Investigating immune recognition of an epithelial stress antigen by human $\gamma\delta$ T cells

And

Investigation into the chemokine profile of Ag-specific T cell responses in multiple myeloma and MGUS patients compared to age-matched controls

Stephen Joyce

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Institute of Cancer Sciences
School of Government and Society
College of Social Sciences
University of Birmingham
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Abstract

Project 1

The ‘lymphoid stress surveillance’ hypothesis proposes a role for γδ T cells in the detection of epithelial stress. TCR mediated recognition of stress ligands by γδ T cells is currently poorly understood. This project aimed to characterise the molecular mechanisms of the interaction between of the \( V_\delta 1^+ \) MAU γδ TCR with the ephrin receptor EphA2. EphA2 is a major target for anti-cancer treatments, and is upregulated on a range of epithelial tumours, a tissue enriched with \( V_\delta 1^+ \) γδ T cells.

We show that EphA2 activates MAU expressing JRT3.5 cells in a TCR dependant manner, by receptor downregulation and phosphorylation of key TCR proximal signalling proteins. It was also found that A-ephrins on the surface of the T cell are essential for the activation of JRT3 MAU by EphA2.

Project 2

Multiple Myeloma (MM) is an incurable haemopoietic malignancy, characterised by the accumulation of malignant plasma cells in the Bone Marrow (BM). MM cells express a group of proteins called Cancer Testis Antigens (CTA) whose expression is limited to the immune-privileged site of the testis in healthy adults. CTA specific CD8\(^+\) T cells in MM patients display poor \textit{in vivo} effectiveness, and are poorly understood.

Immuno dysregulation is a common occurrence in MM, with a dysregulation in the distribution and expression of key chemokines involved in lymphocyte trafficking such as CXCR3 and CXCR4, which may be a defensive mechanism by the tumour to protect against CTA specific T cells.

This project aimed to characterise the chemokine receptor expression on CTA specific CD8\(^+\) T cells in MM patients, and it was discovered that MM patients have a reduction in CXCR3 and CXCR4 expression on CD8\(^+\) T cells in the blood, and that the chemokine receptor expression pattern of different viral specific CD8\(^+\) T cells are affected uniquely by MM.
Discussion .................................................................................................................................. 58
Detection of Viral Peptide and CTA specific CD8\(^+\) T Cells in HD and MM Patients .......... 58
MM Patients Display an Exhausted Memory Phenotype on CD8\(^+\) T Cells ....................... 59
MM Patients Show Reduction of CXCR3 and CXCR4 expression on CD8\(^+\) T Cells in the Blood ............................................................................................................................................... 60
MM Status Effects the Chemokine Expression Profile of CMV and EBV Viral Peptide Specific CD8\(^+\) T Cells Differently .......................................................................................................... 60
Characterisation of CTA Specific CD8\(^+\) Responses in MM Patients ............................... 61
Future Directions ................................................................................................................... 62
Concluding Remarks .............................................................................................................. 62
References ......................................................................................................................................... 64
PROJECT 1

Investigating Immune Recognition of an Epithelial Stress Antigen by Human γδ T Cells
Introduction

γδ T Cells

γδ T cells represent an unconventional subset of T cells which express a γδ TCR at the cell surface, a heterodimer consisting of one γ and one δ chain. In contrast, conventional αβ T cells express an αβ heterodimer. αβ T cells form the majority of the cellular arm of the adaptive immune system, and act via recognition of peptidic antigen presented in the context of MHC (Major Histocompatibility Complex) molecules. Following this recognition, and the subsequent binding of co-stimulatory molecules, a complex signalling cascade is initiated from the TCR/CD3 complex, initially propagated by tyrosine phosphorylation of a series of signalling molecules. This culminates in the initiation of specific effector functions, such as the production of pro-proliferative cytokines, and cell mediated killing.\(^1\)

The genes which encode for all four of the TCR chains (α, β, γ and δ) were discovered simultaneously in the 1980s\(^2\), and yet specific, in depth knowledge of the function and activity of γδ T cells is severely lacking compared to αβ T cells. γδ T cells emerged at the same evolutionary time point as αβ cells, 400-500 million years ago\(^3\), and are conserved in all vertebrates. This suggests that they may have evolved similarly complex functions as αβ T cells, and may perform complementary roles in the vertebrate immune system. Indeed, γδ T cells have been shown to have key roles in immuno-protection, immuno-regulation, tumour recognition and recognition of cellular stress (Reviewed in \(^4\)).

The Lymphoid Stress Response Compartment

The term ‘immune surveillance’ could be defined as the constant monitoring of host cells by the immune system to detect early signs of infection, transformation and other such insults. Conventional myeloid-based immune surveillance involves the recognition of a range of pathogen associated molecular patterns (PAMPs) by germline-encoded pattern recognition receptors (PRRs) on the surface of innate cells\(^5\). PAMPs are derived from a broad spectrum of sources, both microbial and viral in origin, and although PAMPs are not generally specific to a particular pathogen, they are often indicative of an infection. In addition, damage associated
molecular patterns (DAMPs), can also activate innate immune cells. Examples of such molecules include ureate and ATP, which are released from dying cells.

After encountering such antigens, myeloid cells migrate to secondary lymphoid tissue and present antigen to naïve lymphocytes, initiating a specific immune response against the pathogen. This process culminates in an expansion of antigen specific populations of lymphocytes, which then migrate to the damaged tissues. This process of clonal expansion, differentiation and migration takes a significant amount of time to achieve, in the meantime leaving the host vulnerable to the spread and advancement of the infection (*Figure 1*).

![Figure 1: The Role of the γδ TCR in Tissue Stress Surveillance](image)

*(Top)* Conventional, microbe-initiated activation of the local, myeloid cell-mediated innate response and its communication of information to the adaptive T and B cell compartments. *(Bottom)* The lymphoid stress-surveillance response is initiated by many forms of non-microbial as well as microbial stress that upregulate surface expression of ligands for γδ TCR or for NKG2D, to which the T cells are competent to respond because of other receptor-ligand “context interactions”. The question mark denotes that the full scope of this stress-surveillance response has yet to be determined.

*Figure and Caption from 7*
In addition to these myeloid cells, unconventional lymphoid subsets are able to make a key contribution to immune surveillance of stressed tissue, before the adaptive immune system can take effect. These unconventional lymphoid subsets, such as NK and γδ T cells, are able to recognise a set of ‘stress ligands’ which the innate myeloid cells cannot. Examples of such molecules are the MHC Class I Like molecules (e.g. MICA), which are recognised by the NKG2D receptor. Once these cells encounter stress antigens via their receptors (e.g. NKG2D or TCR) they produce a ‘lymphoid stress surveillance response’, in which they respond directly to stressed tissue by processes such as cytolysis. These local effector functions are unlikely to completely eradicate an infection or tumour, due to their lack of adaptive ability. This ‘lymphoid stress surveillance’ hypothesis does however ascribe unconventional T cells a role in maintaining tissue integrity and limiting any development or spread while initiating and integrating with a downstream adaptive immune response. This interaction is likely to be achieved through many actions, such as antigen presentation and the production of cytokines and chemokines to promote the homing of lymphocytes to the site of stress (Figure 1). The ‘lymphoid stress surveillance’ hypothesis therefore proposes a rapid layer of protection to cellular stresses through the actions of specific unconventional lymphocyte subsets, which simultaneously promote and shape a specific downstream adaptive response.

Arguably the prototypic unconventional lymphocyte is the γδ T cell, which can exist as resident, pre-expanded populations at distinct (and often epithelial) anatomical sites, potentially making them ideal candidates for a role in the rapid recognition of both microbial and non-microbial stresses. Given the importance of the proposed role for γδ T cells in the lymphoid stress surveillance response, it is important to achieve a greater understanding of the molecular basis of γδ T cell recognition, specifically the role of the TCR, in the context of the ‘lymphoid stress surveillance’ hypothesis.

**γδ T Cell Subsets, Localisation and Ligand Recognition**

γδ T cell subsets are localised to discrete anatomical sites, where different tissues are enriched with γδ T cells exhibiting particular V-region usages. This is most apparent in the mouse,
which V-region usage can be reliably mapped to distinct sites⁴ (Figure 2). One such association is the Vγ5 Vδ1 subset, expressed by >90% of mouse skin DETC (dendritic epidermal T cells)⁹,¹⁰.

Figure 2: Murine Localisation of γδ T cell Subsets

In humans, the tissue associations of γδ T cell subsets are more poorly understood, with the best characterised being the Vδ2⁺ subset, which comprises 2 to 5% of CD3⁺ cells in the human peripheral blood (PB)⁴, the vast majority of which display the pairing of the Vδ2 chain with the Vγ9 chain (Vγ9 Vδ2 cells)¹¹.

Although the molecular basis of Vγ9 Vδ2 T cell recognition is unclear, they display broad anti-tumour and anti-infectious reactivity. They have been shown to be activated in a TCR dependent manner by low-molecular mass, non-peptidic phospho-antigens (pAg) which are intermediate metabolites from the mevalonate isoprenoid pathway. One such metabolite is isopentenyl PPI (IPP)¹², which is found in all host cells and is essential for mammalian survival¹³. The most potent pAg activator of Vγ9 Vδ2 T cells characterised so far is the bacterial-specific intermediate hydroxy-methyl-butenyl pyrophosphate (HMBPP), which has a 1000 fold stronger stimulating activity of Vγ9 Vδ2 cells than IPP¹⁴. Vγ9 Vδ2 γδ T cells are also capable of recognising and killing a range of myeloma and lymphoma cells in vitro in a TCR dependent manner, such as the Daudi¹⁵ and K562¹⁶ cell lines. Effectiveness of responses to these cells was
increased by the use of amino bisphosphonates (such as Zoledronate) which increase the accumulation of IPP in cells due to inhibition of an enzyme downstream of IPP in the isoprenoid pathway, farnesyl pyrophosphate synthase (FPPS)\textsuperscript{17}. These data suggest Vγ9 Vδ2 cells can recognise both host and microbial pAg, and raises questions as to whether this involves an antigen presenting molecule.

In addition to data on pAg recognition, some data suggest that Vγ9 Vδ2 cells can recognise whole protein antigens via their TCR, such as the mitochondrial F1-ATPase, which is ectopically expressed at the cell surface in a range of cancers\textsuperscript{18}, and may be involved in the processing and presentation of pAgs from non-antigenic precursors (such as triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester (ApppI))\textsuperscript{19}. There is also evidence of direct binding with heat shock proteins\textsuperscript{20}, as well as viral proteins\textsuperscript{21}.

