Immunotherapy and Immunomodulation for Haematological Malignancies

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Abstract

HA22 is an immunotoxin composed of an anti-CD22 variable fragment linked to a 38 kDa truncated protein derived from *Pseudomonas* exotoxin A. The mechanisms of cytotoxicity and resistance of HA22 against Acute Lymphoblastic Leukaemia (ALL) and Burkitt's lymphoma were studied. Using a bone marrow mesenchymal cell culture assay to support ALL cell viability, I? investigated the in vitro cytotoxicity of HA22 against ALL blasts from newly diagnosed and relapsed patients. There was interpatient variability in sensitivity to HA22. There was no significant difference in HA22 sensitivity between diagnosis and relapse samples but peripheral blood ALL blasts were more sensitive to HA22 than those from bone marrow. The mechanisms of resistance to HA22 were studied, using cell lines as a model. The number of CD22 sites/ cell and the rates of immunotoxin internalisation did not affect HA22 cytotoxicity. HA22 mutants with resistance to lysosomal degradation and enhanced targeting to the endoplasmic reticulum had improved cytotoxicity. The role of apoptosis pathways proteins in HA22-mediated cell death was studied. Their role is complex but raised levels of the anti-apoptotic pathway protein Bcl-2 were found in the most resistant NALM6 cell line. Penetration of HA22 into Burkitt's lymphoma masses was studied using a flow cytometric based method. HA22 rapidly penetrated into the lymphoma masses, however a barrier to further uptake is present which could not be overcome by the addition of adriamycin or taxol in the murine xenograft model.

The ability of Acute Myeloid Leukaemia (AML) blasts to create an immunosuppressive niche was investigated using a cell line model and primary patient samples. AML blasts suppress T cell proliferation through altered arginine metabolism, dependent on the enzymes arginase II and iNOS. Small molecule inhibitors to arginase and iNOS restored T cell proliferation in vitro. AML further enhances its immunosuppressive niche by transforming surrounding monocytes into an M2-immunosuppressive phenotype, in an arginase dependent manner. The immunomodulatory protein Serum Amyloid A (SAA) was secreted by AML blasts, and leads to AML chemotaxis, IL-1β production, and release of S100A9 protein. Finally, invariant Natural Killer T cells (iNKT) were shown to be cytotoxic to some AML blasts, in the presence of αGalactosylceramide, and thus able to restore T cell proliferation.

The results provide a strong rationale for the clinical testing of these novel immunotherapeutic and immunomodulatory strategies in patients with haematological malignancies.

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Abbreviations

7-AAD	7-amino-actinomycinD
αGalCer	α-galactosylceramide
ADP	Adenosine DiPhosphate
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
ATP	Adenosine TriPhosphate
ATRA	All-Trans Retinoic Acid
BCR	B Cell Receptor
CD	Cluster Differentiation
CDR	Complementarity Determining Region
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukaemia
CNS	Cranial Nervous System
COG	Children's Oncology Group, USA
CRP	C-Reactive Protein
EBV	Epstein Barr Virus
EF2	Elongation Factor 2
EFS	Event-Free Survival
ELISA	Enzyme-Linked Immunabsorbant Assay
FAB	French-American-British Classification
FLT3	Fms-related tyrosine kinase 3
GVHD	Graft versus Host Disease
HCL	Hairy Cell Leukaemia
HLA	Human Leukocyte Antigen
HSCT	Haematopoietic Stem Cell Transplant
IL-	Interleukin-
iNOS	inducible Nitric Oxide Synthase
ITD	Internal Tandem Duplication
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motifs
JHH	Johns Hopkins Hospital, Baltimore, USA
JMML	Juvenile Myelomonocytic Leukaemia
LMB	Laboratory of Molecular Biology, NIH, USA
L-NMMA	L-N^G-monomethyl Arginine
mAb	Monoclonal Antibody
MDSC	Myeloid-Derived Suppressor Cells
MHC	Major Histocompatability Complex
MLR	Mixed Lymphocyte Reaction
MRC	Medical Research Council, UK
NCI	National Cancer Institute, NIH, Bethesda, USA
NHL	Non-Hodgkin Lymphoma
NIH	National Institutes of Health, Bethesda, USA
NK	Natural Killer
NKT	Natural Killer T cells

iNKT	invariant Natural Killer T cells
NO	Nitric Oxide
NOHA	N^G-hydroxy-L-arginine
Nor-NOHA	N^w-hydroxyl-nor-L-arginine
OS	Overall Survival
PARP	Poly ADP Ribose Polymerase
PCR	Polymerase Chain Reaction
PE	Pseudomonas Exotoxin
Ph+ALL	Philadelphia chromosome positive Acute Lymphoblastic Leukaemia
POG	Pediatric Oncology Group, USA
ROS	Reactive Oxygen Species
SAA	Serum Amyloid A
SJCRH	St. Jude Children's Research Hospital, Memphis, USA
TCR	T Cell Receptor
TDT	Terminal Deoxynucleotidyl Transferase
Th2	T Helper Cells, Type II
TLR	Toll-Like Receptor
UK	United Kingdom
USA	United States of America
VEGF	Vascular Endothelial Growth Factor
VLS	Vascular Leak Syndrome
WHO	World Health Organisation

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In more ways than one completion of this thesis has been a journey. No travels are complete without the people along the way, who make the time memorable and rewarding.

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The Fellowship program encourages its Fellows to become clinician-scientists. Taking advantage of this unique chance, I decided to pursue my interest in the use of novel immunotherapies for acute leukaemias.

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On completion of my Fellowship I returned to the University of Oxford, and redirected my research interests into the potential of using iNKT cells and small molecule inhibitors to modulate a different acute leukaemia – AML. My thanks to Enzo Cerundolo, Director of the MRC Human Immunology Unit, for supporting me in pursuit of a novel research area in his lab and for encouraging me 'to fly high'. Deepest gratitude to Carmen DeSanto for introducing me to the field of MDSCs, and for your guidance, support and Italian catchphrases! Sarah Booth for Geordie good humour and answering my endless questions. To Issa 'Stan' Abu-Dayyeh, Ron Yip, Madeleine Hipp, Jean-Paul Jukes, Nick Platt and Rosie McEwen-Smith for all the encouragement in the pursuit of this DPhil.

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And so this research journey is almost over, from USA to UK, from the NIH to Oxford…. I hope the thesis will be the start of many more scientific endeavours in the future.

Introduction

1.1 Haematologic Malignancies

Leukaemias and lymphomas are a group of disorders characterised by the accumulation of malignant white cells in the bone marrow, lymph nodes and blood. Broadly, leukaemias can be classified as 'acute' or 'chronic', which are then further, subdivided into lymphoid or myeloid. The most common types of acute leukaemia are: Acute Lymphoblastic Leukaemia (ALL) and Acute Myeloid Leukaemia (AML). ALL represents 80% of all childhood leukaemia with a further 15% being AML (4% CML and <1% CLL). In adults, the distribution is markedly different with 36% of leukaemias being CLL, 32% AML, 18% CML, and 14% ALL[1]. Lymphomas broadly can be classified as Hodgkin or Non-Hodgkin lymphomas (NHL), and may then be further clarified using a variety of systems based on pathology, immunophenotype and genetic abnormalities.

The aetiology of leukaemia is a subject of significant study and is likely to be multi-factorial. Hereditary disorders that predispose to leukaemia highlight the importance of genetic factors. Children with Down syndrome are approximately 15 times more likely to develop leukaemia compared to children without Down syndrome.[2] Other genetic disorders such as Fanconi anaemia,[3] Bloom syndrome,[4] and Neurofibromatosis Type I[5] are also reported to have an increased risk of developing leukaemia. Siblings of children with ALL have an approximately two-fold risk of developing ALL compared to unrelated children.[6] The concordance of acute leukaemia in monozygotic twins is approximately 25%.[7] Studies of Guthrie cards have shown that pre-leukaemic clones are present in utero, and that following a secondary event, may proliferate to form overt

leukaemia.[8,9]

In addition to genetics, environmental exposure, carcinogens, prior chemotherapy/ radiotherapy, and infections may contribute to the development of leukaemia. The increased incidence of leukaemia in adult survivors of the Hiroshima atomic bomb is well established and a dose-response relationship was noted.[10] There was, however, no increase in the incidence of leukaemia in children exposed to radiation from the atomic bomb in utero.[11] In contrast the risk of childhood leukaemia is increased significantly following exposure to diagnostic radiation in the first trimester.[12] Chemical exposures, such as to benzene or alkylator agents are associated with increased risk of acute non-lymphoblastic leukaemias and are a cause of secondary leukaemias in cancer survivors.[13,14]

Viral infections have long been suggested as a cause for leukaemia and lymphoma development.[15] Epstein-Barr virus (EBV) is associated with endemic Burkitt lymphoma and human lymphotropic viruses I and II are associated with some cases of adult T-cell and hairy cell leukaemia.[16,17]

The role of immunodeficiencies in the development of haematologic malignancies is unclear. Children with congenital immunodeficiencies such as Wiskott-Aldrich or ataxia-telangiectasias have increased risks of developing leukaemias.[18] However, the role of more subtle changes in immunosurveillance has been difficult to characterise in patients without the hereditary disorders. The role of the immune system in destroying subclinical cancer cells is inferred from evidence that patients on chronic

immunosuppressive treatments have an increased chance of developing leukaemia or lymphomas.[19,20]

In this thesis novel therapies for paediatric ALL, AML, and Burkitt lymphoma are investigated. The next section will provide an overview of the current state of treatment for each of these malignancies to highlight how novel therapies are required to reduce morbidity and mortality. A more detailed overview of the aetiology and diagnostic classification of these diseases is outside the scope of this thesis introduction.

1.2.1 Acute Lymphoblastic Leukaemia

1.2.1.1 Background

ALL is the most common malignancy of childhood and accounts for over 80)% of all cases of childhood leukaemia. A study of childhood leukaemia and lymphomas in the UK reports an age-standardised incidence rate of 26.9 cases per million person-years.[21] The peak incidence occurs between 2 and 5 years of age before decreasing in older children. Previously, ALL classification was based on morphology using the French-American-British (FAB) classification.[22] Although the FAB classification is still used, correlation between morphological and biological or prognostic features is inaccurate. As a result the World Health Organisation proposed that the FAB classification be superseded by the use of immunophenotypic and cytogenetic markers[23]. The classification incorporates clinical evidence with more advanced laboratory diagnostic

techniques in order to stratify ALL into subtypes that can inform treatment decisions.

1.2.1.2 Evolution of treatment regimens for ALL

Children with ALL have seen one of the most dramatic improvements in outcome compared to all cancers, over the last 50 years. A succession of national and international trials has led to stepwise changes in therapy. In the 1960's it was recognised that a small population of patients diagnosed with ALL were able to enter remission with steroids therapy alone.[24] Subsequent studies identified that combining prednisone therapy with other cytotoxic drugs, such as vincristine, asparaginase and cytosine arabinoside, improved the chance of inducing a remisison.[25, 26,27] Building on the early data, the importance of multi-drug chemotherapy approach to treat ALL was investigated at St. Jude's Hospital, USA using a "total therapy" protocol combining prednisone, cyclophosphamide, vincristine, methotrexate and mercaptoputine.[28] This seminal study found that at 5 years 8/37 patients treated were alive, a significant improvement on the almost complete mortality of ALL up until this date. Importantly, the therapy backbone of this trial still forms the basis for treatment used today. Current regimens commence ALL treatment with remission 'Induction', usually composed of the combination of steroid, asparaginase and vincristine with the addition of daunorubicin for higher risk patients.[29]

Although the St. Judge study significantly improved survival a large proportion of patients would relapse, particularly with CNS localised disease.[30,31] The potential of CNS-localised disease to cause relapse was addressed in a second St Jude's trial which demonstrated that cranio-spinal irradiation, significantly improved event-free survival.[32] The need for cranio-spinal prophylaxis was confirmed in the UK by the Medical Research Council UKALL I trial, in which only 1/75 patients treated with CNS

prophylaxis relapsed compared to 26/80 in the untreated group, within the first 18months of the trial.[33] Subsequent findings have confirmed the importance of CNS directed therapy. The use of chemotherapy to treat CNS-localised ALL was addressed in the UKALL III trial. Patients who achieved a haematological remission by chemotherapy, would randomised to either cranio-spinal irradiation plus intrathecal methotrexate or no CNS-directed treatment.[34] Only 1/75 patients who received CNS-directed treatment relapsed with CNS recurrence, compared to 26/80 patients in the group who did not receive CNS prophylaxis. Refinement of CNS directed therapy has continued to takeplace over the last 30 years due to a greater understanding of the late effects of treatment.[35,36] The desire to avoid neurological and cognitive deficits has led to the replacement of cranio-spinal irradiation by intrathecal methotrexate for the majority of patients newly diagnosed with ALL.[37,38,39] The incidence of CNS relapse using current intrathecal chemotherapy based regimens is less that 5% for low-risk patients and 10% across all children with ALL.[40]

Concurrent with the development of CNS-directed therapy, the role of an extended 'maintenance' treatment to target residual, rarely dividing ALL blasts was recognised. The addition of a 'maintenance' treatment phase, centred on orally administered methotrexate and mercaptopurine, has led to a significant improvement in remission rates. Importantly UKALL II identified that changes in timing of mercaptopurine and methotrexate dosing in maintenance, would significantly alter treatment-related toxicity with more prolonged neutropenia in the UKALL I regimen and a more prolonged lymphopenia in UKALL II.[41] Serial trials have led to refinements in the maintenance phase. In a study of 496 children, UKALL VIII randomised patients to receive maintenance chemotherapy with mercaptopurine or methotrexate for 2 or 3 years either

continuously, semi-continuously or as 5-day pulses every 3 weeks. Patients also received prednisone and vincristine during the maintenance phase. The study found that intermittent chemotherapy dosing in maintenance was less immunosuppressive, however was also less effective than continuous mercaptopurine and methotrexate in preventing bone marrow relapse (p=0.002).[42] Current treatment protocols for childhood ALL contain a maintenance phase using oral mercaptopurine and methotrexate, with dexamethasone and vincristine pulses, for approximately 2 years for girls and 3 years for boys.

In addition to induction, maintenance and cranio-spinal prophylaxis a major advance in treatment outcome occurred through the introduction of a delayed intensification phase, post induction.[43,44] This treatment phase has been widely incorporated into international treatment protocols.[39] In the UK the benefit of this post-induction intensification therapy was addressed in UKALL X (1 vs. 2 delayed intensification courses) and UKALL XI (2 vs. 3 delayed intensification courses).[45,46] These 2 studies identified a 20% improvement in 5 year EFS following 3 delayed intensification courses. Whether intensification can be optimised to avoid the long-term toxicities of increased chemotherapy for low risk patients, and in turn made more effective for those patients at high-risk of relapse remains a challenge.[47]

Developments in ALL treatment protocols continue to be made. In Induction prednisone has been replaced by dexamethasone due to the lower risk of relapse brought about through improved marrow and CNS remissions.[48,49,50] Pegylated-

asparaginase has also been incorporated.[51] Etoposide has been removed from ALL treatment regimens due to the association of secondary AML[52] and anthracycline exposure has been reduced in order to decrease the associated risk of cardiac toxicity.

Treatment for ALL has also benefited from increased understanding of the genetic basis for ALL and detection methods of residual blasts, leading to improvements in risk stratification for patients and tailoring of therapy according to the risk of relapse. Early treatment regimens identified prognostic factors to include race, sex and white cell count at presentation.[53,54] Due to recent advances in treatment these factors now have significantly less impact on outcome.[40,55]

The concept of risk stratification, leading to modifications in treatment and improved outcome has been most obviously highlighted through the detection of the BCR-ABL mutation in the blasts of some patients with ALL. Historically this mutation carries an increased risk of relapse, despite allogenic HSCT. However the introduction of the targeted therapy imatinib has led to a 3 year EFS of 80%, a significant improvement compared to patients treated on earlier regimens.[56] Furthermore the use of imatinib has delayed the use of HSCT until after Ph+ALL patients relapse, potentially decreasing the number of patients exposed to the side-effects of transplant. The use of flow cytometry to detect response to induction therapy has also allowed patients to be stratified according to their early response to therapy. This detection of 'minimal residual disease' is now an integral part of modern chemotherapy regimens for paediatric ALL.[57,58,59]

5-year event free survival is now approximately 80% for children with ALL treated on

modern chemotherapy protocols - a remarkable improvement on outcomes compared to only 40 years previously. However the improvement has not been uniform. Infants with ALL, have a very poor outcome despite high dose chemotherapy, with EFS rates of approximately 30%.[59,60] Young adults continue to have an inferior outcome compared to younger adolescents and children with ALL. The reasons for this may be a combination of poor-prognosis cytogenetics,[61] increased treatment related toxicity,[62] and differences in treatment regimens used in adults and children.[63] Failure of remission induction, the presence of more than 1% MRD at the end of induction, t(17;19)(q22;p13.3) and hypodiploidy all mark other subgroups of patients with very poor outcomes despite treatment.[64,65]

1.2.2 Acute Myeloid Leukemia

1.2.2.1 Background

Acute Myeloid Leukaemia (AML) is the second most common leukaemia of childhood but the most frequent leukaemia in adults. The peak incidence of AML in the paediatric setting occurs in the neonatal period.[66] In adults the frequency of AML increases above the age of 55 years old. AML is equally common in males and females. Compared to ALL, AML is heterogeneous in origin, with malignant cells arising from myeloid, monocyte, erythroid, and megakaryocyte precursors. A number of classification systems exist however more recently the WHO classification is most widely used.[23]

1.2.2.2 Evolution of treatment regimens for AML

Treatment for AML is unlike that for ALL but nevertheless treatment centres on induction, and consolidation with CNS prophylaxis. Early treatment regimens were similar to those for ALL but long-term survival was very poor.[67] The lack of CNS-

directed therapy contributed to the dismal.[68,69] A significant departure from ALL based therapy took place with the introduction of cytarabine and daunorubicin based therapy ("7&3"), leading to remission induction in over 75% of patients, but still a unsatisfactory 5 year survival of only 30%.[70] Cranio-spinal radiation was introduced in the same study to target CNS disease.

In contrast to ALL, chemotherapy in AML focused on dose intensification. Initial studies attempted to intensify the 7+3 regimen through the addition of etoposide and dexamethasone, however no improvement in overall survival was seen.[71] The dose of cytarabine was significantly increased in a series of Paediatric Oncology Group (POG) trials in the USA. The addition of high dose cytarabine improved EFS from 29% to 24% (POG 8498), and addition of a second high dose cytarabine cycle improved 3 years EFS from 34.5% to 40.4%.[72,73] Further improvements in overall survival were gained through the addition of etoposide into induction and post-remission cycles.[74] Subsequent trials in the USA and Europe went on to investigate the number and intensity of induction chemotherapy cycles required, with both approaches increasing overall survival rates to over 50%.[75,76,77]

In addition to dose intensification, prolonging exposure of chemotherapy has also been trialled. In the UK, MRC trials AML 9 and AML 10 tested the length of time cytarabine can be given for in combination with daunorubicin (DAT 3+10 and DAT 3+8 regimens). The longer 10 days of cytarabine were found to be too toxic however the addition of etoposide to cytarabine and daunorubicin (ADE 3+10+5) regimen led to an overall survival of 56%. Today 'ADE' remains the backbone of ongoing trials and efforts to reduce the acute and long-term toxicities off treatment are being incorporated into treatment regimens.[78,79,80]

In conjunction with the use of induction regimens, the role of post-induction consolidation therapy has been evaluated. Initial attempts at maintenance chemotherapy resulted in a poor event-free survival of only 20% at 2 years.[71] The MRC AML 9 trial also confirmed that maintenance alone did not improve survival.[81] Instead intensification cycles containing high-dose cytarabine were found to improved 5 year EFS.[82,83] An alternative strategy to post remission intensification developed through trials investigating the roles of autologous or allogeneic haematopoietic stem cell transplant (HSCT) in the treatment of AML.[70] The role of haematopoietic transplant in AML is controversial with conflicting evidence as to the advantages of different strategies. In summary transplant studies performed in the USA have demonstrated a benefit of allogeneic (HLA-matched related donor) stem cell transplants compared to autologous HSCT or chemotherapy.[76,84] European studies, however have not been able to demonstrate the same improvement in overall survivial.[85,77] Importantly, any improvements in survival seen were tempered by the toxicities of allogeneic HSCT, notably acute and chronic GVHD.[86] The role of HSCT in AML requires further study to determine which patients will benefit the most, and how to limit the serious toxicities of treatment.

Risk stratification in order to minimise treatment toxicities or improves survival rates has been employed in AML to stratify the treatment regimen required by patients. Patients with cytogenetics, such as inv(16q) or t(8;21) have a good prognosis using chemotherapy alone, with a 5 year overall survival of around 65%.[87,88] In particular patients with t(15;17) mutation (M3 subtype) have a much improved prognosis since the introduction of all-trans retinoic acid targeted therapy[89,90] or arsenic trioxide

therapy. These novel therapies have resulted in a significant reduction in cytotoxic chemotherapy used in this patient group.[91,92] However patients with monosomy 5 or 7, or residual disease after induction are deemed at a high risk of relapse leading to the use of haematopoietic stem cell transplant as standard of care in such patients.[93,94]

Despite the advances described above, significant numbers of patients with AML relapse. Of the patients with favourable cytogenetics such as t(8;21) and inv(16) approximately 30% will have addition c-KIT mutations increase the risk of relapse.[95] In addition 15% of patients with Acute Promyelocytic Leukaemia will relapse despite all-trans retinoic acid (ATRA) therapy and some patients continue to develop life-threatening infections secondary to the myelosuppression.[96,97] Patients with unfavourable cytogenetics or those who do not enter remission do poorly. The most recent UK AML trial (AML17) aims to improve outcomes through a comparison of 2 induction chemotherapy regimens, the number of consolidation treatment courses required and the use of role of novel agents such as the FLT3 inhibitor CEP-701 and mTOR inhibitors.

1.2.3 Burkitt Lymphoma

1.2.3.1 Background

Burkitt lymphoma is a member of the Non-Hodgkin lymphoma (NHL) 'family', and makes up 40% of the NHLs of childhood. Burkitt lymphoma usually occurs in children between 5 and 15 years old. The WHO classification identifies 3 clinical variants – Immunodeficiency-associated Burkitt lymphoma, Sporadic Burkitt lymphoma, and Endemic Burkitt lymphoma.[98]

Burkitt lymphomas are characterised by a diffusely infiltrative pattern of cells with basophilic round nuclei, and a basophilic cytoplasm containing vacuoles (corresponds to FAB L3 above). Interspersed resident macrophages give the classical 'starry sky' appearance.[99] Burkitt lymphomas express many of the same CD antigens as in ALL, such as CD20, CD22, and CD19, however TdT is not expressed. The majority of Burkitt lymphoma are characterised by the t(8;14) translocation which fuses the c-myc gene to the immunoglobulin heavy chain gene.[100] However in approximately 10% of Burkitt lymphoma no evidence of chromosomal translocations involving MYC are detected.[101,102]

1.2.3.2 Evolution of treatment regimens for Burkitt's Lymphoma

Treatment for Burkitt lymphoma in developed countries has also been shaped through sequential multi-national trials.[103,104] Prognosis is predominantly determined by disease staging however additional features may have an impact on prognosis. Cytogenetic abnormalities such as 13q deletions and evaluation for minimal residual disease may be important.[105,106,107] The most recent European trial FABLMB 96 has shaped the current treatment guidelines, with the ongoing development of treatment stratified according to prognostic grouping of patients. Children with localised disease that has been completely surgically resected (Group A) were treated with 2 cycles of cyclophosphamide, vincristine, prednisolone and doxorubicin (COPAD). Overall survival for this group is excellent at 99.2%, with minimal toxicity of treatment reported to date.[108] In the previous LMB89 study 'intermediate-risk patients' had an excellent 5 year OS at 94%, however this was associated with significant acute and long-term toxicity.[109] As a result, in FABLMB96 corresponding Group B patients with

unresectable Stage I and II disease or stage III disease patients were randomised between 4 treatment arms with differences in the cyclophosphamide dosing and maintenance chemotherapy. As there was no statistical difference in outcome between arms, current treatment for this group is an initial cycle of cyclophosphamide, vincristine and prednisolone (COP) followed by 2 cycles of COPADM and 2 cycles of cytarabine and methotrexate (CYM). Four year overall survival continues to be excellent at 94% however the acute and predicted long-term toxicities were significantly lessened compared to the previous trial.[110] The aim to reduce toxicity whilst preserving outcome is also apparent for high-risk groups of patients. These patients have also benefited from an improved overall survival in previous trials of intensive chemotherapy, however significant morbidities in the acute and longer-term are apparent.[104,111] In the most recent treatment regimen children with CNS or bone marrow involvement (Group C) are given COP, followed by COPADMx2 and then 2 courses of cytarabine with etoposide (CYVE) followed by 4 courses of maintenance therapy. Overall survival for this group of patients is 81% at 4 years.

Although excellent overall survival rates are apparent compared to many high stage paediatric solid tumours, a significant number of Burkitt patients are still dying from disease . In addition patients with progressive or recurrent Burkitt lymphoma have an extremely poor overall survival of 16% at 4 years [112] New therapeutic approaches are required to reduce the morbidity and mortality rates for such patients. The role of rituximab in high-risk and relapsed Burkitt lymphoma is currently being evaluated in the ANHL01P1 trial and adult patient cohorts [113,114] Furthermore the treatment of adults with Burkitt lymphoma remains a significant treatment challenge.[115]

1.3 Targeted therapy for haematological malignancies

The overview above provides an overview of the current state of treatment for paediatric ALL, AML and Burkitt lymphoma. It is clear that over the last 50 years significant improvements in the outcomes for patients with haematological malignancies have been achieved. However, progress has not been universal. In paediatric ALL 5 year overall survival exceeds 90% in patients with low risk disease but it is only 40% in patients determined to be very-high risk.[116,117] For paediatric AML, overall survival is approximately 60% at 5 years with adult patients having a significantly worse outcome.[118,119] Finally in Burkitt lymphoma, overall survival is approximately 90% at 5 years for children.[111,110] However, patients who relapse tend to occur early in therapy with drug resistance being a major obstacle to a successful outcome. Patients who relapse with acute leukaemias or lymphomas often require intensified chemotherapy using myeloablative approaches followed by allogeneic haematopoietic stem cell rescue. Other than treatment failure, acute and long-term toxicities of treatment are significant. 50% of survivors of paediatric ALL will have one or more chronic medical condition secondary to treatment.[120] Even the use of previously-deemed low risk drugs such as dexamethasone, are now recognised as having serious toxicities including increasing the incidence of life-threatening infections, avascular necrosis and neurocognitive sequelae.[121,122] As a result new treatment approaches are required which can improve outcomes whilst minimising toxicities to the patient's healthy tissues.

In the next section 3 types of targeted therapy investigated in this thesis, and applicable to patients with haematological malignancies, will be discussed:

1) **The use of immunotoxins against cell surface antigens to target Acute Lymphoblastic Leukaemia**
2) **The use of small molecule inhibitors to target key enzymes in Acute Myeloid Leukaemia**
3) **The use of invariant Natural Killer T cells to target Acute Myeloid Leukaemia**

1.3.1 Cell surface antigens as therapeutic targets

Cells express a complex pattern of cell surface molecules which are dependent on the differentiation and activation status of the cell. These cell surface antigens are classified according to CD (cluster of differentiation) nomenclature once two specific monoclonal antibodies are shown to bind to the same molecule.[123] To date over 320 CD antigens have been identified. Biologically the roles of each CD antigen are varied and include cell adhesion, second messenger pathway modulation, differentiation, and cell death. In many cases the function of the antigen is unknown.

CD antigens have allowed the identification of cell subtypes through the use of techniques such as immunohistochemistry and flow cytometry. In some cases an individual CD antigen may be unique or more highly expressed on a particular cell type posing an attractive target for cell specific therapies. Since the development of monoclonal antibodies (mAbs), cell surface antigens have become an attractive target

for cancer therapy.[124] Furthermore haematological malignancies provide excellent candidates for monoclonal antibody therapy as the cells are easily accessible in the haematopoietic compartment to the antibodies and the cells express unique antigens dependant on their stage of differentiation.

Unconjugated mAbs can kill target cells through direct or indirect mechanisms. Direct-antibody killing may occur by inducing apoptosis upon antibody binding[125] or by prevention of growth or activation.[126] Indirect mechanisms of mAb killing require other cellular or humoral components such as antibody-dependent cellular cytotoxicity brought about through T-cells and NK cells or complement-mediated cytotoxicity.[127] Antibodies may also help target T cells to the tumour cells[128] or prevent inhibitory signalling on cytotoxic T cells.[129] Over the last 20 years, therapeutic unconjugated monoclonal antibodies which target cell antigens expressed on cancer cells have become widely used and include Rituximab (anti-CD20)[127] for the treatment of non-Hodgkin's lymphoma in adults, Alemtuzumab (anti-CD52)[130] for the treatment of Chronic Lymphocytic Leukaemia and trastuzumab (anti-HER2) for the treatment of breast cancer.[131] Conjugates of monoclonal antibodies with chemotherapy drugs or radionuclides have also been developed with the aim of enhancing cytotoxicity through coupling of an antibody with an effective killing moiety. The anti-CD33 mAb conjugated to the antibiotic calicheamicin (Gemtuzumab ozogamicin) has efficacy in relapsed and refractory paediatric AML and is currently in Phase III trials.[132] Monoclonal antibodies coupled to radionueclides (radioimmunotherapy;RIT) have also been tested in non-Hodgkin lymphoma.[133]

1.3.2 CD22

CD22 is a type I membrane protein member of the sialic-acid binding immunoglobulin like lectin (Siglec) family of adhesion molecules.[134,135] CD22 is expressed on B lineage cells from the pre-B stage through to mature B cells, but is down-regulated on plasma cells.[136]

The physiological ligand of CD22 is unknown. CD22 binds to α2,6 sialic acid residues which are found in cell surface glycoproteins present on a wide variety of cell subtypes as well as in plasma proteins such as haptoglobin.[137] The finding that CD22 is unable to bind sialic acid containing probes unless pre-treated with sialidase also suggests that CD22 may undergo an activation change before it can bind to its target ligand.[138] Immunoprecipitation experiments have identified B cell sIgM and CD45 as possible binding partners, although these results have not been confirmed in other studies.[139]

The result of ligand binding to CD22 remains controversial. In vitro, CD22 cross-linking by anti-CD22 antibodies resulted in proliferation of human tonsillar B cells.[140] However, studies on CD22-deficient mice have demonstrated that CD22 functions predominantly as an inhibitory receptor in vivo. The absence of CD22 led to elevated levels of intracellular calcium in response to stimulation via the B cell receptor (BCR), resulting in reduced B cell proliferation and increased apoptosis.[141] CD22 can inhibit the BCR signalling cascade at several points. Following phosphorylation of cytoplasmic CD22 immunoreceptor tyrosine-based inhibitory motifs (ITIM) by Lyn, CD22 recruits SHP1 which dephosphorylates and inactivates vav-1, BLNK and CD19, resulting in

decreased PI3K activity and reduced Ca^{2+} release from the endoplasmic reticulum.[142, 143] In addition, CD22 deficient mice have reduced numbers of splenic marginal zone B cells and decreased expression of sIgM on peripheral blood B cells, suggesting that CD22 may have a role in B cell maturation.

1.4 Immunotoxins

1.4.1 Immunotoxins – an overview

Immunotoxins are engineered recombinant proteins with a cell antigen binding domain derived from a monoclonal antibody (Mab), and a toxin moiety that induces cell death. A variety of toxins have been conjugated to monoclonal antibodies, including ricin, calicheamicin,[144] diphtheria toxin,[145] and pseudomonas exotoxin.[146] Toxins pose an attractive method of killing cancer cells due because of their high potency.

Of relevance to this thesis immunotoxins coupled to a *Pseudomonas* aeruginosa exotoxin A derivative have been developed and entered clinical trials. Exotoxin A contains three functional domains (Figure 1.1). Domain Ia, located at the N terminus, is the cell binding domain and in its native form binds to the α–2-macroglobulin receptor. Domain Ib has no known function. Domain II allows the translocation of toxin across the endoplasmic reticulum membrane. Domain III, located at the C terminus, catalyses the ADP-ribosylation of elongation factor 2 (EF2) leading to inhibition of protein synthesis and cell death.[146]

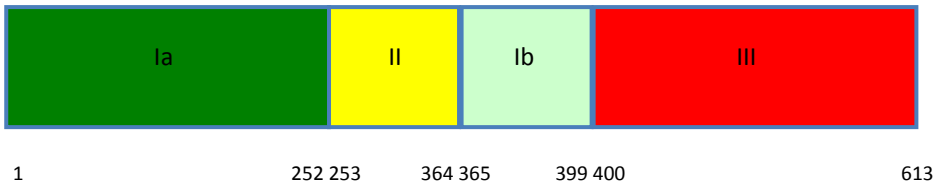


Figure 1.1 Schematic of *Pseudomonas* Exotoxin A. Domain Ia is the physiological binds to the Low-density Lipoprotein-like Receptor. Domain II translocates the toxin to the cytosol after internalisation. Domain III is an ADP ribosylating enzyme that inactivates cytosolic EF-2. Domain Ib has no known function. PE38 is a 38kDa truncated protein composed of amino acids 253-364 and 381-613, used in immunotoxins.

First-generation immunotoxins were produced by chemically connecting the native toxin to antibodies via disulfide bonds.[147] These immunotoxins were limited by poor stability and specificity. Building on this early work with increased understanding of the structure and function of toxin domains, second generation immunotoxins were formed by chemically conjugating truncated toxin derivitives to antibodies.[148] The truncated toxins had the natural cell-binding domains removed so that they could no longer bind to normal cells. As a consequence, it was possible to give increased concentrations of immunotoxin to experimental animals and humans.

Currently, immunotoxins are designed through rational protein engineering and produced using recombinant DNA methodology in E.coli. The result are molecules in which the natural cell binding domain of the toxin is replaced by the Fv portion of an antibody, whilst the translocation and cell-killing domains remain. Large amounts of immunotoxin can be made using this recombinant process.[149] Although the immunotoxins are produced using recombinant technology, toxins are foreign proteins to the human immune system. Patients with normal immune systems, including patients

with solid tumours, can develop neutralizing antibodies which bind circulating immunotoxin and reduce its activity or increase immunotoxin excretion.[150] Patients who are immunosuppressed, either because of the direct effect of leukaemia or lymphoma on the bone marrow or because of chemotherapy, rarely form neutralizing antibodies, thus allowing these patients to receive multiple cycles of immunotoxin therapy.[151]

Immunotoxin toxicity can be non-specific or targeted. Non-specific toxicity includes Vascular Leak Syndrome (VLS) which occurs due to high concentrations of immunotoxin damaging endothelial cells leading to fluid leak from capillary, oedema and hypoalbuminaemia.[152] Specific toxicity occurs when the same target antigen is expressed on normal tissues as well as the cancer cells. The toxicity is more commonly an issue with the treatment of solid tumours in which target antigen may be expressed on normal organs. In haematological malignancies, however, normal B and T cells can be regenerated from haematopoietic stem cells allowing recovery from toxicity.

Immunotoxins have successfully entered Phase I, II and III clinical trials in both haematological malignancies and solid tumours. Antigens and diseases targeted to date include: IL-2 receptor in Cutaneous T cell lymphoma,[153] CD25 in chemotherapy refractory leukaemias and lymphomas,[154] and mesothelin in mesothelioma, ovarian cancer and pancreatic cancer.[150]

1.4.2 Anti-CD22 Immunotoxins

Anti-CD22 recombinant immunotoxins have been developed in the Pastan Laboratory of Molecular Biology, at the National Cancer Institute, for patients with CD22+ B lymphoid malignancies.[150] CD22 is an excellent target for an immunotoxin, because expression is conserved to B lymphoid cells and the CD22 antigen-immunotoxin complex is rapidly internalized by receptor-mediated endocytosis. In the endosomes the Fv fragment is cleaved from the PE38 toxin.[155] The toxin traffics through the endocytic pathway to the endoplasmic reticulum and is translocated across the ER membrane into the cytosol. Once in the cytosol the toxin inhibits protein synthesis through ADP-ribosylation of elongation factor 2 leading to cell death.

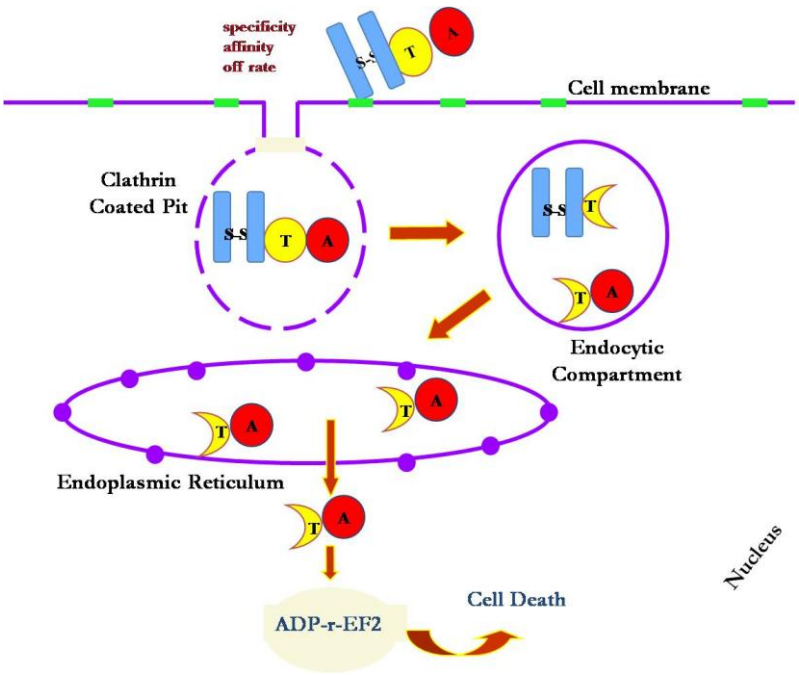


Figure 1.2 Diagram of immunotoxin cytotoxicity. Binding of anti-CD22 immunotoxins to the CD22 antigen results in rapid internalization, lysosomal cleavage, transport to the endoplasmic reticulum and subsequent translocation to the cytosol. Cytosolic EF-2 undergoes ADP-ribosylation resulting in inhibition of protein synthesis.

The first generation anti-CD22 immunotoxin 'BL22' consist of the disulfide-linked Fv portion of the Mab RFB4 fused to PE38. BL22 was found to be cytotoxic to CD22+ Burkitt's lymphoma cell lines and caused complete regression of Burkitt's lymphoma xenografts in irradiated nude mice.[156,157] In a Phase I trial, 46 patients with drug-resistant B cell malignancies (Hairy Cell leukaemia, Chronic Lymphocytic Leukaemia, and Non-Hodgkin's B cell lymphomas) were treated with BL22. In the 31 patients with Hairy cell leukaemia (HCL), Complete Remissions (CR) were achieved in 61% of patients and Partial Remissions (PRs) in 19%. No dose limiting toxicities occurred.[158]

In a paediatric Phase I trial of BL22 in patients with drug-resistant B-lineage acute lymphoblastic leukaemia and non-Hodgkin lymphoma patients were treated with escalating doses and schedules ranging from 10-40ug/kg every other day for three or six doses repeated every 21 or 28 days. The safety profile was acceptable; most adverse events were Grade I and 2 and were rapidly reversible. No maximum tolerated dose could be defined. Transient clinical activity was seen in most subjects, although there were no responses.[159]

The success of BL22 in inducing a high complete remission rate in patients with Hairy Cell Leukaemia confirmed the concept that immunotoxins can produce clinical benefit to patients with drug-resistant malignancies. As a result the Pastan lab aimed to improve the immunotoxin by increasing its affinity and activity. The modifications should improve the efficacy of the immunotoxin against malignancies which expressed

relatively fewer CD22 sites/ cell such as Chronic Lymphocytic Leukaemia (CLL) and ALL. RGYW hotspots (where R=A or G, Y = C or T, and W = T or A nucleotides) are DNA sequences that are frequently mutated during the in vivo affinity maturation of an antibody. Through the use of PCR and small phage-display libraries, random mutations were engineered that resulted in mutations with an increased activity. 'HA22', is a second generation, higher affinity anti-CD22 immunotoxin that was engineered by altering 3 contiguous amino acid resides in the heavy chain CDR3 of BL22.

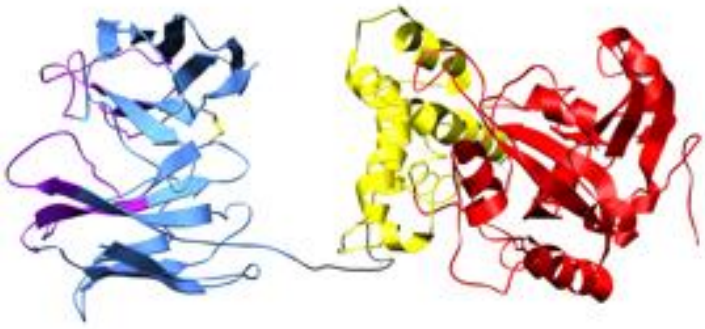


Figure 1.3 HA22 Recombinant Immunotoxin showing the V_L and V_H chains linked by an engineered disulphide bond. The Fv fragment is linked to the PE38 toxin moiety via a short linker peptide. Domain II of PE38 (yellow) enables translocation of the toxin into the cytosol. Domain III of PE38 (red) contains the ADP ribosylating enzyme that inactivates elongation factor 2 in the cytosol.

As a consequence of this modification, HA22 has about a 15-fold increased binding affinity for CD22 compared to BL22.[160] Pharmacokinetic studies of HA22 were performed in mice, rats and cynomolgus monkeys and toxicology studies showed comparable findings to BL22,[161] HA22 is currently undergoing a multi-centre clinical trial in paediatric ALL (*ClinicalTrials.gov number: NCT00659425; http://clinicaltrials.gov/ct2/show/NCT00659425)* and represents new active agent with reduced non-specific toxic therapy.

Studies of other CD22 targeted therapies in paediatric ALL have recently been reported. Epratuzumab, a humanized monoclonal antibody against CD22, has been studied in children with relapsed ALL.[162] Its activity as a single agent in that setting appears to be limited. A Phase I study of a mixture of ricin-based immunotoxins against CD22 and I CD19 (Combotox) was recently conducted for paediatric patients with ALL. In that trial, clinical activity was observed, although this agent was associated with severe adverse events and a high incidence of immunogenicity.[163] Other anti-CD22 targeted agents are in development. For example, a calicheamicin immunoconjugate CMC-544 (Inotuzumab ozogamicin) has been shown to be active in pre-clinical models of ALL and in clinical trials in adults with non-Hodgkin's lymphoma.[164]

1.5 Tumour enzymes as therapeutic targets

Some tumours can be characterised by gene translocations that result in new and tumour-specific proteins. As well as diagnostics, tumour proteins may provide a target for rationally-designed therapy. The first and best known cytogenetic marker is the Philadelphia chromosome, a reciprocal translocation t(9;22)(q34;q11).[165] The result of this translocation is two hybrid genes: bcr/abl on 22q- and abl/bcr on 9q+. In CML bcr/abl plays a major role and encodes a 210-kd tumour-specific protein with unique properties.[166] $P210^{BCR/ABL}$ is a cytoplasmic protein with constitutive tyrosine kinase activity. The action of p210 on other cytoplasmic proteins, including members of the Ras pathway, leads to uncontrolled proliferation of immature myeloid cells. P210 provided the target of the first rationally designed drug against a tumour specific protein. Imatinib mesylate is a small molecule that blocks the binding of ATP to the

bcr/abl protein by occupying the kinase pocket, thus preventing the activation of the tyrosine kinase and its subsequent downstream effectors.[167] Imatinib therefore prevents cell proliferation and leads to apoptosis.[168]

Small molecule inhibitors that target other kinases have also been developed for use in ALL and AML. One of the best characterised targets is FLT-3, a receptor tyrosine kinase that is important in early haematopoiesis.[169] Activating mutations of FLT3 have been found in AML that cause autophosphorylation of the receptor, downstream pathway activation and cell proliferation with decreased apoptosis.[170,171] Furthermore internal tandem duplications (ITD) of FLT3 have prognostic significance in AML.[172] Multiple small molecule inhibitors have been developed which inhibit FLT3.[173,174] Phase II trials of the FLT-3 inhibitor CEP-701 have been encouraging and a Phase III trial in combination with chemotherapy for the treatment of relapsed FLT3-ITD+ AML is underway . Other examples of small molecules that target kinases in ALL and AML include barasertib (an aurora kinase B inhibitor)[175] and ruxolitinib (a JAK kinase inhibitor).[176]

The proteins described above act through intracellular pathways to give the cancer cells a malignant proliferative capacity. In addition, enzymes involved in processing amino acids from the tumour microenvironment are potent targets for new therapy. Methotrexate, one of the most widely used chemotherapy drugs in acute leukaemias and lymphomas, is a structural analogue of folic acid, and acts to inhibit dihydroflate reductase inside tumour cells, leading to depletion of tetrahydrofolate and other

precursors for the synthesis of purines and thymidine.[177] The optimal dose of methotrexate to improve the outcome of patients with leukaemia remains an important subject for investigation.[178] The role of specific amino acids to enhance the survival of patients with leukaemia was most clearly demonstrated by the addition of recombinant L-asparginase to treatment regimens in acute lymphoblastic leukaemia. The addition of L-asparginase to the two-drug combination of vincristine and a glucocorticoid improved the remission induction rate from about 85% to 93% in childhood ALL.[27]

In both solid and haematological malignancies, histopathological analyses suggest that tumour cells reside within a stromal and immunologically protected niche. Although the cells that make up stroma are found in healthy patients, the ability of non-tumour cells to assist the malignant cells in escaping detection, promoting cell survival, and deactivating immune effectors is now clear.[179] Over the last decade the role of a circulating and resident immature myeloid cells, also known as Myeloid-Derived Suppressor Cells (MDSCs), in dampening a patient's immune response through amino acid depletion has been elucidated. In the following section the role of MDSCs in human tumours will be outlined as well as the use of novel drugs to target their mechanism of action.

1.6 Myeloid-derived suppressor cells

1.6.1 Myeloid-derived suppressor cells – an overview

Myeloid-derived suppressor cells are a population of immature myeloid cells composed of macrophage, granulocyte and dendritic cell precursors. Myeloid-derived suppressor cells are best identified by two main characteristics – the expression of myeloid cell surface markers and the ability to inhibit T cell proliferation. In mice, MDSCs can be identified by expression of Gr1 and CD11b cell surface antigens. $Gr1^{+}CD11b^{+}$ cells may be further subdivided based on immunosuppressive activity and variable expression of IL4Rα,F4/80 and CD80.[180] MDSCs have also been identified in human cancer patients characterised by a diverse immunophenotype.[181] The heterogeneous expression of cell surface markers on mouse and human MDSCs is suggestive of a diverse population of cells that are linked through their ability to suppress immune responses.

1.6.2 Myeloid derived suppressor cells in human tumours

Non-lymphoid suppressor cells were first identified as 'natural suppressor cells' over 20 years ago. However their identification as myeloid cells with suppressive activity was first identified in the blood of patients with squamous cell carcinoma of the neck[182].To date MDSCs have been identified in many malignancies. The data are summarised in the following table:

Table 1.1. Phenotypes and function of MDSCs in solid tumours and haematological malignancies in man

Tumour type	No of patients	Reported phenotype	Known data function and mechanism	References
Head and neck cancer	18	$CD34^+$	Correlation between presence of $CD34^+$ and high GM-CSF in SCC	Pak et al. 1995[182]
Head and neck cancer (SCC) NSCLC Breast cancer	13 21 10	Lin^- HLA-DR^-CD33^+ $CD15^-CD13^+$	ImCs directly inhibit antigen specific T cell responses	Almand et al. 2001[183]
Head and neck cancer	7	Not reported	Phosphodiesterase-5 inhibition reverses tumour-induced immunosuppressive mechanisms via downregulation of Arginase-I and nitric oxide synthase-2 expression	Serafini et al. 2006[184]
Head and neck cancer	14	$CD11b^+$ $CD14^-$ $CD33^+$	Tumour localized MDSCs had higher iNOS levels and significantly lower ROS production than those in from peripheral blood	Corzo et al. 2010[185]
Renal cell carcinoma	123	$CD11b^+$ $CD14^-$ $CD15^+$	MDSC with high arginase I levels and a suppressive phenotype	Zea et al. 2005[186]
Renal cell carcinoma	27	$CD33^+$ $CD11b^+$ $CD14^-CD15^+$ $CD66b^+$ $CD11b^+$ $VEGF1^+$	MDSC capable of suppressing antigen-specific T-cell responses *in vitro* through increase in arginase activity, secretion of ROS and nitric oxide.	Rodriguez et al. 2009[187]
Metastatic renal cell carcinoma	18	Lin^- HLA-DR^- CD33+	Substantially elevated numbers of ImCs in the blood of patients. ImCs decreased by ATRA therapy.	Mirza et al. 2006[188]
Metastatic renal cell carcinoma	9	Lin^- HLA-DR^- CD33+ CD18+	MDSCs have arginase and iNOS based suppressive activity	Kusmartsev et al.[189]
Breast carcinoma	50	Lin^- HLA-DR $CD33^+CD11b^+$	MDSCs correlate with clinical stage of cancer and metastatic tumour burden	Diaz-Montero et al. 2009[190]
Colon carcinoma	10	Lin^- HLA-DR^- $CD33^+$ $CD11b^+$	iNOS and arginase mediated suppressive activity	Diaz-Montero et al. 2009[190]
Colorectal carcinoma Breast cancer	25 25	Lin^- $CD11b^+$ HLA-$DR^{low/-}CD33^+$	MDSC subset correlate with patient survival	Solito et al. 2011[191]

Non-Small Cell Lung Cancer	173	$CD11b^+$ $CD14^-$ $CD15^+$ $CD33^+$	Increased pool of MDSCs in peripheral blood correlates with cancer stage. iNOS and arginase detected in MDSCs	Liu et al. 2009[192]
Non-Small Cell Lung Cancer	10	$CD33^+$ $CD11b^+$ (majority $CD15^+$)	MHC II cell-based vaccines can overcome MDSC suppression	Srivastava et al. 2008[193]
Melanoma Colon cancer	14 15	$CD14^+$ $CD15^+$	IL-4Rα expression correlated with suppressive activity	Mandruzzato et al. 2009[194]
Gastrointestinal cancer		Granulocytes by morphology	Hydrogen peroxide dependent inhibition of IFN-γ and TCR-ζ expression on T cells	Schmielau et al. 2001[195]
Hepato-cellular carcinoma	111	$CD14^+$ HLA-$DR^{-/low}$	MDSCs inhibit NK cell cytotoxicity via cell-contact. T cells are suppressed via arginase depletion and increased differentiation of T regulatory cells	Hoechst et al. 2009[196]
Ovarian cancer	17	$CD14^+$ HLA-DR-	MDSCs in patient ascites suppress via IL-10 secretion	Loercher et al. 1999[197]
Prostate cancer	40	$CD14^+$ HLA-$DR^{-/low}$	MDSC numbers correlate with circulating prostate specific antigen levels.	Vuk-Pavlovic et al. 2010[198]
Glioblastoma	28	$CD33^+$ HLA-DR^- $CD15^+$ $CD14^-$	Arginase dependent suppression of T cell proliferation	Raychaudhuri et al. 2011[199]
Multiple myeloma	76	$CD14^+$ HLA-$DR^{-/low}$	Increased MDSCs in the blood of patients	Brimnes et al. 2010[200]
Non-Hodgkin lymphoma	40	$CD14^+$ HLA-$DR^{-/low}$ $CD120^{low}$	Arginase dependent mechanism of suppression	Lin et al. 2011[201]
Melanoma	40	$CD11b^+$ $CD15^+$	Expansion of suppressive IL-10-secreting neutrophils in melanoma patients correlated with elevated amounts of SAA-1 and with stage of disease.	De Santo et al.2010[202]
Melanoma	16 34	$CD14^+$ HLA-$DR^{-/low}$ $CD80^+$ $CD86^+$	Stat3 expression in MDSCs correlates with suppressive activity.	Poschke et al. 2010[203] Filipazzi et al. 2007[204]

ImC, Immature cells; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer.

