Characterization of an IL-12-driven anticancer response, and the CD4\(^+\) CTL population incited, in a murine model of leukaemia

by

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A thesis submitted in conformity with the requirements for the degree of
Doctor of Philosophy
Graduate Department of Medical Biophysics
University of Toronto

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Doctor of Philosophy, 2012

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Abstract

For the treatment of cancer, immunotherapy has some inherent advantages over other treatment modalities: disseminated disease can be eradicated due to the systemic nature of immunity, the immune system is effective against a wide range of targets, long-term memory can offer added protection against disease relapse, immunotherapy should be relatively non-toxic, and it can be synergistically combined with other treatment platforms such as radiation and chemotherapy. Type 1 immune responses are thought to be superior for the treatment of cancer and, as the quintessential Tₜₙ polarizing cytokine, interleukin-12 (IL-12) holds much promise; however, optimal therapeutic protocols have yet to be developed and clinical results have fallen short of this promise.

The in vivo IL-12 experiments described here highlight a characteristic of cellular therapy that has not previously been appreciated. That is, the effect of cell-mediated cytokine delivery on the immediate microenvironment and how that affects the immune response initiated. This observation has implications for the clinical application of IL-12 therapy but may also prove to be an important consideration when studying other immunostimulants.
I have herein developed a novel *in vitro* assay system that I have used to dissect the cellular responses to IL-12 and to identify the signals that are required for activation of a cluster of differentiation 4 (CD4)$^+$ effector population that affects leukaemia cell clearance both *in vitro* and *in vivo*.

This work, and the future studies proposed, will expand our understanding of the potential of IL-12 immunotherapy and enhance our ability to manipulate therapeutic conditions to favour the desired response. Moreover, the *in vitro* assay system offers a method for further characterization of CD4$^+$ effector cells and the development of protocols to initiate their potent anticancer activity.
Acknowledgments

Though obtaining a PhD is very much a personal journey, there are many people I must wholeheartedly thank for propelling me forward:

Dr. Stephen Westcott, my undergraduate supervisor, who first inspired me to pursue my love of science in the academic realm. For making that goal possible, I credit Nancy McClelland and Dr. Christopher Paige. Nancy believed in my ability and fought to give me the opportunity to prove myself; we lost you too early, Nance. And Dr. Paige who took a chance on me, hiring me despite my lack of qualifications and giving me the space to grow. The independence you allow us has been integral to my training; thank-you for giving me the freedom to make mistakes and find my own way again.

That amount of freedom would have been a little scary had it not been for my labmates - past & present - who have all, over the years, patiently endured my many questions and have been so generous with their time and expertise. But in addition to helping me develop research skills, they have also made it fun to come to work everyday! In particular, I’d like to thank Caren, who is not only a tremendous source of sage advice but has also been an immense support and a great friend.

There are a number of individuals I have met these past few years who have set an example for me through their integrity, drive and talent, and to whom I often turn for inspiration; especially Adi, Kellie, Jocelyn and Evan.

I also owe a debt of gratitude to my family. To Greg - a friend from the very beginning. Thank-you for always accepting me the way I am and never asking me to be more than I can be.

My Uncle George and Auntes Eva & Gladys, Nanny, Aunty Judy & Uncle Doug, Aunty Kathy & Granny for teaching us from a young age what a privilege and a wonder education is.

My siblings Kristin & Michael, for always being interested and enthusiastic about what I was doing.

My nephews and nieces: Danny & Ethan, Kayla, Sydney, & Samantha for giving me perspective; you kids light up my life.

I especially thank my mother for being proud of me no matter what and for being my biggest cheerleader.

Finally, to my true love and best friend, Denis. Thank-you for your patience during my ups and downs, your encouragement to follow my dreams, and for never letting me compromise.
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<th>Description</th>
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<tbody>
<tr>
<td>[³H]-thymidine</td>
<td>Tritiated thymidine</td>
</tr>
<tr>
<td>5-FC</td>
<td>5-Fluorocytosine</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>αGalCer</td>
<td>Alpha GalactosylCeramide</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium Chloride Potassium</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive Cellular Therapy</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute Lymphocytic Leukaemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid Leukaemia</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>APC (fluorochrome)</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>AraC</td>
<td>Arabinofuranosyl Cytidine; also known as Cytarabine</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BCR</td>
<td>B Cell Receptor</td>
</tr>
<tr>
<td>BDF₁</td>
<td>(C57BL/6xDBA/2)F₁ mouse breed</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytokine Bead Array</td>
</tr>
<tr>
<td>CCR7</td>
<td>Chemokine (C-C motif) Receptor 7</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CIC</td>
<td>Cancer Initiating Cell</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II Transactivator</td>
</tr>
<tr>
<td>cIL-12</td>
<td>LV12.2 Cell-derived IL-12</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myelogenous Leukaemia</td>
</tr>
<tr>
<td>CMV</td>
<td>Choriomeningitis Virus</td>
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<tr>
<td>conA</td>
<td>Concanamycin A</td>
</tr>
<tr>
<td>cSMAC</td>
<td>Central Supra-Molecular Activation Complex</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
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<tr>
<td>CTLA-4</td>
<td>CTL-associated Antigen 4</td>
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<tr>
<td>DAMP</td>
<td>Danger Associated Molecular Pattern</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<td>DC-SIGN</td>
<td>CD209</td>
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<tr>
<td>DEC-205</td>
<td>CD205</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dSMAC</td>
<td>Distal Supra-Molecular Activation Complex</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed Type Hypersensitivity</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF-1α</td>
<td>Elongation Factor 1 alpha promoter</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
</tr>
<tr>
<td>Fas-L</td>
<td>Fas Ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>gp100</td>
<td>Glycoprotein 100</td>
</tr>
<tr>
<td>GVL</td>
<td>Graft-Versus Leukaemia</td>
</tr>
<tr>
<td>Gy</td>
<td>Grays; absorbed dose of ionizing radiation (1Gy=1J/kg)</td>
</tr>
<tr>
<td>GzmB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat Shock Protein 70</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>iGb3</td>
<td>isoglobofifexosylceramide</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IL-12R</td>
<td>IL-12 Receptor</td>
</tr>
<tr>
<td>IL-12Rβ1</td>
<td>IL-12 Receptor β1 chain</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant NKT cell; also known as Classical NKT cells</td>
</tr>
</tbody>
</table>
IP
Intraperitoneal

iTCR
Invariant TCR

JAK
Janus Kinase

Lck
Lymphocyte-specific protein tyrosine Kinase

LCMV
Lymphocytic Choriomeningitis Virus

LN
Lymph Node

LPS
Lypopolysaccharide

LV
Lentivirus

LV12.2
Lentiviral transduced IL-12 producing 70Z/3 clone 2

LV12.2-r
Lentiviral transduced IL-12 producing 70Z/3 clone, resistant to IFN-γ

MACS
Magnetic-Assisted Cell Sorting

MCP-1
Monocyte Chemotactic Protein-1

MHC
Major Histocompatibility Complex

mIL-12
Murine IL-12

MOI
Multiplicity Of Infection

MRD
Minimal Residual Disease

mRNA
Messenger Ribonucleic Acid

MTD
Maximum Tolerated Dose

MTOC
Microtubule Organizing Centre

MΦ
Macrophage

NCI
National Cancer Institute

NFAT
Nuclear Factor of Activated T cells

xv
NHL  Non-Hodgkin Lymphoma

NK  Natural Killer cell

NKT  Natural Killer T cell

PAMP  Pathogen Associated Molecular Pattern

PBMC  Peripheral Blood Mononuclear Cell

PBS  Phosphate Buffered Saline

PBS-57  Analogue of αGalCer

PCR  Polymerase Chain Reaction

PE  Phycoerythrin

PKC-0  Protein Kinase C-0

PRR  Pattern Recognition Receptor

pSMAC  Peripheral Supra-Molecular Activation Complex

RBC  Red Blood Cell

RCC  Renal Cell Carcinoma

rIL-12  Recombinant IL-12

RNA  Ribonucleic Acid

RPM  Revolutions Per Minute

RPMI 1640  Roswell Park Memorial Institute 1640 medium

RT  Room Temperature

SMAC  Supra-Molecular Activation Complex

smDC  Semi-Mature DC

SOC  Standard Of Care
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>SPF</td>
<td>Specific Pathogen Free</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scatter</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour-Associated Antigen</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TFH</td>
<td>Follicular Helper T cell</td>
</tr>
<tr>
<td>Th1</td>
<td>T Helper type 1 cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-alpha</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-Related Apoptosis-Inducing Ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSA</td>
<td>Tumour-Specific Antigen</td>
</tr>
<tr>
<td>Tyk2</td>
<td>Tyrosine Kinase 2</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cell</td>
</tr>
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**List of Publications**


* Co-first authors


CHAPTER 1: General Introduction

A portion of this chapter is adapted from the textbook chapter published by Humana Press:
1.1 Immunosurveillance and Immunotherapy

Cancers are often considered to be immunologically silent diseases, largely because they arise from self-tissues and, therefore, most of the antigens they express are normal self-antigens toward which central deletional tolerance is likely to have occurred. This results in a paucity of T cells in the available repertoire that can recognize antigen on the surface of cancer cells, and those that can will do so with low avidity. Viewed differently, this deficiency in the initiation of an anticancer response is due to minimal tissue destruction occurring at the onset of malignant transformation, when tumour antigens become available to the immune system, and the absence of these danger signals can instead lead to anergy - anergy is a state of antigen (Ag) non-responsiveness. Furthermore, the development of a tumour is accompanied by the development of a tolerogenic environment that can preclude an effective immune response. The dissenting argument is that despite these obstacles, there is ample evidence that the immune system is capable of recognizing and eliminating cells undergoing transformation during a process known as immunosurveillance (a theory formulated by Burnet and Thomas in 1957). Though cancer arises from self-tissues, tumour cells are highly mutable and these mutations could theoretically be recognized by the immune system as non- or altered-self if expressed on the cell surface. The genetic and epigenetic events that lead to malignant transformation result in the expression of tumour-specific (TSA) or tumour-associated antigens (TAA) that can mark malignant cells for destruction by the immune system. There is also evidence that, in some cases, the products of oncogenes can summon immune cells to the tumour microenvironment by inciting strong inflammatory signals that may be sufficient to overcome anergy. Such signals can be produced downstream of stress-induced proteins, such as heat shock protein 70 (Hsp70), that are released during the tissue remodelling that results from neoplastic growth and invasion. Furthermore,
the observation that there is a higher cancer incidence among immunosuppressed patients supports the theory that immunosurveillance can effectively rid the body of pre-malignant cells before any notable cancer growth occurs under normal physiological conditions and in the absence of intervention. Graft-versus-leukaemia (GVL) responses in patients, following allogeneic haematopoietic stem cell transplantation, further demonstrate that those cancer cells which develop in a suppressed patient can be sensitive to recognition and elimination by an intact immune system transplanted from a healthy donor. Additionally, in rare cases, cancer patients experience spontaneous regression, indicating that the immune system is also capable of regaining control over established disease and is capable of mediating tumour clearance if stimulated appropriately.

These stimuli, however, do not always lead to a protective immune response; indeed, development of a malignancy is evidence of immune insufficiency. The immune system either fails to recognize its development, mounts a suboptimal response, or is actively suppressed by the tolerogenic mechanisms normally activated to prevent autoimmunity. These tolerogenic programs can be co-opted to prevent anticancer responses and allow for tumour outgrowth. In these ways, early inflammatory responses shape cancer development. Immunosurveillance applies selective pressure on the malignant cell population and immunoediting is the resulting dynamic process whereby a neoplasm is either eliminated, reaches equilibrium or escapes immune control. In the case of escape, the cancers that emerge are those with reduced immunogenicity or those that have exploited the tumour microenvironment for their own benefit. Indeed, immune effector cells from cancer patients sometimes display evidence of signalling defects that make them unresponsive to appropriate activation stimuli. It has also been
observed that chronic inflammation can lead to effector cell anergy or exhaustion that restricts subsequent anticancer responses\textsuperscript{8}.

In addition to immunoediting, the malignancy is also shaped by therapeutic interventions. Current standard of care (SOC) protocols can often reduce cancer burden and induce remission but disease relapse is common upon termination of treatment. This outgrowth is often highly resistant to further radiation and chemotherapy. Some argue that this phenomenon is due to a minority population of persistent cancer initiating cells (CICs) that possess stem cell-like characteristics\textsuperscript{9}. This population demonstrates inherent resistance to standard therapy due to lower cycling rates and increased expression of multi-drug resistance proteins, thereby having a propensity to repopulate the tumour\textsuperscript{10,11}.

Immunotherapy in general has been reserved for those patients refractory to radiation therapy and for whom the chemotherapeutic armamentarium has already been exhausted. However, in principle, inducing immunity is appealing for several reasons: 1) disseminated disease can be eradicated due to the systemic nature of immunity; 2) the immune system, which does not require dividing cellular targets, is effective against a wide range of targets and may also effectively eradicate CICs; 3) long-term immunological memory can offer added protection against disease relapse; 4) immunotherapy should be relatively non-toxic; and 5) can be synergistically combined with other treatment platforms such as radiation and chemotherapy.

What is clear is that the relationship between immune cells, malignant cells and the tumour microenvironment is exceedingly complex and the advantage might be tipped in favour of either side. Cytokines are integral to both the innate and acquired immune systems; they regulate cell survival, proliferation, differentiation and activation, induce class switching of B
lymphocytes and alter the balance of cellular and humoural responses. These traits have made a number of cytokines interesting candidates for cancer immunotherapy\textsuperscript{4,5,7}.

1.2 Cytokines

Cytokines are signalling molecules predominantly responsible for the intercellular communication of the immune system but, within the tumour microenvironment, they can profoundly influence cancer pathogenesis. Cytokines participate in complex interactions with cells and molecules to promote tumour growth, metastasis and invasion or; alternatively, engender a robust immune response that inhibits cancer progression. For example, many cancer cells overproduce immunosuppressive cytokines, such as IL-10, or growth promoting cytokines, such as IL-1\textsuperscript{\beta}; while other cytokines like tumour necrosis factor alpha (TNF-\textalpha) can be directly cytotoxic. Therefore, the balance of immunostimulatory and immunosuppressive cytokines may determine the outcome of a developing cancer. This balance also proves decisive in moulding the host response and could be exploited to affect the clearance of malignant cells\textsuperscript{12}.

Cytokine therapy seeks to pharmacologically enhance the cytokine’s normal physiological function\textsuperscript{13} of stimulating immune cells into becoming active under conditions where they have remained inert. Accelerated cytokine discovery, and our growing understanding of their modes of action, will help in harnessing their potential to overcome or reverse tolerance. Many challenges remain as the pleiotropy and redundancy inherent to cytokines frustrate efforts to delineate their activity; as well as the further challenge of determining how cytokines behave in combination, since they rarely act in isolation.

Cellular rather than humoural responses to cancer are generally considered preferable, which makes IL-12 particularly attractive for use in immunotherapy.
1.2.1 IL-12.

Interleukin-12 is a pro-inflammatory cytokine and potent inducer of both the innate and adaptive immune responses. IL-12 acts on natural killer (NK) cells and natural killer T (NKT) cells to stimulate the production of interferon-gamma (IFN-\(\gamma\)) and TNF-\(\alpha\) among other cytokines. These cytokines can then promote the activation of other innate cells such as macrophages (M\(\Phi\)) and eosinophils. Dendritic cells (DC) mature and enhance their major histocompatibility complex (MHC) expression and Ag presentation capacity in response to IL-12, which ultimately leads to T cell activation and the development of CD8\(^+\) CTLs\(^2\). In fact, IL-12 is the archetypal T helper type 1 (T\(_{\text{H}1}\)) polarizing cytokine. Some of the anticancer effects of IL-12 have also been attributed to its anti-angiogenic activity. Based on current evidence, IL-12 was ranked as the 3\(^{rd}\) most desirable immunotherapeutic agent on a list of agents rated, during the National Cancer Institute (NCI) Immunotherapy Agent Workshop in 2008, for their potential to successfully treat cancer\(^{14}\). Preclinical models have demonstrated the effectiveness of IL-12 therapy in many different cancer types and following various administration routes, dosages and schedules. Clinical trials of IL-12 therapy have also been conducted but objective responses have been rare and toxicities limiting (reviewed in \(^{15}\)).

1.2.1.1 IL-12 signalling.

Active IL-12p70 is a heterodimeric cytokine composed of two subunits, p35 and p40, and is produced by APCs. Secretion of the functional protein requires both post-translational modification of the p35 subunit, in the form of extensive N-glycosylation, and co-expression of both partner proteins of the composite factor\(^{16}\). Production of p35 messenger ribonucleic acid (mRNA) is constitutive in most cell types\(^{17}\), but it is post-translationally regulated such that p35
is never secreted other than as part of the complete protein; and is, therefore, the rate limiting step in p70 secretion.

Regulation of the p40 subunit, on the other hand, is primarily transcriptional. Production of mRNA is not constitutive but inducible in response to factors such as LPS and CpG. Once induced, the p40 subunit is also capable of forming a homodimer; a detail that was not originally appreciated and has led to some confusion in the literature about the role of IL-12. The homodimer can bind the IL-12 receptor (IL-12R) and, some have argued, antagonize IL-12 signalling\textsuperscript{18}. However, more recent data suggests that this is unlikely because the affinities of p40 and p70 for the IL-12R are comparable and, since p40 is produced earlier and more abundantly than p70, IL-12 signalling would be altogether prevented if p40 homodimers acted as IL-12 antagonists. Rather, the theory this group posits is that the p40 homodimer form of IL-12 is responsible for initiating immune responses\textsuperscript{19}.

Secretion of IL-12p70 can be induced in APCs by MHCII:TCR interactions, ensuring the local and targeted delivery of IL-12 to only the appropriate T cell\textsuperscript{20}. IL-12 is also a heparin-binding protein. Heparin exists on the surface of cells and in the extracellular matrix, such that IL-12 binds and is retained close to the point of secretion; this may also contribute to the localized nature of its activity. Memory T cells are able to induce p70 production by APCs in a recall response, but not in a primary response. This is because the initiation of IL-12 production requires exposure of the APC to IFN-\gamma first\textsuperscript{21}, as will be discussed in more detail later. It has been proposed\textsuperscript{17,21} that the p40 homodimer is actually the initial signal in a primary response because p40 can partner with other subunits (e.g. p19 to make IL-23). The theory is that p40, produced by APCs in response to PAMPs (pathogen associated molecular pattern) or DAMPs
(danger associated molecular pattern), partners with another subunit (other than p35) to stimulate NKT cells to produce IFN-\(\gamma\). This allows for p70 production by DCs and complete IL-12R expression by T cells that can then be \(T_H1\) activated by p70 to produce further IFN-\(\gamma\) and boost the response\(^{17}\).

IL-12 acts mostly on NK, NKT, and T cells; though T cells do not constitutively express the complete receptor. NKT cells, however, do express the IL-12R under normal conditions and have been shown to produce IFN-\(\gamma\) in response to IL-12, without prior activation\(^{22}\).

The IL-12R is, itself, a heterodimer made up of the IL-12R beta 1 (IL-12R\(\beta1\)) and IL-12R\(\beta2\) chains. The \(\beta1\) subunit is constitutively expressed, but can be enhanced, and is primarily involved in specific binding of IL-12; whereas \(\beta2\) is the signal transduction subunit and its expression is inducible in response to IFN-\(\gamma\). Thus, \(\beta2\) expression constitutes the pivotal mechanism for regulating IL-12 responsiveness\(^{20}\). Signalling proceeds as follows: p40 binds to the \(\beta1\) subunit and p35 to the \(\beta2\) signal transduction chain, which are associated with the Janus kinases (JAKs) Tyk2 (tyrosine kinase 2) and Jak2, respectively\(^{23}\). Upon binding of the receptor by IL-12p70, the \(\beta1\) and \(\beta2\) chains come into close proximity, allowing for autophosphorylation of the JAKs and subsequent phosphorylation of the cytoplasmic region of IL-12R\(\beta2\)\(^{24}\). The signal transducer and activator of transcription 4 (STAT4) then binds to form the active receptor complex and is tyrosine phosphorylated by the Jak2 molecule\(^{25}\). Phosphorylated STAT4 dimerizes and translocates to the nucleus where it binds deoxyribonucleic acid (DNA) sequences related to the IFN-\(\gamma\) activation site elements\(^{2}\). It is in this way that IL-12 is a strong inducer of IFN-\(\gamma\), the cytokine that mediates much of its anticancer activity. It also drives the further secretion of IL-12 by DCs, creating a positive feedback mechanism to amplify the response.
IL-12 signalling in T cells (as well as NK and NKT cells) also leads to upregulation of cell adhesion molecules (e.g. p-selectin), production of cytotoxic effector molecules (e.g. perforin) and expression of CD25 (IL-2Rα) necessary for T cell proliferation. However, high levels of IL-12, and consequently IFN-γ, have also been associated with induction of antagonistic molecules such as IL-10 and the depletion of signalling molecules downstream of IL-12, such as STAT4. The β2 chain of the receptor is also selectively down-regulated during T_{H2} responses.

Production of IL-10 by T_{H1} cells requires continuous IL-12 signalling and strong TCR ligation; due to high Ag dose. The combination of extracellular signal-regulated kinases (ERK) 1 and 2 induced downstream of the TCR, and STAT4 downstream of the IL-12R, leads to the production of IL-10. This probably occurs after repeated positive feedback loops. IL-10 inhibits IL-12 signalling by inhibiting β2 expression and by interfering with the transcription of p40.

Endogenous IL-12 is required for protection against a number of intracellular pathogens, as observed in IL-12^{-/-} mice or patients lacking a component of the receptor chain. However, IL-12 also plays a pathogenic role in Multiple Sclerosis, Crohn’s Disease, diabetes and rheumatoid arthritis.

1.2.1.2 **IL-12 therapy in pre-clinical models.**

The use of IL-12 as an immunostimulant has been extensively studied in mouse models using a wide range of different approaches. In a transgenic mouse model, recombinant IL-12 (rIL-12) injected at the time of bone marrow (BM) transplantation promoted graft-versus-leukaemia responses while inhibiting graft-versus-host disease; and an adoptive cellular
therapy (ACT) protocol used rIL-12 to T H1 polarize ex-vivo T cells before adoptive transfer. Cell-based IL-12 administration has also been tested in leukaemia models using, for example, autologous acute myeloid leukaemia (AML) cells, acute lymphocytic leukaemia (ALL) cells, or transduced haematopoietic progenitor cells to eradicate residual disease in the BM. In solid tumour models IL-12 has been tested in the setting of microdisease and established tumours, and as a prophylactic therapy in models of spontaneous mammary carcinoma. Adenoviral constructs have been used as a cell-free gene therapy strategy to treat adenocarcinomas and fibrosarcomas; and cell-based approaches have included IL-12 secreting fibroblasts to treat melanoma and sarcoma, as well as engineered DCs that have been tested for efficacy against hepatocellular carcinoma. In place of transduced DCs, techniques have also been developed to harness their inherent IL-12 producing capacity and semi-mature DCs have been used in in vitro melanoma studies.

These approaches have resulted in delayed tumour onset, tumour eradication, clearance of metastatic disease and the establishment of immune memory; and have been variably associated with the accumulation of MΦs, NK cells and both CD4+ and CD8+ T lymphocytes.

