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Modulatory role of thymosin-alpha-1 in normal bone-marrow haematopoiesis and its effect on myelosuppression in T-cell lymphoma bearing mice

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Abstract

In continuation with the earlier and ongoing studies on Thymosin-alpha-1 (Tα1) exerting its immunomodulatory effects on various components of the immune system including T-cells, NK-cells, blood lymphocytes and macrophages, the role of Tα1 in normal bone-marrow haematopoiesis has been investigated in the present study. The haematopoietic alterations associated with the growth of murine T-cell lymphoma, Dalton's Lymphoma (DL) and subsequently its restoration by Tα1 was also investigated. It is observed that the non-adherent bone-marrow cells from normal mice (N-BMCs) exhibited enhanced proliferation on in vitro treatment with Tα1 (dose range of 1–100 ng/ml) with maximal response at 100 ng/ml of Tα1. In vitro stimulation with 100 ng/ml of Tα1 also resulted in increased myeloid colony formation, as manifested by the rise in total number of colonies, frequency of the individual colony types and their size. This response was further upregulated in the presence of various colony stimulating factors (CSFs) like MCSF, GMCSF, GCSF and IL-3. Similarly, in vivo administration of Tα1 (a single intraperitoneal injection of 10 μg per mouse) to normal mice also resulted in enhanced proliferation and colony formation by BMCs as compared with BMCs obtained from untreated mice. On the contrary, the progressive growth of T-cell lymphoma in mice led to suppressed myelogenesis, with marked reduction in the total colony numbers and their size. The BMCs from DL-bearing mice (DL-BMCs) displayed a preferential lineage-restricted differentiation towards the granulocytic-type colonies with maximum numbers of CFU-Gs and CFU-GMs, followed by CFU-Ms. However, incubation of DL-BMCs with 100 ng/ml of Tα1, in vitro restored their suppressed proliferation and colony forming ability (CFA) with significantly enhanced total number of colonies and individual colony types, which further increased in the presence of CSFs. In vivo studies with BMCs from DL-bearing mice treated with single intraperitoneal injection of 10 μg Tα1/mouse also resulted in significant enhancement in their proliferative as well as colony forming ability in comparison to that of untreated DL-mice. The present observations suggest that Tα1 can positively modulate the haematopoietic functions of normal murine BMCs, in addition to its myelorestorative role in tumour-bearing mice showing suppressed myelopoiesis.

Keywords: Thymosin-alpha-1; T-cell lymphoma; Bone marrow haematopoiesis cell proliferation; Colony forming ability

1. Introduction

The haematopoietic system is a complex regenerating tissue that supplies mature haematopoietic cells throughout life. Despite the radically different phenotypes of the mature haematopoietic cells, they all are ultimately derived from the pluripotent haematopoietic progenitor cells (HPCs) present in the bone-marrow, the principal site for adult haematopoiesis [1]. It is believed that there exists a complex hierarchy of self-renewing and multipotential HPCs in the bone-marrow, each of which can further be mobilised from their quiescent state to proliferative as well as lineage-restricted differentiated state in response to proper stimuli [1–3]. The proliferation and differentiation of myeloid progenitors in the bone-marrow is an outcome of complex interaction between the progenitor cells themselves, the accessory cells and the cell-derived haematopoietic growth factors, called the colony stimulating factors (CSFs) [4,5].

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Inspite of the increasing knowledge on the role of myelopoiesis, many of the events controlling the self-maintenance of precursor stem cells and their lineage-restricted differentiation into mature cells are not well understood. The analysis of these regulatory interactions have been hampered due to the complexity of the haematopoietic tissues, the low frequency of relevant cells and the heterogeneity of the regulatory factors [6].

As opposed to the well-regulated bone-marrow haematopoiesis in normal hosts, significant modulation in the proliferative and differentiating ability of the HPCs have been reported in case of a variety of pathophysiological states including neoplasia [7,8]. Marked alterations have been observed in the lympho-haematopoietic system with the onset and subsequent growth of spontaneous tumours [8–10]. Depending on the cellular origin of tumour, reports of both upregulation as well as downregulation of haematopoiesis with concomitant reduction in the number of lymphoid, erythroid and myeloid elements in the bone-marrow of tumour-bearers are available [8–11]. Although the mechanism(s) associated with these abnormalities remain to be elucidated, it is clear that discrepancies occur due to the functional disorders/disabilities of the stromal cells, their accessory cells and the effect of immunosuppressive cytokines produced by them on the stem/progenitor cells [12]. Considering that bone-marrow is not only the principal site for adult haematopoiesis but also the most susceptible target for tumour-mediated immunosuppression, complete understanding of the physiological basis for such abnormalities on BMC proliferation/differentiation is a necessity and the search for novel strategies to counteract these effects happens to be a major focus area in cancer therapeutics.

The differentiation and maturation of bone-marrow haematopoietic precursor cells into mature T-lymphocytes occurs in the thymus. Several lines of evidences have shown that thymic humoral factors, as secreted by thymic epithelial cells are crucial to the process of T-cell formation and their maturation [13]. In the past decade, a variety of short peptides of thymic origin have been studied for their immunomodulatory effects and used in preclinical/clinical research for treating inflammatory/viral infections, allergies, autoimmune diseases, AIDS and neoplasia [14,15]. The most promising of all the thymic peptides is Thymosin-alpha-1 (Tα1) which is a 28 amino acid, acidic polypeptide, isolated from thymic humoral factor 5 (TF5), having acetylated amino-terminus [15,16]. Tα1 has also been postulated to be a proteolytic cleavage product of a larger bioactive molecule, Prothymosin-alpha-1 (ProTα1) [17,18]. Accumulating evidences have indicated an extracellular role of immunological nature and an intracellular role related to cellular growth for Tα1 [19]. Tα1 has not only been observed to increase the expression of terminal deoxynucleotide transferase (TdT), Thy 1.2 and Lyt 1,2,3$^+$ cells in mice [20–22] but also plays an essential role in the enhancement and maturation of CD3$^+$CD4$^+$ cells in the thymus and cell division [23–25]. Reports of thymocyte maturation by Tα1, utilising isolated bone-marrow derived CD34$^+$ stem cells further reiterate its active participation in the process of thymopoiesis [23]. In view of the evidences from in vitro and in vivo studies demonstrating Tα1 to be a potential BRM [14,15,27–32] in treating viral infection, cancer, age-related immunosenescence, as adjuvants in vaccination [33–38] and considering the lack of toxicity of this peptide in comparison to other BRMs due to its presence in circulation at basal levels, Tα1 presents an attractive target for therapeutic research.

