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Molecular Profiling of Leukemia

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Expression array profiles of leukemias have recently emerged as useful diagnostic tools, and have provided new insights into the biology of leukemias. For example, expression array analysis has identified gene expression signatures that define novel oncogenic pathways in T-ALL. Expression signatures in T-ALL are indicative of leukemic cell arrest at specific stages of normal thymocyte development, and have clinical prognostic value. For example, HOX11 expression is significantly associated with a favorable prognosis, whereas TAL-LYL1 or HOX11L2 expression confers worse prognosis. In the broader spectrum of all pediatric ALL, expression profiling can reliably identify all known prognostic subtypes of ALL, including T-ALL, E2A-PBX1, TEL-AML1, MLL gene rearrangements, BCR-ABL and hyperdiploid cases. Among infant leukemias, expression profiling has identified MLL rearranged leukemias as a separate subtype of leukemia that is distinct from either ALL or AML. In addition, these analyses identified FLT3 as a potential therapeutic target in MLL rearranged infant leukemia. Finally, gene expression signatures that track biological processes such as differentiation may be useful in drug screens for agents that induce differentiation of leukemic blasts. Expression arrays can thus be added to the armamentarium of complementary techniques that facilitate diagnosis, assessment of prognosis, and inform biology and new drug development.

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The Genes in Childhood ALL Provide Clues to its Origin

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The rising incidence of childhood ALL provides compelling evidence for the presence of contributory extrinsic factors associated with modern life. Although there are no compelling data to implicate any specific types of environmental factors, molecular fingerprinting of ALL is now providing the basis for a highly targeted approach to the epidemiology of ALL. The pre-natal origin of infant ALL is clear, but the structure of the immunoglobulin gene VDJ recombinations suggests origin after approximately 12 weeks of gestation. The strong association of infant ALL with MLL gene translocations points to the involvement of exogenous DNA topoisomerase II inhibitors in its origin. The majority of childhood ALL also show strong evidence for a prenatal origin, with some evidence suggesting an origin between 6-8 weeks of gestation. In contrast, twin studies and the presence of the t(12;21) translocation in normal children at birth implies the contribution of post-natal processes in determining the neoplastic potential of leukaemic clones. The pre-B ALLs associated with t(1;19), show evidence for a postnatal origin. Additional analysis of the molecular features of ALL, together with a more complete understanding of the normal processes of lymphoid development, will refine the time of origin of ALL and direct epidemiological research to the appropriate window of relevance.

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Modelling Leukaemia in Zebrafish

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Zebrafish are recognised as an exciting new model system for cancer research[1,2]. They develop tumours that are histologically similar to human cancers, and pathways involving genes important in tumour formation (including cell cycle regulators, oncogenes and tumour suppressors) are conserved in this species. Advances in zebrafish genetics and genomics have provided a powerful set of tools for studying the biology of cancer and for identifying genes that will form the basis of drug discovery programs. For example, unbiased forward genetic screens can be targeted to cancer pathways and having identified a mutation, enhancer/suppressor screens can be used to find genes that modify the effects of this mutation. Reverse genetic approaches permit the generation of transgenic models where an oncogene might be overexpressed or a tumour suppressor gene inactivated through dominant-negative strategies. The zebrafish is being used to progress understanding of the molecular mechanisms that drive leukaemia and to provide opportunities to conduct screens for new anti-leukaemic drugs[3,4]. We have developed a model of RUNX1-ETO leukaemia in zebrafish, to address the issue of RUNX1-ETO being necessary, but not sufficient in its own right, for the induction of human t(8;21) acute myeloid leukaemia. Forward genetic screens are being undertaken to identify modifiers of *runx1* expression and of the RUNX1-ETO transgenic phenotype. In work by Langenau *et al*., a model of T cell leukaemia has been generated in transgenic zebrafish by expression of c-myc under the control of the *rag2* promoter. These approaches, together with those of other groups using the zebrafish model in leukaemia research, offer the prospect of a better understanding of leukaemogenic pathways and the identification of new targets for drug development.

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2. Stern HM and Zon LI. (2003). Cancer genetics and drug discovery in zebrafish. Nature Reviews 3: 1
3. Kalev-Zylinska *et al*. (2002) Runx1 is required for zebrafish blood and vessel development, fand expression of a human RUNX1-CBF2T1 transgene advances a model for studies of leukemogenesis. Development 129: 2015
4. Langenau *et al*. (2003). Myc-induced T cell leukaemia in transgenic zebrafish. Science 299: 887

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Treatment Strategies for Avoiding Red Blood Cell Blood Transfusions in Chronic Blood Disorders

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Red blood cell transfusions are vital in many situations. There may be no other option in trauma and massive haemorrhage, although other products such as platelet concentrates, coagulation factors and anti-fibrinolytic agents can reduce the blood requirement in selected cases. In chronic blood disorders, ongoing research is defining the mechanisms of the anaemia. This already leads to individually tailored therapy for patients based on analysis of the different contributing factors from their disease and/or co-morbidities. This review highlights available alternative treatments in chronic blood disorders such as

anaemia of chronic disease (ACD), myelodysplasia (MDS), myeloma, myelofibrosis, lymphoproliferative disorders and haemoglobinopathies. These include recombinant erythropoietin (rhEPO) and other growth factors, iron infusions, heme iron, and immune-suppressive agents such as cyclosporin, immunoglobulin and thalidomide. Erythropoietin successfully treats anaemia secondary to malignancy. In patients with lymphoproliferative diseases, rhEPO decreases the need for transfusion, and improves the patients' quality of life. Disadvantages of rhEPO include cost, the variable response, and the delay before maximum benefit is achieved. The anaemia associated with MDS responds less well to rhEPO. In some MDS patients, autoimmune haemolysis may also contribute and responses to cyclosporin and/or other immunosuppressive therapy have been reported. Patients with chronic haematological disorders with coexistent renal impairment and/or ACD may become transfusion independent with rhEPO. The anaemia of chronic disease (ACD) results from the effects of cytokines mediating the immune or inflammatory response. Experience with rhEPO in severely affected patients provides rational and effective treatment. Recent work suggests oral heme iron may be an effective supplement in haemodialysis, as it is absorbed by patients with high ferritin levels, has fewer side effects, and its absorption is stimulated by erythropoietin administration. There is possibly a role for this agent or iron infusions in ACD, to provide bio-available iron. The use of alternative therapies is increasingly important, as the availability of blood donors has become more restricted. Also nucleic acid testing of blood products, leucodepletion, and hospital charges have dramatically increased in recent years to a point where expensive therapies are becoming cost effective. Ideally, patients with chronic blood disorders should often avoid (excess) blood transfusions because of auto- and allo-red cell antibodies, and/or the risk of iron overload. Patients who decline blood products on religious or other grounds also need to be considered.

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Non-invasive Measurement and Imaging of Liver Iron Concentrations Using Nuclear Magnetic Resonance (MRI)

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Iron overload disorders such as thalassaemia and haemochromatosis affect up to 0.5% of the world's population. These disorders result in the accumulation of iron in the form of nanoparticles of iron(III) oxyhydroxide in organs of the body such as the liver, heart, spleen, and pancreas. Knowledge of patient tissue iron concentrations is often essential for appropriate management of iron overload conditions. The currently accepted and widely used method of measurement is chemical analysis of needle biopsy specimens of the liver. However, needle biopsy is an invasive procedure that carries a degree of risk and has associated sampling errors owing to the uneven distribution of iron within tissues. We have developed a method of using nuclear magnetic resonance imaging to measure and image proton transverse relaxation rates (R2)

within the liver. R2 is highly dependent on tissue iron concentration owing to interactions between protons of water molecules and the iron oxyhydroxide nanoparticles within the tissue. The high degree of correlation between R2 and tissue iron concentration has enabled the development of a method for non-invasively measuring and imaging liver iron concentrations. The method has a demonstrated dynamic range of measurement of hepatic iron concentration from 0.3 to 42.7 mg Fe/g dry liver tissue. The unparalleled dynamic range and degree of correlation with needle biopsy measurements together with a demonstrated high degree of reproducibility on multiple magnetic resonance imaging units offers the possibility of readily available non-invasive absolute hepatic iron concentration measurements to the clinical community. Furthermore, there is promise that the ability to image liver iron concentrations may enable the detection of distorted liver architecture such as fibrosis and cirrhosis by virtue of the fact that these distortions result in altered patterns of iron deposition within the tissue.

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Treatment-Related Iron Overload

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Iron overload commonly complicates the clinical course of individuals with thalassemia and sickle cell disease, as well as that of many patients with acquired hematologic disorders. The estimate that approximately 7% of the world's population are carriers for an inherited disorder of hemoglobin highlights the importance of the rational, evidence-based management of this serious complication of treatment in such patients. In general, the toxic manifestations of iron overload in transfused patients depend not only on the amount of excess iron but also on the rate of iron accumulation, the duration of exposure to increased iron, the partition of iron between relatively benign sites in the macrophage and more hazardous deposits in parenchymal cells, ascorbate status, and non-iron-related factors, such as alcohol and viral hepatitis. Data from studies over the past ten years in thalassemia and hereditary hemochromatosis permit a quantitative approach to the management of iron overload, and provide guidelines for iron-chelating therapy. Compliance with effective iron-chelating therapy is the key to prolonged survival and reduction of the complications of iron overload; extended survival free of iron-induced complications, and dramatically improved quality of life, are observed in patients treated with adequate deferoxamine. Yet many practical problems are associated with long-term deferoxamine therapy: the accurate assessment of body iron burden, the initiation of treatment including the maintenance of balance between its effectiveness and toxicity, and the resourceful management of erratic compliance with this difficult regimen. Both direct and indirect means for the assessment of body iron are available; the reference method, measurement of hepatic storage iron, provides a quantitative means of assessing iron burden provided certain cautions are taken. By contrast, while measurement of plasma or serum ferritin is the most commonly used *indirect* estimate of body iron stores, its interpretation is complicated by a variety of conditions, common in iron-loaded patients, that alter concentrations independently of changes in body iron. As a consequence, reliance on this or on other estimates, including serum iron, serum transferrin, urinary iron excretion, and magnetic resonance imaging of various organs, may lead to inaccurate assessment of body iron in individual patients. Magnetic susceptometry using a superconducting magnetometer (SQUID) provides a direct non-invasive measure of hepatic storage iron that, in one US center, has been shown to be quantitatively equivalent to that obtained by chemical analysis of tissue obtained at liver biopsy. Formerly, only two sites worldwide possessed (dissimilar) specialized equipment needed for SQUID measurements; new SQUID machines have

now been launched in other centers worldwide, but the quantitative equivalence of iron concentration determined using these machines to that of hepatic tissue obtained by biopsy is not established. Recently, one group of investigators have contended that iron estimated using particular magnetic resonance imaging techniques ("T2*) are useful in measuring iron within the heart as well as the liver. Unfortunately, no prospective, controlled studies of these techniques have yet provided the evidence for this assertion. The expense and inconvenience of deferoxamine has mandated a twenty-year search for an orally active iron chelator. The evidence arising from trials of the most extensively studied such drug, deferiprone, suggests that in a substantial proportion of patients, over one to eight years, body iron burden is inadequately controlled. Because virtually all studies in the past five years have been conducted with funding from those possessing commercial interests in the commercial licensing of this drug, it bears repeating that, before deferiprone can be considered for clinical use, prospective clinical trials are mandatory to evaluate the possibility of its long-term safety.

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Treatment Strategies in Advanced Hodgkin's Disease

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Approximately 60% of all patients with Hodgkin's disease present with advanced disease. Standard chemotherapy regimens for advanced disease have included MOPP and ABVD, the latter resulting in much less toxicity including infertility and secondary leukaemia. Two successive large randomised trials have shown no advantage of alternating regimens (like COPP/ABVD, MOPP/ABVD, MOPP/ABV) over ABVD, although they proved superior to MOPP. Hence, ABVD was established as the gold standard on the basis of its efficacy and reduced long-term toxic effects. Since then, encouraging results have been reported with some alternative regimens. The Stanford V regimen is a 12-week course of seven active drugs with radiotherapy to sites of initial bulk disease. In a single arm study, 5-year FFS of 89% and 5-year OS of 96% were achieved. Randomised studies of Stanford V versus ABVD are continuing. The German Hodgkin's Study Group have tested their standard alternating regimen of COPP and ABVD with a hybrid regimen (BEACOPP) and a dose-intensified regimen (escalated BEACOPP). At the final analysis of the HD9 study involving 1,195 patients, the rate of FFTF at 5 years was 69% in the COPP-ABVD group, 76% in the BEACOPP group, and 87% in the escalated BEACOPP group ($p=<0.001$ for the comparison between COPP-ABVD and escalated BEACOPP). Rates of overall survival (OS) at 5 years were 83%, 88%, and 91%, respectively ($p=0.002$ for the comparison between COPP-ABVD and escalated BEACOPP). Toxicity concerns with the BEACOPP regimens include anaemia, infertility, and secondary leukaemia. To date, nine cases of AML/MDS have been reported in the escalated BEACOPP arm and four in the baseline BEACOPP arm. Interim analysis of the subsequent HD12 study confirmed the results for escalated BEACOPP seen in the HD9 study, and to date shows no difference in failure-free survival compared to 4 cycles of escalated BEACOPP plus 4 cycles of standard BEACOPP. This trial together with a large randomised EORTC study, also suggest that radiation provides no additional benefit following completion of effective chemotherapy. The EORTC global study is currently assessing whether treatment with 4 cycles of escalated BEACOPP plus 4 cycles of standard BEACOPP is superior to 8 cycles of ABVD in advanced stage patients with International Prognostic Scores of 3 or higher. The ongoing German HD15 study will assess whether fewer cycles of a time-intensified 14-day escalated BEACOPP regimen confers any advantage over the 21-day regimen.

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Adoptive Immunotherapy for Hodgkin's Lymphoma

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We have evaluated the use of polyclonal EBV-specific Cytotoxic T Lymphocytes (EBV-CTL) in patients with EBV+ Hodgkin Disease (HD) with multiple relapses (group A) and with minimal residual disease post stem cell transplant (group B). Of 128 referred patients, 33 (26%) had EBV+ve tumors which are known to express the EBV antigens EBNA1, LMP1 and LMP2. 8 patients with relapsed HD (group A) have been treated on study and have received either $4x10^7$ CTL/m^2 (n=6), or $2x10^7$ CTL/m^2 (n=1) or $1.2x10^8$/m^2 (n=1). In 7 of these patients CTL were gene-marked with the neomycin resistance gene. 5 patients have been treated in group B and 3 received CTL at $4x10^7$/m^2 and 2 received a CTL dose of $1.2x10^8$/m^2. No immediate toxicity was observed post CTL infusions. In group A, 2 patients developed transient flu-like symptoms. Another patient had erosion of tumor through the (L) upper lobe bronchus and died two months after CTL infusion. In situ PCR revealed gene-marked CTL within the tumor but not at the site of bronchus erosion. 5 patients with aggressive disease survived for 8-21 months and 1 patient is alive 4 years after CTL. Using LMP2 peptide tetramers to analyze CTL lines, an average of 1.61% (0.02-7.66%) of CD8 Tcells were +ve for LMP2, which was similar to the frequency seen in CTL lines generated from normal donors. Gene-marked CTL were found in peripheral blood up to 9 months following infusion. Gene-marked CTL were found localized to a malignant pleural effusion in one patient 3 weeks after CTL infusion. Of the 5 patients in group B, 4 remain well 19-37 months post CTL. One patient died 3 months post CTL with progressive disease. One patient in group B had resolution of an abnormal gallium scan post CTL associated with a 4-fold increase in LMP2-CTL detected by ELISPOT assay. Immunotherapy with autologous EBV-CTL is well tolerated in patients with HD and infused cells localize to tumor and persist for up to 9 months. LMP2-CTL increased in the peripheral blood post CTL infusion and we are now developing strategies to increase their frequency, to increase the specificity of the cytotoxic response against EBV+ Hodgkin tumors. However, even if the specificity of the CTL is enhanced, tumor-specific CTL are susceptible to immunosupressive factors, such as TGFβ, secreted by tumor cells. We are therefore developing approaches to genetically modify CTL rendering them resistant to TGFβ, thereby overcoming this powerful immune evasion mechanism.

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T Cell-Mediated Tumor Immunotherapy

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A number of antigens expressed on tumor cells have been identified which represent attractive targets for immunotherapy. The ideal antigens for immunotherapy are expressed selectively on tumor cells and can become associated with major histocompatibility molecules for recognition by the effector cells of the immune system, the CD4+ and CD8+ T lymphocytes. Recent evidence has indicated that the immune system acts as a selective force on tumor development and progression, and that the tumors that ultimately outgrow are those that have developed strategies to evade immune attack. An effective approach to immunotherapy must take this information into account. Several different strategies have been employed to induce the

activation of tumor specific T lymphocytes. Tumor cells have been genetically modified to acquire the capacity to produce cytokines and other immunostimulatory molecules, which in turn cause local inflammation, infiltration of immune cells and activation of T lymphocytes. Tumor-reactive T lymphocytes have been isolated from cancer patients, expanded in culture to very large numbers, and re-infused into the donors. Tumor cells, or tumor cell derived components have been prepared and loaded onto specialized cells of the immune system, the "antigen presenting cells" whose specific function is to direct the activation of T lymphocytes. Each of these approaches has met with some success, demonstrating that the immunotherapy of cancer is feasible and has the potential to succeed. At the same time, success has been sporadic indicating that current approaches must be refined and optimized before they become widely applicable. Data in our Laboratory have shown that effective anti-tumor immunity can be induced by using Antigen presenting cells such as dendritic cell to initiate anti-tumor immune responses. Similarly, transfer of tumor-reactive lymphocytes can delay tumor progression. The long-term survival of the dendritic cells and the anti-tumor lymphocytes, and the enhanced delivery of their effector function at the tumor site are likely to improve the success of this and similar strategies to immunotherapy, and are the focus of our current investigations.

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Targeted Therapy of AML

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AML remains a therapeutic challenge. Most adults who develop AML die from their disease or complications of therapy, and most are still treated with empirically derived cytotoxic chemotherapy. Genetic analysis of acute leukemias has identified a spectrum of mutant alleles that are causally implicated in disease pathogenesis, and present therapeutic opportunities. These mutations appear to fall into two broad complementation groups. One group activates signal transduction pathways that confer a proliferative and/or survival advantage to leukemia cells. Examples include activating mutations in FLT3, RAS and KIT. A second complementation group is typified by mutations that result in loss of function of transcription factors that are important for normal hematopoietic development. These latter gene rearrangements and mutations, exemplified by the AML1-ETO, AML1 point mutants or PML-RARα impair hematopoietic differentiation and confer an immortalization phenotype. Acute leukemias are characterized by the presence of one mutation from each of these complementation groups, such as FLT3 mutations in 30% of acute promyelocytic leukemias (APL) with the PML-RARα fusion gene, or RAS mutations in a high percentage of AML1-ETO leukemias. Murine models have confirmed cooperative effects of these mutations in several different contexts. Many of these acquired mutations have prognostic significance, and represent targets for therapeutic intervention. The use of all-*trans*-retinoic acid (ATRA) in treatment of acute promyelocytic leukemia (APL) ushered in a new era of targeted therapy of leukemia. Other promising targets have recently emerged, such as mutant FLT3 that is present in ~30% of AML cases. Cell based screens have identified several FLT3 selective inhibitors, including PKC412 (Novartis), MLN518 (Millenium), SU11248 (SuGen), and CEP-701 (Cephalon). Each of these is a potent inhibitor of FLT3 kinase activity, and activation of downstream effectors such as RAS/MAPK and STAT5, with a cellular IC50 in the mid-nanomolar range. Although these early stage trials are ongoing, preliminary data indicates that FLT3 inhibitors have activity in reducing blast percentage in relapsed AML patients that have FLT3 activating mutations.

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Results of the ALLG's APML3 Trial of ATRA and Intensive Idarubicin with Molecular Monitoring in Acute Promyelocytic Leukaemia (APL)

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Objective: The impressive response to induction with ATRA plus idarubicin (IDA) in previously untreated APL reported by GIMEMA and PETHEMA prompted the ALLG to evaluate substitution of a second cycle of IDA in place of conventional consolidation. Methods: ATRA 45mg/m^2/d was initiated in 101 eligible patients [age ≥ 18 yrs, t(15;17) and/or PML-RARa +ve] and continued until complete remission (CR). Prednisone 50mg/d was added for leukocytosis (WCC > 10x10^9/L) and/or signs of ATRA syndrome. IDA #1 (12mg/m^2) was given on days 2, 4, 6, and 8, and a second identical cycle (IDA #2) was administered after haemopoietic recovery. All patients in haematological and cytogenetic CR after IDA #2 received 3 cycles of intermittent ATRA, 45mg/m^2/d for 14 days every 28 days. Protocol-defined molecular monitoring for PML-RARa transcripts was employed from the outset, and major endpoints included molecular relapse, haematological/cytogenetic relapse, and death. For patients in CR, detection of molecular relapse mandated reintroduction of therapy. After the first 32 patients, the protocol was amended to include 2 years of maintenance (ATRA 45mg/m^2/d for 14 days every 3 months, oral methotrexate 15mg/m^2/week, and 6-mercaptopurine 90mg/m^2/d). Results: Medians (ranges) for age, WCC and platelets were 41.6yrs (19.4-73.0), 2.3x10^9/L (0.4-106.6) and 25x10^9/L (4-180) respectively. PML breakpoint analysis identified 52 as bcr-1, 11 as bcr-2 (confirmed by sequencing), and 34 as bcr-3. Four patients lacked initial molecular samples but were t(15;17) positive. The proportion of patients still PML-RARa positive after IDA #1, IDA #2 and intermittent ATRA was 69%, 7% and 0% respectively. Eight patients (8%) died within 30 days, 2 patients (2%) were withdrawn with resistant leukaemia, and the remainder (90%) achieved CR. Maintenance therapy significantly increased molecular relapse free survival from 49% to 62% at 3 years ($p<0.03$). Actuarial overall survival (OS) remains outstanding (88% at 5 years). The most significant adverse prognostic factor was an initial WCC ≥ 2.5x10^9/L, which correlated strongly with early death ($p<0.002$) and with OS ($p=0.0005$). Despite the relatively high molecular relapse rate, OS of relapsed patients is 95% at 4.5 years, and contrasts dramatically with survival of comparable relapsed patients initially treated according to PETHEMA's LPA96 and LPA99 protocols (39% survival at 2 years). Conclusion: The APML3 protocol achieved haematological and molecular remission in the vast majority of patients. Freedom from molecular relapse was enhanced by maintenance therapy, and intervention following early detection of molecular relapse enabled successful salvage and contributed to excellent overall survival.

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Mutations within the Protein Z-Dependent Protease Inhibitor (ZPI) Gene are associated with Venous Thromboembolic Disease: a new form of thrombophilia

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ZPI is a recently characterised serpin that has inhibitory activity against factors Xa (in the presence of protein Z) and XIa. Deficiency of ZPI is predicted to enhance coagulation and may be a risk factor for venous thrombosis. To test this hypothesis, we compared the incidence of mutations in the ZPI gene in 149 patients with a history of DVT or PE, with the incidence of the same mutations in 150 healthy controls. dHPLC was used to screen for mutations within the coding region of the ZPI gene. Five PCR products were produced for each patient. Heteroduplex analysis was performed on a WAVE DNA Fragment Analysis System. Once a heteroduplex mismatch was identified, the PCR product was sequenced directly. We identified 16 mutations/polymorphisms within the coding region of the ZPI gene. Two mutations were identified that would be expected to result in the loss of ZPI activity and could result in a thrombotic phenotype. These mutations produced stop codons at arginine 67 and tryptophan 303 and were found in eight patients with thrombosis and two control subjects. Two further non-conservative mutations were identified in two other thrombosis patients. These resulted in a serine to tyrosine (codon 122) and a phenylalanine to leucine (codon 124) change in the region homologous to the D helix of other heparin activated serpins. These changes, involving bulky aromatic amino acid residues, have the potential to disrupt the structure and function of the D helix. We identified a further 12 mutations/polymorphisms (*C454G*, *C574T*, A603G, A647G, *G752A*, A947T, C1276T, G1277A, G1438A, A1617C, *G1789T* and C1811T (italics: not previously described)). Two mutations ((A603G) Lys25Arg and (A647G) Ser40Gly) flank the acidic region of the N terminus of ZPI. This region is homologous to that found in Heparin cofactor II and is thought to play a role in the interaction of HepCoII with glycosaminoglycans and thrombin. Mutations at these sites could potentially alter function. Allelic frequencies for A603G and A647G were calculated from 75 thrombosis patients as 0.8/0.2 and 0.57/0.43 respectively, which were not significantly different from a group of 75 controls. We have identified mutations causing stop codons, which will result in loss of ZPI function, in 5.4% of patients who present with venous thrombosis before the age of 60 years, compared with an incidence of only 1.3% in controls. These results support an association between ZPI deficiency and venous thrombosis. We propose that ZPI deficiency is potentially a new form of thrombophilia.

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Role of Thrombospondin-1 in the Control of von Willebrand Factor Multimer Size in Mice

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Plasma von Willebrand factor (VWF) is a multimeric glycoprotein from endothelial cells and platelets that mediates adhesion of platelets to sites of vascular injury. In the shear force of flowing blood, however, only the ultra large VWF multimers (ULVWF) are effective in capturing platelets. The multimeric size of VWF can be controlled by proteolysis at the Tyr^{842}-Met^{843} peptide bond by ADAMTS13 or cleavage of the disulfide bonds that hold VWF multimers

together by thrombospondin-1 (TSP-1). A severe deficiency of ADAMTS13 is associated with thrombotic thrombocytopenic purpura, a disorder characterized by the persistence of ULVWF multimers in plasma and the thrombotic occlusion of arterioles. To clarify the role of TSP-1 in the control of VWF multimer size in vivo, we have studied the vWF multimer pattern in TSP-1 null mice. Surprisingly, the average multimer size of plasma VWF in TSP-1 null mice was significantly smaller than in wild type mice. In addition, the multimer size of VWF released from endothelium in vivo was reduced more rapidly in TSP-1 null mice than in wild type mice. These findings indicate that TSP-1 inhibits the activity of ADAMTS13 in vivo. TSP-1, unlike ADAMTS13, is stored in platelet α-granules and is released upon platelet activation. Accordingly, platelet VWF multimer size was reduced upon lysis or activation of human and wild type murine platelets but not TSP-1 null murine platelets. This difference was reflected in a significantly faster rate of shear-induced aggregation of the TSP-1 null platelets. These findings indicate that TSP-1 influences plasma and platelet VWF multimeric size differently and may be more relevant for control of the platelet VWF pool.

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Early Measurement of *BCR-ABL* in Imatinib-treated Patients with De-novo chronic Phase CML Identifies 2 Distinct Groups; 1) patients who will achieve stable major molecular remissions and 2) patients with a high risk of progression

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We analysed molecular responses in 55 newly diagnosed patients with chronic phase CML enrolled in a phase 3 study (the IRIS trial) comparing imatinib to interferon-alfa plus cytarabine (IFN+AraC). *BCR-ABL* blood levels were measured by real-time quantitative RT-PCR. The median *BCR-ABL* levels were significantly lower for the imatinib-treated patients up to 18 months (P<0.0001), beyond which time most had ceased IFN+AraC. The median levels for imatinib-treated patients continued to decrease and had not reached a plateau by 24 months. Twenty-four of 26 patients remaining on imatinib at that time still had detectable levels of *BCR-ABL*. All 27 IFN+AraC-treated patients ceased therapy and 24 crossed-over to imatinib at a median of 1.3 years from diagnosis. Only 6 patients did not fail IFN+AraC but they all chose to discontinue and cross-over to imatinib when a protocol amendment allowed. Once imatinib commenced, the median *BCR-ABL* levels were not significantly different to first-line imatinib-treated patients for the equivalent time on imatinib, up to 12 months. It has been reported that a 3-log reduction of *BCR-ABL* (major molecular response (MMR)) is associated with a very low incidence of progression. We determined the prognostic significance of early measurements in imatinib-treated patients in terms of achievement of a MMR or subsequent progression, defined by haematologic, cytogenetic or quantitative PCR criteria. Kaplan Meier analysis estimated that 100% of patients with a 2-log reduction by 3 months will achieve a MMR by 24 months. Those who achieve a 2-log reduction at 6 months have an 86% probability of a MMR. Seventeen patients with a MMR were tested beyond 24 months (median 30, range 27 to 36) and all have ongoing MMR. Nine patients failed to achieve a 2-log reduction by 6 months. None subsequently achieved a MMR and 5 of 9 (56%) progressed. This was significantly higher than those with a 2-log reduction by 6 months where 1 of 26 (3.8%) progressed (P=0.002). We also evaluated 49 imatinib-treated patients for *BCR-ABL* kinase domain mutations, which are highly

associated with resistance. No mutations were detected in first-line imatinib-treated patients, including 4 who progressed. Two cross-over patients had mutations after 20 months of imatinib and both progressed. In conclusion, first and second-line imatinib-treated patients had similar profound reductions in *BCR-ABL*, which significantly exceeded those of IFN+AraC-treated patients. *BCR-ABL* measurement early in imatinib therapy is highly predictive of subsequent achievement of significant reductions and identifies patients at an increased risk of relapse.