1.6.3 Mechanisms of MDSC suppression of immune response

MDSCs block the proliferation and activation of CD4+ and CD8+ T cells.[205] T cells require arginine for activation and one of the best characterized mechanisms of MDSC induced suppression is the ability of MDSCs to deplete arginine from the microenvironment. Arginine is available in the body from dietary intake, secondly de novo synthesis via citrulline recycling, and thirdly from protein turnover.[206] Arginine is the substrate for 5 enzymatic processes: (a) arginyl-tRNA synthetase for protein production, (b) NO synthases (iNOS and eNOS) for nitric oxide species production, (c) arginases I and II for production of prolines, glutamate, polyamines and urea, (d) arginine-glycine amidinotransferase for production of creatine and (e) arginine decarboxylase for production of agmatine. MDSCs contain high intracellular levels of arginase I which depletes arginine, leading to reduced translation of the T cell ζ-chain of CD3 and a decrease in T cell proliferation.[207] In addition MDSC expression of iNOS leads to production of nitrogen and reactive oxygen species. These radicals have been shown to block signaling from the IL-2 receptor, by inhibiting the phosphorylation and activation of Jak3 and STAT5, as well as inhibiting MHC Class II gene expression.[208,209]

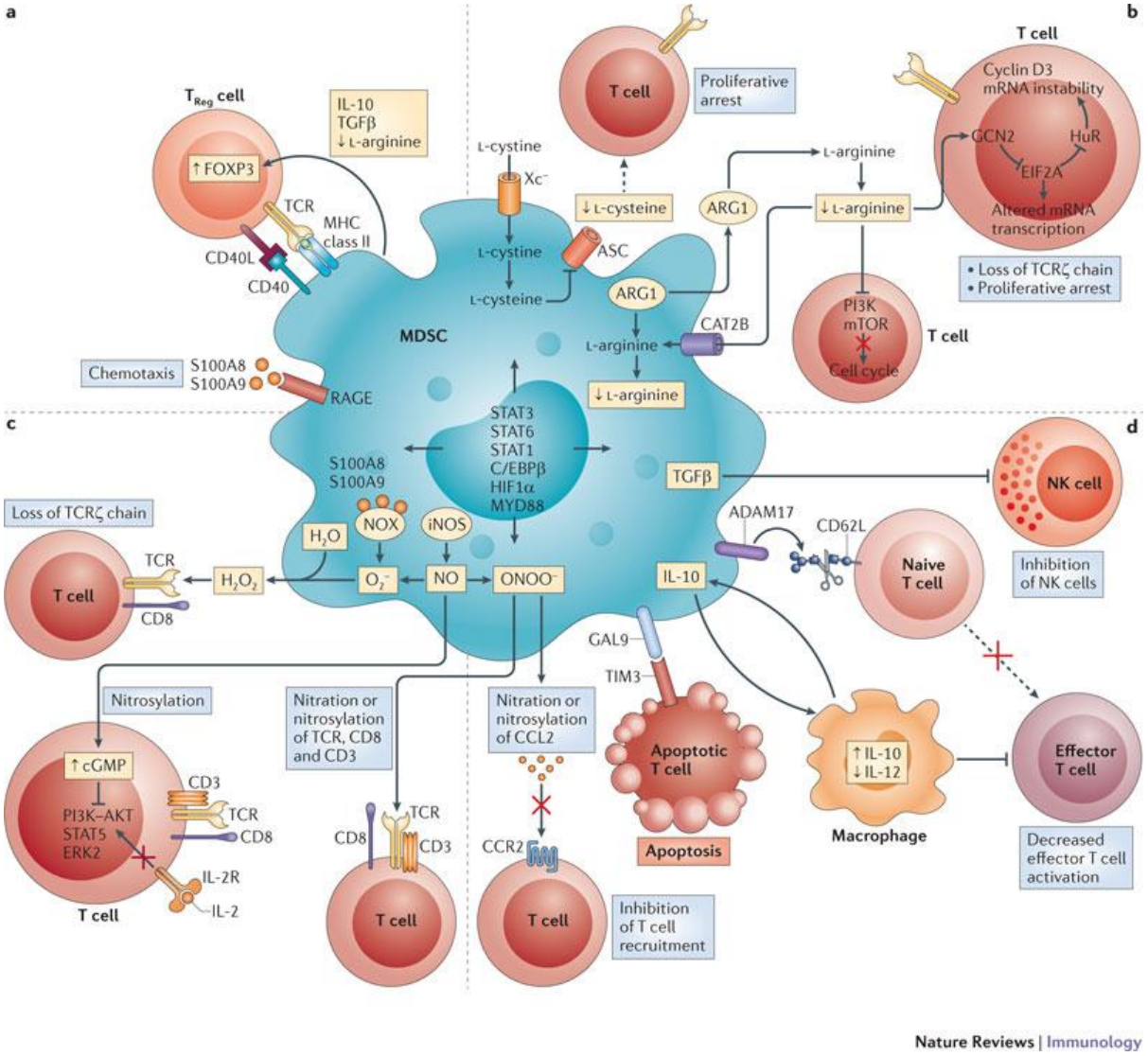


Figure 1.4 Myeloid-derived suppressor cells (MDSCs) can inhibit antitumour immunity. T cell responses through a number of mechanisms. **a** | Tumour-associated MDSCs induce the development of regulatory T (T_{Reg}) cells or expand existing T_{Reg} cell populations. The calcium-binding proteins S100A8 and S100A9 are involved in the chemotaxis of MDSCs and other myeloid cells; these effects are mediated in part through the activation of receptor for advanced glycation end-products (RAGE). At the same time, S100A8 and S100A9 along with gp91phox are part of the NADPH oxidase (NOX) complex that is responsible for the increased production of reactive oxygen species (ROS) by MDSCs. **b** | Tumour-associated myeloid cells deprive T cells of amino acids that are essential for their growth and differentiation. **c** | Tumour-associated myeloid cells release oxidizing molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). Peroxynitrite causes nitration and nitrosylation of components of the T cell receptor (TCR) signaling complex, and H_2O_2 causes the loss of the TCR ζ-chain, thereby inhibiting T cell activation through the TCR. **d** | Tumour-associated myeloid cells can also interfere with T cell migration and viability. The metalloproteinase ADAM17 (disintegrin and metalloproteinase domain-containing protein 17) cleaves CD62L, which is necessary for T cell migration to draining lymph nodes, and galectin 9 (GAL9) can engage T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) on T cells to induce apoptosis. As the induction of the immunosuppressive pathways that are depicted in the figure is regulated by common transcription factors, these pathways can operate in more than one myeloid cell type. ARG1, arginase 1; ASC, asc-type amino acid transporter; CAT2B, cationic amino acid transporter 2 isoform 1 (L-arginine transporter); CCL2, CC-chemokine ligand 2; CCR2, CC-chemokine receptor 2; C/EBPβ, CCAAT/enhancer-binding protein-β; EIF2A, eukaryotic translation initiation factor 2A; ERK2, extracellular signal-regulated kinase 2; FOXP3, forkhead box P3; HIF1α, hypoxia-inducible factor 1α; HuR, Hu-antigen R (also known as ELAVL1); IL, interleukin; IL-2R, IL-2 receptor; iNOS, inducible nitric oxide synthase; mTOR, mammalian target of rapamycin; MYD88, myeloid differentiation primary-response protein 88; NK, natural killer; PI3K, phosphoinositide 3-kinase; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor-β; Xc^-, cystine–glutamate transporter. Figure reproduced from Gabrilovich et al. 2012[210]

More recently other mechanisms for MDSC induced suppression of T cell responses have been identified. Cysteine depletion by MDSCs, plays a role analogous to arginine depletion.[211] Patients with melanoma have been found to have increased numbers of IL-10 secreting neutrophils.[202] IL-10 is known to have multiple immunomodulatory effects including T-regulatory cell induction, downregulation of macrophage IL-12 and promotion of a Th2 type response. Direct cell-cell contact mechanisms have been implicated in altering NK cell tumour surveillance by MDSCs.[212] Finally MDSCs can lead to the development of T_{Reg} cells through a number of different mechanisms.[213,214]

1.6.4 Targeting of enzymatic pathways in MDSCs

Over the last ten years inhibitors to arginase and iNOS have been developed. NOHA (N^G-hydroxy-L-arginine), a physiological intermediate in the NO synthesis pathway, is a potent inhibitor of arginase.[215] A synthetic derivative with increased potency, nor-NOHA (N^w-hydroxyl-nor-L-arginine), has subsequently been produced.[216] Both molecules are competitive inhibitors of arginase that contain N-hydroxyguanidinium moieties that bridge a binuclear manganese cluster within arginase that is required for enzymatic activity. The drugs have been widely used to inhibit arginase in a number of different disease models for both in vitro[217,184] and in vivo experiments.[218,219] L-N^G-monomethyl Arginine (L-NMMA) is a competitive inhibitor for iNOS, with a higher affinity for the binding site than arginine. L-NMMA is hydroxylated by iNOS leading to a number of reactive intermediaries that may further interact with iNOS to reduce enzymatic activity.[220] L-NMMA has been shown to be efficacious in inhibiting iNOS in a number of disease settings, both in vitro[221] and in vivo.[222,223] In vitro testing of another arginine pathway modulator, that has been used in Phase

I and II trials for cardiovascular disease, is ongoing. Nitroaspirin is an aspirin molecule covalently linked to a NO donor group. The drug has been shown to interfere with the inhibitory enzymatic activities of myeloid cells, and orally administered NO aspirin can normalise the immune status of tumor-bearing mice and increase the number and function of tumor-antigen-specific T lymphocytes.[224]

The ability of other drugs to modulate the function of MDSCs from patients has been reported. Eighteen patients were evaluated for the effect of the differentiating agent All-Trans Retinoic Acid (ATRA) in combination with IL-2 on the myeloid and dendritic cells present in metastatic renal cancer (RCC) patients.[225] Immature cells in the blood, defined as $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$, were increased in patients before treatment compared with healthy controls, but decreased after 7 days of ATRA. These results were confirmed when increased numbers of MDSCs were isolated from the blood of RCC patients in a second study.[226]Morphological analysis revealed both 'monocytic' and 'granulocytic' cell types. The addition of L-NOHA and L-NMMA demonstrated that these MDSCs can inhibit IFN-γ production by T cells through reactive oxygen species (ROS) and nitric oxide dependent mechanisms.

High serum VEGF levels are associated with an increase in the number of immature dendritic cells in patients with cancer.[227] Sunitinib, an orally administered tyrosine kinase inhibitor that can inhibit signaling through the VEGF receptor, has been investigated for an effect on MDSCs from RCC patients. In vitro, Sunitinib reduced MDSC suppressive ability and viability. Consistent with these findings, RCC patients treated with Sunitinib had a significant reduction in MDSC numbers in the peripheral blood, which correlated with a reversal of T

cell suppression and a fall in $CD3^{+}CD4^{+}CD25^{high}Foxp3^{+}$ T_{reg} cell numbers. However, in an alternative approach the anti-VEGF antibody Bevacizumab failed to decrease the percentage of peripheral blood MDSCs in RCC patients.[228] Finally, CD14+ immunosuppressive myeloid cells have been identified in the blood of head and neck cancer patients.[184] The study reported that the addition of the phosphodiesterase-5 inhibitor Sildenafil (Viagra) restored T cell proliferation in vitro, despite the presence of MDSCs. The heterogenous nature of MDSCs makes it likely that different therapeutic approaches will be required to target specific MDSC subtypes.

AML develops from immature myeloid cells and thus we hypothesise that the mechanisms by which AML cells escape immune destruction may be similar to those employed by MDSCs. In this thesis the mechanisms of AML induced suppression will be identified and the small molecule inhibitors NOHA and L-NMMA, described above, will be used to target these mechanisms.

1.7 Invariant Natural Killer T cells and Cancer

1.7.1 Invariant NKT cells – an overview

Over the last 30 years the role of invariant NKT (iNKT) cells as a unique population having the capacity to bridge the innate and adaptive immune responses has become increasingly apparent. In contrast to conventional T cells, the invariant T Cell Receptor (TCR) of iNKT cells recognize either endogenous[229] or exogenous (microbial or synthetic) glycolipid antigens[230] presented by the MHC class I-like molecule CD1d.[231] Recent results have led to

the identification of a family of endogenous lipids (beta-glucosylceramides) that is upregulated by TLR signalling events and can activate iNKT cells.[232]

In contrast to endogenous ligand presentation, the ability of iNKT cells to recognize exogenous antigens has been better characterized. The most potent synthetic ligand of iNKT cells to be identified is α-galactosylceramide (αGalCer), a lipid derived from the glycosphinglipid extract of the marine sponge *Agelas mauritanius*.[233] Follow-up studies indicate that the sponge is frequently colonized by *Sphingomonas* bacteria - the likely source of the αGalCer documented earlier.[234] Studies over the last 20 years have demonstrated the importance of iNKT cells in immune surveillance of tumour cells and their potential to control and destroy malignant cells. It is well established that iNKT cells activated by molecules such as αGalCer mediate potent anti-tumour effects in vitro and in vivo.[233] However the physiological role of iNKT cells stimulated by unknown endogenous ligands in tumour surveillance is less clear. An overview of the evidence follows.

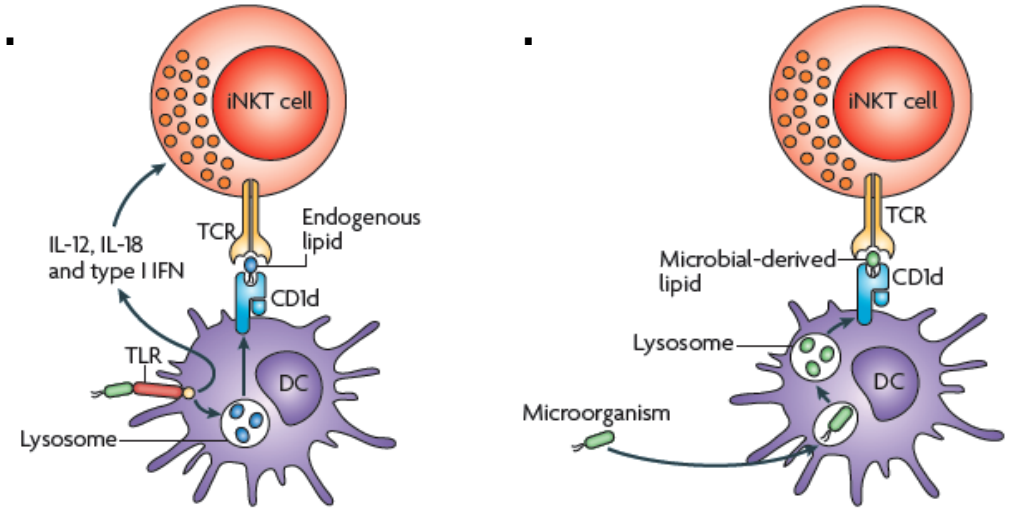


Figure 1.5 iNKT cell activation by endogenous and exogenous lipids (a) Signaling through Toll-like receptors (TLRs) leads to the loading of endogenous (self) lipids onto CD1d molecules. Presentation of the self-lipid, along with TLR-induced IL-12, IL-18 and type I interferon (IFN) secretion by the antigen presenting cell (APC), activates the iNKT cell.[235] The TLR-induced cytokines amplify the activating signals generated by the self-glycolipids, however may activate the iNKT cells irrespective of TCR-lipid-CD1d engagement;[236] **(b)** APCs process and present microbial lipids on CD1d molecules that can activate iNKT cells. DC, dendritic cell *(Adopted from Cerundolo et al Nat Rev Immunol 9 (1), 28-38 (2009).*[237]

1.7.2 The role of iNKT cells in the control of tumours in murine models

Two early studies first identified the role of iNKT cells in methylcholanthrene (MCA)-induced fibrosarcomas in mice. Initially Jα281$^{-/-}$ mice were found to have increased susceptibility to MCA-induced fibrosarcomas compared to wild-type mice.[238] The findings were consolidated in a subsequent study demonstrating that adoptive transfer of liver-derived iNKT cells into Jα281$^{-/-}$ mice significantly reduced the incidence of MCA induced sarcomas by an IFN-γ dependent mechanism.[239] Liver-derived iNKT cells were more potent than thymic or spleen-derived NKT cells in preventing tumour growth, suggesting the existence of functionally distinct subsets of iNKT cells.[240] The study showed that the reduced anti-tumour activity of thymic iNKT cells was due to autologous production of IL-4. In a model of

pulmonary metastases caused by MCA-induced sarcomas, Jα28$^{-/-}$ mice developed increased numbers of pulmonary metastases compared to wild-type mice.[241] The presence of CD4^{+}CD25^{+} T cells inhibited the anti-tumour activity of iNKT cells, indicating a role for T regulatory cells in dampening the physiological role of iNKT cells in preventing tumour growth.

In the studies described above, no exogenous stimulus (for example αGalCer or IL-12) was needed to induce the anti-tumour effect, suggesting that endogenous ligands are able to activate iNKT cells and promote tumour surveillance.

1.7.3 The association of iNKT cells with human tumours

Assessment of iNKT cell frequency in human blood is complicated by the low numbers of circulating iNKT cells. The number of Vα24/Vβ11 iNKT cells, as measured by CD1d tetramers, is often highly variable and close to the lower limit of detection by flow cytometry. As a result, making comparisons between the frequency of circulating iNKT cells in healthy patients and patients with cancer is fraught with inaccuracy. However, studies of patients with HNSCC, prostate cancer, and lung cancer indicate that the frequency of iNKT cells in cancer patients is lower than in healthy controls, and may be associated with disease outcome.[242,243]

Although it has been demonstrated that iNKT cells can protect against tumour development and progression in murine models, evidence for the infiltration of iNKT cells into the tumour microenvironment is limited. The first indirect evidence that iNKT cells can infiltrate tumour

was demonstrated in lung cancer patients. Using quantitative PCR, an increased copy number of Vα24Jαq was found in the malignant sections of lung tissue from cancer patients, compared to non-malignant sections from the same lung.[243] In the same report, patients with primary lung cancer had approximately one-third less Vα24 NKT cells in the peripheral blood than age-matched healthy controls.

Invariant NKT cells have subsequently been identified in tumour tissue from patients with other solid cancers. The number of Vα24 NKT cells was significantly higher in colorectal carcinoma tissue compared to healthy mucosa[244] and patients with higher NKT cell infiltration were found to have improved overall and disease-free survival compared to patients with low NKT cell infiltration. Increased Vα24/Vβ11 iNKT cell frequency was also found in tumour infiltrating lymphocytes of patients with primary or secondary liver tumours compared to peripheral blood lymphocytes, particularly in hepatocellular (HCC) patients.[245] There was no difference found in the frequency of iNKT cells in the blood of the patients compared to that of the healthy controls.

1.7.4 The role of iNKT cells in haematological malignancies

Studies of invariant NKT cells in haematological malignancies are limited. AML and Juvenile Myelomonocytic Leukaemia (JMML) blasts can express CD1d are therefore are a potential target for iNKT cell therapy. iNKT cells activated with αGalCer are cytotoxic to some AML blasts, predominantly via a perforin/Granzyme B mediated mechanism.[246] Human T-cell acute lymphoblastic leukaemia cell lines also express CD1d and can be targeted by Vα24+ iNKT cells in vitro.[247] Indirect evidence for the role of iNKT cells in leukaemia

remission has been elucidated in the post-transplant setting.[248] Paediatric patients treated with HLA-haploidentical haematopoietic stem cell transplants for leukaemia were followed for 6 years post treatment, and the quality of immune reconstitution was investigated. Fourteen patients, out of 22 studied, did not relapse. In these patients iNKT cells were detectable in the blood by 18months post transplant at levels equivalent to those in age-matched healthy controls. The remaining 8 patients relapsed within 18 months of transplant. In these relapsed patients CD4+ and CD8+ T cells were not significantly lower than that of controls, however iNKT cells failed to reconstitute suggesting a role for iNKT cells in maintaining a remission.

In lymphomas the role of iNKT cells is more controversial. In a murine model of T cell lymphoma the absence of iNKT cells correlated with an increased tumour burden, and thus may inhibit anti-lymphoma responses in this disease setting.[249] In a murine model of B-cell lymphoma iNKT cells conferred protection from lymphoma development. MDSCs numbers were increased in the tumours and spleen of iNKT knock-out mice suggesting a cross-talk between these 2 cell populations. Further studies have also demonstrated by flow cytometry that Vα24Jα18/Vβ11 iNKT cells are present within classical Hodgkin lymphoma tissue.[250]

In summary iNKT cells have been shown to have activity against AML cells and to mediate anti-lymphoma responses in murine models. These results are supportive of the therapeutic potential of iNKT cells in haematological malignancies and suggests that they are worthy of further study.

1.8 Introduction Publications

Some of the work in this Introduction has been accepted for publication in the following review:

- **Mussai F***, De Santo C*, Cerundolo, V. Interaction between invariant NKT cells and Myeloid-derived suppressor cells in cancer patients: Evidence and therapeutic opportunities. Journal of Immunotherapy 2012

  *These authors contributed equally to this review

2. Thesis aims

The aims of this DPhil thesis are as follows:

- To investigate the activity of the anti-CD22 immunotoxin HA22 against paediatric Acute Lymphoblastic Leukaemia

- To investigate the factors contributing to sensitivity and resistance of Acute Lymphoblastic Leukaemia and Burkitt's lymphoma to HA22 treatment

- To investigate the mechanisms by which Acute Myeloid Leukaemia suppresses T cell immune responses

- To investigate whether small molecule inhibitors or invariant Natural Killer T cells can target the suppressive activity of Acute Myeloid Leukaemia.

3. Materials

3.1 Tissue Culture

Reagent	Supplier
1x and 10x Phosphate Buffered Saline (PBS)	Lonza
RPMI 1640	Invitrogen
Dimethyl sulphoxide (DMSO)	Sigma
Fetal Bovine Serum (FBS)	Sigma
Fetal Calf Serum (FCS)	Sigma
L-Glutamine (200mM in 0.85% Azide)	Lonza
PEN-STREP (5000U/ml)	Lonza
Sodium pyruvate (100mM)	Invitrogen
Trypan Blue	Sigma
Matrigel	BD Biosciences
Liberase 2	Roche
Hank's solution	Invitrogen
WST-1 Cell Proliferation Reagent	Roche
β-mercaptoethanol (50mM)	GIBCO

3.2 Solutions

Solutions	Composition
0.5% PBST	1× PBS + 0.5% (v/v) Tween-20 (Sigma-Aldrich)
25x Complete Protease inhibitor stock solution	One complete, EDTA-free Protease Inhibitor Cocktail Tablet (Roche), sufficient for 50ml lysis buffer) was dissolved in 2ml of lysis buffer, dispensed into 100µl aliquots and stored at -20ºC
10 % (w/v) SDS	50g SDS (Sigma) was dissolved in 200ml ddH_2O and warmed up to 68°C while stirring. The solution was then made up to 500 ml with ddH_2O and filtered
0.5 M Tris-Cl pH 6.8	6g Trizma Base (Sigma, M_W=121.14g/mol) was dissolved in 60 ml ddH_2O and pH adjusted to 6.8 using 1M HCl; the solution was made up to 100ml with ddH_2O and stored at 4°C.
1.5 M Tris-Cl pH 8.8	90.75g Trizma Base (Sigma, M_W=121.14g/mol) was dissolved in 250ml ddH_2O and the pH adjusted to 8.8 using 1M HCl; the solution was made up to 500ml with ddH_2O and stored at 4°C.
10% APS (w/v)	1g Ammonium persulphate (Sigma) was dissolved in 10ml cold ddH_2O (4 °C); the solution was stored as 200µl aliquots at –20 °C.

3.3 Buffers

Buffer	Composition
Antibody dilution buffer for Western Blot	0.5% PBST + 2.5% (w/v) dried skimmed milk powder.
Blocking buffer for Western Blot	0.5% PBST + 5% (w/v) dried skimmed milk powder.
10x Running Buffer for SDS-PAGE	Tris-Glycine-SDS Buffer 10x Concentrate, Sigma, or 1920mM Glycine, 250mM Trizma Base (all from Sigma) dissolved in 1 l ddH_2O.
1x Running Buffer for SDS-PAGE	100 ml 10x Running buffer mixed with 900ml ddH_2O, or 75 ml 10x Running Buffer, 7.5 ml 10% (w/v) SDS; buffer was made up to 750 ml with ddH_2O
1x Transfer Buffer for Western Blot	100 ml 10× Tris-Glycine Concentrate Buffer (Sigma) made up to 1 litre with ddH_2O, or 100 ml 10× Running Buffer, 10 ml 10% SDS (w/v), 150 ml Methanol; buffer was made up to 1 litre with ddH_2O.
Lysis buffer	RIPA buffer, Sigma
FACS buffer	PBS containing 10% (v/v) FCS, stored at 4 °C.
MACS buffer	PBS containing 2 mM EDTA and 10% (v/v) FCS.

3.4 Protein Gels

3.4.1 Separating Gel

Components	7%	10%	12%	15%
Distilled H_20	5.1ml	4.1ml	3.4ml	2.4ml
1.5M Tris-HCl, pH 8.8	2.5ml	2.5ml	2.5ml	2.5ml
20% (w/v) SDS	0.05ml	0.05ml	0.05ml	0.05ml
30% Acrylamide (w/v) (National Diagnositcs)	2.3ml	3.3ml	4.0ml	5.0ml
10% APS	0.05ml	0.05ml	0.05ml	0.05ml
TEMED	0.005ml	0.005ml	0.005ml	0.005ml
Final Volume	10.005ml	10.005ml	10.005ml	10.005ml

3.4.2 Stacking Gel

Components	Volume
Distilled H_20	3.075ml
1.5M Tris-HCl, pH 8.8	1.25ml
20% (w/v) SDS	0.025ml
30% Acrylamide (w/v) (National Diagnostics)	0.67ml
10% APS	0.025ml
TEMED	0.005ml
Final Volume	5.05ml

Novex 10% and 12% Tris-Glycine Gel 1.0mm, 2D Well (Invitrogen) pre-cast gels were also used.

3.5 Antibodies for Western Blotting

Antibody	Catalogue No.	Supplier
Anti-Mcl-1	4572	Cell Signaling
Anti-Bcl-xl	2764	Cell Signaling
Anti-Bax	06-499	Millipore
Anti-Bak	06-536	Millipore
Anti-Bcl2	2876	Cell Signaling
Anti-BIM	2819	Cell Signaling
Anti-PUMA	4976	Cell Signaling
Anti-actin	8226	Abcam
Anti-Arginase I (N-20), polyclonal	sc-18351	Santa Cruz Biotechnology,
Anti-Arginase II (H-64)	sc-20151	Santa Cruz Biotechnology,
Anti-S100A9	ab75478	Abcam
Anti-actin	A 2066	Sigma
Anti-Goat-IgG-HRP	sc-2020	Santa Cruz Biotechnology
Anti-Mouse-IgG-HRP	NA-931	GE Healthcare
Anti-Rabbit-IgG-HRP	sc-2030	Santa Cruz Biotechnology

3.6 Antibodies for Flow Cytometry and Cell Sorting

Antibody	Conjugate	Catalogue No.	Supplier
Anti-human CD19	FITC	555412	BD Bioscience
Anti-human CD22	PE	347577	BD Bioscience
PE Annexin V Apoptosis Detection Kit	7-AAD and PE	559763	BD Bioscience
Alexa Fluor 647 Protein Labelling Kit	Alexa-647	A20173	Invitrogen
Quantibryte Standard Beads	PE	340495	BD Bioscience
Anti-human CD11b	PE	12-0112-81	eBioscience
Anti-human CD14	APC	555399	BD Bioscience
Anti-human CD15	FITC	11-0159-73	eBioscience
Anti-human CD33	PE	12-0338-71	eBioscience
Anti-human CD34	APC	17-0349-41	eBioscience
Anti-human CD206	PE	555954	BD Bioscience

3.7 Flow Cytometry, Enrichment and Cell Sorting Reagents

Reagent	Supplier
CD11b MACS Beads	Miltenyi Biotec
CD14 MACS Beads	Miltenyi Biotec
CD33 MACS Beads	Miltenyi Biotec
Propidium Iodide	Sigma
Lymphoprep	Axis- Shield
Red Cell Lysis Buffer	Qiagen

3.8 ELISA Buffers and Solutions

Solution	Composition
ELISA coating buffer	0.1 M $NaHCO_3$ (Sigma) in ddH_2O, pH 9, sterile filter.
ELISA stop solution	2 M sulphuric acid.
Substrate solution A for ELISA	10.5 g citric acid monohydrate (Fluka) in 500 ml ddH_2O.
Substrate solution B for ELISA	17 g $Na_2HPO_4 \cdot 2H_2O$ (Fluka) in 500 ml ddH_2O.
ELISA blocking buffer	PBS containing 10% (v/v) FCS, store at 4 °C.
ELISA washing buffer	PBS + 0.1% Tween-20.

3.9 ELISA Antibodies

ELISA Kit	Catalogue No.	Supplier
Human SAA ELISA Kit	KHA0012	Invitrogen
Human CRP ELISA Kit	88-7502-28	eBioscience
Human Arginase I ELISA kit	414563	Antibodies online
Human Arginase II ELISA kit	414194	Antibodies online
Human S100A9 ELISA kit	414828	Antibodies online

Note: TGF-β, IL-1β, and GM-CSF ELISAs were done without a customized kit. See Buffer table for solutions. Antibodies used are listed below.

Antibody	Catalogue No.	Supplier
Anti-human TGF-β (Capture Antibody)	14994381	eBioscience
Biotinylated anti-human TGF-β (Detection Antibody)	13992381	eBioscience
Anti-human IL-10 (Capture Antibody)	554497	BD Bioscience
Biotinylated anti-human IL-10 (Detection Antibody)	554499	BD Bioscience
Anti-human IL-1β (Capture Antibody)	14701885	eBioscience
Biotinylated anti-human IL-1β (Detection Antibody)	13701685	eBioscience
Anti-human GM-CSF (Capture Antibody)	1886339CP	eBioscience
Biotinylated anti-human GM-CSF (Detection Antibody)	866339DT	eBioscience

3.10 Cytokines

Cytokine	Catalogue No.	Supplier
Human IL-10	200-10	Peprotech
Human IL-1β	39-8018-60	eBioscience
Recombinant Human apo-SAA (SAA)	300-13	Peprotech

3.11 Arginine pathway inhibitors

Compound	Catalogue No.	Supplier
N^G-Monomethyl-L-arginine, monoacetate (L-NMMA)	475886	CalBiochem
N^G-Hydroxy-L-arginine, monoacetate (NOHA)	399250	CalBiochem

3.12 Drugs for cell culture assays

Drug	Supplier
Dexamethasone	NIH Veterinary Stores
Cyclohexamide	Sigma
17-AAG	Gift from L. Neckers, NIH
ABT-737	Gift from D. Fitzgerald, NIH
Taxol	NIH Veterinary Stores
Adriamycin	NIH Veterinary Stores
αGalactosylceramide	Cerundolo Lab

3.13 Immunotoxins produced by Pastan laboratory, NIH

Immunotoxin	Antigen Target
BL22	CD22
HA22	CD22
HA22-LR	CD22
HA22-LR-8X	CD22
HA22-KDEL	CD22
SS1P	Mesothelin
LMB-9	Lewis Y

4. Methods

4.1 ALL / Burkitt's Lymphoma cell lines for immunotoxin studies

5 paediatric ALL cell lines and 2 Burkitt lymphoma cell lines were used for immunotoxin experiments. The cell lines were chosen because of known CD22 expression. The cell lines were obtained from the American Tissue Culture Collection (Manassas, VA, USA) and the Deutsche Sammlung vin Mikroorganismen und Zelkuturen GmBH(Braunschweig, Germany).

Name	Disease	Origin	Translocations
KOPN-8	Pre-B ALL	Peripheral blood of a 3 month old girl	t(11:19)(q23:p13) leading to a MLL-ENL fusion gene
EU	Pre-B ALL	Bone marrow of a 1 year old child at 1st relapse	t(4:11)(q21:q23) leading to a MLL-AF4 fusion gene
REH	Pre-B ALL	Peripheral blood of a 15year old female at relapse	t(12:21)(p13;q22) leading to TEL-AML1 gene fusion
NALM-6	Pre-B ALL	Peripheral blood of a 19 year old male at relapse	t(5:12)(q33.2;p13.2) gene fusion
SEM	Pre-B ALL	Peripheral blood of a 5 year old girl at relapse	t(4:11)(q21;q23) leading to a MLL-AF4 fusion gene
CA46	Burkitt lymphoma	Ascites of a patient with EBV-negative Burkitt's	t(8:14)(q23:q32) fusion gene
RAJI	Burkitt lymphoma	Tumour tissue from a 12 year old African male with EBV-positive Burkitt's	the t(8:14)(q24:q32) fusion gene

Table 4.1 Table of ALL and Burkitt Lymphoma cell lines and their characteristics

Cell lines were cultured in RPMI-1640 (Invitrogen, CA, USA) with 10% Foetal Bovine Serum (Sigma-Aldrich, St Louis, MO, USA), Glutamine (1x), Sodium Pyruvate (1x) and Penicillin-Streptomycin. The cell lines were cultured in T-75 flasks, in a humidified atmosphere at 37^0C with 5% CO_2.

4.2 ALL Murine xenografts

Human pre-B ALL cells were derived from 4 murine NOD-SCID-IL2γ^{null} (NOG-SCID) xenografts as follows. Primary leukaemia cells from paediatric patients were cryopreserved and then engrafted by intravenous injection into NOG-SCID mice. One mouse was engrafted with pre-B ALL blasts from a patient at relapse. Three other mice were engrafted with pre-B ALL blasts harvested from patients at diagnosis. After successful engraftment, mice were sacrificed and their spleens were harvested to collect the ALL blasts. At least 92% purity of human pre-B ALL cells was confirmed by flow cytometry and the cells were cryopreserved in RPMI-1640 with 10% FBS and 10% DMSO. Cells were thawed immediately prior to use in the stromal cell assay.

4.3 Patient ALL samples

Peripheral blood and bone marrow samples were obtained from 35 patients with B-lineage ALL treated at the National Cancer Institute (NCI), St. Jude Children's Research Hospital (SJCRH) or Johns Hopkins Hospital (JHH) with informed consent. Twenty-two sets of samples were obtained from patients with multiply relapsed ALL who had been referred to the NCI for enrolment in Phase I clinical trials (Table 4.2). Thirteen patient samples from diagnosis were randomly selected from those available in the tumour banks at St Jude's

TABLE 4.2 Will be inserted here on final print

Children's Research Hospital, Memphis, USA and Johns Hopkins Hospital, Baltimore, USA. Thirty-three cases were characterized as pre-B ALL and two as Burkitt's-type/mature B-cell ALL by immunophenotype. In all cases, >80% blasts expressed CD19 and CD22 antigens. Cells were cryopreserved in RPMI-1640 with 10% FBS and 10% DMSO until use. Institutional review board approval was obtained for the use of these samples in these studies. Cells were thawed immediately prior to use in the stromal cell assay.

4.4 CD22 site density

Antigen site density was quantified by determining the anti-CD22 antibody binding capacity per cell.[251] Samples were stained under saturating conditions with anti-CD22 antibody with 1:1 antibody to phycoerythrin conjugation (BD Biosciences), using the BD Biosciences QuantiBRITE system for fluorescence quantitation. The antibody binding capacity value is the measurement of the mean value of the maximum capacity of each cell to bind the anti-CD22 antibody. QuantiBRITE beads are pre-calibrated standard beads containing known levels of phycoerythrin molecules. QuantiBRITE beads were acquired on a FACSCalibur flow cytometer on the same day at the same instrument settings as the individual specimens. A standard curve comparing the geometric mean of fluorescence to known phycoerythrin content of the QuantiBRITE beads was constructed using QuantiCALC software.

4.5 HA22 cytotoxicity assay

To measure the cytotoxic activity of the anti-CD22 immunotoxins HA22 and BL22, murine xenograft or patient cells were cultured on bone marrow derived mesenchymal cells.[252] Briefly, human bone marrow stromal cells that had been immortalized by telomerase

transfection (developed at St. Jude Children's Research Hospital) were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Sigma-Aldrich, St Louis, MO, USA). $2x10^4$ stromal cells were plated into each well of flat-bottomed 96-well plates and cultured until confluent. Cell lines did not require culture on stromal cells to maintain viability. On day 1 of the assay, ALL cells were resuspended in RPMI-1640, 10% heat-inactivated foetal bovine serum, glutamine (1x) and sodium pyruvate (1x). To each well of stroma-coated plates $3x10^5$ ALL cells were added. On day 2, BL22 or HA22 were added at final concentrations of 0, 0.5, 1, 5, 10, 50, 100 and 500 ng/mL in duplicate wells. SS1P 50 ng/mL, an anti-mesothelin *Pseudomonas* immunotoxin, was used as a negative control as mesothelin is not expressed on ALL cells. The cytotoxicity of dexamethasone (10 mmol/L) was also tested in this assay. Cells were incubated for a further 72 hours in a humidified atmosphere at 37^0C with 5% CO_2 and then analysed as described below.

4.6 Flow cytometric analysis

The cells were harvested by vigorous pipetting, transferred to Falcon tubes (BD Biosciences, San Jose, CA, USA, Cat. No.352052) and labelled with mouse anti-human CD19 antibody conjugated to fluorescein isothiocyanate (BD Pharmingen, San Jose, CA, USA, Cat. No 555412). The cells were resuspended in 1x Annexin Binding Buffer and labelled with 7-AAD and Annexin conjugated to phycoerythrin (PE) (PE Annexin V Apoptosis Detection Kit I, BD Pharmingen, Cat. No. 559763). The numbers of apoptotic cells were analyzed with a FACScalibur flow cytometer in combination with CellQuest (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (Tree Star Inc., Ashland, OR, USA).

Viability was assessed by setting gates based on the light scatter properties of the cells. The relative percentage of viable cells at the end of the assay was calculated using the following formula: (no. of $CD19^+$ viable cells recovered in test well/no. of $CD19^+$ viable cells in untreated well x 100). All results represent the mean of duplicate experiments.

The 50% lethal concentration (LC_{50}) was defined as the concentration of HA22 that kills 50% of the viable cells at the termination of the assay.

Cell death by apoptosis was studied for all samples and late stage apoptosis defined as cells positive for 7-AAD and Annexin-PE by flow cytometry. Non-apoptotic cells are negative for 7-AAD and Annexin-PE.

4.7 Immunotoxins and chemotherapy agents

The recombinant immunotoxins HA22, HA22-LR, HA22-LR8X, BL22 and SS1P were produced as previously described.[253] Dexamethasone was provided by the Division of Veterinary Resources (NIH).

4.8 HA22 internalisation

The time course of HA22 internalisation was compared between the REH and NALM cell lines using fluorescently labelled immunotoxins. HA22 was labelled with Alexa 647 using the Alexa-Fluro 647 Protein Labelling Kit according to manufacturer's instructions (Molecular Probes A20173, Invitrogen, Carlsbard, CA). There are about 3 moles of Alexa dye per mole of immunotoxin. To measure the internalisation rate of HA22, $1x10^6$ REH or

NALM cells were suspended in 500mL of RPMI-1640 (Invitrogen, CA, USA) with 10% Foetal Bovine Serum (Sigma-Aldrich, St Louis, MO, USA), Glutamine (1x), Sodium Pyruvate (1x) and Penicillin-Streptomycin with 1ug/ml HA22-Alexa-647 on ice for 30 minutes. The cells were then moved to a 37^{o} C heat block and incubated for 0, 15, 30, 60, 120, 240 minutes. After incubation the cells were washed with FACS buffer and stripped with 0.2M Glycine-HCl (pH 2.2, with 1mg/ml BSA) on ice for 10 minutes, to remove surface-bound HA22-Alexa-647. The cells were resuspended in 400mL of FACS buffer and analysed by with a FACS Calibur. Cells were incubated with LMB9-Alexa for 240 minutes, using the above method, as a negative control. LMB-9 is an immunotoxin which binds the Lewis Y antigen which is not expressed on ALL cells.

4.9 Protein synthesis inhibition assay

The ability of HA22 to inhibit protein synthesis in the NALM and REH cell lines was compared using a^{3}H-leucine incorporation assay. 1.5x10^{4} cells were plated into 96 well plates in 180ul of RPMI-1640. HA22 was added at final concentrations of 0, 0.1, 0.5, 1, 5, 10, 50, 100 and 500ng/ml (20µl volume). 50ng/ml SS1P was added as a negative control and the protein synthesis inhibitor cyclohexamide (10mg/ml) was added as a positive control of protein synthesis inhibition. The plates were incubated at 37^{0} C for 16hours. After 16hours the cells were pulsed with 20uL of ^{3}H-leucine at 100mCi/mL (diluted in PBS). The plates were incubated for 4 hours at 37^{0}C. After 4 hours the plates were frozen on dry ice for 30minutes, and then thawed at 37C for 1 hour. The 96 well plates were processed in a cell harvester (PerkinElmer). The dry filter mats were collected, saturated in scintillation fluid and incorporation of ^{3}H-leucine was measured using a scintillation counter.

4.10 Immunoblotting

The expression of apoptosis pathway proteins in each of the cell lines was examined by western blotting. Cells were lysed with lysis buffer (20nM Tris-Hcl pH7.5, 150nM NaCl, 2mM EDTA, 1.0% Triton X-100) containing protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IL). Cell lysates were spun at 12000rpm for 30minutes and supernatants were collected. The protein concentration in the lysates was determined by a Bradford assay. Equal amounts of protein were loaded onto 12% Tris-Glycine SDS-PAGE (Invitrogen) and transferred to PVDF membranes (Invitrogen). Hybridisation was carried out using antibodies to Mcl-1 (Cell Signaling #4572), BCl-xL (Cell Signaling #2764), Bax (Millipore/Upstate, NY 06-499), Bak (Millipore/ Upstate, NY 06-536), BCl-2 (Cell Signaling, 2876), BIM (Cell Signaling, 2819), PUMA (Cell Signalling, 4917) or to actin (Abcam, Cambridge, MA, #8226). HRP-conjugated secondary antibodies, goat anti-rabbit (Santa Cruz Biotechnology, sc-2030), sheep anti-mouse (GE Healthcare, NA-931) were used and the blots were developed using ECL substrate (GE Healthcare) and exposed on Kodak film.

In order to compare the relative expression of apoptosis pathway proteins between the cell lines, cell lysates were also loaded onto the same gel membrane. The ratio of apoptosis proteins compared to the actin was determined following analysis using ImageQuant software (BD BioSciences).

The changes in apoptosis protein expression during the first 24hours of HA22 treatment was determined. REH and NALM cell lines were cultured with 100ng/ml HA22 and aliquots of cells were taken at 0h, 4hours, 8 hours, and 24hour time points. Equal concentrations of protein were loaded into gels and immunoblotted as described above.

4.11 Improving HA22 cytotoxicity with 17-AAG and ABT-737

To measure the effect of 17-AAG or ABT-737 on the cytotoxicity of HA22 $3x10^5$ ALL cells (NALM6 or REH) were added to wells of a 96 well plate. HA22 were added at final concentrations of 0, 0.5, 1, 5, 10, 50, 100 and 500 ng/mL in duplicate wells. 1μM 17-AAG or 1μM ABT-737 was added to all wells. SS1P 50 ng/mL, an anti-mesothelin *Pseudomonas* immunotoxin, was used as a negative control as mesothelin is not expressed on ALL cells. Cells were incubated for 72 hours in a humidified atmosphere at 37^0C with 5% CO_2 and then analysed by flow cytometry as previously described.

4.12 Assessment of anti-CD22 immunotoxin uptake in vivo

CA46 cells (Burkitt's lymphoma cell line) were cultured in vitro. $10x10^6$ CA46 cells were injected subcutaneously, in combination with 4mg/ml Matrigel (BD Biosciences. #354234), into 4-6 week old female NOD-SCID mice. The tumours were allowed to grow until approximately $300mm^3$ (200mg). 100ug Alexa-647 (Molecular Probes A20173) labelled HA22-KDEL immunotoxin was administered intravenously (200μL of 0.9% NaCl with 0.2mg/ml BSA). HA22-KDEL is a variant of the HA22 immunotoxin that contains more lysine groups enabling more efficient labelling with Alexa-647, and thus increased resolution for flow cytometry. Where indicated, 50mg/kg Taxol (Division of Veterinary Resources,NIH) or 3mg/kg Adriamycin (Division of Veterinary Resources,NIH) were administered by intraperitoneal injection 72hours before the intravenous injection of HA22-KDEL-Alexa-647, to assess the effect of chemotherapy on immunotoxin penetration.

The mice were sacrificed and tumour xenografts removed surgically 3 hours post immunotoxin injection. The tumours were dissociated gently into a single cell suspension with scissors and incubated with Liberase 2 (28U/ml) (Roche, #11988425001) in 5mL of Hank's Buffered for 15mins with constant shaking at 37C. The cell suspension was washed and incubated with anti-CD19-PE antibody. The percentage of (CD19)+ (HA22-KDEL ALEXA-647)+ cells was analysed using a FACSCalibur.

4.13 AML Cell lines used in the study of T cell suppression

Acute Myeloid Leukaemia (AML) cell lines were used for experiments.

Name	Disease	Origin	Translocations
U937	AML	Pleural effusion of a 37 year old male with refractory disease	t(10:11)(p13;q14) leading to a PICALM-AF10 fusion gene
K562	AML	Bone marrow of a 53 year old female with CML in blast crisis	T(9;22)(q34;q11) leading to a BCR-ABL1 fusion gene
HL-60	AML M3	Peripheral blood of a 35year old female at diagnosis	No specific translocation
KG-1a8	AML M6	Bone marrow of a 59 year old male with erythroleukaemia	No specific translocation
MOLM-16	AML M0	Peripheral blood of a 77 year old female at relapse	No specific translocation
NOMO-1	AML M5a	Bone marrow of a 31 year old female at 2nd relapse	t(9:11)(p22:q3) leading to a MLL-AF9 fusion gene
NB4	AML M3	Bone marrow of a 23 year old female at 2nd relapse	t(15:17)(q22:q11) leading to a PML-RARA fusion gene

Table 4.3 Table of AML cell lines and their characteristics

Cell lines were cultured in RPMI-1640 (Invitrogen, CA, USA) with 10% Foetal Bovine Serum (Sigma-Aldrich, St Louis, MO, USA), Glutamine (1x), Sodium Pyruvate (1x) and Penicillin-Streptomycin. The cell lines were cultured in T-75 flasks, in a humidified atmosphere at 37^0C with 5% CO_2

4.14 Generation of human Dendritic Cells for the Mixed Lymphocyte Reaction

Dendritic cells were generated as previously described.[254] Briefly, blood from healthy donors was obtained from the UK National Blood Transfusion Service (Bristol, UK). Peripheral blood monoculear cells were isolated by density gradient centrifugation over Lymphoprerp. Monocytes were positively selected using anti-CD14 magnetic micro-beads (MACS; Miltenyi Biotec). The recovered cells were >99% CD14+, as determined by flow cytometry with the anti-CD14 antibody. Dendritic cells were generated by culturing monocytes in RPMI 1640, supplemented with 10% FCS, 50ng/ml GM-CSF, and 1000 U/ml Il-14 for 5 days, in a 6 well plate. The CD14- negative fraction was used as the responding cell fraction in mixed lymphocyte reactions, as detailed below.

4.15 Expansion of human iNKT cells

Human iNKT cells were isolated in our lab by Carmela De Santo, as previously described by McCarthy et al[255]. In short, PBMCs were isolated from healthy donors' buffy coats by density

gradient centrifugation over Lymphoprep (Axis-Shield PoC AS). Monocytes were positively selected using anti-CD14 mAb–conjugated magnetic micro-beads (MACS; Miltenyi Biotec). Monocytes were cultured with 50 ng/ml GM-CSF and 1,000 U/ml IL-4 in six-well plates at 4 × 10^5 cells/ml (3 ml/well). After 5 d, maturation was induced using bacterial LPS (1 μg/ml LPS of *Salmonella abortus equi*; Sigma-Aldrich). Immature and mature monocyte-derived DCs were phenotypically analyzed for maturation markers. 2 × 10^5 monocyte-derived DCs were pulsed with αGalCer for 2 h in 24-well plates in 200μl RPMI 1640. 2 × 10^6 autologous lymphocytes were added in 1.8 ml of medium (5% human serum). After 3 days, 25 IU/ml IL-2 was added to cultures. Thereafter, cultures were fed every 3–4 days with fresh medium containing 1,000 U/ml IL-2. iNKT cell frequencies were determined using APC CD1d tetramer and Vα24 antibody (Serotec).

