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Title: Regulation of tumor-associated macrophage (TAM) differentiation by NDRG2 expression in breast cancer cells

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Running Title: Regulation of TAM by NDRG2 expression

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ABSTRACT

Macrophages are a major cellular component of innate immunity and are mainly known to have phagocytic activity. In the tumor microenvironment (TME), they can be differentiated into tumor-associated macrophages (TAMs). As the most abundant immune cells in the TME, TAMs promote tumor progression by enhancing angiogenesis, suppressing T cells and increasing immunosuppressive cytokine production. N-myc downstream-regulated gene 2 (NDRG2) is a tumor suppressor gene, whose expression is downregulated in various cancers. ~~A previous study revealed that overexpression of NDRG2 represses osteoclast differentiation through inhibition of ICAM1 expression in breast cancer cells.~~ However, the effect of NDRG2 on the differentiation of macrophages into TAMs in breast cancer remains elusive. In this study, we investigated the effect of NDRG2 expression in breast cancer cells on the differentiation of macrophages into TAMs. Compared to tumor cell-conditioned media (TCCM) from 4T1-mock cells, TCCM from NDRG2-overexpressing 4T1 mouse breast cancer cells did not significantly change the morphology of RAW 264.7 cells. However, TCCM from 4T1-NDRG2 cells reduced the mRNA levels of TAM-related genes, including MR1, IL-10, ARG1 and iNOS, in RAW 264.7 cells. In addition, TCCM from 4T1-NDRG2 cells reduced the expression of TAM-related surface markers, such as CD206, in peritoneal macrophages (PEM). The mRNA expression of TAM-related genes, including IL-10, YM1, FIZZ1, MR1, ARG1 and iNOS, was also downregulated by TCCM from 4T1-NDRG2 cells. Remarkably, TCCM from 4T1-NDRG2 cells reduced the expression of PD-L1 and Fra-1 as well as the production of GM-CSF, IL-10 and ROS, leading to the attenuation of T cell-inhibitory activity of PEM. ~~which are crucial for TAM activity.~~ These data showed that compared with TCCM from 4T1-mock cells, TCCM from 4T1-NDRG2 cells suppressed the TAM differentiation and activation. Collectively, these results suggest that NDRG2 expression in breast cancer may reduce the differentiation of macrophages into TAMs in the TME.

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INTRODUCTION

Macrophages are major innate immune cells and can be found in almost every organ. Macrophages are mainly known as phagocytic cells and can be divided into two major subsets: classically activated macrophages (M1 macrophages), which have pro-inflammatory and tumor-suppressive activity, and alternatively activated macrophages (M2 macrophages), which have anti-inflammatory and tumor-promoting activity.

The environment around a tumor is the tumor microenvironment (TME). The TME contains immune cells, extracellular matrix (ECM), tumor cells and signaling molecules. The TME profoundly affects tumor progression and immunosuppression via extracellular paracrine signals. Macrophages are one of the abundant immune cells in the TME and are recruited into tumors by various soluble factors, including vascular endothelial growth factor (VEGF) and C-C chemokine ligand 2 (CCL2) (1, 2). They also differentiate into tumor-associated macrophages (TAMs) via their interaction with tumor and tumor-derived factors. In humans, TAMs are defined as $CD14^{hi}$ $CD11b^{+}$ HLA-DR^{+} $CD163^{+}$ cells. In mice, TAMs express CD11b, F4/80, CD206 and CD163 (3, 4). Previous studies have reported that YM1, FIZZ1 and MR1 (CD206) are TAM differentiation markers and that ARG1, iNOS, PD-L1 and PD-1 are TAM activation markers (5-9).

TAMs are similar to M2 macrophages and promote tumor progression via suppression of T cells, generation of immune suppressive cytokines and induction of angiogenesis. In particular, TAMs are well known to suppress immune responses. First, interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) produced by TAMs induce the generation of regulatory T (Treg) cells, suppression of T cells and inhibition of dendritic cell (DC) maturation. TAMs induce metabolic starvation of T cells via secretion of indoleamine 2,3-dioxygenase 1/2 (IDO1/2) and arginase 1 (ARG1). Importantly, they can also suppress the anti-tumor T cell responses through the expression of PD-L1/2. (4, 10). As various types of anti-cancer therapies have side effects, preclinical and clinical studies are focused on immunotherapies that cause fewer side effects than other anti-cancer therapies. However, macrophages recruited in response to immunotherapies tend to differentiate into TAMs in the TME, which is a limitation of these therapies. For this reason, targeting TAMs has emerged as a

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novel strategy for anti-cancer therapies against solid tumors (4).

The N-myc downstream-regulated gene (NDRG) family consists of NDRG1-4. NDRG family proteins are characterized by an α/β hydrolase fold of approximately 220 amino acids and a lipase/esterase/thioesterase active site (11, 12). NDRG2 is known to be a cell stress-related and tumor suppressor gene in multiple solid tumors, including breast cancer (13, 14), colorectal cancer (15), renal cell carcinoma (16) and lung cancer (17). NDRG2 expression can inhibit breast cancer development by decreasing tumor cell proliferation, migration and epithelial-mesenchymal transition (EMT) (13, 14).

Our previous study revealed that NDRG2 overexpression suppresses osteoclast differentiation via the inhibition of ICAM-1 expression in breast cancer (18). This suggests that NDRG2 overexpression may control the differentiation of other immune cells. However, the effect of NDRG2 expression in breast cancer on the differentiation of macrophages into TAMs has not been investigated. Thus, in the present study, we aimed to examine the effect of NDRG2-overexpressing breast cancer cells on the differentiation of macrophages into TAMs.

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RESULTS

Evaluation of NDRG2 expression levels in 4T1-NDRG2 cells prior to TCCM generation

To evaluate whether NDRG2 expression in breast cancer cells affects the differentiation of macrophages into TAMs, tumor cell-conditioned media (TCCM), which are known to mimic the TME, were collected from 4T1 cells. The 4T1 cell line, a mouse mammary carcinoma cell line, was derived from Balb/c mice and has been characterized as a highly metastatic tumor model. 4T1 cells were stably transfected with the pCMV/Tag2B-empty vector (mock) or pCMV/Tag2B-NDRG2 vector. NDRG2 mRNA and protein were confirmed to be expressed at the expected levels by RT-PCR and western blot analysis (Fig. 1A). Difference in cell viability in both cell lines was not observed (Supplementary Fig. 1). In addition, the production of GM-CSF and IL-10 associated with immune suppression in 4T1 cell was significantly decreased by the transfection with NDRG2 (Fig. 1B).

