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Revisiting CLEC12A as Leukaemic Stem Cell Marker in AML: Highlighting the Necessity of Precision Diagnostics in Patients Eligible for Targeted Therapy

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Summary

Targeted therapy directed against rare disease-propagating leukaemic stem cells (LSCs) is a promising prospect for improving the outcome of acute myeloid leukaemia (AML) patients. Thus, distinguishing LSCs from normal haematopoietic stem and progenitor cells (HSPCs) is essential. The CLEC12A receptor has been proposed as a specific marker of LSCs, and consequently as an appealing treatment target. To explore the role of CLEC12A in further detail, we investigated whether a sorting strategy based on the activity of aldehyde dehydrogenase and CLEC12A expression could separate residual normal HSPCs from LSCs in bone marrow from 5 AML patients. We demonstrate that this distinction was possible in 2/5 cases, however with evidence of pre-leukaemic mutations in the CLEC12A- stem cells in one case. In contrast, cytogenetic and/or molecular aberrations were detected in both the CLEC12A+/- cell subsets in 3/5 AML cases studied. Furthermore, targeted next generation sequencing (NGS) of the sorted cell subsets revealed a pronounced clonal heterogeneity in the CLEC12A- cells suggestive of the leukaemia often originating in this immature cell subset. In conclusion, we provide proof-of-concept that precision diagnostics employing targeted cytogenetic/NGS-based analyses on highly purified cell subsets could be a powerful tool for selecting patients eligible for LSC-directed therapy.

Keywords

Leukaemic stem cells, acute myeloid leukaemia, hMICL, ALDH, precision medicine

Introduction

There is an unmet need to improve the outcome of acute myeloid leukaemia (AML) patients, and novel targeted treatment strategies directed against e.g. cell surface proteins, recurrent mutations and signalling pathways, are rapidly being developed and tested in clinical trials (Wei & Tiong, 2017). However, given the extreme biological heterogeneity seen in AML, a prerequisite for such treatments to become

successful will, to a large extent, depend on the selection of the right patients for the relevant combination of targeted therapy.

In AML, the neoplastic cells are believed to be hierarchically organised, with rare disease-propagating leukaemic stem cells (LSCs) residing at the apex, and evidence of the existence and clinical relevance of such LSCs has been subject to an extensive amount of research over the past two decades (Bonnet, 2005; Yanagisawa *et al*, 2016; Thomas & Majeti, 2017). Current literature suggests these particular cells to possess stem cell properties in terms of quiescence, self-renewal capacity and an increased resistance to chemotherapy (Griessinger *et al*, 2014), and LSCs are thought to be responsible for relapse in the vast majority of AML patients (Bonnet, 2005). It is noteworthy that, while the identification of recurrently mutated genes in AML has expanded our view of AML pathophysiology (Cancer Genome Atlas Research Network *et al*, 2013), studies integrating knowledge of the mutational architecture with the hierarchical cancer stem cell model are sparse (Chesnais *et al*, 2017; Klco *et al*, 2014). Thus, LSCs are attractive targets in the development of new treatment modalities in AML (Bruserud *et al*, 2017), and a precise and in-depth characterization of these cells, including an understanding of how they differ from their normal counterparts, is essential.

In this regard, the C-type lectin domain family 12, member A (CLEC12A) receptor, a relevant marker of leukaemic blasts (Larsen *et al*, 2012; Roug *et al*, 2014), has been proposed as a specific marker of LSCs in AML (van Rhenen *et al*, 2007a; Terwijn *et al*, 2014), as the CLEC12A protein is not expressed on normal CD34+CD38- cells, but aberrant expression on this subset of cells has been demonstrated in AML (van Rhenen *et al*, 2007a; 2007b). In addition, we have recently shown the relevance of the marker in myelodysplastic syndrome (MDS) and pointed out important differences in the applicability of the marker in these biologically different disease entities (Toft-Petersen *et al*, 2016). Given this, the CLEC12A receptor is an attractive treatment target in AML, and current developments in this regard include both CLEC12A-CD3 bispecific antibodies (Leong *et al*, 2017; Lu *et al*, 2014) and chimeric antigen receptor (CAR)-engineered T-cells (CAR-T-cells) directed against CLEC12A (Laborda *et al*, 2017; Tashiro *et al*, 2017; Wang *et al*, 2018) and, most recently, the development of a novel antibody-drug conjugate (Jiang *et al*, 2018).

High activity of the cytosolic enzyme aldehyde dehydrogenase (ALDH) has been associated with normal haematopoietic stem and progenitor cells (HSPCs) (Storms *et al*, 1999; Pearce *et al*, 2005; Hess *et al*, 2004; Gentry *et al*, 2007) as well as cancer stem cells (CSCs) in both solid tumours (Ajani *et al*, 2015) and haematological malignancies (Hoang *et al*, 2013; Gerber *et al*, 2012; 2011; Wu *et al*, 2013). Moreover, a high level of ALDH activity has been proposed as a tool for separating LSCs from normal HSPCs (Hoang *et al*, 2015; Schuurhuis *et al*, 2013). Specifically, most cases of AML have a very small fraction of cells with high levels of ALDH activity; these are termed ALDH bright cells ($ALDH^{br}$), and have been shown to represent residual normal HSPCs, while the leukaemic blasts and LSCs display a lower ALDH activity (Gerber *et al*, 2012; Schuurhuis *et al*, 2013). However, , a larger fraction of $ALDH^{br}$ cells is evident in up to 20% of AML samples; in these cases, the separation of LSCs and non-malignant HSPCs has not been possible (Hoang *et al*, 2015).

While early studies of LSCs focused on the immature CD34+CD38- population (Lapidot *et al*, 1994; Bonnet & Dick, 1997), cells with functional LSC properties can also reside in the CD34+CD38+ cell subset (Taussig *et al*, 2008; Sarry *et al*, 2011; Goardon *et al*, 2011) and even in the CD34- compartment (Quek *et al*, 2016; Taussig *et al*, 2010). Moreover, gating of the CD38- stem cell compartment can be challenging, especially in diagnostic AML samples where the blast population often presents as a continuum of CD34+CD38+/- cells.

