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Thymosin α1 promotes the activation of myeloid-derived suppressor cells in a Lewis lung cancer model by upregulating Arginase 1

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ABSTRACT

Thymosin α1 (Tα1) has been tested for cancer therapy for several years, in most cases, the anti-tumor effect of Tα1 was limited, especially when Tα1 was used as a single agent. The role of Tα1 in cancer treatment and the regulatory mechanisms by which Ta1 takes effects are not yet completely understood. Using a Lewis lung caner model, here we report that Tα1 used alone elevated $CD8^+$ T cells, but failed to inhibit tumor growth. Furthermore, immunosuppressive myeloid-derived suppressor cells (MDSCs) showed heightened Arginase 1 production in response to Tα1 treatment, which led to stronger suppression of anti-tumor immunity. In addition, the upregulation of ARG1 was dependent on TLRs/MyD88 signaling, blocking MyD88 signaling abrogated the enhanced ARG1 expression and restored the anti-tumor efficacy of Tα1. This study provides the first demonstration that Tα1 treatment activates but not expands MDSCs via MyD88 signaling, which indicates better immunotherapeutic strategy of Tα1 against cancer.

1. Introduction

Thymosin α1 (Tα1), a 28-amino acid peptide, was first described and characterized by Goldstein et al. [1]. As a biological response modifier, Tα1 acts on immune system through multiple mechanisms, it can promote differentiation and maturation of T cells, activate natural killer (NK) and dendritic cells (DCs), and induce the release of proinflammatory cytokines, it can also upregulate major histocompatibility complex (MHC) class-I antigen expression in normal and transformed cells [2–6]. Tα1 has been in preclinical and clinical tests either used alone or in combination with cytokines and chemotherapeutic agents for malignancies, such as Stage IV malignant melanoma, hepatocellular carcinoma, glioblastoma and breast cancer [7–10], however, the anti-tumor efficacy of Tα1 seems to be controversial [11,12].

Recently, myeloid-derived suppressor cells (MDSCs), as a heterogeneous population of cells that expand in cancer, inflammation and infection, have been characterized as a key factor of immunosuppression [13]. In mice, these cells are characterized by Gr-1 and CD11b. In humans, the markers of MDSCs are not well characterized [14]. MDSCs impair anti-tumor immune responses through multiple mechanisms, including directly suppressing T-cell function by producing arginases (ARG1), reactive species of oxygen (ROS), cyclooxygenase-2 (COX2), inducible nitric oxide synthase (iNOS), immunosuppressive cytokines (IL-6, IL-10) and inducing Treg cells [14,15]. Increased MDSCs level evokes immune-suppressive activities [16], and positively correlates with poor clinical outcome in cancer patients [17,18]. Clinical interventions targeting MDSCs can augment antitumor immune responses and prolong survival significantly [17,19].

Previous studies of Tα1 mainly focused on its effects on immune effector cells, little is known about its action on the immunosuppressive cells, which can modify anti-tumor immunity and cause

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substantial immunosuppression. Given the pleiotropic activity of Tα1, in this study, we focused on the action of Tα1 on MDSCs and found that Tα1 treatment enhanced the suppressive function of MDSCs, which in turn favored tumor progression. Tα1 promoted the activation but not expansion of MDSCs by upregulating ARG1 through MyD88 signaling in a Lewis lung cancer model, leading to its failure in inhibiting tumor growth.

2. Materials and methods

2.1. Mice and cells

C57BL/6 female mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). MyD88$^{-/-}$ mice on a C57BL/6 background were purchased from Jackson Laboratory. Mice (6 week-old) were used in experiments that were performed according to animal experimental ethics committee guidelines of PLA General Hospital. 3LL cells were established in our laboratory and cultured in RPMI1640 with 10% FBS.

2.2. Reagents and antibodies

Tα1 was obtained from Di'ao Group (Cheng du, China). Gemcitabine (GEM) was purchased from Hansoh Pharmaceutical Co., (Nanjing, China). Anti-CD4-FITC, anti-CD8-PE, anti-NK1.1-FITC, anti-CD11c-PE, anti-CD11b-PE, anti-Gr-1-PE/CY5, anti-Ly6C-FITC, anti-Ly6G-PE/CY7, anti-FOXP3-PE, anti-CD25-APC, anti−IFN−γ-FITC and isotype antibodies were obtained from Biolegend (San Diego, USA). MyD88 inhibitor and control peptide were obtained from Imgenex (San Diego, USA).

