1

The Spectrum of Extranodal Non-Hodgkin’s Lymphoma

Graham Young
Royal Prince Alfred Hospital, Sydney, NSW, Australia

Although lymphoma is predominantly a disease affecting the traditional sites of lymph nodes, liver, spleen and bone marrow, it can affect many other sites, which is one of the fascinations of the disease. There are many case reports in the literature of lymphoma affecting unusual sites, and this is particularly true of T/NK cell lymphomas and those associated with HIV infection. Even within the recently revised WHO classification, the histological type of lymphoma may often be dictated by the tissue involved, eg Enteropathy associated T cell lymphoma, Cutaneous T cell lymphoma, Nasal NK cell lymphoma etc.

This talk will explore some of the issues relating to the anatomical localization of lymphoma and why certain lymphomas arise at particular sites.

2

Nongastrointestinal MALT Lymphomas

Pier Luigi Zinzani
Institute of Hematology and Oncology “Seràgnoli”, University of Bologna, Italy

Mucosa-Associated Lymphoid Tissue (MALT) lymphoma only occasionally arises from sites where MALT is normally present, such as the tonsil and Peyer’s patches. The reason for this seems to be that MALT lymphomas generally arise in lymphoid tissue that has been acquired as a result of some pre-existing disorder. Although MALT lymphoma most frequently arises in the stomach, it also presents in a variety of extranodal sites outside the gastric district. Non gastric locations represent 30% to 40% of all MALT lymphomas.MALT lymphoma probably constitutes the in vivo model that best represent how the complex interplay between B lymphocytes and the surrounding microenvironment may lead to a neoplastic disorder. After the seminal discovery of the pathogenic association between Helicobacter pylori and gastric MALT lymphomas, evidence has been provided suggesting the possible involvement of other infectious agents in the development of MALT lymphomas arising at different body sites. In the cases of thyroid, salivary gland, and lung involvement, the lesions known to induce an accumulation of lymphoid tissue are Hashimoto’s thyroiditis, myoepithelial sialoadenitis (MESA) associated or not with Siogren’s syndrome, or lymphoid interstitial pneumonitis, respectively. In addition, several Borrelia strains have been associated with primary cutaneous lymphomas. In Europe, the DNA of Borrelia burgdorferi, the causative agent of Lyme disease, has been detected in distinct primary cutaneous lymphomas: MALT lymphomas (20-50% of cases), follicular lymphomas and diffuse large B-cell lymphomas (15-26%). Recently, Chlamydia psittaci, the etiologic agent of psittacosis, has been suggested as responsible for the antigenic stimulus contributing to the development of ocular adnexal lymphomas.

3

Primary CNS Lymphoma

Peter O’Brien
Newcastle Mater Hospital, Newcastle, NSW, Australia

PCNSL is an uncommon (30 per 10 million population) extra nodal lymphoma with a unique pattern of behaviour and relatively poor prognosis despite its limited extent at presentation. It is most commonly a diffuse large B-cell lymphoma (T-cell more common in patients with HIV) expressing BCL-6 suggesting it is of germinal centre origin. Postulates as to its tropism for the CNS include changes in expression of particular chemokines (BCA chemokine 1) and adhesion molecules.

Treatment has evolved fairly rapidly over the last 15 years as the importance of the use of high dose methotrexate has become widely accepted. Multi and single institution studies have shown that high dose methotrexate combined with cerebral irradiation will result in median survivals of 3 years and cure one third of patients. The initial combined modality studies produced unacceptably high rates of neurotoxicity particularly in patients over 60 years. The aetiology of neurotoxicity in this disease is not particularly well understood and may result from an interaction between...
the disease itself and treatment (chemotherapy alone can also produce neurotoxicity). There is some evidence that changes in radiotherapy dose and scheduling will reduce the risk.

More recent clinical research has focused on the use of chemotherapy alone either with methotrexate as a single agent or as part of combination therapy. Results have varied although with limited follow up in some studies overall survival appears to be equivalent to combined therapy however progression free survival is inferior. Regrettably there are few data to guide clinicians in determining optimal salvage therapy. This presentation will focus on the current issues to be considered in determining the optimal approach for individual patients by trying to balance the potential benefits and morbidities of treatment.

Blood or Marrow for Hematopoietic Stem Cell Transplantation (HSCT)

Alois Gratwohl
Hematology, University Hospital, Basel, Switzerland

Blood and bone marrow present two valid options for HSCT with specific advantages and disadvantages. The activity surveys of the European Group for Blood and Marrow Transplantation (EBMT) from 597 centres in 42 European countries present current status of HSCT in Europe and evolution of use in stem cell source from 1990 to today. In 1990, the vast majority of autologous and all allogeneic HSCT were still bone marrow (BM) transplants. Stem cell source changed rapidly to peripheral blood (PB) for autologous HSCT between 1992 and 1996. In 2004, 97% of autologous HSCT were PB derived. Change to PB for allogeneic HSCT followed 3 years later. In 2004, 68% of all allogeneic HSCT were PB derived. In 2004, BM remains a significant source of stem cells for non-malignant disorders in allogeneic HSCT. Results of nine prospective randomised studies, including 1,111 patients with allogeneic HSCT and analysed in an individual-patient data meta-analysis, showed faster neutrophil and platelet engraftment with PB compared to BMT. PB was associated with a significant increase in the development of extensive chronic GVHD. PB was associated with less relapse, but overall and disease-free survival were only improved in patients with late disease. Side effects for donors differ significantly between PB and BM harvest. Complications with BM are primarily related to the harvest procedure in BMT, they relate to the use of growth factors in PB HSCT. Severe complications were analysed retrospectively in 44,566 donors (28,134 BM, 16,431 PB) for HSCT between 1990 and 2003. There were 4 deaths (1 BM, 3 PB, 28 SAE (12 BM, 16 PB and 14 hematological malignancies in donors, 5 PB. These data illustrate the differences between donor source in allogeneic HSCT.

Donor Selection - Molecular Considerations

Peter Bardy¹, Charles Mullighan²
¹Clinical and Laboratory Haematology TQE & Transplant Services ARCBS-Discovery, and Member Leukaemia BMT Service RAH, Adelaide, SA, Australia
²Pathology, St Jude Children’s Research Hospital, Memphis, TN, USA

Allogeneic Haemopoietic Stem Cell Transplantation (Allo-HSCT) is a vital therapy for hematologic malignancies, but continues to have a strikingly high morbidity and mortality. Despite the increasing use of non-myeloablative preparative regimens, graft-versus-host disease (GVHD) remains a significant problem. Matching for human leukocyte antigen (HLA) complex alleles remains central to donor selection, however not all potential transplant recipients have a fully HLA matched donor. Searching for a fully matched donor may take considerable time during which disease may progress and become less amenable to transplantation. Recent studies have begun to address this interaction between timing of transplantation, stage of disease, the relative “permissibility” of one or two HLA allelic mismatches, and transplant outcome. An analysis of 1874 transplants by the US National Marrow Donor Program concluded that single HLA allelic mismatches were associated with increased mortality, although the effect was modest, and the risk increased further with multiple mismatches (1). These findings were supported by a single centre study of 948 transplants (2). This latter study also found that full molecular HLA matching was beneficial in low risk patients, but not in patients with more advanced disease, suggesting that the benefit of full HLA matching is offset by disease progression. This has important implications for donor selection and timing of transplantation, and suggests that transplanting early in the course of disease with a single HLA allele mismatched donor may be preferable to delaying the transplant in the hope of identifying a fully matched donor.

Even in fully HLA matched transplants, most recipients experience GVHD, suggesting that other allorecognition pathways and immune mediators are also important. What are these factors? Can we type for them and use them in donor selection algorithms? Minor histocompatibility antigens (mHAgS) are peptides presented by HLA molecules that
exhibit polymorphism or differential expression between donor and recipient. A growing number have been identified, some of which may elicit an alloimmune response, and GVHD. Identification of mHAgs is important not only for predicting GVHD, but also for identifying potential targets for immunotherapy to maximise the graft-versus-tumour effect.

A number of groups including our own have investigated the role of polymorphisms in non-HLA genes in influencing the risk of transplant complications. These fall into several groups, such as associations between polymorphisms in inflammatory mediators (e.g. TNF, IL-6) and GVHD, host defence polymorphisms (e.g. in the mannose-binding lectin and myeloperoxidase genes) and infection, and pharmacogenomic factors (e.g. methotrexate metabolic pathway genes and the risk of mucositis). This is a large and complex area, and prospective studies are required before the clinical utility of these observations can be realised.

The importance of Natural Killer (NK) cells in allo-HSCT has been highlighted in the context of haplo-identical transplants. NK cells can mediate important graft-versus-leukemia effects without inciting GVHD. The killer immunoglobulin-like receptor (KIR) genes that encode NK cell receptors are inherited independently of the genes for their ligands, HLA class I. This allows transplantation of donor NK cells into patients without the appropriate ligands even when they are fully HLA matched siblings. The potential utility of such permissible “KIR mismatching” in the context of fully HLA matched transplants is currently being investigated.

In summary we are entering an exciting time where our use of genomics to improve outcome of allo-HSCT will extend well beyond the HLA.

2. Petersdorf et al Blood 2004,104,9,2976-2980

HSANZ / BMTSAA: Stem Cell Transplantation

17
The Importance or Otherwise of Cellular Assays, NK cells and Killer Immunoglobulin-Like Receptors as an Adjunct to Allogeneic Hematopoietic Stem Cell (HSC) Donor Selection

Anthony P Schwarer
BMT Program, Alfred Hospital, Melbourne, VIC, Australia

The mainstay of donor-recipient matching has been and remains typing for HLA. Although, there is clearly more to donor-recipient matching than matching for all 12 HLA because some recipients of HLA-identical sibling HSCs develop severe graft-versus-host disease (GVHD) whereas other patients who receive HSC from donors with known HLA mismatches do not develop GVHD. The explanation for this is poorly understood.

Cellular assays, also termed functional assays, have played an important role in past years. The mixed lymphocyte reaction (MLR) was an important assay prior to more sophisticated HLA-class II molecular typing but nowadays has virtually no role in routine donor-recipient assessment. Several modifications led to the development of a number of assays which seemingly improved on the MLR such as the skin-explant assay and the mixed epidermal cell-lymphocyte reaction. More specific assays, such as the cytotoxic T lymphocyte precursor (CTLp) assay and helper T lymphocyte precursor (HTLp) assay, appeared to improve on earlier assays – more specific and more precise. These assays enjoyed some popularity and did seem to predict, albeit relatively weakly, the risk of developing significant GVHD. This weak predictive ability and the labour-intensive nature of the assays ensured that they did not become widely adopted.

Recent work has shown the cytokine gene polymorphisms play a role in GVHD and transplant outcome. It seems likely that some or all of the predictive ability of functional assays could be measured by these polymorphisms. This is a very complex area which remains to be clarified before it will be useful clinically.

There remains a need for a functional assay that can provide clinically useful information, the readout of which would give a summation of all the various elements that are important in determining patient outcome after allogeneic HSC transplant.

NK cells and NK cell receptors, in recent times, have become trendy – important roles in outcome, particularly relapse and transplant-related mortality, have been reported in haploidentical HSC transplantation as well as allogeneic related and unrelated donor HSC transplantation. NK cells appear to have an important role in other areas of medicine such as pregnancy and a variety of autoimmune diseases. This is an area of rapidly expanding knowledge although much remains to be explained and understood. It is possible typing for NK cell receptors such as KIR will be used as an adjunct to donor selection in the future.
Male Fertility: Effects of Chemoradiotherapy and Strategies for Preservation

Ann Conway
Andrology Department, Concord Hospital, Hospital Rd., Concord, NSW 2139, Australia

Semen cryostorage is a simple process which can and should be offered to all men about to undergo potentially sterilising chemotherapy or radiotherapy who wish to preserve their reproductive potential. Advances in IVF techniques such as intra-cytoplasmic sperm injection [ICSI] make this realistic for any man where at least some viable sperm are present in the ejaculate.

The Andrology Department at Concord Hospital [formerly at RPAH] has been running an elective sperm storage since 1978. In the first 22 years 930 men sought cryostorage and storage was performed in 833 [90%]. Reasons for not storing were; too ill to collect a semen sample [13], no sperm [31] or no motile sperm [53]. Sperm concentrations were lower than a control population of healthy sperm donors, particularly in men with testis cancer where sperm concentrations were approximately half that of men with other cancers, however this could be explained by recent hemicastration. In 692 [74%] men surviving their disease sperm was discarded for 193 men [28% of survivors], usually following fertility recovery [115 men]. Stored sperm was used in 64 men [9% of survivors and material remains in storage without use to date in 431 men [62% of survivors]. Use of stored sperm ranged from 2-180 months after storage [median 36 months]. 85 treatment cycles resulted in 39 births from 29 pregnancies and was most successful were ICSI was used. Of 141 [14%] dying 18 partners requested prolonged storage with only 3 using the material [no pregnancies].

Other strategies such as cytoprotection have not proved successful. Germ cell transplantation may allow fertility insurance to be extended to prepubertal boys but safety and efficacy remain to be established.

Pregnancy During and After Chemo-radiotherapy

Michael Friedlander
Dept of Medical Oncology, The Prince of Wales Hospital, Randwick, NSW, Australia

Cancer develops in about one per thousand women during pregnancy and raises many difficult treatment decisions. The general principle is to offer optimal management to the mother without compromising the chance of cure while at the same time trying to minimize risk to the fetus. There are restrictions on the investigations that can be carried out during pregnancy, particularly those that involve radiation exposure. The teratogenicity of any drug depends on the timing of exposure, and the dose as well as lipid solubility, molecular weight and plasma protein binding of the drug/s. In general the use of chemotherapy in the first trimester increases the risk of spontaneous abortion, stillbirth or fetal malformations and therefore should be avoided. If treatment cannot be safely delayed until the second or third trimester serious consideration should be given to a therapeutic termination. Safe use of chemotherapy during the second and third trimester of pregnancy is possible and there are many reports, which will be summarized during the talk. The evidence to date suggests that many drugs can be administered without definite harm to the neonate and studies are in progress addressing any long term or late effects. Radiation should be avoided during pregnancy. The approach to management of the pregnant woman with cancer, particularly hematological malignancies will be discussed in detail.
Myeloid malignancies are broadly categorized into either acute myeloid leukemia (AML) or chronic myeloid disorder (CMD) depending on the presence or absence, respectively, of AML-defining cytomorphic and cytogenetic features. The CMD are traditionally classified by their morphologic appearances into discrete clinicopathologic entities based primarily on subjective technologies. It has now become evident that most CMD represent clonal stem cell processes where the primary oncogenic event has been characterized in certain instances; BCR/ABL in chronic myeloid leukemia, FIP1L1-PDGFRα or c-kitD816V in systemic mastocytosis, rearrangements of PDGFRβ in chronic eosinophilic leukemia, and rearrangements of FGFR1 in stem cell leukemia/lymphoma syndrome. In addition, recurrent mutations of JAK2 and genes encoding for RAS signaling pathway molecules have been associated with the classic BCR/ABL-myeloproliferative disorders and juvenile myelomonocytic leukemia, respectively. Such progress is paving the way for a transition from a histologic to a semi-molecular classification system that preserves conventional terminology while incorporating new information on molecular pathogenesis. Furthermore, the recent description of the JAK2 V617F mutation in more than 90% of cases with polycythemia vera (PV) and half of those with essential thrombocythemia (ET) has led to refinement of current diagnostic algorithms for these disorders. Hydroxyurea has now been affirmed as the first choice of drug for both ET and PV, the former based on a recently completed randomized treatment trial and the latter based on a carefully executed large cohort study. In myelofibrosis, both stem cell transplantation and new experimental drugs such as lenalidomide are proving to be beneficial in a subset of patients.

HSANZ: Myeloproliferative Disorders

36 Current Status and Future of Abl Kinase Inhibitors in CML

Timothy Hughes
Institute of Medical and Veterinary Science, Adelaide, SA

The last 6 years have been a time of dramatic progress in our management of chronic myeloid leukaemia. The effectiveness of imatinib, the first clinically developed tyrosine kinase inhibitor, in chronic phase patients has been remarkable and there is no doubt that survival has been markedly improved. We are still faced with the challenge of what to do with the 30% of de-novo patients who do not achieve major molecular responses (MMR- a ≥3 log reduction in BCR-ABL) even after 2+ years of therapy. Their risk of resistance and relapse remains significant. Even in patients achieving MMR there is a low level of resistance observed that is mainly related to the emergence of clones with mutations in BCR-ABL. To what extent will the availability of the second generation ABL kinase inhibitors (AKIs) improve this situation? AMN107 (Novartis) and dasatinib (BMS) are both much more potent inhibitors of ABL kinase than imatinib and are active against most of the known ABL mutants that are resistant to imatinib. Will more patients achieve MMR with the new ABL kinase inhibitors? We have evidence that the actual level of in-vivo ABL kinase inhibition achieved is highly predictive for molecular response., making it unlikely that there are other important determinants of response. This suggests that more potent AKIs will achieve more uniform molecular responses. Will less patients develop resistance with the new AKI’s? The spectrum of resistant mutants is much smaller with the two new AKIs so this may be the case. However T315I remains a major problem with all AKIs currently available. Will the new ASKI’s actually eradicate leukaemic stem cells? This will depend on the extent to which the survival of leukaemic stem cells on imatinib is due to the inability of imatinib to completely block ABL kinase activity. The future of CML treatment is likely to include the use of combinations of ABL kinase inhibitors, since the mutant profiles for these inhibitors are overlapping. Our aim for CML patients should be long term stable control but we shouldn’t lose sight of the possibility that well targeted therapy might actually be curative in some cases.

HSANZ Free Communications 1: Lymphoma / HD (Auditorium)

43 Pegfilgrastim Compared to Granulocyte Colony Stimulating Factor (G-CSF) with Hyper-CVAD Chemotherapy Regimen for Aggressive Lymphoid Malignancy

Steven Lane1, Julie Crawford2, Melita Kenealy3, Gavin Cull2, John Seymour3, Paula Marlton1, Devinder Gill1, Peter Mollee1
1Department of Haematology, Princess Alexandra Hospital, School of Medicine, University of Queensland, Brisbane, Queensland, Australia
2Sir Charles Gairdner Hospital, Perth, Western Australia, Australia
3Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia
Aim: Pegfilgrastim (Neulasta®) has proven efficacy as supportive therapy in a variety of chemotherapy regimens, but has not been studied in dose intensive, rapidly cycling regimens such as Hyper-CVAD. We examined whether pegfilgrastim lead to similar kinetics of neutrophil recovery as daily G-CSF.

Methods: Using retrospective case-control analysis, patients receiving pegfilgrastim were matched with controls (G-CSF) by priority for cycle of chemotherapy, age (<60 or >60), diagnosis (ALL or NHL), bone marrow involvement, prior chemotherapy and dose of cytarabine received. The primary endpoint was duration of grade IV neutropenia (ANC <500/ul). Secondary endpoints included time to neutrophil recovery, incidence of febrile neutropenia, positive blood cultures and delay in subsequent chemotherapy.

Results: Between 01/1999 – 06/2005 we identified 89 pegfilgrastim supported cycles in 28 patients. Cases were successfully matched to 89 G-CSF supported cycles from 25 controls. There were no significant differences between cases and controls with regards to age, diagnosis, bone marrow involvement or cytarabine dosage, although there was a trend towards increased proportion of females in the pegfilgrastim group (p=0.054). The median duration of grade IV neutropenia was 4 days in both groups (p =0.39).

<table>
<thead>
<tr>
<th></th>
<th>Pegfilgrastim (range)</th>
<th>G-CSF controls (range)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Grade IV neutropenia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>all cycles</td>
<td>4 days (0-14)</td>
<td>4 days (0-16)</td>
<td>0.39</td>
</tr>
<tr>
<td>A cycles</td>
<td>2 days (0-11)</td>
<td>2 days (0-12)</td>
<td>0.33</td>
</tr>
<tr>
<td>B cycles</td>
<td>6 days (0-16)</td>
<td>5 days (0-14)</td>
<td>0.79</td>
</tr>
<tr>
<td>Time to neutrophil</td>
<td>13 days (9-23)</td>
<td>14 days (8-24)</td>
<td>0.07</td>
</tr>
<tr>
<td>recovery &gt;500/ul</td>
<td>14 days (10-55)</td>
<td>14 days (8-26)</td>
<td>0.06</td>
</tr>
<tr>
<td>Time to neutrophil</td>
<td>14 days (10-55)</td>
<td>14 days (8-26)</td>
<td>0.06</td>
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<tr>
<td>recovery &gt;2000/ul</td>
<td>14 days (10-55)</td>
<td>14 days (8-26)</td>
<td>0.06</td>
</tr>
<tr>
<td>Febrile neutropenia</td>
<td>33%</td>
<td>42%</td>
<td>0.29</td>
</tr>
<tr>
<td>Positive blood cultures</td>
<td>11%</td>
<td>17%</td>
<td>0.30</td>
</tr>
<tr>
<td>Delay in next cycle</td>
<td>46%</td>
<td>45%</td>
<td>0.56</td>
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Conclusions: A single dose of pegfilgrastim is as effective as daily G-CSF for supporting the hyper-CVAD chemotherapy regimen.
An Outpatient Lymphoma Salvage Approach Incorporating Risk Stratification and Treatment Escalation

Andrew Spencer1, Andrew Grigg2, John Catalano3, Michael Leahy4, Craig Underhill5, Chris Arthur6, James D'Rozario7, Ray Lowenthal8, Kerry Taylor9.

1Alfred Hospital, Melbourne; 2Royal Melbourne Hospital, Parkville; 3Frankston Hospital, Frankston; 4Fremantle Hospital, Fremantle; 5Border Medical Oncology, Albury; 6Royal North Shore Hospital, Sydney; 7Canberra Hospital, Canberra; 8Royal Hobart Hospital, Hobart; 9The Mater Adult Hospital, Brisbane.

Aim: We have previously demonstrated significant anti-lymphoma activity using an outpatient-based salvage approach comprising vinorelbine and gemcitabine. We now report results of a similar outpatient approach but incorporating patient stratification and treatment escalation.

Method: Patients were stratified into Group 1 (G1 - good risk - first relapse following durable first remission - follicular NHL>12 months, all other NHL sub-types and HL>6 months); Group 2 (G2 - poor risk – primary refractory, second or subsequent relapse, or non-durable first remission); or Group 3 (G3 - post-transplant). Two chemotherapy regimens were evaluated. VGF (vinorelbine 25mg/sqm days 1 and 8, gemcitabine 1000mg/sqm days 1 and 8, pegfilgrastim 6mg SC day 9); F-GIV (same as VGF but with ifosfamide 3000mg/sqm day 1 with mesna uroepithelial protection). G1 and G3 commenced therapy with VGF and G2 with F-GIV. Following 2 cycles of therapy all patients were planned for interim re-staging. Any patients demonstrating disease progression were removed from trial. Responsive patients (>50% reduction in all previous sites of disease and gallium or FDG-PET negativity where baseline positivity was demonstrated) received 2 further cycles of the same therapy, the remainder ‘escalated’ therapy with F-GIV (G1 and G3) or IVAC (G2) (inpatient treatment with ifosfamide, VP-16 and Ara-C).

Result: 90 patients were accrued between December 2002 and December 2004. Here we report on the first 88 patients (G1 = 26, G2 = 50, G3 = 12), median age of 57 years (range, 17-78). Diagnoses - HL, n = 16 (nodular sclerosing = 13, mixed cellularity = 3) and NHL, n = 72 (diffuse large cell = 41, follicular = 16, others = 15). G1 and G2 have received 79 and 138 cycles of VGF and F-GIV, respectively, with cycle grades 3/4 neutropenia or thrombocytopenia occurring in 24% and 18% (VGF) and 62% and 49% (F-GIV) of patients, respectively. Febrile neutropenia, hospital admission, treatment delay or dose-reductions occurred with 4%, 19%, 4%, 1% and 17%, 34%, 9%, 9% of VGF and F-GIV cycles, respectively. The 12 post-transplant patients (G3) have received 28 and 8 cycles of VGF and F-GIV, respectively, with no unexpected toxicities. Based on published standardised response criteria overall response (CR + CRu + PR) after 2 to 4 cycles of treatment is 53% (n = 47).

Conclusion: Based on this interim analysis we conclude that both VGF and F-GIV can be safely administered on an outpatient basis and show activity against advanced lymphoma. The impact of stratification and escalation remains to be determined.
HL/DLBCL cohort was 65±9% at 12, and 61±9% at 24 months. The 24 month PFS by BCNU or TBI regimen was 63% and 57% respectively (p=0.56). The 24 month PFS for HL or DLBCL treated with either regimen was 54±13% and 68±12% respectively (P=0.34)

Additional groups were too heterogeneous to statistically assess. 1 of 6 patients with aggressive “other” lymphoma maintained a durable remission (39 months) and 4/8 patients with indolent lymphoma have ongoing CR at 9 – 59 months follow up.

Conclusions: Stanford TBI or BCNU conditioning prior to AuSCT are well-tolerated and result in substantial rates of PFS in patients with poor risk HL or DLBCL.