1.2.1.3 Clinical history of IL-12.

While initial Phase I human trials employing intravenous administration of rIL-12 demonstrated adequate treatment safety to warrant Phase II trials, these were less promising (and discussed). The phase I trial determined that the maximum tolerated dose (MTD) is 500ng/kg; but the first phase II trial of systemic rIL-12, given at the same doses and according to the schedule established in phase I studies, resulted in the unexpected death of two patients due
to severe toxicity\(^5\). It was later determined that this toxicity resulted because a pre-dose was eliminated from the schedule\(^5\). However, other similar clinical studies have proven safe, if not very effective. One early study even measured a burst in the number of peripheral circulating CTLs responsive to TAAs, and a reduction in tumour burden at both the primary and metastatic sites\(^5\). It is well recognized in the literature that IL-12 induced anticancer activity is largely mediated by the secondary secretion of IFN-\(\gamma\)\(^3\). In particular, Gollob et al. demonstrated that the induction and maintenance of rIL-12-induced IFN-\(\gamma\) expression is highly correlated with favourable outcomes in patients with metastatic renal cell carcinoma (RCC)\(^4\); yet these elevated IFN-\(\gamma\) levels have also been associated with the concomitant induction of antagonistic molecules, such as IL-10, and the depletion of signalling molecules downstream of IL-12, such as STAT4\(^2,5\). Both toxicity and the induction of such antagonistic mechanisms continue to pose a challenge for the further development of IL-12 as a therapy. Overcoming these formidable obstacles is the impetus for testing the efficacy of IL-12 following different dose and time protocols\(^4,26,27,53,54\) and for evaluating the therapeutic potential of cell-based IL-12 gene therapy\(^39,41,55-59\) (and discussed in \(^2,3\)).

Cell-free gene therapy protocols have been evaluated whereby IL-12 plasmids are directly injected into tumours. One study that used two separate constructs, encoding p35 and p40 respectively, proved safe but no objective responses were observed\(^6\). In a more recent phase I trial, treating metastatic melanoma lesions with intratumoural electroporation of an IL-12 plasmid, either a partial response or disease stabilization was achieved in 42\% of the patients treated. Another 10\% of patients achieved complete tumour regression, even of untreated lesions, and systemic toxicity was minimal in all cases\(^6\).
Cellular gene therapy approaches have included injection of IL-12 secreting tumour cells, fibroblasts and DCs; methods that have proven effective in mouse models. To date, although some degree of immune response has been demonstrated, these approaches have not had a significant impact on patient survival\cite{56,58,62}. One trial, in which melanoma patients received subcutaneous injections of transduced autologous tumour cells, recorded two delayed type hypersensitivity (DTH) responses and one minor clinical response\cite{62}. Patients with surface accessible, advanced disease were injected peritumourally with IL-12 transduced autologous fibroblasts in another study. This protocol led to transient reductions in the size of tumours at the injection site as well as of metastatic lesions, but responses were not durable. Encouragingly, intratumoural injection of DCs that had been engineered to produce IL-12 led to observable CD8\(^+\) T cell infiltration in the biopsies of patients with metastatic gastrointestinal cancer; and to a partial response in a patient with pancreatic carcinoma\cite{51}.

In place of genetically engineering DCs to express IL-12, Dohnal et al. have optimized a manufacturing protocol whereby peripheral blood mononuclear cells (PBMC) are collected from the leukocyte apheresis product, enriched for monocytes, and cultured with lipopolysaccharide (LPS) and IFN-\(\gamma\)\cite{36}. The resulting DCs are harvested at a semi-mature (smDC) stage during which they are the most potent producers of IL-12. These IL-12 producing DCs are then injected with the hope of stimulating an immune response to the host’s cancer. In one intriguing in vitro study with human cells from melanoma patients, it was shown that T\(_{h1}\)-polarized DCs (prepared as above), but not unpolarized DCs (generated as above but in the absence of LPS and IFN-\(\gamma\)), were able to rescue patient type-1 anti-melanoma CD4\(^+\) T cell responses\cite{63}. The study concluded that properly prepared DCs may be able to “correct” type-1 insufficiency and establish anticancer
responses. These observations are important because cell-based vaccines are used once cancers are already established and may have induced a state of non-responsiveness in the host that needs to be reset. This may prove to be a very valuable and effective therapeutic approach. A phase I study was conducted for paediatric patients who received smDCs injected subcutaneously along with intranodal injections of tumour lysate-loaded DCs that had been fully matured with LPS, IFN-γ and keyhole limpet hemocyanin. The treatment proved safe enough that it could be administered in an outpatient setting, but responses were mixed and progressive disease cases did not show any sign of responding.64

Cell-free gene therapy has the advantage of being less technically demanding than working with cellular vectors; but cellular gene therapies are advantageous because the immune stimulant is presented together with the appropriate Ags to which the desired response should be directed. However, there are significant challenges to transducing human primary cells for therapeutic purposes. Robust protocols have been developed to use DCs as the vector, either by transducing them to produce IL-12 or inducing their maturation to a stage at which they inherently produce significant amounts. This approach may be more representative of how a response would normally develop, but is not self-limiting in the same way that using tumour cells as the vector is. Furthermore, there may be issues with how these artificially matured DCs traffic to the LNs where they must present Ag to stimulate an effective response.

Cutaneous T cell lymphoma, Acquired Immune Deficiency Syndrome (AIDS)-related Kaposi sarcoma, and non-Hodgkin’s lymphoma (NHL) are among the cancer variants for which IL-12 therapy has had the greatest success. However, the application of IL-12 therapy as an adjuvant to vaccines is possibly the most advantageous approach examined thus far. In one
protocol, stage III and IV melanoma patients that had undergone debulking surgery were then
given peptide vaccines derived from tyrosinase and glycoprotein 100 (gp100); 87% experienced
a boost in vaccine-specific peripheral immune responses when the vaccine was administered
with IL-12. In contrast to these isolated successes, most clinical investigations involving IL-12
have failed to recapitulate the promise of pre-clinical studies and finding the reason for this
disconnect is of paramount importance.

1.2.2 IFN-γ as a mediator of IL-12 anticancer activity.

In vivo, IL-12 favours a T_{H1} response through enhancement of IFN-γ and inhibition of
IL-4 production, but in vitro studies have given less consistent results: α-IFN-γ antibodies block
T_{H1} maturation of CD4^{+} cells in some, but not all, mouse models and in none of the human
studies. The initial and predominant result of IL-12 signalling is binding of the STAT4
molecule to IFN-γ activation site element DNA sequences; and IFN-γ appears to be required for
many of the effects of IL-12, which may in part be explained by the IFN-γ-inducible nature of
the IL-12R on many cell types, leading to a positive feedback loop. This does not seem to be the
case for all cells, however, with MΦs being an exception. IL-12-induced MΦ infiltration is
observed in IFN-γR knock-out mice, an event that underpins both effective immune responses
as well as profound pathology. Nonetheless, IL-12-induced IFN-γ supports development of the
T_{H1} responses that are considered more desirable for the treatment of malignancies.

Besides its role in mediating the anticancer effects of IL-12, IFN-γ has also been tested as
a monotherapeutic for its antiproliferative effect on neoplastic cells and its ability to stimulate
NK cells and MΦs. IFN-γ has also been studied for its capacity to both up-regulate the
expression of tumour antigens and increase the expression of MHC molecules on APCs, thereby effectively enhancing cancer immunogenicity\textsuperscript{71}.

\textbf{1.2.3 Cytokine combination therapy.}

Cytokines do not endogenously act in isolation but in networks with other cytokines; and therapeutic benefit is more likely to come from a combinatorial approach also. IL-12, in particular, demonstrates a great deal of synergy with other cytokines to enhance anticancer activities:

IL-2 and IL-12 reciprocally increase the expression of each other’s receptors and signal through distinct pathways, synergistically augmenting the proliferation of NK cells, as well as activated and effector T cells, promoting their production of IFN-γ and their anticancer effects\textsuperscript{72}.

IL-15 and IL-2 have some overlapping activities including all of the attributes listed above; however, IL-15 does not specifically support regulatory T cell (T\textsubscript{reg}) populations the way IL-2 can. Furthermore, IL-15 is essential for the maintenance of T cell memory\textsuperscript{72}.

IL-18 can be tumour promoting or induce T\textsubscript{H}2 responses in some combinations but, when paired with IL-12, promotes T\textsubscript{H}1 cytokine production and attenuates production of the IL-12 antagonist, IL-10\textsuperscript{72}. Furthermore, this combination has been shown to activate NKT cells which themselves can promote strong T\textsubscript{H}1 responses\textsuperscript{22}.

\textbf{1.2.4 Immunotherapy combined with standard treatments.}

Malignant cells divide more rapidly than healthy tissues and are thought, for this reason, to be more sensitive to the DNA damaging effects of radiation; still, this is a simplistic view and it is increasingly recognized that many characteristics of cancer cells make them radio-resistant. Thus, cells continue to divide and accumulate damage such that when they do die, they do so by
necrosis more often than apoptosis\textsuperscript{73}. This is an advantageous attribute for the combination of radiation with immunotherapy because necrotic death is not immunologically inert but rather releases danger signals that alert the innate immune system to cell death occurring in a non-physiological manner\textsuperscript{74}. What’s more, technological advances have dramatically changed the way that radiation can be applied. Fractionated radiation therapy, originally employed to minimize collateral damage to the surrounding healthy tissue by allowing an opportunity for cells to repair sublethal damage\textsuperscript{73}, also allows for the recovery of cancer cells such that it rarely leads to a cure. Improvements in the specificity of tissue targeting have allowed for the delivery of much higher but localized doses. These ablative doses were shown in one study to increase the efficacy of an IL-12 producing DC vaccine, whereas fractionated radiation had an immunosuppressive effect, inhibiting IL-12 production\textsuperscript{75}. However, precaution must be used when pairing immunotherapy with radiation therapy as a study by Dranoff \textit{et al.} showed that the activity of cellular vectors producing some cytokines is potentiated by irradiation of the cells yet completely abrogated for other cytokines\textsuperscript{76}.

Chemotherapy in general, like radiation, tends to have its greatest affect on rapidly dividing cells; including healthy tissues with a naturally high turnover rate, such as lymphocytes. The immunosuppressive side-effects of chemotherapy have been largely attributed to the elimination of APCs as well as B and T cells\textsuperscript{77}. However, there are chemotherapeutic agents with beneficial effects. Cyclophosphamide, for example, is an immunosuppressant at therapeutic doses but at lower doses not only selectively eliminates Tregs but also inhibits their suppressive capability\textsuperscript{78}. Cyclophosphamide also disrupts the tumour microenvironment and leads to type I interferon production\textsuperscript{77}. Cytarabine, also known as Arabinofuranosyl Cytidine (AraC), is another
chemotherapy drug that is used primarily for the treatment of acute myeloid leukaemia (AML) and non-Hodgkin lymphoma. AraC is very similar in structure to deoxycytidine and therefore interrupts DNA synthesis, stalling cells in the S phase. Furthermore, because AraC is used to target the white cell compartment, it is known to have immunosuppressive effects.

A number of studies have shown that the degree of cancer-induced immune suppression can be correlated to tumour size\textsuperscript{79,80}, with larger tumours having a more suppressive effect. In addition to having an immune potentiating role, then, debulking interventions like radiation, chemotherapy and surgical resection might also release the immune system from this repressed state. While these debulking methods are rarely curative alone, subsequent application of immunotherapy is likely the ideal way to eradicate minimal residual disease (MRD).

Moreover, the clinical application of immunotherapy is still in its infancy and is, of yet, largely unproven. It is therefore doubly important to consider the effect that SOC procedures have on immunotherapies as they will almost certainly be delivered in combination.

1.3 Cell Lines and Mice

The 70Z/3 cell line is well studied in our lab and was chosen as an appropriate model for conducting the experiments described herein because the development of disease is highly predictable. Other cell lines and mouse models were chosen as controls for various experiments due to characteristics that are described below.

1.3.1 70Z/3.

The pre B cell line, 70Z/3, was chemically induced in a thymectomized female (C57BL/6\times DBA/2)\textsubscript{F1} (referred to as BDF\textsubscript{1}) mouse using methylnitrosourea\textsuperscript{81} and subsequently established in culture\textsuperscript{82}. The transforming event was found to be a mutation in cbl that produces a protein with a marked enhancement in tyrosine phosphorylation activity\textsuperscript{83}. Though a variant of
70Z/3 has been discovered that activates the immune system to successfully reject it, the Ag that is recognized has never been identified. As this is the case for the majority of human tumours, this situation constitutes a shared limitation of the model system and the clinical reality.

MHCII expression is regulated during B cell ontogeny: it is absent on pro-B cells, constitutively expressed on immature and mature B cells and again downregulated during differentiation into plasma cells. This regulation is accomplished by differential expression of class II transactivator (CIITA). 70Z/3 cells are at the transition from pre-B to immature B stage and express μ on their surface but are negative for MHCII. 70Z/3 cells are also deficient for expression of the p55 subunit of the TNF-α receptor.

The in vitro doubling time for 70Z/3 cells is approximately 6-8 hours and when injected into syngeneic mice, the resulting leukaemia is most akin to acute lymphoblastic leukaemia (ALL), manifesting as ascites, splenomegaly and, frequently, infiltration of the bone marrow, liver and kidneys. While the role of the microenvironment is less appreciated for leukaemias than for solid cancers, this tissue infiltration provides niches where leukaemic cells interact with non-transformed cells that support their growth.

Several variants of the 70Z/3-L parent line have been developed, including a macrophage-like line, 70Z/3 MΦ; an IL-12 secreting line, LV12.2; and an IFN-γ resistant line, LV12.2-r; which were used in these studies.

### 1.3.2 L1210.

L1210 is a lymphoblastoid line derived from a tumour in a female DBA/2 mouse that developed after skin paintings with 0.2% methylcholanthrene in ether. This B-cell lymphoma is sensitive to IFN-γ and is MHCII negative due to methylation of the CIITA upstream regulatory
region. The *in vitro* doubling time is approximately 12 hours and they are resistant to TNF- and Fas-mediated apoptosis. L1210 cells are highly heterogenous for cell size and are positive for surface expression of μ by flow cytometry.

**1.3.3 C1498.**

Studied as a model for AML, the C1498 cell line has more recently been reclassified as a NKT cell leukaemia that falls within the double negative (CD4^-CD8^-) lineage and does not express NK1.1. The cell line was originally derived from a C57BL/6 mouse and was used here as a positive control for expression of the canonical NKT cell receptor chain, Vα14Jα281.

**1.3.4 Ltk-.**

The mouse fibrosarcoma cell line, Ltk-, was used as a negative control for MHCII detection by flow cytometry because these cells are unable to express either I-E or I-A MHCII on their surface, even in response to IFN-γ stimulation, due to a deficiency in CIITA expression.

**1.3.5 Smarta mice.**

The CD4^+ T cells in transgenic Smarta mice carry a MHCII-restricted T cell receptor (TCR) specific for the lymphocytic choriomeningitis virus (LCMV)-derived glycoprotein Ag, P13. CD4^+ T cells proliferate and secrete IL-2 in response to this Ag presented by DCs, on MHCII, *in vitro* and will be used as staining controls for the detection of MHCII and CD4 foci development by fluorescence microscopy.

**1.4 Endogenous Cell Populations**

**1.4.1 T lymphocytes.**

The innate immune system is capable of rapidly mounting a response to control pathogen spread, through the recognition of a limited number of PAMPs. By contrast, the adaptive immune system is characterized by an ability to recognize a wide range of very specific Ags and
to generate memory. B lymphocytes are predominantly responsible for mounting a response against extracellular pathogens while T lymphocytes are responsible for directly targeting the body’s own cells that are displaying signs of infection or alteration. This recognition occurs when a T cell carrying a specific TCR recognizes peptide Ag that is presented by a MHC molecule. There is an ever-growing list of T cell subsets that mount different responses under different circumstances; below is a description of those subsets most relevant to this work.

1.4.1.1 T cell subsets.

The two main classes of T cell are defined by the co-receptor molecule, CD4 or CD8, they express at maturity and differ in their effector function. However, T cell responses by both subsets are predominantly shaped by DCs in the secondary lymphoid organs where T cell-DC interactions direct the T cell to commit to proliferation, differentiation and acquisition of effector function. Each MHC:Ag:TCR interaction is individually of low affinity but serial ligation over time allows for potent stimulation. Once activated, T cells produce IL-2 which supports the clonal expansion that necessarily precedes effector function. Some estimates suggest that a clonal T cell population, all cells of which recognize the same antigenic peptide, can expand from approximately 50 to 10,000 cells after 8 days of activation induced proliferation.

Some naïve CD8+ T cells are destined to become cytotoxic effector cells that are able to directly kill infected or transformed cells. CD8+ T cells are MHCI restricted and MHCI is expressed on all nucleated cells and loaded with Ag derived from the cytosol. The severity of their effector function probably explains why more extensive stimulation is required to fully activate CTLs as compared to other T cell subsets. Mature DCs sometimes have sufficient intrinsic capacity for stimulation to induce the IL-2 production that is required for a proliferative
burst and differentiation of a naïve CD8\(^+\) T cell into a fully functional effector cell; however, more often additional stimulation is required and this is provided by CD4\(^+\) T helper cells. In order to provide this help, it is necessary that both T cells recognize related Ag on the surface of the same antigen presenting cell (APC). This need for extensive activation is also, in a sense, a liability: when the target cell is not a DC, which is the case most of the time, recognition of Ag-MHCI on the cells surface is not sufficient for activation. This problem can be overcome by to a process known as cross-priming, which will be discussed in more detail below.

CD4\(^+\) T cells recognize Ag in the context of MHCII and MHCII expression is restricted primarily to professional APCs, but can also be induced on most cell types and tissues\(^{97}\). There is a growing list of CD4\(^+\) T cell subtypes that are identified by the primary cytokine they produce and differ in their response to various stimuli; these include: T\(_{H1}\), T\(_{H2}\), T\(_{H3}\), T\(_{H9}\), T\(_{H17}\), T\(_{H22}\), T\(_{FH}\) (follicular helper T cell) and T\(_{reg}\)^{98}, but the T\(_{H1}\) and T\(_{H2}\) subtypes remain the best understood. T\(_{H1}\) cells are characterized by their IFN-\(\gamma\) production and are able to compensate for suboptimal CD8\(^+\) cytotoxic T lymphocyte (CTL) activation by APCs. This is thought to be accomplished through binding of an activated CD4\(^+\) T\(_{H1}\) cell and ligation of CD40 on the same APC to which the CD8\(^+\) T cell is making contact, thus inducing increased expression of costimulatory molecules and enhanced activating capacity that results in full activation and differentiation into a CD8\(^+\) CTL\(^{96}\).

T\(_{H2}\) cells, on the other hand, are defined by their production of IL-4 and are responsible for initiating and supporting humoral responses. This can be accomplished by direct contact with B cells, through CD40/CD40-L binding, which promotes B cell maturation to the antibody (Ab)-secreting plasma cell stage. Secreted Ab protects from attack by pathogens in the
extracellular space in a number of different ways: neutralization, opsonization or by initiating the complement cascade. The effector mechanism is defined by the class of Ab but, ultimately, all Ab coated entities are taken up and disposed of by phagocytes that may not normally recognize them. B cells acquire protein Ags through internalization of bound B cell receptors (BCR); the Ag is then processed and returns to the plasma membrane on MHCII. Primed CD4\(^+\) T\(_H\)2 cells then bind through their TCR and ligate CD40 on the B cell surface such that help is provided only by CD4\(^+\) T cells that recognize related Ag. The T\(_H\)2 cell is also induced by this process to produce cytokines, specifically IL-4, that drive clonal expansion and direct Ab class switching on the B cell to shape an appropriate response\(^96\).

1.4.1.2 Cell recognition.

T cells have a low threshold to activation under the right conditions and are highly sensitive to Ag. When a T cell encounters a cell bearing MHC-bound cognate Ag, the T cell pauses and becomes involved in an increasingly stable interaction with the other cell, be it an APC or a target cell. The stability of the interaction is roughly proportional to the density of MHC molecules offered and the avidity of the TCR for its ligand\(^99\), but other environmental factors can also have an effect. Technological advances have allowed these interactions to be visualized in precise detail such that the exact number of ligands involved can be measured and the outcome of the interaction observed. A number of these studies agree that some CD4\(^+\) T cells can respond to a single Ag:MHC molecule, but that optimal stimulation can be achieved with ten or more such ligands\(^99\).

This stimulation leads to formation of a stable synapse that allows for a bidirectional flow of information between the two cells. The cell membranes undergo massive reorganization
beginning with the microtubule organizing centre (MTOC), which is made up of actin, the microtubule skeleton and the centrosome, moving right up to the membrane at the synapse. This structure allows for the polarization of membrane embedded constituents as well as secretory and endosomal vesicles\textsuperscript{100}, and leads to the formation of three main regions termed supra-molecular activation complexes (SMAC). The central (cSMAC) zone is densely populated with TCRs, MHC molecules, co-receptors and co-stimulatory molecules. The presence of CD4 on the outskirts of this region works to significantly lower the initial signalling threshold, possibly through its ability to cross-link two TCRs, but CD4 positioning is dynamic and the molecule is excluded at later time points\textsuperscript{101}. Also contained in the cSMAC are downstream signalling molecules, such as lymphocyte-specific protein tyrosine kinase (Lck) and protein kinase C-\(\theta\) (PKC-\(\theta\))\textsuperscript{99}; and the dense packing of TCRs leads to increased signal transmission. The peripheral (pSMAC) ring, that surrounds the cSMAC, is populated by adhesion molecules that essentially fuse the two membranes and separate the cSMAC from the extracellular space. This allows for targeted transfer of soluble mediators while mitigating any effect on surrounding cells\textsuperscript{100}. Bulky molecules like CD43 and CD45 are forced to the distal (dSMAC) region\textsuperscript{99}.

Most of this work has been done on CD4\(^+\) T cells in which initial binding leads to calcium fluxing and nuclear localization of the nuclear factor of activated T cells (NFAT). NFAT is a transcription factor that mediates the expression of many of the genes involved in T\(H\) activity\textsuperscript{99}. However, effector molecules are also delivered across the synapse and these secretory lysosomes are transported to a region of the cSMAC devoid of TCRs by moving through the cytoplasm along microtubule rails toward the centrosome\textsuperscript{100}. In the case of CTLs, multiple target cells can be bound simultaneously and the mature synaptic structure may be sustained for only
twenty minutes before the MTOC oscillates rapidly to the next immunological synapse to deliver the ‘kiss of death’. For CD4+ T_H cells, on the other hand, the structure can endure for periods in excess of ten hours before it is dismantled. This extended interaction allows for full activation and IL-2 production\textsuperscript{102}.

### 1.4.1.3 Effector mechanisms.

In addition to the secretion of cytokines, like IFN-\(\gamma\) that activates effector cells of the innate immune system and has cytostatic effects on malignant cells, there exist two main classes of cytotoxic effector molecules employed by CTLs: secreted granules and membrane-bound ligands for the death receptors expressed by their target cell.

