Comparative analysis of macrophage feeder systems reveals distinct behaviors and key transcriptional shifts in chronic lymphocytic leukemia cells via coculture

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Abstract

In this brief report, we evaluated various macrophage coculture systems for their CLL-feeding potential, phagocytosis capacity, induction of treatment resistance in CLL cells, and their impact on the transcriptional profiles of CLL cells.

Introduction

The development and progression of chronic lymphocytic leukemia (CLL) are driven not only by intrinsic properties of the leukemia cells but also by their complex interactions with the tumor microenvironment.[1] The myeloid compartment, particularly macrophages, plays a crucial role in driving CLL progression and therapy resistance.[2,3] Isolated blood monocytes differentiate *in vitro* under the influence of CLL cells into nurse-like cells (NLCs), which protect leukemic cells from spontaneous apoptosis[4] and promote multidrug resistance.[5] The importance of macrophages in CLL pathogenesis was demonstrated in mouse models by depleting macrophages via either CSF1R signaling blockade or liposomal clodronate, resulting in significant reductions of the leukemic burden.[6,7]

Despite evidence that macrophages play a crucial role in CLL progression, our understanding of the precise mechanisms by which these myeloid cells promote malignant cell survival remains incomplete. To dissect the complex molecular dialog between CLL cells and macrophages, both *in vivo* models and controllable *in vitro* systems are essential. Although some macrophage-CLL coculture systems have been developed,[4,8–10] there remains a need for systematic, simultaneous analyses of these diverse *in vitro* coculture systems. Therefore, we evaluated various macrophage coculture systems for their CLL-feeding potential, phagocytosis capacity, induction of treatment resistance in CLL cells, and their impact on the transcriptional profiles of CLL cells (Figure 1A, Table S1).

Results and Discussion

Several primary and cell line-based macrophage systems derived from human and mice were used. Human systems included primary healthy donor monocyte-derived macrophages (HD-

MDM) differentiated from peripheral blood mononuclear cells (PBMCs), NLCs generated from PBMCs of CLL patients, and the THP-1 cell line[10] that acquires a macrophage-like phenotype after differentiation with phorbol-12-myristate-13-acetate. Murine systems included primary bone marrow derived macrophages[10] (BMDMs), J774A.1[8] and MacCsf1r$^{+/+}$ macrophage[11] cell lines. Whereas primary and THP-1 macrophages do not proliferate after differentiation, J774A.1 cells exhibit robust proliferation and phagocytosis. Thus, J774A.1 macrophages were γ-irradiated to halt proliferation. All macrophage systems were cultured simultaneously with eight treatment-naïve CLL B cell patient samples (Table S2). CLL cell viability was measured over one week on days 0, 1, 3, 5, and 7 by flow cytometry. The viability of CLL cells without any feeding support decreased steadily. In contrast, all macrophage systems significantly supported CLL viability (Figure 1B). Due to the lower NLC count, CLL cells cultured with NLCs showed the lowest viability. When all other macrophage cells were seeded at the same density as the average NLC count (Table S3), the difference in CLL support narrowed considerably (Figure S1A). Interestingly, we observed that using either autologous or allogenic CLL-NLC pairs did not significantly impact CLL support (Figure S1B). After day 3 of coculture with MacCsf1r$^{+/+}$ macrophages, CLL viability started to decrease. In contrast, J774A.1, BMDM and THP-1 cells consistently maintained CLL cell viability throughout the 7-day period.

To assess spontaneous phagocytic capacity – a crucial feature of macrophages, all systems were cocultured with CLL cells for 18 h, and the remaining CLL cells were counted. While HD-MDMs, NLCs, and THP-1 macrophages showed only modest phagocytic activity, the murine macrophages exhibited high phagocytosis rates: non-irradiated J774A.1 cells demonstrated the highest phagocytic rate, followed by MacCsf1r$^{+/+}$ and BMDM (Figure 1C), implying that dead cell clearance by murine macrophages should be carefully considered in CLL coculture.