4.16 AML Patient samples

Blood samples were obtained from 16 patients with Acute Myeloid Leukaemia (AML) treated at the John Radcliffe Hospital, Oxford, Hammersmith Hospital, London or Hillingdon Hospital, London with informed consent. In addition blood from 16 healthy, adult volunteers was obtained from members of the Human Immunology Unit, WIMM, Oxford following informed consent. I am Chief Investigator of the study and applied for and received Regional Ethics Committee (REC Number: 10/H0501/39) and local hospital trust ethics approval for the study. The samples were obtained from patients with newly diagnosed AML before the start of treatment and collected in Lithium Heparin tubes. AML cells were separated from fresh samples using a Lymphoprep gradient followed by purification using CD33 and CD34 Microbead kits and MACS separation columns (Miltenyi Biotec, Bisley UK).

4.17 Mixed Lymphocyte Reaction

The CD14- fraction of cells was prepared from healthy donors as described above. $2x10^5$ CD14- cells were cultured with allogenic irradiated (5000 rad) dendritic cells ($5x10^4$ rad), in 200μl RPMI 5% human serum (Sigma) in 96 well flat bottom plates. Cells were incubated at 37^0C, 5% CO_2 for 4 days and then 1μCi/ well ^{3}H-thymidine (Perkin Elmer Life Sciences) was added for 12-16 hours. ^{3}H-thymidine incorporation was measured using a Wallac Microbeta Jet 1450 reader (Perkin Elmer). AML cell line-mediated inhibition of lymphocyte proliferation was carried out by co-culturing irradiated cell lines together with the CD14- cells and irradiated DCs. The suppressive ability of AML blasts from patients was tested in the same manner, following their isolation as described previously.

To test the ability of iNKT cells to overcome AML-induced suppression of T cell proliferation. $0.25x10^5$ iNKT cells were first co-cultured with AML cell lines or AML blasts from patients for 8hours, in the presence of αGalCer (0.1ug/ml). After 8 hours the cells were washed and CD14- cells and dendritic cells were added to cultures according to the protocol above.

Data are expressed as a percentage of CD14- cell proliferation driven by allogenic irradiated DCs in the presence of AML cells, as compared to allogenic PBMC proliferation in the absence of AML cells (100%).

4.18 Immunophenotyping

AML cell lines and isolated patient-derived AML blasts were stained with anti-CD15-FITC antibody (11-0159-73, eBioscience), anti-CD14-APC antibody (555399, BD Bioscience),

anti-CD33-PE antibody (12-0338-71, eBioscience) anti-CD34-APC antibody (17-0349-41, eBioscience) and anti-CD11b-PE antibody (12-0112-81, eBioscience) on ice for 30minutes. The cells were then washed. Propidium iodide (1:200 final concentration) was added prior to analysis to allow viable cells to be gated. The immunophenotype was assessed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, CA) in conjunction with CellQuest software. Data was analysed using FlowJo 8.8 software for Mac (Tree Star Inc).

4.19 RT-PCR analysis

RT-PCR was used to detect Arginase I, Arginase II, iNOS, FPLR2, TLR2, TLR4 and S100A9 in cell lines and patient-derived AML blasts. RNA was extracted using an RNeasy Mini kit (Qiagen). Briefly cells were disrupted using Buffer RLT and the lysate was homogenised using a 20-gauge needle and syringe. 70% ethanol was added to the homogenised lysate and transferred to an RNeasy spin column, placed in a collection tube. The column was centrifuged for 15s at 8000g. DNAase I was added to the spin column to eliminate any DNA contamination. The column membrane was washed by centrifuging with Buffer RW1 for 15s at 8000g and the repeating twice with Buffer RPE. To elute the RNA the RNeasy spin column was washed with RNase-free water and centrifuged for 1 minute at 8000g.

First strand cDNA was synthesised from the extracted RNA using the following method. 1ul of oligo$(dT)_{20}$ (50μM), RNA (from the above extraction) and 1ul of 10mM dNTP Mix (10mM of dATP, dGTP, dCTP, and dTTP each, at neutral pH) was added to a nuclease-free microcentrifuge tube. The mixture was heated to 65^{o}C for 5 minutes and then kept on ice. To this tube 5x First-Strand Buffer, 0.1M MDTT, 40 units Recombinant RNase Inhibitor and

200 units of SuperScript III RT was added. The mixture was incubated at 50^{o}C for 30minutes, before inactivation of the reaction by heating the mixture to 70^{o}C for 15 minutes. This cDNA can now be amplified in a PCR

A PCR reaction tube was created containing 10X PCR buffer (200nM Tris-Hcl, pH 8.4, 500mM KCl), 50mM $MgCl_2$, 10mM dNTP Mix, Taq DNA polymerase, cDNA, and distilled water. Forward and reverse primers were added to this tube (sequences below). The conditions for PCR amplification were as follows: 94 degrees 5mins followed by 40 cycles of 94 degrees 1 minute, annealing for 1 minute at 60 degrees and elongation for 1.5mins at 72 degrees. Final elongation step for 10minutes at 72 degrees. The PCR products were analysed by gel electrophoresis on a 2% agarose gel and were visualized by staining with ethidium bromide.

Target	Forward Primer	Reverse Primer
Arginase I	5'CTCTAAGGGACAGCCTCGAGGA3'	5'TGGGTTCACTTCCATGATATCTA3'
Arginase II	5'ATGTCCCTAAGGGGCAGCCTCTCGCGT3'	5'CACAGCTGTAGCCATCTGACACAGCTC3'
iNOS	5'CGGTGCTGTATTTCCTTACGAGGCGAAGAAGG3'	5'GGTGCTGCTTGTTAGGAGGTCAAGTAAAGGGC3'
FPLR2	5'GCTGACTTTTCTTTCACGGC3'	5'ACGTAAAGCATGGGGTTGAG3'
TLR2	5'GCCAAAGCTTTGATTGATTGG3'	5'TTGAAGTTCTCCAGCTCCTG3'
TLR4	5'TGCGGGTTCTACATCAAA3'	5'CCATCCGAAATTATAAGAAAAGTC
S100A9	5'CAGCTGGAACGCAACAT3'	5'CCACAGCCAAGACAGTTT3'
GAPDH	5'CCAGCCGAGCCACATCGCTC3	5'ATGAGCCCCAGCCTTCTC3'

Table 4.4 Primers used in RT-PCR analysis of AML cells

4.20 Immunoblotting

The expression of arginase I, arginase II and S100A9 proteins in each of the cell lines was examined by western blotting. Cells were lysed with lysis buffer (20nM Tris-Hcl pH7.5, 150nM NaCl, 2mM EDTA, 1.0% triton X-100) containing protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IL). Cell lysates were spun at 12000rpm for 15 minutes and supernatants were collected. The protein concentration in the lysates was determined by Coomassie blue assay. Equal amounts of protein were loaded onto 12% Tris-Glycine SDS-PAGE (Invitrogen) and transferred to PVDF membranes. Hybridisation was carried out using antibodies to Arginase I (N-20, sc-18351 Santa Cruz), Arginase II (H-64, sc-20151, Santa Cruz), S100A9 (Abcam, ab75478), and actin (Sigma). HRP-conjugated secondary antibodies, goat anti-rabbit (Cell Signalling), and sheep anti-mouse (GE Healthcare) were used and the blots were developed using ECL substrate (GE Healthcare) and exposed on Kodak film.

4.21 Arginase Enzyme activity

The activity of arginase II present within AML blasts from patients or in cell line supernatant was determined by measuring the conversion of arginine into urea. $1x10^6$ cells of each cell line or patient blasts were cultured in RPMI+10% FCS in the presence or absence of L-NMMA (Calbiochem) at 37^0C, 5% CO_2. After 24 hours the supernatants were collected and the cells pelleted. Buffer (0.1% Triton X-100, 5μg pepstatin, 5μg aprotinin and 5μg antipaina) was added to the cell pellets or 50υl of cell supernatants. The samples were placed

on a 37^{o}C heat block for 30minutes before centrifugation at 14000rpm and collection of the supernatants. To activate the arginase enzyme buffer containing Tris-Hcl (25mM) and $MnCl_2$ (10mM) was added to the reaction mixture and heated to 56^{o}C for 10minutes. 0.5M L-arginine (Sigma) was added to each mixture and the samples were heated for 1 hour at 37^{o}C. The hydrolysis of arginine by arginase II was stopped by adding 800µl of an acid solution mixture (H_2SO_4-H_3PO_4-H_2O, 1:3:7). The amount of urea produced was determined using 9% a-isonitrosopropriophenone and compared to a standard curve. Absorbance was measured at a wavelength of 540nm using a spectrophotometer.

4.22 Monocyte polarisation assay

Peripheral blood was collected from healthy donors and mononuclear cells were separated from fresh samples using a Lymphoprep gradient. Monocytes were purified using anti-CD14 magnetic-beads and MACS separation columns (Miltenyi Biotec, Bisley UK). Monocytes were cultured in the presence of absence of AML cells, AML culture supernatants or patient plasma for 48 hours. After 48hours cells were harvested and incubated with anti-CD206-PE (555954, BD Pharmingen) on ice for 30minutes. The cells were then washed. Propidium iodide was added prior to analysis to allow viable cells to be gated. The expression of CD206 on monocytes was assessed by flow cytometry using a FACSCalibur flow cytometer in conjunction with CellQuest software. Data was analysed using FlowJo software.

4.23 Nitric Oxide Species Production

Total nitrite and nitrate concentrations in cell line supernatants or patient-derived AML blast cultures was measured using a colourimetric assay based on the Greiss reaction (780001, Cayman Chemical Company). Briefly, nitrates were converted to nitrites through the addition of nitrate reductase. Nitrites were then converted into a purple compound, through the addition of the Greiss Reagents (Sulfanilamide and N(1-Napthyl) ethylenediamine). Absorbance was measured at 540nm and a standard curve was generated using the absorbance measurements. Sample absorbance was compared to the standard curve to give final concentrations.

4.24 ELISA

Supernatants of cell lines or patient-derived AML blasts cultured in RPMI+10% FCS were collected after 24 hours or serum was diluted accordingly. ELISA kits were used to measure the concentration of SAA (KHA0012, Invitrogen),CRP (88-7502-28 eBioscience),S100A9 (414828, AntibodiesOnline), Arginase I (414563, AntibodiesOnline) and Arginase II (414194, AntibodiesOnline) according to the manufacturers' instructions.

To measure the concentrations of Il-1β, IL-10, GM-CSF and TGF-β sandwich ELISAs were set-up. Briefly, ELISA plates (MAXISORP 96-well immunoplates, Nunc) were coated with primary anti-cytokine antibody diluted in ELISA coating buffer. Plates were incubated overnight at 4^{o}C. The next day plates were washed 5 times with washing buffer and blocked with PBS+10%FCS for 1 hour at 37^{o}C. Cytokine standards were prepared, using serial 1:2 dilutions. Samples and standards were added to the wells and kept overnight at 4^{o}C. Plates

were then washed with washing buffer and biotinylated anti-cytokine detection antibody added to each well. The plates left at room temperature for 1hour in the dark, and then washed 5 times with washing buffer.

To develop the ELISA avidin-peroxidase (eBioscience) was added to each well and the plates incubated for 30minutes in the dark. Plates were washed 5 times in washing buffer, and one further time with PBS. TMB substrate was added and the plate covered. Once colour had developed the reaction was stopped through the addition of H_2SO_4 to each well.

In all cases absorbance was measured at 450nm (unless indicated by manufacturer's instructions) and a standard curve was generated using the absorbance measurements. Sample absorbance was compared to the standard curve to give cytokine concentrations.

4.25 Chemotaxis assay

$5x10^5$ U937 cell lines were labelled with CMDA for 30minutes and dispensed in the upper chamber of 24-transwell plates containing a FluoroBlok membrane with 3um pore-size (VWR, BD Biosciences). RPM+10% FCS with or without recombinant 10ug/ml Serum Amyloid A (Peprotech 300-13) was added to the lower chamber. Plates were incubated for 16hours at 37^oC. Numbers of U937 cells transmigrating through the filter into the lower chamber were recorded at 10 minute intervals on an automated fluorescent multiprobe plate reader (Synergy HT; Bio-Tek) at 37^oC using KC^4 software (Biotech). The fluorescent signal was calibrated against a standard curve and transmigration was as the percentage of cells that migrated from the upper chamber.

4.26 AML Murine Xenografts

Ten to 14 week old NOD/SCID mice were irradiated (100-125 cGy twice, 4 hours apart). 24 hours later 10^2-10^5 AML blasts, from AML patients at the John Radcliffe, were injected into the tail vein of the mice. Mice were killed at time-points up to 12 weeks to assess for different levels of human AML engraftment. Bone marrow from the femurs of the mice was extracted using a syringe and forceps, and re-suspended in RPMI 1640. Human AML engraftment was assessed by flow cytometry gating on CD45+CD33+ cells in conjunction with CellQuest software. Monocytes were gated on by flow cytometry, identifying Ly6C+CD33+ cells and CD206+ expression. Data was analysed using FlowJo software.

5. The cytotoxicity of HA22 against CD22+ haematological malignancies

5.1 Introduction

Immunotoxins represent a potential new approach to the treatment of haematological malignancies. First generation anti-CD22 immunotoxins (BL22) have entered Phase I trials in adults with Hairy Cell Leukaemia and a Phase I trial in children with Acute Lymphoblastic Leukaemia has recently finished. In this Chapter I aim to understand the activity of the second-generation anti-CD22 immunotoxin (HA22) against paediatric ALL.

To do this a stromal-cell based assay which enables the study of ALL blasts from patients in vitro by flow cytometry was established. The activity of HA22 against ALL blasts isolated from patients at diagnosis and relapse and secondly from bone marrow and peripheral blood was compared. In addition the mechanism of cytotoxicity of this novel agent was examined. These results will form the basis of future studies of HA22 in vitro and in vivo, and provide a pre-clinical rationale for a Phase I trial of HA22 in paediatric patients with CD22+ malignancies.

5.2 Results

5.2.1 Assessing ALL cell death by flow cytometry

The change in forward and side scatter properties of ALL blasts when assessed by flow cytometry was used to measure anti-CD22 immunotoxin cytotoxicity. Viable cells have increased cell size (forward scatter) and granulosity (side scatter) compared to cells undergoing necrosis or apoptosis. The flow cytometry method described has been established for the testing of cytotoxic drugs against human ALL.[256] It also allows accurate differentiation of ALL cells from the bone marrow stromal cells used in later murine and human studies. ALL cells can be differentiated from stromal cells by initial labelling with anti-CD19 antibody and then gating to assess viability by cell light scatter properties. Figure 5.1 shows a typical experiment where flow cytometry is used to distinguish viable and non-viable ALL cells in an untreated sample and a sample treated with 500ng/ml HA22.

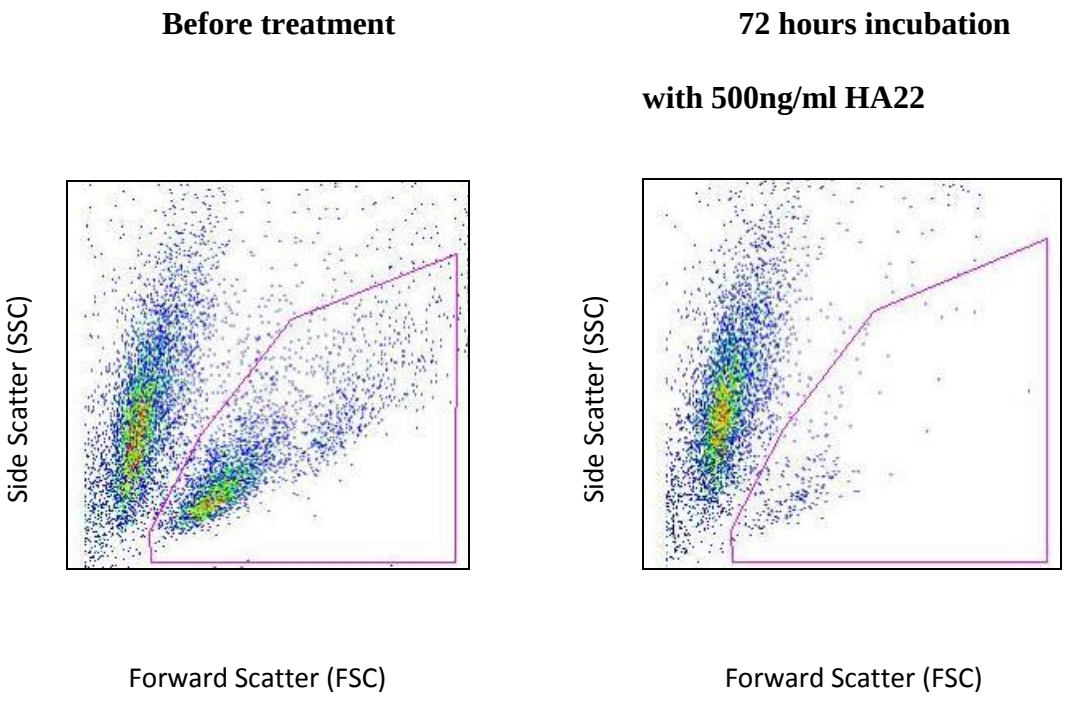


Figure 5.1 HA22 induced cell death of human ALL blasts cultured using a bone marrow stromal cell assay. Assessment of ALL blast viability by flow cytometry. Dot plots of forward light scatter (x axis; cell size) and side light scatter (y axis; cell granularity). A gate is shown imposed over the area representing viable ALL cells. The number of viable ALL cells decreases after 72hours with 500ng/ml HA22

5.2.2 Activity of HA22 against CD22+ cell lines

Cell lines provide a well-established model to test efficacy of novel drugs against haematological malignancies. To determine if HA22 has activity against CD22+ B cell malignancies the cytotoxicity of HA22 against 5 ALL cell lines and 2 Burkitt's lymphoma cell lines was tested. No stromal cell assay is required for these experiments because cell lines can be successfully cultured in tissue culture flasks. All cell lines express CD22 on the cell surface (see later) making them a suitable model for in vitro testing of the immunotoxin. Concentrations of immunotoxins ranging from 0ng/ml to 500ng/ml were chosen, based on

data from paediatric clinical trials of BL22 and HA22 which showed that the majority of patients achieved peak blood levels in this range.

Six cell lines tested were very sensitive to HA22, with LC_{50}s of 0.2-0.6ng/ml (Fig 5.2 and Table 5.1). NALM6 was less sensitive to HA22 with an LC_{50} of 10ng/ml. In all cases there was greater than 95% cell death with 500ngml HA22, indicating that the vast majority of cells were sensitive to HA22-induced cell death.

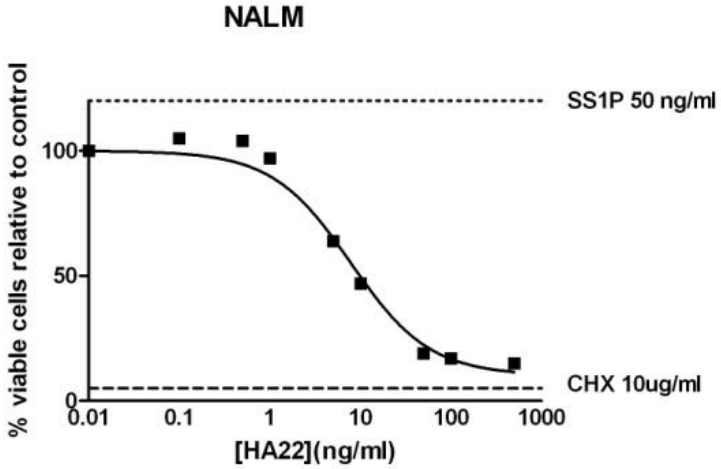


Figure 5.2 Representative cytotoxicity curve for HA22 against the NALM6 cell line, as measured by flow cytometry. 10ug/ml cyclohexamide (CHX) and 50ng/ml SS1P were used as positive and negative controls respectively. Data points are the means of triplicate wells. The experiment was repeated 3 times.

Cell Line	LC_{50} (ng/ml)
KOPN-8	0.2
NALM-6	10
REH	0.5
EU-1	2
SEM	0.15
CA46	0.5
RAJI	2

Table 5.1 Table of LC_{50} for HA22 against CD22+ ALL and lymphoma cell lines

5.2.3 The effect of a stromal cell feeder layer on CD22 site density.

ALL blasts from patients have significantly reduced viability if cultured in vitro, with many cells dying within 24hours of culture. One approach to improve the viability of ALL blasts is to culture the cells on a feeder layer of bone marrow stromal cells.[252] Using a stromal cell assay will reduce spontaneous cell death of human the ALL blasts enabling the specific cytotoxicity of immunotoxins to be tested. The effect of stromal-based culture on ALL cell CD22 site density was assessed before starting cytotoxicity assays. Although there was a decrease in CD22 site density when cell lines were cultured on stroma the effect was not significant for the majority of cell lines ($p=0.27$) (Table 5.2).

Cell line	CD22 sites/ cell	
	No stroma	Grown on stroma
KOPN-8	19,323	16,560
EU1.3	2,000	1,673
REH	10,391	6,726
NALM6	9,907	9,632
SEM	12,591	11,321
CA46	76,281	45,858
RAJI	62,000	54,733

(Stroma is CD22 negative)

Table 5.2. Culture on stroma does not significantly affect CD22 cell density on cell lines. Cell lines were cultured in the presence or absence of stromal layers for 72hours. CD22 site density was determined by flow cytometry using the QuantiBRITE system. Data points are the means of triplicate wells. The experiment was repeated 3 times.

5.2.4 Cytotoxicity of HA22 against human ALL murine xenografts

Murine xenografts provide a readily available source of human ALL cells. ALL blasts from four patients were engrafted in NOG-SCID mice to form human paediatric ALL murine xenograft models. To determine if HA22 had activity against human ALL, ALL blasts were harvested from the spleens of the xenografted mice. The blasts were then cultured in the presence of HA22 using the bone marrow stromal assay.

In the cell line assays, HA22 was incubated with the cells for 72 hours before assessing viability. To assess if the 72 hour time point was also appropriate in the setting of the bone

marrow stromal cell assay, blasts from the xenografts were cultured with HA22 for different periods of time. Figure 5.3a shows the cytotoxicity curves for xenograft 9 incubated with HA22 for different periods of time. The LC_{50}s did not differ significantly (0.35ng/ml, 0.55ng/ml, 0.55ng/ml, p=0.4) and the percentage of viable cells at 500ng/ml was constant at 5%. Therefore 72 hours after treatment with immunotoxin was chosen as the time-point to examine ALL cell death, in human cells, . Importantly HA22 appeared very active in causing death, of ALL blasts derived from human pre-B ALL mouse xenografts, with LC_{50}s between 1-20ng/ml (Fig 5.3b.)

(a) **(b)**

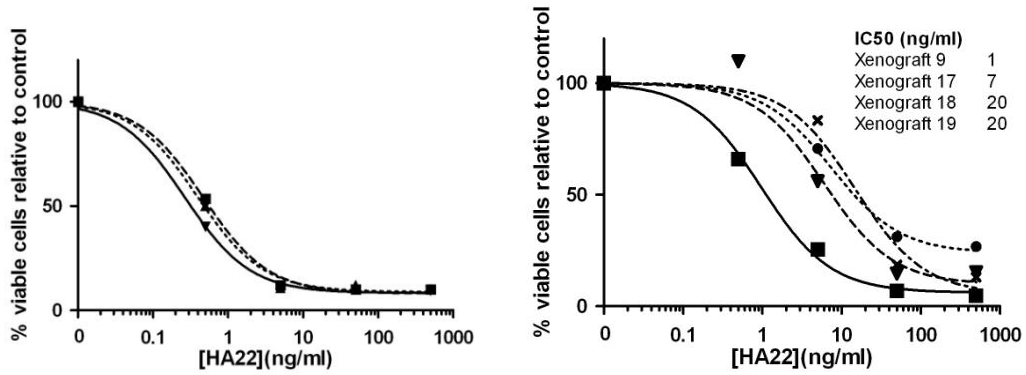


Figure 5.3 HA22 is cytotoxic against ALL blasts from murine xenografts. (a)HA22 cytotoxicity is not time-dependent.Cells from xenograft 9 were incubated with HA22 for 48 hours (dashed line), 72 hours (solid line), 96 hours (dotted line). No significant difference was seen in the LC_{50} (p=0.4) or the percentage of viable cells remaining at each dose. (b) HA22 LC_{50}s are case dependent.Cytotoxicity of HA22 against 4 murine xenografts after 72hours. LC_{50}s: Xenograft 9 (solid line), xenograft 17 (dotted line), xenograft 18 (dashed line), xenograft 19 (dot-dash line).Data points are the means of triplicate wells. The experiment was repeated 3 times.

5.2.5 Cytotoxicity of HA22 against paediatric ALL cells

Having established the efficacy of HA22 against CD22+ cell lines and human ALL blasts from murine xenografts, cytotoxicity against patient ALL blasts was assessed. The cytotoxicity data of HA22 against 35 cryopreserved patient samples is summarized in Fig 5.4. and Fig 5.5 Samples varied in their sensitivity, ranging from 100% cell death to almost complete resistance to death at 500 ng/mL HA22. Representative cytototoxicity curves demonstrating the variation in HA22 response are shown in Fig 5.4. Greater than 75% cell death was achieved in 18 of 33 patient samples tested. There was no significant difference in LC_{50} or the percentage of viable cells after HA22 against blasts from relapsed and newly diagnosed patients (Fig 5.5, p=0.69 and p=0.80, respectively). Of the 22 relapse samples, 8 were very sensitive to HA22 with LC_{50} less than or equal to 5 ng/mL, 7 had a moderate response with LC_{50}s ranging from 18-80 ng/mL and 7 samples were more resistant to HA22 with 50% cell death not achieved. Cells from 13 newly diagnosed patients also showed a range of sensitivities to HA22. LC_{50}s ranged from 0.3-60 ng/mL (median 3 ng/mL) in 9 samples, with 7 showing extreme sensitivity (LC_{50}s $\leq$ 3 ng/mL). Four samples appeared resistant to HA22 and 50% cell death was not achieved at the 500 ng/mL concentration. The median 50% lethal concentration was 20 ng/mL for all patient samples tested. The results indicate that HA22 has efficacy in both chemotherapy naive and drug resistant ALL blasts from patients, at immunotoxin concentrations achievable in patients.

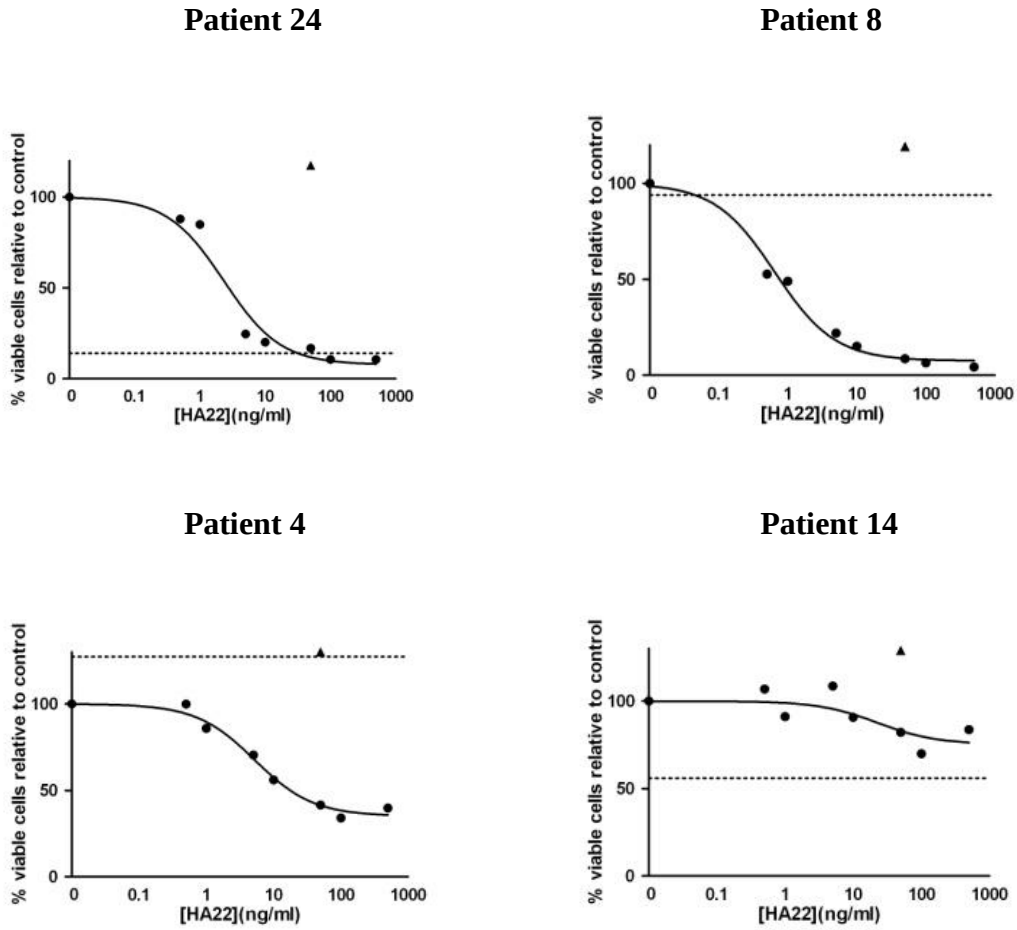


Figure 5.4 Representative cytotoxicity curves showing the patterns of response of ALL patient cells to HA22. ALL blasts from patients were cultured on stromal layers, and the number of viable cells determined after 72hours in the presence of HA22. 10ug/ml cyclohexamide (---) and 50ng/ml SS1P (▲) were used as positive and negative controls respectively. Data points are the means of triplicate wells. The experiment was repeated 2 times.

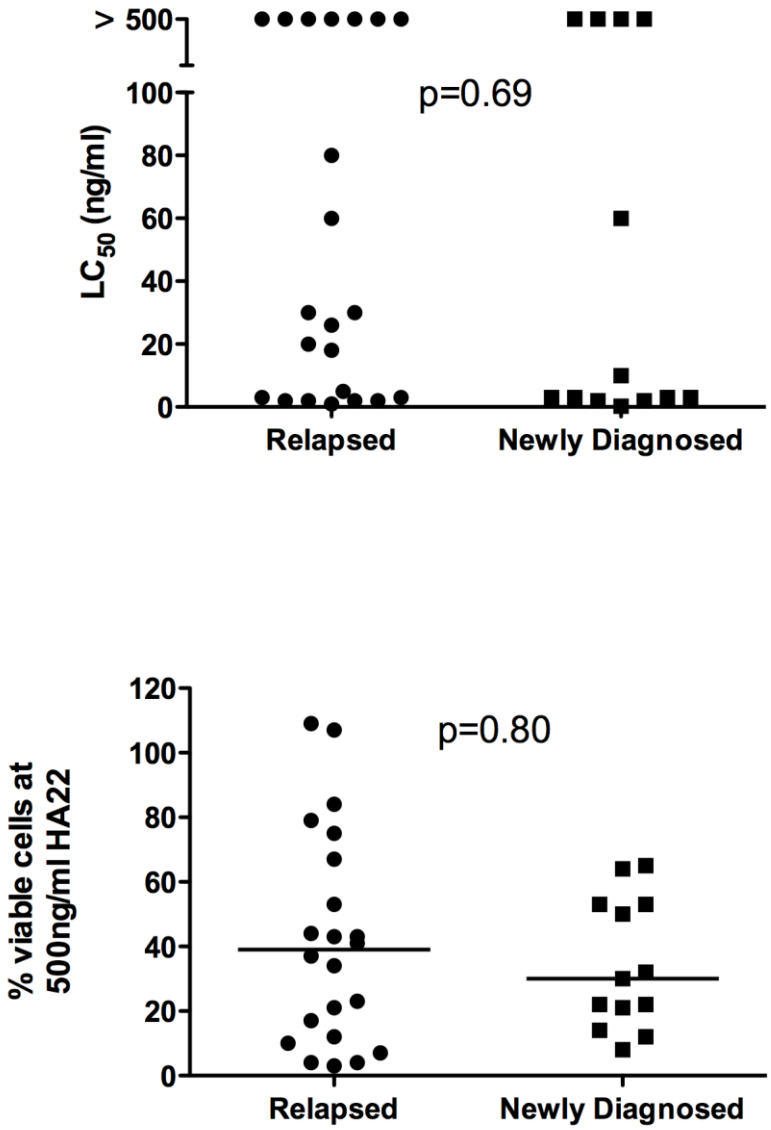


Figure 5.5 HA22 is cytotoxic to the majority of ALL blasts from patients, with no significant difference between blasts from newly diagnosed or relapsed patients. ALL blasts from patients were cultured on stromal layers, and the number of viable cells determined after 72hours in the presence of HA22. LC_{50}s were calculated from plotting cytotoxicity curves. Data points are the means of triplicate wells. The experiment was repeated 2 times.

5.2.6 Relation between sensitivity to HA22 and clinical and cellular features

Having established that HA22 is cytotoxic to patient blasts, correlation between the response to HA22 in vitro and a number of clinical and cellular characteristics was assessed. There was no apparent association between HA22 cytotoxicity and the age, sex, diagnostic white cell count or patient outcome following standard chemotherapy. As CD22 is the cell surface target for the immunotoxin the relationship between response to HA22 and number of CD22 sites per cell was studied. CD22 site density, measured in 19 samples, ranged from 451-15,217 (median 4,063), and was only weakly correlated with HA22-induced cytotoxicity (r=0.33, p=0.16) (Fig 5.6). The result shows that HA22 activity is not dependent on CD22 site density and that cell death is induced even in blasts with low expression of CD22.

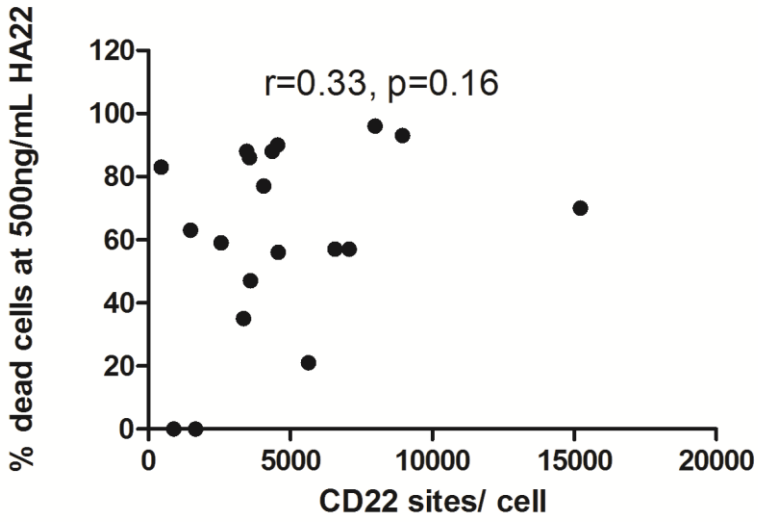
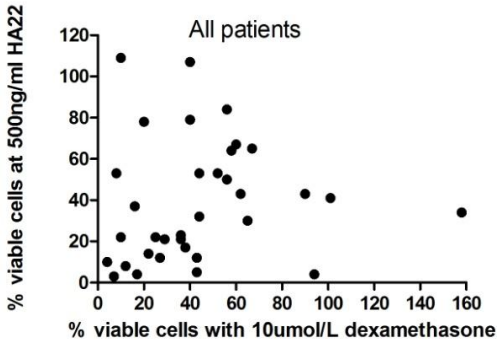


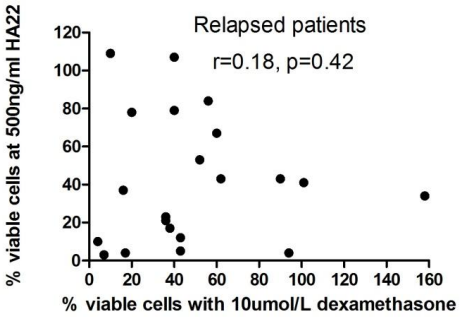
Figure 5.6 CD22 expression in patients is varied and is weakly correlated with HA22 cytotoxicity. CD22 site density was determined by flow cytometry of thawed patient ALL blasts, using the QuantiBRITE system.Data points are the means of triplicate wells. The experiment was repeated 2 times.

Treatment of patients with dexamethasone is a fundamental part of chemotherapy regimens for patients with ALL. Ten mmol/L dexamethasone had previously been shown to induce apoptosis in the majority of ALL patient samples cultured on stromal cells.[257] In addition the resistance of ALL blasts to dexamethasone-induced cell death is known to predict resistance to other chemotherapy agents. The cytotoxicity of HA22 was compared to dexamethasone. Different patterns of cytotoxicity were seen and patient samples showed a range of sensitivities to dexamethasone. The percentage of viable ALL cells at 10 mmol/L dexamethasone ranged from 4%-158%, with a median of 40% (median of 29% for ALL samples from diagnosis and 42% for relapse samples, p=0.2). HA22 was equally or more cytotoxic than dexamethasone in 17 patient cases. Resistance to HA22 did not correlate with dexamethasone resistance (r=0.29, p=0.09) (Figure 5.7a). HA22 can be cytotoxic to both dexamethasone resistant and dexamethasone sensitive ALL blasts in a dose-dependent manner. Resistance to dexamethasone correlated with resistance to HA22 for newly diagnosed patients (r=0.55, p=0.05), but not for relapsed patients (r=0.18, p=0.42) (Fig 5.7b and c). The result is important as it shows HA22 remains active in blasts which are already resistant to conventional chemotherapy, supporting its potential as novel therapy for ALL.

(a)



(b)



(c)

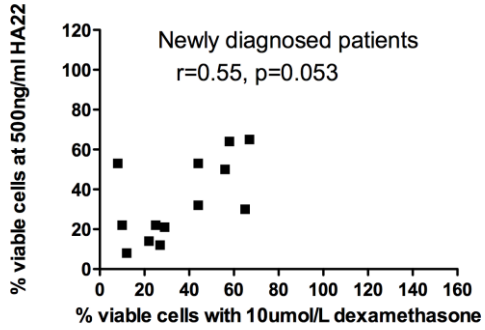
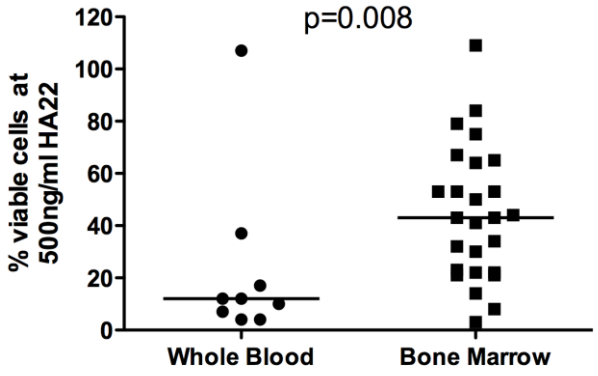


Figure 5.7 Correlation of HA22 and dexamethasone induced cell death for ALL blasts from all patient samples (a), relapsed patient samples (b), and newly diagnosed patient samples (c). ALL blasts from patients were cultured with 500ng/ml HA22 or 10umol/L dexamethasone on stromal cells. The percentage of viable ALL blasts was determined by flow cytometry after 72 hours.Data points are the means of triplicate wells. The experiment was repeated 2 times.

To assess whether the anatomical origin of ALL blasts might affect response to HA22, samples from peripheral blood (9) and bone marrow (26) were compared for cytotoxicity (Fig 5.8). In general, peripheral blood ALL blasts were more sensitive to HA22 than bone marrow blasts (3.6-fold difference in the percentage of viable cells, p=0.008). There was no significant difference in sensitivity of blasts from blood or bone marrow to dexamethasone (p=0.27).

(a)



(b)

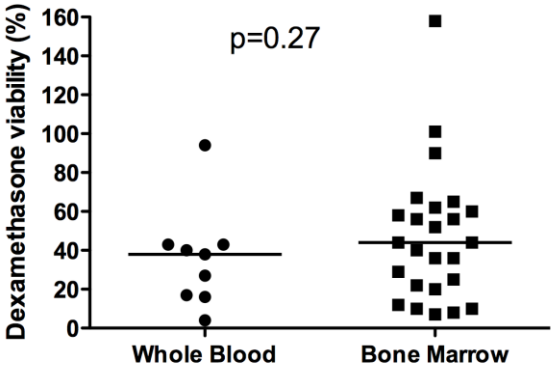


Figure 5.8 Peripheral-blood derived ALL blasts are more sensitive to HA22-induced cytotoxicity (a), but not dexamethasone (b), than bone marrow blasts. ALL blasts collected from peripheral blood or from bone marrow samples of patients at diagnosis and cultured with 500ng/ml HA22 or 10umol/L dexamethasone, on stromal cells for 72hours. The percentage of viable cells after 72 hours was determined by flow cytometry. Data points are the means of triplicate wells. The experiment was repeated 2 times.

To confirm that HA22 and dexamethasone were not cytotoxic to the stromal cells, confluent stromal cells were incubated with these agents for 72 hours (500 ng/mL and 10 μmol/L, respectively). At the end of the assay the stromal cells were incubated with the cell proliferation reagent WST-1 (Roche). There was no difference in stromal cells incubated with HA22 or dexamethasone compared to untreated controls (data not shown). The stromal layers also remained intact, without obvious morphological changes when examined under microscopy.

5.2.7 Specificity of HA22 cytotoxicity and mechanism of action

To ensure that the cytotoxicity observed was due to specific binding of HA22 to CD22, an anti-mesothelin *Pseudomonas* immunotoxin SS1P was used as a negative control. SS1P at 500 ng/mL showed no cytotoxicity.

Cell death following exposure to chemotherapy agents can occur mainly through three mechanisms: necrosis, apoptosis, and more rarely autophagy. Each mechanism is characterised by a unique set of cellular processes and markers. In necrosis there is early loss of the integrity of the plasma membrane, whereas in apoptosis (programmed cell death) membrane integrity persists until much later and there is accompanying nuclear fragmentation. Phosphatidylserine is present almost exclusively on the cytoplasmic surface of the plasma membrane and is exposed early in apoptotic cell death. The protein Annexin V is able to bind with great specificity to phosphatidylserine. 7-amino-actinomycin D (7-AAD) is

a fluorescent compound with strong avidity for DNA once the nuclear membrane has become permeable.

ALL cells were analyzed for externalization of membrane phosphatidylserine and 7AAD binding to measure apoptosis. In all cases HA22 caused a dose-dependent increase in the percentage of cells undergoing apoptosis. A representative plot is shown in Figure 5.9. There was close correlation of the percentage of viable ALL cells after treatment between light scatter properties and annexin/7-AAD staining for all samples. The result identifies apoptosis as the mechanism of HA22 induced cell death in ALL cells.

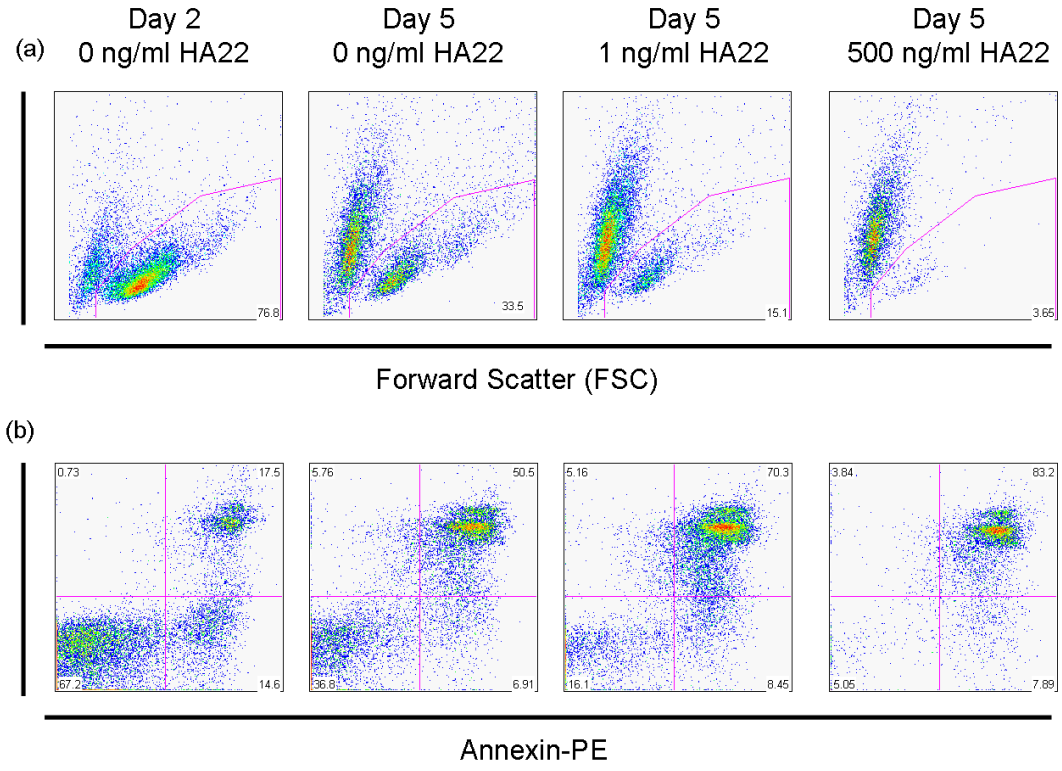


Figure 5.9 HA22 causes dose-dependent apoptosis in paediatric ALL cells as measured by flow cytometry. Example data for one patient. (a) Decreasing percentages of viable ALL cells (gated population) as determined by light scatter properties. (b) Decreasing percentages of viable cells and increasing percentages of apoptotic cells as determined by annexin/ 7AAD staining for the same patient samples. Representative data from 3 separate experiments

5.2.8 Comparison of HA22 with BL22, HA22-LR and HA22-LR-8X

BL22 is a first generation anti-CD22 immunotoxin which has completed clinical trials at the NCI. The activity of HA22 was compared to the lower affinity immunotoxin BL22 in a subset of samples found to be sensitive to HA22. As expected, HA22 was more active than BL22 in all samples tested (Table 5.3).

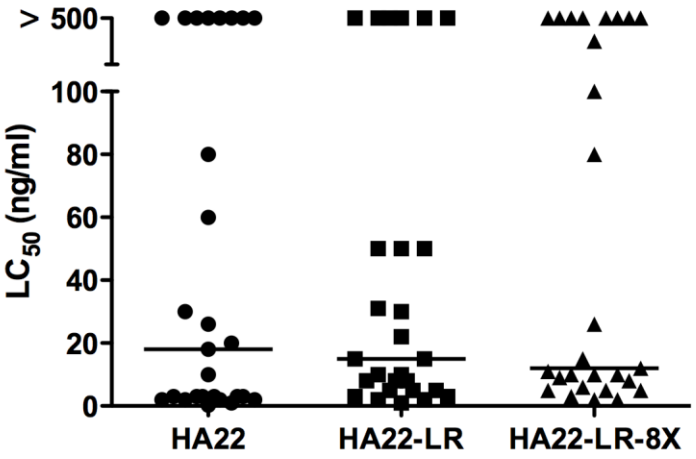
Patient	LC_{50}(ng/ml)		LC_{50} ratio (ng/ml)
	BL22	HA22	BL22/HA22
5	20	3	6.7
9	3	2	1.5
10	3	2	1.5
20	20	5	4.0
22	Not achieved	60	>8.3
29	1	0.3	3.4

Table 5.3 Comparison of BL22 and HA22 cytotoxicity against patient ALL blasts

HA22-LR is a third generation immunotoxin engineered to remove all protease-susceptible cleavage sites from domains II and Ib conferring resistance of the immunotoxin to lysosomal degradation. HA22-LR-8X incorporates the mutations of HA22-LR with additional engineered mutations to remove 8 major B cell epitopes, in order to reduce immunogenicity. Cytotoxicity assays were performed to compare the cytotoxicity of these next generation immunotoxins to HA22 (Figure 5.10). The cytotoxicity of the next generation immunotoxins was compared to HA22 using a one-way ANOVA. There was no significant difference with regard to the LC_{50} ($F= 1.902$, $R^2= 0.06816$, $p=0.1595$). or the percentage of viable cells remaining at 500ng/ml of immunotoxin ($F= 2.025$, $R^2= 0.07224$, $p=0.1423$). The results

indicate that the next generation immunotoxins maintain their cytotoxic activity against ALL blasts but have the advantages of reduced lysosomal degradation and reduced immunogenicity.

(a)



(b)

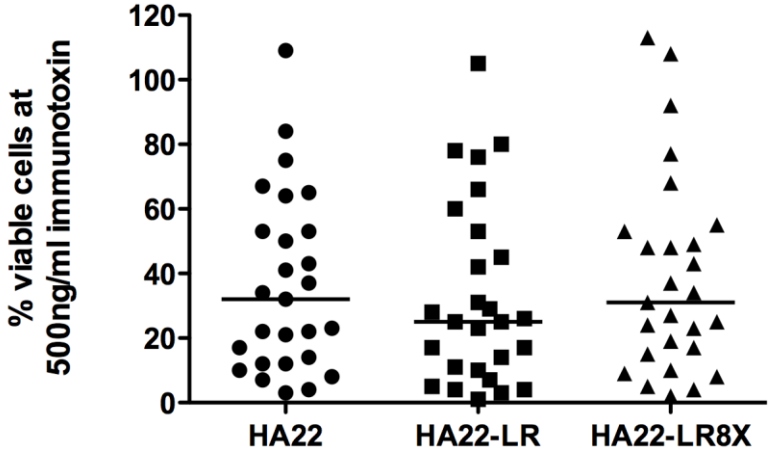


Fig 5.10 Comparison of HA22, to HA22-LR, HA22-LR8x cytotoxicity against human ALL blasts. No significant difference in the cytotoxicity of the next-generation immunotoxins compared to HA22. ALL blasts were cultured on stromal cells in the presence of HA22, HA22-LR, or HA22-LR8X. The LC_{50} (a) and the percentage of viable cells at 500ng/ml of immunotoxin (b) were compared. Data points are the means of triplicate wells. The experiment was repeated 2 times.

5.3 Discussion

The aim of this study was to assess the cytotoxicity of HA22 against B-lineage ALL blasts from paediatric patients. I hypothesised that HA22 would kill paediatric pre-B ALL blasts in a dose-dependent manner at concentrations achievable in patients. The data above is the first in vitro evidence that this novel anti-CD22 *Pseudomonas* immunotoxin is cytotoxic to blasts from children with B-precursor ALL, the most common paediatric cancer subtype. The study also establishes the mechanism of the observed cytotoxicity. The majority of blasts were sensitive to HA22 and cell death occurred via apoptosis. Furthermore, HA22 was cytotoxic to relapsed, newly diagnosed and dexamethasone-resistant patient samples.

