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Comparable restimulation of human T cells activated with CD3/CD28 beads versus soluble antibody complexes

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Ex vivo T-cell activation is critical in both basic immunology and clinical applications, such as CAR-T cell therapy. CD3/CD28 antibody-coated beads and soluble antibody complexes are widely used, yet direct comparisons remain limited and often contradictory. We longitudinally profiled human T cells stimulated with two widely used activation reagents, one bead-based and the other pre-formed soluble antibody complexes, to clarify these differences. Both approaches supported robust expansion and stable CD4/CD8 ratios, indicating comparable proliferative capacity. Beads induced earlier, stronger activation and rapid effector memory differentiation, whereas the soluble antibody complex reagent promoted slower activation and preserved central memory subsets. Upon restimulation, however, both conditions efficiently reactivated and converged toward effector memory differentiation with sustained TIM-3 expression, consistent with chronic stimulation. This convergence suggests that restimulation is a major determinant of long-term phenotype, potentially overriding differences introduced by the initial activation reagent. Together, our findings reconcile prior inconsistencies and demonstrate that while expansion is comparable, activation and differentiation diverge across these commonly used reagents, providing a framework to tailor activation strategies to specific immunotherapy outcomes.

Keywords T-cell activation, Dynabeads, ImmunoCult, T-cell restimulation, CD3/CD28 stimulation

Abbreviations

CM	Central memory
EM	effector memory
IL-2	Interleukin-2
PBMC	Peripheral blood mononuclear cell

T lymphocytes are central mediators of the adaptive immune response, contributing to pathogen clearance, tumor surveillance, and immune homeostasis through their $CD4^+$ helper and $CD8^+$ cytotoxic subsets. Their ability to recognize specific antigens and exert diverse effector functions makes them critical in both physiological and pathological contexts, including infection, cancer, autoimmunity, and transplant rejection. Accordingly, studying T-cell biology is essential for understanding immune mechanisms and developing targeted immunotherapies.

Primary T cells are widely used in both basic and translational immunology. Co-culture models involving primary T cells and tumor cells, for instance, are commonly used to evaluate T-cell-mediated cytotoxicity, immune evasion, or immunomodulatory effects[1–6]. In vitro models allow mechanistic studies of T-cell biology and inform and guide T-cell-based immunotherapy development. T cells notably serve as the cellular backbone of engineered immunotherapies, including chimeric antigen receptor T cells, particularly for hematological malignancies[7,8]; T-cell receptor–modified cells for both hematological and solid tumors[9,10]; and tumor-infiltrating lymphocytes for solid tumors[11,12], all of which depend on efficient ex vivo activation and expansion

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of functional T cells. These applications rely on producing large numbers of functional T cells, highlighting the importance of robust in vitro stimulation methods.

T-cell activation through simultaneous engagement of CD3 and CD28 with agonistic antibodies is a well-established standard[13,14]. CD3 ligation delivers the primary activation signal through the T-cell receptor, while CD28 provides the co-stimulatory signal for proliferation, survival, and differentiation. T cells are commonly stimulated via dual CD3/CD28 targeting using either bead-based stimulation, in which antibodies are immobilized on microspheres, or bead-free stimulation, in which unconjugated free antibodies or pre-formed antibody complexes are provided in soluble form. Bead-based reagents mimic the spherical geometry and high antigen density of antigen-presenting cells, providing strong and synchronous stimulation, though this may come at the cost of non-physiological signal intensity. In contrast, soluble antibodies provide a more diffuse stimulation and possibly asymmetric CD3/CD28 signaling, which may more closely resemble physiological interactions between T cells and antigen-presenting cells.

Several studies have examined how CD3/CD28 antibody-coated beads or soluble antibodies influence T-cell expansion and phenotype[15–19]. In particular, Soltantoye et al.[16] compared soluble versus plate-coated anti-CD3/28 antibodies, the latter providing fixed receptor engagement similar to beads but without a three-dimensional format. The authors reported comparable CD4/CD8 ratios and activation kinetics, but noted faster expansion with immobilized antibodies, a higher CD8 proportion with soluble antibodies, and greater enrichment of central memory (CM) and effector memory (EM) subsets with soluble antibodies. In contrast, Li and Kurlander[15] compared anti-CD3/CD28 beads with soluble anti-CD3 in the presence of mononuclear cells and observed that while CD8 expansion was similar, bead stimulation induced stronger CD4 expansion and rapid EM differentiation, whereas soluble antibodies favoured naïve and CM phenotypes. Taken together, these studies highlight variability in reported outcomes across different bead-based and soluble antibody reagents.

To address these discrepancies, we sought to compare the effects of anti-CD3/CD28 beads and soluble antibody complexes on primary human T cells across both initial activation and subsequent restimulation. Our goal was to define how these two widely used activation reagents influence T-cell activation, proliferation, differentiation, and checkpoint expression over time, and to determine whether restimulation alters reagent-associated differences. Accordingly, this systematic comparison aims to clarify inconsistencies in the literature and provide practical guidance for selecting activation strategies in research and therapeutic applications.

Materials and methods

PBMC and T-cell isolation

Human peripheral blood samples were collected from buffy coats, obtained by a registered biobank (Croix-Rouge de Belgique, Liège, Belgique), that were donated by four healthy anonymous adult donors who provided written informed consent in accordance with institutional guidelines and regulations. The biobank activities and sample utilization are conducted in compliance with the ethical standard and approval of the Human Ethics Committee of UCLouvain. Biobank number: BB210036. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Freiburg, Germany). T cells were then isolated using the EasySep™ Human T Cell Isolation Kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions.

Primary T cell culture, initial stimulation, and restimulation

Isolated T cells were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Lonza), and 100 U/mL penicillin-streptomycin (Lonza). Cultures were maintained at 37 °C in 5% CO_2. For each donor, T cells were divided into two parallel culture conditions following activation: One supplemented with 5 ng/mL human interleukin-2 (IL-2; Peprotech, Neuilly-sur-Seine, France), a supportive but non-excessive dose that maintains T-cell survival without artificially biasing differentiation[20], and one without IL-2. We supplemented cultures with IL-2 alone to promote expansion without the influence of other commonly used cytokines such as interleukin-7 and interleukin-15, which help maintain naïve and central-memory-like states[21], which would have interfered with our ability to compare how the activation reagents themselves shaped differentiation.

T cells were counted 24-, 48-, 72-, and 96-hours post-isolation and subsequently every 2–3 days. Cells were seeded at a density of 5×10^4 cells per well in 200 µL of culture medium in 96-well U-bottom plates (Corning, NY, USA) and were subcultured once cell density exceeded 5×10^5 cells/mL.

On day 0 (initial stimulation), T cells were activated with either bead-based Dynabeads™ Human T-Activator CD3/CD28 (Gibco, Thermo Fisher Scientific) at a 1:1 bead-to-cell ratio or bead-free ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (STEMCELL Technologies), which provides pre-formed soluble complexes of anti-CD3, anti-CD28, and anti-CD2 antibodies, at 25 µL per 1×10^6 T cells according to the manufacturer's instructions. Dynabeads or ImmunoCult were added only at day 0. During subsequent subculturing, cultures received fresh medium, supplemented or not with IL-2, but no further supplementation with the initial activation reagents. This design allowed us to longitudinally monitor cultures under initial stimulation conditions, without confounding effects from repeated re-activation. On day 8 post-isolation (timing selected in line with[22], each culture condition (Dynabeads or ImmunoCult, with or without IL-2) was split into two groups per donor. One group from each condition was then restimulated using the eBioscience™ Cell Stimulation Cocktail (Invitrogen, Thermo Fisher Scientific) following the manufacturer's protocol, while a control group received no additional stimulation (as illustrated in Fig. 1). In total, T cells were cultured for 22 days.