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Isolation and Expansion of Effector Memory T Lymphocytes: potential for restoration of immunity following allogeneic stem cell transplantation

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Aim: Isolation of T lymphocytes that have the capacity to respond to viral antigens, but have a reduced potential to cause graft-versus-host disease (GVHD) may be useful in restoring virus-specific immunity in allogeneic stem cell transplant (SCT) recipients. Two basic T cell subsets exist, naïve and antigen "experienced T lymphocytes. These subsets differ in T cell receptor diversity as well as in the ability to home to secondary lymphoid organs and encounter alloantigens and therefore may be different in their capacity to cause GVHD and respond to viral infection. Therefore, we investigated memory and naïve T cells isolated on the basis of CD45RA and CD62L expression for their ability to respond to allogeneic and viral antigen stimulation. Adoptive transfer of memory T cells may allow restoration of immunity following SCT while minimizing GVHD. Methods: PBMC were isolated from normal donors and labelled with CD62L and CD45RA monoclonal antibodies. The cells were then sorted by flow cytometry into $CD62L^+CD45RA^+$ (naïve, T_N), $CD62L^+CD45RA^-$ (central memory, T_{CM}), $CD62L^-CD45RA^-$ (effector memory, T_{EM}), $CD62L^-CD45RA^+$ (terminal effector, T_T) cell populations. Purified cell populations were then stimulated with anti-CD3 antibody (OKT3) and expanded in media containing IL-2. Cells were then analysed for their ability to recognize allogeneic antigens in proliferation assays and for the presence of CMV-specific CTL by tetramer staining. Cells were also analysed for their capacity to expand after selection. Results: PBMC labelled with CD62L and CD45RA antibodies showed 4 distinct cell populations. T_N, T_{CM}, T_{EM} and T_N lymphocytes were easily selected and purified to >95% by flow sorting. Thymidine proliferation assays indicated that T_{EM} cells had a reduced capacity to proliferate in response to HLA-mismatched and HLA-matched allogeneic stimulator cells compared to T_N. T_{EM} retained their ability to expand in vitro when stimulated with OKT3 and IL-2 (30-fold expansion in 2 weeks). T_{EM} also retained functional CMV-specific CTL (as measured by tetramer) which were slightly enriched in T_{EM} populations and expanded in response to CMV peptide stimulation. T_{EM} were primarily $CD8^+$ (>70%). After expansion, T_{EM} were primarily $CD62L^-$ $CD45RA^-$, however, some $CD62L^+$ $CD45RA^-$ cells were present indicating the generation of T_{CM} lymphocytes. Conclusion: Antigen "experienced" T_{EM} lymphocytes retain the ability to recognize and proliferate in response to viral antigen, but have a reduced capacity to recognize allogeneic antigens. Adoptive transfer of T_{EM} cells following SCT may restore immunity to infectious pathogens while limiting GVHD.

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Developing an Immunocompetent Mouse Leukemia/Lymphoma Model for Testing Cytotoxic Drug Activity on Bcl-2 Overexpressing Tumors

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Objectives: An important step in the development of new cytotoxic agents is to test their efficacy when administered alone or in combination with standard chemotherapy using animal models. To develop such a system we used aggressive B cell leukemia/lymphomas which arose spontaneously in transgenic *Eμ-myc* or bi-transgenic *Eμ-myc-bcl*-2 mice and studied their natural history after transplantation into immunocompetent C57Bl/6/SJL mice. We then studied the response of these transplanted malignancies to chemotherapy by assessing the long term survival of transplanted mice. Methods: Cryopreserved malignant lymphoid tumors from transgenic *Eμ-myc* or bi-transgenic *Eμ-myc-bcl*-2 mice were resuspended at a concentration 10^6 viable cells per 100μl. The tumor suspension was injected intraperitoneally into healthy 6-8 week old male C57Bl/6 mice (average weight 21-23 grams). When the mice were deemed sick, a peripheral blood WCC and whole spleen weight was determined. In chemotherapy response experiments, cyclophosphamide was injected intraperitoneally at doses ranging between 10-150 mg/kg. Kaplan-Meier survival analysis was performed using the GraphPad Prism statistical package. Results: After injection of *Eμ-myc* tumor cells into C57Bl/6 mice, a leukemic syndrome and death resulted with elevated peripheral blood counts (40 x 10^3 cells/μL compared to a normal peripheral blood count of ~3-7 x 10^3 cells/μL) by day 17 (Table 1). Even though mice injected with tumor cells derived from an *Eμ-myc* / *Eμ-bcl*-2 bitransgenic mouse lived longer (death by day 26), there was a greatly elevated peripheral blood count in these mice (~500 x 10^3 cells/μL). Both tumors responded to a single dose of cyclophosphamide intraperitoneally (10-150mg/kg) in a dose-dependent manner. The major finding was that in the *Eμ-myc–bcl*-2 group there were almost no long-term survivors (by day 100) due to documented leukemic relapse despite the highest dose of cyclophosphamide. In contrast, in the *Eμ-myc* group, cyclophosphamide doses of 50mg/kg and over effectively "cured" these animals of disease.

Table 1 Natural history of mice injected with *Eμ-myc* and *Eμ-myc bcl*-2 tumors

Tumor	*Eμ-myc* (n=12)	*Eμ-myc–bcl*-2 (n=14)	Normal mice after 100 days (n=6)
% mice dying of leukemia/lymphoma	100%	100%	0%
Time to death (days);95% CI	16.7 (15.1-18.3)	26.2 (23.4-31)	-
WCC (10^3μl); 95% CI	42.0 (33.7-50.3)	464.3 (410-518)	7.9 (10.1-5.8)
Spleen weight (mg); 95% CI	503 (454-552)	443 (422-464)	105 (94-116)

Discussion: In this study we have developed a mouse model for studying the in vivo effects of chemotherapy in immunocompetent mice using transplantable Eμ-myc and Eμ-myc–bcl-2 tumors which cause disseminated lymphoma and leukemia. This system avoids the confounding effects of malignancy developing in the setting of immunodeficiency in the SCID mouse model. Furthermore, the marked chemoresistance displayed by Eμ-myc-bcl-2 tumors will provide a platform for studying new drugs on a leukaemia refractory to standard chemotherapy. The

measures of peripheral WCC and spleen weight are low cost and reliable endpoints for disease presence and response. In conclusion, transplantable Eμ-myc and Eμ-myc-bcl-2 tumors is an effective system for the testing of new anticancer therapies.

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Anti-CD20 Antibodies Pre- and Post- Transplantation for NHL

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Ritixumab is widely used in Australia for patients with relapsed CD20+ NHL, many of whom proceed to autografting. This presentation will review the literature on the effects of rituximab on:

1. progenitor cell mobilisation
2. in-vivo purging of PBSC in follicular and mantle cell NHL
3. early engraftment and subsequent immune reconstitution

The potential benefits and toxicities of rituximab in the adjuvant setting of minimal residual disease post-autograft and allograft will also be discussed. In addition, the results of a pilot Australian study of rituximab after autografting for MHL will be presented.

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Basic Science of the Immune Response

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Animals and their pathogens have co-existed for a long time. During that time elaborate mechanisms have developed that allow pathogens to recognise and attack the host and for the host to counter the pathogens attack. Initially, innate immune mechanisms that recognised common bacterial motifs (such as lipopolysaccharide and CpG) developed. Using these primitive systems as building blocks more sophisticated specific immune effectors have evolved, along with mechanisms that allow fine tuning of recognition and regulation of immune response. An important aspect of specific immunity is that targets used for recognition of pathogens (by CD4 T-cells) is distinct from the targets used by the specific effector cells (CD8 T-cells and antibody producing cells). This separation makes it difficult for pathogens (and researchers of the immune response) to determine how they are being recognised, thus making attempts to mutate attacked targets futile for long term immune avoidance. Despite the obvious advantages of a specific immune system, innate immunity is still critical in sensing when the body is under attack. It senses when pathogens are dangerous and upregulates the “security level” in the body, lowering the threshold for triggering a specific immune response. It also enhances the elimination of pathogens by recruitment of non-specific effector cells to sites targeted by specific effectors. Understanding the cross-talk of innate and specific immunity is providing insight into pathological immune responses and is important in harnessing the immune response for therapeutic uses.

Inhibitors: bench to bedside - the clinical challenge

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New proposed strategies for the prevention and eradication of inhibitors are emerging from the fields of Immunology and Biochemistry. The perspective of the clinician who is caring for the pediatric and adult patient suffering from the complications of high titer inhibitor development, is that the new therapeutic paradigms cannot be development quickly enough. However, the need for these therapies and their role in inhibitor prevention and eradication must be examined within the context of our current understanding of inhibitor development and the present landscape of immune tolerance. Immune Tolerance / Inhibitor Eradication: Since the seminal observation made by Professor Hans Brackmann over 25 years ago, the mainstay of inhibitor eradication therapy has been the therapy known as immune tolerance. Long-term regular infusions of FVIII/FIX at varying doses and using different dosing regimens induces tolerance in 50 - 90% of congenital hemophilia A patients with high titer inhibitors. The immunological process through which it works is still unclear, but will be further studied in conjuction with the international prospective study of immune tolerance which begun in July 2002. Although considered to be a reasonably successful strategy, immune tolerance is unpredictable in its course and outcome; requires a considerable time commitment from patient/parent and physician; can usually only be accomplished at great medical expense; and requires excellent venous access that can frequently only be secured through the use of central venous catheters. These catheters are themselves associated with a high complication rate. Consequently, other interventions which can either replace or increase the cost-efficacy of immune tolerance are immediately welcome. Despite a relative lack of success with immune modulatory therapy in the past, the rationale for manipulation of the host immune response arises from the immune tolerance outcome predictor data identified through the international immune tolerance database in the 1990's. From these registries, we learned that the nature of the inhibitor and certain characteristics of the host response itself most strongly predicted outcome with immune tolerance (IT) therapy. Good outcome predictors included 1) a low pre IT inhibitor titer; 2) a low historical pre IT peak titer and 3) a low peak titer response to IT. Consequently, as new knowledge about T and B cell interaction emerges, immune strategies can potentially be specifically targeted to favorably modulate the host response. Specific applications will be further discussed within the context of the information provided by the other speakers. Inhibitor Prevention / The Ultimate Strategy: Undoubtedly, the prevention of inhibitor development is the ultimate sought-after strategy. However, to accomplish this successfully will require detailed knowledge about the mechanism of inhibitor development. The mechanistic theories of inhibitor development in congenital hemophilia A have already been discussed from the very important perspective of FVIII antigenicity and immunogenicity as well as the host immune response (or lack thereof) to FVIII exposure. The proposed nature of these interventions will be discussed by the conference participants. However, even these therapies must be viewed within the context of other known mediators of inhibitor

development which may alter the host response to these efforts. For instance, through previous and ongoing investigations by many in the field, the influence of hemophilia and immune system genetics on inhibitor development is highly significant. Further elucidation of its role in antigen presentation and subsequent T and B cell interaction will be crucial to our understanding of the anticipated heterogenicity in the host response to any immune manipulation. Furthermore, clinical experience has demonstrated that the circumstances surrounding patient exposure to clotting factor may also play a large role in inhibitor development and, consequently, prevention. These include 1) age at first exposure; 2) dose of clotting factor used; 3) intensity of exposure over a defined period of time; 4) episodic vs. prophylactic factor administration and 5) inflammatory co-stimuli at the time of factor exposure. Even the role of the type of product administered has not been resolved to uniform satisfaction. Consequently, prospective clinical observation and data collection will continue to play a key role in the solution of this problem. Finally, the ultimate question is whether or not inhibitors can ever be prevented while we still rely on extracellular protein processing by the immune system. Inhibitor prevention may ultimately only be accomplished through gene transfer and intracellular protein expression. Indeed, the problem of inhibitor prevention and eradication is a complex one that will mandate a concerted multidisciplinary investigative approach to its resolution.

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Genetics of Platelet Count Variability in Inbred Mice

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Aim: Thrombocytopenia is a frequent complication of cancer therapy. Pharmacological alleviation of severe thrombocytopenia remains clinically infeasible in those patients receiving myeloablative therapy. It has previously been shown that steady-state platelet count in mice and human is largely under genetic control. To improve understanding of the biology of platelet count regulation, we aim to identify genes responsible for vastly different platelet counts in two healthy inbred mouse strains, CBA/CaH and IQS5. The mean platelet count for IQS5 (1,100 x 10^9/L), is over five phenotypic standard deviations higher than for CBA (600 x 10^9/L). Methods: A genetic linkage-based approach was employed to identify those regions of the genome associated with extreme platelet phenotypes. An IQS5 x CBA F_2 intercross resource of 1,126 animals was bred to scan the genome for quantitative trait loci (QTL) determining the differences in platelet count. Those animals with the highest and lowest platelet counts were selected for genotyping. One hundred and one informative microsatellite markers were selected to cover the mouse genome at an average resolution of 15 centiMorgans. Linkage analyses were performed with the publicly available software package MapMaker and MapMaker/QT. Results: QTL were identified on MMU1 (LOD 6.8, $p< 0.0005$ chromosome-wide) and MMU11 (LOD 11.2, $p<0.0005$) with three other suggestive loci on MMU7, 13 and 17. It is noteworthy that no *a priori* candidate gene for platelet production, including *IL-11*, thrombopoietin (*Tpo*) and its receptor *c-Mpl* among others, corresponded to the QTL identified in this mouse cross. In order to improve the resolution of this linkage-based approach, an Advanced Intercross Line (AIL) mouse resource is being bred to help further refine the locations of these QTL. In addition, Comparative

Genome Hybridization using microarray techniques will be applied to the parental mouse strains as well as a selection of AIL progeny displaying extreme platelet count phenotypes. By combining the results from both approaches, it is expected that each QTL can be localised to 2-3 centiMorgan intervals, and within those intervals the differentially expressed genes relevant to platelet count variability will be identified. Conclusion: We have used a genetic linkage method to identify genetic factors that regulate platelet count. Two novel QTL for platelet count were found in inbred strains of laboratory mice. The QTL do not appear to correspond to known genes that regulate platelet production, confirming an evolving view that the regulation of platelet count may involve multiple hierarchies of control.

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Human Telomerase Reverse Transcriptase (hTERT) Promotes Survival of Factor-Dependent Human TF-1 Progenitor Cells

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To overcome the problems of limited stem cell number and delayed reconstitution following transplantation using umbilical cord blood (UCB), methods need to be developed to allow the *ex-vivo* growth of both haematopoietic stem cells (HSCs) and mature myeloid cells derived from UCB. We are interested in the expression / activation of telomerase as a molecular approach for this purpose. Telomerase is a complex ribonucleoprotein enzyme that is responsible for maintaining telomere length in germ cells and HSCs. The catalytic subunit of telomerase, hTERT, plays a fundamental role in telomere preservation and cell proliferation. We have previously shown that retroviral expression of hTERT in human UCB $CD34^+$ cells leads to an enhanced survival of mature haematopoietic cells[1]. While this pro-survival effect of hTERT was surprising, there is now evidence from others that hTERT can play a pro-survival role independent of telomerase enzymatic activity[2]. The mechanism for this effect of hTERT in haematopoietic cells is not known. The aim of the current study was to directly examine the role of hTERT on haematopoietic progenitor cell survival. A retrovirus co-expressing hTERT and GFP was used to transduce cytokine-dependent, human leukaemia progenitor TF-1 cells. Retrovirus containing GFP alone was used as control. Clonal cell lines were derived and the effect of over-expression of hTERT on survival and cell death in the absence of growth factor was examined. Flow-FISH analysis revealed that telomeres of hTERT-transduced cells were more than three-fold longer than that of control cells (18kb vs. 5kb), confirming expression of catalytically active hTERT. Following withdrawal of cytokine the majority of control cells died within 7 days while the number of hTERT-transduced cells remained relatively constant; overexpression of hTERT allowed TF-1 cells to survive in the absence of cytokine, but did not increase the replicative potential. Trypan-blue and Annexin-V staining in these experiments revealed 50% fewer dead cells in hTERT-transduced cultures compared to control, demonstrating the ability of hTERT to protect cells from death induced by cytokine deprivation. Preliminary experiments suggest an effect of hTERT on the cell-cycle status of TF-1 cells, where in the absence of cytokine cells remain predominantly in G0/G1 with a delayed response to the addition of IL-3. This study demonstrates a novel pro-survival role for hTERT in human haematopoietic progenitor cells, lending support to our hypothesis that modulation of hTERT activity may be of potential benefit for the *ex-vivo* manipulation of human haematopoietic cells.

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Molecular Analysis of a Novel t(5;10) Translocation in a Case of Acute Lymphoblastic Leukaemia

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The molecular dissection of structural chromosome alterations has identified many genes important to the cause or progression of leukaemia. Aside from their diagnostic and prognostic value, the identification of these genes is providing a wealth of new information that is critical to our current understanding of the molecular pathways of leukaemogenesis. This information is a necessary precedent to the design of better treatments. However, the fact remains that the genetic basis of many ALL cases remains unknown. It is now well known that chromosomal translocations typically confer transforming capabilities via the activation of proto-oncogenes. Novel translocations may therefore mark the location of genes potentially rearranged more frequently by cryptic submicroscopic mechanisms in leukaemia patients. To this end, we have targeted the breakpoints of a novel t(5;10)(q22;q24) translocation found as the sole abnormality in the leukaemic cells of a 59 year old male patient diagnosed with acute lymphoblastic leukaemia (ALL). Chromosome band 10q24 is a gene-rich domain and host to a number of cancer, developmental, and neurological genes. Recurring translocations, deletions and mutations involving this chromosome band have been observed in different human cancers and other disease conditions, but the precise identification of breakpoint sites, and detailed characterisation of the genetic basis and mechanisms which underlie many of these rearrangements has yet to be resolved. Studies using a combination of chromosome and interphase fluorescent in situ hybridisation (FISH), bacterial artificial chromosome (BAC) end sequencing and genomic database analyses, have established a definitive genetic map of selected genes and clones in the 10q24 breakpoint region. Until recently, this region has shown considerable volatility through time in published works of scientific journals, within different builds of the same international genomic database, and across the differently constructed databases. These mapping efforts facilitated the selection of yeast artificial chromosome (YAC) clones for FISH screening on patient metaphase chromosomes and enabled the identification of a YAC clone and subsequently a plasmid artificial chromosome (PAC) clone containing the 10q24 breakpoint region. Further Southern hybridisation studies using genomic probes for the analysis of patient DNA resolved a model of the 10q24 breakpoint region. By these positional cloning approaches and Southern analyses, the 10q24 breakpoint site has been mapped within a genomic region containing uncharacterised expressed transcripts in a domain not previously associated with leukaemia. Furthermore, this domain maps within a small cluster of lymphocyte development genes.

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Phenotypic and Functional Changes in Cord Blood Stem Cell Progeny after Cytokine Activation

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Cytokine-mediated expansion of haematopoietic stem cells has been successfully used to increase the total cell number and facilitate engraftment post transplantation. The effects of expansion on cord blood (CB) $CD34^+$ cells at a phenotypic, molecular and functional level are not well understood. The purpose of this study was to characterize changes in the expression of cell cycle regulatory genes, stem cell and lineage markers and adhesion molecules associated with cytokine activation of CB $CD34^+$ cells and correlate the changes with engraftment potential. MACS enriched CB $CD34^+$ cells were cultured with SCF, flt3-L and MGDF (SFM) for up to 7 days. RNA was extracted at day 0 (unexpanded cells) and cells expanded for 1, 3 and 7 days in culture. Expression of $p21^{WAF1/CIP1}$, $p27^{Kip1}$ and D-type cyclin genes were quantified by real time PCR. Unexpanded cells were found to have high $p21^{WAF1/CIP1}$ and low cyclin D1 and D3 gene expression. Expansion led to a decrease in expression of $p21^{WAF1/CIP1}$ and an initial increase in cyclin D1 and cyclin D3, but no significant changes in $p27^{Kip1}$ and cyclin D2**.** We used a high-resolution cell division tracking method to investigate the level of expression of adhesion molecules, lineage markers, cytokine receptors and engraftment potential in vivo after a defined number of cell divisions. $CD34^+$ cells were stained with CFSE, cultured with SFM for up to 7 days and analysed by flow cytometry. CD34 and Thy1 were down-regulated with successive cell divisions. c-kit expression increased in the first division, but was down-regulated thereafter. Myeloid, erythroid, megakaryocytic markers and CXCR-4 were up-regulated after expansion. L-selectin was up-regulated on day 3 but down-regulated after 7 days expansion. CD44, VLA-5 and PECAM-1 expression was high in unexpanded and expanded cells. VLA-4 and LFA-1 were up-regulated in >95% of cells on day 3. Expression of β1-integrin and Mac-1 was up-regulated after 7 days. To investigate engraftment potential, CFSE stained cells were cultured with SFM for 3 days, then sorted according to cell division and transplanted into NOD/SCID mice. BM and spleen were analysed 6 weeks later for human engraftment by flow cytometry. Human cells were detected in all mice groups, but at lower levels in mice transplanted with unsorted expanded and sorted divided cells compared with unexpanded cells. Overall, cytokine-mediated expansion of CB $CD34^+$ cells altered lineage marker, cytokine receptor, adhesion molecule expression and cell cycle gene expression, and caused a decrease in engrafting potential in the NOD/SCID mouse.

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Carboxypeptidase G2 for the Treatment of Severely Impaired Methotrexate Clearance Associated with Methotrexate-induced Renal Dysfunction

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Introduction: Methotrexate (MTX) given at high doses (≥ 1 g/m^2) is a critical component for curative therapy of a variety of malignancies, in particular kinetically aggressive disorders such as Burkitt's lymphoma. MTX clearance is highly renal-dependent. Vigorous hydration, urinary alkalinisation and avoidance of concomitant nephrotoxic agents are essential for the safe

administration of high-dose MTX. Even with these measures MTX can lead to renal dysfunction through tubular precipitation, resulting in prolonged excretion, which invariably leads to severe toxicity. Calcium folinate is an effective cytoprotectant with MTX levels of < 1 μM, but is ineffective against higher MTX concentrations. There have previously been no effective treatments for patients with significantly delayed MTX clearance and levels ≥ 1 μM.
Carboxypeptidase G2 ($CPDG_2$) is a recombinant bacterial enzyme that rapidly and selectively hydrolyzes MTX to the inactive and rapidly cleared metabolites 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA) and glutamic acid. $CPDG_2$ is available through the United States National Cancer Institute (NCI). Stock of $CPDG_2$ is held at the Peter MacCallum Cancer Centre, Melbourne, for distribution upon authorisation by the NCI. Methods: We have collated the data from all patients treated at the Peter MacCallum Cancer Centre who received $CPDG_2$. NCI approval requires risk of life-threatening toxicity following MTX administration secondary to delayed excretion, defined as plasma MTX ≥10μM more than 42 hours after commencement of MTX infusion or serum creatinine ≥ 1.5 x ULN and evidence of delayed MTX excretion (plasma MTX ≥ 2 SD above mean) at least 12 hours following MTX administration. After NCI approval patients were treated with $CPDG_2$ 50units/kg (maximum 2000units) intravenously over 5 minutes, with MTX plasma levels measured post dose. Patients with a plasma MTX concentration ≥ 100μM could receive a second dose 48 hours later. High dose calcium folinate (250mg IV every three to six hours), intensive hydration and urinary alkalinisation were continued during the treatment period. Results: Four patients each received a single dose of $CPDG_2$ between September 2002 and June 2003. No patients required a second dose.

		Creatinine (μM/L)		Plasma MTX levels (μM)			
Patient	MTX dose (g/m2)	Baseline (pre – MTX)	Peak	Highest MTX level	Pre $CPDG_2$	Post $CPDG_2$	Time to 0.05μM from start infusion*
1	1	0.07	0.31	7.29	4.74	0.64	320 hrs
2	1	0.08	0.25	7.94	1.42	0.42	131 hrs
3	1	0.10	0.23	11.79	10.3	0.87	199 hrs
4	3	0.10	0.18	90	50	0.93	288 hrs

* *The inactive metabolite DAMPA cross-reacts in MTX immunoassays, thus the cited results overestimate true MTX levels post $CPDG_2$.*

Grade 4 haematological toxicity was seen in one patient. Non-haematological toxicity of grade 2 mucositis was seen in another patient and grade 2 renal toxicity in all patients. No patients experienced any toxicity directly related to the $CPDG_2$. Conclusion: In patients with delayed MTX clearance $CPDG_2$ had a dramatic effect in rapidly reducing MTX plasma levels and resulted in the avoidance of severe MTX toxicity.

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Opportunities for Improvement in the Care of Asplenic Patients

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Introduction: Overwhelming post splenectomy infection (OPSI) is a serious and well recognized complication of splenectomy. Historically, strategies to reduce the risk of OPSI via education, vaccination and antibiotic prophylaxis have been employed in an ad hoc fashion. More recently

there has been an effort to improve the uniformity and standard of care for asplenic patients by the publication of consensus guidelines. Aims: To perform a baseline analysis of management of splenectomized patients at our institution (a tertiary referral teaching hospital serving as 1 of 2 Victorian state treatment centres for adult road trauma) and to advise on potential strategies to improve compliance with the current recommendations for vaccination published in the Immunization Handbook of Australia[1] and with guidelines published by the Working Party of the British Committee for Standards in Haematology Clinical Task Force[2]. Methods: Patients who underwent splenectomy between 1/7/1998 and 30/6/2001 were identified by Clinical Discharge coding information. 60 patient records have been reviewed to date for documented evidence of vaccination, prescription of antibiotic prophylaxis on discharge from hospital, and provision of education. Results: 53% of splenectomies were performed for trauma, 20% for haematolgical conditions, 12% for intra-abdominal emergencies/operative misadventure, 10% for intra-abdominal malignancy and 3% for miscellaneous conditions. 6 patients died within 48 hours of admission and were not included in any subsequent analysis. 2 patients died in hospital and were excluded from discharge analysis. 53 patients (98%) received the pneumococcal, meningococcal and Haemophilus influenza type b vaccines. Recommendations for revaccination could be found only for pneumococcus and only in 3/53 patients. 1/54 patients was immunized against influenza. 48/52 (92%) patients were discharged on prophylactic antibiotics. The duration of therapy was specified in 50%, with lifelong therapy recommended in 13/48 (27%). There was documented evidence that patients had received information about the risks of asplenism in 4/52 cases (8%). Conclusion: Compliance with pneumococcal, meningococcal, and Haemophilus vaccination (98%) and commencement of antibiotic prophylaxis (92%) was better than that seen in most previously published studies[3,4,5,6]. Clearly, opportunities for improvement exist in the provision of patient education, influenza vaccination and recommendations at discharge with regards to the duration of antibiotic prophylaxis and the timing of revaccination. A registry of asplenic patients is in the process of being compiled at this hospital in the expectation that a proactive, systematic approach to the implementation of 'best practice' guidelines will translate into a reduction in OPSI.

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Recurrent dic(17;20) in MDS/AML Identified by FISH

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Deletion of the long arm of chromosome 20 is a common finding in myelodysplastic syndromes (MDS) and acute myeloid leukaemia (AML) and deletion of 17p, associated with loss of p53, is well recognized in these disorders. We recently described a series of cases of an unbalanced dic(17;20)(p11;q12) in patients with MDS and AML (Patsouris *et al*., 2002). This translocation involves loss of both 20q and 17p in a dicentric rearrangement. To identify further cases of dic(17;20) and to establish the frequency of this unbalanced translocation, we have begun screening MDS and AML cases for further examples of 17;20 rearrangements. An initial search of the database of the Victorian Cancer Cytogenetics Service identified karyotypes from 20 patients diagnosed with either MDS or AML and with apparent monosomies of chromosomes 17 and 20. Metaphases from 12 cases have been studied thus far. In each case, G-banded metaphases were captured via an image analysis system and their location carefully noted on the slide. The slide was then de-stained and fluorescence *in situ* hybridisation performed using chromosomes 17 and 20 whole chromosome painting probes. Two further examples of dic(17;20) have been found. Moreover, no case has been found to be truly monosomic for either 17 or 20 as retained portions of both chromosomes have been identified within either marker chromosomes or unbalanced translocations. There appears to be an unusually high number of dicentric chromosomes present in these complex karyotypes. Chromosomes 17 and 20 appear to have formed dicentrics not only with each other but with a variety of other chromosomes. All of these cases had a complex karyotype with 5 or more chromosome abnormalities and frequent loss of part or all of chromosomes 5 and 7, consistent with poor prognosis disease. In this setting, the deletion of various parts of the genome is well recognized. However, we have observed not only loss of parts of 17 and 20 but, intriguingly, retention of 2 copies of parts of both chromosomes. It is possible that genes critical for the maintenance of the malignant phenotype are located within these retained regions.

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Familial Mutations of the Transcription Factor RUNX1 (AML1, CBFA2) Predispose to Acute Myeloid Leukemia

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RUNX1 (AML1, CBFA2) is mutated in affected members of families with autosomal dominant thrombocytopenia and platelet dense granule storage pool deficiency. Many of those affected, usually by point mutations in one allele, are predisposed to the development of acute myeloid leukemia (AML) in adult life. The syndrome is termed *familial platelet dysfunction with predisposition to acute myeloid leukemia* or FPD/AML (OMIM 601399). The RUNX1 protein complexes with core binding factor beta (CBFB) to form a heterodimeric core binding transcription factor (CBF) that regulates many genes important in hematopoiesis. *RUNX1* was first identified as the gene on chromosome 21 that is rearranged by the translocation t(8;21)(q22;q22.12) recurrently found in the leukemic cells of patients with AML. In addition to the t(8;21), *RUNX1* is rearranged with one of several partner genes on other chromosomes by somatically acquired translocations associated with hematological malignancies. Point mutations of *RUNX1* are also found in sporadic leukemias to reinforce the important position of this gene on

the multi-step path to leukemia. In animal models, at least one functional copy of *RUNX1* is required to effect definitive embryonic hematopoiesis. Cells expressing dominant-negative mutants of RUNX1 are readily immortalised and transformed, and those RUNX1 mutants that retain CBFB binding ability may possess dominant-negative function. However, in some families there is transmitted one mutated allele of *RUNX1* with no dominant-negative function, demonstrating that simple haploinsufficiency of RUNX1 predisposes to AML and also causes a generalised hematopoietic stem cell disorder most recognisable as thrombocytopenia. Here we review clinical, cytogenetic and molecular features providing clear evidence for the causative role of RUNX1 in both sporadic and familial leukemic disorders, with a particular focus on FPD/AML. FPD/AML provides further insight into the normal function of the RUNX1 protein and its role in regulating platelet production and preventing the emergence of AML.