HSANZ Free Communications 1: Lymphoma / HD (Auditorium)

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Adjuvant Rituximab Causes Prolonged Hypogammaglobulinaemia and Neutropenia Following Autologous Peripheral Blood Stem Cell Transplant for Non-Hodgkins Lymphoma

Jake Shortt and Andrew Spencer
Department of Haematology and Bone Marrow Transplant, Alfred Hospital, Melbourne, Victoria, Australia

Aim: To investigate the effects of post-autograft adjuvant rituximab therapy on immune reconstitution in patients treated for high risk non-Hodgkins lymphoma.

Methods: We performed a retrospective analysis of serum immunoglobulin levels and peripheral blood neutrophil counts up to two years following autograft. All patients received high dose chemotherapy (lomustine 200mg/m², etoposide 4g/m² and cyclophosphamide 5.4g/m²) followed by autologous stem cell rescue by a standard protocol, between 1999 and 2004. Eleven patients (mean age 49; range 31-63) had four doses of rituximab 375mg/m² administered at weekly intervals from two months post autograft. Quantitative serum immunoglobulin levels and peripheral blood neutrophil counts from these patients were compared to those from fifteen patients (mean age 51; range 31-67) who did not receive adjuvant rituximab therapy. The incidences of hypogammaglobulinaemia and neutropenia at regular intervals post autograft were compared.

Results: Baseline incidence of neutropenia (peripheral blood neutrophil count below 1.5 x 10^9/L) and hypogammaglobulinaemia (serum immunoglobulin level below reference range) was similar between R+ and R-groups. There was a higher incidence of both neutropenia and hypogammaglobulinaemia in the R+ group from six months autograft. This persisted at two years. Some surviving patients did not have results available for analysis, thus diluting the statistical power of our study. Three out of four R+ patients had low IgG at two years compared to two of seven R- patients (p-value non-significant). One of six R+ patients was still neutropenic at two years; none of the ten patients in the R- group had persisting neutropenia. No patient in either group developed late or atypical infective sequelae.

Conclusions: Rituximab may suppress immunoglobulin production and neutrophil counts for at least two years following autograft. The clinical significance is uncertain, but this may predispose to late opportunistic infections.

HSANZ Free Communications 1: Lymphoma / HD (Auditorium)

48

Risk and Response Adapted Treatment for Early Stage Hodgkin’s Lymphoma (ESHL): Three Year Results of an Australasian Leukaemia & Lymphoma Group/Trans-Tasman Radiation Oncology Group Study

Andrew Wirth, Andrew Grigg, Max Wolf, Sidney Davis, Mark Hertzberg, David Joseph, Carol Johnson, John Reynolds for Australasian Leukaemia Lymphoma Group, Trans-Tasman Radiation Oncology Lymphoma Group

Background: To evaluate risk factor- and response-adapted therapy of ESHL the following study was performed.

Methods: Patients (Pt) with ESHL were prospectively assigned to 1 of 3 treatment groups according to baseline characteristics: Group(Gp) A (Stage I-IIA, no risk factors)-3 cycles ABVD; Gp B (Stage I-IIA with ≥ 1 risk factor (ESR > 50, bulky disease, extra-nodal disease, > 3 sites))-4 cycles ABVD; and Gp C (Stage I-II with B symptoms)-4 cycles ABVD. The protocol specified that involved-field radiotherapy 30 Gy (IFRT) be given for Gps A and B after complete response/unconfirmed (CR/u) or partial response (PR) to initial ABVD, and for Gp C only after CR/u. Lesser responses/major toxicity led to individualised treatment.

Results: There were 148 eligible pt: median age 34 yrs, 52% male, 95% supradiaphragmatic location, 32% stage I, 18% B-symptoms, 20% bulky disease. Five deaths have occurred, 3 from HD. Main results:
### Response to initial ABVD (% evaluable pt)

<table>
<thead>
<tr>
<th></th>
<th>All N=148</th>
<th>Gp A N=75</th>
<th>Gp B N=47</th>
<th>Gp C N=26</th>
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<tbody>
<tr>
<td>CR/u</td>
<td>86%</td>
<td>91%</td>
<td>85%</td>
<td>72%</td>
</tr>
<tr>
<td>PR (%)</td>
<td>10%</td>
<td>9%</td>
<td>7%</td>
<td>20%</td>
</tr>
</tbody>
</table>

### Additional chemotherapy given

- Gp A: 6%
- Gp B: 1%
- Gp C: 4%
- Gp C: 23%

### IFRT given

- Gp A: 94%
- Gp B: 95%
- Gp C: 94%
- Gp C: 92%

### 3 year freedom from progression

- Gp A: 92%
- Gp B: 98%
- Gp C: 88%
- Gp C: 80%

**Conclusions:** Three year results of limited ABVD+IFRT for pt without risk factors are excellent and support further treatment de-escalation studies for this pt group. For pt with B-symptoms / other risk factors, results are less satisfactory, and studies of selective treatment intensification may be warranted.

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**HSANZ Free Communications 2: Myeloma Biology (Meeting Room 2)**

**49**

**Characterisation of a Novel In Vivo Murine Model of Human Multiple Myeloma**

Janelle Sharkey, Tiffany Khong and Andrew Spencer

*Myeloma Research Group, Alfred Hospital, Melbourne, Victoria, Australia*

**Aim:** This study was conducted to determine the ability of the human multiple myeloma cell line (HMCL) KMS-11, which has a t(4;14) translocation and ectopically expresses constitutively active fibroblast growth factor receptor 3 (FGFR3), to engraft in NOD/Scid mice and to characterise the disease induced.

**Method:** 14 male NOD/SCID mice were given a cell transfer of either 1x10⁷ (n=7) or 3x10⁷ (n=7) KMS-11 cells by tail vein injection 24 hours after irradiation (350 cGy whole body irradiation from a ¹³⁷Cs source). Mice were monitored for cachexia or hind limb paralysis and humanely killed when they displayed obvious signs of disease, as determined using the body scoring system of Ullman-Cullere and Foltz. Bone marrow, liver and extramedullary plasmacytomas were isolated and KMS-11 cells were identified by flow cytometry for CD38+ cells. Bones (femur, humerus, vertebrae) and liver were collected from diseased mice at time of death and stained for human CD38 by 3-layer immunoperoxidase staining. Likewise at the time of death, serum β2-microglobulin and bFGF were determined by ELISA. Statistical analysis was performed using Student’s unpaired t-test.

**Result:** KMS-11 cells are able to engraft and induce systemic disease in NOD/Scid mice. Survival time post inoculation with KMS-11 cells was inversely correlated to initial cell dose 1x10⁷ median survival = 39 days, 3x10⁷= 45 days (p-value 0.07). KMS-11 cells (identified as CD38 positive cells) were predominantly present in the liver and bone marrow of diseased mice. β2-microglobulin and basic fibroblast growth factor (bFGF), both secreted by KMS-11 cells, increased in the serum of diseased mice over time.

**Conclusion:** This mouse model of human myeloma may be useful for the evaluation of novel anti-tumour agents with activity against FGFR3.

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**HSANZ Free Communications 2: Myeloma Biology (Meeting Room 2)**

**50**

**Coxsackievirus A21 as a Novel Oncolytic Purging Agent for Multiple Myeloma**

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**Aim:** Oncolytic viruses are currently viewed as attractive biological agents for the control of human malignancy. Our aim was to investigate the capacity of a naturally occurring virus known as Coxsackievirus A21 (CVA21) to target and destroy multiple myeloma (MM) cells while leaving non-malignant cells intact.

**Methods:** We assessed the capacity of CVA21 to target and destroy malignant and pre-malignant plasma cells in vitro using 3 MM cell lines and 10 bone marrow (BM) biopsies collected from patients undergoing routine investigation for MM at the Newcastle Mater Hospital [n = 8 MM (3 at diagnosis, 2 in partial remission and 3 relapsed); n=2 monoclonal gammopathy of undetermined significance (MGUS)]. Flow cytometry was used to establish the presence of the combination of receptor molecules required for successful CVA21 infection, i.e., intercellular adhesion molecule-1 (ICAM-1) and decay-accelerating factor (DAF). Samples were challenged with varying concentrations of CVA21 over 48 hours, viral progeny was quantified by viral endpoint titrations in tissue culture, and viability was assessed by flow
cytometry following CD138 and PI staining. Haematopoietic progenitor cell potential was assessed by CFU-GM assays.

**Result:** All MM and MGUS plasma cells expressed high levels of ICAM-1 and DAF. MM cell lines were remarkably susceptible to CVA21 lytic infection as compared to normal peripheral blood, with MM cells producing 100-1000 fold increases in viral progeny within 48 hours of infection. Incubation of patient BM with CVA21 for 48 hours resulted in specific purging of up to 97.2% of plasma cells in both MM and MGUS samples, with no significant decrease in progenitor cell function.

**Conclusion:** CVA21 virotherapy may have potential applications as a prophylactic treatment for MGUS, a systemic anti-tumor agent for MM, or in the *ex vivo* purging of malignant plasma cells within autografts prior to autologous stem cell transplantation.

**The Carbohydrate Antigen Lewis\(^\text{Y}\) as a Target for Immunotherapy of Multiple Myeloma**

1Dirk Honemann, 1David Westerman, 1Peter Gambell, 1Jenny Westwood, 1Michael Kershaw, 1Ellie Thompson, 1Mark Smyth, 2Andrew Scott, 2Fiona Smyth, 1H Miles Prince

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**Aim/Background:** Antibody mediated immunotherapy of B cell lymphoproliferative disorders has recently been very successful. In contrast, adoptive immunotherapy of Multiple Myeloma (MM) has been hampered by the lack of suitable target antigens, since most B cell antigens are not expressed on plasma cells. However, expression of various carbohydrate molecules on plasma cells has been recognized in the past. We have been examining the expression of the carbohydrate antigen Lewis\(^\text{Y}\) (Le\(^\text{Y}\)) on plasma cells with the aim of testing its suitability as a target for immunotherapy in subsequent experiments.

**Methods/Results:** We examined Le\(^\text{Y}\) expression by flow-cytometry on 47 freshly derived bone marrow aspirates from MM patients. 32 had sufficient PC for analysis and 26 (81.25%) were positive for Le\(^\text{Y}\). The median fluorescence intensity [MFI] of all positive samples was 8.2 as compared to 1.3 on lymphocytes (internal control). We developed a novel construct of a chimeric T-cell receptor which recognises the Le\(^\text{Y}\) antigen in an MHC-independent manner, activates T-cells and confers additional co-stimulatory signals. We have shown efficient retroviral transduction of this construct into T-cells with a transduction efficacy of up to 65%. Functional analysis of transduced T-cells showed specific IFN-\(\gamma\) secretion in response to a co-culture with Le\(^{\text{Ypos}}\) target cells at an effector:target ratio of 10:1 - but not to Le\(^{\text{Yneg}}\) targets or when control T-cells were used. Further, we demonstrated cytotoxicity of transduced T-cells against MM cells with up to 50% specific lysis of target cells in response to co-culture.

**Conclusion:** Le\(^\text{Y}\) is expressed in the majority of cases of MM, albeit at a low to moderate level. Le\(^\text{Y}\) expression on MM cells can serve as a target for adoptive immunotherapy with gene-modified T-cells expressing a chimeric receptor recognising Le\(^\text{Y}\). We are planning a clinical phase I study with gene-modified autologous T-cells in patients with Le\(^\text{Y}\) positive MM.

**Azacytidine Modulates IL-6 and p16 Gene Expression and Induces Apoptosis in Human Multiple Myeloma Cell Lines**

Tiffany Khong, Janelle Sharkey and Andrew Spencer

Myeloma Research Group, Alfred Hospital, Melbourne, Victoria, Australia

**Aim:** Azacytidine (AZA), a DNA methyltransferase inhibitor, has been shown to inhibit cell growth and induce apoptosis in some cancer cells. We determined the impact of AZA on a panel of human myeloma cell lines (HMCL); KMS 12PE, KMS 18, LP-1, NCI-H929, OPM-2, RPMI-8226 and U266 and in an *in vivo* murine model of multiple myeloma (5T33 model).

**Method:** Dose responsiveness to AZA was determined via MTS assays with a range of AZA doses (1-10µM) for 72 hours. FACS and cell cycle analysis were used to evaluate the profile of the cells after exposure to AZA. Western analysis using antibodies against Caspases 3,8,10, PARP, phospho-ERK, ERK, Stat3 and phospho -Stat3 were performed to help characterize the mechanism(s) of cell killing. Similarly inhibitors of caspases 3,8 and 9 were used to determine which apoptotic pathway was being preferentially activated by AZA. Reactivation of p16 gene by AZA-induced hypomethylation was assessed with methylation specific PCR. The level of IL-6 in conditioned media from U266 cells treated with AZA was determined by ELISA assay and addition of exogenous IL-6 was applied to determine if the IL-6 maintained cell viability. AZA was also administered to a 5T33 murine model of multiple myeloma at increasing concentrations (1, 3, 10 mg/kg).
Results: MTS assays demonstrated a dose and time dependent AZA-induced inhibition of HMCL viability with effective concentrations of AZA ranging from 1-10 µM. This was associated with accumulation of cells in the G0/G1 phase with decreasing number of cells in the S and G2/M phases. Cleavage of caspases 3,8,10 and PARP within 24 hours of AZA treatment confirmed early AZA-induced HMCL apoptosis. Inhibitors of both Caspase 3 and 9 effectively abrogated AZA-induced apoptosis. In contrast Caspase 8 inhibitor was less effective which is consistent with AZA acting via the mitochondrial apoptotic pathway. MSP-PCR of the p16 gene indicated a loss of methylation and up-regulated transcription after 48 hours treatment with 5 µM AZA. ELISA demonstrated a rapid fall in autocrine U266 IL-6 production confirmed on RT-PCR to be associated with reduced transcription of the IL-6 gene. This was temporarily associated with the disappearance of upstream phospho -Stat3. Addition of exogenous IL-6 did not rescue U266 from AZA-induced apoptosis. In the 5T33 model mice were tolerant to AZA up to 3 mg/kg but longer survival was not observed. Higher doses of AZA are currently being evaluated. Conclusion: AZA rapidly induced apoptosis in a range of HMCL and is capable of downregulating autocrine IL-6 production by HMCL independent of induction of hypomethylation.

PKC412 Induces c-jun Phosphorylation Mediated Apoptosis in Human Multiple Myeloma Cell Lines

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Aim: This study was conducted to explore the effect and mode of action of the protein kinase C inhibitor PKC412 on proliferation and apoptosis in human multiple myeloma cell lines (HMCL).

Method: MTS assay was used to measure cell proliferation of 6 HMCLs and to examine the effect of co-treatment of HMCLs with PKC412, Bortezomib or the NFkB inhibitor, SN50. PKC412-induced apoptosis was evaluated by western blot (PARP cleavage) and annexin-V-FITC / propidium iodide flow cytometry. Similarly, the level of PKC412-induced apoptosis of HMCLs co-treated with the JNK inhibitor SP600125 was also determined. PKC activity after PKC412 treatment was assessed using the Peptag PKC functional assay and expression of PKC isoforms by HMCLs was determined by western blot. c-fos expression was evaluated by both RT-PCR and western blot. Expression of c-jun and phospho-c-jun was evaluated by western blot. NFkB activation was determined using the NFkB transcriptional assay kit.

Result: Sensitivity to PKC412 varied but some activity was seen against all HMCLs after 72hr treatment at doses of 0.5µM and above. PARP cleavage was observed in all HMCLs tested at both 24 and 72hr following treatment. PKC activity of PKC412 treated HMCLs was decreased in all HMCLs tested but no correlation between PKC isoform expression and sensitivity to PKC412 was found. However, a correlation between sensitivity to PKC412 and the presence of either an activating ras or FGFR3 mutation in HMCLs was observed. When HMCLs were pre-treated with PKC412, the PMA-induced increase in c-fos mRNA was inhibited, and PKC412 down-regulated baseline c-fos mRNA expression over time. PKC412 also down-regulated c-jun mRNA expression in nuclear extracts of HMCLs. c-jun phosphorylation was up-regulated for 24hr after PKC412 treatment and this increase in expression and phosphorylation was inhibited by co-treatment with the JNK inhibitor SP600125. Co-treatment of the HMCL NCI-H929 with PKC412 and SP600125 partially abrogated PKC412-induced apoptosis, indicating that it is partially JNK dependent.

Increased NFkB activation was observed in PKC412 treated HMCLs with the level of increase correlating with PKC412 sensitivity. Enhanced killing of HMCL was seen when NFkB activation was inhibited with either Bortezomib or SN50, suggesting the increase in activation of NFkB may be a PKC412-induced survival response. Conclusion: Our results demonstrate that PKC412 induces apoptosis in HMCLs which is partially dependent on JNK. Furthermore PKC412 is more effective when used in combination with a NFkB inhibitor. The further evaluation of PKC412 in the treatment of multiple myeloma is justified.

Bcl-xL Mediates Resistance to TRAIL-Induced Apoptosis in Multiple Myeloma

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Aim: To elucidate the role of Bcl-xL in resistance to TRAIL-induced apoptosis of human myeloma cell lines (HMCL).

Methods: HMCL were pre-treated with 50uM caspase inhibitors for 30min prior to induction of apoptosis by LZ-TRAIL (1ug/mL) or agonist antibodies (20µg/mL) specific for TRAIL receptors TRAIL-R1 or TRAIL-R2. Cell death was quantified by MTS assay, AnnexinV FITC/propidium iodide flow cytometry and immunoblots to detect PARP cleavage. Bcl-xL levels in HMCL were reduced by sub-lethal treatment with cell-cycle inhibitor flavopiridol (50nM and 100nM) or
overexpressed via transduction of HMCL with murine stem cell retroviral vector (MSCV-GFP-Bcl-xL). HMCL were then treated with LZ-TRAIL or agonist antibodies for 24 hours. Bcl-xL expression was analysed by RT-PCR, immunoblot analysis and flow cytometry.

**Results:** TRAIL-induced apoptosis of TRAIL-sensitive RPMI-8226 (non-mitochondrial) was reduced by caspase 8 inhibitor (p-value=0.001) whilst in the less TRAIL-sensitive NCI-H929 both caspase 8 (p-value=0.02) and caspase 9 (p-value=0.01) reduced TRAIL-induced apoptosis. We examined basal Bcl-xL levels in HMCL and observed that resistance to TRAIL correlated with increased Bcl-xL expression ($R^2=0.75$). Pre-treatment of HMCL with flavopiridol decreased Bcl-xL expression and led to an increase in sensitivity to apoptosis mediated via TRAIL-R2 receptor in NCI-H929 (p-value ≤ 0.05) and U266 (highly TRAIL resistant) (p-value ≤ 0.05). Over-expression of Bcl-xL in RPMI-8226 (non-mitochondrial) and NCI-H929 (mitochondrial-dependant) using a murine retroviral vector (MSCV-GFP-Bcl-xL), protected NCI-H929 (p-value=0.04) but not RPMI-8226 from TRAIL-induced apoptosis mediated via TRAIL-R1.

**Conclusion:** TRAIL induces apoptosis of HMCL via both mitochondrial and non-mitochondrial pathways. The expression of Bcl-xL is an important mediator of resistance in HMCL which undergo TRAIL-induced apoptosis via the mitochondrial-dependant pathway.

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**55 Haemopoietic Stem Cell Transplants for Chronic Myelofibrosis – a Review from the ABMTRR**

*Ian Nivison-Smith¹, Ken Bradstock², Anthony Dodds³, David Ma³ and Jeff Szer⁴*

1: ABMTRR, Darlinghurst NSW Australia. 2: Westmead Hospital, Westmead NSW Australia. 3: St Vincent’s Hospital, Darlinghurst NSW Australia. 4: Royal Melbourne Hospital, Parkville VIC Australia

The Australasian Bone Marrow Transplant Recipient Registry (ABMTRR) has been recording activity and outcome data for haemopoietic stem cell transplants in Australia since 1992 and New Zealand since 1998. Between 1992 and 2004 there were 50 haemopoietic stem cell transplants for chronic myelofibrosis in Australia and 1 in New Zealand. Of these, 46 were allogeneic, 3 syngeneic and 2 autologous; 35 were male and 16 were female. The median age at transplant was 48 with a range of 16 to 71.

The annual number of transplants for this indication is small but increasing. In the five years 2000 to 2004, there were 33 transplants for this indication compared to 14 in 1995 - 1999.

Of the donors for allogeneic transplants, 33 were HLA-identical siblings, 3 were siblings or other relatives with 1 HLA mismatch and 10 were unrelated volunteers. Within the allogeneic transplants performed in 2000 and later, 6 used reduced intensity conditioning.

Two patients had persistent disease, four died prior to 30 days post transplant and it was assumed that all others achieved remission. Four allogeneic and one autologous recipient relapsed, all within 3 years post transplant. For allogeneic transplants, the 5-year overall survival probability was 48% which is comparable to the finding of 47% in a recent EBMT study. Transplant related mortality at 100 days was 19.6%.

There were 16 deaths in the first year post transplant among allogeneic and syngeneic recipients, from infection (7), GVHD (4), organ failure (3) and persistent disease (2). Both autologous recipients died, from septicaemia at 6 months and relapse at 2.3 years post transplant.

The ABMTRR is an important national data resource which enables accurate and timely analysis of transplant activity and outcome, particularly for rare indications that have relatively small numbers.


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**56 Fludarabine/Melphalan Conditioning for Allogeneic Stem Cell Transplants in Older Patients with AML/MDS**

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**Aim:** To assess the tolerability and efficacy of a reduced-intensity, non-TBI based allogeneic stem cell transplant conditioning regimen utilising fludarabine and melphalan (FluMel) in older patients with AML/MDS

**Methods:** Retrospective review of all patients undergoing allogeneic allogeneic transplantation utilising fludarabine (25mg/m2 D-7 to D-3) and melphalan (120mg/m2 D-2) conditioning at our institution was performed. All patients aged ≥ 50 years transplanted for AML or MDS were included in the study cohort. Survival data was calculated utilising the Kaplan-Meier product-limit method.

HSANZ Free Communications 3: Transplantation / Cell Therapy (Meeting Room 5)
Results: A total of 20 patients received a FluMel alloSCT, including 15 for AML and 5 for MDS. Median age at transplant was 60.4 years (range 50.2 to 66.6). Utilising the Intergroup AML cytogenetic risk groups, 7/15 had intermediate risk and 7/15 poor risk (1 had no data available). Transplants for AML were performed in CR1 (n=5), early 1st relapse (n=3), CR2 (n=3), MDS phase post CR1 (n=2), early 3rd relapse (n=1), and primary refractory disease (n=1). All 5 MDS patients were previously untreated; all had INT-1 risk disease on IPSS. Donor source included 14 siblings and 6 unrelated-donors; G-CSF stimulated peripheral blood stem cells were collected from all. Standard GVHD prophylaxis was cyclosporine, plus methotrexate (days +1, 3, 6 and 11). 2 patients died prior to engraftment; one from acute hepatic failure of uncertain aetiology, the other from an idiopathic pneumonia syndrome. All remaining patients achieved durable engraftment. 10/18 evaluable patients developed acute GVHD - 9 Grades II-IV, 3 Grades III-IV. 9/12 evaluable patients developed chronic GVHD, 8 were extensive-stage. At a median follow-up of 2.4 years, both overall and event-free survival at 2 years for the whole cohort is 66%. In addition to the 2 patients above, 2 died from relapsed disease, 1 from acute GVHD, and 1 from multi-organ dysfunction.

Conclusion: FluMel alloSCT’s in patients > 50 years of age with AML/MDS are associated with acceptable treatment-related toxicity and long-term disease-free survival in the majority. Reduced-intensity techniques may increase the feasibility of allogeneic transplantation in patients previously considered unsuitable due to older age, and warrant further study.

HSANZ Free Communications 3: Transplantation / Cell Therapy (Meeting Room 5)

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A Practical Approach for Assessing Donor Natural Killer Cell Alloreactivity in Haploidentical HSCT

Mingus Rose¹, Mary Diviney², Anthony P Schwarer¹
¹ Alfred Hospital Bone Marrow Transplant Programme, Melbourne
² Australian Red Cross Blood Service, Melbourne

Aim: Successful outcome of haploidentical haematopoietic stem cell transplantation (haplo-HSCT) for treatment of myeloid malignancies depends greatly upon the KIR ligand-mismatch-driven graft versus leukaemia action of donor-derived alloreactive natural killer (NK) cells. This study aimed to make the process of identifying donor alloreactive NK clones of interest more efficient by using appropriately mismatched feeder cells for growing clones and by the subsequent selection of clones based on receptor expression prior to functional analysis.

Methods: NK clones were grown from haplo-HSCT donor (n = 6) or healthy volunteer (n = 2) derived peripheral blood lymphocytes by first depleting T cells then diluting and culturing with irradiated feeder cells in medium containing PHA for initial NK activation, then IL-2 and finally IL-15. The feeder cells for each donor were derived from volunteers lacking the same KIR ligand as the prospective transplant recipient and consisted of either PBMC (NK culture day 1) or PHA lymphoblasts (NK culture day 5). Growing colonies were each checked by flow cytometry for NK phenotype (CD56+ CD3-) and expression of inhibitory receptors KIR2DL1, KIR2DL2/3, KIR3DL1, and NKG2A. Receptor expression in conjunction with KIR genotype of the donor was used to select NK clones for functional analysis in cytotoxicity assays (LDH release and flow cytometry-based) against autologous and KIR ligand-mismatched targets.

