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Immunotherapy in Multiple Myeloma

By

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A thesis submitted for the degree of Doctor of Philosophy to the University of Glasgow October 2005

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Abstract

Multiple myeloma (MM) remains essentially incurable by conventional anti-tumour therapy, although the median survival time has increased to 5 years in those who respond well to autologous (auto) haematopoietic stem cell transplant (HSCT). In a small number of patients with MM, long-term disease-free survival has been achieved by harnessing the immune phenomenon, “graft-versus-tumour” effect, induced during allogeneic haemopoietic stem cell transplantation. This has prompted many investigators to examine ways in which a patient’s own immune system can be more effectively directed against their disease, with the ultimate aim of tumour eradication. However, the MM is associated with a number of cytokines and chemokines such as CCL3, IL-6, IL-10, VEGF, and TGFβ that have immunosuppressive effects on the host, and drive disease progression.

The BDCA antibodies allowed reliable measurement of dendritic cell (DC) subsets and B cell numbers in the blood of normal subjects, and patients with MM throughout the disease course. The numbers of blood myeloid DC (BmDC) and blood plasmacytoid DC (BpDC) are low throughout the course of the disease, and only improve for a short period of time following autologous HSCT. Thalidomide therapy of patients with relapsed disease was associated with an increase in BmDC1 and BpDC numbers.

Monocytes, mobilised at the time of stem cell collection, were used to produce mature DC (matDC) from MM patients and normal donors (ND). The matDC produced from MM patients were of poorer quality as compared to those from ND, despite using combinations of GM/IL-4, GM/IL-13, X4 and MIMIC in the production process. The combinations that contained the X4 maturation cocktail produced the best quality matDC.
Pre-stimulation of MM T cells with T cell stimulation beads and IL-2 enhanced their proliferative response to IL-2 and matDC+IL-2, but blocked proliferation in response to matDC alone. Pre-stimulation also blocked T cell cytokine secretion in response to DC alone. Despite this, pre-stimulated matDC primed T cells from MM patients produced the best killing of MM tumour cell targets, followed by un-stimulated matDC primed TC, with un-stimulated matDC primed TC from ND producing poor cytolytic effects.

The fluorescent chemokine uptake assay was a sensitive and specific method for detecting chemokine receptor activity, and was able to track changes during DC development and maturation. CCL3 uptake was low on MM monocytes. Uniquely, mobilised monocytes also demonstrated some CCR7 activity, as indicated by CCL19 uptake. This expression was confirmed by RT-PCR. CCR7 activity was best on ND matDC matured with the X4 cytokine cocktail, and significantly lower on matDC from MM patients. CCR8 expression was significantly higher on MM monocytes and matDC than on ND cells, suggesting that they may be more sensitive to the immunosuppressive effects of CCL1.

In summary, the DC/ T cell system is abnormal in MM patients. Despite this, it is possible to produce antigen loaded mature MoDC from MM patients. When combined with T cell pre-stimulation and IL-2 expansion, these DC are capable of inducing anti-MM cytotoxic T cells, which exhibit considerable anti-MM cytolytic activity. However, the DC from MM patients still display abnormal chemokine receptor expression, which may inhibit their capacity to migrate to lymph nodes in-vivo in order to generate these cytotoxic T cell responses.

These observations will aid in the optimisation of DC based immune therapies for MM, and suggest that a combined immunotherapy approach using pre-stimulated
T cells, MM Ag primed DC and IL-2 may produced better clinical responses in MM patients.
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Related Publications

Harrison SJ. Cook G. Immunotherapy in multiple myeloma - possibility or probability? British Journal of Haematology. 130(3):344-62, 2005 Aug,
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Dedication

This work is dedicated to my wife, Jo, and my children, Ciara, Naomi and Kian.

Without their unfailing love and support, it may never have been completed.
Author’s Declaration

Unless otherwise stated, I declare that all the work presented in this thesis is my own.
### Definitions and abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>3H-thymidine</td>
<td>Tritiated Thymidine</td>
</tr>
<tr>
<td>7AAD</td>
<td>7-amino-actinomycin D</td>
</tr>
<tr>
<td>34^DC</td>
<td>CD 34^ derived dendritic cell</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
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<td>allo</td>
<td>Allogeneic</td>
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<td>auto</td>
<td>Autologous</td>
</tr>
<tr>
<td>BDCA</td>
<td>Blood dendritic cell antigen</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BmDC</td>
<td>Blood myeloid dendritic cell</td>
</tr>
<tr>
<td>BpDC</td>
<td>Blood plasmacytoid dendritic cell</td>
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<tr>
<td>CCL</td>
<td>Chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
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<td>Cytoplasmic tail</td>
</tr>
<tr>
<td>DARC</td>
<td>Duffy antigen receptor for chemokine</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DLI</td>
<td>Donor lymphocyte infusion</td>
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<tr>
<td>DMSO</td>
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<td>Fluoroscein Isothiocyanate</td>
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<td>Forward scatter</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte stimulating colony factor</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte stimulating colony factor</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HSCT</td>
<td>Haematopoietic stem cell transplant</td>
</tr>
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<td>Id</td>
<td>Idiotype</td>
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<tr>
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<td>Interleukin</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>M</td>
<td>Male</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cell</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MGUS</td>
<td>Monoclonal gammopathy of uncertain significance</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple Myeloma</td>
</tr>
<tr>
<td>MoDC</td>
<td>Monocyte derived dendritic cell</td>
</tr>
<tr>
<td>MRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>NM</td>
<td>Non-myeloablative</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
</tbody>
</table>
RT Room Temperature
PBMC Peripheral blood mononuclear cell
pDC Plasmacytoid dendritic cell
PE Phycoerythrin
PGE₂ Prostaglandin E₂
Poly I:C Polyrribosinic-Polyribocytidilic acid
RNA Ribonucleic acid
SCI Subcutaneous injection
sec Second
SSC Side scatter
SE Standard Error
SNBTS Scottish National Blood Transfusion Service
TAP Transporter associated with Ag processing molecules
TM Transmembrane domain
vs Versus
WB Whole blood
1. Introduction and Aims

1.1 Multiple Myeloma

Multiple myeloma (MM) is a clonal B-cell malignancy characterised by an excess of mature plasma cells in the bone marrow (BM), in association with a monoclonal protein in serum and/or urine, decreased normal immunoglobulin (Ig) levels, renal failure and lytic bone disease. It has an incidence of around 4/100,000 in Caucasians and a median age of onset of 70yrs, with the vast majority of patients presenting with fatigue, recurrent infections, bone pain or pathological fractures. MM remains essentially incurable by conventional anti-tumour therapy and the median survival time is 3yrs. However, this may be extended to 5yrs with modern induction therapy and a good response to autologous (auto) haemopoietic stem cell transplantation (HSCT) (Attal and Harousseau 1999, Child, et al 2003). The diagnostic criteria for MM and the other members of the group of disorders known as the monoclonal gammopathies were reviewed recently (International Myeloma Working 2003).

Monoclonal gammopathy of uncertain significance (MGUS) is a pre-malignant condition affecting 1% of people <50yrs and up to 3% of >70yrs. Patients must have a monoclonal protein <30g/l and <10% plasma cells in the bone marrow (BM). Patients with MGUS progress to MM or a related disorder at a rate of 1%/yr. Most MGUS patients have detectable clonal abnormalities, and it is thought that disease progression is the result of accumulating genetic abnormalities within the plasma cell clone. Smouldering MM is defined as the presence of a paraprotein of ≥ 30g/l and/or ≥10% BM plasma cells, with none of the other diagnostic features of MM. Currently there is no evidence that treatment at this stage alters the disease course, but a number of ongoing clinical studies hope to answer this question.
MM can be diagnosed by the presence of > 10% infiltrate of plasma cells in the
BM, in the presence of a monoclonal paraprotein in serum or urine, and evidence
of end organ damage, such as hypercalcaemia, renal failure, anaemia and bone
destruction (CRAB). A small number of patients have no detectable paraprotein,
but meet the other diagnostic criteria, and this is called non-secretory MM. A
number of disease prognostic markers have been defined; raised serum $\beta_2$-
microglobulin; low serum albumin; deletion of chromosome 13 or it's long arm;
t(4;14) and t(14;16) translocations, and they form the basis of the new
International Staging System (Greipp, et al 2005). As the disease progresses, the
neoplastic plasma cell induces a number of changes in the BM microenvironment
(Figure 1-5); increased angiogenesis; suppression of cell mediated immunity
(discussed later); paracrine signalling loops with stromal cells which induce tumour
cell proliferation and resistance to drugs such as Dexamethasone.

The treatment of MM can be divided into 3 phases, induction, consolidation and
maintenance. If patients are not eligible for auto HSCT, then a simple induction
regime of monthly oral Melphalan and Prednisolone until the paraprotein level
stops falling, despite further therapy i.e. plateau is reached, is the treatment of
choice because of the low rate of side effects. Unfortunately, Melphalan is toxic to
stem cells, so younger fitter patients who will go on to have autologous stem cells
collected need to avoid it and other alkylating agents prior to harvest. A number of
alternatives have been the subject of clinical trials, such as high dose
Dexamethasone, VAD (intravenous Vincristine, Dexamethasone and Doxorubicin),
Z-Dex (oral Idarubicin and Dexamethasone) and CTD (oral Cyclophosphamide,
Thalidomide and Dexamethasone) (MRC sponsored Myeloma IX study)
and CTD seem to have superior response and complete remission rates, there is
currently no evidence that they are associated with improved long-term survival.

Following 'induction' therapy, patients go on to have auto stem cells collected for use in auto HSCT consolidation.

As already discussed, the role of auto HSCT, conditioned with 200mg/m² of Melphalan, is well established. However, a number of groups have explored the use of sequential or tandem auto HSCT, and have shown an improvement in EFS and OS (Attal, et al 2003, Barlogie, et al 1997). The use of sequential auto and NM allogeneic (allo) HSCT is also the subject of clinical trials. The role of maintenance therapy following transplant is controversial. At present, there is no convincing evidence to support it's use, but a number of studies (including Myeloma IX) are exploring the use of thalidomide following auto HSCT. Overall MM patients have a relatively poor outlook compared to other low grade lymphoproliferative disorders. This has prompted the development of a number of experimental compounds and treatment modalities

1.2 Allogeneic Haematopoietic Stem Cell Transplantation

The immune system is capable of eradicating malignancies. The first evidence that cancer cells were susceptible to immune attack emerged in the early 1960's from the animal models used for HSCT (Mathe, et al 1965). This was first demonstrated in human HSCT in the late 1980's with the observation that patients who survive T cell replete allo HSCT have a lower relapse rate compared to auto, syngeneic or T cell depleted allo HSCT (Gale, et al 1989, Hughes, et al 1989). This "graft-versus-tumour" effect has also been demonstrated using steady state unprimed donor lymphocyte infusions (DLI) to treat relapse following allo HSCT and in the setting of minimal residual disease (Kolb, et al 1990).
The use of allo HSCT in the treatment of MM has resulted in a higher rate of molecular remission, with lower rates of relapse and disease progression as compared to patients treated with auto HSCT (Bensinger, et al 1996, Corradini, et al 1999). Those patients who survive 1 year have significantly improved disease-free survival (Bjorkstrand, et al 1996). This is partly related to the intensity of the chemo-radiotherapy conditioning regimen, but it is also a result of the graft-versus-myeloma (GVM) effect (Perez-Simon, et al 2003, Tricot, et al 1996, Verdonck, et al 1998). However, these improvements in disease control are achieved at the expense of higher treatment-related morbidity/mortality in the first year and have prompted the experimental use of potentially less toxic NM HSCT (Crawley, et al 2005b, Garban, et al 2001, Perez-Simon, et al 2003, Singhal, et al 2000). The close relationship between Graft-versus-host disease (GvHD) and GVM in published studies suggests that donor allo-reactive T cells directed against minor histocompatibility antigens (Ags) present on both normal and myeloma cells mediates the latter effect. The successful use of DLI in some cases of MM that relapse following allo-HSCT has led to increased interest in the possibility that other forms of immune therapy might be effective in this disease (Lokhorst, et al 1997, Lokhorst, et al 2000). However, the doses of T cells required to induce these remissions are higher in MM than in other DLI responsive diseases such as chronic myeloid leukaemia (CML) (Verdonck, et al 1998), and are associated with an increased incidence of GVHD (Huff and Jones 2002, Salama, et al 2000). This may be partly explained by the influence of the malignant clone on the function of the immune effector cells resulting from both passive and active suppression, although it is possible that MM tumour Ags are less immunogenic compared with those on other malignant cells, as MM is associated with several defects in the host's immune system (Cook and Campbell 1999).
MM tumour cells produce a number of immunologically active agents that can modulate the immune response such as transforming growth factor-beta (TGF-β) (Cook, et al 1999), interleukin-10 (IL-10) (Brown, et al 2004), Fas ligand (FasL) (Villunger, et al 1997), vascular endothelial growth factor (VEGF) (Oyama, et al 1998) and MUC-1 (Agrawal, et al 1998, Gimmi, et al 1996, Treon, et al 1998). It is postulated that by producing these agents the tumour cell modifies both the microenvironment to support growth and differentiation of the clone, and the host immune response to prevent tumour rejection. This duality of function is important in understanding the possible interactions of the malignant clone with the tumour-bearing host, especially if we are to design immunotherapy strategies that will achieve their true potential and result in improved survival in MM.

In an attempt to boost the GVM effect Kwak et al have demonstrated the successful transfer of myeloma idiotype-specific immunity from an actively immunised bone marrow donor to a recipient with MM, demonstrating MHC class I-restricted CD8+ T cell recognition of freshly isolated, recipient myeloma tumour cells (Kwak, et al 1995, Yiwen, et al 2000). It still remains to be seen whether the GVM effect can be separated from GVHD. However, there is evidence that a patients own immune system may play an important role in the control of their disease. It has been shown that T cells specific for MM associated Ags such as Mucin-1 (MUC-1 or CD227) (Beckhove, et al) idiotype protein (Yi, et al 1995) and NY-ESO-1 (van Rhee, et al 2005b) are present in the peripheral blood of MM patients. Joshua’s group have shown that the presence of expanded T cell clones at any time during the disease course is associated with prolonged overall survival (Brown, et al 1997, Raitakari, et al 2003).

Immunotherapeutic strategies attempt to utilise the immune system for disease control and they have mainly been tested in the setting of relapsed or resistant
disease. The use of such strategies in the setting of minimal residual disease following conventional chemotherapy or HSCT offers an increased potential for tumour cell control by adoptively transferring immune effectors at a time when they are likely to have most impact (Hsu, et al 1997, Stevenson, et al 2004).

1.3 Cellular Immunotherapy

Discovered by Steinman and others in the 1970’s (Steinman, et al 1975, Steinman and Cohn 1973, Steinman and Cohn 1974, Steinman, et al 1974), dendritic cells (DC) have been identified as the sentinels of the immune system. As a result it has become theoretically possible to direct the immune response against a specific chosen (tumour) Ag towards immunity or tolerance (Matzinger and Guerder 1989). It is important to consider how the different components of the immune system (DC, T cells and natural killer (NK) cells) develop and interact in-vivo and how this may be altered in patients with MM. Subsequently we will consider how cells of the immune system may be manipulated ex vivo to overcome any MM tumour cell suppressive effects and eradicate the malignant clone.

1.3.1 In-vivo DC development

DC precursors are derived from the bone marrow haematopoietic stem cell (HSC), differentiating into two phenotypically distinct populations. Myeloid DC (mDC) classically arise from the common myeloid progenitor and myelomonocytic precursors, are characterised by the surface phenotype of CD11c++/CD123-/CD1c+, and tend to induce Th1 responses. Plasmacytoid DC (pDC) arise from the common lymphoid progenitor, are CD11c-/CD123++, and induce Th2 responses. However, factors such as the strength of the T cell receptor/MHC class II interaction, Ag density and the microenvironment, are also important in the balance between mounting a Th1 or Th2 T cell response. Recent studies also
suggest that the system for DC generation may exhibit more plasticity than previously thought, in that both myeloid and lymphoid DC may arise from either myeloid or lymphoid progenitors (Shigematsu, et al 2003). DC precursor and circulating blood DC migrate into peripheral tissues, such as skin, portal triads, mucosa and lung, driven by their expression of CCR2 and CXCR4 (Figure 1-1 and 1-3). Here they down regulate CCR2 and up regulate CCR6, which seems to be important for (i) DC circulation to lymphoid tissue like Peyer patches via constitutive expression of CCL20 and (ii) may play a role in the induction of tolerance to self Ags. Immature DC also express CCR1, CCR5 and CXCR2, which allows them to migrate towards areas of inflammation via increased levels of CCL3, CCL5, CXCL8 and CCL20, which may be inducibly expressed in other tissues during an inflammatory response (Austyn and Larsen 1990). The iDC actively sample the environment by phagocytosis and process Ag from bacteria, viruses and apoptotic bodies (Albert, et al 1998, Hengel, et al 1987, Svensson, et al 1997), presenting them in the context of MHC class I and II molecules. To mature, iDC require a second 'danger' signal such as interferon (IFN) α or γ, interleukin-1β or microbial compounds such as bacterial lipopolysaccharide (LPS) (Matzinger 1998). During the ensuing terminal differentiation, DC down regulate their phagocytic and Ag processing functions, up regulate expression of MHC class I and II, costimulatory and adhesion molecules (O'Neil and Bhardwaj 2005). Dendritic cell maturation also down regulates CCR1, CCR5 and CCR6 and up regulates CCR7 and CCR8 expression, thus becoming sensitive to CCL1, CCL19 and CCL21, which are constitutively expressed in lymph nodes (Qu, et al 2004). The mature DC (matDC) then rapidly migrate to the secondary lymphoid tissues via the afferent lymphatic system, attracted by chemokines such as CCL19 and CCL21 (Chan, et al 1999, Dieu, et al 1998), where they efficiently present Ag to T cells, and induce an immunogenic response. If iDC encounter Ag in the absence
of Inflammation, they partially up regulate CCR7 but do not up regulate co-
stimulatory molecules, and constitutively migrate to LN to induce/maintain
tolerance (Matzinger 1998). Naïve and central memory T cells also express CCR7,
thus chemokine expression brings DC and T cells into close proximity for Ag
presentation to occur.

Figure 1-1 In-Vivo DC generation, migration and maturation pathways
DC arise in the bone marrow and migrate from the BM to peripheral tissues via the
blood. Here, iDC encounter Ag, mature and migrate to lymph nodes via the
lymphatics, and present Ag to naïve T cells. Effector (cytotoxic) T cells then
migrate to peripheral tissues via the blood.

1.3.2 Chemokines and their role in DC trafficking
Chemokines (CHEMOtatic cytoKINES) were originally defined as proteins
manufactured by cells and tissues that stimulate movement and activation of
immune cells to the area where the chemokine is produced and their subsequent
activation. However, there is now a considerable body of evidence to show that
the biological effects mediated by chemokines are far more complex. Virtually all cell types, including tumour cells, have the potential to express chemokines and chemokine receptors and this expression may have a direct impact on tumour cell growth, angiogenesis and metastases (Vicari and Caux 2002). At the same time the chemokine expression pattern of the tumour may have a direct bearing on the host immune system's ability to mobilise monocytes, DC, T cells and NK cells against the tumour. The chemokine balance within the patient and the tumour may be crucial in determining the type of immune response that is mounted, in that the tumour may shift the balance from an immunogenic response in to a tolerogenic response by the expression of a different chemokine 'signature'.

**Figure 1-2 General structure of chemokine gene super-family members.**

Schematic representation of the conserved cysteine signature residues present in the primary amino acid structure that distinguish members of the chemokine gene super-family into four basic subfamilies, which can be subdivided based on the presence or absence of additional cysteine residues and ELR (glutamic acid, leucine and arginine) motifs. (Reproduced by kind permission Dr S. McColl, Chemokine Biology Lab, University of Adelaide)

Chemokines are a large family of proteins, with considerable structural homology based on conserved cysteine residues and the binding capacity to particular G protein-coupled receptors (GPCR) (Zlotnik and Yoshie 2000). They have been divided in to 4 major sub-groups based on the relative position of conserved
cysteine residues: CC, CXC, XC and CX3C (also known as β, α, γ and δ respectively) with CC and CXC being by far the most common. In addition

Chemokines can be functionally classified into two groups based on whether they are expressed in a constitutive/homeostatic or inducible fashion. The nomenclature for chemokines is confusing. There are over 40 members of the human chemokine family and a large number have several different names, as a consequence of being described by different groups at different times. In an attempt to rationalise this, a new system to standardise chemokine ligand nomenclature, was introduced after the 1998 Gordon Research Conference on Chemotactic Cytokines (Zlotnik and Yoshie 2000) (Table 1-1).

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Original names</th>
<th>Systematic name</th>
<th>Original names</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL1</td>
<td>I-309</td>
<td>CXCL1</td>
<td>Gro α</td>
</tr>
<tr>
<td>CCL2</td>
<td>MCP-1, MCAF</td>
<td>CXCL2</td>
<td>Gro β</td>
</tr>
<tr>
<td>CCL3</td>
<td>MIP-1α</td>
<td>CXCL3</td>
<td>Gro γ</td>
</tr>
<tr>
<td>CCL4</td>
<td>MIP-1β</td>
<td>CXCL4</td>
<td>PF4</td>
</tr>
<tr>
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<td>RANTES</td>
<td>CXCL5</td>
<td>ENA-78</td>
</tr>
<tr>
<td>CCL7</td>
<td>MCP-3</td>
<td>CXCL6</td>
<td>GCP-2</td>
</tr>
<tr>
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<td>MCP-2</td>
<td>CXCL7</td>
<td>NAP-2</td>
</tr>
<tr>
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<td>Eotaxin-1</td>
<td>CXCL8</td>
<td>IL-8</td>
</tr>
<tr>
<td>CCL13</td>
<td>MCP-4</td>
<td>CXCL9</td>
<td>MIG</td>
</tr>
<tr>
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<td>HCC-1</td>
<td>CXCL10</td>
<td>IP-10</td>
</tr>
<tr>
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<td>HCC-2, Lkn1, MIP-15</td>
<td>CXCL11</td>
<td>I-TAC</td>
</tr>
<tr>
<td>CCL16</td>
<td>HCC-4, LEC</td>
<td>CXCL12</td>
<td>SDF-1</td>
</tr>
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<td>TARC</td>
<td>CXCL13</td>
<td>BLC, BCA-1</td>
</tr>
<tr>
<td>CCL18</td>
<td>MIP-4, PARC, DC-CK1</td>
<td>CXCL14</td>
<td>BRAK</td>
</tr>
<tr>
<td>CCL19</td>
<td>MIP-3β, ELC</td>
<td>CXCL16</td>
<td>CXCL16</td>
</tr>
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<td>CCL20</td>
<td>MIP-3α, LARC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL21</td>
<td>SLC, 6Ckine</td>
<td>CX3CL1</td>
<td>Fractalkine</td>
</tr>
<tr>
<td>CCL22</td>
<td>MDC, STCP-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL23</td>
<td>MPIF-1</td>
<td>XCL1</td>
<td>Lymphotactin</td>
</tr>
<tr>
<td>CCL24</td>
<td>MPIF-2, Eotaxin-2</td>
<td>XCL2</td>
<td>SCM-1β</td>
</tr>
<tr>
<td>CCL25</td>
<td>TECK</td>
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<tr>
<td>CCL26</td>
<td>Eotaxin-3</td>
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<td>CCL27</td>
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<td></td>
</tr>
<tr>
<td>CCL28</td>
<td>MEC</td>
<td></td>
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</tr>
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</table>

Table 1-1 Human chemokine systematic nomenclatures.

No human equivalent of mouse CCL6, CCL9, CCL10, CCL12, CXCL15 has been identified
The function of chemokines on target cells is mediated by binding to their corresponding receptors: CCR, CXCR, XCR and CX3CR, which are members of the huge G-protein coupled seven transmembrane domain receptor superfamily. The complexity of this system is further increased by promiscuity, by which chemokines can bind to a number of different receptors and receptors can bind a number of different ligands (Table 1-2). The result is that there are many possible biological read outs based on the balance of the receptor/chemokine expression patterns.

<table>
<thead>
<tr>
<th>Chemokine receptors</th>
<th>Human chemokine ligands</th>
<th>Ligand expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>CCL3- 5, 7, 14, 15, 16, 23</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCR2</td>
<td>CCL2, 5, 7, 8, 13, 16</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCR3</td>
<td>CCL3, 7, 8, 11, 13, 15, 24, 26, 28</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCR4</td>
<td>CCL17, 22</td>
<td>Constitutive / Inducible</td>
</tr>
<tr>
<td>CCR5</td>
<td>CCL3- 5, 8, 16</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCR6</td>
<td>CCL20</td>
<td>Constitutive / Inducible</td>
</tr>
<tr>
<td>CCR7</td>
<td>CCL19, 21</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CCR8</td>
<td>CCL1</td>
<td>Inducible</td>
</tr>
<tr>
<td>CCR9</td>
<td>CCL25</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CCR10</td>
<td>CCL27, 28</td>
<td>Constitutive / Inducible</td>
</tr>
<tr>
<td><strong>CCR11/CCX CKR</strong></td>
<td><strong>CCL19, 21, 25, CXCL13</strong></td>
<td><strong>? Decoy</strong></td>
</tr>
<tr>
<td>CXCR1</td>
<td>CXCL1, 6, 8</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCR2</td>
<td>CXCL1- 3, 5, 6, 8</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCR3</td>
<td>CXCL4, 9, 10, 11</td>
<td>Inducible</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXCL12</td>
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</tr>
<tr>
<td>CXCR5</td>
<td>CXCL13</td>
<td>Constitutive</td>
</tr>
<tr>
<td>CXCR6</td>
<td>CXCL16</td>
<td></td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CX3CL1</td>
<td>Inducible</td>
</tr>
<tr>
<td>XCR1</td>
<td>XCL1, 2</td>
<td></td>
</tr>
<tr>
<td>D6</td>
<td>CCL2- 5, 7, 8, 11, 13, 14, 17, 22</td>
<td>Decoy</td>
</tr>
<tr>
<td>DARC</td>
<td>CCL3, 5, 7, 11, 13, 14, 17, CXCL1- 4, 7, 8,</td>
<td>Decoy/ Buffer</td>
</tr>
</tbody>
</table>

Table 1-2 Summary of the known human chemokine receptors and their ligands

At the International Union of Pharmacology, XXX, 2002, Update on chemokine receptor nomenclature meeting, the putative receptor CCR11 was disqualified (Murphy 2002) because although high affinity binding to CCL19, CCL21 and CCL25 was identified, there was no detectable signalling or induction of chemotaxis. This placed it in a group of 'silent receptors along with D6 and DARC.
By altering the expression of chemokine receptor genes during differentiation and maturation, cells such as DC and T cells are able to track from the bone marrow, to peripheral tissues and lymph nodes under the influence of homeostatic ligands. Inflammatory processes up-regulate inducible CCRs on leukocytes, inducing chemotaxis towards pro-inflammatory CCLs, such as CCL3, produced during the inflammatory response (Figure 1-3).

Figure 1-3 DC migration and chemokine receptor expression

DC precursors and blood DC migrate into peripheral tissues, driven by their expression of CCR2 and CXCR4. Here they down regulate CCR2 and up regulate CCR6. Immature DC also express CCR1, CCR5 and CXCR2, allowing them to migrate towards areas of inflammation via increased levels of CCL3, CCL5, CXCL8 and CCL20, inducibly expressed in other tissues during an inflammatory response. Dendritic cell maturation down regulates CCR1, CCR5 and CCR6 and up regulates CCR7 and CCR8 expression, becoming sensitive to CCL1, CCL19 and CCL21, which are constitutively expressed in lymph nodes. If iDC encounter Ag in the absence of Inflammation, they partially up regulate CCR7 and constitutively migrate to LN to induce/maintain tolerance. Naive and central memory T cells also express CCR7, thus chemokine expression brings DC and T cells into close proximity for Ag presentation to occur (Gunn 2003, Vicari, et al 2004).
Gene expression, mRNA stability, receptor trafficking and desensitisation regulate the expression of chemokine receptors on the cell surface. Desensitisation may occur as the result of one of two mechanisms. Either, binding of the ligand to the receptor leads to internalisation of the ligand-receptor complex and the cell is refractory to subsequent internalisation, despite continued surface receptor expression. Alternatively, ligands bind to receptors and induce desensitisation of another receptor by transactivation. These processes are known respectively as homologous and heterologous desensitisation. As with the cytokine system, the chemokine system is further complicated by the expression of a number of atypical CCRs (decoy receptors) by the vascular and lymphoid endothelia. The D6 (Jamieson, et al 2005) and CCR11/ CCX CKR (personal communication R.J.B. Nibbs) receptors bind CCLs with high affinity, which are internalised and degraded. However, internalisation is not associated with detectable signalling, chemotaxis, receptor desensitisation or reduced re-circulation to the cell surface (Weber, et al 2004). In contrast, DARC (Duffy antigen receptor for chemokines) may act as a chemokine buffer by binding CCLs present at high concentrations, preventing leukocyte desensitisation, but maintaining a background homeostatic level (Comerford and Nibbs 2005).

Until recently, trafficking of matDC to lymph nodes was thought to be mediated via expression of CCR7 (Ohl, et al 2004). However, there is recent evidence from a transplant model using CCR7 deficient mice to suggest that CCR7 is not essential for graft rejection (Beckmann, et al 2004). Interestingly, other groups have suggested that CCR8 may also be important for matDC migration (Qu, et al 2004) and co-localisation of both effector and regulatory T cells (Sebastiani, et al 2002).

Inflammatory cytokines such as TNFα and IL-1β, and the Pathogen Associated Molecular Patterns (PAMPs), which signal through TLRs, are traditionally thought
of as the main agents responsible for inducing DC maturation. However, it is
becoming clear that chemokines may also influence DC maturation. It has been
shown that *T. Gondii* derived cyclophilin is able to induce IL-12 production by
has been suggested that CCL19 and CCL21 play a role in DC maturation as well
as migration (Marsland, et al 2005). In this study it was shown that the number of
migrating DC isolated from mice genetically deficient in CCL21 and CCL19 (plt/plt)
(Paucity of Lymph node T cell mutation) following Ag priming with FITC conjugated
OVA protein and an LPS maturation signal was significantly reduced. The
migrating DC expressed a partially mature phenotype, HLA class II was normally
up regulated, CD40 was slightly reduced, while expression of CD80 and 86 was
markedly reduced compared to wild type mice. Marsland et al also showed that if
CCL21 or CCL19 was produced in a eukaryotic system, rather than the normal
prokaryotic systems used by the majority of commercial suppliers, they were 100
times more potent at inducing DC migration. They also indicated that these forms
of CCL21 and CCL19 were able to induce DC maturation, i.e. increased
expression of CD86, CD80, CD40 and increased production of IL-1β and IL-12
(and TNFα in the case of CCL19). These results suggest that small glycosylation,
structural or conformational changes in CCLs may radically alter their in-vitro
potency, which may be crucial in the production of DC for clinical use.

Dendritic cells produce large numbers of different chemokines at the various
stages of maturation. For example, iDC are able to produce the inflammatory
chemokines IL8, CXCL10, CCL3, CCL4 and CCL5, which increases the
recruitment of neutrophils, monocytes and perhaps NK cells to sites of infection
and inflammation. Whilst matDC produce CCL17, CCL18, CCL19 and CCL22 to
attract T and B lymphocytes within the lymph node in a paracrine fashion.
As already discussed, MM induces a state of immunosuppression by the secretion of cytokines such as IL-6, IL-10 and TGFβ. It should therefore come as no surprise that tumours manipulate the chemokine system as well (Byrne and Halliday 2002, Vicari and Caux 2002). The migration of DC into tumours is usually driven by the expression of CCL3, CCL4, MCPs and CCL5, which also recruit monocytes and macrophages. Once inside the tumour, DC are presented with Ag in the form of apoptotic bodies, in the absence of a danger signal. In this type of environment, iDC are able to up regulate CCR7 expression, but not co-costimulatory molecules (Gallucci, et al 1999, Sauter, et al 2000). This effect is enhanced by the production of IL-10 and TGFβ by tumour infiltrating macrophages, which block DC maturation. Thus partially mature DC migrate to lymph nodes, deliver a tolerogenic signal to the immune system and induce the production of TH2 and T regs.

