

Transcriptomic and phenotypic analyses reveal time-qualified patterns featuring Chimeric Antigen Receptor-T cells (CAR-T) dynamics

Emanuele Murgo

`emanuele.murgo@unifg.it`

Fondazione IRCCS Casa Sollievo della Sofferenza

Elisabetta de Santis

Fondazione IRCCS Casa Sollievo della Sofferenza

Tommaso Colangelo

University of Foggia

Virginia Manuti

University of Foggia

Anna Alessia Saponaro

University of Foggia

Costanzo Padovano

Fondazione IRCCS Casa Sollievo della Sofferenza

Gaetano Serviddio

University of Foggia

Erich Piovan

Istituto Oncologico Veneto

Vincenzo Giambra

Fondazione IRCCS Casa Sollievo della Sofferenza

Gianluigi Mazzocchi

Fondazione IRCCS Casa Sollievo della Sofferenza

Short Report

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Abstract

Circadian rhythms regulate immune function, yet their role in CAR-T efficacy is unclear. We profiled CD7-CAR T cells across a 24-hour time-course by RNA-sequencing and compared findings against CYCLOPS-inferred circadian phase in the independent Mullighan T-ALL cohort (n = 1,334). CD7-CAR T cells showed a reorganized circadian oscillator, with immune pathways (TNF- α , interferon- γ , IL2-STAT5) peaking in a late-day window, closely mirrored in patient-derived leukemic samples. CAR-T-intrinsic clock biology may drive time-of-day-dependent efficacy, supporting infusion timing as a treatment personalization strategy.

MAIN TEXT

Chimeric antigen receptor (CAR) T-cell therapy is a form of adoptive cellular immunotherapy in which T lymphocytes are genetically engineered to recognize and eliminate cancer cells independently of MHC restriction, overcoming the frequent failure of endogenous T cells to clear malignant cells^{1–3}. Several CAR-T products are currently approved for hematologic malignancies⁴.

Immune system function displays 24-hour (circadian) rhythmicity in the number and activity of immunocompetent cells^{5,6}, generated by molecular clockworks present in virtually all cells, including lymphoid organs and white blood cells⁷. At the intracellular level, the transcription factors ARNTL and CLOCK drive expression of PERIOD (PER1-3) and CRYPTOCHROME (CRY1-2), which in turn repress ARNTL/CLOCK activity, forming an autoregulatory feedback loop that governs rhythmic expression of thousands of genes.^{8,9}

Time of day has emerged as a modifiable factor affecting CAR-T cell clinical performance, with morning infusions associated with higher overall survival and lower neurotoxicity^{10,11}.

We investigated the underlying transcriptional and phenotypic mechanisms that could support circadian-informed CAR-T cell delivery.

CD7-CAR T cells acquire enhanced circadian rhythmicity. To characterize circadian regulation in engineered T cells, CD7-CAR T cells and empty vector (EV) controls were synchronized by serum shock and sampled every 6 hours over a full 24-hour cycle (T0, T6, T12, T18, T24) for RNA sequencing (Fig. 1a). Hierarchical clustering of global gene expression separated CD7-CAR T and EV samples independently of time point, indicating that CD7-CAR expression, rather than time-of-day, is the dominant source of transcriptional variance between samples (Fig. 1b). Principal component analysis reinforced this pattern, with CD7-CAR T and EV samples occupying non-overlapping regions of PCA space across all time points (Fig. 1c). At the level of the core molecular clock, pairwise correlation analysis of core clock gene (CCG) expression including *ARNTL*, *CLOCK*, *PER1-3*, *CRY1-2*, *NR1D1-2*, and related regulators, revealed a substantially more coordinated correlation structure in CD7-CAR T cells than in EV controls (Fig. 1d), suggesting that CD7-CAR expression reinforces the endogenous oscillator. This transcriptional divergence was accompanied by consistent numbers of differentially expressed genes (DEGs, $q < 0.05$)

between CD7-CAR T and EV cells at every time point, with the largest number of DEGs detected at T0 and T24 (Fig. 1E), suggesting that the extent of transcriptional divergence between the two cell populations followed a circadian trajectory.

Immune and proliferative pathways show time of day-dependent enrichment in CD7-CAR T cells.

Having established that the circadian clock is functionally active in both conditions, but more coordinated in CD7-CAR T cells, we next asked whether this pattern extends to the downstream transcriptional program. A temporal structure was evident at the global gene expression level: MetaCycle analysis¹³ showed a broader, evenly dispersed phase distribution in control cells (EV) respected to a concentrated peak in the late time window (16–20 h) observed in CD7-CAR T cells (Fig. 2a).

