Do gut activated immune cells mediate low bone mineral density in paediatric Crohn's disease?

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Copyright statement

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aBMD	areal bone mineral density
ACS	activated cell supernatant
AF	Alexa Fluor™
ALP	alkaline phosphatase
APC	allophycocyanin
APCs	antigen presenting cells
BCM	bone cell media
BM	bone marrow
BMAD	bone mineral apparent density
BMC	bone mineral content
BMD	bone mineral density
BMMSCs	bone marrow mesenchymal stem cells
BMPs	bone morphogenetic proteins
BMU	basic multicellular unit
CD	Crohn's disease
CD*	cluster designation
ConA	concanavalin-A
CRP	c-reactive protein
CTX	c-terminal peptide
Cy	cyanine dye
D-MEM	Dulbecco's Modified Eagle Medium
DCs	dendritic cells
DPD	deoxypyridinoline
DTT	dithiothreitol
DXA	dual X-ray absorptiometry
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acetic acid
EO	endochondral ossification
ESR	erythrocyte sedimentation rate
FBC	full blood count
FI	fluorescent intensity
FITC	fluorescein isothiocyanate
FOXP3	fork-head box P3
FSC	forward scatter
GALT	gut associated lymphoid tissue
HIF	hypoxia induced factor
HRP	horseradish peroxidase
HSC	haematopoietic stem cell
IBD	inflammatory bowel disease
IEL	intra-epithelial lymphocytes
IFN	interferon
Ig	immunoglobulins
IL	interleukin
IO	intramembranous ossification
LP	lamina propria
mAB	monoclonal antibody
Madcam-1	mucosal addressin cell adhesion molecule-1
MEL	mucosal epithelium layer
MHC	major histocompatibility complex
MSC	mesenchymal stem cell
NKKb	nuclear factor kappa-light-chain-enhancer of activated B cells

NOD	nucleotide-binding oligomerization domain-containing proteins
NTx	N-telopeptide crosslinked type 1 collagen
OB	osteoblast
OC	osteoclasts
OPG	osteoprotegerin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PNPP	p-nitrophenylphosphate
PP	Peyer's patch
PRR	pattern recognition receptor
PSC	primary sclerosing cholangitis
Qdot	Quantum dot™
RANK	receptor activator of nuclear factor kappa β
RUNX2	runt-related transcription factor 2
SB	serum buffer
SD	standard deviations
SSC	side scatter
TCR	T-cell receptor
TLR	toll like receptor
TNF	tumour necrosis factor
UC	ulcerative colitis
vBMD	volumetric BMD
VEGF	vascular endothelial growth factor
ViD	viability dye
WCC	white cell count
WNT	wingless-int

Abstract

Background: There has been an increasing awareness of the negative impact of inflammatory bowel disease (IBD) on bone metabolism and bone mineral density (BMD). A number of potential mechanisms have been suggested, including malnutrition and the use of steroids. Although it has been known for some time that the immune system can influence the cells of bone, the specific contribution of interactions between osteoblasts and T cells in the pathogenesis of IBD-associated osteoporosis in children has yet to be clearly demonstrated. This body of work consists of two parts: an *in vitro* study investigating the influence of T cells and their activation products on the growth and alkaline phosphatase activity of the Saos-2 osteosarcoma cell line, and an *in vivo* study comparing the phenotype of lymphocyte subpopulations in the gut and peripheral circulation of children with and without IBD, relating these to measures of bone metabolism and mineralisation.

Methods: For the *in vitro* studies, Saos-2 cells were incubated with increasing numbers of resting and polyclonally activated immune cells, with and without the presence of transwell inserts. The expansion of the Saos-2 cells and their alkaline phosphatase activity were measured, and flow cytometry was used to establish the activation status of T cell subpopulations in the immune cell populations.

The *in vivo* study recruited 30 children undergoing lower gastrointestinal (GI) endoscopy for investigation of GI symptoms aiming to identify 15 patients newly diagnosed with Crohn's disease and 15 healthy controls. Immune cells were isolated from blood and gut biopsy samples, and analysed by flow cytometry. Samples of serum and urine were analysed for bone turnover markers. Confirmed cases of IBD had their bone mineral density measured by dual X-ray absorptiometry (DXA).

Results: The *in vitro* study found that populations of PBMCs and $CD4^+$ lymphocytes had similar effects on the growth of Saos-2 cells, and whilst activated immune cells significantly inhibited Saos-2 cell growth it was also observed that resting immune cells increased Saos-2 cell growth. These effects were lost when immune cells were added after saos-2 had become adherent. There was a reciprocal effect of Saos-2 cells on the activation status of $CD4^+$ lymphocytes, increasing the activation of resting cells and potentially limiting the effects of polyclonal activation.

The *in vivo* study found that children with Crohn's disease had significant reductions in BMD, reductions in both bone resorption and formation, lower total lymphocyte counts and increased expression of CD25. Linear regression showed a positive correlation between the numbers of circulating $CD4^+$ lymphocytes and BMD, and a negative correlation with their expression of CD25. These correlations were not statistically significant, but the power of the study was only 20%

Conclusion: The findings support the hypothesis that gut activated immune cells mediate low bone mineral density in paediatric Crohn's disease. However, the findings also raise the possibility of osteoimmune interactions being important for the immune response and bone metabolism, in both health and disease. Therefore, further study of cellular responses in the bone are necessary to better understand the relationships between these two important systems.

1 Introduction

Inflammatory bowel disease

Clinical features

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gut with two distinct phenotypes: Crohn's disease (CD) and ulcerative colitis (UC) (Bouma et al 2003). CD affects the entire gastrointestinal tract from the mouth to the anus. The mucosa develops a cobblestone appearance with ulcers separated by normal or oedematous mucosa. There is a dense infiltrate of lymphocytes affecting all layers of the bowel wall with the development of granulomas. UC, apart from some ileal involvement, affects only the large bowel. Only the superficial mucosal layers become inflamed and, unlike the cobblestone appearance seen in Crohn's, the pattern of inflammation is continuous. Figure 1-1 compares their histological appearances. Although most patients affected by IBD can be recognised as having either UC or CD, if the features are unclear then the term 'indeterminate colitis' is used.

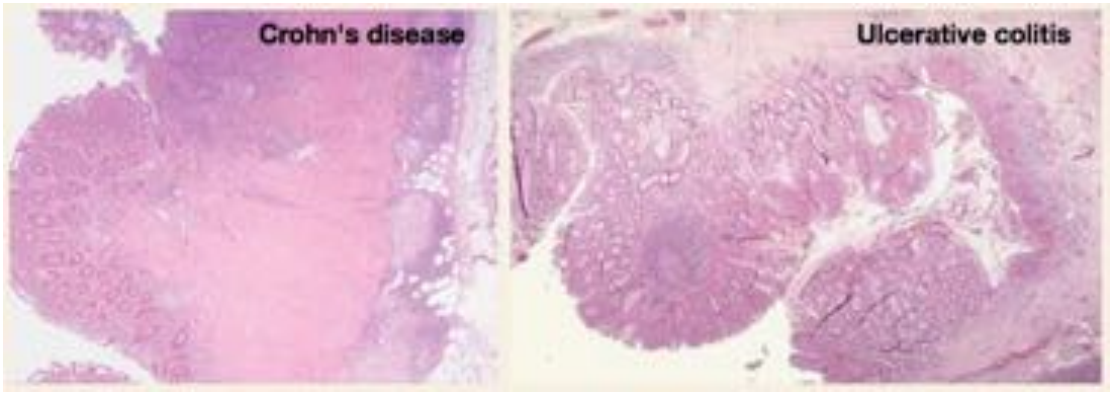

Figure 1-1 Photomicrographs of histological specimens taken from patients with IBD. Photomicrographs of histological specimens taken from patients with IBD. Left – mucosa from a patient with CD showing full thickness inflammation and granulomas towards the serosal surface. Right – mucosa from a patient with UC (at low-power magnification) exhibiting mucosal inflammation and erosion. Reproduced with permission (Bouma et al 2003).

IBD (both CD and UC) is an incurable, life-long condition. It causes significant morbidity including malabsorption, abdominal pain, and diarrhoea. Treatment involves the use of steroids and other immunomodulatory drugs which have significant side effects. In more severe cases surgery (including resection of significant areas of bowel) may be required to control symptoms.

Epidemiology

A systematic review of studies published between 1950 and 2010 showed that the incidence of IBD varies between geographical regions (Molodecky et al 2012). Incidence is lowest in Asia and the Middle East (5 and 6.3/100,000 for CD and UC respectively). Incidence is markedly higher in Europe and North America, but with a higher rate of UC in Europe (24.3 vs 19.2 per 100,000) compared with a higher rate of CD in North America (20.2 vs 12.7/100,000). The incidence of IBD was also shown to vary across Europe (see Figure 1-2). This variation had been previously demonstrated in a European study in which 20 centres across Europe used an agreed methodology to prospectively identify patients (Shivananda et al 1996). Incidence in Northern European countries (including the UK, France and Norway), compared to those in the south (Spain, Greece and Italy), was 40% higher for UC and 80% higher for CD.

Although the majority of studies (60-75%) reporting trends over time showed statistically significant increases in the incidence of both UC and CD (Molodecky et al 2012), the details of these changes varies in individual studies. In Northern France there has been a 29% increase in the incidence of CD from 5.2 to 6.7/100,000, although with a 25% drop in UC from 4.3 to 3.4/100,000 (Chouraki et al 2011). Data collected by the Mayo Clinic over the last seventy years indicates that the incidences of CD and UC have increased from 1.9 to 7.9 per 100,000 population, and from 2 to 8.8 per 100,000 population respectively (Sedlack et al 1972; Sedlack et al 1980; Loftus et al 2007). In Denmark, the incidence of both have increased, but with UC rates 1.5 to 3 times higher than for CD (Binder et al 1982; Vind et al 2006).

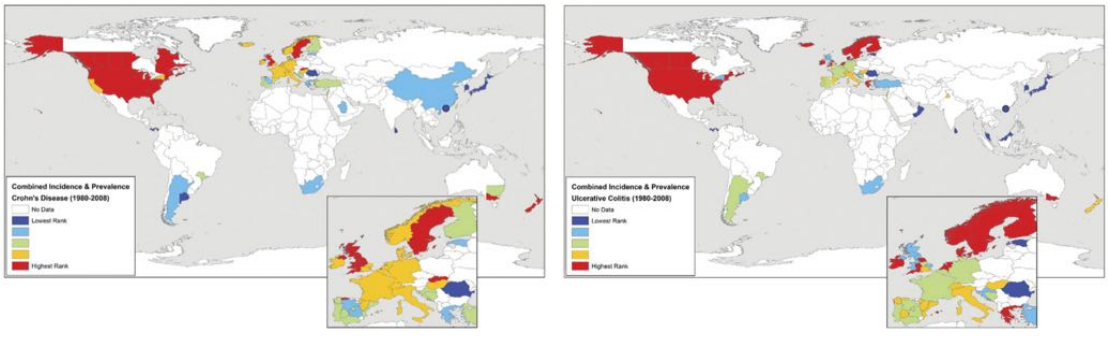

Figure 1-2 Worldwide incidence and prevalence rates of IBD. CD (left) and UC (right) are ranked into quintiles representing low (dark and light blue), intermediate (green) and high (yellow and red) risk of disease. Reproduced with permission (Molodecky et al 2012).

It has been observed that 25% of IBD patients develop the disease during childhood (Kelsen et al 2008). 4% of paediatric IBD is diagnosed before the age of

5 (Kelsen et al 2008), and the incidence gradually increases with age (van der Zaag-Loonen et al 2004) reaching a peak onset in the early twenties (Chouraki et al 2011). A recent review of studies published between 1950 and 2009 found the incidence of paediatric CD has increased but not UC (Benchimol et al 2011), although subsequent studies report increases in paediatric UC (Lehtinen et al 2011; Henderson et al 2012; Malmborg et al 2013; Ashton et al 2014). In Northern France, between 1988 and 2007, the number of children diagnosed with CD between the ages of 10 to 19 years has increased by 71% from 6.5 to 11/100,000 (Chouraki et al 2011).

Some of this temporal and geographic variation may be explained by differing study design, and epidemiological studies will often describe normal variation, but the increasing incidence is real. There are plausible mechanisms by which environmental factors can influence disease expression (Ng et al 2013), and temporal changes are seen in studies published by centres with consistent data collection methods. This means that although IBD already has a significant impact on health outcomes, the global burden of IBD and its associated morbidity will increase.

The gut, its mucosal immune system, and the pathogenesis of inflammatory bowel disease

Structure of the gut mucosa

The gut is exposed to a wide array of antigen, present in the food we consume and the commensal bacteria that reside there. The gut needs to be able to transfer nutrients across the mucosal surface whilst excluding bacteria. The gut's relationship with bacteria is further complicated by a need to clear pathogenic bacteria whilst maintaining an appropriate population of commensal bacteria. The immunological structure of the mammalian gut, as with other mucosal surfaces in the body, has developed in such a way to meet these conflicting demands.

The gastrointestinal tract consists of multiple tissue layers. The innermost mucosa has three: an overlying epithelium in contact with the gut contents; an underlying lamina propria consisting of connective tissue, blood vessels and immune cells; and an outer muscularis mucosa for local movement of the mucosa. Surrounding these is the submucosa containing larger blood vessels, lymphatics and nerves; the muscularis propria for movement of the bowel; and an outer adventitia.

The small intestine is the major absorptive site of the intestine. Both the mucosa and sub-mucosa are thrown up into plicae (folds), increasing the mucosal surface area. The epithelium can be divided into three zones: villi (fingers) extending outwards into the bowel, crypts (tubular glands) extending down from the base of the villi to the muscularis mucosae, and the neck zone where the two meet. Lamina propria is present around the base of the villi and extends up into them. The large intestine lacks villi but tubular glands are still present, extending into the mucosa with lamina propria found at their base. The microvillous brush border secretes mucin and anti-microbial peptides which regulates access of bacteria to the mucosal surface, and tight junctions between the epithelial cells prevent unregulated exposure of antigens to the immune system. Antigen is then processed by an extensive network of organised, gut associated, lymphoid tissue (GALT).

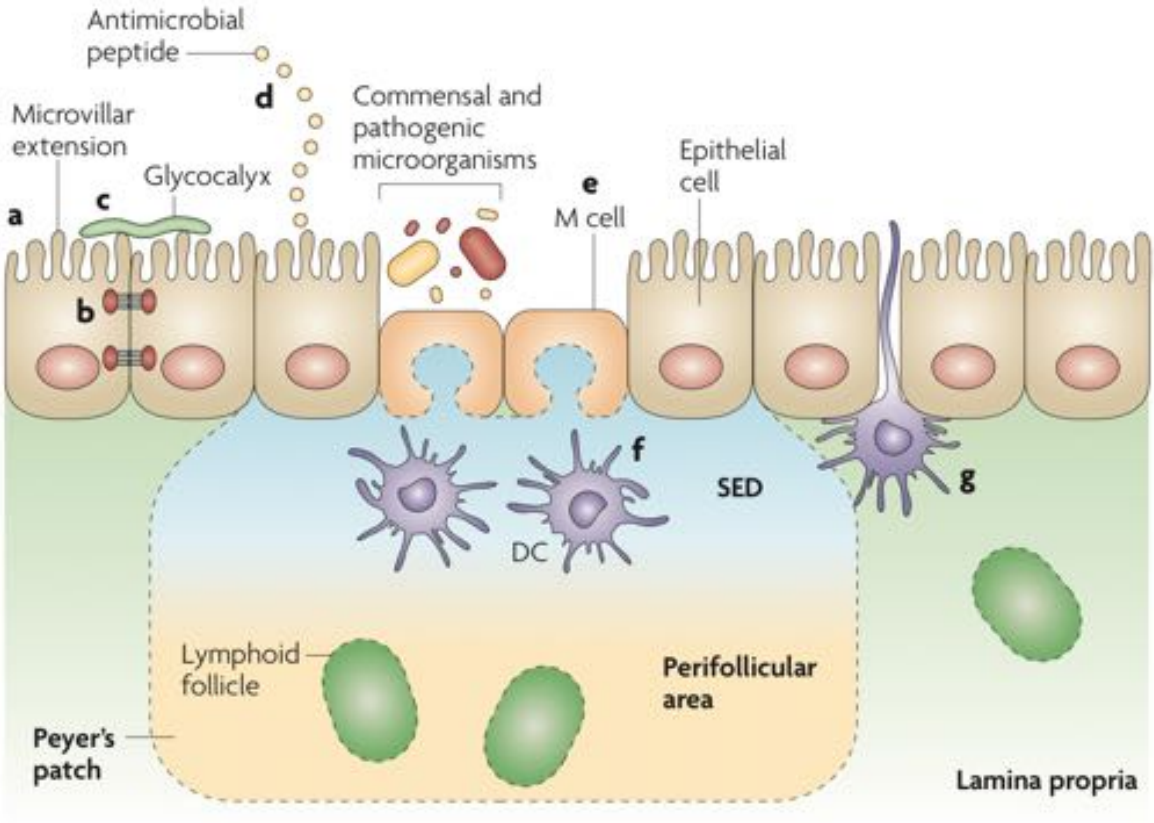

Figure 1-3 The structure of the gut in relation to antigen sampling. Tightly bound epithelial cells separate the immune system from commensal antigen whilst M cells provide a route for dendritic cells (DCs) to sample antigen. Reproduced with permission (Artis 2008).

Peyer's patches (PPs) are specialised lymphoid structures which form part of the GALT (Jung et al 2010). Found primarily in the ileum, PPs become apparent at 24

completed weeks gestation and continue increasing in number into adult life. They consist of aggregated lymphoid follicles, structured to facilitate processing of antigen (see Figure 1-3). Each follicle has a germinal centre containing dendritic cells and B cells; and a sub-epithelial dome consisting of T cells, B cells and dendritic cells. Overlying these aggregates is follicle associated epithelium made up of micro-fold cells (or M cells), specialised in the transport of intact luminal material including protein and bacteria. Efferent vessels connect PPs to the mesenteric lymph nodes and the wider lymphatic system. Evidence of PPs importance to the mucosal immune response is mixed: in one mouse model a lack of PPs limited the development of tolerance to larger protein antigens (Fujihashi et al 2001), but had no effect in a rat model (Enders et al 1986) or ligated murine bowel loops (Kraus et al 2005). Despite these heterogeneous findings specific structures within PPs and lymphoid follicles, pattern recognition receptors, have a clear role in moderating the GALT's response to infective organisms.

Pattern recognition receptors (PRRs) are found on cells within lymphoid follicles and PPs. They recognise specific compounds found on bacteria e.g. lipopolysaccharide. Two types of PRRs, toll like receptors (TLRs) and nucleotide-binding oligomerization domain-containing proteins (NODs), have roles in the innate immune response to pathogens with polymorphisms in their encoding genes associated with chronic inflammatory disorders of the gut. TLRs are transmembrane proteins, homologous to the IL-1R receptor family, first identified in *Drosphila* (Medzhitov et al 2000). Toll-like receptors are necessary in the recognition of commensal bacteria and the development of tolerance (Rakoff-Nahoum et al 2004), and TLR-2 maintains epithelial integrity (Cario et al 2004) and the response to lipopolysaccharides (Cario et al 2000). NODs are cytosolic proteins, similar to R-proteins that form part of plants' innate immunity (Inohara 2002), and expression of NOD2 by epithelial cells in humans is a key component of their antibacterial response (Hisamatsu et al 2003). Mutations in the genes encoding TLR-4 and NOD2 are associated with a greater risk of graft-versus-host-disease (Elmaagacli et al 2006) but also have a role in the pathogenesis of IBD. NOD2 has an important antibacterial role in humans (Hisamatsu et al 2003; Wehkamp et al 2004) and mutations are associated with an increased susceptibility to Crohn's disease (Hugot et al 2001; Ogura et al 2001). Polymorphisms in TLR4 are associated with a greater risk of both UC and CD (Franchimont 2004; Torok et al 2004).

Mucosal lymphocyte populations

Dendritic cells

Dendritic cells (DCs) are included here as they have an important role in determining the response of lymphocytes to antigen (Wyllie et al 2015). DCs sample antigen by two routes: those within a PP sample antigen taken up by M-cells, but there are also DCs outside of PPs which can extend processes between enterocytes to sample antigen on the mucosal surface (Rescigno et al 2001). This antigen is presented to T cells, with co-stimulatory molecules, inducing either a regulatory or effector response. Pathogens require an effector response and their presence in the gut can cause epithelial cells to express pro-inflammatory cytokines, such as TNF-α, which favours the induction of an effector phenotype. Commensal bacteria and food antigens need a regulatory response and intestinal DCs, compared to those in the spleen, produce greater amounts of the regulatory cytokine IL-10. Also, whilst there may be differences in the responses of PP and LP DCs (Strober 2009), they both induce expression of FOXP3 (indicating a regulatory phenotype in lymphocytes) and specific gut homing integrins in naïve T cells (Mora et al 2003), and shift the isotype of B cells towards health-associated IgA production.

Lymphocyte populations

$CD4^+$ lymphocytes

$CD4^+$ T cells (T 'helper' cells) play a key role in the triggering and coordination of immune responses. These cells are able, when exposed to a range of immunoregulatory proteins including cytokines and interleukins, to develop into phenotypically and functionally distinct subtypes. Relevant to IBD was the potential to differentiate into one of two phenotypes: T_H1 cells, secreting IL-12, IFN-γ and TNF-α (Neurath et al 1995); and T_H2 cells secreting IL-4 and IL-13 (Boirivant et al 1998). It has been shown that within the lamina propria of patients with CD, $CD4^+$ lymphocytes express cytokines consistent with a T_H1 phenotype; whereas the T_H2 phenotype predominates in UC (Fuss et al 1996). Two further groups of immunoregulatory $CD4^+$ T cells have been identified: so called T regulatory (T_{reg}) cells and T_H17 cells.

The interleukin-2 receptor alpha chain (IL2R or CD25) was initially identified as being necessary for lymphocyte proliferation (Hatakeyama et al 1989). It was subsequently found that the elimination of $CD4^+CD25^{high}$ lymphocytes in mice resulted in widespread autoimmune disease (Sakaguchi et al 1995). $CD4^+CD25^{high}$ lymphocytes, now

recognised as T regulatory cells or T_{regs}, showed little response to T cell receptor stimulation and suppressed the activation and proliferation of other T cells (Takahashi et al 1998). Whilst initially thought to develop only in the thymus as natural T_{regs} (Itoh et al 1999), exposure of peripherally circulating naïve $CD4^+$ cells can induce them to develop into a population of T regulatory cells referred to as iTregs (Chen et al 2003). The transcription factor forkhead box P3 (FOXP3 or scurfin) plays a specific role in the development and function of the $CD4^+CD25^{high}$ T_{reg} cell population. Retroviral transduction of FOXP3 into $CD4^+CD25^-$ T cells converts them to the $CD4^+CD25^{high}$ T_{reg} cell phenotype (Fontenot et al 2003; Hori et al 2003), and can inhibit the development of IBD in mouse models (Hori 2003). Furthermore, a deficiency of FOXP3 in mice results in hyper-activation of T cells (Fontenot et al 2003). FOXP3 is the gene found to be defective in the Scurfy mouse strain, a mouse model of autoimmune disease (Brunkow et al 2001). In humans, mutations of the FOXP3 gene also underlie the syndrome know as immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance (IPEX) (Bennett et al 2001). IL-10 secreting FOXP3 cells have been identified in the lamina propria of mice with numbers increasing with remission of colitis (Uhlig et al 2006). T_{regs} also produce IL-35, a member of the IL-12 family found to confer a regulatory phenotype in naïve T cells (Collison et al 2007). These observations show the importance of T_{regs} and FOXP3 in regulating the immune response.

In 2005, two groups of investigators successfully identified a type of T helper cell which is now known as the T_H17 cell (Harrington et al 2005; Park et al 2005) which, in contrast to $CD4^+CD25^{high}$ T_{reg} cells, is pro-inflammatory. These cells selectively express IL-23R, CCR6 and the transcription factor RORγt and have been found in the peripheral blood and gut of patients with CD (Annunziato et al 2007). They are also found in the chronically inflamed tissue in psoriasis, rheumatoid arthritis and asthma (Pene et al 2008).

$CD4^+$ lymphocytes are mainly found within the lamina propria, usually at a ratio of greater than 1.5 when compared to $CD8^+$ lymphocytes (Carrasco et al 2013). Table 1-1 summarises the cytokines inducing them to develop and the main cytokines they secrete. However, these lineages are not strictly maintained and there is evidence that T cells can convert between a Th17 and T_{reg} phenotype, and that Th1 and Th17 may have a similar developmental pathway (Zhou et al 2009).

Initiating cytokines	$CD4^+$ lymphocyte type	Produced cytokines
IL-12 IFN-γ T-box transcription factor (T-bet)	Th1	IL-2 IFN-γ
IL-4	Th2	IL-4 IL-5 IL-13
TGF-beta IL-23 IL-6	Th17	IL-17
IL-10 TGF-beta	Treg	IL-10 IL-35

Table 1-1 CD4+ lymphocyte subset cytokine profiles. Cytokines initiating the generation of different $CD4^+$ lymphocytes subtypes, and the cytokines they subsequently secrete.

CD8+ lymphocytes

The intraepithelial layer's main population of lymphocytes are T-cells with no B cells present. Three quarters of the T-cells are $CD8^+$ lymphocytes, consisting of two types (Wyllie et al 2015). The first, type A intra-epithelial lymphocytes (IELs), behave as other peripheral CD8 cells. They are primed in the GALT, drain into the mesenteric and thoracic lymph nodes, enter the systemic circulation, home back to the lamina propria and transfer to the epithelium (Hayday et al 2001). They are cytolytic, produce a Th1-like cytokine profile, and have antigen memory.

The second population of lymphocytes are known as type B IELs. Unique to the gut they are found in the small intestine (Hayday et al 2001). They include $CD4^-CD8^-$-T cells (expressing neither CD4 or CD8), T cells with a γδ T-cell receptor and CD8 cells with an αα T-cell receptor (rather than the usual αβ). Type B IELs develop in the intestine, rather than in the thymus. They seem to have a role in maintaining epithelial immune homeostasis and response to infection (Fujihashi et al 1990; Roberts et al 1996).

B cells

B cells of the intestine are also different from those in the circulation (Wyllie et al 2015). The role of gut derived B cells, also known as B1 cells, seems to be the production of a non-specific immune defence against bacterial invasion. Unlike their circulating counterparts (known as B2 cells) they do not mature into memory cells and produce a non-specific IgM to bacterial carbohydrates. They are found exclusively in the lamina

propria, and their presence in epithelial specimens indicates contamination from the lamina propria (Carrasco et al 2013).

The mucosal immune system in health and disease

The healthy mucosal immune response

The gut needs to develop tolerance to commensal bacteria and dietary antigens. This mechanism is called “oral tolerance”, and like that preventing auto-reactivity of the immune system (Faria et al 2005; Wyllie et al 2015). Exposure to low doses of antigen, presented by antigen presenting cells (APCs), generates T regulatory cells within the gut (induced or iTregs). They migrate from the mucosa to lymphoid organs and, via the systemic circulation, to target organs. They inhibit effector responses to that specific antigen whilst also demonstrating bystander suppression which inhibits the effector response to other antigens present in the same anatomical location. High doses of antigen produce tolerance through clonal anergy or deletion of reactive cells - although there is overlap between this and the response to low doses of antigen. Interventions such as caesarean section, perinatal antibiotics, and use of formula milk means there is an inadequate (or inappropriate) bacterial exposure to produce the necessary development of oral tolerance (Bedford Russell et al 2006) and may explain the increases in both allergic and autoimmune disorders (including IBD).

In germ free mice development of oral tolerance was only possible with bacterial colonisation which had to occur in the neonatal period (Sudo et al 1997). The type of organisms colonising the gut is important and does seem to affect an individual’s risk of allergic disease: colonisation with *B.fragilis* in human children is associated with greater numbers of IgA secreting cells (indicating the appropriate development of an immune response), with those colonised earliest having the highest levels (Gronlund 2000); and colonisation with bifidobacteria was lower at one week of age in children who went on to develop a confirmed allergic disorder (Bjorksten et al 2001). Supporting this is a study in mice which showed that early administration of antibiotics removed bacteria from the gut with subsequent reductions in the number of gut lymphocytes, and a shift in their phenotype from Th1 to Th2 which may increase the incidence of allergy (Oyama et al 2001). There is some evidence to suggest an association between antibiotic use and development of allergy in humans, but a causal link has not been shown (Bedford Russell et al 2006). Probiotics, though, have been shown to reduce the incidence of atopy (Kalliomäki et al 2001). Delivery of human infants by caesarean section delayed bacterial colonisation, compared to those delivered

vaginally (Gronlund et al 1999); and mice delivered vaginally showed a TLR and NFkB (part of a cellular, pro-inflammatory pathway) mediated response of intra-epithelial cells, not seen in those delivered by caesarean section (Lotz et al 2006).

There is a fundamentally different immune response required to clear invasive pathogens (Wyllie et al 2015). Invasive organisms that evade early defences reach epithelial cells causing them to release stress molecules which activate IELs and attract populations of inflammatory cells such as monocytes, dendritic cells, polymorph neutrophils, and lymphocytes. There is a local secretory IgA response, but also systemic immune priming with the generation of cell mediated immunity and serum antibodies which usually clears the pathogen. If these responses are unsuccessful in clearing pathogens, or if disruption of the epithelial barrier exposes the gut to known antigen such as commensal bacteria or dietary antigens, then chronic inflammation can develop.

The loss of mucosal immune tolerance and chronic inflammation

A number of observations in IBD patients show that factors impacting on the ability to develop oral tolerance may underlie the pathogenesis of chronic inflammation. IBD patients have a reduced gut microbiological diversity (Manichanh et al 2006; Frank et al 2007). *Faecalibacterium prausnitzii* has anti-inflammatory effects and is reduced in patients with Crohn's Disease (Sokol et al 2008), whilst there are increases in *E.coli* species (Kotlowski et al 2007) which show greater adherence to epithelial cells (Martin et al 2004). Loss of the mucosal barrier in active disease, with greater attachment of microbes to epithelial cells, has been shown in human CD patients (Swidsinski et al 2007). Humans affected by IBD, and their first degree relatives, have greater gut permeability compared to controls (Hollander 1986), and greater intestinal permeability predicts relapse of IBD (Kiesslich et al 2012). The role of PRRs in IBD has already been mentioned.

The development of the chronic inflammation seen in IBD seems to follow on from an acute inflammatory response and typically has two distinct phenotypes (Wyllie et al 2015). A Th1 or Th17 dominated response activates macrophages to release cytokines such as TNF-α, IL-1β and IL-6. Whilst there is an overlap between Th1 and Th17 responses, the former is typically associated with macrophage mediated Crohn's like lesions and Th17 with a neutrophil dominated or autoimmune response. Both can develop granulomas, formed from aggregates of the inflammatory cells. A Th2

dominated response has two types: IL-4 and IL-3 trigger a humoral response usually seen in UC, with mucosal production of IgG and/or IgE; IL-5 triggers an eosinophilic infiltration observed in allergic disorders and eosinophilic esophagitis.

Compared to controls the proportions of T_{reg} and T_H17 cells are altered in patients with IBD (Eastaff-Leung et al 2010). The numbers of circulating T_{reg} cells are significantly lower in the peripheral blood of adult patients with CD ($5.88x10^3$/ml; p = 0.002) and UC ($5.16x10^3$/ml; p = 0.006) compared with controls ($8.08x10^3$/ml). For cases and controls the upper limit of the confidence interval for the proportion of circulating T_{reg} cells was 0.47 to 0.55%, whilst the lower limit was 0.012% and 0.003% in CD and UC compared to 0.121% in controls. In contrast, the proportion of pro-inflammatory T_H17 cells was found to be *higher* in patients with CD than corresponding controls (0.36% to 1.25% vs 0.1% to 0.49%), as was the absolute number of these cells ($7.67x10^3$/ml vs $15x10^3$/ml; p = 0.0012). They were also significantly increased in UC. These findings demonstrate a shift from a T_H17 to T_{reg} ratio of 1:1, seen in healthy controls, to an excess of pro-inflammatory cells.

A monoclonal antibody to TNF-α (Infliximab) is recommended by NICE for the treatment of IBD (NICE 2010; NICE 2015). Infliximab increases the proportion of $CD4^+$ cells with a T_{reg} phenotype in patients with IBD (2.9% vs 4.4%; p = 0.003) and the capacity of T_{reg} cells isolated from the peripheral blood of these patients to suppress the proliferation of allogenic responder $CD4^+$ T (T_{eff}) cells *in vitro* (28% vs 66%; p = 0.04) (Boschetti et al 2011). This shows that disease remission is associated with a down regulation of the Th1 response and an increase in T_{reg} cells.

Circulation of immune cells

Over forty years ago it was found, using radioactively labelled lymphocytes in rats, that naïve lymphocytes trafficked to secondary lymphoid tissue, whereas activated lymphocytes preferentially trafficked to mucosal tissue (Gowans et al 1964). The passage of lymphocytes from vessels into the tissues involves a series of ligand-specific binding steps including loose tethering, firm adherence and then passage across the endothelium (Butcher 1991). Variations in the expression of selectins and integrins, triggered by activation of lymphocytes, determine which cells enter which tissue and facilitates a system to guide the trafficking of leucocytes (Habtezion et al 2016).

$\alpha4\beta7$ is the receptor for the human mucosal addressin cell adhesion molecule-1 (Madcam-1) (Erle et al 1994), selectively expressed in intestinal venules (Briskin et al 1997). It is expressed by lymphocytes that have been exposed to antigen in the gut of rodents and humans, the latter being demonstrated in an ingenious experiment by Kantele et al (1999). Subjects were exposed, orally or parenterally, to antigen. Lymphocytes from subjects exposed to the oral antigen showed reduced proliferation in response to the same antigen when the $\alpha4\beta7^+$ cells were depleted. Lymphocytes from subjects that had been exposed to the antigen parenterally also responded, but the response was unaffected by the depletion of $\alpha4\beta7^+$ cells. This supports the hypothesis that lymphocytes exposed to antigen in the gut enter the peripheral circulation and express $\alpha4\beta7$, making this an antigen useful in the identification of lymphocytes activated in the gut. Clinical trials of an antibody to $\alpha4\beta7$, Vedolizumab, have demonstrated some success in treating both CD and UC (Behm et al 2009; Cherry et al 2015). Expression of its receptor Madcam-1 is increased in CD and UC (Briskin et al 1997) and, interestingly, this receptor-ligand pair has a role in recruitment of haematopoietic stem cells into bone marrow in mice (Katayama et al 2004).

IBD has a number of extra-intestinal manifestations (Vavricka et al 2015) affecting the skin (erythema nodosum, pyoderma gangrenosum), eyes (uveitis and episcleritis) and mouth (apthous ulcers and periodontitis). The liver is also affected, manifesting as autoimmune hepatitis or primary sclerosing cholangitis (PSC). Liver invading T lymphocytes express the gut-specific chemokine CCL25 which promotes $\alpha4\beta7$ binding to Madcam-1 on the hepatic endothelium and gut activated T cells may mediate PSC (Eksteen et al 2004) as well as the other extra-intestinal manifestations of IBD (Adams et al 2006).

The development and maintenance of bone

Physical structure of bone

The structural components of bone fall into two categories: cortical and cancellous. Cortical bone refers to the thicker, denser, outer portion of bone which itself has three regions: an outer periosteal layer, an inner endosteal layer, and a layer between those consisting of the Haversian canal system carrying blood vessels through the cortical bone. Cancellous bone refers to that contained within the cortex. It consists of

trabeculae, a honey-comb like network of bone which forms a structure more open than that of cortical regions.

Cellular elements of bone

Osteoblasts (OBs) are derived from mesenchymal stem cells under the guidance of specific genes (Datta et al 2008). Runt-related transcription factor 2 (Runx-2, also known as Cbfa1) is a key element in their development, with its absence in mice resulting in a phenotype with no osteoblasts (Komori et al 1997; Otto et al 1997). Expression of RUNX2 is promoted by bone morphogenetic proteins (BMPs) and wingless-ints (WNTS): genetic inactivation of the WNT β-catenin results in the formation of chondrocytes rather than osteoblasts (Day et al 2005), and BMP2 increases expression of Runx-2 (Lee et al 1999; Lee et al 2003). WNTs also mediate the effects of LRP5, a low-density lipoprotein (Mao et al 2001). It is the locus for osteoporosis-pseudoglioma syndrome with low bone formation (Gong et al 2001) and mice lacking this gene have reduced bone formation independent of Runx2 (Kato et al 2002). Interestingly, humans lacking the gene sclerostin (which binds to LRP5) have excessive bone formation which shows it is potentially able to up- and down-regulate osteogenesis (Lewiecki 2014). These observations highlight the importance of WNTS in regulating OB function (Glass et al 2007).

Osteoblasts have three different functional phenotypes. Most commonly they are recognised as the cells able to form osteoid and mineralise it with hydroxapatite. They also go on to become osteocytes, embedded within the bone, and bone lining cells which cover the surface of bone. Together, these two subtypes mediate bone maintenance and repair. The role of OBs is to synthesise bone matrix by producing and mineralising an organic matrix (Florencio-Silva et al 2015). The organic matrix is formed from numerous proteins secreted by the osteoblasts: these include collagen type I, non-collagenous proteins (osteocalcin, osteonectin, bone specific protein II, and osteopontin), and proteoglycans. Mineralisation is initiated by matrix vesicles released from specific regions of the osteoblast membrane. Osteoblasts produce large amount of alkaline phosphatase (ALP) which converts pyrophosphate into phosphate (Yadav et al 2011), whilst annexins enable uptake of calcium into the matrix vesicle (Kirsch et al 2000). These two ions are then nucleated to form hydroxyapatite, released into the organic matrix when the vesicle ruptures (Florencio-Silva et al 2015) and propagating through it (Marotti et al 1972; Boivin et al 2002). Interestingly, whilst chondrocytes do not change into osteoblasts they have a capacity to mineralise during the later stages

of their hypertrophic phase: both mineralising and non-mineralising chondrocytes release vesicles, but those in mineralised cartilage have much stronger alkaline phosphatase (ALP) activity and annexins present in their membrane (Kirsch et al 1997).

Osteoclasts (OCs) are derived from the haematopoietic stem cell line (Datta et al 2008). Whether a promyeloid precursor becomes an OC, a macrophage or a dendritic cell is determined by the presence of specific factors and/or receptors. It was the discovery of a cell surface receptor, its ligand, and an inhibitory secreted factor common to these cellular relatives that generated a large amount of interest in the development of OCs and their interactions with OBs. In a short period of time three research groups separately reported interactions between the ligand receptor activator of nuclear factor kappa β (RANKL) expressed by osteoblasts and T cells, its receptor on osteoclasts (RANK), and a decoy receptor (osteoprotegerin, OPG) involved in T cell signalling, dendritic cell (DC)/T cell signalling and the regulation of osteoclast development (Anderson et al 1997; Simonet et al 1997; Wong et al 1997; Lacey et al 1998). Membrane bound and soluble RANKL is produced by OBs and, in combination with colony stimulating factor 1, induce human peripheral blood mononuclear cells to differentiate into OCs (Lacey et al 1998). OPG has the opposite effect, stopping the development of OCs from spleen cells and protecting ovariectomised rats against bone loss (Simonet et al 1997).

The sequence of cellular events by which OCs resorb bone has been termed the resorption cycle (Vaananen et al 2000). OCs migrate to a specific area of bone where their plasma membrane becomes tightly attached forming a sealed-off zone. Within this sealing zone projections extend from the OC into the bone, forming the ruffled border, and acidic vesicles release hydrochloric acid which dissolves the hydroxyapatite. Proteolytic enzymes, including matrix metalloproteinases, are then able to cleave collagen. The released elements are then taken up and released through the functional secretory domain at the opposite surface of the cell, into the extra-cellular space.

Whilst initial ossification of bone is primarily mediated by OBs, after chondrocytes have built the cartilage skeleton, the processes of modelling and remodelling guide respectively the overall shape and maintenance of bone (Langdahl et al 2016). The RANKL system is involved in both these processes (Boyce et al 2008), but it is not the

only factor and separation of these two cell types by time and location means additional processes are required (Sims et al 2015).

Bone development

Bone develops by endochondral ossification (EO) and intramembranous ossification (IO). EO is the primary process by which bones of the appendicular skeleton (made up of long bones) and the axial skeleton (including vertebrae) develop longitudinally (Cadet et al 2003), with chondrocytes forming an initial cartilage skeleton which is subsequently mineralised by osteoblasts. IO is the process by which sesamoid bones e.g. the patellar, and the craniofacial (or dermal) bones grow. It is also how cortical bone develops on the outer periosteal layer of bone throughout the skeleton, producing radial growth of long bones and vertebrae. IO does not involve a cartilage skeleton and ossification occurs directly from condensations of pre-osteogenic mesenchymal cells (Percival et al 2013), although a cartilage phase has been identified in some bones (Nah et al 2000; Abzhanov et al 2007). This section will focus on EO rather than IO as it is the predominant ossification process in long bones and vertebrae.

The cartilage skeleton

Undifferentiated mesenchymal stem cells (MSCs) reside in a region of the growth plate known as the resting zone (van der Eerden et al 2003), formed from a condensation that develops early in fetal development (Hall et al 2000). The MSCs move out of this resting zone and differentiate into chondrocytes which proliferate and secrete an extracellular matrix (ECM). The ECM produced at this stage consists of type II collagen, and other proteins including aggrecan (a proteoglycan) and hyaluronan (a glycosaminoglycan) (Heinegard 2009). These combine with other proteins in the ECM to provide the growth plate with stability. As the chondrocytes mature they stop proliferating, start to hypertrophy and change their secretory profile (Mackie et al 2011). In the early stages of hypertrophy chondrocytes start to secrete collagen type X, rather than type II (Karsenty et al 2002), and matrix metalloproteinases which remodel the ECM (van der Eerden et al 2003). This remodelling helps form the cartilage skeleton and facilitates angiogenesis which is necessary for mineralisation (Blumer et al 2008).

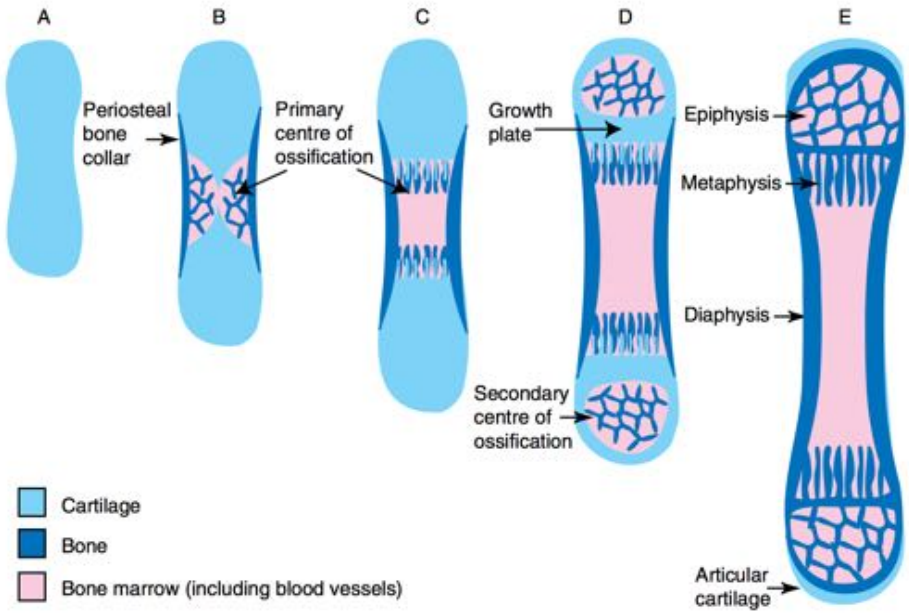

Figure 1-4 Overview of development of a bone by endochondral ossification. (A) The cartilage model of the future bone. (B) A periosteal bone collar has formed and formation of the primary centre of ossification has been initiated. (C) The primary centre of ossification is starting to expand towards the ends of the cartilage model. (D) The secondary centres of ossification have formed at each end of the bone, leaving a cartilaginous growth plate between primary and secondary centres of ossification. (E) Skeletal maturity has been achieved, with complete replacement of the growth plate cartilage by bone. The only cartilage remaining is the articular cartilage at the ends of the bone. Reproduced with permission (Mackie et al 2011).

Mineralisation

Ossification centres are the regions where osteoblasts mineralise the cartilage skeleton converting it into mature bone. Primary ossification centres can be seen in the mid-shaft of human fetal long bones from 9 completed weeks of gestation (Bagnall et al 1982). Secondary centres can be seen in the distal femoral epiphyses as early as 29 weeks, although most appear around 35 weeks (Pryse-Davies et al 1974). Their development is dependent on a blood supply (Colnot et al 2004).

Cartilage is avascular, and whilst that is thought to be true of the growth plate investigators have identified blood vessels supplying it (Tonna 1961; Wirth et al 2002). Although these vessels are dismissed as a transient phenomenon, it is accepted that as chondrocytes proliferate and move away from the resting zone they become increasingly hypoxic. This increases expression of HIF-1α, a member of the hypoxia induced factor gene family (Robins et al 2005). HIF-1α has a role in ensuring chondrocytes produce the cartilage skeleton, with its deletion resulting in abnormal

cartilage formation *in-vivo* and *in-vitro* (Amarilio et al 2007), but is also necessary for angiogenesis (Maes et al 2012).

A number of studies demonstrate that angiogenesis is necessary for mineralisation. Sections of cartilage skeleton, explanted into renal tissue to provide a *de-novo* blood supply, have shown that ossification is dependent on vascular invasion (Colnot et al 2004). Vascular endothelial growth factor (VEGF), an important mediator of angiogenesis, has been shown as necessary for both formation of ossification centres (Maes et al 2002) and trabecular bone (Gerber et al 1999). The growth of blood vessels, arising from the perichondrium and invading the cartilage, deliver pre-osteoblasts which produce the mature skeleton (Colnot et al 2004; Maes et al 2010). When there is a lack of differentiated osteoblasts (OBs), as seen in the Cbfa1 gene knockout mouse, a cartilage skeleton forms but does not mineralise (Komori et al 1997; Otto et al 1997).

Bone modelling

Modelling alters the overall shape of the bone with OCs resorbing bone on the endosteal surface whilst OBs lay down new bone on the periosteal surface (Clarke 2008; Baron et al 2013). This physical separation (see Figure 1-5) may explain why their activities are not considered coupled as they are in the process of remodelling. However, there has been less investigation of modelling, and this may in part reflect a misconception that it only occurred during childhood. Adults who take up tennis after the age of 25, when modelling was thought to have stopped, still show evidence of changes in bone shape (Kontulainen et al 2003). Interestingly, the continuation of appositional growth into adulthood had already been noted in bone from autopsy subjects decades ago (Epker et al 1966). There is also evidence that modelling occurs within the cancellous portion of bone, altering trabecular structure in the iliac crest of adults (Kobayashi et al 2003). It may be disruptions to this system that explain changes in the bone structure of those affected by IBD, only seen with pQCT and bone biopsy but not DXA (Burnham et al 2007; Dubner et al 2009; Ward et al 2010).

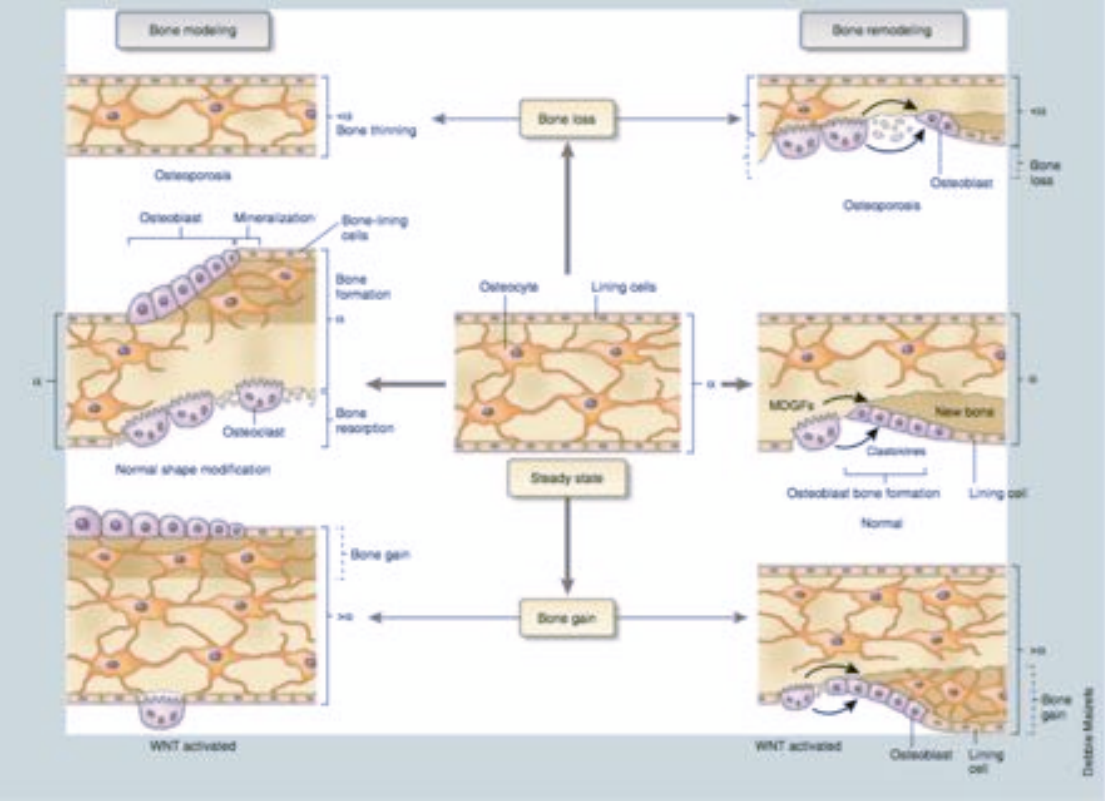

Figure 1-5 Bone modelling and remodelling. On the left is modelling with osteoclasts and osteoblasts on separate bone surfaces, on the right is remodelling with both cell types on the same bone surface. Reproduced with permission (Baron et al 2013).

Bone remodelling

Remodelling is the process by which the body maintains the quality of bone and responds to mechanical stress. It is primarily mediated by osteoblasts (OBs) and osteoclasts (OCs), acting within the basic multicellular unit (BMU). In remodelling, unlike modelling, the actions of OCs and OBs are linked as the two cell types form the basic multicellular unit (Sims et al 2008), although the observation that during childhood bone mass increases and then steadily declines in adulthood (Lips et al 1978) shows that their actions are not necessarily balanced over time. However, in an elegant study by Eriksen et al, the chronology of resorption and formation were mapped out in healthy individuals (Eriksen et al 1984a; Eriksen et al 1984b). They showed that there was an initial resorptive phase of 8 days with increased numbers of OCs, and a formation phase of over 135 days when OBs dominate. As can be seen in Figure 1-6 these two phases are temporally distinct and separated by a “mononuclear phase” with an influx of mononuclear cells. These are thought to be haematopoietic in origin and are present at a period when pre-osteoblasts start to appear.

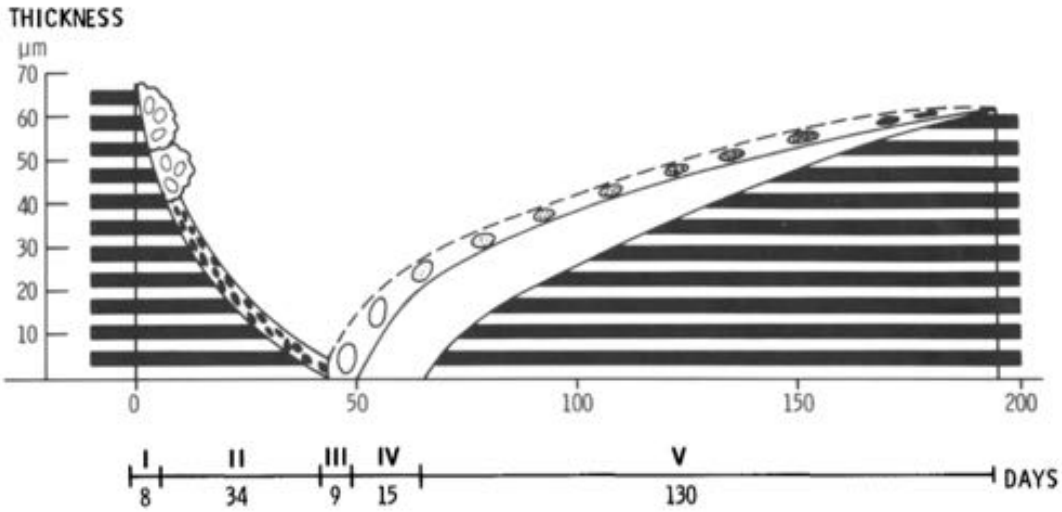

Figure 1-6 Smoothed curves showing the total remodelling period derived from resorption and formation curves. The remodelling period is subdivided into osteoclastic resorption phase (I, 8 days), mononuclear resorption phase (II, 36 days), pre-osteoblastic phase (III, 9 days), initial mineralization lag time (IV, 15 days), and mineralization phase (V, 130 days). Reproduced with permission (Eriksen et al 1984a).

OBs and osteocytes, through RANKL and OPG, start the process of remodelling by initiating the development of OCs and stimulating their resorptive activity (Pivonka et al 2008). Osteocytes seem able to detect mechanical loading and the development of damage, and their apoptosis triggers site specific resorption by OCs (Verborgt et al 2002; Noble et al 2003; Mann et al 2006). Osteocytes may trigger the release of OC precursors from the bone marrow via processes reaching into it (Kamioka et al 2001). Chemoattractants such as osteocalcin and collagen fragments, released from the bone, also mobilise OC precursors (Malone 1982). Bone lining cells, covering the bone surface, release collagenase which exposes the mineralised matrix (Hauge et al 2001). The BMU, when initiated, develops a blood supply (Michael Parfitt 2006). This certainly delivers OCs, and possibly also OBs (Eriksen et al 2006; Sacchetti et al 2007). OCs then trigger bone formation by OBs. There is a reversal phase mediated by mononuclear phagocytes, and the release of growth factors - insulin like growth factor, fibroblast growth factor, transforming growth factor β and bone morphogenic proteins (Sims et al 2008). However, bone formation by OBs can be triggered by the presence of pits alone (Gray et al 1996; Dalby et al 2006).

Bone turnover

OBs and OCs overall activity can be quantified by measuring bone turnover markers, substances released by the cells into the blood stream. In adults, where remodelling is the predominant process, their measurement can predict risk of reduced bone

mineral density and fracture. In children bone development and modelling is greater but, whilst in neither of these the actions of OBs and OCs are linked, measurement of bone turnover does have a role in assessing bone health (Rauchenzauner et al 2007).

Osteoclasts

Collagen, one of the main constituents of bone, has hydroxypyridium crosslinks. One of these, called deoxypyridinoline (DPD), bridges several collagen peptides to provide additional stability. When bone is resorbed, DPD is released into the circulation and the urine (Seibel 2000). Serum levels of DPD have been shown to correlate well with bone resorption (Eastell et al 1997).

Crosslinking is necessary for the formation of collagen and involves specific molecular regions, one of which is the carboxy-terminal telopeptide (Seibel 2000). Measurement of the carboxy-terminal telopeptide of type I collagen in urine, released when bone is resorbed, has been found to correlate with other markers of bone resorption (Gorai et al 1997).

Osteoblasts

Osteocalcin (also known as bone-gla-protein) is a marker of osteoblast activity which is expressed late in the differentiation of osteoblasts (Stein et al 1990). It is secreted by osteoblasts to form bone, but is also secreted into the circulation (Ingram et al 1994). Osteocalcin is considered a useful clinical marker of bone formation (Neve et al 2013).

In bone, collagen is synthesised by osteoblasts in the form of pre-collagen. These molecules can be identified by the presence of a specific amino-terminal propeptide – PINP (Merry et al 1976). Measurement of PINP in the serum correlates with other bone formation markers, and the value of these measurements has increased with the introduction of newer assays which demonstrate a better correlation with bone formation (Seibel 2000).

Alkaline phosphatase (ALP) is a ubiquitous enzyme in the human body which is found in a range of different cell types. Although its precise function is not clear (Harris 1990), its absence is associated with abnormal bone development. ALP has been shown to be an effective marker of bone formation (van Straalen et al 1991).

Bone health in inflammatory bowel disease

Growth

At diagnosis, children with Crohn's disease (CD) have significant reductions in their height, with disease severity seemingly the primary determining factor (Griffiths et al 1993; Hildebrand et al 1994). This is typically ascribed to problems with malnutrition, and may explain why targeted enteral nutrition improves growth (Morin et al 1980; Kirschner et al 1981; Belli et al 1988). An additional mechanism is the effect of the immune response. In a mouse model of IBD lower growth was seen with increased IL-6 and reduced IGF-1, and administration of IGF-1 improved growth (Ballinger et al 2000). It is estimated that inflammation explains 30-40% of growth failure. TNF-α may also affect growth of the cartilage skeleton by inhibiting the differentiation of chondrocytes, as seen in studies using rodent chondrocytes. Supporting this is the observation that Infliximab, a monoclonal antibody to TNF-α, improves growth (Walters et al 2007); although this effect may be due to a reduction in inflammatory cytokines and/or improving absorption of nutrients which would occur with disease remission.