The effects of the chemokine system are not restricted to the immune system, with CCL3 and CCL4 playing predominant roles in driving the increased osteoclast activity that causes MM bone disease. CCL3 and CCL4 are present at higher concentrations in the serum and bone marrow of MM patients, and correlate with the severity of bone disease and prognosis, continuing to rise as the disease progresses (Abe, et al 2002, Choi, et al 2000, Hashimoto, et al 2004, Magrangeas, et al 2003, Terpos, et al 2003). Interestingly, CCR1 and CCR5 are also expressed by MM plasma cells, where they reportedly mediate cell survival, proliferation and migration (Lentzsch, et al 2003, Oyajobi, et al 2003), and induce MM cell adherence to BM stroma, cellular interactions that lead to the production of osteolytic factors by the stroma (Figure 1-5) (Michigami, et al 2000).

It is clear that if we hope to use the immune system to eradicate malignant diseases such as MM, a greater understanding will be needed about the influence of chemokines on normal DC development and trafficking, and how this can be
altered by malignant disease. The results thus far from cellular immunotherapy trials have been disappointing. One of the reasons for this is that mature DC administered in these types of studies fail to migrate to secondary lymphoid tissues in significant numbers (Blocklet, et al 2003, Triozzi, et al 2000). The chemokine system also plays a role in the clinical manifestations of MM, and interfering in these pathways may have significant therapeutic potential.

1.3.3 Antigen Cross Presentation.

CD8+ T cells require Ag to be presented by MHC class I molecules to elicit a CTL response. It was originally thought that only endogenous Ag processed in the cytoplasm of the Ag presenting cell (APC) could gain access to this process, whereas Ag from both endogenous and exogenous sources can be loaded onto MHC class II molecules. It was later found that DC could present Ag acquired from phagocytosed apoptotic bodies and present this in the context of HLA class I, thus generating CTL responses (Albert, et al 1998, Ronchetti, et al 1999). This process has become known as 'cross presentation', although the in-vivo phenomenon was originally described as 'cross priming' by Bevan over 20 years ago (Bevan 1976). Various mechanisms to explain how DC achieve this process have been proposed, including recycling of MHC class I molecules exchanging peptide for those being newly formed in the endosome, translocation of Ag from the cell surface to the cytosol and the passage of Ags from the endosome to the cytosol, which seem to be used mostly by DC (Moron, et al 2003). The Ags are then processed by the proteosome and transported to the endoplasmic reticulum (ER) by transporter associated with Ag processing (TAP) molecules where they can bind to MHC class I molecules. However, it has been suggested that rather than being released directly into the cytosol, the Ags may remain in the phagosome which may either fuse to, or carry elements of, the ER (Guemonprez, et al 2003).
The required TAP, proteosomes and MHC class I may then be recruited (Houde, et al 2003). Other groups have speculated that there may be a specific transport mechanism in DC, which efficiently transports phagocytosed Ag into the cytosol for processing (Rodriguez, et al 1999). Using mechanisms such as these, myeloid DC are able to induce strong CTL responses to exogenous (tumour) Ag.

1.3.4 Dendritic cell, T cell interactions

DC have the unique ability to present Ag to naïve T cells. Techniques such as 2-photon imaging of lymph nodes have shown that this process follows an orderly progression. Within two hours of DC migration T cell behaviour changes from a random stochastic nature, to making short-lived contacts with multiple DC dendrites. During the next 14 hours T cells form stable clusters around DC before breaking down into dynamic swarms, and by 24 hours, T cells proliferate and migrate out of the node (Miller, et al 2004). Elegant studies such as these provide further evidence that the 'immune synapse' is a dynamic, orderly process.

Effective priming of naïve T- cells induces clonal expansion and differentiation into effector and memory cells. This is achieved via the engagement of the T cell receptor (TCR)/HLA-I/II/Ag, CD40 / CD40 Ligand (CD40L) and CD28/CD80 complexes and the secretion of IL-7, IL-12 and perhaps IL-2 (Granucci, et al 2001). CD4⁺ T- cell help is required at the time of priming to generate CD8⁺ effector cells, and is mediated by CD40/CD40L engagement, which induces DC production of IL-12. This CD40 licensing of DC may also be induced by CD40L on some apoptotic bodies (Propato, et al 2001). The CD40/CD40L interaction seems particularly important in that CD40 ligation alone is sufficient to drive the maturation of iDC (Caux, et al 1994), and allows matDC to prime CD8⁺ cytotoxic T
cells (CTL) without further CD4+ T cell help. Of the many other factors important in the T cell response, the maturity of the DC is one of the most important.

Immature DC induce 'abortive proliferation' of T cells, i.e. initial proliferation but short-term survival and perhaps clonal deletion. Mature DC induce T cell survival and differentiation by displaying Ag in the context of the appropriate co-stimulatory molecules (Dhodapkar, et al 2001, Jonuleit, et al 2001b, Liu, et al 2001). Thus it is crucial that DC achieve a fully mature state if they are to be used as an anti-cancer therapy. This may also partly explain how the tumour is able to evade detection by the immune system at an early stage when novel tumour Ags may be encountered by APC in the absence of an inflammatory, pro-maturation environment (danger signal). Thus APC remain in an immature state and a tolerogenic response may be generated (Matzinger 1998).

1.3.5 How can DC be produced/obtained for clinical use?

Circulating blood mDC and pDC (Figure 1-4) can be collected using apheresis machines and enriched with magnetic cell separation protocols in a similar manner to CD34+ stem cells (Fearnley, et al 1997, Lopez, et al 2003). These cells are present at very low concentrations in peripheral blood (Dzionek, et al 2000, MacDonald, et al 2002) but the number of circulating DC can be dramatically increased using FLT-3 (Maraskovsky, et al 1996, Morse, et al 2000).

This is a relatively inexpensive and straightforward process, however, the yield of cells is still relatively modest and there is conflicting evidence relating to whether circulating DC are functionally normal in patients with MM (Brown, et al 2001, Ratta, et al 2002). Brown et al have previously reported that circulating DC from MM patients fail to up regulate CD80 and 86 in response to CD40L, which may be
corrected by co-culture with IL-12, IFN-γ or anti-TGF-β antibodies, suggesting that TGF-β1 and IL-10 are implicated in causing this defect (Brown, et al 2001).

Figure 1-4 Potential cell sources for the production DC intended for clinical use.

Alternatively, DC may be generated from either CD34+ HSC (34+DC) (Caux, et al 1992) or from monocytes (MoDC)(Zhu, et al 2000). 34+DC were first produced using a cytokine cocktail containing GM-CSF and TNFα (Reid, et al 1992, Santiago-Schwarz, et al 1992). Many groups have subsequently refined this by adding cytokines such as FLT-3, SCF and IL-4 (Evans, et al 2000, Strunk, et al 1996). The DC generated by these methods have a mature myeloid phenotype, low phagocytic activity, and express high levels of co-stimulatory molecules such as CD40, CD80, CD83 and CD86 (Ferlazzo, et al 2000). They are also able to induce an Ag specific cytotoxic response from naïve T cells (Caux, et al 1995).
Unfortunately, patients must first undergo HSC mobilisation with either G-CSF alone, or in combination with chemotherapy. Also, the methods by which 34^+ DC can be loaded with Ag, such as transfection or fusion with tumour cells, are not straightforward and are further discussed below.

DC may be generated from monocytes (MoDC) isolated from peripheral blood mononuclear cells (PBMC) separated by either plastic adherence or positive selection of CD14^+ cells using immunomagnetic beads. *In-vitro* culture of MoDC occurs classically in three stages. Immature DC are produced by culture with IL-4 and GM-CSF for 5 to 10 days. These cells are CD1a^+/CD1c^+/CD14^-/CD86^- and HLA-DR^+, and they have a high phagocytic capacity, which facilitates Ag uptake. A number of groups have substituted IL-13 for IL-4 at this stage to produce functional iDC which may have a more stable phenotype compared to those generated with IL-4 (Boyer, *et al* 1999, Morse, *et al* 1999a).

The next stage in the process is to load iDC with Ag. Normal iDC are phagocytic, so they can be simply ‘fed’ Ag as a purified naked form, coated with liposome, crude tumour lysate, tumour derived heat shock protein (HSP), whole apoptotic tumour cells, antibody coated tumour cells or genetically modified Ag (Mylovenge, idiotype-GM-CSF fusion, Dendreon.), as summarised in *Table 1-1* (Galea-Lauri, *et al* 2004).
<table>
<thead>
<tr>
<th>Type of Ag</th>
<th>Method of Ag loading</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour associated proteins e.g. MUC-1, Idiotype protein</td>
<td>Pinocytosis/Phagocytosis</td>
<td>Easy to monitor with tetramers Ubiquitous Ags</td>
<td>Relies on a single Ag for immune response. Some tumour Ags not processed efficiently, e.g. MUC-1</td>
</tr>
<tr>
<td>Tumour Lysate e.g. freeze/thaw, Heat or Sonication</td>
<td>Pinocytosis/Phagocytosis</td>
<td>All tumour Ags available. Simple preparation</td>
<td>May expose DC to immunologically active tumour molecules. Difficult to monitor Need to collect tumour cells at diagnosis from individual patients</td>
</tr>
<tr>
<td>Tumour derive Heat Shock Proteins e.g. HSP70 GP96</td>
<td>Receptor mediated</td>
<td>Ag Preloaded Specific mechanism for Ag uptake and processing</td>
<td>Complex Ag preparation for individual patients. Difficult to monitor Can MM plasma cells be converted to DC? Are tumour DC efficient APC? Tumour cell contamination Difficult to monitor</td>
</tr>
<tr>
<td>Tumour Derived DC</td>
<td>All tumour Ags available.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live tumour cells</td>
<td>Tumour cell/ DC fusion</td>
<td>All tumour Ags available.</td>
<td>Complex Ag preparation for individual patients. Fusion process toxic to DC. Tumour cell contamination Difficult to monitor</td>
</tr>
<tr>
<td>Tumour DNA/ RNA</td>
<td>Transfection</td>
<td>Ubiquitous Ags Easy to monitor with tetramers</td>
<td>Relies on a single Ag for immune response. Poor transfer efficiency Vectors and Tumour DNA may be toxic</td>
</tr>
</tbody>
</table>
However, there is some evidence that DC from patients with malignancy (CML and MM) have reduced phagocytic capacity due to cytoskeletal abnormalities, reduced processing capacity for exogenous Ag and reduced chemokine induced migration (Dong, et al 2003, Ratta, et al 2002). There are at least three approaches that may circumvent this problem. The first is the generation of DC from a malignant precursor cell. This is well characterised in myeloid malignancies such as acute and chronic myeloid leukaemia (AML and CML), and there have been recent reports of the generation of DC-like cells from patients with acute lymphoid leukaemia (ALL) (Blair, et al 2001). These cells appear to have the ability to stimulate naïve T cells, although whether they function as efficiently as normal DC is still open to debate. At present there is no evidence that MM plasma cells can be converted into more efficient APC.

The next approach is to generate normal matDC (maturation is discussed below) and then fuse them with tumour cells by either electroporation or pegylation (Gong, et al 2002, Hao, et al 2004, Raje, et al 2004). These approaches make the assumption that the resulting fusion or malignant DC retain normal function. Such cells must also express all of the relevant tumour Ags, in a state that the immune system is able to recognise as abnormal, so that a cytotoxic response is mounted rather than inducing tolerance. The final approach is to again generate normal matDC but then transfec them with tumour-associated Ags, such as MM idioype protein (Id) or NY-ESO-1 (van Rhee, et al 2005b) using viral vectors (i.e. adenovirus and lentivirus). It is possible to achieve high rates of transfection but these approaches have been hampered by viral effects on DC function and recent reports of leukaemic transformation in 2/9 boys undergoing gene therapy for X-linked severe combined immunodeficiency due to the activation of the oncogene...

Usually, iDC are converted into matDC after Ag loading, using various combinations of cytokines and inflammatory stimulants. Classically, tumour necrosis factor alpha (TNFα) (Thurnher, et al 1997) or monocyte conditioned medium (Romani, et al 1996) are used, but many other compounds and combinations are described including poly (I:C), CD40 Ligand, prostaglandin E2, IL-1β and IL-6 to name but a few. Mature DC up regulate surface expression of the co-stimulatory molecules CD80, CD83, CD40, HLA class I and II, lose their phagocytic ability, increase their migratory capacity to lymph nodes via increased CCR-7 and become much more efficient at inducing responses from naïve T cells (De Vries, et al 2003a, De Vries, et al 2003b, Tarte, et al 2000).  

The production of MoDC (and 34+DC) is costly, labour intensive and requires autologous cells to be generated for each individual patient. Recently groups have been looking at speeding up the process of producing matDC from 1-2 weeks down to 2 days (Fast DC) (Dauer, et al 2003, Xu, et al 2003), which may more closely represent what is happening physiologically during the inflammatory response and has the added benefits of being quicker and less labour intensive.  

Unfortunately, the MM tumour cells are actively producing cytokines (IL-6, IL-10, TGF-β and VEGF) that interfere with this process at many points, resulting in DC being produced in fewer numbers, that are functionally abnormal and fail to mature with appropriate stimulation (Brown, et al) (Figure 1-5). When functionally normal matDC are generated, it will be critical to choose the correct Ag(s) and adjuvants
with which to prime the immune response for clinical use. A number of groups have started clinical trials looking at the use of DC as treatment for MM.

![Image](image_url)

**Figure 1-5 Interaction of MM with the adaptive cellular immune system**

In MM there is a complex interaction between the malignant plasma cell, bone marrow stroma and the immune system. The tumour and stromal cells interact via adhesion and cytokines, such as IL-6, VEGF and TNFα to induce tumour cell proliferation, migration, survival and drug resistance, as well as blood vessel proliferation. IL-6 also blocks the conversion of monocytes into iDC, VEGF,IL-10 and TGFβ prevent DC maturation, and TGFβ also induces T cell anergy.

### 1.3.6 T regulatory cells ($T_{Reg}$ cells)

There is a growing body of evidence that as well as providing immunogenic 'help' to the immune system, CD4$^+$ T cell are also important negative regulators of the immune response. Many of these effects are mediated by a subset of cells known as naturally occurring CD4$^+$/CD25$^+$ regulatory T ($T_{Reg}$) cells, which compose 5-

TReg cells are produced in the thymus by positive selection of CD4+ cells with TCR that have intermediate affinity for MHC class II-self Ag complexes, and exert their immunomodulatory effects in-vitro in a cell contact dependent manner, although there is some in-vivo evidence that immuosuppressive cytokines may also be involved. TReg cells play a role in maternal tolerance to the foetus (Aluvihare, et al 2004), tolerance following transplantation (Wood and Sakaguchi 2003) and they have been implicated in impeding natural anti-tumour immunity and immunotherapy (Sakaguchi, et al 2001). One of the proposed mechanisms for these observations is the interaction between TReg cell CTLA-4 and iDC B7-1 and B7-2 which induces the up-regulation of indolemine 2,3-dioxygenase (IDO) on the DC (Mellor, et al 2003). These IDO-DC inhibit T cell proliferation, induce T- cell apoptosis (Munn, et al 2002) and large numbers of these cells have been found in the tumour-draining lymph nodes of patients with malignancies (Munn, et al 2002). The group from Dana Faber recently reported a significant increase in CD4+/CD25+ TReg in MM patient samples compared to normal donors (23±4% vs. 6±3%) (Prabhala, et al 2004). They found that the proliferation of T cells depleted of TReg cells was significantly lower in MGUS (n=9, SI=12±2) and MM (n=9, SI=28±8) compared with normal donors (n=9, SI=74±9, p<0.01), and LPS was unable to overcome suppression of T cell proliferation by TReg cells in MGUS (49%) and MM (24%) compared to normal controls (110%). These observations
suggest that T_{Reg} cells may play a role in the immune dysfunction seen in MM patients.

### 1.3.7 Natural killer (NK) cells

NK cells are part of the evolutionarily older innate immune system. In contrast to T cells, NK cells do not require pre-activation or immunisation in order to recognise and kill targets such as tumour cells or virally infected cells. NK cells arise from the haematopoietic stem cell (HSC) and develop under the influence of IL-2 and/or IL-15, acquiring effector functions such as lytic ability and cytokine production (IFN-γ, TNF, IL-10, IL-13, Lymphotoxin-β, and GM-CSF) (Williams, et al 1997). Mature NK cells (CD94+ and KIR+) seed to the periphery where they expand, are recruited to tumours or sites of infection (Salazar-Mather, et al 1998, Smyth, et al 2000), where they develop into either specialised cytokine producers (CD94^{+•}CD56^{++•}KIR^{+•}, CD25^{+•}, CCR-7^{+•} and c-KIT^{+•}) or cytotoxic cells (CD56^{+•}, KIR^{++•}, and CD94^{+•}).

NK cells recognise abnormal cells via a number of mechanisms, of which the “missing self hypothesis” is the best understood (Ljunggren and Karre 1990). Tumour and virus-infected cells may lose or down regulate MHC class I expression in an attempt to prevent Ag presentation and evade recognition by CD8^{+} cytotoxic T cells. NK cells detect this via surface receptors, which under normal circumstances engage the MHC class I molecules and deliver an inhibitory signal to prevent NK cells attacking normal healthy cells. There are at least two types of MHC class I inhibitory receptors which either recognise a highly conserved element in the HLA class I signal peptide bound by HLA-E molecules (CD94:NKG2A heterodimer) (Vilches and Parham 2002), or detect down regulation of particular sub-groups via killer Ig like receptors (KIR). KIR binding to
HLA class I molecules is similar to T cell receptor binding of HLA-Ag complexes, with faster binding and detachment kinetics which may facilitate efficient surveillance of the MHC class I repertoire.

More recently activating receptors have been identified on NK cells (Bauer, et al 1999, Diefenbach, et al 2002, Gilfillan, et al 2002). NKG2D recognises structures such as MHC class I chain-related molecule (MIC) A and B, expressed at a significant level when cells are stressed or become transformed, promoting the rejection of abnormal cells (Groh, et al 1996). This effect may be circumvented by abnormal cells producing soluble MIC, which induces down regulation of NKG2D (Groh, et al 1996). The ligands for the Natural Cytotoxicity Receptors (NKp46, NKp30 and NKp44) have yet to be identified, but the process appears to be MHC independent.

There are reports of natural NK cytotoxicity to normal (Ferlazzo, et al 2002) and auto Ag-pulsed DC in patients with MM (Zheng, et al 2002). Immature DC are efficiently targeted by NK cells whilst matDC are protected by the up-regulation HLA class I (HLA-E). The interaction between NK cells and DC at sites of inflammation is complex and was reviewed recently by Ferlazz and Münz (Ferlazzo and Munz 2004). DC are able to activate NK cells via IL-12 and perhaps IL-18 (Andrews, et al 2003) although there is some evidence that cell contact is required via engagement of NKG2D/MICA and B. During this interaction DC maturation is stimulated by IFN-α released by the NK cell. Why should NK cells kill iDC whilst at the same time inducing DC maturation and homing to lymph nodes? It has been suggested that this paradox is a control mechanism used during infective episodes. Both results of DC/NK cell interaction result in the depletion of
DC at sites of inflammation. While this may deprive DC trophic pathogens of their host, it may also be an important feedback mechanism to prevent excessive production of pro-inflammatory cytokines by limiting recruitment of iDC to the matDC pool (Moretta, et al 2003).

Myeloma cells are susceptible to NK cell lysis (Frohn, et al 2002) and there is evidence to suggest that the number and state of activation of NK cells is increased in MM. These cells have inherent anti-MM-cytotoxic activity and drugs such as thalidomide may further augment this effect (EL-Sherbiny, et al 2003, Frohn, et al 2002, Gonzalez, et al 1992, Zheng, et al 2002). These observations provide evidence that there is an active interface between the innate and adaptive arms of the immune response that could be harnessed in future anti-MM NK cell and DC based therapies.

As our understanding of how the immune system regulates itself improves, we should be better able to manipulate it to produce more effective immune therapies. The next section will review the use of immunotherapeutic strategies in the treatment of MM.

1.4 Anti-tumour vaccines in Multiple Myeloma

Vaccination is the most effective intervention modern medicine has developed, and has almost eradicated diseases such as Small Pox and Poliomyelitis. An ideal anti-tumour vaccine would be produced from a tumour-associated Ag (TAA) that is only expressed on tumour cells but is shared between different patients and
tumour types. It should be highly immunogenic, be able to produce both humoral and cellular immune responses and it should be essential for tumour cell survival, thus not susceptible to mutation or deletion. As yet, such ‘ideal’ TAA have yet to been identified in any malignant disease. In B cell malignancies, the only compounds that come close to this ideal are the Id proteins produced by the clone of tumour cells. Unfortunately, these molecules are specific to each individual patient, requiring the vaccine to be tailor-made for each patient and are thus labour intensive and very expensive to produce. Furthermore, Id are weakly immunogenic when administered in-vivo and, in the case of MM, expressed at low levels on the surface of the tumour cells. However, it has been shown that anti-Id antibody and T cells are present in the blood of MM patients (Yi, et al 1995), and experimental data shows that the anti-Id immune response is able to kill MM tumour cells in-vitro and in animal models (Li, et al 2000, Wen, et al 2001). A number of different strategies have been employed to produce an effective Id vaccine. These have employed the use of DNA, purified Id protein or light and heavy chain variable regions (VH and VL), which can then be linked to molecules such as KLH or cytokines like IL-2, IL-12 or GM-CSF in order to render them more immunogenic (King, et al 1998, Osterborg, et al 1998, Rasmussen, et al 2003, Stritzke, et al 2003). DNA acts as a natural vaccine adjuvant. Bacterial DNA is even more potent, due to the 20-fold increase in CpG motifs, recognised by molecules such as the Toll-like receptor 9 (TLR-9) present on cells of both the innate and adaptive immune system (Hemmi, et al 2000).

A number of studies in both MM and B cell lymphoma have demonstrated that it is possible to use these vaccines to boost the immune system but the clinical results so far have been largely disappointing, although most series have been too small
to produce statistically significant results. Coscia et al reported that despite inducing anti-Id antibodies and skin-prick sensitivity in MM patients in first CR following high dose chemotherapy, the residual tumour burden was not eliminated (Coscia, et al 2004). This is in contrast to the study reported by Bendandi, in which Id protein vaccination was able to clear circulating tumour cells in 8 of 11 patients who were otherwise in remission following chemotherapy for follicular lymphoma (FL) (Bendandi, et al 1999). The group in Southampton have developed a DNA fusion vaccine which contains the VH and VL genes of the Id protein assembled as a single-chain (Fv) sequence. To enhance T cell help, this has been fused to either the fragment C (FrC) sequence, a non-toxic part of the Clostridium tetani toxin (Spellerberg, et al 1997), or a plant viral protein coat (PVCP) sequence (Savelyeva, et al 2001). This scFv-FrC fusion gene vaccine has been used in clinical trials in patients with FL and MM (King, et al 1998). Antibody responses to FrC were seen in 8 of 10 FL patients treated, with 5 patients having detectable T cell responses to Id (Stevenson, et al 2004). The trial in MM patients is in its very early stages, but some immune and clinical responses have been reported (Stevenson, et al 2004). This platform continues to be modified in order to induce greater CD8+ mediated immunity by increasing Ag presentation via MHC class I. In a similar approach to Kwak et al (Kim, et al 2003), Stephenson is also conducting a clinical trial of vaccinating normal donors prior to collection of DLI for use in MM patients who relapse following Allo HSCT (Stevenson 2003).

A novel approach has been taken by Cell Genesys with the GVAX® myeloma vaccine. Irradiated, auto MM cells are administered with K562 cells, genetically engineered to produce GM-CSF. Twenty two patients have been enrolled in the phase I/II trial, and 17 patients have received at least one vaccination. Interim data
presented at ASH 2004 demonstrated that chemotherapy followed by auto HSCT and vaccination resulted in six complete responses, five partial responses, three patients with stable disease and two patients with progression (Borrello, et al 2004). Three patients with early progression after transplantation then demonstrated potential antitumor activity following initiation of vaccination, as measured by reductions in the myeloma-associated circulating protein (M-spike) of 92 percent, 37 percent and 25 percent. Treatment with GVAX® myeloma vaccine to date has been well tolerated, with only self-limiting skin rashes (2 patients) and colitis (1 patient) reported.

1.5 Dendritic Cell vaccination in Multiple Myeloma

Immunotherapy in MM may be more effective if it is delivered via professional Ag presenting cells such as DC. A number of studies have examined the use of DC that have been pulsed with tumour-derived Id protein or peptides (Dabadghao, et al 1998, Reichardt, et al 1999, Ridgway 2003, Tarte, et al 1997, Titzer, et al 2000, Zeis, et al 1998). It has been shown that moDC pulsed with purified patient-specific Id can serve as cellular vaccine for MM patients after high dose therapy and auto PBSCT (Lim and Bailey-Wood 1999, Liso, et al 2000, Reichardt, et al 1999, Yi, et al). In the study by the Stanford group, 26 patients were immunized with Id pulsed DC derived from auto monocytes under serum-free conditions and vaccines consisting of mature DCs (HLA-DR+/CD83+/CD80+/CD54+/CD86+, median number of 5x10^6 DC/injection) were administered without serious adverse events. Four patients have demonstrated Id-specific T cell proliferative responses and 2 patients demonstrated the induction of Id-specific T cell cytotoxicity (Liso, et al 2000, Reichardt, et al 1999). As all patients received Id/KLH boosters post-
vaccination, it is not surprising that 24 of 26 patients developed KLH-specific T cell responses after 2 to 3 Id/KLH booster injections. This protocol has been further developed in Tubingen, by the addition of SC GM-CSF, 250ug/m², as an adjuvant to vaccination. Two of twelve patients developed T cell proliferative response and one patient formed a T cell cytotoxic response (Reichardt, et al 2003).

Investigators from Arkansas have also modified this approach by giving Id/KLH loaded DC by weekly intranodal injection for 4 weeks, each followed by low dose SC IL-2 (5x10⁵ IU/injection) (Yi, et al). 7/8 patients developed T cell responses and all enhanced the anti-idiotypic B cell response. The serum paraprotein fell by 30% in one patient and five others continue to have stable disease with longer follow-up required. This group is also examining the use of MM tumour cells lysates as a source of Ag for DC priming (Szmania, et al, Wen, et al 2002) whilst Kim et al are investigating the use of in-vitro priming of allo donor T cells with tumour Id pulsed DC (Kim, et al 2003).

There is some evidence that the route of DC administration, as well as their state of maturation, may influence the ability of DC to migrate and induce an immune response. It has been shown than DC given by intra dermal injection (IDI) are three times more effective at migrating to lymph nodes than those given by subcutaneous injection (SCI), while matDC are eight times better than iDC) (Ridolfi, et al 2004). Intra tumour injection of DC may result in sequestration of DC (Feijoo, et al 2005) mediated by CXCL8.

Dendritic cell based Id vaccination of MM patients is feasible and can induce Id specific immune responses in MM patients. However, the clinical effectiveness of such vaccinations in MM still needs to be proven. These therapies may need to be
'boosted' with adjuvants or other tumour-specific peptides in conjunction with Id-pulsed DC. Such an approach has been taken by Dendreon with the Mylovenge DC vaccine, which uses Id protein fused with GM-CSF, and is currently in phase II trials (Rice and Hart 2002). On-going studies in this area will shed further light on the use of DCs as cellular vaccines but clearly this is an area worthy of further investigation.

1.6 T cells and reversal of tumour induced immune suppression

The effects of the tumour cell population and associated microenvironment in MM is well described (Cook and Campbell 1999). It has been known for many years that patients with MM are immuno-suppressed, being more prone to infections (Glenchur, et al 1959, Perri, et al 1981, Zinneman and Hall 1954), and MM is less responsive to normal T cells when given as DLI to treat relapse post allo HSCT compared to other diseases such as CML (Verdonck, et al 1998). This is at least partly due to MM tumour cells secreting immunologically active compounds such as IL-6, IL-10, VGEF and TGFß among many others. It has been shown that multiple myeloma cell lines and freshly isolated myeloma cells from patients produce excess TGF-ß, and that this agent is responsible, at least in part, for suppressing T cell responses against tumour cells (Campbell, et al 2001, Cook, et al 1999). Using the natural inhibitor to TGF-ß, latency associated peptide (LAP), the TGF-ß suppressive effect against T cells has been shown to be specific and mediated through the inhibition of IL-2 autocrine pathways in the T cells (Campbell, et al 2001, Cook, et al 1999). Ex-vivo activation with using anti-CD3 monoclonal antibody (MoAb) in the presence of exogenous IL-15 is able to
overcome this inhibition and, crucially, the IL-2 autocrine pathways are reinstated in T cells, rendering them resistant to further TGF-β suppression (Campbell, et al 2001). Such a strategy may be employed to reinstate T cell effector function in patients with MM, however we have demonstrated that these cells may be rendered cytokine dependant and fail to respond to Ag's presented by DC in the absence of IL-2/IL-15 (unpublished data).

Other groups have identified that the T cell Vβ TCR diversity in MM patients is severely skewed after auto HSCT (Mariani, et al 2001) and that there is a significant association between survival and lymphocyte recovery post auto HSCT. In the Xcellerate T cell trial (Vij, et al) T cells are activated with anti-CD3 and anti-CD28 MoAb coated beads and infused IV three days following stem cell infusion. Initial results suggest that this process partially corrects the skewing of the Vβ TCR repertoire and also rapidly corrects the lymphocyte count following auto HSCT. It has been previously shown that patients with MM who have less restriction of the T cell receptor Vβ repertoire have a better prognosis (Brown, et al 1997). At present there is little evidence that the cells manipulated by the Xcellerate process have any specific anti-tumour activity. However, the presence of expanded T- cell clones is associated with prolonged overall survival (Brown, et al 1997, Raitakari, et al 2003, Sze, et al 2003), and MM Ag specific T cells circulating in the peripheral blood of MM patients have been identified (Beckhove, et al 2003, van Rhee, et al 2005b, Yi, et al 1995). These cells could now be isolated from the peripheral blood of Myeloma patients using immunomagnetic or flowcytometric methods, expanded ex-vivo using some of the methods described above and re-infused into the patient.
It has been known for some time that immunosuppression with cyclophosphamide may increase the effectiveness of adoptively transferred anti-tumour T cells (Rosenberg, et al 1994b), and recently it has been shown that specific depletion of CD4⁺ CD25⁺ TR cells by anti-CD25 antibodies increases the efficiency of the anti-tumour immune response of tumour bearing animals, although the tumours are not completely rejected (Jones, et al 2002, Vela-Ojeda, et al 2005). It is possible to enhance this effect by using CD25 depletion along with matDC vaccination (Sutmuller, et al 2001).

1.7 Humoral immunotherapy

1.7.1 Monoclonal antibodies

Tumour-directed monoclonal antibody (MoAb) therapy has been the “holy grail” of many Haemato-oncologists since the 1970’s. With the development of compounds such as Rituximab (anti-CD20), Campath-1H (anti-CD52) and Myelotarg (anti-CD33), the potential has developed into a viable treatment for lymphoma and leukaemia. This form of immunotherapy is now beginning to be investigated in the context of MM. One major hurdle in this area has been the selection of a suitable surface Ag that would permit the generation of a MoAb with satisfactory specificity and sensitivity for the targeting of the malignant cell resulting in its destruction. Potential candidate molecules include CD38, CD138 (syndecan-1), CD54 (ICAM), CD40, VEGF (Yang, et al 2003a) and the un-clustered surface type II transmembrane glycoprotein, HM1.24. The use of anti-CD20 MoAb is limited by the fact that CD20 is expressed on <20% of fresh myeloma cells. Despite the success of anti-CD20 MoAb in follicular lymphoma (McLaughlin, et al 1998, Zinzani, et al), diffuse high-grade lymphoma (Coiffier, et al 1998) and
Waldenstrom's macroglobulinaemia (Dimopoulos, *et al* 2002, Gertz, *et al* 2003), to date the experience in myeloma is limited (Lim, *et al* 2004, Musto, *et al* 2003, Treon, *et al* 2001), although it may have a role against the MM 'stem cell' as discussed later.

The use of anti-CD38 MoAb in clinical trials to date has been limited though some clinical efficacy has been demonstrated (Ellis, *et al* 1995). Initial difficulties associated with human-anti-mouse antibodies (HAMA) have largely been temporised with the use of a humanised variant.

