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ORIGINAL ARTICLE

Antitumor Activation of Peritoneal Macrophages by Thymosin Alpha-1

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It was been previously reported that thyα1 can be used to activate monocytes, BMDM and TAM. However, the effect of thyα1 on other tissue macrophages has not been investigated. Moreover, there is no report about the use of thyα1-treated macrophages in adoptive immunotherapy of cancer. In view of these observations in the present study, we checked the response of various tissue macrophages to thyα1 for activation. Tissue macrophages showed differential response to thyα1; moreover, adoptive transfer of peritoneal macrophages treated with thyα1 to mice bearing spontaneous T-cell lymphoma designated as Dalton's lymphoma (DL) resulted in the prolongation of the survival time of tumor-bearing mice. The mechanism of macrophage therapy-dependent tumor regression was enhanced antitumor activity of macrophages in response to thyα1 treatment via their production of macrophage-activating cytokines that act in autocrine manner. These results will help in the development of immunotherapy against tumor based on activation of macrophage with thyα1.

Keywords Macrophages; Activation; Tumor cells; Thymosin alpha-1; Adoptive transfer

INTRODUCTION

One of the proposed approaches to tumor immunotherapy has been to augment an early and a potent antitumor immune response.[1] Although a number of cells of immune system have been demonstrated to participate in host's antitumor immune responses, macrophages play a central role in this process.[2] Resident macrophages need to be activated, for displaying antitumor activity. Therefore, a number of macrophage-activating agents have been described with an aim to identify a perfect blend of endogenously produced cytokines and hormones and exogenous agents with optimum macrophage-activating potential.[3]

Because murine spontaneous tumors are known to resemble human malignancies most closely, using a murine transplantable T-cell lymphoma, designated as Dalton's lymphoma (DL), we have been attempting to understand the host-tumor relationship in a lymphoma-bearing host. We have already reported that DL-bearing hosts show an overall suppression of various immune responses.[4–6] Therefore, we wanted to identify biological response modifiers with the capacity to reverse the DL-associated immunosuppressive effects.[7]

In the recent past, polypeptides or extracts obtained from thymus have gained immense acceptance as immunopotentiators.[8] A number of clinical reports have demonstrated the immunomodulatory role of thymic peptides in diseases such as AIDS, hypersensitivity, infections, and malignancy.[9–12] It has been found that thymosin α1, a 28 amino acid peptide hormone secreted by thymic stromal cells, is a potent immunomodulator with an ability to stimulate the production of cytokines by T cells, potentiate the action of cytokines, and reduce the hematological toxicity of cytotoxic drugs without any known adverse effect.[13–15] Keeping in view the potential of thyα1 in activating the antitumor activity of macrophages, we wanted to develop an immunotherapeutic protocol aimed at regression of tumor using adoptive transfer of thyα1-treated macrophages to tumor-bearing host. Moreover, we have reported that thyα1 can reverse the suppressed antitumor activity of tumor-associated macrophage (TAM).[6,16] TAM, which were shown to respond to thyα1, are not considered suitable for use in adoptive immunotherapy due to two reasons: 1) the low number of TAM harvested from tumor and 2) TAM as such are in a suppressed state.[17] Therefore, we intended to first identify the tissue macrophage population that is easily isolatable in bulk, infiltrate tumor mass, and show optimum response to thyα1 treatment for manifestation of antitumor activity. To the best of our knowledge, none of the earlier studies have reported the effect of thyα1 on the activation of tissue macrophages, which are known to display a tremendous heterogeneity, diversity of phenotypic capabilities acquired in response to local environmental influences.[18] As a consequence, tissue macrophages display a differential response to activation signals.[19]

In view of these observations in the present study, we addressed the following problems for investigations: 1) to

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identify the tissue macrophage population other than TAM with an optimum activation response to thyα1 and 2) to use thyα1-treated tissue macrophage for adoptive immunotherapy of lymphoma-bearing host.