Corticosteroids have a negative effect on linear growth, but when used in the treatment of CD growth does improve (Griffiths et al 1993; Hildebrand et al 1994). This indicates that any negative effect of steroids may be outweighed by improvements in nutrient absorption and reductions in inflammation. Thus, whilst final adult height is reduced not all studies show a significant reduction (Alemzadeh et al 2002; Herzog et al 2014). Perhaps ameliorating the slowing of growth is a delay in puberty, giving greater time for growth (Hildebrand et al 1994).

However, as was discussed previously, growth and mineralisation are distinct processes. The final common pathway for deficits on growth is the impact of the disease on the growth plate (Ballinger et al 2001). Mineralisation, and bone mineral density, is mediated by the action of osteoblasts. Whilst chondrocytes influence mineralisation there are other biological pathways which could have greater effect. For example, whilst IL-6 may inhibit chondrocytes which in turn reduces mineralisation by OBs, the direct effect of IL-6 on osteoblasts may have a far greater effect.

Bone mineral density

Low bone mineral density in IBD is well described in the literature, and the aim of this thesis is to better understand why it occurs. Assessment of bone mineral density

(BMD) in clinical care has been made possible by the development of dual X-ray absorptiometry (DXA). Meaningful interpretation of measurements requires an understanding of the techniques and terminology used.

DXA is well established as a non-invasive method for measuring the mineral density of bone in adults. It calculates the bone mineral density (BMD) based on attenuation of a beam of low dose radiation. As with standard X-rays bones can be identified as areas that are distinct from the surrounding soft tissues (see Figure 1-7). The absorption in those areas is measured and the bone mineral content (BMC) calculated. BMC is then expressed as g/cm^2 (areal bone mineral density, aBMD) by dividing the BMC by the projected area of the bone. Studies using cadavers have established the relationship between absorption of X-rays and BMC, with absorption being greater in more mineralised bone (Ho et al 1990; Edmondston et al 1993).

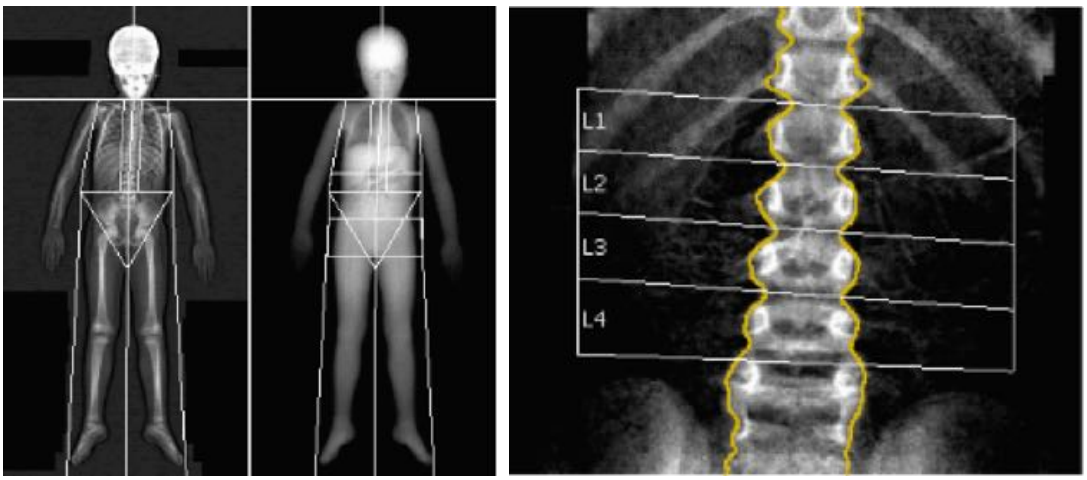

Figure 1-7 Example images of DXA. The left panel shows a whole-body image with the skeleton visible, the right panel shows a higher magnification view of the lumbar spine with image recognition software identifying individual bones.

In adults BMD is reported as a T-score which reflects the number of standard deviations from the mean of a reference population of young, healthy adults. The WHO identifies an individual as having osteoporosis when their BMD T-score is greater than 2.5 standard deviations (SDs) below the mean (Kanis 2002), a value associated with an increased risk of fracture (Marshall et al 1996). In children, the measurement of BMD by DXA is more complicated. DXA uses a 2-dimensional image of 3-dimensional structure and, even if they have the same BMC, a smaller bone will have an apparently lower BMD (Binkovitz et al 2008). Also, during puberty, regional growth of bones differs in boys and girls (Bass et al 1999; Bradney et al 2000). This means SDs must be

adjusted for age and sex, in which case they are referred to as z-scores (Gafni et al 2004).

Studies in adults with IBD have found reduced BMD (Schulte et al 1998; Ardizzone et al 2000; Schoon et al 2000; Jahnsen et al 2004; Leslie et al 2008). In CD 36% to 55% of patients are osteopaenic, and 7% to 50% are osteoporotic. In UC 32% to 67% of patients are osteopaenic and 7% to 18% are osteoporotic. These findings have been confirmed in case controlled studies, although some find no difference between CD and UC (Silvennoinen et al 1995; Lamb et al 2002) whilst one a reduction in only CD (Jahnsen et al 1997). Fracture risk can be increased by as much as 40% (Bernstein et al 2000). This was not confirmed in a similar study (Loftus et al 2002), but screening often identifies asymptomatic fractures (Klaus et al 2002; Stockbrugger et al 2002) and the incidence of fracture may be underestimated.

Case reports describe severe bone disease and fractures in children with CD (Semeao et al 1997; Thearle et al 2000) and UC (Lucarelli et al 2006), but population studies find that fracture rates in children with CD are not increased (Persad et al 2006; Kappelman et al 2011). Studies utilising DXA have shown reductions in BMD in children with IBD but problems with methodology and definitions bring their findings into question.

Semeao et al (1999) measured lumbar spine BMD Z-scores in patients 5 to 25 years of age with Crohn's disease and found that 70% were osteopenic and 38% osteoporotic, but there are two problems with this study. Definitions were based on Z-scores (adjusting for age and sex only) of less than -1 for osteopenia and less than -2 for osteoporosis and The International Society for Clinical Densitometry (ISCD) now state that these diagnoses can no longer be made based on DXA alone (Kalkwarf et al 2014). Semeao et al (1999) also found that "BMD increased with age" despite correcting for bone age which could have occurred because they didn't account for changes in bone size. Because of the way DXA measures BMD as a bone's size increases, even if the true mineral density remains unchanged, the apparent BMD will be greater (Binkovitz et al 2008). This problem has been demonstrated in clinical studies. Ahmed et al (2004) measured lumbar and total body BMD in children with CD and UC. Results were compared to a reference database which included bone area (van der Sluis et al 2002). Ahmed et al (2004) found the adjustment for bone area (a measure of bone size) reduced rates of osteopenia from 65% to 22%, and osteoporosis from 45% to 4%. Walther et al (2006) also found lower rates of

osteoporosis and osteopenia (40% and 13% respectively) when they used they used an established method, recommended by Kroger et al (1992), to adjust BMD for bone size. Also, when measuring BMD by DXA, greater amounts of muscle and fat will increase absorption of X-rays and, hence, the apparent BMD (Bolotin 2007). A study by Burnham et al (2004) measured total body BMC and found a 26% reduction in patients with CD, but this difference disappeared when adjusted for lean body mass (Burnham et al 2004).

Given these findings and their methodological difficulties it is difficult to be sure that the BMD of children with IBD, other than in a few individual patients, is reduced. Another way of measuring BMD is peripheral quantitative computed tomography (pQCT). The advantage of this is that it provides an image of the bone structure which is unaffected by surrounding tissues, and can measure independently the proportion of cortical (outer) and trabecular (inner) bone, unlike the composite absorption image of a DXA (see Figure 1-8). This modality has shown reductions in BMD in children with newly diagnosed CD compared to that of healthy age, sex- and race-matched children (Dubner et al 2009). They found that the tibial trabecular volumetric BMD in children with CD was, on average, on the 10^{th} percentile. They also found an increase in the endosteal dimensions and a marginal reduction in the periosteal surface, which reflects a thinning of the outer cortical bone and in previous studies was associated with greater bone fragility. These findings are supported by a study describing bone biopsies taken from children newly diagnosed with IBD, although they found only reductions in cortical bone width of 23% with no reduction in trabecular bone (Ward et al 2010). Also, it may be that the overall shape of the bone is affected, given the observation of changes in proximal femur geometry in children with IBD (Burnham et al 2007).

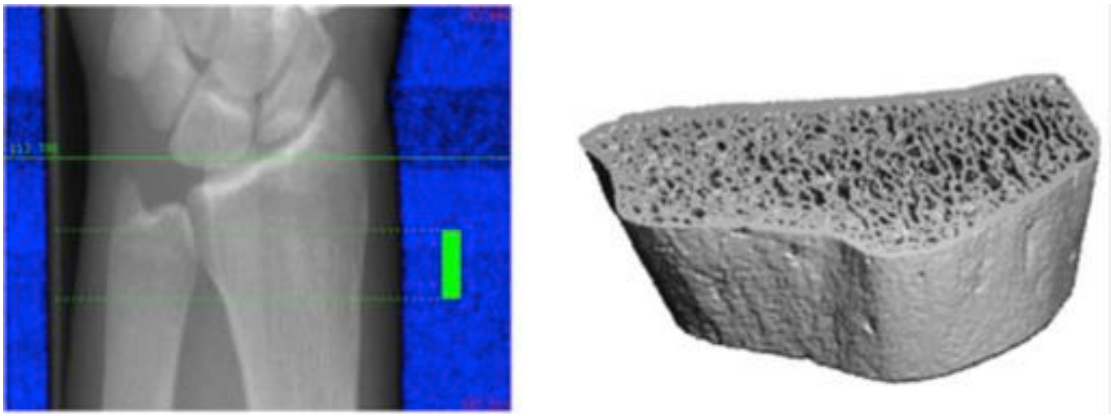

Figure 1-8 Dimensional reconstruction of bone imaged by pQCT. The image shows that this modality enables assessment of both bone volume and its mineral density. Open access article (Cheung et al 2013).

Therefore, whilst bone does appear to be affected in children with IBD, both in terms of mineralisation and structure, this has not been shown to affect short term fracture risk. However, childhood is an important time for bone mineral accrual and development of peak bone mass (Bailey 1997). The observation that children with IBD do not achieve their full height (Sawczenko et al 2006) and have a compromised peak bone mass (Laakso et al 2014) may explain the finding of juvenile onset of CD being associated with lower BMD in later life (Mauro et al 2007) and, potentially, an increased risk of fracture.

Bone turnover

Bone turnover (or bone metabolism) refers to the process of resorption and formation, and bone turnover markers are elements released into the blood which reflect the rate at which these two processes are occurring (Seibel 2000). Greater bone formation would be expected in children who are growing. In adults, whose BMD reduces with age, greater bone resorption would be expected. However, both formation and resorption markers are higher in children (Rauchenzauner et al 2007) indicating higher overall bone metabolism. Their measurement in patients with IBD, either monitoring changes over time or compared with appropriate controls, show that affected individuals have changes in the activity of osteoblasts and osteoclasts and that pattern of change is different in adults and children.

A study recruited 34 adult patients newly diagnosed with IBD over a one year period and measured their BMD by DXA and the bone turnover markers deoxypyridinoline (a measure of osteoclast activity) and osteocalcin (a measure of osteoblast activity) (Lamb et al 2002). They found significant reductions in BMD when compared

to controls with irritable bowel syndrome, no difference in levels of osteocalcin (8.6 vs 7.7μg/L; p = 0.6105), but a significant increase in urinary deoxypyridinoline (7.66 vs 5.70nmol/mmol creatinine; p = 0.0163). This suggests that increased bone resorption was the cause of their lower BMD.

Dresner-Pollak et al (2000) measured serum osteocalcin, bone-specific alkaline phosphatase (a measure of osteoblast activity), urinary NTx (a measure of osteoclast activity) and BMD by DXA in 36 patients with IBD. There was no control group and measurements of bone turnover markers were related to changes in BMD over a 24-month period. It was found that initial urinary NTx levels were inversely correlated with changes in BMD, although only at the lumbar spine and not at the femoral neck. Patients with a urinary NTx in the highest quartile exhibited the lowest increase in spine BMD. No correlations between BMD and serum levels of osteocalcin or bone-specific alkaline phosphatase were observed. This shows that increases in urinary N-telopeptide crosslinked type 1 collagen (NTx, a marker of osteoclast activity) can predict low BMD in adult patients with IBD.

Of note, the treatment of CD patients with infliximab has been shown to reduce levels of urinary NTx, and increase the levels of the bone formation markers bone-specific ALP, osteocalcin and pro-collagen type 1 N-terminal propeptide (P1NP). Franchimont et al (2004) recruited 71 patients with CD who were treated for the first time with infliximab over a six-week period. Prior to treatment, bone turnover markers indicated an increase in resorption and a decrease in bone formation compared to controls. After treatment, levels of bone turnover markers normalised, with all the individual markers being significantly different.

Although these findings suggest that bone resorption is the primary driver of bone loss in adults with IBD, bone biopsies of patients with IBD found primarily a reduction in bone formation (Croucher et al 1993). However, the authors acknowledge that the assessment of resorption is less clear than that of formation, and does not necessarily reflect the balance of bone turnover. The measurement of bone turnover in adults can be used to predict fracture risk independently of actual BMD (Garnero 2000; Kitatani et al 2003).

In children with IBD the pattern of change in bone turnover markers is different. Sylvester et al (2006) measured bone turnover markers in paediatric patients that were newly diagnosed with CD before being started on steroids. The findings demonstrated

that although markers of bone resorption were no different from controls, markers of bone formation were significantly reduced (see Table 1-2). In a follow up study, these markers improved with treatment (Sylvester et al 2007). Whitten et al (2010) also found reductions in ALP, but these weren't significant whilst an increase in bone resorption markers was. The authors point to the work of Sylvester et al (2006) having more peri-pubertal children, a period of life when bone turnover markers change (Cadogan et al 1998) which may explain the differences.

These data suggest that bone formation is reduced in paediatric CD. This is different to the findings in adults with IBD who predominantly exhibit an increase in bone resorption. Sylvester et al (2006) also found that the reduction in bone formation markers positively correlated with levels of the pro-inflammatory cytokine interferon gamma (IFN-γ). Based on this they suggest that activated T cells might mediate the reduction. Although this is a reasonable proposition, it could be argued that levels of IFN-γ are a simplistic measure of what is a complex, predominantly cell-mediated, process.

Marker	Controls	Cases	P value
Collagen type 1 C-terminal propeptide (ng/ml)	358.22	159.82	<0.001
Bone alkaline phosphatase (U/L)	147.8	53.73	<0.001
N-telopeptides of collagen (Nanomole per bone collagen equivalent)	330.56	237	<0.02
Deoxypirridoline (Nanomole per bone collagen equivalent)	20.77	18.4	NS

Table 1-2 Changes in bone turnover markers in children newly diagnosed with Crohn's disease. The table presents data published by Sylvester et al (2006).

Does the immune system cause low BMD in IBD?

Whilst the magnitude of the problem and the risk of fracture is difficult to quantify, especially so in children, the body of evidence presented above shows that IBD has a negative impact on bone metabolism. Understanding why this occurs is important in managing the condition.

Disease severity and/or duration have been identified as risk factors for low BMD (de Jong et al 2003; Sylvester et al 2006; van Hogezand et al 2006; Paganelli et al 2007; Sylvester et al 2007; Lopes et al 2008), although this is not a universal finding (Bartram et al 2006; Pappa et al 2006). There is also improvement in BMD with treatment (Franchimont et al 2004; Dubner et al 2009). However, whilst this association would be consistent with prolonged exposure of the bone to pro-inflammatory cytokines and cells affecting bone metabolism, other possible explanations need to be considered.

Those with more severe disease also have greater problems with nutrition, and this has been suggested as a cause of reduced BMD (Dubner et al 2009; Abraham et al 2014). One mechanism is a reduction in calcium and vitamin D, but studies have not supported this. Calcium and parathyroid hormone levels in patients with IBD are normal (Leslie et al 2008; Abraham et al 2014), calcium intake does not correlate with BMD (Lopes et al 2008), and supplementation does not improve BMD (van Bodegraven et al 2014) even when combined with vitamin D (Bernstein et al 1996; Benchimol et al 2007) or calcitonin (Pappa et al 2011). Vitamin D levels are reduced in those with IBD (Leslie et al 2008; Abraham et al 2014) but do not correlate with low BMD (Silvennoinen 1996; Silvennoinen et al 1996; Jahnsen et al 2002; Sentongo et al 2002; Pappa et al 2006). Reductions in body mass index (BMI), reflecting nutritional status, are associated with reductions in BMD (Siffledeen et al 2004; Bartram et al 2006; Sylvester et al 2007; Cohran et al 2008; Lopes et al 2008). But it may be that the reasons for this association are factors such as patients with less fat and lean tissue absorbing less of the DXA machines X-rays reducing the apparent BMD, as was discussed in the earlier section.

Those with more severe disease also use more steroids, and in children their use has been shown to increase the risk of fracture (van Staa et al 2003). Low BMD in IBD is often ascribed to the use of steroids, and a range of studies in adults (Silvennoinen et al 1995; Jahnsen et al 2004; Siffledeen et al 2004; Bartram et al 2006; van Hogezand et al 2006; Abraham et al 2014) and children (Lopes et al 2008; Ward et al 2010; Laakso et al 2014) support this. But other studies do not find steroid use to be associated with reductions in BMD (Bartram et al 2006; Pappa et al 2006; Walther et al 2006; Paganelli et al 2007; Sylvester et al 2007). In fact, low BMD is often present at diagnosis patients with IBD (Ghosh et al 1994; Habtezion et al 2002; Lamb et al 2002; Gupta et al 2004; Harpavat et al 2005; Walther et al 2006) preceding use of steroids and in some cases improving with treatment (Dubner et al 2009). A

comparison of the effects of steroids in children with IBD and nephrotic syndrome (an immune mediated condition in which the kidneys leak substantial amounts of protein) found that despite equivalent steroid doses the impact on BMD was greater in the IBD group (Leonard et al 2004) and it also affected geometry (Burnham et al 2007), which may be modulated by IL-6 (Kriel et al 2010). Therefore, whilst steroids appear to affect BMD via an effect on bone shape, steroids do not completely explain the finding of low BMD in IBD.

Osteoimmunology – when the immune system talks to the bone

Osteoimmunology refers to interactions between the immune system and bone. As the clinical studies presented above imply, in IBD and other inflammatory disorders the immune dysregulation appears to be associated with low BMD. However, causation cannot be assumed to be due to a number of potential factors affecting BMD in IDB patients. What the clinical data does indicate is that whilst in adults there is an increase in osteoclast mediated bone resorption, in children there is a reduction in osteoblast mediated bone formation. The findings discussed below describe the published literature in relation to laboratory based studies which have examined the effects of the immune system on bone.

Effect of soluble elements

An investigation as to whether humoral factors mediate low BMD in IBD was undertaken by Hyams et al (1997). Fetal rat parietal bones were cultured in serum from healthy paediatric controls, and paediatric patients with CD and UC. Incubation in CD serum resulted in a lower dry weight and calcium content, altered bone histomorphometry as exhibited by a disorganised mineral and osteoid, and distended and misshapen osteoblasts (see Figure 1-9). This effect was subsequently found to be reduced by the presence of IL-6 blocking agents (Sylvester et al 2002). The same group also looked at the effects of serum from patients with CD on rat osteoblast cultures, rather than whole bone. They found that whilst nodule formation and protein expression was reduced, proliferation and viability were unaffected. Blocking of IL-6 did not alter the observed effects (Varghese et al 2002). These findings highlight the complex interactions between humoral elements of the immune system and bone metabolism, also found when looking at the effect of individual cytokines.

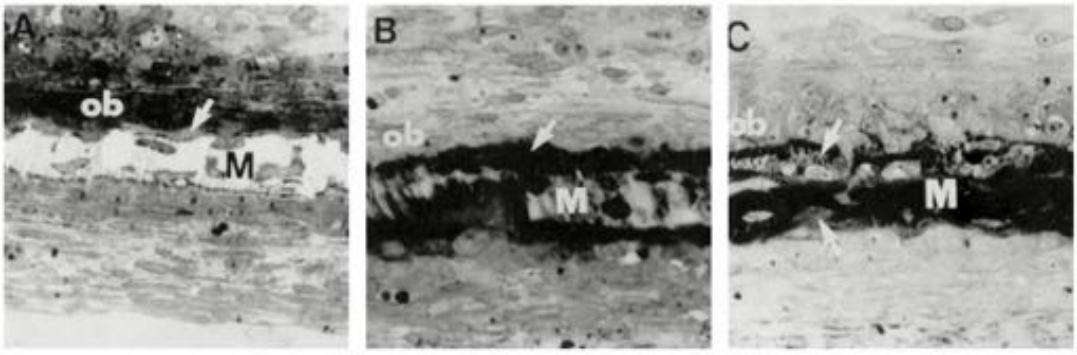

Figure 1-9: Light micrographs showing the effect of soluble immune factors on bone. Fetal rat parietal bones were treated with a 1:100 dilution of serum from a control patient (A) and patients with ulcerative colitis (B) and Crohn's disease (C). In A, the mineralised matrix (M) is covered with a thin layer of osteoid (arrow), which is covered by a thick layer of flattened osteoblasts (ob) and preosteoblasts. In B, no osteoid is apparent and a thick, darkly stained layer of matrix (arrow) covers the mineralised bone (M). The osteoblast layer (ob) is not as compact, and the osteoblasts are ovoid in shape. In C, little mineralised matrix (M) is found and it is uneven. Osteoid (arrows) is prevalent, and the osteoblasts (ob) are misshapen and plump with little organisation. Reproduced with permission (Hyams 1997).

TNF-α

Tumour necrosis factor-α (TNF-α) is a member of the TNF superfamily of 19 ligands and 29 receptors (Aggarwal et al 2012). This family has diverse roles within mammalian bodies with their pro-inflammatory actions mediated, at least in part, by activation of the transcription factor nuclear factor-kB. TNF-α was first isolated from the conditioned supernatants of human promyelomonocytic cell line HL-60. Whilst it has a role in the proliferation of B-cells it has also been implicated in a range of diseases and the effects of this pro-inflammatory cytokine on bone are well established.

TNF-α has been shown to inhibits the differentiation of rat calvarial osteoblasts and their production of mineralised nodules (Gilbert et al 2000). This has also been seen with murine OBs and is mediated by the p55, or type 1, receptor through inhibition of Cbfa1 DNA binding (Abbas et al 2003). Cbfa1, also known as Runx-2, is a key osteoblast differentiation factor. The p55 pathway has a role in apoptosis but a further study on OBs, from p55 knockout mice, confirms whilst the receptor is necessary to mediate the reductions in differentiation there was no evidence of apoptosis (Gilbert et al 2005).

During the life of an osteoblast the response to TNF-α can change. The response of murine mesenchymal stem cells (MSCs) and mature OBs to TNF-α were compared (Sidney et al 2014). MSCs seemed unaffected with no reduction in nodule

formation or alkaline phosphatase activity, and there was no impact on their viability. Primary OBs became less viable, and their ALP activity and nodule formation were reduced. This highlights that observations in MSCs cannot simply be applied to primary OBs. This principle may help explain why in human MSCs TNF-α inhibits the Runx-2 pathway, as seen with murine OBs, whilst ALP activity and mineralisation were increased (Ding et al 2009).

Whilst the effects of TNF-α on OBs is variable it consistently increases OC activity and bone resorption. Human peripheral blood mononuclear cells incubated with macrophage colony stimulating factor (M-CSF) form OCs after 10 days, and in the presence of TNF-α there were greater numbers of OCs (Kudo et al 2002). This was independent of the RANKL pathway as the addition of OPG did not affect numbers. There was also more resorption which could be due to greater OC number and/or OC activity. The blocking of TNF-α reduced both the number of OCs and resorption whilst the blocking of IL1α reduced resorption but not OC number suggesting that TNF-α primarily affect OC number with greater resorption activity requiring IL1α.

IL-17

The DNA signature for IL-17 was identified in 1993 with the identification of its role in inflammation recognised shortly after (Zenobia et al 2015). Ten years later it was shown to be produced by a developmentally distinct subset of $CD4^+$ helper cells, now known as Th17 cells. They have since been shown to have roles in a range of inflammatory disorders but also in protective antimicrobial immunity.

Rat calvarial cells showed reduced bone formation in the presence of IL-17 with reductions in ALP, nodule formation, and expression of the osteogenesis related genes osteocalcin and osterix (Kim et al 2014). The healing of defects created in the calvaria was also inhibited by the presence of IL-17. In contrast to these findings IL-17 increases the osteogenic capacity of human MSCs (Huang et al 2009) through increasing proliferation and subsequent differentiation into OBs expressing ALP (rather than chondrocytes or adipocytes). There was also increased expression of genes for ALP and Runx2.

A co-culture system of mouse haematopoietic cells and primary OBs was used to investigate the effects of IL-17 (Kotake et al 1999). The presence of IL-17 increased formation of OCs and dentine resorption showing its potential to reduce bone mineral

density. IL-17 also increased expression of the gene for osteoclast differentiation factor (ODF, or RANKL), found on the membrane of OBs and necessary for OC formation: separation of OBs from haematopoietic cells by a transwell insert and the presence of a soluble decoy receptor for ODF both reduced IL-17 induced OC formation.

In a study using bone explants from human patients with rheumatoid arthritis the blocking of either TNF-α or IL-17 inhibited production of IL-6 and reduced production of c-terminal peptide (CTX), produced by the breakdown of type 1 collagen, indicating reduced bone resorption (Chabaud et al 2001). However, there was significant variation in the eight patient sample responses. Inhibition in the presence of either TNF-α and IL-17 was only 50% for IL-6 in 25% of samples and for CTX in only 38% of samples. This may explain why combined blockade of both TNF-α and IL-17 increased inhibition overall and indicate that, despite having the same clinical phenotype, the underlying immune process may differ between patients.

IFN-γ

The interferons (IFNs) were first identified as cytokines able to interfere with viral replication (Schroder et al 2004). IFN-γ is the only type II IFN and is produced by a range of cell types, including T cells, B cells and natural killer cells. It was originally called macrophage activating factor and triggers anti-tumour effects, orchestrates leucocyte attraction, and regulates B cell function.

IFN-γ null mice have been shown to have a 45% reduction in bone volume compared to controls (Duque et al 2011). There were reductions in Runx-2 and osteopontin (an acidic phosphoprotein produced by OBs with a role in bone formation) gene expression, the mineral apposition rate, and OC number, indicating reduced bone formation rather than increased resorption.

However, reflecting the complexity of immune pathways, IFN-γ can indirectly increase OC number. A different group isolated OC precursors from the same IFN-γ null mouse and incubated them in culture media from T-cells activated by antigen presenting cells from wild type mice (Gao et al 2007). The addition of IFN-γ to this system, in which OC precursors won't respond to IFN-γ resulted in a threefold increase in OC number. They go on to look at the effects of IFN-γ on resorption in T-cell deficient mice in whom it had no effect on resorption, and bone-loss mouse models in whom it did increase

resorption. This suggests that the pro- and anti-osteoclastogenic effects of IFN-γ on bone resorption are altered by underlying disease processes.

The effects of IFN-γ on OBs is also complex. In a study using cultured human OB-like cells IFN-γ decreased proliferation but increased ALP activity and production of osteocalcin (Gowen et al 1988). TNF-α had the reverse effect with greater proliferation but reduced ALP activity and osteocalcin production. Also, whilst using a combination of IFN-γ and TNF-α, there are differences in the response of MSCs and OBs (Sidney et al 2014). MSCs show a limited reduction in nodule formation but no reduction in ALP activity or viability whilst OBs are significantly reduced in all three aspects.

IL-4 and IL-13

IL-4 is considered an anti-inflammatory cytokine produced by Th2 cells and has been shown to suppress the production of IFN-γ by mononuclear cells (Peleman et al 1989). It is closely related to IL-13 the genes for both being found on chromosome 5 of mice, and shares receptor components (Hilton et al 1996). IL-13 inhibits inflammatory cytokine production by human monocytes (Minty et al 1993) and regulates B cell function by inducing production of IgM and IgG (McKenzie et al 1993). Therefore, whilst in direct comparison they have differing effects on T cell function, unlike the other cytokines discussed in this section they are considered anti-inflammatory (Minty et al 1997).

Mice overexpressing IL-4 show reduced bone formation (Lewis et al 1993) with development of a kyphosis and showing reductions in cortical thickness, trabecular mass and bone strength. Reductions in ALP, osteocalcin and OB number were seen; as were reductions in tartrate resistant acid phosphatase (TRAP, a marker of OC activity). Cortical bone mass is also reduced by a lack of IL-4 (Silfversward et al 2007). Compared to wild type mice, IL-13 knockout mice had normal bone structure whilst male IL-4/IL-13 knockout mice did not.

In a single cell culture system IL-13 can potentially effect OBs, but is less potent than IL-4 (Frost et al 2001). Both reduced OB proliferation whilst increasing ALP activity, IL-4 doing so at much lower doses. They also increased expression of IL-6. Other studies have shown that IL-4 suppresses the formation of OCs from monocytes incubated with M-CSF and RANKL, and OC activity with reduced resorption and inhibition of TRAP expression (Moreno et al 2003; Mangashetti et al 2005). A direct effect of IL-13 on OCs

has not been shown but it can increase expression of OPG by endothelial cells, the conditioned media of which inhibited the functional activity of OCs as measured by an unusual fluorophore-releasing assay (Stein et al 2008).

Effect of cellular elements

The effect of soluble elements of the immune system on bone have been studied extensively, both in terms of bone formation and resorption. With regard to the effect of cellular elements of the immune system on BMD, interactions between lymphocytes and osteoclasts have been well studied. There is an understandable focus on the RANKL system, as discussed earlier, but two specific studies show that the balance of regulatory and pro-inflammatory lymphocytes could influence bone metabolism.

Sato et al (2006) generated Th1, Th2, and Th17 and lymphocytes and included them in an assay for osteoclast differentiation and function. They found that whilst Th1 and Th2 cells inhibited osteoclastogenesis, Th17 cells promoted it by increasing the numbers of osteoclasts. They found that IL-17 was an important mediator of this effect, and that the method of producing osteoclasts (RANKL/MCSF or culture with osteoblasts) had some influence on the outcome.

Interestingly, whilst Sato et al (2006) found no effect of Treg cells on osteoclastogenesis Zaiss et al (2007) found they markedly reduced osteoclast formation. These differences may be due to differences in the methods used, but perhaps of note is that Sato identifies Tregs on CD25 expression alone whereas Zaiss confirms their phenotype based on expression of FOXP3. Therefore, Sato may have had a combination of regulatory and inflammatory lymphocytes in the population and not just Tregs.

These demonstrate the potential for lymphocyte populations to increase bone loss in inflammatory conditions and reduce it as the immune dysregulation is brought under control.

Conclusion

Patient's with IBD have changes in their bone metabolism, affecting the mineral density and possibly leading to a greater risk of fracture. Reduced BMD in IBD is likely to be multifactorial but there is convincing evidence that the chronic inflammation seen in IBD, and other autoimmune disorders, makes a significant contribution. Soluble

elements clearly play a role, and in adults there is an established molecular pathway (RANKL/RANK/OPG) mediating interaction between lymphocytes and osteoclasts leading to the increased bone resorption seen in older IBD patients.

This mechanism cannot be applied to children. Whilst they do have reductions in bone mineral density it is associated with reductions in bone formation rather than increased resorption. This probably reflects the processes of bone growth and development that are predominating, and that process is primarily mediated by osteoblasts. When this thesis began, although the effect of soluble elements had been studied, there was very little published research looking at the effects of immune cells on osteoblasts.

Mucosal immunity in health and IBD involves cells of the adaptive and innate immune system. Locally, $CD4^+$ lymphocytes are the main orchestrators in a variety of guises including T_{reg} and T_h17 phenotypes. Systemically, the trafficking of gut activated immune cells expressing the gut homing integrin $\alpha4\beta7$ potentially mediate the extra-intestinal effects of IBD. This thesis aims to investigate the potential for gut activated immune cells, focussing on $CD4^+$ lymphocytes, to have a negative impact on bone formation.

Hypothesis

The reductions in BMD seen in children with Crohn's disease are, at least in part, mediated by interactions between osteoblasts and activated lymphocytes that have migrated from gut mucosal tissue to the bone.

Aims

This study has two aims. The first is to investigate the effects of lymphocytes, and their secreted factors, on the osteoblast like osteosarcoma cell line Saos-2. The second is to look for associations between the relative proportions and activation status of lymphocytes, including those expressing the gut-activation marker $\alpha 4\beta 7$, in children with Crohn's disease, and changes in their BMD measured by DXA.

Objectives

- Isolate peripheral blood mononuclear cells from healthy volunteers
- Investigate the effects of these cells and their secreted factors on Saos2:
    - Proliferation
    - Alkaline phosphatase activity
- Recruit a cohort of paediatric patients undergoing endoscopy for clinical reasons and collect samples from patients from patients diagnosed with Crohn's disease and those with no identifiable bowel pathology
- Develop a method for the isolation of immune cells from paediatric endoscopic gut biopsy samples
- For these groups:
    - Collect peripheral blood and gut biopsy samples and measure relative proportions and activation status of lymphocytes in the gut and peripheral blood using flow cytometry
    - Collect data on the level of selected markers of bone turnover in peripheral blood and urine
    - Measure bone mineral density of patients with Crohn's Disease using dual-energy X-ray absorptiometry

2 Materials and Methods

The experimental work undertaken for this thesis consisted of two parts. A series of *in vitro* experiments studied specific interactions between immune cells and Saos-2 cells, and a comparative *in vivo* study compared peripheral blood immune cell phenotypes and bone metabolism in children with inflammatory bowel disease to those of healthy controls.

In vitro study of the influence of immune cells on the proliferation and alkaline phosphatase activity of Saos-2 cells

The aim of the *in vitro* experimental work described in this section is to investigate the effects of lymphocytes, and their secreted factors, on the proliferation and alkaline phosphatase activity of the osteoblast like osteosarcoma cell line Saos-2.

Saos-2 cells

The osteoblast-like osteosarcoma cell Saos-2 was used for these experiments (see Figure 2-1), and kindly provided by Professor Alison Gartland of The University of Sheffield's Mellanby Bone Centre. The establishment of the cell line was first described in 1977 (Fogh et al 1977). It was subsequently shown to have several osteoblast characteristics including high levels of alkaline phosphatase (ALP) and the ability to generate a calcified bone-like matrix (Rodan et al 1987). These features were of particular interest as reductions in serum alkaline phosphatase (Sylvester et al 2006) and bone mineral density (Harpavat et al 2005) have been found in children that were newly diagnosed with Crohn's disease.

Other osteosarcoma cell lines (TE85, U2OS and MG63) show very little alkaline phosphatase activity (Murray et al 1987; Pautke et al 2004). It is also important to note that Saos-2 cells show a more mature osteoblast phenotype than U2OS and MG63, consistently producing osteocalcin, bone sialoprotein, decorin, and procollagen-1 (Pautke et al 2004). These proteins are considered characteristic of osteoblasts.

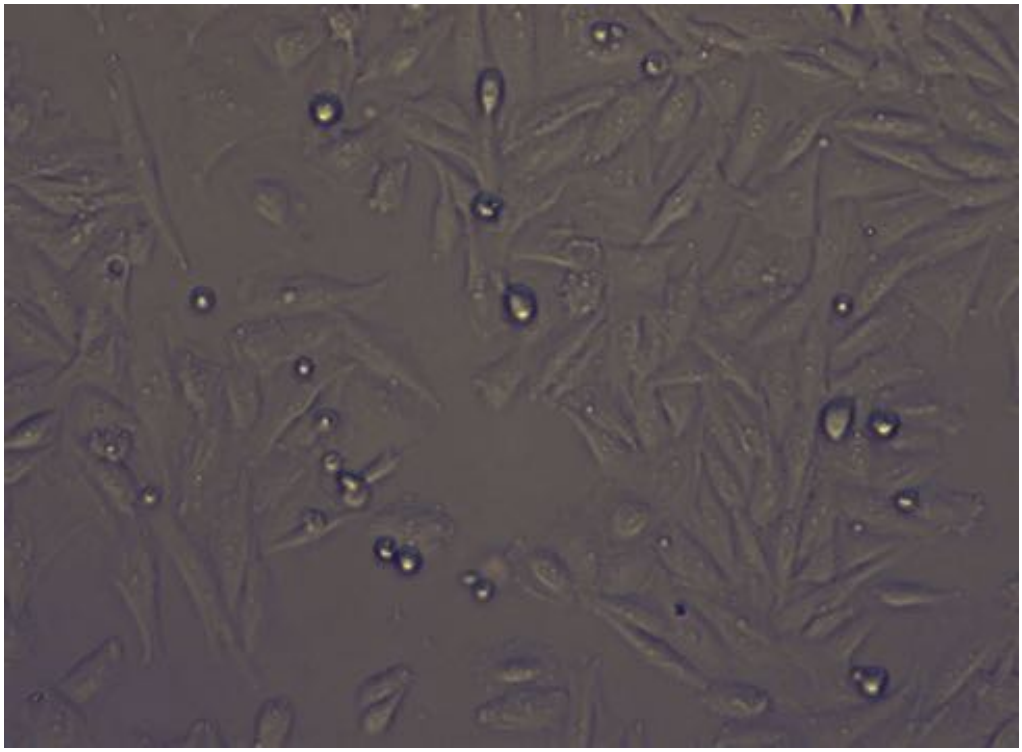

Figure 2-1: Light micrograph of Saos-2 cells (20x magnification).

Maintenance of Saos-2

Saos-2 cells were maintained in Nunc T75 flasks with Dulbecco's Modified Eagle Medium (D-MEM) containing GlutaMAX™, 4500mg/L D-Glucose and sodium pyruvate (GIBCO #31966-021). Medium was supplemented with 10% v/v foetal bovine serum (GIBCO #10270-106), 100units/ml penicillin and 100μg/ml streptomycin (BioWhittaker #DE-17-602E). This will subsequently be referred to as bone cell medium (BCM).

Cells were initially seeded at $1x10^6$ per flask and split once they reached 70-80% confluence, usually on day 3 or 4. To split cells, flasks were washed twice with phosphate buffered saline (PBS) to remove medium and incubated with 2.5 ml of trypsin/EDTA solution (GIBCO #25200-056) for 5 minutes at 37°C. Having ensured that all cells were detached from the base of the flask, 10 ml of BCM was added to stop the enzyme activity. The cell suspension was transferred to a 25 ml Universal container, and after centrifugation at 300g for 5 minutes, the supernatant was discarded and the cells re-suspended in 1 ml of BCM. Viable cells were then counted on the basis of trypan blue dye exclusion (0.4% w/v, Sigma #T8154) using a haemocytometer.

Peripheral blood mononuclear cells, lymphocytes and their isolation

Whole blood comprises erythrocytes, leucocytes and plasma. The cellular components of blood derive from multi-potential hematopoietic stem cells (see Figure 2-2) that, in adult humans, reside in the bone marrow. During haematopoiesis these stem cells develop into erythrocytes (predominantly red blood cells), myelocytes and lymphocytes (leucocytes or white blood cells). Leucocytes consist of two populations: granulocytes and mononuclear leucocytes. Granulocytes are polymorphonuclear cells with stainable cytoplasmic granules. They include neutrophils, basophils and eosinophils; all of which derive from a myeloid progenitor. Mononuclear leucocytes have a single nucleus and lack the stainable granules of the polymorphonuclear cells. This population includes lymphocytes and lymphoid dendritic cells (derived from a lymphoid progenitor), but also monocytes and myeloid-dendritic cells (derived from a myeloid progenitor).

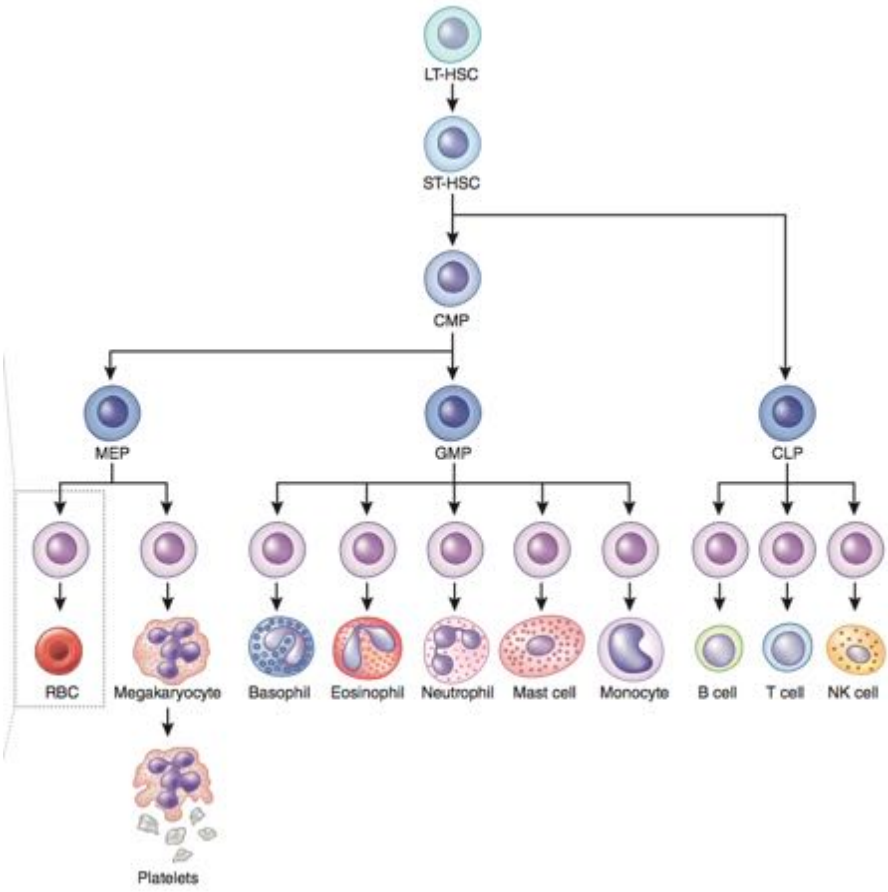

Figure 2-2: The haematopoietic cell line. This diagram shows the development of the various blood cell types from a common, multipotential hematopoietic stem cell. HSC – haematopoietic stem cell, LT – long term, ST – short term, CLP – common lymphoid progenitor, CMP – common myeloid progenitor, MEP – megakaryocyte-erythroid progenitor, GMP – granulocyte-macrophage progenitor. Reproduced with permission (Sankaran et al 2015).

Lymphocytes consist of three subtypes of white blood cell: natural killer (NK) cells, T cells and B cells. T and B cells are the main components of the adaptive immune system recognising non-self-antigens, co-ordinating the immune response and maintaining memory of previously-encountered antigens. B cells mature in the bone marrow and produce antibody in response to foreign antigen. T cells mature in the thymus providing adaptive, cell-mediated immunity. They are an essential part of the immune system; however, if they become reactive to self-antigen autoimmune disease such as inflammatory bowel disease develops. Lymphocytes, like any cell, express proteins on their cell surface which allow them to carry out their functional roles. The use of fluorescently labelled antibodies to specific protein structures enables identification of cell type, and provide information as to their functional status (see later section).

Mononuclear cells can be isolated from whole blood by centrifugation on density gradient media (see Figure 2-3). This approach was first described using a combination of sodium metrizoate and a polysaccharide (Boyum 1968) which is produced commercially as Lymphoprep™. However, this process had been found to alter the relative proportions of lymphocytes, including reductions in the number $CD8^+$ cells (Renzi et al 1987), possibly due to the aggregation of erythrocytes. There was also a concern that the ionic charge on the metrizoate could be toxic to cells (Axis Shield News Bulletin). Therefore, an alternative preparation using isohexol (Nycodenz™), lacking the ionic charge, was introduced in 1982 (Axis Shield News Bulletin). A revised protocol, also by Boyum, used this modified form and was published later (Rickwood 1983).

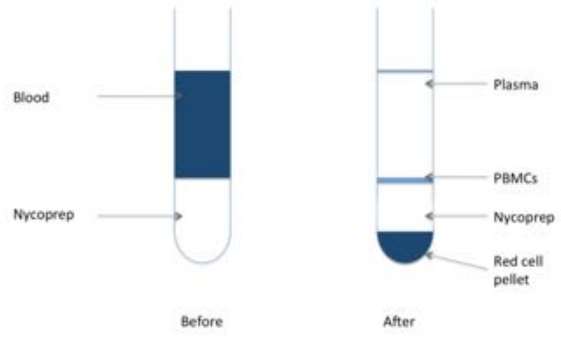

Figure 2-3: Separation of peripheral blood mononuclear cells (PBMCs) using density gradient centrifugation.

The isolation of specific lymphocytes subsets (such as $CD4^+$ T cells) can be achieved by incubating a cell suspension with an antibody to a specific cell-surface protein which is conjugated to either a fluorescent flurophore or a metal oxide micro-bead. Where a flurophore has been used, cells can be separated using a flow cytometer (see later description). If metal-oxide beads are used then cells can be isolated using a magnet (Tomlinson et al 2013). Whilst the former enables more complex cell identification, the use of micro-beads can be cheaper and faster.

Micro-beads and magnets can be used in two ways (see Figure 2-4). For the first, labelled cells pass through a column containing metallic beads within a magnetic field. The labelled cells adhere to these metallic beads, and the unlabelled pass through. For the second, a magnetically-labelled cell suspension is placed in a conical tube. When placed in a magnet, the magnetically labelled cells "attach" to the tube edges, and remain in the tube when it is inverted.

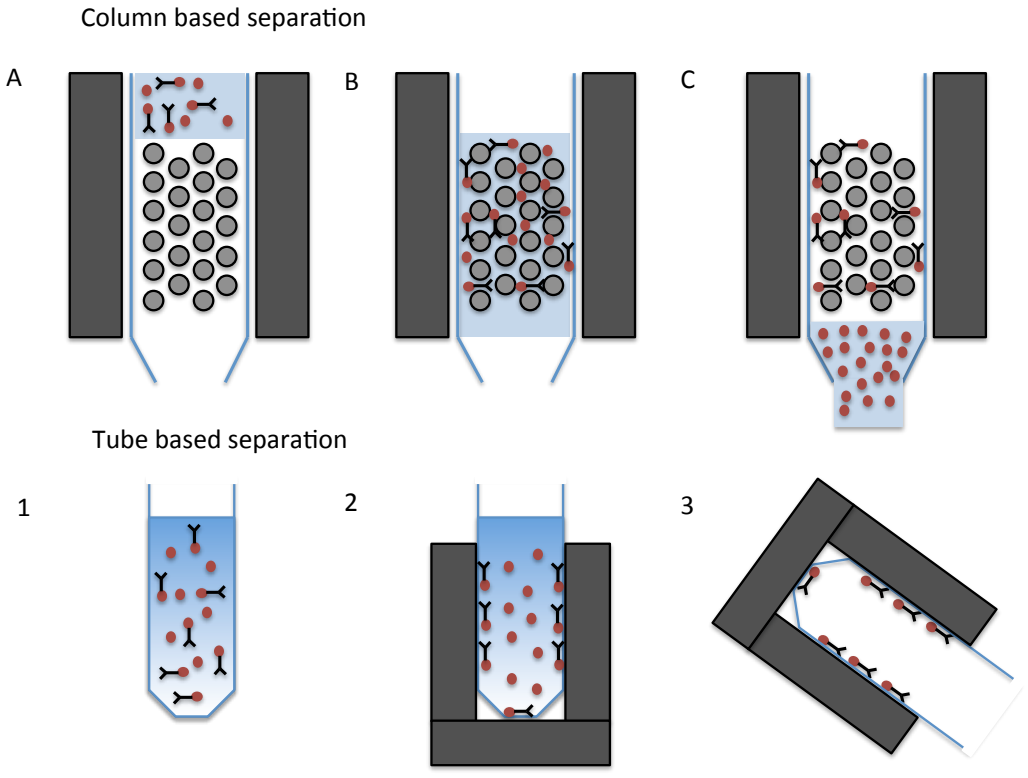

Figure 2-4: Two methods of magnet based cell isolation. In both methods cells of interest are magnetically labelled with antibody. In column based separation the suspension passes through a column of larger beads within a magnetic field, and magnetically labelled cells are retained within the column. In tube based separation the cell suspension is placed within a magnet and magnetically labelled cells remain in the tube when the tube is inverted.

The outcome of both these methods is the same in that labelled cells are removed. However, the cell surface proteins to which the micro-beads bind will usually have a functional role, and the presence of the micro-bead may affect its function and/or future labelling. This makes the labelled cells unlikely to be of use in experiments. It also means the cell markers that are labelled depends on whether the aim is to deplete or enrich a specific population e.g. $CD4^+$ cells. Positive selection can be used to isolate a specific population. The micro-beads are bound by antibody to $CD4^+$ cells, and after passing through the magnet only non-$CD4^+$ cells will remain. Negative selection can be used to enrich a specific cell population. The micro-beads bind to cell surface markers that are not expressed on $CD4^+$ cells (CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and CD235a), and after passing through the magnet only $CD4^+$ cells will remain.

Isolation of peripheral blood mononuclear cells from whole blood

Ethical approval to obtain whole blood from healthy volunteers was given by the University of Sheffield Research Ethics Committee (SMBRER116; related documents in appendix). All participants gave informed consent. PBMCs were isolated from blood by density gradient centrifugation. Peripheral blood was collected into EDTA anticoagulant (BD Biosciences Vacutainer™ tubes, BD Biosciences, Oxford, UK), transferred to a 25 ml Universal container and diluted 1:1 with 0.9% v/v NaCl (Sigma-Aldrich #S57653). The diluted blood was then layered onto Nycoprep™ 1077 (Axis-Shield #1114550) at a ratio of 2:1 (blood: Nycoprep™) ensuring that a clear blood/ Nycoprep™ interface was maintained. Samples were then centrifuged at 400g for 30 min at room temperature, with no brake. This resulted in separation of the blood constituents, as outlined in Figure 2-3.

The mononuclear cell layer at the interface was harvested, transferred into a second container, diluted 1:1 with sterile phosphate buffered saline (PBS; Oxoid #BR0014G) and centrifuged at 600g for 10 minutes. The supernatant was discarded and the cell pellet was washed twice with RPMI growth medium (BioWhittaker #BE12-115F) supplemented with 10% v/v foetal calf serum (Sigma #M5905), 100 units/ml penicillin and 100μg/ml streptomycin (BioWhittaker #DE-17-602E). Viable cells were then counted on the basis of trypan blue dye exclusion (0.4% w/v, Sigma #T8154) using a haemocytometer.

Isolation of lymphocytes subsets from a PBMC population

Lymphocyte subsets were isolated using magnetic cell sorting. For the initial experiments, a negative selection $CD4^+$ T Cell Isolation Kit (Miltenyi Biotech, Bisley, UK) was used following their recommended protocol. All volumes discussed are per 10^7 cells. Aliquots of the PBMC cell suspension were centrifuged at 300g for 5 minutes and the supernatant removed. The cell pellet was re-suspended in 40 µL of PBS with 2% v : v mouse serum (staining buffer, SB), 10µL of Biotin-Antibody cocktail was added and after mixing well was incubated for 10 minutes at 4-8°C. A further 30µL of buffer was added followed by 20µL of Anti-Biotin Microbeads, the suspension mixed well and incubated at 4-8°C for 15 minutes. Cells were again washed in SB at 10-20 times the labelling volume, centrifuged at 300g for 10 minutes and the supernatant aspirated. Viable cells were counted on the basis of trypan blue dye exclusion and were re-suspended in buffer at 500µL per 10^8 cells. A MACS Column was placed in the magnetic field of a MACS Separator and the column rinsed with ice-cold buffer. The cell suspension was then applied to the column and allowed to pass through collecting the effluent. Three volumes of buffer were subsequently applied to ensure optimal efficiency of cell isolation. The cell suspension was washed twice with PBS, the cells re-suspended in RPMI and viable cells were counted on the basis of trypan blue dye exclusion using a haemocytometer.

For the second set of experiments, EasySep™ cell enrichment and isolation kits were used following the manufacturers recommended protocol (StemCell Technologies, Manchester, UK). A $CD4^+$ enriched cell population was generated using a negative selection kit (#19052) and a $CD4^+$ *depleted* cell population was generated using a $CD4^+$ T cell positive selection kit that would bind and extract $CD4^+$ cells (#18052), allowing other cell types to pass through. PBMCs were suspended in Robosep™ buffer at a concentration of 10^8 cells/ml and the appropriate antibody cocktail added. After incubation for 15 minutes at room temperature, magnetic particles which bind to the antibodies were added, followed by a further period of incubation. The cell suspension was gently pipetted into round bottom polystyrene tubes and placed directly into a magnet field. After 5 minutes, the suspension was poured off, and any cells bound to magnetic particles remained within the tube, leaving a suspension that was either enriched or depleted for $CD4^+$ T cells.

Influence of supernatants from activated immune cells on the growth of Saos-2 cells

Activation of lymphocytes

In vivo activation of T cells occurs when antigenic peptides are presented on the major histocompatibility complex (MHC, or human leucocyte antigen, HLA, in humans) of antigen presenting cells (APCs) to appropriately specific receptors on responding T cells. A signal is transduced via the T Cell Receptor (TCR) complex which includes the CD3 protein (Roitt 1996). Full T cell activation requires the concomitant delivery of essential co-stimulatory signals from the APC, in the absence of which T cells can become non-responsive (anergic).

In vitro stimulation of T cells can be achieved in several different ways. T cells can be activated using the lectin protein concanavalin-A (ConA), which activates T cells by binding mannose on the cell surface. The cross-linking of surface carbohydrates induces polyclonal activation in a manner which is antigen and MHC/HLA-independent (Palacios 1982). The binding of specific mAbs to the CD3 component of the TCR, concomitant with binding to CD28 and the consequential delivery of essential co-stimulatory signals, is sufficient to activate T cells (Levine et al 1997). This approach could be considered as being more physiological than ConA co-stimulation. ConA also requires the presence of monocytes to be effective (Hedfors et al 1975), and if a T cell population is purified (for example a $CD4^+$ rich population), ConA will not activate cells (Hedfors et al 1975; Palacios 1982).

Generation of activated immune cell supernatants

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers, from which $CD4^+$ lymphocytes were subsequently isolated using the Miltenyi $CD4^+$ T Cell negative isolation kit. Immune cell populations were centrifuged at 300g for 5 minutes, the supernatant discarded and the cell pellet re-suspended in BCM at $1x10^6$ cells/ml. 1ml of the cell suspension was transferred to wells of a 24 well culture plate (Corning #CLS3524) and $1x10^6$ Miltenyi T_{reg} Suppression Inspector Beads (Miltenyi #130-092-909 subsequently referred to as stimulatory beads) added. These beads are coated with mAbs to CD3 and CD28 and their interaction with T cell populations mimics antigen-induced activation processes. Plates were incubated for 4 days at 37°C with 5% v/v CO_2, at which point the medium was removed and the centrifuged to remove

cells. The supernatant containing secreted factors from the activated PBMCs was stored at -80°C for future use.

Culture of Saos-2 cells in supernatants from activated cells

Saos-2 cells (10^5 per well) were incubated for 5 days with supernatants from activated PBMC populations (50%, 25%, 12.5%, and 6.25% v/v with BCM) in a total volume of 500μl per well (BCM alone as control), after which the medium was removed and stored at -80°C for later cytokine analysis. Cell numbers were assessed for each of the 5 days. For this, 200μl of 0.25% trypsin with EDTA (Invitrogen #25200-056) was added to each well and incubated for 5 min at 37°C. BCM (300μl) was added to each well and pipetted vigorously to ensure all cells were removed. Cells were counted using a Beckman Coulter Vi-CELL™ automated cell counter.

Analysis of cell phenotype, functional status and enumeration using flow cytometry

Flow cytometry was used to assess the phenotypic and functional status of immune cells in the *in vitro* and clinical studies. Additional techniques, relying on the ability of flow cytometry to accurately identify cell types, enabled counting of cells that could not be separated following co-culture.

How a flow cytometer works

A typical flow cytometer uses hydrodynamic focusing to organise particle suspensions into a single stream. It does this by enclosing the central channel, through which the sample is injected, in an outer sheath of faster flowing fluid (see Figure 2-5). When the faster fluid (sheath fluid) flows past the end of the central channel, drag focuses the particle suspension into a single stream within the sheath fluid (Spielman et al 1968; Shuler et al 1972).

Each particle then passes through one or more beams of laser light of different wavelengths. As light is incident upon the cell, it is diffused and measurement of the light at different angles reflects cell characteristics. Light at around a 20° to that of the light source is called forward scatter (FSC), and its intensity reflects the cell size. At 90°, called side scatter (SSC), the light profile reflects granularity or density. The data from each cell (events) can then be plotted on a dot plot of SSC against FSC (see Figure 2-6).

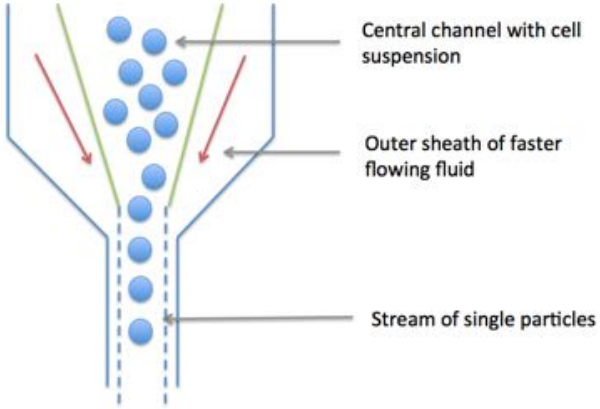

Figure 2-5: Schematic demonstrating hydrodynamic focussing. Particles moving within an inner sheath (shown in green) inside an outer sheath (shown in blue) of faster moving fluid. Where the two fluid streams meet, the particles are focused into a single stream. This enables analysis of individual cells.