One advance in respect to improving efficacy is the engineering of MoAb as carriers of toxin genes. Workers in the Mayo Clinic have generated single-chain variable fragments (scFv) that represent the linkage of the carboxyl terminus of one variable region (IgH) with the amino terminus of the other (IgL), using nucleotides that encode a series of hydrophilic peptides which retain the original antibody specificity (Chen, *et al* 1995). The investigators have attached diphtheria toxin-A plasmid DNA to a scFv directed against CD38, which is internalised into the Ag-expressing cell by receptor-mediated endocytosis. Using this technology, *in-vitro* and animal studies have demonstrated cell suicide by expression of the diphtheria toxin-A and the investigators intend to directly target plasma cells using tissue-specific transcriptional regulatory elements (e.g. Ig heavy chain enhancer) to limit non-specific cell killing. The translation into clinical trials is eagerly awaited using this novel technology.
1.8 Immunologically active drugs

1.8.1 Thalidomide and its analogues

Thalidomide has been successfully used to treat patients with relapsed/resistant myeloma (Singhal, et al 1999). While thalidomide may act as an anti-angiogenic factor in myeloma, several other potential mechanisms of action have been proposed. Firstly, thalidomide may directly inhibit tumour cell growth and differentiation, mediated by reduced cell adhesion interactions and inhibition of cytokine secretion (e.g. TNF-α) or through free-radical generation and apoptosis, either directly or in response to drugs such as dexamethasone. Secondly, thalidomide may inhibit the activity of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF-2), which act as growth and survival factors for myeloma cells. Thirdly, thalidomide may act by promoting a Th1 T cell response resulting in the secretion of interferon-γ and IL-2. Whether, one or all of these mechanisms mediate the clinical effects of thalidomide in myeloma remains to be clarified. Several thalidomide analogues are in the early phases of clinical development aiming to either inhibit cytokines (phosphodiesterase 4 inhibitors) or induce immunomodulation (IMiDs), with fewer side-effects (Corral, et al 1999).

There is tremendous future potential in the use of thalidomide or its analogues and studies such as the MRC Myeloma IX aim to assess the efficacy of thalidomide in combination with induction chemotherapy and as a maintenance strategy following Auto HSCT.
1.8.2 Interleukin-2

IL-2 is a central regulator of immune responses mainly produced by activated CD$^+$ T cells. However, transformed T cells, B-cells, leukaemia cells, LAK cells (lymphokine-activated killer cells) NK-cells and DC may also secrete IL-2. IL-2 is a growth factor for all T cell subpopulations, inducing Ag-unspecific proliferation of T cells by inducing cell cycle progression in resting cells, and clonal expansion of activated T-lymphocytes. IL-2 also promotes the proliferation of activated NK cells and B-cells (but this requires the presence of additional factors, for example, IL-4).

The observation that leukocytes cultured in IL-2 were able to lyse tumour cells (Grimm, et al 1982, Lotze, et al 1981) ultimately led to IL-2 being administered to patients with melanoma, renal cell carcinoma and B-cell lymphoma (Lotze, et al 1986, Rosenberg, et al 1987, Rosenberg, et al 1985) with a number of complete and partial remissions being reported (Yang, et al 2003b). IL-2 has no direct effect on tumours, as high concentrations don't inhibit tumour cell growth in-vitro and the anti-tumour properties are mediated by the cellular immune system i.e. NK and T cells. Investigators such as Rosenberg have combined the use of immune effector cells (tumour infiltrating lymphocytes) and IL-2 in the search for more potent therapies (Dudley, et al 2002).

Intravenous IL-2 is a toxic therapy. The major side effects include hypotension, capillary leak syndrome, organ dysfunction and a treatment related mortality of 1.5%. This has prompted investigators to reduce the dose and change to the subcutaneous route of administration. Although there is debate as to whether this reduces the efficacy of IL-2 therapy (Ravaud, et al 1994, Yang, et al 2003b), Kiss et al have reported that low dose SC IL-2 (1Mu BiD for 1 week, 2Mu BiD for 3
weeks) induced a complete remission of relapsed Hodgkin's disease following sibling allo HSCT complicated by acute skin GVHD (Kiss, et al 2003). Whilst Slavin et al have reported long term remissions using in-vitro activation of DLI and SC IL-2 for the treatment of relapsed leukaemia following Allo HSCT that was unresponsive to DLI alone (Slavin, et al 1996).

These observations suggest that IL-2 may be a useful adjuvant to cellular immunotherapy of MM by providing continuing stimulation of the immune system following the administration of immune effector cells. IL-2 could also be given following allo HSCT in place of, or in addition to DLI to boost any immune mediated effect.

1.9 Efficacy measurements in immunotherapy trials

Progress in the understanding of the basic mechanisms involved in generating immune responses to tumour Ags has never been greater. However, there is a lack of accurate, reproducible and readily transferable measurements of efficacy to assess the immune responses generated in the context of tumour immunotherapy. The role of efficacy measurements in association with immunotherapy trials is fundamental to the full assessment of such novel strategies. It has been assumed that current treatment modalities are themselves curative in patients who achieve long-term disease-free survival. The role of an auto anti-tumour immune response remains to be clarified. It has been shown that it is an auto immune response to the residual disease that prevents relapse in acute leukaemias (Lowdell, et al 1999, Lowdell and Koh 2000, Lowdell, et al 1997) and the data suggests that the immune system has some impact on the disease course of MM patients. Knowing
whether there is an anti-tumour immune response, albeit inadequate, allows one to construct and test hypotheses about how it may be enhanced or induced when absent. Such hypotheses inevitably lead on to the design of appropriate assays of immune function during and after administration of the immunological intervention under trial.

Measurements of immune function have been notoriously unreliable and poorly reflect the true status of the patient. However, recent advances in measurements of cell activation, cytokine production and cell-mediated cytotoxicity have radically changed the reproducibility of such experiments. The identification of relevant peptides from tumour-associated Ags is allowing the construction of peptide/HLA tetramers, which can be used to enumerate and analyse the function of Ag-specific T cells (Howard, et al 1999). In all such assays it remains important that the true measure of a relevant immune response is not that the patient or animal model can be demonstrated to have responded to the vaccine but rather that the response is measurable against the primary tumour. This is plainly easier said than done. Nonetheless it should be an aim at the outset of research so that the primary tumour is collected and stored in an appropriate manner at presentation for use in later experiments. Clearly, robust, reproducible and relevant outcome measurements of immune function are essential to determine the efficacy of any immunotherapy intervention.

1.10 Combination immunotherapy

In view of the disappointing clinical response seen thus far in immunotherapy trials, a number of groups have looked at whether immune therapies may be more
effective if used in combination. Shimizu showed that immune responses to a tumour lysate-pulsed DC vaccine could be increased by the addition of IL-2 in a murine sarcoma model [146]. Yi et al have reported the use of SC low dose IL-2 five days following administration of idiotype-pulsed DC. Anti-idiotype T cell responses developed in four and B cell responses in all five patients, including a 50% reduction in M-protein in one patient (Shimizu, et al 1999, Yi, et al 2002). Whilst in a murine model, 81% of animals treated with Id vaccine, plus FLT-3 ligand (FLT-3L) and IL-2 survived more than 180 days compared to none given the individual therapies, and 27% and 41% given Id plus IL-2 or FLT-3L respectively (Zeis, et al 2002). Another approach has been taken by the group in South Carolina, who have combined Rituximab and low dose SC IL-2 in NHL. Responses correlated with NK cell numbers and the addition of IL-2 resulted in an increase in Ag dependant T cell cytotoxicity in responders (Gluck, et al). However, some of the most promising results to date have come from the NIH, where the combination of vaccination, adoptive transfer of T cells and IL-2 therapy resulted in some long term cures in a mouse melanoma model (Overwijk, et al 2003).

1.11 Discussion

The field of cancer immunotherapy stands at a threshold. There have been great advances in the understanding of the immune system and how it can be manipulated, and in a number of diseases, immune based therapies are beginning to realise their potential. The use of antibody therapy, such as Rituximab, in combination with conventional chemotherapy for the treatment of lymphoma has resulted in improved disease responses and is becoming standard practise. Even more impressive results have been achieved using cellular
immunotherapy for the treatment of post-transplant lymphoproliferative disorder (PTLD) (Bollard, et al 2003). PTLD is a complication of HSCT or solid organ transplantation, associated with regimes that contain antibodies such as anti-lymphocyte or anti-thymocyte globulin, which generate more profound cellular immunodeficiency than those that do not contain such antibodies. This may allow uncontrolled expansion of Epstein-Barr-virus (EBV)-infected B cells. These infected B cells express the full spectrum EBV latent Ags, which are well characterised and highly immunogenic, and there appears to be no additional immune suppression associated with the malignant clone. Adoptively transferred auto or HLA class I matched EBV-specific T cells are able to restore cellular immunity, eradicating the disease without the need for conventional chemotherapy. However, whilst we can consistently generate auto cellular and humoral anti-MM immune responses in-vitro and in-vivo, they have yet to be translated into effective clinical tools capable of inducing sustained disease responses. We have also yet to show that the anti-MM immune effects seen following allo HSCT can be separated from those due to major and minor histo-incompatibility. If immunotherapy of MM is to develop into an effective therapy, a number of crucial questions still need to be answered:

What are the most effective anti-MM Ag's, can they be used alone, or are they more effective in combination?

A number of new and old target Ags are currently under investigation. Of these molecules, Id protein is the most widely studied. We await with interest the results of studies, such as those from Southampton, which look at combining Id with adjuvant compounds to increase the potency of the anti-MM immune response. Newer molecules such as HM1.24 and NY-ESO-1 are in the early phases of
development for the treatment of MM (Chiriva-Internati, et al 2003, Rew, et al 2003). Most MM immunotherapeutic strategies are directed at mature MM cell Ags and will thus be effective against the bulk of the tumour cells, however, as with other targeted therapies such as Glivec in CML, this approach has the potential to leave these tumour stem cells unscathed, resulting in disease relapse (Elrick, et al 2005). MM tumour cells are classically thought of as mature plasma cells (CD45+ve/CD19+ve/CD56+ve/CD138+ve/CD38+ve), with a low proliferative index. However, as with other leukaemia’s, there may be a small population of ‘tumour stem cells’ (CD45+ve/CD19+ve/CD22+ve/CD138-ve) that retain the capacity to self-renew and proliferate (Matsui, et al 2004). These cells are able to repopulate nod/scid mice and if removed by purging with Rituximab, the MM fails to engraft (Jones 2003, Matsui, et al 2004). It may be a mistake to select MM Ags that may only be present on the non-self-renewing cells in a bid to get good clinical responses, when we need to direct our efforts at the cells that contribute to relapse, thus improving long term survival. Further studies of the putative MM stem cell are warranted. Examining their phenotypic and genotypic characteristics may identify novel immunotherapeutic targets. It remains to be seen whether a single Ag can effectively eradicate MM in-vitro. Approaches that combine multiple Ags, such as tumour lysate or apoptotic tumour cells, have the theoretical advantage of being less likely to induce the MM clone to delete the target Ag, a phenomenon known as Ag escape.

Can simple vaccination induce an effective anti-MM response or do we need to deliver the Ag(s) via professional APC generated ex-vivo?

At present, immunotherapy of MM is developing along two main themes, the more traditional vaccination based approach, and ex-vivo generated cellular therapies.
In the future it may be possible to combine these two approaches by pre-conditioning the site of vaccination with growth factors and chemokines in order to recruit APC. Efficient trafficking of these Ag loaded APC to LN could then be induced by vaccines containing adjuvants designed to promote maturation and migration of APC. This type of approach would avoid all the inherent costs and risks of having to collect cells and then ex-vivo generate Ag loaded, mature APC, for each individual patient. In addition, humoral as well as cellular immune responses may be induced.

**How does the MM tumour cell evade immune surveillance?**

The evidence to date indicates that the MM tumour cell is able to disable the immune system at multiple crucial points (Figure 1-5) We are beginning to understand this interplay between the malignant cell and the immune system of the tumour-bearing host. This is prompting researchers to devise strategies that overcome the immunosuppressive effects of the MM tumour cell, such as T cell pre-activation, in order to increase the effectiveness of immune therapy against the malignant clone.

**Can immunotherapies be effective when used alone, or are they best used in combination in the treatment of MM?**

In view of the hostile *in-vivo* environment induced by the MM tumour cell, and the disappointing clinical response seen thus far in immunotherapy trials in MM, it is our view that immune therapies may be more effective if used in combination. A number of groups are examining the effectiveness of combinations of immunotherapy, such as Id pulsed DC and IL-2, CTL and IL-2, and antibody therapy with IL-2. Another approach would be to combine a DC based vaccine to
prime the immune system, pre-stimulation of T cells to correct tumour induced anergy, followed up with low dose subcutaneous IL-2 to maintain the activation of the immune system. As previously discussed a number of groups are examining the use of combinations such as the adoptive transfer of Ag specific T cells and IL-2, and Ag-loaded DC and IL-2.

Until we can answer these questions, effective immunotherapy in myeloma will remain a possibility not a probability. The on-going basic and translational research is encouraging, but the proof of principal in the clinical arena remains to be demonstrated. The design of good clinical trials will be crucial, as is optimal immunological monitoring to measure what, if any, effect such strategies will have on the host’s immune system. As with other biological therapies in haematoncology, it is likely that these immune-based therapies will achieve their maximum effect when the tumour burden is reduced to a minimum. Thus, immune strategies against MM are likely to be useful adjuncts to conventional chemoradiotherapy and auto HSCT.

1.12 Aims

The following aims will be dealt with in turn, within the relevant Results Chapter.

1. To establish and validate that the BDCA assay can be used to measure mDC1, pDC, mDC2 and B lymphocytes in ND and MM patients.

2. To determine whether the number of circulating DC subsets are abnormal in MM patients, and whether they change during the course of the disease and therapy.
3. To establish the method DC production using mobilised monocytes from ND and MM patients.

4. To determine whether the cytokine combinations used during DC production influence the quality of the DC produced from ND and MM patients.

5. To determine whether pre-stimulation of T cells from MM patients alters their interaction with matDC, resulting in improved T cell proliferation and cytolytic activity.

6. To determine whether the Luminex system can detected differences in the level of cytokines produced during the DC/T cell interaction, in cells derived from ND and MM patients using different cytokine combinations.

7. To establish and validate the fluorescent chemokine uptake assay.

8. To determine the chemokine receptor activity on cells from ND and MM patients during MoDC development and maturation.

Each chapter begins with a short, focused introduction to the data and culminates in a brief discussion of the findings.
2. Materials and Methods

2.1 Ethical approval and informed consent

All primary human material was collected with the approval of the Local Research and Ethics Committee, North Glasgow University Hospitals Division. Written informed consent was obtained from each patient and donor prior to obtaining samples.

2.2 Cell handling and culture

2.2.1 Media

PBS/Alba; Phosphate buffered saline (PBS) (Sigma, Poole, Dorset UK) and 0.5% Alba (4.5% human albumin solution, SNBTS, Edinburgh, UK).

PBS/Alba/EDTA; PBS (Sigma) and 0.5% Alba and 2mM EDTA (Sigma).

TC medium; RPMI 1640 media (Sigma) supplemented with 5% human AB serum (SNBTS), 2% L-glutamine (Sigma) and 2% penicillin-streptomycin (Sigma).

DC medium; RPMI 1640 media (Sigma) supplemented with 2% human AB serum, 2% L-glutamine (Sigma) and 2% penicillin-streptomycin (Sigma).

Serum free (SF) medium: RPMI 1640 media (Sigma) supplemented with, 2% L-glutamine (Sigma) and 2% penicillin-streptomycin (Sigma).

Cryopreservation medium; 10% DMSO (Quest Biomedical, Solihull, UK)/Alba
DAMP thawing solution; calcium and magnesium free D-PBS (Sigma), 0.5% Alba, 2.5mM MgCl₂ (Sigma), 10U/ml pulmozyme (Roche Products Ltd, Welwyn Garden City, UK)

FACS-Fix solution; PBS, 1% paraformaldehyde (Sigma) and 0.5% Alba.

2.2.2 Cytokines and chemokines

All cytokines were purchased from PeproTec EC (London, UK), with the exception of IL-6 and GM-CSF (Stem Cell Technologies, Vancouver, Canada). All cytokines were re-constituted as per the manufacturers instructions, and stored at -80°C at a concentration of 1-10mg/ml

All chemokines used in these experiments were kind gifts from Dr R.J.B. Nibbs, Glasgow University.

2.2.3 Cell-lines

All cell culture work was performed in a laminar flow cabinet using aseptic technique. All tissue culture flasks and plates were purchased from Corning life Sciences (Koolhovenlaan, Netherlands).

MM cell lines were obtained from previously cryopreserved stocks at our institution or purchased from The European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, Wiltshire, UK). All MM cell lines were maintained in suspension culture in 15mls of TC medium, in 75cm² tissue culture flasks Cultures were maintained at 37°C in a 5% CO₂ atmosphere. On reaching an approximate cell density of 1x10⁶/ml, they were passaged and re-cultured at 3x10⁵/ml.
The HEK293 cell line, wild type or stably transfected to express either D6 or CCR7 (kind gifts from R.J.B. Nibbs, Glasgow University) were cultured at 37°C, 5% CO₂, in 75cm² tissue culture flasks, and DMEM (Sigma) supplemented with 10% FCS (Sigma), plus 1% streptomycin and penicillin (Sigma), and 4mM glutamine (Sigma). The cells were adherent and had a doubling time of 24-36 hours. Cells were passaged when they became confluent on the bottom of the flask. The supernatant was removed by gentle pipetting and the cells were washed twice with PBS. Five ml of 0.25% trypsin was added to each flask, which was gently agitated until the cells lifted off the bottom of the flask. Twenty ml of DMEM, 10% FCS was added and the cells recovered by centrifugation at 1200 rpm, washed twice and plated out at a 1:10 dilution. The transfected cells were normally maintained in selection culture, but this was not necessary for short-term culture, i.e. less than 7 days.

2.2.4 Harvesting cells collected by apheresis

Normal donors and MM patients had stem cells mobilised for therapeutic use as per the normal referring unit policy. Usually, ND received G-CSF 10μg/kg by subcutaneous injection (SCI) for 5 days, and MM patients were conditioned with cyclophosphamide 1-6gm/m² on day 0, commenced G-CSF 5-10μg/kg SCI on day 4, and collected around day +10, when the peripheral blood CD34⁺ cell count was greater than 20 cells/μl. All subjects had cells collected using COBE® Spectra™ continuous flow Apheresis System (Gambro BCT, Lakewood, Co, USA) and the standard stem cell collection program by staff of the Apheresis Unit, Glasgow Royal Infirmary. The cells for therapeutic use were collected into a bag, which was then heat sealed and removed for processing. The tubing on the collection side of the collection side of the closed circuit was heat sealed and removed from the machine and processed within 4 hours of collection.
All cell manipulations were performed in a laminar flow cabinet using aseptic technique. The collection tubing was flushed with 20mls of TC medium and the cells were collected into a 50ml sterile conical tube (Greiner Bio-One, Longwood, Florida, USA). After gentle mixing by inversion, the cell suspension was carefully layered on top of 3mls of Histopaque®-1077 (Sigma) in 15ml test tubes (Greiner), and spun at 1700rpm for 20mins (density gradient separation). The serum was the pipetted off mononuclear cell layer leaving 0.5cm above the interface, which was then carefully removed and placed in a fresh 15ml tube. The cells were washed three times in fresh TC medium, the first spin was at 1000rpm for 10mins to also remove any excess Histopaque and the next two spins were at 800 rpm for 10mins to leave platelets suspended in the supernatant. The supernatant was removed by careful pipetting and the cells were resuspended by gentle flicking of the tube at 5x10^6/ml TC medium and cultured overnight to rest, at 37°C, in 75cm^2 tissue culture flasks, in preparation for cell sorting.

2.2.5 Red cell Lysis

Red blood cells in peripheral blood mononuclear cell (PBMC) preparations were lysed with ammonium chloride solution (0.083%, Sigma) at 37°C for ten minutes and repeated if required. Cells were washed in PBS and recovered by centrifugation at 1000rpm for 10mins and resuspended in medium or PBS as required.

Red blood cells in whole blood (WB) preparations were lysed with sterile, deionised water for 15secs (at a ratio of 1:10 WB to H2O), the cells were immediately washed in PBS/Alba (1:4, water to PBS) and the whole procedure repeated once. Cells were then suspended in PBS/Alba or appropriate tissue culture medium as required.
2.2.6 Cell counting and assessment of viability

Clinical full blood counts were performed on a Coulter Gen-S analyser (Beckman Coulter, Fullerton, CA, USA) in Glasgow Royal Infirmary and Sysmex SE9500 analyser (Sysmex, Kobe, Japan) at the Western Infirmary, Glasgow. Both analysers use a sample size between 200 and 250μl to produce a full blood count and 5 part differential. Both laboratories participate in the United Kingdom National External Quality Assessment Service (NEQUAS) surveillance scheme for quality assurance.

All other cell counts were performed manually with a haemocytometer and viability was assessed by Trypan Blue (Sigma) exclusion. Briefly, 90μl of 1:10 Trypan Blue was added to 10μl of cell suspension and 10μl was then transferred to a haemocytometer (i.e. cells were diluted 1:10) Blue stained cells were counted as non-viable, and the mean number of unstained cells counted in 5 large squares, multiplied by 10 (the dilution factor), was taken as the viable cell count X10⁴/ml.

2.2.7 Cryopreservation of cells

All cells that required cryopreservation were washed once in cold Alba and resuspended at a concentration of not more than 2x10⁷/ml in DMSO/Alba on ice. A 1ml aliquot of cell suspension was then transferred into 1.5ml Nunc Cryotubes (Nunc A/S, Roskilde, Denmark) and the cells were placed in a polystyrene box surrounded by polystyrene packing beads. The box was then cooled at -20°C for 1 hour and -80°C overnight before the cryotubes were transferred to liquid nitrogen (LN) for storage.
2.2.8 Recovering cells from LN$_2$ cryopreservation

Extreme care was taken to ensure good cell recovery, especially when thawing un-sorted PBMC from apheresis collections, which contain high numbers of granulocytes. On removal from LN, cryotubes were immediately placed in a 37°C water bath until all ice crystals had melted. The cell suspension was the gently transferred into a 15ml test tube and DMP thawing solution was added slowly, drop by drop initially, with constant gentle agitation on ice until 14mls of DMP had been added. The tube was then placed in a 37°C water bath for 10mins to facilitate the action of the enzyme. Cells were then spun at 1000rpm for 10mins, the supernatant removed by pipetting, resuspended in DMP and incubated at 37°C for a further 5mins. Cells were then washed twice in TC medium, counted and checked for viability.

2.2.9 Preparation of Ag

2.2.9.1 Tumour Lysate
Tumour cell lysates were prepared by harvesting cells from culture, and placing them in 15ml test tubes. The cells were recovered by centrifugation at 1200rpm for 10mins, the supernatant discarded and the cells resuspended in 1ml of TC medium. The cells were then counted, and the cell suspension snap frozen at -80°C for 15mins. The cell preparation was rapidly thawed in a 37°C water bath, vigorously vortexed for 2mins, and the process of freezing, thawing and vortexing was repeated 3 times to ensure all cell were disrupted. TC medium was added to adjust the final cell concentration to 1x10$^7$/ml and the freeze/thaw preparation was then stored at -80°C for future use.
2.2.9.2 Induction of tumour cell apoptosis

Tumour cell lines were suspended at 1x10^5/ml in SFM, and placed in a 75cm² culture flask in 15ml aliquots. The cells were exposed to a Philips SLPF15E ultraviolet light source for 30mins and cultured overnight at 37°C to allow them to enter cell cycle and undergo apoptosis. Apoptosis was confirmed using Annexin-V and 7AAD (Viaprobe) staining, and analysed by flow cytometry (as described in 2.3.7). The rates of apoptosis were compared in cells that had been irradiated and cultured overnight in SF medium and TC medium, with un-irradiated cells cultured under the same conditions, Figure 2-1. This confirmed that UV irradiation and overnight culture in SF medium (mean 40.5 ± 3.7, p<0.01) were the optimum conditions to induce apoptosis in U266 cells, as compared to serum free culture (mean 23.7 ± 2.5). UV irradiation alone (mean 12.8 ± 1.8, was not significantly different from the untreated control (mean 13.5 ± 1.6).

Figure 2-1 Apoptosis in irradiated and un-irradiated cells cultured overnight in SF medium and TC medium

The proportion of U266 cells undergoing apoptosis was compared in cells that had been irradiated and cultured overnight in SF medium or TC medium, with un-irradiated cells cultured under the same conditions. Apoptosis was measured using Annexin-V and 7AAD (Viaprobe) staining, and analysed by flow cytometry. Assays were performed in triplicate.
2.2.10 Selection of CD14\(^+\) monocytes and CD3\(^+\) T cells

To obtain monocytes and T cells with a purity of >90\%, cells collected by apheresis were sorted using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). All sorts were performed as per the manufacturers instructions using the LS size column, allowing the selection of up to 1x10\(^8\) positively labelled cells from up to a total of 1x10\(^9\) cells. Cells were placed into 2 15ml test tubes, washed once in cold PBS/EDTA/Alba and resuspended in 70\(\mu\)l of cold PBS/EDTA/Alba per 10\(^7\) cells. We labelled one tube with CD14 microbeads to isolate monocytes, and the other with CD3 microbeads for T cells, both at 20\(\mu\)l/10\(^7\) cells and incubated at 4\(^\circ\)C for 15mins under slow continuous mixing. Cells were washed in cold PBS/EDTA/Alba, passed through a pre-wetted 30\(\mu\)m filter and LS column in a midi-MACS magnet (all Miltenyi Biotec). The column was washed through with 3x3ml of PBS/EDTA/Alba and removed from the magnet. The positively selected cells were then eluted off the column in 5ml of PBS/EDTA/Alba with the supplied plunger. The cells were then counted and checked for viability and an aliquot of 5x10\(^5\) was retained for flow cytometry.

2.2.11 MoDC production

Monocytes, purified from PBMC by CD14 magnetic bead separation, were suspended in DC medium at a density of 5x10\(^6\)/ml and placed in 75cm\(^2\) tissue culture flasks. The DC medium was then supplemented with GM-CSF 50ng/ml (Stem Cell Technologies) and either IL-4 30ng/ml (Peprotech) or IL-13 50ng/ml (Peprotech). Cells were cultured for 4 days at 37\(^\circ\)C and 5% CO\(_2\), counted, checked for viability, and the concentration of cells was adjusted to 1x10\(^6\)/ml by the addition of DC medium supplemented with the appropriate cytokines. The Ag preparation, either apoptotic or freeze-thawed U266 cells suspended at 1x10\(^7\)/ml, was added to
a final ratio of 1 iDC to 1 tumour cell (equivalent to an approximate protein concentration of 1mg/ml) (Thumann, et al 2003). To induce iDC Ag loading and maturation, either the monocyte conditioned medium mimic (MIMIC) combination of TNF-α (10ng/ml) (Peprotech), IL-1β (10ng/ml) (Peprotech), IL-6 (10ng/ml) (Stem Cell Technologies) and PGE₂ (10⁻⁷M) (Sigma) (Feuerstein, et al 2000), or the novel in-house combination X4, TNF-α (10ng/ml), PGE₂ (10⁻⁷M), Poly (I:C) (10ng/ml) (Sigma) and CD40L (250ng/ml) (Peprotech), and the Ag preparation were added at the same time (Thumann, et al 2003), The cells were cultured overnight at 37°C with tissue culture flasks standing up-right to facilitate cell contact and Ag phagocytosis. The antigen-loaded matDC were harvested the following morning, washed in warm TC medium and resuspended in 1ml of TC medium. The mat DC were counted, assessed for viability, and resuspended at a concentration of 1x10⁶/ml. An aliquot of 1x10⁶ matDC was cryopreserved for use in the second DC stimulation step of cytotoxic T cell production.

2.2.12 T cell pre-stimulation with T cell activation beads

T cell activation beads (a kind gift, Dr John Campbell, Miltenyi Biotec), are 3.5μm inert, magnetic beads (2x10⁹/ml) conjugated to anti-biotin monoclonal antibody. This facilitates the beads being loaded with biotinylated molecules, in our case anti-CD2 (100μg/ml), anti-CD3 (100μg/ml) and anti-CD28 (100μg/ml). This was done in accordance with the manufacturer's protocol. Briefly, 500μl of beads, 100μl of biotin-anti-CD2, 100μl of biotin-anti-CD3, 100μl of biotin-anti-CD28, and 200μl PBS/EDTA/Alba were placed in a 1.5ml Eppendorf tube. This mixture was then mixed thoroughly by vortexing and incubated for 2hrs, at 4°C, under constant slow rotation (50-100rpm). This mixture is stable for 4wks at 4°C. Prior to use, the required number of beads was removed, placed in x10 volume of T cell culture medium in an Eppendorf tube, vortex and spun at 1200rpm for 5mins to remove
the excess antibody. The beads were recovered by pipetting off the supernatant and resuspended in 200μl of TC media ready for use.

The manufacturer suggests that when using beads alone to activate T cells, they should be used in the range of 1:1 to 1:2, beads to T cells, in order to avoid activation induce cell death. As the beads were used in conjunction with cytokine, they were added to the T cells (1-5x10^6/ml) at a ratio of 1:4, in 6 well tissue culture plates. The T cell/bead mixture was supplemented with IL-2 (20IU/ml) (MM and ND cells) or IL-15 (15ng/ml) (MM cells only) and incubated at 37°C for 3 days.

T cell activation beads were removed from the T cells as per the manufacturers instructions. Briefly, the T cell/bead culture mix was placed in a 15ml tube and washed in 15mls of cold PBS/EDTA/Alba, recovering the cells by centrifugation at 1200rpm for 5 mins. The supernatant was then completely removed, the cells suspended at a maximum concentration of 2x10^7/ml and placed in the field of a Dynal magnet (Dynal AS, Oslo, Norway) for 2 mins. The supernatant, which contains the cells, was then removed and placed in a fresh tube with the original tube still in the magnetic field to retain the beads against the sidewall of the tube. The original tube was then removed from the magnet, the 2ml of buffer was added to it, vortexed and the bead removal procedure was repeated to optimise cell recovery. The cells were then washed in TC medium, counted, checked for viability, and resuspended at 1x10^6/ml in TC medium ready for use in cytotoxic T cell generation experiments. Cell for use in T cell proliferation assays were cultured overnight in 6 well tissue culture plates to rest.

2.2.13 Cytotoxic T cell generation

Dendritic cells and either un-stimulated or pre-stimulated T cells were co-cultured for 3 days in TC medium at a ratio of 1 DC (1x10^5/ml) to 10 TC (1x10^6/ml) in 24
in 24 well tissue culture plates, with an aliquot of DC cryopreserved for use later in the experiment. After 3 days 50% of the media was removed and replaced with an equal volume of TC media supplemented with IL-2 (40IU/ml). After 7 days, the cells were transferred into 12 or 6 well plates, the aliquot of cryopreserved Ag loaded matDC was thawed, washed, and added to the T cell/DC co-cultures in an equal volume of TC medium. The cultures were fed IL-2 supplemented TC medium, final concentration of 20IU/ml, every 3-4 days until sufficient numbers of T cells were present to perform a cytotoxicity assay, between 3 and 6 weeks.

2.3 Flow cytometry techniques

2.3.1 Antibody labelling

All fluorescently labelled antibodies were purchased from BD/Pharmingen (BD Biosciences, San Jose, CA, USA), with the exception of CD11C-FITC (DakoCytomation Norden A/S, Glostrup, Denmark). All antibodies were titrated to determine the optimal concentration for staining, and pre-loaded into the 75mm Falcon tubes (BD Biosciences). Cells were suspended in PBS/EDTA/Alba at a concentration of 5x10⁵ – 1x10⁶/ml and 100μl was added to each tube. T cells were incubated at 4°C for 30mins in the dark. DC and monocytes were incubated in the dark at room temperature (RT) for 15mins to avoid temperature related down regulation of chemokine receptors. Cells were then washed twice in PBS/EDTA/Alba, the cells recovered by spinning at 1000rpm for 10mins and resuspended in 200μl of PBS/EDTA/Alba for analysis. All samples were analysed within an hour of antibody staining or fixed with 500μl of FACS-Fix solution, stored in the dark at 4°C, and analysed within 3days.
2.3.2 Cell staining

Tumour cells to be used as targets for phagocytosis and cytotoxicity assays were labelled with the PKH26 (red) and PKH67 (green) fluorescent membrane dyes (Sigma) as per the manufacturers instructions. Cells were washed twice in unsupplemented RPMI 1640 to remove any serum, $1 \times 10^7$ cells were suspended in 1ml of diluent C and 2µls of dye was diluted in 1ml of diluent C in a 15ml test tube. The cell suspension was added to the dye and incubated at RT for 5mins. Excess dye was bound by adding 2mls of neat AB serum, incubating for 2mins at room temp and then adding 11mls TC medium. Cells were then washed twice in TC medium and resuspended in TC medium at the concentration required for the assay.

