AML engraftment in the NOD/SCID assay reflects the outcome of AML: implications for our understanding of the heterogeneity of AML.

Short Title: AML NOD/SCID engraftment and prognosis risk group

Daniel J. Pearce1*, David Taussig1/2*, Kazem Zibara1/3, Lan-Lan Smith1/3, Christopher M. Ridler1, Claude Preudhomme4, Bryan D. Young3, Ama Z Rohatiner2, T Andrew Lister2 and Dominique Bonnet1

*DP and DT contributed equally to this work

This work was supported by Cancer Research UK and a National Institute of Health Grant No. HL-64856-03 to D. Bonnet.

1Hematopoietic Stem Cell Laboratory
London Research Institute
Cancer Research UK
London
WC2A 3PX

2Cancer Research UK Medical Oncology Unit,
St. Bartholomew’s Hospital,
West Smithfield,
London,
EC1A 7BE.

3Medical Oncology Laboratory,
Cancer Research UK,
Queen Mary & St Bartholomew’s Medical School,
Charterhouse Square,
London
EC1M 6BQ

4Laboratoire d'hématologie A,
Hôpital Calmette
Bd du professeur Leclercq
59037
Lille,
France

Corresponding Author: Dr Dominique Bonnet
Hematopoietic Stem Cell Laboratory
Cancer Research UK
London Research Institute
44 Lincoln’s Inn Fields
London
WC2A 3PX
Tel: 020 72693281
Fax: 020 72693581
d.bonnet@cancer.org.uk

Heading: Hematopoiesis

Text word count: 4560 excluding references
Abstract word count: 182
Abstract
The non-obese diabetic/severe combined immunodeficient (NOD/SCID) assay is the current model for assessment of human normal and leukemic stem cells. We explored why 51% of 59 acute myeloid leukemia (AML) patients were unable to initiate leukemia in NOD/SCID mice. Increasing the cell dose, using more permissive recipients and alternative tissue sources, did not cause AML engraftment in most previously non-engrafting AML samples. Homing of AML cells to the marrow was the same between engrafters and non-engrafters. FLT-3 ITD and nucleophosmin mutations occurred at a similar frequency in engrafters and non-engrafters. The only variable that was related to engraftment ability was the karyotypically-defined risk stratification of individual AML cases. Interestingly, follow-up of younger patients with intermediate-risk AML revealed a significant difference in overall survival between NOD/SCID-engrafting and non-engrafting AMLs. Hence, the ability of AML to engraft in the NOD/SCID assay seems to be an inherent property of AML cells, independent of homing, conditioning or cell frequency/source, which is directly related to prognosis. Our results suggest an important difference between leukemic initiating cells between engrafting and non-engrafting AML cases that correlates with treatment response.
Introduction

The non-obese diabetic/severe combined immunodeficient (NOD/SCID) xenotransplantation assay is currently the model of choice for assessment of transplantable human hematopoietic stem cells (HSC). This approach has been crucial to our understanding of human hematopoiesis; providing reliable determination of the phenotypes of repopulating cells, and elucidating previously undescribed HSC populations. More recently, a novel mouse strain has been developed by backcrossing β2 microglobulin-null (B2m-/-) mice onto the NOD/SCID background. The resulting B2-/-NOD/SCID strain, in addition to the B-cell, T-cell, complement and partial NK defects that define the NOD/SCID model, has a complete lack of NK cell activity. Hence, this model is reportedly even more permissive to xenotransplantation than the original NOD/SCID strain.

Acute myeloid leukemia (AML) is characterized by a relentless accumulation of immature, abnormal hematopoietic cells in the bone marrow and peripheral blood. It has been postulated that AML is a disease maintained by leukemic stem cells and may be organized in a similar way to normal hematopoiesis. Indeed, only a subset of AML cells are capable of forming colonies in vitro and a smaller fraction can maintain colony production for six weeks whilst on feeder layers. Definitive proof that a small population of leukemic stem cells produce the AML blasts, comes from six-week primary and secondary engraftment experiments in NOD/SCID mice. Further studies have revealed that these SCID-Leukemia initiating cells (SL-IC) share many properties with normal HSC, namely phenotype, quiescence, and in vitro CXCR-4 mediated migration.

AML is an extremely heterogeneous disease and since there are so many different known genetic abnormalities (and probably many more unknown), AML may be thought of as a collection of different diseases that have the same myeloid morphology. Indeed, for patients less than 60 years of age the single most important prognostic factor is the karyotype. AML cases are currently divided via karyotype into the treatment groups of poor, intermediate and favorable prognosis. The majority of patients have an intermediate risk karyotype and the outcome of these patients is variable as well as difficult to predict using prospective tests.

