Heritable Genetic Background Alters Survival and Phenotype of *Mll-AF9*-Induced Leukemias

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Abstract

The MLL-AF9 fusion protein occurring as a result of t(9;11) translocation gives rise to pediatric and adult acute leukemias of distinct lineages, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and mixed phenotype acute leukemia (MPAL). The mechanisms underlying how this same fusion protein results in diverse leukemia phenotypes among different individuals is not well understood. Given emerging evidence from genome-wide association studies (GWAS) that genetic risk factors contribute to *MLL*-rearranged leukemogenesis, here we tested the impact of genetic background on survival and phenotype of a well-characterized *Mll-AF9* knockin mouse model. We crossed this model to five distinct inbred strains (129, A/J, C57BL/6, NOD, CAST), and tested their F1 hybrid progeny for dominant genetic effects on *Mll-AF9* phenotypes. We discovered that genetic background altered peripheral blood composition, with *Mll-AF9* CAST F1 demonstrating significantly increased B lymphocyte frequency while the remainder of the strains exhibited myeloid-biased hematopoiesis, similar to the parental line. Genetic background also impacted overall survival, with *Mll-AF9* A/J F1 and *Mll-AF9* 129 F1 having significantly shorter survival, and *Mll-AF9* CAST F1 having longer survival, compared to the parental line. Furthermore, we observed a range of hematologic malignancies, with *Mll-AF9* A/J F1, *Mll-AF9* 129 F1 and *Mll-AF9* B6 F1 developing exclusively myeloid cell malignancies (myeloproliferative disorder (MPD) and AML) whereas a subset of *Mll-AF9* NOD F1 developed MPAL and *Mll-AF9* CAST F1 developed ALL. This study provides a novel *in vivo* experimental model to evaluate the underlying mechanisms by which *MLL-AF9* results in diverse leukemia phenotypes and provides definitive experimental evidence that genetic risk factors contribute to survival and phenotype of *MLL*-rearranged leukemogenesis.

Introduction

Chromosomal rearrangements involving the *mixed-lineage leukemia 1* (*MLL1*) gene, also known as *Lysine [K]-specific methyltransferase 2A* (*KMT2A*), generate fusion proteins causing aggressive acute leukemias in infants, children and adults. *MLL*-rearranged

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leukemias comprise ~10% of acute leukemias across all age groups[1]. Patients with *MLL*-rearranged leukemias generally have a poor prognosis, with high-risk treatment options and frequent relapse. This underscores an unmet need for novel therapeutic approaches to improve outcomes in *MLL*-rearranged leukemia.

Emerging evidence from GWAS studies suggest that heritable genetic polymorphisms can modify the risk of *MLL*-rearranged leukemia[2–4]. To definitively test causation and build upon these findings toward development of novel therapeutic targets, use of *in vivo* mouse models of *MLL*-rearranged leukemia is ideal. However, the vast majority of genetically engineered mouse models of human leukemia are studied on a single inbred genetic background, C57BL/6, despite genetic variability having been recognized as an important modifier of leukemogenesis in mouse models[5–8].

The first MLL fusion protein to be modeled as an endogenous knockin allele in mice was *Mll-AF9* (t(9;11))[9]. After an early myeloproliferative phase, *Mll-AF9* mice primarily succumb to AML, and only in rare cases to ALL[10,11]. This is notably distinct from human disease, where *MLL-AF9* is found in both B-cell ALL (B-ALL) and AML in infants and children, and AML in adults[12]. Here, we have utilized this well-characterized *Mll-AF9* knockin mouse model to test the extent to which dominant genetic alleles modify *Mll-AF9*-driven leukemogenesis using genetically diverse mouse strains[13,14].