MATERIALS AND METHODS

Reagents and Culture Media

Tissue culture medium, DMEM, and most of the chemicals were obtained from Himedia (Mumbai, India). Thyα1, LPS, MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Culture medium was supplemented with 20 mg/mL gentamycin, 100 mg/mL streptomycin, 100 IU penicillin, and 10% FCS (Himedia). All the reagents were free from endotoxin contamination. The cell cultures were carried out at 37°C in a CO_2 incubator (Sheldon, USA) having 5% CO_2 in air in a humidified atmosphere.

Mice and Tumor Lines

Inbred, apparently healthy male BALB/c mice 8–12 weeks of age and 20–25 g body weight were used for experimentation. DL was maintained in ascitic form by serial transplantation in BALB/c mice. The DL cell line was also maintained by serial culture in vitro and in a cryopreserved state for reference purpose. DL cells were incubated in vitro for 48 hours for culture adaptation and were used while they were in exponential growth phase. L929 cell line (mouse fibroblast) was obtained from the National tissue culture facility in Pune (India).

Isolation of Peritoneal Macrophages (PMO)

Mice were killed by cervical dislocation, and peritoneal exudate cells (PEC) were harvested by peritoneal lavage using chilled serum-free RPMI-1640 as described earlier[20] with slight modifications. PEC were then cultured in plastic tissue culture flask at 37°C in a CO_2 incubator for 2 hours. The cultures were then washed three times to remove all nonadherent cells; 95% of the adherent cell population were macrophages as determined by morphology. These PMO were detached from the tissue culture flask gently by scrapping and plated in a 96-well flat bottom culture plates (1.5×10^5 cells/well).

Isolation of Tumor-Associated Macrophages (TAM)

DL-bearing mice 8 days post-DL transplantation were killed by cervical dislocation, and peritoneal exudate cells were harvested by peritoneal lavage using chilled serum free RPMI-1640 as described earlier.[17] Peritoneal exudate cells were cultured in plastic tissue culture flasks at 37°C in a CO_2 incubator for 2 hours. The cultures were then washed three times with warm serum-free medium with gentle flushing to ensure that all the DL and/or other nonadherent cells were removed; 95% of the adherent cell population were macrophages as determined by morphology. These TAM were detached from the tissue culture flask with a cell scraper and plated in 96-well flat bottom culture plate (1.5×10^5cells/well).

Culture and Isolation of Bone Marrow-Derived Macrophages (BMDM)

Bone marrow-derived macrophages (BMDM) were obtained as described earlier.[21] Briefly, mice were killed by cervical dislocation, and bone marrow cells were flushed from femoral shafts with chilled serum-free medium. A single cell suspension of BMC was washed with serum-free medium and incubated in plastic tissue culture flasks for 4 hours to remove adherent macrophages. Nonadherent BMC were cultured for 3 days in suspension culture in L929CM (20% v/v). After 3 days of incubation, viable cells were washed and plated in flat-bottomed 96-well plastic culture plates (Tarson, India) (2.5×10^5 cells/well) for obtaining BMDM. After 2 hours of incubation, the cultures were vigorously shaken and washed with warm medium to remove nonadherent cells. The cultures were then refed with complete medium. About 60% of the L929CM-treated viable cells adhered to plastic (1.5×10^5 cells/well). More than 95% of the adherent cells were positive for nonspecific esterase staining and showed the typical macrophage morphology.

Isolation of Splenic mACROPHAGES (SMO)

Mice were killed by cervical dislocation, and splenocytes were harvested by a method as described earlier[22] with slight modification. Splenocytes were cultured in plastic tissue culture flasks at 37°C in a CO_2 incubator for 2 hours. The cultures were then washed three times to remove all nonadherent cells; 95% of the adherent cell population were splenic macrophages as determined by morphology. These SMO were detached from the tissue culture flask gently by scrapping and plated in a 96-well flat-bottomed culture plates (1.5×10^5cells/well).

