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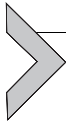

CHAPTER EIGHT

Prothymosin Alpha and Immune Responses: Are We Close to Potential Clinical Applications?

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Abstract

The thymus gland produces soluble molecules, which mediate significant immune functions. The first biologically active thymic extract was thymosin fraction V, the

[2] P. Samara and K. Ioannou contributed equally to this work.

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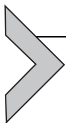
fractionation of which led to the isolation of a series of immunoactive polypeptides, including prothymosin alpha (proTα).

ProTα displays a dual role, intracellularly as a survival and proliferation mediator and extracellularly as a biological response modifier. Accordingly, inside the cell, proTα is implicated in crucial intracellular circuits and may serve as a surrogate tumor biomarker, but when found outside the cell, it could be used as a therapeutic agent for treating immune system deficiencies. In fact, proTα possesses pleiotropic adjuvant activity and a series of immunomodulatory effects (eg, anticancer, antiviral, neuroprotective, cardioprotective). Moreover, several reports suggest that the variable activity of proTα might be exerted through different parts of the molecule. We first reported that the main immunoactive region of proTα is the carboxy-terminal decapeptide proTα (100–109). In conjunction with data from others, we also revealed that proTα and proTα(100–109) signal through Toll-like receptor 4. Although their precise molecular mechanism of action is yet not fully elucidated, proTα and proTα(100–109) are viewed as candidate adjuvants for cancer immunotherapy.

Here, we present a historical overview on the discovery and isolation of thymosins with emphasis on proTα and data on some immune-related new activities of the polypeptide and smaller immunostimulatory peptides thereof. Finally, we propose a compiled scenario on proTα's mode of action, which could eventually contribute to its clinical application.

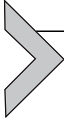


1. INTRODUCTION

Biologic response modifiers (BRMs) are endogenous (ie, naturally produced in the body) or exogenous (administered together with a drug) agents that modulate immunity. BRMs regulate, among others, the type, duration, and intensity of immune responses and are characterized by pleiotropy and redundancy. The thymic polypeptide prothymosin alpha (proTα) has been incorporated in the large family of BRMs, mainly because of its modulating effects on several properties of immune effectors. Its wide distribution in cells, tissues, and organs, its broad phylogenetic dissemination and the lack of a mechanism supporting its secretion, questioned the initial characterization of proTα as "thymic hormone." Now, it is widely acknowledged that proTα possesses an essential intracellular role related to cell survival and growth, and at the same time, extracellularly it enhances the functionalities of diverse subpopulations of the immune system. Several novel functions, beyond immunomodulation, have also been ascribed to proTα.

Accumulated data suggest that its immunopotentiating activity could be therapeutically exploited in various clinical conditions associated with immunodeficiency, immunosenescence, cancer, and autoimmune diseases.

Herein, we present the most prominent effects of the polypeptide, as reported by various research teams for over 30 years, propose a compiled scenario on its mode of action, and provide means, which eventually could lead to its incorporation in clinical trials as an immunostimulant/adjuvant.



2. HISTORICAL OVERVIEW ON ProTα ISOLATION AND PROPERTIES

2.1 Thymosin Fraction V: The First Immunoactive Thymic Extract

The thymus had remained an enigmatic organ for centuries, as its clinical significance and true function were much disputed. Although its lymphopoietic role had been repeatedly demonstrated (Miller, 2002), the long-prevailing opinion claimed that the thymus was a redundant organ with no immunological function. It was not until the early 1960s that Miller, Good, and their colleagues tipped the balance in favor of a crucial and central role for thymus in establishing a competent immune system (Good et al., 1962; Miller, 1961). They were the first to show that neonatally thymectomized mice exhibited a marked deficiency of lymphocytes and impaired immune responses that were further associated with their inability to resist infections and reject histoincompatible transplants. However, even these studies could not fully elucidate the exact mechanism underlying the continued thymic control over lymphocytic function. A year later, it was unequivocally proven that the thymus is actually a gland mediating its function, at least partially, through humoral messages and not merely via cellular interactions (Aisenberg & Wilkes, 1965; Levey, Trainin, & Law, 1963).

In the years to follow, Goldstein and his colleagues isolated and identified a series of hormonal-like agents with immune-restorative capacity (Goldstein, 2007). These initial efforts led to the isolation of a partially purified thymic extract that could enhance in vivo the incorporation of tritiated thymidine into DNA of mouse lymph nodes, indicative of increased lymphocytic proliferation (Klein, Goldstein, & White, 1965). With the invaluable aid of the newly developed in vitro proliferation assay, this soluble factor inducing lymphocytopoiesis was purified, characterized, and named "*thymosin*" (Goldstein, Slater, & White, 1966). However, the major breakthrough was the isolation of *thymosin fraction V* (*TFV*), a further purified preparation with immunoregulatory activity (Goldstein et al., 1981). Using a novel and complex process that enabled its purification in large amounts (Hooper et al., 1975), TFV's properties and activity were explored in several in vitro and

in vivo studies. In vitro, TFV was shown to stimulate lymphocytes deriving from immunosuppressed humans and restore their functions to normal levels (Wara & Ammann, 1975). Additionally, TFV enhanced murine spleen cell responses in mixed lymphocyte reactions (MLRs) and in mixed lymphocyte-tumor cultures (MLTCs; Talmadge, Uithoven, Lenz, & Chirigos, 1984). In vivo, TFV increased the survival of neonatally thymectomized mice (Spangelo, Hall, & Goldstein, 1987), restored graft-vs-host reactivity (Spangelo et al., 1987), and "corrected" T-cell abnormalities in mice with autoimmune disease (Dauphinee, Tala, Goldstein, & White, 1974). Interestingly, TFV also possessed direct antiproliferative properties against malignant cells, as demonstrated in vitro against murine pituitary adenocarcinoma and glioma (Spangelo, Farrimond, Pompilius, & Bowman, 2000), and against human acute T lymphoblastic (Ho, Ma, Price, Hunstein, & HoVbrand, 1983) and promyelocytic leukemia cell lines (Spangelo et al., 2007). Finally, in vivo treatment with TFV conferred resistance to Dunning (Khaw & Rule, 1973) and murine lymphoblastic leukemias (Petro & Watson, 1982).

