RESEARCH ARTICLE

Editorial Process: Submission:09/02/2025 Acceptance:06/14/2026 Published:06/19/2026

Thymosin α1 Augments CD8+ T-Cell Activation and Reverses Exhaustion *In Vitro*

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Abstract

Background: Thymosin alpha 1 (Tα1) is a thymic peptide hormone secreted by the thymus gland with known immunomodulatory properties, yet its specific effects on human CD8+ T-cell function remain incompletely understood. This study investigates the influence of Tα1 on CD8+ T-cell proliferation, activation, cytokine secretion, and exhaustion status *in vitro*. **Methods:** Human CD8+ T-cells were cultured and treated under four conditions: untreated (negative control), CD3/CD28 stimulation (positive control), Tα1 treatment, and combined CD3/CD28 + Tα1 stimulation. Proliferation was measured using carboxyfluorescein succinimidyl ester (CFSE)-based flow cytometry. Surface expression of activation markers (CD69, CD25, HLA-DR) and exhaustion markers (PD-1, TIM-3, LAG-3) was analyzed by flow cytometry. Cytokine secretion (IL-2, IFN-γ, TNF-α, IL-10) was assessed using a multiplex bead-based assay. T-cell exhaustion was induced by repeated CD3/CD28 stimulation before Tα1 treatment. **Results:** Tα1 alone moderately increased proliferation and activation of CD8+ T-cells, while the combination of Tα1 and CD3/CD28 significantly enhanced the proliferation index and surface expression of CD69, CD25, and HLA-DR compared with individual treatments. Cytokine secretion of IL-2, IFN-γ, TNF-α, and IL-10 was elevated in the combination group, indicating enhanced effector function. In the exhaustion model, CD8+ T-cells exhibited overexpression of PD-1, TIM-3, and LAG-3, which was significantly reduced upon Tα1 treatment, suggesting a partial reversal of the exhausted phenotype. **Conclusion:** Tα1 promotes functional activation of CD8+ T-cells and mitigates exhaustion marker expression following chronic stimulation. These findings suggest that Tα1 could potentially serve as a supportive agent in T-cell-based immunotherapies by enhancing activation and partially reversing exhaustion *in vitro*, warranting further *in vivo* validation.

Keywords: Thymosin α1- CD8+ T cells- T cell activation- T cell exhaustion- Immunomodulation

Asian Pac J Cancer Prev, **27 (6)**, 2089-2096

Introduction

In recent years, several T cell-based therapies have emerged and have proven effective against various cancer types, infectious diseases, and autoimmune conditions. These approaches are broadly categorized as immunotherapies and involve the ex vivo modification of T lymphocytes followed by reinfusion into patients, a strategy commonly referred to as adoptive cell transfer (ACT) therapy [1]. ACT harnesses the cytotoxic and regulatory functions of T lymphocytes to mediate targeted immune responses [2]. While these strategies hold significant promise, many remain under investigation, and their clinical efficacy has yet to be fully established. Despite their potential, several key challenges limit their success, including T-cell exhaustion driven by the immunosuppressive tumor microenvironment, inadequate *in vivo* expansion and persistence, poor tumor infiltration, antigen escape, off-target immunogenicity, limited efficacy against solid tumors, suboptimal cytotoxic function, and high manufacturing costs [3]. Addressing these limitations is critical for translating ACT therapies into broadly effective cancer treatments.

To overcome these challenges, diverse approaches such as cytokine supplementation to enhance T-cell signaling, genetic modification to promote survival (e.g., expression of anti-apoptotic genes such as *BCL-2*), enhancement of mitochondrial fitness, immune checkpoint inhibition, and engineered regulatory systems including logic-gated receptors and molecular switches are actively being explored [4, 5]. However, limited and variable success across *in vitro*, *in vivo*, and clinical studies underscores the need for novel complementary strategies to improve T-cell function and durability in ACT-based therapies.

In this study, we investigate the immunomodulatory potential of thymosin alpha 1 (Tα1), a naturally occurring

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immune-modulating peptide derived from the thymus and involved in T-cell maturation and immune regulation [6]. Tα1, also known as thymalfasin, has demonstrated the ability to enhance T-cell activation, proliferation, and survival by modulating key signaling pathways such as NF-κB and by upregulating co-stimulatory molecules, including CD86 [7].

Clinically, Tα1 has shown efficacy in improving immune responses in various infectious diseases and malignancies. It has received marketing authorization for the treatment of chronic hepatitis B and C outside the United States and has been investigated, though not formally approved by the U.S. FDA, for adjunctive use in COVID-19 [8-10]. Despite these therapeutic applications, the molecular and cellular mechanisms underlying Tα1-mediated modulation of T-cell responses remain incompletely understood. In this article, we aim to evaluate the effects of Tα1 on human T lymphocyte function using *in vitro* assays, with the objective of elucidating its role in modulating activation, proliferation, and exhaustion phenotypes.