Flow cytometry staining and analysis

To assess proliferation and viability, live-cell counts were determined using the FACS Canto™ II cytometer (BD Biosciences). A 10 µL aliquot was diluted in 50 µL PBS and stained with 2 µL eBioscience™ 7-AAD Viability

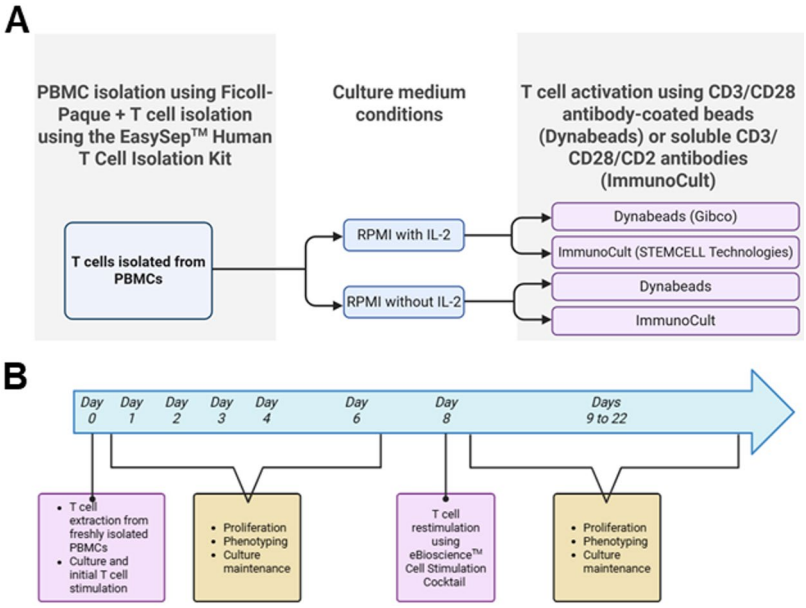

Fig. 1. Experimental workflow. (**A**) Flowchart illustrating experimental conditions. T cells isolated from PBMCs of four donors were cultured in RPMI medium with or without IL-2 and activated with either CD3/CD28 antibody-coated beads (Dynabeads™ Human T-Activator CD3/CD28) or soluble antibody complexes (ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator). (**B**) Timeline showing key experimental steps, including cell counting, flow cytometry phenotyping, culture maintenance, and restimulation.

Staining Solution (Invitrogen, Thermo Fisher Scientific). Viable (7-AAD$^-$) cells were quantified from recorded events. For T-cell phenotyping, cells were stained for 60 min at 4 °C with the following antibodies: CD3-BV605 (UCHT1), CD4-PerCP-Cy5.5 (RPA-R4, Sony Biotechnology, San Jose, CA, USA), CD8-PE-Cy7 (HIT8a), CD45RA-BV510 (HI100, BD Biosciences), CD62L-AlexaFluor700 (DREG-56, Biolegend), CD27-PE (M-T271, BD Biosciences), CD25-BV421 (M-A251, BD Biosciences), HLA-DR-BV650 (L243, Sony Biotechnology), CD69-FITC (L78, BD Biosciences), PD1-BV650 (EH12.2H7, Biolegend), and TIM3-PE-CF594 (7D3, BD Biosciences). Dead cells were excluded by incubating for 10 min at 4 °C with Fixable Viability Dye eFluor™ 780 (1:10,000 dilution; Invitrogen, Thermo Fisher Scientific).

Data and statistical analysis

Flow cytometry data were analyzed using FlowJo™ software (BD Biosciences). Statistical analysis and graph plotting were performed using GraphPad Prism 8.0.1 (GraphPad Software, USA). Data were tested for normality using the Shapiro–Wilk test. For comparisons between two datasets, paired or unpaired Student's t-tests (parametric or non-parametric as appropriate) were used. Repeated-measures two-way ANOVA with Geisser–Greenhouse correction was conducted to evaluate the effects of condition and time, with Šidák post hoc multiple comparisons. Statistical significance was set at $p < 0.05$ and denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Results

T cells were isolated from PBMCs of four healthy donors using the EasySep™ Human T Cell Isolation Kit and then activated with Dynabeads or ImmunoCult (Fig. 1A) in the presence or absence of IL-2 (which showed no measurable effect unless otherwise noted; Figures S1-S5). We then monitored proliferation, viability, CD4/CD8 composition, activation, differentiation, and immune checkpoint expression over time (Fig. 1B).

Comparable proliferation and CD4/CD8 composition following stimulation

We first monitored T-cell proliferation over 22 days to determine whether antibody-coated beads or soluble antibody complexes differentially influenced expansion kinetics. Both reagents induced robust early proliferation, reaching similar cell numbers and viability by day 8 (Fig. 2A, B). Reseeding for restimulation did not impair their proliferative capacity, as cells continued proliferating shortly thereafter. From day 13 onward, cell counts stabilized while viability gradually declined (Fig. 2B), indicating reduced proliferative capacity and the onset of cell death. Notably, restimulation did not promote a renewed proliferation wave, which is consistent with prior reports showing that pharmacological cocktails such as PMA/ionomycin are optimized to trigger short-term effector responses rather than sustained expansion[23,24]. Thus, the initial stimulant was the primary driver of proliferation dynamics.

Having established that proliferation was comparable, we next examined whether the stimulation method would influence the CD4/CD8 subset composition. At baseline, CD4$^+$ T cells comprised ~ 80% of the population and remained relatively stable over time (Fig. 3A). Restimulation caused a brief decline in CD4$^+$ frequency that