In this study, based on the above mentioned current knowledge of LSC immunophenotypes and previous studies proposing CLEC12A to be a highly specific marker of LSCs in AML (van Rhenen *et al*, 2007a; 2007b; Terwijn *et al*, 2014), we employed ALDH activity as a marker of immaturity and investigated whether a sorting strategy based on ALDH activity and (the lack of) CLEC12A expression without pre-selecting the CD34+CD38- cells could identify residual normal HSPCs in bone marrow (BM) from five AML patients. Specifically, we hypothesised that cells with the $ALDH^{br}$CD34+CLEC12A- immunophenotype represent residual normal HSPCs and cells with the $ALDH^{br}$CLEC12A+ (CD34+/-) immunophenotype to represent AML LSCs. We evaluated the purified cell populations in a long-term

colony-initiating cell assay (LTC-IC) and by performing cytogenetic, molecular and targeted next generation sequencing (NGS) analyses on both the sorted cell subsets and the LTC-IC derived colonies. Our data revealed a hitherto unrealised heterogeneity in the applicability of CLEC12A as an LSC marker in AML and strongly indicate the need for thorough and precise diagnostics on biologically relevant cell subsets in order to better select patients suitable for CLEC12A targeted therapy.

Methods

Patient samples

Cryopreserved BM samples from five AML patients and one chronic myeloid leukaemia (CML) patient with myeloid blast crisis (served as a positive control) were obtained as excess material taken as part of the diagnostic process at the Department of Haematology, Aarhus University Hospital. The AML patients were selected to represent various subtypes of AML and, in addition, the selection was based on the presence of known cytogenetic and/or molecular aberrancies obtained by routine karyotyping and screening for mutations in *NPM1, FLT3*-internal tandem duplication (ITD)*, FLT3* D835*, WT1* exon 7*, KIT* and *CEBPA*. Normal BM samples (NBM, n=2) from healthy volunteers served as controls and were obtained in the setting of BM harvest for allogeneic BM transplantation. The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committee. Mononuclear cells (MNCs) were obtained by Lymphoprep (Axis-Shield plc., Dundee, Scotland) separation according to the manufacturer's instructions, cryopreserved in 10% dimethylsulfoxide, and stored in liquid nitrogen. Patient characteristics are given in Table I.

The Aldefluor™ Assay and surface staining

Cryopreserved MNCs were thawed in a 37°C water bath and resuspended in RoboSep Buffer (StemCell Technologies, Vancouver, BC, Canada) with 15% heat-inactivated fetal calf serum (Biochrom, GmbH, Berlin, Germany). Next, cells were stained with 2.5 µl pre-titrated Aldefluor™ reagent in Aldefluor™ Buffer (StemCell Technologies) and incubated at 37°C for 45 min. Subsequently, cells were washed and resuspended in cold Aldefluor™ Buffer, kept on ice, and stained for 45 min with the monoclonal antibodies listed in Table SI.

Fluorescence activated cell sorting

Fluorescence activated cell sorting was performed on a BD FACSAria™ III (BD Biosciences, San Jose, CA, USA). Compensation was set using unstained and Aldefluor™ single stained cells combined with UltraComp eBeads (eBioscience, San Diego, CA, USA) together with the relevant fluorochrome conjugated antibodies. In the gating strategy used (Fig 1 and S1), live cells were identified in a forward scatter (FSC)/side scatter (SSC) plot after which doublet exclusion was done on both FSC and SSC height vs. area plots. As monocytes display a relatively high level of ALDH activity (Storms *et al*, 1999), we selected CD14 negative cells and defined the $ALDH^{br}$ cell subset (Aldefluor bright and SSC low) on this cell population. The lower limit of the $ALDH^{br}$ cells was set, taking the contour of the $ALDH^{low}$ cells into account. Next, in the $ALDH^{br}$ cells, the $ALDH^{br}$CLEC12A+ and $ALDH^{br}$CD34+CLEC12A- gates were defined. The CLEC12A+/- gates were set by use of internal negative controls (lymphocytes), and CLEC12A+ cells were not separated further based on fluorescence intensity. For cultures, two cell subsets were sorted, namely $ALDH^{br}$CLEC12A+ and $ALDH^{br}$CD34+CLEC12A-. The equivalent subsets from two NBM samples were sorted as controls. For patients with a cytogenetic aberration, additional cells from each subset were sorted directly onto poly-L-lysine coated slides (Thermo Fischer Scientific Inc., Waltham, MA, USA), and for patients with a given molecular aberration, cells from each subset were sorted into phosphate-buffered saline (PBS) (Thermo Fischer Scientific Inc) and stored at -80°C for later molecular genetic analyses. Due to the rarity of the cell populations, the purity of the sorted subsets was determined in 9 out of 18 total subsets (Table SII). For the $ALDH^{br}$CLEC12A+ subset, purity was on average 90.3% (n=5). For the $ALDH^{br}$CD34+CLEC12A- subset, purity averaged 96.5% (n=4). Data was analysed using FlowJo Data Analysis Software, version X (BD Biosciences).

Long-term colony initiating cell assay

The LTC-IC assay was performed as described previously (Toft-Petersen *et al*, 2016). In brief, sorted subsets were seeded in 96-well collagen-coated plates and cultured on irradiated M2-10B4 stromal feeder cells (ATCC, Manassas, Virginia, USA) with weekly half-change of medium. After 6 weeks of co-culture, cells were harvested and transferred to MethoCult H4435 (StemCell Technologies) for an

additional 2 weeks. Colony-forming cells (CFCs) were counted and scored for erythroid and myeloid morphology, respectively. Individual CFCs from each subset of the originally seeded cells were picked and either cyto-spinned onto poly-L-lysine coated slides (Thermo Fischer Scientific Inc.) for subsequent fluorescence *in situ* hybridization (FISH) analyses or resuspended in PBS and stored at -80℃ for molecular genetic analyses.