Previous studies have reported that approximately 70% of AML cases will engraft in the NOD/SCID assay. Although many groups have utilized the
NOD/SCID assay, the majority have only assessed the AML cases capable of engraftment.\textsuperscript{12-15} Few studies have addressed the variables that affect engraftment itself.\textsuperscript{16} Various factors affecting normal hematopoietic cell engraftment have been identified and may be applicable to AML NOD/SCID engraftment.

A complex series of interactions of adhesion molecules, cytokines, chemokines and their receptors is responsible for the homing of transplanted human hematopoietic cells from the peripheral injection site to the bone marrow.\textsuperscript{17} A major role in hematopoietic cell homing is attributed to the interaction between the chemokine SDF-1 and its receptor CXCR-4.\textsuperscript{18} Overexpression of CXCR-4 on human CD34\textsuperscript+ cells results in an increased ability to home to and engraft NOD/SCID marrows.\textsuperscript{19} Furthermore, antibody blocking studies have revealed that engraftment of human hematopoietic cells in NOD/SCID mice is dependent on the interaction between CXCR-4 and SDF-1.\textsuperscript{20} In AML, although both \textit{in vitro} transendothelial migration and the level of \textit{in vivo} NOD/SCID bone marrow homing are dependent on CXCR-4, it is not clear whether the actual ability to engraft NOD/SCID mice is dependent on the CXCR-4/SDF-1 axis.\textsuperscript{8,21}

Here, we examined 59 AML patients for their ability to initiate leukemia in NOD/SCID mice. We established via morphology, phenotype, genotype and RNA expression that when AML engrafted, the AML produced was very similar to the patients’ disease. We then investigated variables known to affect normal cell engraftment for their ability to cause AML engraftment. Increasing the cell dose, more intensive conditioning, more permissive recipients and alternative tissue sources (bone marrow), did not cause AML engraftment in previously non-engrafting AML samples. Both the CXCR-4 expression and \textit{in vivo} homing of AML cells were the same between engrafters and non-engrafters. FLT-3 ITD and nucleophosmin mutations occurred at a similar frequency in engrafters and non-engrafters. The only variable, which did seem to be related to engraftment ability, was the karyotype of individual AML cases. Interestingly, follow-up of younger (< 60 years) intermediate-risk AML cases revealed a statistically significant difference in overall survival between NOD/SCID-engrafting and non-engrafting cases of AML.

Hence, the NOD/SCID assay appears to reproduce an AML very similar to the patients’ disease and the ability to engraft seems to be an inherent property of AML cells, that is independent of homing, conditioning or cell dose/source, but is directly related to prognosis.
METHODS

Primary cells. Cells were obtained from newly diagnosed and relapsed patients with AML at St Bartholomew’s Hospital after informed consent. The protocol was approved by the hospital research Ethics Committees. MNCs were obtained by Ficoll-Paque density centrifugation and Ammonium Chloride red cell lysis.

Mice. All animal experiments were performed in compliance with Home Office and institutional guidelines. NOD/SCID mice and B2-/-NOD/SCID mice were originally obtained from Dr Leonard Schultz (Jackson Laboratory, Bar Harbour, ME, USA) and bred at Charles Rivers, Laboratories, UK. They were kept in micro-isolators and fed sterile food and acidified water. Mice aged 8-12 weeks were irradiated at 375 rads ($^{137}$Caesium source) up to 24 hours before intravenous injection of cells.

CXCR-4 expression analysis

Cells were stained with either phycoerythrin (PE)-conjugated or allophycocyanin (APC)-conjugated anti-CXCR-4 antibodies with phycoerythrin-cyanin 5 (PE-Cy5)-conjugated anti-CD34 antibodies for 30 minutes at 4°C (all antibodies from Becton Dickinson (BD) Biosciences (Oxford, UK)). Cells were washed and resuspended in phosphate buffered saline (PBS) with 2% FCS and 4,6-diamidino-2-phenylindoiole (DAPI). Cells were analyzed on a BD LSR flow cytometer. Gates were set up to exclude nonviable cells and debris. The negative fraction was determined using appropriate isotype controls.

Calcium Flux Measurement

Cells were labeled with 2.5μM Indo-1 (Invitrogen, CA) at 37°C for 45mins. Cells were then analyzed on a BD LSR-II for 30 seconds to give background levels before stimulation with 100ng of stromal-cell derived factor – 1 and analysis for a further 4 minutes. Analysis involved detection of fluorescence due to dye bound to Calcium (424/44nm filter used) and fluorescence due to unbound dye (530/30nm filter).