Results and Discussion

To determine the role of genetic diversity in *MLL-AF9* leukemia, we crossed the *Mll-AF9* knock-in mouse model[9] with the five distinct inbred strains A/J, C57BL/6 (B6), 129S1/SvlmJ (129), NOD/ShiLtJ (NOD) and CAST/EiJ (CAST). We studied F1 hybrid mice heterozygous for *Mll-AF9* from these crosses versus the parental genetic background (Fig. 1A). F1 hybrid mice heterozygous for *Mll-AF9* from all crosses were born at expected Mendelian frequencies (Supp. Table 1). The *MLL-AF9* parental strain has been maintained as it was historically, on a mixed B6 and 129 background. While the parental *Mll-AF9* strain has previously been demonstrated to develop leukemia around ~6 months of age, detectable myeloid proliferation has been observed by 8 to 10 weeks of age[9,11]. Consistent with this observation, analysis of peripheral blood (PB) of parental *Mll-AF9* mice compared to *Mll*^WT littermates at 8 weeks of age showed significantly increased myeloid cell frequency with concomitant reduction in T cell frequency (Fig. 1B). This same phenotype was observed in *Mll-AF9* A/J F1, *Mll-AF9* B6 F1, *Mll-AF9* 129 F1 and *Mll-AF9* NOD F1 mice. In contrast, myeloid cell frequency in *Mll-AF9* CAST F1 mice was not significantly different from *Mll*^WT mice but instead a significant increase in B cell frequency and reduction in T cell frequency were observed. To determine whether this observation was based on baseline differences in the CAST genetic background, we examined PB composition in wild-type CAST mice versus the other strains used in this study. We found that wild-type CAST mice have no differences in PB composition compared to the other strains (Supp. Fig. 1A), suggesting that this phenotype may be a consequence of *Mll-AF9* expression. To evaluate baseline differences in PB composition between the F1 strains, we examined PB of *Mll*^WT littermates at 8 weeks of age from a subset of the F1 crosses (*Mll*^WT A/J F1, *Mll*^WT B6 F1 and *Mll*^WT CAST F1). We found that certain F1 strains differ in baseline PB composition

compared to the parental background (Supp. Fig. 1B). Mll^{WT} CAST F1 have reduced frequency of myeloid cells, both Mll^{WT} A/J F1 and Mll^{WT} CAST F1 have higher frequency of B cells, and Mll^{WT} A/J F1 have reduced frequency of T cells. While this suggests that the F1 hybrid strains do differ in baseline PB composition, these differences do not explain the reduced myeloid cell frequency and increased B cell frequency observed uniquely in the *Mll-AF9* CAST F1 mice (Fig. 1B). This observation further supports that differences between strains are *Mll-AF9*-dependent.

Monitoring PB composition until pathology developed revealed that *Mll-AF9* CAST F1 mice maintained significantly reduced frequency of myeloid cells and increased frequency of B cells with aging compared to the parental *Mll-AF9* strain (Fig. 2A). In concordance with previous studies[11], median survival in the parental *Mll-AF9* strain was 225 days. In contrast, *Mll-AF9* CAST F1 had a longer median survival (361 days; $P = 0.079$) and *Mll-AF9* A/J F1 and *Mll-AF9* 129 F1 that had significantly shorter median survival (172 days; $P = 0.0071$, and 178 days; $P = 0.0179$, respectively) (Fig. 2B). Examining *Mll-AF9* transcript expression by real-time PCR in moribund mice across the 6 strains backgrounds revealed no significant differences in *Mll-AF9* expression (Fig. 2C). This data suggests that genetic background can alter the development and progression of leukemia caused by *Mll-AF9*, independent of *Mll-AF9* transcript expression level.

Characterization of the hematologic malignancies that developed in these strains also revealed genetic background-dependent distinctions. While this in-depth phenotyping was performed on only a subset of moribund animals shown in Fig. 2B, no other co-morbidities were observed in any of the *Mll-AF9* mice during post-mortem analysis. Consistent with previous studies[9,11], parental *Mll-AF9* mice developed myelomonocytic AML with 100% penetrance and median latency of 210d (Fig. 3A) characterized by leukocytosis and thrombocytopenia (Fig. 3B), splenomegaly (Fig. 3C), >20% blasts in the BM (Fig. 3D), and abundant myeloid cell infiltration into the spleen and liver (Fig. 3D,E). Strains with shorter median survival (*Mll-AF9* A/J F1 and *Mll-AF9* 129 F1) developed either AML (median latency 65d and 170d, respectively) or a MPD-like disorder (median latency 196d and 182d, respectively) characterized by splenomegaly, <20% immature or blast-like cells in the BM, and high frequency of Gr1-expressing granulocytes in the BM (Fig. 3E). In the *Mll-AF9* CAST F1 strain exhibiting the longest survival, 50% of mice developed AML (median latency 367d) and 50% of mice were found to have ALL (median latency 536d) characterized by leukocytosis, splenomegaly, spleen and liver infiltration, and high frequency of B220[lo] c-Kit^{+} blast cells in the bone marrow and spleen. While CAST mice have not been broadly studied in the context of leukemia or other cancer development, CAST F1 mice do have increased tumor growth in a model of neuroendocrine prostate carcinoma[15], suggesting that the increased survival we have observed is not due to a general tumor-resistant genetic background. Of note, one individual *Mll-AF9* NOD F1 mouse was found in our study to develop MPAL (238d) characterized by leukocytosis, thrombocytopenia, spleen and liver infiltration, and bi-phenotypic B220^{+} CD11b^{+} blast cells in the bone marrow. As NOD mice are a polygenic model for autoimmune type 1 diabetes and exhibit aberrant immunophenotypes it is interesting to speculate that this may influence development of a bi-phenotypic leukemia. As noted above, *Mll-AF9* transcript expression level did not significantly differ between strains (Fig. 2C) and did not significantly differ

