

Expert Opinion

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From lab to bedside: emerging clinical applications of thymosin α_1

Allan L Goldstein[†] & Adam L Goldstein^{*}

[†]The George Washington University School of Medicine and Health Sciences, Washington DC, USA

^{*}Medical School for International Health at Ben-Gurion University of the Negev, Be'er Sheva, Israel

Background: Thymosin α_1 ($T\alpha_1$), a synthetic version of a thymic-derived biological response modifier was the first of the thymosins in clinical use. $T\alpha_1$ is approved in over 35 countries for the treatment of hepatitis B and C, and as an immune stimulant and adjuvant. $T\alpha_1$ is also in late-stage clinical testing in the United States and Europe for hepatitis C and stage IV melanoma. **Objective/methods:** Novel applications and other recently completed trials point to much broader clinical applications of $T\alpha_1$ in the treatment of life-threatening and chronic diseases, and are the subject of this review. **Result/conclusions:** The most recent reports of clinical trials with $T\alpha_1$ are pointing to important, hitherto unrecognized, applications in a number of diseases and disorders, including septic shock, acute respiratory distress syndrome, peritonitis, acute cytomegalovirus infection, TB, severe acute respiratory syndrome, and lung infections in critically ill patients. It is also emerging as a promising chemoprotection agent in patients undergoing chemotherapy.

Keywords: biological response modifiers, cancer, immune modulation, infectious diseases, T-cells, thymosin α_1

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1. Introduction

The isolation and chemical characterization of many of the key molecules and receptors that regulate the immune system and host resistance have now been performed. These molecules, which include cytokines, chemokines, thymosins and growth factors, are collectively termed biological response modifiers (BRMs) [1]. Many of these BRMs, first discovered at the end of the twentieth century, have now made a successful transition from the lab bench to the clinic resulting in the creation of a new and rapidly expanding family of immune-based or adjuvant therapeutics. These synthetic or recombinant forms of natural substances, based on an understanding of the body's own immune system, have provided clinicians with a plethora of potent weapons in the fight against many life-threatening and debilitating diseases from infectious diseases [2] to cancers [3] and rheumatic diseases [4]. What has emerged from an analysis of clinical trials conducted with BRMs over the past decade has been their emergence and recommended use either as first line mono or adjuvant therapies [5-7]. The use of $T\alpha_1$ and other BRMs in combination with conventional therapies and/or other BRMs in order to increase the efficacy of treatment, decrease mortality and lower morbidity currently appears to be more clinically applicable and useful compared with their use as monotherapies [6,7]. A large number of new applications for BRMs are being studied, and their full potential in decreasing the length, toxicities and dosing of current conventional treatments and/or to strengthen the efficacy of current treatments for a variety of diseases remains to be fully established.

2. Discovery of the thymosins

The process that led to the isolation of the thymosins began with research on the thymus initially carried out by A.L. Goldstein and A. White at The Albert Einstein College of Medicine in the early 1960's [8]. Their studies followed shortly after the discovery in 1961 by J.F.A.P. Miller [9], R.A. Good [10], and their colleagues, that the thymus gland actually played a central role as the 'master gland' of immunity instead of the then current belief of the thymus being vestigial and without function. The thymus gland is now recognized to be essential for the development, maturation and education of T-cells and for the maintenance of a healthy immune system [11].

The objective of the initial thymosin studies was to determine if the mechanisms by which the thymus exerted its effects on the immune system were endocrine in nature. This search for thymic hormones, which began in earnest in 1964, led to the isolation of a lymphocytopoietic factor from calf thymus, which was named thymosin [8]. Further purification of this factor led to the isolation of a heat-stable acetone-insoluble preparation, termed thymosin fraction 5 (TF5) [12]. TF5, a highly active preparation with potent immunomodulatory properties, was a mixture of mostly small acidic peptides with molecular weights of less than 15,000 Daltons. TF5 was the first of the thymosin preparations to be prepared on a large scale and standardized for clinical use. It demonstrated a broad range of biological activities in animal models of cancer, immune deficiency diseases and infectious diseases [13].

In 1974, TF5 was the first thymic preparation to receive an Investigational New Drug Application (IND) from the FDA and to enter clinical trials in the US. The first patients treated with TF5 were children with life-threatening primary immune deficiency diseases, such as DiGeorge syndrome [14,15]. The promising results seen with TF5 in increasing T-cell numbers and function, resulting in improved clinical responses in these patients, provided the scientific rationale to actively pursue the isolation and clinical characterization of the molecules in TF5 responsible for the reconstitution of T-cell immunity. Further purification of TF5 quickly led to the complete chemical characterization [16-18] and synthesis [19] of $T\alpha_1$ in 1977, along with a number of other thymosin peptides, such as thymosin β_4 , which is currently in clinical trials in the field of wound healing [20].

3. Pleiotropic properties of thymosin α_1

$T\alpha_1$ is a highly conserved 28 amino acid peptide acetylated at its amino terminus and with a molecular weight of 3108 Daltons and an isoelectric point of 4.2 (Figure 1, Table 1). It is postulated that $T\alpha_1$ is the amino terminus proteolytic cleavage product of a larger molecule termed prothymosin α [21]. $T\alpha_1$'s immune enhancing properties have been fully established in numerous animal models which provided the scientific foundation for the first therapeutic applications in

humans [22]. The highest concentrations of $T\alpha_1$ per mg of tissue are found in the thymus gland, although it is ubiquitously found in smaller amounts in other lymphoid tissues, including the spleen and lymph nodes, along with non-lymphoid tissues such as the lungs, kidneys, and brain. Biologically active $T\alpha_1$ has been successfully synthesized using solid-phase synthesis [19] and recombinant [23] technologies. All of the clinical trials completed and in progress are using a standardized and now routine method of solid-phase synthesis.

$T\alpha_1$ is classified as a potent BRM on the basis of its pleiotropic effects on a broad spectrum of immune responses *in vitro* and *in vivo* in animal and human studies (Figure 2) [16]. Some of the known biological effects of $T\alpha_1$ include enhanced T-cell, dendritic and antibody responses, chemoprotection properties, modulation of cytokine and chemokine production, and the ability to block steroid-induced apoptosis of thymocytes. The molecular mechanism of action of the physiological effects observed are still being defined, but include differentiation of Toll-like receptors [24] and class I MHC expression [25], increase in cytokine receptors [26], and activation of specific transcription factors and signaling pathways [27]. In both *in vitro* and *in vivo* testing, $T\alpha_1$ has been found to be 10 – 1000 times more active than TF5 in stimulating T-cell formation and function [16]. Amino acid sequence analysis of $T\alpha_1$ has revealed structural similarities with members of the glucagon-vasoactive intestinal peptide (VIP)-secretin family of polypeptides such as VIP, secretin, gastric inhibitory peptide, porcine gut peptide, glucagon and both rat and human growth hormone-releasing factors [28]. $T\alpha_1$ has also been found to possess sequence homology with peptide T [29], a region of the gp120 envelope glycoprotein of HIV, as well as a region between amino acids 92 and 109 of the core P17 gag protein of HIV [30]. This region of homology with p17 led, in the early 1980's, to the development of HGP-30, the first HIV vaccine directed toward a highly conserved region of this HIV core protein instead of the envelope proteins [31]. The observation that led to this discovery was the finding that antiserum to $T\alpha_1$ neutralized HIV and blocked its replication in H9 cells. The antiviral activity of the antiserum was due to a region of homology between $T\alpha_1$ and a highly conserved region of p17, a product of the *gag* gene of HIV [31]. Amino acid sequence homology has also been shown with an octapeptide of IFN α and $T\alpha_1$, which effectively competes for common high-affinity receptors on murine T-cells [32].

