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Frequency of Leukemic Initiating Cells does not depend on the xenotransplantation model used

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AML was the first disease where Leukemia initiating cells (LICs) were identified using the NOD/SCID (NS) xenotransplantation model [(1, 2)]. These cells were originally reported to be rare [(2)]. Recent data in melanoma indicate that the frequency of tumor initiating cells (TICs) increases dramatically when more permissive immunodeficient NOD/SCID/ Il2 receptor γ chain null (NSG) mice are used [(3)] and contrary to the findings of Boiko et al. [(4)], TICs in melanoma might not be purified [(5)] thus questioning the existence of TICs in melanoma. A study of pancreatic, non-small cell lung, and head and neck carcinoma shows that despite a 10-fold increase in the frequency of TICs in NSG compared to NS the frequency of TICs remains low[(6)]. Recently, we demonstrated that LICs are also present in the CD34+CD38+ fraction, [(7)] contrary to our initial findings [(2)]. We therefore re-investigated the issue of the frequency of LICs comparing the originally used NS and the new NSG model.

Here, we first report that 14 AML samples that did not engraft in NS mice could still not engraft in NSG (see table supplement 1a, Supplement Figure 1). Despite recent data [(8-10)] including our own showing that higher level of engraftment could be achieved in NSG compared to NS mice, the NSG model does not seem to be significantly more permissive, by enabling the engraftment of "non-engrafter" AML samples tested originally in NS [(11)]. For patients with AML that engraft (see table Supplement 1b), we estimated the LIC frequency using limiting dilution analysis (LDA). Figure 1 shows the results of 11 AML samples where the frequency of LICs was compared side by side between NS and NSG (Figure 1 and table supplement 2). It thus appears that the frequency of LICs varies between patients in both models. For patients where the frequency of LICs in NSG was higher than 1 in 50,000 cells (patients 6 to 11), the absolute number of LICs increased by 12 to 111 fold in NSG compared to NS. This difference was less than 5 fold for patients with lower frequency (LICs > 1 in 50,000) (patients 1 to 5). Our results suggest that when lower numbers of cells were injected a more pronounced difference was seen between the two models. Recently, we showed that the residual immune cells present in NS have a profound effect on the engraftment of antibody labeled and sorted CD38+ cells. When using an anti-NK treatment

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or a more permissive NSG model a drastic reduction of the clearance of these CD38+ cells [7] was seen. Consequently, we decided to investigate the impact of residual immune cells on the difference in frequency observed. For four patients (5, 7, 8 and 9) we compared the LICs frequency using NS versus NS pre-treated with anti-122 antibody versus NSG mice. The frequency of LICs between NS plus anti-CD122 and NSG was similar for patient 7 and 8. No difference was observed between the three mouse models for patient 5 but interestingly for patient 9, even when NS were pre-treated with anti-CD122, they were less sensitive than NSG in detecting LICs. Altogether, this indicates that differences in frequency of LICs between NS and NSG could be at least partially due to the presence of residual innate immune cells in NS (Supplement figure 2 and table supplement 3).

It is also clear that despite differences in absolute frequency of LICs between the two mouse models, the same heterogeneity in the LICs frequency exist between patients. Patients can be classified as having low (patients 1 to 6 in NS and 1 to 5 in NSG) or high frequency of LICs (7 to 11 NS or 6 to 11 in NSG).

Recent data demonstrated the role of the complex CD47/SIRP-a in controlling phagocytosis of normal cells by macrophages and that both mouse and human myeloid leukemia cells have constitutive upregulation of CD47 in order to evade macrophage killing [12]. One possible explanation for the difference observed between “non-engrafter” and “engrafter” groups could be due to differences in the expression level of CD47. Supplement Figure S3A shows that the mean fluorescence intensity of CD47 is variable in both groups with no significant difference between the two groups. We also show that for the “engrafter” patients the mean fluorescence intensity between the bulk mononuclear cells and the LIC fraction is equivalent (Figure S3B) confirming previous data [12]. We thus examine whether the level of CD47 expression could differ between low and high LICs frequency groups. As shown in Figure S3C, CD47 level does not seems to correlate with LIC frequency. Additional experiments will be required to determine the precise requirement of CD47 on human AML-LICs.

One study reported a correlation between patient clinical outcome and the frequency of LICs quantified by phenotype based on the percentage of the $CD34^{+}CD38^{-}$ fraction. [13] However, we recently reported that LICs are not restricted to this phenotypically defined fraction [7,14]. We therefore tested in our cohort of patients, whether we could confirm this correlation. We observed a statistical difference in the overall survival between AML patients that engraft in NSG versus the ones that are unable to engraft confirming our previous data in NS [7]. We nevertheless were unable to find a correlation between the high and low frequencies of LICs (even if we varied the cut off between low and high frequency) (Figure 2). Based on our observations, we suggest that further sub grouping of AML patients based on their absolute frequency of LICs might not be achievable. One might consider that intrinsic properties of LICs (like chemotherapy resistance) rather than absolute number might have a more important impact on the prognosis of the patient.