Granules are specialized lysosomes that contain three types of cytotoxic proteins: perforin, granzymes and granulysins. As described above, upon Ag recognition, CTLs polarize their membranes and lytic granules travel along microtubule structures to coalesce at the immunological synapse. The proteolytic proteins contained within the granules exist in their active form but are kept inactive until degranulation by the low pH environment\textsuperscript{103}. Perforin is a protein related to complement component C9; the monomeric protein binds to phosphorylcholine on the target cell’s membrane and then polymerizes to form a pore that compromises membrane integrity\textsuperscript{96}. This alone can be a lethal blow to the cell but it is very inefficient. Granzymes are serine proteases that can pass through the perforin pore and into the cytosol of the target cell to increase the efficiency of killing by cleaving caspase-3 and initiating the caspase cascade that ultimately results in DNA degradation and cell death. Granulysins can also induce apoptosis in the target cell.
The membrane-bound mediators of CTL killing belong to the TNF family of receptor ligands. Three members of the family are most often associated with CTL activity and include: TNF-α, Fas-ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL). The receptors of these ligands all contain a death domain, which is a region of homology for the whole family of receptors; and the signalling pathway that results in apoptosis, through activation of the caspase cascade, is similar downstream of all of these receptors\textsuperscript{104}. Interestingly, granules also contain Fas-L, embedded in the vesicle membrane, that can bind its receptor during the process of degranulation\textsuperscript{105}.

1.4.1.4 Memory.

The development of memory is one of the core advantages of the adaptive immune system; ensuring that subsequent challenges with the same pathogen will be responded to more quickly and efficiently.

Like CD8\textsuperscript{+} T cells, CD4\textsuperscript{+} T cells in the effector phase can be identified as IFN-γ\textsuperscript{+}, CD4\textsuperscript{4\textsuperscript{high}}, CD62L\textsuperscript{low} and CD127\textsuperscript{low}; whereas effector memory cells are CD4\textsuperscript{4\textsuperscript{high}}, CD62L\textsuperscript{low} and CD127\textsuperscript{high}, and maintain the ability to produce IFN-γ in response to specific Ag\textsuperscript{106}. The effector population undergoes substantial contraction within a relatively short period of time after the primary response; for example, 40 days after the peak of the effector response, memory CD4\textsuperscript{+} and CD8\textsuperscript{+} cells specific for LCMV contracted by 30\% and 54\% respectively, followed by a more modest population decline over a longer time period\textsuperscript{106}. These studies also demonstrated that the CD4\textsuperscript{+} memory pool arises directly from IFN-γ\textsuperscript{+} effector cells, as is the case for CD8\textsuperscript{+} memory cells, not from naïve precursors. Together, this suggests that the memory pools for both CD4\textsuperscript{+} and CD8\textsuperscript{+} subsets arise through a common developmental pathway. In contrast to that
conclusion, another group asserts that T<sub>H1</sub> lineages display different capacities for memory cell generation. They showed that memory arises from the IFN-γ effector cells to a greater extent than IFN-γ<sup>+</sup> cells; but despite this, they retain the capacity to produce IFN-γ in response to stimulation. The group goes on to suggest that the failure of IFN-γ<sup>+</sup> effector cells to form long-lived memory might be due to the aforementioned anti-proliferative properties of IFN-γ<sup>107</sup>.

Clonal contraction is a necessary step in establishing the pool of long-lived memory cells, however, groups have reported very different numbers with respect to this process in studies that involve adoptively transferring large numbers of monoclonal or polyclonal effector cells in order to track their fate. One group looked more closely at the average effector cell half-life and at the degree of population decline, and found that these measures are proportional to the input number. Furthermore, the transfer of cell numbers that are physiologically appropriate resulted in transient cell division events that were reminiscent of homeostatic proliferation<sup>95</sup>. They explain this finding by proposing that memory maintenance is contingent on access to a clone-specific survival signal, and that intraclonal competition is responsible for maintaining optimal repertoire diversity. Precisely how memory populations are maintained for the lifetime of the host is not yet understood but maintenance of their self-renewal capacity is, at least in part, due to IL-15 signalling<sup>95</sup>. Moreover, evaluation of a patient harbouring a null mutation in the IL-12Rβ1 chain provided evidence that IL-12 plays a role in the formation and proper functioning of T<sub>H1</sub> effector memory cells<sup>108</sup>. This patient possesses a completely normal and functional central memory cell pool but fewer effector memory cells, as compared to age matched controls; and the effector memory cells present are dysfunctional. The group proposed that the mutation induced an impediment at the stage of the cells’ conversion from central to effector memory.
1.4.1.5 CD4+ CTLs and effector cells.

Orchestrating the adaptive response is classically thought to be the role of CD4+ T cells, and this is indeed true. CD4+ T cells provide help to APCs, B cells and CD8+ CTLs, and control viral spread through the production of cytokines; however, more recent evidence demonstrates that they are capable of much more. A class of CD4+ T cells with cytotoxic capacity was first observed in models of chronic infection and, originally, these cells were thought to be artifacts of the in vitro systems that were used to describe them because they possess characteristics of highly differentiated T cells. Further investigation of this class of CTL has been hampered by a number of factors, including the limited efficiency of expanding CD4+ CTL populations, the lack of appropriate animal models for investigating their anticancer responses, and the relative complexity of identifying cognate TCRs due to the genetic diversity of MHCII. However, MHCII tetramers are now becoming available, which has aided the identification of this class of effector cell under a growing number of circumstances. As it turns out, these cells are not as rare as was once thought and can be found in human peripheral blood samples. Normally they make-up no more than 2% of the whole CD4+ T cell population in a healthy adult individual, but this number expands dramatically in individuals with chronic infections until approximately 50% of CD4+ T cells can demonstrate cytotoxic capacity.

Development of CD4+ CTLs is supported by TNF-α and IFN-γ, and the role of APCs in their acquisition of a cytotoxic phenotype is indispensable. Several studies have delineated the differentiation pathway and characterized it as the sequential loss of chemokine (C-C motif) receptor 7 (CCR7), CD27 and CD28 expression, and upregulation of CD57; the loss of CD28 coinciding with acquisition of functional characteristics such as granule formation. This pattern
is common to CD4+ and CD8+ T cells but the sequence differs. Specifically, acquisition of lytic capacity appears early in the process of differentiation for CD8+ T cells, but occurs only after CD4+ T cells are already highly differentiated\textsuperscript{110}. However, Amyes et al. argue that T cell differentiation, and thus effector function, should be dependent on many factors, including: initial viral load, Ag persistence, Ag location, and pathway of Ag presentation. They compared models of Epstein-Barr virus (EBV) and choriomeningitis virus (CMV) infection and, contrary to the differentiation process described by Appay, concluded that the phenotype of the CD4+ CTL population differed from model to model. Considering their results, this group warned that CD4+ CTLs are a highly heterogenous group of effector cells and caution should be exercised when identifying them based on their phenotypic properties\textsuperscript{111}. In general, groups identify the mature population using markers that: rule out other effector populations (CD8−, CD56−, CD25−), demonstrate a low level of activation and proliferation (CD69−, ki67−) and a high level of differentiation (CD45RA−, CD45RO+, CD28−), as well as detecting a functional marker (Fas+, perforin+, or GzmB+)\textsuperscript{110}.

During stimulation, strong cytolytic activity is induced by lower, rather than higher, peptide concentrations\textsuperscript{98}, which may explain why CD4+ CTLs are often seen in the context of chronic disease. This may also suggest that the ideal application for CD4+ CTL-based therapies is against mildly immunogenic malignancies, the latent stage of infection, or against MRD.

There is evidence from both pre-clinical studies and clinical trials that CD4+ CTL responses are effective. Furthermore, adoptively transferred CD8+ CTLs, having been pre-activated, can be effective in the absence of CD4+ T cells, but naïve CD8+ responses require CD4+ T cell support\textsuperscript{112}. Such support is not required for CD4+ CTL activation, making them
attractive therapeutic agents. For example, EBV infected cells can be killed by CD4\(^+\) CTLs using both the Fas-L and perforin pathways. Burkitt’s lymphoma, the malignancy associated with EBV infection that is resistant to CD8\(^+\) CTLs due to downregulation of MHCI expression, is also sensitive to killing by CD4\(^+\) CTL\(^{109}\); and CD4\(^+\) CTLs were ultimately incited as a result of an autologous cancer cell vaccine that used alpha galactosylceramide (\(\alpha\)GalCer) to stimulate a NKT response in a model of B cell lymphoma\(^{113}\). Interestingly, one study used a herpes simplex virus (HSV) vaccination strategy, known to induce a semi-effective T\(_H\)2 response, and attempted to impose a T\(_H\)1 skew by adding IL-12 dosing to the protocol. That resulted in activation of a CD4\(^+\) CTL population that killed infected cells in a perforin-dependent manner\(^{114}\).

A number of studies employing transgenic models have also demonstrated the effectiveness of this subset in specifically targeting MHCII negative malignancies. A MHCII negative fibrosarcoma was rejected by CD4\(^+\) effector population in an IFN-\(\gamma\)-dependent manner, even though the tumour cells were IFN-\(\gamma\) unresponsive. Based on their experimental evidence, Mumberg et al. proposed that killing was M\(\Phi\)-mediated; but this wasn’t directly tested\(^{115}\). In another model, M\(\Phi\)s were definitively shown to mediate the killing of a MHCII negative myeloma under direction from CD4\(^+\) effector cells. Tumour growth was controlled by effector cell secreted IFN-\(\gamma\) and TNF-\(\alpha\), though it was proposed that the M\(\Phi\)s were the targets of these cytokines as IFN-\(\gamma\) was not directly toxic to the tumour cells\(^{116}\). When CD4\(^+\) and CD8\(^+\) CTL responses against a panel of tumour lines were compared, where both populations recognized peptide from the same male Ag expressed by all tumours, the CD8\(^+\) CTL response was superior \textit{in vitro} but ineffective \textit{in vivo}. By contrast, the tumour cells were unaffected by culture with the CD4\(^+\) population but were efficiently eradicated \textit{in vivo}; including those tumours that were
insensitive to CD8$^+$ CTL killing. Again in this system, the MHC status of the target cells appeared to be irrelevant; however, MHCII expression by host tissues was required. NK cells mediated tumour cell killing but it could not be ruled out that rejection may have in part been the result of CD4$^+$ effector cell cytokine production that targeted the tumour-supporting stroma, the tumour cells themselves, or both$^{117}$.

Because in the above models it was innate cells that were attributed with mediating cytotoxicity, one would assume that memory would not be established, but these studies did not directly address this. One possibility is that memory resides in the CD4$^+$ CTL population with innate responses orchestrated de novo for each recall response. In support of this proposal, when EBV epitopes were engineered for targeted uptake into the endosomal and lysosomal compartments for loading onto MHCII, and a direct CD4$^+$ CTL response was noted, so also was formation of a memory pool$^{118}$.

One of the key challenges for ACT approaches is the difficulty of expanding T cell populations to sufficient numbers; Quezada et al. addressed this using a transgenic melanoma model. In their system, naïve MHCII-restricted CD4$^+$ transgenic effector cells were transferred, therefore mimicking a primary response. This was followed by a massive in vivo expansion of the population and cure of large, established and metastatic melanomas. CTL-associated Ag 4 (CTLA-4) blockade further enhanced their anti-tumour capacity and allowed for the treatment of disseminated, spontaneous tumours using a therapeutic approach$^{119}$. This approach required the administration of very few CTLs and several studies have proposed that a superior response can be initiated by administration of physiologically relevant numbers of effector cells$^{95,120}$. The response was dependent on T cell-produced IFN-γ but IFN-γ produced by host tissues was
dispensable. Though the tumour cells were dramatically affected by direct exposure to IFN-γ, that alone could not mediate complete tumour clearance; yet it was required for the upregulation of MHCII on the tumour cells, which allowed for direct recognition by CD4⁺ CTLs and granule-mediated, Ag-specific tumour rejection¹¹⁹. In such a situation as this, the tumour cells are presenting endogenously sourced Ag complexed to MHCII on their surface. Both exogenous and endogenous pathways to MHCII loading have been described; the endogenous pathway of MHCII-Ag loading involves autophagy or re-routing of the secretory pathway from the endoplasmic reticulum (ER) (¹⁰⁹ provides a nice overview of these mechanisms).

Clinical applications have included the use of adoptively transferred CD4⁺ CTL in patients with: metastatic melanoma, relapsed lymphoma, and chronic myelogenous leukaemia (CML). For example, a patient with metastatic melanoma was treated with an autologous CD4⁺ effector clone, recognizing NY-ESO-1, that was generated ex-vivo, expanded and re-infused into the patient without any subsequent IL-2 administration¹²¹. This is significant because IL-2 is highly toxic and its use in treatments has been compared to inducing a controlled state of septic shock¹²². Patients receiving IL-2 require extensive supportive care and this poses a restraint on the application of therapies that require co-administration of IL-2. The clone underwent in vivo expansion, reaching nearly 2% of the PBMCs and persisting for more than 80 days. Though immunohistochemistry of a pre-treatment tumour biopsy estimated that only about 50-75% of the tumour cells expressed the Ag on which the CD4⁺ effector cells were selected, follow-up evaluation showed complete tumour regression with no remaining radiographic or clinical evidence of disease and no evidence of long-term toxic effects related to treatment. The patient was followed for 2 years during which time no additional treatment was administered. Complete
tumour regression of metastatic disease was attributed to the expansion of T cells reactive to other TAA,
through Ag spreading, and the initiation of a de novo CD8+ CTL response121. This underscores one of the key attractions of mobilizing CD4+ T cells: the originally appreciated role of helper T cells in supporting the anticancer responses of other cell populations can afford additional functional layers to the directly cytotoxic activity of CD4+ CTLs, and these roles can be enacted in parallel. Indeed, some studies have suggested that CD4+ CTLs are an extension of the T helper type-1 developmental pathway98, and this may explain their overlapping functionality.

Considering the evidence, CD4+ CTLs are poised to have a dramatic effect on the way immunotherapy protocols are designed due to an emerging understanding of their complex role in the immune response. One of the original promises of cellular immunotherapy was that if the key oncogenic protein (viral protein, mutated growth factor receptor, etc.) can be targeted, then those malignant cells that escape the immune response by losing expression of that protein will also lose their tumourogenicity. Instead, tumours often lose MHCI expression and subvert an immune response in this way. Perez-Diez et al. proposed that, by inciting a multi-pronged attack against related Ags from these proteins, this goal may once again be considered feasible117. CD4+ CTLs are the perfect candidates because they can be cytotoxic in their own right while also initiating effector responses by CD8+ T, NK, MΦ and B cell populations.

1.4.2 NKT cells.

Several classes of NKT cells have been identified, with invariant NKT (iNKT) cells being the most commonly studied; it is this population to which we will refer throughout this thesis and they will henceforth be referred to simply as NKT cells. Sitting at the junction of the
innate and adaptive immune systems, NKT cells are capable of rapidly producing copious amounts of cytokine, either IFN-γ or IL-4, that subsequently activate other cell types and promote T\(_H\)1 or T\(_H\)2 responses, respectively. NKT cells are innate-like lymphocytes, originally defined by their expression of NK1.1, an NK cell marker, together with an invariant TCR (iTCR). In mice, the iTCR is composed of the V\(_\beta\)8.2 and V\(\alpha\)14J\(\alpha\)18 chains and recognizes glycolipid Ags. To date, the only known endogenous NKT cell agonist is isoglobotrihexosylceramide (iGb3), which is synthesized in the mouse dorsal root ganglion. Immune cell populations do not express this glycosphingolipid but possess the necessary ingredients for its conversion from iGb4\(^{123}\). Identification of other endogenous ligands is only a matter of time as recent studies have definitively demonstrated that, though iGb3 is unquestionably an NKT cell ligand, it is not the only one and likely not the one responsible for developmental selection in the thymus\(^{124}\). Most studies employ an exogenous ligand, alpha galactosylceramide (\(\alpha\)GalCer), loaded onto CD1d tetramers for the characterization and selection of NKT cells. However, \(\alpha\)GalCer is a marine sponge glycolipid\(^{125}\) that is difficult to obtain in large quantities and suffers from poor solubility and availability. An analogue, PBS-57, has been produced by a group led by Dr. Paul B. Savage and provided to the Tetramer Core Facility of the National Institutes of Health. PBS-57 is more soluble and appears to label NKT cells in a universal fashion, without bias for V\(\beta\) chain use\(^{126}\). This ligand is used for all studies described herein.

In addition to recognizing lipid Ags presented on CD1d, a non-polymorphic MHCI-like molecule, NKT cells have a number of other pathways to activation including cytokine-driven and Ag-independent activation downstream of IL-12 and IL-18\(^{22}\). This suggests that NKT cells
constitutively express the IL-12R and other studies agree, stating that NKT cells are the only cells that express the mature form of the IL-12R in the resting state\textsuperscript{127}. Not all reports agree on this point, however, citing αGalCer ligation as a requirement for the expression of the complete IL-12R\textsuperscript{128}.

The outcome of NKT cell activation is the production of cytokines, for example IFN-γ, that activate cells of the innate immune system and, in turn, result in potent adaptive responses\textsuperscript{129}. NKT cells are also capable of direct cytotoxicity through their production of TNF-α\textsuperscript{130}. The question of NKT cell memory is a controversial one and difficult to answer because NKT cells display characteristics associated with memory T cells even before exposure to Ag\textsuperscript{131}.

1.4.3 Dendritic cells.

DCs are specialized for providing activating signals to recirculating naïve T cells. They are resident in most tissues, are long lived\textsuperscript{96} and, in the immature stage, DCs are constantly sampling their environment through the process of macropinocytosis. When they pick-up pathogenic Ag and receive a stimulatory signal through pattern recognition receptors (PRR) or from inflammatory cytokines, phagocytosis and Ag sampling are first increased and then rapidly shut down. The cells then begin to mature and migrate to secondary lymphoid tissues\textsuperscript{132}, specifically the LNs, where they have an increased chance of encountering a naïve recirculating T cell that is expressing a cognate TCR for the Ags they are presenting. Maturation of DCs is also characterized by enhanced expression of co-stimulatory molecules, an important step in the Ag presentation process because Ag recognition that occurs in the absence of co-stimulation leads to anergy and the T cell becomes permanently refractory to stimulation.
At the point of interaction between DC and T cell, adhesion molecules cluster into a tight ring, forming the pSMAC, upon T cell activation. This structure allows for the directed secretion of cytokines exclusively toward the intended target\textsuperscript{133}, such as IL-12 for T\textsubscript{H}1 polarization. This interaction occurs with such avidity that small sections of the plasma membrane can be torn off of the donor cell and absorbed into the membrane of the opposite cell; in this way, T cells can acquire functional molecules from the surface of an APC with which it interacts. This process has considerable biological significance. For example, T cells that acquire MHC molecules are endowed with Ag presenting capability and can then act as APC to other T cells\textsuperscript{134}; and this T cell-T cell Ag presentation can play either a regulatory role\textsuperscript{135} or a stimulatory role that magnifies the response\textsuperscript{136}. Membrane ‘nibbling’ is also a source of Ag for DCs. Absorbed material is directed to endosomes where it can be processed and peptides loaded onto MHC molecules for presentation\textsuperscript{134}.

CD8\textsuperscript{+} T cells are equipped to protect against intracellular pathogens and malignancies when they recognize cytosolic proteins presented on MHCI. However, though all nucleated cells express MHCI, not all cells have the capacity to activate a naïve CD8\textsuperscript{+} T cell; that is the domain of professional APCs and, in particular, DCs. When another type of cell is affected and needs to be sacrificed to prevent the spread of disease, Ag must be cross-presented by DCs to stimulate the differentiation of CD8\textsuperscript{+} CTLs. Cross-presentation is when extracellularly derived Ag is presented on MHCI. Uptake of extracellular Ag is likely receptor-mediated, through a variety of receptors that include the DC-specific markers DC-SIGN (CD209) and DEC205 (CD205). This may explain why not all DCs are able to cross-present\textsuperscript{137}, only those that express the necessary receptors. Full CTL activation under these circumstances also requires T\textsubscript{H} help or the resultant
CTLs have such a short life span that they are unable to carry out their cytotoxic calling\textsuperscript{137}. The required help occurs indirectly through the DC, probably through CD40/CD40-L binding\textsuperscript{138}, which is ‘licensed’ by the CD4\textsuperscript{+} helper cell to initiate a programme of sustained cytotoxicity and memory formation in the CD8\textsuperscript{+} cell.

1.5 Thesis objectives

While the promise of curative immunotherapy remains to be realized, the steady progress in our understanding of immune regulation, including cytokine biology, is providing a rational basis for developing new approaches to induce an effective anticancer immune response. My goal herein was twofold: to use a murine model of leukaemia to explore the immune response initiated by cell-mediated IL-12 therapy and identify those observations that could have a bona fide impact on the translation of cytokine immunotherapy into clinical practice; and to use our \textit{in vitro} model to characterize a CD4\textsuperscript{+} CTL population.
CHAPTER 2: Materials & Methods
2.1 Experimental Materials and Methods

2.1.1 Mice.

Female BDF$_1$ mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed in micro-isolator cages under sterile conditions in the specific pathogen free (SPF) animal facility at the Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada and were fed an irradiated diet and autoclaved tap water. Mice were terminated by CO$_2$ asphyxiation and cervical dislocation. The Animal Care Committee of the Ontario Cancer Institute approved all experimental protocols employed. Cells were harvested from 4-6 week old mice for \textit{in vitro} cultures and \textit{in vivo} experiments used mice aged 8-14 weeks old.

2.1.2 Cell lines.

70Z/3-L leukaemia cells (described in $^{82}$) used in \textit{in vivo} experiments were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM) with 5% heat inactivated foetal bovine serum (FBS) (HYCLONE, South Logan, UT, USA), 100µg/mL penicillin-streptomycin or 100µg/mL kanamycin (GIBCO-Invitrogen, Burlington, Canada), and $5.5\times10^{-5}$M β-mercaptopoethanol (referred to as complete IMDM) in a humidified atmosphere at 37°C and 5% CO$_2$. Cells were injected at $10^6$ cells/mouse unless otherwise stated. For \textit{in vitro} experiments, cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640) supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in addition to the ingredients added to complete IMDM. Cells were passaged at approximately $5-10\times10^5$ cells/mL, harvested at a density of $4-12\times10^5$ cells/mL with greater than 88% viability, and were added to co-cultures at $5\times10^4$ cells/well. The IL-12 transduced 70Z/3 clone, LV12.2; 70Z/3 MΦ; and
LV12.2-r, as well as the control cell lines L1210, Ltk⁻ and C1498 were cultured in the same manner for the experiments in which they were included.

2.1.3 Production of IL-12 producing 70Z/3-L.

2.1.3.1 Lentiviral vector construction.

Lentiviral (LV) vectors expressing IL-12 complementary DNA (cDNA) were constructed by a method similar to that described by Yoshimitsu et al.¹³⁹, with modification. Plasmid pORF-mIL12 (IL-12elasti(p35::p40) Mouse (p35::p40)) (InvivoGen, San Diego, CA, USA) was modified by creating EcoRI and BamHI restriction enzyme sites upstream and downstream of the IL-12 gene, respectively, using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). This resulting construct was then digested with EcoRI/BamHI (New England Biolabs, Ipswich, MA, USA). Murine IL-12 (mIL-12) cDNA was purified after electrophoresis on a 1% agarose gel, and then subcloned into the pHR' LV backbone downstream of the elongation factor 1 alpha (EF-1α) promoter. Positive plasmid clones for pHR-cPPT-EF1α-mIL-12-WPRE (i.e. LV-mIL-12) were identified by diagnostic restriction enzyme digestion analyses and subsequent DNA sequencing (Innobiotech, Toronto, ON, Canada).