Materials and Methods

Cell culture and enrichment

Peripheral blood mononuclear cells (PBMCs) isolated from healthy adult volunteers (n=10, median age = 30 years) were procured from BioRadius Therapeutic Research Pvt. Ltd (details mentioned in supplementary material). Donors were selected based on availability and absence of known immunological disorders, chronic inflammatory conditions, or acute infections at the time of sample collection. This sample size is consistent with previously published *in vitro* immunological studies investigating T-cell activation, proliferation, and cytokine responses, where biological variability between donors is addressed through paired or repeated-measures statistical analyses. PBMCs were resuspended and cultured in TexMACS media (Miltenyi Biotec). $CD8^+$ T lymphocytes were isolated from freshly thawed human PBMCs using magnetic-activated cell sorting (MACS) with CD8 MicroBeads (Miltenyi Biotec), according to the manufacturer's protocol. Purity of the enriched $CD8^+$ T-cell population was assessed by flow cytometry using an anti-human CD8 PE-conjugated antibody (REAfinity™) and was consistently >80%.

Thymosin treatment

Thymosin alpha 1 (Tα1; thymalfasin) was obtained from MedChemExpress (Cat. No. HY-P0091) as a stock solution of 100 µg/mL. Preliminary dose-titration experiments were conducted during the initial experimental phase to determine an appropriate working concentration of Tα1 by assessing apoptosis across a range of concentrations (1, 5, 10, 25, 50, 100, and 200 µg/mL), which showed comparable trends without dose-dependent cytotoxicity (Supplementary Figure 1). Based on these internal titration experiments and previously published reports, a concentration of 100 µg/mL was selected for all functional assays, as it reproducibly induced immunological activation without measurable cytotoxicity.

The selected concentration was further supported by published *in vitro* studies demonstrating immunomodulatory effects of thymosin alpha 1 at comparable concentrations (e.g., 50 µg/mL) in primary human peripheral blood mononuclear cells without induction of cytotoxicity [11]. $CD8^+$ T cells cultured without stimulation served as the negative control, while $CD8^+$ T cells treated with anti-CD3/CD28 activation beads (Miltenyi Biotec, according to the manufacturer's instructions) served as the positive control. It is important to note that *in vitro* concentrations of thymosin alpha 1 do not directly correspond to clinically administered doses, as cell culture systems lack physiological pharmacokinetics, peptide distribution, and systemic immune amplification mechanisms.

Cytokine and proliferation analysis

CD8+ cells were treated with 100µg/mL Tα1 alone for cytokine analysis and with Carboxyfluorescein succinimidyl ester (CFSE) dye and 100µg/mL Tα1 for cell proliferation assessment. Cytokine analysis (IFN-ϒ, TNF-α, IL-2, and IL-10) was done using MACSPlex Cytokine 12 Kit, human (Miltenyi Biotec) after 24 hours of Tα1 treatment. On day 05, CFSE reading was obtained using flow cytometer (MACSQuant10, Miltenyi Biotec).

T cell activation and exhaustion analysis

CD8+ cells were stained with CD8 Antibody (clone: REA824 | FN50), anti-human, PE, REAfinity™ and activation markers were analyzed using CD69- FITC Antibody (clone: REA824 | FN50), anti-human, REAfinity™; CD25- APC Antibody (clone: REA570 | 3G10), anti-human, REAfinity™; HLA-DR- FITC Antibody (clone: REA805 | L243), anti-human, REAfinity™ antibodies after Tα1 treatment. Exhaustion markers on CD8+ cells were analyzed using CD279 (PD1) Antibody (clone: REA1165 | PD1.3.1.3), anti-human, APC, REAfinity™; CD366 (TIM-3) Antibody (clone: REA635 | F38-2E2), anti-human, Vio® Bright FITC, REAfinity™; and CD223 (LAG-3) Antibody (clone: REA351 | 17B4), anti-human, REAfinity™ *in vitro* T cell exhaustion was induced using CD3/CD28 activation/ expansion beads. Gating strategy defined using Fluorescence Minus One (FMO) control.

Data analysis

The MACSQuant® Analyzer 10 (Miltenyi Biotec) was used to acquire flow cytometry data and evaluated with FlowJo software (version 10.1.0, BD Biosciences). Proliferation index (PI) was calculated by FlowJo (Cell Proliferation Modeling) as the number of cells that went into division divided by the total number of cell divisions. Statistical analyses were performed using GraphPad Prism (version 8.4.2, GraphPad Software), with one-way analysis of variance (ANOVA) to assess statistical significance, followed by Tukey's multiple comparisons test.

Results

Proliferative and Cytokine Responses Reveal Immunomodulatory Effects of Tα1

The Proliferative Index (PI) quantifies the rate of cell division among tumor cells and is calculated using the formula: number of cells that went into division/ total number of cell divisions. This calculation is performed by FlowJo software.

To monitor the immunomodulatory effect of Tα1, $CD8^+$ T cells were isolated from the PBMCs of healthy donors (N = 10), treated with Tα1, and their proliferation was analyzed via CFSE staining. Proliferation was measured and represented as PI, which was notably enhanced in the Tα1 group compared to the untreated control (NC vs. Tα1, $p < 0.0001$), as shown in Fig 1A. Proliferation was also found to be enhanced in the CD3/CD28 + Tα1 group, combination group, relative to the CD3/CD28 stimulation group alone ($p<0.05$), showing a synergistic enhancement.

