Particulate β-glucan regulates the immunosuppression of granulocytic myeloid-derived suppressor cells by inhibiting NFIA expression

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of cells which comprise two subsets: granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). MDSCs involve in tumor-associated immune suppression by remarkably blocking effector T-cell activation and inducing expansion of regulatory T cells in the tumor microenvironment. The treatment that alters the suppression of MDSCs can effectively facilitate the antitumor immune responses. Recently, we showed that the whole β-glucan particles (WGP) are capable of altering the suppression of MDSCs. However, the regulatory mechanism of MDSCs by WGP remains unknown. In this study, we found that the expression of nuclear factor I-A (NFIA), an integral transcriptional component of myeloid differentiation and lineage commitment, was inhibited by WGP in G-MDSCs. The effect of WGP on expression of NFIA was the c-jun molecule dependent via Dectin-1 pathway in vitro. Moreover, NFIA knockdown could alter the suppressive function of G-MDSCs, promote the antitumor immune responses and delay the tumor progression in tumor-bearing mice. Taken together, our results demonstrate a critical role of NFIA during WGP regulating the immunosuppression of G-MDSCs, with potential implications as an antitumor immune therapeutic approach.

Introduction

Immune suppression is a major cause of tumor-associated immune evasion and accumulating evidences have demonstrated that MDSCs are closely associated with tumor-induced immunosuppression.1,2 In mice, MDSCs are a heterogeneous group of cells characterized as CD11b+Gr1+ cells.3 Under physiological conditions, these cells produced in bone marrow can quickly differentiate into granulocytes, macrophages or dendritic cells (DCs). However, in pathological conditions, MDSCs expand dramatically, especially in tumor-bearing hosts.4 MDSCs in mice have recently been divided into two subsets for their different morphology and phenotype: G-MDSCs whose phenotype is CD11b+LY6G+LY6Clow, whereas M-MDSCs are CD11b+LY6G+LY6Chi.5 G-MDSCs which are the majority population of MDSCs in tumor-bearing mice represent nearly 70%–80% of all MDSCs and they suppress the T-cell-mediated antitumor immune response.6 During the close cell-cell contact, Arg1 and ROS released by G-MDSCs can result in the alterations of cell-surface molecules on T cells.4 Additionally, G-MDSCs have also been defined in cancer patients recently.7,8

β-glucans are glucose polymers with a backbone of linear β-1,3-linked D-glucose molecules (β-1,3-D-glucan) and β-1,6-linked side chains of varying sizes with distribution frequency.9 They exist most commonly as cellulose in plants, the brain of cereal grains, the cell wall of yeast, certain fungi, mushrooms and bacteria.10,11 β-1,3-D-glucans with β-1,6 side chains are characterized by the features of high molecular weight, low water solubility and high viscosity. In a recent study, it shows that there exists a unique cyclic structure in Candida albicans hyphal β-glucans while not found in yeast β-glucans.12,13

The biological activity of β-glucans are often determined by the molecular weight, the degree of branching and the secondary structure of these compounds.14 Plenty of research about immunomodulating, antitumor and antiinflammatory effects have conducted essentially for β-glucans. β-glucans belong to pathogen-associated molecular patterns (PAMPs), which are able to induce and enhance innate immune responses efficiently mainly

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via Dectin-1/Syk pathway. Moreover, β-glucans have recently been reported to enhance Ag-specific T cell and B cell responses. In our previous studies, it has been confirmed that WGs accelerate the differentiation of MDSCs as well as inhibiting the suppressive function of MDSCs via Dectin-1/Syk pathway. However, the molecular mechanism of regulation on MDSCs by WGP is still unknown.

NFIA is a member of the nuclear factor I (NFI) family as well as NFI-B, NFI-C, NFI-X. Typical NFI protein contains an N-terminal DNA-binding/dimerization domain and C-terminal transcriptional activation and/or repression domain. Recent studies suggest that NFIA-A is a relevant target of the myeloid regulator miR-223 in promyelocytic leukemia cells as over-expression of NFIA suppresses monocytic and granulocytic differentiation of HPCs but accelerates erythropoiesis. All these data suggest that NFIA is a crucial transcription factor involved in the differentiation of myeloid cells, especially granulocyte.

