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Mn Complex-mediated Enhancement of Antitumor Response Through Modulating Myeloid-derived Suppressor Cells in Drug-resistant Tumor

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Abstract. *Background: The tumor microenvironment (TME) renders tumor cells more resistant to chemotherapy. However, effective immunomodulators for cancer therapy are still elusive. We hypothesized that Mn-N-(2-hydroxyacetophenone) glycinate (MnNG), reported to be an antitumor agent, can modulate the TME. Materials and Methods: Immunomodulatory effects of MnNG were performed through assessing Myeloid Derived Suppressor Cells (MDSCs), Interferon-γ (Ifnγ)- and Interleukin-4 (Il4)-secreting Cluster of Differentiation 4 (Cd4)$^+$ T-cells by annexin V-binding assay in drug-resistant TME and T-cell proliferation following in vitro co-culture assay by flow cytometry. Results: MnNG induced infiltration of Ifnγ-secreting Cd4$^+$ T-cells and reduces MDSC numbers in vivo. Furthermore, it modulated differentiation of MDSCs towards dendritic cells with up-regulation of co-stimulatory molecules and reversed the suppressive function of MDSC's that enhances T-helper cell 1 (Th1) response. MnNG treatment resulted in reduced expression of IL4, but enhanced expression of Ifnγ when Cd4$^+$ T-cells were co-cultured with MDSCs. Conclusion: MnNG modulates MDSCs differentiaton towards dendritic cells and enhances Th1 response in drug-resistant TME, leading to immunomodulatory efficacy.*

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Despite advances in drug discovery, chemotherapeutic agents have not overcome the problem of resistance to cancer drugs in preclinical or clinical scenarios (1). Multiple mechanisms are employed by cancer cells that favor the generation of multidrug resistance (MDR) (2). The immunosuppressive network created by the interactions of tumor cells with non-transformed cells in the tumor microenvironment (TME) is a determining factor in manipulating tumorigenic activities and tumor responses to anticancer drugs (3-5). Among the immunosuppressive cells in the TME, myeloid-derived suppressor cells (MDSCs) are prominent. MDSCs represent a heterogeneous population that encompasses early immature precursors of macrophages, granulocytes, and dendritic cells (6). A large number of studies have suggested that MDSCs are a major culprit in tumor-induced immune dysfunction and cancer progression (6, 7). Multiple evidence has indicated that MDSCs are potent inhibitors of both antigen-specific and non-specific T-cell activation (8). In addition to T-cell suppression, they further impair innate immunity by interacting with macrophages, natural killer cells and natural killer T-cells and thus skew tumor immunity towards tumor-promoting type 2 responses (9-11). Hence to improve the efficacy of chemotherapeutics, it is necessary not only to develop strategies to kill cancer cells efficiently, but also to attempt to stimulate an immune response by subverting or modulating the immunosuppressive TME towards a proimmunogenic type.

Previously, we found a metal complex of Schiff base showed tremendous potential as an immunomodulator against drug-resistant tumor in mice (12-14). Our studies revealed that Cu complex generated a protective antitumor T-helper cell 1 (Th1) response by selectively killing MDSCs from a drug-resistant TME (15). Furthermore, we also studied the antitumor activity of novel Mn-*N*-(2-hydroxyacetophenone) glycinate (MnNG) on doxorubicin-resistant Ehrlich ascites carcinoma (EAC/Dox) cells (16).

Herein we investigated the immunomodulatory activity of MnNG in drug-resistant EAC-bearing mice.

Materials and Methods

Reagents. Penicillin and streptomycin were purchased from Sigma (Sigma Chemical Company, St. Louis, MO, USA). Mouse-specific Fluorescein isothiocyanate (FITC)-conjugated F4/80 monoclonal antibody (mAb) (clone no. BM8), Granulocyte-differentiation antigen-1 (Gr1) (clone no RB6-8C5), cluster of differentiation 4 (Cd4) (clone no. GK1.5), Cd8 (clone no. 53–6.7), Phycoerythrin (PE)-conjugated Cd11b (clone no. M1/70), Interleukin 4 (Il4) (clone no. BVD4-1D11), and Interferon γ (Ifnγ) (clone no. XMG1.2), and anti-mouse Cd3 (clone no. 17A2), Cd28 (clone no. 37.51), FITC-labeled annexin V and recombinant mouse Granulocyte-macrophage colony-stimulating factor (GM-CSF) were obtained from Biolegend (San Diego, CA, USA). Mouse-specific Allophycocyanin (APC)-conjugated Cd80 (clone no.16-10A1), FITC-conjugated rat anti-mouse CD86 (clone no.GL1) were purchased from BD Bioscience (San Diego, CA, USA). RPMI-1640 and Fetal bovine serum (FBS) were purchased from Gibco (Invitrogen Corp., Carlsbad, CA, USA).