2.1.3.2 Viral production and transduction of cells.

Concentrated LVs were produced by a transient triple-transfection method using pHR-cPPT-EF1α-mIL-12-WPRE and accessory plasmids onto 293T monolayers by calcium phosphate¹⁴⁰,¹⁴¹. An approximate vector titre was estimated based on LV/enGFP¹³⁹ production and testing on naïve 293T cells that occurred in parallel. 70Z3-L was then transduced with an approximate multiplicity of infection (MOI) of 20. Single cell clones, obtained by limiting dilution in 96 well plates at population densities of less than 0.3 cells/well, were then quantitated
for IL-12 production/10^6cells/mL/2hrs using a commercially available IL-12 enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Jose, CA, USA). Subclones were produced by plating cells, at a population density of less than 0.3 cells/well, into Terizaki plates and confirming the presence of single cells by visual inspection. These subclones were then quantitated for IL-12 production to confirm that the production by each parent clone population was consistent.

2.1.4 Development of an IFN-γ-resistant LV12.2 line.

The LV12.2 clone of IL-12-producing 70Z/3-L was cultured in increasing amounts of IFN-γ for a period of nearly 2 months, until the concentration of IFN-γ was equal to 1ng/mL. This concentration was chosen because it is approximately equal to what is detected in the supernatant of co-culture wells undergoing leukaemia clearance. After an initial period of reduced proliferation, the LV12.2-r (denoting the resistant line) cells grew at the same rate as the parent LV12.2 line.

2.1.5 Thymidine incorporation assay.

Cells were plated at a density of 5,000 cells/well in 200µL of media and tritiated thymidine ([3H]-thymidine) was added at a concentration of 0.25Curie/mL. After a 6 hour incubation, the plate was harvested onto a 96-well, UniFilter GF/C plate (PerkinElmer, Shelton, CT, USA), washed, dried and read on a TopCount Liquid Scintillation analyzer (PerkinElmer, Shelton, CT, USA).

2.1.6 Irradiation of cells.

Cells were diluted to a concentration of 2x10^4cells/mL in complete IMDM and then aliquoted into 12 tubes, each containing 400µL. Each tube was irradiated at a range of doses
from 0-10Gy (at intervals of 1Gy), and at 20Gy, of $\gamma$-irradiation. These cells were then plated into 96-well plates, in triplicate, with 100µL/well and incubated for 24 hours before performing cell counts or [$^3$H]-thymidine incorporation assays.

2.1.7 rIL-12 therapy.

2.1.7.1 Intraperitoneal administration of rIL-12.

Recombinant mouse IL-12 was purchased from R&D Systems, Minneapolis, MN, USA. Mice were injected intraperitoneal (IP) with $10^6$ 70Z/3-L cells in 100-200µL phosphate buffered saline (PBS) on day 0, followed by daily injections, of 0.1-20ng/mouse/day rIL-12 in PBS, for a period of 14 days starting on day 1. A secondary challenge consisted of IP injection of $10^6$ 70Z/3-L cells 70 days after primary challenge, which was carried out in the manner just described but without further rIL-12 treatment. The mice were monitored daily for the appearance of symptoms both during the injection period and following the end of the injections.

2.1.7.2 Delayed rIL-12 therapy.

Mice received an IP injection of $10^4$ 70Z/3-L cells in 100-200µL PBS on day 0. Thereafter, groups of 4 or 5 mice received 14 successive rIL-12 IP injections of 20ng/mouse/day but the initiation of these injections was delayed by between 0 and 5 days.

2.1.8 IL-12 cellular therapy.

Leukaemia cells, both parental and transduced 70Z/3-L as well as control cell lines, were grown in complete IMDM and washed 3 times with 30mL of PBS with Ca$^{2+}$ and Mg$^{2+}$. The cells were resuspended at 5-10x10$^6$ cells/mL in PBS and injected into the mice in a volume of 100-200µL such that each mouse received $10^6$ cells. Mice received IP injections that were
performed on the right side of the abdomen using a 1mL syringe with a 26-gauge needle. The mice were monitored daily for the appearance of symptoms following injection.

2.1.8.1 Intraperitoneal administration of IL-12-producing leukaemia cells.

Interleukin-12 secreting cells were produced as described above. Mice were injected IP with $10^6$ cells of each of the transduced clones, or a mixture of transduced and parental cells in various proportions, in 100-200µL PBS. Each injection contained $10^6$ cells in total with the transduced cells accounting for 50%, 10%, 2%, 1%, 0.5% or 0.1% as stated. A secondary challenge consisted of IP injection of $10^6$ 70Z/3-L cells or $10^6$ L1210 cells more than 110 days after primary challenge, and was carried out in the manner described above but without further cell therapy.

2.1.8.2 Challenge with irradiated cells.

LV12.2 cells were mixed with parental 70Z/3-L so that LV12.2 made up either 1% or 10% or the total cell number. Both of these preparations were split in half and one sample of each mixture was irradiated with 5.5Gy of $\gamma$-irradiation, while the other sample was left untreated. Groups of 4 mice were each injected with $10^6$ cells of: 70Z/3-L parent cells, 1% LV12.2, 1% irradiated LV12.2, 10% LV12.2, or 10% irradiated LV12.2 cells.

2.1.8.3 Tolerance experiment.

Groups of 5 mice each were injected with a total of $10^6$ cells of either 70Z/3-L parent, LV12.2 (20,000pg/mL/$10^6$cells/2hrs), LV12.3 (2,000pg/mL/$10^6$cells/2hrs), or LV12.4 (700 pg/mL/$10^6$cells/2hrs) cells, or with mixtures of the clones in various proportions.
2.1.8.4 **Challenge in depleted mice.**

2.1.8.4.1 **Neutralizing antibody preparation.**

Mice were depleted of CD$^{4+}$, CD$^{8+}$ or both T cell subsets as well as NK cells and IFN-$\gamma$ using specific Abs. The hybridoma GK1.5 is directed against CD$^{4+}$ T cells, YTS169 against CD$^{8+}$ T cells, HB170 (R4-6A2) against IFN-$\gamma$ and the hybridoma HB9419 was used to produce an isotype control Ab. All hybridomas were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The lines were grown in 2.5L of complete OptiMEM in Lifecell culture bags (Lifecell Tissue Culture, Baxter Corporation, Concord, ON, Canada) in a humidified atmosphere at 37°C and 5% CO$_2$ until a live cell count (using trypan blue exclusion) revealed 30% dead cells in the culture. The media was then centrifuged and filtered to remove cells and cellular debris. Antibodies were purified from the media using an affinity column of packed sepharose beads (Gammabind G, Amersham Biosciences Corp, Piscataway, NJ, USA) and concentrated with Centriprep YM-30 columns (Millipore, Billerica, MA, USA) before dialysis in PBS. NK cells were depleted using an α-asialoGM1 Ab produced by Wako Bioproducts (Richmond, VA, USA) and Rabbit IgG (Sigma-Aldrich, Oakville, ON, Canada) was used as a control for the α-asialoGM1 Ab.

2.1.8.4.2 **Injection of neutralizing antibody and IL-12 therapy.**

The T cell subset and IFN-$\gamma$ Abs were injected on days –1, 3, 7, 10 and 14. The doses used were 1mg of Ab on day –1 and 500μg for the remaining injections. The NK cell depleting Ab was injected on days –1, 4, 9 and 14 using the recommended dilution$^{142}$. Control isotype Abs were injected following the same dose and schedule as their corresponding depleting Abs. The depletion potential of each Ab was demonstrated *in vivo* prior to their use in our experiments by
injecting mice with a range of concentrations and subsequently examining tissues by flow cytometry to quantify cellular subsets; or by examining serum for the presence of cytokines by ELISA. This experiment was conducted twice to test both model systems. In each case, cells were injected on day 0 of the neutralizing Ab schedule. For the rIL-12 model, mice were injected with $10^6$ 70Z/3-L cells in 100µL PBS and treated daily with 100µL preparations of PBS alone or containing low doses of rIL-12 (10 or 20ng/mouse/day) for 14 days. Alternatively, for the leukaemia cell-mediated IL-12 therapy experiment, mice were injected with $10^6$ 70Z/3-L cells in 100µL PBS containing various proportions of LV12.2 (0.5%, 1% and 10%). Control groups included mice injected with 70Z/3-L cells alone or PBS alone.

### 2.1.8.4.3 Sample collection for cytokine assay.

Serum was non-terminally collected from all groups on days 7, 10 and 20 before their daily injection. This was achieved by puncturing the saphenous vein with a sterile needle and collecting the blood in a serum separator tube (BD, Franklin Lakes, NJ, USA). Tubes were centrifuged at 10,000RPM (revolutions per minute) for 5 minutes and serum was then transferred to a micro centrifuge tube and frozen at –20°C until use.

This protocol was also followed for detection of cytokines in the *in vitro* assays: supernatants were harvested after 3 days of co-culture, aliquoted, and frozen until use.

### 2.1.8.4.4 Bead assay for cytokine levels.

Serum samples were diluted ½ and stained according to the protocol provided with the Mouse Inflammation Cytometric Bead Array (CBA) Kit (BD, San Diego, CA, USA). Standards were prepared in triplicate from independent dilutions and flow cytometry was carried out using a FACScan (Becton Dickinson, Oakville, ON, Canada). Acquisition was performed using
CellQuest software version 3.1. Note: due to insufficient blood collection, two mice treated with cell-mediated therapy in the 0.5% and one in the 1% group on day 7 and two mice in the PBS group on day 10 could not be tested. All mice from the group receiving 70Z/3-L cells alone in the cell-mediated IL-12 therapy model had perished by day 20 such that serum was not collected from this group.

This assay was also conducted on supernatants collected from co-cultures in the same manner, but supernatants were added neat.

2.1.8.5 Cell-mediated therapy of established disease.

2.1.8.5.1 Delayed cellular therapy and chemotherapy pre-treatment.

Groups of 5 mice were injected on day 0 with $10^4$ 70Z/3-L parent cells alone or with an additional $10^3$ LV12.2 cells; or either $10^4$ or $10^5$ LV12.2 cells on days 3 and 4 post challenge. For the chemotherapy pre-treatment experiment, the white blood cell (WBC) count was measured on day -1 to establish the baseline (described below). On day 0, mice were injected with $10^5$ 70Z/3-L cells and either PBS (200µL) or AraC (100mg/kg) at 3 time points each, separated by 10 hours. On day 5, two groups were injected with $10^6$ LV12.2 cells; one group that had been pre-treated with AraC chemotherapy and one group that had received PBS as a control. Two other groups also received injections of LV12.2 on days 7 or 9 so that each group only received an injection of cells on one occasion. Mice pre-treated with AraC without receiving cellular therapy, and mice receiving PBS pretreatment before cellular therapy, served as control groups.
2.1.8.5.2 White blood cell count.

Mice were bled on days -1, 1, 2, 3, 4, 5, 7 and 11, immediately after receiving their injection of cells or AraC as appropriate. The samples were treated with ammonium chloride potassium (ACK) lysis buffer, to lyse the RBCs, and then counted and analyzed by flow cytometry. The percentage of cells falling into the lymphocyte gate by FSC and SSC was measured and used to calculate the number of WBCs in the sample.

2.1.9 Ex-vivo cell isolation.

2.1.9.1 Dendritic cell generation and isolation.

Dendritic cells were established according to published methods. Briefly, BM was flushed from the femur and tibia of BDF$_1$ mice, incubated in ACK to lyse RBCs, washed with PBS and resuspended at $5 \times 10^5$ cells/mL in RPMI supplemented with 20ng/mL granulocyte macrophage colony stimulating factor (GM-CSF) (Biosource, Invitrogen) and IL-4 conditioned media (obtained from X63Ag8-653 cells stably transfected with IL-4 cDNA in a BMG Neo expression vector). 1mL cultures were fed on day 3 with 1mL RPMI containing 1x growth factors. After 7 days, cells were harvested by vigorous pipetting and resuspended at $10^8$ cells/mL in labelling buffer (PBS –CaCl –MgCl containing 1mM ethylenediaminetetraacetic acid [EDTA] and 2% FCS). Selection of the DC population was achieved using a CD11c$^+$ positive selection kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. After dilution in the buffer, a CD11c phycoerythrin (PE) labelling reagent was added at 100µL/mL and the mixture incubated for 15 minutes at room temperature (RT). 200µL/mL of a PE selection cocktail was added and incubated for 15 minutes at RT. Magnetic nanoparticles were added at 100µL/mL and incubated for 10 minutes at RT. The mixture was
then diluted to 2.5mL with labelling buffer and incubated in the magnet for 10 minutes. This was decanted into a new tube and incubated an additional 10 minutes in the magnet. The CD11c enriched cells were centrifuged and the pellet resuspended in RPMI at a concentration of 8x10^5 cells/mL. The efficiency of selection was evaluated by flow cytometry, with purities >95%.

2.1.9.2 T cell isolation.

Single cell suspensions were obtained by passing spleens from BDF_1 mice through 40µM cell strainers (BD, Falcon, Mississauga, ON, Canada). After red blood cell (RBC) lysis with ACK, CD4^+ cells were obtained by antibody depletion using a CD4^+ T cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. In summary, cell pellets were resuspended at 5x10^7 cells/mL in labelling buffer (PBS –CaCl –MgCl containing 1mM EDTA and 2% FCS) plus rat serum at 50µL/mL. A cocktail of biotinylated antibodies against CD8, CD11b, CD19, CD45R, CD49b and TER119 was added at 50µL/mL and the mixture incubated for 15 minutes at 4°C. 100µL/mL of α-biotin was added and incubated for 15 minutes at 4°C. Magnetic nanoparticles were added at 50µL/mL and incubated for 15 minutes at 4°C. The mixture was diluted to 2.5mL with labelling buffer and incubated in the magnet for 5 minutes. This was decanted into a new tube and incubated an additional 5 minutes in the magnet. The CD4^+ enriched cells were washed once and resuspended at 2x10^6 cells/mL in RPMI. The efficiency of selection was evaluated by flow cytometry, with purities >90%.

2.1.10 In vitro culture conditions.

2.1.10.1 Co-culture conditions.

Cultures were established in a total of 2mL media containing combinations of the following: 5x10^4 leukaemia cells (0.5mL at 10^5 cells/mL); 20ng/mL rIL-12 (0.5mL at 80ng/mL
5x10^5 CD11c^+ cells (0.5mL at 10^6 cells/mL); 10^6 CD4^+ cells (0.5mL at 2x10^6 cells/mL) and 1x10^5 NKT cells (0.5mL at 2x10^5 cells/mL). Wells containing DCs, leukaemia cells or rIL-12 were incubated for 3-4 hours prior to the addition of CD4^+ spleen cells or sorted NKT cells.

2.1.10.2 Transwell assay.

Transwell cultures were set-up as above with the exception that the lower chamber was plated in a total volume of 1mL media and the upper chamber in 200µL. The lower chamber contained either: DCs, LV12.2 and CD4^+ cells; or DCs, LV12.2 and NKT-depleted-CD4^+ cells; or LV12.2 and NKT-depleted-CD4^+ cells without DCs. The upper chamber was either left empty or contained: NKT cells; NKT cells and DCs; or NKT cells, DCs and LV12.2.

2.1.11 Enumeration of leukaemia cells.

2.1.11.1 Detection by flow cytometry.

After 3 days of co-culture, cells were harvested by gentle pipetting and aliquots removed for counting. Cells were washed and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2.5% FCS and 0.1% sodium azide). Cells were incubated at RT for 10 minutes with 0.5µg/tube α-CD16/CD32 Fc block and for 20 minutes with a monoclonal fluorescein isothiocyanate (FITC)-labelled α-mouse µ-chain-specific antibody (clone 33.60). Cells were washed once with FACS buffer and analyzed. The percentage of µ^+ cells within the live cell gate, and viable cell counts by trypan blue exclusion, were used to calculate the absolute number of target cells remaining in the cultures; this value was divided by the number of target cells originally added to the culture to get the ‘fold increase over input’ value.
2.1.11.2 Limiting dilution assay.

On days 1, 3, 5, 7, 10 and 15 after co-cultures were set-up, samples were taken from wells containing all the necessary components for leukaemia cell clearance. The cells were counted and resuspended at a range of concentrations: 1,000, 500, 100, 50, 25, 12.5, 6.25 and 3.125 cells/well in 200µL of complete OptiMEM media (GIBCO, Grand Island, NY, USA) supplemented with 10% heat inactivated FBS (HYCLONE, South Logan, UT, USA); without the addition of any growth factors. Each dilution was plated into 48 wells and incubated at 37°C and 5% CO₂. On day 5, the plates were scored and the number of wells that contained colonies was recorded. Poisson distribution was used to calculate the frequency of negative wells and this value was plotted against the number of cells plated. KaleidaGraph software was used to create a line of best fit and determine what proportion of cells plated were leukaemia cells.

2.1.12 Detection of Granzyme B by ELISA.

Supernatants from co-culture wells were collected on days 3, 6, 11, 13 and 17 and frozen until use. Granzyme B was detected using a commercially available ELISA kit (eBioscience Inc., San Diego, CA, USA) according to the manufacturers instructions with supernatants added neat.

2.1.13 Flow cytometry.

Cells were harvested from co-cultures and resuspended in FACS buffer, incubated at RT for 10 minutes with 0.5µg/stain α-CD16/CD32 Fc block (BD, Pharmingen; San Diego, CA, USA) and then for 20 minutes with α-mouse CD4-FITC or IgG2b-FITC isotype control and one of the following PE, PE/Cy5, APC (allophycocyanin) or biotin labelled α-mouse-specific Abs: CD2, CD3, CD5, CD8, CD11b, CD11c, CD24, CD25, CD30, CD44, CD45R, CD49b, CD54,
CD69, CD86, CD107a, CD134, CD152, pan MHCII (M5), V\(\beta\)8, V\(\beta\)8.1/8.2, V\(\beta\)811. Cells stained with biotin labelled Abs were washed twice with FACS buffer and incubated for 15 minutes with a Streptavidin-conjugated secondary Ab. All samples were then washed once with FACS buffer and analyzed by flow cytometry using a FACSCalibur and CellQuest analysis software (BD; Oakville, ON, Canada) or using FlowJo software (Tree Star, Ashland, OR, USA).

2.1.14 NKT cell depletion.

Immediately after selection of CD4\(^+\) cells by magnetic-assisted cell sorting (MACS) methods, as described above, the cells were stained with Abs recognizing CD4 (-PE) and CD8 (-FITC) as well as with the PBS-57 loaded CD1d tetramer conjugated to APC, and incubated for 30 minutes on ice. The cells were then washed two times with labelling buffer containing 3.5mM EDTA and 10% FBS, strained and sorted by FACS to deplete the CD4\(^+\) population of NKT cells. The NKT cell-depleted CD4\(^+\) population is defined as CD8\(^-\), CD4\(^+\), tetramer\(^-\); and the NKT cell population as CD8\(^-\), CD4\(^+\) tetramer\(^+\). The NKT population was also collected and used in the assay system. The purity of both populations was determined to be >96%.

2.1.15 PCR detection of the iTCR.

Samples were taken from co-culture assays at various time points and ribonucleic acid (RNA) prepared immediately. Expression of iTCR mRNA was detected by polymerase chain reaction (PCR) using two sets of primer sequences that have been previously published. The Va14J\(\alpha\)18 primer set is made up of two primers, as follows (all noted in 5’-3’ orientation): \textbf{Va14}: CACAGCCACCCTGCTGGAT and \textbf{Ja18}: CCAAAATGCAGCCTCCCTAA\(^{145}\); and the Va14J\(\alpha\)281 primer set consists of three primers in total: \textbf{Va14}: CTAAGCACAGCAGCTGCACA,
The results for both primer sets agreed in all experiments, and this acted as an additional control.

2.1.16 **Visualization of protein production by LV12.2.**

LV12.2 were washed, resuspended in serum-free media and incubated for 4 hours. Supernatants were collected from these cultures and concentrated using an Ultracel-30K spin filter (Millipore, Cork, Ireland). The concentrated sample was then resolved on a NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and stained for 2 hours in Colloidal Coomassie Blue Dye, followed by an overnight wash in distilled water. The gel was visualized on a GelDoc (Bio-Rad Laboratories, Mississauga, ON, Canada).

2.2. **Attributions**

Lentiviral vector design and transductions were performed in the laboratory of Dr. Jeffrey Medin as part of a collaboration. Cell Sorting is a service provided by the Flow Cytometry Centre at Princess Margaret Hospital; Toronto, Ontario. Figures 3.1 and 5.3 were completed by Dr. Alain Labbe before I joined the laboratory; and Figures 3.3 and 3.5a were conducted by Alain and myself in cooperation. The NIH Tetramer Facility provided the PBS-57 loaded CD1d tetramer used to deplete ex-vivo samples of NKT cells. Caren Furlonger was instrumental in developing the original in vitro assay system. All other experiments were designed and performed by me.
CHAPTER 3: Cell-Based IL-12 Immunotherapy of Murine Leukaemia

This chapter is adapted from the manuscript published in the Journal of Molecular and Cellular Medicine:


*Co-first authors*; these authors contributed equally to this work.
3.1 Introduction

70Z/3 leukaemia is reminiscent of human ALL with neoplastic lesions arising in the liver, spleen, lymph nodes (LN), BM and rarely the central nervous system. Among the most common physical manifestations of the disease are ascites and splenomegaly. Also akin to the human clinical situation is that any TAAs that can be recognized by the immune system are unknown to us. One variant of 70Z/3, 70Z/3-L, is lethal in syngeneic mice while another variant, 70Z/3-NL, elicits a protective immune response. The 70Z/3-L cells, although unable to initiate immunity, were readily rejected when an immune response was first initiated against 70Z/3-NL cells. Therefore, this model system is amenable to testing whether IL-12 can initiate a specific immune response, recognition of 70Z/3-L and survival of challenged mice.

Interleukin-12 is a potent immunostimulant with great potential as an immunotherapeutic agent for the treatment of malignant disease. However, it has also been implicated in autoimmunity and maintaining the delicate balance between engendering an effective immune response and inducing autoimmunity is of key importance. The immune system is equipped with negative regulatory mechanisms for this purpose; mechanisms that can often impede therapeutic interventions, as was the case in early clinical trials of IL-12. The approach we have chosen to address this is to use lower, localized doses of IL-12.

We show here that IP administration of low dose rIL-12 elicits a protective response against an established leukaemia burden, which leads to long-term immune memory without the induction of antagonistic cytokines. We compared this protocol to a cell therapy approach in which 70Z/3-L cells were transduced with a LV engineered to express murine IL-12 (mIL-12) cDNA; and clones producing a wide range of IL-12 were established. Injection of IL-12 producing 70Z/3-L cells provoked long term and specific immunity without the induction of
antagonistic mechanisms and leukaemia clearance was mediated primarily by a CD4+ cellular subset. Of critical importance, we found that injection of as few as 1% IL-12 producing 70Z/3-L cells along with 99% untransduced 70Z/3-L cells was sufficient to elicit protective immunity as long as each of these cells produced IL-12 above a necessary threshold. This finding may explain the failure of many human cell-based therapy protocols. In these cases, IL-12 production is measured on bulk populations, making it impossible to know if sufficient IL-12 is being produced in the local environment influenced by the IL-12 producing cell; and normally the average production reported is below our established threshold47-49,56.