Inhibition of MDSCs function is a feasible approach to enhance antitumor immune responses. In this study, we found that NFIA played a critical role in regulating the immunosuppressive capacity of G-MDSCs. WGP downregulated NFIA expression via c-jun-dependent pathway. NFIA knockdown declined the suppression of G-MDSCs and promoted antitumor T-cell responses. These findings indicate NFIA may have potential therapeutic implications as a modulator of MDSCs suppressive function.

Results

WGP downregulates expression of NFIA in G-MDSCs via Dectin-1 pathway in vitro

Our previous studies have demonstrated that WGP is able to reduce the immunosuppression of MDSCs via Dectin-1 pathway. To investigate the regulation of WGP on the expression of NFIA in G-MDSCs, we firstly confirmed whether there existed NFIA expression in G-MDSCs. G-MDSCs were isolated from splenocytes of tumor-bearing or wild-type C57BL/6 mice. qRT-PCR analysis reflected that NFIA expression increased in G-MDSCs isolated from tumor-bearing mice compared to cells from wild-type mice (Fig. 1A). We also found that the expression of NFIA in G-MDSCs sorted from tumor tissue showed no significant difference compared to NFIA level in G-MDSCs derived from splenocytes in tumor-bearing mice (Fig. S1A). Then, WGP was used to stimulate G-MDSCs isolated from spleen and tumor tissue of tumor-bearing C57BL/6 mice in vitro. NFIA mRNA and protein levels were detected with qRT-PCR and protein gel blot respectively. As shown in Fig. 1B, in G-MDSCs isolated from splenocytes of tumor-bearing mice, NFIA mRNA expression was significantly reduced after stimulation with WGP. Analogously, NFIA protein level in G-MDSCs was declined after WGP treatment (Fig. 1C). The analogous results were also detected in G-MDSCs isolated from tumor tissue of tumor-bearing mice (Fig. S1B and C).

β-glucans recognized as PAMPs can mediate the innate immune responses by recognizing Dectin-1, which is a non-Toll-like pattern recognition receptor for β-glucans, and activate Syk or Raf-1 kinase signaling pathway. In order to confirm whether NFIA expression in G-MDSCs was impaired by WGP through Dectin-1 pathway, anti-Dectin-1 antibody was used to block Dectin-1 and it was found that NFIA downregulation induced by WGP was subverted. As shown in Fig. 1D and E, both NFIA mRNA and protein levels in G-MDSCs restored to high levels after Dectin-1 was blocked even treated with WGP. Collectively, these data demonstrate that WGP is able to decrease NFIA expression in G-MDSCs via Dectin-1 pathway.

The attenuated expression of NFIA in G-MDSCs induced by WGP is c-jun dependent

So far, we had already proved that WGP regulated NFIA expression in G-MDSCs via Dectin-1 pathway, and recently, we have found that WGP affects the suppressive function and differentiation of MDSCs mainly through Dectin-1/Syk pathway. To determine the potential molecular mechanism of WGP inhibiting NFIA expression in G-MDSCs, we detected phosphorylation of c-jun in G-MDSCs after treatment with WGP. The results indicated that WGP was capable of promoting the phosphorylation of c-jun molecular obviously in G-MDSCs (Fig. 2A).

Since the phosphorylation of c-jun molecule which is the downstream of Dectin-1 in G-MDSCs was strengthened by WGP, we supposed that c-jun molecule might be involved in the pathway that WGP regulated NFIA in G-MDSCs. We firstly used SP600125, which is the inhibitor of the phosphorylation of c-jun, to suppress the phosphorylation of c-jun molecule in G-MDSCs and then stimulated G-MDSCs with WGP. Interestingly, NFIA mRNA and protein levels were increased manifestly after the phosphorylation of c-jun molecule was inhibited in G-MDSCs (Fig. 2B and C). The data indicate that c-jun molecule is indeed involved in the Dectin-1 pathway that WGP mediates NFIA expression in G-MDSCs.

