at a 1:0 LPMC:T_{reg} ratio, top left panel) or with T_{regs} at a 1:1 LPMC:T_{reg} ratio (bottom left panel). Pooled data showing T_{reg}-mediated suppression of CD154 expression in live LP CD3$^+$ cells (right panel, n=5). **(E)** Representative FACS plots gated on live CD3$^+$ events, showing proliferation of MLN T_{cons} cultured alone (at a 1:0 MLNMC:T_{reg} ratio, top left panel) or with T_{regs} at a 1:1 MLNMC:T_{reg} ratio (bottom left panel). Pooled data showing T_{reg}-mediated suppression of MLN CD3$^+$ proliferation (right panel, n=5). Box and whisker plot shows median, IQR and range.

(D-E) Dotted line shows non-specific suppression from "2X control". Comparisons between observed suppression and non-specific suppression (†p<0.05, ††p<0.01, †††p<0.001, ††††p<0.0001) and observed suppression and no suppression (zero, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001) are shown.



11.9 Discussion

There remains an unmet need to develop novel therapies for CD, as current drug treatments often fail to maintain long-term remission and medication use may be complicated by significant side effects. Cellular therapies are emerging as potentially attractive therapeutic strategies. This study attempts to address a number of perceived barriers to autologous T_{reg}-based cell therapy in CD by attempting to define the optimum precursor population from which to expand T_{regs} *in vitro* from the PB of CD patients, for potential therapeutic use.

T_{regs} are effective in inducing remission or preventing the development of colitis in numerous preclinical models of colitis (Garrett et al., 2007; Huber et al., 2004; Ishikawa et al., 2013; Maloy et al., 2003; Morrissey et al., 1993; Mottet et al., 2003; Powrie et al., 1993; Veltkamp et al., 2006; Watanabe et al., 2008a). Several groups have shown that PB T_{regs} can be expanded *in vitro* using R&D grade reagents from healthy individuals (Battaglia et al., 2006; Golovina et al., 2011; Hippen et al., 2011b; Hoffmann et al., 2006; 2004; Scottà et al., 2013; Strauss et al., 2009; 2007a; Tresoldi et al., 2011) or patients with diabetes (Putnam et al., 2009) or end-stage renal failure (Afzali et al., 2013a). Putnam *et al.* showed that T_{regs} can be expanded *in vitro* from PB using GMP-compatible protocols, while retaining an *in vitro* suppressive function after expansion (Putnam et al., 2013). Other groups have taken this one step further by showing that T_{regs} expanded *in vitro* can be safely infused into humans in phase I clinical trials (Brunstein et al., 2011; Marek-Trzonkowska et al., 2012; Trzonkowski et al., 2009). Data from the present study extend these observations to patients with CD, showing that it is feasible to isolate PBMCs from the PB of patients with CD, enrich these cells for precursor

populations for *in vitro* expansion using MACS or FACS-based technology, and expand these precursor populations *in vitro* a median of 130-873 fold over 24 days. Even after prolonged culture, *in vitro* expanded T_{regs} suppress the proliferation of autologous T_{cons} *in vitro*.

$CD4^+CD25^{hi}CD127^{lo}$ T_{regs} comprise 3-5% of PB $CD4^+$ T cells, or 0.6-1.0% of PBMCs. *In vitro* expansion of T_{regs} is mediated by IL-2. However, other cell populations, including $CD4^+$ T_{cons}, $CD8^+$ lymphocytes, NK cells and eosinophils, can also proliferate in response to IL-2 (Boyman and Sprent, 2012). Consequently, it is necessary to enrich a precursor population for $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} in order to remove IL-2-responsive non-T_{regs} from subsequent culture and minimise the risk of expanding a contaminating population of non-T_{regs}. Rapamycin prevents the outgrowth of contaminating $CD4^+$ T_{cons} and promotes the selective expansion of T_{regs} (Golovina et al., 2011; Putnam et al., 2009; Scottà et al., 2013; Strauss et al., 2009; Tresoldi et al., 2011). In this study, rapamycin was not sufficient to prevent the outgrowth of $CD8^+$ lymphocytes from an initial MACS-enriched $CD8^-CD25^+$ precursor population. In contrast, T_{regs} expanded from FACS-sorted precursor populations were not contaminated by non-$CD4^+$ cells. While the precise contribution of contaminating $CD8^+$ cells to the phenotype of day 24 *in vitro* expanded MACS T_{regs} was not examined in this study, MACS T_{regs} had significantly worse *in vitro* suppression, significantly greater cytokine expression and significantly lower *FOXP3* TSDR demethylation than day 24 *in vitro* expanded $CD45RA^+$ T_{regs}. In future work, it may be interesting to further define the phenotype of $CD4^+$ and $CD8^+$ populations expanded from MACS-enriched $CD8^-CD25^+$ precursors, by FACS-sorting both populations following expansion.

Precise definition of the optimum precursor population from which to expand T_{regs} *in vitro* is required for a program of cell therapy. Hoffmann *et al.* previously showed that a homogenous population of *in vitro* suppressive T_{regs} could be expanded from the CD45RA$^+$ subset of CD4$^+$CD25^{hi} healthy control T_{regs} (Hoffmann et al., 2004). *In vitro* expanded CD4$^+$CD25^{hi}CD45RA$^+$ cells from healthy donors retain CD62L and CCR7 expression following expansion (Hoffmann et al., 2004) and, in contrast to T_{regs} expanded from the healthy control CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^-$ subset, retain a demethylated *FOXP3* TSDR and do not lose FOXP3 protein expression following repeated re-stimulation *in vitro* (Hoffmann et al., 2009). In the present study, T_{regs} expanded *in vitro* from CD PB CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^+$ precursors recapitulated the previously described phenotype of T_{regs} expanded from healthy donor CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^+$ precursors more closely than T_{regs} expanded from other precursor populations. Day 24 *in vitro* expanded CD45RA$^+$ T_{regs} highly expressed CD62L and CCR7, which is associated with therapeutic efficacy in pre-clinical models (Ermann et al., 2005; Issa et al., 2010). Day 24 *in vitro* expanded CD45RA$^+$ T_{regs} also retained *FOXP3* TSDR demethylation despite re-stimulation *in vitro*. This study extends Hoffmann *et al.'s* observations to T_{regs} expanded *in vitro* from patients with CD.

T cell lineage plasticity is well described. A potential barrier to T_{reg} therapy is the possibility that *in vitro* expanded T_{regs} might express pro-inflammatory cytokines and adopt an inflammatory phenotype when adoptively transferred to a CD patient. Thymus-derived T_{regs} can express pro-inflammatory cytokines and transcription factors essential to effector CD4$^+$ lineages, including Th1 (Koch et al., 2009; Oldenhove et al., 2009) and Th17 (Afzali et al., 2013b; Koenen et al., 2008; Voo et al., 2009), both of which are implicated in CD pathogenesis. Indeed, IL-17

expressing T_{regs} have also been identified in non-inflamed human blood and lymphoid tissue (Voo et al., 2009), and inflamed Crohn's mucosa (Hovhannisyan et al., 2011). However, freshly isolated $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} from healthy donors (Miyara et al., 2009) and T_{regs} expanded *in vitro* healthy donor $CD45RA^+$ precursors (Hoffmann et al., 2004) have reduced, or absent, expression of pro-inflammatory cytokines, in contrast to other freshly isolated T_{reg} populations, or T_{regs} expanded *in vitro* from $CD45RA^-$ precursors. This study extends these observations to T_{regs} expanded from CD PB, by showing that T_{regs} expanded from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors did not express IL-17A or other Th17 related genes, even following exposure to Th17-skewing cytokines that may be present in inflamed intestinal mucosa. In contrast, T_{regs} expanded *in vitro* from other CD PB precursor populations (particularly *in vitro* expanded MACS T_{regs}) expressed IFN-γ, IL-17 and TNF to a greater degree than *in vitro* expanded $CD45RA^+$ T_{regs}. Of the three *in vitro* expanded T_{reg} populations studied, these data suggest that *in vitro* expanded $CD45RA^+$ T_{regs} are the least likely to express pro-inflammatory cytokines, which may contribute to CD pathogenesis following adoptive transfer *in vivo*.

Freshly isolated $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} have an epigenetically stable *FOXP3* locus with extensive TSDR demethylation (Baron et al., 2007; Miyara et al., 2009). *FOXP3* TSDR demethylation correlates with stable FOXP3 expression in T_{regs} from fresh healthy donor PB (Baron et al., 2007; Floess et al., 2007; Polansky et al., 2008), in contrast to transient and unstable FOXP3 expression seen in activated T_{cons}, which is not associated with TSDR demethylation (Baron et al., 2007). *FOXP3* TSDR demethylation also correlates with stable FOXP3 expression in T_{regs} expanded *in vitro* from healthy donor $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$

precursors (Baron et al., 2007; Hoffmann et al., 2009), and with T_{reg}-mediated protection from autoimmunity *in vivo* (Barzaghi et al., 2012) in humans. These observations are extended in this study by demonstrating that T_{regs} expanded from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors have complete *FOXP3* TSDR demethylation following 24 days of *in vitro* expansion, while T_{regs} expanded from $CD8^-CD25^+$ MACS-enriched CD PB precursors have an almost completely methylated *FOXP3* TSDR. T_{regs} expanded from $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ CD PB precursors have an intermediate phenotype. This stepwise decrease in *FOXP3* TSDR demethylation seen across the three *in vitro* expanded T_{reg} populations investigated in this study is mirrored by a stepwise decrease in *in vitro* suppressive ability and a stepwise increase in pro-inflammatory cytokine expression. Barzaghi *et al.* recently described a cohort of patients with "IPEX-like syndrome", severe multisystem autoimmunity in the absence of mutations in molecules implicated in T_{reg} function, with decreased TSDR demethylation despite normal T_{reg} numbers and *in vitro* suppression (Barzaghi et al., 2012). Extrapolating these data to the present study, this suggests that *in vitro* expanded T_{regs} with incomplete *FOXP3* TSDR demethylation may have suboptimal biological activity *in vivo*, despite a suppressive function *in vitro*. Taken together, the present study suggests that T_{regs} expanded *in vitro* from CD PB $CD4^+CD25^{hi}CD127^{lo}$ $CD45RA^+$ CD PB precursors are more likely to retain phenotypic stability, are more likely to be biologically active *in vivo*, and are less likely to acquire an effector phenotype following adoptive transfer than T_{regs}, expanded *in vitro* from other precursor populations. This suggests that T_{regs} expanded from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors may ultimately have a more

favourable safety profile, supporting the further development of T_{regs} expanded *in vitro* from this subset as a cell-based therapy for CD.