Analysis of murine bone marrow. Six weeks after transplantation, mice were sacrificed by cervical dislocation. The femurs, tibias and pelvis were dissected and flushed with PBS. Red blood cells were lysed via ammonium chloride. Cells were
stained with human specific FITC-conjugated anti-CD19, PE-conjugated anti-CD33 and PE-Cy5-conjugated anti-CD45 antibodies. Dead cells and debris were excluded via DAPI staining. A BD LSR flow cytometer was used for analysis. More than 100,000 DAPI negative events were collected. Engraftment of AML was said to be present if a single population of CD45⁺CD33⁻CD19⁻ cells was present without accompanying CD45⁺CD33⁺CD19⁺ cells.

**Assessment of engraftment potential of AML.** Samples were screened to assess whether they had the potential to engraft NOD/SCID and B2-/−NOD/SCID mice. 10⁷ MNCs were injected into each mouse. Engraftment of AML was confirmed, where possible, with morphology and fluorescent *in situ* hybridization on human cells FACSorted from engrafted murine marrows.

**Fluorescence activated cell sorting (FACS).** Murine marrow cells were suspended in PBS with 2% FCS at 3 x 10⁷ per ml and stained with human specific, anti-CD45-PE and murine specific anti-CD45-FITC. Cells were washed and resuspended in PBS with 2% FCS and DAPI before sorting on a MoFlo cell sorter (DakoCytomation Colorado Inc, Fort Collins, Colorado). Gates were set up to exclude nonviable cells (DAPI negative) and debris.

**Fluorescent in situ hybridization**

Briefly, FACSorted human cells were swollen in hypotonic (0.075M) KCL solution, fixed in Karnoy’s fixative (3:1 methanol:acetic acid), before dropping onto clean, glass slides. Nuclei were “aged” overnight, before pepsin digestion, dehydration and application of fluorescent probes. Nuclei were incubated with probes overnight at 37°C before analysis at 1000x magnification on a Carl-Zeiss Axioplan-2 microscope equipped with Axiovision software.

**Mutation detection**

Deoxyribonucleic acid was extracted using standard phenol-chloroform methodologies. Primers and precise amplification conditions are available upon request and were derived from previously published studies (*FLT3* exons 14-15^{22}; *FLT3* exon 20^{23}). Polymerase chain reaction products were sequenced directly by use
of ABI 377 and ABI Prism 3730 DNA sequencers (PE Applied Biosystems, Foster City, CA, USA). Before direct sequencing unincorporated primer was removed by ultra-filtration using a Centricon YM-100000 filter device (Millipore Corp. Bedford, MA, USA). Sequencing data were analyzed using DNASTAR (Inc, Madison, WI, USA). Detection of NPM mutations was performed on genomic DNA by PCR as previously described.24

**Treatment for younger (<60 years) intermediate risk patients.**

Patients were treated with one of two protocols. Patients were either treated on the Medical Research Council 15 trial or received the St Bartholomew’s Hospital standard of care protocol. This comprises three cycles of idarubicin 30 mg/m², cytarabine 1400 mg/m² and etoposide 500 mg/m² and one cycle of cytarabine 18g/m². Patients with an appropriate donor underwent allogeneic transplantation in first complete remission. No patents died form treatment related causes.

**Statistics**

Logistic regression was used to assess the significance of factors involved in engraftment. Event free survival (EFS) was defined as survival with no evidence of persistent or recurrent disease. Patients with primary refractory disease (defined as bone marrow blasts greater than 5% in the marrow) were ascribed an EFS of 0 months. The actuarial probabilities of overall survival and EFS were plotted using the methodology of Kaplan and Meier as previously described.25 No significant differences in the number of patients who underwent either treatment protocol could be found between NOD/SCID engrafting and non-engrafting AML cases (data not shown). The student’s paired t-test for significance of no difference was used for all other assessments.

**Affymetrix Array Analysis**

Four samples, which gave high positive engraftment result, were processed for further microarray analysis (patients 9, 17, 19 and 37 in Table 1).
Details of RNA extraction, small sample cRNA target preparation, hybridization and microarray statistical analysis can be found in the Supplementary Methods.
Results

Not all Cases of AML Engraft in NOD/SCID mice

Ten million nucleated cells from 59 different AML patients were injected into NOD/SCID mice (Table 1). Six weeks later, murine bone marrows were assessed via flow cytometry for the presence of human myeloid cells. As reported previously, we found that not all cases of AML can be reproduced in the NOD/SCID model\textsuperscript{11}. We detected human (CD45\textsuperscript{+}), myeloid (CD33\textsuperscript{+}) engraftment without any B-cell (CD19) engraftment in 49\% of cases examined (29/59). Via this simultaneous assessment of both the myeloid and lymphoid lineages, we distinguished normal engraftment from leukemic engraftment. Most previous reports have only assessed the proportion of human CD45\textsuperscript{+} cells in the murine marrow. This approach may have included normal engraftment and hence may have overestimated the proportion of AML cases that engraft in the NOD/SCID model.\textsuperscript{11} Indeed, a significant proportion (\leq 10\%) of our AML patients’ cells produced normal engraftment when 10\textsuperscript{7} cells were injected into NOD/SCID mice.
### Table 1: Summary of Patient's Details.