2.3.3 Flow cytometric analysis

All flow cytometric analysis was performed on a FACScaliber analyser using Cell Quest Pro software (both Becton Dickinson). The cell populations to be analysed were initially gated on using their forward scatter (size) and side scatter (granularity) characteristics with the detectors set on a linear scale. The fluorescence detectors (FL1-4) were set with a Log scale. To ensure the accuracy and reproducibility of the flow data, great care was taken to optimise the cytometer for each experiment. The level of background auto-fluorescence, non-specific staining and day-to-day drift in laser intensity and alignment was compensated for on each experiment. An aliquot of cells was stained with an isotype control antibody for each of the fluorochrome colours to be used and the detector sensitivity was adjusted so that the level of fluorescence fell within the first decade. Cells were deemed positive if their fluorescence level was greater than $10^1$. The spectral overlap of fluorochromes was compensated for by staining an aliquot of
cells with a single positive antibody for the fluorochrome to be measured in FL1, FL2, FL3 or FL4 and with the isotype control antibodies for the other colours to be used in the experiment. The level of the compensation control was set to ensure that cells stained with an antibody measured in each channel, e.g. FL1, only appeared in the positive region of the analysis for that channel, FL1, and no other. This procedure was repeated for each colour of fluorochrome used in the experiment.

Data was analysed using Cell Quest Pro or FlowJo (Tree Star Inc, Ashland, Oregon, USA) software.

2.3.4 Mean fluorescence intensity (MFI)

The MFI of a population of cells was calculated by subtracting the mean fluorescence of the negative control from the mean fluorescence of the target population.

When a blocking was added to an assay, as in 6.1.3, the effect of this was quantified using the equation below.

\[
\frac{(\text{Positive control MFI} - \text{Blocked MFI}) \times 100}{\text{Positive control MFI}} = \% \text{ MFI Blocking}
\]

Equation 2-1 Percentage blocking of MFI

2.3.5 Chemokine uptake assays

This methodology is based on that previously optimised by collaborators I. Comerford and Dr R.J.B. Nibbs (Glasgow University). Biotinylated chemokines were synthesised by Albachem (Gladsmuir, East Lothian), with each molecule carrying an N-terminal biotinylated lysine residue. Other than this residue, Bio-
CCL19 was identical to human CCL19 provided by other commercial suppliers.

The Bio-CCL3 was based on a mutated version of mouse CCL3 that does not aggregate beyond a dimer, and is described elsewhere (Weber, et al 2004). Cells were harvested, resuspended at 5x10^5/ml and allowed to warm to 37°C. For each sample to be tested, 10µl of the following mix was prepared in Falcon tubes and incubated in the dark at RT for 30-45 minutes: For CCL3 receptors, 2.5µl of 0.05mg/ml Biotin CCL3, 3µl streptavidin PE (strep-PE) (Invitrogen, Paisley, UK) and 4.5µl PBS. For CCL19 receptors, 5µl of 0.05mg/ml Biotin CCL19, 3µl strep-PE and 2µl PBS. Control mix, 3µl strep-PE and 7µl PBS. Aliquots of cells (2-500µl) were added to the biotinylated chemokine/Streptavidin-PE mix or strep-PE only control, and incubated at 37°C for 2 hrs with regular gentle agitation. Cells were then washed with ice-cold flow cytometry buffer, to stop the reaction. The cells were retrieved by centrifugation (1200 rpm, 5 min, 4°C), resuspended in 400 µl PBS/EDTA/Alba, and analysed immediately by flow cytometry.

2.3.6 CCL1 surface staining

The CCL1 (I-309) Fluorokine kit, (R and D systems, Minneapolis, MN, USA.) was used to examine the expression of CCR8 on monocytes and DC. Cells were suspended in cold PBS at 1x10^6/ml. An aliquot of cells (100µl) was added to 2 Falcon tubes and 20µl of biotinylated CCL1 (2.5ug/ml) or negative control was added. The samples were incubated for 1 hour at 4°C and then 20µl of avidin-FITC was added and incubated for a further 30mins in the dark at 4°C. Samples were then washed twice in 1x RDF1 buffer and analysed by flow cytometry.

To test the specificity of the staining, 20µl of Bio-CCL1 was incubated with 40µl of anti-human CCL1 blocking antibody, prior to the addition of the cells. The assay was then completed as normal. A competitive binding assay was also performed
by pre-blocking 100μl of test cells with 20μl of 25ug/ml non-biotinylated CCL1 (Peprotech) for 1 hour. The assay was then completed as normal.

**2.3.7 Cytotoxicity assays**

Target cells, U266 MM cell line, were prepared the day before setting up the assay. Debris and dead cells were removed by density gradient centrifugation. The cells were then stained with PKH 26 and cultured overnight in fresh TC medium. The target cells were then washed in fresh warm medium and suspended at 1x10⁶/ml. The T cells were washed in warm TC medium, counted and set up at 8x10⁶/ml and 10 doubling dilutions were set up in Falcon tubes, final volume 200μl. An equal volume of target cells was then added to produce the effector:target cell ratios 8:1, 4:1, 2:1, 1:1 etc. Assays were performed in duplicate. Target cells alone and TC alone were set up to act as negative and gating controls. The tubes were then capped and cultured over night at 37°C. Culturing PKH-26 stained target cells in serum free media after exposure to UV light for 30mins provided positive controls for Annexin-V and 7AAD staining.

The following morning, the tubes were centrifuged at 1000rpm for 10 minutes, the supernatants were removed, and cells resuspended by gentle agitation. We then added 5μl of Annexin-V-FITC (Pharmingen), 10μl of 7AAD (Pharmingen), and 100μl of Annexin-V binding buffer to each tube and incubated for 15mins at RT. The reaction was stopped by adding 400μl of Annexin-V binding buffer to each tube and the assays were read by flow cytometry within 1 hour. A total of 20,000 target cell events were collected per tube and the results for each dilution were expressed as the mean number of dead test cells minus the number of dead cells in the Target alone control.
2.4 T cell proliferation assays

All proliferation assays were set up in 96 well tissue culture plates, with 100μl of T cells (1x10⁶/ml) added to quadruplicate (quad) sets of wells. 100μl of TC medium supplemented with IL-2 (40IU/ml) was then added to each test well. Negative controls were set up by adding 100μl of TC medium to a quad set of T cells. The plates were incubated at 37°C for 3 days, 10 μL of TC media containing a dose of 0.25 μCi ³H-thymidine (Amersham Biosciences, Chalfont St. Giles, UK) was added per well for the final 18 hours. The plates were harvested using a 96 well cell harvester (Micromate 196, Packard, Pangbourne, UK) onto a filtermat, which was dried and read on a beta counter (Matrix 96 Direct Beta Counter, Packard) for 5 minutes. The results were expressed as the mean number of counts of each test quad minus the mean of the control quad.

T cell/matDC proliferation assays were performed by setting up two test quads containing 100μl of T cells (1x10⁶/ml) and 100μl of matDC (1x10⁵/ml). One test set was supplemented with IL-2, final concentration 20IU/ml. Negative controls of matDC and T cells were performed along with a positive control of T cells plus 20IU/ml IL-2 on each occasion. All reactions were performed in 200μl. The assays were then completed as above, and the results were expressed as the mean of each test quad minus the mean of T cell only and DC only quads.

2.5 Luminex assays

A multiplex kit was used to measure GM-CSF, IFN-α, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12p70, IL-15, TNF-α, and VGEF (Biosource International Inc, Camarillo CA, USA.). The assay was performed as per the manufacturers instructions and read on a Luminex 100™ instrument. The working concentrations of all reagents were prepared as follows; a working wash solution (WWS) was prepared by diluting
15mls of x20 wash solution concentrate in 285ml of deionised water. The assay diluent was diluted in an equal volume of TC medium (AD). The 11 detector antibody x10 concentrates were combined and diluted in the biotin diluent, final volume 2.5ml. The Strep-PE x10 concentrate was diluted in 9 parts of Strep-PE diluent, total volume 10ml, for use within 15mins of reconstitution. The capture bead x10 concentrates were combined and diluted in the volume working wash solution, total volume 10mls.

A 96 well filter plate (Millipore, Billerica, MA, USA.) was pre-wet with 200μl of WWS for 30secs and then aspirated with the vacuum manifold (VM) (Millipore) at 5psi. All work was then performed with the plate on the VM, having first released the pressure. The bead solution was vortexed and 25μl was added to each well. The plate was shaded from light from this point onward to prevent bleaching of the fluorochromes. The beads were washed twice with 200μl of WWS (the residual liquid on the underside of the plate was removed after every wash step by blotting with clean paper towels) and 50μl of incubation buffer was added to each well. The standard curve was set up by reconstituting the standard in 150μl of AD, serially diluting this in 300μl of AD (1:3), and adding 100μl of each standard dilution in duplicate to the appropriate wells. Sample (50μl) and AD (50μl) were then added to each sample well in duplicate. The plate was then incubated for 2hours at RT on an orbital shaker (500-600 rpm). The assay liquid was aspirated with the VM, the plate was washed twice with WWS. Biotinylated detector antibody (100μl) was added to each well and incubated for 1hr at RT on an orbital shaker. The assay liquid was aspirated with the VM, the plate was washed twice with WWS. Streptavidin-PE (100μl) was added to each well and incubated for 1hr at RT on an orbital shaker. The assay liquid was aspirated and the plate was washed three
Working wash solution (100μl) was added to each well, placed on the orbital shaker for 2mins and read on a Luminex 100™ instrument. The concentrations of the standard curve samples were determined using the curve fitting software, with a four-parameter algorithm and multiplied by 2 to correct for the 1:2 dilutions. The software then calculated the concentrations of each analyte in each well.

2.6 Molecular techniques

2.6.1 RT-PCR

Cells stored at −80°C as a concentrated cell pellet (5x10⁵) were thawed, and 1ml of TRIzol® added. Following incubation at RT for 5mins, 200μl of chloroform (Sigma) was added, shaken vigorously for 15secs and incubated at RT for 3mins. The samples were microfuged at 12000g and 4°C for 5mins and the supernatant transferred carefully into a fresh Eppendorf tube. The RNA was precipitated by adding 0.5ml of isopropyl alcohol per 1ml original volume of TRIzol®, mixing and incubating at RT for 10mins, and centrifuging at 12,000g for 10mins at 4°C. The supernatant was discarded and the RNA pelleted, washed in 70% alcohol (to an equal volume to the original volume of TRIzol®, and dried. The dried RNA was re-dissolved in 100μl of RNase free water (Sigma). The samples were then treated with DNase1 (DNA free, Ambion, Huntington, UK) according to the manufacturers instructions. The quantity of nucleic acids was analysed spectrophotometrically using an Eppendorf Biophotometer according to the manufacturer's instructions and the sample was diluted in RNase free water to a final concentration of 0.15ug/ml.
The Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used to synthesise cDNA according to the manufacturer's instructions, using 1μl of RNA template in a 20μl reaction.

Aliquots of cDNA (2μl) were subjected to PCR using Reddy Mix PCR tubes (Abgene, Epsom, UK), which contain all PCR components, except primers and Template. The following primer pairs were added to a final concentration of 10ng/μl. Forward and reverse primers for CCR5 (AGATATTTCTCTGGCTCCCAGT and TGGCCTGAATAATTGCAGTA, CCR7 (AACTTGACCGCCGATGAAG and TACCTTGTCACTCATCCGCA) and D6 (CTGGAGTCGCTACGTAGATCC and CTCTCATGCTCAGCCCTAC, all MWG Biotec, Ebersberg, Germany) were added to each reaction for each sample. Samples were incubated at 92°C for 2mins to denature template, then a repeating cycle for 40 cycles of 92°C for 30 secs, 54°C for 30secs, and 70°C for 3mins. The final cycle was 70°C for 10 mins, after which reactions were chilled at 4°C.

The products of the PCR reaction were analysed by conventional gel electrophoresis, using a 1% agarose, 1x TAE (40mMol Tris-acetate, 1mMol EDTA), 1μg/ml Ethidium Bromide gel, to allow UV visualisation of DNA. The gel was placed in 1x TAE buffer, and the samples were electrophoresed at 100mA for 2 hours.

2.7 Statistical analysis

All statistical tests were two sided. Comparisons between paired groups of normally distributed continuous variables were performed using the paired student T-test. Unpaired groups of normally distributed continuous variables were compared using the student T-test. The Mann-Whitney rank sum test was used to compare groups of non-parametric continuous variables. Comparisons of two
groups of repeated measure data (i.e. cytotoxicity experiments) were performed using a two way repeated measures analysis of variance (2 way ANOVA).

2.8 Funding

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3. Results 1

3.1 Enumeration of blood dendritic cells in multiple myeloma patients at presentation and through therapy

3.1.1 Introduction

Although DC play a central role in the adaptive and, as is becoming clear, the innate immune system, they are difficult to quantify in-vivo. This is because iDC reside in the peripheral tissues where they constantly sample the environment. When iDC encounter antigen and a second maturation signal, they then traffic to the secondary lymphoid tissues under the influence of chemokines such as ELC and SLC in order to present antigen to other immune cells such as T lymphocytes. It has been known for some time that there is a small number of DC present in the peripheral blood and they represent a precursor population of conventional myeloid and plasmacytoid DC (Reid, et al 1990). Precursors of the two main DC populations that have been identified in the peripheral blood are becoming known as the pre-myeloid blood DC (blood mDC) and pre-plasmacytoid blood DC (blood pDC), although an increasing amount of plasticity in the DC system has been described recently (Zuniga, et al 2004). Blood DC are classically identified in the peripheral blood by a complex flow cytometry analysis that involve the lineage exclusion of the common blood cell types (myeloid, lymphoid, stem cells and NK cells) and the positive identification of blood mDC using CD11c, with blood pDC expressing CD123 in the absence of CD11c. These experiments are complex and time consuming to optimise because they involve analysis of forward scatter/side scatter characteristics combined with a large number of antibodies (CD3, 19, 20, 33, 34, 14, 16 and 56), as well as being relatively expensive. A number of new antibody staining strategies have been proposed to simplify the identification of blood DC. The CMRF-44 antibody was derived by Hart's group (Hock, et al 1994),
but DC require overnight incubation before they express the relevant antigen. This strategy may be flawed for a number of reasons; firstly it does not directly identify blood mDC, rather an intermediate stage between blood mDC and iDC that is dependant upon maturation in culture. Secondly, in diseases such as MM, DC have problems developing in the normal fashion, thus they may fail to up-regulate surface markers that are dependant upon DC maturation for their expression. Finally, the CMRF-44 antigen is not expressed by blood pDC. More recently, Dzionek described novel antigens that are present on blood DC subsets (Dzionek, et al 2000). BDCA-2 and BDCA-4 are expressed only on CD11c⁺/CD123⁺ blood pDC. BDCA-3 is expressed at high levels on a small population of CD11c⁺/CD123⁻ blood mDC (Blood MDC2), but at low levels on blood pDC, blood mDC, monocytes and granulocytes. CD1c (BDCA-1) is expressed on blood mDC as well as on CD19⁺ B lymphocytes. This degree of co-expression requires the granulocytes, monocytes and B lymphocytes to be gated out of the analysis.

As mentioned earlier, the MM clone is known to affect blood mDC was discussed earlier. There is still some debate as to whether blood mDC in MM patients are present in normal numbers (Brown, et al 2001) or are reduced in number (Ratta, et al 2002). Some of these discrepancies may be explained by the fact that these studies have used different antibodies to identify the blood mDC populations. The study by Brown et al used the CMRF-44 antibody to identify blood mDC, while Ratta employed the more traditional lineage exclusion method. However, there is agreement that blood mDC in MM patients do not function normally. In contrast, little has been published on what happens to circulating blood pDC in MM. Plasmacytoid DC are the main natural IFN-α producing cells and they are involved in regulating both the adaptive and innate immune responses (McKenna, et al 2005), with both immunogenic and tolerogenic effects. Mature pDC seem to amplify mDC responses by initiating DC differentiation from monocytes, promoting
DC maturation and polarising the T cell response to a Th1 phenotype through the secretion of IFN-α and OX40L expression. Plasmacytoid DC also interact with the innate immune system by recruiting and activating NK cells, thus playing a vital role in anti-viral immunity. There has been increasing interest in the part these cells play in the immune response to malignancies (Mohty, et al 2005, Salio, et al 2003). The only study which has reported blood pDC numbers in MM, suggested that they were reduced (Ratta, et al 2002). It is known that in diseases such as HIV/AIDS, the number of both blood blood mDC and blood pDC are reduced (Pacanowski, et al 2001), whilst in infections such as Dengue, a rise in blood pDC seems to modify the severity of the disease (Pichyangkul, et al 2003). The mechanisms by which pDC induce tolerance are poorly understood, and they are not just dependant upon the state of pDC maturation. Thus by preventing pDC development and maturation, tumours such as MM could have a negative impact on both the adaptive and innate immune systems.

The initial aim of our study was to examine the numbers of circulating blood myeloid and plasmacytoid DC subsets in patients with MM as compared to normal individuals. In addition, we wanted to look at how the DC numbers and proportions may change in response to conventional anti-MM chemotherapy, following autologous and allogeneic HSCT, and whether the immunomodulatory drug, thalidomide induced any significant changes in the blood DC compartment. Our secondary aims were to determine whether we could reliably measure a third, recently identified and poorly studied blood DC subset, blood mDC2, in both normal subjects and MM patients, and if we could use this assay to simultaneously measure the CD19 positive B lymphocytes.
3.1.2 Measurement of circulating blood DC by flow-cytometry using the BDCA 1, 2 and 3 antibodies in normal individuals

The normal control population was selected from normal individuals to reflect the mean age of patients with MM in trials (controls 50.2yrs, MM 55-65yrs) and M=F (controls 10:11). The BDCA antibody kit (Miltenyi Biotec) allows the rapid identification of circulating blood DC directly from fresh whole blood collected into a standard EDTA tube, as used for full blood counts in routine clinical practice.

The assays were performed on WB, anti-coagulated with EDTA, within 24hrs of sampling and stored at 4°C if required. The antibody staining was performed on two 150ul aliquots of WB in 12x75 mm Falcon tubes. The test sample was stained with 10ul of BDCA antibody cocktail and the control sample with 10ul of control cocktail. Each sample had 5ul of dead cell discriminator added. Both samples were mixed gently but thoroughly and then incubated for 10 minutes, in a horizontal position on ice, under a 60w white light bulb in order to activate the dead cell discriminator staining. Red cells were lysed twice with sterile water, the sample was resuspended in 300ul of buffer and then analysed by flow cytometry, after having run the appropriate negative and positive compensation controls for channels FL1-4. If the samples could not be analysed immediately, 2.5ul of discriminator stop solution (Deoxyribonucleic acid in 10 mM NaCl, 1 mM EDTA, pH 8) and 150 ul of FACSfix solution was added and the samples analysed within 12 hours.

The control antibody cocktail contains mouse IgG1-FITC, IgG2a-PE and IgG1-APC monoclonal antibodies and CD14-PE-CY5 (mouse IgG2a) CD-19-PE-CY5 (mouse IgG1). This allows the neutrophils, B-lymphocytes and monocytes to be gated out of the target population on an FL3 vs. side scatter plot (Figure 3-1). The control cocktail is also used to set the background level of non-specific staining in
the channels used for measurement of blood pDC (FL1), blood mDC1 (FL2) and blood mDC2 (FL4). The test antibody cocktail contains the same CD14 and CD19 exclusion antibodies, along with the antibodies to identify blood mDC1, blood pDC and blood mDC2, CD1c-PE (BDCA-1)(mouse IgG2a), BDCA-2-FITC (mouse IgG1) and BDCA-3-APC (mouse IgG1) respectively. Dead cells are excluded from the analysis by the addition of a fluorescent photolytic dye to both the control and test samples.

<table>
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<th>Cell Type</th>
</tr>
</thead>
<tbody>
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<td>All live Cells</td>
</tr>
<tr>
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<td>R2</td>
<td>Non B cell, Non monocyte, Non neutrophils</td>
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</tr>
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</tr>
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<tr>
<td>G7</td>
<td>R1+R6</td>
<td>B lymphocytes</td>
</tr>
</tbody>
</table>

Table 3-1 Logic gates for Blood DC analysis
Figure 3-1 Flow-cytometry plots required for the measurement of circulating blood DC subsets in a normal individual.

In plot A, all cellular events are gated (R1) in a forward side (linear) versus scatter (linear) plot composed of all acquired events. Plot B shows CD19 positive B lymphocytes, CD14 positive monocytes dead cells and neutrophils being excluded on an FL3 (log) vs. side scatter plot of all events. Plots C-F show only events in R1 and R2. Plot C shows the isotype controls for FL2 (log) (blood mDC1) and FL1 (log) (blood pDC). Plot D shows CD1c PE positive blood mDC1 in R3 and BDCA2 FITC positive blood pDC in R4. Plot E shows the negative control for blood mDC2 on an FL4 (log) versus FL2. Plot F shows blood mDC2 (R5), which are high expressers of BDCA3 (FL4) versus blood pDC (FL1, BDCA2) which are intermediate expressers of BDCA.

Absolute number of DC's of each DC subset per ml of blood = \( \frac{\text{\% Gated sample} - \text{\% Gated control}}{100} \times \text{WCC} \)

Equation 3-1 Determination of the number of monocytes and blood DC subsets per ml of peripheral blood.
Figure 3-2 Total WCC and circulating B lymphocyte numbers in normal subjects n=21.

The white blood cell count for normal subjects was measured using a Coulter GenS or Sysmex SE9500 haematology laboratory analyser, on the same sample on the same day as the blood DC assay was performed. The B cell population was estimated using the BDCA kit, gating on the CD19 positive cells (G7 = R1 + R2 +R6) and then the absolute number was calculated using Equation 3-1.

Figure 3-2 shows the results of the white blood cell counts (WBCC) and B cell numbers for normal subjects (n=21). The WBCC were performed on the same sample, on the same day as the blood DC assay was performed. The mean value was 6.75 ± 1.67 x10⁶/ml, well within the reference range for both laboratories (4-11 x10⁶/ml). The B cell population was estimated using the BDCA kit, gating on the CD19 positive cells (G7 = R1 + R2 +R6) and then the absolute number was calculated using Equation 3-1, mean 3.35 ± 1.32 x10⁵/ml, again, well within quoted normal ranges (0.6-6.6 x10⁵/ml, HMDS, Leeds. 2.0-6.0 x10⁵/ml, GRI, Glasgow.), validating the use of this method to accurately reflect the number of B cells in the peripheral blood.

The number of each blood DC subset detected in normal individuals is shown in Figure 3-3. The proportion of each blood DC subset out of the total cell population
was measured using the BDCA antibody kit and the gating strategy outlined in Table 3-1. The absolute number of each blood DC subset was calculated using Equation 3-1. The mean number of blood mDC1 was $2.35 \pm 1.37 \times 10^4$/ml, blood pDC $1.65 \pm 0.74 \times 10^4$/ml and blood mDC2 $1.21 \times 10^3$/ml $+0.86 \times 10^3$/ml. These results are comparable with the manufacturers reference range (blood mDC1 1.56-3.28 $\times 10^4$/ml, blood pDC 0.45-2.04 $\times 10^4$/ml and blood mDC2 0-2.16 $\times 10^3$/ml).

![Figure 3-3 Number of DC per ml in the blood of normal subjects n=21](image)

Each blood DC subset was analysed by flow cytometry using the BDCA antibody kit. Blood mDC1 were measured in G4 (R1+R2+R3), blood pDC in G5 (R1+R2+R4) and blood mDC2 in G6 (R1+R2+R4), estimating each population as a percentage of the total cells present. The absolute number of each blood DC subset was calculated using Equation 3-1.

### 3.1.3 Measurement of circulating blood DC by flow-cytometry using the BDCA 1, 2 and 3 antibodies in patients with multiple myeloma

Ethical approval for this study was obtained from the North Glasgow University Trust Hospital (East) ethics committee and all samples were taken with the patients having given prior written informed consent. Samples were obtained from
patients with MM attending the MM clinics at the Western Infirmary, Glasgow and Glasgow Royal Infirmary. Samples were stored and analysed as previously described. Samples were obtained at the time of diagnosis prior to the start of chemotherapy (n=7). As a second control group, samples were also obtained from patients with a plasmacytoma and a detectable paraprotein following radiotherapy or patients with monoclonal gammopathy of uncertain significance (MGUS) (n=12). These diseases are also characterised by the presence of a paraprotein in the peripheral blood, and MGUS is regarded as pre-malignant condition which occurs in 1% of the over 50s and 3% of the over 70's (Kyle and Rajkumar 2003). In contrast to MM, there is much less immunosuppression associated with these disorders such as an increase incidence of infection, reduction in the normal levels of immunoglobulins (immune paresis) or cellular immune defects, but around 1% of patients with MGUS progress to MM each year. Representative flow cytometry plots are shown in Figure 3-4. The number of circulating B lymphocytes in normal subjects vs MM patients at diagnosis and MGUS/Plasmacytoma patients was quantified using the BDCA antibody kit, Figure 3-5.

B lymphocytes were measured in G7, and the number of cells per ml of blood was calculated using Equation 3-1. The number of circulating B lymphocytes in MM patients at diagnosis (mean 0.9 ± 0.3 x10^5 /ml, n=7) was significantly lower (p<0.001) than in the blood of normal subjects (mean 5.2 ± 0.4 x10^5 /ml, n=21). The number of circulating B lymphocytes in MGUS and plasmacytoma patients was also significantly lower (p<0.005) than normal subjects. The clone used for marking B cells is the LT19 (Mason, et al 2002) which is a commonly used for phenotyping normal and malignant B lymphocytes and is not positive on normal or MM plasma cells.
Figure 3-4 Flow cytometry plots from a patient with MM at diagnosis

Blood DC subsets can be readily identified in MM patient samples using BDCA antibodies. Plot D shows blood mDC in R3, blood pDC in R4. In plot F, blood mDC2 present in R5 are identified by their high expression of BDCA 2. Of particular note is the marked reduction in circulating CD19 +ve B lymphocytes seen in plot B.

Figure 3-5 Circulating B cell numbers in normal subjects vs MM patients at diagnosis and MGUS/Plasmacytoma patients

Circulating B lymphocytes were quantified using flow cytometry and the BDCA antibody kit. B lymphocytes were measured in G7, and the absolute number of cells per ml of blood was calculated using Equation 3-1.
Blood DC subsets were quantified in samples from normal subjects, MM patients at diagnosis and patients with MGUS or plasmacytomas using flow cytometry and the BDCA antibody kit, Figure 3-6. The number of blood mDC1 (mean 1.065 ± 0.35 x10^4/ml) and blood pDC (mean 0.454 ± 0.10 x10^4/ml) detected in the blood of MM patients at diagnosis was significantly lower than that seen in normal subjects (p<0.03 and p<0.001 respectively). The number of blood mDC2 in MM patients (mean 0.5 ± 0.016 x10^3/ml) and the number of all DC subsets observed in the blood of patients with MGUS/plasmacytoma, blood mDC1 (mean 1.9650 ± 0.74 x10^4/ml), blood pDC (mean 1.1533± 0.32 x10^4/ml) and blood mDC2 (mean 1.04 ± 0.3 x10^3/ml), was not significantly different from normal subjects.

![Figure 3-6 Circulating DC subsets in MM patients at diagnosis and MGUS/plasmacytoma vs. normal subjects](image)

The numbers of blood mDC1, blood pDC and mDC2 were quantified in samples from normal subjects, MM patients at diagnosis and patients with MGUS or plasmacytomas using flow cytometry and the BDCA antibody kit. Blood mDC1 were measured in G4 (R1+R2+R3), blood pDC in G5 (R1+R2+R4) and blood mDC2 in G6 (R1+R2+R4). The absolute number of each blood DC subset was calculated using Equation 3-1.
3.1.4 Analysis of blood DC subsets in MM patients throughout the course of the disease.

We have shown above that at diagnosis the number of blood DC in MM patients is reduced. During the treatment of their disease, MM patients receive chemotherapy, usually on a monthly basis, until their paraprotein levels stabilise, i.e. plateau phase. Further conventional therapy at this stage has no additional benefit to the patient in terms of length of plateau phase or survival. In order to ascertain whether there was any recovery in the numbers of blood DC during plateau phase, samples were obtained from patients who were in stable plateau phase following induction chemotherapy (n=21). These results were compared with those results obtained at the time of diagnosis (n=7). Samples were also analysed from patients who had relapsed (n=12), prior to commencing rescue therapy. When malignant diseases such as MM relapse, the malignant clone will often gain further mutated genes, which provide it with a further survival advantage. These additional genetic abnormalities may render the clone more resistant to chemotherapeutic agents by a number of mechanisms. These protective mutations include the up regulation of genes that protect the cell from apoptosis, i.e. BCL-2, or proteins that actively pump drugs out of cells, such as MDR1. The tumour burden at the time of relapse is also rapidly increasing. This may suppress normal cell production by simple marrow infiltration, or, as may be more important in MM, by increased production of suppressive cytokines such as IL-6, IL-10, TGF-β and VEGF. These analyses were performed to determine whether any additional suppression of the blood DC system was associated with the relapse phase of the disease and the results are shown in Figure 3-7. No statistically significant increase in the number of B cells or any DC subset was noted between the time of diagnosis (n=7) and the attainment of plateau phase following chemotherapy (n=21). At the time of relapse there was a further significant fall in the number of blood mDC1 compared with diagnosis (p≤ 0.05).
and plateau phase ($p \leq 0.005$). There was an even more striking decrease in the number of blood pDC at the time of relapse compared with diagnosis ($p \leq 0.05$) and plateau phase ($p \leq 0.001$). The number of B cells did not fall further at relapse.

Figure 3-7 Circulating DC subsets in MM patients at strategic points in the disease course

The number of B cells, blood mDC1, blood pDC and mDC2 were quantified in samples from MM patients at diagnosis ($n=7$), in plateau phase following chemotherapy ($n=21$) and at the time of relapse, using flow cytometry and the BDCA antibody kit. The results were compared with those obtained from ND.
3.1.5 Blood DC subsets in MM patients following autologous and allogeneic HSCT.

Stem cell mobilisation and autologous HSCT as consolidation therapy for MM patients who are in plateau following chemotherapy has become the standard of care for patients below the age of 70 years who are otherwise fit enough to tolerate the therapy. This therapy is associated with an over all improvement in response rate, EFS and OS as compared to conventional therapy alone. Patients who have a further significant reduction in their disease burden, i.e. reduction in the level of the paraprotein, have significant improvements in time to progression, EFS and OS (Child, et al 2003, Harousseau and Attal 2002, Harousseau, et al 1995).

These studies were designed to assess two important aspects of the immune response to this therapy. Firstly, is this treatment associated with an improvement in the number of circulating DC subsets as compared with diagnosis or following conventional chemotherapy? Secondly, how rapidly do the DC subsets recover following autologous HSCT?

A small number of patients are suitable to undergo allo HSCT, because of their age (i.e. <55 years) and the availability of a suitable donor (HLA matched sibling or volunteer unrelated donor). This treatment represents the only chance of cure in patients with MM via the GVM effect, but it is associated with significant risks, as discussed in chapter 1. By examining blood DC subsets in individuals who have undergone allo HSCT, we hoped to see whether this therapy was associated with any significant differences in blood DC levels as compared with auto HSCT, which could be attributed to the immune nature of the therapy. This picture may be complicated by the fact that the treatment protocols for allo HSCT may contain antibodies such as Campath1-H as part of the conditioning regimen for non-
myeloablative (NM) and VUD allo HSCT, and immunosuppressive drugs, such as cyclosporin as prophylaxis or treatment for GVHD after myeloablative allo HSCT.

Samples were obtained pre and post auto and allo HSCT from in-patients in the bone marrow transplant unit in Glasgow Royal Infirmary and the haematology ward in Gartnavel General Hospital, Glasgow. Samples were analysed from patients undergoing auto HSCT (n=11) pre-transplant, at the time of engraftment, D100 and 1 year post transplant. Samples from MM patients undergoing allo HSCT (n=3 NM) were analysed at D100. This time point was chosen because by this time, the majority of DC will be of donor origin. There is some evidence that autologous DC are present for some time following allo HSCT, and may contribute to the development of GVHD (Cowing and Gilmore 1992, Duffner, et al 2004). There is no data as to whether blood DC are of donor or host origin at the time of engraftment, but there is one report that blood DC are >99% donor at day 60 (Mohty, et al 2002a). This report also observed that the blood DC subsets in patients undergoing NM HSCT peaked at day 60 with a significant decline noted at day 90 post HSCT in seven patients.