Non-peripheral blood γδ T cells (typically Vδ2- subsets) occupy other sites of the body; for example, use of the Vδ1 chain is greatly increased at epithelial surfaces. These Vδ1\textsuperscript{+} cells comprise part of the intraepithelial lymphocyte (IEL) compartment, and make up 70-90\% of epithelial γδ T cells in humans\textsuperscript{22}. The molecular basis of Vδ1\textsuperscript{+} epithelial ligand recognition is unclear but there is some evidence to suggest that the Vδ1\textsuperscript{+} subset in humans recognises MHC like molecules, such as CD1c, an MHC-like molecule which presents lipids to T cells\textsuperscript{23}, and the MHC class I like proteins MIC A and B, which are ligands for the invariant NK receptor NKG2D, and are expressed in response to cellular stress, such as in both infected and transformed epithelium\textsuperscript{24,25}.

The antigens discussed so far for each of the two subsets share a common theme, in that they have been shown to be associated with both infection and transformation. This, combined with the fact that γδ T cell subsets are distributed to discrete anatomical sites according to their chain usage, has been used to suggest that γδ T cells play a role in recognition of a limited number of tissue associated stress antigens, detected by the limited use of V regions at these distinct anatomical sites\textsuperscript{26}.

The exact molecular basis of γδ TCR/ligand interactions underlying the cellular interactions discussed above is poorly understood, and many of the TCR ligands remain unidentified. In
cases where ligands have been identified, there is little evidence of direct binding available, for example attempts to crystallise Vγ9 Vδ2 TCRs with pAg have failed\(^27\). Some have argued for direct binding between a Vδ1\(^+\) γδ TCR and MICA, although the surface plasmon resonance (SPR) data is unconvincing\(^28\). It is also important to broaden the understanding of the biological context of these interactions, for example how ligand presentation and upregulation is associated with processes such as transformation and infection.

**Clinical Manipulation of γδ T Cells**

There is a growing body of evidence supporting the potential significance of γδ T cells in the prevention and treatment of various cancers\(^29\). γδ T cell-based immunotherapies are particularly attractive as they circumvent the requirement for MHC restriction demanded by conventional αβ based strategies. Promising results from phase I and II clinical trials has fuelled a growing interest in the development of γδ based T cell therapies for cancers\(^33-35\).

In mice, γδ T cells have been shown to play an important role in the protection from carcinogenesis. TCRδ\(^−/−\) mice have a greater risk of chemically-induced skin carcinoma\(^30\), and colorectal carcinoma\(^31\) than γδ T cell-positive controls. More recently, IL-17 producing γδ T cells have been shown to play a pivotal role in the effectiveness of chemotherapy induced anti-cancer immune responses\(^32\). Research is not limited to mouse models however; as there are currently three phase I clinical published using the Vγ9 Vδ2 peripheral blood subset, showing promising results; in each case improved clinical outcomes correlate with the expansion of Vγ9 Vδ2 T cells\(^33-35\). There are also studies showing the importance of the less well characterised Vδ2\(^−\) subset of γδ T cells in cancer prevention, as immunocompromised transplant patients who have an expansion of Vδ2\(^−\) γδ T cells have a decreased risk of post-transplant malignancies in the 6 years following surgery\(^36,37\).

These trials and investigations suggest that there is considerable potential to exploit the tumour surveillance and anti-tumour properties of γδ T cells in the clinic. Ultimately, however, the molecular basis of target cell recognition by γδ TCRs is unclear, and advances in this area are important if we are to understand the mechanism behind their involvement in anti-tumour effects, and potentially manipulate these mechanisms to develop novel cancer treatments.
Expansion of Dually Reactive Vδ2-γδ T Cells Following Renal Transplant

In 2005, Halary et al. reported the expansion of Vδ2- (Vδ1, Vδ2 and Vδ5) γδ T cells in the blood of immunocompromised renal transplant patients, following acute infection with CMV. In these patients, the circulating γδ T cell repertoire was increased from 5% to 50% of total PB T cells, and this increase was directly correlated to the resolution of viremia. This expansion of Vδ2- cells was also linked to a decrease in susceptibility to post transplant cancers which are favoured by the immunocompromised status of the patients.

Clones were generated from the Vδ2- γδ cells which demonstrated strong reactivity to CMV-infected cells in vitro by a TNFα release assay, in an MHC class I-and NKG2D-independent manner. Individual Vδ2- clones also showed strong reactivity to different subsets of cancer cell lines, such as HT29 and 293T, as well as CMV infected fibroblasts. These interactions were suspected to be TCR dependent, as the use of anti CD3/ Cδ antibodies to block TCR engagement prevented reactivity, and the γδ TCR was down regulated following activation, a phenomenon typical of TCR mediated interactions. These suspicions were subsequently confirmed by transfection experiments, in which the TCR genes for the dual reactive clones were transfected into JRT3 cells (a leukemia cell line, with a mutation in the β chain locus of the TCR preventing αβ TCR surface expression). This transfection conferred tumour reactivity to these previously TCR negative cells.

These findings led to the hypothesis that these γδ T clones were recognising stress ligands induced by both viral infection and transformation, via their γδ TCRs. In order to understand and ultimately manipulate these interactions, identification of the ligands recognized was required.

The Identification of Target TCR Ligands for Vδ2-γδ T Cells

Subsequent unpublished studies from the laboratories of Julie Déchanet-Merville and David Vermijlen have shown that there appears to be considerable diversity in the pattern of cancer cell lines these Vδ2- γδ T cell clones were recognising. Three of these clones were selected for further study, designated MAU, LES and POS4. The reactivity of these clones was confirmed by
TCR transfection experiments, in which the TCR genes from each clone were transfected into JRT3 cells (Appendix Figure 1A). The LES clone has the TCR chain usage of Vγ4 Vδ5, MAU has Vγ9 Vδ1 and POS4 has Vγ8 Vδ1.

In order to determine the ligands for the two clones LES and MAU, an antibody blocking screen was conducted by immunising BALB/c mice with the activatory HT29 cell line and CMV infected fibroblasts. Two antibodies discovered in this screen (6G8 for Mau and 2E9 for LES) were shown to block reactivity (of LES an MAU respectively) to activatory cell lines. In each case the antibody stained the surface of the activatory cell and also blocked reactivity in a TCR dependent manner (i.e. 6G8 did not block LES activation and 2E9 did not block MAU reactivity) (Appendix Figure 1A). Based on these observations, a reasonable working assumption was that the ligands for the blocking antibodies 2E9 and 6G8 would be the TCR ligands for LES and MAU respectively.

The 2E9 and 6G8 blocking antibodies were then used in co-immunoprecipitation experiments to isolate their respective ligands (Appendix Figure 1B). The molecules co-immunoprecipitated from activatory cell lysates were analysed by mass spectrometry, and were identified as EPCR (Endothelial Protein C Receptor) and EphA2 (Ephrin Receptor A2) for 2E9 and 6G8 respectively. The simplest explanation considering the evidence thus far was that these molecules were the ligands for the LES and MAU γδ TCRs, respectively.

EPCR is the receptor for activated protein C, and is involved in inhibition of coagulation and endothelial barrier protection. EPCR is expressed on endothelial cells, targets for CMV infection in vivo, and is also over-expressed in epithelial tumours. EphA2 exhibits similar characteristics, and will be discussed in detail in the following sections of this report.

The most striking feature of these two potential ligands was that they are not only expressed on cells infected by CMV in vivo, but also upregulated on transformed epithelial cells. In each case they confirm the central hypothesis that ligands would be present on CMV infected and transformed cells. This raises the possibility that these molecules act as major targets of γδ T cell stress surveillance, or are prototypic of the type of ligand recognised in this context.
EphA2: a Target for Epithelial Stress Surveillance γδ T Cells?

EphA2 is a 130kDa transmembrane protein, belonging to the largest family of tyrosine kinase receptors (RTKs), the ephrin receptors. Ephrin receptors have a diverse range of physiological roles, including in embryonic and nervous system development and angiogenesis.\(^4^0\). The cognate ligands of the ephrin receptors are the ephrins, which can be separated into two groups: A and B ephrins. B-ephrins are transmembrane proteins, with a small cytosolic region. A-ephrins are small proteins, of around 200 amino acids, which are tethered to the cell surface via glycophasphatidylinositol (GPI) anchors, and so do not possess either transmembrane or cytosolic regions. GPI anchors are a post translational modification added to the C-terminal end of a diverse group of proteins during production and processing, and act as a crucial determinant of signalling activity and localisation.\(^4^1\). EphA2 has been shown to bind only to A-ephrins, which are GPI linked. In contrast, EphA4 binds all A-type ephrins like EphA2, but also all transmembrane B-ephrins as well.\(^4^2\).

EphA2 is reported to play an integral role in the formation and progression of many cancers,\(^4^0,4^1,4^3\), and is abundantly overexpressed in many solid tumours.\(^4^0\). Unlike other members of the ephrin receptor family, which are limited to expression during development, EphA2 is primarily located on adult epithelial cells,\(^4^4\), where it is suggested to play roles in the regulation of cell growth, survival, migration, and angiogenesis (reviewed in \(^4^5\)). EphA2 is usually tightly confined to surfaces of cell-cell contacts, and is heavily dependent on E-cadherin for correct localisation and ligand interactions.\(^4^3\).

EphA2’s complex role in the progression and maintenance of malignancies is poorly understood, but seems to depend heavily on cell type and micro-environment.\(^4^6\). It is suggested that EphA2 usually provides a tumour suppressor function, whereby it negatively regulates tumour growth upon binding with its ligands, which results in receptor phosphorylation. Upon transformation, EphA2 displays dramatic alterations in subcellular localisation and is located in membrane ruffling in aggressive tumour cells.\(^4^7\). These unstable cell-cell contacts are thought to prevent effective ligand:receptor interactions, and hence promote the growth of the tumour.\(^4^7\).
This is supported by the finding that EphA2 over expressed on tumours is usually found in an unphosphorylated form, suggesting limited interactions with ligand\textsuperscript{48}.

EphA2 has become a major target for cancer immunotherapy, and a large body of research is dedicated to delivering such strategies. EphA2’s over-expression in a range of solid cancers makes it a very attractive therapeutic target (reviewed in \textsuperscript{40}). There are currently two broad approaches being investigated for the clinical targeting of EphA2. The first is the use of EphA2 as a target for delivery of exogenous anti-cancer compounds and novel chimeric antigen receptors (CAR). For example, use of bacterial toxins bound to ephrinA1 have shown promising results, enabling them to be targeted to bearing tumours \textsuperscript{49}. A second approach is to directly manipulate and inhibit the transforming properties of EphA2 to treat cancers, such as the use of specific mAbs which are able to bind EphA2 and induce receptor phosphorylation and downregulation, restoring tumour suppressive functions\textsuperscript{50}.