Interphase FISH

Fluorescence *in situ* hybridization (FISH) analyses were performed as previously described (Toft-Petersen *et al*, 2016). The following locus-specific directly fluorescent-labelled probe sets were used according to the manufacturer's instructions: t(8;21)(q22;q22)/*RUNX1-RUNX1T1* dual fusion, 11q23/ *KMT2A* (*MLL*) break apart, 18q21/*BCL2* break apart, 7q11.23(*ELN*)/7q31(D7S486,D7S522), t(9;22)(q34;q11)/*BCR-ABL1* dual fusion (all from Abbott Molecular, Des Plaines, Illinois, USA). To estimate the number of FISH-positive cells on cyto-spun slides of individually picked colonies and on cells sorted directly onto slides, a median of 100 interphase nuclei was evaluated (range 5-101) by two independent observers.

Molecular analyses

Genomic DNA was extracted from diagnostic BM MNCs using the Maxwell® 16 Blood DNA Purification Kit on the Maxwell® 16 Instrument (Promega, Madison, WI, USA) or, for sorted cell subsets, by use of the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), both according to the manufacturer's instructions. For individually picked colonies and when ≤ 1031 cells were available from the sorted subsets, whole genome amplification (WGA) was performed prior to downstream molecular analysis using the REPLI-g Single Cell Kit (Qiagen) according to the manufacturer's instructions. Mutations in *NPM1, FLT3, ASXL1, TET2, WT1, IDH1* and *ZRSR2* were investigated by polymerase chain reaction (PCR) with subsequent fragment length analyses (*NPM1, FLT3, ASXL1, TET2, WT1*) or Sanger sequencing (*IDH1, ZRSR2*). Polymerase chain reaction was run on a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) for 15 min at 95℃ followed by 35 cycles of 30 s at 95℃, 1 min at 58℃, and 1 min at 72℃ followed by an elongation step for 60 min at 60℃ (for fragment length analysis) or 10 min at 72℃ (for Sanger sequencing). Sanger sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit

(Thermo Fischer Scientific Inc.). Fragment length analysis and Sanger sequencing were run on a 3500 Genetic Analyzer (Thermo Fischer Scientific Inc.). All primer sequences are listed in Table SIII.

Targeted next generation sequencing

Targeted next generation sequencing (NGS) was performed with the Myeloid Tumour Solution 30 gene panel (SOPHiA Genetics, Saint Sulpice, Switzerland) performed according to manufacturer's instructions and analysed in the SOPHiA DDM® analysis software (SOPHiA Genetics). As the WGA reaction can introduce errors (Gawad *et al*, 2016), the quality of the NGS results obtained from samples that underwent WGA was assessed by calculating the standard deviation (SD) of variant allele frequencies (VAFs) of heterozygous single nucleotide polymorphisms (SNPs) (depth > 2500 reads and 10% < VAF < 90%) in the sequenced regions of the 30 investigated genes identified in the unfractionated BM sample (not exposed to WGA prior to targeted NGS) and the sorted samples (exposed to WGA) (Fig S2, Table SIV). Reference sequences for mutations identified are given in Table SV.

Results

Immunophenotypic characteristics

For the AML patients, CLEC12A was expressed on 59 – 89% of the leukaemic blasts with the highest level of expression on the two CD34- AML cases (AML 4 and 5) (Table I). While the CD34+CD38- cell subsets were devoid of CLEC12A expression in the CD34- cases, the opposite was the case in the CD34+ cases of AML (Table I). The immunophenotypic characteristics of the six patient samples with respect to ALDH activity, CD34 and CLEC12A expression are depicted in Fig 1. Of note, the percentage of $ALDH^{br}$ cells varied between AML samples (range 0.1 - 5.0% of CD14- cells). Thus, AML 1, 4 and 5 displayed low percentages of $ALDH^{br}$ cells (range 0.1 – 0.9% of CD14- cells), while AML 2 and 3 displayed larger fractions of $ALDH^{br}$ cells (range 3.4 – 5.0% of CD14- cells). This finding is in concordance with previous studies of ALDH activity in AML (Hoang *et al*, 2015; Cheung *et al*, 2007). In line with previous findings (Gerber *et al*, 2011), the CML blast crisis patient (CML 1) had a very large percentage of $ALDH^{br}$ cells (41.1% of CD14- cells). By back-gating of the $ALDH^{br}$CD34+CLEC12A- cell subset into a CD34 vs. CD38 plot for all samples

including NBM, we verified that these cells had the immunophenotypic profile of early HSPCs with dim to no expression of CD38 (Fig 1, last column and Fig S1).

In AML, cells with LTC-IC activity are present in the ALDH^{br}CD34+CLEC12A- cell subset

In order to evaluate the stem cell properties of the ALDH^{br}CLEC12A+ and ALDH^{br}CD34+CLEC12A- cell populations in AML, we studied the highly purified cell subsets in the LTC-IC assay. As CML is known to originate in the haematopoietic stem cell compartment (Barnes & Melo, 2006), and BM from CML patients is known to have high levels of ALDH activity (Gerber *et al*, 2011), we also sorted ALDH^{br}CD34+CLEC12A- and ALDH^{br}CLEC12A+ BM cells from a CML blast crisis patient as a positive control. Furthermore, equivalent cell subsets from NBM cultured simultaneously served as a quality control of the assay itself.

In the case of NBM, no CFCs were evident after 8 weeks of culture in the LTC-IC assay when ALDH^{br}CLEC12A+ cells were originally seeded (Fig 2). This suggests that normal ALDH^{br}CLEC12A+ cells do not possess functional stem cell properties and most probably represent normal short-lived committed myeloid progenitors (Bill *et al*, 2018). For the AML samples, the ALDH^{br}CLEC12A+ cells did not produce CFCs in the LTC-IC assay with the exception of AML 1 that gave rise to CFCs that were negative for t(8;21) when evaluated by FISH (Fig 2, Table II). This growth of aberration-negative CFCs from ALDH^{br}CLEC12A+ cells in AML 1 was unexpected, and we cannot formally exclude that the growth of CFCs in this case could be due to impurities of the sorting procedure. Unfortunately, we were not able to assess the purity of the sorted subset in this particular case due to very limited material, but, in general, the average sorting purity of the ALDH^{br}CLEC12A+ cell subset was lower for the ALDH^{br}CD34+CLEC12A- cell subset (Table SII). In contrast to NBM and AML, the ALDH^{br}CLEC12A+ cells from CML 1 produced a large number of CFCs (Fig 2), which were *BCR-ABL1*+ (Table II), indicating the self-renewal potential, and thereby the LSC properties, of these cells.