To provide additional clues on the importance of LICs in driving the disease progression, one will need to study the frequency, phenotype and genotype of the LICs for the same patient overtime. Most of the data on AML patients have been so far generated using patients’ samples at diagnosis. To begin to address this issue, we evaluated the LIC frequency in three patient samples (9, 11 and 12) at both diagnosis and relapse. For patient 9 the frequency of LICs increased significantly between diagnosis and relapse in both mouse models (Supplement Table 4). For patients 11 and 12 no significant differences were observed between diagnosis and relapse. The lack of difference in patients 11 and 12 might be due to the aggressiveness of the leukemia. Further studies with a larger cohort of patients

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might provide clues on the potential clonal evolution and genotype or phenotype changes that might occur in LICs during disease progression.

In AML, the major pitfall of the original NS model has been the underestimation of the presence of phenotypically defined LICs sub fractions other than the $CD34^{+}CD38^{-}$ and thus of the heterogeneity of the LICs [7,14]. Regardless of the LICs phenotype, the differences reported here in the frequency of LICs are also partially due to residual innate immune effect. Beyond this, our data still show that a high heterogeneity in the LICs frequency exists between patients in both mouse models used and even in the most permissive NSG model, LICs remains rare (between 0.28 to 0.00002%). Although NSG is the new “model of choice” for studying TICs, it is certainly not yet optimal for some AML samples (i.e. “non-engrafters”). It is worth mentioning that a new model of transgenic NSG with human SCF-GM-CSF and IL.3 (known as NSGS) has recently been reported [15]. Whether this new model will allow the engraftment of every single AML sample still needs to be evaluated.

In conclusion, the development of the NSG model and the comparison with NS mice have provided important insights into the limitations of using a specific xenotransplant model and demonstrated that caution is required. Despite these limitations, this should not detract from the initial demonstration in NS mice of the existence of LICs in AML. Thus AML, in contrast to melanoma, is driven by rare LICs and their capacity to recapitulate the disease *in vivo* depends on their intrinsic biological properties rather than the xenotranplant model used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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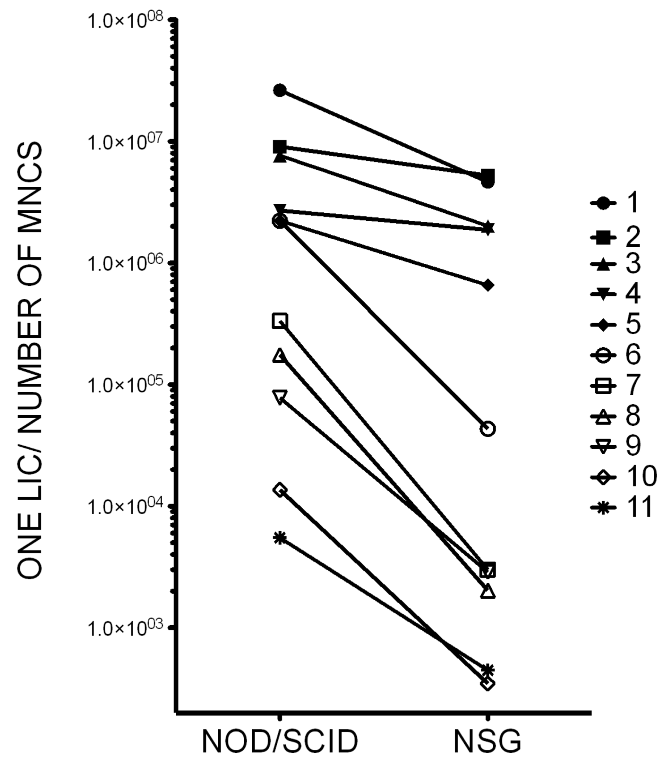

Figure 1.

Frequency of LICs obtained in NOD/SCID and NSG. Five different doses of AML cells were injected in a group of 5 mice per dose. After 12 weeks, the level of engraftment was evaluated by FACS. For each engraftment of human $CD45^{+}CD33^{+}CD19^{-}$ higher than 0.1% the mouse was consider positive. Frequency of LICs was calculated using the extreme limiting dilution analysis (ELDA) software.

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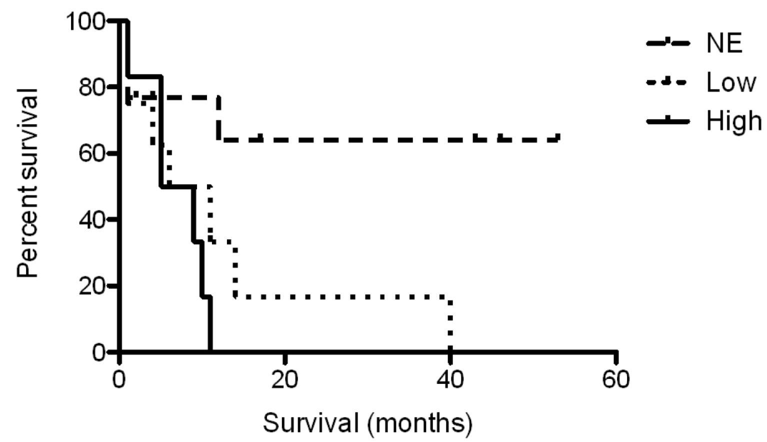

Figure 2.
Overall survival curve of non-engrafters and low and high LICs frequency groups in months. The difference between non-engrafters and low frequency LICs p: < 0.034. No significant difference between low and high frequency of LICs (median 8.5 and 7 months respectively).