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The sequence of the K^+ channel described here is filed at GeneBank/EMBL/DDBJ, accession number 16216. During preparation of this manuscript, a polyclonal antibody to RACTK1 was prepared by M.S. This rat IgG antibody binds to the luminal side of the collecting duct of the rabbit kidney.

A cell initiating human acute myeloid leukaemia after transplantation into SCID mice

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MOST human acute myeloid leukaemia (AML) cells have limited proliferative capacity, suggesting that the leukaemic clone may be maintained by a rare population of stem cells[1–5]. This putative leukaemic stem cell has not been characterized because the available *in vitro* assays can only detect progenitors with limited proliferative and replating potential[4–7]. We have now identified an AML-initiating cell by transplantation into severe combined immune-deficient (SCID) mice. These cells homed to the bone marrow and proliferated extensively in response to *in vivo* cytokine treatment, resulting in a pattern of dissemination and leukaemic cell morphology similar to that seen in the original patients. Limiting dilution analysis showed that the frequency of these leukaemia-initiating cells in the peripheral blood of AML patients was one engraftment unit in 250,000 cells. We fractionated AML cells on the basis of cell-surface-marker expression and found that the leukaemia-initiating cells that could engraft SCID mice to produce large numbers of colony-forming progenitors were $CD34^+CD38^-$; however, the $CD34^+CD38^+$ and $CD34^-$ fractions contained no cells with these properties. This *in vivo* model replicates many aspects of human AML and defines a new leukaemia-initiating cell which is less mature than colony-forming cells.

The success in transplanting normal human haematopoietic cells[8,9] and acute lymphoid leukaemia cells[10,11] into immunodeficient SCID mice[12] suggested that this system might be useful for studying human myeloid leukaemias. But previous experiments indicated that primary AML cells could not be grafted into SCID mice after intravenous injection[13–15], and only a few samples grew locally after implantation into the peritoneum or under the renal capsule[13,14]. As AML cells have stringent cytokine requirements *in vitro*[7], we tested the effect of treating SCID mice, transplanted with peripheral blood cells (PBL) from AML patients newly diagnosed according to the French–American–British classification (FAB) as M1, with cytokine PIXY321 (Fig. 1 legend) and human mast-cell growth factor (MGF). DNA analysis indicated that the bone marrow from mice treated for 30–60 days contained 10–100-fold more human cells than those from untreated control mice (data not shown). We have now examined a large number of samples ($n = 17$) from patients with newly diagnosed AML of different FAB subtypes (AML M1, M2, M4) for their ability to proliferate in SCID mice. The cell source was either fresh bone marrow, fresh PBL, or banked frozen samples. All transplanted mice were treated with growth factors for the duration of the experiment (30–45 days). DNA and cytological analysis indicated that the bone marrow from 60 of 70 mice (86%) contained 10–100% human cells (Fig. 1), leading to almost complete replacement of murine haematopoiesis. Moreover, AML cells from all of the FAB subtypes (16 of 17 patients) engrafted SCID mice to high levels, indicating high reproducibility of the transplant system.

Many morphological and dissemination features characteristic of the donor's disease were reproduced in the SCID-leukaemia mice. The bone marrow of mice transplanted with AML M1 cells was extensively infiltrated with undifferentiated blast cells