Although anti-CD22 immunotoxins have been shown to have activity in adults with hairy cell leukaemia, efficacy against paediatric ALL has not been demonstrated.[258] LC_{50} values ranged from 1-80 ng/mL, which are well below the plasma level achieved in paediatric patients with ALL treated at the upper dose levels on Phase I trials of BL22 (85-500 ng/mL)[159] and HA22 (range 311-586 ng/mL) (Phase I trial ongoing). Additionally, HA22 was cytotoxic to blasts expected to be chemotherapy-resistant, such as those from relapsed patients and those resistant to dexamethasone. These data suggest that the mechanisms of resistance to standard chemotherapy are different than those for PE38-induced death.

HA22-induced cytotoxicity of ALL blasts occurred by apoptosis. PE38-mediated cell death has been shown to occur by induction of apoptosis and by non-caspase mediated mechanisms.[259,260] HA22 had greater activity than BL22, in one case a 10-fold decrease in the

LC_{50}. Increasing the affinity of the immunotoxin for CD22 appears to increase the cytotoxicity and results are consistent with those observed in CLL.[160]

Across the 35 samples tested, a heterogeneous sensitivity to HA22 was observed. In 11 cases less than 50% of the ALL blasts were killed even at 500ng/ml of HA22. Understanding which patients are likely to respond to immunotoxin therapy is important for the clinical development of this agent. To date the reasons why some blasts are more resistant to HA22 have not been investigated. Resistance to immunotoxins could be affected by variation in the rates of internalization between patient samples. Du *et al*. found that for different lymphoma cell lines the majority of BL22 was internalized within 15 minutes. However, in those studies the number of CD22 sites per cell ranged from 26,000-94,000.[261] The number of CD22 sites per cell in these studies ranged from 451 to 15,217 and site density was only weakly correlated with HA22-induced cytotoxicity. Thus, the number of CD22 sites available for immunotoxin binding may be a factor in ALL blast sensitivity to HA22. After internalization the PE38 toxin must be cleaved from the Fv fragment and transported into the cytosol. It is possible that the efficiency of intracellular processing and the localization of immunotoxin to endocytic or lysosomal compartments affect the response to HA22. The importance of lysosomal degradation in ALL and the variation between patient samples is unknown.

Cytotoxicity of blasts from peripheral blood and bone marrow were compared for sensitivity to HA22. HA22 appeared to be more cytotoxic to peripheral blood blasts raising important biological questions. The result should be interpreted cautiously as samples were not paired and the numbers assayed were small. Cell surface receptors, such as CXCR4, are involved in

homing and maintaining blasts in the hematopoietic niche and altered expression may contribute to blast localization to the perivascular space and migration into the peripheral blood. Peripheral blood blasts may lose stromal-cell interactions and/or dependency, which in turn could lead to changes in chemotherapy responsiveness.

The finding that HA22 is cytotoxic to blasts from children with ALL has important clinical implications. Acute and late morbidities associated with standard treatment of ALL remain significant and those individuals who relapse have a poor prognosis. New therapies that can overcome resistance to standard chemotherapy agents without adding significant toxicities are needed. This study shows that HA22 is cytotoxic to blasts from most patients and non-specific toxicities are expected to be less in comparison to chemotherapy. Serial modifications in the *Pseudomonas*-based immunotoxin constructs utilized at the NCI have reduced such non-specific toxicities.[262] Importantly, CD22 is only expressed on cells of B-lineage, further reducing the risk of non-specific side effects. The toxicity profile of anti-CD22 *Pseudomonas* immunotoxins has been encouraging to date.[192,93] The most frequent adverse events have been reversible increases in hepatic aminotransferases and hypoalbuminaemia. In Phase I testing in paediatric subjects, BL22 was associated with an acceptable safety profile. All adverse events were self-limited, most were Grade I and 2, and no dose-limiting toxicities were observed.[94] HA22 appears to have a similar toxicity profile to BL22.

Studies of other CD22 targeted therapies in paediatric ALL have recently been reported. Epratuzumab, a humanized monoclonal antibody against CD22, was studied in children with relapsed ALL by the Children's Oncology Group study AALL01P2.[97] Its activity as a single agent in that setting appears to be limited. A Phase I study of a mixture of ricin-based

immunotoxins against CD22 and CD19 (Combotox) was recently conducted for paediatric patients with ALL. In that trial, clinical activity was observed, although this agent was associated with severe adverse events and a high incidence of immunogenicity.[98] Other anti-CD22 targeted agents are in development. For example, a calicheamicin immunoconjugate CMC-544 (Inotuzumab ozogamicin) has been shown to be active in pre-clinical models of ALL and in clinical trials in adults with non-Hodgkin's lymphoma.[144,263]

In vitro leukaemia-stromal cell assays have been shown to be predictive of treatment outcome,[264] which offers the possibility of utilizing such in the development of individualized leukaemia therapies in the future. This study also establishes the bone marrow stromal cell assay system as a method that can be utilized to further study the biologic basis for sensitivity and resistance of ALL blasts to targeted immunotoxins.

In conclusion, the anti-CD22 immunotoxin HA22 has *in vitro* activity against blasts from children with newly diagnosed and relapsed ALL. These data provide experimental support for ongoing clinical trials of this agent for paediatric ALL (*ClinicalTrials.gov number: NCT00659425; http://clinicaltrials.gov/ct2/show/NCT00659425)* with the potential for a new active agent with reduced non-specific toxic therapy. The next chapter will investigate the mechanisms of resistance to HA22 in paediatric ALL.

5.4 Publications

The work in this Chapter has been published in the following paper:

- Mussai F, Wayne A, Pastan I et al. Cytotoxicity of the anti-CD22 immunotoxin HA22 (CAT-8015) against paediatric acute lymphoblastic leukaemia. British Journal of Haematology 2010; 150(3): 352-358[265]

6. Mechanisms of resistance to HA22 by CD22+ haematological malignancies

6.1 Introduction

In Chapter 1 it was established that HA22 has significant activity against paediatric ALL blasts from patients, in vitro. In addition HA22 was show to be cytotoxic by apoptosis. However ALL blasts from patients showed a range of sensitivity to HA22-induced cell death. In this chapter the factors that contribute to resistance to HA22 will be examined. Major steps in the HA22 processing pathway will be investigated – CD22 expression, HA22 internalisation on binding to CD22, HA22 intracellular processing leading to protein synthesis inhibition, and finally induction of apoptosis. Cell lines are used as a model throughout.

6.2 Results

6.2.1 CD22 expression is higher in Burkitt's lymphoma than pre-B ALL cell lines

To determine if the site density of CD22 correlated with sensitivity to HA22 induced cytotoxicity, CD22 site density was assessed by flow cytometry (Table 6.1). In the 5 paediatric ALL cell lines CD22 site density ranged from 2000 to 19,323 sites/ cell (median 10,391 sites/cell). The CD22 site density ranged from 62,000 to 76,281 sites/cell in the Burkitt's lymphoma cell lines (median 69,141 sites/ cell). The median CD22 site density is over 6 times higher in Burkitt's lymphoma cell lines than in pre-B ALL cell lines. Although in both ALL and Burkitt's cell lines there was a range of CD22 sites/ cell there was no correlation with the LC_{50} for HA22 (r=-0.41, p=0.35). Therefore CD22 site density is unlikely to contribute to resistance to HA22 physiologically.

Cell Line	CD22 sites/ cell	LC_{50} (ng/ml)
KOPN 8	19,323	0.2
EU 1.3	2,000	0.3
REH	10,391	0.6
NALM 6	9,907	10
SEM	12,591	0.2
CA46	76,281	0.5
RAJI	62,000	0.2

Table 6.1 CD22 site density does not correlate with sensitivity to HA22. Cell lines were cultured in T75 flasks and CD22 site density was determined by flow cytometry using the QuantiBRITE system.

To further investigate intracellular factors that may contribute to HA22 resistance, 2 ALL cell lines with similar CD22 site densities but different sensitivities to HA22 were selected for further study - REH and NALM6.

6.2.2 HA22 internalisation is rapid and does not affect cytotoxicity in the REH and NALM6 cell lines

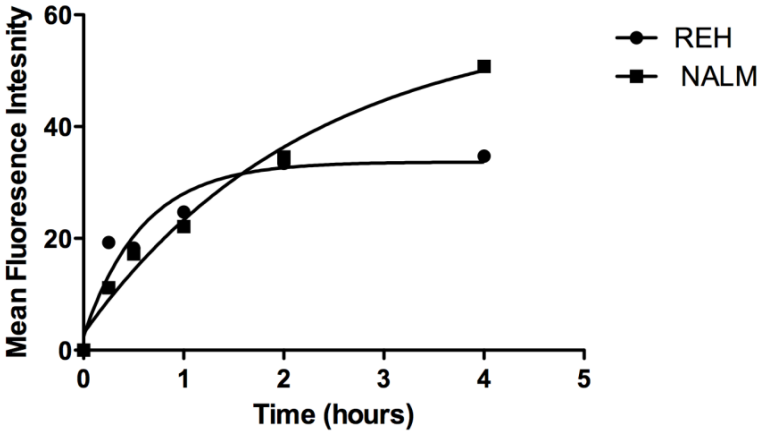
Since CD22 site density does not affect cytotoxicity an alternative explanation for the different HA22 sensitivities must be found. Following binding of HA22 to CD22, the immunotoxin needs to be internalised into the cell to have activity. I hypothesised that, the rate and capacity for REH and NALM6 cell lines to internalise HA22 was different and could lead to the differences in cytotoxicity.

To test the hypothesis, HA22 was labelled with a fluorescent tag and added to cell line cultures. Following incubation, Alexa647-labelled HA22 was rapidly internalized by both cell lines (Fig 6.1a). The rate of internalisation, represented by an increase in Mean Fluorescence Intensity, was quickest within the first 15 minutes for both cell lines. The short time period for internalisation makes it unlikely that the rate of internalisation of HA22 is a limiting factor for cytotoxicity.

To determine if the percentage of surface bound HA22 internalised differed between the two cell lines, surface CD22 was first saturated by binding with labelled immunotoxin on ice (Fig 6.1b). For REH, 80% of cell surface CD22-bound to HA22 was internalised within 1 hour from the start of incubation and plateaus over the next

hour. In contrast NALM6 continues to internalize HA22 up to the 4hour time point. The result is surprising as NALM6 is the less sensitive cell line, yet it would appear able rapidly to internalise HA22 and to have a greater capacity to internalise CD22-bound to HA22. Therefore internalisation characteristics do not explain the difference in LC_{50} between these two cell lines.

a



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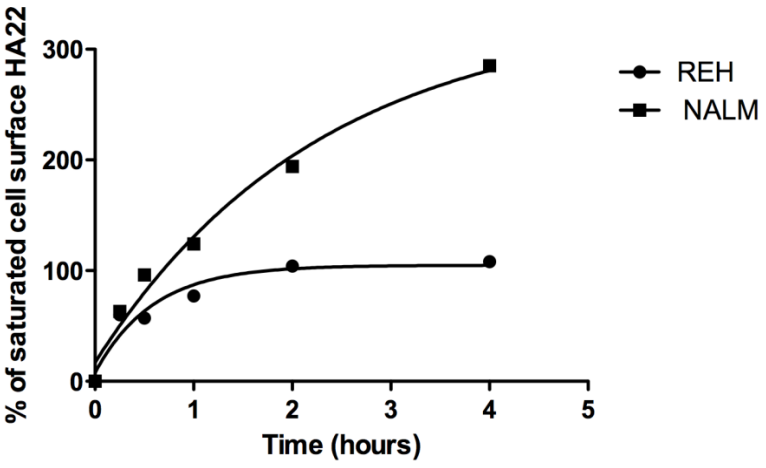


Figure 6.1 The internalisation rate of HA22 is rapid and similar in REH and NALM6 cell lines. Cells were incubated with 1ug/ml HA22-Alexa647 at 37^0C for different lengths of time. The time course of HA22-Alexa647 internalisation and the percentage of surface bound HA22-ALEXA 647 internalised are shown in (a) and (b) respectively. The experiment shown is representative of duplicate experiments.

6.2.3 Comparison of protein synthesis inhibition between REH and NALM6

The previous experiments show that HA22 cytotoxicity is not limited by internalisation. After entering the cell, the PE domains of HA22 passes through the endosomal compartment to enter the cell cytosol, and inhibit protein synthesis through ADP ribosylation of EF-2. To investigate whether the ability of HA22 to inhibit protein synthesis differed in the two cell lines, a ^{3}H-leucine incorporation assay 16 hours after the cells were treated with HA22 was performed (Figure 6.2). HA22 completely inhibits protein synthesis in the REH cell line with an IC_{50} of 3ng/ml. In NALM6 protein synthesis is not fully inhibited even at 500ng/ml HA22 (IC_{50}: 10ng/ml). The result indicates that after internalisation, altered intracellular processing may contribute to the differences in sensitivity of the cell lines.

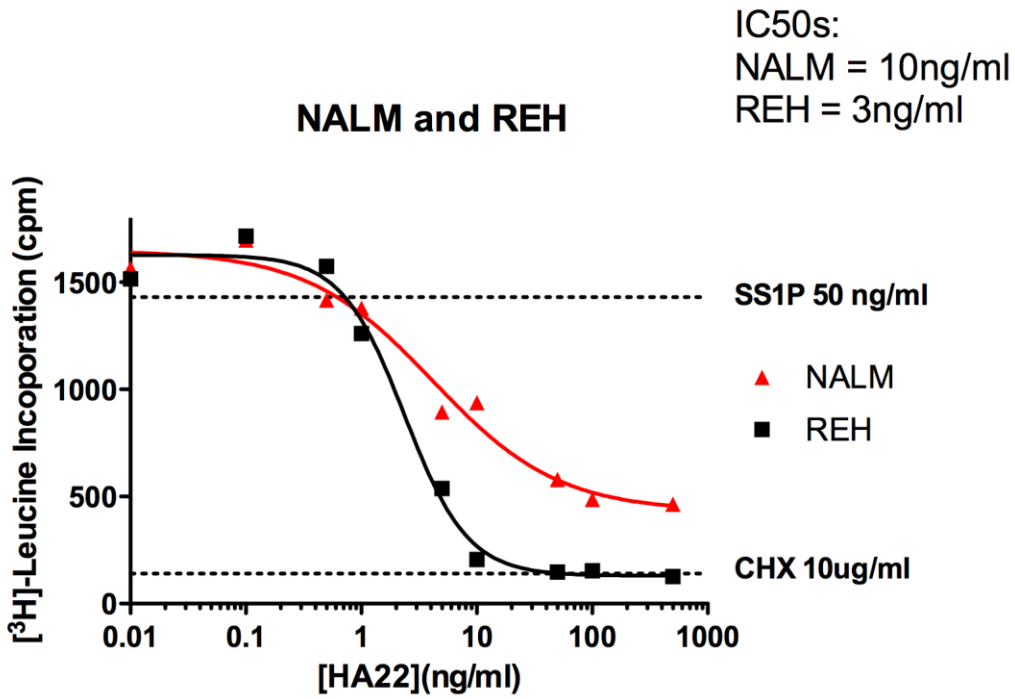


Figure 6.2 Protein synthesis is incompletely inhibited by HA22 in the NALM6 cell line compared to REH. REH and NALM6 cell lines were cultured with different concentrations of HA22 for 16 hours. Protein synthesis was measured by ^{3}H-leucine incorporation. The inhibition of protein synthesis by the anti-mesothelin immunotoxin SS1P and cycloxhexamide is shown as controls. Data points are the means of tri[licate wells. The experiment shown is representative of duplicate experiments.

6.2.4 Inhibition of protein synthesis is improved by HA22-LR or HA22-LR-KDEL

To examine if increased trafficking of the PE domains of HA22, through the endosomal compartment, could improve protein synthesis inhibition HA22 was compared with two HA22 mutants, HA22-LR and HA22-LR-KDEL, in the least sensitive cell line NALM6. HA22-LR is resistant to lysosomal protease mediated cleavage through engineered deletions in Domain II and Ib of PE. HA22-LR-KDEL contains the protease resistant mutations of HA22-LR and also a C terminus KDEL

modification that results in improved trafficking to the endoplasmic reticulum. In this way the contribution of lysosomal degradation and failed trafficking to the ER of HA22, can be examined as mechanisms of resistance. The HA22 mutants inhibited protein synthesis better than HA22, with IC_{50}s of 0.8ng/ml (HA22-LR) and 0.3ng/ml (HA22-LR-KDEL) (Fig 6.3). However, protein synthesis cannot be inhibited completely even with HA22 mutants (500ng/ml), when compared to the cyclohexamide control.

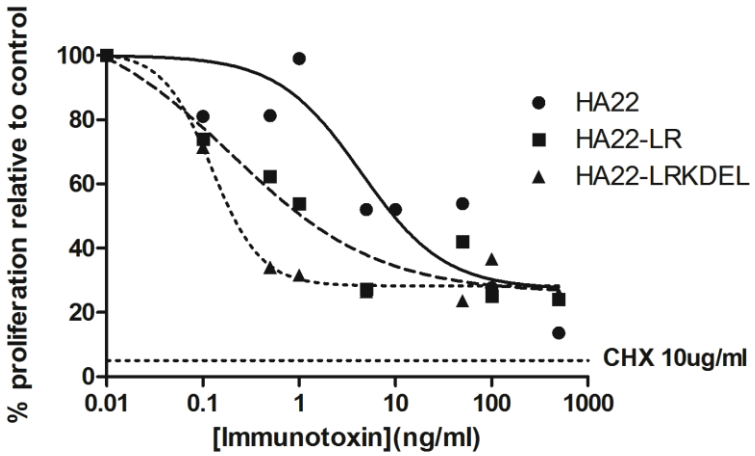


Figure 6.3 Inhibition of protein synthesis is improved by HA22-LR or HA22-LR-KDEL. NALM6 cells were incubated with HA22, HA22-LR, or HA22-LRKDEL for 16hours and protein synthesis inhibition determined by ^{3}H-leucine incorporation. The percentage proliferation relative to untreated control wells is shown.Data points are the means of tri[licate wells. The experiment shown is representative of duplicate experiments.

In summary, HA22 does not fully inhibit protein synthesis in the less sensitive NALM6 cell line. HA22 mutants that alter trafficking of the PE toxin within the endosomal compartment can improve inhibition of protein synthesis in NALM6, but not completely. The result suggests that other mechanisms contribute to HA22 resistance in this cell line.

6.2.5 HA22-LR has similar activity to HA22 against paediatric ALL and Burkitt lymphoma cell lines

Increased inhibition of protein synthesis was demonstrated in the NALM6 cell line by HA22-LR and HA22-LR-KDEL, compared to HA22. Inhibition of protein synthesis by HA22 leads to apoptosis in ALL cell lines. Therefore it was investigated if treatment with HA22-LR and HA22-LR-KDEL leads to improved cytotoxicity against all 5 paediatric ALL cell lines (Table 6.2). All cell lines were sensitive to HA22-LR (median LC_{50}: 0.35ng/ml) and HA22-LR-KDEL (median LC_{50}:0.2ng/ml). Notably HA22-LR and HA22-LR-KDEL had increased cytotoxicity compared to HA22 against NALM6 and REH. In the other 3 cell lines, no improvement in cytotoxicity was seen and this may reflect the intrinsic sensitivity of these cell lines to HA22. In summary paediatric ALL cell lines are very sensitive to HA22, HA22-LR and HA22-LR-KDEL. In cell lines less sensitive to HA22 immunotoxin alterations that improve intracellular processing, lead to improved protein synthesis inhibition, and some increased cytotoxicity.

	LC_{50} (ng/ml)		
	HA22	**HA22-LR**	**HA22-LR-KDEL**
NALM 6	5	1.5	1
REH	1	0.3	0.2
KOPN 8	0.02	0.1	0.3
SEM	0.2	0.2	0.2
EU	0.5	1	0.3

Table 6.2 HA22, HA22-LR and HA22-LR-KDEL are cytotoxic to paediatric ALL cell lines

6.2.6 The balance of pro- and anti-apoptotic pathway proteins contributes to the sensitivity of cell lines to HA22

Results shown earlier revealed that HA22 induces cell death by apoptosis. Apoptosis is dependent on the balance and activation of pro- and anti-apoptotic proteins. In the mitochondrial pathway of apoptosis pro-apoptotic proteins are BAK and BAX, whilst anti-apoptotic proteins are Bcl-2, Bcl-xl, and Mcl-1. Modulators of these proteins are BID, BIM, and PUMA. Therefore the relative baseline expression of pro- and anti-apoptotic proteins in cell lines was investigated to see if this correlated with sensitivity to HA22 (Figure 6.4).

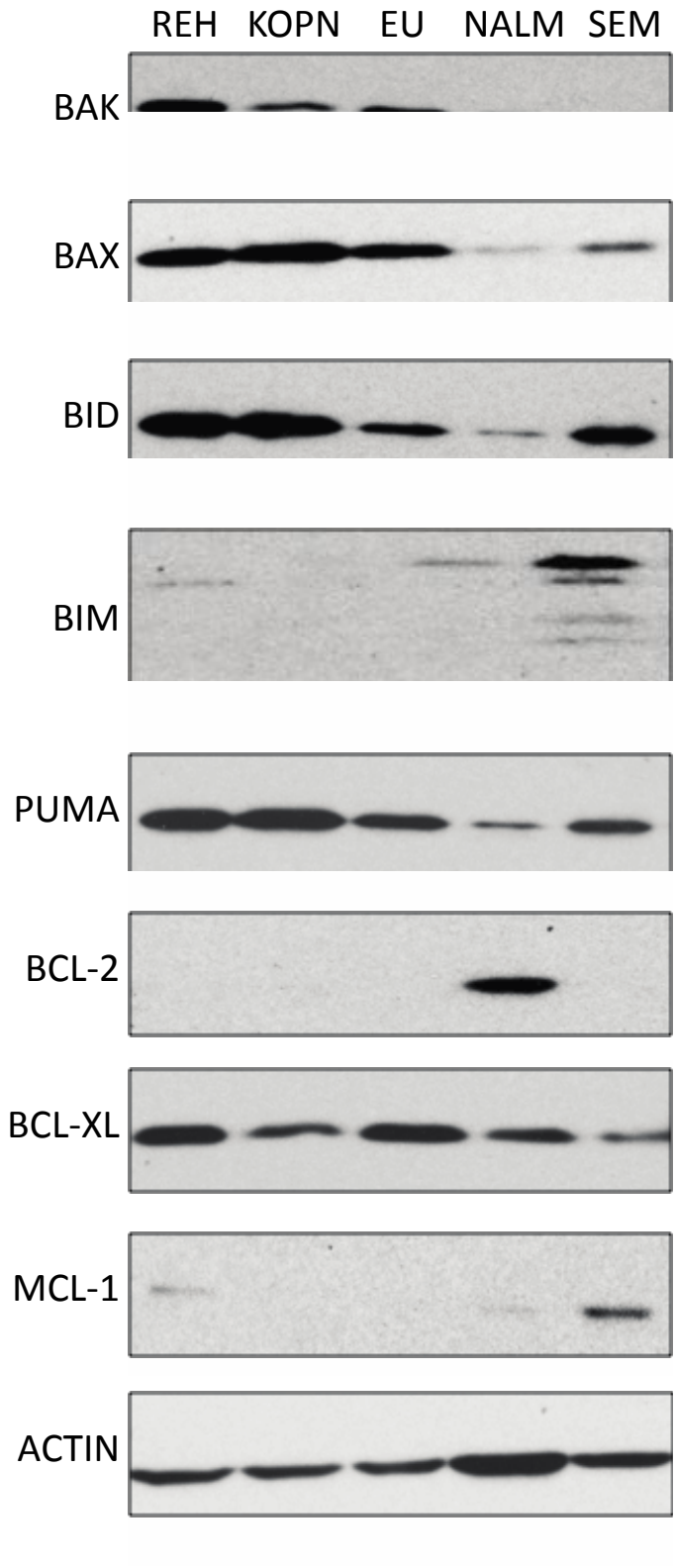


Figure 6.4 Relative expression of pro- and anti- apoptotic proteins in untreated paediatric ALL. Protein expression was detected by western blot. Actin was used as the housekeeping gene ensuring equal loading of protein. Data are representative of 3 experimental repeats.

Levels of pro-apoptotic BAK and BAX varied between cell lines although REH, KOPN, and EU had higher levels of these proteins relative to other cell lines. Anti-apoptotic proteins consist of Bcl-xl, Bcl2, and Mcl-1. The levels of Bcl-xl were similar in each cell line. Bcl-2 was highly expressed in NALM6 and absent in other cell lines. Mcl-1 levels varied and were absent in EU, KOPN, REH and NALM cell lines. Apoptosis pathway modulators BID, BIM and PUMA also had variable expression. BID and PUMA were expressed highest in REH, KOPN, EU and SEM.

To investigate if the balance of the pro- and anti- apoptotic proteins in the model cell lines REH and NALM was related to HA22 sensitivity, ImageQuant densiometry analysis was performed on the western blots to give a value for the expression of each protein compared to the actin control (Table 6.3). REH had a higher ratio of pro-apoptotic proteins compared to NALM6. The increased ratio of pro-apoptotic proteins may contribute to the increased sensitivity of REH, to HA22.

	Bak	**Bax**	**Bcl-2**	**Bcl-xl**	**Mcl-1**	**(Bax+Bak):(Bcl-2+Bcl-xl+Mcl-1)**
REH	1.13	1.16	0	1.23	0	1.86
NALM6	0.74	0.38	1.30	1.68	0	0.37

Table 6.3 Ratio of pro- and anti- apoptotic proteins in REH and NALM 6 cell lines, measured by ImageQuant analysis. Western blots were performed to compare the level of expression of each protein. Blots were scanned and band density determined using ImageQuant software.

6.2.7 HA22 induces apoptosis earlier in REH than NALM6 via a decrease in Bcl-2 levels and caspase cleavage.

The previous experiments indicate that the balance of pro- and anti- apoptotic proteins contributes to HA22 resistance. To understand the changes in apoptosis proteins that occur afters exposure to HA22, cell lysates from REH and NALM cells were analysed at different time points after treatment. Pseudomonas-exotoxin A immunotoxins induce apoptosis via the mitochondrial apoptotic pathway.[200] In this pathway activation of Bak/Bax leads to mitochondrial membrane disruption and the release of proteins that result in activation of effector caspases. Caspase 9 is an initiator caspase that can act on pro-caspase 3, leading to Poly ADP Ribose Polymerase (PARP) cleavage, resulting in cell death. In the more sensitive REH cell line caspases 9, 3 and PARP are cleaved after 24 hours of treatment (Fig. 6.5). In contrast no cleavage was seen in the NALM6 cell line at the 24hour time point. Anti-apoptotic protein Bcl-2 expression in the REH cell line decreased by 4 hours after HA22 and continued to fall over time. In NALM6 Bcl-2 levels did not decrease until 24 hours after treatment. Levels of BAX, BAK, were unchanged in both cell lines. In summary apoptosis occurs earlier in the more sensitive REH cell line, and is associated with falling levels of Bcl-2 and activation of the effector caspases.

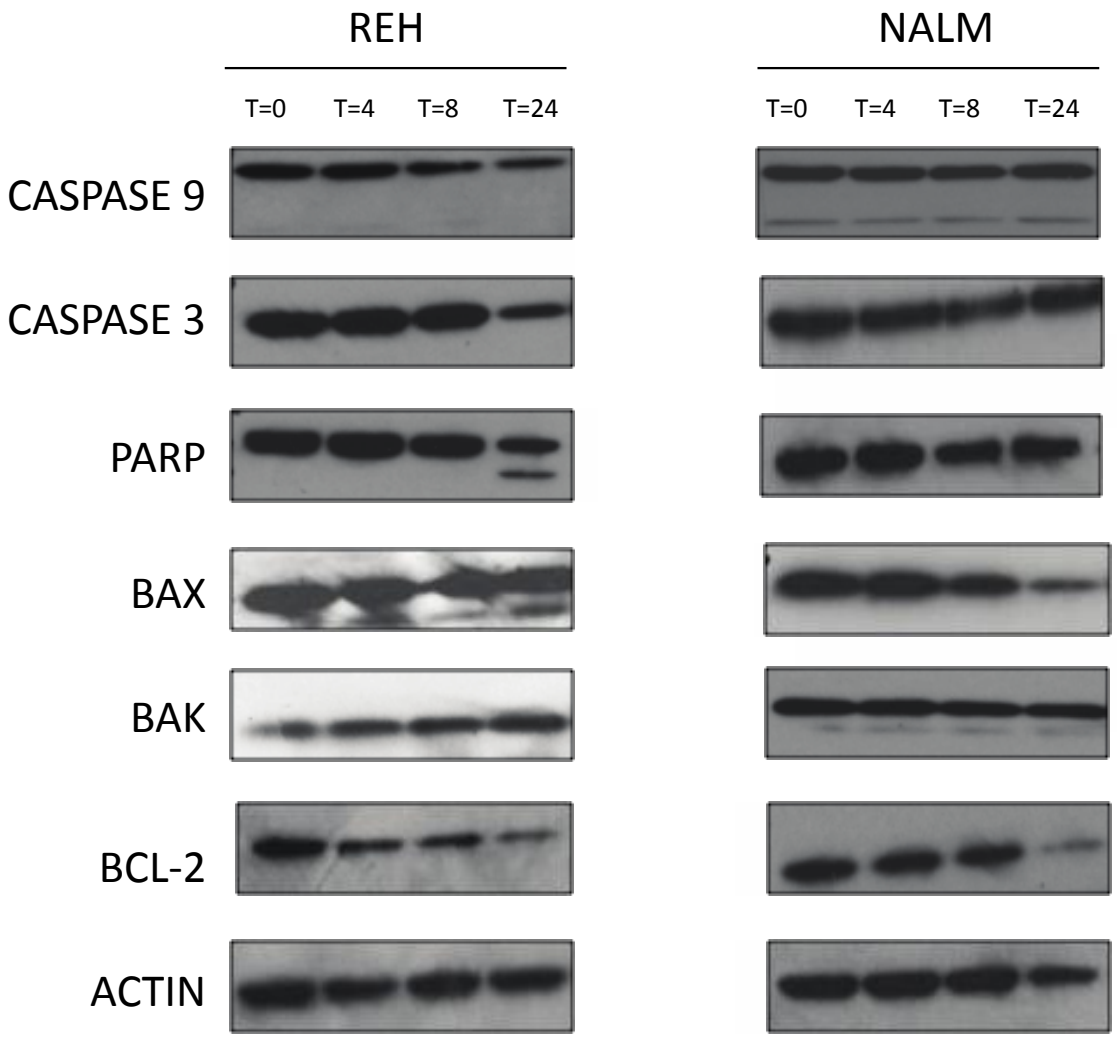


Figure 6.5 Changes in apoptotic protein expression during the first 24hours of treatment with 100ng/ml HA22. Caspase 3 and PARP cleavage occurs earlier in REH than NALM6. Western blots were performed to compare the level of expression of each protein over time. Actin was used as the housekeeping gene to ensure equal loading of protein. Data are representative of triplicate experiments.

6.2.8 17-AAG enhances HA22 cytotoxicity

Heat Shock Protein-90 (Hsp90) is a member of the heat-shock protein family, and acts as chaperone to a number of oncogenic proteins to promote correct intracellular protein folding. 17-AAG is an Hsp90 inhibitor that binds to the ATP binding site of

Hsp90, resulting in the release and proteosome degradation of client proteins which promote apoptosis.[266] To investigate if HA22 cytotoxicity can be improved, REH and NALM6 cell lines were treated with the Hsp90 inhibitor 17-AAG (1μM) in combination with HA22 (Fig 6.6). It was confirmed that 1μM 17-AAG was not toxic alone. The addition of 17-AAG enhanced the cytotoxicity of HA22 against NALM6 (LC_{50} 6ng/ml with HA22 only; LC_{50} 1ng/ml with 17-AAG and HA22). No significant increase in cytotoxicity was observed when we tested the same combination against REH.

6.2.9 ABT-737 does not enhances HA22 cytotoxicity

ABT-737 is a small molecule –BH3 domain mimetic engineered to bind to the anti-apoptotic proteins Bcl-2 and Bcl-xL, leading to BAK release, and precipitating cell death. To investigate if disruption of the apoptosis pathway by ABT-737 (1uM) can enhance HA22 cytotoxicity the combination of these agents against the REH and NALM cell lines was tested . There was no evidence of enhancement in cytotoxicity(Fig 6.6). 1μM ABT-737 did not cause toxicity when added to cultures alone.

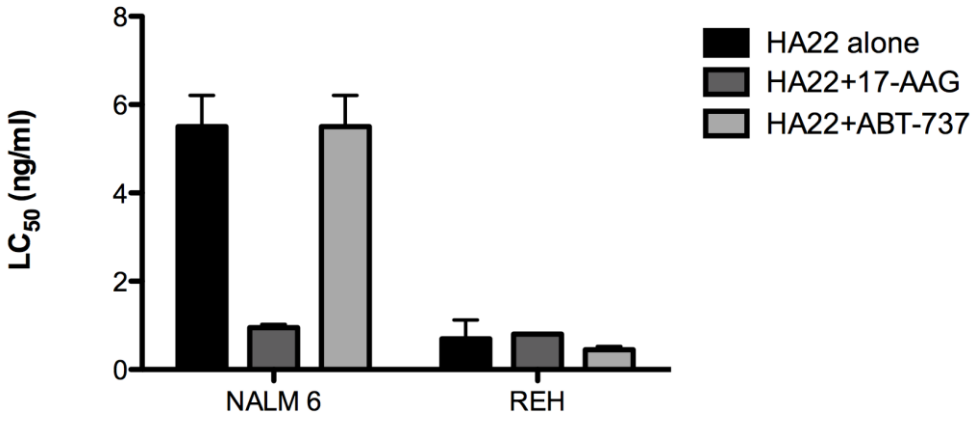


Figure 6.6 Effect of 17-AAG or ABT-737 on HA22 cytotoxicity against NALM6 and REH. 1μM 17-AAG or 1μM ABT-737 was added in combination with HA22 to cultures of REH and NALM6 cell lines. Cell death was determined by flow cytometry. Data are the means of triplicate wells, repeated in 3 separate experiments.

6.2.10 HA22 penetration into Burkitt lymphoma mass

The previous experiments examined the factors contributing to HA22 resistance at the level of individual cells. In the Phase I trial of HA22, the immunotoxin is administered intravenously and distributes throughout the vascular compartment. In leukaemias the majority of the malignant cells are in the blood vessels or bone marrow and thus easily accessible to the immunotoxin. However, in lymphomas HA22 must bind to malignant cells within a tumour mass. The factors affecting penetration of antibodies into lymphomas are not well understood. Therefore the ability of HA22 to be taken up within subcutaneous CA46 tumours in NOD-SCID mice was examined.

6.2.11 Demonstration of in vitro internalisation

The penetration of HA22 into the lymphoma was assessed using a method developed in the Pastan lab.[267] Alexa-647 was conjugated to HA22-KDEL such that approximately 3 moles of ALEXA-647 were bound to every mole of HA22-KDEL (data not shown).

The ability to detect HA22-KDEL-Alexa internalised into individual Burkitt lymphoma cells was first tested in vitro. CA46 cells were incubated with HA22-KDEL-Alexa and following stripping of surface bound immunotoxin, the increase in Mean Fluorescence Intensity (representing internalised immunotoxin) detected by flow cytometry was analysed (Fig 6.7).

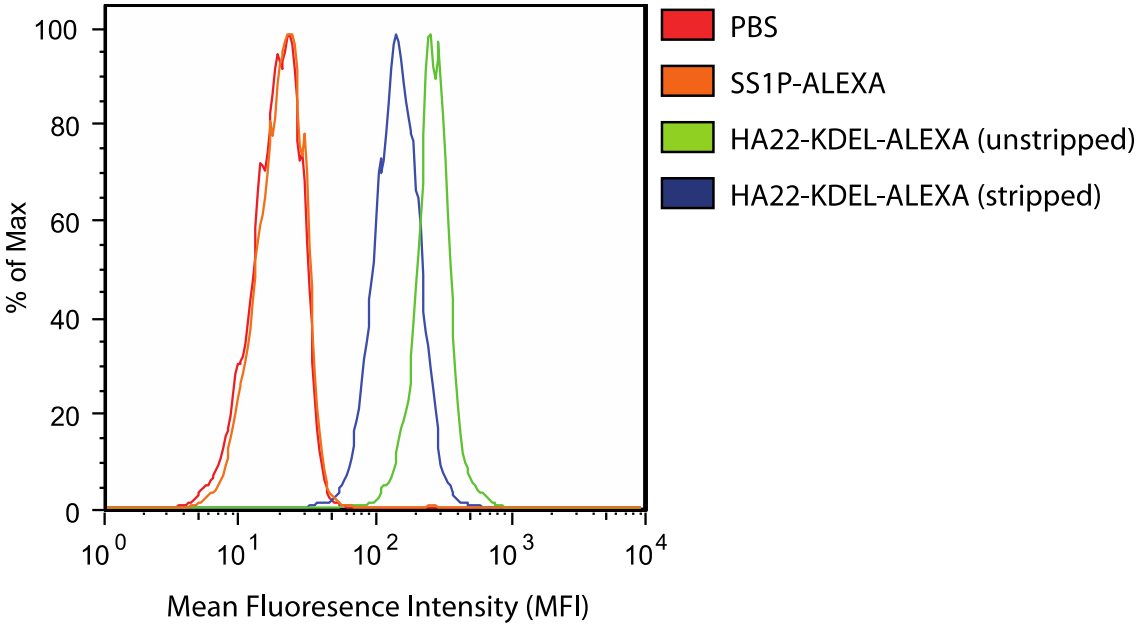
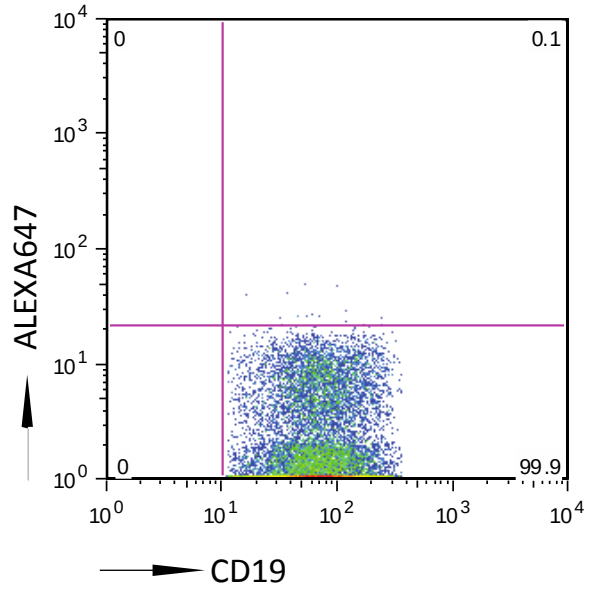


Figure 6.7 CA46 cells that internalise HA22-KDEL-Alexa647 internalise can be detected by flow cytometry. CA46 cells were incubated with HA22-KDEL-Alexa647 or controls on ice. Internalised immunotoxin was detected after stripping of surface-bound immunotoxin. Mean Fluorescence Intensity was measured by flow cytometry.

6.2.12 HA22-KDEL-ALEXA+ CA46 cells can be detected ex vivo.

In the previous experiment it was established that internalised HA22-KDEL-Alexa can be detected within CA46 cells in vitro. To determine if the labelled immunotoxin could be detected ex vivo, NOD-SCID mice were injected with CA46 cells subcutaneously. Once a tumour was established, 100μg of labelled immunotoxin was injected intravenously. 3 hours later the CA46 tumour was harvested and then processed using the method described above. Flow cytometry gating on CD19+ cells confirmed that the CA46 cells had internalised the labelled immunotoxin in vivo and that its presence can be readily detected ex vivo (Fig 6.8). As the digesting enzymes completely removed surface-bound HA22, the method is specifically measured internalised HA22.

(a)



(b)

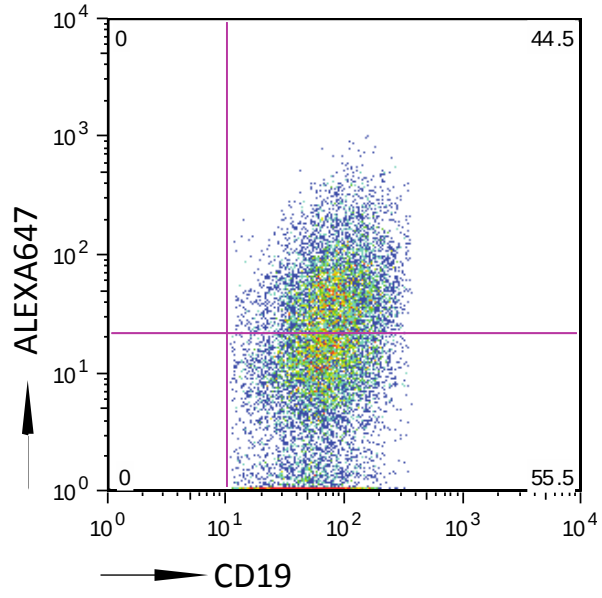


Figure 6.8 Flow cytometry staining of dissociated CA46 murine xenograft tumours following intravenous injection of saline (a) or 100μg of HA22-KDEL-Alexa647 (b). NOD-SCID mice bearing CA46 xenografts were injected iv with either saline or HA22-KDEL-Alexa647. Tumours were extracted after 3 hours and the uptake of labelled immunotoxin into CA46 cells was assessed by flow cytometry of the dissociated tumour.

6.2.13 Penetration characteristics of labelled immunotoxin

To understand whether increasing the administered dose of immunotoxin enhances uptake of labelled immunotoxin, increasing concentrations (0-250μg) of immunotoxin were injected into mice bearing tumours of comparable size. As the dose of HA22-KDEL-Alexa is increased there is an increase in the percentage of cells which have internalised the immunotoxin, but there is a plateau at 53%(Fig 6.9). The results show that within 3 hours of injection the immunotoxin penetrates at least half of all tumour cells. Within this time frame, however, penetration is not improved by increasing the dose beyond 100μg. SS1P-Alexa647 was also injected into mice bearing CA46 tumours, as a control. No Alexa-positive tumour cells were detected in these cells (data not show). The results show that HA22-KDEL-AEXA647 uptake by CA46 cells is specifically mediated by CD22 binding.

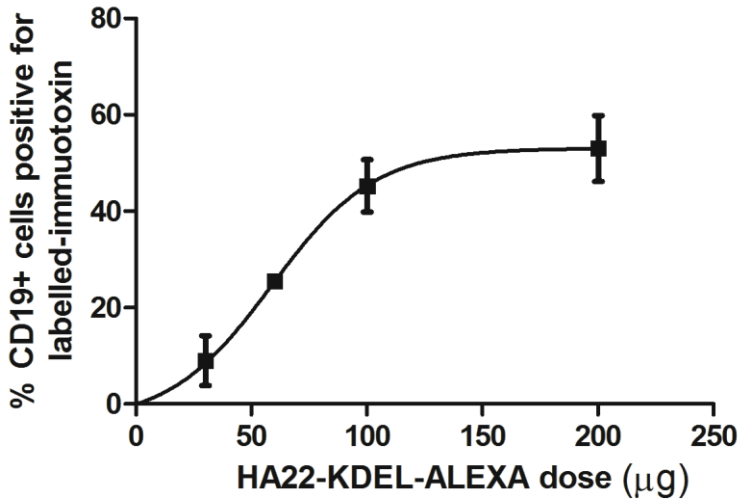
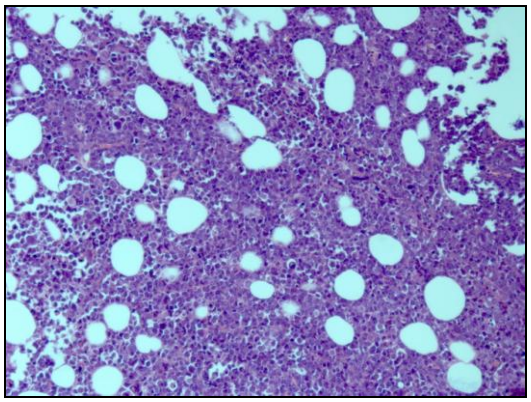


Figure 6.9 Dose-dependent increase in immunotoxin penetration. HA22-KDEL-Alexa647 was administered by intravenous into CA46 tumour bearing mice. Tumours were harvested after 3 hours, dissociated, and the percentage uptake determined by flow cytometry. Data are the mean of 5 mice per data point.

6.2.14 Immunohistochemical analysis of CA46 tumour xenografts

The study above identified that HA22-KDEL-Alexa can penetrate into murine CA46 xenografts. Burkitt lymphoma is known to be composed of tumour cells surrounded by a stroma composed of macrophages, immune cells and vascular endothelium. To determine if the xenografts and immunotoxin penetration were representative of spontaneously occurring Burkitt's lymphomas immunohistochemistry of sections of murine CA46 xenografts was performed (Fig 6.10). The results show that Burkitt's lymphoma xenografts are composed of Burkitt lymphoma cells (CD22+) surrounded by significant numbers of macrophages (F4/80+). Blood vessels are present although appear few in number (PECAM1). The results confirm that this xenograft model is histologically a reasonable representation of spontaneously occurring Burkitttumours. In addition, the histology indicates that for immunotoxin to successfully reach target CD22+ Burkitt lymphoma cells it must traverse through an environment containing altered vascularisation and the presence of tumour-associated macrophages which may engulf free immunotoxin.

a)



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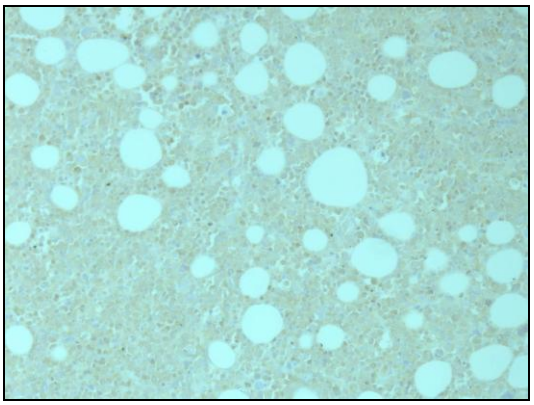
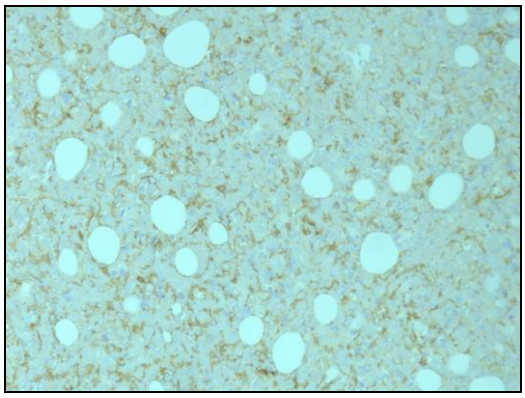


c)



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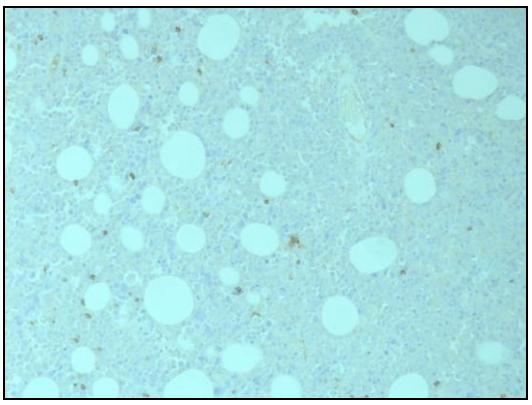


e)

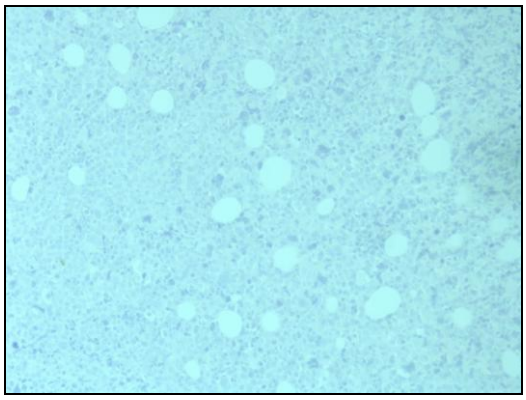
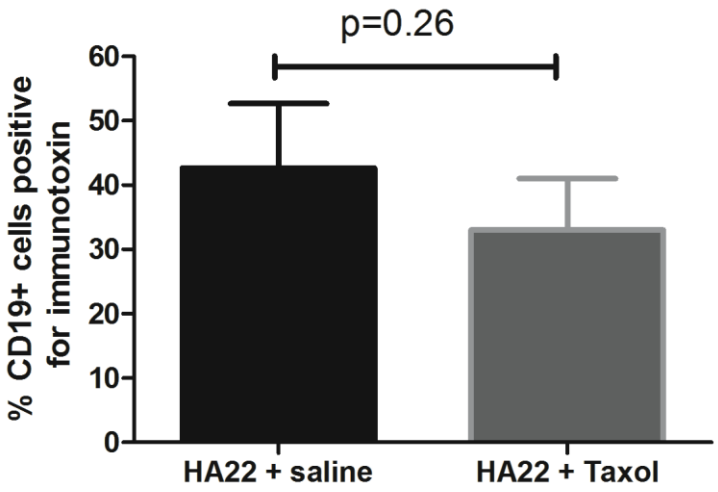


Figure 6.10 CA46 murine xenografts are histologically similar to spontaneously occurring human Burkitt lymphoma. CA46 murine xenografts were harvested and embedded in paraffin. Staining was performed with haematoxylin and eosin (a), CD22 (b), F4/80 (c), and PECAM1 (d). Unstained control (e). Data are representative of 3 xenograft tumours.