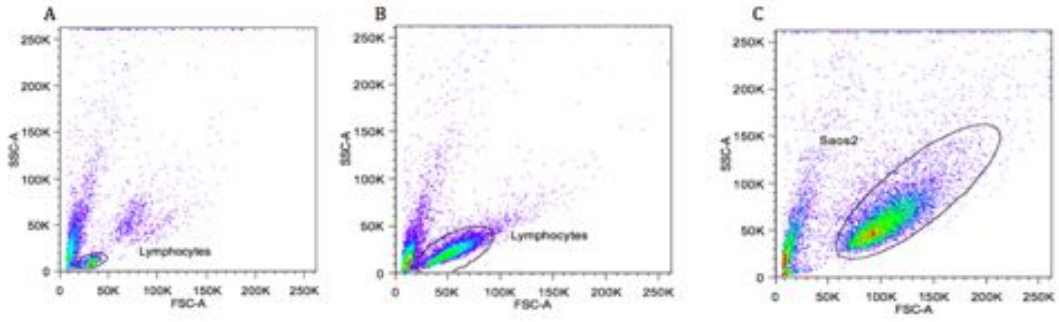

Figure 2-6: Representative flow cytometry scatter dot plots of immune cells and Saos-2 cells. **Panel A** - resting lymphocytes are the small and comparatively agranular. **Panel B** - when activated, lymphocytes enlarge and become more granular. **Panel C** - although Saos-2 cells are larger and more granular than resting lymphocytes, there is some overlap with activated lymphocytes.

When fluorescent molecules (fluorophores) are exposed to light of the appropriate wavelength, energy is absorbed and the molecule is excited. The energy of the excited electrons decays and is re-emitted as light at a different wavelength. The flow cytometer can control the frequency of light which it delivers to cells that are either directly or indirectly labelled with fluorescent molecules using a panel of defined wavelength lasers and this allows cell features and / or the expression of antigens to be determined. Fluorescent molecules are conjugated to monoclonal antibodies (mAbs) that recognise cell-associated antigens of interest (see Figure 2-7).

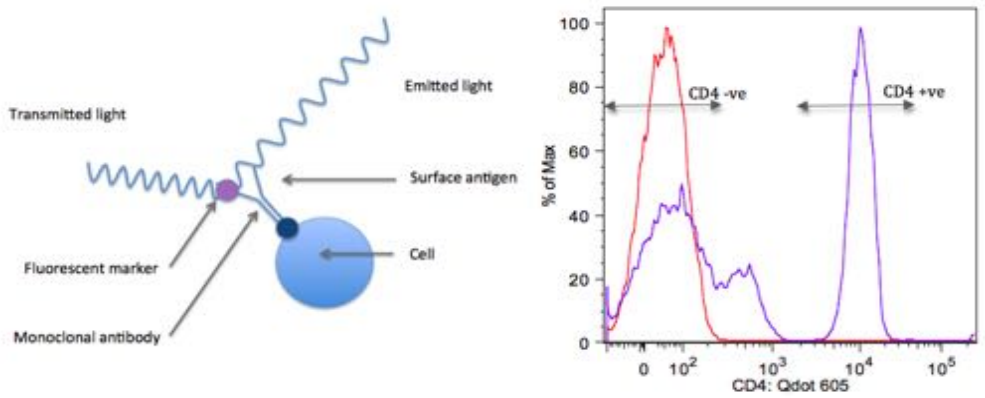

Figure 2-7: Fluorescent labelling of cell surface molecules for analysis by flow cytometry. The diagram on the left shows a cell bearing a surface molecule, in this case CD4. Cells have been incubated with a mAb to which the fluorescent molecule Qdot 605 has been conjugated. Cells which do not express CD4 emit a signal in the region indicated by the red peak on the histogram plot. Cells expressing $CD4^+$ would re-emit the light which would be detected and shown by the blue peak to the far right of the histogram.

Lymphocyte surface markers

PBMC preparations contain sub-populations of cells that express a variety of molecules on their surface. Most of these have a role in enabling specific functions of that cell, but they can also be used to identify phenotypic and functional aspects of the cell. The Cluster of Differentiation or Cluster Designation (CD) system attributes specific numbers to internationally-agreed target molecules (Roitt et al 1996).

CD4 identifies T helper cells that either positively or negatively influence the immune response via interactions with other immune cells. CD8 identifies cytotoxic T cells that can kill virally infected cells (Bierer et al 1988; Roitt et al 1996). These molecules interact with MHC class II and MHC class I molecules that are expressed on the surface of APCs and thereby regulate and target the antigen presentation process to individual T cell sub-populations. CD4 interacts with MHC class II, predominantly found on antigen presenting cells (APCs). CD8 interacts with MHC class I which is expressed on all nucleated cells in the body.

CD19 is expressed on all B cells and some types of DC. It is a member of the immunoglobulin superfamily and forms a transmembrane signalling complex with CD21, CD81 and CD225 and acts as a response regulator for B cell development, activation and differentiation (Tedder et al 1994; Roitt et al 1996). B cells produce antigen specific immunoglobulins (Ig), this being increased when exposed to the appropriate antigen. This can be mediated by direct binding of antigen to the Ig in the

membrane, although the presentation of the antigen on MHC class II to $CD4^+$ cells is a far greater stimulus.

CD45, also known as the leucocyte common antigen, is present on all cells of the haematopoietic cell line (Thomas 1989). It is used to identify immune cells in experiments where they are co-cultured with Saos-2.

CD69 and CD25 are early and late markers of lymphocyte activation respectively. CD25 (the alpha chain of the IL-2 receptor) is also constitutively expressed (at high levels) on a resting population of $CD4^+$ cells that have become known as $CD4^+CD25^{high}$ immunoregulatory T cells (T_{reg} cells). IL-2 plays a central role in the clonal expansion of activated lymphocytes, and binds to the IL-2 receptor (IL-2R) (Minami et al 1993). IL-2R has three chains with the α chain named CD25. CD69 has a role in the early stages of T cell and B cell activation and is one of the earliest antigens expressed after activation by several cell types (Cosulich et al 1987; Testi et al 1989).

Characterisation and identification of cells by flow cytometry

Viability

Antibodies to specific cell markers can non-specifically bind to dead cells. Exclusion of dead cells from the analysis is therefore important when measuring cell populations by flow cytometry. Of course, analysing dead cells is of value when determining potentially cytotoxic responses. Identifying dead cells can be achieved using several approaches, one of which involves amine reactive viability dye (ViD). The dye can penetrate damaged cell membranes and react with amine groups in the cell cytoplasm forming a stable, fluorescent product. They do react with surface amine groups, but the total binding capacity is minimal relative to that of the cytoplasm. For these experiments, a dye excited by blue fluorescence with an ultraviolet emission spectrum was used (LIVE/DEAD® Invitrogen #L23105).

Their effectiveness in identifying dead cells has been demonstrated (Perfetto et al 2006), but how it identifies dead cells was investigated. PBMCs were incubated in increasing concentrations of hydrogen peroxide for 30 minutes, after which they were washed twice in phosphate buffered saline (PBS). Cells were incubated with the ViD for 30 minutes before being washed again with PBS. Cells incubated with propidium iodide (PI) and Annexin V were used as a comparison. PI identified dead cells by penetrating the cells membrane and binding to its DNA. Annexin V identifies apoptotic

cells by binding to phosphotidylserine, expressed on the surface of cells undergoing this process.

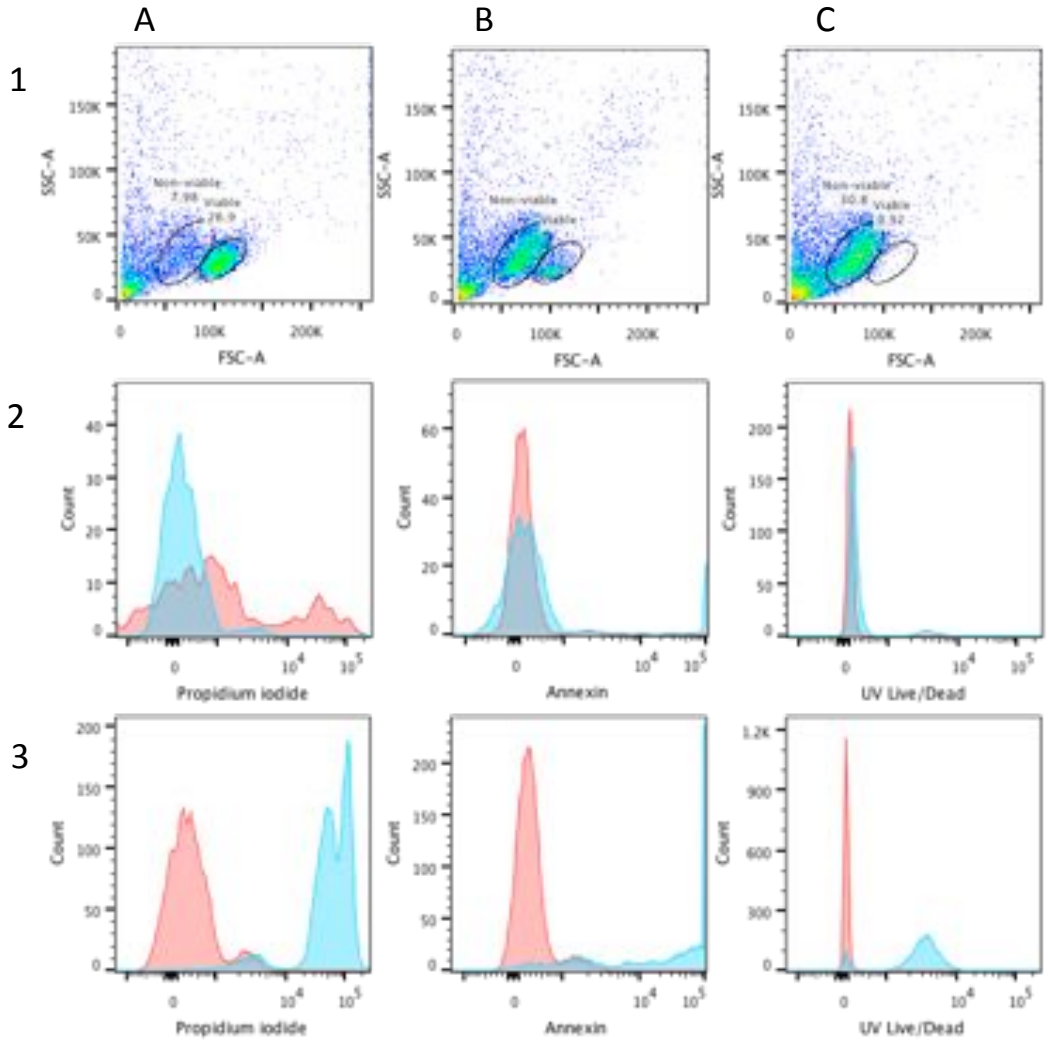

Figure 2-8: Representative histograms of viability staining with propidium iodide, Annexin and and amine reactive dye. **Row 1** – scatter plots of FSC and SSC for cells incubated in increasing concentrations of hydrogen peroxide, with the lowest concentration in A and the highest in C. **Row 2** - histograms of the viable group shown in 1B stained with differing dyes **Row 3** – histograms of the non-viable group shown in 1B stained with differing dyes. On histograms unstained controls are red, stained samples blue.

Figure 2-8 summarises the findings. Row 1 shows that as cells become non-viable their size and granularity change, leading to a change in position on the scatter plot. Row 2 shows the “viable” population in panel 1B, with the three dyes confirming they are mostly viable cells. Row 3 shows the “non-viable” population in 1B, with the three dyes confirming the lack of viable cells. This suggests that the FSC/SSC does identify a significant proportion of non-viable cells. In addition, when staining for flow cytometry, isotype controls will detect some non-specific binding.

Fluorescent labelling of cell surface markers

Fluorescent labelling of cell surface markers enables the identification of specific cell populations (e.g. $CD4^+$ lymphocytes) and their functional status (e.g. expression of CD25 reflecting activation status). The cell surface marker of interest and its pattern of fluorescence influence how the output is interpreted and reported.

It is possible for antibodies to non-specifically bind to cells and result in 'false positives'. This possibility is monitored by including non-specific, isotype-binding controls, in all staining procedures. These controls are immunoglobulins of the same species that are labelled with the same fluorochrome, but which have been demonstrated not to bind to any antigens on the target cell populations. These could be mAbs having specificity to haptens (small irrelevant molecules) or antigens that are expressed by another species. More recently, the concept of fluorescence minus one (FMO) controls which replace the need for isotype controls for multiparameter staining panels has superseded the use of isotype controls.

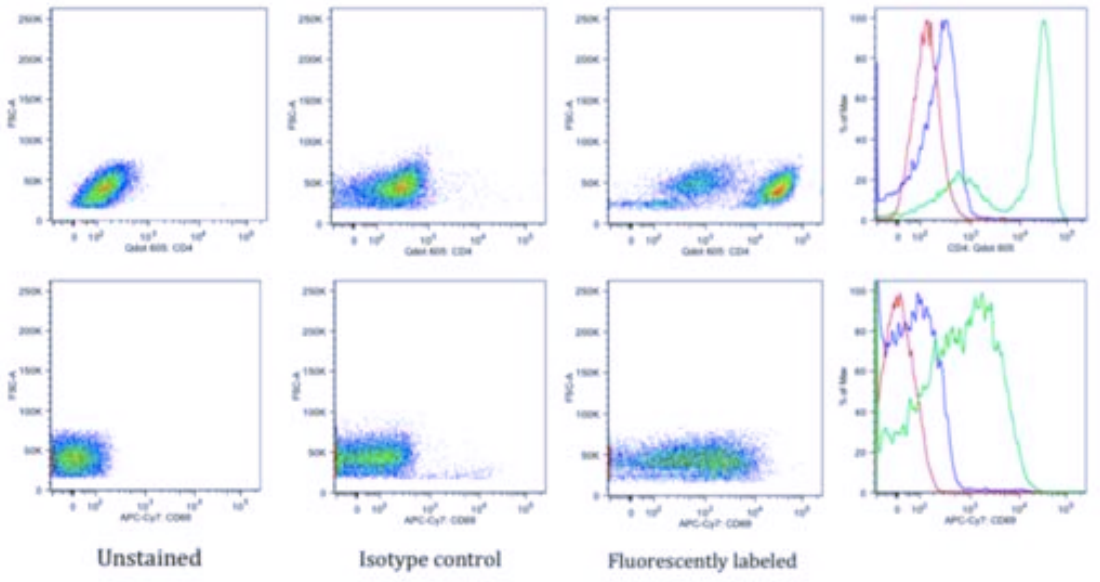

Figure 2-9: Representative examples of staining for CD4 and CD69 expression. The scatter plots (with FSC-A on the Y axis) from left to right compare the appearance of the unstained, isotype control and stained cells. At the far right the same data are presented as overlaid histograms: red (unstained), blue (isotype control) and green (stained).

Figure 2-9 compares the fluorescence produced by $CD4^+$ cells, and expression of CD69. When fluorescently labelled the phenotypic marker CD4 produces a distinct population. Cells are considered simply as being either CD4 positive or $CD4^+$ negative, and this is also true for CD8 and CD19. For the activation marker CD69 no distinct population is produced, but there is an increase in fluorescence from the stained cells.

A similar pattern is seen for CD25. Both CD25 and CD69 can be measured by either the proportion of cells that are positive (as a percentage) and the *intensity* of expression. Both are important parameters providing insight into the amount of a given antigen that is expressed and, in the case of activation antigens, the degree of activation.

Identification of phenotypic markers using fluorescent labelling does not always produce a distinct population. An example of this is the integrin $\alpha 4\beta 7$, which was used to identify immune cells that have been activated within the gut mucosa, and which was used for the *in vivo* study. Identification of $\alpha 4\beta 7^{+}$ cells is described later.

Enumeration of cell populations

A flow cytometer can record the total number of events / cells / particles that pass through the laser beam. This information alone cannot be used to calculate the absolute number of cells in a sample because the total volume of the suspension analysed is not recorded by the instrument which was used in the current study, and so it is only possible to calculate the relative number of cells in each population. However, an absolute cell number can be determined by ensuring the sample volume is accurate and adding a known number of microbeads such as Countbright™ Absolute Counting Beads (Invitrogen #C36950). When analysed, the volume of suspension, and hence the number of cell events, can be calculated using the following formula.

$$\frac{A}{B} \times \frac{C}{D} = \text{Concentration of sample as cells/}\mu\text{l}$$

Where

A = number of cell events

B = number of bead events

C = assigned bead count of the lot (beads/50μl)

D = volume of sample (μl)

Co-culture of immune cells with Saos-2 cells

Series 1 – influence of immune cells on the growth of Saos-2 cells

The primary aim of these experiments was to investigate the effect of activated immune cells on the growth of Saos-2 cells. Four independent variables were included: immune cell type (PBMCs or $CD4^+$ lymphocytes), immune cell number relative to the number of Saos-2, activation status and contact status. The dependent variables were Saos-2 number and changes in activations status of immune cells (based on the expression of CD25 and CD69).

Experimental setup

100,000 immune cells	200,000 immune cells	400,000 immune cells	800,000 immune cells
100,000 immune cells Polyclonal beads	200,000 immune cells Polyclonal beads	400,000 immune cells Polyclonal beads	800,000 immune cells Polyclonal beads
100,000 immune cells Transwell insert	200,000 immune cells Transwell insert	400,000 immune cells Transwell insert	800,000 immune cells Transwell insert
100,000 immune cells Polyclonal beads Transwell insert	200,000 immune cells Polyclonal beads Transwell insert	400,000 immune cells Polyclonal beads Transwell insert	800,000 immune cells Polyclonal beads Transwell insert

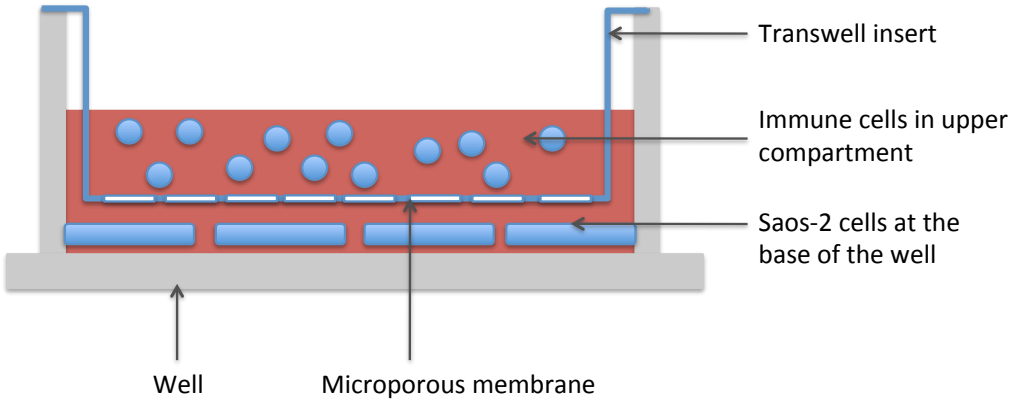

Figure 2-10: Set up for co-culture experiments. **Upper Panel** - grid shows the layout of the variables within the experiment. All wells contained 10^5 Saos-2 cells. **Lower Panel** - representative well including Saos-2, lymphocytes and the transwell insert.

Saos-2 cells (10^5 cells in 500µl BCM) were added to wells of a 24-well culture plate (Corning #CLS3470). Resting and polyclonally activated immune cells were added to the wells at ratios of 1, 2, 4 and 8 immune cells : 1 Saos-2 cell. To establish the effects of immune cell activation on the growth of Saos-2 cells, immune cells were activated using Miltenyi T_{reg} Suppression Inspector Beads (Miltenyi #130-092-909 subsequently

referred to as stimulatory beads) at an immune cell:bead ratio of 1:1. These beads are coated with mAbs to CD3 and CD28 and their interaction with T cell populations mimics antigen-induced activation processes. To establish the effects of cell-cell contact and secreted factors on the observed effects, 0.4μm pore polyester transwell membrane inserts (Corning #CLS3470) were used. These provide a physical separation between the Saos-2 growing on the base of the well and immune cells within the insert, whilst allowing the passage of secreted factors such as cytokines. The details of this are provided in Figure 2-10. Control wells with Saos-2 alone, immune cells alone and immune cells with stimulatory beads were included. For these experiments, cultures were incubated for 4 days at 37°C with 5% v/v CO_2.

Collection of cells from the culture wells

Medium was collected from the wells and the transwell inserts at the end of the experimental period and centrifuged at 300g for 5 minutes. Supernatants were collected and stored at -80°C, and the cell pellet was re-suspended in BCM.

Saos-2 cells were harvested by incubating each well with 200μl of 0.25% v/v trypsin with EDTA (Invitrogen #25200-056) for 5 min at 37°C. Medium (300μl) was added and vigorously pipetted to ensure the Saos-2 were completely removed from the base of the well. The removal of cells was confirmed visually using a standard light microscope. Following centrifugation, cell pellets were combined with the cells isolated from the medium of the corresponding well and washed twice in BCM.

Cells in the Saos-2 and immune cell control wells were counted to give an estimate of maximum cell numbers in the co-culture wells for fluorescent staining. Cells were re-suspended in PBS with 10% v/v FBS and 2% v/v mouse serum at a concentration of $1x10^6$ cells/100μl in preparation for fluorescent staining.

Preparation of samples for flow cytometry analysis

After incubating at room temperature for 15 min in staining buffer with mouse serum, samples were separated into aliquots of 250,000 cells to provide unstained, isotype and stained samples for comparison. Details of the fluorescently-conjugated mAbs used are shown in Table 2-1. Appropriate isotype controls were included in every experiment.

Fluorphore	mAB	Clone	Concentration (1x106 cells/100µl)	Catalogue number
Qdot 605™	anti-CD4	S3.5	1µl	Invitrogen #Q10008
Qdot 705™	anti-CD8	3B5	1µl	Invitrogen #Q100509
Alexa Fluor™	anti-CD19	HIB19	20µl	Biolegend #302226
APC-Cy7™	anti-CD69	FN50	0.5µg	Biolegend #310914
PE-Cy7™	anti-CD25	HI30	0.5µg	Ebioscience #25-0259-42
Pacific Blue™	anti-CD45	BC96	0.25µg	Biolegend #304022
Ultraviolet	LIVE/DEAD®	N/A	1µl	Invitrogen L23105

Table 2-1: Fluorescent antibodies used in the co-culture experiments.

Samples were incubated with mAbs for 30 minutes at 4°C, after which samples were washed once in staining buffer. Unstained and isotype samples were re-suspended in 200µl of staining buffer, and stained samples were re-suspended in 350µl of staining buffer. Samples were kept in the dark on ice and analysed by flow cytometry (BD Biosciences LSRII). Countbright™ Absolute Counting Beads (50µl), Invitrogen #C36950) were added to stained samples immediately prior to analysis. Data were acquired using BD Biosciences FACSDiva™ software and analysed using FlowJo™ analysis software (v8.8.6, Tree Star).

The gating strategy is shown in Figure 2-11. Row 1 shows identification of viable cells. 1A is unstained, 1B is stained and includes the counting beads. In 1C the population "cells" are confirmed as viable. In 1D the population "non-viable" shows a proportion of those cells have taken up the LIVE/DEAD stain. Row 2 shows the identification of haematopoietic cells expressing CD45. In 2A, a population of cells consisting of both immune cells and Saos-2 are identified. 2B shows that population after staining with the isotype control, and 2C shows that same population after staining with a fluorescently labelled antibody to CD45. A distinct population of CD45+ cells can be seen. 2D shows the $CD45^+$ cells identified on the original scatter plot and identified as lymphocytes. 3A-D shows the identification of the lymphocyte subsets $CD4^+$ and $CD8^+$, with isotype and stained scatter plots. 4A and B shows similar scatter plots for $CD19^+$. 4C and D are histograms identifying cells expressing CD25 and CD69.

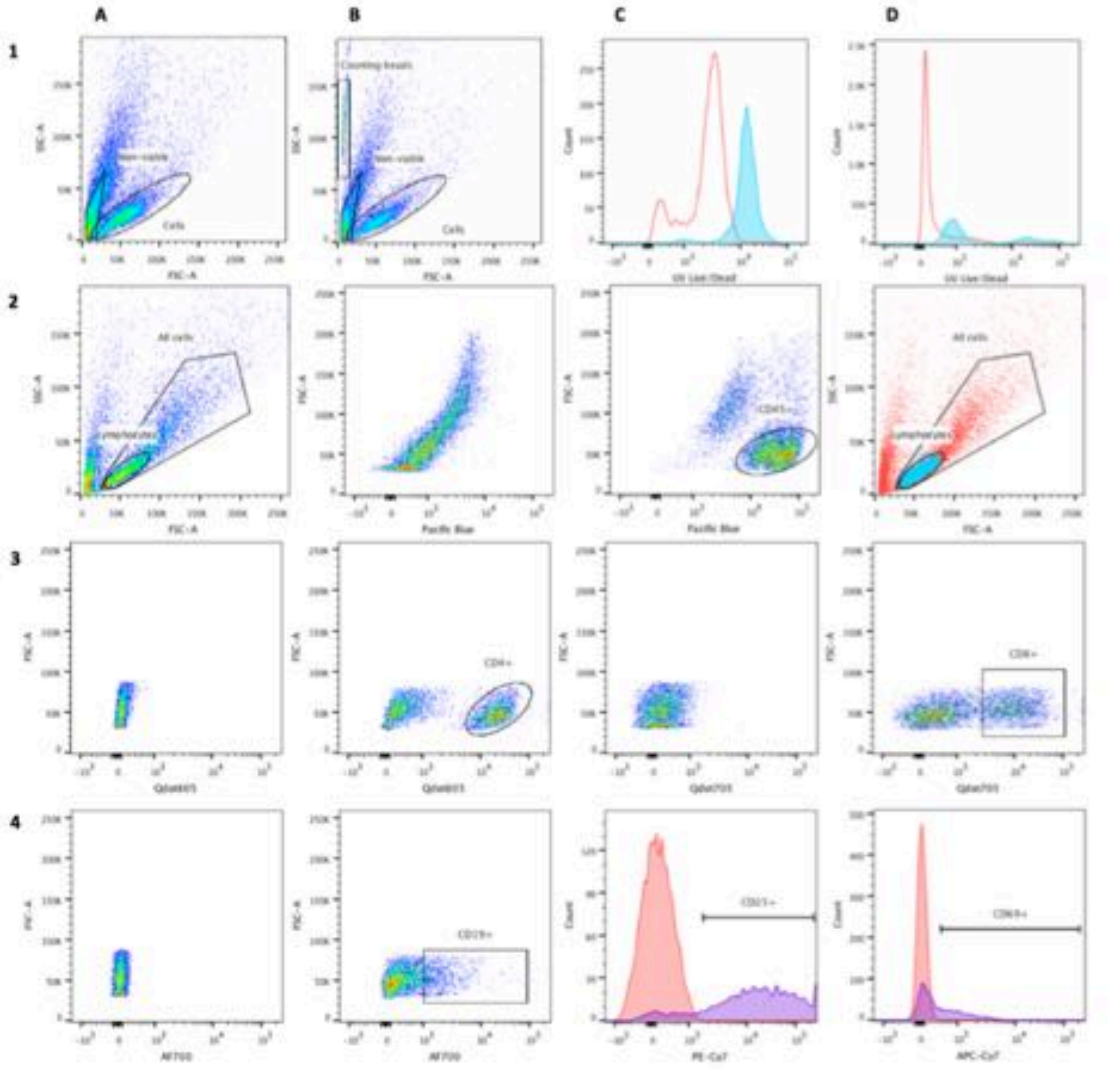

Figure 2-11: Representative flow cytometry plots demonstrating the gating strategy for the co-culture experiments. **Row 1** – identification of viable cells using UV LIVE/DEAD: A Unstained B stained with counting beads present C histogram of cells of interest (red line unstained, blue fill stained) D histogram of presumed non-viable cells (red line unstained, blue fill stained) **Row 2** – identification of haematopoietic cells A scatter plot B "All cells" unstained C "All cells" stained for CD45 D $CD45^+$ cells shown on the scatter plot **Row 3/4** – identification of lymphocyte subsets and their expression of activation markers 3A/B Isotype control and stained for $CD4^+$ 3C/D Isotype control and stained for $CD8^+$ 4A/B Isotype control and stained for $CD19^+$ 4C histogram of CD25 expression (red isotype control, purple stained sample) 4D histogram of CD25 expression (red isotype control, purple stained sample).

Series 2 – influence of immune cells on the ALP activity of Saos-2 cells

The primary aim of these experiments was to investigate the effect of immune cells on alkaline phosphatase (ALP) activity. Three independent variables were considered: cell type (PBMCs, a $CD4^+$ enriched population, and PBMCs depleted of $CD4^+$ lymphocytes), cell number, and activation status. The primary dependent variable was ALP activity, but growth of Saos-2 cells was also studied.

ALP is present in both immune cells and Saos-2 cells, and therefore the two cell types were separated by transwell inserts in all conditions. Immune cells were added 24 hours after the Saos-2 cells, thereby allowing them time to adhere. A $CD4^+$ lymphocyte depleted population was included to further investigate any potential role of these cells in mediating observed effects on Saos-2 cells.

Experimental setup

Peripheral blood mononuclear cells were isolated as described earlier, from which three populations were generated: PBMCs, CD4-enriched and CD4-depleted. Saos-2 cells (10^5 cells in 500μl of BCM) were added to wells of a 24-well culture plate (Corning #CLS3470). After 48 hours, 0.4μm pore polyester transwell membrane inserts (Corning #CLS3470) were placed in each well, to which immune cells were added at ratios of 0.6, 1, 3 and 6 immune cells: 1 Saos-2 cell. Immune cells were activated using Miltenyi T_{reg} Suppression Inspector Beads (Miltenyi #130-092-909 subsequently referred to as stimulatory beads) at an immune cell: bead ratio of 1:1. Control wells with Saos-2 alone, immune cells alone and immune cells with stimulatory beads were included. Cultures were incubated for 4 days at 37°C with 5% v/v CO_2.

Counting of Saos-2 cells and analysis of lymphocytes by flow cytometry

Techniques used here are carried out in the same way as for series 1, unless described otherwise. Immune cells were aspirated from the transwell inserts by pipette and prepared for analysis by flow cytometry. Saos-2 cells were harvested from the wells using trypsin, and counted using an automated cell counter.

Measurement of alkaline phosphatase activity

Alkaline phosphatase (ALP) is a glycoprotein that, in humans, has the protein moiety encoded by four different gene loci: tissue non-specific (or liver/bone/kidney), intestinal, placental and placental-like (Harris 1990). Whilst it is found in serum, it is primarily a membrane-bound protein. Release from the membrane may be mediated by a phosphatidylinositol phospholipase which changes its structure from tetra- to di-meric (Hawrylak et al 1988). As discussed previously, it is strongly expressed by Saos-2. ALP activity can be measured using the substrate p-nitrophenyl phosphate (PNPP), which ALP converts to a soluble end-product (p-nitrophenyl; PN) which absorbs light in the 405nm range (Thompson et al 1991). ALP activity reflects the amount of ALP, which in turn indicates the cells ability to form a mineralised matrix. With greater ALP

activity, more of the soluble end-product PN is produced, and more light is absorbed (see Figure 2-12).

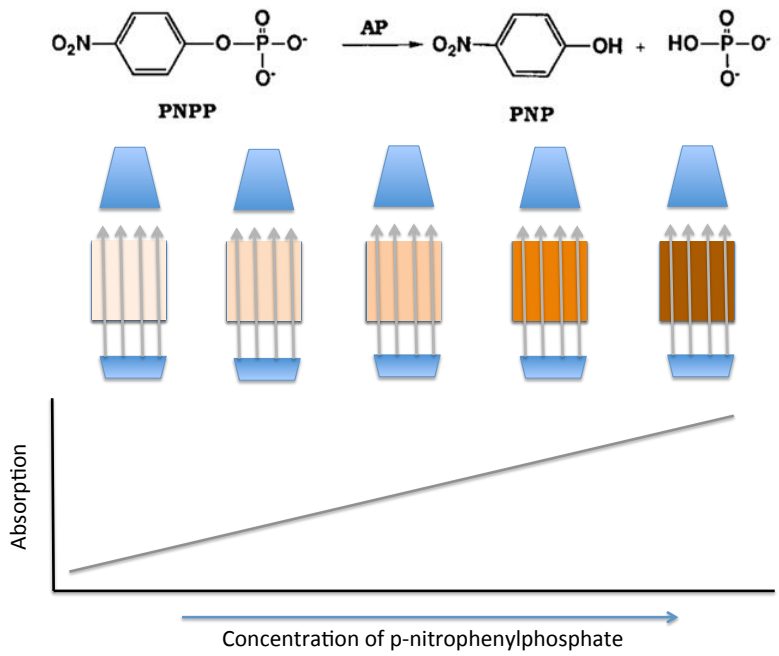

Figure 2-12: Diagram showing the chemical changes as p-nitrophenylphosphate (PNPP) is converted to p-nitrophenyl (PN). The increase in concentration of PN causes a change in colour which reduces the light passing through and increases absorption of light.

Because ALP is membrane bound, the cells needed to be harvested without the use of trypsin as this would remove it from the membrane. Instead, the cells were lysed *in situ* using water and freeze-thaw cycles. The enzymatic activity of ALP in the different experimental conditions was normalised to cell number by measuring the DNA using the Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific #P7589). PicoGreen® is a sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA. It is sensitive to even small amounts of DNA, and so contamination with all other sources of DNA must be excluded.

Once the experiment was complete, medium was removed from the wells, and the remaining cells washed twice with PBS. DNA-free water (0.5 ml) was added to each well and three freeze-thaw cycles undertaken. For each cycle, cells were frozen at -80°C for 45 minutes and then thawed for 15 minutes whilst being agitated on a shaker. This produced a cell lysate retaining ALP.

A buffered solution of PNPP was made by dissolving 10 mg of PNPP (Sigma P4744) in 0.2 ml of 1M TRIS, 0.05 ml of 0.2 M $MgCl_2$, and 10 ml of DNA-free water and adjusting the pH to 8.0-8.3 (termed ALP substrate). Equal volumes of cell lysate and ALP substrate were added to wells of a 96-well plate, including control wells, with DNA-free water being substituted for cell lysate in controls. Absorbance was measured at 405nm using a multi-functional microplate reader (BMG FLUOstar Galaxy™). This was measured immediately and at 5-minute intervals up to 25 minutes.

The PicoGreen® reagents were prepared per the manufacturer's recommended protocol. The kit comes with a TE buffer consisting of 10 mM Tris-HCl and 1 mM EDTA at a pH of 7.5. Samples of each cell lysate were diluted 10 times with the TE buffer in a 96-well plate. A standard DNA solution, provided with the kit, was used to provide a series of wells with a known concentration of DNA to be used as a standard curve. Appropriate volumes of PicoGreen®, diluted in the TE buffer, were added to the cell lysate samples and the standard DNA solutions. After 1 to 2 minutes, fluorescence was measured using the BMG FLUOstar Galaxy™ at an excitation of ~480nm and emission of ~520nm. DNA content was calculated by comparing the measured fluorescent output of the cell lysate samples, minus the reading from the control wells, and comparing it to the standard curve.

In vivo study of immune cell status in children with inflammatory bowel disease and their association with bone metabolism

The aim of this *in vivo* study was to investigate potential associations between the relative proportions and activation status of lymphocytes in children with Crohn's disease and changes in BMD measured by DXA.

Participants and investigative rationale

Children aged 4-16 years of age having an endoscopy for investigation of gastrointestinal symptoms were recruited. Ethical approval for this study was obtained from the Sheffield Research Ethics Committee (08/H1308/275). All participants gave informed consent. Peripheral blood (13 ml) was collected into EDTA anticoagulant (BD Biosciences Vacutainer™ tubes, BD Biosciences, Oxford, UK) for analysis by multicolour flow cytometry. Gut biopsies (10 per participant) were taken using standard size paediatric biopsy forceps (Boston Scientific, Radial Jaw, 2.4mm: Cat no 1340).

Patients were fasted for their general anaesthetic, and their second void urine sample of the day was collected into a plain universal container.

In those with no macroscopic inflammation, 10 biopsies were taken from the colon and the last part of the ileum: 2 ileal, 2 caecal, and 2 each from the ascending, transverse and descending regions of the colon. In patients with evidence of IBD, biopsies were predominantly taken from inflamed areas of bowel, including the upper GI tract. The reason for this approach was to focus on a comparison of inflamed and non-inflamed tissue. This does mean that any differences may reflect the changing location of the biopsy, rather than a pathological process. However, a more consistent approach to biopsy location could potentially limit the option of focusing on inflamed areas of bowel, potentially missing the sampling of areas of greater inflammation. An additional limitation to achieving consistent regional biopsies was that in some cases the technical difficulties of endoscopy meant that specific areas could not be biopsied.

Isolation of lymphocytes from endoscopic mucosal biopsies

Lymphocytes were isolated from the peripheral blood using the same method as for the *in-vitro* experiments. Isolation of lymphocytes from mucosal tissue is an established technique with physical (Clancy 1976) and enzymatic (Bull et al 1977) methods described in the 1970s, and used extensively since to study mucosal immunology. At the time of undertaking this work most studies seemed to use enzymes to isolate immune cells from resected sections of gut, but with no detailed descriptions of an approach to isolation from the smaller pinch biopsies taken with an endoscope some optimisation of a technique for isolation was necessary.

The most recent and comprehensive description at the time used two combinations of enzymes to release cells from the epithelial and lamina propria layers (Weigmann et al 2007) which is necessary given the fundamental differences in the lymphocyte populations normally found in those two compartments. The enzymes do digest cell surface proteins (Abuzakouk et al 1996; Van Damme et al 2000) affecting analysis by flow cytometry. Mechanical isolation methods potentially overcome this problem but may isolate lower numbers of cells (Bland et al 1979) which could also affect analysis given the size of the biopsies. After this work was completed a publication supported and described the use of enzymatic digestion, rather than mechanical, for the isolation of lymphocytes from endoscopic gut biopsies (Carrasco et al 2013).

Optimisation of the isolation technique is described in chapter 4, but the following basic aspects will be covered here. The initial enzymes were those used by Weigmann et al (2007) who used two different solutions in their protocol: a pre-digestion solution to release epithelial cells, and a digestion solution to release cells from the lamina propria. They also used a percoll gradient and passed digested tissue through a cell strainer, both of which were tried. Although an adequate cell yield was achieved there was loss of cell surface proteins so the effects of the individual enzymes were investigated using isolated PBMCs.

Enzymes used for the release of lymphocytes from mucosal gut biopsies

Pre-digestion solution:

- Hanks Balanced Salt Solution (Sigma #H6648)
- 5mM EDTA (GIBCO #15575-020)
- 1mM DTT (Sigma #D0632)

Digestion solution:

- Phosphate buffered saline (PBS)
- 0.05% w/v collagenase type IV (Worthington #LS004186)
- 0.05% w/v DNase 1 (Worthington #LS002004)
- 0.3% w/v dispase (Roche #04942078001)

Ethylenediaminetetraacetic acid (EDTA)

Ethylenediaminetetraacetic acid (EDTA) is a polyamino-carboxylic acid. It is a chelating agent with the ability to bind to, and sequester metal ions. Within mucosa, the epidermal layer is bound to the underlying connective tissue by basal epithelial cell hemidesmosomes, cell-substrate adhesion junctions (Stepp et al 1990). Incubation of oral mucosa in EDTA makes separation of the epithelium from connective tissue possible, and it was suggested this could occur because attachment requires the presence of metal ions which are removed by the EDTA (Scaletta et al 1971).

Dithiothreitol (DTT)

Dithiothreitol (DTT) is a disulfide reducing agent. Its use in mucosal digestion appears to have been serendipitous. When rat skin samples were incubated in a buffer containing DTT it resulted in easier detachment of the epidermis from the dermis. The basement membrane remained attached to the epidermis when mechanical separation

was combined with incubation in DTT, but to the dermis with mechanical separation alone. The authors were unsure of the mechanism for this (Epstein et al 1979).

Collagenase

Collagen is an important constituent of connective tissue. There are at least 27 forms (Eyre 2004), with type I being the most abundant protein in the human body (Di Lullo et al 2002). Although the structure of collagen has been studied since the 1930s, a complete understanding has only recently been described (Orgel et al 2006) and is shown in Figure 2-13. Polypeptide chains, called α chains, have a left-handed helical structure. Three α chains then wind together forming a right-handed triple helix known as the collagen molecule. Three collagen molecules then further combine to form a super-super coil, again a right-handed helix, or sub-fibril. Sub-fibrils then combine to form fibrils.

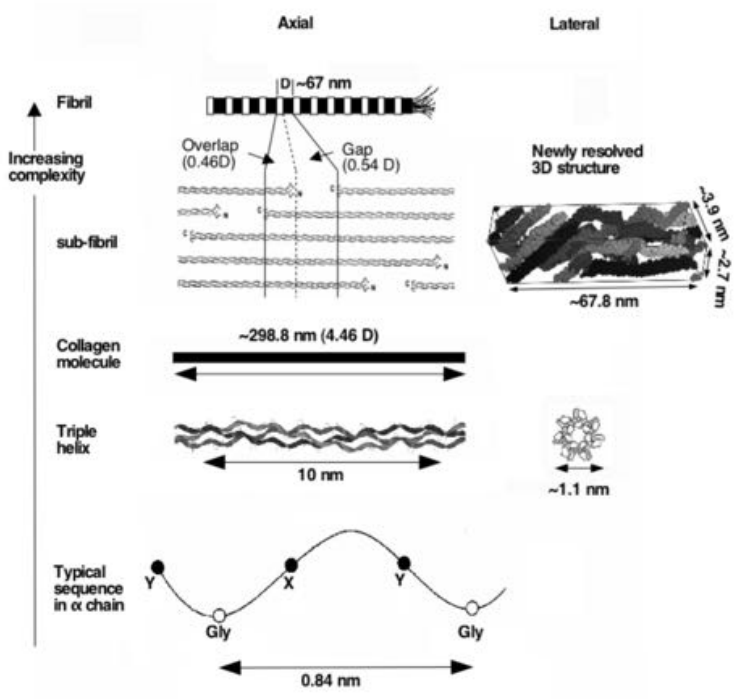

Figure 2-13: The structure of collagen. This shows the alpha chain and its formation into a triple helix known as the collagen molecule. Reproduced with permission (Orgel et al 2006).

Collagenases are endopeptidases (enzymes that break peptide bonds of non-terminal amino-acids) which cleave the triple helical region of the collagen molecule (Gross et al 1974). Collagenase is available in several forms, and one with a low tryptic activity is suggested in the paper by Weigmann et al (2007), as this is thought to have less of an effect on surface proteins. For these experiments, Worthington's collagenase type IV (Worthington #LS004186) was used.

Dispase

Dispase is a neutral protease isolated from culture filtrates of *Bacillus polymyxa.* It has been found to act on fibronectin and type IV collagen, but not laminin or type I collagen (Stenn et al 1989). It has also been used in this protocol, as it is thought to reduce cell clumping without affecting cell viability (Weigmann et al 2007).

Deoxyribonuclease (DNAse)

Deoxyribonuclease (DNAse) was first described as early as 1903 and crystallised by Kunitz in 1950 (Kunitz 1950a; Kunitz 1950b). Its primary role is the breakdown of DNA, although it has also been found to reduce viscosity of mucous secretions in patients with cystic fibrosis (Shak et al 1990). It is this latter effect that may explain its use to reduce cell clumping (Weigmann et al 2007).

Percoll® density gradients

Percoll® density gradient medium enables the separation of cells from tissue based on their density. In this instance, it is intended to separate lamina propria cells from other cell types and debris. The density of the Percoll® solution (GE Healthcare #17-0891-01) was adjusted from 1.13 to 1.124 g/ml. For this, a solution of 1.5M NaCl was made by adding 4.38 g of NaCl (Sigma #57653) to 50 ml of double distilled water. 1.82 ml of the 1.5M NaCl solution (density 1.058 g/ml) was then added to 20 ml of the Percoll® solution, based on the following calculation taken from an Amersham laboratories product sheet:

$$Vx = Vo\ ((Po - Pi)/(Pi - P10)) \quad \text{thus} \quad Pi = (VoPo + VxP10)/(Vx + Vo)$$

Where	Vx	=	volume of diluting medium (ml)
	Vo	=	volume of undiluted Percoll® (ml)
	Po	=	density of Percoll® (1.13 g/ml)
	P10	=	density of 1.5M NaCl (1.058 g/ml)
	Pi	=	density of solution produced (g/ml)

The 1.124 density Percoll® was then mixed with PBS to produce a 40% v/v solution (8.4 ml Percoll® and 11.6 ml PBS) and an 80% solution (8 ml Percoll® and 2 ml PBS).

Effect of digestive enzymes on the expression of cell surface markers

To identify which component was having the greatest effect, the influence of enzymes on cell surface antigen expression was investigated. For this, PBMCs were isolated from the blood of healthy volunteers (as described above). Cells were incubated in RPMI growth medium (BioWhittaker #BE12-115F) supplemented with 10% v/v foetal bovine serum (GIBCO #10270-106), 100units/ml penicillin and 100μg/ml streptomycin (BioWhittaker #DE-17-602E) and concanavalin A (5μg/ml) overnight at 37°C with 5% v/v CO_2 at a concentration of $1x10^6$ cells/ml. Cells were then transferred to medium without the ConA for a further 24 hours.

Digestion enzymes were made up at concentrations corresponding to those used in the biopsy sample digestion process:

Pre-digestion enzymes in HBSS

- EDTA 5mM
- DTT 1M

Digestion enzymes in PBS

- Collagenase type IV 0.1% and 0.01% w/v
- Dispase 0.3% and 0.03% w/v
- DNase 0.05% w/v

PBMCs were incubated in the enzyme solutions for 1 hour at 37°C. Cells were then washed twice with PBS, re-suspended in SB with 2% v/v mouse serum and incubated for 15 minutes at 4°C. Cells were then incubated with appropriate concentrations (as shown in table) of the following mAbs for 30 minutes at 4°C: Qdot™ 605-conjugated anti-CD4, PerCP/Cy5.5™-conjugated anti-CD8, Alexa Fluor™ 700 (AF700)-conjugated anti-CD19, PE/Cy7™-conjugated anti-CD25, APC-conjugated anti-CD69, PE-conjugated anti-α4 integrin, FITC-conjugated anti-β7 integrin. Samples were washed with excess SB and re-suspended in SB. Samples were kept on ice in the dark and analysed within 4 hours.

Fluorescent staining of isolated cells

Most of the cell surface markers of interest were the same as for the co-culture studies: CD4, CD8, CD19, CD25 and CD69. However, the expression of the α4β7 integrin was additionally investigated, as α4β7 is a marker of lymphocytes in the peripheral

circulation that have been exposed to antigen in the gut (as discussed in the introduction). This required separate fluorescent antibodies for α4 and β7.

Fluorescent antibodies

PBMCs were isolated from peripheral blood using the same method as described for the *in vitro* experiments. Development of the technique for isolation of cells from the mucosal biopsies is described in chapter 4. For both, once isolated, the cells were fluorescently labelled as described for the in vitro studies.

Two fluorescent antibody panels were used for these experiments. In the development of the technique for isolation of cells from mucosal samples the panel in Table 2-2 was used for the first three patient samples. An improved antibody panel was determined, shown in Table 2-3, which enabled a fluorescent labelling of a single sample of cells whilst identifying all the necessary cell surface markers.

Fluorophore	mAB	Clone	Concentration (1x106 cells/100µl)	Catalogue number
Alexa Fluor 700	anti-CD4	OKT4	1µg	Biolegend #317426
Alexa Fluor 700	anti CD8a	RPA-T8	1µg	Biolegend #301028
Alexa Fluor 700	anti-CD19	HIB19	1µg	Biolegend #302226
PE	anti-CD25	BC96	5µl	Biolegend #302606
APC	anti-CD69	FN50	5µl	Biolegend #310910
FITC	anti-β7	FIB504	5µl	Biolegend #321212

Table 2-2: Table of mAbs, used initially for the *in vivo* study, and their corresponding fluorophors. Fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), Alexa Fluor™ 700 (AF700), quantum dot (Qdot™), cyanine dye (Cy7™).

Fluorphore	mAB	Clone	Concentration (1x106 cells/100µl)	Catalogue number
Qdot 605™	anti-CD4	S3.5	1µl	Invitrogen #Q10008
Qdot 705™	anti-CD8	3B5	1µl	Invitrogen #Q100509
Alexa Fluor™	anti-CD19	HIB19	20µl	Biolegend #302226
APC-Cy7™	anti-CD69	FN50	0.5µg	Biolegend #310914
PE-Cy7™	anti-CD25	HI30	0.5µg	Ebioscience #25-0259-42
FITC™	anti-β7	FIB27	0.25µg	Biolegend #121010
PE™	anti-α4	9F10	5µl	Biolegend #304303
Ultraviolet	LIVE/DEAD®	N/A	1µl	Invitrogen L23105

Table 2-3: Table of mAbs, used for most of the *in vivo* study, and their corresponding fluorophors. Fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), Alexa Fluor™ 700 (AF700), quantum dot (Qdot™), cyanine dye (Cy7™).

Viability

It is necessary to identify viable cells when using fluorescent labelling, and this is especially true for cells isolated from gut mucosa biopsies since they are subjected to both physical and chemical disruption. Figure 2-14 shows scatter plots for cells isolated from peripheral blood, and the gut epithelial and lamina propria layers. For both gut cell populations, the scatter plot is much more widely distributed than that of the PBMCs. Viability staining shows that both the lymphocyte population, and the larger and more granular cells further out on the scatter plot, are in fact viable.

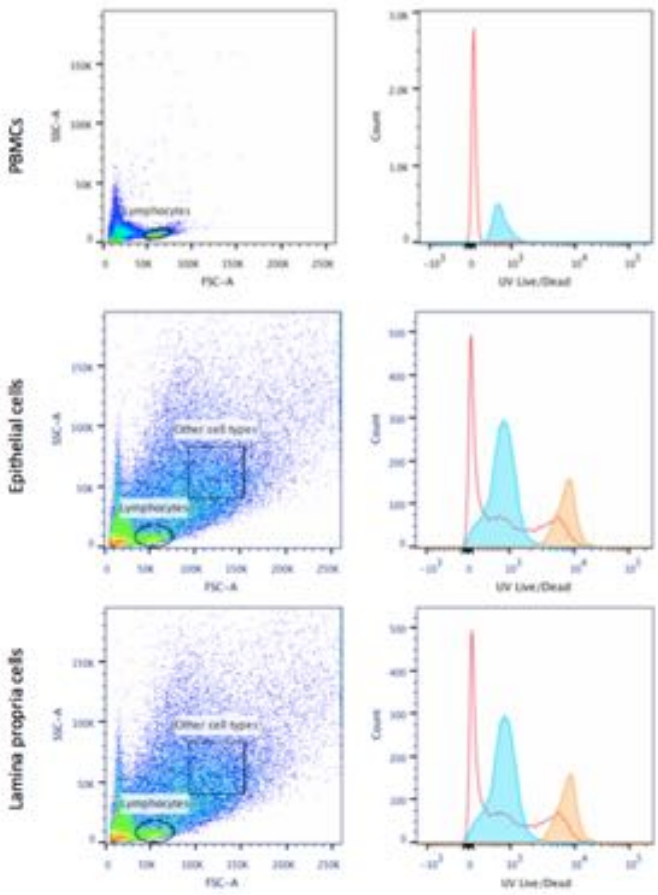

Figure 2-14: Scatter plots for cells isolated from the peripheral blood, gut epithelium and gut lamina propria. Histograms reflect viability staining: open red is unstained, filled blue is the stained "lymphocyte" population, filled orange is the "other cell types" population.

Identification of lymphocyte subsets and their activation status

Enzyme digestion assays

Figure 2-15 shows the gating strategy for the enzyme digestion assays. Panel 1-A identifies the activated lymphocyte population, and panel 1-B identifies the viable cells. Identification of $CD4^+$, $CD8^+$ and $CD19^+$ cells, and their expression of CD25 and CD69, were carried out in keeping with the co-culture experiments.

Identification of $\alpha4\beta7^+$ cells is complicated by the single integrin requiring two separate fluorescent labels. Whilst an antibody to $\alpha4\beta7$ has been produced, it is not commercially available. There are antibodies to human forms of both $\alpha4$ and $\beta7$, but since both can also be expressed with other integrins e.g. as $\alpha4\beta1$, both must be labelled to accurately identify $\alpha4\beta7^+$ cells. Therefore, when identifying those cells a scatter plot of each labels fluorescence is created. On the basis of the isotype control

the scatter plots is divided into 4 sections: $\alpha4^{-}\beta7^{-}$, $\alpha4^{+}\beta7^{-}$, $\alpha4^{-}\beta7^{+}$ and $\alpha4\beta7^{+}$. This can be seen in panel 2-D of Figure 2-15.

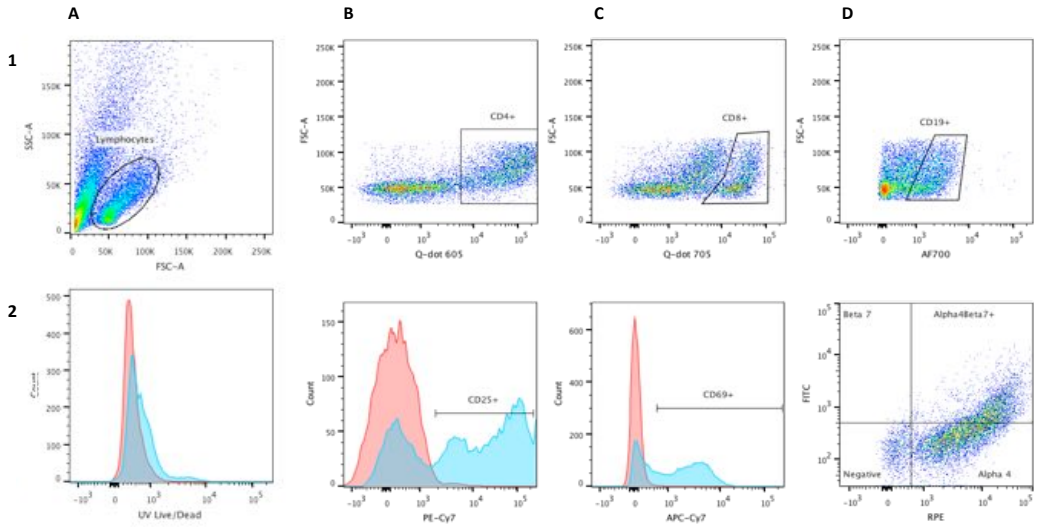

Figure 2-15: Gating strategy for the enzyme digestion assay. **Panel 2 -** A - for this viability stain red filled histogram reflects unstained cells and blue fill reflects stained. **Panel 2 – B and C -** for the activation marker histograms red fill reflects isotype control and blue fill reflects labelled cells.

Patient samples

The gating strategy for identifying lymphocytes in the peripheral blood, gut epithelium, and lamina propria of patients are shown in Figures 2-16 to 2-18. Whilst in the PBMC preparation there is a clear lymphocyte population, the cells isolated from the gut are more diverse and the lymphocyte population less clear. Therefore, for both mucosal epithelium (MEL) and lamina propria (LP) cells, the lymphocyte subsets were identified from all events on the scatter plot. It was then possible to identify $CD4^{+}$, $CD8^{+}$ and $CD19^{+}$ lymphocytes on the original scatter plot. This was then used to locate the lymphocyte gate for subsequent assessment of relative proportions.

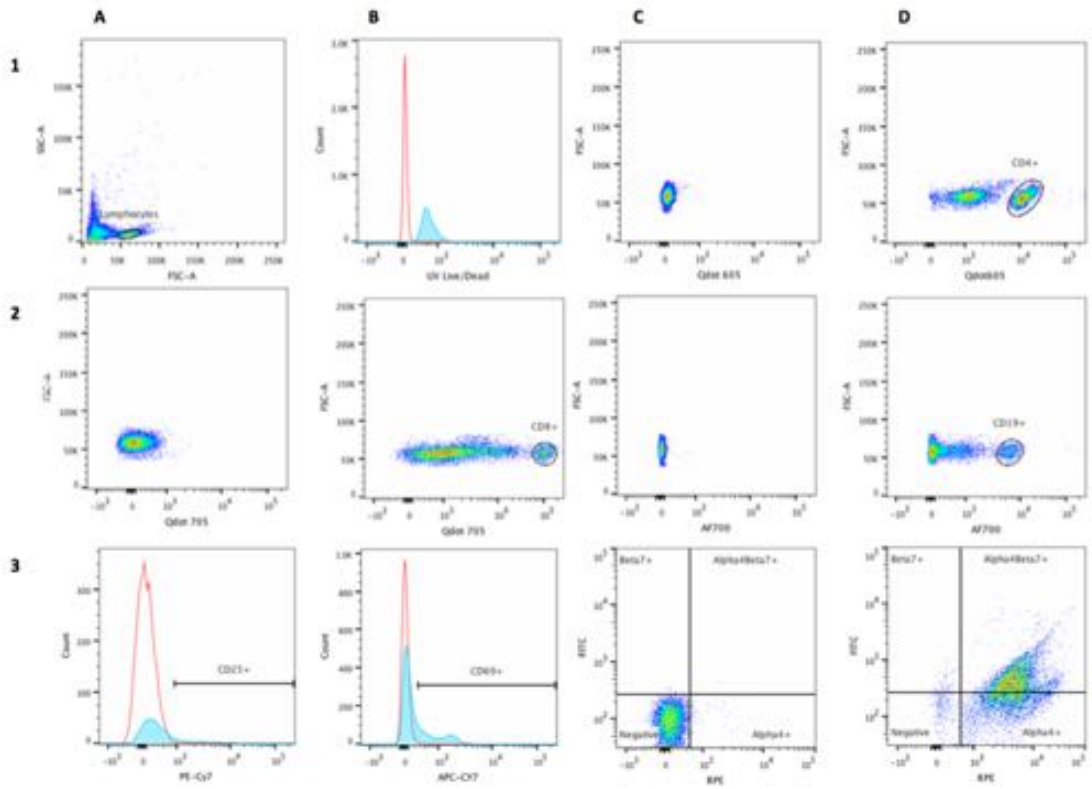

Figure 2-16: Gating strategy for peripheral blood lymphocytes in the *in vivo* study. **Panel 1B -** open red histogram represent unstained cells, and the blue filled histogram stained cells. **Panels 3A to B -** open red histogram represents the isotype control and filled blue the fluorescently labelled cells.