Cell line, tumor implantation and experimental protocol. Swiss albino mice, obtained from the National Institute of Nutrition (Hyderabad, India) and maintained in the institute animal facilities, were used for experimental purpose with prior approval of the Institutional Animal Ethics Committee. The experimental protocols described herein were approved by the Institutional Animal Ethics Committee (Registration No: 175/99/CPCSEA, dated 28 January 2000) in accordance with the ethical guidelines laid down by the Committee for the purpose of Control and Supervision of Experiments on Animals by the Ministry of Social Justice and Empowerment, Government of India. Doxorubicin-sensitive and -resistant EAC cells were maintained in the peritoneal cavity of mice following a previously described method (17-19). Each experimental group comprises of 18 female Swiss albino mice of 6-8 weeks of age having average body weight of 25 grams. Doxorubicin resistant Ehrlich Ascites Carcinoma (EAC/Dox) cells were maintained in doxorubicin-free condition for at least one passage before the start of all the experiments.

Herein, EAC/Dox-bearing mice (nine days following peritoneal inoculation with 1×10^6 EAC/Dox cells) were remained either untreated (control) or treated intraperitoneally (*i.p.*) with our synthesized compound, MnNG (16) solubilized in di-methyl sulfoxide (<0.25%) at day 0 (*i.e.* on 9th day following tumor implantation) at a dose of 15mg/kg. To check the infiltration of MDSCs and Ifnγ$^+$ or Il4$^+$ Cd4$^+$ T-cell population in the tumor site, immune cells were harvested and analyzed on 7th day for one set of experiment and on 15th day for another set following MnNG treatment. In both the cases, treated group were compared with their respective untreated control.

We specifically focused on these time intervals in order to evaluate and compare the modulation of immune parameters when the tumor load is low (*i.e.* at day 7) and when tumor load is high (*i.e.* at day 15). For some experiments, EAC/Dox-bearing mice were treated with MnNG for 15 days.

Treatment. A stock solution of MnNG (10^{-2} M) was prepared just before the experiments dissolving the lyophilized compounds in 0.2% dimethyl sulfoxide (DMSO) following serial dilution in FBS containing medium to reduce the concentration of methanol to nontoxic level *i.e.* less than 0.25% of DMSO concentration. For *in vitro* work FeNG treatments were performed with a concentration range of 10^{-3} M to 10^{-8} at 37˚C in RPMI medium supplemented with FBS. The dose of MnNG and its IC_{50} (4.8×10^{-4} M) value was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay using normal spleen cells (data not shown). For *in vivo* experiments the treatments were given with 15 mg/kg body weight of MnNG, diluting with PBS; equal volumes of medium or PBS were added to untreated cells or injected *i.p.* *in vitro* or *in vivo* experiments respectively.

Isolation and in vitro culture of MDSCs and CD4$^+$ T-cells from tumor site. MDSCs were isolated according to a previously described method (20). Total ascitic fluid was drawn from EAC/Dox-bearing mice and left in a 50-ml sterile tube for at least 2 h to allow settling of the tumor cells. Clear fluid from the upper zone was collected. MDSCs were isolated from this fluid by positive selection with anti-Gr1 (Ly6G$^+$/Ly6C$^+$) Direct Magnet (clone no. RB6-8C5) particle by using BD IMagnet system (BD Bioscience) according to the manufacturer's protocol. Cd4$^+$ T-cells were isolated from ascetic fluid by positive selection with anti-mouse Cd4 Direct magnet (clone no. GK1.5) particle. The purified population was resuspended in RPMI-1640 containing 10% FBS. Flow cytometric data revealed that purity of the separated population was 90%. For *in vitro* experiments, MDSCs were co-cultured with purified CD4+ T-cells in presence or absence of MnNG at a 1:1 ratio in RPMI-1640 supplemented with 10% FBS in an atmosphere with 5% CO_2 at 37˚C.

Measurement of intracellular cytokines from CD4$^+$ T-cells. MDSCs were co-cultured with CD4$^+$ T-cells for three days at a 1:1 ratio in 96-well flat-bottom plates in the presence of antibodies to Cd3 and Cd28. To check intracellular cytokine production, following co-culture, the Cd4$^+$ population was isolated with anti-mouse Cd4 magnetic particle–DM (clone no. GK1.5) then permeabilized with permeabilizing solution (BD Biosciences) and stained with PE-conjugated anti-Ifnγ or anti-Il4. Flow cytometric analysis was performed by using FACS caliber (BD Biosciences) with Cell Quest software.

Carboxyfluorescein succinimidyl ester (CFSE) labeling and T-cell suppression assay. Cd4$^+$ T-cells were purified from EAC/Dox-bearing mice using anti mouse Cd4 magnetic particle-DM (clone no. GK1.5). Following two washes with PBS, purified T-cells were (2×10^6 cells/ml) mixed with 1 μM CFSE solution for 7 min. The reaction was terminated by the addition of equal volumes of FBS and the cells were further incubated for 10 min. The resulting CFSE-Iabeled cells were then washed twice with RPMI-1640 with 10% FBS. In a U-bottom 96-well cell culture plate pre-coated with anti-Cd3 (1 μg/ml) and anti-Cd28 (5 μg/ml), purified T-cells (2×10^5) were cultured in RPMI-1640 supplemented with 10% FBS for 72 h. To determine the impact of MDSCs on T-cell proliferation, CFSE labeled T-cells were cultured alone or co-cultured at 1:1 ratio with purified Gr1$^+$ MDSCs (2×10^5) from doxorubicin resistant Ehrlich Ascites Carcinoma (EAC/Dox) bearing TME and changes in proliferation were assessed by flow cytometry. The level of T-cell proliferation was determined by loss of CFSE fluorescence.

