Single-cell Transcriptome Analysis Uncovers Heterogeneity and Key Regulators in Ibrutinib-resistant Chronic Lymphocytic Leukemia

Hui Jin

The First Affiliated Hospital of Nanjing Medical University

Bin Huang

Nanjing Medical University

Zijuan Wu

First Affiliated Hospital of Nanjing Medical University

Huayuan Zhu

The First Affiliated Hospital of Nanjing Medical University

Hanning Tang

the First Affiliated Hospital of Nanjing Medical University

Jiazhu Wu

The First Affiliated Hospital of Nanjing Medical University

Danling Gu

The First Affiliated Hospital of Nanjing Medical University

Yun Cai

Nanjing Medical University

Luqiao Wang

The First Affiliated Hospital of Nanjing Medical University

Xueying Lu

The First Affiliated Hospital of Nanjing Medical University

Wei Xu

The First Affiliated Hospital of Nanjing Medical University

Kening Li

Nanjing Medical University

Qianghu Wang

Nanjing Medical University

Jian-yong Li (✉ lijianyonglm@126.com)

The First Affiliated Hospital of Nanjing Medical University <https://orcid.org/0000-0002-5878-945X>

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Abstract

Background

Ibrutinib as a widely used Bruton's tyrosine kinase inhibitor has shown outstanding value in clinical therapy for chronic lymphocytic leukemia (CLL). However, the bottleneck of ibrutinib resistance has caused widespread concerns, necessitating the exploration of novel targets.

Methods

Single-cell RNA sequencing (scRNA-seq) was used to characterize the heterogeneity of ibrutinib-sensitive (IBS) and -resistant (IBR) CLL patients and single-cell stemness estimation and metabolic pathway enrichment analysis were performed. Lectin galactoside-binding soluble 1 (LGALS1) and lymphocyte-activating gene 3 (LAG3) were screened as key factors by analyzing the RNA-sequencing data at bulk and single cell levels. Subsequently, pseudo-time trajectory analysis and gene set enrichment analysis were conducted. In addition, an IBR CLL cell line (MEC1-IR) was generated and RT-qPCR, western blotting, and immunofluorescence were performed to detect the expression of LGALS1 and LAG3. OTX008, a selective inhibitor of galectin-1 (Gal-1, encoded by LGALS1) was assessed in CLL cells and CCK8 and apoptotic assays were conducted for functional analysis.

Results

IBR CLL showed significantly different characteristics from IBS in terms of transcriptome expression and energy metabolism. LGALS1 and LAG3 were gradually upregulated in B cells along the evolution trajectory from IBS to IBR. Their expression was verified to be closely related to the prognosis of CLL, as well as sensitivity to ibrutinib. OTX008 could effectively suppress the proliferation and induce apoptosis of CLL cells, especially for those with ibrutinib resistance.

Conclusions

An LGALS1 and LAG3 gene panel is a promising indicator of ibrutinib resistance and a prognostic marker for CLL. OTX008 displays pronounced performance against CLL cells, especially with IBR, and might represent a novel therapeutic strategy for CLL.

Background

Chronic lymphocytic leukemia (CLL) involves leukemic cell infiltration and accumulation in lymphoid organs and is the most common leukemia in Western populations. Although its incidence is lower in China, the frequency of new cases is increasing [1, 2]. The approval of Bruton's tyrosine kinase (BTK) inhibitors such as ibrutinib and acalabrutinib, and the B cell lymphoma-2 (Bcl-2) inhibitor venetoclax have revolutionized the treatment of CLL [3–7]. Whereas these novel agents alone or in combination induce long-lasting and deep remissions in most patients with CLL, drug resistance still occurs which is not well resolved.

BTK, a key B cell receptor (BCR) signaling kinase, is amplified in CLL and activates proliferative and anti-apoptotic signals [8]. Ibrutinib is an orally bioavailable BTK inhibitor that eliminates pro-survival pathway activation [9]. As the first FDA-approved BTK inhibitor, ibrutinib has shown significant clinical benefits for CLL patients [10]. However, current limitations including the development of resistance remain an unresolved clinical issue [11–13]. Mutations in BTK and PLCG2 are recognized as central factors for ibrutinib resistance, yet a low frequency of these mutations has been observed in our center [14–16]. Consequently, a comprehensive understanding of CLL clonal evolution driven by the selective pressure of drug therapy is vital for precise, individualized treatment.

