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The *Mll–AF9* gene fusion in mice controls myeloproliferation and specifies acute myeloid leukaemogenesis

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The *MLL* gene from human chromosome 11q23 is involved in >30 different chromosomal translocations resulting in a plethora of different MLL fusion proteins. Each of these tends to associate with a specific leukaemia type, for example, MLL–AF9 is found mainly in acute myeloid leukaemia. We have studied the role of the *Mll–AF9* gene fusion made in mouse embryonic stem cells by an homologous recombination knock-in. Acute leukaemias developed in heterozygous mice carrying this fusion as well as in chimeric mice. As with human chromosomal translocation t(9;11), the majority of cases were acute myeloid leukaemias (AMLs) involving immature myeloblasts, but a minority were acute lymphoblastic leukaemia. The AMLs were preceded by effects on haematopoietic differentiation involving a myeloproliferation resulting in accumulation of Mac-1/Gr-1 double-positive mature myeloid cells in bone marrow as early as 6 days after birth. Therefore, non-malignant expansion of myeloid precursors is the first stage of Mll–AF9-mediated leukaemia followed by accumulation of malignant cells in bone marrow and other tissues. Thus, the late onset of overt tumours suggests that secondary tumorigenic mutations are necessary for malignancy associated with *MLL–AF9* gene fusion and that myeloproliferation provides the pool of cells in which such events can occur.
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Introduction

Chromosomal translocations are present in many tumours and are involved in the development of the tumours which carry them (Rabbitts, 1994; Look, 1997). The cloning of the genes involved in the chromosomal translocation breakpoints in leukaemias and solid tumours of mesenchymal origin, have shown that a major molecular consequence of these aberrant chromosomes is creation of fusion genes, and therefore of fusion proteins, when the breaks occur between exons of genes on each chromosome. This was first recognized for the fusion of *BCR* and *ABL* genes resulting from the Philadelphia chromosome (Nowell and Hungerford, 1960; Rowley, 1973; de Klein *et al*., 1982; Bartram *et al*., 1983; Groffen *et al*., 1984). Molecular studies of these chromosomal breakpoints demonstrated that two forms of the *BCR–ABL* fusion gene result from distinct chromosomal breakage within the *BCR* gene, linking it to the same coding part of the *ABL* gene (Bartram *et al*., 1983; de Klein *et al*., 1986). More recently, complex situations have been described in subsets of leukaemias with breaks in chromosome 11, band q23 within the *MLL/HRX/ALL-1* gene (Ziemin-van der Poel *et al*., 1991; Djabali *et al*., 1992; Gu *et al*., 1992; McCabe *et al*., 1992; Tkachuk *et al*., 1992; Domer *et al*., 1993) and in sarcomas such as those with breaks within the *EWS* and *FUS/TLS* genes (Delattre *et al*., 1992; Crozat *et al*., 1993; Rabbitts *et al*., 1993; Aman *et al*., 1996; Panagopoulos *et al*., 1996). Under these circumstances, the respective genes become involved with a multitude of different chromosomal translocations which are predominantly associated with a particular sub-type of leukaemia or sarcoma. Furthermore, the *FUS* gene has a role in translocations in both sarcomas and in acute myeloid leukaemia (Ichikawa *et al*., 1994; Panagopoulos *et al*., 1994) depending on the cell type or on the fusion partner. These observations raise issues about possible roles of the fusion genes in tumour type, particularly the role the fusion partner might play in the specificity of the tumour phenotype.

The chromosome 11 region q23 is involved in ~10% of acute myeloid leukaemias (AMLs) and acute lymphoblastic leukaemias (ALLs), as well as in mixed lineage leukaemias and in some lymphomas. In addition, balanced chromosome 11q23 translocations are also observed in therapy-related leukaemias, especially in patients previously treated with inhibitors of topoisomerase II (Ratain *et al*., 1987; Pui *et al*., 1989; Cimino *et al*., 1997; Nasr *et al*., 1997; Rowley *et al*., 1997; Sobulo *et al*., 1997; Atlas *et al*., 1998; Felix, 1998). The cloning of the chromosome 11q23 breakpoint region (Ziemin-van der Poel *et al*., 1991) revealed the *MLL/HRX/ALL-1* gene (Djabali *et al*., 1992; Gu *et al*., 1992; McCabe *et al*., 1992; Tkachuk *et al*., 1992; Domer *et al*., 1993) (herein designated the *MLL* gene), which encodes a 432 kDa protein with several regions of structural homology to other proteins, for instance to *Drosophila* trithorax (Mazo *et al*., 1990; Djabali *et al*., 1992; Gu *et al*., 1992; Tkachuk *et al*., 1992). More than 30 different chromosomal bands have been found in chromosomal translocations with chromosome 11q23, and molecular studies showed rearrangements with the *MLL* gene in all cases (reviewed in Rowley, 1993; Thirman *et al*., 1993; Bernard and Berger, 1995; Rubnitz *et al*., 1996b; Gilliland, 1998). Among the most common reciprocal translocations are

t(4;11)(q21;q23), t(9;11)(p22;q23) and t(11;19)(p13;q23). These fuse *MLL* with *AF4/FEL*, *AF9/LTG9* and *ENL/LTG19* genes, respectively (Gu *et al*., 1992; Tkachuk *et al*., 1992; Chen *et al*., 1993; Iida *et al*., 1993; Morrissey *et al*., 1993; Nakamura *et al*., 1993; Yamamoto *et al*., 1993). There is a strong correlation between the leukaemia phenotype and each of these specific *MLL* translocation fusions (Corral *et al*., 1993; Thirman *et al*., 1993). The translocation t(9;11)(p22;q23) is mainly associated with AMLs (Iida *et al*., 1993; Nakamura *et al*., 1993) while the translocation t(4;11)(q21;q23) is found predominantly in ALLs (Gu *et al*., 1992; Domer *et al*., 1993; Iida *et al*., 1993; Morrissey *et al*., 1993; Nakamura *et al*., 1993; Downing *et al*., 1994). The t(11;19)(p13;q23) translocation on the other hand occurs in both ALL and AML but with higher frequency in ALL (Nakamura *et al*., 1993; Yamamoto *et al*., 1993; Rubnitz *et al*., 1996a). These findings suggest that the incoming fusion partner of MLL helps to specify the cell type of the tumour. Thus, the fusion protein itself may influence tumour type if the chromosomal translocation occurs in a pluripotent precursor cell. It is nonetheless possible that the chromosomal translocation may occur in a committed precursor because the chromosomal region is accessible for the chromosomal translocation event, in which case the protein fusion would not strictly specify tumour type. Several biological models have been established to gain insights into the role of the *MLL* fusions in tumorigenesis. The use of retroviruses encoding the *MLL/HRX–ENL* fusion to infect primitive cells showed that myeloid cell proliferation could be observed and that tumours of this lineage emerged (Lavau *et al*., 1997; Slany *et al*., 1997). These experiments suggest that a gain-of-function mechanism at least partly explains the role of this fusion protein which is made after the chromosomal translocation t(11;19) in humans. The fusion of the *AF9* gene with the *Mll* gene also resulted in the emergence of AML in mice (Corral *et al*., 1996).

