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LETTER

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

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The development and progression of chronic lymphocytic leukemia (CLL) are driven not only by the intrinsic properties of 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] 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] Macrophage depletion in vivo using CSF1R blockade or liposomal clodronate significantly reduced leukemic burden, demonstrating their important role in CLL pathogenesis.[6,7]

However, our understanding of the precise mechanisms by which macrophages promote CLL survival remains incomplete. To dissect the molecular dialog between CLL cells and macrophages, both in vivo models and controllable in vitro systems are essential. Although some macrophage–CLL coculture systems exist,[4,8–10] systematic, simultaneous analyses of these systems are lacking. Therefore, we evaluated various macrophage coculture systems for their CLL-feeding potential, phagocytosis capacity, induction of treatment resistance, and their impact on CLL transcriptional profiles (Figure 1A).

Several human and mice macrophage systems were used. Human systems included THP-1 macrophages[10] differentiated with phorbol-12-myristate-13-acetate, healthy donor monocyte-derived macrophages (HD-MDM) differentiated from peripheral blood mononuclear cells (PBMCs), and NLCs generated from CLL PBMCs. NLC purity was confirmed by flow cytometry and microscopy (Supporting Information: Figure S1). Murine systems included primary bone marrow-derived macrophages[10] (BMDMs), and J774A.1[8] and MacCsf1r$^{+/+}$ macrophage[11] cell lines. Whereas primary and THP-1 macrophages do not proliferate after differentiation, J774A.1 cells show 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 samples (Supporting Information: Table S1). CLL viability was measured on Days 0, 1, 3, 5, and 7 by flow cytometry. All macrophage systems significantly supported CLL viability throughout the 7-day period (Figure 1B) despite interpatient variability (Supporting Information: Figure S2A). Due to the lower NLC count, CLL viability was the lowest in NLC, but this difference narrowed considerably (Supporting Information: Figure S2B) when all macrophage systems were seeded at the same density as the average NLC count (Supporting Information: Table S3). Moreover, fresh and thawed CLL cells showed no significant difference in the viability in cocultures (Supporting Information: Figure S3A), and the coculture systems did not induce CLL proliferation (Supporting Information: Figure S3B). Interestingly, autologous versus allogenic CLL–NLC pairs showed no difference in viability support (Supporting Information: Figure S4), and M2 polarization of macrophages did not enhance CLL viability beyond M0 levels (Supporting Information: Figure S5).

To assess spontaneous phagocytic capacity, 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 modest phagocytosis, murine macrophages showed high rates, with non-irradiated J774A.1 cells demonstrating the highest rate, followed by MacCsf1r$^{+/+}$ and BMDM (Figure 1C), implying that CLL cell clearance by murine macrophages should be particularly considered.

To assess antibody-dependent cellular phagocytosis (ADCP), another key function of macrophages, CLL–macrophage cocultures were exposed for 18 h to the monoclonal anti-CD20 antibody obinutuzumab. We observed no significant difference in ADCP between the systems, except for irradiated J774A.1 (Figure 1D). When combined with the higher spontaneous phagocytosis of murine macrophages, these results suggest that human macrophages are more effective in ADCP than murine ones. Altogether, these assays highlighted fundamental differences between human and murine macrophages and the importance of species-specific immune cell interactions.

