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IL-9 Secreted by Leukemia Stem Cells Induces Th1-Skewed CD4+ T-Cells, which Promote Their Expansion

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Abstract:
In acute myeloid leukemia (AML), leukemia stem and progenitor cells (LSCs and LPCs) interact with various cell types in the bone marrow (BM) microenvironment, regulating their expansion and differentiation. To study the interaction of CD4+ and CD8+ T-cells in the BM with LSCs and LPCs, we analyzed their transcriptome and predicted cell-cell interactions by unbiased high-throughput correlation network analysis. We found that CD4+ T-cells in the BM of AML patients were activated and skewed towards Th1-polarization whereas IL-9 producing (Th9) CD4+ T-cells were absent. In contrast to normal hematopoietic stem cells (HSCs), LSCs produced IL-9 and the correlation modelling predicted IL9 in LSCs as a main hub-gene that activates CD4+ T-cells in AML. Functional validation revealed that IL-9R signaling in CD4+ T-cells leads to activation of the JAK-STAT pathway that induces the upregulation of KMT2A, KMT2C genes resulting in methylation on histone H3 at lysine 4 (H3K4) to promote genome accessibility and transcriptional activation. This induced Th1-skewing, proliferation and effector cytokine secretion, including interferon (IFN)-ɣ and tumor necrosis factor (TNF)-α. IFN-ɣ and to a lesser extend TNF-α produced by activated CD4+ T-cells, induced the expansion of LSCs. In accordance with our findings, high IL9 expression in LSCs and high IL9R, TNF and IFNG expression in BM-infiltrating CD4+ T-cells correlated with worse overall survival in AML. Thus, IL-9 secreted by AML LSCs shapes a Th1-skewed immune environment that promotes their expansion by secreting IFN-ɣ and TNF-α.

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Agreement to Share Publication-Related Data and Data Sharing Statement: Data availability All transcriptomic data compiled for this study have been deposited in NCBI GEO under the accession codes: GSE117090 and GSE249638. Public repository gene expression data was analyzed using the scRNA-seq AML dataset (GSE239721), HOVON-SAKK AML dataset (E-MTAB-3444), NL-Valk AML dataset (GSE6891) and normal karyotype AML "Metzeler" dataset (GSE12417).

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Data availability

All transcriptomic data compiled for this study have been deposited in NCBI GEO under the accession codes: GSE117090 and GSE249638. Public repository gene expression data was analyzed using the scRNA-seq AML dataset (GSE239721), HOVON-SAKK AML dataset (E-MTAB-3444), NL-Valk AML dataset (GSE6891) and normal karyotype AML “Metzeler” dataset (GSE12417).

Competing interests:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

In acute myeloid leukemia (AML), leukemia stem and progenitor cells (LSCs and LPCs) interact with various cell types in the bone marrow (BM) microenvironment, regulating their expansion and differentiation. To study the interaction of $CD4^+$ and $CD8^+$ T-cells in the BM with LSCs and LPCs, we analyzed their transcriptome and predicted cell-cell interactions by unbiased high-throughput correlation network analysis. We found that $CD4^+$ T-cells in the BM of AML patients were activated and skewed towards Th1-polarization whereas IL-9 producing (Th9) $CD4^+$ T-cells were absent. In contrast to normal hematopoietic stem cells (HSCs), LSCs produced IL-9 and the correlation modelling predicted *IL9* in LSCs as a main hub-gene that activates $CD4^+$ T-cells in AML. Functional validation revealed that IL-9R signaling in $CD4^+$ T-cells leads to activation of the JAK-STAT pathway that induces the upregulation of *KMT2A*, *KMT2C* genes resulting in methylation on histone H3 at lysine 4 (H3K4) to promote genome accessibility and transcriptional activation. This induced Th1-skewing, proliferation and effector cytokine secretion, including interferon (IFN)-ɣ and tumor necrosis factor (TNF)-α. IFN-ɣ and to a lesser extend TNF-α produced by activated $CD4^+$ T-cells, induced the expansion of LSCs. In accordance with our findings, high *IL9* expression in LSCs and high *IL9R*, *TNF* and *IFNG* expression in BM-infiltrating $CD4^+$ T-cells correlated with worse overall survival in AML. Thus, IL-9 secreted by AML LSCs shapes a Th1-skewed immune environment that promotes their expansion by secreting IFN-ɣ and TNF-α.

Keywords: Acute myeloid leukemia, AML, leukemia stem cells, LSC, $CD4^+$ T-cells, Th1-differentiation, histone methylation, IFN-ɣ, TNF-α.

Key Points (Each Key Point should be no more than 140 characters, including spaces):

- IL-9 secreted by AML LSCs epigenetically activates $CD4^+$ T-cells and induces Th1-skewing.
- IFN-ɣ and TNF-α produced by activated $CD4^+$ T-cells expand LSCs.

Introduction

Acute myeloid leukemia (AML) is a clonal disease of hematopoietic stem and progenitor cells (HSPCs) which is characterized by a maturation arrest, expansion and abnormal proliferation of undifferentiated progenitor cells and myeloid blasts.[1,2] According to the molecular and cytogenetic profile, AML is grouped into adverse, intermediate and favorable risk categories.[3,4]

Leukemia stem cells (LSCs) have the capacity of self-renewal, are responsible for the disease initiation, propagation and resistance to chemotherapy.[5-10] Similar to hematopoietic stem cells (HSCs), LSCs interact with stromal, endothelial, and immune cells in the bone marrow (BM) microenvironment, that form the niche. Niche cells regulate HSC and LSC homing, quiescence, self-renewal and differentiation.[11]

The BM acts as a primary and a secondary lymphoid organ. Thus, lymphocytes are an important part of the BM environment.[12] In contrast to peripheral blood, the percentage of $CD8^+$ T-cells in the BM is slightly higher than that of $CD4^+$ T-cells.[13,14] Regulatory T-cells (Tregs) in the BM preserve normal hematopoiesis and provide an immune privileged niche for HSCs.[15-17] Sixty percent of $CD8^+$ and $CD4^+$ T-cells are naive, whereas the remainders are memory T-cells.[18] In addition, $CD4^+$ T-cells with Th2-polarization produce many cytokines with important functions in hematopoiesis such as IL-3, IL4, IL-6 and GM-CSF.[19,20] $CD4^+$ and $CD8^+$ T-cells have an important role in hematopoiesis after BM transplantation.[21] T-cell deficient mice have a block in the differentiation of myeloid cells that is restored after transfer of $CD4^+$, but not $CD8^+$ T-cells.[22] During immune activation such as an infection, autoimmunity or cancer, the differentiation and cytokine production of immune cells may change leading to the expansion of Th1-cells that produce IFN-ɣ and TNF-α.[19]

Activated $CD8^+$ T-cells and natural killer (NK) cells have the potential to eliminate AML blasts.[23,24] However, LSCs efficiently avoid the elimination by the immune system through co-localization with Tregs, the expression of immune-inhibitory molecules or downregulation of immunological recognition pathways.[25,26] In contrast to direct cytotoxic effects, activated T-cells produce cytokines such as IL-3, IL-6, IFN-ɣ and TNF-α that induce the expansion of leukemia stem cells.[27-33]

To study the interaction of AML LSCs and LPCs with BM-infiltrating $CD4^+$ and $CD8^+$ T-cells, we performed a comprehensive transcriptomic profiling and unbiased high-throughput correlation network analysis. We recently reported that activated $CD8^+$ T-cells induce the expansion of LSCs by stimulating the production of cytokines, particularly in favorable risk AML.[33] In the present study, we analyzed the interaction of $CD4^+$ T-cells with LSCs/LPCs and $CD8^+$ T-cells. Transcriptomic analysis of $CD4^+$ T-cells in AML revealed an activation of immune-related signaling pathways with skewing towards Th1-polarization and lack of the Th9-immunophenotype. The correlation network analysis identified *IL9* as a crucial hub-gene in AML LSCs regulating the differentiation, activation and proliferation of BM-infiltrating $CD4^+$ T-cells. Functional studies validated that IL-9 secreted by AML LSCs epigenetically activates $CD4^+$ T-cells via JAK/STAT pathway and histone methylation. This induced the expression of the transcription factor T-bet (*TBX21*) leading to Th1-differentiation and secretion of IFN-ɣ and TNF-α. These cytokines expanded LSCs *in vitro* and were associated with worse overall survival in AML patients. Thus, LSCs

shape their surrounding immune-microenvironment by producing IL-9. Increased Th1-cytokines, particularly IFN-ɣ, cause LSC proliferation and expansion.

Materials and methods

Patients and study cohorts

BM aspirates and blood samples from AML patients were collected at the Department of Medical Oncology, University Hospital Bern and the University of Texas MD Anderson Cancer Center. The cantonal ethical committee and the institutional review board at MD Anderson approved the study protocol (KEK 122/14 and 2019-01627). Study participation required a written informed consent. AML risk classification was conducted according to the guidelines of European Leukemia Network (ELN) in 2012[3,34] and updated guidelines in 2022.[4] AML risk categories and immune-phenotype of the AML samples are shown in Supplemental Table 1. The research followed the Helsinki Declaration.

A detailed description of materials and methods is in the Supplemental Data.

Results

BM-infiltrating CD4$^+$ T-cells are skewed towards Th1-polarization.

We characterized the transcriptome of fluorescence-activated cell sorting (FACS)-purified CD4$^+$ and CD8$^+$ T-cells, LSCs and LPCs in the BM of AML patients at the time point of diagnosis.[33] HSCs, HPCs and CD4$^+$ and CD8$^+$ BM T-cells from healthy individuals served as controls (Supplemental Figure 1A, Supplemental Table 1). The gene expression of CD4$^+$ T-cells from AML patients differed from healthy controls and was more heterogeneous (Figure 1A). In total, 386 genes were differentially expressed in BM-infiltrating CD4$^+$ T-cells from AML patients compared to healthy donors (Figure 1B, Supplemental Dataset 1). Gene ontology (GO) analysis revealed that the 154 downregulated genes regulated TOR signaling, antigen presentation via MHC-I or IL-9 production (Supplemental Figure 1B). The 232 upregulated genes were related to T-cell activation, differentiation, IFN-ɣ and TNF-α production, regulation of JAK-STAT signaling and kinase activity, positive regulation of gene expression and cytokine/chemokine signaling (Supplemental Figure 1C). This indicated an activated and inflammatory CD4$^+$ T-cell compartment in the BM of AML patients. Pathway enrichment analysis revealed that important cellular pathways such as HEDGEHOG, JAK-STAT, MAPK-ERK, canonical NF-κB, TGF-BETA and canonical WNT (beta catenin) pathways were activated in CD4$^+$ T-cells from AML patients (Figure 1C). These pathways are involved in the differentiation and activation of T-cells.[35] Additionally, the gene signatures for cytokine signaling (including IL-6, and IL-9 mediated signaling), inflammation pathway, TNF signaling, T-cell receptor signaling and regulation of T-cell activation/proliferation/differentiation were significantly upregulated in AML CD4$^+$ T-cells (Figure 1C). In contrast, most intracellular signaling pathways and immune-related gene signatures were downregulated in AML-derived CD8$^+$ T-cells, consistent with the previously described silenced transcriptomic profile in CD8$^+$ T-cells from AML patients.[33]

According to their cytokine profile and the expression of specific transcription factors, CD4$^+$ T-cells can be grouped in different subsets[36]. BM-derived AML CD4$^+$ T-cells had skewing towards a Th1-immune-phenotype and absence of Th9-polarized CD4$^+$ T-cells in all risk categories (Figure 1D). Single cell RNA sequencing (scRNA-seq) analysis of an independent cohort of AML patients revealed a significantly higher expression of the genes encoding for the Th1 transcription factor, *TBX21,* and for the Th1 cytokines, *IFNG* and *TNF,* in CD4$^+$ T-cells from AML patients (Figure 1E). The scRNA-seq analysis revealed that the frequency of Th1-polarized CD4$^+$ T cells increased approximately 3-fold compared to HD. In contrast, the frequency of CD4$^+$ Treg cells in AML increased only marginally. This results in a substantially higher Th1/Treg ratio, with approximately 35% Th1-polarized CD4$^+$ T-cells and 5% CD4$^+$ Treg cells in AML BM (Figure 1F). These data indicate that BM-infiltrating CD4$^+$ T-cell in AML are activated and preferentially differentiated to a pro-inflammatory Th1-phenotype.

