Immune Dysfunction in Myeloma: Characterisation and Generation of IL-17-secreting Lymphocytes and Immune Manipulation using Oncolytic Virotherapy

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Declaration

The candidate confirms that the work submitted is his own and that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Abstract

Recent years have seen several notable advances in therapy for multiple myeloma (MM), which have translated into impressive improvements in progression-free and overall survival rates. Nevertheless, patients afflicted with this relatively common, incurable cancer can typically expect ever shortening treatment-free intervals between sequential therapies; relapsed, treatment-refractory disease remains common. Myriad experimental and observational studies comprehensively document a state of profound immune dysfunction in MM. Intriguingly, an accruing body of evidence indicates that immune dysregulation is not only progressive and correlated with disease stage, but that it may actually contribute to the pathogenesis of the disease. This thesis will examine the contribution of the recently described Th_{17} subset of T cells to immune dysfunction in MM and the mechanisms of their generation before assessing the use of a novel oncolytic virotherapy as an immunomodulatory therapy for MM.

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Chapter 1 – Introduction and Hypothesis

The work presented in this thesis comprises two broad strands of work and crosses a number of different areas of research. In order to review the relevant literature, the introductory chapter will therefore cover a number of slightly distinct areas in turn: plasma cell dyscrasias and the immune dysfunction seen in these disease states will first be discussed, followed by an overview of Th_{17} cells and then a section on Th_{17} cells in cancer and in myeloma specifically. The second section of the introduction will then discuss oncolytic viruses in general, reovirus specifically and finally murine models of myeloma which might be used to investigate these agents.

Plasma cell dyscrasias and multiple myeloma

Plasma cell dyscrasias are a common group of haematological disorders characterised by the clonal expansion of terminally differentiated B cells (plasma cells, PC) in the bone marrow, that typically produce an intact monoclonal immunoglobulin molecule or immunoglobulin light chain. Early in the natural history of the disease, a malignant PC clone may be present without any evidence of other organ damage; such cases are divided according to the levels of bone marrow infiltration into monoclonal gammopathy of uncertain significance (MGUS; malignant PCs <10% of leukocytes) and asymptomatic myeloma (AMM; ≥10%) (see Appendix 1 for definitions). MGUS is a very common condition, present in approximately 3% of the population above the age of 50[1]. However, in approximately 1% of cases of MGUS, and a higher proportion of those with AMM, the disease progresses to symptomatic myeloma[1], resulting in bone destruction causing pain, fractures and hypercalcaemia, multifactorial renal insufficiency and bone marrow failure[2].

MM has an annual incidence of 3-4 per 100 000, although there is ethnic variation with the disease being commoner in Afro-Caribbean populations. The median age at diagnosis is 65-70 years. It is the second most common form of bone marrow cancer, and represents about 1% of all malignancies. With the exception of petrochemical exposure in a small minority of cases[3], environmental aetiological factors are not known to play a major role in the pathogenesis of this malignancy.

The natural history of MM is extremely heterogeneous, with some patients surviving decades without requiring therapy and others dying within weeks from aggressive

progressive disease. As a consequence a great deal of effort has been expended on developing reliable prognostic systems and clinical scores: ISS[4], cytogenetic assessment[5,6] , flow cytometric phenotyping[7] and gene expression profiling[8] are among the modalities employed clinically at present.

For patients with symptomatic MM requiring therapy, recent years have seen enormous advances. Younger, biologically fit patients are currently treated with intensive chemotherapy, typically with autologous stem cell transplantation as a consolidation therapy – a strategy supported by a wealth of evidence from randomised controlled trials[9,10] (reviewed in[11]). However, since MM is predominantly a disease of the elderly, many patients are insufficiently fit for such an approach and for this large cohort of patients attenuated chemotherapy regimens are typically employed. Patients in both groups have benefitted enormously from the development of a wide range of new agents over the last decade, for example: 'immunomodulatory drugs' (IMiDs e.g. thalidomide, lenalidomide, pomalidomide), proteasome inhibitors (bortezomib, carfilzomib) and monoclonal antibodies (elotuzumab, daratumumab). Unfortunately, MM nonetheless remains incurable for the vast majority and is characterised clinically by ever-shortening cycles of remission and relapse after sequential therapies. There therefore remains a need for effective and, crucially, minimally toxic new treatment modalities for MM.

Immune dysfunction in myeloma

Virtually all aspects of cellular and humoral immunity are impaired in MM as a result of interations between the malignant PC and the host immune system (Figure 1.1), and this immune impairment worsens in association with disease progression[12].

Infections in MM

The increased risk of opportunistic infections in patients with MM is very well described[13] - indeed it is a diagnostic criterion for symptomatic MM[2] (see Appendix 1). Specific immunity against a variety of pathogens is impaired[14]; infections are a common cause of death in MM[15], and are also increased with MGUS, correlating with the paraprotein concentration[16,17]. Interestingly, there may be some reciprocity – i.e. infection may encourage tumour growth: malignant plasma cells express a wider range of toll-like receptors (TLRs, which recognise the pathogen-associated molecular patterns of

microorganisms) than those from healthy donors, and ligation of these induces proliferation and survival of both human MM cell lines (HMCL) and primary samples, partially due to autocrine interleukin-6 (IL-6) production[18,19]. Population-based studies also point to an increased incidence of infections in the years preceding diagnosis of MM[17], although caution is warranted in inferring causality - the association might equally represent an immune manifestation of pre-existing but undiagnosed PC dyscrasia. Medical interventions also contribute to immune compromise (e.g. corticosteroids, chemotherapy, indwelling intravenous lines etc.).

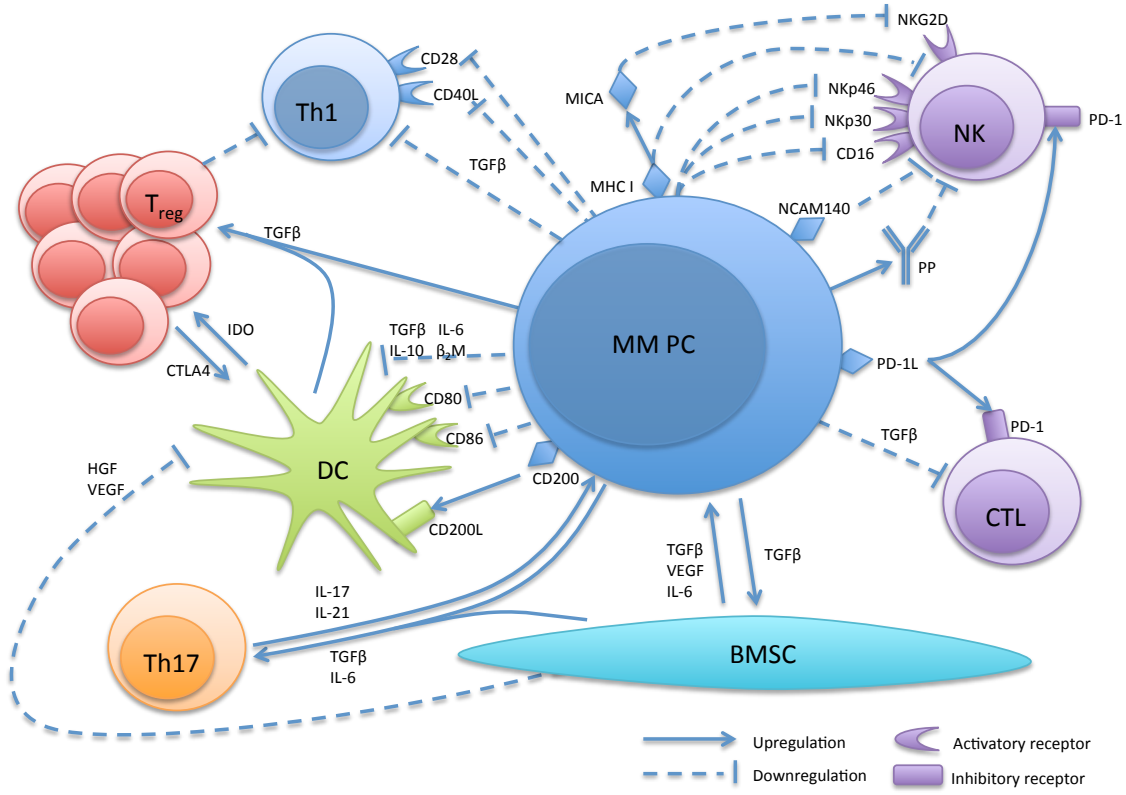


Figure 1.1. Immune dysfunction in multiple myeloma. Adapted from Parrish *et al.* 2013 [12].

Innate immunity in MM

Natural killer (NK) cells have been shown to have cytolytic activity against myeloma PCs [20-22], and may also be increased in number in patients with MM[23]. However, the NK cells present in patients with MM are abnormal, expressing reduced levels of activatory receptors such as NKp46 and NKp30[24], the Ig-Fc receptor CD16 and the coreceptor 2B4/CD244[25], and increased levels of inhibitory receptors such as PD-L1[26]. Functional defects are also apparent in NK cells, and seem to deteriorate as the disease progresses[26-30]. Furthermore, malignant PCs manifest a number of ways to escape NK-mediated killing, for example, upregulation of ligands for inhibitory receptors, such as NCAM140[31] and early haematopoietic zinc finger protein-induced MHC class I expression[32]. Even the myeloma paraprotein itself has been shown to adversely affect NK cell function[23].

NK T cells (NKT) can also mediate anti-cancer effects in several malignancies, and NKT with anti-myeloma activity are detectable in blood from patients with MM[33]. Disease progression from MGUS to MM is associated with a loss of ligand-dependent IFNγ secretion from such cells[28]. Gamma-delta (γδ) T cells, suitably stimulated with aminobisphosphates, have anti-MM activity[34-36], and this can be enhanced by costimulation with MICA produced by PCs in MGUS[37].

T cells in MM

Many aspects of T cell biology are deranged in MM: the CD4:CD8 ratio has been known to be reduced since the 1980s[38], the Th_1:Th_2 ratio was later found to be increased[39] and other subsets of T cells are also affected. The repertoire of T cell receptor (TCR) rearrangements in MM is impaired and correlated with disease stage[40], and oligoclonal expansions are reported[40,41]. Effector T cell responses against tumour cells are inhibited via TGFβ-mediated suppression of IL-2 autocrine pathways[42], and inhibition of expression of perforin, granzyme A, granzyme B, Fas ligand and IFNγ[43]. Co-stimulatory signals such as CD28 and CD40L, signal transduction molecules (e.g. CD3ζ, p56lck, p59fyn, ZAP-70 and PI3K) and stimulation-induced cytokine production are impaired in a disease stage-related manner[44].

Nevertheless, T cells targeting a variety of tumour-associated antigens have been identified in patients with MM[45], and indeed in MGUS, where they may play a role in

combating disease progression[46,47] – such cells are often functionally impaired in MM[47-49]. The myeloma paraprotein represents an obvious target and indeed idiotype-reactive T cells have been demonstrated[45,48,50], which have been shown *in vitro* to kill autologous tumour cells[51] and confer a favourable prognosis[52]. Unfortunately, attempts to harness anti-idiotype immunity for immunotherapy have met with little success, predominantly owing to T cell tolerance, the development of which remains to be fully understood (reviewed in[53]). In the context of allogeneic stem cell transplant (AlloSCT) immunisation of the donor against the idiotype protein can confer specific T cell immunity in the recipient[54-56], highlighting the requirement for a competent host immune system before such strategies can work. T cells specific for other cancer antigens present on MM cells also occur, can attenuate MM growth, and may represent useful avenues for immunotherapy - e.g. cancer testis antigens[57,58] , the mucin MUC1[59] and the embryonal stem cell-associated antigen SOX2[49].

Unsurprisingly, MM cells manifest an assortment of mechanisms to evade T cell-mediated immunity and have proven more resistant to donor lymphocyte therapy than many other haematological malignancies[60]. PD-L1, expressed by MM cell lines, renders cells expressing it less susceptible to cytotoxic T cell lysis[61,62], and exposed T cells may develop an exhausted phenotype and produce IL-10[63]. PD-L1 is not present on MGUS plasma cells (PC)[62] and so this seems to represent another example of the evolution of the immune deficit in MM. Notably, lenalidomide exposure decreases PD-1 expression on T cells *in vitro*[64]. Interestingly, the antigen presentation and costimulatory function of the malignant PCs themselves also seems to deteriorate with disease progression[65].

Our group and others have found regulatory T cells (T_{reg}, $CD4^+CD25^+FoxP3^+$) and $CD8^+CD25^+FoxP3^+$ to be expanded in MM, in a stage-related manner[66-69], although expanded total $CD4^+CD25^+$ with decreased $CD4^+CD25^+FoxP3^+$ populations have also been reported[70]. Regulatory T cells effect immune suppression by myriad mechanisms[71]; circulating T_{reg} have been correlated inversely with survival[72] and reduced BM T_{reg} after treatment are associated with long-term disease control[68]. Non-classical regulatory T cells subsets may also play a role[73]. The recently described Th_{17} subset of T cells, the main focus of the following work, may also be expanded in the blood and bone marrow of patients with MM[74], and are reviewed in detail in a later section.

Immunotherapy in the clinic

Cellular immunotherapy, in the form of allogeneic stem cell transplant, is already in use in the clinic and a range of other cellular therapies are under investigation, including dendritic cell (DC) vaccination, NK, NKT and T cell strategies and humoral immunotherapy[75]. In reality, many of the pharmacological agents already in routine clinical use have immunomodulatory effects that are likely to be at least partially responsible for their action. The prototypic immunomodulatory drug (IMiD), thalidomide, for example, has a stimulatory effect on T cells *in vitro*, augmenting their proliferation[54] and improving their capacity to secrete IFNγ[54,76] (and similar effects on NK cells[77]). Second generation IMiDs, lenalidomide and pomalidomide, have even more potent co-stimulatory actions on T[78,79], NK[77,80,81] and NKT[82] cells, and may be sufficiently potent to empower anti-tumour vaccine strategies[33,83]. Clearly this is a rapidly expanding field at present, which promises to transform therapy for MM over the next few years.

Th_{17} cells – origins and differentiation

For over 20 years, immunologists relied on the Th_1 *vs.* Th_2 paradigm, first proposed by Mosmann and Coffman in 1989[84], to explain skewing of the adaptive immune response in one of two directions via the action of two distinct subsets of effector T lymphocytes: Th_1 & Th_2, the balance of which would configure immune responses[85]. The paradigm was widely accepted for a number of years, being an elegant explanation for many immune phenomena[86]. However, a number of pathological states could not be explained by the model and a large and expanding number of effector T cell subsets has now been described and characterized. Of these, the Th_{17} subset, the principal focus of this thesis, is defined by its production of IL-17.

Cytokine drivers of Th_{17} differentiation

TGFβ, IL-6, IL-1β, IL-21. On binding to its heteromeric transmembrane receptor TGFβ acts through SMAD-dependent and independent pathways[87,88] to inhibit STAT4, T-bet and GATA3[89,90] (so inhibiting Th_1 and Th2 development), and reduce STAT5-mediated IL-2 production in TCR-stimulated cells, which otherwise constrains early Th_{17} differentiation. TGFβ also increases FoxP3 expression and induces development of T_{reg} cells - the addition of pro-inflammatory cytokines such as IL-6, IL-1β, IL-21 and IL-23 (and TNFα in mice) then favours Th_{17} development[91-95] .

In 2007, the role of TGFβ in Th_{17} lineage determination in humans was called into doubt by a small number of studies which reported contradictory information[96-99] - these discrepancies can probably be accounted for by methodological differences. However, subsequent work has demonstrated there are indeed significant differences in Th_{17} lineage determination between mice and humans. Although IL-6 and TGFβ will induce RORC2 (the human homologue of murine RORγt, with equivalent functions[93]), the combination seems insufficient to induce a full effector phenotype or IL-17 production from naive T cells[100], although TGFβ and IL-6 induced during the early stages of an inflammatory response may act on memory T cells to promote IL-17 and IL-21 secretion. In fact, whilst TGFβ does induce RORγt expression, it also paradoxically inhibits RORγt's transcriptional activity via induction of FoxP3[101]. A combination of IL-1β, TGFβ and any of IL-6, IL-21 or IL-23 overcomes this inhibition and is required for IL-17 expression, with IL-1β, TGFβ and IL-23 reportedly being the most potent combination in humans[102]. Some human studies have also reported IL-17 expression in response to IL-1β with or without IL-21[100]. Some recent evidence suggests that in humans (and possibly mice[103]) TGFβ is dispensable but has an indirect role in Th_{17} development through inhibition of Th_1 cells[104]. The level of TGFβ is also probably important, with low levels synergising with IL-6 and IL-21 to promote IL-23R expression and high levels favouring FoxP3 expression and repression of IL-23R expression[101]. To further complicate matters, IL-1β has been reported to induce Th_{17} development in humans independently of RORC2[105].

It is difficult to compose a definitive synthesis of the published data on the drivers of Th_{17} differentiation in humans, and even harder to know which of the proposed cytokine combinations is likely to be of relevance in any given situation. It is likely Th_{17} differentiation usually occurs within secondary lymphoid tissue when antigen-specific naive T cells recognise migrating antigen-presenting cells, and that a number of combinations have activity on naive precursors e.g. TGFβ + IL-21, IL-1β + IL-6 and IL-1β + IL-23[102]. Through T cell plasticity, Th_{17} cells may also be derived from other subsets, notably $FoxP3^+$ T_{reg} cells[106-108].

IL-23. A considerable body of work has now demonstrated the role of IL-23 in stabilization of the Th_{17} phenotype[109-112]. In brief, TGFβ upregulates IL-23R expression (which is absent in naive T cells), so conferring IL-23 responsiveness during Th_{17} development[112]; IL-6 may be able to substitute in this role[103]. Through its receptor IL-23 then induces further expression of its own receptor and RORC2 - a positive-feedback loop that stabilises and maintains the Th_{17} phenotype. IL-23 signalling may also be

required for the up-regulation of T-bet within Th_{17} cells that is necessary for skewing towards a Th_{17-1} profile (i.e. ability to produce both Th_{17} and Th_1 cytokines)[113]. Such data are strongly suggestive that Th_{17} cells induced in the presence of IL-23 are phenotypically altered, having, for example, altered trafficking properties, and may explain both the limited pathogenicity in murine models of Th_{17} cells generated with TGFβ alone and the altered susceptibility to human autoimmune diseases associated with IL-23 gene polymorphisms[114,115] .

IL-2 & other cytokines. Murine work has shown an inhibitory effect of IL-2 on IL-17 expression[102,116,117], but it may have a positive effect in human $CD4^+$ T cell culture. These discrepancies may reflect differences between mouse 'naïve' T cells and $CD45RA^+CD45RO^-$ T cells from human peripheral blood. As might be anticipated, a wide variety of other cytokines also have pro- (GM-CSF[118,119]) and anti-Th_{17} (IL-27[120], IL-25[121], IL-4[122,123] , IL-13[124,125] and IFNγ[123]) properties.

'Signal 2' co-stimulation

A basic tenet of T cell biology is that co-stimulation via CD28 is required for survival and expansion of T cells, the subsequent lineage of which is determined by 'signal 3' factors[126]. However, recent studies have unexpectedly reported that CD28 co-stimulation actually impairs development of Th_{17} cells from naïve T cells[127]; instead Th_{17} cells may be best induced via ligation of the inducible co-stimulator, ICOS[128] or lymphocyte receptors CD5 or CD6[129] (both members of the Scavenger Receptor Cysteine-Rich domains superfamily, SRCR-SF) - the latter effects are antagonised by CD28 co-stimulation. ICOS ligation was shown to up-regulate RORC expression, and to augment human Th_{17} function through induction of c-Maf and IL-21[128]. In the case of CD5 and CD6, this effect was mediated via up-regulation of IL-23R with consequent prolonged STAT3 activation and enhanced RORγt expression. Interestingly the inhibitory effect of simultaneous CD28 co-stimulation suggests optimal Th_{17} induction via CD5 could not be elicited by activated APCs, such as mature mouse bone marrow-derived or human monocyte-derived dendritic cells (DCs), which have high expression of CD80 and CD86, ligands of CD28. This may suggest a role for other DCs, such as mouse $CD103^+$ lamina propria DCs, which have high MHC II expression but do not up-regulate CD80 or CD86 expression after lipopolysaccharide stimulation *in vitro*[130]. Ligands for CD5 and CD6, however, are poorly identified. CD72[131] and CD5 itself[132] are both suggested to be ligands of CD5 and are present on B cells; ALCAM and protein 3A11, ligands of CD6, are present on DCs and B cells[133]. Alternatively CD5 and CD6 might constitute innate immune receptors,

since polysaccharide β-glycan (present on *Candida albicans*) can activate CD5[134], whereas bacterial LPS may activate CD6[135].

Transcriptional regulation

Genome-wide gene expression profiling has yielded large numbers of candidate genes for controlling Th_{17} differentiation and function[136] – some of the more important candidates are discussed below.

RORγt. RORγt, a retinoid orphan nuclear receptor closely related to the retinoic acid receptor and originally defined as a thymic-specific isoform of RORγ[137] is expressed specifically in both human and mouse Th_{17} cells[138], and is driven by stimulation of naive T cells with the cytokines discussed above[97]. Expression of RORγt is necessary and sufficient for induction of IL-17, IL-17F and IL-23R expression[138]. STAT3-deficient mice fail to express RORγt and produce reduced levels of IL-17, and reinstitution of the gene via a retroviral vector rescues the defect[139].

STAT3 & RORα. In addition to RORγt, STAT3 is required for full generation of the Th_{17} phenotype[140,141] ; over-expression of either STAT3 in RORγt-deficient mice or RORγt in STAT3-deficient mice fails to restore normal IL-17 generation[142]. IL-6, IL-21 and IL-23 all induce activation of STAT3[99,143], which binds to the *Il17* promoter, leading to Th_{17} differentiation. RORα, also induced by TGFβ plus IL-6, synergises with RORγt to promote differentiation and function of Th_{17} cells[144]. Simultaneous deficiency of RORγt and RORα results in complete failure of IL-17 production[144].

Other transcription factors. The transcriptional regulation of the Th_{17} lineage is complex and many transcription factors are implicated, all of which cannot be discussed in detail here. Many are concerned with the reciprocal regulation of Th_{17} and Treg development e.g. IRF4[145], RARα [146], BTBD11[136], AHR[147-150] & HIF-1[151]. Peroxisome proliferator-activated receptor γ (PPARγ)[152], B cell activating transcription factor (BATF)[153], ETS1[154], IκBζ[155], growth factor independent 1 (GFI1)[156], Krüppl-like factor 4 (KLF4)[157] and others also play important roles.

Hypoxia-inducible factor 1 (HIF-1) is a key metabolic sensor for the detection of hypoxia and is one of the mediators which has been shown to regulate the balance between T_{reg} and Th_{17} differentiation[151]. This effect is via both direct transcriptional activation of RORγt and through formation of a tertiary complex with RORγt and p300 enhancing recruitment

to the IL17 promoter, as well as by binding of FoxP3 and targeting it for proteasomal degradation.

Plasticity & Th_{17-1} cells

Although it is convenient to classify T cells into subsets, it is increasingly clear that considerable plasticity exists; T cells may move between lineages, or exist in intermediate chimeric states. Genetic and epigenetic mechanisms have been described in the regulation of this plasticity[158,159].

In the case of T_{reg} cells a picture has emerged whereby not only is there antagonism between the effector functions of Th_{17} and T_{reg} populations, but also reciprocal interconnection in the mechanisms giving rise to their differentiation[92]. In fact, it seems likely that RORγt and FoxP3 exist in the same cell; as discussed above, the levels of TGFβ, proinflammatory cytokines[160] and other environmental factors such as hypoxia control the balance[151].

Th_{17-1} cells, that is those able to produce both IL-17 and IFNγ, have been described in a number of settings, often derive from Th_{17} cells on exposure to IL-12[161] and may be important due to their hybrid expression of Th_1 and Th_{17} proteins. For example, attraction by CXCL9, 10 or 11 (ligands of CXCR3, a Th_1 protein) may allow delivery of IL-17 where 'pure' Th_{17} cells cannot access[162]. Th_{17-1} cells are reported in patients with cancer[163] and may mediate significant anti-tumour responses[164].

Cell surface phenotypes of Th_{17} cells

In healthy individuals, roughly 1% of $CD4^+$ T cells are Th_{17}, however, very high levels of Th_{17} cells may be present in pathological micro-environments, possibly because they are recruited to sites of active inflammation via CCR6 and the integrin CD49 (and possibly CD161, although the function of this integrin is poorly characterised)[163]. Th_{17} cells produce multiple other immune mediators (e.g. IL-17F, IL-21, IL-22, CXCL-8 and CXCL-13), and cell surface proteins (e.g. CCR6, CCR4, IL-23R and CD161) but attempts to identify specific cell surface phenotypic markers for the lineage have met with difficulty.

CD161

A C-type lectin receptor, CD161 is induced by RORC2 and is found on human IL-17-producing T cells[165], including Th_{17} cells and others (e.g. $CD8^+TCR\alpha\beta^+$, $CD4^-CD8^-TCR\alpha\beta^+$ and $CD4^-CD8^-TCR\gamma\delta^+$)[166]. It has been proposed as a co-activating receptor promoting

antigen-dependent T cell proliferation upon engagement by the ligand, PILAR (proliferation-induced lymphocyte-associated receptor)[167]. $CD161^+$ umbilical cord blood $CD8^+$ and $CD4^-CD8^-$ T cells, but not their $CD161^-$ counterparts, express RORC2 and IL-23R mRNA and can acquire the ability to express IL-17 under the action of IL-1β and IL-23[166]. It is important to note, however, that the $CD4^+CD161^+$ lymphocytes found in peripheral blood also include Th_1, Th_2 and Th_0 cells and some investigators have even reported $CD161^-$ IL-17 producing lymphocytes in the context of T_{reg} plasticity[168] and cancer [169].

CCR6, CCR4 & IL-23R

CCR6 (CD196), a G-protein coupled receptor whose ligand is CCL20 (MIP-3α), mediates homing to skin and mucosal tissues[170]. It is reportedly expressed on all Th_{17} cells, but also on some $IL\text{-}17^-$ lymphocytes[171]. The same authors report that expression of CCR4, a receptor typically expressed on Th_2 cells and contributing to skin-homing[172], identifies that portion of the $CCR6^+$ population able to produce IL-17. IL-23R, as discussed above, is upregulated on differentiated Th_{17} cells[173], but is expressed also on $Th_{17\text{-}1}$ and to a lesser extent Th_1 cells[173].

Roles of Th_{17} cells in health, cancer and disease

Th_{17} cells are best characterised in the setting of autoimmune diseases (or their animal models), e.g. multiple sclerosis, inflammatory bowel disease, psoriasis and rheumatoid arthritis, where these cells clearly play a central pathogenic role, but which I will not discuss further. The role of immunity in cancer is a complex area; immune responses in this context are often represented as a double-edged sword, with the capacity for inflammatory responses both to provide proliferative and oncogenic stimuli and to mediate anti-tumour responses. Recent years have seen great advances in understanding of this area and an era of clinical immunotherapy has dawned, whereby immune responses are beginning to be manipulated to deliver anti-cancer therapy. In this section I will overview the roles of Th_{17} cells in health and in cancer.

Th_{17} cells in infection

Accumulating evidence shows Th_{17} cells are crucial for specific and effective immune responses against certain pathogens, where Th_1 and Th_2 responses are insufficient.

Roles have been shown in the response to extracellular (e.g. *Klebsiella pneumoniae*[174], *Mycoplasma pneumoniae*[175]) and intracellular (e.g. *Mycobacterium tuberculosis*[176]) bacteria – IL-17 induces neutrophil chemotaxis (within 4-8 hours) and upregulates pro-inflammatory cytokine production by monocytes and macrophages[177], epithelial, endothelial and fibroblastic cells[178]. A crucial role of Th_{17} cells in health is in the response to fungal infections – humans with STAT3 mutations have markedly reduced Th_{17} cells and increased susceptibility to mucosal candidiasis[179,180] and memory T cells specific for *C. albicans* reside in the $CCR6^+CCR4^+$ subset[171].

Th_{17} cells in cancer and in myeloma

Whilst there is an expanding literature concerning Th_{17} cells in cancer, few hard facts about their roles in cancer have been established. Firstly, it is often difficult to tease apart the effects of IL-17, which derives from many tissues, and the role of Th_{17} cells *per se*. It is important to consider that 'IL-17' in fact represents a family of cytokines; the term is most widely used in the published literature to mean any of IL-17A homodimers, IL-17F homodimers and IL-17AF heterodimers, although the family also includes IL-17B, IL-17C, IL-17D and IL-27E (a.k.a. IL-25), which are far less well characterised and almost certainly have disparate functions from IL-17A/F[181-183] . For clarity, in this thesis unless otherwise specified, 'IL-17' is defined by antibody reactivity to the IL-17A subunit and therefore comprises IL-17A homodimers and IL-17AF heterodimers only.

When reviewing the literature, it is tempting to attempt to draw generalisations about the role of Th_{17} cells and their mediators in tumour initiation, metastasis, angiogenesis, lymphangiogenesis and other aspects of neoplasia, however, the evidence from both murine and human work is contradictory and points to both pro- and anti-tumour effects[184-188]. As yet further complication, helper T cells exhibit properties of more than one subset, for example IL-17-secreting T_{reg} cells, or $CD4^+$ cells secreting both IL-17 and IFNγ ($Th_{17\text{-}1}$ cells), are well documented and may have different roles and effects from the 'pure' populations. Crucially it is unwise to extrapolate from one malignancy to another and I will therefore review only MM here.

In 2006, Alexandrakis *et al*. reported increased levels of IL-17 in the sera of patients with MM, correlating with disease stage, vascular endothelial growth factor (VEGF), TNFα and microvessel density[189]. Interestingly, although VEGF receptors are not expressed by myeloma cells, they are present on stromal cells[190]; VEGF signalling has an established role in modulating dendritic cell APC function[191], and autocrine VEGF signalling may

mediate capillarogenesis in MM[192]. Taken together, these findings suggest an angiogenic role for IL-17 in MM, although direct induction of VEGF by IL-17 was not shown. It is notable, however, that whilst some investigators have corroborated such data[193], others report contradictory findings[194]. Observational data suggest bisphosphonates, used extensively in the treatment of MM bone disease and recently shown to affect overall survival in the disease[195], may lower IL-17 levels and might therefore have a suppressive effect on angiogenesis[194]. This in turn raises the question of whether Th_{17} cells in the BM might show anatomical localisation, for example in, or juxtaposed with the periosteal niche.

Added to primary MM cells or human myeloma cell lines (HMCLs), IL-17 has been reported to increase colony size and number, and the fraction of cells in S phase, and to increase adhesion of PCs to bone marrow stromal cells (BMSCs)[74]. After subcutaneous injection of HMCLs into SCID mice, administration of IL-17 was associated with significantly larger tumour size at 3 weeks[74]. Furthermore the majority of patient MM plasma cells and HMCLs express IL-17R, and their growth is blocked by anti-IL-17 antibody[74]. IL-21, also produced by Th_{17} cells, increases proliferation of both cytokine-dependent HMCLs and primary myeloma cells and functions as an anti-apoptotic factor, via JAK1, STAT3 and Erk1/2[196]. In fact, IL-21 was reported to be a more potent growth and anti-apoptotic factor than any other known myeloma cell growth factor, including IL-10, IL-15, TNF and IGF-1[196]

Prabhala *et al.* found Th_{17} cells were increased in the PB and BM of patients with MM compared to healthy donors, by FACS and qPCR, although in BM the difference did not reach statistical significance[74]. Cultures of PB- and BM-derived mononuclear cells in Th_{17}-polarising conditions revealed a greater population of inducible Th_{17} cells in MM patients than in healthy donors. IL-17, IL-1α, IL-21, IL-22 and IL-23, all Th_{17} cytokines, were increased in plasma from myeloma patients. Uptake of apoptotic but not necrotic myeloma cells by DCs leads to enhanced induction of Th_{17-1} cells, which may be expanded in myeloma and correlate with bone disease[197].

There is further evidence of a role of IL-17 in MM bone disease: rhIL-17 treatment of monocytes produces $TRAP^{+}CD51^{+}$ multinuclear cells with actin ring formation (functional and phenotypic properties of osteoclasts), probably mediated via RANKL and TNFα[198]. Noonan *et al.* found reduced T_{reg} and expanded Th_{17} cells in MM BM, and showed this cytokine environment mediated up-regulation of osteoclast activation with consequent lytic bone destruction[199]. Levels of certain Th_{17}-inducing cytokines (IL-6, IL-1β, IL-17)

correlated closely with the extent of bone disease and marrow infiltrating lymphocytes activated under Th_1-polarising conditions were able to reduce formation of mature osteoclasts.

Oncolytic virotherapy

Observations of spontaneous regression of malignancies following naturally contracted viral infections date back to the turn of the nineteenth century and raised interest in the use of viruses as anti-cancer therapies. However, early studies using viral infections in patients with cancer resulted either in arrest of viral infection by competent host immunity, or morbidity and mortality due to overwhelming infection and this line of research was largely abandoned in the 1950s (reviewed in [200]). More recently, advances in immunology and molecular cell biology have cast light upon the mechanisms by which naturally occurring and genetically engineered viruses might preferentially infect and lyse cancer cells and a number of these agents are under investigation in preclinical and early-phase clinical research.

Oncolytic viruses are replication competent viruses with a predeliction for infection of or replication in cancer cells, and that are able to lyse cancer cells after infection. In order to exert this differential effect against malignant cells this group of viruses necessarily rely upon differences in the cellular processes between normal and tumour cells, an effect which can be exploited to deliver anti-cancer therapy. A crucial step forward in the field has been the recognition that the most significant effect of oncolytic virotherapy may result not from the infection and destruction of cancer cells *per se,* but rather through the consequent release of tumour antigens in a pro-inflammatory environment leading to priming of tumour antigen-specific secondary immune responses[201-205]. In turn, this has lead us and others to combination of oncolytic virotherapy with other immunotherapeutic agents[206,207].

Reovirus

Reovirus (derivation: respiratory enteric orphan virus) is a wild-type virus of the family *reoviridae* that is able to infect the human respiratory and gastrointestinal tracts, generally resulting in asymptomatic infection or mild influenza-like symptoms or diarrhoea. The

virus is ubiquitous and seropositivity in the human population is virtually universal[208]. Reovirus is a dsRNA virus consisting of a non-enveloped icosahedral virion capsid with two layers of proteins encoded by 10 segments of RNA.

Preclinical data

The ability of the virus to replicate in tumour and transformed cell lines was noted in the 1970s[209]; subsequent work identified amplified Ras signalling in such cells as the crucial sensitising event. Reoviral infection of healthy cells results in activation of a serine/threonine protein kinase: double-stranded RNA-activated protein kinase (PKR) [210], which through activation of eIF2a induces an interferon response and shutdown of viral protein synthesis; Ras activation prevents PKR phosphorylation and thereby enables unchecked reoviral replication. However, subsequent studies have demonstrated that Ras-activation is not the only enabling abnormality for reoviral replication as some cell lines with wild-type Ras also show susceptibility, for example cells with KRAS mutations[211]. A wide range of malignant cell types have now been demonstrated to be susceptible to reovirus-induced oncolysis[212,213]. Intratumoral injection of reovirus into both syngeneic tumours in immunocompetent animals, and xenografting tumours in immunodeficient animals results in tumour regression, for example in breast cancer[214], colon[215], ovarian[215] and glioma animal models[213]. The actual mechanism of cell death in reovirus-infected cells has been variously reported to be via apoptosis[216-220] and by autophagy[216,221].

Clinical Trials

Following from this work, a number of clinical trials have been completed with reovirus in solid malignancies, with a number more underway at present. In keeping with the usual pattern of clinical exploration for new therapies, the early studies were of cautious dose escalation of single agent virotherapy, initially by intratumoural injection[222]. Clinically significant responses were seen in some patients and side effects seen were mild flu-like illness and headaches; treatment was well-tolerated and grade 3/4 toxicities were not encountered. On the basis of these data, intravenous administration was next appraised, in patients with advanced cancers of multiple histopathological subtypes: again flu-like illness was the dominant side effect, dose-limiting toxicities were not encountered and

viral delivery to tumour sites was demonstrated[223]. Reovirus was next combined with a number of standard chemotherapeutic agents (reviewed in [224]) and with radiotherapy[225]. A recent phase III trial in patients with platinum-refractory head and neck cancers, in combination with paclitaxel and carboplatin (REO 018) has shown improved PFS and OS *vs.* paclitaxel and carboplatin alone.

One of the predominant concerns about the potential of oncolytic viruses for clinical meaningful immunotherapy stems from the development of rapid and potent anti-viral humoral responses. Research has therefore been directed at combination strategies to attenuate anti-viral immune responses, through combination with immunosuppression[226] or chemotherapy[227], and these strategies have seen some efficacy. However, data from human trials delivering virus intravenous have revealed a fascinating phenomenon, that even in the presence of circulating neutralising anti-reovirus antibodies, the virus is able to 'hitch hike' on leukocytes and platelets, allowing evasion of the immune response and access to the tumour bed[228].

Reovirus in haematological malignancy

Building on the successes seen with solid tumours, and the obvious attractions of the potential for intravenous delivery, interest in oncoloytic virotherapy has been growing in the arena of haematological malignancy. Preclinical data have pointed towards efficacy of reovirus against a range of haematological malignancies, including acute myeloid leukaemia[229], lymphoma[230], chronic lymphocytic leukaemia[207] and MM[216,231,232].

The molecular landscape of MM has been increasingly thoroughly characterised over the preceeding decades but in fact RAS pathway mutations including N- and K-RAS mutations have long been known to be among the common disease-driving mutations in myeloma[233-235]; this oncogenic addiction makes myeloma a tempting target for oncolytic virotherapy. Preclinical studies of reovirus in myeloma to date have demonstrated the use of reovirus to 'purge' autologous stem cell grafts of myeloma cells prior to re-infusion to NOD/SCID mice[232], and that reovirus is able to induce ER stress, which can be augmented by combination with proteasome inhibition[218]. A recent phase I trial has also concluded that reovirus monotherapy is well-tolerated and deliverable in patients with relapsed multiple myeloma, but concluded that combination therapy is required to achieve clinically useful disease responses[236].

Murine myeloma models

Considerable effort has been expended over many decades to generate *in vivo* models of human myeloma that will accurately recapitulate the human disease and allow dissection of the aetiology of the disease and testing of potential therapies. The resulting range of approaches has led to a slew of disparate models, each with their own benefits and limitations. The major categories of models will be briefly reviewed, and the reasons for ultimate model selection for the work presented in Chapter 5 will be explained.

Chemically-induced plasmacytoma models

These models rely on injection of pristane oil into the perineum of Balb/c mice[237]; the resulting chronic inflammatory granuloma typically results, after 120 days, in development of a plasmacytoma-like tumour localised to the injection site in 60% of mice. Although these mice are immunocompetent the disease is localised and the model is therefore unsuitable for examination of bone disease or the BM microenvironment, or renal disease. In addition a long lag-time is needed between pristane injection and development of tumours, tumours do not produce IL-6 (which is a typical characteristic of human malignant plasma cells) and tumour outgrowth appears to be driven by t(12;15)(IgH/myc) in 90%, a defect seen in <5% of human myeloma. A pan-European group have recently described a MOPC315.BM model[238], derived through *ex vivo* selection and *in vitro* passage of cells from the original model, that does demonstrate bone marrow tropism and represents an improvement on the original model, but does not circumvent a number of the other drawbacks of this model. In view of the limitations of this model it was not suitable for the work proposed here.

SCID xenograft models

Severe combined immunodeficient (SCID) mice exhibit complete inability to implement V(D)J recombination, resulting in failure of humoral and cellular immunity, and these mice are widely used as model organisms for a range of applications. Due to marked immunodeficiency, human tumour cells can be grafted into these animals with subcutaneous or intravenous injection[239], implantation of human (SCID-hu model[240]) or

rabbit (SCID-rab model[241]) fetal bone chips, implantation of whole BM trephine biopsies (LAGλ-1 model[242]) or intratibial injection of human MM cell lines or primary plasma cell leukaemia samples[243]). Some of these models do offer the potential of exploring a quasi-human bone marrow microenvironment, and may replicate human disease faithfully in some respects (e.g. detectable paraprotein, bony deformity, no widespread organ involvement). Nonetheless, these models are by definition devoid of competent immune effectors and are unsuitable for examination of immunotherapy.

Transgenic models

With the increasing understanding of the molecular underpinnings of myeloma has come a burgeoning of transgenic animal models of the disease. A few of these appear able to reproduce many features of the human disease – for example the Vk*myc ORF model exhibits a slowly progressive monoclonal plasma cell proliferation, a serum paraprotein by 20 weeks, anaemia, myeloma kidney disease, osteoporosis and rare bone lesions[244]. However, most exhibit features quite different from the typical pattern of human plasma cell dyscrasia – for example Eμ-xbp-1a shows prominent activation of proapoptotic tumour suppression mechanisms, which are quite unlike human disease[245], c-MAF mice occasionally develop myeloma, but predominantly generate B cell lymphomas[246], c-Myc/Bcl-X_L mice develop plasmablastic morphology and cytogenetic abnormalities dissimilar to human disease[247,248] . Of course, all animal models are by definition not exactly like their human counterpart, and many of these models will doubtless prove invaluable for studying certain aspects of myeloma pathogenesis and therapy; some would certainly be candidates for conducting the experiments reported here. However, in addition to the concerns outlined, it is also now well-appreciated that human myeloma is a genetically diverse disease with marked intra-tumoral sub-clones which compete in a Darwininan manner under the selection pressures of therapy[249-251] , meaning that models relying on a single genetic driver are likely to be limited in their ability to mirror the responses of human disease to therapy.

5T lineage models

This group of models all derive from a spontaneous myeloma-like disease arising in ageing C57BL/KalwRij mice[252]; cells were subjected to serial autologous passage to evolve a range of cell lines which can be used in this syngeneic model[253]. The 5T2, 5T7,

5T8, 5T13, 5T14, 5T21, 5T33, and 5T41 lines represent different clones isolated from these mice by serial transplantation[253] and exhibit a range of growth characteristics. Stroma-independent subclones, such as 5TGM1 (derived from 5T33), were subsequently isolated and allow *in vitro* passage and thus considerable flexibility for *ex vivo* assays[254-256]. The disease in the 5TGM1 model is largely confined to BM (although spleen, which may represent a haematopoietic organ in adult mice, is also involved), secretes an IgG2bκ paraprotein and shows bone disease similar to humans (lytic lesions, decreased bone mineral density, hypercalcaemia etc.). The 5T lines can develop into isotype-switch variants [257]. This immune competent model offers considerable flexibility to answer the questions posed here and was therefore adopted for this and subsequent work.

Summary

Multiple myeloma is an immunologically complex environment in which anti-tumour immune responses detectable early in the disease are gradually eroded and evaded by the tumour and its microenvironment, and ultimately fail in their battle to restrain the proliferating plasma cell clones. An expanding body of experimental work has elucidated many of the mechanisms underlying these immune responses and their evasion, and characterised the immunomodulatory effects of agents and treatments with clinical activity against the disease.

Evidence points towards an expansion of Th_{17} cells in myeloma, and is suggestive that this population may favour tumour growth and impede anti-tumour immunity; however these cells have not been characterised in great detail, and the potential for beneficial therapeutic manipulation of the responsible mechanisms remains to be explored.

The oncolytic virus, reovirus, have demonstrated pre-clinical and clinical efficacy against a range of solid tumour types, and preliminary evidence also suggests potential against haematological malignancies. The effects of this virus on myeloma cells, and on the host anti-myeloma immune response require further chararcterisation and the potential for combinatorial immunotherapy in this malignancy should now be explored.

Hypothesis, Aims and Objectives

The work contained in this thesis addresses two distinct but related strands of investigation, and the hypothesis, aims and objectives of these will be discussed separately.

IL-17-secreting lymphocytes in multiple myeloma (Chapters 3 & 4)

The hypothesis of this work is that tumour-immune system interactions in multiple myeloma give rise to dysfunctional T cell immunity, manifest as impaired protective immune responses and development of an immune microenvironment which favours tumour outgrowth.

The objectives of this part of the project are to address 3 fundamental questions:

- Do malignant plasma cells in multiple myeloma generate Th_{17} cells?
- If so, how do they do this – what are the key events involved?
- Are there phenotypic or functional differences between Th_{17} cells in health and in multiple myeloma?

Oncolytic virotherapy in multiple myeloma (Chapter 5)

The hypothesis of this section of work is that the oncolytic virus, reovirus, can induce direct and immune-mediated killing of myeloma tumour cells. The work aims to address the following questions:

- Can reovirus kill myeloma cells?
- If so, what are the mechanisms?
- Can this agent represent a useful treatment modality for human MM?

Chapter 2 – Materials and Methods

Reagents and solutions

Phosphate-buffered saline (PBS)	Source: Sigma
Fetal bovine serum (FBS)	Source: PAA laboratories, heat inactivated for 30 minutes at 56°C
Complete medium (CM)	RPMI-1640 (Sigma), 10% heat-inactivated FBS
FACS buffer	490ml PBS, 5ml FBS, 5ml 5% Sodium azide (sterile, filtered)
Hank's buffered salt solution (HBSS)	HBSS-CaCl2-MgCl2 (Sigma)
Lymphoprep	Source: Axis Shield, Norway
MACS buffer	493ml PBS, 5ml FBS, 2ml 0.5M EDTA (sterile, filtered)
Freezing medium	80% RPMI-1640 (Sigma), 10% FBS, 10% DMSO (Sigma)
Brefeldin A	Golgiplug (BD Biosciences, UK)
Intracellular staining buffers	Fixation-Permeabilisation and Permabilisation buffers, source: Miltenyi Biotec (Fixation and Dead Cell Discrimination Kit, 130-091-163)

Table 2.1: Reagents and solutions.

Sample collection from healthy volunteers and patients

Approval was granted by the Local Research Ethics Committee for collection of samples from patients and healthy volunteers (REC: 12/YH/0364, LTHT R&D: HM12/10295). Informed consent was given by all patients and healthy donors. Patients were recruited from the myeloma clinic at St James's Institute of Oncology and gave consent for peripheral blood and, in some cases, bone marrow sample collection (Appendix 2 shows donor information sheets and consent forms). Peripheral blood samples from healthy control donors were collected from both age-matched (relatives of patients) and non-age-matched (University of Leeds staff) donors. Healthy age-matched donor bone marrow samples were collected from patients undergoing hip arthroplasty without haematological comorbidities; matched peripheral blood samples were also taken from these donors. Patient and donor data, such as age, gender, date of diagnosis, and clinical parameters

were obtained and stored in a secure, anonymous manner according to 'good clinical practice' guidelines and the University of Leeds information governance policies.

Venous blood and bone marrow aspiration samples were collected into sterile ethylenediaminetetraacetic acid (EDTA) tubes (Vacutainer, BD Biosciences). Collection tubes were centrifuged at 350g for 15 minutes and the plasma was aspirated and stored at -80°C; the cell pellet was then resuspended in HBSS (twice the original sample volume) for mononuclear cell isolation. Serum samples were collected simultaneously in clotted blood tubes, centrigued at 400g for 20 minutes and rapidly frozen and stored at 20°C until analysis.

Apheresis cones from peripheral blood platelet donations were purchased from National Health Service Blood and Transplant, processed within 6 hours of the initial donation, and flushed out with 50ml of HBSS prior to mononuclear cell isolation (see below).

Isolation of mononuclear cell fractions

Cell solutions were divided into 30ml aliquots and layered onto 15ml of Lymphoprep (Axis Shield, Norway) in 50ml Falcon tubes. The samples were centrifuged at 800g for 20 minutes, with no brake. The interface was aspirated with a wide bore pipette and the peripheral blood mononuclear cells (PBMC) washed twice in HBSS: centrifuging once at 200g for 20 minutes, then once at 300g for 10 minutes. If not required immediately, samples were suspended in freezing medium (10^7 cells per 1ml per cryotube), cooled at -1°C/min and stored at -270°C in liquid nitrogen.

T cell subset enrichment

Magnetic-assisted cell sorting

For some experiments, $CD4^+$ T cells were selected from PBMC by magnetic-assisted cell sorting (MACS), using $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec, UK). In brief, PBMC are first incubated with a cocktail of biotin-conjugated monoclonal antibodies (against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγδ and glycophorin A) for 15 minutes, then anti-biotin monoclonal antibodies conjugated to magnetic beads are added. The magnetically labelled non-CD4+ cells are then depleted by retention on a MACS column

(Miltenyi Biotec) in the magnetic field of a MACS separator (Miltenyi Biotec), while the unlabelled $CD4^+$ cells pass through. The kit was used as per the manufacturer's instructions. Purity checks by flow cytometry revealed >95% purity.

For some experiments, purified naïve ($CD45RA^+$) and memory ($CD45RO^+$) T cell populations were required, which were isolated using the same method. The Naïve $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec, 130-094-131) contains biotin-conjugated monoclonal antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγδ, HLA-DR and glycophorin A. The Memory $CD4^+$ T Cell Isolation Kit (Miltenyi Biotiec, 130-091-893) uses biotin-conjugated monoclonal antibodies against CD8, CD14, CD16, CD19, CD36, CD45RA, CD56, CD123, TCRγδ and glycophorin A.

For some coculture experiments, purified $CD4^+CD25^-$ T cells were required. These were isolated by first selecting $CD4^+$ T cells from PBMC as described above. Following this cells were labelled with anti-CD25 antibody conjugated to magnetic beads (CD25 MicroBeads II, Miltenyi Biotec, 130-092-983), incubated for 15 minutes at 4-8°C and then washed. The cells were then passed through a MACS column as before, resulting in depletion of $CD25^+$ cells by retention within the magnetic field.

Stimulation of T cells

A variety of T cell stimulation modalities were investigated.

Phorbol 12-myristate 13-acetate & ionomycin

The phorbol ester, phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, UK), causes direct activation of Protein Kinase C. Ionomycin (ION, Sigma-Aldrich, UK), an ionophore produced by the bacterium *Streptomyces conglobatus*, induces a rise in intracellular calcium. The combination of these two agents has been widely used to induce T cell stimulation. Unless otherwise stated, cells were suspended at 10^6/ml in complete medium (CM), PMA was added at 25ng/ml and ION at 1µg/ml and cells were incubated for 3 hours at 37°C, 5% CO_2. Golgiplug (BD Bioscience) was then added at 1µl/ml and the cells incubated for a further 3 hours.

T cell activation/expansion kit

This kit is intended for expansion and activation of human T cells by mimicking antigen-presenting cells. Anti-biotin MACSiBead particles (Miltenyi Biotec) were loaded with combinations of biotinylated antibodies against CD3, CD28 (included in the kit), CD5 (BD

Biosicence) and ICOS (eBioscience), under aseptic conditions as follows: MACSiBead particles were first re-suspended by vortexing; 10^8 particles (500µl) were then removed and placed in a sealable 2ml tube. 100µl of each antibody was added, and the mixture was then made up to a total volume of 1ml with MACS buffer. The mixture was incubated at 2-8°C under constant rotation at 4rpm. The loaded particles were then stored for up to 4 weeks at 2-8°C.

MACSiBead particles were added to mononuclear or T cells at ratio of 1 bead : 2 cells. When used for T cell restimulation (to induce cytokine secretion), cells were incubated for 6 hours, with Golgiplug added at 1µl/ml for the last 3 hours, as per the PMA/ION protocol.

Cytostim

Cytostim is an antibody-based reagent that stimulates T cells by crosslinking T cell receptors (TCR) with major histocompatibility complex (MHC) molecules; it acts similarly to a superantigen (although independently of certain Vβ domains of the TCR). Cells were suspended at $10^6 ml^{-1}$ in CM, Cytostim was added at 20µl per ml and the mixture was incubated for 6 hours at 37°C, 5% CO_2; Golgiplug was added for the last 3 hours.

Candida albicans antigens

The Peptivator Candida albicans MP65 reagent (Miltenyi Biotec, UK) is a lyophilised pool of 15aa peptides (with 11aa overlap) covering the sequence of the *Candida albicans* cell wall mannoprotein MP65. Cells were suspended at $10^6 ml^{-1}$ in CM, Peptivator was added at 20µl per ml and the mixture was incubated for 6 hours at 37°C, 5% CO_2; Golgiplug was added for the last 3 hours.

Flow cytometry

Principles of flow cytometry

Flow cytometry, a technique widely employed in immunobiology, allows high-throughput multiparametric analysis of the physical and chemical characteristics of multiple individual cells within a single cell suspension by measurement of their light absorption, scattering and emission characteristics. Through the use of antibodies conjugated to fluorophores (fluorescence chemicals which absorb light of one wavelength and then re-emit light of a

longer wavelength) the method allows assessment of the expression of proteins both on the surface of, and within cells.

In brief, a flow cytometer comprises three components: a fluidic system, an optical system and a computer system. The fluidic system employs hydrodynamic focusing to force cells into a single cell stream, which is then passed at a known speed through the optical system. Here, one or more lasers interrogate the particle stream in turn, causing excitation of fluorophores responsive to that wavelength. A series of dichroic mirrors, bandpass filters and photomultiplier tubes (PMT) then allow detection of transmitted, reflected and re-emitted light within pre-determined ranges of wavelengths during the passage of each particle through each laser. Low angle forward scattered light (forward scatter: FSC, collected at an angle of 15° to the incidence beam, usually measured using the 'blue' laser at 488nm) is detected by a PMT aligned to the laser stream (with an obscuration bar to exclude transmitted light) and is approximately proportional to cell diameter. Light scattered at wide angles (measured at 90° to the incident beam, again usually on the 488nm laser) provides a measure of the granularity of the cell (side scatter: SSC). The remaining PMTs collect light at 90° to their respective incident laser at ranges of wavelengths longer than the excitation laser and therefore allow measurement of light emitted by stimulated antibody-conjugated fluorophores and thus quantitation of the cognate antigen. Since fluorophores emit light across a range of wavelengths and may therefore be detected by more than one detector, a panel of control samples, each stained with only a single fluorophore, is used to compensate for spectral overlap across fluorophores.