Clearly the close association of EphA2 expression to the establishment and progression of epithelial tumours, and association with poor prognosis, increased metastasis and decreased survival (reviewed in \textsuperscript{40}) has established it as a key cancer therapeutic target. It is therefore interesting to speculate that γδ T cells may have evolved to target EphA2 for precisely the same reasons, as part of their role in surveillance of epithelial stress in the context of the ‘lymphoid stress response’ hypothesis. In order to explore this possibility further, this project aims to investigate the molecular basis of the interaction between EphA2 and MAU.

**The Molecular Basis of EphA2 Recognition by the Mau γδ T Cell**

A molecular understanding of EphA2 recognition by MAU is essential to fully appreciate its potential significance in lymphoid stress surveillance. Surface plasmon resonance (SPR) analysis has shown no observable binding between MAU TCR and EphA2 when immobilised on a BIACore chip (C. R. Willcox, unpublished observations). This interesting finding suggested that the interaction between MAU TCR and EphA2 may be too weak to detect by SPR, as is the case for the well documented and physiologically vital interaction between the CD4 ectodomain and class II MHC molecules\textsuperscript{51}. Despite this potentially weak binding, previous evidence also strongly supports the direct role of the TCR in MAU activation by EphA2, such as anti CD3 mAb blocking
experiments, TCR downregulation following activation and the fact that transfection of MAU TCR genes confers EphA2 reactivity to JRT3 cells. In contrast, EPCR and LES were observed to have a binding affinity of 87\(\mu\)M, similar to that of \(\alpha\beta\) TCR/MHC interactions (Willcox C. R, et al. unpublished), confirming that LES is able to directly bind to EPCR, an interaction sufficient to act as the initiator of signalling.

This potentially weak binding affinity between EphA2 and MAU TCR suggested that the interaction between EphA2 and MAU bearing cells may not occur in isolation, and that other co-receptors may be involved. As plate bound EphA2 has been shown to be sufficient to activate MAU cells, attention should first be drawn to the T cell surface for the search for these molecules. Likely candidates are the A-type ephrins, the cognate ligands for EphA2. A-ephrins are present on the surface of Jurkat cells\(^{52}\), and are capable of reverse signalling (i.e. signalling into the T cell) after interacting with EphA2\(^{53}\), and so could potentially play a role in supplementing signalling through the TCR and ultimately lead to T cell activation.

This suggestion raises questions as to how GPI-linked proteins may initiate signalling in T cells, as the exact mechanism by which these proteins signal is not yet fully understood. Because GPI-linked proteins lack a transmembrane and cytosolic region, they cannot signal in a conventional way, for example via phosphorylation of cytosolic tyrosine residues. It has been suggested that GPI-linked ephrins signal via the recruitment of other transmembrane proteins with signalling potential\(^{54}\). These interactions are likely to take place in lipid rafts, discrete micro-domains of the cell surface to which GPI-anchored proteins commonly localise. Lipid rafts have been shown to have key roles in vesicular trafficking and cell signalling\(^{55}\). Molecules implicated in reverse signalling by A-ephrins include the Src family kinases Lck and Fyn, and PI3K\(^{52}\).

**A Mechanistic Model for \(\gamma\delta\) T Cell recognition of EphA2**

The aforementioned considerations suggest a model whereby EphA2 binds to the ephrins on the surface of the T cell, which promotes the formation of a complex with the MAU TCR, possibly complementing a weak TCR interaction with EphA2. This clustering would then initiate a phosphorylation cascade of signalling molecules, leading to T cell activation. The significant role of Lck in TCR signalling potentially suggests a system in which the ephrins fulfil the role of
the co-receptors CD4 and CD8 in αβ T cell recognition in localising Lck to the site of the TCR, to phosphorylate CD3ζ. This system would combine the natural specificity of the ephrins for a surface molecule known to play a major role in transformation of some cancers, with the ability of the γδ TCR to initiate T cell signalling and downstream effector responses.\(^7\)

The suggested model postulates that γδ T cells, such as MAU, may have a tumour surveillance role, using A-ephrins as an ‘invariable receptor’ in combination with the γδ TCR to quickly identify an epithelia associated stress antigen, EphA2. Increased understanding of the molecular nature of this interaction may open up the possibility of identifying other Vδ1^+ T cells capable of recognising EphA2 or conceivably other related epithelia-associated stress ligands. Ultimately, increased understanding in this area will hopefully advance the field of γδ in immunobiology, and potentially facilitate the manipulation of γδ T cells in cancer immunotherapy.
Materials and Methods

Generation of JRT3-MAU and Cell Cultures
cDNA encoding the Vγ and Vδ chains of the MAU (Vγ9 Vδ1) T cell clone was transduced into JRT3.5 cells (ATCC) via a lentiviral vector (Carried out by Willcox et al., unpublished). JRT3-MAU and HT29 cells were maintained in RPMI and DMEM media respectively, containing 8% HI-FCS (Sigma).

Signalling Activation Assays
24 well plates (Iwaki) were coated overnight at 4°C with the following: OKT3 (10μg/ml) and EphA2-Fc (20μg/ml). JRT3-MAU was serum starved by washing in serum-free RPMI (sfRPMI), and culture in sfRPMI overnight.

2x10^6 of pre-warmed JRT3-Mau cells (in 200μl pre-warmed sfRPMI) are added to each well and incubated at 37°C for the following time points: 0, 2, 5, 20 and 30 minutes. Samples were transferred to a pre-chilled eppendorf on ice, and the well washed vigorously with chilled PBS twice and transferred to the eppendorf to ensure all cells are transferred.

Cells were washed twice in chilled PBS and lysed in 100ul of 2.5X SDS Loading Buffer (+0.4M DTT) lacking bromophenol blue with 1x Protease and Phosphatase Inhibitor (PPI) Cocktail (Pierce). Lysates were then boiled (95°C/ 10 minutes) with intermittent vortexing and then frozen. Samples were subjected to repeated freeze thaw cycles until they were no longer viscous, to allow for accurate measuring.

In order to quantify the protein concentration of each sample, they were diluted 1:10 in distilled water and subjected to the colourimetric Biorad DC protein assay in a flat bottomed 96 well plate.

Finally, 1μl of 5X SDS loading buffer containing bromophenol blue was added to visualisation of the sample on the gel and boiled for 10 minutes at 95°C with intermittent vortexing.
**SDS PAGE and Western Blots**

The results from the protein quantification assay were used to calculate the volume of each sample to be loaded into polyacrylamide gels to ensure that the total volume of protein loaded was equal. Samples were normalised so that 5-10µg of total protein was loaded into each well.

Samples were boiled (95°C/10 minutes) and pulsed in the micro-centrifuge for 1 minute before being loaded into the gels. SDS-PAGE was then carried out, and the separated gels were then transferred to PVDF membrane (GE Healthcare) and blocked with 3% BSA.

The membranes were then treated with either of the following primay antibodies: mouse antiphospho CD3ζ (EBioscience) goat anti-phopho Zap70 (T-cell signalling) and anti βactin (origin unknown) and incubated for 1h at RT on a shaker. After washing membranes with TBS + 0.1% Tween-20, secondary antibodies were applied, diluted in 5% milk solution: rabbit anti mouse HRP and goat anti rabbit HRP.

The blots were then treated with EZ-ECL (Biological Industries) for 5 minutes, and developed at various exposures.

**Densitometry**

In order to semi-quantitate the western blot bands obtained, densitometry was used. A detailed protocol of the procedure was procured from 56. Briefly, digitized 8-bit scans of the developed film were obtained. ImageJ software was then used to determine the relative density of dark pixels of the desired bands in each lane compared to the 0 time point control.

**FACS activation**

Flat bottomed 96 well plates (Iwaki) were incubated with either of the following antibodies: OKT3 (Millipore), EphA2-Fc, EphA4-Fc (R&D) and anti-γδ, in 100µl PBS at 10µg/ml, overnight at 4°C. Adherent HT29 cells in DMEM media were also coated onto the wells overnight at 37°C.

Wells were washed out twice with PBS, and 1x10^5 JRT3-Mau (in 100µl) were added and incubated at 37°C for the appropriate time (CD3 downregulation, 0, 15, 30, 45, 60 and 120 minutes, and for CD69 upregulation, 0, 1, 2, 3 and 4h). At each time point, samples were
removed and placed in a pre-chilled 96 well round bottom plate on ice. Wells were washed twice with PBS to remove any residual cells, and transferred to the chilled plate.

Cells were stained in a round bottom 96 well plate by the following antibodies: anti CD69-PeCy5 (BD), anti CD3-FITC (BD) for 30min on ice in 2%FCS-PBS. Cells were then washed and fixed with 1% paraformalydehyde. Samples were stored at 4°C until analysed by flow cytometry (BD LSR II)

**PI-PLC Treatment**

0.8 units of PI-PLC (Sigma) was added to 1x10⁶ JRT3-Mau cells, and incubated for 45 minutes at 37°C. After the incubation the cells were washed and resuspended in the original conditioned media (i.e. the supernatant after spinning down the cells). This was to reduce any stimulus caused by adding fresh media and serum during the 1h incubation, overcompensating for any reduction in ephrin signalling.

Wells were coated overnight as previously described with the following antibodies, OKT3, EphA2-Fc, EphA4-Fc and anti-γδ. PI-PLC, and non-treated cells were added to the wells and incubated for 1h at 37°C

As well as being stained with anti CD69-PeCy5, PI-PLC and non-treated controls were stained with EphA4-Fc , EphA2-Fc and EphrinA1-Fc (and subsequently with goat anti-human AF488 (Invitrogen)) to measure the level of ephrin cleavage also.

**Blocking experiments**

Blocking of the plate bound EphA2-Fc was achieved by incubating with either 6G8 or ephrinA1-Fc (both at 10μg/ml) for 105 minutes. Wells were then washed and incubated for 4 hours with 1x10⁵ JRT3-MAU as detailed previously, and CD69 expression was determined by flow cytometry with an anti CD69-PeCy5 antibody.

Blocking of the ephrins on the cell surface of JRT3-Mau was achieved by incubating JRT3-Mau with EphA4-Fc (20μg/ml) and incubated for 30 minutes on ice. Cells were then washed, resuspended in conditioned media and added to the plate as previously described.
Results

JRT3-Mau is Activated by EphA2

In order to independently confirm that plate bound EphA2-Fc is capable of activating JRT3 Mau, JRT3-MAU was incubated with plate bound EphA2-Fc for up to 4h. Activation was measured by CD69 upregulation, an early activation marker. Over time, the surface expression of CD69 is significantly upregulated on JRT3-MAU after activation, in a similar fashion and timescale as when activated by the activating OKT3 anti CD3ε antibody (Figure 3). CD69 levels continue to rise over the 4h time course, but are significant by 1 hour. There is no significant upregulation of CD69 by plate bound EphA4-Fc, which binds the same ephrins as EphA2, indicating that CD69 upregulation by EphA2 is not a nonspecific effect of cross-linking ephrin receptors on JRT3-MAU.