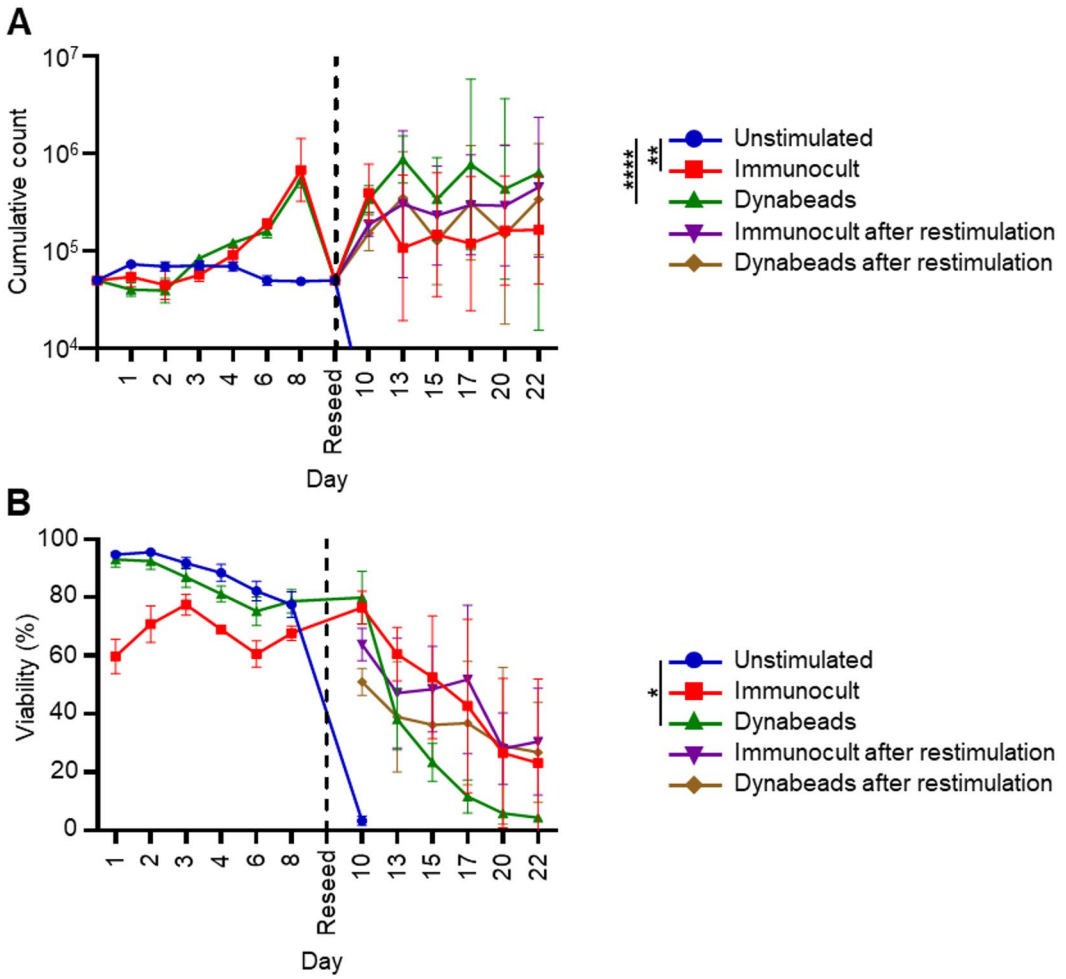

Fig. 2. T-cell proliferation after stimulation with antibody-coated beads (Dynabeads) or soluble antibody complexes (ImmunoCult). (**A**) Cumulative cell counts and (**B**) T-cell viability (%) during the initial stimulation phase (days 0–8), after reseeding in fresh media with or without restimulation on day 8, and throughout the post-restimulation phase (days 10–22). Data represent mean ± SD of four independent donors.)

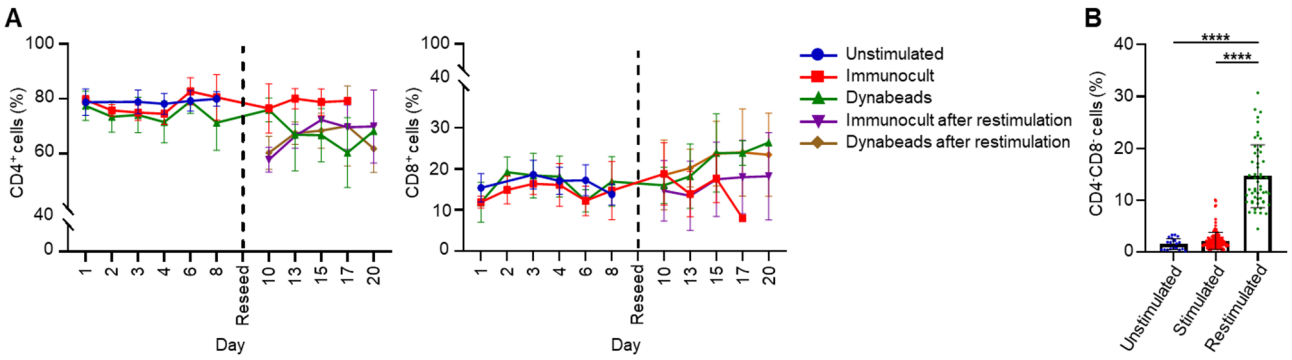

Fig. 3. CD4/CD8 T-cell composition following stimulation with antibody-coated beads (Dynabeads) or soluble antibody complexes (ImmunoCult) (**A**) Percentage of $CD4^+$ (left) and $CD8^+$ (right) T cells during the initial stimulation phase (days 0–8), after reseeding with or without restimulation on day 8, and throughout the post-restimulation phase (days 10–22). (**B**) Bar plots showing the emergence of a $CD3^+CD4^-CD8^-$ population following restimulation, observed consistently across conditions. Data represent mean ± SD of four independent donors.

subsequently stabilized. Similarly, $CD8^+$ T cells (~20% at baseline) remained stable across conditions, showing a modest increase after restimulation with no significant difference between stimulants. Together, these data demonstrate that long-term culture maintained a stable CD4/CD8 ratio, independent of the initial stimulant or restimulation.

Interestingly, a minor but consistent population of $CD3^+CD4^-CD8^-$ (double-negative) T cells emerged across all cultures after restimulation (Fig. 3B), possibly reflecting co-receptor downregulation in chronically activated cells as previously reported for $CD4^+$ T cells subjected to repeated stimulation[25,26] or the emergence of mucosal-associated invariant T, γδ T, or NKT cells[27].

Together, these results demonstrate that CD3/CD28 antibody-coated beads and soluble antibody complexes yielded comparable proliferation and subset dynamics, indicating that both methods can be effectively used for ex vivo T-cell expansion.

Activation, differentiation, and checkpoint dynamics diverge between stimulants

We then sought to determine how each stimulant modulated T-cell phenotype by assessing activation (CD69, CD25, HLA-DR), differentiation ($CD45RA^+CD27^+CD62L^{+/-}$), and immune checkpoint (PD-1, TIM-3) markers. Cultures were monitored until day 17, after which viability fell below 30% (Fig. 3B), precluding reliable analysis at later time points.

Activation markers revealed distinct kinetics between stimulants. CD69, an early T-cell activation marker[28], peaked early and declined rapidly. Specifically, CD69 was significantly higher immediately post-stimulation with Dynabeads than ImmunoCult (Fig. 4A). Levels then decreased in both conditions between days 3 and 6, where they remained low and stable for the duration of the culture. Similar trends were observed for CD69 expression on CD4 and CD8 T cells, highlighting that the reagent did not disproportionately alter CD69 levels (Figure S4A, S5A). Restimulation re-induced CD69 expression, with ImmunoCult showing a trend toward higher expression than Dynabeads over time despite no significant difference between the two stimulants. Thus, Dynabeads induced a stronger initial upregulation in CD69 expression, whereas ImmunoCult-stimulated cells exhibited a more pronounced increase upon restimulation.

CD25, an intermediate-late T-cell activation marker[29], peaked later and persisted longer. In particular, CD25 was upregulated in both conditions with comparable expression levels post-stimulation (Fig. 4B). As with CD69 expression, CD25 peaked earlier with Dynabeads, and peak CD25 levels coincided with decreasing CD69 expression. CD25 expression persisted longer than CD69, declining between days 8 and 10. Additionally, IL-2 influenced expression levels and resulted in significant differences between the two stimulants as well as their IL-2-absent counterparts, possibly by modulating receptor expression (Figure S3B). Nonetheless, in contrast to CD69, CD25 expression was similar between stimulants, suggesting that activation markers differ in sensitivity to stimulation strength. CD25 was also robustly re-induced in both stimulants, peaking immediately post-restimulation before declining. Similar trends were observed in CD4 and CD8 subsets (Figures S4B, S5B), indicating that the initial stimulant did not differentially affect CD25 expression across T-cell lineages.