Though next-generation sequencing [17, 18] has helped to clarify the key gene mutations, an understanding of the precise molecular mechanisms of ibrutinib resistance from a single cell perspective is needed, of which little information exists for CLL. Single-cell RNA sequencing (scRNA-seq) can assess gene expression in individual cells and systematically characterize heterogeneity at a higher resolution [19, 20]. Moreover, this technology can be used to identify different cell subtypes based on relevant transcriptional modules [21, 22], and thus could be used to discover reliable biomarkers for clinical efficacy and drug-resistance.

Here, we characterized and analyzed the difference between CLL patients with ibrutinib-sensitive (IBS) and -resistant (IBR) by bulk and scRNA sequencing. IBR CLL exhibited unique characteristics in terms of transcriptome expression and energy metabolism. Seven distinct ibrutinib-resistant subpopulations were identified and two candidate genes, lectin galactoside-binding soluble 1 (LGALS1) and lymphocyte-activating gene 3 (LAG3) were screened, which were gradually upregulated in B cells along the evolution trajectory from IBS to IBR. LGALS1 is located on chromosome 22 and encodes galectin-1 (Gal-1), a protein involved in regulating physiological and pathological processes like cell transformation, proliferation, and adhesion, blood vessel formation, and immunosuppression [23]. LAG3 is an immune checkpoint molecule that was recently suggested as a novel therapeutic target in CLL [24–26]. A close correlation between LGALS1 and LAG3 expression was observed and these factors were found to be highly expressed in IBR CLL, with diagnostic and prognostic stratification, indicating that they might serve as biomarkers of IBR CLL. Moreover, OTX008 as a Gal-1 inhibitor suppressed proliferation and induced apoptosis in IBS and IBR cells. Together, we hope that these findings might offer a new therapeutic strategy for the treatment of IBR CLL.

Methods

ScRNA-seq data processing

ScRNA-seq libraries were sequenced from 150 bp 3' paired cDNAs using HiSeq Xten instruments. Raw data (BCL files) were converted to fastq files using bcl2fastq software (Illumina). Reads were aligned to a human genome reference (GRCh38) and a digital gene expression matrix built using the STAR algorithm in CellRanger (10x Genomics; v3.0.2). Quality control and further analyses were carried out using Seurat [27] (v3.1.5). To eliminate the effect of abnormal cells and technical noise on downstream analysis, we

removed cells with abnormal gene counts or a high percentage of unique molecular identifiers (UMIs) mapped to mitochondria for each sample. Detailed clinical characteristics are showed in Table S1.

ScRNA-seq sample integration and cell type recognition

After log-normalization and variable feature identification based on variance stabilizing transformation, we integrated filtered cells from three IBR and four IBS CLL patients, as well as two healthy donors using “FindIntegration Anchors” and “IntegrateData” in Seurat with 30 dimensionalities [27], followed by scaling using “ScaleData”. The statistical significance of principal component analysis scores was determined using “JackStraw” and dimension reduction was performed using the first 20 principal components. Data was visualized using T-distributed stochastic neighbor embedding (t-SNE) with “RunTSNE”, whereas cell clusters were identified using “FindNeighbors” and “FindClusters” (resolution = 2). Differentially expressed genes (DEGs) were identified using the Wilcoxon Rank Sum test in “FindAllMarkers”, with significance indicated by an average natural logarithm (fold change) ≥ 0.25 , Bonferroni-adjusted p -value < 0.05 , and minimum percentage ≥ 0.25 . Candidate markers were considered with CD14, CD1C, CD79A, CD3E, and KLRD1, and were used to annotate cell clusters. Single cell copy number variation (CNV) was estimated using inferCNV [28] (v0.8.2), with non-B cells as a reference.

Detailed materials and methods can be found in the supplemental data.