2.3. Tumor models

Mice were injected subcutaneously (s.c.) with 3LL cells (5×10^5). Tα1 (10, 100, 1000 μg/kg, at day 7 for consecutive 7 days), GEM (120 mg/kg, single injection at day 7), PBS (as control) alone or combined were injected intraperitoneally (i.p.). Tumor size was measured every 3 days.

2.4. Cell sorting and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from blood cells by Ficoll−Hypaque density gradient centrifugation. After erythrocyte lysis, splenocytes were stained with antibodies for flow cytometry. Data acquisition and analysis were performed on a FC500 MPL system (Beckman Coulter, USA). For MDSCs sorting, Splenocytes were stained with antibodies to CD11b and Gr-1, and sorted using MoFlo XDP cell sorter (Beckman Coulter, USA). The purity of the sorted cells was >97% as assessed by flow cytometry analysis.

2.5. T cells proliferation and cytokine secretion assay

T cell proliferation and IFN-γ secretion was measured as described [20]. In some experiments, NG-Methyl-L-arginine acetate salt (L-NMMA, Sigma−Aldrich, iNOS inhibitor) was added at 500 μmol/L, Nx-Hydroxy-nor-L-arginine, diacetate salt (nor-NOHA, Calbiochem, Arginase inhibitor) was added at 500 μmol/L.

2.6. GR-1 depletion and MDSCs adoptive transfer

Tumor-bearing mice were injected i.p. with 100 μg of anti-Gr-1 (Bio X Cell) at day 12, 13 and 14 after 3LL challenge, and Tα1 or PBS injection was initiated at day 12 for consecutive 7 days.

At day 27, the GR-1 depleted mice from Tα1 group mentioned above were equally subdivided into 2 groups (A and B), a total of 1×10^7 MDSCs from spleens of non-treated tumor-bearing mice were treated with Tα1 (100 ng/mL) or PBS for 24 h and then injected i.v. into mice of group A or B respectively.

2.7. Quantitative real-time PCR and western blotting

Total RNA was extracted from MDSCs with RNeasy Mini kit (Qiagen, Hilden, Germany) and reverse-transcribed to cDNA with Reverse Transcription System Kit (Takara, Japan). The real-time PCR assay was carried out with ABI7500 Fast Real-Time PCR System (Applied Biosystems, USA). Data are presented as normalized to housekeeping gene β-actin. Western blotting was performed as previously described [21].

2.8. Statistical analysis

Data were expressed as mean ± SD and were analyzed using SPSS version 19.0. Multiple comparisons were made between different groups with the Kruskal−Wallis H nonparametric test. Differences between two groups were compared by unpaired, two-tailed Student's t-test. The survival curves were estimated by Kaplan−Meier method. For all tests, two-sided $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Tα1 alone was insufficient to delay tumor growth

To test the anti-tumor activity of Tα1, gemcitabine (GEM), Tα1, or combination of Tα1 and GEM were used in a mice model of Lewis lung cancer (LLC), PBS was given as control , tumor growth was monitored. We found that treatment of LLC mice with GEM plus Tα1 therapy resulted in a significant reduction in tumor volume, the combined therapy showed stronger anti-tumor effect than GEM alone. However, tumor growth showed no significant differences in Tα1 (100 μg/kg) group, compared with control (Fig. 1A). Furthermore, various doses of Tα1 (10, 100, 1000 μg/kg) alone were used in LLC-bearing mice, the highest dose of Tα1 still showed no inhibition of tumor growth (Fig. 1B) and no differences were observed in tumor weight in all groups either (Fig. 1C). These results suggested that Tα1 synergized with GEM to inhibit tumor growth, but Tα1 alone was insufficient to delay tumor growth, and the anti-tumor effect of Tα1 was limited.