Antibody-dependent cellular phagocytosis (ADCP) is another key function of macrophages in immune defense, facilitating the targeted clearance of antibody-coated cells, including cancerous or infected cells. To assess ADCP, CLL-macrophage cocultures were exposed for 18 h to obinutuzumab – a monoclonal anti-CD20 antibody used in CLL treatment. Human

macrophage systems exhibited significantly higher ADCP rates than murine systems (Figure 1D), mainly influenced by the lower spontaneous phagocytosis without antibody addition. Altogether, spontaneous phagocytosis and ADCP assays highlighted fundamental differences between human and murine macrophages, and the importance of species-specific immune cell interactions.

Based on emerging evidence, we recently proposed that macrophages significantly influence the treatment outcome of CLL patients, particularly in response to first-line therapies.[3] To evaluate the suitability of the coculture systems in drug testing experiments, we treated the human macrophage-CLL cocultures with two commonly used inhibitors: the BCL2 inhibitor venetoclax and the BTK inhibitor ibrutinib. Ibrutinib significantly reduced the survival of CLL cells cocultured with HD-MDMs or NLCs, whereas coculture with THP-1 macrophages appeared to confer resistance to ibrutinib-induced CLL apoptosis, showing no remarkable drop in CLL viability (Figure 1E). Venetoclax was more potent in killing most CLL cells in both mono- and cocultures. However, THP-1 coculture again provided partial protection, resulting in higher CLL cell viability than in cocultures with other feeder systems (Figure 1E). Our finding contradicts a previous report, in which venetoclax-induced apoptosis levels were similar in monocultured- and THP-1 cocultured CLL cells,[9] reflecting possible variations in experimental protocols or read-out timing. Although neither ibrutinib nor venetoclax affected THP-1 viability at the concentrations tested, both inhibitors slightly reduced the viability of HD-MDMs and NLCs (Figures S1D-F). However, these insignificant differences in feeder viability are unlikely to account for the pronounced resistance mediated by THP-1 macrophages. Current studies have showed promising efficacy for immunotherapies such as epcoritamab – a CD3/CD20 bispecific antibody – for patients refractory to BTK and BCL2 inhibitors.[12,13] We tested epcoritamab in a mix culture of CLL-PBMCs with the human macrophage feeders. While HD-MDMs and autologous NLCs exhibited reduced CLL cell viability indicating an epcoritamab-dependent T cell-mediated killing, THP-1 cells abrogated this effect (Figure 1F).

To investigate the molecular mechanisms by which macrophages affect CLL cells, RNA from CLL cells of three patients after five days of coculture with HD-MDMs, THP-1 macrophages, NLCs, and BMDMs were collected and subjected to bulk mRNA sequencing. The transcriptional profile of CLL cells cultured with BMDMs was largely similar to that in monoculture (Figure S2A). In contrast, significant changes were detected in CLL cells cocultured with human macrophages, with 263 genes differentially expressed after coculture with HD-MDMs (Figure 2A), 192 with NLCs (Figure 2B), and 660 with THP-1 (Figure 2C). Of the altered genes, 25 genes overlapped across different systems (Figure S2B), whereas the majority were uniquely altered by each feeder system, underscoring their distinct effects on CLL cells.

By performing gene set enrichment analysis using the hallmark collection from the Molecular Signatures Database, we identified eight hallmarks enriched in all three human systems (Figure 2D). Notably, “inflammatory response”, “IL2 STAT5 signaling”, “IL6 JAK STAT3 signaling”, and “KRAS signaling” hallmarks were consistently upregulated in CLL cells upon coculture. Focusing on the crucial signaling pathways in B cell biology, we used a curated set of gene signatures related to signaling pathways, transcription factor regulation, and key cellular processes such as proliferation and metabolism known to influence CLL pathobiology (Supplemental Gene Set). Again, this analysis revealed an upregulation of “STAT3”, “IL6”, “RAS”, and “Proliferation” across all systems (Figure 2E). Additionally, after HD-MDM- and NLC cocultures, JAK signaling was also elevated in CLL cells, highlighting the IL6/JAK/STAT3 pathway as a key mechanism through which macrophages may drive CLL cell survival.