Inspite of these reports, there exists only scanty information regarding the effect of Tα1 on bone-marrow haematopoiesis. Further, there is no report if Tα1 could positively modulate the altered/suppressed haematopoiesis of tumour-bearing hosts since tumour load could possibly be one of the major causes for the observed immunosuppression and grossly impaired haematopoiesis. In continuation with our earlier studies on the immunostimulatory role/antitumour repertoire of Tα1 in normal and tumour bearing mice [32], the present study has been undertaken to investigate the in vitro and in vivo ability of Tα1 to modulate bone-marrow haematopoiesis under normal conditions as also during progressive ascitic growth of a spontaneous, murine T-cell lymphoma, Dalton's Lymphoma (DL) in BALB/c mice.

2. Materials and methods

2.1. Mice and tumour system

Inbred strains of healthy, pathogen-free, normal BALB/c mice with 8–10 weeks of age and average body weight of 20–22 g have been used to obtain normal bone-marrow cells (N-BMCs).

Dalton's Lymphoma (DL), a spontaneous T-cell lymphoma of the thymus of DBA/2 mice is maintained in the ascitic form by serial transplantation (1×10^5 cells per mouse in 0.05 ml. PBS) in the normal BALB/c mice. The mice normally die within 25–30 days of DL transplantation. Thus, for all experiments, tumour bearing stage of day 14–16 after the transplantation of DL, known as the middle (mid) tumour bearing stage [32] has been used to obtain the bone-marrow cells from the tumour-bearing mice (DL-BMCs).

All the animals were kept under controlled temperature, humidity and 12-h light:12-h dark cycle with food and water ad libidum.

2.2. Culture media and reagents

All cell cultures were maintained in RPMI-1640 medium supplemented with heat-inactivated foetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml) and gentamycin (20 μg/ml) at 37 °C in a humidified incubator containing 5% CO_2. Medium RPMI-1640, Bovine thymosin-alpha-1 and Interferon-gamma (IFN-γ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant murine GMCSF, GCSF, IL-3 and TNF-alpha (TNF-α) were obtained from the National Institute For Biological Standards, UK. Recombinant murine IL-1 was obtained from Collaborative Research Inc. (Bedford, MA, USA). Fetal calf serum (FCS) was purchased from Biological Industries (Israel) and ^{3}H-thymidine (^{3}H-TdR) was obtained from Bhabha Atomic Research Centre (Mumbai, India). TRIzol reagent and ThermoScript RT-PCR system were from Life Technologies Inc., GibcoBRL, USA and One-Step RT-PCR Kit from Qiagen, Germany. All the reagents used were endotoxin-free, as determined by the Limulus Amoebocyte Lysate Assay (sensitivity limit, 0.01 ng/ml).

2.3. Preparation of L929 cell conditioned medium

L929 cell-conditioned medium (LCM) was used as a source of macrophage colony stimulating factor (MCSF) [39]. LCM was prepared according to the method of Hosoe et al. [40]. L929 cells were incubated in RPMI-1640 supplemented with 10% FCS for 5–7 days. Cell-free supernatant was then harvested from the confluent monolayers, passed through 0.22 μm membrane filter and stored at −20 °C until use.

2.4. Cell preparations

Non-adherent bone-marrow cells from both normal (N-BMCs) as well as DL-bearing (DL-BMCs) mice were obtained from the femurs of mice following a method described earlier [41]. Briefly, the mice were killed by cervical dislocation and the bone-marrow cells were flushed from the femoral shafts with serum-free medium. The BMCs were agitated gently to prepare a single cell suspension and then washed thrice with serum-free medium by centrifugation at $1000 \times g$ at 4 °C for 10 min each. The BMCs were then incubated in 25 cm^2 plastic tissue culture flask (A/S Nunc, Denmark) for 2 h at 37 °C to remove the adherent macrophages. The non-adherent BMCs were used for the in vitro proliferation and colony formation assays.

Likewise, two sets each of normal and DL-bearing mice were taken. One set of mice was given a single intraperitoneal injection of Tα1 (10 μg per mouse) whereas the other was injected with an equal volume of sterile PBS, both in case of normal and DL-bearing mice. After 7 days, the BMCs from treated and untreated mice were isolated and assayed for in vitro proliferation and colony formation.

2.5. Bone-marrow proliferation assay

The in vitro proliferation assay was done by measuring the ^{3}H-TdR uptake. The BMCs (1×10^5 cells) from normal and DL-bearing mice were seeded in 100 μl of complete medium or medium containing LCM-derived MCSF in the presence or absence of Tα1 (1–100 ng/ml), as mentioned in the Section 3, in each well of a 96-well tissue culture plate (A/S, Nunc, Denmark) and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. Eighteen h prior to the end of incubation, the cultures were pulsed with 1 μCi/well of ^{3}H-TdR. The cells were then washed three times with PBS and lysed with 100 μl of 0.25% (w/v) SDS. The lysates were counted for radioactivity in a liquid scintillation counter (LKB, Bromma, Sweden). The bone-marrow cell proliferation response was expressed as counts per minute (CPM) of the ^{3}H-TdR incorporated. Similarly, BMCs from in vivo treated and untreated mice were incubated in complete medium with or without LCM-derived MCSF and the incorporation of ^{3}H-TdR was measured. The data from the thymidine incorporation assay was further confirmed by MTT proliferation test (data not shown).