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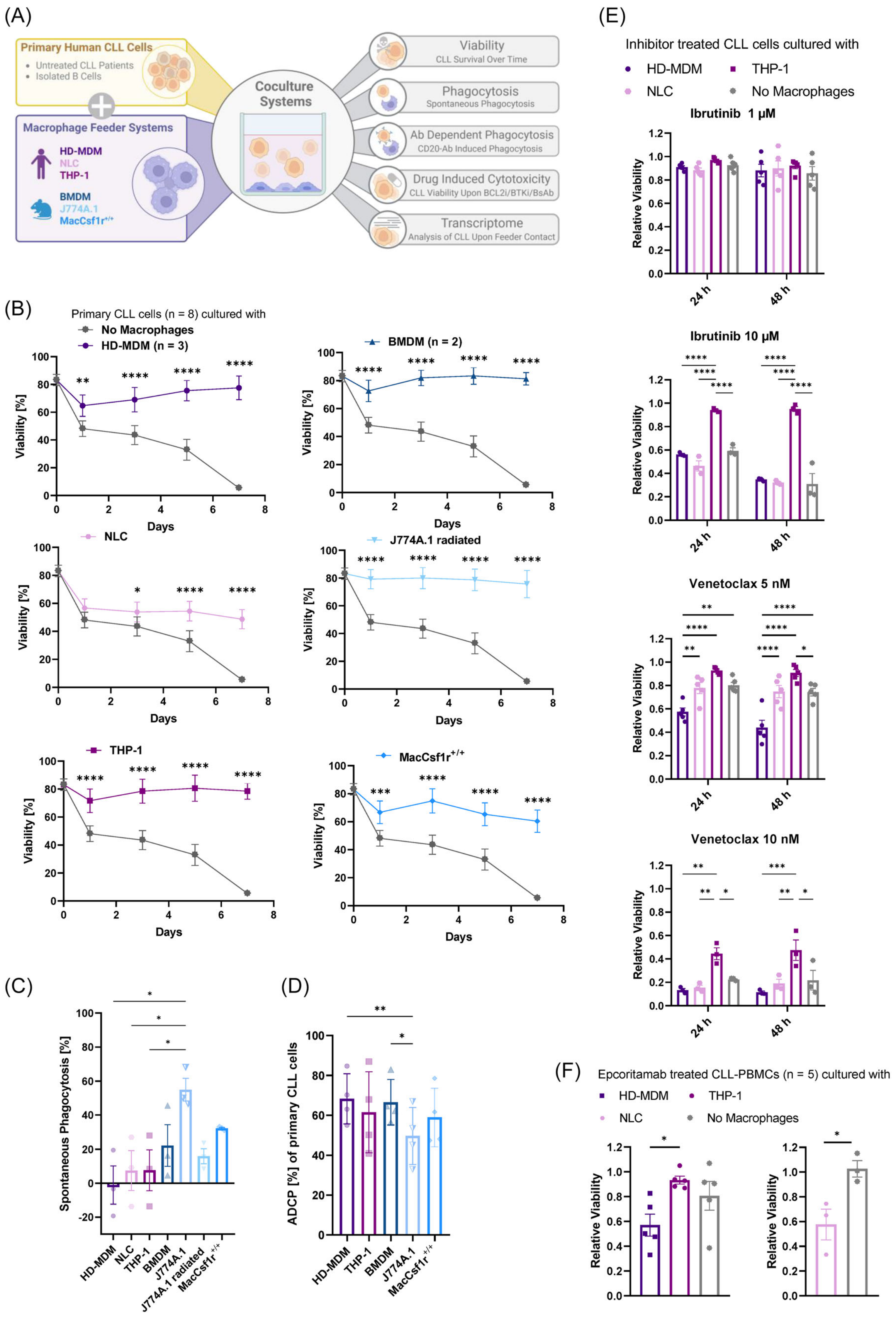

FIGURE 1 (See caption on next page).

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 by a two-way analysis of variance (ANOVA) and Šídák's multiple comparisons test (*p*-values are listed in Supporting Information: Table S1). **(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 Supporting Information: Table S13). **(D)** Obinutuzumab-dependent phagocytosis of primary CLL cells ($n = 4$) was evaluated after 18 h of coculture. 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 Supporting Information: Table S14). **(E)** The effect of venetoclax (5 and 10 nM) and ibrutinib (1 and 10 μM) on CLL viability was evaluated after 24 and 48 h in both coculture and monoculture conditions. The percentage of viable cells was determined to assess treatment efficacy. Statistical testing was performed using a two-way repeated-measures ANOVA test, followed by Šídák's multiple comparisons test, to account for different time points (*p*-values are listed in Supporting Information: Table S15). **(F)** CLL cell viability was measured after 5 days of coculture of CLL PBMCs ($n = 5$) with different feeding systems in the presence of Epcoritamab (500 ng/mL). Viability is normalized to the viability of coculture without Epcoritamab. Statistical testing for THP-1 versus HD-MDMs versus Monoculture was performed using a one-way ANOVA test, followed by Holm-Šídák's multiple comparisons test. NLC versus Monoculture was testing using a paired *t*-test (*p*-values are listed in Supporting Information: Table S16).