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Gene Expression Profiling and Candidate Gene Approach in Dominant Familial Acute Myeloid Leukemia (AML)

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Aim: Studies of large multiple generation families with predisposition to only leukemia are rare but have led to the identification of RUNX1 as the first leukemia susceptibility gene. We aim to identify leukemia-predisposing genes in small to middle-sized pedigrees with familial leukemia using a combination of large scale and high through-put approaches. We hypothesise that the expression profiles can be used to cluster families with leukemia-predisposition of similar genetic origin and that additional expression information can be used in conjunction with other genetic data (e.g. linkage) to generate a prioritized candidate gene list for mutation analysis. Methods: EBV-transformed lymphoblastic cell lines are being established from samples of affected and unaffected leukemia family members obtained by an ongoing collection effort. Expression profiles are generated using Affymetrix human U133 GeneChips. Mutation analysis on candidate genes is performed using denaturing HPLC and direct sequencing for detection of point mutations or insertions/deletions, and a combination of SNP analysis, FISH, Northern Blot and RT-PCR to detect loss of heterozygosity and/or haploinsufficiency. Results: Data from 2 middle-sized families with a similar phenotype of myelodysplastic syndrome/AML has been complemented by data from a previous selective linkage approach. Mutation analysis so far has excluded a mutation and a gross heterozygous deletion in transcription factors CBFβ (RUNX1 cofactor), E2F4, CTCF, NFATC3 and NFAT5, and in AML-associated NQO1, all situated on a 13.7Mb candidate region on chromosome 16q22 identified by candidate region linkage analysis. Depending on the parameters used, expression profiling revealed a distinct clustering of the patients of the 2 families; additional samples should add robustness to this dataset. Stringent statistical analysis identified 99 differentially expressed transcripts between cells of affected and unaffected family members, none of which mapped to 16q22, but revealed candidate regions for a microdeletion on chr2p and chr14q. Functional analysis suggested a perturbation of proliferative and apoptotic pathways in the patients. Conclusions: Using linkage alone in small to medium-sized families with dominant AML may not allow the identification of leukemia-predisposing genes. Additional expression profiling directly reveals new groups of candidate genes that will now undergo mutation analysis and may prove to be a potent approach to study smaller families with an inherited predisposition to leukemia. Identification of novel leukemia predisposition

genes is likely to afford insight into the more common sporadic occurrences of leukemia, providing tools for diagnosis and specific therapeutic strategies.

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Mitochondrial Mutations in Acute Leukaemia

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Mutations in the D loop of the mitochondrial genome were sought in 22 patients with acute myeloid leukaemia (AML) and 26 patients with acute lymphoblastic leukaemia (ALL). All patients were studied by DNA sequencing and 37 by denaturing gradient gel electrophoresis (DGGE). Comparison was made between DNA from marrow cells obtained at diagnosis, at remission, which was regarded as constitutional DNA, and, in 15 patients with ALL, at relapse. Mutations were detected at diagnosis in 7 of the patients with AML (32%) and 15 of the patients with ALL (58%). There was excellent correlation between the results of sequencing and the results of DGGE. When present in a patient, mutations were usually multiple, the number ranging from 1-14 for AML and 1-10 for ALL. The commonest mutations were C→T and T→C and there was evidence for strand bias in their occurrence. The distribution of mutations was non-random, indicating a number of hot-spots along the D loop. However, the nature and location of mutations did not suggest a common aetiology. In 5 of the 6 AML patients and 3 of the 9 patients with ALL who had mutations and had been studied by DGGE, the leukaemic band at diagnosis could still be faintly seen at remission, suggesting that the leukaemic clone was still present at a high level. Of the 9 patients with ALL who relapsed, 4 showed identical mutations at diagnosis and relapse and 5 showed a different pattern at relapse, characterised by disappearance of some mutations and, in 3 of the 5, the appearance of new mutations. Both the pattern of mutations and the DGGE results illustrated the phylogeny of subclonal evolution in leukaemia and indicated that, for at least the 5 patients in whom the mutation spectrum at relapse differed from the spectrum at diagnosis, the relapse clone was already present at diagnosis. Mixing experiments and quantification of mitochondrial genomes/cell suggested that DGGE should be able to detect a level of at least 0.5% of a minor leukaemic population bearing a mitochondrial mutation. We conclude that mitochondrial mutations are common in acute leukaemia, that at least some arise during growth of the leukaemic clone and that, by acting as clonal markers, they provide valuable information on leukaemia biology and they may be useful for detecting low levels of disease.

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Clinical Significance of Soluble CD86 Levels in Acute Myeloid Leukemia and Myelodysplastic Syndrome

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Cell surface expression of CD86 (mCD86) provides a critical costimulatory signal in immune responses. However mCD86 expression by AML blasts is a marker of poor prognosis suggesting the presence of inhibitors of this pathway. The release of soluble forms of CD86 (sCD86) by malignant cells could provide a mechanism by which tumor cells inhibit immune responses. We have previously demonstrated that (1) sCD86 can be detected at ng/ml levels in normal plasma (2) a proportion of AML patients have significantly elevated plasma sCD86 levels (3) changes in sCD86 levels reflect remission/relapse status and (4) AML blasts express sCD86 transcript. These findings led us to analyse the prognostic significance of pre treatment plasma sCD86 levels in AML (n=57) and MDS (n=40) patients. Normal donors (n=52) had a median sCD86 level of 0.74 ng/ml (range 0.22-2.3) whist a wider range of levels was observed in both MDS (median = 0.8 ng/ml, range 0.02-11.5) and AML (median =0.67, range 0.02-10.5) patients. Levels of sCD86 in AML were significantly correlated ($p<0.001$) with WBC count, B_2M and LDH levels. AML patients with elevated sCD86 levels had significantly (P=0.021) lower complete remission (CR) rates than those patients with normal levels. In multivariate analysis using sCD86 as a continuous variable and including the interaction of age and sCD86 as a variable, sCD86 was a significant prognostic factor (p=0.014) independent of cytogenetics. Further analysis demonstrated that in AML patients aged ≤60 years, but not those >60, elevated sCD86 was associated with significantly shorter survival (p=0.04). No significant associations between sCD86 levels and other clinical and laboratory parameters were observed in MDS patients and sCD86 level was not a significant prognostic factor in these patients. These results confirm that sCD86 levels are an independent prognostic factor in AML and suggest that sCD86 may have an important functional role in these patients.

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New Technology for Identifying Leukaemias

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We have developed a CD antibody microarray (Leukaemia Diagnostic Assay, LDA) which provides an extensive immunophenotype of leukocytes from a single analysis. Leukocytes are purified by Histopaque centrifugation and $3x10^6$ cells are incubated over a microarray of CD antibodies on a microscope slide. After 30 min, unbound cells are washed off and the cells captured by the immobilized antibodies of the microarray form a dot pattern which is the immunophenotype of the leukocytes. To advance and commercialize this technology, we have formed a spin-off company called Medsaic. A scanner has been developed which produces a digital file of the dot pattern in less than 10 seconds without staining the captured cells. Software has also been developed which takes the image file of the pattern of captured cells, puts contour

lines for intensity around the dots, integrates the volumes of the dots, presents the data as a histogram and quantitative table, and has a pattern recognition algorithm for diagnosing leukaemias. The program has a library of consensus immunophenotypes for different leukaemias and the dot pattern on the LDA for the unknown leukaemia is matched with each consensus pattern, a probability score is assigned which ranks likely diagnoses. This new technology for identifying leukaemias costs much less than the established technique of flow cytometry, requires less training to perform, and identifies many more CD antigens. Thus, more laboratories could access rapid and comprehensive leukaemia diagnoses. Once cells are captured in patterns on the LDA, they are fixed with formaldehyde and the slides can be dried and stored as an archive for later analysis, or mailed to a laboratory for scanning and diagnosis. The slides are rehydrated by brief immersion in phosphate-buffered saline prior to scanning at a later date. The use of extensive consensus patterns for the immunophenotypes of particular leukaemias should increase the certainty of the diagnosis, and possibly the ability to identify sub-groups of prognostic significance. These extensive immunophenotypes can be analyzed statistically as a Principal Component Plot which shows segregation of disease types in component space. Samples which cluster in such a plot are related and may represent unique leukaemia sub-types. With an extensive database of patient samples, sub-types of some leukaemias such as acute myeloid leukaemia may be reclassified.

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Immunophenotypic Analysis of B-cell Chronic Lymphocytic Leukaemia Using a Novel CD Antibody Microarray

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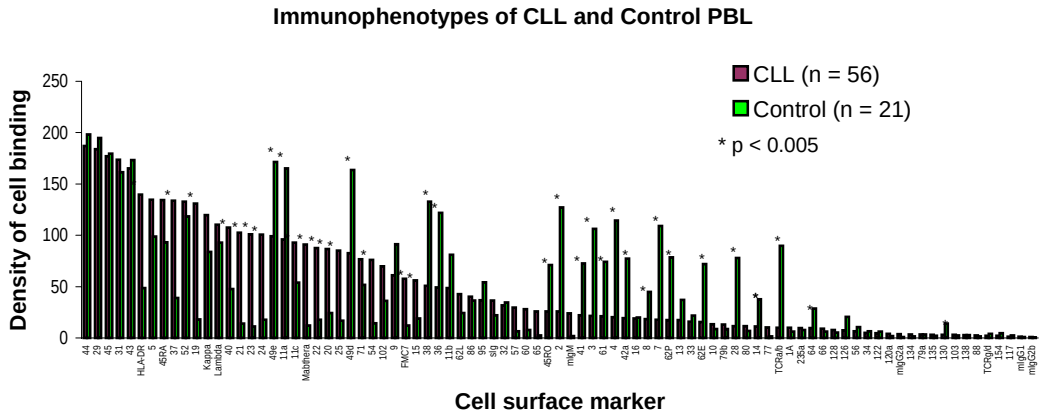
We have developed a CD antibody microarray (Leukaemia Diagnostic Assay, LDA) which provides an extensive immunophenotype (currently 88 CD antigens) by capturing leukocytes with antibodies immobilized on a microscope slide. Leukocytes expressing a specific CD antigen are captured by the corresponding antibody dots (~10 nL) and the resulting dot pattern defines the immunophenotype for that leukaemia. The binding density of cells on a dot is approximately proportional to the density of cells positive for that CD antigen in the sample suspension. Using the LDA, we have determined the immunophenotypes of B-cell Chronic Lymphocytic Leukaemias (B-CLL) and other leukaemias from patients.



Use of the LDA clearly defines the antigenic profile of B-CLL with expression of pan-leukocyte antigens (CD44 and CD45), pan-B cell antigens (CD19, CD20, HLA-DR) usually determined by

flow cytometry, but also CD21, CD24, CD37 and other B-lineage antigens, adhesion markers and others not typically analyzed. "Aberrant" or leukaemia specific phenotypic phenomena, such as expression of CD5 or CD10, are detected in the same analysis using the LDA. A frequency distribution for expression of CD antigens on leukocytes from 56 CLL patients is shown above. The LDA includes antibodies for CD20, CD22 and CD52, potential targets for the therapeutic antibodies rituximab, epratuzumab, and Campath-1H. Studies on serial dilution of B-CLL cells in purified normal lymphocyte populations show the LDA is capable of detecting clonal populations as low as $2.0x10^9$ cells/L, i.e. below the formal diagnostic (NCI) criteria. The extensive data obtained in a single analysis of patient blood with the LDA should provide diagnostic, prognostic and therapeutic information valuable for the management of B-CLL and other leukaemias.

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A Loss-Of-Function Polymorphism in the P2X7 Receptor Gene is Associated with Long Lymphocyte Doubling Time in CLL

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B-cell chronic lymphocytic leukaemia (CLL) is characterised by a continuous accumulation of a CD5+ CD23+ B-cell clone with a highly variable clinical course. The presence or absence of somatic mutations in the variable region of the B-cell receptor heavy chain genes (IgVH) has been proposed to divide CLL into clinically distinct subsets. Patients with unmutated IgVH genes display a progressive clinical course with short overall survival whereas those that are mutated IgVH genes tend to have a more indolent clinical course. Although lymphocyte doubling time (LDT) is widely used in clinical practice, its ability to divide patients into the above prognostic groups is uncertain. P2X7 is a purinoreceptor expressed on the surface of normal and leukaemic lymphocytes. Activation of this receptor by extracellular ATP induces apoptosis and shedding of L-selectin, an adhesion molecule which regulates the rate lymphocyte recirculation. Recently we have identified two single nucleotide polymorphisms (SNP) at position 1513 and 1729 of cDNA in exon 13 of the human P2X7 receptor which abolish function of this receptor. We studied a cohort of 102 CLL patients for their 1513C polymorphism, the IgVH mutational status, as well as LDT to investigate the clinical significance of these SNPs in CLL. Of 44 patients, 36 had mutated and 15 had unmutated IgVH genes. There was no difference between the mutated and unmutated IgVH cases in the function of the P2X7 receptor measured by ATP-induced uptake of ethidium, (4999 and 4766 units respectively). Moreover, there was no difference in the frequency of the 1513C allele between the mutated and unmutated IgVH groups. As expected, LDT was generally longer in the group with mutated IgVH gene (median=1733 days) than for the unmutated group (median= 928 days) although the difference failed to reach significance (P=0.088). However, low P2X7 function and higher frequency of the loss-of-function polymorphisms in P2X7 were associated with longer LDT. One of 27 patients with the 1513C or 1729A allele (4%) had LDT<365 days, while 11 of 54 (20%) wild-type patients had LDT<365 days (P=0.037). Patients with either the 1513C or the 1729A alleles showed a more indolent rise in lymphocyte counts, since LDT for this group was significantly longer than the wild-type patients (median = 1733 and 1155 days respectively, P=0.022). Thus,

the IgVH mutational status, the major predictor of survival, has no correlation with the P2X7 loss-of-function polymorphisms. However, these polymorphisms are strongly associated with LDT implying a role for the P2X7 receptor in regulating lymphocyte movement between the intravascular and extravascular compartments.

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The 1513A>C Polymorphism in the P2X7 Receptor and Familial Chronic Lymphocytic Leukemia

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In indolent forms of chronic lymphocytic leukemia (CLL) mature $CD5^+$ B-lymphocytes accumulate in the peripheral blood due to failure of these cells to undergo apoptosis in vivo. This is generally attributed to over-expression of bcl-2 although a failure of P2X7-mediated apoptosis has been proposed to contribute to this defect. The P2X7 receptor is a cytolytic receptor that is highly expressed on hemopoietic cells of monocytic and lymphoid lineages. On activation by extracellular ATP, this receptor opens a cation-selective channel and triggers a number of signalling events including caspase activation leading to apoptotic death of the cell and also shedding of L-selectin, an adhesion molecule that regulates the rate of lymphocyte recirculation. Several single nucleotide polymorphisms (SNP) have been described in the P2X7 gene which lead to loss-of-function, the most prevalent being the 1513 A→C SNP which has an allele frequency around 0.10 – 0.16 in normal Caucasian populations. The prevalence of this loss-of-function polymorphism has been reported to be higher in a small cohort of patients with CLL than in elderly control subjects, while the median survival of patients heterozygous for the 1513C allele is longer than wildtype 1513A patients. We have studied twenty-five patients with familial CLL in a series of 99 patients diagnosed on the basis of sustained lymphocytosis $>4.0X10^9$/L and a monoclonal population of $CD5^+$ $CD23^+$ B-lymphocytes. Familial CLL was diagnosed in 18 patients from 9 families (3 intergenerational and 6 sibling pairs). A further 7 individual patients with familial CLL were diagnosed on the basis of history and documentary information from their attending physician. Normal subjects were recruited from partners of patients and from staff with no history of hematological disease. Forty cases of histologically proven non-Hodgkins lymphoma were also studied. All patients and normals were white Caucasians to avoid ethnic bias in allele frequencies. Institutional ethics approval was obtained for the study. The frequency of the 1513C allele was significantly higher in familial CLL cases (allele frequency 0.26) than in either normals (allele frequency 0.136, P=0.025) or in non-familial CLL cases (allele frequency 0.156, P=0.040). Moreover there was no difference in the 1513C allele frequencies between normal subjects, lymphoma patients (allele frequency 0.14) and non-familial CLL patients. The earliest manifestation of CLL is an accumulation of B-lymphocytes in blood and bone marrow and it is possible that reduced function of the cytolytic P2X7 receptor plays a role in regulating lymphocyte distribution between intravascular and extra vascular spaces. Alternately this polymorphism may not be causally involved but rather in linkage disequilibrium with the transcription co-repressor gene SMRT/NCOR2, which is 3.5 Mb telomeric to the P2X7 gene. Impaired function of this tumor suppressor gene has been associated with lymphoproliferative disorders and further investigation of the role of these genes in familial CLL is warranted.

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The Majority of MCL and CLL Patients Have Elevated Plasma Levels of a Functional, Soluble Form of CD80

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Cell membrane expression of B7 molecules (CD80, CD86) by antigen presenting cells (APC) is critical for the induction of anti-tumor responses. Consequently the provision of increased B7 expression at the time of tumor antigen presentation is a central theme of current immunotherapy protocols. However, the demonstration that the malignant cells in many haematological malignancies constitutively express membrane B7 suggests that other mechanisms, in addition to cell membrane B7 expression, regulate the delivery of costimulatory signals through these molecules. The release of soluble forms of CD80 (sCD80) provides a potentially powerful mechanism by which tumor cells and/or APC can modulate anti-tumor responses. The *in vivo* presence of sCD80 has not been previously analysed. This study investigated whether circulating sCD80 is present in normal donors and/or patients with hematological malignancies. Circulating sCD80 was detected by ELISA in all normal (0.024-0.318 ng/ml, n=66) and patient (0.02-3.75 ng/ml) blood analysed. The majority of acute myeloid leukemia (13/17) and multiple myeloma (11/12) patients had normal sCD80 levels. Significantly elevated levels were detected in chronic lymphocytic leukemia (CLL, p=0.0001) and mantle cell lymphoma (MCL, p=0.0002) patients. MCL patients had the highest levels with 8/9 having levels > 0.318 ng/ml. Increased sCD80 levels in CLL were significantly associated with poor prognosis markers such as low platelet (p=0.01) and hemoglobin (p=0.002) levels, elevated WBC counts (p=0.03) and expression of CD38 (p=0.048). The immunoreactivity of the sCD80 in both normal and patient plasma was inhibited by the presence of CTLA-4-Ig, suggesting circulating sCD80 is a functional molecule. Levels of sCD80 and soluble CD86 appear to be differentially regulated. This study provides the first evidence of circulating sCD80 that can bind its ligand CTLA-4 and suggests a potential role for sCD80 in modulating costimulatory signals during the malignant process. sCD80 levels may also provide a diagnostic/prognostic marker in CLL and MCL.

107.5

Aggressive Natural Killer – cell leukaemia, report of five cases and review of the literature

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We report five cases of Aggressive Natural Killer-cell Leukaemia (ANKL) diagnosed and treated at Auckland Hospital within a period of five years. Presented are the clinical, haematological, immunophenotypic, immunogenotypic and cytogenetics data. All patients presented with fever and constitutional symptoms. 3 had hepatosplenomegaly and 2 lymphadenopathy. 4 patients had other organ involvement and 3 had CNS infiltration. 4 patients presented with anaemia, all had thrombocytopenia and 2 had leucopenia. All had abnormal liver function tests. Bone marrow showed varying degree of involvement and the trephine biopsies diverse types of infiltration. The leukaemic cells varied morphologically from large blastoid to medium or small classic granular lymphocytes. Haemophagocytosis was prominent in 2 patients. All cases were CD2 positive and CD3 and CD8 negative. CD56 was positive in all but one, who instead expressed CD57. The latter was also CD4 positive. T-cell receptor rearrangement studies were performed on one and showed germline configuration. The two cases tested for EBV DNA in the tumour tissue showed positive results. Cytogenetics was normal in 3 and had complex abnormalities in one. All patients pursued an aggressive fatal course with a median survival of 123 days following

diagnosis. ANKL is a very rare disorder. On review of the English-language literature 68 cases were identified. 71% of them were patients from Asian origin (55% Japanese and 16% Chinese). Caucasians were only 25%. In our series of 5 patients, 3 were Caucasians, which along with one other series from France represented the largest series of non-Asian patients. Contrary to a few publications, including the recent WHO classification, where it is stated that patients are mostly teenagers and young adults, the median age of the disease, as assessed on the current review, is 37 years and the incidence of the disease in patients in their 5th and 6th decade is slightly higher than in patients in their 2nd decade.Comparison was drawn between the historical and present series. No significant differences were found in the clinical presentation. Likewise, haematological findings were similar, except for the less common leukaemic picture in our series. Cell marker studies differed only regarding the CD56 expression, which was uniformly positive in the historic series and was negative in one of our patients. Normal karyotypes were found in 60% of our cases and in 30% of the historical series. The latter had a greater proportion of complex abnormalities, 47% vs. 20% in our series.

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Deletions of the Short Arm of Chromosome 3 in Haematological Malignancies

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Deletions of 3p have been well known in solid tumours, including lung, breast, kidney, cervical, head and neck and ovarian cancer, for many years. Evidence from solid tumour studies suggests that 3p contains several tumour suppressor genes. Loci implicated include the *VHL* gene at 3p25, *RASSF1* at 3p21.3, *FHIT* at 3p14.2 and LCTSGR2 at 3p12. However, there is very little data concerning the incidence of 3p abnormalities in haematological malignancies. A review of the records of the Victorian Cancer Cytogenetics Service over the past 7 years identified 90 patients with interstitial or apparently terminal deletions of 3p, or add(3p) resulting in deletion of the distal segment of 3p. The cases included 27 cases of acute myeloid leukaemia (AML), 17 myelodysplastic syndromes (MDS), 2 myeloproliferative disorders (MPD), 2 chronic myeloid leukaemias (CML), 12 acute lymphoblastic leukaemias (ALL), 15 multiple myelomas (MM) and 15 non-Hodgkin's lymphomas (NHL). The 27 AML, 17 MDS, 2 MPD, 2 CML and 12 ALL cases represented 2.8%, 0.8%, 0.2%, 0.6% and 4.2% respectively of the new cases karyotyped over the last 7 years. The proximal breakpoint of del(3p) or add(3p) in this study was p10-p13 in 15/27 (55.5%) AML, 12/17 (71%) MDS, 2/2 (100%) MPD, 2/2 (100%) CML, 6/12 (50%) ALL and 8/15 (53%) NHL, but only 2/15 (13%) MM. The remaining cases were deleted distal to this region with two-thirds of the myeloma cases showing deletions distal to 3p21. Three AML cases and 7 MDS were therapy-related following chemotherapy for previous malignancies. An AML developed post renal transplant and another 2 AML had transformed from ET and RAEB respectively. In 9/27 AML and 4/17 MDS, the del(3p) appeared in a sideline only; 6/27 AML and 6/12 ALL cases only developed a del(3p) in relapse. These data indicate that loss of 3p occurs in both myeloid and lymphoid malignancies. Fifty percent or more of these cases involved loss of the proximal region of 3p, except for MM, suggesting loss of more distal loci may be important in MM. A significant number of AML and MDS were therapy-related or represented transformation from a previous myeloid disorder. The appearance of del(3p) or add(3p) post-therapy, in relapse and / or as part of a sideline suggests that loss of 3p occurs as a late event in haematological disorders, in association with disease progression and clonal evolution.

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Reduced-Intensity Allogeneic Stem Cell Transplantation for Indolent Non-Hodgkin's Lymphoma

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We have treated 20 patients with relapsed or refractory indolent non-Hodgkin's lymphoma with a reduced-intensity allogeneic stem cell transplant (SCT). Patients included relapsed or refractory follicular lymphoma (FL) (n=11), small lymphocytic lymphoma (SLL) (n=3), mantle cell lymphoma (MCL) (n=5), and diffuse mixed small and large cell lymphoma (n=1). Median time from diagnosis to transplant was 6 years (range=0.7-9.5), while median number of prior treatment regimens was 3 (range=2-8). Four patients had received a prior autologous SCT while three patients were considered chemorefractory. The median age of patients was 43 years (range = 34-58). Sixteen patients underwent a transplant from an HLA-identical sibling, one from a 6/6 HLA-matched aunt, and three from fully matched unrelated donors (MUD). Conditioning therapy consisted of fludarabine (25 mg/m^2 x 5 days) and either cyclophosphamide (60 mg/kg x 2 days) in the related transplants, or, melphalan (140 mg/m^2) and anti-thymocyte globulin (ATGAM 30 mg/kg x 3 days) in the MUD transplants. Prophylaxis against graft-versus-host disease (GVHD) consisted of cyclosporine (CsA) and mycophenolate (MMF) in 14 patients, CsA and methotrexate in four, and CsA alone in two. Median time to achieve an absolute neutrophil count of >1 x 10^9/L and a platelet count of >50 x 10^9/L was 11 days (range=9-17) and 15 days (range=10-98), respectively. Fifteen of 19 evaluable patients exhibited complete donor T-cell chimerism by day 30 post-transplant. Mucositis was minimal, while 7 patients required no red cell transfusions, and 6 required no platelet transfusions. Treatment-related mortality (TRM) was 15% at day100 and 30% at 1 and 2 years. There were six deaths, including two of the three MUD transplant recipients, while the other four patients were > 52 years of age. The incidence of grade II-IV AGVHD was 80%, and that of grade III-IV AGVHD was 55%. Limited chronic GVHD was seen in 4 of 14 evaluable patients (29%), and extensive chronic GVHD in 6 patients (43%). Of the 16 evaluable patients for response, 15 achieved a complete remission (94%) and one a very good partial remission (6%). No patient has exhibited progressive lymphoma and none has required treatment with donor lymphocyte infusions (DLI). The Kaplan-Meier estimate of the proportion of patients alive and event-free at a median follow-up of 15 months is 68% for the entire group, and 74% for the related transplants. This is one of the largest reported series of patients with indolent lymphoma undergoing reduced-intensity allogeneic SCT, and confirms that this treatment modality results in high and durable complete response rates.

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Non-myeloablative (mini-) Allogeneic PBSC Transplantation (PBSCT) using Melphalan and Fludarabine as Conditioning Chemotherapy is Followed by Rapid Establishment of Complete Donor Chimerism

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Mini-PBSCT uses lower intensity conditioning and relies on the graft-versus-tumour effect to cure patients making it more suitable for older patients and those with significant co-morbidities. The clinical significance of the persistence of recipient haemopoietic cells (mixed chimerism, MC) is unclear but may have prognostic significance following mini-PBSCT. We have measured haemopoietic chimerism in 16 mini-PBSCT recipients (9 male and 7 female, median age 51.5 (29-63) years) not eligible for standard PBSCT. There were 5 patients with AML, 2 each with MM, CLL and CML, and 1 each HD, Waldenstrom's macroglobulinaemia, myelofibrosis, NHL, and acute biphenotypic leukaemia. All patients received fludarabine (25 mg/m^2 x5), melphalan (140 mg/m^2 x1) and ATGAM 15 mg/ m^2 from day -4 to day +5. GVHD prophylaxis was cyclosporin 3 mg/kg/day IVI for 3-6 months and mycophenolate mofetil (MMF)15 mg/m^2 BD from day 0 to day +27. Chimerism was measured using minisatellite (VNTR) markers in granulocytes, monocytes ($CD14^+$/$CD15^+$), T-cells ($CD3^+$) and NK cells ($CD2^+$, $CD3^-$) purified immunomagnetically from peripheral blood. VNTRs were amplified by PCR using 100 ng of genomic DNA template and digoxygenin-labeled deoxynucleotide triphosphate precursors. Amplicons detected on Southern blots by enhanced chemiluminescence were quantified by comparison with serially diluted recipient DNA in donor DNA. The sensitivity was between 1 and 5%. This compares favourably with microsatellite based methods. The assay reproducibility was improved by using hot start PCR. Template preparation and post PCR processing were optimized to improve speed of assay. Chimerism was assessed monthly for 6 months and then at 9 and 12 months post transplant. Monitoring of monocyte and NK cells was discontinued in recent patients as it gave the same results as granulocytes and $CD3^+$ cells, respectively. No graft rejections occurred. 15 of 16 patients achieved >90-95% donor chimerism by 1 month post mini-PBSCT. Donor granulocyte engraftment either preceded or was concurrent with that of donor $CD3^+$ cells. Complete and stable donor chimerism was achieved in 14 patients within 2 months. Recipient granulocytes and $CD3^+$ cells (5%) were observed transiently in one CML patient at 5 m. MC at 1 m evident in the myelofibrosis patient (<50% donor) was managed by ceasing MMF; >90% donor chimerism (with severe aGVHD) quickly developed. In summary, rapid and sustained complete donor chimerism was achieved with the use of melphalan, fludarabine and ATGAM as conditioning chemotherapy prior to mini-PBSCT. Monitoring of haemopoietic chimerism by manual PCR offers a robust, relatively inexpensive diagnostic tool which can be implemented in laboratories without access to either automated sequencing facility or to real time PCR based technologies.

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Fludarabine/Melphalan Regimen Results in Low Treament Related Mortality and Low Relapse in Myeloma Patients Undergoing Allogeneic Stem Cell Transplant (BMT)

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From October 1997 to June 2003, 20 patients (pts) with multiple myeloma underwent an allogeneic BMT (alloBMT) using a reduced intensity regimen. All patients received FluMel conditioning (Fludarabine 30mg/m2 x 3 (n=18) or 4 (n=2), on days -5 or –6 to -3, and Melphalan 80 mg/m2 x 2 on days –2 and -1. The graft was G-CSF (10 µg/kg/day x 4) mobilized stem cells from a 6/6 HLA-matched sibling donor given fresh on the day and/or day following collection. GVHD prophylaxis was cyclosporine and reduced dose methotrexate (10mg/m2 on days 3, 6 and 11). Most patients received their transplant as part of initial therapy. Prior treatment was VAD alone (n=13), Radiation and Aredia (n=3), Dexamethasone/Cyclophosphamide (n=1) or Melphalan/Prednisone (n=1), two patients had refractory disease after VAD and autologous BMT. The median age was 50 years (range 39-65 years). Results: All patients engrafted. The median time to ANC engraftment was 12 days (9-18 days). The median time from bone marrow transplant to discharge was 18 days (range 15-21). The median chimerism at day 100 was 99% range 91-100%). Grade II to IV acute GVHD was seen in 55% (11/20) and this was severe (grade III-IV) in 30% (6/20). The median time to acute GVHD onset was 21 days. Chronic GVHD was seen in 13/17 assessable patients and was limited in 6 pts and extensive in 7 pts. There have been 3 treatment-related deaths (fungal infection, interstitial pneumonia, and line sepsis) and 2 relapse deaths. With a median follow-up of 10 months, the Kaplan Meier probability of 3 year overall survival is 82% (95%C.I. 52-95%). The 3 year actuarial progression free survival (PFS) is 40% (95%C.I. 16-68%). There was no difference in relapse or DFS in those with or without extensive chronic GVHD. We conclude, using a reduced intensity regimen (Fludarabine/Melphalan) and peripheral blood stem cells, alloBMT in myeloma patients is well tolerated with low early mortality. Survival compares favorably to autologous bone marrow transplantation and is superior to published data on a 2-step autologous/mini transplant procedure, however relapse remains high.