2.2 Dissecting TFV: Isolation of the First Immunoactive Thymosins

The immune-stimulating and restorative activities attributed to TFV triggered the need to further identify its several components. Thorough fractionation and analysis revealed that TFV consisted of at least 40 different molecules, of which 10–15 were principal and 20 or more were secondary components, and the majority of these molecules were acidic and of varying molecular weights (between 1000 and 15,000 Da). A nomenclature system based upon the isoelectric point (p*I*) of the peptides has resulted in TFV's subdivision into three different groups, each one identified by a Greek letter, ie, alpha (α), beta (β), and gamma (γ). Thus, peptides with p*I*s less than 5.0 were named α-peptides, these with p*I*s of 5.0–7.0 were termed β-peptides, and peptides with p*I*s greater than 7.0 were considered γ-peptides. A subscript number was used to identify the peptides from each group, indicative of the order in which they were isolated from TFV, eg, α_1, α_2, α_3, etc. (Goldstein, 2007).

The first two peptides identified and fully characterized were *thymosin* α_1 (Tα_1; Goldstein et al., 1966) and *thymosin* β_4 (Tβ_4; Low, Hu, & Goldstein, 1981), and their immunopotentiating properties were extensively studied in the years to follow (Spangelo et al., 1987). Subsequently, a number of α- and β-peptides were isolated and sequenced from thymus and other tissues (Hannappel & Huff, 2003). Utilizing a newly developed method that

reduced the effect of proteolysis during peptide isolation, Hannappel and colleagues demonstrated that $T\beta_4$ was the major component of calf thymus extract and that $T\alpha_1$ was absent or present in only trace amounts (Hannappel, Davoust, & Horecker, 1982a). The latter observation led the investigators to hypothesize that the presence of $T\alpha_1$ in TFV was the result of proteolytic cleavage of a precursor peptide. This speculation was further fueled by evidence showing that a previously isolated peptide, $T\beta_8$, was actually the proteolytic product of a longer molecule, $T\beta_9$ (Hannappel, Davoust, & Horecker, 1982b). In addition, the same group isolated two peptides from TFV, whose sequence strongly resembled that of $T\alpha_1$. When compared to $T\alpha_1$, one peptide lacked four amino acids at its carboxy (C)-terminus and it was therefore named des-(25–28)-$T\alpha_1$, and the second had an additional seven amino acids at its C-terminus, and was given the name $T\alpha_{11}$ (Caldarella et al., 1983). Consequently, the search for a larger α-thymosin precursor molecule began.

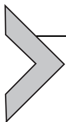
In an effort to isolate the native polypeptide, Haritos and colleagues developed a radioimmunoassay based on an antibody raised against synthetic $T\alpha_1$ (Haritos & Horecker, 1985). By coupling this assay with an isolation procedure designed to minimize any proteolytic activity in cell extracts, they eventually isolated, initially from rat thymus, a polypeptide of 112 amino acids long, which contained the $T\alpha_1$ sequence (amino acid residues 1–28) at its amino (N)-terminus (Haritos, Goodall, & Horecker, 1984). They named this polypeptide *proTα* to indicate that it was the source of $T\alpha_1$ and $T\alpha_1$-related peptide fragments present in TFV preparations.

2.3 ProTα: Major Structural Characteristics and Properties

Human proTα is 109 amino acids long and is encoded by the *PTMA* gene located on chromosome 2 (Szabo et al., 1993). Nearly half of the total residues in proTα are accounted by glutamic and aspartic acid and, as a result, the molecule acquires a particularly low p*I* 3.55. Although it was initially referred to as a "thymic hormone," proTα is not solely expressed in the thymus, but detected in all tissues (Haritos, Tsolas, & Horecker, 1984). It is a noticeably conserved polypeptide characterized by a high degree of sequence homology among mammals (Hannappel & Huff, 2003) and overexpressed in cells with increased physiological (eg, young thymus) or abnormal (eg, malignant) proliferative capacity (Haritos, 1987).

As proTα's acidic residues are found primarily within the central segment of the polypeptide chain, the molecule has no specific secondary structure

and, eventually, adopts a random coil conformation (Gast et al., 1995). However, the possibility that proTα acquires a secondary structure upon interaction with other proteins has not been ruled out (Piñeiro, Cordero, & Nogueira, 2000). In support of this, it has been demonstrated that under specific conditions (low pH and high concentration) the polypeptide forms amyloid fibrils (Pavlov, Cherny, Heim, Jovin, & Subramaniam, 2002). Although proTα is released by necrotic neurons via a unique nonclassical pathway (Halder, Matsunaga, & Ueda, 2012; Matsunaga & Ueda, 2010), the peptide lacks a signal peptide sequence required for secretion. Instead, it bears a bipartite nuclear localization signal which consists of two blocks of basic residues (^{87}KR^{89} and ^{100}TKKQKT^{105}), and is both necessary and sufficient for import of the protein to the nucleus, where it is predominantly located (Manrow, Sburlati, Hanover, & Berger, 1991; Rubtsov et al., 1997). In contradiction, many studies have also supported proTα's cytoplasmic localization (Sburlati, Manrow, & Berger, 1990; Tsitsiloni, Yialouris, Sekeri-Pataryas, & Haritos, 1989). In an effort to address this antiphasis, Enkemann and coworkers demonstrated that proTα is in principle detected at active transcription sites in the nucleus, while a smaller fraction remains in the cytoplasm (Enkemann, Wang, Trumbore, & Berger, 2000). It was additionally suggested that, owing to its small size and its negative charge, proTα may facilitate the movement of other positively charged molecules (eg, of histones) into and within the nucleus, particularly in highly charged environments where there is a need to overcome electrostatic interactions. In terms of proTα's biological role, a vast number of studies have attributed, among others, various and diverse immune-related properties to the polypeptide, the most important of which are analyzed later.