TCCM from NDRG2-overexpressing 4T1 cells reduced TAM differentiation and activation of RAW 264.7 cells

To differentiate macrophages into TAMs, the culture media of 4T1-mock and 4T1-NDRG2 cells were obtained and concentrated. It was previously reported that when macrophages are treated with TCCM from 4T1 cells to induce TAM differentiation, they become elongated (19). RAW 264.7 cells, which are mouse macrophages, were derived from Balb/c mice and are usually used for studies on macrophage differentiation (20). TCCM from 4T1-NDRG2 cells (4T1-N2 CM) and TCCM from 4T1-mock cells (4T1-M CM) altered the elongated morphology of RAW 264.7 cells (Fig. 1C). Since there was little difference in the morphological change in RAW 264.7 cells after treatment with 4T1-N2 CM or 4T1-M CM, the expression of TAM differentiation and activation markers was assessed by RT-PCR (Supplementary Fig. 2) and RT-qPCR (Fig. 1D). ~~Although the expression of certain TAM differentiation markers (YM1 and FIZZ1) was not detected,~~ The expression of ~~another~~ TAM differentiation marker (MR1) and TAM activation markers (IL-10, ARG1 and iNOS) was reduced in cells treated with 4T1-N2 CM compared with those treated with 4T1-M CM. Furthermore, the surface

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expression of CD206, PD-L1 and PD-1 was also suppressed after treatment with 4T1-N2 CM (Fig. 1E). These results suggested that TAM differentiation and activation were attenuated in RAW 264.7 cells treated with 4T1-N2 compared with those treated with 4T1-M CM.

4T1-N2 CM attenuated TAM differentiation and activation in peritoneal macrophages

Peritoneal macrophages (PEMs) have been widely used for studies of macrophage polarization and TAM differentiation (21-23). To examine the effect of NDRG2 expression in 4T1 cells on the differentiation of macrophages into TAMs, murine PEMs were isolated from Balb/c mice. As shown in Fig. 2A, more than 85% of PEMs isolated from the murine peritoneal cavity were F4/80-positive. 4T1-N2 CM slightly altered the elongated morphology of PEMs induced by treatment with 4T1-M CM (Fig. 2B). To compare the effect of 4T1-M CM and 4T1-N2 CM on the induction of TAMs, the expression of TAM differentiation and activation markers was analyzed. TAMs usually exhibit the M2 phenotype, and CD206 has been reported to be a TAM differentiation markers. In addition, PD-L1 and PD-1 have been reported to be TAM activation markers (8, 9). Interestingly, compared with 4T1-M CM, 4T1-N2 CM significantly reduced the expression levels of CD206, PD-L1 and PD-1 on PEMs (Fig. 2C). 4T1-M CM treatment enhanced the mRNA expression of TAM differentiation (MR1, YM1 and FIZZ1) and activation (IL-10 and ARG1) markers (Supplementary Fig. 3). Consistent with the results obtained with RAW 264.7 cells, the mRNA expression level of MR1, TM1, FIZZ1, iNOS and IL-10 was also reduced after treatment with 4T1-N2 CM compared with 4T1-M CM (Fig. 2D-H). These data suggested that compared with 4T1-M CM, 4T1-N2 CM had a diminished ability to induce TAM differentiation and activation.

~~**Compared with 4T1-M CM, 4T1-N2 CM weakly influenced the expression of TAM differentiation and activation markers**~~

~~Next, RT-PCR and quantitative real-time PCR were performed to examine the mRNA expression levels of each marker gene. 4T1-M CM treatment enhanced the mRNA expression of TAM differentiation (MR1, YM1 and FIZZ1) and activation (IL-10 and ARG1) markers. Interestingly,~~

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~~compared with 4T1-M CM, 4T1-N2 CM substantially diminished the expression of MR1, YM1 and FIZZ1 (Fig. 2D-I). Moreover, compared with 4T1-M CM, 4T1-N2 CM reduced the expression of TAM activation markers (ARG1, IL-10, iNOS, PD-L1 and PD-1) (Fig. 2C, D, H and I). Collectively, these results indicated that compared with 4T1-M CM, 4T1-N2 CM had a diminished ability to induce TAM differentiation and activation.~~

The differential effects of 4T1-M CM and 4T1-N2 CM on the differentiation of macrophages into TAMs ~~was not attributed to the difference in cellular secretion of soluble factors~~

Next, to assess whether the two kinds of CM have similar abilities to induce the differentiation of macrophages into TAMs, the expression levels of some common markers were investigated. TGF-β and MMP-9 expressed by TAMs are known to contribute to the immunosuppressive response and tumor metastasis (4). Fra-1 expressed by TAMs is also known to promote the generation of TAMs by binding to the IL-6 promoter (24). Consistent with previous data, treatment of RAW 264.7 cells with 4T1-N2 CM attenuated the mRNA expression of Fra-1 and IL-6 compared with 4T1-M CM (Fig. 3A, B). On the other hand, both types of CM similarly increased the mRNA expression level of TGF-β (Fig. 3C). The protein expression of Fra-1 and PD-L1 was decreased by 4T1-N2 CM, whereas the expression of MMP-9 was increased by the two types of CM to a similar degree (Fig. 3D). Similar results were obtained in experiments using PEM (Fig. 3E, F). These results suggested that, as revealed by downregulation of IL-6, Fra-1 and PD-L1 expression, 4T1-N2 CM increased TAM differentiation and activation to a less extent than 4T1-M CM through downregulation of IL-10, PD-L1 and Fra-1 expression.

4T1-N2 CM induced significantly less activation in peritoneal macrophages than 4T1-M CM

To investigate whether TAM activation in PEM is associated with the production of immune suppressive factors, GM-CSF, IL-10 and reactive oxygen species (ROS) levels were measured in PEM after treatment with TCCM. Compared with 4T1-M CM, 4T1-N2 CM induced low level of GM-CSF, IL-10 and ROS production in PEM (Fig. 4A, B). Finally, we assessed the T cell suppressive

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effect of TCCM-treated PEM by co-culturing with CFSE-labeled mouse $CD3^+$ T cells. As shown in Fig. 4C, 4T1-M CM-pretreated PEM markedly suppressed the proliferation of $CD8^+$ T cells in the presence of anti-CD3/CD28 antibodies compared with untreated PEM, whereas T cell proliferation was weakly inhibited by a treatment of PEM with 4T1-N2 CM. These findings showed that although 4T1-M CM could induce PEM to strongly suppress T cell proliferation, the suppressive effect was diminished greatly by 4T1-N2 CM.