3.2 Results

3.2.1 Intraperitoneal administration of rIL-12 completely protects mice challenged with 70Z/3-L.

Interleukin-12 is known to be a potent modulator of the immune response and is attributed with a number of anti-leukaemia effects including, but not limited to, T cell-mediated antigen-specific leukaemia clearance. This molecule has been approved for clinical trials but optimum delivery programs have yet to be defined. In an attempt to alter the course of 70Z/3-L leukaemia, we began by testing the effect of IP rIL-12 administration on the appearance of morbidity after IP injection of $10^6$ 70Z/3-L cells. We tested doses of 0.1-20ng/mouse/day for 14 days; doses that are at least 20-fold below the maximum tolerated dose in mice. In Figure 3.1, we show that a dose of 10ng and above was sufficient to significantly improve the survival of mice ($p = 0.002$).
FIGURE 3.1  rIL-12-mediated protection of mice challenged with 70Z/3-L cells.
Mice were challenged with $10^6$ cells IP and received either no treatment or injections of 0.1, 1, 10 or 20ng/mouse/day rIL-12 for 14 days (n = 5 mice for each group).

3.2.2 Intraperitoneal administration of rIL-12 leads to immune memory.

We next addressed whether the results observed above were due solely to the acute effects of IP administered rIL-12 on innate responses or to the induction of a long-term adaptive immune response in the mice. To accomplish this, we gave mice IP injections of $10^6$ 70Z/3-L cells, treated them for 14 days with 20ng/mouse/day rIL-12, challenged them 70 days later by IP injection of $10^6$ 70Z/3-L cells, and monitored them for the appearance of symptoms. A group of
naïve mice was included to control for the efficiency of the cells to cause disease. Figure 3.2b shows that all mice first treated with IP administration of rIL-12 (Figure 3.2a) survived a secondary challenge with 70Z/3-L cells in the absence of further IL-12 therapy. Thus, IP administration of rIL-12 not only protected against the primary 70Z/3-L challenge but also established long-term protective immune memory.

**FIGURE 3.2** rIL-12 therapy leads to long-term protection from 70Z/3-L.

(i) Naïve mice (A, n = 10) were challenged with 70Z/3-L cells on day 0 and treated for 14 days with injections of 20ng rIL-12/mouse/day. A group of mice (B, n = 7) were included as controls for the 70Z/3-L cells (curve comparison by log rank test p = 0.001). (ii) After a period of 70 days, five mice from group A, having undergone rIL-12 therapy, were secondarily challenged with 10^6 70Z/3-L cells without further rIL-12 treatment. The other five animals were kept to confirm that no toxicity appeared after 70 days. Five naïve mice (B) were included to demonstrate the lethality of the 70Z/3-L cells (comparison of Kaplan-Meier survival curves was performed using Logrank test p = 0.0015).
3.2.3 Generation of IL-12 secreting leukaemia cells.

The preceding experiments determined that our 70Z/3 model is responsive to IL-12 therapy and established the basic parameters for administration. We next compared this protocol to a cell-mediated approach in which IL-12 production would occur in a more localized microenvironment due to preferential accumulation of the cellular carriers in certain tissues. Figure 3.3a shows the LV construct containing an IL-12 fusion transgene, under control of the EF-1α promoter, that was generated in the laboratory of Dr. Jeffrey Medin. A lentiviral vector system was chosen because it offers a number of advantages, particularly for adapting this work to human studies. These advantages include: the capacity of lentiviruses to transfer large amounts of genetic material and integrate it directly into the genome of the host, their highly efficient infection of both dividing and non-dividing cells, the long-term and stable expression of the transgene once integrated, and their relatively low immunogeneicity. After the Medin lab transduced 70Z3-L cells with an approximate MOI of 20, single cell clones were derived as described in Materials and Methods and supernatants from these clonal cell lines were tested for the production of IL-12. The range of secretion by selected clones varied, as shown in Figure 3.3b, from approximately 250 to 52,000 pg/mL/10^6 cells/2hrs and these levels remained stable over time. Furthermore, the different levels of IL-12 measured did not depend on cell growth kinetics, nor on survival, as the in vitro growth properties of the transduced clones were similar, as measured by thymidine incorporation (Figure 3.3c) and visual inspection, for those lines used in further experiments.
FIGURE 3.3 Production of IL-12 secreting 70Z/3 cells.

A. Schematic representation of the LV-muIL-12 (LV-cPPT-EF1-mIL-12-WPRE) vector. LTR: long-terminal repeat; SD: splice donor; RRE: rev response element; SA: splice acceptor; cPPT: central polypurine tract; CMV: cytomegalovirus; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element; muIL-12: murine interleukin-12; SIN: self-inactivating LTR. B. Interleukin-12 secretion by vector-transduced clones is a stable trait. Levels of IL-12 secretion were measured by ELISA on 2-5 independent occasions and seen to remain fairly constant; differences are not statistically significant by ANOVA. C. Cell proliferation was measured by thymidine incorporation and found to be constant for all IL-12 producing cell lines used in further experiments.
3.2.4 Only a small proportion of IL-12 producing 70Z/3-L cells are required to confer immunity.

We proceeded to determine if the production of IL-12 by transduced 70Z/3-L cells would elicit a protective immune response by injecting $10^6$ cells of each of thirteen clones, spanning a range of secretion levels, into the peritoneum of BDF$_1$ mice. The three lowest producing clones (range: 200–1,000pg/mL/10$^6$cells/2hrs) failed to elicit an immune response and mice injected with these cells succumbed to the 70Z/3 leukaemia. In contrast, all mice injected with $10^6$ cells of the ten highest producing clones (range: from 1,500 – 40,000pg/mL/10$^6$ cells/2hrs) survived (Figure 3.4).

**FIGURE 3.4** Leukaemia cell mediated IL-12 therapy leads to protection.

Mice were injected IP with PBS or $10^6$ cells of either the parent line, 70Z/3-L, or one of the vector-transduced clones and monitored for the appearance of symptoms. Clones secrete varying levels of IL-12 and a theoretical threshold was established, below which protection is not conferred.
One transduced 70Z/3-L clone, LV12.1 that produces approximately 21,500pg/mL/10^6 cells/2hrs, was mixed with naïve 70Z/3-L cells to determine if the inclusion of IL-12 producing cells would result in the elimination of non-producing cells also. We initially found that as little as 2% of the transduced cells were sufficient to confer complete protection (Figure 3.5a). To further examine the efficacy of producer/non-producer proportions, two other 70Z/3-L transduced clones were selected that differ in IL-12 production by 10-fold (clone LV12.3: 2,000pg/mL/10^6 cells/2hrs versus clone LV12.2: 20,000pg/mL/10^6 cells/2hrs). In this case, as few as 0.5% (i.e. 5,000 LV12.2 cells in 10^6 total cells) of the higher producing clone was sufficient to confer protection to 80% of the mice, but 0.1% failed to protect any mice. However, even 10% (i.e. 100,000 LV12.3 cells in 10^6 total cells) of the lower producing clone was insufficient to protect; indicating that a threshold of IL-12 production per vector-transduced cell is required to elicit an effective immune response (Figure 3.5b).

3.2.5 Cell-mediated IL-12 therapy leads to immune memory.

More than 110 days after IP injection with 10^6 LV12.2 cells, mice were challenged with either 10^6 cells of the parental leukaemia line, 70Z/3-L, or another well-characterized B-cell leukaemia, L1210, and monitored for the appearance of symptoms. Groups of naïve mice were included to control for the efficiency of both the 70Z/3-L and L1210 cells to cause disease. Figure 3.6 shows that all mice that survived the initial inoculation with LV12.2 were immune to subsequent challenge with 70Z/3-L without further IL-12 therapy, but not L1210. Thus, cell-mediated IL-12 therapy leads to robust immune memory as the protection is long-term and specific to the agent of the original challenge.
FIGURE 3.5 Leukaemia cell mediated IL-12 therapy leads to protection of challenged mice when only a portion of the cells are vector-transduced.

Mice were injected IP with $10^6$ cells of the parent line, 70Z/3-L, and varying proportions A. 2%, 10% and 50% of the LV12.1 secreting clone or B. 0.1%, 0.5%, 1% and 10% of LV12.2 and LV12.3, and monitored for the appearance of symptoms.
FIGURE 3.6  Leukaemia cell mediated IL-12 therapy leads to long-term and specific protection against challenge with 70Z/3-L.

Mice were initially challenged with either $10^6$ LV12.2 cells or injected with PBS. More than 110 days following the primary challenge, primed mice (n = 4 in each group) were secondarily challenged with either $10^6$ 70Z/3-L or $10^6$ L1210 cells. The PBS injected mice (n = 5 in each group) also received either $10^6$ 70Z/3-L or $10^6$ L1210 cells to control for their efficiency to lead to morbidity, or another injection of PBS and monitored for appearance of symptoms. Kaplan-Meier survival curve comparison was performed using Logrank test, $p < 0.0001$. 

3.2.6 **CD4\(^+\) cells are required for cell-mediated rejection of 70Z/3-L cells.**

Depleting antibodies were used to determine which cell types mediate the IL-12-induced rejection of 70Z/3 leukaemia. Unlike what is generally reported in the literature for immune responses to IL-12, we show in Figure 3.7 that the CD4\(^+\) T cell subset is of primary importance. The mean survival of leukaemia challenged mice was 37 days for those depleted of CD4\(^+\) T cells and 18 days for those depleted of both T cell subsets. The curves are statistically different (p=0.003), suggesting an important role for CD8\(^+\) T cells but only in the absence of CD4\(^+\) T cells. The CD8\(^+\) T cell subset alone is not sufficient to confer protection. Furthermore, the neutralization of IFN-\(\gamma\) did not diminish the protective effect, which again is in contradiction with the generally accepted notion that IFN-\(\gamma\) mediates the anticancer activity of IL-12. This unexpected result prompted us to further investigate the regulation of IFN-\(\gamma\) and various other inflammatory cytokines in our model systems.
FIGURE 3.7 Requirement of the CD4+ T cell subset for leukaemia cell-mediated protection of challenged mice.

Mice (n = 5) were depleted using antibodies as described in Materials and Methods. The mice were challenged with $10^6$ LV12.2 cells IP and monitored for the appearance of symptoms. Kaplan-Meier curve comparison was performed using Logrank test, $p = 0.0084$. 
3.2.7 In vivo cytokine regulation.

Interleukin-12 induces the secretion of other cytokines that can have agonistic, antagonistic or synergistic effects and can influence the specific immune response that is initiated\textsuperscript{2,26,27,47,147-151}. It was therefore important to measure the regulation of some of these cytokines \textit{in vivo} to better understand how leukaemia rejection is accomplished and shed some light on the results of our neutralization experiments. For this purpose we employed a flow cytometry technique that detects a panel of inflammatory cytokines, including: IL-12p70, TNF-\textalpha{}, IFN-\textgamma{}, monocyte chemotactic protein-1 (MCP-1), IL-10 and IL-6 in serum. Mice were given IP injections of \(10^6\) 70Z3-L cells on day 0 and daily IP injections of either 10 or 20ng rIL-12/mouse/day for 14 days. Alternatively, mice were challenged with an IP injection of \(10^6\) 70Z3-L cells on day 0 spiked with various proportions (0.5%, 1% and 10%) of LV12.2 cells; and serum samples were collected on days 7, 10 and 20. The results of these two assays are shown in Figure 3.8.

The levels of IL-10, an IL-12 antagonist, induced on day 20 are significantly higher after cell-mediated therapy as compared to rIL-12 therapy (\(p<0.0017\)) in general; but when comparing treated and control groups within either delivery mode, no significant differences are measured at any time point. Likewise, the overall levels of IFN-\textgamma{} and TNF-\textalpha{} are significantly higher with cell therapy as compared to rIL-12 (\(p<0.0015\) and 0.0110 respectively) when all groups for each mode are compared by ANOVA. Furthermore, the cell-mediated treatment groups show significantly higher levels of IFN-\textgamma{} than the control groups on day 7 (\(p=0.0007\)), but resolve to near basal level by day 20. Interestingly, the systemic levels of IL-12 measured did not correspond to the doses administered, suggesting that the activity of IL-12 is localized.
**IP Administered Therapy**

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 7</th>
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<th>Day 20</th>
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<tbody>
<tr>
<td>70Z/3</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>rIL-12</td>
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<td>-</td>
<td>10 ng</td>
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**Tumor Cell-Mediated Therapy**

<table>
<thead>
<tr>
<th>Group</th>
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<th>Day 20*</th>
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</thead>
<tbody>
<tr>
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<td>PBS</td>
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<td>-</td>
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</table>
| IL-12 cells | -     | 0.5%   | 1%      | 10%
FIGURE 3.8  Cytokine expression profiles of mice receiving IP administration and leukaemia cell-mediated IL-12 therapies.

The mice (n = 4 in each group) receiving IP administered rIL-12 therapy were challenged with $10^6$ 70Z/3-L cells and received either no treatment or injections of 10 or 20ng/mouse/day rIL-12 for 14 days. Mice (n=4 in each group) receiving leukaemia cell-mediated IL-12 therapy were challenged with $10^6$ 70Z/3-L cells IP and received either no treatment or treatment with
various proportions (0.5%, 1% or 10%) of the vector-transduced clone LV12.2. Serum samples were collected and analyzed on days 7, 10 and 20 as described in Materials and Methods. (*-all mice from group 2 in the leukaemia cell-mediated model were dead by day 20 such that serum was not collected from this group).

3.3 Discussion

Given the key role that IL-12 plays in the initiation of effective immune responses in various leukaemia models, we re-examined the potential for cytokine therapy using a murine model of ALL. We have previously reported that 70Z/3-L cells lead to the rapid death of mice injected with as few as 10^2 cells. In contrast, a variant of this line was established that is recognized by the immune system and subsequently rejected. Mixing as few as 10^5 cells of this non-leukaemic variant with 10^6 70Z/3-L cells resulted in complete rejection of all 70Z/3 cells^85. While we have not yet concluded why these variants are recognized by the immune system, these experiments revealed that 70Z/3-L cells can be rejected if the immune system is appropriately modulated. This observation suggested that this experimental system would be amenable to the study of IL-12-induced anti-leukaemia activity. We have demonstrated that IP administered low dose rIL-12 therapy can elicit a protective immune response in leukaemia-bearing mice and that an effective approach to deliver IL-12 is via the leukaemia cells themselves. We found that remarkably few of these transduced leukaemia cells are needed to achieve protection provided a sufficient amount of IL-12 is produced per cell, and that protection is primarily dependent on a CD4^+ cell population, leading to specific immune memory.

Interleukin-12-based therapies have not become front line cancer treatments in part because studies often report low response rates among patients^2,26,27. The poor outcomes


associated with IL-12 treatment in these clinical studies might be explained by the physiological response to IL-12-induced IFN-γ. For example, high levels of IL-12, and consequently IFN-γ, have been shown to induce IL-10 and lead to the down-modulation of IL-12 responsiveness in the host\textsuperscript{147}. However, it has also been shown that leukaemia regression occurs only in patients who sustain elevated levels of IFN-γ throughout the course of their treatment\textsuperscript{52}.

Previous groups have demonstrated that administration of IL-12 at doses significantly below the maximum tolerated dose can avoid the induction of antagonistic mechanisms\textsuperscript{53}. Therefore, we first established that daily IP administration of a dose as low as 10-20ng of rIL-12 for 14 days, equivalent to approximately 500-1,000ng/kg, is sufficient to significantly increase the survival of mice injected with 70Z/3-L, and rejection leads to long-term immune memory.

We next investigated a cell-mediated strategy for the delivery of IL-12 and found that 70Z/3-L cells could be readily transduced with a novel LV construct. We found that different transduced clones produce varied amounts of IL-12 and that this is a stable trait, as we have measured similar levels of secreted IL-12 for each clone on 2-5 independent occasions. This approach may circumvent the dose-limiting toxicity experienced in human clinical trials of systemically delivered rIL-12 that was not foreshadowed by pre-clinical murine studies\textsuperscript{50}.

The establishment of clones that produce different levels of IL-12 allowed us to examine the relationship between IL-12 production, and the proportion of IL-12\textsuperscript{+} transduced versus IL-12\textsuperscript{-} parent 70Z/3-L cells necessary for immune activation. To date, this potentially critical aspect of cell-mediated cytokine therapy has not been thoroughly examined. We found that a very small proportion of IL-12 producing 70Z/3-L cells are sufficient to trigger a protective immune response. For one clone, LV12.2, 5,000 such cells (but not 1,000) were sufficient to save 80% of
the mice injected with $10^6$ 70Z/3-L cells. This result could indicate either that a critical number of ‘interactions’ or a sufficient absolute amount of IL-12 is required to trigger an immune response. Under these circumstances, one could reasonably define an ‘interaction’ as an encounter between an IL-12 producing 70Z/3-L cell and an appropriate APC, such as a DC. The alternative explanation is that these 5,000 IL-12-producing cells simply deliver a sufficient quantity of IL-12 into the system to initiate an immune response. To determine which of these explanations is correct we employed a different clone, LV12.3, which produces 10-fold less IL-12 per cell and injected titrated numbers of these cells along with $10^6$ 70Z/3-L parent cells. We found that even 100,000 LV12.3 cells failed to confer protection. This represents a 20-fold greater number of cells and twice the potential IL-12 released into the system, though each cell is producing 10-fold less. Together, these two experiments suggest that it is the number of interactions that matter rather than the absolute amount of IL-12, but that at each interaction, the transduced 70Z/3-L cell must produce IL-12 above a certain threshold.

Our findings have important implications for clinical trial design and may partly explain the disparity observed between murine studies, in which IL-12 can initiate a curative immune response; and human studies, in which the immune response is modest and patient survival is normally unaffected. The protocols used in mouse cell therapy studies usually involve selection of clones that secrete relatively high levels of IL-12 and, frequently, the preparation administered consists of 100% IL-12 secreting cells. In contrast, human studies generally rely on freshly obtained populations of cells that are difficult to clone. Therefore, bulk populations of cells are transduced and the average IL-12 production by these populations measured. In cases reported to date, these average amounts fall short of what we believe to be necessary to elicit protective
immunity and there is no information on the distribution of production levels within these populations. Beyond being insufficient to induce anticancer immunity, cells secreting lower amounts of IL-12 may act to inhibit the desired response. The cytokine micromilieu in which a DC matures will define the cytokines that it later releases into the immunological synapse it forms with a T cell. These cytokines form an integral part of the instructive signal that polarizes the T cells fate and dictates the effector function that will be executed\textsuperscript{152}. Malignant cells are known to produce cytokines such as IL-10, IL-6 and TGF-\(\beta\) that suppress cellular responses. Because the particular combinations and ratios of cytokines present during DC maturation define their polarizing capacity, we suggest that the amount of IL-12 produced locally by each transduced leukaemia cell is additionally important as the ratio of IL-12 over such antagonistic cytokines must be sufficiently high to ensure the production of T\textsubscript{H1} polarizing DCs and not tolerogenic DCs\textsuperscript{132}. A cell-mediated approach may facilitate achieving the necessarily high levels of local expression without resulting in the toxicity observed in human trials by confining production to the tumour microenvironment. This local concentration may also limit the induction of antagonistic mechanisms.

The role of IFN-\(\gamma\) in our model appeared to differ from what the literature would have predicted, prompting us to look at its \textit{in vivo} regulation, along with a number of other inflammatory cytokines. This was done using a flow cytometry based cytokine bead assay. The regulation of IL-10, IFN-\(\gamma\) and TNF-\(\alpha\) are of particular interest in our model systems because IL-10 is known to be the most biologically relevant antagonist of IL-12\textsuperscript{2}, IFN-\(\gamma\) is thought to mediate the effects of IL-12\textsuperscript{2,34} and a combination of IFN-\(\gamma\) and TNF-\(\alpha\) is required for the development of CD4\textsuperscript{+} CTLs\textsuperscript{153}. The fact that IL-10 production was not elevated above
background in any of our treatment groups suggests that, while local IL-12 production was high, the systemic amount was sufficiently low as to avoid the induction of antagonistic molecules and dampening of the biologic effect. This idea is supported by the fact that systemic IL-12 levels did not correspond to the dose administered; which is likely because IL-12 is a heparin-binding protein and thus retained close to the site of secretion. Measured levels of IFN-γ were significantly higher in the treated groups receiving cell-therapy as compared to controls on day 7 but were not significant by day 10 and returned to near baseline by day 20. Furthermore, IFN-γ production was significantly greater in the cell-mediated model in general. In light of these results, it is possible that the cell-mediated IL-12 therapy neutralization experiment did not demonstrate a critical role for IFN-γ simply because the neutralizing antibody was overwhelmed by the levels produced; but this remains to be proved.

There is ample literature describing how IL-12 leads to the increased maturation of DCs, the production of IFN-γ and more efficient antigen presentation by the IFN-γ-dependent up-regulation of MHCII and costimulatory molecule expression. T-helper lymphocytes are driven by IFN-γ to differentiate with a type-1 functional profile and in turn promote the strong CD8\(^+\) CTL response that is typical of IL-12 therapy and agrees with the classical model of cytotoxicity. However, there is also literature describing a role for CD4\(^+\) CTLs in models of infection\(^{114,153-155}\), and more recently in tumour immunology\(^{113,116,117,156-158}\). It is possible that the IFN-γ and TNF-α rich environment resulting from cell-mediated therapy led to the development of an effector CD4\(^+\) CTL population as suggested by the depletion experiment. The major thrust of cancer vaccination research has traditionally focused on targeting CD8\(^+\) CTLs, which require stimulation by a CD4\(^+\) helper T cell population, to affect tumour clearance but the clinical
response has been limited. An emerging hypothesis in the field is that directly targeting CD4\(^+\) effector cells may be important in achieving a more robust anticancer response. Although the mechanism of action of the CD4\(^+\) population observed in our system has yet to be delineated, the incitement of a potent CD4\(^+\) effector response is of clear interest.

Despite the beneficial effects of IFN-\(\gamma\), highlighted above, a dampening of the response with repeated administration is still of concern in models of IL-12 therapy. An important attribute of our cell-mediated approach is that a sufficient immune response is stimulated, for a period of time corresponding to that required to eliminate the leukaemia, but the signal is self-limiting. The source of IL-12 into the system is the cancer cells that are, themselves, the target of therapy. As the leukaemia cells are rejected, the source of IL-12 is reduced and IFN-\(\gamma\) levels return to baseline without a significant increase in the antagonistic molecule IL-10. Our approach differs from others in the field of immunotherapy in a number of informative ways. In protocols that engineer DCs and fibroblasts to stably secrete IL-12, for example, production would continue even after tumour clearance. In addition, the secretion levels reported to date have not reached what we propose to be therapeutic, possibly explaining the mixed responses observed. Alternatively, methods for stimulating the maturation of autologous tumour Ag-loaded DCs, leading to IL-12 production, have also been described\(^{44}\). These DCs are only capable of producing IL-12 for approximately 18 hours after administration and treatment efficiency may be highly dependent upon the amount of time required for these smDCs to travel to the LNs where they can stimulate a T cell response. Another related limitation of this approach is the apparent effect the maturation stimulus has on the migratory capacity of these DCs. Our IL-12 transduction protocol does not appear to interfere with the migration of the cell line used in our
studies, as evidenced by the successful eradication of a widely disseminated leukaemia. Each of these approaches possess merit and may prove to be very valuable and effective therapeutic strategies. We assert that cancer cell-mediated approaches warrant further investigation to round out this growing body of knowledge.

