A transcriptomic continuum of differentiation arrest identifies myeloid interface acute leukemias with poor prognosis

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Abstract:

Classification of acute lymphoblastic and myeloid leukemias (ALL and AML) remains heavily based on phenotypic resemblance to normal hematopoietic precursors. This framework can provide diagnostic challenges for immunophenotypically heterogeneous immature leukemias, and ignores recent advances in understanding of developmental multipotency of diverse normal hematopoietic progenitor populations that are identified by transcriptional signatures. We performed transcriptional analyses of a large series of acute myeloid and lymphoid leukemias and detected significant overlap in gene expression between cases in different diagnostic categories. Bioinformatic classification of leukemias along a continuum of hematopoietic differentiation identified leukemias at the myeloid/T-lymphoid interface, which shared gene expression programs with a series of multi or oligopotent hematopoietic progenitor populations, including the most immature CD34+CD1a-CD7- subset of early thymic precursors. Within these interface acute leukemias (IALs), transcriptional resemblance to early lymphoid progenitor populations and biphenotypic leukemias was more evident in cases originally diagnosed as AML, rather than T-ALL. Further prognostic analyses revealed that expression of IAL transcriptional programs significantly correlated with poor outcome in independent AML patient cohorts. Our results suggest that traditional binary approaches to acute leukemia categorization are reductive, and that identification of IALs could allow better treatment allocation and evaluation of therapeutic options.

56 **Introduction:**

57 Successful management of acute leukemia is underpinned by accurate diagnostic
58 classification, which provides a basis for treatment allocation, risk stratification and
59 implementation of targeted therapies (1). Although knowledge of the molecular
60 landscape of leukemia has increased enormously over the past decades, contemporary
61 classification remains heavily predicated on simple immunophenotypic resemblance to
62 either myeloid or lymphoid normal hematopoietic precursors (2). While this system has
63 historically been successful, some leukemia categories provide specific diagnostic and
64 therapeutic challenges. The current World Health Organization (WHO) classification (2)
65 recognizes acute leukemias of ambiguous lineage that either lack lineage-specific
66 markers (acute undifferentiated leukemias, AUL) or express a mixture of myeloid and
67 lymphoid antigens (mixed phenotype acute leukemias, MPAL). There is little consensus
68 on the best treatment approaches for these patients, and prognosis is usually poor (3-
69 5).

70 This framework also poses difficulties for some cases of T-acute lymphoblastic leukemia
71 (T-ALL) and acute myeloid leukemia (AML). T-ALL can be subclassified by
72 immunogenotypic and phenotypic resemblance to either immature/ early thymic
73 precursor (ETP), early cortical or late cortical normal T-progenitor equivalents (6, 7).
74 However, the genotypic and phenotypic heterogeneity of immature T-ALLs mean that
75 robust biological classification of this group is not straightforward (8). A subset of these
76 cases harbor mutations that are also commonly seen in AML, suggesting that at least
77 some immature T-ALLs may arise from transformation of a bipotent lympho-myeloid
78 progenitor (9-13). In addition, diagnostic distinction from AML by immunophenotype is
79 often not clear-cut, as immature T-ALLs commonly express myeloid lineage-associated

80 markers (14). Conversely, the most phenotypically immature AML subgroup, M0-AML,
81 is also biologically heterogeneous and expresses lymphoid-associated antigens such as
82 CD7 or TdT in about 50% of cases (15). Immature T-ALLs are frequently chemoresistant
83 and require intensive treatment (10, 14, 16), while M0-AML cases have poor outcomes
84 compared to other AML subgroups (17, 18), so it is clinically important to consider
85 whether improved classification of these cases might allow better therapeutic choices.

86 Current leukemia classification also takes little account of modern advances in
87 understanding of human hematopoiesis, and the recognition of a diverse range of pluri-
88 and multipotent progenitors, as identified by transcriptional signatures and functional
89 assays (19). In particular, traditional notions of an early lymphoid/myeloid dichotomy
90 have been undermined by the discovery of a multitude of lymphoid committed cell
91 types which retain myeloid potential at different stages of differentiation: within the
92 phenotypic stem cell (20) or progenitor compartment (21-25) and in the thymus (26,
93 27). The relevance of these cell types in the context of leukemia is only beginning to be
94 explored (22, 28).

95 Leukemic transcriptome profiling should help to improve categorization, but traditional
96 analytical approaches have their shortcomings. T-ALL can be reproducibly categorized
97 according to a limited number of expression signatures that correlate with the
98 phenotype of differentiation arrest (6, 29, 30). Data may also be interrogated by gene
99 set enrichment analysis (GSEA), which has revealed that immature/ETP-ALLs
100 transcriptionally resemble both normal hematopoietic stem cell (HSC) and immature
101 myeloid precursors (9). However, these approaches rely on comparisons of predefined
102 sample groups, neglect transcriptional heterogeneity of individual leukemias in each
103 group and cannot resolve relationships between groups. These analyses therefore

104 provide limited information about the spectrum of differentiation arrest in acute
105 leukemia.

106 Evolutions in genomic analytical methods provide an opportunity to refine leukemia
107 classification. We have analyzed a series of acute leukemias that comprised a high
108 proportion of immature T-ALLs and AMLs using several approaches, including the novel
109 Iterative Clustering and Guide Gene Selection method (ICGS). This technique, when
110 applied to single-cell RNA-sequencing data, has been shown to infer cellular states from
111 transcriptional data, identify modules of guide genes that are specific to these cellular
112 developmental states in an unbiased, agnostic manner, and infer developmental
113 relationships between these states (31). We show that application of ICGS to global
114 expression data identifies a continuum of differentiation arrest, which includes a group
115 of myeloid/ T-lymphoid interface leukemias that lack clear lineage identity, and which
116 respond poorly to AML treatment regimens.

117

118 **Methods:**

119 ***Microarray data analysis:*** All computational analysis was performed in R (v.3.3.2 or
120 above) unless otherwise specified. Data were normalised with *normalize.quantiles*
121 function from the preprocessCore v1.34.0 package and batch effects between 2
122 independent arrays were corrected using the *ComBat* function (sva package).
123 Hierarchical clustering was performed with the *hclust* function with distance (1-
124 Pearson correlation) and complete clustering method. Principal Component Analysis
125 (PCA) was performed with *prcomp* function. Both hierarchical clustering and PCA were
126 performed on all probes.

127 ***ICGS:*** ICGS was performed with AltAnalyze software v. 2.1.0
128 (http://www.altanalyze.org/) using HOPACH clustering, with default settings for gene
129 expression analysis options (moderated t-test for group comparison and Benjamini-
130 Hochberg false discovery rate <0.05). The gene expression filtering option was set to 2.
131 Cell cycle genes were excluded using the most stringent parameter. From the Liu et al.
132 pediatric cohort (32), all samples were used, whereas from the Chen et al. cohort (33)
133 only adult samples (>18 years) were selected. Heatmap visualization of ICGS data was
134 performed in AltAnalyze.

135 ***Differential expression analysis*:** Differentially expressed genes were derived using the
136 *limma* package (*lmFit* function) for microarray and *DESeq2* for RNA-Seq. Contrast
137 matrices between selected groups are listed in Supplementary Table S1. Genes were
138 considered differentially expressed if Benjamini-Hochberg false discovery rate
139 (FDR) < 0.05. Gene ranking for Gene Set Enrichment Analyis (GSEA) was performed
140 according to *t*-statistic for microarray data or Wald statistic for RNA-seq data. For the

141 thymic subpopulation dataset, most variable genes across all populations were selected
142 as the union of all the probes differentially expressed between any two populations
143 (thymic HVGs, 8751 probes).