Figure 3: Confirmation that plate bound EphA2 activates JRT3 Mau

JRT3-MAU cells were incubated with either plate bound EphA2-Fc, EphA4-Fc or OKT3 for up to 4h. Activation status was measured at various time points by CD69 expression levels.

These results therefore confirm that the ephrin receptor EphA2 is capable of activating JRT3-MAU when plate bound.
Activation of JRT3-MAU by EphA2-Fc Results in Phosphorylation of TCR Signalling Components

Despite showing that transfection of the MAU TCR into JRT3 cells confers reactivity to EphA2, the failure to demonstrate direct TCR:EphA2 binding by SPR could mean that the transfection protocol has altered other pathways in the JRT3 cells, giving the impression of a TCR dependent reaction with EphA2. Therefore, in order to explore whether the TCR is actually involved in JRT3-MAU activation by EphA2, the phosphorylation status of important signalling components of the T cell signalling cascade was determined following activation by EphA2.

![Figure 4: Phosphorylation of TCR Signalling Components following EphA2 activation](image)

**Figure 4: Phosphorylation of TCR Signalling Components following EphA2 activation**

*JRT3 Mau cells were incubated with either plate bound EphA2-FC or OKT3 for up to 30 minutes.*

(A) Representative western blots stained with either anti pCD3ζ or pZap70, with Actin as a loading control.

(B) Representative fold increase of phosphorylation of CD3ζ and Zap70s relative to the zero time point, as measured by densitometry.

CD3ζ and Zap70 were strongly phosphorylated following OKT3 stimulation, with peak intensity between 2 and 5 minutes post activation (*Figure 4A*). Similarly, CD3ζ and Zap70 were also phosphorylated following activation by EphA2, but to a lesser degree.

*Figure 4B shows fold changes in phosphorylation levels relative to the zero time point. All sets show an increase in phosphorylation status of CD3ζ and Zap70 at 2-5 minutes, after which they begin to decline back to basal levels. This pattern is similar to what is conserved in conventional αβ and γδ signalling\(^{52}\), and the OKT3 controls. Thus suggesting that the Mau TCR is involved in*
an active manner in activation of JRT3-Mau by EphA2, resulting in phosphorylation of conventional downstream signalling components.

Activation by EphA2 & HT29 Result in TCR Downregulation from the Cell Surface

A result of TCR activation in conventional \( \alpha \beta \) T cells is the downregulation of the TCR from the cell surface following activation. This process regulates the T cell response, preventing potentially damaging constitutive responses from cells, and has also been observed in \( \gamma \delta \) T cell signalling.

![Figure 5: CD3 is down regulated on JRT3 MAU following activation by EphA2 and HT29](image)

\[ JRT3 \text{ Mau cells were incubated in the presence of OKT3, EphA4-FC, EphA2-FC or a monolayer of HT29 cells. CD3 levels were measured at various time points via flow cytometry.} \]

Following activation by plate bound OKT3, TCR:CD3 complexes are strongly down regulated, reaching maximal downregulation by 45 minutes (Figure 5). No downregulation is observed for EphA4. As expected downregulation is not as complete for EphA2 as for OKT3 cross linking, but occurs in the same timeframe, with maximal downregulation within an hour. This moderate downregulation is consistent with weak affinity interactions, as expected by the weak bending affinity predicted by the lack of SPR data.

JRT3-Mau cells were also incubated with HT29 cells, a human colon adenocarcinoma cell line which expresses EphA2 and activates JRT3-MAU. Incubation of JRT3-MAU with these cells down regulated CD3 in a similar fashion to EphA2, supporting the result in a more physiologically relevant system which should allow the involvement of co-receptors and co-stimulatory ligands,
hence reducing the likelihood of the result being an artefact of an artificial plate bound system. Although the TCR downregulation observed was modest, it was observed to occur following activation with both EphA2-Fc and HT29 cells, and further indicates that the TCR is involved in JRT3-MAU activation by EphA2.

**Activation can be Inhibited by Blocking with Soluble Ephrin A1**

The data previously presented in this report has indicated that EphA2 is stimulating JRT3-Mau via the TCR. Since the affinity of MAU-TCR:EphA2 binding is below the strength detectable by SPR, there could potentially be other co-receptors expressed by JRT3-MAU which are involved in activation. As discussed already, the ephrins are the best candidates for this role, as they are the natural EphA2 ligands. Ephrins have been shown to be expressed on CD4+ T cells, but expression on γδ T cells has not been described. To address weather ephrins are expressed on JRT3-MAU cells, the cells were stained with either EphA2-Fc or ephrinA1-Fc, a control Fc protein, and staining of EphA2-Fc was observed by flow cytometry (*Figure 6*).

![Figure 6](image)

**Figure 6: JRT3 Mau Cells Express Ephrins on the Cell Surface, and are cleaved by PI-PLC**

(A) JRT3 Mau cells were stained with EphA2-Fc and an anti-human Fc AF700 and analysed by flow cytometry. The control is the sample stained with anti-human Fc AF488 antibody alone. (B) Treatment of JRT3 Mau cells with PI-PLC prevents staining by EphA2-Fc, showing that all A-Ephrins are removed from the cell surface.

If the binding of ephrins on the T cell surface to EphA2 is important for activation, then the blocking of the ephrin ligand binding domain (LBD) on EphA2 by ephrin A1-Fc should prevent activation of JRT3 Mau.
Figure 7: Blocking the EphA2 LBD blocks JRT3 MAU activation

*Plate bound EphA2-Fc was pre-blocked with ephrinA1-Fc for 30 minutes at room temperature before incubation with JRT3 Mau cells for 4 hours. Surface levels of CD69 were measured by flow cytometry.*

Blocking the EphA2 LBD with ephrin A1-Fc decreased the activation of JRT3-MAU by EphA2-Fc *(Figure 7).*

Also it would be useful to repeat the experiment using a non Fc bound ephrin-A1 construct. The important activatory domain of EphA2 may be distinct site to the ephrin LBD, but the large Fc construct of an ephrin-Fc bound elsewhere may be sterically hindering interactions at this region of the protein.

**JRT3 Mau Surface Ephrins are Required for Mau Mediated Signalling**

In order to assess whether the ephrins on the surface of JRT3-Mau are important in activation, they were pre blocked with EphA4-Fc to prevent the binding of ephrins to the plate bound EphA2-Fc.
Treatment with sEphA4-Fc did not inhibit activation by EphA2-Fc (Figure 8). This could be because during the 1 hour incubation at 37°C, the ephrins are likely to be internalised from the cell surface, along with the bound EphA4-Fc. This was thought to be minimised by incubating the assay for only 1 h, after which CD69 upregulation is noticeable but no maximal (Figure 3), but it is likely still long enough for ephrin recycling to occur. It would therefore be useful to stain the surface of Mau for the presence of the sEphA4-Fc construct using anti human Fc antibodies by flow cytometry, to determine if levels of EphA4-Fc change significantly over the course of the incubation.

Another method of addressing the importance of ephrins on the surface of JRT3-MAU surface is to remove them completely. EphA2 binds only A-type ephrins, which are tethered to the cell surface by GPI linkages. These ephrins were removed by treatment with PI-PLC, an enzyme which cleaves GPI linkages.
Figure 9: PI-PLC Treatment prevents JR3 Mau Activation by EphA2-Fc

JR3 Mau cells were treated with PI-PLC for 45 minutes to remove GPI linked surface proteins, and then incubated for 1h with plate bound EphA2-Fc. Surface levels of CD69 were determined by flow cytometry.

After treatment with PI-PLC, EphA2 fails to upregulate CD69 on the surface of JR3 Mau (Figure 9). Cleavage of ephrins was shown to be complete by the lack of EphA2-Fc binding following PI-PLC treatment, as detected by fluorescent anti human Ig antibodies [Figure 6B].

PI-PLC treatment is a particularly harsh, nonspecific approach as it indiscriminately cleaves all GPI linked proteins, of which there are many on the cell surface, performing a wide variety of functions. PI-PLC treated cells were still activated by OKT3 and an activatory anti-γδ Ab, which shows that the cell is still capable of signalling after treatment, and the inhibition of EphA2 mediated signalling is not due to non-specific inhibitory consequences of PI-PLC treatment.

These experiments show that the presence of GPI anchored proteins, of which A-type ephrins are an example, are essential for the activation of Mau by plate bound EphA2, as their complete removal completely inhibits activation. A more specific approach would be ideal to complement these experiments, specifically the use of ephrin specific antibodies to block ephrin recognition by EphA2, which would narrow down the cause of the lack of activation to particular ephrins.
**Discussion**

The ‘lymphoid stress surveillance’ hypothesis proposes a role for unconventional T cell subsets in the detection and control of both pathologically and stress induced epithelial damage\(^7\). V\(\delta1^+\) γδ T cells have been shown to play a key role in epithelial lymphoid stress surveillance, but the molecular mechanism of their detection of stress antigens via their TCR is lacking. This study has built upon this framework by firstly confirming that a particular V\(\delta1^+\) γδ T cell clone, MAU, can be activated by an epithelial stress ligand EphA2, in a TCR dependant manner, and then directly addressing the molecular mechanisms underlying this interaction.

This investigation aimed to explore the molecular mechanisms of the interaction between EphA2 and the MAU T cell. Ephrins were postulated to be central to the activation of JRT3-MAU by EphA2 because SPR analysis could not detect binding between MAU TCR and EphA2; suggesting a weak interaction which is dependent on T cell surface ephrins.

In summary, the data firstly confirm that isolated EphA2 is able to activate JRT3-MAU in a TCR dependent manner. Secondly, they have established that activation results in receptor downregulation as well as the phosphorylation of key proximal TCR signalling molecules such as ZAP70 and CD3\(\zeta\). Finally the data suggest that the ephrins on the T cell surface are essential for MAU activation by EphA2. Collectively these data suggest a novel mechanism of T cell activation, possibly involving the formation of a complex between the MAU γδ TCR and ephrins on the T cell surface.

**EphA2 Activates MAU in a TCR Dependent Manner**

Previous studies indicated that the MAU clone was able to recognise target cells in a TCR dependant manner based on recognition of EphA2. Conceivably this mode of recognition could reflect a V\(\delta1\) contribution to epithelial stress surveillance of dysregulated EphA2 on transformed or infected epithelial cells as part of the lymphoid stress surveillance response. JRT3-MAU was incubated with a plate bound EphA2-FC construct and CD69 upregulation, an early activation marker to T cells, was detected on the surface of JRT3-MAU. Importantly, this confirms that EphA2 in isolation is sufficient to activate JRT3-MAU.
It would be useful to complement the detection of this early activation marker with assays to determine whether the CD69$^{\text{HIGH}}$ MAU cells are functionally active. Such assays could include an intracellular cytokine stain, whereby activated cells are screened for the production of pro-proliferate cytokines, such as IL2, or markers of T cell effector function, such as the granulation marker CD107. Effector functions of the activated MAU cells could also be determined by cell killing assays, whereby the ability of activated MAU to kill EphA2 expressing cancer lines could be determined.