when this data was re-analyzed to group samples based on hematologic malignancy diagnosis (MPD vs. AML vs. MPAL vs. ALL) (Supp. Fig. 2). This data supports that genetic background can alter leukemia phenotypes caused by *Mll-AF9*, independent of *Mll-AF9* transcript expression level.

By introducing genetic variation into the *MLL-AF9* knockin mouse model, our work has identified that disease latency and leukemia phenotype are significantly affected by heritable genetic variants segregating among common inbred strains, and suggests the presence of specific, dominant-acting modifier alleles in one or more strains. These findings support that genetic background differences may play a role in how and why leukemogenesis resulting from a common fusion oncogene can result in distinct etiology among different individuals. As epigenetic dysregulation is a critical driver of *MLL*-rearranged leukemia[16,17], we posit that altered survival and leukemia phenotypes may be related to differences in epigenetic or chromatin state in genetically diverse mice. This is also supported by GWAS identification of single nucleotide polymorphisms in *ARID5B*, encoding part of the histone H3K9me2 demethylase complex, that modify risk for *MLL*-rearranged early childhood leukemia[4]. More broadly, apart from the presence of the fusion oncogene, data from our lab and others support that other factors strongly influence the specific outcome, including the cell type-of-origin[18–20], the developmental stage and context in which the chromosome translocation occurs[21,22]. Differing propensities to incur secondary mutations that accelerate *Mll-AF9*-mediated leukemias may be an additional variable, which we have not examined here. Our study also has not definitively determined whether differences in disease latency and leukemia phenotype are mediated by leukemia cell-intrinsic or leukemia cell-extrinsic mechanisms (ex. systemic or immune differences between strains), or both. These cellular and molecular mechanistic studies will be key future work to determine the nature of the differences in leukemia lethality. Importantly, this study included five of the eight founder strains of the Collaborative Cross (CC) and Diversity Outbred (DO) mouse populations[23,24], complementary resources that enable one to model human genetic diversity and map genetic modifiers that underlie phenotype differences in the population. Future studies will take advantage of these powerful tools to map the genetic determinants of leukemia susceptibility and phenotype, with the goal of identifying novel gene targets for the development of new therapies for *MLL*-rearranged leukemia.

Methods

Experimental animals

Kmt2a[tm2(MLLT3)Thr]/KsyJ (referred to as *Mll-AF9*, stock no: 009079) mice were obtained from, and aged within, The Jackson Laboratory. The *Mll-AF9* model was created on a 129P2/OlaHsd background, crossed with C57BL/6NCrl females for four generations, and has been maintained at The Jackson Laboratory since 2012 on a mixed C57BL/6 and 129S1/SvlmJ background by breeding with B6129PF1/J. The *Mll-AF9* original strain was crossed to the A/J, C57BL/6 (B6), 129S1/SvlmJ (129), NOD/ShiLtJ (NOD) and CAST/EiJ (CAST) strains to create F1 generation experimental mice. Male and female F1 progeny from each strain cross were included in the studies and monitored from 8 weeks of age until moribund. Female and male mice were analyzed for PB CBC data at 6 months of age. The Jackson

Laboratory's Institutional Animal Care and Use Committee (IACUC) approved all experiments.

Peripheral blood analysis

PB was collected from mice via retro-orbital sinus and red blood cells were lysed before staining mature lineage markers: B220 (clone RA3-6B2), CD3e (clone 145-2C11), CD11b (clone M1/70), Gr-1 (clone RB6-8C5). Stained cells were analyzed on an LSRII (BD) and populations were analyzed using FlowJo V10. Differential blood cell counts were obtained from PB using an Advia 120 Hematology Analyzer (Siemens).