DISCUSSION

Macrophages have a pivotal role in tumor progression and differentiation by the tumor microenvironment. TAMs in the TME can promote tumor growth and support an immunosuppressive TME. ~~Macrophages can be polarized to towards the M1 and M2 phenotypes by the surrounding environment. M1 macrophages exhibit anti-tumor and pro-inflammatory activity, whereas M2 macrophages exhibit pro-tumor and anti-inflammatory activity. In addition, macrophages can differentiate into TAMs in the TME.~~ TAMs are similar to M2 macrophages, which promote tumor initiation, progression and metastasis. Preclinical and clinical studies have demonstrated that TAM accumulation in tumors correlates with a poor prognosis (10, 25, 26). Hence, targeting TAMs could emerge as a novel strategy for cancer therapy. Given that macrophages are required for the efficacy of cancer immunotherapy, depleting TAMs during cancer therapy may impair the efficacy of immunotherapies. Therefore, instead of depleting them, attempts have been made to repolarize macrophage phenotypes.

NDRG2 expression is decreased in various solid tumors, including breast cancer, colorectal cancer and lung cancer. In addition, NDRG2 inhibits metastasis, angiogenesis and invasion (14, 15, 17). A previous study reported that NDRG2 overexpression in breast cancer suppresses osteoclast differentiation by downregulating ICAM expression (18), suggesting that NDRG2 expression can indirectly modulate the differentiation of other immune cells. However, it has not been reported whether NDRG2 is strongly associated with the differentiation of TAMs in breast cancer. Thus, in this study, we sought to investigate whether NDRG2 expression in breast cancer cells is able to regulate

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the differentiation of TAMs. In the present study, we demonstrated that compared with 4T1-M CM, 4T1-N2 CM significantly reduced TAM differentiation and activation as revealed by the reduction in expression levels of TAM differentiation and activation markers. While the expression of differentiation markers was relatively weak, the difference in the expression of activation markers was very noticeable (Fig. 2C). ~~Although we did not examine the levels of relevant soluble factors in the CM,~~ It has already been reported that monocyte-derived TAMs can be generated reliably using tumor-conditioned medium plus a cytokine cocktail of IL-4, IL-10 and M-CSF (27). In fact, we demonstrated that compared to control vector-transfected cells, NDRG2-transfected monocytes and human histiocytic leukemia U937 cells exhibit significantly decreased IL-10 production (28), supporting the hypothesis that decreased tumor-derived IL-10 production after NDRG2 transfection might play a role in reducing the TAM differentiation of macrophages.

In a previous report, Fra-1 expression increased the production of IL-6 in macrophages by binding to the IL-6 promoter. IL-6 expression induced by Fra-1 can lead to the differentiation of macrophages into TAMs (24). IL-6 also enhances tumor progression by activating EMT and increases tumor resistance to apoptosis (29). Moreover, IL-6 provokes resistance to chemotherapy (30), and high IL-6 expression correlates with poor prognosis in breast cancer (31). Several lines of evidence suggest that PD-L1 and PD-1 expressed by TAMs also contribute to the immunosuppressive function of TAMs by inactivating T cells and inhibiting anti-tumor immunity (4, 8, 9). TGF-β secreted by TAMs promotes tumor metastasis by upregulating the expression of SRY-related high mobility group box 9 (SOX9) (32). MMP-9 secreted by TAMs promotes tumor metastasis via activation of the PI3K/AKT signaling pathway and induces tumor cell invasion (25, 33). Although there was no significant difference in the expression of TGF-β and MMP-9 between 4T1-M CM-treated and 4T1-N2 CM-treated macrophages, compared with 4T1-M CM, 4T1-N2 CM substantially reduced the expression of IL-10, Fra-1 and PD-L1 in macrophages (Fig. 3). These results suggested that compared with 4T1-M CM, 4T1-N2 CM reduced the immunosuppressive function of TAMs. However, further study is required to thoroughly investigate the mechanism by which 4T1-N2 CM diminish TAM activation.

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In summary, we identified in the present study that NDRG2 expression in breast cancer cells inhibits the expression of IL-10, Fra-1 and PD-L1 in TAMs. Downregulation of IL-10, Fra-1 and PD-L1 expression is expected to reduce the immunosuppressive function of TAMs (Fig. 4D). Consequently, TAM activation at the tumor site surrounded by NDRG2-overexpressing breast cancer cells is reduced, leading to the downregulation of IL-10, Fra-1 and PD-L1 expression. These findings suggest that NDRG2 could be a novel regulatory factor that modulates TAM activation in cancer therapy.

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MATERIALS AND METHODS

Cell culture and reagents

Mouse breast cancer cell lines (4T1-mock and 4T1-NDRG2) and a mouse macrophage cell line (RAW 264.7) were cultured in DMEM (Gibco/Invitrogen, Carlsbad, CA) containing 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (Gibco/Invitrogen) at 37°C in a humidified 95% air/5% CO_2 incubator.

Tumor cell-conditioned media (TCCM)

4T1-mock and 4T1-NDRG2 cells were cultured in DMEM containing 3% heat-inactivated FBS for 48 h and the cell supernatants were collected when the cell densities were 3×10^6 cells/plate. ~~the cells were 80% confluent.~~ The supernatants were concentrated at 3,000 × g for 30 min at 4°C using Amicon Ultra-15 centrifugal filter 3K devices (Merck Millipore, Billerica, MA). The concentrated media were diluted with DMEM containing 3% FBS (Gibco/Invitrogen) to produce 30% concentrated media.