Assay for Nitrite Production

Nitrite production in the culture supernatant was determined by a spectrophotometric assay method, as described earlier.[23] Briefly, 100 µl of the sample were collected from the culture supernatants of PMO, SMO, TAM, and BMDM and incubated with an equal volume of Griess reagent (one part of 1% sulfanilamide in 2.5% phosphoric acid plus and one part of 0.1% naphthylethylenediamine dihydrochloride in distilled water, were mixed together and used within 12 hours of use and kept chilled) at room temperature for 10 minutes. The absorbance at 540 nm was determined with an automatic ELISA plate reader (Labsystem, Finland). Nitrite concentration was determined by using sodium nitrite as standard. Data were expressed as µmol nitrite/1.5×10^5 cells. In all the experiments, nitrite contents in wells containing medium without cells was also measured and subtracted.

TABLE 1
Effect of in vivo administration of Thyα1 on NO production by PMO, SMO, TAM, and BMDM

		NO release (μ mol/1.5×10^5 cells)	
Macrophages	In vitro treatments	PBS	Thyα1
PMO	Medium	15.3±0.12	20.8±2.2*
	LPS	20.7±1.9	25.40±3.5#
SMO	Medium	9.2±1.1	17.4±2.6*
	LPS	12.4±1.7	20.3±3.0#
TAM	Medium	16.2±0.19	26.8±3.5*
	LPS	26.2±3.4	34.5±4.4#
BMDM	Medium	7.2±1.0	6.2±0.7
	LPS	8.3±0.9	6.5±0.9

PMO, TAM, SMO, and BMDM were obtained from thyα1- or PBS-administered mice as described in Materials and Methods. 1.5×10^5 PMO were cultured in vitro for 24 hours in medium alone or containing LPS (10 μg/mL), and the culture supernatant was harvested and analyzed for production of NO. Values are mean±SD from a representative experiment done in triplicate. In other experiments, similar results were obtained.

*$p<0.05$ vs values for corresponding control.

#$p<0.05$ vs. values for corresponding control incubated with LPS.

MTT Assay

MTT assay was carried out to estimate tumor cytotoxicity and IL-1 secretion, following a method described by Mosmann.[24] MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] was dissolved in PBS at a concentration of 5.0 mg/mL; 50 μl of the MTT solution was added to each well of the culture plate containing 200 μl medium containing 50 μl of test culture supernatant and 150 μl of medium containing T lymphocytes in the IL-1 assay and for cytotoxicity assay, the macrophage monolayer was washed and supplemented with 200 μl of fresh medium containing 1×10^4 DL cells to which 50 μl of MTT solution were added and incubated at 37°C for 4 hours. The plate was centrifuged for 5 minutes at 100 g at 4°C (Remi, India). Supernatant was then removed carefully without disturbing the dark blue formazan crystals; 50 μl of DMSO were added to each well and mixed thoroughly to dissolve the crystals of formazan. The plates were then read on a microplate reader (Labsystem, Finland) at a wavelength of 540 nm. Readings were presented as OD at 540 nm.

Assay of IL-1 Activity

PMO were incubated in vitro as indicated, and culture supernatants were harvested and checked for IL-1 activity by a standard thymocyte proliferation assay as described earlier.[20] Thymocytes obtained from 4- to 8-week-old mice were incubated at a concentration of 1.5×10^6 cells/well in a 96-well plastic tissue culture plate with medium containing suboptimal doses of concanavalin A (1 μg/mL) and 2-mercaptoethanol (2×10^{-5} mol) along with the culture supernatant of PMO. The cultures were then incubated at 37°C in a CO_2 incubator for 72 hours. Thymocyte proliferation was measured by MTT assay as described above.