Cytokine analysis was done using MACSPlex cytokine 12 kit, human. The analysis revealed a corresponding trend of increased pro-inflammatory cytokine secretion in the activated groups (Figure 1B). IL-2 levels were elevated in all treated groups versus NC ($p < 0.0001$), with the Tα1 + CD3/CD28 group exhibiting the highest levels ($p < 0.0001$ vs Tα1+ CD3/CD28). A similar pattern was noted for IFN-γ, where the combination group showed significantly elevated secretion versus Tα1 ($p < 0.0001$) and CD3/CD28 alone ($p < 0.01$), while Tα1 alone showed no significant modulation when compared with NC.

TNF-α levels were significantly upregulated upon CD3/CD28 stimulation; however, showed no significant change in the combination groups (CD3/CD28 vs CD3/CD28+ Tα1). Interestingly, Tα1 treatment alone resulted in a significant elevation in TNF-α ($p<0.0001$, data mentioned in supplementary materials). For IL-10, a significant elevation was observed in the combination group ($p < 0.0001$ vs NC and Tα1).

Overall, these results demonstrate that Tα1 enhances both proliferative capacity and effector cytokine production of $CD8^+$ T cells, particularly in combination with CD3/CD28-mediated activation, suggesting its potential as an immunomodulatory agent.