2.6. Percentage viability by MTT assay

Percentage viability of BMCs from normal and DL-bearing mice were determined by MTT [3(4,5);dimethyl thiazol-2,5-diphenyl tetrazolium bromide] assay as described earlier [42]. Briefly, the BMCs (10^5 cells per well) as grown in a 96-well tissue culture plate (A/S Nunc, Denmark) were cultured in medium alone or medium containing the recommended doses of the inhibitors/activators and their corresponding vehicles for 12–48 h. Thereafter, 10 μl of 5 mg/ml MTT was added to each well and the cells were incubated for 4 h. A purple formazan product was formed, that was solubilized by the addition of 100 μl of acidic isopropanol (0.04 N HCl in isopropanol). The absorbance of each well was measured with a microplate ELISA reader using a wavelength of 570 nm. The relative cell viability was calculated according to the formula:

$$\text{Relative cell viability} = \frac{\text{Absorbance Experimental}}{\text{Absorbance Control}} \times 100$$

where, 'Absorbance Control' represents BMCs incubated in medium alone and 'Absorbance Experimental' represents BMCs treated with the inhibitor/activator or their vehicles.

2.7. Bone-marrow colony forming assay

Bone-marrow colonies from both the normal and DL-bearing mice were prepared in methylcellulose cultures, as described previously [41]. Briefly, bone-marrow cells (1×10^5 cells per ml, final concentration) as obtained from either normal or DL-mice were suspended in a mixture containing 0.9% (w/v) methylcellulose in complete RPMI-1640 medium, 30% FCS with or without LCM-derived MCSF, GMCSF (200 U/ml), GCSF (200 U/ml), IL-3 (200 U/ml), IL-1 (100 U/ml), IFN-γ (100 U/ml) or TNF-α (100 U/ml) in the presence or absence of 100 ng/ml of Tα1, as mentioned in the Section 3. The mixture was gently vortexed and 1 ml was plated in a 35 mm single well plastic tissue culture plate (A/S, Nunc, Denmark) and incubated at 37 °C in a humidified incubator containing 5% CO_2. The plates were scored for myeloid colonies between 7 and 10 days of culture. An aggregate of more than 50 cells was counted as a single colony-forming unit (CFU) and identified morphologically by microscopy into the following colony types: macrophage-colony forming unit (CFU-M), granulocyte-macrophage colony forming unit (CFU-GM) and granulocytes-colony forming unit (CFU-G).

2.8. RNA isolation, reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated from the BMCs of both normal and DL-bearing mice by TRIzol reagent (GibcoBRL) in accordance with the supplier's instructions and the quantity/purity ascertained spectrophotometrically. Subsequently, the purified RNA was reverse-transcribed using gene-specific primers with 'ThermoScript' Kit (GibcoBRL)/One-step RT-PCR Kit (Qiagen, Germany) and subjected to 30 cycles amplification by PCR. The thermocycle conditions were standardised for each gene as indicated in Table 1. Electrophoresis of the amplified DNA was carried out on 2% agarose gel and stained with ethidium bromide.

An additional extention step at 72 °C for 5 min was included after completion of the core cycles in each of the above cases. The possible contamination of any PCR component was excluded by performing a PCR reaction with these components in the absence of RT product in each set of experiment (negative control). Use of equal amount of RNA template in the RT-PCR reactions was ascertained by running the housekeeping gene, β-globin for each of the reactions in the same tubes.

2.9. Statistical analysis

All the experiments were done in triplicates and repeated at least three times. Results are represented as mean ± S.D. Statistical significance of differences between test groups was analysed by Student's *t*-test (two-tailed).

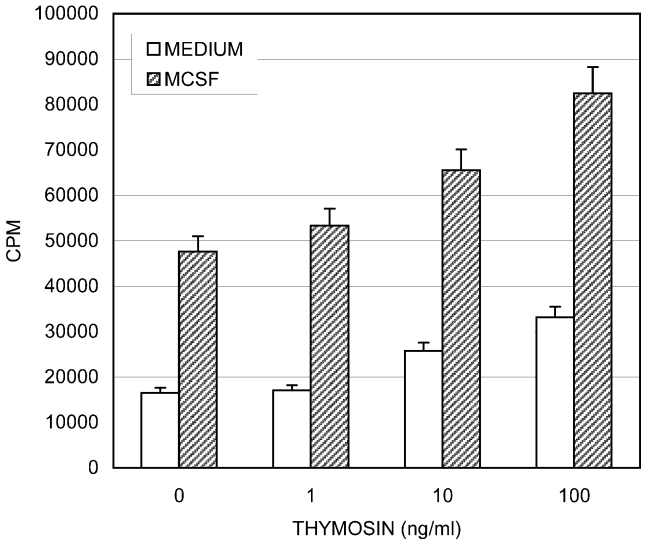


Fig. 1. Dose–response of Tα1 on the in vitro proliferation of normal murine bone-marrow cells. Non-adherent bone-marrow cells (1×10^5 cells per well) were incubated in medium alone or medium containing 20% (v/v) LCM-derived MCSF, in the presence or absence of the indicated doses of Tα1 (1–100 ng/ml) for 48 h. Thereafter, the BMC proliferation was assayed as described in Section 2. The values represent mean ± S.D. of three independent experiments done in triplicates. $^*P < 0.05$ vs. values of untreated N-BMC cultures (without MCSF).

Table 1

Gene		Primer sequences	Cycling conditions
TNF-α	*Forward*	5′-GGCAGGTCTACTTTGGAGTCATTGC-3′	94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min
	Reverse	5′-ACATTCGAGGCTCCAGTGAATTCGG-3′	
GM-CSF	*Forward*	5′-GCCATCAAAGAAGCCCTAAA-3′	94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min
	Reverse	3′-GAAAACACGGACGCATTACT-5′	
IL-10	*Forward*	5′-CCAAAGCCACAAAGCAGCCTTGCAG-3′	94 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min
	Reverse	3′-CTCAGACGACCTGAGGTCCTGGATC-5′	
β-globin	*Forward*	5′-CCTGCAGTGTCTGATATTGTTG-3′	94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min
	Reverse	5′-AACACACCATTGCGATGAA-3′	