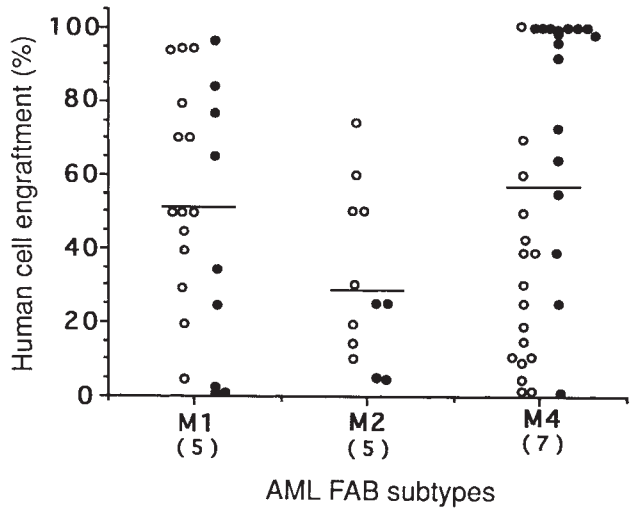

FIG. 1 Summary of human cell engraftment in the bone marrow of SCID mice transplanted with cells from AML patients, FAB subtypes M1, M2 or M4, where: M1 is AML without maturation; M2 is AML with granulocytic maturation; M4, acute myelomonocytic leukaemia; M5, acute monocytic leukaemia[22]. Number of donors is indicated. Engraftment was quantified by DNA analysis (open circles) or by cytology (filled circles) of Wright-stained bone marrow touch preparations 17–45 days post-transplantation. Lines represent mean level of engraftment for each FAB group.
METHODS. Bone marrow and peripheral blood were obtained after informed consent according to procedures approved by the Human Experimentation Committee. Fresh or thawed AML cells were enriched by Ficoll-density gradient centrifugation and washed in IMDM medium containing 10% FCS. For transplantation of human cells, cells (1×10^7–4×10^7) were injected into the tail vein of sublethally irradiated (400cGy, using a ^{137}Cs irradiator) SCID mice according to our established protocols[9]. PIXY321 (a fusion protein of human granulocyte–macrophage colony-stimulating factor with human IL-3) (7 μg) and human mast-cell growth factor (hMGF; c-*kit*-ligand; 10 μg) were administered on alternate days by intraperitoneal injection. Mice were bred and maintained in a defined flora colony (Ontario Cancer Institute). Grafting of human cells was quantified as described[9]. Briefly, 5 μg phenol-extracted DNA was digested with *Eco*RI, blotted onto a nylon membrane (Amersham) and probed with p17H8, a human α-satellite probe specific for human chromosome-17 sequences[23]. The percentage of human cells present in the mouse tissues was estimated by comparison of the intensity of the characteristic 2.7-kb band with standard human/mouse DNA mixtures (0, 0.1, 1.0, 10 and 50% human DNA).

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(Fig. 2*a*); Auer rods were also seen in some leukaemic cells (Fig. 2*b*). SCID mice transplanted with AML M4 cells containing an inversion of chromosome 16 had many characteristically abnormal eosinophils with large basophilic granules (Fig. 2*c*). Flow cytometric analysis, using CD33 and CD13, of leukaemic cells from the engrafted murine bone marrow indicated that they had an immunophenotype identical to the donor leukaemic cells (data not shown). In addition, leukaemic cells were also present in the peripheral blood of engrafted SCID mice (Fig. 2*d*). In contrast to mice transplanted with AML M1 and M2, some with AML M4 cells became sick or died as early as 10–20 days post-transplant, with dissemination of leukaemic blasts to the liver (Fig. 2*e*), lungs, spleen and kidney (data not shown). Clinically, leukaemic blasts from patients with the monocytic subtypes AML M4 and M5 disseminate more extensively to extramedullary sites than those from patients with AML M1/M2, suggesting that the SCID-leukaemia model accurately reflects biological differences between different AML subtypes.