When evaluating the ALDH^{br}CD34+CLEC12A- cell population in the LTC-IC assay, CFCs were produced in both NBM and in all cases of AML (Fig 2). However, when the individually picked CFCs from the 5 AML cases were investigated with

subsequent FISH or molecular analyses these were always negative for the known cytogenetic or molecular aberrancy (Table II). In the case of CML 1, the number of CFCs produced was so high that the discs were uncountable (Fig 2), and all harboured the *BCR-ABL1* aberration (Table II).

The data from the LTC-IC assay thus suggested that residual normal haematopoietic stem cells with self-renewal capacity were present among the ALDH^{br}CD34+CLEC12A- cells in AML BM. However, as the leukaemic AML cells failed to produce CFCs in the LTC-IC setup, we did not obtain functional evidence of their possible LSC nature in either of the two CLEC12A+/- cell subsets. This lack of leukaemic growth in the LTC-IC assay is in line with previous reports (Hoang *et al*, 2015).

In AML, the ALDH^{br}CD34+CLEC12A- cell subset is heterogeneous

Given the fact that the growth of AML stem cells is not always supported in the LTC-IC assay or even in xenograft models (van Gosliga *et al*, 2007; Ailles *et al*, 1999), we next investigated whether the findings from the LTC-IC assay were due to a biased selection of normal HSPCs in the culturing assay. Thus, the sorted uncultured cells from the ALDH^{br}CLEC12A+ and ALDH^{br}CD34+CLEC12A- subpopulations were analysed by FISH or molecular methods (Table II). Interestingly, in the case of AML 1 and 5, we were able to confirm the findings from the LTC-IC results, as the sorted ALDH^{br}CD34+CLEC12A- cells were devoid of the known aberrations characterising the neoplastic cells, while the ALDH^{br}CLEC12A+ cells turned out to be either positive or partially positive. In contrast, in AML 2, 3 and 4, both the sorted ALDH^{br}CD34+CLEC12A- cells and the ALDH^{br}CLEC12A+ cells were positive for the known malignant aberrancy (Table II). However, in AML 3 and 4, the approximate percentages of mutated alleles were lower in the ALDH^{br}CD34+CLEC12A- cell subset than in the ALDH^{br}CLEC12A+ cell subset (Table II). Interestingly, irrespective of whether the ALDH^{br}CD34+CLEC12A- were finally found harbour malignant cells or not, this seemed to be independent of whether the AML blasts and/or the CD34+CD38- cells were CLEC12A positive (Table I). As expected, in the CML patient, both the sorted ALDH^{br}CD34+CLEC12A- and the ALDH^{br}CLEC12A+ cells were *BCR-ABL1*+.

Collectively, the analyses of the sorted cell populations for the *a priori* known aberrancies indicate that ALDH activity and the lack of CLEC12A expression can be useful in defining residual normal HSPCs in selected cases of AML. However, in other cases, this approach is not feasible.

Targeted NGS indicates a hierarchical relationship between the ALDH^{br}CD34+CLEC12A- and ALDH^{br}CLEC12A+ subsets and shows that LTC-IC derived CFCs harbour pre-leukaemic mutations

Given that, on average, AML patients harbour ~5 recurrent mutations (Cancer Genome Atlas Research Network *et al*, 2013), we next sought to perform a more exhaustive analysis of the two sorted subsets by performing targeted NGS of 30 genes known to be recurrently mutated in myeloid malignancies. In the case of AML 3, 4 and 5, material was available from the diagnostic BM sample together with the sorted ALDH^{br}CD34+CLEC12A- and ALDH^{br}CLEC12A+ cell subsets; NGS revealed additional mutations in all three cases (Table III). Thus, in addition to the *FLT3*-ITD and *NPM1* mutations in AML 4, a *WT1* mutation, known to be an AML driver mutation (Lindsley *et al*, 2015), was identified. In AML 3 and 5, mutations often associated with pre-leukaemia and/or antecedent MDS were detected in addition to other recurrent AML driver mutations (Lindsley *et al*, 2015). While this finding was expected in the case of AML 5, who clinically presented with secondary AML (sAML), AML 3 presented clinically as a *de novo* AML (Table I). Overall, the mutations detected in the diagnostic BM sample could also be found in the sorted subsets, apart from the *NPM1* mutation in AML 5, which, as expected, was undetectable in the ALDH^{br}CD34+CLEC12A- cell subset. Interestingly, in all three cases, the VAF of mutations in the ALDH^{br}CLEC12A+ cell subsets were between 45-49% indicating that the vast majority of these cells harbour the heterozygous mutations, except for AML 5, where the *NPM1* mutation had a VAF of 26% in the ALDH^{br}CLEC12A+ cell subset. This was in line with the findings by fragment length analysis (Table II) and indicative of the ALDH^{br}CLEC12A+ cells being only partially *NPM1*-mutated. In contrast, the VAFs of the different mutations in the ALDH^{br}CD34+CLEC12A- varied (Table III).

The finding of pre-leukaemic mutations in AML 3, 4 and 5 prompted us to investigate whether the LTC-IC derived colonies could be positive for these aberrations as well.

Indeed, 5/13 colonies were *ASXL1*-mutated in AML 3 and in AML 5, all of the LTC-IC derived CFCs (6/6) were *TET2* c.1767delT, *TET2* c.1932delA and *ZRSR2* mutation positive. In line with the notion of *WT1* mutations being late events in leukaemogenesis (Krauth *et al*, 2015), the CFCs of AML 4 were negative for this mutation. Of note, despite a VAF of ~44% for the *ASXL1* mutation in AML 3, not all colonies derived from the ALDH^{br}CD34+CLEC12A- subset were pre-leukaemic in nature, indicating the co-existence of residual normal HSPCs with pre-leukaemic and possibly LSCs in the BM.