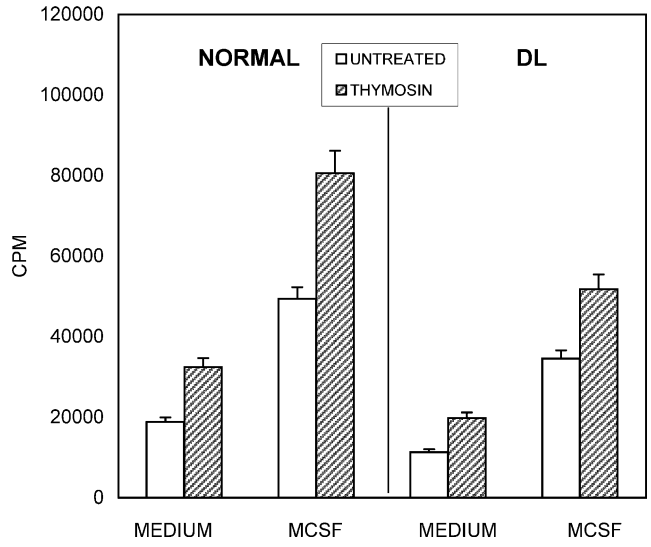


Fig. 2. The effect of in vitro Tα1 treatment on the proliferation of bone-marrow cells from normal and DL-bearing mice. Non-adherent bone-marrow cells (1×10^5 cells per well) obtained from normal and DL-bearing mice were incubated in medium alone or medium containing 20% (v/v) LCM-derived MCSF, in the presence or absence of 100 ng/ml of Tα1 for 48 h. Thereafter, the BMC proliferation was assayed as described in Section 2. The values represent mean ± S.D. of three independent experiments done in triplicates. $*P < 0.05$ vs. values of untreated BMC cultures (without MCSF) from normal mice. $**P < 0.05$ vs. values of untreated BMC cultures (without MCSF) from DL mice.

3. Results

3.1. Effect of in vitro Tα1 treatment on the proliferation of BMCs from normal and DL-bearing mice

The in vitro proliferation of non-adherent BMCs from normal and DL-bearing mice was assessed by the ^{3}H-Thymidine incorporation assay and further confirmed by MTT proliferation test, as described earlier. As shown in Fig. 1, treatment of N-BMCs with Tα1 in vitro enhanced their proliferation in a concentration dependent manner with a dose range of 1–100 ng/ml of Tα1. The maximal proliferation occurred with a dose of 100 ng/ml of Tα1. Culturing the Tα1-treated N-BMCs with MCSF further enhanced the proliferative response dramatically as compared with the N-BMCs treated with Tα1 or MCSF alone. The time kinetic study of Tα1-induced N-BMC proliferation showed maximal proliferation at day 2 of BMC culture, followed by a gradual decline thereafter (data not shown).

In contrast, the untreated non-adherent BMCs obtained from DL-bearing mice showed decreased proliferation in comparison to BMCs from normal mice (Fig. 2). Treatment of DL-BMCs with 100 ng/ml of Tα1 alone caused a significant enhancement in the proliferative response as compared with the untreated DL-BMCs, that was further upregulated in the presence of LCM-

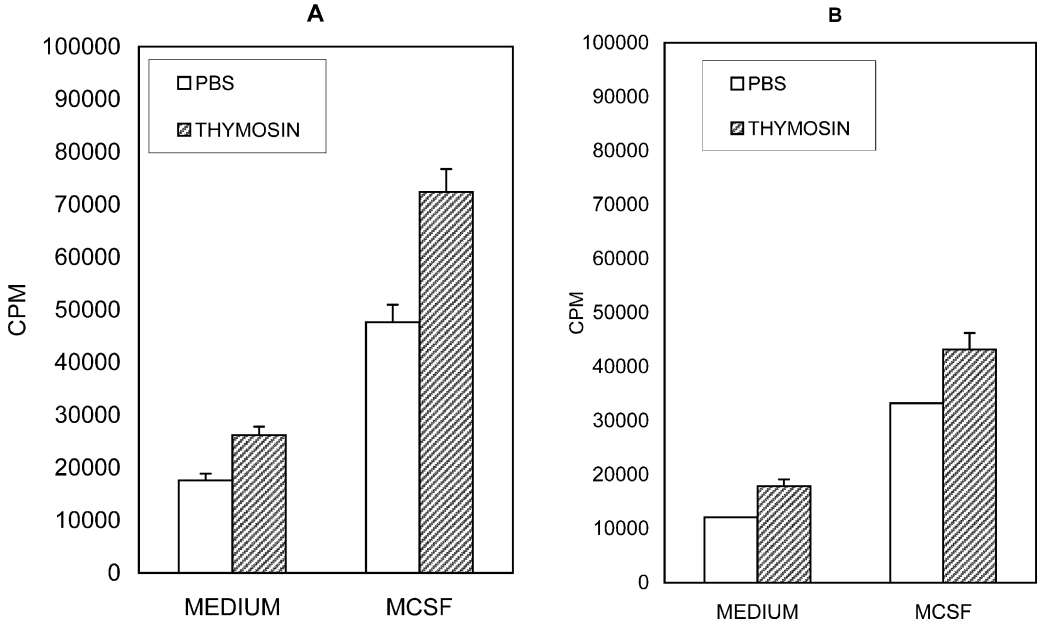


Fig. 3. The effect of in vivo Tα1 treatment on the proliferation of bone-marrow cells from normal and DL bearing mice. Non-adherent bone-marrow cells (1×10^5 cells per well) obtained from (A) normal and (B) DL-bearing mice injected intraperitoneally with 10 μg per mouse of Tα1 or the same volume of sterile PBS were incubated in medium alone or medium containing 20% (v/v) LCM-derived MCSF for 48 h. Thereafter, the BMC proliferation was assayed as described in Section 2. The values represent mean ± S.D. of three independent experiments done in triplicates. $*P < 0.05$ vs. values of untreated BMC cultures (without MCSF) from normal mice. $**P < 0.05$ vs. values of untreated BMC cultures (without MCSF) from DL mice.