Statistical analysis. All data reported are the arithmetic mean±SD of three independent experiments performed in triplicate unless stated otherwise. The unpaired Student's *t*-test was used to evaluate

the significant differences between groups, accepting $p<0.05$ as the level of significance. Data analyses were performed using Prism software (GraphPad, San Diego, CA, USA). Flow cytometric data are representative of at least three independent experiments.

Results

The effect of MnNG on MDSC and T-cell infiltration in the TME. Previously we found that intraperitoneal treatment of MnNG at 15 mg/kg body-weight significantly increased the lifespan of EAC/Dox-bearing mice, which was almost three-fold higher than that of untreated mice (16). On the basis of these previous data, we investigated the immunomodulatory activity of MnNG in drug-resistant EAC-bearing mice. To study the immunomodulatory property of MnNG, we first studied the role of MnNG on MDSC accumulation in theTME. To this end ascitic fluid was drawn-off from MnNG treated and untreated EAC/Dox-bearing mice at 7 and 15 days, and the percentage of $Gr1^+Cd11b^+$ cells were analyzed by flow cytometry. We found that the frequency of $Gr1^+Cd11b^+$ MDSCs in the TME of tumor bearing mice was reduced in a time-dependent fashion (Figure 1A).

The effect of MnNG on T-cell infiltration under a drug-resistant TME were analyzed by studying the frequency of $IFN\gamma^+$ tumor-infiltrating $Cd4^+$ and $Cd8^+$ T-cells. The data summarized in Figure 1B show that injection of 15 mg/kg MnNG *i.p.* to doxorubicin-resistant EAC-bearing mice significantly increased the percentage of Ifnγ-secreting $Cd4^+$ T-cells (day 7, $p=0.0031$; day 15, $p<0.0001$). We also studied the effect of MnNG on Il4-secreting $Cd4^+$ T-cells and found that MnNG treatment markedly reduced the number of these cells (Figure 1C). However no significant difference was observed in Ifnγ-secreting $Cd8^+$ T-cells (data not shown).

MnNG does not kill MDSCs but induces their in vivo differentiation. Treatment with MnNG reduced the number of MDSCs in the TME (Figure 1A). Hence we wanted to investigate whether MnNG converted the immature myeloid cells into mature form or whether it killed MDSCs *in vivo*. To investigate this possibility, MDSCs were isolated from MnNG treated and untreated EAC/Dox-bearing mice on day 7 and 15. The effect of MnNG on differentiation of MDSC into macrophage and dendritic cells was studied by analyzing the expression of Cd11c, F4/80 marker. Flow cytometric analysis revealed that the expression of Cd11c increased significantly following MnNG treatment (Figure 2A). However no changes were observed in F4/80 marker when isolated and compared between MnNG-treated and untreated mice (Figure 2B). The expressions of different co-stimulatory markers (such as Cd80 and Cd86) were also compared by flow cytometric analysis between untreated and treated cancer-bearing mice. We noted a significant increase in the expression of both Cd80 and Cd86 compared to untreated controls at day 15 (Figure 2C and D). However, expression of Cd86 was not comparable at early-day (day 7) treatment (Figure 2D). We further evaluated whether the reduction in number of MDSCs in TME is associated with MnNG-mediated apoptosis of MDSCs. To investigate this, MDSCs were isolated from MnNG-treated or untreated tumor-bearing mice at different times (days 7 and 15) and annexin V binding assay was performed. However, the study revealed no significant differences between MnNG-treated and untreated cells (Figure 2E).

MnNG does not modulate tumor-associated $Cd4^+$ T-cell response in vitro. As mentioned in the previous section, MnNG treatment enhanced the infiltration of $Ifn\gamma^+$ and reduced the number of $Il4^+$ $Cd4^+$ T-cells in the TME (Figure 1B and C). We investigated whether MnNG could also generate a similar effect *in vitro*. Tumor-associated $Cd4^+$ T-cells were isolated from untreated tumor-bearing mice and further cultured *in vitro* for 48 h in the presence and absence of MnNG and then the proportion of $Ifn\gamma^+$ or $Il4^+$ $Cd4^+$ T-cells were analyzed with flow cytometry. MnNG treatment did not alter Ifnγ expression from $Cd4^+$ T-cells (Figure 3A). Furthermore, no significant difference was observed in the Il4-secreting $Cd4^+$ population compared to untreated controls (Figure 3B).