Mice were injected with $10^7$ peripheral blood nucleated cells from the peripheral blood of AML patients. Murine marrows were analyzed six weeks post-transplant for the presence of human hematopoietic cells. AML engraftment was defined as the presence of human CD33+/CD45+ myeloid cells without an accompanying CD19+/CD45+ B-cell population. Patients marked with * were in relapse and patients marked with ** were given supportive care only. Patients marked with *** produced normal engraftment in NOD/SCID mice. Patients marked with § underwent affymetrix analysis. Patients marked with Δ possessed a Flt3-ITD and patients marked with ⊥ had a mutated nucleophosmin gene.
Patients marked with ψ were tested for Flt-3 mutations and were found to be negative. Prognosis risk group was defined as poor, intermediate or good via karyotype according to Grimwade et al 1998. FK = failed karyotype at diagnosis. # = An abnormal stemline clone was detected in 2 out of 10 cells examined, containing a complex rearrangement between chromosomes 2, 10 and 11 resulting in insertion of 11q material in 10p12 with a breakpoint at 11q23. Presentation white blood cell (WBC) count is given as 10⁹ cells/L. Patients in whom the WBC was less than 2 x 10⁹/L also had their bone marrow cells tested for engraftment capacity, with identical results to the peripheral blood data. All AML cases were assessed for NOD/SCID engraftment potential before any chemotherapy.

**Engraftment in NOD/SCID mice reproduces AML**

To confirm the leukemic nature of this myeloid (CD33⁺/CD45⁺/CD19neg) NOD/SCID engraftment, we compared the morphological features identified during diagnosis to the morphology of NOD/SCID engrafted cells. In all cases analyzed, the morphology of NOD/SCID engrafted cells was very similar to the original sample. A representative M2 AML is shown in Figure 1. Similar to the diagnosis smear, a high proportion of the NOD/SCID engrafted cells were myeloblasts. Pathognomic AML Auer rods were also detectable in NOD/SCID mice, confirming the leukemic nature of these cells (arrowed in Figure 1B). Wherever possible, we also performed FISH to detect characteristic genetic abnormalities in NOD/SCID engrafted cells (examples in Figure 1C-F).

![Auer rod](image)

**Figure 1: Confirmation of AML cell growth in NOD/SCID mice.**
Ten million cells were injected into NOD/SCID mice and marrows were analyzed for human, myeloid cell content six weeks later. (A) Diagnostic peripheral blood smear from an AML-M2 patient-10. (B) Murine marrow that was injected with cells from the same AML-M2 patient as Figure 1A. Myeloblasts, featuring Auer rods (arrowed) are present, indicating AML. (C) Dual fusion, dual colour fluorescent in situ hybridisation of a relapsed t[8,21] AML-M2 sample. Cells positive for the re-arrangement exhibit 1 green, 1 red and 2 orange spots. (D, E and F) Examples of NOD/SCID engrafted, FACSorted CD33+/CD45+ cells, exhibiting AML-M2 t[8,21] re-arrangement.

**Figure 2:** Gene expression analysis of engrafted AML cells. Dendrogram is shown from the unsupervised hierarchical cluster analysis of the 8 chips for the 2,260 genes passing the variation filter. Independent of karyotype, AML patients were grouped between before and after engraftment. This means that the AML in the original patient is very much related to the AML that has grown in the mouse. The samples corresponding to before and after engraftment were always adjacent to each other, reflecting a very close relationship between them.

**Gene Expression profile is extremely similar between engrafted AML cells and the original AML sample.**
To ensure that we were reproducing AML correctly in the NOD/SCID model, we examined the expression profiles of 8 AMLs (4 samples before engraftment and 4 samples after) by use of the oligonucleotide U133A arrays containing approximately 22,283 unique genes. An unsupervised hierarchical cluster analysis, performed on 2,476 or 2,260 genes passing the variation filter, grouped samples into 4 groups. On the basis of similarity in the expression pattern, the groups corresponded to the same patient sample before and after engraftment (Figure 2). This indicates that the 2 sets of genes had expression patterns strongly associated with AML sample of origin.