3. THE MULTIFACETED IMMUNE ACTIVITIES OF ProTα

3.1 The Thoroughly Studied Anticancer Activity of ProTα

The potent anticancer activity of proTα was studied in the early 1990s, when it was first reported that it regulates MHC class II expression on human and mouse antigen-presenting cells (APCs; Baxevanis et al., 1992). The significance of this observation was verified in three serial studies in a leukemic in vivo animal model, where mice inoculated with L1210 cells and therapeutically treated with proTα, survived for over 2 months (Papanastasiou, Baxevanis, & Papamichail, 1992). The immunological modifications caused by proTα included the in vivo generation of MHC-restricted tumor-specific CD8+ cytotoxic T lymphocytes (CTLs;

Baxevanis, Gritzapis, Spanakos, Tsitsilonis, & Papamichail, 1995), concomitantly with the enhancement of tumor-reactive NK cell-mediated cytotoxicity (Baxevanis et al., 1994). Both effectors efficiently lysed the syngeneic L1210 tumor cells. The immunoenhancing effect of proTα was exerted upstream lymphocyte activation via an interleukin (IL)-2-dependent manner. Most importantly, proTα shifted antitumor-reactive immune responses toward the stimulation of the most suitable effectors, CTLs or NK cells, depending on the presence or absence of tumor-specific antigenic peptides, respectively.

Animal studies were followed by in vitro human studies in lymphocytes from cancer patients. Cancer induces severe immune dysfunctions, which are further intensified by anticancer therapies administered to patients. ProTα was shown to restore the deficiencies of peripheral blood lymphocytes deriving from patients with advanced solid tumors by enhancing: (1) the allogeneic cell-mediated lympholysis; (2) antigen presentation, as confirmed by the increased values recorded in MLR; and (3) the reduced NK and T-cell cytotoxic activity, by regulating the levels of prostaglandin E2 (PGE2) and IL-2 (Baxevanis, Reclos, & Papamichail, 1993). Two consecutive studies in melanoma and colon cancer patients showed that proTα could act beneficially at early-stage cancers, and when combined with low-dose interferon (IFN)-γ or IL-2, significantly enhanced monocyte, NK and LAK cell tumoristatic and tumorilytic activities (Eckert et al., 1995; Garbin, Eckert, Büttner, Garbe, & Maurer, 1994). ProTα in conjunction with either IFN-γ or IL-2 increased the adhesion of monocytes to tumor targets and the expression of characteristic NK cell markers (eg, CD56, CD16), enhanced the lytic activity of LAK cells, in particular of CD16+CD2− cells, inhibited the secretion of TGF-β and PGE2, and induced the production and secretion of high levels of the proinflammatory cytokines IL-1β and tumor necrosis factor (TNF)-α (Cordero, Sarandeses, López-Rodríguez, & Nogueira, 1995; Eckert, Grünberg, Garbin, & Maurer, 1997; Eckert, Grünberg, Immenschuh, et al., 1997; Garbin, Eckert, Immenschuh, Kreuser, & Maurer, 1997; López-Rodríguez, Cordero, Sarandeses, Viñuela, & Nogueira, 1994). Based on the aforementioned data, the first means of action of proTα was shaped, indicating that the polypeptide restores cancer patient lymphocyte deficiencies by selectively controlling the functions of monocytes. Monocytes, in turn, produce cytokines that generate a favorable cytokine milieu, facilitating lymphocyte activation. To optimize this effect, synergy between proTα and low concentrations of other BRMs was assessed. For example, proTα was combined with a monoclonal antibody

to CD3 (anti-CD3), and this synergy further increased tumor cell-lysis by both MHC- and non-MHC-restricted PBMC effectors (Baxevanis et al., 1999).

All these results were complemented by the elegant study of Voutsas et al. (2000), where, using MLTCs, proTα in synergy with low-dose IL-2 led to expansion of tumor-reactive CD4+ T cells and the subsequent generation of MHC class I-restricted autologous tumor-specific CTLs. This was the first study showing that in order for proTα to fully exert its beneficial effect on CTLs, the concomitant presence of autologous CD4+ T cells and monocytes was required.

Combining all previous data, in an attempt to fill in the gaps, our research team used proteomics to elucidate the mechanism of action of proTα on healthy donor- and cancer patient-derived PBMCs. Based on proteins up- and downregulated upon PBMC treatment with proTα, we were the first to analytically describe the scenario underlying its immunoenhancing activity. We suggested that proTα ligates innate immunity receptor(s) on APCs, leading to formation of strong APC-T-cell synapses, proinflammatory cytokine secretion and thus, the indirect increase in the cytotoxicity of CTLs, NK cells, and other effectors (Skopeliti et al., 2009). We and others further confirmed the realism of this scenario, showing that proTα ligates Toll-like receptor 4 (TLR4) (Mosoian et al., 2010; Omotuyi, Matsunaga, & Ueda, 2015) and matures monocyte-derived dendritic cells (DCs), which express costimulatory molecules (Skopeliti et al., 2009) and secrete proinflammatory cytokines. Moreover, proTα-matured DCs loaded with tumor antigens induced the polarization of T_H1-type tumor-reactive immune responses, resulting in the generation of polyfunctional highly lytic tumor antigen-specific CTLs (Ioannou et al., 2013). Besides DCs, proTα could also activate other immune cell types expressing TLR4, eg, neutrophils to secrete $\cdot O_2^-$ and kill tumor cells in vitro (Samara, Ioannou, et al., 2013), and macrophages (Mosoian et al., 2010). Ex vivo experiments showed that proTα significantly restored the reduced cytotoxicity of immunosuppressed ascites-derived tumor-associated lymphocytes against ovarian tumor cells and inhibited ovarian tumor growth in SCID mice inoculated with human tumors (Voutsas et al., 2013).

3.2 Antiviral Activities of ProTα

Control of viral replication in humans requires the involvement of robust CTL responses, which in turn demand the presence of type I IFNs, that

further support CD8+ T-cell development and function (Welsh, Bahl, Marshall, & Urban, 2012). Interestingly, proTα has been shown to be a regulator and enhancer of type I IFN secretion and as such, its antiviral properties were investigated. Qiu and colleagues provided the first evidence of proTα's capacity to enhance IFN-α secretion by murine macrophages (Qiu et al., 2002). Later, proteomic analysis of human mononuclear cells treated with proTα revealed that the polypeptide enhanced the expression of mixovirus-resistance protein 2, an IFN-α-inducible protein that possesses significant antiviral activity (Skopeliti et al., 2007). Moreover, proTα was identified as a potent suppressor of human immunodeficiency virus (HIV)-1 replication in primary macrophages, when released by virus-infected CD8+ T cells. It was also shown that this HIV-1 inhibition occurs following viral integration, it is not virus specific (Mosoian et al., 2006), and is rather mediated by the upregulation of type I IFNs, following activation of TLR4 by proTα (Mosoian et al., 2010; Teixeira et al., 2015). Similarly, release of proTα upon infection of guinea pigs with an attenuated strain of *Pichinde* virus led to induction of potent antiviral immunity and consequent viral clearance (Bowick et al., 2010). In terms of in vivo applications, proTα has been used as a potent adjuvant for hepatitis B virus DNA vaccines, where it successfully enhanced both humoral and cellular immune responses (Jin et al., 2005). Impressively, a most recent report showed that proTα displays antiviral activity in other classes than mammals, specifically in fish (tongue sole) infected with megalocytivirus (Zhang & Sun, 2015).