Furthermore, targeted NGS on the sorted ALDH^{br}CD34+CLEC12A- and ALDH^{br}CLEC12A+ cell subsets revealed marked differences between the two cell subsets. While the ALDH^{br}CLEC12A+ cells predominantly resembled the diagnostic BM sample, the ALDH^{br}CD34+CLEC12A- cells showed a much greater variation in VAFs of the detected mutations. As exemplified in AML 3, this variation in VAFs may uncover the clonal heterogeneity and hierarchical leukaemic evolution of the different subclones (Fig 3). In addition, the finding of pre-leukaemic mutations in some of the LTC-IC derived colonies supports previous reports of pronounced functional heterogeneity of genetically different subclones (Chesnais *et al*, 2017; Klco *et al*, 2014).

Discussion

In the upcoming era of precision medicine, it is of great importance to know the characteristics of the target cells, yet knowing the details of cell subsets not expressing the target is of equal importance. Firstly, it is necessary to understand which normal cells are spared by such treatment, and not least to determine whether all of the relevant neoplastic cells are indeed targeted as intended. As such, our results should be seen in the context of the increasing need for improved treatment modalities in AML. The CLEC12A receptor holds great promise as a future treatment target and the first clinical trial with a CD3-CLEC12A bispecific antibody in AML has been initiated in Europe (EudraCT number 2015-003704-23). In the present study we therefore explored CLEC12A in further detail in the setting of AML by investigating whether a sorting strategy based on ALDH activity and CLEC12A expression could be useful in distinguishing LSCs from residual normal HSPCs. We demonstrate that this distinction is, indeed, possible in some AML cases, supporting

the current paradigm of CLEC12A as a robust marker of LSCs. However, in 3/5 AML cases, the ALDH^{br}CD34+CLEC12A- cells also harboured the cytogenetic and/or molecular aberrations characterising the malignant cells, and in such cases CLEC12A targeted treatment would probably not be sufficient to eradicate the neoplasm.

Our data should be seen in continuation of a previous study that combined ALDH activity with aberrantly expressed LSC markers, including CLEC12A (Schuurhuis *et al*, 2013). While these authors showed that residual normal HSPCs were ALDH^{br} and LSC-marker negative, their experimentation was devoted to patients with known *FLT3*-ITD or *NPM1* mutation (Schuurhuis *et al*, 2013). Similarly, the earliest studies of CLEC12A as a marker of LSCs were also conducted on AML samples with primarily *FLT3*-ITD or *NPM1* mutations (van Rhenen *et al*, 2007a; 2007b; Moshaver *et al*, 2008). These aberrations are, in most cases, known to be late events in leukaemogenesis, often occurring in committed myeloid progenitors (Shlush *et al*, 2014; Corces-Zimmerman *et al*, 2014). We have recently demonstrated CLEC12A expression on both common myeloid- and granulocyte-macrophage progenitors (Bill *et al,* 2018), and thus in the case of AML 5 (*NPM1*+*,* monosomy 7) our data could indicate that the leukaemic transformation happened in a CLEC12A+ progenitor cell, which would confirm previous findings of Schuurhuis *et al* (2013). This patient had a history of MDS with monosomy 7 and later developed *NPM1*+ sAML. Even though low risk MDS has been shown to propagate from the haematopoietic stem cell (Tehranchi *et al*, 2010; Woll *et al*, 2014), evidence suggests some high-risk MDS subtypes, e.g. monosomy 7 do in fact derive from a later progenitor stage (Dimitriou *et al*, 2016). Thus, despite evidence of pre-leukaemic mutations in the ALDH^{br}CD34+CLEC12A- cell subset, CLEC12A targeted therapy could, theoretically, have been beneficial in this patient with sAML. This notion is supported by a very recent study by Jongen-Lavrencic *et al* (2018), showing that, for AML in complete remission, detection of persistent mutations in *ASXL1*, *DNM3TA* and *TET2* was not correlated with an increased relapse rate. On the other hand, in the case of AML 4, the leukaemic mutations (*FLT3*-ITD+*, NPM1*+ and *WT1+*) must have arisen in a CLEC12A- cell, as the ALDH^{br}CD34+CLEC12A- cell subset also harboured these mutations, albeit in lower VAFs. Hence, CLEC12A-directed therapy would

most likely be insufficient to eradicate the most immature AML cells in this particular patient.

Another study investigating primarily core-binding factor leukaemias has included ALDH activity as a marker of stem cells and found cells with the ALDH^{br}CD34+CD38- immunophenotype to be of non-neoplastic nature (Gerber *et al*, 2012). In line with this, the ALDH^{br}CD34+CLEC12A- cells from AML 1 (t(8;21)) were cytogenetically normal when evaluated by FISH. The majority, but not all of the ALDH^{br}CLEC12A+ cells was also negative for t(8;21) (4/100, Table II), indicating that adding CLEC12A to the sorting scheme actually refined the separation of normal HSPCs from cells with the malignant t(8;21) signature within the ALDH^{br} cell compartment. This contrasts to AML 2, where the sorted ALDH^{br}CD34+CLEC12A- cells were predominantly cytogenetically abnormal (Table II). This patient had a complex karyotype but no history of antecedent MDS. It is worth noticing that both AML 1 and 2 presented with a large fraction of CD34+CD38-CLEC12A+ cells (Table I), which, by current definitions, would indicate CLEC12A as a *bona fide* LCS marker in both cases. However, if one were to choose to target CLEC12A, the predicted outcome would be quite different for AML 1 and AML 2.

While it is known that in a significant proportion of human AML cases, the leukaemic cells do not express CD34, there is no consensus on the definition of CD34- AML (Kanda *et al*, 2000). In a recent work, Zeijlemaker et al (2015) suggested that the distinction between CD34+ and CD34- AML should be based on the presence / absence of aberrant marker expression on the CD34+CD38- cells rather than an arbitrary cut-off value for CD34 expression. Previous reports have shown the small fraction of CD34+ cells in CD34- AML to often represent residual normal HSPCs (Taussig *et al*, 2010). However, the existence of CD34+ LCSs in CD34- AML has recently been demonstrated (Quek *et al*, 2016), and as such the need for a deeper understanding of this subtype of AML remains important. We have previously demonstrated that in CD34- AML, the percentage of blasts expressing CLEC12A is consistently high (Larsen *et al*, 2012). In this study, we have evaluated two cases of CD34- AML (AML 4 and 5; < 1% CD34+ cells). In the case of AML 4, > 80% of the leukaemic CD34- blasts expressed CLEC12A, and it could be tempting to pinpoint such a patient for CLEC12A targeted therapy – which in this case would most

probably fail, as the ALDH^{br}CD34+CLEC12A- cells also harboured *FLT3*-ITD, *NPM1* and *WT1* mutations. As mentioned above, CLEC12A might have been a beneficial target in the other case of CD34- AML (AML 5), and future research is needed to evaluate if CLEC12A could be advantageous as a target in most cases of CD34- and other more "mature" cases of AML, equivalent to what is often seen in CD33-directed therapy (Walter *et al*, 2012).