Emerging evidence suggests that macrophages significantly influence treatment outcome and therapy response of CLL patients.[3] To evaluate the suitability of the coculture systems in drug-testing experiments, we treated human macrophage–CLL cocultures with the BCL2 inhibitor venetoclax and the BTK inhibitor ibrutinib. High-dose ibrutinib (10 μM) significantly reduced the CLL cell survival cocultured with HD-MDMs or NLCs, whereas THP-1 coculture appeared to confer resistance to ibrutinib-induced CLL apoptosis, showing no remarkable decrease in viability (Figure 1E). Both low- and high-dose venetoclax were potent in killing most CLL cells in mono- and cocultures. However, THP-1 coculture again provided partial protection, resulting in higher CLL cell viability than other systems (Figure 1E). Aside from reduced HD-MDM viability at 10 μM ibrutinib, macrophage layers were not markedly affected by drug exposure (Supporting Information: Figures S6). Our finding demonstrates a more significant protection of THP-1 feeder for venetoclax-treated CLL cells than previously reported,[9] suggesting that larger cohorts are needed for a definitive conclusion.

Current studies have shown promising efficacy for the bispecific antibody epcoritamab for refractory patients.[12,13] We tested epcoritamab in a mixed culture of CLL-PBMCs with the human macrophage feeders. While HD-MDMs and autologous NLCs showed reduced CLL cell viability, indicating an epcoritamab-dependent T cell-mediated killing, THP-1 cells abrogated this effect, possibly due to the tumor origin of this cell line (Figure 1F).

To investigate the molecular mechanisms by which macrophages affect CLL cells, sorted CLL cells (Supporting Information: Figure S7) of three patients after 5 days of coculture with HD-MDMs, THP-1 macrophages, NLCs, and BMDMs were collected and subjected to bulk mRNA sequencing. CLL cells cultured with BMDMs showed minimal transcriptional changes (Supporting Information: Figure S8A), whereas coculture with human macrophages led to significant and distinct gene expression shifts: 192 with NLCs (Supporting Information: Figure S8B), 660 with THP-1 (Supporting Information: Figure S8C), and 263 with HD-MDMs (Supporting Information: Figure S8D), highlighting feeder-specific effects on CLL cells.

Gene set enrichment analysis using the hallmark collection from the Molecular Signatures Database identified eight hallmarks enriched in all three human systems (Figure 2A). Notably, “inflammatory response,” “IL2 STAT5 signaling,” “IL6 JAK STAT3 signaling,” and “KRAS signaling” 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 upregulation of “STAT3,” “IL6,” “RAS,” and “Proliferation” across all systems (Figure 2B). JAK signaling was also elevated after HD-MDM- and NLC coculture. Additionally, pathway enrichment analyses using the Gene Ontology and Reactome databases also highlight enhanced ERK/MAPK signaling in CLL cells after cocultures (Supporting Information: Figure S9). Altogether, these analyses highlighted the IL6/JAK/STAT3 and RAS/MAPK pathways as potential key mechanisms through which macrophages may drive CLL cell survival.

In CLL, constitutive activation of NFκB signaling in CLL cells induces IL6 production, which in turn activates the JAK/STAT pathways, creating a feed-forward loop that promotes leukemogenesis.[14] We could confirm the activation of the JAK/STAT3 pathway by immunoblotting of independent CLL samples after cocultures (Figure 2C and Supporting Information: Figure S10), showing increased phospho-STAT3 and phospho-JAK1 across patients in all cocultures. Phospho-JAK1 and total JAK2 levels varied strongly across patients.

Elevated RAS signaling was identified in all cocultured CLL cells, revealing another pathway involved in macrophage-mediated support. Heightened RAS/MAPK activation, often driven by RAS mutations in CLL, correlates with adverse clinical features and shorter treatment-free survival.[15,16] Immunoblotting also confirmed strongly enhanced phosphorylation of the RAS mediators MEK, ERK, and p38 MAPK across patients in THP-1 and HD-MDM cocultures, despite the absence of enhanced BCR activation based on phospho-SYK and phospho-BTK levels (Supporting Information: Figure S10), suggesting alternative upstream activation of ERK signaling such as RAS. Future studies should test whether JAK/STAT or MAPK blockade may disrupt CLL survival support.