MnNG can induce Th1 response by reducing the immune-suppressive activity of tumor-associated MDSCs in vitro. As mentioned (Figure 2), *in vivo* treatment of MnNG helped maturation of MDSCs into dendritic cells. Moreover, MnNG also increased the number of Th1 cells and reduced the $Cd4^+Il4^+$ cells in the TME, whereas *in vitro* treatment did not modulate $Cd4^+$ T-cells stained for either Ifnγ or Il4. Hence we raised the question whether modulation of the $Cd4^+$ T-cell response *in vivo* by MnNG actually depends on the maturation of MDSCs to dendritic cells. To decipher the underlying mechanism, tumor-associated $Cd4^+$ T-cells and MDSCs were isolated from untreated tumor-bearing mice. T-Cells were co-cultured with MDSC at 1:1 ratio for 48 h in presence or absence of MnNG. The $Cd4^+$ T-cells were then analyzed for intracellular Ifnγ and II4. MnNG treatment significantly increased the percentage of $Ifn\gamma^+$ T-cells and reduced the number of II4-secreting T-cells compared to untreated cells (Figure 4A and B). We further investigated whether MnNG could reverse the MDSC-mediated T-cell suppression. To this end CFSE-labeled $Cd4^+$ T-cells were co-cultured with MDSCs isolated from drug-resistant TME at 1:1 ratio and after 72 h of MnNG treatment the changes in proliferation were assessed by flow cytometry. When $Cd4^+$ T-cells isolated from TME were cultured alone without anti-Cd3/Cd28 stimulation, proliferation of T-cells was inhibited. Furthermore, proliferation was inhibited when antibody-stimulated $Cd4^+$ T-cells were cultured with MDSCs in the absence of MnNG. But interestingly, we noted that MDSC-