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Nonmyeloablative Allogeneic Transplants in Australia and New Zealand, 1999 – 2002: a review from the ABMTRR

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Allogeneic haemopoietic stem cell transplants using nonmyeloablative conditioning regimens (NMT) have increased during recent years. The ABMTRR received 24 registrations for adult NMT carried out in Australia and New Zealand in 1999, 37 in 2000, 61 in 2001 and 88 in 2002. Of a total of 210 NMT, 200 were carried out in Australia and 10 in New Zealand; 151 were first transplants and 59 were second or subsequent transplants. The age range of recipients was 17 to 68 years with a median of 50, compared to 40 for fully myeloablative allografts carried out over the same period. The majority of donors were 6/6 HLA-identical siblings (173, 82.4%) with smaller numbers being HLA-identical unrelated volunteers (26, 12.4%), 5/6 matched relatives (5,

2.4%), HLA-identical relatives (4, 1.9%) and 5/6 matched unrelated volunteers (2, 0.9%). Indications for transplant included myeloma (42), non-Hodgkin's lymphoma (37), AML in first remission (23) and beyond (35), CLL (16), CML in first chronic phase (5) and beyond (12), ALL in first remission (3) and beyond (8), and renal cell carcinoma (7). There were 73 deaths from relapse (19), infection (18), persistent disease (13), acute GVHD (10), graft failure or rejection (4), organ failure (4) and other causes (5). There were 39 deaths in the first year post transplant from causes other than relapse or persistent disease - a one-year transplant-related mortality rate of 19%. These included 12 deaths from infection and 10 from GVHD. Disease free survival probability at three years was 56% for the whole group with a median follow-up of 1.3 years for surviving patients. Three-year DFS was higher for first transplants (59%) than for second or subsequent (47%) and higher for recipients from 6/6 HLA-matched siblings (57%) than for other donor types (48%). Compared to myeloablative allografts carried out over the same period, three-year DFS was higher for non-myeloablative procedures for myeloma (53% vs 38%), NHL (72% vs 63%), AML past CR1 (52% vs 38%), and equivalent for AML in CR1 (67% for both). The ABMTRR is an important national data resource providing timely information on transplant activity and outcome across Australia and New Zealand.

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The Impact of the Hyper-CVAD Chemotherapy Regimen on the Ability to Mobilise Peripheral Blood Stem Cells (PBSC) in Patients with Haematological Malignancies

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Introduction: The Hyper-CVAD chemotherapy regimen (JCO 2000;18:547) is being increasingly applied to a number of haematological malignancies. It includes stem cell toxic agents, particularly high-dose cytarabine, which may result in poor and unpredictable PBSC mobilisation. We examined the impact of Hyper-CVAD on PBSC yields. Methods: A retrospective analysis was performed of all patients (pts) treated with Hyper-CVAD in a single centre since Jan'99 in whom an attempt was made to collect PBSC. The regimen consists of alternating cycles of: (A) cyclophosphamide 300mg/m^2 q12hrs for 6 doses d1-3, vincristine 2mg d4&11, doxorubicin 50mg/m^2 d4 and dexamethasone 40mg daily d1-4 and d11-14; and (B) methotrexate 1g/m^2 over 24 hours d1 and ara-C 3g/m^2 q12hrs for 4 doses d2&3. PBSC were generally collected on recovery from one of the B cycles with daily G-CSF 10 g/kg. Target cell number for optimum engraftment is defined at our centre as CD34 $\geq$5 x10^6/kg, while the minimum threshold for proceeding to ASCT is CD34 $\geq$2 x10^6/kg. Pts were analysed in 3 groups according to the extent of prior chemotherapy: mobilised prior to cycle 2A; after 2A and before 3A; and after cycle 3A. Results: 29 pts were identified: median age 35yrs; 79% male; 48% with initial marrow involvement, diagnoses (ALL n=5, lymphoblastic lymphoma n=4, Burkitt's/Burkitt's-like n=6, aggressive NHL n=10, mantle cell n=4). 22 pts were mobilised off the back of cycle B, 3 pts off the back of cycle A, and 4pts after cyclophosphamide. PBSC collection details according to timing of mobilisation are detailed below:

	Timing of mobilisation			
	Before 2A	After 2A & before 3A	After 3A	P value
N	5	16	8	
Total CD34+ (range)	14 (8.9-43.5)	3.6 (0-21.8)	0 (0-5.6)	0.003
% CD34+ $\geq$ 2 x10^6/kg	100%	56%	13%	0.006
% CD34+ $\geq$ 5 x10^6/kg	100%	44%	13%	0.008
Day of 1st apheresis	12 (11-13)	13 (12-19)	14	

Conclusion: Hyper-CVAD is a highly stem cell toxic regimen. The mobilising efficiency can be significantly improved by collecting PBSC earlier in the treatment course, preferably following cycle 1B.

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T-cell Depletion for Paediatric Matched Unrelated Donor Bone Marrow Transplantation

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It has been postulated that T-cell depletion may reduce graft versus host disease (GVHD) and improve overall survival (OS) in patients receiving matched unrelated donor (MUD) bone marrow transplant (BMT). To evaluate the impact of T-cell depletion on GVHD, engraftment, duration of hospitalisation and transplant related mortality we reviewed the clinical course of consecutive paediatric MUD BMT at our institution. Between September 1985 and June 2003, 51 children aged 5 months to 18 years underwent MUD BMT. Donors were sourced through the Australian Bone Marrow Donor Registry and were matched at HLA-A and -B loci by serological methods. Matching at D-loci was by either serological or molecular techniques. Underlying diagnoses included leukaemia and myelodysplasia (n=37), aplastic anaemia (n=6) and genetic or other diseases (n=8). Conditioning regimens contained cyclophosphamide/thiotepa/anti-thymocyte globulin (ATG) and 12cGy total body irradiation (TBI) in 25 patients. In the remaining 26 patients, Cyclophosphamide/ATG $\pm$ busulphan $\pm$ melphalan $\pm$ etoposide $\pm$ fludarabine conditioning was utilised, with 6 patients receiving total lymphoid irradiation in addition. GVHD prophylaxis was cyclosporin/methotrexate (CSA/MTX) in the T-replete group (n=14) or CSA/T-cell depletion (n=37). T-cell depletion was achieved by either sheep red blood cell $\pm$ lectin techniques (n=31) or positive $CD34^+$ cell selection (n=6). Engraftment occurred in all but two patients (one early death on day +2 and one early relapse on day +34). In the remaining children, neutrophils reached 0.5x10^9/L at a mean of 24 (range 10-40) days in the T-replete and 21 (range 11-40) days in the T-cell depleted group. Grade II-IV GVHD occurred in 69% in the T-replete and 22% of the T-cell depleted group. Grade IV GVHD was experienced by 38% in the T-replete and 3% in the T-cell depleted group. Duration of hospitalisation post-transplant was a mean of 54 (range 2-186) and 34 (range 20-67) days for the T-replete and T-cell depleted groups respectively. Of patients alive at day +100, extensive chronic GVHD developed in 63% in the T-cell replete group and 0% in the T-cell depleted patients. OS at 3 years post-transplant was 17% in the T-cell replete group and 46% in the T-cell depleted group. For patients with leukaemia, disease-free-survival at 3 years was 0% for T-cell replete patients, and 47% for those with T-cell depleted marrow. We conclude that paediatric patients receiving MUD BMT have a decreased risk of GVHD and improved OS when marrow is T-cell depleted when compared to unmanipulated marrow and CSA/MTX therapy.

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Organisational Aspects of Establishing a Cellular Therapy Programme

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Cellular therapy programmes entail collection of haemopoietic stem cells (HSC) from the bone marrow, peripheral or cord blood and specialised leucocyte populations from peripheral blood. These cells are then separated, purified, populations enriched or depleted, cultured and cryopreserved as required to enable production of HSC grafts, donor leucocyte infusions, cytotoxic T lymphocytes, natural killer cells and dendritic cells for clinical use. These manipulations require specialised facilities. In England, cellular therapy programmes (CTPs) are based in both hospitals (60%) and blood centres (40%). In the UK the activities of such facilities are guided by the UKBTS/NIBSC guidelines for blood centres, inspected and accredited by the Medicines Healthcare Products Regulatory Agency against a Department of Health Code of Practice. Soon inspection, not only of laboratories but also cell collection and clinical stem cell transplant programmes will required formal accreditation by the Joint Accreditation Committee of ISCT (Europe) and the EBMT. Within the National Blood Service in England there are eight facilities for supplying CTPs. Each has a quality assurance laboratory, cryo laboratory and a GMP cell processing facility. Some of these provide CTPs for several hospitals. A national framework is in place to deliver effective quality management systems. A national and locality-based quality managers support and advise, conducting a programme of internal audit. Total quality systems are in place comprising quality assurance, assessment, control and improvement. The quality management plan describes methods for oversight of all activities including detection of errors, accidents and adverse reactions, significant outcome parameters, the means for review of data on a regular basis and requirements for meetings for new documentation, corrective action and reporting. Staff training forms an essential part of this. The EU Clinical Trials Directive which becomes operative in 2004 will entail a significant change in practice in the way CTPs are developed for clinical use in the future.

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EBV-specific Cytotoxic T Lymphocytes for Post Transplant Lymphoproliferative Disease

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Epstein-Barr virus associated lymphoproliferative disorder (EBV-LPD) is a potentially life-threatening consequence of immune suppression post hemopoietic stem cell transplantation (HSCT). Persistence and reactivation of EBV within the cells of the immune system is fundamental to the disease pathogenesis, which is due to outgrowth of donor-derived EBV-infected B cells. The risk of EBV-LPD varies with different donor sources and HSCT product manipulation with the highest incidence of up to 25% seen in recipients of unrelated donor or mismatched family member transplants where selective T cell depletion is used. Over the past 10 years immunotherapy strategies aimed at reconstituting T cell responses to EBV or targeting B cells have improved the outcome of EBV-LPD post transplant. In addition techniques to detect the disease early have been developed, allowing pre-emptive therapy. From 1993-2000, we generated donor-derived EBV-specific CTL and administered them as prophylaxis and treatment

for this complication. 56 patients received CTL prophylactically. 9 patients on the prophylaxis study had evidence of incipient EBV-LPD prior to CTL infusion. None of the 56 patients in the prophylaxis study developed EBV-LPD compared to an incidence of 11.5% in patients not receiving prophylaxis. 4 patients not enrolled on the prophylaxis study received CTLs as treatment for EBV-LPD. One patient with a mutation in EBNA 3B was resistant to CTL therapy and 2 patients attained remission after CTL therapy. Therefore, polyclonal donor-derived EBV-specific T-cell lines can be used safely to prevent EBV-LPD post allogeneic-BMT with long term persistence of adoptively transferred cells. In addition to cellular therapies, the introduction of modified methods of graft manipulation along with the availability of antibody therapies have also helped reduced the mortality from EBV lymphoma in BMT recipients. The current challenges are how to use monitoring tests to diagnose this complication early and to identify which patients should receive pre-emptive treatment. It is obviously preferable to treat patients with early or incipient disease as treatment of bulky disease is associated with significant morbidity and a higher likelihood of generating escape mutants. However as donor T cells have the risk of alloreactivity and anti-CD20 may increase the risk of infections, it is important to identify patients who have sufficient endogenous EBV-specific immune function to control reactivation. Pre-emptive treatment should be considered in patients with high EBV load and a strong likelihood of developing PTLD such as recipients of T-cell-depleted transplants or patients who have received anti-T-cell antibodies *in vivo* for GVHD.

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Adoptive Cellular Immunotherapy with CMV-specific Cytotoxic T Cells for Prevention of CMV Disease after Allogeneic Hematopoietic Stem Cell Transplantation

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Cytomegalovirus (CMV) is a major opportunistic pathogen in patients undergoing allogeneic hematopoietic stem cell transplantation (AlloHSCT). Despite advances in molecular monitoring techniques, improved anti-viral drugs, and better post-transplant management strategies for CMV, this virus continues to be a challenge to the successful outcome of alloHSCT, particularly for patients with non-sibling donors and those with significant graft-versus-host disease. The cause of CMV disease post-transplant is severe cellular immunodeficiency, particularly CD8+ cytotoxic T cells (CTLs) recognising CMV antigens. Riddell and Greenberg a decade ago showed that transfusion of donor-derived CMV-reactive T cells could restore cellular immunity to CMV post-alloHSCT, opening the possibility that post-transplant immunodeficiency could be rapidly corrected by the use of pathogen-specific donor T cells expanded ex-vivo. Application of this principle however has proved elusive due to biotechnological problems. Along with groups in Heidelberg, London, and Seattle, we have investigated the feasibility of using the immunodominant CMV pp65 antigen as a target for cellular immunotherapy. Using donor monocyte-derived dendritic cells coated with pp65 peptides as antigen-presenting cells, we have shown that large scale expansion of highly purified pp65-reactive CD8+ CTL's from CMV-seropositive HLA-A0201+ donors is feasible over a 4 week period, providing sufficient CTLs for clinical therapy. As part of a phase I clinical trial, we have subsequently transfused 2 alloHSCT patients with pp65-specific CTLs derived from their sibling donor. One patient received only a low specific CTL dose. In the other patient, pp65-specific CD8+ cells were detected by tetramer flow cytometry analysis for 14 days post-transplant, demonstrating that transfused CTLs can persist in vivo, in the absence of CMV reactivation. Further laboratory work has focussed on

extending this strategy to patients who are not HLA-A201-positive. We have demonstrated that CTLs specific for pp65 can be generated from donors with a range of HLA types using DCs pulsed with an Adenoviral construct containing the entire pp65 gene. In addition, this strategy also generates significant numbers of CMV-specific CD4+ cells, which might prove necessary for a sustained in vivo cellular immune response. Other future strategies may include the targeting of other immunodominant CMV antigens, such as IE, or the co-administration to patients of donor DCs coated with CMV antigenic peptides.

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A Rapid Simple Screening Test for Hereditary Spherocytosis: mean sphered cell volume

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Aim: The mean sphered cell volume (MSCV) has been described as a routine screening test for hereditary spherocytosis (HS) available to laboratories with a GenS or LH750 Beckman Coulter haematology analyser. The MSCV is obtained during automated reticulocyte analysis in which red cells are sphered by exposure to a hypo-osmotic solution. The mean cell volume (MCV) of normal red cells is less than the MCSV after osmotic expansion. In hereditary spherocytosis MCV is greater than MSCV possibly due to increased osmotic fragility and subsequent cell fragmentation. Our aim was to assess the utility of the MSCV as a rapid screening test for HS in our unselected population and its sensitivity and specificity in our laboratory. Methods: We analysed the first 500 different patients to have automated reticulocyte analysis on the LH750 analysers newly introduced into our laboratory. Data collected and analysed included haemoglobin, MCV, MSCV, MCH, MCHC, reticulocyte count, immature reticulocyte fraction, blood film findings and clinical diagnoses. Sensitivity, specificity, negative and positive predictive value were also calculated. Results: There was a correlation between MSCV and MCV (R^2= 0.7). A subgroup of 53 patients had MSCV less than MCV. This group included all 5 patients known to have HS. No additional HS cases were identified from blood film examination. The remaining 48 patients with MSCV less than MCV had a variety of conditions including other spherocytic haemolytic anaemias such as autoimmune haemolytic anaemia, post transfusion haemolysis and ABO incompatibility in neonates. Other diagnoses included cold haemagglutinin disease, microangiopathies, acquired sideroblastic anaemia, thalassaemias and haematemesis. The calculated sensitivity and specificity were 100% and 90% respectively. Negative predictive value was therefore 100% but positive predictive value was poor at 0.3%. Conclusions: MSCV is a red cell parameter readily available on new Beckman Coulter haematology analysers when a reticulocyte count is performed. MSCV less than MCV is a very sensitive screening test for HS. However, in an unselected population, it is less specific (90%) occurring in patients with a variety of haemolytic and non-haemolytic anaemias and in a normal pregnancy. It has a high negative predictive value useful for screening but very poor positive predictive value. These characteristics are similar to those seen with more cumbersome tests for HS such as osmotic fragility. MSCV comparison with MCV is thus a rapid simple means of excluding HS in a low prevalence population. Where the MSCV is less than MCV the cause requires further investigation.

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Novel Gene Insertion in a Mâori Patient with Transfusion Dependent Beta Thalassaemia

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A young Mâori woman aged 28 years, current weight 51 kg, has been attending our department[1] for many years for red cell transfusions (to maintain her Hb > 95 g/L) and chelation therapy. Her HbF after transfusions runs at 3%. Her father's latest tests show Hb 156 g/L, MCV 68 fl, RCC 7.14 x 10^{12} /L, HbA2 4.4%, HbF 0.6 %. Her mother's latest results: Hb 138 g/L, MCV 72 fl, RCC 5.9 x 10^{12} /L, HbA2 4.2%, HbF 0.7%. A splenectomy was performed at 7 years of age. Because of difficulties with her therapy during her adolescence, she developed severe iron overload resulting in IDDM, hypogonadotrophic hypogonadism with oestrogen deficiency symptoms, amenorrhea and infertility. In 2002 she developed ventricular tachycardia (which has responded to aggressive chelation and sotalol therapy) at a time when her serum ferritin reached 4534 μg/L. She also suffers from recurrent urinary tract infections and vaginal candidiasis. She is being intensively chelated, currently receiving desferrioxamine (Desferal) 21 g I.V. over 6 days /week through her third portocath with an infusion pump. A liver biopsy in 2002 showed mild periportal fibrosis with grade 4 iron stores; the liver iron was 67,000 μg/g dry tissue (normal 350 – 1250) giving an iron index of 40, at a time when the serum ferritin was 3020 μg/L. The current serum ferritin is 2149. Because of the interest in this patient with respect to her ancestry (β thalassaemia major not described in a Mâori before) and the presumed homozygosity of her defect (parents are first cousins), molecular studies were performed to determine the genetic defect. These revealed homozygosity for a novel 45 bp insertion at the 5' end of the beta globin gene. The insertion involves the duplication of the N terminal sequence and flanking 5' untranslated region so that the novel gene contains two start sites. Translation from the intact normal start codon leads to some normal β globin production whereas translation from the duplicated start site results in no β chain production. Further work is ongoing to determine the relative contribution of each site [2].

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Molecular Spectrum of Deletional Alpha Thalassemia Syndromes - The Singapore General Hospital Experience

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The alpha(α)-thalassemias are the commonest single-gene diseases in the world and are highly prevalent in Southeast Asia. Commonly in more than 95%, they arise from various different deletional defects in the alpha globin gene. We present the incidence of various α-thalassemia deletions in relation to different ethnic groups in 132 cases seen at our institution from 1998 to 2002. Hemoglobin electrophoresis was performed in all cases. Molecular analysis by polymerase chain reaction (PCR) assays was performed for these deletions: $-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, $--^{FIL}$ and $--^{THAI}$.

Table 1

Genotype	Chinese	Malay	Indian	Other ethnic group
$--^{SEA}/\alpha\alpha$	106 (89%)	2 (33%)	0	2*
$--^{THAI}/\alpha\alpha$	1 (1%)	0	0	0
$--^{FIL}/\alpha\alpha$	0 (0%)	0	0	0
$-\alpha^{3.7}/\alpha\alpha$	2 (2%)	2 (33%)	3 (75%)	0
$-\alpha^{4.2}/\alpha\alpha$	2 (2%)	1 (17%)	0	1 (Eurasian)
$-\alpha^{3.7}/-\alpha^{3.7}$	1 (1%)	1 (17%)	1 (25%)	1 (Filipino)
$-\alpha^{3.7}/--^{SEA}$	4 (3%)	0	0	0
$-\alpha^{4.2}/--^{SEA}$	1 (1%)	0	0	0
$-\alpha^{CS}/--^{SEA}$	1 (1%)	0	0	0
Total	118 (100%)	6 (100%)	4 (100%)	4

* 1 case - Filipino, 1 case - Eurasian. CS : Constant spring

Our data suggest $--^{SEA}$ is prevalent in our local Chinese population while in Malays, both $--^{SEA}$ and $-\alpha^{3.7}$ are common. In Indians, the $-\alpha^{3.7}$ appears to be common. The $-\alpha^{4.2}$ mutation is occasionally seen while the $--^{FIL}$ and $--^{THAI}$ deletions were rarely encountered. Due to the heterogeneity of deletional mutations seen and as they are somewhat ethnic-specific, definition of the spectrum of α-thalassemia alleles present in the population is important. It will enable physicians to design a rational PCR-based strategy for rapid screening and identification of the most common α-thalassemia defects seen in the community.

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Successful Pregnancy Management and Outcomes in Beta Thalassaemia Major in a Sydney Hospital 1998-2003

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Thirty years ago, fertility would not have been an issue in female patients with beta-thalassaemia major, who rarely survived past their twenties. Now, with improved transfusion and iron chelation practices, increasing numbers are surviving into their thirties and forties and seek to have a family. We describe six women with transfusion dependent beta-thalassaemia major who became pregnant between 1998-2003, with five live births and one baby due 5/12/03. All required assisted conception (4 ovulation-induction, 4 GIFT, and 2 donor sperm due to haemoglobinopathy in the father), and one woman had two previous miscarriages. The maternal ages ranged from 24-38, with a variety of complications from their thalassaemia; one with insulin-dependent diabetes, three with Hepatitis C, four with previously abnormal left ventricular function on imaging, and all had osteoporosis. Pre-pregnancy ferritins ranged from 2340 g/L-9000g/L; strict transfusion management kept the haemoglobin above 100g/L, and all patients increased their transfusion requirements. No patient conceived while in desferrioxamione, but two re-commenced iron chelation at the end of the second trimester. Pregnancy – related complications were minor – two cases of mild pre-eclampsia and one mild cholestasis of pregnancy. All women were delivered by elective caesarean section at 26-39 weeks, with one foetus showing intrauterine growth

retardation at 35 weeks. Birth weights ranged from 2355g-2700g with Apgars of nine and non neonatal complications. Two mothers developd post-partum endometritis requiring readmission for transfusion and antibiotics. There were no peri-natal deaths, but one mother developed a severe cardiomyopathy three years after delivery and died two years later of cardiac failure. In our experience, intensive medical monitoring and management allowed for successful pregnancy outcomes for this chronically-ill group of patients.

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The High Cost of Transfusions for Patients with Chronic Blood Disorders Should Allow the Trial/Use of Alternative Treatments such as Erythropoietin, Currently Not Funded in New Zealand

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Patients with chronic blood disorders often require considerable support including frequent blood transfusions. In 2001, we reviewed 40 patients with chronic blood disorders attending our Haematology Day Ward, who were transfused on or > two occasions with red cells in that calendar year. Patients with acute conditions (e.g. acute leukaemia or high-grade lymphoma) and those receiving non-red blood products only e.g. Intragam, were excluded from this analysis. The diagnoses included the following: myelodysplasia (n =16; 2 with red cell aplasia [1]), myeloma (8), myelofibrosis (4), haemoglobinopathies (3), lymphoma (3; 2 with red cell aplasia), idiopathic cold agglutinin disease (2), Waldenstroms macroglobulinaemia (2), chronic lymphatic leukaemia and marrow hypoplasia (1 each). Blood products as well as additional related activities including laboratory testing, standard cross matching, additional serological testing and/or red cell elution studies, bed use, nursing/medical staff input, and medications specific to the blood transfusion were costed. The price of blood products supplied by the New Zealand Blood Service or Commonwealth Service Laboratories increased dramatically in 2001 (red blood cell unit: up by 165%, Intragam 12g: 19%, platelet concentrate: 72%.), largely due to the introduction of nucleic acid testing and universal red cell leucodepletion. A standard admission for a two unit transfusion was priced at $611.39 and for three units $771.09. Patients had 2 – 35 admissions (mean 8), with transfusion costs ranging from $NZ1,537 to $47,825 (mean $7,629.). During this period, additional clinical activity was as follows: premedications (12 patients), transfusion reaction investigations (3 episodes), in-patient related admissions (5); these included two episodes for heart failure, one for fever, a portacath insertion for poor venous access, and an episode of severe hypertension after transfusion. Patients received transfusions using a butterfly needle in the majority of admissions (96%); two patients had portacaths, and two received blood through a blood warmer for cold acting antibodies. Seven patients who received more than $12,000 of transfusion therapy during these twelve months were further reviewed with respect to their total transfusions from diagnosis until death or March 31st 2003. The annualised cost of transfusions in this group ranged from $8,534 to $87,631 (median $23,331) which excludes all other treatments, such as chemotherapy, antibiotics etc. The high cost of transfusion therapy in those with chronic blood diseases should allow the use of alternative therapies, which

must be cost effective in selected clinical situations. NZ costs of transfusion therapy are compared with those in other OECD countries.

1. H.A.Blacklock et al. (2003). *Cyclosporin successfully treats red cell aplasia associated with myelodysplasia* . NZ Med Journal

116:386-7

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The Use of Recombinant Human Epoietin Alfa (Eprex) by the Haematology Department: a single institution retrospective audit

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Introduction: Recombinant human epoietin alfa (Eprex) is an established therapy for the management of cancer-related anaemia. Higher haemoglobin levels are associated with better energy levels, improved quality of life assessment & reduced transfusion requirements. Changes in the blood supply may encourage the use of alternatives to allogeneic blood for the treatment of anaemia. Methods & Results: We report a single institution retrospective audit of Eprex use by our department. Pharmacy prescribing records identified 70 haematology patients who were issued Eprex between 1 October 2002 & 31 March 2003. 50 patients had data for evaluation. The response to Eprex is presented below.

Group	Number (N)	Eprex response		Haemoglobin response (number)			Number Tx	Transfusion (Tx) response (number)		
		N	%	Respond	Full	Partial		Respond	Full	Partial
Total	50	46	92.0	43	36	7	35	31	28	3
MDS	16	13	81.3	11	9	2	15	12	10	2
MM	12	12	100	12	9	3	11	11	11	0
NHL	9	8	88.9	8	6	2	4	3	3	0
AML	2	2	100	2	2	0	1	1	1	0
ALL	1	1	100	1	1	0	1	1	1	0
Other	1	1	100	1	1	0	0	0	0	0
ACD*	8	8	100	8	7	1	2	2	2	0
CDA^	1	1	100	0	0	0	1	1	0	1

Legend Haemoglobin response: Full >20g/l increase; Partial 10-<20g/l increase haemoglobin

Transfusion response: Full stopped transfusion; Partial >50% decrease transfusion
*Anaemia of chronic disease, ^Congenital dyserythropoietic anaemia

Serum erythropoietin (EPO) levels were measured in 20 patients (40%) prior to commencing Eprex. 19 (95%) of these had a level <100U/L & all of these patients responded to Eprex. 24 patients (45%) had a calculated serum creatinine clearance (Calc CrCl) <60ml/minute. There was no difference in response rate to Eprex according to Calc CrCl; <60ml/minute 91.7% & >60ml/minute Calc CrCl 92.3% response rate. There was no correlation between Calc CrCl & EPO level. Iron stores should be assessed when

on Eprex. Conclusion: The audit confirms that Eprex is a successful alternative to allogeneic blood transfusion for the management of anaemia. Pre-treatment EPO levels may predict an individual's response to treatment. Creatinine clearance was of no value in predicting response. Use of Eprex avoids the complications of blood transfusion & reduces patient admissions. A prospective audit is proposed.

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Both F-18 FDG PET and Tc-99m Sestamibi are Informative Imaging Modalities which Frequently Aid the Clinical Management of Patients with Multiple Myeloma (MM)

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Objective: Multiple myeloma (MM) may be difficult to assess due to: absent/small volume paraprotein; minimal bone marrow infiltration/biopsy sampling variation; persisting pain or abnormalities on plain skeletal surveys (SS) following therapy which may not represent active disease. In previous small studies, both FDG-PET (PET) and Tc-99m sestamibi scans (MIBI) have identified sites of occult bony and soft tissue disease in MM. This analysis aims to compare the results of PET and MIBI in MM.
Methods: Over Jan 1999–Mar 2003, 36 patients had ³ 1 PET scan, 56 pts had ³ 1 MIBI scan and 23 had concurrent PET and MIBI scans. Medical records and scan results were reviewed to assess: (a) ability of the scans to identify otherwise occult disease; (b) concordance between the scans; and (c) impact on management.
Results: Disease state was: newly diagnosed (12pts), partial/complete remission (6pts), relapsed/refractory (44pts), MGUS (3pts), isolated plasmacytoma (4pts). Thirty-one had scans at diagnosis or as baseline, 30 for suspected progression (PD), 6 for re-staging, and 2 to investigate persistent fever. Thirty-one (45%) had difficult to assess disease with: small volume/absent paraprotein (24pts), extramedullary disease only (3pts), disproportionate osteoporosis (2pts) and discordant response to treatment (2pts). In 18/36 cases (50%), PET identified additional sites of disease not seen on routine SS. Additional sites were soft tissue in 5 cases, bony in 11, and both bony and soft tissue in 2 cases. Thirteen of these 18 cases had known active disease at other sites. Five cases showed unexpected additional sites in patients thought to have limited/stable disease. Additional sites were confirmed to be positive for disease in 10/11(91%) cases that were further assessed with imaging (CT or MRI) ± biopsy, with one false positive site. In 37/56 cases (66%), additional sites of disease not seen on SS were identified on MIBI. Twenty-seven of 37 cases had known active disease at other sites. Ten cases showed unexpected additional sites in patients thought to have limited/stable disease. Additional sites were confirmed to be positive for disease in 18/21(86%) cases that were further assessed with imaging ± biopsy, and false positive in 3/21 cases. MIBI generally detected more disease sites than PET: median number of sites/scan for MIBI was five (range 0-15) versus one (0-10) for PET (P<0.0001). PET and MIBI were concordant in 8 cases (6 both negative scans). In 12 (52%), MIBI detected additional sites to PET, mostly bony (11 bony, 1 bony and soft tissue). In three cases, PET detected additional sites to MIBI (2 bony, 1 soft tissue). In 23/69 cases (33%), scan results impacted on management (33% of PET and 30% of MIBI scans). In two cases a diagnosis of MGUS was confirmed. In eight cases, stable or responsive disease was confirmed and patients continued their treatment plan. In three cases, radiotherapy fields were altered to encompass confirmed active disease sites. In six cases, treatment was changed due to detection of progressive disease. In four cases of presumed MGUS or isolated plasmacytoma, myeloma was diagnosed and systemic therapy commenced.