Correlation network analysis identifies hub-genes in LSCs that regulate target genes in BM-infiltrating CD4$^+$ T-cells

To study possible interactions of CD4$^+$ T-cells with paired AML LSCs, LPCs and CD8$^+$ T-cells in the BM, we conducted an unbiased comprehensive correlation network analysis. Within all mapped networks, a

node was defined as a gene expressed in any of the studied cell populations, and a hub-gene correlated significantly with more than 15 different genes in the other cell type (Supplemental Figure 2A). Three categories of correlation networks were mapped: 1) 'appear', a correlation was present in AML and was absent in controls, 2) 'disappear', a correlation was detected in controls and not in AML, and 3) 'flip', where the sign of the correlation changed.

Most hub-genes and nodes were detected in the appear networks (Figure 2A). Since we recently described that AML LSCs with adverse-risk constellation are less dependent on the interaction with $CD8^+$ T-cells than LSCs from favorable or intermediate risk patients, we analyzed the correlation network for $CD4^+$ T-cells in different prognostic groups (Supplemental Dataset 2). Independent of AML risk groups, the highest number of hub-genes was detected in the appear network in $CD4^+$ T-cells and LSCs (Figure 2B, Supplemental Figure 2B**).** All hub-genes identified in LSCs correlated with target genes in $CD4^+$ T-cells, but not vice versa (Supplemental Dataset 2).

These observations indicate that the detected hub-genes in LSCs were involved in the regulation/activation of lymphocytes. *IL9* is an important growth factor for different immune cells including T-cells, *IFI30* is involved in antigen-processing and *CD1C* in antigen-presentation.[37,38] In contrast, the correlation networks in $CD4^+$ T-cells with LPCs or $CD8^+$ T-cells suggested a bidirectional communication as hubs occur in both cell populations and the number of correlating genes was more balanced (Figure 2C). Overall, the network analysis suggests that LSCs regulate $CD4^+$ T-cells that interact with LPCs and $CD8^+$ T-cells.

IL-9 expands LSCs in an autocrine loop

To study the role of IL-9 produced by LSCs, we quantified the IL-9 concentrations in the BM-fluid of AML patients and healthy donors. IL-9 levels were very low in healthy controls, but significantly increased in AML patients (Figure 3A). IL-9 protein was only detected in the supernatant of FACS-purified LSCs but not of $CD4^+$ or $CD8^+$ T-cells from AML patents after 48h *in vitro* culture (Figure 3B). This is consistent with the absence of *IL9* mRNA expressing $CD4^+$ T-cells in AML (Th9-cells; Figure 1D). In healthy individuals, $CD4^+$ T-cells secreted IL-9 while normal HSCs did not (Figure 3B). In AML patients, the *IL9* gene was expressed similarly in French-American-British (FAB) and in molecular subtypes (Supplemental Figure 3A-B). Interferon regulatory factor 4 (*IRF4*) expression, the main transcriptional regulator of the *IL9* gene, was significantly higher in AML LSCs than in healthy HSCs. In contrast, AML $CD4^+$ T-cells expressed significantly less *IRF4* than $CD4^+$ T-cells from healthy controls (Figure 3C). *IRF4* was similarly expressed in different AML subtypes according to FAB classification (Supplemental Figure 3A).

The IL-9 receptor (IL-9R) gene and protein expression was significantly increased in AML LSCs and $CD4^+$ T-cells (Figure 3D-E, Supplemental Figure 3C). Two *IL9R* protein-coding splice variants have been identified (Supplemental Figure 3D). Only the gene expression of long isoform was increased in AML and correlated with IL-9R protein expression (Supplemental Figure 3E). AML cells in BM can be hierarchically classified as primitive-, granulocyte macrophage progenitors (GMP)-, or differentiated-like

cells, whereas cells in the immune-niche include T-, B-, NK-cells and monocytes (Figure 3F).[39] The scRNA-seq analysis of BM cells from an independent cohort of AML patients revealed that primitive-like AML cells, representing LSCs, had the highest expression levels of *IL9* signaling genes compared to more differentiated AML cells (Figure 3G). In BM-infiltrating immune cells of AML patients and healthy individuals, T-cells showed the highest expression of *IL9* signaling genes, followed by NK-cells (Figure 3G). Within AML $CD4^+$ T cells, *IL9* signaling was most active in Th1/Th2 differentiated subtypes, while in healthy $CD4^+$ T cells, it was primarily active in Th2/Th17 skewed T-cells (Figure 3H). Further analysis of *IL9* gene expression in FACS-purified cell populations confirmed that it is predominantly expressed in LSCs/LPCs in AML BM and $CD4^+$ T-cells in HD BM (Figure 3I).

As previously reported, IL-9 stimulated the proliferating capacity of AML LSCs.[40] Pre-incubation with anti-IL-9 neutralizing antibody (αIL-9) reduced the colony forming capacity of LSCs but not HSCs (Figure 3J-K). In contrast, the capacity of LSCs to form colonies in secondary plantings in the absence of αIL-9 Ab was not impaired. This indicates that blocking IL-9/IL-9R signaling before plating AML LSCs in methylcellulose did reduce the number of stem cells but not their function to form colonies in secondary platings. Since AML LSCs produce IL-9 endogenously, additional short/long-term treatment with rhIL-9 did not increase their colony forming capacity (Supplemental Figure 3F-G). These results indicated that IL-9 secreted by LSCs induced their expansion in an autocrine loop.

_IL-9_ expression in AML LSCs correlates with the expression of histone lysine methyltransferase genes in $CD4^+$ T-cells.

IL9 was identified as a potential hub-gene in LSCs of AML patients, regulating BM-infiltrating $CD4^+$ T-cells (Figure 2C). Additionally, gene set enrichment analysis (GSEA) revealed that IL-9R signaling is similarly active in $CD4^+$ T-cells from AML patients across all molecular risk groups (Figure 4A). This data suggests that paracrine IL-9/IL-9R signaling may regulate $CD4^+$ T-cell function independently of the molecular risk groups.

The correlation network modeling identified 88-target genes in $CD4^+$ T-cells that correlated with *IL9* expression in LSCs (Supplemental Table 4). These 88-target genes positively regulate gene expression, histone methylation (specifically H3 lysine-4 methylation), cell cycle and cell growth, response to TNF and negatively regulate adaptive immune responses (Figure 4B). Eight of these 88-genes were part of two or more GO-predicted biological pathways (Figure 4C).

Two members of KMT2 complex, *KMT2A/C,* were associated with five identified biological pathways, including positive regulation of gene expression and histone methylation. Since, the KMT2 complex has core family members (KMT2A-E),[41] we examined their gene expression in $CD4^+$ T-cells from different AML risk groups. *KMT2A and C, but not KMT2B, D or E* genes were expressed at significantly higher levels in $CD4^+$ T-cells of AML patients (Figure 4D, Supplemental Datasets 1). We observed a significant positive correlation between *KMT2A and KMT2C* gene expression in AML $CD4^+$ T-cells, but not for other KMT2 complex genes (Figure 4E). This data suggests that IL-9 secreted by LSCs activates $CD4^+$ T-cells by regulating *KMT2A/C*.

IL-9 secreted by AML LSCs activates BM-infiltrating CD4$^+$ T-cells.

To investigate the role of IL-9 signaling on BM-infiltrating CD4$^+$ T-cells in AML patients, we stimulated FACS-purified CD4$^+$ T-cells with rhIL-9 or co-cultured them with paired LSCs and performed RNA-seq transcriptomic analysis. CD4$^+$ T-cells stimulated with rhIL-9 or co-cultured with paired LSCs had distinct gene expression patterns from untreated or co-cultured CD4$^+$ T-cells with paired LSCs and IL-9 neutralization antibody (αIL-9) (Figure 5A). *IL9R* but not *IL9* was highly expressed in purified CD4$^+$ T-cells from AML patents (Supplemental Figure 4A). Both, rhIL-9 and LSC co-culture activated *IL9* signaling in CD4$^+$ T-cells (Supplemental Figure 4B). After stimulation with rhIL-9, 1073-genes were upregulated and 81-genes were downregulated (Fig 5B, Supplemental Dataset 3). GSEA indicated a positive enrichment of T-cell activation, proliferation and differentiation in rhIL-9 stimulated CD4$^+$ T-cells from AML patients (Figure 5C).

To analyze whether co-culture of CD4$^+$ T-cells with paired LSCs induced IL-9/IL-9R signaling, we defined an IL-9 stimulated_up signature, including the identified 1073-genes upregulated upon rhIL-9-stimulation (Figure 5B). GSEA analysis of CD4$^+$ T-cells co-cultured with paired LSCs revealed a significant enrichment for this IL-9 stimulated_up gene signature compared to both untreated or CD4$^+$ T-cells co-cultured with LSCs in the presence of αIL-9, indicating that LSCs regulate CD4$^+$ T-cells through IL-9/IL-9R signaling (Figure 5D). The expression profile of key cytokine/transcription factor genes for Th-polarization in IL-9-stimulated CD4$^+$ T-cells or co-cultured with AML LSCs, indicated a skewing towards Th1-immune-phenotype (Figure 5E). To determine whether the observed changes in gene expression reflect the situation in AML patients, we created a signature of the 386 DE-genes in CD4$^+$ T cells in AML patients vs. healthy controls, as shown in Figure 1B. This signature was then assessed in CD4$^+$ T-cells treated with rhIL-9 or co-cultured with LSCs, in comparison to untreated CD4$^+$ T-cells. Our analysis revealed a significant positive enrichment of these DE genes, implying that activating CD4$^+$ T-cells with IL-9 or co-culture with LSCs may induce a transcriptomic phenotype similar to that initially observed in AML CD4$^+$ T-cells (Figure 5F). Incubation with hIL-9 and co-culture with LSCs resulted in a significant upregulation of *KMT2A*/C/E, *JAK1*/2, *STAT1*/3/5B, *TNF, IFNG* and the Th1-transcription factor (*TBX21*) (Supplemental Figure 4C, Supplemental Dataset 3). GO analysis of upregulated intersection genes revealed a positive regulation of transcription, histone methylation, JAK-STAT and cytokine signaling (e.g., IFN-ɣ, TNF-α, IL-1 and IL-6 production) (Supplemental Figure 4D).

Next, we quantified 12-key cytokines in the culture supernatant of CD4$^+$ T-cells upon stimulation with rhIL-9 or co-cultured with paired LSCs *in vitro*. CD4$^+$ T-cells stimulated with rhIL-9 or co-culture with LSCs produced more IFN-ɣ and TNF-α (Figure 5G, Supplemental Figure 4E). In addition, the concentration of IFN-ɣ and TNF-α was significantly higher in the BM-fluid of AML patients than healthy donors (Figure 5H).

