

1 **A multidimensional analysis reveals distinct immune phenotypes and the**
2 **composition of immune aggregates in pediatric acute myeloid leukemia**

3 Joost B. Koedijk^{1,2}, Inge van der Werf^{1,3}, Livius Penter^{4,5,6,7}, Marijn A. Vermeulen¹, Farnaz
4 Barneh¹, Alicia Perzoli^{1,2}, Joyce I. Meesters-Ensing¹, Dennis S. Metselaar^{1,8,9}, Thanasis
5 Margaritis¹, Marta Fiocco^{1,10,11}, Hester A. de Groot-Kruseman¹, Rubina Moeniralam¹, Kristina
6 Bang Christensen¹², Billie Porter¹³, Kathleen Pfaff¹³, Jacqueline S. Garcia^{4,6}, Scott J. Rodig¹⁵,
7 Catherine J. Wu^{4,5,6,14}, Henrik Hasle¹⁶, Stefan Nierkens^{1,17}, Mirjam E. Belderbos¹, C. Michel
8 Zwaan^{1,2#}, Olaf Heidenreich^{1,18#*}

9 ¹Princess Máxima Center for Pediatric Oncology, 3584 CS Utrecht, The Netherlands.

10 ²Department of Pediatric Oncology, Erasmus MC/Sophia Children's Hospital, 3015 GD
11 Rotterdam, The Netherlands.

12 ³Oncode Institute, 3521 AL, Utrecht, The Netherlands

13 ⁴Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

14 ⁵Broad Institute of Massachusetts Institute of Technology and Harvard University,
15 Cambridge, MA, USA

16 ⁶Harvard Medical School, Boston, MA, USA

17 ⁷Department of Hematology, Oncology, and Tumorimmunology, Campus Virchow Klinikum,
18 Berlin, Charité - Universitätsmedizin Berlin, Freie Universität Berlin and Humboldt-Universität
19 zu Berlin, Berlin, Germany

20 ⁸Hopp Children's Cancer Center Heidelberg (KiTZ), Heidelberg, Germany

21 ⁹Division of Pediatric Neurooncology, German Cancer Research Center (DKFZ) and
22 German Consortium (DKTK), Im Neuenheimer Feld 280, Heidelberg, Germany

23 ¹⁰Mathematical Institute, Leiden University, Leiden, The Netherlands

24 ¹¹Department of Biomedical Data Sciences, Leiden University Medical Center, Leiden, The
25 Netherlands.

26 ¹²Department of Pathology, Aarhus University Hospital, Aarhus, Denmark

27 ¹³Center for Immuno-Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

28 ¹⁴Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA

29 ¹⁵Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA

30 ¹⁶Pediatrics and Adolescent Medicine, Aarhus University Hospital, Aarhus, Denmark

31 ¹⁷Center for Translational Immunology, University Medical Center Utrecht, 3584 CX Utrecht,
32 The Netherlands.

33 ¹⁸Wolfson Childhood Cancer Research Centre, Newcastle University, Newcastle upon Tyne
34 NE1 7RU, UK.

35 #Equal contribution

36 *Corresponding author and lead contact: Olaf Heidenreich, Princess Máxima Center for
37 Pediatric Oncology, 3584 CS Utrecht, The Netherlands. Phone: 088-9727272; E-mail:
38 o.t.heidenreich@prinsesmaximacentrum.nl

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56

57

58 **Abstract**

59 Because of the low mutational burden and consequently, fewer potential neoantigens,
60 children with acute myeloid leukemia (AML) are thought to have a T cell-depleted or ‘cold’
61 tumor microenvironment and may have a low likelihood of response to T cell-directed
62 immunotherapies. Understanding the composition, phenotype, and spatial organization of T
63 cells and other microenvironmental populations in the pediatric AML bone marrow (BM) is
64 essential for informing future immunotherapeutic trials about targetable immune-evasion
65 mechanisms specific to pediatric AML. Here, we conducted a multidimensional analysis of
66 the tumor immune microenvironment in pediatric AML and non-leukemic controls. We
67 demonstrated that nearly one-third of pediatric AML cases has an immune-infiltrated BM,
68 which is characterized by a decreased ratio of M2- to M1-like macrophages. Furthermore,
69 we detected the presence of large T cell networks, both with and without colocalizing B cells,
70 in the BM and dissected the cellular composition of T- and B cell-rich aggregates using
71 spatial transcriptomics. These analyses revealed that these aggregates are hotspots of
72 CD8⁺ T cells, memory B cells, plasma cells and/or plasmablasts, and M1-like macrophages.
73 Collectively, our study provides a multidimensional characterization of the BM immune
74 microenvironment in pediatric AML and indicates starting points for further investigations into
75 immunomodulatory mechanisms in this devastating disease.

76

77 **Keywords:** spatial transcriptomics, trephine, tertiary lymphoid structures, ectopic T cell
78 networks, hematological malignancies.

79

80 Introduction

81 The abundance and phenotype of intratumoral T cells are crucial for the effectiveness of T
82 cell-directed immunotherapies such as immune checkpoint inhibitors (ICIs) and bispecific T
83 cell-engagers¹⁻⁴. Because of the low mutational burden and consequently, fewer potential
84 neoantigens, children with acute myeloid leukemia (AML) are thought to have a T cell-
85 depleted or 'cold' tumor microenvironment and therefore may have a low likelihood of
86 response to ICIs and bispecific T cell-engagers⁵⁻⁸. Although clinical trials in adult AML
87 patients treated with ICIs and bispecific T cell-engagers, whether as mono- or combination
88 therapy, have largely been unsuccessful, a small subset of patients showed exceptional
89 responses^{4, 9-15}. This suggests that there may be specific subgroups that could benefit from
90 these T cell-directed immunotherapies. To pave the way for successful ICI- and bispecific T
91 cell-engager immunotherapies in both adult and pediatric AML, a better understanding of the
92 heterogenous landscape of bone marrow (BM)-infiltrating T cells and the surrounding tumor
93 microenvironment is needed. While recent studies have provided insights on this matter in
94 adult AML, such as the identification of a relatively low presence of exhausted CD8⁺ T cells
95 in the tumor microenvironment compared to cancers that respond well to ICIs^{16, 17}, there is a
96 paucity of data on the BM immune microenvironment in pediatric AML^{11, 18-20}. In addition to
97 the need for a quantitative and qualitative assessment of the pediatric AML BM immune
98 microenvironment, emerging research in solid cancers has shown that the spatial
99 organization of the immune response is highly relevant for ICI efficacy²¹. For instance,
100 'excluded' tumors, where the immune response cannot invade the tumor bed, show poor
101 responses to ICI²². Furthermore, the presence of intratumoral immune aggregates, such as
102 tertiary lymphoid structures (TLS), is associated with improved ICI responses in many solid
103 cancers²³⁻²⁸. Despite its potential importance, the spatial organization of the immune
104 response in hematological cancers remains understudied. Therefore, we generated a
105 multidimensional view of the tumor immune microenvironment in treatment-naïve *de novo*
106 pediatric AML, to inform future immunotherapeutic trials about potentially targetable immune-
107 evasion mechanisms specific to this patient population. We identified distinct BM immune
108 phenotypes and dissected the composition of immune aggregates in the BM of pediatric
109 AML, which encourage further investigations into immunomodulatory mechanisms in this
110 devastating disease.

111 Materials/Subjects and Methods

112 Ethical regulation

113 This study complies with all relevant ethical regulations and was approved by the
114 Institutional Review Boards of the Princess Máxima Center for Pediatric Oncology

115 (PMCLAB2021.207 & PMCLAB2021.238), the Scientific Committee of the Dutch Nationwide
116 Pathology Databank (PALGA: lzv2021-82)²⁹ and at participating sites of the ETCTN/CTEP
117 10026 study^{12, 13}. All patients treated at the Princess Máxima Center, Aarhus University
118 Hospital, and Dana-Farber Cancer Institute provided written consent for banking and
119 research use of these specimens, according to the Declaration of Helsinki.

120 **Human patient samples**

121 FFPE BM biopsies taken from the crista of children with treatment-naïve *de novo* AML and
122 non-leukemic controls (Supplementary Methods) were obtained from the Princess Máxima
123 Center Biobank (n=15), biobanks of 10 other Dutch hospitals (n=28; mediated by the Dutch
124 National Tissue Portal) and the biobank of Aarhus University Hospital (n=29). BM biopsies of
125 adult AML cases treated on the ETCTN/CTEP 10026 study (n=6) were collected at Dana-
126 Farber Cancer Institute^{13, 17}. Details on other pediatric and adult AML datasets,
127 immunohistochemistry/immunofluorescence, digital image analysis, spatial transcriptomics,
128 and immune-related gene expression profiling are provided in the Supplementary Methods.

129 **Statistical analyses**

130 Statistical analyses were performed with GraphPad Prism V.9.3.0 (GraphPad Software, LA
131 Jolla, CA, USA). Details on the various statistical tests used are provided in the
132 Supplementary Methods. For spatial transcriptomics data, $P < 0.01$ was considered
133 statistically significant to correct for measuring multiple regions from the same biopsy. For all
134 other comparisons, $P < 0.05$ was considered statistically significant.

135 **Results**

136 **A subset of pediatric AML patients has high T cell infiltration in the bone marrow**

137 To determine whether the BM microenvironment in pediatric AML is characterized by high
138 ('hot') or low ('cold') T cell infiltration, we performed immunohistochemistry (IHC) with
139 antibodies against CD3 and CD8 on 82 formalin-fixed and paraffin-embedded (FFPE) BM
140 biopsies from pediatric patients with treatment-naïve *de novo* AML (n=72), and age- and
141 sex-matched non-leukemic individuals (n=10; **Fig. 1A**; representative images shown in **Fig.**
142 **1B-C**; collectively referred to as 'primary study cohort'). Patient characteristics are depicted
143 in **Table S1**. We found a trend towards a decreased abundance of the number of T cells and
144 a significant decrease in CD8⁺ T cells in pediatric AML cases in comparison to non-leukemic
145 controls ($P=0.11$ and $P=0.011$, respectively; **Fig. 1D-E**), similar to other observations in adult
146 and pediatric AML^{20, 31, 32}. The T- and CD8⁺ T cell infiltration ranged from 55 to 9832
147 cells/mm² between individual pediatric AML cases, with a subset showing high T- and CD8⁺

148 T cell infiltration in the BM (above median of non-leukemic controls; n=22 and n=18,
149 respectively; **Fig. 1D-E**).

150 We next explored whether this heterogeneity in T cell infiltration reflected inherent
151 differences in disease biology. Notably, eleven out of nineteen patients with *KMT2A*-
152 rearranged AML (58%) and five out of eight patients with complex karyotype AML (63%) had
153 high BM T cell infiltration (**Fig. 1F**). However, substantial heterogeneity in T cell infiltration
154 was also present within both the *KMT2A*-rearranged and complex karyotype AML groups
155 (**Fig. 1F-G**). Thus, both high and low levels of BM T cell infiltration were noted among
156 *KMT2A*-rearranged and complex karyotype AML patients. Most cases in the other
157 cytogenetic subgroups had low overall T- and CD8⁺ T cell infiltration (**Fig. 1F-G**). Specifically,
158 twelve out of thirteen cases (92%) with normal karyotype AML had low total T- and CD8⁺ T
159 cell infiltration. Among these thirteen cases, nine (69%) had both low T cell infiltration and a
160 *FLT3*-ITD and/or *NPM1* mutation (**Table S1**), which have been associated with low T- and
161 NK cell infiltration in adult AML³³. Using diagnostic flow cytometry data of an independent
162 cohort of 20 pediatric cases with normal karyotype AML (n=12 wildtype, n=8 *FLT3*-ITD
163 and/or *NPM1* mutation), we confirmed this association ($P=0.013$; **Fig. S1A**).