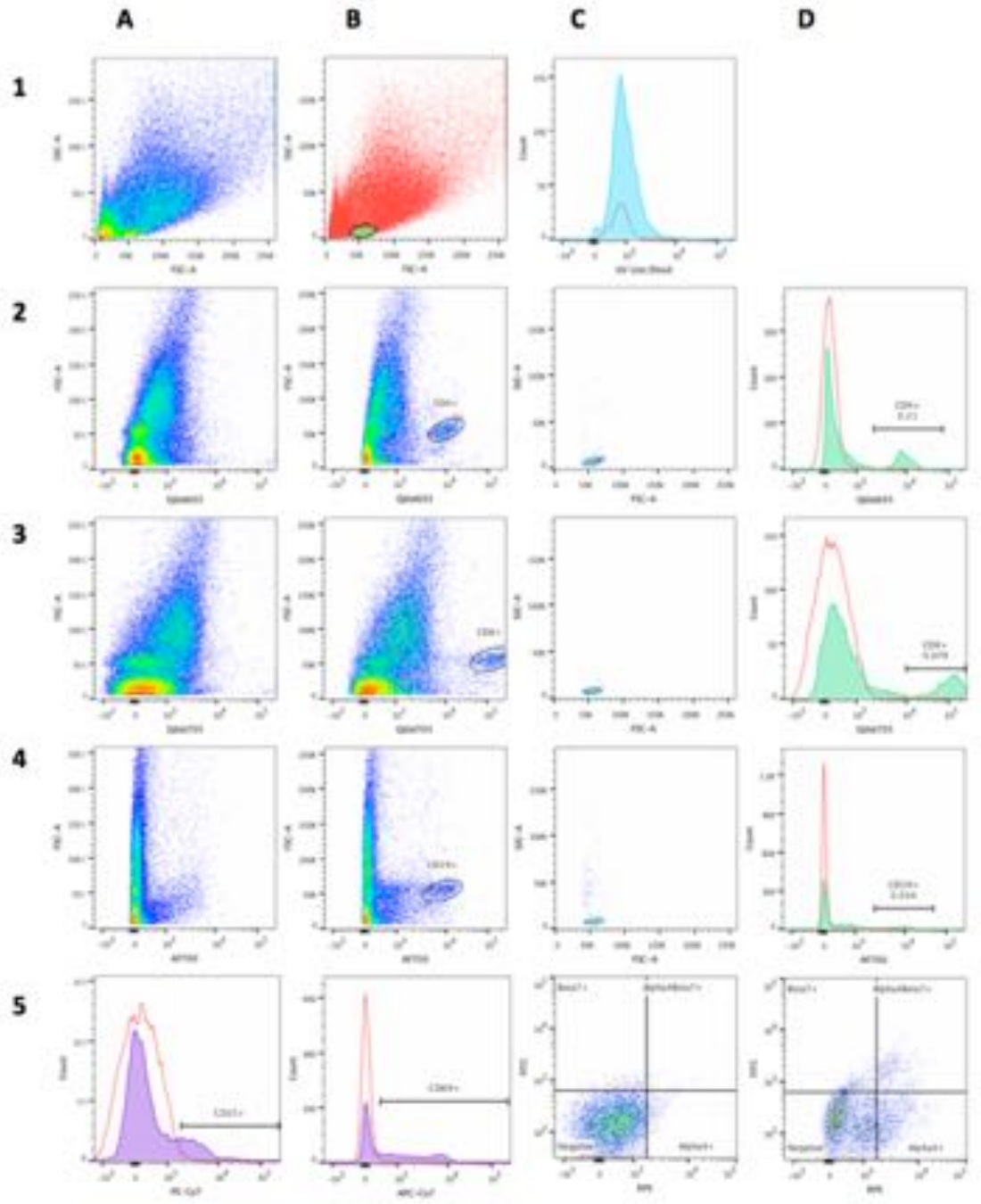

Figure 2-17: Gating strategy for epithelial lymphocytes. **Panel 1A -** scatter plot of all cells isolated from the epithelial layer. **Panel 1B -** populations identified in B2-3 shown on the original scatter plot, identifying the location of the lymphocyte population. **Panel 1C -** open red histogram represents unstained cells, whilst the filled blue represents stained cells. **Panels D2 and 3 –** open red histograms where open red represents the isotype control and filled green the fluorescently labelled cells. **Panels 5A and B –** open red histograms represent the isotype control and filled purple the fluorescently labelled cells.

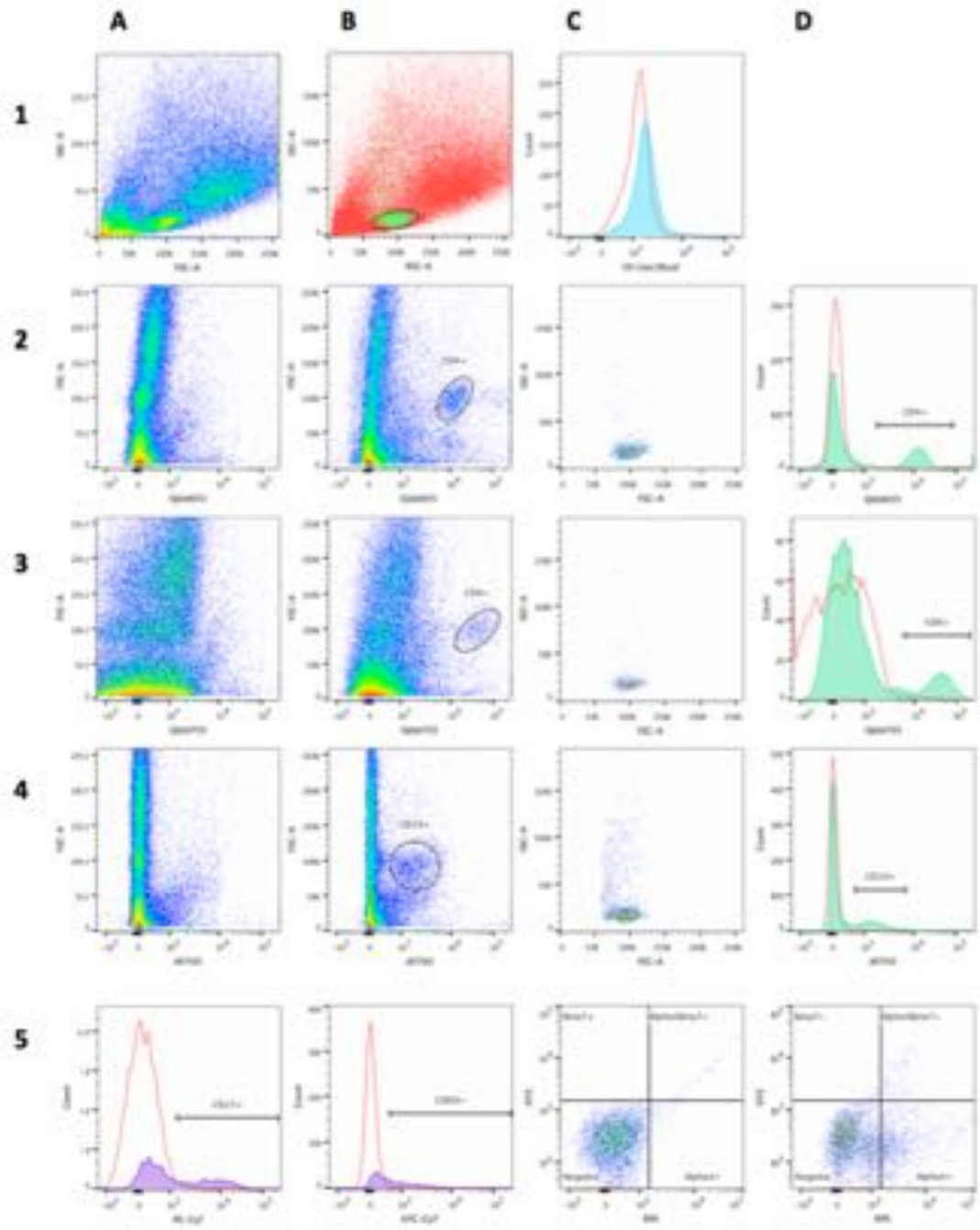

Figure 2-18: Gating strategy for lamina propria lymphocytes. **Panel 1A -** scatter plot of all cells isolated from the epithelial layer. **Panel 1B -** the populations identified in B2-3 shown on the original scatter plot, identifying the location of the lymphocyte population. **Panel 1C –** open red histogram represents unstained cells, whilst the filled blue represents stained cells. **Panels D2 and 3 –** open red histograms represent the isotype controls whilst filled green represent the fluorescently labelled cells. **Panels 5A and B –** open red histograms represent the isotype control and filled purple the fluorescently labelled cells.

Measurement of bone turnover markers

Bone metabolism refers to activity of osteoblasts and osteoclast activity in terms of formation and resorption of bone. Organic compounds released by these processes can be measured in blood and urine, and used to assess bone metabolism (as

discussed in the Introduction). Analysis of bone turnover markers in blood and urine was carried out by the Bone Analysis Laboratory of the University of Sheffield and the biochemistry laboratory at Sheffield Teaching Hospitals NHS Trust. Details of the methods used are given below.

Type 1 procollagen amino-terminal propeptide.

Bone primarily consists of type 1 collagen which is derived from type 1 pro-collagen synthesised by fibroblasts and osteoblasts. Type 1 pro-collagen has amino (N) and carboxy (C) terminal extensions (both propeptides) that are removed during the conversion of pro-collagen to collagen. The propeptide with amino terminal extensions, also known as P1NP, is most commonly measured. Once released and has entered the blood stream thermal degradation effects convert the trimeric form to monomeric.

Serum levels were measured using an Elecsys total P1NP assay (Roche #03141071), utilising electrochemoluminescence. For this immunoassay, serum is incubated with a biotinylated monoclonal P1NP-specific antibody, a ruthemium labelled monoclonal P1NP-specific antibody and streptavadin-coated micro-particles. The biotin labelled antibody binds to the streptavidin of the micro-particle. The P1NP molecule then binds to two types of antibody: one bound to a micro-particle, the other bound to a ruthenium molecule. The complex can be isolated by application of a magnet allowing non-bound molecules to be removed. Application of voltage then induces chemoluminescence by the ruthenium complex $[Ru(Bpy)_3]^{2+}$, with the molecule being regenerated by tripropylamine (TPA). The luminescence was measured using a Cobas E411 automated immunoassay analyser.

Osteocalcin

Osteocalcin is the most important non-collagen protein in bone matrix. It is a bone-specific, calcium binding protein which is dependent on vitamin K. It is synthesised by osteoblasts in response to vitamin D3. Osteocalcin is assimilated into bone, and released into the blood stream where it can be measured.

Serum osteocalcin levels were measured using an Elecsys™ N-MID osteocalcin assay (Cobas #12149133). This method utilises electrochemoluminescence as described above for the measurement of P1NP, the only difference being the use of a biontinylated monoclonal osteocalcin specific antibody.

N-telopeptide of type 1 collagen

N-telopeptide of type 1 collagen, NTx, is a marker of bone resorption and increases when there is increased osteoclast activity.

Patients were starved overnight, prior to their endoscopy, and their second urine sample of the day collected. Samples were analysed for NTx, corrected for the urine creatinine level to account for any dilution effect, by the biochemistry laboratory of Sheffield Teaching Hospitals NHS Foundation Trust using a competitive immunoassay (Vitros #680 0030). In brief, greater concentrations of NTx in the urine reduce the binding of luminogenic substrates and decrease the amount of light emitted.

Wells are coated with a synthetic NTx peptide able to bind a mouse monoclonal anti-NTx antibody conjugated to a horseradish peroxidase (HRP). HRP generates luminescence by catalysing the oxidation of a luminol derivative, and the effect is prolonged by the addition of an electron transfer agent. The sample is added to the peptide coated wells with the HRP-conjugated antibody. The greater the concentration of NTx in the urine sample the less HRP will bind to that on the well. The liquid portion is removed, thus discarding the HRP bound to the urinary NTx leaving only that bound to the synthetic NTx peptide which remains within the well. The luminescence reagent (containing the luminol derivative and electron transfer agent) is added to the well generating luminescence proportional to the amount of HRP bound to the well. This, in turn, is inversely proportional to the concentration of NTx in the urine sample. The concentration of NTx will be affected by the how much urine the subject has produced, thus results are corrected for the concentration of creatinine in the urine.

Measurement of BMD by DXA

As discussed in the introduction, dual X-ray absorptiometry (DXA) provides a non-invasive method of measuring bone mineral density (BMD). One important aspect of its use in children is that it expresses the measured BMD per unit area, which for smaller bones leads to an underrepresentation of the bones true mineral content (see earlier discussion). A more accurate value for BMD would be expressed per unit of volume, and so several different methods have been used to calculate the volume of bone from its projected area.

13 patients with confirmed IBD had a DXA scan within 3 months of their enrolment into the study. DXA scans were obtained using the GE Healthcare Lunar iDXA. Volumetric

BMD (vBMD) was calculated using the method of Carter as described in the Alphabet Study (Crabtree et al 2017). In this study 3598 children aged 4 to 20 years were recruited and their BMD measured by GE-Lunar and Hologic scanners across 7 centres. The data were used to construct reference values for measures of BMD in keeping with the 2013 ISCD recommendations (Kalkwarf et al 2014). They recommend that in children with short stature or growth delay (seen in children with IBD) should be reported as either bone mineral apparent density (BMAD) or TBLH adjusted for the subject height Z-score. For this study BMAD was used as described by Carter et al (Carter et al 1992):

$$\text{Lumbar spine BMAD (g/cm3)} = \frac{(BMC_1+BMC_2+BMC_3+BMC_4)}{(V_1+V_2+V_3+V_4)}$$

Where V_n is the volume of the n^{th} individual vertebrae = $AP_n^{1.5}$ (AP_n = projected vertebral area of the n^{th} vertebra).

BMC_n is the bone mineral content of the n^{th} vertebrae.

The calculated BMAD of cases was compared to three healthy controls, identified from the Sheffield database which contributed to the Alphabet Study, matched for age and sex.

Statistics

The section describes the statistical tests used to analyse the data presented within this thesis. Advice was provided by Maths and Statistics Help (MASH) of the University of Sheffield, and Laerd Statistics (https://statistics.laerd.com) an online guide to statistics and the software programme SPSS.

Independent samples T-test

The independent samples T-test is used to determine if a difference exists between the means of two independent groups on a continuous dependent variable. It has three assumptions in relation to the study design:

1. There is one dependent variable measured at the continuous level
2. There is one independent variable that consists of two categorical and independent groups

3. There is independence of observations

The dependent variable is usually the outcome measure, and continuous indicates it is measured as a scale e.g. the number of Saos-2 cells. The independent variable is the aspect of the experiment which is adjusted. An example would be whether the Saos-2 cells are cultured in a PBMC or $CD4^+$ lymphocytes activated cell supernatant.

There are also three assumptions about the data

4. There should be no significant outliers
5. The dependent variable should be normally distributed
6. There is homogeneity of variance

Any deviation from these three assumptions, and interventions to resolve them, are discussed in the results section. Outliers were looked for by inspection of boxplots. Normality was assessed by the Shapiro-Wilk test for normality. Whilst it is true that to run a T-test data should be normally distributed, the test remains accurate even when this assumption is not met (Wilcox 1995). Considered more important is the variance of the data, which needs to be similar between the two variables. This is measured by Levene's test of equality of variance. Both normality and variance can be adjusted by transforming the data using square function, square root, inverse or $\log^{10}$.

It was assumed that the experimental data would be normally distributed. This was in part because of central limit theorem which argues that with enough observations the data will be normally distributed (Läärä 2009). For the *in vitro* experiments, each well of Saos-2 would have many cells, all with very similar characteristics, thus each experiment was carried out with a large "population". T-tests were also used for the flow cytometry data as each value was based on analysis of large numbers of individual cells.

ANOVA

Analysis of variance (ANOVA) has very similar requirements to the independent samples T-test, including the need for normally distributed data. It is used when there are more than two groups of data to compare because multiple comparisons increase the chance of finding a false positive or type 1 error. Thus, it is an omnibus statistic reflecting the data overall, rather than comparing the means of each group. It is therefore usually followed by post-hoc testing which compares all the groups to each

other, such as Tukey's. This can sometime produce conflicting results with the ANOVA finding no difference, but the post hoc test finding one. Given that specific predictions were not made for these experiments, when such a conflict occurred the findings of the ANOVA were given priority.

The basic requirements are the same as that of the independent samples T-test (see above), except that the number of independent variables is increased which affects the type of ANOVA.

One-way ANOVA

This is used when there is one independent variable with two or more categorical variables. An example of its use would be to compare the number of Saos-2 cells (the dependent variable) each day over a five-day period (one independent variable with five groups). The one-way ANOVA would say whether the cells grew overall, and post hoc testing would compare the differences between each individual day (a total of ten comparisons). When there is more than one independent variable ANOVA can be used, but the interpretation becomes more complex.

Two-, three-, and four-way ANOVA

The co-culture experiment investigated the effects of three independent variables on Saos-2 growth (or four variables if also considering the type of immune cell population) and statistical analysis was carried out using two-, three- and four-way ANOVA. The use of multiple ANOVAs is not usual in biological experiments but in this instance made it possible to consider the combined effects of all the independent variables on the growth of the Saos-2 cells. The effect of each variable could have been quantified statistically using a series of T-tests, as used in studies quoted in this thesis (Kawakami et al 1997; Yamaza et al 2008), but there are three limitations to this approach.

The first is the occurrence of type 1 errors resulting from repeated analyses. Whether this is a significant issue depends on whether you consider each well of the plate an individual experiment or part of a whole experiment. However, biological studies do sometimes use a one-way ANOVA rather than a series of T-tests (Rifas 2006; Croes et al 2016). Whilst the one-way ANOVA has important differences from a multiple ANOVA one shared feature is they both account for multiple analyses and, whilst the papers don't discuss their rationale, this may be why they chose to use ANOVA.

The second limitation is that using a series of T-tests only allows consideration of the effect of one variable at a time, and if there are two or more "independent" variables they may be inter-dependent. For example, in this study it was found that increasing the number of resting immune cells increased the growth of Saos-2 cells whilst increasing the number of activated immune cells inhibited growth, with both acting in a dose dependent manner. Therefore, it is potentially misleading to consider (both statistically and mechanistically) the effects of cell number and their activation status independently.

The third limitation then arises is if interactions between the variables assessed by T-test are determined by a comparison of p values. One group found that 50% of 157 articles from 5 top ranking journals, where interactions had been assessed, used this method (Nieuwenhuis et al 2011). They found what they describe as an erroneous approach was most common in cellular and molecular neuroscience, and it potentially led to incorrect conclusions in two thirds of all articles.

Interpretation of multiple ANOVA analysis

Whilst multiple ANOVA has advantages it is a more complicated analysis, but this is necessary when considering multiple variables and wouldn't necessarily be considered in a series of T-tests.

Figure 2-19 shows three representative graphs, each with two independent variables. One reflected by the y-axis, and has three groups indicated by the data points (dependent variables). The other is reflected by the trend lines, and has two groups indicated by the line colour (independent variables).

In graph A the two trend lines (solid blue and green) are parallel which means they don't interact. If the immune cell number is carried on the x axis and the immune cell activation status shown by the two solid trend lines, the red dotted line represents the average immune cell number and excludes the effect of their activation status. Since the lines are all parallel it is possible to statistically analyse the effect of immune cell number alone, and is referred to as a "main effect".

In graphs B and C, the two trend lines are not parallel indicating an interaction between the independent variables, and the red dotted line (or average) no longer reflects either of the trend lines. Therefore, the only way to statistically analyse the effect of immune cell number, is to make a series of comparisons based on both immune cell number

and the activation status. Unhelpfully in these circumstances, despite the analysis being more complicated, the analysis is referred to as the “simple main effects”.

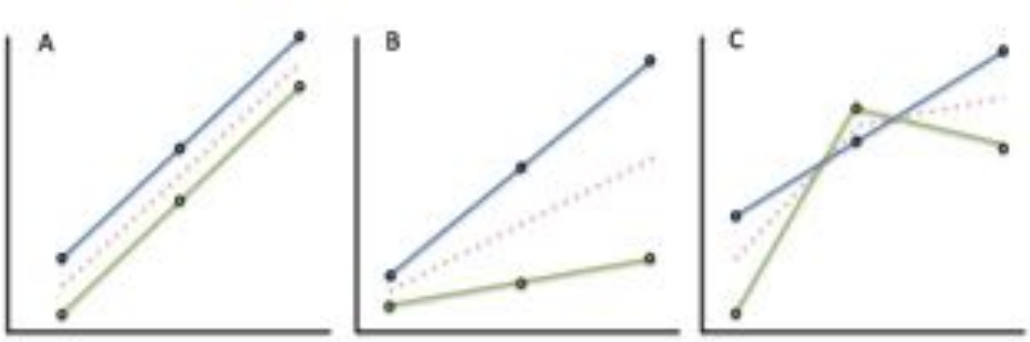

Figure 2-19: Representative graphs demonstrating interactions between independent variables. The y-axis represents the dependent variable, the x-axis one independent variable, and the coloured (solid) trend lines the second independent variable. Dashed lines represent the average of the two trend lines. Graph A shows no interaction, graph B shows an ordinal interaction, graph C shows a disordinal interaction.

The Mann-Whitney U-test

Whilst described as the non-parametric equivalent to the T-test, the more important difference is that it compares the distribution of two populations rather than their means. Therefore, the distribution of data does not need to be “normal”, although if the distributions are similar then the medians of the two populations can be compared.

This test was used for statistical analysis of the patient data that were not expected to be normally distributed, such as BMD and bone turnover markers.

Linear regression

This test was used to assess relationships between immune cell populations and BMD in patients. It produces a value “R” which reflects the strength of the association between two variables, the higher the value the stronger the association, and whether it is direct or inverse, the latter giving a negative value for R. Linear regression also measures how much of the variation is due to the association between the two variables. As an example of this, a population of people will have a range of body weights. An observational study might find an inverse relationship between exercise and body weight, but only explain 10% of the populations variation in body weight.

3 *In vitro* results

Effect of immune cells and their secreted factors on the growth and functional activity of Saos-2 cells

The following experiments used immune cells isolated from blood samples provided by healthy volunteers. Each experiment was repeated three times on different days using blood from different volunteers, but experiments for each donor were not replicated.

Characterisation of lymphocyte populations and Saos-2 cells

Relative proportions of lymphocyte subsets in the PBMC and $CD4^+$ enriched populations

The two populations of immune cells were different in respect to their proportions of lymphocytes (see Figure 3-1). The enrichment process increased the proportion of $CD4^+$ lymphocytes from 45% to 93.5%, with the proportions of $CD8^+$ and $CD19^+$ cells being reduced from 24% to 2.9% and 7.6% to 1.1% respectively. The relative proportions of lymphocytes were unchanged following activation with Miltenyi T_{reg} Suppression Inspector Beads.

Effects of activation on the proliferation and phenotype of immune cells

Cell number

A 4-day incubation of immune cells with Miltenyi T_{reg} Suppression Inspector Beads increased numbers of PBMCs (443,333 ± 74,461 resting vs 1,660,000 ± 530,031 activated) and $CD4^+$ lymphocytes (390,667 ± 87,351 resting vs 1,102,250 ± 475,546 activated). However, these differences were not statistically significant, primarily due to variability in the data (see Figure 3-2). This variability might result from the need to use counting beads and the repeated incubation and washing steps that were used for the fluorescent staining of the cells for flow cytometric analysis, during which cells are lost.

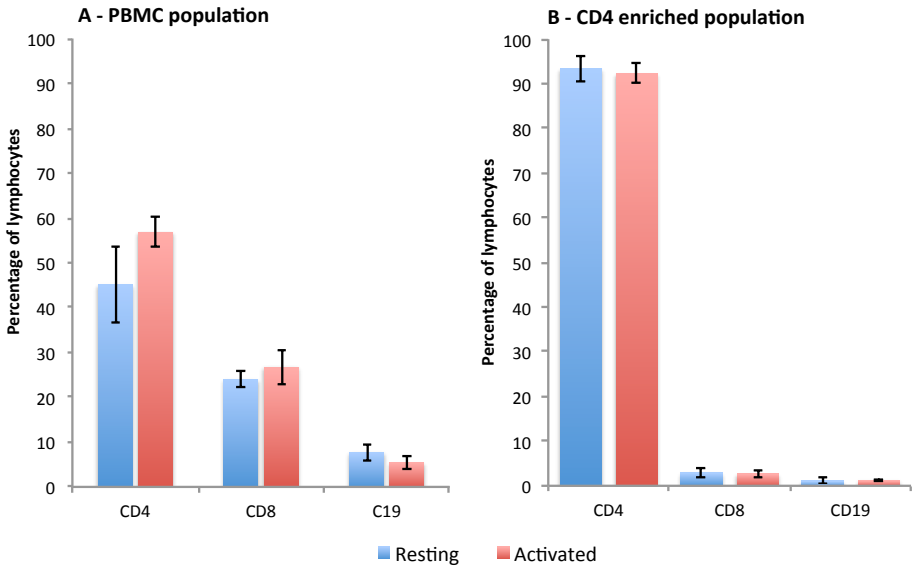

Figure 3-1: A comparison of the relative proportions of lymphocytes within a PBMC and a CD4 enriched population, and the effects of polyclonal activation. **Panel A** - the proportion of lymphocytes in a PBMC population before and after activation with Miltenyi T_{reg} Suppression Inspector Beads. **Panel B -** the proportion of lymphocytes in a $CD4^+$ enriched population before and after activation with Miltenyi T_{reg} Suppression Inspector Beads. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

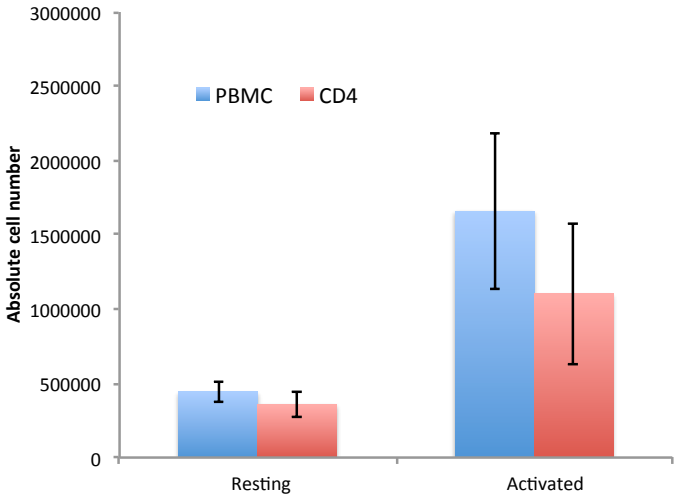

Figure 3-2: Effect of polyclonal activation on the proliferation of lymphocytes in PBMC and $CD4^+$ enriched immune cell populations. The graphs compare incubation over a 4-day period in medium alone (resting) with the addition of with Miltenyi T_{reg} Suppression Inspector Beads (activated). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Size and granularity

Changes in forward scatter (FSC) and side scatter (SSC), describing respectively the size and granularity of cells, have previously been shown to correlate with

activation (Webster et al 1995). In these experiments, visible changes in the side and forward scatter of the activated lymphocyte populations were apparent (Figure 3.3).

Activation with Miltenyi T_{reg} Suppression Inspector Beads increased the FSC and SSC of cells, and could be visualised using dot plots (Figure 3-3, panel A) or histograms (Figure 3-3, panel B). Numerical values for SSC and FSC were generated to allow statistical comparison. Activation with Miltenyi T_{reg} Suppression Inspector Beads statistically increased the FSC and SSC of PBMCs and $CD4^+$ populations (Figure 3-3, panels C and D).

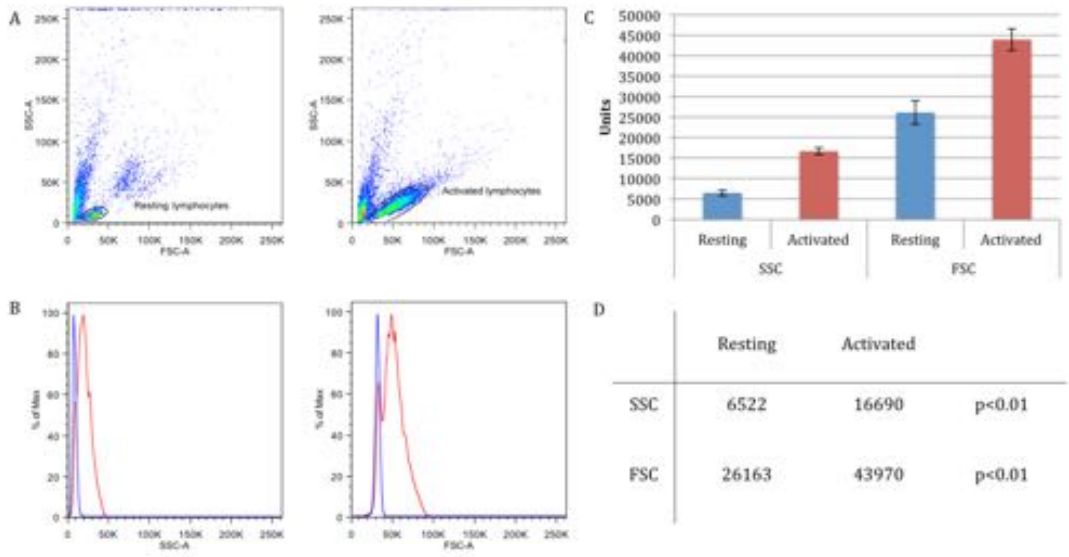

Figure 3-3: Effects of polyclonal activation on the FSA and SSC of lymphocytes. **Panel A** - representative scatter plots of resting and activated PBMCs illustrating the effects of activation with Miltenyi T_{reg} Suppression Inspector Beads on FSC and SSC, with the lymphocytes population highlighted. **Panel B** - overlay histograms comparing the SSC and FSC of the resting (blue) and activated (red) lymphocytes. **Panels C and D -** Numerical values for SSC and FSC. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Influence of activation with Miltenyi T_{reg} Suppression Inspector Beads on the expression of the activation markers CD69 and CD25

In these experiments, incubation of PBMCs with Miltenyi T_{reg} Suppression Inspector Beads increased both the percentage of cells expressing CD25 and CD69 and the overall intensity of their expression (see

Figure 3-4). This was seen across all the lymphocyte subsets ($CD4^+$, $CD8^+$ and $CD19^+$), but only the increases in percentage of positive lymphocytes were statistically significant. Apart from expression of CD25 by $CD19^+$ B cells, the increases in fluorescent intensity were not statistically significant. However, the fluorescent intensity

of CD69 expression increased by a factor of approximately 1.5, whereas the expression of CD25 increased by a factor of up to 12, depending on the subset (5.9 for $CD4^+$ T cells, 12 for $CD8^+$ T cells and 4 for $CD19^+$ B cells).

What these experiments do not demonstrate is the variation of expression over the 4-day incubation period, and since CD69 is considered an early activation marker the intensity may change over that period. What can be seen is that all three subsets up-regulated their expression of both activation antigens, and that appears to be greater for CD25 than CD69, at least at the 4-day time-point.

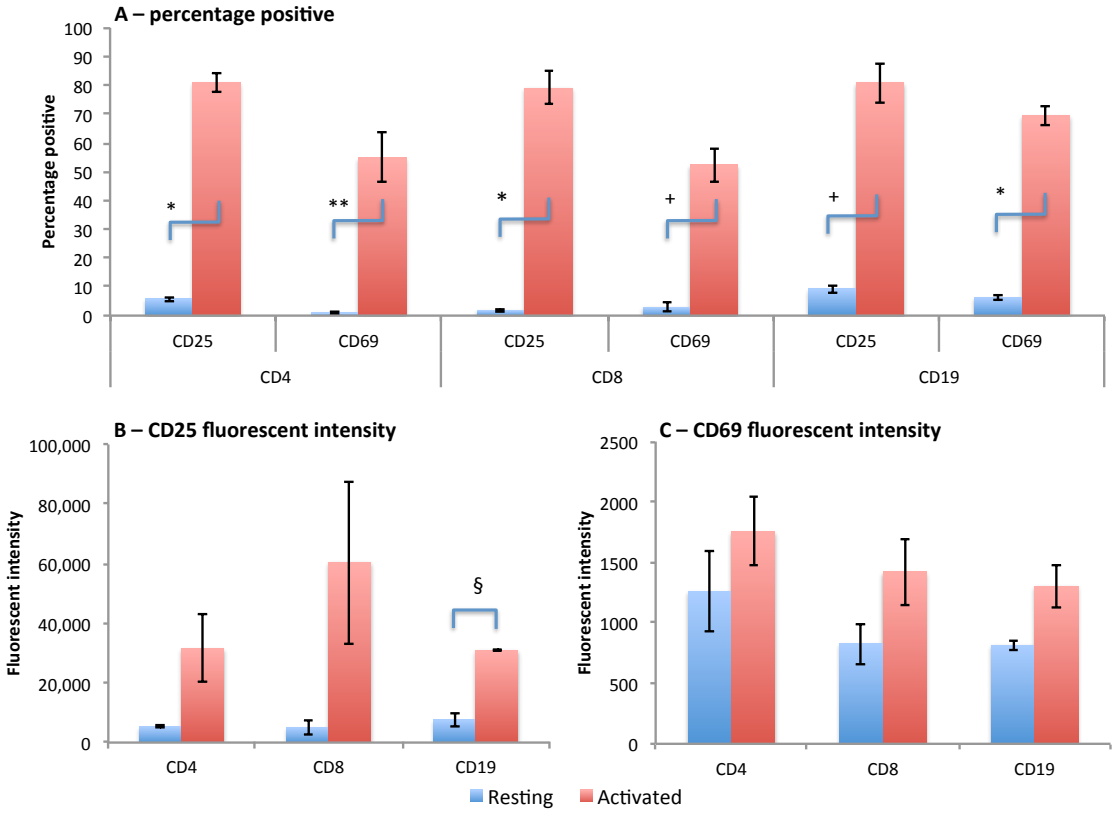

Figure 3-4: Effect of polyclonal activation on the expression of CD25 and CD69 by lymphocyte subsets within a PBMC population. **Panel A** – percentage of lymphocyte subsets expressing CD25 and CD69 * $p < 0.001$ ** $p = 0.001$ + $p = 0.003$. **Panel B** – fluorescent intensity of CD25 expression by lymphocyte subsets § $p = 0.006$. **Panel C** – fluorescent intensity of CD69 expression by lymphocyte subsets. Comparing panels B and C, there is a more marked difference in expression of CD25 between resting and activated cells, than for CD69. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

A similar pattern was seen for the $CD4^+$ cells in a population which was enriched for these cells. As shown in

Figure 3-5, activation with Miltenyi T_{reg} Suppression Inspector Beads induced statistically significant increases in the expression of CD25 (6.6% vs 73.4%; p = 0.013) and CD69 (1.4% vs 64%; p = 0.002), when measured as the percentage of positive cells. Activation also induced a statistically significant increase in the intensity of CD25 expression (3550.7 vs 46104; p < 0.001), but not the intensity of CD69 expression (1449.0 vs 2334.7; p = 0.344), at least at this 4-day time-point.

Expression of the Major Histocompatibility (MHC) antigens by Saos-2 cells

Major histocompatibility antigens / clusters (MHCs) are expressed on the surface of many human tissue cells. Whereas MHC (human leukocyte antigen, HLA in the humans) class I is expressed on all nucleated cells in humans, MHC class II expression is more restricted and is selectively expressed on antigen presenting cells (APCs) such as dendritic cells (DCs), monocytes/macrophages and B cells, as well as on certain populations of activated cells such as activated T cell populations and endothelial cells. MHC class II interacts with CD4 on T cells and, if this is allogeneic to the responding T cell, then potent T cell activation can be triggered. This process is known as the mixed lymphocyte reaction (MLR) if the triggering and responding cells are lymphocytes isolated from genetically dissimilar donors, and underpins the potent early rejection response that can be observed following organ transplantation. Given that

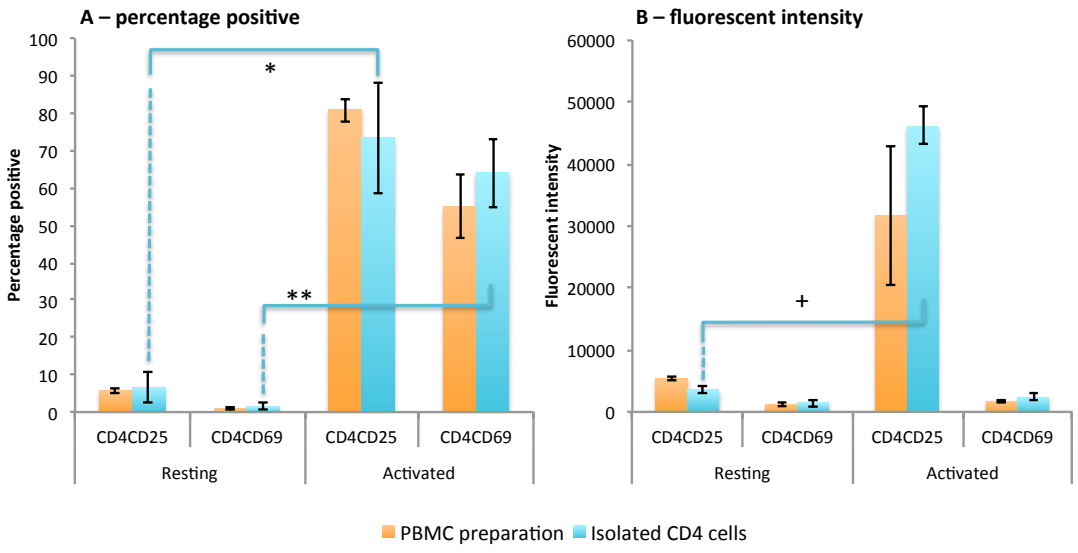

Figure 3-5: Effects of polyclonal activation on the expression of CD25 and CD69 by $CD4^+$ lymphocytes in PBMC and $CD4^+$ enriched populations. **Panel A** - percentage of lymphocytes positive * p = 0.013 ** p = 0.002. **Panel B** - fluorescent intensity + p < 0.001. Statistical significance of differences in the PBMC population are not repeated here. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

MHC (HLA) class II expression on Saos-2 cells could therefore be a potent trigger of T cells in the co-culture experiments, it was important to determine the expression of HLA class I and class I by the Saos-2 cells.

As the histograms in Figure 3-6 show, the Saos-2 cells expressed MHC class I but not MHC class II. Although the absence of MHC class II removes one potential confounding factor with regards to their potential ability to activate $CD4^+$ lymphocytes, the presence of MHC class I could provide a mechanism by which $CD8^+$ lymphocytes in the PBMC preparations are influencing the measure of proliferation of the Saos-2, in that $CD8^+$ T cells could lyse Saos-2 target cells via the recognition of HLA class I. Although the frequency of cells that have the potential to kill Saos-2 cells would be expected to be extremely low and so the level of killing would be minimal, the possible influence of such responses on the measurements made and the interpretation of the data needs to be appreciated.

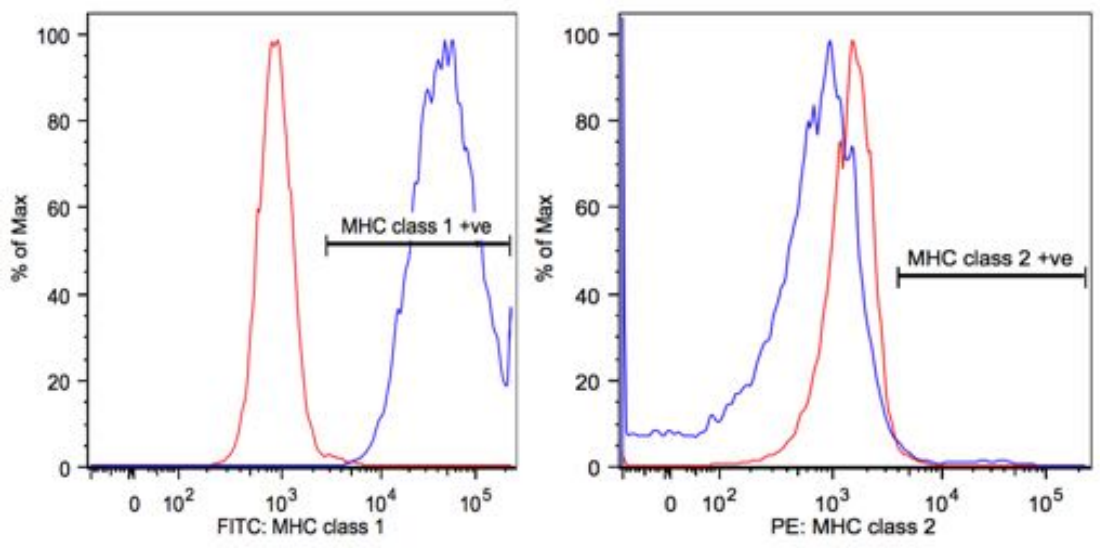

Figure 3-6: Representative fluorescence histograms showing the expression of MHC class I and II by Saos-2 cells. MHC class I (left panel – fluorescein [FITC] fluorophore), but not MHC class II (right panel – phycoerythrin [PE] fluorophore), expressed by Saos-2 cells. Red histograms represent isotype controls, blue histograms represent stained cells.

Effect of supernatants-derived from activated immune cell populations on the growth of Saos-2 cells

The aim of these experiments was to determine the effects of factors secreted by PBMCs or $CD4^+$ lymphocytes on the growth of Saos-2 cells. For these experiments, PBMCs or $CD4^+$ lymphocytes were incubated with Miltenyi T_{reg} Suppression Inspector

Beads in bone cell media (BCM) for four days, after which the cells and beads were removed to leave the activated cell supernatant (ACS). Saos-2 cells were then cultured for 5 days in fresh medium with increasing concentrations of the supernatant.

Growth profile of Saos-2 cells

Before undertaking experiments looking at the growth of Saos-2 cells, their growth in bone cell medium (BCM – as described in Methods and Materials) was studied. This was undertaken to establish their proliferation profile over the course of the experiments and identify lag and plateau phases.

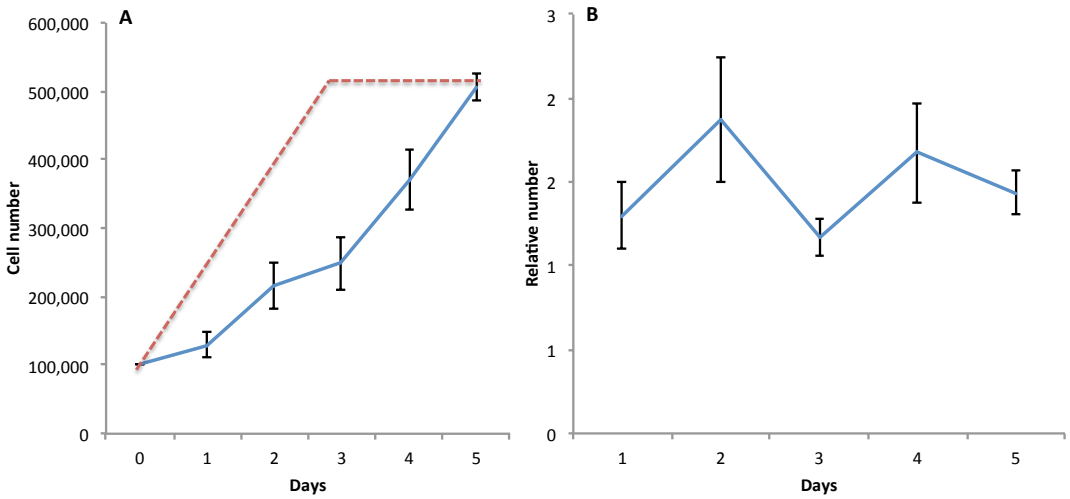

Figure 3-7: Saos-2 growth characteristics under normal culture conditions. **Panel A** - number of Saos-2 cells on days 1 to 5 when cultured in BCM; dashed red line indicates a theoretical population reaching its size limit. **Panel B** - cell number relative to that of the day before. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

The solid blue line of the graph in panel A in Figure 3.7 shows the absolute number of Saos-2 cells on each of the 5 days of the culture period. Cell numbers increased steadily over that period with no apparent lag or plateau phase. On day 5, the Saos-2 cells were still in a phase of growth, thereby indicating that culture periods of up to 5 days would be appropriate to assess the influence of any potential factor using kinetic-based analyses. If, for example, growth reached a plateau on day 3 (as indicated by a dashed red line on panel A in Figure 3-7) this could not be done. Panel B in Figure 3-7 plots the cell number relative to that of the day before, showing rate of growth on each day, and was calculated by dividing the cell number on day *x* by that of the day before. The relative number, although varying between 1.17 and 1.88, was not significantly

different for each day (t-test, p>0.05), thereby supporting the conclusion that the cells were in a steady growth phase.

Effect of activated PBMC and CD4$^+$ cell supernatants on the proliferation of Saos-2 cells

This analysis compared the number of Saos-2 cells after 5 days of culture in different concentrations of activated cell supernatants (ACS; 0-50% v/v) derived from activated PBMCs or CD4+ lymphocytes. ACS from activated PBMCs (ANOVA; p = 0.017) and CD4+ lymphocytes (ANOVA; p = 0.028) similarly inhibited the proliferation of the Saos-2 cells in a dose-dependent manner (Figure 3-8). Since only the concentration of ACS is seen to affect Saos-2 number, data were analysed using a one-way ANOVA. Although inhibition of proliferation was seen at a 6% v/v concentration, the inhibitory effect only reached statistical significance at a 50% v/v concentration for both media preparations, when compared to the medium alone control (PBMC 527,500 vs 275,317 p = 0.015; CD4 485,150 vs 213,000 p = 0.021).

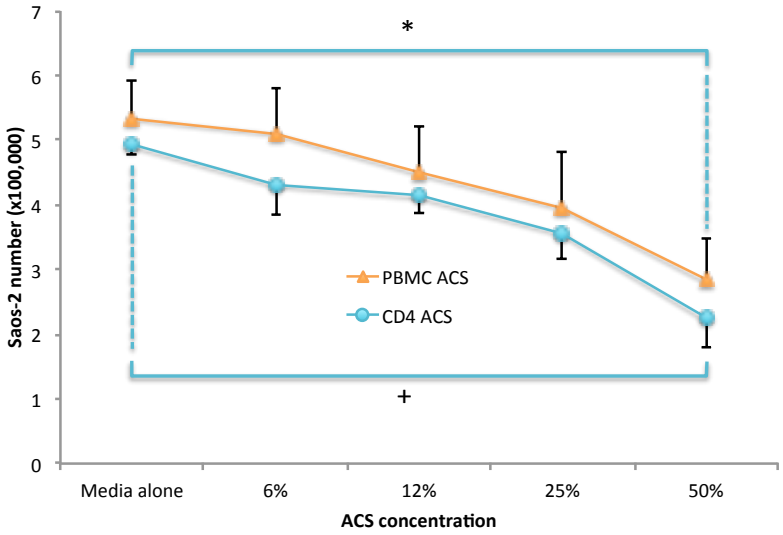

Figure 3-8: Effect of supernatants from polyclonally activated PBMCs and CD4$^+$ lymphocytes on the growth of Saos2 cells. Saos2 cells were cultured in medium alone or increasing concentrations of activated cell supernatant (ACS) for 5 days, at which time cell numbers were determined. Both forms of ACS dose-dependently inhibited the proliferation of Saos-2 cells, with the inhibitory effect becoming of statistical significance at 50% v/v for both ACS types. * p = 0.015 + p = 0.021. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

The influence of ACS on the number of Saos2 cells was also determined on each day of the 5-day culture period (Figure 3-9). Both ACS types had a negative impact on the numbers of Saos-2, with the effects being visible as early as day 1 or 2. These observations indicate that cell number (the dependent variable) is potentially

influenced by the two variables, time and ACS type, and so the data were analysed using two-way ANOVA. Although these are independent variables the growth curves indicate that their effects may be inter-dependent (called an interaction). As discussed in the Materials and Methods, this influences the statistical analysis. In brief, if the two variables interact then their effects must be considered individually (known as the simple main effects).

The two-way ANOVA comparing the PBMC and CD4-ACS to their respective controls revealed a statistically significant interaction between time and being cultured in ACS for both the PBMC-ACS ($p = 0.02$) and the CD4-ACS ($p = 0.02$) and the simple main effects are reported below for both ACS conditions.

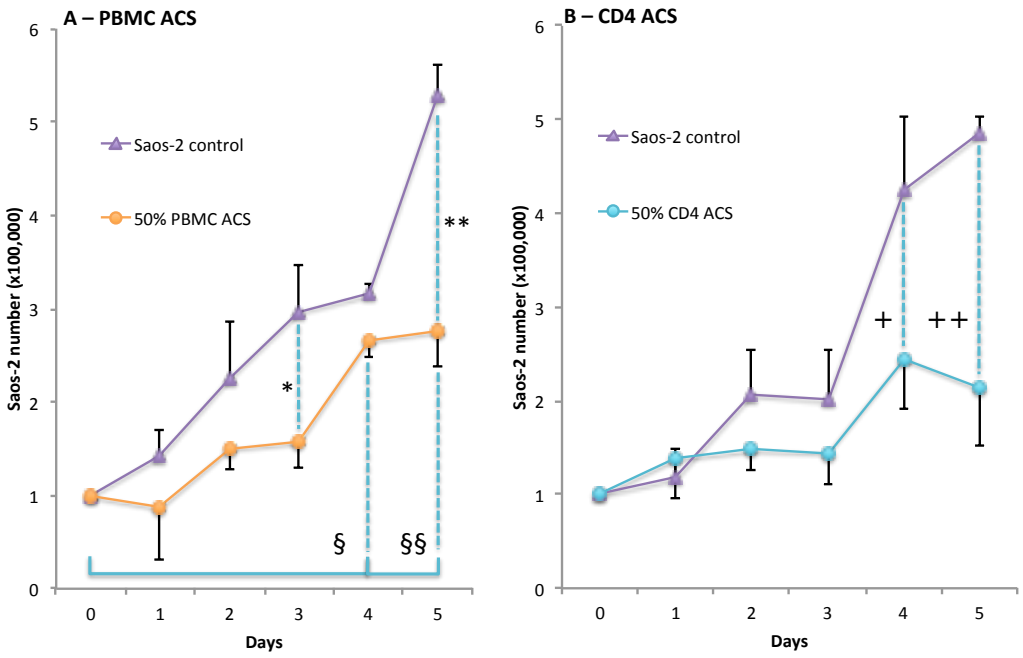

Figure 3-9: Effect of 50% v/v supernatants from polyclonally activated PBMCs and $CD4^+$ lymphocytes on the growth of Saos-2 cells. **Panel A -** the growth of Saos2 cells in the presence of ACS derived for activated PBMCs, with no statistical interaction between the variables presence of ACS and time. * $p = 0.017$ ** $p < 0.001$ § $p = 0.033$ §§ $p = 0.022$. **Panel B -** the growth of Saos2 cells in the presence of ACS derived from activated $CD4^+$ lymphocytes, with a statistical interaction between the variables presence of ACS and time + $p = 0.015$ ++ $p = 0.001$. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Cells grew in control media, the increase reaching significance on day 5 compared to day 1 (142,383 vs 527,500; $p < 0.001$). Saos-2 cells grew in PBMC-ACS ($p = 0.009$), increasing in number significantly by day 4 (87,683 vs 265,583; $p = 0.033$) through to day 5 (87,683 vs 275,317; $p = 0.025$) compared to day 1. However, they grew more slowly than those in the control medium, with significant reductions in the number of

Saos-2 cells on days 3 (297,183 vs 157,300; $p = 0.017$) and 5 (527,500 vs 275,317; $p < 0.001$).

In the CD4-ACS experiment, the Saos-2 cells grew in the control medium ($p < 0.001$), the increase in cell number compared to day 1 reaching significance on day 4 (117,883 vs 424,383; $p = 0.002$) and continuing to day 5 (117,883 vs 485,150; $p < 0.001$). However, in the 50% v/v CD4-ACS, cell numbers increased, but the difference was not significant ($p = 0.422$). This led to significant differences between the two conditions on day 4 (424,383 vs 244,933; $p = 0.015$) and day 5 (485,150 vs 213,000; $p = 0.001$).

Despite there being differences in the pattern of statistical significance between the two experiments, on inspection the growth profiles appear broadly similar showing slower growth in the ACS condition of both experiments, relative to its control, over the five-day culture period. Comparing the growth curves of Saos-2 cells in ACS derived from activated PBMCs and $CD4^+$ lymphocytes using a two-way ANOVA revealed no significant difference between the two conditions. There was growth overall ($p = 0.006$), with increased numbers of cells on day 4 (mean difference 142,141; $p = 0.016$) and day 5 (mean difference 131,041; $p = 0.030$) compared to day 1.

In summary, these data indicate that ACS derived from both PBMCs and $CD4^+$ lymphocytes slows the growth of Saos-2 cells, and this effect is cumulative over time. However, no clear difference between the ACS types was demonstrated.

Series 1 - influence of resting and activated immune cell populations on the growth of Saos-2 cells

The data so far indicate that supernatants from activated PBMCs and $CD4^+$ cells inhibit the growth of Saos-2 cells. Although this supports the hypothesis that inflammatory processes could affect bone metabolism, more complex cell-mediated processes may be involved *in vivo*. Therefore, a series of experiments were undertaken to investigate a series of potential factors: immune cell type i.e. PBMC or $CD4^+$ lymphocyte enriched, activated or resting, contact between immune and bone cell, and the relative proportion of Saos-2 cells to immune cells. The growth of Saos-2 cells in response to these factors was measured, as were changes in the activation status of the immune cells.

Identification of Saos-2 cells and PBMCs in mixed cultures

One practical problem with this series of experiments was that whilst resting PBMCs and Saos-2 cells form distinct populations on FSC vs. SSC plots, activated PBMCs become larger, denser, and begin to 'encroach' into the light scatter region for the Saos-2 population (see Figure 3-10). This makes it difficult to identify the two cell types in a mixed population based on their FSC vs. SSC characteristics alone. It was therefore necessary to identify cells on the basis of discriminating surface antigen expression.

CD45 is a surface antigen expressed only by cells of the haematopoietic system. Osteoblasts are mesenchymal in origin and so do not express this marker. CD45 expression was therefore selected as a potential cell surface antigen which can distinguish the two different cell types in a mixed population, and a Pacific Blue™-conjugated mAb was used. This approach allowed the two cell types to be identified in a mixed population (see Figure 3-11). All the cells in the lymphocyte populations express CD45, as the histogram of stained lymphocytes has shifted to the right for the stained cells. Saos-2 did not express CD45, as the histograms for the isotype controls and stained cells overlay each other. The overlay histograms for one experiment (Experiment 3) exhibited some overlap of the Saos-2 and activated lymphocytes with regards to Pacific Blue™ fluorescence. The reasons for this are not clear.

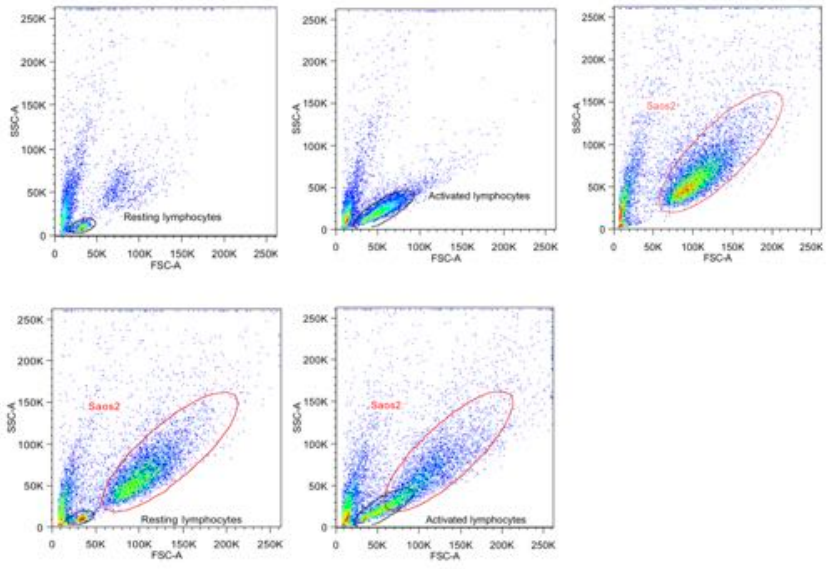

Figure 3-10: Representative FSC vs. SSC scatter plots demonstrating the encroachment of activated lymphocytes into the light scatter region of Saos-2 cells.