Assay of TNF Activity

The activity of TNF in the culture supernatant of PMO was measured by dye uptake assay as described earlier.[20] Briefly, 3×10^4 L929 cells in 100 μl of medium were grown in wells of a 96-well tissue culture plate in the presence of 1 μg/mL of actinomycin D and 100 μl of the test culture supernatant. After 18 hours of incubation, the plates were washed, and cell lysis

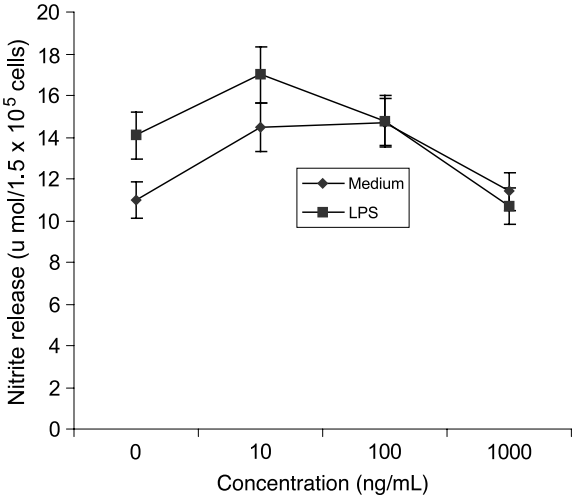


FIG. 1. PMO obtained were incubated for 24 hours in medium alone or with different concentrations of thyα1. NO production was estimated as described in Materials and Methods. Values are means±SD from a representative experiment done in triplicate. *$p<0.05$ vs. values of corresponding controls.

was determined by staining the plate with a 0.5% (w/v) solution of crystal violet in methanol/water (1:4 v/v). The OD was measured at 540 nm; % cytotoxicity was calculated as follows:

$$\%\,\text{Cytotoxicity} = \text{C} - \text{T}/\text{C} \times 100$$

where C is the absorbance of wells containing L929 cells incubated in medium alone, and T is that of those wells in which L929 cells were incubated with culture supernatant of PMOs.

Adoptive Transfer Experiments

Adoptive transfer of PMO obtained from thyα1 administered mice was carried out by following the method described by Jalali et al.[25] DL (1×10^5 cells/mouse)-transplanted mice in a group of six each were administered with a single i.p. injection of 1×10^6 PMO in 500 μL of PBS on day 8 posttumor transplantation, and the progression of tumor was monitored as per survival of the tumor-bearing mice. The results were analyzed on a standard Kaplan-Meier plot.

Detection of Macrophage Colony-Stimulating Factor (M-CSF) Expression by Semiquantitative Reverse Transcription-Polymerase Chain Reaction (PCR)

Total cellular RNA was isolated from normal macrophages by using the Qiagen RNeasy mini kit (batch no. 4061509). The Primer sequences for M-CSF used were M-CSF forward primer 5′-CGGGCATCATCCTAGTCTTGCTGACGGTGTGGG 3′ and for reverses primer 5′-AAATAGTGGCAGTATGTGGGGGGCATCCT 3′ (Qiagen). Before use, the integrity and purity of RNA were checked by electrophoresis. The RT-PCR reaction was carried out by following a method described elsewhere.[26] The reaction mixture contained 1 μL of cDNA, 50 ng of forward and reverse primer sequences of M-CSF, 1× PCR buffer (Genetix), 0.2 mM each of dNTPs (MBI fermentas), 2 mM $MgCl_2$, and 0.5 U of Gold Taq DNA Polymerase (Genetix) in a total volume of 20 μL. The PCR was run on a programmable thermocycler (Techne, UK) as follows: an initial denaturation step of 5 minutes at 94°C followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 2 minutes. The PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. The gel images were captured on a Gel doc image analysis system (Biorad, Australia).