164 Given the relatively small number of samples in some cytogenetic subgroups, we
165 also reanalyzed a large bulk RNA-sequencing dataset of treatment-naïve pediatric AML BM
166 aspirates (TARGET-AML cohort, n=159 patients; **Fig. 1H**)³⁴. Using the immune
167 deconvolution tool CIBERSORTx^{17, 35, 36}, we estimated the absolute abundance scores of T-
168 and CD8⁺ T cells, which ranged from 0.15 to 5.15 and 0 to 1.43, respectively (**Fig. 1H-J**). We
169 categorized patients into overall T- and CD8⁺ T cell-high and -low groups based on the
170 median estimated BM T- and CD8⁺ T cell abundance in four non-leukemic pediatric controls
171 (BM bulk RNA-sequencing data³⁷). The TARGET-AML cohort showed substantial
172 heterogeneity in overall T- and CD8⁺ T cell levels across all cytogenetic subgroups, with only
173 nine out of 27 *KMT2A*-rearranged AML cases (33%) having high T cell infiltration (**Fig. 1I-J**).
174 Consequently, we hypothesized that specific *KMT2A*-rearrangements may be associated
175 with different levels of T cell infiltration in the BM. We therefore compared the levels of T-
176 and CD8⁺ T cells among cases with *KMT2A*-rearrangements with at least three samples per
177 group (TARGET-AML cohort). This analysis suggested a lower abundance of CD8⁺ T cells in
178 *KMT2A::ELL* AML compared to *KMT2A::MLL3* AML ($P=0.044$; **Fig. S1B-C**), although the
179 small sample size precluded firm conclusions. Among cases with complex karyotype AML,
180 thirteen out of 24 cases (54%) had high T cell infiltration (**Figure 1I**). However, we were not
181 able to identify any commonalities between those with high or low T cell infiltration. Other
182 clinical factors including the abundance of leukemic blasts and AML differentiation stage
183 showed no or only marginal correlation with T- and CD8⁺ T cell infiltration (**Fig. S1D-K**).

184 To further characterize the immune profiles of pediatric AML cases with a high
185 abundance of T cells in the BM, we categorized AML cases in the primary study cohort into
186 two groups using the non-leukemic controls' median CD3⁺ T cell abundance as the cut-off
187 value (1133 cells/mm²; **Fig. 2A**). Focused IHC analyses of CD20 expression showed that
188 patients in the CD3⁺ T cell-high group also had a significantly higher abundance of CD20⁺ B
189 cells in comparison to the CD3⁺ T cell-low group ($P<0.001$; **Fig. 2B**). Accordingly, we termed
190 the two groups 'immune-infiltrated' and 'immune-depleted' (n=22 and n=50, respectively).
191 We subsequently investigated whether these groups demonstrated differences in clinical
192 outcomes upon standard chemotherapy treatment (Supplementary Methods), as seen in
193 many other cancer types¹. In our cohort, we did not detect differences in overall survival
194 (OS) between the immune-infiltrated and immune-depleted group (**Fig. S2A**). To increase
195 statistical power, we repeated the analysis in the TARGET-AML cohort. Again, we did not
196 detect differences in OS between the immune-infiltrated (CD3⁺ T cell-high, n=66) and
197 immune-depleted (CD3⁺ T cell-low, n=93) groups (**Fig. S2B**), together suggesting that the
198 extent of T cell infiltration in the BM at diagnosis is not critical for patient survival in pediatric
199 AML cases treated with standard chemotherapy regimens. Taken together, we identified
200 wide heterogeneity in T cell infiltration in the BM of pediatric AML with nearly one-third of
201 cases (31%) showing notably high T cell infiltration. In addition, our findings suggest that
202 specific genetic alterations may be linked to the extent of T cell infiltration in the BM of
203 pediatric AML, although the exact underlying mechanisms remain to be unraveled^{38, 39}.

204 **The ratio of M2- to M1-like macrophages is linked to the extent of T cell infiltration in** 205 **the pediatric AML bone marrow**

206 To elucidate mechanisms driving high- and low immune infiltration in the BM of pediatric
207 AML, we examined the expression of immune-related genes in a cytogenetically
208 representative subset of immune-infiltrated (n=6) and immune-depleted cases (n=17) using
209 the NanoString PanCancer IO 360TM panel (**Fig. 2A**). Using differential gene expression
210 analysis, we identified genes related to T- and/or NK cells to be significantly upregulated in
211 immune-infiltrated compared to immune-depleted pediatric AML (**Fig. 2C**), confirming our
212 categorization of cases into these two groups. Regarding factors that either promote or
213 restrict T cell infiltration, we found that immune-infiltrated cases demonstrated significantly
214 higher expression of genes related to T cell-attracting chemokines (*CXCL9*, *CXCL10*), and
215 their corresponding receptor (*CXCR3*; **Fig. 2C**). In line with this, pathway analysis using the
216 GO biological processes and WikiPathways gene sets indicated that immune-infiltrated
217 cases were enriched in T cell migration and chemokine signaling, suggesting that immune-
218 depleted cases lack signals that attract T cells to the BM (**Fig. 2D**; full list of pathways in
219 **Table S2**). In solid cancers, macrophages have been described as key players in attracting

220 T cells⁴⁰. Specifically, pro-inflammatory M1-like macrophages are known to be a primary
221 source of T cell-attracting chemokines, while anti-inflammatory M2-like macrophages restrict
222 T cell infiltration into the tumor^{40, 41}. Previously, a gene expression-based score that reflects
223 the ratio between M2- and M1-like macrophages (a score > 0 indicates M2-predominance,
224 while a score < 0 indicates M1-predominance; collectively termed M2-predominance score)
225 has been developed for five cancer types including AML⁴². We applied this score to our
226 cohort and found that the M2-predominance score was negatively correlated with the BM T-
227 and CD8⁺ T cell abundance ($r=-0.75$ [95% CI: -0.89 to -0.48], $P<0.001$; $r=-0.50$ [95% CI: -
228 0.76 to -0.12], $P=0.014$, respectively; **Fig. 2E,F** and **Fig. S2C**). Concordantly, we identified a
229 significantly decreased M2-predominance score in immune-infiltrated compared to immune-
230 depleted cases ($P=0.001$; **Fig. 2G**). Likewise, in the TARGET-AML cohort, we detected a
231 (subtle) negative correlation between the M2-predominance score and the T- and CD8⁺ T
232 cell abundance in the BM ($r=-0.25$ [95% CI: -0.39 to -0.10], $P=0.002$; $r=-0.20$ [95% CI: -0.35
233 to -0.05], $P=0.011$, respectively; **Fig. 2H** and **Fig. S2D**), and a significantly decreased M2-
234 predominance score in the immune-infiltrated compared to the immune-depleted group
235 ($P<0.001$; **Fig. 2I**). Altogether, our data suggest that the M2:M1-like macrophage ratio is
236 linked to the extent of T cell infiltration in the BM of pediatric AML, in line with data from
237 (preclinical) studies in other cancers^{40, 42}.

238 T cells cluster into aggregates in the bone marrow of pediatric AML

239 Several studies in solid cancers have identified the presence of immune aggregates in the
240 tumor microenvironment, such as T- and B cell-rich structures resembling secondary
241 lymphoid organs (TLSs) and T cell-rich structures lacking B cells^{23-25, 43, 44}. These aggregates
242 may represent sites of priming or re-activation of anti-tumor immune responses and have
243 been associated with better responses to immune checkpoint inhibitors in many cancers
244 types^{23-28, 45, 46}. As TLSs and other immune aggregates often develop at sites of chronic
245 inflammation²³⁻²⁵, we next asked whether similar structures were present in the BM of
246 immune-infiltrated pediatric AML cases. Accordingly, we first investigated the number of T
247 cell networks in pediatric immune-infiltrated (n=22), immune-depleted (n=50), and non-
248 leukemic BM biopsies (n=10). In this analysis, T cell networks were defined as at least ten
249 directly interacting T cells (≤ 10 μm between adjacent nuclei), to avoid classifying randomly
250 dispersed T cells as networks (**Fig. 3A**)⁴⁴. We found that T cell networks were present in
251 both AML groups and controls, although they were significantly more frequent in the BM of
252 immune-infiltrated compared to immune-depleted cases ($P<0.001$; **Fig. 3B** and **Table S1**;
253 representative image of a T cell network shown in **Fig. 3C**). Moreover, controls had
254 significantly more T cell networks in comparison to immune-depleted cases ($P=0.027$; **Fig.**
255 **3B**; representative images of immune-depleted biopsies shown in **Fig. S3A-B**).

256 In addition, we identified large T cell networks, defined as at least 100 directly
257 interacting T cells (**Fig. 3D**), to be more abundant in the BM of immune-infiltrated compared
258 to immune-depleted cases ($P<0.001$; **Fig. 3E**; representative image shown in **Fig. 3F**).
259 *KMT2A*-rearranged (9/17 cases; 53%) and complex karyotype AML (3/17 cases; 18%) were
260 the most common cytogenetic subgroups among cases with large T cell networks ($n=17$ in
261 total; **Table S1**). These large networks often colocalized with a dense network of B cells in
262 immune-infiltrated cases (termed ‘lymphoid aggregates’; LA; **Fig. 3G**; 9/15 cases; 60%);
263 representative images shown in **Fig. 3H-I**, **Fig. S3C-D**; 0/2 immune-depleted cases).
264 However, the LAs identified in pediatric AML patients were not organized in a fashion seen
265 in mature TLSs with an inner zone of B cells surrounded by T cells²³⁻²⁵. Instead, T- and B
266 cells appeared to be mixed throughout the aggregate, as seen in immature TLSs and other
267 immune aggregates (representative images shown in **Fig. 3H-I** and **Fig. S3C-D**)^{25, 43}. In the
268 BM of non-leukemic control biopsies, we did not identify LAs or large T cell-dominant
269 networks with no or sparse B cells (representative image shown in **Fig. S3E**). Taken
270 together, these data show that large networks of T cells, both with and without colocalizing B
271 cells, are frequent in the BM of treatment-naïve immune-infiltrated pediatric AML cases.

272 Given that TLSs and other immune aggregates may function as sites of intratumoral
273 immune priming that can lead to successful anti-tumor immunity in solid cancers^{23-28, 45-48},
274 they may also serve as markers of leukemia-specific immune responses in hematological
275 malignancies. Consequently, we performed pilot work to explore whether responses to
276 immune checkpoint inhibitors were associated with the presence of LAs and/or large T cell-
277 dominant networks in the AML BM. As data from pediatric AML cases treated with such
278 therapies were not available, we applied multiplex immunofluorescence to pre- and post-
279 treatment BM biopsies of transplant-naïve and post-transplant adult AML cases (three
280 responders and three non-responders) treated with both ipilimumab and decitabine in the
281 context of a clinical trial (NCT02890329^{13, 17}; **Fig. S4A**). Accordingly, BM biopsies collected
282 at baseline, time of best response, and end of treatment were stained with antibodies
283 against CD3, CD20, and CD34 ($n=17$ in total; representative images of LAs and large T cell-
284 dominant networks are shown in **Fig. S4B-D**; patient characteristics are depicted in **Table**
285 **S3**). LAs were only observed in responders (response definitions in Supplementary
286 Methods), although their time of appearance differed (**Fig. S4E-J**). Large T cell-dominant
287 networks were present in two responders at baseline and at time of best response
288 (AML1002 + AML1006; **Fig. S4E,G**). Among non-responders, one patient had large T cell-
289 dominant networks at baseline (AML1010; **Fig. S4I**). Like pediatric AML, T- and B cells in
290 lymphoid aggregates were not organized in distinct zones (**Fig. S4B-C**). Thus, in this
291 exploratory analysis, we found that large T cell-dominant networks were present both at

292 baseline and at time of best response in two out of three responders, whereas LAs were
293 present in all three responders but at different time points. Although the patient
294 heterogeneity and small cohort size preclude conclusions regarding the predictive utility of
295 lymphoid aggregates and T cell dominant networks, our preliminary findings encourage
296 future investigations into the association between immune aggregates and ICI response in
297 both adult and pediatric AML, as done in solid cancers^{26-28, 45}.