3.2. The effect of Tα1 on the frequency and function of MDSCs

To investigate the reason accounting for the failure of Tα1 in inhibiting tumor growth, we analyzed the frequency of splenic CD4$^+$ T cells, CD8$^+$ T cells, NK cells, and DCs, and found that only CD8$^+$ T cells was elevated significantly in Tα1 and Tα1 plus GEM group, compared with GEM or PBS group (Fig. 2A), no measurable changes were observed in the numbers of CD4$^+$ T cells, NK cells, and DCs (data not shown). GEM treatment showed no significant effect on all these cell types, including CD8$^+$ T cells. CD8$^+$ T cells level was comparable between Tα1 and Tα1 plus GEM group (Fig. 2A). Compared with GEM alone, Tα1 plus GEM treatment induced higher CD8$^+$ T cells (Fig. 2A), and showed stronger anti-tumor effect (Fig. 1A). Nevertheless, Tα1 alone did not inhibit tumor growth compared with control group (Fig. 1A), albeit higher CD8$^+$ T cells (Fig. 2A).

In cancer immune-suppressive network, MDSCs and regulatory T (Treg) cells are major components and influence tumor progression profoundly [22]. In previous report, GEM was capable of

selectively eliminating MDSCs level in tumor-bearing mice [23], here we found the frequency of MDSCs was decreased significantly in GEM and GEM plus Tα1 groups, but not changed in Tα1 groups; no differences in MDSC frequency were observed between GEM and GEM plus Tα1 group (Fig. 2B), indicating Tα1 had no effect on the expansion of MDSC. Then MDSCs from tumor-bearing mice in PBS, Tα1, GEM and Tα1 plus GEM groups (PBS-, Tα1-, GEM-, and Tα1 plus GEM-MDSCs) were sorted and cocultured with CFSE-labeled naïve PBMCs stimulated with anti-CD3/anti-CD28 or PMA/ionomycin respectively, we found the level of $CD8^+$ T cells and interferon-γ (IFN-γ) producing-$CD8^+$ T cells was significantly lower in Tα1-MDSCs and Tα1 plus GEM-MDSCs group but not changed in GEM-group, no differences were found between Tα1-MDSCs and Tα1 plus GEM-MDSCs group (Fig. 2C, D). These results suggested that Tα1 could strengthen the immunosuppressive ability of MDSCs, whereas GEM showed no effect on the suppressive function of MDSCs. We also examined the frequency and immunosuppressive function of Treg, but no changes were observed (data not shown).

To test whether MDSCs and its enhanced immunosuppressive function were responsible for the poor anti-tumor efficacy of Tα1, MDSCs in tumor-bearing mice were depleted by anti-Gr-1 antibody. The MDSCs-depleted mice were treated with Tα1 or PBS. We found tumor growth was significantly delayed in Tα1 group, compared with PBS group (Fig. 2E). Furthermore, MDSCs from non-treated tumor-bearing mice were sorted and cultured in the presence of Tα1 or PBS for 24 h, then adoptively transferred into the MDSCs-depleted mice, we found Tα1-treated MDSCs significantly promoted tumor growth compared with PBS-treated MDSCs (Fig. 2E). These results indicated that MDSCs impaired the anti-tumor effect of Tα1, the enhanced immunosuppressive function of MDSCs caused by Tα1 facilitated tumor progression.

3.3. *Tα1 enhanced the immunosuppressive ability of MDSCs by upregulating Arginase 1 through MyD88 signaling*

To identify molecular mechanisms that underlie the increased immunosuppressive function of MDSCs, we examined the relative gene expression of MDSC-related molecules by quantitative RT-PCR. The levels of ARG1 and iNOS were significantly elevated in MDSCs from Tα1 group compared with PBS group, whereas no significant differences were found in the expression level of indoleamine 2,3-dioxygenase (IDO), transforming growth factor-β (TGF-β), IL-6, IL-10, programmed death ligand 1 (PD-L1), NADPH oxidase (NOX1) and COX2 (Fig. 3A). To further identify whether elevated level of ARG1 or iNOS was responsible for the enhanced suppressive ability, MDSCs from mice of Tα1 or PBS group were pretreated with iNOS inhibitor L-NMMA or ARG1 inhibitor nor-NOHA and then cocultured with PBMCs from naïve mice, we found that nor-NOHA significantly abrogated the immunosuppressive effect of MDSCs and restored $CD8^+$ T cells proliferation and IFN-γ production. More importantly, after nor-NOHA but not L-NMMA treatment, the suppressive ability of MDSCs from Tα1 group was reduced to the same level as MDSCs from PBS group; a comparable level of $CFSE^{low}$ $CD8^+$ and IFN-γ^+ $CD8^+$ T cells was yielded between two groups (Fig. 3B), suggesting that the increased ARG1 level was responsible for the enhanced suppressive ability of Tα1-treated MDSCs.