A key aim of this investigation was to determine the significance of the MAU $\gamma\delta$ TCR in the activation of MAU by EphA2, but CD69 upregulation is not exclusive to TCR mediated activation, and may also be induced by other activatory stimuli, such as anti CD2 mAbs, activators of protein kinase C and phytohemagglutinins (PHA) (Reviewed in $^{59}$). Therefore, in order to exclude the possibility that the CD69 upregulation detected is a result of a non-TCR mediated interaction between EphA2 and another molecule on the surface of JRT3 MAU, direct involvement of the TCR in the activation needs to be shown.

The phosphorylation of key signalling components of the T cell signalling cascade, particularly CD3$\zeta$ and ZAP70, are among the earliest events in TCR mediated activation of lymphocytes. Therefore the observed increased phosphorylation of these key proteins following JRT3-MAU activation by EphA2 strongly suggests that the TCR is indeed involved in the activation.

In terms of the experiments conducted, an omission is the lack of a negative control; the use of which would exclude the possibility that the observed effects are non-specific. EphA4 would serve as an ideal control, as it is able to bind the same ephrins as EphA2, but has been ben demonstrated to activate JRT3-MAU.

The experimental design could also be optimised by altering the method by which phosphorylation status is quantified. The current process of digitising the images and performing densitometry adds a relatively large potential for errors and subjectivity, which is well documented$^{60}$. Briefly, the main difficulty is in determining a suitable background level, which can vary due to a myriad of factors both within individual blots and across the groups of experiments$^{60}$. To reduce these inherent errors, a system such as the LiCore Odyssey infrared
imaging system could be used, which directly detects up to two membrane bound antibodies conjugated to an IR emitting fluorochrome. This system is capable of detecting two different bound antibodies on the same western blot, and combined with the lack of requirement to develop photographic film will make it possible to make direct comparisons of absolute phosphorylation levels between samples. This process has been used successfully by other groups in detecting ZAP70 phosphorylation.61

Another key indication of TCR mediated activation is the downregulation of TCR from the T cell surface immediately following activation. Following incubation of JRT3-MAU cells with either plate bound EphA2-FC or a HT29 monolayer, a modest TCR down regulation was observed. The consistency between these two experimental approaches is promising, indicating that the down regulation, however modest, is consistent between two different experimental approaches, supporting that it is a genuine observation. Previous studies have shown that αβ T cells expressing TCRs with low affinity for specific pMHC complexes show similarly low levels of TCR downregulation post activation, while a significant amount of specific killing is still detected58,62. These studies support our data, as the lack of binding between MAU TCR and EpHA2 detected by SPR suggests a weak interaction, and so TCR down regulation would be expected to be modest. They also indicate that despite relatively low TCR downregulation, low affinity pMHC/αβ TCR interactions are still able to elicit significant effector functions.

Collectively, these data suggest that the MAU TCR is both required and actively involved in the activation of JRT3-MAU by EphA2. This complements the findings by Halary et al. in 2005 that the recognition of CMV infected and transformed cells by the MAU clone is TCR dependent, as detected by TCR downregulation and mAb TCR blocking assays11.

**Ephrins on the T Cell Surface Play a Key Role in Activation by EphA2**

Thus far, the data collected in this project have shown that EphA2 activates JRT3-MAU in a TCR dependent manner. The project also aimed to determine the molecular nature of this previously uncharacterised interaction. The suspected low affinity interaction between MAU TCR and EphA2 lead us to postulate the involvement of additional molecules in the activation process, specifically the A-ephrins, the cognate ligands for EphA2, on the surface of MAU. In
addition, GPI linked A-ephrins have been shown to have the capacity to propagate signals in the cells they are expressed on, as a result of complexing with proteins possessing a cytosolic region with signalling potential\textsuperscript{54}.

The ligand binding domain (LBD) of EphA2 appeared to be required for activation, as blocking of the LBD with an ephrinA1-Fc construct reduced the amount of CD69 upregulation detected compared to the upregulation detected in untreated control samples. The experimental approach could be refined however, as the presence of the human FC region on the ephrin-A1 construct does not make the assay specific enough. Conceivably, steric effects could be caused by the Fc region, such as blocking interactions at sites distinct from the LBD that are involved in activation. Use of an ephrin-A1 construct lacking the Fc moiety could exclude this possibility, and would substantiate the role of the EphA2 LBD in activation.

The suggestion that the LBD of EphA2 is required for activation of JRT3-MAU by EphA2 supports the role of ephrins in the interaction. In order to clarify the importance of ephrins on the T cell surface, experiments whereby the JRT3-MAU surface ephrins were pre-blocked with an EphA4-Fc construct prior to incubation with EphA2-Fc showed no inhibition of the activatory capacity of EphA2 on JRT3-MAU. These experiments are inconclusive and should be repeated with several considerations in mind. Ephrins are actively involved in signalling processes, and are likely to be down-regulated from the cell surface following binding\textsuperscript{63}, leading to removal of blocked ephrins from the cell surface, and their replacement with \textit{de novo} unbound counterparts. A more robust way to test the importance of A-ephrins on the surface of JRT3-MAU was to treat cells with PI-PLC, an enzyme which non-discriminately cleaves GPI-linked proteins from the cell surface. PI-PLC treated, A-ephrin\textsuperscript{negative} cells were not activatable by plate-bound EphA2, whereas their ability to be activated by both anti γδ and anti CD3 mAbs was unaffected. This therefore suggests that JRT3 surface ephrins are required for the activation of JRT3-MAU by EphA2.

The suggested importance of A-ephrins in JRT3-MAU activation by EphA2 is supported by previous studies, in which reverse signalling via A-ephrins has been shown to be able to activate Lck, a key kinase in the initiation of the αβ TCR signalling cascade\textsuperscript{52}. This therefore suggests a
role for ephrins to recruit Lck to the EphA2/MAU TCR complex, which then could initiate the earliest stages of TCR mediated signalling, specifically CD3ζ phosphorylation.

**Future Directions**

In order to expand upon the findings of this investigation and explore the new potential ideas it exposes, more research into the nature of the interaction of MAU TCR with EphA2 is needed. In addition to the improvements to the experimental designs already mentioned, different approaches could also be used.

In order to expand upon the idea that MAU TCR, A-ephrins and EphA2 localise together and form a signalling complex, several approaches could be used. Firstly, immunofluorescence based approaches such as Fluorescence Resonance Energy Transfer (FRET) analysis could be used to visualise the location of the different components during both activatory and non activatory conditions. To supplement these localisation experiments, a co-immunoprecipitation (Co-IP) could be conducted in order to detect any potential interactions between the molecules. This approach could also be used to identify any further molecules which may be complexing with EphA2/MAU TCR; as the precipitated complex could be analysed by mass spectrometry.

In order to expand these findings beyond the artificial Jurkat system for increased biological relevance, the first step would be to analyse *ex vivo* Vδ1+ γδ T cells for ephrin expression, and how they change in response to common signals of infection, such as TNFα and IFNγ.

**Concluding Remarks**

This investigation has supplemented previous work suggesting that EphA2 can activate JRT3-MAU cells via its γδ TCR. We have also shown that ephrins on the surface of the JRT3 cell are essential for full activation or JRT3-MAU by EphA2.

The lymphoid stress surveillance hypothesis proposes a role for epithelium resident Vδ1+ γδ T cells in the detection and control of both infection and stress mediated damage. This suggests that EphA2, an antigen heavily unregulated on epithelial cells in response to stress, is capable of
activating the Vδ1+ JRT3-MAU T cell via its γδ TCR. This activation was found to also be dependent on JRT3 surface A-ephrins; a finding which begins to deconstruct the molecular mechanism of this interaction.

This increased understanding will not only form the basis of further research into this specific ligand/receptor interaction, but will hopefully also aid the discovery and characterisation of further Vδ1+ γδ TCR interactions with other epithelial stress related ligands, exposing potential new targets for therapeutic approaches.
Appendix

Appendix Figure 1: Identification of 6G8 ligand

(A) Graph showing the TCR specific blocking of JRT3 Mau activation by 6G8 with the activatory U373 cell line. (B) Silver nitrate stain showing the identification of a 130Kda band in MAU activatory cell lines following immunoprecipitation with 6G8.
References

PROJECT 2

Investigation into the Chemokine Profile of Ag-Specific T Cell Responses in Multiple Myeloma and MGUS Patients Compared To Age-Matched Controls
Introduction

Multiple Myeloma

Multiple Myeloma (MM) is a haemopoietic malignancy which is characterised by the accumulation of a monoclonal population of malignant plasma cells in the bone marrow. MM is the second most common cancer of the blood, accounting for 13% of haematological malignancies\(^1\), with around 3000 new cases a year in the UK\(^2\). The disease is often associated with the disruption of the bone marrow environment, osteolytic bone lesions, renal disease, immuno-deficiency and an increase in chemokine levels. Although the cause of the disease is unknown, many cases arise from the asymptomatic condition monoclonal gammopathy of uncertain significance (MGUS), which is considered a pre-malignant stage of the disease\(^3,4\). The rate of progression from MGUS to MM is 1% a year\(^5\), a process which is thought to be exacerbated by the breakdown of immunosurveillance, but this remains unclear\(^6\). MM is a multi-step disease, consisting of relapse and plateau phases, with successive relapses becoming more difficult to treat, and death usually occurring within 3-5 years\(^1\).

Although there have been many improvements in treatments for MM, it is still considered an incurable disease, and so there is desperate need for effective novel treatments. Examples of such treatments include peripheral blood stem cell transplantation (PBSCT) and the use of pharmacological agents such as thalidomide, bortezomib, and lenalidomide\(^7\). A common approach for the targeting of tumours for immunotherapy is through the recognition of tumour antigens, either common to a range of tumours (tumour associated antigens, TAA) or to a specific malignancy (tumour specific antigens, TSA). A range of TAAs expressed on MM cells have been discovered, such as Wilms Tumour 1 (WT1)\(^8\), MUC1\(^9\) and idiotype protein\(^10\). The expression of these proteins is not exclusive to tumour tissue however, and so effective T-cell immunity towards these antigens may result in healthy tissue damage\(^11\). A group of TSAs whose expression is limited to transformed cells were discovered on MM cells, which have since been identified as cancer testis antigens (CTA). This therefore means that immunotherapeutic approaches targets CTAs would not be effective against host tissue, and be specifically aimed towards cancerous cells.
Cancer Testis Antigens are Expressed on Multiple Myeloma Cells

CTAs are a large group of proteins whose expression is restricted to the testis and the placenta in adults, where their functions are largely unknown. CTAs are also expressed on a wide range of tumours, such as melanoma, lung cancer and ovarian cancer (reviewed in ). The exact role played by CTAs in the progression and maintenance of cancers is currently unknown. The expression of CTAs on MM cells was first demonstrated by van Baren et al. in 1999 by IHC and RTPCR approaches. The group detected a significant expression of genes of the MAGE, BAGE, GAGE, and LAGE-1/NY-ESO-1 families in a high proportion of bone marrow samples from patients with advanced MM. CTAs were then identified as potential T cell targets in 2000, when it was observed that a MAGEA3 peptide, presented by HLA-DP4 on tumour cells was capable of eliciting a cytolytic CD4+ response.