298 **Spatial transcriptomics unravels the composition of lymphoid aggregates in the bone** 299 **marrow of pediatric AML**

300 The identification of LAs prompted us to explore whether the profiles of these aggregates
301 shared similarities with TLSs in solid cancers (i.e. several T cell subsets, germinal center B
302 cells, plasma cells, dendritic cells)²³⁻²⁵. To accurately identify TLSs, several transcriptomic
303 signatures have been proposed²⁵: a 12-gene chemokine signature⁴⁹ ('12chem'; reflective of
304 TLSs independent of their maturation stage), an 8-gene follicular helper T cell signature⁵⁰
305 ('Tfh'; reflective of mature TLSs), and a 29-gene TLS imprint signature²⁷ (reflective of mature
306 TLSs). We aimed to examine the expression of these signatures in both LA and BM regions
307 without such aggregates. Towards this aim, we performed spatial transcriptomics using the
308 GeoMx spatial transcriptomics platform³⁰ on eight BM biopsies: four immune-infiltrated AML
309 biopsies with LAs, and four reference biopsies (two immune-infiltrated AML biopsies without
310 LAs and two non-leukemic control biopsies; patient characteristics are depicted in **Table S1**).
311 Regions of interest (ROIs) included areas with LAs, 'mixed' areas containing leukemic cells
312 and various other populations, and 'control' areas in the non-leukemic BM (region types are
313 illustrated in **Fig. 4A-C**; representative images of ROI selection are shown in **Fig. S5A**). In
314 total, we successfully sequenced 143 regions with an average of 335 cells per region (range:
315 126 to 826 cells): 35 LA regions, 92 mixed regions, and 16 control regions. Our analysis
316 revealed two main clusters of regions in the UMAP: cluster 1 contained both regions with
317 LAs and mixed regions from biopsies with LAs, while cluster 2 consisted of mixed regions
318 from biopsies without these aggregates, and control regions (**Fig. 4D**). Consequently, we
319 divided mixed regions in two distinct groups: areas from biopsies with LAs (MIXED1, N=48),
320 and those from biopsies without such aggregates (MIXED2, N=44). All three TLS-specific
321 gene signatures were significantly enriched in LA regions in comparison to both mixed- and
322 control regions (**Fig. 4E-F** and **S5B**). Thus, while the identified LAs lacked the typical
323 organization of mature TLS, we did identify enrichment of the Tfh- and TLS imprint
324 signatures representative of mature TLSs.

325 To gain insight into differences in immune and stromal cell types between LA and
326 other BM regions, we performed immune deconvolution by integrating spatial transcriptomic

327 data with single-cell (sc) and flow-sorted bulk RNA-sequencing data of microenvironmental
328 cell populations⁵¹. This analysis revealed that LA regions had an increased abundance of
329 CD4⁺ T cells, CD8⁺ T cells, non-plasma B cells, plasma cells, and macrophages (**Fig. 4G-K**).
330 In both LA and MIXED1-regions, the CD4⁺:CD8⁺ T cell ratio was lower compared to
331 MIXED2- and control regions (approximately 1:1 in both vs. 2:1 and 3:1, respectively; **Fig.**
332 **S5C**), indicating a relatively higher abundance of CD8⁺ T cells in the former regions. When
333 considering macrophage polarization, we noted a significantly lower M2-predominance score
334 in LA regions compared to both mixed regions, consistent with the negative correlation
335 between M2-predominance and BM T cell infiltration observed above (**Fig. 4L; Fig. 2F**).
336 Moreover, endothelial cells and fibroblasts were enriched in LA regions compared to
337 MIXED1-regions (**Fig. S5D-E**). Additionally, NK- and dendritic cells were distributed evenly
338 across AML biopsies, while MIXED2 regions had a significantly higher abundance of
339 neutrophils compared to both LA and MIXED1-regions (**Fig. S5F-H**). To confirm our
340 deconvolution results, we performed differential gene expression analysis between LA,
341 mixed, and control regions. In LA regions compared to non-aggregate regions within the
342 same biopsies (MIXED1), we found upregulation of many T- and B cell genes (e.g., *CD3D*,
343 *CD8B*, *CD79A*), as well as genes associated with TLS formation in solid cancers (*LTB*,
344 *CCL19*) and B cell recruitment (*CXCL13*; **Fig. S6A**). Furthermore, immunoglobulin-related
345 genes (*IGHG1*, *IGHG3*, *IGHG4*, *IGKC*) were significantly enriched in LA compared to
346 MIXED1-regions, consistent with an increased abundance of plasma and/or memory B cells
347 (**Fig. S6A**). Similar results were observed when comparing LA regions with MIXED2- and
348 control regions (**Table S4**).

349 Given the combination of a lack of distinct T- and B cell zones and an increased
350 estimated abundance of plasma cells in these LAs, we next sought to perform a more in-
351 depth characterization of the B cells in these regions. To do so, we again applied immune
352 deconvolution by integrating our spatial transcriptomic data with another scRNA-sequencing
353 reference dataset (**Fig. 5A**). In this case, we used a comprehensive scRNA-sequencing
354 dataset of pediatric tonsillar B cells⁵², which included naïve B cells, germinal center B cells,
355 plasmablasts, and memory B cells, as a reference. In line with the lack of a follicle-like
356 morphology of LAs, we did not identify germinal center B cells in these regions (**Fig. 5B**).
357 Similarly, naïve B cells were absent. The most abundant B cells were memory B cells, while
358 a smaller fraction consisted of plasmablasts, together indicating the presence of
359 differentiated B cell subsets in these aggregates (**Fig. 5B**). Since LA regions showed
360 increased expression of the cytotoxicity-related genes *GZMA* and *GZMK* compared to
361 neighboring regions (**Fig. S6A**), we also aimed to investigate the cytotoxic capacity of CD8⁺
362 T cells in these regions. Accordingly, we performed immune deconvolution of our spatial

363 transcriptomic dataset using a scRNA-sequencing dataset of BM CD8⁺ T cells from adult
364 AML patients¹⁶. This reference dataset included naïve-, memory-, cytotoxic- (CTL), mucosal-
365 associated invariant T- (MAIT), and ‘dysfunctional’ CD8⁺ T cells. The CD8⁺ T cells classified
366 as dysfunctional were characterized by increased expression of multiple immune checkpoint
367 receptor (IR) genes, such as *PDCD1*, *LAG3*, and *HAVCR2*, and reduced expression of
368 cytotoxicity-related genes such as *GZMB*, *GNLY*, and *PRF1*. Notably, deconvolution
369 revealed that these potentially dysfunctional CD8⁺ T cells were enriched in LA regions
370 compared to mixed- and control regions, at the expense of both naïve- and cytotoxic CD8⁺ T
371 cells (**Fig. 5C-D**). Moreover, memory CD8⁺ T cells were not identified in any of the biopsies.
372 The increased proportion of potentially dysfunctional CD8⁺ T cells in LA regions prompted us
373 to investigate whether these T cells were truly dysfunctional (i.e. no cytotoxic potential).
374 Accordingly, we examined the expression of the cytotoxicity marker granzyme B (GZMB) in
375 CD8⁺ T cells that expressed at least two immune checkpoint receptor markers (IR^{++/+++}; PD-
376 1, LAG3, and/or TIM-3) in a subset of the LA-rich biopsies that had been characterized using
377 spatial transcriptomics (n=2 biopsies with six LAs in total). Using multiplex
378 immunofluorescence with antibodies against CD3, CD8, PD-1, LAG3, TIM-3, and GZMB, in
379 combination with standard IHC for CD20, we identified that 58% (range: 30 to 100%) of
380 CD3⁺CD8⁺IR^{++/+++} T cells in LAs expressed GZMB (representative images shown in **Fig. 5E**;
381 **Fig. 5F**), suggesting that more than half of the potentially dysfunctional CD8⁺ T cells still had
382 cytotoxic capacity. In addition, since TLS-associated regulatory T cells (Tregs) have been
383 found to attenuate the positive prognostic effect of TLS-associated CD8⁺ T cells in human
384 non-small cell lung cancer, colorectal cancer, and soft tissue sarcoma^{45, 53, 54}, we assessed
385 the presence of putative Tregs (CD3⁺FOXP3⁺) in LAs in the same subset of biopsies (n=2).
386 In the six examined LAs, we observed very few Tregs (<1%), suggesting that Tregs are
387 hardly present in LAs in pediatric AML (representative image shown in **Fig. S6B**; **Fig. S6C**).
388 Taken together, our integrative spatial analyses demonstrate that LAs in the BM of pediatric
389 AML are specifically enriched for CD8⁺ T cells, memory B cells, plasma cells and/or
390 plasmablasts, and M1-like macrophages. Despite the lack of germinal center B cells and
391 separate T- and B cell zones, the presence of memory B cells, plasma cells and/or
392 plasmablasts, and immunoglobulin gene expression suggests that LAs in pediatric AML are
393 sites of B cell differentiation. Furthermore, the presence of CD8⁺ T cells expressing multiple
394 immune checkpoint markers, yet not fully exhausted, within LAs, encourages future
395 investigations into the potential of leveraging these LA-associated T cells for
396 immunotherapeutic efficacy.

397 Discussion

398 In this study, we performed a multidimensional assessment of the tumor immune
399 microenvironment in pediatric AML. We demonstrated that nearly one-third of pediatric AML
400 cases has an immune-infiltrated BM, which is characterized by a decreased M2-:M1-like
401 macrophage ratio. Furthermore, we detected the presence of large T cell networks, both with
402 and without colocalizing B cells, in the BM and dissected the cellular composition of T- and B
403 cell-rich aggregates using spatial transcriptomics. These analyses revealed that these LAs
404 are hotspots of CD8⁺ T cells, memory B cells, plasma cells and/or plasmablasts, and M1-like
405 macrophages. Collectively, our study provides a multidimensional characterization of the BM
406 immune microenvironment in pediatric AML and indicates starting points for further
407 investigations into immunomodulatory mechanisms in this devastating disease.

408 Understanding the prevalence of distinct immune phenotypes and associated
409 targetable immune-evasion mechanisms in the BM of pediatric AML is key for better
410 prospective selection of patients in future immunotherapy trials^{33, 45}. Although we identified a
411 subset with high abundance of T cells, many pediatric AML cases had an immune-depleted
412 BM microenvironment. We observed that *FLT3*-ITD and/or *NPM1* mutations were associated
413 with low T cell infiltration in the BM of pediatric AML, consistent with data in adult AML³³.
414 Although we did not investigate the direct consequences of these molecular alterations, such
415 as alterations in the expression of immunomodulatory surface molecules^{38, 39}, we identified
416 M2-like macrophage predominance in immune-depleted cases. The observed negative
417 correlation between the M2-:M1-like macrophage ratio and the extent of T cell infiltration is
418 consistent with data from (preclinical) studies in other cancers, suggesting a role for M2-like
419 macrophages (or a lack of M1-like macrophages) in limiting T cell infiltration to the leukemic
420 BM^{40, 41}. Consequently, targeting M2-like macrophages (e.g., using checkpoint blockade) to
421 overcome their immunosuppressive functions could be an attractive therapeutic strategy for
422 immune-depleted AML^{55, 56}.

423 In addition, our integrative spatial analyses showed that T cells in the BM can be
424 organized into multicellular aggregates. Although T- and B cell-rich LAs did not contain
425 distinct T- and B cell zones and germinal center B cells typically seen in mature TLSs in solid
426 cancers, the localized enrichment of memory B cells, plasma cells and/or plasmablasts, and
427 immunoglobulin gene expression suggests that these aggregates are sites of B cell
428 maturation. Moreover, the 1:1 ratio of CD4⁺ and CD8⁺ T cells observed in LAs has also been
429 associated with mature TLSs⁵⁷. Notably, CD8⁺ T cells in LAs were found to be in different
430 cell states, including a subset that expressed multiple immune checkpoint markers. GZMB-
431 expression was identified in more than half of these cells, suggesting that these CD8⁺ T cells
432 are not fully exhausted and may be susceptible to ICI therapy⁵⁸. In addition to T- and B cell-
433 rich immune aggregates, we detected large T cell networks with sparse B cells. Since these

434 networks have also been associated with ICI response in solid cancers, further
435 investigations into the different types of immune aggregates in AML and their relevance for
436 immunotherapy response are needed^{43, 46, 59, 60}. For instance, do large T cell-dominant
437 networks relate to LAs, and do they (differentially) associate with ICI efficacy in AML? Since
438 TLS are considered to be sites where tumor-specific T- and B cell immune responses may
439 be generated²³⁻²⁵, future research also needs to investigate whether immune aggregates in
440 AML are tumor-directed.

441 Altogether, our analyses deepen the understanding of the BM immune
442 microenvironment in AML and provides an impetus to explore how intratumoral immune
443 aggregates could be exploited for improving immunotherapy outcomes. Further, our work
444 provides a framework for leveraging spatial transcriptomics to interrogate the spatial
445 organization of the leukemic BM. Advances in spatial transcriptomic techniques now allow
446 for investigating the spatial dimension of hematological malignancies at subcellular
447 resolution, opening an exciting path towards new discoveries in the field of AML^{61, 62}.

