Single-cell characterisation of the hematopoietic bone marrow interactome in health and disease

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1 Abstract

The bone marrow (BM) is a complex microenvironment and the primary site of hematopoiesis, coordinating the production of billions of blood cells every day. Despite the essential role of the hematopoietic niche in maintaining hemostasis and its relevance to hematopoietic diseases, many aspects of this environment remain poorly characterised due to experimental hurdles. Here we present a high-resolution characterisation of the niche in health and acute myeloid leukemia (AML) by establishing a comprehensive single-cell gene expression database of nearly 340,000 BM constituent cells encompassing all disease stages (healthy BM, AML at diagnosis, remission and relapse). We characterised the cell type composition of the BM and found that the proportions of both myeloid and lymphoid lineage cell types are significantly altered in AML. We also determined broadscale dysregulation of gene expression in almost all BM cell types upon establishment of AML, indicating that the entire niche is disrupted by the disease. Given the importance of interactions between hematopoietic cells and their microenvironment in regulating their function and properties, we determined all possible ligand-receptor interactions between hematopoietic stem and progenitor cells (HSPC) and every other BM constituent cell type. This analysis revealed a remarkable expansion of HSPC interactions in AML involving multiple BM constituent cells that can drive dysregulated HSPC-cell adhesion, immunosuppression and enhanced cytokine signalling. These interactions shed light on potential mechanisms of osteoblast loss, enhanced leukemic stem cell competitiveness and an overall, skewed microenvironment fostering AML growth.

2 Introduction

The hematopoietic niche within the human BM consists of a diverse range of hematopoietic and non-hematopoietic (such as endothelial, mesenchymal and osteolineage) cells [1]. The role of the niche is to regulate hemostasis by coordinating the homing, self-renewal, proliferation and differentiation of hematopoietic stem and progenitor cells (HSPCs) and orchestrate the daily production of myeloid, lymphoid and erythroid lineage blood cells. This environment is tightly regulated through reciprocal interactions between HSPCs and the niche [2]. For example, C-X-C motif chemokine ligand 12 (CXCL12)-expressing mesenchymal stromal cells play a crucial role in supporting HSPCs by tethering them in the BM via interaction with CXCR4, thus facilitating HSPC quiescence and cell survival [3, 4]. These regulatory interactions are known to be disrupted in leukemia, where malignant cells shape the alter the niche to support their proliferation and survival. At the same time, these changes make the microenvironment less hospitable to normal HSPCs, leading to their egress and impaired blood cell production [5].

This is also the case in acute myeloid leukemia (AML), an aggressive blood cancer characterised by rapid accumulation of myeloid lineage precursor cells in the BM. Mouse models of AML have shown that the BM-engrafting AML cells gradually erode the stroma leading to an over 90% non-erythroid stromal cell loss in advanced disease [6]. This skewed microenvironment is known to drive drug resistance and consequent relapse [7], but the mechanisms and the responsible cellular interactions are not well understood [8]. Better characterisation of the cell-cell interactions in the BM and how they change in AML may provide opportunities for a "two-pronged" treatment approach by directly targeting the cancer cells with chemotherapy while also disrupting their interactions with the microenvironment so that it becomes inhospitable to malignant cells.

Advancements in technology, such as improved mouse models and the development of single-cell and spatial transcriptomics have increased our understanding of the biology of the normal hematopoietic niche, particularly in mice [9, 10]. However, a large-scale characterisation of the composition and behaviour of the niche in humans and its perturbation in malignancy is lacking. Although single-cell RNA-sequencing (scRNA-seq) has been applied to the study of the BM and has provided valuable insights [9–18], due to economic and experimental hurdles, most of these studies have remained restricted to mouse models or cells from few donors. Also, as these experiments are typically performed on BM aspirates cells that are more adherent, such as bone marrow stromal cells (BMSC) are under-represented [19]. Another challenge when using scRNA-seq to characterise the leukemic BM is the very high degree of heterogeneity between patients. Because these studies typically only include tens of patients, it is difficult to identify unifying mechanisms of leukemogenesis, allowing only patient-level analysis [13–15].

Here we report a large-scale analysis of the bone marrow cell-cell interactome in health and upon development of AML. By integrating multiple scRNA-seq datasets (Table 1), a large database has been generated (339,381 cells from 63 donors) that enabled a comprehensive analysis of the HSPC-interactome in the hematopoietic niche. Using this dataset we established a baseline reference of healthy cells and then identified changes in cell type composition, gene expression and ligand-receptor interactions of HSPCs that occur during the development of AML. The results highlight the widespread alterations in the BM cellular interactome in AML, especially impacting cell adhesion and cytokine signalling.

3 Results and Discussion

3.1 A harmonised single-cell atlas of healthy and AML BM

To generate a single-cell atlas of the BM in health and AML, we performed droplet-based scRNA-seq on BM aspirates from 10 AML patients collected at key stages of disease progression (diagnosis, post-treatment/remission and relapse, clinical information available in Supplementary Table 1) and integrated the data with other, openly available scRNA-seq datasets of healthy and AML BM. We identified 4 suitable datasets [11–14] and processed each of them through a uniform pre-processing pipeline to minimise any study-specific effects (Table 1, and further information on each individual sample is provided in Supplementary Table 2).

Table 1: Summary of datasets included in this study.

Dataset no.	**No. of donors**		**No. of samples**	**No. of cells**	**Reference**
	Healthy	AML			
1	0	10	28	111,347	Current study
2	3	0	4	20,344	Granja et al., 2019 [11]
3	20	0	25	69,264	Oetjen et al., 2018 [12]
4	4	5	9	100,407	Petti et al., 2019 [13]
5	5	16	41	38,019	van Galen et al., 2019 [14]
Total	**32**	**31**	**107**	**339,381**	

To ensure optimal batch correction of the datasets and enable simple reuse and sharing of the atlas, we used scArches (Supplementary Figure 1) [20] to generate a harmonised reference atlas of the BM. The atlas consists of a total of 339,381 cells from 63 donors (Figure 1). Cell types within the dataset were labelled using a combination of predictive and manual annotations. First, cell type labels were predicted for each cell using the dataset published by Granja and colleagues as a reference [11]. We then confirmed the predicted labels and annotated cell types that were not included in the Granja dataset based on expression of well-known marker genes (Figure 1d, 1e). Overall, we identified 19 different cell types including all expected hematopoietic lineage cells, such as hematopoietic stem and progenitor cells, and multiple myeloid, lymphoid and erythroid populations (Figure 1d). Due to the large size of the dataset, we were also able to identify clusters of rare and under-represented cell types such as BM mesenchymal stromal cells and megakaryocytes which could not be identified in any of the individual datasets prior to integration. The integrated dataset can be downloaded from the Zenodo repository accompanying this publication, along with a Google colab notebook demonstrating how to explore and reuse it.