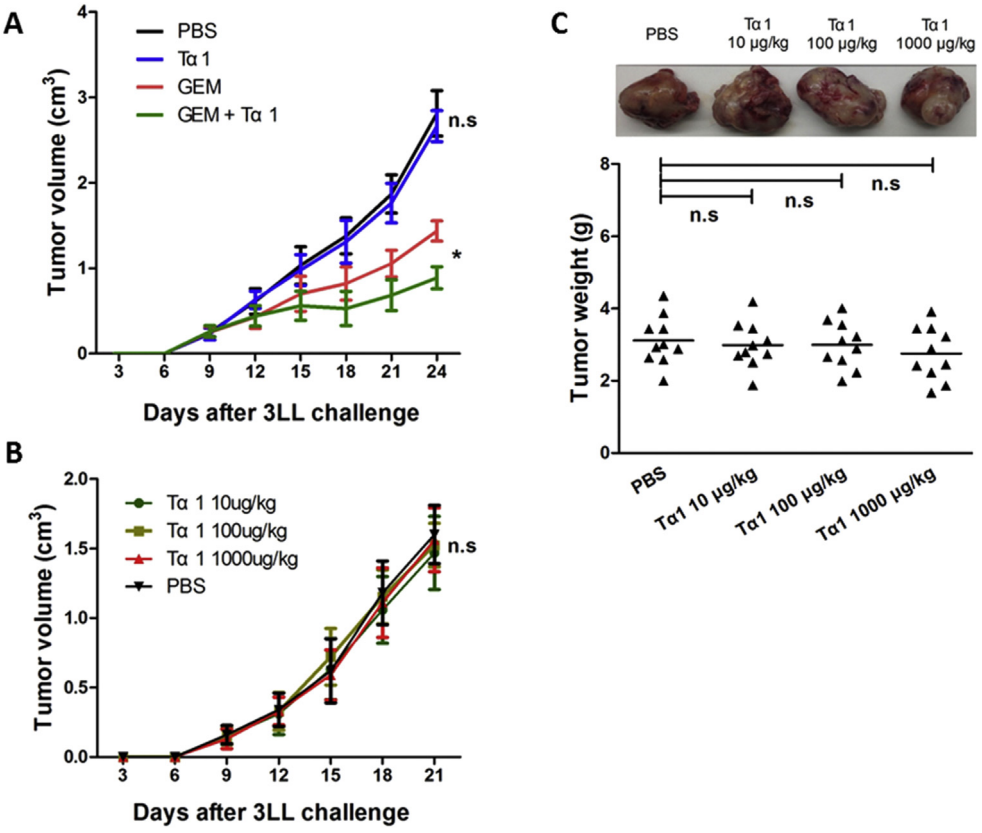

Fig. 1. Tα1 alone was insufficient to delay tumor growth. 3LL cells (5×10^5) were implanted s.c. into C57/BL6 mice, PBS, Tα1, GEM, and GEM plus Tα1 were injected respectively as described in Materials and Methods. (A) Tumor size in mice treated with PBS, Tα1 (100 μg/kg), GEM (120 mg/kg), or GEM (120 mg/kg) plus Tα1 (100 μg/kg). Various doses of Tα1 (10, 100, 1000 μg/kg) was given to tumor-bearing mice, (B) tumor size was assessed every 3 days; (C) at day 21 after 3LL cells inoculation, all mice were sacrificed and tumor weight was measured. (* = $P < 0.05$; n.s = not significant).