Fluorochome-conjugated antibodies

The antibody-fluorochrome conjugates and DNA dyes employed in these experiments were as follows:

Target	Fluorochrome	Excitation laser (wavelength /nm)	Maximum emission wavelength /nm	Manufacturer	Clone (product ID)
Live/Dead discriminator	PI-like	Blue (488)	625	Miltenyi Biotec	(130-091-163)
CD4	FITC	Blue (488)	520	BD	RPA-T4 (555346)
CD4	APC-Cy7	Red (633)	785	BD	RPA-T4 (557871)
CD8	APC-Cy7	Red (633)	785	Biolegend	SK1 (344714)
CD161	APC	Red (633)	660	BD	DX12 (550968)
CD25	PE-Cy7	Blue (488)	785	BD	M-A251 (557741)
CD183 (CXCR3)	PE-Cy7	Blue (488)	785	BD	1C6/CXCR3 (560831)
CD184 (CXCR4)	PE-Cy7	Blue (488)	785	BD	12G5 (560669)
CD194 (CCR4)	AF647	Red (633)	668	BD	1G1 (557863)
CD196 (CCR6)	PE	Blue (488)	578	BD	11A9 (559562)
IL-17	V450	Violet (405)	448	BD	N49-653 (560610)
IFNγ	V500	Violet (405)	500	BD	B27 (561980)
RORγt	PE	Blue (488)	578	BD	Q21-559 (563081)
RORγt	PE	Blue (488)	578	eBioscience	AFKJS-9 (12-6988)
T-bet	PE	Blue (488)	578	BD	O4-46 (561268)
FoxP3	AF488	Blue (488)	495	BD	236A/E7 (561181)
FoxP3	APC	Red (633)	660	eBioscience	236A/E7 (17-4777)

Table 2.2: Fluorochrome-conjugated antibodies for flow cytometry. IL-17: interleukin 17, IFNγ: interferon gamma, RORγt: retinoic acid-related orphan receptor gamma t, T-bet: T-box gene expressed in T cells, FoxP3: forkhead box P3, PI: Propridium iodide, FITC: Fluorescein isothiocyanate, APC-Cy7: Allophycocyanin-cyanine dye 7, APC: Allophycocyanin, AF488: Alexa Fluor 488, AF647: Alexa Fluor 647, PE: R-phycoerythrin, PE-Cy7: phycoerythrin-cyanine dye 7, BD: Becton Dickinson,

Cell staining and assay protocol

Live cells were transferred to U-bottomed 96 well plates (Corning Incorporated, USA) at $\leq 10^6$ in 100µl per well and the plate was centrifuged at 300g for 3 minutes (all further spins used these settings). After removal of supernatant, cells were resuspended in 50µl of FACS buffer per well and 1µl of Dead Cell Discriminator (Miltenyi Biotec, UK) was added. The plate was then incubated on ice under a 60W tungsten filament bulb for 10 minutes to photo-label cells. From this point onwards, all incubations were performed in the dark and on ice. Thereafter conjugated monoclonal antibodies against surface proteins, or their corresponding isotypes, were added and cells were incubated for 30 minutes. Wells were then washed with 200µl of Fixation-Permeabilisation buffer (Miltenyi Biotiec). 1µl of Dead Cell Discriminator stop (Miltenyi Biotec, UK) was added and cells incubated for 10 minutes. Cells were then washed once in 100µl of FACS buffer and once in 100µl of Permeabilisation buffer (Miltenyi Biotec, UK), before resuspension in 100µl of permeabilisation buffer. Conjugated monoclonal antibodies against intracellular proteins, or their corresponding isotypes, were then added and cells incubated for a further 30 minutes. Finally, cells were washed in FACS buffer, and resuspended in either 200µl FACS buffer per well in the 96 well plate, or 300µl in 5ml polypropylene FACS tubes, and analysed immediately on the BD LSR II flow cytometer.

Analysis, gating strategy and fluorochrome compensation

Analysis was performed with FACS DIVA versions 6 and 7 (BD Biosciences). The lymphocyte population was identified by forward and side scatter characteristics and dead cells were excluded using fluorescence in the dead cell discriminator channel. Where there was spectral overlap between fluorochromes, this was corrected using compensation values calculated from single stain controls. When all compensation was completed, $CD4^+$ T cells were gated on and categorised as Th_{17}, Th_1 or $Th_{17\text{-}1}$ on the basis of expression of IL-17, IFNγ or both respectively. The phenotype of these subsets was then characterised by their expression of additional markers.

Flow cytometric cell sorting

Flow cytometric sorting was performed by the University of Leeds Core Facilities FACS sorting service using a BD FACS Aria cell sorter. In principle this machine is a standard flow cytometer, which has in addition a vibrating mechanism able to fracture the single stream of cells into individual droplets after it has passed through the flow cell, with each droplet containing a single cell . An electrostatic charge is then applied to each droplet, which subsequently passes through an electrostatic deflection system which directs each

drop into a selected collection tube. In this way, each individual cell can be directed to a particular collection tube according to gating on their flow cytometric characteristics, allowing collection of one or more specific populations of cells. For cell sorting experiments, cell staining was performed according to the same protocols described above.

Luminex® assays

A Luminex assay (R&D Systems: Human HS Cytokine B Pre-Mixed Mag Luminex Performance Assay) was employed for multiplex cytokine assays, and was used according to the protocol supplied with this propriety kit. The selection of beads used is detailed in the Chapter 3 methods section. In brief, donor plasma samples were loaded into 96-well plates and incubated with spectrally encoded beads conjugated with antibodies against the analytes of interest. Serial dilutions of control samples were loaded into the same plate and treated similarly. Secondary biotinylated detection antibodies specific for the same analytes were then added, followed by phycoerythrin (PE)-conjugated streptavidin. The plate was then read using a Luminex 100 flow cytometry analyser, which employs dual lasers to simultaneously classify the bead and determine the magnitude of the PE-derived signal. The results were subsequently analysed using Luminex IS software. By comparison of the PE-derived signal associated with each bead in each sample against a standard curve, determination of the concentration of each analyte in each sample can be determined.

Human myeloma cell (HMCL) & bone marrow stromal cell lines (BMSC)

The human myeloma cells H929, JIM1, JIM3, JJN3, KMS11, KMS18, OPM2, RPMI8226, U266B (in house), the human bone marrow stromal cells HS-5 and HS-27, the murine bone marrow stromal cell M2-10B4, the CD40L-transduced human fibroblast line L929 and the Epstein Barr virus-transformed B cell line IM9, were maintained in CM in ventilated tissue culture flasks at 37°C and 5% CO_2 in a humidified incubator. CM was replaced every 3 to 4 days. 1% trypsin was used to loosen the plastic-adherent BMSC lines prior to passage. HMCL characteristics are shown in Appendix 3.

PBMC / HMCL co-cultures

All tissue culture work was performed under sterile conditions in laminar flow hoods. Proliferation of myeloma cell lines was reduced by treatment with mitomycin-C at a concentration of 50µg/ml for 30 minutes. Cells were then washed twice in CM, centrifuging at 300g for 10 minutes. PBMC and HMCLs were mixed at a 1:1 ratio at a concentration of $10^6 ml^{-1}$ in CM. Unless indicated, cocultures were incubated for 7 days at 37°C, 5% CO_2 before cells were harvested for analysis by flow cytometry.

For cultures where HMCL lysates were used, 10^6 HMCL were resuspended in 100µl of PBS and subjected to 5 freeze-thaw cycles, alternating between -80°C for 20 minutes and 37°C for 10 minutes. 100µl of this solution was then added to 10^6 PBMC. Cultures were then treated and analysed as above.

Western blotting

Loading buffer, running buffer and blot transfer buffer were made as listed in Appendix 4. Cell cultures were harvested and then centrifuged in a 15ml Falcon tube at 1000g for 10 minutes. The supernatants were removed and the cell pellet resuspended in 50µl of loading buffer, mixed thoroughly and transferred to 1.5ml Eppendorf tubes.

A 10% SDS-PADE resolving gel with a 5% stacking gel was prepared and immersed in running buffer. 25µl of pre-stained standard protein ladder (Invitrogen) was placed in the first well. Protein samples were heated to 100°C for 5 minutes before being placed on ice, and 25µl of each sample was then loaded into well. A potential difference of 200V was applied across the gel for 3 hours.

A piece of polyvinylidene difluoride membrane (Hybrid P, Sigma) was activated in methanol for 20 seconds and then immersed in transfer buffer. The SDS-PAGE gel was then placed on top, the two sandwiched in blotting paper (Whatman paper, 3M), placed within a transferred cassette and 120mA current was applied across the cassette for 12 hours.

The membrane was first blocked in PBS/TWEEN containing 10% Marvel for 1 hour at room temperature with continuous rocking. The membrane was then transferred to

PBS/TWEEN containing 1% Marvel, and the primary antibody (rabbit anti-bcl2, 50E3, Cell Signalling Technologies) was added at 1:1000 before incubation for 3 hours at room temperature with continuous rocking. The membrane was next washed 3 times in PBS/TWEEN (10 minutes per wash, with continuous rocking) and then placed in PBS/TWEEN containing 0.1% Marvel; secondary antibody was then added (HRP-conjugated anti-rabbit, Cell Signalling Technologies) at 1:3000 and the membrane incubated for 1 hour at room temperature with continuous rocking. The membrane was then washed 3 times in PBS/TWEEN (10 minutes per wash, with continuous rocking) before electrochemiluminescence ECL solutions 1 and 2 were added at a ratio of 1:1, and the blot transferred to a light-proof cassette. In a darkroom, CL-XPosure film (Thermo Scientific) was then inserted into the cassette and exposed for a variety of times, before being developed using an automated developer.

Statistical analysis

Statistical analysis was performed using Graphpad Prism version 6. Unless stated otherwise a p value of <0.05 was considered statistically significant. Cell phenotyping data is expressed as median values and co-culture readouts as mean values, unless otherwise stated.

Chapter 3 – Th_{17} cells in multiple myeloma

Introduction

As reviewed in detail in Chapter 1, the role played by Th_{17} cells in cancer remains the subject of debate. Considerable discrepancy exists between published experimental findings and a range of reasons might account for this. Firstly, there are likely to be true differences in the prevalence and biological roles of these T cells between different types of cancer; clearly their contribution is potentially complex, with both pro- and anti-tumour roles ascribed. However, in addition, variations in experimental approach may contribute to the disparate findings. For example, these cells are defined by secretion of IL-17, however since this is often a difficult property to assay, a range of surrogate markers have been used to identify the cells, ranging from expression of their signature transcription factor, RORC, to cell surface markers said to be characteristic of the subset; IL-17 is also sometimes quantified and a T cell source inferred[258-260].

This chapter of work aims firstly to develop a robust methodology for identification, quantitation and phenotypic assessment of Th_{17} cells in peripheral blood and bone marrow samples, and then to employ this protocol to assess the prevalence, phenotype and function of Th_{17} cells in healthy donors and patients with plasma cell dyscrasia.

Materials and Methods

Patients and normal donors

Samples from newly diagnosed patients were taken prior to any treatment of disease; samples from patients with relapsed disease were taken prior to any treatment of relapse. All patients on maintenance therapy were taking lenalidomide, with or without dexamethasone. Donor characteristics are shown in Table 3.1.

Luminex assay

A Luminex assay (R&D Systems: Human HS Cytokine B Pre-Mixed Mag Luminex Performance Assay) was employed for cytokine assays. Microparticles used are shown in Table 3.2.

	PB donors	Apheresis donations	MGUS & AMM	Newly diagnosed MM	MM on maintenance	MM in remission	Relapsed MM
N	22	10	14	5	5	4	4
Gender male:female	10:12		8:6	3:2	2:3	2:2	2:2
Age (range)	46 (22-79)		74 (46-90)	66 (55-81)	71 (58-88)	68 (59-74)	68 (63-75)
Paraprotein type							
IgG			12	4	4	4	3
IgA			1	0	1	0	0
IgM			0	0	0	0	0
LC			1	0	0	0	1
NS			0	1	0	0	0
Paraprotein level /gl^{-1} (range)			8 (0-38)	16 (0-58	4 (0-10)	1 (0-5)	18 (10-27)

Table 3.1: Donor characteristics. PB: peripheral blood, MGUS: monoclonal gammopathy of uncertain significance, AMM: asymptomatic myeloma, LC: light chain disease, NS: non-secretory disease

Analyte	Sensitivity /$pgml^{-1}$	High standard value /$pgml^{-1}$	Microparticle region
IFNγ	0.029	260	30
IL-1β	0.146	4,240	20
IL-2	0.386	4,850	19
IL-6	0.135	2,650	25
IL-17A	0.349	10,370	36
IL-17F	4.54	36,750	37
IL-22	0.529	6,700	38
IL-23	9.52	70,000	39
TNFα	0.551	4,920	12

Table 3.2: Luminex microparticles used for cytokine assays on serum samples.

Results

Quantitation and phenotyping of Th_{17} cells in normal individuals – stimulation of cytokine secretion

Since cytokine secretion must be induced to identify the Th_{17} population, IL-17 production by $CD4^+$ T lymphocytes was assayed after a range of polyclonal stimulation modalities: PMA and ION, anti-CD3/28 MACSiBeads and Cytostim. As reported in the published literature, Th_{17} cells represented only a very small proportion of T cells, often less than 1%; representative dot plots are shown in Figure 3.1. The combination of PMA and ION (PMA/ION) was optimal for stimulation of IL-17 secretion, however, PMA also resulted in loss of CD4 from the cell surface in a time-dependent manner (Figure 3.2). Use of lower concentrations impaired cytokine secretion but did not satisfactorily abrogate loss of CD4 (data not shown). Phosphorylation and modulation of cell surface expression of CD4 in response to PMA has been reported previously[261] and result in internalisation and lysosomal degradation of the protein[262]. CD8 was also affected (Figure 3.2). Addition of fluorochrome-conjugated anti-CD4 antibody at the intracellular staining stage reliably identified cells having internalised CD4 in response to PMA and this protocol was therefore adopted for the remainder of the project.

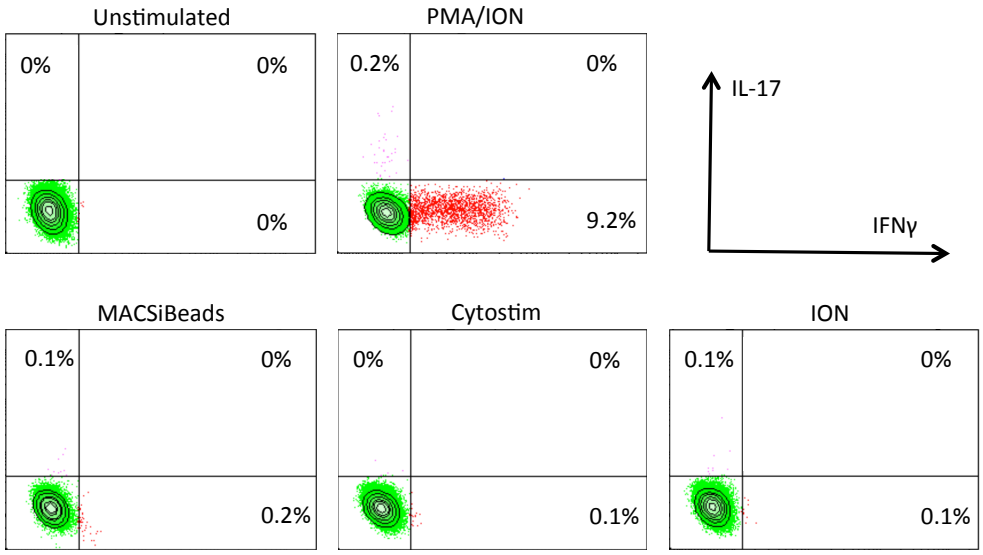


Figure 3.1. Representative dot plots of IL-17 and IFNγ secretion by CD4⁺ T cells. PBMC from a healthy donor were stimulated for 6 hours with PMA and ION, ION alone, anti-CD-3/28 MACSiBeads or Cytostim. All cells were treated with Golgiplug for the last 4 hours of stimulation. Percentages of total CD4⁺ cells shown.

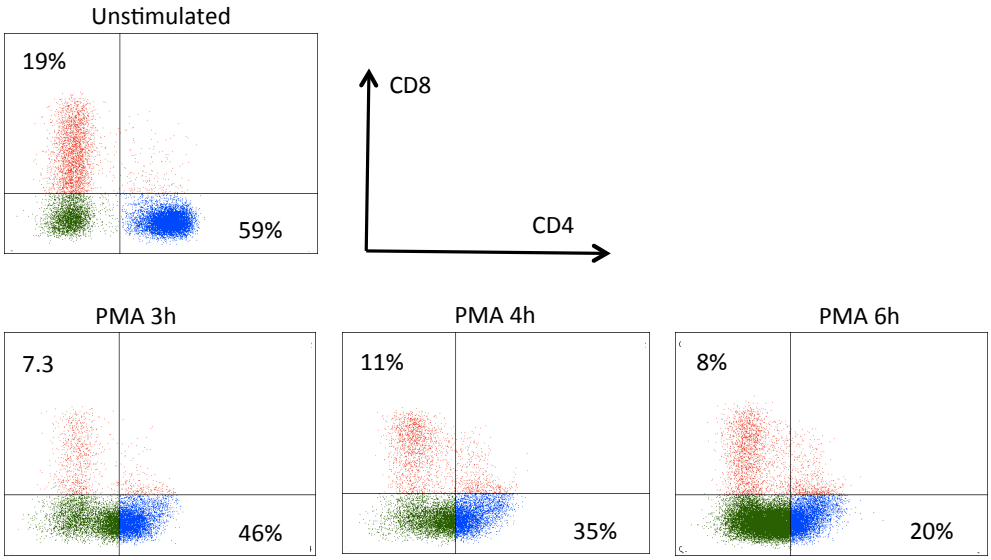


Figure 3.2. Representative dot plots of surface CD4 and CD8 staining on CD3⁺ cells after incubation of unselected PBMC with PMA for 6, 4 or 3 hours at 25ng/ml. All conditions were treated with Golgiplug for the last 3 hours of incubation. Percentages are the proportion of CD3⁺ cells expressing CD4 and CD8 on their surface.

Circulating Th_{17} cells in healthy donors

PBMC were isolated from healthy volunteers (n=22, median age 46.9 years) and stained to quantify and phenotype $CD4^+$ T cell subsets, as described above. The median frequency of $CD4^+$ T cell subsets was as follows (Figure 3.3): Th_{17} cells: 0.21% of CD4 cells (range 0-2.94), Th_{17-1}: 0.04% (0-0.85), Th_1: 11.39% (0.08-49.67).

$CD4^+$ T cell subsets from peripheral blood apheresis cones (n=10) were also analysed and showed: Th_{17}: 0.64% (0.20-2.852), Th_{17-1}: 0.13% (0-1.06), Th_1: 28.27% (10.99-43.42). When peripheral blood (PB) and apheresis cone (AC) samples were compared (Figure 3.4), there was no significant difference between the frequency of any measured T cell subsets in peripheral blood donors and apheresis cone cells (unpaired student t test, p=NS). These results indicate that apheresis cones are a suitable substitute for peripheral blood samples, and the two sources of mononuclear cells were thereafter considered equivalent and apheresis cones were used where large numbers of cells were required.

There was a small, but significant, negative correlation between donor age and Th_{17} cells as a percentage of $CD4^+$ cells (r^2=0.19, p=0.04, Figure 3.5A). However, there was no significant correlation between donor age and Th_1 (Figure 3.5B) or Th_{17-1} cell prevalence (Figure 3.5C). Since MM is predominantly a disease of the elderly, these results indicate age-matched healthy donors are required as a control for assessing Th_{17} in patients with MM.

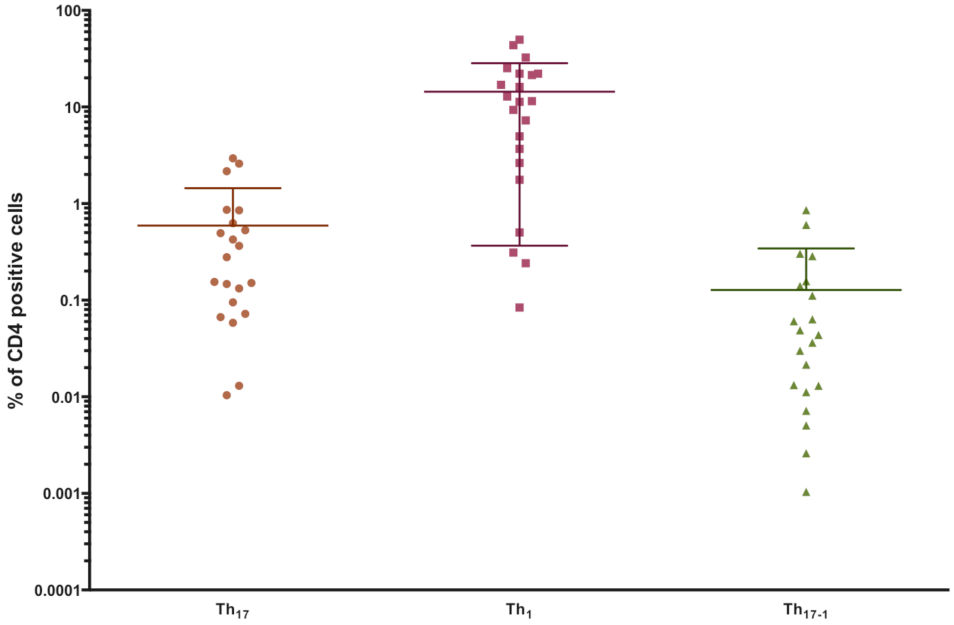


Figure 3.3. Th_{17}, Th_1 and Th_{17-1} subsets as a percentage of $CD4^+$ cells in peripheral blood samples from healthy donors. Horizontal lines show mean and standard deviation (which crosses the x axis for the Th_{17} and Th_{17-1} groups). N=22

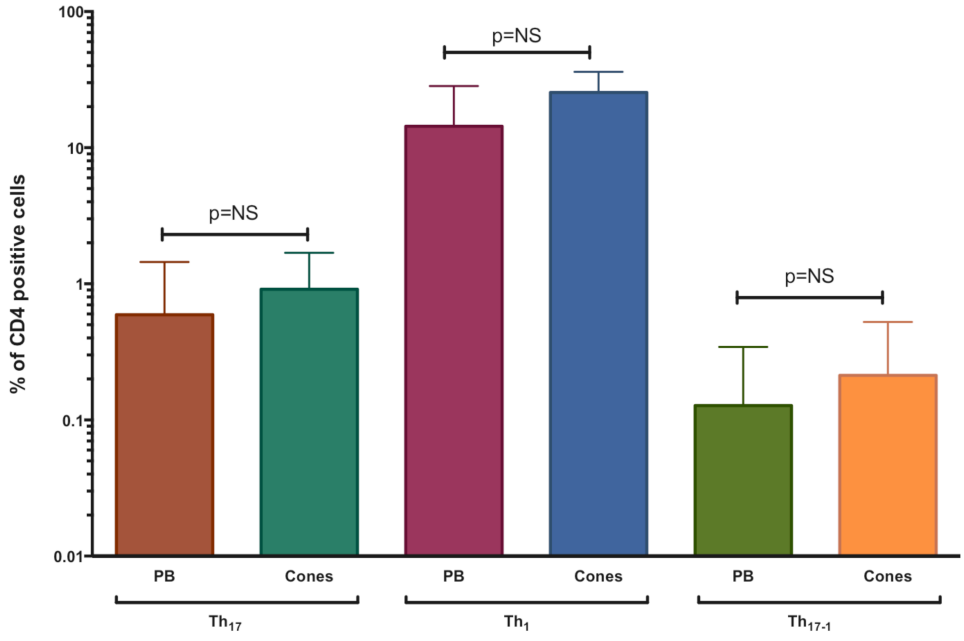


Figure 3.4. Comparison of T cell subsets in peripheral blood (PB) of healthy donors and apheresis cone derived cells. Horizontal lines show mean and standard deviation. There was no difference in the numbers of Th_{17}, Th_1 or Th_{17-1} cells between PB and Cones (unpaired t test, p=NS). There was also no difference in the expression of CD161, CCR4 of CCR6 on Th_{17} cells from PB or Cones (data not shown). Healthy donors n=22, cones n=10.

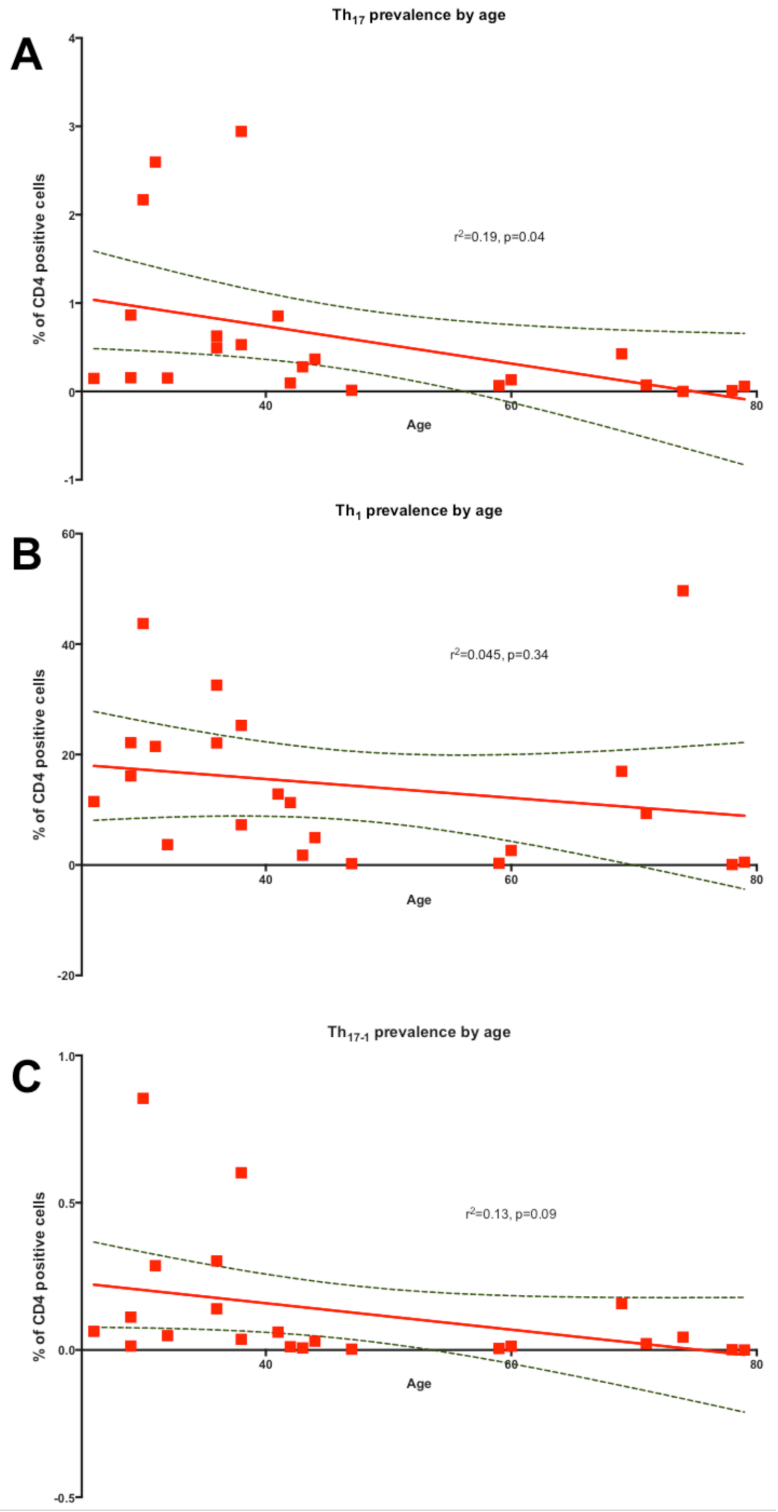


Figure 3.5. Th_{17} (panel A), Th_1 (B) and $Th_{17\text{-}1}$ (C) cells as a percentage of all $CD4^+$ cells as a function of donor age. The red lines show linear regression fits and the dotted lines show the 95% confidence intervals. There was a small but significant correlation (r^2=0.19, p=0.04) between age and percentage of Th_{17} cells, but no significant correlation between age and percentage of Th_1 cells (r^2=0.045, p=NS), or $Th_{17\text{-}1}$ cells (r^2=0.13, p=0.09). N=22.

Th_{17} cell phenotype in health

Since retinoic acid-related orphan receptor gamma t (RORC) is the signature transcription factor for the Th_{17} lineage, it was assumed that this would represent a sensitive and specific marker of the population by flow cytometry. Unfortunately only two commercial antibodies against RORC (the human homologue of RORγt) are currently available. Despite testing with a range of staining protocols and reagents these antibodies were found to be nonspecific, staining almost all $CD4^+$ cells, with no significant difference seen in either percentage positivity or median fluorescence intensity between those $CD4^+$ cells producing IL-17 and those not (data not shown). The cell surface and functional properties of Th_{17} cells were therefore investigated, in order to attempt to define a phenotypic profile for this rare set of T cells.

Th_{17} cells from PB and AC samples were examined for their expression of cell surface proteins (CCR6, CD161, CD127, CXCR4, CXCR3, CCR7, CCR4, CD25), cytokines (IL-17, IFNγ, IL-10) and transcription factors (RORγt, T-bet, FoxP3).

Representative flow cytometry plots of CD161 expression on T cell subsets is shown in Figure 3.6, collated data for multiple donors in Figure 3.7. Although there was a significant difference in CD161 expression between Th_{17} and Th_1 cells (p=0.0001) and between Th_{17} cells and $CD4^+IL\text{-}17^-IFNg^-$*FoxP3*$^-$ T cells (p=0.0001), there was no difference between Th_{17} cells and either $Th_{17\text{-}1}$ or T_{Reg} cells (p=NS). Furthermore, CD161 was expressed to some extent on all subsets (by comparison to isotype control staining), with wide variation in the degree of expression in all subsets. Consequently, gating on $CD4^+CD161^+$ cells yielded a population considerably enriched for IL-17-producing lymphocytes (5.1% *vs.* 1.2% in the un-gated population for the donor shown), but which still contained Th_1 cells, as well as $CD4^+$ cells producing neither cytokine (Figure 3.8).

Expression of CCR6 was seen on a median of 93.4% of Th_{17} cells, although once again expression was noted in a subpopulation of Th_1 cells (Figures 3.9 & 3.10); CCR4 expression was recorded in median 80.6% of Th_{17} cells, but expression of the marker was found to be even less specific for the subset (Figure 3.11). As with CD161, neither of these markers was therefore suitable as a surface marker for Th_{17} cells in isolation. The remaining markers examined were found to be minimally expressed on Th_{17} cells (data not shown).

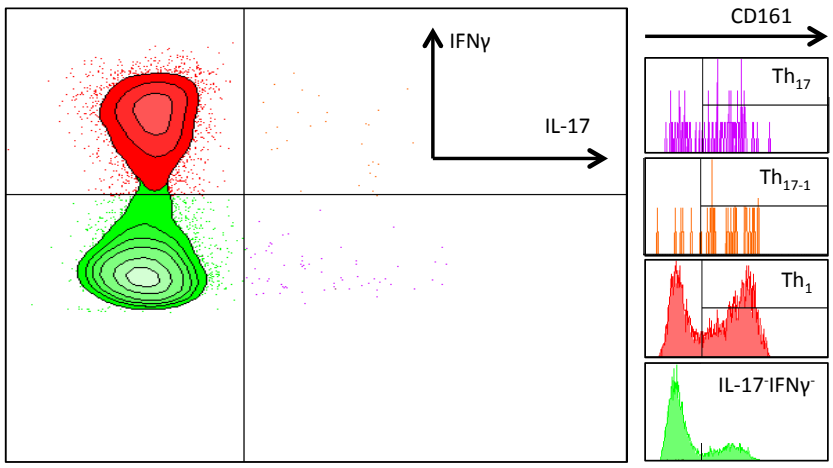


Figure 3.6. Representative dot plots of CD161 expression in T cell subsets in a healthy donor. The left panel shows IL-17 and IFNγ secretion in $CD4^+$ cells after stimulation with PMA & ION and Golgiplug. The 4 panels on the right show CD161 expression on Th_{17}, Th_{17-1}, Th_1 and $IL\text{-}17^-IFN\gamma^-$ $CD4^+$ cells.

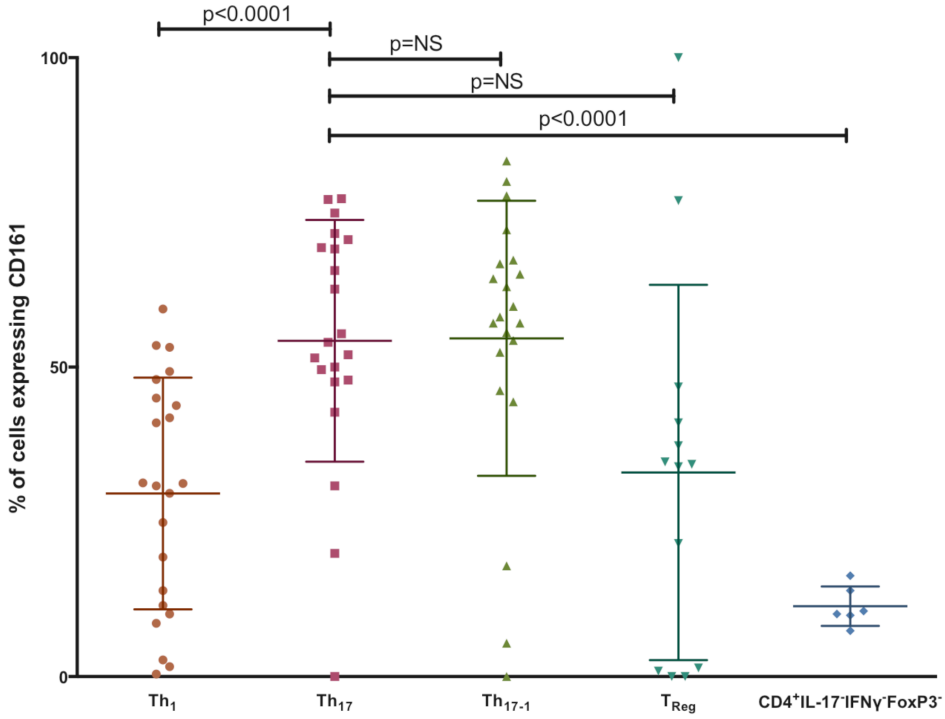


Figure 3.7. Expression of CD161 on Th_{17}, Th_1, Th_{17-1} and T_{reg} ($CD4^+CD25^+FoxP3^+$) cells in healthy donors. CD161 expression on $CD4^+IL\text{-}17^-IFNg^-FoxP3^-$ cells is shown for comparison (n=6). Median percentages of cells expressing CD161 were: Th_{17}: 54.0% (range 0-77.2%), Th_1: 31.0% (0.4-59.4%), Th_{17-1}: 58.1% (0-83.3%). T_{Reg} ($CD4^+CD25^+FoxP3^+$): 34.3% (0-100%), $CD4^+IL\text{-}17^-IFNg^-FoxP3^-$: 10.35% (7.4-16.3%). Plots show mean and standard deviation. p values are two-tailed paired t tests. N=22.

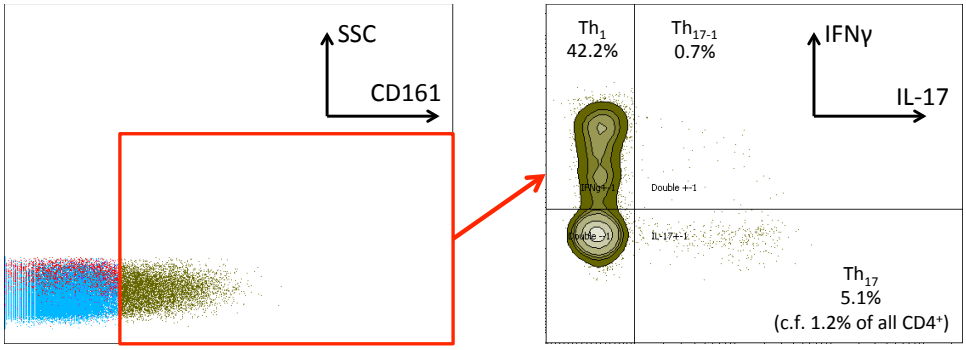


Figure 3.8. Representative dot plots of cytokine production in CD161⁺ cells. The left hand plot shows CD161 *vs.* side scatter, with the red square indicating a gate on CD161+ cells (gate set according to corresponding isotype control). The right plot shows IL-17 and IFNγ production in the gated population.

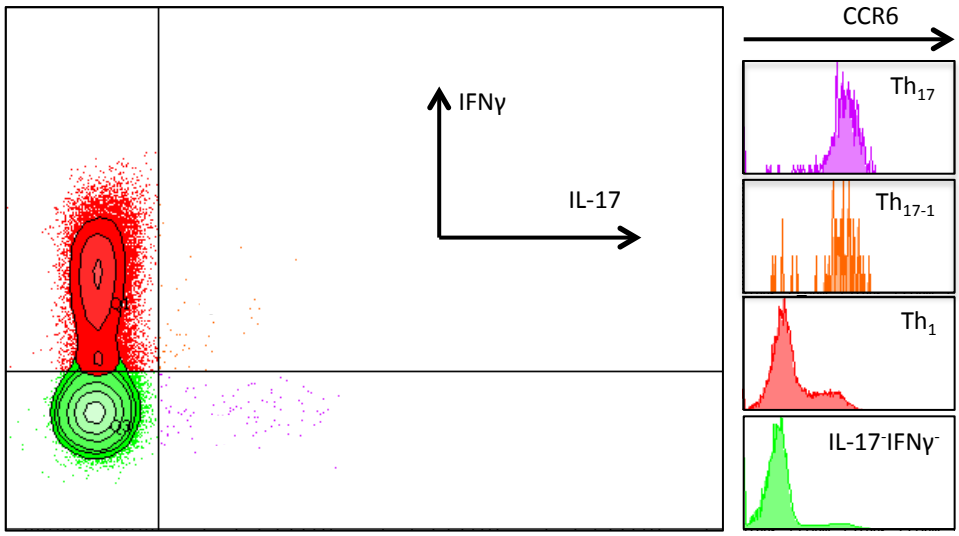


Figure 3.9. Representative dot plots of CCR6 expression in T cell subsets in a healthy donor. The left panel shows IL-17 and IFNγ secretion in $CD4^+$ cells after stimulation with PMA & ION and Golgiplug. The 4 panels on the right show CCR6 expression on Th_{17}, Th_{17-1}, Th_1 and $IL\text{-}17^-IFN\gamma^-$ $CD4^+$ cells.

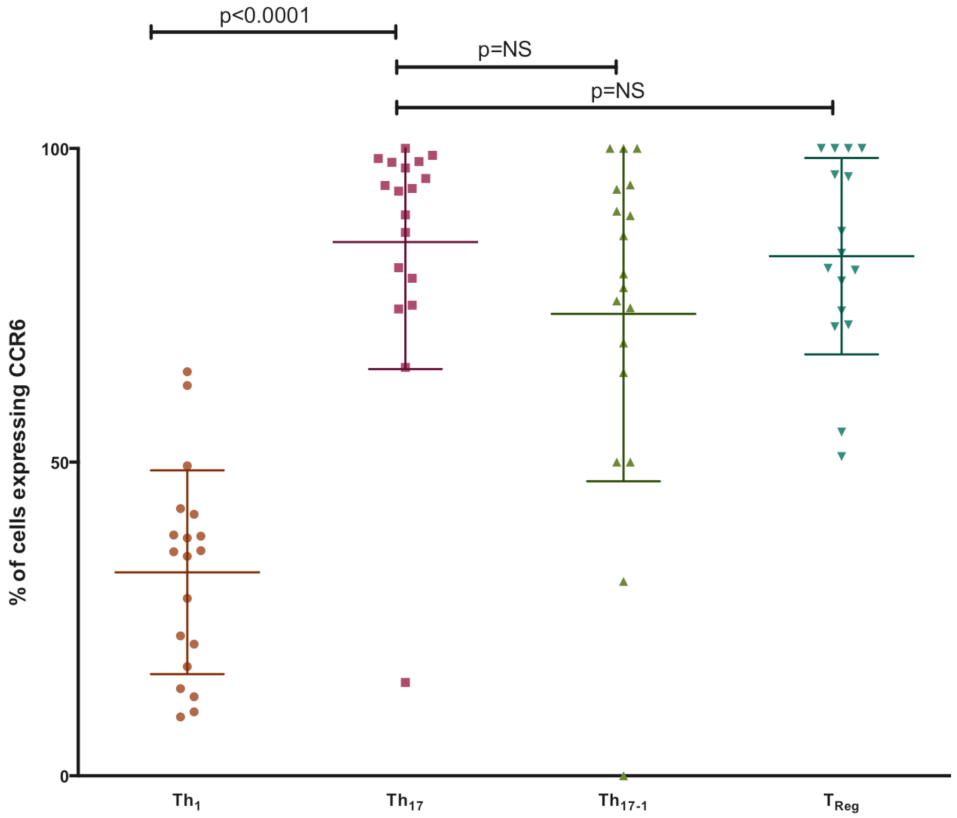


Figure 3.10. CCR6 expression on T cell subsets in healthy donors. Mean and standard deviations shown. P values are for two-tailed paired t tests. N=18.

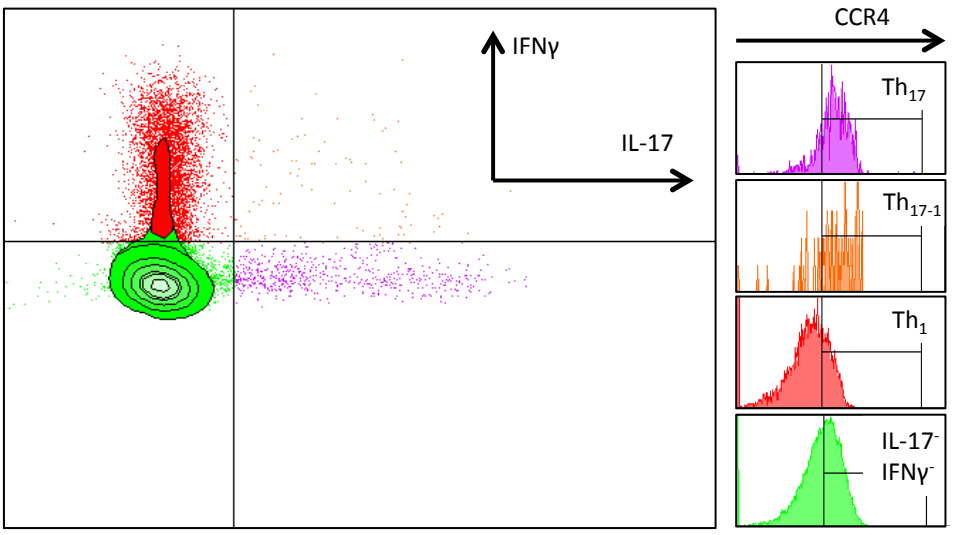


Figure 3.11. Representative dot plots of CCR4 expression in T cell subsets in a healthy donor. The left panel shows IL-17 and IFNγ secretion in $CD4^+$ cells after stimulation with PMA & ION and Golgiplug. The 4 panels on the right show CCR4 expression on Th_{17}, Th_{17-1}, Th_1 and $IL\text{-}17^-IFN\gamma^-$ $CD4^+$ cells.

These results indicated that none of the cell surface markers used individually were sufficiently selective or specific for Th_{17} cells to be used to reliably identify the population. Staining for combinations of these proteins was therefore evaluated. Figure 3.12 shows a representative example of this gating strategy. Here, PBMC from a healthy donor were stimulated as previously, and the production of IL-17 and IFNγ by live $CD4^+$ cells is shown in the left panel – 0.3% of this population produced IL-17. In the right panel an alternative gating is shown whereby the live $CD4^+CD161^+CCR6^+$ population was assessed for production of IL-17 and IFNγ – 1.4% of this population produced IL-17. Therefore, combination of markers in this way resulted in an approximately five-fold enrichment for Th_{17} cells, although an increase in the proportion of cells producing IFNγ was also seen and the vast majority of cells produced neither cytokine. Addition of CCR4 to the gating strategy did not improve purity. For these reasons, it was not possible to use staining of the available surface antigens to define Th_{17} cells, and for the remainder of this thesis Th_{17} cells are therefore identified by their determining characteristic – production of IL-17 (i.e. $CD4^+$IL-17^+ cells).

In light of the correlation seen between Th_{17} prevalence and age, the interplay of age and expression of CD161 and CCR6 was examined; linear regression analysis indicated no significant correlation in both cases (data not shown).

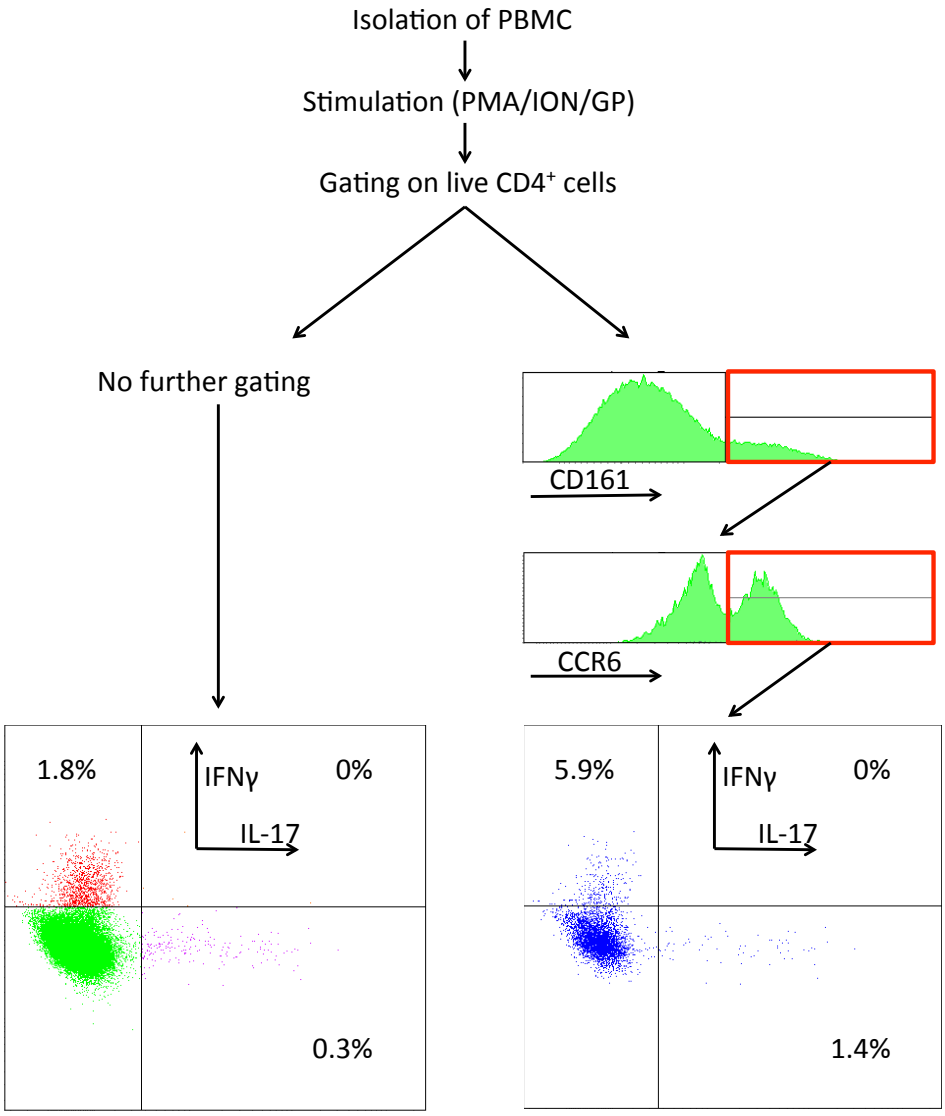


Figure 3.12. Representative example of two gating strategies for identification of Th_{17} cells. PBMC from a healthy donor was stimulated as described and flow cytometric gates were used to identify live $CD4^+$ cells. In the left panel, the production of IL-17 and IFNγ by these cells in shown. In the right panel, the $CD4^+$ cells were further selected for expression of both CD161 and CCR6, and production of IL-17 and IFNγ by this subpopulation is then shown in the bottom right panel.

Quantitation of Th_{17} cells in the peripheral blood of myeloma patients

Th_{17} cells ($CD4^+IL\text{-}17^+$) were enumerated in the peripheral blood of patients with MM and MGUS and compared to healthy controls; a significant reduction in Th_{17} cells was noted in the patient group compared to healthy donors: 0.39% (range 0-2.94%) *vs.* 0.69% (range 0-1.86%), p=0.0331; Figure 3.13. This is in contradiction to published data also comparing with non-age-matched healthy donors[74]. In contrast, Th_1 cells and $Th_{17\text{-}1}$ cells were not significantly different between health and disease (p=NS, student t-test, data not shown).

Impact of Age

Since donor age had been shown to correlate inversely with prevalence of Th_{17} cells in the peripheral blood of healthy donors, comparisons were next made between patients with plasma cell dycrasias (median age 73, range 46-90) and a cohort of age-matched healthy donors (median age 70 years, range 47-79). In this age-matched cohort, 0.1% of $CD4^+$ T cells produced IL-17 – significantly less than the 0.39% seen in the patient samples (p=0.05, Figure 3.14)

The effect of age on prevalence of Th_{17} cells in the peripheral blood of patients with plasma cell dyscrasias was next examined (Figure 3.15). Surprisingly, unlike in the case of healthy donors, the age of patients did not correlate with the prevalence of Th_{17} cells in their peripheral blood (r^2=0.0005, p=0.9009); when the impact of age was assessed in the age-matched healthy donor cohort (shown in blue on Figure 3.15), there was no significant correlation (r^2=0.0001, p=0.9766).

These data indicate that although Th_{17} cell frequency was lower in peripheral blood of MM patients than in an unselected healthy donor population, when a more appropriate age-matched healthy donor cohort was used, levels were actually found to be higher in MM than in health. An age-matched healthy donor cohort was therefore included for the proceeding work.

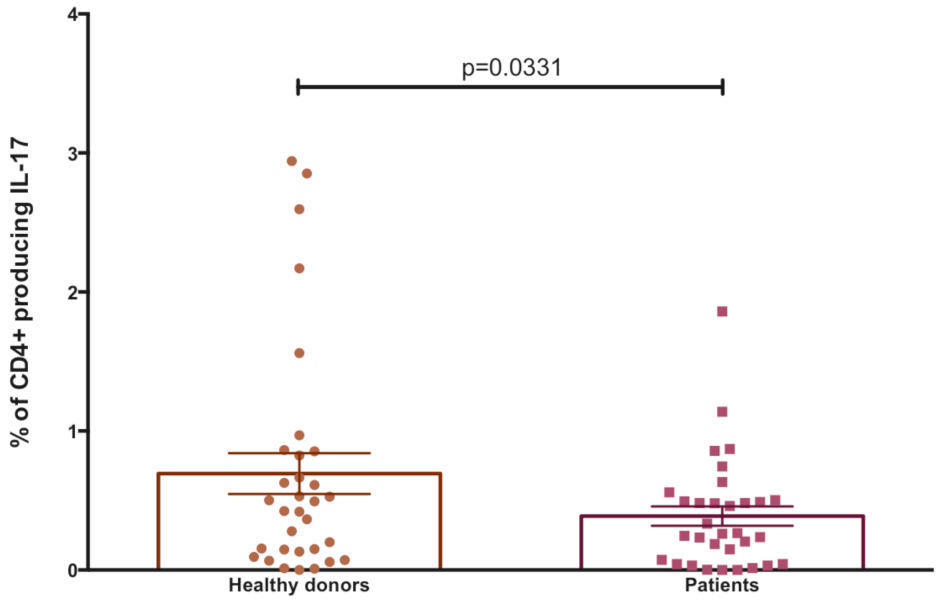


Figure 3.13. Th_{17} cell frequency in the peripheral blood of healthy donors and patients with plasma cell dyscrasias. Healthy donors: n=32, Patients: n=32. Mean and standard errors of the mean shown. Two-tailed student t-test shown.

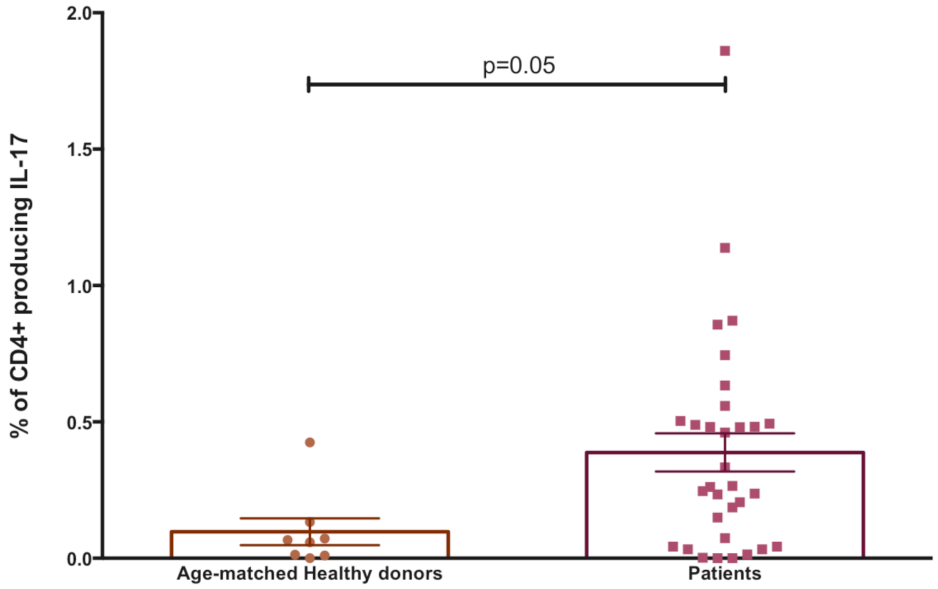


Figure 3.14. Th_{17} cell frequency in the peripheral blood of age-matched healthy donors and patients with plasma cell dyscrasias. Healthy donors: n=8, Patients: n=32. Mean and standard errors of the mean shown. Two-tailed student t-test shown.

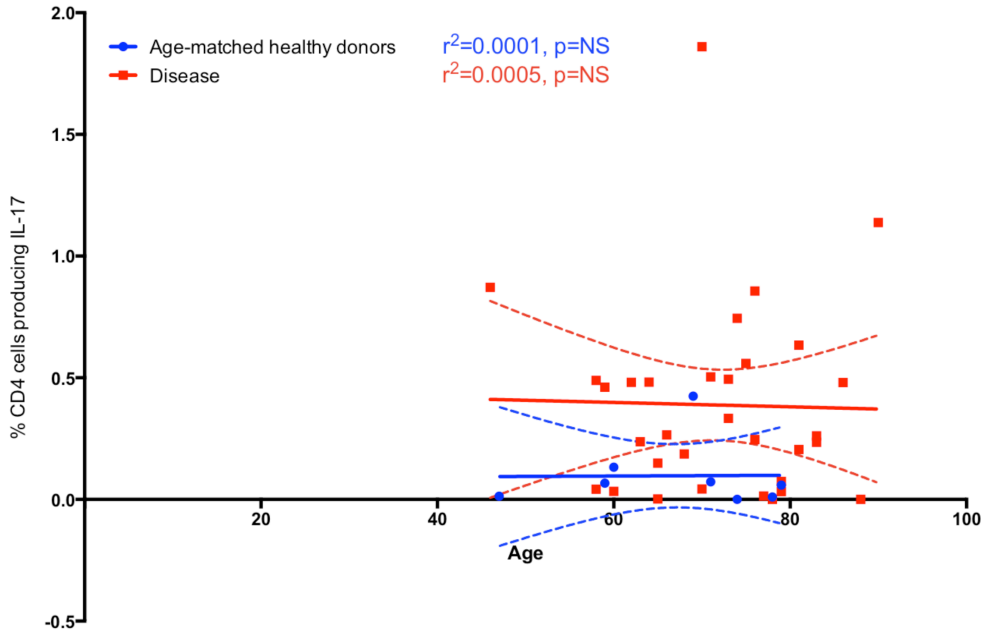


Figure 3.15. Th_{17} cell frequency as a percentage of all $CD4^+$ cells in the peripheral blood of patients with plasma cell dyscrasias (red, n=32), and age-matched healthy controls (blue, n=8), plotted as a function of age. The solid lines show linear regression fits, and the dotted lines 95% confidence intervals for the linear regressions.

Subdivision by disease stage

When patients were subdivided by disease category (for disease category classifications, see Appendix 1), differences in the prevalence of IL-17- and IFNγ-secreting $CD4^+$ T cells were noted between stages of disease (Figures 3.16-18). Interestingly, although all patient groups had higher levels of Th_{17} cells than age-matched controls, the highest levels were seen in patients with pre-symptomatic disease (MGUS and asymptomatic myeloma), and the lowest in those with 'active' disease (newly diagnosed or relapsed).

When analysed by disease classification, $Th_{17\text{-}1}$ cells were significantly increased in patients with MGUS & asymptomatic MM compared to age-matched healthy controls; there were no other significant differences (Figure 3.17). Th_1 cells were increased in patients at relapse compared to healthy donors (age-matched or of all ages), newly diagnosed myeloma and patients in remission, but no other significant differences between groups were found (Figure 3.18).