To identify the pathways underlying this temporal organization we performed Gene Set Enrichment Analysis (MSigDB)¹⁸ on genes differentially expressed between CD7 CAR-T and EV cells at each time point, which displayed enrichment of hallmark immune and proliferative gene sets (TNF- α /NF- κ B, IL2-STAT5, interferon- γ response, inflammatory response, allograft rejection, MYC targets, E2F targets, G2M checkpoint; Fig. 2b), with enrichment degree and significance varied substantially across the 24h time-course. Focusing on genes common to all time points within the TNF- α /NF- κ B, IL2-STAT5, and interferon- γ pathways -encompassing both up and down regulated transcripts- revealed oscillatory expression across the 24-hour cycle, corroborating these findings at the single-gene level (Fig. 2c). We next asked whether this pathway level divergence reflects circadian coordination, rather than a static difference in expression by applying phase set enrichment analysis (PSEA)¹⁶, which confirmed hallmark pathways of CD7-CAR T and EV cells peaked at distinct circadian phases (Fig. 2d). Notably, several of these pathways, including TNF- α /NF- κ B signaling, interferon- γ response, allograft rejection, and MYC targets, overlapped with those independently identified by MSigDB (Fig. 2b), indicating that this convergent set of pathways is not only differentially regulated between the two populations, but is organized in a coordinated circadian manner.

CD7-CAR T effector function is under circadian control. As a functional validation of these transcriptional programs, cytotoxic activity, cytokine secretion, and exhaustion phenotype were assessed by flow cytometry across the 24 hours time-course. Rhythmicity outline and significance were assessed by fitting a cosinor model to each parameter raw data. Cytotoxic activity oscillated significantly in both EV (acrophase 4.9 h, $p = 0.015$) and CD7-CAR T cells (acrophase 6.7 h, $p = 0.027$), with markedly greater amplitude in CD7-CAR T cells (Fig. 3a). TNF- α secretion showed significant rhythmicity only in CD7-CAR T cells (acrophase 15.7 h, $p = 0.041$; EV: $p = 0.176$) (Fig. 3b), while IFN- γ secretion showed an oscillatory pattern in CD7-CAR T cells (acrophase 17.0 h, $p = 0.0022$), markedly more robustly than in EV cells, which showed a trend at a closely aligned phase (acrophase 17.3 h, $p = 0.060$) (Fig. 3c). Strikingly, in CD7 CAR-T cells, both TNF- α and IFN- γ secretion peaked close to the 16-20h phase windows identified by PSEA (Fig. 2d), indicating concordance between transcriptional and functional circadian organization that was not observed in controls. Exhaustion marker expression showed a similar time-of-day trend in both cell populations, (EV: acrophase 7.7 h, $p = 0.053$; CD7-CAR T: acrophase 8.9 h, $p = 0.067$) (Fig. 3d), while the proportion of cytotoxic T cells varied by time of day cosinor parameter without reaching significance in

both cell populations. (EV: $p = 0.084$; CD7-CAR T: $p = 0.091$) (Fig. 3e). Collectively, these data showed that CD7-CAR T cells effector function fluctuates rhythmically across the day, with more robust rhythmicity than the EV control cells across tested readouts.