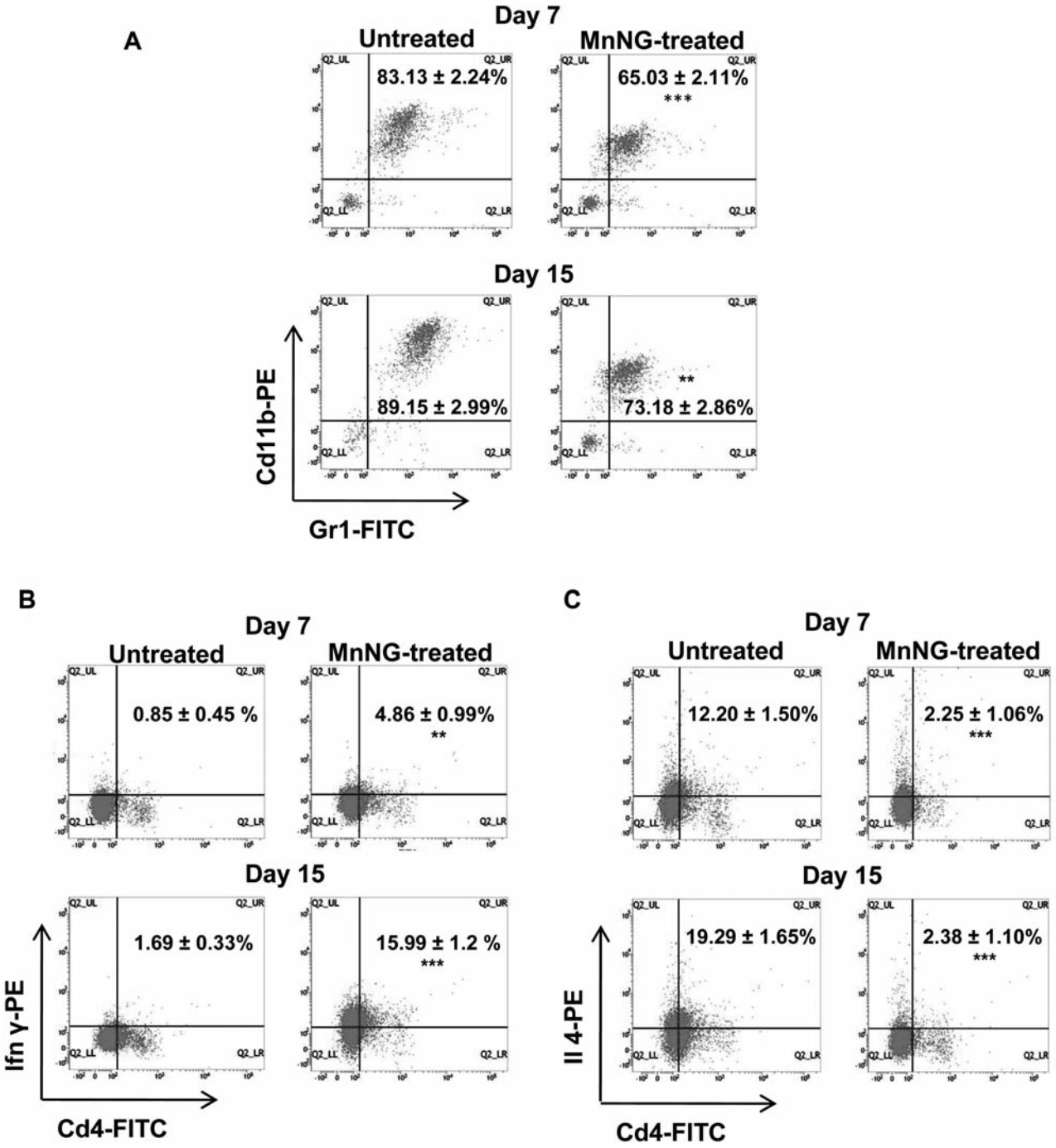

Figure 1. *Mn-N-(2-hydroxyacetophenone) glycinate (MnNG) mediated reduction of Myeloid Derived Suppressor Cells (MDSCs) and Interleukin 4 (Il4)-secreting Cluster of Differentiation 4 (Cd4)$^+$ T-cell number and augmentation of Interferon γ (Ifnγ)-secreting Cd4$^+$ T-cell infiltration in Doxorubicin resistant Ehrlich Ascites Carcinoma (EAC/Dox)-bearing mice. Ascitic fluid from MnNG-treated or untreated EAC/Dox-bearing mice was drawn off after two different time points (7 and 15 days) and total cell populations were collected (see materials and methods for details). Cells were labeled with Fluorescein isothiocyanate (FITC)-conjugated anti-Granulocyte-differentiation antigen-1 (Gr1) and Phycoerythrin (PE)-conjugated anti-Cd11b to check MDSC infiltration (A). Total cells were labeled with either FITC-conjugated anti-Cd4 and PE-conjugated anti-Ifnγ, or FITC-conjugated anti-Cd4 and PE-conjugated anti-Il4 and analyzed by flow cytometry to determine the status of Th1 (B) and Th2 (C) cells in the tumor microenvironment. Immunofluorescence analysis was performed by using FACS caliber (BD Biosciences) with CellQuest software. All data are presented as the mean±standard deviation of three independent studies. Statistically significant difference at **p<0.01, ***p<0.001; ns: not statistically significantly different (p>0.05).*