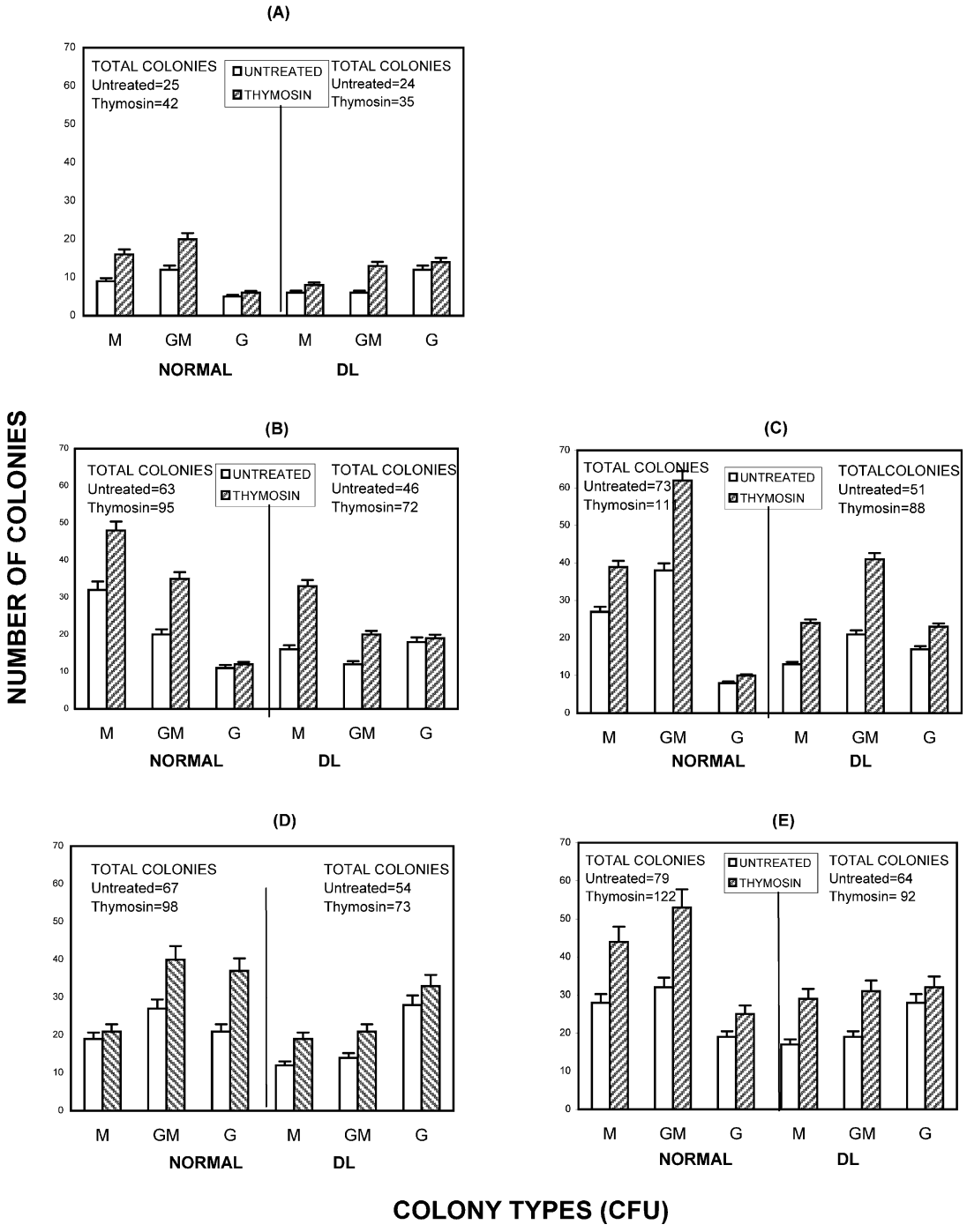


Fig. 4. The effect of in vitro Tα1 treatment on the colony forming ability of bone-marrow cells from normal and DL-bearing mice. Non-adherent bone-marrow cells (1×10^5 cells per ml) obtained from normal and DL-bearing mice were incubated in 0.9% (w/v) methylcellulose in medium alone or medium containing 100 ng/ml of Tα1, with or without LCM-derived MCSF, GMCSF (200 U/ml), GCSF (200 U/ml) or IL-3 (200 U/ml), as indicated in the figure. The colonies were scored after 7 days, as described in Section 2. (A) Medium; (B) MCSF; (C) GMCSF; (D) GCSF and (E) IL-3. The values are mean ± S.D. of three independent experiments done in triplicates.