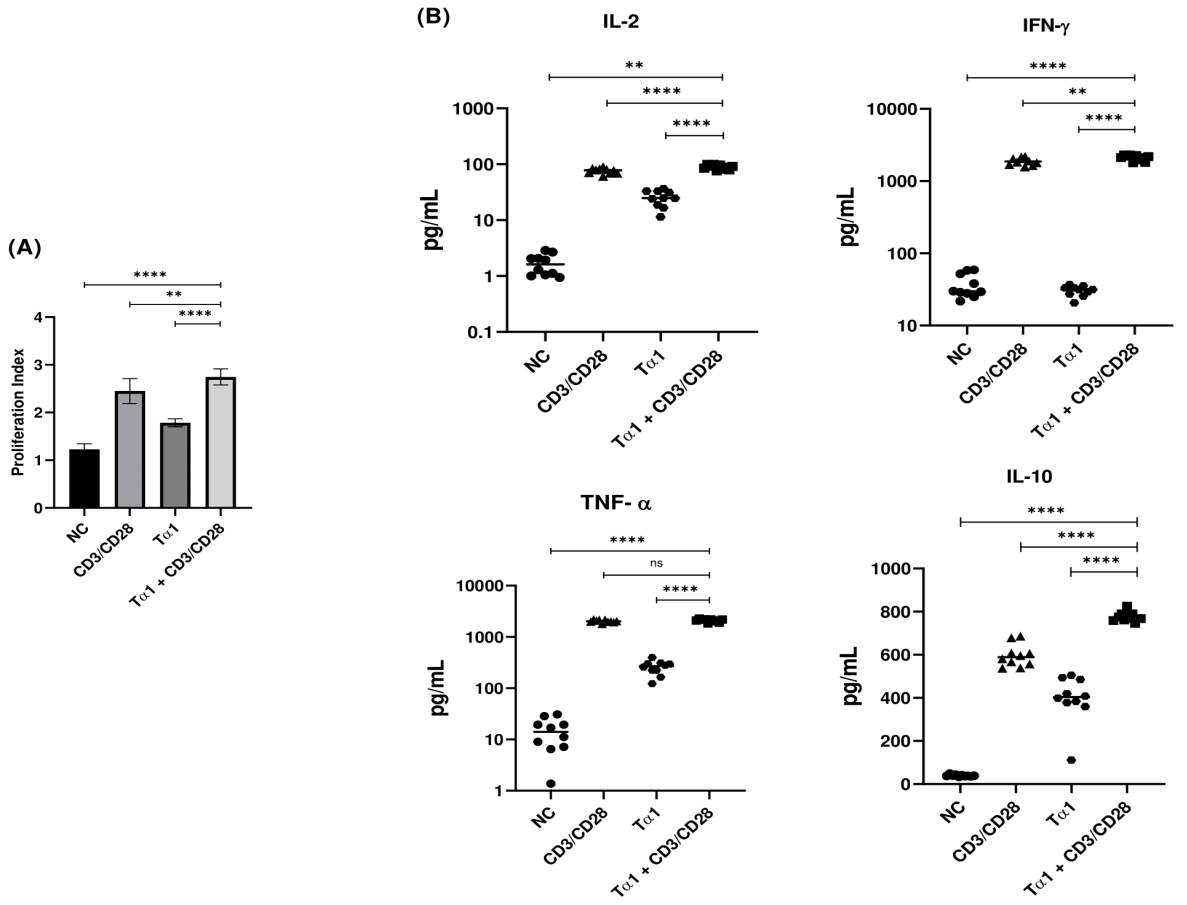

Figure 1. Sorted CD8+ Cells (N=10) were Subjected to Tα1 Treatment and Analyzed for Cell Proliferation and Cytokine Secretion. (A) Proliferation index (PI) calculated as number of cells that went into division/ total number of cell divisions, hence PI represents the proliferation of $CD8^+$ T cells after stimulation. CD3/CD28-stimulated cells show higher PI than Tα1 alone. Combination of CD3/CD28 + Tα1 showed higher PI than CD3/CD28 alone ($p<0.05$). (B) Cytokines; IL-2, IFN-ϒ, TNF-α and IL-10 were analysed after Tα1 treatment. IL-2 secretion was significantly upregulated in CD3/CD28 + Tα1 group in comparison to CD3/CD28 group alone ($p<0.05$). IFN- ϒ also showed significant upregulation in CD3/CD28 + Tα1, combination group than CD3/CD28 alone ($p<0.05$). Both TNF-α and IL-10 showed no significant difference in secretion (CD3/CD28 vs CD3/CD28 + Tα1). Data presented as mean ± SEM. One-way ANOVA with Tukey's post hoc test was used. **$p < 0.05$, ****$p < 0.0001$.

Tα1 Enhances CD8⁺ T Cell Activation Marker Expression

CD8+ sorted cells were analyzed using flow cytometry from 10 healthy donors. The assessment of activation of CD8⁺ T lymphocytes following treatment with CD3/CD28, Tα1, or their combination was assessed (Figure 2A).

Quantification of activation marker expression (Figure 2B) revealed a substantial increase in $CD8^+CD69^+$ T lymphocytes frequency in the CD3/CD28 group in comparison to the negative control ($p < 0.0001$), with maximum expression noted in the Tα1 + CD3/CD28 group ($p < 0.0001$ vs all groups). Tα1 alone moderately upregulated CD69 expression relative to NC ($p<0.005$).

Similar trends were observed for $CD8^+CD25^+$ T cells, where the combination of Tα1 and CD3/CD28 resulted in significantly higher expression than CD3/CD28 treatment ($p < 0.05$). similarly, a moderate increase in $CD8^+CD25^+$ T cells was seen in Tα1 group alone ($p=0.001$, vs NC).

HLA-DR expression on CD8⁺ T lymphocytes showed a sharper increase in the Tα1 + CD3/CD28 group in comparison to other groups ($p < 0.0001$). These results imply that Tα1 augments CD8⁺ T cell activation, especially when combined with CD3/CD28 stimulation, as evidenced by enhanced expression of early (CD69) and intermediate (CD25) activation markers, with a high impact on late activation marker HLA-DR.

Tα1 Treatment Attenuates the Expression of Exhaustion Markers in In Vitro Exhausted CD8⁺ T Cells

To investigate whether Tα1 can modulate T cell exhaustion, CD8⁺ T cells were initially subjected to repeated stimulation using CD3/CD28 beads to induce an exhausted phenotype *in vitro*. Following the induction phase, cells were treated with Tα1, and flow cytometry was used to measure PD-1, TIM-3 and LAG-3 expression (Figure 3A).

Analysis showed a robust induction of exhaustion, as indicated by significantly elevated PD-1, TIM-3, and LAG-3 expression in the exhausted T cell group in comparison to the untreated negative control group ($p < 0.0001$ for all markers) (Figure 3B). Notably, treatment