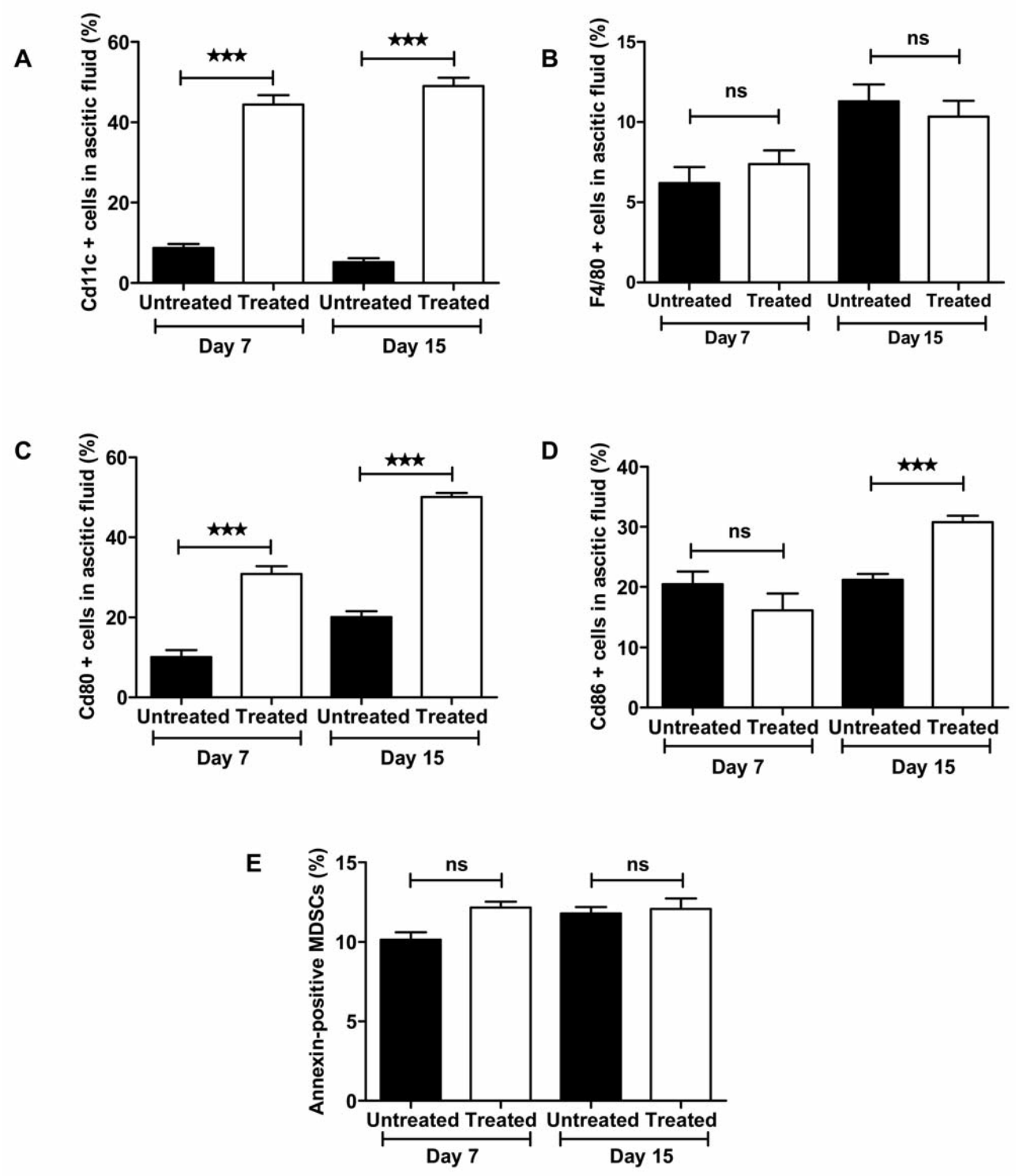

Figure 2. *Mn-N-(2-hydroxyacetophenone) glycinate (MnNG) does not kill Myeloid Derived Suppressor Cells (MDSCs) but rather induces their differentiation in vivo. Ascitic fluid was taken from both MnNG-treated and untreated Doxorubicin resistant Ehrlich Ascites Carcinoma (EAC/Dox)-bearing mice at different time intervals and total cells were labeled with either Phycoerythrin (PE)-conjugated anti-F4/80 or Allophycocyanin (APC)-conjugated anti-Cluster of differentiation (Cd) 11c. Prevalence of F4/80+ macrophages (A) and Cd11c$^+$ dendritic cells (B) along with co-stimulatory molecules Cd80 (C) and Cd86 (D) present in the ascitic fluid was determined by flow cytometry. Apoptosis of MDSCs was estimated by annexin V binding assay using flow cytometry (E). Representative data of three independent experiments are presented. Statistically significant difference at *p<0.05, ***p<0.001; ns: not statistically significantly different (p>0.05).*

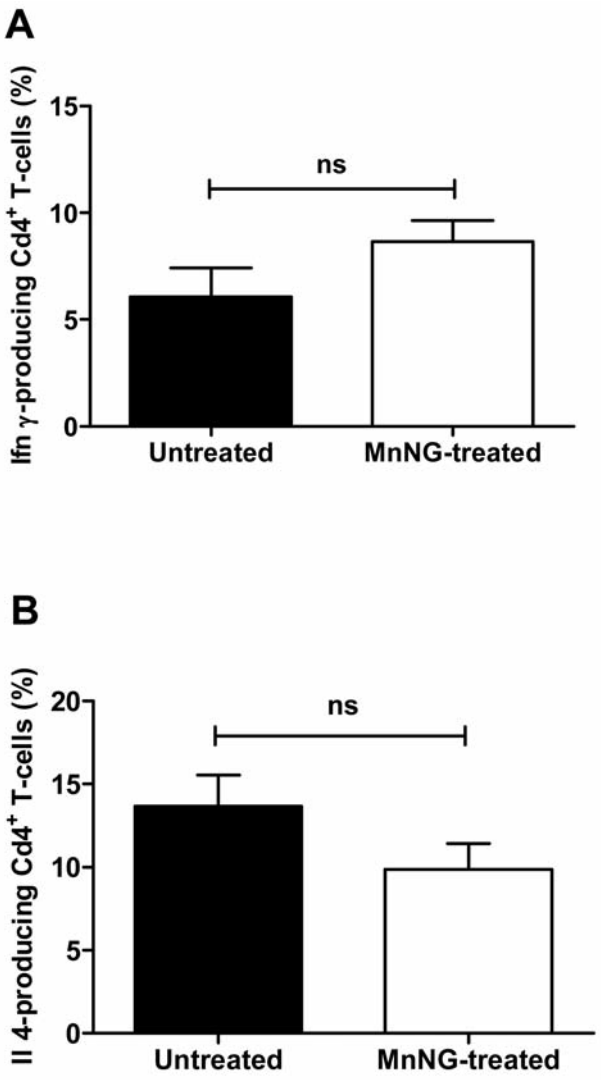

Figure 3. *In vitro Mn-N-(2-hydroxyacetophenone) glycinate (MnNG) treatment does not alter of cytokines profile of tumor associated Cluster of differentiation 4 (Cd4)+ T-cells. In vitro MnNG treatment (4.8×10^{-4} M) did not change Interferon γ (Ifnγ) (A) or Interleukin 4 (Il4) (B) production from Cd4+ T-cells of tumor associated lymphocytes of untreated doxorubicin resistant Ehrlich Ascites Carcinoma (EAC/Dox)-bearing mice. Immunofluorescence analysis was performed by flow cytometry. Representative data of three independent experiments are presented. ns: not statistically significantly different (p>0.05).*

mediated T-cell suppression was abrogated when MnNG was applied under co-culture conditions (Figure 4C).

Discussion

The ability of tumor cells to acquire resistance to cytotoxic drugs is a major obstacle for successful application of cancer treatment. Numerous mechanisms have been suggested to explain the development of MDR phenotype in cancer cells, such as a change in the specific target of a drug (1), reduced uptake or increased efflux of a drug (21, 22), differential compartmentalization (23, 24), increased rate of drug detoxification (25), increased ability to repair DNA damage (26, 27), gene amplification and an increase in the activity of survival proteins and reduced capacity to undergo apoptosis (28-30). In addition, recent studies highlight the role of the TME as an important factor significantly contributing to the emergence of therapeutic resistance (31, 32). The complex interactions between cancer cells and host immune cells in the TME create an immunosuppressive network that promotes tumor growth, protects the tumor from immune attack and attenuates therapeutic efficacy (33). Among immune cells infiltrating the TME, MDSCs play one of the most important roles in mediating immune suppression. These cells directly stimulate tumorigenesis, as well as tumor growth and expansion, by inhibiting the protective antitumor response. A number of strategies have been employed to eliminate or alter the function of MDSCs in order to break the immunosuppressive network (34-39). One is to promote differentiation of MDSCs into mature cells that no longer possess suppressive functions. All *trans*retinoic acid (ATRA), a vitamin A metabolite promotes differentiation of myeloid progenitor cells into DCs and macrophages (that do not have suppressive functions) (40). Although recent studies have identified one MDSC differentiating agent Sunitinib (41), the emergence of compounds capable of modulating the suppressive network by inducing the differentiation of MDSCs is of great clinical importance.