While earlier findings have noted BCR activation upon NLC contact,[17] BCR signaling was downregulated in CLL cells cocultured with HD-MDMs and NLCs in our GSEA and in THP-1 coculture in immunoblot (Figure 2B and Supporting Information: Figure S10). This is further confirmed by our extended analysis across multiple time points and culture conditions (Supporting Information: Figure S11A), confirming enhanced BCR activation of CLL cells during the NLC generation phase (Supporting Information: Figure S11B), but unchanged or even reduced phospho-SYK and phospho-BTK during cocultures of allogeneic CLL cells with the fully differentiated macrophages (Supporting Information: Figure S11C). This finding illustrates the context-dependent nature of BCR regulation in CLL, and indicates that survival support provided by fully differentiated macrophages can occur independently of BCR activation.

The CLL lymph node environment was shown to enhance the “Proliferation” signature, associated with increased BCR, NFκB, and NOTCH signaling in CLL cells.[18–20] Although we detected elevated NFκB and NOTCH signaling in CLL cells cocultured with HD-MDMs and THP-1 macrophages (Figure 2A,B), the lack of BCR activation and leukemic proliferation in our systems indicates that macrophage cocultures are insufficient to induce the prominent lymph node signatures, underscoring the collaborative role of different microenvironmental components.

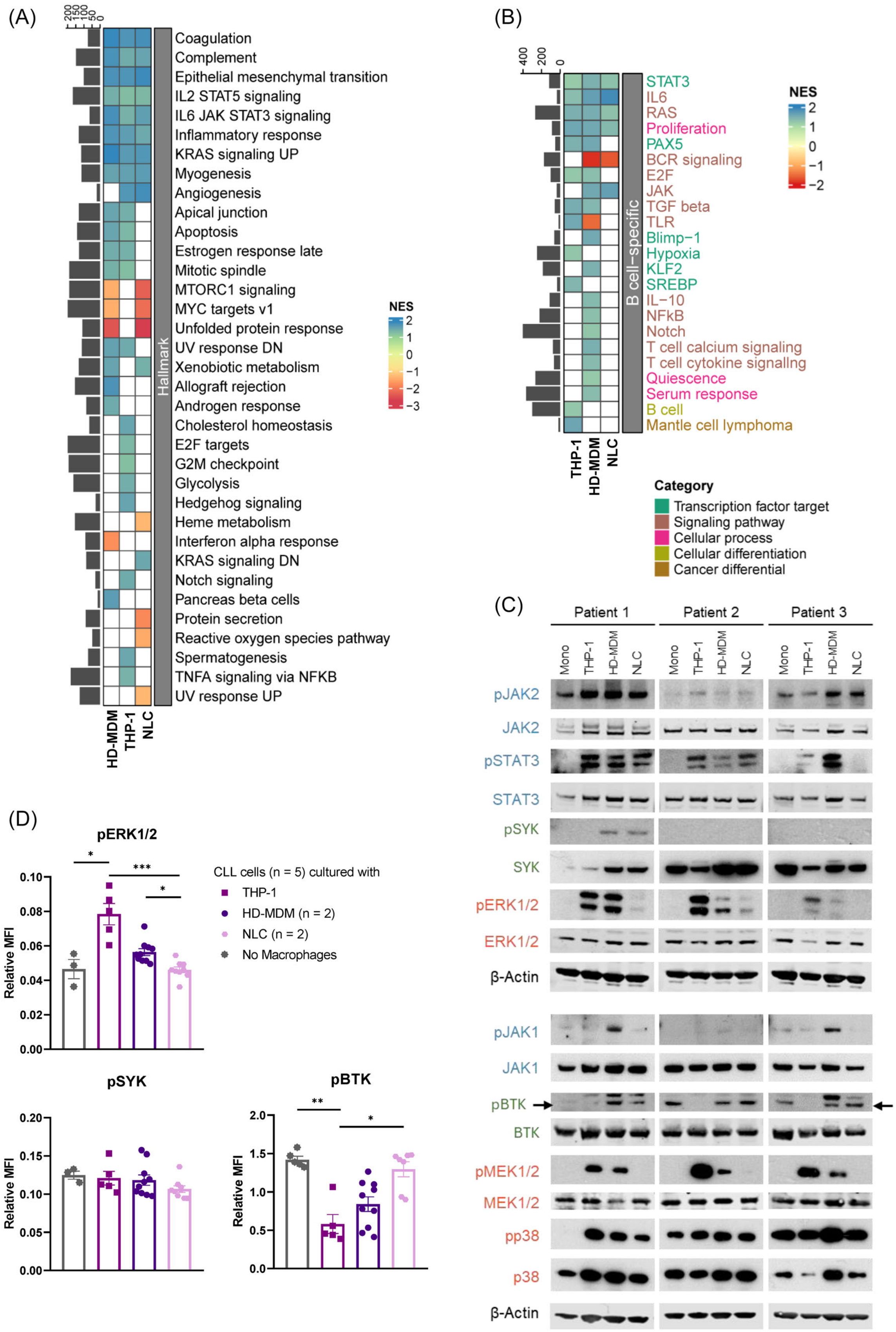

FIGURE 2 (See caption on next page).

FIGURE 2 (**A**) 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 (NES) depicted as color gradients. NES and adjusted *p*-values can be found in Supporting Information: Table S6. **(B)** 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. A positive score indicates enrichment in cocultured samples. NES and adjusted *p*-values can be found in Supporting Information: Table S7. **(C)** CLL cells ($n = 3$) were lysed after 5 days of monoculture or coculture with THP-1 cells, HD-MDMs, or NLCs and analyzed by immunoblotting. Blots were probed for phosphorylated and total forms of JAK2, STAT3, SYK, ERK1/2, JAK1, BTK, MEK1/2, and p38MAPK. β-Actin served as a loading control. Members of the JAK/STAT pathway are marked in blue, members of the RAS/MAPK pathway are marked in orange, and members of BCR signaling are marked in green. Quantification of western blot signals including 2 more patients is shown in Supporting Information: Figure S10. **(D)** CLL cells ($n = 5$) were analyzed after 5 days in monoculture or coculture with THP-1 cells, HD-MDMs, or NLCs using phospho-flow cytometry. Levels of pSYK, pBTK, and pERK1/2 were normalized to the respective total protein levels. Statistical analysis was performed using a Kruskal–Wallis test, followed by Dunn's multiple comparisons. (*p*-values are reported in Supporting Information: Table S17).