Expression of CTAs in adults is limited to the immunologically privileged site of the testis, and are not expressed in somatic tissue. Immune privileged sites are able to tolerate the induction of antigens without the induction of an inflammatory response, a mechanism thought to protect key tissues from potentially harmful immune interactions. As a result of this, tolerance is not established to CTAs during development, and so they are immunogenic when expressed in adult tissues because they are not recognised as self-proteins. Expression of CTAs is epigenetically controlled, and it is thought to be the breakdown of this epigenetic control via demethylation, a common feature in many cancers, which leads to CTA expression.

CTA Specific CD8+ T Cells are Detected in Multiple Myeloma Patients

MM cells express a range of CTA proteins, which are found exclusively on cancerous tissue, and to which immune system has not become tolerant to. It would therefore be expected that host immune responses would be raised to these antigens, and lymphocytes specific for CTA epitopes could be detected experimentally.

Experiments conducted by Goodyear et al. in 2005 detected specific CD8+ T cell responses to 12 peptide epitopes from a range of CTAs in MM patients. CTA specific CD8+ T cells were isolated from 15 out of 37 patients, ranging between 0.0004% and 0.1% of the total CD8+ population.
CTA specific CD8$^+$ T cells were isolated from these patients and cloned, and one such clone for the B7 restricted MAGE-A1$_{289-298}$ (RVRF) epitope shows high avidity for, and is capable of killing, MAGE-A1 expressing myeloma cell lines$^{19}$.  

The magnitude of the CTA specific CD8$^+$ T cell response was also found to closely correlate with clinical progression, with the magnitude of CTA specific responses coinciding with decreases in paraprotein during recovery phases of the disease. During relapses, the magnitude of the response has also been shown to increase. Patients who demonstrated CTA specific T cell responses also have improved clinical outcome, with a hazard ratio between CTA positive and negative groups is 0.457, indicating a significant increase in survival in CTA CD8$^+$ positive patients$^{19}$.  

Despite these ex vivo responses, CD8$^+$ CTA specific T cells are not capable of clearing the tumour, and do not ultimately prevent patient death. Therefore, the strong correlations between expanded CTA specific CD8$^+$ T cell population and clinical status may just be a response to the tumour's progression and status, while not being able to elicit an effective immune response.

This is an interesting finding, as ex vivo studies have shown CTA specific CD8$^+$ T cells have a high avidity for, and are capable of killing CTA expressing myeloma cell lines, and yet in vivo they seem to be incapable of mounting an immune response to, and ultimately clear the tumour. A possible reason for this is that a common association with MM is global immune dysregulation of the cellular immune response, and so any potential anti-tumour immune responses may be repressed by this immune dysfunction$^{20}$.  

**Multiple Myeloma Patients Exhibit Global Immune Dysfunction**  
An essential factor for the development maintenance of MM is the tumour microenvironment in the BM. This is maintained by interactions between the bone marrow stroma, myeloma plasma cells and immune cells via adhesion molecules, cytokines and chemokines, creating an immunologically hostile microenvironment, while simultaneously promoting tumour growth. This microenvironment promotes tumour cell survival, development of drug resistance,
angiogenesis and disordered bone metabolism. Various interactions are shown to decrease the host’s immune response, such as by inadequate presentation via MHC and co-stimulatory pathways, as well as promotion of regulatory T cells ($\text{T}_{\text{reg}}$) by the production of TGF-$\beta$.

It is well documented that MM patients exhibit global immune dysfunction, a status which not only promotes the growth and development of the tumour and chemotherapy resistance, but also leads to increased morbidity and mortality from infections. Examples of immune dysregulation include hypogammaglobulinemia, impaired lymphocyte function and neutropenia. Impairment of the cellular immune response is well documented and plays a key role in the maintenance of disease. For example, a decrease in the number of CD4$^+$ and CD8$^+$ T cells correlates with decreased survival rates, indicating a link between cellular immune response and the control of the disease. Similarly, positive clinical responses are observed following the dose of infused lymphocytes, and lymphocyte recovery phases following peripheral blood stem cell transplantation (PBSCT). Other T cell aberrations include an inversion of both the CD4$^+$ and CD8$^+$ ratio and Th$_1$:Th$_2$ CD4$^+$ ratio. Abnormal T cell functional responses are also common in MM patients, as it has been frequently observed that key molecules in T cell signalling, such as CD3$\zeta$ and ZAP70, are down regulated in T cells from MM patients.

**Chemokines Play a Key Role in the Progression and Maintenance of MM**

Another key characterisation of MM is the increase in global chemokine levels, which have been shown to contribute towards MM proliferation and survival. Chemokines and their receptors play key roles in the migration, homing and development of T cells.

Chemokines are typically small secreted proteins, with a highly conserved secondary structure. Chemokine receptors are a diverse group of transmembrane G protein-coupled receptors which interact with their chemokine ligands. In humans, there are currently 44 chemokines and 17 chemokines receptors which have been characterised. Unpublished data from Goodyear et al. show that there are global alterations in the distribution of the chemokine receptors CCR4 and CXCR3 between T cell subsets in the BM and PB. There was also found to be an increase in the expression of CCR4 and CXCR3 ligands in the BM of MM patients. Ultimately, this change in
chemokine gradients within the tumour microenvironment leads to an alteration in the
distribution of T cell subsets between the BM and blood in MM patients.

This dysregulation in chemokine receptor distribution may contribute towards to
immunologically impaired status of MM patients, although its exact role has not yet been
determined.

**Does Dysregulation of Chemokine Receptor Expression on CTA Specific CD8⁺ T Cells
Inhibit their Function in vivo?**

Previous research has established that CTA specific CD8⁺ T cells are present in a group of MM
patients, which despite showing strong affinity and avidity towards CTA expressing myeloma
cells *in vitro*, are ultimately incapable of clearing the tumour effectively *in vivo*.

As a common observation in MM patients is global immune dysregulation, including aberrations
to the cellular arm of the immune system, such as global alterations in chemokine receptors, as
well as in chemokine receptor expression on CD8⁺ T cells.

It is therefore hypothesised that the expression pattern of chemokine receptors on CTA specific
CD8⁺ T cells may be inhibiting them from mounting effective immune responses against CTA
expressing myeloma cells *in vivo*. Specifically, as chemokine receptors are heavily involved in
the trafficking and localisation of T cells, it is expected that an alteration on chemokine
receptors would result in localisation of CTA specific CD8⁺ T cells to sites where they are unable
to interact directly with the cancerous tissue.

The aim of this project therefore was to characterise the expression of the chemokine receptors
CXCR6, CXCR4, CCR5 and CXCR3 on the surface of EBV and CMV peptide specific CD8⁺ T cells in
both healthy donors and MM patients. These expressions will be compared to the expression
profile of CTA specific CD8⁺ T cells in order to determine any major differences between the
cohorts.

It is hoped that this investigation will provide insight into the nature and function of CTA
specific CD8⁺ T cells in MM patients. Increased knowledge of their homing properties, and
ultimately their manipulation, may open up new possibilities for much needed novel immunotherapeutic approaches using CTA specific CD8\(^+\) T cells in patients with MM.
**Materials and Methods**

**Patient Samples**

Patients were recruited from outpatient clinics at Heartlands Hospital. Informed written consent was obtained from all patients and local ethical permission (South Birmingham Local Regional Ethics Committee) was obtained prior to the study. Peripheral blood samples (20 mL) were prospectively taken from patients with multiple myeloma at various stages of disease. Where possible, samples were taken at monthly intervals and mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). Unless otherwise stated, freshly isolated cells were used in assays. Control subjects were obtained from healthy volunteers without a history of malignant disease. It is worth noting that due to the lack of information available about the samples, HDs were not able to be accurately age matched. However donors were matched to the typical age of disease carriers as closely as possible.

**Antibodies and Reagents**

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<tr>
<td><strong>Chemokine Receptors</strong></td>
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<td><strong>CTA Specific Dextramers</strong></td>
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<tr>
<td>MLMA-PE (A2)</td>
<td>Immudex</td>
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<tr>
<td>YLQL-PE (A2)</td>
<td>Immudex</td>
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<td>RVRF-PE (B7)</td>
<td>Immudex</td>
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</tbody>
</table>
Table 1: Table of surface antibodies and CTA specific Dextramers used in the Study (Brackets indicate HLA restriction of CTA Peptide)

Lymphocytes were stored and washed in RPMI Media. TCS Media was used for the incubation of T lymphocytes and consists of RPMI, 10% Human Sera (Heat Inactivated), 200mM Glutamine and Penicillin Streptomycin (All Gibco-Invitrogen). A list of antibodies used through the investigation is shown in Table 1.

Production of HLA-Peptide Tetramers
HLA-peptide tetramers were generated using standard protocols. Briefly, HLA-A*0201 and B*0702 molecules were refolded in vitro with β2- microglobulin and peptides ELK, NLV, CLG and RAK. Refolded HLA-peptide monomer complexes were enzymatically biotinylated and then purified by gel filtration and ion exchange. Monomer complexes were then multimerized using streptavidin-PE at a molar ratio of 4:1 (molecular process). For staining, cells were washed, resuspended in MACS buffer, and incubated for 15 minutes at 37°C with tetramer.

Generation of CTA-Specific T Cell Peptide Lines
2.5x10⁶ freshly isolated PBMCs were resuspended in 200μl RPMI. CTA peptide was added to the cell suspension at a final concentration of 10μM, and incubated for 1h at 37°C for 1.5 hours with intermittent agitation. Cells were then washed and resuspended in 1ml of RPMI +10% human serum containing IL7 (at 25ng/ml) and IL15 (at 2ng/ml) and transferred to a 24 well plate and stored at 37°C. After 5 days, 1ml of RPMI +10% human serum containing IL2 (at 40U/ml). After 4 days, cells were washed and stained with CTA specific dextramers, as with fresh cells.