RNA isolation, quantitative real-time reverse transcription-polymerase chain reaction and RT-PCR

All cells were lysed using TRIzol reagent (Molecular Research Center Inc, Cincinnati, OH), and total RNA was isolated according to the manufacturer's instructions. ~~After incubation for 5 min at room temperature (RT), 200 μl of chloroform was added. The mixtures were inverted and incubated for 12 min at RT. After centrifugation at 12,000 rpm for 12 min at 4°C, the supernatants were transferred to new Eppendorf tubes and the same volume of isopropyl alcohol was added to the samples. The mixtures were incubated for 10 min at RT and then, the mixtures were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were removed after centrifugation. The pellets were washed using 400 μl of 80% EtOH and the mixtures were centrifuged at 12,000 rpm for 5 min at 4°C. Then, the EtOH was eliminated, and the pellets were dried and resuspended in DEPC-treated water (Invitrogen).~~ Total RNA (5 μg) was reverse transcribed into cDNA. For the reverse transcription (RT) reaction, first-strand cDNA was generated using M-MLV reverse transcriptase (Promega, Madison, WI), oligo-

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(dT) primers and dNTPs (Bioneer, Daejeon, Republic of Korea). ~~First, RNA and oligo (dT) were incubated at 70°C for 5 min and incubated at RT for 10 min. Then, RT buffer (Promega, Madison, WI), M-MLV reverse transcriptase (Promega) and dNTPs (Bioneer, Daejeon, Republic of Korea) were added and the RT mixtures were incubated at 37°C for 2 h. The synthesized cDNA was boiled for 3 min.~~ The cDNAs were amplified with 10 mM dNTPs, 0.5 U of Taq DNA polymerase and 10 pmol each primer (Bioneer). The PCR products were electrophoresed on 1% agarose gels containing ethidium bromide. Quantitative real-time PCR was performed using an ABI StepOnePlus™ real-time PCR thermal cycler with Power SYBR Green PCR Master Mix. The experiments were performed in triplicate. The primer sequences for quantitative real-time PCR and RT-PCR are listed in the Supplementary Table 1.

Isolation of peritoneal macrophages

Female Balb/c mice at 6 to 8 weeks of age were purchase from Daehan Bio link (Eumseong, Republic of Korea). The mice were maintained in specific pathogen-free facilities at Sookmyung Women's University. The animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee after approval by the Institutional Ethical Committee of Sookmyung Women's University (Resolution No. SMWU-IACUC-1912-024-1). The method used to isolate peritoneal macrophages from mice was described previously (34). In order to isolate peritoneal macrophages, Balb/c mice were sacrificed, and 10 ml of cold RPMI (Gibco/Invitrogen) medium was injected into the abdominal cavity. The medium was withdrawn and centrifuged (1,500 rpm for 5 min at 4°C), and the cell pellet was resuspended in 1 ml of RPMI medium. These peritoneal exudate cells were cultured in dishes (> 4 h at 37°C). After 2 h, the non-adherent cells were removed.

Western blot analysis and enzyme-linked immunosorbent assay (ELISA)

All cells were lysed in protein extraction solution (iNtRON Biotechnology, Seongnam, Republic of Korea) ~~for 20 min on ice. The supernatant fractions were transferred to new Eppendorf tubes after centrifugation at 12,000 rpm for 5 min at 4°C.~~ The proteins were mixed with 5X sample buffer and

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separated by electrophoresis on gradient SDS-PAGE gels (8% to 15%). Then, the proteins were transferred onto PVDF membranes (Amersham Biosciences, ~~Little Chalfont,~~ Buckinghamshire, UK). The membranes were blocked with Tris-buffered saline plus 0.05% Tween-20 (TBST) with 5% skim milk for 30 min and incubated with primary antibodies overnight at 4°C. After incubation, the membranes were washed and incubated with HRP-conjugated secondary antibodies. The blots were visualized with an enhanced chemiluminescence system using an Ez-Capture MG system (ATTO Corporation, Tokyo, Japan). Specific antibodies against NDRG2, β-actin and Fra-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD-L1, α-tubulin and MMP-9 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). For the measurement of IL-10 (Cat No: 900-TM53) and GM-CSF (Cat No: 900-M55) levels, the culture supernatants were collected and sandwich ELISA kits (Peprotech Inc., Rocky Hill, NJ) were used according to the manufacturer's protocol.

Flow cytometry

The cell surfaces was stained with antibodies at 4°C for 30 min. Specific antibodies against CD11b (M1/70), F4/80 (BM8.1), CD206 (MR6F3), PD-1 (J43) and PD-L1 (MIH5) were purchased from eBioscience (San Diego, CA). The samples were washed using PBS and fixed with 4% paraformaldehyde. The samples were analyzed using FACSCantoII™ flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed using FlowJo software (Tree Star, Ashland, OR). Cell apoptosis was measured using a double staining with FITC-labeled Annexin V/Propidium Iodide (PI) Apoptosis Detection Kit (BD Biosciences) according to the manufacture's instruction. The apoptosis levels were examined by flow cytometry. For reactive oxygen species (ROS) measurement, the cells were pretreated with 10 mM NAC (Sigma-Aldrich, St. Louis, MO) for 24 h. Then, 10 μM DCF-DA (Sigma-Aldrich) was added to the cells for 30 min at 37°C, and DCF fluorescence was measured by flow cytometry.

In vitro T cell proliferation assay

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Mouse $CD3^+$ T cells were isolated from spleens using a S3™ Cell Sorter (Bio-Rad Laboratories, Hercules, CA). Sorted $CD3^+$ T cells with the high purity (>95%) using an antibody (145-2C11, eBioscience) were labeled with carboxyfluorescein succinimidyl ester (CFSE) (2.5 μM; Invitrogen) according to the manufacturer's guideline and plated at 1×10^5/well in 96-well plates containing 2 μg/ml anti-CD3 (17A2, eBioscience) and 2 μg/ml anti-CD28 (37.51, eBioscience) mAbs. After 3 days, the cells were stained with CD8 antibody (53-6.7, eBioscience), and then the $CD8^+$ T cell proliferation was determined by flow cytometry at the PEC/T cell ratio of 1:2.

Statistical analysis

Statistical significance was analyzed by one-way ANOVA (Tukey's post hoc test for multiple comparisons) with GraphPad Prism software version 5 (GraphPad Software, San Diego, CA). The values are presented at the mean ± SEM. ~~A value of~~ $P < 0.05$ was considered significant. ~~*p < 0.05, **p < 0.01, ***p < 0.001.~~

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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FIGURE LEGENDS