Activation of the JAK/STAT3 pathway contributes significantly to the formation of an inflammatory microenvironment and plays an important role in tumorigenesis.[14] In CLL, IL6 in both plasma and tissue microenvironment is crucial in supporting CLL cell survival. Constitutive activation of NFκB signaling in CLL cells induces IL6 production, which in turn activates the B cell receptor (BCR) and JAK2/STAT3 pathways, creating a feed-forward loop that promotes leukemogenesis.[15]

Elevated RAS signaling was observed in all cocultured CLL cells, revealing another pathway involved in macrophage-mediated support. Heightened activation of the RAS/MAPK pathway, often driven by RAS mutations in CLL, correlates with adverse clinical features and shorter treatment-free survival.[16,17] It is intriguing that macrophage contact may upregulate the RAS pathway in CLL cells, which warrants further investigation.

In transcriptome analyses of paired blood- and lymph node CLL samples, the lymph node environment was shown to enhance “Proliferation” signature, associated with increased BCR, NFκB, and NOTCH signaling in CLL cells.[18–20] In line with these observations, we detected elevated NFκB and NOTCH signaling in CLL cells cocultured with HD-MDMs (Figure 2E) and THP-1 macrophages (Figure 2D). Interestingly, BCR signaling was downregulated in CLL cells cocultured with HD-MDMs and NLCs (Figure 2E), suggesting that macrophage-mediated support operates independently of the BCR. This also indicates that macrophages alone are insufficient to induce the prominent lymph node signatures, underscoring the collaborative role of different microenvironmental components. Finally, the distinct signaling pathways identified across these macrophage systems point to the unique impact of each feeder system.

Conclusion

Collectively, all tested macrophage feeder layers effectively maintained CLL cell viability *in vitro* despite notable differences in their spontaneous- and antibody-dependent phagocytic capacity. Transcriptome analyses revealed that human macrophages prominently upregulate IL6/JAK/STAT3 and RAS signaling pathways in CLL cells. Furthermore, inhibitor-testing experiments highlighted that HD-MDMs mimic the functional and molecular influence of NLCs on CLL cells. Notably, among the tested systems, THP-1 macrophages induced the most substantial transcriptional changes in the CLL cells and conferred CLL resistance to treatment-induced apoptosis. Given the complexity of the CLL microenvironment and the pivotal role of macrophages in CLL pathogenesis and progression, our study highlights the importance of selecting an appropriate *in vitro* coculture system to obtain reliable mechanistic insights.

Authors' Contribution

V.K. conceptualized the study, performed experiments and analyses and wrote the manuscript. H.J. performed experiments and analyses and wrote the manuscript. R.R.R. performed computational analyses. T.T.T., R.Z., L.D.S., A.v.L., A.F.v.S. performed experiments. M.H. provided critical conceptual input and supervision. P.-H.N. conceptualized and supervised the study and wrote the manuscript. All authors read, revised and approved the manuscript.

Disclosures

All authors declare no conflict of interest.

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Figure 1A was created with BioRender.com.