RESULTS

Effect of In Vivo Administration of Thyα1 in Multiple Doses on NO Production by PMO, SMO, TAM, and BMDM

First, we wanted to check if macrophages obtained from different anatomical sites were responsive to thyα1 for production of NO. Thyα1 at a concentration of 5 μg/mouse, which has been reported earlier to be optimum for in vivo immunomodulatory effect,[8] was administered for 3 consecutive days to normal or tumor-bearing mice. PMO, SMO, TAM, and BMDM, obtained as described in Materials and Methods, were incubated in vitro for 24 hours in medium alone or containing LPS (10 μg/mL), and the culture supernatants were checked for NO contents. Results are shown in Table 1. A significant increase in NO production was observed in PMO, TAM, and SMO obtained from mice administered with multiple doses of thyα1 even in the absence of in vitro treatment with LPS, but there was no activating effect on BMDM.

Effect of In Vitro Thyα1 Treatment on NO Production by PMO

To understand the mechanism if PMO could be directly activated by thyα1, PMO were incubated in medium alone or containing different concentrations of thyα1 in the presence or absence of LPS for 24 hours prior to collection of culture supernatant for estimation of NO. Results are shown in Figure 1. Maximum production of NO was observed on incubation of PMO with thyα1 at a concentration of 10 ng/mL. NO production of PMO increased further in the presence of LPS.

Effect of In Vivo Administration of Thyα1 on PMO-Mediated Tumor Cytotoxicity

In this experiment, we checked if in vivo administration of thyα1 could activate PMO-mediated tumor cytotoxicity. PMO obtained were incubated in vitro, in medium alone or

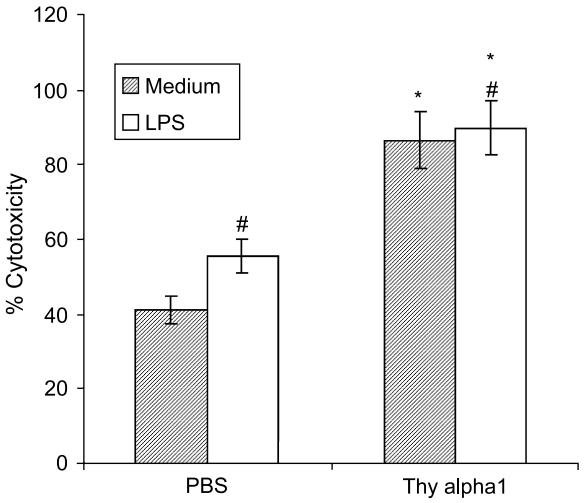


FIG. 2. PMO were obtained from mice administered with thyα1 in multiple doses (5 μg/mouse) administered for 3 consecutive days, and the % cytotoxicity against tumor cells was assayed. Values are means ± SD from a representative experiment done in triplicate. *$p < 0.05$ vs. values for corresponding control of PMO incubated without LPS. #$p < 0.05$ vs. values for corresponding control incubated with LPS.

TABLE 2
Effect of in vivo administration of Thyα1 on the IL-1 and TNF-α production by PMO

	In vivo treatments			
	Thymocytes proliferation (O.D. at 570 nm)		% Cytotoxicity against actinomycin D treated L 929 cells	
In vitro treatments	PBS	Thyα1	PBS	Thyα1
Medium	2.144	3.004*	5±0.4	18±0.9*
LPS	3.085*	4.029*	8±1.4*	21±1.2*

PMO were obtained from thyα1- or PBS-administered mice as described in Materials and Methods. 1.5×10^5 PMO were cultured in vitro for 24 hours in medium alone or containing LPS (10 μg/mL), and the culture supernatant was harvested and analyzed for production of IL-1 and TNF-α. Values are means±SD from a representative experiment done in triplicate. In other experiments, similar results were obtained.

*$p<0.05$ vs. values for corresponding control.

containing LPS (10 μg/mL) for 24 hours. The cells were then washed three times with warm medium and coincubated with DL cells, as indicated in Materials and Methods, for estimation of PMO-mediated cytotoxicity. Results are shown in Figure 2. PMO obtained from mice administered with PBS showed little tumor cytolytic activity compared with PMO obtained from mice administered with multiple doses of thyα1. Treatment in vitro with LPS could not further augment the macrophage tumoricidal activity of PMO obtained from mice administered with thyα1.