As expected for a late activation marker[30], HLA-DR rose gradually and peaked late, with increasing HLA-DR expression coinciding with declining CD69 expression (Fig. 4C). HLA-DR levels also rose earlier and higher with Dynabeads than with ImmunoCult but reached comparable levels between days 6 and 8. Interestingly, unstimulated cells displayed transient HLA-DR expression early in the culture, which may reflect stress-induced upregulation[31]. Expression levels rapidly increased in both conditions between days 6 and 8 and remained high and stable until day 17, reflecting late activation. CD4 and CD8 subsets showed similar patterns (Figures S4C, S5C), indicating that the initial stimulant did not disproportionately alter HLA-DR expression. Following restimulation, HLA-DR levels were moderate and stable, with expression levels similar to those observed during the initial stimulation, suggesting that restimulation reset T cells to an early-intermediate activation state rather than sustaining a late activation profile.

Since stimulant type affected the expression of activation markers, we then sought to assess whether they also altered T-cell phenotype. Naïve T cells rapidly decline under long-term stimulation with CD3/CD28 agonists, particularly in the absence of cytokines such as interleukin-7 and interleukin-12[32,33]. We therefore focused on memory ($CD45RA^-CD27^+$) T cells and classified them into two main categories: $CD62L^+$ CM and $CD62L^-$ EM (Fig. 5A and B)[34]. ImmunoCult maintained almost equal distributions of CM and EM cells throughout the duration of the culture period, apart from the phase immediately post-stimulation and between days 6 and 10. In contrast, EM cells predominated after Dynabeads exposure, especially immediately post-stimulation. The EM frequency then steadily declined over time until reseeding, possibly due to media replenishment. Restimulation then led to an almost complete conversion to EM cells in both conditions. The rapid conversion to EM cells with Dynabeads is consistent with the strong activation observed in this condition, whereas ImmunoCult, which induced milder activation, preserved a greater proportion of CM cells. Like with activation, CM and EM proportions remained similar between CD4 and CD8 T cells, highlighting that the initial stimulant did not disproportionately affect CD4 or CD8 differentiation (Figure S8A, S8B). Together, these findings demonstrate that the two activation reagents differed in the extent of CM or EM proportions, with Dynabeads-stimulated cells exhibiting a more pronounced early shift toward EM phenotypes than cells stimulated with soluble antibody complexes.

Lastly, we assessed immune checkpoint expression to determine whether long-term stimulation induced features of T-cell dysfunction. We focused on TIM-3 and PD-1, as these receptors are frequently co-expressed on chronically stimulated T cells[35] and are key targets of immune checkpoint blockade therapies. At the start of the culture, TIM-3 expression was low but significantly higher with ImmunoCult than Dynabeads immediately post-stimulation, although Dynabeads-stimulated cells reached comparable levels around day 6. Thereafter, TIM-3 expression declined in both conditions but remained slightly elevated in ImmunoCult cultures with IL-2 between days 10 and 17 (Figure S9A). This pattern is consistent with prior reports showing antigen-independent

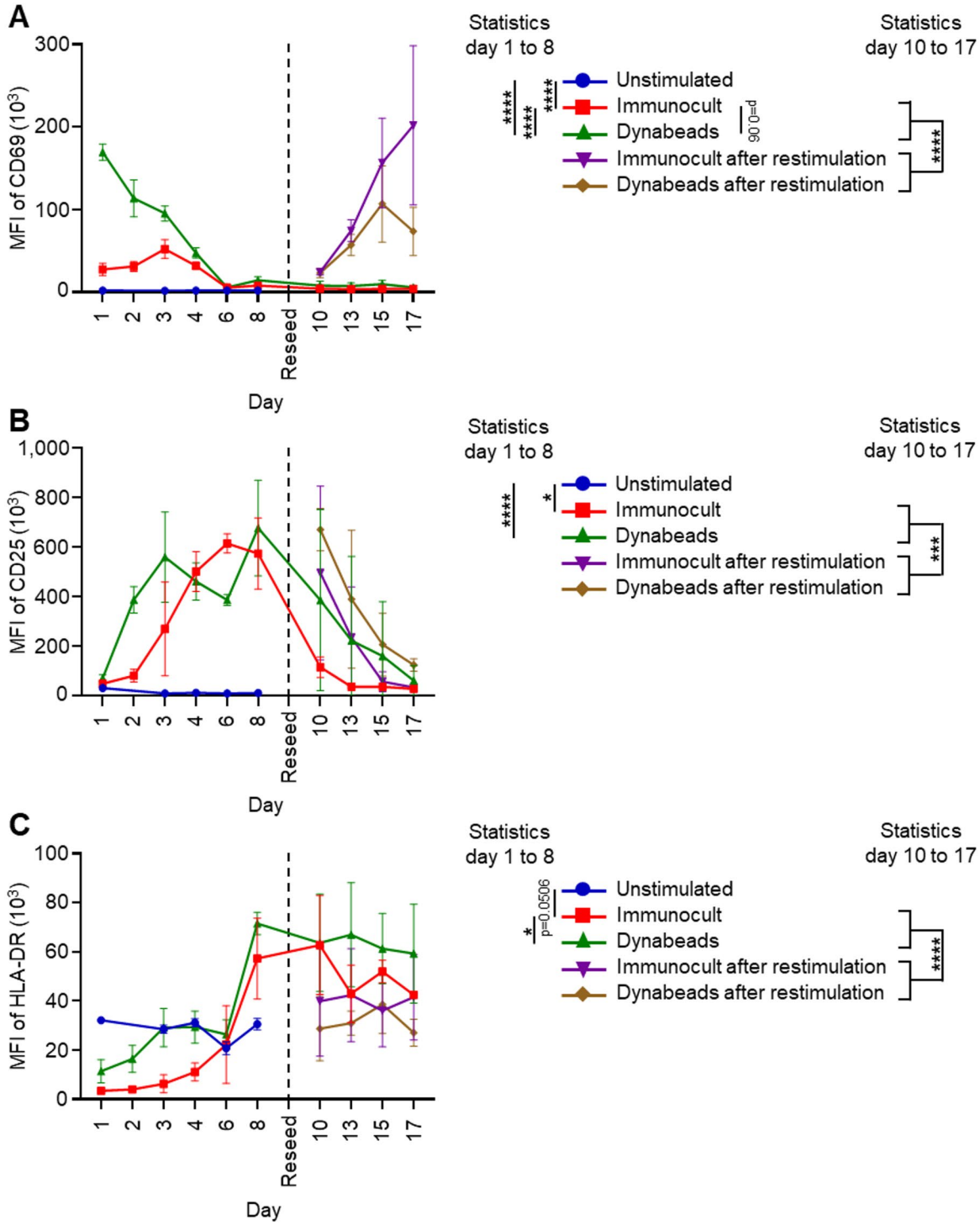

Fig. 4. T-cell activation dynamics after stimulation with antibody-coated beads (Dynabeads) or soluble antibody complexes (ImmunoCult) during the initial stimulation phase (days 0–8), after reseeding in fresh media with or without restimulation on day 8, and throughout the post-restimulation phase (days 10–22). Activation marker expression was measured as the Mean Fluorescence Intensity (MFI) of (**A**) CD69 (early activation) (**B**) CD25 (intermediate-late activation), and (**C**) HLA-DR (late activation). Data represent mean ± SD of four independent donors.