Results: To date, NK clones have been grown from all subjects using no more than four 96-well culture plates per donor. Phenotypic analysis shows a diverse clonal distribution of inhibitory receptors, as has been previously described by others. Interestingly, KIR repertoire of NK clones appears to be influenced by HLA class I (KIR ligand) repertoire of the feeder cells, although numbers are too small at this stage to allow meaningful analysis. Target killing could not always be predicted by effector cell inhibitory receptor expression, suggesting a greater complexity in the interaction than that accounted-for or detectable using cytotoxicity alone as a measure of cell function.

Conclusion: The strategy for small-scale culture of NK clones using feeder cells with a specific KIR ligand mismatch results in NK clones with diverse KIR repertoire that doesn’t always predict cytotoxic function.

HSANZ Free Communications 3: Transplantation / Cell Therapy (Meeting Room 5)

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KIR-ligand Mismatched Haploidentical Haematopoietic Stem Cell Transplantation (haploHSCT) for Advanced Haematological Malignancies: Alfred Hospital Experience

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Introduction: Haploidentical HSCT is an experimental form of transplantation where the donor and recipient are matched for only one HLA haplotype. Recent technological advances have improved transplant-related mortality: “megadoses” of CD34+ stem cells have decreased graft rejection and effective T cell depletion has largely abrogated GVHD. Some groups have shown that patients with AML whose donors are KIR-ligand mismatched with them may
have a 50% chance of cure. These outstanding results appear to be due to a wave of NK cell recovery after engraftment – the NK cells recognize the KIR-ligand mismatch to generate a potent GVL effect.

Methods: Patients with advanced myeloid malignancies were conditioned with ATGAM 15 mg/kg/day (day -10 to -6), melphalan 140 mg/m^2 (day -9), thiota 5 mg/kg/day (day -8 to -7) and fludarabine 40 mg/m^2/day (day -6 to -2) followed by infusion on day 0 of CD34+ cells purified using the ClinIMACS device. No post haploHSCT immunosuppression was given. Post transplant G-CSF was not given.

Results: Seven patients without any other curative option for advanced myeloid malignancies (AML = 5; CML-BC = 1; MDS = 1) underwent haploHSCT. Median age was 35 (22-58) yrs. Two female and 5 male. Two patients had previously failed autologous transplantation. Median CD34+ cell dose was 4.2 (1.1-5.1)x10^6 per kg and median CD3+ cell dose was 0.05 (0.01- 0.4)x10^4 per kg. One patient died early (day +9) of Scedosporium infection. One patient died at 9 months of renal failure/GVHD/infection. One patient died at 9 months of renal failure/GVHD/infection. One patient relapsed at 3 months. Four patients (57%) are alive and well at a median of 7 months (day +3 to 5 yrs). Recurrent, particularly viral, infections have been frequent. Two additional patients with T-ALL underwent haploHSCT – both relapsed and died.

Conclusion: HaploHSCT is a relatively well tolerated transplant procedure which appears to be able to control advanced and refractory myeloid haematological malignancies.

59 Prophylactic Infusion of CMV pp65 Specific Cytotoxic T Lymphocytes (CTL) Following Haemopoietic Stem Cell Transplantation (HSCT)

Ken Micklethwaite1, Anna Hansen2, Aaron Foster1, Vicki Antonenas2, Mary Sartor1, Mary McGurgan3, Cameron Turtle1, Ken Bradstock3 and David Gottlieb1
1 Westmead Millennium Institute, University of Sydney at Westmead Hospital, Sydney, NSW
2 Sydney Cellular Therapies Laboratory, Westmead Hospital, Sydney, NSW
3 Blood and Marrow Transplant Service, Westmead Hospital, Westmead, NSW

Aim: CMV reactivation in patients after HSCT is common and life threatening. CMV specific CTL are effective therapy for CMV reactivation occurring after allogeneic transplantation. We aim to demonstrate safety of prophylactically administered adoptively transferred CTL following allogeneic HSCT.

Methods: We are engaged in a phase 1 clinical trial of donor-derived CMV specific CTL for prophylactic infusion post HSCT. Monocyte derived dendritic cells are loaded with an HLA-A2 restricted epitope of the pp65 protein and co-cultured with donor mononuclear cells in vitro. The resulting cell population is >90% CD3+, of which the majority were CMV-specific. These are infused after day 28 of HSCT in the absence of CMV reactivation. Recipients are monitored for infusion-related adverse events, CMV specific immune reconstitution and CMV reactivation.

Results: Five patients have been infused with CMV specific CTL with no adverse events observed within 24 hours. Recipient one is two years post transplant with no CMV, acute graft versus host disease (GVHD) or infections. The second participant died at day +117 with acute GVHD and TTP secondary to cyclosporin. The third has had multiple viral infections and acute GVHD, but no CMV reactivation. The fourth is day +130 with no CMV reactivation, acute GVHD or infections. The fifth participant had primary graft failure, requiring further conditioning and stem cell infusion. She has acute GVHD and will receive a second CTL infusion. A sixth recipient is awaiting CTL infusion. Two patients have demonstrated increases in CMV specific cells in the peripheral circulation following CTL infusion.

Conclusion: Generation of CMV specific CTL for use in the clinical setting is feasible and is not associated with immediate adverse events. Long term safety and efficacy remain to be demonstrated with accrual of greater participant numbers. This technique may decrease morbidity and mortality associated with CMV in HSCT recipients.

60 Ex-Vivo Expansion of CMV Specific CTL for Therapeutic Use in Allogeneic Haemopoietic Stem Cell Transplantation (HSCT)

Anna Hansen1, Ken Micklethwaite2, Aaron Foster2, Vicki Antonenas1, Mary Sartor3, Cameron Turtle3, Ken Bradstock3 and David Gottlieb1.2.3
1 Sydney Cellular Therapies Laboratory, Westmead Hospital, Sydney, NSW
2 Westmead Millennium Institute, University of Sydney at Westmead Hospital, Sydney
3 Blood and Marrow Transplant Service, Westmead Hospital, Westmead, NSW

Aim: We aim to produce CMV-specific T cells in high number and quality for transfer to HSCT patients for the prevention of CMV reactivation following allogeneic HSCT.

HSANZ Free Communications 3: Transplantation / Cell Therapy (Meeting Room 5)

59 Prophylactic Infusion of CMV pp65 Specific Cytotoxic T Lymphocytes (CTL) Following Haemopoietic Stem Cell Transplantation (HSCT)

Ken Micklethwaite1, Anna Hansen2, Aaron Foster1, Vicki Antonenas2, Mary Sartor1, Mary McGurgan3, Cameron Turtle1, Ken Bradstock3 and David Gottlieb1
1 Westmead Millennium Institute, University of Sydney at Westmead Hospital, Sydney, NSW
2 Sydney Cellular Therapies Laboratory, Westmead Hospital, Sydney, NSW
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Methods: We are engaged in a phase 1 clinical trial of donor-derived CMV specific CTL for prophylactic infusion post HSCT. Monocyte derived dendritic cells are loaded with an HLA-A2 restricted epitope of the pp65 protein and co-cultured with donor mononuclear cells in vitro. The resulting cell population is >90% CD3+, of which the majority were CMV-specific. These are infused after day 28 of HSCT in the absence of CMV reactivation. Recipients are monitored for infusion-related adverse events, CMV specific immune reconstitution and CMV reactivation.

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HSANZ Free Communications 3: Transplantation / Cell Therapy (Meeting Room 5)

60 Ex-Vivo Expansion of CMV Specific CTL for Therapeutic Use in Allogeneic Haemopoietic Stem Cell Transplantation (HSCT)

Anna Hansen1, Ken Micklethwaite2, Aaron Foster2, Vicki Antonenas1, Mary Sartor3, Cameron Turtle3, Ken Bradstock3 and David Gottlieb1.2.3
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3 Blood and Marrow Transplant Service, Westmead Hospital, Westmead, NSW

Aim: We aim to produce CMV-specific T cells in high number and quality for transfer to HSCT patients for the prevention of CMV reactivation following allogeneic HSCT.
Methods & Results: CMV-specific CD8+ cytotoxic T cells (CTL) can be generated in vitro using peripheral blood mononuclear cells (PBMC) isolated from CMV seropositive transplant donors. CTL are cultured in IL-2 after two stimulations with monocyte-derived dendritic cells pulsed with an HLA-A2 restricted CMV peptide. Procedures adhere to Good Manufacturing Practice (GMP) guidelines and are performed within a clean room facility (class 350), following an SOP written for this procedure. With starting frequencies of around 1% CMV specific tetramer+ CTL, expansion of between 400-8000 fold occurs over 3 weeks of culture. The resulting infusion is predominantly CD3+ CD8+ T cells, the majority of which are CMV-specific. Specific secretion of IFNγ in response to stimulation with CMV antigen is observed. CTL are cryopreserved and stored in liquid nitrogen prior to infusion, with appropriate quality assurance including sterility, post-thaw viability and mycoplasma testing. To date, 6 patients have been enrolled in the first phase of clinical trials. There have been no immediate infusion related adverse effects. Further work is continuing on the long-term safety and efficacy of this therapy, including the use of in vitro assays for the assessment of alloreactivity and CMV-specific cytotoxic activity.

Conclusion: CMV-specific CTL suitable for use in allogeneic HSCT patients have been generated under GMP conditions and are currently being used in a Phase 1 safety trial.

HSANZ Free Communications 4: Leukaemia Biology (Skyline Room 1)

61 Predictive Value of Two Assays Measuring Kinase Inhibition Induced by Imatinib in CML Patients

Deborah White1, Verity Saunders1, Susan Branford2, Kevin Lynch3, L Bik To1 and Timothy Hughes1

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We have measured imatinib-induced inhibition of BCR-ABL kinase activity by assessing the level of Crkl phosphorylation (p-Crkl) in in-vitro and in-vivo assay. %p-Crkl was assessed in presentation CML patients using western blot and the value of both assays for predicting molecular response was compared statistically. In the in-vitro assay (IC50imatinib) a significantly higher proportion of patients with kinase inhibition above the median achieved rapid major molecular response (MMR). Conversely, the majority of patients with kinase inhibition below the median had delayed response. Interestingly analysis at 24 months revealed responses were equivalent.

For the in-vivo assay blood was collected at 7 day intervals for the first 28 days of imatinib treatment, and kinase inhibition monitored. We found a significantly higher percentage of patients achieved MMR in the group who had in-vivo kinase inhibition above the median by day 21-28. In contrast to the in-vitro assay the in-vivo assay identified patients with superior molecular response at all time points. This data suggests that the in-vitro assay predicts the initial slope of the leukaemic cell reduction, perhaps because it reflects the sensitivity of the more mature leukaemic cells. In contrast the in-vivo assay is a better predictor of later response because it reflects the net effect of dose intensity, GI absorption, metabolic heterogeneity and intrinsic sensitivity of the CML cells. Thus assays of in-vivo levels of kinase inhibition may provide the best early predictor of long term molecular response.

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62 CXCL12 Synergises with Cytokines to Enhance the Proliferation of Pre-B ALL

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CXCL12 has been described as a B cell growth factor and is reported to support B cell progenitor ALL cell survival in vitro. CXCL12 has also been reported to synergise with cytokines including TPO and SCF. The aim of this study was to examine the interaction between CXCL12 and cytokines known to affect ALL cell biology. The combination of CXCL12 with IL-3 and IL-7 and/or Flt-3L enhanced survival in 7 of 21 and the proliferation of 3 of 21 cases of B cell progenitor ALL over 4 days in serum free culture. In the presence of marrow stromal cells, 9 of 12 cases demonstrated enhanced proliferation in the presence of exogenous CXCL12, IL-3 and IL-7. All cases examined displayed CXCL12-dependent proliferation on stroma, with inhibition of CXCL12 resulting in a 44.7±20.4% reduction in proliferation.
(p<0.03, n=12). The addition of IL-3 or IL-7 to cells cultured on stroma enhanced proliferation in 4 and 4 of 12 cases respectively. When CXCL12 was blocked in these cultures, IL-3 and IL-7 stimulated the growth of 3 and 7 of the 12 cases. In 2 of 12 cases clear synergistic interactions between CXCL12 and IL-7 were observed, while only 1 of 12 cases showed synergy between IL-3 and CXCL12. No synergistic interactions were observed between Flt-3L and CXCL12. The synergistic interactions between IL-7 and CXCL12 were associated with enhanced signalling through the MEK/ERK, PI-3K/AKT and p38 MAPK pathways. Inhibition of signalling through PI-3K and p38 MAPK, but not MEK, was able to inhibit synergistic proliferation, suggesting that the PI-3K and p38MAPK pathways are the primary mechanism responsible for synergistic proliferative responses between IL-7 and CXCL12 in B cell progenitor ALL. This study demonstrates for the first time the growth factors influencing pre-B ALL proliferation, and provides new directions for targeted therapy.

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Simplified Gene Expression Profiles Identified by Microarray Suggest a Potential Diagnostic Tool in Paediatric Acute Lymphoblastic Leukaemia

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Aim: The optimal treatment of patients with childhood acute lymphoblastic leukaemia (ALL) depends on establishing accurate diagnosis. Recently, researchers have attempted to assess global transcription using microarray technology to identify gene expression ‘signatures’ that correlate to patient outcome. In this study we seek to strategically develop the application of microarray gene expression profiling to identify ALL patients with clinically homogenous presentations but which may respond differently to established treatment regimens.

Methods: We describe our analysis of both Affymetrix (HU133A) and cDNA (10.5K) microarray gene expression profiles generated from diagnostic bone marrow (BM) from >100 ALL patients. Data analysis focused on a novel and innovative statistical technology, Gene-RaVE, a multinomial regression model using Bayesian variable selection which leads to the generation of a parsimonious and simple diagnostic signature, but which provides increased predictive ability over current analysis approaches.

Results: Gene expression profiles from a cohort of ALL patients, identified as ‘standard risk’ at diagnosis, were compared on the basis of their overall clinical outcome: relapse within 2 yrs vs non-relapse. Using a range of analyses including t-test, Gene-RaVE, discriminant analysis approaches and principle component analysis, we have discovered that small subsets of genes (<10), all of which included Nedd4BP3 and Ribosomal Protein L38, can be used to distinguish the two outcome groups. Subsequent validation using real time PCR supports of the findings. The Gene-RaVE algorithm also provides a generic framework for survival analysis and indicates that the expression of these small numbers of genes can be used to build a survival ‘index’ which correlates with the time to a relapse event in standard risk childhood ALL patients.

Conclusions: Our results are suggestive of a way forward in the development of an informative, yet efficient diagnostic tool for this childhood malignancy using microarray gene expression analysis technology.

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The Wnt/beta-catenin Pathway Promotes Growth and Survival of Acute Lymphoblastic Leukaemia (ALL) Cells in Vitro

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Wnt signalling is known to play an important regulatory role in self-renewal of haematopoietic stem cells as well as development of normal lymphocyte progenitors. Recently this pathway was implicated in a number of haematological malignancies including AML, CML, MM and CLL. However its role in acute lymphoblastic leukaemia is not clear. We have previously shown that primary pre-B ALL cells and cell lines express various Wnt family members, receptors and downstream transcription factors. We extended these studies further to elucidate a functional role of Wnt signalling in leukemic cells. Exposure to Wnt3a, a ligand inducing the canonical Wnt signal cascade, resulted in stabilisation of beta-catenin and its nuclear translocation in all growth factor independent pre-B cell lines (n=3), stromal-dependent cell line (n=1) and primary cases (n=3) examined. Wnt3a treated pre-B ALL cell lines and a patient sample were more
resistant to apoptosis induced by serum deprivation (viability on day 4, 51.3±18.4% Wnt3a treated vs 29.5±19.9% control treated, p-value <0.05). H-thymidine incorporation studies revealed 4.0±1.7 fold increase in proliferation which was associated with increased cell cycle entry as determined by Ki-67 staining. Cell cycle analysis confirmed an increase in the proportion of cells in S and G₂M phases of cell cycle. These effects were associated with increased transcriptional activity of topoisomerase II-alpha as assessed by semi-quantitative PCR and protein expression. Taken together our results indicate that Wnt-dependent beta-catenin signalling pathway is active in pre-B ALL cells and promotes their growth and survival in vitro.

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Assembling the Players for Evaluation of Anti-leukaemic CTL Activity in NOD-SCID Mice

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Acute leukemia patients who relapse after haematopoietic stem cell transplantation have a poor prognosis with few therapeutic options. An immunotherapeutic approach that enhances the graft versus leukemia effect may improve the survival of relapsed patients. We postulate that dendritic cells (DC) derived from cord blood CD34⁺ stem cells will be ideal and propose using RNA as a source of antigen to induce T cell responses to poorly immunogenic tumours. We have shown that CD34⁺ cells can expand and differentiate into DC and function as professional antigen presenting cells. In addition, we regularly achieve >90% transfection efficiency with >90% cell viability when CD34⁺-DC are electroporated with eGFP mRNA. Electroporation of CD34⁺-DC with flu mRNA results in processing, translation and presentation of epitopes by the DC, which are recognised by specific flu Cytotoxic T Lymphocytes (CTL). We have induced specific CTL using leukaemic total RNA from the HLA-A2⁺ Nalm-6 cell line. We have established a NOD-SCID mouse model of primary human ALL to test the efficacy of the anti-leukaemic CTL in vivo. Mice engraft within 4-6 weeks of transplantation. Mice transplanted with ALL3 had 93.9±0.8% (BM) and 84.3±4.7% (spleen) CD45⁺ cells at sacrifice. Mice transplanted with ALL7 had 79.1±5.5% and 89.9±1.2% CD45⁺ cells in the BM and spleen respectively. Cells from the BM and spleen were cryopreserved and total RNA extracted for later CTL generation. New experiments aimed at replicating the minimal leukaemia burden post transplant, show that the kinetics of appearance of human cells in the blood and the time of palpable splenic engraftment relates to the cell dose. This suggests that while the leukaemic burden is low, the kinetics of growth are identical and thus, by varying the inocula we model emerging relapse. We thus have an effective model to test the efficacy of leukaemic CTL to control in vivo leukaemic growth.

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CXCR4 Antagonists Mobilise Acute Lymphoblastic Leukemia Cells into the Peripheral Blood and Inhibit Engraftment in Mice

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The role of CXCL12 in the growth of B cell progenitor acute lymphoblastic leukaemia (ALL) and the homing of these cells to the bone marrow has been well established. However the effect of modulating CXCL12/CXCR4 interactions on the growth of ALL cells in vivo has not been examined. In this study we used specific peptide and small molecule antagonists of CXCR4 to examine the importance of CXCL12/CXCR4 interactions in the development of leukaemia in an in-vivo murine model of ALL. CXCR4 antagonists induced mobilization of human and murine B cell progenitor ALL cells into the peripheral blood, with a 3.8±1.9 and 6.5±3.3 fold increase in leukaemic cells/ml one hour after administration of the antagonist respectively, similar to that observed for normal progenitors. Daily administration of AMD3100 commencing the day following the injection of cells and continuing for 21 days resulted in a mean reduction in peripheral blood white cell count of 50±12% and the leukaemic cell count of 63±4%. There was also a significant reduction in both the total cells in the spleen of 58±11% and the leukaemic cell number in this organ of 75±11%. A significant reduction in leukaemic cell numbers in the bone marrow was observed in one (44% reduction) case. There was reduced infiltration of other organs including kidney, liver and skeletal muscle. This study demonstrates that disrupting the CXCL12/CXCR4 axis in B cell progenitor ALL reduces the tumour burden. Whether this is due to direct inhibitory effects on proliferation and survival, or results from disruption of the leukaemic cell interactions within the bone marrow remains to be determined.

HSANZ Orals ASM 2005
Haematopoietic Stem Cells

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Haematopoietic stem cells reside in the bone marrow in a quiescent state, then become rapidly activated given appropriate signals to regenerate the haematopoietic system. Little is known about how decisions between quiescence, self-renewal, and differentiation are regulated in haematopoietic stem cells (HSC). To begin to study this, we have profiled gene expression changes that occur in adult murine HSC during activation, self-renewal, and return to quiescence, after perturbing the haematopoietic system with 5-fluorouracil. These data were compared to differences between naturally proliferating foetal HSC and their quiescent adult counterparts. Bioinformatics strategies enabled the identification of signatures of quiescent and dividing stem cells, and gene ontologies were used to characterise the broad classes of genes involved and revealed a model of the HSC activation cycle. Initially, quiescent HSC evince a state of readiness. The proliferative signal induces a preparative state, which is followed by early, then late, proliferation phases. Re-induction of quiescence involves changes in adhesion molecule expression, prior to reestablishment of homeostasis. These data will be of use in attempts to recapitulate the HSC self-renewal process for therapeutic expansion of stem cells, and our model may correlate with acquisition of self-renewal characteristics by cancer stem cells.

Prospective Isolation of Mesenchymal Stem Cells in Man and Mouse

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Mesenchymal stem cells (MSC) represent a second population of bone marrow (BM)-resident stem cells in adult mammalian bone marrow. There is considerable interest in the use of MSC as a cellular therapy for the treatment of not only of musculoskeletal defects and diseases but also in a range of other clinical applications such as cardiovascular repair. In contrast to the well-defined cell and molecular features of hematopoietic stem cells (HSC), the corresponding properties of MSC are much less well understood. Much of our current knowledge of MSC has been gained through in vitro assays and culture manipulations. In defining MSC by their in vitro properties much remains unknown about the cellular identity, ontogeny and anatomical location of MSC in the marrow in vivo. In addition, a physiological role for MSC has not been established. Seeking to address these issues we have sought to develop methodologies to prospectively isolate MSC in highly enriched form from primary hematopoietic tissues in order to explore the biological properties of these cells in an unmanipulated state, unaltered by culture epiphenomena. For the isolation of MSC from human BM antibody STRO-1 has proved to be a key reagent in purifying these rare cells. In addition, culture conditions have been developed to allow the ex vivo expansion of MSC under cGMP compliant conditions. Based in part on experience with human MSC, recent studies have culminated in the first successful prospective isolation of MSC from murine bone marrow. Notably, these studies indicate that bone tissue is the major reservoir of MSC in the mouse and that these precursors are associated with the vasculature. In addition, we have performed the first transcriptional profiling of prospectively isolated MSC from any mammalian species and are thus well positioned to explore the genetic program of these poorly characterized cells.

G-CSF and an RARα Specific Agonist, VTP195183, Synergise to Enhance the Mobilisation of Haematopoietic Stem and Progenitor Cells

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Failure to mobilise adequate numbers of haematopoietic stem cells (HSC) is an important clinical problem that needs to be resolved. We have previously shown that bone marrow (BM) neutrophils have a central role in HSC mobilisation. We therefore hypothesised that retinoic acid receptor alpha (RARα) agonists, which are known to enhance granulopoiesis, would synergise with G-CSF to enhance HSC mobilisation via increasing the pool of BM granulocytes. We tested the potential of the pan-RAR agonist, all-trans retinoic acid (ATRA) or an RARα specific agonist, VTP195183 to enhance G-CSF-mediated mobilisation of haematopoietic progenitor cells (CFU-GEMM and CFU-GM) and stem cells (HSCs) in two inbred mouse strains representing “poor” and “good” mobilisers (C57Bl/6J and BALB/c respectively). The combination of retinoid and G-CSF enhanced PB leukocyte counts, CFU-GEMM and CFU-GM compared to G-CSF alone. Unlike ATRA, VTP195183 also synergised with G-CSF to enhance the mobilisation of early engrafting HSCs, as shown in both non-competitive and competitive repopulating transplant assays. Pretreatment with VTP195183 was shown to be crucial to its synergistic effects with G-CSF, and this pretreatment increased the numbers of granulocyte/macrophage progenitors in the BM. Continued treatment with VTP195183 and G-CSF was then accompanied by enhanced levels of active neutrophil proteases in the BM extracellular fluid compared to G-CSF treatment alone. Hence, we have demonstrated that mobilisation of HSCs can be enhanced by increasing the pool of BM immature granulocytes prior to treatment with agents such as G-CSF.

Tissue factor (TF), the essential cofactor for FVIIa, is required for activation of FX and FIX to generate thrombin. Transmembrane TF resides in a cryptic configuration on the cell surface with low procoagulant activity, however TF can be rapidly switched to an active configuration on exposure to certain stimuli. The nature of this switch is unknown. The extracellular part of TF consists of 2 fibronectin type III domains. The disulphide-bond in the membrane proximal domain (Cys186-Cys209) is atypical for fibronectin domains in that it links adjacent strands in the same β-sheet, a cross-strand bond. The Cys186-Cys209 TF bond has the same unusual configuration as the disulphide-bond in the second domain of CD4, which controls CD4 function by switching between oxidized (disulphide) and reduced (dithiol) states (Matthias et al. Nature Immunol. 3, 727, 2002). Ablation of the cross-strand bond severely impairs procoagulant activity (Rehemtulla et al. J. Biol. Chem. 266, 10294, 1991). Labeling with a biotinylated maleimide, we demonstrate that cryptic tissue factor is reduced at the domain 2 disulfide and oxidised on activation. In HL60 cells, membrane based tissue factor procoagulant activity is blocked by the mono-thiol alkylator N-ethylmaleimide but increased by formation of the disulfide via the thiol oxidiser, HgCl₂ or thiol cross-linkers, bismaleimidohexane and bismaleimidoethane. Using the VIC7 anti-TF antibody which recognises an epitope between aa181-214 in TF (Magdolen et al Biol Chem 1998) we demonstrate that activation of cryptic TF on HL60 cells correlates with a change in the conformation of the TF region that is constrained by the cys186-cys209 disulfide. These results indicate that the activation of TF involves a change of conformation of the domain 2 of extracellular TF caused by formation of the cross strand cys186-cys209 disulfide bond. This is likely to be the physiological change that facilitates productive binding of FIX and FX in coagulation.