6.2.15 Effect of chemotherapy on penetration of immunotoxins

It has previously been shown that administration of chemotherapy agents in conjunction with immunotoxins can enhance the penetration of immunotoxin into tumours.[203] In addition, prior treatment of CA46 tumour bearing mice with either adriamycin or paclitaxel has a synergistic effect in reducing tumour size (Pastan laboratory, unpublished data). To understand the effect described, the ability of prior administration of adriamycin (3mg/kg ip) or paclitaxel (50mg/kg ip) to increase the uptake of HA22 into CA46 tumours was investigated. Tumour bearing mice treated with paclitaxol or adriamycin 72 hours before the immunotoxin, did not have increased uptake of labelled immunotoxin compared to mice treated with saline ($p=0.96$ and $p=0.26$ respectively) (Fig 6.11a and 6.11b).

(a)



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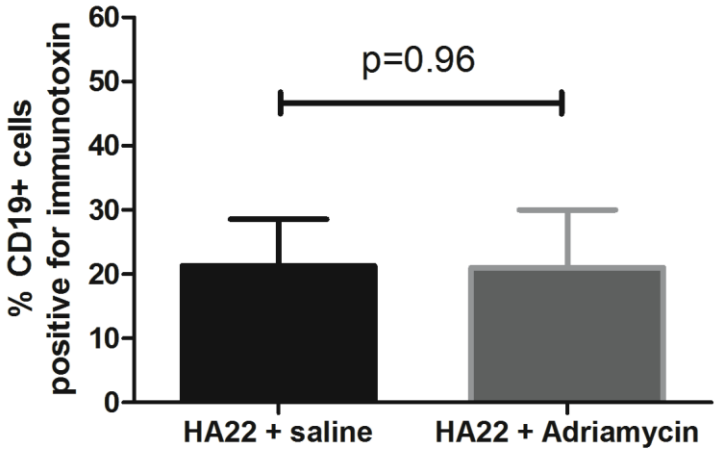


Fig 6.11 No difference in HA22-KDEL-Alexa uptake following treatment with taxol of adriamycin, in CA46 tumour bearing mice. NOD-SCID mice bearing subcutaneous CA46 Butkitt's lymphoma xenografts were injected with saline or 50mg/kg paclitaxel (Taxol) (a) or 3mg/kg adriamycin (ip) (b). 72hours later HA22-KDEL-ALEXA-647 was injected (iv) and tumours harvested 3 hours later. Uptake of labelled immunotoxin into the tumour cells was assessed by flow cytometry. Data are the mean of 5 mice per treatment.

6.3 Discussion

The aim of this study was to understand the factors contributing to sensitivity and resistance of paediatric ALL blasts to HA22. I hypothesised that differences in internalisation intracellular processing and expression of pro- and anti-apoptosis proteins would affect the cytotoxicity of HA22. Two cell lines that had different sensitivities to HA22 (>10fold difference in LC_{50}) were used as a model. The study concluded that in the less sensitive cell line NALM6, protein synthesis is not fully inhibited by HA22. Degradation of internalised HA22 by lysosomal enzymes and the balance of pro- and anti-apoptotic proteins contributed to reduced HA22 activity. CD22 site density and the capacity to internalise HA22 did not play a significant role in resistance. Finally, the study investigated the penetration of HA22 into Burkitt's lymphoma xenografts. HA22 penetrates in a dose-dependent manner but reached a plateau within 3 hours from administration that is not improved by pre-treatment with chemotherapy agents.

The last chapter determined that for patient-derived ALL samples CD22 site density only weakly correlated with HA22 cytotoxicity. The result was confirmed using ALL and Burkitt lymphoma cell lines. The EU1.3 cell line that has the lowest CD22 site density (2000 sites/ cell) is highly sensitive to HA22. CD22 immunotoxins were successfully developed for the treatment of hairy cell leukaemia which expresses high levels of CD22. The finding that HA22 is also active against leukaemic blasts with much lower CD22 site densities is highly encouraging. Whether ALL cells down-regulate CD22 in vivo, following treatment with HA22, is not determined. However, the results suggest that CD22 site density would have to fall to very low levels before

an effect on cytotoxicity is seen. The data supports the ongoing development of HA22 as a novel therapy in relapsed ALL.

The role of target antigen site density in immunotoxin sensitivity is variable between diseases studied and immunotoxins used. In an earlier study of chronic lymphocytic leukaemia (a B cell malignancy), cells expressing 400-1900 sites/ cell were less sensitive to the first generation anti-CD22 immunotoxin BL22 than cells expressing 4000-15,000 sites per cell.[260] Consequently, CD22 site density was proposed to predict in vitro response to BL22.[268] Similarly, a study of the anti-CD33 immunotoxin Gemtuzumab ozogamicin found that cell surface expression of CD33 is a limiting factor for the cytotoxicity against AML blasts.[204] In contrast a study of another anti-CD22 immunotoxin, CMC-544, found that cytotoxicity was not dependent on the level of CD22 expression levels.[269] CD19 site density, another ALL-associated surface antigen, also does not correlate with cytotoxicity of the anti-CD19 immunotoxin HD-37-dgRTA.[270] The increased avidity of HA22 compared to BL22 may have overcome any earlier limitations imposed by low site-density or intracellular mechanisms of resistance may also differ between ALL and other malignancies.

Following binding to surface HA22 the next step in processing is internalisation. The capacity of cells to internalise anti-CD22 immunotoxins has been confirmed as an important determinant in cytotoxicity.[144] This study found that HA22 is rapidly internalised in ALL blasts within 30minutes. No differences in internalisation rates were present between the least sensitive (NALM6) and more sensitive cell line

(EU1.3). The findings are supported by a study from Du et al. showing that CD22 is rapidly internalised into B cell lymphoma cell lines within 15 minutes of BL22 binding.[261] Although there are reports of differences in unconjugated antibody internalisation depending on histological subtypes for lymphoma no evidence exists in ALL.[271] Therefore it is reasonable to conclude that the rate of HA22 internalisation does not affect the cytotoxicity of HA22 and is unlikely to be important physiologically.

NALM6 was able to continue internalising HA22 for significantly longer than REH. The result indicates that after the CD22-HA22 complex is internalised, HA22 is released, and CD22 is recycled to the cell surface to allow further HA22 to enter the cell. The studies of Du et al. and de Vries et al both suggest that a continuing loop of CD22 internalisation and re-expression contributes to immunotoxin efficacy and that an intracellular pool of CD22 molecules may exist. As the MFI in this study continues to increase beyond that of 100% initial cell surface saturation, the result also confirms that the HA22 dissociates in the cell and does not get exocytosed. Drug-efflux pumps such as MRP and p-glycoprotein are known to play a role in the resistance of ALL blasts to standard chemotherapy drugs.[272] In adult patients these drug-efflux pumps have been shown to reduce the effectiveness of the anti-CD22 immunotoxin inotuzumab ozogamicin in CLL and the anti-CD33 immunotoxin gemtuzumab ozogamicin in AML.[269] [273] However there is no evidence for drug-efflux pumps mediating immunotoxin resistance in paediatric ALL.[274]

In the study a significant difference in the ability of HA22 to inhibit protein synthesis in NALM6 compared to EU was observed. Having established that differences in internalisation do not contribute to resistance, the intracellular processing of HA22 was compared. Following internalisation the anti-CD22 scFv is cleaved from the *Pseudomonas* toxin domains in a furin mediated step within the endosomes.[155] Before *Pseudomonas* exotoxin (PE) can enter the cytosol it must traffic through the endosomal compartment to the endoplasmic reticulum. Lysosomes are a significant degredative pathway for internalised molecules and immunotoxins must avoid lysosomal degradation in order to remain active. Lysosomal enzyme activity is known to be significantly higher in the blasts of ALL patients compared to lymphoid cells from healthy controls.[275] In the study the role of lysosomal degradation in the resistance of NALM6 was investigated by comparing HA22 with an engineered lysosomal resistant variant HA22-LR. HA22-LR was more potent at inhibiting protein synthesis and inducing cell death in NALM6 suggesting that lysosomal degradation does contribute to ALL resistance. Similar findings have been reported in CLL.[90]

To assess the role of the endoplasmic reticulum in limiting transport of the toxin into the cytosol, another immunotoxin mutant, HA22-LR-KDEL that contains the protease resistant mutations of HA22-LR and also a C terminus KDEL modification was assessed. Soluble proteins that are targeted to and retained in the endoplasmic reticulum (ER) frequently contain a K/RDEL motif at the C terminus of the protein.[276] Proteins containing the motif bind to KDEL receptors normally present throughout the Golgi, and are transported to the endoplasmic reticulum.[277] Once inside the ER the change in pH releases the protein ligand. Physiologically, Pseudomonas exotoxin exploits this system by containing a KDEL-like motif (REDLK) that allows for

targeting and retention in the ER. The REDLK motif has been shown to be important for PE cytotoxicity and replacement of the motif with KDEL enhances potency.[278,279] In vitro, PE has been shown to associate with immobilised KDEL receptors.[280] This study demonstrated that the mutant HA22-LR-KDEL showed increased activity against NALM6 suggesting that improved cytotoxicity can occur by enhancing trafficking of the toxin moiety to the endoplasmic reticulum. In turn the result suggests that some of the dissociated toxin from unmodified HA22 may fail to reach the ER, and thus contribute to decreased efficacy against some ALL cells.

Following transport across the ER membrane Domain III of Pseudomonas exotoxin inactivates elongation factor-2 (EF-2), by adenosine diphosphate (ADP) ribosylation of a modified histidine residue, called dipthamide. Protein translation is halted, triggering cell death.[281] The dipthamide residue is generated by the modifications of a histidine residue at position 715, by five proteins Dph1 to Dph5.[282] [218] As dipthamide sits at the tip of a loop in EF2 that mimics the tRNA anticodon loop it has been suggested that dipthamide stabilises the mRNA-codon interactions for translation. The incomplete inhibition of protein synthesis seen in NALM6 may be due to a failure in the inactivation of EF-2, perhaps through dipthamide mutations that render the catalytic activity of the pseudomonas exotoxin domains inactive. Engineered mutations in Dph2 have been shown to protect cells against toxicity from pseudomonas exotoxin.[283] The Pastan laboratory is continuing to study the physiological role of dipthamide mutations as a mechanism of resistance to HA22.

In Chapter 1 it was shown that HA22 induces cell death through apoptosis. The

mitochondrial pathway initiates apoptosis in response to cell stressors that lead to cell damage. Stressors can include insufficient growth factors,[284] DNA damage,[285] unfolded protein response[286] and increased intracellular reactive oxygen species.[287] The balance of pro- and anti- apoptotic members of the BCL2 family of proteins controls the response of cells to these stressors.[288] Anti-apoptotic members of the BCL family are: Bcl2, Bcl-xl, Bcl-w, and Mcl-1 which all share four Bcl2 homology domains. The pro-apoptotic members are BAX and BAK which dimerise to form a pore in the mitochondrial membrane. BAX and BAK have three BCL2 homology domains. Finally there are the 'BH3-only' group of proteins which bind to and inhibit the other members of the BCL2 family, thus regulating their ability to trigger apoptosis. 'BH3-only' protein members are BID, BIM, PUMA, BMF, BAD and NOXA. The exact role and specificity of action of the BH3-only group of proteins is controversial however BIM appears essential for apoptosis related to growth factor withdrawal[224], and PUMA is important for apoptosis induced by DNA damage.[289] PUMA has also recently been implicated in playing an important role specifically in the sensitivity of ALL blasts chemotherapy, by priming the mitochondria for apoptosis.[290]

Mutations and defects in the apoptosis pathway are common in malignant cells, including haematological malignancies. Bcl2 was originally identified in follicular B cell lymphoma as the gene deregulated by the t(14:18) chromosomal translocation.[291] Bcl-2, Bcl-xL and Bax expression correlated with sensitivity to irradiation-induced apoptosis in paediatric ALL.[292]

In this study the balance between pro- and anti-apoptotic cell lines, and in particular the levels of Bcl2, correlated with resistance to HA22. The role of apoptosis pathway proteins in ALL resistance to therapy is unclear. No association with features at presentation, response to drugs, or clinical outcome has been shown for the expression levels of Bcl-2, Bcl-xl, Mcl-1, Bax, Bad, and Bak.[228] In particular Bcl-2 levels also did not correlate with the response to induction chemotherapy or rate of relapse for paediatric ALL.[293,294,295] Despite these reports, Bcl-2 has previously been shown to be a major contributor to PE immunotoxin based resistance. In a study of the B cell malignancy Mantle Cell lymphoma higher Bcl-2 expression was detected in immunotoxin resistant cell lines compared to sensitive cell lines.[296] Furthermore Bcl-2 inactivation increased the susceptibility of the resistant cell line to BL22.

Du et al. have shown that there is an absolute requirement for Bak and almost no requirement for Bax in PE-mediated apoptosis of Mouse Embryonic Fibroblast cells.[297] In addition Du et al. showed PE treatment causes Mcl-1 degradation and initiation of apoptosis via Bak released from Mcl-1-Bak complexes. In this study Mcl-1 levels in ALL blasts were notably low or absent in untreated cells. The finding may explain why ALL blasts are generally very sensitive to HA22, compared to other leukaemias or solid tumours which require higher concentrations of PE-based immunotoxins to induce cell death.

Having established the role of intracellular processing and apoptosis proteins in mediating HA22 resistance, the ability of 2 novel agents to increase the cytotoxicity of HA22 was determined. 17-allylamino-17-demethoxygeldanamycin (17-AAG,

tanespimycin) is a synthetic derivative of the naturally occurring Hsp90 inhibitor geldanamycin.[298] Hsp90 is a chaperone protein that acts to stabilise oncogenic proteins within malignant cells.[299,300] Geldanamycin has been shown to inhibit Hsp90 activity by blocking the binding of ATP to Hsp90, and thus leads to the depletion of oncogenic proteins by ubiquitin-mediated proteosomal destruction. 17-AAG has entered Phase I and II clinical trials for a number of malignancies. In particular 17-AAG has shown activity against HER2+, trastuzamab-refractory breast cancer.[301] 17-AAG is well tolerated with predominantly Grade 1 and 2 toxicities described. 17-AAG has also entered clinical trials for the haematological malignancy myeloma, in combination with bortezomib.[302] In the study 17-AAG was effective in overcoming the resistance of NALM6 to HA22 and lowered the LC_{50}. The role of Hsp-90 in mediating resistance to chemotherapy in ALL is unclear. Yufu et al. reported that HSp-90 expression is increased in ALL blasts from patients compared to non-malignant lymphoyctes.[303] Hsp-90 inhibitors are able to induce apoptosis in Philadelphia chromosome positive ALL through induction of the proteosome degradation of the Bcr-ABL fusion protein.[304] The NALM6 cell line expresses an ETV6-PDGFRB t(5;12)(q33.2;p13.2) fusion protein that may similarly be degraded following 17-AAG treatment and lead to increased HA22 sensitivity.

The second drug examined in this study is ABT-737. ABT-737 is a small molecule – BH3 domain mimetic that can bind to a hydrophobic groove in Bcl-2 proteins and release the pro-apoptotic Bax and Bak proteins, enhancing apoptosis.[305] ABT-737 has very low affinity for Mcl-1, but high affinity for Bcl-2, Bcl-xl, and Bcl-w. Studies have shown that ABT-737 works effectively with agents that cause Mcl-1 degradation, to cause cell death.[306,307] As ALL has low levels of Mcl-1 and HA22

inhibits protein synthesis, leading to the rapid loss of Mcl-1, ABT-737 could prove effective in enhancing immunotoxin sensitivity. In this study ABT-737 did not enhance HA22 induced cytotoxicity for the NALM6 cell line. Traini et al. have shown that ABT-737 can overcome resistance to other PE-based immunotoxins in epithelial cell lines by enhancing apoptosis and disruption of the ER leading to increased toxin in the cytosol.[308] It is possible that as HA22 is still able to induce apoptosis in NALM 6 at low concentrations compared to other tumours, no further improvement in cytotoxicity is possible using BH3-mimetcs. In addition the other mechanisms of resistance described may be the main contributors to HA22 resistance and alterations in apoptosis proteins play little effect in ALL. Further work to understand the complex role of apoptosis proteins in ALL, using patient samples, is required.

Having established some of the intracellular factors that contribute to HA22 resistance the penetration of HA22 into lymphoma xenografts was investigated. In leukaemias the malignant cells reside in the vascular compartment and thus are readily accessible to intravenous-based therapies. However in lymphomas the malignant cells are present in lymph nodes, which often have abnormal vasculature or are poorly vascularised in areas of malignant cell expansion. Other barriers to immunotoxin activity may include a high interstitial fluid,[309] high collagen composition,[310] an antigen barrier,[311] and the presence of shed tumour antigens.[312] In this study an approach developed in the Pastan laboratory to study internalised immunotoxin inside tumour cells, within a tumour mass, was used. The results showed that as the dose of HA22 injected intravenously is increased there is increasing uptake of HA22 into the lymphoma xenografts. The result is significant at it shows that HA22 is taken up by

about half the target lymphoma cells within a short time period of time. However above 100μg there was no further increase in uptake at the 3hour time point. The mechanisms preventing further HA22 uptake are not known and include those described above. CD22 is known to be cleaved and shed into the blood in hairy cell leukaemia however soluble CD22 has not been demonstrated in lymphomas.[313]

One approach to improve the efficacy of immunotoxins is through combination treatment with chemotherapy. HA22 has been shown to synergise with Taxol or adriamycin in killing CA46 xenografts in mice (Pastan et al., unpublished data). In this study no increase in the penetration of HA22 into lymphoma xenografts was seen in mice co-treated with either taxol or adriamycin. The result replicates the finding that taxol has a synergistic anti-tumour effect with SS1P (an anti-mesothelin *Pseudomonas* exotoxin immunotoxin) against A431/K5 murine xenografts but does not improve uptake within the tumour mass.[314] Mathematical modelling of the SS1P data predicts that shed antigen is actually a favourable biological process for immunotoxin therapy of sold tumours (manuscript in press). The model predicts that shed antigen acts to protect the immunotoxin from the binding site barrier and enables a more uniform distribution of the immunotoxin within the tumour mass. Taxol may therefore not improve immunotoxin penetration, because it decreases the amount of shed antigen carrying immunotoxin within the tumour. Taxol or adriamycin may synergise at the single-cell level to enhance HA22 induced cell death.

The study has improved our understanding of why some ALL blasts are resistant to HA22. In addition the experiments demonstrated that the use of next-generation

immunotoxins or co-treatment with other agents can overcome resistance. The results strongly support the further development of anti-CD22 PE immunotoxins against ALL and Burkitt's lymphoma and offer new hope for a cure for these malignancies.

6.4 Publications

Some of the work in this Chapter contributed to the following paper:

- Fitzgerald DJ, Moskatel E, Ben-Josef G, Traini R, Tendler T, Sharma A, Antignani A, **Mussai F**, Wayne A, Kreitman RJ, Pastan I. Enhancing immunotoxin cell-killing activity via combination therapy with ABT-737. Leukaemia & Lymphoma 2011; 52: 79-81[315]

7. A cell line model of AML-induced immunosuppresion

7.1 Introduction

Chapters 1 and 2 examined the cytotoxicity and mechanisms of resistance of the anti-CD22 immunotoxin HA22 against paediatric Acute Lymphoblastic Leukaemia (ALL) and Burkitt's lymphoma. Chapters 3 and 4 investigate the immunobiology of another haematological malignancy, Acute Myeloid Leukaemia (AML).

The mechanisms by which AML blasts can inactivate the immune system of patients to expand and proliferate has been poorly understood. In this chapter I aim to understand how AML blasts can suppress T cells, a key immune activator and effector against malignant cells. In turn I examine how small molecule inhibitors can be used to target AML-derived immune suppression. Cell lines are used as a model throughout this Chapter.

7.2 Results

7.2.1 AML blasts suppress T cell proliferation and express markers of Myeloid-derived Suppressor Cells

To investigate whether AML can suppress T cell proliferation 7 AML cell lines were cultured with allogenic T cells in a Mixed Lymphocyte Reaction (MLR) assay. U937, K562 and HL-60 cell lines strongly inhibit T cell proliferation at a 1:1 ratio (AML: T cells). MOLM16, NB4T and KG1A8 did not suppress T cell proliferation by more than 50% even at a 1:1 ratio. The NOMO cell line had no suppressive activity (Fig7.1)

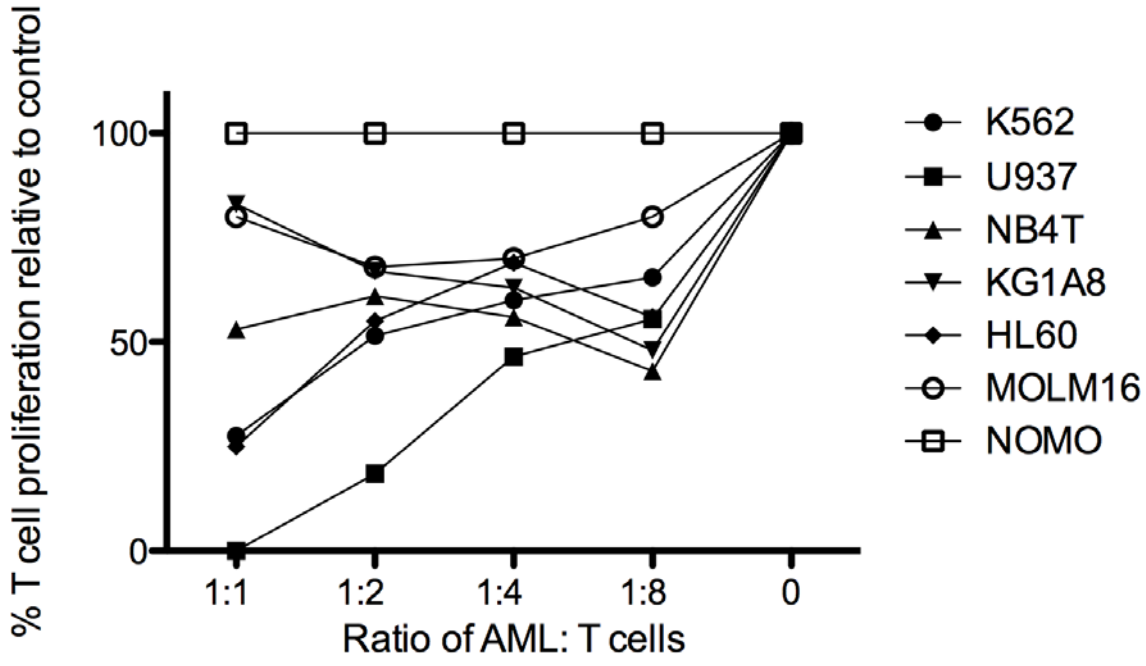


Figure 7.1 Suppressive activity of AML cell lines in an allogenic mixed lymphocyte reaction. Irradiated AML cell lines were cultured with allogenic T cells and dendritic cells to form a mixed lymphocyte reaction assay. The ratio of AML cells: T cells ranged from 0 to a 1:1 ratio. T cell proliferation was measured by ^{3}H-thymidine incorporation after 4 days. Data are the mean of triplicate wells. The experiment was repeated 3 times.

Non-malignant immature myeloid cells, called Myeloid Derived Suppressor Cells (MDSCs) have been described in cancer and inflammation, with an ability to suppress T cell proliferation. MDSCs are a heterogeneous population of cells characterised in humans by the immunophenotype $CD11b^{+}CD33^{+}HLA\text{-}DR^{low/-}$ and by the expression of the monocytic marker CD14 or the granulocytic marker CD15. AML is also heterogeneous in origin, with malignant cells arising from myeloid, monocyte, erythroid and megakaryocyte precursors. Therefore it was investigated if the suppressive cell lines U937, K562 and HL-60 share the same immunophenotype as MDSCs. Cell lines were stained for a panel of myeloid surface markers, and showed that U937 cells are $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}CD15^{+}CD14^{-}$, K562 cells are $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}CD15^{+}CD14^{-}$, and HL-60 are $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}CD15^{+}CD14^{-}$ (Fig 7.2). The immunophenotype of all cell lines is shown in Table 7.1. The result indicates that AML cells can share the same suppressive ability and immunophenotype as MDSCs.

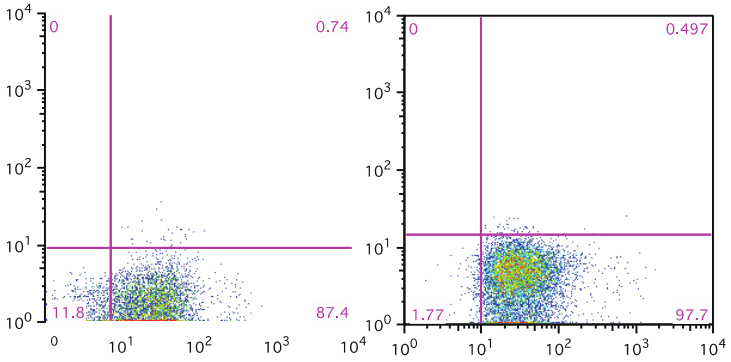


Figure 7.2 Immunophenotype of U937, K562 and HL-60 cells by flow cytometry. U937, K562 and HL-60 cell lines were stained with fluorescent antibodies against myeloid cell markers. Surface antigen expression was assessed by flow cytometry. FACS plots showing the percentage of cells expressing each marker.

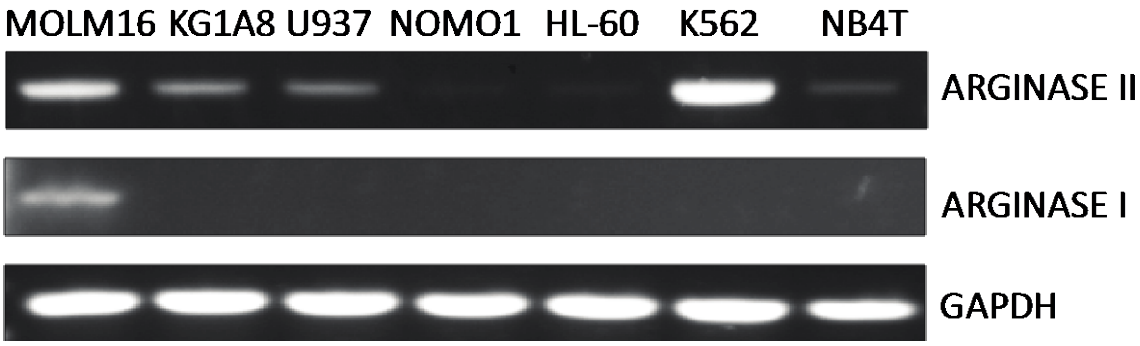
	CD11b	CD33	CD34	HLA-DR	CD14	CD15
U937	+	+	-	-	-	+
K562	+	+	-	-	-	+
HL-60	+	+	-	-	-	+
KG1A8	+	+	-	-	-	+
MOLM-16	+	+	+	-/low	-	-
NB4T	+	+	-	-	-	-
NOMO	+	+	-	+	-	+

Table 7.1 Immunophenotype of AML cell lines. AML cell lines were stained with fluorescent antibodies against myeloid cell markers. Surface antigen expression was assessed by flow cytometry.

7.2.2 The role of Arginase II in suppression of T cell proliferation

It has been reported in the literature that MDSCs suppress T cell proliferation by reducing arginine concentrations in the cellular microenvironment and increasing nitric oxide species, through the synergistic activation of Arginase I and iNOS enzymes. Following this concept the ability of AML cell lines to suppress T cell proliferation by the same mechansisms identified in MDSCs was investigated. Firstly RT-PCR was used to show that the 7 AML cell lines expressed Arginase II(Fig 7.3a) and confirmed by western blotting (Fig 7.3b). Arginase I was only detected in the MOLM16 cell line (Fig 7.3a).

a)



b)

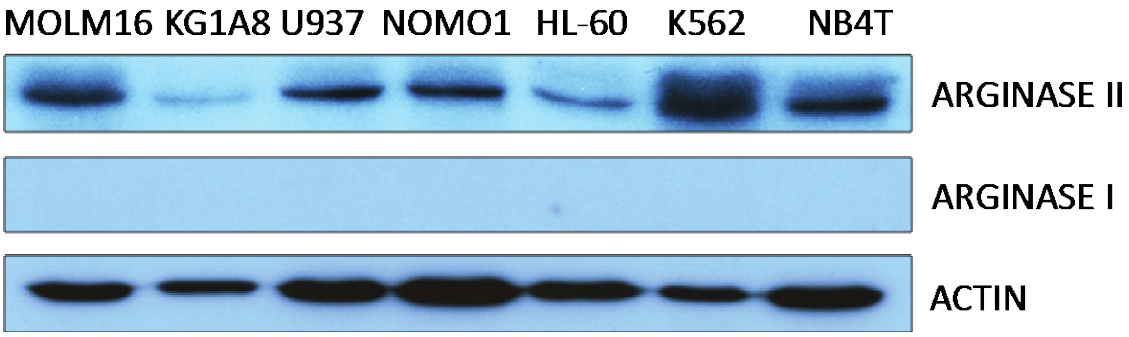


Figure 7.3 AML cell lines express Arginase II but not Arginase I. Expression of arginase I and II in AML cell lines as determined by (a) RT-PCR and (b) Western Blotting. GAPDH and actin were used as the house-keeping genes to ensure equal loading. Data are representative of 3 repeat experiments.

Therefore to investigate if the Arginase II present in AML cell lines was functional, the ability of cell lines to convert arginine into urea was tested by a colourimetric assay (Fig 3.4). K562 had the highest arginase activity (35 miliunits of arginase/ $1x10^6$ cells) compared to other cell lines. U937 also had significant arginase activity (3 miliunits of arginase/ $1x10^6$ cells) whereas the other cell lines, which do not inhibit T cells, have a very low endogenous arginase activity. The specificity of cell line arginase to convert arginine into urea in the assay was confirmed by adding the arginase inhibitor N^G-Hydroxy-L-arginine (NOHA, 0.5mM) to cell lysates. For K562 the arginase activity

was reduced 82% in the presence of the inhibitor (6.5 miliunits of arginase/ $1x10^6$ cells). In U937 the arginase activity was reduced 80% in the presence of NOHA (0.6 miliunits of arginase/ $1x10^6$ cells).

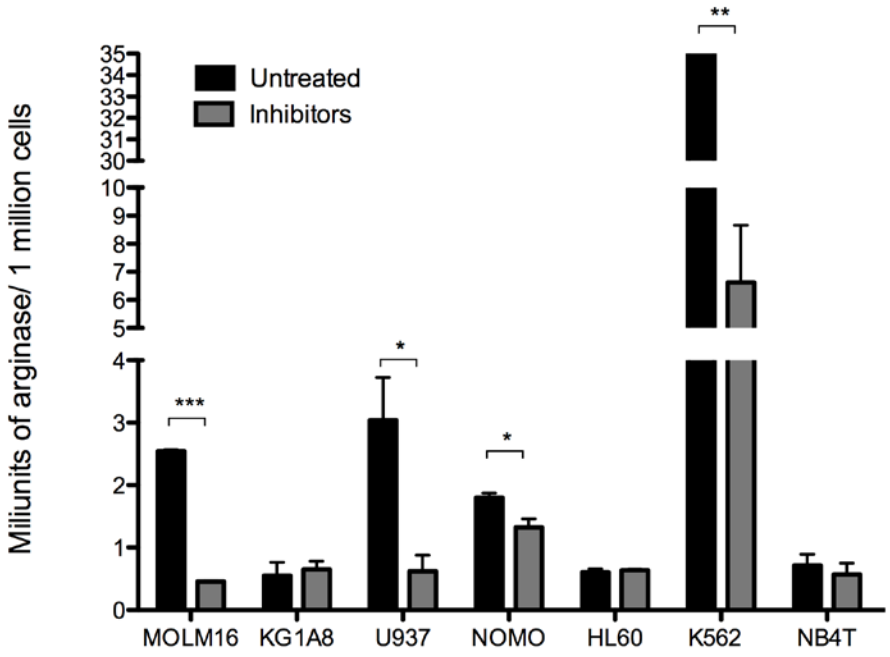


Figure 7.4 AML cell lines have increased arginase activity which is inhibited by the addition of NOHA. (* p<0.0001, ** p<0.03, * p<0.05).** Cell lysate of AML cell lines was tested for arginase activity using a colourimetric assay based on the conversion of arginine to urea. Data are the mean of triplicates. The experiment was repeated 3 times.

AML cells are metabolically highly active cells and are able to release molecules into their microenvironment. To investigate if Arginase II was released from AML cell lines the ability of cell line supernatants to convert arginine into urea was tested. Supernatants from all cell lines had significantly increased arginase activity compared to media alone (Fig 7.5). Supernatant from K562 cell cultures had the highest arginase activity, producing the most amount of urea (3micromoles).

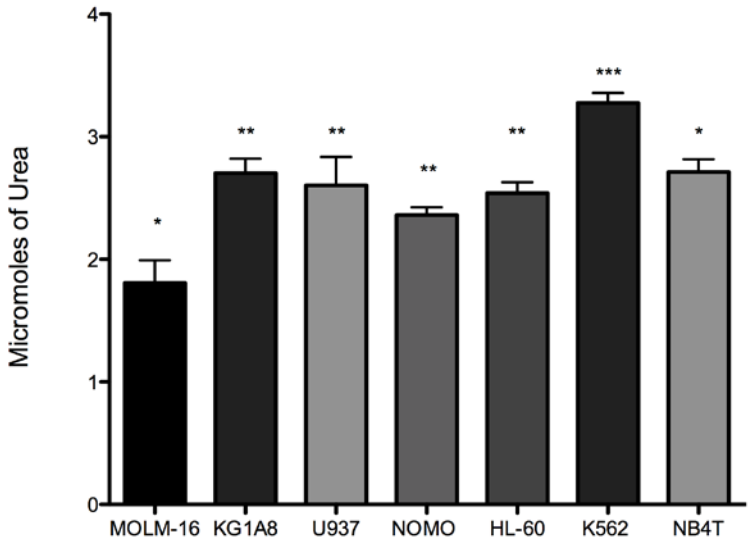
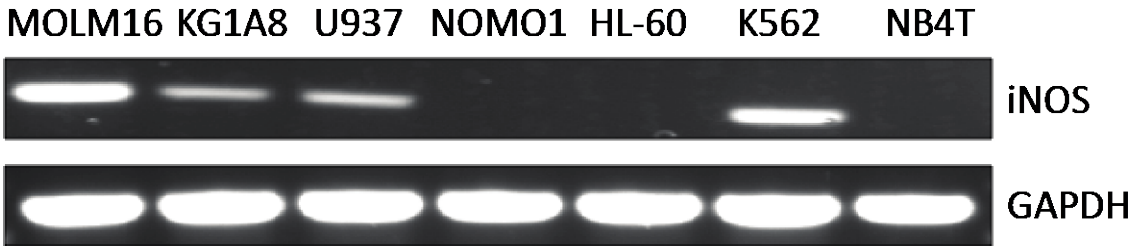


Figure 7.5 Supernatants from cell line cultures have increased arginase activity compared to media alone. (* p<0.0001, ** p<0.03, * p<0.05)** AML cell lines were cultured overnight. 50ul of cell supernatant was tested for arginase activity using a colourimetric assay based on the conversion of arginine to urea. Data are the mean of triplicates. The experiment was repeated 3 times.

7.2.3 The role of iNOS in suppression of T cell proliferation

Arginine metabolism can also lead to the production of reactive nitric oxide species through the enzyme iNOS. Using RT-PCR it was shown that iNOS is expressed by K562, U937, MOLM and KG1A8 cell lines (Fig. 7.6a), whereas it was not expressed by NOMO, HL-60, and NB4T cell lines. Nitrates produced, by the iNOS activity, are released into the tissue microenvironment and are able to inhibit T cell proliferation. Therefore supernatants from cell line cultures were tested for nitrate concentrations. Nitrate concentrations were significantly higher in supernatants of all cell lines compared to media alone (Fig. 7.6b). K562 supernatant contained the highest concentrations of nitrates (15uM).

a)



b)

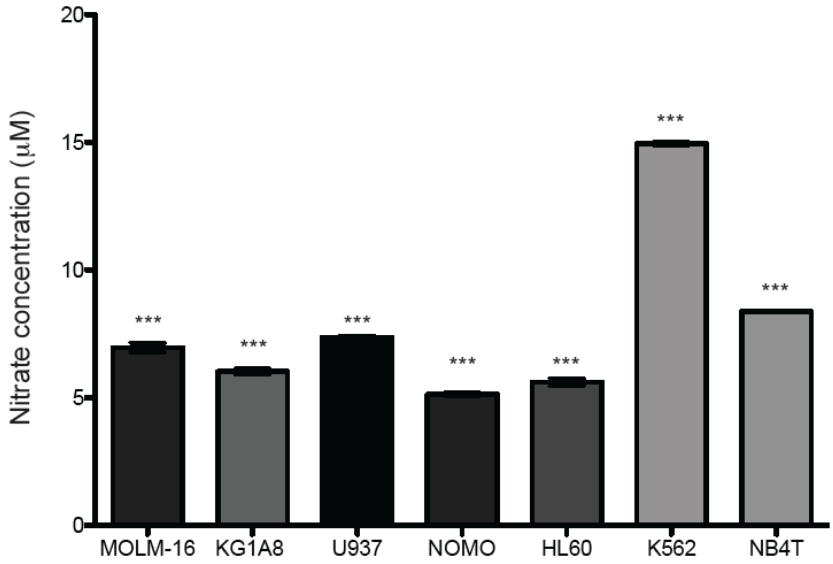
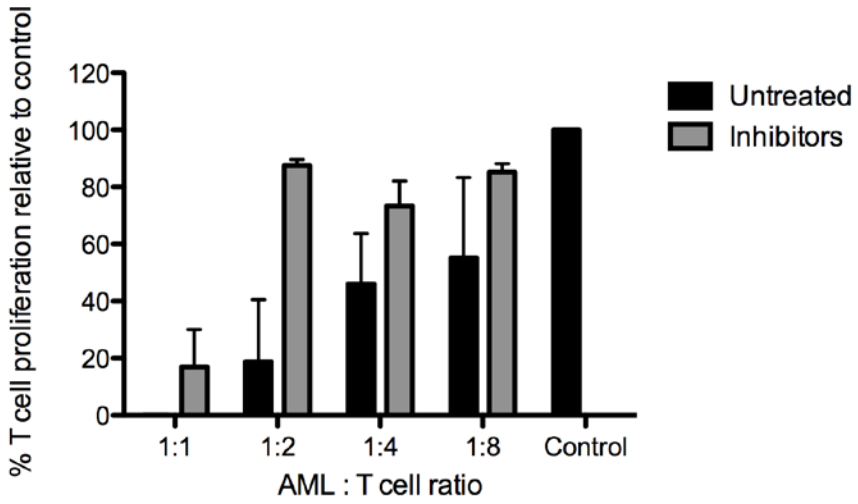


Figure 7.6 iNOS produces reactive nitric oxide species in AML cell lines (a) AML cell lines express iNOS by RT-PCR. GAPDH was used as a loading control (b) Nitrate levels are increased in the supernatants of cell line cultures after 24hours – as measured by the Greiss reaction colourimetric assay ($p<0.0001$ compared to media alone). Data are the mean of triplicates. The experiment was repeated 3 times.

Thus far the model established that AML cells contain functional arginase II and iNOS which can potentially suppress T cell proliferation. To confirm that AML cells do suppress T cell proliferation through these mechanisms the iNOS inhibitor N-G-monomethyl–l-arginine (L-NMMA, 0.5mM) was added with the arginase inhibitor N-G -hydroxy-L-l-arginine (NOHA, 0.5mM) to MLR cultures in the presence of U937 or K562 cell lines. For both cell lines T cell proliferation was restored by the combination of L-NMMA and NOHA (Fig. 7.7)

U937



K562

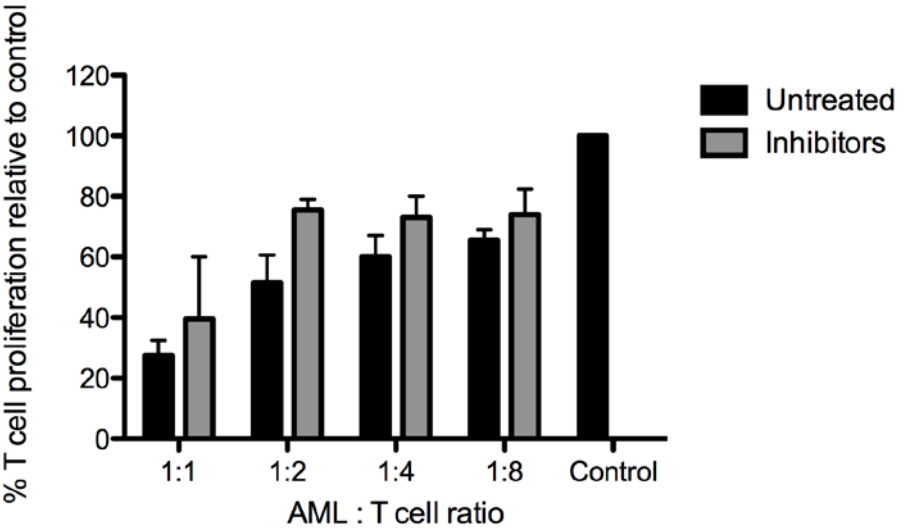


Figure 7.7 Culture of U937 and K562 cell lines in the presence of arginase and iNOS inhibitors restores T cell proliferation in a mixed lymphocyte reaction. Irradiated AML cell lines were cultured with allogenic T cells and dendritic cells to form a mixed lymphocyte reaction assay. The ratio of AML cells: T cells ranged from 0 to a 1:1 ratio. Arginase (NOHA, 0.5mM) and iNOS (L-NMMA, 0.5mM) inhibitors were added on day 1. T cell proliferation was measured by ^{3}H-thymidine incorporation after 4 days.Data are the mean of triplicates. The experiment was repeated 3 times.

7.2.4 The immunomodulatory proteins Serum Amyloid A and S100A9 are produced and released by AML cells

In addition to arginase and nitrates the release of another immunomodulatory molecule by AML cells was dertemined. It has previously been found, in our laboratory that Serum Amyloid A (SAA) can induce the immunosuppressive activity of neutrophils from melanoma patients.[202] In addition SAA is reported to have multiple, physiological roles including improving cell viability,[316] inducing chemotaxis of myeloid cells[317] and creating a pre-metastatic niche for myeloid cells.[318]

The expression of SAA by AML cell lines was investigated. SAA was released into the supernatants of 5/7 cell lines, but not by U937 and K562 (Fig 7.8a). The result was confirmed, using confocal microscopy, that although U937 and K562 do not spontaneously release SAA, they do express SAA (R. McEwen-Smith, Cerundolo Laboratory).

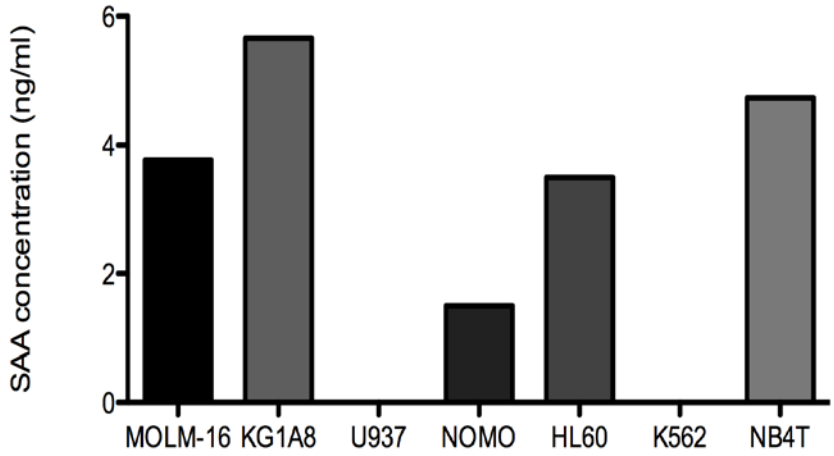


Figure 7.8 SAA is produced by AML cell lines. AML cell lines were cultured for 24hours in vitro. SAA levels in the supernatant were determined by ELISA.Data are the mean of triplicates. The experiment was repeated twice.

SAA is reported to act through a number of cell surface receptors including FPLR2, TLR2, and TLR4. The expression of these receptors in AML cell lines was confirmed using RT-PCR (Fig 7.9).

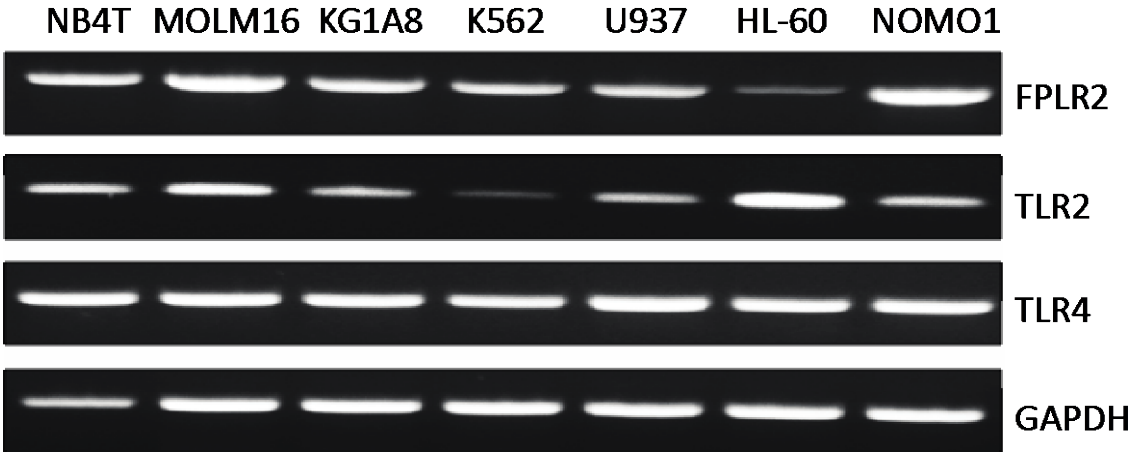


Fig 7.9 Expression of FPLR2, TLR2, and TLR4 by AML cell lines using RT-PCR. GAPDH was used as the housekeeping gene to ensures equal loading. The experiment was repeated 3 times. Representative data is shown.

As the receptors for SAA are expressed by AML cells, the ability of SAA to increase the viability of AML cell lines was investigated. AML cell lines were treated with 10ug/ml of SAA for 24hours in 1% serum containing media. No significant difference in the viability of the 7 AML cell lines, as measured by Propidium Iodide staining on flow cytometry, was found whether cultured in the presence or absence of SAA (Fig 7.10).

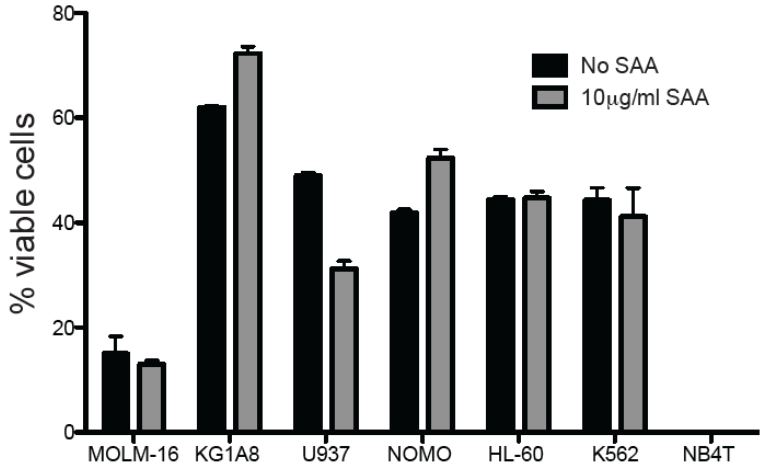


Figure 7.10 SAA does not improve the viability of AML cell lines cultured in serum-deprived media (1% FCS). AML cell lines were cultured in RPMI containing 1% FCS with or without 10ug/ml of SAA. Viability of AML cells after 24hours was assessed by PI uptake using flow cytometry.Data are the mean of triplicates. The experiment was repeated twice.

Finally the ability of SAA to induce the chemotaxis of AML cells was investigated. Labelled U937 cells were placed in the top of a transwell chamber, with 10ug/ml SAA in the lower chamber. The migration of U937 cells was followed for 16 hours. The results show that SAA induces the chemotaxis of U937 cells, compared to media alone (Fig 7.11).

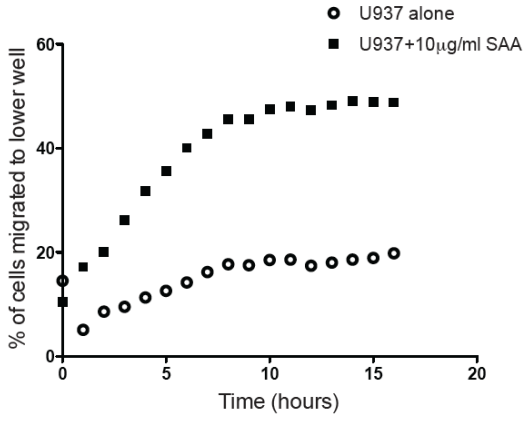


Figure 7.11 SAA induces chemotaxis of AML cell line U937. U937 was labelled with CMDA and cultured in the upper chamber of a transwell system. 10μg/ml SAA was added to the lower chamber. Migration of labelled U937 cells was measured by luminometer over a 16hour period. Data are the mean of triplicates. The experiment was repeated 3 times.

A recent publication shows that SAA can act with S100A8 and S100A9 proteins in establishing a pre-metastatic niche in a murine model of lung cancer.[318] In this study S100A8 and S100A9 lead to the expression and release of SAA from tumour-promoting myeloid cells. In turn, the released SAA acts as a chemo-attractant to other circulating myeloid cells. S100A9 is a member of the S100 family of calcium-binding proteins and has been recently described as a marker for human monocytic MDSCs.[319] S100A9 can act in an autocrine loop on MDSCs, via TLR4, to induce its own expression and release. S100A9 can also serve as a chemoattractant for MDSCs.[320]

To establish if S100A9 may also be part of a signalling cascade with SAA for AML cells, S100A9 expression was measured in the presence or absence of SAA. AML cell lines were cultured with 10ug/ml SAA for 24hours and the release of S100A9 was analysed by Western Blot (Fig 7.12). The result showed a significant increase of S100A9 in the supernatant of SAA-treated AML cell lines. Recombinant S100A9 is not commercially available and as it acts through multiple receptors further analysis of this protein's function in AML is challenging. However, the result does suggest that SAA can act in an autocrine and paracrine manner, to induce S100A9 and encourage the migration of AML cells.