TIM-3 induction by IL-2 via the PI3K pathway[36,37]. Restimulation resulted in high TIM-3 expression, likely reflecting chronic stimulation (Fig. 6A).

PD-1 expression followed a different pattern: levels were comparable between stimulants, peaking around days 3–4 before steadily declining over the remainder of the culture period. Restimulation also induced a transient

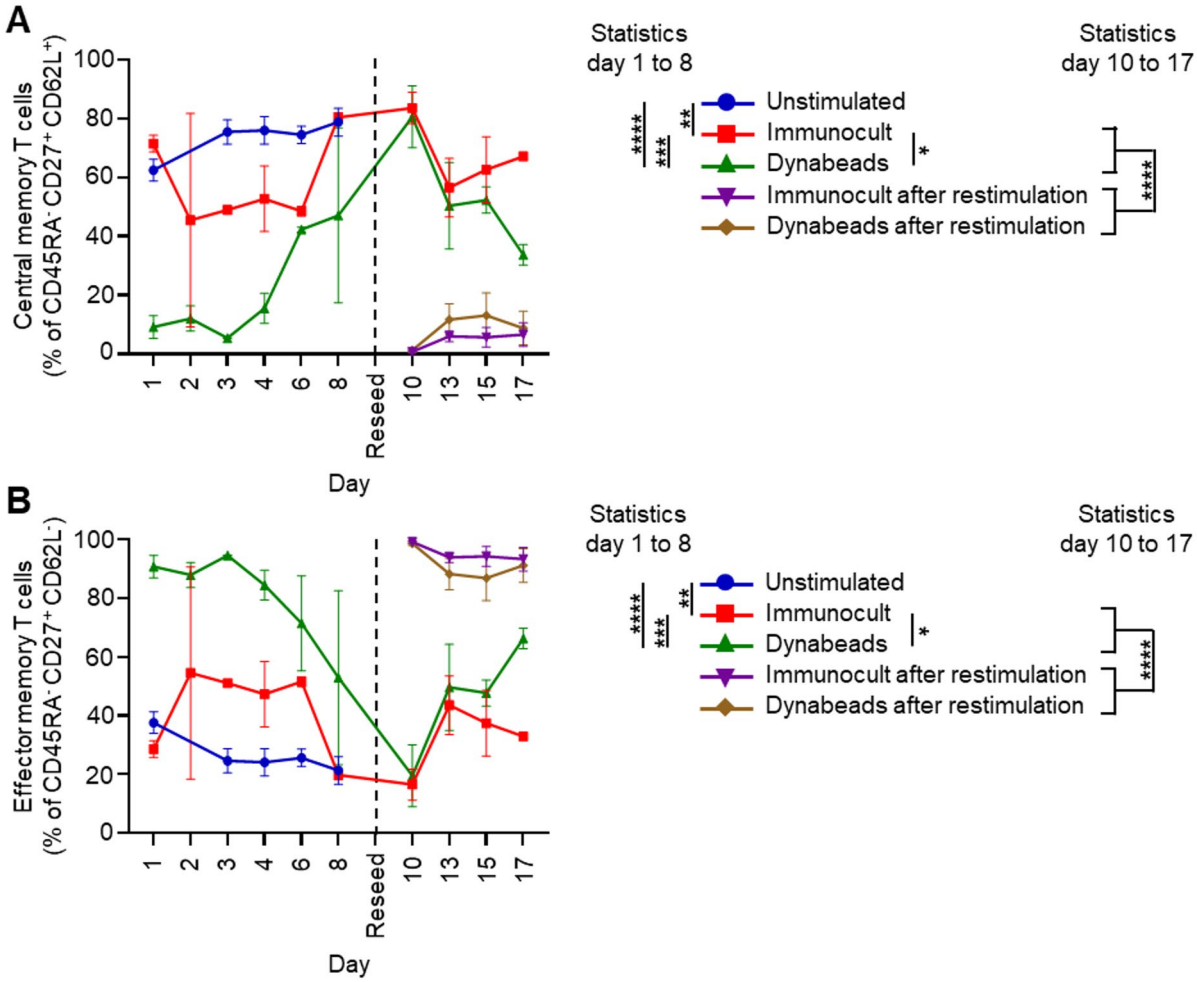

Fig. 5. T-cell differentiation dynamics after stimulation with antibody-coated beads (Dynabeads) or soluble antibody complexes (ImmunoCult) during the initial stimulation phase (days 0–8), after reseeding in fresh media with or without restimulation on day 8, and throughout the post-restimulation phase (days 10–22). Memory T cells were categorized as (**A**) central memory (% of $CD45RA^{-}CD27^{+}CD62L^{+}$) or (**B**) effector memory (% of $CD45RA^{-}CD27^{+}CD62L^{-}$). Data represent mean ± SD of four independent donors.

increase in PD-1 expression, followed by a gradual downregulation (Fig. 6B). Although we did not directly assess effector function, the induction of TIM-3 is consistent with features of T-cell dysfunction described in chronic stimulation models, while those of PD-1 closely aligned with early-intermediate activation marker expression patterns. These findings indicate that both stimulants elicit transient checkpoint upregulation, but restimulation is associated with sustained TIM-3 induction. As PD-1 and TIM-3 were measured only in the bulk T-cell population, we were unable to assess reagent-specific effects on these checkpoints within CD4 and CD8 subsets.

Collectively, these results show that the two activation reagents differed in activation kinetics and differentiation, whereas checkpoint expression followed similar trajectories in both conditions, with restimulation associated with increased TIM-3 expression consistent with chronic stimulation.

Discussion

The method used to activate T cells is a critical determinant of their phenotype and functional potential, with implications for both basic research and clinical manufacturing. Here, we compared two CD3/CD28 agonists to examine how these two widely used reagents influence proliferation, activation, differentiation, and immune checkpoint expression over time.

Unlike previous reports[35,38], we found that bead-based and soluble antibody complexes induced comparable proliferation. Consistent with Soltantoye et al.[16], both stimulants maintained similar CD4/CD8 proportions. In contrast, Li and Kurlander[15] reported stronger CD4 expansion with beads. This discrepancy may reflect differences in soluble reagents: Li and Kurlander[15] compared beads to soluble anti-CD3 alone, which lacks co-stimulation and has been associated with preferential CD4 expansion, whereas our soluble condition included CD28/CD2, supporting balanced CD4 and CD8 proliferation. Thus, differences in reagent composition likely account for contradictory findings across studies.