GrANuleocyte-Colonv stimulating factor (G-CSF) is widely used to obtain progenitor/stem cells for clinical transplantation in the treatment of haematological malignancies. Recently, G-CSF has been trialed in the treatment of ischemic heart disease (IHD). However, its safety and efficacy in such patients remains unclear.
Aim: To assess the safety and efficacy (in causing neovascularisation and hence reducing ischaemia) of G-CSF in patients with chronic IHD.

Methods: After baseline cardiac assessment (CA) [Seattle Angina Questionnaire (SAQ), Exercise stress test (EST), persantin-Seastamibi and dobutamine-echocardiographic imaging], stable ‘no-option’ chronic IHD patients received open-label G-CSF 10µg/kg for 5 days, with an EST (to facilitate myocardial cytokine generation and stem cell trafficking) on the 4th and 6th days. After 3 months, CA and the same regimen of G-CSF and ESTs was repeated, but in addition, leucopheresis and a randomised double-blinded intracoronary-infusion of either CD133+ or unselected cells was performed (randomised data remains blinded). Final CA was 3 months thereafter. Haematologic parameters were monitored daily during G-CSF administration.

Results: Thirteen patients (12 male, 1 female, mean age 62) received 21 cycles of G-CSF. There were no deaths, Q-wave myocardial infarctions or any complications with long-term sequelae. Mean CD133+ cell count rose from 1.28 to 56.12 x1012/L (p=0.001). There was no trend towards lower numbers of G-CSF mobilised CD34+ cells in the IHD patients, as compared to younger (<60y.o.) normal (control) male donors (n=66). Overall, SAQ angina frequency score improved 46 points (p=0.003). This was reflected by reduced anginal frequency and nitrate use (both p<0.005). SAQ physical limitation score improved 26 points (p=0.0003), and EST time improved 97 seconds (p=0.005).

Conclusions: G-CSF and intracoronary cell infusion is safe in chronic ‘no-option’ IHD patients. In this phase-I study G-CSF improved anginal frequency, nitrate use and EST performance. A placebo controlled phase-II trial investigating G-CSF in IHD patients is warranted.

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A Common Ancestral Glycoprotein (GP) IX Gene Mutation (Asn45Ser) Causes Bernard-Soulier Syndrome (BSS) in European Families from Northern Europe and Australia

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Bernard-Soulier syndrome (BSS) is an extremely rare hereditary bleeding disorder, caused by mutations occurring in the Glycoprotein (GP) Ibα, GPIbβ and GP9 genes that encode for the corresponding subunits of platelet GPIb-V-IX adhesion receptor complex. BSS has been reported in many populations, mostly behaving in an autosomal-recessive manner. While the great majority of BSS mutations are unique to a single individual or family, the GP9 1828A>G Asn45Ser single nucleotide substitution, which we have identified in an undocumented Australian Caucasian, has already been reported in multiple unrelated Caucasian families from various Northern and Central European countries. Consequently, we performed a haplotype study to determine whether the GP9 1828A>G Asn45Ser mutation is an ancient ‘mutation’ in the European population, or indicates a ‘hot-spot’ for mutagenesis in the GP9 gene. The haplotypes of GP9 1828A>G Asn45Ser mutation carriers and 100 Caucasian controls were established by single nucleotide polymorphism screening using allele-specific PCR and DHPLC techniques. Haplotype analysis of 19 BSS patients from 15 unrelated Northern European families (including 2 compound heterozygote siblings from a British family previously published, and 17 Asn45Ser homozygotes), showed that 14 of these BSS patients from 11 of the Asn45Ser homozygote families share a common haplotype at the chromosomal region 3’ to the GP9 gene. Hence, the results suggest that the GP9 1828A>G Asn45Ser mutation in these families is ancient, and its frequent emergence in the European population is the result of a founder effect rather than recurrent mutational events. Association of the GP9 1828A>G Asn45Ser mutation with variant haplotypes in 4 other Northern European BSS families raised the possibility of a second founder event, or rare recombinations in these families. Additional members from these ‘atypical’ lineages would be needed for haplotype screening to resolve this question.

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A Global Role for EKLF in Definitive and Primitive Erythropoiesis

HSANZ & ASTH Presidential Symposium

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A Global Role for EKLF in Definitive and Primitive Erythropoiesis

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Aim: Erythroid Kruppel-like factor (EKLF or Klf1) is an erythroid specific C2H2 zinc-finger transcription factor which is essential for definitive erythropoiesis and β-globin gene expression. The absence of EKLF results in fatal anaemia but correction of globin chain imbalance does result in rescue, suggesting the existence of additional EKLF target genes. The aim of this study was to search for such genes by expression profiling.

Method: We performed profiling on fetal livers from wild-type versus EKLF null litter mates, and also EKLF null erythroid cell lines containing an inducible EKLF-ER fusion construct. Hybridisations were performed on microarray slides printed with a 23K oligo library from Compugen. Target gene validation was performed by real-time RT-PCR, chromatin immuno-precipitation (ChIP) and promoter-reporter assays.

Results: A large number of genes were down regulated in the absence of EKLF but few were up regulated, suggesting EKLF acts primarily as a transcriptional activator in vivo. One hundred genes were EKLF dependent in both systems. These include heme synthesis enzymes, red cell surface proteins including Rh and the transferrin receptor, and erythroid transcription factors. Two interesting highly EKLF-dependent genes are α-haemoglobin stabilising protein (AHSP), a key chaperone for free -globin chains, and dematin (band 4.9) which links the cytoskeleton to the red cell membrane. A search for EKLF binding sites within the dematin and AHSP genes demonstrated a number of phylogenetically conserved sites, and ChIP demonstrated in vivo EKLF occupancy at some but not all of these. Promoter-reporter assays showed EKLF directly activates dematin gene transcription through two promoters containing these sites. Lastly, investigation of EKLF target genes in the yolk sac lead to the discovery of unexpected defects in the embryonic red cell membrane and cytoskeleton.

Conclusion: EKLF regulates global erythroid gene expression which is critical for development of primitive as well as definitive red cells.
The Addition of Celecoxib to Thalidomide Improves Outcome in Patients with Relapsed and Refractory Multiple Myeloma

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Background: Pre-clinical data indicates that cyclo-oxygenase (COX)-2 inhibition impairs plasma cell growth and potentially synergises with thalidomide.

Methods: We performed a trial in previously treated patients with myeloma using thalidomide up to a maximum dose of 800 mg/day with celecoxib (400mg bid). Outcomes were compared to a prior trial of thalidomide.

Results: 66 patients, with median age 67 (range 43-85) received a median dose of thalidomide and celecoxib of 400mg/day and 800mg/day, respectively with median duration s of treatment of 27 weeks and 13 weeks, respectively. The most common toxicities associated with premature discontinuation of celecoxib (n = 30/53, 57%) were fluid retention and deterioration of renal function. Overall RR was 42% and with 20 months median follow-up, the actuarial median PFS and OS were 6.8 and 21.4 months, respectively. Unlike our prior study, age > 65 years was not predictive of inferior RR, due to improvement in RR in older patients with the combination (37% v 15%, P = 0.08). The RR was superior in patients who received a total dose of celecoxib exceeding 40g in the first eight weeks of therapy (62% vs 30%, P = 0.021). PFS and OS was also significantly improved. Other predictors for inferior PFS were age >65 years (P = 0.016) and elevated beta2microglobulin (P = 0.017).

Conclusion: This study provides evidence that the addition of high-dose celecoxib adds to the anti-myeloma activity of thalidomide but this comes with unacceptable toxicity. Future studies should utilize newer COX-2 inhibitors with thalidomide, or their respective derivatives.

Increased Marrow CD57+ Cytotoxic T Cells is a Powerful Prognostic Marker for Survival in Patients with Relapsed Multiple Myeloma (MM) Receiving Thalidomide

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Background: Thalidomide (T) alters the marrow (BM) microenvironment and also has an immunoregulatory role. We assessed various laboratory and clinical parameters to examine potential prognostic markers and to assess for changes during T therapy.
Methods: Specimens from 75 patients with relapsed MM receiving T on trial were examined (Blood 2003;102:69-77). Platelet-poor plasma (PPP) and BM were obtained pre-T and 3 monthly. Immunohistochemistry for CD34, VWF, mast cell tryptase and CD57 was performed on BM biopsies. Flow cytometry on BM aspirates was used to define the CD57+ population (T cell subsets, NK, NKT markers). Vascular endothelial growth factor (VEGF), basic-Fibroblast growth factor (bFGF), interleukin-6 (IL-6) and Hepatocyte growth factor (HGF) were measured in PPP. Objectives were to examine for effect on response rate (RR), progression-free (PFS) and overall survival (OS).

Results: Overall RR was 28% with 55% stable disease. Only VEGF predicted RR-no responses seen with a level of 0, cf 34% RR in those with VEGF > 0 (p=0.015). Microvessel density (MVD) did not predict for RR, PFS or OS. CD57+ cells did not predict for RR, however on univariate analysis elevated numbers were the major predictor of better OS (p=0.003). Flow cytometry confirmed that CD57+ cells were predominantly cytotoxic T cells. Predictors for inferior OS were raised baseline levels of IL-6 (p=0.014) and HGF (p=0.016). Multivariate analysis for OS, which incorporated clinical variables, demonstrated that age >65 (p=0.009), raised LDH (p=0.008) and zero baseline CD57+ cells (p=0.011) predicted inferior OS. MVD and VEGF fell significantly in responding patients although CD57+ cells remained unchanged.

Conclusion: VEGF and MVD decline in T responders. However, high baseline angiogenic activity was not necessary to obtain a response. Increased age and elevated LDH are important predictors of poorer OS, with elevated baseline levels of CD57+ cells being an independent predictor of superior outcome.

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In Vitro Anti-Myelomatous Activity of the Proteosome Inhibitor Bortezomib is Highly Schedule Dependent

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Aim: Multiple myeloma (MM) cells are malignant plasma cells that accumulate in the bone marrow (BM), exhibit impaired apoptosis and invariably develop pronounced resistance to standard therapeutic approaches. Bortezomib (B) is a first-in class proteosome inhibitor that displays significant single-agent activity against MM but presents significant challenges in terms of both drug scheduling and toxicity. We have evaluated the impact of drug combinations and scheduling on the efficacy of Bortezomib to kill the human myeloma cell line (HMCL) NCI-H929 and primary myeloma tumours (PMT).

Method: The dose response of NCI-H929 to Bortezomib (B) and a variety of partner drugs (Adriamycin, Dexamethasone, Etoposide and Flavopiridol) was determined by MTS assay. B-drug combinations were sequenced as either B-drug or drug-B with a 24 hour interval. Cell viability was then determined a further 24 hours later by MTS assay. PMT were collected from patients following informed consent. 20,000 BM mononuclear cells were plated per well in 96 well plates and B-drug/drug-B combinations were added and evaluated as described above.

Results: With dose-escalation all combinations were effective against both NCI-H929 and PMT. Sub-optimal doses of all drugs and B were identified (50ng/ml Adriamycin, 12.5µM Dexamethasone or 5nM Bortezomib - 100% cell viability; 5µM Etoposide and 50nM Flavopiridol - 47% and 86% cell viability, respectively). Treatment with 5nM Bortezomid followed by drug (B-drug) demonstrated little evidence of relevant synergy with cell viabilities of 84% B-Adriamycin, 90% B-Dexamethasone, 41% B-Etoposide and 60% B-Flavopiridol. In contrast, reverse scheduling (drug-B) demonstrated enhanced cell killing with viabilities 55% Adriamycin-B, 61% Dexamethasone-B, 6% Etoposide-B and 35% Flavopiridol-B. A similar pattern of enhanced schedule-dependent anti-MM activity was seen when tested against PMT.

Conclusion: Scheduling of Bortezomib subsequent to other anti-MM agents improves cell killing even with demonstrably sub-optimal (single-agent) dosing. These data provide a rational basis for the clinical use of Bortezomib in schedule-dependent drug combinations at lower, potentially less toxic doses.

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Efficacy of Single-Agent Bortezomib versus Thalidomide in Patients with Relapsed or Refractory Multiple Myeloma: a Systematic Review

Miles Prince1, Michael Adena2, Dell Kingsford Smith2, Judy Hertel3
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2. Covance Pty Ltd, North Ryde, NSW, Australia
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In Vitro Anti-Myelomatous Activity of the Proteosome Inhibitor Bortezomib is Highly Schedule Dependent

Tiffany Khong and Andrew Spencer
Myeloma Research Group, Alfred Hospital, Melbourne, Victoria, Australia

Aim: Multiple myeloma (MM) cells are malignant plasma cells that accumulate in the bone marrow (BM), exhibit impaired apoptosis and invariably develop pronounced resistance to standard therapeutic approaches. Bortezomib (B) is a first-in class proteosome inhibitor that displays significant single-agent activity against MM but presents significant challenges in terms of both drug scheduling and toxicity. We have evaluated the impact of drug combinations and scheduling on the efficacy of Bortezomib to kill the human myeloma cell line (HMCL) NCI-H929 and primary myeloma tumours (PMT).

Method: The dose response of NCI-H929 to Bortezomib (B) and a variety of partner drugs (Adriamycin, Dexamethasone, Etoposide and Flavopiridol) was determined by MTS assay. B-drug combinations were sequenced as either B-drug or drug-B with a 24 hour interval. Cell viability was then determined a further 24 hours later by MTS assay. PMT were collected from patients following informed consent. 20,000 BM mononuclear cells were plated per well in 96 well plates and B-drug/drug-B combinations were added and evaluated as described above.

Results: With dose-escalation all combinations were effective against both NCI-H929 and PMT. Sub-optimal doses of all drugs and B were identified (50ng/ml Adriamycin, 12.5µM Dexamethasone or 5nM Bortezomib - 100% cell viability; 5µM Etoposide and 50nM Flavopiridol - 47% and 86% cell viability, respectively). Treatment with 5nM Bortezomid followed by drug (B-drug) demonstrated little evidence of relevant synergy with cell viabilities of 84% B-Adriamycin, 90% B-Dexamethasone, 41% B-Etoposide and 60% B-Flavopiridol. In contrast, reverse scheduling (drug-B) demonstrated enhanced cell killing with viabilities 55% Adriamycin-B, 61% Dexamethasone-B, 6% Etoposide-B and 35% Flavopiridol-B. A similar pattern of enhanced schedule-dependent anti-MM activity was seen when tested against PMT.

Conclusion: Scheduling of Bortezomib subsequent to other anti-MM agents improves cell killing even with demonstrably sub-optimal (single-agent) dosing. These data provide a rational basis for the clinical use of Bortezomib in schedule-dependent drug combinations at lower, potentially less toxic doses.

HSANZ Free Communications 5: Myeloma Therapy

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Efficacy of Single-Agent Bortezomib versus Thalidomide in Patients with Relapsed or Refractory Multiple Myeloma: a Systematic Review

Miles Prince1, Michael Adena2, Dell Kingsford Smith2, Judy Hertel3
1. Department of Haematology and Medical Oncology, Peter MacCallum Cancer Centre, VIC, Australia.
2. Covance Pty Ltd, North Ryde, NSW, Australia
3. Janssen-Cilag Pty Ltd, North Ryde, NSW, Australia
Aim: To systematically review the efficacy of monotherapy with bortezomib versus thalidomide in patients with relapsed or refractory multiple myeloma.

Methods: Published English literature from 1966 to June 2005 (MEDLINE, EMBASE, Cochrane library), publication reference lists, Janssen-Cilag Pty Ltd data-on-file, and recent abstracts from multiple myeloma conferences were reviewed. Prospective studies containing at least a single arm of any treatment group with n \( \geq 30 \) and using continuing or variable thalidomide dosing were included. Studies adding dexamethasone for non-responders were excluded. Outcomes were analysed on an intent-to-treat basis rather than a per-protocol basis. Statistical pooling was performed where possible for the following outcome measures: primary outcome of response rate, defined by a serum M-protein reduction \( \geq 50\% \) (A) and strict (e.g. EBMT) criteria (B), and for the secondary outcomes of overall survival and progression-free survival.

Results: One bortezomib (n=333, APEX, NEJM 2005, 352; 2487-98) and 15 thalidomide (n=1007) studies were included. Patient baseline characteristics including age, gender, IgG:IgA, disease duration and \( \beta_2M \), were well matched except that 48% of bortezomib patients had received prior thalidomide. Reanalysed on an intent-to-treat basis, the overall estimate for response rate (A) was 53% for patients receiving bortezomib versus 32% for thalidomide (p<0.001, n=10 studies). For response rate (B) the estimate was 36% for patients receiving bortezomib versus 22% for thalidomide (p<0.001, n=4 studies). One-year survival was 81% for patients receiving bortezomib versus 67% for thalidomide (p<0.001, n=6 studies). Due to differences in disease monitoring and definitions of progression, it was not possible to compare results for progression-free survival.

Conclusion: In patients with relapsed or refractory multiple myeloma, bortezomib achieved significantly higher response rates and one-year survival than thalidomide, despite 48% of bortezomib treated patients having received prior thalidomide.

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Health-Related Quality of Life (HRQL) Associated with Bortezomib Compared with High-Dose Dexamethasone in Relapsed Multiple Myeloma (MM): Results from APEX Study

Stephanie J Lee,1 Paul G Richardson,1 Pieter Sonneveld,2 Michael Schuster,3 David Irwin,4 Joe Massaro,5 Bruce Crawford,6 Ravinder Dhawan,7 Sanjay Gupta,6 Kenneth C Anderson1

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Aim: In a large, randomized phase 3 study, bortezomib showed significant clinical benefits in terms of time to progression, response, and survival, compared with high-dose dexamethasone in relapsed multiple myeloma (MM) (APEX; Blood 2004;104: Abstract 1479). The present study compared health-related quality of life (HRQL) in these patients.

Methods: Two HRQL instruments (EORTC QLQ-C30 and FACT/GOG-Ntx) were administered at baseline, weeks 6, 12, 18, 24, 30, 36, and 42, or until discontinuation of protocol treatment. Data were analysed based on modified intention-to-treat analysis; 44 of 642 patients were excluded due to no baseline or no follow-up HRQL data. At baseline, the two treatment groups were comparable on demographic and clinical characteristics, FACT/GOG-Ntx score, and on most EORTC QLQ-C30 scores. There were substantial missing HRQL data (from 12.5% at week 6 to 75.6% at week 42), primarily due to premature closure of the study and disease progression. Extensive statistical analyses were undertaken to correct for potential bias related to the missing data. The a priori primary endpoint for the HRQL analyses was the Global Health Status score of the EORTC QLQ-C30. All other scales and symptom scores were considered secondary endpoints.

Results: Patients on bortezomib had better Global Health Status (P = 0.0005), Physical, Role, Cognitive, and Emotional Functioning (adjusted P values < 0.05), and better symptom scale scores of Nausea, Dyspnea, Sleep, Diarrhoea and Financial Impact (adjusted P values < 0.05) compared with patients on dexamethasone over 42 weeks, assessed using generalized estimating equations of multiply imputed datasets. There was no HRQL domain for which high-dose dexamethasone was superior over time. Using alternative methods to adjust for potential informative censoring, both a Sun and Song analysis and a Pattern-Mixture model analysis supported the generalized estimating equations conclusions.

Conclusions: Bortezomib is associated with better HRQL than high-dose dexamethasone in relapsed MM.
Lenalidomide (Revlimid) Achieves a High Response Rate in Patients with Relapsed or Refractory Multiple Myeloma: Local Analysis of the International MM010 & MM012 Studies

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²Alfred Hospital, Melbourne, Australia

Background: Lenalidomide (L; Revlimid, Celgene NJ), an analogue of thalidomide, has in Phase I and II trials demonstrated substantial activity in myeloma, with greater tolerability. Here we describe our combined local experience with L in 25 myeloma patients as part of a recent international double-blind placebo randomised clinical trial (RCT) (MM010).

Methods: Patients were randomised on MM010 to either L oral capsules (25mg/day for 21/28day cycle) plus dexamethasone (dxm; 40mg d1-d4, 9-12, 17-20 q28d) or pulse dxm alone. Patients who progressed were subsequently unblinded and those receiving dxm were offered L as part of a ‘follow-on’ study (MM012). We report a retrospective analysis of patients who were known to have actually received L (n=25).

Results: Median age was 62yrs (range: 43-83). M:F = 19:6. Median number of prior therapies was 2.5 (range: 1-5); 14/25 had previous high dose therapy and 68% thalidomide. Median B2M was 4.6; 54% had albumin <35g/L. The response rate was 64% with an additional 28% stable disease (SD). Median duration of treatment was 4 mo (range: 0.5-15+) with median PFS of 8 mo. 8 remain on treatment free of progression with patients discontinuing L due to disease progression (12), change in treatment with SD (3), G4 desquamating rash (1) or G3 pancytopenia (1). Overall L was well tolerated with cytopenias being the primary reason for dose reduction, transfusion or G-CSF (11). Other SEs included pruritis/skin rash (9), cramps (5), neuropathy (2) and hypothyroidism (1). Two patients had recurrence of DVT.

Conclusion: L demonstrates a RR of 64% with a TTP of 8 months, consistent with the preliminary results of the RCT. L has a toxicity profile quite different to thalidomide, with cytopenias the most common treatment limiting toxicity, although the thrombosis rate of 8% is notable.

Isolation of Common Lymphoid Progenitors from Adult Bone Marrow/Peripheral Blood CD34+ Cells with In Vitro T Lineage Differentiation Capacity

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A better understanding of the characteristics of the Common Lymphoid Progenitor (CLP), the bone-marrow stem cell-derived cell which seeds the thymus, would be a foundation for safe and effective stem cell transplants.

Aim: 1. To isolate CLPs from the adult bone marrow and mobilized peripheral blood CD34+ compartment. 2. To examine their growth potential in a thymic culture system.

Method: We analysed cell surface markers on HSCs from bone marrow and mobilized peripheral blood to determine the phenotype of cells likely to enter the thymus, using a standard primary gating strategy of CD34+, CD45mid, Lineage-. Gated cells were analysed for a panel of secondary cell surface markers (e.g. CD7, CD127, Notch). We tested FACS-sorted cells for growth potential in a model thymic culture system which uses OP9 cells expressing Delta-like1 as support T cell development (Schmitt & Zuniga-Pflucker (2002) Immunity 17:749).

Result: A population of cells which were CD38- Kit+ decreased with age as a proportion of primary gated (CD34+ etc) cells. The correlation was significant (n=21 patients, age range 22-71, p<0.05). CD132 (c, intracellular) increased significantly with age (n=16 p<0.05). Based on these results we selected a gating strategy for isolation by FACS cell sorting of pre-thymic precursors from mobilized peripheral blood. Sorted CLPs were CD45mid, CD34+, Lineage-, and either CD38+ or CD38-. Mobilized stem cells from adults (n=4) differentiated to CD4+ CD8+ double positive cells, as well as CD4+ and CD8+ SP cells. Both CD38+ and CD38-selected cells developed to the CD4+ phenotype via a CD38- stage, but CD38+ were slower to mature.

Conclusion: The OP9 coculture assay is a useful model to analyse development potential of adult HSCs in vitro and is likely to help determine the characteristics of the CLP.
Populations of B Cells Lacking Surface Immunoglobulin L Chain Expression are Highly Associated with B-Lineage Lymphomas

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Aim: We investigated the incidence and significance of subpopulations of B cells with absent surface immunoglobulin (sIg) light chain expression in lymph node biopsies.

Method: We undertook a retrospective review of the immunophenotyping and histology of consecutive lymph node biopsies submitted for diagnosis over the period from January 2003 to June 2005.

Results: Both histology and immunophenotyping results were available for a total of 229 lymph nodes. Of 89 nodes which were reported reactive on histology the proportion of light chain negative B cells ranged from 0 – 15%, and the kappa:lambda ratio varied between 0.75 – 2.3. Only one case showed a population of light chain negative cells (29%) on a secondary analysis of a sub-population of large cells as defined by the forward scatter pattern. For further analysis, cases in which the number of B cells was >20% and the proportion of sIg negative B cells was >20% were selected. 17 cases fulfilled these criteria. The histologic diagnoses were DLBL (n=6), Follicular lymphoma (n=6), SLL (n=3), Post-transplant Lymphoproliferative disorder (n=1), Nodular Sclerosing Hodgkins Lymphoma (n=1). The proportion of light chain negative cells varied from 21% - 99% with 10 cases having >40% sIg light chain negative B cells. In 8 cases the kappa:lambda ratio was in the normal range. In 5 cases, PCR was performed for IgH chain rearrangements and in 4 clonality was confirmed.

Conclusion: The presence of populations of B cells in which sIg light chain expression is absent is uncommon, accounting for 7.5% of lymph nodes studied. However, when this phenomenon is present it is highly associated with B-lineage lymphomas even when the kappa:lambda ratio is within normal limits. This is relevant in the interpretation of FNA specimens submitted for immunophenotyping, and the finding of L chain negative populations should prompt further investigation to confirm a malignancy.

