

Research Article

TOX Phase Separation Locks T Cells in a Terminal Exhausted State, and Disrupting It Can Revive Anti-Tumor Immunity

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Abstract

One major obstacle limiting the efficacy of tumour immunotherapy is terminal exhaustion of T cells. Although the molecular mechanisms sustaining the tired state are still unclear, the transcription factor TOX is thought to be a crucial driver of this process. The purpose of this study was to determine whether TOX can “lock in” terminal fatigue by forming condensates through liquid-liquid phase separation and to evaluate the possibility of targeting phase separation to restore anti-tumor immunity. We discovered that the N-terminal intrinsically disordered region (IDR) of TOX can spontaneously form droplet-like condensates through sequence analysis and in vitro recombinant assays. This property is markedly amplified in the nucleus of tired T cells after persistent antigen stimulation. We verified that TOX condensates are dynamic and capable of enriching transcriptional co-repressor complexes using methods such as optogenetic clustering and fluorescence recovery following photobleaching. By creating a phase-separation-deficient mutant without the IDR (TOX-IDRm) and adoptively introducing it into tumor-bearing mice, the percentage of terminally fatigued T cells was considerably decreased while effector cytokine production and proliferation were restored. TOX condensates directly occupy and maintain chromatin in an open state at important exhaustion genes like *Pdcd1* and *Havcr2*, according to single-cell transcriptomic and epigenomic investigations. In melanoma models, interfering with TOX phase separation not only inhibited tumour growth but also shown a synergistic impact with anti-PD-1 antibodies, greatly increasing animal survival. In conclusion, TOX serves as the foundation for sustaining terminal exhaustion by physically solidifying the exhaustion transcriptional program by phase separation. The functional limitations on T cells can be effectively lifted by targeting TOX phase separation, offering a new potential target for the development of immunotherapy techniques akin to “phase separation intervention”.

Keywords

TOX, Liquid-liquid phase separation, T cell exhaustion, Antitumor immunity, Immunotherapy

1. Introduction

The development of immune checkpoint blockade (ICB) therapy has significantly changed how cancer is treated. ICB can help some patients achieve long-lasting remission by partially restoring the activity of depleted T lymphocytes in the tumour microenvironment by targeting inhibitory receptors such CTLA-4 and PD-1/PD-L1. Nevertheless, the great majority of cancer patients either develop acquired resistance or do not respond to ICB. The primary barrier is that T cells experience “terminal exhaustion”, a severely compromised functional state. Terminally exhausted T cells (T_{tex}) exhibit diminished effector cytokine production, loss of proliferative potential, and chronically elevated expression of inhibitory molecules like PD-1, TIM-3, and CD39. Even when antigens are removed or inhibitory signals are blocked, their transcriptional profile is fundamentally altered and exhibits strong epigenetic stability, making reversal challenging. Therefore, one of the most pressing fundamental problems in the field of cancer immunotherapy is figuring out the molecular mechanisms that sustain the terminal exhaustion state and identifying the important nodes that drive exhaustion locking.

Thymocyte selection-associated high mobility group box, or TOX, is the transcription factor that drives T cell exhaustion programs. TOX is strongly expressed and quickly induced in tumour models and chronic viral infections. While overexpression of TOX is sufficient to encourage the transition of progenitor exhausted T cells to terminal exhaustion, its lack can impede the development of exhausted T cells. By enlisting epigenetic repressive complexes and controlling exhaustion-related gene networks, TOX mechanistically enforces exhaustion-specific transcriptional programs at the chromatin level. Nevertheless, there is still a significant gap: even tho TOX can start the exhaustion process, the terminally fatigued condition is quite stable once it is reached. Uncertainty surrounds the precise physical process by which TOX spatially “locks in” the fatigue transcriptional program, making it harder for cells to avoid this fate.

A new knowledge of the exact spatiotemporal regulation of gene transcription has recently been made possible by liquid-liquid phase separation (LLPS), a basic mechanism for the development of membrane-less compartments within cells. Low-complexity intrinsically disordered regions (IDRs) have been found in many transcription factors. These IDRs can mediate protein phase separation to form dynamic condensates, which can enhance local transcriptional activity, silence gene expression, or enrich transcriptional co-factors. Interestingly, bioinformatics investigation indicates that TOX can form homomeric or heteromeric multimers and that the amino-terminal region of the protein contains a highly disordered IDR motif. It is a possible phase-separating protein because of these characteristics.

Based on this, we speculate that TOX may experience liquid-liquid phase separation thru its IDR in response to long-term antigen stimulation. This would result in the formation of functional condensates in the nucleus that spatially anchor exhaustion core gene loci in a repressive transcriptional regulatory environment, locking the terminal exhaustion state in the form of a “physical lock”.

This study first thoroughly investigated TOX’s phase separation capability and the sequence modules needed for it in order to verify this theory. We then investigated the production kinetics and roles of TOX condensates using *in vivo* exhaustion models and *in vitro* reconstitution. We fully examined the structural biological basis of TOX condensates in sustaining the terminal exhaustion program by creating phase separation-deficient mutants and combining them with single-cell multi-omics data. In order to provide theoretical underpinnings and experimental support for the development of novel immunotherapeutic strategies based on interfering with protein phase separation, we finally assessed the effects of disrupting TOX phase separation on reversing T cell exhaustion and enhancing anti-tumor immunity in various mouse tumour models.

2. Literature Review

T cell exhaustion is a distinct state of differentiation in which T cells progressively lose their ability to function in tumour microenvironments and chronic infections. It is primarily defined by persistently high expression of inhibitory receptors, decreased effector cytokine secretion, and decreased proliferative capacity (Blank et al., 2019; Wherry, 2011). Recent developments in single-cell transcriptomics and epigenomics have advanced our knowledge of depleted T cell heterogeneity. TCF-1⁺ precursor/progenitor exhausted T cells (Tpex) and TIM-3⁺ terminally exhausted T cells (Ttex) are the two primary subgroups of exhausted T cells according to the current mainstream concept. While the latter is in a highly dysfunctional state and has virtually lost its anti-tumor potency, the former still has some capacity for self-renewal and the ability to react to immune checkpoint blockade (Miller et al., 2019; Pauken et al., 2016). It is particularly noteworthy that Beltra and colleagues (2020) discovered in 2020 that the Ttex population has a distinct chromatin accessibility landscape thru integrated single-cell multi-omics research, indicating that severe epigenetic barriers limit its development. Tpex can only be momentarily activated by simply blocking PD-1 signalling, and a significant reversal of Ttex is improbable. All of these results indicate to a crucial bottleneck: once the terminal exhaustion state is established, it is difficult to break, and the molecular underpinnings of this “locking” effect are still unclear.

One of the main causes of T cell fatigue is the transcription factor TOX. According to three separate research conducted nearly simultaneously in 2019, TOX is necessary for the generation of fatigued CD8⁺ T lymphocytes in tumour models and chronic lymphocytic choriomeningitis virus

(LCMV) infection. While maintaining their original phenotype, T cells deficient in TOX are unable to efficiently create exhausted populations and exhibit overactivation and death (Alfei et al., 2019; Scott et al., 2019). Among these, Khan and others (2019) demonstrated that TOX binds to fatigue-related genes such as *Pdcd1* and recruits epigenetic modifiers to force the exhaustion transcription program at the chromatin level. According to further research, TOX frequently collaborates with transcription factors from the NR4A family and TOX2 to create a transcriptional network that propels the development of exhaustion (Chen et al., 2019; Seo et al., 2019). There is still much to learn about how TOX physically “locks in” the exhaustion transcription program into an irreversible state, ultimately cementing terminal exhaustion, despite the fact that the aforementioned research has demonstrated TOX’s critical role in initiating and sustaining exhaustion early on.

A completely new understanding of the spatial and temporal regulation of gene expression is provided by liquid-liquid phase separation, which is the physicochemical basis for the development of membrane-less organelles (biomolecular condensates) inside cells (Hnisz et al., 2017; Shin & Brangwynne, 2017). Numerous studies demonstrate that the intrinsically disordered regions of coactivators and transcription factors can drive multivalent weak interactions, leading to phase separation in particular genomic regions and the formation of high-concentration condensates, which significantly increases local transcription efficiency (Boija et al., 2018; Sabari et al., 2018). Phase separation under TCR stimulation allows signalling molecules such as LAT and SLP-76 to form functional signalling clusters during T cell activation. The physiological significance of phase separation in adaptive immunity was initially demonstrated by this research (Su et al., 2016). After attaching to DNA, cGAS can also experience phase separation in innate immunity, serving as a catalytic platform to increase the creation of cGAMP (Du & Chen, 2018). These developments demonstrate the extensive involvement of phase separation in immunological signal transduction and effector function execution. From the standpoint of structural biology, phase separation is mostly dependent on low-complexity regions, or IDRs, in protein sequences. The material characteristics and assembly dynamics of condensates are determined by the amino acid composition of these regions, such as the distribution of polar or aromatic residues (Li et al., 2012; Martin & Mittag, 2018).

According to bioinformatics studies, the TOX protein’s N-terminal has a low-conservation IDR sequence, and it is a member of a subfamily of transcription factors that may form homopolymers and have an HMG-box domain (O’Flaherty & Kaye, 2003). Phase separation is often caused by the combination of IDRs and this type of multivalent domain (Boija et al., 2018; Li et al., 2012). Furthermore, research has shown that low-complexity domains can create persistent regulatory hubs by selectively anchoring the transcription machinery at particular genomic locations (Chong et al., 2018). These hints suggest that TOX may employ its IDR to undergo phase separation and create inhibitory molecular condensates at exhausted core gene locations, therefore “locking in” the

terminally exhausted state. Nevertheless, no research has yet specifically examined whether TOX may phase separate, much less examined the implications for T cell fatigue.

Targeting biomolecular condensates has emerged as a new approach to medication development in recent years. It is possible to actively intervene in aberrant phase separation processes in therapy, as studies have demonstrated that small molecule medicines can accumulate in particular transcription factor condensates, altering their pharmacological behaviour (Klein et al., 2020). Interventions that target pathological phase separation are being actively investigated since aberrant regulation of phase separation is directly linked to the development of many tumours (Zheng et al., 2023). In this regard, if TOX phase separation indeed serves as the structural foundation for terminal fatigue, then interfering with this mechanism may let spent T cells function again, perhaps leading to a completely different strategy for cancer immunotherapy.

In conclusion, despite the fact that previous research has consistently demonstrated the crucial role of TOX in exhaustion-driven transcription programs and demonstrated the significance of liquid-liquid phase separation in immune modulation, a number of important problems remain unresolved: Is it possible for TOX to separate into liquid and liquid phases under physiological settings? Do TOX condensates directly contribute to the chromatin state locking of genes linked to terminal exhaustion? Can anti-tumor immunity be restored by focusing on TOX phase separation and breaking the “physical lock” of terminal exhaustion? This study’s contribution is that it is the first to identify the molecular mechanism by which TOX sustains T cell terminal exhaustion thru phase separation mediated by its IDR. It also demonstrates that interfering with this phase separation process can greatly increase the effectiveness of immune checkpoint blockade therapy, offering a novel target and theoretical foundation for the development of phase separation-based tumour immunotherapy strategies.