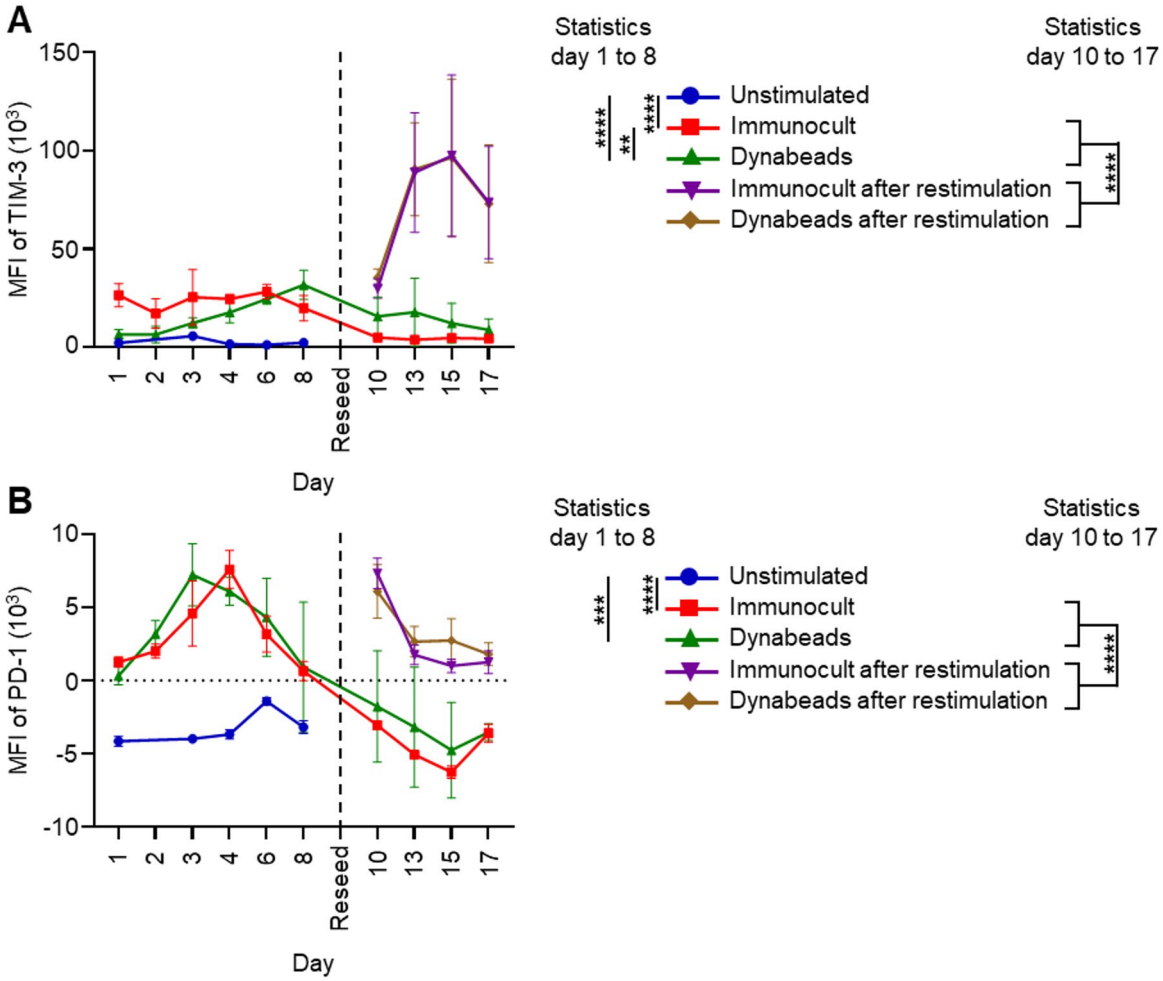

Fig. 6. Immune checkpoint receptor dynamics after stimulation with antibody-coated beads (Dynabeads) or soluble antibody complexes (ImmunoCult) during the initial stimulation phase (days 0–8), after reseeding in fresh media with or without restimulation on day 8, and throughout the post-restimulation phase (days 10–22). Checkpoint marker expression was measured as the Mean Fluorescence Intensity (MFI) of (**A**) TIM-3 or (**B**) PD-1.

In agreement with Li and Kurlander[15], beads were associated with rapid and robust activation, whereas soluble antibody complexes showed slower activation with greater reactivation capacity. This early activation with beads coincided with accelerated differentiation, as EM cells predominated soon after stimulation, while soluble antibody complexes preserved a larger CM fraction. These results contrast with Soltantoye et al.[16], who reported distinct differentiation dynamics under similar conditions. Together, our findings suggest that although proliferation outcomes converge, activation strength and differentiation trajectories are stimulant-dependent.

Both stimulants used in this study also induced transient checkpoint expression. PD-1 peaked early, consistent with early activation, and TIM-3 rose more prominently upon restimulation, consistent with chronic stimulation. These results suggest that checkpoint upregulation is shaped more by the temporal context (early vs. chronic) than by the specific activation reagent. Prior reports vary: some describe PD-1/TIM-3 co-expression as a hallmark of exhaustion[39–41], while others highlight divergent kinetics with TIM-3 persisting under chronic stimulation[42,43]. Our findings support the latter, showing PD-1 as a marker of early activation and TIM-3 as a feature of chronic stimulation.

IL-2 supplementation did not alter activation, proliferation, or differentiation phenotypes, indicating that differences between the reagents, rather than cytokine support, primarily shaped the observed phenotypes. However, IL-2 did modulate long-term TIM-3 expression, consistent with reports of cytokine-driven persistence via PI3K signaling[36,37].

Despite differences in activation and differentiation, expansion outcomes were broadly similar between both reagents, with comparable proliferation and stable CD4/CD8 ratios. Thus, the choice of stimulant may depend less on proliferative capacity and more on desired timing and cell fate. Soluble antibody complexes may be advantageous for applications requiring long-lasting memory T-cell responses, such as adoptive transfer or long-term co-culture models, whereas beads may be preferable when rapid differentiation is prioritized, such as in clinical manufacturing or short-term co-culture models[18,38,44].

Additionally, consistent with Li and Kurlander[15], we observed brief cell expansion (approximately 40 h) followed by gradual cell death following restimulation. However, unlike their report of greater death and poorer expansion in bead-stimulated cells, our restimulation yielded comparable cell death across conditions. This difference may reflect differences in restimulation reagents: we used a PMA/ionomycin cocktail to model a receptor-independent "second hit" for uniform short-term reactivation, whereas Li and Kurlander[15] restimulated with the same reagent initially applied. Moreover, their soluble condition lacked CD28 co-stimulation, which could account for the divergent outcomes. By standardizing restimulation, our study provides novel evidence that both reagents ultimately converge toward EM differentiation with high TIM-3 expression, highlighting shared consequences of repeated stimulation.

Beyond Li and Kurlander[15], several studies have examined the impact of repeated T-cell stimulation[18,45–47]. Early adoptive therapy work demonstrated progressive loss of proliferative capacity with repeated antigen exposure, while later studies highlighted skewing toward EM and reduced persistence. Our findings add a unique perspective by showing that, despite differences in initial activation kinetics, both reagents ultimately converge toward EM differentiation with high TIM-3 expression.

Several caveats warrant consideration. The emergence of a $CD3^+CD4^-CD8^-$ double-negative population following restimulation complicates interpretation of subset frequencies, as antibody-based quantification may underestimate true proportions. Similar populations have been described in repeatedly stimulated $CD4^+$ cultures, attributed to activation-induced co-receptor downregulation[25,26]. Our findings suggest that a comparable process in mixed T cell populations, though the functional significance remains to be determined. Single-cell RNA-seq could help clarify the mechanisms underlying this phenomenon.