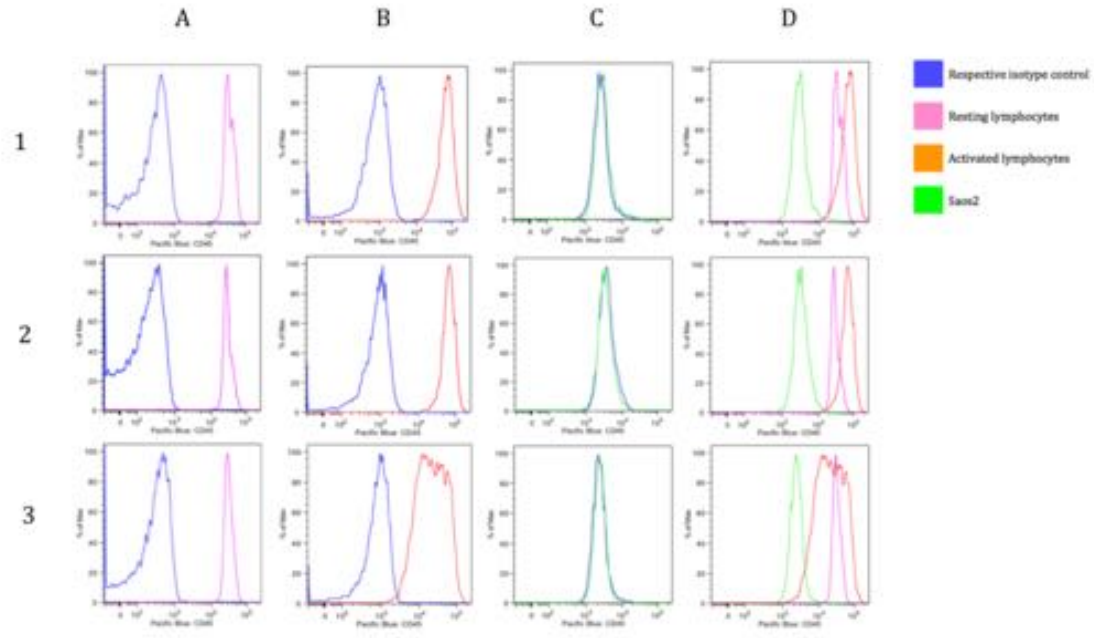

Figure 3-11: Representative fluorescence histograms of CD45 expression by lymphocytes and Saos-2 cells. Resting lymphocytes (column A), activated lymphocytes (column B) and Saos-2 cells (column C) with their respective isotype controls. Overlay histograms for the three cells types in each experiment are illustrated in Column D.

Influence of resting and activated PBMCs on the growth of Saos-2 cells

Increasing numbers of activated immune cells had a negative effect on Saos-2 growth (see Figure 3-12), a finding consistent with the effects of the activated cell supernatant. Resting PBMCs had an opposite effect, and the overall effect was to *increase* the growth of Saos-2, which was an unexpected finding. The presence of a transwell insert appears to promote the growth of the Saos-2 in both the resting and activated conditions.

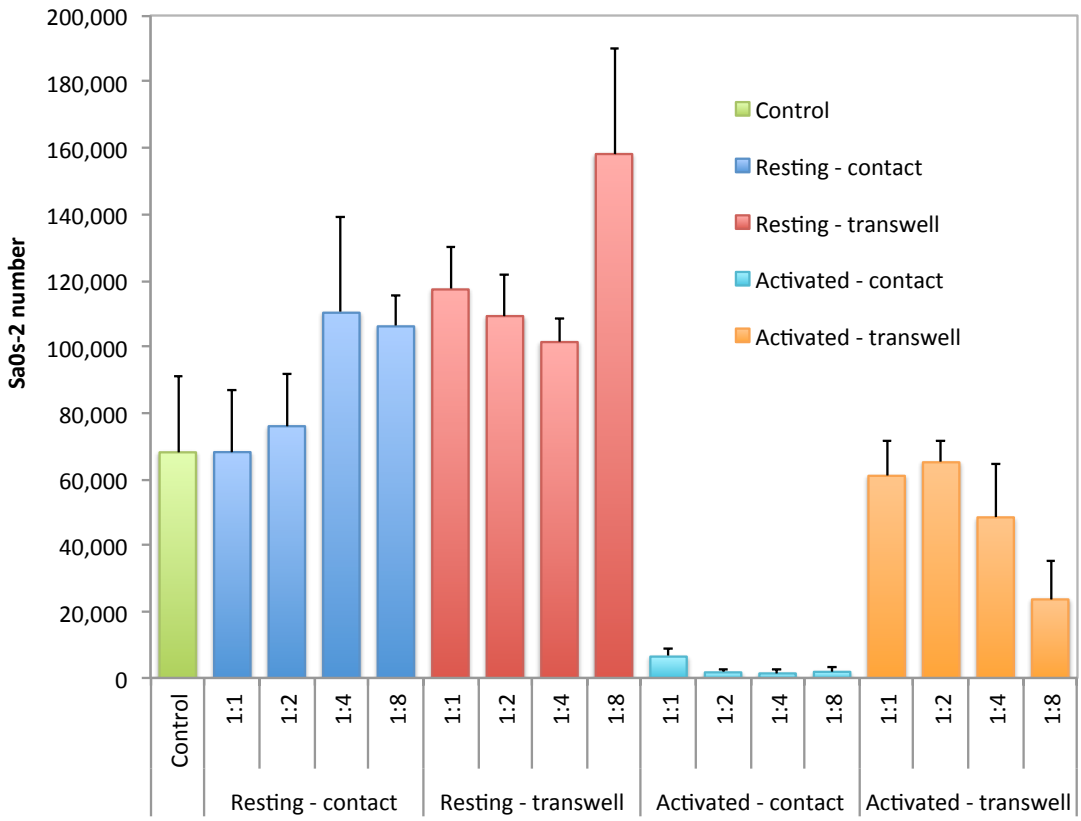

Figure 3-12: Effects of resting and activated PBMCs on the growth of Saos-2 cells. Several factors are presented: activation status (resting or activated), contact status (in contact or separated by a transwell insert) and PBMC number (ratios are Saos-2 to immune cells e.g. 1:8 represents 100,000 Saos-2 cells and 800,000 PBMCs). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

The data was normally distributed but there was excessive variance (Levene's test for homogeneity of variances, $p = 0.002$), addressed by carrying out a square-root transformation of the data (so that $p = 0.088$). There was no statistically significant interaction between the three independent variables of cell number, activation status and contacts status ($p = 0.204$). However, there were significant interactions between each possible pairing of independent variables.

Two-way interaction between activation status and number of immune cells

There was a statistically significant interaction between activation status and the number of immune cells ($p < 0.001$) and their effects are shown in Figure 3-13. Increasing the number of resting PBMCs increased the growth of Saos-2 cells ($p = 0.024$), and at a Saos-2 cell: PBMC ratio of 1:8 the increase was statistically significant when compared to control (mean difference -106 [95% CI -198 to -14.7]; $p = 0.013$). Increasing the number of activated PBMCs progressively inhibited the growth of Saos-2 cells ($p < 0.001$), and at a Saos-2 cell: PBMC ratio of 1:2 the decrease was

statistically significant (mean difference 106 [95% CI 15 to 198]; p = 0.013). At all PBMC to Saos-2 ratios there was a significant difference in the number of Saos-2 cells in the activated and resting conditions (p < 0.001 for all ratios).

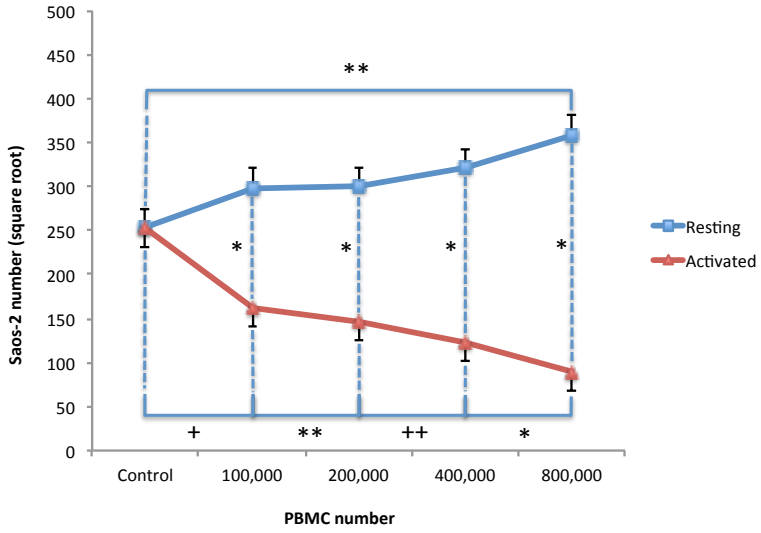

Figure 3-13: The two-way interaction between activation status and PBMC number. *p < 0.001 ** p = 0.013 $^{+}$ p = 0.05 $^{++}$ p = 0.001 (values are Bonferroni adjusted for multiple comparisons; α = 0.05). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Therefore, in keeping with the finding of an interaction, the effect of cell number is dependent on the activation status of the PBMCs. Resting PBMCs promote Saos-2 growth whilst activated cells are inhibitory, with the latter having a greater overall effect.

Two-way interaction between contact status and number of immune cells

There was a statistically significant interaction between contact status and the number of immune cells (p = 0.027) and their effects can be seen in Figure 3-14. It shows that in the contact condition the presence of PBMCs reduced growth, but increased growth when separated by transwell insert.

Increasing the PBMC number had a statistically significant effect on the growth of Saos-2 cells when in contact (p = 0.025), but not in the presence of a transwell insert (p = 0.632). Although increasing the number of PBMCs in contact reduced the growth of Saos-2 the difference was only statistically significant at a ratio of 2:1 compared to control (mean difference 97.588 [95% CI 6.16 to 189]; p = 0.029), but there is no dose response apparent. For all PBMC to Saos-2 ratios the presence of a transwell insert significantly increased the number of Saos-2 cells.

Therefore, it appears that when PBMCs are in contact with Saos-2 they have a negative effect on growth, but when separated by a transwell insert the effect is positive.

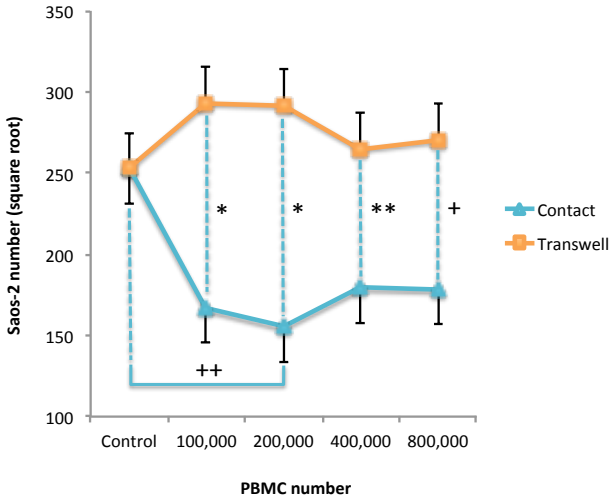

Figure 3-14: The two-way interaction between contact status and PBMC number. * $p < 0.001$ ** $p = 0.008$ + $p = 0.005$ $p = 0.029$ (p values are Bonferroni adjusted for multiple comparisons, $\alpha = 0.05$). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Two-way interaction between activation status and contact status

There was a statistically significant interaction between the activation status of the immune cells and the contact status ($p = 0.001$).

As can be seen in Figure 3-15 the presence of a transwell insert significantly increased the number of Saos-2 cells in both the resting (mean difference 40.729 [95% CI -80 to -1]; $p = 0.043$) and activated (mean difference -136 [95% CI 175 to 97]; $p < 0.001$) conditions, with the effect being greater in the activated condition. Activated PBMCs significantly reduced the number of Saos-2 cells when in contact (mean difference 199 [95% CI 160 to 239]; $p < 0.001$), and in the presence of a transwell insert (mean difference 104 [95% CI 65 to 144]; $p < 0.001$). The effect was greatest in the contact group.

This shows that the overall effects of resting and activated PBMCs i.e. positive or negative, are not affected by contact status; but Saos-2 number are greater in the presence of a transwell insert.

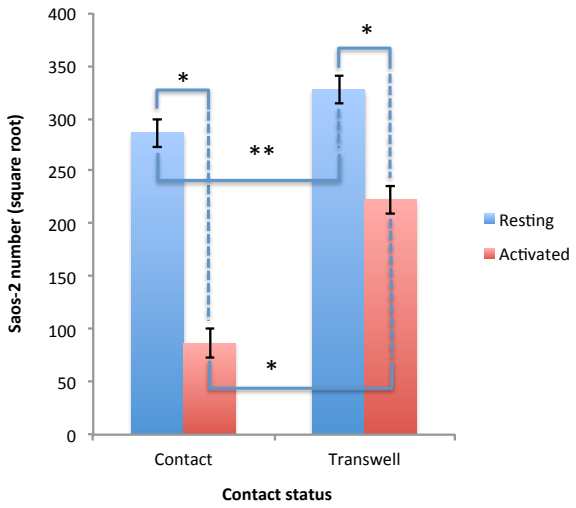

Figure 3-15: Two-way interaction between activation status and contact status in the PBMC co-culture. * $p < 0.001$ ** $p = 0.043$ (values are Bonferroni adjusted for multiple comparisons; $\alpha = 0.05$). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Influence of resting and activated $CD4^+$ lymphocytes on the growth of Saos-2 cells

The co-culture experiments were repeated using a $CD4^+$ cell-enriched population.

Figure 3-16 illustrates all three experimental factors and their effect on Saos-2 number at the end of the culture period. The findings are like those for the PBMC experiments, with resting $CD4^+$ cells seemingly promoting the growth of Saos-2, and activated $CD4^+$ cells inhibiting growth. In the activated conditions, the presence of a transwell insert appeared to lessen the inhibitory effect. As for the PBMC experiments, data were analysed by three-way ANOVA. Although data were normally distributed and there was homogeneity of variance, it was square root transformed to be treated in a similar way to the PBMC data.

There was no statistically significant three-way interaction between cell number, activation status and contacts status ($p = 0.283$). Of three potential two-way interactions, activation status/cell number ($p < 0.001$) and activation status/contact status ($p = 0.001$) were statistically significant. The interaction between contact status and cell number was the only non-significant interaction.

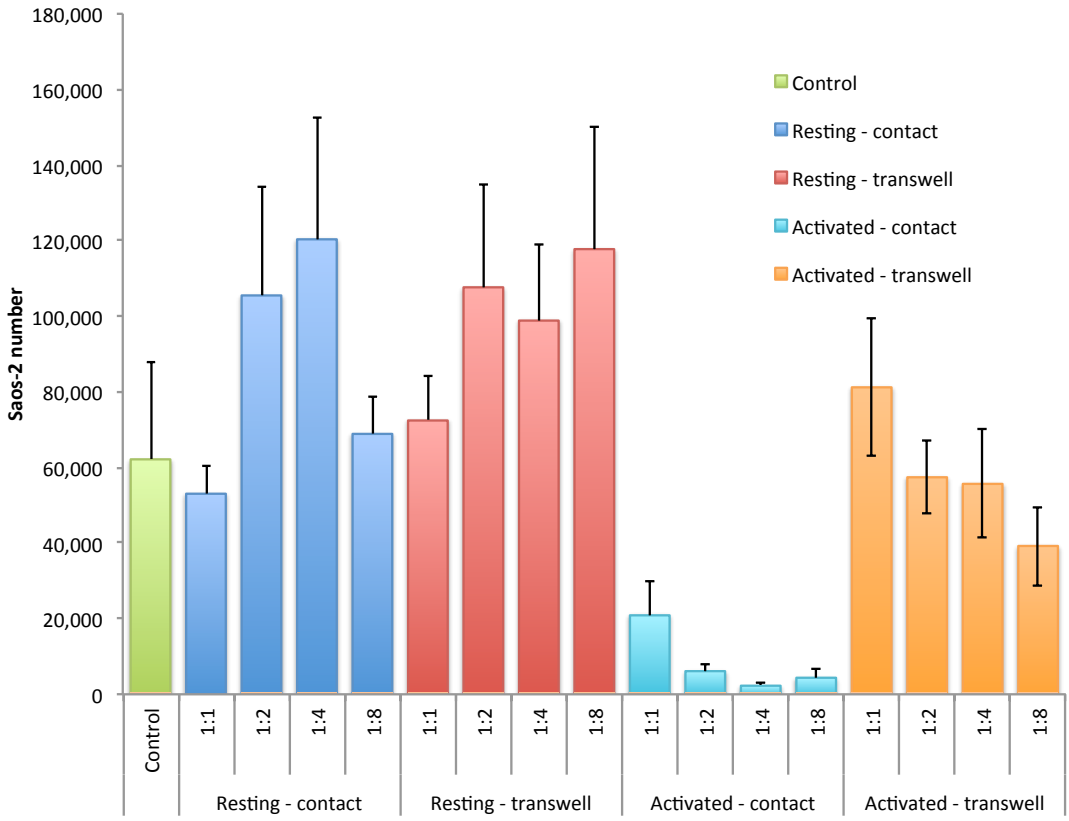

Figure 3-16: Effects of a $CD4^+$ lymphocyte enriched population on the growth of Saos-2 cells. Three factors are presented: activation status (resting or activated), contact status (in contact or separated by a transwell insert) and $CD4^+$ lymphocyte enriched population cell number (ratios are Saos-2 cell to $CD4^+$ lymphocyte enriched population e.g. 1:8 represents 100,000 Saos-2 cells and 800,000 $CD4^+$ lymphocyte enriched population). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Two-way interaction between activation status and number of $CD4^+$ lymphocytes

There was a statistically significant interaction between activation status and the number of $CD4^+$ lymphocytes ($p < 0.001$).

The number of $CD4^+$ lymphocytes significantly influenced Saos-2 cell growth, increasing it in the resting condition ($p = 0.035$) and decreasing it in the activated condition ($p = 0.002$), with an apparent dose response effect (see Figure 3-17). However, whilst the difference relative to control reached significance in the activated condition at 400,000 Saos-2 cells (mean difference 110.1 [95% CI 8.33 to 211.8]; $p = 0.026$) and 800,000 (mean difference 112.0 [95% CI 10.23 to 213.7; $p = 0.022$), none of the differences to control were statistically significant in the resting condition.

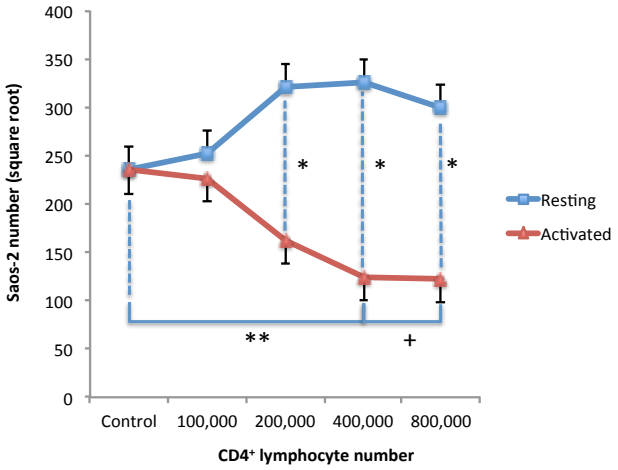

Figure 3-17: Two-way interaction between activation status and $CD4^+$ lymphocyte number. * $p < 0.001$ ** $p = 0.026$ + $p = 0.022$ (values are Bonferroni adjusted for multiple comparisons; $\alpha = 0.05$). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

A reciprocal analysis of the effect of activation at differing numbers of $CD4^+$ lymphocyte enriched population cells found that activation status caused a statistically significant difference in Saos-2 cell number at Saos-2 cell: $CD4^+$ lymphocyte enriched population cell ratios of 1:2 and above.

Therefore, in keeping with the finding of an interaction, the effect of cell number is dependent on the activation status of the $CD4^+$ lymphocyte enriched population with resting cells seemingly promoting Saos-2 growth and activated cells inhibiting it. This is similar to finding for the PBMCs.

The two-way interaction between activation status and contact status

There was a statistically significant interaction between the activation status of the $CD4^+$ lymphocyte enriched population cells and the contact status ($p = 0.001$) shown in Figure 3-18.

For the resting $CD4^+$ lymphocytes presence of a transwell insert resulted in a non-significant increase in the growth of Saos-2 cells (mean difference -16 [95% CI -59.710 to 27.830]; $p = 0.466$), but a significant increase in the growth of Saos-2 cells in the activated condition (mean difference -120.652 [95% CI -164.423 to -76.882]; $p < 0.001$).

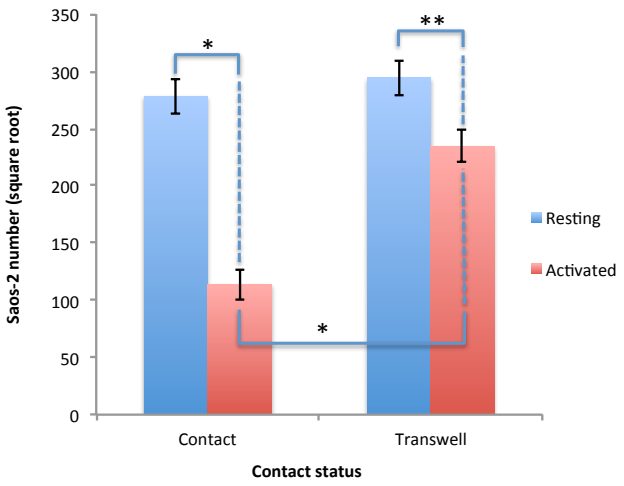

Figure 3-18: Two-way interaction between activation status and contact status in the $CD4^+$ lymphocyte enriched population co-culture * $p < 0.001$ ** $p = 0.008$ (values are Bonferroni adjusted for multiple comparisons; $\alpha = 0.05$). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

The reciprocal analysis found that for both cells in contact, and those separated by a transwell insert, activation depleted cell numbers significantly. In the contact condition, activation numbers fell by 165 (95% CI 121 vs 208; $p < 0.001$), whereas in the transwell condition they fell by 60 (CI 16 to 104; $p = 0.008$).

The two-way interaction between contact status and cell number

There was no statistically significant interaction between contact status and the number of $CD4^+$ lymphocyte enriched population cells. On inspection of the graph in Figure 3-19, contact appears to reduce Saos-2 cell numbers relative to control, and the presence of a transwell insert increases Saos-2 cell number.

In both the contact and transwell groups, there was no statistically significant difference in proliferation compared to their respective controls. However, there are visible differences between the two groups on each day. They reach statistical significance at 100,000 (197 vs 282; $p = 0.017$), 200,000 (192 vs 259; $p = 0.017$) and 800,000 (159 vs 263; $p = 0.004$).

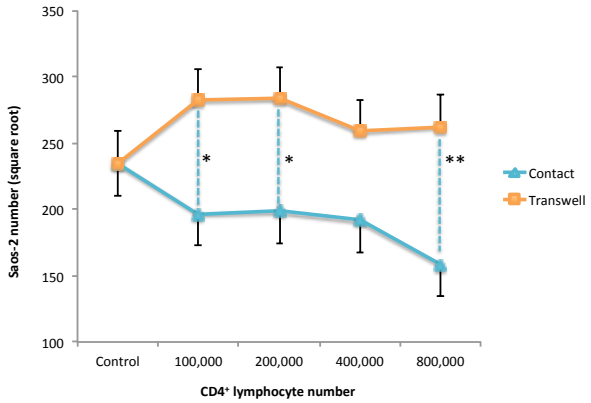

Figure 3-19: Two-way interaction between contact status and $CD4^+$ lymphocyte number. * p = 0.017 ** p = 0.004 (p values are Bonferroni adjusted for multiple comparisons, α = 0.05) Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Comparison of the effect of PBMCs and $CD4^+$ lymphocytes on the growth of Saos-2 cells

The purpose of this section is to bring together the results of the two co-culture experiments. The experiments were, independently, complex and considered several inter-dependent variables. The statistical analysis reflects this, with it not being possible to discuss the effects of each independent variable due to interactions between them. It is also desirable to compare the effects of PBMCs and $CD4^+$ lymphocytes on the growth of Saos-2 cells. Figure 3-20 repeats all the graphs in Figures 3.12 to 3.19, placing them next to each other for comparison. The patterns for each corresponding graph appear similar, indicating that there is little difference in the effect of PBMCs and $CD4^+$ cells on Saos-2 cell growth. Table 3-1 summarises the statistical analyses and, whilst there are differences, the effects are broadly similar.

The two immune cell subtypes were compared. A T-test established that there was no statistically significant difference between the PBMC and $CD4^+$ lymphocyte enriched population controls (68,117 vs 58,060; $p > 0.05$). After this, a series of two-way ANOVAs were carried out comparing the groups. Excessive variance in the data precluded 4- and 3- way ANOVA, despite square root transformations (maintained for consistency with the previous analyses). There were no statistically significant two-way interactions, although interactions are seen in the graphs (see Figure 3-21). Comparing the effects of PBMCs and $CD4^+$ lymphocyte enriched population cells on Saos-2 number, a difference was only seen in the activated contact group (45 vs 82; $p = 0.008$), for all others there was no significant difference.

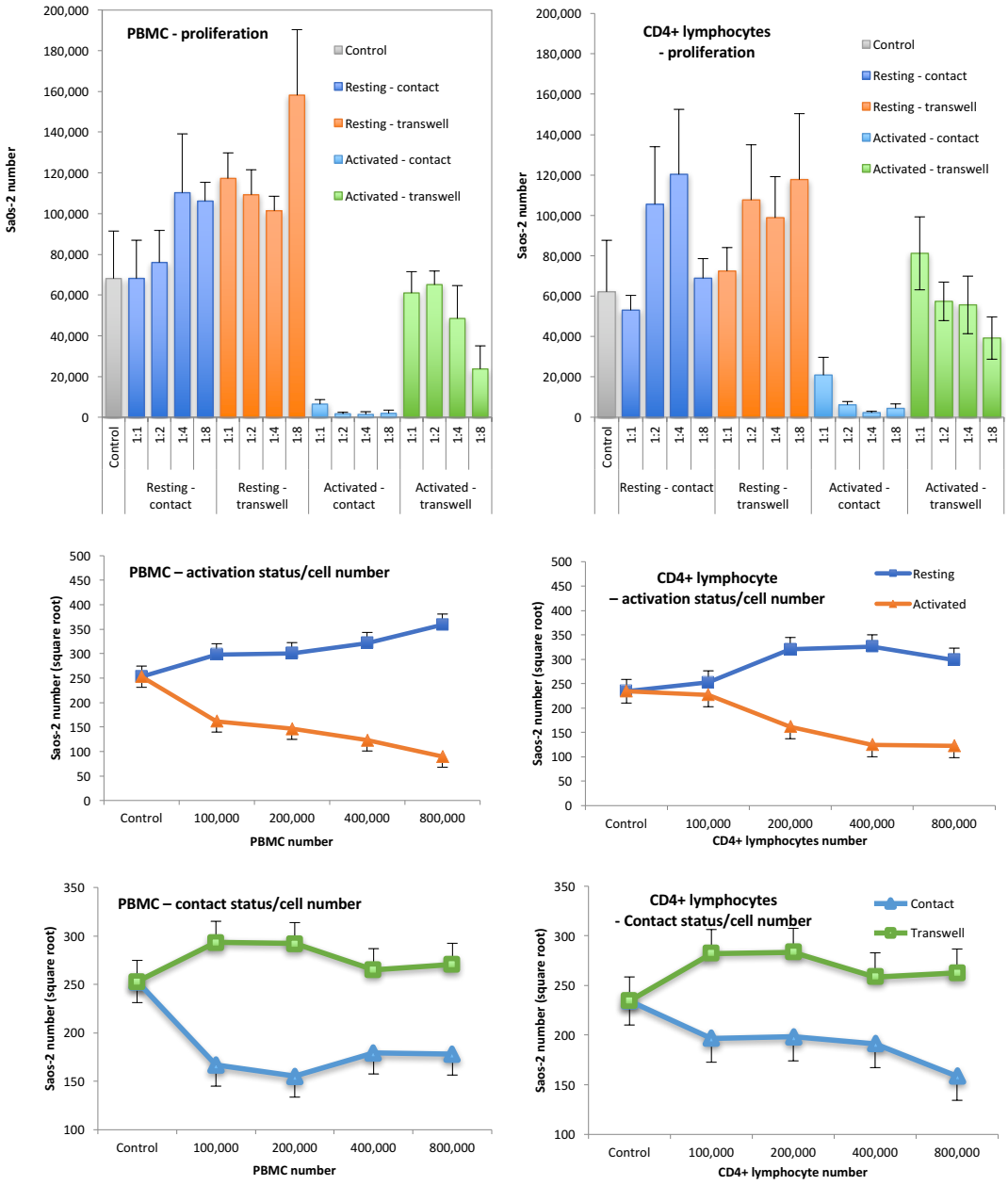

Figure 3-20: Comparison of data patterns in the PBMC and CD4$^+$ lymphocytes experiments. The graphs are those seen in Figures 3.12-19, repeated here to enable comparison of data patterns between the two immune cell populations.

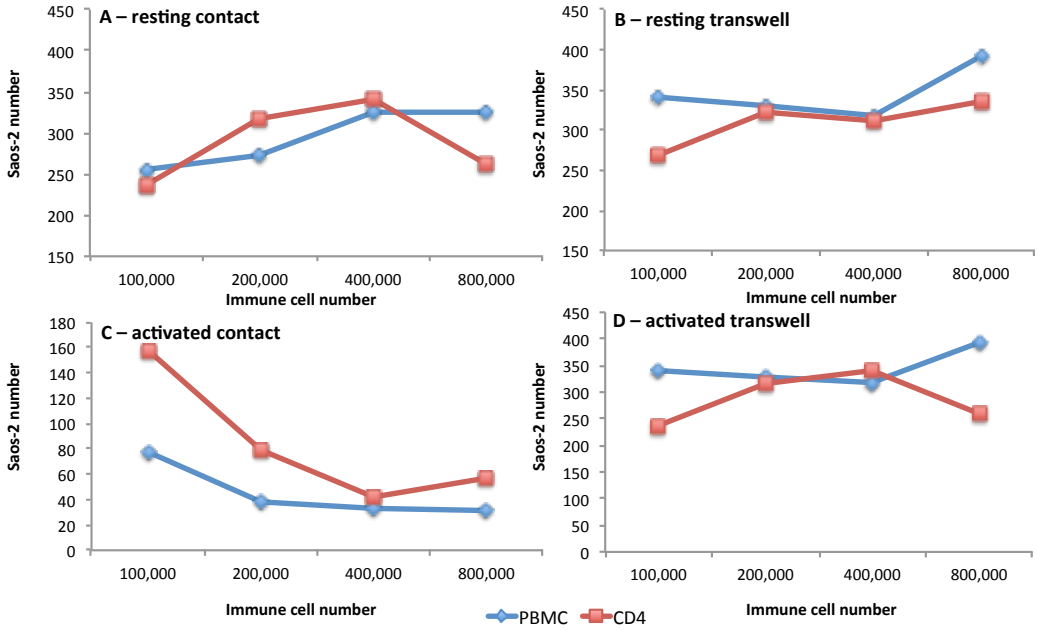

Figure 3-21: Direct comparisons of the effects of PBMCs and $CD4^+$ lymphocyte enriched population cells on Saos-2 proliferation. Y axes are the square root of Saos-2 number and X axes the number of immune cells. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means. Error bars are not included due to overlap precluding meaningful interpretation.

Simple main effect	Levels	PBMC	CD4
Cell number	Activation status	$p = 0.024$ in resting $p < 0.001$ in activated, suggesting greater effect in activated	Only increasing activated cell number affected Saos-2
	Contact status	Only 200,000 contact condition different from control, suggesting no real effect	No significant interaction
Activation status	Cell number	Activation reduced Saos2-number for all ratios, suggesting strong effect of activation	At ratios 1:2 and above, activation reduced Saos-2 number
	Contact status	Activation reduced Saos2-number for both contact states, suggesting strong effect of activation	Activation reduced Saos2-number for both contact states, suggesting strong effect of activation
Contact status	Cell number	Presence of a transwell insert increases Saos-2 number at all ratios	No significant interaction
	Activation status	Presence of a transwell insert increases Saos-2 number in both activation conditions	Presence of a transwell insert increases Saos-2 number in only the activated condition

Table 3-1: Summary of the findings from both three-way ANOVAs. It shows the six combinations of the three variables, and their outcome in the PBMC and CD4 conditions.

Effect of Saos-2 cells on the activation status of lymphocytes

Whilst the primary aim of this experiment was to look at the effects of immune cells on Saos-2 cell growth, using flow cytometry it was also possible to investigate the reciprocal effects of Saos-2 cells on the activation status of immune cells.

Effect of Saos-2 cells on expression of activation markers by lymphocytes within a PBMC population

Within the PBMC population it was possible to examine changes in expression of CD25 and CD69 by $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes (see Figure 3-22). Expression of activation markers, as measured by the percentage of cells positive, within both the resting and activated conditions, follows a similar pattern for all three lymphocyte types. In the resting conditions, Saos-2 cells induce expression of activation markers, whereas in the activated condition the reverse is seen, with Saos-2 cells inhibiting expression.

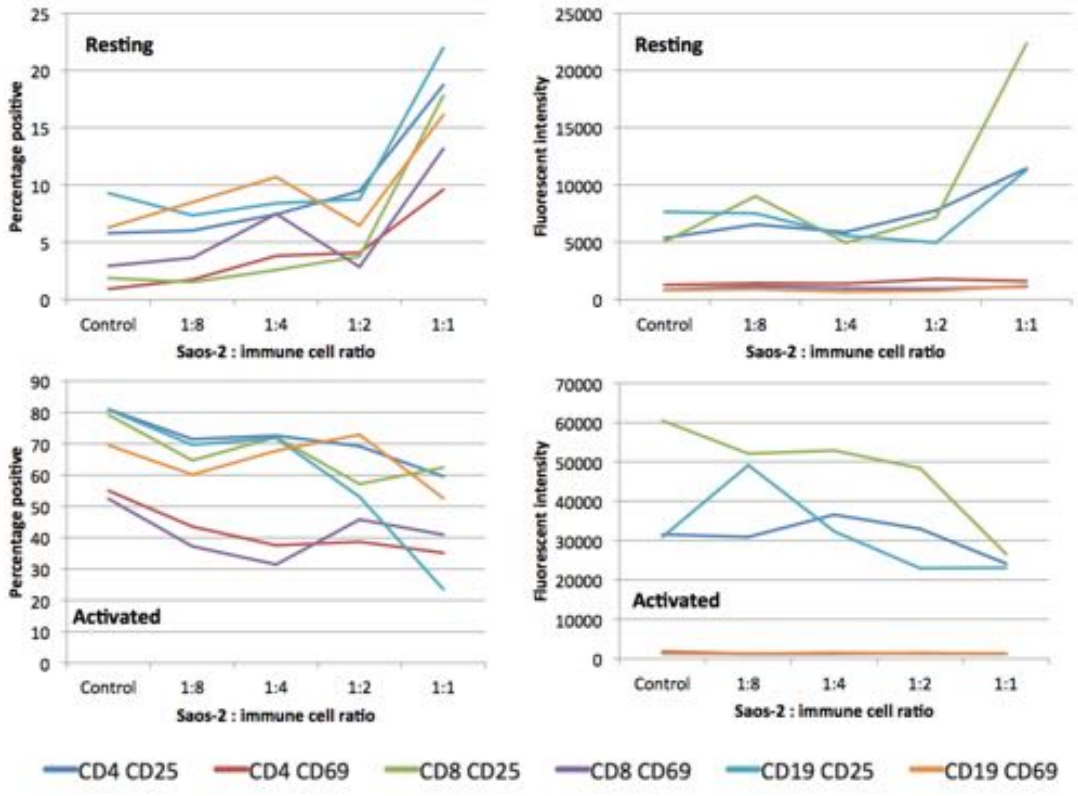

Figure 3-22: Influence of Saos-2 cells on the expression of CD25 and CD69 by $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes within a PBMC population. Expression of markers is measured on the basis of the percentage of cells positive (left), and the intensity of expression (right). The upper graphs show the findings in the resting PBMC populations, the lower graphs the findings in the activated condition. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means. Error bars are not shown due to their overlap precluding meaningful interpretation.

When measured by intensity of the fluorescent signal, expression of CD25 follows the same pattern as when measured by the percentage of cells positive. The expression of CD69 measured by fluorescent intensity is not affected by the increasing number of immune cells, and is markedly lower than that of CD25 (see Figure 3-22). This may be because CD69 is an early activation marker and the intensity of expression reduces over the 4-day period, although this does not explain why the percentage of cells expressing CD69 is still affected by Saos-2 number. It may be that different activation pathways are triggered.

Activation of $CD4^+$ve lymphocytes within a PBMC population

Figure 3-23 show changes in expression of CD25 and CD69 by $CD4^+$ lymphocytes in a resting PBMC population, as measured on the basis of fluorescent intensity (FI) and the proportion (%) of cells that are positive for the antigen, in relation to the ratio of Saos-2 cells to PBMCs and their contact status. In the resting PBMC conditions, an increasing number of Saos-2 cells was associated with an increase in expression of CD25, as measured by FI and percentage of cells positive. For CD69 expression, there was an increase in the proportion of cells expressing the activation antigen, but not the intensity of expression. The presence of a transwell insert had no effect.

In the activated condition, the effect of Saos-2 cell number was less clear (see Figure 3-24). Although a dose-response effect in relation to the percentage of $CD4^+$ lymphocytes that were positive for CD25 and CD69 expression was observed, no dose-response effect was seen for the intensity of CD25 and CD69 expression. As for the resting PBMC conditions, contact status has no apparent effect.

A two-way ANOVA was used compare the effect of contact status and Saos-2 cell number on the expression of activation markers by $CD4^+$ cells in the resting PBMC population, but no statistically significant differences were found. This is surprising given the appearance of the data, but large variances within the data, which could not be resolved by either square root or log transformations, made establishing differences difficult.

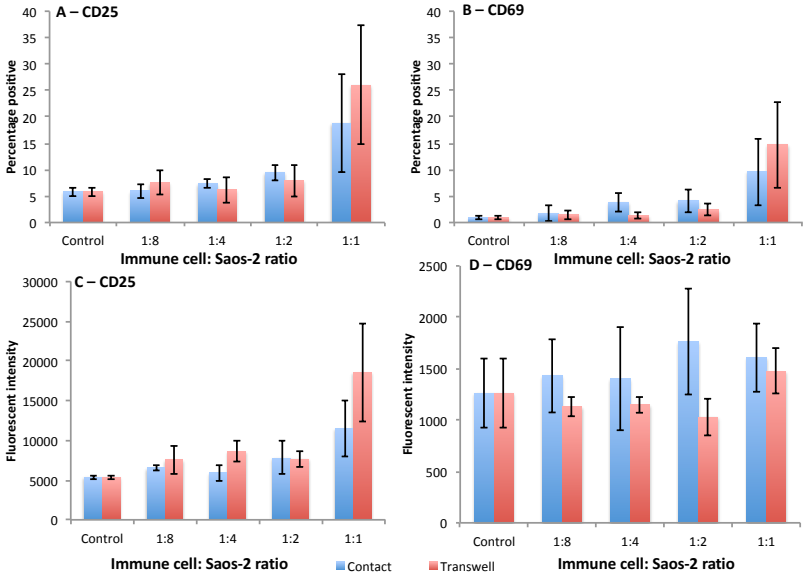

Figure 3-23: Relationship between ratios of Saos-2 cells to resting PBMCs, and the effect of contact status, on expression of CD25 and CD69. A Saos-2: PBMC ratio of 1:8 indicates 1 Saos-2 cell for every 8 PBMCs. Saos-2 cells appear to induce expression of the activation markers CD25 and CD69 by $CD4^+$ lymphocytes, within a population of resting PBMCs. As the proportion of Saos-2 cell increase, there is an increase in the percentage of $CD4^+$ lymphocytes expressing CD25 (panel A), CD69 (panel B) and the fluorescent intensity of CD25 expression (panel B). The fluorescent intensity of CD69 expression does not show the same pattern (panel D). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

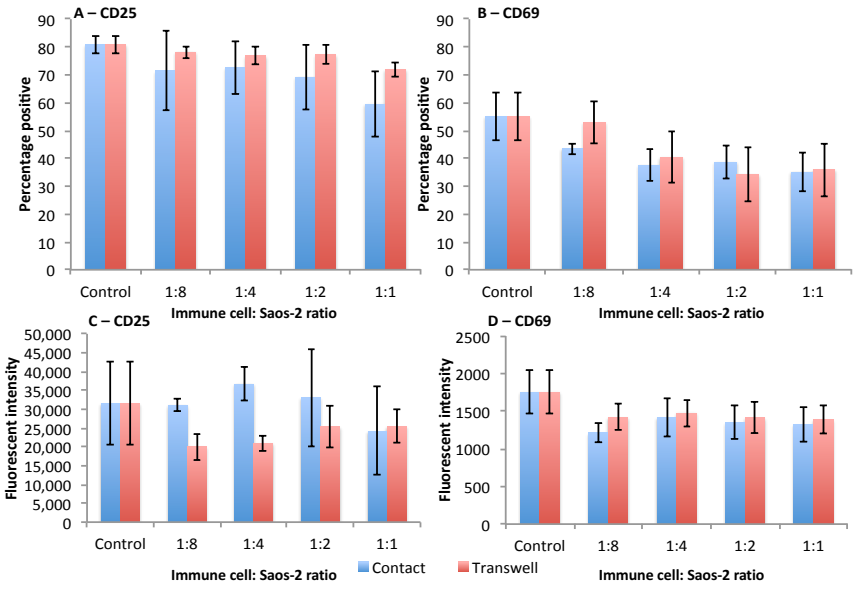

Figure 3-24: Relationship between ratios of Saos-2 cells to activated PBMCs, and the effect of contact status. A Saos-2: PBMC ratio of 1:8 indicates 1 Saos-2 cell for every 8 PBMCs. Saos-2 cells inhibited the expression of activation markers by $CD4^+$ lymphocytes in an activated PBMC population, although the effect is not as clear as that seen for the resting PBMCs. As numbers of Saos-2 cells increase the proportion of cells expressing CD25 (panel A) and CD69 (panel C) is reduced in a dose-dependent manner. No effects on the intensity of CD25 (panel B) and CD69 (panel D) were observed. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Activation of $CD4^+$ lymphocytes within a $CD4^+$ lymphocyte enriched population

Figure 3-25 show the changes in expression of activation markers by resting $CD4^+$ lymphocytes in a $CD4^+$ lymphocyte enriched population, in relation to Saos-2 cell number and cell contact status. On inspection, a dose-response can be seen for all four measures of activation markers i.e. percentage of cells positive and FI. Also, in relation to the percentage of $CD4^+$ lymphocytes expressing CD25, the presence of a transwell insert appeared to increase CD25 expression.

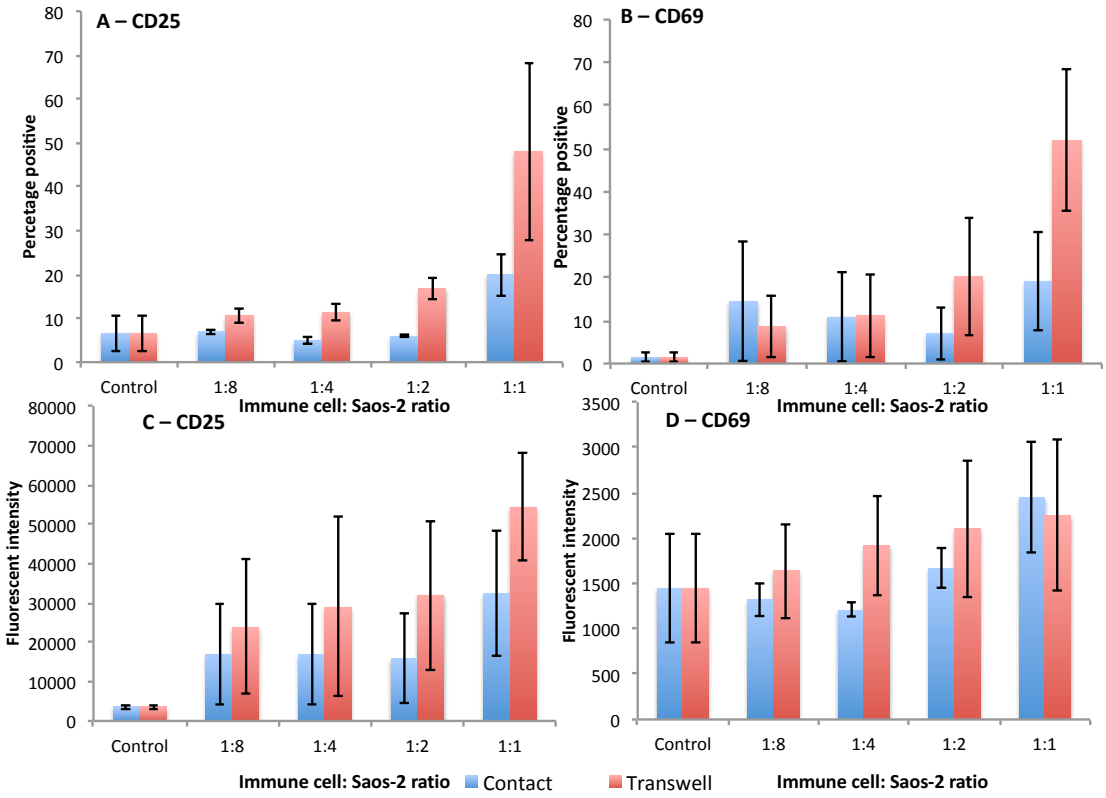

Figure 3-25: Expression of activation markers by a resting, purified $CD4^+$ cells in a $CD4^+$ lymphocyte enriched population is induced by co-culture with Saos-2 cells. Increasing proportions of Saos-2 cells (ratios are Saos-2: CD4+ve) resulted in increased percentage of $CD4^+$ cells expressing CD25 (panel A) and CD69 (panel C), and the fluorescent intensity of CD25 expression (panel B). Fluorescent intensity of CD69 does appear to show an increase in expression, although the differences are not statistically significant. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Figure 3-26 shows the same variables for the activated immune cell condition. Associations between activation marker expression and Saos-2 cell number are much less clear, and whilst it could be said that all four measures of surface marker expression were reduced at a 1:1 ratio of Saos-2 cell : $CD4^+$ lymphocyte enriched population cells the simple dose relationship is variable. In fact, expression of surface markers is initially increased (for all except the percentage of cells positive for CD25).

Although there are differences between the groups in contact and the groups where a transwell insert is present, a clear relationship is difficult to describe.

Two-way ANOVA was used compare the effect of contact status and Saos-2 cell number on expression of activation markers by $CD4^+$ cells in the resting $CD4^+$ lymphocyte enriched population. A Log_{10} transformation of the data resolved the high variance, and there were no two-way interactions. At a $CD4^+$ lymphocyte enriched population : Saos-2 cell ratio of 1:1 there was an increase in the percentage of cells positive for CD25 (0.542 vs 1.438; p = 0.002) and CD69 (-0.11 vs 1.38; p = 0.029), and an increase in the FI of expression of CD25 (3.54 vs 4.55; p = 0.015). Although for most measures contact status had no effect, the presence of a transwell insert increased the percentage of $CD4^+$ cells that expressed CD25 (mean difference -0.263 [95% CI -0.521 to -0.005]; p = 0.046).

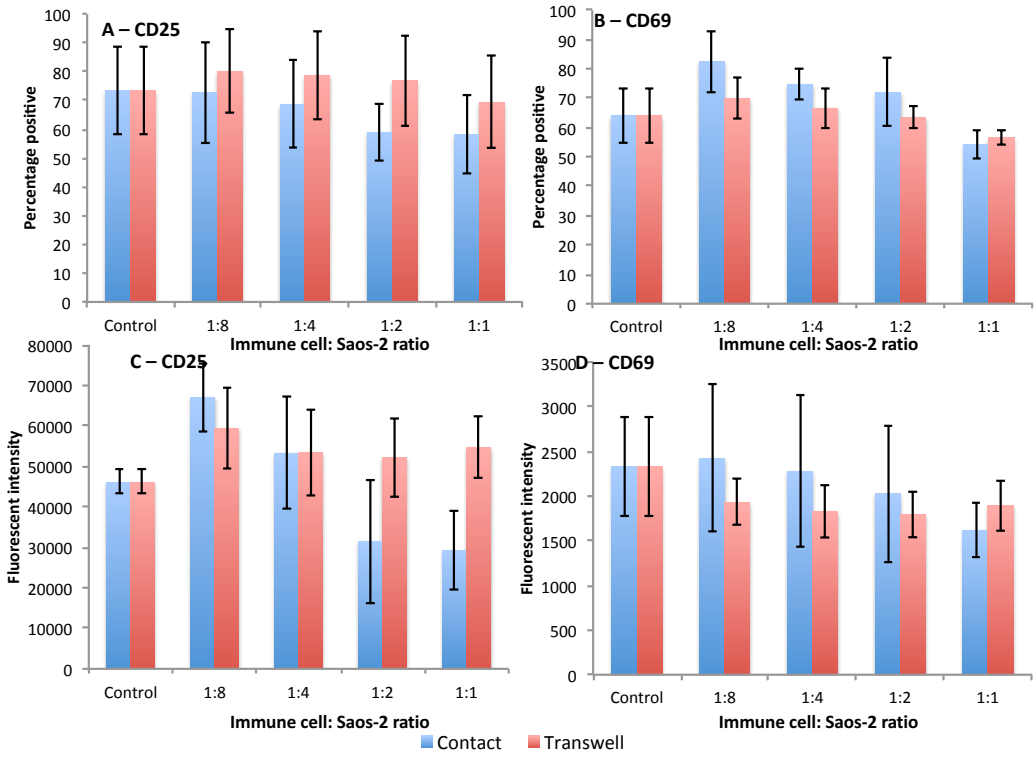

Figure 3-26: Effect of Saos-2 cells on expression of CD25 and CD69 by purified population of polyclonally activated $CD4^+$ lymphocytes. Saos-2 cells had no effect on expression of activation markers. There were no clear differences in the percentage of those positive for CD25 (panel A) or CD69 (panel C), or in fluorescent intensity of CD25 (panel B) or CD69 (panel D). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Summary of findings

Saos-2 growth

These findings show significant effects of immune cells on the growth of Saos-2 cells. There is no strong evidence to suggest a difference between the effects of PBMCs and $CD4^+$ lymphocytes on the growth of Saos-2 cells. Activation of immune cells has a consistently negative impact on the growth of Saos-2 cells, seen in both the PBMC and $CD4^+$ lymphocyte enriched population conditions. The effect of a transwell insert overall was to increase Saos-2 cell number, although the effect appears greatest in the activated conditions. Cell number does appear to be important, with both resting and activated immune cells showing evidence of a dose-response effect. However, the effect of contact does not appear to be affected by cell number.

Effect of Saos-2 cells on the activation of status if lymphocytes

These findings demonstrate an effect of Saos-2 cells on the activation status of lymphocytes. Increasing numbers of Saos-2 cells activated resting immune cells. Although a dose-response effect was seen in both PBMCs and $CD4^+$ lymphocytes, only in the latter did that reach statistical significance. Inspection of the graphs of expression of CD25 and CD69 by activated immune cells does suggest that with increasing numbers of Saos-2 cells there is a slight reduction activation. This more apparent in the PBMC population than in the $CD4^+$ lymphocyte population, but neither reached statistical significance.

Series 2 - influence of resting and activated immune cell populations on the alkaline phosphatase activity of Saos-2 cells

The aim of these experiments was to further investigate the influence of PBMCs and a $CD4^+$ lymphocyte enriched population on the growth and function of Saos-2 cells. Functional aspects of Saos-2 cells include alkaline phosphatase (ALP) activity and mineralisation. Functional studies require the Saos-2 cells to be growing, however previous experiments indicate that higher numbers of activated lymphocytes inhibit growth. Therefore, changes to the experimental design for these experiments were made.

Firstly, all the experiments use transwell inserts as even small numbers of activated immune cells were shown to dramatically inhibit growth in the earlier experiments. Furthermore, keeping cells separate during culture enabled the analysis of ALP activity

as all cells contain ALP. An additional consideration was the time at which to add immune cells. Doing this at the start of the experiment means they are introduced before the Saos-2 have adhered, therefore it could be said that two processes – adherence and proliferation – are being assessed. Waiting 48 hours before adding the immune cells allows the adherence and growth of Saos-2 cells to be established, thereby excluding any potential effect of treatment on adherence indirectly influencing measures of ALP generation. With regards to cell numbers, ratios between 6:1 to 0.6:1 were used (immune cell : Saos-2 cell).

Preparation of $CD4^+$ enriched and $CD4^+$ depleted cell populations

For these experiments, a population which had been depleted of $CD4^+$ cells was used, in addition to the PBMC and $CD4^+$ enriched populations. The $CD4^+$ enriched population was isolated using a negative CD4 selection kit designed to remove as many $CD4^-$ cells as possible, leaving a suspension of $CD4^+$ cells. The $CD4^+$ depleted population was generated using a positive selection $CD4^+$ selection kit which binds $CD4^+$ cells to the magnetic beads. For these experiments the "waste" population, depleted of $CD4^+$ cells, was used. The purity and content of the different cell preparations are illustrated in Figure 3-27 and Figure 3-28.

Figure 3-28 illustrates that the enrichment was effective, resulting in a population with over 90% $CD4^+$ cells. The $CD4^+$ depletion reduced the proportion of $CD4^+$ cells from 55% to 30%, and increased the relative proportion of $CD8^+$ cells from 30% to over 60%. However, the expectation from the $CD4^+$ depletion was to remove all $CD4^+$ cells. One reason this may not have happened is that the positive selection kit was not designed to do this. It is designed to produce a pure population of $CD4^+$ using positive selection.

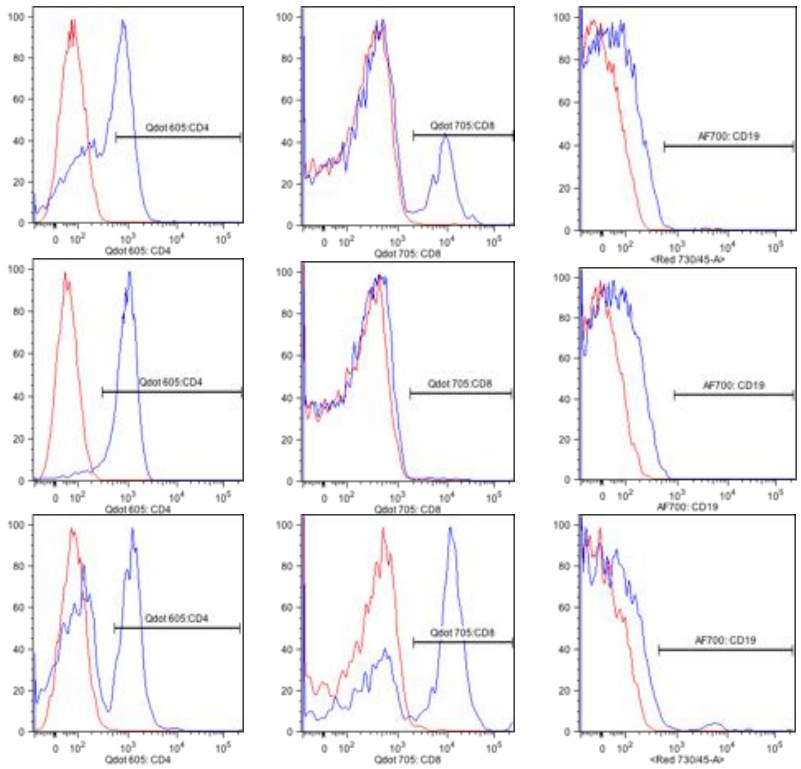

Figure 3-27: Representative histograms of identification of lymphocyte subsets. They show differing proportions of $CD4^+$ and $CD8^+$ cells in PBMCs (top row), $CD4^+$ enriched (middle) and $CD4^+$ depleted (bottom). The PBMC population contains more $CD4^+$ than $CD8^+$, and the $CD4^+$ enriched contains no $CD8^+$ cells. The $CD4^+$ depleted population still contains $CD4^+$ cells, but the proportion is altered, relative to the $CD8^+$. $CD19^+$ cells are absent in all populations, for reasons that are currently unclear.