The cluster dendrogram gave similar results with the 2 lists of genes, with or without the variation filter. A statistical group comparison approach was used to identify genes with statistically significant differences in expression levels between groups of samples before and after engraftment. By using a T-test analysis on normalized data, we could not identify any genes differentially expressed between before and after engraftment. Indeed, gene expression profiling on these samples showed highly consistent profiles. This was the same on normalized data directly or after logging (to log2) the normalized data.

Hence, expression profiling reveals no fundamental biological differences in acute myeloid leukemia samples before and after engraftment. Independent of karyotype, patients AMLs were grouped between before and after engraftment. The samples corresponding to before and after engraftment were always sitting adjacent to each others, reflecting a very close relationship between them. This means that the gene expression profile of AML in the original patient is very similar to the AML that has grown in the mouse.

**Increasing the cell dose and utilizing alternative cell sources does not increase the number of engrafting AML samples.**

To investigate factors that affect AML NOD/SCID engraftment, we repeated certain engraftment assessments, increasing the cell numbers and utilizing alternative tissue sources (bone marrow). Six-week NOD/SCID engraftment could not be achieved with up to $10^8$ cells from five, previously non-engrafting AML samples. Interestingly, although in two patients (53 and 59) AML engraftment was not observed with $10^7$ cells, apparently normal multilineage engraftment was seen when $10^8$ cells were injected. Taken together, these results suggest that the NOD/SCID
assay is working correctly and that the reason some AML samples are incapable of engraftment is independent of cell dose.

All previous engraftment screening was performed on peripheral blood. Occasionally, we obtained both peripheral blood samples (PB) and bone marrow (BM) samples from the same patients. The engraftment potential from either PB or BM cells was compared in paired experiments from ten different AML patients. Six patients demonstrated engraftment from both sources (Patients 6, 11, 16, 17, 18 and 42), whereas 4 patients (35, 48, 49 and 50), which did not engraft from the PB, also did not engraft when BM cells were injected.

**Most cases of non-engrafting AML do not engraft in a more permissive xenotransplantation model**

To investigate the influence of the murine microenvironment on AML engraftment, we examined engraftment in the more permissive B2-/-NOD/SCID model. Samples were injected into both the NOD/SCID assay and the B2-/-NOD/SCID model in paired experiments. Generally, samples that engrafted in the NOD/SCID assay, engrafted at higher level in the B2-/-NOD/SCID model. However, the majority (10/12) of AML cases that failed to engraft in the NOD/SCID model, could not be modelled in the B2-/-NOD/SCID assay. Only two samples (Patients 36 and 37), which failed to engraft in the NOD/SCID model, engrafted in the B2-/-NOD/SCID assay (Figure 3). Since the major difference between the B2-/-NOD/SCID and NOD/SCID models is probably NK cell activity, one may suggest that in most cases of AML, the reason for NOD/SCID non-engraftment is not immune mediated.  

[4]
Figure 3: Most cases of non-engrafting AML do not engraft in the B2-/-NOD/SCID model. Ten million mononuclear cells from 23 different AML patients were injected into both NOD/SCID and B2-/-NOD/SCID mice in paired experiments. Six weeks later, bone marrow engraftment was assessed via flow cytometry. AML engraftment was recorded if human CD33+/CD45+ myeloid cells were present without an accompanying CD19+/CD45+ B-cell population. 10 of 12 AML cases that failed to engraft in the NOD/SCID assay, did not engraft in the B2-/-NOD/SCID model.

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Table 2: Percentage of CXCR-4 Expression on CD34⁺ cells. AML cells from 21 different patients were labelled with antibodies to CD34 and CXCR-4. CXCR-4 expression is displayed as a percentage of CD34⁺ cells. There did not seem to be a difference in CXCR-4 expression levels between AML cases capable of NOD/SCID engraftment and those not able to do so. Of note, the highest CXCR-4 expression was observed in a non-engrafter and the
lowest was seen in an engrafting AML sample. Samples marked with * were tested for intracellular calcium release from CD34+ cells when stimulated with 100ng/ml SDF-1 as described in the methods section. Student paired T-test : 1.0.

**Figure 4: Calcium flux in cells from both NOD/SCID engrafting and non-engrafting AML cases.** Samples were labelled with Indo-1 dye as described in the methods section. The ratio of fluorescence due to dye bound to Calcium over fluorescence due to un-bound dye is displayed against time. Data was collected for 30 seconds, before addition of 100ng/ml of SDF-1 and further analysis. All samples analyzed produced detectable intracellular Calcium upon SDF-1 stimulation and no differences could be detected between NOD/SCID engrafting and non-engrafting AML cases.