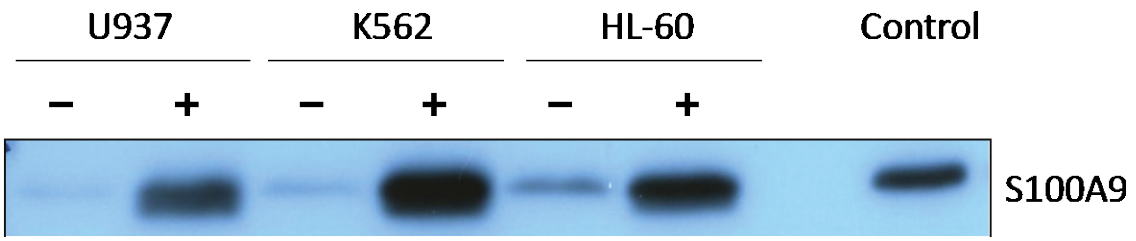


Figure 7.12 S100A9 is released from AML cell lines after stimulation with SAA. AML cell lines were cultured with 10ug/ml SAA for 24hours in vitro. Levels of S100A9 in the culture supernatant were measured by immunoblotting. Representative data shown of 2 experimental repeats.

This study is the first evidence that AML cell lines can created an immunosuppressive microenvironment through altered arginine metabolism. Although MDSCs canonically express Arginase I, AML cell lines act through Arginase II and additionally release this enzyme into the microenvironment. Arginase II acts in concert with iNOS activity to drive the suppressive phenotype of AML cell lines. The use of arginase and iNOS inhibitors can overcome AML suppressive mechanisms and rescue T cell proliferation, pointing at a potential therapeutic target for future study. In addition the study identifies that AML cell lines secret immunomodulatory molecules, as SAA and S100A9, which may act in an autocrine and paracrine manner. The results of this chapter provide a model which can now be applied to patient samples.

8. The immunosuppressive microenvironment created by AML blasts from patients

8.1 Introduction

Chapter 3 established that AML cell lines can suppress T cell proliferation in an arginase II and iNOS-dependent manner, in vitro. Small molecule inhibitors of arginase and iNOS were shown to overcome this suppressive activity. Furthermore, AML cell lines can secrete immunomodulatory proteins, such as Serum Amyloid A, which may play an important role in AML pathogenesis.

In this Chapter I seek to understand whether the cell line model is applicable to AML blasts from patients. I will investigate whether arginase II and iNOS are the mediators of suppressive activity and examine the potential of small molecule inhibitors of these enzymes to restore T cell proliferation. In addition I will aim to understand how AML cells can create an immunopriviledged niche through the interaction with surrounding monocytes in vitro and in vivo. Finally I will explore the activity of invariant Natural Killer T cells against AML blasts, to understand their therapeutic potential.

8.2 Results

8.2.1 AML blasts from patients suppress T cell proliferation

Patients diagnosed with AML frequently present with pancytopenia, including lymphopenia. The previous chapter demonstrated that AML cell lines are able to suppress T cell proliferation, in the setting of an allogenic MLR. To investigate if AML blasts from patients could be directly responsible for inhibiting T cell proliferation a MLR using AML blasts purified from patients' peripheral blood was performed. Samples from 16 patients were tested for suppressive activity (Fig 8.1). 12 out of 15 patients were able to suppress T cell proliferation significantly, even at the lowest AML: T cell ratio. Patients P5 and P11 had weaker suppressive activity, whereas P8 suppress T cell proliferation only by 10%.

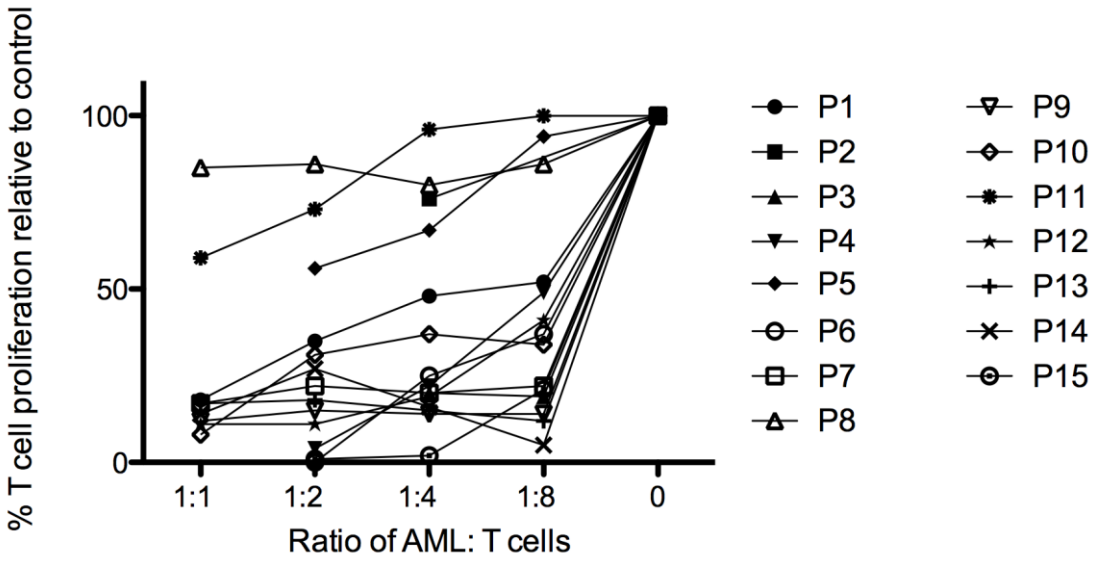


Figure 8.1 AML blasts from 15 patients were cultured with allogenic T cells in a Mixed Lymphocyte Reaction. The ratio of AML cells: T cells ranged from 0 to a 1:1 ratio. T cell proliferation was measured by ^{3}H-thymidine incorporation after 4 days. AML blasts from 12/ 15 patients strongly suppressed T cell proliferation. Patients are identified by unique symbols.Data are the mean of triplicate wells.

8.2.2 The role of arginase II and iNOS in suppression of T cell proliferation

In the AML cell lines the suppressive activity of arginase II and iNOS were responsible for the inhibition of T cell proliferation. Therefore the role of these two enzymes in the suppressive phenotype of AML cells patients was assessed. Firstly RT-PCR was used to examine the expression of arginase II, arginase I and iNOS in AML cells purified from patients, as described in the methods. Arginase II and iNOS are expressed by the majority of AML blasts, whereas arginase I was not detected (Fig 4.2). The expression of Arginase II, but not Arginase I has been confirmed by confocal microscopy (R.McEwen-Smith, Cerundolo Laboratory). The results demonstrate that Arginase II and iNOS are expressed in patient AML patients' blasts at diagnosis.

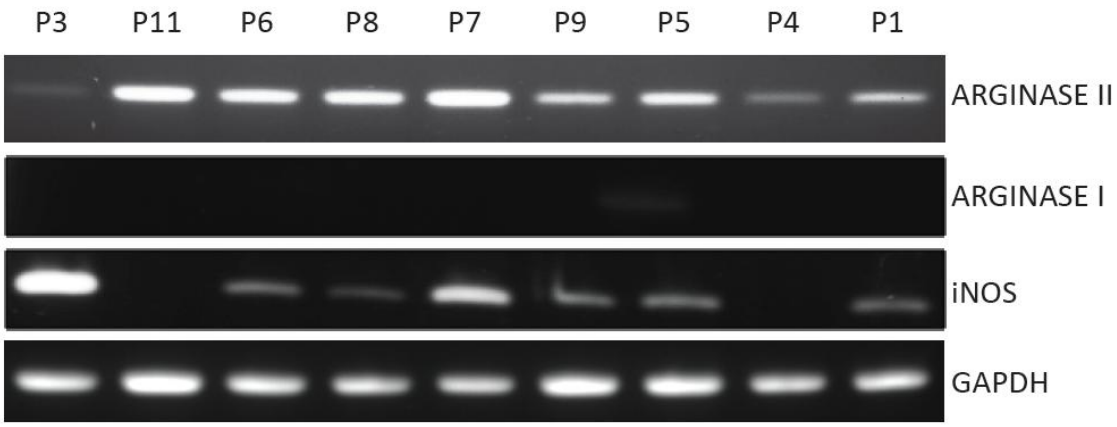


Figure 8.2 Arginase I, Arginase II and iNOS expression by RT-PCR in AML blasts from patients. Arginase I expression is absent for all samples. Representative data from 9 patients is shown. GAPDH was used as the housekeeping gene to ensure equal loading. Representative data of 3 experimental repeats.

In addition to the gene and protein expression the activity of the arginase II in patients' AML blasts was assessed. $1x10^6$ AML blasts were purified from patients and tested for

arginase activity using a colorimetric assay. The results showed high arginase activity by AML blast comparable to the activity of the cell lines (range 4.5-12.4 miliunits of arginase/ 1 million cells) (Fig 8.3).

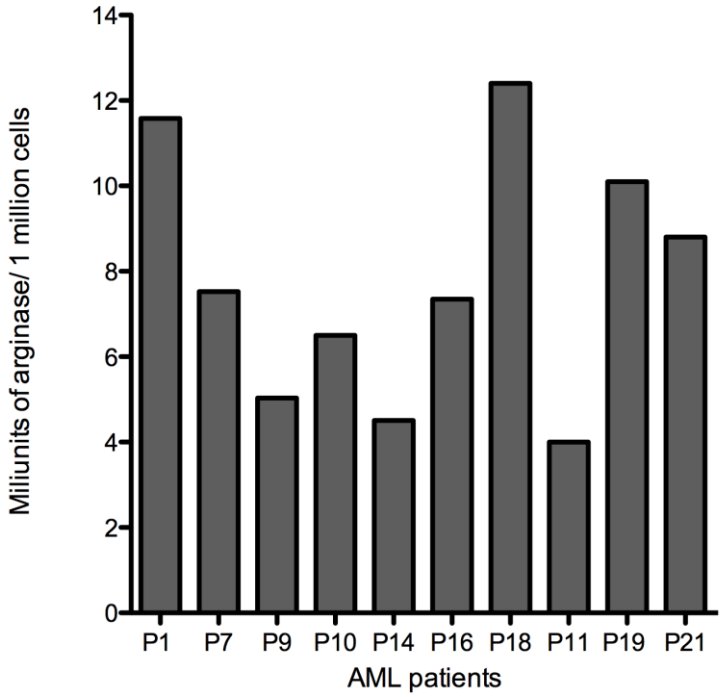


Figure 8.3 Arginase activity of patient-derived AML cells. Whole cell lysates of AML blasts from 10 patients was tested for the ability to convert arginine into urea using a colourimetric assay. Arginase activity is found for all patients. Means of triplicate wells, repeated in 2 experiments.

The reactive nitric species generated by iNOS cannot be directly measured as nitrates in culture media are well established as invalidating the results of the assay. To demonstrate that arginase and iNOS are responsible for patients‘ AML suppressive phenotype inhibitors were added to the AML-T cell co-culture: NOHA (0.5mM) and L-NMMA (0.5mM), arginase and iNOS inhibitors respectively. The combination of the two inhibitors rescued T cell proliferation in the mixed lymphocyte reaction in the

presence of AML blasts(Fig 8.4). The result confirms the specificity of arginase and iNOS activity in AML blasts as the mediators of T cell suppression.

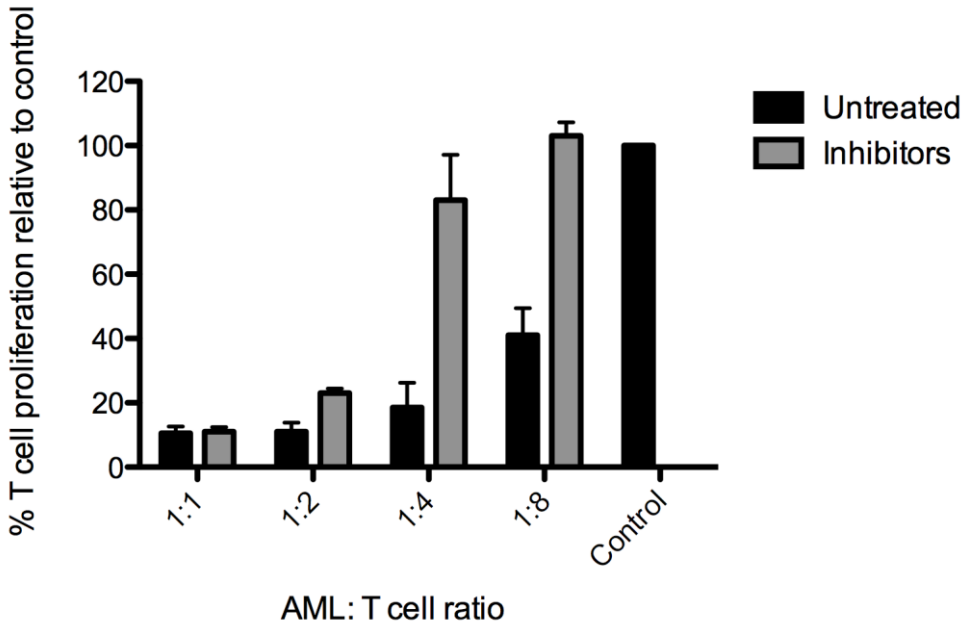


Figure 8.4 Arginase and iNOS inhibitors restore T cell proliferation. AML blasts from patients were cultured with allogenic T cells in a mixed lymphocyte reaction in the presence of the enzyme specific inhibitors for arginase (NOHA, 0.5mM) and iNOS (L-NMMA, 0.5mM). The ratio of AML blasts: T cells ranged from 0 to a 1:1 ratio. T cell proliferation was measured by ^{3}H-thymidine incorporation after 4 days. Culture with the enzyme inhibitors restores T cell proliferation. Representative data from one patient is shown.

8.2.3 Release of arginase II from AML blasts enhances the immunosuppressive microenvironment through modulation of surrounding monocytes

T cells activity can not only be directly inhibited by immunosuppressive cells, but also via modifying the phenotype of surrounding myeloid cells. In patients with solid tumours monocytes/macrophages with an immunosuppressive (M2) phenotype have been documented.[321,322] M2 like monocytes/macrophages are characterised by the

expression of CD206 (Mannose Receptor) in humans[323] and by the increase of arginase I expression[324] and IL-10 secretion.[325]

The ability of AML blasts to induce monocytes into an M2 phenotype and thus contribute to the immunosuppressive microenvironment in which AML resides was addressed. Monocytes from healthy donors were co-cultured with (2:1 ratio) AML blasts for 48 hours and afterwards analysed the expression of CD206 on monocytes. CD206 expression is very low on unactivated monocytes. The expression of CD206 was significantly upregulated on monocytes co-cultured with AML blasts. The upregulation occurred even if AML blasts and monocytes were separated by the transwell, indicating that the factors polarising monocytes to the M2 phenotype were soluble molecules released from the AML blasts (Fig 8.5). The reasons why CD206 expression is higher in the monocytes in the transwells compared to those in direct contact with the blasts is unclear. It is possible that direct contact between the AML blasts and monocytes leads to other signalling events that modulate CD206 expression.

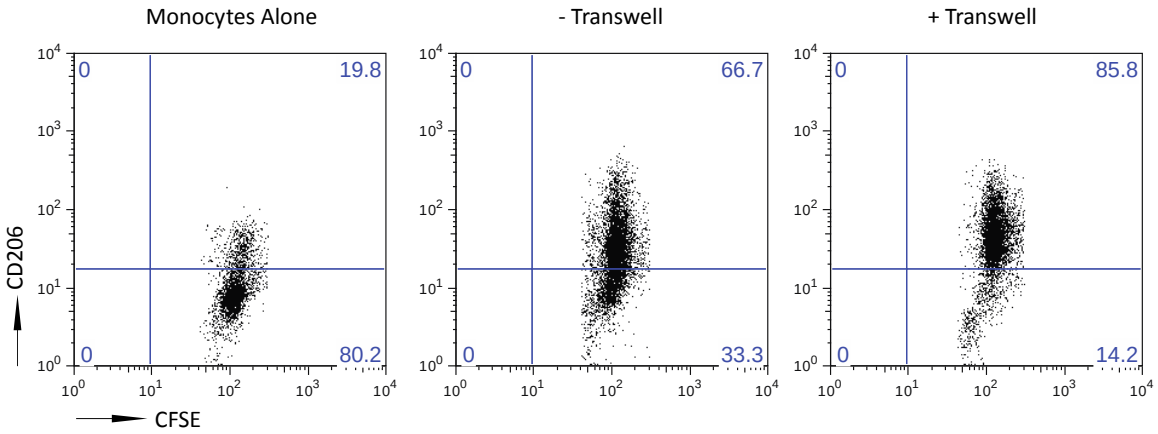


Figure 8.5 AML blasts increase CD206 expression on monocytes. Transwell culture of CFSE-labelled monocytes from healthy patients with AML blasts from patients upregulates CD206 expression on monocytes. Monocytes from healthy patients were placed in the lower well and AML blasts from patients were placed in the upper well of a transwell assay system. Representative flow cytometry plots from 1 patient of 11 tested are shown.

It has been demonstrated that IL-10, GM-CSF and TGF-β are able to polarise monocytes to a M2 like phenotype.[326,327] We investigated, by ELISA, whether AML blasts can spontaneously release IL-10, GM-CSF and TGF-β into culture supernatants. All supernatants were negative for these cytokines (data not shown). Instead a high level of arginase II was detected by ELISA (Fig 8.6).

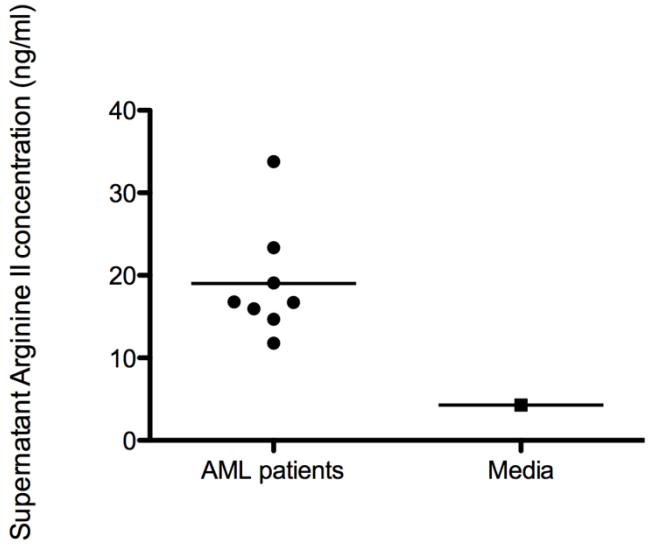


Figure 8.6 Arginase II concentrations are increased in the supernatants of AML cell cultures. AML cells were cultured overnight in a 96 well plate. Arginase II concentrations were measured by ELISA in culture supernatants and compared to media (RPMI-1640+10%FCS) alone. Data are mean of triplicates.

The role of arginase II released from AML cells in CD206 upregulation on healthy donor monocytes was investigated. Monocytes from healthy patients were cultured with supernatants from AML blasts with or without arginase inhibitors, or supplementation with arginine. The results demonstrated that L-NMMA (0.5mM) and NOHA(0.5mM) significantly reduced the expression of CD206 on monocytes towards M2 (Fig 8.7).

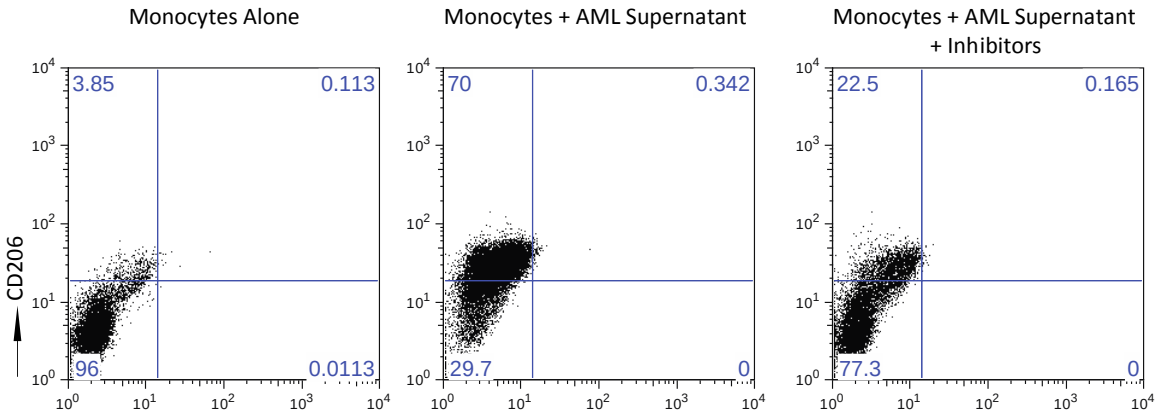


Figure 8.7 Polarisation of M2 monocytes is arginase dependent. Co-culture of CFSE-labelled monocytes from healthy patients with supernatants from AML blasts upregulates CD206 expression on monocytes. Culture in the presence of L-NMMA and NOHA significantly reduces CD206 expression Representative flow cytometry plots gating on CFSE positive cells are shown.

To investigate if AML blasts could polarize monocytes to an M2 phenotype in vivo the expression of YM-1, a murine M2 marker, on the monocytes of mice engrafted with different percentages of AML blasts was examined. Bone marrow was harvested and the percentage of YM-1 expressing monocytes (Ly6C+) and AML cells (human CD45+) cells were assessed by flow cytometry. YM-1 is significantly upregulated by the monocytes of mice engrafted with AML (Fig 8.8). Furthermore the percentage of monocytes expressing YM-1 in the bone marrow increased as the percentage of AML engraftment increased (Fig 8.9). To confirm if the model was relevant in humans, the presence of CD206+ monocytes in AML patients was investigated. Bone trephines of AML patients at diagnosis were stained for CD206. Significant expression of CD206+ cells was found, with morphology consistent with monocytes, in the bone marrow of newly diagnosed AML patients (Fig 8.10). Flow cytometry confirmed that CD206 is not expressed on AML blasts.

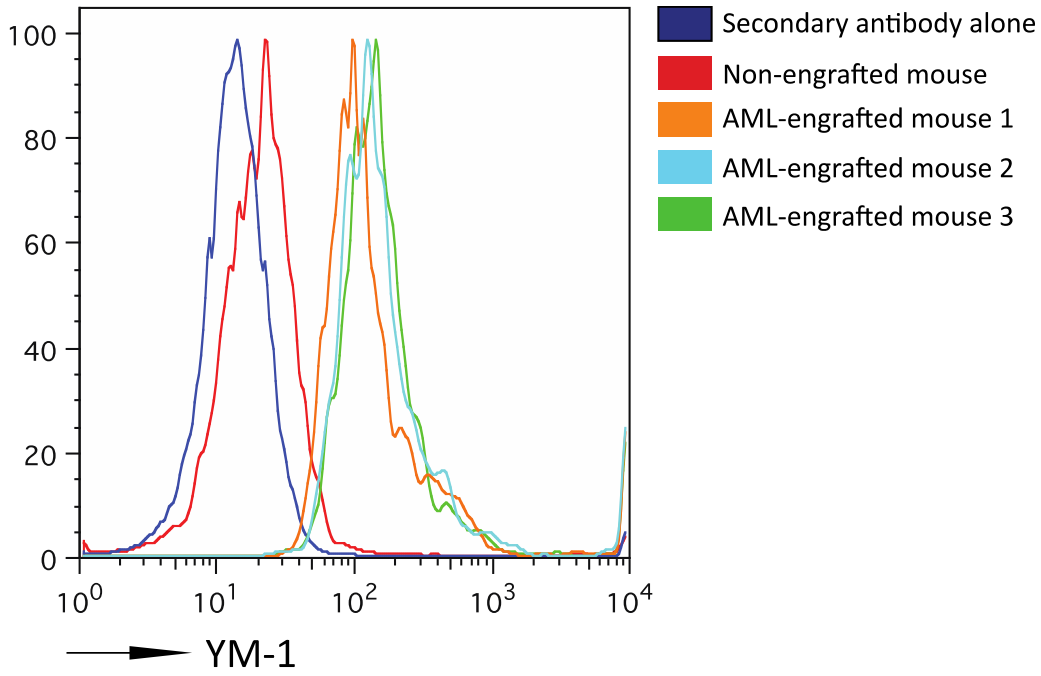


Figure 8.8 Monocytes from NOD-SCID mice engrafted with human AML express increased YM-1, indicating M2 polarisation. NOD-SCID mice were injected with AML cells from patients. Following engraftment, bone marrow was harvested from the femurs, and the expression of YM-1 on murine monocytes (Ly6C+) was assessed by flow cytometry.

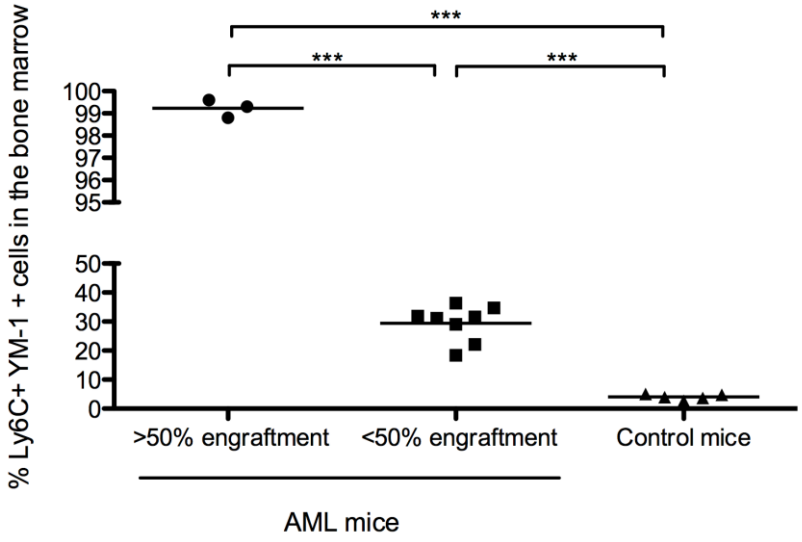


Figure 8.9 Increased percentage of YM-1 positive monocytes in NOD-SCID mice engrafted with human AML. NOD-SCID mice were injected with AML cells from patients. Following engraftment, bone marrow was harvested from the femurs, and the percentage of YM-1+ Ly6C+ murine monocytes was assessed by flow cytometry. (*** $p<0.001$, ** $p<0.03$, * $p<0.05$)

a) b)

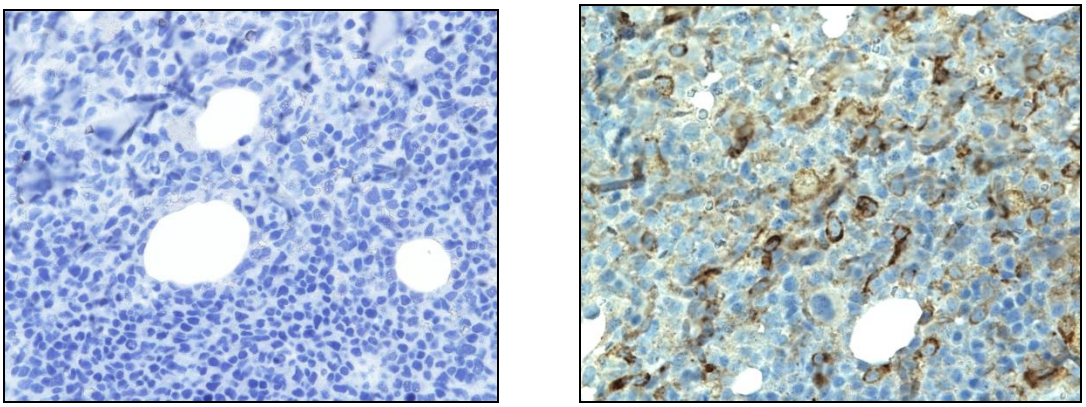


Fig 8.10 CD206+ monocytes are present in the bone marrow of patients with AML at diagnosis. Bone marrow trephines, embedded in paraffin, from an AML patient at diagnosis stained with DAPI alone (control) (a) and CD206 expression (b). Representative of bone marrows from 6 patients. Staining on each bone marrow section was performed twice.

8.2.4 Arginase II concentrations are increased in the plasma of AML patients

The previous experiments demonstrated that AML blasts spontaneously release high amount of arginase into culture supernatants. I hypothesized that arginase will therefore accumulate in the plasma of patients with AML. We analysed plasma from 17 patients with AML and 23 healthy controls for arginase I and arginase II by ELISA. Increased concentrations of arginase II in plasma from AML patients (Fig 8.11) was observed, whereas arginase I was not detected (Fig 8.12).

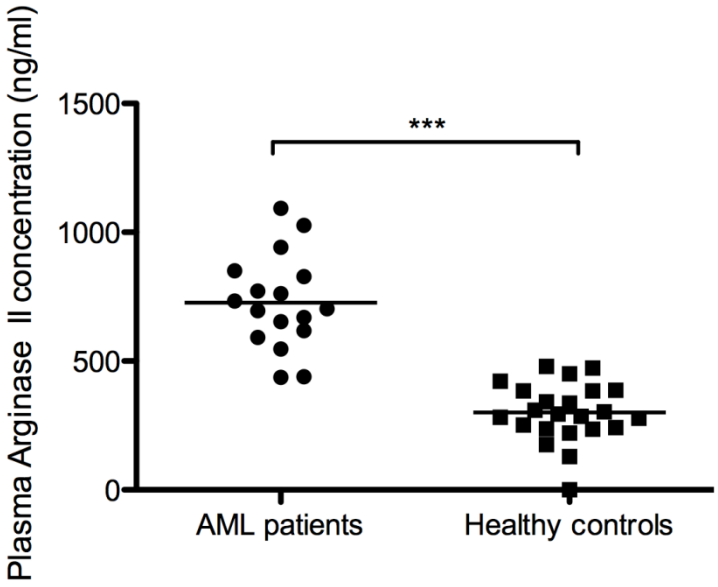


Figure 8.11 Arginase II is present in increased concentrations in the plasma of AML patients compared to healthy controls. Plasma from AML patients and healthy controls was analysed by ELISA for arginase II ($p<0.001$). The ELISA is specific for arginase II and does not cross-react with arginase I. Data points are the mean of triplicates.

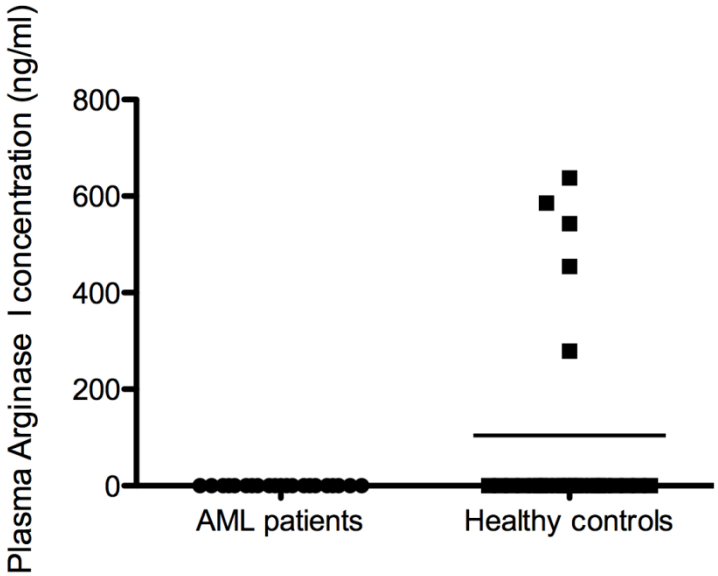


Figure 8.12 Arginase I is not increased in the plasma of AML patients compared to healthy controls. Plasma from AML patients and healthy controls was analysed by ELISA for arginase I. The ELISA is specific for arginase I and does not cross-react with arginase II. Data points are the means of triplicates.

To confirm that the arginase released is active, the arginase activity of the plasma from AML patients and healthy volunteers was compared(Fig 8.13). The arginase activity of patient plasma was significantly higher than that of healthy volunteers. The result suggests that AML blasts also create an immunosuppressive environment in the blood of patients, through increased arginine metabolism.

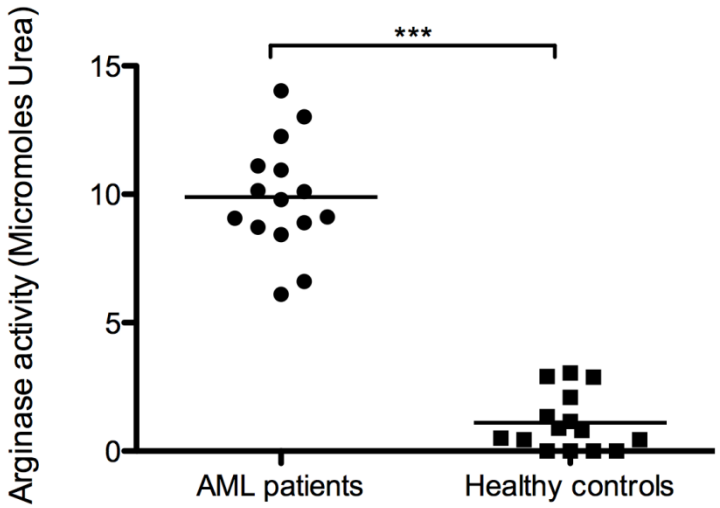


Figure 8.13 Arginase activity is increased in plasma from AML patients compared to healthy controls. Plasma obtained from AML patients at diagnosis and controls was tested (50µl) for arginase activity using a colourimetric assay based on the conversion of arginine to urea. (*** $p < 0.001$). Data points are the means of triplicates.

To confirm if the plasma of patients was indeed suppressive to T cell proliferation, plasma from AML patients at diagnosis or healthy volunteers was added to MLR cultures. AML patient plasma significantly inhibited T cell proliferation compared to that from healthy controls (Fig 8.14).

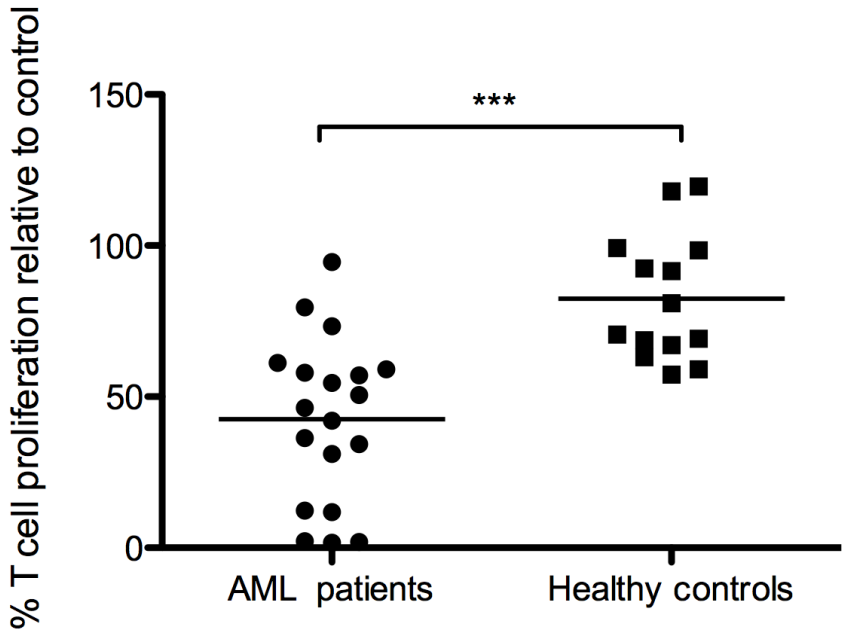


Figure 8.14 Plasma (50μl) from AML patients at diagnosis inhibits T cell proliferation in a mixed lymphocyte reaction compared to plasma from healthy controls. T cell proliferation was measured by ^{3}H-thymidine incorporation after 4 days (*** $p<0.001$). Data points are the means of triplicates.

8.2.5 AML patient plasma polarizes monocytes to an M2 phenotype in an arginase dependent manner

Following the previous experiments which showed that AML cells can polarize monocytes to an M2 phenotype, the ability of arginase II contained in the plasma of AML patients to increase CD206 expression on healthy monocytes was assessed. Healthy patient monocytes were co-cultured with plasma from AML patients, resulting in up-regulation of CD206 on monocytes, indicating a switch to an M2 phenotype (Fig 8.15).

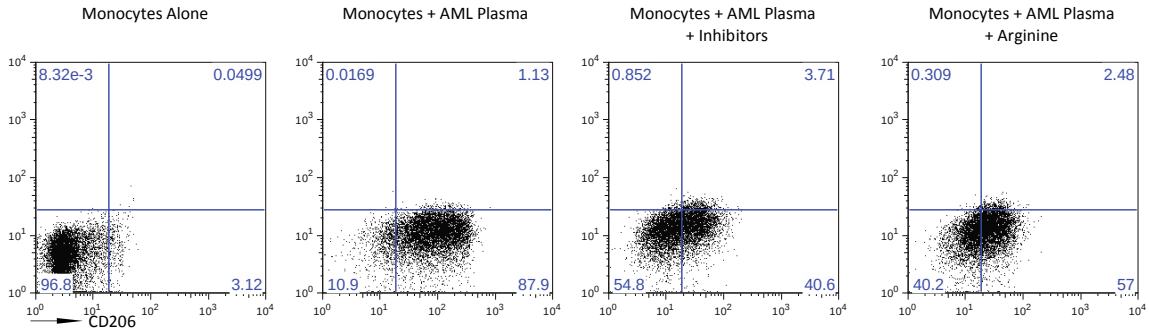


Figure 8.15 Plasma and AML blasts from patients can polarise monocytes to an immunosuppressive M2 phenotype in an arginase-dependent manner.

Culture of monocytes from healthy patients with plasma from AML patients upregulates CD206 expression on monocytes. Culture in the presence of L-NMMA (0.5mM) and NOHA (0.5mM) or the addition of arginine (100ug) significantly reduces CD206 expression. Representative flow cytometry plots from 1 patient of 18 tested are shown.

The role of arginase and nitric oxide species as the mediators of monocyte polarisation was confirmed through the addition of L-NMMA and NOHA to supernatants of AML cultures, or the addition of arginine (Fig 8.15). The arginase and iNOS inhibitors and exogenous arginine significantly reduced the expression of CD206 on monocytes.

8.2.6 AML spontaneously secretes the immunomodulatory protein Serum Amyloid A

The experiments above demonstrate that AML creates an immunosuppressive microenvironment partly through the release of immunomodulatory mediators such as arginase and reactive nitrite species. To investigate if AML blasts produce other immunomodulatory molecules ELISAs were performed on AML culture supernatants for TGF-β, IL-10 and GM-CSF. None of these cytokines were detected by ELISA,

however the levels of the acute phase protein Serum Amyloid A were significantly raised (Fig 8.16).

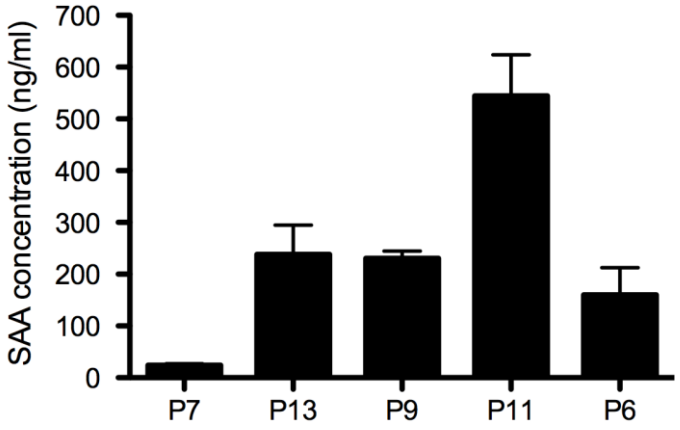


Figure 8.16 SAA is spontaneously produced by AML blasts from patients AML blasts were cultured for 24hours in vitro. SAA levels in the supernatant were determined by ELISA. Data points are the means of triplicates.

As large numbers of AML cells can be present in a patient's blood the level of SAA in patients' plasma was investigated. SAA levels were significantly raised compared to healthy controls, with cases over 100μg/ml (Fig 8.17).

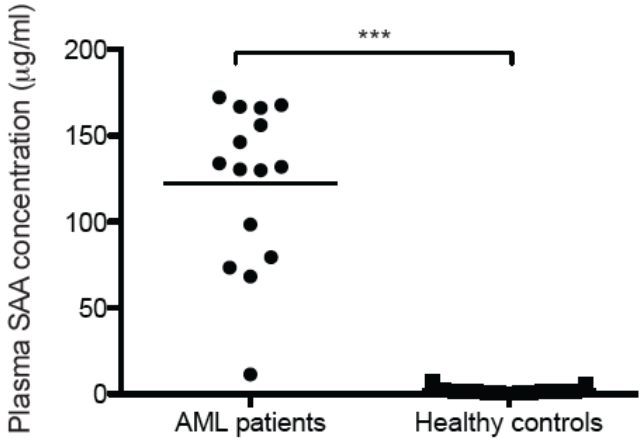


Figure 8.17 SAA is increased in the plasma of patients with AML SAA levels in plasma from AML patients and healthy donors was determined by ELISA. (*** $p<0.001$) . Data points are the means of triplicates.

It has been reported that LPS and inflammatory cytokines (IL1-β, IL-6 and TNF-α) induce SAA expression in hepatocytes, in tissue macrophages and synoviocytes. Although the data above demonstrated that SAA can be directly secreted from AML blasts the concentrations of another canonical hepatic-derived acute phase protein, C-Reactive Protein (CRP), in the supernatants of AML cultures was also measured. Interestingly, CRP was found in the supernatants of AML cultures after 24hours, albeit at significantly lower levels than SAA (Fig 8.18)

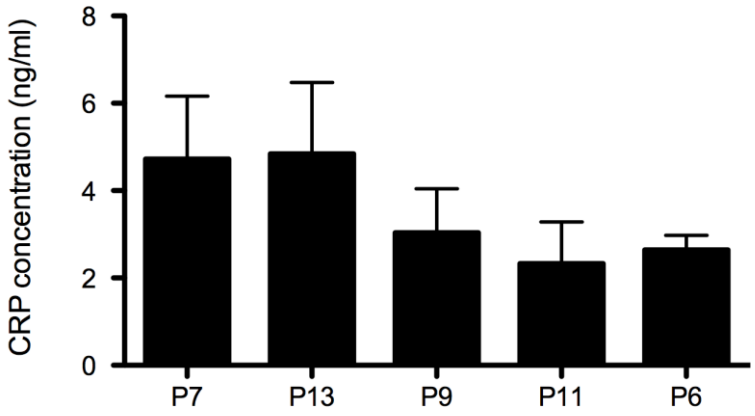


Figure 8.18 CRP is spontaneously produced at low concentrations by AML blasts from patients AML blasts were cultured for 24hours in vitro. CRP levels in the supernatant were determined by ELISA. Data points are the means of triplicates.

To investigate the levels of CRP in AML patient plasma compared to healthy controls ELISA were performed on patient plasma at diagnosis. Levels of CRP in patients' plasma were significantly higher than those of healthy controls, however were still 10,000 fold lower than for the SAA (Fig 4.19). It should be noted that the levels of CRP found in the AML patients would be classed as within the normal range of clinical laboratory standards.

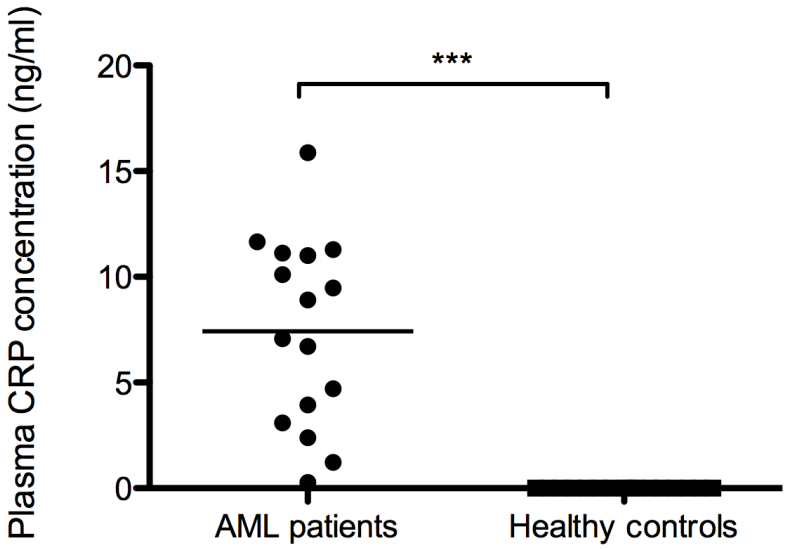


Figure 8.19 CRP is increased in the plasma of patients with AML. CRP levels in plasma from AML patients and healthy donors was determined by ELISA (*** $p<0.001$). Data points are the means of triplicates.

8.2.7 Serum Amyloid A augments the immunosuppressive environment through IL-1β and S100A9

As SAA is significantly raised in the plasma of patients and is directly secreted from AML cells the functions SAA can have in AML were investigated. It has been reported that SAA has numerous proinflammatory actions: it is chemotactic to neutrophil, monocytes and T lymphocytes, causing leukocyte infiltration and promoting neutrophil adhesion to endothelial cells, and stimulates monocytes and neutrophils to release cytokines, tissue factor, and matrix metalloproteinases.[328] In Chapter 3 SAA induced migration of U937 cells to a greater extent than media alone, indicating that SAA can function as a chemotactic protein. As SAA levels are high in patient plasma, the migration of U937 in response to plasma from AML patients or healthy controls was

investigated. AML cells migrated significantly in response to patient plasma, compared to media alone or plasma from healthy patients (Fig 8.20).

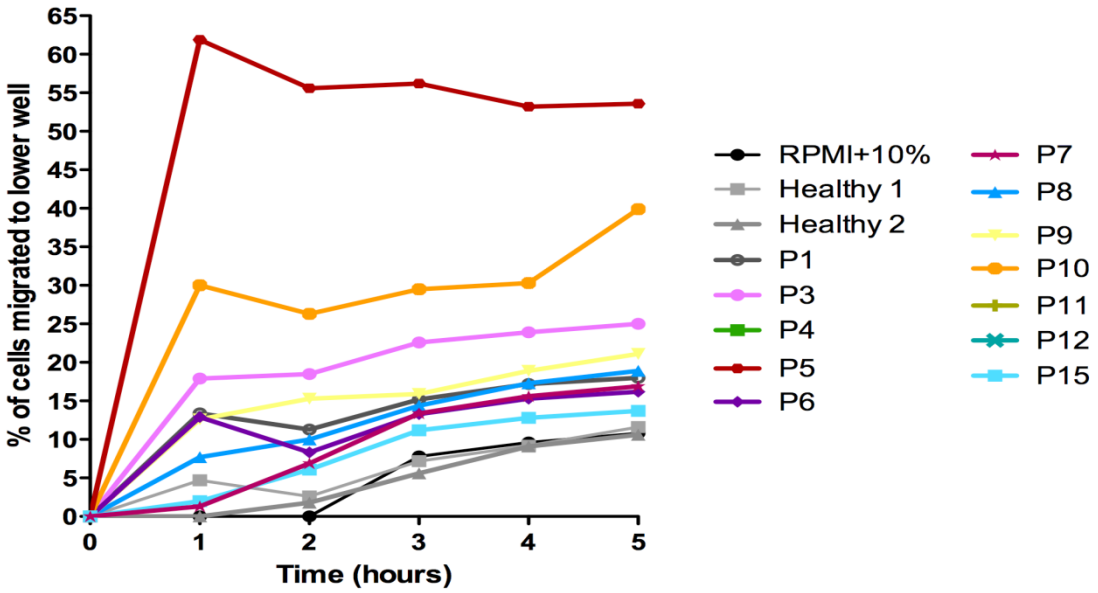


Figure 8.20 AML patient plasma induces AML cell line migration. The AML cell line U937 was labelled with CMDA and cultured in the upper chamber of a transwell system. Plasma (700ml) from AML patients or healthy controls was added to the lower chamber. Migration of labelled U937 cells was measured by luminometer over a 5 hour period. Data points are the means of duplicates.

As described in Chapter 3, SAA is reported to act via a signalling cascade with IL-1β and S100A9 proteins to promote tumourigenesis.[318] SAA can act through multiple cell surface receptors which are expressed by AML blasts from patients (Fig 8.21).

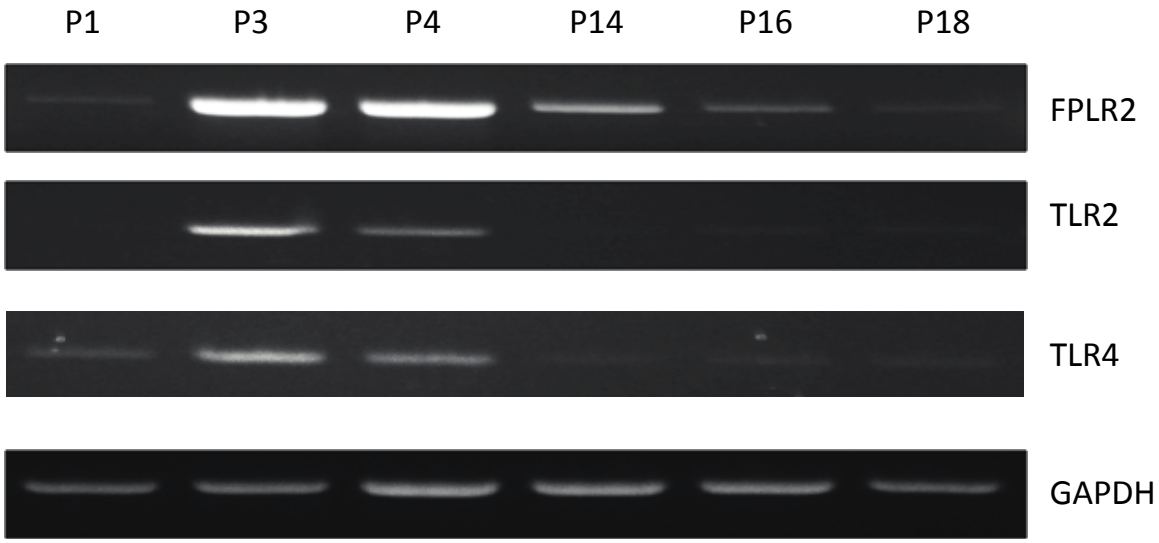


Figure 8.21 FPRL2, TLR2, TLR4 and S100A9 expression by RT-PCR in AML blasts from patients. Representative data from 6 patients is shown. GAPDH is the housekeeping gene used to ensure equal loading.