144 ***Pathway and Gene Set Enrichment Analysis:*** GSEA was performed with GSEA software
145 (http://software.broadinstitute.org/gsea/index.jsp) using the C2.all.v6.1 collection of
146 genesets from MSigDB (http://software.broadinstitute.org/gsea/msigdb/index.jsp) or a
147 collection of custom genesets (Supplementary Table S1) derived from datasets
148 generated here or publicly available (19, 23, 24, 34-36). When specific genesets were
149 derived from published data, differential expression analysis was performed as
150 indicated above using the contrasts indicated in Table S1. Differentially expressed genes
151 were then ranked by *t*-statistic for microarray data or by Wald statistic for RNA-seq
152 data and the top 500 genes (or all genes with FDR < 0.05 if <500 genes had FDR<0.05)
153 were selected as genesets to be tested by GSEA. GSEA outputs were either visualised
154 with the EnrichmentMap plugin (FDR Q-value cutoff 0.05) of Cytoscape (v.3.2.0), or with
155 heatmaps generated with Prism software (v.7). ClueGO analysis was performed with the
156 ClueGO plugin (v.2.1.6) of Cytoscape (v.3.2.0), using the GO Term Fusion option and
157 otherwise default parameters.

158 **Data availability:** All gene expression data have been deposited in the GEO portal
159 under the accession numbers GSE131180 (thymic populations isolated from neonatal
160 thymi), GSE131184, GSE131207 (AML and T-ALL samples). All relevant data are also
161 available from the authors.

162 Other experimental methods are described in the **Supplemental Data**.

163

164 **Results:**

165 ***Transcriptional profiling identifies an AML-like subset of T-ALL***

166 We performed transcriptional profiling of a series of 124 acute T-lymphoid and myeloid
167 leukemias (See Supplementary Methods). The 48 T-ALLs included a high proportion
168 (54.2%) of immature cases, as defined by T-receptor immunogenotype (37), comprising
169 9 IM0 (germline *TR*), 9 IMD (*TRD* rearrangement only) and 8 IMG (*TRG* and *TRD*
170 rearranged but absent or incomplete *TRB* rearrangement) leukemias. Similarly, 28/76
171 AML samples (40.8%) were categorized as M0-AML. Patient details are shown in
172 Supplementary Table S2.

173 Unsupervised hierarchical clustering (HC) analysis of the expression data revealed that
174 T-ALL and AML samples largely formed two distinct groups (HC cluster 1 and HC cluster
175 2, Figure 1A). Strikingly, 8/48 T-ALLs (16.7%, henceforth 'AML-like T-ALL') segregated
176 in the AML cluster in this unsupervised analysis, and clustered together when HC was
177 restricted to T-ALLs (Supplementary Figure S1A). When visualized by Principal
178 Component Analysis (PCA), T-ALL and AML samples were distributed differently along
179 the first principal component. Notably, T-ALL samples clustering with AMLs by HC
180 overlapped with AML samples (Supplementary Figure 1B).

181 Not all of these AML-like T-ALLs exhibited immunogenotypic immaturity (6/8) or had
182 an ETP-ALL immunophenotype (4/7 fully-phenotyped samples) (14), indicating that
183 AML-like transcription features are not restricted to previously identified categories of
184 less differentiated T-ALLs.

185

186 ***AML-like T-ALL is enriched for myeloid progenitor transcriptional signatures***

187 We next examined the transcriptional differences between AML-like cases and the rest
188 of the T-ALL cohort. 2274 genes (Supplementary Table S3) were significantly
189 differentially expressed between the two groups (FDR <0.05), with 1213 and 1061
190 respectively upregulated and downregulated in AML-like T-ALLs. Pathway analysis
191 revealed that AML-like T-ALLs had elevated expression of genes involved in cell cycle
192 and mitochondrial, amino-acid and pyruvate metabolism, and high levels of interferon-
193 related genes, MYC, HOXA, MEIS1 and GATA2 targets (Figure 1B). Gene-sets that were
194 previously reported to be upregulated in AML in independent datasets were also
195 significantly over-represented. In contrast, TCR, NOTCH1 and TNF signaling were all
196 downregulated.

197 We then sought to better characterize AML-like T-ALLs similarity to normal stem and
198 progenitor cells, by performing GSEA using normal umbilical cord blood (UCB)
199 hematopoietic progenitor transcriptional signatures that we previously reported (38).
200 AML-like T-ALLs were significantly enriched for megakaryocytic-erythroid progenitor
201 (MEP) and granulocyte-monocyte progenitor (GMP), but not hematopoietic stem cells
202 (HSC) signatures. These leukemias were also enriched for a GMP signature from an
203 independent data-set (23), and resembled lymphoid-mono-dendritic progenitors
204 (LMDP) from an UCB-derived humanized murine model of early lymphoid development
205 (24) (Figure 1C). To confirm transcriptional similarity to myeloid progenitors, we
206 combined the gene expression of the T-ALL samples with that of highly purified stem
207 and progenitor populations (38) on a 2D PCA map. Consistent with the GSEA results,
208 AML-like T-ALLs localized in the HSPC differentiation space, near GMPs (Figure 1D).

209

210 ***AML-like T-ALL transcriptionally resembles immature thymic progenitors***

211 While previous analyses of ETP-ALL have evaluated transcriptional proximity to normal
212 ETP cells (9), comprehensive transcriptional comparisons of T-ALL and normal thymic
213 subpopulations are lacking. We performed transcriptional profiling of six
214 phenotypically defined T-lymphoid progenitor groups isolated from a series of human
215 thymi (Supplementary Figure S2A).

216 The genes most differentially expressed in each subpopulation (Supplementary Figure
217 S2B and Supplementary Table S4) were consistent with known T-lymphopoietic
218 transcriptional patterns. PCA also reflected this developmental progression
219 (Supplementary Figure S2C), which was similar to an *in-vitro* system of human
220 thymocyte differentiation from UCB CD34+ cells (39) (Supplementary Figure S2D).

221 PCA identified 3 main clusters: a rare (Supplementary Figure S2A) 'early' thymic group
222 comprising CD34+CD1a-CD7- samples, a 'middle' thymic group comprising CD34+CD1a-
223 CD7+, CD34+CD1a+ and CD4+ ISP samples and a 'late' thymic group encompassing the
224 transcriptionally similar CD4+CD8+DP/TRLow and CD4+CD8+DP/TRHigh samples. We
225 derived specific gene expression signatures for each of these clusters and used these in
226 GSEAs to assess the transcriptional similarity of AML-like T-ALLs to normal thymocyte
227 subsets. Strikingly, AML-like T-ALLs were strongly positively enriched for genes that
228 were specifically expressed by the most immature CD34+CD1a-CD7- thymic
229 subpopulation (Figure 1C). Of note, this signature differed from an ETP transcriptional
230 profile that we previously reported, which was derived by comparison to CB stem and
231 progenitor cells (38) (Supplemental Figure 2E-2G). Conversely, when compared with
232 the rest of the T-ALL cohort, AML-like T-ALL samples were negatively enriched for 'late'

233 thymic discriminating genes (Figure 1C). Taken together, these results indicate that
234 AML-like T-ALLs share gene expression programs with both UCB-derived myeloid-
235 competent progenitors and the most immature thymic precursors, which also retain
236 myeloid differentiation potential (27).