4. Mechanism of action

$T\alpha_1$ acts primarily by increasing the efficiency of T-cell maturation [33-35] by increasing the ability of T-cells to produce specific cytokines such as IFN- α , IFN- γ , IL-2, IL-3, IL-6, IL-7, IL-10, IL-12 and IL-15 following antigen or mitogen activation and by upregulating specific cytokine receptors such as high-affinity IL-2 cytokine receptors [22,26,36-43]. $T\alpha_1$

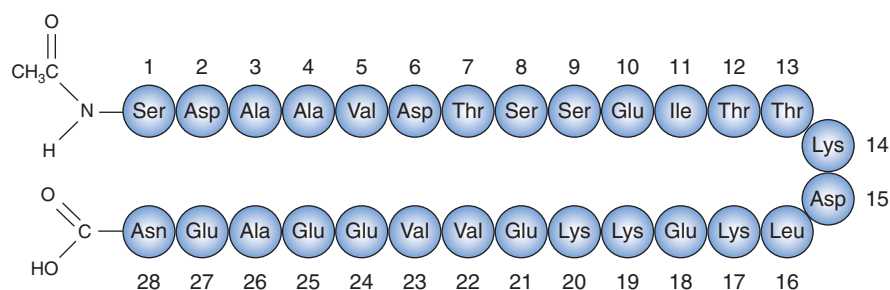


Figure 1. Structural formula of thymosin α_1 .

Table 1. Characteristics of thymosin α_1 .

Originally isolated from thymus

The peptide responsible for reconstruction of immune function in neonatally thymectomized and/or immunosuppressed animal models

Enhances resistance to a number of viral, fungal, and mycobacterial pathogens

Enhances anti-tumour activity via stimulation of T-cell, NK-cell, and dendritic cell function

Synthetic $T\alpha_1$ is active in both animal studies and human trials

Non-glycosylated, N-terminal-acetylated, 28 amino acids

Highly conserved sequence (humans and various animal species)

Comprises the N-terminus of human prothymosin (109 amino acids)

has been found to trigger the differentiation of human $CD34^+$ stem cells into $CD3^+ 4^+$ cells and induce dendritic cell differentiation from $CD14^+$ monocytes [34,38]. The ability of $T\alpha_1$ to act on both lymphoid stem cells and more mature T-cells to produce cytokines and to induce cytokine receptors may be the reason why $T\alpha_1$ exhibits such a wide range of bioactivities, and may explain its synergism with cytokines such as $IFN-\alpha$ and $IL-2$ in animal and human studies [24,44-53]. $T\alpha_1$ also increases MHC class I expression in both lymphoid [25] and non-lymphoid cells [54]. Upregulation of MHC-1 has also been seen with cancer cells potentially making them more visible to the immune system and providing a mechanism to explain some of the anti-tumor properties of $T\alpha_1$ [55-57].

As illustrated in Figure 3, $T\alpha_1$ augments T-cell function through engaging Toll-like receptors, such as TLR9, leading to increases in cytokine expression through gene expression of a number of immune proteins affected by changes in the NF- κ B signaling pathway [24]. These effects include increasing the efficiency of T-cell maturation and increasing the ability of T-cells to produce the Th1 cytokines, $IFN-\gamma$, $IL-2$, and $IL-3$. $T\alpha_1$ has also been found to induce $IL-6$ expression through the inhibitor of NF- κ B (I- κ B) kinase (IKK), NF κ B, and TNF-receptor-associated factor 6 (TRAF6) signaling pathway [58]. Although a specific $T\alpha_1$ membrane receptor has yet to be isolated, $T\alpha_1$ has also been shown to antagonize dexamethasone + $CD3$ -induced apoptosis of $CD4^+ CD8^+$ thymocytes through the activation of cAMP and protein-kinase-C-dependent second messenger pathways [59].

5. Anti-tumor and anti-fungal properties of $T\alpha_1$ at the cellular and molecular level

Based on the triggering of specific signaling pathways in lymphoid cells, $T\alpha_1$ would be expected to augment an anti-tumor immune response in several ways. First, $T\alpha_1$ has been shown to increase lymphocytic infiltration to sites of disease [45], an effect which may be facilitated by the release of certain chemokines shown to be stimulated by $T\alpha_1$. A correlation between tumor-infiltrating lymphocytes and prognosis for patients with stage IV melanoma, for example, has been documented [56]. Second, $T\alpha_1$ mediates maturation and differentiation of dendritic cells and their expression of the specific cytokines leading to an increase in anti-tumor T-cells [60-62]. Anti-melanoma T-cells have been shown to correlate with tumor regression and improved survival of patients with melanoma [57].

At the molecular level, the effect of $T\alpha_1$ in stimulating anti-fungal T helper type 1 (Th1) resistance against pulmonary aspergillosis also depends on Toll-like receptor (TLR/myeloid differentiation primary response gene 88 protein (MYD88)) signaling of primed dendritic cells (DCs) and against cytomegalovirus infection by plasmacytoid DCs (pDC) via the TLR9/MYD88-dependent viral recognition which leads to the activation of the interferon regulatory factor 7 and promotion of the $IFN-\alpha/IFN-\gamma$ -dependent effector pathways [24,27]. A new application of $T\alpha_1$ in inducing transplantation tolerance and reducing inflammation allergy has been reported to occur via induction of indoleamine 2, 3-dioxygenase (IDO)

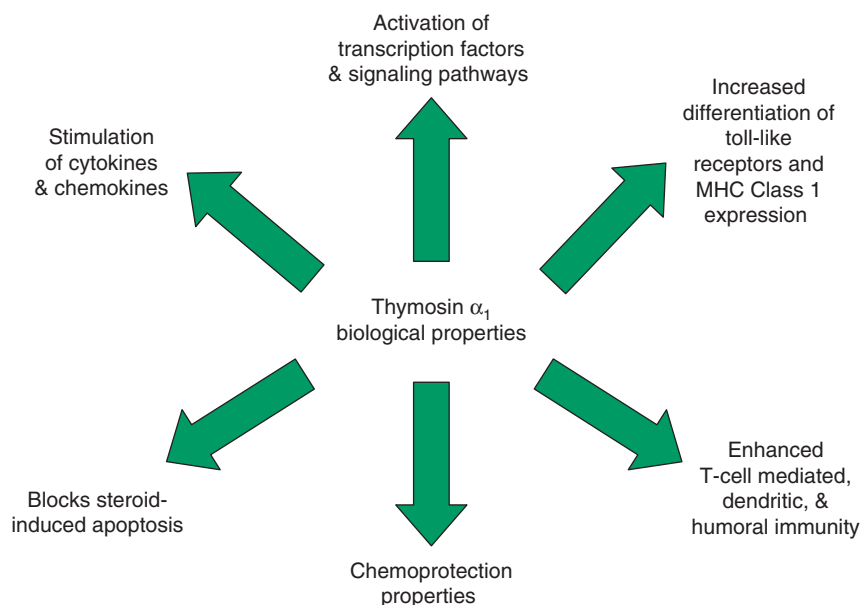


Figure 2. Pleiotropic properties of thymosin α_1 .

activity in DCs [63]. Discovering the molecular mechanism of actions of $T\alpha_1$ has provided new insights into the importance of specific transcription factors in triggering immune responses following $T\alpha_1$ treatment.