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Figure Legends

Figure 1

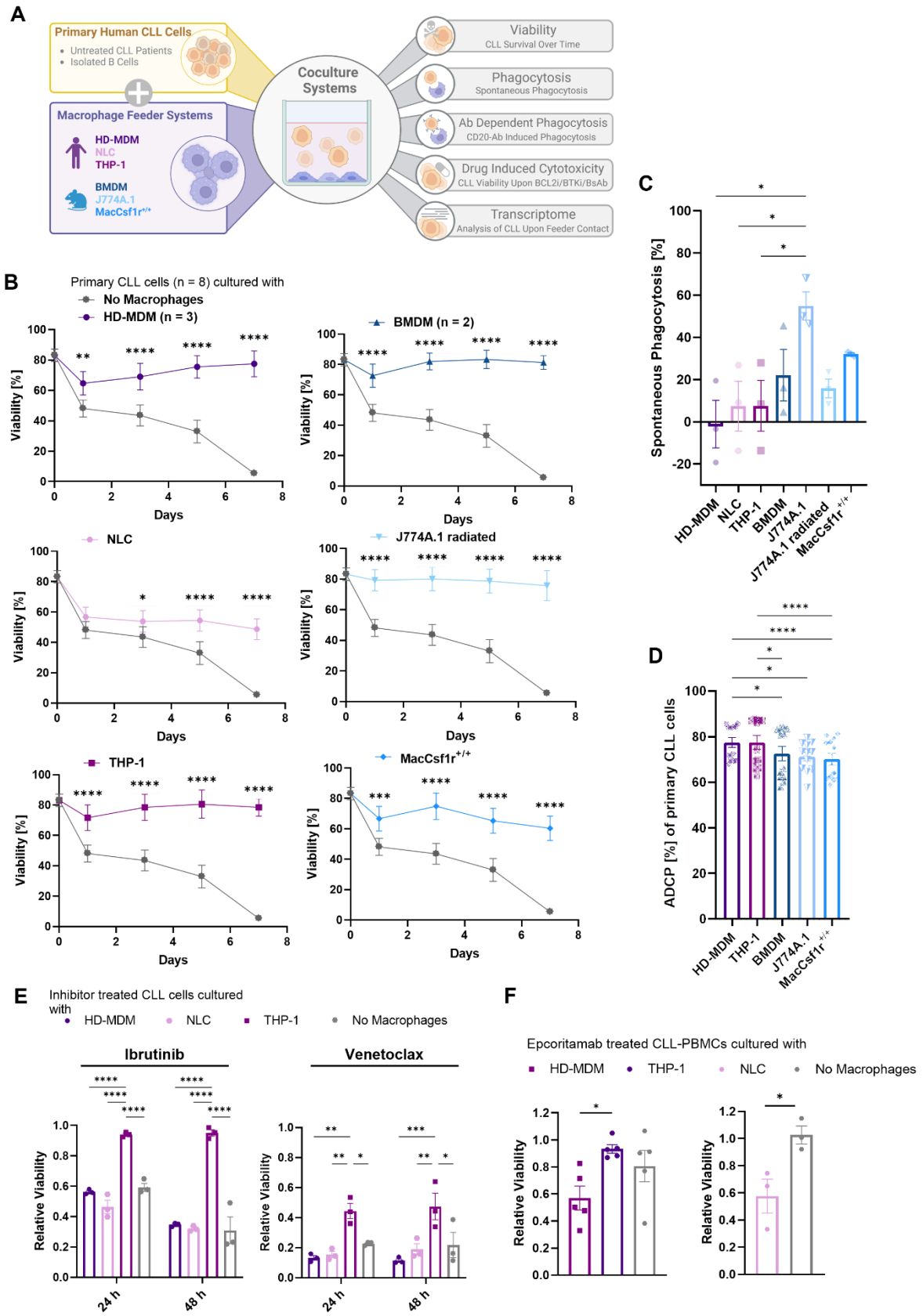
(**A**) Schematic overview of the macrophage systems and experimental readouts utilized in this study. (**B**) The viability of chronic lymphocytic leukemia (CLL) cells (n = 8) was assessed at 1, 3, 5, and 7 days in coculture with various macrophage feeder systems compared to CLL monoculture. Results are expressed as the percentage of viable CLL cells over time. All systems showed significantly prolonged CLL cell survival analyzed with a two-way ANOVA and Šídák's multiple comparisons test (p-values are listed in Table S4). (**C**) Phagocytosis of CLL cells (n = 3) after 18 h of coculture was quantified. Statistical analysis was performed using an ordinary one-way ANOVA followed by Tukey's multiple comparisons test to assess differences between groups. (p-values are listed in Table S5) (**D**) Obinutuzumab-dependent phagocytosis of primary CLL cells (n = 2) was evaluated after 18 h of coculture. Antibody-dependent cellular phagocytosis (ADCP) of primary CLL cells by human macrophages was significantly higher compared to murine macrophage systems. THP-1 cells also exhibited higher ADCP compared to murine systems. Statistical significance was determined via repeated measures ANOVA to account for differences between patients followed by Tukey's multiple comparisons (p-values are listed in Table S6). (**E**) The effect of 10 nM venetoclax and 10 µM ibrutinib on CLL viability was evaluated after 24 and 48 hours in both coculture and monoculture conditions. The percentage of viable cells was determined to assess treatment efficacy. Within each day, THP-1 showed significantly more survival support for CLL cells under treatment than other systems. Statistical testing was performed using a two-way repeated measures ANOVA test followed by Šídák's multiple comparisons test to account for different timepoints (p-values are listed in Table S7). (**F**) CLL cell viability was measured after 5 days of coculture of CLL PBMCs with different feeding systems under the presence of Epcoritamab (500 ng/ml). Viability is normalized to the viability of coculture without Epcoritamab. While NLCs (autologous setting) and HD-MDMs seem to have a proficient effect on T-cell killing, THP-1 macrophage coculture does not show reduced CLL cell viability.