Another perceived barrier to T_{reg} therapy in CD is that effector T cells from inflamed CD mucosa may be resistant to the suppressive action of T_{regs}. Indeed, T_{cons} isolated from inflamed CD mucosa overexpress Smad7, an inhibitor of TGF-β signalling, rendering them resistant to suppression by freshly isolated LP T_{regs} (Fantini et al., 2009; Monteleone et al., 2005a). While it was not formally shown for T_{regs} expanded from CD PB in this study, *in vitro* expansion in the presence of rapamycin enhances the *in vitro* suppressive ability of T_{regs} isolated from patients with other diseases, compared with freshly isolated PB T_{regs} from the same donor (Afzali et al., 2013a; Cao et al., 2010). In the present study, T_{regs} generated *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors in the presence of rapamycin suppress activation of MLN and LP T cells, and suppress proliferation of MLN T cells obtained from inflamed Crohn's resection specimens. This result contradicts the prediction that *in vitro* expanded T_{regs} will not suppress T_{cons} obtained from inflamed CD mucosa due to T_{con} resistance to T_{reg}-mediated suppression (Fantini et al., 2009; Monteleone et al., 2005a). These data also suggest that T_{regs} expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors may modulate immune responses in niches directly relevant to the pathogenesis of CD. Future work will need to determine if *in vitro* expansion in the presence of rapamycin enhances the suppressive ability of T_{regs} obtained from CD PB, compared with freshly isolated CD PB T_{regs}, in addition to formally demonstrating that T_{regs} expanded *in vitro* from CD PB $CD4^+CD25^{hi}CD127^{lo}$ $CD45RA^+$ precursors suppress proliferation of LP T_{cons} obtained from inflamed CD mucosa.

In order to be therapeutically effective in CD, adoptively transferred T_{regs} may need to traffic to intestinal lymphoid tissue or LP. Some groups have used T cell receptors (TCRs) specific for luminal antigens to direct T_{regs} to the intestinal mucosa, such as T cells with transgenic Cbir1 flagellin-specific TCRs (Feng et al., 2011) or IL-10-producing T cell clones selected on the basis of ovalubumin specificity (Desreumaux et al., 2012). An alternative approach has been to expand T_{regs} in the presence of ATRA which induces $\alpha_4\beta_7$ integrin expression but also increases expression of effector cytokines, such as IL-17 and IFN-γ, limiting its potential use in cell expansion for therapeutic purposes (Golovina et al., 2011; Scottà et al., 2013; Tresoldi et al., 2011). The present study shows that T_{regs} expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors in the presence of IL-2 and rapamycin highly express CD62L and CCR7, allowing homing to and anatomical orientation within lymphoid tissue (Debes et al., 2002; Zhang et al., 2009). T_{reg} CD62L expression is also required for T_{reg}-mediated cure of GvHD in murine models (Ermann et al., 2005). *In vitro* expanded $CD45RA^+$ T_{regs} also expressed CCR4, required for T_{reg}-mediated prevention of $CD45RB^{hi}$ colitis (Yuan et al., 2007). However, homing to the LP may not be required in order to exert an immune regulatory effect in the gut. T_{regs} lacking β_7 integrin have defective intestinal homing but home to MLN and prevent $CD45RB^{hi}$ colitis (Denning et al., 2005). Consequently, the ability to home to MLN is highly desirable in potentially therapeutic cells.

In vitro expanded $CD45RA^+$ T_{regs} also express $\alpha_4\beta_7$ integrin and CXCR3, indicating an ability to home to LP and sites of inflammation, respectively. Moreover, the present study shows for the first time that *in vitro* expanded

$CD45RA^+$ T_{regs} from CD PB home to inflamed human gut in a murine human SB xeno-transplant model. In this model, human foetal SB segments transplanted on the dorsum of a C.B17 *Scid* mouse mature into tissue that is morphologically and functionally identical to normal gut and is capable of peristalsis and nutrient absorption (Golan et al., 2009; 2010; Howie et al., 1998). The xenografts also possess a chimeric endothelium that expresses human MAdCAM-1 (Winter et al., 1991), providing a potential mechanism for human $\alpha4\beta7$ integrin-mediated homing to the xeno-transplanted SB. This is the first demonstration that this model can be used in the assessment of immune cell homing.

Future work will include establishing this model on an NOD-*Scid*-$\gamma_c^{-/-}$ (NSG) background, in order to remove the requirement for anti-mouse granulocyte treatment following adoptive transfer of human immune cells. Other groups have successfully reconstituted human skin xeno-grafts transplanted on the dorsum of *RAG2*$^{-/-}\gamma_c^{-/-}$ or NSG mice by adoptively transferring IV human PBMCs (Issa et al., 2010; Putnam et al., 2013; Sagoo et al., 2011). A similar approach could be taken to reconstitute SB xeno-grafts with human PBMCs. As the xeno-transplanted SB lumen is sterile, it may be interesting to ask if a luminal microbiome is required for optimum immune reconstitution of the xeno-transplanted SB LP. It may also be interesting to determine if adoptively transferred $CD34^+$ stem cells can be used to reconstitute SB xeno-grafts with human immune cells. The requirements for human immune cell homing to the SB xeno-grafts can also be determined during these experiments.

Xeno-graft inflammation has been induced in this model using intraluminal injection of enteropathogenic bacteria, such as *Mycobacterium avium*

paratuberculosis (Golan et al., 2009) or EPEC (Golan et al., 2010). SB xeno-grafts reconstituted with HLA-mismatched $CD4^+CD25^{hi}$-depleted PBMCs are likely to develop GvHD. This model can also be used to determine if *in vitro* expanded T_{regs} can prevent or treat intestinal GvHD in humans.

In conclusion, these observations suggest that T_{regs} expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors are likely to be the most suitable T_{reg} subset for cellular therapeutics in CD. In contrast to other *in vitro* expanded T_{reg} populations tested, T_{regs} expanded from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors have a demethylated *FOXP3* TSDR and do not express pro-inflammatory cytokines, suggesting that they will stably express FOXP3 and are unlikely to acquire a pro-inflammatory phenotype following adoptive transfer. These cells express a repertoire of homing receptors suitable for intestinal and lymphoid homing and traffic to inflamed gut *in vivo.* Importantly, T_{regs} expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors suppress activation of LP and MLN T_{cons}, and proliferation of MLN T_{cons}, obtained from an inflamed CD resection specimen. Furthermore, the present study suggests that the general conclusions of earlier studies on both freshly isolated $CD45RA^+$ T_{regs} from healthy donors (Miyara et al., 2009) and T_{regs} expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors obtained from healthy donors (Baron et al., 2007; Hoffmann et al., 2006; 2009) can be extended to the equivalent T_{reg} populations obtained from CD patients. These data pave the way for further the development of T_{regs} expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors as a cell-based therapy for CD.

12 General Discussion and Conclusions

12.1 Introduction

T_{regs} are currently the subject of intensive investigation as a potential cell-based therapy for a range of immune-based diseases in humans. In humanised immunodeficient mice, *in vitro* expanded human T_{regs} prevent both the rejection of human skin transplants following adoptive transfer of allogeneic PBMCs (Issa et al., 2010; Sagoo et al., 2011) and arteriosclerosis in xeno-transplanted internal mammary artery grafts (Nadig et al., 2010). *In vitro* expanded T_{regs} also prevent GvHD in humanized mouse models (Ermann et al., 2005; Scottà et al., 2013).

However, T_{reg} doses at a 1:1 ratio with adoptively transferred effector cells are required to suppress GvHD in murine models (Hoffmann et al., 2002). As PB T_{regs} comprise only 1% of all PBMCs, multiple groups have focussed on developing techniques to expand these cells *in vitro* for subsequent infusion at ratios close to 1 T_{reg} per PBMC. Many groups have shown that human T_{regs} can be expanded *in vitro* while maintaining stable *FOXP3* expression and a suppressive phenotype *in vitro* (Battaglia et al., 2006; Golovina et al., 2011; Hippen et al., 2011b; Hoffmann et al., 2006; 2004; Putnam et al., 2009; Scottà et al., 2013; Strauss et al., 2007b; 2009; Tresoldi et al., 2011). Furthermore, the first protocol for clinical-grade *in vitro* T_{reg} expansion was recently published (Putnam et al., 2013).

Proof-of-principle studies have recently been performed in humans. A recent case series (Trzonkowski et al., 2009) and a phase I clinical trial (Brunstein et al., 2011) suggest that *in vitro* expanded T_{regs} are safe in the prevention of GvHD following HSCT. Similarly, freshly isolated T_{regs} may also be safe in the prevention

of GvHD (Di Ianni et al., 2011). Another recent phase I clinical trial safely infused autologous *in vitro* expanded T_{regs} to children with recently diagnosed T1DM (Marek-Trzonkowska et al., 2012). In addition, clinical trials of *in vitro* expanded T_{regs} with the intention of reducing the burden of immunosuppression following renal or liver transplantation are currently planned (Leslie, 2011; Putnam et al., 2013).

Data from immunodeficient mouse models, in which adoptively transferred T_{regs} can modulate immune-mediated intestinal inflammation (Garrett et al., 2007; Huber et al., 2004; Ishikawa et al., 2013; Maloy et al., 2003; Morrissey et al., 1993; Mottet et al., 2003; Powrie et al., 1993; Veltkamp et al., 2006; Watanabe et al., 2008a), and data from humans with rare monogenetic immunodeficiencies, in which defects in T_{reg} function may be associated with intestinal inflammation (Glocker et al., 2009; Moran et al., 2013; Wildin et al., 2000), suggest that T_{regs} play a role in intestinal immune homeostasis. While the vast majority of IBD patients do not have cell-intrinsic T_{reg} defects, LP T_{cons} obtained from CD patients have been shown to be resistant to autologous LP T_{reg}-mediated suppression of proliferation *in vitro*, mediated by LP T_{con} overexpression of Smad7, an inhibitor of TGF-β signalling (Fantini et al., 2009). These observations suggest that it is reasonable to explore the possibility of developing T_{regs} as a possible cell-based therapy for IBD.

This thesis presents results in two chapters. The first results chapter validates a rapid assay of T_{reg} function for *in vitro* expanded T_{regs}, with the aim of ultimately developing this as a functional assay to aid in the assessment of *in vitro* expanded T_{reg} lots. The second results chapter compares methods of isolating precursor populations for subsequent *in vitro* expansion of putative T_{reg}

populations that are consistent with currently available GMP cell isolation and enrichment techniques. It suggests that T_{regs} expanded *in vitro* from FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors have significant advantages over T_{regs} expanded *in vitro* from MACS-enriched $CD4^+CD25^-$ and FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ precursors.

12.2 A future role for a rapid assay of T_{reg} function

Quantification of T_{reg}-mediated inhibition of T_{con} proliferation by T_{reg}-mediated inhibition of CFSE dilution or thymidine incorporation in T_{cons}, is the current gold standard for the *in vitro* assessment of T_{reg} function. The present study validates a novel, commercially available 7-hour assay of T_{reg} function by showing excellent correlation with the results of 96-hour CFSE dilution assays, for both freshly isolated and *in vitro* expanded T_{regs}. This provides an additional platform for the functional T_{reg} assessment. As illustrated in Section 11.8 (page 235), the 7-hour assay might be most useful in testing T_{reg} function on responder cells obtained from highly inflamed tissue that might not remain viable for the 72-96 hours required for a CFSE dilution assay. The 7-hour assay could also be incorporated into protocols for functional assessment of T_{regs} during the pre-clinical development of T_{reg} cell products.