Quality Cancer Treatments – Web-Based Protocols

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Purpose: This presentation demonstrates the Quality Cancer Treatments web-based application, and describes the governance and review processes employed to offer best-practice treatment including drug calculation to oncology/Haematology nurses and doctors across NSW.

Abstract: The process of reviewing and updating standard Haematology treatment protocols requires expert and detailed attention drawing considerable resources away from administering treatment and providing patient care. This process is currently replicated across each Haematology treatment unit, although some hospitals lack the specialist expertise and resources to maintain such protocols to internationally acceptable standards.

The Cancer Institute (NSW) disseminates evidence-based protocols for cancer specialists, nursing staff, allied health, and general practitioners, via a secure web-based application. The application was developed at St Vincent’s Hospital, and includes:

- An overview of each treatment protocol
- A summary of evidence including references, survival graphs and cost for each protocol
- An episode calculator and print out generated from each protocol and patient parameters
- A “patient view” of protocol information including plain language description of treatment and side effects.

Expert Reference Groups representing, Haematologists, Haematology nurses nurses, pharmacists and consumers review and approve the protocols. This means

- Information on standard, evidence-based treatment protocols are available via the internet to specialist clinicians and GPs across NSW.
- Background information about cancer treatments and their side effects is provided for patients via the internet in plain English.

HSANZ Free Communications 6: Lymphoma / Lymphopoiesis

HSANZ Free Communications 6: Lymphoma / Lymphopoiesis

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The program is not intended to replicate or replace the knowledge, skills and experience of trained Haematology health professionals nor is it a substitute for clinical judgement and advice, nor is it designed to replace the relationship that exists between a patient and their physician or other qualified health professional.

**Note:** The presentation comprises a live demonstration of the application

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**The Clinical Utility of a Lymphoma Outcomes Registry in the Management of Aggressive Lymphoma**

**David Joske, Helen Lund, Bradley Augustson, Gavin Cull**

**Sir Charles Gairdner Hospital, Perth, Western Australia**

**Aim:** Since 1996, details of successive cases of lymphoma referred to and managed at Sir Charles Gairdner Hospital have been entered onto a Hospital-Based Cancer registry. We recently completed a look-back exercise to analyse our outcomes.

**Methods:** The Registry software, developed in-house, is a Microsoft Access–based program that includes demographic, clinical, staging, IPI, and treatment information fields exceeding the recommended national minimum data set. Patient information has been collected on a variety of cancers, including breast, colo-rectal, lung carcinomas and lymphomas since 1996. Over this time the Haematology Department has pursued an evolving strategy to manage diffuse large cell lymphomas (DLCL): CHOP 21/physician preference (until 2001), CHOP-14 (2001-2004) and most recently, R-CHOP-14. Follow-up data on patient outcomes (remission, relapse, or death) have been collected, by scrutiny of medical records.

**Results:** Between 1996 and 2003, cases of aggressive lymphomas managed in the Haematology department (n=181) have included anaplastic large cell NHL (ALCL, n=8), DLCL (121), Burkitt’s (10), peripheral T-cell (12), and mantle cell NHL (17). Median survival for ALCL was 57.3 months; DLCL 40.9 months; peripheral T-NHL 14.8 months; for mantle cell NHL 42.5 months; and for Burkitt’s not reached with approx. 50% long term survival. Outcomes for DLCL when assessed by IPI at presentation were significantly different (p<.0001 on log-rank test), and essentially mirrored published survival curves. For DLCL patients treated with a regimen other than CHOP (e.g. elderly; use of MACOP-B for primary mediastinal NHL, etc) the outcome was worse with a median survival of 24 months. Early analysis shows super-imposable survival curves for CHOP-21 and CHOP-14.

**Conclusions:** (1) The poor outcome of T-NHL suggested in the literature is confirmed. (2) Our outcomes with CHOP chemotherapy are in accord with published results from multi-centre trials. (3) At our institution, patients with DLCL who were treated with an alternative regimen did worse. This observation led to a change in policy. (4) Our outcomes with CHOP-21 will serve as historical controls for subsequent cohorts treated with CHOP-14 and R-CHOP-14 and this data should prove extremely valuable.

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**HSANZ Free Communications 6: Lymphoma / Lymphopoiesis**

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**The Emerging Role of PET-CT in Primary Staging of Lymphoma**

**Michael Fulham¹, Melinda Gibson², Judith Trotman², Ilona Cunningham², Graham Young³, Armin Mohamed¹**

**Departments of PET & Nuclear Medicine¹, Haematology at Concord² and Royal Prince Alfred³ Hospitals, Sydney South West Area Health Service, Sydney, Australia**

**Aim:** To assess the impact of FDG PET-CT on the clinical management of patients staged for previously untreated Hodgkin’s Disease (HD) and non-Hodgkin’s lymphoma (NHL).

**Method:** A retrospective analysis was done on all patients with newly diagnosed, histologically proven lymphoma (n=177; 85 women, 92 men; mean age 52 yrs) scanned between June 2003 and May 2005. All patients had an FDG PET-CT (LSO Biograph). Thirty three had HD and 144 NHL. Referring doctors provided details of the patients’ stage and management plan prior to the PET scan. Subsequently they were asked to provide details of the patients’ post PET management plan and additional clinical parameters. We compared the pre and post PET stage and management plans to measure the impact of PET-CT.

**Result:** In the study period 1150 lymphoma patients (compared to 243 in the period 1993 to June 2003) were scanned. Primary staging comprised 177 (15%) while other indications included re-staging, assessment of residual mass and suspected recurrence. Patients were referred by 52 specialists (73% were haematologists). PET-CT changed the primary staging in 46.3%: 31.6% of patients were up-staged and 14.7% were down-staged. The post PET management plan changed in 34% of cases. PET-CT tended to detect many more lesions than were suspected
pre PET. Although FDG uptake was markedly increased in the high-grade lymphomas there was also heterogeneous FDG uptake within patients and across histologies (30% in NHL, 36% in HD).

**Conclusion:** PET-CT improves primary staging of lymphoma and changes patient management. The mechanism for the variation of FDG uptake within individual patients is uncertain. Further sub-population analysis on this and the effect of PET-CT on the IPI and FLIPI is being undertaken.

**HSANZ Free Communications 6: Lymphoma / Lymphopoiesis**

**128 A Mid-Treatment FDG-Positron Emission Tomography (PET) Scan Is Highly Predictive Of Subsequent Treatment Failure In Patients With Diffuse Large B-Cell Lymphoma (DLBCL)**

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**Aims:** Computerised Tomography (CT) scanning is the primary modality for assessment of therapeutic response in DLBCL. We evaluated whether functional imaging utilising FDG-PET may provide additional prognostic information in response assessment prior to completion of chemotherapy for patients with DLBCL.

**Methods:** We performed a retrospective, single-centre study of patients with DLBCL who received anthracycline-based chemotherapy +/- radiotherapy (RT) and had PET prior to completion of therapy.

**Results:** From 1996–2004 there were 45 eligible patients. Median age was 59 years (range 26-82) with disease stage I-II (n=25) or III-IV (n=20). Median IPI was 2[IPI 0-2(n=27), IPI 3-4(n=13)] in 40 evaluable patients. Therapy included full-course CHOP/CHOP-like therapy (n=26); HyperCVAD (n=6) and limited (3–4 cycles) CHOP/CHOP-like chemotherapy with RT (n=13). Rituximab was used concurrently in 18 patients. Planned RT was administered in 23 patients.

13 (29%) patients were PET positive after a median of 3 chemotherapy cycles (range 1-5). Of these, 7 (54%) progressed a median of 7.2 months following completion of therapy. Of the 6 progression-free, 4 demonstrated residual low-grade activity at sites of prior bone involvement (median follow-up 50.8 months), while 2 patients (stage I and IV disease) had limited follow-up (<8 months).

Of 32 patients who were PET-negative, only 3 have progressed (median 7.7 months follow-up); 2 with stage IV (IPI 1 and 2 respectively) and 1 with stage I disease.

Overall the Positive predictive value (PPV) of a positive-interim PET was 58.3% [Hazard Ratio of 6.67 (95% C.I. 2.6 – 46.3)] and Negative PV was 90.9%. The median number of chemotherapy cycles after which PET was performed was equivalent according to interim PET status (3 cycles) as was median baseline IPI [IPI 2(PET-positive), IPI 1.5(PET-negative); p=0.17].

**Figure 1. Log-rank analysis**

Patients in remission

![Diagram showing patients in remission](image)

* p=0.0012

**Conclusions**

Patients with DLBCL receiving chemotherapy who have persisting PET-positivity during their therapy have a greatly increased risk of treatment failure and should be considered for treatment intensification.

Bone lymphoma may represent a distinct entity with a good prognosis despite some minor persisting PET-positivity prior to completion of therapy.

**HSANZ Free Communications 7: Transplantation - Practical Considerations**

**HSANZ Orals ASM 2005**
Establishment of a Hospital Based Information Session for Patients, Families and Friends Prior to Stem Cell Transplantation (SCT)

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Aims: When medical procedures exceed a certain level of complexity, providing comprehensible information to patients and families becomes difficult. As a result, problems arise in obtaining meaningful informed consent and discussing attitudes to adverse outcomes. To address these issues, we have established an information session prior to SCT aimed at providing information, reducing anxiety, facilitating informed consent and introducing concepts relating to withdrawal of treatment in the event of adverse clinical outcomes.

Methods: For the last two years, we have invited patients scheduled to undergo autologous or allogeneic BMT, their friends and family to a structured Information Session that we hold every two months at Westmead Hospital. The group is addressed by members of the BMT unit including the transplant coordinator, transplant and infectious disease physicians, nursing staff, clinical psychologist, dietitian, dentist and social worker. A patient who has had a bone marrow transplant relates his/her experiences to the group. Patients and families receive written material including copies of slides as well as printed information about transplantation. An evaluation is performed at the end of each session.

Results: Information Sessions are well attended and highly rated by participants for alleviation of anxiety and provision of information. Preliminary data from prospective interviews with patients and their carers suggests that these sessions also have other benefits including boosting patient morale through providing reassurance regarding the “presence” and expertise of clinical staff. The interviews also raise important questions about the limits of information-giving and suggest ways in which understanding can be evaluated.

Conclusion: A structured Information Session is a resource efficient way of providing a large amount of information simultaneously to patients, family and friends. This method may alleviate anxiety, enable setting of realistic goals by patients and their carers and help promote understanding, and where this is not possible, trust.

Correlation of Serum Concentrations of Cystatin C and Creatinine in Assessment of Glomerular Filtration Rate in Patients Undergoing Allogeneic Stem Cell Transplantation

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Division of Laboratory Medicine, Royal Perth Hospital

Aim: To determine the most useful method of assessing glomerular filtration rate(GFR) by measurement of serum cystatin C and creatinine in patients undergoing allogeneic stem cell transplantation and to evaluate the validity of cystatin C in predicting patients at risk of developing renal impairment as creatinine has been recognized as unreliable in these patients due to variables of age; weight; muscle mass; race and protein intake.

Method: Serum creatinine and cystatin C levels were measured simultaneously in 10 patients with haematological malignancies undergoing allogeneic stem cell transplants at Royal Perth Hospital from January to July 2005. 24 hour urinary creatinine clearances were measured on a weekly basis. GFRs were calculated for each patient using the Cockcroft-Gault equation. Data on patients’ other results were collected. Data analysis was undertaken with the use of SPSS software. In the evaluation of results, pearson correlation coefficients between serum creatinine, cystatin, GFR and urinary creatinine clearance were calculated and the significance of difference between correlation coefficients estimated.

Results: Cystatin C levels range from 0.62mg/L to 6.74mg/L (mean 1.60mg/L; male 1.81; female 1.24; reference range 0.53 to 0.94mg/L) and creatinine range from 38 micromol/L to 378 micromol/L (reference range 45-90 for females and 60-105 for males). Calculated GFR ranges from 19 to 155ml/min for males (mean 72.54ml/min) and for females, 25 to 196ml/min (mean 98ml/min). The high correlation (r=0.8) between cystatin C and serum creatinine supports the concept that they have similar properties as markers of GFR. Cystatin C progressively increases as GFR decreases. The reciprocal of cystatin significantly correlated with GFR (r=0.665; p<0.01) and also correlated with urinary creatinine clearance (r=0.756; p<0.01). The diagnostic sensitivity for GFR <80ml/min appears to be greater for cystatin as 99% of patients had an abnormally increased cystatin (>0.94mg/L), whereas only 36.4% of patients had increased creatinine.

Conclusion: Cystatin C appears to be an alternative and probably more accurate marker of GFR than creatinine in adults undergoing allografts. The rise of cystatin C levels precedes creatinine rise and allows earlier detection of renal impairment.
Optimum Temperature for Maintaining the Viability of CD34+ cells During Storage and Transport of Fresh Haematopoietic Progenitor Cells

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Background and Aim: The optimum storage and transport of freshly harvested haemopoietic progenitor cells (HPC) in the liquid state is not specified in the JACIE and FACT guidelines. Depending upon the transplant centre, there is a range of reported ideal temperatures (1°C to 24°C), for the storage and transport of HPC before infusion or processing. There is very little data documenting the viability of CD34+ cells stored under these conditions. Due to the limitations of Trypan Blue viability assays and CFU-GM colony assays, we used a no-lyse, CD34 assay (Bone Marrow Transplantation June 2005) to determine the optimum storage and transport temperature for maintaining viability of CD34+ stem cells in freshly harvested HPC.

Method: Samples were aseptically removed from 46 fresh HPC harvests (34 PBSC & 12 BM) and stored at refrigerated temperature (2°C -8°C), room temperature (18°C- 24°C) and 37°C, for up to 72 hours. Samples were analysed for viable CD34+ cells/µl at 0, 24, 48 and 72 hours.

Results: The mean viable CD34+ yield prior to storage was 7.7x10^6/kg (range: 0.7 – 30.3). The mean % loss of viable CD34+ cells can be summarized as follows:

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<tr>
<th>Time</th>
<th>Refrigerated Temperature</th>
<th>Room Temperature</th>
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</tr>
<tr>
<td>72 hrs</td>
<td>29</td>
<td>-28.0</td>
</tr>
</tbody>
</table>

There were no viable CD34+ cells after storage at 37°C for 24 hours.

Conclusion: These results demonstrate that the optimum temperature to maintain the viability of CD34+ stem cells, during overnight storage and transport of freshly harvested HPC, is 2°C- 8°C.

Determining Acceptable Microbial Contamination Rates for PBSC Processing

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¹Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia

Aim: Patients undergoing haemopoietic stem cell transplantation are at high risk of developing life-threatening infections. Stem cell products represent a potential infection source - reported microbial contamination rates of peripheral blood progenitor preparations range from 0 – 4.5%. Bone marrow stem cell collections are not performed at Peter Mac, however reported contamination rates range between 2 – 42%. Our objective was to retrospectively investigate the Peripheral Blood Stem Cell (PBSC) processing system at Peter Mac to determine its effectiveness in contamination prevention and monitoring and to establish whether a previously determined contamination rate limit is appropriate.

Method: The Peter Mac Cryopreservation Laboratory employs multiple systems directed at PBSC contamination prevention. A 4-point microbial testing system allows identification of contamination sources when they occur. All contaminated products are reported and contamination points identified. Preventative actions to avoid further episodes are developed where relevant. Our unit has imposed a process-associated contamination rate limit of 1% of all procedures. A retrospective analysis of processing data from May 2001 – June 2005 allowed us to collate all positive microbial events and classify as process-associated or derived from other sources to identify our overall laboratory contamination rate, and determine acceptability.

Results: 1770 PBSC collections were processed over the 4-year period (May 2001-June 2005). Overall, 22/1770 (1.2%) PBSC products were contaminated, with 4 /1770 (0.2%) deemed as process associated. The remainder resulted from other sources, including endogenous (13/1770, 0.7%), broken bag at infusion (1/1770, 0.06%) and false positives (4/1770, 0.2%).

Conclusions: With appropriate quality systems, we have shown that a process-associated contamination rate limit of 1% is achievable. Additionally, we have demonstrated overall contamination rates should fall within reported range of
0 – 4.5%. Our quality systems are adequate to achieve acceptable PBSC contamination rates, they allow adequate investigation of failures in asepsis and they encourage continuous improvement when required.

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Reduction in Cell Numbers Reported in Unrelated Donor Products Between Collection Facility and Transplant Hospital

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Background and Aim: Over the past 5 years there has been a remarkable increase in the use of peripheral blood stem cells (PBSC) for unrelated donor transplantation. Collections are frequently performed overseas or at distant national sites. To our knowledge, there has been no correlation performed between haematopoietic progenitor cell (HPC) counts performed by the collecting facility at the time of cell harvest and the transplant hospital at the time of cell infusion. The aim of this study was to compare these values for unrelated donor products provided to our laboratory for infusion between 2002 and 2005. We used a standard unit of measurement, either total nucleated cell count (TNC) for unrelated BM collections or viable CD34+ cell count for unrelated PBSC collections

Method and Results: We report the outcome of HPC count variability of 30 PBSC collections and 19 BM collections from unrelated local and overseas donors. The viable CD34 counts for PBSC and TNC counts for BM, as determined by the collection hospitals, were consistently reduced when products were reanalysed by our laboratory. CD34 counts from PBSC harvests obtained by our laboratory were an average of 20% less than those provided by collection hospitals (95% CI 11-42%, p<0.001). For unrelated BM harvests, TNC counts were an average of 11% less than those provided by collection hospitals (95% CI 8-20% p= 0.04). In some cases reductions resulted in infusion of cell numbers considered inadequate for routine transplantation. Relevant factors underlying these findings could include the transport/storage conditions of HPC during transfer to our centre and variations in laboratory analysis of TNC or CD34 cell counts. The latter include sampling, gating strategies and other methods of data analysis.

Conclusion: Standardisation of transport conditions and analytic techniques would be of value in determining accurate numbers of unrelated donor HPC available for transplantation.

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Mobilisation of Haemopoietic Progenitors/Stem Cells in a Nonhuman Primate Model

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Aim: Since 2002 we have sought to establish a nonhuman primate (NHP) model to test novel agents for their ability to mobilise haemopoietic progenitors/stem cells. We have tested the efficacy of Pegylated G-CSF (Peg G-CSF) in mobilising haemopoietic progenitors/stem cells in this model by directly comparing it to G-CSF. We wished to compare these results to data from human mobilisations to further validate the NHP for future experiments.

Methods: Four male baboons aged between 7 and 11 years of age were mobilised twice: once with G-CSF and once with Peg G-CSF with a minimum period of 12 weeks between mobilisations. G-CSF was administered at the dose of 100mcg/kg/day subcutaneously for 5 days based on data previously published and our own experience. Pegylated G-CSF was administered at the dose of 300mcg/kg subcutaneously as a single dose. Blood counts, peripheral blood CD34 positive cells and colony forming cells (CFC) were quantitated at baseline and at day 5 for both cytokines. These were also quantitated on days 3, 7 and 10 for Peg G-CSF. SCID repopulating cell (SRC) frequency was quantitated at baseline from PBMNCs and on day 5 using cells harvested by leucapheresis.

Results: In all 4 animals, following a single dose of Peg G-CSF, CD34 and CFC peaks occurred on day 5, compared to the neutrophil peak which occurred on day 3. The mean CD34 fold-increase for G-CSF and Peg G-CSF at day 5 compared to baseline was 37.06 and 32.7 respectively (p=0.8850), and the mean CFC fold-increase was 29.88 and 27.13 respectively (p=0.8897). The mean SRC frequency fold-increase was 33.5 and 35.5 for G-CSF and Peg G-CSF respectively. These results are highly comparable to published data in humans, thereby emphasising the accuracy of this NHP model and paving the way for more ambitious experiments to be outlined.

HSANZ Orals ASM 2005
**Conclusion:** Using this model, we have demonstrated that a single dose of PEG G-CSF is as good as G-CSF in mobilising CD34 positive cells, CFC and SRC.

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An Evaluation of Predictive Factors for Adequate Peripheral Blood Stem Cells (PBSC) Collection

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¹BMT Program, Alfred Hospital, ²Monash University, Melbourne, Victoria

**Aim:** Rapid engraftment depends on CD34+ stem cell dose. We know that PB CD34 > 20/ul is strongly correlated with good CD34 yield. However there are other factors, which also influence stem cell yield. We have conducted this retrospective analysis to determine various factors that may affect PBSC yield and we hypothesize that even at low PBCD 34 level useful numbers of stem cell can be collected.

**Method:**
- 583 leukapheresis procedures from 1 Jan 2003 to 15 Jun 2005.
- 228 patients and 61 donors
- Median age 52.7 (16.1 to 71) years;
- M/F=165/123
- Mobilization: chemotherapy plus G-CSF n=114 patients; G-CSF alone (5 to 20 ug/kg) n=169 donors & patients.
- Machine type: Cobe Spectra/ Baxter CS3000 (400/175 procedures).
- Factors analysed: patient demographics, disease, previous treatment, mobilization and collection related factors.

**Results:**

**Multivariate analysis of factors predicting CD34 Yield**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB CD34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Collection Efficiency (CE)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Volume</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Collection duration</td>
<td>0.0020</td>
</tr>
</tbody>
</table>

PB CD34 and CE explain almost 90% variation in peripheral blood CD34 yield.

**Analysis of CD34 yield when PBCD34 < 20/ul**

<table>
<thead>
<tr>
<th>PBCD 34/ul</th>
<th>No of Collections</th>
<th>Collections &gt; 0.5 x 10^5/kg (%)</th>
<th>Median (range) CD34 yield X10^5/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 to 20</td>
<td>58</td>
<td>36 (62%)</td>
<td>0.73 (0.51 to 1.41)</td>
</tr>
<tr>
<td>&lt;10</td>
<td>21</td>
<td>2 (9.5%)</td>
<td>0.3 (0.03-0.65)</td>
</tr>
<tr>
<td>Below sensitivity**</td>
<td>156</td>
<td>55 (35%)</td>
<td>0.749 (0.51-4.2)</td>
</tr>
</tbody>
</table>

**Below sensitivity:** Less than 100 positive events were collected per 100,000 total events (limitation set by ISHAGE protocol)

We have developed simple equation to predict stem cell yield

Log yield = -2.38 + log PB CD34 + Log CE

**Conclusions:**
- PB CD34 on day of collection and collection efficiency are strong predictors of stem cell yield and by above simple equation we could predict the yield.
- We found that useful numbers of PBSC were collected when PBCD34 were below 20/ul.
Deletions of chromosomes 17 (17p) and 20 (20q) are both common findings in acute myeloid leukaemia (AML) and myelodysplastic syndromes (MDS). Following our report of a recurrent unbalanced dicentric 17;20 translocation in AML and MDS incorporating loss of both 17p and 20q, we reviewed cases received at the Victorian Cancer Cytogenetics Service for further examples of this abnormality. Twelve cases were identified with unbalanced translocations which combined deletion of the critical regions on chromosomes 17p and 20q, with a 17;20 translocation. These cases were studied using fluorescence in situ hybridization (FISH) with a combination of locus specific probes, multicoloured karyotyping (M-FISH) and multicoloured banding (m-BAND).

Six of the 12 cases contained apparently identical dic(17;20) translocations but m-BAND showed that the breakpoints varied considerably. Given this variation, there was no evidence for the formation of a critical fusion gene. Of particular interest was the fact that all 12 cases, even those without dicentric chromosomes present, had apparently originally contained a dicentric 17;20 translocation. However, it was clear that not all these dicentric chromosomes were stable and various complex rearrangements had occurred subsequently including loss of one or other centromere.

Dicentric chromosomes are unusual, and so consistent formation of a dicentric chromosome during 17;20 rearrangement is notable. The formation of the dic(17;20) ensures the retention of pericentric segments of both 17 and 20 whilst deleting regions of 17p and 20q presumed to contain critical tumour suppressor genes. We hypothesise that there are regions on 17p and 20q which are always retained during chromosome 17 or 20 deletion in AML and MDS, and may contain genes which are required for the development of these malignancies.

**HSANZ Free Communications 8: Genetics of Leukaemia & Myeloproliferative Disorders**

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**Breakpoint Clustering and Alternative Splicing in the Bcr2 Breakpoint Subtype of PML/RARA**

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On behalf of the Australasian Leukaemia & Lymphoma Group
Institute of Haematology, Royal Prince Alfred Hospital, Sydney, NSW, Australia.

Acute promyelocytic leukaemia (APL) is characterized by the occurrence of gene rearrangements that invariably involve the RARA gene and most frequently the PML gene. A combination of alternate splicing and breakpoint clustering in PML results in several forms of PML-RARA fusion transcripts being expressed. Breakpoint clustering in regions known as bcr1 and bcr3 produces long and short variants of the fusion transcripts, respectively. The bcr2 (or variable) breakpoint subtype results from genomic breakpoints within PML exon 6 (P6) and RARA intron 2 (R2). Characterization of the PML breakpoint subtype at diagnosis facilitates minimal residual disease detection in serial samples, and in bcr2 cases this requires DNA sequencing to exclude non-specific amplification and to accurately size the PCR product.