The present study describes the immunomodulatory property of MnNG, a metal chelate of manganese which posses a number of valence states. The study reveals that MnNG possess the ability to modulate the immunosuppressive TME by reducing the number of tumor-associated MDSCs in drug-resistant EAC cell-bearing mice (Figure 1A).

T-cells present in TME are less capable of Ifnγ production (42) and in TME, Th2 Il4–secreting $Cd4^+$ cells promote increased tumor cell growth and invasion (43). In consequence the host immune system is unable to control the growing tumor *in vivo* that ultimately leads to the failure of most immune therapies. Modulation of such events may hint at improved therapeutic efficacy. In this context, we observed that MnNG treatment enhanced Th1 responses (evident from increase in Ifnγ production from $Cd4^+$ T cells) and diminished Th2 responses (decrease in Il4 production from $Cd4^+$ T cells) in TME (Figure 1B and C). However, Ifnγ production from $Cd8^+$ T-cell was not significantly enhanced (data not shown) following MnNG treatment.

Previous reports disclosed that MDSCs under normal differentiation condition differentiates into mature dendritic cells, macrophages, and/or granulocytes. In tumor-bearing hosts, the presences of tumor-derived factors block such

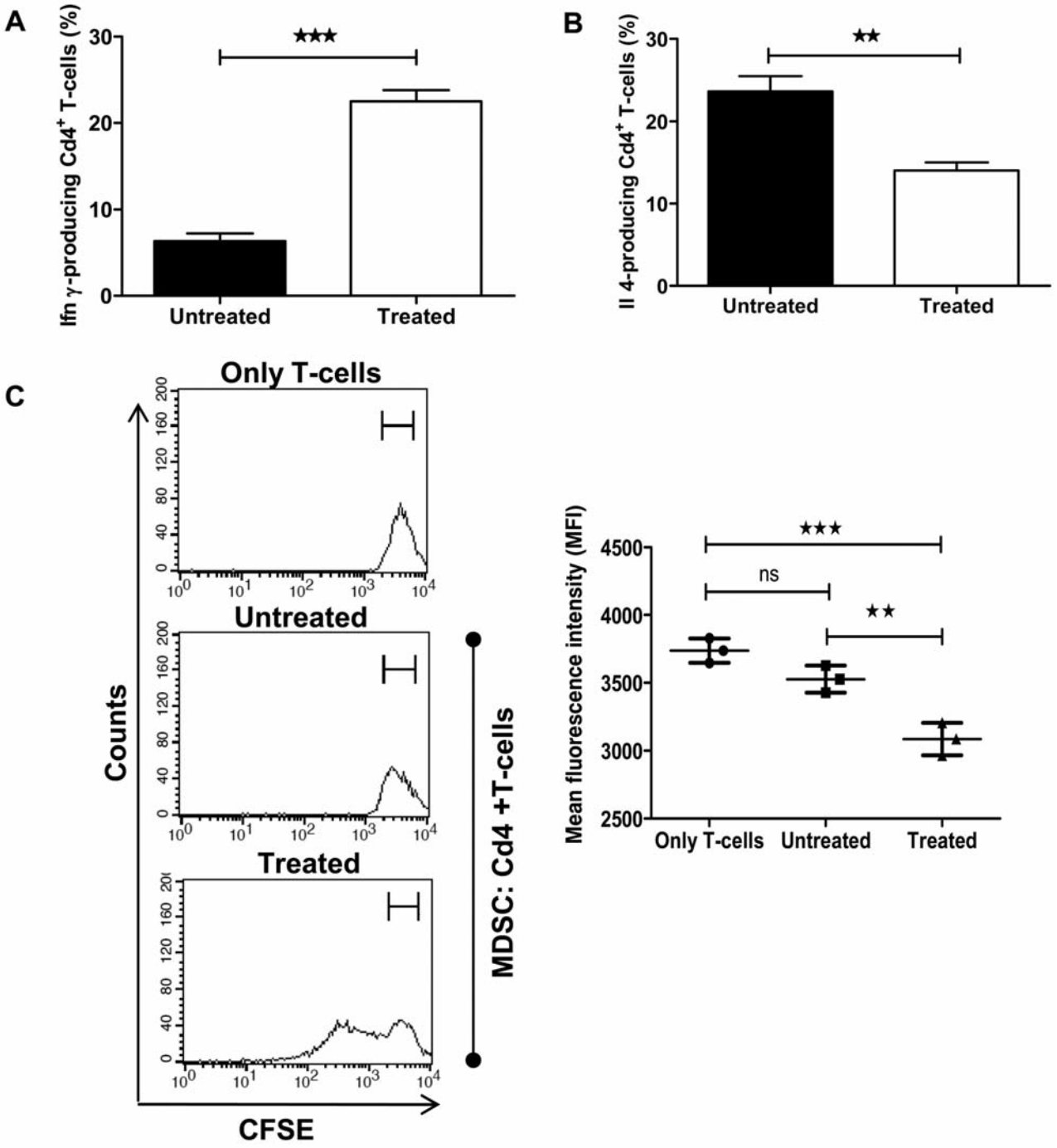

Figure 4. *Mn-N-(2-hydroxyacetophenone) glycinate (MnNG)-mediated modulation of Myloid Derived Suppressor Cells (MDSCs) in Doxorubicin resistant Ehrlich Ascites Carcinoma (EAC/Dox)-bearing mice redirects tumor-associated Cluster of differentiation 4 (Cd4)$^+$ T-cells toward Th1 type by reversion of T-cell suppression. MDSCs were isolated with Granulocyte-differentiation antigen-1 (Gr1) Direct Magnet (DM) particle and co-cultured with Cd4$^+$ population for 72 h at 1:1 ratio in 96-well flat-bottom plates with/without MnNG (4.8×10^{-4} M). For intracellular cytokines, the Cd4$^+$ population was labeled with FITC-conjugated anti-Cd4 and treated with permeabilizing solution, and stained with PE-conjugated anti-Ifnγ (A), PE-conjugated anti-Il4 (B). For T-cell proliferation assay (C), tumor associated Cd4$^+$ T-cells and MDSCs were isolated from doxorubicin-resistant mice. MDSCs (2×10^5/well) were added to Carboxyfluorescein succinimidyl ester (CFSE)-labeled naive Cd4$^+$ T-cells (2×10^5/well) either unstimulated (left panel) or activated (middle and right panel) with plate-bound anti-Cd3 (1 μg/ml) and anti-Cd28 (5 μg/ml). Cells were co-cultured for 72 h, then the proliferation of T-cells was determined in presence or absence of MnNG by loss of CFSE fluorescence. Immunofluorescence analyses were performed by using FACS caliber (BD Biosciences) with CellQuest software. All the data are presented as the mean±SD of three independent studies. Statistically significant difference at **p<0.01, ***p<0.001; ns: not statistically significantly different (p>0.05).*