To determine whether immature leukaemic blast colony-forming units (AML-CFU) were present in the bone marrow of highly engrafted mice, single-cell suspensions of marrow were plated in methylcellulose cultures. AML-CFU were present in mice transplanted with 11 of 11 donor samples, regardless of the FAB classification (Fig. 3*a*), and no progenitors of normal lineages were detected. Leukaemic cells, before and after transplantation into SCID mice, were plated at limiting dilution to compare the frequencies of AML-CFU. The assay was linear, and similar frequencies were obtained from the patient sample and the mouse bone marrow, 0.9 versus 0.3% respectively (Fig. 3*b*). Interestingly, the response in culture to interleukin-3 (IL-3) and human MGF of AML-CFU from the patient and the transplanted mouse was identical, indicating that neither the murine environment nor exogenous cytokine treatment selected for clones with altered responses to growth factors (Fig. 3*b*). Kinetic experiments were done to measure the number of human cells and AML-CFU present in the murine bone marrow over 28 days following transplantation of either 10^6 or 1.3×10^7 cells. At both cell doses, AML-CFU increased by >100-fold over 14–28 days relative to the number detected in bone marrow one day after transplantation (Fig. 3*c*). The total number of human cells increased by 1,000-fold over the same period (data not shown). The presence of large numbers of growth-factor-responsive AML-CFU and their extensive expansion in the bone marrow of cytokine-treated mice implied that a leukaemic stem cell more immature than AML-CFU was maintaining the progenitor pool.

Flow-sorting was used to characterize and purify the leukaemia-initiating cells. CD34 is a cell-surface marker normally expressed on a small population of bone marrow cells, including progenitor cells and pluripotent stem cells[16]. Expression of CD38 on $CD34^+$ cells is an important marker for lineage commitment and therefore the $CD34^+CD38^-$ phenotype defines an immature human cell in normal bone marrow. Although the expression of CD34 and CD38 on AML cells is very heterogeneous[17], the $CD34^+CD38^-$ phenotype is present on immature AML-CFU[18]. Peripheral blood cells from an AML M1 patient were separated into CD34-positive and -negative fractions and transplanted into SCID mice. Leukaemic cell proliferation and high levels of AML-CFU were observed in the bone marrow of mice transplanted with $CD34^+$ cells, as in mice transplanted with unsorted populations (Fig. 3*d*). By contrast, four mice transplanted with $CD34^-$ cells were poorly engrafted (0–0.1%) and contained no AML-CFU, except for one mouse that had been transplanted with a high cell dose and contained only a few colonies (Fig. 3*d*). In two further experiments mice were transplanted with $CD34^+CD38^-$ or $CD34^+CD38^+$ cells purified from the same donor. Leukaemia cell proliferation and high levels of AML-CFU were only seen in mice transplanted with $CD34^+CD38^-$ cells (Fig. 3*d*). Interestingly, both populations contained comparably high numbers of