These data show differences in the relative prevalences of Th_{17} and Th_1 cells, with Th_{17} cells being raised in MGUS/AMM and falling to the lowest levels in relapsed disease where Th_1 cells in contrast reached the highest levels in relapsed MM. These findings are novel and indicate that the loss of Th17 cells with disease progression is not merely a consequence of disease- or treatment-related lymphopenia but instead suggests a specific depletion of the T cell subset in active disease.

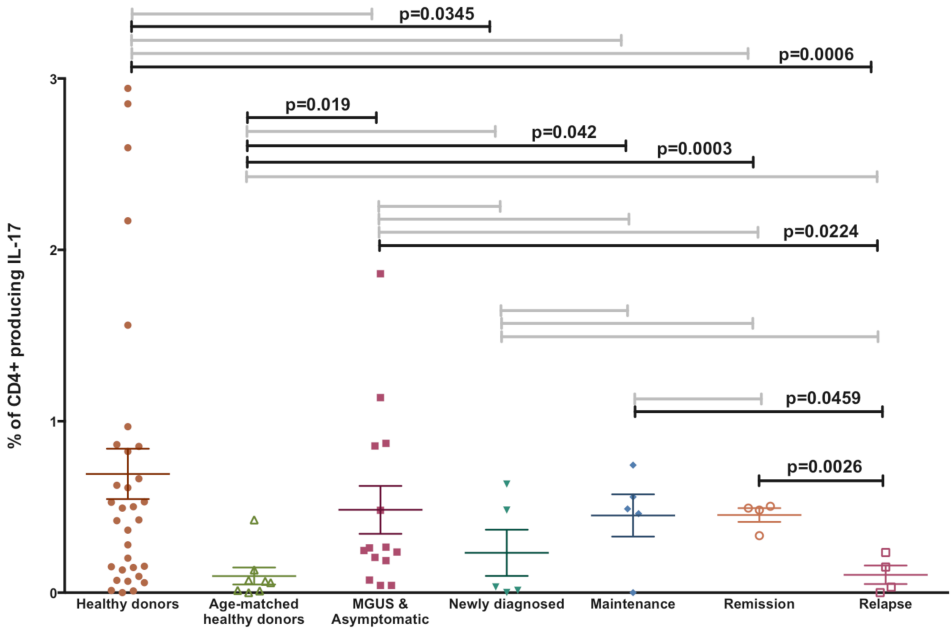


Figure 3.16. Th_{17} cell frequency in the peripheral blood of healthy donors and patients. Mean frequencies were as follows: healthy donors of all ages: 0.69% (range 0-2.94%, n=32), age-matched healthy donors: 0.10% (0-0.42%, n=8), MGUS & asymptomatic MM: 0.48% (0.04-1.86%, n=14), newly-diagnosed MM: 0.233% (0.002-0.63%, n=5), MM on maintenance therapy: 0.45% (0-0.74%, n=5), MM in remission: 0.45% (0.33-0.50%, n=4), relapsed MM: 0.10% (0-0.23%, n=4). Mean and standard errors of the mean shown. P values shown are for unpaired student t tests with Welch's correction; where not shown, p=NS.

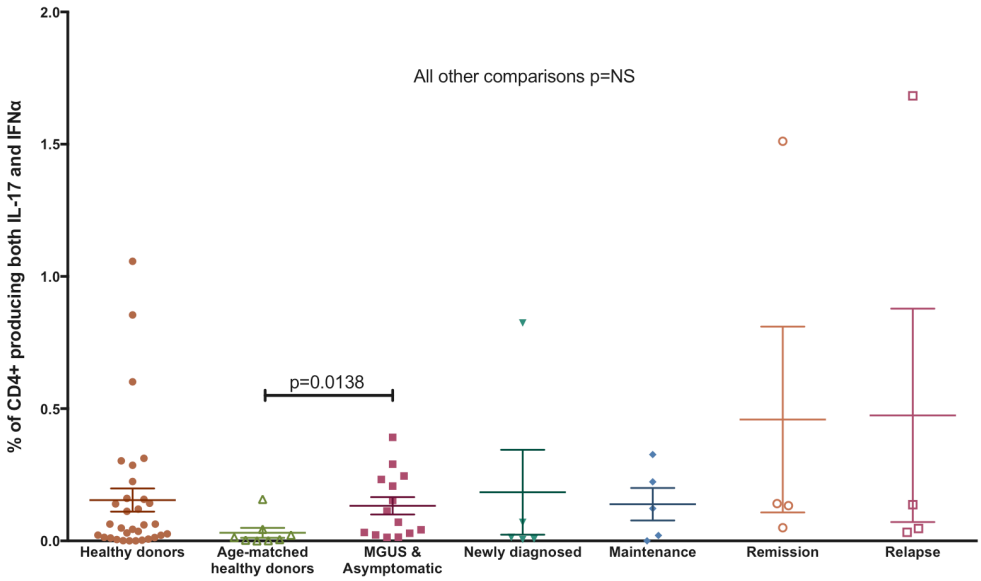


Figure 3.17. Th_{17-1} cell frequency in the peripheral blood of healthy donors and patients. Healthy donors: n=32, Age-matched healthy donors: n=8, MGUS & asymptomatic MM: n=14, newly diagnosed MM n=5, MM on maintenance therapy n=5, remission n=4, relapse n=4. Mean and standard errors of the mean shown. P values shown are for unpaired student t tests with Welch's correction.

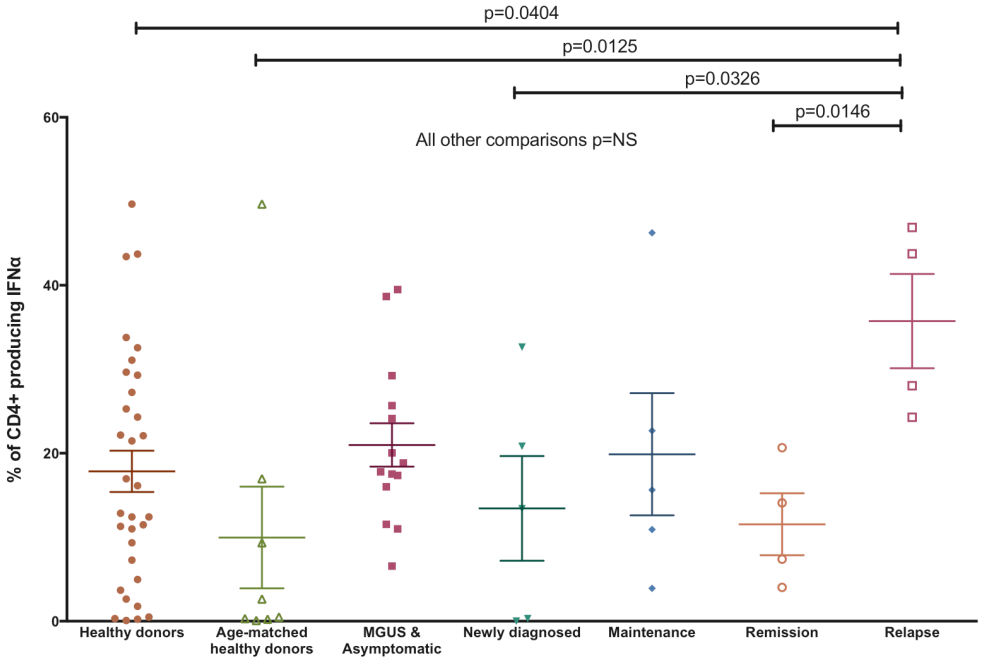


Figure 3.18. Th_1 cell frequency in the peripheral blood of healthy donors and patients. Healthy donors: n=32, Age-matched healthy donors: n=8, MGUS & asymptomatic MM: n=14, newly diagnosed MM n=5, MM on maintenance therapy n=5, remission n=4, relapse n=4. Mean and standard errors of the mean shown. P values shown are for unpaired student t tests with Welch's correction.

Quantitation of Th_{17} cells in bone marrow of myeloma patients

Bone marrow samples from age-matched healthy donors and patients with plasma cell dyscrasias were examined for Th_{17} cell frequency; figure 3.19 shows representative flow cytometry dot plots from analysis of the bone marrow of a healthy donor and a patient with newly-diagnosed MM. Although a rare subset in all marrow samples examined, there was a significantly higher frequency of Th_{17} cells in patients than in healthy donors (mean 0.69% *vs.* 0.12%, p=0.0185, Figure 3.20A). Th_1 cells were increased in patients compared to healthy donors (mean 22.24% *vs.* 6.28%, p=0.0426, Figure 3.20B), whereas $Th_{17\text{-}1}$ cells were not significantly different between the two populations (mean 0.45% *vs.* 0.14%, p=NS, Figure 3.20C). The ratio of Th_{17}:Th_1 cells was not significantly different between the groups (Figure 3.20D).

These results indicate that Th_{17} cells are expanded in the bone marrow as well as the peripheral blood of patients with plasma cell dyscrasias. Since the bone marrow is the tumour bed in MM this is further evidence to suggest the expanded Th_{17} cells derive from interactions between the tumour and the host immune system. Th_1 cells were also expanded in the bone marrow, but since the number of samples was insufficient for meaningful subdivision of patients by disease stage, it is not clear whether this reflects an increase in Th_1 cells in association with disease relapse, as was seen in the peripheral blood.

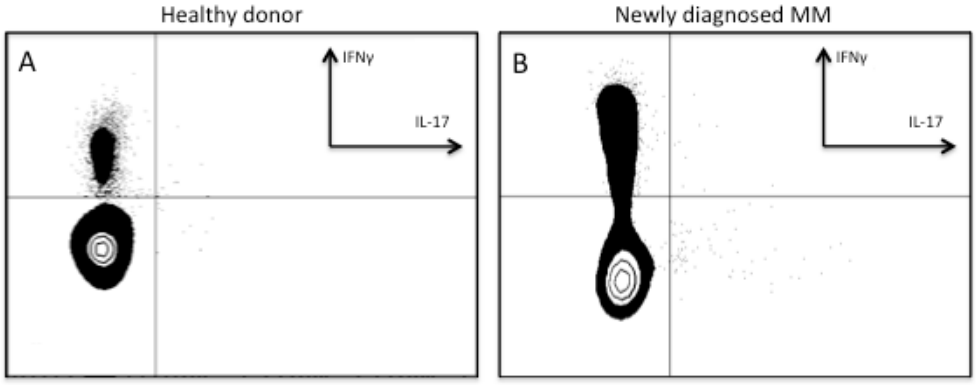


Figure 3.19. Representative dot plots of IL-17 and IFNγ expression in $CD4^+$ cells from bone marrow of a healthy donor (A) and a patient with newly diagnosed MM (B).

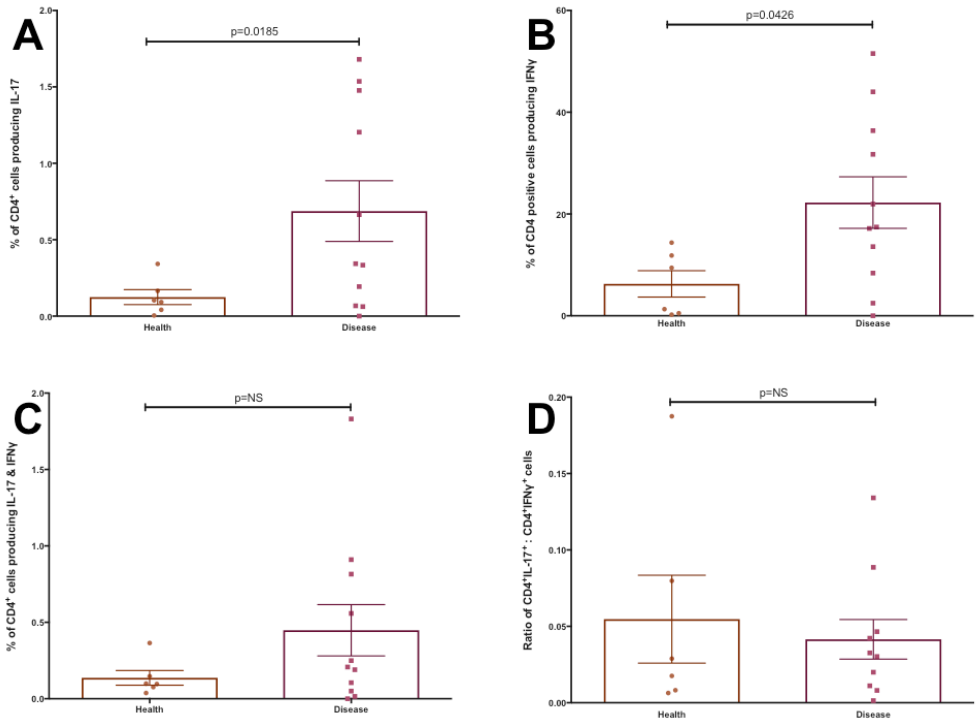


Figure 3.20. Th_{17} (panel A), Th_1 (B), $Th_{17\text{-}1}$ (C) cell and Th_{17}:Th_1 ratio (D) in the bone marrow of healthy donors and patients with plasma cell dyscrasias. Healthy donors: n=6, Patients: n=11. Mean and standard errors of the mean shown. Two-tailed student t-tests shown.

Impact of disease-specific variables

A number of disease-specific variables were next examined for their effect on the frequency of Th_{17} cells. Patients whose paraprotein was of lambda isotype were found to have a higher frequency of Th_{17} cells than those with a kappa paraprotein (0.59% *vs.* 0.28%, p=0.0301). Those with an IgG heavy chain had a lower frequency of Th_{17} cells than those with an IgA paraprotein (0.25% *vs.* 0.56%, p=0.02) or light chain disease (0.69%, p=0.02). Level of paraprotein, number of prior lines of therapy, previous exposure to thalidomide, lenalidomide or bortezomib, prior stem cell transplant, percentage of normal or malignant plasma cells in the bone marrow, serum creatinine, myeloma plasma cell phenotype (CD56, CD27 and CD45 expression) and myeloma cytogenetic and molecular abnormalities (del 13q and IgH rearrangement) were uncorrelated with Th_{17} frequency.

Caution is warranted in making inferences from these results, since the numbers of patients falling into some subgroups was small, and multivariate analysis was therefore not undertaken. Results for these analyses are shown in Appendix 5.

Functional phenotype of Th_{17} cells in MM patients – IL-17 production

The mean fluorescence intensity (MFI) of Th_{17} cells for IL-17, which can be considered a surrogate marker of IL-17 production, was compared across healthy and disease states (Figure 3.21A). Since MFI varies between experiments according to the flow cytometer setup and calibration, it was necessary to normalise data in order to compare samples analysed at different points throughout the project. In order to achieve this the MFI of Th_{17} cells for IL-17 was divided by the MFI of IL-17-negative $CD4^+$ lymphocytes for IL-17, measured in the same sample.

When patient peripheral blood Th_{17} cells were compared to healthy donor Th_{17} cells they were found to have significantly lower expression (student t-test, p=0.0181). However, when the MFI of Th_1 cells for IFNγ (similarly normalised to $IFN\gamma^-$ $CD4^+$ cells) was compared between health and disease, this was also found to be reduced in patients with plasma cell dyscrasias compared to healthy donors (Figure 3.21B). When BM samples were compared, no significant difference was seen in MFI of either Th_{17} cells for IL-17 or Th_1

cells for IFNγ between healthy donors and those with plasma cell dyscrasia (data not shown).

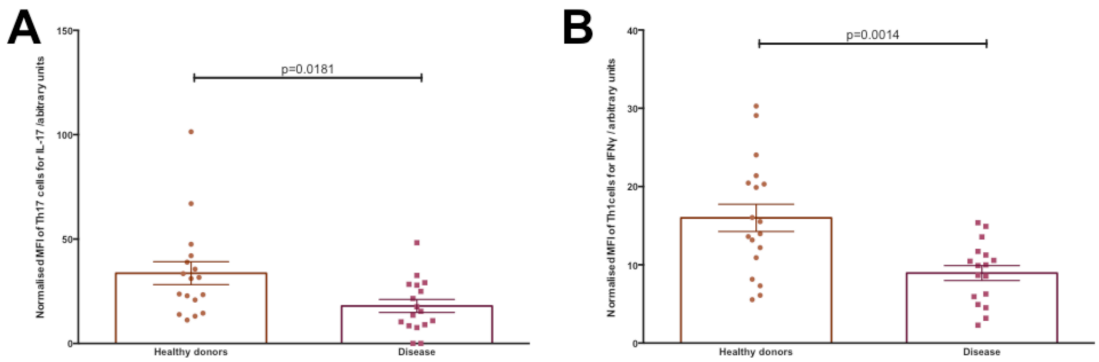


Figure 3.21. Normalised mean fluorescence intensity of Th_{17} cells for IL-17 (panel A) and Th_1 cells for IFNγ (panel B) in healthy donors and patients with MM. P values are for unpaired two-tailed t tests. N=17 each.

These data suggest that Th_{17} cells in MM are abnormal not only in frequency, but also in their ability to produce IL-17 upon stimulation. In order to further examine this issue, concentrations of a range of cytokines were next examined in serum samples from patients with multiple myeloma and age-matched healthy donors, using a Luminex® assay.

Firstly, cytokines produced by Th_{17} cells were assessed: IL-17A, IL-17F, IL-22, IL-23 and TNFα. Median levels of IL-17A, IL-17F and IL-22 are shown in Figure 3.22, TNFα in Figure 3.23. IL23 was below the limit of detection in almost all samples (<9.52pg/ml). No significant differences between healthy donors and patients were noted for any of these cytokines (unpaired t tests).

Cytokines implicated in the generation of Th_{17} cells were next assessed: IL-1β, IL-2 and IL-6 (Figure 3.24). IFNγ was also assessed, but was below the limit of detection in the majority of samples (<0.029pg/ml). No significant differences were noted between healthy donors and patients in any of these cytokines (unpaired t tests).

In summary, the levels of cytokines detected in all samples were low, and whilst median levels of Th_{17} cytokines were lower in the patient samples in keeping with the flow cytometry data, the differences between patient and healthy donor samples did not reach significance.

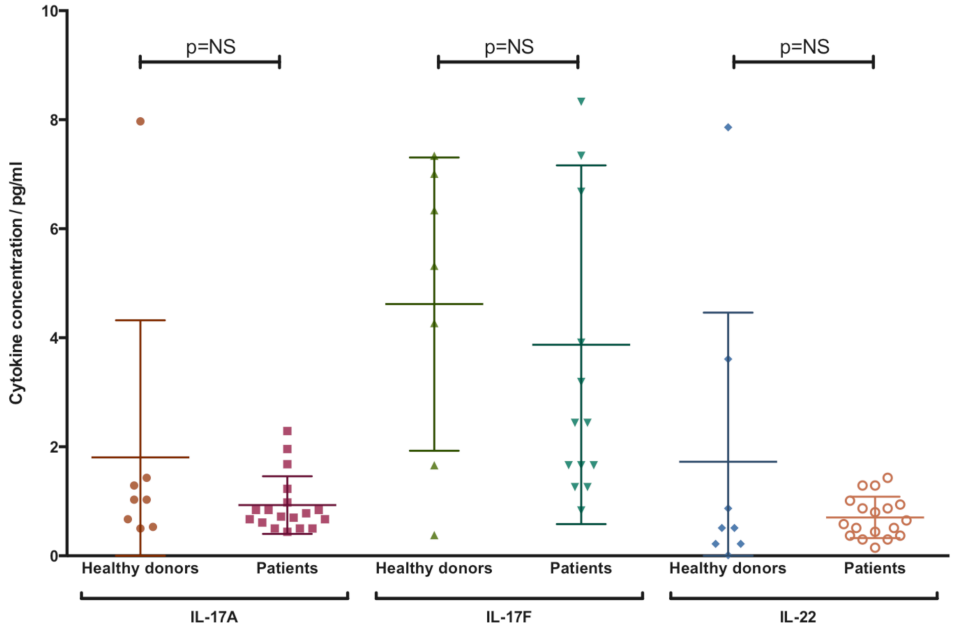


Figure 3.22 Concentrations of Th_{17} cytokines in the serum of healthy donors (n=8) and patients with MM (n=18). Median levels in healthy donors and patients were as follows: IL-17A: 1.030pg/ml (range: 0.5-7.97) *vs.* 0.75pg/ml (0.44-2.29), IL-17F: 5.32 pg/ml (0.38-7.34) *vs.* 2.44pg/ml (0.83-11.54), IL-22: 0.51pg/ml (0.01-7.86) *vs.* 0.62pg/ml (0.15-1.43). Mean and standard deviations shown. p values are for unpaired student t tests.

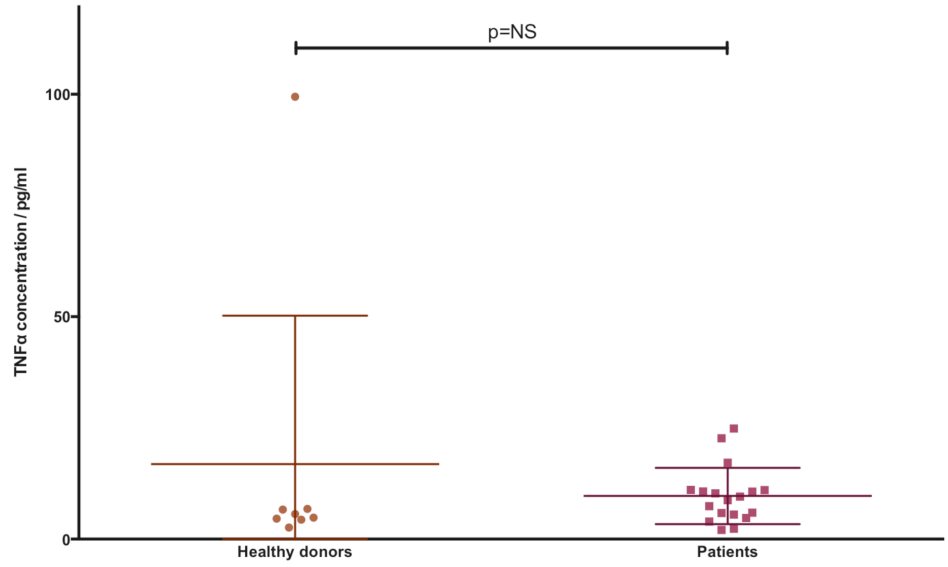


Figure 3.23 Concentration of TNFα in the serum of healthy donors (n=8) and patients with MM (n=18). Median levels in healthy donors and patients were as follows: TNFα: 5.25pg/ml (2.61-99.4) *vs.* 9.15pg/ml (2.08-24.9). Mean and standard deviations shown. p value is for unpaired student t test.

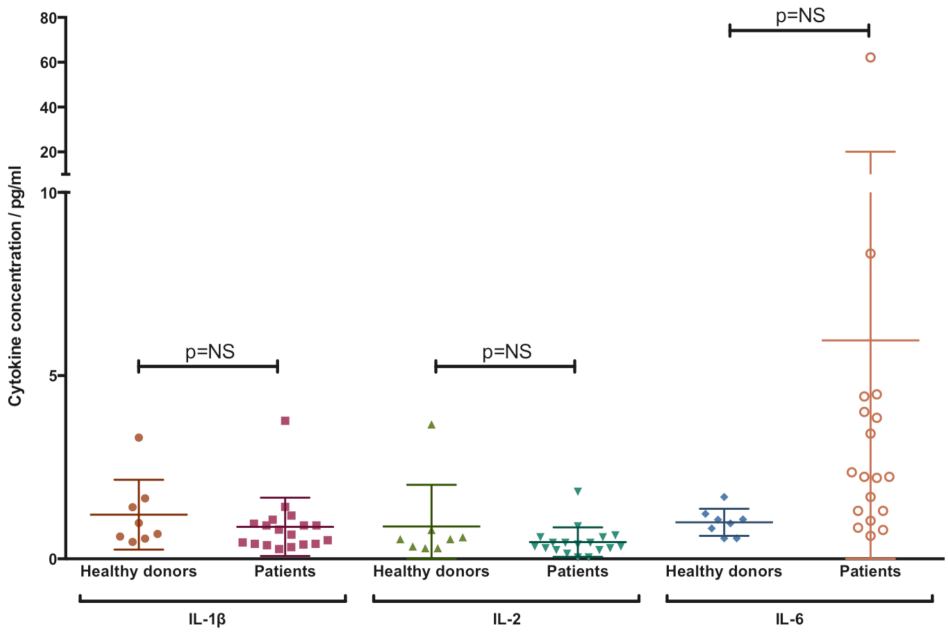


Figure 3.24 Concentrations of cytokines implicated in induction of Th_{17} cells, in the serum of healthy donors (n=8) and patients with MM (n=18). Median levels in healthy donors and patients were as follows: IL-1β: 0.83pg/ml (range 0.46-3.31) *vs.* 0.73pg.ml (0.27-3.77), IL-2: 0.54pg/ml (0.29-3.67) *vs.* 0.37pg/ml (0.04-1.83), IL-6: 1.02pg/ml (0.56-1.69) *vs.* 2.24pg/ml (0.63-62.15). Mean and standard deviations shown. p values are for unpaired student t tests.

Cell surface proteins, transcription factors and other cytokines

The phenotype of Th_{17} cells in patients with myeloma and related disorders was compared to that of healthy donors with respect to CD161, CCR4, CCR6, CD127, CXCR4, CXCR3, CCR7, *RORC, FoxP3, T-bet*, IL-10 and IFNγ expression. Expression of CD161 and CCR6 were significantly reduced on Th_{17} cells from patients compared to healthy donors (Figure 3.25). Furthermore, subgroup analysis of CD161 and CCR6 expression by disease status revealed an association between advancing disease and progressive loss of cell surface expression (Figures 3.26 & 3.27). CCR4, CD127, CXCR4, CXCR3, CCR7, *RORC, FoxP3, T-bet* and IL-10 expression were not found to be significantly different between patients and healthy donors (data not shown).

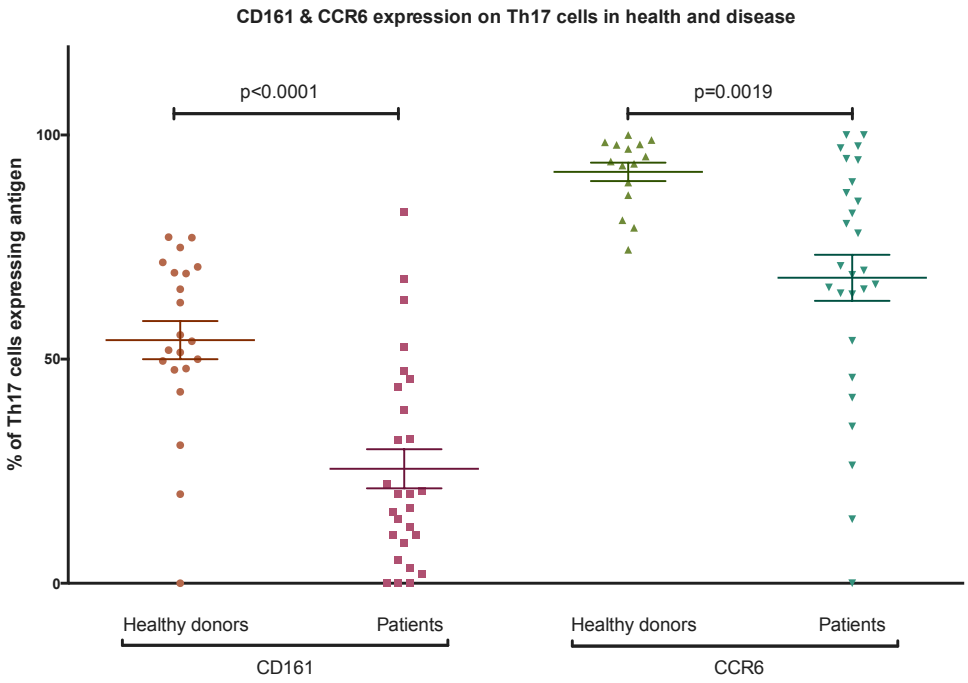


Figure 3.25. CD161 and CCR6 expression on PB Th_{17} cells in healthy donors (n=21) and patients with plasma cell dyscrasias (n=27). CD161: patients 25.54% (range 0-82.8%), healthy controls: 54.26% (0-77.2%) p<0.0001; CCR6: patients 68.18% (0-100%), healthy controls: 91.78 (74.4-100%), p=0.0019. Medians and standard errors of the mean indicated. P values are for unpaired, two-tailed t tests.

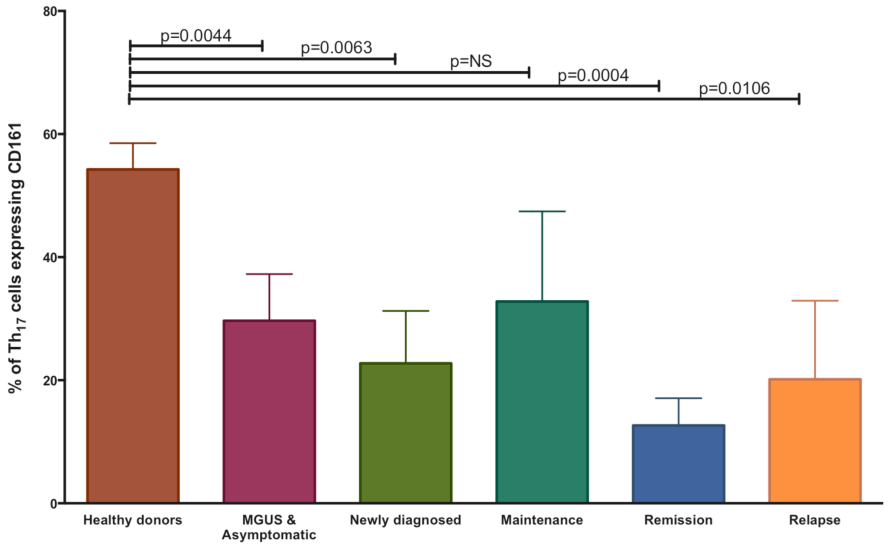


Figure 3.26. CD161 expression on PB Th_{17} cells in healthy donors (n=21), MGUS & asymptomatic MM (n=12), newly diagnosed MM (n=4), MM on maintenance therapy (n=4), MM in remission (n=4) and relapsed MM (n=3). Medians and standard errors of the mean indicated. P values are for unpaired, two-tailed t tests.

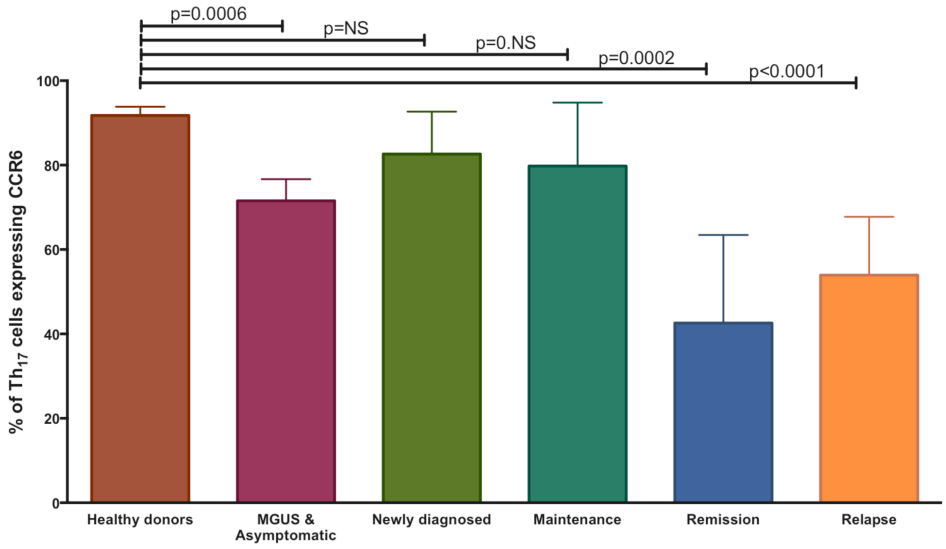


Figure 3.27. CCR6 expression on PB Th_{17} cells in healthy donors (n=21), MGUS & asymptomatic MM (n=12), newly diagnosed MM (n=4), MM on maintenance therapy (n=4), MM in remission (n=4) and relapsed MM (n=3). Medians and standard errors of the mean indicated. P values are for unpaired, two-tailed t tests.

Expression of CD161 and CCR6 on Th_{17} cells in the BM of healthy donors and patients were next examined. Th_{17} cells in the BM of healthy donors expressed CD161 at a lower level than in the PB of the same donors (mean ± SD: 14.02% ± 10.65% *vs.* 42.32% ± 10.61%, p=0.0245, representative single patient flow cytometry plots shown in Figure 3.28, collated data in figure 3.29). A similar pattern was seen when CCR6 was examined (43.34% ± 20.25% *vs.* 80.58% ± 11.41%, p=0.0294, Figures 3.28 and 3.29).

When expression of CD161 and CCR6 on Th_{17} cells from BM and PB of patients was compared, CD161 expression was not significantly different (23.14% ± 24.12% *vs.* 17.39% ± 14.31%, p=NS) whereas CCR6 expression was lower in BM than PB (38.48% ± 33.49% *vs.* 68.66% ± 22.38%, p=0.047); representative flow cytometry plots for a single patient shown in Figure 3.30, collated data from all donors in Figure 3.31.

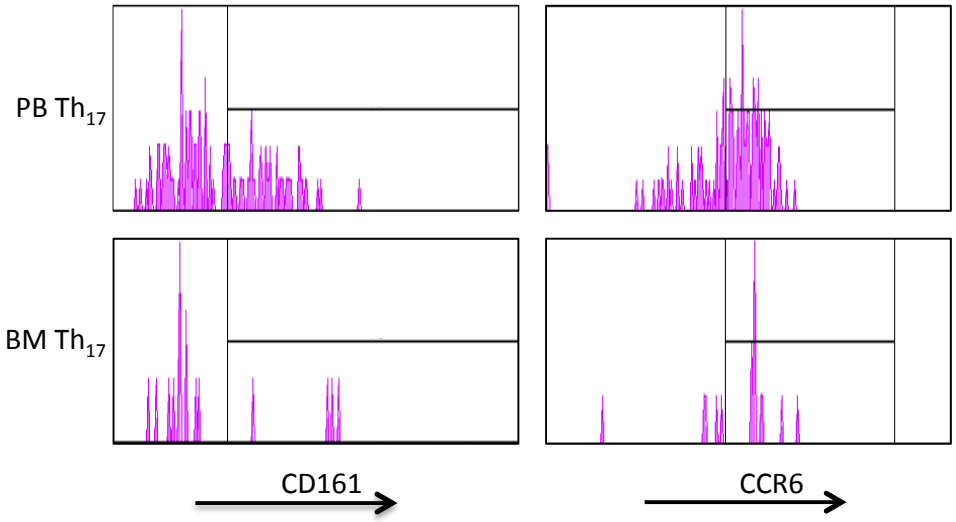


Figure 3.28. Representative histograms of CD161 and CCR6 expression on Th_{17} cells from PB and BM of a single healthy donor.

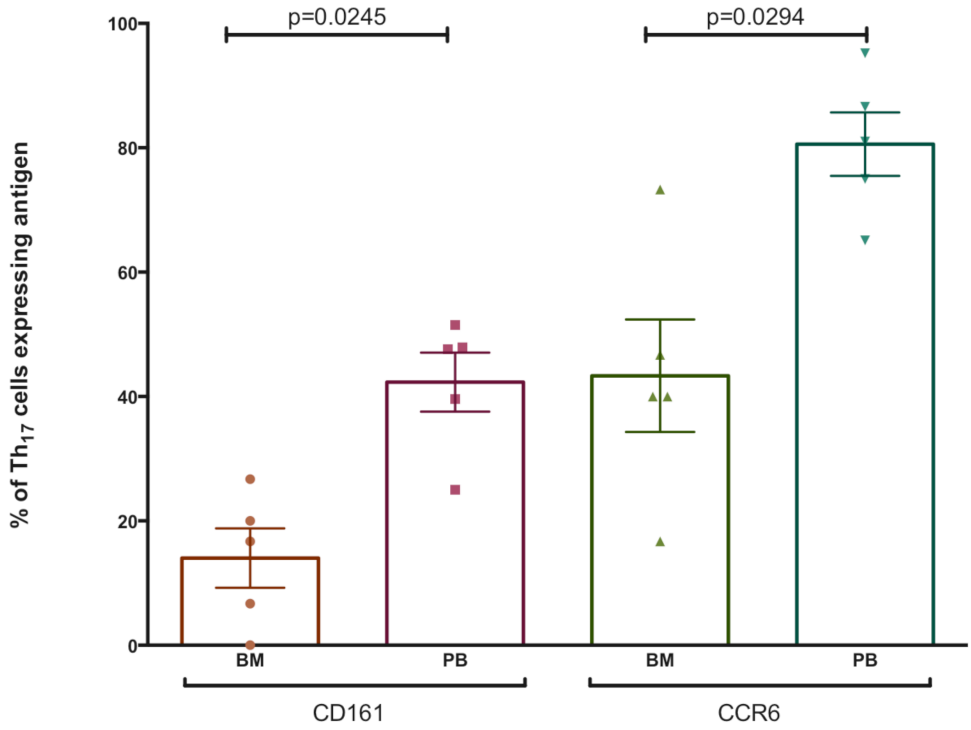


Figure 3.29. CD161 and CCR6 expression on Th_{17} cells in the BM and PB of healthy donors. N=5. P values are for paired student t tests. Histogram shows means ± SEM.

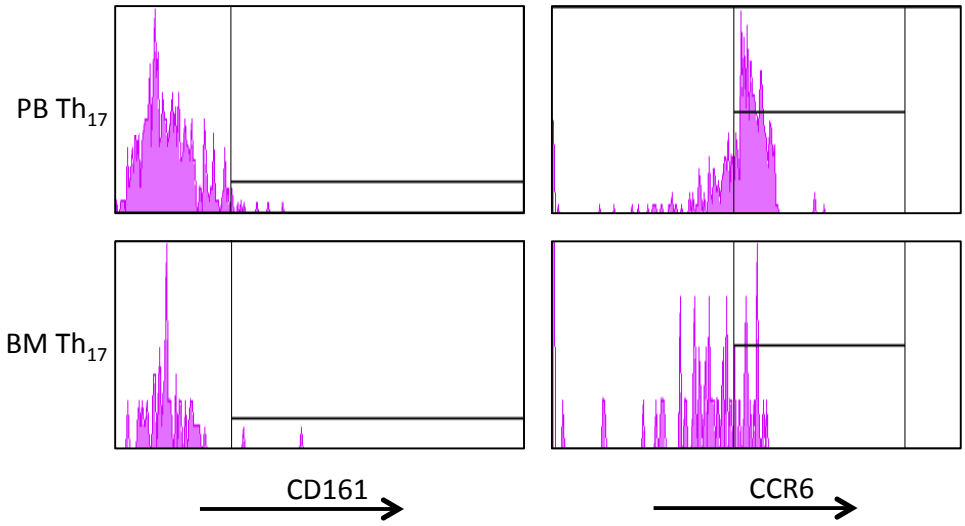


Figure 3.30. Representative histograms of CD161 and CCR6 expression on Th_{17} cells from PB and BM of a single patient with newly-diagnosed MM.

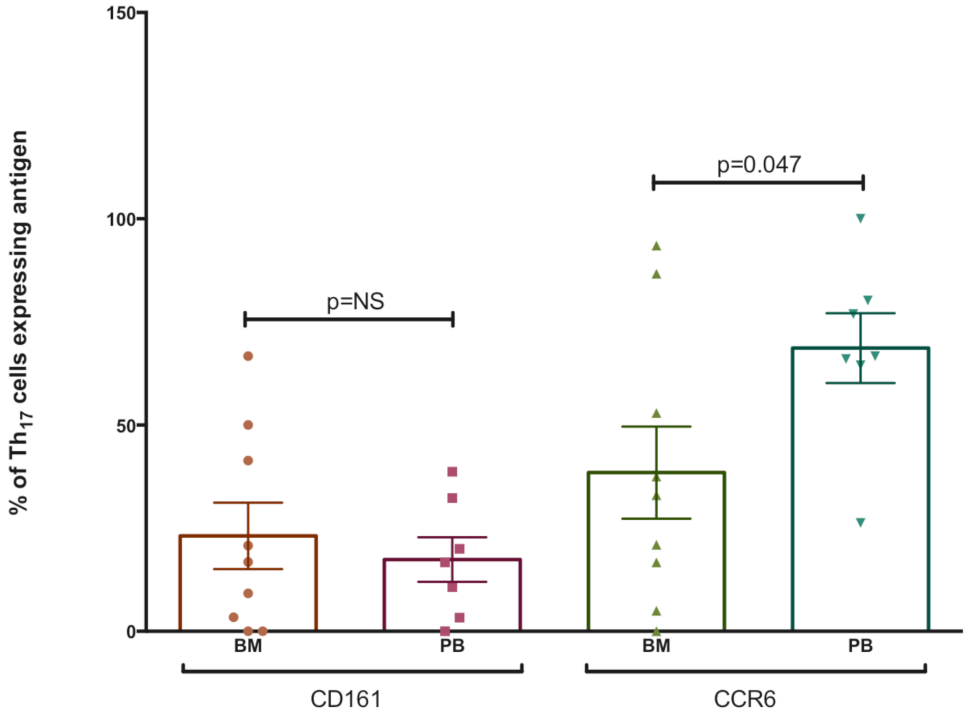


Figure 3.31. CD161 and CCR6 expression on Th_{17} cells in the BM and PB of patients with plasma cell dyscrasias. N=9. P values are for paired student t tests. Histogram shows means ± SEM.

Expression of neither CD161 nor CCR6 on BM Th_{17} cells was significantly different between healthy donors and patients (mean and SD): CD161: 14.0 ± 10.7% *vs.* 23.1 ± 24.1%, CCR6: 43.3 ± 20.3% *vs.* 38.5 ± 33.5% (Figure 3.32).

Since CD161 expression on PB Th_{17} cells and MFI for IL-17 in PB Th_{17} cells were both reduced in patients in a disease stage-dependent manner, these two variables were next assessed for correlation. Linear regression analysis revealed no significant correlation between the two: r^2=0.048, p=NS (Figure 3.33).

These findings reveal a complicated pattern of antigen loss in MM. Both CD161 and CCR6 were found to be lost in MM, in a disease stage-related manner. Nonetheless, CCR6 expression on Th_{17} cells in MM shows the normal overall pattern in terms of higher level expression on PB than BM, albeit it at lower absolute levels in both sites. In contrast CD161 expression is specifically reduced in the PB compared to BM.

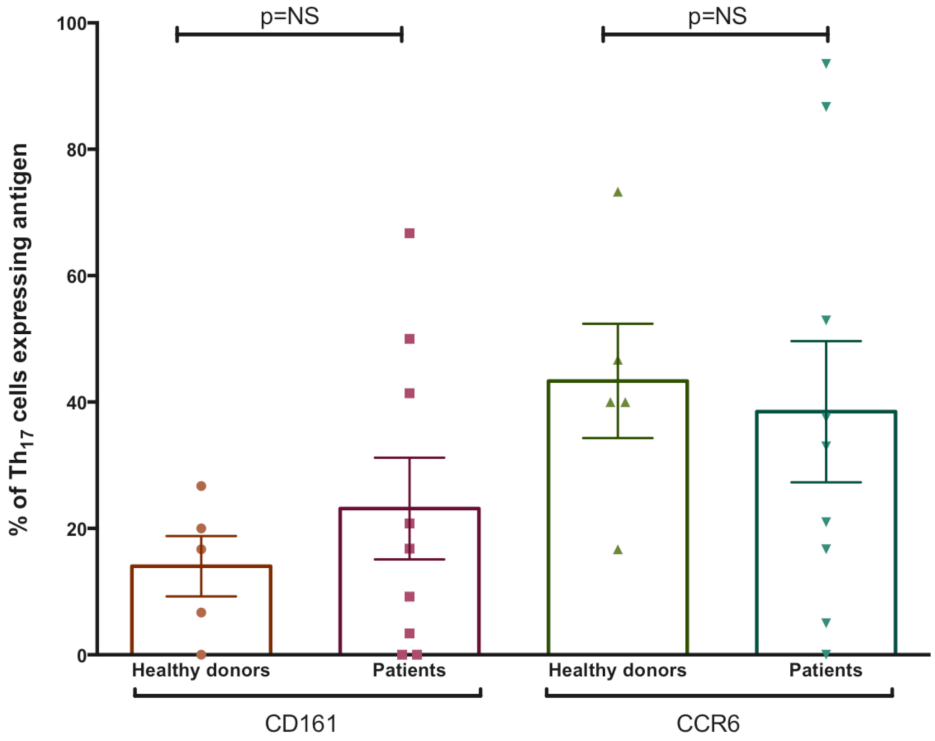


Figure 3.32 CD161 and CCR6 expression on Th_{17} cells in the BM of healthy donors and patients with plasma cell dyscrasias, n=5 healthy donors, n=9 patients. P values are for paired student t tests. Histogram shows mean ± SEM.

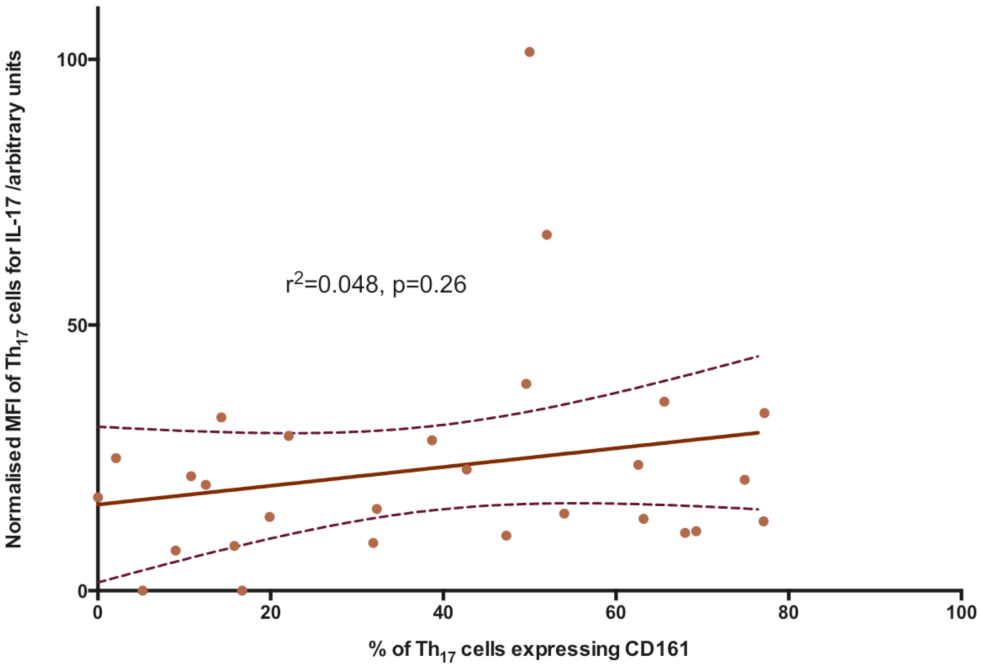


Figure 3.33 Normalised MFI of Th_{17} cells for IL-17 as a function of fraction of Th_{17} cells expressing CD161. The solid line shows a linear regression (r^2=0.048, p=0.26) and the dotted lines the 95% confidence interval for the linear regression.

Th_{17} cell responses to fungal antigens in health and disease

The IL-17 family of cytokines has a well-described role in the immune response to fungal infections, and Th_{17} cells are able to respond to fungal peptides. The proportion of Th_{17} cells able to respond in this way in the peripheral blood of healthy donors and patients was therefore assessed using a pool of lyophilised peptides, consisting mainly of 15-mer sequences with 11 amino acid overlap, covering the sequence of the *Candida albicans* mannoprotein MP65 protein (Miltenyi Biotec, UK), hereafter referred to as MP65. 0.7% of healthy donor $CD4^+$ T cells produced IL-17 in response to stimulation with PMA/ION (SD: 0.205%, n=7). Surprisingly, a mean of 0.15% (SD: 0.156%) of $CD4^+$ T cells from the same donors produced IL-17 in response to stimulation for 6 hours with MP65, indicating that 21.4% ± 20.7% of Th_{17} cells responded to this oligoclonal stimulus (representative flow cytometry plots: Figure 3.34, collated data: Figure 3.35). As anticipated in view of the reduced frequency of total Th_{17} cells in patients compared to controls, there were fewer cells able to produce IL-17 in response to MP65 (mean 0.13% *vs.* 0.15% in controls, p=NS, n=3 patients, representative flow cytometry plots: Figure 3.34). However, interestingly there was also a trend towards a reduction in the percentage of total Th_{17} cells apparently able to respond to the oligoclonal stimulus, although this did not reach significance (7.4% ± 10.5% in disease *vs.* 21.4% ± 20.7% in health, p=NS, Figure 3.35).

These data indicate that in addition to being aberrant in frequency and in their level of IL-17 secretion, Th_{17} cells in myeloma are additionally compromised in their ability to respond to a physiologically appropriate stimulus and are therefore functionally impaired.

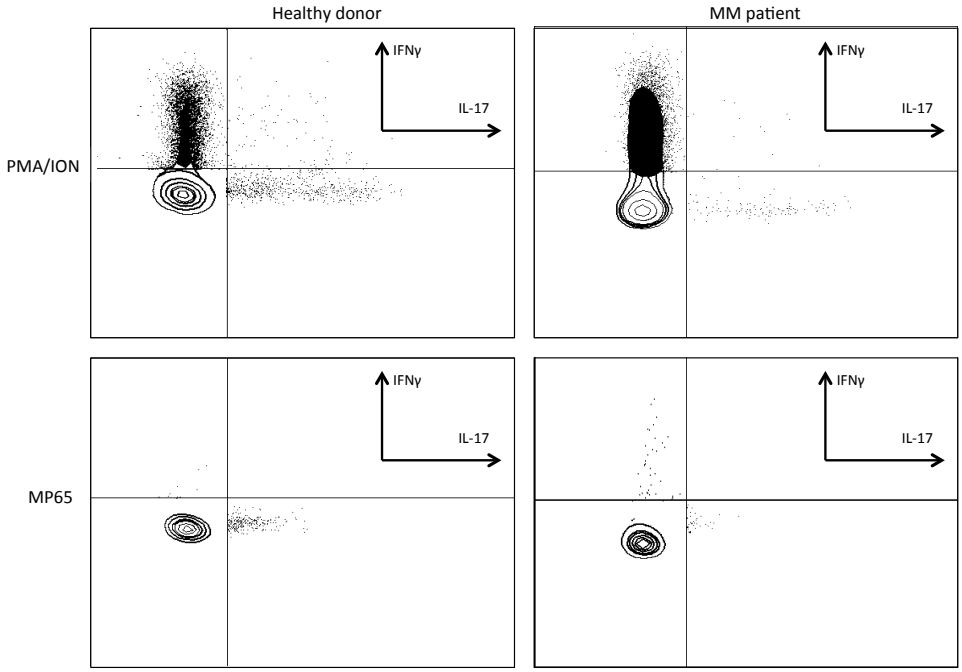


Figure 3.34. Representative dot plots of IL-17 and IFNγ secretion by PB Th_{17} cells from a healthy donor (left) and a patient with MM (right) after stimulation with PMA and ION (top) or MP65 (bottom).

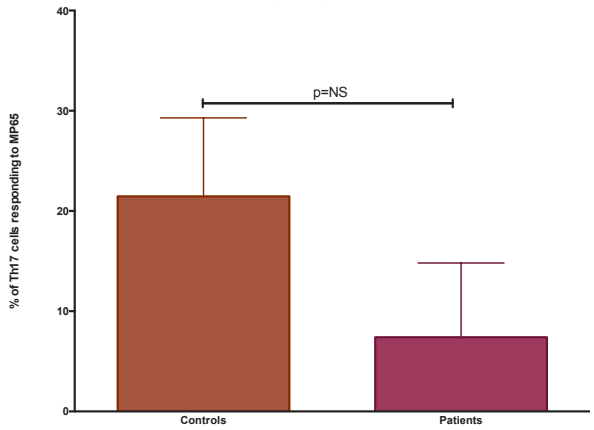


Figure 3.35. Proportion of Th_{17} cells producing IL-17 after stimulation with MP65 (expressed as a percentage of cells from the same sample producing IL-17 after stimulation with PMA & ION) in healthy donors (n=7) and patients (n=2). All cells were stimulated for 6 hours and treated with Golgiplug for the last 3 hours. P value is for an unpaired two-tailed t test.

Discussion

Testing a range of methods of T cell stimulation revealed PMA with ION to be most potent for induction of IL-17 secretion from the very small percentage of $CD4^+$ T-cells able to produce it, and in combination with intracellular staining for CD4, this allows reliable identification of Th_{17} cells. An optimised protocol of stimulation and flow cytometric staining was thereby devised for the identification and phenotypic characterisation of Th_{17}, Th_{17-1} and Th_1 cells in health and disease. Th_{17} and Th_{17-1} cells were found to comprise median 0.21% (range 0-2.94%) and 0.04% (range 0-1.06%) of $CD4^+$ T-cells in a cohort of healthy donors, which is in keeping with published work[171], thus validating the method.

When the prevalence of T cell subsets in healthy donors was examined as a function of age, a weak but significant negative correlation was noted between age and Th_{17} frequency, whereas when Th_1 and Th_{17-1} cells were examined the effect of age was smaller and not significant. In the context of myeloma, a disease with a median age of presentation of around 70 years, this is clearly an important observation. Nonetheless, linear regression analysis yielded r^2=0.19, p=0.04, indicating that only 19% of the variance seen in Th_{17} frequency is expected to be accounted for by age alone. Interestingly, one previous study reported an association between increasing age and increased incidence of $CD4^+IL23R^+$ cells (employed in that study as a surrogate marker for Th_{17} cells), but decreased production of IL-17 by $CD4^+$ cells after stimulation[263]. It is therefore tempting to speculate that the changes in immune responses associated with ageing might include an impaired ability of $RORC^+CD4^+$ cells to produce IL-17 upon stimulation; further work would be required to clarify this issue.

Experiments were next undertaken to examine the phenotype of Th_{17} cells in health, which as discussed previously has been extensively reported to be $CD4^+RORC^+CD161^+CCR4^+CCR6^+$. When assessed by flow cytometry, expression of RORC, the signature transcription factor for Th_{17} cells was found to be non-specific for the subset, staining almost all $CD4^+$ cells to some extent. Clearly, RORC is extremely well characterised in this role by multiple publications. The failure of this method might then represent a problem associated with the specificity and sensitivity of the available antibodies, but might also reflect the presence of RORC at a range of concentrations in a range of $CD4^+$ cells, the cytokine secretion phenotype of which depends on the relative concentrations of other lineage-determining transcription factors (e.g. T-bet, GATA-3). It does not appear to be a technical issue with detecting nuclear proteins as both T-bet and

FoxP3 staining yielded expected results. Alternative methods (e.g. PCR, Western blotting) would be needed if this were to be examined further. Nonetheless, the fundamental defining characteristic of Th_{17} cells is their ability to produce IL-17 upon stimulation, and this more basic, functional definition was therefore adopted for the remainder of the work presented here.

CD161 was expressed on a significantly higher proportion of Th_{17} cells than Th_1 or $CD4^+IL\text{-}17^-IFN\gamma^-FoxP3^-$ cells. However, two important caveats exist. Firstly, despite previous reports[165], by flow cytometry at least, this marker was not expressed on all IL-17-producing $CD4^+$ cells – indeed only 54% of these cells expressed the marker in healthy donors, with considerable inter-donor variability seen; importantly age was not found to be correlated with CD161 expression on Th_{17} cells. Three different fluorophore-conjugated antibodies were tested, with similar results, suggesting this is not a reagent-related problem. Secondly, CD161 was also expressed on $Th_{17\text{-}1}$ and T_{Reg} cells and the level of expression by these cells was not significantly different from that of Th_{17} cells. When flow cytometry gating was employed to focus on $CD4^+CD161^+$ cells for analysis, a relative enrichment in Th_{17} cells was seen, but the effect was modest and unfortunately these data indicate that despite a great deal of enthusiasm in the published literature[165], CD161 is unsuitable for selection of un-stimulated Th_{17} cells e.g. by FACS sorting. CCR6 and CCR4 were similarly found to be expressed at higher levels on Th_{17} cells than on Th_1 cells, but did not allow differentiation between Th_{17} cells and $Th_{17\text{-}1}$ or T_{Reg} cells. Combinations of these markers did not greatly improve specificity or sensitivity for Th_{17} cells, leading to the conclusion that it is presently not possible to identify Th_{17} cells reliably without redress to stimulation and functional assay – i.e. measurement of IL-17 production.