Statistical testing for THP-1 vs HD-MDMs vs Monoculture was done using a one-way ANOVA test followed by Holm-Šídák's multiple comparisons test. NLC vs Monoculture was testing using a paired t-test (p-values are listed in Table S8).

Figure 2

Volcano plots depicting the log2 fold-change (FC) in gene expression from bulk RNA-seq analysis of CLL cells cocultured for 5 days with (**A**) HD-MDMs, (**B**) NLCs, and (**C**)THP-1 macrophages compared to CLL cells in monoculture. The y-axes represent the –log10 p-value. Differentially expressed genes (DEGs) are highlighted in color (q-value < 0.05 and 1 ≤ log2-FC ≤ −1). The top 10 genes ranked by q-value in each coculture system are labeled. (**D**) Heatmap of enriched hallmark pathways identified by the Gene Set Enrichment Analysis (GSEA) across the different systems. The bar plots adjacent to the heatmap represent the total number of genes contributing to each enriched term, with normalized enrichment scores (NSE) depicted as color gradients. (**E**) Heatmap of enriched gene signatures identified through GSEA using a curated list of B-cell-specific gene sets. The gene signatures are grouped into categories, with the names color-coded according to their respective categories. The bar plots adjacent to the heatmap represent the total number of genes contributing to each enriched term, with NES depicted as color gradients.