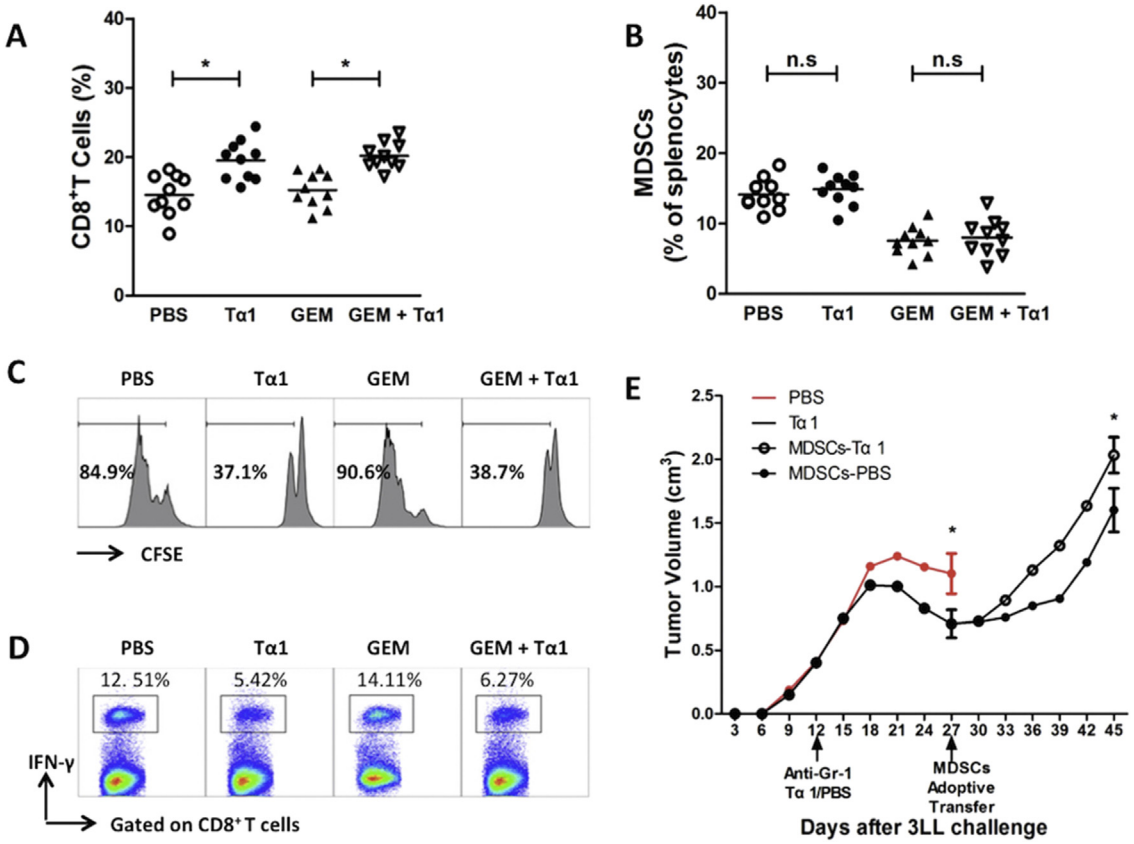

Fig. 2. The effects of Tα1 on frequency and function of MDSCs. Splenocytes from tumor-bearing mice were stained and subjected to flow cytometry. The frequencies of (A) $CD8^+$ T cells and (B) MDSCs were determined. MDSCs from Tα1 or PBS treated tumor-bearing mice were sorted and cocultured with CFSE-labeled PBMCs from naïve mice at ratio 1:10, (C) the proliferation of anti-CD3/anti-CD-28 stimulated $CD8^+$ T cells was assessed by CFSE diluent assay, and (D) IFN-γ producing-$CD8^+$ T cells after PMA/ionomycin stimulation was analyzed by flow cytometry. (E) Tumor-bearing mice were treated with Gr-1 antibody from day 12 after 3LL inoculation to deplete MDSCs, and then Tα1 or PBS was injected as described in materials and methods. At day 27, Gr-1-depleted tumor-bearing mice from Tα1 group were divided into two groups, MDSCs from tumor-bearing mice were sorted and treated with Tα1 (MDSCs-Tα1) or PBS (MDSCs-PBS) for 24 h and then injected i.v. into mice of two groups mentioned above respectively, tumor growth was evaluated every 3 days. (* = $P < 0.05$; n.s = not significant).

To further confirm the elevated ARG1 expression was induced by Tα1, MDSCs were sorted from splenocytes of tumor-bearing mice, and treated with PBS or various doses of Tα1 respectively in vitro. 24 h later, we detected a significant induction of ARG1 in Tα1 group in a dose-dependent manner (Fig. 3C).