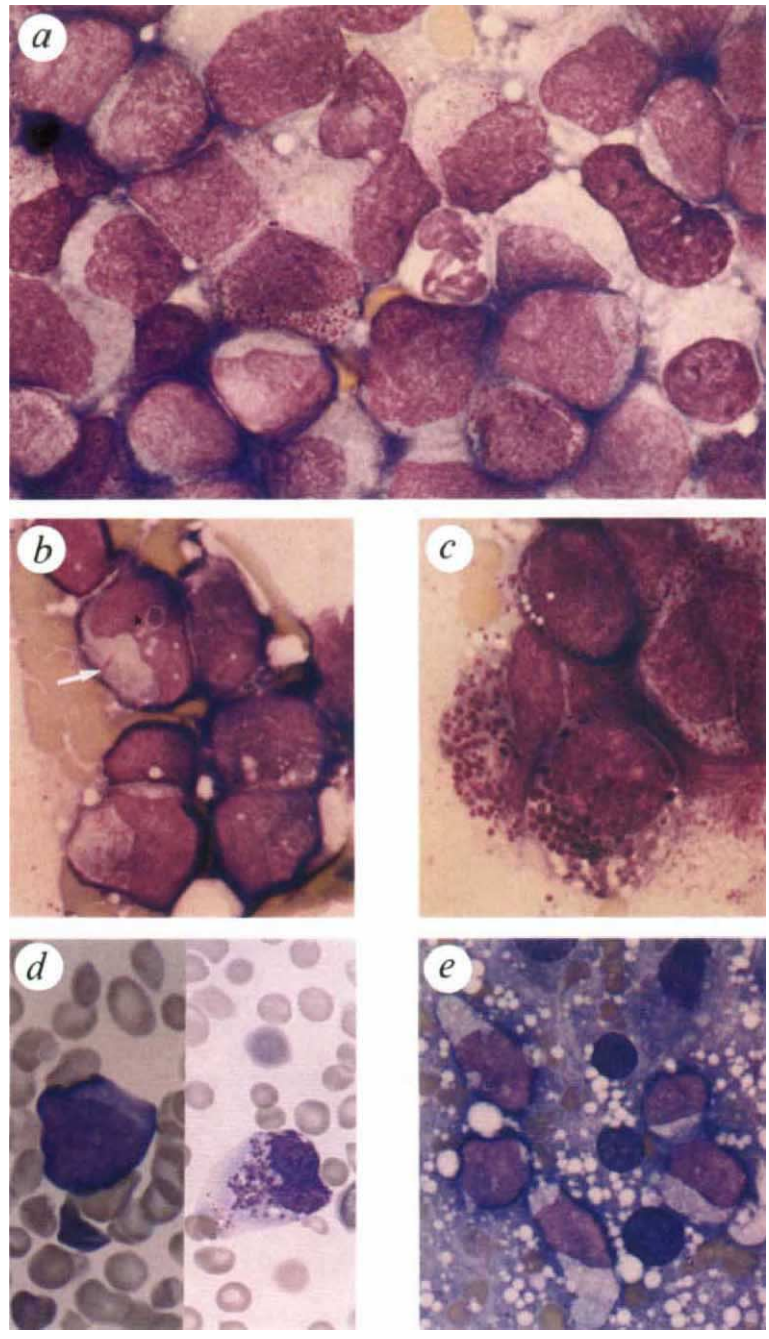

FIG. 2 Histology of cytokine-treated SCID mice injected with AML cells 3–5 weeks post-transplantation. *a*, Bone marrow touch preparation of a mouse highly infiltrated with AML M1 cells. Only one mouse neutrophil with a ring-shaped nucleus is present among the characteristic AML M1 blast cells. *b*, Mouse marrow repopulated with cells from a different AML M1 patient containing an Auer rod (arrow). *c*, Mouse marrow repopulated with cells from an AML M4 (with eosinophils and inversion of chromosome 16) patient containing characteristic eosinophils with large basophilic granules. *d*, Peripheral blood smear of a mouse repopulated with AML M4 cells. Human blast cells (left) and eosinophils (right) were present in the circulation of the mouse. *e*, Liver touch preparation of a mouse engrafted with AML M4 cells. Infiltrating leukaemic blast cells can be seen among mouse hepatocytes.

AML-CFU before injection into SCID mice, providing evidence that at least $CD34^+CD38^+$ AML-CFU could not engraft SCID mice. $CD34^+CD38^-$ cells transplanted into SCID mice did not produce any detectable normal human progenitors; all of the colony-forming cells tested (40/40) contained the same t(2;4) chromosomal translocation found in the patient's leukaemic clone.

We next investigated whether there was a linear relationship between the number of cells injected and leukaemic engraftment, in order to develop a quantitative assay for AML-initiating cells. Peripheral blood cells from AML patients were diluted in a tenfold series from 2×10^7 cells to 2×10^4 cells before transplanting into SCID mice. In a representative experiment, DNA analysis indicated that as few as 2×10^5 cells were sufficient to initiate leukaemic proliferation (Fig. 4). Statistical analysis of the proportion of mice that had leukaemic proliferation at each cell