Conclusions: PET and MIBI are useful additional diagnostic tools for detecting otherwise occult MM sites. The use of MIBI ± PET should particularly be considered in the evaluation of a patient with a presumed solitary plasmacytoma to exclude the presence of other disease sites. Their use may also be valuable in providing an overall assessment in patients with 'difficult-to-assess' myeloma.

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Thalidomide and Celecoxib for Patients with Multiple Myeloma (MM) – a promising combination

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Introduction: Thalidomide (Thal) has proven efficacy in the management of MM. However response rate (RR) when used as a single agent is low. Thal has been shown to down regulate COX-2 mRNA, and COX-2 inhibitors have anti-angiogenic effects. Commencing August 2001, we performed a prospective Phase-II open-label, multi-centre(n=7) study combining Thal and Celecoxib (Thal/Cel) in patients(pts) with relapsed/refractory MM. The primary objective was to determine the RR and compare this with our previous trial using single agent Thal ± interferon (Mileshkin et al, Blood in press). Further objectives were to determine event-free (EFS) and overall survival (OS), as well as the toxicity profile. Methods: Eligible pts had relapsed or refractory MM, platelets $\geq 50 \times 10^9$/L and serum creatinine $\leq$ 1.5 x upper limit of normal. Pts commenced Cel 400mg bid plus Thal 200 mg/d. After 14 days, Thal was escalated by 200 mg/d every 14d to a maximum of 800 mg/d, or an individually maximum tolerated dose (iMTD) <800mg. The combination was to continue while tolerated or until progressive disease (PD). Cel was dose reduced for oedema or elevations in creatinine. Pts continue on Thal alone, if Cel is ceased due to toxicity. The trial is ongoing. Results: An interim analysis was performed in February 2003. 39 pts (17 females, 22 males) with a median age 68 (range: 43-84) had completed a median 20 weeks treatment (range 1-67). Median follow-up was 8 months (2-17). Median WHO performance status was 1 (0-2) with median prior chemotherapy regimens of 2 (range: 1-8). At study entry, 15 pts (38%) had an elevated B2M, elevated CRP (n=20:51%), elevated LDH (n=5:13%), ANC $<1.5 \times 10^9$/L (n=7:18%), Hb <100 g/L (n=30:77%). Median iMTD of Thal was 400mg/d and mean daily dose of Cel was 446mg. Responses for all pts were: PR 36%, stable disease 56%, progressive disease 3%, not evaluable 5%. Estimated predicted RR at 6 months was 26% (SE 7%). Median EFS and OS have not been reached, with 25 pts (64%) alive without progression at the time of analysis. Estimated 12 month EFS was 57% (95%CI:42-78) and 12 month OS was 63% (95%CI:47-86) for all pts. By comparison, our previous trial of Thal ± interferon reported an ORR of 28%, with estimated 12 month EFS of 23% (95%CI:14-34) and 12 month OS of 56% (95%CI:44-67). At the time of analysis, 22 pts had ceased Thal due to PD in 45% and toxicity in 23%. 22 pts had ceased Cel, due to toxicity in 50%. Important grade 3/4 toxicities seen above those expected of single-agent Thal were elevated creatinine (10%) and oedema (5%). There were no treatment-related deaths. Less than expected constipation was seen. Conclusions: These preliminary results suggest Thal/Celeb is tolerable in the majority, but elevations in creatinine and oedema are important additional toxicities over Thal alone. Although this was not a direct comparison with the Thal ± interferon combination in our previous trial,

given the promising RR (36% vs 28%) and predicted 1 year EFS (57 vs 23%), the trial will continue to accrue to 65 pts.

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A Phase II Study of Liposomal Doxorubicin (Caelyx) in Combination with Vincristine and Dexamethasone in Patients with Multiple Myeloma

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Doxorubicin is an effective drug in the treatment of multiple myeloma. Liposomal doxorubicin has several advantages over doxorubicin (1) longer half life of 40 hours, approximately 100-fold over doxorubicin, therefore enabling chemotherapy to be given in one day rather than a four day infusion (2) liposomal encapsulation may overcome multi-drug resistance and (3) concentration of the drug within tumours. We treated 30 patients over three years with Caelyx 40 mg/m2 IV over 2 hours, vincristine 1.4 mg/m2 stat. and dexamethasone 40 mg/d po for four days. Cycles were repeated every 28 days for four to six cycles. Pyridoxine 150 mg/d po was given to prevent muco-cutaneous toxicity (palmar plantar erythrodysaesthesia or PPE). Twenty seven (27) patients are evaluable after three or more courses. Patient demographics are as shown.

Sex M/F	Age	Salmon Durie Stage	New / Relapse
20/7	Med 63 (35-79)	1-5; 2-15; 3-7	17/10

Response after 3 cycles (PR is defined as >50% reduction in monoclonal protein and / or bone marrow infiltrate)

	Newly diagnosed (17)	Relapse (10)
CR	0	0
PR	8 (47%)	6 (60%)
SD	8 (47%)	2 (20%)
PD	0	0
NE	1 (6%)	2 (20%)

Adverse Effects-non haematologic
PPE- All grades 35%, grade 3-12%, febrile neutropenia 12%, nausea and vomiting 46%, allergic reactions 12%, one patient was withdrawn after an anaphylactic reaction. Adverse reactions –haematologic Anemia Hb<80-27%, platelets <75-46%, neutrophils<1.5-80%. Conclusion: Caelyx VD is not superior to the VAD regime and has substantial greater toxicity. Particularly PPE, allergic reactions and neutropenia.

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Retrospective Analysis of Response To Vincristine-Doxorubicin-Dexamethasone (VAD) Chemotherapy Before Autologous Transplantation in Multiple Myeloma

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Aim: VAD is commonly used as initial chemotherapy in myeloma patients proceeding to high dose therapy. We retrospectively analysed the monoclonal protein responses and survival data in 62 patients treated in this way. The aim was to assess the relative response to components of the pre-transplant chemotherapy and its relation to the overall disease response. Methods: Clinical and laboratory data were reviewed for the 62 patients. Skeletal imaging results were not available and so response rates are referred to as unconfirmed. Defined events for survival analysis were rising paraprotein, commencement of salvage regimen or death. The median age of patients was 55 years. Only two patients had prior chemotherapy. Results: The average reduction in paraprotein after the first cycle of VAD was 37% in serum and 69% in urine. After the third cycle, the reduction was 59% and 89% respectively. The unconfirmed complete remission rate (uCR) was 6% and for partial remission (uPR) 60%. Following cyclophosphamide mobilisation the uCR rate rose to 11%, while the uPR rate was unchanged. Greater than 50% reduction in monoclonal protein after the first cycle of VAD was observed in 40% of patients. This group had a significantly better event-free survival (EFS at 3 years 54% vs. 12%; p=0.05) than those with <50% reduction. The same association was no longer significant when comparing response groups after the third cycle of VAD. The OS curves for these two groups did not differ at 3 years. There was no significant correlation between uCR, uPR or non-responders with regard to event-free or overall survival. Discussion: These results indicate that the greatest measurable response in paraprotein is observed after the first cycle of chemotherapy. This initial response appears to be predictive of EFS and may be used as a marker for risk stratification. As most of these patients were treated de novo it is likely that this response reflects the underlying chemosensitivity of the disease. The absence of correlation between early paraprotein response and overall survival is likely to reflect the small number of patients. Also, efficacy of salvage regimens means that longer follow-up is required to establish effect on overall survival. We conclude that monoclonal protein response is a potential prognostic marker which is readily available and which may be used in conjunction with established markers such as cytogenetics and β_2 microglobulin to guide alternative treatment in patients identified as high-risk.

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The Use of Thalidomide for the Treatment of Multiple Myeloma in New Zealand: 1999-2002

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Aim: Thalidomide has been shown to have significant anti-tumour activity in multiple myeloma and has been used for this purpose in New Zealand since 1999. Because thalidomide is not registered in New Zealand, an individual approval must be granted by the Ministry of Health's Medsafe unit for each patient using this drug. Therefore, all patients who have received thalidomide treatment for multiple myeloma in New Zealand can be identified, allowing a complete audit of this form of therapy. The aim of this study was to review the use of thalidomide for the treatment of multiple myeloma in New

Zealand and compare the efficacy and toxicity with reported data in the literature. Methods: We identified every patient who had a special authority application approved for thalidomide use in multiple myeloma between March 1999 and December 2002. A questionnaire was then sent out to the treating clinicians to collect demographic and response data. Results: Questionnaires were sent out on 231 patients. Interim results are available for 69 patients for whom thalidomide had been taken for at least one month and response data are available. The median age at starting thalidomide was 65 years (range 36-86 years) with the median number of previous chemotherapy regimens used 2 (range 0-5). Thirty-three patients (48%) achieved a major response to thalidomide, defined as a 50 percent or greater reduction in paraprotein levels, whilst a further thirteen patients (19%) had a reduction in paraprotein levels of between 25 and 49 percent. The median time to maximum response was 4 months (range 1-22 months) with the median dose of thalidomide at the time of maximum response 100mg (range 50-800mg). The median duration of response was 7 months (range 1-43 months). Fifty patients (72%) reported mild (grade I or II) adverse events, whilst 4 patients (6%) had a significant (grade III or IV) adverse event. No adverse events) were reported in 15/69 patients (22%). Conclusions: The overall response rate (ORR) of 67% (greater than 25% improvement in paraprotein levels) compares favourably to overseas experience (ORR 37-69%). The doses used to achieve this were generally low and adverse events, although common, were generally mild and tolerable.

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Autologous Stem Cell Transplantation (ASCT) in Primary Systemic Amyloidosis (AL): The Impact of Selection Criteria on Outcome

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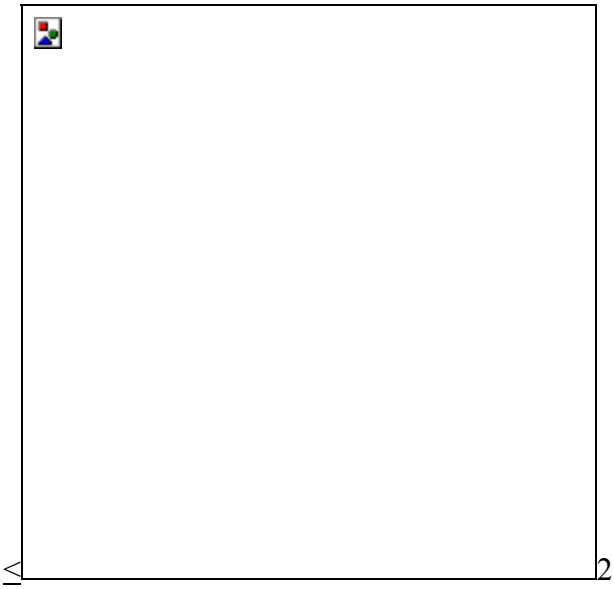
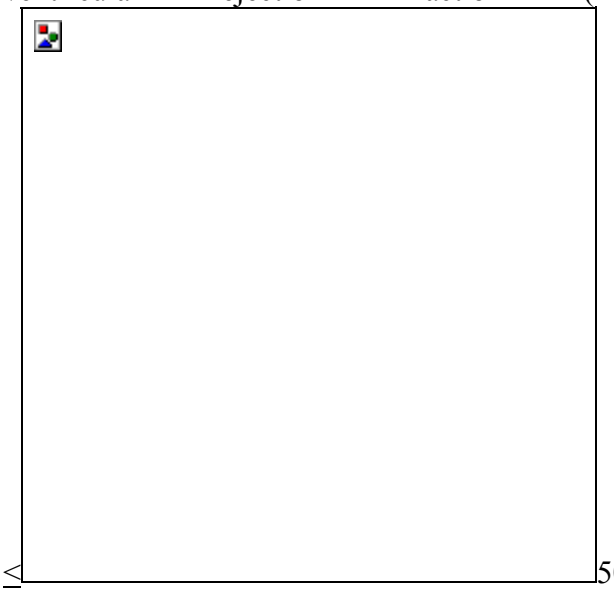
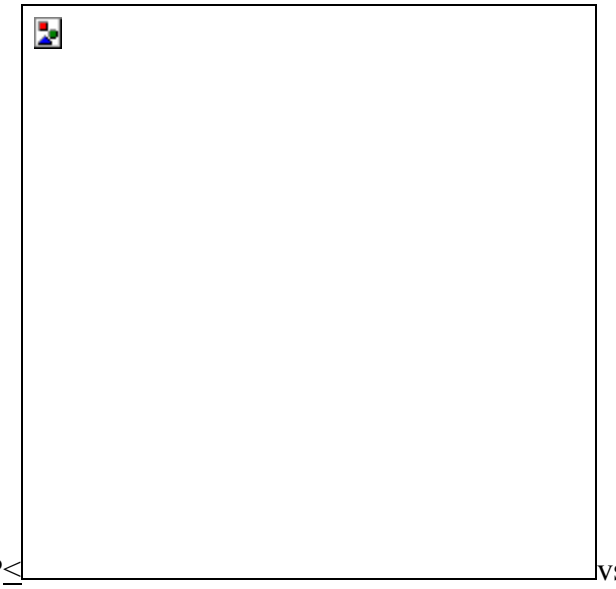
Introduction: ASCT has produced higher response rates than conventional chemotherapy in patients (pts) with AL. However, transplant-related mortality (TRM) remains high and reported series are subject to selection bias. Methods: We report a retrospective analysis of all pts with symptomatic amyloid referred to a single centre from '96-'01, identifying prognostic variables and the impact of ASCT in the entire population. Results: 48 pts had primary AL: median age 58 yrs; median time from diagnosis to referral 1.8months; and prior therapy in 27%. Organ involvement: renal 68%; cardiac 77%; liver 42%; and neurologic 19%. The 26 pts who were deemed ASCT candidates were younger (55 vs 63 yrs, p=0.001), had a better performance status (96% vs 73% with ECOG score



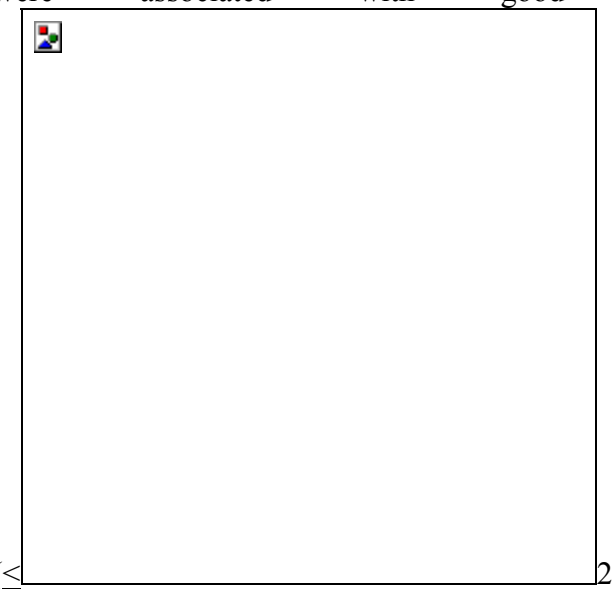
<2, p=0.02) and a trend to a better left ventricular ejection fraction (27% vs 45% with EF



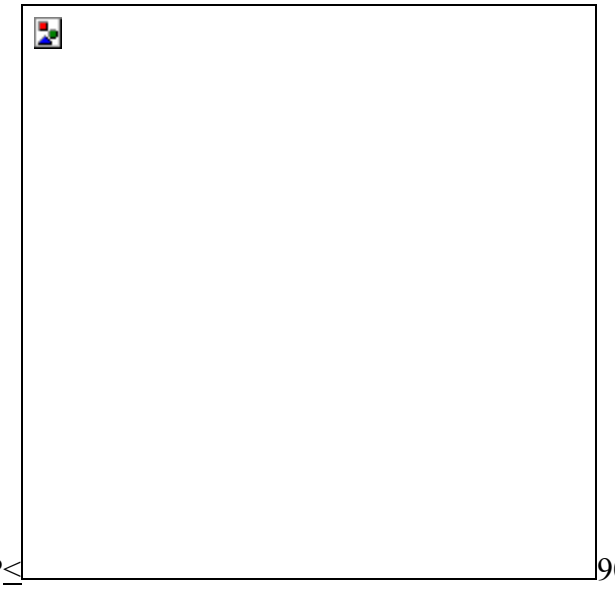
<50%, p=0.2) and less syncope (4% vs 18%, p=0.11). Five pts died during the peri-collection period from progressive disease or mobilisation complications and one failed to mobilise stem cells. 20 pts underwent ASCT following high-dose melphalan. TRM was 35%; however, since Jan'99 TRM has fallen from 50% to 20%. Intent-to-treat organ responses were renal 46%, cardiac 25% and liver 50%. The 3yr OS post-ASCT was 56% with improved outcome predicted by a better performance status (p=0.08), normal ALP (p=0.08), nephrotic syndrome (p=0.01) and absence of severe hypotension (3yr OS 0% vs 75% for systolic



BP≤vs>90mmHg, p<0.001). The 3yrOS for all 48 referred pts (i.e.whether transplanted or not) was 44%, and this was not significantly better for transplant candidates. Improved outcomes for all referred pts were associated with good ECOG performance status



(≤2 vs >2, p=0.001), the presence of nephrotic syndrome (p=0.06), no increase in septal wall thickness (p=0.07) and a systolic BP>90mmHg (p<0.001). Conclusions: Selection of appropriate candidates for transplantation of primary AL is important and can result in avoidance of excessive TRM. Those with significant hypotension (systolic



BP< 90mmHg) and poor performance status (ECOG >2) have an exceedingly high mortality and should not be transplanted. For those undergoing ASCT, organ response rates appear promising, but overall only small numbers benefit. Conclusive evidence of improved survival for this select group of pts is still lacking and will require randomised trials.

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Interleukin-2 and Lactate Limit *In Vitro* Large-Scale T-Lymphocyte Expansion for Clinical Immunotherapy Trials

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Aim: Recent clinical trials exploiting adoptive transfer of antigen-specific cytotoxic T--lymphocytes (CTL) have demonstrated the potential to treat viral and malignant disease. However, this strategy utilises large cell numbers to achieve efficacy, 10^9 to10^{11}, making routine clinical application impractical. T-lymphocytes have high substrate uptake rates and, in both resting and activated states, rapidly deplete culture media of basic nutrients. Also, proliferating T-lymphocytes internalise IL-2, with IL-2/IL-2 receptor (IL-2R) binding events determining the cell cycle progression rates. As a result, limiting conditions may arise in large-scale expansion at modest cellular densities ($5x10^6$cells/mL). In this study, we investigated the uptake and production rates of growth promoting and limiting factors found in cell culture media in an effort to identify key variables for: (1) predicting the outcome of large-scale CTL expansion and (2) development of a mathematical model for bioreactor design and optimisation. Methods: PBMC were isolated from healthy donors and exposed to PHA and saturating concentrations of IL-2. Activated PBMC were then used to inoculate suspension bioreactors run in parallel operating either batch or fed-batch modes (48hr half-media exchange). Cultures were analysed for cell number, activation status, phenotype, apoptotic fraction, IL-2, glucose and lactate during the 4 day expansion. Results: We found that PHA stimulated PBMC could be expanded >10-fold in 4 days. The phenotypic analysis indicated the populations were >95% $CD3^+$ at this time. The inoculum, upon seeding, was expressing high levels of CD25 and CD71 and observed a steady down regulation during the expansion. The apoptotic fraction in the batch operation was

markedly higher than the fed-batch expansion mode. Also, rapid reduction in IL-2 concentration was observed during expansion with concentrations falling below the saturation level within 2 days of culture. The proliferation rates decreased (double time (DT) ~30hr to ~50hr) over the first 2 days of culture. The lactate exposure decreased the cell proliferation rate, (DT ~1.5 and ~1.3-fold increase in the batch and fed-batch operation respectively), over the 4 day expansion. Conclusion: This study indicates that lactate production and IL-2 consumption limit the proliferation rate of T-lymphocytes in culture. Also, monitoring of cell density, activation status, metabolic sources and sinks and IL-2 during large-scale expansion is integral in maximising both quality and quantity of CTL. Finally, the large substrate uptake and release rates observed also signify a need to develop a flexible feeding program to minimise exposure of the cells to lactate and limiting concentrations of IL-2.

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Generation of CMV-Specific T Cells in HLA-A2$^-$ Donors by Stimulation with CMVpp65 Transduced or Whole CMV Lysate-Pulsed Dendritic Cells

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Aim: Allogeneic stem cell transplant (SCT) recipients lack cellular immunity shortly after transplant and are susceptible to viral infection. Cytomegalovirus (CMV), in particular, is a common infection which is associated with increased mortality and morbidity following SCT. Adoptive transfer of CMV-specific T cells can reduce the risk of developing CMV disease. However, conventional techniques to produce these cells have several shortcomings including targeting single CMV antigens, lack of MHC class II antigens and the use of HLA-restricted peptides. Therefore, we investigated whether dendritic cells (DC) loaded with lysate derived from CMV infected cells, or DC transduced with the pp65 gene could be used to generate CD4$^+$ and CD8$^+$ CMV-specific T cell cultures regardless of HLA haplotype. Methods: PBMC were collected from normal HLA-A2$^-$, CMV-seropositive donors. Monocytes were enriched through plastic adherence and cultured in the presence of GM-CSF and IL-4 for 5 days. On day 5, DCs were transduced with adenovirus encoding CMVpp65 (Ad5pp65GFP) or pulsed with CMV viral lysate in combination with the DC maturation cytokine, TNF-α. On day 7, antigen-loaded DCs were cocultured with autologous PBMC. The cultures were restimulated on day 14, and expanded in the presence of IL-2. T cell cultures were then tested for cytotoxicity against CMV-loaded target cells and cytokine production. In some experiments, the reactivity of both CD4$^+$ and CD8$^+$ T cells in cytotoxicity and cytokine production assays was confirmed by cell sorting. Results: PBMC from CMV seropositive, HLA-A2$^-$ individuals cocultured with CMV lysate-loaded or pp65 transduced DC showed a 10-fold increase in cell number over a period of 21 days. Similar to HLA-A2$^+$ cultures, CMV lysate stimulated cultures were predominantly CD4$^+$ (>75%) while Ad5pp65 stimulated T cells showed a mixed phenotype (40% CD4$^+$, 45% CD8$^+$). T cells from both cultures efficiently killed chromium labeled DC presenting CMV antigens (either CMV lysate or Ad5pp65 transduced targets), but failed to recognize control DCs. Furthermore, T cells sorted into CD4$^+$ and CD8$^+$ T cell populations showed recognition of CMV antigens in IFN-γ secretion assays, indicating the presentation of both MHC class I and II antigens in this culture system. Discussion: Both CMV lysate and pp65-gene transduction allows DCs to process and present MHC class I and II CMV antigens for a variety of HLA haplotypes. These antigen preparations can be used to generate CMV-specific CD4$^+$ and CD8$^+$ T cells regardless of HLA tissue type.

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Development of Human Dendritic Cell Subsets in NOD/SCID-huHSC Chimeric Mouse Model

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Dendritic cells (DCs) are becoming the focus of serious clinical investigations as the key antigen presenting cells in allogeneic haematopoietic stem cell transplantation (HSCT) generated graft-versus-host-diseases (GVHD) and for cancer immunotherapy. Key issues as to human DC differentiation, migration and activation cannot be studied *in vitro* or readily in patients. We have developed a NOD/SCID-huHSC chimeric mouse model to examine the development and function of donor-derived $CD11c^+$ DC and $BDCA2^+$ DC. NOD/SCID mice received 325 cGy of whole body radiation followed by reconstitution with cord blood $CD34^+$ cells ($2x10^5$ cells, i.v.). Eight weeks post transplantation donor-derived $CD11c^+$ DC and $BDCA2^+$ DC were analysed in murine bone marrow (BM), peripheral blood and spleen. High levels of human engraftment were observed in BM, blood and spleen of NOD/SCID-huHSC chimeric mouse (human $CD45^+$ cells: 90.7±3.4%, 39.4±11.4%, 68.4±8.8%, respectively, n=10). Human $CD11c^+$ DCs accounted for 0.3%-1.1%, 1%-7.7% and 0.4%-4.4% and BDCA-2^+ DCs for 2.3%-6.5%, 1.3%-17% and 1%-5% of human $CD45^+$ cells in murine BM, blood and spleen, respectively (n=10). In murine BM and spleen human $CD11c^+$ DCs have a phenotype resembling activated DC with expression of CD40 and CD86 antigens. Human DCs generated in the NOD/SCID-huHSC chimeric mice were functional as they were able to i) respond to microbial products (LPS, poly I:C) by producing inflammatory cytokines (IL-6 and IL-12) and ii) induce proliferation of allogeneic naïve $CD4^+$ T-cells. These data show for the first time that the NOD/SCID-huHSC chimeric mouse model provides an opportunity to follow the development, migration and function of donor-derived DCs after HSCT. As such this model provides a revolutionary opportunity to analyse the contribution of donor-derived DC to GVHD and clinical immunotherapy protocols.

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Profiling Dynamics of EBV-specific CTL responses in Hodgkin's Disease, and the development of *in-vitro* expansion protocols for LMP-specific CTL.

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A small but significant proportion of patients with Hodgkin's disease (HD) will relapse or have refractory disease, and less than half of these patients will respond to current salvage strategies. Recently, there is increasing emphasis to develop immunotherapeutic strategies to target EBV latent-antigens expressed in Hodgkin-Reed-Sternberg (HRS) cells. These are expressed in 30-50% of HD cases, with expression restricted to EBNA1, LMP1 and LMP2. HRS cells present LMP, and strategies to boost LMP-specific CTL activity may be beneficial. LMP sequences are poorly immunogenic in healthy EBV carriers, suggesting that HRS cells provide a weak LMP-specific stimulus to evade immunosurveillance. We performed a prospective multi-centre analysis to precisely map the EBV-specific CTL responses in HD patients, and to design LMP-specific CTL expansion strategies for the future treatment of relapsed/refractory EBV-HD. 71 patients were enrolled: 28F: 43M; median age 29yrs [range 6-54); 24 newly diagnosed [ND], 8 relapsed [R], 39 long-term survivors [LTS]; NLPHD 12%; NS 57%; MC 29%; LR 2%; LD 0%. 97% were EBV seropositive. Immunohistochemistry +/- ISH revealed 47% cases were EBV-HD. In ND and R cases, blood was taken at day 0 and 12 months; in LTS median time from diagnosis to blood sample was 15yrs (range 5-33). Samples were analysed for γ-interferon CTL activity by ELISPOT assay using a panel of 23 different HLA class I-restricted CTL epitopes within LMP1, LMP2, EBNA and lytic proteins. These epitopes are presented by a wide range of HLA class I alleles covering >90% Caucasians. Strikingly, mean LMP-specific CTL activity was only 34.9 [SE 11.7] spot-forming cells [SFC] x 10^6 at day 0 in ND patients, rising to 137.1 SFC [29.1] at 12 months (p=0.0010), and 130.2 SFC [36.1] in LTS (p=0.008). There was no significant difference in CTL activity between ND at 12 months and LTS, with values similar to those seen in healthy EBV-seropositive carriers, indicating that remission is associated with sustained recovery of LMP-specific CTL activity. Analysis of LMP-specific CTL function, phenotype and comparison with healthy virus carriers is on-going. Polyclonal EBV-specific CTL expansion protocols preferentially stimulates CTL specific for EBNA antigens rather than LMP. Therefore, we developed protocols using an adenoviral construct encoding 13 LMP epitopes, and autologous PBMC coated with LMP peptides, to successfully expand LMP-specific CTL from the peripheral blood of healthy viral carriers and HD patients. These studies will allow the highly efficient expansion of LMP-specific CTL for adoptive immunotherapy in relapsed/refractory EBV-HD patients.