3. Materials and Methods

3.1. Experimental animals and tumor models

Beijing Vital River Laboratory Animal Technology Co., Ltd. supplied female SPF-grade C57BL/6J mice that were 6–8 weeks old. Jackson Laboratory provided CD45.1⁺ congenic mice (B6.SJL-Ptprca Pepcb/BoyJ) and OT-I T cell receptor transgenic mice that specifically recognised OVA257-264/H-2Kb. The mice’s right flank was subcutaneously injected with either 1×10⁵ B16-OVA melanoma cells or 2×10⁵ MC38 colon cancer cells that had been resuspended in 100 µL PBS. The formula $V = (\text{length} \times \text{width}^2)/2$ was used to compute the tumour volume. When the tumour volume reached 1500 mm³ or ulceration developed, the mice were put to death. Mice were given an intraperitoneal injection of 2×10⁶ PFU of LCMV Clone 13 to create a chronic infection. On days 30-35 after infection, the mice’s spleens were taken for the examination of fatigued T cells.

3.2. Plasmid construction and retroviral transduction

OT-I cell cDNA was used to amplify mouse TOX cDNA (NM_019926.2). Amino acids 1–210 at the N-terminus of TOX were predicted to be an IDR using the disorder prediction algorithms PONDR VL-XT and IUPred2A. The beginning methionine and the HMG-box domain (211–288aa), produced via overlapping extension PCR, were retained whereas a fragment encoding amino acids 2–209 was removed from the whole TOX sequence. The retroviral vectors MSCV-IRES-Thy1.1 and MSCV-IRES-GFP were used to clone full-length TOX and TOX-IDRm, respectively. TOX-IDR (1–210aa) was fused with mEGFP or mCherry for intracellular phase separation visualisation; CRY2olig (PHR domain, 1–498aa) was fused with mCherry-TOX-IDR and built into the pLVX-EF1 α vector for optogenetic aggregation investigations. Sanger sequencing was used to verify each plasmid. After co-transfecting MSCV constructs into Plat-E packaging cells with the pCL-Eco helper plasmid, viral supernatants were gathered 48 hours later. After being activated for 24 hours, freshly obtained OT-I CD8⁺ T cells were infected by spin infection with viral supernatant containing 8 μ g/mL polybrene (2000g, 32°C, 90min). Positive cells were sorted using Thy1.1 magnetic beads or GFP 48 hours after infection.

3.3. T cell activation and in vitro exhaustion induction

A magnetic negative selection kit was used to purify naive OT-I CD8⁺ T cells from the lymph nodes and spleen (purity >95%). The cells were cultivated in RPMI 1640 media with 10% FBS, 55 μ M β -mercaptoethanol, and 100 U/mL IL-2 after being activated on plates coated with anti-CD3 (5 μ g/mL) and anti-CD28 (2 μ g/mL). OVA257–264 peptide-loaded EL4 thymoma cells treated with mitomycin C were co-cultured with OT-I T cells in a 1:1 ratio. For five to seven rounds of stimulation, new stimulator cells and IL-2 (20 U/mL) were introduced every 48 hours, and phenotyping was assessed before to each round.

3.4. Recombinant protein expression and in vitro phase separation experiments

Using a 6 $\times$ His tag and mEGFP at the N-terminus, clone the cDNA of TOX-IDR (1–210aa) and full-length TOX into the pET-28a(+) vector. Express in E. Coli BL21 (DE3) for 16 hours at 16°C using 0.2 mM IPTG. Use Superdex 200 gel filtration and Ni-NTA affinity chromatography to purify the protein, then store it in buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM DTT). Purified EGFP-TOX-IDR protein (final concentration 10 μ M) should be added to a buffer containing 10% (w/v) PEG-8000 or 100 ng/ μ L salmon sperm DNA. The mixture should be incubated for 30 minutes at room temperature in a 384-well glass-bottom plate, and droplet formation should be observed using a confocal microscope (Nikon A1R+) with 488 nm excitation. To encourage phase separation for full-length TOX, add 10% PEG-8000 and 10 nM Cy5-labeled double-stranded DNA (containing 2 $\times$

TOX binding motifs). To assess phase separation tendency, measure absorbance at 340 nm in protein solutions with varying PEG-8000 concentrations. Choose droplets larger than 1 μm in diameter, bleach the center with a 405 nm laser at full power, take pictures every 2 seconds for five minutes, and use NIS-Elements software to compute fluorescence recovery rates.

3.5. Intracellular phase separation detection and light-controlled aggregation experiment

After being fixed and permeabilized, T cells generated by in vitro exhaustion or tumor-infiltrating T cells sorted following adoptive transfer were stained with anti-TOX antibody (Santa Cruz, sc-374164) and Alexa Fluor 647 secondary antibody with DAPI counterstaining. A confocal microscope was used to get Z-stack pictures, and Imaris 9.0 was used for 3D reconstruction and spot counting. After five minutes of treatment with 3% 1,6-hexanediol at 37°C, the cells were fixed to track the dispersion of TOX condensates. Glass-bottom culture dishes were seeded with OT-I T cells transduced using CRY2olig-mCherry-TOX-IDR. The in situ aggregation and dissolution kinetics of mCherry fluorescence were observed by illuminating a tiny area in the cell nucleus with a 488 nm laser (10% power) for 30 seconds under a confocal microscope. The control was non-fused mCherry-TOX-IDR.

3.6. Adoptive T cell transfer and combination therapy

After being activated in vitro for 48 hours, OT-I CD45.1⁺ T cells transduced with MSCV-Thy1.1-TOX or - (TOX-IDRm) were adoptively transferred by tail vein into tumor-bearing B16-OVA CD45.2⁺ recipient mice (1 $\times$ 10⁶ cells per mouse). One group of mice received intraperitoneal injections of either isotype control or anti-PD-1 antibody (RMP1-14, BioXCell, 200 μg per animal) once every three days for a total of four injections on day five following transfer. Tumor-infiltrating lymphocytes (TILs) were separated by mechanical dissociation and Percoll gradient centrifugation on days 8–10 after the mice were killed. After that, donor-derived Thy1.1⁺CD45.1⁺ T cells were sorted for additional examination.

3.7. Flow cytometry and functional analysis

We stained the following surface molecules after the single-cell suspension was incubated with an Fc receptor blocker: CD8 (53-6.7), CD45.1 (A20), Thy1.1 (HIS51), PD-1 (29F.1A12), TIM-3 (RMT3-23), LAG-3 (C9B7W), and CD39 (Duha59). We performed intracellular labelling of TOX (TXRX10), TCF-1 (C63D9), and Ki-67 (SolA15) following fixation and permeabilization. PMA (50 ng/mL), ionomycin (1 $\mu\text{g/mL}$), and brefeldin A were used to stimulate cells for four hours in order to identify cytokines. The cells were subsequently fixed and permeabilized in order to stain IFN- γ (XMG1.2), TNF- α (MP6-XT22), and Granzyme B (GB11). A BD LSRFortessa was used to gather the

data, and FlowJo v10 was used for analysis.

3.8. Single-cell transcriptomics and single-cell ATAC-seq

Following the official 10x Genomics protocols, Thy1.1⁺ donor CD8⁺ T cells selected from B16-OVA tumours (TOX and TOX-IDRm groups) were submitted to scRNA-seq (using the Chromium Single Cell 5' Library Kit) and scATAC-seq (using the Chromium Single Cell ATAC Library Kit). The Illumina NovaSeq 6000 was used to sequence libraries. After using Cell Ranger (v6.1.2) to match scRNA-seq data to the mm10 reference genome, the data were filtered, normalised, subjected to PCA dimensionality reduction, then visualised with UMAP using Seurat (v4.3). Known markers were used to identify cell subpopulations. The Wilcoxon rank-sum test ($|\log_2FC| > 0.25$, adjusted $P < 0.05$) was used to identify differential genes. Cell Ranger ATAC was used to process the scATAC-seq data, and ArchR (v1.0.1) was used for clustering, peak calling, and differential accessibility analysis. Gene activity scores and regulatory networks were constructed by integrating scRNA-seq and scATAC-seq data.

3.9. ChIP-seq and ATAC-seq

ChIP-seq: After five rounds of in vitro exhaustion stimulation of OT-I cells transduced with TOX or (TOX-IDRm), 1×10^7 cells were collected for cross-linking, lysis, and sonication. As previously noted, isotype IgG or anti-TOX antibody were used for immunoprecipitation. DNA underwent elution, library preparation, and sequencing. MACS2 (v2.2.7.1) was used to identify enriched peaks, while HOMER (v4.11) was used for motif analysis. The Omni-ATAC procedure was used to prepare 5×10^4 cells for ATAC-seq, bowtie2 was used to align the sequencing data, and MACS2 was used to name peaks. DiffBind (v3.6) was utilised for differential peaks analysis, while ChIPseeker (v1.34) was used to identify the distribution of genomic regions.

3.10. Statistical analysis

The results are displayed as mean $\pm$ standard error of the mean (SEM), and each experiment was independently repeated at least three times. Two-tailed unpaired Student's t-test was used to compare two groups; one-way or two-way ANOVA with Tukey's post hoc test was used to compare multiple groups. The Kaplan-Meier method was used to plot survival curves, and the log-rank test was used to assess differences. T-tests were used to compare the areas under the curve (AUC) of tumour growth curves. R 4.2.2 and GraphPad Prism 9 were used for the analyses.

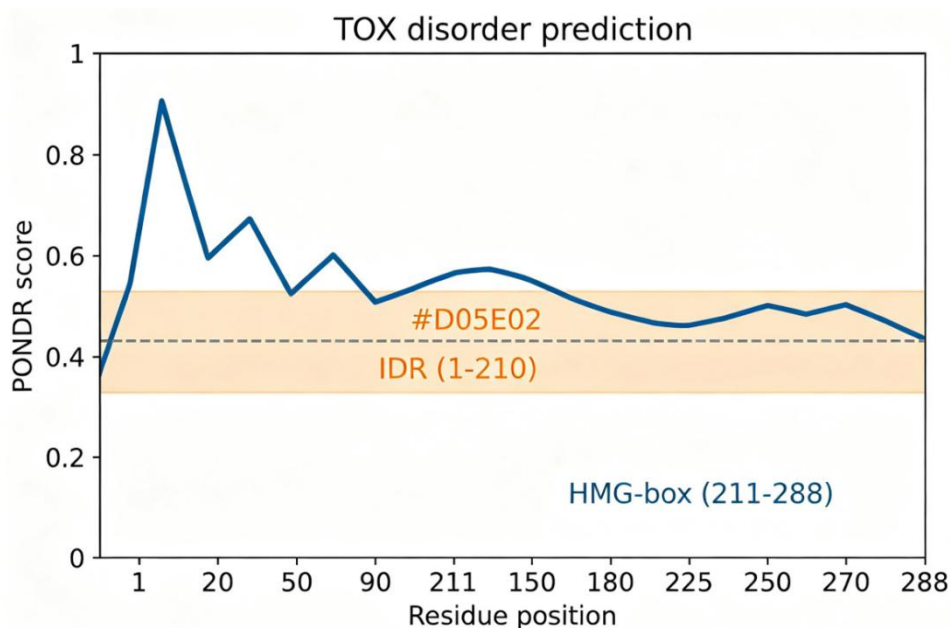
4. Experimental Results

4.1. The N-terminal intrinsically disordered region of TOX protein has the ability to drive liquid-liquid phase separation