237 ***Iterative Clustering and Guide Gene Selection analysis identifies a continuum of***
238 ***leukemic differentiation arrest***

239 The recently described ICGS method employs serial iterative clustering with pattern-
240 specific guide genes to define coherent transcriptional patterns between samples and
241 then groups these samples into cellular states that recapitulate developmental
242 trajectories (31). We reasoned this method could help resolve stages of differentiation
243 arrest in leukemia. To test the feasibility of applying this approach to leukemic datasets,
244 we initially used ICGS to analyze two published series of adult (33) and pediatric (32) T-
245 ALL. For both cohorts, the ICGS algorithm unbiasedly identified guide gene modules
246 enriched for human stem and progenitor cells (HSPCs, CD34+), myeloid cells and
247 thymocytes (Supplementary Figure S3A and S3C and Supplementary Table S5), and
248 ordered the T-ALL samples in clusters along a continuum of expression of these genes.
249 Along this spectrum, adult T-ALLs attributed to ICGS clusters with the lowest expression
250 of thymic-associated genes (Groups A and B), but with high expression of HSPC and
251 myeloid genes, were enriched for the ETP-ALL immunophenotype (10, 12-14). For the
252 pediatric cohort (32), ICGS ordering recapitulated in an unsupervised manner the
253 classification the authors had derived linking mutations to thymic developmental stages
254 (Supplementary Figure S3C and S3D). We thus concluded that ICGS allows unbiased
255 classification of leukemic samples according to their stage of differentiation arrest.

256 We then used ICGS to analyze our patient cohort. ICGS classified these leukemias into
257 five developmental clusters that were defined by the levels of expression of a limited
258 number of guide genes (Figure 2A and Supplementary Table S5) that again
259 predominantly comprised transcripts that discriminate hematopoietic cell types. The
260 proportions of different leukemic phenotypes within each cluster are shown in Figure
261 2B. Cluster 1 was defined by high expression of thymic- and lymphoid-related genes
262 (e.g. *TCF7*, *LCK*, *BCL11B*), and comprised T-ALL cases exclusively. Conversely, Clusters 4
263 and 5 were effectively restricted to AML cases, with concentration of Core Binding
264 Factor (CBF)-AMLs in cluster 5. These clusters exhibited increased expression of factors
265 that define myeloid transcriptional modules (e.g. *MPO*, *CEBPE*, *CSF3R*). The intermediate
266 Clusters 2 and 3 were characterized by heterogeneous guide gene expression, and
267 included one third of T-ALL cases (16/48, 33.3%). Notably, the most immature M0
268 subtype AMLs were predominantly found in these two clusters (24/28, 85.7%), as
269 compared with 14/48 (29.2%, $p<0.001$ by Fisher test) of non-M0-AML. Also, virtually all
270 AML-like T-ALL samples that were defined by HC (7/8, 87.5%) were found in either
271 Cluster 2 (n=4) or 3 (n=3). ICGS therefore provides a means of classifying leukemias
272 along a spectrum of hematopoietic ontogeny, which in our cohort included a significant
273 number of cases at the interface between T-lymphoid and myeloid lineages. Broadly,
274 these 'interface' acute leukemias (IAL) either showed no clear evidence of mature T-
275 lymphoid or mature myeloid identity (Cluster 2), or had a partial HSPC/mature myeloid
276 signature (Cluster 3).

277 ***Mutational analysis of ICGS-defined clusters***

278 We performed targeted next generation sequencing (NGS) of the 79/124 cases (34 T-
279 ALLs and 45 AMLs) where diagnostic material was available. The NGS panel

280 (Supplementary Table S6) had a predominance of genes that are more often altered in
281 T-ALL, including mutations typically found in the immature subgroup that overlap with
282 those seen in AML (9, 10, 12, 40). Comprehensive results are in Supplementary Table
283 S7, and all mutations detected in ≥ 2 patients are shown in Figure 2C.

284 Some results were in keeping with the spectrum of differentiation observed. Cluster 1
285 was enriched for T-ALL type NOTCH pathway-activating mutations ($p<0.0001$, all
286 comparisons below by Fisher test), while *KIT* mutations correlated with the
287 concentration of CBF-AMLs in Cluster 5 ($p=0.0007$). However, Cluster 1 was also
288 enriched for mutations in *SUZ12* ($p=0.004$), *WT1* ($p=0.0044$) and genes encoding
289 IL7R/JAK/STAT pathway members ($p=0.0364$), which are normally more frequent in
290 immature T-ALLs (9, 10, 41). Other mutations usually found in less differentiated
291 leukemias (13, 42, 43) were more common in interface cases. Notably, T-ALLs with
292 alterations in DNA methylating factors *DNMT3A*, *IDH1* and *IDH2* (including 4 with
293 double *DNMT3A*/*IDH* mutations) were confined to cluster 2 ($p=0.0267$). *RUNX1*-
294 mutated AMLs were restricted to interface clusters 2 and 3 ($p=0.0015$). Surprisingly,
295 AML-like T-ALLs in clusters 1 and 2 had frequent *PTEN* mutations, which are usually
296 found in more differentiated T-ALLs (44). Overall, AML-like T-ALLs were significantly
297 more likely to have *PTEN* mutations than the rest of the T-ALL cases analyzed by NGS
298 (3/6, 50% v 2/28, 7.1%, $p=0.0287$). Taken together, these results suggest that the
299 spectrum of differentiation arrest defined by ICGS is not directly paralleled by
300 underlying mutational genotype, but may throw light on the stage of arrest associated
301 with well-recognized somatic mutation patterns.

302 ***ICGS identifies myeloid leukemias with early lymphoid transcriptional signatures***

303 Having found that ICGS permits classification of acute leukemias along a spectrum of
304 hematopoietic differentiation, we went on to more precisely characterize the
305 transcriptional identity of individual clusters by GSEA. Analysis of the two published T-
306 ALL cohorts (32, 33) revealed that the least differentiated clusters were enriched for
307 transcriptional signatures from a series of immature myeloid and lymphoid progenitor
308 populations, in addition to HSCs (Supplementary Figure S3F).

309 Within our cohort, Cluster 1 T-ALLs were strongly enriched for mid- and late-thymic
310 expression profiles, and negatively enriched for both early thymic and UCB HSC and
311 myeloid progenitor signatures. AMLs in Clusters 4 and 5 had broadly converse patterns
312 of positive and negative enrichment (Figure 3A).

313 Transcriptional differences in IAL Clusters 2 and 3 were less clear-cut. Cluster 2 IAL
314 (comprising 7 T-ALL, 16 M0-AML and 4 non-M0 AML) were enriched for both HSC and a
315 series of lymphoid progenitor signatures, including MLP, LMDP, early B-cell
316 progenitors, T-oriented CD127- Early Lymphoid Precursors (ELPs) and CD34+CD1a-
317 CD7- early thymic cells (Figure 3A . Cluster 3 cases (9 T-ALL, 8 M0-AML and 10 non-M0-
318 AML) were more likely to be enriched for myeloid profiles (MEP, GMP and UC-derived
319 monocyte-dendritic cell progenitors, MDCP), but also showed transcriptional
320 resemblance to several lymphoid subpopulations, including LMDP and both early and
321 mid-thymic signatures (Figure 3A).

322 We considered whether this heterogeneity might be driven by differing transcriptional
323 contributions of T-ALLs and AMLs within each cluster. Further analysis of Cluster 2
324 revealed the surprising finding that while T-ALLs were mostly negatively enriched for
325 lymphoid signatures, AMLs had expression patterns that resembled several lymphoid-

326 competent populations, including MLPs, T-oriented CD127- and B-oriented CD127+
327 ELPs and early B-cell progenitors (Figure 3B). Similarly, Cluster 3 AMLs showed
328 significant enrichment for LMDP and mid-thymic signatures, while T-ALLs in the same
329 group were more likely to resemble myeloid populations, including GMPs and MDCPs
330 (Figure 3C). These data suggest that interface AMLs demonstrate significant lymphoid
331 orientation, which can be more pronounced than the T-ALLs with which they co-cluster
332 by ICGS. Enrichment for B-lymphoid transcription was particularly evident when
333 expression of genes related to B-cell development was compared in interface and non-
334 interface AMLs (Figure 3D).