We also observed inter-donor variability, consistent with the heterogeneity of human immune responses. As all donors were healthy adults, it remains unclear whether these dynamics extend to patient-derived or disease-associated T cells. Larger cohorts and patient samples will be needed to validate these conclusions.

Our comparison was also limited to one bead-based and one soluble antibody complex reagent; testing additional reagents will determine whether the observed differences reflect general features of bead versus soluble antibody stimulation or are reagent-specific. Moreover, another consideration is that both activation reagents used herein incorporate different anti-CD3 and anti-CD28 clones, which could partly account for the phenotypic differences we observed. In addition, ImmunoCult also provides CD2 co-stimulation, introducing an additional variable that may have influenced our findings. Finally, because each reagent was used according to its manufacturer-recommended conditions, the effective antibody concentrations are not directly matched; therefore, the observed differences likely reflect the combined effects of antibody composition, co-stimulatory signals, and dosing rather than format alone. To conclusively compare the effects of antibody presentation from clone-specific properties, future work will benefit from using identical CD3 and CD28 clones presented in different formats, such as soluble antibodies, plate-bound stimulation, or bead-coupled systems.

Future studies would also benefit from assessing the effects of different cytokine cocktails.

Overall, the two CD3/CD28 activation reagents used herein yielded broadly similar expansion outcomes, with comparable proliferation and stable CD4/CD8 ratios, but differ in activation, differentiation, and checkpoint regulation. By comparing beads with a soluble reagent, we reconcile prior inconsistencies and show that checkpoint dynamics are influenced by timing rather than by the reagent used. Importantly, our standardized restimulation experiments underscore the shared consequences of repeated stimulation. These insights support practical guidance: the two reagents differed in activation kinetics and differentiation trajectories, with Dynabeads-stimulated cells showing faster EM acquisition and soluble antibody complexes maintaining CM subsets longer, enabling tailored strategies for immunotherapy and research applications.

Data availability

No datasets were generated or analysed during the current study.

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References

1. Li, Y., Su, R. & Chen, J. Co-culture systems of drug-treated acute myeloid leukemia cells and T cells for in vitro and in vivo study. *STAR Protoc.* **1**(2), 100097. https://doi.org/10.1016/j.xpro.2020.100097 (2020).
2. Kubicka, E., Lum, L. G., Huang, M. & Thakur, A. Bispecific antibody-targeted T-cell therapy for acute myeloid leukemia. *Front. Immunol.* **13**(November), 1–17. https://doi.org/10.3389/fimmu.2022.899468 (2022).
3. Mutascio, S. et al. $CD8^+$ T cells promote HIV latency by remodeling $CD4^+$ T cell metabolism to enhance their survival, quiescence, and stemness. *Immunity* **56**(5), 1132-1147.e6. https://doi.org/10.1016/j.immuni.2023.03.010 (2023).
4. Kießling, M. et al. Chronic inflammation degrades CD4 T cell immunity to prior vaccines in treated HIV infection. *Nat. Commun.* **15**(1), 1–14. https://doi.org/10.1038/s41467-024-54605-3 (2024).
5. Pacini, C. P., Soares, M. V. D. & Lacerda, J. F. The impact of regulatory T cells on the graft-versus-leukemia effect. *Front. Immunol.* **15**(April), 1–11. https://doi.org/10.3389/fimmu.2024.1339318 (2024).
6. Jiang, H., Fu, D., Bidgoli, A. & Paczesny, S. T Cell subsets in graft versus host disease and graft versus tumor. *Front. Immunol.* **12**(October), 1–15. https://doi.org/10.3389/fimmu.2021.761448 (2021).
7. Montagna, E., de Campos, N. S. P., Porto, V. A., da Silva, G. C. P. & Suarez, E. R. CD19 CAR T cells for B cell malignancies: A systematic review and meta-analysis focused on clinical impacts of CAR structural domains, manufacturing conditions, cellular product, doses, patient's age, and tumor types. *BMC Cancer* https://doi.org/10.1186/s12885-024-12651-6 (2024).
8. Shi, Y. & Sadelain, M. Targeting systemic IL-2 to BCMA CAR-T cells. *Nat. Immunol.* **25**(11), 1988–1990. https://doi.org/10.1038/s41590-024-01993-9 (2024).
9. Ma, P. et al. Toward a comprehensive solution for treating solid tumors using T-cell receptor therapy: A review. *Eur. J. Cancer* https://doi.org/10.1016/j.ejca.2024.114224 (2024).
10 Shao, W. et al. Novel insights into TCR-T cell therapy in solid neoplasms: Optimizing adoptive immunotherapy. *BioMed Central* **13**(1), 37 (2024).