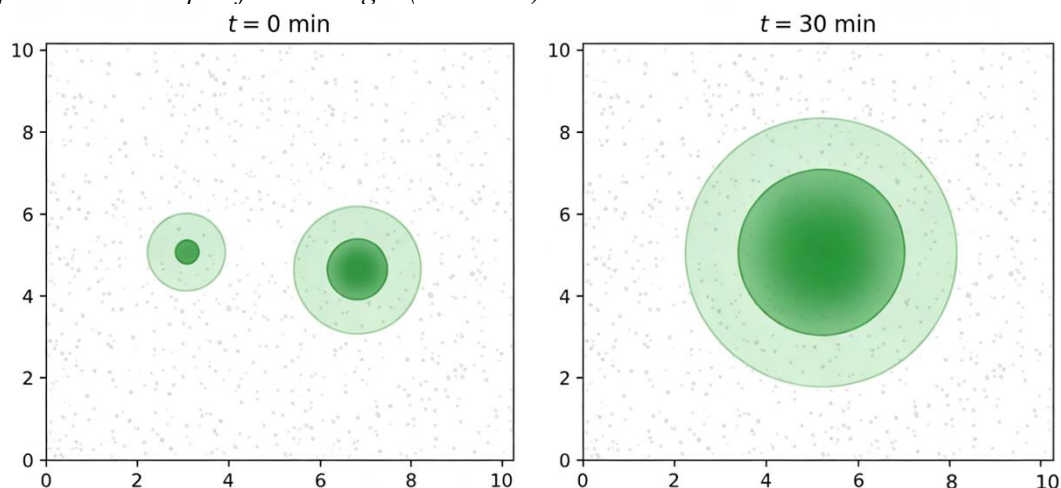
We first examined the disorder tendency of the mouse TOX protein sequence in order to determine whether TOX has a structural foundation for phase separation. The C-terminal HMG-box domain (211-288aa) of TOX is highly ordered, whereas the N-terminal portion (amino acids 1–210) is extremely intrinsically disordered, according to the PONDR VL-XT algorithm (Figure 1A). We created and produced an EGFP-fused TOX-IDR (1-210aa) recombinant protein in order to directly evaluate whether this disordered region can cause phase separation. The EGFP-TOX-IDR protein rapidly produced spherical, fusible liquid-like droplets at physiological salt content and neutral pH following the addition of 10% PEG-8000 as a molecular crowding agent (Fig. 1B). Turbidity measurements further demonstrated that the protein's phase separation is concentration-dependent, and when the PEG-8000 concentration increases, the turbidity signal dramatically gets stronger (Fig. 1C). Nucleic acids may function as multivalent scaffolds to facilitate phase separation, as evidenced by the formation of TOX-IDR condensates at lower protein concentrations under circumstances with 100 ng/μL salmon sperm DNA (Figure 1D). With a half-recovery time ($t_{1/2}$) of 25.3 ± 4.1 s and a recovery rate of $82.6 \pm 5.2\%$ (Fig. 1E, Table 1), fluorescence recovery after photobleaching (FRAP) experiments demonstrated that the fluorescent signal inside the condensates recovered rapidly after bleaching, confirming that the condensates have liquid-like mobility. In contrast, the FRAP recovery rate decreases to $12.4 \pm 3.8\%$ (Table 1), which is indicative of liquid-liquid phase separation, when the condensates are totally dissolved by treatment with 3% 1,6-hexanediol. When double-stranded DNA with the TOX-binding motif is introduced, the full-length TOX protein can similarly produce liquid-like condensates (Figure 1F). The aforementioned findings demonstrate that TOX's N-terminal IDR can cause liquid-liquid phase separation, and nucleic acid binding can enhance this process.

Figure 1

A: TOX disorder prediction

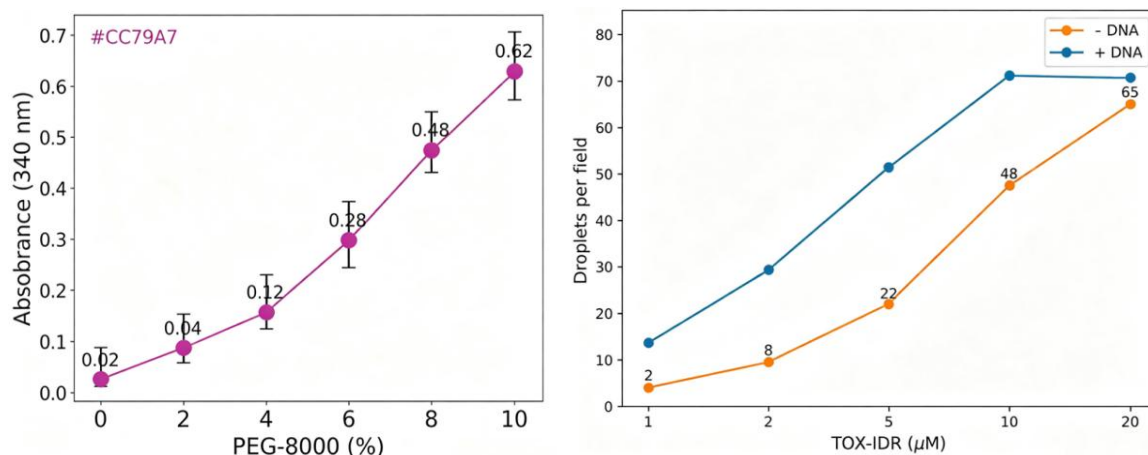


B: Representative droplet fusion images (simulated)



C: Turbidity assay (PEG-8000 dependent)

D: DNA lowers the threshold for phase separation



E: FRAP recovery curves

F: Full-length TOX forms droplets with dsDNA

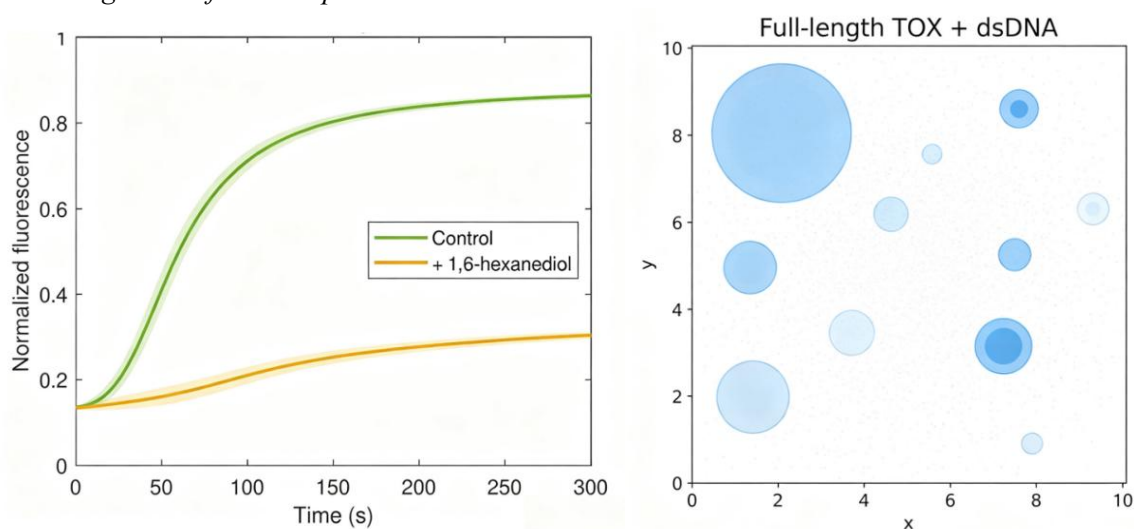


Table 1

FRAP kinetics parameters of TOX-IDR condensates

Experimental conditions	Half-recovery time $t_{1/2}$ (s)	Recovery Rate (%)	Droplet count/field of view
10% PEG-8000	25.3 ± 4.1	82.6 ± 5.2	48.2 ± 7.6
10% PEG-8000 + DNA	18.7 ± 3.5	89.1 ± 4.7	62.5 ± 8.3
10% PEG-8000 3% 1,6-hexanediol	—	12.4 ± 3.8	2.1 ± 1.2

Note. For $n = 3$ independent experiments, the data are displayed as mean $\pm$ SEM. The majority of the droplets disintegrated after being treated with 1,6-hexanediol, and recovery was computed from the insoluble aggregates that remained.

4.2. Exhausted T cells form TOX condensates in the nucleus with phase separation characteristics

We used immunofluorescence labelling on OT-I CD8⁺ T cells that were depleted by prolonged stimulation in vitro to determine if TOX can form aggregates in primary T cells. Following seven rounds of chronic stimulation, TOX protein showed characteristic punctate clusters in the nucleus, with an average of 6–10 puncta measuring 0.5–1.2 μm in diameter in each nucleus, according to confocal microscope imaging (Figure 2A). The nuclear TOX puncta nearly entirely vanished and dispersed widely throughout the nucleoplasm following a brief treatment with 3% 1,6-hexanediol (Figure 2B), indicating that hydrophobic interactions are necessary for the maintenance of these aggregates, which is consistent with the properties of phase-separated condensates. We fused the CRY2olig photosensitive oligomerisation domain with mCherry-TOX-IDR using an optogenetic aggregation approach and expressed it in OT-I T cells to confirm the innate cellular drive for TOX phase separation. mCherry fluorescence rapidly accumulated at the lit region, generating visible condensates, following 30 seconds of 488 nm laser illumination of a tiny area in the cell nucleus. The condensates gradually disintegrated about 180 seconds after the laser was turned off (Figure 2C-D). Under the same circumstances, mCherry-TOX-IDR without CRY2olig does not produce any punctate aggregates as a control (Figure 2E). Additionally, T cells transduced with full-length EGFP-TOX displayed distinct nuclear punctate patterns, whereas the phase separation-deficient mutant EGFP-(TOX-IDR_m) (with the IDR region deleted) was entirely distributed throughout the nucleoplasm, with a sharp decrease in the number of condensates per cell from 8.7 ± 1.2 to 0.3 ± 0.1 (Table 2). Overall, this evidence clearly demonstrates that TOX can use its IDR to promote liquid-liquid phase separation in fatigued T cell nuclei, resulting in the formation of functional

condensates.

Table 2

Formation of nuclear aggregates in exhausted T cells with different TOX constructs

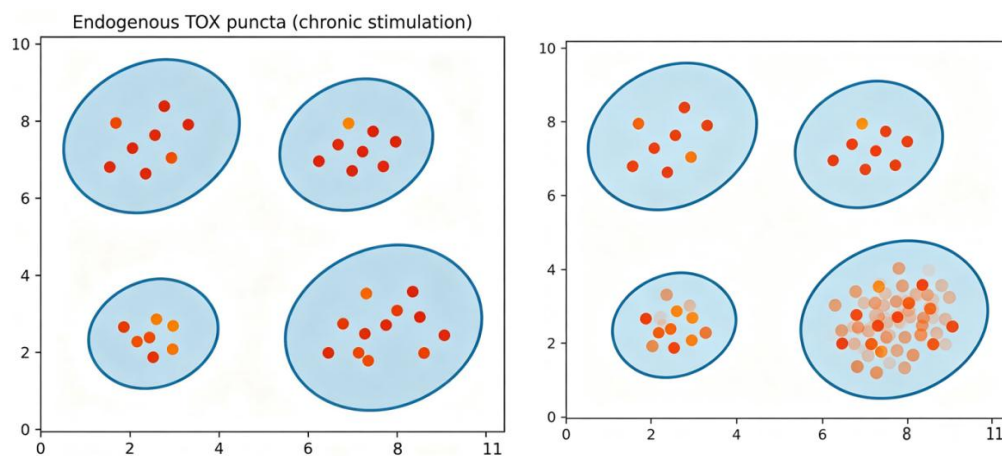
Construct body	Average number of aggregates per cell	Aggregate cell positivity rate (%)
EGFP-TOX (full length)	8.7 ± 1.2	92.3 ± 4.5
EGFP-TOX- IDR	0.3 ± 0.1	3.1 ± 1.4
EGFP-TOX 3% 1,6-hexanediol	0.5 ± 0.2	5.2 ± 2.1

Note. The experiment was conducted three times separately, with each group counting fifty cells. Dot-like signals with a diameter more than $0.3 \mu\text{m}$ and fluorescence intensity greater than three times the nucleoplasm background are referred to as aggregates.