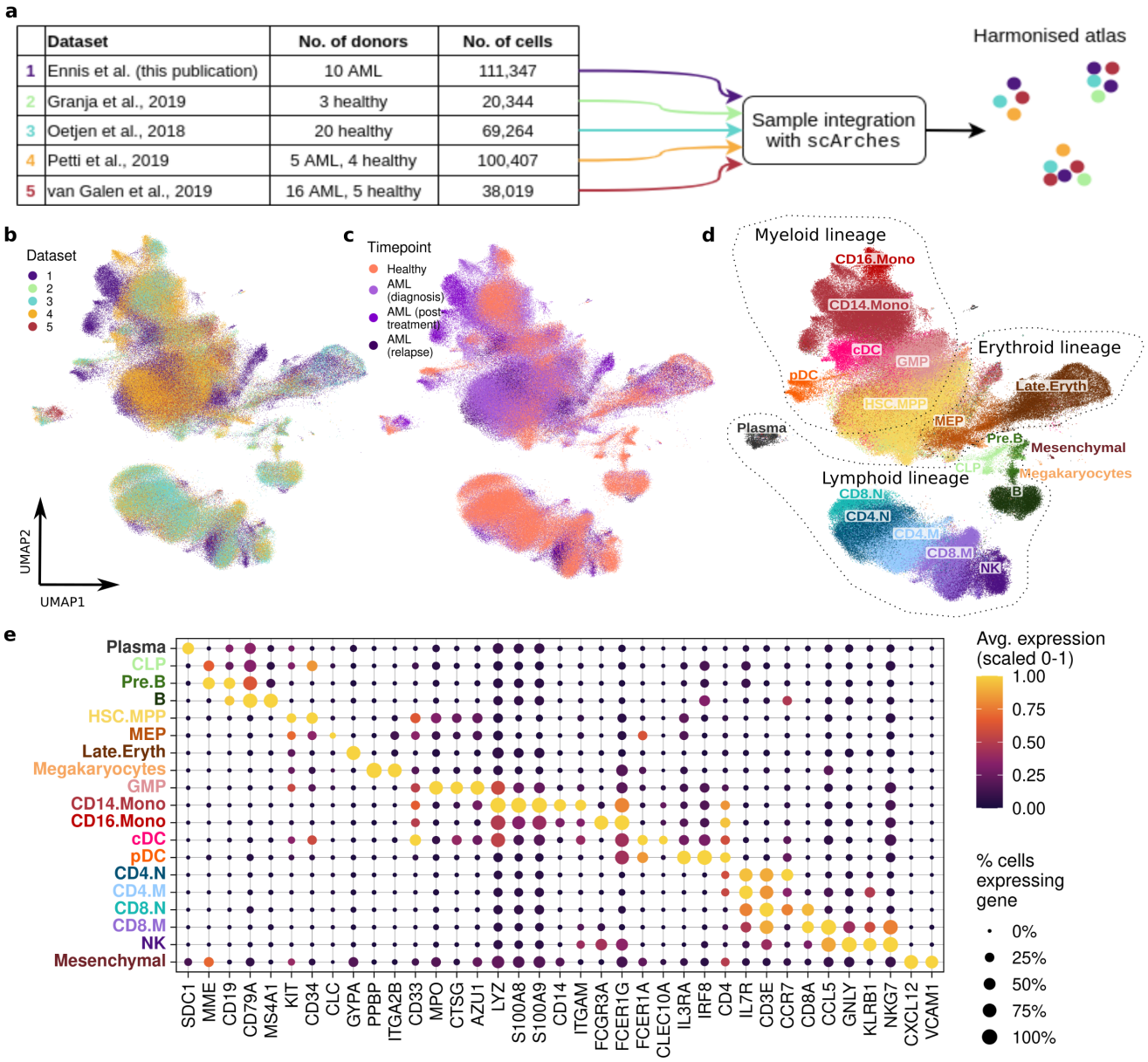

Figure 1: A harmonised single-cell atlas of healthy and AML BM. a Schema of dataset generation. **b-d** UMAP was used for dimensionality reduction and visualisation of the dataset. Cells are coloured by dataset (**b**), timepoint (**c**) and cell type (**d**). **e** Dotplot depicting the expression of known marker genes for each identified cell type. The size and colour of each dot represents the percentage of cells expressing the gene and the normalised expression level in each cell type respectively. *HSC.MPP = Hematopoietic stem cell/multipotent progenitor, GMP = granulocyte monocyte precursor, MEP = megakaryocyte erythrocyte precursor, CLP = common lymphoid progenitor, cDC = conventional dendritic cell, pDC = plasmacytoid dendritic cell, CD4/8.N = CD4/8 naive T, CD4/8.M = CD4/8 memory T, NK = natural killer cell.*

3.2 Cell type proportions in the BM are significantly altered during the establishment and progression of AML

To understand the HSPC interactome in the BM, first we determined how its cellular composition changes in health and at different stages of AML progression. We used the `propeller` function from the `speckle` R package to test whether there are statistically significant changes in cell type proportions during development and progression of AML [21]. The analysis showed that in healthy BM, over 60% of cells are lymphoid lineage cells, around 30% are myeloid cells and the rest are erythroid and other types (Figure

2a). As expected, a significant shift in cell type proportions takes place at the onset of AML with myeloid cells making up over 75% of BM cells (Figure 2a). In particular, the more primitive myeloid progenitor populations (hematopoietic stem cell/multipotent progenitor (HSC.MPP), granulocyte monocyte precursor (GMP), megakaryocyte erythrocyte precursor (MEP)) become enriched at diagnosis. Post-treatment, during remission, these proportions return to levels similar to the healthy condition but at relapse, as expected, enrichment of myeloid progenitors reappears (Figure 2b).

In addition to these expected changes, profound changes occur in the lymphoid lineage. The proportion of common lymphoid progenitors (CLP) drops upon development of AML and fails to return to pre-disease levels in remission. The loss of CLP was associated with a profound drop in its progeny, especially pre-B cells (Pre.B), mature B cells, and to a lesser extent of CD4+ and CD8+ naïve T cells (CD4.N, CD8.N) and CD4+ memory T cells (CD4.M). These results reveal that the adaptive immune system suffers long-term damage either due to the disease or to the chemotherapy. Corroborating this notion, both B and T cells have been previously reported to display impaired function in AML [22, 23]. The impaired function of lymphoid cells exacerbated by their reduced numbers may be important to consider, given that new treatments based on harnessing T cells are currently in pre-clinical and clinical development [24].

A further indication of impaired immune tumour surveillance is that cytotoxic lymphoid cells (CD8+ memory T cells (CD8.M) and natural killer (NK) cells) are enriched at relapse, compared to diagnosis. This may indicate that although cytotoxic cell numbers recover during remission, they fail to eliminate the leukemic cells or may in fact play a role in supporting AML cells thus allowing relapse (Figure 2b).

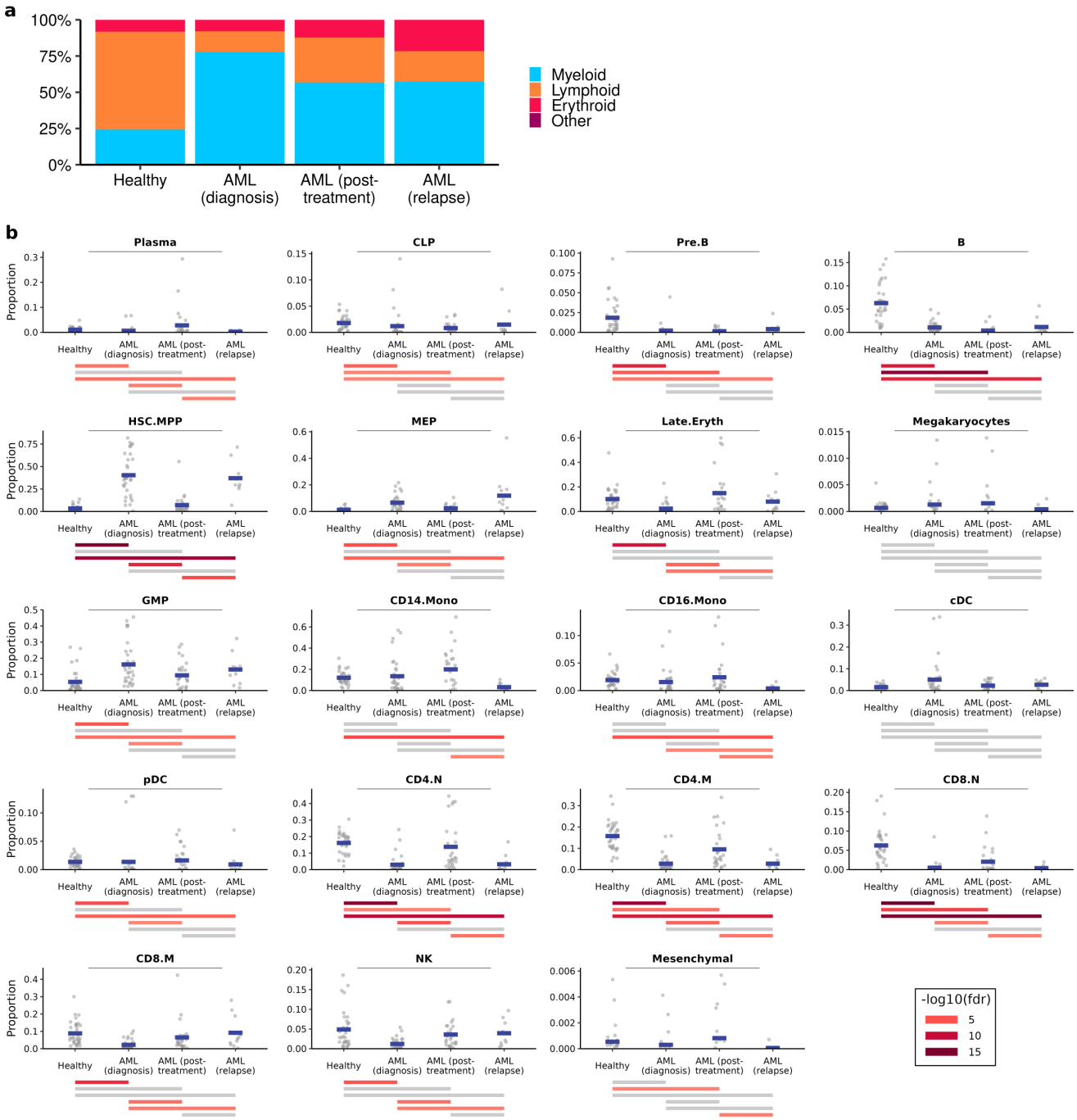

Figure 2: Cell type proportions in the BM are significantly altered during the establishment and progression of AML. a Percentage of myeloid, lymphoid, erythroid and other cell types at each timepoint. **b** The blue bars show the `propeller` estimated proportion of each cell type at different timepoints. The grey dots represent the proportions of each cell type in individual samples. The lines below each plot represent the significance level of the change in proportions between each pair of timepoints. Cell types that showed a significant change in proportions between a given pair of timepoints are represented by coloured lines and grey lines indicate FDR ¿= 0.05. *HSC.MPP = Hematopoietic stem cell/multipotent progenitor, GMP = granulocyte monocyte precursor, MEP = megakaryocyte erythrocyte precursor, CLP = common lymphoid progenitor, cDC = conventional dendritic cell, pDC = plasmacytoid dendritic cell, CD4/8.N = CD4/8 naive T, CD4/8.M = CD4/8 memory T, NK = natural killer cell.*

3.3 Impact of AML on gene expression in the BM

To gain insight into functional changes in BM cell types at the onset of AML, we determined changes in gene expression for each cell type between AML diagnostic samples and healthy samples. To minimise false discoveries, differentially expressed genes (DEGs) were calculated using a pseudobulk approach, implemented in the Libra R package [25] and the DEGs were then used as input for gene ontology (GO) enrichment analysis. Most cell types showed broad scale changes in gene expression, with a median of 2,368 genes being differentially expressed ($|\text{avg. logFC}| \geq 1$, adj. p value ≤ 0.05) (Figure 3a). Of all cell types, pre.B, cDC, CD4.M, CD14.Mono and HSC.MPPs had the highest number of DEGs. Notably, pre.B cells showed downregulation of genes mediating B cell maturation (e.g. VPREB1, TCL1A), and upregulated heat shock protein expression (HSPA1A, HSPA1B), indicating cellular stress (Supplementary Figure 2). In HSC.MPPs, genes associated with HSC self-renewal and differentiation (PBX1 [26, 27]), angiogenesis (VEGFA, NRP1, PDGFA) and myeloid cell differentiation (CSF1R, PTBP3, IL11) were some of the most notable differentially expressed genes (Figure 3c). Importantly, the top upregulated gene in HSC.MPP cells, interleukin 1 receptor accessory protein (IL1RAP, avg. logFC = 3.79, adj. p value = 1.94e-33) is a gene mediating IL-1β signaling. This pathway has been shown to promote the growth and survival of malignant cells in AML and to play a central role in remodelling the BM microenvironment into a niche favoring leukemogenesis over normal hematopoiesis by driving pro-inflammatory signalling [28].