**A lack of AML engraftment is not due to an obvious homing defect.**

To investigate the mechanism of homing, we first examined the expression of the CXCR-4 receptor on various NOD/SCID engrafting (n=11) and non-engrafting (n=10) AML cases. Although the range of CXCR-4 expressions was large, we could
not identify any obvious differences in the average CXCR-4 expression between the NOD/SCID engrafting and non-engrafting AML cases (Table 2). Indeed, one sample with over 90% CXCR-4 expression did not engraft (Patient 39) and conversely one of the engrafting AMLs possessed hardly any CXCR-4 expression (Patient 9).

To investigate whether this detected CXCR-4 was functional, certain AML samples (indicated on Table 2) were stimulated with SDF-1 and analyzed for intracellular calcium release. All samples analyzed released significant amounts of calcium from CD34+ cells upon SDF-1 stimulation. Indeed, there was no statistically significant difference in the amount or speed of calcium release between AML samples capable of NOD/SCID engraftment and those not capable (Example in Figure 4).

We then looked directly at the homing of PKH-26 labelled AML cells to the marrows of NOD/SCID mice. Six million PKH-26 positive cells from 3 NOD/SCID engrafting and 4 non-engrafting AML cases were injected into NOD/SCID mice. Sixteen hours later, murine marrows were assessed via flow cytometry for PKH26 bright cells. There was no significant difference (p=0.83) between engrafting groups in the proportion of labelled cells injected that homed to the marrow (data not shown, also confirmed in B2-/−NOD/SCID with patients 36 and 37). In addition for one sample (a B2-/−NOD/SCID-only engrafter, patient 37), the percentage of PKH26 bright cells that homed to the marrow was identical in B2-/−NOD/SCID and NOD/SCID, indicating that although they are not capable of six-week engraftment, cells still home to the NOD/SCID marrow. To confirm this result, we repeated the engraftment assessment of four previously non-engrafting samples, but injected the cells directly into the bone marrow as previously described. In all four patients (46, 38, 43 and 35), no AML engraftment was observed.

When combined, these data suggest that the SL-IC from non-engrafting AML samples home normally to the marrow and that a lack of NOD/SCID engraftment is not due to an obvious homing defect.

**Engraftment in NOD/SCID assay does not correlate with white blood cell count.**

The level of engraftment in NOD/SCID mice is thought to correlate with white blood cell count within AML samples capable of NOD/SCID engraftment. No study to date has investigated the relationship between the actual ability to engraft and the presentation white blood cell count. The median WBC in our study was very
similar for cases capable and incapable of NOD/SCID engraftment (39.8 and 44.5 x 10⁹/L, respectively) and hence, no statistically significant difference could be detected.

**Engraftment in NOD/SCID assay correlates with karyotypically defined prognostic group.**

The samples we describe here represent a broad spectrum of AML cases, including *de novo* and therapy related leukemia (tAML) from FAB groups 0, 1, 2, 3, 4 and 5. We excluded eight patients from karyotypic analysis, as five were relapse samples, two as the karyotype failed at diagnosis and one in which the karyotype was not performed. All of the remaining poor prognosis patients we analyzed engrafted in the NOD/SCID assay (5/5), whereas none of the previously untreated, good prognosis patients did (0/11). Of the intermediate risk, *de novo* patients we analyzed, 50% (18/36) engrafted. Using logistic regression analysis, the only factor that was significantly associated with engraftment was the karyotypically defined prognosis group reported in 2001 by Grimwade *et al* (see Table 3 for a summary; white cell count p=0.85; FAB group p=0.302; risk group p=0.0002).¹⁰

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**Table 3:** De novo AML patients were organized into poor, intermediate and good prognosis risk groups according to karyotype definition. Four patients (7, 9 10, 2) were excluded due to relapse, one not done (Patient 22) and two karyotypes (Patients 4 and 5) failed at diagnosis. Engraftment correlates with poor prognosis and conversely all favorable prognosis patients did not engraft.

**There is no absolute correlation between two frequent mutations and NOD/SCID engraftment**

To examine these two groups of AML cases further, we examined two genes that are frequently mutated in AML: Flt-3 and nucleophosmin. We detected the Flt3-ITD mutation in 6 (out of 29 tested) of our AML samples, a proportion similar to the
18% that was previously published.\textsuperscript{28} Four of these Flt3-ITD samples were capable of NOD/SCID engraftment and two were not (indicated by $\Delta$ in Table 1).