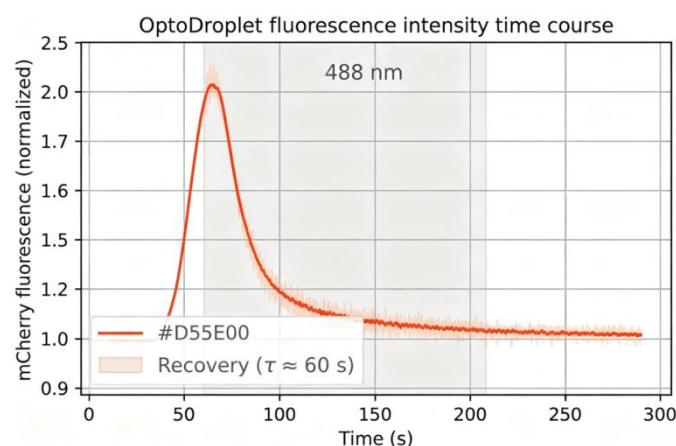
Figure 2

A: Nuclear TOX puncta in exhausted T cells (simulated)

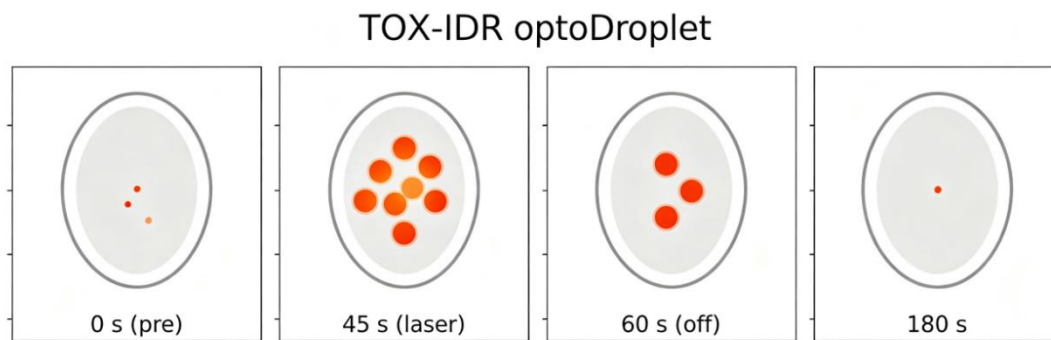
B: 1,6-hexanediol dissolves TOX condensates



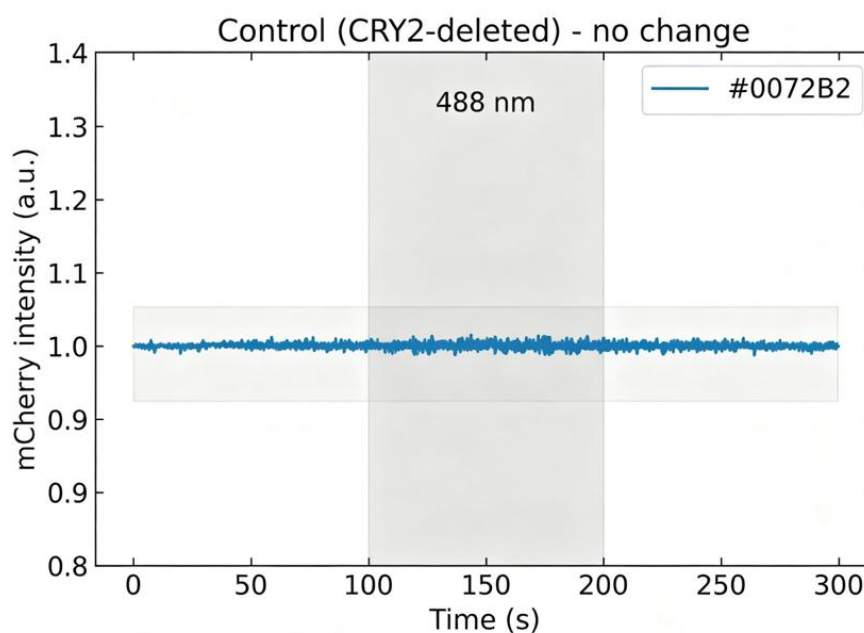
C: OptoDroplet fluorescence intensity time course



D: Representative time-lapse images of optoDroplet assay



E: Control (CRY2-deleted) - no change



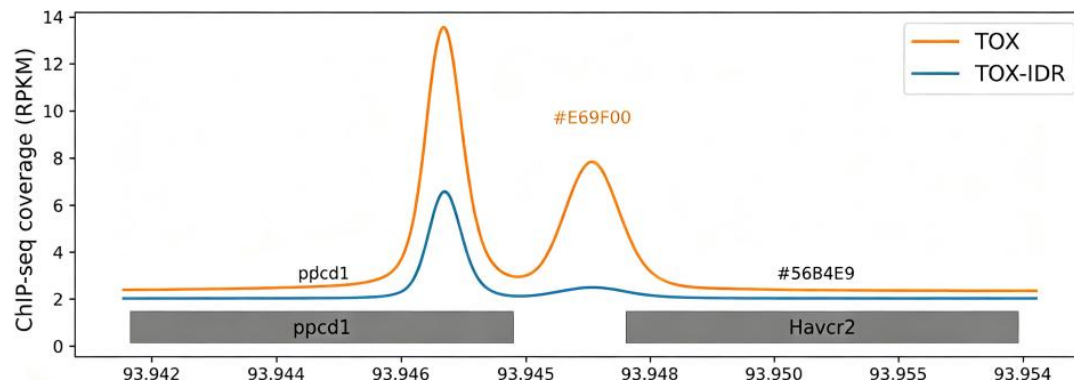
4.3. TOX aggregates directly anchor and maintain an open chromatin state at terminal exhaustion gene sites

We used TOX ChIP-seq and ATAC-seq analysis in exhausted OT-I T cells transduced with either full-length TOX or TOX-IDRm to determine if TOX condensates directly affect the transcriptional environment of core exhaustion genes. Full-length TOX exhibited significant enrichment peaks in the promoter and enhancer regions of traditional exhaustion effector genes such as *Pdcd1*, *Havcr2*, *Tox*, and *Cd244a*, according to the ChIP-seq results (Figure 3A). Significantly reduced binding signals were observed at these gene locations in the IDR-deleted TOX-IDRm mutant, indicating that phase separation-mediated condensate formation is essential for TOX's persistent occupancy at target genes. Accordingly, chromatin accessibility at terminal exhaustion marker genes like *Pdcd1* and *Havcr2* dramatically decreased in TOX-IDRm cells, while accessibility at loci like *Tcf7* (precursor exhaustion gene encoding TCF-1) and *Il7r* significantly increased (Figure 3B, Table 3). 1,283 peaks in the

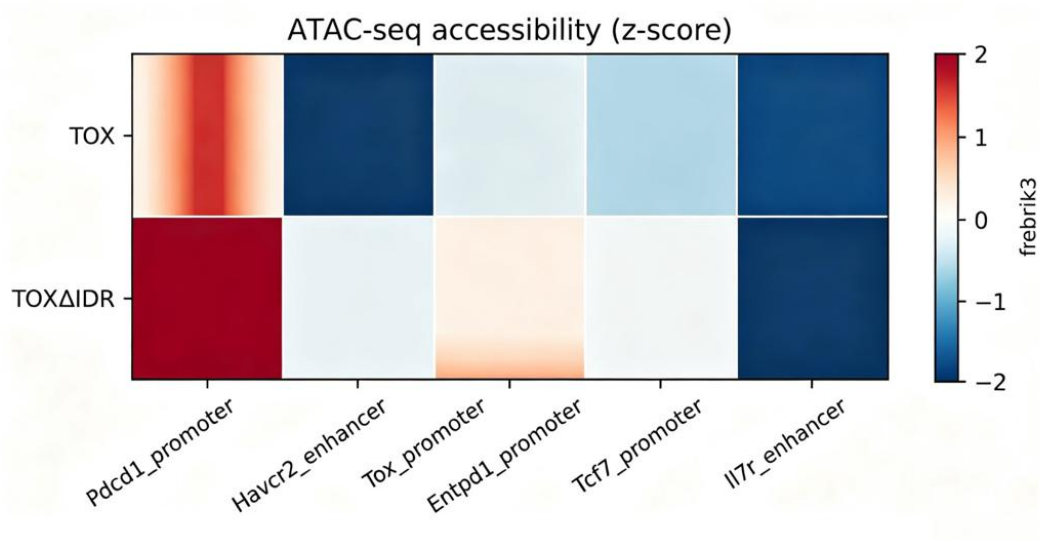
TOX-IDRm group had decreased accessibility, according to genome-wide distribution analysis; these peaks were primarily enriched at loci indicative of terminal exhaustion (Figure 3C).

Figure 3

A: ChIP-seq tracks at Pcdcl and Havcr2 loci (simulated)



B: ATAC-seq heatmap of key exhaustion loci



C: Pie chart of differential ATAC peaks (TOXΔIDR vs TOX)