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Analysis of Cellular Immune Response to Melanoma Antigens Using Allogeneic HLA-Matched Donors as Sources of Immune Effector Cells

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Aim: The Graft-versus-tumour (GvT) effect is an important therapeutic mechanism of allogeneic haematopoietic stem cell transplantation (allo-HSCT) against leukaemias and solid tumours. The aim of this study was to determine if GvT T lymphocytes could be generated from HLA-matched donors against human melanomas. Methods: Melanoma cell lines expressing HLA Class I and II were established from two patients and transfected with retroviruses encoding B7.1 and/or B7.2. Lymphocytes were collected from HLA-matched sibling donors and stimulated weekly with irradiated melanoma cell lines in mixed lymphocyte-tumour cultures (MLTCs) with 10 U/mL of IL-2. The expansion, phenotype, cytotoxicity and cytokine production of the lymphocytes were assessed. Lymphocyte clones were isolated from MLTCs by limiting dilution and reacted against donor EBV-LCLs transfected with melanoma antigens to identify the

antigens recognised by lymphocyte clones. Results: Fold expansion of T cells after four weeks of stimulation by the two donor-recipient pairs were as follows: original cell line 1.7±0.5 (pair 1) / 6.8±1.8 (pair 2); B7.1-transfectant 131.5±8.4 / 217.3±17.7; B7.2-transfectant 68.7±15.4 / 90.1±30.4; B7.1+B7.2 double transfectant 116.7±13.5 / 312.2±16.2. Therefore, B7.1-transfectants were superior to B7.2-transfectants in stimulating T lymphocyte expansion. B7.1-transfectants also stimulated higher cytotoxicity against melanoma cells than B7.2 transfectants. All MLTCs were predominantly $CD4^+$ (72.5%-95.7%) and most lymphocytes secreted INF-γ but not IL-4 or IL-10. No MLTCs showed cytotoxicity or INF-γ-release against recipient-derived EBV-LCLs, suggesting they recognised melanoma-associated antigens rather than minor-histocompatibility antigens. Several $CD4^+$ T lymphocyte clones were generated from the MLTCs. These clones exhibited specific cytotoxicity and secreted INF-γ against the recipient melanoma cells but not recipient-derived EBV-LCLs. To identify the antigens recognised by these $CD4^+$ clones, donor EBV-LCLs were transduced with MAGE-2, MAGE-3 and melan-A which were expressed by the patient melanoma cells. However, none of the T lymphocytes recognised EBV-LCLs transduced with these antigens nor EBV-LCLs pulsed with MAGE-3-derived peptides, suggesting they recognised antigens other than MAGE-2, MAGE-3 or melan-A. Further work is required to elucidate the antigens recognised by these clones. Conclusions: Transduction of B7 costimulators to melanoma cells stimulated greater T cell expansion and cytotoxicity against recipient melanomas, with B7.1 being superior to B7.2 in stimulating both expansion and cytotoxicity. No evidence of a graft-versus-host effect was found in the T lymphocytes raised. T lymphocyte clones did not recognise known melanoma antigens. Elucidation of melanoma antigens recognised by T lymphocytes could enable more specifically directed immunotherapy to be used in conjunction with allo-HSCT to enhance the GvT effect.

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Issues of Psychological Control during Haematological Transplantation: early findings from a study in progress

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Control issues, and more generally, the ways people prefer to be in control of their lives are thought to be important in predicting psychological adjustment to cancer, patient compliance with treatment regimes, and subsequent quality of life. Although 'locus of control' measures have dominated this particular research, recent conceptual reviews point to the multifaceted nature of control, and accordingly, the importance of employing more sophisticated measures. Using one such measure, the purpose of this prospective study is to describe the control profile of a clinical population (n = 60-80) as they undergo and recover from a bone marrow or stem-cell transplantation. Specifically, the study investigates the relationship between psychological control (Shapiro Control Inventory), quality of life (EORTC QLQ-C30), and psychological distress (HADS) during transplant planning and then again one week after transplant, at hospital discharge, and 100 days after the transplant. While recruiting of participants continues for another year, early results after one year indicate that pre-transplant depressive symptoms are

significantly associated with impaired beliefs in ability to attain control, a sense of loss of control, and impaired control in the domains of body, mind, self, and wider impulses. The point at which there is the greatest risk of clinically significant mood or anxiety symptoms is at hospital discharge. Issues such as 'locus of control', desire for control, or the methods by which people gain control, were not significantly associated with psychological distress. If these early trends are sustained, this would have implications for the form, content, and timing of psychological consultations with people undergoing haematological transplantation.

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Ganciclovir Responsive Post-Engraftment HHV-6 Infection Following High Dose Melphalan Conditioning Autologous Peripheral Blood Stem Cell Transplant for Multiple Myeloma

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Human herpesvirus 6 (HHV-6) infection has been increasingly recognised after bone marrow transplantation, with a higher frequency of detection in allogeneic transplantation compared with autologous transplantation. Epidemiological and clinical characteristics were analysed retrospectively in 18 patients with multiple myeloma undergoing autologous peripheral blood stem cell transplantion (PBSCT) during a 12 month period (may2002-2003). All patients received a conditioning regimen of melphalan 200mg/m2 at D-1 and subsequently received filgrastim from D+1. 4 of the 18 patients developed a clinical syndrome characterised by lethargy, persistent high fever, abnormal liver function tests and subsequent HHV-6 PCR positivity. Clinically, these 4 patients developed a new onset, non-neutropenic and culture negative febrile illness within 72 hours of engraftment (ANC $>0.5x10^9$/L). This followed a period of no, or low grade, fevers within 24 hours of engraftment. These patients typically experienced a high, swinging fever between 38 and 39.5^OC unresponsive to empirical antibacterial and antifungal therapies. The duration of the fever prior to ganciclovir therapy was between 3-5days. At the onset of the fever, the neutrophil count ranged between 1.4-6.1x10^9/L. There were no focal symptoms or localising signs to suggest an alternative infectious cause of the illness. All 4 patients described lethargy and 2 of the 4 developed a fine, erythematous rash, 1 on the torso, and 1 on the face. 2 of the 4 patients also developed a raised GGT and ALP. All 4 patients were found to be HHV-6 PCR positive during the febrile illness. 3 of the 4 patients were HHV-6 PCR negative prior to the transplant, 1 patient having an invalid result due to PCR inhibition. 3 of the 4 patients were HHV-6 PCR negative post resolution of their symptoms, 1 patient was not retested. Of the 14 patients who did not develop HHV-6 infection, 10 were HHV-6 PCR negative prior to or during the transplant. 1 patient became HHV-6 positive at D18 during an episode of antibiotic responsive pneumonia. The remaining 3 patients did not receive HHV-6 testing. Neutrophil and platelet engraftment (ANC>0.5x10^9/L and plt>20x10^9/L) were more rapid in the HHV-6 positive group versus the non HHV-6 positive group, with a median of day 11 and day 13.5 for neutrophils ($p=0.05$ Mann-Whitney U test), and D18.5 and D20 for platelets ($p=0.28$), respectively. All 4 patients were treated with intravenous ganciclovir (5mg/kg bd) resulting in a dramatic resolution of their fever within 24-72 hours. The duration of the illness, from onset, ranged between 5 to 9 days. We conclude that HHV-6 infection post autologous PBSCT for multiple myeloma may be more common than previously recognized, and represents a readily treatable cause of febrile illness following autologous PBSCT.

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CMV Viraemia after Allogeneic Haemopoietic Stem Cell Transplantation in Patients Receiving Prophylactic Ganciclovir: impact of CMV PCR testing, from 1999-2003

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Cytomegalovirus (CMV) disease remains a significant cause of morbidity and mortality following allogeneic haemopoietic stem cell transplantation (HSCT). High-risk patients may be managed using either prophylactic or pre-emptive therapy with potent anti-viral agents such as intravenous ganciclovir. There are few studies of the incidence, risk factors and outcome of CMV infection in patients who receive prophylaxis, particularly since the introduction of polymerase chain reaction (PCR) techniques to detect CMV viraemia. This retrospective study reviews 84 consecutive patients receiving allogeneic HSCT at St Vincent's Hospital, Sydney in the four years from April 1999, when PCR testing for CMV became available at the hospital. Of the 84 patients, 69 (82%) were at high-risk for CMV disease (recipient or donor CMV IgG positive pre-transplant) and their transplant protocols included prophylactic ganciclovir as described previously (Atkinson *et al.*, 1991). CMV viraemia was detected using quantitative DNA PCR and qualitative RNA PCR. Testing was performed on 60 of 84 patients (71%), based on clinical suspicion of CMV infection. Twenty-one of 84 patients (25%) developed a positive DNA PCR at a median time of 67 days post-transplant (range, 28-181 days). Peak CMV DNA titres ranged from 315 to 127,000 copies per mL of plasma. On multivariate analysis, significant risk factors for CMV viraemia in this study were recipient CMV seropositivity ($p = 0.01$) and the use of a matched unrelated donor ($p= 0.02$). Of the 21 DNA PCR positive patients, 4 had biopsy proven CMV enteritis (5% of all allogeneic HSCT patients). There were no cases of CMV interstitial pneumonitis in the group. Management of significant CMV viraemia +/- disease consisted of 10-14 days of intravenous, treatment dose ganciclovir or foscarnet. At day 100 post-transplant, the actuarial survival probability in the CMV PCR positive group was 87%, comparable to the rate of 83% for the patients in whom CMV viraemia was not diagnosed. At one year, however, the survival of CMV PCR positive patients was 38%, compared to 67% for the patients who were not PCR positive. The results of this review support the role of PCR testing in detecting early CMV viraemia in HSCT recipients, in particular those receiving matched unrelated donor transplants. The late mortality of the CMV PCR positive patients emphasises the need for prolonged surveillance of these patients beyond day +100.
Reference: Atkinson et al. (1991) Br J Haematol 79:57-62.

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Cells, Cytokines and Prediction of Graft-versus-Host Disease

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By analysing donor T-cells, their specific subsets and the cytokines they produce, in response to antigenic stimulation by the recipient, a profile was developed that could assist definition of those recipients of HLA-identical sibling marrow that might develop acute GvHD after transplantation. Ninety-six bone marrow transplants recipients and their HLA-identical sibling donors were assessed from 6 different transplant centres. All recipients received unmanipulated bone marrow transplants in the period January 1990 to

June 2000. Pre-transplant the frequencies of IL-2, IL-4, IL-10 and INFγ releasing T effector cells were determined in the donors when stimulated by their respective HLA identical sibling recipients. The CTLL-2 bioassay was used to detect IL-2 production while Elispot technology was used to measure the other cytokines Cytokine producing T lymphocyte precursors were analysed for statistical relevance (Fisher's Exact Test) to development of acute GvHD after transplant. For IL-2 detection the HTLp assay, with a two-sided P value of <0.0001, was considered extremely significant. IL-10 production was negatively correlated with the post transplant development of severe acute GvHD. The two sided p value was 0.0048 and considered very significant. In this study group, IL-4 and IFN γ could be detected in 15-20% of all donors when stimulated by their sibling recipients, however, these donor cells did produce cytokines in response to PHA. Additionally an extremely significant difference was found between those recipients that developed grade 0-I when compared with those that developed grade II and higher when the response of recipient versus 3rd party HLA disparate was analysed. As a result of this study, 2 cytokine and cellular profiles have been developed. For those recipients of HLA identical sibling bone marrow that are more likely to develop acute (grade II-IV) GvHD there will be a higher frequency of IL-2 producing T cells in response to sibling and 3rd party HLA disparate alloantigen, combined with a lower frequency of IL-10 producing cells. For those recipients more likely to develop grade 0-I the frequency of IL-2 production is low but high for IL-10. The latter scenario is a typical cytokine profile for T regulatory cells. Profiling recipients in this way should prove useful as a guide for immunotherapy and to assist donor selection.

Abbreviations: HTLp Helper T lymphocyte precursor

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Enhanced Hemopoietic Recovery Following Transplantation with *Ex Vivo* Expanded Mobilized Blood CD34$^+$ Cells

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This study evaluated the efficacy of ex vivo expanded, CD34$^+$-selected peripheral blood progenitor cells (PBPC) to support repetitive cycles of high-dose chemotherapy (HDC) for treatment of metastatic breast cancer. The protocol involved 3 cohorts, each consisting of 3 patients with each receiving 3 cycles of HDC supported by PBPC with or without ex vivo expanded CD34$^+$ cells. Apheresis collections were either cryopreserved as an unmanipulated product or as an Isolex-300i selected CD34$^+$ fraction. Each HDC cycle was supported by transplantation with unmanipulated PBPC and/or ex vivo expanded (EXE) CD34$^+$ cells. CD34$^+$ cells were cultured for 12 days in stericell flasks containing X VIVO-10 media supplemented with 0.5% human serum albumin, G-CSF, SCF and MGDF (each at 100ng/ml) in GMP conditions. The final ex vivo expanded cell product was washed in the cleanrooms of the GMP facility by use of a Haemonetics Hemolite 2 blood salvage machine to yield between 250-300ml of cells. All patients received G-CSF post transplant until the absolute neutrophil count (ANC) was greater than 1,500 on two consecutive days. Infusion of EXE cells ($5.82 \times 10^9 \pm 3.23$, mean±SD, n=21) resulted in fewer days with ANC $<0.1 \times 10^9$/L (2.0±1.3 vs 4.5±1.6, mean±SD, p=0.0056; expanded vs unmanipulated PBPC), faster neutrophil recovery and fewer episodes of

febrile neutropenia. Regarding the impact on platelets (plt), the ex vivo expanded product contained 15.5±8.75% (mean±SD) megakaryocytic cells ($CD61^+CD42a^+$) and CTP cycles supported by EXE cells (n=21) required fewer plt transfusions with 29% of cycles requiring no plt support (cf to 0% of the 6 unmanipulated cycles and 0% in a historical control group, n=124). Furthermore, time to last plt transfusion (7d v 9d; p=0.036) and time to spontaneous platelet recovery (8d v 12d; p=0.043) was improved. Number of days to platelets to $>20x10^9$/L was no different (med=12d, p=0.6). Those patients transplanted with *ex vivo* expanded cells alone exhibited equivalent rates of neutrophil and platelet recovery to those transplanted with unmanipulated PBPC and expanded cells, suggesting that interaction between accessory cells within an unmanipulated PBPC graft and the expanded cells is not required to confer rapid haemopoietic recovery. These data demonstrate proof-of-concept that *ex vivo* generated haemopoietic cells alone, can enhance not only neutrophil, but also platelet recovery.

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A Phase I Study of PV701, a Milk Protein Extract for the Prevention of Mucositis in Patients Undergoing High-dose BCNU, Etoposide, Cytosine Arabinoside, Melphalan (BEAM) Chemotherapy and Autologous Peripheral Blood Stem Cell Transplantation

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Purpose: Grade 2-3 oral mucositis (OM) is a common side effect of BEAM chemotherapy. This study describes the use of PV701 mouthwash (PV701) for prophylaxis of OM in lymphoma patients (pts). PV701 contains a number of naturally occurring growth factors identified in the whey fraction of milk including members of the TGFβ, PDGF, IGF and FGF families. Previous *in vitro* and *in vivo* animal studies demonstrated its activity in stimulating epidermal cell growth and protection against cytotoxicity, and a Phase I study in normal human volunteers had demonstrated safety as an oral MW. Patients and methods: BEAM was administered on d-6 to d-2, autologous peripheral blood stem cells on d0, and PV701 (15 ml) was administered 6 times/day for 12 days, commencing on d-6 through to end d+5. The initial dose of PV701 was 13.5 mg PV701/ml and dose de-escalation was planned if dose-limiting toxicities (DLT) were experienced (in ≥ 2 pts/cohort). OM was graded according to the NCI-CTC (for Bone Marrow Transplant pts) by independent trained observers. The duration of enteral/parenteral feeding, the requirement for intravenous (i.v.) opiates and any admission to ICU were also recorded. Outcomes were compared to a group of 89 historical controls who received BEAM in the preceding 10 years. Results: 7/9 trial patients completed protocol treatment and the remaining 2 pts ceased PV701 early because of nausea related to BEAM. No DLT were seen, PV701 was well tolerated and no dose reduction was required. No increase in antibodies to PV701 was detected. When compared to the 89 historical control pts, PV701 treated pts had significantly less severe grade 2 and/or 3 OM (100% v 78%, p=0.0006) and had grade ≥ 3 OM for an estimated 5 fewer days than historical controls (median 10d v 5d, p=0.0003). There was also a further 2 days less grade 2 (developing/resolving) OM observed in the trial patients (median 13d v 11d, p=0.002). There was a significant reduction in the need for enteral/parenteral feeding (91% v 66%, p=0.012) and its duration (median 12d v 3d, p=0.010) and similarly, there was a lower likelihood of receiving i.v. opiates (98% v 78%, p=0.022), and a reduced duration of treatment (median 9d v 4d, p=0.0006). Conclusion: We conclude that PV701 mouthwash at a dose of 13.5 mg/ml is easily administered with minimal side effects. The efficacy data from this study are very encouraging and there is now a need for a randomized trial.

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New Agents in Myeloma

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Thalidomide: Thalidomide (T) can still be considered a 'new agent' in myeloma (MM) although the first comprehensive report of its activity in MM now dates back to 1999. Since then, a number of Phase II studies have confirmed a response rate in patients (pts) with relapsed disease of approximately 30%, with a further 50% demonstrating disease stabilization. Predictors of response include age <65yrs, normal LDH, absence of Chrom13 deletion and normal platelet count. Neurotoxity remains the most problematic side effect (SE) particularly for pts who have received >12 mo treatment. Remissions are generally short-lived with the median duration of 6-9 mo however there are pts that have remissions beyond 4yrs. The most contentious issue is appropriate dose – indeed, the only data on tolerability is the evidence that most pts post-autograft cannot tolerate doses in excess of 200mg. Moreover, most investigators agree (despite definitive data) that doses in excess of 400mg are rarely needed. Phase II data supports a synergistic effect with dexamethasone (D), with PR rates of approx 70% when used as front-line therapy. There are 2 ongoing international Phase III studies examining this question. A worrisome aspect of combination therapy is venous thromboembolism that occurs with T alone (3%), with D+T (10-15%) and T+ athracyclines (10-25%). The role of T in maintenance therapy or in non-progressive MM, is the subject of current studies. IMiDs: These molecules are derivatives of T and appear to work through similar, but non-identical, mechanisms. Revemid is the lead molecule and in Phase II studies achieves PR in approximately 30% of pts with a further 50% achieving disease stabilization. Of note 60%+ of pts studied to date had previously received T. Although neurotoxicity appears to be spared, cumulative haemopoietic toxicity is the major SE. Phase III studies are underway to compare revemid to D in relapsed disease. Combination studies with chemotherapy, D, or bortezomib are promising. Bortezomib (Velcade): Previously known as PS341, this is a proteasome inhibitor and has recently been approved in the US for the treatment of pts with relapsed MM. It appears that its ability to reduce NFkB-induced gene transcription of anti-apoptotic proteins is its major, but not sole, mechanism of action. A large 200 patient phase II study demonstrated it to achieve PR rates of 35% (with a median duration of response of 12 months) in heavily pre-treated pts. Significant toxicities include, nausea, fatigue, neuropathy, haemopoietic toxicity (and cost!). Oblimersen sodium (Genesense): The bcl-2 antisense is well tolerated and effective in combination with chemotherapy and steroids and a large phase III study in relapsed disease is nearing completion. Arsenic Trioxide: Unlike its action in APML, this molecule appears to have its major mechanism by inducing apoptosis through induction of intracellular hydrogen peroxide. Concomitant ascorbic acid appears to increase its efficacy by depleting reduced intracellular glutathione levels. To date, only small phase II studies have been performed but it frequently induces disease stabilization. Current studies are exploring shorter administration schedules and drug combinations (steroids, melphalan). Farnesyl Transferase Inhibitors: Amongst a variety of actions, the FTI's inhibits prenylation of the Ras protein thus inhibiting a variety of cell signalling pathways. Although true 'responses' are rare with Zanestra, disease stabilization is frequent and combination with chemotherapy is under investigation.

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Platelet Function Analysis - what is the clinical role?

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The laboratory investigation of a bleeding disorder manifesting as a disorder of platelet function typically requires a progression of testing processes. The initial investigation requires a clinical review (personal and family bleeding history, physical examination and a medication/drug review) together with basic laboratory testing which should include a full blood count (especially assessment of platelet count and potentially morphology, but also haematocrit and other parameters). Routine coagulation tests (PT, APTT, etc) may be useful but will be non-diagnostic. Testing of von Willebrand factor (VWF, including VWF-activity assays and factor VIII) is usually warranted to exclude von Willebrand disorder (VWD), since this disorder may explain the majority of clinically suspected 'platelet-like defects'. Subsequent or parallel testing of 'platelet function' may or may not be warranted, depending on the results of the above preliminary testing and the strength of the clinical suspicion. In this case, there are three main options: (a) (skin) bleeding time (SBT); (b) a platelet function screening test such as the PFA-100; and/or (c) full platelet function (aggregation) studies. We and most other laboratories no longer provide SBT tests, as these are neither sensitive nor specific to disorders of platelet function, nor do they predict bleeding risk. The decision to perform a platelet function screening test and/or full aggregation studies should be based on its effect on future clinical support. If the test result will not affect clinical management, is there really a need to perform it? The PFA-100 is certainly sensitive to severe VWD and severe disorders of platelet dysfunction (eg Glanzmann's Thrombasthaenia, Bernard-Soulier Syndrome, and pseudo/platelet-type VWD), but is only moderately sensitive to mild VWD and mild disorders of platelet dysfunction (eg storage pool deficiencies, secretion disorders and Herman-Pudlak syndrome). In any case, whilst a normal PFA-100 will exclude severe defects, and an abnormal PFA result may suggest a defect, the test is non-diagnostic, and full aggregation studies may be required in order to diagnose the disorder. Nevertheless, there are often advantages to starting the diagnostic process with the PFA, as this may provide a therapeutic monitoring tool later (eg an initially abnormal PFA corrected after DDAVP therapy will provide some clinical reassurance). Alternatively, will full aggregation studies providing evidence of mild platelet dysfunction such as secretion defects really provide any clinical utility? If the answer is no, which it often is, then is performance of the test indicated or justifiable? So, what is the clinical role of platelet function analysis? The answer is somewhat complicated; it depends on what the purported defect may be, what form the clinical management may take, and ultimately, will the test result affect the patient's future management? In our own experience, full aggregation studies are rarely justifiable, and the PFA-100 more often forms a part of the laboratory investigation.

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What Have Factor IX Mutations Taught Us?

Harper P

Studying mutations in Haemophilia has made a great contribution to understanding the cause of genetic diseases. As a model disease to study genetic disorders, haemophilia has two advantages over many other conditions; it is sex-linked and in severe cases has virtually a hundred percent penetrance. This means that affected males are easily identified and have a single copy of the affected gene. Over the last twenty years a large number of mutation in the factor IX gene have been identified. On the international

database (version 12) there are 2,511 patient entries with 896 unique molecular events. The list contains 523 different amino acid substitutions including mutations at nine of the twelve □-carboxylation sites and all 22 cysteine residues confirming the structural importance of the disulphide bonds. In many cases it is easy to understand how a specific mutation can result in deficiency. For example it is obvious that a large deletion or insertion will result in defective protein synthesis; there are 180 short insertions or deletions reported in the factor IX gene. Where haemophilia has proved particularly useful is in understanding less severe disease. Only about 25% of mutations reported are for cases of mild or moderate disease. One group of particular interest are the splice site mutations which account for about 9% of mutations in haemophilia B. In this talk I will give an overview of mutation in the factor IX gene with particular reference to splice site mutations. I will review the mechanism of RNA splicing and, using cases identified in New Zealand, illustrate how mutations at the splice site junction or within the polypyrimidine tract in the factor IX gene can result in both severe and mild haemophilia B.

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Motexafin Gadolinium (MGd) is Cytotoxic to Steroid and Chemotherapy-resistant Myeloma Cell Lines: enhancement of apoptosis by redox regulation

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The novel agent, MGd, is a metallo-porphyrin that has been studied as a radiation and chemotherapy sensitizing agent in tumor cell lines and in patients with metastatic solid tumors. MGd accumulates in tumor cells, and the mechanism of action is related to its electron affinity (reducibility) so that it catalyzes the oxidation of several intracellular reducing metabolites forming hydrogen peroxide and other reactive oxygen species (ROS). MGd has not been previously studied in hematopoietic malignancies. We postulated that redox regulation with MGd would result in cell cytotoxicity in myeloma cell lines by apoptotic mechanisms. We studied cell viability and mechanism of cell death in the sensitive myeloma cell line, C2E3, the steroid resistant cell lines, 1-310 and 1-414, the chemotherapy-sensitive cell line, 8226, and the highly chemotherapy-resistant cell line, DOX-10V. Cellular viability was measured by microculture tetrazolium and trypan blue dye exclusion following treatment with 25πM, 50πM, 75πM and 100πM of MGd from 24 to 72 hours. To assess the mechanism of MGd cytotoxicity in myeloma cells, we investigated the redox potential by measuring ROS by flow cytometric analysis and intracellular glutathione (GSH) by colorimetric analysis. Mitochondrial membrane potential (MMP) and Annexin V activity were evaluated by flow cytometry as indicators of apoptosis. Our results showed dose and time-dependent cytotoxicity in all cell lines (at clinically achievable doses) with maximal killing noted at 24 hours. After 24 hour 75□M MGd exposure, more than 60% cytotoxicity was seen in the C2E3, 1-414, 1-310 and DOX-10V cell lines. We demonstrated a 12 fold increase in ROS in C2E3, greater than 20 fold increase in 8226, 4 fold increase in 1-414 and a 6 fold increase in the DOX-10V cell line following 24 hour exposure with 50□M of MGd. Furthermore, in the C2E3 cell line, GSH was depleted by more than 60% following 24 exposure with 50□M MGd. In subsequent experiments, a 4 and 5 fold decrease in MMP with concomitant 8 and 10 fold increase in Annexin V was demonstrated in the C2E3 and 1-310 cell lines, respectively, following 50□M to 75□M exposure of MGd. We also

demonstrated, using fluorescence microscopy, that there was co-localization of MGD and ROS in the mitochondria. We conclude that MGd has significant cytotoxicity in steroid and chemotherapy-sensitive and steroid and chemotherapy-resistant myeloma cell lines. Moreover, the mechanism of cytotoxicity in these cell lines is related to apoptosis, presumably induced by redox regulation. Further study of the mechanism of action of MGd and related porphyrin derivatives is warranted, and clinical studies of synergy with MGd and redox active agents (doxorubicin and bleomycin) or radiation in hematopoietic malignancies are logical consequences of these observations.

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Cyclin D1 Overexpression in Myeloma Does Not Affect Survival, while Increased MMSET Expression may be Associated with a Favourable Prognostic Effect

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Chromosome 14q32 translocations occur in up to 80% of myeloma, but their pathogenic significance is not yet clear. The most frequent translocation partners are chromosomes 11q13, 4p16 and 16q32. We examined the expression of 2 candidate oncogenes on chromosomes 11q and 4p, Cyclin D1 (CND1) and MMSET respectively, in purified myeloma plasma cell (MPC) populations, using real-time RT-PCR with β-actin as internal control, Sybr Green I as fluorophore and standards containing $10^3 – 10^7$ cDNA copies. PCs were purified by flow sorting on the basis of CD38hi and CD138 expression. CND1 was quantitated in 18 MPC populations, with CND1: β-actin cDNA ratios ranging from 0.01 to 314. CND1 is not expressed in normal PCs (CND1: β-actin ≈ 0). In 11/18 samples, CND1: β-actin was 0.01 - 1.4. Seven samples demonstrated increased ratios of 8.2 to 314, indicating CND1 overexpression. There was no significant difference between the 2 groups in survival, β_2 microglobulin (4.2 and 6.0 mg/L respectively, normal range 0 – 2.4 mg/L) or mean PC labelling index (PCLI) (5.0% and 5.8% respectively, normal range 0 – 4% measured by flow cytometry). The correlation between CND1 expression and FISH detection of t(11;14) has been confirmed in 11 samples. The t(4;14) translocation was detected by nested RT-PCR for "hybrid" cDNA containing IgH and the candidate oncogene MMSET, using myeloma cell lines carrying t(4;14) as internal controls. Of 32 samples examined, 7 were positive. Survival of the 2 groups also showed no significant difference. In a separate group of 12 patients, MPCs were examined for MMSET expression using real-time RT-PCR. MMSET: β-actin cDNA ratios ranged from 0.1 to 22. MMSET expression has also been reported to be low or undetectable in normal PCs. Eight of the 12 MPCs examined showed MMSET: β-actin ratios of 0 to 1.6. The other 4 samples demonstrated higher ratios of 9 to 22, indicating increased MMSET expression. Survival of these 4 patients appeared to be superior compared with the other 8 patients, although not significant due to small sample size. The mean PCLI of the 2 groups with low and high MMSET expression were 2.4% and 7.4% respectively (normal range 0 – 4%). In summary, expression analysis of candidate genes in chromosome 14q32 translocations in purified MPCs has shown no effect of CND1 overexpression on survival, while a possible favourable prognostic effect of MMSET upregulation will be further examined in a larger cohort.

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Significance of Microaggregates Detected by Immunohistology in Plasma Cell Proliferative Disorders

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Objectives: Monoclonal gammopathy of undetermined significance (MGUS) is distinguished from plasma cell myeloma (PCM) in the WHO classification by paraprotein <30g/L, marrow plasmacytosis <10% and the absence of end-organ damage and bone lesions. Complete remission (CR) after chemotherapy for PCM is defined as less than 5% marrow plasma cells and undetectable monoclonal protein by immunofixation. Frequent relapses despite CR suggest the need for better indicators. Detection of residual disease by flow cytometry, interphase FISH and PCR requires high quality aspirate samples often lacking post-chemotherapy. Furthermore, such methods are not applicable to all patients. Bone marrow trephine immunohistology (IH) for plasma cell microaggregates (MA) is an alternative method for the diagnosis of early PCM and for the detection of residual disease after chemotherapy. It allows the pathological clustering of plasma cells in the marrow to be examined with greater reliability than H&E staining. The method is applicable to all patients with plasma cell dyscrasia and is independent of aspirate quality. Methods: 44 patients diagnosed according to WHO criteria were included; MGUS (23), PCM (9) or CR after chemotherapy (12). B5 fixed paraffin sections were stained with CD138 (Clone MI15), VS38c and Bcl-2 (Clone 124) antibodies (DAKO). MA were defined as contiguous, non-perivascular, inter-fat space collections of 10-30 plasma cells on IH. Results: Table 1 shows that 13% of MGUS patients had MA. In contrast, 100% patients with myeloma (10-15% plasma cell infiltration) had MA. After chemotherapy, 40% with <5% plasma cells and no paraprotein on immunofixation (IF) had MA. In those with positive IF, all had MA.