Nucleophosmin (NPM) is a nucleo-cytoplasmic-shuttling protein. Approximately a third of normal karyotype AML cases (35%) have an abnormality in the C-terminus of the protein that causes it to be present in the cytoplasm of affected cells rather than restricted to the nucleus.\textsuperscript{29} Although gene array analysis has revealed an upregulation of genes associated with stem cell function,\textsuperscript{30} the NOD/SCID engraftment potential of this group remains to be determined. The most interesting aspect of abnormal nucleophosmin expression is that a subset of normal karyotype AML cases are identified. We tested 10 of our normal karyotype AML samples for the presence of the altered NPM gene. We found a higher proportion (7/10) of samples than has been previously reported (35%) contained the altered gene but this may be explained by our small number of samples (indicated by $\perp$ in Table 1). Consistent with previous reports, we did observe the co-incidence of 3/7 of our NPM mutations with Flt3-ITD (indicated by $\Delta\perp$ in Table 1).\textsuperscript{30} Within the ten patients analyzed, we cannot report a definite correlation with NOD/SCID engraftment (3/7 engraft in NOD/SCID mice).

**Follow-up analysis confirms the relationship between NOD/SCID engraftment and disease behaviour**

The majority of patients with AML have an intermediate risk karyotype. Within this group are patients with AML that is refractory to treatment and actually have a poor prognosis.\textsuperscript{10} To examine if NOD/SCID engraftment could provide prognostic information, we prospectively screened 25 consecutive samples from younger (<60 years) patients with de novo intermediate risk AML who underwent intensive chemotherapy. Four patients underwent an allograft in first remission and these were censored at the time of allograft. As presented in Figure 5, overall survival was significantly reduced in AML capable of NOD/SCID engraftment when compared to AML cases that were not capable of NOD/SCID engraftment. Indeed, the 2-year actuarial overall survival of younger patients (<60 years) with intermediate risk AML treated with intensive chemotherapy was 31% (95% confidence interval (CI) 8-59%) and 76% (95% CI 33-94%) for engrafting and non-engrafting AMLs, respectively (p=0.02). The 2-year actuarial event free survival of younger patients (<60 years) with intermediate risk AML treated with intensive chemotherapy was
12% (95% CI 1-40%) and 62% (95% CI 27-84%) for engrafting and non engrafting AMLs, respectively (P=0.07).

**Figure 5a: Overall survival data of NOD/SCID-engrafting and non-engrafting AML samples.** The overall and event-free survival data of 25 *de novo*, intermediate risk AML cases (<60 years old) that received intensive multi-agent chemotherapy is presented above. Four cases were censored at allograft in first complete remission (two in each group). NOD/SCID engrafting AML cases had a poor overall survival that was statistically lower than NOD/SCID non-engrafting AML cases.
Figure 5b: Event-free data of NOD/SCID–engrafting and non-engrafting AML samples.
The event-free survival data of 25 *de novo*, intermediate risk AML cases (<60 years old) that received intensive multi-agent chemotherapy is presented above. Four cases were censored at allograft in first complete remission (two patients in each group). NOD/SCID engrafting AML cases had a poor event-free survival when compared to non-engrafting AML cases, though this did not reach statistical significance.

Discussion

This work describes the assessment of primary human AML in NOD/SCID and B2-/NOD/SCID mice. Via comparison to diagnostic smears, FISH and gene expression analysis we confirmed that both the NOD/SCID and B2-/NOD/SCID assays reproduce the same AML as in the original patient. Although it has been reported that the phenotype of 10/16 AMLs changes during engraftment, this was at a different time-point of engraftment and hence may have represented cells derived from less primitive cells than those in our study.4,27

Contrary to previous studies which had investigated variables that affect the level of AML engraftment,11,27,28 here, we studied the factors that are associated with whether or not individual AML cases engraft in the NOD/SCID model. We report here that approximately 50% of AML cases examined produced leukemic engraftment.
Since our results suggest that the inability of certain AMLs to engraft in the NOD/SCID model is not due to AML SL-IC frequency, immune rejection or tissue source, we progressed to examine the effect of homing. As mentioned above, the interaction between CXCR-4/SDF-1 plays a major role in hematopoietic cell homing in NOD/SCID mice.\(^\text{17}\) We investigate here whether CXCR-4 expression and function was the same between NOD/SCID engrafting and non-engrafting AML cases. A recent study reports that within engrafting AML samples, homing to the marrow may be inhibited by anti-CXCR-4 antibodies.\(^\text{21}\) The examination of the mean percentage of CXCR-4 expression on CD34\(^+\) cells in our study is consistent with published values.\(^\text{27}\) There was no statistically significant difference in CXCR-4 expression or in calcium release upon SDF-1 stimulation between NOD/SCID-engrafting and non-engrafting AML samples. Hence, when the process of homing is circumvented completely (direct BM injection), engraftment still cannot be achieved with previously non-engrafting AML samples, indicating that a homing defect is not the reason for the incapacity of some AML samples to engraft in NOD/SCID mice.