Results

Single-cell landscape of PBMCs in patients with IBR and IBS CLL

To study PBMC heterogeneity in CLL patients treated with ibrutinib and the underlying mechanism of ibrutinib resistance, we collected PBMCs from three IBR, four IBS CLL patients and two healthy donors and performed scRNA-seq using a droplet-based 10× Genomics platform. After quality control, we obtained 42,288 cells with a mean number of 4,760 cells per sample. The median number of unique molecular identifiers and genes per cell were 3,570 and 1,108 respectively. T-distributed stochastic neighbor embedding (t-SNE) visualization of filtered cells following integration and unsupervised clustering, revealed that cells from IBR and IBS samples were differently distributed in clusters, suggesting that IBR cells display a unique transcriptional pattern (Fig. 1A). To further differentiate these cells, we sorted them into 36 clusters (Fig. 1B) and annotated them as five major categories, specifically CD79A⁺ B-cells, CD3E⁺ T-cells, KLRD1⁺ NK-cells, CD14⁺ monocytes, and CD1C⁺ dendritic cells (Fig. 1C, D; Fig. S1).

First, we calculated the proportion of cell types in each sample (Table S2). B cells exhibited significantly increased expansion in CLL patients compared to that in healthy donors. (Fig. 1E). As copy number variation (CNV) is a common phenomenon in CLL [29], we predicted InferCNV in IBR and IBS patients using scRNA-seq data Fig. 1F). We found that one IBR patient (R3) carried a deletion of the chromosome 8 short arm, consistent with the findings of Burger [30]. Two IBR patients (R2 and R3) carried a deletion of

the chromosome 9 long arm and one IBR patient (R3) carried a deletion of the chromosome 4 short arm, implying that del(9q) and del(4p) might be associated with ibrutinib resistance. Obviously, we found that chromosome 6p was often amplified, consistent with the results of Brown [31].

B cells from IBR patients exhibit a unique transcriptional pattern

Next, we investigated B-cell heterogeneity in IBR and IBS patients by re-clustering the 30,417 identified B cells into 21 clusters (Fig. 2A). B cells from IBR patients exhibited different distributions compared with those from IBS patients. CytoTRACE analysis revealed that B cells in IBR CLL patients had higher stem index scores, implying that abnormal B cell stemness might be involved in the resistance to ibrutinib ($p < 2.2\text{e-}16$, Fig. 2B and 2C).

According to the proportion of B cells from IBR samples, B-cell clusters were categorized into three main subgroups, namely IBR (> 50 % of B cells from IBR samples) and IBS (> 70 % of B cells from IBS or NC samples). Other clusters were defined as shared clusters and clusters with < 200 cells were filtered (Fig. 2D). To elucidate the functional differences between IBR and IBS/shared clusters, we performed metabolic enrichment analysis. IBR clusters were significantly enriched in glycolysis and gluconeogenesis, which supply energy and support cancer cell growth (Fig. 2E, Table S3). We also found that many glycometabolism pathways, such as the pentose phosphate pathway, pentose and glucuronate interconversion, and fructose and mannose metabolism, were enriched in IBR clusters. Further, we found that glutathione metabolism was enriched in IBR clusters, consistent with Zhang who reported that elevated glutathione levels can increase leukemia cell survival and protect them against drug-induced cytotoxicity [32]. Together, these findings suggest that B cells from IBR patients exhibit a unique transcriptional and metabolic pattern compared with B cells from IBS patients.

Difference between intercellular interactions in the microenvironment of IBR and IBS cells

Intercellular communication mediated by ligand–receptor complexes is essential for coordinating biological processes, such as differentiation and inflammation. Therefore, we studied the interaction between B cells and other types of cells from IBR and IBS clusters by performing cell–cell communication analysis using CellphoneDB. IBR-B cells displayed more interactions with monocytes, NK, T, and dendritic cells than IBS-B cells, suggesting that IBR-B cells could actively build connections with other cells to reshape the protective niche, which would be beneficial for cell survival (Fig. 2F). Previous studies have shown that co-culturing with stromal NK cells can increase the oxidative phosphorylation of CLL cells, promoting their proliferation [33]. Kurtova, et al. [34] also found that bone marrow stromal cells provide survival and drug resistance signals for CLL cells against spontaneous and drug-induced apoptosis.