Detection of CTA-Specific T cells using CTA Specific Dextramers
2x10⁷ freshly isolated PBMCs were CD8⁺ enriched using the CD8⁺ T cell isolation kit (Miltenyi Biotec) and the AutoMACs Pro separator (Miltenyi Biotec), as documented in the documentation. The enriched population is then stained with CTA specific dextramers (Immudex) for 20 mins at room temperature. Cells are washed twice in MACs buffer then stained with the surface antibodies for 20 minutes at 4°C. Cells were then analysed by flow cytometry on the LSR II (BD Bioscience).
Statistics

Graphs were plotted in Prism 5 (GraphPad, CA) and unless otherwise noted the histogram represents the mean with the error bars representing the standard error of the mean (SEM).
**Results**

Detection of Virus Specific CD8\(^+\) T Cells in HD and MM Patients.

In order to identify CD8\(^+\) T cells specific for CMV or EBV derived peptides in healthy donors (HD), the PBMCs of four donors were isolated by density gradient centrifugation and analysed by flow cytometry. Virus specific CD8\(^+\) T cells were identified by staining with a PE conjugated MHC tetramer containing one of four viral peptides, two of which were EBV derived, and two of CMV origin. The detected responses ranged from between 0.5% and 4.5% of the total CD8\(^+\) T cell population. An example of the gating strategy used to characterise these cells is shown in Figure 10.

![Figure 10: Gating Strategy for the detection of viral specific CD8\(^+\) T cells](image)

PBMCs were isolated from donors, stained and analysed by flow cytometry. i) Gate P1 selects single cells ii) gate P2 selects the lymphocyte population. iii) Gate P3 selects cells which are negative for the Viability Dye, which is taken up only by dead cells. iv) Gate P4 selects for CD8\(^+\) cells which have stained positively with viral MHC tetramer. In order to select the entire CD8\(^+\) population, P4 is extended to include the PE negative CD8\(^+\) cells also.

Example shown for donor HD-DD stained with the NLV CMV MHC tetramer. P4=0.7% of CD8\(^+\) T cells.
Virus specific CD8\(^+\) T cell responses were also identified in MM patients by identical methods. Viral responses were detected in the cohort of MM patients, 3 EBV peptide specific responses and 3 CMV peptide specific responses. An example of the detection of an EBV specific response in a MM patient is shown in Figure 11.

![Figure 11: Detection of Virus Specific CD8\(^+\) T cells in MM patients.](image)
PBMCS were isolated and gated as in Figure 10. Example patient: PIN04 for the RAK EBV MHC Tetramer. P4=0.5% of CD8\(^+\) T cells

**Detection of CTA Specific CD8\(^+\) T cells in MM Patients**

Building on previous work by Goodyear *et al.* CTA specific CD8\(^+\) T cells were detected in the blood of MM patients by using PE conjugated CTA peptide specific dextramers. Because CTA responses are characteristically small\(^{18}\), PBMCs isolated from MM patients were enriched for CD8\(^+\) cells using a magnetic negative selection protocol in order to increase the concentration of dextramer within the assay and subsequently increase its sensitivity. The enrichment protocol consistently yielded population with a >90% CD8\(^+\) content. Again, because CTA responses are characteristically small, a negative control dextramer was used in each example, consisting of a PE conjugated dextramer containing either a HLA-A2 or HLA-B7 molecule bound to a nonsense peptide in order to account for nonspecific background staining of PBMC populations. Table 2 shows the samples previously characterised as having CTA specific responses by Goodyear *et al.*, as well as the patients analysed in this investigation.
A CTA specific response was detected in one patient, out of five of the previously characterised as positive responders of the samples which were available during the investigation. 0.02% of CD8\(^+\) T-cells isolated from PIN 04 \textit{ex vivo} PBMCs were specific for the CTA derived RVRF peptide. (Figure 12).

In order to screen for small CTA specific responses in MM patients, a peptide line is established in order to expand the population of CTA specific CD8\(^+\) cells \textit{in vivo}. Patient PBMCs are incubated for 10 days in the presence of CTA peptide and growth promoting cytokines. The cultures are then stained with CTA specific dextramer in order to detect a potential response.

Unfortunately, due to limited availability of samples, it was not possible to develop a peptide line of patient PIN 04 in order to confirm the finding in the peptide line \textit{in vivo} setting.

<table>
<thead>
<tr>
<th>Patient Identification Number (PIN)</th>
<th>Previous Studies</th>
<th>This Project</th>
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<tbody>
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<td>CTA Stain \textit{ex vivo}</td>
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<tr>
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Table 2: Table of Patients and their CTA responses from this study and previous work by Goodyear \textit{et al.}
Figure 12: Detection of CTA specific CD8⁺ T cells inpatient PIN 04

Isolated PBMCs were stained with a CD8 Ab and CTA specific dextramer conjugated to PE. The left hand panel shows the response with a HLA-B7 control dextramer, containing a nonsense peptide. The right hand panel is stained with the RVRF MHC dextramer, and P4 represents 0.02% of CD8⁺ T cells.

It is for similar reasons further characterisation of other samples was not possible, as the high number of cells needed to perform each assay meant that there were not sufficient cells available to perform parallel studies.

It is interesting to note that four patients who had previously been characterised as having CTA specific CD8⁺ responses were negative for these responses in these assays Table 2. CTA response levels have been shown to correlate with stages of disease progression, resulting in an increase and decrease in their numbers. Considering the time between the two investigations, the disease status may have changed, and so the CTA response may have diminished below the detectable threshold of the assays.

It is important to note that the CTA response detected in PIN 04 has not been validated with the peptide line protocol, and represents a small number of events, 0.02% of the total CD8⁺ population. The comparisons made in the following sections using these data are therefore
expressed to give an indication of potentially important phenotypes in order to help to shape future investigations

**There is Global Dysregulation of Memory Phenotype of CD8\(^+\) T Cells in MM Patients**

In order to determine the memory phenotype of the CD8\(^+\) T cell population, samples were gated in a similar fashion as in Figure 10, but gate P4 was extended to include all CD8\(^+\) cells. This gated population was then characterised into memory phenotypes by virtue of their CCR7 and CD45RO expression. The percentage of cells of the total CD8\(^+\) population for each phenotype was then calculated(Figure 13).

![Figure 13: Memory Phenotype of the CD8\(^+\) Population of HD and MM patients](image)

**A)** The memory phenotype of the CD8\(^+\) population was determined by their expression of CCR7 and CD45RO. CM= Central memory and EM = Effector Memory. **B)** Percentage of CD8+ cells expressing the memory phenotype in HD and MM patients.

The results suggest that MM patients display a less varied distribution of memory phenotypes, with over 80% of CD8\(^+\) T cells being classified as TEMRA, compared to less than 60% for healthy donors. MM patients also express a much lower number of naive and central memory CD8\(^+\) T cells than healthy donors, whereas levels of effector memory cells are not significantly altered.
Figure 14: Gating Strategy and Characterisation of Chemokine Expression Pattern

(A) Events isolated in gate P4 from Figure 10 are separated into one of four gates in the first panel, by virtue of their expression of CCR5 and CXCR6. (B) Each quadrant represents one of the four possible combinations of these chemokines. Each quadrant is then analysed in the subsequent panels for their expression of CXCR4 and CXCR6. Each of the 16 quadrants of the final four panels therefore represents one of the 16 possible combinations of the four chemokines CXCR4, CXCR6, CCR5 and CXCR3. This example characterises the expression pattern of HD-DD NLV specific CD8⁺ T cells.
There is Global Dysregulation of Chemokine Receptor Expression of CD8⁺ T Cells in MM Patients

In order to characterise the chemokine receptor expression profile of both global and virus specific CD8⁺ T cell populations, HD and MM patient PBMCs were stained with a panel of fluorescently labelled antibodies specific for the chemokine receptors CXCR4, CXCR3, CXCR6 and CCR5. Populations of either all CD8⁺ T cells or PE stained viral specific peptides where then characterised for their chemokine receptor expression profile. Figure 14 demonstrates the gating strategy used. In order to include the global CD8⁺ T cell population, gate P4 from Figure 10 was extended to include all CD8⁺ cells.

The expression levels of the chemokine receptors CXCR6, CXCR4, CXCR3 and CCR5 was determined on both the PB CD8⁺ T cell population and viral/CTA peptide specific CD8⁺ T cells in both HD and MM patients. The percentage of cells in the global CD8⁺ T cell population expressing each of the chemokines previously mentioned was calculated and is shown in Figure 15.

![Figure 15: Expression levels of Chemokine Receptors on CD8⁺ Cells in HD and MM Patients](image-url)
CXCR6 expression is low on the CD8⁺ T cell population of both HD and MM patients, being expressed on less than 5% of cells in each case. There is no significant difference in the expression of CCR5 between the two cohorts, with levels consistently between 30% and 40%.

The results show a significant decrease in the expression levels of CXCR3 and CXCR4 on the global CD8⁺ T cell population. The percentage of cells positive for CXCR4 decreases from 30% to 10% in MM patients.

**MM Patients Exhibit Altered Chemokine Receptor Expression on Viral Specific CD8⁺ T Cells**

As well as dysregulation of the chemokine receptors expressed on the global CD8⁺ T cell population, their expression is uniquely altered on CMV and EBV viral peptide specific CD8⁺ T cells in MM patients. EBV peptide specific CD8⁺ T cells exhibit decreased expression of CXCR3 compared to HD controls. Levels of CXCR3 on CMV peptide specific cells are not affected however (Figure 16A).

CMV specific CD8⁺ T cells show a significant decrease in CXCR4 levels compared to HD controls, whereas conversely MM patients appear to have increased levels of CXCR4 on CMV specific CD8⁺ T cells, however this observation is not statistically significant. Similarly to the global CD8⁺ T cell population, CCR5 levels do not differ between HD and MM patients.
In order to investigate the different combinations of chemokine receptors on viral/CTA peptide specific CD8\(^+\) T cells between HD and MM patients, combinations of CXCR4, CCR5 and CXCR3 were analysed. CXCR6 was excluded from the analysis because its expression on PB CD8\(^+\) T cells in both HD and MM samples is very low (Figure 15).

Combinations of these chemokines on viral peptide specific CD8\(^+\) T cells were analysed for combinations which showed the biggest differences between the two populations, and other potentially important combinations (Figure 16B).
The most common phenotype across both of the cohorts is CXCR4^−CCR5^−CXCR3^−, meaning the majority of cells do not express any of the three chemokines analysed. Several combinations of chemokine receptors are different for each of the cohorts however, for example the percentage of cells expressing the CXCR4^−CCR5^+CXCR3^+ phenotype is lower in MM patients than in controls, whereas the expression of CXCR4 single positive cells are increased in MM patients, despite global CXCR4 levels being shown to be decreased in MM patients.
**Discussion**

A proportion of MM patients possess a subset of CD8<sup>+</sup> T cells which are specific for CTA derived peptides expressed on the surface of MM cells. Despite these cells eliciting an effective immune response against CTA expressing myeloma cell lines *in vitro*, effective *in vivo* responses have yet to be documented. The further characterisation of these CTA specific responses in MM patients is an important area to explore, as their manipulation may potentially lead to improvements in the immunotherapeutic approaches used to treat the disease.