GO (Biological Process) terms enriched among up- and downregulated genes for each cell type were clustered into groups based on similarity. There were 6 clusters of similar GO terms enriched among downregulated genes and another 6 clusters of GO terms enriched among genes upregulated upon development of AML (Figure 3b 3c). Three clusters of GO terms enriched among downregulated genes were broadscale changes, affecting most BM-resident cell types. These included processes associated with DNA repair, transcription and oxidative metabolism (Cluster 1), lymphocyte functionality (Cluster 3) as well as oxygen levels (hypoxia), detoxification and necrotic cell death (Cluster 5) (Figure 3b). Importantly, the most significantly enriched GO terms among downregulated genes were pathways of mitochondrial respiration (with downregulation of several genes of the mitochondrial electron transport chain: NDUFA4, UQCRB, COX6B1). This was detectable in all BM-constituent cell types, except the HSC.MPP, MEP and megakaryocyte populations, indicating widespread hypoxia and/or metabolic reprogramming of nearly all non-malignant BM cell types. This supports the notion that leukemia impacts the on entire microenvironment.

Downregulation of genes associated with cell division was also present in multiple cell types, but mostly affected CLP and pre.B cells (Cluster 2). CLP and pre.B cells also displayed impaired mRNA transportation and protein maturation, signs of proteostatic cellular stress (Cluster 4, Figure 3b).

The first group of processes upregulated in AML centred on morphogenesis and cytokine signalling (Cluster 1) predominantly affecting pre.B cells, the malignant population-encompassing HSC.MPP, MEP and GMP populations and pDCs. Of note, there was little overlap in the specific GO terms across these cell types. For example the enriched pathways for MEPs and GMPs differed from each other (e.g. 'regulation of interleukin-6 production' enriched among genes upregulated in MEPs but not GMPs).

The second cluster of processes enriched among upregulated genes involved the phosphatidylinositol 3-kinase (PI3K) and signal transducer and activator of transcription (STAT) pathways, especially in CLP and pre.B, where these pathways drive cell proliferation and cell survival before and after immunoglobulin heavy chain and light chain recombination, during which the cells are non-proliferative [29]. Downregulation of mitotic activity (cluster 2 of downregulated pathways) and simultaneous induction of proliferative signalling in these cell cohorts indicates deregulation of B cell receptor assembly (via Ig-recombination) and thus early B cell maturation in AML.

The largest cluster of GO terms enriched among upregulated genes (Cluster 3) is made up of processes related to T cell differentiation, where CLP, Pre.B, HSC.MPP, and pDC cells showed upregulation of genes that drive helper T cell differentiation (IL6R, IL18). This indicates that the leukemic microenvironment

may skew lymphoid progenitor maturation towards T cell generation over B cell generation and could partly explain the significant B cell depletion observed at all AML timepoints compared to healthy samples (Figure 2b).

Other clusters of terms enriched among upregulated genes (Clusters 4 & 5), particularly in HSC.MPPs were related to TGF-β signalling and cell adhesion pathways, two pleiotropic pathways regulating multiple facets of hematopoiesis.

Only a few genes were identified as differentially expressed in mesenchymal stromal cells, likely due to their high level of heterogeneity, encompassing multiple different subtypes and the relatively low number of them present in the dataset (n healthy = 30, n AML = 42). This limited the power to detect DEGs, particularly given the stringent fold-change threshold applied ($|\text{avg. logFC}| \geq 1$), and does not necessarily indicate that this cell population does not change upon AML development.

Figure 3: Impact of AML on gene expression in the BM. For each cell type, DEGs were identified by comparing gene expression in AML diagnostic samples to healthy samples. **a** Volcano plots showing number of DEGs for each cell type. **b-c** GO terms enriched among genes significantly down- (**b**) and up-regulated (**c**) in AML. All significantly enriched GO terms are on the vertical axis and the pink heatmap shows the adj. p value for the enrichment of each term in each cell type. The white/purple matrix represents the similarity between GO terms with clusters of similar terms highlighted with coloured boxes. The word clouds beside each cluster show the keywords enriched in that cluster of terms. **d** Enrichment of terms related to metabolism and respiration among genes downregulated in AML. *HSC.MPP = Hematopoietic stem cell/multipotent progenitor, GMP = granulocyte monocyte precursor, MEP = megakaryocyte erythrocyte precursor, CLP = common lymphoid progenitor, cDC = conventional dendritic cell, pDC = plasmacytoid dendritic cell, CD4/8.N = CD4/8 naive T, CD4/8.M = CD4/8 memory T, NK = natural killer cell.*

3.4 Interactions between HSPCs and their niche

Cell-cell interactions are central in regulating the hematopoietic BM niche and HSPC maintenance. The role of the niche in controlling HSPC self renewal, expansion and differentiation are well recognised. There

is also accumulating evidence that during development of AML, cell-cell interactions change substantially. To gain a comprehensive understanding of HSPC-niche communication pathways and how they change upon development of AML, we predicted ligand-receptor interactions between HSC.MPP cells and the other BM-resident cell types by looking at the expression of genes that code for ligands and their interacting receptors. We used the `liana` R package [30] to run a combination of interaction prediction methods with the Omnipath database of known ligand-receptor interactions [31] and generated a list of high-confidence, well-curated interactions in healthy BM and upon development of AML.

A total of 485 and 905 interactions were identified in healthy and AML diagnostic samples, respectively (Figure 4a). Of the total 1,390 interactions, only 354 were common to both timepoints, demonstrating the extent of the impact that leukemia has on the interactome of hematopoietic cells (Figure 4a). Of the interactions that were common to both timepoints, 34 had a higher interaction score in healthy samples, 43 had a higher score in AML and 277 remained unchanged (Supplementary Table 5, Supplementary Figure 3a). Notably, HSC.MPPs could participate in a large number of interactions in AML which were not present in healthy BM, involving the all BM constituent cell types (Figure 4a). This may not be because leukemic HSC.MPPs expressed a higher range of ligands and receptors in comparison to normal HSC.MPPs, instead, it likely reflects the intra-patient heterogeneity of AML where different AML sub-populations co-exist in individual patients and form varied, partially overlapping interactions.

The predicted interactions also varied on a broad scale with regards to how specific they were to given cell types (Figure 4c). There were 13 ubiquitous interactions (Figure 4d), where the interacting partner of the HSC.MPP-expressed ligand or receptor was present in at least 14 other cell types (over 75% of BM-constituent cell types). On the other end of the scale, 159 interactions were cell type-specific i.e. the partner ligand or receptor was only present in one cell type (Figure 4e-g). The number of HSC.MPP-interacting cells for the remaining interactions varied, but showed trends where cell types within each hematopoietic cell lineage tended to participate in similar interactions.

The ubiquitous interactions were dominated by cell-adhesion mediators, namely, integrin beta 1 (ITGB1), galectins (LGALS1, LGALS9, LGALS3BP) and CD44. Additionally, angiopoietin-1 (ANGPT1) and TGF-β1 (TGFB1) signalling was detected (Figure 4d). There was a pronounced shift in these ubiquitous interactions upon development of AML. Firstly, in AML, ITGB1 could form interactions with a broader spectrum of ligands, including the disintegrin and metalloprotease family members, ADAM-15 and -17, as well as the growth factor, angiopoietin-1 (ANGPT1). In addition to enabling adhesion, these interactions could allow HSC.MPPs to receive pro-survival signals from nearly all BM cell types [32]. Another striking change was in the TGFB1-TGFB1 receptor-2 (TGFBR2) interaction, which became widespread in AML (associated with TGFBR2 upregulation in AML-HSC.MPPs), with CD14.mono cells being a major interacting partner. TGFB1 is a pleiotropic regulator of all stages of hematopoiesis and excessive TGFB1 expression has been linked to proliferation of leukemic cells and failure of normal hematopoiesis in chronic lymphocytic leukemia [33]. In AML, TGFB1 has been linked to leukemia stem cell/leukemia-initiating cell quiescence [4] as well as bone marrow fibrosis [34].

The opposite trend was seen for the interaction between amyloid precursor protein (APP) and the plasma membrane molecular chaperone, CD74, which was widespread in healthy BM, but became almost undetectable in AML. CD74 mediates trafficking of APP into endocytotic vacuoles thus preventing the production of amyloid β peptide, the main constituent of senile plaques in Alzheimer's disease [35]. Loss of the interaction in AML was due to significant downregulation of CD74 expression, which occurred in 13 out of the 19 BM cell types. Loss of CD74 expression may contribute to enhanced fitness of leukemic HSC.MPPS, as CD74 has been reported to regulate HSPC maintenance and its deficiency in mice caused accumulation of HSPCs in the BM due to their increased potential to compete for BM niches [36]. Alternatively, as an MHC-II chaperone, reduced CD74 expression may play an immunosuppressive role, causing reduced MHC-II complex expression affecting antigen presentation and thus enabling immune escape [37].