The cytokine profile of AML supernatants, following treatment with SAA, was examined and raised levels of Il-1β were found (Fig 4.22). IL-1β was not detected in the plasma of patients (data not shown). It is established in the literature that IL-1β cannot be detected in the plasma of patients with AML.[329]

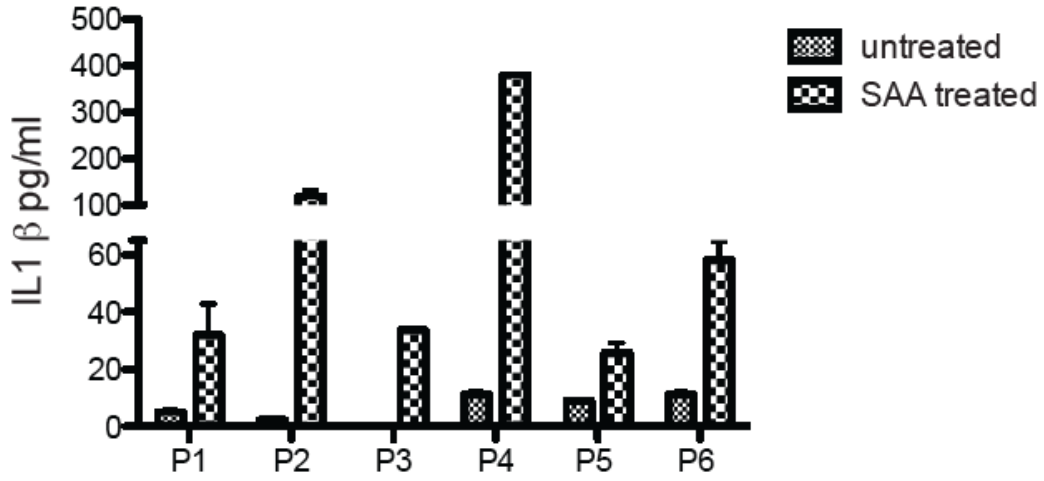


Figure 8.22 SAA induces IL-1β secretion from AML blasts. Blasts from AML patients were cultured with 10mg/ml SAA for 24hours in vitro. Levels of IL-1β in the culture supernatant were measured by ELISA. Data are the means of triplicates.

The results in Chapter 3 showed that AML cell lines can secrete the immunomodulatory protein S100A9 in response to SAA. S100A9 has also been used as a marker of immunosuppressive myeloid cells.[319] AML cells from patients were treated with SAA and confirmed that S100A9 is released into the culture supernatants. (Fig 8.23).

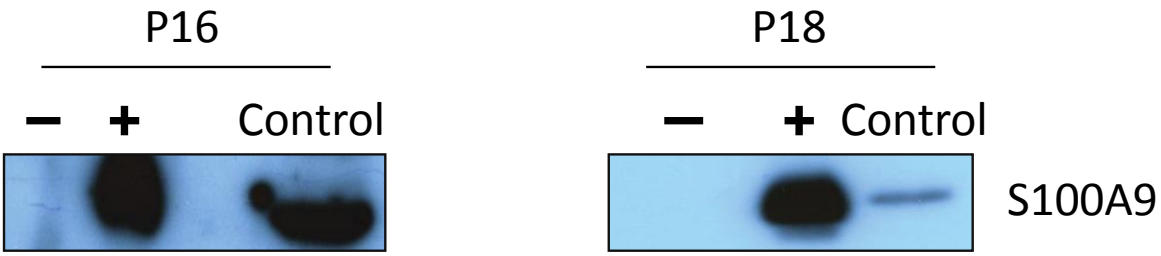


Figure 8.23 AML cells release S100A9 in response to SAA. AML blasts from patients were cultured with 10μg/ml SAA for 24hours in vitro. Levels of S100A9 in the culture supernatant were measured by immunoblotting. Representative blots from 2 patients are shown.

The plasma of 7 AML patients was analysed and found moderately high levels of S100A9 protein (mean 28.2ng/ml) (Fig 8.24). This is higher than levels reported in healthy controls (3.48ng/ml in healthy volunteers).[330]

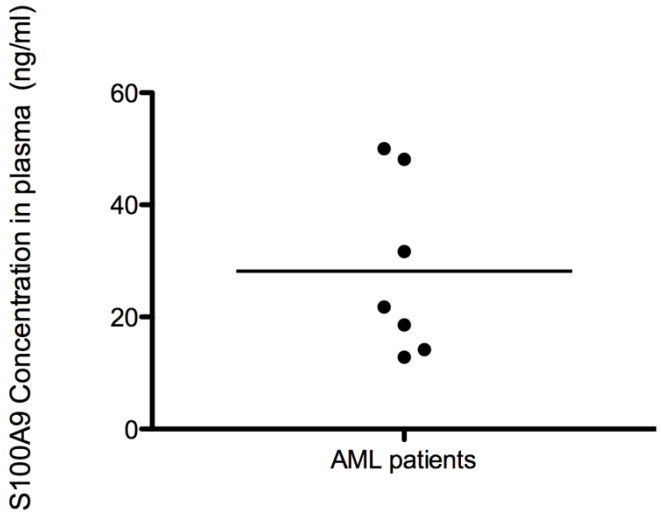


Figure 8.24 S100A9 is raised in the plasma of AML patients. Levels of S100A9 in the plasma of 7 AML patients was measured by ELISA. Data are means of triplicate wells.

8.2.7 Activated iNKT cells overcome the suppressive activity of AML blasts and restore T cell proliferation

The above results demonstrate that AML blasts are able to significantly impair T cell proliferation and thus inhibit the immune response. iNKT cells have previously been shown to modulate the activity of immunosuppressive neutrophils[202] and also to be cytotoxic to AML cells.[181] Therefore the ability of iNKT cells to modulate the immunosuppressive capacity of AML blasts was investigated. iNKT cells require activation through recognition of endogenous or exogenous CD1d restricted antigens on

antigen-presenting cells or target cells. In the presence of the iNKT agonist αGalCer, AML cells activate the iNKT cells as demonstrated by IFN-γ secretion (Fig 8.25).

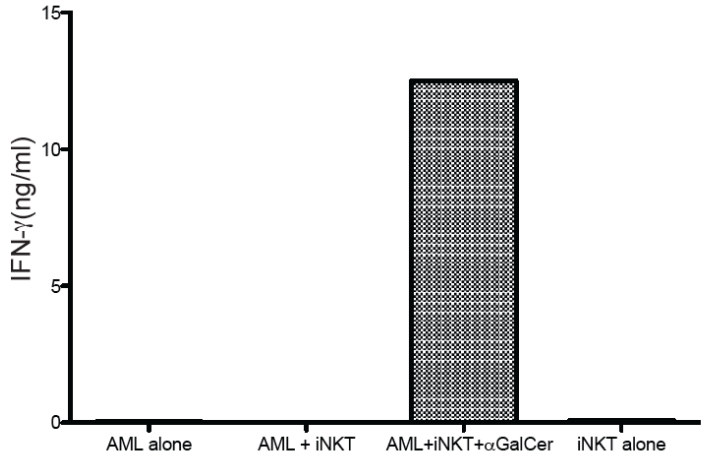
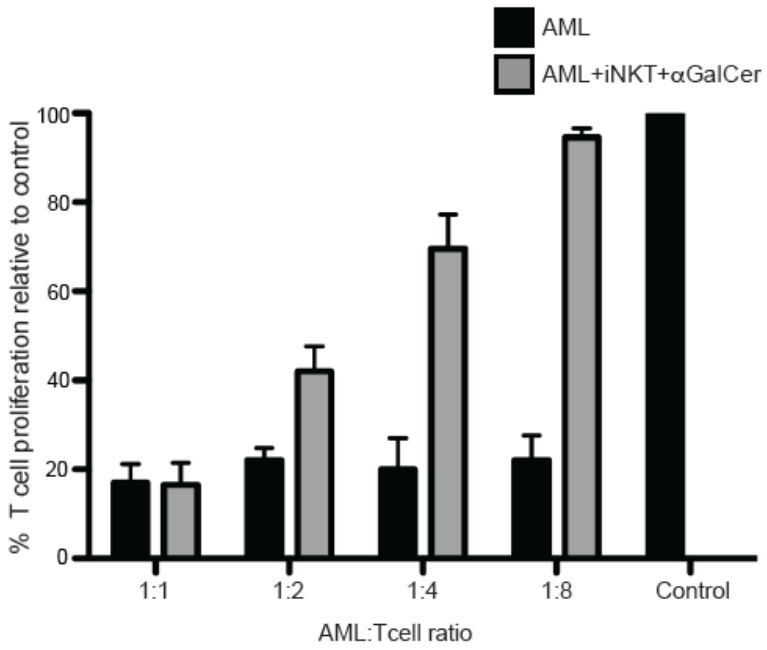


Figure 8.25 AML blasts activate iNKT cells in the presence of αGalCer. AML blasts were cultured with iNKT cells (ratio 4:1) in the presence or absence of αGalCer (100ng/ml) for 8 hours. IFN-γ secretion was measured by ELISA. Representative data from one patient (of 5) is shown.

As iNKT cells can be activated, in the presence of αGalCer, by AML blasts the ability of iNKT cells overcome the suppressive activity of the AML blasts, to restore the T cell proliferation, was investigated(Fig 8.26). In 2 cases T cell proliferation was restored to greater than 80% of the control, and in 2 other patients T cell proliferation was improved to a lesser extent. The results indicate that activated iNKT cells can restore T cell proliferation, although the effect is patient dependent.

a)



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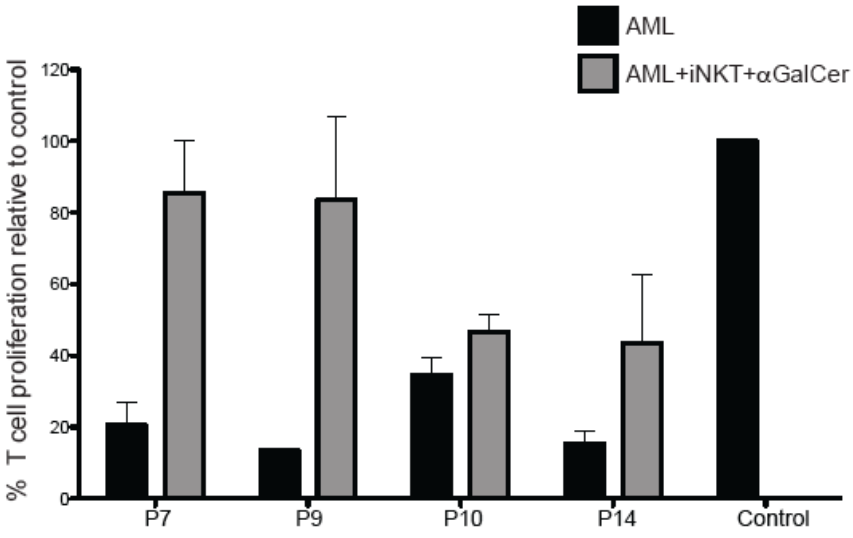

Figure 8.26 Ability of iNKT cells to restore T cell proliferation in an allogenic MLR. AML blasts were cultured with iNKT cells (1:16 ratio) in the presence of αGalCer for 8 hours. After 8 hours the cells were washed and allogenic dendritic cells and T cells were added to the assay to form a Mixed Lymphocyte Reaction. The ratio of AML cells: T cells ranged from 0 to a 1:1 ratio. T cell proliferation was measured by ^{3}H-thymidine incorporation after 4 days. MLRs from a single patient with changing AML: T cell ratio in the presence of activated iNKT cells (a) and from 4 patients tested at a fixed ratio of 1:8 AML: T cell ratio (b). Data are the mean of duplicates.

To investigate the mechanism in which iNKT cells restore T cell proliferation cultures were analysed by flow cytometry to assess AML viability after culture in the presence of iNKT cells. Staining with propidium iodide revealed that following culture with activated iNKT cells, the numbers of viable AML cells was significantly decreased. Therefore activated iNKT cells restore T cell proliferation through induction of AML cell death (Fig 8.27).

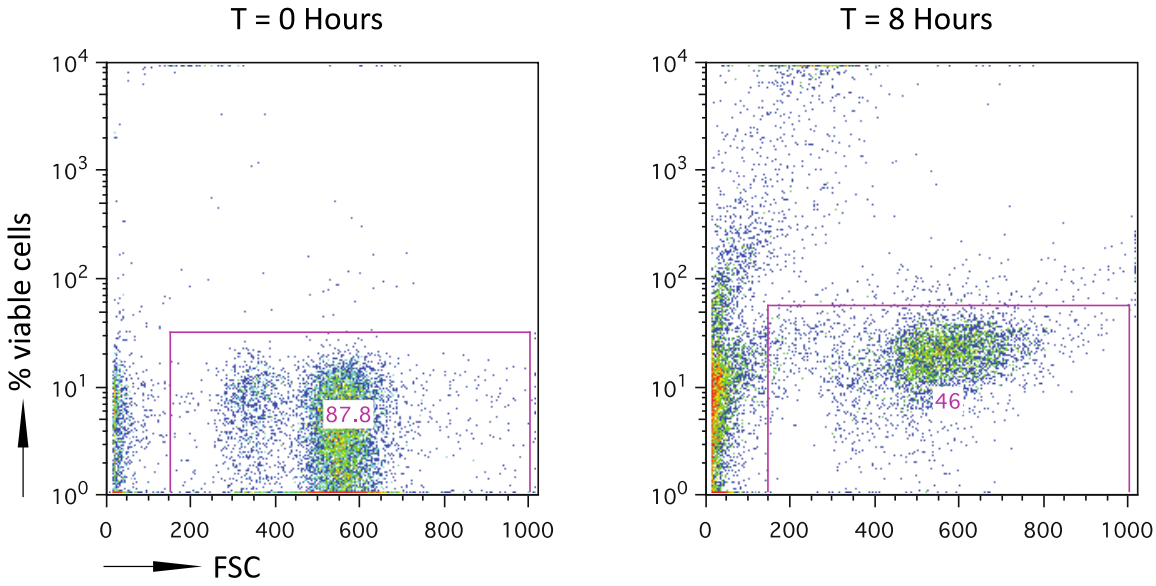

Figure 8.27 Decreased numbers of viable AML cells following culture with activated iNKT cells. Flow cytometry plots showing the percentage of viable AML cells (gated population) after 8 hours culture with iNKT cells in the presence of αGalCer. Data from one representative patient (of 5) is shown.

In summary I have shown that AML creates a unique immunosuppressive

microenvironment. AML blasts can suppress T cell proliferation through arginase II and iNOS dependent mechansims. In turn AML blasts can co-opt surrounding monocytes/macrophages to develop an alternatively activated phenotype, contributing to an immunopriviledged niche. Therapeutically the inhibition of T cell proliferation can be restored through the use of drugs (specific arginase and iNOS inhibitors) or activated iNKT cells. Finally AML blasts secrete SAA, which enhances the

immunosuppressive microenvironment through the stimulation of AML chemotaxis and the induction of the immunomodulatory molecules IL-1β and S100A9.

8.3 Discussion: Chapters 3 and 4

In Chapter 3 I identified that the AML cell lines U937, K562, and HL-60 can suppress T cell proliferation in vitro. The suppressive activity is mediated through Arginase II expression and activity and the production of reactive nitric oxide species. Inhibitors to arginase and iNOS can overcome these suppressive mechanisms to restore T cell proliferation in vitro. Finally I found that AML cell lines can express serum amyloid A and S100A9. The above results demonstrated that AML cell lines can provide a model which could be tested on primary AML blasts from patients.

In Chapter 4 I hypothesised that aimed that AML blasts from patients had the same immunosuppressive properties identified in the AML cell line model. The study first showed that patient-derived AML blasts suppress T cell activity in an Arginase II/ iNOS dependent manner. AML blasts extend their immunosuppressive microenvironment through the release of Arginase II, which I showed to be highly concentrated in the plasma of leukaemia patients. In addition AML blasts polarise surrounding monocytes into a suppressive M2 phenotype to create an immunosuppressive niche. Interestingly, I demonstrated that AML blasts, like cell lines, produce and release the acute phase protein Serum Amyloid A. SAA can act in an autocrine or paracrine fashion to induce the migration of AML blasts, and AML blasts stimulated with SAA release the immunomodulatory proteins IL-1β and S100A9. Finally the study demonstrated that the above immunosuppressive activity of AML blasts can be modulated through the use of small molecule inhibitors against arginase and iNOS, and also through the cytotoxic capacity of invariant NKT cells.

The ability of patient-derived AML blasts to create an immunosuppressive microenvironment has been previously studied. T cells and NK cells cultured in AML supernatants have decreased proliferation but no loss in cytolytic activity.[331] Similarly the supernatants of AML blast cultures were shown to hold T cells in the G0 phase of cell division and inhibit the production of Th1 cytokines.[332] Although both these studies identified that AML supernatants were able to suppress T cell responses, no causative molecule was identified. A subsequent study identified that in approximately 50% of patients examined, AML cells express the tryptophan catabolising enzyme IDO, which induces T-reg $CD4^{+}CD25^{+}Foxp3^{+}$ invitro and in vivo.[333] Higher serum kynurenine/tryptophan ratios were also reported in patients with AML than healthy controls.[334] Interestingly, analysis of the blood of AML patients reveals that the absolute number of autologous T cells is actually increased at diagnosis.[335] However, gene expression profiling of the autologous T cells reveals an altered expression profile, particularly in genes related to the actin cytoskeleton, compared to healthy controls. In addition, the study showed that T cells from AML patients are less able to form immunologic synapses with the AML blasts and recruit intracellular signalling molecules. Together the results suggest that a microenvironment suppressive to T cell responses does exist in patients with AML. Despite these studies it is clear that the mechanism by which AML cells can suppress T cell proliferation and create an immunopriviledged niche has not been fully identified.

I demonstrated that AML blasts isolated from the majority of patients at diagnosis are strongly suppressive to T cell proliferation, in the setting of a mixed lymphocyte

reaction. AML blasts are a malignant expansion of immature myeloid cells and thus are reminiscent of the myeloid-derived suppressor cells identified in a number of human diseases and tumours.[336] AML blasts express the same immunophenotype described for MDSCs: $CD33^{+}HLADR^{-}$ $CD14^{+}CD15^{+}$ suggestive of their common developmental origin.[180] This concept is supported by a recent study in which a human promyelocytic-like population was found to be the population responsible for immune suppression mediated by MDSCs.[126]

One of the most well characterised mechanisms of MDSC immunosuppression is through altered arginine catabolism and the production of reactive nitric species. It is well established that Arginase I expression in myeloid-derived suppressor cells is responsible for the suppressive activity of these cells in cancer patients. This study found that Arginase I is not expressed by the majority of analysed AML blasts, but instead they express Arginase II, another isoform of arginase that has been less well characterised in the context of immunosuppression. The Arginase II in the AML cells is active, and able to convert arginine to urea. 2 isoforms of arginase exist in mammals as a likely result of gene duplication during evolution. Arginase I is located in the cytosol, whilst arginase II is located in the mitochondria.[337] The two enzyme isoforms are the products of distinct genes located at band q23 on the long arm of chromosome 6 (Arginase I)[338] and band q24 on chromosome 14 (Arginase II).[339] Both enzymes catalyse the same reaction converting arginine into ornithine, with urea as a by-product. In healthy patients Arginase I is predominantly localised to the liver with little or no expression in other tissues. Arginase II is expressed in a more diverse range of cells including kidney, vascular endothelium, lung, and pancreas.[340] The role of Arginase II in inhibiting immune responses has only been studied in a limited fashion - Arginase 2 expression by prostate cancer cells has been shown to inhibit T cell proliferation.[341]

This study therefore identifies a novel mechanism through which cells can deplete arginine from microenvironment leading to impaired T cell responses.

The ability of AML blasts to create an immunosuppressive microenvironment is extended through the finding of increased Arginase II concentrations in the plasma of AML patients. Furthermore I showed that the arginase present in plasma is active, and therefore contributes to a whole body depletion of arginine. It has previously been reported that concentrations of Arginase I and increased arginase activity is found in cancer patients with increased numbers of MDSCs.[122] This study is the first to report increased plasma Arginase II levels in patients with a malignant disease. The combination of increased intracellular arginase activity and plasma arginase activity halts T cell proliferation, and may contribute to the lymphopenia found at diagnosis. In addition Arginase II levels and activity may serve as an important biomarker in patients with AML. Plasma levels of other enzymes are already routinely studied in clinical labs to indicate tissue damage and disease progression (AST, ALT). Patients with AML, who have high-risk disease or relapse, are often treated with allogenic bone marrow transplant. Measurement of Arginase II levels may serve as an indicator of whether a patient will undergo a successful immune reconstitution post transplant, or the potential of a relapse.

The study identified that AML blasts and accordingly the supernatants of AML cultures can polarise surrounding monocytes to an immunosuppressive M2 phenotype. The finding was confirmed in vivo. In this model increased numbers of YM-1 expressing monocytes were found in the bone marrows of NOG-SCID mice engrafted with human

AML blasts. Furthermore CD206+ monocytes were found in the bone marrow of AML patients at diagnosis. The polarization of monocytes to a M2 phenotype was confirmed to be arginase dependent by the addition of arginase inhibitors. The ability of monocytes to be polarised to an M2 phenotype by tumour cells builds on work developed in the Cerundolo lab (unpublished data).

It is well established in the literature that macrophages can play a critical role in tumour progression, and can be associated with a poor clinical outcome.[342] M2 macrophages are associated with the expression of YM-1 in mice[343] and CD206 in humans.[344] This mechanism may be particular important in the early development and maintenance of AML in which AML can co-opt surrounding monocytes to create an immunosuppressive niche. There is growing evidence that AML can influence the normal bone marrow cells to create a niche, even when the disease burden is low.[345] Further work is required to understand if AML stem cells may remain quiescent and immunologically undetected by switching local monocytes to an M2 phenotype.

Inhibitors of arginase and iNOS (NOHA and L-NMMA respectively) have been used to confirm the roles of these enzymes in mediating immunosuppressive activity in studies of MDSCs.[346,347] In this study I showed that a combination of the arginase and INOS inhibitors can relieve the suppressive activity of AML. The requirement for inhibitors of both enzymes in order to restore T cell proliferation is well established in the literature and points to a more complex interplay between arginase and iNOS in catabolising arginine to cause immunosuppression.[348,349]

The finding may have important clinical consequences. One of the difficulties in curing patients of AML is that residual AML stem cells remain in the bone marrow. Currently treatment is reliant on using multiple cycles of multi-drug chemotherapy and the use of allogenic bone marrow transplant. If the immunosuppression created by AML blasts can be counteracted, it is possible that a patient's own immune system or a graft-vs.-leukaemia effect (post allogenic transplant) may enable the detection and killing of any residual disease. In theory, therapy targeting this immunosuppressive activity of AML could be given as a maintenance course post AML consolidation chemotherapy or post bone marrow transplant. However, limitations remain. NOHA and L-NMMA have not entered clinically trials due to concerns that NOHA may inhibit the urea cycle in healthy cells, and that L-NMMA can be toxic to mice in high doses. Alternate strategies are being developed including the use of nitroaspirin[224] and phosphodiesterase-5 inhibitors.[184] The in vitro effect of the phosphodiesterase-5 inhibitor sildenafil (Viagra) on AML immunosuppression was investigated in this thesis. No restoration in T cell proliferation was seen (data not shown) indicating that therapies which may be effective in murine MDSC models may not be effective in this unique setting of AML.

Having established that Arginase II concentrations and activity is increased in culture supernatants and patient plasma, I investigated if other immunomodulatory factors were also altered in patients with AML. No increase in TGF-β, IL-10, and GM-CSF was found compared to healthy controls. The study confirms results found by other groups, in which these cytokines are not raised in the plasma of AML patients and their concentration has no prognostic significance in patients with AML.[350,351,329] However, I

found that levels of the acute phase protein Serum Amyloid A are raised in the plasma of patients with AML. SAA is an acute phase reactant, produced by the liver in the setting of acute inflammation.[352] To establish the source of SAA in our patients the plasma levels were compared with C-Reactive Protein, another hepatic derived acute phase protein. Although CRP levels were raised in patient plasma compared to healthy controls, they were approximately 1000 fold lower than for SAA– making the liver a less likely source of such high levels of SAA seen in our patients. Instead I found that AML cells themselves can produce SAA, and to a lesser extent CRP. The extra-hepatic production of SAA has been previously reported in other disease settings.[202] In addition SAA has been reported to be secreted from the AML cell line THP-1, following stimulation with dexamethasone or IL-1β, adding to the findings of SAA production by the AML cell lines tested in Chapter 3.[353] The finding has important clinical and biological consequences. Clinically SAA levels in the plasma may serve as a biomarker for AML. This could be particular important in the setting of minimal residual disease, or in detecting relapse, where current diagnostic techniques require a bone marrow aspirate to be performed. The potential of SAA as a cancer biomarker was first suggested over 30 years ago[354] and has been investigated in a number of human cancer patients since[355].

Biologically SAA may also play an important physiological role. SAA can induce the migration of myeloid cells into the blood[328] and induce the accumulation of myeloid cells in pre-metastatic lungs.[318] SAA acts through multiple cell surface receptors – FPLR-2,[356] TLR2,[328] and TLR4. These receptors are expressed by AML cells and in turn that SAA is able to induce chemotaxis of the U937 cell line. Furthermore, I

demonstrated that AML patient plasma, in which SAA is present in high concentrations, can cause a significant chemotaxis using a transwell assay. The raised levels of SAA in the plasma of patients may play a part in the migration of AML cells from the bone marrow and into the blood.

SAA forms part of a signalling pathway with S100 proteins and IL-1b and IL-6, promoting tumour progression. S100 are a family of calcium-binding proteins including S100A8, S100A9, and S100A12 that are released by cells of myeloid origin. The proteins form hetero- or homo-dimers to attract[357,320] and enhance the immunosuppressive activity of myeloid cells. S100 proteins are able to bind to a number of cell surface receptors: TLR4,[358] heparan sulfate,[359] and RAGE.[360] The S100 proteins can act in an autocrine feedback loop, alongside IL-1β to induce the secretion of SAA from myeloid cells.[328] This study first confirmed that AML blasts express TLR2, TLR4 and FPLR-2 receptors. Furthermore it showed that SAA can induce the production of IL-1β by AML cells. As IL-1β concentrations are not raised in the plasma of patients, it is most likely that IL-1β acts in an autocrine or paracrine fashion on other AML cells and surrounding cells. In addition SAA can induce the production of S100A9 from AML blasts. Interestingly S100A8 expression is associated with a worse prognosis in AML.[361] This study found that S100A9 concentrations are raised in the plasma of AML patients at diagnosis. The findings point to a novel network of pro-inflammatory mediators in AML, which may further support the immunosuppressive environment created by AML. The role of other S100 proteins was not studied in this thesis due to time and funding constraints however further study is required to elucidate these pathways more clearly.

To investigate the potential of cell-based therapies to target AML, the ability of invariant Natural Killer T cells (iNKT) to overcome the suppressive AML activity was investigated. iNKT cells can restore T cell proliferation in vitro. iNKT cells are activated by the agonist αGalCer when presented by target cells in the context of the invariant T-cell receptor CD1d. Some AML cell lines and AML blasts from patients are known to express CD1d.[246,362] These studies have also demonstrated that iNKT cells can be cytotoxic to AML blasts (in about 40% of cases), if activated by αGalCer, in a CD1d-restricted manner. The study confirmed the ability of iNKT cells to kill some patient-derived AML blasts when stimulated by αGalCer. Furthermore the results extend the previous findings by showing that in killing AML cells, the immunosuppressive microenvironment is abolished and T cell proliferation is restored. The result has important clinical consequences if iNKT cells can be activated in patients.

Physiologically, the role of iNKT cells in immunosurveillance against leukaemia has been suggested in a recent study of paediatric patients treated with haematopoietic stem cell transplant. In all patients who relapsed iNKT cells failed to reconstitute post transplant. iNKT cells may therefore play a role when AML blast numbers are at their lowest, for example in the setting of minimal residual disease or post-bone marrow transplant. In addition this may be the time when reducing the immunosuppressive microenvironment may be most effective in allowing T cells to proliferate. The pivotal role played of αGalCer in activating iNKT cells to produce IFN-γ and stimulate other immune cells to control tumour cells is well documented in the literature.[363]

Inparticular, iNKT cells can promote the cytotoxic T cell response against tumour-associated antigens, thus enhancing the anti-tumour response.[364]

One of the first indications that αGalCer had therapeutic potential came from a murine model of melanoma [365]. In the B16 melanoma model, mice injected with αGalCer had prolonged survival compared to controls. The results were supported in models of EL-4 lymphoma and colon adenocarcinoma in which tumour metastases were inhibited following injection with αGalCer.[366] In preclinical models αGalCer has been administered intravenously using doses of up to 220mg/kg (mice), 440mg/kg (rats), and 660mg/kg (monkeys) without significant side effects. However, higher doses of αGalCer can induce iNKT anergy and thus abolish any anti-tumour activity that could be of benefit to patients. [367,368]

Based on preclinical data the first Phase I clinical trial of αGalCer was carried out in patients with refractory solid tumors.[369] Patients were treated in 4 weekly cycles on days 1, 8 and 15 with intravenous αGalCer. The Maximum Tolerated Dose (MTD) was not reached and toxicity was acceptable (no greater than Grade 3 fever on the NCI-CTC). αGalCer pharmacokinetics were linear over the dose range.[367] Although clinical outcome data should be interpreted cautiously from Phase I trials, seven patients showed a stabilization of their disease.

Preclinical models show that iNKT cells require αGalCer to be presented in the context of the CD1d molecule on antigen presenting cells (APC), in order to be activated. As

described earlier CD1d is not expressed on all AML blasts thus, one alternative approach is to pulse APCs with αGalCer ex vivo and then inject the loaded APCs intravenously. In the context of haematological malignancies, a Phase I clinical trial was performed, in which five patients with myeloma were immunized three times intravenously with mature DCs.[370] Injection of αGalCer-loaded DCs led to an increase of more than 100-fold in the number of iNKT cells in some patients, which was detectable for up to 6 months after vaccination. In addition, the investigators detected increased serum levels of the cytokines that are associated with DC maturation, such as IL-12, and an increase in the frequency of cytomegalovirus-specific $CD8^+$ T cells. These data indicate that injection of DCs loaded with αGalCer can lead to the activation of APCs *in vivo*, resulting in enhanced antigen-specific T-cell responses, and suggests that iNKT-cell agonists may also facilitate the expansion of antigen-specific responses in humans, as was described previously in mice. The study also showed that following immunization with αGalCer-loaded DCs, three patients had clinically relevant decreases in the level of monoclonal immunoglobulins (M protein) in the serum or urine and one patient showed stabilization of the disease. To date no Phase II trials have been completed to assess the efficacy of αGalCer pulsed APCs in the treatment of human leukaemias. Future directions could include the development of alternative strategies to activate iNKT cells in AML patients. Activation of iNKT cells with another exogenous antigen, threitol ceramide, is currently being developed in the Cerundolo laboratory.

In summary I demonstrated that AML blasts create an immunosuppressive microenvironment through a number of mechanisms. The results improve our

understanding of the immunobiology of AML as well as highlight clinically relevant biomarkers. Finally the potential for small molecule inhibitors of arginase or the use of iNKT based therapy to modulate the immunosuppressive activity of AML, may offer new therapeutic approaches to cure patients with AML.

9. Concluding remarks

Despite significant financial investment, thousands of man-hours of research, and the care of medical staff, many patients with cancer fail to be cured. As the therapeutic benefit of current chemotherapy agents and multi-modality treatment regimens become maximised, new approaches will be required to improve the outcomes for patients.

This thesis study has investigated the effect of novel immunotherapies against Acute Lymphocytic Leukaemia, Acute Myeloid Leukaemia and Burkitt's lymphoma. In addition the work has shed new insights into the underlying biology through which haematological malignancies avoid destruction from a patient's immune system or by targeted therapies.

I hope these investigations can provide a small stepping-stone towards my goal of finding a cure for children and adults with haematological malignancies.

10. References

[1] Ries LAG, Smith MA, Gurney JG, Linet M, Tamra T, Young JL, Cancer incidence and survival among children and adolescents: United States SEER program 1975-1995 (1999)

[2] Hasle, H., Clemmensen, I. H., and Mikkelsen, M., Risks of leukaemia and solid tumours in individuals with Down's syndrome. *Lancet* **355** (9199), 165 (2000).

[3] Alter, B. P. and Olson, S. B., Wilms tumor, AML, and medulloblastoma in a child with cancer prone syndrome of total premature chromatid separation and Fanconi anemia. *Pediatr Blood Cancer* **54** (3), 488; author reply 489.

[4] German, J., Bloom, D., and Passarge, E., Bloom's syndrome. V. Surveillance for cancer in affected families. *Clin Genet* **12** (3), 162 (1977).

[5] Miles, D. K. et al., Patterns of hematopoietic lineage involvement in children with neurofibromatosis type 1 and malignant myeloid disorders. *Blood* **88** (11), 4314 (1996).

[6] Infante-Rivard, C. and Guiguet, M., Family history of hematopoietic and other cancers in children with acute lymphoblastic leukemia. *Cancer Detect Prev* **28** (2), 83 (2004).

[7] Greaves, M. F., Maia, A. T., Wiemels, J. L., and Ford, A. M., Leukemia in twins: lessons in natural history. *Blood* **102** (7), 2321 (2003).

[8] Wiemels, J. L. et al., In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. *Blood* **99** (10), 3801 (2002).

[9] Greaves, M., Pre-natal origins of childhood leukemia. *Rev Clin Exp Hematol* **7** (3), 233 (2003).

[10] Ozasa, K. et al., Risk of cancer and non-cancer diseases in the atomic bomb survivors. *Radiat Prot Dosimetry* **146** (1-3), 272 (2011).

[11] Delongchamp, R. R., Mabuchi, K., Yoshimoto, Y., and Preston, D. L., Cancer mortality among atomic bomb survivors exposed in utero or as young children, October 1950-May 1992. *Radiat Res* **147** (3), 385 (1997).

[12] Brill, A. B., Tomonaga, M., and Heyssel, R. M., Leukemia in man following exposure to ionizing radiation. A summary of the findings in Hiroshima and Nagasaki, and a comparison with other human experience. *Ann Intern Med* **56**, 590 (1962).

[13] Khalade, A., Jaakkola, M. S., Pukkala, E., and Jaakkola, J. J., Exposure to benzene at work and the risk of leukemia: a systematic review and meta-analysis. *Environ Health* **9**, 31 (2010).

[14] Hijiya, N., Ness, K. K., Ribeiro, R. C., and Hudson, M. M., Acute leukemia as a secondary malignancy in children and adolescents: current findings and issues. *Cancer* **115** (1), 23 (2009).

[15] Hjalgrim, H. and Engels, E. A., Infectious aetiology of Hodgkin and non-Hodgkin lymphomas: a review of the epidemiological evidence. *J Intern Med* **264** (6), 537 (2008).

[16] Prevot, S. et al., Analysis of African Burkitt's and high-grade B cell non-Burkitt's lymphoma for Epstein-Barr virus genomes using in situ hybridization. *Br J Haematol* **80** (1), 27 (1992).

[17] Gallo, R. C. and Wong-Staal, F., Current thoughts on the viral etiology of certain human cancers: The Richard and Hinda Rosenthal Foundation Award lecture. *Cancer Res* **44** (7), 2743 (1984).

[18] Gatti, R. A. and Good, R. A., Occurrence of malignancy in immunodeficiency diseases. A literature review. *Cancer* **28** (1), 89 (1971).

[19] Connell, W. R. et al., Long-term neoplasia risk after azathioprine treatment in inflammatory bowel disease. *Lancet* **343** (8908), 1249 (1994).

[20] Melosky, B. et al., Lymphoproliferative disorders after renal transplantation in patients receiving triple or quadruple immunosuppression. *J Am Soc Nephrol* **2** (12 Suppl), S290 (1992).

[21] Feltbower, R. G. et al., Epidemiology of leukaemia and lymphoma in children and young adults from the north of England, 1990-2002. *Eur J Cancer* **45** (3), 420 (2009).

[22] Bennett, J. M. et al., Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol* **33** (4), 451 (1976).

[23] Vardiman, J. W. et al., The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* **114** (5), 937 (2009).

[24] Suzman, M. M., Goldberg, B., and Hirsch, H., Cortisone in acute lymphoblastic leukemia. *Lancet* **1** (6658), 760 (1951).

[25] Frei, E., 3rd et al., The effectiveness of combinations of antileukemic agents in inducing and maintaining remission in children with acute leukemia. *Blood* **26** (5), 642 (1965).

[26] Hardisty, R. M., McElwain, T. J., and Darby, C. W., Vincristine and prednisone for the induction of remissions in acute childhood leukaemia. *Br Med J* **2** (5658), 662 (1969).

[27] Ortega, J. A. et al., L-Asparaginase, vincristine, and prednisone for induction of first remission in acute lymphocytic leukemia. *Cancer Res* **37** (2), 535 (1977).

[28] Pinkel, D., Five-year follow-up of "total therapy" of childhood lymphocytic leukemia. *JAMA* **216** (4), 648 (1971).

[29] Gaynon, P. S. et al., Modified BFM therapy for children with previously untreated acute lymphoblastic leukemia and unfavorable prognostic features. Report of Children's Cancer Study Group Study CCG-193P. *Am J Pediatr Hematol Oncol* **10** (1), 42 (1988).

[30] Evans, A. E., Gilbert, E. S., and Zandstra, R., The increasing incidence of central nervous system leukemia in children. (Children's Cancer Study Group A). *Cancer* **26** (2), 404 (1970).

[31] Aur, R. J. et al., Central nervous system therapy and combination chemotherapy of childhood lymphocytic leukemia. *Blood* **37** (3), 272 (1971).

[32] Aur, R. J., Simone, J. V., Hustu, H. O., and Verzosa, M. S., A comparative study of central nervous system irradiation and intensive chemotherapy early in remission of childhood acute lymphocytic leukemia. *Cancer* **29** (2), 381 (1972).

[33] Hustu, H. O. et al., Prevention of central nervous system leukemia by irradiation. *Cancer* **32** (3), 585 (1973).

[34] Aur, R. J. et al., Comparison of two methods of preventing central nervous system leukemia. *Blood* **42** (3), 349 (1973).

[35] Blatt, J. et al., Reduced pulsatile growth hormone secretion in children after therapy for acute lymphoblastic leukemia. *J Pediatr* **104** (2), 182 (1984).

[36] Meadows, A. T. et al., Declines in IQ scores and cognitive dysfunctions in children with acute lymphocytic leukaemia treated with cranial irradiation. *Lancet* **2** (8254), 1015 (1981).

[37] Littman, P. et al., Central nervous system (CNS) prophylaxis in children with low risk acute lymphoblastic leukemia (ALL). *Int J Radiat Oncol Biol Phys* **13** (10), 1443 (1987).

[38] Sullivan, M. P. et al., Equivalence of intrathecal chemotherapy and radiotherapy as central nervous system prophylaxis in children with acute lymphatic leukemia: a pediatric oncology group study. *Blood* **60** (4), 948 (1982).

[39] Tubergen, D. G. et al., Prevention of CNS disease in intermediate-risk acute lymphoblastic leukemia: comparison of cranial radiation and intrathecal methotrexate

and the importance of systemic therapy: a Childrens Cancer Group report. *J Clin Oncol* **11** (3), 520 (1993).

40 Pui, C. H. et al., Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N Engl J Med* **360** (26), 2730 (2009).

41 MacLennan, I. C., Kay, H. E., Festenstein, M., and Smith, P. G., Analysis of treatment in childhood leukaemia. II. Timing and the toxicity of combined 6-mercaptopurine and methotrexate maintenance therapy. *Br J Haematol* **33** (2), 179 (1976).

42 Chessells, J. M., Durrant, J., Hardy, R. M., and Richards, S., Medical Research Council leukaemia trial--UKALL V: an attempt to reduce the immunosuppressive effects of therapy in childhood acute lymphoblastic leukemia. Report to the Council by the Working Party on Leukaemia in Childhood. *J Clin Oncol* **4** (12), 1758 (1986).

43 Henze, G. et al., [Acute lymphoblastic leukemia therapy study BFM 79/81 in children and adolescents: intensified reinduction therapy for patients with different risk for relapse]. *Klin Padiatr* **194** (4), 195 (1982).

44 Ritter, J. et al., Childhood leukemia: cooperative Berlin-Frankfurt-Munster trials in the Federal Republic of Germany. *J Cancer Res Clin Oncol* **116** (1), 100 (1990).

45 Chessells, J. M., Bailey, C., and Richards, S. M., Intensification of treatment and survival in all children with lymphoblastic leukaemia: results of UK Medical Research Council trial UKALL X. Medical Research Council Working Party on Childhood Leukaemia. *Lancet* **345** (8943), 143 (1995).

46 Wheeler, K. A. et al., Bone marrow transplantation versus chemotherapy in the treatment of very high-risk childhood acute lymphoblastic leukemia in first remission: results from Medical Research Council UKALL X and XI. *Blood* **96** (7), 2412 (2000).

47 Harris, M. B. et al., Consolidation therapy with antimetabolite-based therapy in standard-risk acute lymphocytic leukemia of childhood: a Pediatric Oncology Group Study. *J Clin Oncol* **16** (8), 2840 (1998).

48 Kaspers, G. J. et al., Comparison of the antileukemic activity in vitro of dexamethasone and prednisolone in childhood acute lymphoblastic leukemia. *Med Pediatr Oncol* **27** (2), 114 (1996).

49 Bostrom, B. C. et al., Dexamethasone versus prednisone and daily oral versus weekly intravenous mercaptopurine for patients with standard-risk acute lymphoblastic leukemia: a report from the Children's Cancer Group. *Blood* **101** (10), 3809 (2003).

50 Silverman, L. B. et al., Improved outcome for children with acute lymphoblastic leukemia: results of Dana-Farber Consortium Protocol 91-01. *Blood* **97** (5), 1211 (2001).

51 Dinndorf, P. A. et al., FDA drug approval summary: pegaspargase (oncaspar) for the first-line treatment of children with acute lymphoblastic leukemia (ALL). *Oncologist* **12** (8), 991 (2007).

52 Pui, C. H. et al., Acute myeloid leukemia in children treated with epipodophyllotoxins for acute lymphoblastic leukemia. *N Engl J Med* **325** (24), 1682 (1991).

53 Sather, H. N., Age at diagnosis in childhood acute lymphoblastic leukemia. *Med Pediatr Oncol* **14** (3), 166 (1986).

54 Pui, C. H. et al., Sex differences in prognosis for children with acute lymphoblastic leukemia. *J Clin Oncol* **17** (3), 818 (1999).

55 Silverman, L. B. et al., Long-term results of Dana-Farber Cancer Institute ALL Consortium protocols for children with newly diagnosed acute lymphoblastic leukemia (1985-2000). *Leukemia* **24** (2), 320 (2010).

56 Schultz, K. R. et al., Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. *J Clin Oncol* **27** (31), 5175 (2009).

[57] Hann, I. et al., Benefit of intensified treatment for all children with acute lymphoblastic leukaemia: results from MRC UKALL XI and MRC ALL97 randomised trials. UK Medical Research Council's Working Party on Childhood Leukaemia. *Leukemia* **14** (3), 356 (2000).

[58] Campana, D., Minimal residual disease in acute lymphoblastic leukemia. *Semin Hematol* **46** (1), 100 (2009).

[59] Campana, D., Role of minimal residual disease evaluation in leukemia therapy. *Curr Hematol Malig Rep* **3** (3), 155 (2008).

[60] Pui, C. H. et al., Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. *Lancet* **359** (9321), 1909 (2002).

[61] Pulte, D., Gondos, A., and Brenner, H., Improvement in survival in younger patients with acute lymphoblastic leukemia from the 1980s to the early 21st century. *Blood* **113** (7), 1408 (2009).

[62] Nachman, J. B. et al., Young adults with acute lymphoblastic leukemia have an excellent outcome with chemotherapy alone and benefit from intensive postinduction treatment: a report from the children's oncology group. *J Clin Oncol* **27** (31), 5189 (2009).

[63] Ribera, J. M. et al., Comparison of the results of the treatment of adolescents and young adults with standard-risk acute lymphoblastic leukemia with the Programa Espanol de Tratamiento en Hematologia pediatric-based protocol ALL-96. *J Clin Oncol* **26** (11), 1843 (2008).

[64] Silverman, L. B. et al., Induction failure in acute lymphoblastic leukemia of childhood. *Cancer* **85** (6), 1395 (1999).

[65] Pui, C. H., Robison, L. L., and Look, A. T., Acute lymphoblastic leukaemia. *Lancet* **371** (9617), 1030 (2008).

[66] McKinney, P. A., Alexander, F. E., Cartwright, R. A., and Ricketts, T. J., The leukaemia research fund data collection survey: the incidence and geographical distribution of acute myeloid leukemia. *Leukemia* **3** (12), 875 (1989).

[67] Choi, S. I. and Simone, J. V., Acute nonlymphocytic leukemia in 171 children. *Med Pediatr Oncol* **2** (2), 119 (1976).

[68] Baehner, R. L. et al., Improved remission induction rate with D-ZAPO but unimproved remission duration with addition of immunotherapy to chemotherapy in previously untreated children with ANLL. *Med Pediatr Oncol* **7** (2), 127 (1979).

[69] Chard, R. L., Jr. et al., Increased survival in childhood acute nonlymphocytic leukemia after treatment with prednisone, cytosine arabinoside, 6-thioguanine, cyclophosphamide, and oncovin (PATCO) combination chemotherapy. *Med Pediatr Oncol* **4** (3), 263 (1978).

[70] Nesbit, M. E., Jr. et al., Chemotherapy for induction of remission of childhood acute myeloid leukemia followed by marrow transplantation or multiagent chemotherapy: a report from the Childrens Cancer Group. *J Clin Oncol* **12** (1), 127 (1994).

[71] Wells, R. J. et al., Treatment of newly diagnosed children and adolescents with acute myeloid leukemia: a Childrens Cancer Group study. *J Clin Oncol* **12** (11), 2367 (1994).

[72] Ravindranath, Y. et al., High-dose cytarabine for intensification of early therapy of childhood acute myeloid leukemia: a Pediatric Oncology Group study. *J Clin Oncol* **9** (4), 572 (1991).

[73] Capizzi, R. L. et al., Treatment of poor risk acute leukemia with sequential high-dose ARA-C and asparaginase. *Blood* **63** (3), 694 (1984).

[74] Bishop, J. F. et al., Etoposide in acute nonlymphocytic leukemia. Australian Leukemia Study Group. *Blood* **75** (1), 27 (1990).

[75] Lange, B. J. et al., Outcomes in CCG-2961, a children's oncology group phase 3 trial for untreated pediatric acute myeloid leukemia: a report from the children's oncology group. *Blood* **111** (3), 1044 (2008).

[76] Woods, W. G. et al., A comparison of allogeneic bone marrow transplantation, autologous bone marrow transplantation, and aggressive chemotherapy in children with acute myeloid leukemia in remission. *Blood* **97** (1), 56 (2001).

[77] Creutzig, U. et al., Improved treatment results in high-risk pediatric acute myeloid leukemia patients after intensification with high-dose cytarabine and mitoxantrone: results of Study Acute Myeloid Leukemia-Berlin-Frankfurt-Munster 93. *J Clin Oncol* **19** (10), 2705 (2001).

[78] Creutzig, U. et al., Less toxicity by optimizing chemotherapy, but not by addition of granulocyte colony-stimulating factor in children and adolescents with acute myeloid leukemia: results of AML-BFM 98. *J Clin Oncol* **24** (27), 4499 (2006).

[79] Gibson, B. E. et al., Treatment strategy and long-term results in paediatric patients treated in consecutive UK AML trials. *Leukemia* **19** (12), 2130 (2005).

[80] Tukenova, M. et al., Role of cancer treatment in long-term overall and cardiovascular mortality after childhood cancer. *J Clin Oncol* **28** (8), 1308 (2010).

[81] Rees, J. K., Gray, R. G., Swirsky, D., and Hayhoe, F. G., Principal results of the Medical Research Council's 8th acute myeloid leukaemia trial. *Lancet* **2** (8518), 1236 (1986).

[82] Woods, W. G. et al., The role of timing of high-dose cytosine arabinoside intensification and of maintenance therapy in the treatment of children with acute nonlymphocytic leukemia. *Cancer* **66** (6), 1106 (1990).

[83] Ritter, J., Creutzig, U., and Schellong, G., Treatment results of three consecutive German childhood AML trials: BFM-78, -83, and -87. AML-BFM-Group. *Leukemia* **6 Suppl 2**, 59 (1992).

[84] Michel, G. et al., Allogeneic bone marrow transplantation vs aggressive post-remission chemotherapy for children with acute myeloid leukemia in first complete remission. A prospective study from the French Society of Pediatric Hematology and Immunology (SHIP). *Bone Marrow Transplant* **17** (2), 191 (1996).

[85] Burnett, A. K. et al., The value of allogeneic bone marrow transplant in patients with acute myeloid leukaemia at differing risk of relapse: results of the UK MRC AML 10 trial. *Br J Haematol* **118** (2), 385 (2002).

[86] Ferrara, J. L., Levine, J. E., Reddy, P., and Holler, E., Graft-versus-host disease. *Lancet* **373** (9674), 1550 (2009).

[87] Byrd, J. C. et al., Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood* **100** (13), 4325 (2002).

[88] Delaunay, J. et al., Prognosis of inv(16)/t(16;16) acute myeloid leukemia (AML): a survey of 110 cases from the French AML Intergroup. *Blood* **102** (2), 462 (2003).

[89] Alcalay, M. et al., Translocation breakpoint of acute promyelocytic leukemia lies within the retinoic acid receptor alpha locus. *Proc Natl Acad Sci U S A* **88** (5), 1977 (1991).

[90] Grimwade, D. and Lo Coco, F., Acute promyelocytic leukemia: a model for the role of molecular diagnosis and residual disease monitoring in directing treatment approach in acute myeloid leukemia. *Leukemia* **16** (10), 1959 (2002).

[91] Lazo, G. et al., Use of arsenic trioxide (As2O3) in the treatment of patients with acute promyelocytic leukemia: the M. D. Anderson experience. *Cancer* **97** (9), 2218 (2003).

[92] Chen, G. Q. et al., In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. *Blood* **88** (3), 1052 (1996).

[93] Grimwade, D. et al., The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood* **92** (7), 2322 (1998).