NFIA knockdown alters the suppressive capacity of G-MDSCs in vitro

As described previously, WGP can regulate the suppressive function of MDSCs via Dectin-1 pathway. Meanwhile, NFIA acting as a crucial nuclear transcription factor has the ability to mediate lineage commitment and biology of myeloid cells. Since NFIA expression in G-MDSCs was declined after WGP treatment, we hypothesized that NFIA may be related to the suppressive function of G-MDSCs. To determine a link between NFIA expression and the immunosuppression of G-MDSCs, G-MDSCs isolated from spleen and tumor tissue were transfected with NFIA siRNA respectively to inhibit NFIA expression. It was observed that compared with the control, NFIA protein expression in G-MDSCs transfected with siNFIA was inhibited apparently (Fig. 3A; Fig. S2A). In addition, we found G-MDSC-mediated T-cell suppression was altered after NFIA knockdown (Fig. 3B; Fig. S2B).

It has been reported that MDSCs suppress T-cell-induced antitumor immune responses through a variety of mechanisms, especially Arg1 activity, production of NO and ROS. NO and Arg1 are the main effector molecules of M-MDSCs while
G-MDSC-induced suppression relies on ROS and Arg1. NADPH oxidase complex which consists of phox p47 and gp91 appears to be a major source of ROS. Here, p47 and gp91 mRNA levels were chosen to represent the ROS. As shown in Figure 3C; Fig. S2C, after transfected with siNFIA, Arg1 and ROS (p47 and gp91) mRNA levels were reduced in G-MDSCs sorted from spleen and tumor tissue. Meanwhile, in G-MDSCs derived from spleen of tumor-bearing mice, Arg1 activity was declined but ROS produced by G-MDSCs showed no significant difference (Fig. 3D). However, Arg1 activity and ROS production were both decreased in G-MDSCs isolated from tumor tissue after NFIA knockdown (Fig. S2D).

Together, these data indicate that NFIA expression is closely associated with the suppressive capacity of G-MDSCs in vitro.

NFIA knockdown declines the capability of G-MDSCs to accelerate the tumor progression and inhibit the antitumor immune responses

G-MDSCs isolated from spleen of tumor-bearing C57BL/6 mice were transfected with NFIA siRNA in vitro. Then, 3 × 10^6 G-MDSCs transfected with siNFIA were mixed with 0.9 × 10^6 Lewis lung carcinoma (LLC) cells, and these mixed cells were implanted s.c into C57BL/6 mice. As shown in Fig. 4A, the tumor growth rate and tumor weight were significantly reduced.
Administration of siNFIA into tumor tissue delays tumor progression and promotes antitumor T-cell responses

In order to investigate whether NFIA could impair the antitumor immune responses directly, we firstly prepared C57BL/6 mice implanted with $0.9 \times 10^6$ LLC cells. After palpable tumors were formed, tumor-bearing mice were treated by intratumoral injection of NFIA siRNA every 3 d for 2 weeks. At the third week, G-MDSCs in tumor tissue were isolated and NFIA expression along with Arg1 activity and ROS level in G-MDSCs was measured. As shown in Fig. 5A and B, along with the proportion of CD11b$^+$Ly6G$^+$ cells declined, NFIA expression was dropped. At the same time, Arg1 activity and ROS production were also reduced in G-MDSCs sorted from tumor tissue after NFIA knockdown.

Besides that, tumor volume and weight as well as survival rate were detected. As shown in Fig. 5C and D, NFIA knockdown could delay tumor progression and enhance the survival. To confirm the malignant degree of tumor, Ki67 antigen which is associated with carcinoma cells proliferation was detected. We found Ki67 expression was declined in tumor tissue after NFIA knockdown (Fig. 5E). We also found NFIA knockdown was capable of reinforcing the CD4$^+$IFN$\gamma^+$Th1 priming in spleens, draining lymph nodes and tumor tissue. However, NFIA knockdown enhanced CD8$^+$IFN$\gamma^+$CTL priming only in tumor tissue (Fig. 5F). The results suggest that NFIA knockdown enhances the antitumor T-cell responses.