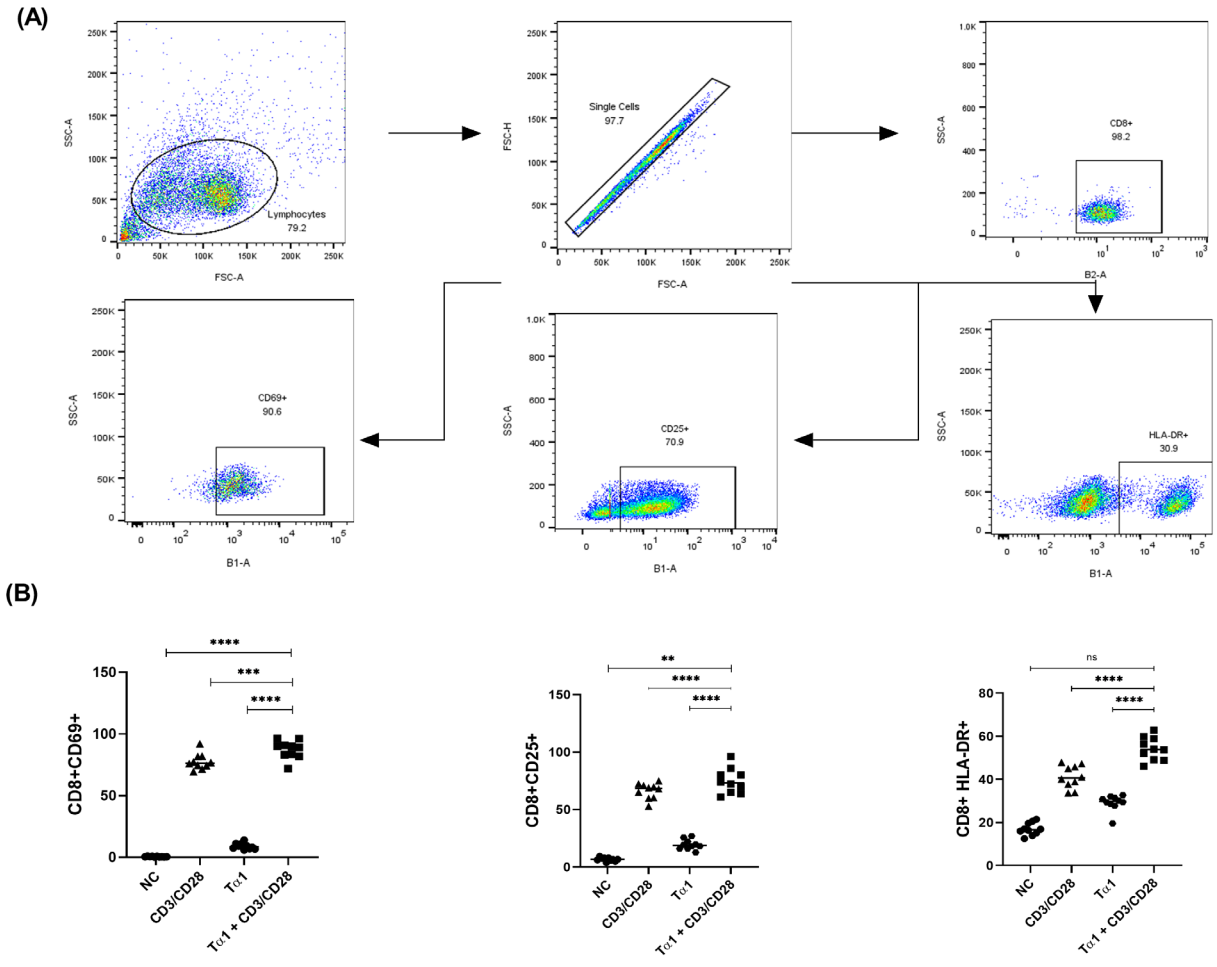

Figure 2. Tα1 Enhances CD8⁺ T Cell Activation, Marked by Increased Expression of Surface Activation Markers on CD8 Cells from 10 Healthy Donors (N=10). (A) Representative flow cytometry gating strategy showing sequential gating of lymphocytes, single cells, CD8⁺ T cells, and subsequent analysis of activation markers CD69, CD25, and HLA-DR. (B) Quantitative analysis of activation marker expression on CD8⁺ T cells across four experimental groups: negative control (NC), CD3/CD28 stimulation, Tα1 treatment alone, and combined CD3/CD28 + Tα1 treatment. Tα1 alone moderately increased CD69 and CD25 expression, while CD3/CD28 stimulation significantly upregulated all markers. Co-treatment with Tα1 and CD3/CD28 further enhanced $CD8^+CD69^+$, $CD8^+CD25^+$, and $CD8^+HLA\text{-}DR^+$ populations compared to either treatment alone. Data represented as mean ± SEM. One-way ANOVA with Tukey's post hoc test was used. $^{**}p < 0.05$, $^{****}p < 0.0001$.