Tα1 can activate the MyD88-dependent pathway of TLRs signaling in DCs [5], and TLRs/MyD88 signaling pathway also plays a role in the expansion of MDSCs [24,25]. We therefore tested whether the upregulation of ARG1 by Tα1 is dependent on TLR/MyD88 signaling. MDSCs were sorted from spleens of tumor-bearing mice and TLRs expression on MDSCs was assessed by conventional RT-PCR; we found that TLR1-9 except TLR3 and TLR5 were expressed on MDSCs (Fig. 3D). As a pivotal adaptor protein, MyD88 binds to all TLRs with the exception of TLR3 [26]. To investigate whether MyD88 was involved in the upregulation of ARG1 by Tα1, sorted MDSCs from tumor-bearing mice were treated with various doses of Tα1 for 24 h, then we assessed the level of MyD88 protein in MDSCs, and found Tα1 treatment significantly increased the expression of MyD88 (Fig. 3E). Furthermore, sorted MDSCs from tumor-bearing mice were pretreated with MyD88 inhibitor (MyD88i) or control peptide for 24 h before subjected to Tα1, we found that MyD88i abrogated the elevated expression of ARG1 (Fig. 3F), and $CD8^+$ T cells proliferation was also enhanced (Fig. 3G). Taken together, these results suggested that the upregulation of ARG1 induced by Tα1 is dependent on MyD88 signaling.

3.4. Blockade of MyD88 signaling inhibits Tα1-mediated MDSC activation in vivo and enabled Tα1 to delay tumor growth

To test the effect of MyD88 blockade in vivo, 3LL was injected s.c. into $MyD88^{-/-}$ mice, and Tα1 was used to treat tumor-bearing mice. We found Tα1 inhibited tumor growth significantly compared with PBS group (Fig. 4A).

Although Tα1 showed effective anti-tumor effect in $MyD88^{-/-}$ tumor-bearing mice, all tumor-bearing mice died eventually within 2 months after tumor transplantation (Fig. 4B). Thus we further added GEM to Tα1 therapy to eliminate MDSCs, the combined therapy significantly inhibited tumor growth (Fig. 4A) and prolonged the survival of $MyD88^{-/-}$ tumor-bearing mice compared with Tα1 therapy (Fig. 4B), moreover, when wild-type (W.T.) tumor-bearing mice were treated in the same manner, although the survival of W.T. tumor-bearing mice was improved by Tα1 plus GEM therapy, $MyD88^{-/-}$ tumor-bearing mice treated with the combined therapy showed significantly prolonged survival compared with W.T. tumor-bearing mice (Fig. 4B).

4. Discussion

Although Tα1 was reported to act on immune effector cells and even tumor cells to inhibit tumor progression [11,27], its anti-tumor effect is controversial and still needs to be clarified. The underlying regulatory mechanisms of Tα1 in cancer therapy and its effects on