differentiation of the MDSCs; thereby reduce the number of dendritic cells and macrophages with an increase in MDSCs accumulation (44-46). Hence to ascertain the cause of MnNG-mediated reduction in MDSCs number, we report the interesting observation that MnNG treatment in EAC/Dox-bearing mice promotes MDSC differentiation into dendritic cells but not towards macrophages (Figure 2A and B). However, the underlying mechanism of MnNG-induced transformation of MDSCs into dendritic cells rather than macrophage remains unexplored. Moreover, MnNG treatment helps in up-regulation of co-stimulatory molecules (Cd80 and Cd86) (Figure 2C and D) in the process which seems to be important as MDSCs express low level of co-stimulatory molecules (Cd80 and Cd86) and enhancement of these molecules ultimately activate effector T cell response more prominently (47). We have also checked the other possibility that whether MnNG treatment induces apoptosis in MDSCs. Our study shows that the MnNG treatment does not induce apoptosis in MDSCs as evident from annexin V binding assay (Figure 1E).

However, our investigation subsequently revealed that *in vitro* application of MnNG does not have any direct effect on $Cd4^+$ T cells isolated from EAC/ Dox bearing mice (Figure 3A and B). This finding provides the hint that MnNG mediated modulation of MDSCs may be involved in determining the fate of T cell response that ultimately elicits host protective Th1 response. Recent studies also reveal that MDSCs being a major suppressive element of the immune inhibitory network prime Th2 type cytokine programming, block T cell proliferation in the tumor microenvironment and when reduction of MDSCs is achieved in the TME, the protective Th1 response is enhanced (6, 9, 10, 41). Therefore we planned our experiments to reveal the relation between differentiation of MDSCs towards dendritic cells and induction of Th1 response. We found that in presence of MnNG, MDSCs augments Th1 cytokine (Ifnγ) and diminishes Th2 cytokine (Il4) production from $Cd4^+$ T cells *in vitro* (Figure 4A and B). These findings are consistent with the present *in vivo* observations that Th1 response is increased and Th2 response is decreased when MDSCs are modulated by MnNG (Figure 1). Furthermore MDSCs mediated suppression of $Cd4^+$ T cell proliferation is restored following MnNG treatment (Figure 4C) is quite exiting as MDSCs in most of the cancer models suppress antitumor immunity by blocking the activation of $Cd4^+$ and $Cd8^+$ T-cells (45).

Present work thus disclosed that MnNG may help in differentiation of immunosuppressive MDSCs towards dendritic cells and with up-regulation of co-stimulatory molecules enabling them to act as better antigen-presenting cells (APCs) that ultimately generates protective anti-tumor Th1 response by reducing tumor-promoting Th2 response in doxorubicin-resistant EAC-bearing model.

Our studies provide *in vivo* evidence in a drug-resistant animal model that MnNG having potential to eradicate cancer cells and also modulate the TME by converting suppressive MDSCs towards non-suppressive dendritic cells and are likely to have an additive or synergistic effect leading to higher therapeutic efficacy for drug-resistant cancer. However, the plausible molecular mechanism underlying MnNG mediated modulation of MDSCs in TME is still elusive and warrants further investigations.

Acknowledgements

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