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490 **Author contributions**

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492 M.A.V., M.F., S.N., M.B., C.M.Z., O.H. Data acquisition: J.B.K., M.A.V., F.B., J.I.M.-E. H.G.-
493 K., R.M, K.B.C., B.P., K.P., H.H. Data analysis and interpretation: J.B.K., I.W., L.P., F.B.,
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496 **Competing interests:**

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509 **Data availability**

510 Sequencing data can be accessed from the Gene Expression Omnibus (nCounter data:
511 GSEXXX; GeoMx data: GSEXXX; both normalized counts [GSE IDs will be available upon
512 approval]) and from the European Genome-phenome Archive (EGA) database ([https://ega-](https://ega-archive.org/studies/EGAXXX)
513 [archive.org/studies/EGAXXX](https://ega-archive.org/studies/EGAXXX); raw nCounter and GeoMx data [idem]).

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699 **Table Legends (tables are provided in a separate excel file)**

700 **Supplementary Table 1. Clinical characteristics of the primary study cohort,**
701 **supplemented with information on immune-infiltration, performed assays, and T cell**
702 **networks.**

703 **Supplementary Table 2. List of significantly up- or downregulated pathways**
704 **comparing immune-infiltrated versus immune-depleted cases using ssGSEA.**

705 **Supplementary Table 3. Clinical characteristics of the adult AML cohort treated with**
706 **ipilimumab-based therapy.**

707 **Supplementary Table 4. Differentially expressed genes (DEGs) between regions in the**
708 **spatial transcriptomics (tx) dataset identified by linear mixed-effect modelling (LMM).**

709 **Supplementary Table 5. Target antigens, antibody clones and suppliers of antibodies**
710 **used for pediatric AML bone marrow IHC/multiplex immunofluorescence.**

711 **Supplementary Table 6. Target antigens, antibody clones, suppliers, dilution of**
712 **markers, diluents, and antigen retrieval conditions used for adult AML bone marrow**
713 **multiplex immunofluorescence.**

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729 **Figure Legends**

730 **Figure 1. Characterizing T cell infiltration in pediatric AML cases and non-leukemic**
731 **controls.** (A) Schematic overview of the study population, used techniques, and the digital
732 image analysis pipeline. AML cases are categorized in immune-infiltrated (red) and immune-
733 depleted (blue) groups according to their T cell infiltration levels (above or below median of
734 non-leukemic controls). (B-C) Representative bone marrow biopsy images from a treatment-
735 naïve pediatric AML case (B) and a non-leukemic control (C) showing H&E staining, CD3⁺ T
736 cells, and CD8⁺ T cells. White lobules indicate adipocytes. (D-E) Comparison of the
737 normalized abundance of CD3⁺ T cells (D) and CD8⁺ T cells (E) in the bone marrow between
738 pediatric AML cases and non-leukemic controls using the Mann-Whitney test. The dashed
739 lines indicate the median CD3⁺ (D) and CD8⁺ (E) T cell abundance in non-leukemic controls,
740 respectively. (F-G) Normalized abundance of CD3⁺ (F) and CD8⁺ T cells (G) per cytogenetic
741 subgroup. 'Normal' indicates normal karyotype, while 'Others' is a merge of cytogenetic
742 abnormalities different from the five defined cytogenetic subgroups. See Table S1. 'Complex'
743 indicates cases with complex karyotype AML (≥ 3 chromosomal abnormalities). The dashed
744 lines shown in Figure 1D-E are also shown in Figure 1F-G. (H) Schematic overview of the
745 TARGET-AML cohort, the additional non-leukemic control group, the performed analysis
746 (CIBERSORTx), and the subsequent categorization of patients into the immune-infiltrated or
747 immune-depleted groups (based on median T- and CD8⁺ T cell abundance in non-leukemic
748 controls). (I-J) Estimated absolute (ABS) abundance of T- (I) and CD8⁺ T (J) cells in the
749 bone marrow of treatment-naïve pediatric AML cases in the TARGET-AML cohort. The
750 dashed lines shown in Figure 1I-J indicate the estimated median bone marrow T- and CD8⁺
751 T cell abundance in four non-leukemic controls.

752 **Figure 2. Transcriptional differences between immune-infiltrated and immune-**
753 **depleted pediatric AML.** (A) Schematic overview of the study population, used techniques,
754 and analyses performed in Figure 2B-D. (B) Comparison of the normalized abundance of
755 CD20⁺ B cells in the bone marrow of pediatric AML (CD20 stains available for 69 cases) with
756 CD3⁺ T cell levels above or below the median of non-leukemic controls (later referred to as
757 immune-infiltrated and immune-depleted, respectively; Mann-Whitney test). (C) Volcano plot
758 of differentially expressed genes between immune-infiltrated (n=6) and immune-depleted
759 (n=17) pediatric AML bone marrow biopsies, identified using *DEseq2* with an FDR cut-off of
760 0.05 and minimum fold change (FC) of 2. (D) Single-sample gene set enrichment analysis of
761 differentially expressed genes between immune-infiltrated and immune-depleted cases using
762 the GO Biological Processes gene set with an FDR cut-off of 0.05. WikiPathways-related
763 results are shown in Table S2. (E) Schematic overview of the study populations for which
764 gene-expression data was available (primary study cohort and TARGET-AML cohort), and

765 the associated analysis. (F) Correlation plot of the negative correlation between the M2-
766 predominance score and the normalized number of CD3⁺ T cells, calculated using Spearman
767 correlation. (G) Comparison of the M2-predominance score between immune-infiltrated
768 (n=6) and immune-depleted (n=17) cases using the Mann-Whitney test. (H) Correlation plot
769 of the negative correlation between the M2-predominance score and the estimated
770 abundance of T cells in the bone marrow of TARGET-AML cases, calculated using
771 Spearman correlation. (I) Comparison of the M2-predominance score between immune-
772 infiltrated (n=80) and immune-depleted (n=79) cases using the Mann-Whitney test.

773 **Figure 3. T cell networks in the bone marrow of pediatric AML.** (A) Illustration of the
774 identification of directly interacting T cells (above) and T cell networks (below) using
775 Delaunay Triangulation. (B) Comparison of the normalized abundance of T cell networks
776 between immune-infiltrated (n=22), immune-depleted (n=50), and non-leukemic control
777 biopsies (n=10; Kruskal-Wallis followed by Dunn's multiple comparisons test). (C)
778 Representative image of a T cell network in a treatment-naïve pediatric AML patient. (D)
779 Schematic of the definition of a large T cell network. (E) Comparison of the number of T cell
780 networks with at least 100 T cells/network between immune-infiltrated (n=22), immune-
781 depleted (n=50), and non-leukemic control biopsies (n=10; Kruskal-Wallis followed by
782 Dunn's multiple comparisons test). (G) Schematic of the definition of a lymphoid aggregate.
783 (H-I) Representative images of lymphoid aggregates.

784 **Figure 4. Composition of lymphoid aggregates in immune-infiltrated pediatric AML.**
785 (A-C) Overview of the different types of regions profiled using spatial transcriptomics in
786 immune-infiltrated biopsies with lymphoid aggregates (A), immune-infiltrated biopsies without
787 lymphoid aggregates (B), and non-leukemic biopsies (C). Green cells indicate T cells, blue
788 cells indicate B cells, and pink/purple cells indicate AML blasts or normal myeloid cells.
789 These examples do not necessarily reflect the actual abundance of these subsets. LA:
790 lymphoid aggregate. (D) UMAP of transcriptomic profiles of various region types, organized
791 into two separate clusters. (E-F) Comparison of the expression of the 'Tfh' (E), and 'TLS
792 imprint' (F) signatures across different region types (Kruskal-Wallis followed by Dunn's
793 multiple comparisons test). (H-L) Deconvoluted abundance of various cell subsets and the
794 M2-predominance score compared across different region types (Kruskal-Wallis followed by
795 Dunn's multiple comparisons test). In case of multiple p-values, the upper one is associated
796 with the Kruskal-Wallis test, while the lower one(s) reflects the result of Dunn's multiple
797 comparison test.

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801 **Figure 5. In-depth characterization of T- and B cells in lymphoid aggregates.** (A)
802 Schematic overview of the analysis approach applied to the spatial transcriptomics dataset
803 and the subsequent multiplex immunofluorescence (IF). scRNA-seq: single-cell RNA-
804 sequencing. (B) Proportions of memory B cells, plasmablasts, and unassigned B cells in
805 lymphoid aggregates (LA). (C) Proportions of naïve-, cytotoxic (CTL), mucosal-associated
806 invariant T (MAIT)-, and potentially dysfunctional CD8⁺ T cells in lymphoid aggregate, mixed,
807 and control regions. (D) Comparison of the deconvoluted proportions of potentially
808 dysfunctional CD8⁺ T cells in lymphoid aggregate, mixed, and control regions (Kruskal-Wallis
809 followed by Dunn's multiple comparisons test). In case of multiple p-values, the upper one is
810 associated with the Kruskal-Wallis test, while the lower ones reflects the result of Dunn's
811 multiple comparison test. (E) Representative image of the multiplex immunofluorescence
812 analysis of a lymphoid aggregate. The names below each image indicate which antibodies
813 are shown. The green boxes on the lower row are zoomed in on the part of the biopsy in the
814 green box in the upper left image. (F) The proportion of CD3⁺CD8⁺ T cells that expressed
815 two or three inhibitor receptors (IR^{++/+++}) positive (or not) for granzyme B (GZMB).

816 **Supplemental information titles and legends**

817 **Supplementary Figure 1. AML blasts and differentiation stage in relation to T- and**
818 **CD8⁺ T cell infiltration in the bone marrow.** (A) Comparison of the percentage of CD3⁺ T
819 cells out of all bone marrow (BM) cells between diagnostic pediatric AML cases with normal
820 karyotype and no identified molecular aberrations (wildtype) and those with a *FTL3*-ITD
821 and/or *NPM1* mutation (*NPM1*mut; Mann-Whitney test). The T cell proportions were
822 retrieved from diagnostic flow cytometry reports. These patients are part of an independent
823 cohort, not the primary study cohort. (B-C) Comparison of the CIBERSORTx-based
824 estimated absolute (ABS) abundance of T- (B) and CD8⁺ T cells (C) between pediatric AML
825 patients (TARGET-AML cohort) with different *KMT2A*-rearrangements. The names on the x-
826 axis indicate the *KMT2A*-fusion partners. The dashed lines indicate the median estimated
827 abundance of T- and CD8⁺ T cells in four non-leukemic controls. Examined using the
828 Kruskal-Wallis test followed by Dunn's multiple comparisons test. In case of multiple p-
829 values, the upper one is associated with the Kruskal-Wallis test, while the lower one(s)
830 reflects the result of Dunn's multiple comparison test. (D-E) Correlation plot between the
831 normalized number of CD3⁺ (D) and CD8⁺ (E) T cells and leukemic blasts in the bone
832 marrow (blast % available for 65 cases), calculated using Spearman correlation. (F-G)
833 Comparison of the normalized abundance of CD3⁺ (F) and CD8⁺ (G) T cells in the bone
834 marrow across AML differentiation stages (FAB-classifications; Kruskal-Wallis followed by

835 Dunn's multiple comparisons test). Data are presented as mean plus range. The dashed
836 lines indicate the median T- and CD8⁺ T cell abundance in non-leukemic controls. (H-I)
837 Comparison of the estimated abundance of T- (H) and CD8⁺ (I) T cells in the bone marrow of
838 TARGET-AML cases across AML differentiation stages (FAB-classifications; Kruskal-Wallis
839 followed by Dunn's multiple comparisons test). Data are presented as mean plus range. The
840 dashed lines indicate the estimated median T- and CD8⁺ T cell abundance in non-leukemic
841 controls. (J-K) Correlation plot between the estimated number of T- (J) and CD8⁺ (K) T cells
842 and leukemic blasts in the bone marrow (blast % available for 154 cases) of TARGET-AML
843 cases, calculated using Spearman correlation.