In the present study we show that an *Mll–AF9* gene fusion [made in embryonic stem (ES) cells by homologous recombination] carried in the mouse germline contributes to AML. In both chimeras and heterozygous mice, the majority of mice developed AML and a small percentage developed ALL. The features of this disease reflect those of the disease which develops in humans carrying the *MLL–AF9* translocation. Furthermore, a selective proliferation of Gr-1-positive myeloid bone marrow cells was observed in heterozygous animals before the symptoms of leukaemia occurred. The propensity for myeloid tumour formation therefore seems to be a consequence of the advantageous growth of myeloid precursors caused by the *Mll–AF9* fusion, thereby providing a pool of cells in which secondary mutations, necessary for overt tumour development, can occur.

Results

Mll–AF9 mice develop acute leukaemia

An *Mll–AF9* fusion gene was created in mouse ES cells, by knock-in homologous recombination (Corral *et al*., 1996), in which an *AF9* cDNA was fused into exon 8 of one allele of the endogenous *Mll* gene (a diagram of the targeted allele is shown in Figure 1A). These ES cells were injected into C57Bl/6 blastocysts and chimeric mice

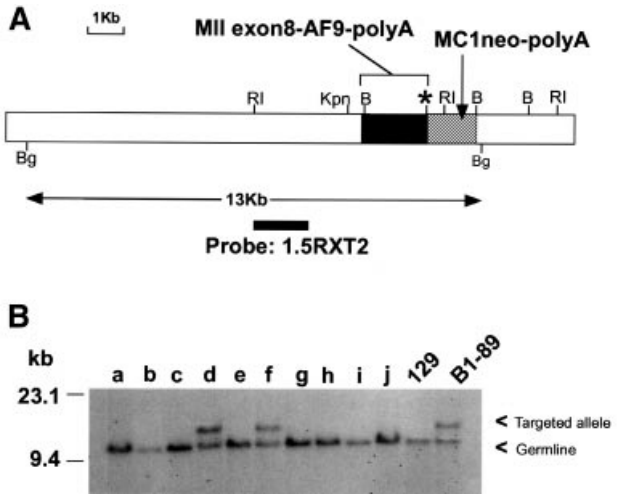

Fig. 1. Germ-line transmission of the Mll–AF9 targeted allele in mice. (**A**) A diagrammatic map of the Mll–AF9 targeted allele is shown (Corral *et al*., 1996). The human AF9 cDNA sequences were fused at exon 8 of *Mll* and MC1-neo-poly(A) cassette (Thomas and Capecchi, 1987) was cloned into the targeting vector as a positive selection marker. The restriction fragments corresponding to the wild-type *Mll* and the targeted *Mll–AF9* genes are 10 and 13 kb *Bgl*II fragments, respectively. The probe used to detect homologous recombination events [1.5RXT2 (Corral *et al*., 1996)] is indicated. (**B**) Filter hybridization of tail biopsy DNA. Lanes a–j show hybridization of DNA extracted from a litter of Mll–AF9 mice produced by crossing a male chimera with a wild-type mouse. The DNA was digested with *Bgl*II and hybridized with the *Eco*RI–*Xho*I fragment from clone p1.5RXT2. Two heterozygous carrier mice were present. 129 DNA corresponds to a 129 mouse liver and B1-89 represents DNA from the original Mll–AF9 targeted ES clone. Germline and targeted alleles are indicated.

were produced which developed haematopoietic tumours, mainly AML, after 6 months (Corral *et al*., 1996). Germline transmission of this *Mll–AF9* fusion gene was obtained by crossing chimeras with wild-type females. Heterozygotes obtained from these crosses were identified by filter hybridization of somatic DNA using an internal probe from the targeting region (1.5RXT2 probe, Figure 1A). The probe detects a 10 kb germ-line *Mll* band and a 13 kb band corresponding to the homologous recombination fusion gene (Figure 1B shows the hybridization of DNA from one litter, designated a–j, compared with 129 liver DNA and DNA from an initial targeted ES clone B1-89). No homozygous Mll–AF9 mice were found in any litter from a heterozygous cross (41 pups from four litters were analysed), indicating embryonic lethality of the knock-in gene as reported for the *Mll* null mutant mice (Yu *et al*., 1995).

Our previous report on the *Mll–AF9* fusion gene expressed in chimeric mice showed that most animals succumbed to acute leukaemia within one year (Corral *et al*., 1996). Whilst many of these were diagnosed as myeloid, a complete analysis was not performed in all cases. A detailed investigation of the leukaemias in Mll–AF9 heterozygous and chimeric mice has now been undertaken to define more precisely the type of malignancy and to address issues relating to the mode of onset of overt malignancy. Cohorts of Mll–AF9 mice were compared with mice which had been made with an epitope tag fused (this line of mice has been designated Mll–myc; Corral *et al*., 1996) at the same *Mll* exon 8 position at

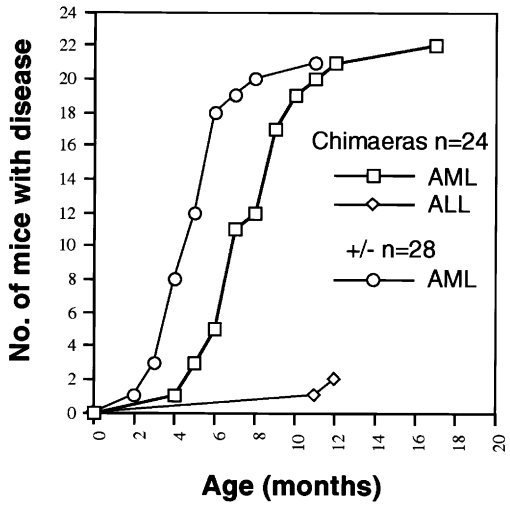

Fig. 2. Acute leukaemia occurrence in Mll–AF9 mice. The rate of leukaemia occurrence (age in months of detection of disease versus number of mice with disease) in Mll–AF9 chimeric and heterozygous mice. Diagnosis of leukaemia was obtained by histological analysis of bone marrow, spleen, thymus and liver, and in most cases by FACS analysis of cell surface marker expression. AML developed in 21 out of 28 heterozygous Mll–AF9 mice (circles), in 22 out of 24 Mll–AF9 chimeras (squares) and ALL developed in two out of 24 Mll–AF9 chimeras (diamonds). No Mll–myc heterozygous mice developed a malignancy in the 18 month period of this experiment (four tumours were detected in the cohort of 27 Mll–myc mice but these occurred at 28, 29, 32 and 32 months).

which the AF9 sequences were fused. These groups were analysed over a period of 18 months, and during this time both the Mll–AF9 chimeras and heterozygotes began to show signs of distress. No disease was observed in Mll–myc mice within the 18 month experimental period (there were 27 Mll–myc mice in the control group; four developed AML tumours but these occurred after two years suggesting that they were of sporadic origin rather than due to the manipulation of the *Mll* gene).