This assay was initially validated with the intention of providing a method to assess the function of *in vitro* expanded T_{reg} lots on the same day as infusion. While a rapid assay makes same-day assessment of T_{reg} function feasible, the relationship between *in vitro* suppressive function and subsequent biological activity of adoptively transferred T_{regs} in humans is unclear. It may be worthwhile

to incorporate functional assessment of *in vitro* expanded T_{reg} lots into future clinical trial protocols in order to define the relationship between *in vitro* suppressive activity and *in vivo* therapeutic activity. These data could then be used to determine if functional assessment has a role in identifying potentially non-therapeutic *in vitro* expanded T_{reg} lots prior to infusion, followed by confirmation in a prospective clinical trial. Functional assessment of *in vitro* expanded T_{regs} by CFSE dilution has been reported in one recent clinical trial (Brunstein et al., 2011). At present, *in vitro* expanded T_{reg} products must currently meet pre-specified "lot release criteria", relating to cell purity and the sterility of the manufacturing process, before being released for infusion. It is unlikely that an assay of T_{reg} function will be incorporated into, or supersede conventional lot release criteria in the absence of a prospective clinical trial confirming the utility of the functional assay in excluding T_{reg} lots that are unlikely to have biological activity *in vivo*.

Assessment of *FOXP3* TSDR demethylation might be a complementary, or alternative approach to a rapid assay of *in vitro* expanded T_{reg} function. Demethylated *FOXP3* TSDR correlates with stable FOXP3 expression and a regulatory phenotype in T_{regs} obtained from healthy donor PB (Baron et al., 2007; Floess et al., 2007; Miyara et al., 2009; Polansky et al., 2008) and also in *in vitro* expanded T_{regs} (Baron et al., 2007; Hoffmann et al., 2009). However, as with the 7-hour T_{reg} functional assay, a correlation between *FOXP3* TSDR demethylation status and *in vivo* biological activity will have to be determined. This will be particularly interesting, as TSDR demethylation status is inversely proportional to cytokine expression in freshly isolated PB T_{regs} from healthy donors, whereas evidence from murine models suggests that T_{regs} co-opt the cellular machinery associated with Th1 (Koch et al., 2009; Oldenhove et al., 2009), Th2 (Zheng et al.,

2009) and Th17 (Chaudhry et al., 2009) polarisation to home to inflamed sites and suppress Th1, Th2 or Th17-mediated inflammation, respectively.

12.3 $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors can be expanded *in vitro* from Crohn's disease patients' peripheral blood to yield an *in vitro* suppressive and phenotypically stable cell population

In order to develop T_{regs} as a cell-based therapy for CD, it is necessary to determine that precursor cells can be isolated from the PB of CD patients, that these cells can be expanded *in vitro*, that the expanded cells have a regulatory phenotype *in vitro* and are phenotypically stable, that these cells have the potential to home to the intestine, and that these cells can suppress intestinal inflammation. The second results chapter attempts to define the precursor population from which to expand a T_{reg} population from CD PB *in vitro*, that meets the above criteria.

Freshly isolated human PB $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} can be further sub-divided on the basis of $CD45RA^+$ expression (Hoffmann et al., 2004; Miyara et al., 2009). Hoffmann *et al.* previously showed that a homogenous population of *in vitro* suppressive T_{regs} could be expanded from the $CD45RA^+$ subset of $CD4^+CD25^{hi}$ healthy control human T_{regs} and that this expanded cell population is resistant to the expression of pro-inflammatory cytokines (Hoffmann et al., 2004). Hoffmann *et al.* then showed that putative T_{regs} expanded *in vitro* from $CD4^+CD25^{hi}CD45RA^+$ precursors have a demethylated *FOXP3* TSDR and retain stable expression of FOXP3 protein despite repeated stimulation *in vitro* (Hoffmann et al., 2009). Based

on these observations, it was hypothesised that $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} may be suitable population from which to expand T_{regs} from CD PB *in vitro*.

Precursor populations were then enriched using enrichment techniques that were compatible with currently available GMP technology. The characteristics of cell populations expanded from MACS-enriched $CD4^+CD25^+$ precursors, or FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ or $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ precursors were then compared.

This study shows that it is technically feasible to isolate each of these precursor populations from the PB of CD patients, and to expand these cells *in vitro*. MACS-enriched precursors expanded to a greater degree than FACS-enriched precursors. Cells expanded from each of these precursor populations suppressed *in vitro* proliferation of autologous T_{cons}. However, cells expanded *in vitro* from FACS-sorted $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors had significant advantages over cells expanded from other precursor populations. In contrast to T_{regs} expanded from other precursor populations, and consistent with the results of previous studies on freshly isolated, or *in vitro* expanded, $CD45RA^+$ T_{regs} from healthy controls (Hoffmann et al., 2006; Miyara et al., 2009), T_{regs} expanded *in vitro* from CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors were resistant to the induction of pro-inflammatory cytokines. Similarly, consistent with results of previous studies on freshly isolated, or *in vitro* expanded, $CD45RA^+$ T_{regs} from healthy controls (Baron et al., 2007; Hoffmann et al., 2009), T_{regs} expanded *in vitro* from CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors have a completely demethylated *FOXP3* TSDR, which is associated with stable FOXP3 expression. Taken together, these data suggest that T_{regs} expanded *in vitro* from CD PB

$CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors are likely to be phenotypically stable and maintain a T_{reg} phenotype. These data also suggest that T_{regs} expanded *in vitro* from CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors may be less likely than T_{regs} expanded *in vitro* from other precursor populations to express pro-inflammatory cytokines upon adoptive transfer to CD patients, and thus potentially contribute to CD pathology. Interestingly, these data also suggest that observations made on T_{regs} obtained from healthy controls also appear to apply to equivalent populations of T_{regs} obtained from CD patients, suggesting that it may be possible to extrapolate conclusions made with T_{regs} from healthy controls to patients with CD.

12.4 The potential for gut tropism in T_{regs} expanded *in vitro* from Crohn's disease patients' peripheral blood $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors

In order to be therapeutically effective , adoptively transferred T_{regs} may need to home to CD LP or MLN. T cell homing is mediated by the expression of cell surface homing receptors that interact with glycoproteins expressed in high endothelial venules of target organs. T_{regs} expanded *in vitro* from CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors express $\alpha_4\beta_7$ integrin and CCR4, which may facilitate homing to the intestine. However, CCR9 expression was not detected in *in vitro* expanded T_{regs}, either by FACS or rtPCR. CCL25, the ligand for CCR9, is expressed in both non-inflamed and inflamed small bowel. The CCR9-CCL25 interaction induces a conformational change in $\alpha_4\beta_7$ integrin, allowing a stable adhesion of $\alpha_4\beta_7$ to MAdCAM-1 (Thomas and Baumgart, 2012). Antibody-mediated blockade of CCR9 prevents lymphocyte homing to the ileum of

SAMP1/YitFc mice, preventing the induction of ileitis in these animals (Rivera Nieves et al., 2006). Genetic absence of CCR9 is associated with impaired ileal homing of regulatory $CD4^+CD25^+$ and $CD8^+CD103^+$ lymphocytes and worse ileitis in $TNF^{\Delta ARE}$ mouse model, compared with CCR9 sufficient $TNF^{\Delta ARE}$ mice (Wermers et al., 2011). CCR9 is also likely to be relevant to human biology. A phase II clinical trial of an orally active CCR9 antagonist yielded promising results in CD (Keshav et al., 2013), although phase III studies were discontinued early and results are not yet published. These results suggest that CCR9 expression may be required for optimal homing of adoptively transferred T_{regs} to small bowel.

Small bowel tropism mediated by $\alpha_4\beta_7$ integrin and CCR9 expression can be imprinted on murine lymphocytes *in vitro* by co-culture with Peyer's patches dendritic cells in a retinoic acid-dependent manner (Mora et al., 2003). $\alpha_4\beta_7$ integrin and CCR9 expression is also imprinted in MLN *in vivo*, *via* MLN stromal cell production of retinoic acid (Hammerschmidt et al., 2008). Consequently, it is possible that adoptively transferred T_{regs} generated from CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors may be induced to up-regulate $\alpha_4\beta_7$ integrin and CCR9 expression in MLNs upon activation *in vivo*.

Scottà *et al.* wished to determine if gut tropism could be imprinted on *in vitro* expanded T_{regs} during culture (Scottà et al., 2013). MACS-enriched $CD4^+CD25^+$ PB lymphocytes from healthy donors were expanded *in vitro* for 28 days in the presence of rapamycin, ATRA or rapamycin plus ATRA, and CCR9 and $\alpha_4\beta_7$ integrin expression was then assessed. Approximately 10% of rapamycin-expanded T_{regs} expressed CCR9, while approximately 20% expressed $\alpha_4\beta_7$ integrin. A possible reason for the discrepancy in CCR9 expression seen between Scottà *et*

al. and the present study may be that $CCR9^+$ cells expanded from the $CD45RA^-$ subset of the precursor population. Culture in the presence of ATRA or ATRA/rapamycin increased the approximate expression of CCR9 to 50% and 25%, respectively; and the expression of $\alpha_4\beta_7$ integrin to 97% and 95%, respectively. While it is clear that culture in the presence of ATRA increases CCR9 expression, this is at the cost of increased IL-17 and IFN-γ expression, compared with T_{regs} cultured with rapamycin alone (Golovina et al., 2011; Scottà et al., 2013; Tresoldi et al., 2011), which may be disadvantageous for the reasons stated above. Furthermore, T_{regs} expanded in ATRA alone do not suppress cytokine production in co-cultured T_{cons} *in vitro*(Golovina et al., 2011). Nevertheless, it may be worthwhile to expand CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors in the presence of rapamycin alone or rapamycin plus ATRA, in order to determine if ATRA increases CCR9 and $\alpha_4\beta_7$ integrin expression in T_{regs} expanded from CD PB.

The immunological site, or sites, at which *in vitro* expanded T_{regs} might exert their immune modulating effect *in vivo* in humans is not known. T_{regs} expanded *in vitro* from CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors also expressed other homing receptors for gut (CCR4), for lymphoid tissue (CD62L, CCR7) and inflammatory sites (CXCR3). Interestingly, murine T_{regs} do not require $\alpha_4\beta_7$ integrin-mediated gut tropism in order to prevent colitis in the $CD45RB^{hi}$ adoptive transfer model (Denning et al., 2005). Adoptively transferred $\beta_7^{-/-}$ T_{regs} have defective intestinal homing but are identifiable in MLN and prevent the development of $CD45RB^{hi}$ colitis (Denning et al., 2005). Murine T_{regs} also require CD62L-mediated lymphoid homing to prevent murine GvHD (Ermann et al., 2005). Consequently, the lymphoid tropism expressed by T_{regs} expanded *in vitro* from CD

PB $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{+}$ precursors may be desirable in potentially therapeutic cells.