Taken together, IL-9 secretion by LSCs activates BM-infiltrating CD4$^+$ T-cells and induces a Th1-skewing with the production of IFN-ɣ and TNF-α.

AML LSC-secreted IL-9 activated CD4$^+$ T-cells through JAK-STAT signaling and histone H3 methylation.

IL-9 stimulation and co-culture with LSCs induced signaling pathways in CD4$^+$ T-cells that are associated with positive regulation of gene expression, histone methylation and JAK-STAT signaling (Supplemental Figure 4D). We identified the top 10-genes involved in $\geq$4 of these pathways. *KMT2A/C/E* genes were significantly upregulated in CD4$^+$ T-cells and associated with positive regulation of gene expression, histone methylation as well as transcription factor and promoter-specific chromatin binding (Figure 6A). GSEA comparing the transcriptome of CD4$^+$ T-cells incubated with or without IL-9 revealed a significant positive enrichment for the gene expression patterns regulating histone H3 lysine-4 (H3-K4) and histone H3 lysine-27 (H3-K27) methylations (Figure 6B). A similar enrichment pattern was identified in CD4$^+$ T-cells co-incubated with LSCs in the presence or absence of αIL-9 mAbs (Figure 6B). IL-9 induced di- and trimethylation H3-K4 signatures (Supplemental Figure 5A). To validate these findings, we quantified 21-diverse histone H3 modifications in purified histones from AML CD4$^+$ T-cells after *in vitro* IL-9 stimulation or LSC co-culture. In both conditions, the levels of di- and trimethylation on H3-K4 and H3-K27 significantly increased, with H3-K4 methylation showing particularly pronounced enhancement (Figure 6C, Supplemental Table 4).

IL-9R signaling is mainly mediated through the JAK/STAT pathway.[42] In accordance, an *in silico* pathway analysis predicted that IL-9/IL-9R signaling triggers the expression of *KMT2A/C* mainly through JAK-STAT signaling (Figure 6D). GSEA revealed a significant positive enrichment of JAK-STAT and NF-κB signaling in IL-9 stimulated or LSC co-cultured CD4$^+$ T-cells (Supplemental Figure 5B-C).

To confirm the transcriptomic finding, we individually analyzed the gene expression profile of 5-members of the KMT2 complex and key genes of JAK-STAT, Phospho Erk, canonical NF-κB signaling pathways as well as *TBX21*, *IFNG* and *TNF* in CD4$^+$ T-cell from AML patients or healthy donors upon IL-9 stimulation or co-cultured with LSCs. Stimulation with IL-9 or co-culture with AML LSCs significantly increased the expression of *KMT2A*/*C/E,* JAK-STAT, canonical NF-κB signaling, *TBX21*, *IFNG* and *TNF* in CD4$^+$ T-cell (Figure 6E). Similarly, cell culture supernatant from AML LSCs or LPCs induced the expression of those genes in AML CD4$^+$ T-cells, indicating that a soluble factor is mediating the observed effect (Supplemental Figure 5D). Given that healthy CD4$^+$ T-cells produce IL-9 at high concentrations (Figure 3B), neither treatment with rhIL-9 nor co-culture with AML LSCs increased the expression of crucial target genes (Figure 6E). In contrast, αIL-9 treatment reduced the expression of those target genes. Thus, IL-9 produced by LSCs does not regulate gene expression in healthy CD4$^+$ T-cells, because these cells already produce high amounts of IL-9.

We functionally validated the predicted effect of IL-9 signaling on the JAK-STAT pathway and histone methylation on CD4$^+$ T-cell activation and proliferation. Incubation of CD4$^+$ T-cells from primary AML with rhIL-9 increased the expression of *KMT2A*/C/E, genes of the JAK-STAT and the canonical NF-κB signaling pathways as well as *TBX21*, *IFNG* and *TNF*. Treatment with si*KMT2A*, si*KMT2C* but not si*KMT2E* prevented the increase in NF-κB related genes, *TBX21*, *IFNG* and *TNF* (Figure 6F). Treatment with the JAK1/JAK2 inhibitor, ruxolitinib, reduced the expression of *KMT2A/C* but not *KMT2E,* as well as the NF-κB related genes, *TNX21*, *IFNG* and *TNF* (Figure 6G). In contrast, treatment with the NF-κB

inhibitor, BAY 11-7082, only prevented the IL-9 induced upregulation of *IFNG* and *TNF* without affecting the JAK-STAT pathway or the expression of *TBX21* or KMT2-complex genes (Figure 6G). Furthermore, rhIL9 induced the proliferation and replication of $CD4^+$ T-cells from AML patients (Figure 6H, I). KMT2A/C knockdown and treatment with ruxolitinib or BAY 11-7082 reduced the IL-9-induced proliferation of $CD4^+$ T-cells.

To confirm the possibility of histone H3 lysine methylation at the promoter region of *TNF*, *IFNG* and *TBX21* genes, we examined the ChIP-seq histone methylation data of $CD4^+$ T-cells in the Roadmap Epigenomics Mapping Database. H3K4me3 peaks were detected at the promoter region of *TNF*, *IFNG* and *TBX21* genes (Supplemental Figure 6A-C). This suggests that histone H3 lysine methylation (especially at H3K4me3) may regulate the gene expression of *TNF*, *IFNG*, and *TBX21* in $CD4^+$ T-cells. To functionally validate the impact of H3K4me3 on promoter accessibility and the increased expression of target genes, we assessed H3K4me3 occupancy at the promoter regions of NF-κB targets (*NFKB1* and *RELA*), *TBX21, IFNG,* and *TNF* genes in $CD4^+$ T-cells from AML patients in the presence or absence of rhIL-9, using ChIP-quantitative real-time (q)PCR. H3K4me3 occupancy at these promoter regions was observed exclusively in IL-9-stimulated $CD4^+$ T-cells, while it was absent in untreated cells (Figure 6J).

In summary, IL-9/IL-9R signaling activates JAK-STAT signaling, leading to the upregulation of *KMT2A/C* in $CD4^+$ T-cells. This, results in H3 lysine-4 methylation, which activates $CD4^+$ T-cells through *TBX21* and NF-κB signaling, promoting cell proliferation and cytokine production.

TNF-α and IFN-ɣ secreted by activated $CD4^+$ T-cells expand LSCs and correlate with disease progression.

To investigate the functional role of TNF-α and IFN-ɣ produced by AML $CD4^+$ T-cells on LSCs, we cultured primary AML LSCs with rhTNF-α or rhIFN-ɣ and performed a colony-forming and re-plating assay. Treatment with rhTNF-α or rhIFN-ɣ significantly increased colony formation in the first plating. LSCs stimulated with rhTNF-α did not show a significant increase in colony formation in the secondary platings, whereas the number of rhIFN-ɣ stimulated colonies even increased (Figure 7A). This indicates TNF-α and IFN-ɣ expanded LSCs that retained the capacity of self-renewal in re-platings. Our findings were confirmed by culturing primary LSCs with the supernatant of IL-9-activated $CD4^+$ T-cells with or without αTNF-α or αIFN-ɣ neutralization antibodies. The addition of $CD4^+$ T-cell supernatant increased colony forming capability by 3-fold and the expanded LSCs maintained their capacity to from colonies in secondary re-platings. Treatment with αIFN-ɣ antibody reduced the colony numbers to levels of non-treated LSCs. In contrast, the addition of αTNF-α antibody did not significantly reduce the colony forming capacity (Figure 7B). The results indicate that although both effector cytokines have the potential to expand LSCs *in vitro*, $CD4^+$ T-cells mainly expand LSCs by secreting IFN-ɣ. This may be due to the fact that the level of TNF-α produced by $CD4^+$ T-cells in co-cultures with LSCs is rather low (Figure 5G).

To investigate the prognostic value of IL-9/IL-9R signaling on overall survival, we used 3-independent datasets containing gene expression values of non-fractionated BM cells from AML patients (HOVON-SAKK dataset: E-MTAB-3444[43]; NL-Valk dataset: GSE6891[44,45]; Metzeler dataset: GSE12417[46]). AML

patients with lower expression of *IL9* or *IL9R* had a significantly better overall survival in all three datasets (Figure 7C, Supplemental Figure 7). Although the prognostic value of *IL9* and *IL9R* in total BM cells in these large datasets is consistent with our hypothesis, it does not allow concluding on the role of IL-9/IL-9R signaling in $CD4^+$T-cells. Thus, we examined the prognostic value of *IL9* and *IL9R* in purified AML LSCs and $CD4^+$ T-cells (Figure 7D). The expression of *IL9* in LSCs and *IL9R* in $CD4^+$ T-cells correlated with worse overall survival, although not significant due to the small sample size. In addition, LSCs in the *IL9*-high group patients had a significant higher expression of 16 LSC-signature genes than patients in the *IL9*-low group (Figure 7E-F). Although IFN-ɣ secreted by $CD4^+$ T-cells from AML patients expands LSCs *in vitro*, we did not detect *IFNG* as a hub-gene in $CD4^+$ T-cells in our network modeling (Figure 2). However, a positive correlation between *TNF* and *IFNG* expression in $CD4^+$ T-cells with 16 LSC-signature genes in purified AML LSC suggests that $CD4^+$ T-cells regulate LSCs through IFN-ɣ and TNF-α secretion (Figure 7G).

Since IL-9/IL-9R signaling in AML $CD4^+$ T-cells increases the secretion of IFN-ɣ and TNF-α, we investigated their correlation with prognosis. Higher *TNF* and *IFNG* expression in purified $CD4^+$ T-cells correlated significantly with worse outcome (Figure 7H). Taken together, LSC induce $CD4^+$ T-cells to secrete IFN-ɣ and TNF-α that induces their proliferation and expansion.

Discussion

The immune-microenvironment plays a crucial role in leukemia development, progression and control. For solid tumors, it is well documented that cancer cells shape their immune microenvironment. In contrast to solid tumors, LSCs develop and expand in the BM within a secondary lymphoid structure, in close proximity to naïve and memory lymphocytes.[47] $CD8^+$ and $CD4^+$ T-cells contribute to the immunosurveillance, recognize leukemia associated antigens and lyse leukemia cells.[47] Nevertheless, $CD8^+$ T-cells are often dysfunctional in myeloid leukemia.[33,48] Furthermore, many $CD4^+$ T-cell subsets with distinct functions on leukemia cells have been described.[49] $CD4^+$ Tregs are important part of the HSC niche and their frequency has been shown to increase in leukemia.[50] They frequently cluster with $CD8^+$ T-cells in the BM. They have a main function in the immune escape of LSC by inhibiting adaptive immune responses.[50,51] Increased numbers of BM $CD4^+$ Tregs in AML correlate with poor prognosis.[17] Although the BM contains all cytokine-producing subsets (Th1, Th2, Th9, Th17 and Th22), their roles in AML development remain poorly understood. Our gene expression analysis of BM-infiltrating $CD4^+$ T-cells in AML identified a skewing towards the pro-inflammatory Th1-subset, while Th9-cells were notably absent. We also found an increase in Treg cells in AML, but the expansion of the Th1-subset was much more pronounced, resulting in a higher proportion of inflammatory Th1-cells compared to immunosuppressive Tregs.

IL-9 is an essential cytokine for $CD4^+$ T-cell proliferation and function.[52] IL-9 is produced by a variety of different cells including mast cells, NK cells, T-cells, ILCs and different Th-subsets including specialized Th9-cells.[53] In AML, *IL9* gene expression was absent in $CD4^+$ T-cells. In contrast, IL-9 was produced by AML LSCs independent of molecular risk groups, genetic aberrations or FAB classification. Due to the high number of LSPCs, IL-9 concentrations in the BM fluid of AML patients were significantly elevated. IL-9 is an important growth factor not only for immune cells but also for malignant cells including AML. Thus, IL-9 promotes LSC expansion and proliferation in an autocrine loop.