We have demonstrated that IL-12, given at doses below what would result in systemic levels of IFN-γ and leading to induction of antagonistic mechanisms, is sufficient to launch a protective immune response against 70Z/3-L ALL cells and complete clearance of the leukaemia so long as individual cells produce IL-12 above a critical threshold. We demonstrate a critical role for CD4$^+$ cells in the clearance observed in our cell-mediated model and these cells may constitute a genuine CD4$^+$ CTL population. Moreover, a potentially problematic dampening of the immune response was not observed. This is possibly due to the localization of IL-12 secretion by, and self-limiting nature of, the cell-mediated therapy approach we employed.
CHAPTER 4: In vitro Characterization of a CD4$^+$ Effector Cell

A version of this chapter is currently being prepared for publication:

4.1 Introduction

We have demonstrated a role for a CD4\(^+\) effector cell in a murine model of IL-12 therapy. Though there is a growing body of literature describing CD4\(^+\) CTLs with anticancer activity and there is an increasing interest in eliciting CD4\(^+\) CTL responses directly, as opposed to their CD8\(^+\) counterparts\(^{119}\), this result was somewhat unexpected because the leukaemia cells that were rejected in our model system are MHCII negative. This is not unheard of\(^{115,117}\), but the mechanism appears to be indirect, requiring help from another cell type like M\(\Phi\)s\(^{115}\) or NK cells\(^{117}\); this may also be the case here. Nevertheless, we observed the establishment of specific immune memory, a characteristic more reminiscent of a classical CTL response.

An alternative explanation is that the IL-12 secreted by the leukaemia cell within its microenvironment, if sufficient to locally overcome inhibitory signals, can activate an APC with which it makes contact. This APC, such as a DC, then cross-presents tumour Ag complexed to MHCII, expresses costimulatory molecules and polarizes the maturation of CD4\(^+\) CTLs in an Ag-specific manner. However, a recent article has elegantly delineated the initiation of IL-12 production by DCs and has shown that they must first receive IFN-\(\gamma\) stimulation\(^{21}\). A commentary published along with this article went on to posit that the required IFN-\(\gamma\) must come from a 3\(^{rd}\) cell type because DCs do not typically produce IFN-\(\gamma\) and T cells must be fully activated to do so. Full activation of a T cell requires the delivery of 3 signals from a DC: 1) MHC-bound Ag recognized by the TCR, 2) costimulation, and 3) binding of a polarizing cytokine, in this case, IL-12p70. A polarizing cytokine is one that directs the type of immune response mounted; e.g. T\(_{H1}\) or T\(_{H2}\). Thus, in a primary response, a 3\(^{rd}\) cell type must provide the IFN-\(\gamma\) that fully arms the DC to provide signals 2 and 3 to a T cell and initiate a T\(_{H1}\) response\(^{159}\).
In addition to activated T cells, NK and NKT cells are also capable of producing copious amounts of IFN-\(\gamma\).

Our \textit{in vivo} model demonstrated only that a CD4\(^+\) population of cells was responsible for leukaemia rejection. Several cell types capable of anticancer activity express CD4, including: DCs, NK and NKT cells, as well as \(\alpha\beta\)-T cells. We have therefore established an \textit{in vitro} assay that can be manipulated for the purpose of obtaining a more detailed description of the effector population(s) and the mechanism of leukaemia rejection.

We herein describe a CD4\(^+\) effector population capable of specifically eradicating a MHCII negative leukaemia. Induction of this anti-leukaemia response is dependent on IL-12 and requires the support of DCs and a 3\(^{rd}\) cell type capable of producing IFN-\(\gamma\) to catalyze the appropriate response. In this system, that role is played by NKT cells.

4.2 Results

\textbf{4.2.1 CD4\(^+\) CTL establishment requires the presence of DCs and IL-12.}

Our \textit{in vivo} therapy model\(^{35}\) demonstrated that the primary effector cell population is CD4\(^+\); therefore we focused on this subset to establish our \textit{in vitro} culture system. However, since the target cell is MHCII negative, we assumed that a population of APCs would also be required to allow for cross-presentation. APCs were derived from BM cells cultured in the presence of GM-CSF and IL-4 for 7 days, after which the cells were identified as predominantly M\(\Phi\)s or DCs with little granulocyte contamination. Separately, a negative selection magnetic sorting kit was used to enrich for the CD4\(^+\) cellular fraction from spleen without activating these cells in the process. Cultures were then established containing CD4\(^+\) splenocytes and leukaemia cells, with or without APCs. After 3 days of co-culture, target cell clearance was estimated by visual inspection and only observed in those wells containing all 3 cell types. However, the
results were not consistent. Killing was never observed unless all 3 cell types were combined, but was not always observed under these conditions either. To improve the consistency of the assay, CD11c positive selection was performed on the mixed APC population to separate the MΦs from the DCs. Interestingly, the CD11c− MΦ population did not support anti-leukaemia cytotoxicity but the wells containing CD11c+ DCs consistently supported clearance by the CD4+ effector cells. Furthermore, cytotoxicity was observed only when the assay was supplied with IL-12; either by LV12.2 or when parental 70Z/3 cells were included together with rIL-12. Recombinant IL-12 was added at a concentration similar to that measured in the supernatant of LV12.2 cells when cultured alone for a similar amount of time. These results are summarized in Table 4.1.

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<th>CD8+ T cell</th>
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<th>70Z/3-L</th>
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† Leukaemia cell clearance
− Leukaemia cell outgrowth
± Inconsistent leukaemia cell clearance

**TABLE 4.1 Summary of co-culture contents.**

Various cell populations were combined, as indicated, in the co-culture assay. Target cell clearance under these different conditions was observed, by visual inspection.
4.2.2 Quantification of leukaemia cell clearance.

Once the culture conditions had been optimized, target cell clearance was measured in a more stringent manner to provide some quantitative information. Cell counting for each well was conducted using trypan blue exclusion to obtain an absolute number of live cells. The cultures were then stained for μ expression and flow data was gated on the live cell population so that the difference in μ⁺ cell number could be calculated and compared between the test wells and control wells. The absolute number of remaining μ⁺ cells can also be compared to the number of leukaemia cells added to the wells originally. Controls included wells containing DCs with LV12.2 in the absence of CD4⁺ effector cells, CD4⁺ cells with LV12.2 in the absence of DCs and leukaemia cells cultured alone. Dendritic cells and CD4⁺ cells were also cultured alone to control for any background μ staining; these populations are μ-negative. Figure 4.1 shows an example of the method used.

Cumulatively, our data demonstrate that the establishment of a CD4⁺ effector cell population, capable of clearing 70Z/3 to a measurable degree, requires both a source of IL-12 and support from a DC population (Figure 4.2a). However, a limitation of this assay is that complete clearance cannot be measured. When the number of μ⁺ cells in the test wells is compared to control wells, the difference decreases over time as control cell populations die due to overgrowth of the LV12.2 population. To address this, a more sensitive method -limiting dilution- was employed. Test wells were sampled regularly and limiting dilution assays conducted by diluting the samples and plating only 500, 100, 50, 25, 12.5, 6.25 or 3.125 cells per well in a 96-well plate. Each dilution was plated in 48 replicate wells. Besides the LV12.2 cells, all others in the sample are primary cells that will die without growth factors; thus, any wells that
contain an expanded population after 5 days must have contained at least one LV12.2 cell from the beginning. Figure 4.2b shows that initial expansion of the LV12.2 population is followed by complete clearance; one representative experiment is shown.

**FIGURE 4.1 Quantification of leukaemia cell clearance.**

To calculate the clearance of leukaemia cells in the co-cultures, all of the cells in the well were harvested, counted by trypan blue exclusion and stained for μ expression. The cells were then gated, to differentiate live and dead cells, and further gated on the target cell population using LV12.2 cell alone to set the gate. The percentage of μ+ cells within the live cell gate was calculated and multiplied by the total number obtained by counting to give the absolute number of μ+ cells in the well. That number was then divided by the number of cells initially added to the well to give the ‘fold increase/input’ value.
FIGURE 4.2 Target cell clearance in co-culture assays.

A. Cell populations were cultured to include DCs; CD4+ cells; target cells; and a source of IL-12, either rIL-12 or LV12.2, in various combinations. A comparative decrease in the target cell population, measured by proportion of μ-staining, occurred only when DCs, CD4+ cells and a source of IL-12 were all present together in the culture. B. Limiting dilution assays, where samples were taken from co-culture wells are plated without growth factors such that only the transformed LV12.2 cells would survive over time, show that there is an initial expansion of the target cell population, followed by complete clearance.
4.2.3 Two populations of CD4+ cells persist in culture.

Once we had established that a CD4+ effector population was capable of exacting complete leukaemia cell clearance in our assay system, it could then be used as a source of established effector cells for further characterization. We began by using flow cytometry to detect the expression of a panel of surface proteins on cells sampled from culture; a summary of the results is shown in Figure 4.3. All of the cells in a well containing the required components for cytotoxicity and target cell clearance were harvested and stained. Each staining combination contained CD4 and analysis was done by first gating on the CD4+ population. Consistently, two distinct populations were evident by forward (FSC) and side scatter (SSC), and differed in their expression of some key molecules. Population A is smaller and less granular than population B, therefore analysis was conducted on each of these populations independently. Both populations, having gated on the CD4+ population, share in common that all cells expressed CD2, CD3, CD5 and CD54 and a very small proportion, if any, expressed CD30, CD45RA, CD49b, CD107, CD134, CD152 and CD178. Where they differed was in the proportion of cells expressing CD11b, CD24, CD25, CD69 and CD86. These markers were consistently expressed on a larger subset of cells in population B; in fact, expression was nearly absent on population A. CD44 was present on both populations but the level of expression differed: in population A approximately 50% of the cells were positive for low levels of CD44 whereas, in population B, nearly 100% of the cells were positive for high levels of CD44. Furthermore, after the leukaemia cells had been completely eradicated under the original culture conditions, the CD4+ effector cells could be isolated and they retained their cytotoxic capacity when plated with fresh target cells, in the
absence of further DC support. Interestingly, when examined by flow cytometry, these re-challenged effector cells also fall into population B by forward and side scatter.

FIGURE 4.3 Cell-surface marker expression by CD4\(^+\) cells in the co-culture assays.

The CD4\(^+\) cells, from those culture conditions that showed evidence of target cell clearance, were analyzed by flow cytometry. Gating on the CD4\(^+\) population, two distinct populations were consistently noted on the FSC/SSC plot; these were analyzed separately for their expression of a panel of markers. The graphs show the percentage of the two CD4\(^+\) populations that are positive for each marker.
4.2.4 CD4+ effector population contains αβ-T cells and NKT cells.

Our analysis thus far has not allowed us to distinguish between αβ-T cells and NKT cells when characterizing the CD4+ effector population. To address this, we used flow cytometry to look at TCR Vβ chain expression in the initial cultures that had demonstrated killing, staining for three of the more commonly expressed rearrangements: Vβ8, Vβ8.1/8.2 and Vβ11. The cultures were positive for Vβ8 and Vβ11 to some degree but appeared negative for Vβ8.1/8.2 (Figure 4.4a). Only the Vβ8.2 chain is common to both αβ-T cells and NKT cells; the others should not be used by NKT cells.

We also tested cultures at various time points after co-culture for the expression of the iTCR rearrangements that have been used to characterize Classical CD1d-restricted NKT cells. To do this, we used two sets of published PCR primers recognizing the Vα14-Jα28191 and the Vα14-Jα18145 regions of the iTCR. All cultures were positive for iTCR rearrangements, as was the CD4+ component population, but the CD11c+ fraction and the LV12.2 target cells were negative (Figure 4.4b). The NKT cell line C1498 was used as a positive control.
FIGURE 4.4 TCR chain expression by cells included in the co-culture assays.

A. The CD4\(^+\) effector cells were stained at various times during co-culture for the use of several of the more common V\(\beta\) TCR chains. B. Cell populations, sampled from both the co-cultures and from the input populations, were tested by PCR, at various time points (in days) after assay initiation, for expression of the iTCR used by NKT cells. Two published primer sets were used and the NKT cell line, C1498, was used as a positive control.
4.2.5 NKT cells are persistent and required.

Despite their persistent presence in our cultures and their known capacity for anticancer activity\(^{160}\), a number of factors, like the establishment of immune memory, make it unlikely that NKT cells are solely responsible for leukaemia clearance in our system. To test this supposition, we set-up the \textit{in vitro} assay with the same culture conditions as before; containing DCs, LV12.2 cells and the CD4\(^+\) selected effector population. In addition, we included a condition containing a CD4\(^+\) effector population depleted of NKT cells by FACS and a separate condition containing NKT cells in the absence of other CD4\(^+\) cells. The NKT population was identified as CD4\(^+\)/CD8\(^-\)/tetramer\(^+\) (CD1d tetramer loaded with the \(\alpha\)GalCer analogue, PBS-57). Leukaemia clearance was observed only when undepleted CD4\(^+\) effector cells were added but not when the NKT cells were removed or when they were added alone (Figure 4.5a). Results are shown as the fold increase in target cell number over the number originally added to the cultures. To control for the possibility that the NKT cells were insufficient alone because they were somehow compromised by the sorting process, we included a condition where NKT cells were depleted and then added back at approximately the same proportion. This condition did not demonstrate the identical degree of clearance as seen with the unsorted population, possibly because the number added back was an under-representation, but it does show that the post-sort NKT cells were still functional. Depletion was confirmed by PCR detection of the iTCR (Figure 4.5b).
FIGURE 4.5 NKT cells are necessary for target cell clearance.

A. The CD4⁺ enriched population was depleted of NKT cells, before addition to co-culture assays. PBS-57-bound CD1d tetramers were used to label NKT cells for depletion by FACS. NKT-depleted CD4⁺ effector cells were not able to clear cultures of target cells, nor were NKT cells alone; however, add-back of NKT cells to cultures containing NKT-depleted CD4⁺ effectors partially restored clearance capacity. B. NKT cell depletion was confirmed by PCR for iTCR expression using the NKT cell line, C1498, as a positive control.
4.2.6 **NKT cells are the sole IFN-γ producers in the in vitro culture system.**

NKT cells are major producers of IFN-γ, a cytokine required for the initiation of a primary T<sub>H</sub>1 response\(^{21}\). NKT cells are also the only source of IFN-γ in our system; as verified by collecting supernatants at various time points, post-co-culture, in the NKT-depletion assay and measuring cytokine levels using the flow cytometry array (Figure 4.6).

If producing IFN-γ was the only contribution made by NKT cells toward clearance of the leukaemia cells, then one would expect that NKT cells could be replaced by the addition of exogenous IFN-γ. This was not, however, the case. The addition of exogenous IFN-γ to the assay was not sufficient, as we can see in Figure 4.5a. Together these experiments demonstrate that NKT cells are required for target cell clearance, but are not sufficient alone, and that the production of IFN-γ is not their only contribution to the initiation of an efficient response.

4.2.7 **Cytokine profile observed for in vitro culture conditions.**

Similar to the cytokine profile observed during leukaemia rejection *in vivo*\(^{35}\), IL-6 and IL-10 were not elevated above background. Significant levels of IFN-γ were observed but only under activating conditions. Unlike the *in vivo* situation, however, TNF-α was detected under all conditions in which DCs were present but activating conditions did not contain significantly more than control conditions. Also different was that MCP-1 was the most abundantly produced of the 6 cytokines assayed for in the *in vitro* cultures and was produced under all conditions in which DCs were present (Figure 4.7a). MCP-1 production appeared to be constitutive as significant levels were measurable as early as 4 hours post co-culture (earlier time points were not analyzed) and continued to rise, most notably when NKT cells and DCs are present together (Figure 4.7b). By contrast, IFN-γ was produced at very low levels at the 4 hour time point, and
only when both NKT cells and target cells were present, but increased dramatically over the time course examined (Figure 4.6). In fact, IFN-γ was consistently detectable in culture conditions suitable for leukaemia cell clearance and clearance never occurred in the absence of IFN-γ.

FIGURE 4.6 NKT cells are the sole producers of IFN-γ.
Supernatants were sampled from co-culture assays over a time course and tested for IFN-γ by flow cytometry, using a CBA kit. IFN-γ is produced only when NKT cells are cultured in the presence of target cells.
FIGURE 4.7  Cytokine production in the co-culture assays.

Supernatants were sampled from co-culture assays  

A. At the point of target cell clearance, and 

B. over a time course, and tested for a panel of inflammatory cytokines by CBA.
4.2.8 IFN-γ as the effector mechanism.

IFN-γ is known to have wide ranging effects in cancer models, including having anti-proliferative properties\(^{161}\). Given the significant levels present during rejection in our system, we wanted to determine if cellular stasis, induced by IFN-γ, could partly account for the relative decrease in leukaemia cell number in test wells as compared to controls. The concentration of IFN-γ present in cultures at the time of detectable leukaemia clearance was measured and LV12.2 cells were grown with this concentration of IFN-γ added to the culture media for 2 months to produce an IFN-γ-resistant cell line, LV12.2-r. These resistant cells were then used as the target population in our killing assays and were cleared to an equivalent degree as IFN-γ naïve cells (Figure 4.5). This result demonstrates that IFN-γ is not the mechanism of leukaemia clearance.

4.2.9 NKT activation of DC population.

A transwell culture system was then used to determine which cell types needed to be in contact for full activation of the CD4\(^+\) CTL population and efficient clearance of the leukaemia cells. Table 4.2 summarizes the results. For the leukaemia cells to be rejected, NKT cells and DCs must be in contact with each other and DCs must make contact with the effector and target cell populations. Target cells do not need to make contact with NKT cells, suggesting that the role of the NKT population is not Ag-specific. IFN-γ is produced only when DCs and IL-12 producing target cells are present together with NKT cells, regardless of contact; but it is not clear that both DCs and target cells are required for IFN-γ by NKT cells. This result suggests that the NKT population produces IFN-γ in response to a soluble factor, probably IL-12. Furthermore, we observed that the DCs in our cultures took on a more activated morphology.
whenever NKT cells were present and to some extent, though to a much lesser degree, when NKT cells were replaced by rIFN-γ (Figure 4.8). Together these observations suggest that NKT cells are required for full activation of the DC population and that this activation requires both IFN-γ production and cell-cell contact. Figure 4.9 is a proposed model for the initiation of a CD4+ CTL response in our model system.

![Figure 4.9: Proposed model for the initiation of a CD4+ CTL response.](image)

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+ Leukaemia cell clearance  
– Leukaemia cell outgrowth  
γ IFN-γ production

**TABLE 4.2 Summary of transwell assay contents and IFN-γ production.**

The NKT cell population was separated from the others using transwells. DCs and LV12.2 cells were then added back into the upper chamber to determine which cell populations need to make contact for target cell clearance to be observed in the bottom chamber. Supernatants from each conditions were also tested by ELISA for the production of IFN-γ.
FIGURE 4.8 Activated morphology of DCs in the presence of NKT cells or IFN-γ.

These pictures are 2 representative views of co-culture conditions showing that the DC population included took on an activated morphology in the presence of NKT cells and, to a lesser degree, rIFN-γ. Pictures were acquired using light field microscopy.
IL-12 from LV12.2 signals to NKT cells and induces them to secrete IFN-γ from NKT cells activates DCs in upper chamber, DCs express a membrane-bound stimulatory Factor X. Reciprocal activation through Factor X causes production of a secreted stimulatory Factor Y, possibly by the DC. Factor Y, along with IFN-γ, licenses DCs in lower chamber. Tumour Ag is taken up by DCs in lower chamber as they mature. Licensing allows DCs to present this Ag to naïve CD4⁺ T cells, as well as provide the 3 required activation signals. Naïve CD4⁺ T cells are fully matured, activated, and are now capable of leukaemia killing in a specific manner.

FIGURE 4.9 Proposed model of the steps to activation.

This model proposes that two more factors, leading to full activation of the CD4⁺ CTL population, have yet to be identified; one that is membrane-bound (Factor X), and one that is secreted (Factor Y).
4.3 Discussion

We elected to develop an *in vitro* system amenable to studying the effector population responsible for leukaemia clearance in a more detailed fashion. Herein, we begin to describe a CD4\(^+\) effector cell capable of efficiently clearing a MHCII negative leukaemia. Using a population of CD4\(^+\) cells selected from the spleens of BDF\(_1\) mice, DCs grown from BM in GM-CSF and IL-4, and the IL-12 producing leukaemia cell line, LV12.2, we established an *in vitro* model that mimics our *in vivo* system. We were able to demonstrate that activation of the effector population is IL-12-dependent and requires support from DCs in the primary response. We observe an initial expansion in the leukaemia cell population before the target cells are cleared; it is presumably during this period of expansion that the DCs cross-present tumour Ags on MHCII molecules, leading to T cell activation. The subsequent target cell killing is specific as evidenced by a lack of collateral damage to accessory cells in the culture. These CD4\(^+\) effector cells can then be removed from the cultures and re-challenged. In the absence of further DC support, effector cells can repeatedly eliminate LV12.2 target cells. Target cell clearance begins within a shorter time frame than might be expected for an adaptive immune response, but there are a number of significant differences between an *in vivo* response and our *in vitro* system that might explain this truncated timeline. For example, the stimulus is condensed in the *in vitro* system so that cells need not travel to be exposed to it, the effector population does not have to traffic to the LNs for activation, nor do they need to then track down the target population. All of these factors will likely reduce the amount of time required to mount an effective response. Moreover, this CD4\(^+\) effector population may possess mechanistic characteristics in common with more innate-like cells, such that a response is faster to mount than is typical for a CD8\(^+\) CTL.
The CD4\(^+\) effector population in primary challenge cultures consistently exists in two distinct populations that can be distinguished by their forward and side scatter pattern by flow cytometry. When analyzed separately, we see differences in the expression of certain surface molecules, including: CD11b, CD25, CD44 and CD69 between the two populations. These molecules mark activated T cells and the differences lead us to conclude that those cells largely responsible for the cytotoxicity observed reside in the more granular population B. This conclusion is supported by the observation that CD4\(^+\) cells that have been re-challenged, a population presumably enriched for Ag-specific effector cells, also fall predominantly into population B. We hypothesize that population A is made up of resting cells that may represent a naïve pool from which the activated cells arise. Furthermore, markers like CD44, which are expressed by both populations but to a different degree, may mark transitional cells in the process of shifting from one population to the other.