Circadian phase is prognostic in a large T-ALL patient cohort. To assess clinical relevance, we utilized the publicly available Mullighan cohort (T-ALL patients)¹⁴ selected for its large sample size ($n = 1,334$), treatment-naïve status at sample collection, and availability of matched clinical outcome data, allowing us to test whether the circadian organization identified in our CD7-CAR T model reflects biological mechanisms rather than a feature restricted to CAR-engineered cells. Because sample collection times were not recorded, circadian rhythms could not be reconstructed using the time-based approach applied to our in vitro model. We therefore inferred circadian phase using CYCLOPS¹⁷, an algorithm with potential application in circadian medicine¹⁹. Inferred phases showed a defined core clock gene correlation structure and a non-uniform distribution across the 24-hour cycle (Fig. 4a-b), supporting the biological validity of the phase assignment. PSEA identified enrichment of hallmark pathways strikingly similar to the in vitro CD7-CAR T model (IL2-STAT5, TNF- α /NF- κ B, MYC targets, interferon- γ response), concentrated within the 20–24 h phase window (Fig. 4c), indicating that the experimentally identified circadian program is also detectable in patient-derived leukemic samples. Stratifying patients by circadian phase quartile (Q1-Q4), overall survival did not differ significantly ($p = 0.122$), but event-free survival was significantly worse in quartile Q4 versus the remaining quartiles ($p = 0.011$) (Fig. 4d). For each pathway shared between the in vitro and patient-derived PSEA analyses, we calculated the phase difference (Δt) between its acrophase in the experimental system and its acrophase in the Mullighan cohort. Comparing peak phases (Δt) of shared pathways between systems revealed pathway-specific concordance for CAR-T cells, but no for control cells (Fig. 4e), suggesting that some, but not all, circadian-regulated programs are preserved during CAR-T engineering. Together, these findings suggest that circadian phase at diagnosis is not merely a passive biomarker, but reflects a biologically active state, partially conserved between the experimental and clinical systems -possibly related to the same circadian dynamics associated with CAR-T efficacy when infusion timing is considered- reinforcing the rationale for circadian-informed treatment timing in adoptive cell therapy.

CAR T-cell therapy success rates remain variable and achieving durable responses represents a major clinical challenge^{20,21}. A growing body of evidence points to the temporal dimension as a relevant determinant of efficacy across different immunotherapy modalities, including immune checkpoint inhibitors and CAR-T cell therapy²², positioning chronotherapy as increasingly paradigm in cancer immunotherapy.

In large B-cell lymphoma patients cohort, earlier (morning) CAR T-cell infusion has been independently associated with improved progression-free survival, while later delivery was linked to reduced expansion and increased inflammatory toxicity⁴. These associations have so far been interpreted primarily through recipient physiology. Our data suggest an additional explanation: the CAR-T product itself appears to retain a reorganized, functionally active circadian architecture, with greater rhythmicity and amplitude

across multiple effector readouts as cytotoxicity, TNF- α , and IFN- γ secretion, compared to unmodified T cells. This raises the possibility that CAR engineering does not simply preserve the endogenous oscillator, but reinforces its functional output, suggesting that the CAR-T product itself, and not only the recipient, may represent a relevant target for circadian-informed intervention.

The concordance between pathways enriched in our engineered CD7-CAR T cells and those associated with clinical outcome in the independent, treatment-naïve Mullighan cohort is, one of the more notable observations of this study. Circadian phase at diagnosis was associated with event-free survival, with patients in the fourth phase quartile (N = 333 per quartile) having significantly worse outcomes ($p = 0.011$), and the hallmark pathways underlying this association closely resembled those identified in our in vitro model. This convergence, observed across two independent systems supports the biological relevance of circadian organization in T-ALL and its CAR-T-directed treatment, and suggests that circadian phase- defined by a coherent, pathway signature- may hold value as a stratification variable in this setting, pending further validation.

Several limitations warrant consideration. The in vitro model relies on a single donor-independent cell system, and the Mullighan cohort is cross-sectional in nature, precluding direct causal inference between circadian phase and outcome.

Taken together, these findings support a model in which CAR-T cell-intrinsic circadian biology, together with host physiology, may contribute to the time of day- dependency of clinical outcomes already reported for adoptive cell therapy. Because both patient and product appear to carry exploitable circadian signals, treatment timing could potentially be optimized without modifying the CAR-T product itself, simply by aligning infusion with a favorable circadian phase for both host and cell therapy, positioning infusion timing as a low-cost, readily testable chronotherapeutic strategy for adoptive cell therapy, warranting prospective validation.

MATERIALS AND METHODS

Generation and synchronization of CD7-CAR T cells

Human peripheral blood pan-T cells (StemCell Technologies) were expanded for three days in TexMACS™ Medium (Miltenyi Biotec) with T-cell TransAct™, then underwent CRISPR/Cas9-mediated CD7 knockout to prevent fratricide, as previously described¹⁰ (efficiency confirmed by flow cytometry). Cells were transduced with a lentiviral vector encoding a second-generation CD7-CAR, with CAR expression assessed via the co-expressed GFP reporter.

Cells were synchronized by serum shock¹² (2 h in 50% serum, then serum-free medium, defining T0) and sampled at 0, 6, 12, 18, and 24 h post T-0.