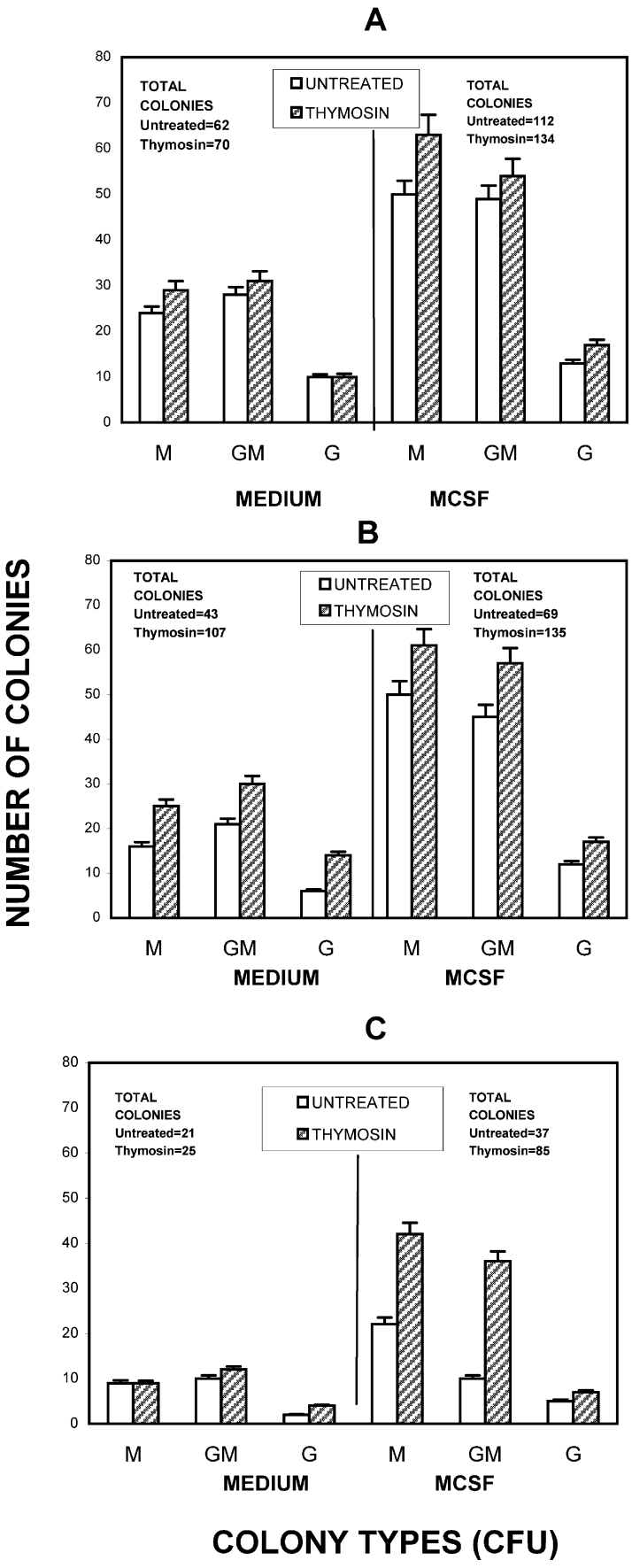
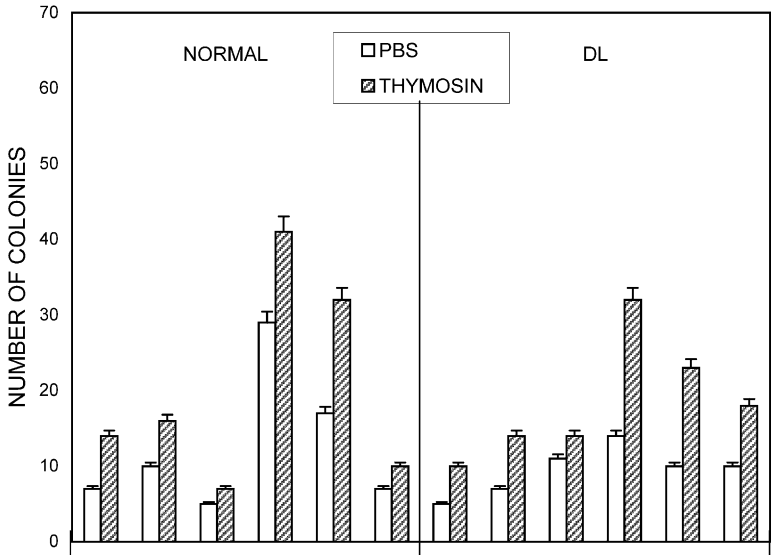

A
TOTAL COLONIES Untreated=62 Thymosin=70
UNTREATED
THYMOSIN
TOTAL COLONIES Untreated=112 Thymosin=134
M GM G M GM G
MEDIUM MCSF
B
NUMBER OF COLONIES
TOTAL COLONIES Untreated=43 Thymosin=107
TOTAL COLONIES Untreated=69 Thymosin=135
C
TOTAL COLONIES Untreated=21 Thymosin=25
TOTAL COLONIES Untreated=37 Thymosin=85
COLONY TYPES (CFU)

Fig. 5



TOTAL COLONIES	MEDIUM			MCSF			MEDIUM			MCSF		
	M	GM	G	M	GM	G	M	GM	G	M	GM	G
PBS	22			53			23			34		
THYMOSIN	37			83			38			73		

Fig. 6. The effect of in vivo Tα1 treatment on the colony forming ability of bone-marrow cells from normal and DL-bearing mice. Non-adherent bone marrow cells (1×10^5 cells per well) obtained from normal and DL-bearing mice injected intraperitoneally with 10 μg per mouse Tα1 or the same volume of sterile PBS were incubated in methylcellulose (0.9% w/v) with medium alone or medium containing LCM-derived MCSF, as indicated in the figure. The colonies were scored after 7 days as described in Section 2. The values are mean ± S.D. of three independent experiments done in triplicates.

derived MCSF. The proliferative ability of the Tα1-treated DL-BMCs was, however, much less than that of the Tα1-treated N-BMCs.

3.2. *In vivo treatment of Tα1 induces proliferation of BMCs from normal and DL-bearing mice*

The efficacy of in vivo administration of Tα1 (i.p. injection of 10 μg per mouse) to enhance the proliferative capacities of N-BMCs and DL-BMCs was studied. As shown in Fig. 3, it is observed that the BMCs obtained from the Tα1-treated normal and DL-bearing mice displayed significantly enhanced proliferation in contrast to the untreated mice. The proliferative ability of BMCs from Tα1-treated mice (normal and DL-bearing) was further upregulated in the presence of MCSF. However, the Tα1-treated or untreated DL-BMCs showed reduced proliferation as compared with BMCs from normal mice, both in the presence or absence of LCM-derived MCSF.

3.3. *Induction of myeloid colony formation by in vitro Tα1 treatment of BMCs from normal and DL-bearing mice*

It is observed that Tα1 alone or in combination with the individual CSFs, upregulated the colony forming ability of the N-BMCs as manifested by the increase in the overall number of colonies as well as the size and frequency of the individual colonies. Although, the N-BMCs treated with Tα1 in the absence of any CSFs resulted in a modest increase in the colony formation, the presence of the individual CSFs synergised with Tα1 to dramatically upregulate this process. The presence of individual CSFs, i.e. MCSF, GMCSF or GCSF in the untreated or Tα1-treated BMC cultures resulted in the preferential differentiation into M-type (CFU-Ms),

Fig. 5. The effect of IL-1, IFN-γ and TNF-α on the Tα1-induced in vitro colony forming ability of normal murine bone-marrow cells. Non-adherent bone-marrow cells (1×10^5 cells per ml) obtained from normal mice were incubated in 0.9% w/v methylcellulose in medium alone or medium containing 100 ng/ml of Tα1, with or without 20% (v/v) LCM-derived MCSF, in the presence of (A) IL-1 (100 U/ml), (B) IFN-γ (100 U/ml) and (C) TNF-α (100 U/ml), as indicated in the figure. The colonies were scored after 7 days as described in Section 2. The values are mean ± S.D. of three independent experiments done in triplicates.