Notably, the LGALS9–CD47 interactions only existed between T cells and IBR-B cells, and not IBS cells (Fig. 2G, Table S4), which has not previously reported in CLL. We also found that other B cell–T cell interactions such as MIF-TNFRSF14 and LILRA4-BST2 were specifically enriched in IBR clusters. These findings indicated that IBR B cells might reprogram intercellular interaction patterns in CLL patients.

Therefore, the interaction between CLL cells and other microenvironment cells might be involved in the development of drug resistance.

LGALS1 and LAG3 are associated with the transition between IBS and IBR

Although genome-level features have been extensively studied in IBR patients with CLL [35, 36], the transcriptional characteristics are rarely reported. To improve our understanding of the underlying mechanism of ibrutinib resistance, we performed bulk RNA sequencing with PBMCs from 6 IBR and 40 IBS CLL patients. Ninety genes were significantly upregulated in the IBR group compared to levels in the IBS group, among which LGALS1, LAG3, and PTMS were the top three upregulated genes (Fig. 3A, Table S5). In addition, we identified highly expressed genes in each IBR cluster at the single cell level (719 genes; Table S6), including LGALS1, LAG3, and PTMS. Moreover, we investigated the developmental trajectory of B cells in IBR and IBS patients using pseudo-time analysis. Remarkably, B cells from IBR samples were distributed at the end of one state track, indicating that IBR cells might evolve from IBS cells (Fig. 3B). According to the pseudo-time analysis, we some genes were found to be gradually upregulated along the trajectory from IBS to IBR (Fig. 3C). LGALS1 was highly expressed in five single-cell IBR clusters (Fig. 3D). LGALS1 and LAG3 expression increased along the transition trajectory, whereas PTMS did not coincide with this trend (Fig. 3E). Interestingly, PTMS and LAG3 were found to be located adjacent to each other on chromosome 12p13.31 and bulk-RNA data indicated the co-expression of these genes.

LGALS1 and LAG3 typically act as immune checkpoints that repress innate and adaptive immune programs [37–39]. To further elucidate the function of LGALS1 and LAG3 in CLL patients, we analyzed the bulk RNA-seq data. Genes co-expressed with LGALS1 were identified, including S100A4, S100A6, and COX5B, which are associated with cancer cell invasion and proliferation [40] (Fig. 3F, Table S7), as well as genes co-expressed with LAG3, such as HSPG2, which is associated with poor prognosis in acute myeloid leukemia [42] (Fig. S2, Table S8).

GO analysis revealed that genes co-expressed with LGALS1 were significantly enriched in mitochondrial ATP synthesis-coupled electron transfer and oxidative phosphorylation (Fig. 3G, Table S9). LGALS1 might play an important role in the proliferation of CLL cells. In contrast, genes co-expressed with LAG3 were enriched for pathways related in glucose metabolism (Fig. S3). Together, LGALS1 and LAG3 are related to the metabolic pathways and might be involved in cell resistance to ibrutinib.

High expression of LGALS1 and LAG3 closely correlates with poor outcome in CLL

Next, we detected and analyzed the overall survival (OS) of CLL patients, finding that patients with higher LGALS1 and LAG3 expression showed poorer OS (Fig. 4A, left and middle). Moreover, Kaplan-Meier analysis demonstrated that patients with concurrent high expression of LGALS1 and LAG3 exhibited a worse OS compared to those with high LGALS1 or LAG3 expression respectively (Fig. 4A, right). To further validate the survival analysis results, we also analyzed GSE22762 data from the GEO database [43]

which is comprised of 107 CLL patient samples (Fig. 4B). As expected, these results both demonstrated that an LGALS1 and LAG3 gene panel is associated with poor prognosis for CLL patients.