3.3 ProTα Exerts Neuroprotective Functions

Neuronal death, induced by stroke or trauma, occurs via both necrosis and apoptosis. While apoptosis is a more regulated process that limits the damage in the brain, necrosis tends to expand and is therefore being targeted for stroke treatment (Ueda, 2009). Based on this premise, in an effort to identify soluble molecules that could inhibit necrosis, Ueda and colleagues isolated and identified proTα as a unique antineuronal necrosis factor in the conditioned medium of cortical neurons (Ueda, Fujita, Yoshida, Matsunaga, & Ueda, 2007). In more detail, proTα could reverse the rapid decrease in survival of cortical neurons, abolish the typical necrosis features in these cells, and switch the cell death mode from necrosis to apoptosis (Ueda et al., 2007). Later on, proTα's potent neuroprotective functions were also demonstrated in in vivo experimentally induced cerebral and retinal ischemia (Fujita & Ueda, 2007; Fujita, Ueda, Fujiwara, & Ueda, 2009). As for the mechanism underlying proTα's neuroprotective role, it has been shown that

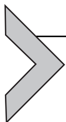
the polypeptide is released via a nonclassical manner under ischemic stress conditions and its extracellular release is facilitated by its interaction with a cargo molecule, namely S100A13 (Halder et al., 2012; Matsunaga & Ueda, 2010). Upon release, proTα activates the TLR4–TRIF-signaling pathway, inducing the expression of neuroprotective factors (Halder, Matsunaga, Ishii, & Ueda, 2015).

3.4 Miscellaneous Functions Reported for ProTα

Given its neuroprotective properties, the effect of proTα on cardiomyocytes during ischemic injury was also investigated (Cannavo et al., 2013). In vitro treatment of cardiomyocytes with recombinant proTα during simulated ischemia significantly decreased the apoptotic response and enhanced cell survival. Consistent with the in vitro findings, in vivo administration of proTα following myocardial infarction successfully reduced the infarct size in mice, when compared to untreated controls, an effect that was mediated by an Akt-dependent mechanism.

Li and colleagues had initially reported that proTα transgenic mice exhibited the polycystic kidney disease phenotype, as well as emphysema-like changes in the lung (Li, Shiau, Chiou, Yo, & Wu, 2005). Further studies by the same research group showed that proTα enhanced the acetylation of histones and nuclear factor-kappa B (NF-κB), or inhibited the TGF-β/Smad signaling, and thus contributed to the development of emphysema (Su et al., 2013; Su et al., 2016). Furthermore, high levels of proTα were positively correlated with the severity of emphysema, both in transgenic mice and in emphysema patients (Su et al., 2013).

Finally, the polypeptide's involvement in the induction of insulin resistance was investigated, as proTα reportedly regulated some inflammatory responses and oxidative stress, features that are associated with diabetes (Su et al., 2015). Patients with type 2 diabetes had significantly higher levels of serum proTα compared to normal individuals and proTα transgenic mice exhibited an insulin-resistant phenotype. Exploitation of the underlying mechanism revealed that proTα induces insulin resistance through a TLR4–NF-κB-dependent pathway.



4. THE IMMUNOSTIMULATORY ACTIVITY OF ProTα-DERIVED PEPTIDES

Since $T\alpha_1$ was the first thymosin isolated and identified from TFV and proTα was the natural precursor of $T\alpha_1$ and other smaller α-thymosins

(Haritos, Goodall, et al., 1984), $T\alpha_1$ was initially considered as the major immunoactive fragment of the polypeptide. However, data accrued in particular over the last years revealed that proTα exerts its immunomodulating role through diverse fragments, such as the C-terminal fragment spanning residues 100–109 (Skopeliti et al., 2009) and the central, negatively charged region 50–89 (Mosoian et al., 2010). Two points that we consider very important are: (1) when compared to its fragments, intact proTα seems to perform better at least in most immune-based assays; and (2) different areas of the molecule seem to be responsible for proTα's diverse activities (Table 1).

Table 1 Prothymosin Alpha Fragments with Distinct Activities

ProTα Fragment	Type of Activity	Reported in:
ProTα(1–28), referred to as "$T\alpha_1$"	Immunomodulatory; DC activation; anticancer; antiviral; antifungal; vaccine enhancement	Camerini and Garaci (2015)
ProTα(1–35), referred to as "$T\alpha_{11}$"	Antifungal	Hannappel and Huff (2003)
ProTα(49–78) (P30) ProTα(52–60) (P9)	Neuroprotective	Halder, Sugimoto, Matsunaga, and Ueda (2013)
ProTα(50–89), referred to as "Mosoian domain"	Anti-HIV-1	Mosoian et al. (2010)
ProTα variants (p7 [proTα(32–49)] and isoB [proTα(32–41), proTα(51–55), proTα (56–61), proTα(65–71)])	Anti-HIV-1	Teixeira et al. (2015)
ProTα(100–109), referred to as "Skopelitian domain"	Immunomodulatory; anticancer; DC maturation; enhancement of phagocytosis, respiratory burst, and cytotoxicity of human neutrophils	Skopeliti et al. (2009), Voutsas et al. (2013), Ioannou et al. (2013), and Samara, Ioannou, et al. (2013)
ProTα(1–100)	Cardioprotective	Cannavo et al. (2013)

4.1 The Amino-Terminal Peptide Tα_1 Has Shown Some Immune Activity

The 28-amino acid-long N-terminal proTα fragment Tα_1 was reported to enhance human cell-mediated immunity and stimulate endothelial cell migration, angiogenesis, and wound healing (Malinda et al., 1998). Being the only α-thymosin tested in man, Tα_1 has been used in a broad range of clinical applications, both as a single agent and in combination with other standard treatments (eg, chemotherapeutics), showing an excellent safety profile (Romani et al., 2012). Among other clinical cases, Tα_1 has been used for treating infectious diseases including viral (chronic hepatitis B and C, AIDS), fungal (aspergillosis in bone marrow-transplanted patients) and bacterial (*Pseudomonas aeruginosa*) sepsis, severe lung pathologies (eg, chronic obstructive pulmonary disease, acute respiratory distress syndrome, and severe acute respiratory syndrome), age-related deficiencies, cancer, and as a vaccine enhancer (Camerini & Garaci, 2015). However, despite the promising data generated so far, the clinical benefit of Tα_1 administration in these specific pathologies is still disputed and needs to be verified in larger well-designed randomized clinical trials.