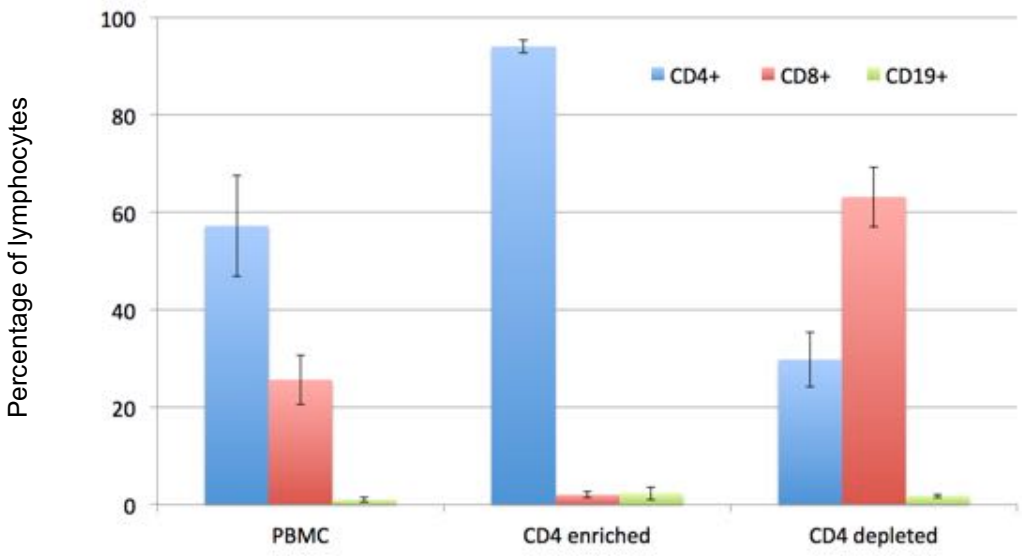

Figure 3-28: Relative proportions of lymphocytes subsets in three populations in PBMC, $CD4^+$ enriched and $CD4^+$ depleted preparations. 30% of the cells in the $CD4^+$ depleted population are $CD4^+$. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Effect of PBMCs, $CD4^+$ enriched and $CD4^+$ depleted cell populations on Saos-2 cells

Growth

After 5 days of co-culture with PBMCs, $CD4^+$ enriched and $CD4^+$ depleted cell populations, the Saos-2 cells were counted using an automated cell counter. Results presented in Figure 3-29 show that, with increasing numbers of resting immune cells, the growth of Saos-2 cells initially increases, and then begins to fall. This decline in growth only falls below that of controls following culture with resting PBMCs, and remained higher than the untreated controls following culture with the $CD4^+$ enriched and depleted populations (Figure 3-29).

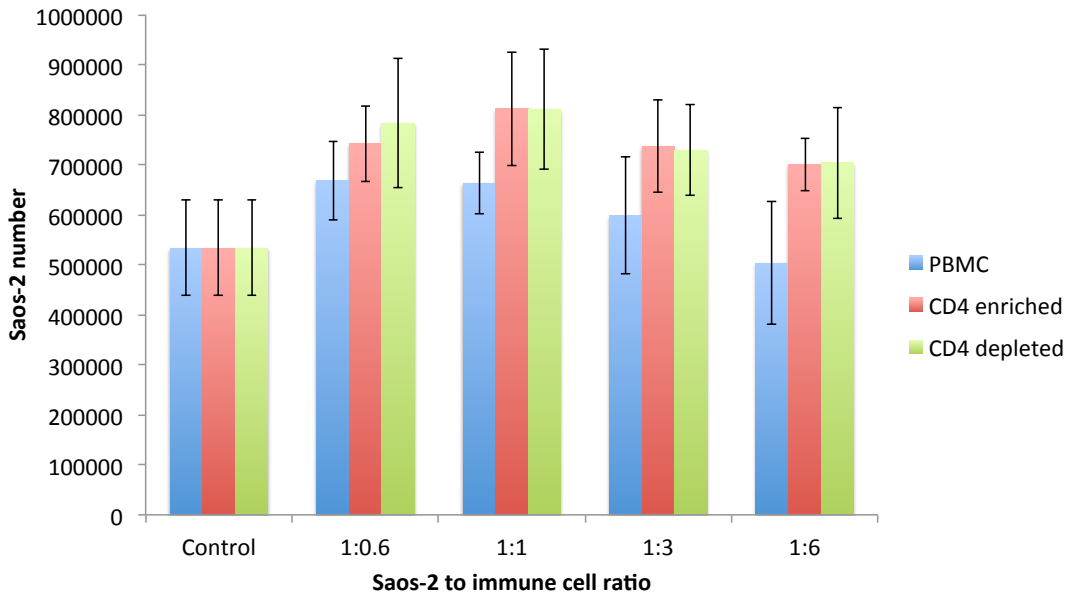

Figure 3-29: Effect of increasing numbers of resting PBMCs, $CD4^+$ enriched and $CD4^+$ depleted cell populations on the growth of Saos-2 cells. Ratios are Saos-2 cells : immune cells. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Although culture with activated PBMCs, $CD4^+$ enriched and $CD4^+$ depleted cell populations influenced the growth of Saos-2 cells, the effects were variable and quantitatively small (Figure 3-30). Analysis by three-way ANOVA found that any apparent differences in either the resting or activated conditions were not statistically significant.

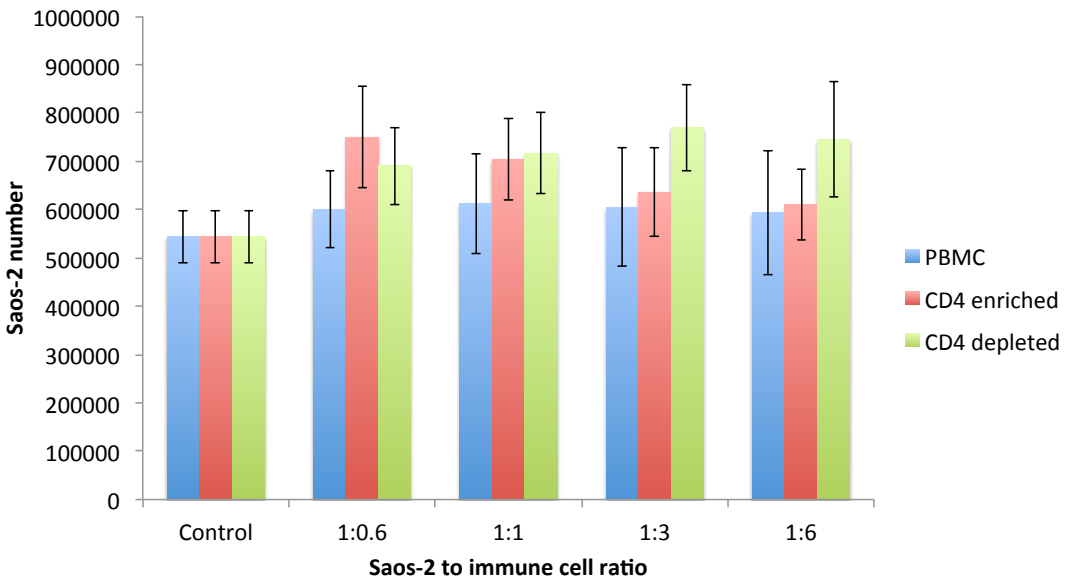

Figure 3-30: Effect of increasing numbers of activated PBMCs, $CD4^+$ enriched and $CD4^+$ depleted cell populations on the growth of Saos-2 cells. Ratios are Saos-2 cells : immune cells. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Alkaline phosphatase activity

Alkaline phosphatase activity is a useful measure of Saos-2 cell function, given its importance in bone formation by osteoblasts. Resting and activated PBMC and the $CD4^+$ enriched populations increased ALP activity in a dose-dependent manner (Figure 3-31).

Data were analysed by three-way ANOVA on the basis of three factors: immune cell number, immune cell type and activation status. There were no outliers (as determined by inspection of boxplots), and although the data were normally distributed, they were Log_{10} transformation to reduce variance. There was a significant three-way interaction found ($p < 0.001$). This was followed up with simple two-way interactions, which describe the interactions between two factors at all levels of the third factor e.g. the presence of an interaction between activation and immune cell type for the different immune cell numbers. This is slightly different to the three-way ANOVA analysis of the original co-culture data, for which the two-way interactions (not defined as “simple”) would, for example, compare two factors with no reference to the third.

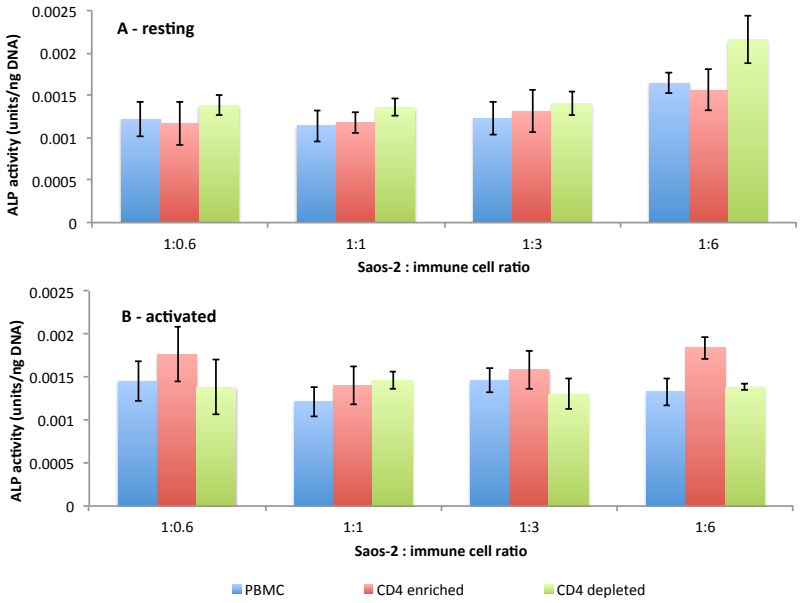

Figure 3-31: Effect of increasing numbers of activated PBMCs, $CD4^+$ enriched and $CD4^+$ depleted cell populations on ALP activity of Saos-2 cells. **Panel A –** resting immune cells. **Panel B –** activated immune cells. Ratios are Saos-2 cells : immune cells. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Activation status and cell number had a significant interaction in the $CD4^+$ depleted condition ($p < 0.001$), but not in the PBMC or $CD4^+$ enriched experiments. Cell type and activation status had a significant interaction for all immune cell numbers ($p < 0.001$). Cell type and cell number had a significant interaction in the activated conditions ($p < 0.001$), but not in the resting conditions.

Simple two-way interaction: the effect of cell number relative to cell type and activation status

There was a significant overall effect of cell number on ALP activity in both the $CD4^+$ enriched and $CD4^+$ depleted populations, regardless of activation status. Changes in ALP activity induced by culturing with PBMCs were not significant. The detailed comparisons for cell number, that were statistically significant in relation to cell type and activation status, are summarised inFigure 3-32.

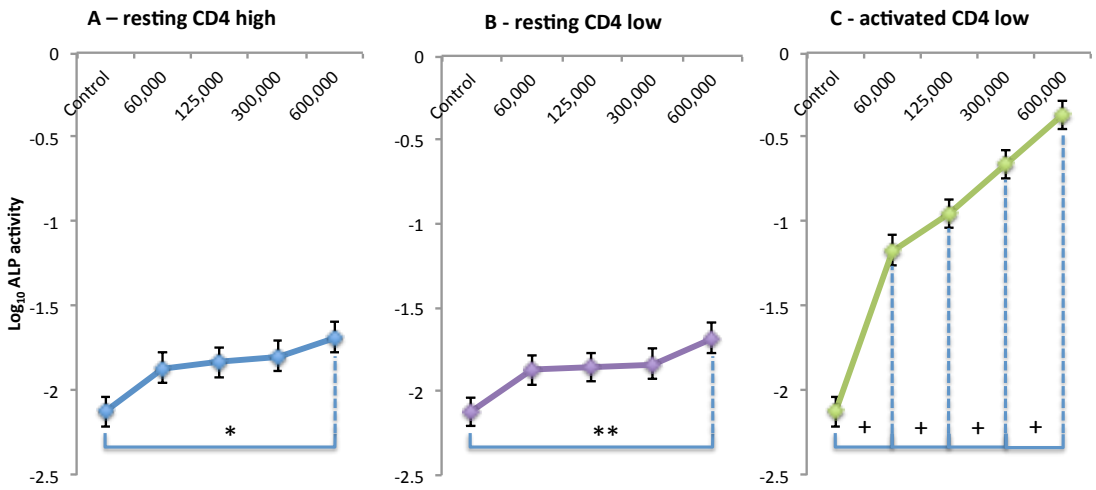

Figure 3-32: Influence of $CD4^+$ enriched and $CD4^+$ depleted populations on ALP activity in Saos-2 cells showing only those comparisons that had statistical significance. Panel A – ALP activity increases with the number of resting $CD4^+$ enriched cells * p = 0.012 Panel B - ALP activity increases with the number of resting $CD4^+$ depleted cells. ** p = 0.008. Panel C - ALP activity increases with the number of resting $CD4^+$ depleted cells $^+$ p = 0.001 (p values are Bonferroni adjusted for multiple comparisons, α = 0.05). Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Cell number of the resting (p = 0.019) and activated (p = 0.043) $CD4^+$ enriched populations influenced ALP activity. Pairwise comparisons revealed that the only significant difference in ALP activity was seen for the 600,000-cell resting condition when compared to control (mean difference -0.421 [95% CI -0.784 to -0.058]; p = 0.012). The individual comparisons for the resting $CD4^+$ enriched population cell number are not shown in Figure 3-32. For the $CD4^+$ depleted population, cell number overall affected ALP activity in the resting (p = 0.018) and activated (p < 0.001), but as can be seen in Figure 3-32 the detailed comparison reveals they are more marked in the activated group.

Simple two-way interaction: the effect of activation status relative to immune cell type and number

The activated $CD4^+$ depleted population increased ALP activity (p < 0.001) an effect not seen with the other two cell types. Detailed analysis showed significant increases in ALP activity in the activated condition relative to the resting at all cell numbers, with evidence of a dose-response effect (see Figure 3-33).

Simple two-way interaction: the effect of cell type relative to immune cell number and activation status

There was a statistically significant effect of cell type on ALP activity, when the immune cells were activated ($p < 0.001$). Detailed analysis showed that it was the activated $CD4^+$ depleted population that affected ALP activity, with significant increases at all immune cell ratios compared to the other cell types (see Figure 3-34). However, the significant differences in the effects of the resting immune cell populations on ALP activity.

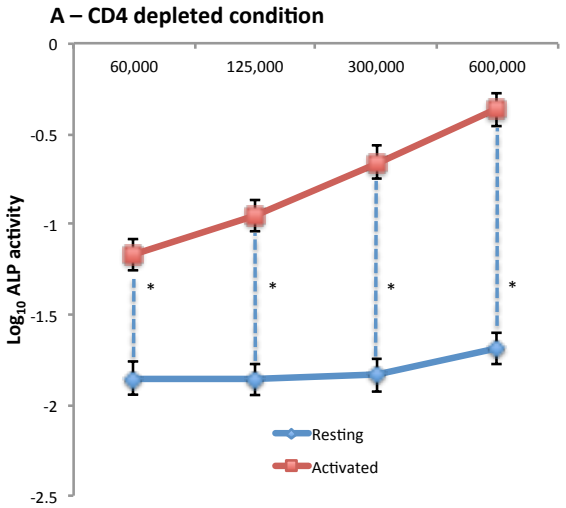

Figure 3-33: Influence of resting and activated $CD4^+$ depleted populations on ALP activity. There were no significant differences seen in the other conditions $p < 0.001$ (p values are Bonferroni adjusted for multiple comparisons, $\alpha = 0.05$) Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

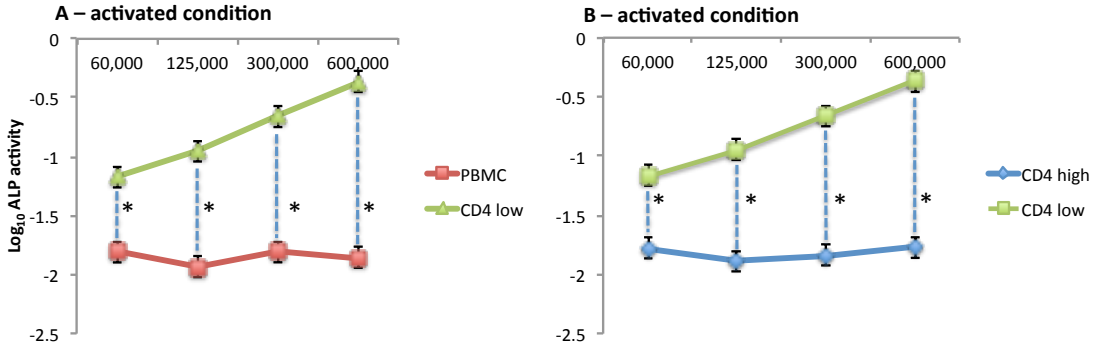

Figure 3-34: Influence of activated PBMCs, $CD4^+$ enriched and $CD4^+$ depleted populations on ALP activity in Saos-2 cells. * $p < 0.001$ (p values are Bonferroni adjusted for multiple comparisons, $\alpha = 0.05$) Data are derived from three independent experiments with a log10 transformation and presented as means ± 1 standard error.

4 Optimisation of lymphocyte extraction from mucosal tissue

Since completing this work a comprehensive method for the isolation of lymphocytes from mucosal biopsies has been published (Carrasco et al 2013), at the time of undertaking this study no method for their isolation from small biopsy samples had been published - a method therefore had to be developed. Given that the isolation of lymphocyte populations from gut biopsies required the use of enzymes, a pre-requisite to these studies was to demonstrate that the use of these enzymes had no influence on the phenotype of the isolated cells.

Patient sample preparation and analysis

Initial method

Biopsy samples were collected into a 25ml Universal container containing ice cold PBS. The biopsy specimens were transferred from the PBS to 5ml of pre-digestion solution, which had been pre-warmed to 37°C, in a 25ml Universal container and placed in a water-bath at 37°C with gentle rotation for 20 minutes. The contents of the tube were then filtered through a 100μm cell strainer (BD Falcon #352360) and the effluent was reserved. The biopsy pieces were incubated in fresh pre-digestion solution for 20 minutes, followed by filtering and collection of the effluent, a further two times.

After the third filtering step, the effluent from the pre-digestion stages contained no visible cells, and so was discarded. Biopsy pieces were placed in digestion solution, pre-warmed to 37°C, and after 20 minutes the solution was passed through a 100μm filter. Gentle pressure with a syringe plunger was applied to encourage cell release. The tissue readily dissociated and so further digestion steps were not carried out.

The digested gut biopsy solution was centrifuged for 10 minutes at 500g, the supernatant was discarded, the cell pellet re-suspended in cold serum buffer (PBS with 10% v/v foetal bovine serum (FBS, GIBCO #10270-106) and centrifuged at 300g for 5 minutes. The cell pellet was re-suspended in 10ml of 40% v/v Percoll® in a 25ml Universal container. 5ml of the 80% v/v Percoll® solution was drawn up using a 22g needle and a 10ml syringe. This was then introduced under the 40% v/v Percoll® by placing the needle end in the base of the tube and gradually depressing the syringe

plunger. The 80% v/v Percoll® stayed underneath the 40% and a clear dividing line was visible. This was then centrifuged at 1000g for 20 minutes with no brake. Once complete, cells at the interface were aspirated using a Pasteur pipette, placed in an excess of PBS and centrifuged at 300g for 5 minutes. The cell pellet was added to 5 ml of RPMI with 10% v/v FBS and kept overnight in an incubator at 37°C.

The next day cells were counted on a haemocytometer using trypan blue exclusion. There were typically a total of $1.5x10^6$ cells, of which more than 95% were viable. Cells were suspended in SB with 2% v/v mouse serum (Sigma #M5905) at $1x10^6$ cells/100μl. After incubating for 15 minutes at 4°C, fluorescent mAbs were added and incubated for a further 30 minutes at 4°C. Cells were then washed in an excess of SB and the cell pellet re-suspended in SB for analysis. Unfortunately, the fluorescent signal was not clear (see Figure 4-1).

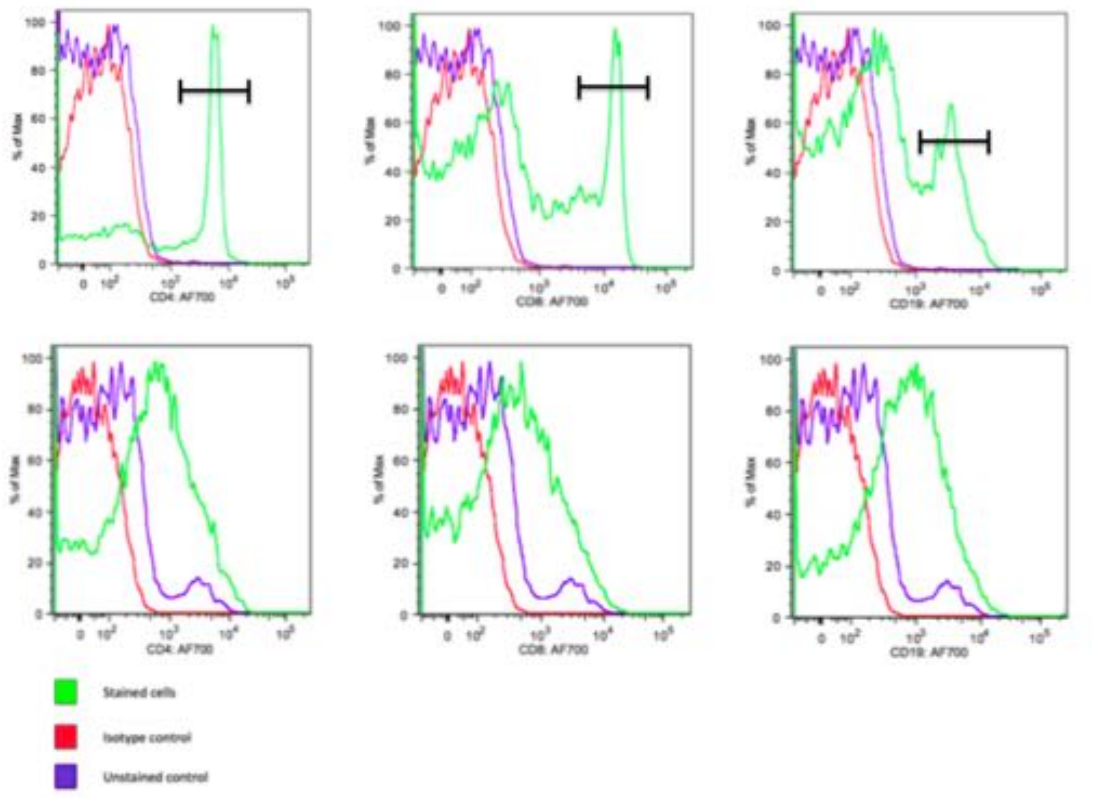

Figure 4-1: Fluorescent signal of the first population of immune cells isolated from gut mucosal biopsies using Method 1. Whilst the PBMCs (top row) show a clear peak representing the indicated populations of cells (indicated by black bars), but no such defined populations were apparent in the biopsy-derived samples (bottom row).

Method 2

At the digestion solution stage, biopsies were for incubated for 40 minutes then 20 minutes, rather than using 3 x 20 minute incubations. At the end of each digestion stage, biopsy pieces were washed over a 40μm cell strainer. After diluting the biopsy suspension a lower-force 5 minute 300g centrifugation was undertaken, thereby omitting the higher force step. After completing the Percoll® stage, cells were visible at the interface, but were only 500,000 in number when counted. This was inadequate for staining and may have occurred because the higher force centrifuge step was missed.

Method 3

Having not seen any cells being released during the pre-digestion stages, only the digestion solution was used. After 40 minutes uninterrupted incubation, no breakdown of the tissue was seen, as a consequence of which the biopsy samples were incubated in pre-digestion solution for 20 minutes. Although incubation in the digestion solution did start to break up the tissues only 600,000 cells were present after Percoll® stage and analysis was not possible.

Method 4

Two patients were recruited on the same day and their samples processed and analysed under the same conditions. The method was adjusted to use a different incubator with a flat rubber platform. The incubator was not compatible with 25ml Universal container and so a 6-well culture plate was used instead. Biopsy specimens were placed in the 6-well plate (Corning #3516) with 3 ml of warmed pre-digestion solution and placed in an incubator with gentle shaking. After 20 minutes the tissue was removed and placed in fresh pre-digestion solution. The used solution was transferred to a microfuge tube, centrifuged at 300g for 5 minutes and the resultant pellet re-suspended in medium. The tissue underwent 3 x 20 minute incubations in pre-digestion solution, followed by 3 x 20 minute incubations in digestion solution using this process. Once complete, cells were centrifuged on a Percoll® gradient. Although this procedure generated cells that could be analysed by flow cytometry, the surface antigens were not reliably maintained and no CD4 or CD8 expression signal was visible. CD19 expression was maintained, as was some expression of CD69, albeit of very low intensity (see Figure 4-4).

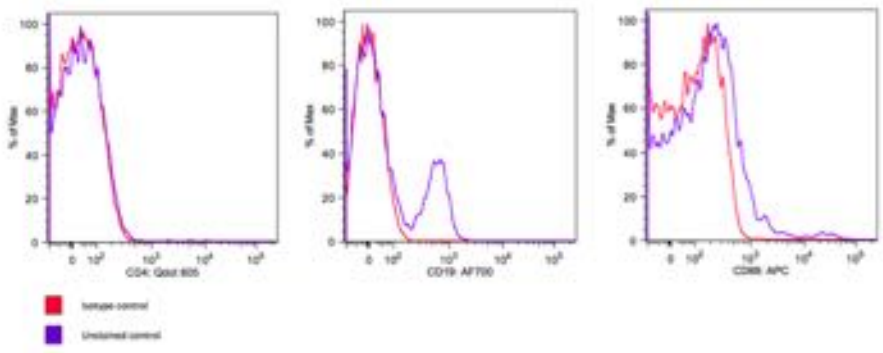

Figure 4-2: Fluorescent signals for gut immune cells isolated using Method 4. Although there is a clear population of $CD19^+$ cells (middle panel), there is no evidence of a $CD4^+$ or $CD8^+$ population.

Method 5

Samples were processed using the same method as for the two individuals whose samples were processed using Method 4 above, except that that the concentration of collagenase was reduced to 0.01% v/v and that of the dispase to 0.1% v/v. Only 200,000 cells were isolated and, although they were stained for analysis by flow cytometry, no cells were visible.

Method 6

The aim of this approach was to minimise the number of transfers between different vessels. For this, biopsies were placed in a microfuge tube, to which 1 ml of the pre-digestion solution was added. Tubes were placed in the incubator at 37°C with moderate agitation. After 20 minutes, the tube was shaken vigorously by hand and biopsy specimens were allowed to settle. The supernatant looked much cloudier than previous attempts and was transferred to a fresh microfuge tube. Pre-digestion solution (1 ml) was added to the biopsy specimens and returned to the incubator. The supernatant was centrifuged at 300g for 5 minutes and, after discarding the supernatant, cells were re-suspended in 1 ml of D-MEM medium. This process was repeated for a total of three incubations in pre-digestion followed by digestion solution.

Although there were sufficient cells for analysis by flow cytometry (including unstained and isotype controls), there was no visible fluorescent signal when analysed by flow cytometry.

Method 7

For this, the process was repeated except this time, on the basis of experiments looking at the effects of the various enzymes on the surface markers (see next Section)

dispase was not used. This approach yielded 800,000 cells during the pre-digestion stage and ultimately sufficient cells for the flow cytometric analysis (including unstained and isotype controls) after the digestion stage. Importantly, the expression of surface markers (as determined by flow cytometry) was maintained. The data for Methods 6 and 7 are presented in the Results section.

Effect of digestive enzymes on immune cell surface markers

In developing the technique for isolation of immune cells from gut biopsy samples the effect of digestive enzymes that were required to break down gut tissue and release the immune cells was investigated on the expression of surface antigens on resting and activated lymphocyte populations was investigated. Expression of CD25 and CD69 was increased by incubating isolated PBMC populations in concanavalin A (as described in Methods and Materials). Means were compared by independent samples T-test.

Effect of digestive enzymes on the expression of lymphocyte phenotypic markers

The treatment of PBMCs with EDTA, dithiothreitol (DTT), deoxyribonuclease (DNAse) and 0.1% w/v collagenase had no effect on the percentage of $CD4^+$, $CD8^+$ and $CD19^+$ cells (see Figure 4-3). In contrast, treatment of PBMCs with 0.3% w/v dispase reduced

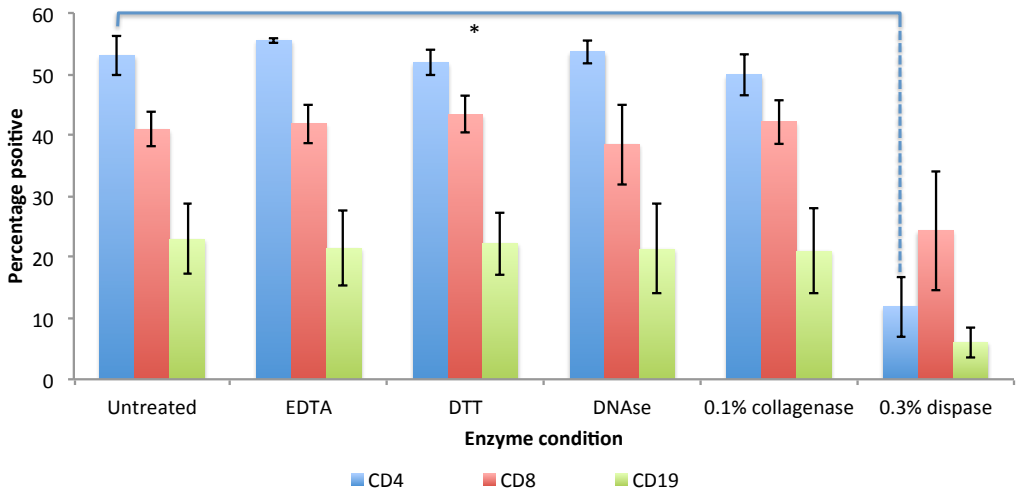

Figure 4-3: Effect of treatment with digestive enzymes on the relative proportions of $CD4^+$, $CD8^+$ and $CD19^+$ lymphocyte subsets in activated PBMC preparations. * p = 0.002. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

the proportion of $CD4^+$ cells (53% vs 11.9%; p = 0.002), $CD8^+$ (41% vs 24%; p= 0.177) and $CD19^+$ (23% vs 6%; p = 0.053), as determined using flow cytometry. Although reducing the concentration of dispase from 0.3% to 0.03% reduced this effect, a significant reduction in the percentage of $CD4^+$ cells remained (53% vs 15 %; p = 0.002).

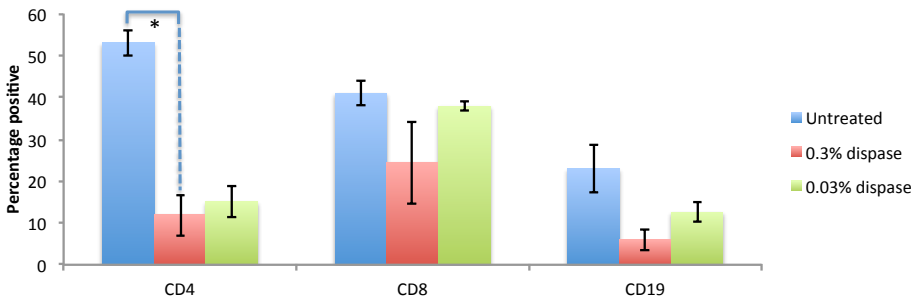

Figure 4-4: Effect of dispase treatment (0.3%, 0.03% w/v) on the relative proportions of $CD4^+$, $CD8^+$ and $CD19^+$ lymphocyte subsets in PBMC preparations. * p = 0.002. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Effect of digestive enzymes on the expression of lymphocyte activation markers

The effect of digestive enzymes on expression of CD25 and CD69 by activated PBMCs were also studied. As discussed previously, their expression can be measured by the percentage of positive cells and the overall fluorescent intensity of expression.

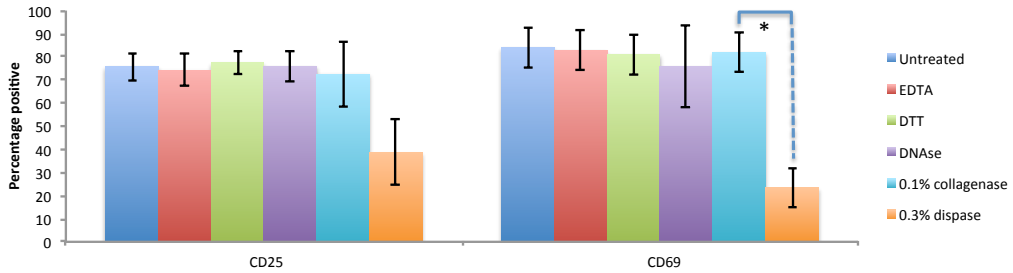

Figure 4-5: Effect of digestive enzyme treatments on the proportion of PBMCs expressing CD25 and CD69. * p = 0.008. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Dispase (0.3% w/v) reduced the proportion of $CD25^+$ and $CD69^+$ cells (from 75% to 38% and 84% to 23% respectively, see Figure 4-5). However, only the reduction in the proportion of $CD69^+$ was of statistical significance (p = 0.008). Reducing the concentration of dispase to 0.03% w/v significantly reduced its effect on the proportion of $CD25^+$ and $CD69^+$ cells (23% vs 70.2%; p = 0.015; see Figure 4-6), and its effect on

the proportion of $CD69^+$ cells was no longer of statistical significance relative to the untreated control.

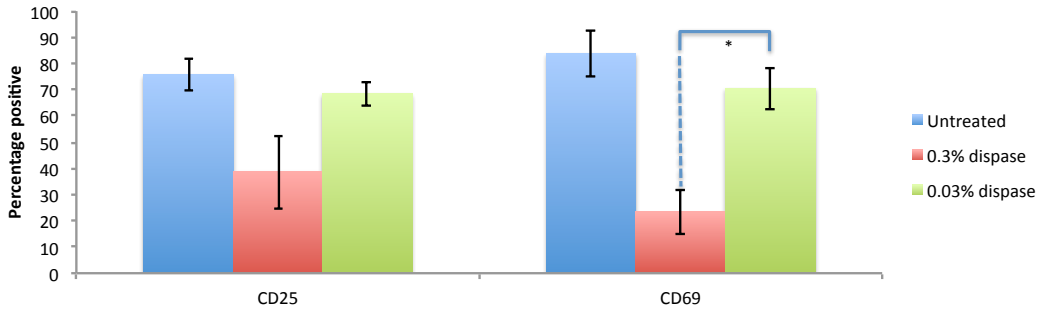

Figure 4-6: Effect of dispase treatment (0.3%, 0.03% w/v) on the proportion of $CD25^+$ and $CD69^+$ in PBMCs. * p = 0.015. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Although EDTA, dithiothreitol (DTT), deoxyribonuclease (DNAse) and 0.1% w/v collagenase had no effect on the intensity of CD25 and CD69 expression, dispase treatment did reduce the intensity of expression intensity (see Figure 4-7 and Figure 4-8).

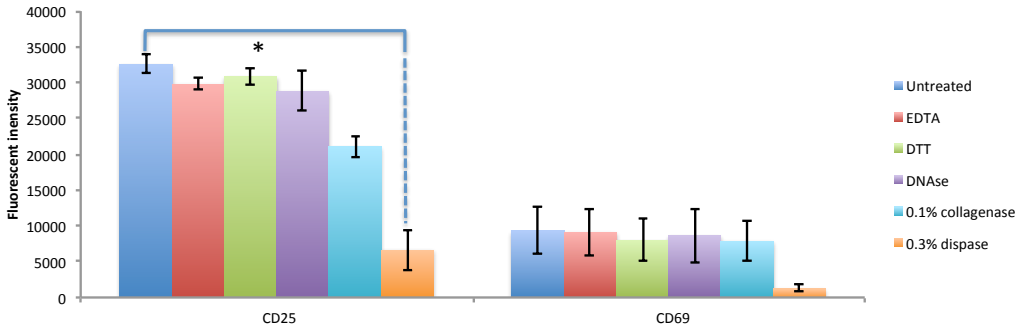

Figure 4-7: Effect of digestive enzyme treatments on the intensity of CD25 and CD69 expression by activated PBMCs. * p = 0.001. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

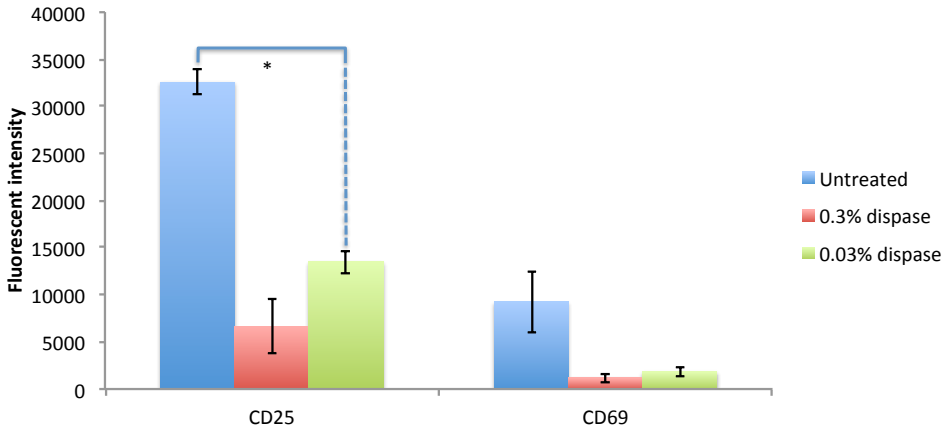

Figure 4-8: Effect of dispase treatment (0.3%, 0.03% w/v) on the intensity of CD25 and CD69 expression by activated PBMCs. * $p < 0.001$. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Effect of digestive enzymes on the expression of α4 and β7 integrins

The α4β7 integrin identifies lymphocytes that have been primed in the gut. Given that a single antibody to this surface marker is not commercially available, a combination of two separate antibodies was necessary. Expression of both integrins was measured on the basis of the proportion of positive cells since the intensity of expression is not considered in the published literature, unlike for CD25 and CD69.

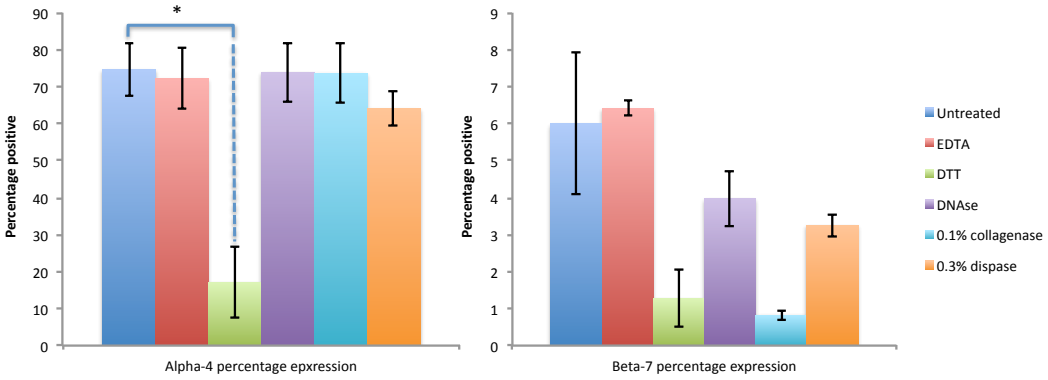

Figure 4-9: Effect of digestive enzyme treatments on the proportion of activated PBMCs expressing α4 and β7 integrins. * $p = 0.009$. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Treatment with DTT reduced the expression of both integrins (Figure 4-9), despite it not having had any effect on the expression of the other surface antigens that were examined in this study. The expression of β7 was also reduced by treatment with 0.1% w/v collagenase and 0.3% w/v dispase. Reductions in the concentrations of dispase

and collagenase had little or no effect on the reduction in expression of β7 (see Figure 4-10).

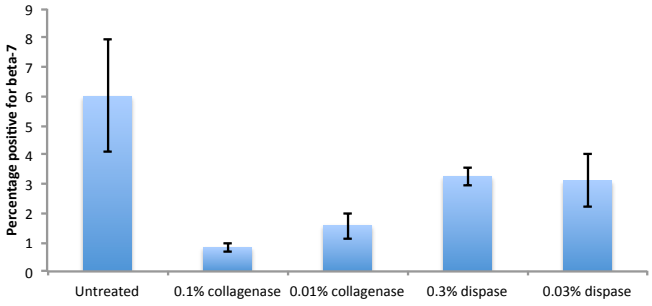

Figure 4-10: Effect of collagenase (0.1%, 0.01% w/v) and dispase (0.3%, 0.03% w/v) treatment on the proportion of activated PBMCs expressing β7 integrin. Data are derived from three independent experiments, from three donors with no technical repeats, and are presented as means ± 1 standard error.

Summary of findings

Although the use of digestive enzymes to liberate cells from gut mucosa has the potential to affect expression of surface markers, the limited availability of the clinical material precluded the use of physical techniques, rather than enzyme digestion. Although expression of the integrins was the most sensitive to treatment, their expression on circulating lymphocytes (which are not isolated using enzyme digestion) is arguably the more important parameter. It should be note that these experiments did not examine the effects of enzyme combinations. However, as will be shown, a reasonable signal could be obtained from isolated cells. Even if reduced, comparisons between patients could still be made.

5 *In vivo* results

Phenotypic characterisation of intestinal and peripheral blood leucocytes in children with Crohn's disease, and their relationship(s) to bone metabolism and mineralisation

The hypothesis of this thesis is that the reductions in bone mineral density (BMD) seen in children with Crohn's disease are, at least in part, mediated by interactions between osteoblasts and gut-activated lymphocytes that have migrated from mucosal tissue to the bone. The aim of this *in vivo* study is to look for associations between the relative proportions, and activation status of, lymphocytes in children with Crohn's disease; changes in biochemical markers of bone turnover; and changes in measures of BMD measured by dual X-ray absorptiometry.

Children undergoing endoscopy for investigation of gastrointestinal were approached with an aim of recruiting 15 with Crohn's disease and 15 with no identifiable pathology. Peripheral blood was taken for measurement of the serum bone turnover markers P1NP and osteocalcin, and isolation of peripheral blood mononuclear cells. 10 gastrointestinal mucosal biopsies were obtained, from which cells were isolated by enzymatic digestion. The isolated cells were analysed by flow cytometry to establish the proportions of the various phenotypes, and expression of CD25, CD69 and $\alpha 4\beta 7$. Finally, confirmed cases of IBD had a DEXA scan to measure their BMD.

To date there are no published studies investigating the association between lymphocyte populations and their cell-surface activation markers with BMD in children with IBD. This work has the potential to lead to further studies aiming to identify the presence of gut-activated immune cells in the bone of IBD patients, and their effects on osteoblasts and bone metabolism.

The impact of inflammatory bowel disease on the immune cell profile and measures of bone metabolism

Patient demographics

30 patients were recruited in total, 16 controls and 14 cases. Controls were younger (9.3 vs 13.4 years; $p = 0.003$). The cohort consisted of 19 males and 11 females: 10

males and 6 females in the control group, 9 males and 5 females in the cases group (Table 5-1).

Sample	Controls	Cases
Routine bloods	14	13
Peripheral blood lymphocytes	8	12
Mucosal epithelial lymphocytes	4	11
Lamina propria lymphocytes	4	11
Serum bone turnover markers	8	9
Urine bone turnover markers	8	9
DEXA	N/A	13

Table 5-1: Samples taken from, and investigations performed, in cases and controls.

A decision regarding diagnosis, based on mucosal appearances, was made at the time of endoscopy. A final diagnosis was made on the basis of histology as part of their routine clinical assessment. Of the 14 cases, 10 were new diagnoses of Crohn's disease, 2 were known cases of Crohn's disease, one was a new diagnosis of ulcerative colitis and one a new diagnosis of indeterminate colitis.

Figure 5-1 and Table 5-2 compare basic clinical data. Patients had a full blood count (FBC), and measurements of the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels and serum albumin levels were taken. Cases had a significantly higher ESR (29.33 vs 12.5; $p = 0.018$). The mean CRP was raised in cases, and although this difference was not of statistical significance, values less than 7 are not reported which will have affected the statistical analysis. The total white cell count (WCC) was similar for the two groups. The proportion of lymphocytes, as a percentage of the total white cell count, was significantly lower in cases (32.86% vs 21.65%; $p = 0.022$). The total lymphocyte count was lower in cases, although the difference did not reach significance (1.81×10^9/L vs 2.52×10^9/L; $p = 0.053$). Serum albumin was significantly lower in cases (37.0 vs 42.5; $p = 0.002$).

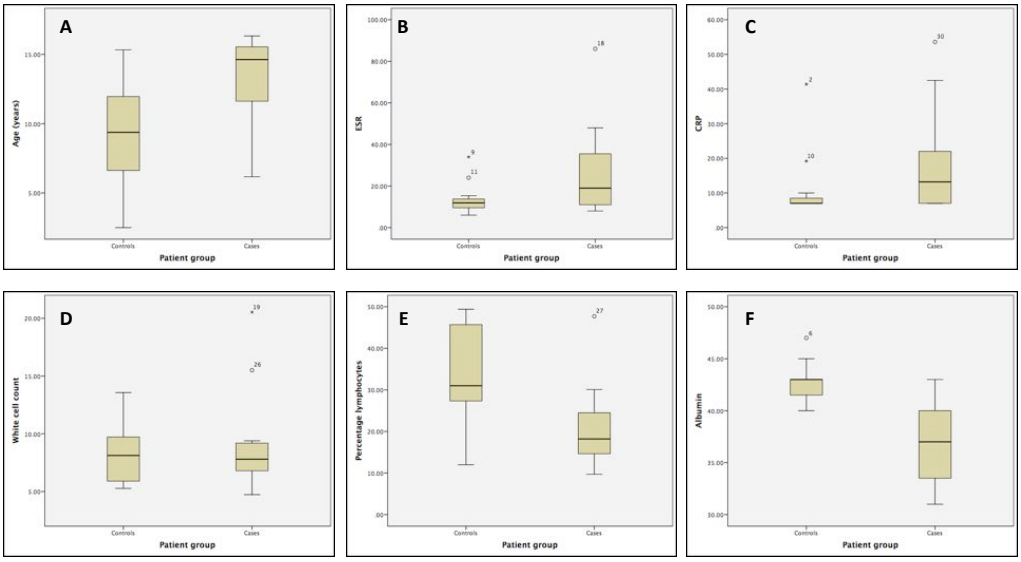

Figure 5-1: Box and whisker plots comparing the distributions of basic patient data for cases and controls. **Panel A –** age at time of endoscopy higher for cases (p = 0.003) **Panel B –** ESR raised in cases (p = 0.018). Panel C = CRP. **Panel D** - white cell count (WCC) higher in cases. **Panel E –** lymphocytes as percentage of total WCC lower in cases (p = 0.022). **Panel F –** albumin lower in cases (p = 0.002). Sample size as in Table 5-1.

	Controls	Cases	p
Age (years)	9.30 (0.90)	13.43 (0.86)	0.003
ESR	12.54 (1.66)	29.33 (6.63)	0.018
CRP	11.51 (3.19)	20.62 (4.73)	0.091
White cell count (10^9/L)	8.27 (0.67)	9.24 (1.18)	0.793
Percentage lymphocytes	32.86 (3.41)	21.65 (3.01)	0.022
Lymphocyte count (10^9/L)	2.52 (0.19)	1.81 (0.23)	0.053
Albumin	42.54 (0.58)	37.0 (1.32)	0.002

Table 5-2: Comparison of clinical data in cases and controls. Statistically significant findings are highlighted in red (α = 0.05). Data are presented as means ± one standard error.

Phenotypic characterisation of intestinal and peripheral blood leucocytes

Immune cells were isolated and the relative proportions of lymphocyte subsets ($CD4^+$, $CD8^+$, $CD19^+$) and their expression of CD25 and CD69 were measured as described in the Materials and Methods. The presence of lymphocytes expressing $\alpha4\beta7$ (indicating their activation in the gut) was also determined.

Mucosal immune cell populations

Mucosal lymphocyte subsets

The proportions of $CD4^+$ and $CD8^+$ cells are equivalent (15.8% vs 16.2%) within the lamina propria (LP) of controls, with the predominant cell type being $CD19^+$ B cells (27.8%, Figure 5-2, panel A). In contrast, $CD4^+$ cells predominate in the LP of cases relative to $CD8^+$ T cells (26.7% vs 14.6%; p = 0.032) and $CD19^+$ B cells (26.7% vs 13.6%; p = 0.037).

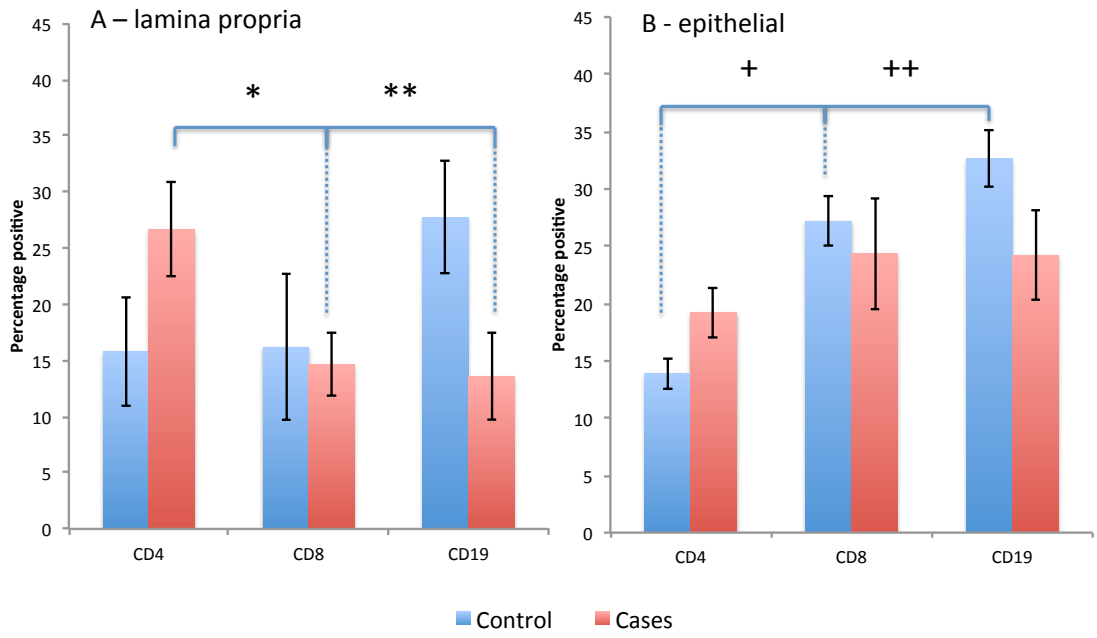

Figure 5-2: Relative proportions of $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes subsets within the lamina propria and gut mucosal epithelial layer (MEL) of cases and healthy controls. **Panel A -** proportions of lymphocyte subsets with the LP * p = 0.032 ** p = 0.037 **Panel B -** proportions of lymphocyte subsets with the MEL. + p = 0.002 ++ p = 0.001. Data are presented as means ± 1 standard error, sample size as in Table 5-1.

The composition of cells within the mucosal epithelial layer (MEL) was distinct to that of the LP compartment (Figure 5-2, panel B), with a significantly lower proportion of $CD4^+$ cells than either $CD8^+$ cells (27.2% vs 13.9%; p = 0.002), or $CD19^+$ cells (32.6% vs 13.9%; p = 0.001) in controls. In cases, the proportion of $CD4^+$ cells is higher (13.8% vs 19.2%; not significant), and this is accompanied by a lower proportion of $CD8^+$ cells (27.2% vs 24.3%; not significant) and $CD19^+$ cells (32.6% vs 24.2%; not significant).

In summary, these data indicate that for healthy controls, $CD19^+$ B cells predominate within the gut mucosa of healthy controls, with the LP and MEL compartments exhibiting differences in the proportions of $CD4^+$ to $CD8^+$ cells. In cases, there is an

infiltration of $CD4^+$ cells, primarily (but not exclusively), into the LP. This is accompanied by corresponding reductions in the proportions of $CD8^+$ and $CD19^+$ cells. However, none of the differences when comparing proportions of each individual lymphocyte subset between cases and controls, and within the LP and MEL were statistically significant ($p > 0.05$; Figure 5-1).

Mucosal $\alpha4\beta7^+$ lymphocyte subsets

Mucosal lymphocytes expressing $\alpha4$ and $\beta7$ were identified in the LP and MEL of cases and controls, and quantified as a percentage of the total number of lymphocytes (Figure 5-3).

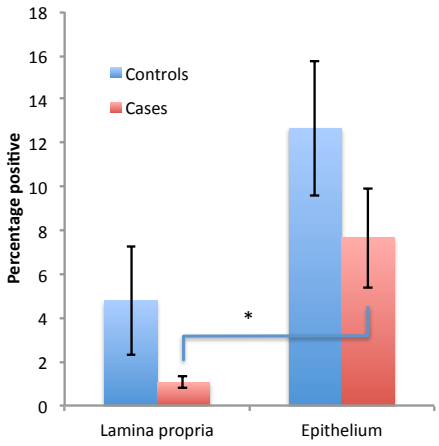

Figure 5-3: Proportion of mucosal lymphocytes expressing $\alpha4\beta7$. A comparison between LP and MEL in cases and healthy controls. * $p = 0.006$ Data are presented as means $\pm$ 1 standard error, sample size as in Table 5-1.

There were a greater proportion of $\alpha4\beta7^+$ lymphocytes within the MEL of controls than in the LP (12.7% vs 4.8%, not significant, Figure 5-3). A similar pattern was seen for cases (1.3% vs 8.4%; $p = 0.006$, Figure 5-3). Although the proportion of $\alpha4\beta7^+$ lymphocytes within each mucosal compartment was lower in the cases, the differences were not of statistical significance. As the data have been analysed as proportions, rather than as cell numbers, differences may reflect either an increase in $\alpha4\beta7^-$ lymphocytes or a decrease in $\alpha4\beta7^+$ lymphocytes.

The pattern of subset distribution for $\alpha4\beta7^+$ lymphocytes in the mucosal compartments was visibly different to that seen for all lymphocytes (Figure 5-3). $\alpha4\beta7^+$ $CD8^+$ cells predominate in both the LP and MEL of healthy controls, with $\alpha4\beta7^+$ $CD19^+$ B cells being the next most prevalent cell type. In the MEL (Figure 4.12, panel B), the

proportions of $\alpha4\beta7^+$ $CD4^+$ to $\alpha4\beta7^+$ $CD8^+$ (13.1% vs 56.5%; $p < 0.001$) and $\alpha4\beta7^+$ $CD19^+$ (13.1% vs 39.3%; $p = 0.046$) were significant, but not in the LP.

For patients with IBD, the proportion of $\alpha4\beta7^+CD4^+$ lymphocytes in both the LP (50.29% vs 19.76%; $p = 0.001$) and the MEL (26.59% vs 13.12%; $p = 0.02$) was significantly greater than in healthy controls. However, although the difference between the proportions of $\alpha4\beta7^+CD4^+$ and $\alpha4\beta7^+CD8^+$ cells remained significantly different in the MEL (26.6% vs 67.1%; $p < 0.001$; § Figure 5-4, panel B), the significance of the corresponding difference within the LP was lost.

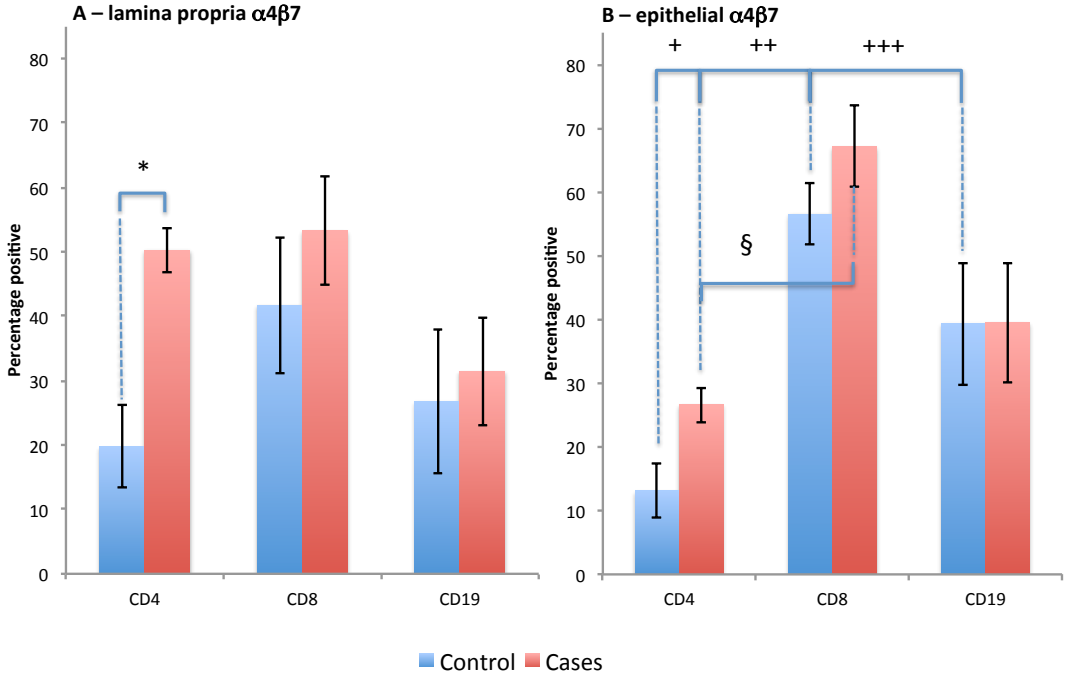

Figure 5-4: Relative proportions of $\alpha4\beta7^+$ lymphocytes subsets within the lamina propria (LP) and epithelial layers (MEL) of the gut mucosa of cases and healthy controls. **Panel A** - the proportions of $\alpha4\beta7^+$ lymphocytes subsets. * $p = 0.001$. **Panel B -** shows the proportions of proportions of $\alpha4\beta7^+$ lymphocytes subsets in the MEL. $^+$ $p = 0.002$ control $\alpha4\beta7^+$ $CD4^+$ vs case CD4, $^{++}$ $p < 0.001$ control $\alpha4\beta7^+$ $CD4^+$ vs control $\alpha4\beta7^+$ $CD8^+$, $^{+++}$ $p = 0.046$ control $\alpha4\beta7^+$ $CD4^+$ vs control $\alpha4\beta7^+$ $CD19^+$. Data are presented as the mean ± one standard error, sample size as in Table 5-1.