To evaluate and confirm the clinical relevance of LAG3 and LGALS1 in CLL, we analyzed their expression and the clinical features of CLL patients (Fig. 4C). Patients with higher level of LAG3 and LGALS1 were found to have higher mutation rates for SF3B1 ($p = 0.035$) or NOTCH1 ($p = 0.038$). Immunoblots were then performed to compare protein levels between IBS and IBR patients, and the results revealed higher levels of Gal-1 and LAG3 in IBR patients (Fig. 4D, Fig. S4A). As Gal-1 is a secretory protein, we determined its plasma concentration as well as that of LAG3 in 16 treatment-naïve, refractory and/or relapsed (R/R) CLL patients using ELISA. As expected, Gal-1 and LAG3 levels were significantly higher in R/R CLL patients (Fig. S4B). Collectively, these results indicate that elevated expression of LGALS1 and LAG3 is associated with poor prognosis in CLL.

LGALS1 and LAG3 are upregulated in the established ibrutinib-resistant cell line

As LGALS1 and LAG3 indicated poor prognosis in CLL patients, we sought to explore the reasons for this with the acquired ibrutinib-resistant cell line (MEC1-IR, Fig. 5A). CCK8 assays indicated that MEC1-IR cells were significantly more resistant to ibrutinib than the parental cells (Fig. 5B). Annexin-V/PI staining also revealed a pronounced increase in cell death in MEC1 cells (Fig. 5C, D). Next, we examined the expression of LGALS1 and LAG3 in parental and resistant cells, revealing that they are markedly higher in MEC1-IR cells (Fig. 5E). Expectedly, immunoblotting and ELISA showed consistent results (Fig. 5F-H). These results demonstrate that LGALS1 and LAG3 are upregulated in IBR cells.

The Gal-1 inhibitor OTX008 effectively inhibits the proliferation of CLL cells

Recent studies have demonstrated the efficacy of OTX008, a Gal-1 inhibitor, in preclinical models of multiple tumors [44]. Therefore, we studied the anti-tumor effect of OTX008 on IBS and IBR CLL cells. First, we examined the expression of Gal-1 and LAG3 with OTX008 treatment. We found that OTX008 could decrease LAG3 mRNA levels (Fig. 6A). Meanwhile, the protein levels of Gal-1 and LAG3 were decreased in a dose-dependent manner in both CLL cell lines and primary cells (CLL-1) (Fig. 6B). This observation was validated by immunofluorescence (Fig. 6C). Though MEC1-IR cells displayed higher proliferation than MEC1 cells, the proliferative capacity of both groups was almost completely inhibited by OTX008 (Fig. 6D). Subsequently, flow cytometric analysis revealed that MEC1-IR cells underwent markedly increased apoptosis compared to that in MEC1 cells (Fig. 6E). Primary CLL cells were then treated with OTX008 and trypan blue staining revealed that OTX008 was more effective against the IBR group than IBS (Fig. 6F). Overall, our findings demonstrate the potential clinical utility of OTX008 for CLL, and particularly for IBR patients.

Discussion

CLL is a highly heterogeneous disease that can be treated using ibrutinib, a BTK inhibitor that has profound activity against this disease. However, the issue of ibrutinib-resistance has not been well solved

yet. This study elucidated the transcriptional landscape of CLL patients using scRNA-seq. We identified IBR subpopulations and revealed their transcriptional and metabolic characteristics, and intercellular communication, finding that LGALS1 and LAG3 were upregulated in IBR primary cells and cell lines. LGALS1 and LAG3 expression was strongly associated with the prognosis of CLL patients and the panel of the two genes could be a potential biomarker of ibrutinib-sensitivity. Furthermore, the Gal-1 inhibitor OTX008 induced apoptosis in and displayed particular efficacy against IBR CLL cells, indicating its potential clinical value for CLL.

Considerable evidence has shown that LGALS1 is often upregulated in tumor tissues [45] and correlates with tumor aggressiveness and treatment resistance [46]. Croci, et al. [47] indicated that Gal-1 contributes to the modulation of BCR signaling and is associated with poor outcomes in CLL. Here, we revealed that Gal-1 plays a critical role in CLL and might participate in the development of ibrutinib resistance. Moreover, OTX008 directly targeted Gal-1 to inhibit proliferation and promote apoptosis of CLL cells, especially with IBR-CLL cells. This could provide new treatment strategies to improve the clinical efficacy for patients with ibrutinib-resistant CLL.