Our correlation network analysis and the functional validation revealed that AML LSCs secrete IL-9 to regulate $CD4^+$ T-cell skewing, proliferation, activation and cytokine production. IL-9 activates and expands T-helper cells.[54] Furthermore, IL-9 suppresses the immunological response to cancer cells by enhancing the function of $Foxp3^+$ Tregs, or the growth of myeloid-derived suppressor cells (MDSCs).[55-57] Our study now indicates that IL-9/IL-9R signaling in AML induces T-bet expression, which differentiates and expands Th1-cells. In addition, *IL9* signaling genes were also expressed in Th2- and NK-cells in the BM of AML patients. This suggests that different immune cells are dependent on the secretion of the growth factor IL-9 by LSCs.

We confirmed that IL-9R signaling activates the Jak-STAT pathway and increased *STAT1/3/5B* expression. STAT1 is critical for Th1-polarization, whereas STAT3 induces RORγt and Th17-differentiation. Given the lack of Th1-cytokines and the specific transcription factor, T-bet, in the BM of healthy individuals, it is likely that in AML naïve $CD4^+$ T-cells are activated and skewed towards a Th1-phenotype. Inhibiting JAK-STAT signaling blocked *TBX21* expression, indicating that IL9/IL9R signaling affects Th1-skewing. In addition, IL-9 may also induce the proliferation of already differentiated Th-subsets.

IL-9 expression in LSCs correlates with the expression of genes involved in histone methylation (*KMT2A/C*) in $CD4^+$ T-cells. Histone methylation regulates $CD4^+$ T-cell growth, function, selection and maturation.[58-60] Methylation of histone H3 at lysine 4 (H3K4me) increases gene expression, activation and T-cell differentiation into effector cells.[59] Histone H3 lysine 27 methylation (H3K27me) induces the development of Tregs.[61,62] We showed that IL-9/IL-9R signaling in $CD4^+$ T-cells of AML patients increased histone methylation, particularly at H3K4, leading to $CD4^+$ T-cell activation. It has been shown that histone methylation is regulated by JAK/STAT signaling during $CD4^+$ T-cell activation.[61] Accordingly, inhibiting JAK/STAT reduced *KMT2A*/*C* expression, but not *KMT2E*, as well as $CD4^+$ T-cell activation and proliferation, *TBX21*, *TNF* and *IFNG* expression. Moreover, silencing *KMT2A*/*C*, but not *KMT2E* reduced $CD4^+$ T-cell activation and proliferation, as well as *TNF* and *IFNG* expression. This indicates that IL-9 induces IFN-ɣ and TNF-α production in $CD4^+$ T-cells by regulating *KMT2A/C* histone methylation genes.

IFN-ɣ and TNF-α are key inflammatory cytokines secreted by activated $CD4^+$ Th1-cells.[57,63] These cytokines stimulate inflammation, recruit immune cells, and alter the tumor microenvironment in cancer.[58] IFN-ɣ and TNF-α may boost the immune response to cancer cells.[64,65] In addition, IFN-ɣ upregulates MHC-I and II.[66] For example, Flotetuzumab and CAR-T-cells targeting surface antigens on AML cells, upregulate MHC-II expression on AML cells by increasing IFN-ɣ secretion leading to improved graft-versus leukemia effects post-transplantation.[67] IFN-ɣ also induces immunosuppression by promoting the synthesis of indoleamine-2,3-dioxygenase (IDO) and immune checkpoint inhibitory molecules.[68,69] We found that CTL-secreted IFN-ɣ promotes disease progression by expanding LSCs.[70] The effect of IFN-ɣ on LSCs might be dose-dependent. Low-doses of IFN-ɣ promote AML LSC self-renewal and disease progression, while high-doses have anti-AML effects.[71] We now documented that the IFN-ɣ secreted by IL-9-activated $CD4^+$ T-cells expand LSCs.

Elevated IL-9 levels in the blood have been associated with poor prognosis therapy outcomes in lymphoma and leukemia.[72-74] We found that patients with lower *IL9* or *IL9R* expression in non-fractionated BM cells had a significantly improved overall survival. In addition, *IL9* expression in purified LSCs as well as *IFNG* and *TNF* expression in purified BM-infiltrating $CD4^+$ T-cells predicted poor overall survival in our patient cohort. Thus, IL-9 secreted by AML LSCs activates $CD4^+$ T-cells in the BM and skews them towards pro-inflammatory Th-subsets, mainly Th1. Activated $CD4^+$ T-cells secrete IFN-ɣ and TNF-α, expand LSCs and correlate with poor prognosis. The autocrine and paracrine effects of IL-9 secreted by LSCs may enable a novel therapeutic approach by blocking IL-9R signaling.

Additional information

The complete list of differentially expressed genes in $CD4^+$ T-cells from AML patients versus healthy controls is shown in Supplemental Datasets 1. The correlation analysis including the list of predicted hub-genes and their correlations (edges) are listed in Supplemental Dataset 2. The complete list of differentially expressed genes from *in vitro* stimulated $CD4^+$ T-cells is shown as Supplemental Datasets 3.

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Authorship

Contribution: R.R. designed and performed experiments, analyzed and interpreted data and wrote the manuscript. C.S., B.W. and H.A.A. analyzed the data. C.R. designed experiments and analyzed data. A.F.O. designed experiments, wrote the manuscript and supervised the project.

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Figure legends

Figure 1. **The transcriptomic analysis of BM-infiltrating CD4$^+$ T-cells in AML indicates T-cell activation and Th1-polarization. A)** PCA based on the transcriptomic profile of BM-infiltrating CD4$^+$ T-cells from AML patients (n=30) and controls (n=7). **B**) Heatmap illustrating differentially expressed genes in CD4$^+$ T-cells (AML vs. Ctrl). **C**) Balloon plots illustrating significantly up/downregulated pathways (left panel) or crucial immune-related signaling (right panel) within CD4$^+$ and CD8$^+$ T-cells based on gene set enrichment analysis (GSEA) and normalized enrichment scores (NES). **D)** Heatmap illustrating the expression profile of key genes and transcription factors regulating CD4$^+$ T-cell polarization (Th1, Th2, Th9, Th17, Th22 and Tregs). **E)** Gene expression analysis of *IFNG*, *TNF* and *TBX21* in BM-infiltrating CD4$^+$ T-cell from AML patients (n=20) and healthy donors (n=9) assessed by single cell RNA sequencing (scRNA-seq). **F)** Frequency of Th1 CD4$^+$ T-cells (*IFNG*, *TNF* or *TBX21* expressing CD4$^+$ T-cells) and Tregs (*FOXP3* expressing CD4$^+$ T-cells) from AML patients (n=20) and healthy donors (n=6) analyzed by scRNA-seq. Statistics: Two-sided Wilcoxon test (E-F).

Figure 2. Detection of hub-genes in different cell populations. A) Visualization of nodes (genes) in the AML appear network that have more than two connections. Node fill color indicates the cell population; node and label size indicate the node degree (number of correlations). Hub-genes are annotated with their gene name. **B)** Number of hub-genes in LSCs, LPCs, CD8$^+$ and CD4$^+$ T-cells for the appear network across AML risk groups. **E)** Summary of significantly correlated hub-genes in the different studied cell populations (LSCs, LPCs, CD8$^+$ and CD4$^+$ T-cells).

Figure 3. LSCs but not BM-infiltrating CD4$^+$ T-cells in AML produce IL-9 that expands LSCs *in vitro*.

A) Quantification of IL-9 protein in BM-fluid of AML patients (n=5) and healthy donors (n=12). BM-fluid from healthy donors was either collected from BM aspirates (n=7) or from orthopedic patients who underwent vertebroplasty (n=5). **B)** Quantification of IL-9 protein in culture supernatants of FACS-purified LSCs/HSCs, BM-infiltrating CD4$^+$ and CD8$^+$ T-cells (n=6 AML patients and 3 healthy donors (HD)). **C)** Gene expression levels of *IRF4* in HSCs/LSCs and CD4$^+$ T-cells (n=28-30 AML and 7 HD). Fold differences represented as AML vs. HD. **D)** Gene expression levels of different isoforms of *IL9R* in HSCs/LSCs and CD4$^+$ T-cells (n=9 AML and 5 HD). **E)** IL-9R protein quantification in LSCs/HSCs and CD4$^+$ T-cells (n=9 AML patients and 5 HD; ΔMFI: MFI αIL-9R – MFI isotype). **F)** Uniform manifold approximation and projection (UMAP) representation of primitive-, GMP- and differentiated-like cells as well as T-, B-, NK-cells and monocytes in the BM of AML patients (scRNA-seq; n=20 AML), or HSC, GMP, T-, B-, NK-cells and monocytes in the BM of HD (scRNA-seq; n=6 HD). **G)** The reactome *IL9* signaling signature, which includes nine genes (*IL9*, *IL9R*, *IL2RG*, *JAK1*, *JAK3*, *STAT1*, *STAT5A*, *STAT3*, and *STAT5B*) in various cell subsets studied in scRNA-seq for both AML and HD. It was scored using AUCell in R, which calculates the area under the curve of gene set enrichment scores for each cell. **H)** The reactome IL9 signaling signature in scRNA-seq analysis in different subtypes of CD4$^+$ T-cells in the BM of AML patients and HD (*TBX21* pos. Th1, *GATA3* pos. Th2, *RORC* pos. Th17, *FOXP3* pos.

Treg and *SELL/LEF1* pos. Naïve). **I)** *IL9* gene expression in FACS-purified BM-infiltrating LSC/HSC, LPC/HPC, $CD4^+$ T-cells, $CD8^+$ T-cells, $CD19^+$ B-cells, NK-cells, or $CD14^+$ monocytes assessed by qPCR (n = 4 AML and 3 HD). **J-K)** Colony forming assays using FACS-purified AML LSCs (I) or HSCs from healthy donors (HD, J), cultured overnight in the presence or absence of IL-9 neutralization antibody (αIL-9; 1µg/ml) before plating in methylcellulose in the primary plating. In secondary platings, rhIL-9 was not added (n=6 AML and 4 HD; each value indicates the average of three replicates of one individual patient sample). Statistics: Two-sided Wilcoxon test (G-H), two-tailed unpaired t-test (J-K). **P*<0.05, *****P*<0.0001.

Figure 4. IL-9 secreted by AML LSCs upregulates histone lysine methyltransferase genes in BM-infiltrating $CD4^+$ T-cells. A) GSEA of the *IL9* signaling signature in AML $CD4^+$ vs. CTRL $CD4^+$ T-cells within the entire cohort of AML samples (n=30) or in each respective risk group. **B)** GO analysis of the IL-9 regulated 88-target genes in $CD4^+$ T-cells. GO enrichment score of ≥3 indicates significant changes. **C)** Circus plot illustrating the top 8-genes involved in several of the dysregulated pathways in the GO analysis (4B) **D)** Heatmap illustrating the expression of important family members of Histone–Lysine N-Methyltransferase 2 (KMT2) complex in $CD4^+$ T-cell in the different AML risk groups. **E)** Correlation matrix of lysine methyltransferase complex genes in AML $CD4^+$ T-cell. Statistics: Pearson correlation.