12.5 Intestinal homing of adoptively transferred T_{regs} in the human intestinal xeno-graft model

Demonstration of intestinal homing of adoptively transferred T_{regs} *in vivo* is an entirely novel use of the human intestinal xeno-graft model. This model was originally described as a means of investigating foetal intestinal development (Angioi et al., 2002; Howie et al., 1998; Savidge et al., 1995; Shmakov et al., 1996; Winter et al., 1991). It was then further developed as a model to investigate the biology and pathogenesis of pathogens in the human gut, such as *Mycobacterium avium paratuberculosis* (Golan et al., 2009)and enteropathogenic *Escherichia coli* (Golan et al., 2010).

The model involves subcutaneous implantation of germ-free human foetal small bowel tissue on the dorsum of a *Scid* mouse. Following initial ischaemic necrosis of luminal villi, angiogenesis occurs in the xeno-transplanted intestinal segment, which is associated with epithelial healing (Savidge et al., 1995). By 4-8 weeks after xeno-transplantation, an extensive circumferential vascular array develops, supplying all layers of the foetal gut (from serosa to epithelium) (Savidge et al., 1995). By this time, intestinal segments are morphologically similar to foetal gut of 7-10 weeks gestational age. Transplanted intestinal segments have functional pluripotent epithelial stem cells and thereafter display conventional, tissue-specific spatio-temporal patterns of cell proliferation, lineage commitment and differentiation, including the development of goblet cells and Paneth cells

(Shmakov et al., 1996). By 10-12 weeks after transplantation, intestinal segments develop into tissue that is morphologically and functionally identical to normal paediatric gut and is even capable of peristalsis and nutrient absorption (Angioi et al., 2002).

This study shows that T_{regs} expanded *in vitro* from CD PB $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{+}$ precursors home to human small bowel when adoptively transferred to *Scid* mice bearing an inflamed human intestinal xeno-graft. The xeno-transplanted gut possesses a chimeric endothelium: with both murine- and human-derived endothelial cells (Winter et al., 1991). Furthermore, the xeno-transplanted gut endothelium has been shown to express human MAdCAM-1 (Winter et al., 1991), which is a ligand for both $\alpha_4\beta_7$ integrin and CD62L (Thomas and Baumgart, 2012), providing a potential mechanism for intestinal homing in this model.

A potential criticism of this model is that a relatively small number of T_{regs} were identified in the lamina propria of the xeno-transplanted intestine, by FACS. This made it technically impossible to retrieve adoptively transferred cells from the xeno-transplanted LP in order to demonstrate that they remained suppressive *in vitro* following isolation. Sharif-Paghaleh *et al.* recently used a Single Photon Emission Computed Tomography (SPECT) gene reporter system to track the homing of *in vitro* expanded T_{regs} that were adoptively transferred to C57BL/6 mice (Sharif-Paghaleh et al., 2011). Twenty four hours after adoptive transfer, T_{regs} primarily localised to the spleen. This is consistent with ready identification of anti-human $CD45^{+}CD3^{+}$ events in the spleen of xeno-transplanted mice at 24 hours, in the present study (shown in Figure 11.9 [page 231]). Further work will

focus on defining the tempo of adoptively transferred T cell homing to xeno-transplanted gut in this model. SPECT would be an ideal non-invasive method to localise adoptively transferred human T cells at various time-points *in vivo*. Further work will also focus on determining the mechanism of intestinal homing in this system. The expression of integrin and CCR ligands on xeno-transplanted gut endothelium will be defined by immunohistochemistry and rtPCR. Furthermore, the physiological relevance of these markers to homing of adoptively transferred T cells to non-inflamed and inflamed human gut will be defined in antibody-blocking experiments.

Another potential criticism of this model is that the acute EPEC infection used to induce graft inflammation is dissimilar to the chronic, or acute-on-chronic mucosal inflammation seen in human IBD. EPEC infection of xeno-grafts in this model results in significant epithelial damage and haemorrhagic colitis within 8 hours (Golan et al., 2010). *Scid* mice also lack T and B cells, in addition to lacking NK cells in this anti-NK antibody treated model. Induction of xeno-graft mucosal inflammation by acute *E. coli* infection does not result in a prominent immune cell infiltrate. Consequently, further work will focus on developing this system in order to more closely model human IBD in a murine host. This is further discussed in Section 12.6 (page 260), below.

The human intestinal xeno-graft experiments described in this thesis were technically complex and required travel to a collaborating laboratory abroad. They were also limited by the availability of foetal material. This limited the experimental approach available. For example, it was not possible to assess the homing of T_{regs} expanded *in vitro* from CD PB $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{-}$

precursors in this model, due to a limited number of mice available. The interesting questions raised by these experiments are sufficiently wide in scope as to be separate projects in themselves, and will require separate project grant funding. However, these results provide proof-of-principle that the human foetal intestinal xeno-graft model may be a useful platform to investigate T cell homing to human gut in a pre-clinical system.

12.6 Future work demonstrating the *in vitro* suppressive activity of *in vitro* expanded T_{regs}

In developing a regulatory cell population for potential therapeutic use, it is important to demonstrate that the putative regulatory cell population can suppress effector actions of responder cells *in vitro*. This is particularly important in relation to CD, due to pre-clinical evidence that effector T cells from the diseased mucosa of CD patients may be resistant to the suppressive action of T_{regs} (Fantini et al., 2009; Monteleone et al., 2005a). This study shows that T_{regs} expanded *in vitro* from CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors suppress early activation of MLN and LP responder $CD3^+$ cells obtained from an inflamed CD terminal ileal resection. In addition, in a parallel experiment, these *in vitro* expanded T_{regs} also suppressed proliferation of MLN responder $CD3^+$ cells obtained from the same CD resection. These results are statistically robust, as each experiment tests the *in vitro* suppressive activity of at least 5 T_{reg} lines, while minimising variability by using responder cells from a single resection. However, results for T_{reg}–mediated suppression of LPMC $CD3^+$ proliferation are not shown, as responder LPMCs died during the 96-hour incubation required for this co-culture experiment. This demonstrated the importance of having earlier optimised and validated the 7-hour

assay of T_{reg} suppressive function, and underlines the novel use of this assay here. Nevertheless, these experiments should be repeated using responder cells obtained from other CD intestinal resections in order to formally show that *in vitro* expanded T_{regs} can suppress the proliferation of LP $CD3^+$ responder cells *in vitro*, and to confirm the generalisabilty of these results.

In vitro culture of T_{regs} in the presence of rapamycin enhances their *in vitro* suppressive ability, compared with freshly isolated PB T_{regs} obtained from the same donor. This has been demonstrated for T_{regs} obtained from healthy controls and from patients with rheumatoid arthritis, systemic lupus erythematosus, asthma and end-stage renal failure (Afzali et al., 2013a; Cao et al., 2010). The present study did not formally demonstrate this for freshly isolated, and *in vitro* expanded, T_{regs} obtained from CD patients. Future work will address this question.

12.7 Future work demonstrating the *in vivo* suppressive activity of *in vitro* expanded T_{regs}

It would be ideal to support the *in vitro* suppression data described above with a demonstration that T_{regs} expanded *in vitro* from CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors modulate intestinal inflammation in a humanised pre-clinical model, *in vivo*. Adoptively transferred murine T_{regs} modulate intestinal inflammation in both adaptive (Morrissey et al., 1993; Powrie et al., 1993; Watanabe et al., 2008a) and innate (Garrett et al., 2007) immune-mediated intestinal pathology in mice. However, concerns exist relating to the direct applicability of findings in murine models to human disease. Seok *et al.* recently examined the transcriptional responses to inflammatory stresses from

burns, trauma and endotoxaemia in humans and murine models (Seok et al., 2013). Further *in silico* analysis revealed significant differences in the time courses of transcriptional responses, and in the pathways that were activated and suppressed, between humans and murine models of human disease. Furthermore, mechanisms of drug action may be different across species, which has implications for the conclusions that can be drawn from pre-clinical testing of therapeutic agents across species barriers, as highlighted in recent reports on the mechanism of action of anti-TNF antibodies in IBD (Atreya et al., 2011) and oral anti-CD3 antibody in T1DM (Waldron-Lynch et al., 2012). Pre-clinical testing may also yield results that allow falsely reassuring conclusions to be drawn across species barriers. In 2006, 8 patients participated in a double-blind, randomised, placebo controlled trial of TGN1412, an anti-CD28 antibody (Suntharalingam et al., 2006). Six participants received the study drug. TGN1412 infusion was associated with the development of systemic inflammatory response syndrome in all 6 recipients, due to cytokine release from effector memory $CD4^+$ lymphocytes (Eastwood et al., 2010; Suntharalingam et al., 2006). Subsequent work showed that CD28 was not expressed on effector memory $CD4^+$ lymphocytes in non-human primate species used in pre-clinical testing. Finally, there is often poor concordance between the results of murine models of human disease and subsequent clinical trials in humans based on these models (Perel et al., 2007). These points illustrate the need to develop pre-clinical models of IBD that more closely mimic IBD in humans.

Humanised mice are immunodeficient mouse strains that have been adoptively transferred with human immune cells with the intention of recapitulating a human immune response in a pre-clinical model. This allows the characterisation of human immune responses in a pre-clinical setting. Humanised

mice more closely model human immune responses than murine models of human disease (Waldron-Lynch et al., 2012). Humanised mice have been used to demonstrate that *in vitro* expanded human T_{regs} can treat xeno-GvHD *in vivo* (Scottà et al., 2013). Humanised mouse xeno-transplant models have been used to demonstrate that *in vitro* expanded human T_{regs} can prevent rejection of human skin transplants (Issa et al., 2010; 2012; Sagoo et al., 2011) or arteriosclerosis (Nadig et al., 2010), mediated by HLA-mismatched human PBMCs. However, at the time of writing, robust and reproducible humanised models of IBD are not widely available. Nolte *et al.* recently described oxazolone-induced colitis in NSG mice engrafted with human PBMCs from healthy donors, and colitis induced by intra-rectal ethanol instillation in NSG mice engrafted with PBMCs from patients with UC (Nolte et al., 2013). Goettel *et al.* recently described the development of enteritis in NSG mice injected with anti-human $CD3^+$ antibody (OKT3) (Goettel et al., 2013). In another recently described model, NSG mice lacking MHC-II, but expressing the human HLA-DR transgene under the control of the MHC-II promoter, were engrafted with human $CD4^+$ lymphocytes and treated with intra-rectal TNBS, following cutaneous sensitisation (Goettel et al., 2013). The induction of TNBS-induced colitis in this model was dependent on the presence of human $CD4^+$ lymphocytes. Future work may involve collaboration with these groups in order to determine if adoptively transferred *in vitro* expanded human T_{regs} modulate intestinal inflammation in these novel humanised models.

Future work will also focus on humanising the human intestinal xeno-graft model in order to recapitulate a human immune system in the xeno-grafted gut. A first step will be to recapitulate the xeno-graft model on a NSG or $RAG2^{-/-}$ $\gamma_c^{-/-}$ background in order to remove the requirement to ablate NK cells with anti-asialo

GM1 antibody. Allogeneic PBMCs will then be adoptively transferred in order to determine if these cells induce GvHD in the intestinal xeno-graft. *In vitro* expanded T_{regs} can then be transferred to determine if these cells suppress intestinal GvHD *in vivo*. Adult PB or neonatal UCB $CD34^+$ stem cells will be adoptively transferred and engraftment assessed by FACS, in order to determine if human stem cells can recapitulate an immune system in the xeno-grafted intestine. As the xeno-transplanted SB lumen is sterile, it may be interesting to ask if a luminal microbiome is required for optimum immune reconstitution of the xeno-graft. The requirements for human immune cell homing to the SB xeno-grafts can also be determined during these experiments.