ScRNA-seq also identified pronounced LAG3 expression in IBR clusters. Many studies have illustrated the role of LAG3 in immunotherapeutic regulation [48]. Dual PD1 and LAG3 immune checkpoint targeting has been shown to successfully control CLL development in preclinical mouse models [49]. According to the sequencing analysis and clinical relevance of Gal-1 and LAG3, we propose that a combination of OTX008 and anti-LAG3 monoclonal antibodies might achieve better efficacy for CLL, especially for IBR patients. However, further *in vivo* studies are required to determine the synergistic effect and toxicities. In addition, more clinical samples from patients with resistance to ibrutinib and other BTK inhibitors need to be collected.

Collectively, the present study comprises an in-depth analysis of the heterogeneity and microenvironment characteristics of IBR and IBS CLL. Our findings provide, for the first time, evidence that an LGALS1 and LAG3 gene panel could be used as an indicator of ibrutinib sensitivity, as well as a prognostic marker and potential therapeutic target for CLL patients. Further, we demonstrate that the Gal-1 inhibitor OTX008 could help to overcome ibrutinib-resistance, offering a promising combinatorial therapeutic strategy for IBR CLL patients.

Conclusions

Together, our findings demonstrate that IBR CLL cells exhibit a unique transcriptional pattern and indicate the prospect for future combination therapy. We propose that ibrutinib-resistance is mediated by LGALS1 and LAG3, two promising prognostic biomarkers and potential therapeutic targets in CLL, and that OTX008 might help to overcome the resistance to ibrutinib.

Abbreviations

BCR B cell receptor

BTK Bruton tyrosine kinase

CLL chronic lymphocytic leukemia

CNV copy number variation

DEGs differentially expressed genes

Gal-1 galectin-1

IBR ibrutinib-resistant

IBS ibrutinib-sensitive

LGALS1 lectin galactoside-binding soluble 1

LAG3 lymphocyte-activating gene 3

OS overall survival

PBMC peripheral blood mononuclear cell

scRNA-seq single-cell RNA sequencing

t-SNE T-distributed stochastic neighbor embedding

Declarations

Ethics approval and consent to participate

The human sample study was approved by the Medical Ethics Committee of First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from all participants.

Consent for publication

Written informed consent was obtained from all patients.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the GSA under accession number HRA000773.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HJ, KNL, ZJW, and HYZ designed and performed most of the experiments; BH, KNL and YC performed bioinformatics analysis of scRNA-seq and bulk RNA-seq data; HYZ, HNT and DLG collected the clinical samples; ZJW, JZW, LQW, and XYL collected and analyzed the clinical data; HJ, BH, and ZJW drafted the manuscript; HJ, KNL, QHW, and JYL critically revised the manuscript; JYL, KNL, QHW, and WX supervised the project. All authors read and approved the final manuscript.

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Supplemental Figures

Supplemental Figures are not available with this version.

Figures

Figure 1

Expression profiling of 42288 single cells from the PBMC of nine samples. A T-SNE plot of all single cell data (10× Genomics). R1-3 represents three ibrutinib-resistant (IBR) cases. S1-4 represents four ibrutinib-sensitive (IBS) cases. N1-2 represents two normal donors. B Result of the unsupervised clustering of all cells. C Cell-type annotation in the t-SNE plot. D Highly expressed genes in different cell types. Dot size indicates the gene expression percentage in the same cell type. Color indicates the average gene expression in the same cell type. E PBMC components in nine samples. F Large-scale copy number variation (CNV) in single cells inferred based on scRNA-seq data.