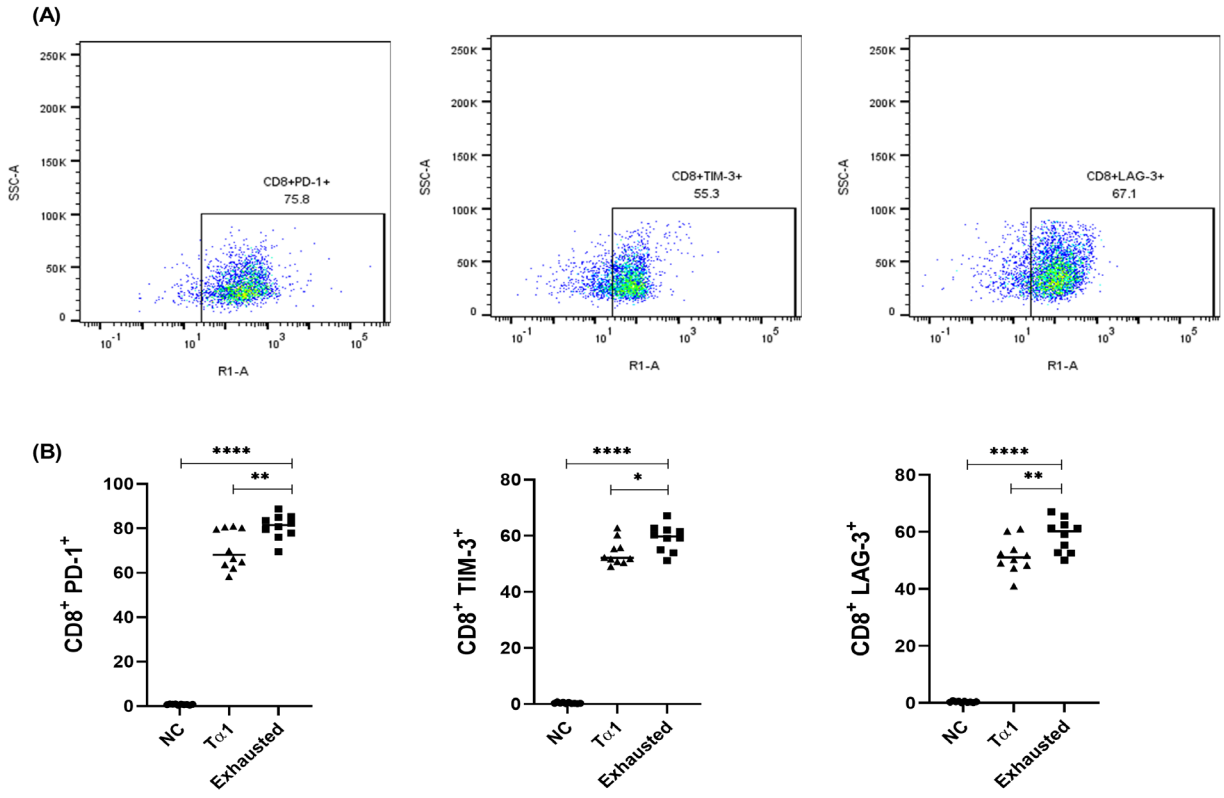

Figure 3. Thymosin alpha 1 Attenuates Exhaustion Marker Expression in CD8+ T Cells Following *in vitro* Exhaustion. (A) Representative flow cytometry plots showing expression of PD-1, TIM-3, and LAG-3 on CD8+ T cells in the exhausted group. Exhaustion was induced in vitro by repeated CD3/CD28 stimulation, followed by Tα1 treatment. (B) Quantitative analysis of exhaustion marker expression across three groups: negative control (NC), exhausted, and Tα1-treated exhausted CD8+ T cells. The exhausted group showed significantly elevated levels of PD-1, TIM-3, and LAG-3 versus the NC group (****$p < 0.0001$). Tα1 treatment caused a significant decline in expression of all three markers compared to untreated exhausted cells, indicating partial reversal of the exhausted phenotype. Data presented as mean ± SEM. Statistical analysis performed using one-way ANOVA with Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

with Tα1 led to lowered expression of these exhaustion markers. PD-1 expression significantly decreased in the Tα1-treated group versus the untreated exhausted group ($p < 0.01$), indicating a partial reversal of the exhausted phenotype. Similarly, TIM-3 and LAG-3 levels were also reduced following Tα1 treatment ($p < 0.05$ and $p < 0.01$, respectively), though expression remained higher than in the negative control.

These findings demonstrate that Tα1 is capable of downregulating key exhaustion markers in CD8+ T cells, suggesting its potential to serve as an immunomodulatory agent to restore T cell function in exhausted states.

Discussion

Tα1, being a short peptide and having important functions to play in T cell development and maturation, holds immense potential as an immunomodulatory agent [12]. Its use in therapeutics such as treatment of hepatitis B, hepatitis C, COVID-19, and some cancer types makes it particularly significant to understand the immunological changes that can be attributed to Tα1 [13-15].

So far, we know that Tα1 interacts with TLR-2 present on precursor T cells and helps in advancing its response against viral infection and tumor cells [16]. This study focused on the response of key T cell biomarkers associated with their activation, exhaustion and overall T cell response to Tα1. The present study was designed as an exploratory mechanistic investigation to evaluate the immunomodulatory effects of thymosin alpha 1 on human CD8+ T cells, rather than as a powered clinical study. We showed that CD8+ cells exhibit higher proliferation when treated with Tα1 in combination with CD3/CD28 stimulation. Interestingly, Tα1 alone showed higher proliferation when compared with unstimulated CD8+ cells. Hence, Tα1 shows the capability of inducing T cell proliferation, which is also reflected by our results of IL-2 cytokine secretion after Tα1 treatment (Fig. 1A and B). A recent study has reported similar results wherein Tα1 promoted the proliferation of effector T cells isolated from COVID-19 patients [17]. The proposed mechanism of T cell proliferation by Tα1 is via TLR 2 and TLR 9, Mitogen-activated protein kinase (MAPK), NFκB and IL-2 secretion [18, 19]. We also observed a significant upregulation of IFN-ϒ between the CD3/CD28 control group and the CD3/CD28+ Tα1 combination group ($p<0.05$). It has been previously shown that PBMCs from chronic hepatitis patients demonstrated increased IFN- ϒ

production when treated with Tα1, *in vitro* [20]. This study not only analyzed IFN-ϒ but also IL-2 and concluded that there is a shift in Th1 phenotype as indicated by elevated IL-2 and IFN-ϒ, however, we found an elevation in IL-2 and IFN-ϒ by CD8+ cells, which directly points towards cytotoxic T cell activation.