Post-mortem examination of Mll–AF9 mice showed consistent evidence of haematological disease. Signs included pale femurs, splenomegaly, heptomegaly and pale kidneys. All the mice analysed were diagnosed as having acute leukaemias and no tumours of other tissues were observed, despite widespread activity of the *Mll* promoter (Yu *et al*., 1995; Corral *et al*., 1996). The development of these acute leukaemias is shown in Figure 2. The disease was classified as predominantly AML with rare examples of ALL, mirroring the spectrum and ratio of acute leukaemias found in humans with the chromosomal translocation t(9;11). The rate of development of AML was overall slightly faster in heterozygous animals compared with the chimeras (Figure 2), the point at which 50% of each cohort had succumbed to AML being 5 and 7 months for heterozygotes and chimeras, respectively. Twenty-two of the 24 chimeras and 21 of the 28 heterozygous Mll–AF9 mice developed AML in the 18 month period. Two Mll–AF9 chimeras developed ALL rather than AML.

Acute myeloid and lymphoblastic leukaemia in Mll–AF9 mice

Almost all Mll–AF9 heterozygous and chimeric mice developed malignancy within one and a half years. Detailed tissue histology was carried out on mice as pathological signs of disease developed, to classify the types of leukaemia present and to establish a diagnosis of disease. This analysis confirmed the presence of acute leukaemias in all the mice with symptoms and showed the occurrence of either myeloid or lymphoid malignancies. Marked infiltration of leukaemic cells was seen in the bone marrow, peripheral blood and liver of Mll–AF9 mice. Figure 3 shows a comparison of the histology of tissues from an Mll–myc mouse (Figure 3A) with those from Mll–AF9 mice. The AML was observed in two different forms, overt myeloid leukaemia and extramedullary leukaemia. Mice exhibiting overt myeloid leukaemia were distinguished by $\geqslant$30% of nucleated cells in the bone marrow being blasts. Heavy infiltration of the peripheral blood with myeloblasts was observed in these mice (either chimeras or heterozygotes). The myeloblasts were characterized by their large, granular appearance and were frequently observed in the liver (Figure 3B shows histology from a heterozygous mouse and Figure 3C shows histology from a chimeric mouse, both with AML). Other tissues were also involved at this stage, for instance kidney. The alternative form of myeloid disease was extramedullary leukaemia (Figure 3D), in which extensive extramedullary infiltration of myeloblasts was found in the liver, with peri-vascular deposits of malignant cells. In the example shown (histology from a chimeric mouse), the bone marrow shows significantly $<$30% infiltration with myeloblasts. Similarly, few abnormal cells were observed in the peripheral blood in the extramedullary stage of the malignancy.

Detailed analysis of tissue histology in the Mll–AF9 mice (one example is shown in Figure 3E) indicated that two ALLs arose. In these mice the bone marrow samples were hypercellular, with normal haematopoiesis suppressed, and $>$90% of the mononuclear cells were lymphoblasts with many active mitoses seen. An enlarged thymus was evident in these mice in addition to a pale femur, enlarged, pale spleen and pale kidneys. Extra-medullary haematopoiesis was evident in the liver which showed the characteristic peri-vascular accumulation of leukaemic cells. The circulating blood of the mouse shown is packed with lymphoblasts. In addition, the peripheral blood film indicates that this mouse was also suffering from anaemia.

Immunoglobulin gene rearrangement analysis showed that both of the ALLs were of B cell origin. Filter hybridization was carried out with an immunoglobulin heavy chain probe and spleen cell DNA of the two ALL-bearing mice compared with a myeloma cell line (J558) and a T cell line (BW) and kidney DNA from C57Bl/6 and 129 mice (Figure 4A). The probe detects a 6.5 kb *Eco*RI germ-line band in the kidney DNA samples and the BW (T cell) DNA, but detects two rearranged bands in the myeloma (B cell) DNA representing heavy-chain gene rearrangements. DNA from both of the Mll–AF9 tumours similarly had two distinct rearranged bands together with faint germ-line bands, indicative of clonal B cell tumours in these mice. Rearrangement of T cell receptor β-chain genes was also investigated with Jβ2 and Cβ1 probes, but no rearrangements were found in the tumour DNA from either ALL mouse (data not shown). FACS analysis of bone marrow cells from mouse 1