Total RNA isolation and sequencing

Total RNA was isolated using the RNeasy Mini kit (Qiagen) and quantified via Equalbit dsDNA BR Assay Kit (Vazyme). For RNA-seq of CAR-T EV and CD7-CAR T cells, libraries were prepared from 500 ng of total RNA (VAHTS Universal V10 RNA-seq Library Prep Kit, Vazyme) and sequenced on a DNBSeg G-400 (MGI Tech) using a paired-end, 100-cycle strategy; raw data were analyzed with the CLC Genomics Workbench 26 (Qiagen) RNA-seq pipeline.

Killing assay and flow cytometry

Cytotoxic activity was assessed by co-culturing CAR-expressing T cells with CD7 high BFP-labeled target cells (CUTLL-1, 3:1 target: effector ratio) in TexMACS Medium supplemented with IL-7/IL-15 (40 IU/mL each), comparing GFP+/BFP+ populations at baseline and 24 h across all time points.

Surface immunophenotyping (CD3, CD4, CD8, CD45, LAG-3, PD-1, TIM-3) and viability (PI/DAPI/DRAQ7 exclusion) were assessed by flow cytometry; intracellular IFN- γ and TNF- α were detected after fixation/permeabilization. Acquisition and sorting were performed on a BD FACSCanto™ II and MoFlo Astrios™ (Beckman Coulter), respectively, with data analyzed in FlowJo (v10.10.1) following standard doublet, morphological, and viability-based gating.

The exhaustion score was calculated as the sum of PD-1, LAG-3, and TIM-3 MFI, each normalized to GFP MFI (a surrogate for CAR expression) to restrict the readout to bona fide CAR+ cells and correct for inter-experimental variability:

$$[(\text{MFI PD-1} / \text{MFI GFP}) + (\text{MFI LAG-3} / \text{MFI GFP}) + (\text{MFI TIM-3} / \text{MFI GFP})]^{10}$$

Circadian analyses

Rhythmic transcripts (24 ± 4 h periodicity) were identified using the MetaCycle R package¹³ applied to normalized expression data for cell lines and median-normalized data for Mullighan cohort¹⁴ (period range 20–28 h; significant rhythms at $p < 0.05$). Cosinor analysis was additionally used to model rhythmicity and generate cosinor graphs for selected transcripts and functional readouts¹⁵.

Circadian-related pathways were determined by Phase Set Enrichment Analysis (PSEA)¹⁶ on statistically significant ($q < 0.05$) circadian transcripts against MSigDB gene sets. Acrophases (rounded to the full hour) were compared to a uniform background distribution to identify significant circadian-related gene sets ($q < 0.05$).

Circadian phase in the cross-sectional, unsynchronized Mullighan/Polonen T-ALL cohort ($n = 1,334$) was estimated using CYCLOPS¹⁷ an algorithm reconstructing a circular sample ordering along an inferred circadian axis based on core clock gene covariation, without requiring known collection times. Each sample's continuous phase estimate ($0-2\pi$) was converted to an estimated internal time-of-day ($0-24$ h).

Declarations

Ethics approval and consent to participate

Not applicable. The study was conducted according to the guidelines of the Declaration of Helsinki and European legislation, with no need for approval by any local ethics committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no conflicts of interest with respect to the authorship and/or publication of this article.

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Author Contribution

GM, EM, VG, conceived the study; EM, EdS, TC, VM performed in vitro experiments and acquired data; VG, EP, CP, EM, TC performed statistical and bioinformatics analysis; VG, CP, VM, EP, EM, AAS, GS, TC, GM analyzed data and wrote the manuscript. All authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved. All authors have read and approved the journal's authorship agreement.

Data Availability

Raw and processed RNA-sequencing data generated in this study are available in the Gene Expression Omnibus (GEO) repository (accession number GSE346256). Gene expression and clinical outcome data for the Mullighan T-ALL cohort were obtained as described in the original publication by Pölönen et al., where data access details are provided.