Our study did not provide functional evidence of LSCs with recognised AML driver mutations (e.g. *FLT3*-ITD or *NPM1*) in the LTC-IC assay. However, we confirmed the findings of previous studies, showing a preponderance of LTC-IC activity in cells with early leukaemogenic events, e.g. in epigenetic or chromatin modifying genes, such as *TET2* and *ASXL1* (Chesnais *et al*, 2017). In addition, the data from targeted NGS on highly purified cell subsets strongly suggested a hierarchical relationship between the ALDH^{br}CD34+CLEC12A- and ALDH^{br}CLEC12A+ cell subsets, indicating that the pre-leukaemic and/or founding clones often arise in the most immature ALDH^{br}CD34+CLEC12A- cells with additional driver mutations emerging either in a ALDH^{br}CD34+CLEC12A- cell or in a more mature ALDH^{br}CLEC12A+ cell. This is most evident in the case of AML 3, where the ALDH^{br}CLEC12A+ cell harboured all 7 identified mutations in VAFs of 45-49%, indicating that the vast majority of these cells harboured all of the mutations, while although all 7 mutations were present in the ALDH^{br}CD34+CLEC12A- cells, the VAFs varied, indicating a linear acquisition of mutations (Fig 3). In further support of the accumulative nature of the leukaemic events, the LTC-IC derived CFCs were negative for the *IDH1* mutation, while 38% of the evaluated colonies were *ASXL1*+. Furthermore, although AML 3 clinically presented as *de novo* AML, the presence of *ASXL1* and *EZH2* mutations is highly indicative of a subclinical antecedent MDS phase (Lindsley *et al*, 2015), and the finding of neoplastic CLEC12A- cells would be in accordance with our previous study of CLEC12A in MDS (Toft-Petersen *et al*, 2016). Similarly, in the case of AML 5, the colonies from LTC-IC harboured founding mutations in *TET2* c.1767delT, *TET2* c.1932delA and *ZRSR2*, while being devoid of the *NPM1* mutation. Taken together, targeted NGS analyses on rigorously purified cell subsets, in contrast to bulk BM analysis, can reveal knowledge of the clonal evolution in a given patient sample and thus help determine – if not the LSC, as this by strict definitions requires functional studies – then the most immature cell subset to harbour a given set of mutations.

Naturally, as our results are based on the evaluation of very few cells, the lack of a specific mutation in a given cell subset should, of course, be interpreted with caution. Nevertheless, we feel that the presented method, if standardized and optimized to the clinical setting, could be very useful. For example, in the setting of a clinical trial testing a given targeted treatment, this technique could prove valuable in the evaluation of the efficacy of such therapies based on information of the clonal architecture in different AML stem cell compartments. Ultimately, assuming that LSC marker positivity/negativity in immature AML cells and mutational status in the respective cell compartments is correlated with treatment response, a similar methodology could indeed serve as a prerequisite for successful identification of the particular patients likely to benefit from a given treatment directed against any cell surface molecule.

In conclusion, our study adds to the understanding of CLEC12A in AML and cautions that a detailed determination of the leukaemic cell of origin "up front" will be necessary to predict treatment response and direct the right patients for CLEC12A targeted therapy. Thus, we predict a future need for a refinement of the initial diagnostics where cytogenetic and molecular analyses ideally should be performed on sorted cell subsets based on relevant markers and/or treatment targets. Such an approach indeed appears to be essential in order to practice personalised medicine in a complex disease such as AML.

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Authorship contributions

M.B., L.N., A.S.R. and P.H. designed the study. M.B., A.A., E.K. and L.N. performed the experiments and analysed the data. M.B. drafted the manuscript and received input and critical reviews from L.N., A.A., E.K., A.S.R. and P.H.. P.H. provided the financial background for the study. M.B. has previously published under her maiden name Toft-Petersen. All authors read and approved the final manuscript.

Disclosure of conflicts of interest

The authors declare no competing interests.

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Table I. Patient characteristics

Clinical AML presentation	Age	Blast % in BM	Blast CD34 phenotype	% CLEC12A+ blasts	% CLEC12A+ CD34+CD38- cells	Karyotype	Molecular aberration
De novo	32	~ 50%	CD34 high	78%	54%	46,XX,t(6;10)(q22;q25)c[14] / 46,idem,t(8;21)(q22;q22)[11]	-
De novo	79	> 80%	CD34 + / -	59%	21%	43,XX,-3,der(3;11)(q10;q10), -11,-14,-18,+mar[25]	-
De novo	75	70-80%	CD34 + / -	69%	6%	46,XY[25]	*IDH1* R132C
De novo	86	> 90%	CD34 - *	81%	0.7% (2 events)	46,XY[25]	*NPM1* mut A *FLT3*-ITD
sAML (MDS)	80	> 95%	CD34 - *	89%	2.7% (2 events)	45,XY,-7[22/25]	*NPM1* mut A
CML-BC	67	~ 40%	CD34 +	53%	13%	ND	BCR-*ABL1*

* less than 1% CD34+ cells

AML: acute myeloid leukaemia; BM: bone marrow; CML-BC: chronic myeloid leukaemia in blast crisis; ITD: internal tandem duplication; MDS: myelodysplastic syndrome.ND: not done; NK: normal karyotype; sAML: secondary acute myeloid leukaemia.