844 **Supplementary Figure 2. Overall survival and M2-like macrophage abundance in**
845 **immune-infiltrated and immune-depleted cases across two cohorts.**

846 (A-B) Overall survival between immune-infiltrated and immune-depleted pediatric AML cases
847 in our primary study cohort (A) and in the TARGET-AML cohort (B). (C-D) Correlation plots
848 of the correlation between the M2-predominance score and the normalized (C; our cohort) or
849 estimated (D; TARGET-AML cohort) abundance of CD8⁺ T cells, calculated using Spearman
850 correlation.

851 **Supplementary Figure 3. Representative images of bone marrow biopsies of immune-**
852 **infiltrated and immune-depleted pediatric AML, and a non-leukemic control.**

853 (A-B) Representative images of CD3⁺ T cells in the bone marrow of immune-depleted cases.
854 (C-D) Representative images of large T cell networks (C) that colocalized with a dense
855 network of B cells (lymphoid aggregates) (D). (E) Representative image of CD3⁺ T cell
856 infiltration in the bone marrow of a non-leukemic control.

857 **Supplementary Figure 4. Immune aggregates in the bone marrow of adult AML cases**
858 **treated with ipilimumab-based treatment.** (A) Schematic overview of adult AML cases that

859 were treated with ipilimumab and decitabine, the used techniques, and the examined
860 variables. The table indicates the patient IDs, whether patients responded (yes=green,
861 purple=no) to these therapies, whether the patients had primary AML (AML) or secondary
862 AML (sAML), and whether they were treated in the treatment-naïve or post-stem cell
863 transplantation (SCT) setting. (B-D) Representative multiplex immunofluorescence images of
864 lymphoid aggregates (B-C) and large T cell-dominant networks with sparse B cells (D). (E-J)
865 Longitudinal overview of lymphoid aggregates, T cell-dominant networks, and AML blasts in
866 bone marrow biopsies before treatment according to the clinical trial (baseline), at time of
867 best response, or at end of treatment (EOT). Presented blast percentages are the average
868 of the aspirate and biopsy blast percentages. EC=end of course; the number (e.g., EC1)
869 indicates which course was given (e.g., EC1 indicates that the biopsy was taken after course

870 1). Green IDs indicate responders, purple IDs indicate non-responders. The AML blast
871 percentage was the average of the percentages in the aspirate and the core biopsy.
872 CR=complete morphologic remission with or without complete count recovery and no signs
873 of leukemia elsewhere. *AML1003 had an aplastic bone marrow without hematologic
874 recovery, and AML1011 had 5% blasts by histology.

875 **Supplementary Figure 5. Composition of lymphoid aggregates in immune-infiltrated**
876 **pediatric AML.** (A) Representative images of the region of interest (ROI) selection on the
877 GeoMx Digital Spatial Profiling platform. On the left, both the CD3 (T cell) and CD20 (B cell)
878 stains are shown (DAB, both in brown). On the right, magnifications of immune-aggregate
879 regions (above) and mixed regions (below) are shown. Selection of ROIs was further aided
880 by overlaying stains of CD34 and CD117 (Supplementary Methods). (B) Comparison of the
881 expression of the '12chem' signatures across different region types (Kruskal-Wallis followed
882 by Dunn's multiple comparisons test). (C) Proportions of CD4⁺ and CD8⁺ T cells in lymphoid
883 aggregate (LA), mixed, and control regions. (D-H) Deconvoluted absolute abundance of
884 various cell subsets across several region types (Kruskal-Wallis followed by Dunn's multiple
885 comparisons test). In case of multiple p-values, the upper one is associated with the Kruskal-
886 Wallis test, while the lower one(s) reflects the result of Dunn's multiple comparison test.

887 **Supplementary Figure 6. Upregulated genes and the presence of regulatory T cells in**
888 **lymphoid aggregates in immune-infiltrated pediatric AML.** (A) Volcano plot of genes
889 differentially expressed in lymphoid aggregates compared to MIXED1 regions, generated
890 using linear mixed-effect modelling. FC: fold change. (B) Representative images of putative
891 Tregs (CD3⁺FOXP3⁺) in lymphoid aggregates (CD3⁺ T cells). The image on the right reflects
892 a zoom of the region in the green box in the left image. The names below the images reflect
893 the antibodies and the associated colors in the images. (C) Proportion of putative Tregs
894 among all CD3⁺ T cells in lymphoid aggregates (LA).

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Figure 1

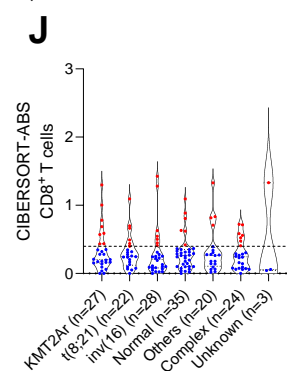
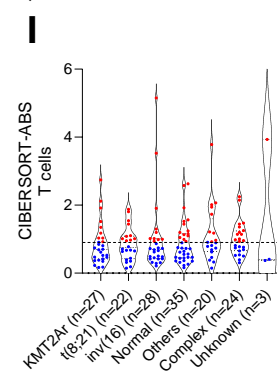
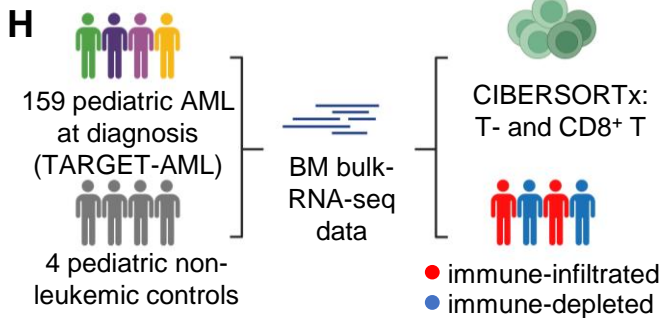
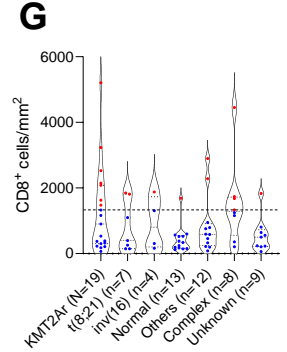
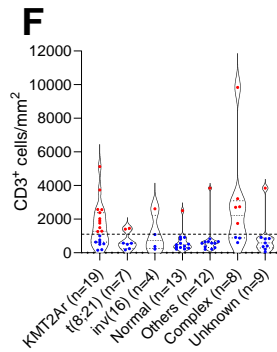
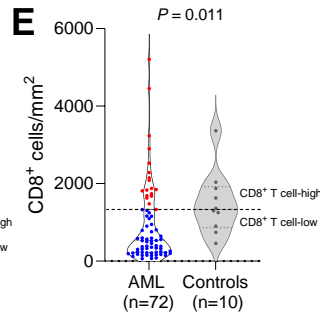
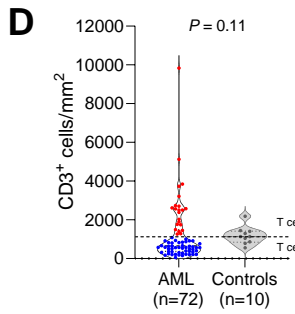
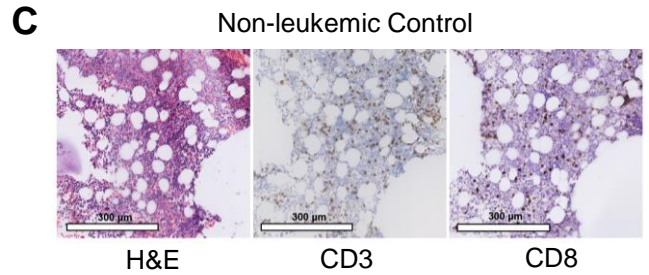
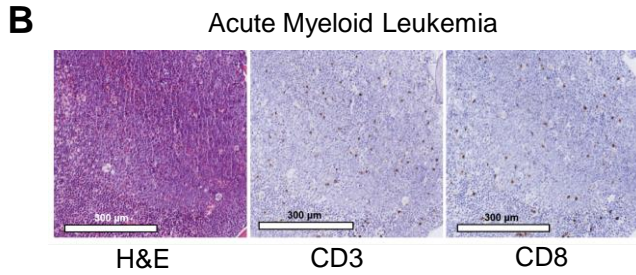
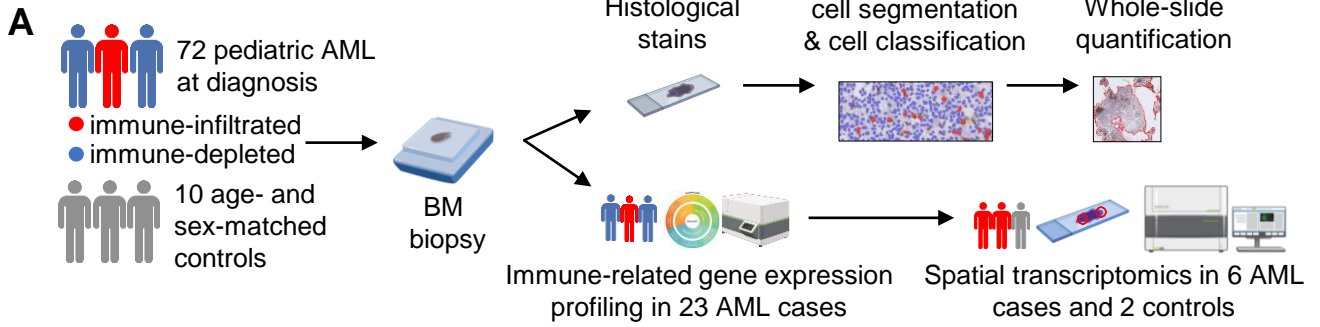