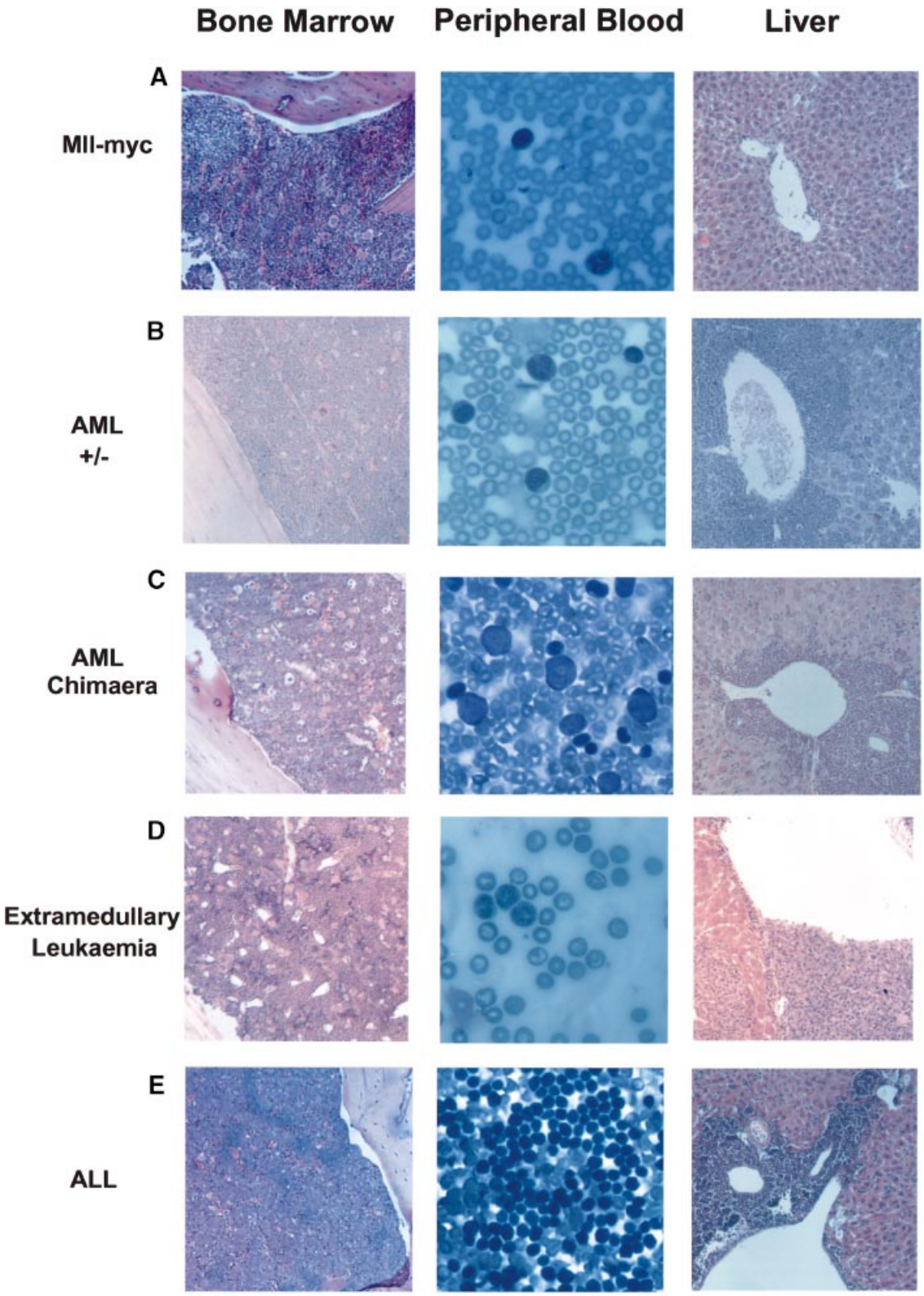

Fig. 3. Histology of leukaemias arising in Mll–AF9 mice. Histology was carried out on tissues from all mice with symptoms of disease. Tissues were dissected and fixed in 10% formalin. Sections were prepared from wax-embedded specimens and stained with haematoxylin and eosin (H&E). Blood films were stained with May–Grünwald–Giemsa (MGG). Representative examples of histology are shown. (**A**) Mll–myc mouse 2021 (age 24 months): this mouse had no symptoms of disease and serves as a control. (**B**) Mll–AF9 heterozygous mouse 4134 (age 6 months) with overt AML. In this example, the bone marrow is extensively infiltrated with myeloblasts and the liver has significant deposits. The peripheral blood has many circulating myeloblasts. (**C**) Mll–AF9 chimera 4039 (age 7 months) with overt AML similar to heterozygous mouse 4134. (**D**) Mll–AF9 chimera 4019 (age 12 months) showing signs of extramedullary leukaemia with myeloblast deposits in the liver but normal bone marrow pattern. (**E**) Mll–AF9 chimera 4005 (age 12 months). This mouse has ALL, with lymphoblasts in the bone marrow and peripheral blood. The liver has significant lymphoblast infiltration.

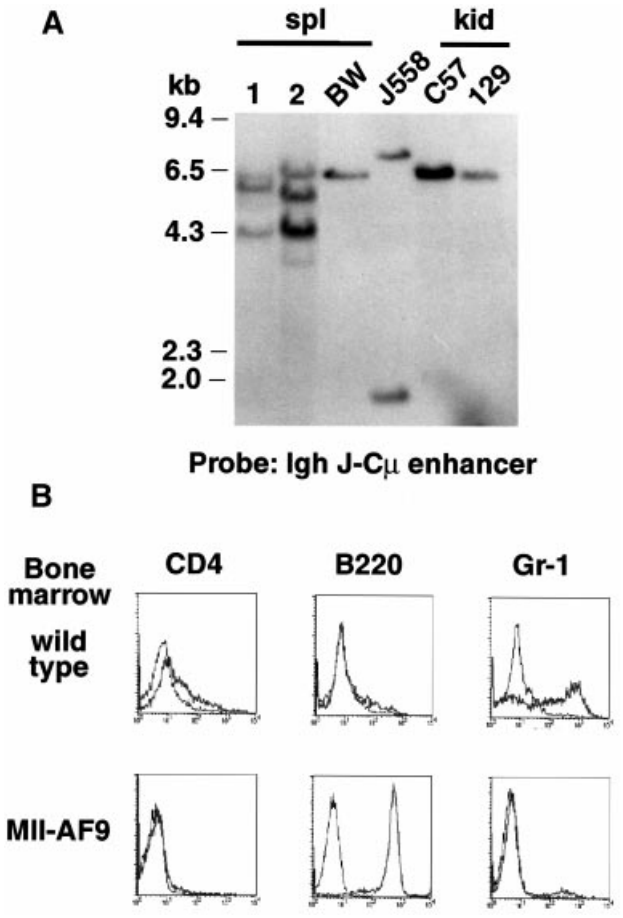

Fig. 4. Mll–AF9 mice develop ALL at low frequency. Two Mll–AF9 chimeric mice showed acute leukaemia (ages 11 and 12 months) with cells morphologically identified as lymphoblasts (see Figure 3E); mouse 1: number 4005, mouse 2: 4020. Bone marrow cells were examined by gene rearrangement using filter hybridization (A) and lymphoid surface marker expression (B) to specify the lineage. (**A**) Filter hybridization. DNA was extracted from the spleen of Mll–AF9 mice, of J558 (myeloma) or BW (T) cells and of C57Bl/6 or 129 kidney. DNA was digested with *Hin*dIII, separated by gel electrophoresis and transferred to nylon membranes. The membranes were hybridized with a radiolabelled immunoglobulin enhancer probe (Neuberger and Williams, 1986) and the washed filters autoradiographed at –70°C with pre-fogged film. (**B**) FACS analysis of Mll–AF9 mouse 4005 (mouse 1): bone marrow cells were prepared as single cell suspensions from mouse 4005 or an age-matched wild-type mouse and were stained with fluorescent antibodies recognizing the T cell marker CD4, the B cell marker B220 and the myeloid marker Gr-1. Graphs represent cell number (*y*-axis) versus fluorescence intensity (*x*-axis). Similar data were obtained with Mll–AF9 mouse 4020 (mouse 2). No evidence of mixed lineage phenotype was found for either of the ALL tumour-bearing Mll–AF9 mice.

confirmed that the tumour was comprised of predominantly B220 antigen-expressing B lymphocytes, which are usually found in low numbers in the bone marrow. In contrast, the normal constituents of the bone marrow (Figure 4B) such as Gr-1- or CD4-positive cells are depleted compared with wild-type mouse controls. A similar pattern of surface marker expression was observed with bone marrow for ALL mouse 2 (data not shown). No evidence of mixed lineage tumours was obtained. Glucose phosphate isomerase (GPI) analysis (Papaioannou and Johnson, 1993) was performed on spleen samples from the two mice with ALL to estimate the contribution originating from the CCB-derived ES cells, injected into the C57Bl/6 blastocysts. Both mice had significant CCB ES cell contribution in the spleen (data not shown) suggesting that the lymphoblastic tumours are of ES cell origin.