When patient and healthy donor PB samples were compared, a significant reduction in Th_{17} cells was seen, below even the low levels seen in healthy donors. However, in view of the correlation between age and Th_{17} frequency in healthy donors, patient samples were then compared to an age-matched cohort and were found to have a significantly higher level of Th_{17} cells than this, more appropriate control group. When patient samples were subdivided according to clinical disease stage, those with MGUS and asymptomatic disease (who had the highest levels), MM on lenalidomide maintenance therapy and MM in remission showed significantly raised Th_{17} levels compared to age-matched controls, whereas those with newly diagnosed and relapsed disease had levels not significantly different from age-matched controls. $Th_{17\text{-}1}$ cells were increased only in those with MGUS

& asymptomatic MM compared to age-matched controls and Th_1 cells were raised only at relapse – significantly by comparison to almost all other groups including age-matched controls.

The MM disease stage-related differential in IL-17-secreting T cells has not been reported previously, and the high levels of Th_{17} and $Th_{17\text{-}1}$ cells seen in those with MGUS and asymptomatic MM are of particular interest and warrant further exploration. Th_{17} cytokines such as IL-17 and IL-21 have certainly been reported to serve as growth factors for MM cells[74,196] (although attempts to recapitulate those experiments have not been successful to date; data not shown), however levels of Th_{17} cells were actually lower in newly diagnosed, symptomatic MM than in MGUS & asymptomatic MM, suggesting the relationship between malignant plasma cells and Th_{17} cells is more complex than a simple positive feedback loop. In addition, the role of $Th_{17\text{-}1}$ cells is unclear: these cells have been linked with effective anti-tumour responses in adoptive transfer models[164] – an effect which is critically dependent on both Th_{17} polarisation and secretion of IFNγ, and it may be that the ability to traffic according to a Th_{17} cell surface phenotype but deliver IFNγ confers a particular significance on these cells; unfortunately numbers of these cells were so small that detailed *ex vivo* characterisation was not possible. The significance of the raised levels of Th_1 cells seen at relapse is also unknown, although clearly in the past IFNγ immune responses have been widely associated with anti-cancer effects. On the basis of these results, and the known presence of anti-tumour immune responses in MM [45,47] , it is tempting to speculate that Th_{17} or $Th_{17\text{-}1}$ cells might be playing a role in anti-cancer immunity in early disease, but a large number of further experiments are required to examine this question.

Th_{17} cells in MM were also noted to have a lower normalised MFI for IL-17 in MM, and serum levels of IL-17A, IL-17F, IL-22 and TNFα were lower in MM samples than healthy donors, although the differences in cytokine levels did not reach significance. Low levels were expected in all samples in view of the rarity of Th_{17} cells, and are in keeping with the levels reported in other malignancies [264,265] , though they are significantly lower than those reported in the only paper to examine this in myeloma [74] and this might reflect differences in sample storage and assay protocols. When BM samples were examined, Th_{17} cells were increased in patients compared to healthy donors, as were Th_1 cells; the Th_{17}:Th_1 ratio, employed as a surrogate for the balance of T cell polarisation towards Th_{17} cells, was not significantly different. However, Th_{17} cells in MM did exhibit reduced responsiveness to a more physiologically relevant stimulus – Candidal wall proteins,

suggesting a physiologically relevant functional defect. Since Th_{17} cells have been ascribed roles in immune responses against not only fungal infections [180,266], but also extracellular bacteria [174,175,267,268] and intracellular bacteria [176], the loss of Th_{17} cells with disease progression may be contributory to the increased risk of a range of infections seen in MM.

These data are not directly in line with published work. Prabhala *et al.* examined PB from 11 newly-diagnosed MM patients and 12 healthy donors (not age-matched) and BM from 4 patients and 3 healthy donors, and noted expansion of the Th_{17} population in both the PB and BM of patients compared to controls[74]. These authors report considerably higher levels of Th_{17} cells in both health and disease than those seen here – for example 2.05% ± 0.3% of T cells are Th_{17} in normal donors and 4.49% ± 0.78% in MM patients. Prabhala *et al.* also report increased levels of IL-17, IL-21, IL-22 and IL-23 in the serum of patients with MM, in contrast to the data presented here.

Some of this discrepancy might be accounted for by differences in the gating strategies employed. For example, the FACS protocol used here incorporates a dead cell discriminator stain to allow exclusion of dead cells, which otherwise appear to produce IL-17 due to autofluorescence. This is of considerable importance when dealing with such a small population and may account for the lower numbers of Th_{17} cells in my data – the exemplar FACS plots in the published paper do suggest a degree of autofluorescence which might artificially increase Th_{17} numbers[74]. Cytokine levels measured by Luminex here were low, and it is possible that the blood collection or sample freezing procedure employed was not optimal for preservation of cytokines, although the same technique was used for all samples, including healthy donors. The levels of Th17 signature cytokines in health and disease might be further examined through qRT-PCR, and RNA from relevant samples has been isolated and stored for this purpose.

Surprisingly, unlike with healthy donors, no correlation was seen between age and Th_{17} frequency in MM patients, strengthening the assertion that Th_{17} changes are a consequence of disease. Interestingly, the prevalence of Th_{17} cells did vary according to some disease-specific variables, with lower levels seen in those with kappa light chains, and with IgG paraproteins; the causes and significance of these observations is unknown and since multivariate analysis could not be conducted, caution is warranted in avoiding over-interpretation. Nonetheless, the data highlight the importance of the use of age-matched controls, which has been overlooked by a number of the published studies of Th_{17} cells in myeloma and cancer more widely.

The increased levels of Th_{17} cells in BM raises the possibility that myeloma cells are able to induce Th_{17} cell differentiation or survival – a number of the cytokines required for Th_{17} polarisation (e.g. IL-1β, TGFβ, IL-6) are known to be abundant in the bone marrow microenvironment [12,269] and it is possible these mediators might lead to expansion of Th_{17} cells in MM; this issue will be explored in the following results chapter. It is uncertain why Th_{17} cells should be increased in the BM to a considerably greater extent than in the PB; alterations in trafficking, sensitivity to stimulation, longevity, peripheral plasticity or other mechanisms might account for this, and again warrant further evaluation. Of potential relevance is the fact that IL-1β, IL-2 and IL-6 were not found to be raised in the peripheral blood of MM patients compared to healthy controls, strengthening the hypothesis that microenvironmental changes may be responsible for the localized expansion of Th_{17} cells in the bone marrow.

When examining patient samples it was noted that CD161 is absent on a large portion of Th_{17} cells, in a disease-stage dependent manner. A 2011 publication reported reduced CD161 expression on Th_{17} cells from patients with head and neck cancer[169], at all sampled sites but particularly in metastatic lymph nodes, suggesting this may be a more generalised cancer-related phenomenon. It remains to be clarified whether CD161 has functional importance in this context, or indeed in general, as the role of the protein is unknown, although PILAR-signalling through CD161 has been shown to support CD3 antibody-dependent and antigen specific T-cell proliferation[167], meaning it is possible loss of CD161 might contribute to the relative paucity of Th_{17} cells in the peripheral blood of MM patients. A weak correlation was noted between expression of CD161 and MFI for Th_{17}. In interpreting this finding it should be borne in mind that for the reasons discussed earlier IL-17 secretion was here assessed by supra-physiological, antigen-independent activation of T cells by PMA and ionomycin. If CD161 does indeed function as a costimulatory receptor or other mediator in the context of normal immune synapse formation, the effect of loss of CD161 might reasonably be expected to exert a far more profound effect on the ability of Th_{17} cells to respond appropriately to stimulation *in vivo*. Experiments using T cell re-stimulation using MACSiBeads loaded with and without a stimulating anti-CD161 antibody might be used to examine this issue further.

Since Th_{17} cells are thought to derive only from $CD161^+$ precursors in health, two possibilities exist to explain the presence of significant numbers of $CD161^-$ Th_{17} cells in MM: either $CD161^+$ Th_{17} cells lose expression of CD161 through interactions with MM or the microenvironment, or $CD161^-$ T cells are abnormally induced to become Th_{17} cells

through such interactions. This issue will be addressed in the subsequent results chapter. Further characterisation of the phenotypic and functional properties of $CD161^+$ and $CD161^-$ Th_{17} cells may also be illuminating, and many basic immunology and translational questions remain to be explored in this area. For example, does ligation of CD161 affect production of IL-17 after a physiological stimulus such as Candidal antigen? If so, are the $CD161^+$ and $CD161^-$ cells different in terms of their capacity to conduct their normal physiological antifungal role or to upregulate neutrophil migration and epithelial immune responses[270-272]? Further examination of some of these issues may cast light on the basic immunological questions about the natural role of CD161. Of course, in the context of myeloma it will also be of interest to know whether these cells respond differently to immune synapse formation with MM plasma cells, and whether $CD161^-$ Th_{17} cells either lose anti-tumour efficacy or can even support the tumour clone. The latter point is of interest, since Prabhala *et al.* have reported an ability of IL-17 to augment growth of HMCLs in culture[74], although as mentioned previously, attempts to reproduce these experiments have thus far failed.

CCR6 expression was also reduced on patient Th_{17} cells. This protein mediates attraction of Th_{17} cells to CCL20 and human β-defensin[273], thereby mediating homing to skin and mucosal tissues where Th_{17} cells have an increasingly well-described role in mucosal immunity[270,272,274]. CCR6-expression has been reported in high proportions of $CD4^+$ cells in inflamed sites in juvenile arthritis[171], and $CCR6^+$ γδ T cells are more responsive to TCR-stimulation than their $CCR6^-$ counterparts[273], suggesting the loss of CCR6 expression in MM might contribute to the increased risk of infections in MM.

Restimulation with Candidal antigens revealed that, surprisingly, 20% of Th_{17} cells in healthy donors were able to respond to this oligoclonal stimulus. Although previous studies have demonstrated T cells with TCR specificity for *C. albicans* antigen are preferentially or exclusively present in the $CCR6^+$ subset[171], the experiments presented here were performed in the absence of antigen presenting cells (APCs), implying TCR-mediated antigen specificity is not the mechanism of response. This raises the possibility that Th_{17} cells might instead be stimulated via pathogen-associated pattern recognition receptors such as C-type lectins[275,276], or even CD5[134]. In light of the loss of CD161, a C-type lectin receptor, in patient-derived Th_{17} cells this is an exciting possibility and requires exploration, particularly in light of the decreased responsiveness to MP65 seen in patient Th_{17} cells.

A large number of additional avenues of further investigation could follow from this work. The TCR specificity of the Th_{17} cells has not been addressed by the preceding studies and T cell priming/recall experiments might be used to examine this issue further. Development of further functional assays of Th_{17} cell function are another natural extension of this work – for example neutrophil migration assays (since Th_{17} cytokines are chemoattractant to neutrophils during normal immune responses[277]) or examination of innate endothelial and epithelial immune responses such as upregulation of defensins[270] or cytokine secretion[178]. Th_{17} cell localisation within the BM has not yet been assessed, and the interplay between tumour cells, immune cells, hypoxia and the tumour niche warrants examination, perhaps initially by immunohistochemical appraisal of myeloma BM trephine samples. Th_{17} cells, through their cytokine mediators, may also favour growth of the malignant clone. Th_{17} cells may therefore play a contributory role in both the pathogenesis and manifestations of the disease and represent a rational therapeutic target. The ability of established immune-active and immunomodulatory MM therapies to interrupt and reverse the generation of Th_{17} cells could next be investigated. Monoclonal antibodies targeting IL-17[278-281] and IL-23[282] have been used in clinical trials for inflammatory diseases in humans, and an anti-IL-21R antibody is similarly under investigation[283], and the potential for these drugs to disrupt Th_{17}-MM interactions might also be explored. Th_{17} cells and their mediators are therefore clinically viable targets and further characterisation of the derangements in MM is warranted.

The work presented here points towards an impairment of Th_{17} immune function in MM, in terms of prevalence, phenotype and function, and this may contribute to the risk of infection in the disease. The following results chapter will employ an *in vitro* model to address a number of the questions arising from this work: do myeloma plasma cells induce formation of Th_{17} cells, and if so how? What are the precursor cells, and what effect do tumour interactions have on the expression of CCR6 and CD161? And importantly, what role does the bone marrow microenvironment play in these processes?

Chapter 4 – *In vitro* modelling of Th_{17} generation in multiple myeloma

Introduction

The data presented in Chapter 3 demonstrate that Th_{17} cells are expanded in the peripheral blood and bone marrow of patients with multiple myeloma, have an abnormal cell surface phenotype and are compromised in their ability to respond to stimulation. A number of possibilities might account for the abnormal numbers and phenotypes of Th_{17} cells in MM. For example, Th_{17} cells might be induced in the bone marrow through interactions with tumour cells, either directly or via other cellular/chemical mediators such as bone marrow stromal cells (BMSCs), cytokines and chemokines. Alternatively, Th_{17} cells from peripheral blood might traffic to the bone marrow microenvironment (BMME) through BM-expressed surface proteins or chemokines, and be retained there. A considerable body of evidence points to plasticity between Th_{17} cells and regulatory T cells (T_{reg}), which have been shown to be expanded in patients with MM[67] and might serve as a reservoir for peripheral Th_{17} generation. The longeivity of Th_{17} cells is also unknown; if Th_{17} cells are not generated per se by myeloma, it is possible they may be protected from apoptosis by the BMME or MM-produced cytokines.

The cell surface marker CD161 a C-type lectin receptor, induced by *RORC2* and previously reported to be present on all human IL-17-producing cells, be they Th_{17} cells or others (e.g. $CD8^+TCR\alpha\beta^+$, $CD4^-CD8^-TCR\alpha\beta^+$ and $CD4^-CD8^-TCR\gamma\delta^+$)[166], was found in the previous chapter to be downregulated on the surface of Th_{17} cells in MM. Two likely explanations exist for this: either these Th_{17} cells might have arisen via an abberant pathway from $CD161^-$ precursors (which should normally be unable to give rise to IL-17-producing cells), or the Th_{17} cells may have developed normally and subsequently lost their expression of CD161 due to interactions with the tumour microenvironment. Since it has been proposed that CD161 can act as a co-activating receptor, allowing antigen-dependent T cell proliferation upon engagement by its ligand, the loss of this antigen may have functional repercussions for immunity in MM.

In the work reported in this chapter, a co-culture model is developed to examine the cellular interactions between T cells, peripheral blood immune subsets and tumour cells

that might give rise to the expanded Th_{17} cell population identified in patients. This model is then developed to examine the contribution of bone marrow stromal cells and the impact of $CD4^+$ starting populations, including the importance of initial CD161 expression. Finally, a protocol is developed for *in vitro* generation of Th_{17} cells to examine the propensity to Th_{17} generation in health and in MM, and factors that may affect this.

Materials and Methods

Preparation of cells for co-culture

PBMC and Mitomycin C-treated HMCL were isolated and maintained as described in Chapter 2, before mixing at a 1:1 ratio. Where HS-5 and HS-27 were used in cocultures, these were seeded in 24 well plates at 10^5 cells/well for 24 hours at 37°C, 5% CO_2 before washing the plates to remove dead or non-adherent cells prior to addition of HMCL and T cells. BMSCs were added at a BMSC:T cell ratio of 1:10.

Flow cytometric cell sorting

For the experiments described under 'characterisation of parental population', flow cytometric sorting of $CD4^+$ cells according to expression of CD161 and CCR6 was undertaken. $CD4^+$ T cells were first isolated using MACS, and then stained with CCR6-PE and CD161-APC, as described in Chapter 2. Cells were then resuspended at 10^7/ml in MACS buffer and sorting using a BD Influx 5 laser cell sorter (principles described in Chapter 2) into CM at 37°C. Samples of sorted populations were re-examined by flow cytometry and were found to be >90% pure in all cases.

T cell stimulation protocols

TCR and signal 2 stimulation was provided using a T Cell Activation/Expansion Kit (Miltenyi Biotec: 130-091-441), modified to provide alternative signal 2 stimuli. In brief, 10^8 anti-biotin MACSiBead particles in 500µl MACS buffer were mixed with combinations of 100µl of 100µg/ml each of anti-CD3-Biotin (included in kit), anti-CD28-Biotin (included in kit), anti-CD5-Biotin (BD Biosciences) and anti-ICOS-Biotin (eBIosience), the total volume made up to 1ml with further MACS buffer, and incubated for 2 hours at 4°C under constant gentle rotation, yielding MACSiBead particles at $10^8 ml^{-1}$ and a final antibody concentration of 10µg per antibody per ml. Loaded MACSiBead particles were then added to T cells at a 1:1 ratio.

Recombinant human cytokines and cytokine-neutralising antibodies were obtained and used as shown in Table 4.1. All cytokines were certified endotoxin level <0.1ng/µg of protein.

Cytokine	Manufacturer	Source	Final concentration	Product ID & Lot
IL-1β	Peprotech	E. coli	10ng/ml	200-01B 1202B95R1
IL-2	Peprotech	E. coli	200IU/ml	2002-02 020812ST
IL-6	Peprotech	E. coli	20ng/ml	200-06 090916
TGFβ1	Peprotech	HEK 293	5ng/ml	100-21 1012209
Anti-IL-12	eBioscience	Mouse	1ng/ml	BMS152 B-T21

Table 4.1. Recombinant cytokines and cytokine-neutralising antibodies.

Culture conditions for Th_{17} polarisation

Unless otherwise stated, incubations were performed in CM at 37°C, 21% O_2 and 5% CO_2 in a humidified tissue culture incubator. Hypoxic cultures were incubated at 37°C, 5% O_2 and 5% CO_2 in a humidified tissue culture incubator (Galaxy R CO_2 Incubator; Scientific Laboratory Supplies Ltd.). Where Iscove's modified Dulbecco's medium (IMDM, Sigma: I3390) was used, this was supplemented with 10% heat-inactivated FBS). Where stated, 11µg/ml ι-tryptophan was added to culture media prior to incubations. For experiments where 'dark' was specified, culture media and culture vessels were stored in opaque containers and exposure to natural and artificial light minimised.

Results

Development of a co-culture model

Since Th_{17} cells were expanded in bone marrow samples from myeloma patients, relative to peripheral blood, and published work reports expansion of Th_{17} cells in bone marrow from myeloma patients compared to normal marrow, a simple *in vitro* model was developed to allow examination of the requisite cellular interactions. Initial attempts to culture HMCLs with negatively-selected $CD4^+$ T cells at ratios ranging from 1:16 to 1:1 resulted in high levels of cell death in the cultured T cells, and absence of cytokine secretion (data not shown). Since this was associated with, and presumably consequent upon, marked expansion of the HMCL population and acidic cell cultures (determined by phenol red), the model was modified to incorporate mitomycin C (MitC) treatment of HMCLs prior to co-culture. PBMC and MitC-treated HMCL were mixed at a 1:1 ratio and the timescale of interactions between $CD4^+$ T cells and HMCLs was examined.

As discussed in the previous results chapter, considerable inter-donor variability is seen in terms of the prevalence of Th_{17} cells in the peripheral blood. In order to correct for this variation, cocultures were repeated 5 times, and the percentages of $CD4^+$ cells producing IL-17 throughout coculture were normalised to the pre-coculture levels for that donor, and thus expressed as a fold change. IL-17 production by $CD4^+$ cells was found to increase throughout the culture, whereas IFNγ secretion decreased (dot plots for a single donor are shown in Figure 4.1, collated data: Figure 4.2). After 4 days of isolated culture of $CD4^+$ cells alone, the fraction of cells producing IL-17 was unchanged (95.6% of baseline), whereas when cocultured with U266B, a mean 2.9-fold increase in the fraction of CD4 cells producing IL-17 was seen ($p=0.043$, paired student t-test). In contrast, the fraction of $CD4^+$ cells producing IFNγ after 4 days decreased under both culture conditions and was not significantly different between the two (28.5% and 45.4% respectively, p=NS).

After 7 days of culture, a 2.75-fold increase in Th_{17} cells was seen in isolated culture, and an 11.7-fold increase under coculture conditions ($p=0.004$, paired student t-test). After 7 days of culture, IFNγ production by $CD4^+$ cells was minimal under both isolated and coculture conditions, and not significantly different between the two (1.8% and 5.1% of baseline respectively, p=NS).

Attempts to continue co-culture beyond day 7 resulted in a high rate of cell death in $CD4^+$ cells, presumably due to depletion of nutrients within the culture media. Since renewal of culture media at this point would dilute any soluble mediators of coculture interactions, culture was not continued beyond this point for future experiments and the 7 days time point was selected for further characterisation. As anticipated from the *ex vivo* assessment of samples from patients with MM, very few $Th_{17\text{-}1}$ cells were identified under any culture conditions (data not shown).

These results indicate that addition of the HMCL U266 to $CD4^+$ T cell cultures results in an increased proportion of Th_{17} cells in a time-dependent manner. The range of possible explanations for this effect includes induction of Th_{17} cells from naïve precursors, expansion of memory T cells and enhanced survival of existing Th_{17} cells compared to IL-17^- T cells.

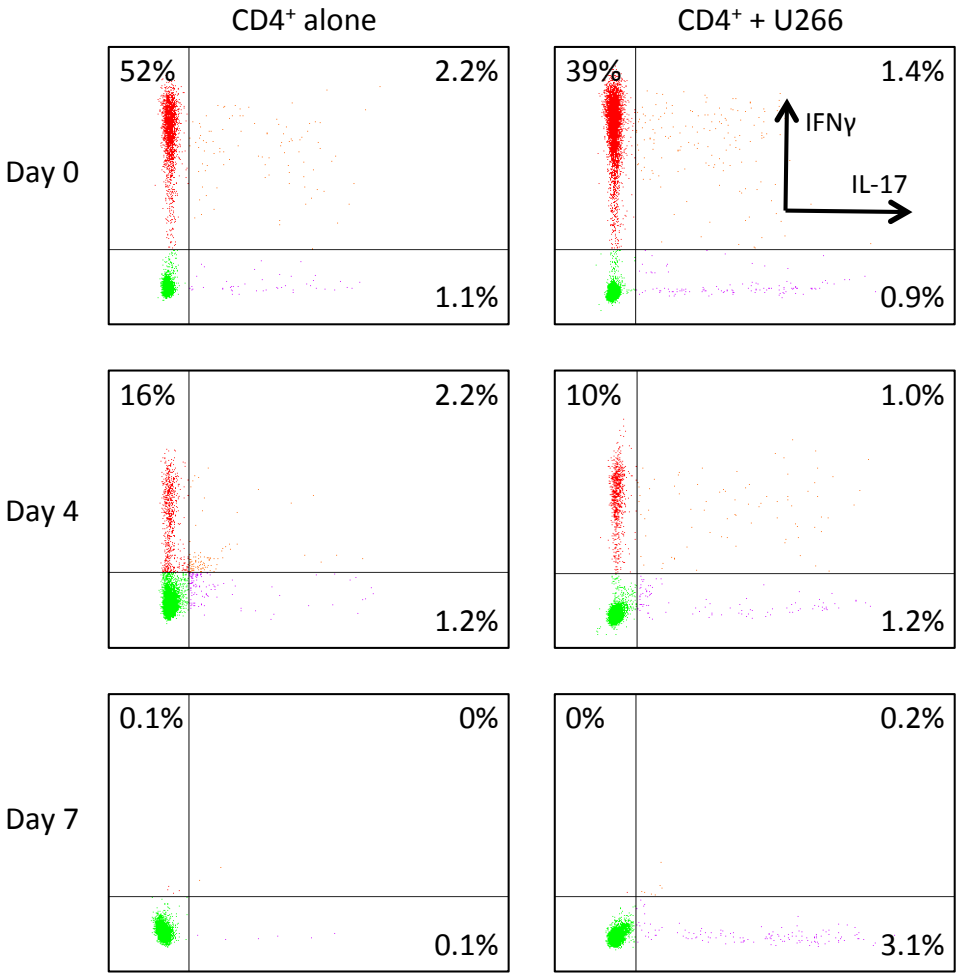


Figure 4.1. Representative dot plots of IL-17 and IFNγ produced by $CD4^+$ cells from a single healthy donor cultured either alone or with U266 for 0, 4 and 7 days.

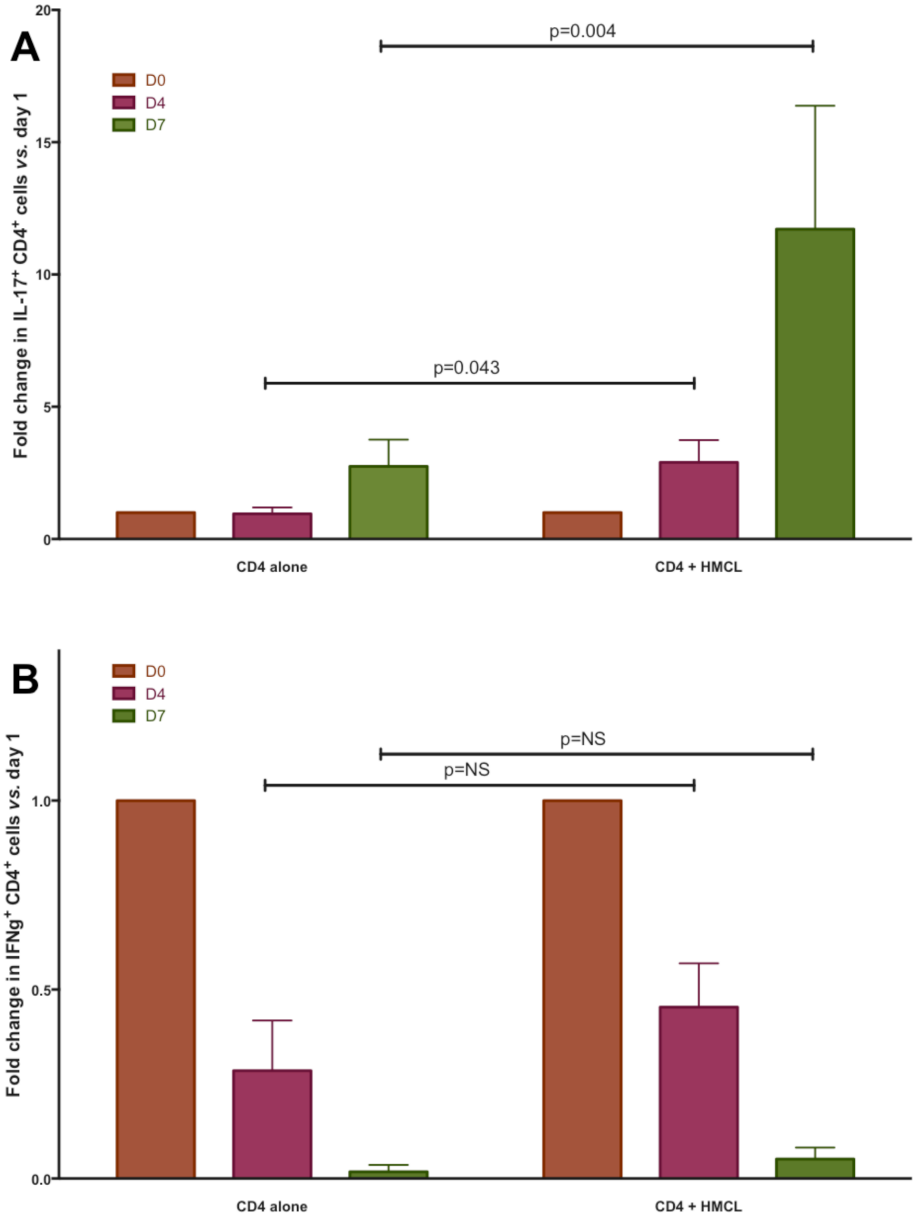


Figure 4.2. Fraction of CD4⁺ cells producing IL-17 (upper panel) and IFNγ (lower panel) after culture either alone (left) or with the HMCL U266B (right) for 0, 4 or 7 days. Percentages of cells are normalised to D0 levels for each donor and expressed as a fold change. Mean values +/- SEM shown, n=5. P values are for paired student t-tests.

In order to confirm this phenomenon was representative of a range of HMCLs, and not an idiosyncratic property of the cell line U266, these experiments were next repeated with a variety of HMCLs (Figure 4.3). After 7 days of coculture, it was found that the majority of HMCLs tested were able to increase the percentage of $CD4^+$ cells able to produce IL-17, compared to isolated culture of $CD4^+$ cells. The EBV-transformed B cell line IM9 was employed as a control and did not increase Th_{17} cells.

On average, U266 was the most effective cell line at inducing Th_{17} cells, whereas JIM3's effect was slightly smaller but more reproducible; these two cell lines were therefore selected for further characterisation.

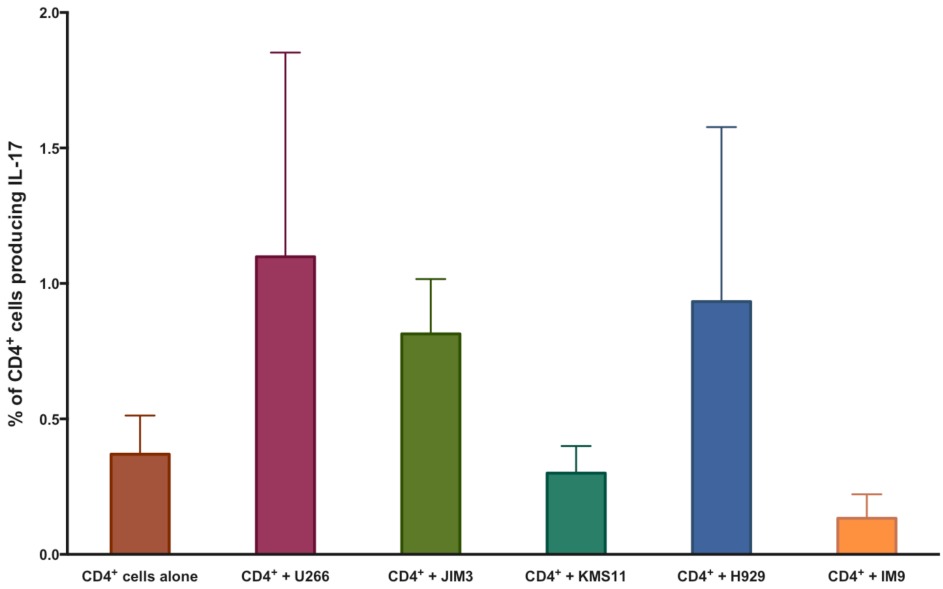


Figure 4.3. Th_{17} cells after culture of $CD4^+$ T cells either alone or in combination with the HMCLs U266, JIM3, KMS11 or H929, or the B-cell line IM9, at a 1:1 ratio for 7 days. Th_{17} cells as a percentage of total $CD4^+$ cells were enumerated as described previously. Mean values were as follows: $CD4^+$ alone: 0.37%, U266 coculture: 1.1%, JIM3 coculture: 0.8%, KMS11 coculture: 0.3%, H929 coculture: 0.9%, IM9 coculture: 0.1%. Fold change *vs.* $CD4^+$ alone: U266 coculture: 2.97, JIM3 coculture: 2.20, KMS11 coculture: 0.81, H929 coculture: 2.52, IM9 coculture: 0.36. The histogram shows mean values +/- SEM, n=5.

Contribution of other immune subsets of peripheral blood

Having established the ability of HMCL to drive differentiation of $CD4^+$ T cells into Th_{17} cells, the contribution of other peripheral blood immune subsets was next investigated by comparison of cocultures of HMCL with either unselected peripheral blood mononuclear cells (PBMC) or negatively-selected $CD4^+$ cells. There were no significant differences in Th_{17}, $Th_{17\text{-}1}$ or Th_1 cells as a percentage of total $CD4^+$ cells, or in fold change, between PBMC and $CD4^+$ in any of the conditions examined (Figure 4.4). Addition of the adherent fraction of the mononuclear cell population (which is enriched for monocytes) to coculture of $CD4^+$ cells with HMCL also had no statistically significant effect on Th_{17} cells (p=NS, student's t test, data not shown).

These results indicate that the generation of Th_{17} cells in the bone marrow microenvironment in MM can result from direct interactions between MM PCs and T cells, although interactions with other BM-resident cells, such as bone marrow stromal cells, and non-T cell peripheral blood immune subsets remain possible.

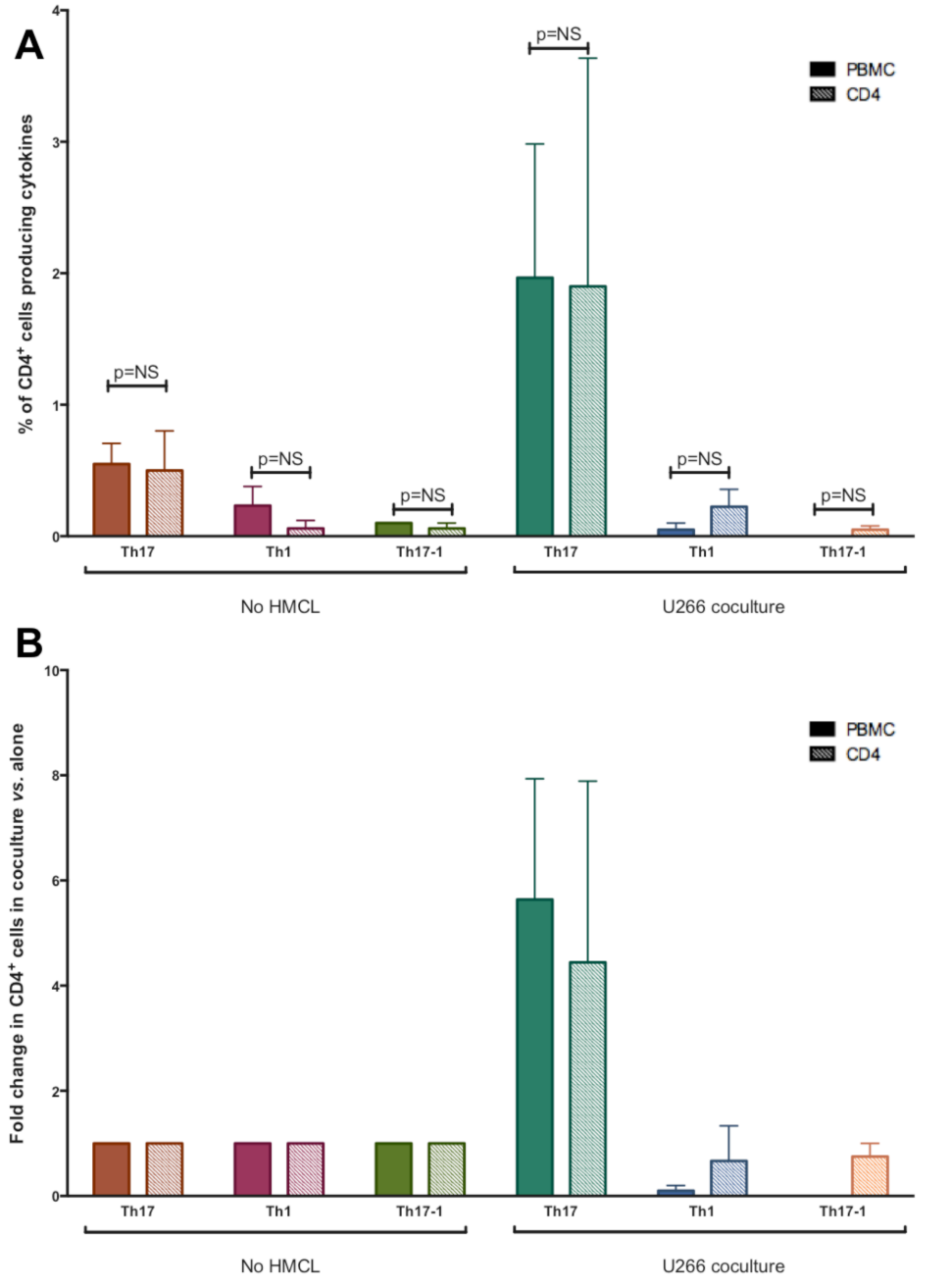


Figure 4.4. Comparison of T cell subsets present after 7 days of culture of PBMC or CD4⁺ cells, either alone or co-cultured 1:1 with U266B. A: absolute number of Th_{17} (IL-17-producing), Th_1 (IFNγ-producing) and $Th_{17\text{-}1}$ (IL-17- and IFNγ-producing) cells; p values are for paired two-tailed t tests. B: fold change in subsets after coculture *vs.* cells cultured alone. Histograms show means and standard deviations.

Contribution of Naïve and Memory T cells

The Th_{17} cells seen in the previous experiments, and in the patients' samples might derive either from expansion of existing memory T cells, or by *de novo* generation from naïve T cells. In order to determine more specifically the origin of the Th_{17} cells identified, the $CD4^+$ starting population was further selected, by MACS, for expression of CD45RA or CD45RO to enrich for naïve and memory T cells respectively. Co-culture starting with these populations yielded similar numbers of Th_{17} cells from $CD4^+$ and $CD4^+CD45RO^+$ populations, with a trend towards a reduced number when starting with $CD4^+CD45RA^+$ cells, although this did not reach statistical significance (Figure 4.5 shows a representative single donor and Figure 4.6 collated data). As expected, addition of adherent mononuclear cells to these cultures did not alter the proportion of Th_{17} cells (data not shown). These results, though not conclusive, suggest that the observed *in vitro* effect of Th_{17} generation after coculture of HMCL with $CD4^+$ T cells is a consequence of expansion of an existing $CD45RO^+$ memory population rather than generation from $CD45RA^+$ naïve precursors.

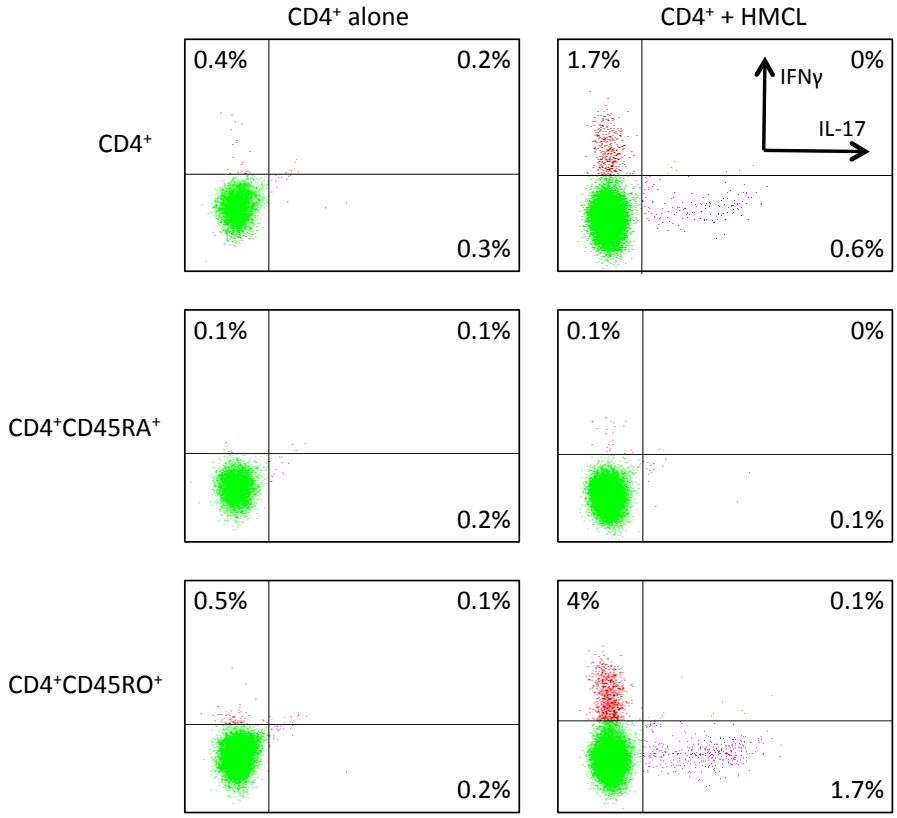


Figure 4.5. Representative dot plots of IL-17 and IFNγ production by $CD4^+$ T cells after 7 days coculture of JIM3 with $CD4^+$, $CD4^+CD45RO^+$ or $CD4^+CD45RA^+$ cells from a single healthy donor.

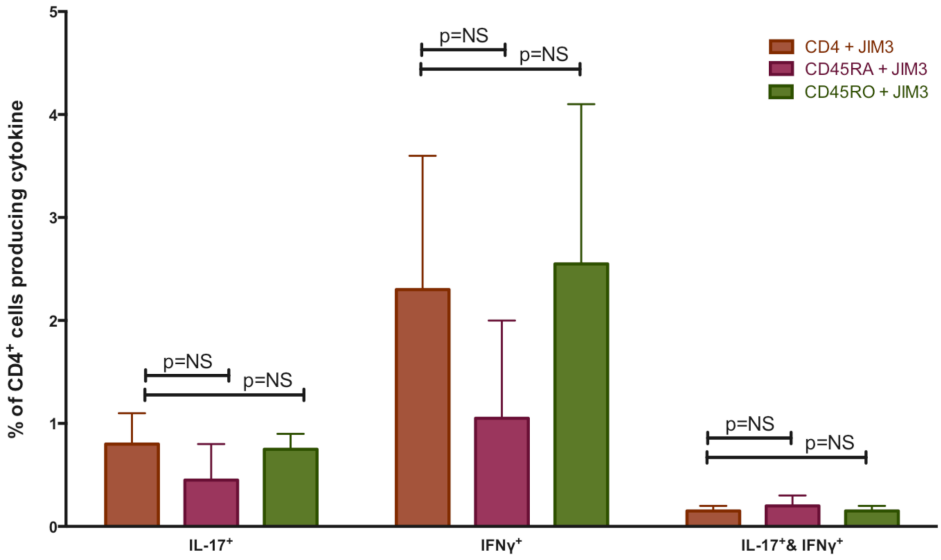


Figure 4.6. Cytokine production by $CD4^+$ T cells after 7 days of coculture of JIM3 with $CD4^+$, $CD4^+CD45RO^+$ or $CD4^+CD45RA^+$ cells. N=2, p values are for paired two-tailed t tests.

Contribution of bone marrow stromal cells

The potential contribution of bone marrow stromal cells to *in vivo* generation of Th_{17} cells in the bone marrow microenvironment was next assessed through incorporation of bone marrow stromal cells into the *in vitro* coculture model. For these experiments, the human bone marrow stromal cells lines HS-5 and HS-27 were used. Stromal cells were placed in culture plates and incubated for 24 hours; each well was then washed to remove non-adherent cells before HMCL and $CD4^+$ T-cells were added as before. Figure 4.7 shows representative dot plots from a single donor; collated data are shown in Figure 4.8.

Coculture of $CD4^+$ T-cells with HS-5 or HS-27 without HMCLs was found to have no significant impact on the presence of IL-17 producing cells (0.19% and 0.2% respectively, compared to 0.16% of $CD4^+$ cells cultured alone; fold changes 1.43 and 1.51 for addition of HS-5 and HS-27 respectively). As previously, addition of JIM3 or U266 to $CD4^+$ induced generation of Th_{17} cells (1.06% and 0.37% respectively; fold changes: 8.38 and 2.56 respectively). However, incorporation of HS-27 into the culture hugely augmented Th_{17} generation by both JIM3 and U266 (5.89% and 5.36% respectively); fold changes: 39.34 and 37.86 respectively); HS-5 increased Th_{17} generation by JIM3, but not by U266 (3.81% and 0.37% respectively; fold changes: 26.59 and 2.63 respectively). Whilst a trend was seen, after 4 repetitions, statistical significance was not reached for either absolute numbers or fold-change in Th_{17} cells, reflecting wide variation in the propensity to Th_{17} generation between donors – indeed some donors generated almost no Th_{17} cells under any culture conditions tested.

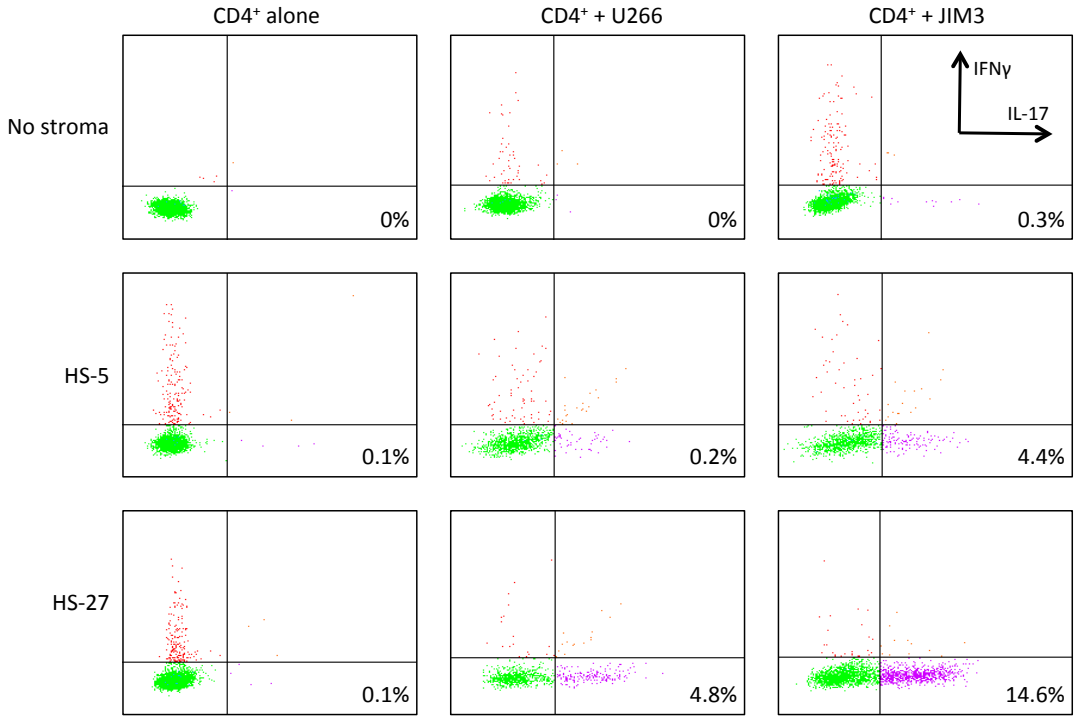


Figure 4.7. Representative dot plots of IL-17 and IFNγ production by $CD4^+$ T cells after 7 days coculture of $CD4^+$ T cells from a single donor with HMCLs (U266 or JIM3) and stromal cells (HS-5 or HS-27).

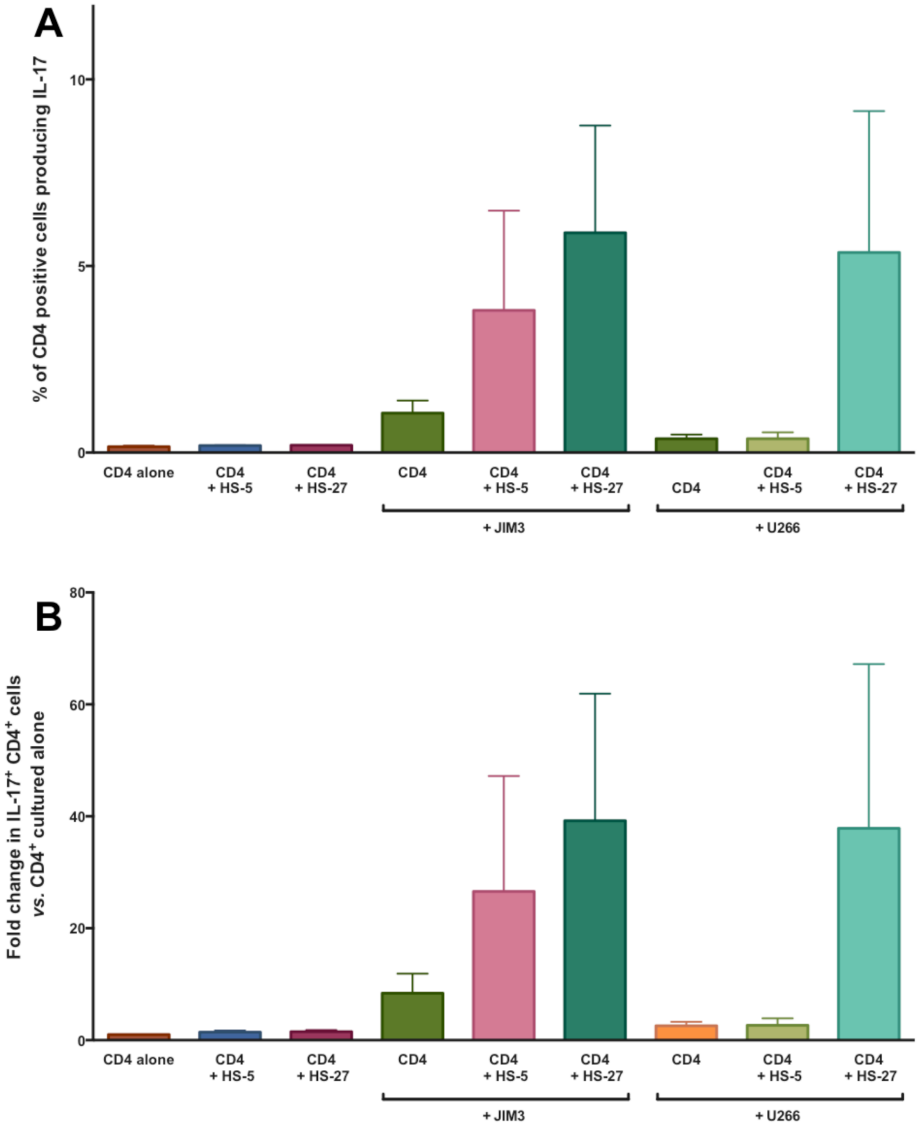


Figure 4.8. Prevalence of Th_{17} cells as a fraction of $CD4^+$ T cells (A) and fold change over $CD4^+$ cultured alone (B) after culture either alone or in combination with the HMCLs U266 and JIM3 and/or the human bone marrow stromal cells HS-5 and HS-27. Histograms show mean values +/- SEM, n=4.

Characterisation of parental population

A broad consensus of literature describes the phenotype of Th_{17} cells as being $CD4^+CD161^+CCR4^+CCR6^+$, however, as demonstrated in Chapter 3, the phenotype of Th_{17} cells in patients with myeloma is aberrant, with attenuated expression of CD161 and CCR6. It was therefore important to determine whether Th_{17} cells in myeloma patients derive from $CD4^+$ cells previously expressing those markers, in which expression of the surface antigens was subsequently down-regulated as a result of immune-tumour interactions, or from surface antigen-negative populations, which have not previously been reported to give rise to Th_{17} cells under any physiological or *in vitro* culture conditions.

In order to address this question, $CD4^+$ cells were labelled with fluorochrome-conjugated antibodies against CD161 and CCR6 and separated using flow cytometric cell sorting into 4 populations: $CD161^-CCR6^-$, $CD161^-CCR6^+$, $CD161^+CCR6^-$ and $CD161^+CCR6^+$ (Figure 4.9). The post-sort purity of sorted populations was >85% in all cases (gates for purity checks were identical to the pre-sort gating; fluorophore bleaching is likely to account for the slight loss of signal intensity seen after sorting). Sorted cells were then cultured alone, with and without JIM3 and/or BMSCs (at 10 T-cell : 10 JIM3 : 1 BMSC ratio). After 7 days of culture, cells were harvested, stimulated with PMA/ION/GP, labelled with fluorochrome-conjugated antibodies and examined by flow cytometry to assess the proportion of $CD4^+$ cells producing IL-17 (Figures 4.10 and 4.11).

Surprisingly, no significant difference in the proportion of IL-17-secreting lymphocytes was found between the four different starting $CD4^+$ populations. Furthermore, the trend that was seen, was towards increased IL-17^+ cells in those cultures starting with $CD161^-CCR6^-$ cells, although due to inter-donor variability, this difference did not reach significance. As previously, coculture with HMCL and BMSC was found to result in the highest proportion of IL-17-secreting $CD4^+$ lymphocytes irrespective of starting $CD4^+$ population. Figures 4.10B and 4.11B show the generation of Th_{17} cells expressed as a fold increase over the same starting T cell subset cultured alone; error bars are not shown on some histograms since Th_{17} cells were often completely undetectable after isolated T cell culture meaning a fold increase could not be calculated.

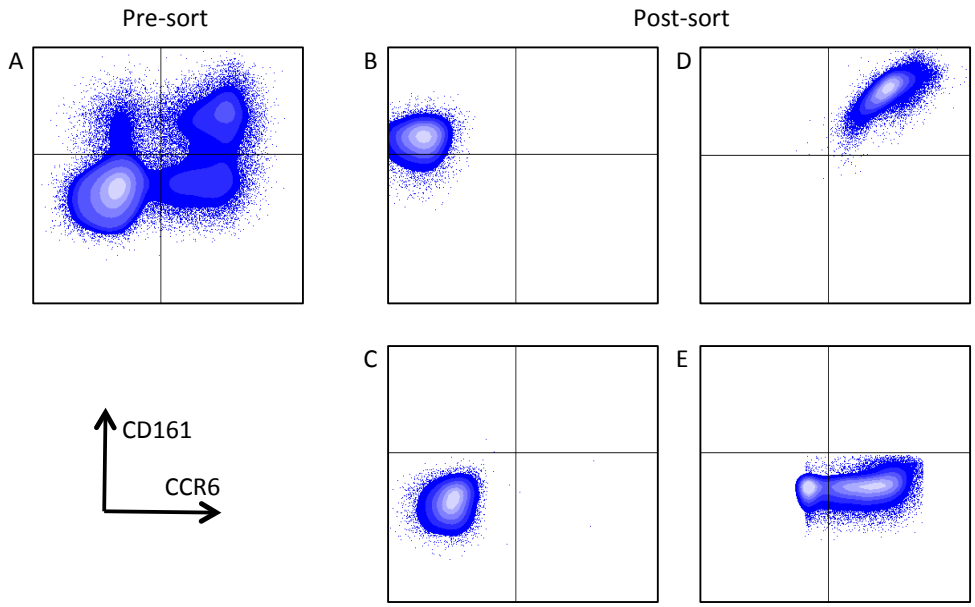


Figure 4.9. Expression of CD161 and CCR6 on all $CD4^+$ T cells before (A) and sorted populations after (B-E) sorting by FACS.