6. Clinical applications

Recent clinical trials with $T\alpha_1$ point to a broad and rapidly increasing spectrum of clinical applications as either a mono or adjuvant therapy in the treatment and diagnosis of diseases associated with deficiencies and/or imbalances of the immune system. These clinical studies have benefited from a deeper understanding of the molecular pathology of diseases associated with a dysfunctional immune system and advances in unraveling the mechanism of action of naturally occurring $T\alpha_1$ in host resistance [64]. This continuum of knowledge has led to an increasing spectrum of emerging clinical applications of $T\alpha_1$, as illustrated in Figure 4.

7. Infectious diseases

$T\alpha_1$ has been effective in improving immune responses and in arresting the progression of many viral, bacterial, and fungal infections in mice [5]. In preclinical and clinical studies, $T\alpha_1$ has increased T-cell numbers and functions and activated other antiviral lymphokines and macrophage cell populations. In addition, a significant number of clinical trials have been reported that indicate that $T\alpha_1$ alone and/or in combination with other BRMs is clinically effective in treating human viral diseases, such as viral hepatitis, cytomegalovirus, and influenza, and in the treatment of some bacterial, mycobacterial and fungal diseases [22].

Many of these infections develop in patients with secondary immunodeficiencies and from the toxic side effects of chemotherapies. A number of clinical trials have also evaluated the efficacy of $T\alpha_1$ as an adjuvant in enhancing the efficacy of the hepatitis and influenza vaccines in patients treated with kidney dialysis. Evidence for impairment of cellular immunity and the ability to respond effectively to vaccines is a widespread problem in hemodialysis patients. In randomized double-blind placebo-controlled clinical trials, it has been observed that the administration of $T\alpha_1$ to kidney dialysis patients significantly enhances the ability to respond to either the influenza vaccine or the hepatitis vaccine in patients that were previously non-responsive [65,66].

7.1 Severe sepsis

In the US, severe sepsis is still one of the leading causes of death and accounts for an estimated 750,000 new cases every year and about 10% of admissions to intensive care units [6,7]. The mortality rates for patients with sepsis, severe sepsis and septic shock has been approximately reported to be 16, 20 and 46%, respectively. A large number of experimental agents including numerous anti-inflammatory and immunomodulatory drugs have been tried, but few, if any, have yet been found to significantly reduce the risk of death as single agents [67]. Although bacteria play a significant role in the onset of sepsis, in many cases the primary cause of death is due to the paralysis and dysregulation of the immune system and the severe pro-inflammatory response due to a cascade of inflammatory cytokines and chemokines that are multi-organ in nature.

Given the established effects of $T\alpha_1$ as an immunomodulatory agent, a number of interesting clinical trials have been initiated

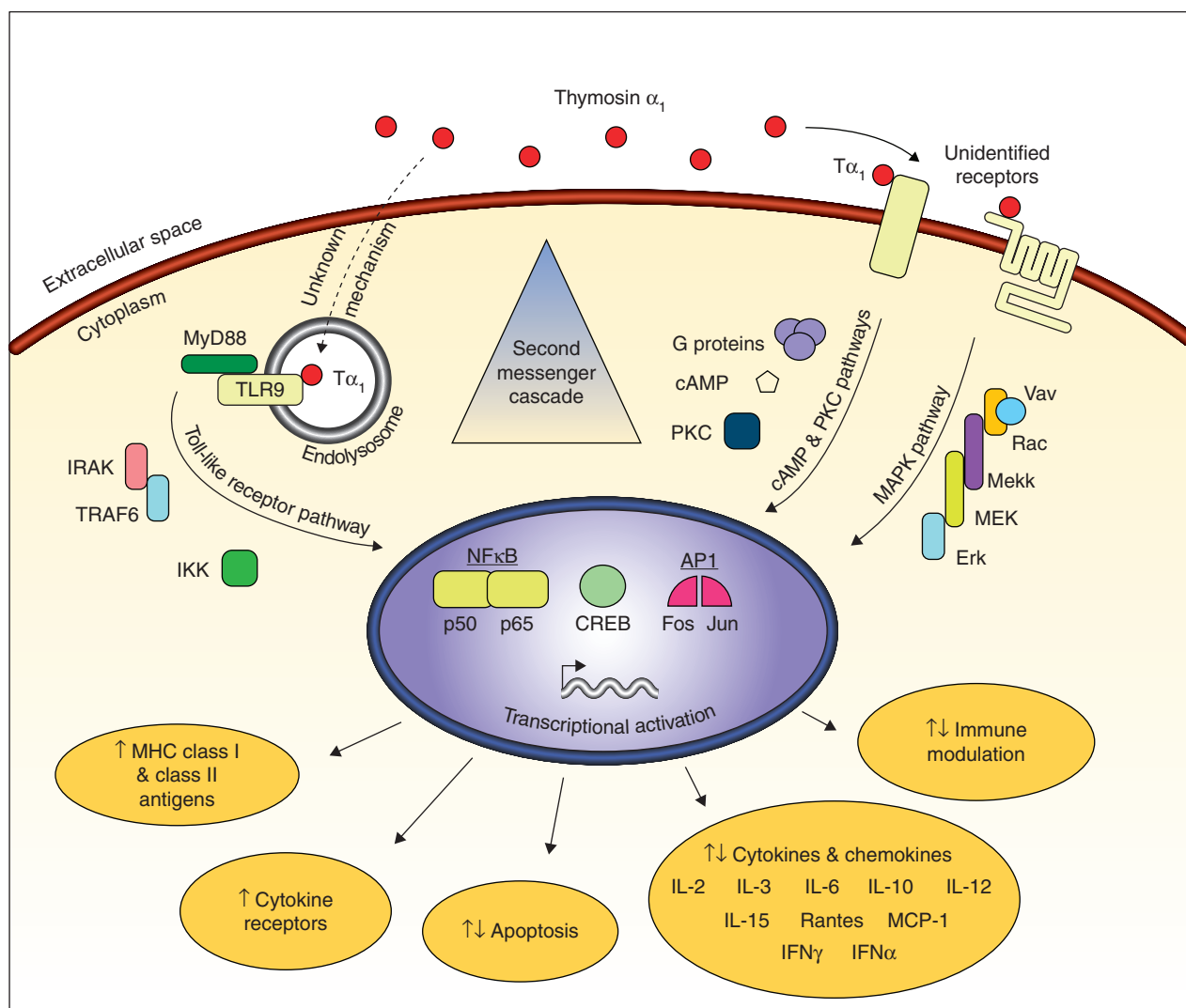


Figure 3. Thymosin α_1 : mechanism of action, second messenger cascade, and transcriptional activation.