4.2 The Carboxy-Terminal ProTα Peptides Exhibit Improved Immune Functions

The C-terminal decapeptide proTα(100–109) (TKKQKTDEDD) was identified by our research team as the immunoactive area of the polypeptide and a potent lymphocyte stimulator (Skopeliti et al., 2006). ProTα(100–109) has been shown to stimulate PBMC proliferation and cytotoxicity and to promote the phenotypic maturation of DCs (Skopeliti et al., 2009), and, consequently, it improved the functionality of immunogenic peptide-pulsed DCs, induced T_H1-type immune response polarization (Ioannou et al., 2013), augmented basic properties of human neutrophils (Samara, Ioannou, et al., 2013), enhanced the depressed cytotoxicity of tumor-associated lymphocytes against autologous tumor cells in vitro, and retarded tumor growth in vivo (Voutsas et al., 2013). Using as control a scrambled decapeptide with the same amino acid composition but a different primary structure, the immunoenhancing activity of proTα(100–109) was shown to be sequence-specific and comparable to that of intact proTα. Most recently, we reported that proTα(100–109) radiolabeled with (99m)Tc binds on the surface of human neutrophils via a complex

involving TLR4 (Karachaliou et al., 2015) and selectively accumulates in sites of experimentally induced inflammation (C.E. Karachaliou et al., personal communication).

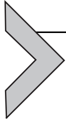
Based on the fact that the decapeptide proTα(100–109) is in vivo generated upon caspase cleavage of proTα during apoptosis (Enkemann et al., 2000; Evstafieva et al., 2003), we developed a highly sensitive and specific competitive ELISA for proTα(100–109), using high affinity-purified polyclonal antibodies (Samara, Kalbacher, et al., 2013). The decapeptide was quantified in the serum of healthy humans, where it was detected at very low concentrations (≤0.5 ng/mL; P. Samara et al., unpublished data), whereas higher levels were recorded in the serum of mice infected with the bacterium *Streptococcus pyogenes*, suggesting a correlation between proTα(100–109) levels and the progress of bacterial infection.

Two more C-terminal immunomodulatory peptide fragments of proTα have been reported. In an earlier study, our research group identified a slightly smaller segment, namely proTα(103–109), which was also effective in vitro in restoring the immune functions of PBMCs derived from cancer patients (Skopeliti et al., 2006). Most recently, gravimetric assays and molecular dynamics simulation revealed that proTα, via its C-terminal segment (91–111) and similar to LPS, biophysically interacts in vitro with the TLR4/MD-2 complex at overlapping LPS-binding positions (Omotuyi et al., 2015).

4.3 The Immune Activity of ProTα Middle Segment Peptides

The research group of Mosoian introduced the synthetic peptide spanning amino acids 50–89 of proTα, designated proTα(50–89), in an attempt to comprehend how proTα mediates its anti-HIV-1 activity and induces type I IFN production. Incubation of human macrophages and myeloid DCs with proTα or proTα(50–89) stimulated IFN-α1 and IFN-β production, as shown by the increase in type I IFN mRNA (Mosoian et al., 2010). Furthermore, the same research team recently reported that proTα variants (p7 and isoB), spanning middle segment sequences of the polypeptide (amino acids 32–41, 32–49, 51–55, 56–61, and 65–71), also induced mRNA expression of type I and type III IFNs in human macrophages, suggesting that these peptides possess strong antiviral activities, responsible for the registered suppression of HIV-1 replication in this cell type (Teixeira et al., 2015).

In addition, Ueda and colleagues reported that a 30-amino acid middle peptide sequence of proTα [termed P30; proTα(49–78)] exerts substantial neuroprotective activity in vitro and in vivo and inhibits cerebral blood vessel damage caused by ischemic stress in retina and brain. The minimum neuroprotective sequence encompasses the 9-amino acid peptide 52–60 (P9), which was shown to comprise the full neuroprotective effect of proTα in cultured cortical neurons under ischemic conditions (Halder et al., 2013).



5. EVIDENCE SUPPORTING A DUAL—INTRACELLULAR AND EXTRACELLULAR—ROLE FOR ProTα

It has long been disputed whether a strictly intracellular, in principle nuclear molecule like proTα, could modulate immune responses and several researchers initially rejected the idea that the polypeptide could exert an extracellular role. The wide distribution of proTα in tissues and cells strongly supported the sole implication of the polypeptide in crucial intracellular processes.

5.1 The Intracellular Role of ProTα

Indeed, in normal cells, proTα was shown to be important for survival and proliferation. High levels of proTα mRNA were detected in lymphocytes (Eschenfeldt & Berger, 1986; Szabo, Ehleiter, Whittington, & Weksler, 1992) and in NIH3T3 cells stimulated to divide (Wu, Shiau, & Lin, 1997), whereas proliferation of myeloma cells was hindered in the presence of proTα antisense oligomers (Sburlati, Manrow, & Berger, 1991). Over the years, research on the intracellular activity of proTα revealed its implication in: (1) *gene transcription*; proTα was reported to localize in nuclear sites of active transcription, physically interact with the CREB-binding protein (CBP), bind to histone H1, and stimulate transcription (Karetsou, Kretsovali, Murphy, Tsolas, & Papamarcaki, 2002); (2) *DNA remodeling*; during proliferation proTα increased the accessibility of micrococcal nuclease to chromatin (Gomez-Marquez & Rodriguez, 1998); (3) *inhibition of apoptosis*; proTα was shown to bind Apaf-1 and inhibit apoptosome formation (Jiang et al., 2003); and (4) *protection against oxidative stress*; proTα interacted with INrf2 leading to its nuclear translocation, where it mediated the degradation of Nrf2 and promoted cell survival and growth (Niture, Kaspar, Shen, & Jaiswal, 2010) (Fig. 1).