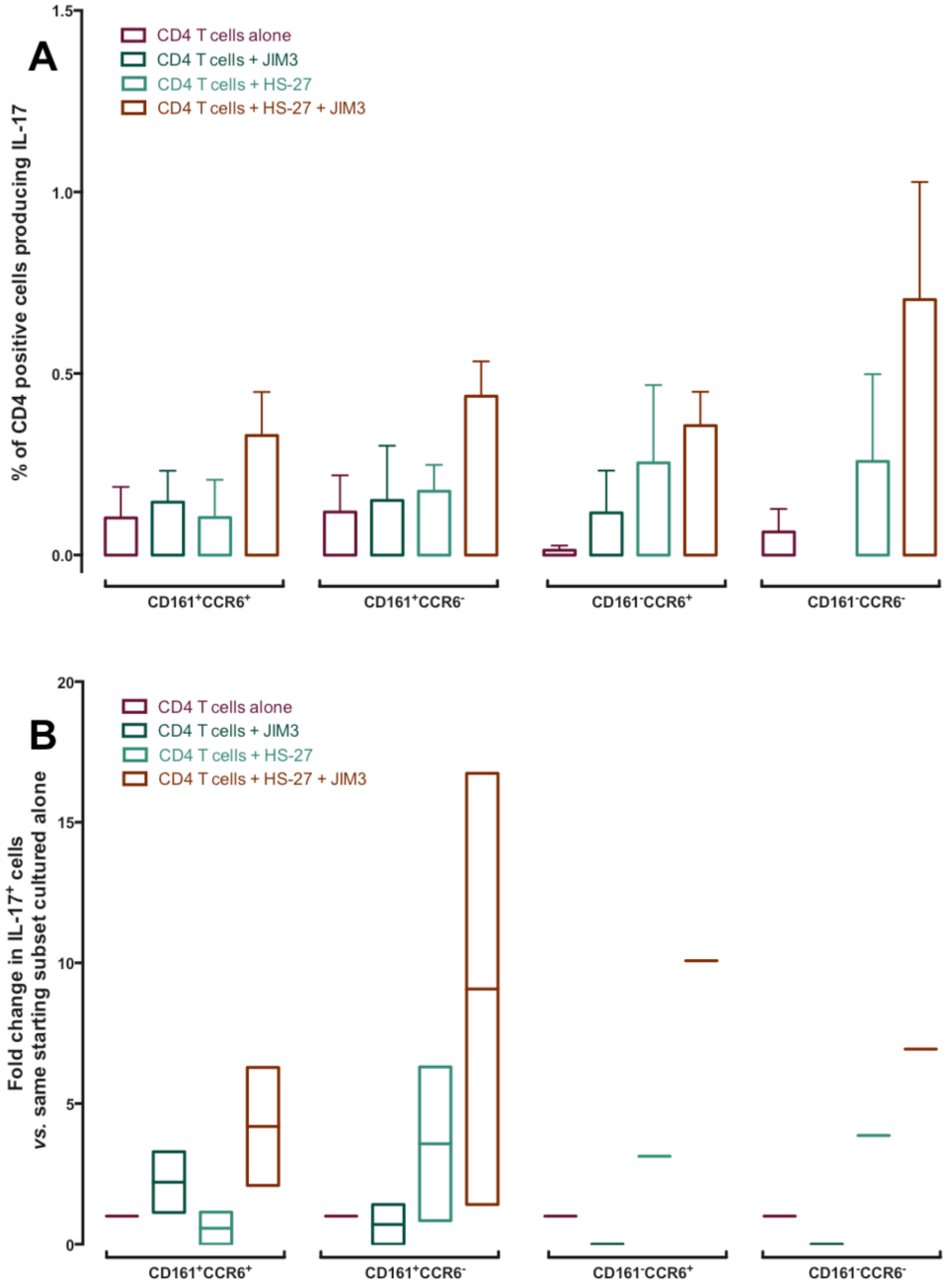


Figure 4.10. Expression of IL-17 by CD4⁺ T cells sorted by expression of CCR6 and CD161 (starting population showed with square brackets on x-axis), and cultured either alone or with JIM3 and/or HS-27. After 7 days of culture, cells were harvested, stimulated with PMA/ION/GP, stained with fluorochrome-conjugated antibodies against CD4, IL-17 and IFNγ, and assessed by flow cytometry. Percentage of CD4⁺ cells producing IL-17 (A) and fold change in IL-17-producing cells (B) are shown, histograms show means, error bars indicated SEM, n=3.

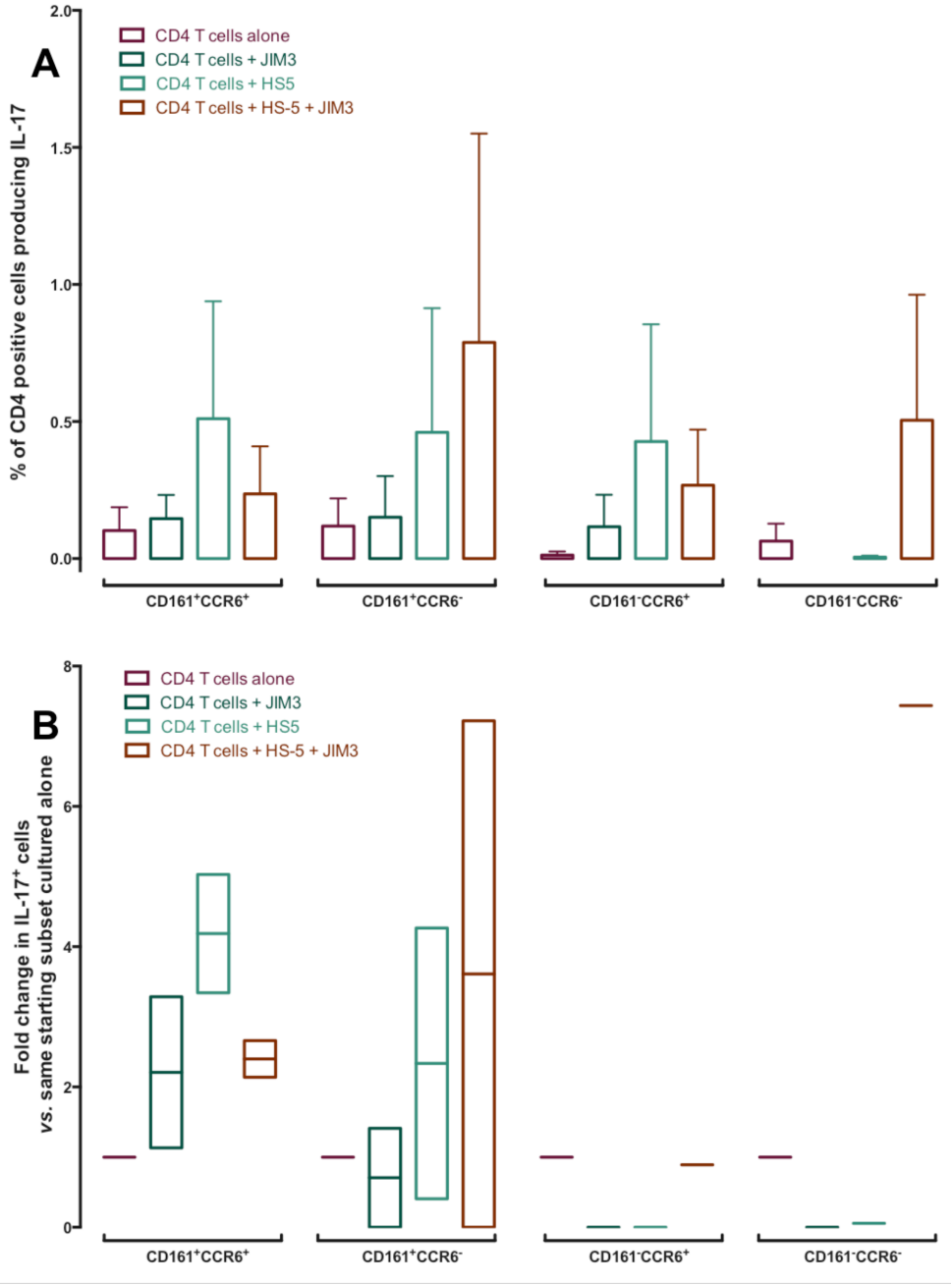


Figure 4.11. Expression of IL-17 by CD4⁺ T cells sorted by expression of CCR6 and CD161 (starting population showed with square brackets on x-axis), and cultured either alone or with JIM3 and/or HS-5. After 7 days of culture, cells were harvested, stimulated with PMA/ION/GP, stained with fluorochrome-conjugated antibodies against CD4, IL-17 and IFNγ, and assessed by flow cytometry. Percentage of CD4⁺ cells producing IL-17 (A) and fold change in IL-17-producing cells (B) are shown, histograms show means, error bars indicated SEM, n=3.

In view of the abnormal expression of CD161 seen on peripheral blood Th_{17} cells in myeloma, CD161 expression by $CD4^+$ cells after each of the culture conditions was next examined (Figure 4.12). Interestingly, although purity checks had confirmed successful sorting of populations according to CD161 and CCR6 expression (Figure 4.9), CD161 expression was noted to be low after isolated culture of $CD4^+$ T cells, irrespective of CD161 status at the beginning of co-culture ($CD161^-CCR6^-$ starting population: 2.69%, $CD161^-CCR6^+$: 3.16% $CD161^+CCR6^-$: 8.5%, $CD161^+CCR6^+$: 18.6%, p=NS). This might reflect internalisation of CD161 after ligation by an antibody for sorting.

Addition of JIM3 to culture conditions had no effect on CD161 expression at the end of coculture. However, addition of either HS-5 or HS-27 to culture conditions significantly increased CD161 on T cells at the end of coculture, irrespective of both starting population and presence of JIM3 (p=0.0262, 2-way ANOVA).

These results suggest the stromal cell lines HS-5 and HS-27 are able to induce CD161 expression on $CD161^-$ T cells; addition of JIM3 may augment this effect, although the latter effect did not reach significance. It is conceivable that stromal cells might instead have lead to differential expansion of contaminating $CD161^+$ cells, although this appears unlikely since the levels of $CD161^+$ cells after coculture might then be expected to be higher in the cocultures starting with $CD161^+$ cells.

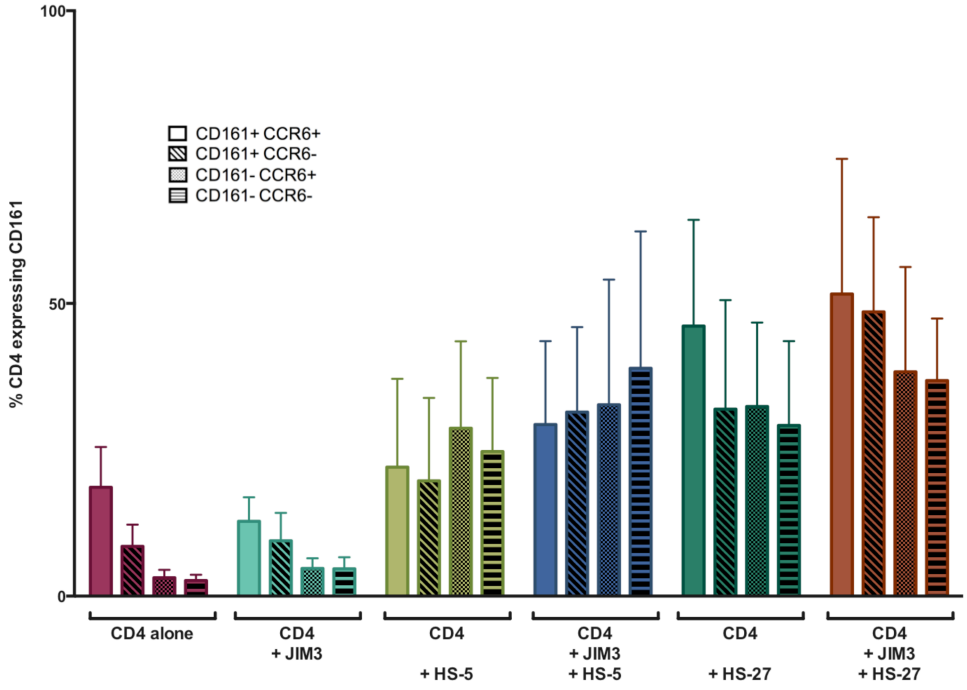


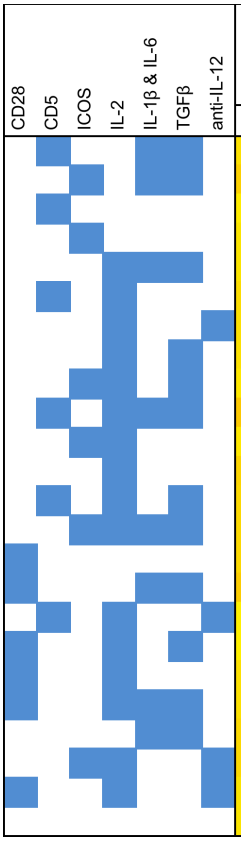
Figure 4.12. Expression of CD161 after culture by CD4⁺ T cells initially sorted on expression of CCR6 and CD161, and cultured either alone, or with JIM3 and/or HS-27 or HS-5. After 7 days of culture, cells were harvested, stimulated with PMA/ION/GP, stained with fluorochrome-conjugated antibodies against CD4, IL-17 and CD161, and assessed by flow cytometry. Histograms show median, error bars indicated SEM, n=3.

Development of protocol for Th_{17} generation/expansion from peripheral blood $CD4^+$ T cells

In order to begin to assess the mechanisms through which the differentiation or expansion of Th_{17} cells might be driven, and to allow comparison of the effects of these mechanisms in health and disease, it was next necessary to develop protocols to drive differentiation of Th_{17} cells *in vitro*. As discussed previously, an extensive literature over the last ten years has attempted to delineate the optimal conditions for Th_{17} generation. However, a number of differences are evident between 'optimal' protocols, probably due to discrepancies in techniques, reagents, starting populations and definitions of Th_{17} cells. For these reasons, the optimal conditions for Th_{17} generation were reassessed experimentally.

Initially, a range of culture conditions were selected from existing published work and tested for their ability to drive differentiation/expansion/preferential survival of Th_{17} cells from the peripheral blood of a healthy donor. In keeping with the classical tenet of T cell immunology that the fate of T cells depends on the integration of T cell receptor stimulation (signal 1), co-receptor ligation (signal 2) and the prevailing cytokine milieu (signal 3), each of these signals was varied (Figure 4.13).

For this initial screen, peripheral blood mononuclear cells were isolated from a healthy donor using Lymphoprep density centrifugation. $CD4^+$ T cells were then isolated using MACS ($CD4^+$ T cell Isolation Kit II, Miltenyi Biotec, UK), and cultured at 10^6/ml in CM; 10^6 $CD4^+$ cells were seeded for each condition and time point. T cell receptor stimulation and signal 2 stimulation were provided by anti-biotin MACSiBead particles (Miltenyi Biotec) loaded with combinations of biotinylated antibodies against CD3 and CD28, CD3 and CD5, or CD3 and ICOS. A range of cytokines was also added: IL-2 (200IU/ml), IL-1β (10ng/ml), IL-6 (20ng/ml), TGFβ (5ng/ml), and anti-IL-12 neutralising antibody (1ng/ml). After 24, 48, 72 and 168 hours, cells were harvested, stimulated with PMA/ION/GP and assessed by flow cytometry for total number of live cells, and for expression of IL-17 by $CD4^+$ cells (Figure 4.13).



% of CD4 cells producing IL-17				Total number of CD4 cells			
24 hours	48 hours	72 hours	7 days	24 hours	48 hours	72 hours	7 days
0.39	0.64	0.15	3.53	2852	5032	1311	16170
0.64	0.58	0.68	3.34	17988	5895	8227	10397
0.24	0.12	0.19	2.16	5916	5685	8356	6946
0.37	0.44	0.11	1.97	7554	11584	8292	7004
0.37	0.11	0.65	1.71	22632	9001	19336	5452
0.49	0.76	0.78	1.19	5140	6091	8624	50361
0.42	0.29	0.09	0.99	12720	15057	13993	6560
0.41	0.19	0.13	0.86	16460	22709	27042	9208
0.29	0.42	0.03	0.85	13150	5500	5802	118157
0.73	0.52	0.3	0.63	5234	3060	675	124188
0.32	0.42	0.29	0.63	15862	9282	1369	56734
0.58	0.4	0.17	0.61	20423	9437	21430	10027
0.5	0.88	1.35	0.48	4774	6486	8386	104629
0.54	0.95	0.35	0.45	13712	6641	5164	107085
0.5	0.22	0.87	0.39	5946	3168	13162	63268
0.71	0	1.66	0.32	12944	1631	11117	70240
0.17	0.53	1.25	0.31	5856	4912	2795	81727
0.18	0.58	1.37	0.27	6122	2394	7826	65447
0.43	0.1	0.05	0.21	10864	5801	5783	78032
0.41	1.47	1.84	0.21	12185	2378	13431	31120
0.53	0.18	0.08	0.12	20525	14993	19907	8880
0.51	0.65	0.36	0.11	19356	12043	6128	49036
0.37	0.86	0.41	0.1	13346	5445	9066	38309
0.37	0.23	0.05	0.06	7538	15916	29995	8499

Figure 4.13. Total cell numbers and IL-17 production after stimulation of CD4⁺ T cells with combinations of TCR ligation, signal 2 and cytokines (left panel) and culture for 24, 48, 72 and 168 hours. At the end of culture, cells were restimualted with PMA/ION/GP and assayed by flow cytometry for the percentage of cells producing IL-17 (middle panel) and the total number of CD4⁺ cells (right panel).

Considerable variation was noted between culture conditions in terms of both the total numbers of $CD4^+$ T cells present at the end of culture, and the percentage of them producing IL-17 after re-stimulation. As expected, those conditions giving rise to the largest number of $CD4^+$ cells in total were not the same as those producing the maximal proportion of $CD4^+$ cells making IL-17. IL-2, well-described as a growth factor for $CD4^+$ T cells was present in many of the combinations giving rise to the largest numbers of $CD4^+$ cells, but was absent from the top four combinations for Th_{17} percentage yield. CD3/28, the TCR and signal two combination conventionally used for stimulation of T cells did indeed appear to produce high numbers of $CD4^+$ cells in most cases. However, surprisingly, this combination was not effective in producing a high proportion of Th_{17} cells, which was instead maximal after signal two stimulation targeting either CD5 or ICOS. As predicted from the published literature, the cytokine combination of IL-1β, IL-6 and TGFβ further enhanced the fraction of $CD4^+$ cells producing IL-17 after CD3/5 or CD3/ICOS stimulation, although was ineffective without TCR stimulation. Blockade of IL-12 with neutralising antibodies appeared to have little effect on the cultures. Unsurprisingly, but importantly, considerable interactions were apparent between the different variables assessed.

On the basis of these preliminary data, experiments were next designed to assess in turn each of the most significant variables on a larger panel of donors. TCR/signal 2 combinations were first assessed with and without the 'Th_{17} cytokine cocktail' (IL1β, IL-6 and TGFβ). As with the initial screen, CD3/ICOS with the 'Th_{17} cocktail' was found to be the most potent inducer of Th_{17} cells (Figure 4.14). As previously, considerable variation was noted between donors in terms of their propensity to form Th_{17} cells after culture, and as a consequence results did not reach statistical significance.

The fraction of $CD4^+$ cells producing IFNγ (Th_1 cells) under the same conditions was next examined, and the ratio of cells producing IL-17 to those producing IFNγ calculated (Th_{17}:Th_1 ratio; Figure 4.15). This indicated that whilst all other combinations tested decreased the IL-17 to IFNγ ratio, the CD3/ICOS 'Th_{17} cocktail' combination actually increased the proportion of Th_{17} cells relative to Th_1 cells.

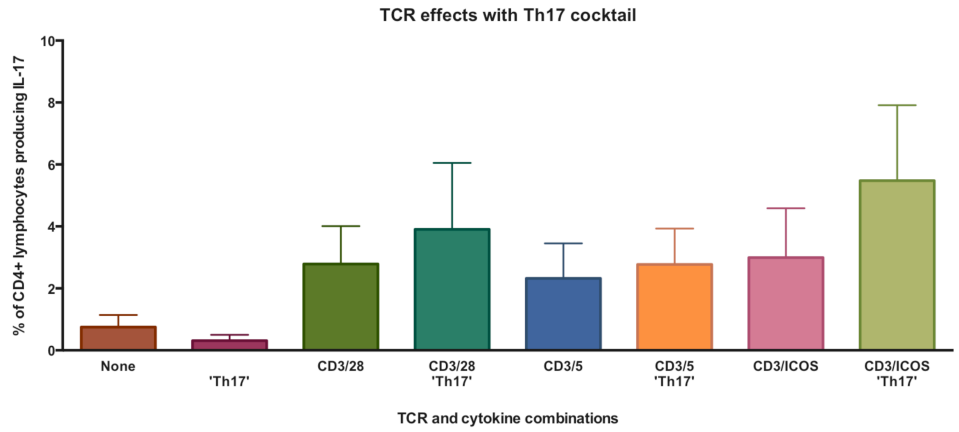


Figure 4.14. Percentage of $CD4^+$ T cells producing IL-17 on restimulation after prior culture with combinations of TCR and signal 2 stimulation, with and without Th_{17}-polarising cytokines ('Th_{17}'; IL-1β 10ng/ml, IL-6 20ng/ml and TGFβ 5ng/ml). N=3

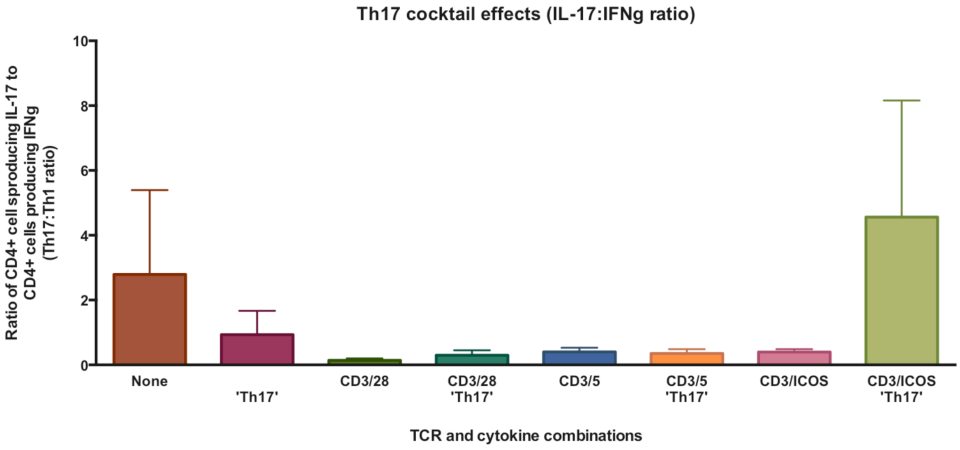


Figure 4.15. Ratio of $CD4^+$ T cells producing IL-17 to $CD4^+$ cells producing IFNγ on restimulation after prior culture with combinations of TCR and signal 2 stimulation, with and without Th_{17}-polarising cytokines ('Th_{17}'; IL-1β 10ng/ml, IL-6 20ng/ml and TGFβ 5ng/ml). N=3

The interactions between IL-2 and the other variables tested was next assessed. Whilst IL-2 had previously been found to increase the total yield of $CD4^+$ T cells at the end of culture, the addition of IL-2 decreased the ratio of IL-17-secreting to IFNγ-secreting $CD4^+$ cells under all conditions tested (Figure 4.16).

A number of recent publications have examined other variables that may affect Th_{17} polarisation, and the effect of a number of these factors on IL-17-secretion by $CD4^+$ T cells after culture with CD3/ICOS stimulation and the 'Th_{17} cytokine cocktail' was next examined (Figure 4.17). CD25-depletion, culture in the dark or in IMDM instead of RPMI and addition of tryptophan all actually decreased the fraction of $CD4^+$ cells producing IL-17 at the end of culture, although this did not reach significance. Culture under controlled hypoxic conditions was found to increase the generation of Th_{17} cells; again, this did not reach statistical significance.

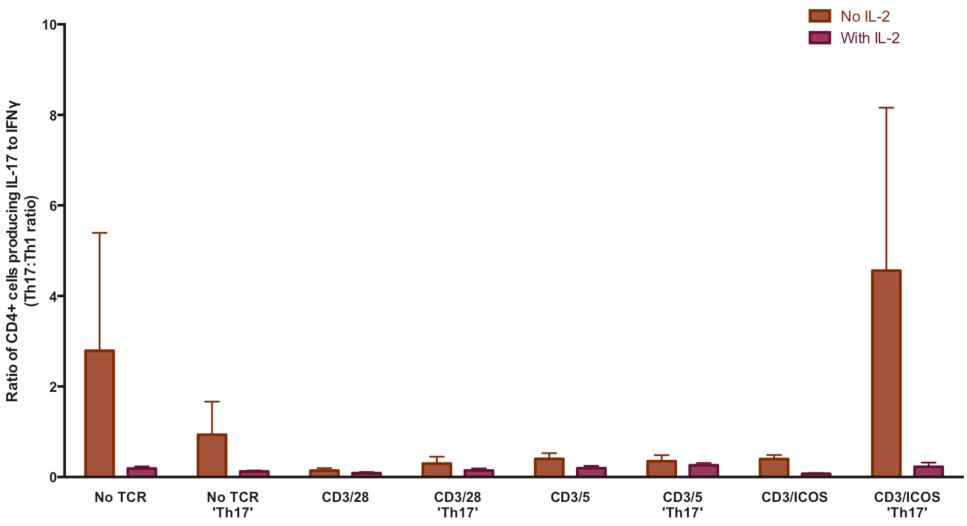


Figure 4.16. Ratio of $CD4^+$ T cells producing IL-17 to $CD4^+$ cells producing IFNγ on restimulation after prior culture with combinations of TCR and signal 2 stimulation, Th_{17} polarising cytokines ('Th_{17}'; IL-1β 10ng/ml, IL-6 20ng/ml and TGFβ 5ng/ml) and IL-2 (200IU/ml). N=3

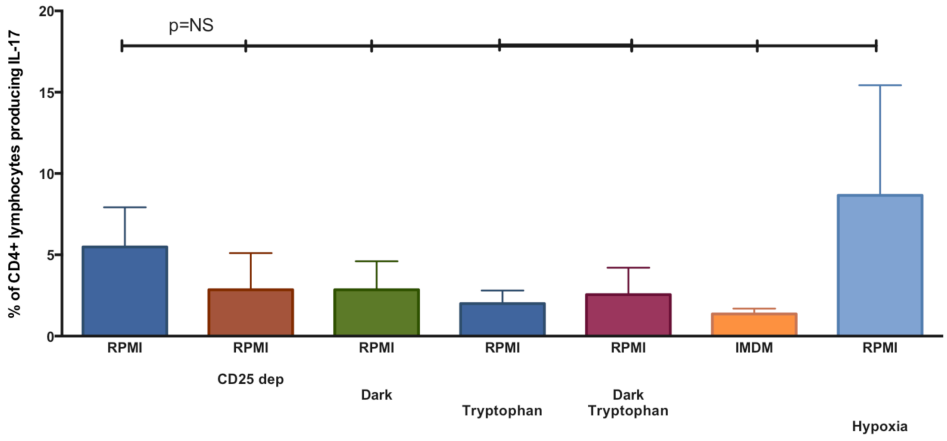


Figure 4.17. Percentage of $CD4^+$ T cells that produced IL-17 on restimulation, after culture with combinations of culture media (RPMI or IMDM), CD25 depletion, avoidance of light exposure and tryptophan supplementation. N=3, p=NS for all comparisons.

On the basis of the preceding experiments, the conditions chosen for optimal Th_{17} expansion from unselected $CD4^+$ T cells were CD3/ICOS stimulation, IL-1β, IL-6, TGFβ in RPMI, at 21% O_2 without tryptophan supplementation. Peripheral blood $CD4^+$ T cells from healthy donors and patients with multiple myeloma were next cultured under these conditions and the fraction of Th_{17} cells at the end of culture enumerated (Figure 4.18 shows dot plots from two representative donors and figure 4.19 collated data). With healthy donors, culture under polarising conditions increased the Th_{17} fraction from 0.63% to 1.75% (p=NS; fold change: 2.78), whereas when the myeloma samples were cultured under these conditions, Th_{17} cells were increased from 0.8% to 3.78% (p=0.01; fold change: 4.73).

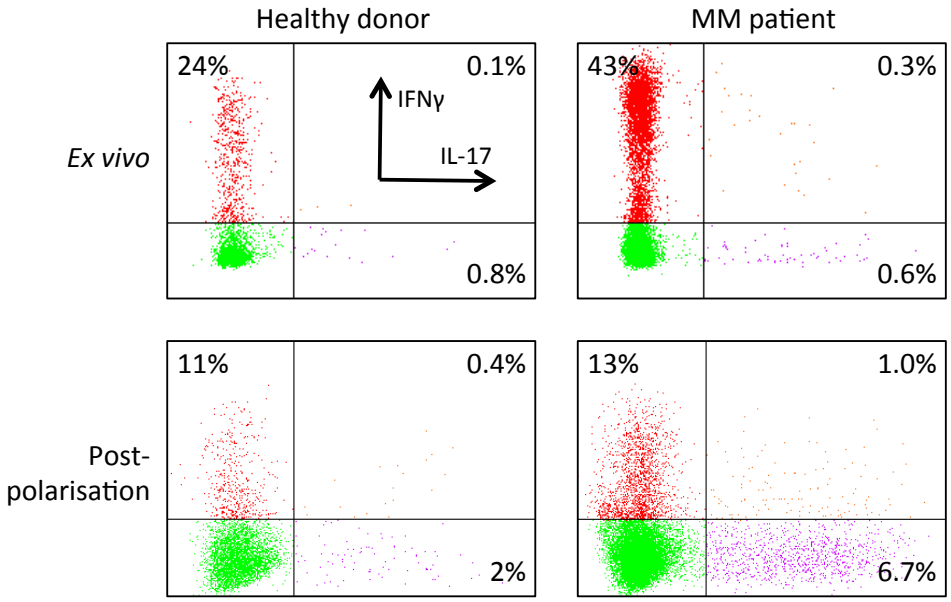


Figure 4.18. IL-17 and IFNγ secretion by CD4⁺ T cells from a healthy donor and a patient with MM, before and after culture under Th_{17} polarisation conditions.

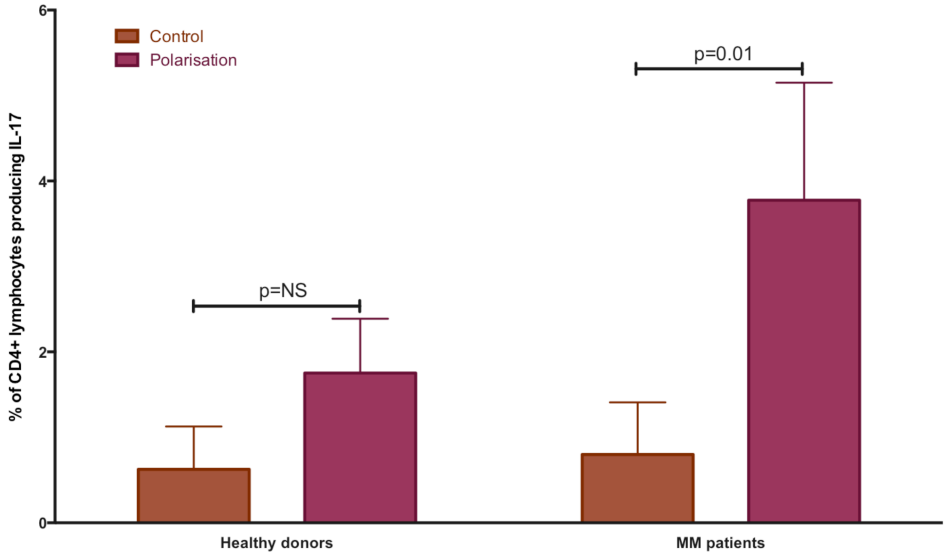


Figure 4.19. Percentage of CD4⁺ T cells from healthy donors (left) and patients with myeloma (right) that produced IL-17 on restimulation after 7 days of culture in CM alone (orange) or with polarisation (CD3/ICOS stimulation, IL-1β, IL-6 & TGFβ; purple). Healthy donors n=10, MM patients n=8. P values are for paired student T tests.

Discussion

The co-culture experiments described here clearly demonstrate an ability of human myeloma plasma cells to modulate T cell lineage determination to increase the frequency of Th_{17} cells after co-culture. This is in keeping with the data presented in the previous chapter, and those published by other authors[74], showing an expansion of Th_{17} cells in the bone marrow of patients with multiple myeloma. In contrast, Th_1 cells, characterised by secretion of IFNγ on re-stimulation, were reduced by co-culture with tumour cells. Sub-selection of $CD4^+$ cells by expression of CD45 isoforms indicated that Th_{17} cells were derived predominantly from $CD45RO^+$ activated and memory T cells.

Taken in conjunction, these results are suggestive of redirection of T cell lineage determination from Th_1 to Th_{17}, however it is also possible that co-culture might provide conditions optimal for survival and expansion of existing Th_{17} cells and/or leading to death or loss of secretory function of Th_1 cells. The lack of a definitive cell surface phenotype for Th_{17} cells, discussed in the previous chapter, means that they can be identified only through stimulation and assay of cytokine secretion, most easily by flow cytometry. Unfortunately, as discussed previously, the only robust method for Th_{17} stimulation was found to be using PMA, ionomycin and brefeldin A, and although the use of cytokine capture-based FACS sorting was tested (data not shown), Th_{17} cells so harvested were found to have extremely low viability and were thus unsuitable for subsequent cell culture. Co-culture of CD161/CCR6-sorted $CD4^+$ cells, which would be expected to enrich or deplete starting populations of Th_{17} cells, revealed no difference in the final proportions of Th_{17} cells, providing further evidence that HMCLs can drive modulation of lineage determination.

Interestingly, whilst a range of HMCLs were found to have this capability to induce Th_{17} cells, the line KMS11 did not display this effect. Analysis of the cell surface and cytokine secretion phenotype of this cell line may be one avenue of investigation into the mechanisms underlying the effect.

Modification of the co-culture model to incorporate other cell types demonstrated that the other immune subsets of peripheral blood had no effect on polarisation of T cells. However, the addition of human bone marrow stromal cells to the cultures yielded marked augmentation of the generation of Th_{17} cells. Since co-culture of $CD4^+$ lymphocytes and BMSC without HMCLs did not result in generation of Th_{17} cells, these

experiments indicate three-way cellular interactions between the cell types are responsible. Although the experiments reported here do not allow dissection of the underlying mechanisms it is of course well known that bone marrow stromal cells can create a permissive microenvironment for MM development[284], and that MM cells in turn may modify the stromal niche to support their own prolfieration[285]; the BMME plays a key role in the pathophysiology of myeloma, tumour cell quiescence, support of cancer 'stem cells' and development of relapse[286]. A number of the known reciprocal positive-feedback interactions between BMSC and MM plasma cells, involving such mediators as Dkk1[284], VEGF[190], IL-6[190] and TGFβ[287] are likely candidates here – the latter two in particular have well-described roles in the polarisation of Th_{17} cells. Future experiments using cytokine supplementation/blocking and transwells to abrogate cell surface interactions could be used to further examine this phenomenon.

When $CD4^+$ cells were sorted by FACS on their surface expression of CD161 and CCR6 prior to culture with HMCLs, no significant difference was seen in the prevalence of Th_{17} at the end of culture. This is surprising, since a robust body of previous evidence indicates that under normal circumstances, human IL-17 producing cells arise exclusively from $CD161^+$ precursors[165,166,288-291]. In the context of rheumatoid arthritis, IL-26-stimulated monocytes have been reported to switch $CD161^-$ memory T cells into $ROR\gamma t^+$ Th_{17} cells, through secretion of IL-1β [292], suggesting that in other inflammatory disease states, some abnormal plasticity may exist. Whilst IL-1β was not found to be increased in the patient cohort examined here by Luminex, other authors have reported increased levels of this cytokine in MM[293-295], correlating this with disease stage[294,296,297]. Furthermore, polymorphisms in IL-1β have been associated with risk of myeloma[298] and outcomes after treatment[299,300]. The role of IL-1β and IL-26 are relatively unexplored in MM and warrant further exploration.

Recently published work also indicates that IL-34- and M-CSF-induced macrophages (which have many similarities to tumour-associated macrophages) are able to induce reconfiguration of $CD4^+CCR6^-CD161^-IL23R^-$ T cells into Th_{17} cells, through membrane IL-1α[301]. However, in contrast to those reports, CD161 expression was found to be low on the Th_{17} cells induced by the co-culture, irrespective of initial CD161 status. This low level of CD161 expression after coculture is intriguing since sort purities were good meaning CD161 was known to be expressed in the appropriate cells at the beginning of the cultures. Certainly, it is tempting to suggest MM cells or BMSC might reduce CD161 expression, which would be in keeping with a previous publication reporting

downregulated CD161 on Th_{17} cells in patients with head and neck cancer, and suggests alternate mechanisms may exist for Th_{17} generation in cancer. However, in fact, CD161 expression was lowest of all in those $CD4^+$ cells cultured alone after sorting, and alternate mechanisms are therefore probably responsible. For example, ligation of CD161 by monoclonal antibodies prior to FACS may lead to internalisation of the antigen. It is unclear whether this is a physiologically relevant phenomenon.

In the context of the observational data presented in the previous results chapter, the coculture experiments yield some interesting observations. Firstly, they corroborate the hypothesis that the expansion of Th_{17} cells seen in patients with MM results from interactions between the tumour clone, BMSC and $CD4^+$ T cells in the marrow. This also provides a putative explanation for the relatively greater expansion seen in BM compared to PB. Secondly, with respect to the loss of cell surface expression of CD161 and CCR6 on Th_{17} cells in MM, the coculture experiments are strongly suggestive that MM-BMSC-$CD4^+$ interactions are able to subvert the normal rules of Th_{17} lineage determination and induce IL-17 production from $CD161^-$ cells.

These observations in turn lead to a number of further questions. Firstly, modification of the coculture model will allow more detailed dissection of the actual mechanisms leading to Th_{17} generation. For example, use of contact-free culture systems (e.g. Transwells), culture supernatants (e.g. tumour-conditioned media), cytokine supplementation and specific cytokine and cell surface blocking will allow determination of the relative contributions of contact- and soluble factor-mediated mechanisms. This is an important question, particularly since this model is based on a modified one-way mixed lymphocyte reaction and is therefore allogeneic

Secondly, the *in vitro* work conducted to date does not compare the functional characteristics of Th_{17} cells derived from tumour cell cocultures and those derived from healthy donor peripheral blood or induced by cytokine polarisation. This line of investigation could firstly employ stimulation with the MP65 protein, and then build upon the functional assays postulated in the previous results chapter (e.g. neutrophil migration, epithelial immune responses and trafficking in response to chemokine gradients). It should be noted that since Th_{17} cells have proven so difficult to isolate from either patients or healthy donors, characterisation of the effects of naturally occurring Th_{17} cells from the two states may not prove possible, but assessment of the cytokines individually or in combination through coculture is a natural extension of this work.

Thirdly, assessment of the effects of CD161 engagement, e.g. by monoclonal antibodies, might also be revealing, as may examination of MM BM by immunohistochemistry to assess localisation of Th_{17} cells in relation to tumour cells and BMSC. Effects of IL-17 on osteoclast function have also been reported[199] and immunohistochemistry may provide a useful avenue for examining this further.

Finally, and perhaps most importantly of all, work should now be undertaken to examine in detail the effect of Th_{17} cells, and their cytokines, on the growth of tumour cells, and their survival after chemotherapeutic attack (in addition to IL-17, other Th_{17} cytokines such as IL-22, IL-21 and TNFα might also play a role[196]). These experiments will likely prove difficult to conduct using HMCLs since the majority of these are highly proliferative, grow in the absence of cytokine supplementation and respond poorly to a number of chemotherapy strategies. Prabhala *et al.* reported acceleration of cell division in HMCLs treated with IL-17 [74], although the concentrations used were not specified and attempts to repeat these experiments have demonstrated no effect on growth of a range of HMCLs at a range of concentrations of rhIL-17 (data not shown). Development of a protocol for harvesting and *in vitro* culture of primary tumour cells may therefore be necessary to conduct these experiments.

In order to examine the propensity of $CD4^+$ T cells to become Th_{17} cells and to begin to dissect the mechanisms that might underlie the generation of Th_{17} cells in myeloma, a second group of experiments were next undertaken to create an optimised protocol for driving Th_{17} generation *in vitro*. In addition, since Th_{17} cells are such a rare population, it was envisaged that *ex vivo* expansion might facilitate future work such as more detailed phenotyping and assessment of the role and functional capacity of Th_{17} cells in health and disease. A body of work by multiple authors has examined the issue of Th_{17} cell expansion, often with conflicting findings[91-99,102,109-112], and the data presented here may shed some light on the potential reasons for this.

It was apparent from the initial screening experiments that those culture conditions giving rise to the largest numbers of $CD4^+$ cells were quite distinct from those producing the highest proportion of IL-17-secreting $CD4^+$ cells. This is not surprising, but underlines the importance of considering the starting population when determining the optimal polarisation cocktail. For example, IL-2 has long been recognised as a major promoter of clonal expansion in T cells, but its role in Th_{17} polarisation protocols has been the subject of some debate in the literature with the majority of authors advocating its use[302-305] and others not incorporating or even blocking it[306], or using it only for the maintenance of

previously sorted populations[129]. In the polarisation experiments, IL-2 was found to augment total T cell numbers, and therefore potentially the total number of Th_{17} cells, but potently reduced the proportion of $CD4^+$ cells producing IL-17 – i.e. the purity of the population. It is likely therefore, that the effects of IL-2 may depend heavily on the starting population with a potential favourable role in purified populations, and adverse effects seen in mixed populations where it may allow other subsets (e.g. Th_1 cells) to out-compete Th_{17} cells, or may even directly suppress IL-17 production. The recognition that, in mice at least, STAT5 signalling, which is up-regulated by IL-2, can oppose Th_{17} differentiation[117], and of the inability of Th_{17} cells to produce and their reduced ability to respond to, IL-2[307], provide additional support for this conjecture.

When TCR and signal 2 stimulation was examined, Th_{17} cells were found to expand optimally not after stimulation with CD3 & CD28, but rather with either CD3 and CD5 or CD3 and ICOS stimulation. CD5 co-stimulation has previously been reported as promoting Th_{17} development, through sustained STAT3 activation and up-regulation of IL-23R expression[129]. Similarly, ICOS has been shown to be superior to CD28 co-stimulation in driving differentiation of both Th_{17} and Th_{17-1} cells [128]. These findings may partly reflect the inability of Th_{17} cells to produce IL-2 in response to CD3/28 stimulation[307], although the signalling involved in these alternate signal 2 pathways is also probably discrete – for example ICOS induces both c-MAF and IL-21 thereby augmenting Th_{17} function[128], and also appears to have a role in polarisation of Th_{17-1} cells[128,308] , although these were not seen at appreciable levels in the polarisation experiments. The central role of ICOS in Th_{17} generation is of interest in light of previous work from our group which has shown ICOS/ICOS-L interactions to be central to the generation of regulatory T cells in myeloma[309], and offers a further likely mechanism for the generation of Th_{17} cells by myeloma plasma cells – this will require exploration in further work.

As predicted from the majority of the body of published work on Th_{17} polarisation[91-99,102,109-112], the combination of TGFβ with pro-inflammatory cytokines such as IL-1β & IL-6 was found to enhance Th_{17} generation. IL-23 has also been ascribed a role in stabilisation of the Th_{17} phenotype, however, currently available sources of rhIL-23 were found to be financially prohibitive and this was therefore not investigated further. Interestingly, blockade of IL-12, an often-employed component of Th_{17} polarisation protocols, was found to have no significant efficacy. Whilst this may relate to the particular reagent or concentrations used, these were in keeping with published work and

the result might equally reflect minimal generation of IL-12 under the best Th_{17}-polarising conditions due to minimisation of Th_1 proliferation and cytokine secretion.

Hypoxia-inducible factor 1 (HIF-1) is a key metabolic sensor that is known to be one of the mediators regulating the balance between regulatory T cell (T_{reg}) and Th_{17} differentiation via multiple mechanisms[151]. It has previously been shown that HIF-1α mRNA is upregulated in murine T cells under Th_{17}-skewing conditions, in a STAT3-dependent manner[151]. Since it is now well-known that the bone marrow microenvironment in myeloma may be hypoxic[310], experiments were undertaken to examine the effect of culture in hypoxic conditions (5% O_2, 37°C); this modification of the coculture tended to increase generation of Th_{17} cells, although this did not reach significance. Unfortunately, further exploration of this avenue of investigation was not possible at the time of these experiments due to technical problems with the hypoxic incubator used, but this may warrant revisiting. Immunohistochemical analysis of bone marrow trephine biopsies for colocalisation of IL-17 (or RORC) and HIF-1α, or for localisation of Th_{17} cells within the micro-anatomy of the marrow might also be a useful way of assessing the true contribution of hypoxia to Th_{17}-generation in the marrow of patients with MM.

Th_{17} cell differentiation is also known to be modulated by activation of the aryl hydrocarbon receptor (AHR); Veldhoen *et al.* found that the presence of endogenous AHR agonists present in cell culture medium was thereby able to modulate the proportion of T cells able to produce IL-17 after culture, with Iscove's modified Dulbecco's medium (IMDM), which is richer in aromatic amino acids that give rise to AHR agonists, resulting in consistently greater Th_{17} expansion in both human and murine cells[306]. Supplementation of RPMI with ι-tryptophan was found to increase Th_{17} polarisation. Interestingly at least one tryptophan metabolite, FICZ, a high-affinity AHR ligand, is known to be produced through exposure of tryptophan to normal laboratory light conditions. However, in the experiments reported here, no impact of IMDM use, light exposure or tryptophan supplementation was seen. Whilst these factors might have provided useful means of increasing the yield of Th_{17} cells to allow more detailed experimental characterisation, they seem less likely to play a role in myeloma and were not examined further.

Finally, since there is considerable evidence of reciprocal interactions in the development of T_{reg} and Th_{17} cells, as well as suppression of IL-17 secretion by T_{reg} populations[305], CD25 depletion was undertaken, as described previously, to examine the effect of removal of the natural T_{reg} population on Th_{17} numbers. This modification resulted in a

non-significant decrease in Th_{17} cells, possibly reflecting removal of T_{reg} which through T cell plasticity might provide one portal for Th_{17} generation.

When the optimised Th_{17} polarisation protocols were applied to a larger number of samples from healthy donors, a modest and non-significant increase in Th_{17} cells was noted. The difficulty in generating significant numbers of Th_{17} cells reflects the published experience, and is a very significant hurdle to the characterisation of these cells in health and disease. Nonetheless, when peripheral blood samples from patients with myeloma were treated with the sample protocol, a significant increase in Th_{17} cells was seen. This suggests that not only do MM patients have increased levels of circulating Th_{17} cells, but that in addition their non-committed T cells have a higher propensity to become differentiated Th_{17} cells. This may partly reflect the increased numbers of circulating T_{reg} cells in MM patients[309], which are known to exhibit the plasticity typical of T cell subsets. In addition, a number of the key cytokines in Th_{17} development are known to be increased in the peripheral blood of patients with myeloma[189,190,311], which may render T cells 'primed' for Th_{17} generation. Addition to cocultures of the Th_{17} polarisation cocktail and/or neutralising antibodies against the cytokines, may yield further insights into the mechanisms of Th_{17} generation in the cocultures. Use of the cocktail on CD161/CCR6-sorted populations may also allow further clarification of whether HMCLs are able to induce Th_{17} cells from a population otherwise unable to develop in this way, as appears to be the case from the data presented.

The data presented in this chapter provide explanations for many of the observations detailed in the previous chapter. The increase in Th_{17} cells seen in patients with MM is explained by cellular interactions occurring within the BMME, which lead to relative expansion or preferential survival of Th_{17} cells relative to other T cell subsets. Moreover, it appears that these interactions may lead to expansion of Th_{17} cells from precursor populations previously thought unable to give rise to this lineage of cells. A number of potential avenues of further investigation are apparent and will form the basis of future work.

Chapter 5 – Oncolytic virotherapy in Multiple Myeloma

Introduction

Reovirus in myeloma

Following from the work presented in the preceding results chapters, the abilities of a range of potential therapeutic agents to modulate the immune dysfunction seen in MM were investigated. The oncolytic virus, Reovirus, was found to have a number of interesting properties with respect to both myeloma and immune cells and more detailed characterisation was therefore undertaken and will be reported in this results chapter.

Reovirus (originally respiratory enteric orphan virus) is a naturally occurring double-stranded RNA virus and a ubiquitous but benign human pathogen, to which the majority of the population have been exposed by the age of five[312]. The virus has been infrequently associated with upper respiratory symptoms, resembling a mild influenza-like illness, and gastrointestinal symptoms such as diarrhoea; close to 100% of human adults are seropositive[208,313], although interestingly this does not appear to abrogate the effect of the virus since cellular carriage can sequester virus from neutralizing antibodies allowing delivery to tumour targets [314]

Reovirus, along with a number of other naturally occurring and genetically engineered viruses, has become of considerable interest to the field of oncology, due to its ability to enter, replicate in, and selectively lyse cancer cells, possibly due to the dysregulation of Ras signalling which renders such cells unable to appropriately phosphorylate double-stranded RNA-activated protein kinase (PKR) and so clear viral infection[210,315,316]. An expanding body of preclinical data, reviewed in the main introduction to this thesis, demonstrates the efficacy of the virus in a number of settings, predominantly solid malignancies.

Some work has been conducted on reovirus in MM to date, much of which has been published whilst the experiments here were being conducted. The virus has been shown to be capable of *in vitro* lysis of myeloma cell lines[216], to mitigate tumour growth in NOD/SCID models[216] and to selectively purge peripherally harvested stem cells of tumour cells[232]. It has been postulated as a clinically useful agent[216] and shown to be safe when

employed as a single agent in a phase I trial in patients with relapsed refractory myeloma[236]. However, almost all of the work published to date on reovirus in myeloma has focused on the direct tumour-mediated lytic effects of virotherapy, ignoring the secondary immune-mediated effects which are beginning to prove so important for oncolytic virotherapy[204,317], and furthermore have not examined combination therapies which might potentiate such effects. The experiments presented within this chapter therefore attempt to expand existing evidence for the direct oncolytic effects of the virus in myeloma, assess immune activatory potential, and yield preliminary data for subsequent work examining rational reovirus-based combination therapies for clinical use.

Materials and Methods

General techniques used in this chapter have been described in chapter 2. Specific techniques employed for this work are outlined below.

Reagents and solutions

Reovirus Type 3 Dearing strain	Reolysin® donated by Oncolytics Biotech Inc., stored at -80°C for long term storage, or in PBS in the dark at 4°C (maximum 4 weeks)
Paraformaldehyde	4% solution (Sigma)
Lenalidomide	Donated by Celgene Corporation, stored at -20°C
zVAD-FMK	Source: BD Pharmingen (550377)
Dexamethasone	Source: Sigma (D1756, 50-02-2), stored at -20°C
ACK Buffer	500ml PBS, 4.01g ammonium chloride (0.15M), 0.5g $KHCO_3$ (10mM), 0.02g EDTA (0.1mM).

Table 5.1 Reagents and solutions.

Live/Dead flow cytometry assays

Tumour cells were treated as described in individual experiments. An intracellular amine stain was added (1µl per 106 cells in 100µl of FACS buffer) and cells incubated for 30 minutes on ice. Cells were then fixed in 4% paraformaldehyde for 10 minutes to inactivate any remaining reovirus before flow cytometric analysis as described previously.

NK cell degranulation assays

This assay operates on the principle that the lysosomal protein CD107a becomes expressed on the cell plasma membrane as a consequence of degranulation of NK cells. HMCL were labeled using a cell tracker dye (CellTracker™ Blue CMAC, Invitrogen) – briefly, tumour cells were incubated with the cell tracker at 1µM for 30 minutes in RPMI media without FCS, washed and incubated for a further 30 minutes in RPMI media without FCS, and then resuspended in CM. Whole PBMC were treated as detailed in

individual experiments. Both tumour cells and PBMC were then resuspended at $2x10^6$/ml and combined at a range of PBMC to tumour cell ratios (9:1, 3:1 and 1:1) in round-bottomed 96-well plates, always with $2x10^5$ cells in total in 100µl of medium per well. Cells were then incubated for 1 hour at 37°C, before the addition of GolgiStop and GolgiPlug, each at 1:1000 (both Miltenyi Biotec) and a fluorophore-conjugated antibody against CD107a (see table 5.2). After a further 2 hours, antibodies against CD3 and CD56 and a dead cell discriminator were added (table 5.2) and cells incubated for a further 30 minutes at room temperature. Finally, cells were centrifuged at 300g, resuspended in FACS buffer and assayed by flow cytometry as described previously.

Target	Fluorochrome	Excitation laser (wavelength /nm)	Maximum emission wavelength /nm	Manufacturer	Clone (product ID)
Live/Dead discriminator	PI-like	Blue (488)	625	Miltenyi Biotec	(130-091-163)
µLIVE/DEAD Fixable Red		Blue (488)	615	Life Technologies	(L231012)
CellTracker™ Blue CMAC	CMAC	UV (350)	466	Invitrogen	C2110
CD3	PE	Blue (488)	578	Miltenyi-Biotec	BW264/56 (130-091-374)
CD56	APC	Red (633)	660	Miltenyi-Biotec	REA196 (130-100-698)
CD107a	FITC	Blue (488)	520	Miltenyi-Biotec	H4A3 (130-095-518)
JAM1	PE	Blue (488)	578	BD Pharmingen	M.Ab.F11 (552556)

Table 5.2. Fluorophore-conjugated antibodies and cell stains used for flow cytometry.

Cytotoxicity assays on stromal cell layers

For these assays stromal cells were seeded into sterile 48-well tissue culture plates at a density of 10^5 cells per well in 1ml of CM and incubated at 37°C and 5% CO_2 in a humidified incubator for 24 hours. Media and non-adherent cells were then removed and 2ml of fresh CM added containing 10^5 HMCLs, resulting in incubation at a 1:1 ratio and an initial cell density of $2x10^5$ cells in 2ml in each well. These cultures were then incubated for a further 48 hours prior to assessment of cell death in tumour cells as described previously.

C57BL/KaLwRijHsd 5TGM1 murine model

All animal work was conducted under an appropriate Project License (License holder: Prof Alan Melcher), by Home Office licensed and appropriately trained individuals. All murine experiments were conducted in collaboration with Dr Fiona Errington-Mais. C57BL/KaLwRijHsd mice were purchased at age 6-10 weeks from a colony already established at the University of Leeds, which was in turn originally purchased from Harlan Laboratories (Indianopolis). Mice were ear-notched for identification and housed in groups of 4-5 in OPTIcages, until injected with reovirus, when they were transferred into ISOcages and kept separately from uninfected animals. All animals were monitored daily, and any mice exhibiting hind limb paralysis, hunched posture, reduced activity levels, skin ulceration, weight loss, difficulty breathing or other distress were removed from the colony and euthanized via cervical dislocation; at the end of experiments animals were similarly euthanized via cervical dislocation.

The 5TGM1 and 5TGM1-eGFP cell lines were obtained from Dr Oyajobi, Department of Molecular Medicine, University of Texas Health Science Centre, San Antonio, under an appropriate material transfer agreement, and cultured in CM. Cell lines were passaged *in vivo* in C57BL/KaLwRijHsd mice and bone-resident tumour cells isolated and cultured *in vitro* for 2-3 passages prior to use in *in vivo* experiments. The method and vector employed for generation of the eGFP-transduced line are described[318].

After sacrifice of animals, post-mortem dissection was undertaken to isolated both femora and tibias, and the spleen. Bone marrow cells were isolated by removal of the epiphyses and flushing of the shaft of the long bones with media. These cells were then processed as described in individual experiments. Spleens were crushed in a Falcon cell strainer (BD Biosciences) using the plunger from a 12ml syringe (BD Biosciences) into CM. Cells were then centrifuged at 400g for 5 minutes and resuspended in ACK red cell lysis buffer

(5ml per spleen) for 2 minutes. Excess cold medium was then added, before centrifuging at 400g for 5 minutes, resuspension in CM and assessment by flow cytometry as described previously. Table 5.3 shows the antibodies used for flow cytometry for murine experiments.

Target	Fluorochrome	Excitation laser (wavelength /nm)	Maximum emission wavelength /nm	Manufacturer	Clone (product ID)
µLIVE/DEAD Fixable Red		Blue (488)	615	Life Technologies	(L231012)
CD4	APC	Red (633)	660	Miltenyi	GK1.5 (130-102-594)
CD8	PECy5	Blue (488)	655	BD Pharmingen	53-6.7 (553034)
DX5	PE	Blue (488)	578	Miltenyi	DX5 (130-102-337)
CD69	FITC	Blue (488)	520	Miltenyi	H1.2F3 (130-103-950)
CD138	PE	Blue (488)	578	Miltenyi	REA104 (130-102-580)

Table 5.3. Fluorophore-conjugated antibodies and cell stains used for flow cytometry for murine experiments.