Figure 2

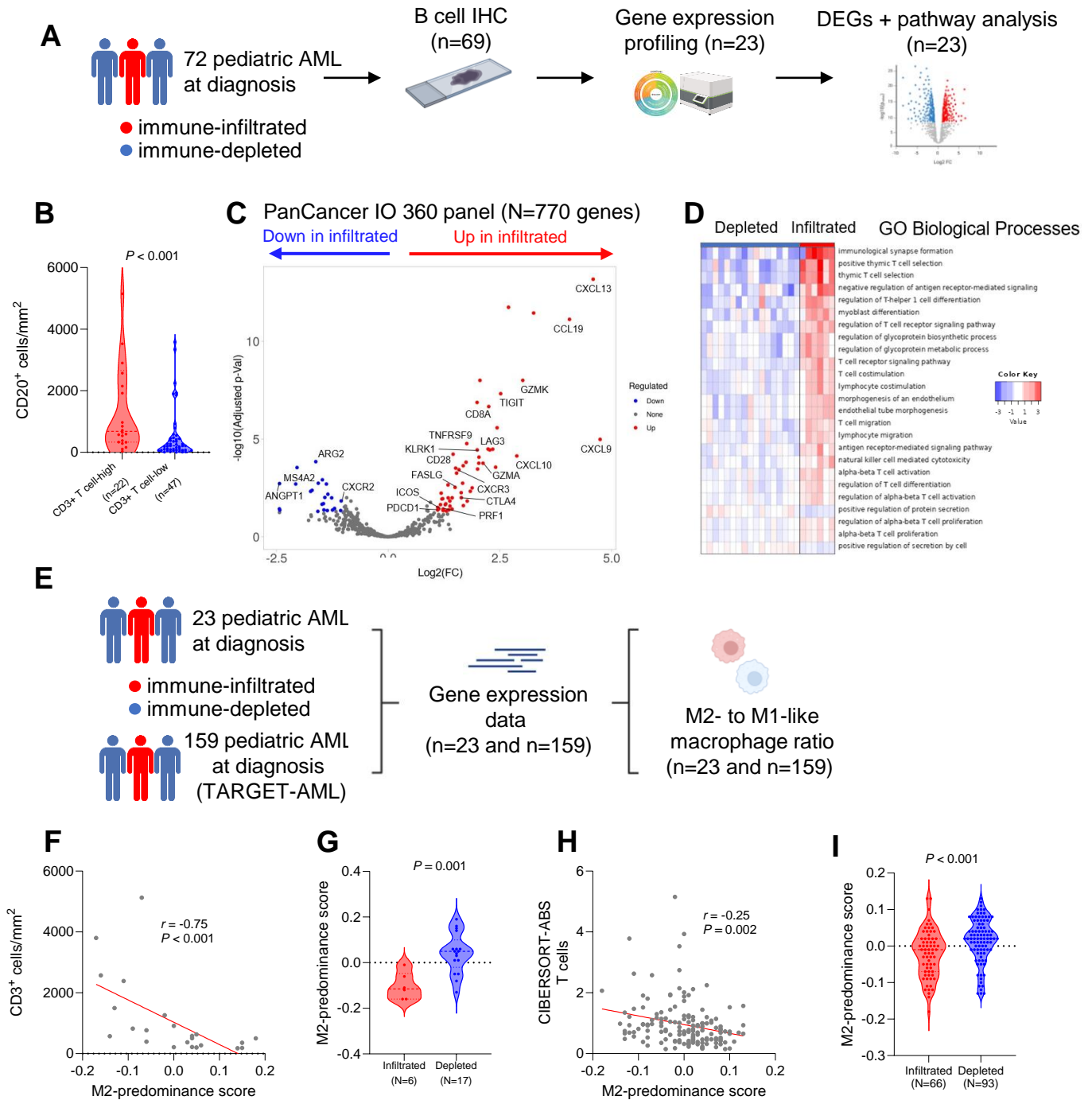
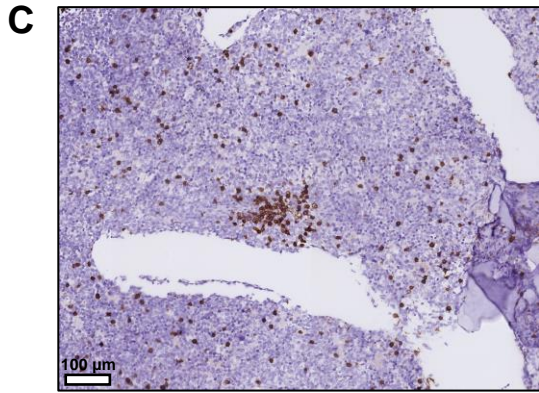
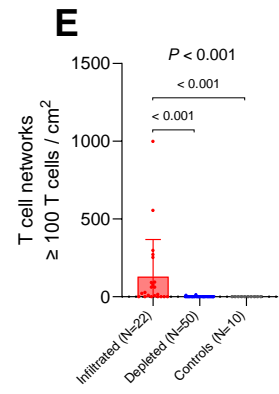
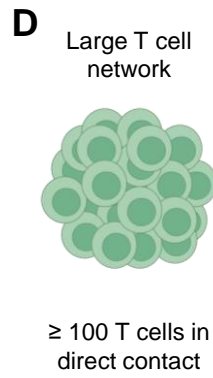
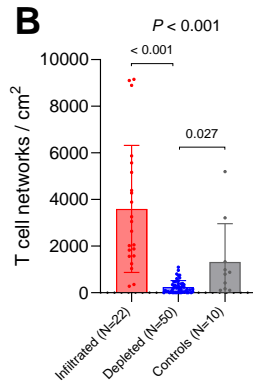
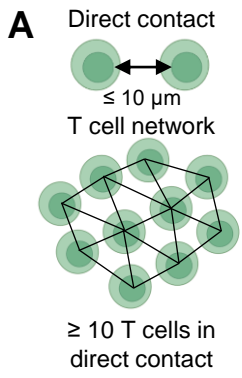
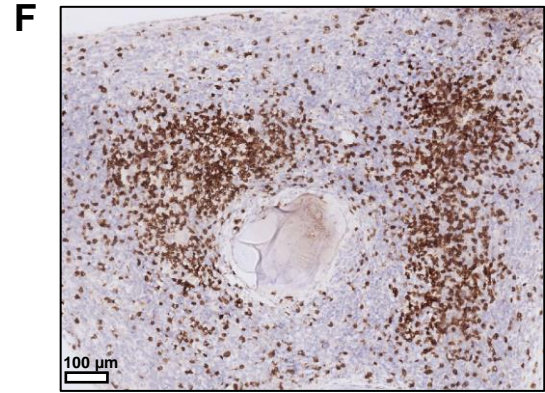


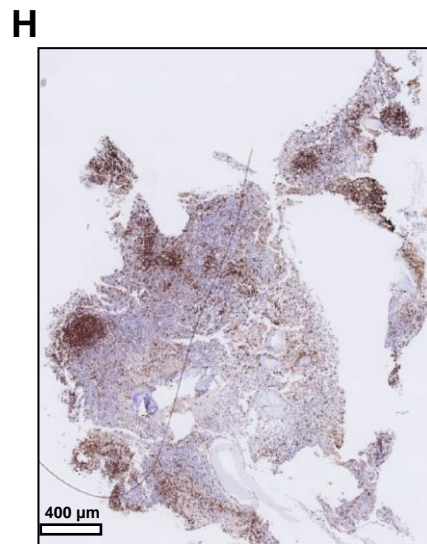
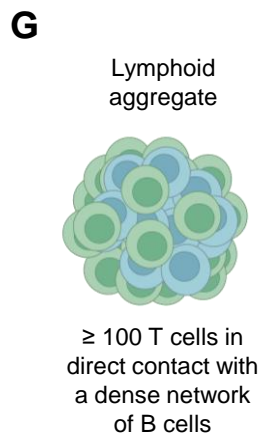
Figure 3



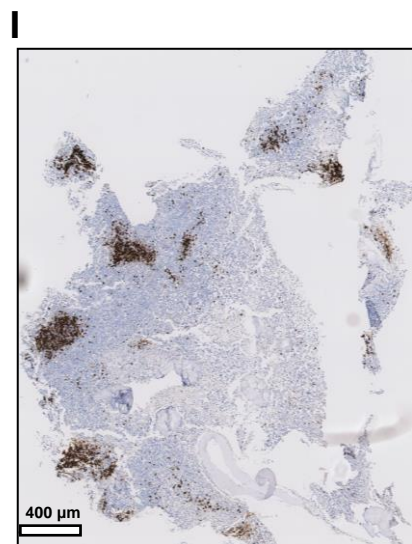
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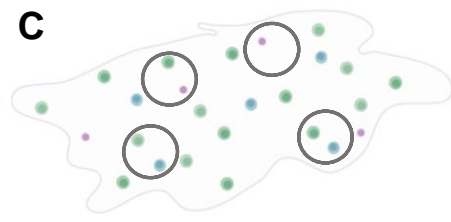
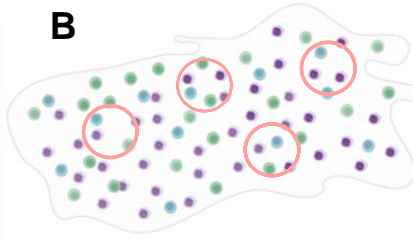
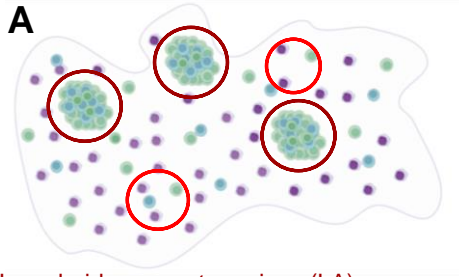


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Figure 4



Lymphoid aggregate regions (LA)

Mixed regions in aggregate-rich biopsy (MIXED1)

Mixed regions in biopsy without aggregates (MIXED2)

Control regions in non-leukemic control biopsy (CONTROL)