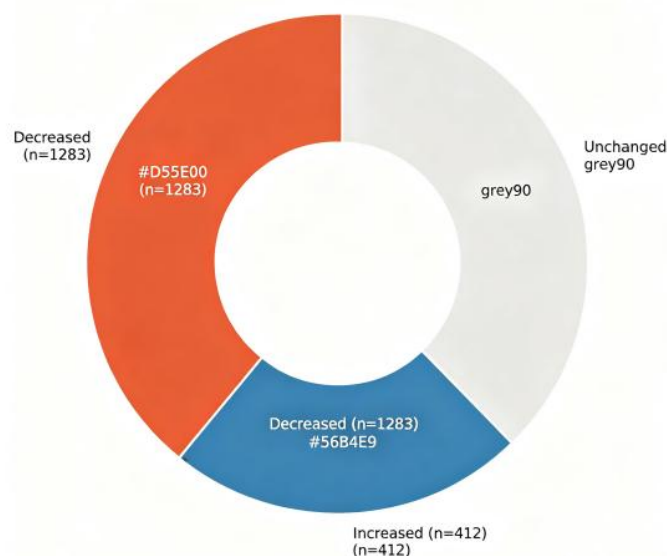


Table 3

Changes in ATAC-seq chromatin accessibility at key exhaustion-related gene sites

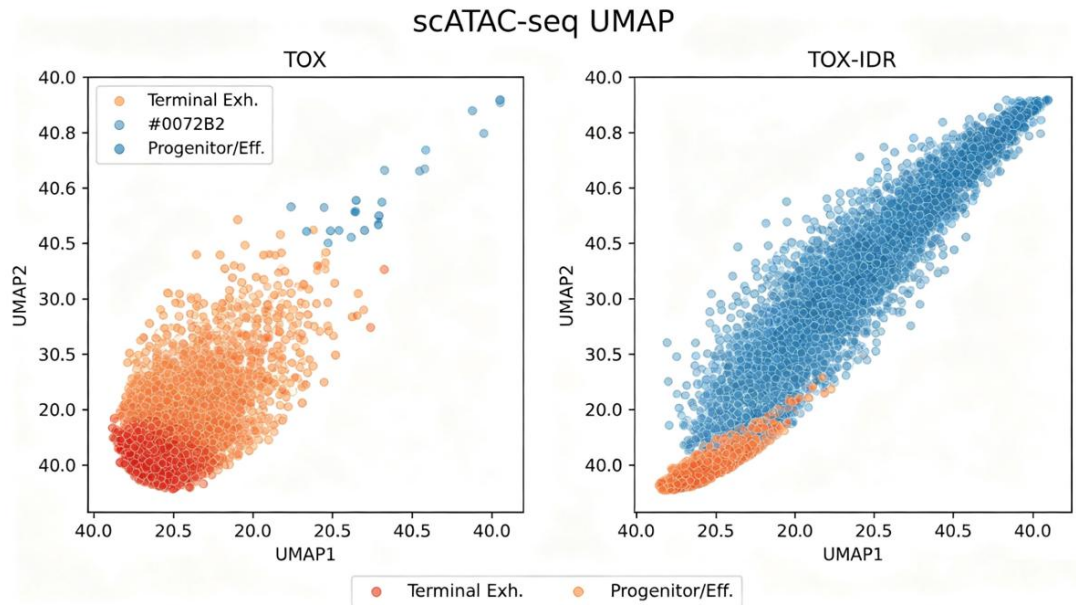
Genelocus	Full-lengthTOXRPKM (TOX-IDRm)	RPKM	FoldChange(IDR/TOX)	AdjustedP-value
Pdcd1 (promoter)	12.34 ± 1.56	3.21 ± 0.89	0.26	<0.001
Havcr2 (enhancer)	9.87 ± 1.23	2.45 ± 0.67	0.25	<0.001
Tox (promoter)	8.56 ± 0.98	5.12 ± 0.76	0.60	<0.01
Entpd1 (promoter)	7.43 ± 0.88	1.98 ± 0.54	0.27	<0.001
Tcf7 (promoter)	2.34 ± 0.45	6.78 ± 1.12	2.90	<0.001
Il7r (enhancer)	1.56 ± 0.32	4.21 ± 0.68	2.70	<0.01

Note. RPKM data are displayed as n=3 separate biological replicates, mean ± SEM. Fold Change is the ratio of full-length TOX group signal strength to (TOX-IDRm). Benjamini-Hochberg was used to correct adjusted P values after DiffBind was used for differential peak analysis.

We conducted an integrated scATAC-seq and scRNA-seq investigation on donor CD8⁺ T cells with TOX and TOX-IDRm transplanted into B16-OVA tumours in order to further investigate the epigenetic regulatory role of TOX condensates at the single-cell level. T cells in the TOX group were primarily concentrated in the terminally exhausted cluster, according to UMAP clustering, whereas cells in the TOX-IDRm group clearly migrated toward the precursor exhausted and effector-like clusters (Figures 4A-B). Pdcd1, Havcr2, and Entpd1 activity scores were 2-5 times lower in TOX-IDRm cells than in the TOX group, but Tcf7 and Slamf6 activity increased 3-4 times, according to gene activity scoring (Figure 4C). This tendency was further confirmed at the transcriptional level by scRNA-seq data, which showed that effector molecule expression and memory/precursor-related genes were elevated while terminal exhaustion marker genes were considerably downregulated (Figure 4D). When combined, these findings consistently show that TOX condensates physically occupy the terminal exhaustion-specific chromatin landscape, “locking” it, and that IDR deletion-induced phase separation disruption can directly remove this transcriptional restriction.

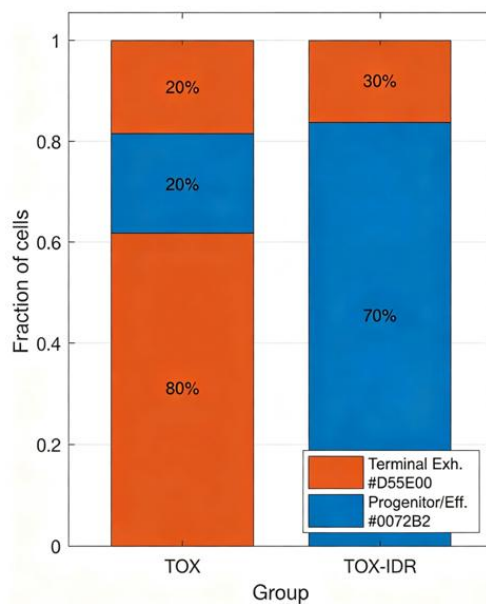
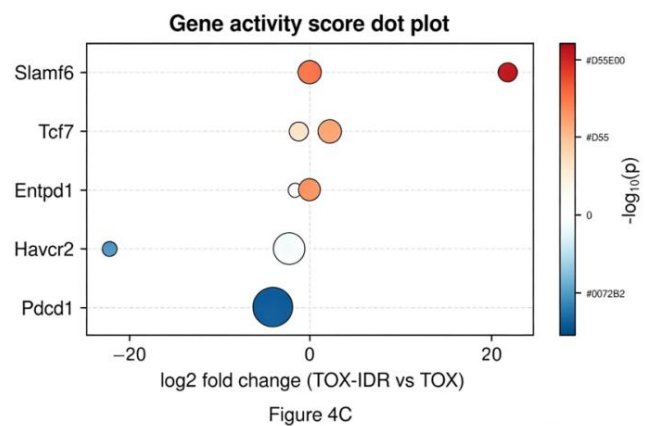
Figure 4

A: UMAP projection of scATAC-seq cells

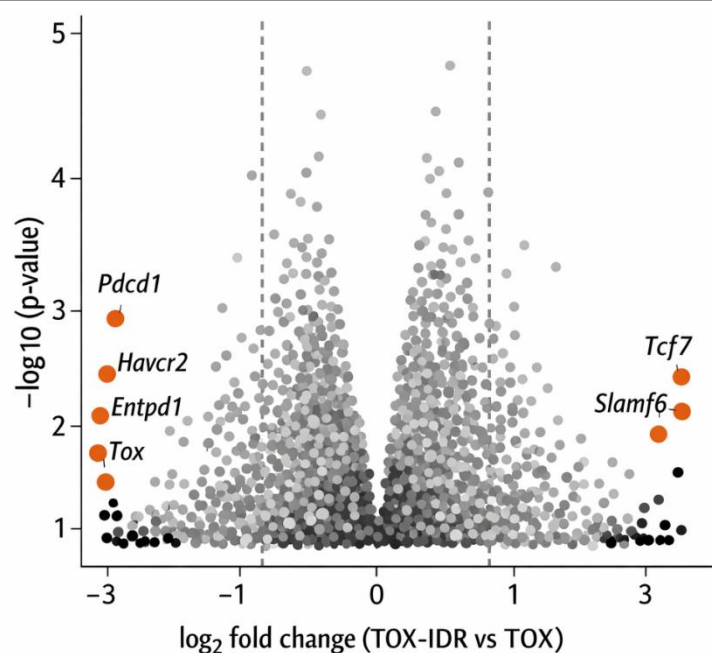


B: Cluster proportion bar chart

C: Gene activity score dot plot



D: Volcano plot scRNA-seq

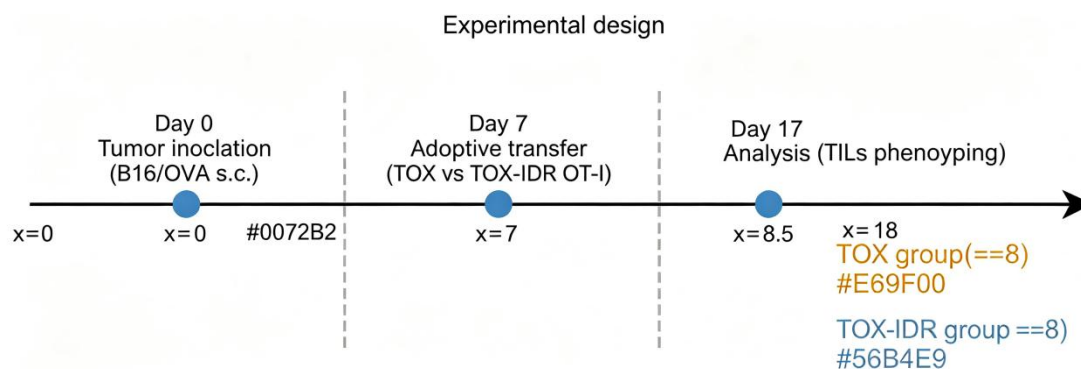


4.4. Disrupting TOX phase separation reprograms T cell fate and reverses terminal exhaustion phenotype

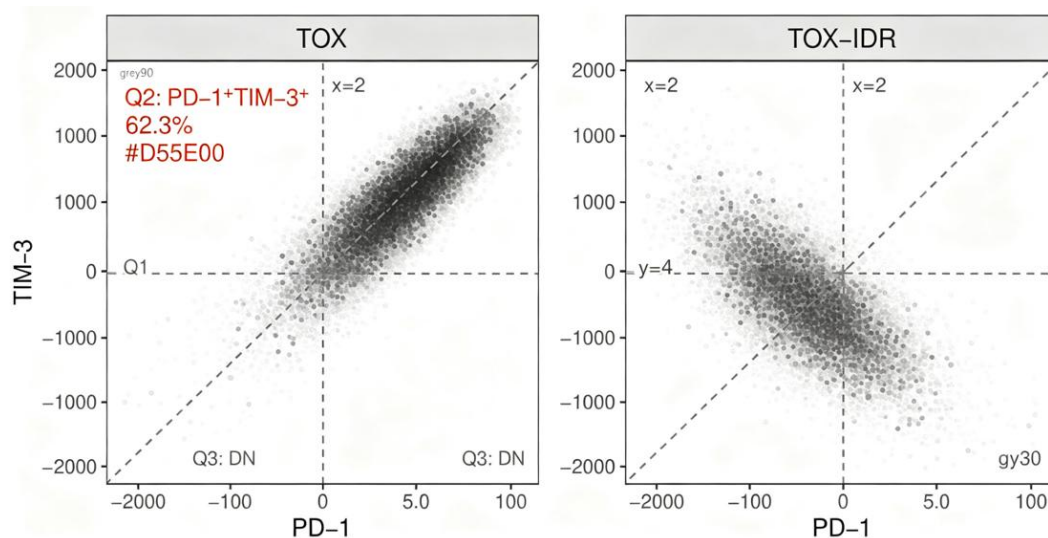
These results prompted us to investigate directly if terminal fatigue could be broken at a functional level by interfering with TOX phase separation. On day 10 following transfer, we examined tumor-infiltrating donor T cells in CD45.2⁺ recipient mice with B16-OVA tumours using OT-I CD45.1⁺ T cells expressing either full-length TOX or TOX-IDRm (Figure 5A). According to flow cytometry analysis, the number of TCF-1 precursor fatigued cells grew dramatically from 12.1% to 38.7% in the TOX-IDRm group, while the proportion of PD-1⁺TIM-3⁺ terminally exhausted cells decreased sharply from 62.3% in the TOX group to 23.5% (Figures 5B-C, Table 4). Functional assays revealed that upon PMA/ionomycin re-stimulation, (TOX-IDRm) T cells produced IFN- γ and TNF- α at rates of 42.5% and 29.3%, respectively, which is roughly 2.8 and 3.3 times greater than the TOX group; the granzyme B positive rate also increased from 13.2% to 35.6% (Figures 5D-F, Table 4). Concurrently, the percentage of Ki-67⁺ proliferating cells in the (TOX-IDRm) group increased from 18.4% to 56.2%, suggesting that the cells had recovered their ability to proliferate (Table 4). More significantly, TIM-3 and CD39 expression levels were markedly decreased, exhibiting typical characteristics of functional revival, even tho PD-1 expression intensity (MFI) on (TOX-IDRm) T cells was still present (Figure 5G). According to these findings, terminally tired T cells can be significantly reprogrammed into functional precursor/effector-like states in vivo by using phase separation-defective mutants to impair TOX condensate formation.