Results

Expression of JAM1 of human myeloma cell lines

Junctional adhesion molecule 1 (JAM1) is known to be the cell surface receptor for reovirus[209,213,216] and the expression of this protein on the surface of human myeloma cell lines was therefore evaluated first. A panel of human myeloma cell lines (HMCLs), and the erythroleukaemia cell lines K562, were cultured in complete media. Live cells were purified by density centrifugation on Lymphoprep, stained with a PE-conjugated antibody against JAM1 (or with an isotype-matched control antibody), and then assessed for expression of this protein by flow cytometry. Representative examples are given in Figure 5.1; Figure 5.2 shows compiled results for all HMCLs examined (n=3). A proportion of all cell lines tested was found to express the marker, although a range of levels of expression was seen – KMS11, KMS18, RPMI 8226 and U266 as well as K562 expressed JAM1 on close to all cells, whereas OPM2 demonstrated only low level expression with intermediate results seen on other lines.

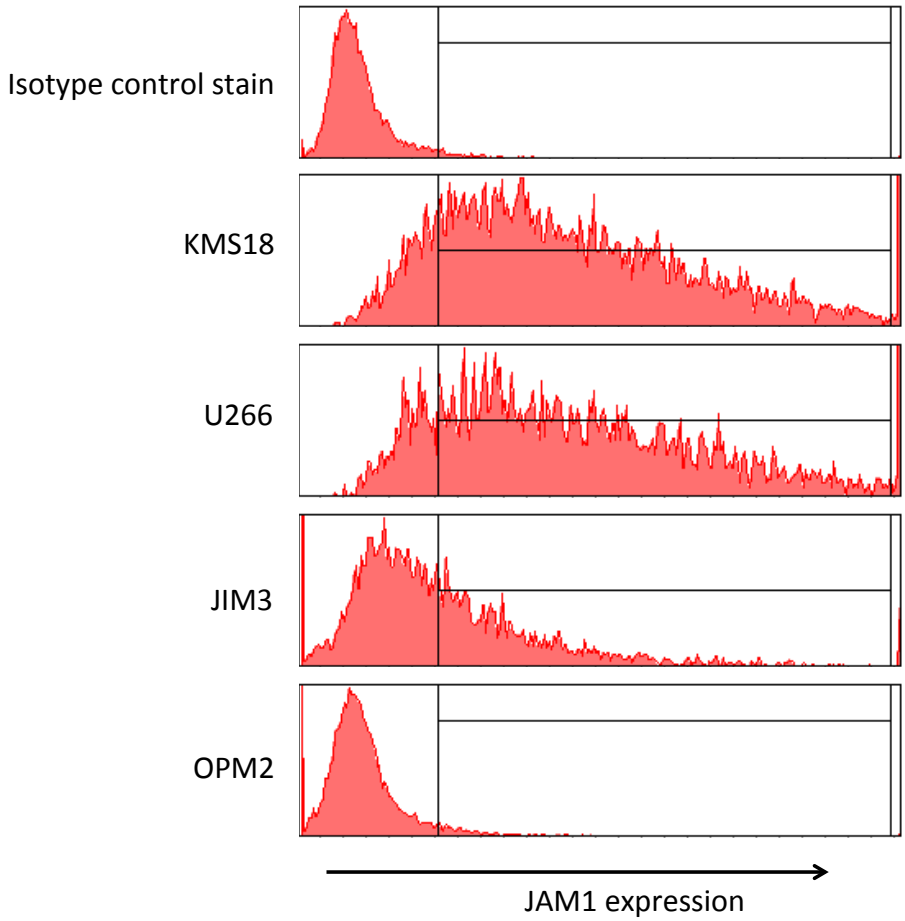


Figure 5.1. Mean fluorescence intensity of JAM1 staining on the HMCLs KMS18, U266, JIM3 and OPM2.

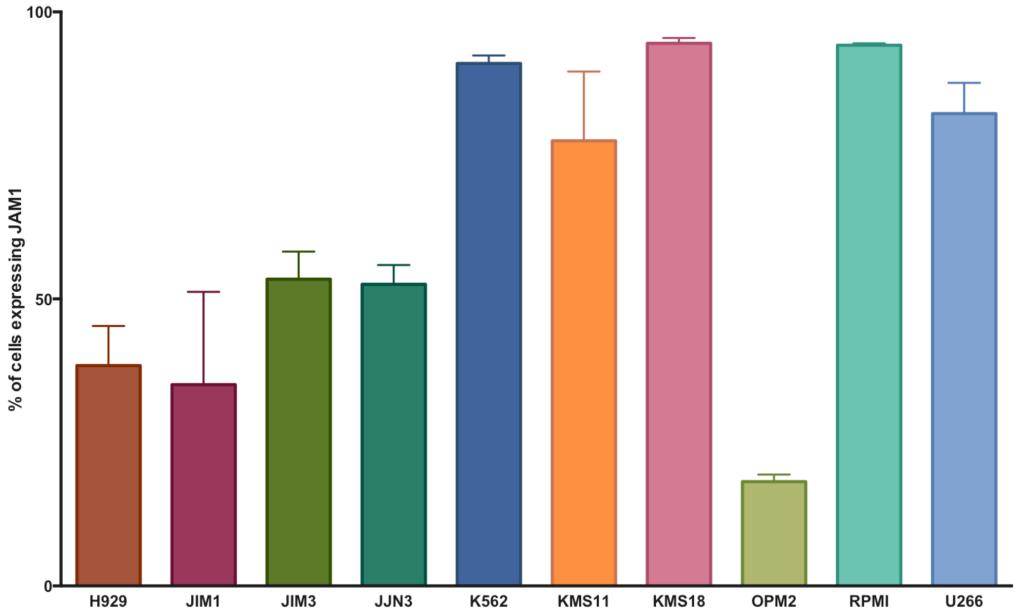


Figure 5.2. Percentage of HMCLs with surface expression of JAM1. Histograms show medians and standard deviations, n=3.

Direct reovirus-induced oncolysis of HMCLs

Direct cytotoxic effects of reovirus type 3 Dearing strain (reovirus hereafter) treatment on HMCLs were next assessed. For these experiments HMCLs were incubated with reovirus at a range of multiplicities of infection (MOI) for a range of periods of time, then the dead cells labelled using an intracellular amine stain (LIVE/DEAD® Fixable Dead Cell Stain Kits, Invitrogen) and quantified by flow cytometry. Figure 5.3 shows representative example flow cytometry scatter plots and histograms, in this case for treatment of U266B with reovirus for 72 hours. Treatment with reovirus was associated with a dose- and time-dependent alteration in forward and side scatter characteristics and uptake of LIVE/DEAD discriminator, indicating cell death.

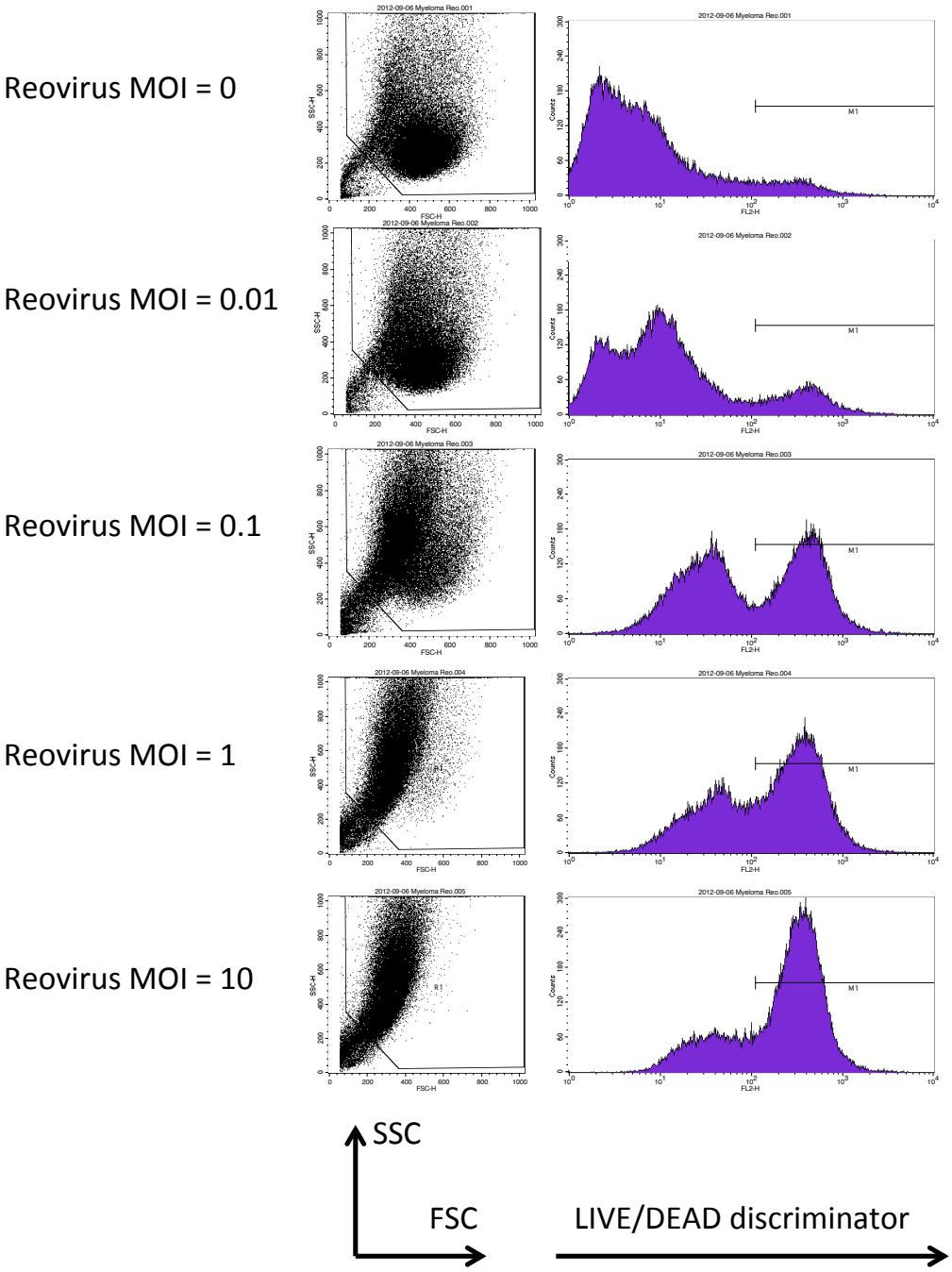


Figure 5.3. Representative scatter plots of forward scatter (FSC) and side scatter (SSC, left panel) and intracellular amine staining (right panel) of U266 after treatment for 72 hours with a range of multiplicities of infection with reovirus.

Collated data for, JIM3, U266B and OPM2 are shown in Figures 5.4-5.6. H929, JIM3, KMS11 and U266B all exhibited time- and MOI-dependent killing by reovirus, $p<0.0001$ for both variables by 2-way ANOVA in all cases. As expected, some variation was seen between these lines in terms of sensitivity to the virus, with H929 being the most susceptible line tested. Further cell lines (H929, KMS11, JJN3, KMS18 and RPMI8226) were found to be similarly sensitive to reovirus treatment (data not shown). One HMCL, OPM2 (previously demonstrated to have only low level surface expression of JAM1), showed no change in viability even after treatment with the highest tested MOI of reovirus for 96 hours (2-way ANOVA, p=NS).

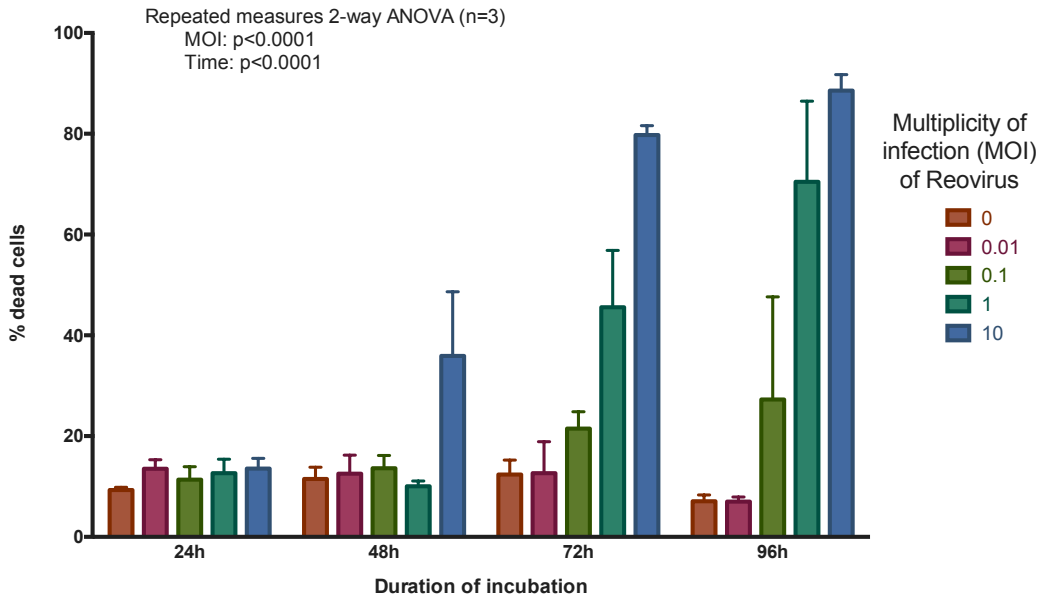


Figure 5.4. Fraction of JIM3 cells dead after incubation with reovirus at a range of multiplicities of infection (MOIs) for the durations shown, as determined by intracellular amine staining and flow cytometry. N=3. Mean and standard deviations shown. p values are for 2-way ANOVA.

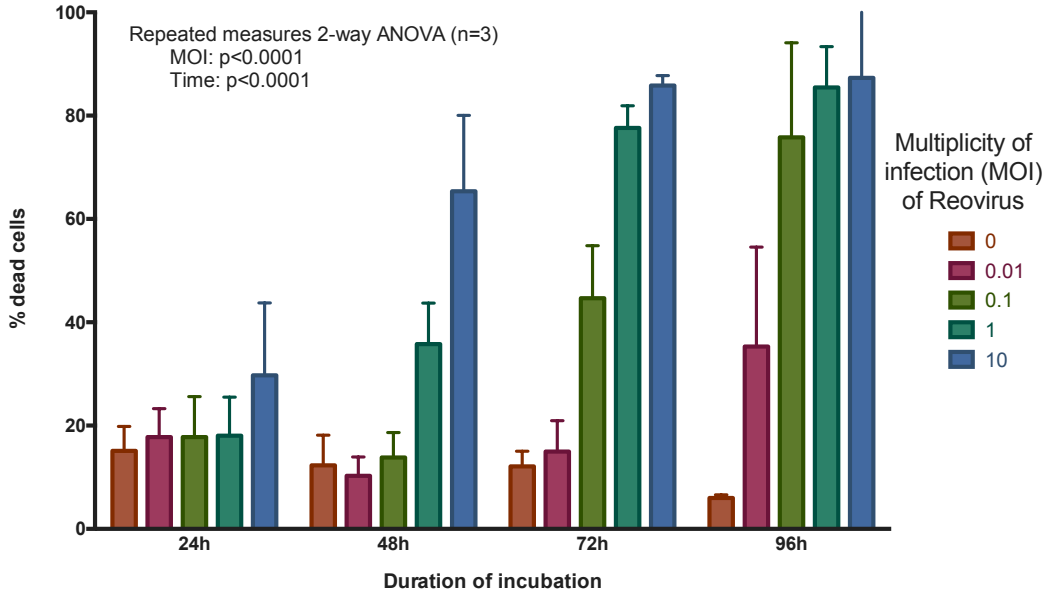


Figure 5.5. Fraction of U266B cells dead after incubation with reovirus at a range of multiplicities of infection (MOIs) for the durations shown, as determined by intracellular amine staining and flow cytometry. N=3. Mean and standard deviations shown. p values are for 2-way ANOVA.

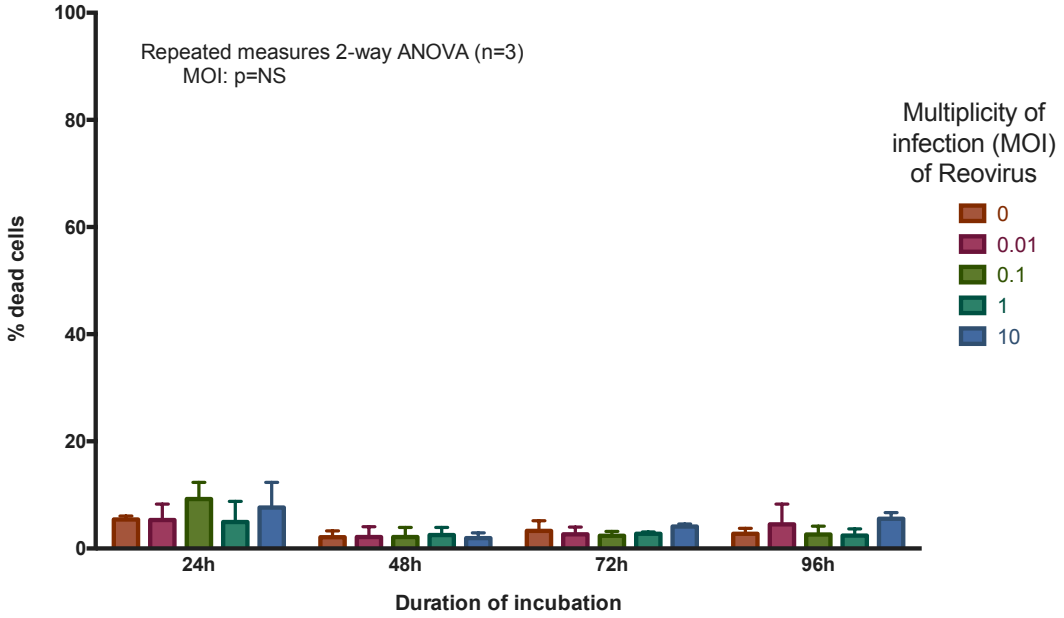


Figure 5.6. Fraction of OPM2 cells dead after incubation with reovirus at a range of multiplicities of infection (MOIs) for the durations shown, as determined by intracellular amine staining and flow cytometry. N=3. Mean and standard deviations shown. p values are for 2-way ANOVA.

Reovirus has previously been reported to induce apoptosis in susceptible tumour lines; in order to assess whether this may be mechanism of action in HMCLs, a selection of cell lines were cultured with and without reovirus treatment (MOI=1) for 96 hours, cell lysates made and Western blots performed for the anti-apoptotic protein bcl2 (Figure 5.7). Equal cell numbers were loaded in each lane.

Reovirus treatment was seen to reduce bcl2 expression in a range of cell lines. The tumour cell line JIM3 was then incubated for 96 hours with reovirus, as previously, with or without the addition of the cell-permeable general caspase inhibitor zVAD-FMK 50μM, and the proportion of dead cells assayed as previously (Figure 5.8). Addition of the caspase inhibitor was seen to militate against reovirus-induced killing.

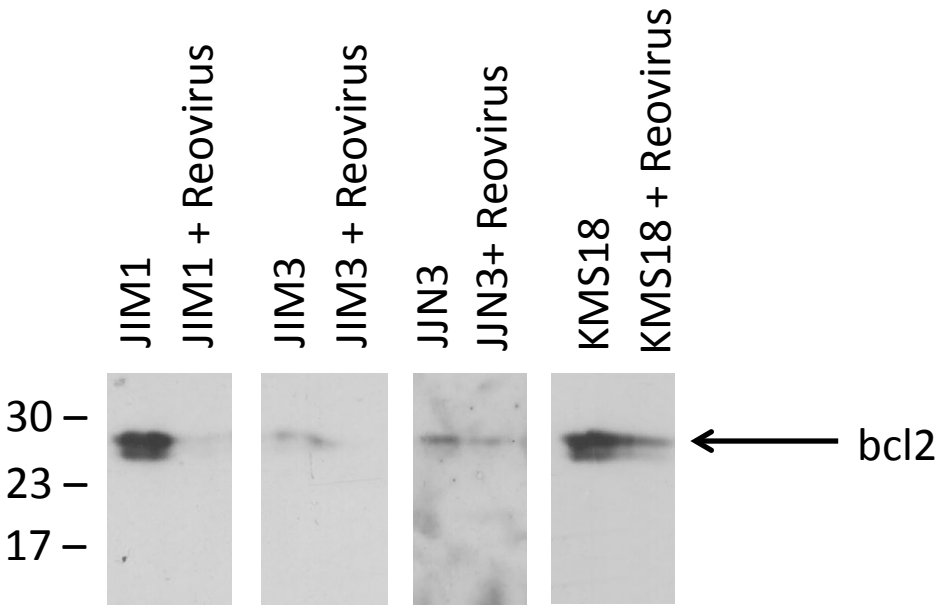


Figure 5.7. Western blots for bcl2 on cell lysates from JIM1, JIM3, JJN3 and KMS18 with and without Reovirus treatment. Cells were treated for 96 hours at Reovirus MOI=1.

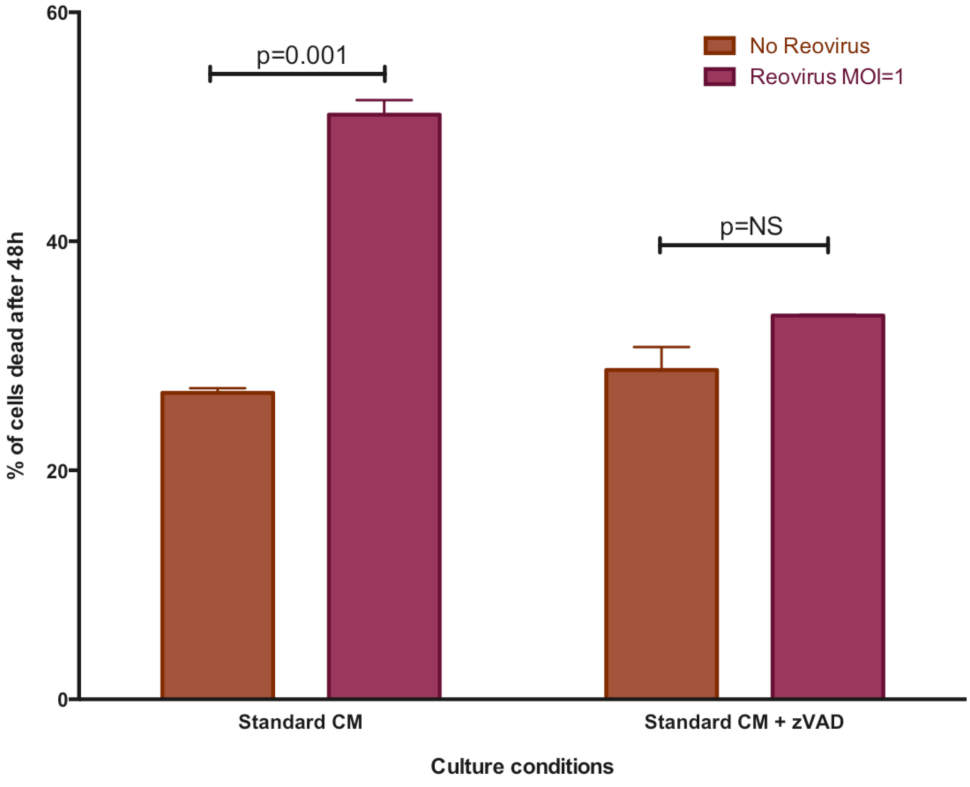


Figure 5.8. Fraction of JIM3 cells dead after incubation with and without reovirus at MOI=1 for 48 hours in CM or CM with zVAD. N=3. Mean and standard deviations shown. P values are for two-tailed paired student t tests.

Oncolysis in combination with immunomodulatory agents

Since it was the intention of this work to examine the potential for combination of reovirus with immunomodulatory agents (IMiDs), particularly in light of the immunological effects of reovirus discussed later, the effects of combination with the IMiD lenalidomide on direct cytotoxicity was next examined. Firstly, a panel of HMCLs, and the erythroleukaemia cell lines K562, were cultured in complete media or complete media with lenalidomide 10μM for 96 hours. Live cells were then purified by density centrifugation on Lymphoprep, stained with a PE-conjugated antibody against JAM1 (or with an isotype-matched control antibody), and then assessed for expression of this protein by flow cytometry. The experiment was repeated in triplicate (Figure 5.9). Treatment with lenalidomide did not significantly affect the fraction of cells expressing JAM1 for any of the cell lines tested. Mean fluorescence intensity for JAM1 expression was similarly unaffected by lenalidomide treatment, indicating no change in the level of expression of the protein on those cells already expressing it at baseline (Figure 5.10)

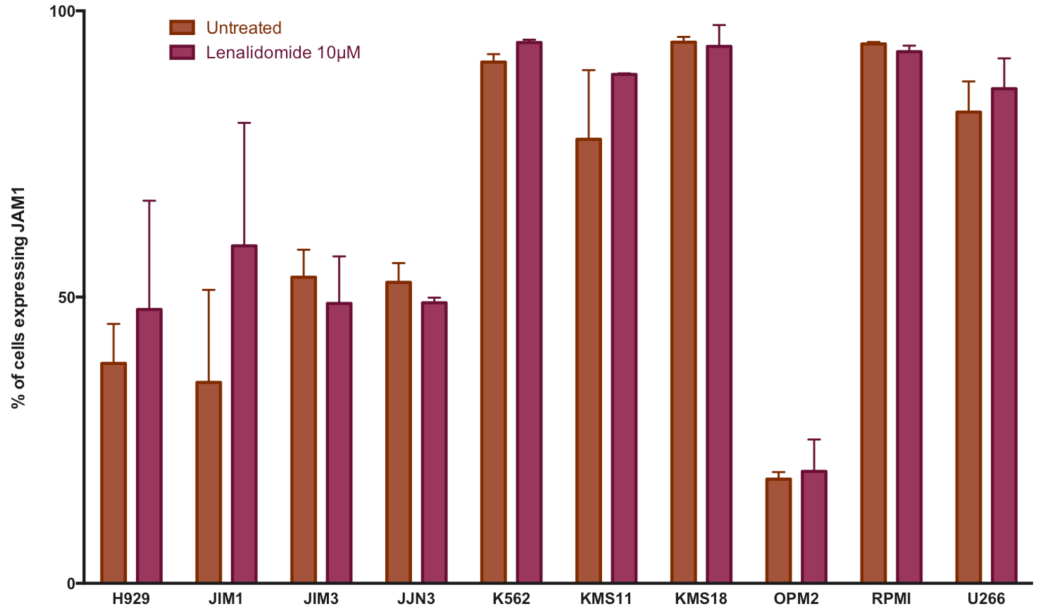


Figure 5.9. Percentage of HMCLs with surface expression of JAM1 as determined by flow cytometry, after culture for 96 hours in RPMI with 10% FBS, with or without addition of 10μM lenalidomide.

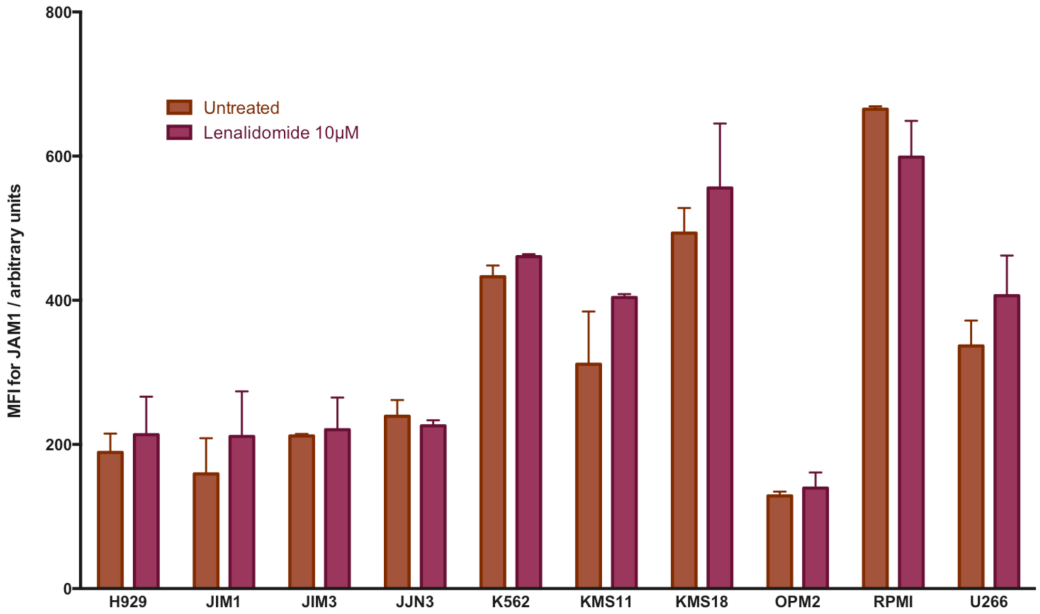


Figure 5.10. Mean fluorescence intensity of JAM1 staining on a range of HMCLS, as determined by flow cytometry, after culture for 96 hours in RPMI with 10% FBS, with or without addition of 10μM lenalidomide.

The effect of combination treatment of cells lines with reovirus and lenalidomide was next investigated. Since in clinical use lenalidomide is usually combined with corticosteroid therapy, at least for a number of cycles of chemotherapy before tapering, the effects of dexamethasone treatment were also examined. The cell lines KMS11, JIM3 and OPM2 were therefore treated with combinations of reovirus (at the previously examined range of MOIs), lenalidomide 10μM and dexamethasone 5μM; cultures were incubated for periods known from the previous experiments to result in suboptimal reovirus-induced killing for KMS11 and JIM3 (48 hours and 72 hours respectively) and for 96 hours for OPM2, which is resistant to reovirus. Cell death at the end of cultures is shown in Figures 5.11-5.13.

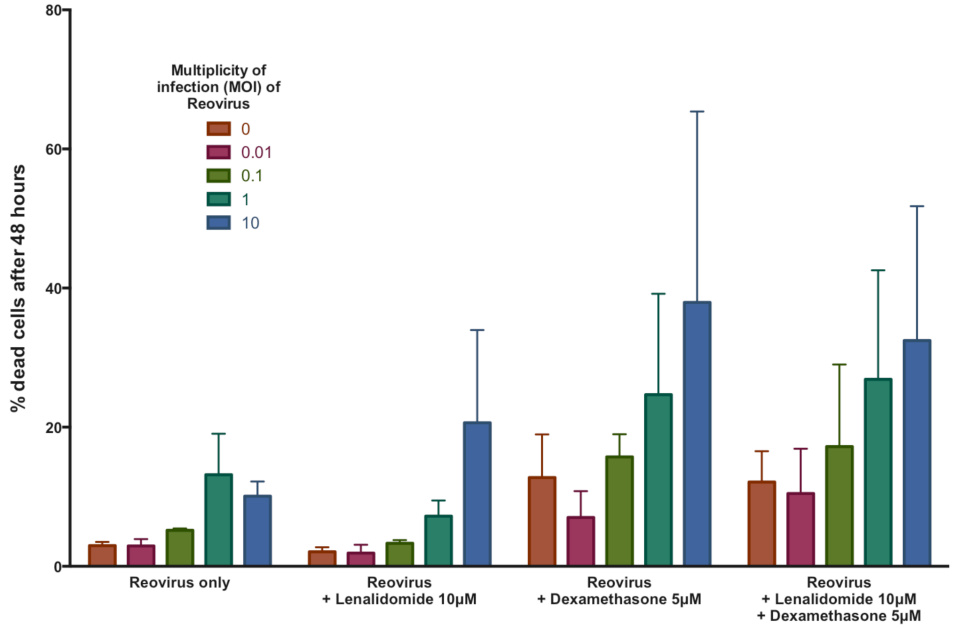


Figure 5.11. Fraction of KMS11 cells dead after incubation for 48 hours with reovirus at a range of MOIs and/or lenalidomide and dexamethasone. N=3. Mean and standard deviations shown. 2-way ANOVA: p=0.0094 for reovirus MOI, p=0.3016 for lenalidomide/dexamethasone, p=0.8377 for interaction.

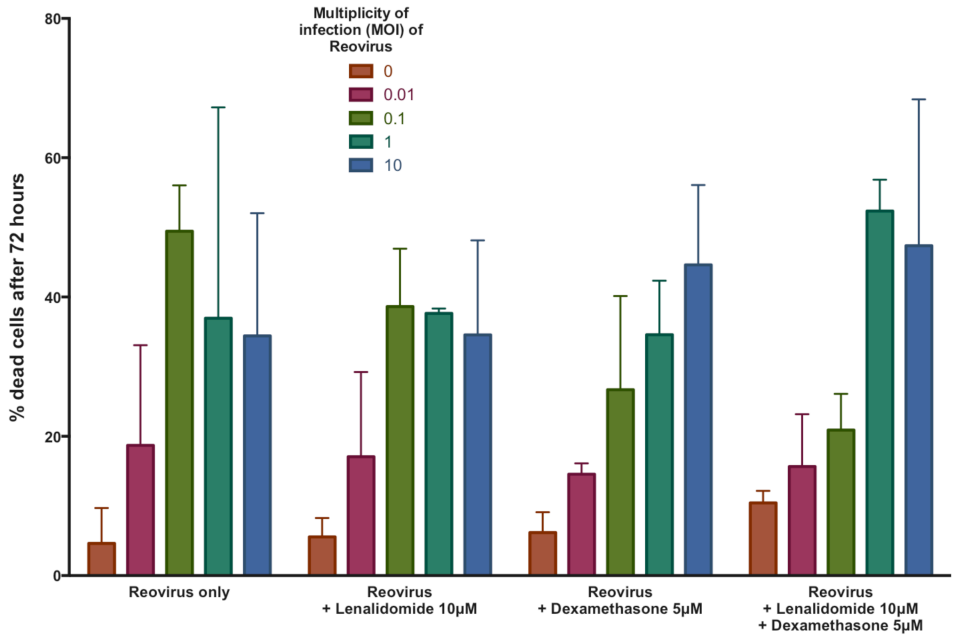


Figure 5.12. Fraction of JIM3 cells dead after incubation for 72 hours with reovirus at a range of MOIs and/or lenalidomide and dexamethasone. N=3. Mean and standard deviations shown. 2-way ANOVA: p=0.0014 for reovirus MOI, p=0.9369 for lenalidomide/dexamethasone, p=0.229 for interaction.

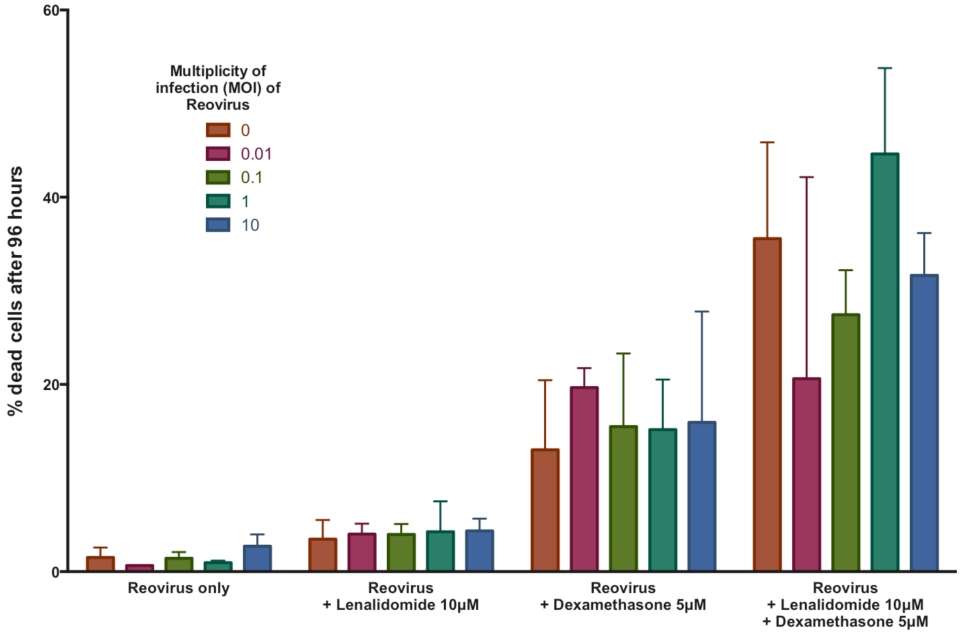


Figure 5.13. Fraction of OPM2 cells dead after incubation for 96 hours with reovirus at a range of MOIs) and/or lenalidomide and dexamethasone. N=3. Mean and standard deviations shown. 2-way ANOVA: p=0.3776 for reovirus MOI, p=0.0063 for lenalidomide/dexamethasone, p=0.3902 for interaction.

The effects of these combination therapies were next tested on a primary bone marrow sample from a patient with multiple myeloma; the fraction of all bone marrow cells expressing $CD138^+$ after treatment for 48 hours and the cell death in $CD138^+$ cells are presented (Figure 5.14).

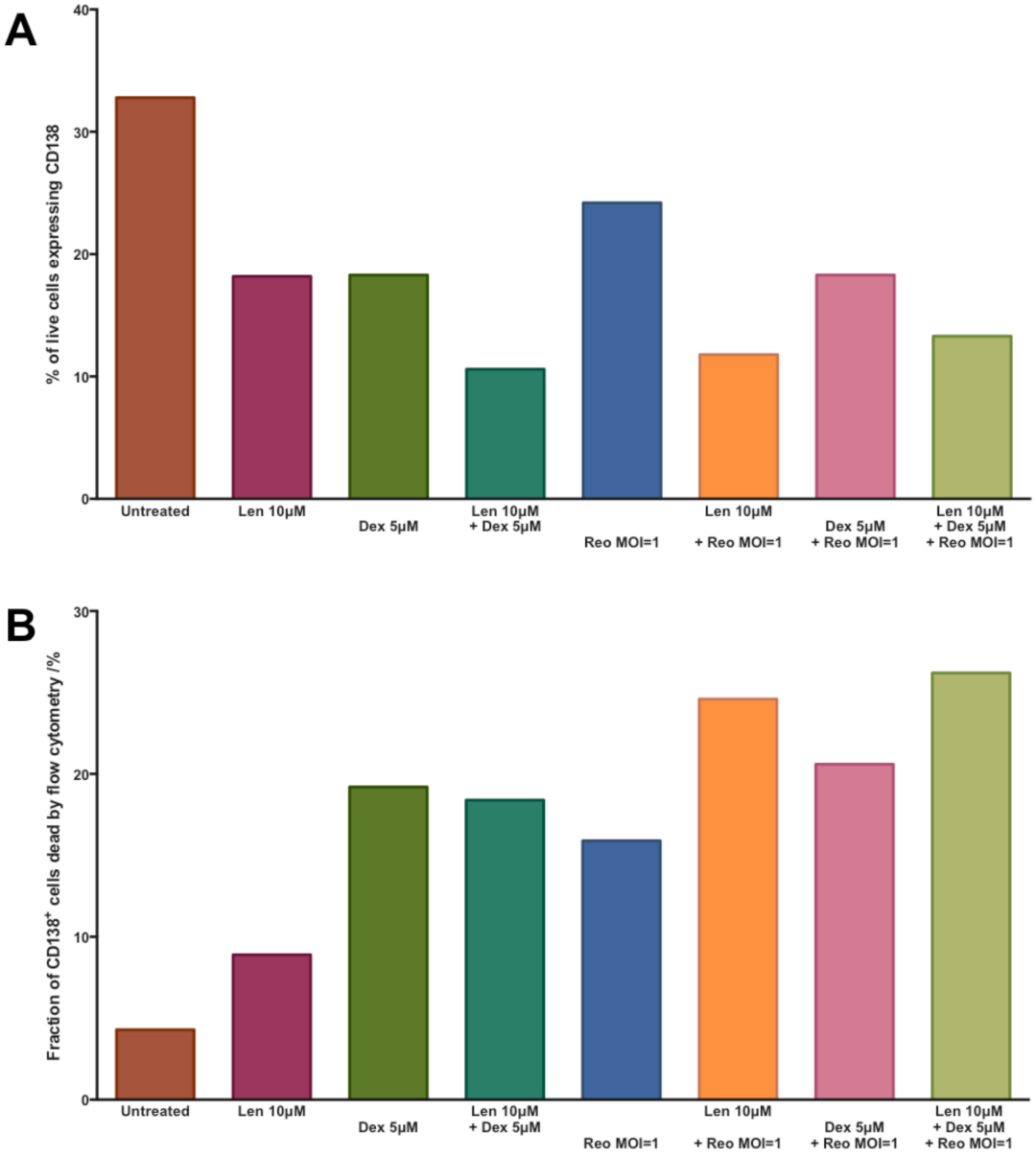


Figure 5.14. Number of CD138$^+$ cells (A) and cell death in CD138+ cells (B) after treatment of a MM BM sample with reovirus, dexamethasone and lenalidomide.

Reovirus-induced activation of peripheral blood immune cells

The effects of reovirus treatment on peripheral blood immune cells was next examined. For these experiments PBMC were isolated from a healthy donor and incubated with reovirus at MOI=0.1 or 1, and/or lenalidomide 10μM; dexamethasone was found to be markedly lympholytic in this context and was not evaluated further. Activation of peripheral blood immune subsets at 24 hours and 7 days was estimated using surface CD69 expression (Figure 5.15). It should be noted that since CD69 expression is induced on activation, but then down-regulated by 72 hours, expression of CD69 at day 7 is likely to represent ongoing activation.

Since promising results were seen in this pilot experiment using PBMC from a healthy donor, the more relevant question of activation of immune subsets in myeloma patients, who have marked immuneparesis, was next explored. Here PBMC were isolated from patients with MM and were incubated with reovirus alone for 1, 4 or 7 days, and activation of $CD4^+$, $CD8^+$ and $CD3^-CD56^+$ (NK cells) subsets estimated by CD69 expression (Figure 5.16-5.18); since immune subset activation at baseline was found to vary markedly between donors, CD69 expression was normalised against expression in the untreated group at D1 and expressed as a fold increase.

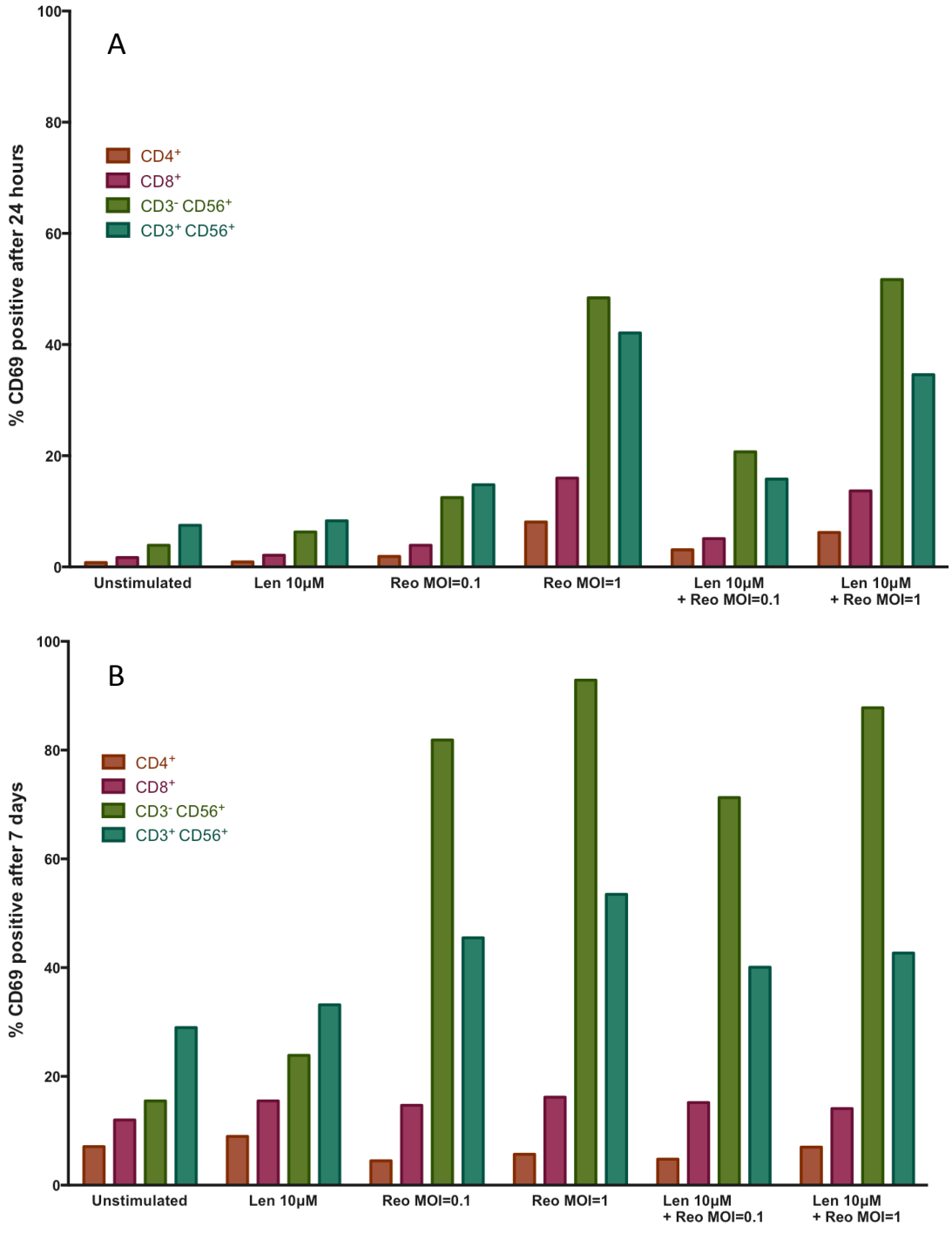


Figure 5.15. CD69 expression on peripheral blood immune subsets after incubation of healthy donor PBMC with reovirus and lenalidomide combinations at the concentrations shown for 24 hours (panel A) and 7 days (panel B).

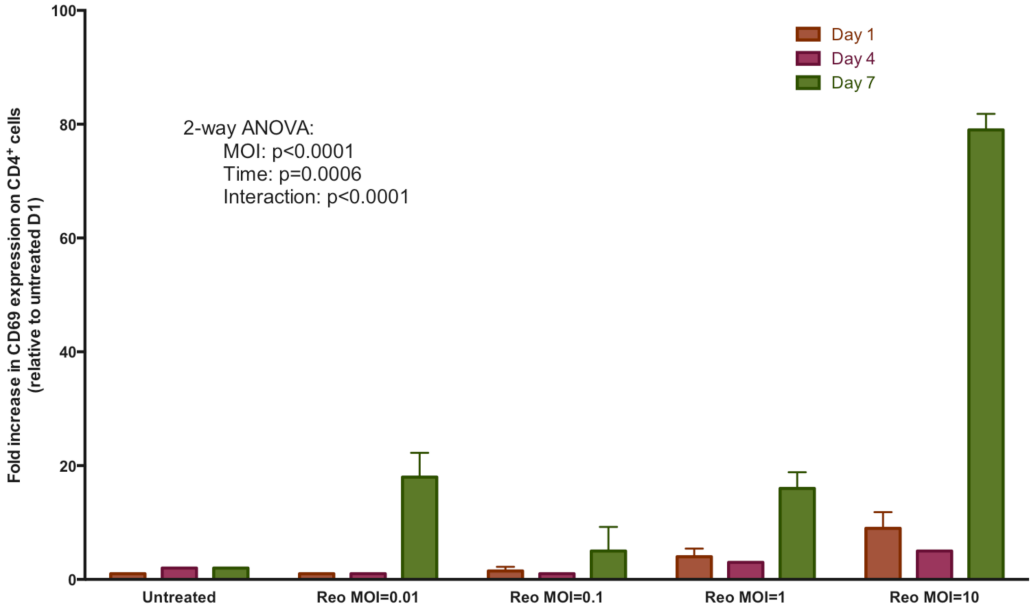


Figure 5.16. CD69 expression on peripheral blood CD4⁺ cells after incubation of PBMC from patients with MM with reovirus at the MOIs shown. CD69 expression is normalised to expression in the untreated sample at D1 for each donor. n=3, histogram shows means and standard deviations. 2-way ANOVA: MOI: p<0.0001, time: p=0.0006, interaction: p<0.0001.

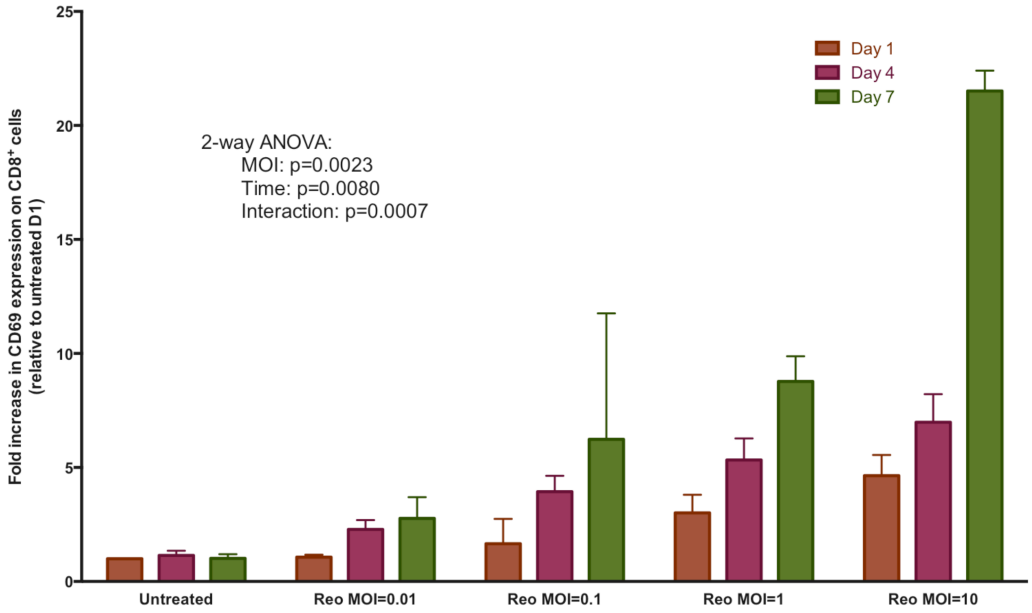


Figure 5.17. CD69 expression on peripheral blood CD8⁺ cells after incubation of PBMC from patients with MM with reovirus at the MOIs shown. CD69 expression is normalised to expression in the untreated sample at D1 for each donor. n=3, histograms show means and standard deviations. 2-way ANOVA: MOI: p=0.0023, time: p=0.0080, interaction: p=0.0007.

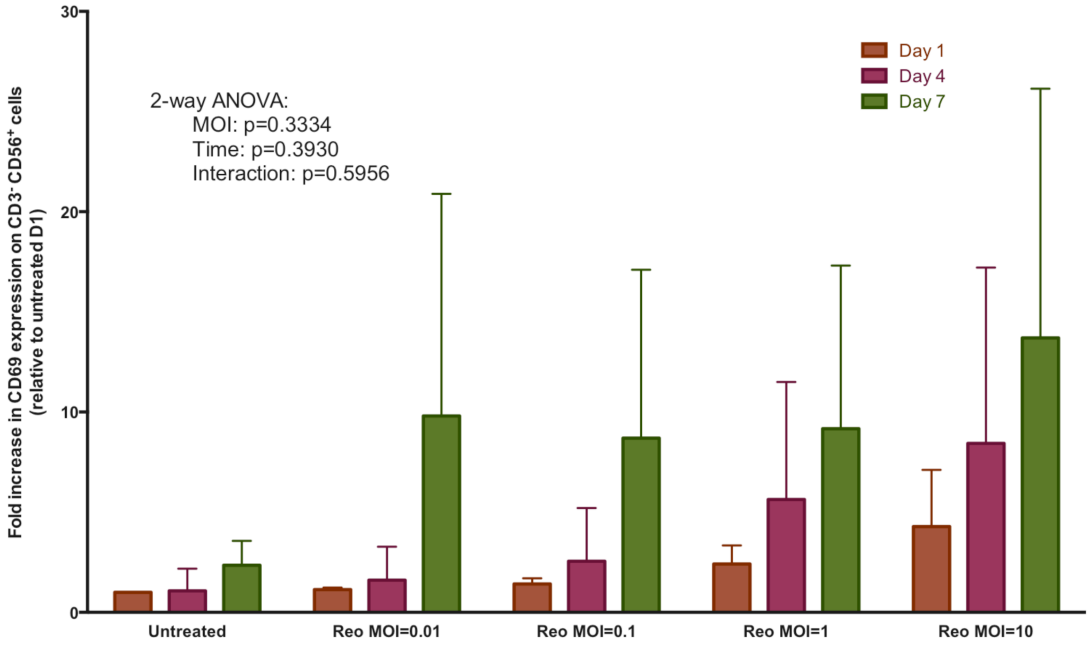


Figure 5.18. CD69 expression on peripheral blood $CD3^-$ $CD56^+$ cells after incubation of PBMC from patients with MM with reovirus at the MOIs shown. CD69 expression is normalised to expression in the untreated sample at D1 for each donor. n=3, histogram shows means and standard deviations. 2-way ANOVA: MOI: p=NS, time: p=NS, interaction: p=NS.

The effect of addition of lenalidomide to these cultures was next investigated. In order to allow for potential for synergy between the agents, the doses of reovirus MOI 0.1 and 1, and lenalidomide 1μM ad 10μM were selected. Incubations were again conducted for 1, 4 and 7 days (Figure 5.19-5.21).

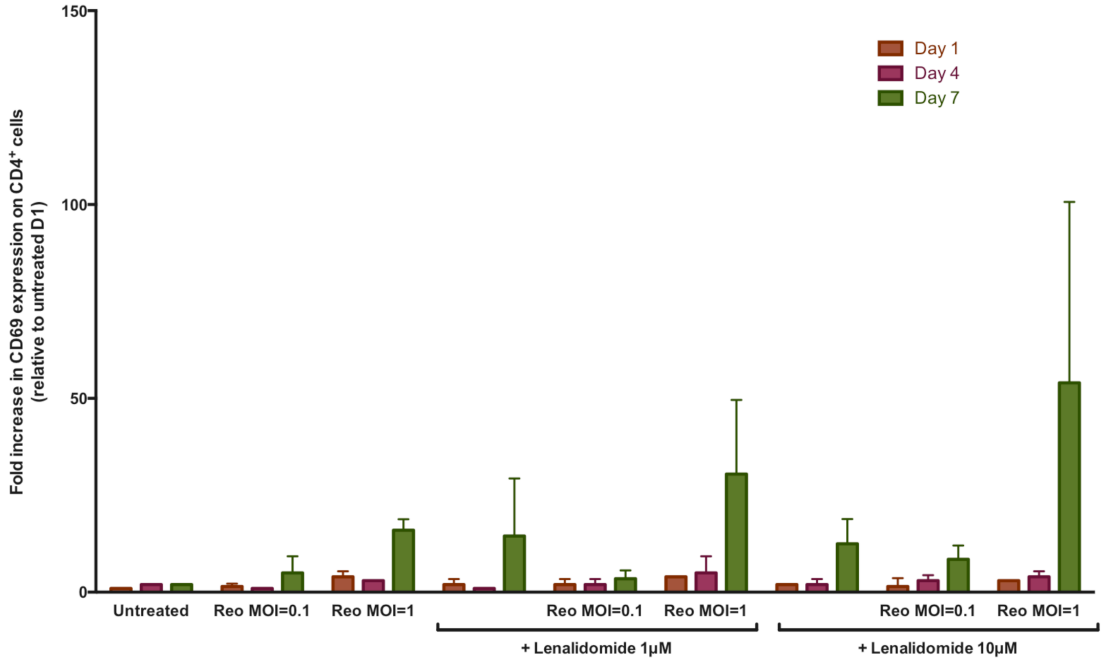


Figure 5.19. CD69 expression on peripheral blood CD4⁺ cells after incubation of PBMC from patients with MM with reovirus and/or lenalidomide at the doses shown. CD69 expression is normalised to expression in the untreated sample at D1 for each donor. n=3, means and standard deviations shown.