Were improved engraftment of the xeno-transplanted intestine required, the model can be recapitulated on an autologous "BLT" ("bone marrow, liver and thymus") background (Melkus et al., 2006; Pearson et al., 2008). This will involve implantation of autologous foetal liver and thymus under the kidney capsule at the same time as establishment of the intestinal xeno-graft. Autologous $CD34^+$ stem cells will be reserved and stored. Three weeks after implantation, the mice will receive sub-lethal irradiation, followed by adoptive transfer of the reserved $CD34^+$ stem cells.

Once a reproducible system to engraft the xeno-graft with human immune cells has been characterised, methods to induce xeno-graft inflammation will then be developed. These may include intraluminal injection of pathogenic bacteria (Golan et al., 2009; 2010) or chemicals (Wirtz et al., 2007) to the xeno-graft. Once these models are characterised, it will be possible to determine if adoptively

transferred T_{regs} expanded from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors suppress human intestinal inflammation *in vivo*.

12.8 Conversion from R&D-grade to GMP-grade cell manufacturing

A significant challenge in the further development of these cells as a potential cell therapy for CD will be the conversion of the manufacturing process from R&D-grade reagents in a "standard" biosafety level 2 laboratory to GMP-grade reagents in a GMP-licensed facility. The GMP facility at Guy's Tower currently has the technical and administrative staff in post to facilitate this process, in addition to appropriate licensing from the Medicines and Healthcare Products Regulatory Agency (MHRA) and the Human Tissue Authority (HTA). A summary of the potential GMP workflow is illustrated in Figure 12.1 (page 267).

In contrast to R&D-grade cell culture, GMP-grade manufacturing occurs in a closed system, using automated devices. It is likely that process development will be split into three pieces: (i) Whole blood volume reduction and $CD4^+$ enrichment; (ii) GMP-compatible staining with antibody-conjugated fluorochromes, and subsequent cell sorting; and (iii) cell culture. A significant challenge will be to define the optimal device and reagents to use in each piece.

The GMP facility currently has access to three devices to reduce whole blood volume and to isolate PBMCs. These include the Sepax S2 Cell Processor (Biosafe, Eysins, Switzerland), the SynGenX Cell Processor (SynGen, Sacramento, CA, USA) and the CliniMACS Prodigy device (Miltenyi). A significant advantage of the CliniMACS Prodigy is that it can undertake the volume reduction, PBMC isolation and subsequent $CD4^+$ MACS-based enrichment in a single device.

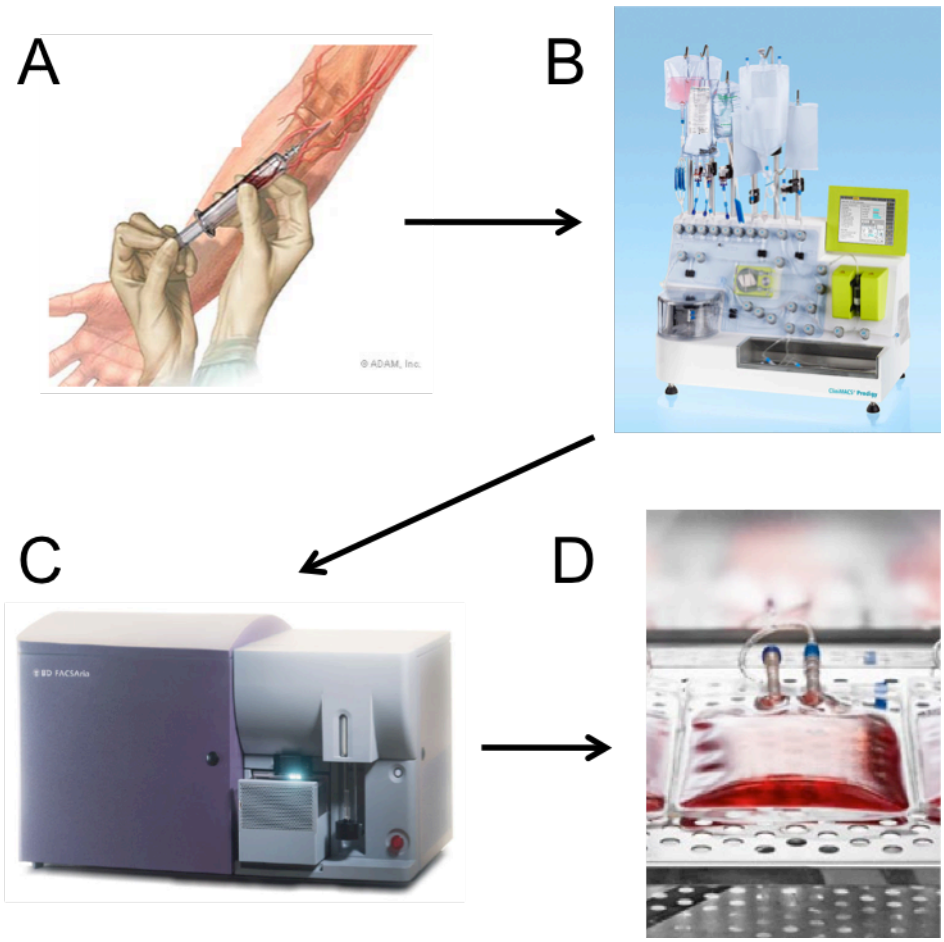
The GMP facility is currently identifying the optimum device with which to sort enriched $CD4^+$lymphocytes into $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors for *in vitro* expansion. Possible devices include the FACSAria II or Influx (both BD) or the MACSQuant Tyto (Miltenyi). The BD Influx contains disposable fluidics tubing, which will prevent cross-contamination between patient samples. The MACSQuant Tyto utilises a sterile, closed and disposable sorting cartridge, which will maintain the sterility of the sorting process also prevent cross-contamination. It is likely that the final decision on the sorter to be acquired will be supported by a collaboration agreement with the manufacturer. Such an agreement may include the provision of GMP antibodies and support for MHRA licensing of the device.

A challenge with cell culture will be the conversion from plate-based, small volume culture in the laboratory to culture bag-based, larger volume cell culture in the GMP facility. This may be the most straightforward aspect of the GMP conversion process, as the GMP facility technical staff already have experience in GMP cell culture of other T_{reg} cell products.

Once the optimum manufacturing process has been defined and scaled up, the sterility of the manufacturing process will be confirmed using a broth transfer validation.

Figure 12.1: A strategy for GMP enrichment and expansion of $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} from the PB of CD patients.

(A) Whole blood or apheresis material will be obtained. **(B)** This material will undergo red cell depletion or volume reduction, prior to labelling with anti-$CD4^+$ magnetic beads and $CD4^+$ enrichment. A CliniMACS Prodigy (Miltenyi, shown here), although other devices that can perform some of these steps are commercially available. **(C)** Cells will then be sorted to $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ events on a GMP cell sorter. A FACSAria II is shown here, although other devices are available to be validated and licensed to perform this step. **(D)** $CD45RA^+$ T_{regs} will then be expanded using GMP reagents, followed by quality control (QC) for each lot prior to infusion, or storage in liquid nitrogen for later use.



12.9 Towards a phase I clinical trial to test the safety of autologous T_{regs} expanded *in vitro* from peripheral blood $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors in patients with Crohn's disease

Funding for a phase Ib/IIa clinical trial is currently being sought in order to test the hypothesis that T_{regs} expanded *in vitro* from autologous CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors are safe in subjects with moderate-to-severe CD.

12.9.1 Aims of the proposed clinical trial

The primary aim of this proposed study will be to identify the maximum tolerated dose (MTD) of the cell product. Secondary aims will include determination of the safety of the cell product in subjects with moderate-to-severe CD (i.e. a description of the number of serious and non-serious adverse events in the study cohort). Subjects will also be comprehensively immune-phenotyped during the clinical trial in order to characterise the immune response to T_{reg} therapy and to correlate this immune response with clinical parameters. While phase I clinical trials are not powered to determine efficacy, it is possible that an early signal of efficacy may be seen.

12.9.2 Summary of the inclusion and exclusion criteria

Inclusion criteria will include (i) Age ≥ 18 years, (ii) A diagnosis of CD ≥ 3 months duration, made by standard clinical, endoscopic and radiological criteria, (iii) Moderate-to-severe disease activity with a Crohn's Disease Activity Index ≥ 220 (Best et al., 1976), (iv) Objective evidence of inflammation, e.g. raised C

Reactive Protein, raised faecal calprotectin or endoscopic evidence of mucosal inflammation and (iv) Failure to tolerate or failure to respond to at least 1 standard course of CD medication prescribed with the intention of inducing or maintaining remission. This can also include corticosteroid dependency.

Exclusion criteria will include (i) A requirement for immediate surgical, endoscopic or radiological intervention related to CD, (ii) Positive stool test for *Clostridium difficile*, and (iii) Current medically significant infection, or positive screening tests for tuberculosis, HIV, HBV or HCV. Additional inclusion and exclusion criteria may be defined in the study protocol.

Rules for concomitant medications will be specified in the clinical trial protocol.

12.9.3 Description of the study design

This will be an open-label phase Ib/IIa clinical trial to determine the maximum tolerated dose (MTD) of a single parenteral dose of T_{regs} expanded *in vitro* from autologous CD PB $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{+}$ precursors. The study will have an adaptive study design, utilising a Bayesian "Continual Reassessment Method" (CRM) (O'Quigley et al., 1990; Sweeting et al., 2013). This method allows the "best estimated" relationship between 5 dose levels and their safety to be continually reassessed based on the occurrence of dose-limiting toxicity (DLT) in preceding subjects across dose levels within the clinical trial. The first subject be allocated to the lowest dose level. Depending on the toxicity response status by dose level from all previous subjects, it will be determined whether each new patient, singly in turn, will have escalation by one dose, will stay at same dose

level, or will de-escalate by one dose. Subjects' toxicity responses will allow the monotonic ("one-parameter power", or stretch-S-shaped) relationship of the risk of DLT across the dose levels to be continually reassessed and updated for the next patient's dose allocation. Based on the experience that will accumulate after every single patient, successively recruited patients will individually receive the "best indicated" of the five dose levels of autologous in vitro expanded Tregs. The rationale for choosing this study design and its performance compared with a traditional 3+3 dose escalation study design are discussed in Section 12.9.5 (page 272).