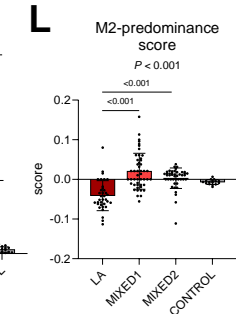
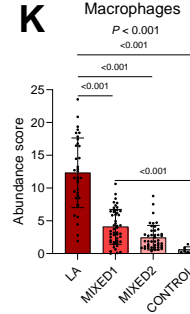
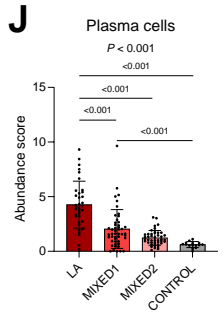
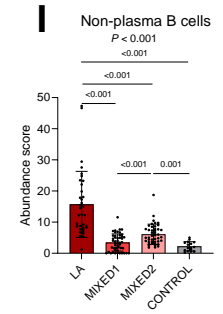
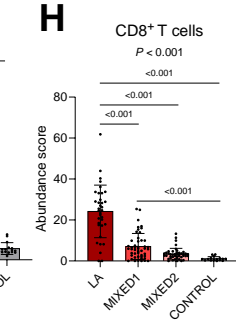
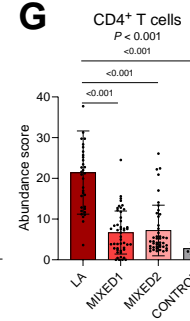
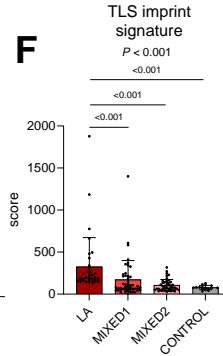
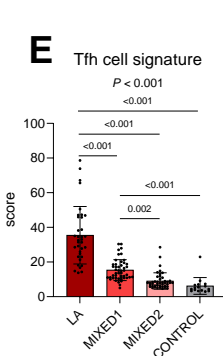
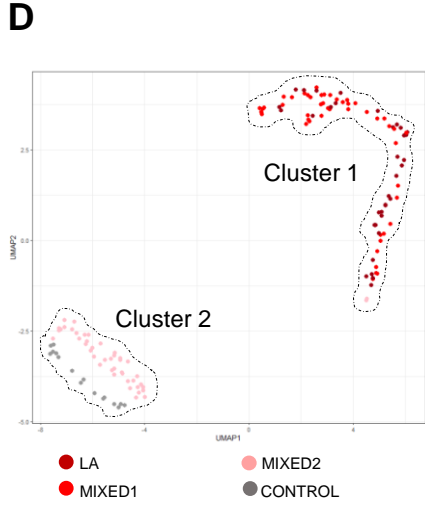
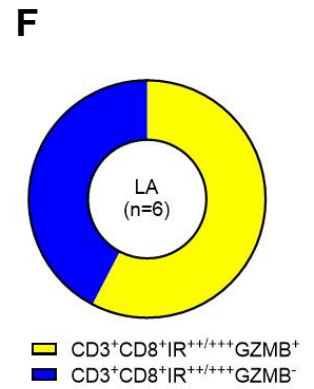
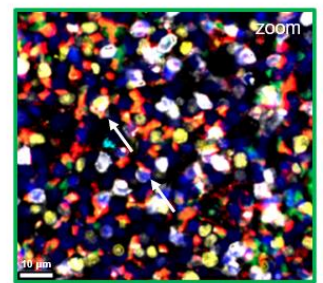
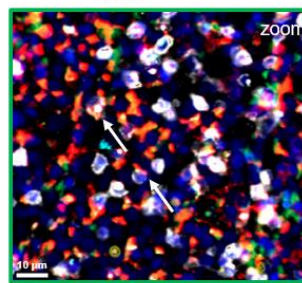
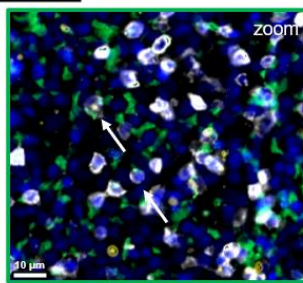
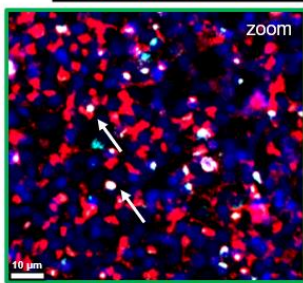
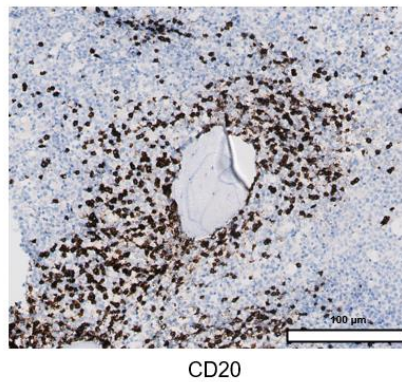
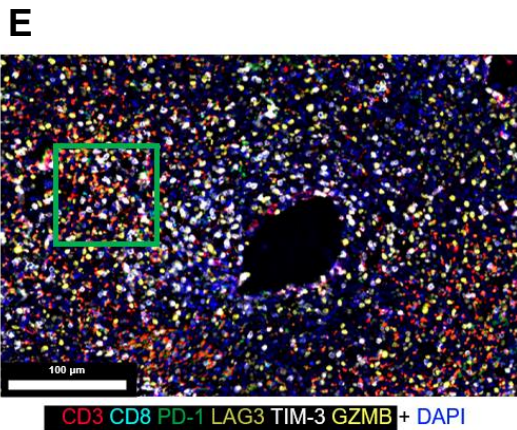
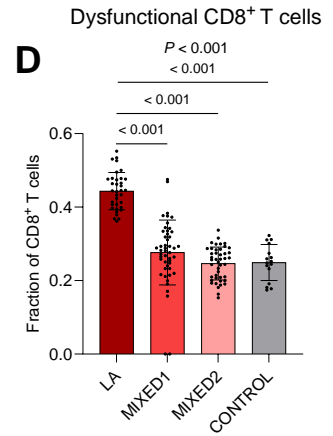
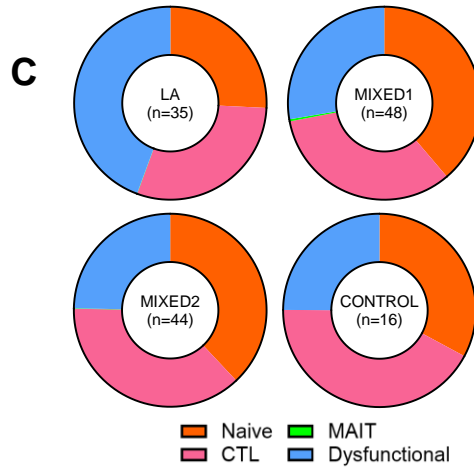
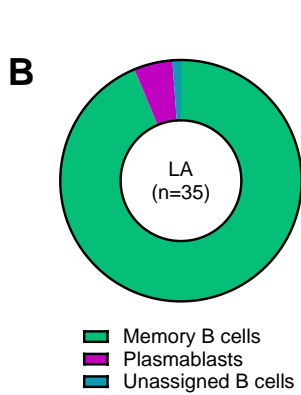
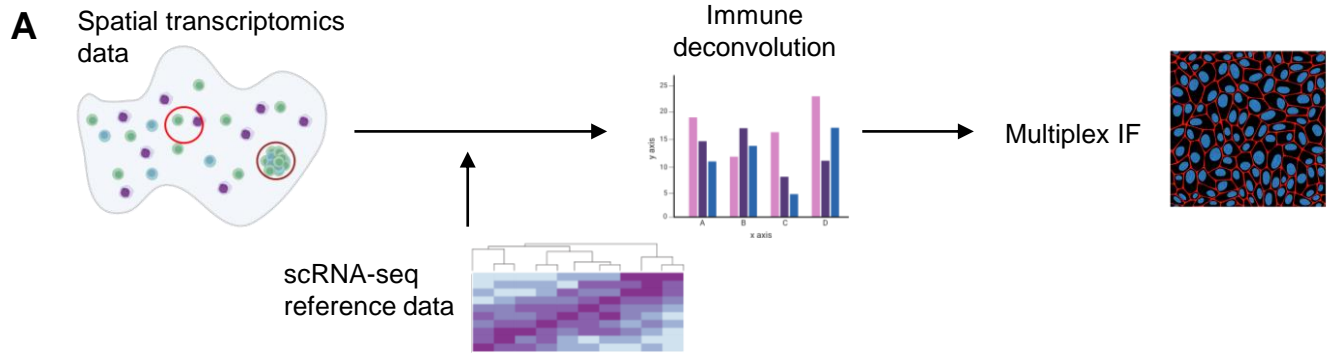
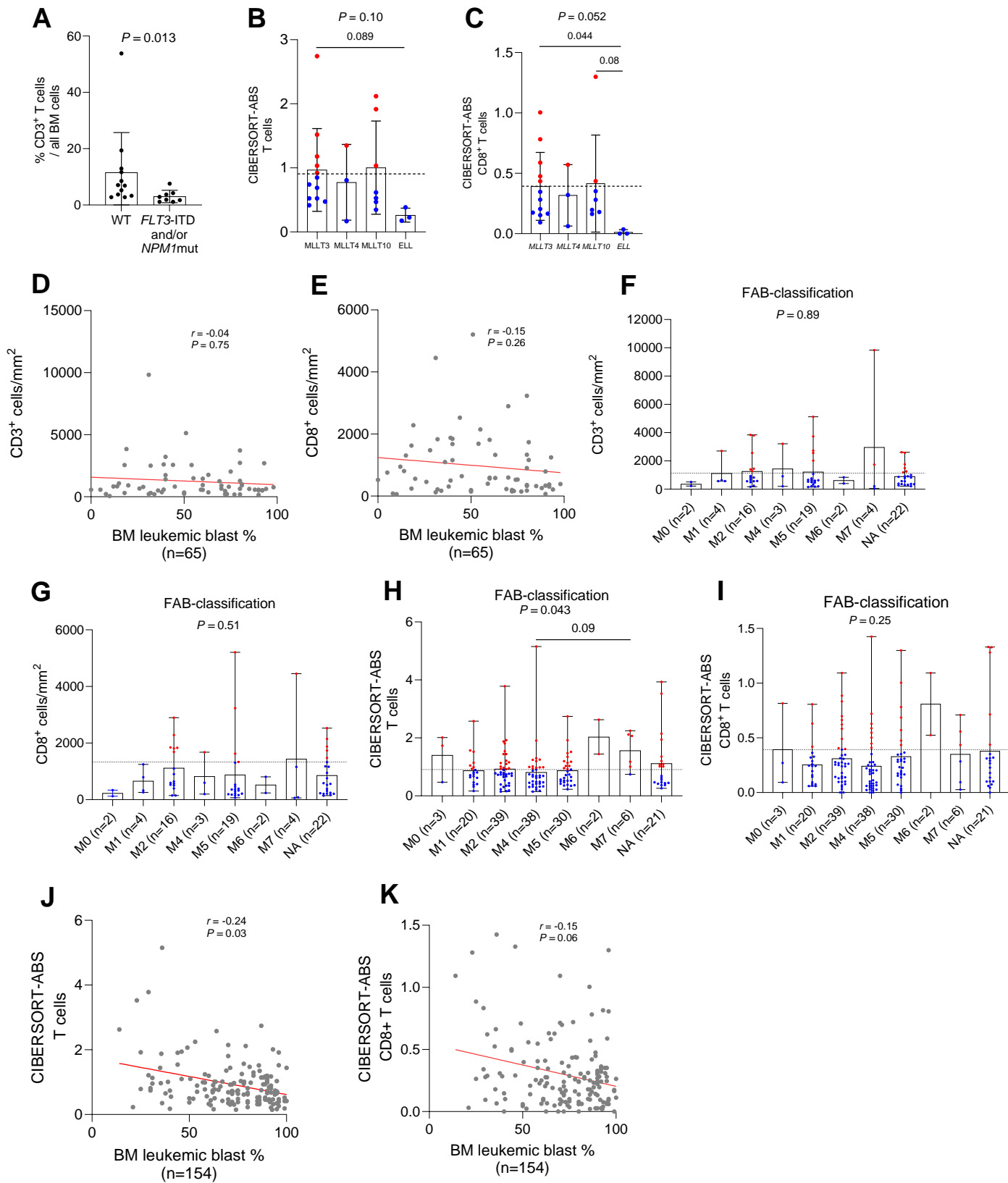


Figure 5

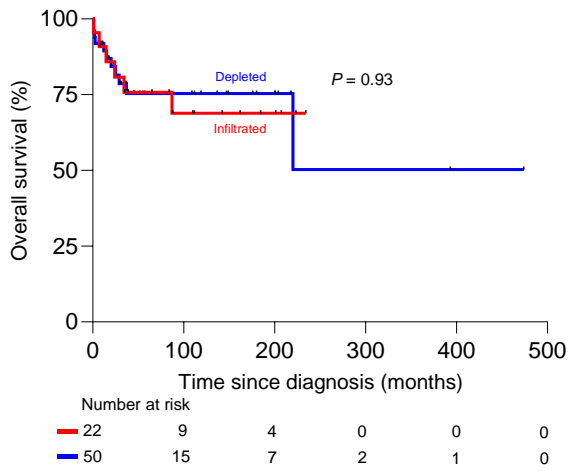


Supplementary Figure 1

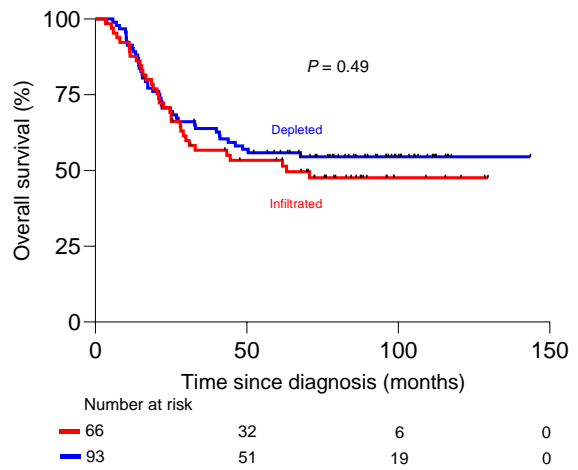


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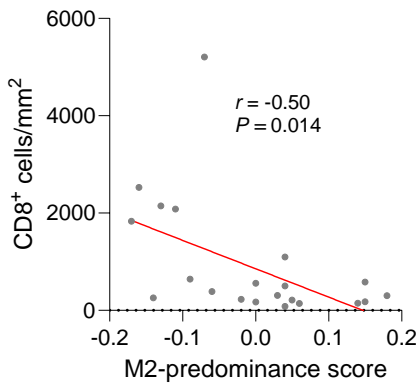
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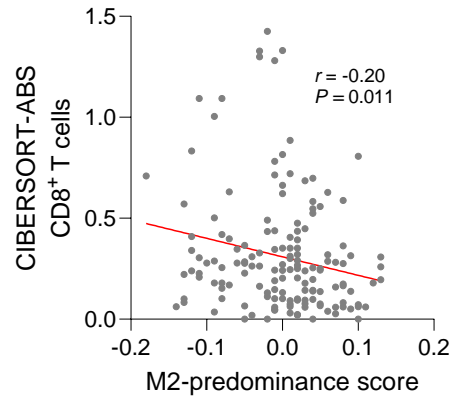
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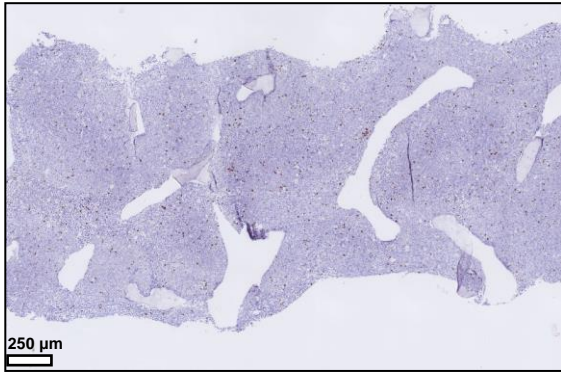