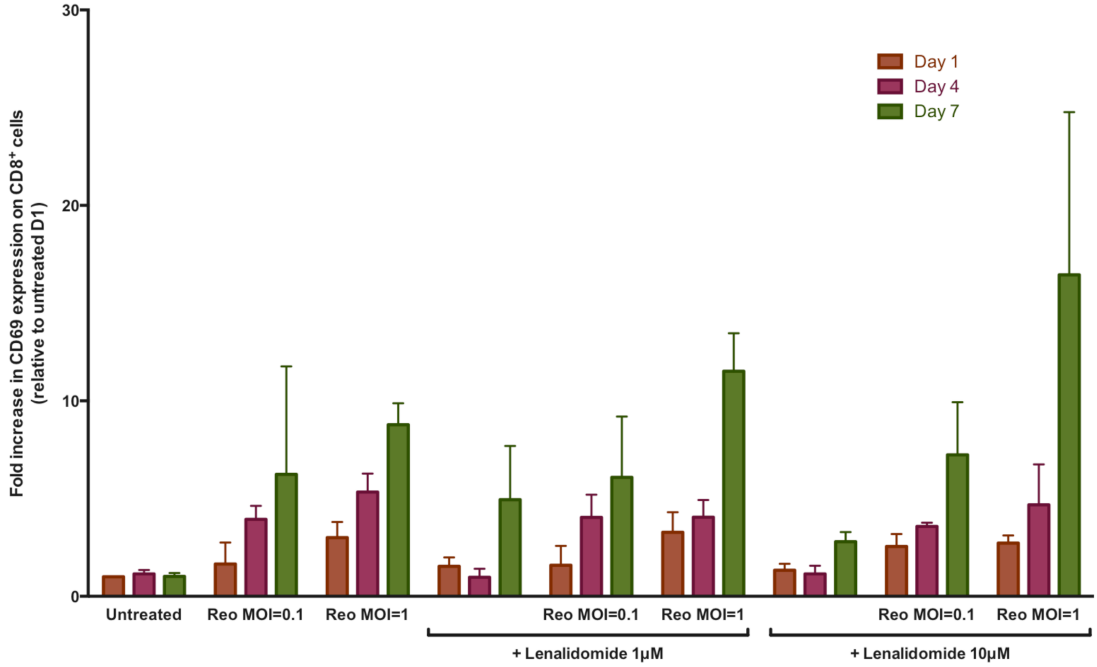


Figure 5.20. CD69 expression on peripheral blood CD8⁺ cells after incubation of PBMC from patients with MM with reovirus and/or lenalidomide at the doses shown. CD69 expression is normalised to expression in the untreated sample at D1 for each donor. n=3, means and standard deviations shown.

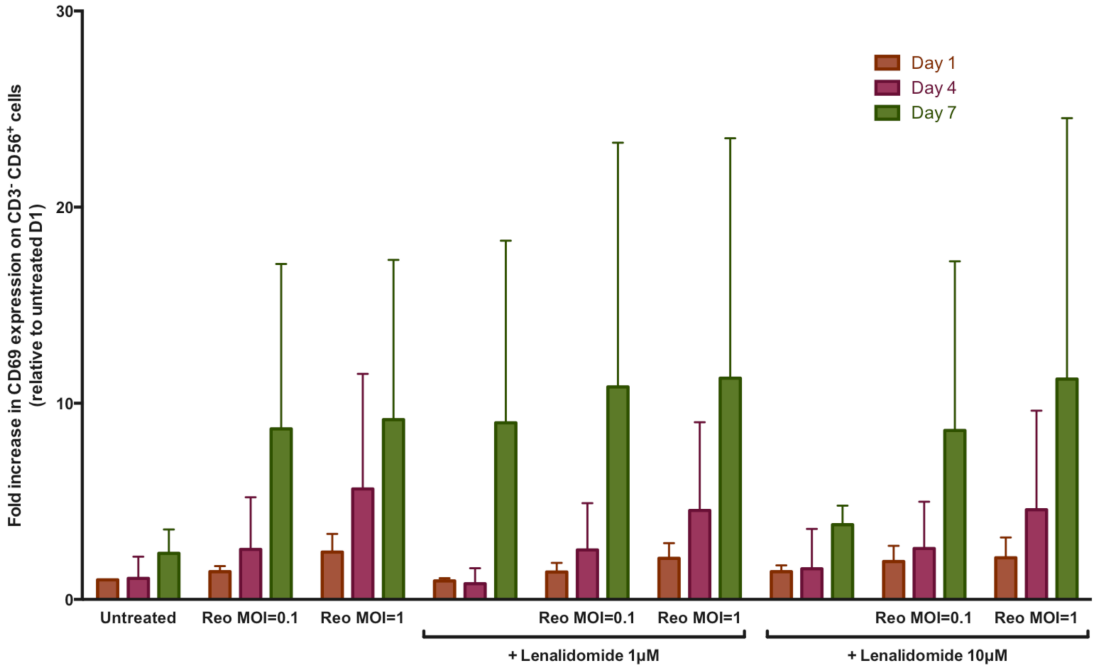


Figure 5.21. CD69 expression on peripheral blood $CD3^-$ $CD56^+$ cells after incubation of PBMC from patients with MM with reovirus and/or lenalidomide at the doses shown. CD69 expression is normalised to expression in the untreated sample at D1 for each donor. n=3, means and standard deviations shown.

Reovirus-induced, NK cell-mediated tumour killing

In view of the demonstrated ability of reovirus to activate peripheral blood immune cells, the implications of these findings for immune-mediated secondary tumour killing were next investigated, in particular the ability of NK cells so activated to induce lysis of tumour targets.

PBMC were incubated for 48 hours with and without Reovirus (MOI=1) prior to NK cell degranulation assays conducted as described in the methods section of this chapter. The cell line K562, an erythroleukaemia line known to be sensitive to NK cell-mediated lysis, was first examined to validate the assay protocol (Figure 5.22); H929, JIM3 and OPM2 were then assessed for susceptibility to NK cell-mediated lysis and the ability of reovirus to augment this (Figures 5.23-5.25).

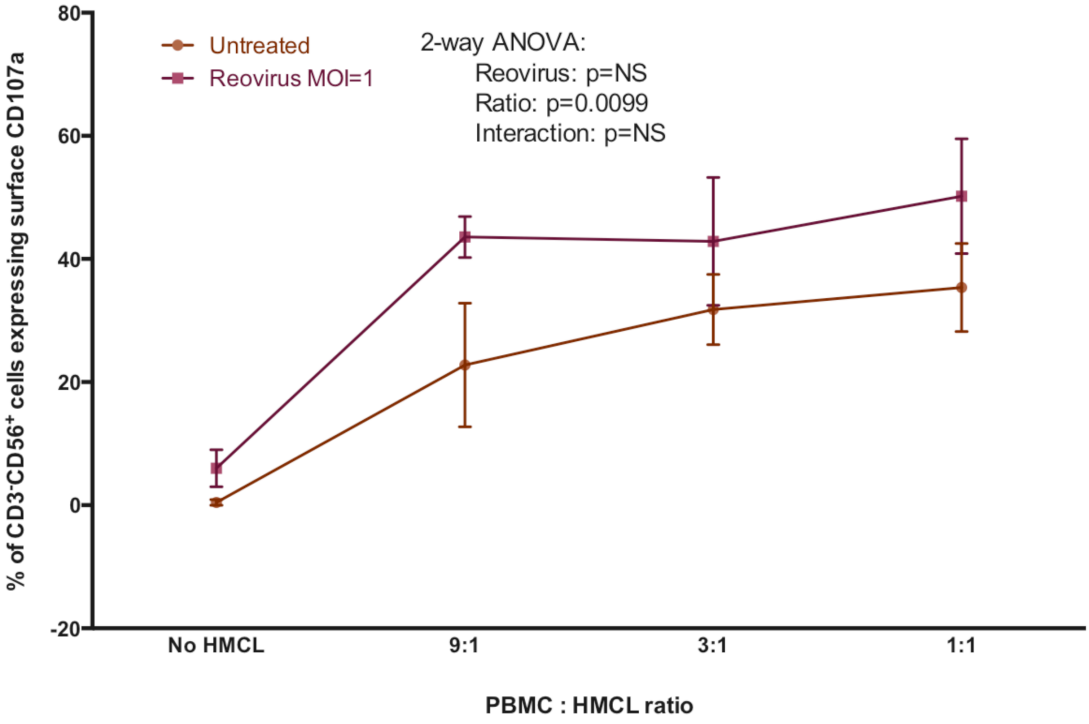


Figure 5.22. NK cell degranulation assay against K562. The fraction of NK cells (defined as $CD3^+CD56^-$) expressing surface CD107a at the end of the assay is shown . N=3. Means and SEM shown.

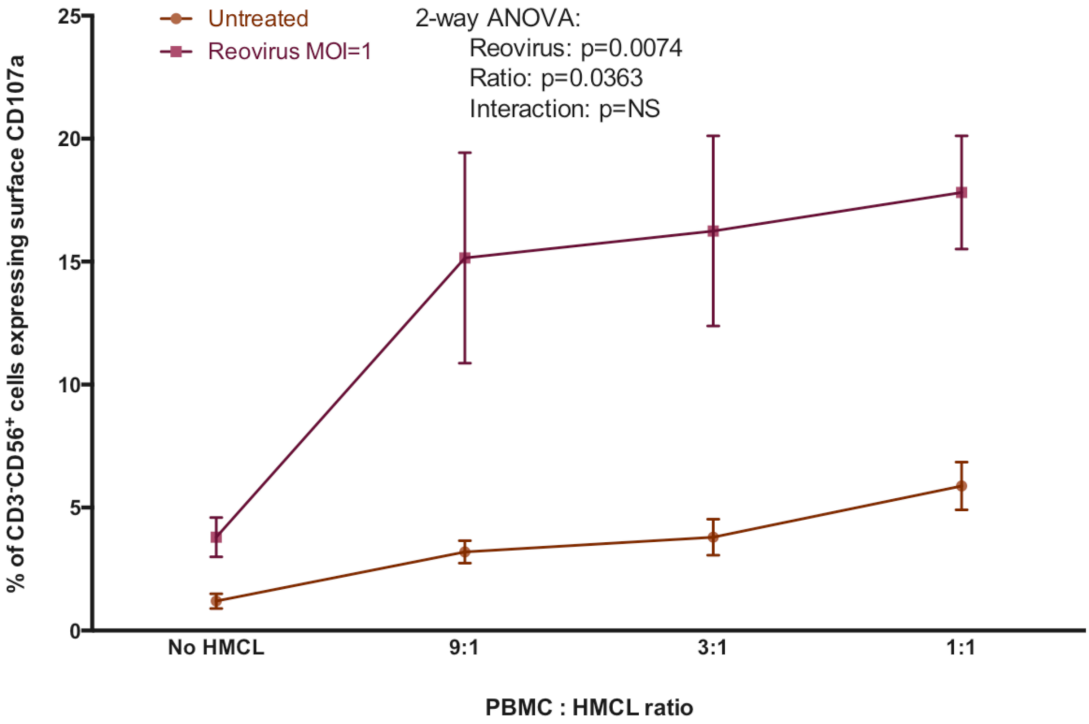


Figure 5.23. NK cell degranulation assay against H929. The fraction of NK cells (defined as $CD3^+CD56^-$) expressing surface CD107a at the end of the assay is shown . N=3. Means and SEM shown.

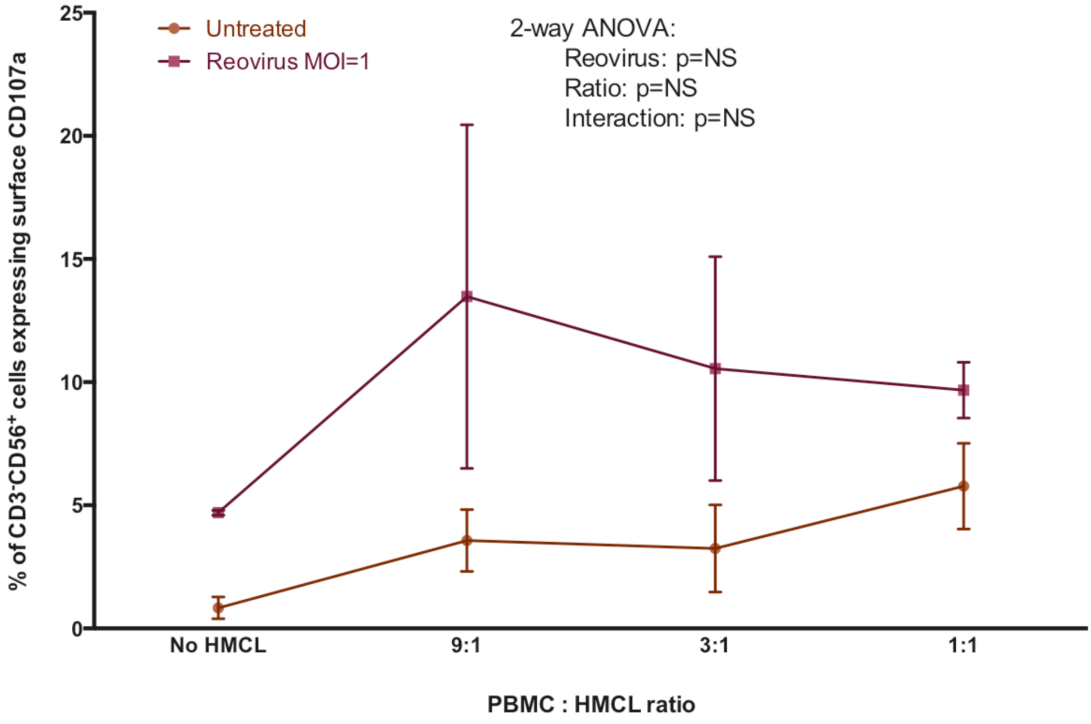


Figure 5.24. NK cell degranulation assay against JIM3. The fraction of NK cells (defined as $CD3^{+}CD56^{-}$) expressing surface CD107a at the end of the assay is shown . N=3. Means and SEM shown.

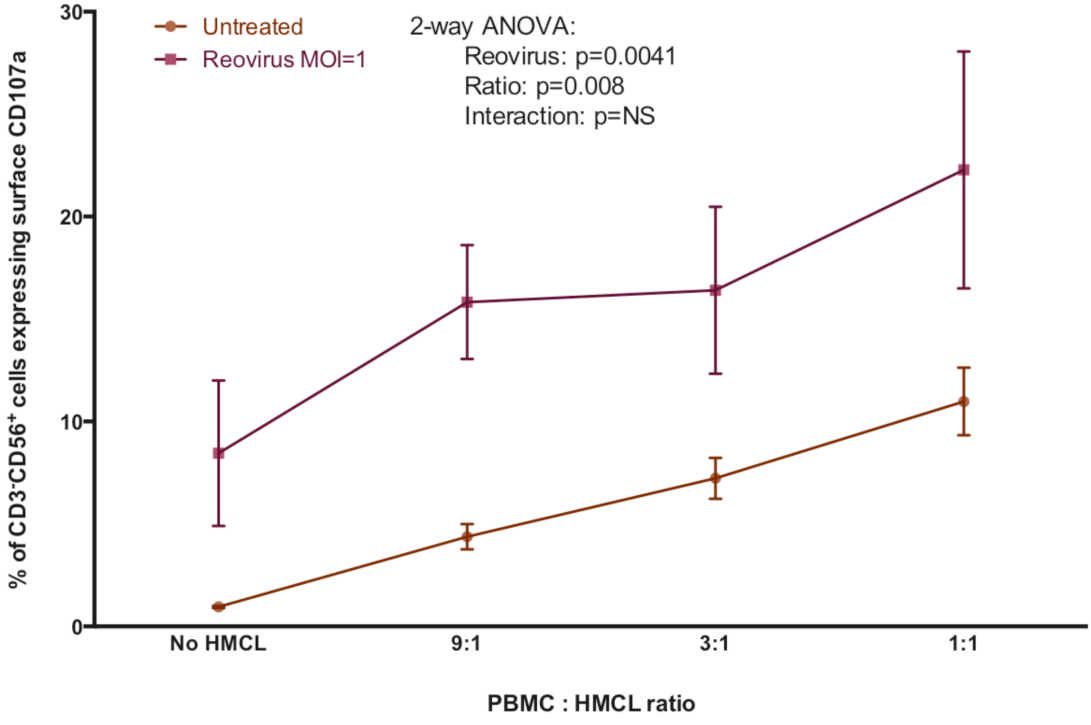


Figure 5.25. NK cell degranulation assay against OPM2. The fraction of NK cells (defined as $CD3^{+}CD56^{-}$) expressing surface CD107a at the end of the assay is shown . N=3. Means and SEM shown.

NK degranulation assays were next conducted after treatment of PBMC with both reovirus and lenalidomide for 48 hours. Lenalidomide was found not to increase NK cell degranulation above that already seen with reovirus treatment; Figure 5.26 shows the results for H929 – other HMCLs yielded similar results (data not shown).

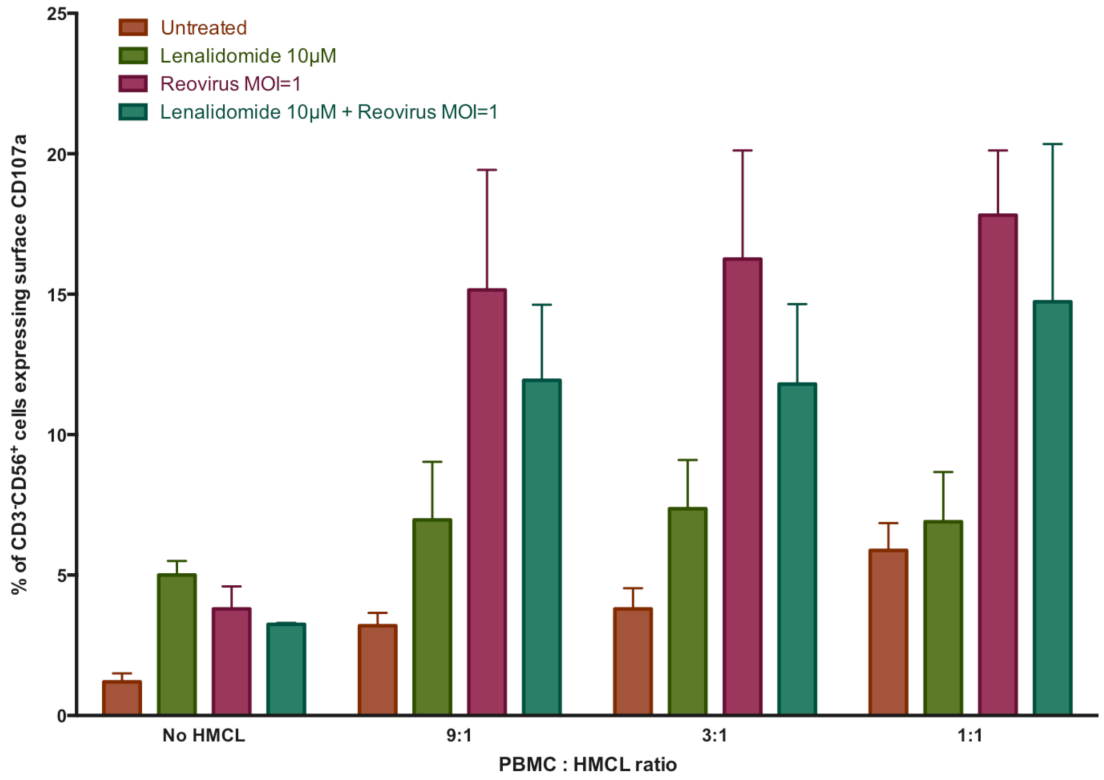


Figure 5.26. NK cell degranulation assay against H929 with and without prior lenalidomide treatment of PBMC. Histograms show the fraction of NK cells (defined as $CD3^{+}CD56^{-}$) expressing surface CD107a at the end of the assay. N=6. Histograms show medians and standard deviations.

Attempts were next made to assess the impact of NK cell degranulation in these assays on death of tumour cells. Tumour cells were labeled with cell tracker prior to the degranulation assay, as described previously, and cell death in the labeled cells was then determined by intracellular amine staining after the assay. Figures 5.27 and 5.28 show cell death in H929 and OPM2 respectively.

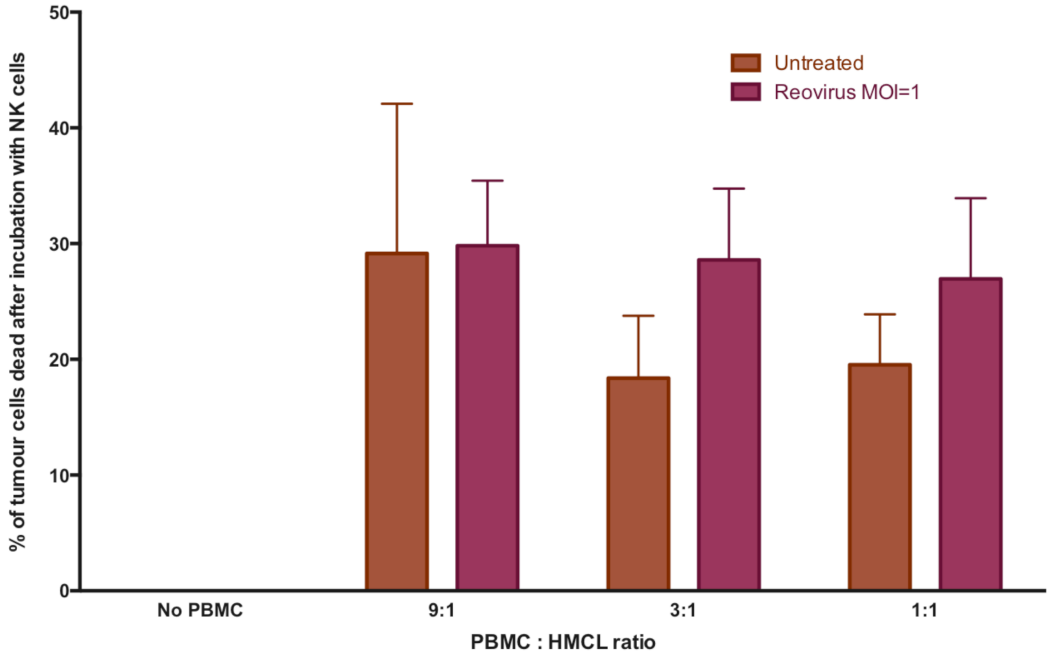


Figure 5.27. Cell death in H929 after NK cell granulation assay. Histograms show the fraction of tumour cells dead after 3 hours of incubation with NK cells, which were either pretreated with reovirus for 48 hours (red) or not (orange). Histograms show medians and standard deviations; n=4.

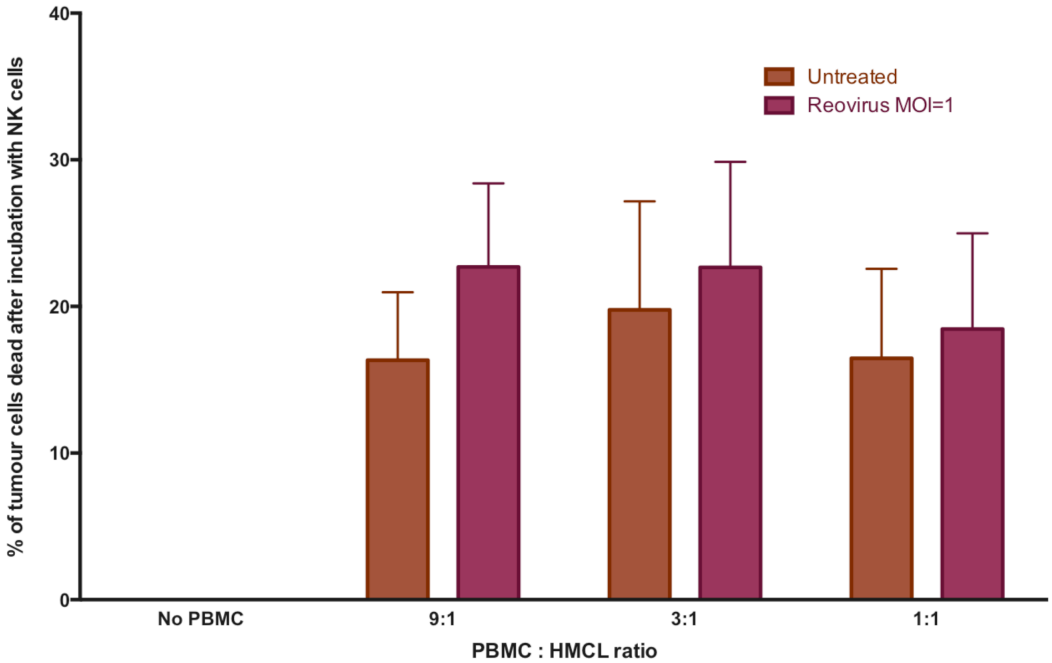


Figure 5.28. Cell death in OPM2 after NK cell granulation assay. Histograms show the fraction of tumour cells dead after 3 hours of incubation with NK cells, which were either pretreated with reovirus for 48 hours (red) or not (orange). Histograms show medians and standard deviations; n=3.

Oncolysis of HMCLs on stromal cell layers

A body of evidence points to an ability of bone marrow stromal cells to confer protection to tumour cells against cytotoxic agents. For this reason, it was important to determine whether the lytic effect of reovirus on HMCLs would be abrogated by the presence of bone marrow stromal cells. Tumour cells were therefore cultured either alone, or on a basal layer of stromal cells; the human bone marrow stromal cell lines HS-5 and HS-27, the murine bone marrow stromal cell line M2-10B4 and a $CD40L^+$ human fibroblast line L929 were used for these assays. Cultures were then treated with reovirus, lenalidomide or dexamethasone for 48 hours as described previously.

When U266 was treated in the absence of stromal cells (Figure 5.29), both dexamethasone and reovirus treatments induced a significant increase in cell death ($p=0.04$ and $p=0.013$ respectively), although lenalidomide did not. However, when cultured with HS-27, HS-5 or M2-10B4, dexamethasone did not induce a significant increase in cell death above the untreated control; reovirus on the other hand induced significant cell death in HMCLs cultured on both HS-27 and HS-5 ($p=0.009$ and $p=0.011$ respectively). Both dexamethasone and reovirus induced significant killing of cells cultures on the L929 cell line ($p=0.004$ and $p=0.002$ respectively).

Figure 5.30 shows the results of treatment of H929 with lenalidomide, dexamethasone and reovirus during culture on stromal cell layers. In these experiments, neither dexamethasone nor lenalidomide resulted in a significant increase in cell killing even in the absence of stromal cells, so little can be inferred from their failure to induce killing of HMCLs on stromal cell layers. Nonetheless, reovirus was able to induce significant killing of H929 under all conditions, irrespective of the presence of stromal cell layers.

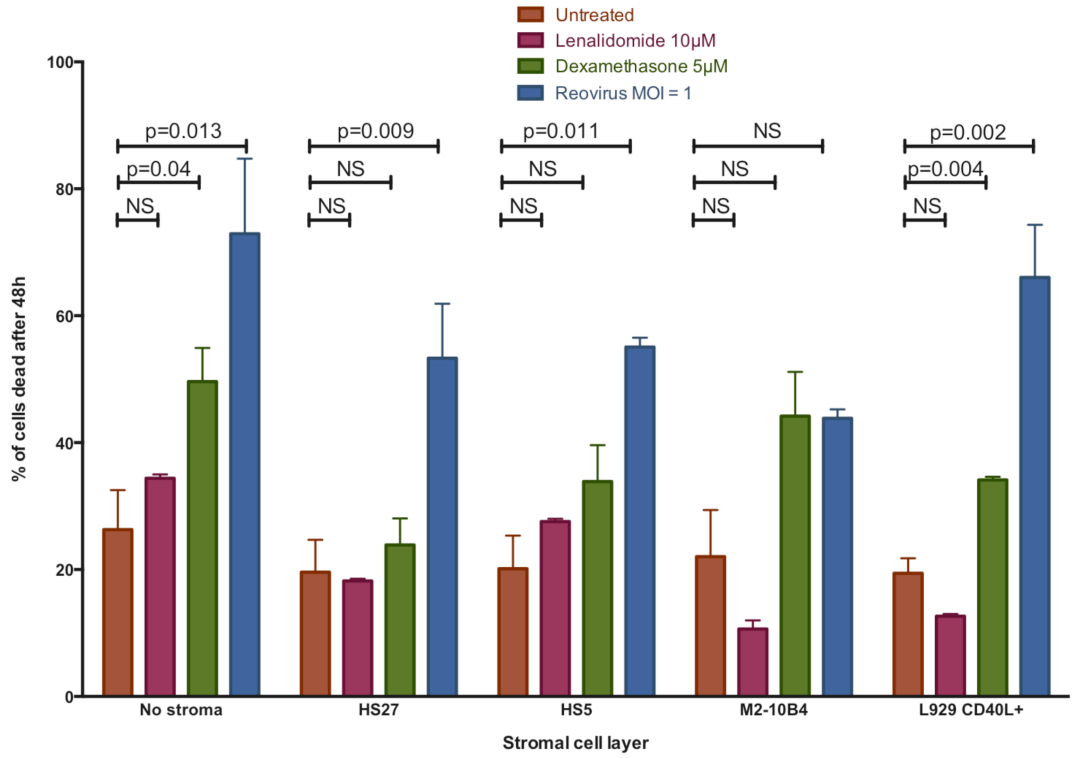


Figure 5.29. Cell death in U266B after culture for 48 hours either alone or on the stromal cell lines HS-5, HS-27, M2-10B4 or L929, in the presence of reovirus, lenalidomide, or dexamethasone at the doses shown, or untreated. Histograms indicate medians and standard deviations; n=3. P values are for two-tailed paired student t tests.

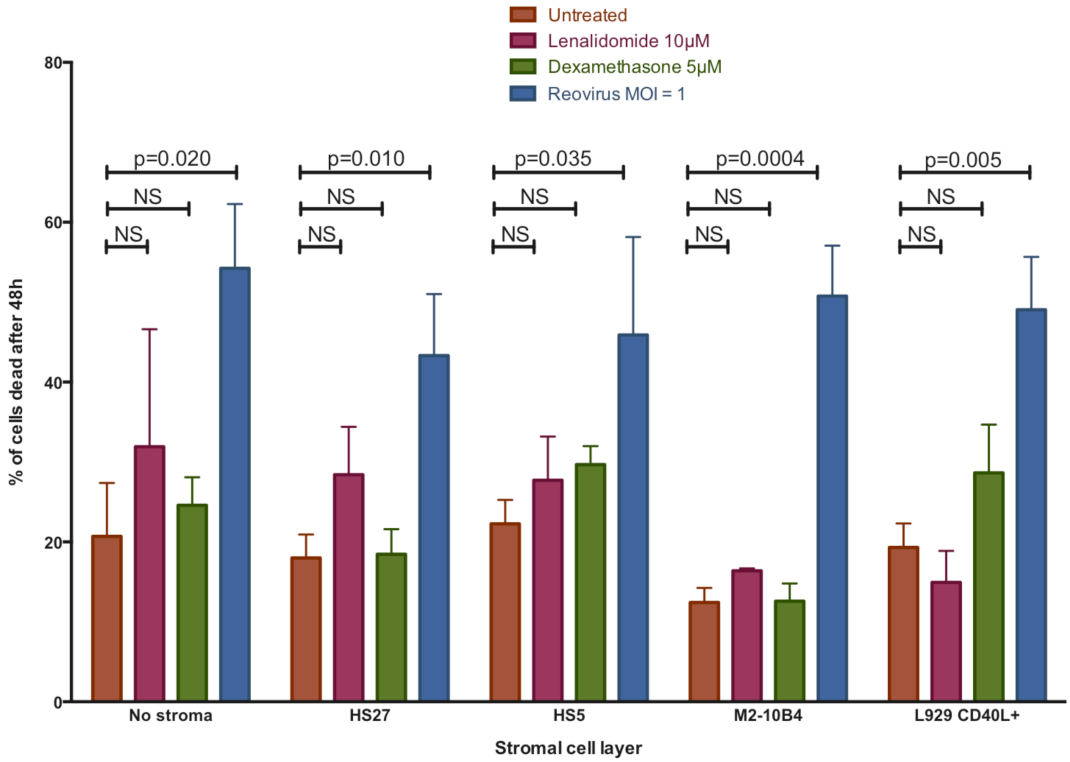


Figure 5.30. Cell death in H929 after culture for 48 hours either alone or on the stromal cell lines HS-5, HS-27, M2-10B4 or L929, in the presence of reovirus, lenalidomide, or dexamethasone at the doses shown, or untreated. Histograms indicate medians and standard deviations; n=3. P values are for two-tailed paired student t tests.

Development of an in vivo model

The experiments described thus far provide compelling evidence that reovirus may represent a useful agent for therapy of myeloma, exhibiting multiple mechanisms of action and an ability to overcome stromal-mediated cytoprotection. In order to allow further elucidation of the mechanism of action of reovirus in myeloma, and elaboration of clinical strategies – for example examination of dosing and combination chemotherapy strategies – an *in vivo* model was therefore required. A number of models were considered (discussed in introduction) and the C57BL/KaLwRijHsd model with the 5TGM1 cell line selected.

C57BL/KaLwRijHsd mice aged 6-10 weeks were injected with $2x10^5$ 5TGM1 tumour cells. After 21 days, bone marrow was harvested and unselected bone marrow cells were stained using a Live/Dead intracellular amine stain and an antibody against CD138. The percentage of live $CD138^+$ cells present was assessed by flow cytometry and compared to wild type mice; Figure 5.31 shows representative flow cytometry histograms and collated data. Engraftment of myeloma plasma cells was seen with 46.6% *vs.* 1.0% $CD138^+$ cells seen ($p<0.0001$). Since splenic haematopoiesis is seen in mice, and some extramedullary myeloma is expected in this model, splenic cells were next assessed in the same way. Splenic plasma cells were expanded in diseased mice compared to control mice: 33.9% *vs.* 2.1% ($p=0.017$, Figure 5.32).

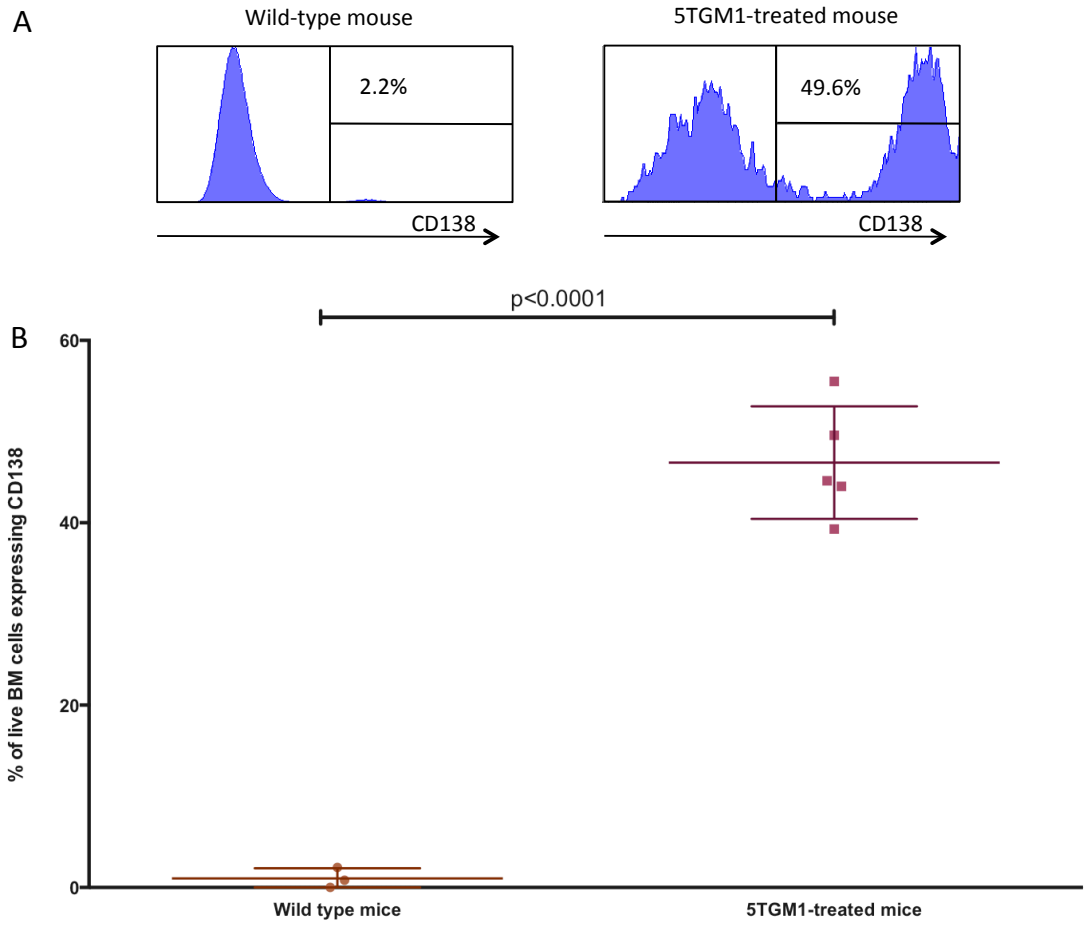


Figure 5.31. Fraction of live BM cells expressing CD138 in wild-type and 5TGM1-bearing C57BL/KaLwRijHsd mice. Representative histograms (panel A) and collated data (panel B) are shown. Mean and standard deviation are shown in panel B, p value is for unpaired student t-test.

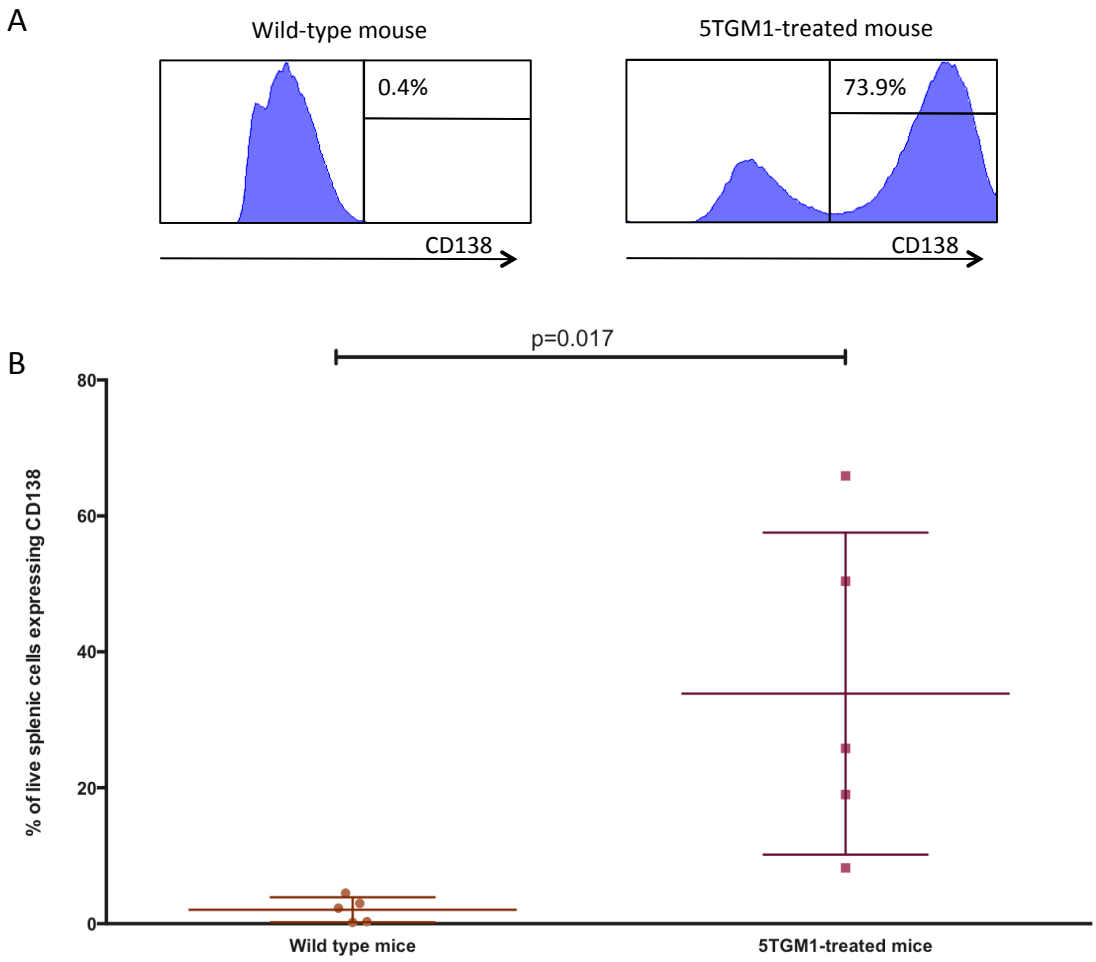


Figure 5.32. Fraction of live splenic cells expressing CD138 in wild-type and 5TGM1-bearing C57BL/KaLwRijHsd mice. Representative histograms (panel A) and collated data (panel B) are shown. Mean and standard deviation are shown in panel B, p value is for unpaired student t-test.

The susceptibility of the 5TGM1 cell line to treatment with reovirus was next explored. C57BL/KaLwRijHsd mice aged 6-10 weeks were injected with $2x10^5$ 5TGM1 tumour cells. After 21 days, bone marrow was harvested and unselected bone marrow treated *ex vivo* with reovirus, dexamethasone and/or lenalidomide and incubated for 48 hours at 37°C and 5% CO_2 in a humidified incubator. Cells were then harvested, stained using a Live/Dead intracellular amine stain and an antibody against CD138, and the percentage of live cells expressing $CD138^+$ was assessed by flow cytometry (Figure 5.33).

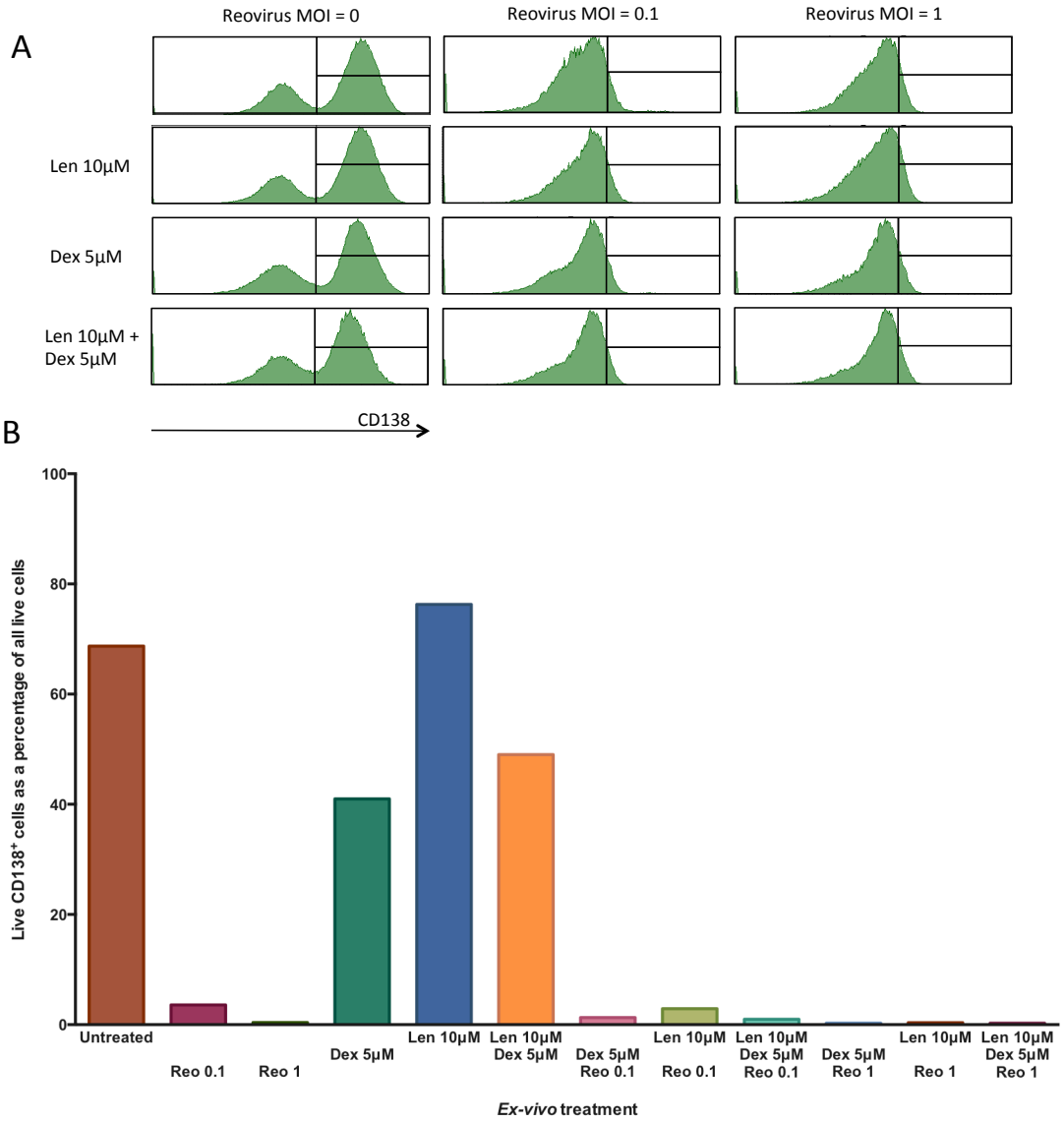


Figure 5.33. CD138⁺ cells as a fraction of live cells after *ex-vivo* treatment of bone marrow samples from 5TGM1-bearing C57BL/KaLwRijHsd mice with lenalidomide, dexamethasone and reovirus in the combinations and at the doses indicated. Flow cytometry histograms (panel A) and percentages of CD138⁺ cells as a fraction of live cells (panel B) are shown. n=1.

Since this pilot experiment revealed marked purging of $CD138^+$ cells from the BM by *ex vivo* reovirus therapy, protocols for *in vivo* therapy were next developed. The remaining work presented here represents pilot data, which will form the basis of planned ongoing experimental work.

C57BL/KaLwRijHsd mice aged 6-10 weeks were divided into 3 groups:

- Group 1: naïve mice (n=5)
- Group 2: 5TGM1 (n=6)
- Group 3: 5TGM1 + reovirus (n=10)

Mice in groups 2 and 3 were injected with $2x10^5$ 5TGM1 tumour cells in 100µl of PBS via tail vein injection (Day 0). On days 12, 14 and 16, mice in group 3 were injected with $2x10^7$ plaque forming units (PFU) reovirus. On day 28, one animal in each of groups 2 and 3 developed hind limb paralysis, at which point all animals were sacrificed.

Body and spleen weights were not significantly different between groups, although a trend towards higher splenic mass was seen in the tumour-bearing animals, which was reduced by reovirus treatment (Figure 5.34). The mean fraction of bone marrow cells expressing the plasma cell marker CD138 was as follows: Group 1: 0.82% (SEM 0.10%), Group 2: 33.42% (SEM: 3.93%), Group 3: 21.89% (SEM: 2.33%), with a significant decrease seen in the reovirus treated cohort (group 3) compared to untreated (group 2): p=0.017; unpaired two-tailed student t test (Figure 5.35). The mean fraction of splenocytes expressing the plasma cell marker CD138 was as follows: Group 1: 2.06% (SEM: 0.82%), Group 2: 31.98% (SEM: 8.82%), Group 3: 20.03% (SEM: 3.94%), the reduction associated with reovirus treatment did not reach significance here (p=0.173, unpaired two-tailed student t test, Figure 5.35).

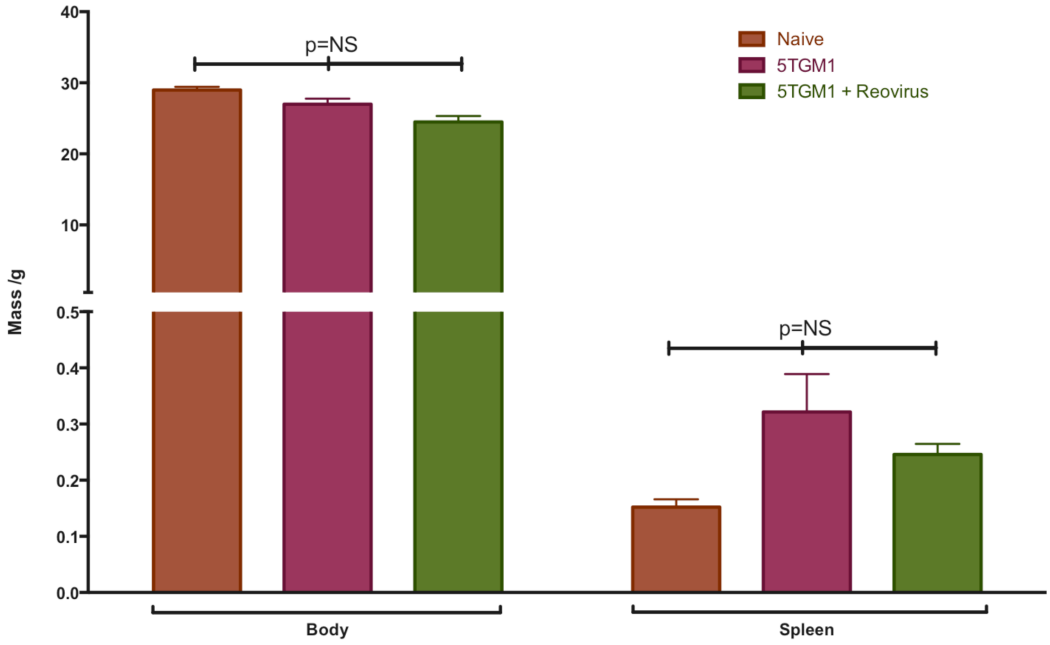


Figure 5.34 Post-mortem body and splenic masses in naïve (n=5), 5TGM1-bearing (n=6) and 5TGM1-bearing *in-vivo* reovirus-treated (n=10) C57BL/KaLwRijHsd mice. Histograms represent means and standard deviations; p values are for un-paired two-tailed student t-tests.

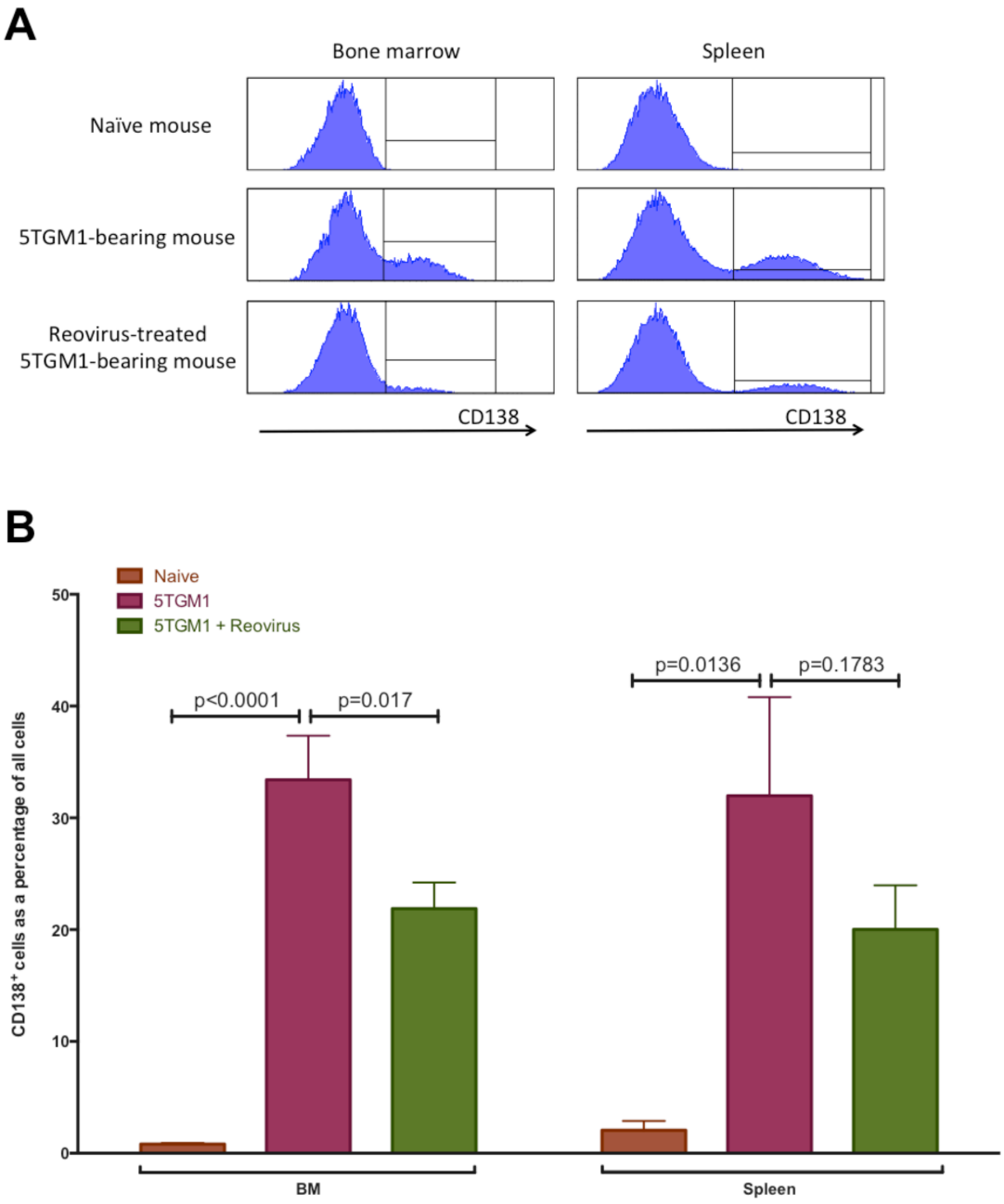


Figure 5.35 Fraction of bone marrow cells expressing CD138 in naïve (n=5), 5TGM1-bearing (n=6) and 5TGM1-bearing *in-vivo* reovirus-treated (n=10) C57BL/KaLwRijHsd mice. A: representative flow cytometry histograms, B: collated data showing means and standard deviations; p values are for un-paired two-tailed student t-tests.

The activation status of bone marrow- and splenic-resident $CD4^+$, $CD8^+$ and $DX\text{-}5^+$ (NK cell) lymphocytes, as measured by surface CD69 expression, was next assessed (Figure 5.34). $CD4^+$ cells were found to be activated in Group 2 *vs* Group 1 in both BM and spleen ($p<0.0001$ in both cases), $CD8^+$ cells in BM ($p<0.0001$) but not spleen (p=NS) and NK cells in spleen ($p=0.0021$) but not BM (p=NS). This suggests the induction of a host immune response by the presence of tumour cells. The reovirus-treated group, group 3, exhibited significantly increased CD69 expression on BM NK cells compared to group: 62.82% (SD 9.25%) *vs.* 46.19% (21.69%), indicating further activation of NK cells by reovirus treatment.