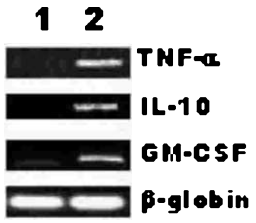


Fig. 7. The expression of TNF, GM-CSF and IL-10 mRNA in murine bone-marrow cells in response to in vitro Tα1 treatment. Non-adherent bone-marrow cells (1×10^5 cells per well) were incubated in medium alone or in medium containing 100 ng/ml of Tα1 for 12 h. Thereafter, total RNA was isolated and RT-PCR was carried out using gene-specific primers as described in Section 2. The figure is a representative of three independent experiments with similar results.

GM-type (CFU-GMs) and G-type (CFU-Gs) colonies, respectively, whereas the addition of IL-3 (Multi-CSF) in the presence or absence of Tα1 led to an increase in almost equal numbers of all the three colony types (Fig. 4).

The DL-BMCs showed an overall reduction in the total number of colonies formed as compared with the N-BMCs. However, the proportion of the granulocytic lineages (as represented by CFU-Gs and CFU-GMs) was enhanced in case of DL-BMCs. The colony forming ability of the DL-BMCs in the presence of CSFs was significantly enhanced on treatment with 100 ng/ml of Tα1, though it remained lower than that of the treated N-BMCs (Fig. 4).

3.4. Effect of IL-1, IFN-γ and TNF-α on the colony forming ability of normal murine BMCs in response to in vitro Tα1 treatment

IL-1 (100 U/ml), IFN-γ (100 U/ml) or TNF-α (100 U/ml) was added in the methylcellulose cultures to study the effect on the Tα1-induced in vitro colony formation by the N-BMCs, in the presence or absence of MCSF. As shown in Fig. 5A and B, the presence of IL-1 or IFN-γ in the N-BMC cultures resulted in an upregulation of the Tα1-induced myeloid colony formation in comparison to the untreated N-BMCs (Fig. 4A), which was further augmented in the presence of MCSF.

In contrast, TNF-α showed myelosuppressive effect on the N-BMC colony formation. As noted in Fig. 5C, the presence of TNF-α with or without MCSF resulted in the suppression of Tα1-induced BMC colony formation, as evident from the decreased number of total as well as individual myeloid colony types.

3.5. Effect of in vivo Tα1 treatment on the colony formation by BMCs from normal and DL-bearing mice

It is observed that the N-BMCs from Tα1-treated mice have greater ability of colony formation than that of the untreated mice. Both the number as well as the size of the colonies was significantly upregulated in case of the Tα1-treated mice (Fig. 6), which is further augmented in the presence of MCSF.

The DL-BMCs from the Tα1-treated mice displayed an enhanced ability of colony formation as compared with the control mice. Both the number as well as the size of the colonies showed a marked increase in contrast to the Tα1-untreated mice. The most prolific increase in the colony formation is observed when the DL-BMCs from Tα1-treated mice were cultured in the presence of MCSF. The myeloid colony formation by the DL-BMCs was less than the BMCs from normal mice.

3.6. Expression of TNF, GM-CSF and IL-10 mRNA in the BMCs from normal and DL-bearing mice

Non-adherent BMCs from normal and DL-bearing mice were incubated for 12 h in medium alone. Thereafter, the expression of TNF, GM-CSF and IL-10 mRNA in the BMCs was studied by RT-PCR. It is observed that BMCs from DL mice exhibited enhanced expression of TNF, GMCSF and IL-10 mRNA as compared with the BMCs from normal mice where the expression of these genes was not detectable/negligible (Fig. 7). However, the expression of the housekeeping gene, β-globin was observed to be equal in case of both normal and DL-BMCs.

4. Discussion

The role of Tα1 in innate immunity as well as T-cell-mediated immunity and its therapeutic potential in a broad and diverse range of clinical situations have been reported [14,15]. However, the mechanism(s) of Tα1 action that is crucial in defining its role in therapeutic applications is not well-understood. The present work is an attempt to elaborate upon the role of Tα1 in the normal bone-marrow haematopoiesis and its modulation in tumour-bearing mice.

The continuous production of mature blood cells from the haematopoietic progenitor cells in the bone-marrow is an outcome of the interacting roles of the progenitor cells, the accessory cells and the haematopoietic growth factors [4]. Among the principal haematopoietic factors that regulate the growth, survival and differentiation of myeloid progenitor cells in the bone-marrow microenvironment are the CSFs, which synergize with each other to regulate their lineage-restricted differentiation [4,5]. The effect of Tα1 on in vitro myeloid haematopoiesis was presently studied using murine non-adherent BMCs in the presence or absence of various CSFs. It is observed that the in vitro treatment of murine BMCs with Tα1 results in a dose-dependent increase in BMC proliferation (Fig. 1) and enhanced differentiation into mature myeloid colonies

(Fig. 4) with maximum response at a dose of 100 ng/ml of Tα1. The BMCs treated with Tα1 in the presence of other CSFs showed a dramatic increase in their proliferation and colony formation as compared with the BMCs treated with CSFs alone (Figs. 2 and 4). The effect of Tα1 on in vitro BMC colony formation was manifested by an increase in the size of the individual colonies, the frequency of the individual colony types as well as the total number of colonies formed. The addition of the CSFs, i.e. MCSF, GMCSF and GCSF to the Tα1-treated or untreated NABMCs mobilised them to differentiate preferentially into the macrophage (CFU-Ms), granulocyte-macrophage (CFU-GMs) and granulocyte (CFU-Gs) lineage respectively, while the addition of IL-3 led to an overall enhancement in almost all the three colony types. Supporting the above observations, the induction of the haematopoietic cell proliferation is known to require synergising factors that induce the viable precursor/progenitor cells to proliferate [43,44]. Such a view is also favoured under in vivo conditions where it is known that lineage-restricted differentiation of myeloid progenitors in the bone marrow is regulated by the presence of CSFs [5]. The effect of in vivo Tα1 treatment (single i.p. injection) on the BMC proliferation and differentiation was similar to the in vitro observations.