A comprehensive survey of bcr2 sequence data was performed from cases in the literature (n=26) and bcr2 cases referred to our laboratory (n=20). Our sequence analysis of bcr2 transcripts identified 9 new breakpoint sites within P6 including a new common breakpoint 63 bp from the 3’-end of P6 (~63break). Inserted sequences in 12 cases, ranging from 7 to 156 bp, were demonstrated to be derived from sequences directly 3’ to the R2 genomic breakpoints. One case with a ~64break expressed a splice variant of the insert sequence. Cases with the previously recognised common ~54break often express out-of-frame transcript variants. Non-canonical cryptic splice donor sequences were often used in splice variants. Deduction of the PML-RARA genomic breakpoints from the RT-PCR products in cases with R2-derived inserts allows the design of genomic PCR assays for detection of the patient specific breakpoint. We have shown in one patient that this strategy is feasible and it has the potential to simplify minimal residual disease detection in patients with bcr2 breakpoints.
Aim: Quantitative PCR for BCR-ABL in the peripheral blood (RQPCR) provides an accurate measure of response to therapy in chronic myeloid leukaemia (CML). We analysed a large group of imatinib-treated patients to assess what additional clinical information was provided by routine cytogenetic monitoring.

Method: CML patients were treated with imatinib at centres throughout Australia and New Zealand with RQPCR monitoring at our laboratory. Patients were in morphological chronic phase, but may have had clonal evolution prior to starting imatinib. Data were available for 183 patients undergoing 828 simultaneous RQPCR and cytogenetic analyses. Metaphase karyotyping was performed in the patient’s local laboratory. Real-time reverse transcriptase PCR results were expressed as a ratio of BCR-ABL/BCR.

Results: Paired blood RQPCR and marrow cytogenetics showed a good correlation (Spearman r = 0.765 p<0.001). We defined clinically relevant cytogenetic abnormalities (CRA) as Ph(+) clonal evolution, sustained loss of CCR or an increase of >=20% Ph(+) cells. CRA occurred in 24/183 (13%) patients, but there were none among patients with 3-log reduction in RQPCR. In 20/24 patients RQPCR results doubled at or before CRA. All of the remaining 4 patients had failed to achieve 2-log reduction in RQPCR by 6 months. One patient proceeded directly to allogeneic transplantation, while the remaining three had doubling of RQPCR within 3 months after CRA. Clonal abnormalities were detected in Ph(-) metaphases in 12/183 patients (7%), with overt myelodysplasia in one. RQPCR results did not increase unless there was simultaneous progression of the Ph(+) clone.

Conclusion: Routine cytogenetic monitoring detected no clinically relevant abnormalities which would not have been detected by selective monitoring determined by molecularly-based criteria – doubling RQPCR values, or failure to achieve 2-log reduction by 6 months. We conclude that for most patients routine bone marrow cytogenetics is not indicated.

Haemopoietic Cells Bearing the Jak2V617F Mutation Are Present at Very Low Levels in the Peripheral Blood in Some Patients with ET

Aim: The Jak2V617F mutation has recently been recognised in the majority of patients with polycythemia rubra vera (PRV) and approximately half of the patients with essential thrombocythemia (ET) and idiopathic myelofibrosis (IM). We have examined for the Jak2V617F mutation in DNA derived from purified peripheral blood granulocytes in fifty patients with different myeloproliferative disorders using three different molecular assays for the mutation.

Method and Results: Direct sequencing of the PCR product amplified from DNA identified the mutation in 5 of 5 patients with PRV, 3 of 5 with IM, 5 of 8 patients with a mixed myeloproliferative disorder, but only 6 of 32 patients with ET. DHPLC is a potentially more sensitive assay which can be used in a semi-quantitative fashion to detect heteroduplex formation between mutant DNA bearing the Jak2V617F mutation and wild-type Jak2 DNA. Using this method we discovered that patients with ET have very variable ratios of mutant Jak2V617F DNA to wild-type DNA in their peripheral blood. DHPLC was more sensitive than direct sequencing identifying a further 4 ET patients with the mutation. The most sensitive method to detect the mutation was found to be a PCR-based enrichment assay where DNA was amplified by PCR, a restriction enzyme was then used to selectively cut the PCR product containing wild-type DNA prior to a second round of PCR amplification with subsequent mutation detection. This method identified an additional 7 patients that were found to harbour very low levels of mutant DNA in their peripheral blood granulocyte pool.

Conclusion: While the clinical significance of very low levels of mutant Jak2 DNA in ET is unknown at this time, care should be taken to select a sensitive molecular assay when testing peripheral blood in patients with a potential diagnosis of ET.
Background: The point mutation A2446T (D816V) in the c-kit gene commonly found in systemic mastocytosis (SM) leads to constitutive activation of tyrosine kinase activity and confers resistance to the tyrosine kinase inhibitor imatinib mesylate (IM) at concentrations achievable with standard doses.

Aim: In SM, tumour burden is typically low and below the threshold for detection by most mutation methods. A rapid and sensitive method was developed to more accurately determine the mutation status of patients and subsequent eligibility for a Phase II molecularly targeted protocol with IM (INDY study, Novartis Pharmaceuticals, Australia).

Methodology: Two methods were developed and compared with direct sequencing. Enriched sequencing in which the mutant fragment of a BsmAI cleaved PCR product is sequenced. A modification of the allele-specific competitive blocker PCR (ACB-PCR) method in which three primers are combined: a normal primer that targets the normal allele is tagged with a 3’ phosphate to block amplification, a mutant primer that targets the mutant allele with mismatches to ensure that the normal allele is not amplified and a common reverse primer.

By analysing DNA extracted from the D816V carrying human leukaemic cell line (HMC-1) diluted on a background of normal DNA, enriched sequencing and ACB-PCR were sensitive to the level of 1% and <0.1% respectively. No false positives were determined analysing 19 different control DNA samples.

Results: Bone marrow samples from 24 patients under investigation for SM were analysed by the above novel methods and standard direct sequencing. Using ACB-PCR, 16/24 patients were positive for the D816V mutation. Of the 16 positive cases, direct sequencing detected 19% (3/16) and enriched sequencing detected 69% (11/16). Additionally, enriched sequencing detected another 2 mutations D816Y and D816N. Overall, 18/24 patients were positive for a point mutation in the c-kit gene.

Conclusion: These results indicate that the ACB-PCR method enhances D816V mutation detection in systemic mastocytosis patients and a similar methodology maybe applied for sensitive mutation detection in other malignancies.

secondary gene rearrangements in FIP1L1/PDGFRA positive hypereosinophilia syndrome

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Aim: A subset of patients with idiopathic hypereosinophilia syndrome (HES) respond to treatment with imatinib mesylate (Glivec). In the majority of cases, it has been demonstrated that PDGFRA is constitutively activated as a result of a FIP1L1-PDGFRA (FP) gene rearrangement formed by an interstitial deletion of chromosome 4. We sought to molecularly characterize FP+ve patients referred to our laboratory.

Method: The fully nested RT-PCR assays1 for FIP1L1, PDGFRA and FP were used. FP PCR products from positive patients were cloned using the TOPO cloning system and plasmids were sequenced to identify the breakpoint type.

Results: RT-PCR amplification of FIP1L1 and PDGFRA transcripts indicates that PDGFRA is expressed at a much lower level than FIP1L1. Four FP+ve patients were identified in our laboratory from 44 cases with HES or other eosinophilic disorders referred to us for FP testing (see Table).

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex, Age, Initial Diagnosis</th>
<th>Breakpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M, 31, HES</td>
<td>FIP1L1 intron 10; 15bp insert;</td>
</tr>
<tr>
<td>2</td>
<td>M, 70, HES</td>
<td>FIP1L1 intron 9; no insert;</td>
</tr>
<tr>
<td>3</td>
<td>M, 59, HES</td>
<td>FIP1L1 intron 8; no insert;</td>
</tr>
<tr>
<td>4</td>
<td>M, 37, HES</td>
<td>FIP1L1 intron 13; 7bp insert;</td>
</tr>
</tbody>
</table>

All four positive patients responded to treatment with Glivec and two cases have been serially monitored for FP by RT-PCR. The FIP1L1 breakpoint found in case 4 has not been previously described. Two cases (1 & 4) also expressed minor out-of-frame transcripts that indicated the presence of secondary FP genomic rearrangements. However, there is a possibility that amino-truncated PDGFRA peptides might be translated from the altered open reading frames. In case 1, the secondary rearrangement was a genomic deletion (of ~29kb) within the FIP1L1 portion of the fusion gene; in case 4, it involved a FIP1L1 exon 8 breakpoint and a more 3’ PDGFRA breakpoint.

Conclusion: We have demonstrated the presence of minor secondary genomic rearrangements in a subset of FP+ve patients. In these cases, in addition to the formation of a fusion protein, a secondary mechanism of PDGFRA activation may be occurring as a result of expression of an amino-truncated peptide.

Reference
A Pilot Study of the Effect of Imatinib Mesylate on Porphyrin Elimination

Silvia Ling, Victor Poulos, John D Allen
1. Centenary Institute of Cancer Medicine and Cell Biology
2. Department of Biochemistry, Royal Prince Alfred Hospital.

Aim: To study the effect of Imatinib on the elimination of porphyrins

Background: Imatinib mesylate is a potent inhibitor of the transporter ABCG2, which is instrumental in the efflux of porphyrins, particularly protoporphyrin IX from erythrocytes. Mice lacking functional Abcg2 have a ten fold rise in erythrocyte protoporphyrin IX and develop photosensitivity when fed a diet rich in plant chlorophyll. Indeed, cases of photosensitivity in patients receiving Imatinib have been reported.

Methods: Blood and urine samples from 18 patients with CML were analysed by spectrofluorimetry for total red blood cell, plasma and urine porphyrins. Informed consent was obtained. Results were compared to reference range established by Blake et al 1992 using Student’s t tests.

Results: The mean red blood cell porphyrin was 1.5 fold higher in patients treated with Imatinib than the mean of the reference range (p=0.003, Figure 1). Although statistically significant, this degree of elevation was not clinically significant. The mean red blood cell porphyrin in patients taking high dose Imatinib (> 400mg, n=3) was higher than the group on 400mg or less (p=0.036), but more patients are needed to confirm this trend (Figure 2).

Conclusions: This study suggests that Imatinib causes accumulation of red blood cell porphyrins. Although low dose Imatinib alone is unlikely to cause a porphyria-like syndrome, it highlights the clinical awareness of the potential drug interaction between inhibitors of ABCG2. It also suggests a dose relationship between Imatinib and porphyrin accumulation and larger studies is warranted to confirm this data. Furthermore, ABCG2 may play a role in the variable clinical penetrance of genetic porphyria.

Rapid Clinical and Molecular Response to Dasatinib (BMS 354825) in a CML Patient with Myeloid Blast Crisis and Imatinib Resistance Not Due to Kinase Domain Mutation

Hanlon Sia, Sue Branford, Deborah White, Timothy Hughes
Royal Adelaide Hospital and Institute of Medical and Veterinary Science, Adelaide

A 61 year old woman presented in May 2001 with accelerated phase chronic myeloid leukaemia (CML). Peripheral blood (PB) real-time Q-PCR for BCR-ABL/BCR was 89% at presentation. She was treated with imatinib 600mg/d without significant side effects. A >3 log reduction in BCR-ABL was achieved by 6 months. She remained well until March 2005 when her BCR-ABL increased markedly to 63%. Imatinib was increased to 800 mg/d. A screen for BCR-ABL kinase domain mutations was negative. In May 2005 she presented with myeloid blast crisis with rapidly growing left tibial lytic bone lesion and soft tissue chloroma. She had 12% blood and 24% marrow blasts. Cytogenetics revealed an extra copy of the Ph chromosome and other abnormalities. She had severe hypercalcaemia and acute renal failure. Imatinib therapy was discontinued and SRC-ABL kinase inhibitor (dasatinib) was started at 70mg twice daily. BCR-ABL level has decreased markedly from 326% to 1.8% in 2 months. Marrow aspirate shows chronic phase morphology with no Ph+ cells. Dasatinib (BMS-354825) is an orally available dual SRC-ABL kinase inhibitor with 325 fold greater potency than imatinib and activity against all but one of the common imatinib-resistant BCR-ABL mutants. The cause of resistance in this case is not defined. We have found very low uptake of radiolabelled imatinib into CML.
cells taken from this patient pre-dasatinib relative to her de-novo CML cells, suggesting acquisition of an influx or efflux defect as the cause of resistance. This case report demonstrates the efficacy of this new SRC-ABL kinase inhibitor in a patient with resistance not due to kinase domain mutations. The mechanism of imatinib resistance and reason for dasatinib sensitivity in this case are currently being investigated.

HSANZ Free Communications 9: Experimental Therapeutics

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Pathways of All-Trans-Retinoic Acid Resistance in the Acute Promyelocytic Leukaemia Cell Line NB4

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Aim: Resistance to all-trans-retinoic acid (ATRA) develops in approximately 30% of APML sufferers, for which therapeutic options are limited. In order to develop new treatment strategies, the aim of the current project was to characterise growth, differentiation, morphology and gene expression pathways involved in development of ATRA-resistance in the PML/RARα+ NB4 cell line.

Methods: Three ATRA-resistant cell lines were developed (NB4-8, NB4-7 and NB4-6) by long term dose escalation of ATRA in NB4 cell culture media (final concentrations=10^-8M, 10^-7M and 10^-6M, respectively). Differentiation was characterised by nitroblue tetrazolium (NBT) reductase assay, surface expression of CD54 and CD11b, and morphological/nuclear changes visualised by light microscopy and hoechst staining. Proliferation was assessed by MTT assay following 96h ATRA treatment. Gene expression analysis was performed using a Compugen 19K oligonucleotide Micro-array.

Results: ATRA resistance was characterised by significant morphological alterations, notably the production of microvilli, resulting in increased adhesiveness and clumping of cell cultures. This differentiation was accompanied by increased expression of the granulocyte markers CD54 (doubled to 90%) and CD11b (5-fold increase to 30%). In contrast, NBT reduction was decreased by 50-90% in resistant cells compared to parental cells when treated with 10^-5M ATRA. Resistant cells required up to 8x10^-4M ATRA to produce a 50% decrease in growth compared to parental NB4 cultures which were equally sensitive to just 1x10^-7M. Micro-array analysis showed a dose response between ATRA resistance and expression of at least two genes involved in proliferation, namely calcium homeostasis endoplasmic reticulum protein (CHERP) and adaptor protein containing PH domain, PTB domain, and leucine zipper motif (APPL or DIP13alpha) as well as the anti-apoptotic glutaminyl-tRNA synthetase (QRS).

Conclusions: ATRA-resistant NB4 cells displayed morphological features of differentiation but over-expressed genes involved with proliferation and survival, and did not undergo growth arrest in response to therapeutic doses of ATRA.

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Eliminating Tumor Cells with Small Molecule Inhibitors of Pro-Survival Bcl-2 Proteins

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The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3050, Australia.

Aim: Overactivity of pro-survival Bcl-2 proteins promotes neoplasia and enhances resistance of malignant cells to cytotoxic therapies, a common cause of treatment failure. A promising approach for overcoming chemo-resistance is to directly target the pro-survival proteins by mimicking their physiological antagonists, the BH3-only proteins. The aim of this study is to determine the mechanism of action of putative BH3 mimetic compounds and to determine their optimal application.

Methods: We compared the activity of seven putative BH3 mimetic compounds with the physiological BH3-only proteins. Killing by the BH3-only proteins require the action of the essential cell death mediators, so it is anticipated that true mimics of their action will not kill cells lacking Bax and Bak. We used these and other genetically modified cells to ascertain how putative BH3 mimetic agents act and confirm these findings in tissue culture and in whole animal systems.

Results: Among seven putative BH3 mimetics, however, we found that only the recently described ABT-737 (Abbott Laboratories), like BH3-only proteins, requires the pro-apoptotic protein Bax or Bak to induce apoptosis. The cytotoxicity of ABT-737 alone is often modest, however, probably because it only targets selected pro-survival proteins (Bcl-2, Bcl-xL, Bcl-w), and apoptosis usually also requires neutralization of Mcl-1. Indeed, targeting of Mcl-1 with the BH3-only protein Noxa, or Mcl-1 down-regulation by RNAi, cytokine deprivation or genotoxic agents, allowed ABT-737 to efficiently kill diverse cell types, even when Bcl-2 was over-expressed.

Conclusions: We conclude ABT-737 is a highly selective and specific BH3 mimetic compound that should prove highly efficacious in tumors where Mcl-1 is low, or when combined with agents that down-regulate Mcl-1.
In Vivo Tracking of Macrophage Activated Killer Cells to Sites of Metastatic Ovarian Carcinoma

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1Department of Haematology and Medical Oncology, Peter MacCallum Cancer Centre, Melbourne; 2Department of Diagnostic Imaging, Peter MacCallum Cancer Centre, Melbourne; 3Immuno-Designed Molecules, Paris, France; 4University of Melbourne, Australia

Aim. To assess radio-labelling as a means of tracking cellular immunotherapy in patients with metastatic cancer. 

Method: Radio-labelling of blood cells is an established technique for evaluating in vivo migration to sites of pathology such as infection and haemorrhage. We describe the use of two methods of cell labelling for tracking the destination of autologous-derived macrophage activated killer (MAK) cells linked to the bi-specific antibody MDX-H210 delivered either by intravenous (iv) or intraperitoneal (ip) injection in ten patients with peritoneal relapse of epithelial ovarian carcinoma.

Results: Our results demonstrate the feasibility of generating high numbers and purity of GMP quality MAK cells, which can be efficiently radiolabeled. MAK cell administration produced minimal infusional toxicity and demonstrated a reproducible pattern of in vivo distribution and active in vivo tracking to sites of known tumor following either iv (8 of 16 infusions) or ip (4 of 6 infusions) using either 18F-FDG or 111In-labelled cells. The addition of MDX-H210 monoclonal antibody did not alter the distribution of cells to tumour sites, but did alter the in vivo distribution of cells administered by iv injection.

Conclusion: This study demonstrates that cellular cancer immunotherapies may be successfully delivered to sites of active tumour and allows selection of those patients who may benefit from these approaches.

Development of Acute Myeloid Leukemia NOD/SCID Mouse Model to Assess CD300 Antibodies as Therapeutics for AML

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AML is the most common type of leukemia in adults. Despite advances in treatment, it remains fatal in 70% of cases. Currently, the only antibody-mediated therapy for AML is Mylotarg, a reagent that targets the normal myeloid cell surface marker, CD33. Whilst Mylotarg has promising results, associated fatal veno-occlusive disease has been identified as a complication of the treatment. Our laboratory identified CD300 family, a group of leukocyte surface molecules, similar to CD33. Four members of this family are restricted to the myeloid lineage. We hypothesize that these molecules, CD300e, 35-L3, 35-L4 and 35-L5, have potential as targets for antibody mediated therapy of AML. We have developed antibodies against these molecules and are now assessing them in vitro and in vivo systems.

As an in vivo system, we have developed a human AML chimeric model in NOD/SCID mice. We have optimized a number of parameters to ensure consistent engraftment and found that irradiation at 250cGy 48 hours prior to injection and 5 x 10^6 cell dose was optimal. We observed no difference between using fresh or frozen cells. Bone marrow and peripheral blood mononuclear cells preparations were compared and both engrafted. Using these conditions engraftment was observed for most leukemias between 5-10 weeks. We were able to detect engraftment in BM and spleen by flow cytometry, however, no human cells were detected in the mouse peripheral blood. Analysis by flow cytometry indicated that the phenotype of the human cells in engrafted mice were the same as that of the injected AML. U937 cells can also be reliably engrafted into NOD/SCID mice using the conditions we have outlined here. This provides us with a useful in vivo leukemia model that allows assessment of our CD300 monoclonal antibodies as targeting vehicles.
Recently, several laboratories have shown that after bone marrow transplantation, small numbers of donor-derived cells could be found in a wide variety of tissues, including heart, skeletal muscle, and liver, as well as others. This suggested that bone marrow transplantation might be able to be used for therapy of non-haematopoietic disease. However, the origin of the engrafting cells was not clear, and the efficiency low. In order to examine this possibility, we identified the cells involved in non-haematopoietic regeneration after transplant. By transplanting single haematopoietic cells into mice, we showed that progeny could unequivocally be found in both skeletal muscle, liver, and heart. Using a cre-lox lineage marking strategy, we showed that myeloid cells, most likely macrophages, were most likely the major cell type that is directly involved via direct fusion with regenerating differentiated cells. While the process is generally inefficient, selection for the hybrid cells can lead to remarkable conversion of a mutant liver to a wild-type phenotype. Recent data indicate that fusion, while rare, is not uncommon. Limiting the efficiency of the putative therapy is also reprogramming of the donor cell nucleus. Therefore, improvements to reprogramming, as well as to specific cell fusion, will be required to facilitate the use of bone marrow transplantation as a therapy for non-haematopoietic disease.

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Differentiation of Human Embryonic Stem Cells

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This presentation will discuss progress in the development of controlled systems for directed differentiation of human embryonic stem cells (hESC) in vitro. High resolution immunotranscriptional analysis of the early differentiation hierarchies in hESC cultures shows that paracrine interactions between cells act to control stem cell fate. Neural differentiation occurs spontaneously in hESC cultures, but may be driven at high efficiency by modulation of these endogenous signalling pathways. Neural precursors derived from hESC have been shown to have a beneficial effect in ameliorating disease symptoms in preclinical models of Parkinson’s Disease. Better outcomes in future studies will require control of the commitment and differentiation of these neural precursors. Other in vitro differentiation systems have produced cells of hematopoietic, cardiac, and endothelial lineages from hESC; while these systems have relied mainly on the selection and expansion of progenitors from spontaneously differentiating cultures, further analysis will enable the design of more efficient protocols. The accelerating pace of discoveries in hESC biology is pushing this technology faster towards preclinical assessment and ultimately early phase trials, but careful assessment of safety and efficacy will be essential before humans receive hESC-based therapies.

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Stem Cell Therapies for Heart Diseases – Past Progress, Future Challenges

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Ischaemic heart disease is a major cause of death and disability in the Western world. Heart muscle-cell death results from coronary artery occlusion (myocardial infarction). Given the limited regenerative capacity of heart cells, healing is by scar-formation, resulting in compensatory hypertrophy of remaining viable cells, with activation of genes that cause apoptotic cell death, further scaring and heart failure – a process known as remodelling. Even severe flow-limitation without complete coronary occlusion accelerates apoptotic cell-loss and causes contractile dysfunction. Heart failure, therefore, is a muscle-cell-deficiency disorder and is increasing exponentially - already effecting 23 million people worldwide. To redress this problem, stem cells are being evaluated as a novel approach to augment myocardial repair and limit remodelling. Animal and human studies have provided proof-of-principle for use of skeletal muscle and bone marrow-derived stem cells (BMSCs), although in vivo few if any differentiate into cardiomyocytes, or couple with them electrically. Moreover, whether the potential beneficial effects of BMSCs are due to their engraftment and differentiation into cardiomyocytes or other cell-types, or to generation of cytokines, which stimulate proliferation and differentiation of endogenous cells, remains unclear. Nevertheless, administration of cytokines that can mobilise BMSCs, or that have direct myogenic effects, have also been explored. Yet another approach is the use of potential bone marrow-derived endothelial precursors cells to promote new blood vessel formation. Finally, resident cardiac stem cells, which have been identified within the myocardium and retain regenerative capacity, are being actively evaluated. Although, their numbers are small, their identity still controversial and their ability to regenerate myocardium, still largely untested. Their enormous potential as a tool for cardiac regenerative therapy makes them attractive as a target for further intense evaluation. These advances into the application and utility of stem cells as a therapy for heart disease will be considered in this presentation.
Aplastic Anaemia (AA) in its acquired form is considered today an autoimmune disease. Viruses, drugs, toxins or hormonal changes can trigger the disease. In most cases the cause remains unknown. A close relation is observed between AA, myelodysplastic syndromes and paroxysmal nocturnal hemoglobinuria (PNH). Standards of care are an allogeneic haematopoietic stem cell transplant (HSCT) for young patients with an HLA-identical sibling donor and immunosuppression with antithymocyte globulin (ATG) and cyclosporin A for older patients and those without an adequate donor. Survival rates are similar for both treatment forms. The pattern of response differs strikingly. Response to ATG is slower, relapse is more frequent with ATG, early mortality and graft-versus-host disease reduce quality and adjusted life after HSCT, as shown in our single institution study of 430 patients with AA. Better prognostic criteria and novel approaches are required. The Working Party Aplastic Anaemia of the European Group for Blood and Marrow Transplantation (EBMT) is currently investigating in a prospective randomised study the role of the growth factor, G-CSF, as adjunct to immunosuppression and the role of early re-treatment with ATG in patients with no donor. In a retrospective analysis of over 2,000 patients transplanted from an HLA-identical sibling main risk factors for outcome were identified. Survival is better in younger patients transplanted earlier in their disease, given bone marrow as stem cell source compared to peripheral blood and given a transplant from a same gender donor. Male donor transplants are more frequently rejected in female recipients and female donor transplants induce more severe GvHD and more transplant related mortality in male recipients. The application of ATG appears to attenuate these effects. Treatment algorithms for AA should include risk profile of the disease, risk of HSCT for the donor and quality of life aspects.

Critical Determinants of Quality of Life (QoL) in Long-term Australian Survivors of Allogeneic Bone Marrow Transplantation (alloBMT)

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Aims: We aimed to identify the critical determinants of QoL in an Australian cohort of long-term survivors of alloBMT.