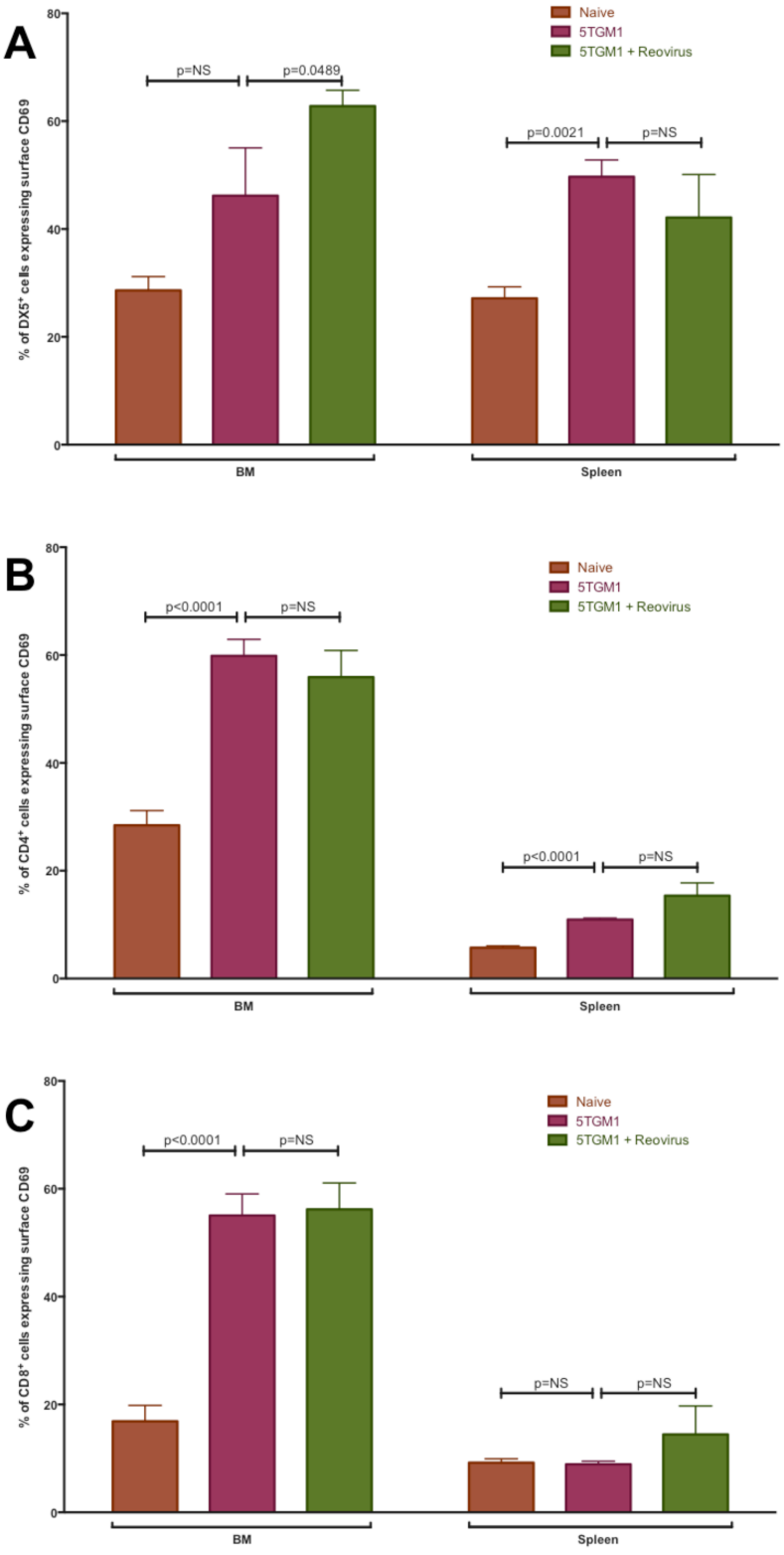


Figure 5.36. Fraction of bone marrow and splenic NK (DX5⁺, panel A) CD4⁺ (panel B), and CD8⁺ (panel C) cells expressing the activation marker CD69 in naïve (n=5), 5TGM1-bearing (n=6) and 5TGM1-bearing *in-vivo* reovirus-treated (n=10) C57BL/KaLwRijHsd mice. Histograms represent means and standard deviations and p values are for un-paired two-tailed student t-tests.

As a refinement of this model, C57BL/KaLwRijHsd mice aged 6-10 weeks were injected with $2x10^5$ eGFP-transduced 5TGM1 tumour cells in 100µl of PBS via tail vein injection. After 21 days, mice were sacrificed, shaved, depilated and imaged using an IVIS200 live imaging system with excitation at 465nm and emission collected at 520nm. A representative image, overlayed on a standard monochrome visible light image is shown in figure 5.35. Mouse A had a clinical evident plasmacytoma, which is clearly illuminated on the imaging. Some illumination of the axial skeletons is visible, despite subsequent flow cytometric examination of BM from these mice revealing only MGUS-levels of BM infiltration (<5% $CD138^+$ cells in all three cases). Splenic uptake is seen in mouse C.

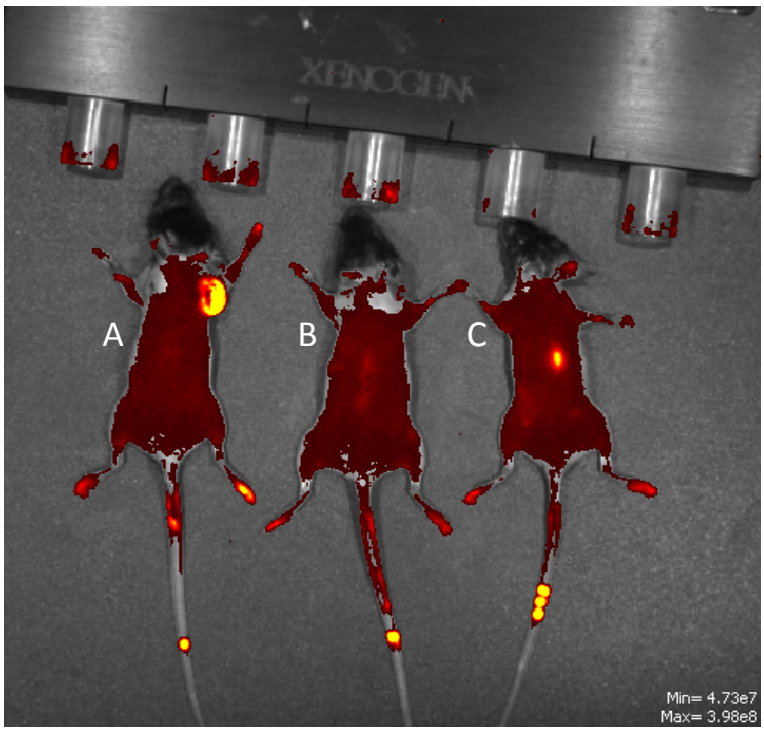


Figure 5.37. Composite image of monochrome visible light photograph and luminescent imaging (excitation: 465nm, emission filter: 520nm) of three C57BL/KaLwRijHsd mice injected with eGFP-transduced 5TGM1 tumour cells. Images taken after 21 days.

Discussion

The data presented here demonstrate multifaceted activity of reovirus type 3 Dearing strain against multiple myeloma. Firstly the majority of HMCLs tested were found to express junctional adhesion molecule 1 (JAM1) a widely expressed cell surface protein that in addition to regulating endothelial and epithelial tight junctions and serving as an integrin ligand, also functions as a receptor for reovirus. Interestingly, one cell line, OPM2, was found to express the receptor only at very low levels. A panel of HMCLs was then shown to be susceptible to direct killing by the virus, with only the JAM1^{low} cell line OPM2 proving resistant.

Preliminary experiments presented here are suggestive that death in the tumour cells is via apoptosis, since bcl2 is downregulated by reovirus, and the pan-caspase inhibitor zVAD-FMK abrogated reovirus-induced killing of JIM3. This is in keeping with the bulk of published work [220,319,320], but it remains to be clarified whether this is the case for all HMCLs, and whether other mechanisms of cell death, such as autophagy, necroptosis or direct virally-programmed lysis, which have been reported to result from reovirus infection in certain cell types, might also be contributory. Some data in the published literature are conflicting on this point[221,320] , and further elaboration of the mechanisms is warranted, particularly since this may provide for rational combination therapies, for example with clinically available bcl family inhibitors[321]. Furthermore, preliminary work examining bcl2 levels after reovirus infection, by Western blotting (data not shown), have suggested that reovirus might actually reduce bcl2 levels even in the resistant HMCL OPM2, suggesting that the most obvious explanation for resistance – that the virus is unable to enter cells due to inadequate expression of JAM1 – may be overly simplistic and that examination for dysregulation of apoptotic or other pathways in this cell may be central to understanding the mechanism of resistance. Further work will examine the modulation and role of other members of the bcl2 and other pathways (e.g. bcl-xl,LPARP)

When HMCLs were incubated with lenalidomide, no effect was seen on expression of JAM1 across a wide range of lines. Furthermore, when HMCLs were treated with combinations of reovirus, lenalidomide and dexamethasone, no significant interaction between their treatment effects was noted by 2-way ANOVA analysis, indicating no synergism or potentiation of the effects of one by the other. For example, with KMS11, which is sensitive to lenalidomide, dexamethasone and reovirus, additive effects were seen, with maximal killing induced by the top dose of reovirus in combination with either

dexamethasone or both lenalidomide and dexamethasone. JIM3, which is resistant to lenalidomide and dexamethasone, showed a dose-dependent sensitivity to reovirus which was unaffected by lenalidomide and dexamethasone dosing, whereas OPM2 exhibited sensitivity to both lenalidomide and to dexamethasone, maximum death when treated with the two in combination and no effect of reovirus on any combination.

Of course, there are considerations in assessing the efficacy of lenalidomide and dexamethasone treatments using a simplistic *in vitro* assay such as this. Firstly, this assay only assesses direct drug-induced effects on the tumour cells – other cellular interactions are deliberately excluded, and some of the later work presented in this chapter addresses this issue. Scheduling effects are also ignored – for example it is currently unknown what effect either dexamethasone or lenalidomide may have on the replication cycle of reovirus, and it is conceivable that treatment at different points in the cycle may have different effects. Dosing of lenalidomide and dexamethasone for *in vitro* assays is also difficult, since detailed data on the clinically relevant concentrations (i.e. in the bone marrow microenvironment of patients on tolerable treatment doses) are not available. The cell lines used for these assays are also necessarily primarily derived from patients with end-stage disease, usually plasma cell leukaemia, often with atypical features such as ascites or pleural effusion – it is likely therefore that these HMCLs may misrepresent any potential for synergy in less heavily pre-treated tumour clones. When whole bone marrow from a patient with MM was treated with reovirus, effective purging of $CD138^+$ cells from the culture and induction of death in plasma cells was seen, particularly when reovirus was used in combination with lenalidomide and dexamethasone, and further study of such 'whole marrow' cultures may prove a useful avenue of further investigation.

Reovirus was also shown to activate immune cells from both healthy donors and patients with myeloma, and it was further found that this effect was augmented by combination with lenalidomide. On the basis of this activation, NK cell degranulation assays were undertaken which demonstrated increased degranulation of NK cells against tumour targets when stimulated by reovirus, and furthermore that this degranulation translated into increased NK-mediated killing of tumour targets; from previous work it is likely this NK activation is mediated through monocyte-derived IFNα[207]. Interestingly, in the assays conducted here, addition of lenalidomide was not found to increase reovirus-induced NK degranulation against tumour targets, although again it will be worth assessing the effect

of combination therapy on NK cell degranulation in the context of less selected populations, for example, on whole marrow cultures.

A considerable body of evidence now points to an ability of bone marrow stromal cells to confer protection to tumour cells against chemotherapeutic strategies through myriad mechanisms, for example upregulation of cell cycle regulating and anti-apoptotic proteins[322], tumour cell entrapment within the marrow[323,324], and secretion of MM-supporting cytokines[325-327]. It was therefore of considerable interest to assess the impact of stromal cells on the susceptibility of HMCLs to reovirus-induced lysis. Reovirus was able to induce significant killing of two tumour lines under almost all culture conditions, irrespective of the presence of 'protective' stromal cell layers. Further studies in this area might include cellular adhesion assays[328,329], and assessment of viral replication in and modulation of stromal cells.

In concert with these *in-vitro and ex-vivo* data, the C57BL/KaLwRijHsd murine model seeded with the 5TGM1 cell line offers platform for elaborating a number of aspects of this work. As an immunocompetent model, it will allow dissection of the contribution of immune cells to both the anti-tumour action of reovirus and of course clearance of the organism by the host, and in particular will allow dissection of these interactions in the context of a fully-populated immune-competent myeloma-bearing bone marrow microenvironment. The preliminary data presented here indicate upregulation of CD69 on immune cells induced by the presence of tumour cells and therefore suggest this model may also be useful to examine the interactions between tumour and host immunity during the evolution of myeloma from MGUS – perhaps allowing elucidation of mechanisms that determine whether elimination, equilibrium or escape occurs. A number of preliminary experiments have been conducted, and are presented. Firstly an *ex-vivo* purging experiment confirms that the virus is able to induce death in the murine myeloma cells and provides further evidence of the ability of the virus to purge BM of malignant plasma cells. The *in-vivo* therapy experiment reveals the same ability in the intact bone marrow microenvironment and well as providing further evidence of the immune activatory properties of reovirus, which require further investigation. Finally, a eGFP-transduced modification of this model was assessed, and this will provide a platform for *in vivo* live imaging to assess tumour responses to experimental therapies.

A number of lines of evidence now indicate that for a number of oncolytic viruses (OV) being investigated as therapy of a range of solid and haematological malignancies, the primary 'lytic' phase of oncolytic action may in fact be far less important than a

subsequent secondary phase of immune-mediated oncolysis. There may be multiple mechanisms by which this occurs, involving both innate and adaptive immunity. For example, we have recently shown in CLL that reovirus is able to enhance antibody-dependent cytotoxicity against tumour cells, when used in conjunction with anti-CD20 antibodies[207]. The experiments presented in this thesis show NK cell function against MM can also be augmented by the virus.

However, true adaptive immune responses are probably still more important - in fact, in *in vivo* use, the primary viral infection and oncolysis may serve primarily to induce a highly immunogenic death in a proportion of cancer cells that serves to prime a far more potent adaptive immune response against cancer-associated antigens. A number of preclinical models have demonstrated that the efficacy of OV is dependent on generation of systemic antitumour immunity[203,204,330,331], and there is additionally evidence of secondary immune responses from human trials, for example a phase II trial demonstrated regression of metastases following intratumoural injection of a primary melanoma lesion with herpes simplex virus (HSV) expressing granulocyte-macrophage colony stimulating factor (GM-CSF)[332]. A recent manuscript has reported durable remissions in two patients after therapy of relapsing refractory MM with the engineered measles virus MV-NIS[333] – both of these responding patients developed a severe cytokine storm-like syndrome after infusion, in contrast to a large body of non-responding patients, who experienced no benefit from the virus, probably due to the presence of neutralizing antibodies; it seems likely that here too a secondary immune effect may have mediated the impressive responses in these patients. Understanding in more detail these 'priming' effects and adaptive immune responses in the context of reovirus therapy of myeloma are likely to be extremely important in devising rational therapeutic strategies including the agent.

Reovirus has recently been examined as a single agent in a phase I trial in patients with relapsed multiple myeloma [236], which demonstrated safety in this population. Building on the data presented within this chapter, an early phase clinical trial to examine the agent in combination with lenalidomide is now in the setup stage. In conjunction with this trial, promising avenues of investigation to inform future trials will now be undertaken using murine modeling, including examination of combination therapies with monoclonal antibodies and checkpoint blockade, as well as 'sub-optimal' initial therapy to examine the use of reovirus alone or in combination in the relapse setting after treatment with other agents.

Chapter 6 – Conclusions and Future Directions

In Chapter 3, a robust protocol for identification and phenotypic assessment of Th_{17}, Th_{17-1} and Th_1 cells in healthy donors and in patients with MM has been presented. A weak but significant correlation between Th_{17} frequency and age in health was demonstrated. This clearly has importance in the context of MM, a disease of the elderly – it casts light on interpretation of the validity of previously published work, which often did not employ age-matched controls. However, there are also wider implications in terms of immune senescence, particularly in a dawning age of immunotherapy, where selection of treatment modalities may increasingly depend upon characterisation of patients' underlying immune function. On the basis of published data on Th_{17} phenotypes, the expression of a range of cell surface markers was assessed. Disappointingly, CD161 and CCR6 were found not to be robust methods for identification of the subset, even in health, although they did allow relative enrichment of IL-17-producing populations.

Examination of patient peripheral blood samples revealed an increase in Th_{17} cells in MM compared to age-matched healthy donors, and this was found to occur in a disease stage-related manner. This defect was associated with a decrease in the level of IL-17 secretion by those Th_{17} cells still present under both supraphysiological and physiological stimulation, and a trend towards decreased serum levels of Th_{17} signature cytokines. When bone marrow samples were assessed, Th_{17} cells were seen to be relatively expanded in MM-bearing marrow compared to health, albeit with an unaffected Th_{17}:Th_1 ratio. The latter data were subsequently corroborated by coculture experiments and indicate MM plasma cells and the associated BM microenvironment are able to induce Th_{17} differentiation or survival. Th_{17} cells in MM were also demonstrated to have an aberrant cell surface phenotype, with relative loss of CD161and CCR6 expression relative to health.

In Chapter 4, *in vitro* coculture of $CD4^+$ T cells with HMCLs revealed tumour-driven expansion of Th_{17} cells, predominantly from $CD45RO^+$ activated and memory T cells, and suppression of the Th_1 population. Interestingly, and somewhat unexpectedly, selection of starting populations for CD161 and CCR6 expression had no significant effect on final numbers of Th_{17} cells derived from coculture whereas incorporation of bone marrow stromal cells in the culture system resulted in marked augmentation of Th_{17} polarisation. A T cell polarisation protocol was devised, and when $CD4^+$ cells from healthy donors and

patients with MM were treated with this protocol an increased propensity to Th_{17} differentiation was seen in the patient samples.

These data add to the currently published knowledge by documenting an additional aspect to the profound immune dysfunction seen in MM, and in turn lead to a number of further questions which may warrant further study. The quantitative and qualititative defects seen in Th_{17} immunity have a number of important implications. Firstly, the normal role of Th_{17} cells lies predominantly in delivering anti-infective immune responses, in particular against fungal pathogens, and the phenotypic abberancy and functional compromise shown herein may contribute to the increased predisposition to infections seen in patients with MM. Further assessment of this defect could follow a number of directions. Initially, the secretion of peripheral blood Th_{17} signature cytokines in health and disease might first be corroborated by another method, for example qRT-PCR – this is likely to be important since my data are contradictory to published work, as discussed in detail earlier. The functional consequences might also be examined, for example by assaying the capacity of cell culture supernatants to induce neutrophil activation, migration and recruitment or upregulation of endothelial defensins, or indeed their impact on the NK cell degranulation assays reported in Chapter 5.

Secondly, the relative expansion of PB Th_{17} cells seen in MGUS and AMM, but lost by the time of progression to symptomatic MM or at time of relapse warrants further examination. Clearly the interaction between $CD4^+$ cells and the tumour clone is more complex than a simple positive feed-forward loop. It is of interest, for example, that certain HMCLs are unable to drive Th_{17} generation. It is tempting to speculate, in light of a wealth of published work suggesting MGUS may represent a state of immune equilibrium during which the tumour clone is controlled by immune responses, that Th_{17} cells in this situation might even mediate anti-tumour responses; certainly there is evidence from the published literature that $Th_{17\text{-}1}$ cells have efficacy in the context of adoptive cellular therapy. Mapping of the TCR repertoire of Th_{17} cells in each of these states might therefore be illuminating, and indeed serial examination of the repertoire within patients could be considered. An interesting aside here is that a very large proportion of Th_{17} cells were seen to respond to Candidal antigens, suggestive either of a highly restricted TCR repertoire within the subset, or perhaps more likely an ability to respond via a PAMP receptor of some sort. In view of the relative expansion of Th_{17} cells seen in MM BM (in contrast to the peripheral blood findings), immunohistochemistry to

examine the localisation of Th_{17} cells in the BM may also be a useful method to begin to infer which additional cellular players may be contributing to lineage determination.

Balanced against such postulated anti-tumour immunity are published data indicating IL-17, and indeed other Th_{17} signature cytokines, may act as growth factors for MM cells [74]. Importantly, I have been unable to corroborate these experiments and this should be further examined using additional methods to assess the effect of IL-17 on HMCLs e.g. thymidine incorporation, Ki67 staining, serum starvation of cell lines, and use of primary MM plasma cells.

Thirdly, the loss of CD161 and CCR6 on Th_{17} cells in MM is intriguing, and this body of work might be developed in a number of directions. The role of CD161 remains poorly characterised, so one group of experiments could aim to examine the contribution of CD161 to the immune synapse, for example testing the effect of CD161 ligation on IL-17 production after physiological stimulation (e.g. with fungal antigens), or as a costimulatory molecule with TCR stimulation. The issue of whether CD161 is downregulated by Th_{17} activation also remains to be clarified. Building upon the functional assays already proposed, the capacity of $CD161^+$ and $CD161^-$ Th_{17} cells, in health and disease, to exert normal physiological effects on immunity could also be tested. It would also be interesting to examine the responses of these two groups of cells to immune synapse formation with MM cells, in particular whether one group is more supportive than the other of the tumour clone. CCR6 was also found to be lost on Th_{17} cells in MM, and the implications for this in terms of Th_{17} cell migration might also be assessed.

Fourthly, the coculture and polarisation data suggest several avenues of work exploring both deranged Th_{17} immunity in MM and the role of costimualtory pathways in T helper cell lineage determination. An increased propensity to Th_{17} differentiation in MM was seen and the underlying reasons for this are not known. Plausibly this might result from circulating cytokines that prime $CD4^+$ cells for Th_{17} differentiation, which could be examined for example through modification of the coculture system to include additional cytokine supplementation or neutralising antibodies. In light of the clear contribution of bone marrow stromal cells to the T cell modulating immune interactions, the role of additional cell types should now be assessed in coculture – for example osteoclasts and osteoblasts, cell types known to play a key role in the development of bone disease so characteristic of MM. It is known from work previously undertaken in our laboratory that T_{reg} cells are expanded in patients with MM and are expanded by interactions between HMCLs and $CD4^+$ T cells, and a large body of literature addresses the plasticity between

Th_{17} and T_{reg} cells. In light of this, investigation of the Th_{17}:T_{reg} balance in the coculture model, potentially including the effect of therapy within the model (e.g. immunomodulatory agents, proteasome inhibitors etc.), might help to illuminate whether Th_{17} cells are predominantly pro or anti-tumour immune effectors.

The polarisation data also suggest some questions of more fundamental immunological interest. The publications to date examining polarisation cocktails designed for driving Th_{17} differentiation have yielded a range of different recommendations, and often conflicting results. My data highlight the importance of considering the starting population – conditions giving rise to the largest total number of Th_{17} cells were often distinct from those yielding the purest population and this may be partly due to expansion of contaminating populations, such as Th_1 cells, which subsequently negatively regulate Th_{17} development. Such contaminating cells might for example explain the widely disparate effect of IL-2 reported in the literature. In addition, when signal 2 combinations were assessed, CD5 and ICOS were found to induce Th_{17} differentation more potently than the more traditionally employed CD28. Previous work has shown ICOS/ICOS-L interactions to be important in driving T_{reg} generation[309], and further work to examine the contribution of signal two in determining lineage determination may be warranted. It is likely from the data presented here that there are complex interactions between TCR stimulation, signal two and the cytokine microenvironment so that, for example, a particular signal 2 may have different effects on T cell fate depending on the cytokine costimulation.

Chapter 5 focused on the potential for the use of the oncolytic virus reovirus for treatment of multiple myeloma. HMCLs were seen to express JAM1, and to be susceptible to reovirus-mediated lysis *in vitro*, probably via an apoptotic mechanism; additive effects were seen when lenalidomide was added. Reovirus was also noted to induce marked immune activation of peripheral blood immune effectors from both healthy donors and patients with MM, an effect that was again augmented by the inclusion of lenalidomide. Reovirus was able to upregulate NK cell-mediated killing of tumour cells. Finally, a murine model was developed to allow further examination of reovirus as an *in vivo* therapy, and preliminary data were presented confirming effective *ex vivo* purging of MM-bearing muring marrow, *in vivo* immune activation and effective clearance of MM cells from BM and spleen by *in vivo* reovirus therapy.

These data on reovirus are extremely exciting and indicate a potential novel immunotherapy for MM. On the basis of this work a phase I/IIa clinical trial has been devised to examine reovirus in combination with lenalidomide or pomalidomide in patients

developing serological relapse on IMiD therapy. Further preclinical work, and planned companion translational studies for the trial, will now be undertaken to characterise the immune effect of reovirus-based combination therapy, and to develop rational treatment combinations to augment reovirus treatment. The mechanisms of cell death induced by reovirus should be investigated in more detail, incorporating assays and blocking of apoptosis, necroptosis and autophagy pathways – this would then allow subsequent evaluation of combination therapy strategies with inhibitors of these pathways to optimise direct reovirus-induced cell lysis. The mechanisms by which reovirus is able to augment NK cell-mediated tumour cell lysis also warrant further examination. Previous work indicates this may be via monocyte-derived IFNα [207], and furthermore that monocytes are capable of reovirus carriage [334]; combination of reovirus with cellular therapies or cytokine conditioning may therefore have the potential to augment tumour killing. Preliminary experiments, not presented herein, also suggested soluble factors from reovirus-treated PBMC can kill HMCL, meaning a large body of work to characterise in more detail the effect of reovirus on PBMC may yield strategies for augmenting immune-mediated tumour killing. The continued efficacy of reovirus even in the context of stromal-mediated protection is also of interest. This work could be developed in a number of directions, for example the replication in, and modulation of, stromal cells by reovirus remains to be characterised. Cellular adhesion assays may also be of interest as one possible explanation is that reovirus is able to disrupt the interactions between HMCL and their stromal support. Of course, the murine model now offers a platform for *in vivo* testing of any promising potential combination strategies.

The work presented in thesis documents a new aspect of immune dysfunction in MM, with implications for anti-infective and anti-tumour immunity. Furthermore a novel immunotherapeutic strategy against multiple myeloma has been described and this is currently in translation to an early phase clinical trial. Subsequent pre-clinical and translational work will now allow rational development of further combination immunotherapy strategies.

Glossary

AC	Apheresis cone; used as a source of peripheral blood mononuclear cells from normal donors
AHR	Aryl hydrocarbon receptor
AlloSCT	Allogeneic stem cell transplant
APC	Antigen presenting cell
β_2M	Beta-2-microglobulin
BM	Bone marrow
BMSC	Bone marrow stromal cell(s)
BMME	Bone marrow microenvironment
CM	Complete medium
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-assisted cell sorting; used within the text to indicate both cell analysis and physical cell sorting using the technique
FBS	Fetal bovine serum
FoxP3	Foxhead box P3; the signature transcription factor for regulatory T cells (T_{reg})
HBSS	Hank's buffered salt solution
HMCL	Human myeloma cell line
ICOS	Inducible T cell co-stimulator; CD278
IFNγ	Interferon gamma
IMiD	Immunomodulatory drug

ION	Ionomycin
IRF4	Interferon-regulatory factor 4
MACS	Magnetic-assisted cell sorting
MACS buffer	Solution used for MACS (see reagents and solutions)
MFI	Mean fluorescence intensity; a flow cytometric parameter considered a surrogate for level of antigen expression
MGUS	Monoclonal gammopathy of uncertain significance (see Appendix 1)
MHC	Major histocompatibility complex
MitC	Mitomycin C; used to impair HMCL proliferation in coculture
MM	Multiple myeloma
MNC	Mononuclear cells
MP65	*Candida albicans* mannoprotein 65, used within the text to indicate the Peptivator® lyophilised pooled peptide product derived from MP65 (Miltenyi Biotec).
NK	Natural killer cell
NKT	Natural killer T cell
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PC	Plasma cell
PMA	Phorbol 12-myristate 13-acetate
PP	Paraprotein; M-protein; myeloma clonal immunogloublin
RARα	Retinoic acid receptor alpha
RORC	The human homologue of RORγt

RORγt	Thymic isoform of RAR (retinoic acid receptor)-related orphan receptor gamma – RORC is the human homologue; the signature transcription factor of Th_{17} cells
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells; the signature transcription factor of Th_1 cells.
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
T_{reg}	Regulatory T cell
VEGF	Vascular endothelial growth factor

Appendices

Appendix 1 – Plasma cell disease classification

(adapted from [2])

Diagnostic criteria for MGUS, Asymptomatic and symptomatic MM

	MGUS	Asymptomatic myeloma	Symptomatic myeloma
Monoclonal protein in serum	<30g/l	≥30g/l AND/OR	Present in serum and/or urine at any concentration†
Bone marrow clonal plasma cells	<10% and low level of plasma cell infiltration in a trephine biopsy if done	≥10%	Present, or biopsy-proven plasmacytoma
Related organ or tissue impairment*	None (including bone lesions)	None (including bone lesions); no symptoms	Present

Table A1.1 Diagnostic criteria for MGUS, asymptomatic and symptomatic myeloma.

† A small percentage of patients have no detectable M-protein in serum or urine but do have myeloma-related organ impairment and increased bone marrow plasma cells (non-secretory myeloma)

Myeloma-related organ or tissue impairment

Clinical effect	Definition
Hypercalcaemia	Corrected serum calcium >0.25 mmol/l above the upper limit of normal or >2.75 mmol/l
Renal insufficiency	Creatinine >173µmol/l
Anaemia	Haemoglobin ≥2g/sl below the lower limit of normal, or <10g/dl
Bone lesions	Lytic lesions or osteoporosis with compression fractures (magnetic resonance imaging (MRI) or computed tomography (CT) may clarify)
Other	Symptomatic hyperviscosity Amyloidosis Recurrent bacterial infection (>2 episodes in 12 months)

Table A1.2 Myeloma-related organ or tissue impairment

Appendix 2 – Donor Information and Consent Forms

Patient Information Sheet (Patient)

THE HOST-IMMUNE CONFLICT IN MULTIPLE MYELOMA

You have been invited to take part in a research study, but before you decide to take part, 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. Ask the doctor informing you of the study, if there is anything that is not clear to you 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 this study?

The immune system is the body's defence not only against infection but also abnormal cells and tissues. We are investigating the role of immune cells in a condition called multiple myeloma. Through these studies we wish to understand how to manipulate the diseased (tumour) cells to increase their susceptibility and potential killing by the patient's own immune system. Immune cells, found both in the blood and bone marrow, are important in helping to control myeloma. This study aims to determine whether there are enough of these cells or whether there is a problem with how these cells work and thus do not fight the myeloma. It is by studying this, that we aim to develop a way in which we can help the patient's own immune system to fight the myeloma cells.

This study aims to detail the different type of immune cells found in both the blood and bone marrow, in particular whether there is any evidence of cells that could potentially attack the myeloma cells. The ultimate aim of this research is to gain a better understanding of what effect myeloma has on the immune system so that we can design treatment plans which may help to boost the patient's immunity whilst setting groundwork for a new therapeutic system for multiple myeloma. The study you are being invited to participate in is to investigate if any defects exist in the immune system. It does not involve any specific experimental treatment and will not affect any treatment being planned by you doctor.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and will be asked to sign a consent form, to indicate that you are willing to take part in this study. You will also be given a copy of that consent form. You can always withdraw from the study at any time without giving a reason. A decision not to take part or to withdraw from the study will not affect the standard of care you receive.

What will happen to me if I take part?

If you agree to take part in this study, once you have read and understood this patient information sheet, you will be asked to sign your written consent form. This is a paper record that will show the authorities that you voluntarily agreed to take part in this study.

If you agree to participate in this study, a small amount (5 ml) of extra bone marrow will be taken at the same time as your scheduled bone marrow. We would also take one extra sample (30-40 mls) of blood on the same day as your bone marrow, which may take an additional 30 minutes of your time at this visit. If you are scheduled for a procedure called vertebroplasty, a small amount of the bone marrow tissue extracted will be taken aside for this study.

All samples will be taken to the Research Laboratory where the different cell types of interest to the study will be analysed. Information collected will be stored on a database, which will only record your age, sex and disease type. The data will be anonymous (i.e. will not have your name connected with this data) and will only be available to the principal investigator of this study (Prof G Cook) and his researchers. No external organisation, charity or commercial organisation will be permitted access to this data. Samples taken will be stored in a freezer so that follow-on experiments can be performed to verify the research results, if required, over the period of the study. With your permission, any surplus samples may be used in any other future myeloma research. You will not be identified in any future research.

Why have I been chosen?

You have been chosen to take part in this study because you have been diagnosed of myeloma or a related condition called MGUS.

What are the possible benefits of taking part?

There is no immediate benefit to you if you decide to take part. However, the information we get from this research may lead to development of treatment strategies for patients with multiple myeloma in the future.

What are the possible risks of taking part?

There is a small risk of bruising and discomfort, and a very small risk of infection at the site of the blood sample. If you are listed for a bone marrow or vertebroplasty, there is no additional risk to what has already been explained to you for the procedure. Taking an additional sample does not significantly prolong the time of the procedure.

Will my taking part in the study be kept confidential?

If you consent to take part in this study then your medical records will only be inspected by your doctors. Laboratory generated information will be kept on a data base in an anonymised fashion (i.e. your name will not be associated with any laboratory information) which will only be available for scrutiny by the principal investigator or his researchers. Sections of your

medical notes may be looked at by responsible individuals from regulatory authorities. No external body, charity or commercial organisation will have access to this data. Your name will not be disclosed outside of the hospital/clinic.

What will happen to the results of the research study?

Once the study has finished, the information will be analysed and a report will be written to record your results. It is hoped that the results from this study will be suitable for publication in a scientific journal or presented at scientific meetings. Your doctor will keep you informed of the results from the study.

Who is organising and funding the research?

This study is being conducted under the guidance of the principal investigator, Professor Cook, by the Transplant Immunology Group based at the University of Leeds. This study has been funded by the Leeds Teaching Hospitals Charitable Trust Foundation and the Yorkshire Cancer Network.

Will I receive any expenses for taking part in the study?

No.

Who has reviewed this study?

This study has been reviewed by NRES Committee Yorkshire & the Humber - Leeds East.

Contact for further information:

If you have any questions regarding how this study will be conducted you may contact: **Professor Gordon Cook (telephone 0113 2068433).**

Thank you for reading this information sheet and for considering taking part in this study.

Volunteer Consent Form

THE HOST-IMMUNE-CONFLICT IN MULTIPLE MYELOMA.

Principle Investigator: **Prof G Cook**

PLEASE INITIAL BOX

1. I confirm that I have read and understand the information sheet dated……. (version……..) for the above study and 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, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I understand that relevant sections of my medical notes (if applicable and data collected during the study may be looked at by individuals from regulatory authorities and/or Leeds Teaching Hospitals NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records" ☐
5. I give permission for my samples of blood and bone marrow to be stored and use in future scientific research linked to this study. ☐
6. I understand that my samples will be anonymised and that strict confidentiality will be maintained. ☐
7. I agree to take part in the above study ☐

Name of Volunteer	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

1 copy for the donor, 1 for the researcher and 1 to be kept in the hospital notes

Version 2 01 September 2012

Appendix 3 – Human myeloma cell line characteristics

Name	Source	Cell surface markers	Cytogenetics	Molecular abnormalities	Notes
H929	62 year old caucasian with IgAκ-producing plasmacytoma,	$CD38^+$ $HLA\text{-}DR^-$	hypodiploid 43-46, X t(4;14)	c-myc rearranged myc RNA expressed, NRAS G13D het	Secretes large amounts of IgA
JIM1	Myeloma plasma cells derived from pleural fluid of patient with advanced MM	$CD38^+$	t(4;14)	TP53 R273C hom	Homoplastic with JIM3
JIM3	Myeloma plasma cells derived from pleural fluid of patient with advanced MM	$CD38^+$	t(4;14)	FGFR3 not expressed KRAS G12D het TP53 R273C homo	Homoplastic with JIM1
JJN3	Plasma cell leukaemia in 57-year old women, IgAκ-producing.	$CD138^+$ $HLA\text{-}DR^+$	hypotriploid 58-67, XX t(14;16)	TP53 homo del	2 copies of t(14;16) associated with c-maf activation
KMS11	Pleural effusion in female with myeloma	$CD138^+$	t(4;14), t(14;16)	FGFR3 het TP53 homo del TRAF3 homo del.	κ light chain-secretor
KMS18	58 year-old male	$CD138^+$	t(4;14)	BIRC2/3 homo del FGFR3 G382D het	Patient had hyperammoniaemia and produces ammonia in culture
OPM2	Plasma cell leukaemia of 56-year old woman, IgGλ	$CD138^+$ $HLA\text{-}DR^-$	hypertriploid 77-82, XX t(4;14)	CDKN2C homo del FGFR3 K650E het TP53 R175H homo	Cryptic t(4;14) associated with IgH-FGFR3 fusion
RPMI8226	IgGλ plasma cell leukaemia in 61-year old man	$CD38^+$ $CD138^+$ $HLA\text{-}DR^+$	hypotriploid 62-67, XXY t(14;16)	CDKN2C homo del KRAS G12A het TP53 E285K homo TRAF3 homo del	Secretes λ light chains only
U266B	Plasma cell leukaemia in patient with IgEλ MM	$CD38^+$ $CD138^+$	t(11;14)	BRAF K601N het TP53 A161T homo TRAF3 K550IfsX3 homo RB1 E419X homo RB1 K228R homo	Secretes IL-6 and IgE

Table A3.1 Human myeloma cell line characteristics.

Appendix 4 – Western Blotting Solutions

Basic solutions:

Acrylamide: 30g acrylamide, 0.8g bis acrylamide, 100ml H_20.

1M Tris-HCl pH 8.8: 60.6g Tris, pH adjusted to 8.8 with HCl, 500ml H_20

0.25M Tris-HCl pH 6.8: 15.5g Tris, pH adjusted to 6.8 with HCl, 500ml H_20

5% SDS: 5g SDS (sodium dodecyl sulphate), 100ml H_20

10% APS: 0.5g APS (ammonium persulphate), 5ml H_20

PBS/TWEEN: 500ml phosphate-buffered NaCl, 0.1% TWEEN 20

ECL (electrochemiluminescence) solution 1: 400µl luminol, 176µl P-coumaric acid, 4ml Tris pH 8.8, 40ml distilled H_20

ECL solution 2: 24µl H_2O_2, 4ml Tris pH 8.8, 40ml distilled H_20.

Loading buffer (2x), for 50ml:

0.25M Tris-HCl pH6.8	25ml
SDS	2g
Bromophenol Blue	1mg
Glycerol	20ml
Distilled H_20	5ml

Gel Running Buffer (10x), for 1 litre:

0.025M Tris	30.3g
0.192M Glycine	144g
0.1% SDS	10g
Distilled H_20	1000ml

Blot Transfer Buffer (10x), for 1 litre:

25mM Tris	30.3g
0.192M Glycine	142.6g
20% Methanol	200ml
Distilled H_20	800ml

10% Resolving gel, for 50ml (2 gels):

Acrylamide	16.7ml
Tris-HCl pH8.8	18.75ml
Distilled H_20	13.3ml
5% SDS	1ml
10% APS	200µl
TEMED (Tetramethylethylenediamine)	50µl

Stacking gel, for 20ml (2 gels):

Acrylamide	3ml
Tris pH6.8	10ml
Distilled H_20	6ml
5% SDS	0.4ml
10% APS	100µl
TEMED	20µl

Appendix 5 – Th_{17} frequency and disease-specific variables

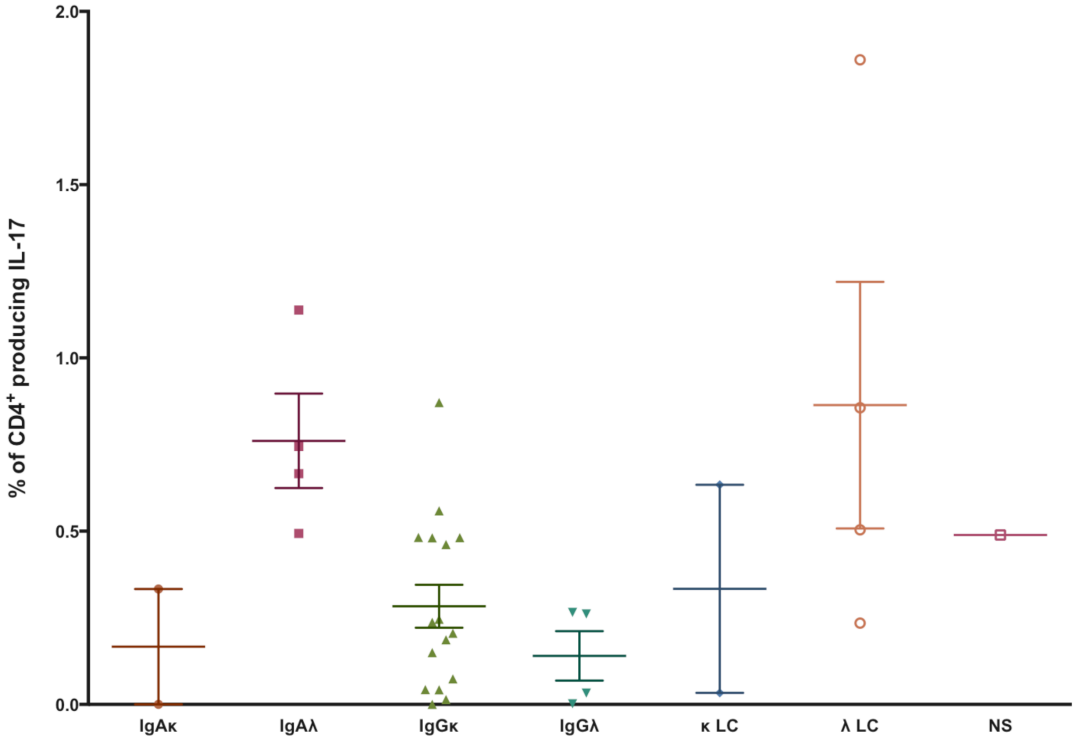


Figure A3.1. Fraction of $CD4^+$ T cells producing IL-17 according to patient paraprotein subtype. LC: light chain disease, NS: non-secretory disease.

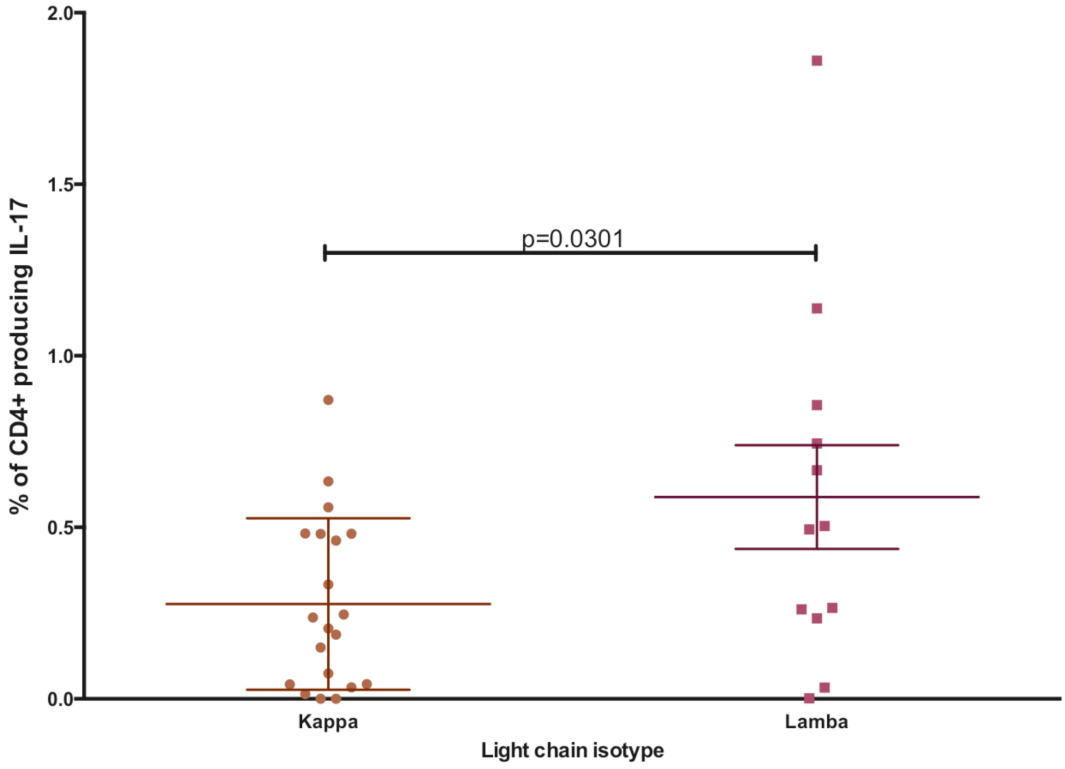


Figure A3.2. Fraction of CD4⁺ T cells producing IL-17 according to patient paraprotein light chain subtype.

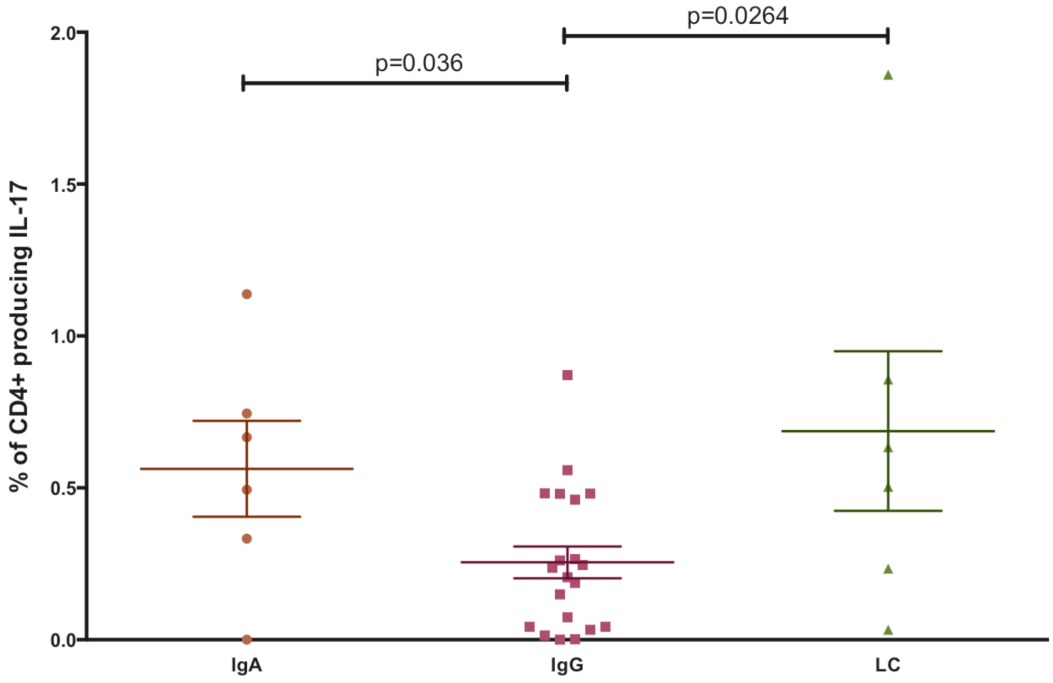


Figure A3.3. Fraction of CD4⁺ T cells producing IL-17 according to patient paraprotein heavy chain subtype. LC: light chain disease only (no intact paraprotein).

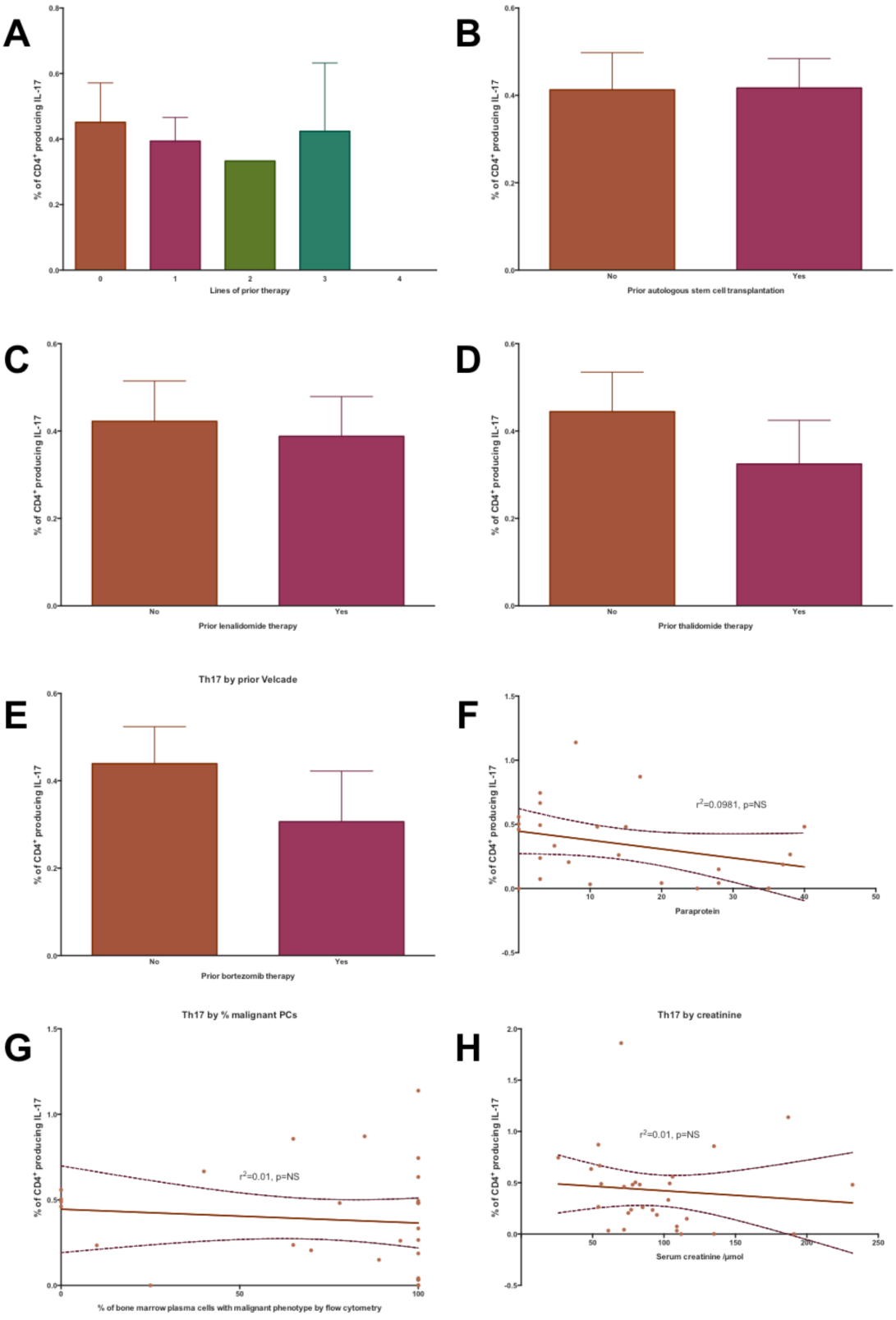

A
% of CD4+ producing IL-17
Lines of prior therapy
B
% of CD4+ producing IL-17
No
Yes
Prior autologous stem cell transplantation
C
% of CD4+ producing IL-17
No
Yes
Prior lenalidomide therapy
D
% of CD4+ producing IL-17
No
Yes
Prior thalidomide therapy
Th17 by prior Velcade
E
% of CD4+ producing IL-17
No
Yes
Prior bortezomib therapy
F
% of CD4+ producing IL-17
r2=0.0981, p=NS
Paraprotein
Th17 by % malignant PCs
G
% of CD4+ producing IL-17
r2=0.01, p=NS
% of bone marrow plasma cells with malignant phenotype by flow cytometry
Th17 by creatinine
H
% of CD4+ producing IL-17
r2=0.01, p=NS
Serum creatinine µmol

Figure A3.4. Fraction of $CD4^+$ T cells producing IL-17 according to: A: prior lines of therapy, B: prior autologous stem cell transplantation, C: prior treatment with lenalidomide, D: prior treatment with thalidomide, E: prior treatment with bortezomib, F: patient paraprotein level, G: fraction of bone marrow plasma cells with a malignant phenotype, H: serum creatinine. Lines on scatter plots show linear regression best fits, with 95% confidence intervals. P=NS for histograms.

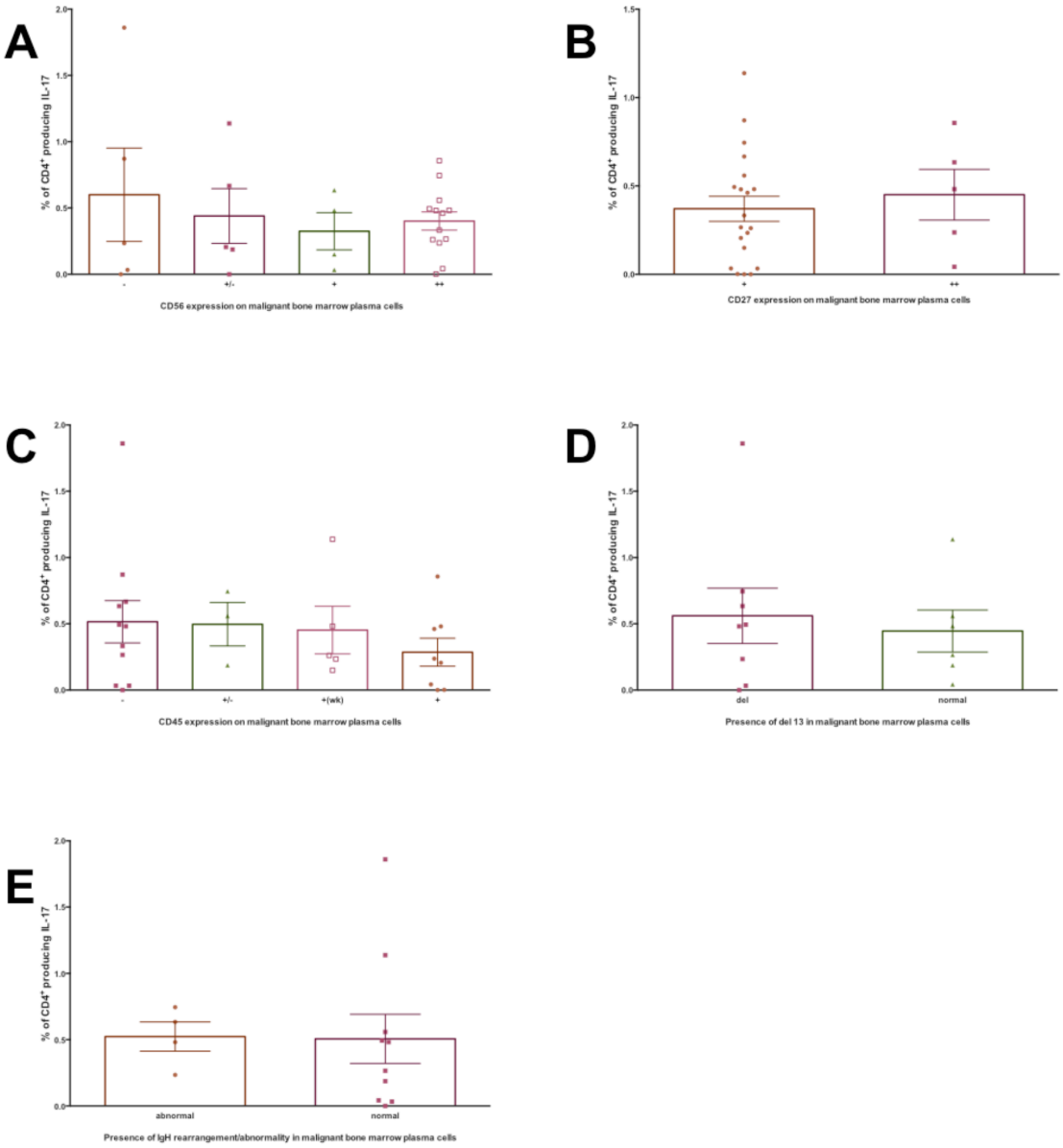


Figure A3.5. Fraction of $CD4^+$ T cells producing IL-17 according to: A: expression of CD56, B: expression of CD27, C: expression of CD45, D: presence of del13, E: presence of IgH rearrangement or IgH abnormality in malignant bone marrow plasma cells. P=NS for all comparisons.

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