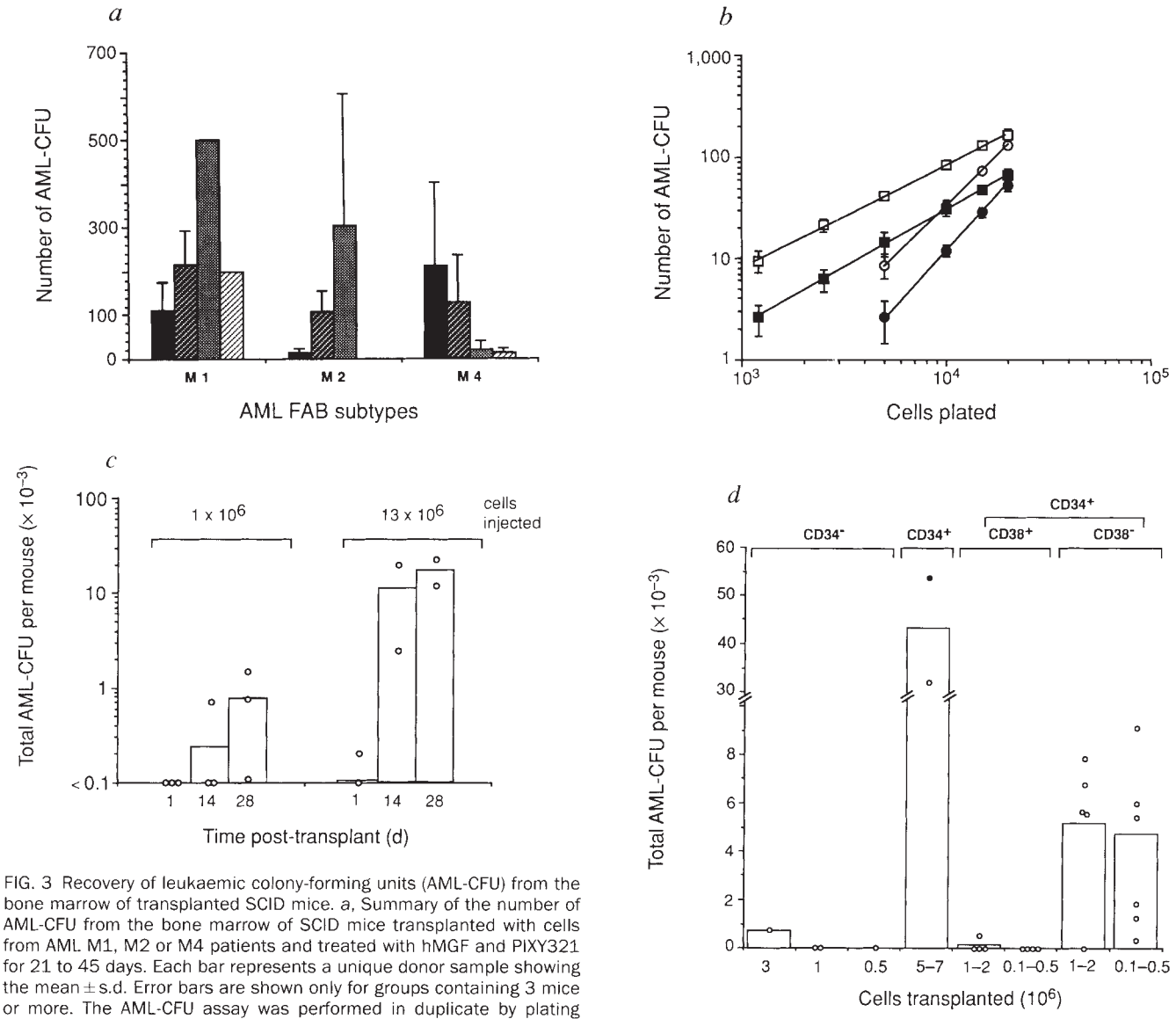

FIG. 3 Recovery of leukaemic colony-forming units (AML-CFU) from the bone marrow of transplanted SCID mice. *a*, Summary of the number of AML-CFU from the bone marrow of SCID mice transplanted with cells from AML M1, M2 or M4 patients and treated with hMGF and PIXY321 for 21 to 45 days. Each bar represents a unique donor sample showing the mean ± s.d. Error bars are shown only for groups containing 3 mice or more. The AML-CFU assay was performed in duplicate by plating 2×10^5 bone marrow cells in 0.9% methylcellulose-containing fetal bovine serum (15%), human plasma (15%), hMGF (50 ng ml^{-1}), PIXY 321 (5 ng ml^{-1}), hGM–CSF (1 U ml^{-1}), hIL-3 (10 U ml^{-1}) and human erythropoietin (2 U ml^{-1}). At day 7, leukaemic blast colonies were scored and their leukaemic identity confirmed by cytology and chromosomal analysis where applicable. No murine colonies were obtained under these selective culture conditions[8,9]. *b*, Frequency of AML-CFU before and after transplantation. Limiting dilution analysis of AML-CFU present in the PBL of an AML M1 patient before (open symbols) and after (filled symbols) transplantation. Cell numbers indicated were plated in methylcellulose culture containing either hIL-3 (1 nM) alone (circles) or in combination with hMGF (323 pM) (squares). Bone marrow from the transplanted mouse was analysed 35 days after transplantation and treatment with hMGF and PIXY321. *c*, Expansion of AML-CFU in the bone marrow