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Figures

Figure 1

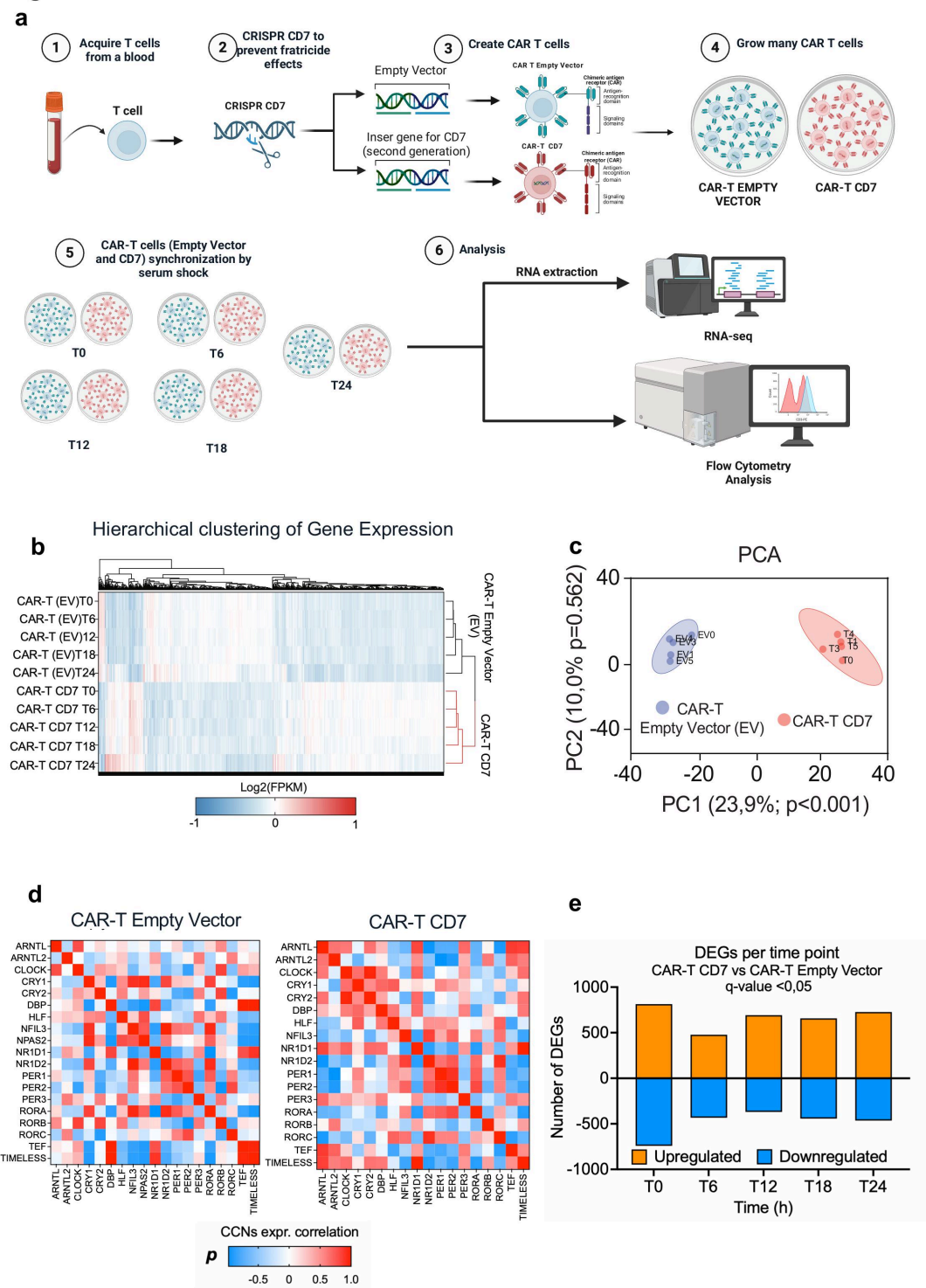


Figure 1

Experimental design and global transcriptomic characterization of CD7-CAR T cells versus empty vector controls. (A) Workflow: CD7 knockout (CRISPR/Cas9, to prevent fratricide) followed by transduction with second-generation CD7-CAR or empty vector (EV); both synchronized by serum shock and sampled T0-T24 for RNA-seq/flow cytometry. (B-C) Hierarchical clustering and PCA showing

EV/CD7-CAR T separation across all time points. (D) Correlation heatmap of core clock gene (CCG) expression, EV (left) vs CD7-CAR T (right). (E) DEGs ($q < 0.05$) in CD7-CAR T vs EV at each time point.