335 ***ICGS-defined interface AMLs transcriptionally resemble mixed phenotype leukemia***

336 Further GSEA revealed that interface Cluster 2 was significantly enriched for a myeloid
337 leukemic stem cell (LSC) transcriptional signature (34), and that this enrichment was
338 shared by both T-ALLs and AMLs in this group (Figures 3E and 3F). AMLs in interface
339 Cluster 3 (Figure 3G), and AML-like T-ALLs (NES=1.92; FWER=0.003) were also
340 enriched for the LSC signature, suggesting that expression of leukemia stemness genes
341 is a common feature of IAL cases.

342 As interface leukemias share expression profiles with a range of progenitors of
343 multipotent lineage capacity, we next tested whether there was any transcriptional
344 similarity to MPALs of either T-lymphoid/myeloid (T/M MPAL) or B-lymphoid/
345 myeloid (B/M MPAL) phenotype in children (35) and adults (36). We found that
346 interface Clusters 2 and 3 were enriched for B/M MPAL and T/M MPAL signatures
347 respectively, and that enrichment was driven by the AML cases in each group (Figures
348 3F and 3G). Therefore, in keeping with the results observed in normal progenitor

349 comparisons, transcriptional resemblance to the earliest stages of lymphoid orientation
350 appears to be driven by interface AMLs rather than T-ALLs.

351 ***Interface AMLs have poor outcomes***

352 The fact that interface AMLs exhibit markedly different transcription to other AML cases
353 led us to speculate that these leukemias may have specific biology which in turn might
354 affect clinical behavior. We therefore evaluated the outcome of interface AMLs in two
355 independent studies (45, 46). To identify these cases, we calculated an interface AML
356 (IAL) score based on gene expression differences between interface and non-interface
357 AMLs in our cohort (Supplementary Methods and Table S8). Outcome analyses revealed
358 that AMLs with high IAL scores had significantly shorter survival in both studies
359 (Figures 4A and 4B). Within the ALFA-1701 group, we found that high IAL scores
360 predicted lack of response to gemtuzumab ozogamicin (Figure 4C), which in keeping
361 with our previous results (47), correlated with reduced expression of CD33 in high IAL
362 cases (Figure 4D). Importantly, multivariate analysis of the ALFA-0701 cohort (46)
363 revealed that IAL score predicted outcome independently of other prognostic variables,
364 including cytogenetic classification and the recently described LSC17 score (34) (Table
365 1). Consistent with this, our IAL signature had almost no overlap with the LSC17
366 signature, or the extended 48 gene signature that was reported in the same paper (34)
367 (Supplementary Figure S4A and S4B). Full comparison of clinicobiological and
368 mutational profiles of ALFA-0701 patients with high and low IAL scores is shown in
369 Supplementary Table S9. Finally, we evaluated whether IAL High cases had evidence of
370 lymphoid transcriptional activation. In keeping with our earlier results (Figure 3), we
371 found that IAL High cases in both AML cohorts were significantly enriched for both MLP
372 signatures and B-lymphoid gene expression (Supplementary Figure S4C-S4G).

373 **Discussion:**

374 In keeping with modern concepts of a hematopoietic progenitor framework that
375 comprises a spectrum of differentiation potential, integrated transcriptional analysis of
376 AMLs and T-ALLs revealed a continuum of leukemic developmental arrest. While AMLs
377 and T-ALLs at either end of the spectrum were specifically enriched for the
378 transcriptional signatures of the corresponding lineage, interface leukemias had
379 evidence of both myeloid and lymphoid precursor gene expression, with early lymphoid
380 signature enrichment being driven by interface AML cases. Specifically, while interface
381 Cluster 3 AMLs had T-lymphoid transcriptional enrichment, interface Cluster 2 AMLs
382 more closely resembled B-oriented lymphoid precursors including early B progenitors,
383 MLPs and CD127+ ELPs (24, 38), and B/Myeloid MPAL (35, 36). This cluster comprised
384 a high proportion of *RUNX1*-mutated M0-AMLs, reported to show B-cell gene activation
385 (48). Overall, these results suggest that these leukemias may be more likely to arise
386 from lymphoid-oriented progenitors and/or be arrested at an early stage of lymphoid
387 orientation (prior to CD19 expression) than is currently recognized.

388 ICGS clustering presented several important differences with accepted methods of T-
389 ALL categorization by phenotype, immunogenotype or mutational profile (9, 14, 37).
390 For example, the majority of immature T-ALLs defined by *TR* rearrangement (37)
391 (16/26, 61.5%) or ETP-ALL phenotype (12/20, 60%) (14) were in Cluster 1, including
392 those with JAK-STAT pathway mutations (Supplementary Table S7). In addition, IALs
393 had low percentages of *WT1* and *SUZ12* mutations that are typical of ETP-ALLs (9, 10)
394 and positive enrichment for *PTEN* alterations that are more frequent in mature T-ALLs
395 (44, 49). We also noted differences in mutational cooccurrence in these groups. While
396 *PHF6* mutations were always accompanied by *NOTCH1* alterations in Cluster 1, 3/5

397 *PHF6*-mutated IALs (1/3 T-ALL and 2/2 AML) were *NOTCH1* wild-type. This pattern
398 was also reported in MPAL (35, 50), and suggests that the leukemic phenotype of *PHF6*
399 mutation may correlate with co-expression of other oncogenes, as shown for *TLX3* (51).
400 Interestingly, PHF6 has been shown to regulate B/T lineage plasticity, at least on a *BCR-*
401 *ABL* leukemic background (52). Interface AMLs were also not restricted to
402 immunophenotypically immature M0 cases, since they included 29% of non-M0 AMLs.

403 Our description of myeloid/T-lymphoid IALs provides support for recent proposals to
404 define acute myeloid/T-lymphoblastic leukemia (AMTL) as a distinct diagnostic entity
405 (11), but our results also indicate that this group comprises significant molecular and
406 lineage heterogeneity, particularly with regard to lymphoid gene expression. It is also
407 striking that B-lymphoid transcription correlated with poor response to AML treatment
408 regimens. *RUNX1*-mutated AML-M0 cases in our cohort showed B-lymphoid identity,
409 which is consistent with previous reports (48). Intriguingly, *RUNX1*-mutated AMLs have
410 recently been shown to be sensitive to glucocorticoids (53), which form the backbone of
411 ALL induction treatment. Our findings therefore suggest that the poor response of these
412 cases to AML therapy in both adults (54) and children (55) might be improved by better
413 treatment allocation, and would plead against the recent provisional classification of
414 *RUNX1*-mutated AML-M0 with AML (2). Finally, we hope that these data will provide
415 further impetus to include these and other IALs in shared myeloid/lymphoid protocols
416 that might provide better treatment options for patients with these poor-risk leukemias.