Figure 5. IL-9 secreted by LSCs activates BM-infiltrating $CD4^+$ T-cells and induces Th1-skewing. A) PCA based on the transcriptomic profile of $CD4^+$ T-cells upon stimulation with rhIL-9 or untreated $CD4^+$ T-cells as controls as well as $CD4^+$ T-cells co-cultured with paired AML LSCs and $CD4^+$ T-cell co-cultured with AML LSCs together with neutralizing antibody for IL-9 (αIL-9) (n=3 biological replicates per condition). **B)** Heatmap of differentially expressed genes in $CD4^+$ T-cells + rhIL-9 vs. untreated $CD4^+$ T-cells. **C)** GSEA of T-cell activation, proliferation and differentiation-related gene signatures in $CD4^+$ T-cells + rhIL-9 vs. untreated $CD4^+$ T-cells. **D)** GSEA of the IL-9 stimulated_up signature (1073 genes, Figure 5C) in $CD4^+$ T-cells co-incubated with LSCs vs. untreated or $CD4^+$ T-cells co-culture with LSCs in the presence of neutralizing αIL-9 antibody. **E)** Heatmap illustrating the expression profile of key genes for $CD4^+$ Th-cell polarization. **F)** GSEA of AML CD4 vs. CTRL_DE gene signature (386 genes, Figure 1B) in $CD4^+$ T-cells treated with rhIL-9 or co-incubated with LSCs vs. untreated $CD4^+$ T-cells. **G)** Protein quantification of IFN-ɣ and TNF-α in supernatants of FACS-purified AML $CD4^+$ T-cells in the presence or absence of rhIL-9 and co-cultured with LSCs in the presence or absence of neutralizing αIL-9 antibody (n=3 AML patients). **H)** Protein quantification of IFN-ɣ and TNF-α in BM-fluid of AML patients (n=5) and healthy donors (n=12). Statistics: Two-tailed unpaired t-test. **P*<0.05, ***P*<0.01, *****P*<0.0001.

Figure 6. IL-9 secreted by AML LSCs activated $CD4^+$ T-cells and induced a Th1-phenotype through JAK-STAT signaling and histone H3 methylation. A) Circus plot illustrating the top 10-upregulated genes involved in several of the dysregulated pathways based on the GO analysis (Figure 5E) ($CD4^+$ T-cells + rhIL-9 vs. untreated $CD4^+$ T-cells). **B)** GSEA of H3-K4 and H3-K27 methylation gene signatures in $CD4^+$ T-cells treated with hIL9 vs. untreated and in $CD4^+$ T-cells co-incubated with LSCs

vs. $CD4^+$ T-cells co-cultured with LSCs in the presence of neutralizing αIL-9 antibody. **C)** Quantification of 21-histone H3 modifications (15-different lysine methylation, 4-different lysine acetylation and 2-different serine phosphorylation) in extracted histones from FACS-purified in the same experimental groups (n=3 biological replicates per condition). The dotted square lines represent significant changes. **D)** *In silico* prediction of *IL9* and *KMT2A* or *KMT2C* gene interactions (GeneMANIA database). **E)** Expression levels of crucial genes for lysine methyltransferases, JAK-STAT, Phospho Erk, canonical NF-κB signaling as well as *IFNG* and *TNF* genes assessed by qPCR (n=3 AML and 3 HD per condition). Fold differences in gene expression, as depicted in bar charts, were calculated in comparison to unstimulated, untreated $CD4^+$ T-cells. Corresponding heatmaps, illustrating the relative expression levels of the analyzed genes in individual samples. **F)** Heatmap demonstrating fold differences in gene expression for lysine methyltransferases, JAK-STAT, important genes of canonical NF-κB signaling, as well as *TBX21*, *IFNG* and *TNF* in $CD4^+$ T-cells from AML patients after gene silencing using si*KMT2A*, si*KMT2C* or si*KMT2E* in the presence or absence of rhIL-9. The fold changes in gene expression were calculated according to siCtrl treated $CD4^+$ T-cells without addition of rhIL-9 as the control condition (n=4 AML patients per condition). **G)** Heatmap demonstrating fold differences in gene expression in $CD4^+$ T-cells from AML patients after *in vitro* treatment with rhIL-9 plus NF-κB inhibitor (BAY 11-7082) or JAK1/JAK2 inhibitor (Ruxolitinib). The fold changes in gene expression were calculated using vehicle (Veh) treated cells as the control condition (n=4 AML patients per condition). **H-I)** Proliferation of $CD4^+$ T-cells from BM of AML patients treated *in vitro* with or without rhIL-9 plus si*KMT2A*, si*KMT2C*, si*KMT2E*, NF-κB (BAY 11-7082) or JAK1/JAK2 (ruxolitinib) inhibitors. Following the CFSE staining, the proliferation and replication indexes were determined using Flowjo's proliferation modeling module. The proliferation and replication indices were compared to control $CD4^+$ T-cells treated with siCtrl or veh without addition of rhIL-9 (n=3-4 AML patients per condition). **J)** H3K4me3 occupancy at the promoters of *NFKB1*, *RELA*, *TBX21, IFNG* and *TNF* by ChIP-qPCR in FACS-purified AML $CD4^+$ T-cells cultured 48h in the presence or absence of rhIL-9. H3K4me3 occupancy at the *GAPDH* promoter was used as a positive control (n=4 AML samples). Statistics: Two-way ANOVA with multiple comparisons and Dunnett's Post Hoc Test (E), two-tailed unpaired t-test (H-J). *P<0.05, **P<0.01, ***P<0.001 ****P<0.0001.

Figure 7. IL-9/IL-9R signaling as well as TNF-α and IFN-ɣ expression in $CD4^+$ T-cells correlate with worse prognosis in AML patients.

A) Colony forming assays with primary LSCs treated overnight in the presence or absence of rhTNF-α (100 pg/mL) or rhIFN-ɣ (100 pg/mL), before plating in methylcellulose in the primary plating. Secondary plating was without addition of rhTNF-α or rhIFN-ɣ (n=6 AML patients; each value indicates the average of three replicates of one individual patient sample). **B)** Colony forming assays performed with primary LSCs treated overnight with or without supernatant of IL-9 activated AML $CD4^+$ T-cells in the presence or absence of neutralizing αTNF-α (1 µg/mL) or αIFN-ɣ antibodies (1 µg/mL), before plating in methylcellulose in the primary plating. No further treatment was added for secondary platings (n=3 AML patients; each value is average of three replicates of one individual patient sample). The results were normalized to the colony numbers of LSCs that have been treated with vehicle (A) or left untreated (B)

(dotted line). **C)** Kaplan-Meier plots of overall survival (OS) for pooled AML patients from 3-independent AML cohorts (HOVON-SAKK dataset: E-MTAB-3444; NL-Valk dataset: GSE6891; Metzeler dataset: GSE12417), according to the *IL9* and *IL9R* gene expression (n=1339 AML patients). The cut-off for high or low gene expression was assessed by the X-tile program. **D)** Kaplan-Meier plots of OS according to the *IL9* gene expression in LSCs or *IL9R* gene expressions in FACS-purified BM-infiltrating $CD4^+$ T-cells from AML patients (n=28 LSCs and n=30 $CD4^+$ T-cells). **E)** GSEA of 16 LSC signature genes (*IL9* high vs. *IL9* low in purified LSCs, as determined by the X-tile program cut-off (D)). **F)** Heatmap of 16 LSC signature genes. **G)** Correlation between *TNF and IFNG* in $CD4^+$ T-cells and the geometric mean of 16 LSC signature genes in purified LSCs. **H)** Kaplan-Meier plots of OS according to the *TNF* and *IFNG* gene expressions in FACS-purified BM-infiltrating $CD4^+$ T-cells from AML patients (n=30 AML). Statistics: Two-way ANOVA with multiple comparisons and Dunnett's Post Hoc Test (A-B) and log-rank test (C, D and H), Pearson correlation (G). **P*<0.05, ***P*<0.01, ****P*<0.001 *****P*<0.0001.