of transplanted SCID mice. SCID mice were transplanted with AML M1 cells at the cell numbers indicated and killed at 1, 14 and 28 days post-transplant. The total number of AML-CFU per mouse was determined by multiplying the AML-CFU per 2×10^5 cells plated by the total number of bone marrow cells present in the mouse. The limit of detection was 100 AML-CFU per mouse. *d*, Measurement of AML-CFU from SCID mice transplanted with AML cells fractionated according to CD34 and CD38 expression. Three independent cell-sorting experiments were done on thawed cells containing 75% $CD34^+$ cells from an AML M1 patient; of these 40% were $CD38^-$. Cells were purified by fluorescence-activated cell sorting (FACStar^{PLUS}; Becton–Dickinson) or using a CD34 affinity column (Cell-Pro) (filled circle). In the first experiment, cells were separated on the basis of CD34 expression; the $CD34^+$ and CD34$^-$ populations were each 98% pure; in experiments 2 and 3, respectively, the $CD34^+/CD38^-$ cells were 88 and 97% pure, and the $CD34^+/CD38^-$ cells were 80 and 73% pure, being contaminated with 10 or 15% $CD34^-/CD38^-$ cells. The $CD34^-/CD38^-$ and the $CD34^+/CD38^-$ fractions from both experiments 2 and 3 contained an average of 6,525 and 8,736 AML-CFU per 2×10^5 cells, respectively. Mice were transplanted with the indicated number of purified cells and treated with cytokines. After 45 days (experiment 1) or 30 days (experiment 2 and 3), the total number of AML-CFU was determined.