Fig. 1. The expression of TAM differentiation and activation markers in RAW 264.7 cells was decreased by 4T1-N2 CM. (A) The mRNA and protein levels of NDRG2 in 4T1 cells were measured by RT-PCR and western blot analysis. (B) The 4T1-M and 4T1-N2 cells were cultured for 48 h, and then IL-10 and GM-CSF levels in the supernatants were determined by sandwich ELISA kits. (C) To generate TAMs, RAW 264.7 cells were treated with 4T1-M CM or 4T1-N2 CM for 24 h. Cell morphology images were captured using a microscope (x20). (D) The mRNA expression levels of TAM differentiation markers (MR1) and activation markers (IL-10, iNOS and ARG1) were measured by ~~(C) RT-PCR and (D)~~ quantitative real-time PCR. (E) The CD206, PD-L1 and PD-1 expression in TAM was quantified by flow cytometry. The results are presented as the mean ± SEM. The data are representative of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Fig. 2. Compared with 4T1-M CM, 4T1-N2 CM inhibited TAM differentiation and activation in PEMs. (A) To confirm the purity of the obtained PEMs, the cells were stained with anti-F4/80. The purity of PEMs was analyzed by flow cytometry. (B) PEMs were seeded in a 6-well plate (8×10^5) and treated with 4T1-M CM or 4T1-N2 CM for 24 h or 48 h. Cell morphology images were captured using a microscope (×20). (C) The cells were stained with specific antibodies aganist ~~F4/80,~~ CD11b, F4/80, CD206, PD-1 and PD-L1 for cell analysis. The expression levels of TAM differentiation marker (CD206) and TAM activation markers (PD-L1 and PD-1) were measured by flow cytometry, and these data were analyzed using FlowJo software. (D-H) The mRNA expression levels of TAM differentiation markers (MR1, YM1 and FIZZ1) and activation markers (IL-10 and iNOS) were measured by ~~RT-PCR and (E-I)~~ quantitative real-time PCR. The results are presented as the mean ± SEM. The data are representative of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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Fig. 3. 4T1-M CM and 4T1-N2 CM differentially increased the expression of other TAM activation markers. RAW 264.7 or PEM cells were treated with 4T1-M CM or 4T1-N2 CM for 24 h. (A-C) The mRNA expression levels of TAM activation markers (IL-6, Fra-1 and TGF-β) in RAW 264.7 cells were measured by quantitative real-time PCR. (D and F) The protein levels of TAM activation markers (Fra-1, PD-L1 and MMP-9) were measured by western blot analysis. (E) The mRNA expression level of Fra-1 in PEM was measured by quantitative real-time PCR. The results are presented as the mean ± SEM. The data are representative of at least three independent experiments. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

Fig. 4. Comparative analysis of immunosuppressive effects of PEM treated with 4T1-M CM or 4T1-N2 CM. (A) PEMs were treated with 4T1-M or -N2 CM for 48 h, and then cultured in serum-free medium. After 48 h, the supernatant was collected for ELISA. (B) The cells pretreated in the absence or presence of 10 mM NAC were cultured with 4T1-M or -N2 CM for 24 h and were treated with 10 μM DCF-DA for 30 min. Harvested cells were analyzed by flow cytometry. (C) *In vitro* generated PEM was co-cultured with CFSE-labeled $CD3^+$ T cells at 1:2 (PEM:T) ratio for 72 h. The $CD8^+$ T cell proliferation was measured by flow cytometry. *$p < 0.05$, ** $p < 0.01$. (D) Model demonstrating the effect of NDRG2 expression in breast cancer on TAM activation. NDRG2 expression in breast cancer cells reduces the expression levels of IL-10, PD-L1 and Fra-1 in TAMs and attenuates the immunosuppressive function of TAMs.