High level of NFIA in human lung cancer tissue

Finally, to define whether NFIA expression has clinical outcome in human tumor biology, we detected the NFIA expression in tumor and adjacent tissues from lung cancer patients. We found that NFIA and Arg1 expression significantly increased in tumor tissue compared with adjacent tissue derived from lung cancer patients (Fig. 6A and B). Moreover, NFIA expression was positively correlated with the expression of Arg1 in tumor tissue of lung cancer patients (Fig. 6C). The data suggest that NFIA expression is associated with MDSC-derived factor in human lung cancer.

Figure 2. The attenuated expression of NFIA in G-MDSCs induced by WGP is c-jun molecule dependent. (A) G-MDSCs sorted from splenocytes of tumor-bearing mice were treated with or without WGP (100 $\mu$g/mL) for indicated times. Protein gel blot analysis developed with anti-phospho-c-jun antibody and anti-c-jun antibody, histone 3 served as a loading control. The amount of phospho-c-jun protein was calculated by gray scanning. (B, C) G-MDSCs sorted from splenocytes of tumor-bearing mice were pretreated with SP600125 (20 $\mu$mol/mL) which is the inhibitor of the phosphorylation of c-jun or DMSO at 37°C for 1 h and then stimulated with 100 $\mu$g/mL WGP. After 24 h/48 h stimulation, cells were collected. (B) The NFIA mRNA expression in collected G-MDSCs was detected by qRT-PCR. (C) Protein gel blot detection of NFIA protein in G-MDSCs. The amount of NFIA protein was calculated by gray scanning. **$p < 0.01$, *$p < 0.05$. 

in mice implanted with G-MDSCs transfected with siNFIA compared with mice implanted with G-MDSCs transfected with RNA control. Analogously, mice in group that implanted with G-MDSCs transfected with siNFIA survived longer compared with the group implanted with G-MDSCs transfected with RNA control (Fig. 5B).

As T cells are the main effector cells suppressed by MDSCs in tumor microenvironment, we further investigated whether the alteration of NFIA expression in G-MDSCs could regulate the Th1 and CTL priming in vivo. Thus, the proportions of CD4$^+$IFN$\gamma^+$ Th1 and CD8$^+$IFN$\gamma^+$ CTL cells in spleens, draining lymph nodes and tumor tissue were detected by flow cytometry, we found that mice in group that implanted with G-MDSCs transfected with siNFIA had augmented Th1 and CTL cells responses compared with the group implanted with G-MDSCs transfected with RNA control (Fig. 5C).
Discussion

β-glucans have been defined as the immunomodulator for a long time.\(^{25}\) β-glucans belong to PAMPs which can bind to their receptors such as Dectin-1 and CR3 and effectively induce immune responses.\(^{26,27}\) β-glucans involved in antitumor immune responses mainly depend on their capacity of regulating the release of cytokines, nitric oxide and arachidonic acid.\(^{28}\) It has been demonstrated that β-glucans, acting as immunomodulators, can promote antitumor effect and inhibit metastasis.\(^{29}\) During antitumor immune responses, β-glucans can activate macrophages and neutrophil complement receptor 3 (CR3) to