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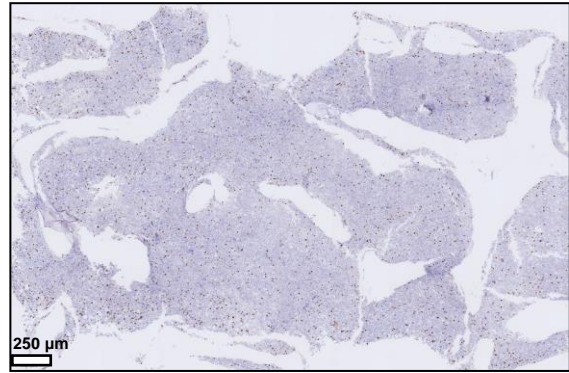
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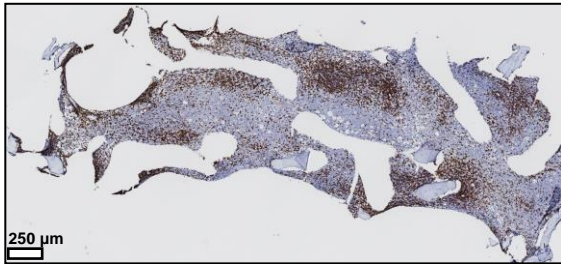
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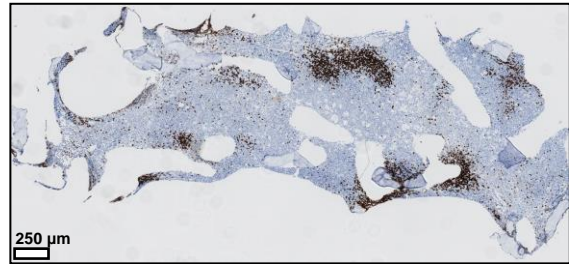
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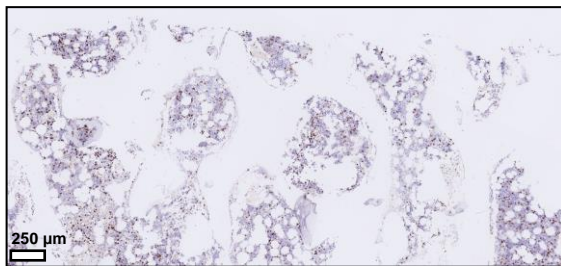
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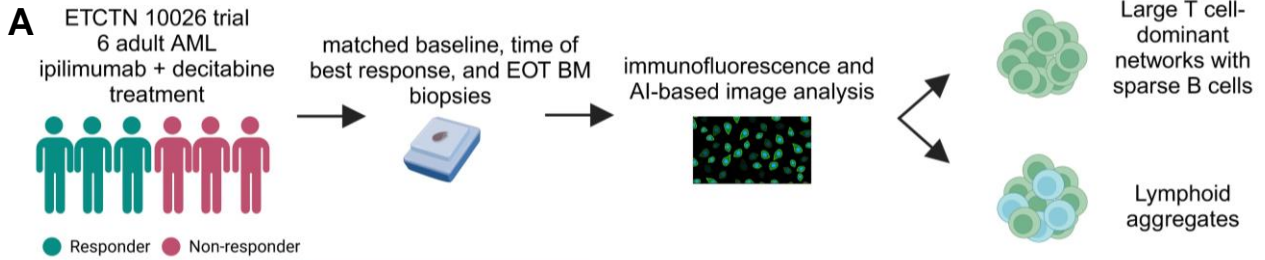
CD20

E

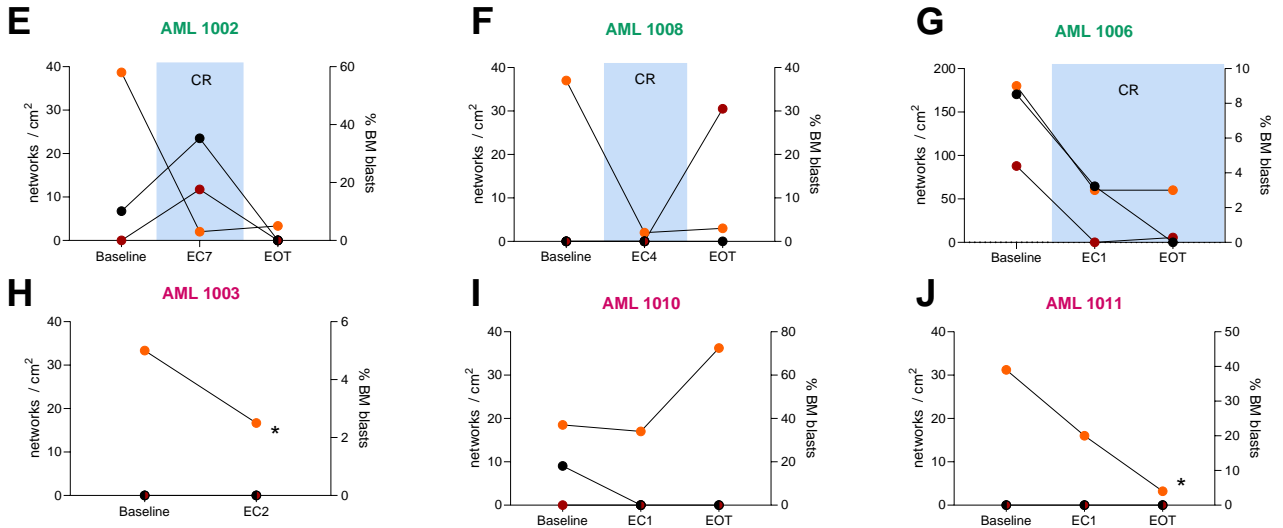
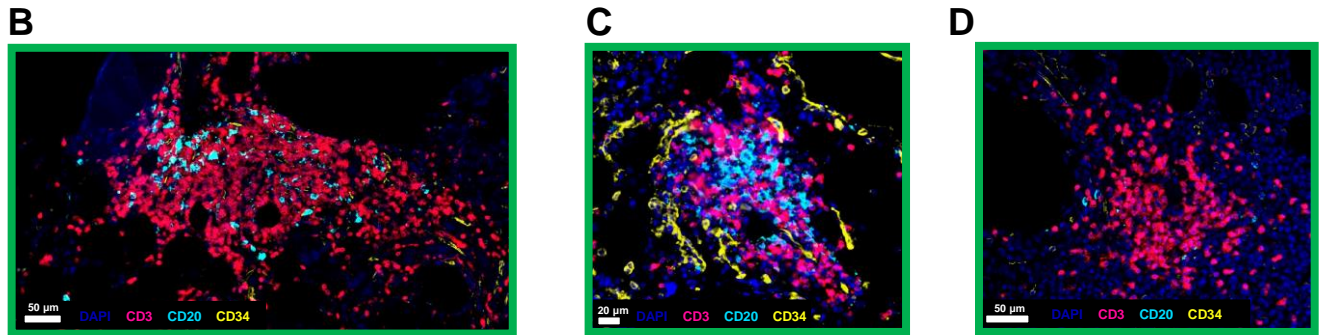


CD3

Supplementary Figure 4



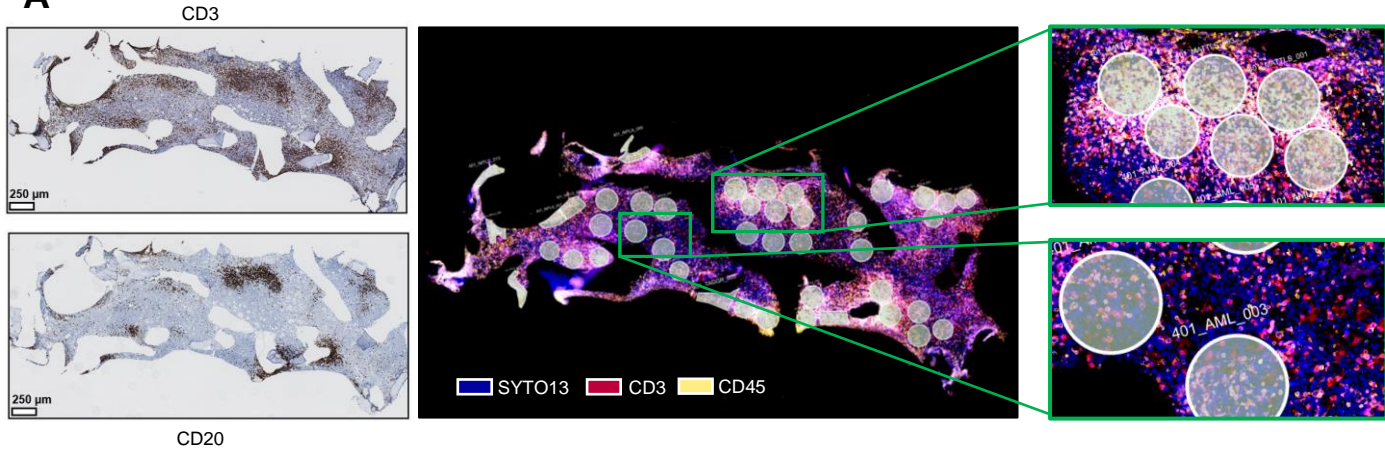
AML1002	AML1008	AML1006	AML1003	AML1010	AML1011
sAML treatment-naive	sAML treatment-naive	sAML post-SCT relapse	AML post-SCT refractory	AML post-SCT relapse	AML post-SCT relapse



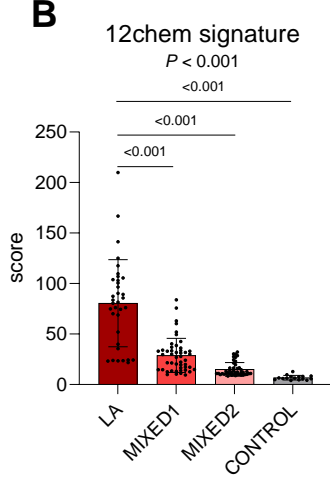
● Lymphoid aggregates ● Large T cell-dominant networks with sparse B cells ● AML blasts

Supplementary Figure 5

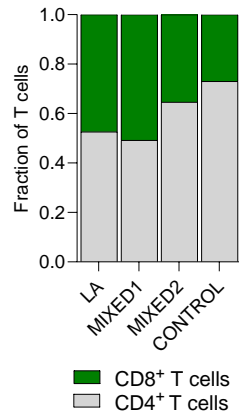
A



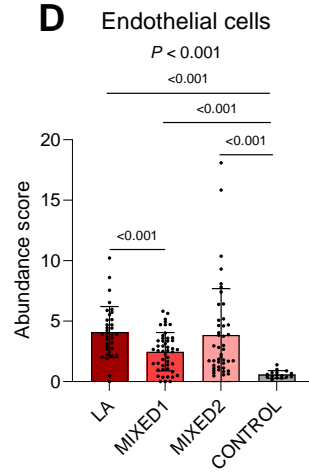
B



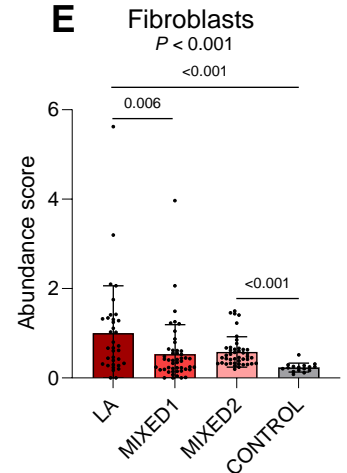
C



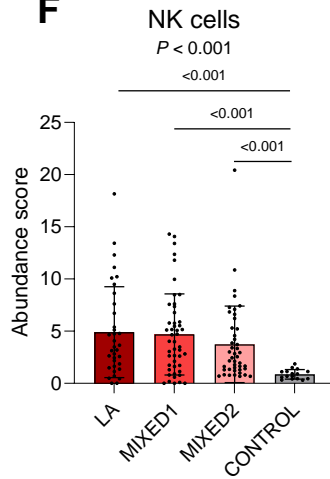
D



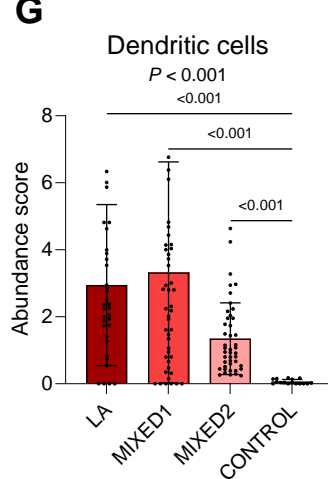
E



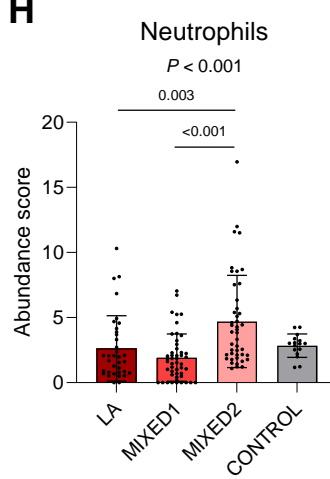
F



G



H



Supplementary Figure 6