12.9.4 Dose levels in previous cohorts, and planned dose levels in this study

One phase I clinical trial and one case report using adoptively transferred *in vitro* expanded T_{regs} to prevent or treat GvHD have been reported. Brunstein *et al.* expanded T_{regs} obtained from 1 unit of healthy donor UCB and infused this cell product to 23 patients receiving UCB transplantation, with the intention of preventing GvHD in recipients (Brunstein et al., 2011). Planned dose levels were 0.1, 0.3, 1 and 3 x 10^6 UCB-derived T_{regs}/kg, administered 24 hours after UCB transplantation. An additional cohort received 3 x 10^6 UCB-derived T_{regs}/kg on day 15. Dose escalation occurred after each subject, unless dose-limiting toxicity (DLT) or inadequate T_{reg} expansion to meet the target dose level occurred. Thirteen (56%) received the highest planned dose of 6 x 10^6 T_{regs}/kg, divided between two infusions. Eighteen (78%) patients received a total dose of 3 x 10^6 T_{regs}/kg. Five subjects received less than the planned T_{reg} dose due to insufficient T_{reg} expansion, while one subject received less than the planned dose due to peri-transplant morbidities unrelated to T_{reg} therapy.

Trzonkowski *et al.* administered *in vitro* expanded T_{regs} to one subject with steroid-dependent chronic GvHD (1 x 10^6 T_{reg}/kg) and one subject with treatment-refractory acute GvHD (3 x 10^6 T_{regs}/kg in total over three infusions) (Trzonkowski et al., 2009).

One phase I clinical trial using adoptively transferred *in vitro* expanded T_{regs} to treat newly diagnosed T1DM in children has been reported (Marek-Trzonkowska et al., 2012). Marek-Trzonkowska administered 10 x 10^6 T_{regs}/kg to 4 subjects, followed by 20 x 10^6 T_{regs}/kg to an additional 6 subjects. These infusions were well-tolerated.

The optimum T_{reg} dose required to modulate intestinal inflammation in human CD is unknown. However, optimal T_{reg}-mediated prevention of $CD45RB^{hi}$ colitis in mice occurs when T_{regs} are adoptively transferred at a 1:1 ratio with effector T cells (Morrissey et al., 1993; Powrie et al., 1993). Optimal T_{reg}-mediated prevention of GvHD also occurs when T_{regs} are adoptively transferred at a 1:1 ratio with effector cells (Hoffmann et al., 2002).

In view of the safety profile observed in previous studies and the feasibility of applying the top dose, dose finding will proceed across the following five dose levels: 1.0, 4.5, 6.0, 8.0, and 10.0 x 10^6 T_{regs}/kg. These dose levels have been informed by the safety profile of T_{regs} seen in previous studies, balanced against a desire to minimise potentially sub-therapeutic dose levels. Brunstein *et al* were able to escalate their per-protocol T_{reg} dose to 1 x 10^6 T_{regs}/kg by the fifth patient (Brunstein et al., 2011). Consequently, the planned starting dose of 1 x 10^6 T_{regs}/kg is likely to be well tolerated. The top dose planned for this trial is 50% of the top dose shown to be well tolerated in the treatment of paediatric T1DM (Marek-

Trzonkowska et al., 2012). To put the dose levels in biological context, the PB T_{reg} pool ranges from 50-100 x 10^6 cells (assuming 1-2 x 10^6 PBMCs/ml, 20% of which are $CD4^+$, and 5% of which are T_{regs}). The cell doses described range from 70-700 x 10^6 T_{regs} for a 70kg individual.

12.9.5 Statistical considerations in clinical trial design

Organ toxicities will be assessed at scheduled clinical visits and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events v4.0 (National Cancer Institute, 2010). DLTs will include anaphylaxis, any grade 4 toxicity (i.e. life-threatening toxicity) or development of a life-threatening infection. Clinical exacerbation of CD requiring surgical intervention will also be considered a DLT. Even though variable disease course is well-described in active CD, "investigational new drugs" intended to treat CD have occasionally resulted in disease exacerbation, e.g. secukinumab (Hueber et al., 2012). The MTD will be defined as the dose that has a 30% risk of DLT in the wider population of eligible patients.

A 3+3 dose escalation study design accrues 3-6 subjects at a single dose level and allows the dose to be escalated to the next dose level if no more than 1 DLT occurs at that level (Le Tourneau et al., 2009). This process is reiterated at each dose level. In contrast, a Bayesian "Continual Reassessment Method" (CRM) study design can allow dose escalation to take place with each subject, based on the side-effect profile of the preceding subject (O'Quigley et al., 1990; Sweeting et al., 2013). Professor Toby Prevost (KCL) performed statistical modelling to

compare the performance each of these study designs in correctly identifying the MTD.

First, the performance of the 3+3 design was assessed by simulating 3,000 trials with a sample size of 30, for four hypothetical scenarios with differing underlying DLT risks across cell doses: Scenarios A-D (Table 12.1 [page 274]). In Scenario A, the MTD is at the 3rd dose level, while the highest dose level has an adversely high DLT risk of 50%. Only 20.3% of trials correctly detected the MTD, and only 59.7% of trials recommended a dose within 10% of the MTD rate (20% to 40%). 1.7% of trials overstated the MTD by 10%. Modelling the MTD at the 4th or 5th dose level (Scenarios B and C) gave similar results. Scenario D described a scenario where the MTD was at the 3rd dose, but risks were less separated from each other (10% for Scenario A *vs.* 5% for Scenario D). This resulted in poorer performance for Scenario D compared with Scenario A.

The CRM study design was then assessed for the same hypothetical scenarios (Table 12.2 [page 274]). Overall, the CRM study design performed better than the 3+3 dose escalation design in correctly identifying the MTD. In the 3+3 study design, the probability of correctly recommending the MTD ranged from 15.1% to 25.1%, whereas this ranged from 24.1% to 73.7% for the CRM study design. In the 3+3 study design, the probability of recommending a dose within 10% of the MTD ranged from 48.5% to 59.9%, whereas this ranged from 87.6% to 95.5% for the CRM study design.

Table 12.1: Performance of a 3+3 study design in detecting the maximum tolerated dose.

Probability of DLT at each dose level, in four scenarios.	Probability of identifying each dose level as the MTD (dose x 10^6 T_{regs}/kg) (%)					Probability of correctly recommending the MTD (%)	Probability of recommending within 10% of MTD rate (%)	Probability of recommending >10% over MTD rate (%)
	1.0	4.5	6.0	8.0	10.0			
A: (10,20,**30**,40,50)%	28.6	33.3	20.3	6.1	1.7	20.3	59.7	1.7
B: (5,10,20,**30**,40)%	12.4	27.7	32.2	18.1	9.6	18.1	59.9	-
C: (5,10,15,20,**30**)%	12.2	17.1	21.8	23.8	25.1	25.1	48.9	-
D: (10,25,**30**,35,45)%	49.0	26.8	15.1	6.6	2.5	15.1	48.5	2.5

Table 12.2: Performance of a CRM study design in detecting the maximum tolerated dose.

Probability of DLT at each dose level, in four scenarios.	Probability of identifying each dose level as the MTD (dose x 10^6 T_{regs}/kg) (%)					Probability of correctly recommending the MTD (%)	Probability of recommending within 10% of MTD rate (%)	Probability of recommending >10% over MTD rate (%)
	1.0	4.5	6.0	8.0	10.0			
A: (10,20,**30**,40,50)%	1.5	25.3	44.7	24.4	4.2	44.7	94.3	4.2
B: (5,10,20,**30**,40)%	0	1.5	24.8	44.7	28.9	44.7	98.5	-
C: (5,10,15,20,**30**)%	0	0.5	4.0	21.8	73.7	73.7	95.5	-
D :(10,25,**30**,35,45)%	0.4	33.2	24.1	26.3	12.4	24.1	87.6	12.4

The 3+3 study design stopped on average with 15 or 18 patients (range 3 to 30 across trials) over the scenarios, but underestimated the MTD for the available sample of 30 patients. This indicated that implementation of the 3+3 design would underestimate the MTD and lead, with high probability, to premature stopping, due to the dominance of chance fluctuations in observed DLT rates in small samples.

Table 12.3: Percentage of subjects expected to receive each dose level by scenario and study design.

Probability of DLT at each dose level, in four scenarios.	**3+3 design** Dose x 10^6 T_{regs}/kg **1.0**	**4.5**	**6.0**	**8.0**	**10.0**
A: (10,20,**30**,40,50)%	38.5%	32.0%	19.7%	7.9%	1.9%
B: (5,10,20,**30**,40)%	24.8%	27.0%	25.5%	16.3%	6.4%
C: (5,10,15,20,**30**)%	24.1%	24.5%	22.4%	18.1%	10.9%
D: (10,25,**30**,35,45)%	42.0%	32.9%	16.2%	6.8%	2.1%
Probability of DLT at each dose level, in four scenarios.	**CRM design** Dose x 10^6 T_{regs}/kg **1.0**	**4.5**	**6.0**	**8.0**	**10.0**
A: (10,20,**30**,40,50)%	11.4%	24.8%	32.8%	21.1%	9.9%
B: (5,10,20,**30**,40)%	5.2%	8.9%	24.3%	32.2%	29.4%
C: (5,10,15,20,**30**)%	5.1%	6.3%	9.0%	19.9%	59.7%
D: (10,25,**30**,35,45)%	15.1%	28.4%	18.1%	20.5%	17.9%

The proportion of subjects receiving each dose level by scenario and study design is shown in Table 12.3, above. The percentage of patients receiving doses below the MTD is an absolute 30% higher under the 3+3 study design compared with the CRM study design, reflecting underestimation of the MTD with the 3+3 study design.

Conversely, the percentages of patients receiving doses above the MTD are consistently higher in the CRM study design, reflecting an absence of the basic underestimation of the MTD seen with the 3+3 study design, and also due to natural dose exploration both sides of the MTD. For Scenario A, 9.9% (a mean of 3 patients in 30) are expected to be exposed at risk level 50%, which is two doses above the MTD. For Scenario B, there are 29.4% patients above MTD, but all limited to an excess of 10% risk (i.e. at 40% risk). For Scenario C, there is by

definition no dose above the MTD. For Scenario D, the dose-DLT risk relationship is flatter due to more closely space risks of 5%, and patients at high doses could be reduced, but by increasing the overall sample size.

The scenarios presented are likely to err on the side of caution, because they represent a steep relationship and because it is anticipated that the DLT rate will be relatively rare based on past studies and on experience in other conditions. Conservative Scenarios A and B (MTD at 6.0 or at 8.0) suggest that 1-in-3 subjects will receive the estimated MTD, and Scenario C suggests that 60% will receive the MTD. This means that between 10 and 18 of the 30 subjects will receive the estimated MTD, and that there will be a high probability that this will be close to the true MTD.

12.9.6 Target sample size

Based on the modelling described above, the target sample size will be a minimum of 30 evaluable subjects from 33 subjects who receive the cell product. The effect size of autologous *in vitro* expanded T_{reg} therapy in CD is currently unknown. In a recent clinical trial using a single infusion of ovalbumin-specific IL-10^+ $CD4^+$ lymphocytes, 8/20 subjects achieved a clinical response, including 6/8 subjects who received the highest planned dose (Desreumaux et al., 2012). Three of the 8 subjects who received the highest planned dose achieved clinical remission.

12.9.7 Subject screening and evaluation

The summary of the evaluation of subjects throughout the clinical trial is illustrated in Figure 12.2, below. A summary of required data at each encounter within the clinical trial is given in Table 12.4 (page 278).