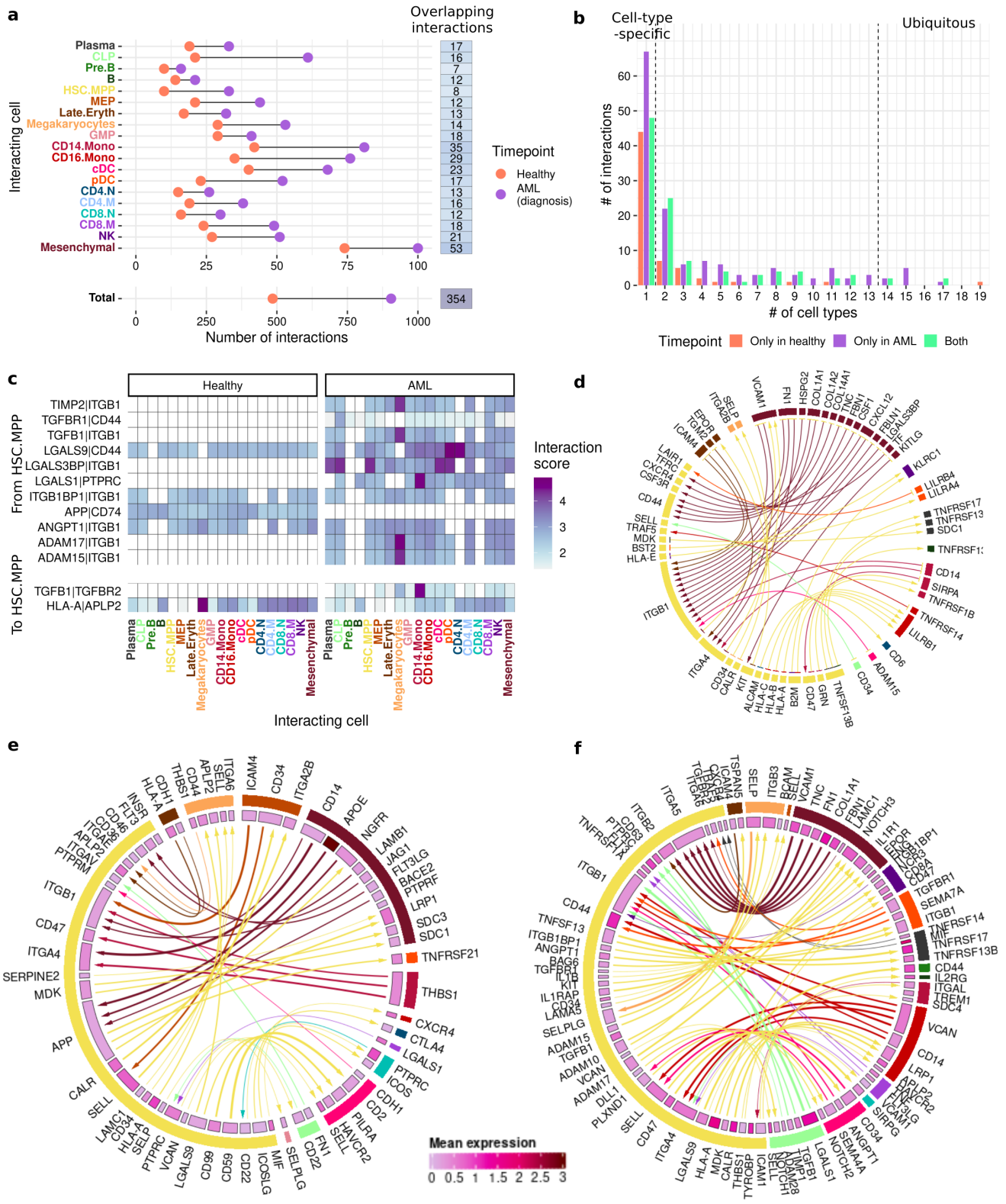

Figure 4: Interactions between HSPCs and their niche. a Ligand-receptor interactions identified between HSC.MPP cells and the other cell types in healthy and AML BM. **b** Histogram showing the number of cell types each interaction was identified in. **c** Genes and cell types involved in ubiquitous interactions ($\geq$ 14 cell types). **d-f** Chord diagrams of cell-type-specific interactions identified at both timepoints (**d**), or exclusively in healthy (**e**) or diagnostic (**f**) samples. The colour of the outer sectors represents cell type (colours same as in **a**) and links are coloured by cell type of the sending cell. In **e-f** the inner sectors represent the mean expression of the ligand/receptor gene and the width of links is proportional to the interaction score. *HSC.MPP = Hematopoietic stem cell/multipotent progenitor, GMP = granulocyte monocyte precursor, MEP = megakaryocyte erythrocyte precursor, CLP = common lymphoid progenitor, cDC = conventional dendritic cell, pDC = plasmacytoid dendritic cell, CD4/8.N = CD4/8 naive T, CD4/8.M = CD4/8 memory T, NK = natural killer cell.*

With regards to the most active HSC.MPP-interacting cell types, a similar pattern can be seen in healthy and AML BM – mesenchymal cells can form the highest number of interactions with HSC.MPPs, followed by CD14+ and CD16+ monocytes (CD14.Mono, CD16.Mono) (Figure 4b). These cell types are known to play important roles in regulating the hematopoietic niche, particularly mesenchymal cells which are essential for HSPC maintenance [2, 38]. The top 50% of interactions between HSC.MPP cells and monocytes and mesenchymal cells at each timepoint are shown in Supplementary Figure 4.

Since BM mesenchymal stem cells (BMSCs) are key regulators of processes involved in HSPC maintenance, ranging from HSPC quiescence, proliferation, homing and migration to survival, detailed analysis of the BMSC-HSC.MPP interactome was carried out. HSC.MPPs were found to be able to form broad-scale interactions facilitating cell-cell and cell-ECM adhesion with adhesion molecules and ECM components expressed by BMSCs (Figure 5). Interactions between the adhesion receptors, ITGA4, ITGB1 (forming the very late antigen (VLA)-4 complex) and CD44 of HSC.MPPs with collagens, fibronectin, laminins and other BMSC-produced ECM components as well as the cell-cell adhesion ligands VCAM1, VCAN and galectins (LGALS1, LGALS3BP) expressed on the surface of BMSCs were detectable, facilitating HSC.MPP engraftment in the niche. Reciprocally, HSC.MPPs also expressed adhesion ligands, such as galectins (LGALS1, LGALS9), ICAM3 and TIMP1 that could bind to adhesion receptors of BMSCs (CD44, ITGB1, ITGB2, CD63). All these interactions were stable and retained in AML.

In AML, additional cell-ECM and cell-cell adhesion interactions became apparent. AML-HSC.MPPs showed increased expression of ITGA5 (avg. logFC = 2.1, adj. p val = 6.42e-14) enabling VLA-5 assembly (ITGA5-ITGB1 complex), ITGA6 induction (avg. logFC = 1.04, adj. p value = 1.16e-4) as well as adhesion ligands, most notably members of the ADAM family (ADAM15, -17). This enabled the formation of further adhesion interactions with ECM proteins (via ITGA5, ITGA6) and BMSC-expressed adhesion proteins (VCAM1 and VCAN via ITGB2), emphasising that engraftment of HSC.MPPs in the hematopoietic niche remains essential or it is strongly beneficial to AML cells. Of note, ITGA5 and ITGA6 are also recognised markers of leukemic stem cells [39]. The analysis revealed that they mediate interaction with fibronectin (FN) and tenascin C (TNC), an ECM protein important for hematopoiesis and known to co-distribute with FN and collagen III surrounding HSPCs [32, 40].

Overall, the high number of adhesion interactions can increase the competitiveness of AML-HSC.MPPs to engraft in protective niches that provide pro-survival signals contributing to chemoresistance. In addition to cell-matrix and cell-cell adhesion, BMSCs and HSC.MPPs could engage in a large number of cell-cell and cytokine-cell communication interactions (Figure 5). Many well-established interactions between HSC.MPPs and BMSCs were detected, including CXCL12-CXCR4, KITLG-KIT (Kit ligand and its receptor) and CSF-CSF3R (colony stimulating factor-3/G-CSF and its receptor). In addition, interactions between MIF (macrophage migration inhibitory factor), a pleiotropic cytokine regulating innate immunity and one of its receptors, CD74; CALR and LRP1 (calreticulin and low density lipoprotein related protein 1), and between MDK and four receptors (SDC (syndecan)-1, SDC3, LRP1 and NOTCH2) were also detected both in healthy and AML BM. The MIF-CD74 interaction was reciprocal, where both HSC.MPPs and BMSCs expressed both the ligand and the receptor, providing pro-survival signal via ERK and PI3K signaling [41]. CD74 also interacts with CD44 and participates in CD44-dependent signal transduction [42].

MDK (midkine) is a heparin-binding growth factor that can bind to multiple receptors and mediates anti-apoptotic, mitogenic and chemotactic signaling. While HSC.MPP-expressed MDK interactions were present both in healthy- and AML-BM, BMSCs in AML expressed MDK that could bind to NOTCH2 and facilitate NOTCH2-signaling in HSC.MPPs. In addition to MDK, AML-BMSCs also induced expression of ADAM17. ADAM17 can also interact with NOTCH2 facilitating its proteolytic processing and activation, which has been shown to reduce myeloid differentiation and enhance long- and short-term HSPC repopulation upon stress hematopoiesis [43]. NOTCH signaling was not only affected in HSC.MPPs, but also in AML BMSCs. ADAM10, 15 and 17 expressed by AML-HSC.MPPs could form interactions with NOTCH2 and NOTCH3 expressed by BMSCs driving NOTCH2/3-activation, which has been shown to block BMSC differentiation and osteogenesis and to enhance BM-mediated drug resistance [44, 45].

In the leukemic BM, a number of interactions that were not present in healthy BM developed. Firstly, IL1B (interleukin-1 β) produced both by HSC.MPPs and BMSCs could activate inflammatory signaling via binding to IL1R1 (IL1 receptor 1) and IL1RAP (IL1 receptor associated protein 1) on BMSCs and HSC.MPPs, respectively. Interleukin-1 β levels are fine tuned in the BM and chronic IL-1B exposure restricts HSPC lineage output and weakens healthy HSPC self-renewal capacity [46]. This cytokine also promotes AML blast proliferation by induction of growth factors and cytokines like CSF3 [47].