Figure 1

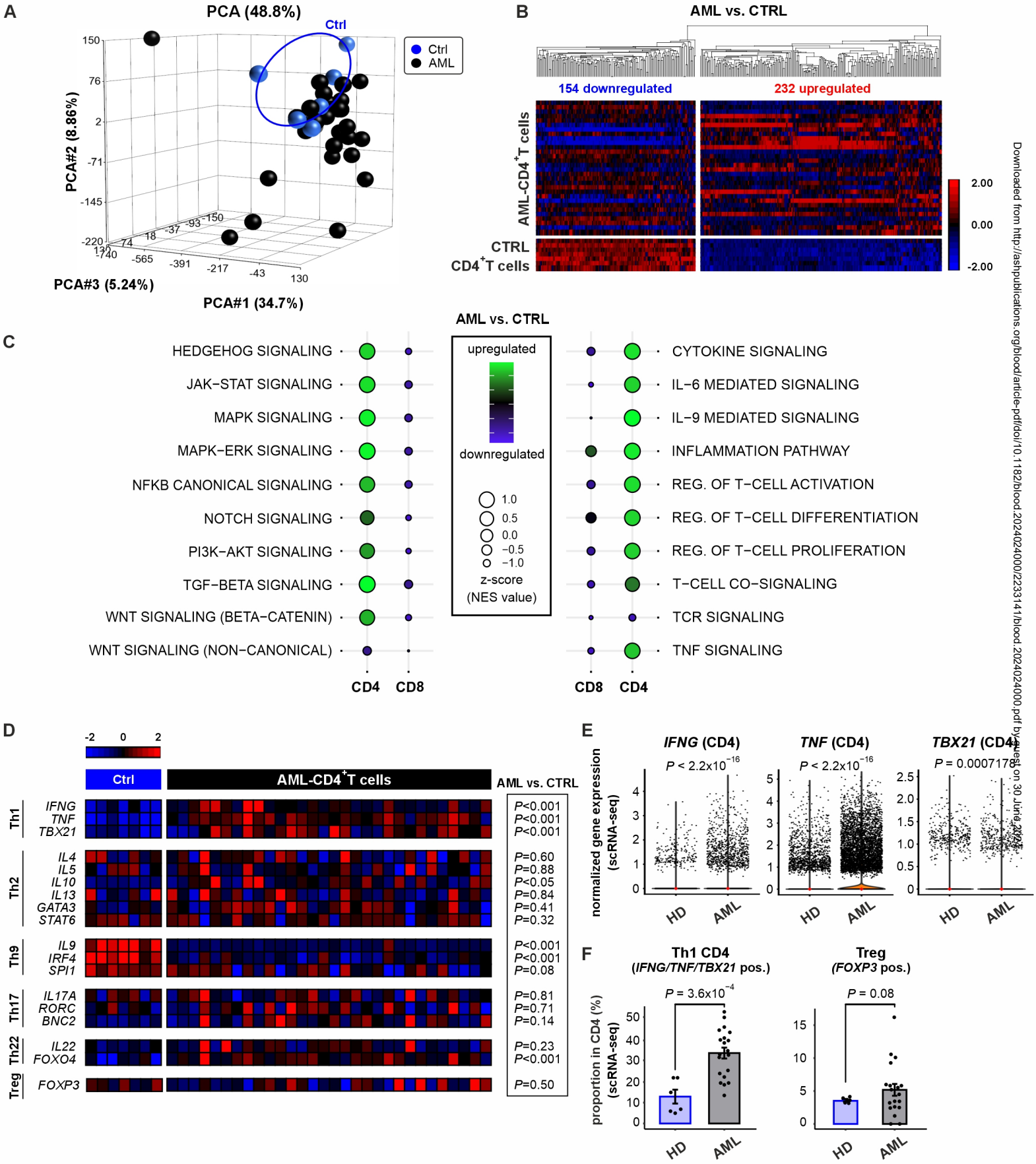
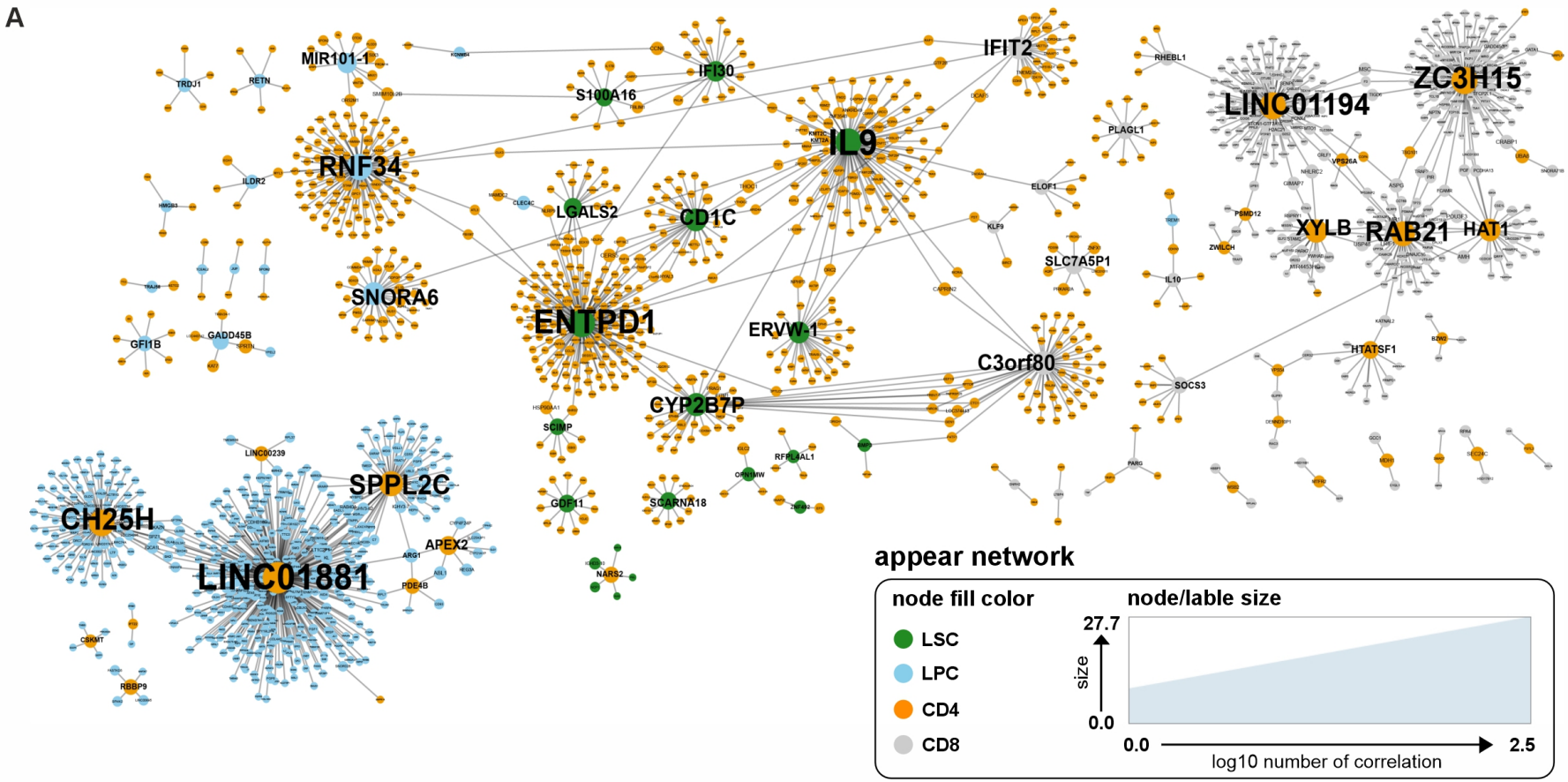
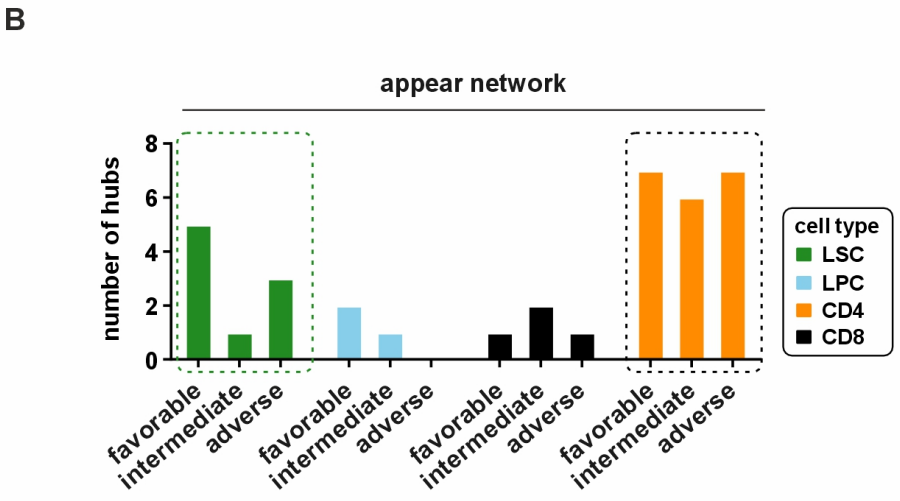
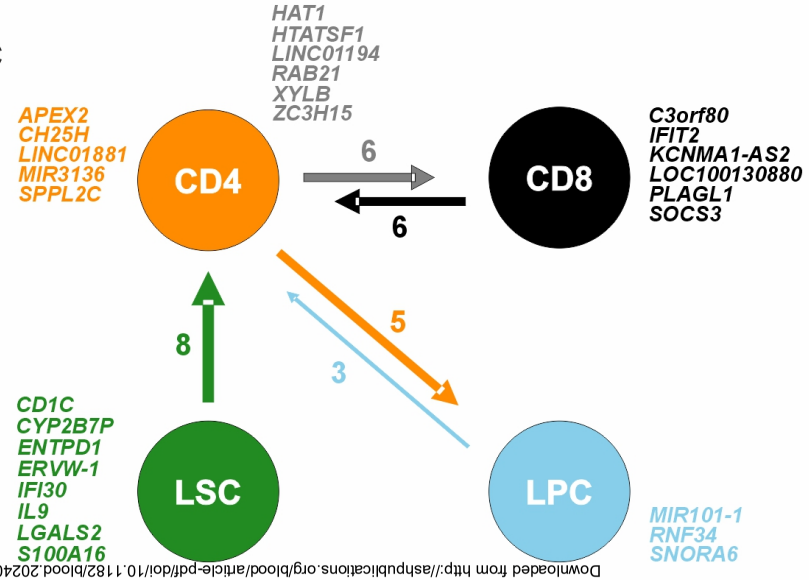


Figure 2







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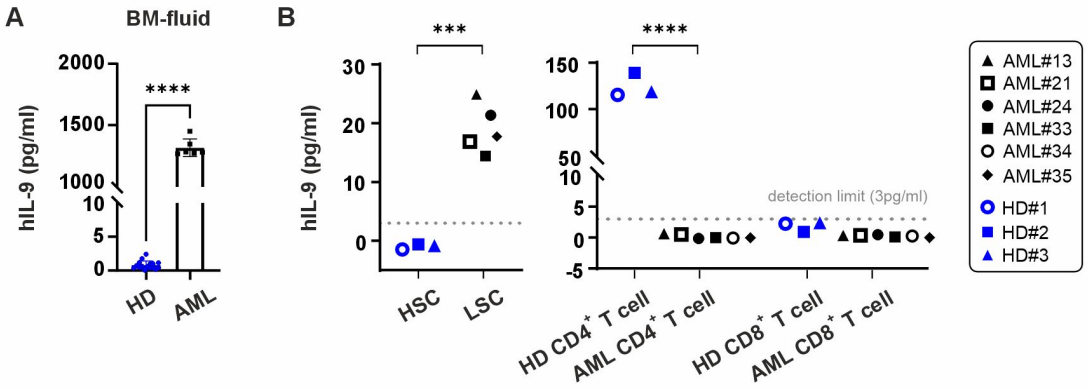
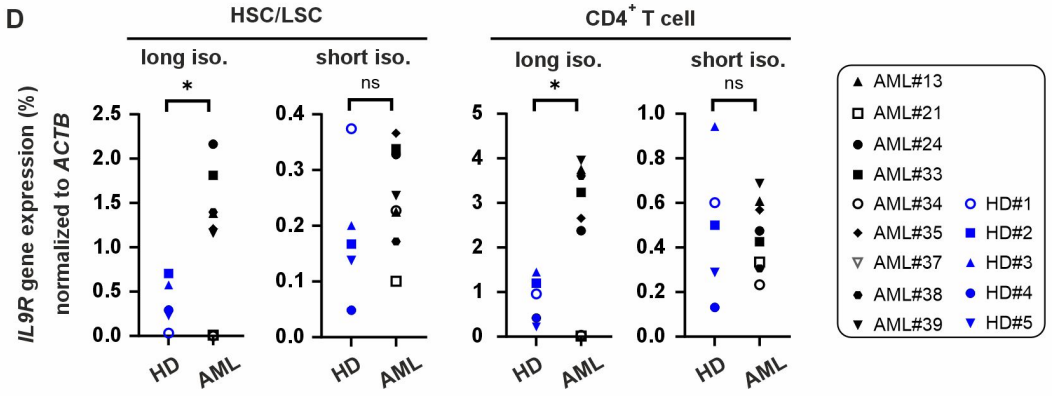
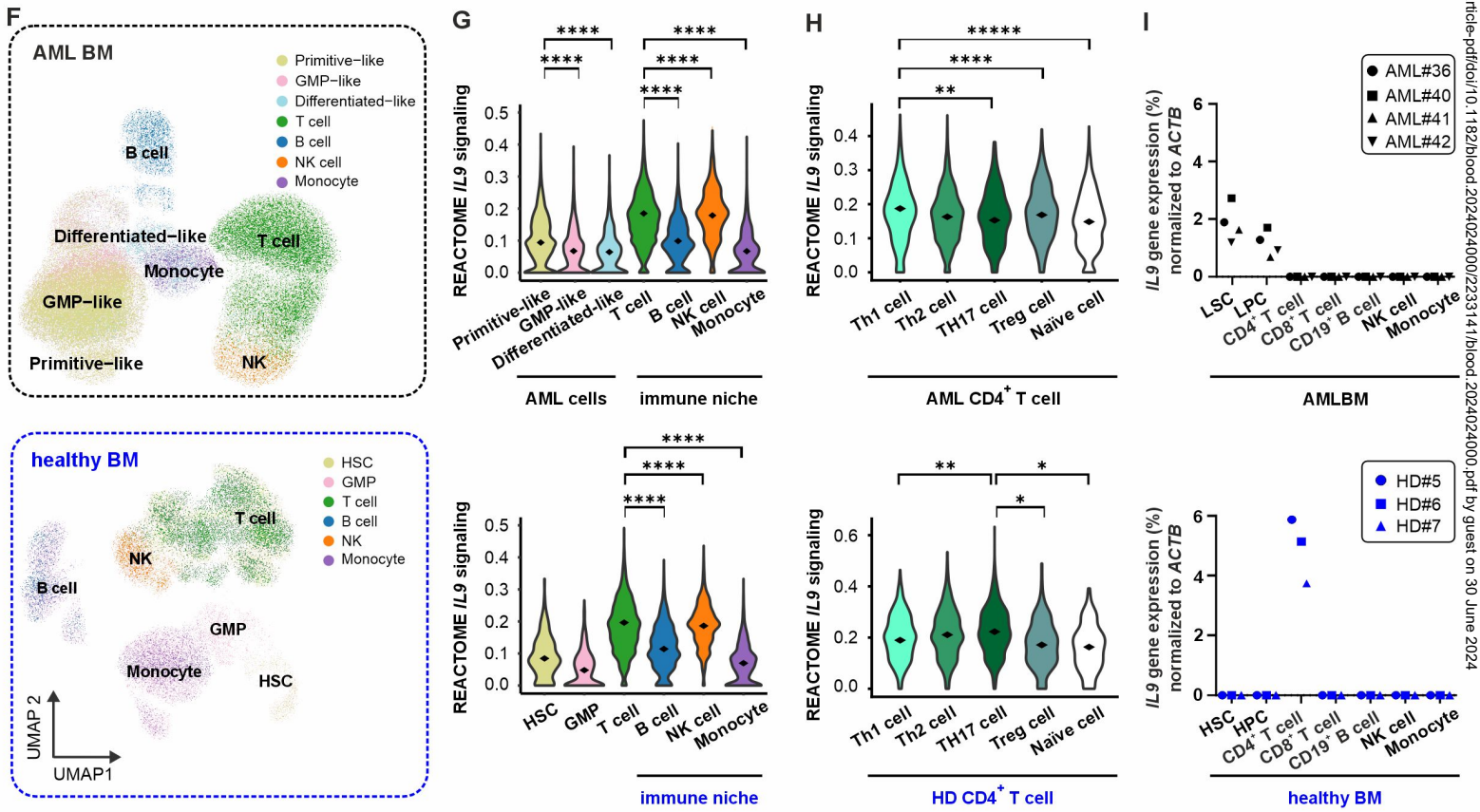