Mll-AF9 transcript expression

RNA was isolated from whole BM from moribund mice using RNeasy mini kit (Qiagen) and quantitative PCR was performed using RT^2 SYBR Green ROX qPCR Mastermix (Qiagen) on a QuantStudio 7 Flex (Applied Biosystems). *Mll-AF9* expression level was calculated relative to the housekeeping gene *B2M*. Primer sequences: *MllAF9* For: 5'-TGTGAAGCAGAAATGTGTGG, *MllAF9* Rev: 5'-TGCCTTGTCACATTCACCAT, *B2M* For: 5'-TTCTGGTGCTTGTCTCACTGA, *B2M* Rev: 5'-CAGTATGTTCGGCTTCCCATTC.

Analysis of moribund mice

Moribund mice identified by declining health status were euthanized and PB, spleen, liver, and BM harvested. Single-cell suspensions of PB, spleen, and BM were analyzed by flow cytometry for mature lineage markers and c-Kit (clone 2B8), using an LSRII (BD) and populations were analyzed using FlowJo V10. Differential blood cell counts were obtained from PB using an Advia 120 Hematology Analyzer (Siemens). Cytospin preparations of whole BM MNCs were stained with May–Grunwald–Giemsa stain. Liver and spleens were fixed for 24 h in 10% buffered formalin phosphate, embedded in paraffin, and sections were stained with H&E. Histological images of stained BM, liver, and spleen were captured on a Nikon Eclipse C*i* upright microscope with SPOT imaging software (v.5.6).

Statistical analysis

Overall survival, Log-rank (Mantel–Cox) test was performed on Kaplan–Meier survival curves. Statistical analysis of non-survival data was performed by Brown-Forsythe one-way ANOVA test followed by Dunnett's multiple comparisons test. All statistical tests, including evaluation of normal distribution of data and examination of variance between groups being statistically compared, were assessed using Prism 8 software (GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- *Mll-AF9* knockin mice crossed to five inbred strains, tested F1 progeny

- Genetic background alters peripheral blood lineage composition

- Genetic background impacts overall survival

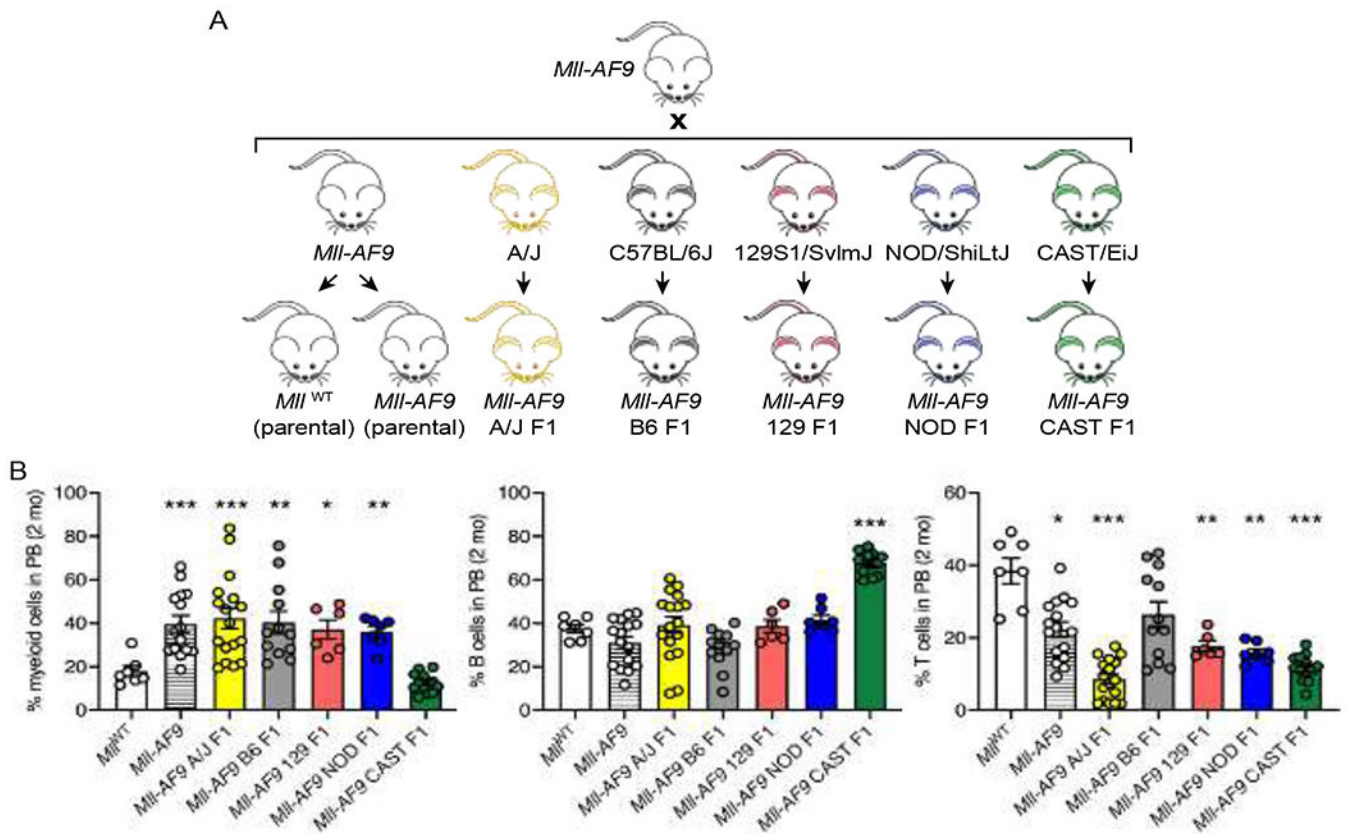- Genetic background alters types of hematologic malignancies observed