417

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437

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617

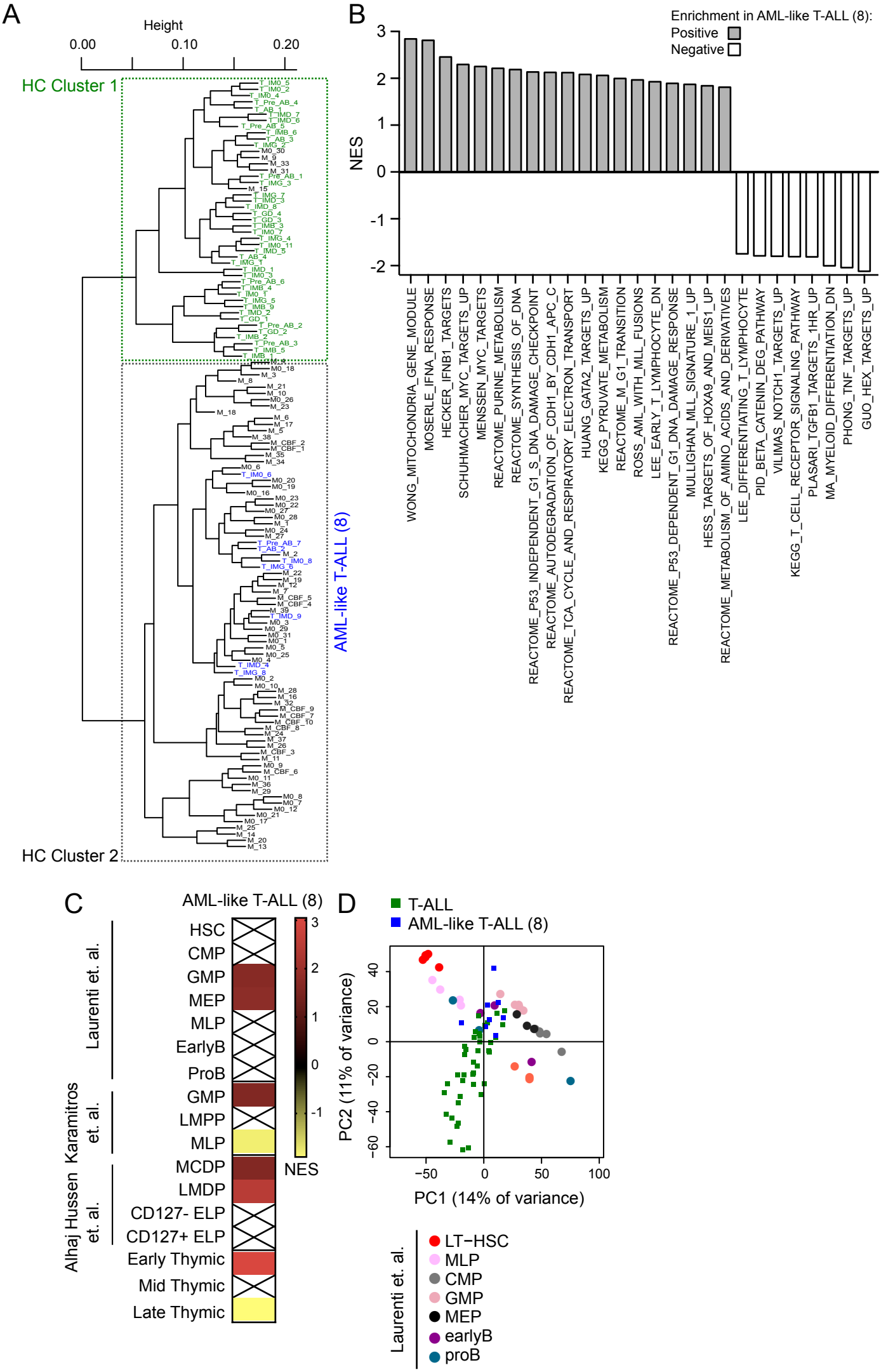
618

Variable	Univariate			Multivariate		
	HR	**95% CI**	**p**	**HR**	**95% CI**	**p**
GO Arm	0.82	0.61-1.10	0.19	0.85	0.59-1.22	0.38
Adverse cytogenetics	2.89	2.06-4.06	**<0.001**	2.17	1.41-3.36	**<0.001**
High LSC17 score*	2.45	1.71-3.53	**<0.001**	2.11	1.42-3.15	**<0.001**
***NPM1* mutation**	0.67	0.48-0.94	**0.019**	1.24	0.78-1.97	0.37
High IAL score	**1.73**	**1.21-2.46**	**0.002**	**1.58**	**1.07-2.32**	**0.021**

Table 1. Prognostic impact of IAL score on Overall Survival in the ALFA-0701 trial.

Covariates selected for multivariate analyses were selected based on the results of univariate analyses (full results in Supplementary Table S10), with additional retention of GO (gemtuzumab ozogamicin) treatment arm. *The LSC17 score was described in Ng *et al* (34). HR = Hazard Ratio. Statistically significant differences are shown in bold.