Figure 5

A: Experimental design schematic

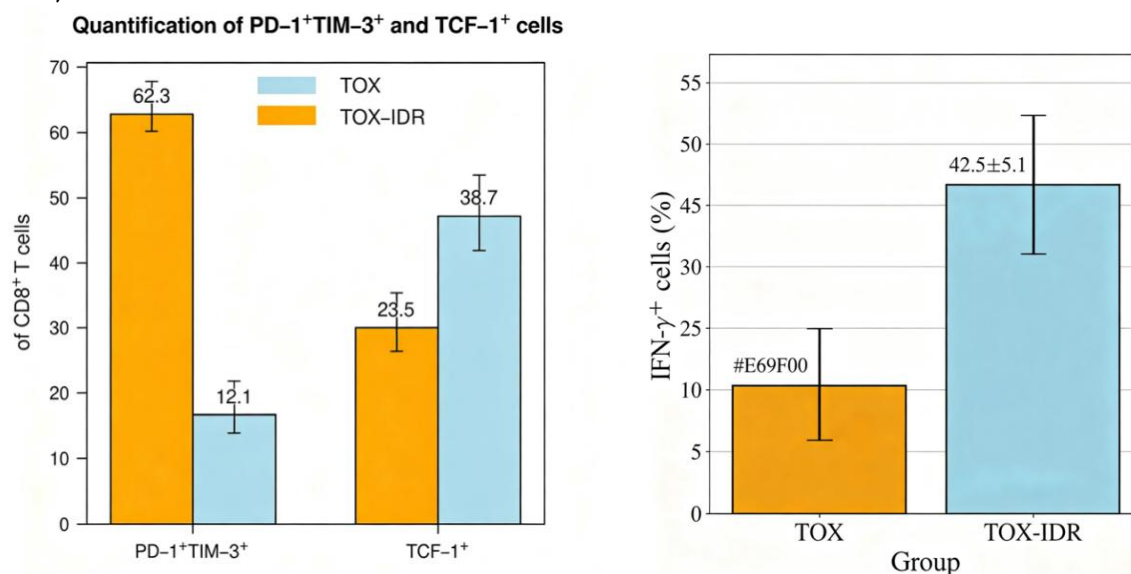


B: Flow cytometry pseudocolor plots (PD-1 vs TIM-3)



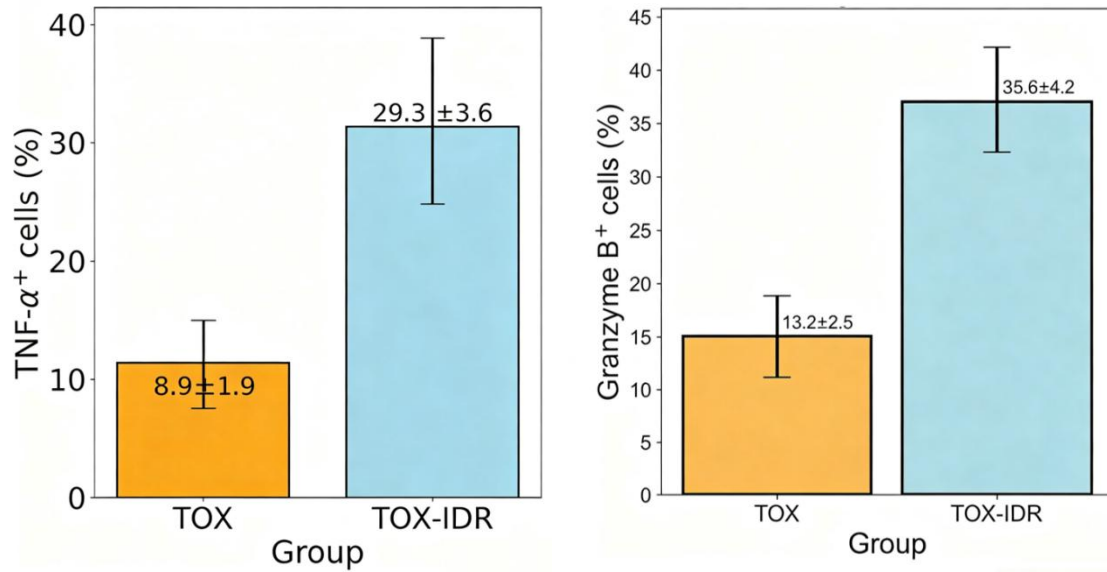
C: Quantification of PD-1+TIM-3+ and TCF-1+ cells

D: IFN- γ + cells



E: TNF- α + cells

F: Granzyme B+ cells



G: Inhibitory receptor MFI (simulated as bar chart)

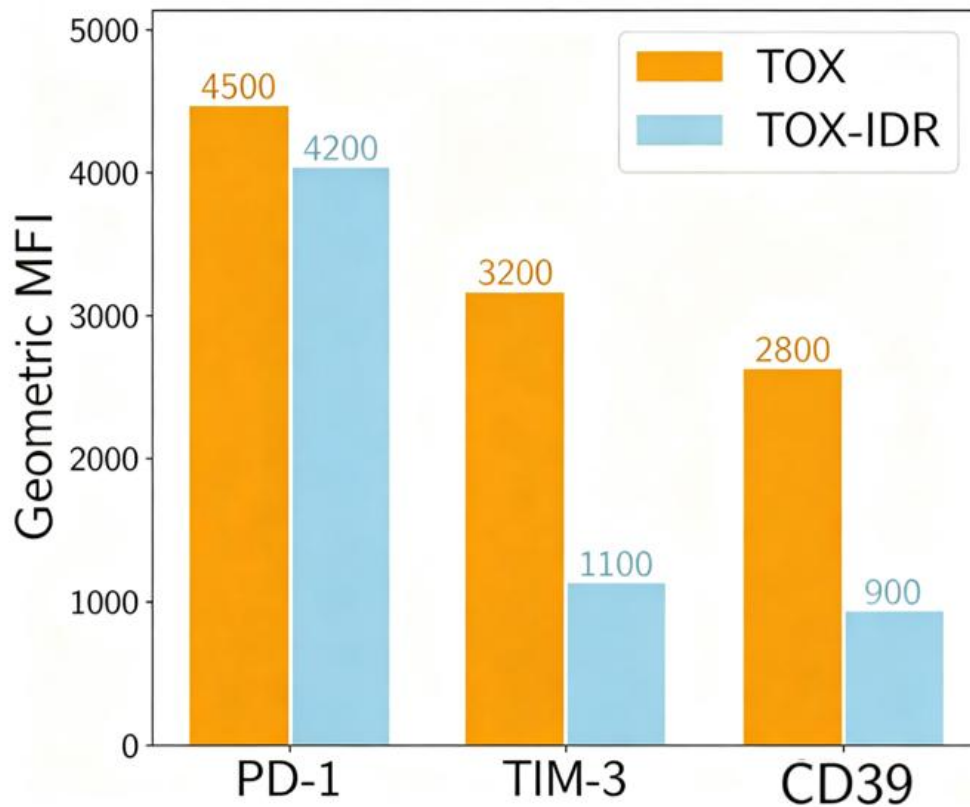


Table 4

Donor CD8⁺ T cell phenotype and functional indicators of tumor-infiltrating donors

Indicator	TOX Group	(TOX-IDRm) group	P-value
PD-1 ⁺ TIM-3 ⁺ (%)	62.3 ± 4.5	23.5 ± 3.2	<0.0001
TCF-1 ⁺ (%)	12.1 ± 2.3	38.7 ± 3.8	<0.0001
IFN-γ ⁺ (%)	15.2 ± 2.8	42.5 ± 5.1	<0.001

TNF- α^+ (%)	8.9 $\pm$ 1.9	29.3 $\pm$ 3.6	<0.001
Granzyme B ⁺ (%)	13.2 $\pm$ 2.5	35.6 $\pm$ 4.2	<0.001
Ki-67 ⁺ (%)	18.4 $\pm$ 3.1	56.2 $\pm$ 4.7	<0.0001

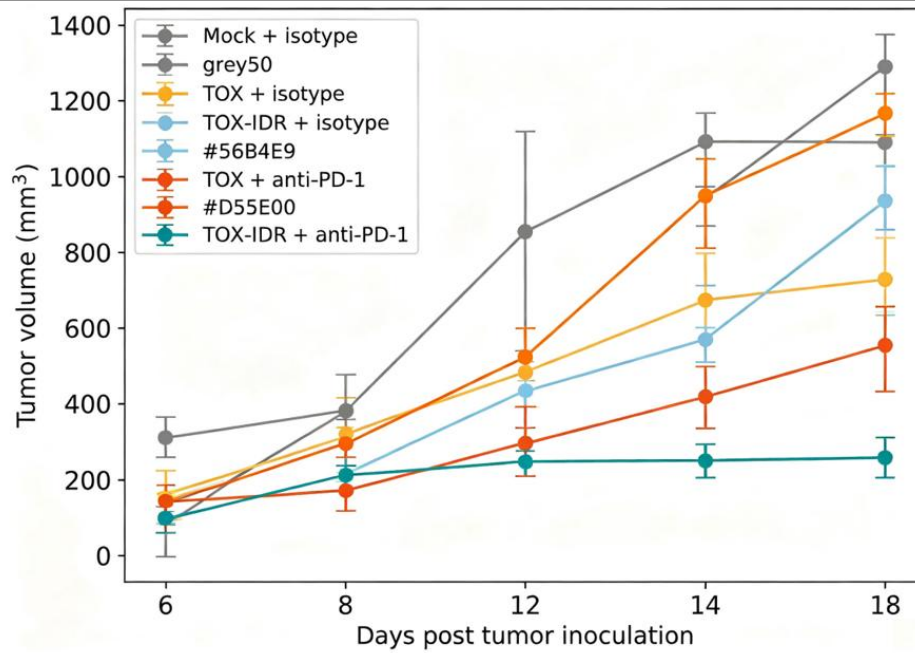
Note. Data from three separate trials, with n = 8 mice per group, are displayed as mean $\pm$ SEM. A two-tailed unpaired t-test was used for the statistical analysis.