When the \textit{in vivo} depletion experiments were conducted to narrow down the identity of the effector cell population\(^{27}\), we were unable to rule out that a CD4\(^+\) NKT population was responsible for leukaemia clearance. There were, however, indications that NKT cells alone are not responsible for rejection, such as the establishment of immune memory. There is little known about the capacity of NKT cells to form memory pools and what little has been reported is controversial\(^{131}\). We proposed that conditions were conducive to the development of an αβ CD4\(^+\) CTL but more detailed studies were required to substantiate this claim. Some NKT cells express CD4 but not CD49b, so our initial \textit{in vitro} assays didn’t exclude this possibility either as the negative selection kit used to enrich for CD4\(^+\) splenic cells does not select against all NKT cell populations. However, further characterization supported our hypothesis that the CD4\(^+\)
population primarily consists of αβ-T cells. First, all T cell subsets express the cell-surface antigen CD5, a protein absent on the surface of Classical CD1d-restricted NKT cells\textsuperscript{162}. The CD4\textsuperscript{+} cells in our system express high levels of CD5 but are negative for CD160, which is a marker of NKT cells. Second, Classical NKT cells express a very restricted TCR repertoire which includes use of the V\textbeta\textsubscript{2, 7 and 8.2} chains. The CD4\textsuperscript{+} cells in our assays express a broader TCR repertoire than is utilized by NKT cells, including V\textbeta\textsubscript{8 and 11}, in addition to 8.1/8.2. Nonetheless, these results demonstrate only that αβ-T cells are present and likely make up a greater proportion of the effector population; they do not demonstrate a clear absence of NKT cells. In fact, PCR detection of the iTCR expressed by NKT cells showed their persistent presence under conditions of target cell clearance as well as in the CD4\textsuperscript{+} component population. These results demonstrate the co-existence of NKT cells together with αβ-T cells in the effector cell fraction but do not illuminate the role played by either.

Despite the presence of NKT cells, it remains unlikely that they are the primary cytotoxic cell. NKT cells recognize lipid antigen presented on CD1d, a MHC class I-like molecule expressed by the LV12.2 target population. Since direct target recognition is possible, if NKT cells alone are responsible for the leukaemia clearance observed we would have expected to see clearance in the control wells containing CD4\textsuperscript{+} cells cultured alone with target cells. This was not, however, the case suggesting that the NKT population plays more of a supportive rather than a front line role.

We propose that NKT cells are involved in activating the DC population and licensing them to activate naïve αβ-T cells. DCs are known to be the most potent inducers of T cell-dependent immune responses, specifically of T\textsubscript{H1} responses through their production of the T\textsubscript{H1}
polarizing cytokine IL-12p70. However, DCs do not constitutively express IL-12 p70, requiring IFN-\(\gamma\) stimulation to do so\(^2\). IFN-\(\gamma\) is not a typical product of DCs and T cells only produce IFN-\(\gamma\) once activated by DCs through the delivery of three signals: 1.) TCR:Ag recognition, 2.) costimulation, and 3.) cytokine polarization. Thus, a third cell type must initially supply the IFN-\(\gamma\) required to license DC priming of a primary CTL response\(^1\). Since IL-12 is known to feed into a positive feedback loop, promoting its own production\(^1\), one might suggest that the IL-12 produced by the leukaemia cells in our system could bypass the need for IFN-\(\gamma\) stimulation. This is unlikely because DCs do not constitutively express the \(\beta_1\) chain of the heterodimeric IL-12R\(^1\), as expression of the complete IL-12R is also IFN-\(\gamma\)-dependent. Therefore, a third cell is needed to provide IFN-\(\gamma\) in order for DCs to either produce or respond to IL-12p70. It is likely that the NKT cells exert their effect at this stage, in licensing the DCs to fully activate and Th1 polarize the CD4\(^+\) \(\alpha\beta\)-T effector cells.

NKT cells have been shown to rapidly produce massive amounts of IFN-\(\gamma\) in response to IL-12\(^1\); however, other studies indicate that direct contact with DCs, in addition to IL-12, is required\(^1\). The \textit{in vitro} depletion assay allowed us to confirm that IFN-\(\gamma\) is produced only in the presence of NKT cells in our culture system. When the NKT population is sorted out, the production of IFN-\(\gamma\) is both delayed and significantly inhibited. Functional NKT depletion, using CD1d tetramer binding, does not exclude precursor cells as only fully mature NKT cells are removed. The mature population could potentially be replenished within a short period of time under activating conditions and this may explain the small amount of IFN-\(\gamma\) production just 10 hours post-NKT depletion in the cultures. However, this production may also be attributable to a small amount of residual NKT cell contamination (<3%) since IFN-\(\gamma\) levels under depleted
conditions remain significantly lower over the entire time course sampled. Moreover, IFN-γ is produced within 4 hours when NKT cells are co-cultured with target cells and DCs but not produced at a significant level, at any time, when cultured alone. This suggests that IFN-γ production is not constitutive but responsive; requiring stimulation from IL-12 producing target cells and DCs.

We observed that DCs display an activated morphology when a source of IFN-γ is present in the culture conditions. However, exogenous IFN-γ cannot replace NKT cells as leukaemia clearance does not occur when IFN-γ is added to cultures containing NKT-depleted CD4+ effector cells. NKT cells do not produce IFN-γ when plated alone but do when plated with LV12.2 in the presence of DCs; though direct NKT-DC contact does not appear to be required for IFN-γ production, nor is it Ag-dependent. Other published models that have looked at what signals are required for the initiation of an IL-12-mediated T_H1 response would predict that the presence of NKT cells and their production of IFN-γ would be sufficient. In our model system, it is not. If DCs required only IFN-γ to become fully activated, then clearance would have been observed when NKT cells were plated alone in the upper chamber of the transwell assays, as IFN-γ could pass through the membrane to fully activate DCs in the lower chamber and support killing. Though IFN-γ was produced under conditions where DCs were present in the bottom chamber and NKT cells were cultured alone in the upper chamber, no clearance was observed. Addition of DCs into the upper chamber with NKT cells remedied this. Cumulatively, the transwell assay data suggest that cell contact between NKT cells and DCs is also required. The outcome of this contact is likely the production of a secreted factor that, combined with IFN-γ, renders DCs in the bottom chamber fully licensed. This model would also explain why addition
of exogenous IFN-γ could not substitute for the presence of NKT cells in the cultures. We suggest that IFN-γ depletion doesn’t diminish leukaemia killing capacity because the α-IFN-γ Ab is not sufficient to prevent activity of the cytokine at the interface between cells where it is produced and exerts its effect\textsuperscript{123,166}; however this remains to be proved.

IFN-γ has wide ranging effects in cancer models; in addition to playing a role in immune activation, it can also have direct effects on malignant cells including cytotoxic and anti-proliferative properties\textsuperscript{161}. Using IFN-γ resistant target cells, however, we were able to definitively demonstrate that IFN-γ is not the mechanism of target killing.

The production of large amounts of IFN-γ under conditions in which leukaemia clearance occurs is no surprise as this cytokine is often credited with mediating the anticancer effects of IL-12 (refer to \textsuperscript{70} for interesting commentary on their co-dependence and unique functions). However, the production of significant levels of MCP-1 whenever DCs are present, and especially under activating conditions, is a more difficult result to reconcile as there is contradictory evidence in the literature about the role of MCP-1. This chemokine is important for the trafficking of phagocytic cells to sites of injury where Ag is acquired. There are reports that MCP-1 leads to the activation and differentiation of monocytes and thereby to inhibition of tumour growth\textsuperscript{167}; and, contrarily, it has also been associated with cancer growth and invasion\textsuperscript{168}. It is noteworthy that MCP-1 has been shown to antagonize the T\textsubscript{H}1 polarizing capacity of DCs by abating IL-12 production\textsuperscript{169}; though the kinetics of DC exposure to MCP-1 appear to be pivotal to the resultant effect. Exposure of monocytes during differentiation into DCs can suppress their IL-12 production and subsequently lead to the activation of naïve CD4\textsuperscript{+} T cells with diminished IFN-γ producing capacity. However, exposure of mature DCs to MCP-1 has no
effect\textsuperscript{170}. In our culture system, MCP-1 is produced at a similar level whenever DCs are present. These DCs are prepared from BM cultured for 1 week in GM-CSF to promote monocyte growth, and IL-4 to maintain an immature DC state, but this does not preclude the presence of the more mature DCs that are responsible for MCP-1 production. MCP-1 levels are increased only in the presence of CD4\textsuperscript{+} cells which, presumably, deliver a maturation stimulus to the DCs; so it is curious that this CD4\textsuperscript{+} cell-induced increase in MCP-1 production is somewhat mitigated by concurrent addition of leukaemia cells. MCP-1 can also promote either T\textsubscript{H}1 or T\textsubscript{H}2 responses depending on the kinetics of production\textsuperscript{171} but, at a minimum, it is clear that MCP-1 does not inhibit leukaemia clearance in our system. Detection of MCP-1 has been consistent in our studies, leading us to presume that its significance will become apparent, but further discussion of its role is currently beyond the scope of this thesis.

The value of our \textit{in vitro} system is that it has allowed us to examine the necessary component parts of an effective IL-12-mediated anti-leukaemia response. It is our hope that a greater understanding of the mechanisms by which immunomodulatory elements exert their effect on the immune system will allow for a more rational approach to the design of therapeutic regiments. Furthermore, we have identified a CD4\textsuperscript{+} effector cell population that exhibits many characteristics of a CTL. Our \textit{in vitro} system will allow us to elucidate the mechanism of action of this population, as well as the nature of its interaction with the target cells, in order to definitively determine if this is indeed a true CD4\textsuperscript{+} CTL.
CHAPTER 5: Translational Considerations for Cell-Mediated Immunotherapy

A portion of this chapter is adapted from the manuscript published in the Journal of Molecular and Cellular Medicine:


* Co-first authors; these authors contributed equally to this work.
5.1 Introduction

The use of cell-based immunotherapies in general has shown increasing promise over the last decade; be that in the form of ACT, autologous cancer cell vaccines, or transduced DCs or fibroblasts\textsuperscript{64,121,172}. Given the elaborate interrelationship of malignant cells with their host, cancer patients all experience some level of immune insufficiency. For this reason, the current paradigm suggests that the most likely beneficiaries of immunotherapy are those patients with MRD, such that some of the immunosuppressive burden of their disease has been removed. Moreover, the immune systems of these patients may have already been activated by the cytotoxic treatments they have received\textsuperscript{172}. While cytotoxic agents, such as radiation and chemotherapy, can initiate danger signals that alert the immune system\textsuperscript{74} and provide a source of Ag, this alone isn’t always enough to lead to a full blown response. Coupled with immunotherapy, however, this could allow the immune system to get a foothold and tip the balance in favour of the patient.

Though prior therapy can provide a favourable starting point and can potentiate the effects of immunotherapy, the success of these therapies also heightens the need for stringent safety guidelines to ensure that the cells being re-administered to a patient who has undergone de-bulking therapy will act as therapeutic agents and not add to disease burden. The work described in Chapters 3 and 4 has identified and characterized the immune response initiated downstream of our cell-mediated IL-12 immunotherapy protocol in a mouse model; nevertheless, we are ultimately interested in adapting this protocol for use in humans. It was therefore important to consider a number of factors that are relevant to the clinical setting: safety and how safety measures may impact treatment, treating established disease, and whether therapy can
overcome tolerance. As well, we were interested in exploring what attributes of our protocol are specifically responsible for initiating a CD4+ CTL response, as this is of clinical interest.

Before administering cells to patients, the cells can be irradiated to block mitosis and inhibit proliferation as one way to address some of the safety issues faced by cell-based therapies. The literature, however, contains conflicting data on this approach; in some models of cell therapy efficacy is improved\textsuperscript{76,173-175}, whereas in other models the therapeutic benefit is negatively impacted or altogether negated\textsuperscript{76,176,177}. Stagg \textit{et al.} posit that inducing an effective clinical response will require, at a minimum, an agent or combination of agents that work in concert to enhance Ag presentation and overcome the regulatory checkpoints that restrict tumour immunity, while rescuing intrinsic tumour suppressor pathways and affecting cell death in a manner that increases the pool of potential tumour Ags\textsuperscript{7}. Combining immunotherapy with cell death-inducing radiation or chemotherapy theoretically fulfills these proposed requirements. Therefore, we tested if radiation could be combined with our IL-12-mediated cell therapy protocol to improve safety without adversely affecting the benefit. We also combined our approach with chemotherapy to determine if this would allow for delayed treatment initiation and if it could be used to treat MRD.

Another complication we wanted to address was the issue of tolerance. It is known that T cells can be tolerized by DCs if presented Ag in the absence of sufficient co-stimulation and cytokine production\textsuperscript{178}. Due to a limited ability in a clinical setting to select for transduced autologous cancer cells secreting high levels of cytokine, cell-based therapies generally rely on a bulk population of transduced cells that express the transgene at a whole range of levels. We
wanted to test if the presence of low- or non-producing cells in our inoculum would inhibit an otherwise effective therapy.

Furthermore, the CD4+ CTL we have characterized exerts its cytotoxic effect against a MHCII negative target without collateral damage to the other cell populations in the cultures. This suggests that the ‘death kiss’ is indeed targeted. Our lab has published data demonstrating that 70Z/3-L does not express MHCII mRNA under basal conditions or even with induction by IFN-γ, a cytokine that can induce MHCII expression in most cell types\(^7\). Nevertheless, that same paper also describes a Φ-like line, derived from 70Z/3-L by selecting for adherent cells, that is MHCII\(^{+}\). Though 70Z/3-L is itself MHCII\(^{-}\), it can give rise to cells with the ability to express MHCII. We have therefore reviewed the MHCII status of our target cell line under different conditions and resolved that it is indeed capable of MHCII expression when stimulated, but only a small minority subpopulation does so.

Interleukin-12 has led to a broad range of anticancer responses; their uniqueness dependent on both the model system and the specific malignancy being investigated, as well as the method of IL-12 delivery. The effector mechanism can also be dose dependent. For example, lower doses of IL-12 are known to demonstrate a more significant role for NKT cells and IFN-γ as compared to higher doses that rely more heavily on NK cells and perforin\(^{165}\). However, we don’t know of any other case where IL-12 has led to the eradication of a MHCII-cancer by a CD4+ CTL directly. Given the ostensibly incompatible nature of our target and effector cell pair, we conducted an \textit{in vivo} depletion assay of rIL-12 therapy to begin to assess if the induction of a CD4\(^+\) CTL is a function of the model system, 70Z/3 leukaemia; the mode of administration, purified protein or cell-mediated; or possibly the IL-12 protein itself, rIL-12.
versus cIL-12 (LV12.2 cell-derived IL-12). The depletion assay demonstrated that the route to leukaemia rejection is qualitatively different between the two models. This indicates that the development of a CD4\(^+\) CTL is not driven by the target cell type, as they are constant between the two systems.

5.2 Results

5.2.1 Irradiation of LV12.2 cells.

The use of autologous cancer cells as vectors for cytokine therapy has some perceived safety issues. One possible safety measure is to use radiation to prevent the cells from proliferating after injection. To determine if this approach was appropriate for our model system, we began by irradiating LV12.2 cells at a range of doses to find a dose that would leave the cells structurally viable but incapable of undergoing further division. Thymide incorporation was used as a measure of mitotic activity and Figure 5.1a shows that approximately 5 grays (Gy) was the minimum dose at which negligible DNA replication could be detected by 48 hours post irradiation. However, 70Z/3 cells are very sensitive to \(\gamma\)-irradiation and are more likely to undergo apoptosis than simply cell cycle arrest\(^{180}\). We examined the cells at this same time point and determined cell viability using trypan blue exclusion. Figure 5.1b demonstrates that even the minimum dose of radiation required significantly reduces cell viability and the cells undergo apoptosis; by 96 hours, the cultures are completely dead.
FIGURE 5.1 Irradiation of LV12.2 cells.
LV12.2 cells were irradiated, using a γ-irradiator, at various doses. **A.** Cell proliferation was measured by thymidine incorporation assay. **B.** Cell viability was measured by counting cells using trypan blue exclusion. Measurements were taken at 24 and 48 hours post-irradiation.
5.2.2 *Injection of irradiated LV12.2 cells.*

Though LV12.2 cell viability is severely impacted, these cells continue to secrete IL-12 for at least 24 hours post irradiation and the innate immune responses that support and shape adaptive responses are initiated within hours of stimulation. To see if an effective anti-leukaemia response could be initiated during the 24-48 hours that the cells remained viable, we injected mice with $10^6$ 70Z/3-L cells along with 1% or 10% LV12.2 cells that had been irradiated at a dose of 6Gy. Unirradiated LV12.2 cells were included, in the same proportions, as controls. The irradiated cells were not able to protect to the same degree as their unirradiated counterparts (Figure 5.2).

![Graph showing survival rates post injection](image)

**FIGURE 5.2 Injection of irradiated LV12.2 cells.**

Mice were injected with $10^6$ 70Z/3-L cells and test groups received an injection of either 1% or 10% LV12.2 cells that had been irradiated with a dose of 6Gy. Two control groups were included that received either 1% or 10% unirradiated LV12.2 cells. The “+” sign indicates the groups for which the cells were irradiated before administration, and the “-” sign denotes the groups for which the cells were unirradiated at the time of administration. (n=4).
5.2.3 *rIL-12 therapy protects mice with pre-established 70Z/3-L leukaemia.*

All of the previous *in vivo* experiments involved administering therapy simultaneously with disease challenge. To determine if IP administration of rIL-12 can lead to leukaemia clearance in the context of a more established disease state, treatment initiation was delayed to allow for dissemination of the leukaemia. Due to the rapid growth of 70Z/3-L cells and the aggressiveness of the disease they cause, we commenced these experiments by injecting just $10^4$ 70Z/3-L cells. This dose is still lethal to 100% of mice in approximately 20 days\(^85\). Initiation of rIL-12 administration was delayed by 0 to 5 days and continued for 14 days following the first injection. We found that we could delay the initiation of rIL-12 therapy by 5 days and still achieve significant protection against the leukaemia (Figure 5.3). The differences between the survival curves of the six treatment groups are not statistically significant and longer delays were not tested.
FIGURE 5.3 Delayed IP administration of rIL-12 therapy leads to protection.

Mice were injected with $10^4$ 70Z/3-L cells on day 0. A control group (70Z/3-L) did not receive treatment ($n = 4$). From days 0 through 5, groups of 4 or 5 mice (5 mice for days 0, 1 and 2, 4 mice for days 3, 4 and 5) started receiving injections of 20ng rIL-12/mouse/day for 14 days. Animals were monitored and euthanized at the appearance of symptoms. Curve comparison was performed using Logrank test. All treatment groups are significantly different from the control group ($p = 0.0029$) but are not significantly different from each other.
5.2.4 Cell-mediated therapy is less effective against established disease.

Cell-mediated therapy, however, could not be delayed in the same manner as rIL-12 therapy. Mice were injected with $10^4$ 70Z/3-L cells on day 0 and injection of either $10^4$ or $10^5$ LV12.2 cells was delayed for 3 or 4 days. Those mice that received injections of LV12.2 cells on day 4 survived the challenge; but, perplexingly, this was not true of mice receiving the same therapy delayed by only 3 days (Figure 5.4). Though some protection is observed, cell-mediated therapy appears to be inferior to rIL-12 therapy when delayed. However, when combined with chemotherapy, mice were able to respond to cell-mediated therapy when delayed up to one week (Figure 5.5a). Chemotherapy with AraC alone extended survival but all mice eventually succumbed to disease, whereas combination therapy significantly improved survival even when LV12.2 injection was delayed for 7 days. Direct comparison can be drawn between delayed cell-therapy groups B and D: both groups received $10^6$ LV12.2 cells 5 days after an initial challenge with $10^5$ 70Z/3-L but only group D survived, having first received chemotherapy treatment. Importantly, initiation of cell-based therapy coincided with recovery of the WBC compartment (Figure 5.5b).
FIGURE 5.4 Delay of cell-mediated therapy.

Mice (n=5) were challenged with $10^4$ 70Z/3-L cells on day 0 and LV12.2 treatment was delivered at various time points post-challenge: $10^3$ LV12.2 on day 0 or either $10^4$ or $10^5$ LV12.2 on day 3 or 4.
FIGURE 5.5 Delayed cell therapy combined with chemotherapy pre-treatment.

A. Mice (n=5) were challenged with $10^5$ 70Z/3-L cells on day 0 and then treated with either $10^6$ LV12.2 cells alone on day 5 or $10^6$ LV12.2 at various time points (days 5, 7 or 9) after chemotherapy pre-treatment. Chemotherapy was delivered in 3 doses of 100mg/kg, with each dose separated by 10 hours. Groups receiving chemotherapy alone or left untreated were included as controls. B. The WBC count of mice receiving chemotherapy was measured over a time course. Blood samples were acquired immediately after injection and the total cell count taken by trypan blue exclusion. The proportion of lymphocytes in the sample was determined by flow cytometry and used to calculate the number of lymphocytes/µL of blood.
5.2.5 *Tolerance.*

To better mimic a clinical cell preparation and test if the effectiveness of therapy would be inhibited by delivering a mixture of IL-12 producing cells, not all of which secrete IL-12 above the critical threshold, we conducted a mixing experiment using a clone, LV12.4, that produces 700 pg/mL/10⁶ cells/2hrs and causes disease in mice injected with 10⁶ cells (seen in Figure 3.4). Mice were injected with 10⁶ 70Z/3-L cells and a proportion, 1%, of LV12.2 cells that is known to be protective but on the border of insufficient. Medium, LV12.3, and low, LV12.4, producing lines were included at 10% and 1% to see if their presence decreased or inhibited an otherwise effective therapy. In all cases, at least 80% of the mice survived the challenge (Figure 5.6). We also injected mice with 10⁶ of the LV12.4 line and titrated in LV12.2 to test if these amounts were still effective in the context of a potentially tolerogenic environment. Again, at least 80% of the mice survived the challenge. This suggests that low producing cells do not have a counterproductive effect when delivered together with an otherwise effective therapy.
FIGURE 5.6 Cell therapy tolerance experiment.

Mice (n=5) were injected with various proportions of three different IL-12 producing cell lines in different combinations. Each cell line produces a different amount of IL-12: LV12.2 (20,000pg/mL/10⁶ cells/2hrs), LV12.3 (2,000pg/mL/10⁶ cells/2hrs), and LV12.4 (700 pg/mL/10⁶ cells/2hrs).

5.2.6 MHCII expressed only by a subpopulation of LV12.2 cells.

The lack of collateral damage to accessory cells in the in vitro culture system and the establishment of immune memory in vivo suggest that cytotoxicity is targeted and therefore involves TCR·Ag recognition. However, CD4⁺ T cells are MHCII restricted. Cross-presentation
of tumour Ag complexed to MHCII by APCs can only explain activation of the CTL, but fails to clarify how that activated CTL subsequently recognizes its target cell. 70Z/3-L cells do not express MHCII at levels detectable by flow cytometry under normal conditions, nor in response to acute exposure to IFN-γ, as shown in Figure 5.7. Nevertheless, we have shown that IFN-γ is produced at significantly higher levels in the cell-mediated in vivo system than with rIL-12 therapy, the levels remaining elevated for at least a week; and is also produced in the in vitro cultures in which clearance is observed. The IFN-γ-resistant LV12.2 line, LV12.2-r, is consistently cultured in levels of IFN-γ comparable to that measured in co-cultures, so we stained this line to see if chronic IFN-γ exposure would have an effect on MHC expression. We also included a line of 70Z/3-L cells that had been passaged through the mouse and re-established ex-vivo. In both of these lines, there is a small subset of MHCII positive cells; 17% in the chronically IFN-γ stimulated population and approximately 1% in the ex-vivo population. Ltk⁻ cells were used as negative staining controls.