Figure 1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Isolated B Cells
Macrophage Feeder Systems
HD-MDM
NLC
THP-1
BMDM
J774A.1
MacCsf1r+/+
Coculture Systems
Viability
CLL Survival Over Time
Phagocytosis
Spontaneous Phagocytosis
Ab Dependent Phagocytosis
CD20-Ab Induced Phagocytosis
Drug Induced Cytotoxicity
CLL Viability Upon BCL2i/BTKi/BsAb
Transcriptome
Analysis of CLL Upon Feeder Contact
B
Primary CLL cells (n = 8) cultured with
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HD-MDM (n = 3)
BMDM (n = 2)
NLC
J774A.1 radiated
THP-1
MacCsf1r+/+
Viability [%]
Days
C
Spontaneous Phagocytosis [%]
HD-MDM
NLC
THP-1
BMDM
J774A.1
J774A.1 radiated
MacCsf1r+/+
D
ADCP [%] of primary CLL cells
E
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NLC
THP-1
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Ibrutinib
Venetoclax
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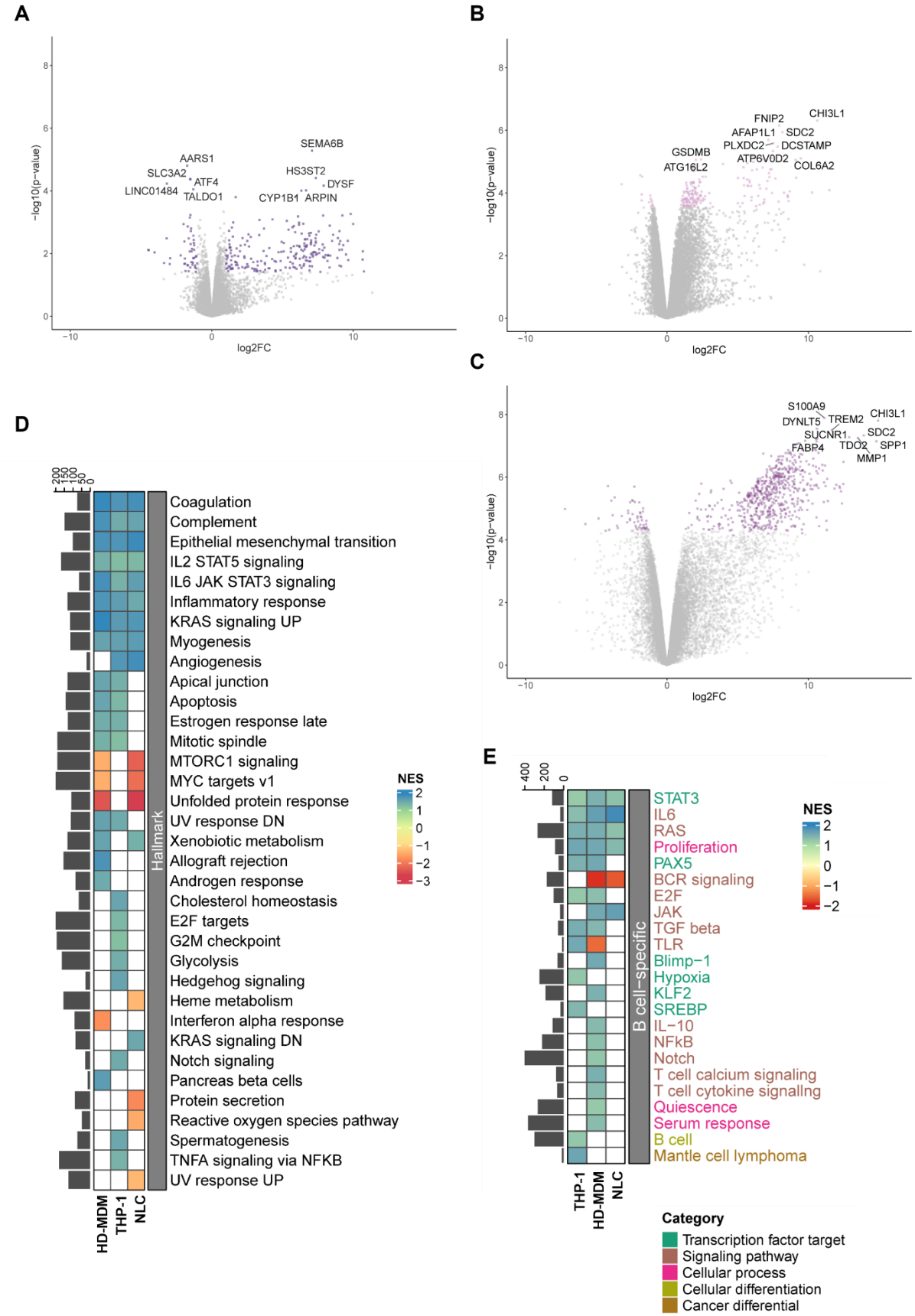
NLC
No Macrophages
Relative Viability

Figure 2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Hallmark
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Complement
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IL6 JAK STAT3 signaling
Inflammatory response
KRAS signaling UP
Myogenesis
Angiogenesis
Apical junction
Apoptosis
Estrogen response late
Mitotic spindle
MTORC1 signaling
MYC targets v1
Unfolded protein response
UV response DN
Xenobiotic metabolism
Allograft rejection
Androgen response
Cholesterol homeostasis
E2F targets
G2M checkpoint
Glycolysis
Hedgehog signaling
Heme metabolism
Interferon alpha response
KRAS signaling DN
Notch signaling
Pancreas beta cells
Protein secretion
Reactive oxygen species pathway
Spermatogenesis
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UV response UP
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THP-1
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