AP-1: Activator protein 1, CREB: cAMP response element binding protein, Erk: Extracellular regulated MAP kinase, Fos: FBJ osteosarcoma oncogene, IKK: Inhibitor of NF- κ B kinase, IRAK: Interleukin-1 receptor-associated kinase, Jun: v-Jun sarcoma virus 17 oncogene homolog; MCP: Monocyte chemoattractant protein, MEK: MAPK kinase, Meck: MEK kinase, MyD88: Myeloid differentiation primary response gene 88 protein; PKA: Protein kinase A, RAC: Rac protein kinase alpha, RANTES: Regulated upon activation, normally T-expressed, and presumably secreted, TLR: Toll-like receptor, TRAF: TNF-receptor-associated factor, Vav: Vav oncogene product.

in recent years using $T\alpha_1$ alone and /or in combination with trypsin protease inhibitors (PI) in addition to antimicrobial therapy in an attempt to improve both survival and immune responses [68-73]. Using $T\alpha_1$ as a single agent, Chen *et al.* [70] have reported that $T\alpha_1$ upregulated immune function in patients with severe sepsis. In this first, to our knowledge, published trial using $T\alpha_1$, in a group of 42 patients with severe sepsis, 21 patients who received $T\alpha_1$ (1.6 mg twice daily for a week) in combination with standard antimicrobial therapy (with a 28 day follow-up) had a significantly higher cumulative survival rate (18 out of 21) than did 21 patients receiving antimicrobial and a placebo alone (12 out of 21). There was a significant difference between the two groups ($p < 0.01$). Also of interest was the shortened time of

mechanical ventilation, use of antibiotics, and stay in the intensive care unit (ICU). Zhao *et al.* [74] have reported similar results. In addition, there were significant improvements in the number of lymphocytes and CD14⁺ monocytes and HLA-DR ($p < 0.05$).

In a large, cooperative group trial of 342 patients with severe sepsis [69], patients were treated with standard anti-microbial therapy +PI + $T\alpha_1$ (600,000 units PI + 3.2 mg $T\alpha_1$ per day \times 7 days). Compared with controls receiving only antimicrobial therapy, it was found that the mortality of patients decreased from 38.32 to 25.14% ($p = 0.0088$), at day 28 and from 52.10 to 37.14% ($p = 0.0054$) versus controls at day 90. At 90 days, acute physiology and chronic health evaluation II (APACHE II) scores decreased

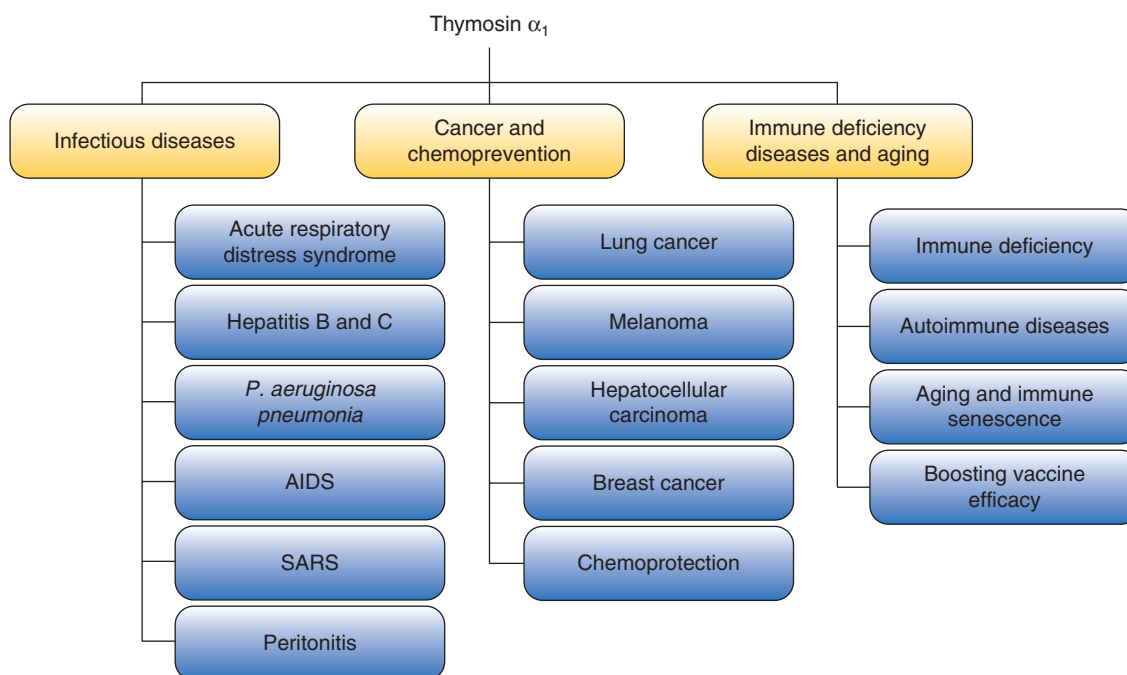


Figure 4. Emerging clinical applications of thymosin α_1 .

significantly from 14.32 to 12.7% ($p = 0.0382$) while monocytes (HLA-CR/CD14+ cells) increased from 40.13 to 51.65% ($p = 0.0092$). The results of this study clearly indicated that combining anti-inflammatory and immune-enhancing agents with standard anti-microbial therapy can significantly improve the outcome of severe sepsis and that the response is dose dependent.

In an additional trial of 120 patients with sepsis due to carbapenem-resistant bacteria it was similarly found that a combination of antibiotics plus $T\alpha_1$ reduces mortality [71]. In this trial, 1.6 mg $T\alpha_1$, two times per day for 3 days followed by a dose of PI (100,000 units three times per day for 3 days) plus 1.6 mg $T\alpha_1$ once a day for 4 successive days resulted in normalizing the balance between pro-inflammatory cytokines and anti-inflammatory cytokines and increased the cumulative survival versus the placebo group by 17.8% at day 28, 25.9% at day 60, and 27.4% at day 90.

An additional trial of $T\alpha_1$, in combination with PI, has been reported [72]. In this prospective, double-blinded, clinical trial, 56 septic patients were randomized to receive either a combination of $T\alpha_1$ and PI with conventional antibiotic therapy or conventional therapy and a placebo. At 28 days, 78% of the patients in the $T\alpha_1$ group survived versus 60% in the group treated with the placebo. In this study, the authors reported that the survival differences were mirrored by improved acute physiology and chronic health evaluation scores. There was also an improvement in leukocyte and lymphocyte counts, coagulation parameters, and cytokine levels.

While it is clear from the clinical trials done to date that the addition of $T\alpha_1$ in combination with a PI, decreases the

morbidity and mortality seen in patients with severe sepsis, there still remain a number of important questions to be addressed. These questions include what is the effect of $T\alpha_1$ alone in patients with sepsis and what stage of sepsis responds the best to immune modulation. Most recently, Yu *et al.* [73] reported a meta-analysis of five randomized controlled trials of 198 patients with sepsis, blood poisoning, septicemia or multiple organ dysfunction. They reported that $T\alpha_1$ improved T-cell numbers, $CD4^+/CD8^+$ ratios and the overall immune state of the patients significantly reduced the APACHE II score, and shortened the time on mechanical ventilation and the duration of time spent in the ICU. The authors also concluded that there was not yet enough evidence of decreased mortality and recommended additional controlled trials including dosage, duration, and timing of $T\alpha_1$.