Contrary to the steady-state haematopoiesis maintained in normal subjects, periods of decreased haematopoiesis or haematopoietic abnormalities have been well documented in humans and animal models for a variety of tumours [8–10]. Coherent with these reports, the present in vitro and in vivo studies demonstrate that the progressive growth of a non-metastasing, murine T-cell lymphoma, Dalton's Lymphoma (DL) results in profound alterations in the bone-marrow myelogenesis. The untreated non-adherent BMCs obtained from DL-bearing mice (DL-BMCs) showed a marked reduction in the proliferation and CFA in vitro as compared with the BMCs from normal mice (N-BMCs). The reduced myeloid colony formation by the DL-BMCs was evident by the decreased formation of total number of colonies, but was accompanied by an increased percentage of differentiation into the granulocytic lineage (CFU-Gs plus CFU-GMs) of the total colonies formed. In contrast, treatment of DL-BMCs with Tα1, both in vitro and in vivo, resulted in a significant increase in the BMC proliferation and myeloid colony formation, an effect that was further potentiated in the presence of CSFs. This response was, however, significantly less than that observed for the Tα1-treated N-BMCs.

Although the factors responsible for the haematological and immunological alterations associated with neoplasia are still not well characterized, high levels of growth stimulatory cytokines like GM-CSF [45–48] as well as tumour-derived inhibitory molecules like TGF-β, PGE_2, IL-10, TNF-α and gangliosides in the systemic circulation [49–57] have been implicated for several tumour models. Further, the enhanced expression of IL-10, GMCSF and TNF mRNA in the BMCs of DL-bearing mice in the present study (Fig. 7), provide additional support. Therefore, it is conceivable that the unresponsiveness of the DL-BMCs to the lineage-restricted differentiation mediated by the individual CSFs could be an outcome of the presence of high levels of these molecules that possibly overshadow the otherwise stimulatory effect of the CSFs.

Considering the reports of thymic involution and thymic atrophy, particularly in case of DL-bearing mice [58,59] and our present observations on the restoration of haematopoietic defects by in vitro and in vivo Tα1 administration, a decrease in the circulating levels of thymic peptides in tumour-bearers may possibly be another reason for the observed immunosuppression. In support, a decline in the serum levels of Tα1 is observed for a number of tumour models and its subsequent correction by the in vivo administration of thymic hormones has been reported [15,60–62].

The precise mechanism(s) for the immunorestorative property of Tα1, as reported here and elsewhere is unclear. The effect of Tα1 on in vitro bone-marrow haematopoiesis may be direct or indirect. Favouring a direct effect on haematopoiesis are reports on the Tα1-induced differentiation of $CD34^+$ precursors [24–26] and the reversal of apoptosis [63–65]. Our data on the stimulatory effect of Tα1 alone on BMC proliferation and colony formation assays supports this hypothesis. Alternatively, Tα1 action on myelogenesis could also be an indirect effect, whereby, a favourable blend of cytokines are generated by the immune cells in response to the initiating/priming signal of Tα1 stimulation that probably renders the BMCs responsive to differentiation signals of the various haematopoietic growth factors, which in turn, upregulates the functioning of the haematopoietic machinery. In agreement, in vivo administration of Tα1 to nude mice is reported to result in increased colony formation and high levels of IL-3 secretion by bone-marrow cells [66–68]. As additional support, the production of cytokines like IL-1, TNF-α and IFN-γ has already been shown in bone-marrow derived macrophages (BMDMs), NK cells and T-lymphocytes in response to Tα1 [14,32]. Recently, we observed that in vitro treatment of murine BMDMs with Tα1 resulted in significantly enhanced expression of T-cell specific chemokines, MIP-1β and RANTES (unpublished data). Both these chemokines have been reported to promote the proliferation of CFU-GM myeloid progenitor cells [69].

Moreover, the exogenous addition of IL-1, IFN-γ and TNF-α in vitro has been observed to markedly modulate the colony forming ability of Tα1-treated murine BMCs with IL-1 and IFN-γ showing significant upregulation, while TNF-α causing myelosuppression (Fig. 5). IL-1, a

pleiotropic cytokine with multiple immunologic and inflammatory functions has already been implicated in haematopoietic regulation, the dual role of which involves the activation of early haematopoietic progenitors, the initiation of their proliferation and upregulation of receptors to other CSFs on one hand and induction of CSF production by different accessory cells on the other hand [70–73]. The stimulatory activity of IL-1 with other growth factors has been demonstrated both in vitro and in vivo [71]. Tα1 has also been implicated in the production of IFN-γ by activated lymphocytes [14]. The functional synergism between IFN-γ and Tα1 as observed here, is reflected in the recent reports on their use in combination therapies for viral infections and malignant diseases [30,33,74]. Likewise, the observed myelosuppressive effect of TNF-α on Tα1-induced colony formation is well in accordance with other reports [75,76], whereby it induces haematopoietic inhibitory activities via endogenous production of other cytokines [77].

Inspite of these studies, several aspects of Tα1 action on the bone-marrow haematopoiesis still need to be clarified. The present study is the first to extend the immunopotentiating/immunomodulatory role of Tα1 to myelopoiesis, in addition to its well-documented role in thymopoiesis. Additionally, the present data also demonstrates altered haematopoiesis in tumour-bearing mice and the myelorestorative effect of Tα1, both in vitro and in vivo. Therefore, this study provides a new insight in the area of thymosin research and suggests yet another therapeutic aspect of Tα1 to enable designing effective clinical protocols to treat neoplasia and haematological disorders.

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