Figure 1. Genetic background-dependent differences in hematopoietic lineage composition in *Mll-AF9* knockin model.

(A) Breeding strategy to create cohorts of genetically distinct F1 animals heterozygous for the well-characterized *Mll-AF9* knockin allele[9]. (B) Frequency of myeloid, B and T cells in the peripheral blood (PB) of indicated strains at 2 months of age. Dots represent individual mice (*Mll*WT, n=7; *Mll-AF9*, n=15; *Mll-AF9* A/J F1, n=17; *Mll-AF9* B6 F1, n=12; *Mll-AF9* 129 F1, n=6; *Mll-AF9* NOD F1, n=7; *Mll-AF9* CAST F1, n=13). Bars represent mean +/− SEM. *P<0.05, **P<0.01, ***P<0.001 compared to *Mll*WT parental values as determined by Brown-Forsythe ANOVA with Dunnett's T3 multiple comparisons test.
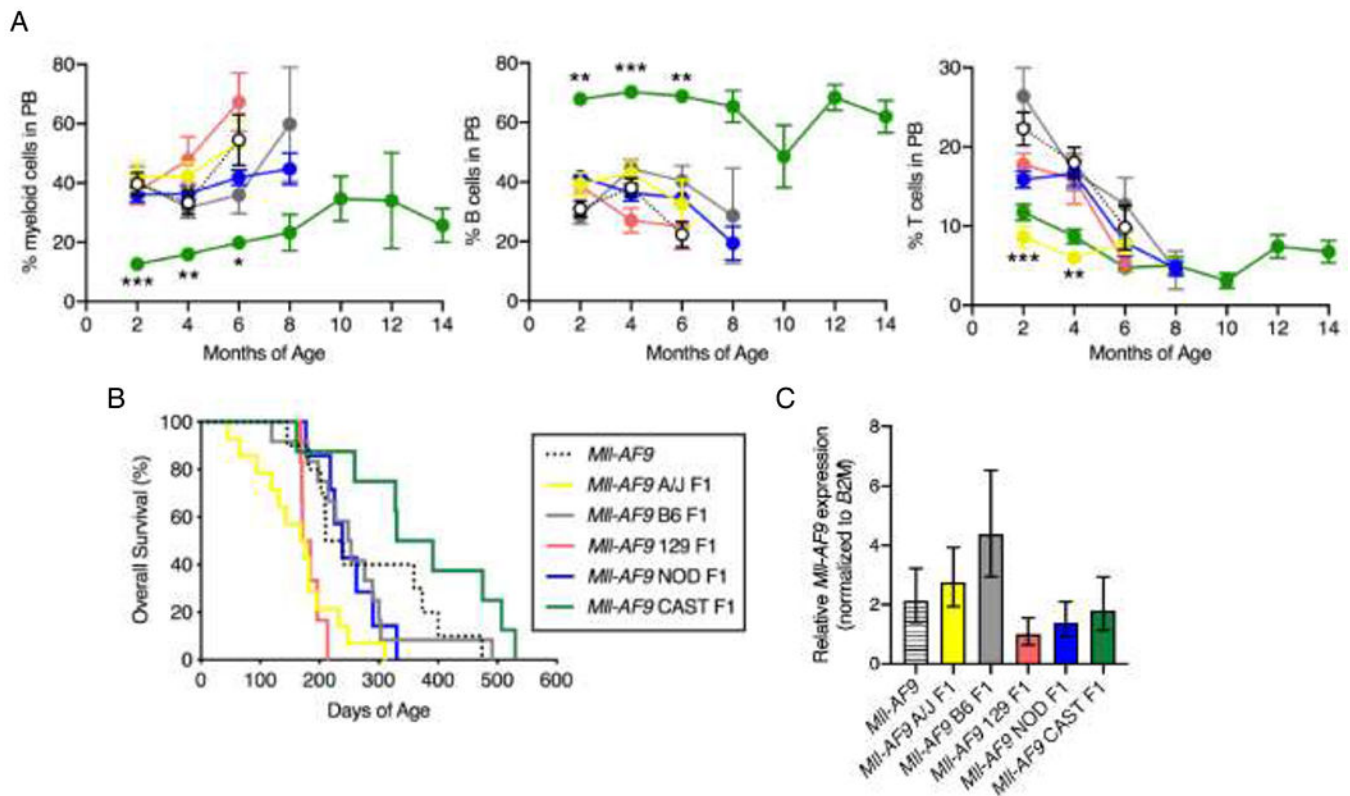
Figure 2. Genetic background-dependent progression to leukemia and overall survival mediated by *Mll-AF9*.