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**Figure 3.** NFIA knockdown alters the suppressive capacity of G-MDSCs in vitro. G-MDSCs sorted from splenocytes of tumor-bearing mice were transfected with NFIA siRNA (100 nM). (A) Protein gel blot analysis confirmed NFIA knockdown in G-MDSCs transfected with siNFIA. (B) G-MDSCs transfected with NFIA siRNA, then cells were harvested and cocultured with CD4+ T cells at different ratios in the presence of anti-CD3 mAb and anti-CD28 mAb for 72 h. 3H-thymidine incorporation was used to detect T cells proliferation. (C) NADPH oxidase complex which consists of phox p47 and gp91 appears to be a major source of ROS. Here, p47 and gp91 were chosen to represent the ROS production. Arg1 and gp91/p47 mRNA levels in G-MDSCs transfected with siNFIA were detected by qRT-PCR. (D) Arg1 activity of G-MDSCs transfected with siNFIA was measured. Production of ROS in G-MDSCs was analyzed by flow cytometry. ***p < 0.001, **p < 0.01, *p < 0.05, ns: no significance, Geo MFI: geometric mean fluorescent intensity.
kill iC3b-opsonized tumor cells. Furthermore, it has been recently reported β-glucans enhance Ag-specific T cells and B cells responses by promoting the maturation and antigen presentation of DCs. Thus, β-glucans play a key role in the innate and adaptive immune responses. We have indicated that WGP can regulate the differentiation and immunosuppression of MDSCs as this NF-κB dependent regulation is through Dectin-1/Syk pathway. However, there are still many problems unsolved about the mechanism that WGP mediates MDSCs. Here, we firstly demonstrate that NFIA plays a critical role during WGP mediating the suppressive function of G-MDSCs and involves in Dectin-1 pathway. In this study, we observed that either NFIA mRNA or protein expression in G-MDSCs was significantly inhibited by WGP through Dectin-1 pathway.

As another important receptor that β-glucans can recognize and bind to, CR3 composed with CD11b/CD18 is an integrin dimer which is widely expressed by monocytes, DCs, macrophages, natural killer (NK) cells and neutrophils. CR3 acts as the main receptor which is responsible for phagocytosis of complement-opsonized particles through binding complement component iC3b. β-glucans recognize the lectin domain on the αM chain of CR3 and enhance the innate immune responses. However, the consociation of CR3 and Dectin-1 is indispensable for neutrophil responses to β-glucans when macrophage recognition by β-glucans only relies on the Dectin-1. During this procedure, β-glucans recognize Dectin-1 and facilitate Vav1 activation which results in enabling CR3 binding and internalization of β-glucans by neutrophils. Moreover, soluble β-glucans binding to human neutrophils depend on iC3b-mediated β-glucans opsonization which can accelerate CR3 binding. All these imply the importance of CR3 involved in the β-glucans recognition. Whether its cooperation with Dectin-1 may participate in the process WGP regulates the immune suppressive function of G-MDSCs will be defined in our following work.

After β-glucans recognize and bind to Dectin-1 on the surface of myeloid cells such as DCs, monocytes/macrophages and

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**Figure 4.** NFIA knockdown declines the capability of G-MDSCs to accelerate the tumor progression and inhibit the antitumor immune responses. Groups of mice were injected s.c. with the cells mix of G-MDSCs transfected with siNFIA and LLCs. (A) Tumor volume and weight were measured at indicated time. (B) Survival of mice in different groups. (C) The proportions of CD4^+ IFN^+^ Th1 and CD8^+ IFN^+^ CTL cells from spleens, draining lymph nodes and tumor tissue were analyzed by flow cytometry. ***p < 0.001, **p < 0.01, *p < 0.05.
neutrophils, Syk and Raf-1 kinase signaling pathways are activated, resulting in the phosphorylation of MAP kinase, AP-1 and NF-κB, which finally cause a variety of cellular responses. When trying to determine the specific mechanism that WGP-mediated NFIA expression in G-MDSCs, we found c-jun molecule which is a component of AP-1 could cooperate with NFIA.

Figure 5. Administration of siNFIA into tumor tissue delays tumor progression and enhances antitumor immune responses. Groups of mice bearing established LLC were intratumorally injected with siNFIA every 3 d for 2 weeks. (A) The proportion of CD11b^+ Ly6G^- G-MDSCs in tumor tissue was analyzed by flow cytometry. (B) G-MDSCs from tumor tissue were isolated, NFIA protein level was detected by Protein gel blot. Arg1 activity and ROS production in G-MDSCs sorted from tumor tissues were detected. (C) Tumor volume and weight were measured at indicated time. (D) Survival of mice in different groups. (E) Ki67 expression in tumor tissue was detected by qRT-PCR. (F) The proportions of CD4^+ IFNγ⁺ Th1 and CD8^+ IFNγ⁺ CTL cells from spleens, draining lymph nodes and tumor tissue were analyzed by flow cytometry. **p < 0.01, *p < 0.05, ns: no significance, Geo MFI: geometric mean fluorescent intensity.
Besides that, NFIA is also identified as a molecular target of mutually antagonistic TGF-β and TNF-α regulation while TGF-β and TNF-α mediate NFIA expression in NIH3T3 cells is probably via c-jun molecule.Interestingly, NFIA mRNA and protein levels in G-MDSCs were increased after the inhibition of the phosphorylation of c-jun even treated with WGP. These results indicate the attenuated expression of NFIA in G-MDSCs induced by WGP is c-jun molecule dependent, and NFIA may be associated with the procedure that WGP mediates the function of G-MDSCs. Additionally, NFIA siRNA was used to downregulate the NFIA expression in G-MDSCs, and the immunosuppression of G-MDSCs was significantly declined after NFIA knockdown. Thus, NFIA knockdown can inhibit the suppressive function of G-MDSCs in vitro.