Table II. Molecular genetics and fluorescence *in situ* hybridization on sorted subsets and single colonies from LTC-IC

Patient sample	Investigated aberration	ALDH^{br}CLEC12A+		ALDH^{br}CD34+CLEC12A-	
		Sorted cells	LTC-IC aberration-positive colonies (n)/ total colonies evaluated (N)	Sorted cells	LTC-IC aberration-positive colonies (n)/ total colonies evaluated (N)
AML 1	*RUNX1-RUNX1T1*[#]	4/100 (4%)	0/8 (0%)	0/44 (0%)	0/7 (0%)
AML 2	11q23/*KMT2A*[#]	94/100 (94%)	No colonies in LTC-IC	85/100 (85%)	0/6 (0%)
	18q21/*BCL2*[#]	90/100 (90%)	No colonies in LTC-IC	91/100 (91%)	0/6 (0%)
AML 3	*IDH1* mutation[$]	*IDH1* mutation+ (20.000 sorted cells) ~ 45%	No colonies in LTC-IC	*IDH1* mutation+ (833 sorted cells) ~ 30%	0/16 (0%)
AML 4	*NPM1* mutation A[§]	*NPM1* mutation A+ (9722 sorted cells) ~ 45%	No colonies in LTC-IC	*NPM1* mutation+ (1031 sorted cells) ~ 25%	0/16 (0%)
	FLT3-ITD[§]	*FLT3*-ITD+ (9722 sorted cells) ~ 50%	No colonies in LTC-IC	*FLT3*-ITD+ (1031 sorted cells) ~ 15%	0/16 (0%)
AML 5	7q31.23/*ELN*[#]	90/90 (100%)	No colonies in LTC-IC	0/5 (0%)	0/6 (0%)
	NPM1 mutation A[§]	*NPM1* mutation A+ (6093 sorted cells) ~ 45%	No colonies in LTC-IC	*NPM1* WT (357 sorted cells)	0/6 (0%)
CML 1	t(9;22)(q34;q11)[#]	99/100 (99%)	5/5 (100%)	97/100 (97%)	3/3 (100%)

Investigated by: [#] fluorescence *in situ* hybridization; [§] fragment length analysis; [$] Sanger sequencing

AML: acute myeloid leukaemia; CML: chronic myeloid leukaemia; ITD: internal tandem duplication; LTC-IC: long-term colony-initiating cell assay; NK: normal karyotype; WT: wild type.

Table III. Targeted NGS on sorted subsets and single CFCs derived from LTC-IC

Patient sample	Targeted NGS 30 Gene Panel		ALDH^{br}CLEC12A+		ALDH^{br}CD34+CLEC12A-	
	Mutations identified in unfractionated BM	VAF (%)	Sorted cells VAF (%)	LTC-IC aberration-positive colonies (n)/ total colonies evaluated (N)	Sorted cells VAF (%)	LTC-IC aberration-positive colonies (n)/ total colonies evaluated (N)
AML 3	*IDH1* c.394C>T	45	46	N/A	30	0/16 (0%)$^{\$}$
	ASXL1 c.1934dupG	38	48		44	5/13 (38%)*$^{\#}$
	EZH2 c.1331T>A	42	49		32 %	ND
	EZH2 c.394C>A	46	45		20	ND
	RUNX1 c.508 + 2T>C	41	48		19	ND
	RUNX1 c.1283_1288delins10	38	47		16 %	ND
	JAK2 c.1849G>T	43	45		33	ND
AML 4	*FLT3*-ITD c.1770_1793 dup	47	50	N/A	9	0/16 (0%)*
	NPM1 c.860_863dupTCTG	41	46		23	0/16 (0%)*
	WT1 exon 9 c.1379delT	46	51		6	0/16 (0%)*
AML 5	*NPM1* c.860_863dupTCTG	38	26	N/A	0	0/6 (0%)*
	TET2 c.1767delT	47	51		45	6/6 (100%)*
	TET2 c.1932delA	49	46		55	6/6 (100%)*
	ZRSR2 c.827+ 1G>A	98 §	100 §		100 §	6/6 (100%)$^{\$}$

Investigated by * fragment length analysis or $^{\$}$ Sanger sequencing

$^{\#}$ three colonies were inconclusive § male patient with ZRSR2 mutation on chromosome X

AML: acute myeloid leukaemia; BM: bone marrow; CFC: colony forming cell; ITD: internal tandem duplication; LTC-IC: long-term colony-initiating cell assay; N/A: not applicable, ND: not done; NGS: next generation sequencing; VAF: variant allele frequency

Legends

Figure 1. Gating strategies and immunophenotypic characteristics of AML 1-5 and CML 1. In the left column, gating of the $ALDH^{br}$ subset on the CD14- cells is shown for each patient sample with back-gating of the $ALDH^{br}$CLEC12A+ (blue) and the $ALDH^{br}$CD34+CLEC12A- (red) cell subsets. In the centre column, the distribution of the $ALDH^{br}$CLEC12A+ and $ALDH^{br}$CD34+CLEC12A- cells subsets are shown in a CLEC12A vs. CD34 plot. In the right column, back-gating of the $ALDH^{br}$CLEC12A+ (blue) and the $ALDH^{br}$CD34+CLEC12A- (red) cells into CD14- cells displayed in a CD38 vs. CD34 plot is shown in order to view the expression of CD38 on the two cell subsets.

Figure 2. Colony forming cells from the LTC-IC assay. A. CFCs/500 initially seeded $ALDH^{br}$CLEC12A+ cells. B. CFCs/500 initially seeded $ALDH^{br}$CD34+CLEC12A- cells.

AML: acute myeloid leukaemia; BFU-E: burst forming unit-erythroid; CFC: colony-forming cell; CFU-E; colony-forming unit erythroid; CFU-GEMM: mixed lineage colony-forming unit; CFU-GM: colony-forming unit granulocyte-macrophage; CML: chronic myeloid leukaemia; LTC-IC: long-term colony-initiating cell assay; NBM: normal bone marrow; UC: uncountable due to over-plating; UI: unidentifiable colony.

Figure 3. Proposed hierarchical relationship of subclones in the CLEC12A+/- subsets in AML 3. The VAFs of the 7 identified mutations were equally high in the $ALDH^{br}$CLEC12A+ cell subset, while the VAFs varied in the $ALDH^{br}$CD34+CLEC12A- cell subset, indicating a linear accumulation of mutations in the most immature cells.