11. Matsueda, S., Chen, L., Li, H., Yao, H. & Yu, F. Recent clinical researches and technological development in TIL therapy. *Cancer Immunol. Immunother.* **73**(11), 1–20. https://doi.org/10.1007/s00262-024-03793-4 (2024).
12. Qiu, X. et al. Advances and prospects in tumor infiltrating lymphocyte therapy. *Discov. Oncol.* https://doi.org/10.1007/s12672-024-01410-5 (2024).
13. Dixon, J. J. F. J. F. & Law, J. L. Activation of human T lymphocytes by crosslinking of anti-CD3 monoclonal antibodies. *J. Leukoc. Biol.* https://doi.org/10.1002/jlb.46.3.214 (1989).
14. Valitutti, B. S., Müller, S., Salio, M. & Lanzavecchia, A. Complexes after antigenic stimulation. *J. Exp. Med.* **185**(10), 1859–1864 (1997).
15. Li, Y. & Kurlander, R. J. Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: Differing impact on CD8 T cell phenotype and responsiveness to restimulation. *J. Transl. Med.* https://doi.org/10.1186/1479-5876-8-104 (2010).
16. Ghaffari, S. et al. Optimizing interleukin-2 concentration, seeding density and bead-to-cell ratio of T-cell expansion for adoptive immunotherapy. *BMC Immunol.* **22**(1), 1–9. https://doi.org/10.1186/s12865-021-00435-7 (2021).
17. Cieri, N. et al. IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors. *Blood J. Am. Soc. Hematol.* **121**(4), 573–584. https://doi.org/10.1182/blood-2012-05-431718 (2013).
18. Kim, S. D. et al. Eight-day point of care CAR T-cell manufacturing on Clinimacs Prodigy from healthy donors as a proof-of-concept study. *Blood* https://doi.org/10.1182/blood-2021-154013 (2021).
19. Jung, C. R. T., Schauer, U., Heusser, C. & Neumann, C. Detection of intracellular cytokines by flow cytometry. *J. Immunol. Methods* https://doi.org/10.1016/0022-1759(93)90158-4 (1993).
20. Lee, J. H. et al. Single-cell RNA sequencing identifies distinct transcriptomic signatures between PMA/ionomycin-and αCD3/αCD28-activated primary human T cells. *Genomics Inform.* **21**(2), 1–11. https://doi.org/10.5808/gi.23009 (2023).
21. Lee, M. S. et al. Human MAIT cells undergo clonal selection and expansion during thymic maturation and aging. *Exp. Mol. Med.* https://doi.org/10.1038/s12276-025-01509-x (2025).
22. Cibrián, D. & Sánchez-Madrid, F. Europe PMC Funders Group CD69: From activation marker to metabolic gatekeeper. *Eur. J. Immunol.* **47**(6), 946–953. https://doi.org/10.1002/eji.201646837.CD69 (2017).
23. Adamczyk, M. et al. The expression of activation markers CD25 and CD69 increases during biologic treatment of psoriasis. *J. Clin. Med.* **12**(20), 6573. https://doi.org/10.3390/jcm12206573 (2023).
24. Petrov, S. A. et al. The influence of metabolites of microorganisms of the genus *Bacillus* from permafrost rocks on T lymphocyte differentiation. *Bull. Exp. Biol. Med.* **177**(4), 488–192. https://doi.org/10.1007/s10517-024-06212-x (2024).
25. Ni, M. et al. Dual roles of $CD11b^{+}CD33^{+}HLA\text{-}DR^{-/low}CD14^{-}$ myeloid-derived suppressor cells with a granulocytic morphology following allogeneic hematopoietic stem cell transplantation: From inflammation promoters to immune suppressors within 90 days. *Front. Immunol.* **15**, 1–17. https://doi.org/10.3389/fimmu.2024.1403272 (2024).
26. Alvarez-Fernández, C., Escribà-Garcia, L., Vidal, S., Sierra, J. & Briones, J. A short CD3/CD28 costimulation combined with IL-21 enhance the generation of human memory stem T cells for adoptive immunotherapy. *J. Transl. Med.* **14**(1), 4–13. https://doi.org/10.1186/s12967-016-0973-y (2016).
27. Williams, C. et al. CD28 and TCR differentially impact naïve and memory T cell responses. *Discov. Immunol.* https://doi.org/10.1093/discim/kyaf006 (2025).
28. Gattinoni, L., Speiser, D. E., Lichterfeld, M. & Bonini, C. T memory stem cells in health and disease. *Nat. Med.* **23**(1), 18–27. https://doi.org/10.1038/nm.4241 (2017).
29. Li, J. & Ferris, R. L. Differential expression of PD-1 and Tim-3 marks activation versus exhaustion status of T cells in the tumor microenvironment. *J. Immunother. Cancer* **2**(S3), 2014. https://doi.org/10.1186/2051-1426-2-s3-p220 (2014).
30. Francis, N. et al. Development of an automated manufacturing process for large-scale production of autologous T cell therapies. *Mol. Ther. Methods Clin. Dev.* **31**(December), 101114. https://doi.org/10.1016/j.omtm.2023.101114 (2023).
31. Soltantoye, T., Akbari, B., Mirzaei, H. R. & Hadjati, J. Soluble and immobilized anti-CD3/28 distinctively expand and differentiate primary human T cells: An implication for adoptive T cell therapy. *Iran J. Allergy Asthma Immunol.* **21**(6), 630–637. https://doi.org/10.18502/ijaai.v21i6.11521 (2022).
32. Lustig, A. et al. Lipid microbubble-conjugated anti-CD3 and anti-CD28 antibodies (microbubble-based human T cell activator) offer superior long-term expansion of human naive T cells in vitro. *ImmunoHorizons* **4**(8), 475–484. https://doi.org/10.4049/immunohorizons.2000056 (2020).
33. Turtle, C. J. et al. CD19 CAR–T cells of defined $CD4^{+}$:$CD8^{+}$ composition in adult B cell ALL patients. *J. Clin. Invest.* **126**(6), 2123–2138. https://doi.org/10.1172/JCI85309 (2016).
34. Fraietta, J. A. et al. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. *Nat. Med.* **24**, 563–571. https://doi.org/10.1038/s41591-018-0010-1 (2018).
35. Grishkan, I. V., Ntranos, A., Calabresi, P. A. & Gocke, A. R. Helper T cells down-regulate CD4 expression upon chronic stimulation giving rise to double-negative T cells. *Cell. Immunol.* **284**(1–2), 68–74. https://doi.org/10.1016/j.cellimm.2013.06.011 (2013).
36. Jelley-Gibbs, D. M. et al. Repeated stimulation of CD4 effector T cells can limit their protective function. *J. Exp. Med.* **201**(7), 1101–1112. https://doi.org/10.1084/jem.20041852 (2005).
37. Mujib, S. et al. Antigen-independent induction of Tim-3 expression on human T cells by the common γ-chain cytokines IL-2, IL-7, IL-15, and IL-21 is associated with proliferation and is dependent on the Phosphoinositide 3-Kinase Pathway. *J. Immunol.* **188**(8), 3745–3756. https://doi.org/10.4049/jimmunol.1102609 (2012).
38 Tomkowicz, B. et al. TIM-3 suppresses anti-CD3/CD28-induced TCR activation and IL-2 expression through the NFAT signaling pathway. *PLoS ONE* **10**(10), e0140694. https://doi.org/10.1371/journal.pone.0140694 (2015).
39. Li, J. & Ferris, R. L. Differential expression of PD-1 and Tim-3 marks activation versus exhaustion status of T cells in the tumor microenvironment. *J. Immunother. Cancer* **2**(Suppl 3), 220. https://doi.org/10.1186/2051-1426-2-S3-P22 (2014).
40. Avery, L., Filderman, J., Szymczak-Workman, A. L. & Kane, L. P. Tim-3 co-stimulation promotes short-lived effector T cells, restricts memory precursors, and is dispensable for T cell exhaustion. *Proc. Natl. Acad. Sci. U. S. A.* **115**(10), 2455–2460. https://doi.org/10.1073/pnas.1712107115 (2018).
41. Zhou, Q. et al. Coexpression of Tim-3 and PD-1 identifies a $CD8^{+}$ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood* **117**(17), 4501–4510. https://doi.org/10.1182/blood-2010-10-310425 (2011).
42. Yan, Z. et al. TIM-3 teams up with PD-1 in cancer immunotherapy: Mechanisms and perspectives. *Mol. Biomed.* **6**, 27. https://doi.org/10.1186/s43556-025-00267-6 (2025).
43. Romero, D. PD-1 says goodbye, TIM-3 says hello. *Nat. Rev. Clin. Oncol.* **13**, 203. https://doi.org/10.1038/nrclinonc.2016.40 (2016).
44. Riddell, S. R. et al. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science* **257**(5067), 238–241. https://doi.org/10.1126/science.1352912 (1992).
45. Berger, C. et al. Adoptive transfer of effector $CD8^{+}$ T cells derived from central memory cells establishes persistent T cell memory in primates. *J. Clin. Invest.* **118**(1), 294–305. https://doi.org/10.1172/JCI32103 (2008).
46. Gerdemann, U., Vera, J. F., Rooney, C. M. & Leen, A. M. Generation of multivirus-specific T cells to prevent/treat viral infections after allogeneic hematopoietic stem cell transplant. *J. Vis. Exp.* **27**(51), 2736. https://doi.org/10.3791/2736 (2011).
47. Gattinoni, L. et al. A human memory T cell subset with stem cell–like properties. *Nat. Med.* **17**, 1290–1297. https://doi.org/10.1038/nm.2446 (2011).

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Author contributions
MJ, BES, and GE designed the study. MJ and BES performed all cell culture experiments, functional assays, analyses, and data interpretation. MJ and BES wrote the manuscript, and the final manuscript was edited and approved by all authors under equal supervision from both GE and JC.

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Declarations

Competing interests
The authors declare no competing interests.

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