Finally, an interaction between the semaphorin family member, SEMA4D expressed by HSC.MPPs and its receptor, PLXNB2 on BMSCs became apparent in AML. SEMA4D is known to inhibit osteoblast differentiation in multiple myeloma patients, where bone resorption is abundant. This process may be aggravated by TGFB1 signaling, which also became enhanced in AML, where both HSC.MPPS and BMSCs expressed TGFB1 and could activate TGFBR2 on the other cell type. While the effects of TGFB1 on BMSCs are multifold and not fully understood, TGFB1 can reduce osteoblastic differentiation and facilitate the development of a myelodysplastic syndrome (MDS)/AML-like BMSC phenotype [48].

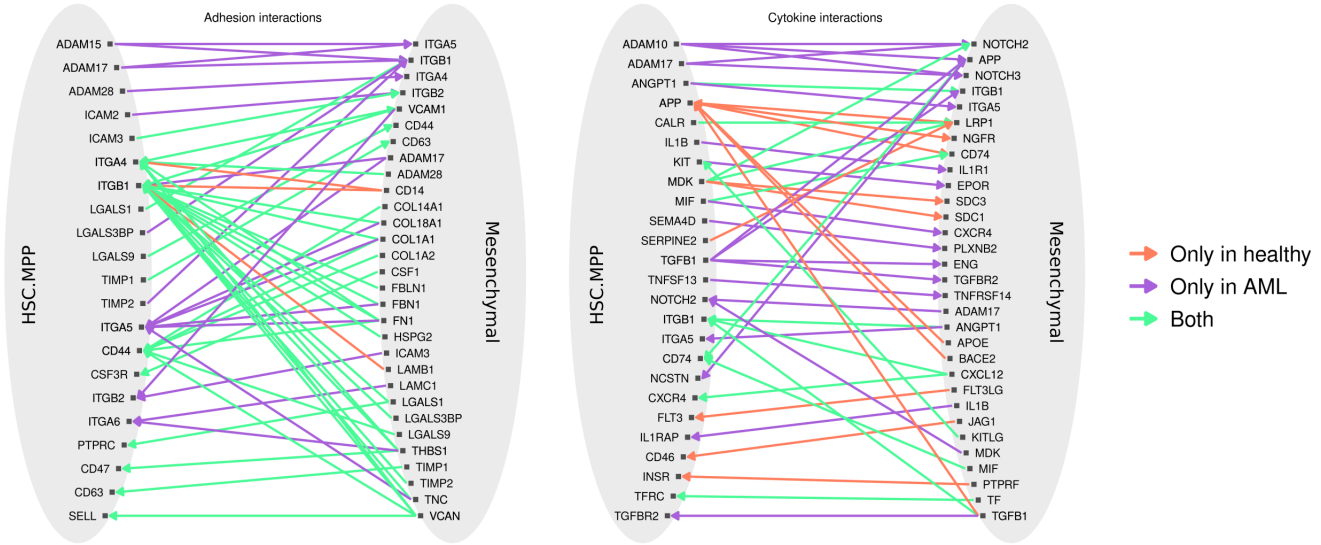

Figure 5: Interactions between HSPCs and BM mesenchymal cells. Interactions were grouped into those involved in cell adhesion and those involved in cytokine signalling. The colour of the arrows represents which timepoint each interaction was identified at. *HSC.MPP = Hematopoietic stem cell/multipotent progenitor*

3.5 Altered cell adhesion and cytokine signalling in AML

To determine how the identified interactions regulate cell-cell communication in the BM and thus regulate the functioning of the hematopoietic niche, ligand and receptor genes were linked with KEGG pathway annotations and filtered to only include interactions where both the ligand and the receptor had the same annotation (Figure 6a). The identified pathways centred around four processes: 1. cell adhesion and migration (including *Cell adhesion molecules CAMS, ECM receptor interaction, Focal adhesion, Adherens junction* and *Leukocyte transendothelial migration*), 2. hematopoiesis and leukocyte maturation (including *Hematopoietic cell lineage, NOTCH signaling pathway* and *Antigen processing and presentation*), 3. cytokine and kinase signalling (*Cytokine cytokine receptor interaction, TGF beta signaling pathway, JAK-STAT signaling pathway, MAPK signaling pathway, Insulin signaling pathway* and *Adipocytokine signaling pathway*) and finally 4. cancer-related pathways (including *Pathways in cancer, Chronic myeloid leukemia, Colorectal cancer* and *Pancreatic cancer*) (Figure 6a).

The pathways that the identified ligand-receptor interactions could mediate were largely cell type/cell lineage-specific in healthy BM (detected only in healthy BM and interactions that did not change upon

development of AML). Upon development of AML, only a small number of these interactions were lost, instead an expansion both in the number of possible interactions and participating cell types were detected. Additionally, via newly formed interactions, pathways which were not detected in healthy BM were activated, predominantly regulating cancer-related pathways, cell adhesion and cytokine signaling (Figure 6a).

To gain insight into the impact of these interaction on HSC.MPP function and the BM microenvironment in AML, the participating ligand-receptor pairs were analysed for the cell type origin of the signal, the target of the signal if it originated from HSC.MPPs (Figure 6b) and how the interaction changed between healthy and AML BM (Figure 6b).

The role of cell adhesion interactions in normal hematopoiesis and in AML is well-documented [49]. Adhesion of AML cells to the BM niche can confer protection from apoptosis and enhance chemoresistance and targeting cell adhesion molecules to mobilise malignant cells from the BM has been shown to improve drug sensitivity in AML (e.g. CXCR4 inhibitors) [50]. Accordingly, a broad spectrum of interactions mediating cell adhesion were detected, involving nearly all cell adhesion molecule (CAM) families, namely the immunoglobulin superfamily of CAMs (with members ICAMs, VCAM, ALCAM), integrins (ITGAL (forming LFA-1 when binding to ITGB2), ITGAM (forming Mac-1 with ITGB2) and ITGB2), C-type lectin-like domain proteins (SELL, SELP) and proteoglycans (SELPLG, VCAN) (Figure 6b).

Importantly, cell adhesion interactions between selectin genes (SELL, SELP) and CD34 and the proteoglycan selectin counter-receptor, SELPLG (selectin P ligand/PSGL1) were either upregulated or identified exclusively in AML samples. SELE is typically expressed by endothelial cells in the BM and is known to interact with SELPLG expressed on the surface of leukocytes, as well as AML blasts. Inhibiting SELE and thus preventing AML cell-endothelium/BM interactions (GMI-1271, uproleselan) has been shown to improve AML patient response to therapy [51, 52]. Interestingly, another selectin interaction, which so far received less attention has also been identified. The interaction between SELPLG (expressed by HSC.MPP cells) and SELP (expressed by megakaryocytes) was confidently detected in AML samples but not healthy samples (Figure 6b). The therapeutic potential of SELL and SELP is supported by recent studies that showed that platelets can "cloak" malignant cells via SELP to protect them from NK cell-mediated cytotoxicity in multiple myeloma [53], and high soluble SELE and SELL expression is associated with a high probability of AML relapse [54].

Other identified cell adhesion interactions involved the integrin family (ITGAL, ITGAM, ITGA4, ITGB1, ITGB2), especially the leukocyte integrins, ITGAL, ITGAM and ITGB2. HSC.MPPs expressed both a ligand (ICAM1-3) and ITGB2, enabling reciprocal interactions between HSC.MPPs and multiple BM-constituent leukocytes. Notably, these interactions substantially expanded in AML. For example, while no interactions were detected for HSPC-expressed ICAM2 in healthy BM, ICAM2-expressing AML-HSC.MPPs could form cell-cell interactions with 13 out of the 19 BM cell types expressing ITGB2. Interactions via AML-HSC.MPP-expressed ITGB2, ICAM2 and ICAM3 also became widespread in AML, involving 15 cell types (Figure 6b). This expansion of integrin signalling is likely to play a role in promoting survival of the malignant cells [55, 56].

With regards to cytokine signalling, a number of interactions known to be important in the development of AML were identified. For example, IL1B signalling has been shown to promote the growth and survival of malignant cells in AML [28]. Accordingly, ligands and receptors involved in IL1B signalling (IL1B, IL1R1, IL1RAP) were broadly expressed in HSC.MPP cells, monocytes (CD14.Mono, CD16.Mono) and mesenchymal cells (Figure 6b), corroborating the presence of inflammatory signalling in AML-BM.

The analysis also identified TGF-β1 interactions between HSC.MPP and multiple different cell types specific to AML. As mentioned in the previous section, TGF-β1 signaling became widespread in AML, possibly due to the upregulation of the two receptors - TGFβ-RI (TGFBR1, avg. logFC = 1.27, adj, p value = 1.54e-7) and TGFβ-RII (TGFBR2, avg. logFC = 1.15, adj. p value = 9.75e-9) - in HSC.MPP cells. TGF-β1 signalling is an important regulator of hematopoiesis and has pleiotropic effects depending on the context [57]. In healthy HSPCs, binding of TGF-β1 to TGFβ-RI and TGFβ-RII is an important

inhibitory stimulus that prevents cell proliferation via induction of the cyclin-dependent kinase inhibitor CDKN1C [58, 59]. However, in the case of malignancy, HSPCs may become less sensitive to the homeostatic effects of TGF-β1 [60]. It has also been shown that certain subtypes of HSPCs may respond differently to TGF-β1 signalling and that it's more likely to stimulate proliferation in myeloid-biased HSPCs compared to lymphoid-biased HSPCs [61]. Our data suggest that TGF-β1 signalling is particularly active in AML and should be investigated further to establish whether the HSPCs are resistant to TGF-β1, or whether it's acting as a pro-survival signal.