Figure 2

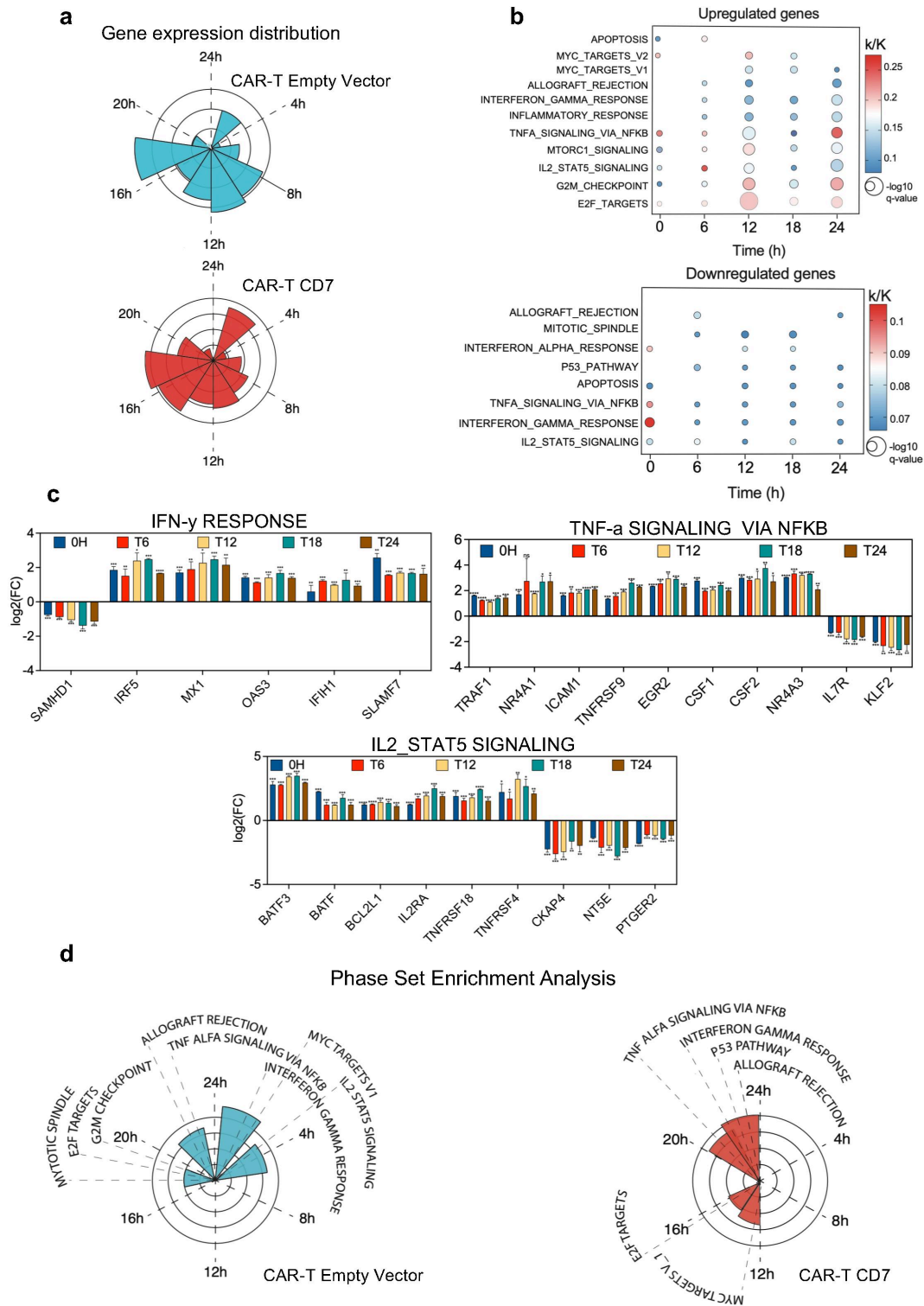


Figure 2

Temporal dynamics of transcriptional programs and phase set enrichment analysis.(A) Radar plot of gene acrophase distribution (MetaCycle), EV vs CD7-CAR T; petal length and size reflects the density of

rhythmic transcripts peaking within each bin. (B) Bubble plot of hallmark pathways enriched in CD7-CAR T vs EV per time point; size = $-\log_{10}(\text{q-value})$, color = k/K ratio. (C) Expression profiles (log2 fold-change) of TNF- α /NF- κ B, IL2-STAT5, and interferon- γ signature genes across time points; data are presented as the mean $\pm$ SD. ns, not significant. * $p < 0.005$, ** $p < 0.01$, *** $p < 0.001$. P values were calculated using Student's t-test. (D) PSEA of hallmark pathways, EV (left) vs CD7-CAR T (right), peak phase on polar axis; size = vector-average magnitude.