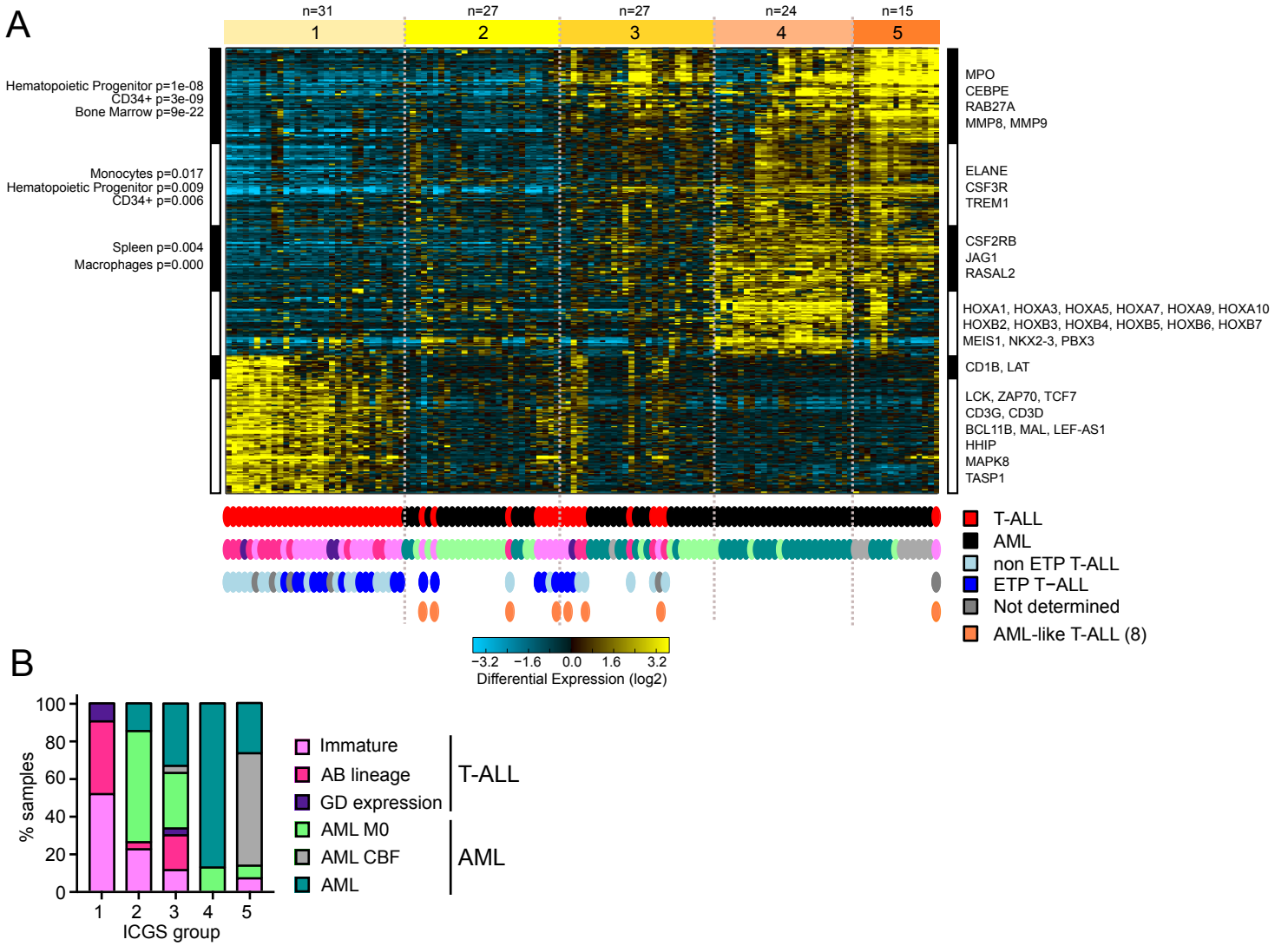
Figure 1


A
Height
0.00
0.10
0.20
HC Cluster 1
HC Cluster 2
AML-like T-ALL (8)
B
Enrichment in AML-like T-ALL (8):
Positive
Negative
NES
3
2
1
0
-1
-2
WONG_MITOCHONDRIA_GENE_MODULE
MOSERLE_IFNA_RESPONSE
HECKER_IFNB1_TARGETS
SCHUHMACHER_MYC_TARGETS_UP
MENSSEN_MYC_TARGETS
REACTOME_PURINE_METABOLISM
REACTOME_SYNTHESIS_OF_DNA
REACTOME_P53_INDEPENDENT_G1_S_DNA_DAMAGE_CHECKPOINT
REACTOME_AUTODEGRADATION_OF_CDH1_BY_CDH1_APC_C
REACTOME_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT
HUANG_GATA2_TARGETS_UP
KEGG_PYRUVATE_METABOLISM
REACTOME_M_G1_TRANSITION
ROSS_AML_WITH_MLL_FUSIONS
LEE_EARLY_T_LYMPHOCYTE_DN
REACTOME_P53_DEPENDENT_G1_DNA_DAMAGE_RESPONSE
MULLIGHAN_MLL_SIGNATURE_1_UP
HESS_TARGETS_OF_HOXA9_AND_MEIS1_UP
REACTOME_METABOLISM_OF_AMINO_ACIDS_AND_DERIVATIVES
LEE_DIFFERENTIATING_T_LYMPHOCYTE
PID_BETA_CATENIN_DEG_PATHWAY
VILIMAS_NOTCH1_TARGETS_UP
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY
PLASARI_TGFB1_TARGETS_1HR_UP
MA_MYELOID_DIFFERENTIATION_DN
PHONG_TNF_TARGETS_UP
GUO_HEX_TARGETS_UP
C
AML-like T-ALL (8)
Laurenti et. al.
Karamitros et. al.
Alhaj Hussen et. al.
HSC
CMP
GMP
MEP
MLP
EarlyB
ProB
GMP
LMPP
MLP
MCDP
LMDP
CD127- ELP
CD127+ ELP
Early Thymic
Mid Thymic
Late Thymic
3
2
1
0
-1
NES
D
T-ALL
AML-like T-ALL (8)
PC2 (11% of variance)
40
20
0
−20
−40
−60
−50
0
50
100
PC1 (14% of variance)
Laurenti et. al.
LT−HSC
MLP
CMP
GMP
MEP
earlyB
proB

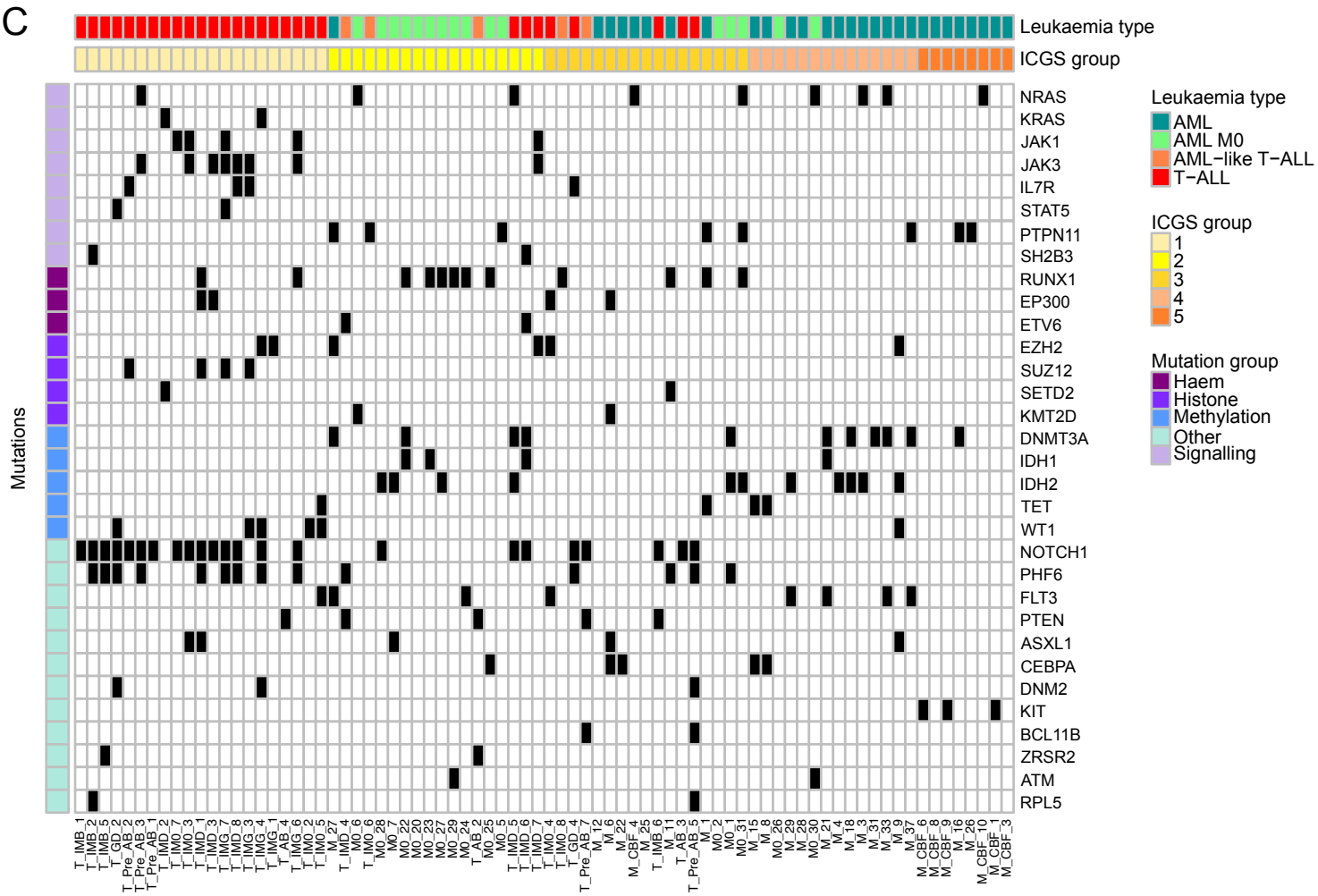
626 **Figure 1: Transcriptional profiling identifies AML-like T-ALLs that are enriched**
627 **for immature myeloid and thymic progenitor transcriptional signatures. (A)**
628 Unsupervised hierarchical clustering (HC) of the transcriptional profiles of 124 acute
629 leukemias, comprising 48 T-ALLs and 76 AMLs. A subset of T-ALL cases segregates with
630 the AML cluster. **(B)** GSEA analysis of pathways significantly enriched in AML-like T-
631 ALLs vs the rest of the T-ALL cohort. The MSigDB C2 collection of genesets was used and
632 only selected genesets with FDR < 0.05 are shown**.** NES = Normalized Enrichment Score.
633 **(C)** Enrichment of selected normal hematopoietic progenitor transcriptional signatures
634 derived from the indicated published datasets or our own analysis of thymic
635 subpopulations (genesets provided in Supplementary Table S4) in AML-like T-ALLs by
636 GSEA. NES = Normalized Enrichment Score, crossed out boxes indicate genesets that are
637 not significantly enriched (FDR > 0.05). HSC = Hematopoietic Stem Cell, CMP = Common
638 Myeloid Progenitor, GMP = Granulocyte-Monocyte Progenitor, MEP = Megakaryocytic-
639 Erythroid Progenitor, MLP = Multi-Lymphoid Progenitor, LMPP = Lymphoid-Primed
640 Multipotent Progenitor, MDCP = Monocyte-Dendritic cell Progenitor, LMDP =
641 Lymphoid-Mono-Dendritic Progenitor, ELP = Early Lymphoid Precursor. **(D)** 2D PCA
642 map of umbilical cord blood stem and progenitor populations and T-ALL gene
643 expression patterns (38); distribution of AML-like T-ALLs (blue squares) is significantly
644 different to that of other T-ALLs (PC1: p= 0.003; PC2: p= 4.1×10^{-5} by two-sided t-test).