Myeloproliferation is observed in Mll–AF9 mice prior to leukaemia occurrence

The normal function of Mll appears to be related to the embryonic developmental plan by affecting *Hox* gene expression profiles (Yu *et al*., 1995) and it may also have a specific role in some aspects of haematopoiesis (Fidanza *et al*., 1996; Hess *et al*., 1997). One of several possible roles for the tumour-specific MLL–AF9 fusion protein is an influence on the molecular interactions which are important for haematopoietic differentiation, which could thus partly explain the predominant association of the chromosomal translocation t(9;11) with myeloid malignancies. A corollary of this is that the lineage from which the majority of tumours arise (i.e. the myeloid lineage) is selectively increased in the heterozygous Mll–AF9 mice. In order to assess this situation, we first examined the surface antigen phenotype of the myeloid tumours. Antibodies binding to surface proteins of myeloid cells (Mac-1 and Gr-1), B-lymphocytes (B220) and T lymphocytes (CD3 and CD4) were used to detect antigen expression on spleen and bone marrow cells. On the whole, tumour cells were found in bone marrow and in spleen, and in mice with AML malignant cells typically expressed Mac-1 and Gr-1 surface markers. Figure 5 shows FACS analysis of the bone marrow and spleen cells from a typical Mll–AF9 heterozygous mouse (mouse number 4299) with AML. The infiltration of Gr-1-positive tumour cells into the spleen population is very marked with the concomitant loss of $B220^+$ B cells and $CD4^+$ T cells, compared with splenocytes from a wild-type mouse. In the bone marrow there was also a striking difference between AML-bearing mice and wild-type mice. In the latter, there are usually ~50% $Gr\text{-}1^+$ cells exhibiting a narrow fluoresence profile corresponding to mature myeloid cells, the fluorescence profile of the Mll–AF9 mouse had a broad appearance consistent with there being mainly immature myeloid cells (Figure 5). A small population of $B220^+$ bone marrow cells found in the wild-type mouse was absent from the Mll–AF9 mouse.

The examination of the $Gr\text{-}1^+/Mac\text{-}1^+$ population in young heterozygous Mll–AF9 mice was used as a basis for determining whether a pre-leukaemic effect on myeloid cell differentiation resulted from the presence of the fusion gene. FACS analysis was carried out on young Mll–AF9 mice using Gr-1 and Mac-1 (myeloid markers), Ter119 (erythrocyte marker), B220 (B cell marker), CD4 (T cell marker), Thy 1.2 (T-lymphocytes, monocytes, B-lymphocytes), Sca-1 (multipotent haematopoietic stem cells, mature myeloid cells), CD44 (leukocytes, erythrocytes, epithelia) and c-Kit (haematopoietic progenitor cells and mast cells). Only effects on the myeloid markers were detectable. Bone marrow from Mll–AF9 heterozygous mice of either 6 days or 5 weeks of age were analysed and compared with either wild-type or Mll–myc mice of similar age (Figure 6). While there was a modest increase in Mac-1/Gr-1 double-positive cells in the Mll–AF9 bone marrow compared with the two controls at 6 days of age

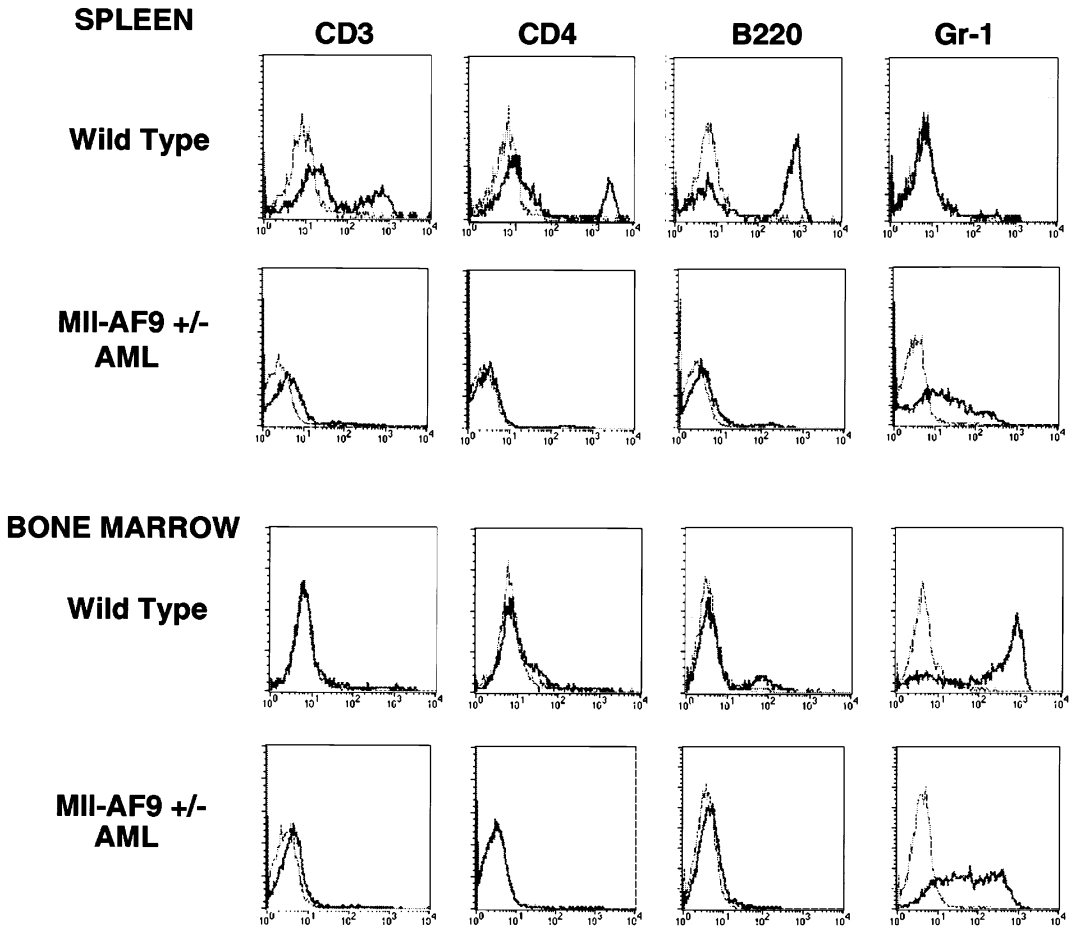

Fig. 5. Surface phenotype of AMLs in Mll–AF9 mice. Mll–AF9 mice with signs of disease were sacrificed and single cell suspensions were made from the spleen and bone marrow. Cells were stained with fluorescent antibodies recognizing the myeloid marker, Gr-1, the T cell markers, CD3 and CD4, and the B cell marker, B220. Twelve out of 24 Mll–AF9 chimeras and 15 out of 28 Mll–AF9 heterozygotes were analysed. In the representative example shown, the Mll–AF9 heterozygous mouse 4299 developed AML (age 4 months) and FACS analysis was performed and compared with a wild-type mouse of comparable age. Graphs represent cell number (*y*-axis) versus fluorescence intensity (*x*-axis).

(35% compared with 21 and 15% for wild-type and Mll–myc mice, respectively), a much larger population (~85%) was observed in the Mll–AF9 mice at 5 weeks compared with either the wild-type (48%) or the Mll–myc (54%) mice. The Gr-1^+ cells in the Mll–AF9 heterozygotes seem to represent a true increase in myeloid lineage cells, rather than overtly leukaemic cells. This is indicated by the narrow fluorescence profile which is similar to those of the control mice (wild type or Mll–myc; Figure 6), as compared with the broad profile found with AML bone marrow.