[94] Wells, R. J. et al., Mitoxantrone and cytarabine induction, high-dose cytarabine, and etoposide intensification for pediatric patients with relapsed or refractory acute myeloid leukemia: Children's Cancer Group Study 2951. *J Clin Oncol* **21** (15), 2940 (2003).

[95] Stone, R. M. et al., Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood* **105** (1), 54 (2005).

[96] Sanz, M. A., Tallman, M. S., and Lo-Coco, F., Tricks of the trade for the appropriate management of newly diagnosed acute promyelocytic leukemia. *Blood* **105** (8), 3019 (2005).

[97] Castagnola, E. et al., Incidence of bacteremias and invasive mycoses in children with acute non-lymphoblastic leukemia: results from a multi-center Italian study. *Pediatr Blood Cancer* **55** (6), 1103 (2010).

[98] World Health Organisation, Swedlow S, *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues - IARC WHO Classification of Tumours v.2*. (World Health Organisation, 2008).

[99] Fujita, S. et al., Early stage of Epstein-Barr virus lytic infection leading to the "starry sky" pattern formation in endemic Burkitt lymphoma. *Arch Pathol Lab Med* **128** (5), 549 (2004).

[100] Yustein, J. T. and Dang, C. V., Biology and treatment of Burkitt's lymphoma. *Curr Opin Hematol* **14** (4), 375 (2007).

[101] Lindstrom, M. S. and Wiman, K. G., Role of genetic and epigenetic changes in Burkitt lymphoma. *Semin Cancer Biol* **12** (5), 381 (2002).

[102] Dave, S. S. et al., Molecular diagnosis of Burkitt's lymphoma. *N Engl J Med* **354** (23), 2431 (2006).

[103] Patte, C. et al., High survival rate in advanced-stage B-cell lymphomas and leukemias without CNS involvement with a short intensive polychemotherapy: results from the French Pediatric Oncology Society of a randomized trial of 216 children. *J Clin Oncol* **9** (1), 123 (1991).

[104] Patte, C. et al., The Societe Francaise d'Oncologie Pediatrique LMB89 protocol: highly effective multiagent chemotherapy tailored to the tumor burden and initial response in 561 unselected children with B-cell lymphomas and L3 leukemia. *Blood* **97** (11), 3370 (2001).

[105] Nelson, M. et al., An increased frequency of 13q deletions detected by fluorescence in situ hybridization and its impact on survival in children and adolescents with Burkitt lymphoma: results from the Children's Oncology Group study CCG-5961. *Br J Haematol* **148** (4), 600 (2010).

[106] Lovisa, F. et al., IGH and IGK gene rearrangements as PCR targets for pediatric Burkitt's lymphoma and mature B-ALL MRD analysis. *Lab Invest* **89** (10), 1182 (2009).

[107] Mussolin, L. et al., Minimal disseminated disease in high-risk Burkitt's lymphoma identifies patients with different prognosis. *J Clin Oncol* **29** (13), 1779.

[108] Gerrard, M. et al., Excellent survival following two courses of COPAD chemotherapy in children and adolescents with resected localized B-cell non-Hodgkin's lymphoma: results of the FAB/LMB 96 international study. *Br J Haematol* **141** (6), 840 (2008).

[109] Aubier, F. et al., Male gonadal function after chemotherapy for solid tumors in childhood. *J Clin Oncol* **7** (3), 304 (1989).

[110] Patte, C. et al., Results of the randomized international FAB/LMB96 trial for intermediate risk B-cell non-Hodgkin lymphoma in children and adolescents: it is

possible to reduce treatment for the early responding patients. *Blood* **109** (7), 2773 (2007).

111 Reiter, A. et al., Improved treatment results in childhood B-cell neoplasms with tailored intensification of therapy: A report of the Berlin-Frankfurt-Munster Group Trial NHL-BFM 90. *Blood* **94** (10), 3294 (1999).

112 Cairo, M. S. et al., Results of a randomized international study of high-risk central nervous system B non-Hodgkin lymphoma and B acute lymphoblastic leukemia in children and adolescents. *Blood* **109** (7), 2736 (2007).

113 Mohamedbhai, S. G. et al., Rituximab in combination with CODOX-M/IVAC: a retrospective analysis of 23 cases of non-HIV related B-cell non-Hodgkin lymphoma with proliferation index >95%. *Br J Haematol* **152** (2), 175.

114 Corazzelli, G. et al., RD-CODOX-M/IVAC with rituximab and intrathecal liposomal cytarabine in adult Burkitt lymphoma and 'unclassifiable' highly aggressive B-cell lymphoma. *Br J Haematol* **156** (2), 234 (2011).

115 Galicier, L. et al., Intensive chemotherapy regimen (LMB86) for St Jude stage IV AIDS-related Burkitt lymphoma/leukemia: a prospective study. *Blood* **110** (8), 2846 (2007).

116 Holleman, A. et al., Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *N Engl J Med* **351** (6), 533 (2004).

117 Heerema, N. A. et al., Hypodiploidy with less than 45 chromosomes confers adverse risk in childhood acute lymphoblastic leukemia: a report from the children's cancer group. *Blood* **94** (12), 4036 (1999).

118 Smith, F. O. et al., Long-term results of children with acute myeloid leukemia: a report of three consecutive Phase III trials by the Children's Cancer Group: CCG 251, CCG 213 and CCG 2891. *Leukemia* **19** (12), 2054 (2005).

119 Creutzig, U. et al., Treatment strategies and long-term results in paediatric patients treated in four consecutive AML-BFM trials. *Leukemia* **19** (12), 2030 (2005).

120 Oeffinger, K. C. et al., Chronic health conditions in adult survivors of childhood cancer. *N Engl J Med* **355** (15), 1572 (2006).

121 Hurwitz, C. A. et al., Substituting dexamethasone for prednisone complicates remission induction in children with acute lymphoblastic leukemia. *Cancer* **88** (8), 1964 (2000).

122 Waber, D. P. et al., Cognitive sequelae in children treated for acute lymphoblastic leukemia with dexamethasone or prednisone. *J Pediatr Hematol Oncol* **22** (3), 206 (2000).

123 Bernard, A. and Boumsell, L., The clusters of differentiation (CD) defined by the First International Workshop on Human Leucocyte Differentiation Antigens. *Hum Immunol* **11** (1), 1 (1984).

124 Kohler, G. and Milstein, C., Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256** (5517), 495 (1975).

125 Merchant, M. S. et al., Interferon gamma enhances the effectiveness of tumor necrosis factor-related apoptosis-inducing ligand receptor agonists in a xenograft model of Ewing's sarcoma. *Cancer Res* **64** (22), 8349 (2004).

126 Kolb, E. A. et al., R1507, a fully human monoclonal antibody targeting IGF-1R, is effective alone and in combination with rapamycin in inhibiting growth of osteosarcoma xenografts. *Pediatr Blood Cancer* **55** (1), 67 (2010).

127 Anderson, D. R. et al., Targeted anti-cancer therapy using rituximab, a chimaeric anti-CD20 antibody (IDEC-C2B8) in the treatment of non-Hodgkin's B-cell lymphoma. *Biochem Soc Trans* **25** (2), 705 (1997).

128 Lev, A., Novak, H., Segal, D., and Reiter, Y., Recruitment of CTL activity by tumor-specific antibody-mediated targeting of single-chain class I MHC-peptide complexes. *J Immunol* **169** (6), 2988 (2002).

[129] Leach, D. R., Krummel, M. F., and Allison, J. P., Enhancement of antitumor immunity by CTLA-4 blockade. *Science* **271** (5256), 1734 (1996).

[130] Hale, G. et al., Removal of T cells from bone marrow for transplantation: a monoclonal antilymphocyte antibody that fixes human complement. *Blood* **62** (4), 873 (1983).

[131] Pegram, M. D. et al., Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J Clin Oncol* **16** (8), 2659 (1998).

[132] Arceci, R. J. et al., Safety and efficacy of gemtuzumab ozogamicin in pediatric patients with advanced CD33+ acute myeloid leukemia. *Blood* **106** (4), 1183 (2005).

[133] Bethge, W. A. et al., Radioimmunotherapy with yttrium-90-ibritumomab tiuxetan as part of a reduced- intensity conditioning regimen for allogeneic hematopoietic cell transplantation in patients with advanced non-Hodgkin lymphoma: results of a phase 2 study. *Blood* **116** (10), 1795 (2010).

[134] Sgroi, D., Varki, A., Braesch-Andersen, S., and Stamenkovic, I., CD22, a B cell-specific immunoglobulin superfamily member, is a sialic acid-binding lectin. *J Biol Chem* **268** (10), 7011 (1993).

[135] Stamenkovic, I. and Seed, B., The B-cell antigen CD22 mediates monocyte and erythrocyte adhesion. *Nature* **345** (6270), 74 (1990).

[136] Smith, K. G., Hewitson, T. D., Nossal, G. J., and Tarlinton, D. M., The phenotype and fate of the antibody-forming cells of the splenic foci. *Eur J Immunol* **26** (2), 444 (1996).

[137] Powell, L. D. et al., Natural ligands of the B cell adhesion molecule CD22 beta carry N-linked oligosaccharides with alpha-2,6-linked sialic acids that are required for recognition. *J Biol Chem* **268** (10), 7019 (1993).

[138] Razi, N. and Varki, A., Masking and unmasking of the sialic acid-binding lectin activity of CD22 (Siglec-2) on B lymphocytes. *Proc Natl Acad Sci U S A* **95** (13), 7469 (1998).

[139] Peaker, C. J. and Neuberger, M. S., Association of CD22 with the B cell antigen receptor. *Eur J Immunol* **23** (6), 1358 (1993).

[140] Tuscano, J., Engel, P., Tedder, T. F., and Kehrl, J. H., Engagement of the adhesion receptor CD22 triggers a potent stimulatory signal for B cells and blocking CD22/CD22L interactions impairs T-cell proliferation. *Blood* **87** (11), 4723 (1996).

[141] O'Keefe, T. L., Williams, G. T., Davies, S. L., and Neuberger, M. S., Hyperresponsive B cells in CD22-deficient mice. *Science* **274** (5288), 798 (1996).

[142] Smith, K. G. et al., Inhibition of the B cell by CD22: a requirement for Lyn. *J Exp Med* **187** (5), 807 (1998).

[143] Sato, S., Jansen, P. J., and Tedder, T. F., CD19 and CD22 expression reciprocally regulates tyrosine phosphorylation of Vav protein during B lymphocyte signaling. *Proc Natl Acad Sci U S A* **94** (24), 13158 (1997).

[144] de Vries, J. F. et al., The novel calicheamicin-conjugated CD22 antibody inotuzumab ozogamicin (CMC-544) effectively kills primary pediatric acute lymphoblastic leukemia cells. *Leukemia* **26** (2), 255 (2012).

[145] Frankel, A. E. et al., Anti-CD3 recombinant diphtheria immunotoxin therapy of cutaneous T cell lymphoma. *Curr Drug Targets* **10** (2), 104 (2009).

[146] Pastan, I., Hassan, R., Fitzgerald, D. J., and Kreitman, R. J., Immunotoxin therapy of cancer. *Nat Rev Cancer* **6** (7), 559 (2006).

[147] Krolick, K. A. et al., Selective killing of normal or neoplastic B cells by antibodies coupled to the A chain of ricin. *Proc Natl Acad Sci U S A* **77** (9), 5419 (1980).

[148] Myers, D. E. et al., Production of a pokeweed antiviral protein (PAP)-containing immunotoxin, B43-PAP, directed against the CD19 human B lineage lymphoid differentiation antigen in highly purified form for human clinical trials. *J Immunol Methods* **136** (2), 221 (1991).

[149] Kreitman, R. J. et al., Single-chain immunotoxin fusions between anti-Tac and Pseudomonas exotoxin: relative importance of the two toxin disulfide bonds. *Bioconjug Chem* **4** (2), 112 (1993).

[150] Kreitman, R. J., Hassan, R., Fitzgerald, D. J., and Pastan, I., Phase I trial of continuous infusion anti-mesothelin recombinant immunotoxin SS1P. *Clin Cancer Res* **15** (16), 5274 (2009).

[151] Kreitman, R. J. et al., Phase II trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with hairy cell leukemia. *J Clin Oncol* **27** (18), 2983 (2009).

[152] Avarbock, A. B. et al., Lethal vascular leak syndrome after denileukin diftitox administration to a patient with cutaneous gamma/delta T-cell lymphoma and occult cirrhosis. *Am J Hematol* **83** (7), 593 (2008).

[153] Olsen, E. et al., Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. *J Clin Oncol* **19** (2), 376 (2001).

[154] Chaudhary, V. K. et al., A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin. *Nature* **339** (6223), 394 (1989).

[155] Weldon, J. E. et al., A protease-resistant immunotoxin against CD22 with greatly increased activity against CLL and diminished animal toxicity. *Blood* **113** (16), 3792 (2009).

[156] Mansfield, E., Amlot, P., Pastan, I., and FitzGerald, D. J., Recombinant RFB4 immunotoxins exhibit potent cytotoxic activity for CD22-bearing cells and tumors. *Blood* **90** (5), 2020 (1997).

[157] Kreitman, R. J., Wang, Q. C., FitzGerald, D. J., and Pastan, I., Complete regression of human B-cell lymphoma xenografts in mice treated with recombinant anti-CD22 immunotoxin RFB4(dsFv)-PE38 at doses tolerated by cynomolgus monkeys. *Int J Cancer* **81** (1), 148 (1999).

[158] Kreitman, R. J. et al., Phase I trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with B-cell malignancies. *J Clin Oncol* **23** (27), 6719 (2005).

[159] Wayne, A. S. et al., Anti-CD22 immunotoxin RFB4(dsFv)-PE38 (BL22) for CD22-positive hematologic malignancies of childhood: preclinical studies and phase I clinical trial. *Clin Cancer Res* **16** (6), 1894 (2010).

[160] Salvatore, G. et al., Improved cytotoxic activity toward cell lines and fresh leukemia cells of a mutant anti-CD22 immunotoxin obtained by antibody phage display. *Clin Cancer Res* **8** (4), 995 (2002).

[161] Alderson, R. F. et al., CAT-8015: a second-generation pseudomonas exotoxin A-based immunotherapy targeting CD22-expressing hematologic malignancies. *Clin Cancer Res* **15** (3), 832 (2009).

[162] Raetz, E. A. et al., Chemoimmunotherapy reinduction with epratuzumab in children with acute lymphoblastic leukemia in marrow relapse: a Children's Oncology Group Pilot Study. *J Clin Oncol* **26** (22), 3756 (2008).

[163] Herrera, L. et al., A phase 1 study of Combotox in pediatric patients with refractory B-lineage acute lymphoblastic leukemia. *J Pediatr Hematol Oncol* **31** (12), 936 (2009).

[164] Advani, A. et al., Safety, pharmacokinetics, and preliminary clinical activity of inotuzumab ozogamicin, a novel immunoconjugate for the treatment of B-cell non-Hodgkin's lymphoma: results of a phase I study. *J Clin Oncol* **28** (12), 2085 (2010).

[165] Nowell, P. C. and Hungerford, D. A., Chromosome studies in human leukemia. II. Chronic granulocytic leukemia. *J Natl Cancer Inst* **27**, 1013 (1961).

[166] Ben-Neriah, Y. et al., The chronic myelogenous leukemia-specific P210 protein is the product of the bcr/abl hybrid gene. *Science* **233** (4760), 212 (1986).

[167] Schindler, T. et al., Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. *Science* **289** (5486), 1938 (2000).

[168] Gambacorti-Passerini, C. et al., Inhibition of the ABL kinase activity blocks the proliferation of BCR/ABL+ leukemic cells and induces apoptosis. *Blood Cells Mol Dis* **23** (3), 380 (1997).

[169] Small, D. et al., STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD34+ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. *Proc Natl Acad Sci U S A* **91** (2), 459 (1994).

[170] Carow, C. E. et al., Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. *Blood* **87** (3), 1089 (1996).

[171] Tse, K. F., Mukherjee, G., and Small, D., Constitutive activation of FLT3 stimulates multiple intracellular signal transducers and results in transformation. *Leukemia* **14** (10), 1766 (2000).

[172] Kottaridis, P. D. et al., The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood* **98** (6), 1752 (2001).

[173] Levis, M. et al., A FLT3 tyrosine kinase inhibitor is selectively cytotoxic to acute myeloid leukemia blasts harboring FLT3 internal tandem duplication mutations. *Blood* **98** (3), 885 (2001).

[174] Levis, M. et al., A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo. *Blood* **99** (11), 3885 (2002).

[175] Lowenberg, B. et al., Phase 1/2 study to assess the safety, efficacy, and pharmacokinetics of barasertib (AZD1152) in patients with advanced acute myeloid leukemia. *Blood* **118** (23), 6030 (2011).

[176] Eghtedar, A. et al., Phase 2 study of the JAK kinase inhibitor ruxolitinib in patients with refractory leukemias, including postmyeloproliferative neoplasm acute myeloid leukemia. *Blood* **119** (20), 4614 (2012).

[177] Chu, E. et al., Mechanism of thymidylate synthase inhibition by methotrexate in human neoplastic cell lines and normal human myeloid progenitor cells. *J Biol Chem* **265** (15), 8470 (1990).

[178] Matloub, Y. et al., Escalating intravenous methotrexate improves event-free survival in children with standard-risk acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Blood* **118** (2), 243 (2011).

[179] Li, H., Fan, X., and Houghton, J., Tumor microenvironment: the role of the tumor stroma in cancer. *J Cell Biochem* **101** (4), 805 (2007).

[180] Peranzoni, E. et al., Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol* **22** (2), 238 (2010).

[181] Greten, T. F., Manns, M. P., and Korangy, F., Myeloid derived suppressor cells in human diseases. *Int Immunopharmacol* **11** (7), 802 (2011).

[182] Pak, A. S. et al., Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34(+) cells which suppress immune functions within cancers that secrete granulocyte-macrophage colony-stimulating factor. *Clin Cancer Res* **1** (1), 95 (1995).

[183] Almand, B. et al., Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* **166** (1), 678 (2001).

[184] Serafini, P. et al., Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J Exp Med* **203** (12), 2691 (2006).

[185] Corzo, C. A. et al., HIF-1alpha regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J Exp Med* **207** (11), 2439 (2010).

[186] Zea, A. H. et al., Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res* **65** (8), 3044 (2005).

[187] Rodriguez, P. C. et al., Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res* **69** (4), 1553 (2009).

[188] Mirza, N. et al., All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res* **66** (18), 9299 (2006).

[189] Kusmartsev, S., Nefedova, Y., Yoder, D., and Gabrilovich, D. I., Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J Immunol* **172** (2), 989 (2004).

[190] Diaz-Montero, C. M. et al., Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol Immunother* **58** (1), 49 (2009).

[191] Solito, S. et al., A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood* **118** (8), 2254 (2011).

[192] Xiang, X. et al., Induction of myeloid-derived suppressor cells by tumor exosomes. *Int J Cancer* **124** (11), 2621 (2009).

[193] Srivastava, M. K. et al., Lung cancer patients' CD4(+) T cells are activated in vitro by MHC II cell-based vaccines despite the presence of myeloid-derived suppressor cells. *Cancer Immunol Immunother* **57** (10), 1493 (2008).

[194] Mandruzzato, S. et al., IL4Ralpha+ myeloid-derived suppressor cell expansion in cancer patients. *J Immunol* **182** (10), 6562 (2009).

[195] Schmielau, J. and Finn, O. J., Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res* **61** (12), 4756 (2001).

[196] Hoechst, B. et al., Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. *Hepatology* **50** (3), 799 (2009).

[197] Loercher, A. E. et al., Identification of an IL-10-producing HLA-DR-negative monocyte subset in the malignant ascites of patients with ovarian carcinoma that inhibits cytokine protein expression and proliferation of autologous T cells. *J Immunol* **163** (11), 6251 (1999).

[198] Vuk-Pavlovic, S. et al., Immunosuppressive CD14+HLA-DRlow/- monocytes in prostate cancer. *Prostate* **70** (4), 443 (2010).

[199] Raychaudhuri, B. et al., Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. *Neuro Oncol* **13** (6), 591 (2011).

[200] Brimnes, M. K. et al., Increased level of both CD4+FOXP3+ regulatory T cells and CD14+HLA-DR(-)/low myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma. *Scand J Immunol* **72** (6), 540 (2010).

[201] Lin, Y. et al., Immunosuppressive CD14+HLA-DR(low)/- monocytes in B-cell non-Hodgkin lymphoma. *Blood* **117** (3), 872 (2011).

[202] De Santo, C. et al., Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. *Nat Immunol* **11** (11), 1039 (2011).

[203] Poschke, I. et al., Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign. *Cancer Res* **70** (11), 4335 (2010).

[204] Filipazzi, P. et al., Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol* **25** (18), 2546 (2007).

[205] Bronte, V. et al., Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. *J Immunol* **161** (10), 5313 (1998).

[206] Morris, S. M., Jr., Enzymes of arginine metabolism. *J Nutr* **134** (10 Suppl), 2743S (2004).

[207] Rodriguez, P. C. et al., Regulation of T cell receptor CD3zeta chain expression by L-arginine. *J Biol Chem* **277** (24), 21123 (2002).

[208] Schmielau, J., Nalesnik, M. A., and Finn, O. J., Suppressed T-cell receptor zeta chain expression and cytokine production in pancreatic cancer patients. *Clin Cancer Res* **7** (3 Suppl), 933s (2001).

[209] Mazzoni, A. et al., Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol* **168** (2), 689 (2002).

[210] Gabrilovich, D. I., Ostrand-Rosenberg, S., and Bronte, V., Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* **12** (4), 253 (2012).

[211] Srivastava, M. K. et al., Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res* **70** (1), 68 (2010).

[212] Li, H. et al., Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. *J Immunol* **182** (1), 240 (2009); Elkabets, M. et al., IL-1beta regulates a novel myeloid-derived suppressor cell subset that impairs NK cell development and function. *Eur J Immunol* **40** (12), 3347.

[213] Serafini, P., Mgebroff, S., Noonan, K., and Borrello, I., Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res* **68** (13), 5439 (2008).

[214] Hoechst, B. et al., Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells. *Blood* **117** (24), 6532 (2011).

[215] Boucher, J. L. et al., N omega-hydroxyl-L-arginine, an intermediate in the L-arginine to nitric oxide pathway, is a strong inhibitor of liver and macrophage arginase. *Biochem Biophys Res Commun* **203** (3), 1614 (1994).

[216] Custot, J. et al., The new α-amino acid Nω-hydroxy-nor-L-arginine: a high affinity inhibitor of arginase well adapted to bind to its manganese cluster. *J Am Chem Soc* **119**, 4086 (1997).

[217] Kusmartsev, S. et al., Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. *Clin Cancer Res* **14** (24), 8270 (2008).

[218] Jeyabalan, G. et al., Arginase blockade protects against hepatic damage in warm ischemia-reperfusion. *Nitric Oxide* **19** (1), 29 (2008).

[219] Bagnost, T. et al., Treatment with the arginase inhibitor N(omega)-hydroxy-nor-L-arginine improves vascular function and lowers blood pressure in adult spontaneously hypertensive rat. *J Hypertens* **26** (6), 1110 (2008).

[220] Olken, N. M., Rusche, K. M., Richards, M. K., and Marletta, M. A., Inactivation of macrophage nitric oxide synthase activity by NG-methyl-L-arginine. *Biochem Biophys Res Commun* **177** (2), 828 (1991).

[221] Serafini, P. et al., Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol Immunother* **53** (2), 64 (2004).

[222] Pullamsetti, S. S. et al., Effect of nitric oxide synthase (NOS) inhibition on macro- and microcirculation in a model of rat endotoxic shock. *Thromb Haemost* **95** (4), 720 (2006).

[223] Momohara, Y. et al., Roles of endogenous nitric oxide synthase inhibitors and endothelin-1 for regulating myometrial contractions during gestation in the rat. *Mol Hum Reprod* **10** (7), 505 (2004).

[224] De Santo, C. et al., Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. *Proc Natl Acad Sci U S A* **102** (11), 4185 (2005).

[225] Mirza, N. et al., All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res* **66** (18), 9299 (2006).

[226] Kusmartsev, S. et al., Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. *Clin Cancer Res* **14** (24), 8270 (2008).

[227] Almand, B. et al., Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* **166** (1), 678 (2001).

[228] Rodriguez, P. C. et al., Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res* **69** (4), 1553 (2009).

[229] Zhou, D. et al., Editing of CD1d-bound lipid antigens by endosomal lipid transfer proteins. *Science* **303** (5657), 523 (2004).

[230] Fischer, K. et al., Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc Natl Acad Sci U S A* **101** (29), 10685 (2004).

[231] Bendelac, A. et al., CD1 recognition by mouse NK1+ T lymphocytes. *Science* **268** (5212), 863 (1995).

[232] Spencer, J. S. et al., Analysis of antibody responses to Mycobacterium leprae phenolic glycolipid I, lipoarabinomannan, and recombinant proteins to define disease subtype-specific antigenic profiles in leprosy. *Clin Vaccine Immunol* **18** (2), 260 (2010).

[233] Kawano, T. et al., CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* **278** (5343), 1626 (1997).

[234] Dieckmann, R. et al., Rapid screening and dereplication of bacterial isolates from marine sponges of the sula ridge by intact-cell-MALDI-TOF mass spectrometry (ICM-MS). *Appl Microbiol Biotechnol* **67** (4), 539 (2005).

[235] Mattner, J. et al., Liver autoimmunity triggered by microbial activation of natural killer T cells. *Cell Host Microbe* **3** (5), 304 (2008); Brigl, M. et al., Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol* **4** (12), 1230 (2003); Paget, C. et al., Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. *Immunity* **27** (4), 597 (2007); Salio, M. et al., Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation. *Proc Natl Acad Sci U S A* **104** (51), 20490 (2007).

[236] Nagarajan, N. A. and Kronenberg, M., Invariant NKT cells amplify the innate immune response to lipopolysaccharide. *J Immunol* **178** (5), 2706 (2007).

[237] Cerundolo, V., Silk, J. D., Masri, S. H., and Salio, M., Harnessing invariant NKT cells in vaccination strategies. *Nat Rev Immunol* **9** (1), 28 (2009).

[238] Smyth, M. J. et al., Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* **191** (4), 661 (2000).

[239] Crowe, N. Y., Smyth, M. J., and Godfrey, D. I., A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med* **196** (1), 119 (2002).

[240] Crowe, N. Y. et al., Differential antitumor immunity mediated by NKT cell subsets in vivo. *J Exp Med* **202** (9), 1279 (2005).

[241] Nishikawa, H. et al., CD4+ CD25+ T cells responding to serologically defined autoantigens suppress antitumor immune responses. *Proc Natl Acad Sci U S A* **100** (19), 10902 (2003).

[242] Molling, J. W. et al., Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. *J Clin Oncol* **25** (7), 862 (2007).

[243] Motohashi, S. et al., Preserved IFN-alpha production of circulating Valpha24 NKT cells in primary lung cancer patients. *Int J Cancer* **102** (2), 159 (2002).

[244] Tachibana, T. et al., Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin Cancer Res* **11** (20), 7322 (2005).

[245] Bricard, G. et al., Enrichment of human CD4+ V(alpha)24/Vbeta11 invariant NKT cells in intrahepatic malignant tumors. *J Immunol* **182** (8), 5140 (2009).

[246] Metelitsa, L. S., Weinberg, K. I., Emanuel, P. D., and Seeger, R. C., Expression of CD1d by myelomonocytic leukemias provides a target for cytotoxic NKT cells. *Leukemia* **17** (6), 1068 (2003).

[247] Takahashi, T. et al., Valpha24+ natural killer T-cell responses against T-acute lymphoblastic leukaemia cells: implications for immunotherapy. *Br J Haematol* **122** (2), 231 (2003).

[248] Dellabona, P. et al., On the use of donor-derived iNKT cells for adoptive immunotherapy to prevent leukemia recurrence in pediatric recipients of HLA haploidentical HSCT for hematological malignancies. *Clin Immunol* **140** (2), 152 (2011).

[249] Renukaradhya, G. J. et al., Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *Int J Cancer* **118** (12), 3045 (2006).

[250] Xu, C. et al., Expression of CD1d and presence of invariant NKT cells in classical Hodgkin lymphoma. *Am J Hematol* **85** (7), 539 (2010).

[251] Zenger, V. E. et al., Quantitative flow cytometry: inter-laboratory variation. *Cytometry* **33** (2), 138 (1998).

[252] Manabe, A. et al., Bone marrow-derived stromal cells prevent apoptotic cell death in B-lineage acute lymphoblastic leukemia. *Blood* **79** (9), 2370 (1992).

[253] Brinkmann, U. et al., A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc Natl Acad Sci U S A* **90** (16), 7538 (1993).

[254] Sallusto, F. and Lanzavecchia, A., Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* **179** (4), 1109 (1994).

[255] McCarthy, C. et al., The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. *J Exp Med* **204** (5), 1131 (2007).

[256] Campana, D., Manabe, A., and Evans, W. E., Stroma-supported immunocytometric assay (SIA): a novel method for testing the sensitivity of acute lymphoblastic leukemia cells to cytotoxic drugs. *Leukemia* **7** (3), 482 (1993).

[257] Ito, C. et al., Comparative cytotoxicity of dexamethasone and prednisolone in childhood acute lymphoblastic leukemia. *J Clin Oncol* **14** (8), 2370 (1996).

[258] Kreitman, R. J. et al., Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. *N Engl J Med* **345** (4), 241 (2001).

[259] Keppler-Hafkemeyer, A., Kreitman, R. J., and Pastan, I., Apoptosis induced by immunotoxins used in the treatment of hematologic malignancies. *Int J Cancer* **87** (1), 86 (2000).

[260] Kreitman, R. J. et al., Cytotoxic activity of disulfide-stabilized recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) toward fresh malignant cells from patients with B-cell leukemias. *Clin Cancer Res* **6** (4), 1476 (2000).

[261] Du, X., Beers, R., Fitzgerald, D. J., and Pastan, I., Differential cellular internalization of anti-CD19 and -CD22 immunotoxins results in different cytotoxic activity. *Cancer Res* **68** (15), 6300 (2008).

[262] Onda, M., Vincent, J. J., Lee, B., and Pastan, I., Mutants of immunotoxin anti-Tac(dsFv)-PE38 with variable number of lysine residues as candidates for site-specific chemical modification. 1. Properties of mutant molecules. *Bioconjug Chem* **14** (2), 480 (2003).

[263] Ogura, M. et al., Phase I study of anti-CD22 immunoconjugate inotuzumab ozogamicin plus rituximab in relapsed/refractory B-cell non-Hodgkin lymphoma. *Cancer Sci* **103** (5), 933 (2012).

[264] Galderisi, F. et al., Flow cytometric chemosensitivity assay as a predictive tool of early clinical response in acute lymphoblastic leukemia. *Pediatr Blood Cancer* **53** (4), 543 (2009).

[265] Mussai, F. et al., Cytotoxicity of the anti-CD22 immunotoxin HA22 (CAT-8015) against paediatric acute lymphoblastic leukaemia. *Br J Haematol* **150** (3), 352 (2010).

[266] Neckers, L., Development of small molecule Hsp90 inhibitors: utilizing both forward and reverse chemical genomics for drug identification. *Curr Med Chem* **10** (9), 733 (2003).

[267] Zhang, Y. et al., A flow cytometry method to quantitate internalized immunotoxins shows that taxol synergistically increases cellular immunotoxins uptake. *Cancer Res* **70** (3), 1082 (2010).

[268] Decker, T. et al., Induction of caspase-dependent programmed cell death in B-cell chronic lymphocytic leukemia by anti-CD22 immunotoxins. *Blood* **103** (7), 2718 (2004).

[269] Takeshita, A. et al., CMC-544 (inotuzumab ozogamicin) shows less effect on multidrug resistant cells: analyses in cell lines and cells from patients with B-cell chronic lymphocytic leukaemia and lymphoma. *Br J Haematol* **146** (1), 34 (2009).

[270] Herrera, L. et al., Immunotoxins against CD19 and CD22 are effective in killing precursor-B acute lymphoblastic leukemia cells in vitro. *Leukemia* **14** (5), 853 (2000).

[271] Beers, S. A. et al., Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for antibody selection. *Blood* **115** (25), 5191 (2010).

[272] Swerts, K. et al., Prognostic significance of multidrug resistance-related proteins in childhood acute lymphoblastic leukaemia. *Eur J Cancer* **42** (3), 295 (2006).

[273] Walter, R. B. et al., CD33 expression and P-glycoprotein-mediated drug efflux inversely correlate and predict clinical outcome in patients with acute myeloid leukemia treated with gemtuzumab ozogamicin monotherapy. *Blood* **109** (10), 4168 (2007).

[274] Lehne, G., De Angelis, P., den Boer, M., and Rugstad, H. E., Growth inhibition, cytokinesis failure and apoptosis of multidrug-resistant leukemia cells after treatment with P-glycoprotein inhibitory agents. *Leukemia* **13** (5), 768 (1999).

[275] Sippell, W. G., Antonowicz, I., Lazarus, H., and Shwachman, H., Lysosomal and mitochondrial enzyme activities in human lymphoid cell lines obtained from children with acute lymphoblastic leukemia and controls. *Exp Cell Res* **91** (1), 152 (1975).

[276] Munro, S. and Pelham, H. R., A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48** (5), 899 (1987).

[277] Townsley, F. M., Wilson, D. W., and Pelham, H. R., Mutational analysis of the human KDEL receptor: distinct structural requirements for Golgi retention, ligand binding and retrograde transport. *EMBO J* **12** (7), 2821 (1993).

[278] Chaudhary, V. K., Jinno, Y., FitzGerald, D., and Pastan, I., Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc Natl Acad Sci U S A* **87** (1), 308 (1990).

[279] Seetharam, S., Chaudhary, V. K., FitzGerald, D., and Pastan, I., Increased cytotoxic activity of Pseudomonas exotoxin and two chimeric toxins ending in KDEL. *J Biol Chem* **266** (26), 17376 (1991).

[280] Kreitman, R. J. and Pastan, I., Importance of the glutamate residue of KDEL in increasing the cytotoxicity of Pseudomonas exotoxin derivatives and for increased binding to the KDEL receptor. *Biochem J* **307 (Pt 1)**, 29 (1995).

[281] Deng, Q. and Barbieri, J. T., Molecular mechanisms of the cytotoxicity of ADP-ribosylating toxins. *Annu Rev Microbiol* **62**, 271 (2008).

[282] Robinson, E. A., Henriksen, O., and Maxwell, E. S., Elongation factor 2. Amino acid sequence at the site of adenosine diphosphate ribosylation. *J Biol Chem* **249** (16), 5088 (1974).

[283] Roy, V., Ghani, K., and Caruso, M., A dominant-negative approach that prevents diphthamide formation confers resistance to Pseudomonas exotoxin A and diphtheria toxin. *PLoS One* **5** (12), e15753 (2012).

[284] Petronini, P. G. et al., Cell susceptibility to apoptosis by glutamine deprivation and rescue: survival and apoptotic death in cultured lymphoma-leukemia cell lines. *J Cell Physiol* **169** (1), 175 (1996).

[285] Zhao, Q. L., Kondo, T., Noda, A., and Fujiwara, Y., Mitochondrial and intracellular free-calcium regulation of radiation-induced apoptosis in human leukemic cells. *Int J Radiat Biol* **75** (4), 493 (1999).

[286] Uckun, F. M. et al., Inducing apoptosis in chemotherapy-resistant B-lineage acute lymphoblastic leukaemia cells by targeting HSPA5, a master regulator of the anti-apoptotic unfolded protein response signalling network. *Br J Haematol* **153** (6), 741 (2011).

[287] Khan, N. I. et al., Para-NO-aspirin inhibits NF-kappaB and induces apoptosis in B-cell progenitor acute lymphoblastic leukemia. *Exp Hematol* **40** (3), 207 (2012).

[288] Youle, R. J. and Strasser, A., The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* **9** (1), 47 (2008).

[289] Erlacher, M. et al., BH3-only proteins Puma and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells in vivo. *Blood* **106** (13), 4131 (2005).

[290] Ni Chonghaile, T. et al., Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science* **334** (6059), 1129 (2012).

[291] Hua, C. et al., Consequences of the t(14;18) chromosomal translocation in follicular lymphoma: deregulated expression of a chimeric and mutated BCL-2 gene. *Oncogene Res* **2** (3), 263 (1988).

[292] Findley, H. W., Gu, L., Yeager, A. M., and Zhou, M., Expression and regulation of Bcl-2, Bcl-xl, and Bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. *Blood* **89** (8), 2986 (1997).

[293] Campos, L. et al., Expression of BCL-2 proto-oncogene in adult acute lymphoblastic leukemia. *Leukemia* **10** (3), 434 (1996).

[294] Coustan-Smith, E. et al., Clinical relevance of BCL-2 overexpression in childhood acute lymphoblastic leukemia. *Blood* **87** (3), 1140 (1996).

[295] Wuchter, C. et al., Constitutive expression levels of CD95 and Bcl-2 as well as CD95 function and spontaneous apoptosis in vitro do not predict the response to induction chemotherapy and relapse rate in childhood acute lymphoblastic leukaemia. *Br J Haematol* **110** (1), 154 (2000).

[296] Bogner, C. et al., Immunotoxin BL22 induces apoptosis in mantle cell lymphoma (MCL) cells dependent on Bcl-2 expression. *Br J Haematol* **148** (1), 99 (2010).

[297] Du, X., Youle, R. J., FitzGerald, D. J., and Pastan, I., Pseudomonas exotoxin A-mediated apoptosis is Bak dependent and preceded by the degradation of Mcl-1. *Mol Cell Biol* **30** (14), 3444 (2010).

[298] Whitesell, L. et al., Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc Natl Acad Sci U S A* **91** (18), 8324 (1994).

[299] Normant, E. et al., The Hsp90 inhibitor IPI-504 rapidly lowers EML4-ALK levels and induces tumor regression in ALK-driven NSCLC models. *Oncogene* **30** (22), 2581 (2011).

[300] Peng, C. et al., Inhibition of heat shock protein 90 prolongs survival of mice with BCR-ABL-T315I-induced leukemia and suppresses leukemic stem cells. *Blood* **110** (2), 678 (2007).

[301] Modi, S. et al., HSP90 inhibition is effective in breast cancer: a phase II trial of tanespimycin (17-AAG) plus trastuzumab in patients with HER2-positive metastatic breast cancer progressing on trastuzumab. *Clin Cancer Res* **17** (15), 5132 (2011).

[302] Richardson, P. G. et al., Tanespimycin and bortezomib combination treatment in patients with relapsed or relapsed and refractory multiple myeloma: results of a phase 1/2 study. *Br J Haematol* **153** (6), 729 (2011).

[303] Yufu, Y., Nishimura, J., and Nawata, H., High constitutive expression of heat shock protein 90 alpha in human acute leukemia cells. *Leuk Res* **16** (6-7), 597 (1992).

[304] Tong, W. G. et al., The synthetic heat shock protein 90 (Hsp90) inhibitor EC141 induces degradation of Bcr-Abl p190 protein and apoptosis of Ph-positive acute lymphoblastic leukemia cells. *Invest New Drugs* **29** (6), 1206 (2011).

[305] van Delft, M. F. et al., The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* **10** (5), 389 (2006).

[306] Chen, S. et al., Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. *Cancer Res* **67** (2), 782 (2007).

[307] Andersson, Y., Juell, S., and Fodstad, O., Downregulation of the antiapoptotic MCL-1 protein and apoptosis in MA-11 breast cancer cells induced by an anti-epidermal growth factor receptor-Pseudomonas exotoxin a immunotoxin. *Int J Cancer* **112** (3), 475 (2004).

[308] Traini, R. et al., ABT-737 overcomes resistance to immunotoxin-mediated apoptosis and enhances the delivery of pseudomonas exotoxin-based proteins to the cell cytosol. *Mol Cancer Ther* **9** (7), (2007).

[309] Griffon-Etienne, G. et al., Taxane-induced apoptosis decompresses blood vessels and lowers interstitial fluid pressure in solid tumors: clinical implications. *Cancer Res* **59** (15), 3776 (1999).

[310] Netti, P. A. et al., Role of extracellular matrix assembly in interstitial transport in solid tumors. *Cancer Res* **60** (9), 2497 (2000).

[311] Juweid, M. et al., Micropharmacology of monoclonal antibodies in solid tumors: direct experimental evidence for a binding site barrier. *Cancer Res* **52** (19), 5144 (1992).

[312] Zhang, Y. and Pastan, I., High shed antigen levels within tumors: an additional barrier to immunoconjugate therapy. *Clin Cancer Res* **14** (24), 7981 (2008).

[313] Matsushita, K. et al., Soluble CD22 as a tumor marker for hairy cell leukemia. *Blood* **112** (6), 2272 (2008).

[314] Zhang, Y. et al., Synergistic antitumor activity of taxol and immunotoxin SS1P in tumor-bearing mice. *Clin Cancer Res* **12** (15), 4695 (2006).

[315] Fitzgerald, D. J. et al., Enhancing immunotoxin cell-killing activity via combination therapy with ABT-737. *Leuk Lymphoma* **52 Suppl 2**, 79 (2011).

[316] Filippin-Monteiro, F. B. et al., Serum amyloid A is a growth factor for 3T3-L1 adipocytes, inhibits differentiation and promotes insulin resistance. *Int J Obes (Lond) (2010)*.

[317] Liang, T. S., Wang, J. M., Murphy, P. M., and Gao, J. L., Serum amyloid A is a chemotactic agonist at FPR2, a low-affinity N-formylpeptide receptor on mouse neutrophils. *Biochem Biophys Res Commun* **270** (2), 331 (2000).

[318] Hiratsuka, S. et al., The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nat Cell Biol* **10** (11), 1349 (2008).

[319] Zhao, F. et al., S100A9 a new marker for monocytic human myeloid-derived suppressor cells. *Immunology* **136** (2), 176 (2012).

[320] Sinha, P. et al., Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. *J Immunol* **181** (7), 4666 (2008).

[321] Laoui, D. et al., Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. *Int J Dev Biol* **55** (7-9), 861 (2011).

[322] Umemura, N. et al., Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. *J Leukoc Biol* **83** (5), 1136 (2008).

[323] Roca, H. et al., CCL2 and interleukin-6 promote survival of human CD11b+ peripheral blood mononuclear cells and induce M2-type macrophage polarization. *J Biol Chem* **284** (49), 34342 (2009).

[324] Briken, V. and Mosser, D. M., Editorial: switching on arginase in M2 macrophages. *J Leukoc Biol* **90** (5), 839 (2011).

[325] Whyte, C. S. et al., Suppressor of cytokine signaling (SOCS)1 is a key determinant of differential macrophage activation and function. *J Leukoc Biol* **90** (5), 845 (2011).

[326] Standiford, T. J. et al., TGF-beta-induced IRAK-M expression in tumor-associated macrophages regulates lung tumor growth. *Oncogene* **30** (21), 2475 (2011).

[327] Dominguez-Soto, A. et al., Dendritic cell-specific ICAM-3-grabbing nonintegrin expression on M2-polarized and tumor-associated macrophages is macrophage-CSF dependent and enhanced by tumor-derived IL-6 and IL-10. *J Immunol* **186** (4), 2192 (2010).

[328] He, R. L. et al., Serum amyloid A induces G-CSF expression and neutrophilia via Toll-like receptor 2. *Blood* **113** (2), 429 (2009).

[329] Tao, M. et al., SCF, IL-1beta, IL-1ra and GM-CSF in the bone marrow and serum of normal individuals and of AML and CML patients. *Cytokine* **12** (6), 699 (2000).

[330] Boso, M. et al., Alterations of circulating endogenous secretory RAGE and S100A9 levels indicating dysfunction of the AGE-RAGE axis in autism. *Neurosci Lett* **410** (3), 169 (2006).

[331] Orleans-Lindsay, J. K., Barber, L. D., Prentice, H. G., and Lowdell, M. W., Acute myeloid leukaemia cells secrete a soluble factor that inhibits T and NK cell proliferation but not cytolytic function--implications for the adoptive immunotherapy of leukaemia. *Clin Exp Immunol* **126** (3), 403 (2001).

[332] Buggins, A. G. et al., Microenvironment produced by acute myeloid leukemia cells prevents T cell activation and proliferation by inhibition of NF-kappaB, c-Myc, and pRb pathways. *J Immunol* **167** (10), 6021 (2001).

[333] Curti, A. et al., Acute myeloid leukemia cells constitutively express the immunoregulatory enzyme indoleamine 2,3-dioxygenase. *Leukemia* **21** (2), 353 (2007).

[334] Corm, S. et al., Indoleamine 2,3-dioxygenase activity of acute myeloid leukemia cells can be measured from patients' sera by HPLC and is inducible by IFN-gamma. *Leuk Res* **33** (3), 490 (2009).

[335] Le Dieu, R. et al., Peripheral blood T cells in acute myeloid leukemia (AML) patients at diagnosis have abnormal phenotype and genotype and form defective immune synapses with AML blasts. *Blood* **114** (18), 3909 (2009).

[336] Montero, A. J. et al., Myeloid-derived suppressor cells in cancer patients: a clinical perspective. *J Immunother* **35** (2), 107 (2012).

[337] Jenkinson, C. P., Grody, W. W., and Cederbaum, S. D., Comparative properties of arginases. *Comp Biochem Physiol B Biochem Mol Biol* **114** (1), 107 (1996).

[338] Sparkes, R. S. et al., The gene for human liver arginase (ARG1) is assigned to chromosome band 6q23. *Am J Hum Genet* **39** (2), 186 (1986).

[339] Gotoh, T., Araki, M., and Mori, M., Chromosomal localization of the human arginase II gene and tissue distribution of its mRNA. *Biochem Biophys Res Commun* **233** (2), 487 (1997).

[340] Morris, S. M., Jr., Bhamidipati, D., and Kepka-Lenhart, D., Human type II arginase: sequence analysis and tissue-specific expression. *Gene* **193** (2), 157 (1997).

[341] Gannon, P. O. et al., Androgen-regulated expression of arginase 1, arginase 2 and interleukin-8 in human prostate cancer. *PLoS One* **5** (8), e12107 (2010).

[342] Steidl, C. et al., Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. *N Engl J Med* **362** (10), 875 (2010).

[343] Ho, V. W. and Sly, L. M., Derivation and characterization of murine alternatively activated (M2) macrophages. *Methods Mol Biol* **531**, 173 (2009).

[344] Burt, B. M. et al., Circulating and tumor-infiltrating myeloid cells predict survival in human pleural mesothelioma. *Cancer* **117** (22), 5234 (2011).

[345] Frisch, B. J. et al., Functional inhibition of osteoblastic cells in an in vivo mouse model of myeloid leukemia. *Blood* **119** (2), 540 (2012).

[346] Nagaraj, S. et al., Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* **13** (7), 828 (2007).

[347] Bronte, V. et al., L-arginine metabolism in myeloid cells controls T-lymphocyte functions. *Trends Immunol* **24** (6), 302 (2003).

[348] Gabrilovich, D. I. and Nagaraj, S., Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* **9** (3), 162 (2009).

[349] Gallina, G. et al., Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J Clin Invest* **116** (10), 2777 (2006).

[350] Sallerfors, B. and Olofsson, T., Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) in serum during induction treatment of acute leukaemia. *Br J Haematol* **78** (3), 343 (1991).

[351] Tsimberidou, A. M. et al., The prognostic significance of cytokine levels in newly diagnosed acute myeloid leukemia and high-risk myelodysplastic syndromes. *Cancer* **113** (7), 1605 (2008).

[352] Urieli-Shoval, S., Linke, R. P., and Matzner, Y., Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states. *Curr Opin Hematol* **7** (1), 64 (2000).

[353] Yamada, T., Wada, A., Itoh, K., and Igari, J., Serum amyloid A secretion from monocytic leukaemia cell line THP-1 and cultured human peripheral monocytes. *Scand J Immunol* **52** (1), 7 (2000).

[354] Rosenthal, C. J. and Sullivan, L. M., Serum amyloid A to monitor cancer dissemination. *Ann Intern Med* **91** (3), 383 (1979).

[355] Dowling, P. et al., Analysis of acute-phase proteins, AHSG, C3, CLI, HP and SAA, reveals distinctive expression patterns associated with breast, colorectal and lung cancer. *Int J Cancer (2012)*.

[356] Dufton, N. et al., Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation. *J Immunol* **184** (5), 2611 (2010).

[357] Srikrishna, G. et al., Two proteins modulating transendothelial migration of leukocytes recognize novel carboxylated glycans on endothelial cells. *J Immunol* **166** (7), 4678 (2001).

[358] Vogl, T. et al., Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med* **13** (9), 1042 (2007).

[359] Robinson, M. J., Tessier, P., Poulsom, R., and Hogg, N., The S100 family heterodimer, MRP-8/14, binds with high affinity to heparin and heparan sulfate glycosaminoglycans on endothelial cells. *J Biol Chem* **277** (5), 3658 (2002).

[360] Ghavami, S. et al., S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP kinase-dependent pathway. *J Leukoc Biol* **83** (6), 1484 (2008).

[361] Nicolas, E. et al., Expression of S100A8 in leukemic cells predicts poor survival in de novo AML patients. *Leukemia* **25** (1), 57 (2011).

[362] Metelitsa, L. S. et al., Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol* **167** (6), 3114 (2001).

[363] Berzofsky, J. A. and Terabe, M., A novel immunoregulatory axis of NKT cell subsets regulating tumor immunity. *Cancer Immunol Immunother* **57** (11), 1679 (2008).

[364] Bontkes, H. J. et al., Tumor associated antigen and interleukin-12 mRNA transfected dendritic cells enhance effector function of natural killer cells and antigen specific T-cells. *Clin Immunol* **127** (3), 375 (2008).

[365] Kobayashi, E. et al., KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* **7** (10-11), 529 (1995).

[366] Nakagawa, R. et al., Treatment of hepatic metastasis of the colon26 adenocarcinoma with an alpha-galactosylceramide, KRN7000. *Cancer Res* **58** (6), 1202 (1998).

[367] Crul, M. et al., Population pharmacokinetics of the novel anticancer agent KRN7000. *Cancer Chemother Pharmacol* **49** (4), 287 (2002).

[368] Parekh, V. V. et al., Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* **115** (9), 2572 (2005).

[369] Giaccone, G. et al., A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* **8** (12), 3702 (2002).

[370] Chang, D. H. et al., Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* **201** (9), 1503 (2005).