4.5. Targeting TOX phase separation inhibits tumor growth and works together with anti-PD-1 antibodies

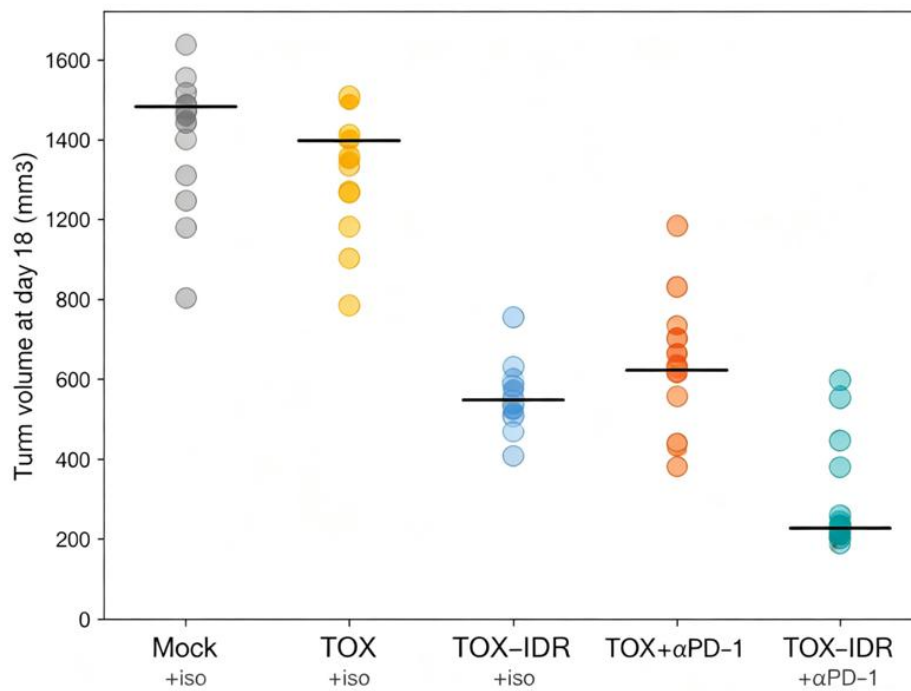
Lastly, we assessed the possibility of interfering with TOX phase separation in tumour immunotherapy. Mice that received adoptive transfer of (TOX-IDRm) OT-I T cells in the B16-OVA melanoma model exhibited noticeably slower tumour growth than mice that got full-length TOX T cells. The average tumour volume in the (TOX-IDRm) group was only 520 mm³ by day 18 following tumour inoculation, compared to 1180 mm³ in the TOX group (Fig. 6A, Table 5). More significantly, the (TOX-IDRm) anti-PD-1 group demonstrated a potent synergistic tumor-suppressive impact when paired with anti-PD-1 antibody treatment. The average tumour volume decreased to 180 mm³ by day 18, and in six of the eight animals, the tumours almost vanished (Figure 6A-B). A substantial synergistic impact between TOX-IDRm and anti-PD-1 was established by analysis of the tumour development curve area ($P < 0.001$, two-way ANOVA). 75% of the mice in the (TOX-IDRm) anti-PD-1 group were still alive at the experimental endpoint of 45 days, which was significantly better than the TOX anti-PD-1 group (median survival 32 days) and all single-agent control groups (Fig. 6C, Table 5). Survival analysis revealed that the median survival in the (TOX-IDRm) group was extended to 34 days. The MC38 colon cancer model also showed a comparable synergistic anticancer effect (Fig. S1). These findings imply that interfering with TOX phase separation can both partially overcome resistance to PD-1 blocking and independently restore the anticancer activity of tired T cells, greatly increasing the efficacy of immune checkpoint inhibition.

Figure 6

A: Tumor growth curves



B: Day 18 tumor volume dot plot



C: Kaplan-Meier survival curves

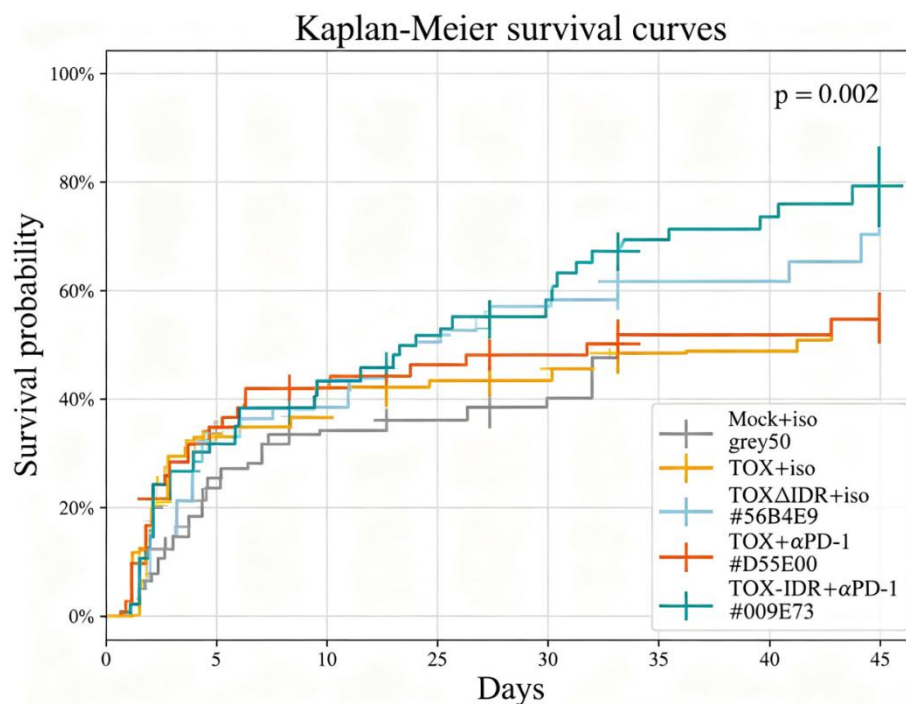


Figure S1

Tumor growth curves in MC38 colon carcinoma model

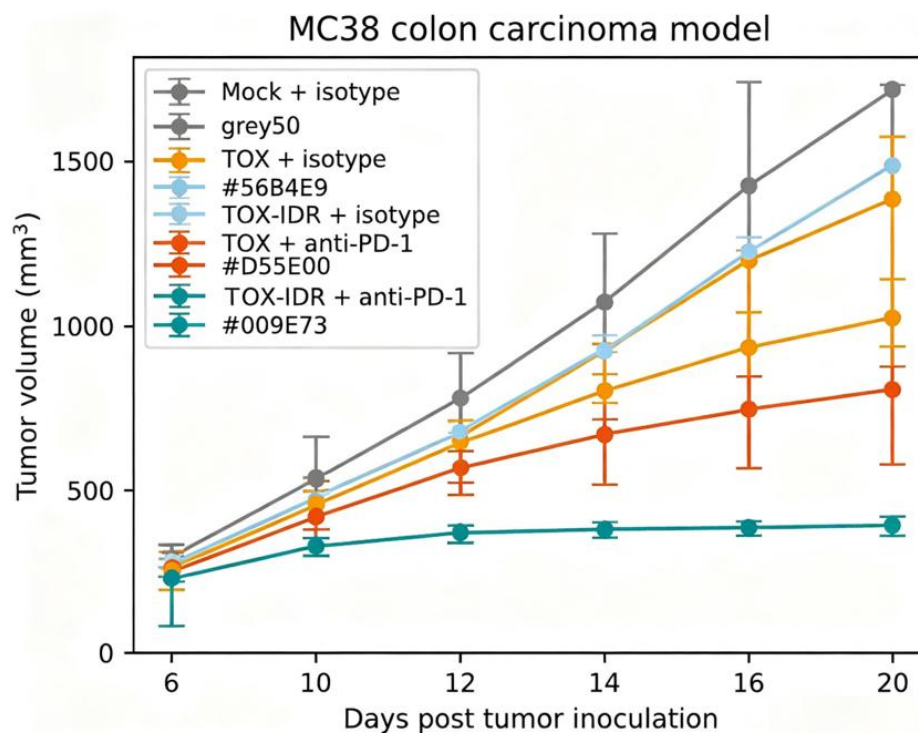


Table 5

Tumor volume and survival of B16-OVA tumor-bearing mice

Group	Tumor volume on day 18 (mm ³)	Median survival (days)	Survival Rate on Day 45 (%)
Mock + Isoform comparison	1250 ± 156	25	0

TOX + Isoform comparison	1180 ± 134	26	0
(TOX-IDRm) + Isoform comparison	520 ± 78	34	0
TOX + anti-PD-1	640 ± 92	32	0
(TOX-IDRm) + anti-PD-1	180 ± 45	Not reached (>45)	75

Note. With $n = 8$ mice per group, tumour volume is displayed as mean $\pm$ SEM. The Kaplan-Meier method was used to calculate median survival, and the log-rank test revealed that the anti-PD-1 group (TOX-IDRm) was significantly different from all other groups ($P < 0.001$).

5. Discussion

For the first time, this study demonstrates that the transcription factor TOX experiences liquid-liquid phase separation via its intrinsically disordered N-terminal region, creating functional condensates in exhausted T cell nuclei. This serves as a physical foundation to ‘lock’ the terminal exhaustion transcription program into an irreversible state. In addition to resetting the chromatin accessibility of fatigue-related genes at the epigenetic level, disrupting TOX’s phase separation ability also functionally reprograms T cell destiny, releasing them from terminal exhaustion and reinstating their anti-tumor activity. More significantly, in several mice tumour models, targeting TOX phase separation results in a substantial synergistic impact with PD-1 immune checkpoint suppression, attaining tumour control beyond what either therapy alone can do. These results offer proof of concept for the development of tumour immunotherapy techniques based on interfering with protein phase separation, in addition to revealing a novel molecular mechanism sustaining terminal T cell depletion.

The introduction of liquid–liquid phase separation, a biophysical phenomenon, into the framework of T cell exhaustion control is the primary innovation of this work. In the past, TOX was widely acknowledged as the primary transcription factor responsible for CD8⁺ T cell exhaustion. However, the traditional transcriptional regulation paradigm, which involves binding DNA and recruiting epigenetic modifiers to activate or silence target genes, was primarily responsible for its functional mechanism. Nevertheless, this linear model is unable to adequately account for the epigenetic stability specific to terminally exhausted T cells; even after antigen removal or PD-1 signal blocking, Ttex’s chromatin landscape is still challenging to rebuild. According to our research, TOX condensates create a “physical lock” by spatially anchoring exhaustion core genes like *Pdcd1*, *Havcr2*, and *Entpd1* within an inhibiting transcriptional milieu. Once created, the entire condensate network cannot be destroyed by merely interfering with individual proteins or blocking upstream signals. This shifts the focus of exhaustion research from a one-dimensional map of transcription factor binding to a three-dimensional subnuclear compartmentalisation perspective, offering a structural explanation for the irreversibility of terminal exhaustion.

When we consider the results of this work in the context of the developing field of phase separation and immune modulation, we find that it engages with a number of significant developments. Phase separation serves as a catalytic platform in the cGAS-STING innate immune pathway to increase signal output and has been demonstrated to be crucial in TCR signalling by the production of LAT-SLP-76 signal clusters. In order to stimulate gene expression at the transcriptional regulatory level, coactivators like BRD4 and MED1 and transcription factors like OCT4 and GCN4 create condensates at super-enhancer areas during phase separation. However, phase separation's significance in gene silencing and locking in terminal differentiation receives less attention than how it triggers gene expression or amplifies signals. According to our research, TOX condensates serve as a prolonged inhibitory platform that keeps exhaustion gene chromatin open while blocking retrograde transcription factors, rather than only acting as transient hubs for transcription activation. This implies that, depending on the kinds of molecules abundant in the condensate, phase separation may play a dual role in cell fate decisions, either driving activation or locking in a terminal state.