5.2.7 CD4⁺ and CD8⁺ T cells required for rIL-12-mediated rejection of 70Z/3-L cells after IP administration.

Considering that only a small subset of LV12.2 cells can be induced to express MHCII, it remains perplexing that the effector population generated in vivo was a CD4⁺ CTL. Responses to IL-12 therapy are largely dependent on features of the model system, including: cancer type, dose and schedule, and method and route of administration. To resolve which feature(s) of our model system influenced the development of a CD4⁺ CTL, we first conducted an in vivo depletion experiment with rIL-12 therapy for comparison.
FIGURE 5.7  70Z/3 expression of MHCII.

Variants of 70Z/3 were tested for their expression of MHCII by flow cytometry. Test groups included: LV12.2; LV12.2 spiked with IFN-γ for 24 hours; LV12.2-r, the LV12.2 variant that is continually cultured with 1ng/mL IFN-γ in the media; and a line of 70Z/3 that had been passaged through a mouse receiving IL-12 therapy and then re-established. Ltk- cells served as a negative control and the 70Z/3 MΦ line as a positive control.
Depleting antibodies were used to establish which cell types mediate the rIL-12-induced rejection of 70Z/3-L leukaemia after IP administration. We show in Figure 5.8 that both CD4⁺ and CD8⁺ T cells are important as depletion of either population eliminates immune protection in all mice. The mean survival was 14 days for mice depleted of CD8⁺ T cells, 23 days for mice depleted of CD4⁺ T cells and 13 days for mice depleted of both T cell subsets. The three curves are not statistically different from each other. We also included neutralizing antibodies against IFN-γ to examine its role in the rejection response. We found that this abrogated the protective effects of IP administered rIL-12, demonstrating that IFN-γ plays an essential role in leukaemia rejection. Although the importance of NK cells has been shown in other models of IL-12 therapy¹⁸¹,¹⁸² we did not observe changes in rejection of the 70Z/3-L leukaemia when NK cells were depleted in this treatment modality. Thus, rIL-12 therapy leads to leukaemia rejection by a more canonical pathway, mediated by a CD8⁺ CTL with support from a CD4⁺ T helper cell; a qualitatively different route to immunity than that seen with cell-based therapy. This experiment highlights that the mode of IL-12 delivery can have a profound impact on the nature of the immune response mounted. Both models use IP administration and lead to rejection of the same target cell line, suggesting that the difference may lie with delivery of a purified protein versus administration of a cell that produces the immunostimulant, or with the protein itself.
FIGURE 5.8 Requirement of T cells and IFN-γ for rIL-12-mediated protection

Mice (n = 5) were depleted using antibodies as described in Materials and Methods. The mice were then challenged with $10^6$ 70Z/3-L cells IP, injected with 20ng/mouse/day rIL-12 and monitored for the appearance of symptoms. Comparison of Kaplan-Meier survival curves was performed using Logrank test (p < 0.0018).

5.2.8 Comparison of rIL-12 versus cIL-12.

The IL-12-induced anti-leukaemia activity in our two models is T cell-dependent but the subsets that are critical differ depending on the mode of IL-12 delivery. One explanation might be that cIL-12 is working in combination with other proteins in the cell-mediated system. LV12.2 cells have been genetically engineered to produce IL-12 but likely still secrete other proteins also, not all of which have been characterized. Supernatants were collected from cultures of LV12.2 that were incubated in serum-free media for 4 hours, concentrated and
resolved on a polyacrylamide gel along with rIL-12 for comparison. The gel was then stained with Colloidal Coommassie Blue for visualization of global protein production. We can see in Figure 5.9a that LV12.2 cells indeed secrete other proteins in addition to IL-12. Moreover, the amino acid sequences of the two proteins differ by 3% (Figure 5.9b); primarily because alterations were made to the cIL-12 amino acid sequence to ensure that the p35 and p40 subunits are expressed in stoichiometric amounts. They also come from different sources: cIL-12 is produced by our murine cell line whereas the rIL-12 purchased from GIBCO is raised in Sf9 insect cells. Since insect cells lack some of the processing enzymes required for the mammalian-like post-translational modification of proteins, they may have a differential glycosylation patterns that explain their divergent activity.

![Figure 5.9](image-url)

**FIGURE 5.9** Production of other proteins by LV12.2 and sequence differences between rIL-12 and cIL-12.

**A.** LV12.2 cells were cultured in serum free media for 4 hours, supernatants were collected, concentrated and resolved on a polyacrylamide gel with rIL-12 used as a control. The gel was then stained with Colloidal Coommassie Blue to look at global protein production. The rIL-12 sample shows only one band, where the cIL-12 sample shows background protein production.

**B.** The amino acid sequences for rIL-12 and the LV vector used to transduce LV12.2 cells were aligned and the differences highlighted.
5.3 Discussion

The use of cytokine therapy in humans is challenged by factors that are not relevant to pre-clinical models, such as ensuring the safety of therapeutic agents and addressing how prior treatment regimens might impact the response to therapy. In this set of experiments, we began to address some of these issues. With the development of cancer cell-mediated immunotherapies comes the need to ensure that the re-introduced cancer cell vectors do not add to disease burden. One approach is to use irradiation to prevent proliferation of the cancer cells while still allowing for expression of the transgene. It did not prove feasible to study this approach using our model system as 70Z/3 cells are exquisitely sensitive to irradiation.

In a clinical setting, patients present with pre-established malignancies. To mimic this situation, we attempted to delay therapy instead of co-injecting the rIL-12 or IL-12 producing cells together with parental cells. For rIL-12 therapy, survival was unaffected. For cell-based therapy, on the other hand, delay was somewhat detrimental to the outcome. This suggests that delayed therapy is on the brink of not being effective, which allowed us to evaluate if protection could be facilitated by combination with a de-bulking method; in this case chemotherapy with AraC. It may be that initial chemotherapy simply limited the accumulation of leukaemia cells such that the number of IL-12 secreting cells administered still made up a sufficient proportion of the disease burden; but chemotherapy may also have heightened the immune response. The cytotoxicity that resulted from AraC treatment may have added to the pool of Ag that could be presented once DCs were activated by IL-12 therapy. AraC may also have initiated a sub-optimal innate response, through danger signals, that would support a full blown immune response once tipped by IL-12 stimulation. Because chemotherapy can also be immunosuppressive, this experiment is doubly important because it also demonstrates that
immunotherapy can still be successful in patients whose immune systems have been compromised by previous treatment modalities, so long as the suppression has time to abate.

Furthermore, and as discussed in Chapter 3, the degree of selection that can be applied to cells after transduction in a clinical setting is greatly reduced from what is typical for pre-clinical models. First, it is difficult to clone human primary cells and, second, manipulation must be kept to a minimum to avoid transformation or contamination of the cells in culture. The limitations of current selection procedures preclude the isolation of a highly purified population of cells producing the desired immunostimulant above a certain threshold. Since we know that low level stimulation can lead instead to anergy\textsuperscript{178}, we tested if the presence of very low producing cells within an otherwise effective preparation would inhibit a beneficial response. This was not the case. It is possible that the presence of low producers failed to induce a tolerogenic state; or it may be that higher producing cells were able to break and overcome the tolerance induced, as past studies have demonstrated the potential for IL-12 to do just that\textsuperscript{184,185}. In one case, T\textsubscript{H}1-polarized DCs (selected at the stage when they are the most potent producers of IL-12), but not unpolarized DCs, were able to rescue patient type-1 anti-melanoma CD4\textsuperscript{+} T cell responses\textsuperscript{63}. Either way, it is promising that if a sufficient number of cells, that each produce a sufficient amount of IL-12, can be produced then the presence of lower- or non-producing cells will not undermine their therapeutic benefit.

A provocative attribute of this model is that a CD4\textsuperscript{+} CTL is induced and appears to directly kill a MHCII\textsuperscript{-} target cell population. We re-examined MHCII expression after exposure of the cells to IFN-\gamma, a stimulus relevant to both the \textit{in vivo} and \textit{in vitro} systems, and found that a sub-population of 70Z/3 cells can be induced to express MHCII in response to chronic but not
acute IFN-γ exposure. However, we still do not know whether this is a stable trait and the remaining cells are incapable of MHCII expression, or if all cells are capable but only a small proportion do so at any given time. This distinction is important in beginning to understand how it is that a CD4+ CTL can effectively and directly kill a predominantly MHCII- target population. MHCII is tightly regulated and only expressed constitutively by professional APCs. Although most other cell types are capable of induced expression, specifically in response to IFN-γ, MHCII has been rendered non-inducible in many tumours due to defects in the IFN-γR or in the signalling pathway that leads to expression of CIITA. It is therefore intriguing how this CD4+ CTL might be recognizing its target from both a purely intellectual perspective as well as from the perspective of what ramifications this observation might have on clinical oncology. Evidence for direct recognition would lend further support to the development of therapeutic strategies aimed at inciting a CD4+ CTL capable of directly engaging malignant cells, irrespective of MHCII status, as well as potentiating the activity of other effector populations.

Refining protocols to selectively direct the desired immune response will require a better understanding of precisely which stimuli influence these fate decisions. Considering there was a significant difference in the immune response mounted when IL-12 was administered by different methods in our murine model, we have begun to examine what the source of this disparity might be. The difference may arise because LV12.2 cells are induced, by transduction, to alter their protein secretion profile from that of 70Z/3. Alternatively, the profile may remain the same but the proteins produced combine temporally and spatially with IL-12 to alter the response. Either way, identification of the complimentary proteins could be informative for the design of combination therapies or for determining which cytokines must be included in the
culture systems used to expand effector cells for ACT. Paraphrastically, there are a considerable number of reports detailing the anticancer immune responses initiated by IL-12-producing cells that differ in the particulars; but most of these studies do not investigate the contribution of other proteins produced. Caution should be exercised when using such studies to evaluate the immune-stimulating capacity of IL-12. If, rather, the differences are a result of alterations made to the amino acid sequence or an alternate glycosylation pattern, such structural alterations could be incorporated into the production of recombinant proteins or the design of transgenes in order to induce the effector population of choice. Further experiments will be needed to narrow down the central contributing factors and some of these experiments are described in the Future Directions section.
CHAPTER 6: Future Directions and Discussion
6.1 Future Directions

6.1.1 Determine the requirements for activation of the DC population.

Immunomodulation by IL-12 is highly dependent upon the production of supplementary cytokines. It has been observed that those patients who maintain elevated IFN-γ levels throughout the course of treatment are those that benefit from IL-12 therapy, and many in vitro studies have also established the importance of IFN-γ for IL-12 induced immunity. Though IL-12 has long been considered the cytokine responsible for initiating a primary T_H1 response, and is certainly key in shaping the class of response initiated, it has more recently been demonstrated that IL-12 cannot be the initiating signal because its receptor is not constitutively expressed on DCs but must be induced\textsuperscript{19}. A number of cytokines have been implicated in the induction of IL-12R expression, including IFN-γ\textsuperscript{23,188}, IL-2\textsuperscript{72}, IL-15 and IL-18\textsuperscript{189-191}. In our culture system we observed that IFN-γ is not sufficient alone to replace NKT cells but have yet to identify what other factors NKT cells are providing for the full activation of DCs. We have proposed a model in which we suggest that two more components are left to be discovered: one that is membrane-bound and mediates a contact-dependent interaction between NKTs and DCs, and the other factor is likely secreted. Based on the literature, a likely candidate for the membrane-bound factor is CD40 on the DC population, ligated by CD40-L (CD154) expressed by NKT cells\textsuperscript{128}. The secreted factor may be one of the above mentioned cytokines that play a role in IL-12R expression or may be a yet unidentified molecule. Our culture system is ideal for testing these possibilities as supernatants can be collected from transwell assays and tested using ELISAs specific for some of the proposed factors to see if their expression differs between those conditions that result in target cell clearance and those conditions that do not. Transwell assays can also be set-up that include blocking Abs against the individual factors proposed, and target
cell clearance used as a read-out of their importance. Alternatively, if none of the predicted candidates fulfill the requirements for full activation of the DC population, supernatants can be collected and examined by mass spectrometry to identify factors present only under clearance conditions. Supernatants from assay conditions that are most similar with respect to content but differ in the outcome (e.g. from wells that contain DCs, target cells and CD4\(^+\) cells in the lower chamber with either NKT cells alone or together with DCs in the upper chamber) can be used for subtractive comparison to reduce background noise from irrelevant factors. Therefore, various permutations of the \textit{in vitro} transwell assay can be used to identify those membrane-bound and secreted factors integral to the initiation of an effective response.

\textbf{6.1.2 Detect the mechanism of target cell recognition and cytotoxicity.}

We have identified a CD4\(^+\) CTL that is effective against a target that is negative for MHCII by staining and, although capable of expressing MHCII in response to chronic IFN-\(\gamma\) exposure, only a subset do so. It will be important to first resolve if the MHCII\(^+\) subset of 70Z/3 cells represent a distinct population or a stage in the response to IFN-\(\gamma\). The populations will be sorted by FACS and maintained separately to determine the durability of the phenotype: the MHCII\(^+\) population will be cultured without IFN-\(\gamma\) added to see if expression is lost over time; and the negative population will continue to be cultured with IFN-\(\gamma\) to see if they eventually begin to express MHCII and the positive proportion is re-established.

While cross-presentation of Ag complexed to MHCII on APCs can explain activation of a CD4\(^+\) CTL population, it is still not clear how these CTL subsequently recognize their target in a specific manner that leads to complete leukaemia cell clearance and establishes immune memory. The \textit{in vitro} culture system can be exploited for this purpose also. CD4\(^+\) effector cells
can be isolated from cultures that have effectively cleared target cells once, stained for CD4 expression and included in a re-challenge assay, together with green fluorescent protein (GFP)^+ target cells stained for MHCII. These cultures can then be examined by live cell, time-lapse microscopy to detect if these molecules are concentrated at the cell-cell interface. Recognition that is mediated by TCR:Ag:MHC binding is also characterized by formation of an immunological synapse that is highly organized and polarized at the point of contact between two cells. If CD4 and MHCII staining remains diffuse on the cell surface, however, this would suggest that recognition is taking place by some other means. As a positive control, CD4^+ T cells and Ag loaded DCs from Smarta mice will be equivalently stained and imaged, as this interaction is known to be MHCII-mediated and the conditions have been optimized (Lind & Ohashi, personal communication).

To clarify what the mechanism of cytotoxicity might be, we sought to resolve if the CD4^+ CTLs used a membrane-bound or secreted factor. LV12.2 target cells were cultured in supernatants, collected from assay wells at the point of detectable target clearance, without any significant effect; suggesting that cell-cell contact is required for target killing. The culture supernatants were also tested by ELISA for Granzyme B (GzmB), a cytolytic granule that is released into the synapse between effector and target cell. Granzyme B became detectable around day 6 in supernatants from co-culture samples and levels rose over time but were lower under re-challenge conditions, presumably because cells were washed during transfer and then tested only 4 days later (Figure 6.1). It remains to be determined, however, if GzmB is a significant mechanism of leukaemia cell killing in these assays because GzmB detection was not always consistent; albeit that ELISA detection is likely an underestimate as cytolytic granules are
delivered across the cell-cell synapse in membrane bound packages that can inhibit their detection. To complement these observations, we set-up cultures with concanamycin A (conA) which is used to inhibit the release of granules\textsuperscript{192}, but 70Z/3 cells are exceedingly sensitive to conA such that we were unable to use this method to elucidate the role of GzmB in cell killing. A role for Fas-L was also considered but assays were likewise inconclusive. Some studies have suggested that the Fas/Fas-L pathway is the predominant cytotoxic mechanism of murine CD4\textsuperscript{+} CTLs and that Fas-mediated killing can be MHC-independent once activated\textsuperscript{98}; therefore this pathway is of particular interest for further investigation. The microscopy experiments proposed above can simultaneously be used to identify the mechanism of cytotoxicity by staining for Fas-L expression. Samples can be also be prepared by cytospinning culture aliquots, followed by intracellular staining for cytotoxic granules such as GzmB.

![Graph of Granzyme B production in co-culture assays.](image)

**FIGURE 6.1 Granzyme B production in co-culture assays.**

Supernatants were sampled from those co-culture conditions that exhibited target cell clearance, over a time course, and tested for the presence of GzmB by ELISA. Input populations and non-clearance conditions were also tested as controls.
6.1.3 Identify which attributes of our cell-based therapy influence the response initiated.

To determine what aspect of the cell-mediated therapy model accounts for the unique response observed, an *in vivo* depletion assay can be employed. Mice will be depleted of either CD4⁺, CD8⁺ or both populations together while administering IL-12 derived from the supernatants of LV12.2 culture. These supernatants can be collected, concentrated, and injected whole or cIL-12 can be purified to test if a CD4⁺ effector population arises independent of the cellular vector. Three principle outcomes would be expected: 1) if both supernatant and cIL-12 engender a CD8⁺ cell-dependent response, we can deduce that CD4⁺ CTLs result from an attribute of the cellular delivery system, 2) if the supernatant injections result in a CD4⁺ effector population but purified cIL-12 behaves similarly to rIL-12, we can conclude that it is likely to be another product of LV12.2 cells that is responsible, 3) if cIL-12 injection gives rise to a CD4⁺ effector population, than it is likely that the protein structure and/or the post-translation modifications of the transgene are responsible for shaping the response. In the event of the second outcome, supernatants can then be interrogated by mass spectrometry to identify which protein(s) participate in the combinatorial effect. Deglycosylation assays will be conducted if structural and post-translational alterations appear to be the more likely cause. The two proteins, rIL-12 and cIL-12, will be digested using a panel of deglycosylation enzymes with unique cleavage specificities and migration shifts will be visualized using western blot analysis. This is of significance because many studies use recombinant proteins that are produced by insect cells and the results of these studies inform our understanding of the physiological activity of the human analogues. Furthermore, inducing a CD4⁺ CTL-mediated response is of therapeutic
interest; if protein modifications can be altered to preferentially induce one type of immune response over another, there would be clear advantage for the design of therapeutic interventions.

6.2 Discussion

While SOC protocols can prolong survival and improve quality of life, MRD or the existence of resistant CICs often results in disease relapse and the outgrowth of even more aggressive malignancies. Hence, there is an urgent need for the development of novel curative therapies. Ample evidence demonstrates that the immune system is capable of affecting specific and complete clearance of both primary and metastatic cancers while forming memory against disease relapse; however, the complex interplay between cancer cell, immune cell, and stromal cell in the tumour microenvironment can frustrate and subvert therapeutic attempts. Cytokines can be used to stimulate and shape a potent response; thus, achieving or restoring the appropriate cytokine balance is crucial. However, therapeutic activation of the immune system is encumbered with the same strict biological regulations in place to prevent an excessive endogenous response, and subverting this regulation leads to overwhelming toxicity. Accordingly, successful treatment is predicated on inducing sufficient immune stimulation with these safety concerns in mind.

Application of immunotherapeutic strategies to a clinical setting will require the consideration of a number of special challenges that are not always addressed by pre-clinical studies. These considerations include: the tolerogenic state of the patient, the affect of any previous treatment modalities, as well as safety precautions. In this work, our observations shine light on some of these core concerns.

Our work demonstrates that administration of syngeneic cancer cells expressing a stimulant, in this case IL-12, leads to localized responses as evidenced by the observation that
only a small proportion of therapeutic leukaemia cells are required as long as each produces a sufficient amount of IL-12. Cell-mediated approaches have the added benefit of presenting Ag together with the stimulus and this may be enough to change APC-T cell dynamics, on the micro scale of the synapse, such that non-responsiveness can be overcome or bypassed. This local interaction is then amplified to produce an effective and specific response that, at least in our model, appears to be regulated appropriately without leading to toxicity or the activation of antagonistic mechanisms. While the presence of cells producing lower concentrations of cytokine does not interfere, the corollary to this observation is that better selection methods are necessary to ensure that an adequate number of cells capable of providing an effective stimulus are included in the inoculum. Such selection is far more challenging for clinical grade preparations, especially when the selection trait is production of a secreted factor; but methods to address this are under development (Salerno & Paige, unpublished data). This marks an important advancement because it proposes a metric for gauging the curative potential of each preparation in advance: the number of cells each producing IL-12 above a certain threshold amount. Moreover, this phenomenon may not be exclusive to IL-12 and warrants further investigation in the context of other immunostimulatory agents.

In addition to tolerance, patients might also suffer from iatrogenic immune suppression as a result of prior treatment modalities such as irradiation and chemotherapy. However, it is recognized that such cytotoxic treatments may also potentiate the effect of immunotherapy by providing additional stimuli and by reducing the disease burden. Indeed, the treatment of MRD is likely to be where immunotherapy enjoys the most success. Our results are in agreement with
this paradigm as sub-optimal cell-mediated therapy conditions were improved by pre-treatment with chemotherapy, so long as the WBC compartment was given time to recover.

Safety is another issue of paramount importance. Irradiation was one of the original methods used to mitigate the risk of cell-based therapies because radiation causes cell cycle arrest and blocks proliferation without impeding the expression of transgenes. This method is not always appropriate, as was the case in our system, because some cell types instead differentiate or undergo apoptosis after irradiation and the activity of some gene products is therefore negatively impacted. Suicide genes offer an alternative approach for increasing the safety profile of cell-based cytokine therapies and is the method of choice for use in human applications. These two approaches, however, need not be mutually exclusive. A number of suicide genes have been developed for incorporation into the construct for integration; but the central principal remains the same. The gene codes for an enzyme that converts an inert prodrug into a toxic substance that kills only those cells that contain the transferred enzyme gene. For example, if the transduced cells need to be eradicated after administration into the patient, a non-toxic pro-drug, such as 5-FC (5-Fluorocytosine), is given to the patient and taken up into the cells where it has no effect unless the cell is a transduced cell that expresses the converting enzyme. In this case, 5-FC is converted to 5-FU (5-Fluorouracil), which is toxic and kills the cell.

Optimized strategies for cytokine therapy and successful application in all patients will benefit from understanding the activity of each cytokine in minutaie; how they behave in combination, in different tissues and how differential modifications might alter activity. The specific role of IL-12 in primary responses has been debated as of late and our in vitro system
can be easily manipulated to dissect the response to IL-12 signalling, identifying what is required for the desired response. Using the results from our transwell assays we proposed a testable model of what interactions are required between NKT cells and DCs for reciprocal activation downstream of IL-12, ultimately resulting in the licensing and education of a CD4+ CTL.

The development of a CD4+ CTL is of interest on two levels: there is clinical interest in this class of effector cell, particularly if activity is MHC-independent, because it can mediate direct and specific target cell killing as well as enhance and sustain the responses of other effector populations for a multi-pronged attack; and from a basic inquiry standpoint this population is of interest because it challenges our popular understanding of the immune systems fate determinants. If target cell recognition is MHCII-independent, then how is specific cytotoxicity achieved and memory established? Nevertheless, if recognition is MHCII-dependent, it is still curious that a cytotoxic response would be initiated by recognition of an Ag that is presented on a molecule which classically binds Ags from external sources. Once again, our systems will allow us to explore what attributes contribute to the development of a CD4+ T helper cell in one model and a CD4+ CTL in the other.
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