The data illustrated in Figure 3-8 shows that following autologous HSCT, blood mDC1 show a rise back into the normal range (mean 1.52 ± 0.28 x10^4 /ml) (p>0.05), which is maintained at D100 (mean 1.55 ± 0.24 x10^4 /ml), but by one year the numbers (mean 0.98 ± 0.36 x10^4 /ml) are back to levels seen at diagnosis (mean 1.07 ± 0.35 x10^4 /ml). The numbers of blood pDC show a rise at the time of engraftment, although not back into the normal range, but then decline at D100 and one year. The numbers of blood mDC2 show a similar trend to blood mDC1. At D100 following NM allo HSCT, the numbers of blood mDC1 (0.4600 ± 0.04 x10^4 /ml), blood pDC (0.31 ± 0.11 x10^4 /ml) and blood mDC2 (0.350 ± 0.07 x10^3 /ml)
were reduced as compared to normal, MM at diagnosis and post treatment, but due to the small sample size these data are difficult to interpret.

Figure 3-8 Circulating blood DC subsets in MM patients following auto and NM allo HSCT

Using flow cytometry and the BDCA antibody kit, we quantified the number of blood mDC1, blood pDC and mDC2 were in samples from MM patients undergoing autologous HSCT (n=11) and NM allo HSCT (n=3). The auto group were analysed pre-conditioning, at the time of engraftment and at D100 following stem cell re-infusion. Samples from the NM allo HSCT group were analysed at D100 to ensure that DC were of donor origin. The results were compared with those obtained from at MM patients at diagnosis (n=7), following chemotherapy (n=21), at the time of relapse (n=12), and ND (n=21).
3.1.6 Blood DC subsets in MM patients following therapy for relapse with thalidomide and bortezomib.

At the time of relapse, a number of treatment options are available. Thalidomide is increasingly one the first line drugs to be used to treat relapsed MM patients, either alone or in combination with steroids and conventional chemotherapeutic agents such as cyclophosphamide. The mode of action of thalidomide is complex and is not fully understood. It’s anti-MM properties include inhibition of angiogenesis, induction of MM plasma cell apoptosis in combination with steroids, prevention of adhesion and cytokine signaling and enhancement of the TH1 immune response. By measuring blood DC subsets in patients taking this drug we hoped to gain a further insight into the immunostimulatory properties of thalidomide, and whether they may be mediated in some way by the DC system.

Samples were obtained from patients at the time of relapse (n=12) and when they were taking more than 100 mg of thalidomide for 1-3 months (n=12), the normal starting dose being 50mg, which is then usually increased to 100mg after 2-4 weeks if the patient is able to tolerate the therapy.

A small number of samples were obtained from patients with refractory MM who were entered into the bortezomib (Velcade®) phase IIIB clinical study (n=5). Velcade is a proteosome inhibitor that has been shown to be active against MM in earlier studies (Richardson, et al 2003) and has recently been granted a licence for use in MM patients at the time of relapse. There is currently no data that bortezomib has any direct effects on the DC system, although there is a report that bortezomib mediated cancer cell apoptosis preferentially induces DC maturation (Demaria, et al 2005).

There was a significant increase in the number of blood mDC1 (mean 1.1247 ± 0.2122 x10⁴/ml) in patients who were taking more than 100mg of thalidomide for 1-
3 months (n=12) as compared to patients at the time of relapse (n=12) (p<0.005),

**Figure 3-9.** There was no significant change in the number of blood pDC (mean $0.3271 \pm 0.0808 \times 10^4$/ml) and blood mDC2 (0.78 ± 0.16 x10³/ml) in patients taking thalidomide. The patients in the bortezomib study (n=5) showed no change in the number of blood mDC1 (mean $0.5856 \pm 0.3490 \times 10^4$/ml) and blood pDC (mean $0.8274 \pm 0.7142 \times 10^4$/ml). There was a statistical significant fall in the number of blood mDC2 (0.129 ± 0.048 x10³/ml) in the bortezomib group as compared to the relapse and thalidomide patients (p<0.05).

![Figure 3-9 Blood DC subsets at the time of relapse and following therapy with thalidomide and bortezomib](image)

Using flow cytometry and the BDCA antibody kit, we quantified the number of blood mDC1, blood pDC and mDC2 in samples obtained from MM patients when they had been taking more than 100 mg of thalidomide for 1-3 months (n=12), and 1 month after receiving bortezomib (n=5). These results were compared to those obtained in patients who had relapsed disease (n=12).
3.1.7 Maturation of blood DC subsets from normal subjects and MM patients

The DC system in MM patients is functionally abnormal. They fail to up-regulate costimulatory molecules such as CD80 and CD40, and they are inefficient at priming naïve T cells against tumour antigen. However, in the study by Brown et al, blood mDC from MM patients were identified using the CMRF-44 antibody following overnight culture and 30% of these cells were already expressing CD80 on their surface, prior to maturation. In the Ratta study all blood DC were examined for their expression of maturation markers after 72 hours of culture in medium without any specific maturation stimulus and then enrichment by sequential density gradient centrifugation. They showed a significant reduction in the expression of CD40, CD80 and CD86 in MM blood DC compared to time 0, but no data was collected on the state of maturation of each DC subset and the data represents three repeat experiments on cells from the same patient.

These types of experiments are difficult to perform. They require a large number of very rare cell populations, which do not express exclusive surface markers, thus they are obtained by a number of negative and positive selection steps. In order to simplify this process, we examined whether we could combine blood DC subset identification using the BDCA antibodies with an assay which would show the DC's ability to up-regulate the surface expression of co-stimulatory molecules such as CD40, CD80, CD86 along with expression of phenotypic markers CD1a and CD123. Mononuclear cells were isolated by density gradient centrifugation and placed in DC culture medium. A sample of cells was analysed for co-expression of BDCA, co-stimulatory and phenotypic markers at time zero. The remainder of the cells were divided into two aliquots. One was cultured in medium alone, while the other had a maturation stimulus added in the form of TNF-α (20ng/ml) and PG E2
(10^{-7} \text{M}). Cells from each culture condition were analysed after 4 hours and overnight culture.

Cells were analysed by flow cytometry using the BDCA kit having run the appropriate negative and compensation controls. As well as analysing the control and test samples, 5 additional tubes were stained with the BDCA test antibody cocktail, with the addition of an extra FL4 antibody to measure the surface expression of CD1a, CD40, CD83, CD86 and CD123. These experiments were repeated on three occasions on samples from normal subjects.

Figure 3-10 BDCA2 and CD123 double staining of blood pDC control and after 4 hours culture

At time zero, blood pDC were positive for the additional FL4 CD123, as expected, and this antibody was included in the panel to act as a positive control. None of the other additional antibody stains were positive prior to culture. After 4 hours of culture in medium the level of CD123 staining is reduced on the blood pDC (100% vs 14%)
Figure 3-11 Blood DC expression of CD83 and CD86 after 4 hours culture in medium supplemented with TNF-α and PGF2.

After 4 hours culture in DC medium alone and DC medium supplemented with TNF-α and PGF2, the control (plot A) and Test (plot B) are still able to identify the blood mDC and blood pDC populations. Plot C and D shows blood pDC expression of CD 83 and 86 respectively. Plots E and F show blood mDC expression of CD 83 and 86. At 4 hours the number of cells expressing these markers of DC maturity has increased from 0% pre-culture, to between 3% and 7% of each of the DC subset population.

At 4hrs we were able to show that there is a small but detectable increase in the levels of CD83 and CD86 surface staining on both blood mDC and blood pDC9.

Figure 3-11 and Table 3-2. There was no increase in the level of CD1a or CD40 at 4 hours. The surface expression of CD123 on blood pDC was almost completely down regulated (-84.64 ± 5.2) after 4 hours of culture in both supplemented and plain DC medium. However, following overnight culture in
medium alone or supplemented with TNF-α and PGE₂, the numbers of cells that remained in the blood mDC and blood pDC gates were so low that no meaningful estimate of surface expression of other markers could be made.

<table>
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Table 3-2 Change in the mean fluorescence intensity of maturation markers in blood mDC and blood pDC following 4 hours culture in TNF-α and PGE₂ supplemented DC medium

Figure 3-12 BDCA 1 and 2 surface expression after overnight incubation in medium supplemented TNF-α and PGE₂

Plot A and B show BDCA 1 and 2 surface expression following overnight culture in DC medium (A) or DC medium supplemented with TNF-α (20ng/ml) and PGE₂ (10⁻⁷M) (B).
3.1.8 Discussion

The presence of DC subsets in the blood gives a unique window into the DC system in both normal subjects and those patients with malignant disease, such as MM. Using the BDCA kit we were able to reliably measure the number of blood mDC, blood pDC and blood mDC2 in the peripheral blood of normal subjects, with our results being comparable with the manufacturers reference range. We also validated the use of the kit to reliably measure the number of B lymphocytes in the blood of normal subjects and our results are within a number of published reference values (HMDS, Leeds) (Glassman and Bennett 1977, Reichert, et al 1991). Having validated its use, we went on to use the BDCA antibody kit to analyse samples from patients with MM at a number of time points during the disease course.

Using this assay, we found that the number of B lymphocytes in the blood of MM patients (mean 0.9386 ± 0.2750 x10^5 /ml) was significantly lower than in normal subjects (p<0.001). There is some debate in the published literature as to whether B lymphocyte numbers are suppressed in MM, with various groups reporting normal, low and heterogeneous numbers of B lymphocyte in the blood of MM patients (Kay, et al 1997, Pilarski, et al 1984, Rasmussen, et al 2000, Schutt, et al 2005). Why should B lymphocyte numbers be reduced in MM? MM is a clonal disorder of the B cell compartment, and as with other clonal disorders, this may represent a simple replacement of the normal cells by the malignant clone.

Another possibility is that the immunosuppressive cytokines, such as IL-6, IL-10, TGF-β and VEGF secreted by the (pre-) malignant MM plasma cell are actively suppressing the development of normal B lymphocytes. A third possibility is that epitopes expressed in the CD19 molecule are altered in MM and MGUS patients by the cytokines secreted by the malignant clone, preventing some antibodies such as Leu-12, FMC-63 and B4 from detecting CD19 molecules. This may go
some way to explain the differences in B cell numbers reported in MM patients in the literature. The antibody used in our analysis is produced by the LT19 clone, which is commonly used for phenotyping CD19+ cells and there are no reported discrepancies between normal and MM B cell staining. We also found that the number of B cells in the blood of patients with MGUS and plasmacytoma (1.5883 ± 0.3405 x10^5 /ml) was also lower than in normal subjects (p<0.005). This last finding was unexpected, as there are no previous reports of cellular immune defects in MGUS. However, there are a number of reports of clonal B cell populations in the blood of MGUS patients (Pilarski, et al 1996). There is speculation as to whether this represents a pre-malignant clone that is awaiting a further genetic mutation (2nd hit) to become truly malignant, or a malignant clone that is being held in check by the immune system which is allowed to progress when the immune system is down regulated by mechanisms we have discussed previously. We feel that these observations warrant further investigation, as they may reveal further mechanisms by which the MM clone evolves and induces an immunosuppressive state in its host.

The number of blood mDC1 and blood pDC detected in the blood of MM patients with the BDCA antibodies was significantly lower than in the control group. This effect was even more pronounced at the time of relapse. The number of blood pDC seemed to be particularly affected, especially at the time of relapse. Standard induction chemotherapy made no significant improvement in the number of blood mDC1 or blood pDC seen in the blood of MM patients. The number of blood mDC2 seen throughout the course of the disease was not significantly different from the normal population. The number of DC improve in the short term after auto HSCT, with the numbers of blood mDC1 returning to within the normal range at the time of engraftment (mean 1.52 ± 0.28 x10^4 /ml) (p>0.05) and subsequently maintained up to D100, but then fall back again at one year post auto HSCT. The
improvement in blood pDC and blood mDC2 after HSCT is less impressive and again the levels were low at one year post auto HSCT. These findings are encouraging, as they reflect the improved survival data that suggest auto HSCT has an important role in further reducing the tumour burden in MM patients as compared to conventional therapy alone. In the future, these kinds of data may be important in deciding upon the timing of immune based therapies, which should have the best chance of inducing significant benefits when the disease burden, and hence its immunosuppressive potential, is at its lowest. Our data on the recovery of blood DC following NM allo HSCT was limited due to the very small numbers of patients that are eligible for this treatment option. We found that the level of all blood DC subsets were low at D100, which appears to be at odds with data generated within our group from patients undergoing NM allo HSCT for the treatment of CML, which showed that at D100 blood mDC1 numbers were 27-fold higher than ND, and the other blood DC subsets were relatively normal (Harrison, et al 2003). Both of these findings offer only snap shots of complex, dynamic and rapidly evolving interactions between the prolonged effects of conditioning therapy (i.e. Campath 1H), the process of immune reconstitution, response to infections and the underlying malignant disease. The true picture seems to be one where the numbers of blood DC subsets vary dramatically throughout the first year following allo HSCT in a series of peaks and troughs (Hart, personal communication). The process of blood DC recovery following HSCT will only become better understood by studying these aspects of immune reconstitution in a formal, longitudinal study.

Thalidomide is playing an increasingly important role in the management of patients with MM. In the MRC UK myeloma IX study, it is being used in both the induction regime for new patients and as single agent maintenance therapy following autologous HSCT. Of the many putative modes of action of thalidomide, its immuno-stimulatory properties are some of the most interesting. Our finding
that the number of blood mDC1 in patients taking thalidomide was significantly higher than patients at relapse suggests that thalidomide is able to either stimulate the DC system or allow it to recover somewhat from the added insult of disease relapse. There are no published data on the clinical effect of thalidomide on blood DC, but our data are in keeping with the in vitro data which suggests that thalidomide is able to improve the ability of MoDC to induce TH1 type responses (Mohty, et al 2002b). The number patients in the bortezomib study were small and they had been heavily pre-treated, making the data difficult to interpret. Even so, it was interesting to note that the blood mDC1 and pDC numbers were similar to the relapse group, while the blood mDC2 numbers were significantly lower that at relapse and following thalidomide treatment. As this drug becomes more widely used it will be important to assess the impact that it has on the immune system in MM and other malignant diseases.

In the final set of experiments we attempted to use the BDCA antibodies to monitor the blood DC response to a maturation signal in a mononuclear cell population. We were able to show early up regulation of surface expression of the costimulatory molecules CD83 and CD86 on blood mDC and blood pDC and down regulation of CD123 on blood pDC at 4 hours. Following overnight culture we were not able to reliably identify the blood DC subsets due to down regulation of CD1c and BDCA2. These experiments were able to give some insight into the timing of changes in the surface phenotype of blood DC. In order to take these experiments further, formal isolation of the DC subsets by either FACS or magnetic bead sorting would need to be performed prior to culture. These experiments require a large number of starting cells because of the small target populations and the magnitude of cell loss encountered at each step.
In summary, The BDCA antibodies allowed us to reliably measure the DC subsets and B cell in the blood of normal subjects and patients with MM throughout the disease course. The numbers of blood mDC and blood pDC are low throughout the course of the disease, and only improve for a short period of time following autologous HSCT. Thalidomide is able to increase blood mDC1 and blood pDC numbers in patients with relapsed disease.
4. Results 2

4.1 MoDC Generation in NDs and MM patients

4.1.1 Introduction

Multiple myeloma is not curable in the majority of patients, however the immune system is able to affect the cure of a small number of young patients via the GVM effect seen in allo BMT, but at the cost of increased toxicity. The introduction of non-myeloablative allo BMT has attempted to reduce the toxicity of allo BMT and thus make it a more viable treatment option in older patients (Shaw, et al 2003). However, this may also reduce the effectiveness of the immune component of the therapy against the disease, especially in groups with advanced and heavily pre-treated disease (Crawley, et al 2005a). Many groups around the world are therefore attempting to use the autologous immune system to eradicate residual MM following standard and high dose conventional chemotherapy. Dendritic cells are seen as one of the most promising tools that we can manipulate in order to increase a patient's immunity against their residual tumour cells. In the previous chapter it was shown that circulating BDC in MM patients are quantitatively abnormal. As previously discussed, these cells are also qualitatively abnormal in MM patients, thus it is difficult to justify using BDC from MM patients in the treatment of their disease. It is possible to produce iDC from steady state or mobilised monocytes from ND and patients with malignant disease (Morse, et al 1999b, Syme, et al 2001). Alternatively, monocytes can be separated by plastic adherence or CD14 selection (Motta, et al 2003, Suen, et al 2001) and they are then cultured in medium supplemented with cytokines such as GM-CSF, IL-4, IL-13 and IFN-α (Della Bella, et al 2004, Gabriele, et al 2004, Motta, et al 2003). To date, there are few comparative studies to show if these methods are equally
effective and there are almost none to show whether they are equivalent when using monocytes from patients with diseases such as MM (Syme, et al 2001, Tarte, et al 1998). In addition, we are only just beginning to explore how therapeutic interventions and the level of residual disease may influence our ability to produce immune therapies in the context of malignant disease states. For example, Schutt has shown that if MM patients are taking thalidomide, dendritic cells produced by in vitro culture from monocytes have a reduced expression of DC surface markers such as CD1a, CD40, CD83, and HLA-DR (Schutt, et al 2005). The question on how to mature iDC is also confused. There are many published cytokine combinations containing TNFα, PGE2, IL-1β, IL-6, CD40L, Poly(I;C), IFNs (Luft, et al 2002b) etc. in numerous combinations and doses that can be used to produce matDC from Ag-loaded iDC, but there are few comparative studies in MM (Curti, et al 2004).

It is therefore conceivable that in view of the hostile environment induced by the MM tumour cell, with high levels of TGFβ etc, monocytes from MM patients may produce overall less efficient MoDC. Thus, culture conditions may be even more critical for the production of DC for clinical use, which may eventually translate into more effective immunotherapy for use in clinical trials, when these cells are injected back into the patient and a hostile cytokine environment.

The aim of the experiments described in this chapter was to examine whether the method of iDC production from monocytes influenced the quality and function of immature MoDC produced from MM patients vs. ND. We also examined whether different maturation cytokine combinations were able to overcome any blockade of maturation in MM patient derived DC as compared to cells from ND.
4.1.2 A comparison of iDC generation using GM-CSF and IL-4 or IL-13 from mobilised monocytes collected from NDs and MM patients

In order to examine whether monocytes from NDs and MM patients could equally be used to produce iDC using the cytokine combinations GM-CSF and IL-4 or GM-CSF and IL-13, monocytes were obtained from subjects who gave informed consent and were undergoing stem cell collection at the Apheresis Unit, GRI. NDs were conditioned with growth factor (G-CSF) alone, while most MM patients were mobilised with growth factor (G-CSF) following myelosuppressive chemotherapy.

Excess cells that remained in the apheresis harness were harvested immediately following the procedure. The PBMC were obtained by density gradient centrifugation and then cultured overnight in DC medium to allow the cells to rest following the mobilisation and apheresis procedures. The following morning, monocytes were purified from PBMC by CD14 magnetic bead separation (Miltenyi Biotech). An aliquot of monocytes (2x10⁶) was retained for flow cytometric analysis of surface phenotype (Figure 4-1), while the remainder were suspended in DC medium at a density of 5x10⁶/ml and placed into 75cm² tissue culture flasks (Corning Life Sciences). The DC medium was then supplemented with GM-CSF 50ng/ml (Stem Cell Technologies, Vancouver, Canada) and either IL-4 30ng/ml (Peprotech) or IL-13 50ng/ml (Peprotech). Cells were cultured for 4 days at 37°C and 5% CO₂, and then assessed by flow cytometry for surface expression of markers of DC phenotype (Figure 4-2).
Monocytes from NDs (n=10) and MM patients (n=10) were collected during stem cell mobilisation and purified by density gradient centrifugation and CD14 magnetic bead separation. Cells were cultured overnight in DC medium and the surface phenotype was analysed by flow cytometry prior to culture in DC medium supplemented with GM-CSF and IL-4 or IL-13. The results are expressed as the percentage of total cells that were positive for the marker.

The purity achieved by CD14 magnetic bead separation was similar in both the ND and MM patient samples (mean 93.8 ± 1.8% vs. 89.9 ± 3.2%). The proportion of cells that expressed CD86 (mean 74.4 ± 6.2% vs. 44.7 ± 10.2%, p<0.03), HLA-DR (mean 83.6 ± 5.2% vs. 55.8 ± 8.8%, p<0.05) and CCR5 (mean 45.1 ± 6.5% vs. 21.6 ± 10.0%, p<0.05) on their surface was significantly higher in the ND monocytes as compared to MM cells. There was also a small, but significant increase in the surface expression of CD40 on MM monocytes compared to ND cells (mean 7.0 ± 1.9% vs. 3.1 ± 0.7%, p<0.05).
An aliquot of ND (n=20) or MM (n=17) iDC (1-2x10^6) was removed after 4 days in culture with GM-CSF (50ng/ml) and either IL-4 (30ng/ml) or IL-13 (50ng/ml). The number of cells expressing DC markers were analysed by flow cytometry.

Following 4 days culture in DC medium supplemented with GM/IL-4 or GM/IL-13, all iDC down regulated CD14, with no significant difference between ND and MM cells. There was an up regulation of the surface expression of CD86, HLA-DR, CCR5, CD1a, and CD1c on both ND and MM iDC as compared to monocytes. The
The proportion of ND iDC that expressed CD1c (mean 63.4 ± 4.6% vs. 36.0 ± 6.5%, \(p \leq 0.003\)), CD86 (mean 87.2 ± 3.0% vs. 70.9 ± 3.4%, \(p \leq 0.001\)), and CCR5 (mean 49.1 ± 5.6% vs. 34.2 ± 6.3%, \(p \leq 0.05\)) was significantly higher as compared to MM iDC. The MFI of HLA-DR (mean 223.0 ± 40.7 vs. 126.3 ± 20.2, \(p < 0.05\)) and CD1a (mean 34.3 ± 7.2 vs. 11.3 ± 2.0, \(p < 0.001\)) surface expression was also significantly higher on ND iDC as compared to MM iDC (Figure 4-2).

There were no significant differences in the surface expression of CD1a, CD40, CD86, HLA-DR and HLA-class I between iDC from ND and MM patients generated with GM-CSF and IL-4, as compared with cells generated with GM-CSF and IL-13. These findings are summarised in Table 4-1.

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<td>90.3 ± 3.2%</td>
<td>81.5 ± 4.0%</td>
<td>83.7 ± 6.0%</td>
</tr>
</tbody>
</table>

**Table 4-1 Surface phenotype of iDC from ND and MM patients generated with GM/IL-4 or GM/IL-13**

The surface phenotype of iDC from ND and MM patients generated with GM/IL-4 (n=11 and 9) or GM/IL-13 (n=9 and 8) was expressed as the mean percentage of positive cells ± SEM (top line) and mean MFI ± SEM (bottom line).
ND iDC generated with GM-CSF and IL-4 expressed significantly higher levels of CD1c (68.6±5.6%, MFI 20.1±3.4) as compared with iDC generated with GM-CSF and IL-13 (57.2±7.5%, MFI 12.9±2.8) (p<0.03 and p<0.005). Immature DC generated from MM patients had significantly higher expression of CD40 when generated with GM-CSF and IL-4 (MFI 10.5±2.9) compared with those generated with GM-CSF and IL-13 (MFI 11.7±3.6, p<0.01). In contrast, MM iDC cultured with GM-CSF and IL-13 had a higher proportion of CCR5 positive cells (43.9±9.61%) compared with those produce with GM-CSF and IL-4 (27.0±7.8%, p<0.03). Thus iDC from patients with MM were consistently of poorer quality than cells from ND irrespective of the cytokines used to generate them.

4.1.3 Phagocytosis of Ag by ND and MM patient iDC generated using GM-CSF and IL-4 or IL-13

One of the main functions of iDC is to continuously monitor the in-vivo environment for Ag. Immature DC encounter Ag in the peripheral tissues in many forms, such as apoptotic bodies, cell debris following necrosis, bacteria, food and purified proteins following vaccination. The main way in which iDC take up Ag is by simple phagocytosis. Immature DC may also employ a number of more specific mechanisms that enhance the efficiency of Ag loading such as CD68 mediated uptake of antibody coated particles, HSP uptake via CD91 and the uptake of apoptotic bodies via various receptors, including the phosphotidylserine receptor, vitronectin receptor and CD36. In order to assess whether iDC from ND and MM patients generated with GM/IL-4 and GM/IL-13 were equally efficient at phagocytosing particulate Ag, we performed phagocytosis assays using 1 or 2μm latex beads that were coated with PE (Sigma).
Dendritic cells were suspended in DC medium at a concentration of $1 \times 10^6$/ml, and 100µl was added to a Falcon tube. Latex-PE beads (1mg/ml) were diluted 1:100, and 10µl was added to each sample. The assay was then incubated at 37°C for 1 hour. The cells were counter stained with CD11c-FITC, which was added to the assays for the final 10mins of the incubation. Excess antibody and non phagocytosed beads were removed by washing the cells in 5mls of cold PBS. The cells were recovered by centrifugation at 800 rpm for 10 minutes, before completely removing the supernatant. This was repeated and the cells were suspended in 300µl of PBS prior to analysis by flow cytometry, having first run the appropriate compensation controls (i.e. unstained cells, Isotype control PE and FITC cells, CD11c-FITC positive cells, PE bead positive cells) (Figure 4-3).

**Figure 4-3 iDC bead phagocytosis assays**

ND iDC generated with GM-CSF and either IL-4 or IL-13, were incubated with PE labelled 1 or 2 um latex beads and incubated for one hour. Immature DC were counter stained with CD11cFITC, washed twice in PBS and analysed by flow cytometry. Cells may phagocytose more than one bead, and this produces an incremental increase in the fluorescence of the cell per bead, as illustrated by the arrows and the characteristic 'stripes' within the plots.
Immature DC generated from ND (mean 59.4 ± 3.9%) were significantly better at phagocytosing fluorescent latex beads compared to those generated from MM patients (mean 20.1 ± 2.1%, p<0.001). The cytokines used to generate the iDC made no significant difference to the ability of ND (IL-4 mean 57.2 ± 6.6%, IL-13 mean 61.6 ± 4.6%) or MM iDC (IL-4 mean 19.5 ± 0.6%, IL-13 mean 20.8± 2.3%) to phagocytose fluorescent beads.

**Figure 4-4 ND and MM iDC latex bead phagocytosis assays**

ND (n=7) and MM iDC (n=4) were generated with GM/IL-4 and GM/IL-13. Fluorescent latex bead phagocytosis assays were performed on D4 of culture. Live iDC were gated on by forward and side scatter and CD11c expression, and the percentage of cells that have phagocytosed at least one bead are expressed as the mean ± SEM.

In order to assess the ability of iDC to phagocytose tumour Ag we modified the phagocytosis assay by using MM tumour cells, labelled with fluorescent dye, in place of PE labelled latex beads. The U266 myeloma tumour cells (HLA-A2) were labelled with the PKH-67 green fluorescent dye, which is detected in FL1 and washed three times in DC medium. Labelled tumour cells were added to the iDC at a ratio of 5:1 to ensure that the number of available tumour cells did not limit the rate of iDC phagocytosis, and the cells were incubated for 1 hour at 37°C.
Dendritic cells were counter stained with CD11c-PE. The cells were washed in PBS and the results were analysed immediately by flow cytometry (Figure 4-5).

![Figure 4-5 Fluorescent tumour cell phagocytosis assay](image)

ND iDC generated with GM-CSF and either IL-4 or IL-13, were incubated with PKH-67 labelled U266 tumour cells and incubated for one hour. Immature DC were counter stained with CD11c-PE, washed twice in PBS and analysed by flow cytometry.

The results from the fluorescent tumour cell assay were in keeping with the bead phagocytosis assay, although iDC were better able to take up tumour-derived material as compared to the latex beads. ND derived iDC were more efficient at phagocytosing tumour cell Ag (mean 70.2 ± 3.9%) as compared to iDC generated from MM patients (mean 34.2 ± 6.9%, p<0.001). Once again, the cytokine combination that was used to generate the iDC had no significant impact on the ability of ND (IL-4 mean 69.8 ± 4.2%, IL-13 mean 70.7 ± 5.3%) or MM iDC (IL-4 mean 32.5 ± 10.3%, IL-13 mean 36.0 ± 10.7%) to phagocytose tumour cell derived Ag (Figure 4-6).
4.1.4 Maturing iDC from ND and MM patient iDC using the MIMiC and X4 cytokine combinations

It has been known for some time that iDC do not induce potent anti-tumour responses, and may even induce tolerance when administered to patients as immune therapy. Thus it is crucial to any immunotherapeutic strategy that DC achieve a state of full maturity, i.e. up regulate co-stimulatory molecules such as CD40, CD80, CD83, CD86 and HLA class I and II prior to being given to patients (Nieda, et al 2003). The process of maturation also alters the chemokine receptor profile, i.e. down regulation of CCR5 and up regulation of CCR7, which facilitates the migration of matDC from peripheral tissues to lymph nodes where they are brought into close proximity to naïve T cells for Ag presentation to occur. As previously discussed BDC from MM patients do not up regulate co-stimulatory molecules such as CD40.
molecules in response to maturation signals such as TNF-α and PGE₂ as compared to cells from normal subjects. There is some debate as to whether MoDC from MM patients mature normally in response to standard maturation stimuli (Ratta, et al 2000). A number of strategies have been shown to induce DC maturation. One of the first methods that was shown to induce effective DC maturation was the use of monocyte conditioned medium (Bender, et al 1996), however these media are time consuming to produce and are subject to marked inter batch variation in responses. Cytokines such as TNF-α can be used alone to induce DC maturation, but may be used more effectively in combination with PGE₂, which seems particularly important in the up regulation of CCR7 (Scandežla, et al 2002). The interaction between CD40 and CD40L is particularly important in the induction of DC maturity in-vivo, and it has been shown that it can be used alone or in combination with other cytokines such as IL-1β and IFN-α to induce a potent maturation of iDC in-vivo (Caux, et al 1994, Luft, et al 2002a, Luft, et al 2002b).

The aim of the next set of experiments was to compare the ability of two cytokine combinations to induce the maturation of iDC from ND and MM patients generated with GM/IL-4 and GM/IL-13. We compared the monocyte conditioned medium mimic (MIMIC) combination of TNF-α (10ng/ml) (Peprotech), IL-1β (10ng/ml) (Peprotech), IL-6 (10ng/ml) (Stem Cell Technologies) and PGE₂ (10⁻⁷M) (Sigma) (Feuerstein, et al 2000) with the novel in-house combination X4, TNF-α (10ng/ml), PGE₂ (10⁻⁷M), Poly (I;C) (10ng/ml) (Sigma) and CD40L (250ng/ml) (Peprotech). The X4 combination was rationally designed to maximise the stimulus to the MM iDC. In particular, by incorporating the signalling of CD40L via CD40 with the more traditional TNF-α signalling via CD120, PGE₂ via the eicosanoid receptors 2 and 4 (EP2 and 4), and finally polyriboinosinic polyribocytidylic acid (poly I:C), which is a member of the pathogen-associated molecular patterns (PAMP) group of
molecules and signals through Toll-like receptor 3, X4 may overcome the relatively poor expression of co-stimulatory molecules on MM iDC and the impairment of their up regulation seen in MM BDC, as discussed in the previous chapter.

Immature DC were prepared from CD14 selected monocytes from MM patients and ND cultured for 4 days in DC medium supplemented with GM/IL-4 or GM/IL-13. The iDC were then counted and fresh DC media, supplemented with the appropriate cytokines, was added so that iDC were at a final concentration of 1x10^6/ml and placed in two 25cm² tissue culture flasks. The Ag preparations, either apoptotic or freeze-thawed U266 cells suspended at 1x10^7/ml, were added to a final ratio of 1 iDC to 1 tumour cell (equivalent to an approximate protein concentration of 1mg/ml) (Thumann, et al 2003). The X4 or MIMIC maturation cytokine combinations were added immediately and the cells were cultured overnight at 37°C (Thumann, et al 2003), with tissue culture flasks standing upright to improve cell contact and phagocytosis. The following morning 2x10^6 matDC generated with the X4 and MIMIC cytokine combinations were removed for flow cytometric analysis of their surface phenotype (Figure 4-7a and b).

The results of the phenotypic analyses of matDC from ND and MM patients are summarised in Table 4-2. In ND, both X4 (n=15) and MIMIC (n=14) induced up regulation of the costimulatory molecules CD40, CD80, CD83, CD86, HLA-DR and HLA-Class I, with X4 producing significantly higher expression of CD80 (mean 17.1 ± 1.8) compared to MIMIC (mean 9.7 ± 1.5, p<0.02). The expression of CCR7 in both groups was disappointing, but the proportion of positive cells and the level of expression were higher in the X4 treated matDC (mean 12.1 ± 2.3%, mean 3.3 ± 0.9) as compared to the MIMIC treated group mean (mean 9.4 ± 1.8% p<0.05, mean 1.8 ± 0.6 p<0.05).
Figure 4-7 Surface phenotype of ND matDC matured overnight with the X4 (A) and MIMIC (B) cytokine combinations
DC were gated on using forward and side scatter, and expression of CD11c.