The TOX phase separation-dependent maintenance mechanism identified in this work presents a novel concept for tumour immunotherapy from the standpoint of translational medicine: directly interfering with protein condensates. Immune checkpoint blockade therapy is currently ineffective for Ttex cells, whose enormous accumulation is a major cause of immune evasion in the majority of solid tumours, and can only partially restore the function of Tpex cells. We showed that terminally tired T cells can restore proliferation and effector capabilities by only interfering with TOX's capacity to undergo phase separation. Additionally, this "unlocking" effect is amplified when anti-PD-1 antibodies are added, and in certain animals, it even results in total tumour remission. Developing small molecules or peptides that specifically disrupt TOX condensates without affecting other normal phase separation processes may become a precise new strategy to "unlock" exhausted T cells, given that small molecules can concentrate in particular transcription factor condensates and affect their function. More generally, this strategy might be used for other diseased conditions that are preserved by phase separation, like aberrantly activated T cell populations in autoimmune disorders.

However, there are a number of limitations to this study that should be carefully taken into account. First, the IDR deletion mutant (TOX-IDRm) is necessary for the main experiment demonstrating TOX's phase separation function. We cannot totally rule out the possibility that the IDR loss may impact TOX's interactions with other unidentified proteins, resulting in symptoms that are independent of phase separation, even tho this mutant keeps the intact HMG-box DNA binding domain. To better analyse the phase separation-specific functions, future research should employ more accurate mutation techniques, such as point mutations of hydrophobic residues within the IDR to diminish phase separation without changing the sequence length. Second, adoptively transplanted TCR transgenic T cells and subcutaneous tumour models are the primary tools used in this study's in vivo

investigations. These systems are distinct from the intricate tumour microenvironment of endogenous T cell responses, despite their obvious antigen specificity and controllability. Furthermore, the IDR sequence in human TOX is less conserved, despite the fact that we verified the phase separation phenomena in mouse TOX. Further research is required to determine whether the human protein has a similar capacity for phase separation and whether phase separation abnormalities can similarly revitalise depleted T cells in clinical samples.

The exact composition and dynamic management of TOX condensates are another matter that need further investigation. We still need to use immunoprecipitation in conjunction with mass spectrometry to determine whether co-exhaustion factors, such as TOX2 and members of the NR4A family, cooperate within the same condensates to lock in the exhausted state. We have not yet identified the precise protein partners enriched in TOX condensates aside from DNA. Simultaneously, it's worthwhile to look into which upstream signals control phase-separated condensates *in vivo*, such as whether metabolic products, ROS levels, or calcium signals can dynamically modify the construction and disassembly of TOX condensates. Lastly, delivering the “phase separation intervention” strategy to tumor-infiltrating T cells specifically, preventing off-target toxicity by interfering with phase separation in other normal cells (such as neurones or stem cells), and assessing pharmacodynamic endpoints are just a few of the many obstacles that still need to be overcome before it can be used in clinical settings.

In conclusion, this work challenges the current belief that exhaustion is irreversible by inventively revealing the “physical lock” mechanism of TOX phase separation in sustaining terminal T cell depletion. It also offers novel targets and combination methods for cancer immunotherapy. This finding adds a crucial note on the various roles of biomolecular condensates in adaptive immunity while also deepening our understanding of the temporal and geographical regulation of T cell fate decisions. In the future, it may be possible to overcome immune therapy resistance and enhance long-term benefits for cancer patients by focusing on TOX condensates and investigating the integration of phase separation interventions with current immunotherapies.

6. Conclusion

This work shows for the first time that the transcription factor TOX forms functional condensates in the nuclei of fatigued T cells by driving liquid-liquid phase separation thru its inherently disordered N-terminal region. This creates an irreversible condition for the terminal exhaustion transcriptional program, akin to a “physical lock.” Disrupting TOX's phase separation ability can significantly restore exhausted T cell proliferation and effector functions in addition to reprogramming T cell fate and resetting chromatin accessibility of core exhaustion genes at the epigenetic level. When paired with anti-PD-1 immune checkpoint suppression, targeting TOX phase separation successfully suppresses

tumour development and extends survival in a range of mouse cancer models. These discoveries provide new molecular targets and intervention techniques to overcome immunotherapy resistance while also elucidating the physical basis for sustaining terminal T cell exhaustion.

Developing small molecules or cell-penetrating peptides that can specifically disrupt TOX condensates and testing their safety and effectiveness in preclinical models; verifying the phase separation characteristics of human TOX and their pathological significance in patient tumor-infiltrating T cells, helping to translate basic discoveries to clinical applications; and exploring the application of this strategy in engineered T cells (such as CAR-T cells) by decreasing or altering TOX's phase separation tendency to increase the persistence and anti-tumor efficacy of adoptive cell therapy. In conclusion, this work establishes the theoretical and experimental groundwork for the development of cancer immunotherapies of the “phase separation intervention” kind by introducing liquid-liquid phase separation into the realm of T cell exhaustion regulation.

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Author contributions

Conceptualization, Gang Liu and Jia Wang; methodology, Jia Wang; validation, Gang Liu; formal analysis, Jia Wang; investigation, Gang Liu and Jia Wang; resources, Gang Liu; data curation, Jia Wang; writing—original draft preparation, Jia Wang; writing—review and editing, Gang Liu; visualization, Jia Wang; supervision, Gang Liu; project administration, Gang Liu. All authors have read and agreed to the published version of the manuscript.

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The authors declare that there is no conflict of interest.

Ethics approval

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Consent to participate

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Consent for publication

All authors have given their consent.

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References

- [1] Alfei, F., Kanev, K., Hofmann, M., et al. (2019). TOX reinforces the phenotype and longevity of exhausted T cells in chronic viral infection. *Nature*, 571(7764), 265-269. <https://doi.org/10.1038/s41586-019-1326-9>
- [2] Beltra, J. C., Manne, S., Abdel-Hakeem, M. S., et al. (2020). Developmental relationships of four exhausted CD8⁺ T cell subsets reveals underlying transcriptional and epigenetic landscape control mechanisms. *Immunity*, 52(5), 825-841.e14. <https://doi.org/10.1016/j.immuni.2020.04.014>
- [3] Blank, C. U., Haining, W. N., Held, W., et al. (2019). Defining 'T cell exhaustion'. *Nat Rev Immunol*, 19(11), 665-674. <https://doi.org/10.1038/s41577-019-0221-9>
- [4] Boija, A., Klein, I. A., Sabari, B. R., et al. (2018). Transcription factors activate genes through the phase-separation capacity of their activation domains. *Cell*, 175(7), 1842-1855.e16. <https://doi.org/10.1016/j.cell.2018.10.042>
- [5] Chen, J., López-Moyado, I. F., Seo, H., et al. (2019). NR4A transcription factors limit CAR T cell function in solid tumours. *Nature*, 567(7749), 530-534. <https://doi.org/10.1038/s41586-019-0985-x>
- [6] Chong, S., Dugast-Darzacq, C., Liu, Z., et al. (2018). Imaging dynamic and selective low-complexity domain interactions that control gene transcription. *Science*, 361(6400), eaar2555. <https://doi.org/10.1126/science.aar2555>
- [7] Du, M., & Chen, Z. J. (2018). DNA-induced liquid phase condensation of cGAS activates innate immune signaling. *Science*, 361(6403), 704-709. <https://doi.org/10.1126/science.aat1022>
- [8] Hnisz, D., Shrinivas, K., Young, R. A., Chakraborty, A. K., & Sharp, P. A. (2017). A phase separation model for transcriptional control. *Cell*, 169(1), 13-23. <https://doi.org/10.1016/j.cell.2017.02.007>
- [9] Khan, O., Giles, J. R., McDonald, S., et al. (2019). TOX transcriptionally and epigenetically programs CD8⁺ T cell exhaustion. *Nature*, 571(7764), 211-218. <https://doi.org/10.1038/s41586-019-1325-x>
- [10] Klein, I. A., Boija, A., Afeyan, L. K., et al. (2020). Partitioning of cancer therapeutics in nuclear condensates. *Science*, 368(6497), 1386-1392. <https://doi.org/10.1126/science.aaz4427>



- [11] Li, P., Banjade, S., Cheng, H. C., et al. (2012). Phase transitions in the assembly of multivalent signalling proteins. *Nature*, 483(7389), 336-340. <https://doi.org/10.1038/nature10879>
- [12] Martin, E. W., & Mittag, T. (2018). Relationship of sequence and phase separation in protein low-complexity regions. *Biochemistry*, 57(17), 2478-2487. <https://doi.org/10.1021/acs.biochem.8b00008>
- [13] Miller, B. C., Sen, D. R., Al Abosy, R., et al. (2019). Subsets of exhausted CD8⁺ T cells differentially mediate tumor control and respond to checkpoint blockade. *Nat Immunol*, 20(3), 326-336. <https://doi.org/10.1038/s41590-019-0312-6>
- [14] O'Flaherty, E., & Kaye, J. (2003). TOX defines a conserved subfamily of HMG-box proteins. *BMC Genomics*, 4(1), 13. <https://doi.org/10.1186/1471-2164-4-13>
- [15] Pauken, K. E., Sammons, M. A., Odorizzi, P. M., et al. (2016). Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. *Science*, 354(6316), 1160-1165. <https://doi.org/10.1126/science.aaf2807>
- [16] Sabari, B. R., Dall'Agnese, A., Boija, A., et al. (2018). Coactivator condensation at super-enhancers links phase separation and gene control. *Science*, 361(6400), eaar3958. <https://doi.org/10.1126/science.aar3958>
- [17] Scott, A. C., Dündar, F., Zumbo, P., et al. (2019). TOX is a critical regulator of tumour-specific T cell differentiation. *Nature*, 571(7764), 270-274. <https://doi.org/10.1038/s41586-019-1324-y>
- [18] Seo, H., Chen, J., González-Avalos, E., et al. (2019). TOX and TOX2 transcription factors cooperate with NR4A transcription factors to impose CD8⁺ T cell exhaustion. *Proc Natl Acad Sci U S A*, 116(25), 12410-12415. <https://doi.org/10.1073/pnas.1905675116>
- [19] Shin, Y., & Brangwynne, C. P. (2017). Liquid phase condensation in cell physiology and disease. *Science*, 357(6357), eaaf4382. <https://doi.org/10.1126/science.aaf4382>
- [20] Su, X., Ditlev, J. A., Hui, E., et al. (2016). Phase separation of signaling molecules promotes T cell receptor signal transduction. *Science*, 352(6285), 595-599. <https://doi.org/10.1126/science.aad9964>
- [21] Wherry, E. J. (2011). T cell exhaustion. *Nat Immunol*, 12(6), 492-499. <https://doi.org/10.1038/ni.2035>



- [22] Zheng, L. W., Liu, C. C., & Yu, K. D. (2023). Phase separations in oncogenesis, tumor progressions and metastasis: A glance from hallmarks of cancer. *J Hematol Oncol*, 16(1), 123.
[https://doi.org/ 10.1186/s13045-023-01522-5](https://doi.org/10.1186/s13045-023-01522-5)