Our study also showed how Tα1 affected TNF-α and IL-10 (Fig. 1B). TNF-α showed a non-significant increase between CD3/CD28 group and CD3/CD28+ Tα1 groups. Interestingly, NC vs Tα1 alone showed significantly higher expression of TNF-α ($p<0.0001$, data provided in supplementary materials). In our study, even though we found an elevation of TNF-α in NC vs Tα1, we also found insignificant modulation in the combination group (CD3/CD28 vs CD3/CD28+ Tα1). This implies that Tα1 not only activates the immune response but also regulates it in case of overexpression. This finding is also corroborated by IL-10 secretion data, as shown in Figure 1B, where significantly higher expression of IL-10, an immunoregulatory cytokine, is observed across all the groups ($p<0.0001$). Another study analyzed the effect of Tα1 on IL-10 secretion from PBMCs of COVID-19 patients and found a significant rise in IL-10 and proposed that Tα1 might be involved in mitigating cytokine storm in patients [21].

We also demonstrated that Tα1 treatment leads to CD8+ cell activation by significantly enhancing CD69, CD25 and HLA-DR populations. Very few studies have analyzed the effect of Tα1 on CD8 cell activation; this study lays a solid foundation for understanding the true effect of Tα1. In our study, treatment with thymosin alpha 1 led to a significant upregulation of early (CD69), intermediate (CD25), and late (HLA-DR) activation markers on $CD8^+$ T cells, indicating a shift toward a functionally activated phenotype. CD69 is one of the earliest surface molecules expressed upon T cell receptor engagement [22], CD25 represents the high-affinity IL-2 receptor alpha chain crucial for proliferation and survival, and HLA-DR is a marker of late or sustained activation [23]. The coordinated increase in these markers suggests that Tα1 not only initiates early activation but may also support continued T cell responsiveness.

Previous studies have reported immunopotentiating effects of Tα1 in both innate and adaptive compartments, including enhanced T cell-mediated responses in viral infections and cancer [16, 24]. Tα1 has shown to influence T cell function via TLR-mediated signaling, activation of the NF-κB and STAT5 pathways, and increased expression of IL-2 receptors [7], all of which may contribute to the observed upregulation of CD25 and CD69. For example, Garaci and colleagues proposed that Tα1 augments T-cell activation through the upregulation of co-stimulatory molecules and modulation of cytokine signaling networks [25].

Taken together, our findings provide further evidence that Tα1 enhances $CD8^+$ T cell activation at multiple stages, supporting its potential use as an immunomodulatory agent to improve T cell function, particularly in settings that require robust activation, such as cancer or chronic infection. Unlike classical mitogens, Tα1 appears to fine-tune activation without inducing overt hyperinflammation, consistent with its dual role as an immune enhancer and modulator.

We acknowledge that CD3/CD28 antibody-coated bead stimulation provides a supra-physiological activation signal compared to antigen-specific stimulation and does not fully recapitulate disease-relevant T-cell priming. However, this approach was intentionally used to establish a standardized and highly reproducible activation and exhaustion model, enabling controlled assessment of the immunomodulatory effects of thymosin alpha 1 across donors. Importantly, the use of CD3/CD28 stimulation allowed us to evaluate whether Tα1 could further enhance T-cell function or attenuate exhaustion even under conditions of strong activation. Future studies employing defined tumor-associated or viral antigens will be essential to validate these findings in more physiologically relevant antigen-specific systems.

In our study, repeated CD3/CD28 bead stimulation of $CD8^+$ T cells successfully induced an exhausted phenotype, as evidenced by significant upregulation of atypical exhaustion markers PD-1, TIM-3, and LAG-3. Interestingly, treatment with thymosin alpha 1 (Tα1) led to a statistical decline in the expression of all three markers, suggesting that Tα1 can partially reverse exhaustion and restore immune functionality. These findings are noteworthy, particularly in terms of chronic infection and cancer, where T-cell exhaustion limits the benefit of immunotherapy.

Past findings revealed that Tα1 plays a complex role in modulating immune homeostasis, with several studies demonstrating its ability to enhance T cell responses under immunosuppressive conditions [21]. For example, Tα1 has been reported to activate transcriptional programs associated with immune recovery, including those regulating cytokine signaling and apoptosis resistance [26]. In a transcriptomic study by Garaci and colleagues, Tα1 was shown to modulate immune regulatory genes and promote the restoration of effector function in immunocompromised settings [27]. Additionally, a recent study on a post-COVID immunopathology model suggested that Tα1 rebalanced CD4/CD8 differentiation and reduced hyperactivation and exhaustion profiles, providing further support for its immunorestorative potential [28].

The mechanisms by which Tα1 mediates these effects are not yet fully elucidated, but existing evidence points toward its interaction with innate immune receptors, particularly Toll-like receptors (TLRs), causing downstream activation of NF-κB and STAT signaling pathways that govern co-stimulatory molecule expression, cytokine receptors, and survival genes [6]. It is plausible that through these mechanisms, Tα1 enhances mitochondrial fitness or downregulates inhibitory receptor signaling, thereby restoring effector functions in exhausted $CD8^+$ T cells. Although previous studies have reported the involvement of TLR2 and TLR9 signaling and downstream NF-κB and STAT pathways in thymosin alpha 1–mediated immune modulation, the present study did not directly assess receptor engagement or downstream signaling events. Accordingly, we did not

perform TLR expression analysis, pathway inhibition, or phospho-signaling assays. Future studies incorporating receptor-specific blockade, signaling pathway inhibitors, or phospho-protein analyses will be required to definitively delineate the molecular mechanisms underlying Tα1-mediated modulation of CD8+ T-cell function.