These data indicate that an early proliferation of cells in the myeloid lineage, driven by the Mll–AF9 fusion protein, occurs prior to the development of malignancy. The increase in proportion of Mac-1/Gr-1 double-positive cells in Mll–AF9 heterozygous mice at 6 days of age compared with wild-type or heterozygous Mll–myc mice was verified using cells obtained from 6 day pups from litters obtained by mating heterozygous Mll–myc or heterozygous Mll–AF9 mice. The bone marrow cells were analysed by FACS and the percentage of Mac-1/Gr-1 double-positive cells determined (Figure 7A). In these litters, there were four Mll–AF9 heterozygous mice, three of which had high Mac-1/Gr-1 double-positive cell counts compared with the +/– Mll–myc or +/+ litter mates. Thus, a high Mac-1/Gr-1 double-positive count is predictive of Mll–AF9 heterozygosity soon after birth. As the number of Mac-1/Gr-1 bone marrow cells naturally increases as mice mature, we examined groups of littermates at between 5 and 12 weeks of age in which environmental disturbances should be less significant. In this analysis, we examined bone marrow cells from seven Mll–AF9 +/– mice, eight wild-type +/+ mice (Figure 7B) and 14 Mll–myc +/– mice (Figure 7C). Of the seven Mll–AF9 heterozygous mice analysed, the proportion of Mac-1/Gr-1 double-positive cells in the bone marrow ranged from 71–93%, with an average of 82%. These figures are consistently higher than those we observed for wild-type mice (Figure 7B), in which the average number of Mac-1/Gr-1 double-positive cells is 52%; one mouse had 77% double-positive bone marrow cells but this was exceptional. Furthermore, the 14 Mll–myc heterozygous mice had an average of 50% Mac-1/Gr-1 bone marrow cells, ranging from 43–60%. The standard errors from these cohorts of mice were calculated at 3, 4 and 1.2% for Mll–AF9, +/+ wild-type and Mll–myc mice, respectively, indicating a significant proliferative advantage for the myeloid cell compartment in the bone marrow of the Mll–AF9 mice. Only the

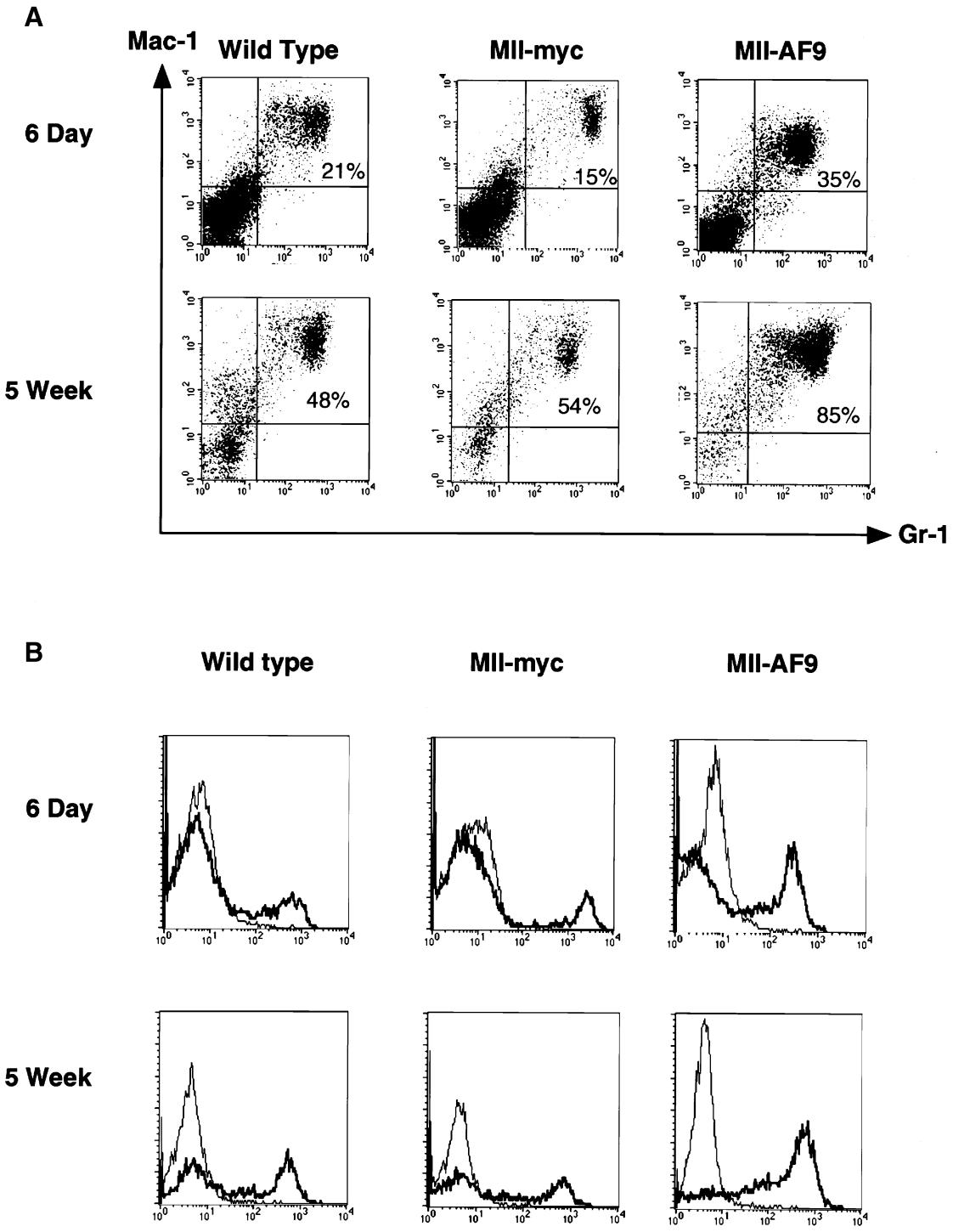

Fig. 6. Young Mll–AF9 mice exhibit increased myeloproliferation. Mll–AF9 heterozygous mice were analysed at 6 days or 5 weeks of age to determine the status of haematopoietic differentiation, prior to the development of overt leukaemia. Mice were selected from litters of Mll–AF9, Mll–myc and wild-type mice at 6 days or 5 weeks of age and single cell suspensions were made from the bone marrow. Cells were stained with an anti-Mac-1 antibody coupled with phycoerythrin (PE) together with an anti-Gr-1 antibody coupled with fluorescein isothiocyanate (FITC) to detect the myeloid population of cells. (**A**) Populations of bone marrow cells expressing both Mac-1 and Gr-1 markers are depicted. Percentage values are shown for the upper right-hand quadrant of Gr-1/Mac-1 double-positive cells. (**B**) Fluorescence profiles of the same bone marrow cell populations for anti-Gr-1 antibody binding. The peaks of fluorescence intensity for wild-type and Mll–AF9 mouse bone marrow cells are similar indicating these are not transformed myeloid cells (compared with the AML bone marrow shown in Figure 5) at the stage of analysis, but rather there is an increase in myeloid cell compartment size.

presence of the *Mll–AF9* gene fusion was capable of eliciting this change, and not the truncation of the *Mll* gene by addition of the myc epitope. Thus, one function of the *Mll–AF9* fusion gene appears to be to confer a proliferative advantage for cells of the myeloid lineage, prior to the onset of AML.