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Figure 1

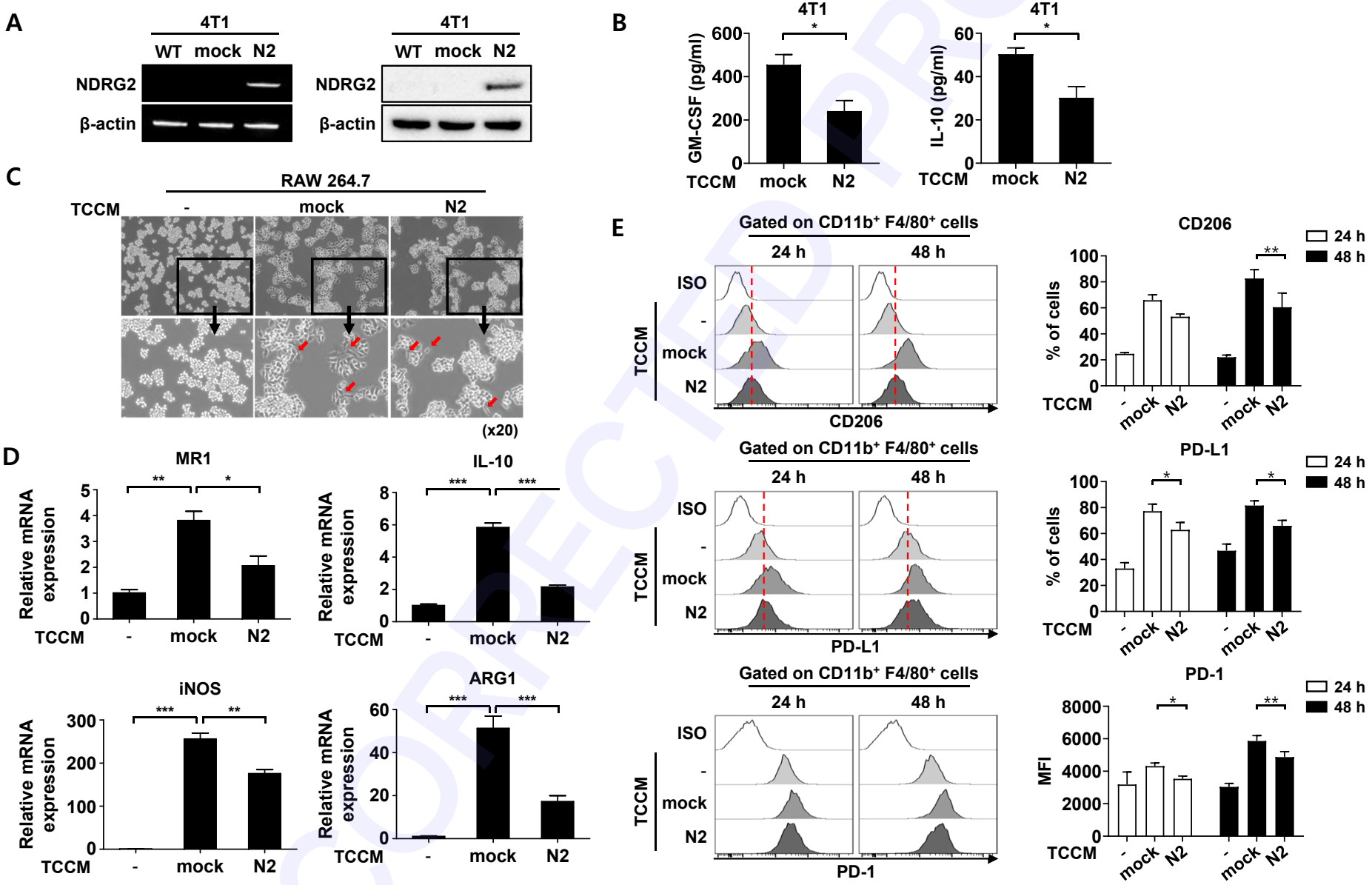

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Figure 2

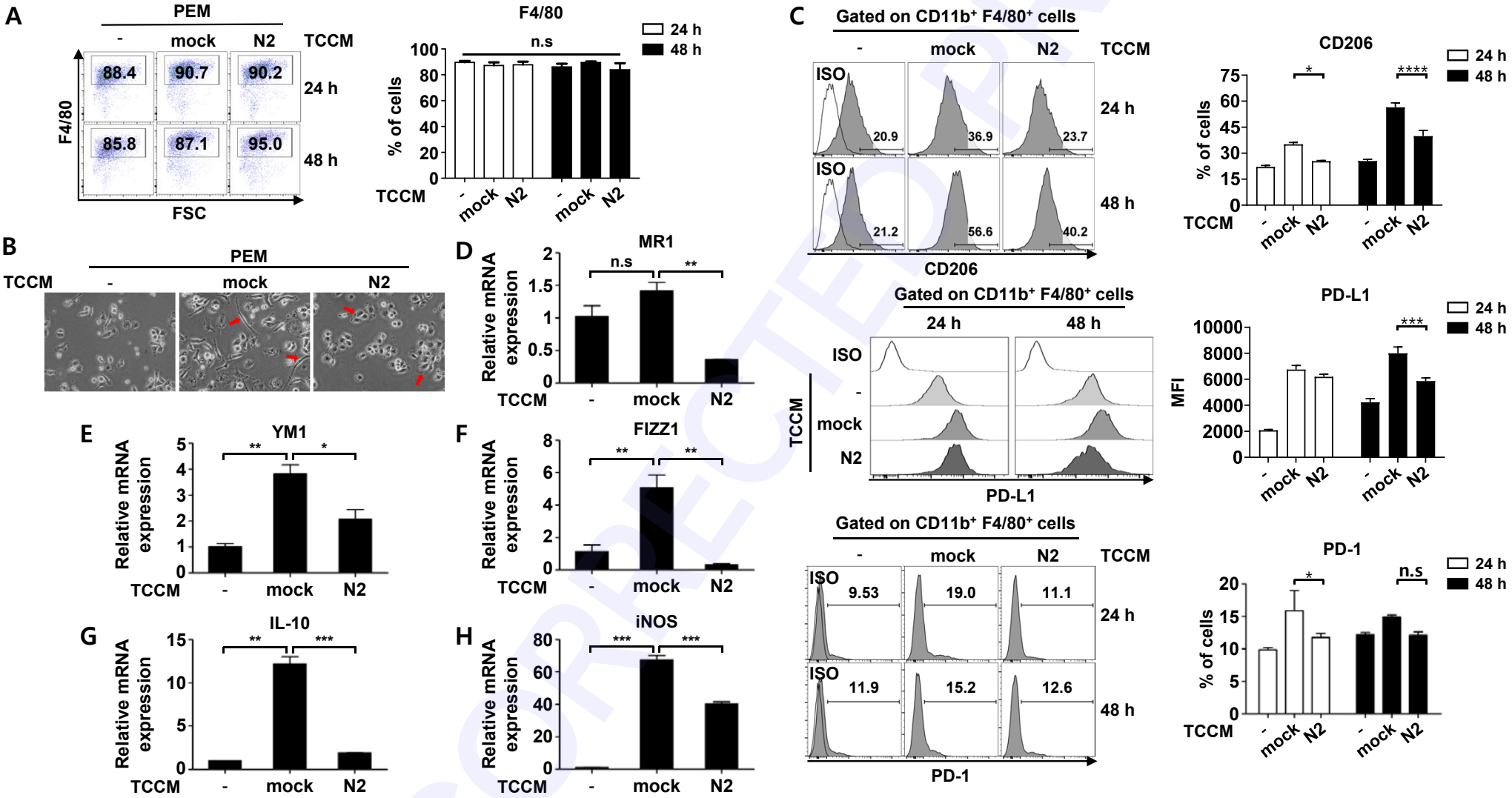
A
PEM
-
mock
N2
TCCM
88.4
90.7
90.2
24 h
85.8
87.1
95.0
48 h
F4/80
FSC
F4/80
n.s
24 h
48 h
% of cells
TCCM
B
PEM
TCCM
-
mock
N2
C
Gated on CD11b+ F4/80+ cells
ISO
20.9
36.9
23.7
21.2
56.6
40.2
CD206
CD206
PD-L1
MFI
Gated on CD11b+ F4/80+ cells
24 h
48 h
PD-L1
Gated on CD11b+ F4/80+ cells
9.53
19.0
11.1
11.9
15.2
12.6
PD-1
PD-1
n.s
D
MR1
Relative mRNA expression
E
YM1
F
FIZZ1
G
IL-10
H
iNOS