7.2 Acute respiratory distress syndrome

Acute respiratory distress syndrome (ARDS) has an extremely high mortality rate of over 52%, reaching almost 100% in patients on ventilation [75]. In the first reported clinical trial of $T\alpha_1$ in patients with ARDS 21 renal transplant patients were used. They all met the criteria for ARDS and needed mechanical ventilation because of severe pneumonia due to either CMV (43%), bacterial (33%), or fungal (5%) etiology. The results of the study showed that 13 of 21 patients (61.9%) that received $T\alpha_1$ survived, a significant increase over their historical controls. The authors concluded that their aggressive systemic protocol, based on rebuilding the body's immune system with $T\alpha_1$ may improve the outcome of ARDS after renal transplantation [76]. In a follow up trial,

Ji *et al.* [77] studied another group of 46 renal transplant patients in which CMV pulmonary infection led to ARDS. In this study, 32 patients received $T\alpha_1$ and 14 served as controls. The success rate was significantly higher in the $T\alpha_1$ group than in the control group (78% versus 50%), and the death rate was significantly reduced (21.9% versus 50%). The authors also found that $T\alpha_1$ significantly increased the number of CD4⁺ and CD8⁺ lymphocytes, suggesting that repairing cellular immunity reinforces resistance to CMV.

7.3 SARS

Severe Acute Respiratory Syndrome (SARS) is caused by a novel coronavirus that was first identified in Asia in 2003 and quickly spread globally to many countries in Europe, North America and South America before it was contained. The genome of this virus has been characterized [78]. Although first carried in birds, it is readily transmitted to humans and has a high mortality. Gao *et al.* [79] was among the first to report out a study of 41 SARS cases, which included patients receiving $T\alpha_1$. They reported that timely administration of a cocktail of antivirals, steroids and immuno-enhancers, including IFN α -2b and $T\alpha_1$, to SARS patients was efficacious in helping to control the development of the disease and in improving patients' prognosis and controlling the spread of SARS. The trial was not designed to specifically define the role of each experimental agent.

7.4 AIDS

In the US and Italy, *in vivo* and *in vitro* studies suggest that thymosin may be useful in treating AIDS, particularly in combination with antivirals. A pilot study [80] in human immunodeficiency virus (HIV)-positive patients with helper/suppressor ratios of < 1.2 showed that 60 mg of TF5 daily for 10 weeks was effective in significantly increasing functioning immune responses; however, other surrogate markers were not changed. In multicenter studies [81], a combination of $T\alpha_1$ and zidovudine (AZT) with IFN- α was found to improve and maintain CD4 counts and to reduce HIV virus titers, as measured by polymerase chain reaction, in patients with AIDS. In a pilot Phase II randomized controlled open-label trial of $T\alpha_1$ in combination with highly active antiretroviral therapy (HAART) Chadwick *et al.* [82] studied the safety and efficacy of $T\alpha_1$ in stimulating immune reconstitution. Thirteen patients received $T\alpha_1$ and seven served as controls. In this 12-week study it was found that there were no serious adverse effects. The most interesting finding of this study was a significant increase in peripheral blood mononuclear cell (PBMC) signal joint T-cell receptor circles (sjTREC) levels at week 12. sjTREC levels have been shown to correlate with recent thymic emigration and thymopoiesis and to be a biomarker for *de novo* T-cell synthesis. Unfortunately, the short duration of the trial precluded detection of clinical benefits and the long-term consequences of enhanced immune reconstitution remain for future trials in this patient population.

7.5 *Pseudomonas aeruginosa* pneumonia

Pseudomonas aeruginosa infections are a major clinical problem in the lungs of immunocompromised and aging populations and account for about 10% of nosocomial infections [83]. The first clinical report of $T\alpha_1$ in critically ill hospitalized patients with tracheotomies was carried out by Huang *et al.* [84]. In this study, of 42 patients who were randomly divided into a treatment group that received 1.6 mg $T\alpha_1$ daily for 7 days and a control group, they found a significant decrease of infection rate, white blood cell (WBC) count, C-reactive protein, TNF α , and IL-6 in the treatment group in comparison with the control group. The authors concluded that $T\alpha_1$ can be effective for prevention and treatment of lung infections and improve patients' immunity and prognosis. In a follow up study of drug-resistant *P. aeruginosa* pneumonia in a group of 37 older patients treated with Sulperazone and 1.6 mg $T\alpha_1$ for 5 days or just Sulperazone Li *et al.* [85] reported that $T\alpha_1$ improved immunological function and reduce inflammation and had a synergistic effect on drug-resistant *P. aeruginosa* pneumonia.

7.6 Hepatitis B and C, severe chronic hepatitis, and spontaneous peritonitis

The most clear-cut clinical indications for $T\alpha_1$ in infectious diseases to date have been in patients with chronic hepatitis B and hepatitis C. Since the approval of $T\alpha_1$ for the treatment of hepatitis B (HBV) in Singapore in 1993 and in a number of other countries in Asia, Europe, Central and South America, a large number of trials using $T\alpha_1$ alone or in combinations have been reported [9,22]. In the first reported trial in the US a 12-patient placebo-controlled pilot study, $T\alpha_1$ was found to significantly improve the remission rate in advanced chronic hepatitis B [86]. These initial results stimulated further development of $T\alpha_1$ for use in the treatment of HBV. In a meta-analysis of the results of five placebo-controlled trials enrolling 353 patients, Chan *et al.* [87] reported that the odds ratio of $T\alpha_1$ over placebo at the end of treatment and at 6 and 12 months was found to be 0.56, 1.76, and 2.67 respectively, and with a significantly increasing virological response over time after the discontinuation of $T\alpha_1$ ($p = 0.02$). $T\alpha_1$ in combination with IFN- α and/or antivirals have also shown promise [88].

Based on these promising results with $T\alpha_1$ therapy in HBV the Asian Pacific Association for the study of Liver (APASL) have included $T\alpha_1$ as one of its recommended approved drugs for treatment of HBV [89]. A significant advantage of $T\alpha_1$ appears to be the fixed duration of therapy and the minimal side effects.

Among 109 patients with HCV in whom the remission rates with interferon alone were significantly greater than in placebo controls, the remission rates in patients treated with $T\alpha_1$ in combination with interferon were greater than those obtained with interferon alone [90]. In addition, end of treatment biochemical responses (normalization of alanine amino transferase (ALT) and aspartate aminotransferase (AST)) was

seen in 37.1% of patients treated with combination therapies, 16.2% of patients treated with IFN- α , and 2% of untreated controls ($p = 0.04$).

An interesting pilot trial in patients with severe chronic hepatitis (SCH) has been reported by Gao *et al.* [91] In this trial, patients received 1.6 mg T α_1 , three times per week for 2 weeks and then twice a week for 6 weeks in addition to conventional therapy. The 3-month survival was 83.3% (10 of 12). The results were significant in allowing the authors to conclude that T α_1 is beneficial for the treatment of SCH. A major complication of SCH is bacterial infection, with a mortality rate of over 90%. The origin of infection and cause of death is often spontaneous bacterial peritonitis with subsequent hepatic coma. In still another patient population with HBV, Ma *et al.* [92] reported in a study of 64 patients with spontaneous peritonitis (SP) in cirrhotic patients with HBV that the addition of T α_1 with conventional antibiotics significantly increased the efficacy seen with antibiotics alone and also enhanced survival.

8. Cancer

Many cancers are associated with significant deficiencies in cellular immunity [3]. In addition, standard treatments for cancer (i.e., surgery, radiotherapy and chemotherapy) usually depress cellular immunity. In animal studies, T α_1 has been shown to restore immunity and increase resistance to progressive tumor growth, and reverse or ameliorate the immunosuppressive effects of chemotherapy or radiotherapy [49,93].