Effect of In Vivo Administration of Thyα1 on the Production of IL-1 and TNF

The effect of in vivo administration of thyα1 on IL-1 production by PMO was investigated. Results are shown in Table 2. Little IL-1 activity was observed in culture supernatant of PMO obtained from PBS-administered mice on incubation in vitro in medium without LPS. LPS treatment of these PMO increased the production of IL-1. PMO obtained from mice administered with thyα1 showed significantly enhanced production of IL-1 in vitro on incubation in medium without LPS compared with PMO of PBS-administered mice treated with LPS in vitro. In vitro treatment with LPS further augmented IL-1 activity of PMO obtained from thyα1 administered mice. Moreover, PMO isolated from mice administered with thyα1 showed a significant level of TNF production, even in the absence of LPS treatment in vitro. Treatment of these PMO in vitro with LPS resulted in a further augmentation of TNF production.

Adoptive Transfer of PMO Obtained from Thyα1 or PBS Administered Mice on the Survival Rate of DL-Bearing Mice

PBS containing 1.5×10^6 PMO obtained from thyα1- or PBS-administered mice were transferred to DL-bearing mice, and the survival was monitored. Results are shown in Figure 3. Survival of DL-bearing mice on transfer of PMO obtained from thyα1-administered mice was higher than that

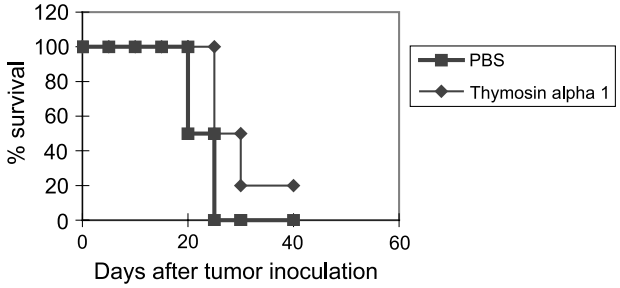
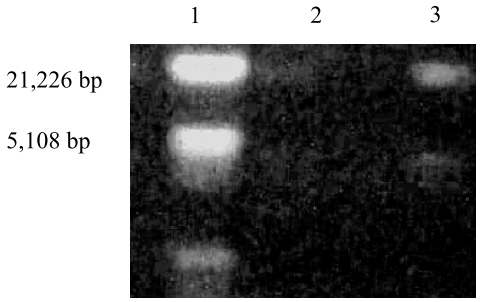


FIG. 3. PMO (1×10^6 cells) obtained from PBS or thymosin alpha1-administered mice in 500 μL. of PBS were transferred into the peritoneal cavity of DL-bearing mice on day 8 of DL transplantation. Kaplan-Meier plot of the survival of mice was made. The experiment was performed three times.



Lane 1 DNA bp
Lane 2 Control
Lane 3 Thymosin alpha1treated

FIG. 4. PMO obtained from mice administered with PBS or thyα1 (5 μg/mice) were incubated in vitro for 24 hours in medium alone or containing LPS (10 μg/mL). mRNA was extracted as described in Materials and Methods. Data presented for reverse transcription PCR are representative of two experiments.

of DL-bearing mice administered with PMO obtained from PBS-administered mice.

Effect of In Vivo Administration of Thyα1 on the Expression of M-CSF in PMO

To determine whether thyα1 induces the expression of M-CSF gene by PMO, a RT-PCR was carried out. PMO isolated from PBS-treated mice did not show expression of M-CSF gene, whereas PMO isolated from thyα1-treated mice showed expression of M-CSF gene (Figure 4).