Figure 12.2: Schema illustrating the flow of a single patient through the clinical trial.

(A) Following written informed consent, subjects will be screened at least 4 weeks prior to a planned infusion date. **(B)** PBMCs will be obtained and $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{+}$ T_{regs} isolated and expanded *in vitro*. Expanded cell lots will undergo quality control and either be frozen for later use, or infused fresh. **(C)** Subjects will receive baseline clinical and endoscopic evaluation (E1) within 48h prior to infusion, followed by T_{reg} infusion at Guy's Hospital's clinical research facility. The first three subjects (or first subjects at each dose level) will be admitted overnight following infusion. Each of these first three subjects will receive cells sequentially and complete a 5-week per protocol observation period following infusion prior to infusing the next subject. **(D)** Subjects will be assessed frequently during the 12-week follow-up period. A repeat endoscopy will be performed at week 5 (E2) to assess mucosal healing in response to T_{reg} therapy. In addition to frequent assessment of clinical parameters (to determine safety and clinical response), extensive immune phenotyping will be performed on PB and LP samples to correlate changes in immune phenotype with clinical outcome. This will allow the cell types and immune responses associated with clinical response to be determined.

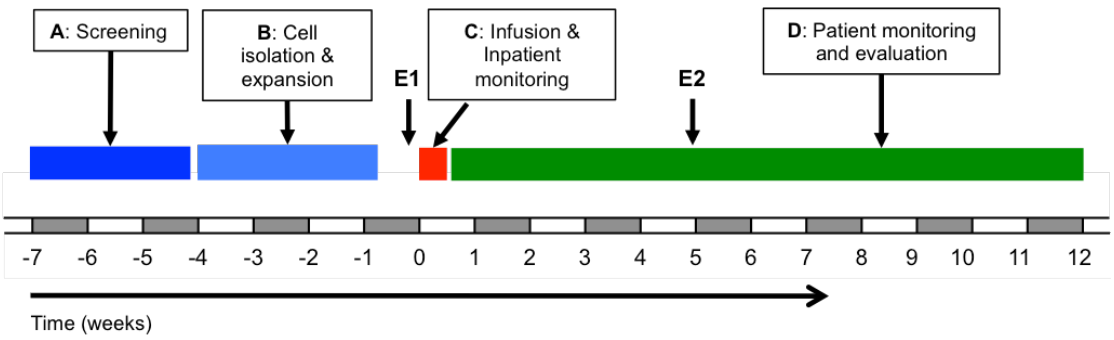


Table 12.4: Summary of required data throughout clinical trial.

	Screening (Pre-treatment)	Days 0, 1* Weeks 1, 2, 3, 5	Week 8	Week 12
Medical history	✓	✓*	✓	✓
Physical examination	✓	✓	✓	✓
ECG	✓	✓		
Pregnancy test	✓			✓
Colonoscopy	✓		✓	
Radiology (CT, MRI, U/S)	✓	✓[1]		
Viral testing: HIV, HBV, HCV	✓			
Screening for TB	✓			
Stool for C&S, O&P, *C. difficile*	✓			
Colonoscopy & biopsy, endoscopic disease score (CDEIS)	✓	✓[1]		
FBC & differential	✓	✓	✓	✓
ESR, CRP, Calprotectin	✓	✓	✓	✓
Serum chemistry	✓	✓	✓	✓
Thyroid function tests	✓		✓	
Serum & biopsy cytokine analysis	✓	✓[1]		✓
CDAI calculation	✓	✓	✓	✓
IBDQ calculation		✓[2]	✓	✓

Notes. *: History and physical examination only at day 1, unless additional investigations indicated clinically. 1: Week 5 only. 2. Weeks 2, 5, 8 and 12 only.

12.9.8 Study endpoints

The safety of the cell product will be reported by enumerating the number of DLTs, serious and non-serious adverse events experienced by study subjects during the 12-week follow-up period. The MTD will be determined using the dose escalation strategy described above.

Efficacy will be determined at weeks 5 and 8, according to current best practice (Committee for Medicinal Products for Human Use, 2009; Desreumaux et al., 2012). Clinical response will be identified by a decrease in the CDAI of ≥100 points from baseline, while clinical remission will be identified by an absolute CDAI of <150 points (Best et al., 1976). The severity of mucosal disease at

endoscopy will be evaluated by an independent observer using the Crohn's Disease Endoscopic Index of Severity (CDEIS), a validated endoscopic disease activity score (Ferrante et al., 2013). Mucosal healing will be defined as absence of ulceration in the colon and terminal ileum, while endoscopic response will be determined by a decrease in CDEIS at the second endoscopy.

12.9.9 Evaluation of the immune response to autologous *in vitro* expanded T_{reg} therapy

Because the investigators will have access to PB and LP from subjects receiving autologous *in vitro* expanded T_{reg} therapy, the proposed clinical trial will offer an exciting opportunity to evaluate immune responses adoptively transferred T_{regs} *in vivo* in humans. To enable this, multi-parameter flow cytometric panels will be developed to allow detailed delineation of immune cell subsets, and the activation status of immune cells within these subsets. The cytokine expression profile of immune cell populations will be assessed by FACS and assessment of cytokine expression in 24-hour cell culture supernatants. PBMCs, LPMCs or fractionated cell populations will be collected and stored in RNALater, which will allow subsequent qPCR or microarray analysis, in order to define transcriptional profiles associated with disease and clinical response. In addition to defining immune responses to T_{reg} therapy, this approach offers an opportunity to correlate immunological parameters with clinical response following treatment, which may provide and insight into the immune pathways that are associated with a clinical improvement in CD.

The anatomic location at which adoptively transferred T_{regs} might exert their effect in order to modulate immune responses in CD is currently unknown. Were it possible to label adoptively transferred T_{regs} in a subset of recipients, so that their anatomic location could be identified at various time-points (particularly with sufficient resolution to discriminate between localisation in the intestinal mucosa *vs.* MLN), this question could be addressed during this proposed clinical trial. *In vivo* tracking of T_{regs} is currently the subject intensive research within the division (Sharif-Paghaleh et al., 2011).

12.10 Overcoming clinical and economic barriers to incorporating autologous T_{reg} cell therapy within the armamentarium of therapeutics for Crohn's disease

It is likely that cell-based therapies will have a role in the treatment of CD in the future. At the time of writing, multiple cell products are in development for the treatment of CD, including autologous HSCT (ASTIC study, ClinicalTrials.gov identifier: NCT00297193; Hawkey and Trialists, 2014), allogeneic bone marrow-derived mesenchymal stem cells (MSC) ("Prochymal", Mesoblast/Osiris; ClinicalTrials.gov identifier: NCT00482092), allogeneic adipose tissue-derived MSC ("Cx601", TiGenix; ClinicalTrials.gov identifier: NCT01541579) and ovalbumin-specific IL-10^+ $CD4^+$ lymphocytes (Desreumaux et al., 2012).

Barriers currently exist to the widespread clinical acceptance of cell-based therapy for CD. In order for cell therapy to become a clinically acceptable alternative to currently available pharmaceutical therapies, clinical trials will have to robustly demonstrate efficacy and allow the calculation of estimates of effect

size in comparison with "gold standard" treatments, such as biologics. Identification of the mechanism of action of cell therapies in humans will add to their biological plausibility amongst clinicians, and perhaps allow patient stratification by focusing therapy on patient groups that are most likely to respond.

Financial barriers also exist to the development of cell therapy, including high costs for entry into this market and high costs for the development and production of cell therapy products. For example, the establishment of a GMP facility at Guy's Tower required a capital investment of over £10M. The hidden costs of this facility include the capital investment required to establish co-located support facilities, including a flow cytometry core and a clinical research facility. Furthermore, development of the underpinning science and subsequent development of the cell therapy is time-consuming and expensive. These factors will limit participation in cell therapy development and manufacture to a small number of academic centres and early phase pharmaceutical or device manufacturing companies. The high cost of entry and subsequent development has driven extensive collaboration between academic centres and industry in the development of cell therapy products. For example, the "ONE study" consortium is a trans-Atlantic collaboration between 8 academic centres and 5 companies, that aims to define the optimum cell therapy products to minimise maintenance immunosuppression following solid organ transplantation. Multi-centre collaborations are also likely to be required in order to recruit patients in sufficient numbers to conduct clinical trials of cell therapy in the future.

The immaturity of the cell therapy market represents another barrier to the development of cellular therapeutics. Cell therapies for CD are currently novel, and a clinical role for cell therapy has not yet been fully defined. Cell therapy production has also not yet achieved economies of scale. Consequently, the business model for cell therapy in IBD is evolving. These uncertainties have meant that the infrastructure for cell therapy manufacture and the on-going cost of development has been heavily supported by public funding. The UK Government has responded to this need by establishing the Cell Therapy Catapult, located at Guy's Tower. The Cell Therapy Catapult aims to support and develop the UK cell therapy industry by providing clinical, technical and regulatory expertise, in addition to developing business plans and pricing in the development of cell therapy products. This will have the effect of reducing the risks of participating in this sector.

The proposed clinical trial outlined in Section 12.9 (page 268) has the potential to establish the safety of autologous *in vitro* expanded T_{regs} in CD. Were this trial to be successful, subsequent phase II and phase III clinical trials may provide evidence of clinical effectiveness. Infliximab therapy for CD was first used with a "bottom up" approach in patients with moderate-to-severe disease, who had failed other therapies (Targan et al., 1997). It took a further 10 years for good evidence for the success of a "top-down" approach to therapy to become available and widely accepted (Colombel et al., 2010; D'Haens et al., 2008). It is reasonable to assume that clinical indications for *in vitro* expanded T_{regs} will follow the same paradigm.

At present, the projected cost of manufacturing an autologous *in vitro* expanded T_{reg} cell product is £16,500. This is comparable with the cost of a 1-year supply of infliximab for the induction and maintenance of remission in CD: £16,800 for an 80 kg individual. It is reasonable to anticipate that the cost of cell therapy manufacture will fall as the technology matures and economies of scale arise.

12.11 Conclusions

In conclusion, these data suggest that the T_{reg} population that can be expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors is likely to be an appropriate population for further development as a potential autologous T_{reg} cell therapy for CD. This conclusion is based on observations presented in this thesis that this *in vitro* expanded T_{reg} population exhibits phenotypic stability and stable FOXP3 expression following *in vitro* expansion, is resistant to the induction of a pro-inflammatory phenotype, homes to human small bowel *in vivo* in a novel pre-clinical model, and suppresses proliferation of responder T cells isolated from inflamed CD LP and MLN.

These observations suggest that the conclusions of earlier studies on both freshly isolated $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} from healthy donors (Miyara et al., 2009) and T_{regs} expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors obtained from healthy donors (Baron et al., 2007; Hoffmann et al., 2006; 2009) can be extended to the equivalent T_{reg} populations obtained from CD patients. Consequently, it is likely that the general conclusions of T_{reg} literature derived from healthy control samples can be applied to CD T_{regs}. This suggests that it may

not be necessary to recapitulate experimental data originally derived from healthy control T_{regs} using CD patient samples, unless the results are required for the development of novel therapeutics and a subsequent licensing application, or to address a specific safety issue related to future CD therapeutics.