The MM matDC had broadly similar expression of costimulatory molecules and CCR7. The only exception was that the X4 group had significantly higher
proportion of CD83 positive cells than the MIMIC treated matDC (mean 65.5 ± 5.6% vs. 46.3 ± 8.3%, p<0.05). The level of CD83 expression was also higher in the X4 matDC, but this failed to reach statistical significance.

### Table 4-2 Phenotype of ND and MM matDC generated with X4 and MIMIC cytokine combinations

<table>
<thead>
<tr>
<th></th>
<th>Normal Donor</th>
<th></th>
<th>MM Patient</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>All</td>
<td>X4</td>
<td>MIMIC</td>
<td>All</td>
</tr>
<tr>
<td>CD40</td>
<td>76.6 ± 3.9%</td>
<td>79.7 ± 4.6%</td>
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<td>CD80</td>
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<tr>
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<td>CD83</td>
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<td>30.7 ± 4.0</td>
<td>36.4 ± 6.6</td>
<td>24.6 ± 3.7</td>
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<tr>
<td>CD86</td>
<td>91.9 ± 3.1%</td>
<td>94.1 ± 3.3%</td>
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<td>CCR7</td>
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<td>HLA-DR</td>
<td>95.6 ± 1.5%</td>
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<td>371 ± 65.1</td>
<td>419 ± 75.5</td>
<td>213 ± 31.4</td>
</tr>
<tr>
<td>HLA-Class I</td>
<td>96.4 ± 1.0%</td>
<td>96.3 ± 1.7%</td>
<td>96.5 ± 1.2%</td>
<td>87.2 ± 2.6%</td>
</tr>
<tr>
<td></td>
<td>169 ± 18.4</td>
<td>181 ± 24.3</td>
<td>155 ± 28.3</td>
<td>123 ± 22.5</td>
</tr>
</tbody>
</table>

Immature DC from ND (n=11) and MM (n=9) patients were loaded with Ag and simultaneously matured by overnight culture with the X4 (ND s=15, MM s=19) or MIMIC cytokine combination (ND s=14, MM s=7). The results are shown as the mean percentage expression ± SEM (top line) and the mean MFI ± SEM (bottom line).

The results of the surface phenotype of MM matDC were compared to those from ND. It was found that the levels of CD40 (mean 24.8 ± 3.3 vs. 43.3 ± 6.1, p<0.002), CD83 (mean 58.9 ± 4.9% vs. 72.1 ± 4.4%, p<0.05 and 15.8 ± 2.3 vs. 30.7 ± 4.0, p<0.002), CD86 (mean 156.9 ± 32.1 vs. 366.3 ± 57.0, p<0.002), HLA-DR (mean 213.0 ± 31.4 vs. mean 394.0 ± 48.9, p<0.005) and HLA-Class I (mean...
87.2 ± 2.6% vs. 96.4 ± 1.0%, p<0.002) expression were significantly reduced (Figure 4-8). The level of CD80 and CCR7 expression was similar in the both groups.

Figure 4-8 Co-stimulatory markers on ND and MM matDC

Mature DC were generated from ND and MM patient iDC by loading with Ag and overnight incubation with the X4 or MIMIC combination of cytokines. Samples were analysed for surface expression of co-stimulatory molecules by flow cytometry.
Although the expression of the co-stimulatory molecules on both ND and matDC following either the X4 or MIMIC cytokine combination was satisfactory, the level of CCR7 up regulation was poor. Therefore, the expression of CCR7 on ND matDC was examined after an additional 24hrs incubation in X4 maturation medium (n=4) or after 24 hours interacting with autologous T cells (n=6) isolated from the apheresis sample by anti-CD3 magnetic bead isolation and cryopreserved in LN$_2$ until the day of the assay. The cells were placed in 24 well plates (Corning) at a ratio of 1DC (1x10$^5$/ml) to 10 T cells (1x10$^6$/ml) in TC medium. The surface expression of CCR5 and 7 was analysed by flow cytometry (Figure 4-9) with live matDC gated by forward and side scatter in both analyses, as there was a clear separation between matDC and T cells in the co-culture experiments.

![Figure 4-9 CCR5 and 7 expression on ND matDC after an extra 24 hrs culture in X4 supplemented media or co-culture with autologous T cells](image)

Immature DC were cultured in X4 supplemented DC medium for overnight. Cells were divided into two aliquots. The first was cultured in X4 supplemented medium for a further 24 hrs (left), while the second aliquot was cultured with autologous T cells at a ratio of 1DC to 10 T cells for 24hrs (right). The surface phenotype of the cells was then analysed by flow cytometry.
There was a highly statistically significant up regulation of CCR7 expression in ND mat DC culture for 42 hours in X4 (32.4 ± 15.7% and 13.4 ± 6.3) as compared with overnight culture (10.8 ± 1.4% and 2.6 ± 0.6, p<0.001). Extended culture in maturation media, or 24hrs co-culture with autologous T cells produced no significant differences in the level of CCR7 expression (13.4 ± 6.3 vs. 12.7 ± 2.4) or the proportion of positive cells (32.4 ± 15.7% vs. 28.69 ± 7.6%), as shown in Figure 4-10. There was no significant increase in the level of expression of the other co-stimulatory molecules in either the extended X4 or T cell matDC. These findings indicate that iDC rapidly up regulate surface expression of co-stimulatory markers during maturation. However, the level of CCR7 expression continues to increase with prolonged exposure to a maturation stimulus, such as cytokine or T cells.

![Graph showing CCR7 expression](image)

**Figure 4-10** CCR7 expression on ND mat DC after overnight maturation compared with an additional 24hrs maturation or 24 hrs co-culture with autologous T cells

ND iDC were incubated with X4 overnight to induce maturation. The surface expression of CCR7 was measure by surface antibody staining and flow cytometry (A). Two aliquots of cells were culture for a further 24hrs in either X4 or in co-culture with auto T cells, isolated by CD3 magnetic bead isolation, at a ratio of 10 T cell to 1 DC and the CCR7 expression measured by flow cytometry (B).
4.1.5 Discussion

Dendritic cell based immune therapy of MM has thus far failed to produce significant benefits to patients in terms of time to progression and OS. If we are to generate meaningful clinical responses, we must learn how to induce autologous matDC from MM patients that are able to migrate efficiently to lymph nodes using chemokine receptors. Once there, anti-MM matDC should prime an immunogenic response to the tumour Ags by the interaction of matDC and T cell via the HLA-Ag complex and T cell receptor, and driven by the matDC co-stimulatory molecule expression profile. During this process, the therapeutic DC should be resistant to the hostile in-vivo cytokine environment generated by the MM tumour plasma cell.

These experiments shown that, as with the circulating BDC, monocytes from MM patients are already different from ND monocytes. They have reduced expression of CD86, HLA-DR and CCR5, thus the building blocks of the cellular therapy would seem to be abnormal. These changes are almost certainly induced by cytokines, such as IL-6 and TGF-β, produced by the MM plasma cells. The reduction of CCR5 is interesting in that it's ligand, MIP-1α, is present in increased amounts in MM patients and it correlates with the extent of MM bone disease and survival (Terpos, et al 2003). The increased MIP-1α levels may directly lead to a homeostatic down regulation of CCR5 expression on MM monocytes, interfering with their ability to respond to pro-inflammatory chemokines.

Using CD14 positively selected monocytes collected during stem cell mobilisation, iDC were produced using either GM-CSF/IL-4 or GM-CSF/IL-13 from both ND and MM patients. Within the two subject groups, the cytokines used to generate the iDC made little impact on the quality of the cells that were produced in terms of their phenotype and phagocytic function. However, when MM iDC were compared
with ND iDC they were found to be of inferior quality. The surface expression of CD1a, CD1c, CD86, HLA-DR and CCR5 was significantly reduced on MM iDC, and their ability to phagocytose Ag was also significantly impaired, as measured by the fluorescent latex bead and tumour cell assays. It was not possible to overcome these deficiencies in the MM iDC using either of the cytokine combinations chosen to generate iDC. The question that was addressed next was whether the X4 or MIMIC maturation cytokine combinations were able to produce improved maturation of the MM iDC.

The maturity of the DC used in immunotherapy is crucial. Immature DC will tend to induce a tolerogenic rather than immunogenic T cell response. It was hoped that by maximising the maturation stimulus it might be possible to overcome the inhibition of MM DC maturation previously reported. The X4 and MIMIC cytokine combinations were given to the iDC from ND and MM patients in overnight culture at the same time as the Ag preparations for two reasons; firstly to speed up the process of DC production, and secondly to give the Ag in the context of a pro-inflammatory environment, in a bid to increase the chances of generating an immunogenic rather than a tolerogenic response to the chosen Ag. ND matDC generated with X4 had an increase in the expression of CD80 and CCR7 compared to MIMIC matured matDC, while the MM X4 matDC had an increase in CD83 expression. Unfortunately, MM matDC had significantly reduced CD40, CD83, CD86, HLA-DR and Class I expression compared to ND matDC irrespective of the maturation cytokine combination used. The level of CCR7 expression was disappointing in both ND and MM patient cells. We went on to show that this continues to up regulate for 24hrs when ND DC are cultured with cytokines or autologous T cells, thus increasing the time in the cytokine combination media seems to have no benefit over placing the mat DC in culture with T cells in-vitro. However in-vivo, DC need to migrate to lymph nodes in order
to encounter naive T cells to interact with. Thus when producing matDC for use in patients, extending the time spent in maturation media is likely to be of benefit, as the DC need to be administered in a state that enables them to migrate immediately to lymph nodes.

Overall these results show that MoDC from MM patients are phenotypically and functionally abnormal at all stages of the production process, including the freshly isolated monocytes. It was not possible to completely overcome these defects using the various combinations of cytokines, however, cells generated with IL-4 or IL13, and matured with X4, produced the best quality matDC. In order to further improve the quality of DC obtained from MM patients a number of strategies could be investigated. Monocytes were obtained from MM patients that had been harvested following chemotherapy and G-CSF, rather than G-CSF alone, as in the ND group. In order to examine the impact of the mobilisation regimen on DC production on cells from MM patients' further, chemo-mobilised cells from could be compared with monocytes obtained with G-CSF alone or collected in a steady state. It is not ethical to mobilise cells from ND using chemotherapy. To examine the impact of MM on the quality of DC produced using chemo-mobilised cells, it may be better to compare MM cells to those collected from patients with malignant diseases such as Non-Hodgkin’s Lymphoma (NHL), as these conditions are not thought to have a significant negative impact on the host immune system.

The experiments described in this chapter, cells were collected at an unspecified level of residual disease. Therefore it is not possible to say whether cells collected from patients with a higher level of residual disease produced DC that were of poorer quality than those from patients with undetectable disease. This is an obvious comparison to perform in future studies. Finally, there are other compounds such, as IFN-α, that have been used for MoDC production, and the
use of more novel cytokine combinations for DC production could be examined in MM.

In conclusion, matDC were produced from MM patients and ND using monocytes mobilised at the time of stem cell collection. The matDC produced from MM patients were of poorer quality as compared to those from ND, despite using combinations of GM/IL-4, GM/IL-13, X4 and MIMIC in the production process. The combinations that contained the X4 maturation cocktail produced the best quality matDC. In the next chapter the functional interaction of MM matDC with autologous MM T cells will be discussed.
5. Results 3

5.1 Optimisation of autologous anti-MM cytotoxic T cell production using matDC and T cell pre-stimulation

5.1.1 Introduction

Dendritic cells are crucial to the initiation and co-ordination of the immune response. However, one of the most important, complex, and perhaps least understood parts of this process, is the interaction of DC with immune effector cells such as T, NK and NKT cells. These interactions require close cell-to-cell contact and the interface between DC and T cells via receptors and cytokines has become known as the immunological synapse (Creusot, et al 2002). In this chapter we will concentrate on the interaction between DC and T cells, one of the main effector cells of the adaptive immune system. Naïve T cells can be primed by DC to differentiate into antigen specific cytotoxic T cells, and this is the main goal when using DC as an anti-tumour immune therapy, where the antigen is an tumour specific one. Unfortunately, this is not the only possible outcome of this interaction. Depending on the state of DC maturation (Benvenuti, et al 2004), the affinity of the T cell receptor for the antigen being presented, the availability of T cell help/regulation and the cytokine environment, DC may induce abortive T cell proliferation, T regs (Fiore, et al 2005) or even clonal deletion. T cells are also susceptible to the immunosuppressive effects of the MM tumour cells induced by cytokines such as IL-6, IL-10, TGF-β and VEGF.

Previously, our group has described a defect in MM T cells, induced by TGF-β, which prevented normal T cell proliferation in response to IL-2 (Cook, et al 1999). It was also reported that pre-stimulation with anti-CD3 antibody and IL-15 reinstates the MM T cell proliferative response to IL-2. It is not known whether this
method of reinstating the T cell response to IL-2 can be transferred to the DC:T
cell interaction, thus improving the proliferative capacity of T cells in response to
DC stimulation alone, or combined with IL-2. It is also not known whether this can,
in turn, increase the ability of antigen primed MM T cells to kill MM tumour cells.

The aims of the experiments described in this chapter are to answer the following
questions.

1. Can MM T cell pre-stimulation improve the proliferative response to DC
alone and in combination with IL-2?

2. Do MM matDC produced with the MIMIC or X4 cytokine combinations
have the same ability to induce MM T cell proliferation?

3. Does the cytokine profile produced during the DC:T cell interaction vary
with T cell pre-stimulation?

4. Are pre-stimulated, DC primed MM T cells better able to kill MM tumour
   cells compared to un-stimulated and ND T cells?

5.1.2 ND and MM T cell proliferation in response to IL-2 with and
without T cell pre-stimulation.

In the published experiments that showed pre-stimulation of MM T cells with anti-
CD3 antibody and IL-15 improved the proliferative response of MM T cells to IL-2,
we used tissue culture plates pre-coated with anti-CD3 antibody. This is a
relatively inefficient method to stimulate T cells, as the antibody is only available in
one plane of the T cell at a time. Physiologically, T cells are stimulated in 3-
dimensions, and grow best when allowed to form cell balls or clusters. To improve
the efficiency of our pre-stimulation system we substituted the pre-coated plates
with T cell activation beads (a kind gift, Dr John Campbell, Miltenyi Biotec), which are inert, magnetic particles conjugated to anti-biotin monoclonal antibody. This facilitates the beads being loaded with biotinylated molecules, in our case anti-CD2, anti-CD3 and anti-CD28.

T cells were collected from ND and MM patients at the time of stem cell harvest and prepared by density gradient separation and anti-CD3 magnetic bead isolation. Sixty percent of the T cells were cryo-preserved, while 40% were pre-stimulated with beads and either IL-2 or IL-15 (MM cells only). Prior to re-stimulation with IL-2, the beads were removed from the T cells, resuspended at 5x10^6/ml in T cell medium and cultured overnight in 6 well tissue culture plates to rest. The cryo-preserved T cells were recovered, washed and cultured overnight in T cell medium, in a 6 well plate at a density of 5x10^6/ml. Both cryopreserved and Pre-stimulated T cells were then used in T cell proliferation and DC co-culture experiments.

The following morning, the rested T cells were harvested into 15ml tubes, centrifuged at 1200rpm for 5mins and resuspended in fresh warm T cell medium at 5x10^5/ml. The proliferation assays were then set up with 2 quad sets of 200μl samples for each test condition. Interleukin-2 was added to one set of samples, final concentration 20μ/ml, while the other set of samples acted as an un-stimulated control. The results were analysed using the mean values of the total counts for each sample quad minus the mean of the background rate of proliferation in the un-stimulated control quad (Figure 5-1).

There was no statistical difference between the un-stimulated ND (mean 13329±2569) and MM (mean 10573±2228) T cell proliferative response to IL2. The IL-2 proliferative response of either ND (mean 22643±3403) or MM (mean
24772± 5250) T cells pre-stimulated with beads and IL-2 was significantly enhanced as compared to un-stimulated cells (p<0.05 and p<0.01 respectively). The response of the IL-15 and bead pre-stimulated MM T cells (mean 12525±3130) was not improved.

![Box plot showing T cell proliferation](image)

**Figure 5-1 ND and MM T cell proliferation in response to IL-2 with and without pre-stimulation**

T cells from ND and MM patients were isolated using anti-CD3 magnetic beads at the time of stem cell harvest. An aliquot of cells was pre-stimulated with T cell activation beads and either IL-2 or IL-15 (MM patients only) for 3 days. The remaining cells were cryopreserved and recovered on day 3. Both the activated and cryopreserved cells were rested overnight and then set up at 1x10^6/ml in a T cell proliferation assay with and without IL-2 (20IU/ml) and read on day 3 having been exposed to ^3H-thymidine for the final 18hrs.

These findings would appear to be at odds with our previously published results. However, our previous results were obtained using cells from MM patients at the time of diagnosis. The cells used in these experiments were obtained at a time when the patients have had a significant amount of therapy, and the disease
burden is likely to be at a much lower level. Consequently, any suppressive effects of tumour cell derived TGFβ may be less pronounced or absent.

5.1.3 The proliferative response of ND and MM cryopreserved and pre-stimulated T cells to autologous matDC in the presence and absence of IL-2

In our next set of experiments we examined the T cell proliferative response, with and without pre-stimulation, to autologous matDC from ND and MM patients in the presence and absence of exogenous IL-2. T cells and monocytes were obtained at the time of stem cell harvest, and purified by density gradient centrifugation and magnetic bead isolation. Mature dendritic cells were generated with GM/IL-4 or GM/IL-13 and simultaneously loaded with antigen (U266 apoptotic cells or lysate preparation) and matured with the X4 or MIMIC cytokine combination. T cells were pre-stimulated with T cell activation beads, and either IL-2 or IL-15 for 4 days, then rested overnight. An aliquot of T cells was cryopreserved to act as an un-stimulated control.

A proliferation assay was set up with a DC:TC ratio of 1:10 in the presence and absence of IL-2 (20IU/ml), with matDC alone and T cells + IL-2 as controls. The cultures were analysed after 3 days incubation at 37°C and the results are expressed as the mean number for DC:TC or DC:TC:IL-2 quads minus the means of the DC and T cell quads alone, as shown in Figure 5-2.

The most striking observation was that pre-stimulation of T cells blocks the ability of ND (mean 3815 ± 1632) and MM (mean 2492 ± 874) T cells to proliferate in response to matDC as compared to un-stimulated T cells (ND mean 12794 ± 1455 and MM 10890 ± 2542). The proliferative response of un-stimulated ND and MM T
cells to matDC alone was similar (mean 12794 ± 1455 vs. mean 10890 ± 2542) as was the result obtained using pre-stimulated T cells and matDC in the presence of IL-2 (ND mean 31501 ± 3362 vs. MM 30583 ± 2397).

Figure 5-2 ND (A) and MM (B) patient un/pre-stimulated T cell proliferative response to autologous matDC in the presence and absence of IL-2

ND and MM T cells and monocytes were isolated at the time of stem cell harvest. An aliquot of T cells was cryopreserved to act as controls; the remainder were stimulated for 4 days with T cell activation beads and either IL-2 or IL15 and the rested overnight. Monocytes were cultured with GM/IL-4 or GM/IL-13 for 4 days, and then simultaneously loaded with antigen and matured with the X4 or MIMIC cytokine combinations. The Proliferation assays were set up in quadruplicate at a DC:TC ratio of 1:10 ± IL-2 (20IU/ml) with stimulated and un-stimulated T cells. T cells ± IL-2 and DC alone were used as positive and negative controls. The results represent the mean of each test quad minus the means of the T cells and DC alone quads.

There was a trend for the ND T cell +DC + IL-2 proliferation (mean 31259 ± 3838) to be higher than that seen in the MM patients (mean 24246 ± 4381), but this did not achieve statistical significance. The pre-stimulated MM T cells proliferated better in response to matDC and IL-2 (mean 30583 ± 2397), as compared to the un-stimulated MM T cells (mean 24246 ± 4381), but this again failed to achieve statistical significance.
We went on to examine whether the DC maturation cytokine combination or the cytokine used for the T cell stimulation had an impact on the proliferative response of the T cells to DC in the presence and absence of IL-2 (Figure 5-3).

Figure 5-3 ND and MM patient un-stimulated and pre-stimulated T cell proliferative response to autologous X4 and MIMIC matDC

ND and MM T cells and monocytes were isolated at the time of stem cell harvest. An aliquot of T cells was cryopreserved to act as controls; the remainder were stimulated for 4 days with T cell activation beads and IL-2 or IL15 and the rested overnight. Monocytes were cultured with GM/IL-4 or GM/IL-13 for 4 days, and then simultaneously loaded with antigen and matured with the X4 or MIMIC cytokine combinations. The Proliferation assays were set up in quadruplicate at a DC:TC ratio of 1:10 ± IL-2 (20IU/ml) with stimulated and un-stimulated T cells. T cells ± IL-2 and DC alone were used as positive and negative controls. The results represent the mean of each quad, minus the means of the T cells and DC alone quads.
Although the proliferation of un-stimulated T cells was greater with MIMIC than X4 matDC in both the ND and MM patients, there were no significant differences in the ability of ND or MM patient matDC generated with X4 or MIMIC to induce control or pre-stimulated T cell proliferation and the results are summarised in Table 5-1.

<table>
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<th>Pre Stim DC TC</th>
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<td>± 5279</td>
<td>± 1363</td>
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<td>5032</td>
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<td>± 3326</td>
<td>± 5045</td>
<td>± 1115</td>
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<td>27107</td>
<td>2476</td>
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<td>± 2972</td>
<td>± 5981</td>
<td>± 1462</td>
<td>± 4104</td>
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Table 5-1 ND and MM patient mean and SEM of control and pre-stimulated T cell proliferation in response to X4 and MIMIC matDC

We found that there was a trend for the proliferation of MM T cells pre-stimulated with beads and IL-2 in response to matDC, in the presence of IL-2 (mean 33626 ± 2936) to be greater than MM T cells stimulated with beads and IL-15 (mean 23737 ± 7458), but this just failed to achieve statistical significance (p=0.055), Figure 5-4.

In summary, we found that T cells collected at the time of stem cell harvest are able to proliferate relatively normally in response to IL-2. Pre-stimulation of ND and MM patient T cells with anti-CD2, anti-CD3 and anti-CD28 coated beads, and IL-2, significantly increases their proliferative response to IL-2, but pre-stimulation of MM T cells with beads and IL-15 had no significant effect.
Figure 5-4 The proliferative response of ND and MM patient T cells pre-stimulated with beads and IL-2 or IL-15 to matDC in the presence of IL-2

ND and MM T cells and monocytes were isolated at the time of stem cell harvest. An aliquot of T cells was cryopreserved to act as controls; the remainder were stimulated for 4 days with T cell activation beads and IL-2 or IL-15 and the rested overnight. Monocytes were cultured with GM/IL-4 or GM/IL-13 for 4 days, and then simultaneously loaded with antigen and matured with the X4 or MIMIC cytokine combinations. The Proliferation assays were set up in quadruplicate at a DC:TC ratio of 1:10 + IL-2 (20IU/ml) with stimulated and un-stimulated T cells. T cells + IL-2 and DC alone were used as positive and negative controls. The results represent the mean of each quad minus the means of the T cells and DC alone quads.

Pre-stimulation blocks the proliferative response of ND and MM patient T cells to autologous matDC alone. Although the proliferation of pre-stimulated T cells in the presence of IL-2 and mat DC was higher than the un-stimulated T cells, it did not achieve statistical significance. The proliferation of MM T cells pre-stimulated with beads and IL-2 induced by autologous mat DC in the presence of IL-2 was significantly better than that achieved by those pre-stimulated with beads and IL-15.
5.1.4 The measurement of cytokines secreted during the T cell matDC interaction by Luminex technology from MM patients and ND

The interaction between T cells and DC is complex, and involves both contact and cytokine dependant processes. Cytokines, such as IL-2, may be secreted within the immunological synapse, while others may be secreted in a more generalised manner. Traditionally these cytokines have been detected using a solid phase (96 well plate) ELISA for each analyte to be measured, making it difficult to measure a number of cytokines because of cost, the size of the sample required and the time taken for each ELISA. Luminex technology allows multiple different ELISAs to be performed concurrently on the same sample in the same well (Carson and Vignali 1999).

This has been achieved by linking the capture antibody for each analyte to a polystyrene bead that has a unique and fixed fluorescence signature (Vignali 2000). This signature is achieved by mixing two fluorescent dyes in up to 100 different ratios to allow labelling of 100 different analytes simultaneously. The detection antibody is biotinylated, and the binding of a streptavidin–PE conjugate to the detection antibody allows the system to identify beads that have bound to their specific analyte. The detection system is based around two lasers and a flow cell similar to that of a flow cytometer. The sample is passed through the beams of each laser sequentially in a stream that allows one bead to be interrogated by the laser at a time. The first laser determines the identity of the bead (i.e. which analyte is being detected), and the second laser determines whether there is analyte bound to the bead, i.e. is there Strep-PE bound to the bead? The machine aspirates a fixed volume of sample and the concentration of beads is fixed,
allowing the analysis software to calculate the concentration of each analyte to be measured.

The DC and T cells used in these experiments were prepared as described above in 5.1.3, washed once in fresh T cell medium and co-cultured at a ratio of 1 DC (1x10^5/ml) to 10 T cells (1x10^6/ml) in 24 well tissue culture plates. The supernatants were collected after 3 days, snap frozen in 1.5ml Eppendorf tubes and stored at minus 80°C. A multiplex kit was used to measure GM-CSF, IFN-α, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12p70, IL-15, TNF-α, and VEGF. The assay was performed as per the manufacturers instructions and read on a Luminex 100™ instrument. Each sample was analysed in duplicate and the results were expressed as the mean of the two observations. Data was obtained from 38 DC T cell supernatants, from 6 ND and 4 MM patients and is shown in Figure 5-5. The T cells from 2 ND and MM patients had been pre-stimulated with beads and IL-2, and un-stimulated T cells were used from all 10 subjects.

The most striking observation was that there was almost no IL-2 detected in the supernatant from the reactions in which T cells that had been pre-stimulated with T cell stimulation beads (mean 0.3pg/ml ± 0.1), as compared to un-stimulated cells (mean 98.2pg/ml ± 33.42), and this was highly statistically significant (p ≤ 0.001). The level of IL-10 detected in the un-stimulated T cell group (mean 62pg/ml ± 32) was significantly higher than the bead-stimulated cells (mean 2pg/ml ± 2, p ≤ 0.01). The amount of GM-CSF (mean 348pg/ml ± 168, p ≤ 0.05) and TNF-α (mean 991pg/ml ± 470, p ≤ 0.01) was also significantly higher in the un-stimulated T cell reaction (mean 24.8pg/ml ± 14.6, mean 18.6pg/ml ± 9.4 respectively) as compared to the bead-stimulated cells.
Figure 5-5 IL-2, IL-10, GM-CSF and TNF-α levels detected in the supernatants of ND and MM DC and T cell co-cultures by Luminex™ technology

To facilitate antigen presentation, DC from ND (n=6) and MM patients (n=4) produced from monocytes collected at the time of stem cell harvest, cultured with GM/IL-4 or GM/IL-13, Ag primed with U266 cell lysate and matured with X4 or MIMIC cytokine combination were co-cultured with T cells pre-stimulated with beads and IL-2 (n=4, MM=2, ND=2) or cryopreserved un-stimulated cells (n=10, MM=4, ND=6). The supernatants were harvested after 3 days and the levels of IL-2, IL-10, GM-CSF and TNF-α were measured using the Luminex system.

There was some concern that these levels could, at least in part, have been due to the cytokines added to the DC cultures during the generation of maturation phase. However, all cells were washed prior to the co-culture experiments, and the DC used in each arm of the experiments had had identical treatment and cytokine exposure.

The level of IL-6 detected in the un-stimulated T cell group (mean 48.8pg/ml ± 22.5) was similar to the bead stimulated T cell group (mean 31.0pg/ml ± 4.8)
**Figure 5-6.** However, a statistically higher amount of IL-6 was detected in the supernatants from DC matured with X4 cocktail (mean 57.05 pg/ml ± 9), which contained no IL-6, as compared with MIMIC matured cells (mean 31.8 pg/ml ± 6, \( p < 0.005 \)), which were exposed to 10 ng/ml IL-6.

**Figure 5-6 IL-6 levels detected in the supernatants of ND and MM DC and T cell co-cultures by Luminex™ technology**

To facilitate antigen presentation, DC from ND and MM patients produced from monocytes collected at the time of stem cell harvest, cultured with GM/IL-4 or GM/IL-13, Ag primed with U266 cell lysate and matured with X4 or MIMIC cytokine combination were co-cultured with T cells pre-stimulated with beads and IL-2 or cryopreserved un-stimulated cells. The supernatants were harvested after 3 days and the levels of IL-6 were measured using the Luminex system.

We did not detect any significant levels of IL-4, IL-7, IL-12p70, IL-15, IFN-α or VEGF. Interestingly, no differences in cytokine levels were detected between ND and MM cells, or between the cytokines used to generate the DC.

In summary the Luminex™ system is a reliable, straightforward method to detect multiple cytokines simultaneously in small volume samples. Pre-stimulation of T cells with T cell activating beads and IL-2 significantly reduces the amount of GM-CSF, IL-2, IL-10 and TNF-α detected in the supernatants of T cell, DC co-cultures, as compared to those using un-stimulated cells. The IL-6 detected was higher in the X4 matured DC cells. No significant amounts of IL-4, IL-7, IL-12p70, IL-15, IFN-α or VEGF were found.
5.1.5 *In-vitro cytotoxicity of ND and MM T cells following co-culture with autologous matDC and the effect of T cell pre-stimulation with T cell activation beads and IL-2*

The anti-tumour effects of DC based immune therapies are mainly mediated via cytotoxic T cells, although a number of groups are investigating effects that may be induced by the interaction of DC with NK and NKT cells. In order to quantify the potency of the cytotoxic T cells generated from ND and MM patients, it was decided to use a modification of the flow cytometry based assay in which fluorescently stained target tumour cells are co-incubated with un-labelled effector cells. This allows the target cells to be easily gated and then the number of dead target cells can be quantified by annexin-V (a marker of early apoptosis) and either PI or 7AAD (necrotic) staining (Derby, et al 2001, Fischer, et al 2002). In these analyses 7AAD was used rather than propidium iodide because it has much less spectral overlap and is therefore much easier to compensate.

The first set of experiments investigated whether the pre-stimulation of ND and MM patient T cells with T cell activation beads and IL-2, and then further expansion with IL-2 alone, was sufficient to induce significant cytotoxicity using the annexin-V/7AAD assay. T cells were stimulated for 3 or 4 days with T cell activating beads and IL-2. The beads were then removed and the T cells were expanded in IL-2 supplemented TC medium for 2-4 weeks. Prior to use in the cytotoxicity assay dead cells were removed by density gradient centrifugation. Cytotoxicity assays were set up in duplicate with effector: target cell ratios ranging from 16:1 to 1:16, cultured for 18hrs and the assays were read by flow cytometry within 1 hour of annexin-V and 7AAD staining. A total of 20,000 target cell events were collected per tube and representative plots are shown in Figure 5-7.
Figure 5-7 Cytotoxicity assay gating for controls and 2 dilutions

The left-hand plot shows that only cellular events are gated in R1. The middle plot shows that T cells are excluded from the analysis using R2. The right-hand plot shows that >95% are alive control target cells in the logic gate G3 (R1 and R2), less than 1% of T cells appear in the analysis, and that the cytotoxic effects of the T cells are dose dependant.
The results for each dilution were expressed as the mean number of dead test cells minus the number of dead cells in the Target alone control and the results are shown in Figure 5-8.

![Graph showing percentage target cell kill vs effector target cell ratio for ND and MM bead pre-stimulated T cell cytotoxicity assay.](image)

**Figure 5-8 ND and MM bead pre-stimulated T cell cytotoxicity assay**

T cells from ND (n=4) and MM (n=5) patients were collected at the time of stem cell harvest, pre-stimulated with T cell activating beads and IL-2 and expanded with IL-2 for 2-4 weeks. They were placed in culture with PKH-26 labelled U266 MM cell targets at effector target ratios between 16:1 and 1:16, incubated overnight, and the degree of target cell kill was assessed using Annexin-V and 7AAD staining by flow cytometry.