Methods: 168 potential subjects in remission at least 2 years after alloBMT at the Royal Melbourne Hospital (RMH) were sent a patient information form, a demographics questionnaire, two instruments specifically designed to evaluate QoL in this setting: the QLQ-C30 questionnaire and the FACT-BMT questionnaire and a specific sexuality questionnaire. Another mail-out occurred one month later. Responses were combined with abstracted clinical data. The data were analysed against the following factors: age, marital status, employment, education, disease, transplant conditioning regimen, survival time since transplant, graft-versus-host-disease (GVHD), severe infection, sicca syndrome, osteoporosis-related fractures, pulmonary dysfunction, sexual dysfunction and infertility. The sexuality data will be subject to a separate analysis. This study was approved by the Melbourne Health Human Research Ethics Committee.

Results: 118 subjects returned survey packages; 101 were assessable (60% of targeted population). Median age was 41 years (range 18 to 64). These factors had a significant adverse association with post-transplant QoL: age at transplant > 40 years, lack of employment and low education level. Pre-transplant clinical factors did not predict post-transplant QoL. Number of years survived beyond 2 years did not affect QoL. Only the following complications appeared to significantly affect post-transplant QoL: presence of acute GVHD, severity of acute GVHD and extent of chronic GVHD (all p < 0.05).

Conclusions: This landmark Australian study confirms that GVHD is the critical reversible determinant of post-transplant QoL in long-term Australian survivors of alloBMT. However, strategies addressing the barriers to re-employment of survivors may further improve QoL in these individuals.
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Expression of Dendritic Cell Activation Marker, CMRF-44, on Blood CD11c+ LIN-, HLA-DR+ Dendritic Cells is Closely Associated with the Onset of Acute GVHD After Allogeneic Haematopoietic Stem Cell Transplant

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Aim: Dendritic cells (DC) are considered central to the development of graft versus host disease (GVHD) following allogeneic haematopoietic stem cell transplantation (alloHSCT). Both donor and host DC are thought to initiate allogeneic immune responses by presenting host antigens to donor T lymphocytes. We studied the potential of CMRF-44, a novel monoclonal antibody identifying activated circulating blood DC, as a predictive marker of acute GVHD.

Methods: Peripheral blood was taken from 34 patients twice weekly up to 100 days post-alloHSCT. Circulating myeloid (CD11c+) and plasmacytoid (CD123+) DC were enumerated as described previously (J Immunol Methods 2004; 284:73) and the expression of CMRF-44 was assessed on CD11c+ DC by four colour flow cytometry. Statistical analyses were performed using a non-parametric Mann-Whitney U-test.

Results: Following alloHSCT, the severity of acute GVHD correlated with the number of total DC in the blood (p=0.035). Furthermore, low myeloid and plasmacytoid DC numbers were significantly associated with grade 2-4 acute GVHD (p=0.046 and 0.017 respectively). In 34 alloHSCT patients, 22 developed acute GVHD. CMRF-44 was expressed on CD11c+ DC in all cases prior to the onset of acute GVHD. Of the 12 patients without GVHD, 8 had no circulating CMRF-44+ CD11c+ DC. CMRF-44 expression was independent of the reconstitution of myeloid DC (p=0.73). Patients who had CMRF-44+ CD11c+ DC in more than 20% of their post-transplant monitoring samples were more likely to develop acute GVHD (p=0.001, OR=37.1). In addition, patients with more severe grade 2-4 GVHD had significantly higher percentages of CMRF-44+ CD11c+ DCs (p=0.001).

Conclusion: CMRF-44 expression on blood CD11c+ DC is highly associated with the onset of acute GVHD. These results suggest that CMRF-44 may be used as a predictive tool to direct therapy. Furthermore, it reinforces the potential application of suitably engineered CMRF-44 antibodies (Transplantation 2003; 75:1723 and Collin et al Transplantation 2005; 79:722-725) for the prevention or treatment of graft versus host disease.

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Resting B Cells Suppress CD8+ T Cell Function and Prevent the Induction of Graft Versus Host Disease

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B cells have been variously shown to induce direct tolerance of antigen specific CD8+ T cells, induce T cell anergy via TGF- production, down regulate IL-12 production by dendritic cells (DC) and influence Th1/Th2 differentiation via the production of regulatory cytokines. Through these mechanisms, B cells can exert a regulatory function in in vivo models of T cell immunity including, experimental autoimmune encephalitis (EAE) and rheumatoid arthritis (RA). Recently, B cells have been shown to be essential in the prevention of effector T cell differentiation in a model of autoimmunity.

We have previously shown that resting B cells inhibited tumor protection induced by dendritic cells vaccination. Inhibition of DC immunity by B cells was independent of presentation of major histocompatibility molecule (MHC) class-I bound tumor antigen but dependent on the expression of class-II MHC. Furthermore the inhibitory effect of B cells was lost if the B cells were activated by CD40L or if CD4+/CD25+ regulatory T cells (Treg) were depleted. These studies have been further extended to examine the role of resting B cells on the induction and severity of graft versus host disease (GVHD) induced in a major MHC mismatch model. We have found that mice transplanted with B cell depleted marrow revealed more rapid CD8+ T cell engraftment, higher IL-2 and IFN- production and more severe GVHD. Conversely, those who received additional resting B cells at the time of marrow infusion were substantially protected from GVHD. These findings indicate that resting B cells may regulate T cell activation, in part via the suppressive effects of Treg, but also through their important role in T cell homeostasis. Resting B cells may therefore...
limit the efficacy of DC based immunotherapy or alternatively be used therapeutically to limit CD8+ T cell autoimmunity including GVHD.

**HSANZ Free Communications 10: Transplantation / Graft vs Host Disease**

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*In vivo Dendritic Cell Depletion to Attenuate Graft versus Host Disease in a Murine Transplant Model*

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Graft versus host disease (GVHD) and treatment related mortality (TRM) are major limitations to the widespread application of allogeneic haematopoietic stem cell transplantation (HSCT) for haematological and non-haematological malignancies. Current treatment options for GVHD focus mainly on the effector T-cell and whilst numerous, all result in systemic immunosuppression. Our intention is to shift the focus from elimination of activated T-cells to manipulation of the dendritic cells (DC) that activate T-cells. We propose that the strategic administration of DC depleting antibodies could be an effective means to control GVHD. We have previously shown immunoregulatory DC subsets to exist in mice that make good targets for manipulation in a transplant setting. We have also shown one DC subset in particular, that of mature plasmacytoid DC, to be increased in mouse spleen after conditioning by radiation. Intrapерitoneal (ip) injection of N418, a monoclonal antibody against mouse leukocyte integrin CD11c is able to deplete murine DC *in vivo*. Our preliminary data show elimination of 50% of DC after injection of N418 (500 g). Subsequent experiments show that 1mg of N418 is sufficient to significantly delay, but not prevent, GVHD in a full MHC mismatched model of HSCT (p=0.025). The action of N418 is specific, as DC depletion was not seen in mice treated with 1 mg Hamster Ig. Together, these observations suggest that increasing antibody concentration and prolonged administration may be required to prevent GVHD. Proposed experiments specifically address this question. The successful application of DC depletion to control GVHD will improve the safety of HSCT for patients with leukaemia and extend the immune benefits of this curative therapy to the wider patient population.

**HSANZ Free Communications 10: Transplantation / Graft vs Host Disease**

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*Efficacy of Soluble TNFα Inhibitor (Etanercept) for Treatment of Steroid Refractory Acute Graft Versus Host Disease Post Allogeneic Stem Cell Transplantation*

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**Aims:** To review the efficacy of the soluble TNFα inhibitor etanercept in treatment of steroid refractory acute graft versus host disease (GVHD).

**Methods:** All patients who received etanercept for treatment of steroid refractory acute GVHD complicating allogeneic transplantation at our institution were retrospectively reviewed. Etanercept dose was 0.4mg/kg (max 25mg) SC twice per wk for 4 wks, then once per wk for 4 weeks. Response was assessed at end of etanercept therapy as complete response (CR) if complete resolution of GVHD had occurred, partial response (PR) if incomplete resolution but reduction in overall GVHD grade was noted, and no response (NR) if no reduction in grade of GVHD occurred. Standard prophylaxis used for HSV / VZV, PCP and fungal infection was valaciclovir, bactrim / pentamidine and fluconazole respectively.

**Results:** In total 17 patients had received etanercept therapy. All patients suffered grade 2-4 GVHD, with predominant organ affected gut in 9 cases, skin in 5 and combination gut and skin in 3. In 3 cases etanercept was administered if NR occurred >1mth after both 1st line therapy of prednisolone 2mg/kg/day and 2nd line therapy of ATG + tacrolimus +/- mycophenolate mofetil (MMF). NR and no survivors occurred in these 3 cases. 14 patients received etanercept as part of 2nd line therapy, administered within approximately 2wks of commencing ATG + tacrolimus +/- MMF. Responses in these 14 patients were 10 CR (72%), 1 PR (7%) and 3 NR (21%), with 7 patients (50%) alive at median FU of 6mths (range 3-30mths) post commencing the drug. Causes of death included progressive GVHD in 4 patients and infectious complications in 6. Overall infective complications observed included bacterial sepsis (6/17), CMV reactivation (8/17, including CMV pneumonitis in 1), other viral infections (3/17), new possible or proven aspergillosis (3/17) and progression of previously documented fungal infection (2/17). Also, TTP developed in 4/17 patients (24%).

**Conclusions:** Our experience suggests that when used in combination with ATG, etanercept has significant activity in the treatment of steroid refractory acute GVHD, with high response rates and significant survival observed. Better strategies to reduce infectious complications in this setting are needed.
The Effect of HSCT Transplant Conditioning Regimens on Murine Dendritic Cell Subsets, Inflammatory Cytokines and Endotoxin Levels

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Haematopoietic stem cell transplantation (HSCT) has traditionally relied on conventional conditioning (CC) to ablate the underlying haematological malignancy and control graft rejection post transplant. Pre-transplant conditioning is vital for HSC engraftment, however it up-regulates inflammatory cytokine and endotoxin levels, activates dendritic cells (DC) and initiates graft versus host disease (GvHD). The use of non-myeloablative, but highly immunosuppressive, reduced intensity conditioning (RIC) regimens has increased in response to treatment related mortality (TRM) associated with CC. Reducing the intensity of the conditioning regimen reduces TRM and delays the onset of GvHD, but the overall incidence of GvHD remains unchanged. We hypothesise this is due to increased persistence of host DC. While there is information on the effects of CC on DC, there is no information as yet on the effects of RIC on DC. Preliminary experiments have defined murine models of conditioning (CC = Cyclophosphamide [CY] + 800cGy and RIC = Fludarabine [FLU] + CY + 200cGy). The effects on DC numbers, activation and composition, cytokine and systemic endotoxin levels were studied on each day of the conditioning regimens. Results indicate a significantly higher proportion of pDC in mice receiving CC as opposed to RIC (p<0.001). However, mice that received RIC have significantly higher absolute numbers of host pDC than CC mice. Preliminary data shows no difference in endotoxin levels in mice receiving RIC or CC without HSCT. However, there is a transient increase in endotoxin levels in mice after 2 FLU injections (p=0.12). No such increase was seen after CC. There were significantly higher levels of TNF-α (p=0.02) and IL1-β (p=0.03) in mice receiving RIC rather than CC without HSCT. This observation in combination with the higher total numbers of host pDC in RIC mice may be a mechanism for the delayed onset of GvHD seen in patients.

The Matutes Scoring System Remains a Valuable Diagnostic Tool

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Background: The Matutes scoring system for CLL was originally proposed in 1994 to distinguish CLL from other lymphoproliferative disorders based on immunophenotype. Scores are derived from the expression pattern of CD5, CD23, FMC7, CD22 and surface immunoglobulin (sIg) with scores ranging from 5 (typical of CLL) to 0 (atypical of CLL). Since the Matutes score was first described, the biological and prognostic significance of markers such as CD20 and CD38 has been established and 3-4 colour flow cytometry developed.

Aim: To assess the modern-day utility of the Matutes scoring system.

Methods: The immunophenotype of 99 cases of lymphoproliferative disorders were reviewed. B-cell antibodies were directed against CD10, CD19, CD20 and CD38 in addition to the Matutes markers. FISH, molecular studies and immunostaining for cyclin D1 were used to validate results in some cases.

Results: In 66 CLL cases, 63 scored 4-5 and 3 scored 3. In 16 Mantle Cell Lymphoma (MCL) cases 3 scored 4-5, 6 scored 3 and 7 scored 0-2. In 14 Follicular Lymphoma cases 2 scored 3 and 12 scored 0-2 and in 3 Hairy Cell Leukaemia cases all scored 0-2. Equivocal scores of 3 and 4 in cases of MCL were associated with atypical features such as low intensity of sIg and weak CD23 or CD22 expression. In such cases the expression of CD38 and CD20 intensity were distinguishing features and the diagnosis was confirmed in several cases with positive cyclin D1 immunostaining.

Conclusion: The Matutes score remains a reliable real time diagnostic tool with the addition of CD38 valuable in some equivocal cases. Ancillary tests such as immunohistochemistry for cyclin D1 are particularly important in atypical cases. The use of 3 colour flow cytometry has the potential to enhance the utility of the Matutes score by defining the target B-cell population more precisely.
Flow-cytometric Zap-70 Analysis in Chronic Lymphocytic Leukaemia (CLL): The Peter Mac Experience

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Background: The mutational status of the immunoglobulin heavy chain variable region (IgVH) provides pivotal prognostic information in CLL. Zap-70 expression using diagnostic immunophenotypic methods is reported as being highly correlated with IgVH mutational status.

Aim: To assess and highlight the technical issues and pitfalls of Zap-70 analysis by flow cytometry encountered during validation at Peter Mac.

Methods: During Jan-July 2005 peripheral blood (n=19) and bone marrow (n=2) immunophenotyping on 21 patients was performed using a Becton Dickinson FACSCalibur, subsequent analysis using Cytomics™ RXP software. Cells were surface labelled with CD2/CD19/CD45 (BD MoAbs) and cytoplasmic Zap-70 (SB70 clone 0.5ug/test – Southern Biotech) using the permeabilization method (DAKO Fix & Perm). Incubation was for a minimum of 15 min with anti-Zap-70 FITC. An IgG2b FITC control was used and ≥2000 lymphoid events accrued per case. T & B cell Zap-70 expression was tested on 20 samples from healthy controls. A comparison between the Crespo (N Engl J Med. 2003) and Orchard (Lancet 2004) gating methodology was performed. Logarithmic mean fluorescent intensity (MFI) of Zap-70 was assessed on the T- and B-cell populations.

Results: Controls showed an incubation time dependent MFI on T-cells; 15 min median log MFI = 6.5 (range 4.8-8.6), >15min median log MFI = 15.8 (range 11.9-26.4), B-cell median log MFI = 1.6 (range 1.0-2.4). The median log MFI of T-cells in 21 CLL cases = 8.1 (range 4.1-16.1), median log MFI B-cells with CLL = 1.7 (range 1.0-4.1). Using the Crespo gating strategy 8/21 cases were Zap-70 positive and 12/21 positive using the Orchard methodology. Discordance between methods was seen in 6/21 cases.

Conclusion: Considerable technical finesse is required for accurate Zap-70 validation and analysis impeding its role in routine service laboratories. Validation of the method with a “gold standard” of quantitative mRNA expression is essential while multicentre collaboration and a formal QAP program is recommended.

Pax-5 Demonstrates Residual B-cells in Bone Marrow Trephines Post Anti-CD20 Immunotherapy

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Background: Immunohistochemical stains for CD20 are reliable in both formalin and mercuric-based fixed tissues. Anti-CD20 (rituximab (R)) immunotherapy for B-cell malignancies has negated the usefulness of CD20 immunohistochemistry post-therapy. Many other routine B-cell immunohistochemical stains are unreliable in mercuric-based fixatives. Thus, a reliable B-cell stain is needed post-R treatment. Pax-5 is a transcription factor specific for B-cells that is required for normal B-cell development. It is expressed from the Pro-B stage through to mature B-cells and is expressed on both normal and malignant B-cells. The Pax-5 immunohistochemical stain has proven to be reliable in formalin-fixed tissue. Its role in mercuric-fixed tissue has yet to be determined.

Aim: To demonstrate residual CD20-negative B-cells, using the Pax-5 immunohistochemical stain, on mercuric-fixed bone marrow trephines following R treatment.

Methods: We performed CD20 and Pax-5 immunostains on bone marrow trephines in patients who had received combined chemoimmunotherapy with R. Patients underwent restaging bone marrows midway through treatment (range 2-4 cycles) and at the end of treatment (range 6-8 cycles). We divided analysis into two groups; 1. Patients with overt residual disease on H&E stains. 2. Patients with morphologic complete remission on trephine.

Results: Patient diagnoses included: CLL/SLL n=20; Follicular lymphoma n=2; Mantle cell lymphoma n=1; Waldenstrom macroglobulinaemia n=3. Patients’ morphologic responses were: complete remission (CR) n=5; partial remission (PR) n=9; nodular partial remission (nPR) n=10; no response (NR) n=2. In the non-CR group (PR, nPR, NR) 4 patients were positive for CD20 and all 21 patients were positive for Pax-5. In the CR group 0 patients were positive for CD20 and 5 patients were positive for Pax-5.

Conclusion: Pax-5 is a robust and reliable immunohistochemical stain that demonstrates B-cells post anti-CD20 immunotherapy. While it stains both normal and malignant B-cells, it may prove to be a useful tool in assessing minimal residual disease following immunotherapy.
Accelerated Delivery of Rituximab is Safe on an Out-Patient Basis

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Aim: A prospective study to determine the safety of the accelerated delivery of rituximab.

Methods: Eligible patients were aged 18–80 years. First doses of rituximab were delivered as per prescribing guidelines. Patients without infusion related toxicity were eligible for accelerated rituximab in second or subsequent infusions. In the first 4 patients the infusion was administered at 100mg/hr, increasing to 400mg/hr after 15 minutes in the absence of a reaction. All subsequent patients commenced the infusion at 400mg/hr. All patients received premedication with paracetamol, promethazine and hydrocortisone unless contra-indicated. Patients with Chronic Lymphocytic Leukaemia, circulating lymphoma cells, bulky disease (>7.5 cm) or a previous grade III/IV infusion related toxicity were excluded.

Results: 20 patients were enrolled. Data is available for 45 infusions - median 2 doses per patient (range:2-4). Median age was 64 years (range:28-80). 70% were male. Diagnoses included non-Hodgkin's Lymphoma- 17 patients (DLCL-8, FL-7, MCL-1, LPL-1), Immune Thrombocytopenic Purpura-2 and Dermatomyositis-1. 45% received single agent rituximab and 55% received it in combination. The median dose of rituximab was 700mg (range:600-800mg). There were 2 adverse events with no grade III/IV infusion related toxicity. One patient experienced grade I hypothermia. A second patient experienced grade II fevers and rigors which required interruption of the infusion. The patient was subsequently found to have pneumonia. The infusion was completed without further incident. Excluding data from the latter patient, median infusion time was 1 hour and 55 minutes and 73% of infusions were completed within 2 hours.

Conclusion: For second or subsequent infusions, the accelerated delivery of rituximab is safe and well tolerated allowing shorter outpatient stays and improving the efficiency of resource utilisation.

Delivery and Toxicity of Fludarabine-Based Combination Chemotherapy Regimens in Patients Aged 60 and Older

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Fludarabine (F)-based combination chemotherapy regimens have been shown to be highly effective in a range of indolent lymphoproliferative disorders. However, despite the prevalence of such disorders in older patients, the deliverability of these regimens in patients aged ≥60 has not been assessed.

We analysed the delivery and toxicity of three F-based regimens, all using F 25 mg/m²/dx3 q28d, in 82 adults aged ≥60 years, and compared this with the same regimens in 99 adults aged < 60. The sample comprised 66 patients (32 ≥ 60) with F and cyclophosphamide (C; 250 mg/m²/dx3); 29 with F and mitoxantrone (M; 10 mg/m² x1; 12 ≥ 60); and 86 with FC and rituximab (R; 375 mg/m² x1; 38 ≥60). The age cohorts were well matched for baseline characteristics other than age, including gender, performance status, disease type, and number and type of prior therapies (each P > 0.2). 349 cycles in older patients were compared with 393 cycles in younger patients for haematologic nadirs, infectious complications and organ toxicity. The median number of cycles received was 4 in both groups. The treatment-related mortality rate was <1% for both cohorts (P > 0.5).

Overall, older patients had a slightly higher rate of infections (18%/cycle versus 15%/cycle), though this was not statistically significant (P = 0.28). This difference was more prominent for the FCR regimen; 28%/cycle versus 17%/cycle (P = 0.02). For severe (grade ≥3) infections the difference was minimal: 6% versus 7% (P > 0.5). The rates of neutropenia < 1.0 and 0.5 were 13% and 22% versus 11% and 20% for older and younger patients, respectively (all P values >0.1). The rates of thrombocytopenia < 100 and < 50 were 21% and 5% versus 16% and 5% for older and younger cohorts (all P values > 0.1). Other organ toxicities were uncommon with all three regimens, and showed no difference between age groups).

These results demonstrate that F-based regimens are well tolerated and can safely be delivered to older patients, with a modestly increased rate of infectious morbidity, but no increased treatment-related mortality.
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The Risk of Myelodysplasia (MDS) and Secondary Acute Myeloid Leukemia (sAML) Among Patients Treated with Fludarabine & Cyclophosphamide (FC), FC-Rituximab (FC-R) or FC-Mitoxantrone-Rituximab (FCM-R)

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Introduction: The risk of MDS and sAML following fludarabine combination chemotherapy remains poorly defined (McLaughlin Blood 105:4573,2005). Herein, we report all cases of MDS or sAML encountered at the Peter MacCallum Cancer Centre among consecutive cohorts of patients treated with either FC, FCM-R or FC-R.

Methods: Between 10/96 and 06/05, 136 patients underwent 154 treatment-episodes with either FC (F25mg/m²x3, C250mg/m²x3; n=66), FC-R (FC + R375mg/m²x1; n=83) or FCM-R (FC-R + M10mg/m²; n=5) as initial (n=39) or salvage therapy (n=115) for chronic lymphocytic leukemia (CLL; 40%), follicular lymphoma (FL ;32%), Waldenström macroglobulinemia (WM; 12%) or other indolent lymphoid malignancies. Diagnostic marrow specimens for all cases of MDS or sAML were reviewed according to the WHO Classification, and clinical outcomes reported.

Results: During the follow-up period of up to 91 months (median 24), there were seven cases of MDS/sAML at a median of 45 (range 8 – 86) months post therapy. The rate of MDS/sAML was 5% and 2.5% for pretreated & previously untreated patients respectively (P = 0.68), with a trend to increased number of prior therapies (median 3 vs 2, p=0.16) and longer time from diagnosis to fludarabine combination therapy (median 96 vs 41 months, p=0.08) among MDS/sAML patients. 5 of 6 cases tested had karyotypic abnormalities and frequently involved deletions of chromosomes 5 or 7 (n=4). Median IPSS was 1.0 (range 0.5-2.0). At follow-up, two patients have died (one unknown cause, one from progression to sAML); three are alive with progressive disease at 9, 15 and 23 months; and two are alive with stable disease at 5 and 19 months.

Conclusion: MDS/sAML are observed at a low rate among patients receiving fludarabine combination therapy as initial or salvage therapy. Previous alkylating agent exposure among heavily pretreated patients is likely to be contributory.

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Zebrafish Kruppel-like Factor, KLF4, is Essential for Primitive Haematopoiesis

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Aim: EKLF (Klf1) plays an essential role in definitive erythropoiesis and β-globin gene expression, but primitive (yolk sac) erythropoiesis is reportedly normal. Since expression of embryonic globin genes is dependent upon promoter CACC elements, additional KLFs must function during the embryonic wave of erythropoiesis. Like mammals, zebrafish undergo 3 waves of blood development and produce haematopoietic stem cells which generate a full gamut of differentiated blood cell types. KLFs have been described in zebrafish. One of these, zKLF4, is an early marker of lateral plate mesoderm (LPM), the site of origin of HSCs. Our aim was to test the function of zKLF4 by morpholino (MO) gene knockdown in zebrafish.

Methods: Gene knockdown was performed by microinjection of two zKLF4 MOs or a control. Phenotypes were analysed by WISH and real time RT-PCR for blood markers.

Results: There is a 2-3 cell wide continuous band of zKLF4 expression within the LPM extending around the entire embryo. Expression precedes that of stem cell markers, scl and flk1. Expression in the posterior LPM persists as it migrates medially in a zipper-like movement to generate the intermediate cell mass, the site of embryonic haematopoiesis. MO knockdown of zKLF4 results in moderate anaemia which resolves as definitive erythropoiesis replaces primitive erythropoiesis at 48-72 hpf. zKLF4 morphants show down-regulation of GATA-1 (>100 fold) and embryonic globin (>10 fold) by real time RT-PCR and WISH. On the other hand there is a dramatic up-regulation (>100 fold) in the level of myb expression in zKLF4 morphant compared to control embryos at 72 hpf, which is consistent with expansion of the secondary definitive wave of erythropoiesis, possibly in response to anaemia.