8.1 Lung cancer

To date there have been at least nine trials of T α_1 in patients with a variety of lung cancers reported in the literature. The first randomized double-blind Phase II trial of T α_1 in humans was carried out by Schulof *et al.* in 1985 [94]. In this trial, T α_1 as an adjuvant to conventional radiation therapy was found to significantly prolong survival in patients and increase the disease-free interval, particularly in patients with nonbulky tumors, and with NSCLC. A follow-up Phase III trial confirmed this finding in patients with non-bulky squamous cell NSCLC [95]. Similar beneficial effects of T α_1 as an adjuvant to chemotherapy and IFN- α have been reported, along with an added ability to reduce the toxicity of conventional chemotherapy, in patients with NSCLC [96,97].

8.2 Melanoma

There have been a number of small clinical trials of T α_1 reported from the labs of Enrico Garaci, Guido Rasi, and colleagues [98-100] studying the immunological and clinical effects of T α_1 patients with late-stage metastatic melanoma. Late-stage metastatic melanoma is resistant to most forms of therapy. Only a small number of chemotherapeutic agents have demonstrated antitumor activity against this disease. Dacarbazine (DTIC) still remains the most active single agent, with response rates of ~ 17 – 30%, usually of short

duration, and without impact on patient survival. Combination therapy with other cytotoxic therapies have not proven to be clearly superior to DTIC alone. In contrast, combination studies using BRMs which include T α_1 have been promising. A growing body of scientific evidence has demonstrated that rejection of established tumors can be mediated in part by vigorous cellular immune responses. Experimental and clinical studies of IL-2, for example, have shown that high doses of IL-2 can induce tumor regression of established micro-metastases in lung [101-104] and have demonstrated reproducible responses in patients with metastatic melanoma, with response rates of 10 – 25% using several doses and schedules of administration. Potential therapeutic BRM combinations have included the combination of DTIC and BRMs such as IL-2, IFN and T α_1 . In the first trial of T α_1 by Rasi *et al.* [98] they studied T α_1 in combination with DTIC and natural IFN- α . Patients were treated with DTIC followed by a course of T α_1 (1 mg) and IFN- α (3 MIU) in a dosing regimen that repeated every 28 days. Results on 26 treated patients evaluated by WHO criteria showed an overall response rate of 50%, with a mean duration of response of 13.5 months. In the second trial with T α_1 Lopez *et al.* [99] administered T α_1 in combination with DTIC and IL-2. Patients were treated with DTIC followed by a course of T α_1 (2 mg) and IL-2 (18 MU/m²) in a dosing regimen that repeated every 3 weeks. Results reported on 42 evaluable patients showed an objective response rate of 36% (2 complete responses and 13 partial responses). The median time to progression was 5.5 months and median survival was 11 months. When compared with historical controls, both combinations showed a greater overall response over DTIC alone, or DTIC with IFN or IL-2 minus T α_1 . In both of these trials, patients received chemotherapy and either IFN- α or IL-2. In these studies T α_1 was found to significantly increase T-cell levels, natural killer (NK) activity, and survival obtainable with regimens of dacarbazine in combination with IFN- α or IL-2 [47].

Based on the promising increase in survival and response ratios of patients with stage IV melanoma compared with historical controls, a large follow-up dose-finding study has been conducted in Europe [104]. In this Phase II multi-center, randomized open-label study a total of 488 patients with stage IV melanoma were enrolled at 64 European clinical sites. The trial was designed to evaluate different dose levels of T α_1 in combination with DTIC chemotherapy, with and without low-dose IFN- α , as a first-line treatment for stage IV melanoma. Most patients enrolled in the trial had liver and other metastases and the remaining patients had lung metastases and skin or lymph node metastases. T α_1 at all dose levels was well-tolerated in all treated patients, with no serious adverse events attributed to the drug. In this trial, T α_1 achieved its primary endpoint in treating patients diagnosed with stage IV melanoma. Reported results show that T α_1 , in combination DTIC chemotherapy, tripled the overall response rate and extended overall survival by nearly 3 months

compared with patients treated with the current standard of care of DTIC, combined with IFN- α . Based on the promising results of this trial, the US FDA has most recently approved the design of a Phase III registration trial for T α_1 as a potential treatment for stage IV melanoma [105].

Although the specific mechanism by which T α_1 appears to act on melanoma is yet to be defined, it is known that suppression of the growth of immune-sensitive tumors such as melanoma have been shown to be dependent on a strong and aggressive immune response, including a large number of activated effectors such as tumor-infiltrating lymphocyte cells (TILs) and specific anti-melanoma cytotoxic T lymphocytes (CTL). As shown in Figure 3, T α_1 's potential beneficial role in the treatment of melanoma may derive from its demonstrated direct activation of these various arms of the immune system, including increases in TILs and CTLs, and via its indirect effects on expression of MHC Class I and tumor-specific antigens. Part of T α_1 's multiple activities arise through the activation of a Toll-like receptor 9 [24], an increased activity of the nuclear factor NF- κ B and by activating Jun activating transcription factor-2 (ATF-2) and activator protein 1 (AP1) [58]. Evaluation of T α_1 's utility in animal models of melanoma has confirmed effective anti-tumor activity and provided the scientific foundation for current human trials in this indication.

8.3 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the top three leading causes of cancer death and has a worldwide annual incidence of approximately 1 million new cases. There is a strong connection between HCC and chronic hepatitis B and C [106]. The first Phase II trial of T α_1 in patients with HCC was reported in Italy by Stefanini *et al.* in 1998 [107]. The addition of T α_1 following chemotherapy with doxorubicin significantly increased the survival obtained by transcatheter arterial chemoembolization (TACE) in this patient population from 41 – 82% at 7 months. The survival advantage with T α_1 was still significant at 18 months and maintained its significant increase in survival at the 3 years study endpoint. A number of other trials from the US [108] and China [109-111] have been reported which compare the safety and efficacy of T α_1 plus TACE. In the US Phase-II open label pilot trial, 25 patients with unresectable HCC who received T α_1 five times a week plus TACE for 24 weeks or TACE alone, did not yield significant ($p = 0.44$) results, but showed a trend toward increased survival. The median survival time was 774 days for patients treated with T α_1 plus TACE, compared to a median survival of 399 days for patients receiving TACE alone. Of interest in this trial was that more T α_1 treated patients (4 of 14) became eligible for liver transplants than patients treated with TACE alone (0 of 11). Also of interest was the decrease of bacterial infections (0 of 14) in patients receiving TACE and T α_1 versus (4 of 11) in the group receiving TACE alone.

HCC is endemic in most of Asia and is the second most common cause of cancer mortality there. A number of interesting clinical reports are emerging from China describing use of T α_1 in combination with TACE in patients with advanced HCC. In an open-label study of 32 patients, Li [109] was the first to study the efficacy of T α_1 in combination with TACE in patients with advanced HCC in comparison with a historical group of 26 matched patients treated with TACE alone and matched for age, gender and Okuda staging. In the T α_1 group survival rates were 91,88 and 78% respectively at 6, 9 and 12 months while in the TACE group they were 77,58 and 46%. Survival rates at all three time points were significantly higher ($p < 0.05$) in the group receiving T α_1 . This report concluded that treatment with T α_1 resulted in a significant increase in survival and immune parameters in HCC patients versus TACE alone.