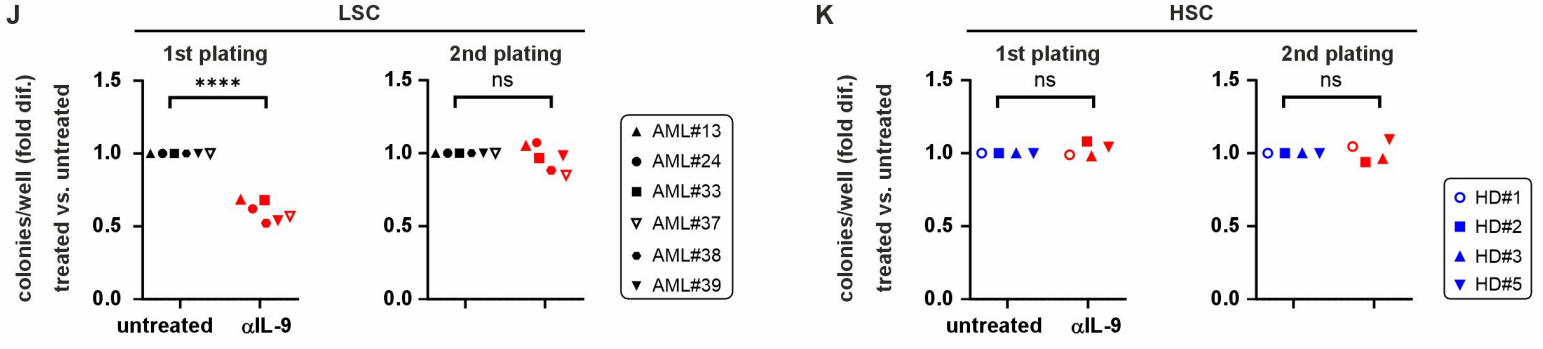
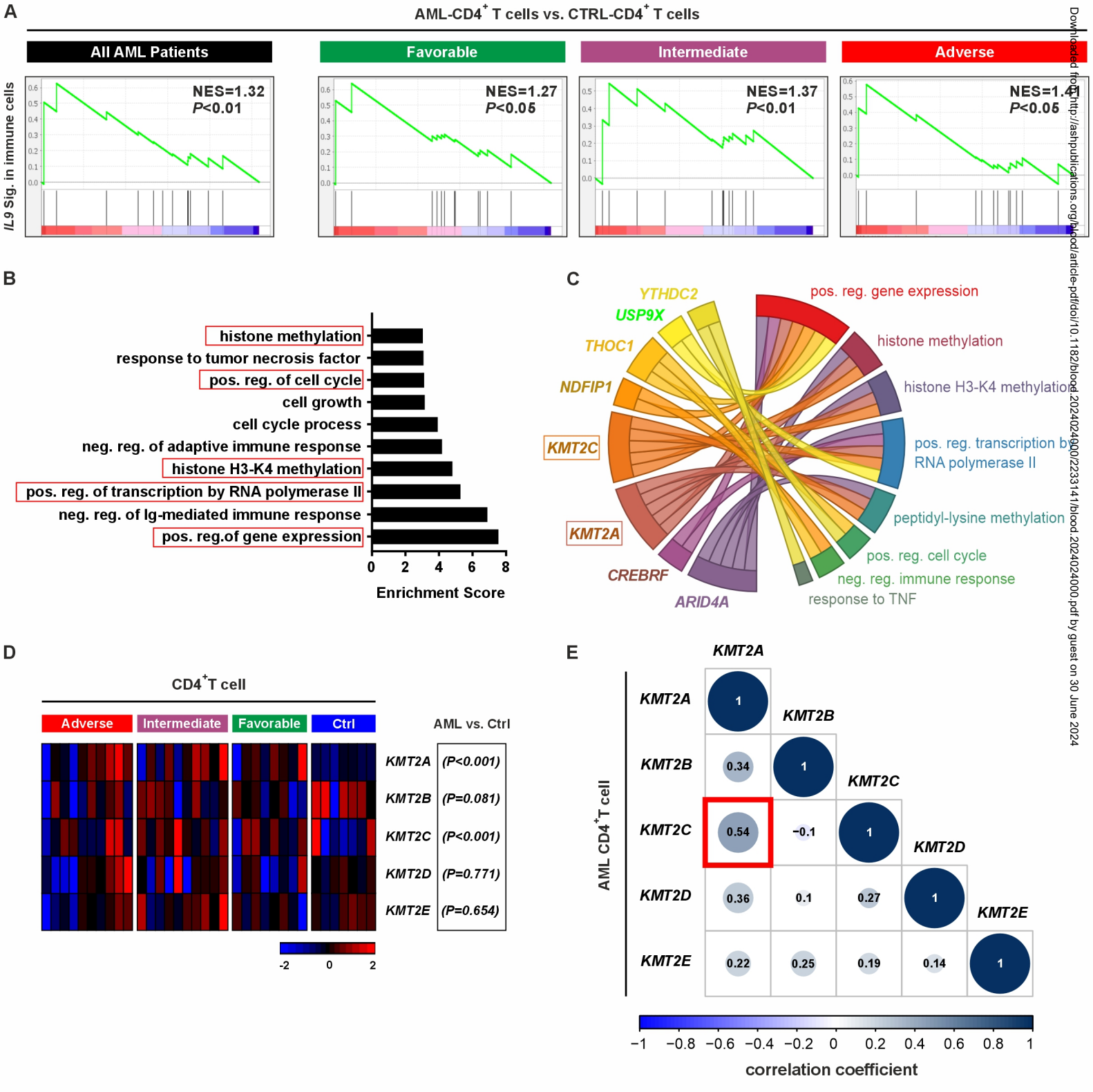


Figure 4

Figure 4



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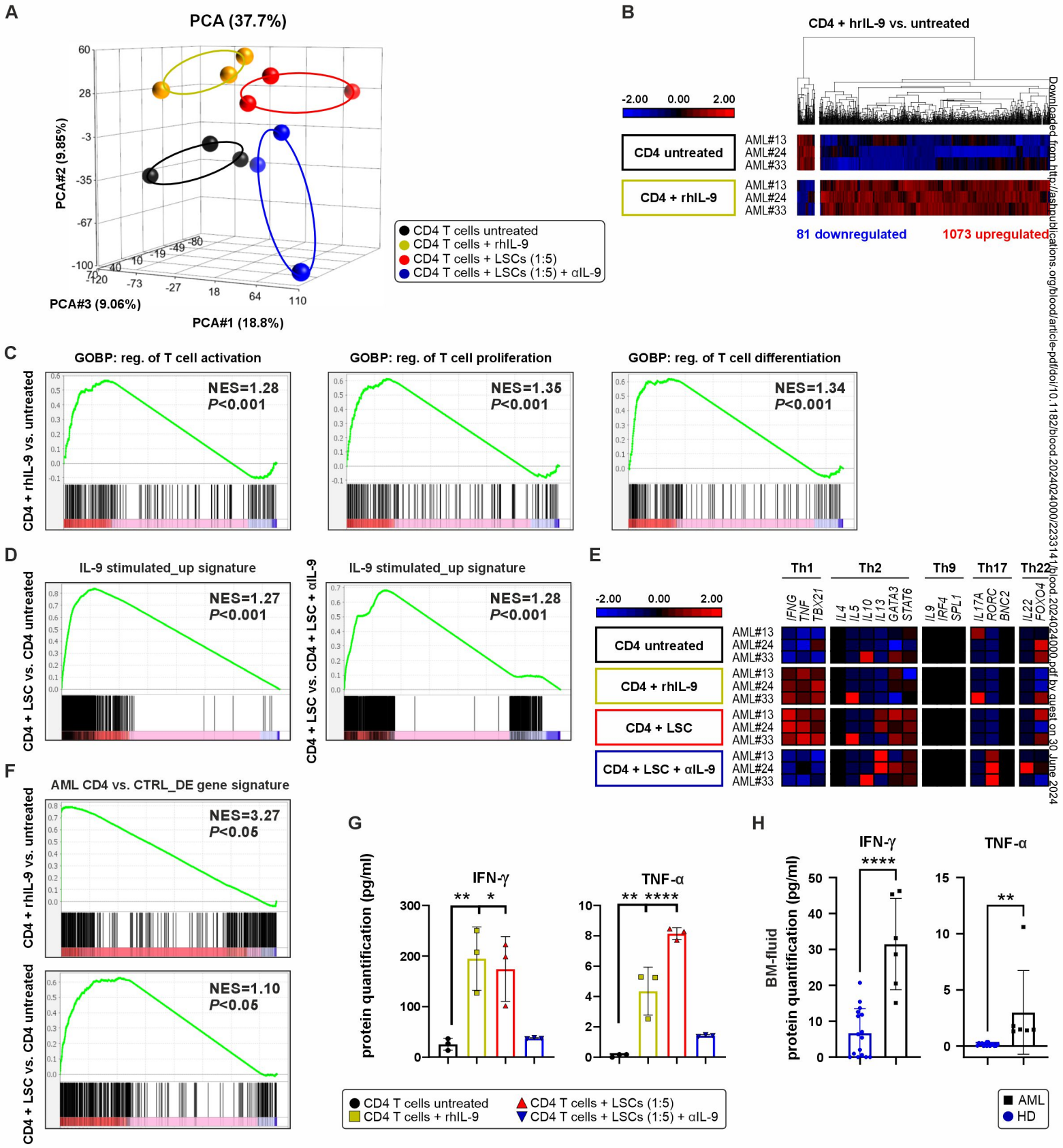