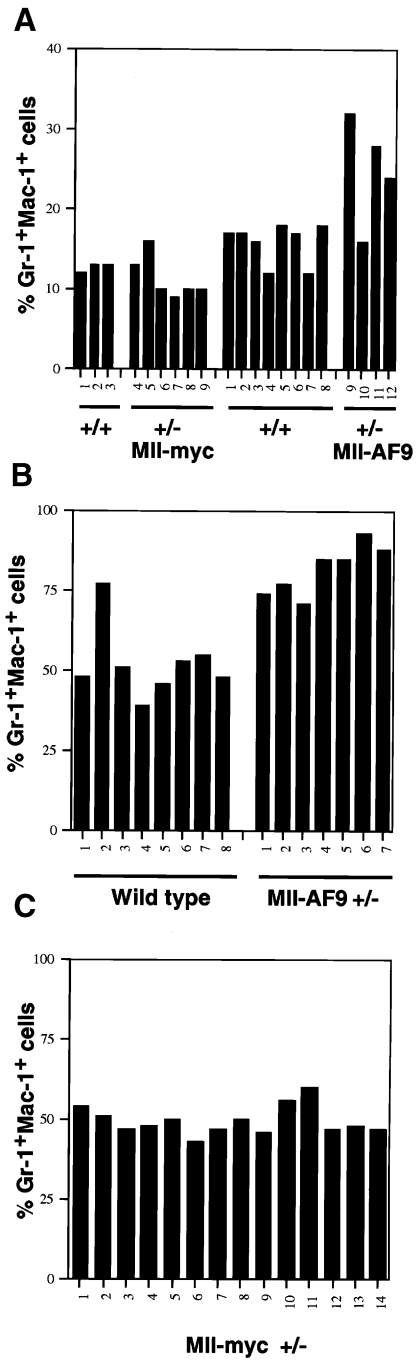

Fig. 7. Mll–AF9 mice exhibit myeloproliferation prior to development of overt leukaemia. The cell populations of bone marrow, from mice aged 6 days or between 5 and 12 weeks, were analysed by flow cytometry. Single cell suspensions were made from the bone marrow and cells stained with an anti-Mac-1 antibody coupled with PE together with an anti-Gr-1 antibody coupled with FITC. Data are expressed as the percentage of double staining cells. Genotypes were determined by filter hybridization of DNA from tail biopsies. (**A**) Histogram showing the percentage of Gr-1$^+$Mac-1$^+$ cells in the bone marrow of individual 6 day old mice from one litter of a heterozygous Mll–myc cross (nine pups) and one litter of Mll–AF9 mated with a wild-type mouse (12 pups). (**B**) Histogram showing the percentage of Gr-1$^+$Mac-1$^+$ cells in the bone marrow of 5- to 12-week-old wild-type (eight mice) or Mll–AF9 mice (seven mice). (**C**) Histogram showing the percentage of Gr-1$^+$Mac-1$^+$ cells in the bone marrow of 5- to 12-week-old Mll–myc mice (14 mice).

Discussion

In vivo fusion of Mll and AF9 in mice provides a model for human cancer caused by chromosome 11q23 translocations

In vivo models of cancer development occurring after chromosomal translocations are needed for a comprehensive understanding of the biological consequences of these aberrant chromosomes and as a means to test possible therapies. The propensity of the *MLL* gene to form fusion genes after chromosomal translocations is important because >15% of all acute leukaemias in man have *MLL* gene fusions and because there is an association of particular *MLL* fusions with particular subtypes of acute leukaemia. For instance, *MLL–AF4/FEL* fusions are typically found in lymphoblastic tumours, whereas MLL–AF9 fusions are typically found in myeloid tumours (Djabali *et al*., 1992; Gu *et al*., 1992; Domer *et al*., 1993; Iida *et al*., 1993; Morrissey *et al*., 1993; Nakamura *et al*., 1993). This implies a specific role for the chimeric protein in determination of the type of leukaemia which develops. Nonetheless, an alternative possibility is based purely on mechanism and accessibility of genes in chromosomes. Thus the ability of *MLL* and *AF9* or *MLL* and *AF4* genomic regions to take part in an inter-chromosomal translocation may be greater in committed myeloid or lymphoid cells, respectively. The alternative models can be assessed using animal models of disease in which gene fusion is either controlled by specific interchromosomal events (Smith *et al*., 1995; van Deursen *et al*., 1995) or by knock-in of the fusion gene partner (Thomas and Capecchi, 1987; Corral *et al*., 1996). We have developed and analysed a model for MLL–AF9-induced leukaemia using homologous recombination to knock-in the *AF9* into the endogenous mouse *Mll* gene (Corral *et al*., 1996). In this model system, we find that the fusion causes tumours in both chimeric mice and mice heterozygous for the *Mll–AF9* fusion gene. The majority of tumours observed in these mice were myeloid as has been reported in humans with chromosomal translocation t(9;11). Two acute lymphoblastic tumours also arise reflecting the relative occurrence of AML:ALL in humans. In the knock-in mice, the ALL cases occurred with a longer latency than the acute myeloid tumours.

The pathological features of the haematopoietic malignancies in the Mll–AF9 mice were very similar to those of human MLL–AF9 associated tumours, providing a striking counterpart between the pathology of the murine and human leukaemias associated with the t(9;11). In man, the most common French-American-British (FAB) subtypes found in this context are AML, M5 (monoblastic/monocytic), M4 (myelomonocytic), and occasionally M1 or M2 (Bain, 1993). Comparable morphologies were evident in the mice used in the investigation. The pattern of organ infiltration described in the mice has close similarities to the clinical findings in man. Thus, enlargement of the superficial lymph nodes, liver and spleen is common in human AML, with 90% of cases showing infiltration of the liver and spleen at autopsy. Extramedullary infiltration of leukaemic myeloblasts is most common in monocytic or myelomonocytic leukaemia. Although usually diffuse, disease may occur as tumorous deposits (granulocytic sarcoma). This may be the initial manifesta-

tion of AML, with the appearance of the disease in the bone marrow following later if the patient remains untreated. Hepatic infiltration by leukaemic myeloblasts in the absence of heavy marrow disease was a recurrent feature of the t(9;11) leukaemia in our mice. Since the mice in our experimental group develop a malignancy which mimics the disease occurring in humans with the translocation t(9;11), they could be used to test therapies which might later be applicable to human cancer management.