Our findings expand upon previous literature by demonstrating, for the first time in this context, that Tα1 not only promotes activation of CD8+ T cells under stimulatory conditions but also mitigates exhaustion-associated phenotypes. This dual role underscores the therapeutic potential of Tα1 as an immunomodulatory agent capable of both enhancing T cell responses and rejuvenating dysfunctional T cell populations, a property of high relevance in the design of adoptive T cell therapies and cancer immunotherapy regimens. It should be noted that the present study was designed as an exploratory mechanistic investigation to evaluate the immunomodulatory effects of thymosin alpha 1 on human CD8+ T cells, rather than as a powered clinical study.

While the current study provides novel insights into the immunomodulatory effects of Tα1 on human CD8+ T cells, several important aspects remain to be addressed in future investigations. A limitation of the current study is the absence of comprehensive dose–response analyses for each functional endpoint (proliferation, activation markers, cytokine secretion, and exhaustion markers). Although our findings suggest that Tα1 promotes functional activation and attenuates exhaustion, we did not include experiments using non-functional Tα1 mutants or neutralizing antibodies to confirm the specificity of these effects. Given the reported interaction of Tα1 with TLR2 and TLR9, assessing the expression levels of these receptors in CD8+ T cells, as well as employing knockdown or knockout strategies, would help elucidate the underlying signaling pathways. Since CD8+ T cells were enriched rather than flow-sorted to absolute purity, the relative contribution of direct versus accessory cell–mediated effects to Tα1-induced functional modulation was not dissected in the present study and warrants further investigation.

The CD8+ T cell compartment is heterogeneous, comprising subsets such as naïve, effector, memory, and regulatory populations, each with distinct functional profiles. We did not characterize the subset composition in this study, which may explain differential cytokine patterns, including the potential contribution of regulatory CD8+ T cells to IL-10 production. Finally, direct functional assays to evaluate cytotoxic capacity, such as target cell killing or degranulation assays, were not included in the present study and would provide further insight into the extent to which thymosin alpha 1 modulates CD8+ T-cell effector function. Incorporation of such assays in future studies will be important to validate the functional relevance of the observed activation and exhaustion phenotypes.

The findings from this *in vitro* study provide a rationale for evaluating thymosin alpha 1 as an adjunct immunomodulatory agent in *in vivo* settings characterized by CD8+ T-cell dysfunction. Future studies could employ tumor-bearing mouse models, chronic viral infection models, or adoptive T-cell transfer systems to assess the capacity of Tα1 to enhance T-cell persistence, functionality, and resistance to exhaustion in a physiological context. Such *in vivo* validation will be essential to determine the durability, safety, and translational relevance of Tα1-mediated modulation of CD8+ T-cell responses.

In conclusion, this study provides compelling evidence that thymosin alpha 1 (Tα1) enhances the functional capacity of human CD8+ T cells *in vitro*. Tα1 treatment led to a significant increase in proliferation, activation marker expression (CD69, CD25, HLA-DR), and cytokine secretion (IL-2, IFN-γ, TNF-α, and IL-10), particularly when used in combination with CD3/CD28-mediated stimulation. Importantly, Tα1 also reduced the expression of exhaustion markers PD-1, TIM-3, and LAG-3 in repeatedly stimulated CD8+ T cells, indicating a potential role in reversing T cell dysfunction. These findings suggest that Tα1 acts as a potent immunomodulator, capable of both enhancing activation and restoring effector function in exhausted CD8+ T cells. Collectively, our results support the potential utility of Tα1 as an adjunct to T cell-based immunotherapies, particularly in clinical settings characterized by T cell exhaustion, such as chronic infections and cancer.

Author Contribution Statement

S.M. and G.T. conceptualized and designed the study. S.M. performed the experiments, acquired the data, and conducted flow cytometry and cytokine analysis. G.T. and A.S. contributed to data processing and statistical analysis. S.M. wrote the initial manuscript draft. S.B., R.S., and A.K. provided scientific guidance, reviewed data, and critically revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Acknowledgements

The authors gratefully acknowledge the technical assistance and infrastructure support provided by BioRadius Therapeutic Research Pvt. Ltd., Pune, India, where all experimental work was conducted. The authors also thank the laboratory and quality teams for facilitating the use of shared resources and instrumentation.

Ethical Statement

Peripheral blood mononuclear cells (PBMCs) used in this study were procured from BioRadius Therapeutic Research Pvt. Ltd., Pune, India, from anonymous healthy donors with prior informed consent, in accordance with institutional ethical policies. No human or animal intervention was involved in this study.

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request. No publicly archived datasets were generated during this study.

Conflict of Interest

The authors declare no commercial or financial conflict of interest related to this work.

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