Figure 2

Distinct transcriptional pattern of ibrutinib-resistant (IBR) B cells and intercellular interactions among multiple cell types. **A** Left: unsupervised clustering of B cells showed in t-SNE plot. Right: t-SNE plot displays B cells from different types of samples. Red dots represent B cells from IBR patients; green dots represent B cells from ibrutinib-sensitive (IBS) patients; blue dots represent B cells from normal donors. **B** Stem score visualization for B cell in the t-SNE plot. **C** Comparison of the stem scores of B cells from IBR patients, IBS patients and normal donors. Significance was calculated using the Wilcoxon rank-sum test. **D** Upper: proportion of cells derived from three sample types in each cluster. Red represents IBR patients. Green represents IBS patients. Blue represents normal donors. The dashed lines represent 0.3 and 0.5, respectively. Lower: bar plot shows the number of cells in each cluster. The dashed line represents 200. **E** Metabolic pathway enrichment in each cluster. Red represents IBR clusters. Green represents IBS clusters.

Orange represents shared clusters. F Graph showing interactions among different cell types. Line thickness represents the number of significant interactions. G Graph displaying the summary of selected ligand–receptor interactions between different cell types. Circle size represents p-values (permutation test). Color gradient indicates the score of interaction.

Figure 3

Identification of key regulators of ibrutinib resistance based on bulk and single cell transcriptomic data. A Left: heatmap representing the differential gene expression between ibrutinib-resistant (IBR) and ibrutinib-sensitive (IBS) patients based on bulk RNA-seq data. Red indicates IBR patients. Green represents IBS patients. Right: volcano plot depicting differentially expressed genes (DEGs). The significant up-regulated genes in IBR patients are shown with red, and the significant down-regulated genes in IBR patients are shown with green. B Developmental trajectory from IBS to IBR B cells based on scRNA-seq data. C Dynamic changes in gene expression from IBS to IBR. D Graph showing up-regulated genes in single-cell IBR clusters. E Graph showing dynamic changes in LAG3, LGALS1, and PTMS expression along the developmental trajectory from IBS to IBR. Color gradient indicates pseudo-time scores. F Heatmap visualization of genes co-expressed with LGALS1 based on bulk RNA-seq data. Patients were sorted by the expression of LGALS1. G Functional enrichment analysis of genes co-expressed with LGALS1. Dot size represents the number of enriched genes in the pathway. Color gradient represents the adjusted p-value.

Figure 4

Increased LGALS1 and LAG3 expression correlates with poor survival in chronic lymphocytic leukemia (CLL). A Kaplan–Meier analysis of CLL patient samples based on LGALS1 and LAG3 expression (n = 59). * $p \leq 0.05$, ** $p \leq 0.01$. B Survival analysis of patients from GSE22762 stratified based on LGALS1 and LAG3 expression. C LGALS1 and LAG3 mRNA levels in CLL patients. Schematic diagram of diverse clinical features and prognostic patterns in CLL samples. β2-MG, β2-microglobulin; del, deletion; IGHV, immunoglobulin heavy variable gene. D Gal-1 and LAG3 protein levels in four ibrutinib-sensitive (IBS) CLL patients and four ibrutinib-resistant (IBR) CLL patients. S, sensitive; R, resistant.

Figure 5

High LGALS1 and LAG3 expression in cells with acquired ibrutinib resistance. A Schematic diagram of ibrutinib-resistant chronic lymphocytic leukemia (CLL) cell line (MEC1-IR) establishment and cell viability throughout. B Relative proliferation of MEC1 and MEC1-IR cells treated with different ibrutinib concentrations for 48 h. IC₅₀ values were calculated from dose-response curves for four biologically-independent samples. The IC₅₀ for each cell line is indicated in parentheses. C, D MEC1 and MEC1-IR cell apoptosis following exposure to ibrutinib (ibrutinib-resistant; IBR) for 48 h. E-H LGALS1 and LAG3 RNA and protein levels in MEC1 and MEC1-IR cells estimated using qRT-PCR (E), western blotting (F), and ELISA (G, H). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

percentage of Annexin-V and PI double-negative cells. F Freshly isolated PBMCs from CLL patients were incubated with 10 μ M ibrutinib and 30 μ M OTX008 for 48 h. Cell viability was assessed using trypan blue staining. Cells with > 20 % cytotoxicity at 10 μ M ibrutinib were regarded as IBS (blue, n = 6) and those with < 20 % were IBR (red, n = 9). CLL, chronic lymphocytic leukemia; IBR, ibrutinib-resistant; IBS, ibrutinib-sensitive.

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