In cancer cells, which are highly proliferative and divide constantly, increased proTα content was also reported. High proTα levels were

detected in human colon (Mori et al., 1993), colorectal (Zhang et al., 2014), lung (Sasaki, Nonaka, et al., 2001), bladder (Tsai et al., 2014), and liver cancers (Wu, Habib, et al., 1997); and in neuroblastoma (Sasaki, Sato, et al., 2001), rhabdomyosarcoma (Carey et al., 2006), thyroid carcinomas (Kashat et al., 2010; Letsas, Vartholomatos, Tsepi, Tsatsoulis, & Frangou-Lazaridis, 2007), pituitary tumors (Pawlikowski et al., 2014), head and neck cancers (Tripathi et al., 2011), gastric adenocarcinomas (Leys et al., 2007), hepatocellular (Ha, Song, Hwang, Cho, & Park, 2015), and urinary tract transitional cell carcinomas (Jou et al., 2009). We and others reported that proTα was elevated in breast tumors (Tsitsiloni et al., 1993) and tumor proTα levels correlated with the disease outcome (Dominguez et al.,

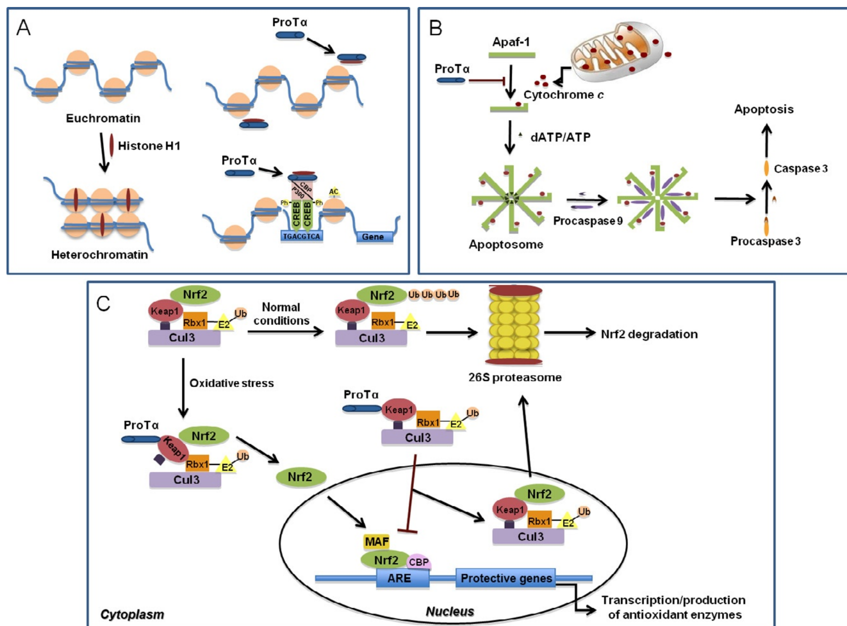


Fig. 1 The intracellular role of proTα. (A) In the absence of proTα, histone H1 binds to nucleosomes and induces condensation of euchromatin to heterochromatin. ProTα interacts with histone H1, mediates its transfer from and to chromatin, and leads to the formation of CREB–CBP–p300 complex, chromatin remodeling, and gene transcription. (B) Apoptotic stimuli induce the release of cytochrome *c*, which binds to Apaf-1 and forms the apoptosome. The subsequent activation of caspase 9 results in conversion of procaspase 3 to caspase 3, leading to apoptosis. ProTα hinders binding of cytochrome *c* to Apaf-1, and the apoptotic cascade is inhibited. (C) Normally, the Nrf2–Keap1–Cul3–Rbx1 complex is ubiquitinated and degraded by proteasomes. Under oxidative stress, proTα binds to the complex via Keap1, and Nrf2 is released, migrates to the nucleus, and promotes gene transcription and antioxidant enzyme production.

1993), the metastatic potential of the tumor (Tsitsilonis et al., 1998), and the risk of recurrence and death (Magdalena, Dominguez, Loidi, & Puente, 2000). It is worth mentioning that high proTα levels detected in prostate cancer samples (Klimentzou et al., 2008) were shown to progressively increase as prostate tissue progressed from normal epithelium, through prostatic intraepithelial neoplasia to carcinoma, and correlated with Gleason's score (Suzuki et al., 2006). Therefore, following the appropriate validation, proTα tumor content and/or its serum levels may be used as a surrogate tumor biomarker for cancer development, prognosis, and follow-up.

5.2 The Extracellular Role of ProTα

Nevertheless, evidence supporting the extracellular activity of proTα was also reported by in vivo studies in animals, which showed that proTα can protect mice against opportunistic infections, in particular with *Candida albicans* (Pan et al., 1986). Numerous in vitro studies further confirmed the extracellular role of the polypeptide. As already aforementioned, peripheral blood T cells stimulated with proTα produced high amounts of IL-2 and increased the expression of IL-2 receptor on their surface (Baxevanis et al., 1990; Cordero et al., 1991), while APCs and DCs activated with proTα upregulated MHC class II (Baxevanis et al., 1992) and costimulatory molecules (CD11b, CD80, CD83, CD86, and CD40; Skopeliti et al., 2009), respectively. NK and LAK cells cultured in the presence of proTα augmented their cytotoxicity (Cordero, Sarandeses, López, & Nogueira, 1992; López-Rodríguez et al., 1994), and proTα-activated neutrophils showed increased chemotaxis, produced high amounts of ROS, and became cytotoxic against cancer cell targets (Heidecke, Eckert, Schulze-Forster, & Maurer, 1997; Samara, Ioannou, et al., 2013). All these multiple immunological responses suggested that proTα acts pleiotropically, especially in immunosuppressed environments.