Figure 3

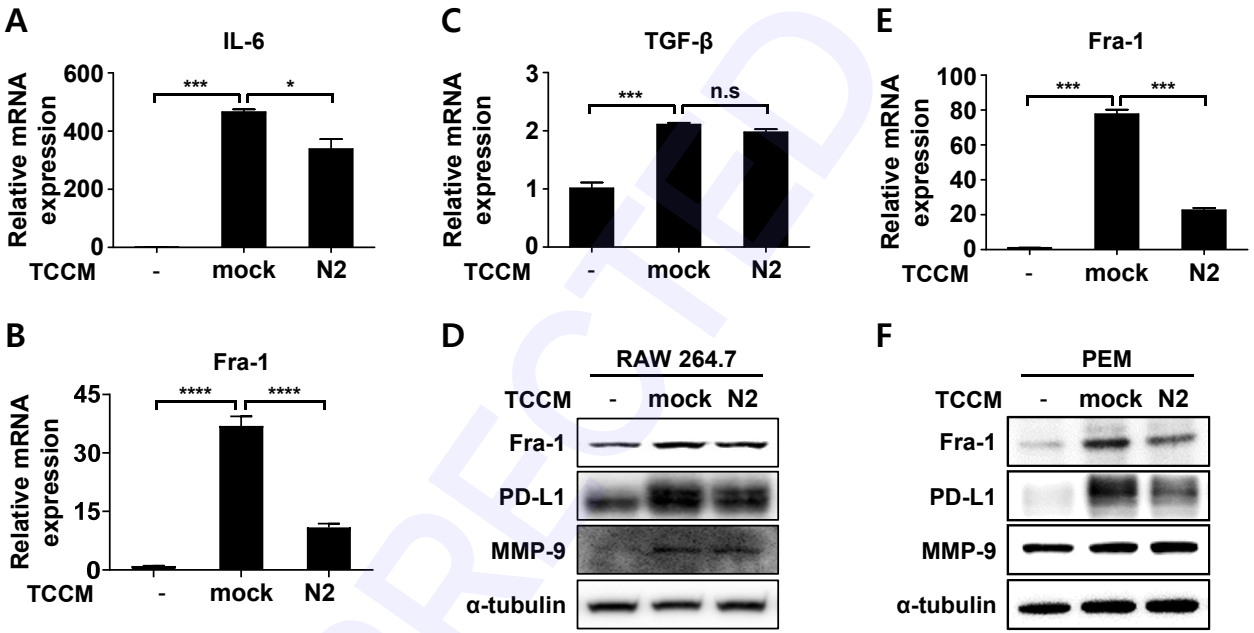

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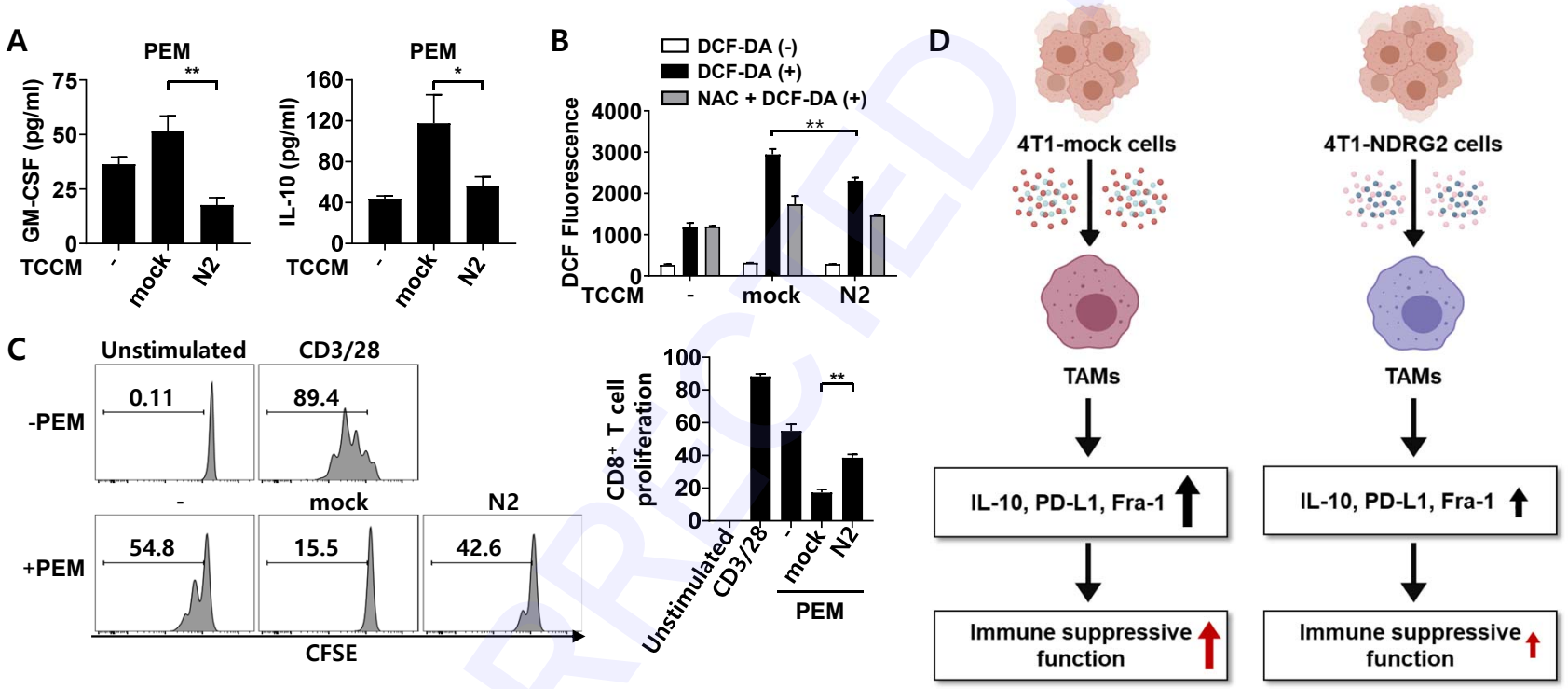
Figure 4


A
PEM
GM-CSF (pg/ml)
IL-10 (pg/ml)
TCCM
mock
N2
B
DCF-DA (-)
DCF-DA (+)
NAC + DCF-DA (+)
DCF Fluorescence
C
Unstimulated
CD3/28
-PEM
+PEM
0.11
89.4
54.8
15.5
42.6
CFSE
CD8⁺ T cell proliferation
D
4T1-mock cells
4T1-NDRG2 cells
TAMs
IL-10, PD-L1, Fra-1
Immune suppressive function

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Supplementary Table 1. Quantitative PCR primer sequences and RT-PCR primer