DISCUSSION

The first aim of this investigation was to identify the tissue macrophage population showing optimum response to thyα1 for displaying antitumor activity. This was done for the reason that until now, the macrophage-activating potential of thyα1 had been checked only with monocytes,[8,27] BMDM, and in our laboratory on TAM, but none of these macrophage types actually represent the true functions of tissue macrophages. The results of the present investigation indicate that peritoneal macrophages show optimum activation response to thyα1 treatment next to the response of TAM. We selected PMO for further experiments in this study instead of TAM and other macrophages considering several advantages of using PMO. It is difficult to isolate TAM in bulk from tumor mass compared with the normal peritoneal macrophages. Moreover, we showed earlier that with the progression of tumor growth, TAM become unresponsive to signals of activation.[28]

PMO are the macrophage population that infiltrate into the ascitic growth of lymphoma by virtue of their presence in the peritoneal cavity; Gjomarkaj et al.[19] also showed that PMO display functional and phenotypic features with respect to morphology, biochemistry, secretory products, and functional phenotypes, which are different from macrophages isolated from other anatomical locations. Moreover, the sterile anaerobic environment of peritoneal cavity imparts altogether a different set of constraints on its macrophages (e.g., peritoneal cavity macrophages do not express high levels of MHCII antigens and 5′ nucleotidase activity, whereas the opposite is found in other tissue macrophages).[29] Other studies using monoclonal antibodies against membrane-associated markers of macrophages found that the expression of cell surface antigens in PMO differs from other tissue macrophages.[18]

To the best of our knowledge, to date, no known receptor for thyα1 on macrophages has been identified.[30] The difference in response of BMDM and PMO to thyα1 may not be, therefore, attributed to the differential expression of thyα1 receptor on macrophage cell surface. Another reason for the higher response of PMO to thyα1 could be attributed to their interaction with signals derived from lymphocytes and DC in the peritoneal compartment. It has been suggested that lymphocytes and DC present in the peritoneal cavity may contribute to the intrinsic difference of PMO from other macrophages as a result of their priming through the release of cytokines.[19] However, because SMO showed a better response of activation to thyα1 administration, the effect of thyα1 may therefore be systemic in nature rather than being local.

The second aim of the study was to understand if adoptive transfer of thyα1 treated macrophages to DL-bearing host could cause tumor regression. Adoptive transfer experiments using PMO obtained from thyα1-administered mice showed prolongation of the survival time of DL-bearing mice. There could be multiple causes for the observed prolongation of the survival of DL-bearing mice on adoptive transfer of thyα1-treated PMO. Some of the possibilities were considered. Our results show that PMO obtained from thyα1-administered mice show enhanced production of tumoricidal molecules, such as TNF, NO, and IL-1, and also display direct tumor cytotoxicity. Thus, the reason for the prolongation of the survival of DL-bearing mice on transfer of thyα1-treated PMO could be the direct cytotoxic/cytostatic effect of these macrophages on tumor cells. Moreover, IL-1 and TNF produced by thyα1-treated PMO could activate dendritic cells in the peritoneal cavity and lead to an altered cytokine balance in situ, augmenting the potential of other cells of immune system involved in the host antitumor response. We have shown that thyα1 can also activate the antigen-presenting ability[31] of macrophages by virtue of which specific antitumor immune response can also be augmented. The cytokine dependence of macrophage for activation is also evident from the fact that differentiation of macrophages into M1 and M2 phenotype, which show differential response to activation signals are dependent on the presence of the Th1 or Th2 cytokines for their differentiation.[32]

Moreover, the results of the present study show that thyα1-treated PMO also showed an enhanced expression of M-CSF. M-CSF production by macrophages has been reported by other workers also.[33] M-CSF has shown autocrine actions on macrophages to activate them.[34] This could be another reason for the better antitumor response of thyα1-treated PMO leading to the prolongation of the survival of DL-bearing mice.

This study shows for the first time that 1) different macrophage populations show a differential response to thyα1 for activation and 2) thyα1-treated PMO showing enhanced antitumor activity can prolong survival of tumor-bearing host on adoptive transfer. These results will, therefore, have long-lasting implications in tumor immunotherapy using macrophages and thyα1.

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