At that time, it was obvious that the cytokine-like activities of proTα would be justified if specific-binding sites (receptors?) on immune cell surface were discovered. In an initial attempt, Cordero and colleagues radiolabeled proTα and searched for binding sites on the surface of lymphoblasts. Two binding sites, of low and high affinity, were identified (Cordero, Sarandeses, & Nogueira, 1996). Five years later, the same researchers demonstrated, by affinity cross-linking and chromatography, the existence of three binding partners for proTα on the membrane of PHA-activated lymphoblasts, which were associated with lipid rafts (Piñeiro, Begoña

Bugia, Pilar Arias, Cordero, & Nogueira, 2001; Salgado, Piñeiro, Canda-Sánchez, Lojo, & Nogueira, 2005). However, a specific proTα receptor was never cloned. In 2007, based on the detailed proteomic analysis of protein changes induced by proTα on immune cells and on the upregulation of the signaling protein IRAK4, we proposed that proTα ligates TLRs (Skopeliti et al., 2007). In 2010, Mosoian and colleagues confirmed that proTα ligates TLR4 and signals through both the TRIF-dependent and the MyD88 pathways for induction of IFN-β and proinflammatory cytokines, respectively (Mosoian et al., 2010). Most recently, proTα was shown to bind to the TLR4/MD-2 complex and activate the TRIF–IRF3-signaling pathway downstream TLR4 (Halder et al., 2015; Omotuyi et al., 2015).

5.3 Evidence Suggesting That ProTα May Act as an Alarmin

Although more data need to be generated to specifically define the pathways activated in response to proTα, it is more than tempting to speculate that the polypeptide probably belongs to the vague family of molecules termed damage-associated molecular patterns (DAMPs) or else "alarmins" (Bianchi, 2007), and as such, can simultaneously and distinctively exert both an intracellular and an extracellular role.

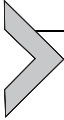
With the term "alarmins" we practically refer to endogenous pathogen-associated molecular patterns (PAMPs), or in more detail, to multifunctional endogenous constitutively available molecules, passively released from damaged cells or rapidly secreted by stimulated leukocytes and epithelial cells following tissue destruction. Alarmins activate both innate and adaptive immune responses. Uncontrolled and excessive release of alarmins extracellularly induces concomitantly the recruitment and activation of APCs through pattern recognition receptors, such as TLRs. In the absence of such stimuli, alarmins exert their vital intracellular roles.

The alarmin family is rapidly growing and, as for now, its best-characterized members are high-mobility group protein B1 (HMGB1) and some heat-shock proteins (Bianchi, 2007). Thymosins were suggested as candidate alarmins, although, to our knowledge, direct comparison of their characteristics with known alarmins has not been performed. Herein, we compile evidence that proTα possesses several attributes to be considered an alarmin (Table 2). Among others and in comparison to HMGB1, proTα is a ubiquitously expressed, nonhistone nuclear protein with a marked intracellular physiological role in regulating transcription. It is released via a nonclassical pathway upon ischemic stress, and when

localized extracellularly, proTα can recruit and activate innate immune cells, promoting inflammation and cytokine secretion, similar to HMGB1. In addition, proTα signals through TLR4, activates leukocytes and orchestrates immune responses (Ioannou et al., 2012). Finally, it is implicated in neuro- and cardio-tissue repair, suggesting the regenerative potential of the polypeptide (Cannavo et al., 2013; Halder et al., 2012).

Table 2 Characteristics Ascribed to Alarmins (HMGB1) and Comparison with Reported Properties of Prothymosin Alpha

Characteristic	**HMGB1 (Chan et al., 2012; Schiller et al., 2013; Telusma et al., 2006)**	**ProTα (Cannavo et al., 2013; Halder et al., 2013; Ioannou, Samara, Livaniou, Derhovanessian, & Tsitsilonis, 2012)**
Origin	Nonhistone nuclear protein	Nonhistone nuclear protein
Expression	Expressed in all cells	Expressed in all cells
Physiological intracellular role	DNA organization, transcriptional regulator	DNA organization, transcriptional regulator, antiapoptotic and oxidative stress regulator
Extracellular role	Cytokine/inflammatory mediator	Cytokine/inflammatory mediator
Release mechanism	Passive release and active secretion	Release upon ischemic stress
Receptors	TLR2, TLR4, TLR9, TIM3, and RAGE	TLR4
Regenerative potential	Cardiac and nervous cell regeneration, skin wound healing, bone repair, skeletal muscle repair	Cardiac and nervous cell regeneration
Implication in diseases	Cancer, rheumatoid arthritis stroke, atherosclerosis, sepsis	Cancer, autoimmune diseases, ischemic stroke, viral infections
Additional similarities		
Immunoactive peptides	Hp-106, Hp-31, Hp-91, and Hp-16	ProTα(100–109), proTα (50–89), $T\alpha_1$
Intracellular mobility	Translocation from nucleus to cytoplasm; during apoptosis, translocation into apoptotic cell-derived membranous vesicles	Translocation from nucleus to cytoplasm; during apoptosis, NLS cleavage by caspases and generation of proTα(100–109)



6. PROPOSED SCENARIO FOR THE MECHANISM OF ACTION OF ProTα

Taking all the above into consideration, we propose a scenario depicting the mode of action of proTα. In *normal healthy cells*, proTα is localized in the nucleus (Eschenfeldt & Berger, 1986), albeit a fraction of it is assigned to remain in the cytoplasm (Tsitsiloni et al., 1989). In the nucleus, proTα regulates gene transcription (Karetsou et al., 2002), shapes DNA remodeling, and promotes cell proliferation (Gomez-Marquez & Rodriguez, 1998). In the cytoplasm, proTα protects cells from apoptosis (Jiang et al., 2003) and, due to its negative charge, facilitates the transportation of molecules in the nucleus, protecting the cell from insults, eg, oxidative stress (Niture et al., 2010). In hyperproliferative cells, like *cancer cells*, proTα's gene expression and protein content are increased and this contributes to uncontrolled proliferation and induction of resistance to apoptosis and oxidative stress. Cells receiving death stimuli die violently by necrosis or undergo programmed apoptotic death. *During necrosis*, cell swelling and membrane disruption lead to abrupt uncontrolled release of internal cell components, including proTα, which acts as alarmin, alerts cells of the innate arm of immunity expressing TLR4, and initiates immune responses (Ioannou et al., 2012). *During apoptosis*, the major proportion of proTα is transferred to the cytoplasm, where activated caspases cleave the molecule at its C-terminus, generating among other fragments, the decapeptide proTα(100–109). The negatively charged fragment proTα(1–99) remains in the cytoplasm, where most probably is degraded or, as suggested, is exposed on apoptotic cell surface where it acts as an "eat-me" signal (Evstafieva et al., 2003). The decapeptide proTα(100–109), or a proportion of it, is protected from degradation as it polymerizes adopting a β-sheet conformation (Skopeliti et al., 2009) and, consequently, is excreted from dying cells via an unknown mechanism. In both necrosis and apoptosis, exocytosed proTα and proTα(100–109) are sensed as DAMPs by innate immune cells and ligate TLR4 (Ioannou et al., 2013; Mosoian et al., 2010). Stimulated innate immune cells, such as macrophages, neutrophils, monocytes, and DCs, respond by initiating molecular signaling pathways leading to NF-κB activation, chemokine, IFN, and proinflammatory cytokine secretion. Additionally, stimulated macrophages and neutrophils increase their phagocytosis and produce TNF-α and $\cdot O_2^-$, respectively (Mosoian et al., 2010; Samara, Ioannou, et al., 2013). Stimulation of APCs (DCs and monocytes) increases antigen (eg, shed from dying cancer cells)