In a second trial from China in patients with HCC, Cheng *et al.* [110] studied 57 hepatectomy patients who were randomly divided into three groups receiving either hepatectomy and TACE plus T α_1 , hepatectomy plus TACE only, or hepatectomy only. The time of tumor recurrence was 7.0, 5.0 and 4 months, respectively, and median survival was 10.0, 7.0 and 8 months ($p < 0.002$) respectively. Although the one-year recurrence rate did not decrease, postoperative treatment with TACE and T α_1 significantly prolonged survival time. In a follow-up trial, Cheng *et al.* [112] studied a group of 84 patients with HCC plus tumor thrombus in the portal vein (PVTT) post HCC resection that were divided into five groups that received either HCC resection plus PVTT removal, plus postoperative TACE and T α_1 , or HCC resection plus PVTT removal plus postoperative TACE, or HCC resection plus PVTT removal, TACE only, or conservative treatment only. These authors observed median survival times of 10.0, 7.0, 8.0, 5.0 and 2.0 months, respectively, and a one year survival rate of 44.4%, 15%, 14.3%, 10.5% and 0%, respectively, suggesting a beneficial improvement in survival by the addition of TACE and T α_1 .

Clinical evidence is also emerging from China that a combination of anti-viral therapy and T α_1 may be helpful in preventing the recurrence of HCC with coexisting Hepatitis B (HB). In a group of 33 patients post hepatectomy with HCC coexisting with active HB, Cheng *et al.* [112] has reported that the administration of lamivudine plus T α_1 versus hepatectomy alone, the one year HBV-DNA suppression rate was 100% versus 6% ($p < 0.01$) the recurrence time was 7 versus 5 months ($p < 0.01$) and the median survival 10.0 versus 7.0 months respectively ($p < 0.01$). These studies indicate that antiviral therapy plus T α_1 may delay the recurrence and prolong the survival of patients with HCC with coexisting active HB.

8.4 Chemoprotection during lung and breast cancers treatments

In spite of significant advances in treatment over 160,000 deaths due to lung cancer and over 40,000 from breast cancer still

occur every year in the US alone. The potential of using $T\alpha_1$ as a novel chemopreventative agent is gaining support from animal models of mammary and lung carcinogenesis. For example, in two animal models of mammary carcinogenesis (*N*-methyl urea (NMU) in Fisher rats or a transgenic mouse model, C3(1) SV40T), the administration of $T\alpha_1$ was found to significantly enhance median survival times and decrease the mammary tumor burden [93]. These animal studies have provided the scientific rationale for clinical trials with $T\alpha_1$ in the area of chemoprotection.

A number of clinical studies with $T\alpha_1$ in patients with lung and breast cancers have been reported. In these trials, the addition of $T\alpha_1$ therapy appears to offer protection from some of the toxicities of chemotherapy. For example, in a study by An *et al.* [113] in 22 patients with advanced lung or advanced breast cancer treated with chemotherapy, it was found that $T\alpha_1$ given daily for four days before chemotherapy and twice weekly after chemotherapy reduced neurotoxicities significantly in 45% of the patients. The authors concluded that $T\alpha_1$ may protect patients from chemotherapy-induced neurotoxicities. In another 60-patient study focusing on quality of life (QoL) issues in patients receiving chemotherapy, it has been reported by Chen [114] that comparing patients receiving $T\alpha_1$ between cycles of chemotherapy versus patients not receiving $T\alpha_1$ resulted in a significant increase in QoL scores in the combined chemotherapy plus $T\alpha_1$ group in terms of appetite, sleep, fatigue, daily activity and overall feeling of well being, and reduced depression [114]. Collectively, these studies are pointing to hitherto unexpected action of $T\alpha_1$ as a chemoprotective agent.

9. Immune deficiencies

Thymosins modulate immune activity and may be used to restore depressed immune systems. Most of the congenital immune deficiencies are T-cell-dependent; that is, the defective immune activity is due to a partial or complete absence of mature T-cells, resulting from a deficiency of thymic hormone production [14,15]. TF5 has been used to stimulate the formation and development of an effective T-cell immunity in newborns with thymic aplasias, such as DiGeorge, in which full functioning of the thymus was disrupted during embryonic development due in part to genetic defects [14]. Overall, the response to therapy of patients with primary immunodeficiency diseases has been variable depending on the nature of the defect. Patients with diseases where the defect lies at the level of the stem cell (e.g., severe combined immunodeficiency diseases (SCID)) have not responded, but those with thymic deficiencies have. A subgroup of patients, such as those with DiGeorge syndrome, and some patients with combined immunodeficiency disorders have been found to respond to TF5 [14]. Randomized trials are difficult because of the rarity of the diseases. A patient with DiGeorge syndrome has been treated with $T\alpha_1$ and has responded favorably [115]. Using a human thymus/bone marrow coculture, a clear effect of $T\alpha_1$

has also been demonstrated in enhancing CD34⁺ T-cells and in producing IL-7, a cytokine critical in the maturation of thymocytes [38].

9.1 Autoimmune diseases

Controlled clinical trials with $T\alpha_1$ have not yet been initiated in patients with autoimmune diseases. Given the biological properties of $T\alpha_1$ observed in animal and human studies, $T\alpha_1$ may be particularly useful in the treatment and control of certain autoimmune diseases. Autoimmune diseases are distinguished by an abnormally sensitive and over-responsive immune system, one that attacks not only harmful organisms and malignant T-cells but also targets normal tissues. A frequent finding in many of these diseases is a deficiency of suppressor T-cells. In preclinical studies, $T\alpha_1$ restored immune balance and increased the level of suppressor T-cells. Studies with $T\alpha_1$ in animals suggest that juvenile-onset diabetes and other similar endocrine abnormalities have autoimmune components. Since $T\alpha_1$ has been found to be safe and without either significant side effects or a narrow therapeutic index, patients with those diseases may be candidates for experimental clinical studies with $T\alpha_1$ or other thymic hormones. In pilot clinical studies, TF5, the partially purified thymic preparation which contains $T\alpha_1$, has relieved symptoms in rheumatoid arthritis, systemic lupus erythematosus and Sjögren's syndrome [116].

9.2 Aging and immune senescence

It is well known that immunity declines with age. Contributing factors include a decline in thymus-related cellular function, an accumulation of memory cells, and dysregulated inflammatory mediators. Of all the cellular components of the immune response, T-cells, are most affected by age. This may be linked to the universally occurring age-associated thymus gland involution and resultant shrinkage of the naïve T-cell compartment [9,117].

In influenza, for example, although the attack rate is highest among young children, the elderly account for the great majority of hospitalizations and over 90% of the influenza-related deaths each year [117,118]. In addition to death resulting directly from influenza, cardiopulmonary conditions and other diseases can be exacerbated and lead to death in susceptible persons, particularly those of advanced age.

A decline in T-cell immunity and possibly serum levels of $T\alpha_1$ appears to correspond with an increase in immune senescence associated with aging [117]. These studies suggest that it may be possible to improve immunologic responses in the elderly by manipulating the blood levels of thymic hormones, such as $T\alpha_1$. A number of key studies in aged mice have provided support for this hypothesis and provided the scientific foundation for clinical trials with $T\alpha_1$ in geriatric medicine [118,119].