There was a small cytotoxic effect detected at the highest effector target ratio 8:1 using MM bead T cells, with a mean of 29.5% ± 8.3 of targets killed, and at 16:1 with ND bead T cells, 18.4% ± 7.4. At the effector target ratio of 8:1, MM pre-stimulated T cells produced statistically more tumour cell kill than ND pre-stimulated T cells (p < 0.005). There was no difference in the cytotoxic activity of pre-stimulated T cells from ND or MM patients at any other dilution.
We next compared the cytotoxic activity of MM pre-stimulated T cells with T cells that had also had been primed with antigen loaded autologous DC, and unstimulated T cells that had been primed with antigen loaded autologous DC, Figure 5-9.

![Graph showing cytotoxicity of pre-stimulated MM T cells, DC primed pre-stimulated MM T cells and DC primed un-stimulated MM T cells.](image)

**Figure 5-9 Cytotoxicity of pre-stimulated MM T cells, DC primed pre-stimulated MM T cells and DC primed un-stimulated MM T cells**

T cells from MM patients (n=5) were collected at the time of stem cell harvest. An aliquot of cells was cryopreserved and the remainder were pre-stimulated with T cell activating beads and IL-2 for 3-4 days. An aliquot of pre-stimulated T cells was expanded with IL-2 for 2-4 weeks as an antigen negative control. The remainder of the pre-stimulated cells, and the cryo-preserved un-stimulated T cells, were primed with autologous antigen loaded DC, expanded in IL-2, re-stimulated with DC after 7 days and expanded in IL-2 for 3-6 weeks. T cells were placed in culture with PKH-26 labelled U266 MM cell targets at effector target ratios between 8:1 and 1:8, incubated overnight, and the degree of target cell kill was assessed using Annexin-V and 7AAD staining by flow cytometry.
Pre-stimulated MM T cells primed with autologous DC were significantly more effective at killing target cells, compared to un-stimulated, DC primed, MM T cells with \( p < 0.05 \). Pre-stimulated T cells that had been primed with autologous DC were significantly more effective at killing target cells at all ET ratios compared to T cells that had only been pre-stimulated with beads and IL-2 \( p < 0.05 \). Although un-stimulated DC primed T cells were more effective at killing targets compared to pre-stimulated only T cells, this did not reach statistical significance at any effector target ratio.

The results obtained using ND pre-stimulated T cells were then compared with those generated using ND un-stimulated T cells, primed with autologous antigen loaded DC, Figure 5-10. Again no difference was observed between the two groups at any effector target ratio.

**Figure 5-10 Cytotoxicity of pre-stimulated ND T cells and DC primed un-stimulated ND T cells**

T cells from ND \( (n=5) \) were collected at the time of stem cell harvest. An aliquot of cells was cryopreserved and the remainder were pre-stimulated with T cell activating beads and IL-2 for 3-4 days and expanded with IL-2 for 2-4 weeks as an antigen negative control. The cryo-preserved un-stimulated T cells were primed with autologous antigen loaded DC, expanded in IL-2, re-stimulated with DC after 7 days and expanded in IL-2 for 3-6 weeks. T cells were placed in culture with PKH-26 labelled U266 MM cell targets at effector target ratios between 8:1 and 1:8, incubated overnight, and the degree of target cell kill was assessed using Annexin-V and 7AAD staining by flow cytometry.
In the final analysis the effectiveness of MM pre-stimulated and un-stimulated T cells were compared with un-stimulated ND T cells, which had all been primed with autologous antigen loaded DC, Figure 5-11.

![Graph showing cytotoxicity comparison](image)

Figure 5-11 Comparing the cytotoxicity of DC primed pre-stimulated MM T cells (n=19) with DC primed un-stimulated MM (n=14) and ND T cells (n=20)

T cells from ND (n=5) and MM patients (n=5) were collected at the time of stem cell harvest. T cells were cryopreserved. An aliquot of MM T cells were pre-stimulated with T cell activating beads and IL-2 for 3-4 days and then rested overnight. All T cells were primed with autologous antigen loaded DC, expanded in IL-2, re-stimulated with DC after 7 days and expanded in IL-2 for 3-6 weeks. T cells were placed in culture with PKH-26 labelled U266 MM cell targets at effector target ratios between 8:1 and 1:8, incubated overnight, and the degree of target cell kill was assessed using Annexin-V and 7AAD staining by flow cytometry.

Multiple myeloma DC primed, pre-stimulated T cells were significantly better at killing target tumour cells compared to ND un-stimulated DC primed T cells.
T cells from MM patients that had only been primed with DC were significantly better at killing targets at the highest two effector target ratios \( p < 0.001 \).

In summary, it was found that the flow cytometry based cytotoxicity assay was an effective way of determining the killing ability of T cell line generated by pre-stimulation with T cell activating beads and/or antigen loaded autologous DC. The most effective cytotoxic cells were MM patient T cells that had been pre-stimulated and primed with DC, followed by MM patient T cells that had only been primed with DC. T cells from ND primed with DC, MM patient and ND T cells pre-stimulated with T cells activating beads and IL-2 were relatively ineffective at killing target cells in this assay. This observation may be linked to the conditions under which the cells were originally collected.

### 5.1.6 Discussion

Immune based therapies are normally considered for use in patients who have malignant disease, such as MM. When designing the methods for the production of these therapies we must take into account that the immune systems in these patients will almost certainly not be normal. This may be due to a number of factors, such as immune suppression by the malignant clone, the effects of previous therapy and more general issues such as nutrition and co-morbidities.

The cells used in these experiments were collected at a time when the MM patients had received at least 6 courses of MM induction therapy and a course of intravenous cyclophosphamide prior to the start of G-CSF for the mobilisation of stem cells. In contrast, the previously published experiments (Campbell, *et al* 2001) used cells from MM patients who were at diagnosis and so the immune systems were likely to have been subject to a greater disease burden at that time.
This is likely to be at least part of the explanation for the current finding that there was no difference between the abilities of ND and MM patient T cells to proliferate in response to IL-2. Thus we should aim to collect immune effector cells at a time when the disease is either in remission, or is at its lowest level during the disease course. This observation may be crucial in determining the success of immune therapies for MM patients, but also for patients with other malignant diseases that induce immune suppression by secreting cytokines such as TGF-β, IL-10 and IL-6.

Pre-stimulation of MM T cells with T cell activation beads and IL-2 significantly up-regulated the T cell proliferative response to IL-2, in a similar manner to that seen in ND cells. In contrast to the previous experiments, we did not see an improved response when MM T cells were pre-stimulated with beads and IL-15. This is again likely to be due to the more normal baseline responses of the MM T cells that we used in these experiments. However, the activation beads deliver a co-stimulatory signal via CD28 as well as cross-linking T cell CD3 in three dimensions. This is a more physiological stimulus compared to plate bound anti-CD3 alone, and this may contribute to the improved level of proliferation of MM T cells that were pre-stimulated with beads and IL-2, compared to beads and IL-15. It was hoped that this would translate into a better response to autologous antigen loaded DC.

There was a normal proliferative response by MM un-stimulated T cells to autologous antigen loaded DC in the presence and absence of IL-2. However, the pre-stimulated T cells completely failed to proliferate in response to autologous DC alone. The same cells proliferated normally in response to autologous DC in the presence of IL-2, suggesting that these cells had been rendered IL-2 dependant. This is of some concern if these cells were to be used as part of an immune based therapy in MM patients as IL-2 would be need to be given in conjunction to ensure
an adequate initial proliferative response to antigen presenting cells. It is likely that this would have to be continued for a time afterward to ensure the survival of any T cell clones that were produced by this interaction. We do not currently know what the minimum concentration of IL-2 is which ensures this response, or whether this is safely achievable in patients. IL-2 has been given to patients by both IV infusion and SC injection, however the IV route is associated with a number of potentially fatal side-effects, making the SC route the only feasible option. These observations were important when interpreting the data obtained by the Luminex™ analysis of the cytokine levels in the supernatants from the DC T cell interactions.

The Luminex™ system is a convenient way to examine the levels of multiple cytokines in small volume samples. This method provided another way in which the DC T cell interactions could be investigated to see how the pre-stimulation of T cells modifies the process. This system was able to measure levels of IL-2, IL-6, IL-10, GM-CSF and TNF-α in the supernatants obtained from DC: TC co-cultures. With the exception of IL-6, the level of these cytokines in the supernatants collected from the interactions between DC and pre-stimulated T cells were significantly lower than that seen with DC and un-stimulated T cells. This suggests that the pre-stimulation process is interfering with the DC T cell interaction. It is most likely that both pre-stimulated CD4+ and CD8+ cells are unable to secrete these cytokines in the absence of IL-2.

The level of IL-6 detected did not vary in respect to the T cell pre-stimulation; rather it varied in respect to the maturation stimulus received by the DC. DC matured by X4 produced significantly more IL-6 as compared to MIMIC mature DC. This is in keeping with data from Fillatreau's group in Berlin, who have shown that DC actively secrete IL-6 in response to microbial products or CD40L, and was
highest in cells that had received both (www.drfz.de/pdf/ar04/aq_fillatreu04.pdf and personal communication).

At first glance this combination of cytokines may seem a little confusing, being a combination of TH1 and TH2 type responses. When the pattern is examined as a whole it becomes clear that the cells are attempting to induce a pro-inflammatory environment favouring the development of a CD8 cytotoxic T cell response, **Figure 5-12.** Pre-stimulation of the T cell blocked the secretion of all T cell derived cytokines, but had no effect on matDC production of IL-6. Currently, there is no data to determine whether supplementation of the matDC: pre-stimulated T cell co-cultures is able to reinstate the cytokine response of the T cells, or whether 20IU/ml of IL-2 simply over-rides these mechanisms and directly induces T cell proliferation, enhanced by the DC antigen and co-stimulatory signals. However, in view of the fact that matDC primed, pre-stimulated T cells produce the best target cell kill in the cytotoxicity assay, it can be speculated that when IL-2 was added to the matDC: T cell co-cultures, at least some of these mechanisms were re-instated.

The flow cytometry based cytotoxicity assay was easy to handle, as compared to the more traditional radioactive chromium release assay, although great care is needed when setting up the initial compensations. Both ND and MM patient pre-stimulated T cells produced some target cell killing at the highest effector: target cell ratios. This is most likely due to effects mediated by minor histocompatibility antigen mismatch, as all effector cells were HLA-class I (HLA-A2) matched with the U266 cell line (Kloosterboer, et al 2005). Interestingly, pre-stimulated MM T cells produced statistically better killing than ND pre-stimulated T cells at the highest E:T ratio. There are a number of likely explanations for this observation.
Figure 5-12 Cytokine interactions during co-culture of matDC and un-stimulated T cells to facilitate antigen presentation

Mature DC present antigen to T cells in the context of co-stimulatory markers. This activates both CD4 and CD8 T cells in an antigen dependant manner. The CD4 cells produce IL-2, IL-10 and TNF-α, which along with IL-6 from the matDC, preferentially induce the CD8 T cells to proliferate and differentiate into cytotoxic T cells. The CD8 cells also secrete IL-10, which recruits other CD8 cells by chemotaxis. In-vivo, the GM-CSF produced by activated CD8 T cells and other cells, such as fibroblasts and endothelial cells, under the influence of TNF-α, induce monocytes to differentiate into DC, completing the loop.

It is known that MM patients have circulating T cells that have anti-MM activity (Beckhove, et al 2003, Yi, et al 1995). Thus the pre-stimulation process may simply be un-masking this activity. However, the MM cells were collected after a number of courses of chemotherapy and immediately after a course of IV cyclophosphamide, a very powerful T cell immunosuppressive agent (Winkelstein 1973). It is possible that these pre-treatments are depleting the MM patients of regulatory cells, thus allowing greater anti-MM cytotoxic activity. It may be possible
to differentiate between these two possibilities by repeating these experiments using cells mobilised with a similar regime in non-MM patients, such as those with follicular lymphoma, who should not be able to mount an anti-MM memory T cell response. These two explanations may also help us understand the results obtained using ND and MM patient matDC primed T cells.

The cytotoxic activity of ND matDC primed un-stimulated T cells was significantly less than that seen in cells from MM patients. The ND T cells and monocytes were collected after G-CSF alone. This is known to induce T cell hypo-responsiveness (Jun, et al 2004) and increase the number of tolerogenic DC present in the graft (Lonial, et al 2004), which may go some way to explain the reduced incidence of acute GVHD following the use of mobilised stem cells. The cytotoxicity of the MM cells was further enhanced by the pre-stimulation step. Again this could be because we are simply un-masking and enhancing the inherent anti-MM potential of the MM patient T cells, but there was a substantial DC dependant increase in the target cell killing ability of the MM patient T cells. This would favour the hypothesis that collecting the T cells following immunosuppressive chemotherapy has down regulated any suppressive effects induced by the G-CSF and the disease.

In summary, pre-stimulation of MM T cells with beads and IL-2 enhanced their proliferative response to IL-2 and matDC+IL-2, but blocked proliferation in response to matDC alone. Pre-stimulation also blocked T cell cytokine secretion in response to DC alone. Despite this, pre-stimulated matDC primed T cells produced the best killing of MM tumour cell targets, followed by un-stimulated matDC primed T cells, with ND un-stimulated matDC primed T cells producing poor cytotoxic effects. These results suggest that a combined immunotherapy
approach using pre-stimulated T cells, MM Ag primed DC and IL-2 may produce better clinical responses in MM patients.
6. Results 4

6.1 Chemokine receptor expression/activity on monocytes and monocyte derived dendritic cells assessed by fluorescent labelled chemokine uptake

6.1.1 Introduction

The interaction between chemokines and their receptors is vital to the function of the immune system. The homeostatic migration of cells such as DC and T cells, from the bone marrow to peripheral tissues and then lymph nodes, is governed by changes in of chemokine receptor gene expression during differentiation and maturation, and their interaction with ligands that are constitutively expressed by tissues. Inflammatory processes up-regulate inducible CCL, inducing leukocyte chemotaxis towards pro-inflammatory CCLs, such as CCL3 (Figure 1-3). Professional antigen presenting cells, such as DC, mature during these interactions. This induces down regulation pro-inflammatory CCRs, and up regulation of CCRs, such as CCR7, that promote APC migration to lymph nodes, facilitating Ag presentation by the co-localisation of naive T cells, which also express CCR7.

The chemokine expression pattern of the tumour may also have a direct bearing on the host’s ability to mobilise monocytes, DC, T cells and NK cells against the tumour. The chemokine balance within the patient and the tumour may be crucial in determining the type of immune response that is mounted, in that the tumour may shift the balance from an immunogenic response to a toleragenic response by the expression of a different chemokine ‘signature’.

The study of the chemokine system is made more difficult by the structural constraints imposed by the GPCR, which makes it difficult to produce good quality
antibodies for use in experimental work. In addition, antibody staining does not give any indication of functional receptor activity. During the experiments in the previous chapters, the level of CCR7 expression on matDC, as detected by surface antibody staining, was not as good as expected in view of the level of expression of the other co-stimulatory markers such as CD40, CD80, CD83 and CD86. This led us to explore other ways in which we could examine chemokine receptor expression and function on normal and MM DC and monocytes.

Biotinylated chemokines (biokines), such as CCL3 (Bio-CCL3) and CCL19 (Bio-CCL19), can be synthesized with an additional biotinylated lysine residue on the C terminus (Ravkov, et al 2003, Weber, et al 2004). These biokines retain the full functional activity of the wild type molecules, and they can be labelled with streptavidin-linked fluorochromes. The labelled biokine retains its ability to bind to CCR on the surface of the target cell and, crucially, to be internalised. This leads to accumulation of fluorochrome within the cell, which can be detected by fluorescence microscopy or flow cytometry. The main advantage of these techniques is that they allow analysis to be performed on a cell-by-cell basis, rather than on the population as a whole, as is the case when using radio labelled CCL. These reagents have been used to great effect in the analysis of D6 and CXC-CKR by local collaborator Dr R.J.B. Nibbs (Weber, et al 2004) and others (Ravkov, et al 2003). This method can be combined with other fluorescent surface markers, allowing further characterisation of cells taking up the fluorescent ligand.

In view of the recent evidence, the expression of CCR8 on MM monocytes and DC compared to cells from normal individuals was of considerable interest (Qu, et al 2004, Sebastiani, et al 2002). There were no reliable anti-CCR8 antibodies available at the time these experiments were performed. Therefore alternative
methods to detect the level of CCR8 surface expression are required. To address these issues, the use of biotinylated chemokines was explored.

### 6.1.2 Optimisation of fluorescent chemokine uptake in CCR transfected cell lines and monocyte derived DC

Firstly, to establish these approaches in the lab, HEK293 cells that had been stably transfected to express D6 or CCR7 were assessed for their ability to take up Bio-CCL3 or Bio-CCL19 respectively. These biolkines were mixed with PE-coupled streptavidin and these complexes or Strep-PE alone were fed to harvested cells. As can be seen in Figure 6-1, D6 or CCR7 expressing cells became strongly fluorescent after incubation with Bio-CCL3/PE or Bio-CCL19/Pecomplexes respectively. Parental untransfected HEK293 cells failed to internalise these tagged chemokines, (Data not shown). These assays act as positive controls for the subsequent experiments on primary human material.

**Figure 6-1 Fluorescent chemokine uptake in HEK cells stably transfected with D6 and CCR7**

HEK 293 cells transfected with D6 (A) or CCR7 (B) were analysed for their ability to take up fluorescently labelled biokines, CCL3 (A) and CCL19 (B), as compared to control samples stained with strep-PE alone.
For the next experiment, iDC were generated from CD14 selected monocytes, by culture in GM-CSF and IL-4 for 4 days. On day 4 an aliquot was removed for analysis and the remaining cells were fed with GM-CSF and IL-4 supplemented media, pulsed with U266 lysate as a source of antigen and cultured over night. On day 5, an aliquot of antigen pulsed iDC was removed for analysis and the remaining cells were then divided in half for maturation. The antigen-loaded iDC were cultured with either the MIMIC or X4 maturation cocktail and incubated overnight prior to analysis. The biokine uptake assays were all performed in 500ul, on the same day as the aliquots were removed from the parent culture. The surface expression CD1a, 1c, 14, 40, 80, 83, 86, HLA class I, HLA DR, CCR5 and CCR7 of each sample was determined by surface antibody staining and flow cytometry. The results of the surface phenotype and fluorescent chemokine uptake are shown in Figures 6-2a-d.

Figure 6-2a Fluorescent chemokine uptake and surface phenotype of iDC

Immature DC, generated from CD14 positive monocytes were analysed after 4 days of culture in GM-CSF and IL-4 supplemented DC medium. Live cells have been gated on forward and side scatter. The uptake of fluorescent Bio-CCL3 and Bio-CCL19 is shown in the upper histogram plots and the surface antibody staining is shown in the lower dot plots.
Figure 6-2b Fluorescent chemokine uptake and surface phenotype of iDC post antigen loading with tumour lysate

Immature DC were loaded with tumour antigen by adding U266 cell lysate to the culture medium, incubated overnight and analysed on D5 of culture. Live cells have been gated on forward and side scatter. The uptake of fluorescent Bio-CCL3 and Bio-CCL19 is shown in the upper histogram plots and the surface antibody staining is shown in the lower dot plots.

Figure 6-2c Fluorescent chemokine uptake and surface phenotype of MIMIC matured DC

Antigen loaded iDC were given the MIMIC cytokine cocktail as a maturation stimulus and analysed after overnight culture. Live cells have been gated on forward and side scatter. The uptake of fluorescent Bio-CCL3 and Bio-CCL19 is shown in the upper histogram plots and the surface antibody staining is shown in the lower dot plots.
Antigen loaded iDC were given the X4 cytokine cocktail as a maturation stimulus and analysed after overnight culture. Live cells have been gated on forward and side scatter. The uptake of fluorescent Bio-CCL3 and Bio-CCL19 is shown in the upper histogram plots and the surface antibody staining is shown in the lower dot plots.

In chapter 4, iDC and antigen-loaded iDC were found to strongly express CCR5 (a CCL3 receptor) by surface antibody staining (Table 4-1). However, using the experimental conditions optimised for use with transfected cell lines, the uptake of Bio-CCL3 was poor (Figure 6-2a+b). In contrast, there was significant uptake of Bio-CCL19 by the matDC, with 61% of the X4 (Figure 6-2d) and 37% of the MIMIC (Figure 6-2c) matured cells becoming positive indicating that these cells were expressing CCR7 or, less likely, CCR11, another receptor capable of binding CCL19. A small number (17%) of antigen-loaded iDC were positive for Bio-CCL19 uptake, whilst still being CCR5 positive by surface antibody staining (Figure 6-2b). This indicated that tumour lysate alone was sufficient to initiate the up regulation of CCR7 activity, but a stronger signal is required to complete the process of DC maturation, a matter discussed earlier.

With regard to the suitability of biokine usage, it is first clear that the Bio-CCL19 approach provides a sensitive method for detecting CCL19 receptors, which is
arguably better than CCR7 antibodies. CCL3 uptake was disappointing, as there was little BioCCL3 uptake despite the presence of surface CCR5, as indicated by anti-CCR5 antibody staining.

The amount of fluorescence accumulated by a cell during the biokine uptake assay is dependant upon receptor activity during the assay, biokine concentration and time. In order to optimise the Bio-CCL3 uptake in primary human material, a fluorescent Bio-CCL3 assay was performed with an increased incubation time of 4hrs. A second assay was performed with an increased concentration of fluorescent Bio-CCL3, by reducing reaction volume of the assay from 500μl to 200μl.

Figure 6-3 Reducing the reaction volume from 500μl to 200μl significantly improves Bio-CCL3 uptake by CD14 selected monocytes (n=3)

Normal monocytes were obtained by CD14 selection and suspended in DC medium at 1x10^6/ml. Fluorescent chemokine assays were performed on 200μl and 500μl aliquots of cells. The 500μl aliquot samples were incubated for 2 (plot shown in Figure 6-2a) or 4hrs (above), the 200μl samples were incubated for 2hrs. Controls (Strep-PE alone) were performed for each culture condition, and each condition was set up in triplicate. Samples were analysed by flow cytometry with a negative gate set on 99% of cells in each control sample.
Reducing the reaction volume to 200µl improved the fluorescent chemokine uptake (Figure 6-3), to a level more in keeping with results from surface antibody staining. Increasing the incubation time from 2-4hrs for the 200µl assays only served to increase the level of non-specific uptake of the control. It was decided at this point that all further assays would be performed in 200µl for both Bio-CCL3 and Bio-CCL19 uptake assays, as there was no significant change in the Bio-CCL19 uptake using 200µl samples (data not shown).

6.1.3 Specificity of fluorescent chemokine uptake by competitive blocking with non-biotinylated chemokines

As previously discussed, chemokine receptor-ligand binding is uniquely promiscuous in the way ligands and receptors can bind in a number of combinations. In order to determine whether the uptake of Bio-CCL19 was via CCR7 or CCR11, a blocking step was added to the assay. Aliquots of matDC were pre-stimulated with either non-biotinylated CCL19, to block uptake via CCR7 and CCR11, or CCL25, which should specifically block uptake via CCR11. These unlabelled chemokines were added at a concentration x10 greater than the concentration of CCL19 used in the fluorescent biokine uptake assay. Cells were incubated at 37°C for 1 hour, and then washed twice in warm DC medium. A fluorescent biokine uptake assay was then performed as previously described with a negative control and an untreated sample of matDC (Figure 6-4).
Mature DC, generated from CD14 selected monocytes, cultured in GM-CSF and IL-4 supplemented DC medium for 4 days. The iDC were pulsed with U266 tumour cell lysate overnight and then matured overnight with the X4 cytokine cocktail. Two aliquots of matDC were pre-stimulated for 1 hour with non-biotinylated CCL19 to block uptake via CCR7 and CCR11, or CCL25 to block uptake via CCR11. Unblocked cells were incubated for 1 hour in medium alone. All cells were then washed in warm medium and the fluorescent chemokine assay was performed on blocked and unblocked matDC. Repeat experiments gave similar results.

The CCL19 and CCL25 pre-blocking experiment confirmed that Bio-CCL19 uptake was mediated via CCR7. Pre-stimulation with CCL25 had no effect on Bio-CCL19 uptake (79.22 ± 1.0%, positive, MFI 54.55) as compared to the unblocked positive control (75 ± 0.85%, MFI 47.66). Pre-stimulation with CCL19 significantly reduced Bio-CCL19 uptake (8.74 ± 0.2% positive, MFI 9.67) (p< 0.001).

The task to determine receptor specificity for Bio-CCL3 uptake is a more complex one, as CCL3 can bind to CCR1, CCR3, CCR5 and the decoy receptor D6. To attempt to address this, fluorescent Bio-CCL3 uptake assays were set up as normal with the addition of a ten times excess of unlabelled human (h)CCL3L1, murine (m)CCL3, hCCL3L1-2, hCCL3L1-4 and hCCL4. There are two naturally
occurring isoforms of CCL3 in humans, called CCL3 and CCL3L1. CCL3L1 is viewed as the true human homologue, and binds CCR1, CCR3, CCR5 and D6 (Nibbs, et al 1999). Confusingly, CCL3L1 is also the true functional homologue of murine CCL3. The truncated form CCL3L1-2 is missing the final 2 amino acid residues from the N terminal, and has increased specificity for CCR1 and CCR5, and no ability to bind CCR3 and D6. The CCL3L1-4 protein is missing the N terminal 4 amino acid residues. It retains its ability to bind CCL1, has reduced specificity for CCR-5 and no ability to interact with CCR3 or D6. Finally, CCL4 was included because it binds to CCR5 and D6. The results are shown in Figure 6-5 and it was hoped that the use of these chemokines would give an indication of the Bio-CCL3 receptors present on the cells. The amount of blocking of Bio-CCL3 uptake in each assay was calculated using Equation 2-1.

Significant inhibition was achieved with all CCL3 variants, mCCL3 (mean 57.05 ± 16.24), hCCL3L1 (mean 48.68 ± 13.53), hCCL3L1-2 (mean 45.64 ± 15.64) or hCCL3L1-4 (mean 36.86 ± 12.54). Overall, these data suggest that Bio-CCL3 uptake was mediated by either CCR5 or CCR1. To further elucidate which of these two receptors was responsible for Bio-CCL3 uptake, blocking would need to be performed using CCL7, which has specificity for CCR1, and CCL8, which only binds CCR5. Another method would be to use cell that genetically lack one of the receptors, such as Δ32 homozytes who do not express CCR5. None of these reagents was available at the time these assays were performed. Interestingly, competition with CCL4 did not block Bio-CCL3 uptake but rather induced a significant up regulation of Bio-CCL3 uptake (mean 116.78 ± 17.57, p< 0.005) and the mechanisms that may induce this phenomenon will be discussed later.
Figure 6-5 Competitive blocking of Bio-CCL3 with non-biotinylated chemokines, which have varying CCR specificities, suggests uptake is mediated by CCR5

Monocytes from normal individuals (n=4) were purified by density gradient centrifugation and CD14 magnetic bead selection. The fluorescent chemokine uptake assay was performed for Bio-CCL3 uptake with the addition of 12.5ul of 0.1ug/ml (x10 excess) of the non-biotinylated blocking chemokines hCCL3L1, mCCL3, hCCL3L1-2, hCCL3L1-4 and hCCL4. Samples were incubated for 2 hours and analysed by flow cytometry. Samples were set up in duplicate and the results represent 4 separate experiments. The data shown is the mean percentage blocking of the positive control MFI induced by each chemokine and was calculated as shown in Equation 6-1.

The competitive blocking experiments for Bio-CCL3 uptake shown in Figure 6-5 did not show complete inhibition, as seen when matDC were pre-stimulated with CCL19, prior to a Bio-CCL19 assay being performed (Figure 6-4). Thus, the impact of CCL3 pre-incubation prior to Bio-CCL3 uptake was assessed. Pre-stimulation induces receptor desensitisation, making the cells refractory to subsequent ligand interactions. A pre-stimulation blocking experiment of Bio-CCL3 uptake was performed in triplicate using normal monocytes, cultured with a ten
times excess of hCCL3L1, mCCL3, hCCL3L1-2, hCCL3L1-4 and hCCL4 for an hour at 37°C. The fluorescent Bio-CCL3 uptake assays were performed as previously described. The data in Figure 6-6 shows that pre-stimulation with CCL3L1 (mean 86.03 ± 2.78%, p < 0.03) and CCL3L1-2 (mean 77.94 ± 1.80%, p < 0.03) increased the degree of inhibition of Bio-CCL3 uptake compared to competitive co-blocking (Figure 6-5). There was also a higher level of blocking with mCCL3 (mean 88.0 ± 1.15%) and CCL3L1-2 (mean 81.62 ± 1.01%) pre-stimulation compared with the competitive blocking, but this failed to achieve statistical significance. Pre-stimulation with CCL4 again increased the Bio-CCL3 uptake (mean 20.54 ± 10.54%), but this was significantly less than that seen with competitive blocking alone (p < 0.03).

![Figure 6-6 Pre-stimulation of normal monocytes with non-biotinylated chemokines further reduces Bio-CCL3 uptake](image)

Aliquots of normal monocytes used in one of the experiments in Figure 6-8 and 6-9 were incubated with a X10 excess of non-biotinylated CCL3L1, mCCL3, CCL3L1-2, CCL3L1-4 and CCL4. They were incubated for 1 hour and then a Bio-CCL3 uptake assay was performed in triplicate, in the presence of the non-biotinylated chemokines. The assays were then incubated at 37°C for 2 hours and analysed by flow cytometry. The assays were set up in triplicate and compared with the results of the assays incubated at 37°C to calculate the inhibition of Bio-CCL3 uptake as shown in Equation 6-1.
In summary, biokines can be used to assess the capacity of primary human cells to take up chemokines such as CCL3 and 19, giving an indication of active, functional receptor expression on these cells. Pre-stimulation blocking assays, using CCL19 and 25, showed that the uptake of Bio-CCL19 was mediated by CCR7. Of the possible receptors that could have mediated the uptake of Bio-CCL3, CCR1 and 5 are the most likely candidates. However, the lack of receptor specificity in the Bio-CCL3 assay may have advantages. It may be used to simultaneously probe for the presence of all pro-inflammatory CCL3 receptors (CCL1, 3, 5) on target cells, avoiding the use a number of reagents, as would be the case when using antibodies. Indeed, in many cases, particularly in species other than humans, high quality antibodies are often unavailable for chemokine receptors. Biokines may represent a suitable alternative. Moreover, the biokine methodology is a useful approach when examining the shift from an iDC to a matDC phenotype, as it will give a better overall view of the changes in the cell's capacity to interact with CCL3.

6.1.4 Tracking Chemokine Receptor activity in normal and MM MoDC production using the fluorescent chemokine uptake assay

As discussed previously, DC migrate under the control of different chemokine/receptor interactions at various stages of development. These mechanisms are vital to the DC's ability to generate an immunogenic response. They encounter antigen at sites of inflammation, then mature and migrate to lymph nodes, where DC present these antigens to immune effector cells in the context of appropriate co-stimulation. The fluorescent chemokine uptake assay was used to track changes in CCR7 and CCL3 receptor activity during the different phases of MoDC production. Monocytes were collected from MM patients and ND undergoing stem cell mobilisation, and purified by density gradient centrifugation
and CD14 magnetic bead selection. Immature DC were produced from monocytes by 4-day cultures in DC medium supplemented with GM/IL-4 or GM/IL-13. Immature DC were simultaneously matured and loaded with antigen by overnight culture in medium supplemented with either the X4 or MIMIC maturation cocktail, and U266 tumour cell lysate. This study has revealed a number of fundamental changes in CCR7 and CCL3 receptor expression during MoDC development and maturation that vary between ND and MM patients.

A significant number of monocytes isolated from ND following stem cell mobilisation were found to have CCR7 activity using the chemokine uptake assay (mean 31.37 ± 9.91%) (MFI 10.93 ± 5.75), shown in Figure 6-7. This expression is maintained by iDC and up regulated following maturation (mean 49.35 ± 6.12%) (p< 0.05). The MFI of CCR7 activity is markedly up regulated during maturation (mean 27.86 ± 4.12%) (p< 0.001). The activity of CCL3 receptors progressively down regulates during MoDC production (monocytes 44.28 ± 8.22%, iDC 19.41 ± 4.00% and mat DC 15.8 ± 3.9%) (p< 0.01).

Figure 6-8 shows the MFI and percentage positive cells for monocytes, iDC and mat DC produced from MM patients undergoing chemotherapy and G-CSF stem cell mobilisation. Monocytes from MM patients show a reduced level of CCL3 receptor activity (mean 20.12 ± 4.26%) as compared to monocytes from normal donors (*p< 0.01). The pattern of change seen in the iDC and mat DC is similar in MM patients compared to normal donors. However, the level of expression of CCR7 in MM mat DC (30.33 ± 6.81%) is lower than that seen in matDC from normal donors (mean 49.35 ± 6.12%), but this fails to achieve statistical significance (+ p=0.055).
Figure 6-7 CCL3 receptor and CCR7 activity during MoDC production from normal stem cell donors, measured using the fluorescent chemokine uptake assay.