Another cytokine pair highlighted by the analysis was TNFSF13 (A Proliferation-Inducing Ligand, APRIL) and TNFSF13B (B cell activating factor of the TNF-family, BAFF), two key regulators of B cell maturation and survival [62]. TNFSF13B/BAFF is produced by myeloid cells, and exerts its effect by binding to three receptors, TNFRSF13B (Transmembrane Activator And CAML Interactor, TACI), TNFRSF13C (BAFF-R) and TNFRSF17 (B Cell Maturation Antigen, BCMA) [62]. Interaction between TNFSF13B and its three receptors were detectable between HSC.MPPs and plasma cells or B cells in healthy BM and were retained in AML. On the other hand, expression of TNFSF13/APRIL is reportedly very low in healthy tissues [63], but it is often induced upon malignant transformation where it can drive tumour cell growth [64]. Indeed, our analysis identified significant upregulation of APRIL in 13 BM cell types, particularly myeloid lineage cells. APRIL, similar to BAFF/TNFSF13B can also bind to TNFRSF13B and TNFRSF17, while TNFRSF13C is a BAFF-specific receptor. Interaction between TNFSF13/APRIL and its two receptors became apparent in AML-BM corroborating reports that APRIL expression is enhanced in AML cells where it supports leukemic cell proliferation [65]. Underlining the importance of APRIL in malignant transformation, high APRIL expression is also associated with chemoresistance [66] and immunosuppression via facilitating the formation of regulatory B and T cells [67].

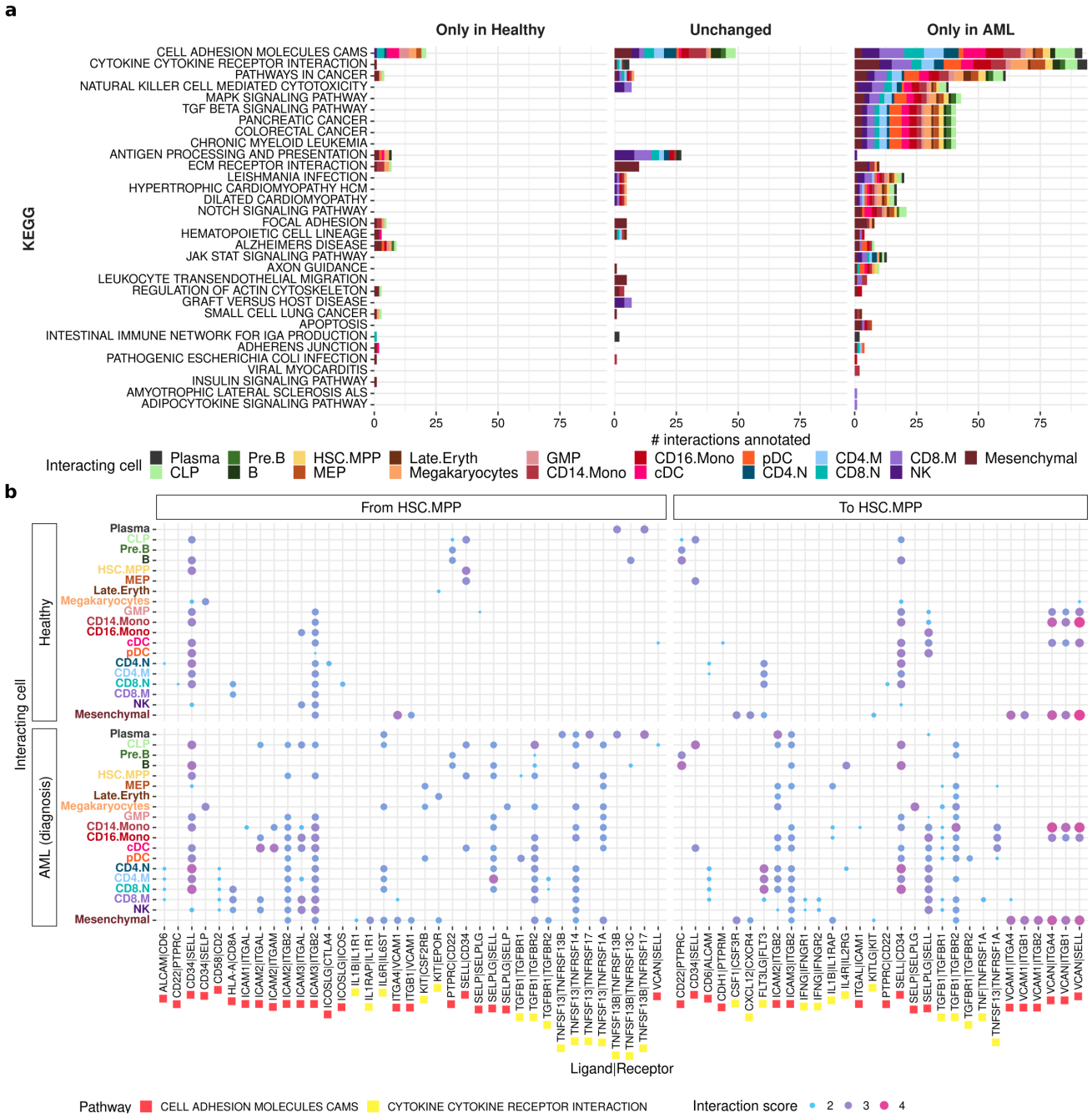

Figure 6: Altered cell adhesion and cytokine signalling in AML. a Functional annotation of interactions. Interactions were annotated with KEGG pathway annotations and filtered to interactions where the ligand and receptor gene both had the same annotation. **b** Cell types and signalling molecules involved in interactions annotated with *"CELL ADHESION MOLECULES CAMS"* or *"CYTOKINE CYTOKINE RECEPTOR INTERACTION"*. The size and colour of each dot represents the interaction score which is a measure of the confidence with which that interaction was identified at a given timepoint. *HSC.MPP = Hematopoietic stem cell/multipotent progenitor, GMP = granulocyte monocyte precursor, MEP = megakaryocyte erythrocyte precursor, CLP = common lymphoid progenitor, cDC = conventional dendritic cell, pDC = plasmacytoid dendritic cell, CD4/8.N = CD4/8 naive T, CD4/8.M = CD4/8 memory T, NK = natural killer cell.*

4 Conclusions

It is well-established that reciprocal interactions between hematopoietic cells and their niche are essential in regulating their properties and functions, and that these interactions are skewed in AML generating a microenvironment that fosters leukemogenesis. In this study, we provide several advances towards deciphering the interactome of hematopoietic cells in health and AML. By compiling the largest collection of single-cell gene expression data of the human BM to date, we were able to robustly characterise this complex environment. Our study leverages several previously generated datasets [11–14], along with a novel dataset to perform a comprehensive analysis of the hematopoietic niche.

Using this dataset, we determined changes in cell type proportions, cell-type-specific gene expression and putative cell-cell interactions HSC.MPP form with the other BM constituent cell types. The results highlight several recurring themes that provide insights into how the microenvironment is corrupted by AML. Firstly, B cell maturation is permanently damaged in AML, shown by the clear decline in the number of CLP and their progeny, including pre.B and B cells, at all AML timepoints. Unlike T cells, whose numbers partially recover during remission, the significant depletion of B cells persists post-treatment, which likely has implications for the functioning of the adaptive immune system in AML patients even during disease remission. Gene expression changes indicated skewed lymphoid lineage maturation towards T cells rather than B cells and deregulated early B cell maturation, shedding light on the potential reasons for B cell loss in AML.

The predicted HSC.MPP interactions revealed key cell-cell communication pathways that can provide a competitive advantage to AML cells over normal hematopoietic cells including both well-established mechanisms (e.g. CXCL12-CXCR4, FLT3LG-FLT, KITLG-KIT) and several novel ones (SELPLG-SELP, SEMA4D-PLXNB, MIF-CD74, MDK and its receptors, etc). These interactions can drive:

1. Enhanced engraftment of AML cells in protective niches, via enhanced cell-adhesion mediated via integrins (VLA-5, LFA-1, Mac-1), CD44, selectins as well as ADAMs
2. Immunosuppression, for example by reduced antigen presentation, and the cytokine, APRIL
3. Impaired BMSC functions/bone resorption via enhanced Notch, TGFB1 and SEMA4D signalling
4. Establishment of an inflammatory environment (IL1B and IL6)

Many of these interactions are novel and represent potential therapeutic targets to disrupt AML-niche interactions, and warrant further investigation and validation.

Inferring ligand-receptor interactions between cells from gene expression data comes with certain limitations as surface protein expression is not always reflected at the level of mRNA. Ideally, many of the novel cell-cell interactions we have predicted here would also be validated at the protein level. In addition, future studies using newer technologies that allow characterisation of spatially resolved gene expression will be critical to adding more precise spatial information about the interactome in the BM, as it is a highly spatially organised tissue.

In conclusion, this study provides a comprehensive single-cell characterisation of the interactome of HSCs in the hematopoietic BM niche in health and AML. The study provides a comprehensive view of cell-cell communication pathways in the hematopoietic niche and reveals the scale of niche alterations that take place in AML.