645

Figure 2


A
n=31
n=27
n=27
n=24
n=15
1
2
3
4
5
Hematopoietic Progenitor p=1e-08
CD34+ p=3e-09
Bone Marrow p=9e-22
Monocytes p=0.017
Hematopoietic Progenitor p=0.009
CD34+ p=0.006
Spleen p=0.004
Macrophages p=0.000
MPO
CEBPE
RAB27A
MMP8, MMP9
ELANE
CSF3R
TREM1
CSF2RB
JAG1
RASAL2
HOXA1, HOXA3, HOXA5, HOXA7, HOXA9, HOXA10
HOXB2, HOXB3, HOXB4, HOXB5, HOXB6, HOXB7
MEIS1, NKX2-3, PBX3
CD1B, LAT
LCK, ZAP70, TCF7
CD3G, CD3D
BCL11B, MAL, LEF-AS1
HHIP
MAPK8
TASP1
T-ALL
AML
non ETP T-ALL
ETP T−ALL
Not determined
AML-like T-ALL (8)
−3.2
−1.6
0.0
1.6
3.2
Differential Expression (log2)

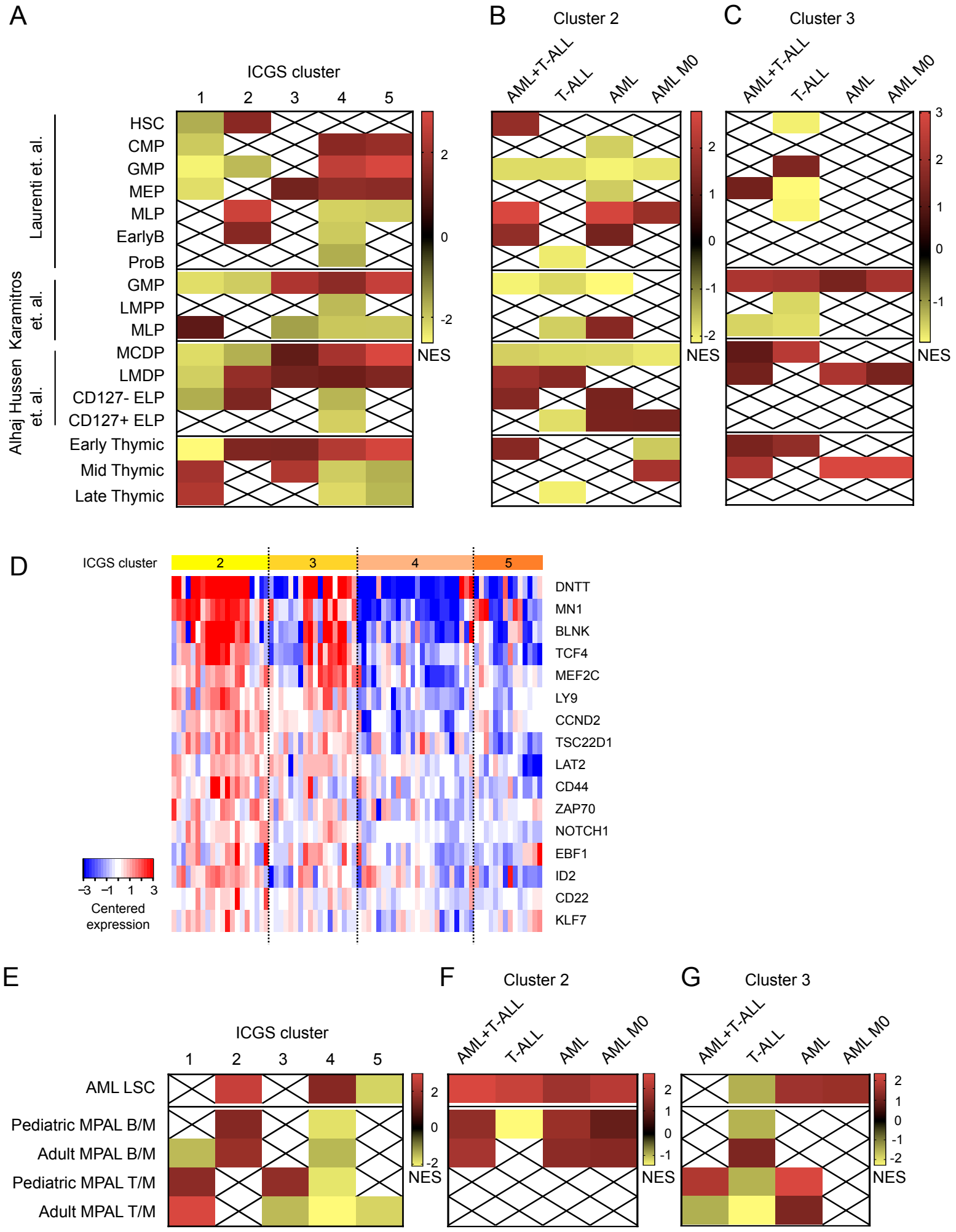
B
100
80
60
40
20
0
% samples
1
2
3
4
5
ICGS group
Immature
AB lineage
GD expression
T-ALL
AML M0
AML CBF
AML
AML


C
Leukaemia type
ICGS group
Mutations
NRAS
KRAS
JAK1
JAK3
IL7R
STAT5
PTPN11
SH2B3
RUNX1
EP300
ETV6
EZH2
SUZ12
SETD2
KMT2D
DNMT3A
IDH1
IDH2
TET
WT1
NOTCH1
PHF6
FLT3
PTEN
ASXL1
CEBPA
DNM2
KIT
BCL11B
ZRSR2
ATM
RPL5
Leukaemia type
AML
AML M0
AML−like T−ALL
T−ALL
ICGS group
1
2
3
4
5
Mutation group
Haem
Histone
Methylation
Other
Signalling

646 **Figure 2: Iterative Clustering and Guide Gene Selection (ICGS) analysis identifies a**
647 **continuum of leukemic differentiation arrest. (A-B)** ICGS analysis of adult and
648 pediatric T-ALLs (n=48 samples) and AMLs (n=76 samples) identifies 5 acute leukemia
649 clusters (top). **(A)** Heatmap of expression of guide genes selected by ICGS. Columns
650 represent individual samples. Bars on the top identify ICGS clusters. Rows represent
651 genes, and bars on the side represent blocks of correlated genes. Selected enriched gene
652 ontology groups are shown. Full gene lists are provided in Supplementary Table S5.
653 Leukemic phenotypes are indicated in the bars below the heatmap. **(B)** Proportions of
654 leukemic phenotypic groups in each ICGS cluster. **(C)** Mutations observed in T-ALL
655 (n=34) and AML (n=45) samples ordered according to ICGS analysis in (A). Only
656 mutations found in at least 2 samples are shown.