uptake and presentation through MHC class I molecules directly to CD8+ cytotoxic T cells. Upregulation of MHC class II expression by the thymic peptides on monocytes and DCs increases antigen presentation and their synapse with CD4+ helper T cells, which produce T_H1-type cytokines (eg, IL-2, IFN-γ), providing a favorable environment for enhancing specific

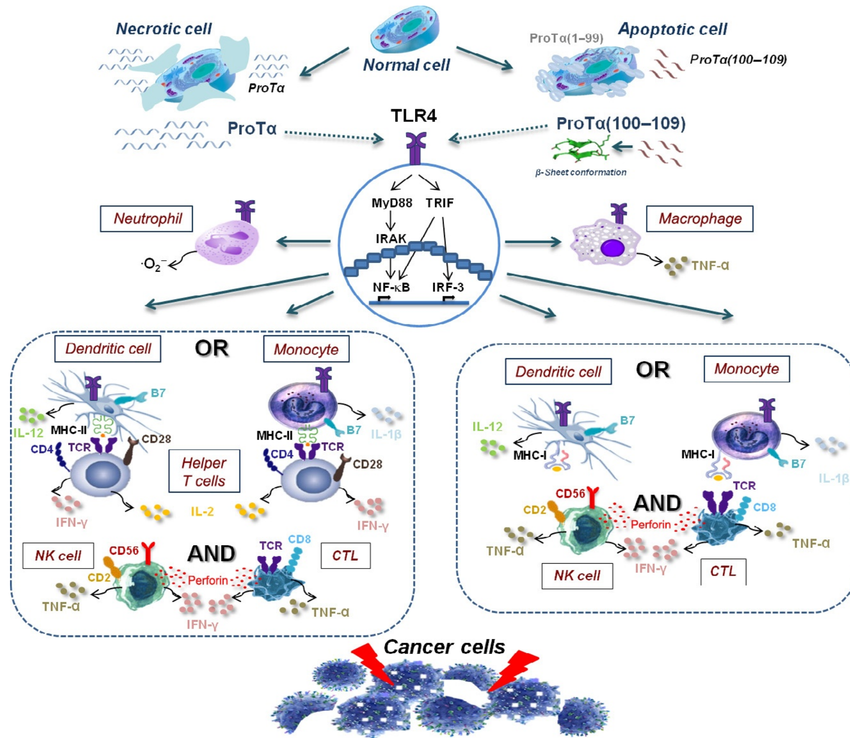
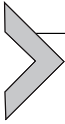


Fig. 2 Proposed scenario on proTα's dual role. In normal cells, proTα regulates gene expression and cell proliferation in the nucleus. Under abnormal conditions, cells die via necrosis or apoptosis. During necrosis, cell membrane is ruptured and intact proTα is released out of the cell. During apoptosis, proTα is relocated to the cytoplasm and is cleaved at its C-terminus by activated caspases, and proTα(100–109) is generated. The decapeptide adopts a β-sheet conformation and is excreted. Extracellularly, both proTα and proTα(100–109) activate innate immune cells expressing TLR4 (macrophages, neutrophils, DCs, and monocytes) and signal through the MyD88 and TRIF pathways. Cytotoxic responses are enhanced through antigen presentation with MHC class II molecules and synapsis with helper T cells which secrete T_H1-type cytokines, and/or with MHC class I molecules, leading to activation of cytotoxic effectors (NK cells and CTLs). In both cases, effector cells upregulate adhesion molecule expression (eg, CD2) and produce lytic molecules (eg, perforin), mediating cell binding and cell destruction, respectively (eg, cancer cell targets, as shown here).

and nonspecific cytotoxicity. CTLs and NK cells produce lytic molecules (perforin), express adhesion molecules (CD2), secrete TNF-α and IFN-γ, and kill cell targets (eg, cancer cells) (Fig. 2).



7. CONCLUSIONS AND FUTURE DIRECTIONS

ProTα, although initially characterized as a thymic hormone, is a ubiquitously expressed polypeptide that exerts pleiotropic immunostimulatory adjuvant effects. Nevertheless, and despite extensive and intensive research on its mode of action, the efficacy of proTα has not yet been tested in humans. In our opinion, the main reason stands in our lack of understanding of its dual role and in elucidating how the same molecule promotes cell proliferation and mediates immunity. This paradox is most surprising and contradictory in the case of cancer. We know that increased proTα gene transcription correlates with carcinogenesis, whereas proTα ligation to TLR4 promotes anticancer-reactive immune responses. In view of recent progress in elucidating the complex machinery of immune responses and the concept that the immune system responds to both pathogens (PAMPs) and danger signals (DAMPs), the adjuvant mode of action of proTα points toward its integration in the family of alarmins. As such, under specific conditions related to danger (resulting from cell damage, destruction, or death), proTα and/or smaller fragments of the molecule [eg, proTα(100–109)] are relocalized extracellularly, ie, at a different site than inside the cell, where they physiologically function. Innate immune cells like DCs that are programmed to sense danger respond to proTα, and the cascade of an immune response is initiated.

Recent evidence additionally suggests that proTα is a TLR agonist. At present, driving closer to optimal orchestration of the immune machinery, several TLR agonists have reached the clinical setting. We believe that proTα has adequately proven its potent immunostimulating capacity in vitro and in animals in vivo, by generating the appropriate cytokine milieu promoting the activation of effector cells. Although it should be studied in more detail, it is also known that the polypeptide when administered in animals at relatively high concentrations does not induce toxicity or severe adverse effects. Therefore, the next step should be to test the adjuvant effectiveness of proTα or smaller immunoactive fragments thereof in clinical studies, aiming to strengthen deficient immune responses.

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