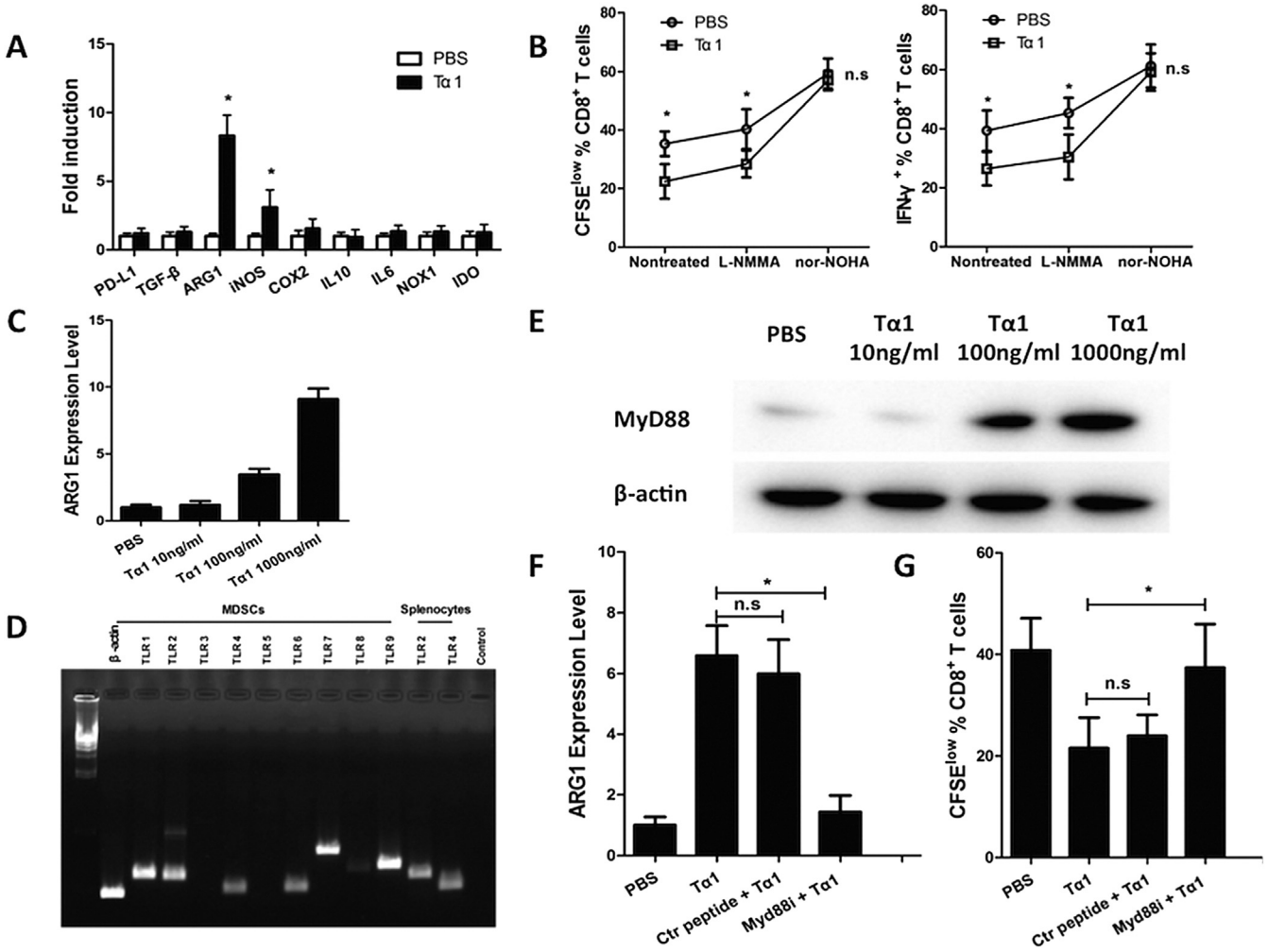

Fig. 3. Tα1 upregulates ARG1 expression of MDSCs through MyD88 signaling. (A) Splenic MDSCs were sorted from PBS or Tα1 treated tumor-bearing mice, and MDSC-related molecules were assessed by quantitative RT-PCR. (B) MDSCs from PBS or Tα1 group were pretreated with L-NMMA (iNOS inhibitor, 500 μmol/L), nor-NOHA (Arginase inhibitor, 500 μmol/L) respectively and then cocultured with PBMCs from naive C57/BL6 mice, the immune-suppressive function of MDSCs was assessed by $CD8^+$ T cells proliferation and IFN-γ production. (C) MDSCs from tumor-bearing mice were treated with PBS or different doses of Tα1 for 24 h; ARG1 expression of MDSCs was assessed by quantitative RT-PCR. (D) TLRs expressions in MDSCs were determined by conventional RT-PCR. (E) After various doses of Tα1 treatment, MyD88 level of MDSCs was assessed by western blotting. (F) The effect of MyD88i or control peptide on ARG1 level. (G) The effect of MyD88i or control peptide on immune-suppressive function of MDSCs, proliferation of $CD8^+$ T cells was assessed by CFSE diluent assay. (* = $P < 0.05$; n.s = not significant).