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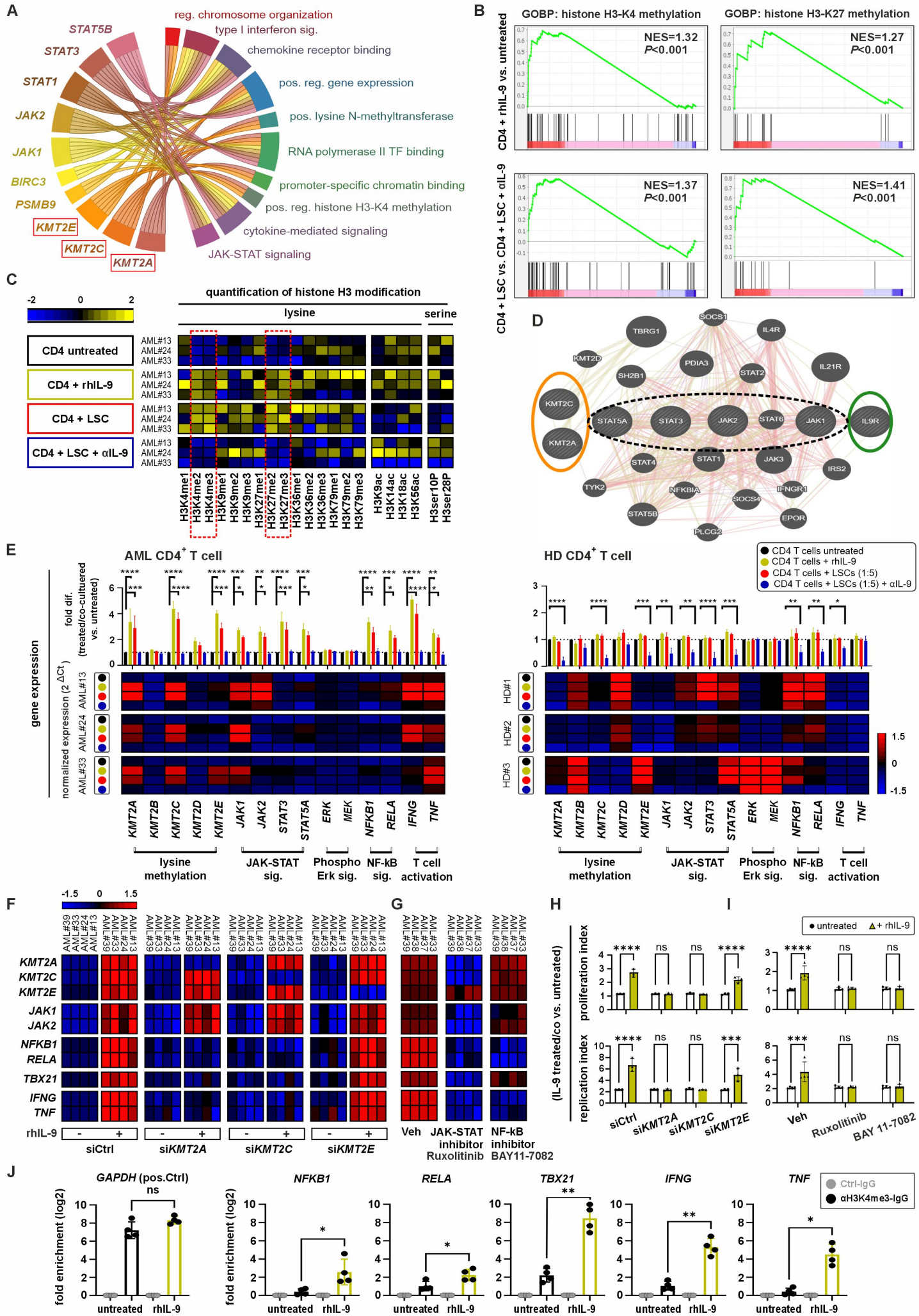
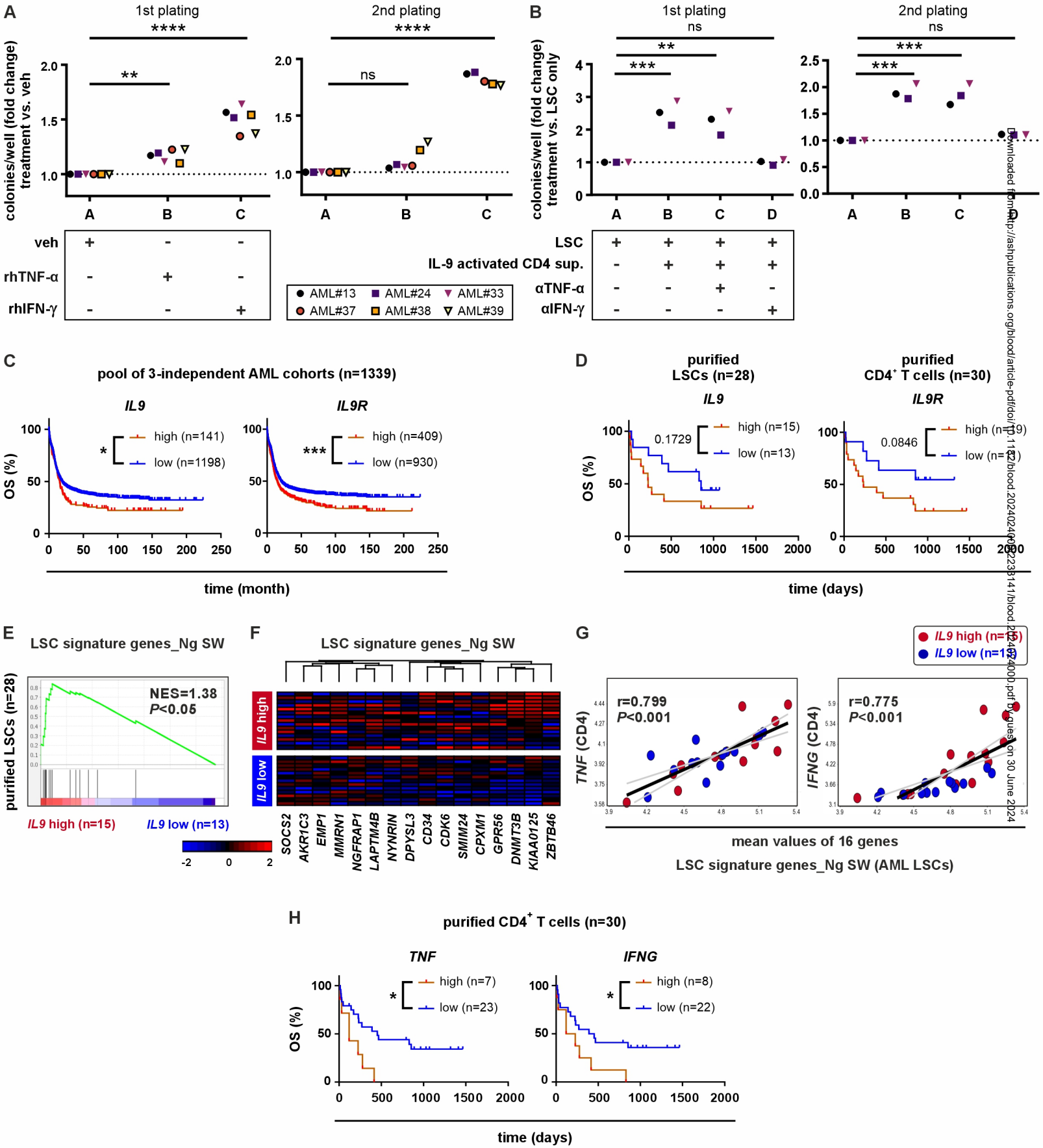


Figure 7

Figure 7



IL-9 Secretion by Leukemia Stem Cells (LSCs), Epigenetic Activation of CD4+ T-Cells, and Th1 Skewing in Acute Myeloid Leukemia (AML)

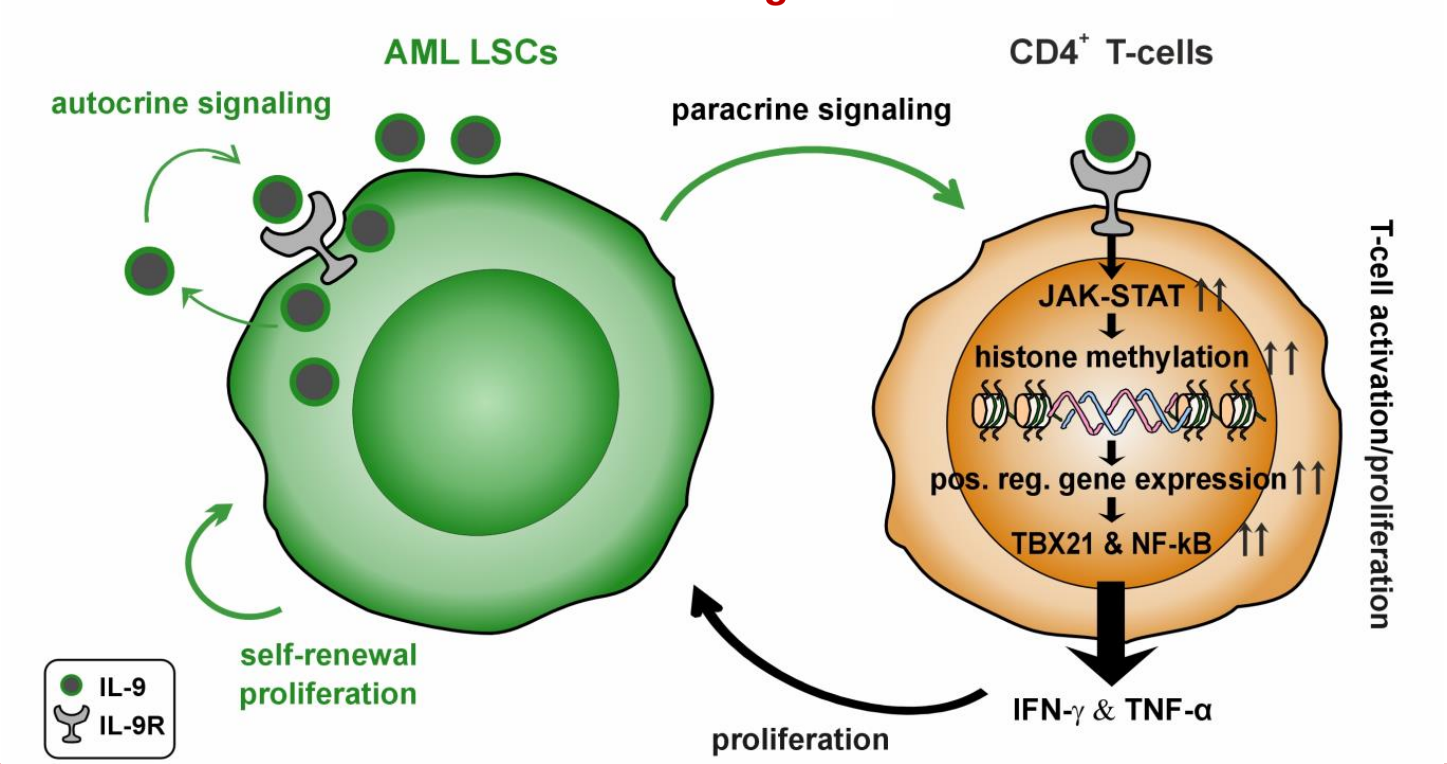
Context of Research

LSCs interact with various cell types in the bone marrow to initiate and propagate AML

Aim of This Study

To study the interaction of CD4+ and CD8+ T-cells with LSCs in the bone marrow of AML patients

Findings



Conclusion: IL-9 secreted by AML LSCs epigenetically activates CD4+ T-cells and induces Th1 skewing. IFN-ɣ and TNF-α produced by activated CD4+ T-cells expand LSCs.

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blood Visual Abstract