Figure 3

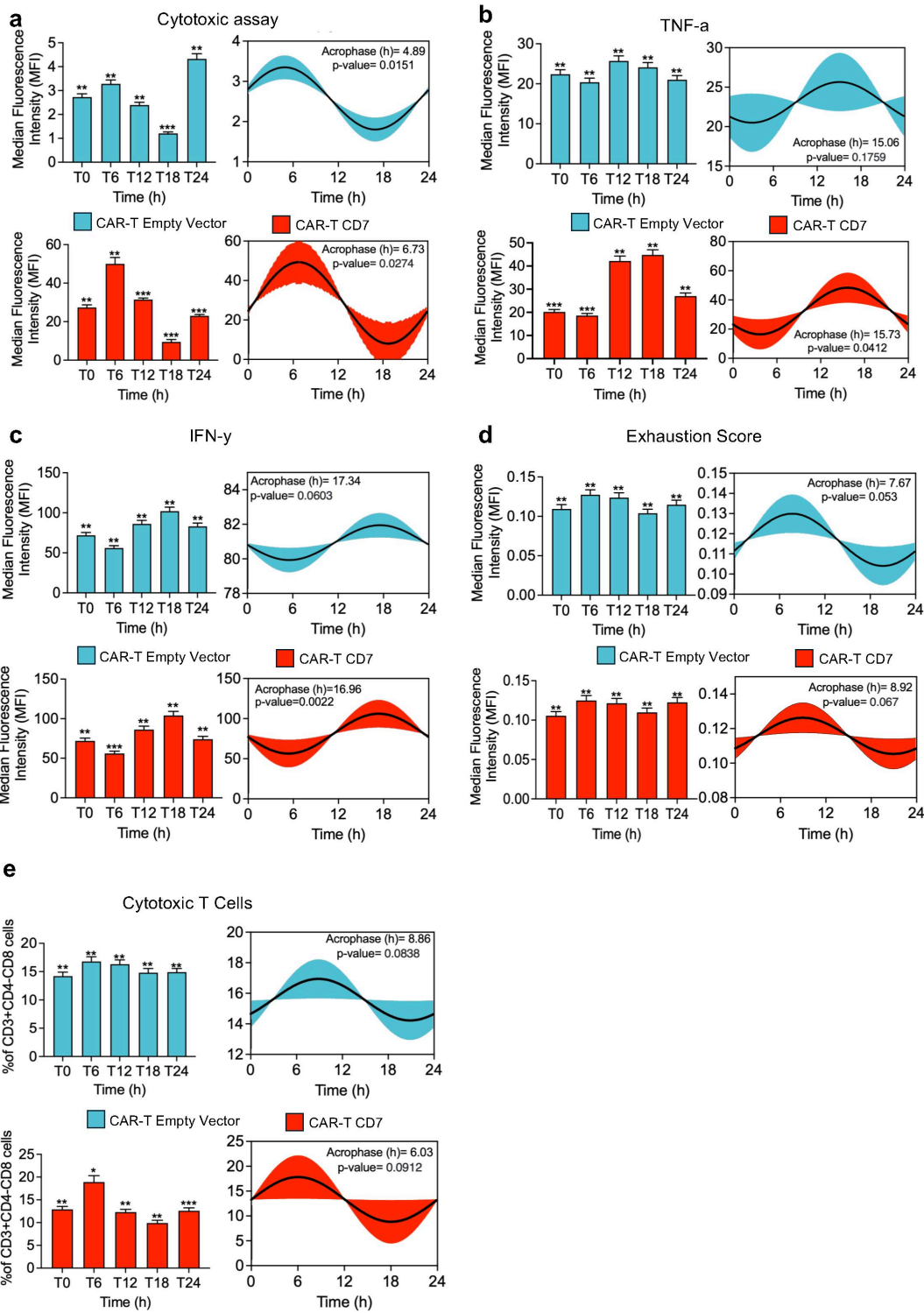


Figure 3

Circadian rhythmicity of CD7-CAR T effector function. (A) Cytotoxic activity, (B) TNF- α , (C) IFN- γ , (D) exhaustion score, (E) proportion of cytotoxic T cells in EV vs CD7-CAR T across time points, with MFI values (bars) and cosinor fit/acrophase/p-value (curves) for EV (teal) and CD7-CAR T (red). Data are presented as the mean $\pm$ SD. ns, not significant. * $p < 0.005$, ** $p < 0.01$, *** $p < 0.001$. P values were calculated using Student's t-test.