It is difficult to explain why, despite the increase in the proportion of $\alpha4\beta7^+CD4^+$ lymphocytes in cases there is also an increase in $\alpha4\beta7^+CD8^+$ lymphocytes, with the sum of the subsets being greater than 100%. It is possible that this reflects the presence of $CD4^+CD8^+$ lymphocytes, which has indeed been reported in cases of IBD (Senju et al 1991b; Das et al 2003), although not in relation to $\alpha4\beta7^+$ lymphocytes.

It is important to note that there was no difference in the proportions of $\alpha4\beta7^+CD8^+$ and $\alpha4\beta7^+CD19^+$ in the LP and MEL between cases or controls. Significant differences were found in the proportion of $\alpha4\beta7^+CD4^+$ lymphocytes in the LP and MEL of cases and controls (as discussed), but also in the proportion of $\alpha4\beta7^+$CD4+ lymphocytes in the LP and MEL of cases (49.6% vs 26.6%; $p < 0.001$ – see Figure 5-4).

Expression of activation markers by mucosal $CD4^+$ lymphocytes

Overall, there is very little difference between cases and controls in expression of CD25 and CD69 by $CD4^+$ cells isolated from the LP and MEL of the gut mucosa (Figure 5-5). The percentage of $CD4^+$ cells in the MEL expressing CD69 was significantly increased in cases compared with healthy controls (57.8% vs 27.3%; $p = 0.043$). The intensity of CD25 expression by $CD4^+$ cells in the LP of cases was higher than that in controls (363.9 vs 211.0; $p = 0.015$). Otherwise, although there is an overall trend for increased expression of activation markers, the differences between cases and controls are not of statistical significance ($p > 0.05$). However, compared with expression by peripheral $CD4^+$ lymphocytes, expression is markedly higher. In the peripheral blood the percentage of $CD4^+$ lymphocytes expressing CD25 or CD69 is consistently less than 10%, with fluorescent intensities of 50 to 250. In the LP and MEL, the percentage of $CD4^+$ lymphocytes expressing CD25 or CD69 is consistently 10-20%, with fluorescent intensities of 1000 to 3000. This observation indicates greater activation of lymphocytes within the gut mucosa.

A comparison of the proportion of $CD25^+$ and/or $CD69^+$ cells in the LP or MEL revealed that there were no significant differences between cases and controls, apart from expression of CD69 in the MEL (Figure 5-5 B). With regards to the intensity of CD25 and CD69 expression, the former was higher in the LP of cases as compared to the control (Figure 5-5 C; $p = 0.015$), whilst the latter was higher in the MEL of cases as compared to the control but the difference was not of statistical significance (Figure 5-5 D).

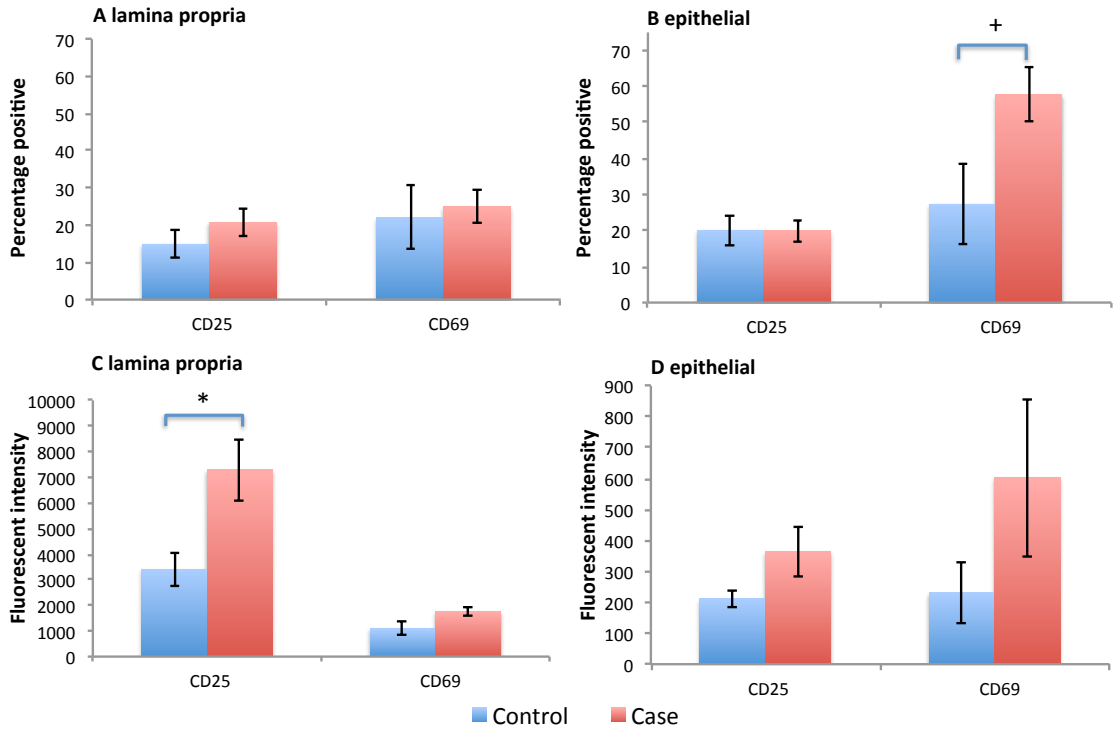

Figure 5-5: Expression of activation markers by $CD4^+$ cells within the lamina propria and epithelial layers of the gut mucosa. **Panel A -** percentage expression by lamina propria cells; **Panel B -** percentage expression by epithelial cells, + p = 0.043. **Panel C –** fluorescent intensity of expression by lamina propria cells, * p = 0.015. **Panel D –** fluorescent intensity of expression by epithelial cells. Data are presented as means ± 1 standard error, sample size as in Table 5-1.

Expression of activation markers by mucosal $\alpha4\beta7^+CD4^+$ lymphocytes

The expression of activation markers by mucosal $\alpha4\beta7^+CD4^+$ lymphocytes isolated from cases and healthy controls were compared (Figure 5-6). Expression of CD25 and CD69 on $\alpha4\beta7^+CD4^+$ cells was higher in both the LP and MEL of cases compared with controls, with both compartments showing similar patterns in cases and controls. Within the LP, the increase in expression was significant for the percentage of $\alpha4\beta7^+CD4^+$ cells expressing CD69 (65.88% vs 22.97%; p = 0.021; Figure 5-6, panel A), and the FI (243.15 vs 1528.33; p = 0.029; $^+$ Figure 5-6, panel C). The increases in CD25 expression were not significant. Within the MEL, the higher proportion of $\alpha4\beta7^+CD4^+$ cells expressing CD69 (20.18% vs 73.94%; p = 0.007; ** Figure 5-6, panel B), and the FI (1528.33 vs 273.27: p = 0.021; $^{++}$ Figure 5-6, panel D) were significant. In contrast, the higher CD25 expression by $\alpha4\beta7^+CD4^+$ cells was not statistically significant.

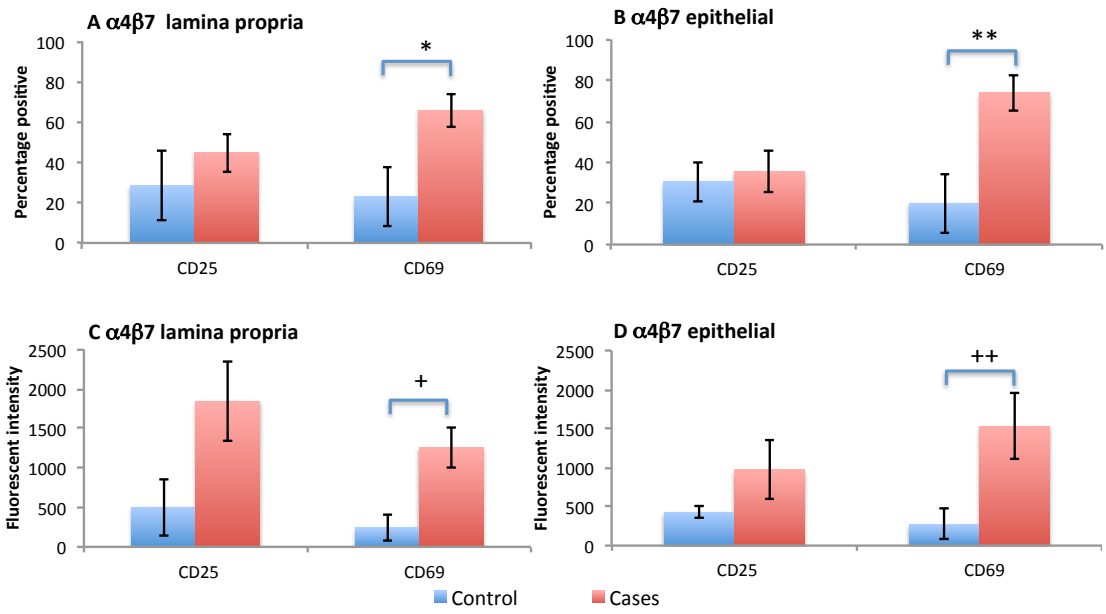

Figure 5-6: Expression of activation markers by $\alpha4\beta7^{+}CD4^{+}$ cells within the lamina propria and epithelial layers of the gut mucosa. **Panel A -** percentage expression by lamina propria cells, * p = 0.021. **Panel B -** percentage expression by epithelial cells, ** p = 0.007. **Panel C –** fluorescent intensity of expression by lamina propria cells, + p = 0.029. **Panel D –** fluorescent intensity of expression by epithelial cells, ++ p = 0.021. Data are presented as the mean ± one standard error, sample size as in Table 5-1.

These data show that there is a selective increase in the expression of activation markers by $\alpha4\beta7^{+}CD4^{+}$ lymphocytes within the mucosa of patients with IBD. This would suggest greater stimulation of cells activated in the gut mucosa (and thus expressing $\alpha4\beta7^{+}$).

Peripheral blood immune cell populations

Peripheral blood lymphocyte subsets

Figure 5-7 shows the profile of $CD4^{+}$, $CD8^{+}$ and $CD19^{+}$ lymphocytes and $\alpha4\beta7^{+}CD4^{+}$, $\alpha4\beta7^{+}CD8^{+}$ and $\alpha4\beta7^{+}CD19^{+}$ lymphocytes in the peripheral blood of cases and controls. Whilst there was no difference in the proportion of $\alpha4\beta7^{+}$ cells in cases and controls (35.2% vs 36.5%), they are markedly higher than in mucosal samples. $\alpha4\beta7^{+}$ lymphocytes constitute only 1% to 5% of LP cells, and only 8% to 13% of MEL cells (see Figure 5-3).

For the lymphocytes isolated from peripheral blood there is a significantly greater proportion of lymphocytes expressing CD4 than CD8 or CD19 in both the case and control groups (p values shown in Figure 5-7). Comparing cases and controls, a difference in $CD19^{+}$ lymphocytes is statistically significant (26.97% vs 17.92%; p =

0.043). There are greater proportions of $CD4^+$ lymphocytes and lower proportions of $CD8^+$ lymphocytes, but these differences were not statistically significant.

Comparing the lymphocytes isolated from controls there is a difference in the relative proportion of subsets between all lymphocytes and those expressing $\alpha4\beta7$. Whereas for all lymphocytes $CD4^+$ cells predominate, for $\alpha4\beta7^+$ lymphocytes $CD19^+$ cells predominate. Whilst the differences between the control group $\alpha4\beta7^+$ lymphocyte subsets are not statistically significant, comparisons with all lymphocytes from the control group are. Proportions of $CD8^+$ lymphocytes are higher in the $\alpha4\beta7^+$ group (29.54% vs 39.94%; p = 0.044), as are proportions of $CD19^+$ lymphocytes (17.92% vs 29.16%; p = 0.037). These data are not shown in Figure 5-7.

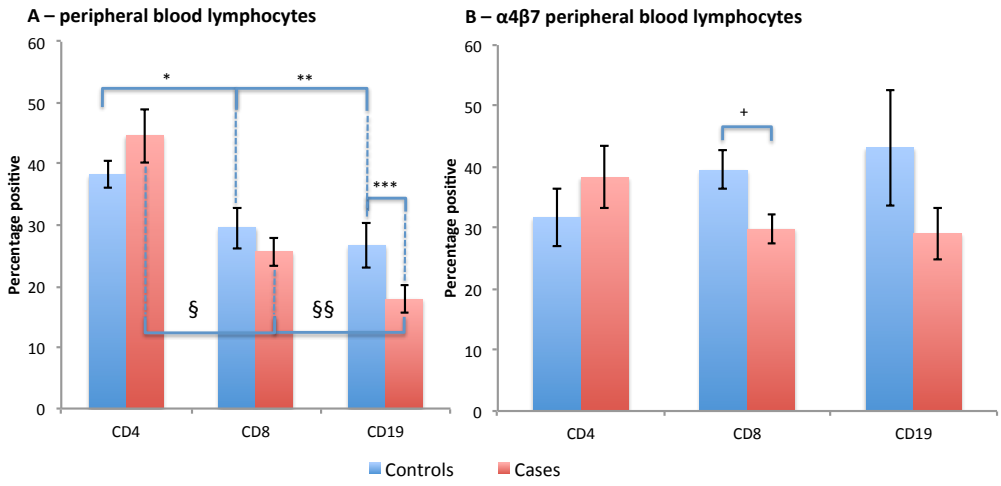

Figure 5-7: Lymphocyte subsets in the peripheral blood of cases and controls. **Panel A –** subsets as a percentage of all lymphocytes * p = 0.042 ** p = 0.017 *** p = 0.043 § p = 0.002 §§ p < 0.001 **Panel B –** subsets as a percentage of lymphocytes expressing $\alpha4\beta7^+$ $^+$ p = 0.018. Data are presented as the mean ± one standard error, sample size as in Table 5-1.

Comparing $\alpha4\beta7^+$ lymphocytes isolated from cases and controls there is a difference in the relative proportions of lymphocyte subsets. As stated above, $CD19^+$ lymphocytes predominate in controls, whereas $CD4^+$ lymphocytes are increased and come to predominate in cases. This difference is concomitant with reductions in the proportion of $CD8^+$ and $CD19^+$ lymphocytes. However, the only statistically significant difference is the lower prevalence of $CD8^+$ lymphocytes in cases (39.5% vs 29.8%; p = 0.018).

Expression of activation markers by peripheral blood $CD4^+$ lymphocytes

The proportion of lymphocytes, both $CD4^+$ and $\alpha4\beta7^+CD4^+$, expressing CD25 was higher in cases; although this difference was only significant for the $CD4^+$ lymphocytes

(p = 0.006). The fluorescent intensity of CD25 expression was also increased in cases, with the differences being statistically significant for both $CD4^+$ lymphocytes (p = 0.019) and $\alpha4\beta7^+CD4^+$ lymphocytes (p = 0.005).

The proportion of lymphocytes, both $CD4^+$ and $\alpha4\beta7^+CD4^+$, expressing CD69 was higher in cases. The increases were statistically significant for both $CD4^+$ lymphocytes (p = 0.004) and $\alpha4\beta7^+CD4^+$ lymphocytes (p = 0.031). There was no difference in the fluorescent intensity of CD69 expression in either the $CD4^+$ or $\alpha4\beta7^+CD4^+$ populations between cases and controls.

These data are presented in Figure 5-8. In the peripheral blood lymphocytes there is, overall, greater statistical significance in the differences between cases and controls than is seen in those isolated from the gut mucosa (see Figure 5-3). There is also a difference in the pattern of activation marker expression by lymphocytes in the gut and peripheral blood. In the peripheral blood, increased expression of CD25 by lymphocytes of cases predominate. In the gut mucosa, and particularly for $\alpha4\beta7^+$ $CD4^+$ lymphocytes, increases in CD69 expression predominate.

In the gut mucosa of controls, the expression of CD25 and CD69 is much closer, with cases having a relatively greater increase in the expression of CD69 compared with the increases in CD25. In the peripheral blood of controls, expression of CD25 is relatively higher than that of CD69, with cases having a relatively higher greater increase in expression of CD25 compared to that of CD69.

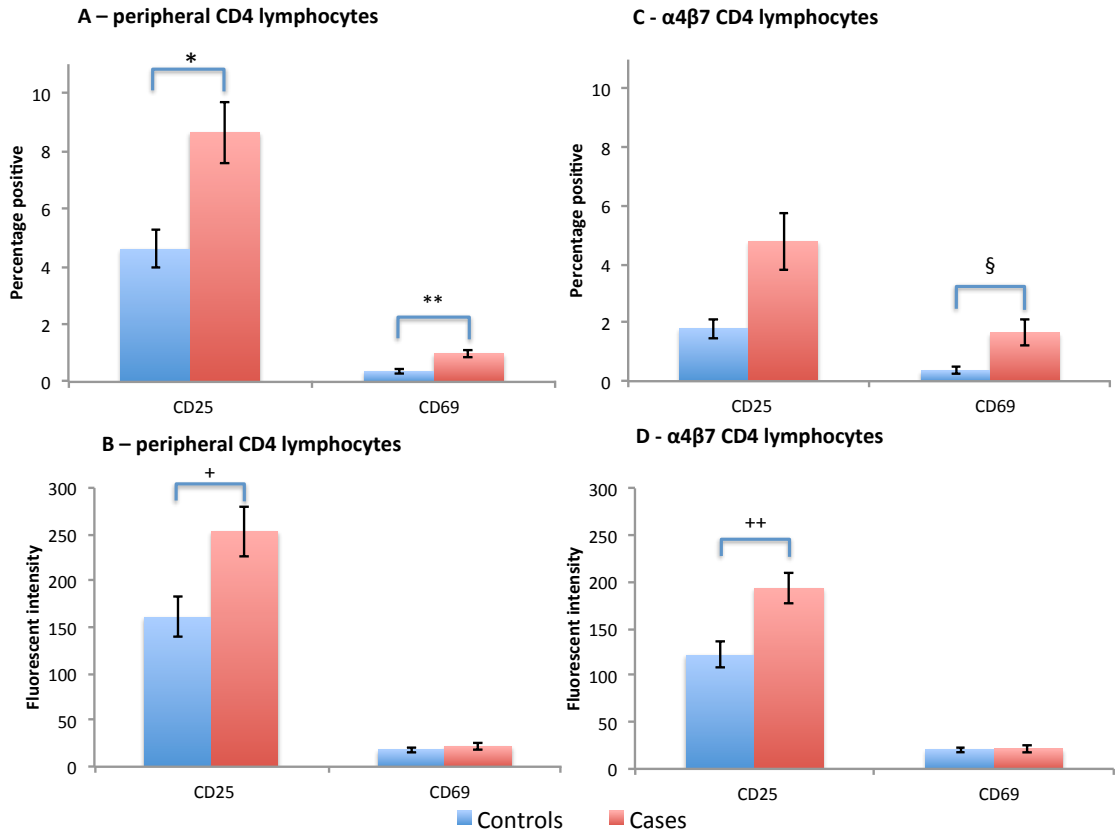

Figure 5-8: Expression of CD25 and CD69 by $CD4^+$ and $\alpha4\beta7^+CD4^+$ cells isolated from peripheral blood. **Panel A –** percentage expression by peripheral blood $CD4^+$ cells, * p = 0.006 ** p = 0.004. **Panel B –** percentage expression by peripheral blood $\alpha4\beta7^+CD4^+$ cells, § p = 0.031. **Panel C –** fluorescent intensity of expression by peripheral blood $CD4^+$ cells, + p = 0.019; **Panel D –** fluorescent intensity of expression by peripheral blood $\alpha4\beta7^+CD4^+$, ++ p = 0.005. Data are presented as means ± 1 standard error, sample size as in Table 5-1.

Bone metabolism and bone mineral density

The primary reason for this element of the study was to examine measurements of bone metabolism. Bone turnover markers were measured in all participants, and confirmed cases of IBD were subjected to a DXA scan.

Bone turnover markers

Figure 5-9 compares the measured bone turnover markers in cases and controls. Comparing cases to controls urine NTX levels (236.4nM bone collagen equivalent/mM creatinine ± 46.2 vs 325.7 ± 40.7) and serum osteocalcin levels (67.99ng/ml ± 11.56 vs 78.06 ± 7.93) were both lower in cases but the differences was not statistically significant. P1NP levels were lower with the difference reaching statistical significance (211.60ng/ml ± 51.45 vs 407.50 ± 54.26; p = 0.012).

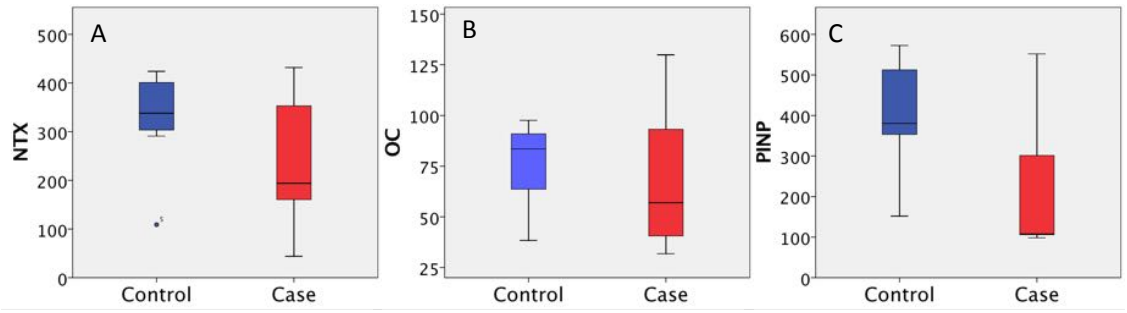

Figure 5-9: Boxplots showing comparing bone turnover markers in cases and controls. Panel A **-** urine NTX lower in controls. Panel B **-** serum osteocalcin lower in cases. Panel C **-** serum PINP lower in cases p = 0.012. Data are presented as medians and inter-quartile range, sample size as in Table 5-1.

Overall, these data demonstrate reductions in bone formation in children with IBD and a possible reduction in bone resorption. These findings are consistent with those of Sylvester et al (2006).

Bone mineral density

All patients diagnosed with IBD underwent a DXA to measure their bone mineral density (BMD). The control group was taken from a cohort of healthy children who had undergone DXA in a separate study, the data kindly provided by Dr Paul Arundel of The Sheffield Children's Hospital. Three age and sex matched patients were selected for each case. As discussed in the Materials and Methods, there are several different approaches to reporting the BMD in children. The method chosen herein is that of Carter (Crabtree et al 2017) which calculates an approximate volume for the lumbar

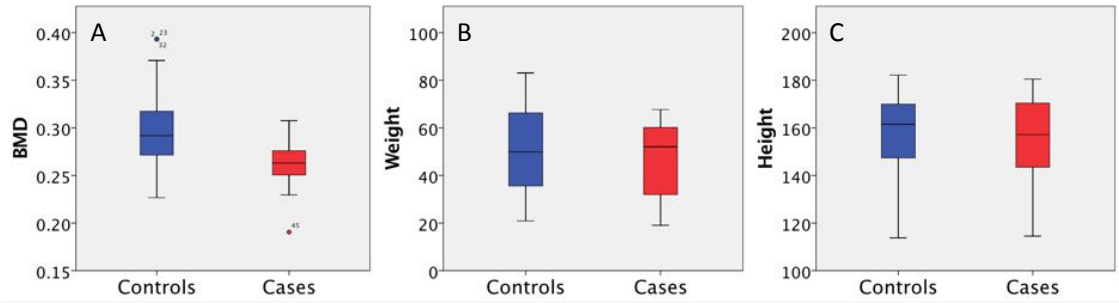

Figure 5-10: Boxplots comparing BMD, height and weight in cases and controls. **Panel A -** BMD (g/cm^3) median 0.292 vs 0.263 p = 0.002 **Panel B -** weight (kg) median 49.9 vs 52.1 p = 0.448 **Panel C -** height (cm) median 161.5 vs 157.2 p = 0.847. Data are presented as medians and inter-quartile range, sample size as in Table 5-1.

vertebrae, on the basis of their visible area, and relates to the measured bone mineral content (BMC) to give the BMD in g/cm^3. As can be seen in Figure 5-10, BMD was significantly lower in cases (median BMD 0.2918 vs 0.263; p = 0.002). Height and

weight, recorded routinely when undertaking a DEXA, were also compared as it has been suggested they may influence measurement of bone mineral content. As can be seen in Figure 5-10 there was no difference in either parameter in terms of median, or in the distribution.

Associations between the immune system and bone metabolism in children with inflammatory bowel disease

The aim of this element of the study was look for associations between the immune response and bone metabolism in children with IBD. The laboratory studies showed that $CD4^+$ lymphocytes may be the mediators of low bone mineral density, with resting $CD4^+$ cells supporting the growth of Saos-2 cells, and activated cells inhibiting growth. Changes in the profile of lymphocytes (proportions, phenotypes and activation status) in patients with IBD were compared to measures of BMD using linear regression which measures the strength of the association between the two variables, expressed as "R". The higher the value of R the stronger the association, and the value can be positive (for a direct relationship) or negative (for an inverse relationship). R^2 (which is not simply the value of R, squared) can be used to indicate how much of the variation in the dependent variable (in this case BMD) is due to changes in the independent variable, and can be expressed as a percentage.

Peripheral blood immune cells subsets

$CD4^+$ cells are important mediators of inflammatory bowel disease, and the laboratory studies indicate that they could potentially explain a significant proportion of the PBMC-mediated effects on Saos-2 cells.

White cell counts (WCC) in cases and controls were not significantly different, but the proportion of lymphocytes as a percentage of the WCC was. Whilst there was a weakly positive correlation between the proportion of lymphocytes and BMD (r = 0.186) which explained 3.4% of the variability in BMD, this relationship was not statistically significant (p = 0.585, Panel A of Figure 5-11).

The full blood count quantified the number of lymphocytes within the peripheral blood. A direct comparison between cases and controls (see earlier section) found that whilst numbers were lower in cases, the difference did not reach significance (p = 0.053). There was a moderately positive correlation (r = 0.345) between the lymphocyte count

and bone mineral density (BMD) explaining 12% of the variation, but the correlation was not significant (p = 0.298; Panel B of Figure 5-11).

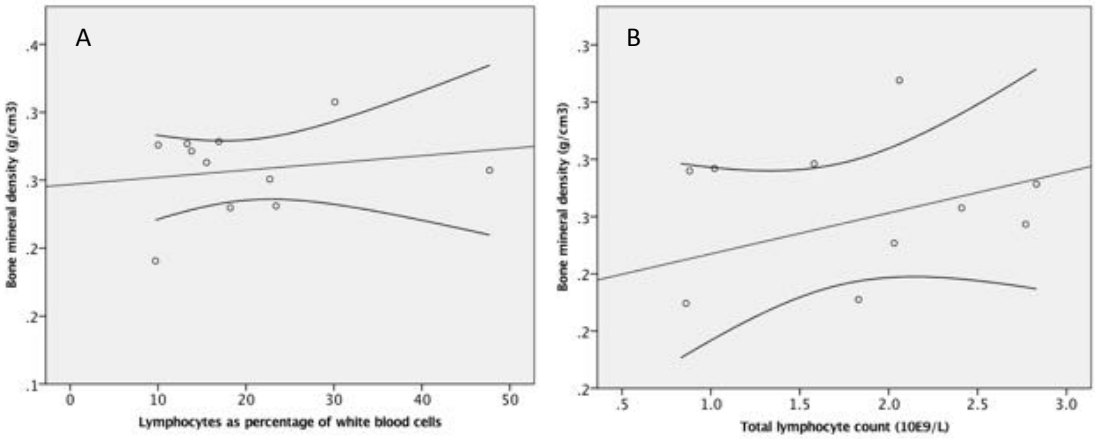

Figure 5-11: Correlations between total lymphocyte numbers and BMD. **Panel A -** correlation between BMD and the lymphocytes as a percentage of the total white cell count (11 patients; r = 0.186; p = 0.585) **Panel B -** correlation between BMD and the total lymphocyte count (10 patients; r = 0.345; p = 0.298).

The flow cytometry analysis determined the proportion of $CD4^+$ cells, and these data could be combined with the lymphocyte count from full blood counts to convert the percentage to a concentration:

$$lymphocyte\ count\ x\ (percentage\ CD4^+/100)\ = CD4\ count\ (cellsx10^9/L)$$

Density gradient isolation of PBMCs may change the relative proportions of lymphocytes from that in whole blood, but this approach still provides a more meaningful measure of the $CD4^+$ cell number than the proportion alone since it better reflects numbers of circulating lymphocytes.

Using this method there was a positive correlation between the $CD4^+$ lymphocyte count and BMD (r = 0.403; p = 0.219) and explained 16% of the variation in BMD. Applying a similar approach, there was a weakly positive correlation for $CD8^+$ cells (r = 0.152; p = 0.655), and a moderately positive correlation for $CD19^+$ cells (r = 0.434; p = 0.183), although neither of these correlations were statistically significant. A similar analysis identified moderately positive correlations between BMD and the number of $\alpha4\beta7^+CD4^+$ cells (r = 0.331; p = 0.320), $\alpha4\beta7^+CD8^+$ (r = 0.313; p = 0.349) and $\alpha4\beta7^+CD19^+$ (r = 0.435; p = 0.181) respectively explaining 11%, 9.8% and 19% of the variation in BMD. Scatterplots for these comparisons are shown in Figure 5-12.

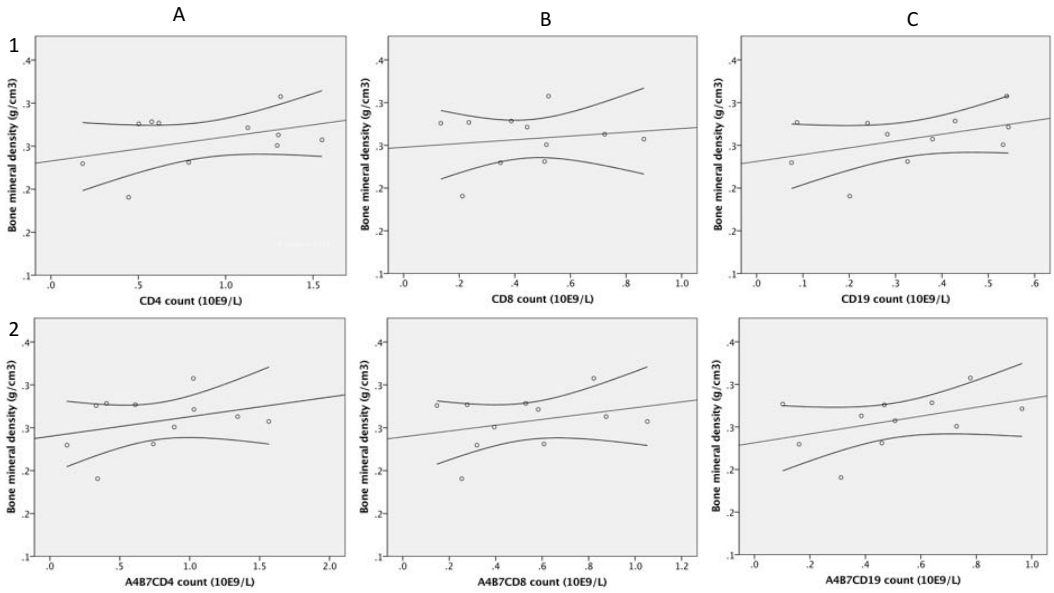

Figure 5-12: Correlations between BMD and absolute counts of peripheral blood lymphocyte subsets. **Panels 1A to 1C** - $CD4^+$, $CD8^+$ and $CD19^+$ cells. **Panels 2A to 2C** - $\alpha4\beta7^+CD4^+$, $\alpha4\beta7^+CD8^+$ and $\alpha4\beta7^+CD19^+$ cells. Sample size 11 for each analysis.

Mucosal immune cell subsets

Interpreting immune cell populations and their relation to BMD is difficult. In the peripheral blood studies above, it can be confidently assumed that the numbers and proportions seen in the samples taken reflect (broadly) those throughout the circulation. This means the lymphocyte populations in the samples will reflect that in the patient's circulation. However, this is not the case with the mucosal samples.

The distribution of immune cells throughout the gut changes in different regions to reflect functionality. However, the distribution will vary even within a functionally distinct region (especially if taking small biopsies) due to the structural elements of the gut. This could have been partially overcome by having a strict series of locations for biopsy, but this would risk missing areas of inflammation. Finally, it has not been practical to quantify immune cell numbers within the gut mucosa samples.

Despite these the findings still potentially add valuable information. There is a dynamic flow of immune cells between the gut and peripheral blood, and whilst it was possible to establish numbers in the blood, this flow of cells is not accounted for. Furthermore, IBD is primarily a mucosal disease and therefore the immune response within the bowel could be a better marker of disease severity.

Within the mucosal epithelium (Figure 5-13) there was a very weakly positive correlation between BMD and the percentage of $CD4^+$ cells (r = 0.049; p = 0.893), a moderately positive correlation with $CD8^+$ cells (r = 0.422; p = 0.225) and a moderately negative correlation with $CD19^+$ cells (r = 0.316; p = 0.373). Lymphocytes expressing $\alpha4\beta7$ also showed no significant correlations.

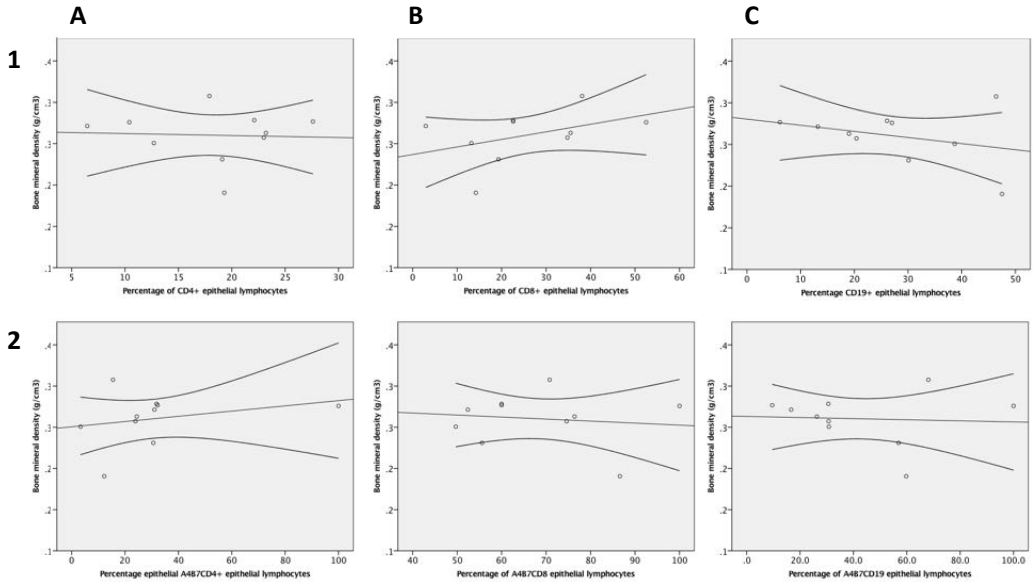

Figure 5-13: Correlations between BMD and the percentage of lymphocyte subsets in the mucosal epithelium. Panels 1A to C - $CD4^+$ (r = 0.049; p = 0.893), $CD8^+$ (r = 0.422; p = 0.225) and $CD19^+$ (r = 0.316; p = 0.373) cells. Panels 2D to E - $\alpha4\beta7^+CD4^+$ (r = 0.263; p = 0.463), $\alpha4\beta7^+CD8^+$ (r = 0.125; p = 0.732) and $\alpha4\beta7^+CD19^+$ (r = 0.058; p = 0.873) cells. Sample size 10 for each analysis.

Within the lamina propria, whilst none of the correlations were statistically significant, increases in the percentage of cells expressing CD4, CD8 and CD19 were all positively associated with BMD whereas amongst $\alpha4\beta7^+$ cells the same correlations were consistently negative. In fact, the correlation between the percentage of $\alpha4\beta7^+CD8^+$ lamina propria cells and BMD almost reaches statistical significance (r = 0.615; p = 0.059).

It is worth noting that whilst 7.7% of lymphocytes within the epithelial layer were $\alpha4\beta7^+$, only 1% of lymphocytes in the lamina propria were $\alpha4\beta7^+$. This is compared to 36.5% in the peripheral blood. Therefore, to find that changes in the proportion of $\alpha4\beta7^+CD8^+$ lymphocytes within the lamina propria potentially influences BMD is surprising.

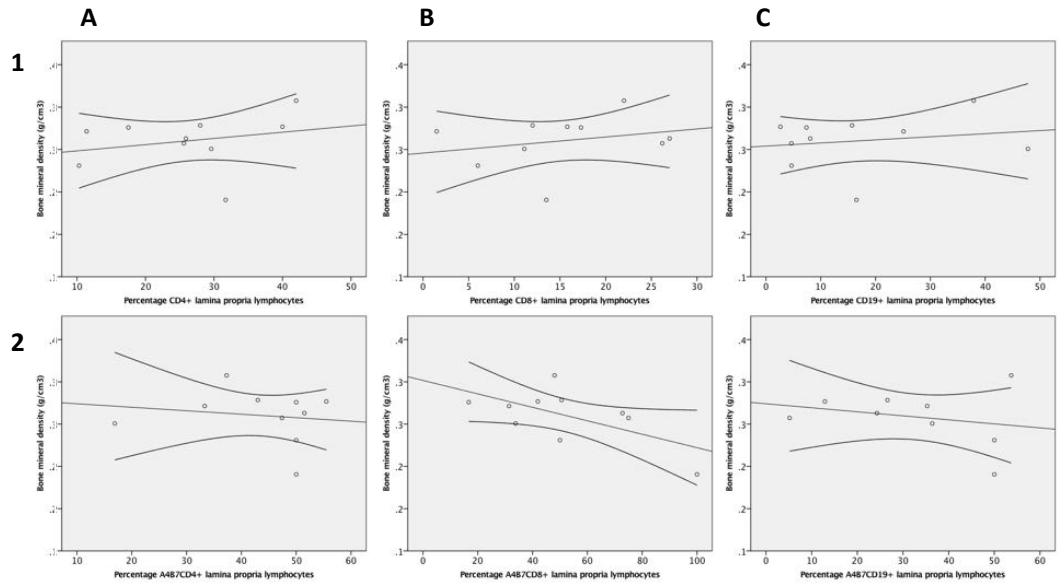

Figure 5-14: Correlations between BMD and lymphocyte subsets in the lamina propria. **Panels 1 A to C** - $CD4^+$ cells (r = 0.25; p = 0.489), $CD8^+$ cells (r = 0.25; p = 0.492) and $CD19^+$ cells (r = 0.178; p = 0.623) **Panels 2A to C** - $\alpha4\beta7^+CD4^+$ cells (r = 0.150; p = 0.678), $\alpha4\beta7^+CD8^+$ cells (r = 0.615; p = 0.059) and $\alpha4\beta7^+CD19^+$ cells (r = 0.245; p = 0.525). Sample size 10 for each analysis.

Activation of circulating and gut derived lymphocytes

The hypothesis is that immune cells, activated in the gut, negatively affect bone metabolism leading to osteoporosis. This was supported by the laboratory work which found that $CD4^+$ lymphocytes, potentially, mediated the observed inhibition of Saos-2 cells. Comparisons between cases and controls in this *in vivo study* showed primarily increased expression of CD25 by $CD4^+$ cells in IBD patients, with some increase in CD69 expression. Therefore, correlations between expression of CD25 and CD69 by $CD4^+$ lymphocytes (including those expressing $\alpha4\beta7$) and BMD were investigated. This included lymphocytes isolated from the peripheral blood and gut mucosa.

Figure 5-15 shows scatter plots for expression of CD25. There was a weak, negative correlation between the percentage of $CD4^+$ lymphocytes expressing CD25 and BMD (r = 0.264p p = 0.432), and $\alpha4\beta7^+CD4^+CD25^+$ lymphocytes and BMD (r = 0.190, p = 0.575). When the absolute number of $CD4^+$ lymphocytes and $\alpha4\beta7^+CD4^+CD25$ lymphocytes was calculated, based on the total lymphocyte count, both the correlations became positive. For $CD4^+$ lymphocytes r = 0.367 (p = 0.267) and for $\alpha4\beta7^+CD4^+$ lymphocytes r = 0.220 (p = 0.516).

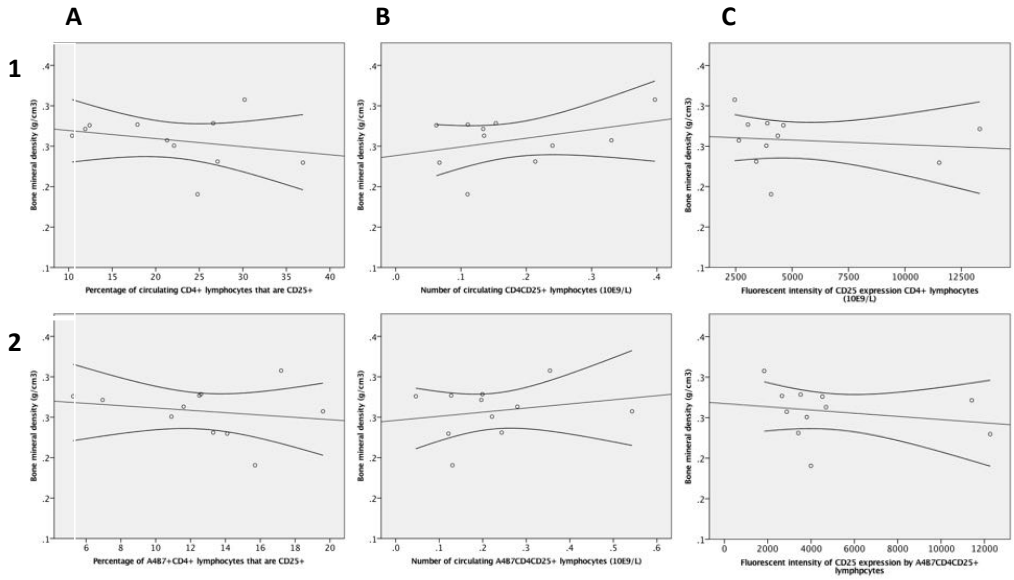

Figure 5-15: Correlations between expression of CD25 by $CD4^+$ lymphocytes and BMD in patients with IBD. **Panel 1A -** r = 0.264, p = 0.432 **Panel 1B -** r = 0.367, p = 0.267 **Panel 1C -** r = 0.132, p = 0.698 **Panel 2A -** r = 0.190 p = 0.575 **Panel 2B -** r = 0.220 p = 0.516 **Panel 2C -** r = 0.219 p = 0.517. Sample size 11 for each analysis.

The intensity of CD25 expression by $CD4^+CD25^+$ lymphocytes showed a weakly negative correlation (r = 0.132, p = 0.698), similar to that for $\alpha4\beta7^+CD4^+$ lymphocytes (r = 0.219, p = 0.517). However, as can be seen in the scatterplots in panels 1C and 2C of Figure 5-15, there are 2 outliers with markedly higher expression of CD25. Removing these outliers resulted in a stronger correlation (see Figure 5-16). They still

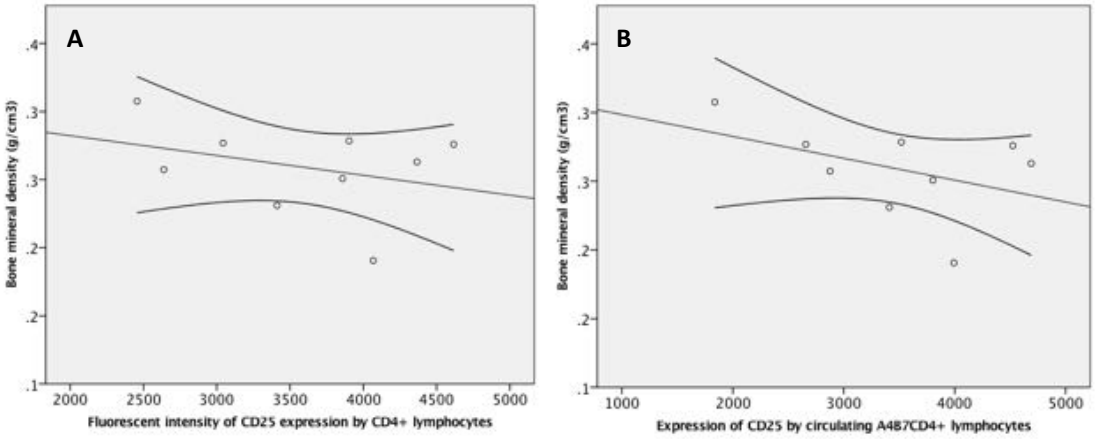

Figure 5-16: Correlations between the intensity of CD25 expression by circulating lymphocytes and BMD, with outliers removed. **Panel A:** Intensity of CD25 expression by $CD4^+CD25^+$ lymphocytes (r = 0.330; p = 0.386). **Panel B:** Intensity of CD25 expression by $\alpha4\beta7^+CD4^+CD25^+$ lymphocytes (r = 0.435; p = 0.241). Sample size 9 for each analysis.

did not reach statistical significance, but the fluorescent intensity explained more of the variation in BMD. For $CD4^+$ lymphocytes R^2 increased from 1.8% to 10.9%, and from 4.8% to 19% for $\alpha4\beta7^+CD4^+$ lymphocytes.

Relationships between CD69 expression and BMD was also studied, but no statistically significant correlations were found. Expression of CD69 by circulating $CD4^+$ cells showed a negative correlation when measured by both the percentage of cells expressing CD69 ($r = 0.505$; $p = 0.113$), and their fluorescent intensity ($r = 0.059$; $p = 0.864$). Similarly for $\alpha4\beta7^+CD4^+$ cells, BMD was negatively correlated with expression of CD69 when measured by the percentage of cells expressing CD69 ($r = 0.245$; $p = 0.467$) and the fluorescent intensity ($r = 0.246$, $p = 0.466$).

			r	r2	p
Epithelial mucosa	Percentage positive	CD4CD25	0.205	0.042	0.570
		CD4CD69	0.283	0.080	0.428
		A4B7CD4CD25	0.225	0.050	0.561
		A4B7CD4CD69	0.124	0.015	0.732
	Fluorescent intensity	CD4CD25	0.140	0.020	0.700
		CD4CD69	0.790	0.625	0.006
		A4B7CD4CD25	0.818	0.669	0.007
		A4B7CD4CD69	0.021	0.000	0.955
Lamina propria	Percentage positive	CD4CD25	0.072	0.005	0.844
		CD4CD69	0.017	0.000	0.962
		A4B7CD4CD25	0.033	0.001	0.932
		A4B7CD4CD69	0.402	0.162	0.250
	Fluorescent intensity	CD4CD25	0.144	0.021	0.691
		CD4CD69	0.270	0.073	0.450
		A4B7CD4CD25	0.186	0.034	0.632
		A4B7CD4CD69	0.622	0.386	0.055

Table 5-3: Output of statistical analysis of correlations between expression of activation markers by gut $CD4^+$ cells and bone mineral density. Significant findings are highlighted in red $\alpha = 0.05$.

Studying further expression of CD25 and CD69 by mucosal $CD4^+$ cells the majority of analyses were not statistically significant (see Table 5-3). Two analyses were significant: the fluorescent intensity of CD69 expression by epithelial $CD4^+CD69^+$ cells, and the fluorescent intensity of CD25 expression by epithelial $\alpha4\beta7^+CD4^+CD25^+$ cells. The former showed a positive correlation with BMD, and the latter a negative

correlation (see Figure 5-17). However, although statistically significant, even if it is not a false positive, it is difficult to describe a reason why the association would be causative.

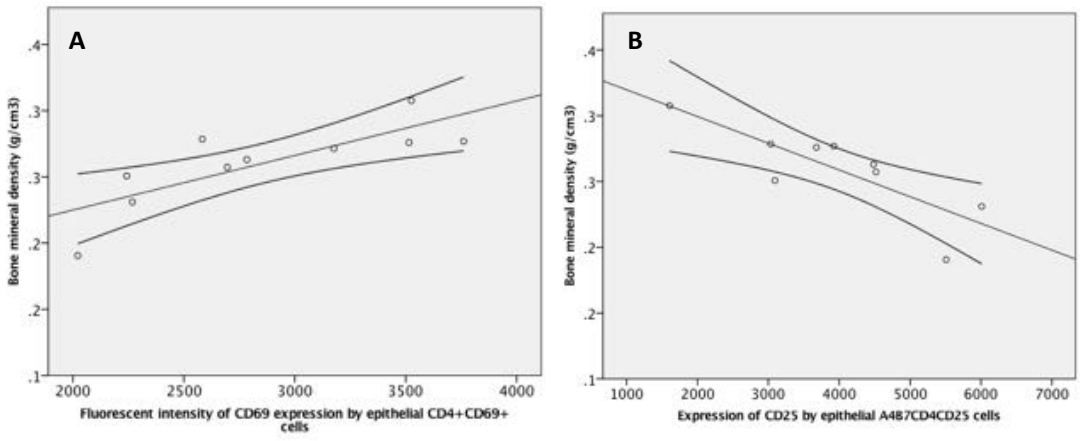

Figure 5-17: Correlations between expression of activation markers by gut $CD4^+$ lymphocytes and bone mineral density. **Panel A –** Fluorescent intensity of CD69 expression by epithelial $CD4^+CD69^+$ lymphocytes (r = 0.625; p = 0.006). **Panel B -** Fluorescent intensity of CD25 expression by epithelial $\alpha4\beta7^+CD4^+CD69^+$ lymphocytes (r = 0.669; p = 0.007). Sample size 10 for A, 9 for B.

Conclusion

In this cohort of children with IBD there was increased expression of CD25 by circulating $\alpha4\beta7^+CD4^+$ cells and reductions in BMD, compared with healthy controls. However, whilst expression of CD25 was negatively correlated with BMD, and the number of lymphocytes was positively correlated with BMD, neither of these correlations were statistically significant.

6 Discussion

The hypothesis of this thesis is that reduced bone mineral density (BMD) in children with Crohn's disease (CD) is, at least in part, mediated by interactions between activated lymphocytes that have migrated from the gut mucosal tissue to the bone where they come in contact with osteoblasts. An *in-vivo* study looked for correlations between gut derived and circulating lymphocytes, and measures of bone metabolism. An *in-vitro* study investigated the effects of immune cells on the growth and alkaline phosphatase activity of Saos-2 cells.

Results

In-vitro study

The first two co-culture experiments looked at the effect of immune cell populations (one PBMC, one $CD4^+$ lymphocyte enriched), cell number, activation status, and cell contact, on the growth of Saos-2. These two experiments had some differences in their results but, broadly, the effects of PBMCs and $CD4^+$ lymphocytes on the growth of Saos-2 were similar. Activated immune cells i.e. both the PBMCs and the $CD4^+$ lymphocytes, inhibited the growth of Saos-2 whilst resting immune cells increased their growth. These opposing effects were potentiated by increasing numbers of immune cells. The presence of a transwell insert increased the number of Saos-2 in both the activated and resting conditions, indicating that contact with immune cells has a consistently negative effect on their growth. Saos-2 also appeared to alter the expression of CD25 and CD69 by immune cells, reducing the expression of these activation markers in the activated condition whilst increasing expression in the resting condition. These changes in expression were potentiated by increasing numbers of Saos-2 in a dose-dependent manner.

Numbers of Saos-2 cells at the end the co-culture period, counted after fluorescent labelling, were in the hundreds of thousands when the expected number is nearer a million. Rather than indicating apoptosis, this may be due to the method of counting the fluorescently labelled cells. Fluorescent labelling requires cell to be washed i.e. diluted, centrifuged, and the supernatant aspirated; potentially leading to a loss of cells. This could have been overcome by having separate samples for counting and labelling; however, since the loss of cells during wash steps would have been proportional, valid comparisons can still be made between the experimental conditions.

The third experiment introduced a $CD4^+$ lymphocyte depleted population, and studied how the three immune cell populations influenced the growth and alkaline phosphatase (ALP) activity of Saos-2. ALP is present on the membrane of Saos-2 cells and within immune cells so could not easily be measured if these cells had been cultured in contact: analysed together the ALP activity of both cell types would be measured, but if separated prior to analysis ALP on the membrane of the Saos-2 would be disrupted. It also wasn't practical to measure Saos-2's generation of a mineralised matrix: the prolonged incubation time would have required the generation of a series of immune cell populations.

An unexpected finding of the third experiment was that immune cells no longer influenced the growth of Saos-2 cells, which can only be explained by the immune cells addition after the Saos-2 had adhered. There was a small increase in Saos-2 growth with increasing immune cell number, but the marked and opposing effects of activation and resting immune cells was lost. ALP activity was largely unaffected by the PBMCs or the $CD4^+$ enriched population, but was increased by the presence of both the resting and activated $CD4^+$ lymphocyte depleted populations. This was an unexpected finding and one possible explanation is that cell stress increases ALP activity and can induce mineralisation, but why it was associated with a depletion of $CD4^+$ lymphocytes is not clear. It may indicate a significant role for $CD4^+$ lymphocytes in supporting the survival of Saos-2 cells.

There are limitations to these experiments which limit generalisation of the findings. Whilst each experiment was repeated at three time points there were not any replicates at each of those time points. Independent repeats are important in ensuring the experiment is reproducible, but replicates are necessary to provide internal quality control for each repeat (Vaux et al 2012). It is also possible that the effects seen were a non-specific interaction between the cells, rather than reflecting a specific osteoimmune effect. The experiment cultured immune cells with a tumour cell line, and when normal cells become tumour cells their interactions with the immune system are altered. Also, the two cell types are from different hosts; and whilst the culture period is short there is the potential for alloimmune reactions. However, as will be discussed below, our results are consistent with those published elsewhere.

One group, investigating interactions between T cells and osteoblasts in rheumatoid arthritis, cultured PBMCs with human primary osteoblasts and the human osteoblastic cell line MG63 and showed that Fas (a protein known to be involved in cell apoptosis)

was necessary for T cell induced apoptosis of osteoblasts (Kawakami et al 1997). They found that activated PBMCs and their secreted factors induced apoptosis of both primary osteoblasts and MG63 cells, blocked with the addition of a Fas chimeric protein. The different cell types were not autologous, but they do not say if the osteoblasts had adhered before exposure to immune cells and their secreted factors. It seems possible that Fas mediated apoptosis of the Saos-2 cells occurred in the experiments presented here, and this apoptotic pathway is not specific to these osteoimmune interactions. But, as the authors point out, it may still have a role in mediating bone loss in inflammatory diseases.

The Fas ligand pathway also mediates apoptosis of murine bone marrow mesenchymal stem cells (BMMSCs) by T cells (Yamaza et al 2008). Mouse BMMSCs cultured with autologous lymph node T cells, pre-activated with monoclonal antibodies (mAbs) to the CD3 component of the T Cell Receptor, resulted in the death of the BMMSCs. This effect was dependent on cell contact (it was not observed with conditioned media from activated T cells, nor in the presence of a transwell insert) pre-activation of the T-cells. It did not occur in experiments using T cell deficient mice, nor in the presence of a CD3 or a Fas ligand blocking agent. Anti-TNF-α and anti-IFN-γ antibodies did not stop cell death. This study confirms the Fas pathway's role in mediating T cell induced death of BMMSCs. The importance of cell contact, also seen in our studies, further supports the potential for Fas ligand binding as the mechanism mediating the negative effects of contact between immune cells and Saos-2 in this thesis. That the cells were autologous overcomes one limitation of other cell culture experiments, and may explain why the effects were only seen in the presence of cell contact.

The experiments presented in this thesis show activated immune cells *in-vitro* having a predominantly negative effect of on the growth of Saos-2, but mineralisation doesn't just depend on growth. Osteoblasts can be generated from the differentiation of human BMMSCs and, once mature osteoblasts, ALP activity reflects their capacity to mineralise. It has been shown that factors secreted by activated immune cells may promote both growth and mineralising capacity (Rifas et al 2003). Human BMMSCs and osteoblasts were cultured in immune cell supernatants, generated from activated and resting $CD4^+$ lymphocytes. The activated cell supernatant increased ALP activity by both human BMMSCs and osteoblasts, an effect not seen with the non-activated cell supernatant. Human BMMSCs increased their expression of Runx2 (an osteoblast transcription factor) and osteocalcin, confirming their osteoblast phenotype. This

suggests activated $CD4^+$ lymphocytes may improve BMD, and the authors cite evidence that in rheumatoid arthritis there is increased osteoblast activity, ALP activity and osteocalcin; although this is the context of lower BMD. The findings of the data presented in this thesis, supported by others (Sylvester et al 2006), show in children with CD reduced bone formation and reductions in serum osteocalcin levels. It may be that the effects seen are due to cell stress which increases ALP activity and could, theoretically, also induce differentiation. That does not, however, exclude the possibility that cell stress *in-vivo* influences BMD.

A more recent publication provides a more comprehensive investigation of interactions between immune cells and BMMSCs (Croes et al 2016), but their findings may still reflect non-specific cell interactions. BMMSCs were isolated from human bone marrow, and lymphocytes from the blood of different donors. Immune cells populations (PBMC, $CD4^+$ lymphocyte enriched and $CD8^+$ lymphocyte enriched) were activated prior to their introduction to BMMSCs, and whilst it appears they were added after the BMMSCs had become adherent this is not explicitly stated. Activated immune cells increased the ALP activity of the BMMSCs, indicating their development of an osteogenic phenotype. The presence of a transwell insert did not change the effects of the $CD4^+$ lymphocytes. Lymphocytes ($CD4^+$ and $CD8^+$) had a greater effect than PBMCs, and conditioned media from $CD4^+$ lymphocytes produced the greatest increase in the production of a mineralised matrix. Resting $CD4^+$ lymphocytes also increased ALP activity. That both resting and activated T cells reduced growth of the BMMSCs could be due to less favourable culture conditions, but the effect was not statistically significant. Interestingly, although co-culture with a population enriched in Th17 cells showed no osteogenic effect, conditioned medium from those same cells markedly increased osteogenic differentiation of the BMMSCs. They suggest it may be the BMMSCs inhibiting proliferation of the TH17 cells, and they demonstrate that expression of CD25 by T cells was reduced by the BMMSCs.