Table 1.

	MGUS	Early PCM (10-15% plasma cells)	Post-chemotherapy (<5% plasma cells and IF **negative**)	Post-chemotherapy (<5% plasma cells and IF **positive**)
Patients (%) with MA on IH	3/23 (13%)	9/9(100%)	2/5(40%)	7/7(100%)

Discussion: In this study the immunohistological detection of plasma cell microaggregates was universal in early myeloma. The presence of MA in 13% of MGUS patients is interesting. Whether it is predictive of progression to myeloma will require a large study with extensive follow-up. This will determine whether it is a more reliable marker of early myeloma than the current diagnostic criteria. After chemotherapy for PCM, it appears that the presence of MA is a better marker of residual disease than the percentage of plasma cells. In contrast to flow cytometric, cytogenetic and molecular analyses, the detection of MA as a marker of residual disease is applicable to all patients and is not reliant on aspirate quality. In summary, the immunohistological detection of plasma cell microaggregates is a useful marker of early myeloma and residual disease after chemotherapy.

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Xenobiotic Gene Polymorphisms Alter Susceptibility to Multiple Myeloma

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Aim: Epidemiological studies have suggested that the incidence of multiple myeloma (MM) is associated with both environmental exposure to carcinogens and inheritance of as yet unidentified genetic risk factors. Genetic variations in the activity of xenobiotic enzymes responsible for the detoxification of environmental carcinogens may predict individual susceptibility to this disease. We therefore conducted a case-control study to determine if xenobiotic gene polymorphisms were associated with increased risk of MM. Methods: Polymerase-chain-reaction based methods were used to detect gene polymorphisms for glutathione S-transferases mu and theta (GST M1 and GST T1), paraoxonase (PON1) and N-acetyltransferases 1 and 2 (NAT1 and NAT2) and cytochrome P-450 (CYP1A1) in 90 patients with MM and 205 healthy Australian Caucasians. Genotype frequencies were compared using Fisher's exact tests (two-tailed) and unconditional logistic regression. Results: There was a significant increase in incidences of GST T1 null, PON1 BB and NAT2 slow acetylation genotypes in MM cases compared with controls (24% vs. 14%, p= 0.04; 18% vs 11%, p=0.04; and 66% vs 50%, p=0.01) respectively. Multivariate analysis (Table 1) revealed that GST T1 null was the most significant risk factor for MM (p=0.008, adjusted OR=2.47, 95% CI, 1.26 – 4.80), followed by PON1 BB (p=0.02, adjusted OR=2.66; 95% CI, 1.20 – 5.88) and NAT2 slow acetylation genotypes (p=0.02, adjusted OR=1.93; 95% CI, 1.13 – 3.30).

Table 1: Comparison of GST T1, PON1 and NAT2 polymorphism frequencies in cases and controls (multivariate analysis).

polymorphism		**MM patients n = 90**		**Controls n = 205**		**Odds ratio**	**95% confidence interval**	**P value**
		n[a]	(%)	n[a]	(%)			
GST T1	**pos**	68	(76)	176	(86)	**2.47**	1.26-4.80	**0.008**
(null vs pos)	**null**	22	(24)	29	(14)			
PON1	**AA**	33	(37)	103	(52)	**2.66**	1.20-5.88	**0.02**
(BB vs AA)	**BB**	16	(18)	22	(11)			
NAT2[a]	**Rapid**	31	(34)	98	(50)	**1.93**	1.13-3.30	**0.02**
(slow vs rapid)	**Slow**	59	(66)	98	(50)			

[a]NAT2 rapid acetylators consist of all geneotypes containing allele 1 (ie.,1/1, 1/2, 1/3, 1/4), whereas any other combination of the four alleles produces a slow acetylation phenotype.

Conclusions: This study supports the notion that environmental exposure to particular chemicals may contribute to the development of MM, and further suggests that risk of disease may be dependent on inherited polymorphisms in genes responsible for metabolising these substances, namely GST T1, PON1 and NAT2.

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IL-12 and IFN- Corrects the Dendritic Cell Defect of Patients with Myeloma

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Development of effective immunotherapy strategies in multiple myeloma will depend on a thorough understanding of any immune defect in patients with the disease. Binding of B7 to CD28 on T cells is an essential costimulatory signal during antigen presentation. The strength of this signal determines the fate of the cell - anergy, apoptosis or productive immunity. We have previously shown that high potency (CMRF44+ve) dendritic cells (DCs), in the peripheral blood of patients with multiple myeloma do not upregulate CD80 *in vitro*, in response to huCD40LT. This defect is attributed to TGF-β and IL-10 produced by malignant plasma cells. We have also shown *in vitro* that anti-TGF-β is effective at neutralizing this effect. However, as anti TGF-β is not suitable for use *in vivo* it is therefore necessary to find a clinically acceptable agent. We enumerated high potency DCs (CMRF44+ve CD14-ve CD19-ve PI-ve) and found no significant difference between patients with multiple myeloma (0.03-0.8% of MNCs) and normal controls (0.05-0.8% of MNCs). CD80 and CD86 expression on the DCs of patients with myeloma (29±17% and 85±10%) was also not significantly different to normal controls (29±17% and 86±17%). Normal control DCs and B-cells upregulated CD80 in response to huCD40LT, although DCs from patients with myeloma demonstrated either no increase or less than expected increase in CD80 expression. Furthermore, rhTGF-β dose dependently inhibited CD80 upregulation by huCD40LT. Less than 10% of malignant plasma cells expressed CD80 and these cells failed to upregulate expression of CD80 in response to huCD40LT. CD86 (B7.2) expression was high, preceding (86%) and following (89%) stimulation with huCD40LT. IL12 and IFN-γ were investigated as possible alternatives to anti-TGF-β as they are suitable for *in vivo* use. IL12 and IFN-γ, were capable of neutralising inhibition of CD80 upregulation by TGF-β. Subset analysis was performed on DCs stimulated by huCD40LT. DCs were predominantly CD11c+ve CDw123-ve identifying them as myeloid and therefore suggesting that they are capable of mounting a Th1 immune response. Thus, IL12 and IFN-γ are capable of neutralising inhibition of CD80 upregulation in high potency myeloid DCs and may present suitable alternatives to anti-TGF-β which is unsuitable for *in vivo* use.

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RNA-loading of CMRF-56 positive blood dendritic cells is a promising strategy for multiple myeloma immunotherapy.

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Immunologic responses to the malignant plasma cells of multiple myeloma (MM) patients are being investigated for their ability to prevent disease relapse after autologous and allogeneic haematopoietic stem cell transplantation. Dendritic cells (DC) are specialized leukocytes that have the capacity to prime and direct an immune response against tumour-associated antigens (TAA). We have used new Trucount® technology to evaluate the whole blood DC subset composition of healthy donors and MM patients. A CMRF-56 monoclonal antibody-based immunomagnetic selection procedure was used to enrich blood DC for functional studies from the peripheral blood mononuclear cells of healthy donors and MM patients. The CMRF-56^+ blood DC preparation is able to present MHC class I- and II-restricted peptide antigens and has been used to generate cytotoxic T lymphocytes (CTL) against a MM-related TAA. CMRF-56^+ blood DC from MM patients are efficiently activated *ex vivo* and induce autologous and allogeneic mixed lymphocyte responses. We have optimised the loading of CMRF-56^+ blood DC preparations with antigen-encoding mRNA and have shown that enhanced green fluorescent protein mRNA is rapidly translated after electroporation into blood DC. In addition, influenza matrix protein (FMP) mRNA-loaded blood DC can process and present antigen to FMP-specific CTL clones and prime FMP-specific CTL responses in whole PBMC populations. We are currently in the process of generating responses against total RNA extracted from MM cell lines prior to initiating a clinical trial of RNA-loaded CMRF-56^+ blood DC in patients.

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The Utility of Whole Peripheral Blood FISH in the Quantitation of BCR/ABL in CML Patients: a comparison with bone marrow FISH and conventional cytogenetics

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Aim: The hallmark of CML is the BCR/ABL fusion gene that is usually formed as a result of the t(9;22) translocation leading to the formation of the Philadelphia chromosome. Conventional cytogenetic analysis has been the standard method for monitoring the Philadelphia (Ph) chromosome, however, evaluation of the BCR/ABL fusion using Interphase Fluorescence in Situ Hybridisation (FISH) on peripheral blood may allows more frequent and less invasive follow up in CML patients. The objective of this study was to compare the utility of whole peripheral blood FISH (without CD3 negative cell selection) versus bone marrow FISH and conventional cytogenetics in patients with CML following treatment with imatinib mesylate. Methods: 30 sets of peripheral blood and bone marrow aspirate samples (taken simultaneously) from 30 Ph +ve CML patients at various stages of disease were assessed. The bone marrow samples were processed by the standard cytogenetics procedures and G-banded analysis of at least 20 metaphases per sample was performed. Interphase FISH on the peripheral blood and bone marrow samples was carried out by scoring positive signals in 600 nuclei in each sample using BCR/ABL dual fusion or extra signal probes (Vysis). For each test, the percentage of the Ph +ve cells was reported. Bland and Altman plots were constructed to assess the level of agreement between these tests. The mean differences (with 95% confidence intervals) and the Spearman Rank Correlations were then measured. Results: The mean difference in the percentages of the Ph +ve cells measured by BM FISH exceeded the PB FISH by 5.4% (-0.27, 11.15) with a rank correlation of 0.36 (P: 0.051), when we compared the mean difference in the percentages of cytogenetics to BM and PB FISH, there was a tendency for cytogenetics to be 2.7% (-0.52, 5.95)

higher than BM FISH and 8.2% (1.56, 14.75) higher than PB FISH with rank correlations of 0.41 (P: 0.021) and 0.56 (P: 0.001) respectively. Conclusion: There is good agreement of PB FISH with BM FISH and BM FISH with cytogenetics in monitoring patients with CML following therapy. There is though a statistically significant difference between cytogenetics and PB FISH values; this may be related to the lack of the CD3 negative cell selection when measuring the Ph +ve cells in the PB samples. The above difference needs to be considered when monitoring patients by PB FISH studies.

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Imatinib Produces Substantial Molecular Remissions In Interferon Treated Chronic Phase (CP) Chronic Myeloid Leukemia (CML) In Longstanding bcr-abl Positive Cytogenetic Remission (CCR)

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Alpha-interferon (IFN) based therapy was formerly standard initial therapy for de novo CP CML not immediately proceeding to allograft. While producing survival benefit and CCR rate of 10-30%, IFN was associated with substantial early and late toxicity, low molecular remission rate (<5%) and threat of late relapse including blast crisis. Imatinib has become the standard of care and produces significantly greater reduction in bcr-abl in de novo CML. The ALLG explored Imatinib in 22 longstanding IFN-treated CP patients in CCR or near CCR (<10% Ph+ve metaphases). Patients had been in CCR/near CCR for 30 months (6-117) and were 63 (22-176) months from diagnosis. IFN was stopped and Imatinib 400mg daily commenced. Patients were assessed for toxicity and efficacy with serial 3 monthly bone marrow cytogenetic and quantitative bcr-abl/bcr analyses on peripheral blood and marrow. No patients experienced significant toxicity with Imatinib being well tolerated. 15 patients (12- CCR, 3 – near CCR) are assessable following 9 months of Imatinib therapy: six patients have achieved molecular remission (peripheral blood bcr-abl/bcr analysis) while three patients have reduced levels and six patients stable levels on peripheral blood bcr-abl/bcr analyses since study commencement. This latter group tended to contain those who have already exhibited major molecular response on Interferon whereas patients who achieved molecular remission or significant reduction tended to be those who had not achieved such pre-trial responses. There was no significant difference between the median peripheral blood and bone marrow values at each assessment timepoint emphasizing suitability of peripheral blood for molecular monitoring. Crossover to Imatinib in long term IFN-treated patients is safe and can produce significant molecular remissions without toxicity. The efficacy in this setting supports studies investigating combined or sequential IFN-Imatinib approaches in chronic phase.

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Imatinib Mesylate is Effective Treatment for Idiopathic Hypereosinophilia (IH)

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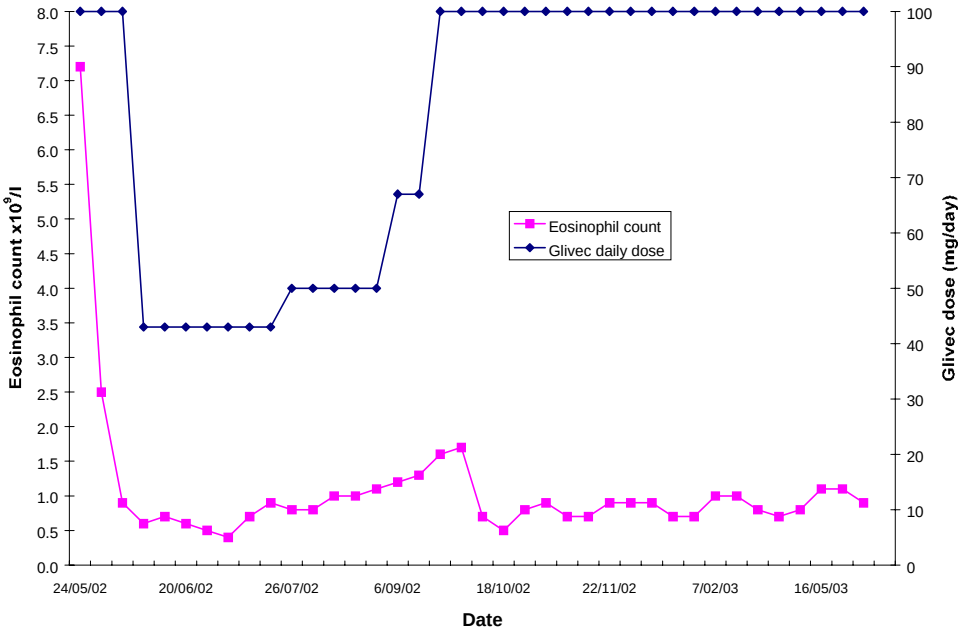
Idiopathic hypereosinophilia (IH) is a rare disorder with sometime significant clinical effects on the skin, gastrointestinal tract and heart along with other body symptoms. The thymidine kinase inhibitor imatinib mesylate (Glivec) has been reported as having a significant response rate in IH. Case reports to date have suggested that a significantly lower dose than that needed for the control of chronic myeloid leukaemia is required. In addition, it is unclear what the mechanism of action might be as the usual gene targets of imatinib do not seem to be involved in the process. This is a report of a patient with refractory IH achieving prolonged control of his condition and clinical manifestations with the use of low dose imatinib. Case report: A 52 year old man presented in 1996 with persistent diarrhoea. Investigations revealed a peripheral blood eosinophilia of $10x10^9$/l and lower gastrointestinal biopsies confirmed eosinophilic enteritis. No infective pathogen was confirmed and the bone marrow was consistent with hypereosinophilic syndrome with normal cytogenetics. Prednisolone and hydroxyurea improved the diarrhoea but made little impact on the blood with eosinophils rising to $30x10^9$/l. In May 1997 he presented with cardiac failure and endocardial thrombosis. The eosinophils were $70x10^9$/l. He was anticoagulated and treated with vincristine for 18 months and then vinblastine for a further 2 years. Eosinophils varied between 1.8 and $6.5x10^9$/l. Peripheral neuropathy and loss of haematologic control led to a trial of imatinib (patient funded) with doses and control as shown in the figure below. Now stable on 100mg per day, the eosinophils remain less than $1x10^9$/l, diarrhoea is not present and corticosteroids have been able to be withdrawn for the first time. IL-5 levels have dropped somewhat on treatment (diagnosis: 637.05pg/ml, prior to imatinib 131.42 pg/ml and after 4 months of imatinib 97.22pg/ml). Conclusions: Imatinib is an effective therapy for this condition but comes at a significant financial price. Funding mechanisms for non-approved indications of expensive medications require a rethink.



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Inhibitory Effect of Imatinib on the In Vitro Development of the Human Monocyte Macrophage Lineage

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The anti-leukaemic tyrosine kinase inhibitor, imatinib (Glivec, formerly STI571), is a 2-phenylaminopyrimidine derivative that has been reported to specifically inhibit the growth of *bcr-abl* expressing CML progenitors. This is achieved at levels of 0.1-5.0μM imatinib, by blocking the ATP binding site of the kinase domain of *bcr-abl*. Inhibition of the *c-abl*, platelet derived growth factor receptor and stem cell factor receptor (*c-kit*) tyrosine kinases by imatinib has also been reported. Here, we used semi-solid agar colony assays and non-specific esterase staining to demonstrate that the addition of imatinib to cultures stimulated with IL-3, IL-6, G-CSF and GM-CSF significantly decreased the number of monocyte/macrophage colonies, whilst eosinophil and neutrophil colonies were less affected. This inhibition was observed at concentrations of imatinib as low as 0.3μM. The use of GM-CSF and M-CSF (alone or in combination) to stimulate colony growth increased the number of monocyte/macrophage colonies and confirmed the inhibitory affect of imatinib on the growth of these cells. Liquid culture assays demonstrated and confirmed an inhibition of cell proliferation without affecting viability, following treatment with imatinib, although cells remained too immature to determine if a particular cell type was preferentially inhibited. The maturation of peripheral blood monocytes into macrophages by stimulation with M-CSF or GM-CSF was also found to be inhibited following treatment with 1.0μM imatinib or greater. This inhibition was seen morphologically following staining with naphthol acetate esterase, and phenotypically following flow cytometric analysis of surface expression of CD14, CD11c, HLA-DR and carboxypeptidase M. Furthermore, functional studies revealed that as a consequence of imatinib-induced inhibition of monocyte maturation, the ability of these cells to

respond to stimuli and produce the proinflammatory cytokines IL-6 and TNF-α was diminished, as was the ability to phagocytose zymosan particles. Imatinib was found to inhibit the monocyte/macrophage development in cultures stimulated with and without M-CSF, suggesting that inhibition of the M-CSF receptor, *c-fms*, may not be responsible for the effects observed in culture. Imatinib may therefore have inhibitory activity for other tyrosine kinases that play a role in monocyte/macrophage differentiation. The inhibition of normal monocyte/macrophage development and maturation was observed at concentrations of imatinib achievable pharmacologically, suggesting that imatinib or closely related derivatives may have potential for the treatment of diseases where monocytes/macrophages contribute to pathogenesis.

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GM-CSF Can Modulate the Anti-proliferative Effect of Imatinib on CML CD34+ Cells

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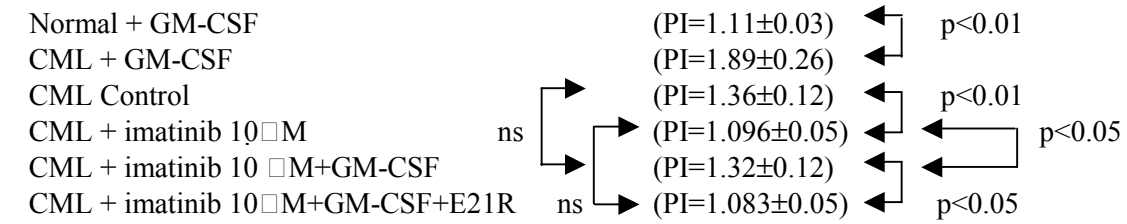
CML cells gain a survival advantage over normal cells due to dysregulated tyrosine kinase activity of BCR/ABL protein, which phosphorylates a number of proteins, potentially activating multiple signal transduction pathways. Imatinib specifically targets BCR/ABL protein and results in anti-proliferation and apoptosis. Even in the absence of mutation, the leukaemic clone may not be eradicated by imatinib, suggesting that progenitors may not be absolutely reliant on BCR/ABL activity for survival. Reports on GM-CSF production by CML cells suggest a possible autocrine role, therefore we examined if GM-CSF could protect CML CD34+ cells from imatinib. CD34+ cells from peripheral blood of patients with CML in chronic phase (n=5) and from normal bone marrow donors (n=5), were labelled with CFSE (**C**arboxy-**F**luorescein diacetate **S**uccinimidyl **E**ster) to enable tracking of cell division. Normal and CML samples were cultured with and without GM-CSF (300pg/ml) to assess response to this cytokine as a single agent. CML CD34+ cells were cultured for 3 days in serum deprived medium with imatinib alone, GM-CSF (300pg/ml) + imatinib, GM-CSF (300pg/ml) + E21R (a GM-CSF analogue able to block cytokine binding) (10□g/ml), and GM-CSF (300pg/ml) + E21R (10□g/ml) + imatinib. In each condition, imatinib was titrated over the range of 0 to 10□M. Cultures were analysed by flow cytometry to evaluate the proliferation index (PI), a ratio of final cultured cells to precursor cell number, where a PI of 1 represents no proliferation, and a PI of 2 corresponds to approximately three division cycles on average. Data are summarised in the following list.



GM-CSF induced strong proliferation in all CML, but not normal samples. Imatinib reduced proliferation of CML CD34+ cells at 1□M and above, and the addition of GM-CSF reduced this anti-proliferative effect at concentrations of imatinib up to 10□M. The protective effect of GM-CSF was clearly blocked using E21R. As the GM-CSF receptor and BCR/ABL may share common signalling pathways, when BCR/ABL activity is blocked by imatinib, GM-CSF may be able to compensate to maintain cell viability and proliferation. This finding has implications for optimising imatinib therapy by manipulating cytokine signalling. We are currently examining if apoptosis induction by imatinib can be modulated by GM-CSF.

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The Value of Imatinib Sensitivity Studies as Predictors of Cytogenetic and Molecular Response in CML Patients.

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Imatinib mesylate (Glivec, Novartis) blocks the binding of ATP to the kinase domain of the Bcr-Abl fusion protein, preventing further downstream signalling and hence reversing the clinical and haematological abnormalities of chronic myeloid leukaemia (CML). While remission rates are high with imatinib therapy, not all patients achieve cytogenetic responses and relapse is a problem, especially in advanced phases of the disease. In this setting, the development of assays that predict primary and secondary resistance, would be valuable. We are currently assessing several assays. Two of them assess the phosphotyrosine content of the adaptor protein CrkL, which is phosphorylated by Bcr-Abl. CrkL binds Bcr-Abl directly and is thought to be important in downstream effector signalling. We have demonstrated in-vitro dose-dependent inhibition of CrkL phosphorylation in the presence of imatinib in 10 patients. In a further 3 patients who developed secondary resistance due to mutations we have shown a rise in the in-vitro dose of imatinib required to reduce the level of p-CrkL by 50% (IC50). In the current Australian study of 100 newly diagnosed CML patients treated with imatinib 600mg/day (TIDEL) we are studying the level of p-CrkL at presentation, assessing the IC50, and assessing the effect of imatinib on p-CrkL levels in the blood weekly over the first 28 days. Phosphorylated CrkL has been analysed in 60 patients. The median level of p-CrkL at presentation was 56% within a range of 23 to 80%. The median IC50 was 0.75µM with 6 patients showing an IC50 greater than 1µM. The median decrease in the level of p-CrkL after 7 days of imatinib therapy was 38% (n=23 patients). After 3 to 4 weeks of therapy the median decrease in p-CrkL was 52% (n=25). The decrease in p-CrkL at 3 to 4 weeks showed a correlation (R^2 = 0.62) with the decrease in the percentage of Bcr-Abl transcript at 1 month. In the third assay using potent haemopoietic growth factors as stimuli the metabolic activity of cells as determined by the conversion of NAD to NADPH is monitored in response to varying doses of imatinib. To date 9 patients on the TIDEL trial have been examined. The median IC50 in this assay was 2.25µM with a range of 0.75 to 7.5µM. Clinical, cytogenetic and molecular data at 1, 3 and 6 months will be examined as each time point is reached to assess the prognostic value of these assays of imatinib sensitivity.

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Detection of Antigen Specific T cells in the Blood of Patients with Myeloma using MHC Tetramers containing Immunodominant Peptides to CMV and Tumour Antigens

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There has been much interest in the development of immunotherapy strategies for patients with myeloma. Patient idiotype protein produced by malignant plasma cells, would seem an ideal tumour antigen, although it is unclear as to whether idiotype specific T cells are tolerised or deleted. Previous studies have used assays of proliferation, cytokine release and cytotoxicity to detect antigen specific T cells. More recently the use of MHC Class I tetramers has allowed the

direct detection of populations of antigen specific CD8+ve T cells. We sequenced both heavy and light chain hypervariable regions of the immunoglobulin genes from 6 HLA-A*0201+ve patients with expanded T cell clones and IgG_1 idiotype. Immunoglobulin sequences were translated into amino acid sequences and 2 separate bioinformatic algorithms were used to predict that 3/6 patients had immunodominant peptides. Peptides from 2 surviving patients, that scored highly using bioinformatic algorithms, were used to synthesise fluorochrome conjugated MHC Class I tetramer complexes, which were used to search for patient idiotype specific T cells. Initial staining, according to manufacturers instructions, failed to detect idiotypic T cells in the peripheral blood of either patient. T cells specific for MUC-1 and MAGE-3 were also not detected in a further 10 patients. CMV tetramers were used to optimize additional staining conditions. A modified tetramer staining strategy based on conditions of the cytotoxicity assay, detected idiotype specific T cells in an activated T cell population, however failed to detect MAGE-3 and MUC-1 specific T cells in either the non-activated or activated T cell population. Tetramer staining at 4ºC, peptide inhibition of the TCR:CD3 complex and cross over control studies suggest that T cells detected after modified staining have low specificity and avidity. This study has identified a strategy by which to select idiotype specific T cells, monitor them during idiotype vaccination and potentially isolate them for adoptive immunotherapy. We have demonstrated that immunodominant peptides are rare in patients with multiple myeloma and we have also demonstrated that T cells specific for idiotypic peptides may be deleted, which could explain the poor response to immunotherapy.

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A Study Using F-18 FDG PET AND SPECT to Track the Destination of Macrophage-Activated Killer (MAK) Cell Therapy in Patients with Epithelial Ovarian Cancer

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Objective: Clinical trials are in progress to evaluate the efficacy of MAK cell immunotherapy in adjuvant and relapsed epithelial ovarian cancer. Despite demonstrated efficacy in Phase II trials, little is known about the *in vivo* distribution of these cells following intraperitoneal (i.p.) or intravenous (i.v.) injection, or how well the cells localise to tumour sites. The objective of this study was to track the destination of MAK cells using PET or SPECT imaging after delivery via i.v. or i.p. injection to patients with recurrent epithelial ovarian cancer. PET scanning allows excellent spatial resolution while SPECT allows prolonged tracking of cells. Methods: MAK cells were produced by in-vitro culture of autologous monocytes collected via apheresis from patients with recurrent ovarian carcinoma. The cells obtained by apheresis were stimulated in culture by a combination of the cytokines γ interferon and GM-CSF. Following culture, the cells were bound to an antibody (hMAb), which binds to a receptor (HER2) on the surface of ovarian cancer cells, and has the potential to improve tumour cell recognition by MAK cells. All processing was performed using the MAK Cell Processor under GMP conditions. Prior to injection, the MAK cells were labelled with either F-18 Fluorodeoxyglucose (FDG) or Indium-111. Patients then underwent serial PET or SPECT scans respectively, to track the destination of injected cells following i.v. or i.p. injection. Results: To date, 9 infusions have been performed in 5 patients, 4 of whom received both i.v. and i.p injections of MAK cells. Following i.v injection cells were successfully tracked through the lungs and spleen. Tracking of cells to sites of intraperitoneal tumour have been demonstrated in 2 patients following i.v. injection, using both PET and SPECT. Both of these patients had moderate intraperitoneal disease bulk. Some tracking to tumor was also seen in these 2 patients following i.p. injection, however there appeared to be significant amounts of residual free intraperitoneal cells that did not localize to

tumour. No tracking to tumour was observed in 2 patients with low-volume disease. Intraperitoneal distribution of MAK cells following i.p. injection was limited in two patients due to the presence of adhesions. Conclusion: We have demonstrated that it is possible to label MAK cells being used for immunotherapy, with radiotracers and track their progress *in vivo* following injection using both PET and SPECT nuclear medicine imaging techniques. Tracking of cells to the tumour target appears to be most efficient after i.v. injection in results to date and the trial is continuing.

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The Use of Adenoviral Gene Transfer to Augment the Immune Response to Human Myeloid Leukaemic Cells

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Myeloid leukaemic cells rarely induce a significant T-cell response *in vitro*. Transduction of leukaemic cells with genes encoding cytokines or other immunomodulatory molecules may enhance their immunogenicity. We have previously demonstrated the ability of the chimeric Ad5f35 vector to deliver the EGFP gene to myeloid leukaemic cells. In the present work we have assessed the ability of Ad5f35 to deliver immunomodulatory genes encoding IL12, CD40L, IL2 and CD80/B7.1 to leukaemic cells. We have also evaluated the potential of adenoviral gene transfer to facilitate the generation of leukaemia reactive T-cells in allogeneic settings. AML and CML samples were transduced with the Ad5f35 vector encoding the different immunomodulatory genes. Following 24 hour infection of 8 AML and 3 CML samples with Ad5f35B7.1, all samples expressed CD80 in over 20% of cells with a range between 21-96% (mean 67 %). Expression of CD40 ligand after 24 hour infection with Ad5f35CD40L was observed in 6 of the 7 myeloid leukaemic samples tested (6 AML, 1 CML) with a range between 32-92% (mean 64%). Eight leukaemic samples (6 AML, 2 CML) transduced with Ad5f35fIL12 produced between 0.15 and 1 ng (mean, 0.54 ng) of IL12 per 10^6 cells per 24 hours. Ad5f35IL2 infection of 9 AML samples resulted in between 0.01 and 0.83 ng (mean, 0.27 ng) of IL2 production per 10^6 cells per 24 hours. Infection of myeloid samples with a combination of two vectors encoding different genes (B7.1 and IL12 or B7.1 and IL2) did not reduce the transduction efficiency of leukaemic cells. To analyze the effect of these immunomodulatory genes on the generation of *in vitro* immune responses, we set up mixed lymphocyte reactions using an MHC unrelated normal donor. Increased cell proliferation was observed when donor lymphocytes were stimulated with leukaemic cells transduced with Ad5f35fIL12 alone or in combination with Ad5f35B7.1. Absent or lower levels of proliferation were detected when leukaemic cells remained untransduced or were transduced with Ad5f35IL2, Ad5f35B7.1 or an empty Ad5f35 vector. Our results suggest that IL12 potentiates the immunogenicity of human myeloid leukaemic cells. We are currently assessing the ability of T cells stimulated with Ad5f35-transduced leukaemic cells to recognize untransduced blasts. Transfer of genes encoding cytokines and co-stimulatory molecules may promote the

activation of different components of the immune system, enhancing an immune anti-leukaemic effect.