Figure 4

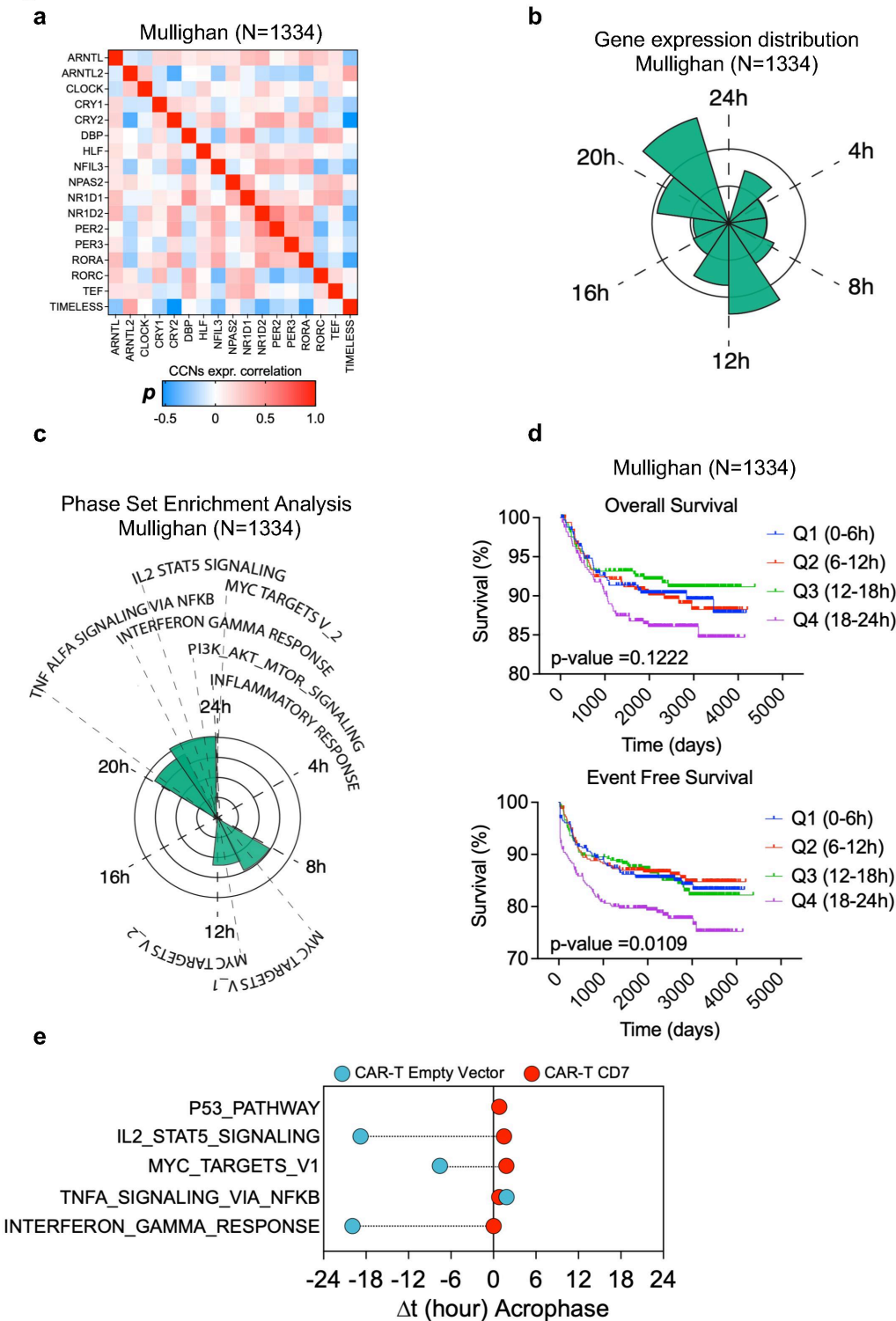


Figure 4

Clinical relevance of circadian phase in a T-ALL cohort (Mullighan, n=1,334). (A) Core clock gene correlation heatmap. (B) Radar plot of CYCLOPS-inferred phases; petal length and size reflects the density of rhythmic transcripts peaking within each bin. (C) PSEA of hallmark pathways. (D) Kaplan-Meier overall (top) and event-free (bottom) survival by circadian phase quartile (Q1-Q4, 6-h bins); p values were determined by log-rank test (E) Peak phase difference (Δt) vs Mullighan cohort for selected (PSEA) pathways, EV (teal) vs CD7-CAR T (red).