An early effect of Mll–AF9 on myeloproliferation

Various fusion partners can be linked to the *MLL* gene after the formation of the abnormal chromosomes (reviewed in Rowley, 1993; Bernard and Berger, 1995; Rubnitz *et al*., 1996b; Gilliland, 1998). However, it is not clear whether the timing of the translocation is important for determining the type of leukaemia which develops or whether the incoming fusion partner has an important role in determining the nature of the tumour. We have found that myeloproliferation occurs prior to the acute myeloid disease that arises in most Mll–AF9 mice. These data therefore suggest that the disease in the majority of animals initiates in a proliferating pool of myeloblasts from which leukaemic precursors arise as a result of secondary mutations. The surviving Mll–AF9 mice without tumours are presumbaly ones in which secondary mutations have not occurred. Thus, our observations with the Mll–AF9 knock-in mice suggest that the role of the *MLL–AF9* fusion is to specify lineage. However, a number of other factors need to be taken into account. No signficant numbers of tumours arose in Mll–myc mice (Corral *et al*., 1996) and further study showed that four out of 27 mice developed AML after two years. Similarly, no significant numbers of mice developed tumours when the *lacZ* gene was fused to exon 3 of *Mll* (Corral *et al*., 1996). However, we have recently found that fusion of *Mll* exon 8 with *lacZ* results in the occurrence of AML (C.L.Dobson, A.J.Warren, A.Forster, R.Pannell and T.H.Rabbitts, unpublished) but with a much longer latency than seen with *Mll–AF9* fusion mice. This implies that formation of a stable MLL fusion protein per se is sufficient for tumorigenesis and that fusion of *Mll* with *AF9* in mice has an accelerating function due to specification of the myeloid lineage cell population.

A further complicating issue is that a small proportion of the Mll–AF9 mice develop lymphoblastic malignancies, as do humans with the t(9;11) translocation. This would suggest that the chromosomal translocation does not usually occur in a committed myeloid progenitor cell but rather in an early progenitor, which retains the ability to differentiate along lymphoid or myeloid lineages and thus form tumours of either type. The latency of the tumours in Mll–AF9 mice suggests that secondary mutations must be occurring prior to overt leukaemia. The corollary is that different mutations are likely to accompany lymphoid as opposed to myeloid leukaemia. Furthermore, at least one of these must occur early for a lymphoid tumour to arise, as the myeloproliferation conferred by the *MLL–AF9* fusion would presumably commit the majority of cells beyond the boundary for lymphoid tumour development. These arguments suggest that other major recurring MLL fusions, such as with AF4/FEL or ENL/LTG19 also function to specify the lineage of the tumours, which explains why there is such a strong association between these fusions and lymphoblastic tumours (Gu *et al*., 1992; Domer *et al*., 1993; Morrissey *et al*., 1993; Nakamura *et al*., 1993; Yamamoto *et al*., 1993; Downing *et al*., 1994; Rubnitz *et al*., 1996a). In the case of *MLL–ENL* fusions, retroviral transduction experiments with mouse bone marrow cells have clearly shown that a gain-of-function mechanism is operational (Lavau *et al*., 1997; Slany *et al*., 1997) and that myeloid differentiation and myeloid transformation are paramount. Therefore, the spectrum of mutations in other genes, which must occur in cells with chromosomal translocations involving the *MLL* gene, may well differ in the context of tumour types with the different *MLL* fusions. The nature of these mutations, the pattern of perturbation of gene expression and the biological consequences of *AF4* fusion with *MLL* are now our major objectives.

Materials and methods

Construction of the targeting vectors and analysis of targeted ES cells

The plasmid constructs for gene targeting have been described (Corral *et al*., 1996). Mll–AF9 comprises a knock-in of the *AF9* short form (Iida *et al*., 1993; Nakamura *et al*., 1993) into exon 8 of the mouse *Mll* gene whilst Mll–myc comprises a knock-in of a short epitope tag into the same location in exon 8. CCB ES cells were transfected with the Mll–AF9 targeting vector DNA as described previously (Warren *et al*., 1994). Homologous recombination was assessed by filter hybridization of *Bgl*II digested DNA using the *Eco*RI–*Xho*I fragment from clone p1.5RXT2 as a probe. The presence of a single insertion site was confirmed with a neomycin probe. Targeted ES cell clones were injected into C57Bl/6 blastocysts, which were transferred into recipient females. Chimeric mice thereby generated were estimated by coat colour. Heterozygous mice were obtained for both *Mll–AF9* and *Mll–myc* fusion genes by crossing chimeric males with wild-type MF1 females.

Mouse pathology

Leukaemia development in mice was characterized by indolent habit, hunched gait and ruffled hair. On developing sickness, mice were culled and a post-mortem carried out. Tissues were taken for analysis and were fixed in 10% formalin and embedded in paraffin wax. Blocks were sectioned and slides were processed and stained with haematoxylin and eosin (H&E) by routine techniques. Blood films were stained with May–Giemsa–Grünwald (MGG) stain. Slides were photographed using Kodak Tungsten 160 film. Glucose phosphate isomerase (GPI) isozyme analysis was performed on spleen samples as described previously (Papaioannou and Johnson, 1993).

Immunoglobulin gene rearrangement assays

DNA was prepared from various tissues using standard procedures. The DNA was digested with *Hin*dIII, and fractionated on 0.8% agarose gels, transferred to nylon membranes (Southern, 1975) and hybridized with randomly labelled probe (Feinberg and Vogelstein, 1983). An immunoglobulin heavy chain enhancer probe originating from the intron between *JH* and *C*μ gene segments (Neuberger and Williams, 1986) was used. For T cell rearrangement, DNA was digested with *Eco*RI or *Hin*dIII and filter hybridizations carried out with probes for either Jβ2 or Cβ1 (Malissen *et al*., 1984).

Flow-cytometric analysis of cell surface marker expression

Single-cell suspensions were prepared from mouse thymus, spleen and bone marrow. Cells (100 μl; 5×10^7/ml) were incubated at 4°C for 20 min in phosphate-buffered saline (PBS) with 5% fetal calf serum (FCS) with the following antibodies (PharMingen, San Diego, CA): anti-Gr-1 and anti-Mac-1 (granulocytes), anti-B220 (B cells), anti-CD3 and anti-CD4 (T cells), anti-Ter119 (erythrocytes), anti-c-Kit (haematopoietic progenitor cells), anti-Thy 1.2 (T lymphocytes, monocytes, B lymphocytes), anti-CD44 (leukocytes, erythrocytes, epithelia) and anti-Sca-1 (multipotent haematopoietic stem cells). Antibodies were conjugated with either fluorescein isothiocyanate (FITC), or phycoerythrin (PE).

Isotype-matched fluoresceinated antibodies were used as controls for non-specific immunofluorescence. After antibody labelling, cells were washed once with PBS containing 5% FCS and resuspended in 1 ml of PBS/5% FCS. Flow cytometric analysis was performed using a FACSCALIBUR (Becton Dickinson, San Jose, CA). Data analysis was performed with Cell Quest (Becton Dickinson). Each phenotype analysis was generated by analysis of 10 000 cells.

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