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6 Methods

6.1 Datasets

6.1.1 Generation of scRNA-seq dataset

Longitudinally-collected BM aspirates were obtained for 10 AML patients from the time of diagnosis, post-treatment/remission and at relapse from The Finnish Haematology Registry and Clinical Biobank. The mononuclear cell fraction was isolated using Ficoll density gradient separation. Red cell lysis and dead cell removal (Miltenyi Biotec) was carried out prior to generation of single cell droplets with 10X Genomics Chromium Controller. Cell counting and viability assessments were conducted using To-Pro-3 viability dye. Thereafter, GEM generation & barcoding, reverse transcription, cDNA amplification and 3' gene expression library generation steps were all performed according to the Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Single Index) User Guide (10x Genomics CG000204 Rev D) with all stipulated 10x Genomics reagents. Generally, 16.5 µL of each cell suspension (1,000 cells/µL) and 26.5 µL of nuclease-free water were used for a targeted cell recovery of 10,000 cells. GEM generation was followed by a GEM-reverse transcription incubation, a clean-up step and 11 cycles of cDNA amplification. The resulting cDNA was evaluated for quantity and quality using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851) and an Advanced Analytical Fragment Analyzer System using a Fragment Analyzer NGS Fragment Kit (Agilent, DNF-473), respectively. Thereafter, 3' gene expression libraries were constructed using a sample index PCR step of 14-15 cycles. The generated cDNA libraries were tested for quantity and size using fluorometry and capillary electrophoresis as described above. The cDNA libraries were pooled and sequenced with a loading concentration of 300 pM, paired end and single indexed, on an illumina NovaSeq 6000 sequencer using a NovaSeq 6000 S1 Reagent Kit v1.5 (100 cycles; illumina, 20028319) and a S4 Reagent Kit v1.5 (200 cycles; illumina, 20028313). The read set-up was as follows: read 1: 28 cycles, i7 index: 8 cycles, i5: 0 cycles and read 2: 91 cycles. The quality of the sequencing runs was assessed using illumina Sequencing Analysis Viewer (illumina version 2.4.7) and all base call files were demultiplexed and converted into FASTQ files using illumina bcl2fastq conversion software v2.20. At least 50,000 reads/cell were generated for each sample. All steps post cell-suspension preparation were performed at the Next Generation Sequencing Platform, University of Bern. Reads were aligned to the GRCh38 reference genome and quantified using CellRanger (version 6.0.0).

6.1.2 Public datasets

When looking for datasets to include for this analysis, we excluded datasets with fewer than 20,000 genes as we wanted to ensure we captured as much of the transcriptome as possible. Table 2 shows where each dataset was obtained from.

6.2 Analysis

6.2.1 Pre-processing and normalisation

Datasets were analysed using a uniform pre-processing pipeline to ensure consistency. In the case where cell quality control needed to be performed, this was done using the ScanPy python module [68] according to current best-practices [69]. Counts were normalised using the `computeSumFactors` function from the scran R package [70]. The SingleR R package [71] was used to predict cell type labels for each dataset using the Granja et al., 2019[11] dataset as a reference. This dataset was chosen as the reference dataset as it is a CITE-seq dataset with expression values for several cell surface proteins and so the cell type labels should be reliable. Doublets were identified using Scrublet [72] and removed. All datasets were then merged into a single matrix for batch correction.

Table 2: Summary of the publically available datasets included in this study and where they were obtained from.

Dataset	Downloaded from	Comments
Granja et al., 2019 [11]	Data was downloaded in the form of raw count matrices from GEO (GSE139369) and cell metadata was downloaded from the github repository (GreenleafLab/MPAL-Single-Cell-2019) accompanying the publication.	Only the samples from healthy donors (BMMC_D1T1, BMMC_D1T2, CD34_D2T1, CD34_D3T1) were included in this analysis.
Oetjen et al., 2018 [12]	Data was downloaded in the form of raw count matrices from GEO (GSE120221).	
Petti et al., 2019 [13]	Data was downloaded in the form of raw count matrices from the zenodo link accompanying the publication (https://doi.org/10.5281/zenodo.3345981 `expression_matrices.tar` file).	
van Galen et al., 2019 [14]	Data was downloaded in the form of raw count matrices from GEO (GSE116256).	The 2 samples from AML cell lines (OCI-AML3 and MUTZ3) were excluded from this analysis.

6.2.2 Batch integration

Batch correction was performed using scArches [20] (2000 highly variable genes, 20 latent dimensions) and a nearest neighbour graph was constructed from the latent dimensions (n neighbours = 15) to generate a UMAP graph. A NextFlow [73] pipeline was developed to test different values for number of highly variable genes, latent dimensions and neighbours, to determine which values would yield the optimal integration results using the scPOP R package [74] to evaluate the results on the basis of Adjusted Rand Index (ARI), Normalized Mutual Information, Silhouette Width and Local Inverse Simpson Index (LISI). The results are shown in Supplementary Table 2.

6.2.3 Cell type proportions

The `propeller` function from the `speckle` R package was used to test whether there are statistically significant changes in cell type proportions between the different conditions [21]. Samples that were sorted prior to sequencing (n = 6) were excluded from this analysis.

6.2.4 Differential gene expression

For each cell type, differentially expressed genes were identified by comparing gene expression in healthy samples to gene expression in AML (diagnosis) samples. To avoid calling too many false positives, the `Libra` R package [25] was used to implement a pseudobulk approach to calculate differential expression and genes with absolute average log fold change ≥ 1 and an adjusted p value ≤ 0.05 were labelled as differentially expressed. Significant DEGs for each cell type were used as input for a Gene Ontology enrichment analysis using the `clusterProfiler` R package [75].

6.2.5 Cell-cell interactions

For the prediction of cell-cell interactions, healthy and diagnosis samples were isolated from the larger dataset and 1000 cells (or all cells if n $\leq$ 1000) of each cell type were subsampled. The liana R package [30]

was used to run 5 different methods for predicting cell-cell interactions (cellphonedb (squidpy), cellchat, NATMI, iTALK and SingleCellSignalR) using the Omnipath database resource of ligand-receptor interactions [31]. Results were filtered to interactions where either HSC.MPP cells were expressing either the ligand or receptor gene. To avoid calling false positive interactions, only interactions with `curation_effort` ≥ 3 were considered. The `liana_aggregate` function was used to generate an aggregate rank score for each interaction and interactions with a score ≤ 0.05 were considered to be high-confidence interactions. The `OmnipathR` R package was used to import functional annotations (KEGG and CellChat) for all genes involved in ligand-receptor interactions [31].

7 Code and data availability

The scRNA-seq data generated as part of this study is available on GEO under the accession number GSEXXX. The code to reproduce the results is available at https://github.com/Sarah145/bone_marrow_analysis.

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8 Supplementary materials

8.1 Supplementary Tables

- Supplementary Table 1: Clinical information for newly sequenced patients
- Supplementary Table 2: Sample information for all samples
- Supplementary Table 3: scPOP results
- Supplementary Table 4: DEG results
- Supplementary Table 5: Cell-cell interaction results

8.2 Supplementary Figures

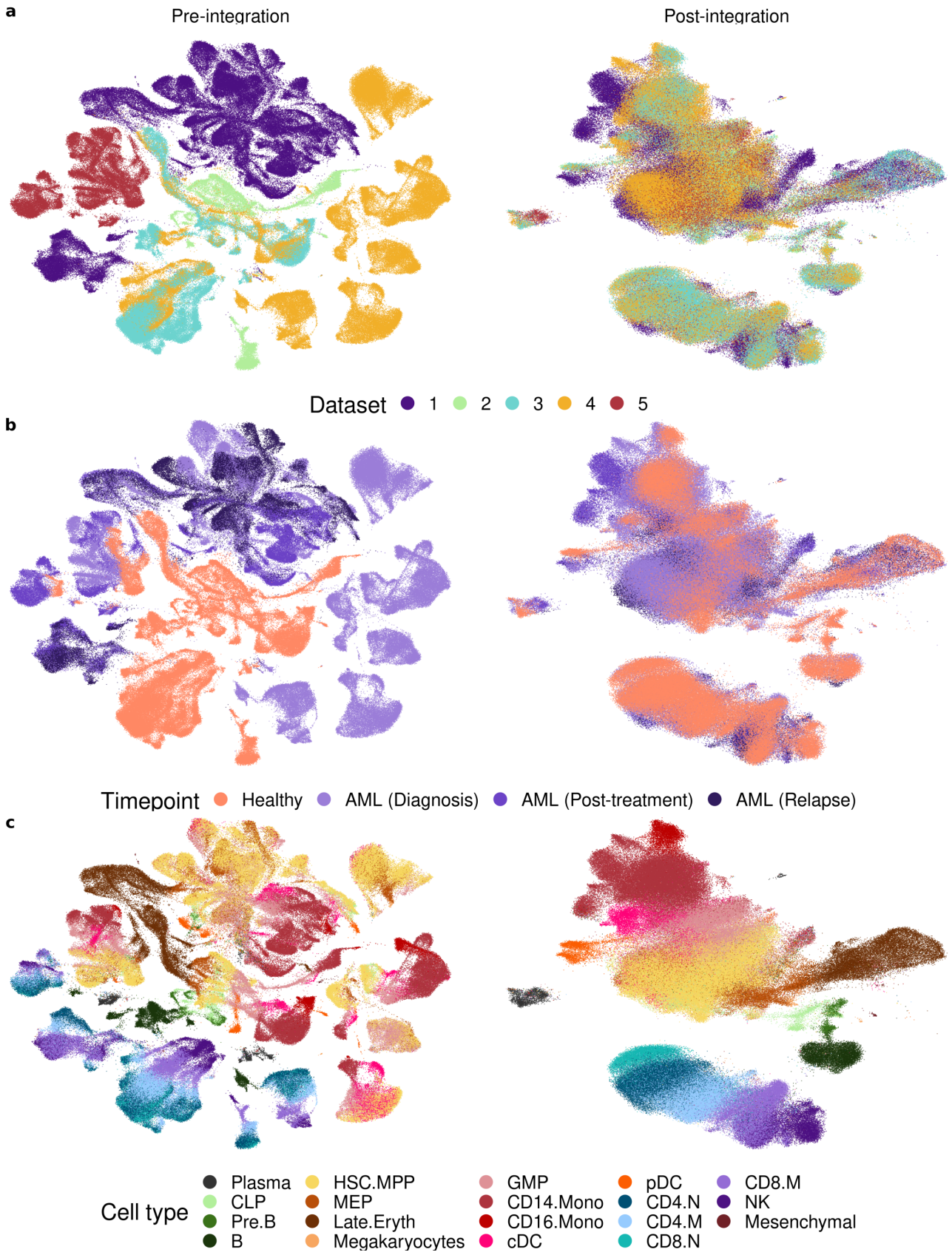

Figure 1: UMAP graphs pre-/post-integration.