(A) Frequency of myeloid, B and T cells in the PB of indicated strains from 2 months of age until moribund. Dots represent mean +/− SEM (*Mll-AF9*, n=15; *Mll-AF9* A/J F1, n=17; *Mll-AF9* B6 F1, n=12; *Mll-AF9* 129 F1, n=6; *Mll-AF9* NOD F1, n=7; *Mll-AF9* CAST F1, n=13). **P<0.01, ***P<0.001 compared to parental *Mll-AF9* values as determined by Brown-Forsythe ANOVA with Dunnett's T3 multiple comparisons test. (B) Overall survival of *Mll-AF9* mice (*Mll-AF9*, n=10; *Mll-AF9* A/J F1, n=14; *Mll-AF9* B6 F1, n=12; *Mll-AF9* 129 F1, n=6; *Mll-AF9* NOD F1, n=7; *Mll-AF9* CAST F1, n=8). (C) Relative *Mll-AF9* expression assessed by real-time PCR. Bars represent mean +/− SEM (*Mll-AF9*, n=3; *Mll-AF9* A/J F1, n=3; *Mll-AF9* B6 F1, n=3; *Mll-AF9* 129 F1, n=2; *Mll-AF9* NOD F1, n=3; *Mll-AF9* CAST F1, n=3). *P*=0.3480 by Brown-Forsythe ANOVA.
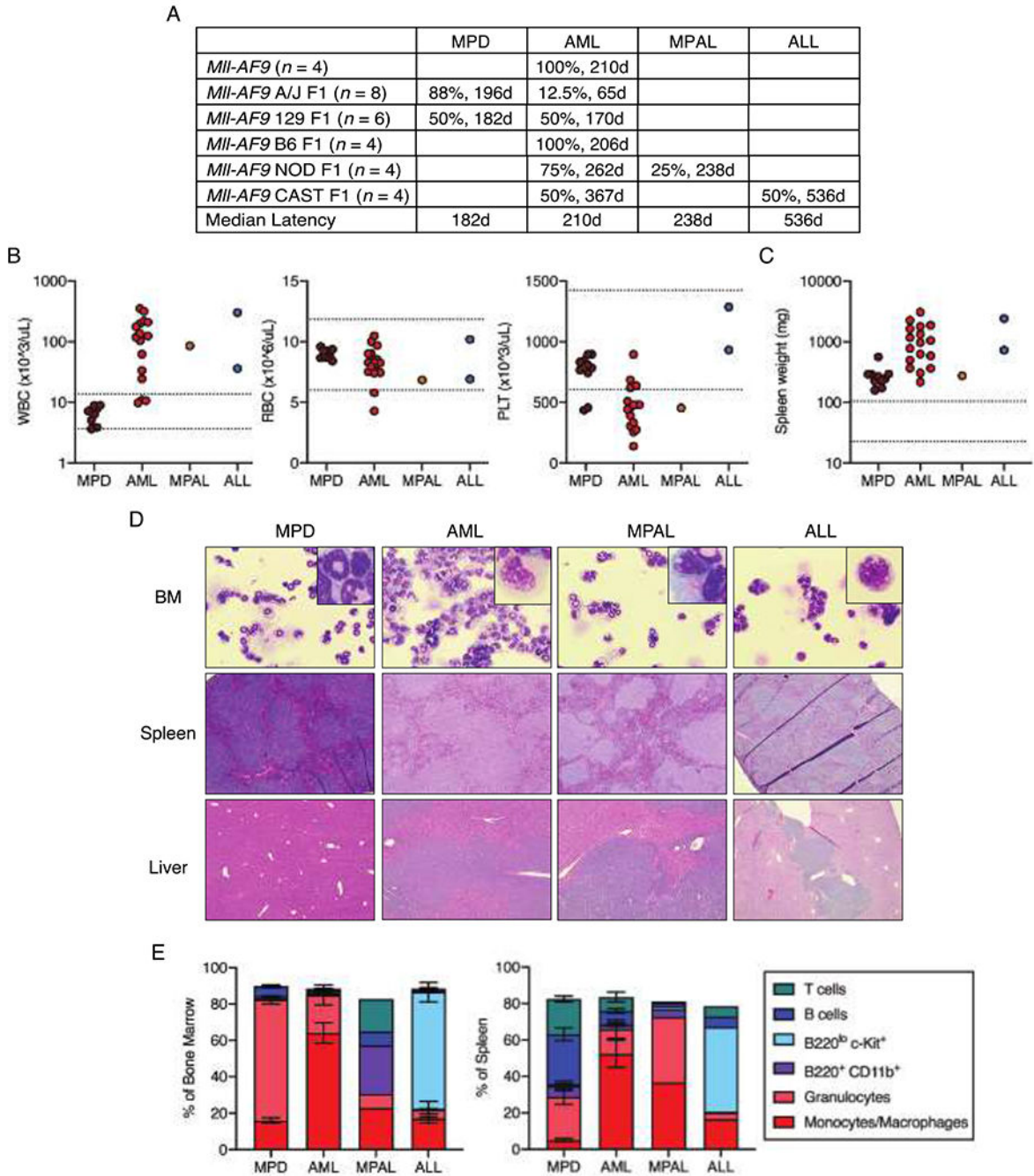
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	MPD	AML	MPAL	ALL
Mll-AF9 (*n* = 4)		100%, 210d		
Mll-AF9 A/J F1 (*n* = 8)	88%, 196d	12.5%, 65d		
Mll-AF9 129 F1 (*n* = 6)	50%, 182d	50%, 170d		
Mll-AF9 B6 F1 (*n* = 4)		100%, 206d		
Mll-AF9 NOD F1 (*n* = 4)		75%, 262d	25%, 238d	