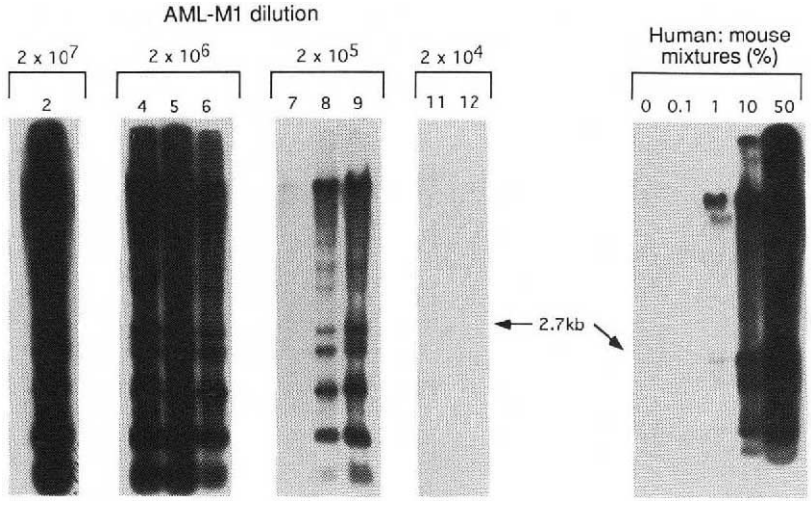

FIG. 4 Determination of the frequency of the SCID leukaemia-initiating cell (SL-IC) engraftment unit by limiting dilution analysis. PBL cells from an AML M1 patient were thawed and different cell doses (2×10^4, 2×10^5, 2×10^6 and 2×10^7 cells) were transplanted into groups of 3 or 4 mice. Mice were treated with hMGF and PIXY321 for 1 month, after which DNA from the bone marrow was analysed for human cells as described for Fig. 1. The Southern blot is representative of four experiments with four different donors. SCID mice containing >5% leukaemic cells in the bone marrow were considered positive for the statistical analysis used to determine the frequency of SL-IC by the method of Porter and Barry[24]. The negative mice contained from 0.1% to undetectable human cells. Ethidium bromide staining indicated that equal amounts of DNA were loaded in each gel lane.

dose, using data from four different donors transplanted into 40 mice, indicated that the frequency of the leukaemia-initiating cell in the PBL of AML-M1 patients was 1 engraftment unit per 2.5×10^5 cells (range: 1 in 1.2×10^5 to 1 in 5.3×10^5); the engraftment followed single-order kinetics as measured by the χ^2 test (95% confidence limit).

We have identified an AML-initiating cell on the basis of its ability to establish human leukaemia in SCID mice (the SCID leukaemia-initiating cell, or SL-IC). Three pieces of evidence suggest that there may be a hierarchy of leukaemic stem cells in human AML, where SL-IC are more immature than AML-CFU. First, the frequency of SL-IC in the PBL of AML M1 patients is at least 1,000-fold lower than the frequency of AML-CFU. Second, only mice transplanted with $CD34^+CD38^-$ cells developed leukaemia whereas $CD34^+CD38^+$ mice did not, despite the fact that similar numbers of AML-CFU were present in both populations before transplatation. Third, based on the low proliferative capacity of AML-CFU in liquid or long-term cultures[4,19,20] even with maximal growth-factor stimulation, it is likely that their large expansion in SCID mice for >45 days post-transplantation is due to the proliferation and differentiation of SL-IC. In future, autologous transplantation with purged cells may address the relationship between SL-IC and the leukaemic stem cell that maintains the disease in patients. But the fact that SL-IC shares a $CD34^+CD38^-$ expression pattern similar to normal stem cells indicates that purging strategies may be difficult to develop. It will also be possible to create complementary DNA libraries[21] from single cells to characterize genes that are expressed in SL-IC and compare them with those from normal stem cells and the more differentiated AML-CFU. Finally, a SCID-leukaemia model that reproduces many features of human AML should help us to understand the processes governing the transformation and progression of leukaemic stem cells and to test new therapeutic strategies. □

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Selectivity of MHC-encoded peptide transporters from human, mouse and rat

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MAJOR histocompatibility complex (MHC) class I molecules present peptides from degraded intracellular antigens to $CD8^+$ T cells[1]. These peptides are translocated in an ATP-dependent fashion[2–4] into the lumen of the endoplasmic reticulum (ER) for binding to class I molecules[5,6] by means of the MHC-encoded transporters associated with antigen processing, TAP1 and TAP2. These are members of a family of proteins containing an ATP-binding cassette and form heterodimers in the ER membrane[7–10]. Defects in the genes encoding TAP1 or TAP2 account for impaired class I assembly and antigen presentation in several human and rodent cell lines[7,11–13]. Whereas MHC class I molecules select peptides according to binding motifs[14–17], it is not clear to what extent the TAP1–TAP2 transporters have peptide sequence and length specificity. Previous studies of the rat MHC class I molecule, RT1A[a], suggested a specific conveyance of peptides by rat TAP1–TAP2 (ref. 18). Here we substitute the amino- and carboxy-terminal and the penultimate amino-acid residues of model peptides to