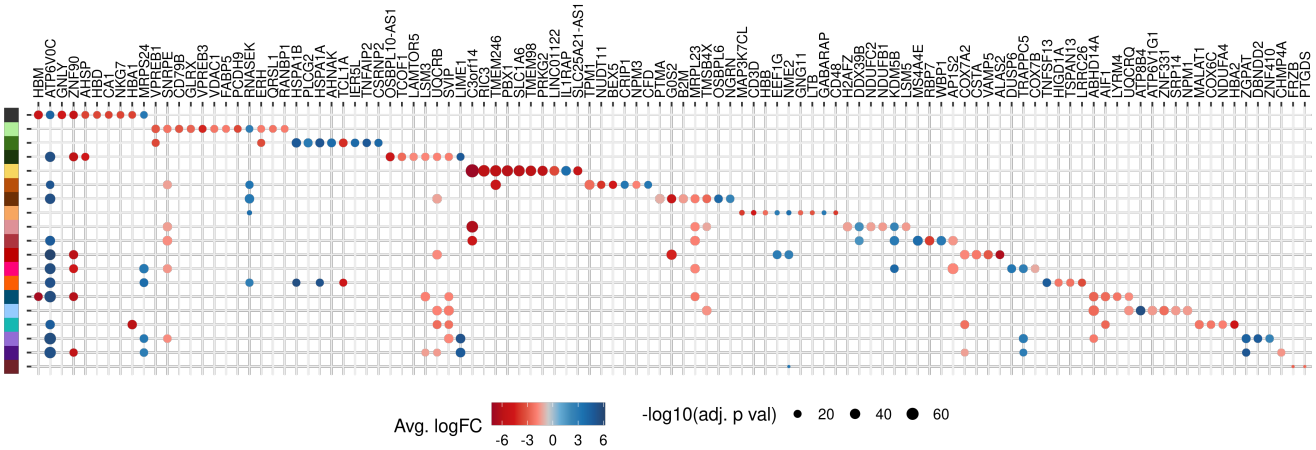

Figure 2: Top 10 DEGs for each cell type.

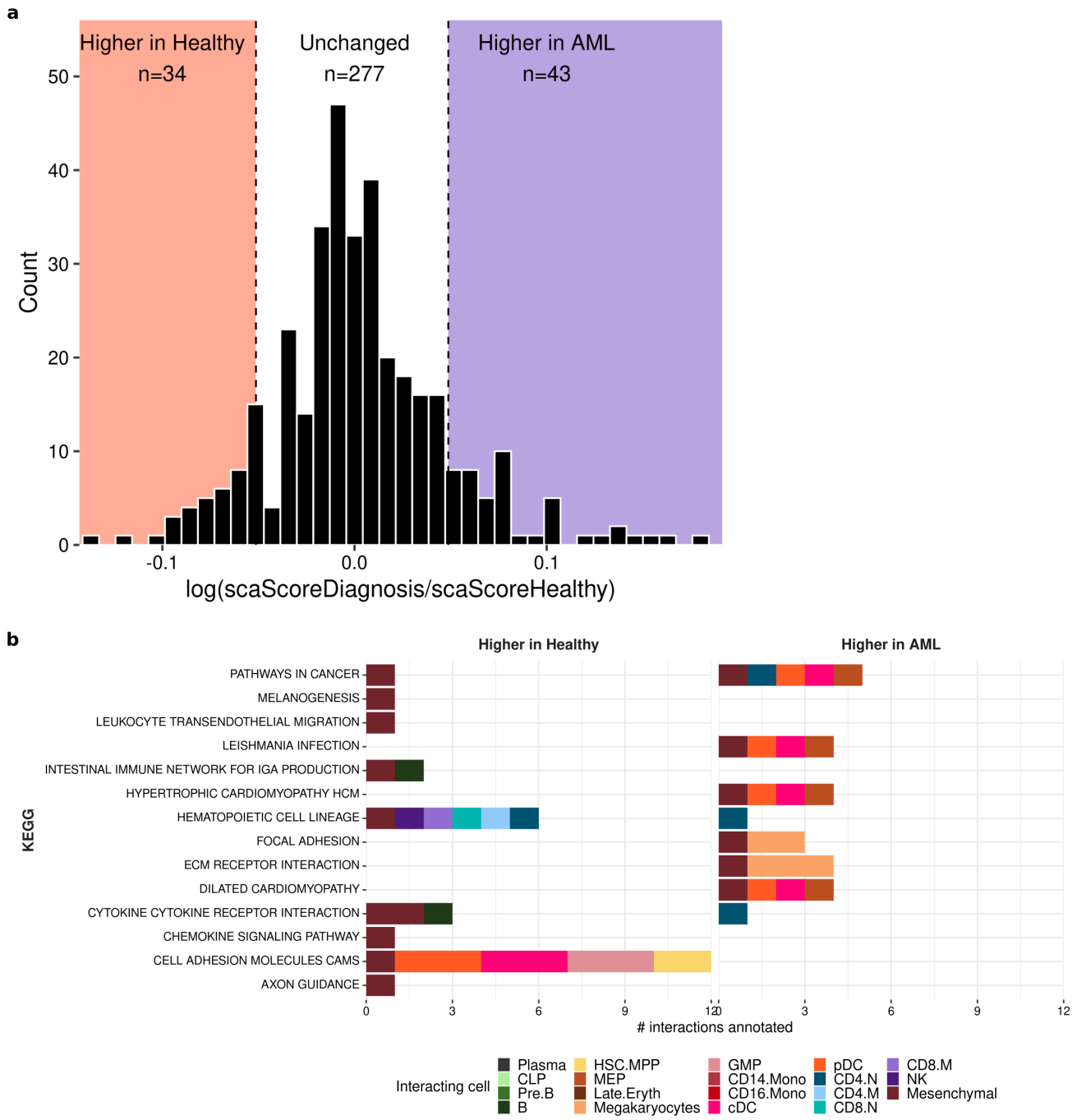

Figure 3: Interactions that were higher in healthy/AML.

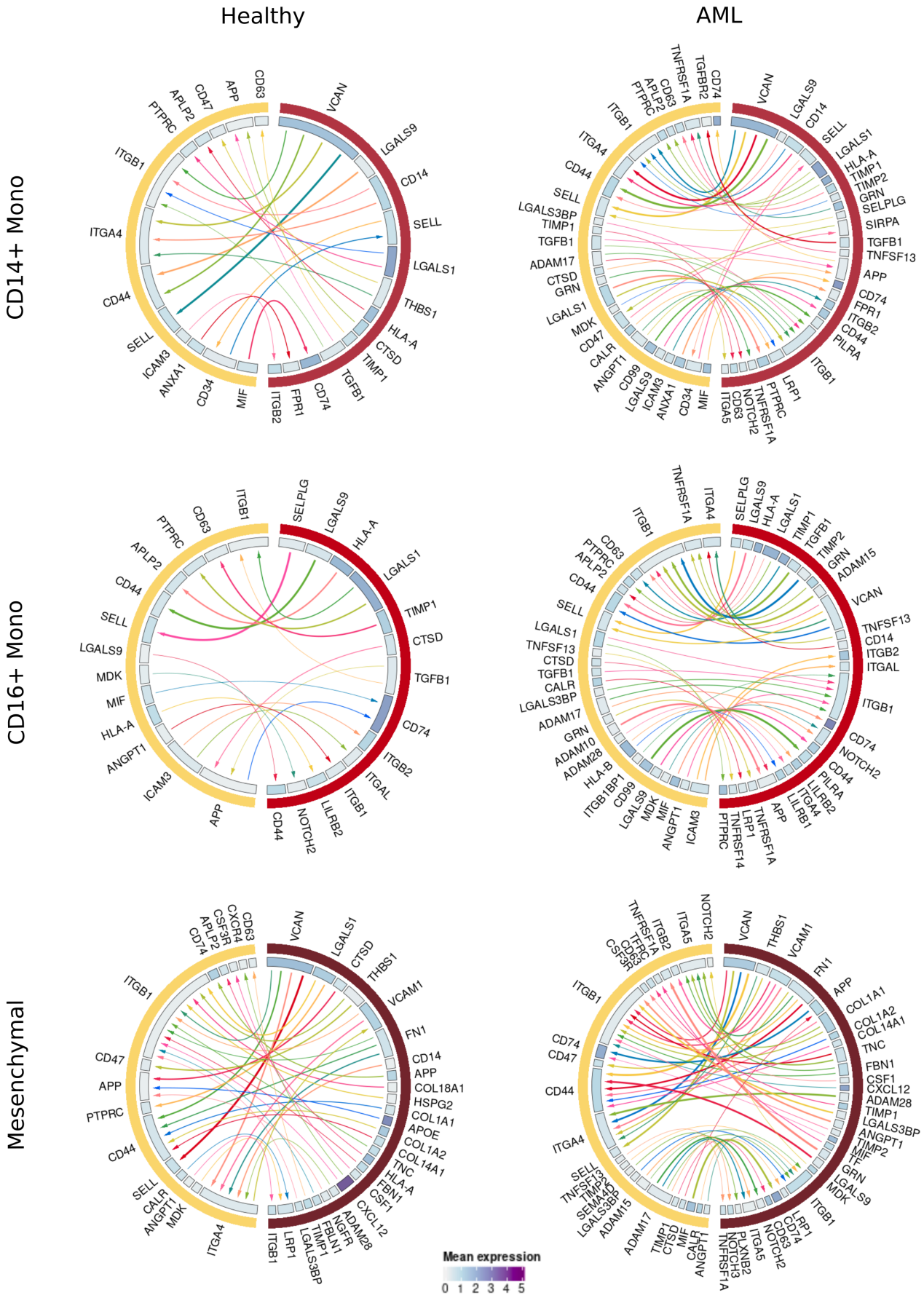

Figure 4: Interactions between HSC.MPPs and CD14.Mono, CD16.Mono and Mesenchymal cells.