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Ex Vivo Expansion of Mobilised Blood CD34+ Cells for Therapy: laboratory aspects

Haylock, D

We have recently completed a study to determine whether *ex vivo* expanded $CD34^+$ cells enhance the rate of neutrophil and platelet recovery following repetitive high-dose chemotherapy (HDC) for treatment of metastatic breast cancer. The protocol involved 3 cohorts, each consisting of 3 patients with each receiving 3 cycles of HDC supported by PBPC with or without *ex vivo* expanded $CD34^+$ cells. Patients were mobilised with HDC and G-CSF (Filgrastim, 10ug/kg/day) then apheresis collections were either cryopreserved as an unmanipulated product or as an Isolex-300i selected $CD34^+$ fraction. *Ex vivo* culture of cryopreserved $CD34^+$ cells was performed in GMP cleanrooms of the Centre for Blood Cell Therapies at the Peter Mac. Cells were cultured for 12 days in stericell flasks containing X VIVO-10 supplemented with 0.5% human serum albumin (Buminate), G-CSF, SCF and MGDF (each 100ng/ml). At the completion of culture, *ex vivo* generated cells were concentrated and assessed for microbial sterility. A total of 12 Isolex procedures were performed to yield 306±165.7 x 10^6 (mean±SD) $CD34^+$ cells with 92±35.6% (mean±SD) and 94.38±5.17% (mean±SD) $CD34^+$ cell recovery and purity, respectively. *Ex vivo* cultures (9 patients, 21 episodes) established in multiple stericell flasks (5-10 litres/culture), resulted in 41.1±20.49 (mean±SD) fold cell expansion. The final cell product, processed using a Haemonetics Haemolite-2 cell salvage device and reduced to 250-300ml, contained 5.82±3.23 x 10^9 cells comprising 9.25±5.76% $CD34^+$, 63.9±9.25% neutrophilic precursors ($CD15^+$,$CD11b^+$,$CD61^-$) and 15.5±0.8% megakaryocytic cells ($CD15^-$,$CD11b^-$, $CD61^+$,$CD42a^+$). Although *ex vivo* culture was performed within a GMP facility, a bacterial contamination occurred on one occasion in a single stericell flask (1 of 170 flasks in the total study), highlighting the need for stringent product assessment before release for transplantation. All patients transplanted with *ex vivo* expanded cells demonstrated a significantly ($p=0.0052$) increased rate of neutrophil recovery and reduced need for platelet transfusions. There was no correlation between dose of particular *ex vivo* generated cell types and the time to haemopoietic recovery, although in 94% of cases where patients received greater than 3.0 x 10^9 *ex vivo* expanded cells, there were 3 days or less of neutropenia (ANC of <0.1).

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Large-Scale Expansion of CMV-Specific Cytotoxic T Lymphocytes in a Stirred Suspension Bioreactor

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Aim: Recent clinical trials have shown adoptive transfer of antigen-specific cytotoxic T lymphocytes (CTL) to be an effective method of reducing viral and malignant disease. However, the large cell number required to achieve efficacy, 10^9 to 10^{11}, makes routine application of adoptive immunotherapy impractical. Stirred suspension bioreactors are one such method which

may more readily allow clinical scale expansion. Suspension cultures offer advantages over conventional static culture methods, including providing homogeneous culture environment and the potential for optimisation and control of culture conditions. In this study, we generated cytomegalovirus (CMV)-specific CTL and investigated the potential of stirred bioreactor systems for expansion of large cell numbers. Methods: CMV-specific CTL lines were generated from HLA-$A2^+$, CMV seropositive donors by weekly stimulation with CMVpp65 peptide-pulsed dendritic cells. After testing for specificity by tetramer and cytotoxicity assays, the CTL were cryopreserved for later expansion. Cryopreserved CTLs were then thawed and restimulated with OKT3 in the presence of IL-2 and allogeneic feeder cells. Activated CMV-specific CTL were then inoculated into suspension bioreactors or into standard T-flasks. Cultures were analyzed on a daily basis for cell number, activation status, phenotype (including specificity to CMVpp65 by tetramer staining) and glucose and lactate concentration. Subsequent to expansion, CTL cultures were tested for cytotoxicity against peptide-loaded target cells. Results: We found that CTL can be readily expanded (>200-fold) from cryopreserved stocks by non-specific stimulation in the presence of allogeneic feeder cells and IL-2. Activated CTL inoculated into either suspension or static cultures could be subsequently expanded 10-fold, and showed similar growth kinetics and metabolism independent of the culture vessel used. Furthermore, CTL remained specific for CMV pp65 peptide through the expansion phases, as demonstrated by pp65-tetramer staining (>95% $tetramer^+$) and by cytotoxicity assays. Conclusion: This study indicates that suspension reactor systems may be useful in the large-scale expansion of antigen-specific CTL lines or clones, and may facilitate the advancement of routine adoptive immunotherapy.

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Mobilisation, Apheresis, Selection and Coronary Artery Infusion of CD34+ Angioblasts for Stable Chronic Ischaemic Heart Disease

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The primary objective for this phase I study was to determine the safety and feasibility of G-CSF mobilisation and coronary artery infusion of $CD34^+$ selected angioblasts in patients with stable chronic ischaemic heart disease (CIHD). Patients with a prior history of myocardial infarction and symptoms of stable exertional angina pectoris of at least 6 months duration unresponsive to optimal medical or interventional therapy were eligible for study. Mobilisation was induced by subcutaneous administration of 10ug/kg/day, G-CSF for four consecutive days, followed by apheresis on a COBE Spectra. $CD34^+$ cells were selected on an Isolex 300i and held overnight in cryocyte bags at room temperature prior to infusion. Stress myocardial perfusion imaging was performed prior to and 3 months after infusion of mobilised $CD34^+$ angioblasts. Four patients (1 female, 57yr and 3 males, 67, 79, 62yrs) were mobilised and infused with Isolex selected $CD34^+$ cells. The first patient had 8 previous angioplastys over a 5-year period for class IV angina (NYHA criteria). The 3 male patients were diabetic and had experienced between 6-15 years of CIHD and class III-IV angina, with 2/3 having previous bypass surgery. Despite their severe cardiac impairment, G-CSF infusion and apheresis was well tolerated. A three-hour apheresis was performed at a blood flow rate of 40ml/minute on the 4^{th} day of G-CSF administration, when the leucocyte count and blood $CD34^+$ cell levels were 40.1, 35.4, 38.3 and 45.2 x 10^9 /L and 68, 26, 36 and 39 x 10^6/L, respectively. A total of 2.0, 2.5, 0.68 and 4.9 x 10^{10} leucocytes were processed on the Isolex 300i to yield 130.5, 51.8, 23 and 64 x 10^6 $CD34^+$ cells. Immunophenotyping revealed that 55.3-68% of $CD34^+$ cells expressed the angioblast associated cell surface markers CD133 and CD117. Cells were administered intracoronary through a 70 micron filter. One patient had a small troponin I rise to 0.2 following the procedure, but no chest pain. All others had no complications. Of the 4 patients so far treated only 2 can be evaluated. Both have exhibited a 30-40% improvement in exercise duration before onset of angina. The first

patient now experiences less than one episode of angina per week as compared to 3-4/day prior to angioblast infusion, the second patient none at all. This study demonstrates the feasibility of G-CSF induced angioblast mobilisation, apheresis and coronary artery infusion in a population of elderly patients with stable CIHD.

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Results of EPOCH Chemotherapy in the Treatment of HIV Lymphoma

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Recent reports have indicated favourable results for continuous infusion chemotherapy in the treatment of HIV-related, systemic, non-Hodgkins lymphoma (HIV-NHL). We report the results of combination etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin (EPOCH) continuous infusion chemotherapy in 11 patients (pts). Eleven male pts, median age 47years (25-66) received EPOCH for HIV-NHL commencing July 1999 with follow-up till June 2003 (minimum 6 months). Duration of HIV infection ranged from one to 19 years (median 11) with 4 pts naive for HAART. While histology suggested a poor prognosis (6 with Burkitts/Burkitt-like, 5 high grade large cell type) other prognostic factors at diagnosis were reasonable with 10 pts stage I-IIA/E and only one pt stage IVA, median CD4 cells 380 (200-777) and only one pt with a previous AIDS defining illness (KS). Serum LDH was elevated in 5 pts. HAART was continued or commenced for all pts. Delivered dose intensity was 100% for all pts with 8 receiving 6 cycles and 2 pts, 4 and 5 treatment cycles respectively. One pt had only one cycle and changed therapy to CHOP because of complications associated with central line insertion. Nine pts developed grade 3-4 neutropenia with 2 developing fever needing intravenous antibiotics. All pts needed G-csf. Generally treatment was well tolerated with major complications seen in 2 pts. One had massive upper gastrointestinal bleeding secondary to tumour erosion and the other multiple infections, small bowel perforation, pulmonary embolus and massive biliary bleeding. There were no treatment related deaths. Complications of central lines included infection and thrombophlebitis. Subclavian lines were better tolerated than PICC lines. All pts achieved complete remission (CR) of their lymphoma and remain alive and disease free with 6 to 42 months follow-up. Even allowing for the better prognostic features of this cohort these results are remarkable when compared to historical results. Our results confirm those recently reported for EPOCH in a larger pt cohort. EPOCH should be considered as standard of care for HIV-NHL.

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Outpatient-based Fractionated ICE Chemotherapy for Both Salvage and Stem Cell Mobilisation in Transplant-eligible Patients with Non-Hodgkin's Lymphoma and Hodgkin's Disease

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We have treated 60 transplant-eligible patients with relapsed or refractory lymphomas using an outpatient-based regimen of ifosfamide, carboplatin, and etoposide (ICE) for both salvage and peripheral blood stem cell (PBSC) mobilisation. Patients included relapsed or refractory diffuse large B-cell lymphoma (DLBCL) (n= 26), follicular

lymphoma (FL) (n=20), NK/T-cell lymphoma (n=3), mantle cell lymphoma (n=3) and Hodgkin's disease (n=8). Twenty-three percent of patients were chemorefractory. Cycles of ICE were administered every 21 days and consisted of: ifosfamide 5,000 mg/m^2 iv fractionated into 3 equally divided doses on days 1 to 3, carboplatin (mg dose = 5 x AUC) iv on day 1; and etoposide 100 mg/m^2 iv daily on days 1 to 3. Subsequently, filgrastim 5 µg/kg sc was administered daily from day +5. The majority of patients with indolent lymphomas also received Rituximab with ICE (R-ICE). The median age of patients was 50 years (range 26-70 years). Patients received a mean of 2.8 cycles (range 1-4) of ICE. There were no toxic deaths, while two patients experienced grades III-IV non-haematological toxicities. Haematological toxicity included grade IV thrombocytopenia and grade IV neutropenia with at least one cycle of ICE in 72% and 73% of patients, respectively. The median time to PBSC harvest was 14 days (range = 10-20d), while the median CD34 cell yield was 5.1 x 10^6/kg (range = 2.3 to 37.8). Three patients (6%) failed to mobilise PBSCs. The overall response rate to ICE was 88%, comprising 18 patients (30%) who achieved a CR and 35 (58%) who achieved a PR. Seven patients (12%) were considered refractory to ICE and did not proceed to stem cell transplantation (SCT). A total of 46 patients have undergone autologous SCT while 6 indolent lymphoma patients have received a reduced-intensity allogeneic SCT (one received both). Nine patients did not proceed to transplantation because of disease progression in six, organ toxicity in two, and failure to collect stem cells in one. The Kaplan-Meier estimate of the proportion of patients alive and event-free at a median follow-up of 14 months is 67% and 49%, respectively. The event-free survival (EFS) and overall survival (OS) for patients who achieved a CR after ICE is 55% and 82%, respectively, versus 42% and 64% for those who achieved a PR after ICE. These data confirm the efficacy and tolerability of fractionated ICE chemotherapy as both a salvage and mobilisation regimen that can be readily delivered in an outpatient setting.

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Fludarabine, Cyclophosphamide and Rituximab is Highly Effective Treatment in Patients with Indolent Lymphoid Malignancies

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Fludarabine (F), cyclophosphamide (C) and rituximab (R) are each highly active drugs in the treatment of indolent lymphoid neoplasms. Preclinical data has shown synergism between F and C, as well as between R. In this report, we present our results with the combination of FCR in the treatment of patients (pts) with indolent lymphoid malignancies. R 375mg/m^2 is given on d1 of each course, followed by F 25mg/m^2 and C 250mg/m^2 daily d1 – 3; growth factors were not used routinely. From 12/00 - 6/03, 39 pts were treated; 16 with CLL, 18 "low-grade" non-Hodgkin's lymphoma ((LG-NHL); including 13 follicular, 4 mantle cell and 1 marginal zone), and 5 Waldenstrőm's Macroglobulinaemia (WM). One third of pts were previously untreated, and two-thirds pre-treated (median two previous therapies; range 1 – 6). Pt characteristics: median age 62 years (range 30 – 73); male 64%; median performance status 1; stage IV disease 92%; median IPI score 2 (range 0 – 5); elevated LDH 18%; elevated β_2-microglobulin 60%. A median of 4 cycles were delivered (range 1-6). Six pts received F with mitoxantrone 8 mg/m^2 and C reduced to 200

mg/m^2 (FCM-R). Median follow-up period is 9 months (1 – 31). The overall response rate is 89% (44% CR and 45% PR), 100% of previously untreated pts responded (64% CR 36% PR) as did 84% of previously treated pts (36% CR 48% PR) (p= 0.19). Overall response rates did not vary significantly by histology. Two of 3 evaluable pts with mantle-cell NHL attained CR. The CR rate was higher among pts with follicular NHL (85%) than CLL (21%; $p = 0.001$). Elevated LDH was predictive of lower response (60% vs 93%; $P = 0.03$), but β_2-M was not. When compared to a prior cohort of 64 pts treated with FC without R, the overall response rate did not differ (85%; $P = 0.3$) but the addition of R lead to a higher CR rate (44% vs 26%; $P = 0.06$). The median remission duration is 22.2 months and is numerically longer than those treated with FC without R (13 months) (p = 0.3). Grade ≥ 3 or 4 neutropenia were seen in 43% and 22% of cycles, respectively; 3% cycles had grade 3 thrombocytopenia (not different from FC treatment). 29% of first cycles were complicated by infusional reactions to R, the majority were mild with only 5% first cycles complicated by grade 3+ infusional reactions. Severe infections complicated only 7% of cycles (versus 6% for FC), including 3 episodes each of Gram-negative septicemia and febrile neutropenia, two severe pneumonia, one varicella-zoster infection and one CMV viraemia. There were no cases of *Pneumocystis carinii* despite the absence of routine prophylaxis. There were no treatment-related deaths. 3 patients have died – one of cardiac causes in ongoing remission, and two of pneumonia in context of active disease 8 – 18 months post therapy. The median overall survival has not been reached and the 2-year actuarial survival rate is 68%. In summary, FCR is a highly active and well-tolerated regimen in patients with indolent lymphoid malignancies.

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Single Dose Per Cycle Pegfilgrastim Successfully Supports Full Dose Intensity CHOP-14 in patients over 60 years with Non-Hodgkin's Lymphoma (NHL)

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CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) administered at 21 day intervals for 6 to 8 cycles has been the standard treatment for patients with aggressive NHL, although emerging data suggest that dose-dense CHOP given every 14 days (CHOP-14) may produce better outcomes. Dose intensity of cytotoxic regimens can be augmented by both increasing drug dose and by reducing the intervals between treatment cycles. Preliminary efficacy and safety of a CHOP-14 regimen was assessed for patients over the age of 60 years supported by a single dose of Pegfilgrastim (6mg SC) on day 2 of each cycle. Pegfilgrastim, a pegylated form of Filgrastim, has a sustained-duration effect relative to Filgrastim, as a result of decreased renal clearance of the Pegfilgrastim molecule. An interim analysis was conducted on the first 20 of the total 30 enrolled patients. Previously untreated patients with aggressive (intermediate or high-grade) NHL of any histological subtype or grade were eligible. The mean age of patients is 68 years

(62-74) with 9 males and 11 females. A total of 103 cycles (83 cycles 2-6) has been administered to 20 patients. Cycles were delivered on day 15 on 71/83 occasions (87%). Only two cycle delays were specific to haematological toxicity (one neutropenia, and one neutropenia & thrombocytopenia). Ten other delays were attributed to at least two toxicities of which three cases included febrile neutropenia, and one case included neutropenia. Four patients experienced grade 3 febrile neutropenia and one patient experienced grade 3 thrombocytopenia. One other patient experienced grade 3 neutropenia in 4 cycles. Full chemotherapy dose was delivered in 88% (91/103) of cycles. There were 12 dose reductions due to febrile neutropenia (7), GI toxicity (1), sepsis (1), weight loss (1), administrative (1) and cellulitis of the hand (1). There were two instances of bone pain (1 mild, 1 moderate). The lowest neutrophil count at the beginning of any cycle was 1.9 x 10^9/L. Responses were seen in 15 patients (75%): 7 CR and 8 PR. One patient was not assessable because of complications following bowel perforation. There were no deaths on study, no withdrawals due to haematological toxicities, no grade 4 haematological toxicities and no unanticipated toxicities. These interim results support the use of once-per-cycle pegfilgrastim (6 mg SC) for safe and effective on-time delivery of a dose dense regimen with CHOP-14 in an elderly population with aggressive NHL.

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Treatment of Patients with Advanced Mycosis Fungoides and Sézary Syndrome with Alemtuzumab

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Background: CD52 is expressed on normal B and T-lymphocytes and in most malignant B and T-cell disorders. Alemtuzumab (Campath-1H, ILEX Pharmaceuticals, L.P., San Antonio USA), a monoclonal anti-CD52 antibody, has been reported to be effective in the treatment of a range of hematological malignancies, including cutaneous T-cell lymphomas (CTCL). Aims: To evaluate the safety, tolerability and efficacy of alemtuzumab in patients with relapsed or refractory advanced stage mycosis fungoides (MF) and Sézary syndrome (SS). Methods: Patients with advanced stage (IIB – IV) relapsed and / or refractory MF / SS were enrolled in a phase II, open label study. Alemtuzumab was administered intravenously 3 times a week, commencing at 3mg / infusion and rapidly dose escalated to 30mg / infusion if tolerated. Planned treatment duration was 12 weeks, or, if disease was still responding at 12 weeks, until maximum response and /or for a further 2 weeks thereafter. Anti-fungal, PCP and HSV prophylaxis was routinely administered throughout treatment until 2 months post completion of therapy. Results: A total of 8 patients (4 male, 4 female) were enrolled, 7 with mycosis fungoides / Sézary syndrome (MF/ SS) and 1 with large-cell transformation of MF. Median age was 48 years (range 30-62). Patients were heavily pre-treated, with 7 of 8 patients refractory to multiple (≥4) previous therapies. The overall response rate was 38% (95% confidence interval 9-76%), with 3 partial remissions, 2 cases of stable disease, and 3 cases of progressive disease (PD). Median treatment duration was six weeks (range 2-13). Cessation of treatment was due to PD (4 patients), infectious

complications (2 patients), infusion-related side effects (1 patient), and completion of therapy (1 patient). Median time to progression was short (9.5 weeks, range 3-16); response duration in the 3 PR patients was <3 months in all 3 cases. Overall survival was poor, with 6 of 8 patients dying a median of 4 months (range 1-22) after commencing treatment. Significant hematological and immunosuppressive toxicity was also observed, with both grade 3-4 cytopenias and significant infectious complications occurring in a majority of cases. Conclusions: Our findings suggest that in heavily pre-treated, refractory, advanced stage MF / SS, although alemtuzumab has biological activity, it is associated with significant toxicity and only modest clinical utility. We suggest that if used in the treatment of MF / SS, alemtuzumab should be considered relatively early in the disease course, prior to developing disease refractory to multiple other therapies. Given the partially non-overlapping toxicities with other active therapies, combination regimens incorporating alemtuzumab also merit further investigation in this difficult to treat patient group.

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Treatment of Acute Myeloid Leukemia with Fludarabine and High Dose Cytarabine Followed by G-CSF (FLAG). the Peter Mac experience 1996-2002

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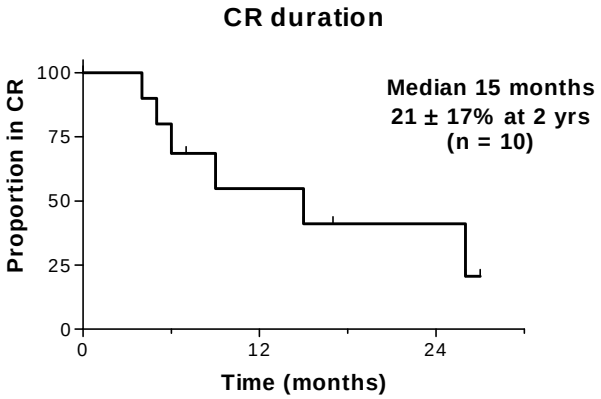
From Jan 1996 to May 2003 we treated 35 AML patients with FLAG – fludarabine 30 mg/m2, followed by cytarabine 2g/m2 for five consecutive days, followed by G-CSF 5 mcg/kg daily from day 6 till neutrophil recovery. Consolidation treatment was two further cycles of FLAG. The 35 patients are shown in the table

	Number	Age	Prior diagnosis	Cytogenetics Fav/Inter/Poor	Response CR/PR/NR/ED
Newly diagnosed	10	74 (58-79)		0/8/2	7/0/3/0
s/t-AML	14	66 (51-75)	t-AML 3; MPD 2; CMML 2, MDS 7	0/12/2	3/3/7/1
Relapse/refractory	11	52 (21-70)	* 3 pat auto		1/2/7/1

Results: Overall 11 of 35 patients (31%) attained complete response (CR); 7 of 10 (70%) new AML patients, 3 of 14(21%) s/t-AML patients and 1 of 11 (9%) relapse / refractory patients. In addition in the s/t AML group there were 3 partial responses with improvements in blood counts and two partial responses in the relapse/refractory group. There were only 2 (6%) early deaths within 28 days in this elderly or poor prognosis group. Only 2 of the first 27 patients had GI symptoms with diarrhoea and abdominal distension suggestive of neutropenic entercoloitis. Duration of the 7 CR's in newly diagnosed AML ranged from 4-27+ months, median 15 months (4,5,7+, 9, 15,17+ and

27+ months). Duration of the three CR's in the s/t-AML was 5+,6 and 26 months. A Kaplan Meyer plot of CR duration is shown below



Conclusion: FLAG is safe with minor incidence of neutropenic enterocolitis compared to anthracycline and cytarabine chemotherapy. Response is as expected for this group of poor prognosis patients. In newly diagnosed patients >60 years it is a safe and effective induction and consolidation treatment for AML.

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Legal and Ethical Issues in Industry-Funded Trials

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Biomedical research in publicly-funded institutions, which provide expertise and infrastructure, is increasingly supported by private industry which controls and profits from what is studied: creating a situation described as the "privatization of profits, and a socialization of losses." All researchers should be increasingly concerned about the implications of for-profit companies, the tight linking of research money to industry, and the often incautious licensing of drugs and food. The pharmaceutical industry now finances most of North America's drug research and determines the direction of much basic academic research. Modern researchers may adapt their conduct and interpretations to the corporate funding provided, and those who do not so adapt are likely to suffer great hardship for refusing to do so. Furthermore, alteration, omission, and suppression of data is not rare in scientific research. Under demands by their industry paymasters to publish, researchers are pressured to deliver 'new', 'positive' results: for example, that a new drug being tested is superior to an old drug, or to placebo. At the least, an unconscious bias in favour of any new drug may emerge. Moreover, in drug company-sponsored research, many researchers believe that the company 'owns' the data, and that, if refused permission to publish, one can do nothing. Many scientists may be persuaded that, whilst public accountability is one thing in the context of medical care, there is a lessening of ethical responsibility to patients in research trials. This belief may have a direct effect on patient morbidity and mortality. If the public cannot rely upon the integrity of its scientific community, in whom can it repose trust? If the scientific community forfeits public trust, can it expect that the public will continue to support research in universities and hospitals? While an ethical duty for all researchers to disclose all findings in industry-sponsored research exists – even those potentially adverse to commercial interests - there exists little legal protection for the individual who discloses such findings against the wishes of a commercial sponsor, or her academic institution. In part because of this lack of protection, retaliation toward so-called 'whistleblowers' of research misconduct usually derails careers, an

outcome that is rarely reversible, despite ultimate vindication. Legal protections recently introduced in the UK and USA may improve the future for those who recognize this ethical duty but, arguably, scientific and professional organizations should protect those who honor their ethical responsibilities.

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Molecular Targets for Myeloproliferative Disorders

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The myeloproliferative disorders are a heterogeneous group of hematopoietic dyscrasias characterized by enhanced proliferative/survival capacity of various myeloid lineage cells. Syndromes have been described that encompass virtually all types of terminally differentiated myeloid cells, including mast cells (systemic mast cell disease, SMCD), red cells (polycythemia vera, PCV), platelets (essential thrombocythemia, ET), eosinophils (hypereosinophilic syndrome, HES), neutrophils (chronic myeloid leukemia, CML) and monocytes (chronic myelomonocytic leukemia, CMML). These diseases are most often sporadic, and both cytogenetic and X-inactivation analysis indicate that these are clonal hemopathies that are the consequence of acquired somatic mutation in hematopoietic progenitors. Initial insight into the genetic cause of myeloproliferative diseases was gleaned from cloning of recurring chromosomal translocations associated with a particular disease phenotype. The prototypic example is the t(9;22) translocation giving rise to the BCR-ABL fusion associated with CML. The causal role of the constitutively activated BCR-ABL kinase in pathogenesis led to rational drug design of the tyrosine kinase inhibitor imatinib (Gleevec). Imatinib is a selective inhibitor of the ABL, ARG, PDGFRA, PDGFRB and KIT receptor tyrosine kinases. The efficacy of imatinib in therapy of CML has led to FDA approval for this indication. Cloning of other chromosomal translocations associated with a CMML phenotype has invariably identified fusion tyrosine kinases, including the TEL-PDGFRB, TEL-ABL, H4-PDGFRB, RBTPN-PDGFRB, HIP1-PDGFRB and TEL-JAK2 fusions. Each of these is a constitutively activated tyrosine kinase that causes a CMML-like disease in murine models of disease. Furthermore, the majority of these are imatinib sensitive fusions, and efficacy of imatinib in patients that harbor PDGFRB fusions have been reported. These examples are similar to rationally-based imatinib therapy of gastrointestinal stromal cell tumors harboring activating mutations in KIT. Imatinib has also been used as empiric treatment of cancers. Among these, responses of a subset of HES patients to imatinib have been quite dramatic. These observations led to the cloning of the FIP1L1-PDGFRA fusion gene that explains the clinical response of HES patients to imatinib. A subset of SMCD patients have KIT D816V activating mutations that are unfortunately not imatinib sensitive, but SMCD with associated eosinophilia often have FIP1L1-PDGFRA fusion that are imatinib sensitive. Thus, individual molecular genotyping may be important in selecting targeted therapies. Taken together, these data indicate that the myeloproliferative diseases are the consequence of unregulated activation of signal transduction pathways that confer a proliferative/survival advantage without affecting differentiation, and suggest that screens for this class of mutations may be useful in defining the etiology of other MPD such as ET and PV.

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Implications of *BCR-ABL* Mutations in Imatinib-treated CML Patients

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Imatinib is highly effective in the treatment of chronic myeloid leukaemia (CML) patients in chronic phase and less effective in more advanced phases of CML. The major limitation of therapy is acquired resistance, which is mainly, but not exclusively, observed in advanced phase patients. Acquired resistance is commonly associated with *BCR-ABL* kinase domain mutations. It is unclear whether patients who remain sensitive to imatinib also have a significant incidence of mutations. We evaluated 144 imatinib-treated patients for *BCR-ABL* kinase domain mutations by direct sequencing. They were selected on the basis that they had received at least 6 months of imatinib, regardless of their response or whether they had evidence of resistance. The survey included 40 accelerated phase (AP), 64 late chronic phase (≥12 months from diagnosis, late-CP) and 40 early-CP patients. Mutations were detected in 27 patients at 17 different residues, 13/40 (33%) in AP, 14/64 (22%) in late-CP, and 0/40 in early-CP. Acquired resistance was evident in 24/27 (89%) patients with mutations. Twelve of 13 patients (92%) with mutations in the ATP binding loop (P-loop) died (median survival of 4.5 months after the mutation was detected). In contrast, only 3/14 (21%) patients with mutations outside the P-loop died (median follow-up of 11 months). As the detection of mutations was strongly associated with imatinib resistance we analyzed features that predicted for their detection. Patients who commenced imatinib >4 years from diagnosis had a significantly higher incidence of mutations (18/44, 41%) compared to those treated within 4 years (9/100, 9%), $P<0.0001$. Lack of a major cytogenetic response (MCR) after 6 months of imatinib was also associated with a higher likelihood of detecting a mutation; 19/50 (38%) patients without a MCR had mutations compared to 8/94 (8.5%) with an MCR, $P<0.0001$. In conclusion, the detection of kinase domain mutations using a direct sequencing technique was almost always associated with imatinib resistance and patients with mutations in the P-loop had a particularly poor prognosis.