Quantitative real-time PCR primer sequences	
GENE	**Sequence '5 → 3'**
ARG1 (sense)	AAGCCTGGTCTGCTGGAAAAA
ARG1 (anti-sense)	CTGGTTGTCAGGGGAGTGTT
CYCLOPHILIN (sense)	ATGGTCAACCCCACCGTGT
CYCLOPHILIN (anti-sense)	TGCTGTCTTTGGAACTTTGTC
FIZZ1 (sense)	TCCCAGTGAATACTGATGAGA
FIZZ1 (anti-sense)	CCACTCTGGATCTCCCAAGA
FRA-1 (sense)	CGGAGACTGACAAACTGG
FRA-1 (anti-sense)	CTCAGGTTCAAGCACAGG
INOS (sense)	GGGCAGCCTCTGAGACCTT
INOS (anti-sense)	GGGCAGCCTCTGAGACCTT
IL-10 (sense)	GATGCCCCAGGCAGAGAA
IL-10 (anti-sense)	CACCCAGGGAATTCAAATGC
MR1 (sense)	CAAGGAAGGTTGGCATTTGT
MR1 (anti-sense)	CCTTTCAGTCCTTTGCAAGC
TGF-β (sense)	GGACTCTCCACCTGCAAGAC
TGF-β (anti-sense)	GACTGGCGAGCCTTAGTTTG
YM1 (sense)	GAAGGAGCCACTGAGGTCTG
YM1 (anti-sense)	CACGGCACCTCCTAAATTGT
RT-PCR primer sequences	
GENE	**SEQUENCE '5 → 3'**
ARG1 (sense)	CAGAACCTGCTGTCCTGTGA
ARG1 (anti-sense)	TCCATGAGTGCTTGAACAGG
FIZZ1 (sense)	CTTGTTCCCTTCTCATCTGC
FIZZ1 (anti-sense)	CAGTGGTCCAGTCAACGAGT
IL-10 (sense)	CTATGCTGCCTGCTCTTACT
IL-10 (anti-sense)	GTAGACACCTTGGTCTTGGA
INOS (sense)	AGATCCCGAAACGCTTCACT
INOS (anti-sense)	GAAGTAGGTGAGGGCTTGGC
MR1 (sense)	GCAAATGGAGCCGTCTGTGC
MR1 (anti-sense)	CTCGTGGATCTCCGTGACAC
YM1 (sense)	GGGCATACCTTTATCCTGAG
YM1 (anti-sense)	CCACTGAAGTCATCCATGTC

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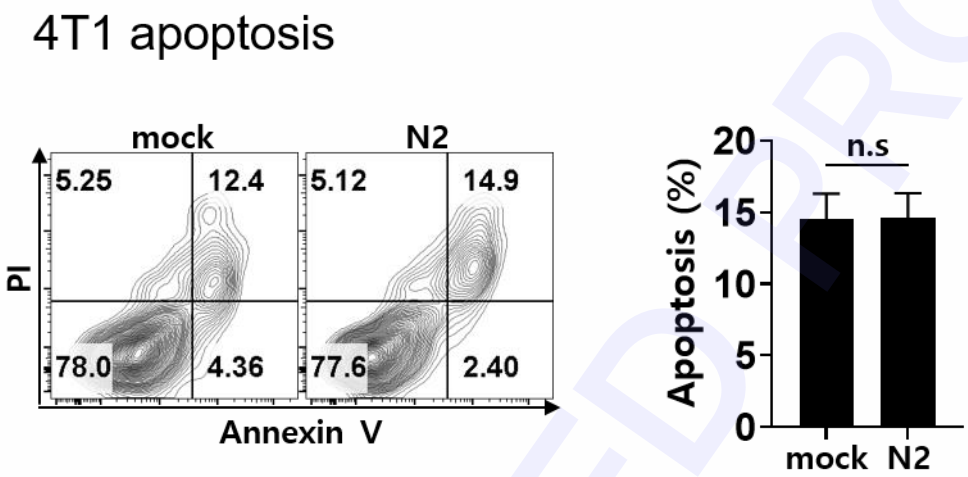
Supplementary Figure 2.



Supplementary Figure 1. Cell apoptosis was measured using a double staining with FITC-labeled Annexin V/Propidium Iodide (PI) Apoptosis Detection Kit (BD Biosciences) according to the manufacture's instruction. The apoptosis levels were examined by flow cytometry (n=3).

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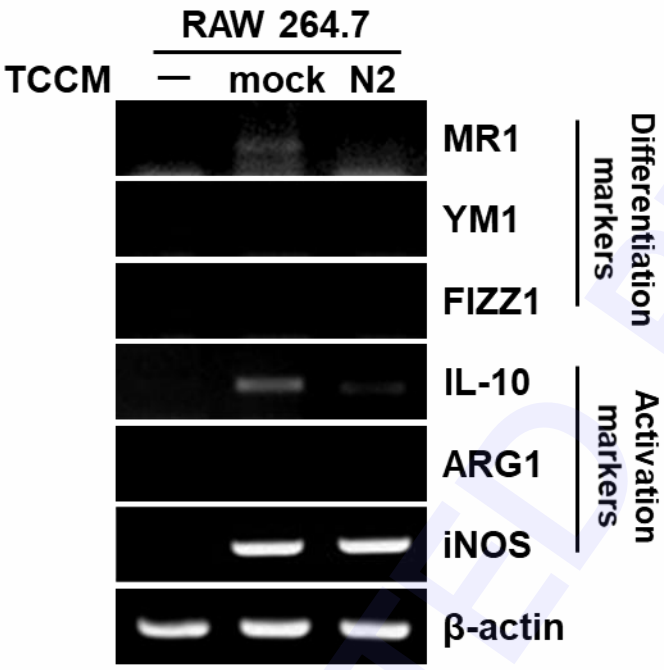
Supplementary Figure 2.



Supplementary Figure 2. The mRNA expression levels of TAM differentiation markers (YM1, FIZZ1 and MR1) and activation markers (IL-10, ARG1 and iNOS) were measured by RT-PCR.

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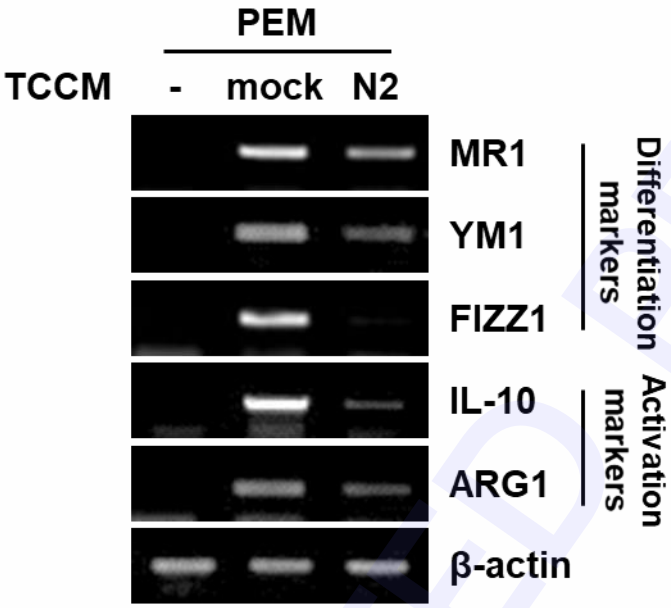
Supplementary Figure 3.



Supplementary Figure 3. The mRNA expression levels of TAM differentiation markers (YM1, FIZZ1 and MR1) and activation markers (IL-10, ARG1 and iNOS) were measured by RT-PCR.

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