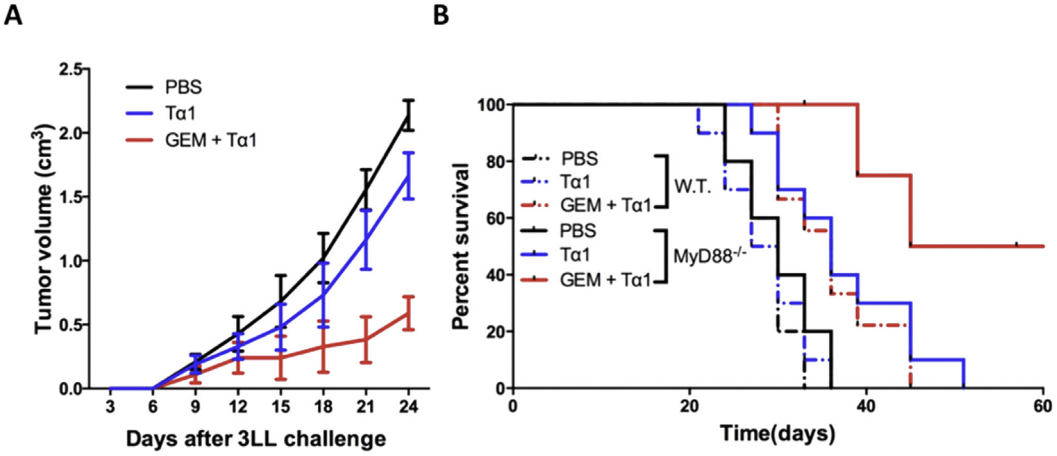

Fig. 4. Tα1 shows anti-tumor efficacy in $MyD88^{-/-}$ tumor-bearing mice. 3LL cells (5×10^5) were implanted s.c. into $MyD88^{-/-}$, Tα1 (100 μg/kg), or GEM (120 mg/kg) plus Tα1 (100 μg/kg) was injected i.p. as described in Materials and Methods. (A) Tumor growth was measured every 3 days. (B) Survival curve of $MyD88^{-/-}$ and wild-type (W.T.) tumor-bearing mice after Tα1, or Tα1 plus GEM treatment , survival curve of W.T. tumor-bearing mice was shown as a comparison. (* = $P < 0.05$).

immunosuppressive cells remain unknown. Using a murine model, we report that Tα1 treatment alone failed to inhibit tumor growth, when administrated in tumor-bearing mice, Tα1 not only facilitated the increase of $CD8^+$ T cells, but also enhanced the immune-suppressive function of MDSCs, leading to inhibition of T-cell response and ultimately impaired anti-tumor efficacy.

MDSCs-mediated T-cell suppression has been associated with many immune-suppressive factors [14], our data showed that the upregulation of ARG1 in Tα1-treated MDSCs was responsible for the enhanced immune-suppressive function. Although no specific membrane receptors have been identified, Tα1 was reported to interact with Toll-like receptors and activate MyD88 signaling in DCs [5,28,29]. In this study, we demonstrate that Tα1 can also activate TLRs/MyD88 pathway in MDSCs and increase ARG1 expression, blocking MyD88 signaling abrogated the upregulation of ARG1 and restored anti-tumor efficacy of Tα1.

TLRs/MyD88 signaling can initiate both protumorigenic and anti-tumor response [30], although TLR2/4 agonist BCG and TLR9 agonist CpG, oligodeoxynucleotides(ODN) have shown efficacy in cancer treatment, most TLR agonists used as single agent failed to inhibit tumor progression [30–32]. Here we found that Tα1 also acted as a TLR agonist and activated MDSCs through TLRs/MyD88 signaling. In inflammation and cancer, MyD88 signaling is responsible for the activation and expansion of MDSCs [25,33], however, we didn't observe a measurable increase in MDSCs after Tα1 treatment in tumor-bearing mice. Furthermore, bone marrow cells of tumor-bearing mice were isolated and treated with Tα1, and no significant expansion of MDSCs was found either (data not show). These results suggest that activation of TLRs/MyD88 signaling by Tα1 and the exact mechanisms by which MyD88 signaling acts on MDSCs still need to be further investigated.

In conclusion, our data show a new perspective of Tα1 able to activate MDSCs through MyD88 signaling and restrain immune response in a Lewis lung cancer model. Furthermore, the enhanced immune-suppressive function of MDSCs induced by Tα1 presents a new variable that needs to be considered in the future design of Tα1 involved preclinical and clinical trials. Better Tα1 combined therapies need to be designed to maximize the anti-tumor efficacy and to minimize the immunosuppression induced by Tα1.

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