Collectively, all tested macrophage feeders effectively maintained CLL cell viability in vitro, despite notable differences in phagocytosis, ADCP capacity, or drug responses. Our transcriptomic and protein-level analyses revealed that human macrophages prominently upregulate JAK/STAT and MAPK pathways in CLL cells. Given the complexity of the CLL microenvironment and the pivotal role of macrophages in disease pathogenesis, our study highlights the importance of selecting an appropriate coculture system to obtain reliable mechanistic insights. THP-1 macrophages induced the strongest transcriptional changes in the CLL cells and conferred resistance to inhibitors, warranting caution in drug-testing applications. Notably, HD-MDMs closely mirrored NLC behaviors across our assays, supporting their use as a more accessible alternative model. Cross-species systems should be interpreted with caution due to elevated spontaneous phagocytosis. Finally, future studies examining macrophage polarization and the feeder system-specific differences in gene expression and cytokine production can further refine model selection and mechanistic understanding.

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AUTHOR CONTRIBUTIONS

Viktoria Kohlhas: Conceptualization; investigation; funding acquisition; writing—original draft; methodology; writing—review and editing; project administration; visualization; data curation. **Hendrik Jestrabek**: Investigation; methodology; writing—original draft; writing—review and editing; visualization; data curation. **Rocio Rebollido-Rios**: Investigation; writing—review and editing; formal analysis; data curation; visualization. **Thanh Tung Truong**: Investigation; writing—review and editing. **Rebekka Zölzer**: Investigation; writing—review and editing. **Luca D. Schreurs**: Visualization; investigation; writing—review and editing. **Duc Pham**: Investigation; writing—review and editing. **Alexander F. vom Stein**: Visualization; writing—review and editing; investigation. **Michael Hallek**: Resources; supervision; writing—review and editing; funding acquisition. **Phuong-Hien Nguyen**: Conceptualization; funding acquisition; writing—original draft; writing—review and editing; methodology; project administration; supervision; resources.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the BioStudies database (http://www.ebi.ac.uk/biostudies) at https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-15 353.

ETHICS STATEMENT

All ethics approvals and patient consents are provided in the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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