Conclusions: zKLF4 is essential for embryonic haematopoiesis. Mammalian orthologs such as KLF4 or KLF2 may play roles in primitive erythropoiesis and embryonic globin gene expression in mammals.
**HSANZ Free Communications 12: Haemopoiesis / Stem Cells**

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**IFI16: A Novel TPO Induced Gene Identified by Microarray**

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**Aim:** Thrombopoietin (TPO) is the primary cytokine responsible for both the proliferation and differentiation of megakaryocytic progenitors, and the eventual production of mature megakaryocytes (Mks) and platelets. Using microarray analysis, this study aimed to comprehensively identify genes involved in the induction of megakaryopoiesis by TPO.

**Method:** The human megakaryoblastic cell-line UT7-EPO/Mpl acquires a Mk phenotype in response to TPO treatment. Using human 19K oligonucleotide microarrays, differential gene expression from TPO treated cells was compared to non-treated cells.

**Results:** Approximately 50 genes were identified as being up-regulated in response to TPO treatment. We identified IFI16, an interferon responsive gene, as being up-regulated by TPO at the RNA level by microarray, and confirmed this result using real-time PCR. We have also shown that IFI16 protein is expressed in all Mk cell-lines examined. Promoter analysis has revealed that the Jak/STAT signalling pathway is involved in regulating the induction of IFI16 by TPO. Using EMSA analysis we have shown that TPO treatment induces the binding of STAT-1 and STAT-3 to the IFI16 promoter, and that mutation of the relevant STAT site reduces the TPO inducibility of the IFI16 promoter. To determine the functional importance of this gene we are currently using siRNA directed towards IFI16, and preliminary data suggests that IFI16 may play a role in Mk proliferation.

**Conclusion:** IFI16 has not previously been reported to be a target of TPO signalling, nor present in the megakaryocytic lineage. Elucidation of the functional significance of IFI16 in megakaryopoiesis may provide important insight into the molecular mechanisms driving normal Mk development.

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**Models of Thrombocytosis Dependent on Genetic Changes in the Thrombopoietin Pathway**

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**Aim:** The recent discovery of the Jak2V617F mutation has significantly increased the understanding of the molecular pathology underlying the myeloproliferative disorders. However, approximately half of the patients diagnosed with ET do not appear to have mutations in Jak2 indicating other genes may be involved in the thrombocytosis exhibited by these patients.

**Method and Results:** As part of our ongoing program to identify other candidate genes, we have studied a mutant mouse strain, called plt2, with autosomal recessive thrombocytosis unrelated to mutations in Jak2, thrombopoietin or the thrombopoietin receptor, c-Mpl. plt2/plt2 mice have platelet counts of 2038±347x10⁶/L (n=107) increased by 47% compared to wild-type mice (1386±223, n=107; p<0.001). This thrombocytosis was associated with increased thrombopoietin (TPO) production and hyperplasia of megakaryocytes and megakaryocyte progenitor cells. Serum thrombopoietin levels were elevated by 76% in plt2/plt2 mice (4930±1309pg/mL, n=18 compared to 2802±1031, n=15; p=0.001) and the TPO content of whole liver lysates was increased by 50% (p=0.03). To examine how the plt2 mutation interacts with other components of the TPO pathway, plt2/plt2 mice were intercrossed with mice lacking the TPO receptor (Mpl−/−). Mpl−/−plt2/plt2 mice have thrombocytopenia with platelet counts that are indistinguishable from Mpl−/− mice confirming that the plt2 mutation causes thrombocytopenia by acting through the Mpl receptor. Surprisingly, Mpl−/−plt2/plt2 mice, with only one copy of the Mpl gene, had marked thrombocytosis with platelet counts of 2656±459x10⁶/L (n=25) increased by 92% over wild-type mice.

**Conclusion:** These Mpl−/−plt2/plt2 compound mutant mice demonstrate an additive effect of increased TPO production and decreased Mpl gene dosage on platelet count. These finding are potentially important in the understanding of thrombocytosis in patients with ET, PRV and myelofibrosis as both TPO and Mpl are upstream of Jak2 in the hemopoietic cytokine signalling pathway and Mpl protein has previously been demonstrated to be decreased in many patients with myeloproliferation.
Specific Activation of Human but not Murine β-globin Gene Expression by the Transcription Factor Ikaros

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2. John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia

Aim: The zinc finger transcription factor Ikaros is a key regulator of lymphocyte differentiation and a tumour suppressor. Recently generated dominant negative mutants have hinted at a broader role in haematopoietic stem cell generation. Most recently, a mouse strain, IkarosPlastic, with a point mutation in Ikaros that disrupts DNA binding but preserves efficient assembly of Ikaros protein complexes, is embryonically lethal due to severe defects in erythrocyte differentiation (Papathanasiou P, et al., Immunity, 2003). These mice display normal murine globin gene expression in the fetal liver. However the human globin locus is under alternative regulatory control compared with the mouse locus, particularly with respect to the fetal-to-adult globin switch.

Methods: To determine if Ikaros plays a role in human globin switching, we crossed IkarosPlastic mice with mice transgenic for a YAC with sequences of the full-length human β-globin locus, which show a developmentally normal human fetal to adult globin switch. Embryos were harvested from E12.5 to E15.5 and globin gene expression was determined by real-time PCR (relative to actin).

Results: At all time points human gamma-globin gene expression was not significantly altered by the presence of the IkarosPlastic mutation (relative expression Ikaroswt/wt 1±0.11, IkarosPlastic/Plastic 0.82±0.12). In contrast, over the course of development, human β-globin gene expression was significantly down-regulated in IkarosPlastic fetal livers (relative expression Ikaroswt/wt 1±0.14, IkarosPlastic/Plastic 0.18±0.07). Interestingly, neither murine α- nor β-globin gene expression was significantly different in IkarosPlastic/Plastic compared to Ikaroswt/wt mice.

Conclusion: The transcription factor Ikaros plays a specific role in activation of the human β-globin gene during development. The mechanism by which this occurs remains to be elucidated. One possibility is Ikaros acts as a potentiator of erythroid specific transcription factors that are known to play a role in β-globin gene expression, such as EKLF. Experiments to address this will be presented.

Erythroid Kruppel-Like Factor Regulates the Cell Cycle in Erythroid Cells

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Aim: Kruppel-like factors are C2H2 zinc finger transcription factors involved in terminal differentiation of many cell types. Indirect evidence also suggests they are tumour suppressors. Erythroid Kruppel-Like Factor (EKLF) is expressed specifically in erythrocytes. It activates β-globin gene expression via binding to CACC box motifs in the proximal promoter. Experiments demonstrating repression of proliferation upon EKLF induction suggested a role in cell cycle regulation. Our aim was to find and characterize key EKLF dependent cell cycle genes.

Methods: Expression profiling was performed in EKLF null versus wild type fetal livers and cell lines containing a tamoxifen inducible EKLF-ER<sup>TM</sup> fusion protein. The gene lists were examined for cell cycle genes. EKLF-dependent regulation was validated by real time RT-PCR, and direct regulation studied by chromatin immuno-precipitation (ChIP) and promoter-reporter assays. DNAse1 hypersensitivity and gel shift assays were also performed.

Results: The cyclin dependent kinase inhibitor, p18, and the S phase transcription factor, E2F4, were both down regulated in EKLF null fetal liver. Interestingly, The E2F4 knockout has a similar lethal erythroid phenotype to the EKLF knockout. Scanning of the p18 and E2F4 genes found phylogenetically conserved CACC boxes in key regulatory regions. There are two CACC sites in the p18 promoter which drive EKLF dependent transcription in vitro and are also bound by EKLF in vivo, as determined by ChIP. The E2F4 gene contains a conserved CACC site in intron 5 that is closely associated with two conserved GATA1 binding sites. This region is also occupied by EKLF in vivo, and thus is a likely to be an EKLF-dependent enhancer.

Conclusions: EKLF regulates p18 and E2F4 gene expression by different mechanisms. This is likely to be responsible for its role in cell cycle arrest. These results suggest a mechanism by which KLFs act as tumour suppressors in many common human cancers.
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Pluripotent Characteristics of Human Bone Marrow Derived Mesenchymal Stem Cells in Long Term Culture

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Aim: Recently pluripotent capabilities have been assigned to adult stem cells, particularly bone marrow-derived mesenchymal stem cells (MSCs), which were previously thought to be lineage-restricted. However, it is not known whether long-term in vitro culture will affect the pluripotent nature of MSCs. To address this, we examined characteristics of stemness and lineage differentiation at early and late culture stages by immunophenotyping and quantitative gene expression analysis.

Method: Whole bone marrow was obtained from 4 normal donors and MSCs were isolated using antibody-mediated negative selection and Ficoll density gradient centrifugation, followed by plastic adherence. MSCs at passage 2, and every subsequent third passage until passage 11, were analysed by examination of cell morphology, growth kinetics, cell viability, and the expression of markers of pluripotency and the three germ lineages (ectoderm, endoderm, mesoderm) by real-time RT-PCR and/or immunofluorescent staining.

Result: MSCs exhibited only minor morphological changes in the first 8 weeks (6 passages) of culture. During this time, MSCs showed the greatest and most consistent proliferative potential and cell viability. Beyond this time, MSCs gained larger cell morphology and displayed decreased proliferation rates. MSCs expressed markers of pluripotency (Oct4, Nanog), as well as lineage markers from the ectoderm (Nestin, NG2, MAP2) and mesoderm (Collagen I), at both mRNA and protein levels, while, endodermal markers (FoxA2, Albumin) were mostly undetectable. A trend of decreasing expression was observed for Nestin and Alkaline Phosphatase; while Collagen I expression was stable during the first 8 weeks, and subsequently showed an increasing trend; CD44 expression was stable or showed a generally increasing trend during the culture period.

Conclusion: Bone marrow-derived MSCs maintain a stable phenotype in vitro during the first 8 weeks of culture, and expressed ectodermal and mesodermal markers suggesting the potential for differentiation into these lineages.

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CD 138 Immunostaining of Bone Marrow Trephine Specimens is the Most Sensitive Method for Quantifying Marrow Involvement in Patients with Plasma Cell Dyscrasias

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Aim: In this study plasma cell quantitation comparing four modalities comprising morphology of the aspirate, flow cytometry, H&E trephine section examination and bone marrow immunohistology was undertaken to determine the most sensitive technique.

Methods: 70 patients with a plasma-cell dyscrasia were retrospectively analysed. Plasma-cell quantitation was performed on Romanowsky-stained aspirate slides by a 200-cell differential. Flow cytometric quantitation of plasma-cell burden was performed by identifying CD38/138 co-expressing cells. Trephine specimens were analysed after H&E and CD138 staining.

Statistical analysis was performed using a two-tailed paired t-test.

Results:
Table 1. Two tailed Paired t-test analysis of Quantitation of Plasma Cell burden by various modalities

<table>
<thead>
<tr>
<th></th>
<th>Flow Cytometry</th>
<th>Aspirate</th>
<th>H&amp;E</th>
<th>Immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean % of plasma cells</td>
<td>5.6</td>
<td>18.9</td>
<td>23.6</td>
<td>29.7</td>
</tr>
<tr>
<td>Paired t-test (two tailed)</td>
<td>-24.12</td>
<td>-11</td>
<td>-6.14</td>
<td>-</td>
</tr>
<tr>
<td>Mean Difference compared to CD138 immunostaining</td>
<td>-30.5 to -17.8</td>
<td>-15.8 to -6.1</td>
<td>-8.34 to -3.9</td>
<td>-</td>
</tr>
<tr>
<td>95% C.I.</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>-</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Discussion: Significant discrepancy was noted between the four methods for determining plasma-cell infiltration. CD138 immunohistochemical staining of paraffin embedded trephine specimens was the most sensitive modality. Flow-cytometric analysis underestimated the degree of marrow infiltration. Morphology of the aspirate
sample may also underestimate plasma-cell burden as sampling may be affected by patchy marrow infiltration as well hypoplastic, fibrotic marrows or clotted specimens.
Quantification of plasma cells based on H&E stained trephine specimens is less sensitive, and is dependent on the technical quality of the specimen and observer experience.
Immunostaining has the advantages of improved plasma-cell identification compared to H&E sections and avoids the problems inherent in the analysis of aspirate samples.
In conclusion, CD138 immunostaining is the most sensitive method for quantifying plasma-cell burden in the bone marrow.

HSANZ Free Communications 13: Myeloma / Amyloidosis

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Peripheral Blood Stem Cell Transplantation (PBSCT) for Multiple Myeloma: Factors that Influence Engraftment

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¹BMT Program, Alfred Hospital, ²Box Hill Hospital, ³Monash University
Melbourne, Victoria, Australia

Aim: We have analyzed factors that influence engraftment in homogenous group of multiple myeloma patients who had <12 months of prior therapy and melphalan 200mg/m² as a conditioning chemotherapy.

Methods: Between 1992 and 31 Dec 2004, patients of multiple myeloma who underwent PBSCT at our and associated institutes were evaluated for inclusion criteria. Engraftment was defined as days to neutrophils > 0.5x 10⁹/L or > 1.0x 10⁹/L on two consecutive days and platelet ≥ 20 x 10⁹/L or platelet ≥ 50 x 10⁹/L for two consecutive days without platelet transfusion.

Univariate analysis was done by Pearson correlation coefficient and multivariate analysis by Cox regression analysis

Results:
Ninety-two patients with multiple myeloma were included for this analysis and their median age was 57(30 to 70) years. Twelve patients had PCAB & 6 had melphalan during prior treatment. Median CD34 dose infused was 4.04 x 10⁹/kg (2.01 to 14.22) and forty-two patients received GCSF on D+1 following PBSCT.

On Multivariate Analysis of Factors

<table>
<thead>
<tr>
<th>Variables</th>
<th>Days to Neu. &gt;0.5</th>
<th>Days to Neu.&gt;1.0</th>
<th>Days to PLT &gt;20</th>
<th>Days to PLT&gt;50</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCAB</td>
<td>0.0004</td>
<td>&lt;0.0001</td>
<td>0.0156</td>
<td>0.0017</td>
</tr>
<tr>
<td>PLT on 1st day of leukapheresis</td>
<td>0.0006</td>
<td>0.0003</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Post transplant G-CSF</td>
<td>0.0206</td>
<td>ns</td>
<td>0.0014</td>
<td>0.0046</td>
</tr>
</tbody>
</table>

Stem cell dose was not significantly associated with either neutrophil or platelet engraftment.

Conclusions:
- Patients who had less than 12 months of prior therapy, CD34 > 2 X10⁶/kg is adequate and there is no difference in engraftment kinetics with higher doses.
- Prior PCAB treatment is toxic to stem cells and it affects not only quantity of CD34 yield but also delays engraftment even when adjusted for stem cell dose.
- Higher platelet count on 1st day of stem cell collection is associated with more rapid engraftment, which may indicate better the quality of stem cells.
- Post transplant G-CSF on D+1 is associated with rapid neutrophil engraftment but delays platelet recovery.

HSANZ Free Communications 13: Myeloma / Amyloidosis

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Tracking Dendritic Cell In-vivo in Patients with Multiple Myeloma

Dominic Wall¹, Mick Thompson¹, Linda Mileskin¹, Bruce Loveland², Judi Coverdale¹, P Xing², Rachel Taylor², Rod Hicks¹, H Miles Prince¹
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HSANZ Orals ASM 2005
Aim: This study aimed to determine the in-vivo distribution of dendritic cells (DC) in patients (pts) with stable Multiple Myeloma (MM) with MUC-1 expression, via intravenous-IV, intranodal-IN, intradermal-ID and subcutaneous-SC injections using ex-vivo In111Oxine labelled DC produced with a VacCell® processor from autologous monocytes.

Method: Cells were cultured for 7 days with GM-CSF and IL-13 on days 0 and 4 and were pulsed with MUC1-Mannan protein (24h) prior to use. DC were non-matured (imDC, n=3), or matured (mDC, n=3) with FMKp and γIFN (6h) prior to labelling. Cells were labelled with In111 (20 M bq IV or 0.7 M bq each of IN, ID and SC) and Pts received DC by IN, SC, ID simultaneously and two weeks later IV. Pts were serially imaged for 4 days using a planar gamma camera.

Results: Mean DC labelling efficiency was 86% (54-95, n =12). Mean IV injection was 1.5 x 10^8 cells (0.33-2.5 x 10^8, n = 6), and IN, SC, ID was 2.0 x 10^7 cells (1.3-3.6 x 10^7, n = 6).

IV: At 1 & 4h cells were predominantly in the lungs. After 24h -72h lungs were clearing with DC migrating to liver, spleen and bone marrow.

ID and SC: % injected cells detected at proximal lymph node (LN)

<table>
<thead>
<tr>
<th>4hr</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>mDC SC</td>
<td>0.06%</td>
<td>0.22%</td>
<td>0.74%</td>
</tr>
<tr>
<td>mDC SC</td>
<td>0.10%</td>
<td>0.40%</td>
<td>0.67%</td>
</tr>
<tr>
<td>imDC ID</td>
<td>0.01%</td>
<td>0.14%</td>
<td>0.30%</td>
</tr>
<tr>
<td>mDC ID</td>
<td>0.02%</td>
<td>0.26%</td>
<td>0.43%</td>
</tr>
<tr>
<td>mDC ID</td>
<td>0.36%</td>
<td>0.80%</td>
<td>0.83%</td>
</tr>
</tbody>
</table>

IN injections were retained within the node, but in 1/3 imDC and 2/3 mDC there was early and substantial drainage into an afferent LN (<4 hours, >3% of cells).

Conclusion: ID and SC images suggest mDC track more consistently to LN, whilst IV mDC cleared more slowly from lungs at 24h compared to imDC.

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**HSANZ Free Communications 13: Myeloma / Amyloidosis**

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**Successful Second Mobilization of Peripheral Blood Stem Cells using Recombinant Human Stem Cell Factor (SCF) and Granulocyte Colony Stimulating Factor (G-CSF) in Patients with Multiple Myeloma who Failed Prior Mobilization**

*Devendra Hiwase¹, Anthony P Schwarer¹, Joe Mckendrick², Geraldine Bollard¹, Smita Hiwase¹ and Michael Bailey³*

¹Bone Marrow Transplant Program Alfred Hospital, ²Box Hill Hospital, ³Monash University, Melbourne, Victoria, Australia

Aim: Autologous stem cell transplant is a standard of treatment for multiple myeloma patients younger than 65 years. However 5 to 20% patients fail to mobilize enough stem cells for transplant. We have conducted this analysis to assess efficac SCF in combination with G-CSF patients who failed first mobilization.

Methods: Between Jan 1998 and Aug 2004, multiple myeloma patients who had failed to collect more than ≥ 2 x10^6/kg CD34 cells during 1st mobilization were remobilized using combination of SCF (20 ug/kg) and G-CSF (5 to10 ug/kg) with or without chemotherapy. Data were collected for 1st mobilization and second mobilization.

Result: There were 12 (M/F, 7/5) patients who had 2nd mobilization, median age 57.5 (30 to 70) years. Eight patients had prior melphalan and 3 had PCAB treatment (Prednisone, Cyclophosphamide, Doxorubicin, BCNU). Eight patients had cyclophosphamide during 1st mobilization whereas only 3 had cyclophosphamide during 2nd mobilization and G-CSF doses were similar during 1st & 2nd mobilization.

**Stem cell collection data shown in the table**

<table>
<thead>
<tr>
<th>Stem cell collection parameters</th>
<th>1st Mobilization</th>
<th>2nd Mobilization</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CD34/kg median (range)</td>
<td>0.615 (0.230 to 1.39)</td>
<td>1.95 (0.610 to 3.68)</td>
<td>0.0049</td>
</tr>
<tr>
<td>Total CD34/kg/L median (range)</td>
<td>0.09 (0.03 to 0.2)</td>
<td>0.26 (0.087 TO 0.53)</td>
<td>0.0049</td>
</tr>
<tr>
<td>No of patients &gt;2 x 10^6/kg</td>
<td>0</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>Total of 1st &amp; 2nd Mobilization</td>
<td>0.615 (0.230 to 1.39)</td>
<td>2.65 (0.840 to 4.99)</td>
<td>-</td>
</tr>
</tbody>
</table>

Conclusion: This study shows SCF Plus G-CSF is effective in more than >50% of patients who had failed a prior mobilization. 9/12 patients underwent successful peripheral blood stem cell transplant SCF can be used in combination with GCSF +/- chemotherapy for patients who failed 1st mobilization.
Utility of the Serum Free Light Chain Assay in Patients with AL Amyloidosis

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² QHPS, Brisbane, Qld, Australia
³ Gold Coast Hospital, Gold Coast, Qld, Australia

Aim: To assess the utility of the serum free light chain assay (FLC) in diagnosis and monitoring of patients with AL amyloidosis.

Methods: FLC concentration was measured in serial serum samples collected from patients and normal controls using a kit assay (The Binding Site Ltd., Birmingham, UK). Monoclonal paraprotein was detected by serum and/or urine protein electrophoresis and immunofixation. Paraprotein, FLC and organ responses were assessed according to standard criteria. Patients who died before assessment of organ response at 3 months were deemed to have no response.

Results: 27 patients with FLC measured at diagnosis were identified. 9(33%) had a quantifiable paraprotein, 8(30%) had a trace (immunofixation only) of monoclonal serum light chains, 6(22%) had BJP only in the urine, and 4(15%) had no detectable serum or urine paraprotein. 26(96%) had an abnormal FLC ratio with raised absolute monoclonal light chain measurement. In contrast 23 normal controls and 12 patients with renal impairment all had normal FLC ratios with higher absolute FLC levels in the renal failure patients.

Of 22 treated patients with follow-up data (autotransplant 9, melphalan/dexamethasone 12, dexamethasone alone 1), 18 were assessable for FLC response (4 early deaths). FLC response rates were: CR 44%, PR 39%, <PR 17%. Organ response occurred in 8 (38%) and was 71%, 43% and 0% for patients achieving CR, PR and <PR according to FLC response criteria, respectively(p=0.2). 2yrOS was 63%(range,39-80%). FLC response was predictive of survival (p=0.04) with 2yrOS being 100%, 69% and 0% for patients achieving CR, PR and <PR, respectively. For this same group of 18 patients, the paraprotein response was predictive of organ response (p=0.05) but not survival (p=0.4).

Conclusions: Local experience with the FLC assay reproduces similar results to those published by overseas centres. Measurement of FLC is an important component of managing patients with AL amyloidosis.

Primary eosinophilia is operationally classified into two categories; clonal and idiopathic. Clonal eosinophilia occurs in the context of a spectrum of otherwise classified hematologic malignancies including acute lymphocytic leukemia, acute myeloid leukemia, myelodysplastic syndrome, chronic myeloid leukemia, and both classic and atypical myeloproliferative disorders (MPD). Atypical MPD that are sometimes associated with clonal eosinophilia include systemic mastocytosis (SM), chronic myelomonocytic leukemia (CMML), and unclassified MPD (UMPD). Chronic eosinophilic leukemia (CEL) is a sub-category of UMPD where the eosinophilic process is substantially more conspicuous than the myeloproliferative features of the disease. As such, it is usually the presence of a clonal cytogenetic/molecular marker that distinguishes CEL from idiopathic eosinophilia. A subset of the cases with clonal eosinophilia are now molecularly characterized; FIP1L1-PDGFRα SM, PDGFRB-rearranged UMPD or CEL, and the 8p11 syndrome that is associated with FGFR1 rearrangement. Hypereosinophilic syndrome (HES) is a sub-category of idiopathic eosinophilia and is characterized by an absolute eosinophil count of ≥1.5 x 10⁹/L for at least 6 months as well as eosinophil-mediated tissue damage. The presence of either PDGFRA or PDGFRB mutations warrants the therapeutic use of imatinib mesylate in clonal eosinophilia. In HES, prednisone, hydroxyurea, and interferon-α constitute first line therapy whereas imatinib, cladribine, and monoclonal antibodies to either interleukin-5 (mepolizumab) or CD52 (alemtuzumab) are considered investigational. Currently ongoing studies are looking into the potential value of the second generation kinase inhibitors including AMN107 and dasatinib in the treatment of HES and clonal eosinophilia.
Optimising Fludarabine-Containing Regimens in Follicular Lymphoma

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Early trials using single-agent fludarabine produced response rates of 30-50% in previously treated follicular lymphoma (FL) patients. More recent phase II trials focusing on the use in untreated patients of fludarabine-containing combination regimens, including agents like mitoxantrone, cyclophosphamide or idarubicin, have reproducibly yielded overall response rates of 70–100%, with complete response rates of 60-90%. In addition, encouraging data have been reported on the ability of front-line treatment with fludarabine plus mitoxantrone (FM) to induce molecular response: in pre-treated patients with bcl-2 gene rearrangement the FM regimen led to PCR negativity in over 50% cases. Another promising therapeutic option is the chimeric mouse/human anti-CD20 antibody, rituximab. Single-agent treatment with rituximab is capable of inducing CR and molecular responses (a detectable gene rearrangement converted to PCR-negativity in the peripheral blood or bone marrow following rituximab) in pretreated and previously untreated FL. Moreover, rituximab appears to exert synergistic activity with cytotoxic chemotherapy, leading to improved CR and molecular response rates. We performed a multicenter randomized trial to compare the front-line efficacy—in terms of both CR and molecular response—and toxicity of the FM and CHOP chemotherapy regimens with/without sequential immunotherapy with rituximab (elected on the basis of clinical/molecular restaging). The encouraging results of this trial indicate that FM is superior to CHOP for front-line treatment of FL, and that rituximab is an effective sequential treatment option.