657

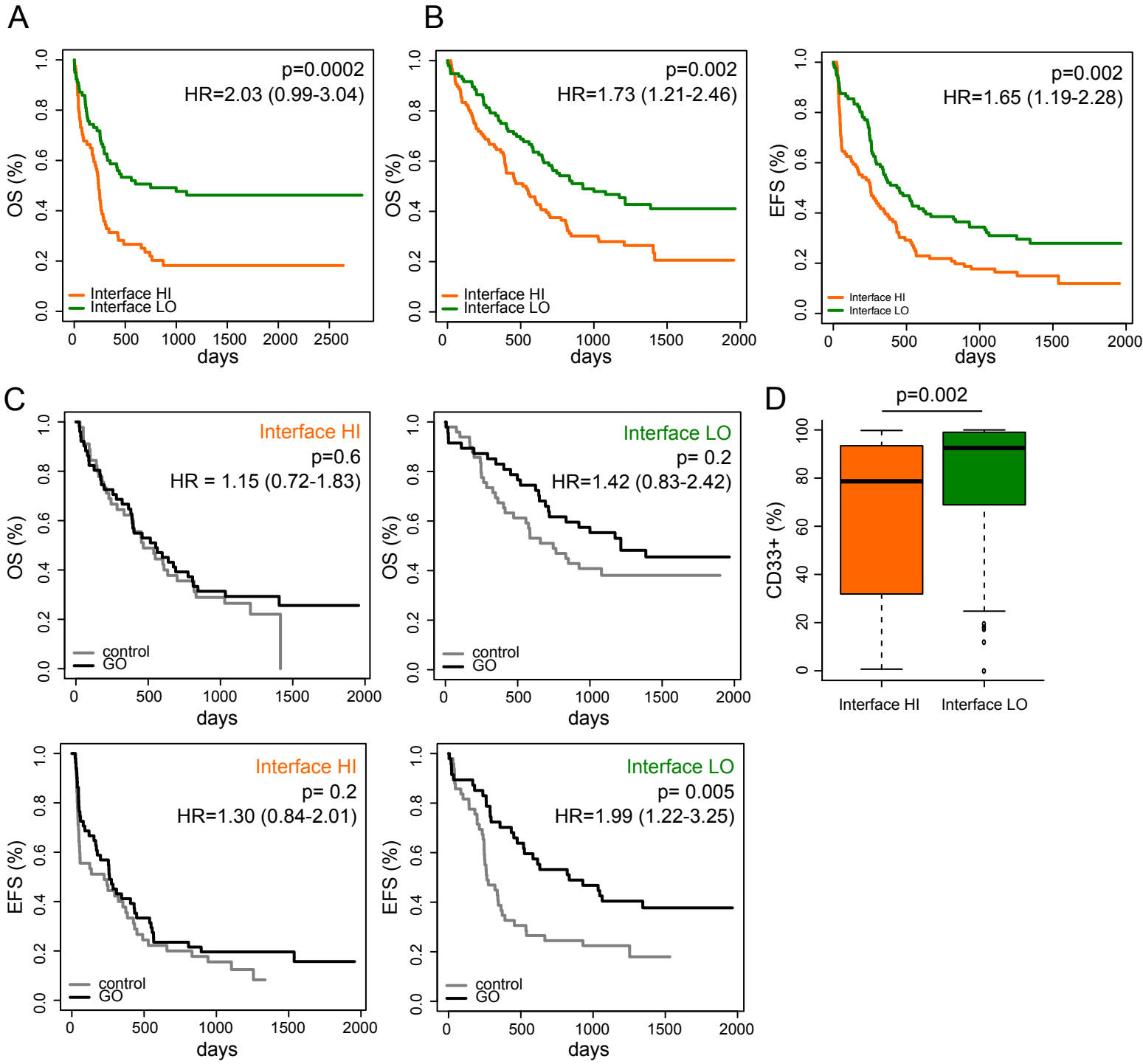
Figure 3


A
ICGS cluster
1 2 3 4 5
HSC
CMP
GMP
MEP
MLP
EarlyB
ProB
GMP
LMPP
MLP
MCDP
LMDP
CD127- ELP
CD127+ ELP
Early Thymic
Mid Thymic
Late Thymic
Laurenti et. al.
Karamitros et. al.
Alhaj Hussen et. al.
NES
B
Cluster 2
AML+T-ALL
T-ALL
AML
AML M0
C
Cluster 3
D
ICGS cluster
2 3 4 5
DNTT
MN1
BLNK
TCF4
MEF2C
LY9
CCND2
TSC22D1
LAT2
CD44
ZAP70
NOTCH1
EBF1
ID2
CD22
KLF7
Centered expression
E
AML LSC
Pediatric MPAL B/M
Adult MPAL B/M
Pediatric MPAL T/M
Adult MPAL T/M
F
G

658 **Figure 3: Transcriptional characterization of ICGS-defined clusters.** GSEAs using
659 normal hematopoietic precursor transcriptional signatures of **(A)** all clusters, **(B)**
660 interface cluster 2 and **(C)** interface cluster 3. Analyses restricted to either T-ALL, AML-
661 like T-ALL, non-M0-AML and M0-AML are shown. Crossed out boxes indicate genesets
662 that are not significantly enriched (FDR > 0.05). **(D)** Comparison of expression of genes
663 related to B-cell development in interface and non-interface AMLs. **(E) – (G)** Enrichment
664 of leukemic stem cell (LSC) (34) and mixed phenotype acute leukemia (MPAL) (35, 36)
665 transcriptional signatures by GSEA of **(E)** all clusters, **(F)** interface cluster 2 and **(G)**
666 interface cluster 3. Analyses restricted to either T-ALL, AML-like T-ALL, non-M0-AML
667 and M0-AML are shown.

668

Figure 4


A
p=0.0002
HR=2.03 (0.99-3.04)
OS (%)
days
Interface HI
Interface LO
B
p=0.002
HR=1.73 (1.21-2.46)
OS (%)
days
Interface HI
Interface LO
p=0.002
HR=1.65 (1.19-2.28)
EFS (%)
days
Interface HI
Interface LO
C
Interface HI
p=0.6
HR = 1.15 (0.72-1.83)
OS (%)
days
control
GO
Interface LO
p= 0.2
HR=1.42 (0.83-2.42)
OS (%)
days
control
GO
Interface HI
p= 0.2
HR=1.30 (0.84-2.01)
EFS (%)
days
control
GO
Interface LO
p= 0.005
HR=1.99 (1.22-3.25)
EFS (%)
days
control
GO
D
p=0.002
CD33+ (%)
Interface HI
Interface LO

669 **Figure 4: Interface IALs have poor outcomes**. Survival comparisons of AMLs with
670 high and low IAL scores in independent cohorts, **(A)** Metzeler *et al* (45) and **(B)** ALFA-
671 1701 (46). OS = Overall Survival. EFS = Event-free Survival. Hazard ratios (HR) and 95%
672 Confidence Intervals for each event and p values are indicated. **(C)** Outcome
673 comparisons according to IAL score and treatment with gemtuzumab ozogamicin (GO)
674 in the ALFA-1701 cohort. **(D)** Comparison of CD33 expression in IAL High and Low
675 cases in the ALFA-1701 cohort. Boxes indicate median, interquartile range and whiskers
676 the 95 percentile.

677