A common observation in MM patients is global immune dysregulation, and the progression of disease appears to bring about changes in chemokine expression levels, and certain chemokine receptors on CD8<sup>+</sup> T cells. Studies have shown that CTA specific T cells are enriched in the PB of MM patients, with reduced numbers in the BM, the site of the tumour. The disruption of chemokine and chemokine receptor expression gradients between the BM and PB may be a technique employed by the tumour in order to divert potentially anti-tumour CD8<sup>+</sup> T cells away from the tumour site.

In order to explore this hypothesis further, the aim of this investigation was to characterise the chemokine receptor expression profile of CTA specific CD8<sup>+</sup> T cells in MM patients, and compare them with the expression profile of viral peptide specific CD8<sup>+</sup> T cells from HD and MM patients. Although only one CTA response was detected in the investigation, the chemokine receptor expression profile of global as well as viral specific CD8<sup>+</sup> T cells was analysed in HD and MM patients. It was found that globally, CXCR4 and CXCR3 levels are reduced on CD8<sup>+</sup> T cells in MM patients, as well as unique alterations in chemokine receptor expression of EBV and CMV specific CD8<sup>+</sup> T cells between the two cohorts. It is hoped that these finding will shape further research into this area, and ultimately lead to the better understanding of these potentially therapeutically useful CTA specific CD8<sup>+</sup> T cells in MM patients.

**Detection of Viral Peptide and CTA specific CD8<sup>+</sup> T Cells in HD and MM Patients**

CD8<sup>+</sup> T cells specific for either virus derived peptides or CTA peptides were identified in HD and MM patients. Six virus specific responses were detected in the MM patient cohort and 4 in the
HD cohort, with half of each being specific for EBV, and the other half for CMV peptides. It would be interesting to determine if there is any significant differences between the response levels of the two groups of peptides between the two cohorts, a the progression of the disease may alter the levels of specific CD8⁺ responses, some of which may have anti-tumour properties, as a method of protecting the tumour. There is a great variation however between responses over a cohort of patients, and even between the same patients over time, so in order to determine this, a much larger number of samples would be required. For this reason, no conclusion was drawn in this investigation.

**MM Patients Display an Exhausted Memory Phenotype on CD8⁺ T Cells**

The memory phenotype of T cells is important in determining their potential responses, as well as playing a key role in their distribution and localisation. Data collected in this investigation suggest that MM patients have a less diverse repertoire of CD8⁺ T cells compared to HD controls. In HD controls, 20% of the PB CD8+ T cells exhibit the naïve phenotype of CD45RO⁻CCR7⁺. These naïve cells have yet to encounter their cognate antigen, and so are trafficked to the lymph node by virtue of their CCR7 expression, where they are presented with antigen by antigen presenting cells (APCs). Within the MM cohort however, a significantly smaller proportion of the CD8⁺ population (less than 3%) are naïve. The majority of the CD8⁺ T cells in MM patients are TEMRA cells, which have already been exposed to antigen, and so are therefore unable to contribute towards a response to newly induced antigens. This combination of cells represents an immunologically exhausted immune phenotype, resulting in reduction in naïve CD8⁺ cells will lead to the host being less able to mount effective immune responses to newly induced antigens, potentially leaving an infection or tumour to persist and spread unchecked by the cellular immune response. This alteration may be a technique employed by the tumour in order to reduce the host’s ability to respond to rapidly changing tumour antigens, ultimately allowing the tumour to progress unhindered by the immune system. This data builds on previous findings that MM patients exhibit monoclonal expansions of subsets of T-cells, which would lead to a decrease in the numbers of other subsets, due to the limited habitable niche available, ultimately leading to a reduced ability to adapt to new immunogenic threats. This phenomenon is also observed during the ageing process. For
example, there are expansions of CMV specific subpopulations of T cells in older patients, which is known to decrease the host’s ability to adapt to new antigens.

**MM Patients Show Reduction of CXCR3 and CXCR4 expression on CD8⁺ T Cells in the Blood**

Chemokine receptors on T cells provide a central role in the trafficking of cells around the body and to the tissues where they are required, based on the distribution of their ligands. This investigation found that CD8⁺ T cells in PB of MM patients express less CXCR4 and CXCR3 on their surface than equivalent cells in HD controls. Combined with the data from the unpublished studies by Goodyear *et al.* which have shown an increase in the expression of CXCR3 ligands in the BM of MM patients, this suggests the distribution of CXCR3 and its ligands may contribute towards the alteration in the trafficking of T cells between the BM and PB.

The decrease in CXCR3 expressing CD8 T cells in the blood may represent skewing of the distribution of these T cells towards the BM, as CXCR3 ligands are increased there in MM. An alternative interpretation is that CXCR3 and CXCR4 are down regulated on the surface of T cells in order to reduce their ability to home to the site of the tumour in the BM, hence shielding the tumour from these cells.

In order to explore these finding further, it would be necessary to obtain matched BM samples from these patients, and compare the proportion of CXCR3 and CXCR4 expressing T cells between the BM and blood of the two cohorts of HD and MM patients. An increase in CXCR3 and CXCR4 expressing CD8⁺ T cells in the BM would suggest that the BM is recruiting these cells, potentially as a method to combat the tumour.

**MM Status Effects the Chemokine Expression Profile of CMV and EBV Viral Peptide Specific CD8⁺ T Cells Differently**

CD8⁺ T cells specific for different virus derived peptides express a unique pattern chemokine receptors, and are also affected differently by MM status. Patients with MM appear to increase CXCR4 expression on EBV peptide specific CD8⁺ T cells, whereas CXCR3 expression is decreased. Conversely, CXCR4 expression is decreased on CMV specific CD8⁺ T cells in MM patients.
This differential alteration between specific CD8⁺ T cells in the same cohorts indicates that MM status can effect different CD8⁺ subsets in unique ways. This indicates that MM may be able to selectively recruit and dismiss certain subsets of CD8⁺ T cells which would benefit the tumours survival and progression. For example, CTA specific CD8⁺ T cells may be exclusively excluded from the tumour site, perhaps by a decrease in chemokine receptors, whose cognate ligand levels are increased in the BM, such as CXCR3.

**Characterisation of CTA Specific CD8⁺ Responses in MM Patients**

CTA specific CD8⁺ T cells effectively target and kill CTA expressing myeloma cells *in vitro*, yet effective responses *in vivo* have not been observed. CTA responses are characteristically low, usually representing between 0.0004% and 0.1% of the total CD8⁺ population in a subset of MM patients. In this short study, a limited number of MM samples were available, with limitations on clinical availability of the patients. Samples from 17 MM patients were collected during the course of the study, many of which had previously been characterised as not having CTA specific CD8⁺ response. As a result of this, only one CTA response was detected and characterised. Unfortunately subsequent samples were not obtained in order the confirm the findings by means of repeats or the establishment of peptide lines in order to expand any potential responses.

Interestingly, four patients who had previously been characterised as positive responders for CTA specific CD8⁺ T cells failed to show a response in these assays. It has been shown that CTA response levels can change and track the stage of the disease, which fluctuates between plateau and relapse phases. As such, responses detected previously could likely disappear, or reduce to a level not detectable due to the sensitivity of the assays used.

Because of these reasons, no significant comment can be made on the responses observed, as they represent an unconfirmed subset of CTA specific T cells in one patient. In order to expand upon this investigation to be able to characterise these CTA specific responses, greater numbers of patient samples would be required. These patients are available to the group, and have an extensively documented clinical history, and so are ideal for the study. It was only time restraints and clinical availability which limited the scope of this investigation, and the assays
and techniques used have been refined and demonstrated to work effectively. Therefore, given more time, expansion of this investigation would hopefully yield significant, novel data in this area.

**Future Directions**

In order to expand upon the foundations laid by this investigation, several approaches have been considered. Firstly, in order to improve the quality and reliability of data obtained in this study, a greater number of patient and donor samples is required. This would allow the characterisation of multiple CTA specific T cell responses, generating statistically robust data which can be analysed and reliably compared to controls.

As mentioned previously, obtaining paired BM samples from patients and controls would allow for the comparison between the chemokine receptor expression patterns on CD8 T cells between the two sites. This would allow for us to determine the distribution of these subsets see if a particular chemokine receptor expression profile is favoured at each site. This increased understanding of the potential role of chemokine receptors in the localisation of CTA specific CD8 T cells in MM.

Another further direction to expand upon this research would be to obtain samples from a cohort of MGUS patients. MGUS is the pre-malignant a-symptomatic precursor of MM, and is currently poorly understood in terms of risk factors and triggers for progression into malignant disease. Exploration of the role of CTA specific CD8 T cells in MGUS and its progression into MM may lead to an understanding of the significance of these responses and their role in the maintenance of disease. Trafficking of T cells which have a role in immune-surveillance or inhibition of tumour growth make play a key role in the development of the disease, and increased understanding may shed some light on potential therapies to inhibit this transition.

**Concluding Remarks**

MM is the second most common cancer of the blood, accounting for 13% of haematological malignancies, with around 3000 new cases a year in the UK. MM is an incurable disease, and although many treatments are currently in use in the clinic, novel curative treatments are
desperately needed. There is currently massive interest in the area immunotherapy as a method for treating a range of cancers, with both trial and clinical applications proving successful. There is a growing interest in the characterisation and potential manipulation of CTA specific CD8$^+$ T cells in MM patients, and the understanding of their potential role in persistence of the disease is key to this development. Recent evidence has suggesting that the localisation and trafficking of these cells, as a result of chemokine and chemokine receptor expression may be an important factor in their lack of ability to mount an effective immune response against the tumour \textit{in vivo}.

This investigation has aimed to build on these findings, and has refined and expanded upon techniques to analyse chemokine receptor expression patterns antigen specific T cells in patients, such as viral peptide and CTA specific CD8$^+$ T cells. The data collected in this investigation suggest that globally, MM patients have lower CXCR3 and CXCR4 expression on CD8$^+$ T cells in PB, likely as the cells expressing high levels of CXCR3 have been trafficked to the BM, where CXCR3 ligand expression is increased in MM. This study also found that MM effects the chemokine expression profile of antigen specific CD8$^+$ T cells in different ways, indicating that perhaps CTA specific T-cells may be specifically trafficked in a manner which protects the tumour site from their potentially power anti-tumour functions.

Ultimately it is hoped that this project will aid in the development of future research into this field, leading to the characterisation and manipulation of these responses to improve clinical outcomes of MM patients.
References