The chemokine uptake assays were performed using monocytes (n=9), iDC (n=12) and matDC (n=18) isolated from normal donors (n=9) undergoing stem cell mobilisation with G-CSF 10μg/kg for 5 days. Plot A show the mean fluorescence intensity and plot B shows the percentage of positive cells. * Denotes significant difference from MM patients, + denotes a trend towards a difference.
Figure 6-8 CCL3 receptor and CCR7 activity during MoDC production from MM patients undergoing stem cell mobilisation, measured using the fluorescent chemokine uptake assay.

The chemokine uptake assays were performed on monocytes (n=9), iDC (n=12) and matDC (n=18) isolated from MM patients (n=9) undergoing stem cell mobilisation with chemotherapy and G-CSF 5-10μg/kg for 5 days. Plot A shows the mean fluorescence intensity and plot B shows the percentage of positive cells. * Denotes significant difference from ND, + denotes a trend towards a difference.
Immature DC analysed in the above analyses had been produced using one of two cytokine cocktails, GM-CSF combined with either IL-4 or IL-13. Thus, they were examined separately. The iDC produced from ND (n=9) showed no significant difference in chemokine receptor activity between the cells produced by GM/IL-4 (n=5) (mean 38.1 ± 6.9%) (MFI 9.8 ± 1.7) and GM/IL-13 (n=7) (mean 26.2 ± 1.6%) (MFI 8.0 ± 0.8). However, the percentage of cells positive for Bio-ELC uptake and the level of activity, as indicated by the MFI, were significantly lower in the MM patient (n=9) iDC produced with GM/IL-13 (n=5) (mean 7.56 ± 2.67%) (MFI 5.05 ± 1.33) as compared to those produced with GM/IL-4 (n=4) (mean 23.79 ± 5.29%, p<0.01) (MFI 10.95 ± 1.16, p < 0.05), (Figure 6-9).

![Figure 6-9 CCR7 activity on iDC from MM patients](image)

Immature DC were produced from MM patient (n=9) monocytes collected following stem cell mobilisation. Samples were cultured for 4 days in DC medium supplemented with GM-CSF and either IL-4 (n=4) or IL-13 (n=5). CCR7 activity was analysed using Bio-CCL19 uptake as a marker of receptor activity.
Dr Simon J Harrison, 2005

In order to assess the quality of the matDC induced by the X4 and MIMIC maturation stimulus, we compared the results of Bio-CCL19 uptake in cells that had been matured with the two maturation cocktails. Samples from all nine ND were matured with both MIMIC and X4, and results are shown in Figure 6-10. A lower proportion of ND MIMIC matured matDC exhibited CCR7 activity (mean 38.63 ± 6.33%) compared to those matured with X4 (mean 58.86 ± 9.3%, p<0.05).

![Figure 6-10](image)

**Figure 6-10 CCR7 activity is lower in normal matDC matured with the MIMIC cytokine cocktail compared to those matured with X4**

Mature DC, produced from normal donors (n=9), were analysed to ascertain whether the maturation stimulus they received influenced the level of CCR7 activity. Aliquots from all Nine ND were matured with MIMIC (n=9) and X4 (n=9).

The majority of samples from MM patients were matured with X4 (8/9). Thus we compared the level of CCR7 activity in these cells with those from normal donors that had been matured with X4 (n=9) (Figure 6-11). This analysis showed the MM matDC matured with X4 had a significantly lower percentage of Bio-CCL19 positive cells (mean 30.32 ± 6.81) as compared to the normal donor controls (mean 58.86 ± 9.3%, p ≤ 0.02).
Dr Simon J Harrison, 2005

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Figure 6-11 CCR7 activity is higher in normal matDC matured with X4 cytokine cocktail compared to MM matDC matured with X4

The uptake of Bio-CCL19 by normal donor matDC matured with X4 (n=9) was compared with MM matDC matured with X4 (n=8) in order to assess the quality of MM matDC.

These experiments have shown that the biokine uptake assay can be used to monitor the level of CCR7 and CCL3 receptor activity during MoDC development. The level of receptor activity can vary depending upon the method of DC production, and between cells from ND and patients with malignant disease, such as MM. DC produced with GM/IL-4 and matured with X4 may be of better quality, with regards to CCR7 activity and homing to lymphoid tissue, than those produced with GM/IL13 or matured with MIMIC. However it will be important to correlate these findings with those from migration and chemotaxis assays before deciding whether this is the preferred method for DC production for patients with MM.

Monocytes were found to express a significant level of CCR7 activity in these assays. The level of CCR7 activity on MM monocytes is much more variable than that seen on ND monocytes. This could be due to the different regimes used to mobilise cells, or to inherent differences in MM monocytes.


6.1.5 Analysis of the expression of the chemokine receptors CCR5, CCR7 and D6 by reverse RT-PCR

Intriguingly, the fluorescent chemokine uptake assay indicates that monocytes, collected during stem cell mobilisation, and iDC have some detectable CCR7 activity. This is at odds with the data in the published literature generated by surface antibody staining, which suggest that these cells do not express detectable CCR7. In order to confirm this data, RT-PCR was used to determine CCR5 and CCR7 gene expression in monocytes, iDC pre and post antigen loading and matDC. In addition, parallel CCR5 expression was gauged.

Dendritic cells were generated from ND monocytes with GM/IL4 and X4. At each stage of production process, 5x10^5 cells were stored at -80°C as a concentrated cell pellet. RNA was isolated from cells and used to synthesise cDNA. The relative expression of chemokine mRNA was then analysed using a single set of primers, within a single PCR reaction. The products of the PCR reaction were analysed by conventional gel electrophoresis and visualised by UV light.

The PCR gel shown in Figure 6-12 shows that in keeping with the cytokine uptake assay, there was expression of CCR7 in monocytes collected at the time of stem cell mobilisation, as well as in iDC pre and post antigen loading. Although the PCR that was performed was not quantitative, this expression seemed to up regulate in mature DC. The monocytes used in this experiment were >93% pure, so it is possible that the CCR7 mRNA detected was due to T cell contamination. However, expression was maintained in iDC, which were in culture for 4 or 5 days in the absence of any cytokine that would maintain T cell viability. Alongside the Bio-CCL19uptake data, these experiments suggest CCR7 expression by monocytes.
Figure 6-12 Gel electrophoresis of PCR products for CCR5, CCR7 and D6

RT-PCR reactions for CCR5, CCR7 and D6 were performed, with and without reverse transcriptase (RT), on monocytes harvested at the time of stem cell mobilisation (Gel A), iDC produced by culture in DC medium supplemented with GM/IL-4 (Gel B), iDC post antigen loading with U266 tumour cell lysate (Gel C) and matDC following the X4 maturation cocktail (Gel D).

The expression of CCR5 appeared highest in monocytes and iDC, with apparent down regulation in iDC following antigen loading, and matDC. These findings need to be interpreted in light of the contamination in the CCR5 –RT control lane. This could be due to contamination of the sample with DNA that has not been completely destroyed by the DNAse neutralisation step. This emphasises the importance of –RT controls, which are often omitted by others. Finally, there was no evidence of D6 expression in any of the test cells in this experiment. Thus the
Bio-CCL3 uptake observed in the experiments discussed in 6.1.4 is unlikely to be mediated by the D6 decoy receptor.

6.1.6 **CCR8 expression during MoDC production measured by surface marking with biotinylated CCL1**

There is recent evidence that CCR8, as well as CCR7, has a role in the migration of CD40 licensed matDC from peripheral tissues to lymph nodes. The CCL1 (I-309) Fluorokine kit, (R and D systems) was used to examine the expression of CCR8 on monocytes and DC during MoDC production. CCL1 is the main ligand for CCR8. The molecules employed in this method had been biotinylated after production. As a result, each CCL1 molecule has multiple biotin sites, and they form large complexes when streptavidin-bound fluorochromes are added. This prevents this type of molecule from being pre-labelled with streptavidin-bound fluorochromes and so they are not suitable reagents to measure ligand internalisation. However, it does allow sensitive and specific surface quantification of the cell surface receptor expression.

Aliquots of cells were suspended in cold PBS, and labelled with Bio-CCL1 or negative control. The samples were incubated for 1 hour at 4°C, the Strep-FITC was then added and the samples incubated for a further 30mins, in the dark, at 4°C. Samples were then washed twice and analysed immediately by flow cytometry. The specificity of the staining was tested by pre-blocking Bio-CCL1 with anti-human CCLI antibody, and performing a competitive binding assay by pre-incubating test cells with a ten times excess of non-biotinylated CCL1 for 1 hour. The assays were then completed as normal (Figure 6-13). All assays were performed in triplicate.
Figure 6-13 CCR8 surface marking with Bio-CCL1 is blocked by anti-human CCL1 antibody, and partially inhibited by competition with un-biotinylated CCL1

Normal monocytes were prepared by density gradient centrifugation and CD14 selection. Samples were analysed for CCR8 expression by CCL1 surface staining (plot B) and compared with negative control samples (A). Blocking with an antihuman CCL1 anti-body (C) and pre-stimulation with x10 excess of un-biotinylated CCL1 (D) determined specificity of marking. Samples were analysed by flow cytometry. The M1 contains 99% of the negative control cells and M2 is the positive gate.

Monocytes were positive for CCR8 (mean 95% ± 1.14%) and this was completely blocked by anti-human CCL1 antibodies. Pre-stimulation with un-biotinylated CCL1 reduced the MFI by a mean of 50% ± 2%. This suggested that the Bio-CCL1 surface staining was both a sensitive and specific method for detecting CCR8 on the surface of primary human cells (Figure 6-13).

We went on to examine the CCR8 levels on monocytes, iDC and matDC generated from normal donors (n=6) and MM patients (n=6), who were undergoing stem cell collection. Monocytes from normal donors had a lower MFI (mean 28.34 ± 10.80) and percentage of CCR8 positive cells (mean 43.17 ± 5.80%) compared to MM monocytes (mean 124.69 ± 54.0, p < 0.001) and (mean 64.79 ± 13.90%, p<0.03) (Figure 6-14). There was no statistical difference between CCR8 levels on normal donor or MM patient iDC. However, the expression of CCR8 on MM matDC (163.0 ± 18.5) was statistically higher than on ND matDC (163.0 ± 18.5, p ≤ 0.01).
Figure 6-14 CCR8 levels on normal and MM monocytes, IDC and matDC

CCR 8 levels were assessed using the Bio-CCL1 surface-marking assay. Monocytes were isolated from normal donors and MM patients undergoing stem cell collection. Immature DC were produced by culture in DC medium supplemented with GM/IL4 or GM/IL13. Maturation was induced using the MIMIC or X4 maturation cocktails. Plot A shows the MFI data and plot B shows the percentage of cells that were CCR8 positive. Samples were analysed from 6 ND (n=12) and 6 MM patients (n=6).

The difference in the level of CCR8 expression on cells from MM patients and ND may be constitutive, or due to the differing stem cell mobilisation regimes. This may be the case for monocytes, but it is much less likely to be the explanation for the different levels of expression seen on matDC. Thus it is more likely that cells from MM patients have an inherently higher expression of CCR8. This may be further clarified by measuring CCR8 levels on cells from ND and MM patients obtained under steady state conditions.
6.1.7 Discussion

Chemokines and their receptors are crucial for the efficient function of the immune system. They provide a homeostatic mechanism by which cells such as DC and T cells can migrate from the bone marrow to the periphery and then on to lymphoid tissues. Chemokines also play a role in the regulation of the inflammatory response, by recruiting cells through the up regulation of inducible chemokines and then facilitating APC antigen priming of naïve T cells, through their co-localisation in lymph nodes, mediated by CCR7 expression. Unfortunately tumours can use the chemokine system to down regulate the immune system either directly or by recruiting regulatory cells into the tumour site.

We have shown that the fluorescent biokine uptake assay is a sensitive and specific method of detecting chemokine receptor activity on immune cells such as monocytes and DC from normal donors and patients with malignant disease, such as MM.

The uptake of Bio-CCL-3 was mediated via CCR1 or CCR5, and uptake generally decreased as cells differentiated into matDC. This is consistent with the reported reduction of pro-inflammatory chemokine receptors reported as DC mature. Interestingly, the level of Bio-CCL-3 uptake activity was lower on MM monocytes as compared to ND. This could be simply due to the fact that MM patients have stem cells mobilised following chemotherapy, rather than growth factor alone as in ND. However, there may be an underlying effect due to the malignant disease, and it may be possible to differentiate between the two by analysing monocyte expression of CCR1 and CCR5 on cells from MM patients or ND collected under steady state conditions. It is known that CCL3 levels are elevated in the serum and BM supernatants of MM patients. These levels also correlated with the severity of MM bone disease and prognosis, and increase with as the disease progresses.
We postulate that these elevated levels of CCL3 may directly induce the down regulation of pro-inflammatory chemokine receptors by desensitisation, preventing efficient monocyte recruitment to sites of inflammation. Strangely, the level of Bio-CCL3 uptake was dramatically increased by co-stimulation with CCL4. This observation is in keeping with a number of recent reports that have shown that some chemokines, such as CCL21 and CXCL13, and CXCL8 and CXCL6, may produce synergistic effects on target cell migration (Paoletti, et al 2005, Struyf, et al 2005). In addition Paoletti et al have suggested that these effects may be mediated by the formation of chemokine heterodimers. It is already known that CCL3 and CCL4 are secreted in the form of heterodimers by monocytes (Guan, et al 2001), and we postulate that these two mechanisms may be responsible for the increase in Bio-CCL3 uptake observed by co-stimulation with CCL4. These observations are of interest to those who are designing immune-based therapies, as it is clear that receptor-ligand interactions alone are not enough to trigger functions such as migration (Randolph, et al 2005). Dendritic cell migration also requires cells to be primed with a balance of other stimuli, such as PgE2 and CD40L, in order for migration and not cytokine production to be triggered.

Interestingly, this assay showed some CCR7 activity on monocytes collected during stem cell mobilisation, which was similar in cells collected from MM patients and ND. This expression was confirmed on PCR analysis. To my knowledge, CCR7 expression has not been previously reported on monocytes. It is possible that this receptor expression may allow limited monocyte access to lymphoid tissue, where they could differentiate into macrophages or DC, known to be present in these organs. The biokine assay was also used to track the up regulation of CCR7 activity through DC development.
It is not surprising that the chemokine receptor profile of MoDC may be influenced by the cytokines used to generate them. Immature DC from MM patients generated using GM/IL-13 had a lower expression of CCR 7 compared to those generated by GM/IL-4. The maturation cocktail also influenced the level of CCR7 expression. Normal donor DC matured with X4 had higher level of CCR7 expression compared with normal donor cells matured with MIMIC and MM DC matured with X4, again suggesting an inherent difference between MM and ND derived cells.

CCR8 expression could be measured using the Bio-CCL1 surface-marking assay, and this expression was shown to up-regulate during MoDC production. Monocytes and matDC from MM patients were shown to have a significantly higher expression of CCR8 as compared to normal donors. This observation suggests that cells from MM patients may be more sensitive to the potential immunosuppressive effects of CCL1 reported recently (Batten, et al 2005). Batten et al showed that mesenchymal stem cells (MSC) directly inhibited T cell proliferative responses to allo PBMCs, and failed to induce the proliferation of primary and primed T cells, through the production of CCL1. Furthermore, MSCs also induced T cell production of Th2 cytokines and chemokines (IL-3, IL-5, IL-10, IL-15 and CCL1), accompanied by down regulation of Th1 cytokines (IL-1α, IL-β, IFNγ and TNFα).

A greater understanding of how malignant diseases and their therapies interact with the chemokine system is required if the DC system is to be used to produce effective immune therapies for patients. The changes in the MoDC system detected in cells from MM patients suggest that monocytes are unable to home to sites of inflammation, matDC have impaired ability to home to LN and immunosuppressive chemokines, such as CCL1, may have increased potency on
MM monocytes and DC. The correlation between the expression of CCRs on MM patient cells with the level of chemokines in the plasma and bone marrow may provide further insights into these processes.

Observations such as these may help to explain why DC used in immunotherapy trials have failed to home effectively to lymph nodes, or have any major impact of the course of malignant diseases, such as MM.
7. Discussion and Conclusions

The field of tumour immunotherapy is still in its infancy. It is becoming clear that the human immune response is the result of highly complex, continuously evolving interactions between cells of the adaptive and innate arms of the immune system, the internal and external environments, and normal and abnormal cells (i.e. malignant or virally infected cells). Despite the considerable advances in our knowledge over the past 30 years, we still seem to have only scratched the surface of the complexities of the immune system and its interaction with malignant disease. It is therefore not surprising that the results from immunotherapy trials have so far been disappointing. Although we and others are able to generate immune effector cells in the laboratory that are capable of killing tumour cells in-vitro, with the notable exception of PTLD (Bollard, et al 2003), this has not translated into improved outcomes for patients with malignant disease who have participated in clinical trials of tumour specific immunotherapy. There are a number of factors that are likely to contribute to this situation:

1. As with most new therapies, the majority of immunotherapy trials have been conducted in patients who have advanced malignant disease. Given that patients who undergo allo HSCT have improved survival when transplanted early in the disease course (Gratwohl, et al 1998), and respond best to DLI when it is given for relapse at the molecular level (van Rhee, et al 1998) the poor results so far in the immunotherapy trials are hardly surprising. It remains unclear whether the negative impact of disease burden is the result of disease suppression of the immune system or if the increased amount of treatment that these patients will have received also contributes to their poor outcomes. Clearly it will be advantageous to use immunotherapy treatments earlier in the disease course, preferably at a time when the
tumour burden is at its lowest (Hsu, et al 1997), such as following autologous HSCT.

2. Thus far it has been difficult to identify tumour specific, immunogenic antigens with which we can direct the immune system against the malignant clone. The success of immunotherapy in the treatment of PTLD is largely attributable to the fact that the immune therapy is direct against a few well characterised, highly immunogenic viral (i.e. EBV) Ags. In the majority of other malignancies in which immunotherapy is being explored, the potential target antigens that have been employed so far are normal, often early developmental antigens, that are expressed in an aberrant location and to a much higher degree than normal (i.e. wrong place, wrong time and wrong amount), such as MUC-1, NY-ESO-1 and Id protein (Barratt-Boyes 1996, Timmerman, et al 2002, van Rhee, et al 2005a). Many groups, including our own, have opted for a simpler approach. Rather than trying to identify tumour specific antigens with which to prime the immune system, whole tumour cell preparations can be used, such as freeze thaw tumour cell lysates and apoptotic tumour cells (Hayashi, et al 2003, Kotera, et al 2001), leaving the APC and immune effector cells to identify the most immunogenic epitopes. While this method runs the risk of potentially useful antigens being ignored by the immune system because of the antigen immuno-dominance phenomenon (Berkower, et al 1982, Gapin, et al 1998, Milazzo, et al 2003), it is less likely that a broad based approach such as this will allow the tumour to escape immune based therapies by simple down-regulation or deletion of a single target antigen. This approach does not get around the theoretical problem of the potential anti-tumour Ags being only weakly immunogenic, due to the fact that the host may have developed tolerance to them at some stage during development. However,
in Figure 5-9, it was shown that this type of antigen preparation is capable of producing tumour cell kill at relatively low effector: target ratios \textit{in-vitro}. It remains to be seen whether this success can be replicated \textit{in-vivo}.

3. In diseases such as MM, which induce a hostile environment in which to attempt to generate an immune response (Brown, \textit{et al} 2001, Cook, \textit{et al} 1999), it is unclear when and how the cells that are to be used to generate APC should be collected. Furthermore, there is still debate about which cells should be used i.e. circulating blood DC, MoDC or indeed whether the whole of this process can be conducted \textit{in-vivo} via antigen vaccination with the addition of adjuvants to attract APC to the vaccination site and then promote maturation, migration and immunogenic antigen presentation to immune effectors (Adams, \textit{et al} 2005). In order to reduce the negative impact of the tumour on the immune based therapy, it would again seem logical to collect the cells and/or vaccinate at a time when the tumour burden is at its lowest (Hsu, \textit{et al} 1996). In the treatment of the majority of patients with myeloma this would be after the patients had recovered from autologous HSCT (Attal and Harousseau 1999).

4. In order to generate a cytotoxic immune response, vaccinated DC need to migrate from the vaccination site, i.e. the skin, to secondary lymphoid tissues such as draining lymph nodes. There is evidence that matDC used in clinical studies fail to migrate in significant numbers (Blocklet, \textit{et al} 2003) despite seemingly adequate expression of chemokine receptors that are thought to mediate DC migration to LN, such as CCR7. Recent evidence suggests that the factors governing DC migration are not as straight forward as originally thought. Other chemokine receptors, such as CCR8, and the context in which they are expressed may also be important in DC migration.
to LN (Qu, et al 2004, Randolph, et al 2005). Some groups have tried to circumvent this process by direct intranodal injection of DC (Yi, et al 2003). However, these approaches have thus far not produced significant improvements in the potency of the anti-tumour effect in the treatment of patients with MM. A greater understanding of how the chemokine system controls DC and effector cell migration, and how the hostile tumour induced environment may inhibit this process is required if we are to translate the encouraging *in-vitro* anti-tumour effects demonstrated in Figure 5-11 into effective therapies for MM patients. Tools such as the fluorescent chemokine uptake and staining assays described in Chapter 6 will prove useful in this process. They provide a rapid, straightforward, direct functional measure of chemokine receptor activity on target cells. Thus it is no longer necessary to rely upon phenotypic data alone or undertake complex migration experiments in order to assess chemokine receptor expression and activity.

5. Immune effector cells are also suppressed in malignant diseases such as MM (Campbell, et al 2001, Cook, et al 1999). Data from studies such as those described in Chapter 5 and the Xcelerate™ trial (Vij, et al 2003) should help us decide whether it is possible to improve/boost any inherent anti-tumour immune response (as shown in Figure 5-8), or reverse any tumour-induced suppression of effector cells in order to improve the efficacy of immune base therapies (as suggested by Figure 5-9). It would also seem sensible to collect immune effectors for use in immune therapies at a time when the disease burden is at its lowest. Given that this cytotoxicity data showed the best tumour cell kill was achieved using MM T cells collected after cyclophosphamide and G-CSF, it would be reasonable to use this type of protocol, rather than mobilising with G-CSF alone. This is in
keeping with published data, which suggest that G-CSF alone is associated with a greater proportion of DC2 as compared with chemotherapy and G-CSF mobilisation (Bolwell, et al 2003), and G-CSF mobilised cells contain an increase proportion of T\textsubscript{Regs} (Vela-Ojeda, et al 2005). It is not known whether the improved cytotoxicity data was as the result of altering the proportions of T cell subsets that were collected, i.e. suppressing T\textsubscript{Regs}. It remains to be seen whether this method is preferable to collecting cells under steady state conditions, without any formal mobilisation. These questions warrant further investigation, as the cellular composition of the starting material with which an immunotherapy is built may have a dramatic impact on the end results. There is good experimental evidence to suggest that depleting subjects of T\textsubscript{Regs} prior to vaccination with DC based immunotherapy may improve the effectiveness of these approaches (Ghiringhelli, et al 2005, Sakaguchi, et al 2001). Whether this can be achieved using a simple in-vivo T cell suppressive therapy such as cyclophosphamide (Rosenberg, et al 1994a), or requires a more specific approach using an anti-CD25 monoclonal antibody, such as denileukin diftitox (Ontak) (Olsen, et al 2001), to specifically deplete the subject of CD25\textsuperscript{+} cells remains to be seen.

When designing immune therapies for use in patients, it is important to consider that the building blocks of the treatment, i.e. the cells of the immune system, may be numerically and functionally abnormal. The blood DC system in MM and MGUS patients is compromised at diagnosis (Brown, et al 2001, Ratta, et al 2002) and Figure 3-6. However, following autologous HSCT or thalidomide therapy for the treatment of relapse, there is an improvement in the numbers of circulating blood DC, albeit short lived (Figure 3-8 and 3-9). This would suggest that the best time for collecting cells for use in immune based therapies is following high dose...
chemotherapy and stem cell rescue. This is in keeping with other published data, which suggest that the best time to use immune base therapies is a time when the disease burden is at its lowest (Hsu, et al 1997, Stevenson, et al 2004). The immune system in MM patients following these types of therapies does not return to normal, in that the B cell and pDC numbers remained suppressed (Data not shown and Figure 3-8). Along with the data from other groups which has shown that the blood DC do not mature normally (Brown, et al 2001, Ratta, et al 2002), it is becoming clear that blood DC are not ideal for use as immune therapies for MM.

Generating DC from monocytes is a more encouraging prospect. Monocytes are more readily available than blood DC, and the use of CD34+ cells is more technically demanding, with respect to the collection and purification of CD34+ cells in sufficient quantity, and Ag loading of CD34+ derived DC. However, even when monocytes were collected following a course of anti-MM induction therapy and stem cell mobilisation, MoDC generated from MM patients were still found to be phenotypically and functionally abnormal at all stages of development (Figure 4-2, 4-4, 4-6 and 4-8). Cells that were matured with the X4 cytokine combination were found to be superior to those cells matured with the MIMIC combination, in that a higher proportion of X4 matured cells expressed CD83 (Table 4-2) and had greater CCR7 activity (Figure 6-10). It is also likely that prior to being used as therapy, MoDC would need to be matured for 48 hours in order to fully up regulate chemokine receptors, such as CCR7, and efficiently migrate to lymph nodes in order to interact with T cells (Figure 4-10). It is currently unclear whether the increase in CCR8 expression on MM matDC has a negative or a positive impact on MoDC migration following vaccination (Figure 6-14). Overall, these findings suggest that it may be crucial to optimise MoDC production for each disease state in which they are to be used, in order to maximise the chances of inducing an effective anti-tumour response. The functional activity of chemokine receptors may
best be studied using a combination of ligand uptake, surface antibody staining and *in-vitro/in-vivo* migration assays to determine the optimal timing of MoDC Ag loading, maturation and administration.

Despite the limitations imposed by using functionally abnormal cells, MM patient MoDC can be used to generate anti-MM cytotoxic T cells *in-vitro*. The cytotoxic activity of unprimed MM patient T cells was better than that observed with ND cells, and this may be related to the fact that the MM cells were collected after cyclophosphamide and G-CSF, while the ND cells were collected following G-CSF alone (*Figure 5-8*) and the potential differences in the T cell populations in the respective collections, as discussed above. The proliferative response to IL-2 + matDC and the cytotoxic activity of MM T cells was boosted by pre-stimulating with T cell activating beads and IL-2, prior to antigen presentation (*Figure 5-2* and *Figure 5-9*). This was despite the fact that this process seemed to render the T cells IL-2 dependant, in that they did not proliferate or secrete cytokines in the presence of DC alone (*Figure 5-2* and 5-5). It remains to be seen whether these effects can be transferred *in-vitro* given the difficulties that surround the use of IL-2 in patients (Escudier, *et al* 1994, Farag, *et al* 2002).

The observations described above could form the basis for a novel clinical immunotherapy trial in MM patients, using both autologous moDC, pre-stimulated T cells and followed by low dose IL-2 as consolidation immunotherapy following induction chemotherapy and autologous HSCT (*Figure 7-1*). Monocytes and T cells could be collected by formal cyclophosphamide and G-CSF mobilisation, following autologous HSCT, at a time when the disease burden is at its nadir.
Figure 7-1 Total Immunotherapy for Multiple Myeloma using antigen loaded autologous DC, pre-stimulated T cells and SCI IL-2

Collecting immune cells after induction chemotherapy and auto HSCT should reduce the tumour burden to its lowest level, reducing any immunosuppressive effects on the immune system and the therapeutic cells when given back to the patient. Monocyte derived DC generated with GM-CSF and IL-4, loaded with autologous tumour cell antigen and matured with X4 could then be given in 2 weekly aliquots by intra-dermal injection. T cells collected during the same procedure, marked to allow tracking studies, pre-stimulated with activating beads and IL-2 would be administered simultaneously by I.V. infusion. This should enable the antigen loaded matDC and pre-stimulated T cell to migrate to lymph nodes in the same time frame. IL-2 would be administered by SCI the next day and daily for the next 2 weeks to provide cytokine growth support to the pre-stimulated cells.
The aim of collecting cells at such a late time point in the treatment of MM is to minimise any disease related immunosuppressive effects on the collected monocytes and T cells. A secondary effect of collecting T cells at this point may be a relative depletion of memory and regulatory T cells, whilst enriching for naïve T cells as the immune system repopulates itself following autologous HSCT. These hypotheses form the rational basis for the use of autologous HSCT for the treatment of autoimmune diseases such as rheumatoid arthritis and Immune thrombocytopenic purpura (Huhn, et al 2003, Snowden, et al 2004). Monocytes would be cultured with IL-4 and GM-CSF, primed with an autologous tumour cell preparation (i.e. tumour cell lysate) collected at the time of diagnosis, and matured with the X4 cytokine combination. At the same time T cells could be pre-stimulated with anti-CD2, 3 and 28 coated beads and IL-2, in order to reverse T cell anergy and regain T cell responsiveness to IL-2. Both cell types could be administered on the same day, DC by ID and T cells by IV injection, in order to promote rapid co-localisation in lymph nodes. Subcutaneous low dose IL-2 would be given the following day to maintain T cell viability, increase the T cell proliferation stimulus and stimulate the adaptive and innate immune systems (Aladdin, et al 2000, Dudley, et al 2002, Gonzalez-Barca, et al 1999).

In a similar vein, although MM is susceptible to the GVL effect, and patients who have relapsed following allo HSCT do respond to DLI. However, it is far less potent strategy for patients with MM when compared to other diseases, such as CML (Verdonck, et al 1998). This may be in part due to the timing of administration, as it is known that patients with molecular relapses from CML respond better to DLI as compared to patients with haematological relapse. Up until now it has been difficult to monitor MM with this level of sensitivity, but the use of techniques such as serum free light chain quantification, high sensitivity chimerism assays and rapid PCR MRD monitoring may improve this situation.
(Rasmussen, et al 2004). As previously discussed, it is also likely that the donor lymphocytes are being infused into a hostile immunosuppressive environment induced by the MM tumour cell. Pre-stimulation of donor lymphocytes may render the cells less sensitive to the effects of the MM tumour cells. When given in combination with IL-2 to provide cytokine support to these pre-stimulated cells, there may also be more generalised immuno-stimulatory effects on the transplanted immune system, which may promote an increase in the GVL effect (Kiss, et al 2003, Slavin, et al 1996). This combination may warrant further investigation, and could form the basis of a more potent treatment for MM relapse following allo HSCT.

We are about to enter an exciting phase in the development of immune based therapies for malignant diseases such as MM when we can build on the results of the phase I studies and the advances in basic immunology. A greater understanding of how the adaptive and innate arms of the immune system interact and regulate each other may be crucial in this process and form the basis of some of the next generation of clinical immunotherapy studies (Ritchie D, personal communication). It may also be possible to engineer our cellular therapies so that a number of different cell types are administered in the correct state of maturation, allowing them to rapidly co-localise in lymph nodes and interact efficiently, whilst being resistant to the suppressive effects of the tumour.

Ultimately, it may be possible to avoid having to process cells in-vitro by using cytokines and chemokines to manipulated cells of the immune system in-vivo. In theory, it should be possible precondition an area of skin so as to attract or generate APC ready to receive tumour specific antigen delivered by simple vaccination. The addition of adjuvant compounds would then promote efficient APC maturation and migration to lymph nodes where they would present antigen
to immune effector cells, such as naive T cells. A CD8 cytotoxic T cell response could be promoted by further administration of cytokines, such as IL-2. However, while we are developing and testing the next generation of immunotherapies we should remember that even if the disease burden is reduced to a very low level, it may still have a disproportionate suppressive effect on the host immune system. Thus these in-vivo approaches would seem to be a very long way off for the treatment of diseases such as MM.

In summary, the results described in this thesis have confirmed that the DC system is highly abnormal in MM patients, but does recover to some degree following autologous HSCT. Although the mature MoDC produced from MM patients seem to be of poorer quality than those from ND, when combined in-vitro with pre-stimulated autologous T cells, the resultant cytotoxic T cells had superior killing ability compared to ND and un-stimulated T cells. However, the CCR7 and 8 chemokine activity of mature MoDC when measured using the fluorescent ligand uptake and staining assays was abnormal, suggesting that when administered in-vivo, MM matDC may not migrate as efficiently to lymph nodes and be more susceptible to immunosuppressive chemokines as compared to normal DC. These results should help in the development of effective immune based therapies for the treatment of MM and other malignant diseases.
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