Whilst Saos-2 were shown in this thesis not to express MHC class II, that may change in the presence of activated immune cells. It has been shown that culture of Saos-2 in the cytokine IFN-γ resulted in expression of MHC class II (Stanley et al 2006). Saos-2 cells were also able to present antigen to T-cells and increased the proliferation of the latter, with this effect lost in the presence of an MHC class II blocking agent. This demonstrates the potential for bidirectional signalling between these mature cells of the haematopoietic and mesenchymal cells.

Therefore, whilst the *in-vitro* experiments have a number of limitations and can only be considered preliminary, they are in keeping with the findings of other well established research groups. Activated and resting immune cells can interact with osteoblasts and BMMSCs in ways that could, both positively and negatively, affect bone metabolism. It is also possible for cells of mesenchymal origin to influence immune cells.

In-vivo study

Peripheral blood lymphocytes

In the peripheral blood of IBD patients there was increased expression of CD25 and CD69 by $CD4^+$ lymphocytes, indicating greater lymphocyte activation. There was no difference between cases and controls in the percentage of lymphocytes expressing $\alpha4\beta7$, but was up to 18 times greater in peripheral blood compared to the mucosal layers. The phenotypic profile of $\alpha4\beta7^+$ lymphocytes in controls showed a slight predominance of $CD19^+$ lymphocytes over $CD4^+$ with the pattern reversed in cases, although these differences were not statistically significant. Activation marker expression by $\alpha4\beta7^+$ lymphocytes was similar to that of all lymphocytes i.e. showed greater expression of CD25 and CD69.

These findings are consistent with those of other investigators. The number of $CD4^+CD25^+$ lymphocytes in adult patients with IBD has been found to be higher (18% vs 11%) (Chamouard et al 2009), and also in children with IBD (10% vs 5%) (Cseh et al 2010). Cseh et al (2010) also found that the proportion of $CD4^+$ lymphocytes was not increased in children with IBD. In adult IBD patients, the total lymphocyte count has been shown to be reduced, whilst the ratio of $CD4^+$ to $CD8^+$ is unchanged (Selby et al 1983; Senju et al 1991a), with Senju et al (1991a) additionally observing reductions in the number of $CD19^+$ lymphocytes. Chamouard et al (2009) also looked at expression of the $\beta7$ integrin by $CD4^+$ lymphocytes, and found no difference between cases and controls (although they did not differentiate between $\alpha4\beta7^+$ and $\alpha E\beta7^+$, the latter indicating cells activated in the epithelial layer). Meenan et al (1997) found around 50% of circulating $CD4^+$ lymphocytes expressed $\alpha4\beta7$, with no difference between cases and controls (Meenan et al 1997). There was an increase in the percentage of $CD4^+\alpha4\beta7^+$ lymphocytes expressing CD25 from 4% in controls to 7% in CD and 5.2% in UC. They also found that $CD4^+CD25^+$ correlated with disease severity with steroids having no effect on these measures.

If expression of CD25 was used as a marker of T regulatory cells an increase in the number of $CD4^+$ lymphocytes taken from patients with IBD and expressing this marker, as found in this thesis and the work of others (Chamouard et al 2009; Cseh et al 2010), may seem counterintuitive. However, in isolation CD25 is not specific to T regulatory cells and, as discussed earlier in this thesis, it is also a marker of immune cell activation. This may explain why multiple markers, including CD127 and FOXP3, need to be included to differentiate activated $CD4^+$ lymphocytes from T regulatory cells (Santegoets et al 2015).

Mucosal lymphocytes

Numerous studies have reported on cell populations within the gut mucosa of healthy individuals and those affected by IBD and, as discussed in the introduction, help understand the mucosal immune system. One objective of this thesis was to compare gut mucosal lymphocyte populations in cases and controls, and to look for associations between them and measures of bone metabolism. This is much more difficult with mucosal samples than with blood for several reasons.

Within both the LP and epithelial layers of controls, $CD19^+$ lymphocytes predominated; but in the lamina propria (LP) of cases there was a marked increase in the proportion of $CD4^+$ lymphocytes, thereby making it the predominant lymphocyte subset. The distribution of lymphocyte subsets with the epithelial layer of cases and controls did not differ. Numbers of $\alpha4\beta7^+$ lymphocytes were greater in the epithelial layer than in the LP, although the difference was only significant in cases. In the LP and epithelial layer of controls, $\alpha4\beta7^+$ lymphocytes were predominantly $CD8^+$, and in cases the only difference was an increase in the percentage of $CD4^+$ lymphocytes in the LP. In the mucosal samples taken from cases, LP lymphocytes showed increased expression of CD25 whilst epithelial layer lymphocytes showed increased expression of CD69. When selected on the basis of $\alpha4\beta7$, it was only expression of CD69 by both LP and epithelial layer lymphocytes that was significantly increased.

Selby et al (1984) isolated lymphocytes from the gut mucosa of patients with IBD using dithiothreitol (DTT) and ethylenediamine tetra-acetic acid (EDTA) with an overnight incubation in collagenase, as well as immunohistochemistry. They found 80% of lymphocytes in the epithelial layer were $CD8^+$, and 60% in the LP were $CD4^+$. In a separate paper, the same group found no difference in the ratio of $CD4^+$ to $CD8^+$ lymphocytes between cases and controls. Comparing immunohistochemistry and

isolated immune cells, the latter showed relative reductions in the prevalence of $CD8^+$ lymphocytes. Senju et al (1991c) isolated LP cells from the gut mucosa of patients with IBD, but did so using no collagenase. Cells were incubated in DTT and EDTA, but the process completed by passing the digested tissue through a nylon mesh. They found an increase in the proportion of $CD8^+$ lymphocytes in patients with Crohn's, and no increase in the percentage of lymphocytes expressing CD25. Yacyshyn (1993) studied B cell populations in the colonic mucosa of patients with IBD, isolating cells using EDTA and an overnight incubation in collagenase. They found around 10% LP lymphocytes were $CD19^+$, with no differences between controls and IBD. Meenan et al (1997) isolated immune cells from endoscopic biopsies, incubating for one hour in collagenase. They found that in healthy controls 72% of colonic T-cells expressed $\alpha4\beta7$, with this percentage reduced to around 50% in IBD. However, as was shown by Farstad et al (1996), the proportion of lymphocytes expressing $\alpha4\beta7^+$ are higher in the lamina propria (around 70%) and lower in the epithelial layer (at 30-50%).

Whilst some of the findings are consistent with those in the published literature, there are also many differences which may reflect the difficulties in studying lymphocyte populations in gut mucosa. The first problem is where samples are taken from. Immune cell populations within a blood sample are likely to be similar regardless of which larger blood vessel it is taken from. There may be some variation, but it is much less than for samples of gut mucosa in which it will vary depending on the location (Mowat et al 2014). The relative proportions of $CD4^+$ lymphocytes and $CD8^+$ lymphocytes changes along the GI tract with a decrease in $CD8^+$ lymphocytes (17 to 12%) and an increase in $CD4^+$ lymphocytes (36 to 42%) (Tauschmann et al 2013). Then each region of the gut mucosa contains regions of organised lymphoid tissue, such Peyer's Patches in the terminal ileum, which contain different populations to the surrounding mucosa. Finally, in patients with IBD populations will differ in inflamed and non-inflamed mucosa.

In this study biopsy samples were not taken in a consistent way. One important reason for this was the difficulty in ensuring the obtaining research samples did not have any detrimental effect on the patient, but also some uncertainty about the yield from small biopsy samples. Having developed the technique, in future studies a more structured approach could be used. It would be possible to take sufficient samples from only the terminal ileum, and in cases to do so from areas of inflamed and non-inflamed mucosa. However, the distribution of inflammation can vary between patients.

Secondly, the technique of immune cell isolation used affects the result. Flow cytometry allows multiple cell surface markers to be analysed but, as was shown in this works optimisation, whilst enzyme methods cleave the cell surface proteins mechanical methods resulted in non-specific binding that prevented meaningful analysis. The use of different enzymes is described as a method to release separately cells from the epithelial and lamina propria layers from large sections of mouse bowel (Weigmann et al 2007), but a study published after completion of this work highlights the problems with using this technique on endoscopic biopsy samples (Carrasco et al 2013). The investigators isolated lymphocytes using "smooth" and "intense" enzyme digestion solutions to epithelial cells (using EDTA and DTT) then lamina propria cells (using collagenase). Immunohistochemistry was used to establish the effectiveness of epithelial layer removal. Their conclusion is that a more intense enzyme digestion method achieves the best, but not complete, epithelial layer removal; whereas as a gentler method preserves the surface proteins of lamina propria cells. Van Damme et al (2000) found similar problems with enzymes cleaving surface proteins.

In this thesis the finding of CD19+ lymphocytes in the epithelial samples means there was contamination with cells of the lamina propria. Whilst the technique could be further optimised the work of Carrasco et al (2013) found a smooth enzyme protocol (low dose enzymes for a longer period) resulted in 42% of epithelial leucocytes being CD19+, whilst a more intense protocol (high dose enzymes for a shorter period) reduced that to 16%. Immunohistochemistry could be used but it does also have limitations. The sections are smaller than the endoscopic biopsy, being less than one cell thick, and a series taken from one biopsy may show quite different populations. Also, fewer cell surface markers can be studied than with multi-colour flow cytometry. This may explain why Carrasco et al (2013) don't make any effort to compare the lymphocyte populations in samples analysed by flow cytometry to those analysed by immunohistochemistry. Both techniques have their role, but in the context of this study looking for correlations between gut immune cell populations and measures of bone mineral density is inherently problematic.

The finding in this study of a greater number of $\alpha 4\beta 7$+ lymphocytes in the epithelial layer conflicts with the published work of Farstad et al (1996) who found greater numbers in the lamina propria (LP). This is unlikely to reflect contamination of the epithelial population with LP cells as it shouldn't be able to reverse the ratio overall, but simply reduce the difference. It may reflect problems with using two separate

fluorescent antibodies, one for α4 and one for β7. This requires gating based on the fluorescent intensity of two different fluorphores, and whilst that is done using appropriate isotypes it introduces the possibility of greater error. A more stringent gating strategy may have addressed this, but the same strategy was used for both mucosal and peripheral lymphocytes and the proportion of circulating lymphocytes expressing α4β7 was comparable to that of Meenan et al (1997). There is an alternative explanation for the finding. It was shown in the experiments assessing the effects of enzymes on cell surface markers that α4β7 was especially sensitive to those used for both epithelial and LP digestion. The epithelial population was exposed to enzymes for a shorter period than those of the LP, and the latter were additionally exposed to collagenase. This could have resulted in greater degradation of α4β7 altering the relative proportions creating the appearance of lower numbers in the LP. It would explain the magnitude of difference in expression of α4β7 between mucosal and peripheral lymphocytes.

Bone turnover and bone mineral density

The measurement of bone turnover markers showed reductions in markers of bone formation and resorption. Of the two formation markers, although both PINP and osteocalcin were lower in cases, only the reduction in PINP was significant. The reduction in NTX, a marker of resorption, was not significant; but this contrasts with increased bone resorption in adult IBD. The DEXA scans did show a significant reduction in bone mineral density (BMD) in cases compared with controls. This is in keeping with numerous studies of BMD in IBD (discussed in the introduction), although reductions are not always found in IBD patients. These findings would suggest that the reductions in BMD have occurred due to low bone formation, with a possible reduction in bone resorption.

The influence of immune cells on bone mineral density

No statistically significant correlations between the proportion of circulating lymphocyte subsets and their activation status with BMD were found. This was also true for most gut mucosal immune cells, and whilst two measures were positive (epithelial $CD4^+$ cell expression of CD69, and $\alpha4\beta7^+CD4^+$ cell expression of CD25) the limitations discussed mean they must be interpreted with caution.

Of all the measures looked at, circulating $CD4^+$ cells expression of CD25 seems to be the cell surface marker most likely to be associated with changes in BMD so its measurement by fluorescent intensity was used in a power calculation. Based on the variability of CD25 expression by $CD4^+$ lymphocytes and BMD, and the number of subjects, the power of the study was only 20%. To achieve the necessary power of 80% a cohort of 80 cases would be required, and so it was unlikely that this study would have been able to find a significant correlation. Therefore, whilst associations between immune cells expressing $\alpha4\beta7$ and BMD are not seemingly different when expression of $\alpha4\beta7$ is not considered, it is still possible that gut activated immune cells could have a greater effect.

Even though there were no statistically significant correlations between the peripheral immune cells and BMD there are some potentially interesting observations. BMD was positively correlated with the circulating lymphocyte count, but negatively correlated with expression of CD25 by $CD4^+$ cells (both the percentage of cells positive, and the fluorescent intensity). Similar patterns were seen in $\alpha4\beta7^+CD4^+$ cells, and in expression of CD69. This fits with the observations in the laboratory study of resting $CD4^+$ cells supporting bone formation, and activated $CD4^+$ cells inhibiting growth.

Statistical analysis

The use of 3- and 4-way ANOVA to analyse the co-culture experiments data was unusual, and such a complex analysis may not have been appropriate given the quality of the data. A simpler approach could have been taken and individual data points compared by a series of T-tests. This is how data is usually analysed, although the one-way ANOVA is sometimes seen. But laboratory experiments are typically set up to compare only two variables, and the co-culture experiments in this thesis compared 4 or 5. The use of 3- and 4- way ANOVA considers, and statistically measures, how those variables affect each other. The relative merits of these two approaches is discussed in chapter 2 of this thesis.

Summary

The findings of this thesis support the hypothesis that activated immune cells can have a negative impact on bone formation. The *in-vitro* work shows that activated lymphocytes have a negative impact on the proliferation of osteoblasts, although only if the two cell types are combined before the cells of mesenchymal origin have become adherent. Resting immune cells seem able to support the growth of osteoblasts, and

there is evidence of bi-directional signalling with Saos-2 influencing the activation status of resting and activated lymphocytes. The overall effects of PBMCs and $CD4^+$ lymphocytes were not different.

The *in-vivo* work found children with IBD had reduced bone mineral density, with reductions in both formation and resorption. Expression of CD25 was negatively correlated with BMD whilst the number of lymphocytes was positively correlated with BMD, which can be said to be in keeping with the *in-vitro* observations that immune cells can both increase and decrease bone formation. Associations between bone mineral density and lymphocyte populations cannot be said with any confidence to differ based on expression of $\alpha 4\beta 7$.

So far there has been limited discussion of whether gut-activated lymphocytes traffic to bone, and if they do whether they enter the bone marrow or reach the mineralised bone tissue. In setting up this study it was assumed to occur, given the evidence for interactions between T cells and osteoclasts. This will now be discussed prior to describing how best to take this work forward. In addition, whilst there are significant limitations to the experimental work presented here, it raises the potential importance of osteoimmune interactions in both inflammatory disorders and the normal functioning of both bone and the immune system.

The broader context of osteoimmune interactions

Bone marrow and its relationship with mineralised bone tissue

The bone marrow is found within the cancellous portions of bone, coming in contact with the trabeculae and the endosteal surface (Guillerman 2013). Bone marrow consists of “red marrow” (with a significant blood supply) and “yellow marrow” (less blood supply and more fat). Whilst in young children all cancellous bone contains red marrow, by adulthood this has been replaced by yellow marrow in peripheral bones. In central bones, such as the vertebrae, red marrow persists throughout life.

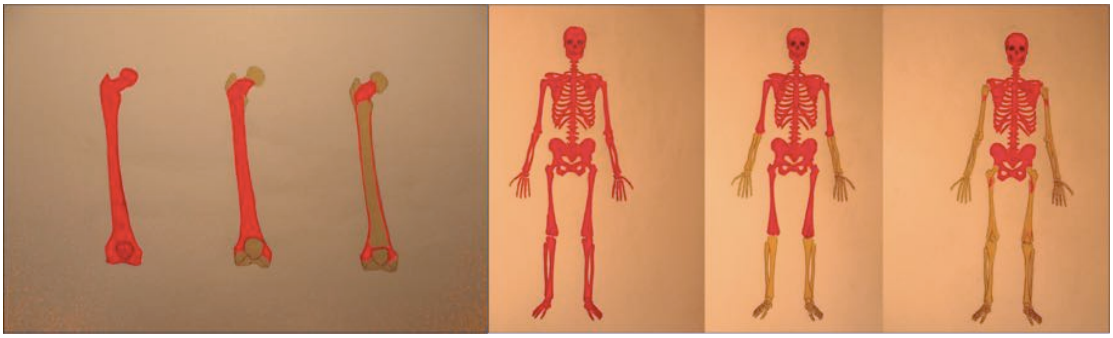

Figure 6-1: Distribution of red and yellow marrow from childhood to adulthood. Reproduced un creative commons license (Malkiewicz et al 2012).

Whilst mineralised bone and the bone marrow are considered separate organs they function as a single unit (Compston 2002). Bone histomorphometry is technically challenging, but an important study by Hauge et al (2001) was able to describe the interface between bone marrow and bone. The two were separated by a single layer of bone lining cells expressing osteoclacin, osteonectin and alkaline phophatase which, during remodelling, lift away from the mineralised bone surface. The article discusses observations of small tubular extensions reaching from the surface of bone marrow sinusoids through this layer of bone lining cells, and that these may be a direct connection between marrow vessels and mineralised bone. Whether these extensions deliver osteoblasts to the bone surface is still debated (Parfitt 2001; Compston 2002).

Bone is the third most common site for metastases (Ottewell 2016), and their dissemination to bone could be analogous, but the close association between bone marrow and mineralised bone makes it difficult to establish how they come into contact with OBs. Expression of CXCL12 by haematopoietic and endosteal niches enables attachment of CXCR4 expressing breast and prostate tumour cells. Whilst metastases do induce the development of bone resorbing and bone forming lesions the aggressive nature of tumour cells may simply erode through the thin cell layer lining the bone, rather than reflecting a physiological route by which cells can access the bone surface. Perhaps more telling is the consistent requirement for a blood supply for bone to form. This has been discussed in the introduction to this thesis, but is also necessary for tissue engineering of bone (Pirraco et al 2010). Given the difficulties in identifying the various niches and small blood vessels by immunohistochemistry (Parfitt 2001; Ottewell 2016) a synthetic model, like that used for tissue engineering, may help.

The role of osteoimmune interactions in normal bone metabolism and immune function

It has been shown, at least in mice, that OB precursors and their secreted factors regulate haematopoiesis within the stem cell niche (Calvi et al 2003; Zhang et al 2003). Within bone HSCs are found primarily at the endosteal surface, entering bone by its central blood vessel (Nilsson et al 2001; Xie et al 2009), although more recently they have been shown to be more prevalent in the central bone marrow (Acar et al 2015). It may also be that the stem cell niches exist around arterial and venous sinusoids, which reach to the endosteal surface (Calvi et al 2015).

Haematopoiesis seems to be dependent on the presence of OBs on the endosteal and trabecular surfaces of bone. A mouse model was developed in which, following a course of ganciclovir, it was possible to ablate osteoblast lineage cells lining the endosteal and trabecular surfaces with a decrease in osteoclast numbers (Visnjic et al 2001). This had no effect on the ability of MSCs, isolated from these mice after treatment with ganciclovir, to successful undergo osteogenic differentiation. What they did find was a marked loss of cellular elements of the bone marrow in those regions where OBs were lost. The group went on to show that it was B-cells and erythroid cells that were most affected, with an associated initiation of extra-medullary haematopoiesis and no loss of function in secondary lymphoid organs (Visnjic et al 2004).

MSCs may have a role in maintaining haematopoiesis within the stem cell niche. Having identified a population of bone marrow cells expressing the green fluorescent protein (GFP) regulated by the nestin promoter (nestin is an intermediate filament expressed by neural cells) (Mignone et al 2004) that met the requirements of a stromal cell. The group went on to show that these cells co-located with HSCs in a perivascular distribution adjacent to the bone (Mendez-Ferrer et al 2010). These cells went on to form both osteoblasts and chondrocytes, and their depletion resulted in a reduction in HSCs.

Conversely, haematopoietic cells appear necessary for normal bone development with one group having carried out a series of studies of bone in T cell and B cell knockout mice. They were initially interested in the role of T cells as mediators of bone loss due to oestrogen deficiency and studied BMD in ovariectomised, athymic mice (Cenci et al 2000). When sacrificed at 9 weeks of age (when they are still classed as young, and

their bone potentially in a growth/modelling phase), they found the athymic mice to have normal BMD whilst their euthymic controls' BMD was reduced by 30%. Bone loss was associated with a reduction in osteoclast number. To establish the role of T-cells in mediating bone loss in rheumatoid arthritis, athymic mice were again used. This time it was shown that administration of IL-7 to euthymic mice resulted in a reduction of BMD and increased CTX (very similar to NTX measured in this theses patient group), indicating increased OC activity, but had no effect in athymic mice (Toraldo et al 2003). One observation was that, in the placebo groups, athymic mice had a lower BMD than euthymic. This suggests that whilst T cells mediate pathological bone loss, under physiological conditions they are necessary for normal bone development. A third paper by this group showed that both B cells and T cells are necessary for bone homeostasis and achievement of peak bone mass (Li et al 2007). B cell KO mice at 16 weeks (when peak bone mass is achieved) were osteopaenic because of elevated bone resorption with trabecular bone more affected than cortical. They also found reductions in BMD in athymic mice at 12 weeks, with on-going losses to 16 weeks of age.

Lymphocytes also appear to be important for bone healing following fracture, reflecting processes out with those that influence early development, although specific populations may have delay healing. After fracturing the bone of wild type mice there is an influx of lymphocytes there is an influx of T-cells within 24 hours, shifting the balance from $CD8^+$ to $CD4^+$ predominance localising to the endosteal surface initially but then appearing in the areas of woven bone (Konnecke et al 2014). The lymphocytes that migrated to the bone were predominantly B cells. There was evidence of close contact between immune cells and bone cells, with B cells in direct contact with OBs, and T cells in close contact with OCs. Another study investigating the role of lymphocytes in bone healing after fracture identified, in humans, that a greater proportion of $CD8^+$ effector memory cells was associated with delayed healing (Reinke et al 2013). Conditioned media from those $CD8^+$ effector memory cells inhibited osteogenic differentiation of MSCs, and their depletion in a mouse model reduced fracture healing times.

It is also likely that proliferation of lymphocytes, as opposed to lymphopoiesis, is influenced by MSCs. Bone marrow stromal cells (BMSCs), isolated from human iliac crest bone marrow aspirates, inhibited the proliferation of $CD4^+$ and $CD8^+$ T cells (Li Pira et al 2006). Proliferation was induced by DCs, and the BMSC mediated this by inhibiting cell contact between DCs and T cells. Monocytes also mediate umbilical

MSCs suppression of T cell proliferation (Cutler et al 2010). Whilst there was evidence that MSCs affected monocyte activation, MSCs had no direct on T cell proliferation in the absence of monocytes. But BMSCs can induce the proliferation of regulatory T cells. The culture of a highly-purified population of $CD8^+$ lymphocytes with BMSCs, in the presence anti-CD3 monoclonal antibodies, resulted in the generation of a population of $CD8^+$ able to inhibit the proliferation of lymphocytes with peripheral blood mononuclear cell population (Poggi et al 2008).

These studies demonstrate the inter-dependency of the haematopoietic and mesenchymal cell lines, and how this relationship is important in maintaining BMD and normal immune function.

The potential for immune cell trafficking to bone

There is evidence in the literature that memory T cells, carrying memory of antigen to which the immune system has been exposed, reside in the bone marrow. A number of studies infect mice with specific viruses, and then study the response of immune cells to antigen from that virus to study T cell memory responses. $CD8^+$ lymphocytes isolated from the blood, spleen and bone marrow of infected mice all respond equally to virus re-exposure (Slifka et al 1997). Adoptive transfer of bone marrow $CD8^+$ lymphocytes conferred immunity in the recipient. Amongst those antigen experienced $CD8^+$ lymphocytes some circulate through lymphoid tissue, but a phenotypically distinct population circulate through non-lymphoid tissue where they are maintained for longer and still present 60 days after the infection (Masopust et al 2001). It was subsequently shown that antigen experienced $CD8^+$ lymphocytes were still present in the bone marrow 7 months after infection, but were no longer present in the spleen (Di Rosa et al 2002). Compared with those from the spleen and lymph nodes, a greater proportion of memory $CD8^+$ lymphocytes from the BM were proliferating (Parretta et al 2005). A similar fate for memory $CD4^+$ lymphocytes activated in secondary lymphoid organs has been shown, but with the additional observation that they were found within the bone marrow stroma 3-8 weeks after infection (Tokoyoda et al 2009).

This process has also been demonstrated in humans. Okhrimenko et al (2014) noted the observation of Tokoyoda et al (2009) that memory T cell populations in secondary lymphoid organs are gradually depleted if not exposed to antigen and in BM dock with stromal cells to support their survival. They isolated cells from the blood and BM of healthy adults and found that $CD4^+$ memory T-cells to CMV and measles are prevalent

in the bone marrow in a resting state (Okhrimenko et al 2014). Memory $CD8^+$ lymphocytes, responding to CMV infection, are almost exclusively found in the BM (Letsch et al 2007).

The presence of these cells in the BM may have a role in the wider functioning of the immune system. In mice DCs in the bone marrow have been shown able to activate BM resident memory T cells (Cavanagh et al 2005), with one mechanism being they present antigen to $CD8^+$ lymphocytes which then express CD69 and proliferate (Milo et al 2013). In splenectomized mice, no longer filtering antigen, the BM took up the role of the spleen generating an appropriate primary immune response (Tripp et al 1997). Whilst an interesting observation, they acknowledge that the changes seen in lymphoid organs during the generation of a primary immune response may well be undesirable in the bone marrow. In a mouse model of colitis, induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice, Nemoto et al (2007) found that $\alpha4\beta7^+CD4^+$ memory T cells migrated to the bone marrow. These cells produce a Th1 cytokine response and are more actively diving than those in mesenteric lymph nodes and the lamina propria (Nemoto et al 2007). Adoptive transfer of colitic cells from the BM induced colitis in the recipient.

Whilst it has been suggested that bone could function as a secondary lymphoid organ (Warnawin et al 2005) this may be an incorrect use of terminology. Although it is correct that memory cells have been found in bone but, unlike a lymph node, it remains a primary lymphoid organ. An editorial by Ceredig (2009) discusses the evidence for the return of memory T cells to the thymus. He points to work suggesting that the purpose of this is to support the process by which the thymus regulates production of new lymphocytes. This is nicely demonstrated in the image shown in Figure 6-2. The retention of memory cells in the bone marrow appears to have a slightly different role, perhaps acting as a memory bank. Therefore, whilst bone marrow is not a secondary lymphoid organ, antigen-experienced lymphocytes do seem to enter reside there.

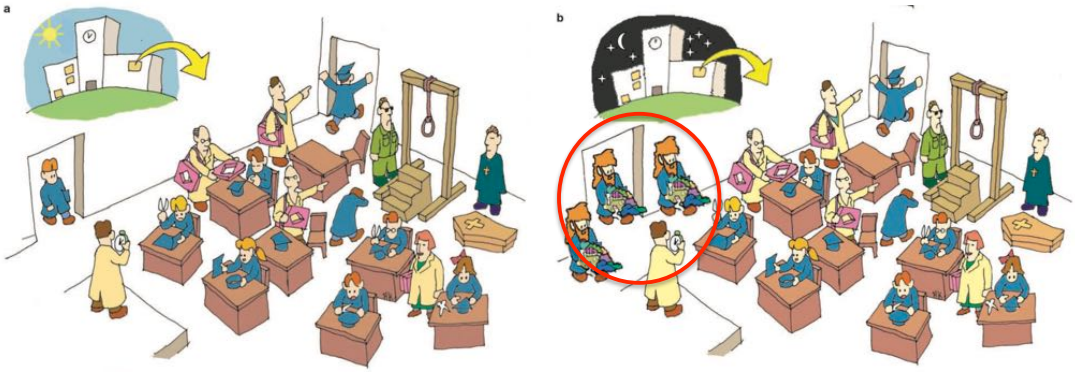

Figure 6-2: Cartoon representation of the thymus, and in B it can see that experienced pupils return to help educate their naïve colleagues. Reproduced with permission (Ceredig 2009).

Further work

Whilst the experiments undertaken for this thesis could be repeated with greater numbers, to do so seems unlikely to be worthwhile. The *in-vivo* study as it is only looks at associations and the *in-vitro* study, compared to other published studies, is relatively crude. More pressing is the need to establish whether lymphocytes, including those activated in the gut, come into contact with OBs at a location which means they affect the mineralisation of bone.

This could be done by conducting a study similar to that presented in this thesis, but to also take a bone biopsy as was done by Ward et al (2010). However, this is a highly invasive procedure which can lead to some pain and discomfort, and without clinical benefit doing so may not be ethical. The study of Ward et al (2010) had undergone external review, and consent was given by parents and patients, but they do not indicate any formal review by an ethics board. Also, as already discussed, even it was approved, the separation of mineralised tissue and bone marrow by a single layer of cells means it may not be possible to establish the exact location of OBs and lymphocytes.

An alternative approach would be to recruit patients who are undergoing orthopaedic surgery or bone biopsy for other reasons. This would overcome the problem of taking bone sample solely for research, and would be appropriate since it seems likely that osteoimmune interactions are relevant in healthy individuals. Of course, that they are having a procedure undertaken on their bone suggests they are not entirely "healthy", but there may be circumstances where the micro-anatomy is not expected to be

abnormal. This methodology could be used to identify $\alpha4\beta7^+$ lymphocytes, providing evidence for trafficking of gut activated immune cells to the bone. However, given the disruption of blood vessel, bone marrow, and mineralised tissue, it may be difficult to establish whether any identified $\alpha4\beta7^+$ lymphocytes have passed from blood vessels to the bone marrow *in-vivo* or whilst obtaining the sample.

Animal models could also be used. The problems of identifying the exact location of lymphocytes and osteoblasts, and where they come into contact, will still be difficult as the separation of bone and bone marrow is presumably no different to that in humans. But the smaller size of the bones, and the ability to remove the whole for analysis, may make it possible to overcome some of the technical difficulties. For example, a whole bone could be removed and a stabilising agent injected, before preparing histological sections. With animal models there is then greater potential to interrogate the osteoimmune system by using gene knockout species, blocking specific pathways and labelling of cells.

There is also a role for more refined co-culture experiments. It would be possible to isolate cells of both haematopoietic and mesenchymal origin, from single human subjects or animals, to overcome the problems of alloimmune reactions when considering their interactions. MSCs and OBs may need a period of expansion to generate sufficient numbers for co-culture experiments, and this may mean either taking a later blood sample for immune cells, or maintaining or storing them in some way. It would be possible to study the influence of lymphocytes on cells as they mature from MSC to OB. This could be undertaken using 3D collagen scaffolds or other tissue engineering approaches to study this in the context of bone development. There is the potential to alter the lymphocyte populations and their activation status, but it would also be interesting to further study why the time point at which lymphocytes were added had such a profound effect on the outcome. It is also important to consider how cells of mesenchymal origin affect those of haematopoietic origin.

The following questions warrant further investigation:

- Do $\alpha 4\beta 7$+ lymphocytes enter the bone marrow after being exposed to antigen in the gut?

- Do antigen experienced lymphocytes, including those expressing $\alpha 4\beta 7$, interact directly with osteoblasts?

    - Does this occur in the bone marrow, on mineralised bone tissue, or both?

    - How is that interaction mediated?

    - What are the roles of cell contact and humoral factors?

    - At what stage of differentiation from MSC to osteoblast does the interaction occur?

    - How does this interaction alter the outcome for both the cell lineages involved?

- How do these interactions influence bone development, modelling and re-modelling? Are there reciprocal effects on immune function?

Perhaps the most exciting aspect of this work is to provide the opportunity for researchers from different disciplines (immunology and bone biology) to work together with clinicians to better understand what appears to be the fundamental role of osteoimmune interactions in bone health and immunocompetence.

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8 Appendix

In-vitro study

Ethical approval

The University Of Sheffield.

School Of Medicine & Biomedical Sciences.

Professor A P Weetman, Pro VC Medicine

Medical School Office
Ms Sara Watkinson
Research Administrative Officer
Beech Hill Road
Sheffield S10 2RX

Telephone: +44 (0) 114 226 1458
Fax: +44 (0) 114 271 3960
Email: s.watkinson@sheffield.ac.uk

Dr Gareth Penman
Academic Unit of Child Health
Children's Hospital
Western Bank
Sheffield

28th April 2009

REF: SMBRER116

Dear Gareth

A systematic analysis of immune and bone interactions

I am pleased to inform you that on *28th April 2009* the School's Ethics Reviewers **approved** the above-named project on ethics grounds, on the basis that you will adhere to and use the following documents that you submitted for ethics review.

i) Ethics form (revised) [27.04.09]
ii) Participant Information Sheet [27.04.09]
iii) Consent Form [27.04.09]
iv) Subject Information Sheet [12.03.09]

If during the course of the project you need to deviate from the above-approved documents please inform mew. The written approval of the School's Ethics Review Panel will be required for significant deviations form or significant changes to the above-approved documents. If you decide to terminate the project prematurely please inform me.

Yours sincerely

Sara Watkinson
School Research Ethics Administrator

SCHOOL
OF
MEDICINE
& BIOMEDICAL SCIENCES.

Immunobiology Research Unit
School of Medicine
University of Sheffield

PARTICIPANT INFORMATION SHEET

A systematic analysis of immune and bone cell interactions

You are being asked to provide a sample of blood for research being conducted by staff of the Immunobiology Research Unit on behalf of the University of Sheffield. Before you decide to provide this sample, it is important for you to understand why the research is being done, what it will involve and the possible benefits, risks and discomforts. Please take time to read the following information carefully and discuss it with others if you wish. Do not hesitate to ask us if there is anything which is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this…..

What is the purpose of the study?

The importance of bone and its cells (known as osteoblasts and osteoclasts) in the immune system has been increasingly recognized over the last decade. Osteoclasts and immune cells share a common cellular origin originating from the bone marrow. But it was the discovery of cell surface proteins common to both of these cell types, with the potential to mediate their interactions, which pointed toward an ongoing relationship between these two apparently very different types of cells.

One important area this affects is bone metabolism in autoimmune disease. Inflammatory bowel disease (IBD) in adults is associated with reduced bone strength, and in children with the additional problem of reduced growth. Whilst there are a number of possible reasons for IBD to affect the bones e.g. malnutrition, reduced physical activity, the role of interactions between the immune system and bone are increasingly being investigated.

We want to investigate how the immune system affects bone cells. Osteoblasts, which build bone, are currently being grown in the lab. We also want to grow osteoclasts, which breakdown bone. This can be done by taking blood from normal healthy adults, extracting a particular type of immune cell (known as a monocyte) and helping it grow into an osteoclast in a test tube. In addition, we want to see how the immune cells affect the growth and function of the bone cells. This can be done by incubating the various cell types together.

What will happen if I take part?

We will check that you are suitable for the study and that you have not had any recent serious illnesses or anaemia, or recently received any medication which is likely to affect the function of your immune (white blood) cells, including anti-inflammatory pain killers. A blood sample will be taken from the vein in your arm (a maximum of around 50 mL, or about 3 tablespoons) using a syringe and needle or similar device. The blood will only be used for the research outlined above. You should not donate more than 200 mL of blood in any 1-month period and so you should let us know if you have donated blood in the last month. The blood testing is not designed to detect any blood abnormalities.

What do I have to do?

You will need to attend the premises of the University of Sheffield. Blood will be taken within the Immunobiology Research Unit by a person qualified to do so and should take no more than 15 minutes of your time.

What are the possible side effects, risks and discomforts of taking part?

Taking blood with a syringe and needle or a similar device from a vein in the arm can cause some pain and bruising.

What are the possible benefits of taking part?

You will not obtain any benefit from taking part in this research. We hope that the information obtained from the research will be of benefit to patients in the future.

What if something goes wrong?

There are no special compensation arrangements should you be harmed by taking part in this research. If you are harmed due to someone's negligence, then you might have grounds for a legal action, but you may have to pay for this. Regardless of this, if you wish to complain or you have any concerns about any aspect of the way in which you have been approached or treated during the course of this research, then the normal University of Sheffield complaints mechanism should be available to you.

What will happen to the results of the research?

The results of the research may be published in the scientific and medical literature. Your identity will not be revealed in any publication arising from this work.

Who is organising and funding the research?

The Research is funded by the University of Sheffield, charitable institutions and Research Councils. None of the staff involved in the research study will be paid for including you in it.

Who has reviewed the project?

This research programme has been reviewed by the University of Sheffield Research Ethics Committee

Who should I contact if I need more information or help?

In case of a study-related injury, or whenever you have questions about the research, please contact either of the following:

Dr Gareth Penman **Tel:** 07974 836033
Specialist Registrar/Clinical Fellow
Immunobiology Research Unit

Professor Graham Pockley **Tel:** 0114 271 2027 (secretary)
Professor of Immunobiology
Immunobiology Research

Expenses

No payment can be made.

You will be given a copy of this Information Sheet to keep if you decide to take part in this research.

Thank you for your help with this research project.

SCHOOL
OF
MEDICINE
& BIOMEDICAL SCIENCES.

Immunobiology Research Unit
School of Medicine
University of Sheffield

CONSENT FORM

Title of Project: A systematic analysis of immune and bone cell interactions

	Please initial box
1. I confirm that I have read and understand the information sheet for the above project dated 10th March 2009, and that I have had the opportunity to ask questions.	
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving reason.	
3. I agree to take part in the above project.	

Name of Participant: ……..……..

Date: ……

Signature: ……

Name of Person Taking Consent: ……..……..

Date: ……

Signature: ……

Copies: Participant
Immunobiology Research Unit

Version 1: 10th March 2009

Ethical approval

Sheffield Research Ethics Committee
Yorkshire and Humber REC Office
First Floor, Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Tel: 0113 3050122
Fax:

09 September 2010

Dr David Campbell
Consultant Paediatric Gastroenterologist
Sheffield Children's Hospital
Western Bank
Sheffield
S10 2TH

Dear Dr Campbell

Study title:	**A comparative phenotypic characterisation of intestinal and peripheral blood leukocytes in children with and without Crohn's disease.**
REC reference:	**08/H1308/275**
Protocol number:	**Awaited**
Amendment number:	**3**
Amendment date:	**11 August 2010**

Thank you for submitting the above amendment, which was received on 11 August 2010. It is noted that this is a modification of an amendment previously rejected by the Committee (our letter of 14/7/2010 refers).

The modified amendment was reviewed by the Sub-Committee in correspondence. A list of the members who took part in the review is attached.

Ethical opinion

Favourable Opinion

I am pleased to confirm that the Committee has given a favourable ethical opinion of the modified amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved are:

Document	Version	Date
Modified Amendment		11 August 2010

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

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.

08/H1308/275:	**Please quote this number on all correspondence**

Yours sincerely

Mrs Elaine Hazell
Committee Co-ordinator

E-mail: Elaine.hazell@leedsth.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Mrs Wendy Swann, Sheffield Children's NHS Trust Research Department*

Sheffield Children's NHS
NHS Foundation Trust

PARTICIPANT INFORMATION SHEET
FOR YOUNG PEOPLE

Study title

Growing the Bones of It!

Part 1 – to give you first thoughts about the project

1. Invitation paragraph

We would like you to help us with our research study. Please read this information carefully and talk to your mum, dad or carer about the study. Ask us if there is anything that is not clear or if you want to know more. Take time to decide if you want to take part. It is up to you if you want to do this. If you don't then that's fine, you'll be looked after at the hospital just the same.

This research is helping one of the doctors involved to learn more about carrying out research, and the information that is gathered will be used in his final assessment for a qualification from the University of Sheffield.

2. Why are we doing this research?

Some children have a condition called inflammatory bowel disease. Their bowel becomes inflamed and they suffer from diarrhoea and abdominal pain. Inflammatory bowel disease can affect the bones making them weaker and more likely to break. We want to know why this happens so we can find the best way to make their bones stronger.

3. Why have I been asked to take part?

You have been chosen because you are having an endoscopy, where we look inside your bowel with a long flexible camera, whilst under a general anaesthetic. We would like 30 children and young people to take part in this project.

4. Do I have to take part?

No! It is entirely up to you. If you do decide to take part:

- you will be asked to sign a consent form to say that you agree to take part

- you will be given this information sheet and a copy of your signed consent form to keep.

You are free to stop taking part at any time during the research without giving a reason. If you decide to stop, this will not affect the care you receive whilst in hospital.

5. What will happen to me if I take part?

Having an endoscopy involves a general anaesthetic so you sleep through the whole thing. It can be done in a day so you shouldn't have to stay overnight, and you could go back to school the next day.

You'll come to hospital in the morning. We'll give your parents a container to collect the second wee you do that day. Once you get to the ward a cream that numbs the skin will be put on the back of your hand. One of the doctors will put a small tube, called a cannula, in the back of the hand. This is to give medicines to make you sleep, but will also be used to take 15 ml (about 3 teaspoons) of your blood. Once you are asleep a long, flexible camera is used to look at the upper and lower parts of your bowel, and small pieces of your bowel (about the size of a breadcrumb) are taken to be looked at under a microscope. The doctors then start to wake you up and you're taken out of the theatre. This takes about 40-60 minutes. You then stay in hospital until you are awake and able to eat and drink. Taking part means you will be under a general anaesthetic for about an extra 10 minutes. You won't be aware of this and it won't take any longer for you to wake up, or mean you need to stay in hospital any longer. Also we will record details of your symptoms, blood test results, and what we see with the camera.

6. What will I be asked to do?

If you're happy to be involved in the study we'll ask you to sign a form confirming we can take the additional samples. You won't need to do anything else.

7. Is there anything else to be worried about if I take part?

Lots of teenagers have an endoscopy and it is very unusual for there to be any problems. However very occasionally (about once in every 1000 endoscopies) the endoscope makes a hole in the wall of the bowel. Since we are taking some extra biopsies this risk is increased, but the increase is very small and if it was thought to be too great we wouldn't be allowed to take the samples.

If we find out something that we think is important about you we will talk to your mum, dad or carer and ask them if they want to come back and have you checked again at the hospital.

8. Will the study help me?

No, but the information we get might help us to better treat young people with inflammatory bowel disease in the future.

9. What happens when the research study stops?

We will collect all the information together and see if we are able to identify cells that have passed through the gut, and how they differ between patients with and without inflammatory bowel disease. If we are successful we will organise further studies to look at the effects of these cells on bone.

10. Contact for further information

If you would like any further information about this study you could contact:

Name:	Dr David Campbell
Designation:	Paediatric Gastroenterology Consultant
Hospital/Department:	Sheffield Children's Hospital
Tel:	0114 2717000
Email:	David.Campbell@sch.nhs.uk

Thank you for reading so far - if you are still interested, please go to Part 2:

Part 2 - more detail – information you need to know if you still want to take part.

11. What if I don't want to do the research anymore?

Just tell your mum, dad, carer, doctor or nurse at any time. They will not be cross with you. You will still have the same care whilst you are at hospital.

12. What if there is a problem or something goes wrong?

Tell us if there is a problem and we will try and sort it out straight away. You and your mum, dad or carer can either contact the project co-ordinator:

Name:	Dr David Campbell
Designation:	Paediatric Gastroenterology Consultant
Hospital/Department:	Sheffield Children's Hospital
Tel:	0114 2717000
Email:	David.Campbell@sch.nhs.uk

Or the hospital complaints co-ordinator:

Mrs Linda Towers
Patient Advice & Liaison Co-ordinator
Sheffield Children's NHS Foundation Trust
Tel: 0114 271 7594
Email: Linda.Towers@sch.nhs.uk

13. Will anyone else know I'm doing this?

The people in our research team will know you are taking part. The doctor looking after you while you are in hospital will also know. Your medical notes may also be looked at by other people who work at the hospital to check that the study is being carried out correctly.

We keep a record of your name, date of birth and copies of the consent form in one place along with a unique number. We then use this number for all the other information we collect e.g. your gender, whether you have any other illnesses and what we find in the samples we take. We need to keep a record of your personal details in case we need to identify who gave a particular sample, we keep this information but only a few people in the research team can access it. The unique number allows us to keep track of who all the other information belongs to but the rest of the research team, and anyone we discuss the results with, won't know who you are. Once the study is complete all information will kept, but we will be keeping your details separately from any information we get from the samples we take.

14. What will happen to any samples I give?

The samples you give will be taken to a science lab where we will look at the cells. Samples don't have your name on, and can only be traced back to you one of the doctors in the lab. Anything we don't use we would keep in case we found new tests we

could do on them. We ask you to sign a consent form for this now, but we may need to contact you to speak to you about it in the future. We would speak to our ethics committee before doing any extra tests.

15. What are genetic tests and will any be done?

Genetic tests look at the molecules which control your cells. For this research we don't need to do any genetic tests.

16. What will happen to the results of the research study?

When the study has finished we will present our findings to other doctors, and we will put the results in medical magazines and websites that doctors read. We would also like to put a brief summary on the hospital research website so that you will be able to read about our results too. This will be available at the end of the study on www.sheffieldchildrenscrf.nhs.uk. The results will also be included as part of the chief investigator's educational qualification. They will be anonymous, which means that you will not be able to be identified from them.

17. Who is organising and funding the research?

The research is being organised by Sheffield Children's NHS Foundation Trust and paid for by Sheffield Children's Hospital Charity.

18. Who has checked the study?
Before any research goes ahead it has to be checked by a Research Ethics Committee. This is a group of people who make sure that the research is OK to do. This study has been looked at by North Sheffield Research Ethics Committee.

It has also been checked by the Research Department at this hospital.

19. How can I find out more about research?
The Clinical Research Facility at this hospital has an **Information for families** section on its website *(http://www.sheffieldchildrenscrf.nhs.uk/info_families.html)*, or you could contact the hospital Clinical Research Facility:

Mrs Tracy N'Diaye
R&D Manager
Sheffield Children's NHS Foundation Trust
Tel: 0114 226 7904
Email: tracy.ndiaye@sch.nhs.uk

Thank you for taking the time to read this – please ask any questions if you need to.

Sheffield Children's NHS
NHS Foundation Trust

PARENT/LEGAL GUARDIAN INFORMATION SHEET

Study title

Growing the Bones of It!

Part 1 – to give you first thoughts about the project

1. Invitation paragraph

You and your child are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you and your child if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you want your child to take part.

This research is helping one of the doctors involved to learn more about carrying out research, and the information that is gathered will be used in his final assessment for a qualification (a Phd) from the University of Sheffield.

2. What is the purpose of the study?

Inflammatory bowel disease is a condition where the bowel becomes inflamed causing diarrhoea and abdominal pain. Children and adults with inflammatory bowel disease can develop osteoporosis (brittle bones). This can make it more likely their bones will break (or fracture) and in children can affect the growth and development of their skeleton.

There is a lot we don't know about osteoporosis in inflammatory bowel disease, including why it occurs and how best to prevent or treat it. We in the gastroenterology team want to try to understand more about why brittle bones occur. This will help us decide the best way to prevent and treat the problem, improving patients bone health and reducing problems of fracture and poor growth and development.

We believe that it may be cells from the gut which travel to the bone and interact with bone cells resulting in less healthy bones. In order to investigate this problem we want

to identify cells which have passed through the gut and entered the bloodstream, and investigate how these relate to the activity of bone cells.

3. Why has my child been chosen?

Your child has been chosen as they are having an endoscopy to investigate symptoms related to the bowel. To do this a flexible camera is used to look inside the bowel. We are interested in looking at biopsies (small pieces) of the bowel from children who have a condition called Crohn's disease, one of the two types of inflammatory bowel disease, and from children who's bowel appears normal. That way we can see whether there are any important differences between the two.

4. Does my child have to take part?

No. It is up to you and your child (wherever possible) to decide whether or not to take part. You are both free to withdraw from the research at any time and without giving a reason. Your decisions about this will not affect the standard of care your child will receive.

If you are happy to take part, and are satisfied with the explanations from the research team, you will be asked to sign a consent form. If your child is able to understand the research and is happy to take part and can write their name, they will be asked to sign a consent form with you, if they want to. You will be given a copy of the information sheet and the signed consent forms to keep for your records.

5. What will happen to my child if we agree to take part?

Children who are having an endoscopy usually need to take laxatives, at home, to empty the bowel. During this time they can only drink clear fluids. They will then come to hospital on the day and will be seen by the doctors and nurses looking after them. This will includes asking how they are and explaining what will happen. They will be taken to the operating theatres where the anaesthetist puts a cannula (a small flexible tube) into the back of the hand so that medicines can be given. To make it less painful an cream is used which numbs the back of the hand. Once your child is asleep a long flexible camera is used to look into the upper and lower parts of the bowel, and small pieces of the bowel wall (biopsies) are taken to be looked at under a microscope. This normally takes around 40 minutes. Afterwards your child will be woken up and can take up to an hour to stop feeling sleepy. You would usually be allowed home 2 to 3 hours after the procedure, provided your child felt well enough.

If you and your child agreed to be in the study we would need to collect a few additional samples, and if your child is on the afternoon list then we would ask you to come to hospital 2 hours earlier. Doing this shouldn't cause any additional distress, but the endoscopy was in the afternoon we might need you to come to hospital two hours earlier.

Firstly we would need a urine sample which has to be the second one of the day. We would give you a container for it and ask you to bring it along on the day. Secondly we need to take a 13 ml blood sample before 11am. This would mean having the cannula

put in earlier, but we would still use the cream, and the cannula would be used to give the anaesthetic so there would not be any additional needles required. Finally, during the endoscopy, we would take an additional ten biopsies of the gut. The most difficult part of the procedure is guiding the endoscope through the bowel, so the extra biopsies would add at most 10 minutes to the time under anaesthetic. However it wont affect how long it takes to recover from the anaesthetic, or how long your child spends in hospital. All these samples would then be taken to the laboratory to be analysed.

In normal circumstances biopsies of the bowel are sent to be looked at under a microscope. The majority of the time this result confirms our diagnosis. However it is possible that we may take the additional samples and analyse them, but then discover that the microscopic appearance changes our diagnosis. If the diagnosis was one that we're not looking at in this study we would still like to use the results.

6. What will we have to do?

We would ask you to collect your child's second urine sample of the day. If the endoscopy is in the afternoon then we would need you to come to hospital by 10am, about 2 hours earlier than usual. We would then collect the samples as described above. Otherwise nothing else is required, and your child shouldn't need to stay any longer in hospital after the procedure.

If you wanted to be involved in the study, but were unable to come to hospital earlier, we would still want to collect some blood and the additional gut biopsy samples. If you wanted to be involved in the study and you were able to come hospital earlier we would give you £5 to cover the cost of parking and some refreshments for the parents.

7. What are the possible disadvantages and risks of taking part?

Diagnostic endoscopy is a relatively safe procedure with a low risk of complications. There is a 1 in 1000 risk of the endoscope making a hole in the wall of the bowel. This mostly occurs as a result of the endoscope moving through the bowel, and is more likely in patients who have unhealthy bowel. Taking additional biopsies could increase the possibility of making a hole. But this increase would be small, and taking double the number of biopsies doesn't double the risk. Otherwise, taking part should not affect your child in any way.

8. What are the possible benefits of taking part?

Your child will not benefit from being part of this study. However the information we collect may help us to better treat future patients with inflammatory bowel disease.

9. What happens when the research study stops?

We will collect all the information together and see if we are able to identify cells that have passed through the gut, and how they differ between patients with and without Crohn's disease. If we are successful we will organise further studies to look at the effects of these cells on bone.

10. What if there is a problem?

Any complaint about the way you or your child have been dealt with during the study or any possible harm you or your child might suffer will be addressed. The detailed information on this is given in Part 2.

11. Will my child's taking part in the research project be kept confidential?

Yes. We will follow ethical and legal practice and all information about your child will be handled in confidence. The details are included in Part 2.

12. Contact for further information

If you would like any further information about this study you could contact:

Name:	Dr David Campbell
Designation:	Paediatric Gastroenterology Consultant
Hospital/Department:	Sheffield Children's Hospital
Tel:	0114 2717000
Email:	David.Campbell@sch.nhs.uk

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2 - more detail – information you need to know if you still want to take part.

13. What will happen if we don't want to carry on with the research?

If you withdraw from the study we will destroy all your child's identifiable samples if you wish; but we will need to use the data collected up to their withdrawal.

14. What if there is a problem?

Complaints

If you have any cause to complain about any aspect of the way in which you or your child has been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study. If you have any complaints or concerns please contact either the project co-ordinator:

Name:	Dr David Campbell
Designation:	Paediatric Gastroenterology Consultant
Hospital/Department:	Sheffield Children's Hospital
Tel:	0114 2717000
Email:	David.Campbell@sch.nhs.uk

Otherwise you can use the normal hospital complaints procedure and contact the following person:

Mrs Linda Towers
Patient Advice & Liaison Co-ordinator
Sheffield Children's NHS Foundation Trust
Tel: 0114 271 7594
Email: linda.towers@sch.nhs.uk

Harm
If your child is harmed by taking part in this research project, there are no special compensation arrangements. If your child is harmed due to someone else's fault, then you may have grounds for a legal action – but you may have to pay for it.

15. Will taking part in this study be kept confidential?

All information which is collected about your child during the course of the research will be kept strictly confidential. Any information about your child which leaves the hospital will have their name and address removed so that your child cannot be recognised from it.

Once the study is complete information about your child (name, date of birth) and copies of the consent forms you have signed will be kept securely for five years in a locked cabinet, and then placed in an archive. Only members of the research team will be able to access this. Information obtained from the samples your child has given will be kept indefinitely, but separately from details that would identify your child. This data

would be shared in various formats with people outside of the research team. We will use a numbering system to allow us to trace who particular data came from. This would only be accessible by the main members of the research team.

Our procedures for handling, processing, storage and destruction of data are compliant with the Data Protection Act 1998.

Your child's medical notes may also be looked at by other people within the hospital involved in the running and supervision of the study to check that it is being carried out correctly.

16. What will happen to any samples my child gives?

In order to maintain confidentiality each participant is given a unique number. When the samples are taken this number, not your child's name or date of birth, is used to identify the sample. However we do keep a separate record of this number with your child's details in case we need to identify who gave the sample. Your child's data would only be accessible to the two principal members of the research team.

Sometimes when we analyse samples we discover new things to look for. Therefore we would, with your permission, keep the samples for future testing. If any of this testing could have implications for your child, if testing found something relating to your child's health, or a research team other than us was going to use the samples, we would contact you.

17. Will any genetic tests be done?

No genetic testing will be done.

18. What will happen to the results of the research study?

When the study has finished we will present our findings to other doctors, and we will put the results in medical magazines and websites that doctors read. We would also like to put a brief summary on the hospital research website so that you will be able to read about our results too. This will be available at the end of the study on www.sheffieldchildrenscrf.nhs.uk. The results will also be included as part of the chief investigator's educational qualification. They will be anonymous, which means that your child will not be able to be identified from them.

19. Who is organising and funding the research?

The research is being organised by Sheffield Children's NHS Foundation Trust and paid for by the Sheffield Children's Hospital Charity.

20. Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by North Sheffield Research Ethics Committee. It has also been approved by the Research Department at this hospital.

21. How can we find out more about research?
The Clinical Research Facility at this hospital has an **Information for families** section on its website *(http://www.sheffieldchildrenscrf.nhs.uk/info_families.html)*, or you could contact the hospital Clinical Research Facility:

Mrs Tracy N'Diaye
R&D Manager
Sheffield Children's NHS Foundation Trust
Tel: 0114 226 7904
Email: tracy.ndiaye@sch.nhs.uk

If you and your child decide to take part in this study, you will be given this information sheet and signed consent and assent forms to keep.

Thank you for taking the time to read this information sheet.

Sheffield Children's NHS Foundation Trust

Patient study number:

PARENT/LEGAL GUARDIAN CONSENT FORM

Title of project: *Growing the Bones of it!*

Names of researchers: Dr David Campbell, Dr Gareth Penman

Please initial box

1. I confirm that I have read and understand the information sheet dated 04/01/2010 (version 4) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my child's participation is voluntary and that I am free to withdraw my child at any time, without giving any reason, without my child's medical care or legal rights being affected. ☐

3. I understand that sections of any of my child's clinical record may be looked at by researchers and those involved in the running and supervision of the study from Sheffield Children's NHS Trust where it is relevant to my child taking part in research. I give permission for these individuals to have access to my child's records. ☐

4. I agree to my child taking part in the above study. ☐

4. I agree to my child having the following samples taken

- Urine sample ☐
- Additional 13mls of blood ☐
- Additional gut biopsies ☐

6. I agree to any samples not used being stored for further testing as outlined in the information sheet ☐

7. I understand that I will be informed if any of the results of the medical tests done as part of the research are important for my child's health. ☐

8. I know how to contact the research team if I need to, and how to get information about the results of the research. ☐

9. I agree that if I wish to withdraw from this study, any data or samples already collected can be used as described in the information sheet. ☐

Name of Parent/Guardian	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

Would you like to be sent information about the progress of this project?

Yes	No
☐	☐

1 copy for parent; 1 copy for researcher; 1 copy to be kept with hospital notes