HSC: haematopoietic stem cell; LSC: leukaemic stem cell; LTC-IC: long-term colony-initiating cell assay; VAF: variant allele frequency

Figure 1

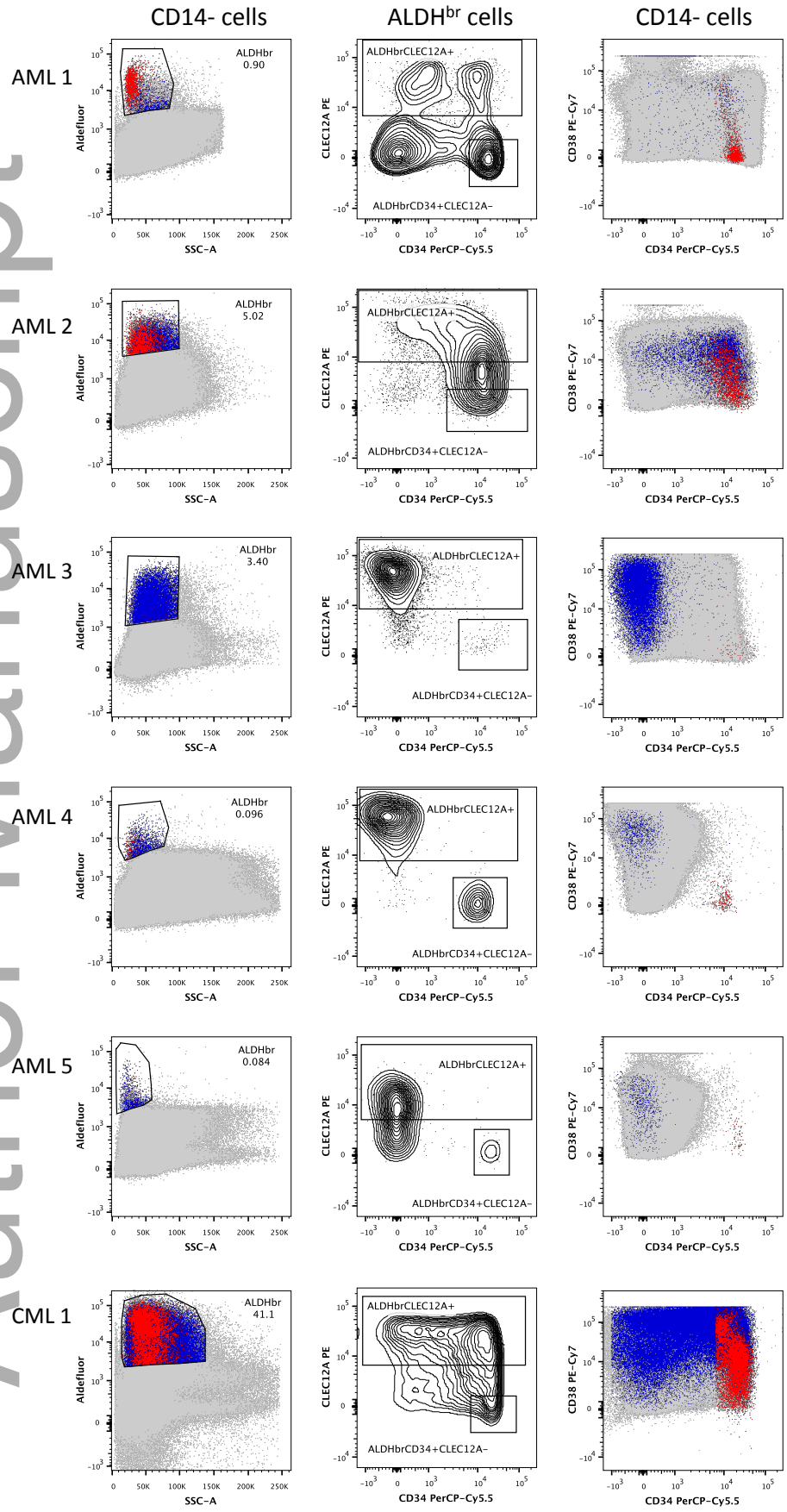

Figure 2

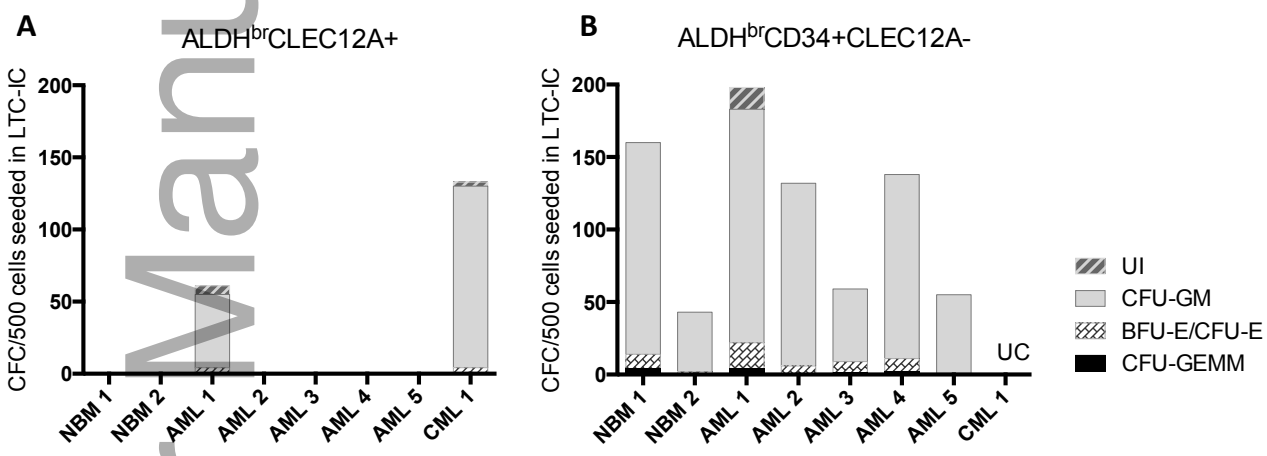

Figure 3