Since our results suggest that the reason that some AML samples do not engraft is independent of AML SL-IC frequency, CXCR-4 expression/homing or tissue source, we then tested for other potential correlations.

NOD/SCID engraftment correlated statistically with the karyotypically defined prognosis groups described by Grimwade et al. in 1998.\(^\text{10}\) This is consistent with suggestions postulated by other authors working with AML and the NOD/SCID assay, but we can now confirm this association with a larger sample of consecutive, previously untreated, AML patients that were screened prospectively.\(^\text{16,31}\) For instance, Monaco et al (2004) included both treated and untreated patients as well as patients with variable risk stratification in their follow-up data; whereas we studied a more homogenous group of patients that were <60 years old, with intermediate risk karyotype that had not been previously treated.

This karyotypic assessment of leukemic cells is the most widely used and powerful prognostic factor in AML. Although cytogenetic analysis allows the definition of the hierarchical groups with favorable, intermediate and poor prognosis, the intermediate risk group contains patients with variable outcomes.\(^\text{10}\) Assessing the prognosis of this large group of patients is currently difficult.

However, in this study, intermediate-risk AML cases that engrafted in the NOD/SCID assay had a poorer overall survival that was statistically significant when
compared to AML cases that were incapable of NOD/SCID engraftment. Hence, the NOD/SCID assay may be used to identify poor risk AML cases and in conjunction with array technology may be a useful tool to identify other pathogenic but subtle abnormalities within the intermediate risk AML group.

Although many factors have been identified that affect the engraftment of hematopoietic cells in NOD/SCID mice, the most important factors may be the injected cells’ self-renewal, proliferation and differentiation potentials. Cells that have limited potentials (such as CD34+/CD38+ cells) cannot engraft at six weeks in the NOD/SCID model, whereas more primitive cells with greater cell potential (CD34+/CD38+/−) can still produce engraftment at six weeks. Leukemic engraftment in the NOD/SCID model also discriminates between cells with a primitive (CD34+/CD38low/−) and mature (CD34+/CD38+) phenotype, presumably due to the same intrinsic cellular factors.

It is extremely interesting to note that although the NOD/SCID model assesses AML independent of the response to chemotherapy, engraftment still correlates with the response to this treatment (prognosis group). A possible explanation is that NOD/SCID engraftment reflects the stem cell nature of each individual AML case. AML cases that engraft in the NOD/SCID assay at six weeks may represent diseases driven by potent leukemia-initiating cells with stem cell-like self-renewal and proliferation abilities whereas non-engrafting AML cases may involve less potent leukemia-initiating cells with more restricted progenitor-type self-renewal and proliferation abilities.

Our data may give clues as to the cellular origin of the transformation event in individual AML cases. It is possible that AML cases that engraft in the NOD/SCID assay (and have a poor prognosis) are derived from a transformation in a hematopoietic stem cell whereas AML cases that do not engraft in the NOD/SCID assay (and have a more favorable prognosis) are derived from a progenitor-type cell. The AML initiating cell in cases derived from normal stem cells may well inherit other biological properties that confer an increased chemoresistance when compared to AML cases in which the initiating cell is derived from a more progenitor-type cell. Specifically, AML cases derived from normal stem cells would presumably have an increased ability to efflux and inactivate chemotherapy agents due an increased expression of various pumps and detoxification enzymes. Further studies examining this hypothesis are currently underway.
In conclusion, engraftment of AML in the NOD/SCID assay seems to be dependent on an inherent ability of the cells, which correlates well with disease prognosis.

ACKNOWLEDGEMENTS
We thank the patients for providing samples and Dr J Amess for providing diagnostic data. We also thank Derek Davies, Gary Warnes, Ayad Eddaoudi and Kirsty Allen of the FACS Lab at Cancer Research UK for their invaluable expertise. This work would not have been possible without Julie Bee, Clare Millum and Ella Smallcombe of our Biological Resource Unit. Mathew Smith kindly performed mutation analysis. We also thank Spyros Skoulakis for his statistical analysis of the patients’ follow-up data.
References
13. Blair A, Hogge DE, Sutherland HJ. Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)/CD71(-)/HLA-DR. Blood. 1998;92:4325-4335


AML engraftment in the NOD/SCID assay reflects the outcome of AML: implications for our understanding of the heterogeneity of AML

Daniel J Pearce, David Taussig, Kazem Zibara, Lan-Lan Smith, Christopher M Ridler, Claude Preudhomme, Bryan D Young, Ama Z Rohatiner, T A Lister and Dominique Bonnet