Mll-AF9 CAST F1 (*n* = 4)		50%, 367d		50%, 536d
Median Latency	182d	210d	238d	536d

Figure 3. *Mll-AF9*-driven leukemia phenotype varies based on genetic background.
(A) Frequency and median latency in mice diagnosed with myeloproliferative disorder (MPD), acute myeloid leukemia (AML), mixed phenotype acute leukemia (MPAL), and acute lymphoblastic leukemia (ALL). (B) WBC, RBC and platelet (PLT) counts in moribund mice. Dots represent individual mice. MPD, n=10; AML, n=16; MPAL, n=1; ALL, n=2. Dotted horizontal lines represent range of values considered to be normal in C57BL/6J mice[25]. (C) Spleen weight in moribund mice. Dots represent individual mice. MPD, n=10; AML, n=17; MPAL, n=1; ALL, n=2. Dotted horizontal lines represent range of values

considered to be normal in C57BL/6J mice[25]. (D) Representative histological staining of bone marrow (BM) (Giemsa-stain, 40x, inset: 100x), spleen (H&E, 4x) and liver (H&E, 4x) in mice with MPD, AML, MPAL or ALL. (E) Flow cytometric analysis of indicated cell types in bone marrow and spleen of moribund mice. Bars represent mean +/− SEM of MPD, n=10; AML, n=14; MPAL, n=1; ALL, n=2.