The 7-hour assay of T_{reg} *in vitro* suppressive function was originally validated in comparison with a 96-hour proliferation assay for *in vitro* expanded T_{regs} with the intention of providing a novel technique to rapidly assess the function of *in vitro* expanded T_{reg} lots. This might ultimately be a complementary approach to the assessment of *FOXP3* TSDR demethylation in *in vitro* expanded T_{reg} lots. It may be useful to include these assays for information in forthcoming clinical trials of T_{reg} cell therapy, as correlation between the results of these assays and *in vivo* biological activity may provide insights into the biology of adoptively transferred *in vitro* expanded T_{regs} in humans. As additional data become available, a role for these assays in the assessment of *in vitro* expanded T_{reg} lots prior to infusion to humans may become apparent.

Future work will focus on the development of humanised models of IBD, which will provide platforms for pre-clinical testing of *in vitro* expanded T_{regs}, in addition to other novel therapeutics. Funding is currently being sought to convert the isolation and expansion of PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} to a fully GMP process. A phase I clinical trial of this cell product will then be initiated. These data pave the way for further development of T_{regs} expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ CD PB precursors as a cell-based therapy for CD.

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14 Appendix: Research Ethics Committee and National Health Service Trust R&D approvals

14.1 "Regulatory T cells in inflammatory bowel disease". (South East London REC 1 reference 10/H0804/65).

This REC approval letter is reproduced on the following pages.

NHS
National Research Ethics Service
South East London REC 1
(Formerly Guy's REC)
Governor's Hall Suite
St Thomas' Hospital
London
SE1 7EH

Telephone: 020 7188 2260
Facsimile: 020 7188 2258

20 December 2010

Professor Graham Lord
Professor of Medicine
King's College London
Lord Laboratory
5th Floor, Tower Wing
Guy's Hospital
Great Maze Pond
London SE1 9RT

Dear Professor Lord

Study Title: **Regulatory T cells in inflammatory bowel disease.**
REC reference number: **10/H0804/65**
Protocol number: **2**

Thank you for your letter of 23 October 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research

governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Protocol	2	23 October 2010
Response to Request for Further Information		23 October 2010
Participant Information Sheet: Healthy Control	2	23 October 2010
Covering Letter		13 July 2010
GP/Consultant Information Sheets	1	09 July 2010
REC application	Parts A - D	12 July 2010
Participant Information Sheet: IBD Patients	2	23 October 2010
Participant Consent Form	2	23 October 2010
Evidence of insurance or indemnity	KCL	01 August 2010
Investigator CV	Professor Graham Lord	09 July 2010
Investigator CV	Dr James Canavan	09 July 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

10/H0804/65	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Professor David Bartlett
Chair

Email: stephanie.hill@gstt.nhs.uk

Enclosures: "After ethical review – guidance for researchers" *SL- AR2 for other studies*

Copy to: *Mr Keith Brennan, KCL*

R&D office, GSTFT

14.2 "Regulatory T cells in inflammatory bowel disease". (Guy's & St Thomas' NHS Foundation Trust R&D reference RJ110/N321)

This NHS R&D approval letter is reproduced on the following pages.

Guy's and St Thomas' NHS
NHS Foundation Trust

Research & Development
16th Floor Tower Wing
Guy's Hospital
Great Maze Pond
London SE1 9RT
Tel: 020 7188 7188

Dr Jeremy Sanderson
Consultant Gastroenterologist
Guy's and St. Thomas' NHS Foundation Trust
Department of Gastroenterology
St. Thomas' Hospital
Westminster Bridge Road
London SE1 7EH

04 January 2011

Dear Dr Sanderson

Title: Regulatory T cells in inflammatory bowel disease

In accordance with the Department of Health's Research Governance Framework for Health and Social Care, all research projects taking place within the Trust must receive a favourable opinion from an ethics committee and approval from the Department of Research and Development (R&D) prior to commencement.

- **Ethics Number: 10/H0804/65**
- **Sponsor: KCL/GSTFT**
- **Funder: NIHR**
- **End Date: 31/10/2012**
- **Protocol: Version 2**
- **Site: GSTT**
- **R&D Approval Date: 04 January 2011**
- **Chief Investigator: Professor Graham Lord**
- **CSP: 59947**

NHS permission for the above research has been granted on the basis described in the application form, protocol and supporting documentation as listed in the Research Ethics Committee favourable opinion letter dated 20 December 2010. I am pleased to inform you that we are approving the work to proceed within Guy's and St Thomas' NHS Foundation Trust and has been allocated the Trust R&D registration number **RJ110/N321**. Please quote the R&D registration number in any communications with the R&D Department regarding your project.

Conditions of Approval:

- The principal investigator must ensure that the recruitment figures are reported.
- The principal investigator must notify R&D of the actual end date of the project.
- R&D must be notified of any changes to the protocol prior to implementation.
- The project must follow the agreed protocol and be conducted in accordance with all Trust Policies and Procedures especially those relating to research and data management.
- Members of the research team must have appropriate substantive or honorary contracts with the Trust prior to the study commencing. Any additional researchers who join the study at a later stage must also hold a suitable contract.
- **Storing tissue at the end of the study:** If tissues will be stored pending ethical approval for use in another project, then at the end of this study REC approval for the additional study must be in place, or the tissue has to be stored in HTA licensed premises.

If appropriate it is recommended that you register with the Current Controlled Trials website; http://isrctn.org/

Data Protection:
Please ensure that you are aware of your responsibilities in relation to The Data Protection Act 1998, NHS Confidentiality Code of Practice, NHS Caldicott Report and Caldicott Guardians, the Human Tissue Act 2004, Good Clinical Practice, the NHS Research Governance Framework for Health and Social Care, Second Edition April 2005 and any further legislation released during the time of this study.

The Principal Investigator is responsible for ensuring that Data Protection procedures are observed throughout the course of the project.

If the project is a clinical trial under the European Union Clinical Trials Directive the following must also be complied with:

1. The EU Directive on Clinical Trials (Directive 2001/20/EC) and UK's implementation of the Directive: The Medicines for Human Use (Clinical Trials) Regulations 2004;
2. The EU Directive on Principles and Guidelines for Good Clinical Practice (EU Commission Directive 2005/28/EC); and UK's implementation of the Directive: The Medicines for Human Use (Clinical Trials) Amendment Regulations 2006;

Amendments:
Please ensure that you submit a copy of any amendments made to this study to the R&D Department.

Annual Progress Report:
It is obligatory that an annual report is submitted by the Chief Investigator to the research ethics committee, and we ask that a copy is sent to the R&D Department. The yearly period commences from the date of receiving a favourable opinion from the ethics committee.

Please submit a copy of the progress report on the anniversary of the Ethics favourable opinion **(20 December)**

Should you require any further information please do not hesitate to contact us.

In line with the Research Governance Framework, your project may be randomly selected for monitoring for compliance against the standards set out in the Framework. For information, the Trust's process for the monitoring of projects and the associated guidance is available from the Trust's intranet or on request from the R&D Department. You will be notified by the R&D Department if and when your project has been selected as part of the monitoring process. No action is needed until that time.

Thank you for registering your research project and with best wishes for the success of your study.

Yours sincerely

Samantha Roper
Research Governance Associate
Guy's and St. Thomas' NHS Foundation Trust

14.3 "Development of protocols for the generation *in vitro* of clinical grade T cells for adoptive cell therapy for use in autoimmune, inflammatory and malignant diseases". (Hammersmith and Queen Charlotte's & Chelsea REC reference 09/H0707/86).

This REC approval was obtained by Professor Mark Peakman. The REC approval letter is reproduced on the following pages.

National Research Ethics Service
Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee
Room 4W/12, 4th Floor
Charing Cross Hospital
Fulham Palace Road
London
W6 8RF
Telephone: 020 3311 7258
Facsimile: 020 3311 7280

Professor Mark Peakman
Professor of Clinical Immunology & Honorary Consultant Immunologist
Department of Immunobiology
2nd Floor, Borough Wing
Guy's Hospital,
London SE1 9RT

21 December 2009

Dear Professor Peakman

Study Title:	**Development of protocols for the generation in vitro of clinical grade T cells for adoptive cell therapy for use in autoimmune, inflammatory and malignant diseases**
REC reference number:	**09/H0707/86**
Protocol number:	**2**

Thank you for your letter of 04 December 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

NHS
National Research Ethics Service

Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
REC application		25 September 2009
Investigator CV	Prof Peakman	
GP/Consultant Information Sheets	1	29 September 2009
Referees or other scientific critique report	delete	26 September 2009
Evidence of insurance or indemnity	King's College (AON)	
Letter from Sponsor		26 September 2009
Protocol	2	06 November 2009
Participant Information Sheet: Haemochromatosis	2	06 November 2009
Participant Information Sheet: Diabetes/transplantation/cancer	2	06 November 2009
Participant Information Sheet: Healthy Volunteers	2	06 November 2009
Participant Information Sheet: Leukapheresis	1	09 November 2009
Participant Consent Form: Leukapheresis	1	09 November 2009
Participant Consent Form	2	06 November 2009
Advert: Kidney Transplant	2	06 November 2009
Advert: Healthy Volunteers	1	06 November 2009
Advert: Type 1 Diabetes	2	06 November 2009
Advert: prostate cancer	2	06 November 2009
Response to Request for Further Information		04 December 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views

known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

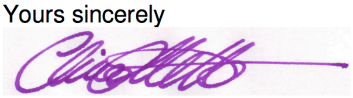
- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H0707/86	**Please quote this number on all correspondence**

Yours sincerely



Professor A George
Chair
Email: clive.collett@imperial.nhs.uk
Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mr Keith Brennan*

14.4 Human Tissue Authority license to King's College London .

This is reproduced on the following page.

HTA
Human Tissue Authority

Licensing Number	**12521**
Licensed Premises	**King's College London Guy's Campus London SE1 1UL**
Licence Holder	**King's College London**
Designated Individual	**Dr Cheryl Gillett**

This licence is granted under Section 16 (2) (e) (ii) of the Human Tissue Act 2004.

This licence authorises the storage of relevant material which has come from a human body for use for the following scheduled purposes:

- Determining the cause of death
- Establishing after a person's death the efficacy of any drug or other treatment administered to him
- Obtaining scientific or medical information about a living or deceased person which may be relevant to any other person (including a future person)
- Public display
- Research in connection with disorders, or the functioning, of the human body
- Clinical audit
- Education or training relating to human health
- Performance assessment
- Public health monitoring
- Quality assurance

The licensed activity should be carried on only at the licensed premises specified above, and under the supervision of the Designated Individual.

This licence is subject to the conditions set out in the Annexes accompanying this licence as may be subsequently varied pursuant to an application under paragraph 8 of Schedule 3 to the Human Tissue Act 2004.

This licence is valid from the date specified below and will remain in force until revoked.

.....
Baroness Diana Warwick
Chair

.....
Craig Muir
Chief Executive

Dated **26 August 2010**