In addition to the suboptimal antibody responses to vaccination in elderly subjects, similarly diminished antibody responses have been reported in patients with end-stage

renal disease. The evidence for impairment of cell-mediated immunity in hemodialysis patients has been attributed to incompetence in T-cell-mediated immune responses that worsen with age. Hepatitis B viral infection has historically been a major complication in patients receiving chronic hemodialysis, as well as a threat to the staff working in the dialysis unit. A high percentage of dialysis patients have become chronic carriers of hepatitis B surface antigen (HBsAg) after infection, and most remain highly infectious, as indicated by a high prevalence of the hepatitis B antigen. Control of this infection in the dialysis unit by vaccination has not been successful since the hepatitis B vaccine does not provide the immunogenicity and efficacy in hemodialysis patients that has been documented in healthy adults. Therefore, in addition to current control measures, a more effective vaccination regimen would provide greater control of the hepatitis B viral infection among dialysis patients and attending staff. One strategy for augmenting vaccine efficacy has been temporary immunological reconstitution with $T\alpha_1$ administered in conjunction with the vaccine. This approach was taken in several studies to determine whether $T\alpha_1$, when administered subsequently to influenza or hepatitis B vaccine, would be associated with greater serum antibody levels in an elderly or hemodialysis-patient population.

An early example of the use of $T\alpha_1$ as an adjuvant to boost the efficacy of a vaccine in an immunodeficient patient population with renal disease on hemodialysis was carried out by Shen *et al.* [65] This was a double-blind, placebo-controlled trial of 23 patients who received $T\alpha_1$ as an adjuvant to hepatitis vaccine. Historically, these patients have an increased incidence of hepatitis and have low response rates to the vaccine because of the immunosuppressive effects of chronic renal failure. Seven of eleven patients who had previously failed to respond to the vaccine and who were given hepatitis vaccine and $T\alpha_1$ responded positively with protective antibody levels to the hepatitis vaccine, while only 2 of 12 patients given the vaccine and placebo responded positively. A 100-patient trial of the same design in patients on chronic renal dialysis, in which patients were randomized to receive influenza vaccine and either $T\alpha_1$ or placebo, similarly found that $T\alpha_1$ as an adjuvant to the influenza vaccine could significantly boost immune response [66]. The trial showed that $T\alpha_1$ increased the number of patients who responded to the vaccine by approximately 50%.

10. Boosting vaccine efficacy

Ershler *et al.* [118] found that they could significantly enhance antibody responses and immunity to influenza with $T\alpha_1$. Effros *et al.* [120] were able to confirm and extend the findings that $T\alpha_1$ increased immune responses in a mouse model of influenza infection. A strategy of using $T\alpha_1$ to stimulate immunity and to enhance vaccine responses in geriatric populations has been developed based on these animal studies in aged mice.

In the first human trial in geriatric patients, a small series of nine patients (age range 65 – 99 years) who were known to be non-responsive to the prior year's influenza vaccine, were vaccinated and then treated twice weekly with $T\alpha_1$ (0.9 mg/m²) for 5 weeks. Six of the nine subjects met criteria for vaccine response (fourfold rise in hemagglutination (HA) titer) [119].

This trial was followed by a double-blind, randomized placebo controlled study conducted by Gravenstein *et al.* [121]. In this trial, 90 male veterans 65 years and older (mean age, 77.3 years) were randomized to receive either $T\alpha_1$ (0.9 mg/m² twice weekly for 4 weeks) or placebo injections (twice weekly for 4 weeks) with the first injection immediately after influenza vaccine. Of the 45 patients receiving $T\alpha_1$, 31 (69%) achieved a fourfold HA titer rise compared with 21 of 40 (52%) in the placebo group ($p = 0.023$).

A third, larger randomized trial was undertaken by McConnell *et al.* [122] to determine if a 4-week course of $T\alpha_1$ was required to realize the enhanced vaccine response. For this, 330 elderly volunteers from the United States Soldiers' and Airmen's Home in Washington, D.C., were randomized to one of three treatment groups. All subjects received the influenza vaccine. Group 1 subsequently received twice weekly $T\alpha_1$ (0.9 mg/m²) for a total of eight doses; group 2 received the same dose of $T\alpha_1$ but only for four doses (2 weeks) followed by four doses of placebo for the subsequent two weeks; and group 3 received eight doses of placebo over 4 weeks. Antibody levels were greater in group 1 than either of the two other groups ($p = 0.015$) [123]. There was no toxicity observed in any of the research subjects in any of these three trials related to the vaccine, $T\alpha_1$, or placebo administrations. In the third trial, subjects were followed weekly until completion of the influenza season (18 weeks after vaccine). $T\alpha_1$ did not reduce the appearance of respiratory symptoms and only modestly (but significantly) reduced the number of cases of influenza, but what was interesting was that for those receiving $T\alpha_1$ who developed influenza, the symptoms were more likely to be mild or nonexistent (i.e., be asymptomatic and detected only by sero-conversion) [123]. In contrast, in the group receiving the vaccine plus placebo, all subjects with influenza reported symptoms. Thus, $T\alpha_1$, perhaps by enhancing vaccine responses, or perhaps by augmenting T-cell-effector mechanisms, appeared to ameliorate some of the consequences of influenza observed in this patient population. The beneficial effects of $T\alpha_1$ appears to be age-related, with the increases in antibody titers and improvement in pulmonary infections increasing with the age of the reported subjects [122,123].

11. Conclusions and expert opinion

The advances in our knowledge of the potential clinical applications of $T\alpha_1$ alone or in combination with other BRMs or standard therapies offer a myriad of opportunities to augment and regulate the immune network and to influence

therapy. Recent clinical studies indicating that T α_1 can synergize with other BRMs, select proteases, and conventional treatments are rapidly expanding our knowledge of the potential use of T α_1 and are providing broad and novel insights into understanding the underlying mechanisms utilized by neoplastic and infectious agents to evade destruction by the immune system. We are beginning to see evidence that T α_1 , in combination with selected cytokines, may allow for lower doses of cytokines to be effective, thus reducing their potential associated side effects and toxicities. The growing ability to intervene in a number of serious life-threatening and/or chronic diseases with one or more BRMs in combination with T α_1 or conventional therapies is rapidly expanding the body of knowledge concerning the ways in which the immune system affects disease processes.

The myriad of clinical trials being reported from countries like China and Italy, where T α_1 has been approved for over 10 years, is significantly expanding the number of potential applications of T α_1 in the treatment of diseases in which modification of the immune system appears to improve outcome. Coupled with the advances that have been made in our understanding of the molecular biology of T α_1 using genomics, proteomics and stem cell approaches, we are amplifying our understanding of the important role played by the thymus and the soluble mediators of immunity in health and disease. It is the authors' expert opinion that this

increased understanding of the role of T α_1 on immune function, either alone or in combination with other BRMs and conventional therapies, will, over the next decade, translate into the successful treatment of many infectious diseases and cancers that have a high morbidity and mortality.

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Affiliation

Allan L Goldstein^{†1} & Adam L Goldstein²

[†]Author for correspondence

¹The George Washington University School of Medicine and Health Sciences,

Department of Biochemistry & Molecular Biology, 2300 I St., N.W., Room 438,

Washington, DC, USA

Tel: +1 202 994 3171; Fax: +1 202 994 8974;

E-mail: bcmalg@gwumc.edu

²Medical School for International Health at

Ben-Gurion University of the Negev,

Be'er-Sheva, Israel