Next, we investigated the relationship between NFIA in G-MDSCs and the antitumor immune responses in vivo. The cells mixed with G-MDSCs transfected with NFIA siRNA and LLCs were implanted to C57BL/6 mice, the tumor progression and both CD4^+IFNγ^+ and CD8^+IFNγ^+ T cells proportions in spleens, draining lymph nodes and tumor tissues were detected. NFIA knockdown declines the capability of G-MDSCs to accelerate the tumor progression and inhibit the antitumor immune responses. Then, to confirm whether NFIA could directly influence the antitumor immune responses, NFIA siRNA was intratumorally injected into tumor tissue and we found NFIA knockdown could delay the progression of tumor and enhance the priming of CD4^+IFNγ^+ T cells in spleens, draining lymph nodes on the Use of Animals for Research and Teaching.

**Materials and Methods**

**Cell line, mice and tumor models**

The LLC cells were obtained according to American Type Culture Collection. Specific pathogen-free male C57BL/6 mice which were 6–8 weeks old were purchased from Yangzhou University. Mice were implanted s.c. with LLC (0.9–3 £ 10^6/mouse) to construct the tumor models. All experiments were approved by the Institutional Committee.

**Whole β-glucan particles (WGs)**

WGP (Biothera, Eagan, M N) purified from the cell wall of S. cerevisiae via a strand of alkaline and acid extractions to generate hollow yeast cell wall ghosts is constituted mainly by β-1,3/1,6-glucan. The constituents of WGP contain β-glucan (>85%), protein (<3.5%), mannan (< 0.01%), and moisture (<8%) as described previously. WGP was kindly provided by Dr Jun Yan from the University of Louisville School of Medicine.

**Isolation of G-MDSCs**

Murine G-MDSCs from the spleen and tumor tissue of tumor-bearing mice were isolated using a mouse MDSC isolation kit (Miltenyi Biotec, Auburn, CA) following the manufacturer’s protocol.

**Reverse transcript PCR and quantitative real-time PCR**

The RNA isolation, reverse transcript PCR and quantitative real-time PCR (qRT-PCR) were performed as described previously. The sequences for the primers used are: NFIA, 5’-TGGCCAAGTTACGGAAAGAT-3’ (forward), 5’-GGCGTCGGCCTACATC-3’ (reverse); Arg1, 5’-ATGGAAGACCTTTCAGTAC-3’ (forward), 5’-GCTG

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**Figure 6.** High level of NFIA in human lung cancer tissue. NFIA expression (A) and Arg1 expression (B) of tumor and adjacent tissues from lung cancer patients were detected by qRT-PCR. (C) Correlation of the expression of NFIA and Arg1 in tumor tissue from lung cancer patients. **p < 0.01, *p < 0.05.**
TCTTCCAAAGAGGTTGGG-3′ (reverse); p47, 5′-GCCAAAAGATGGCAGAATAC
CG-3′ (forward), 5′-CTCGTCGGGACTGTCAAGG-3′ (reverse); gg91, 5′-GAAAGC
CTACAGAGAAGCCAAA-3′ (forward), 5′-TGGTCATC
CCTACGTGGAAAAG
-3′ (reverse); Ki67, 5′-CAAGGAAGTGGTTGCGAC-3′ (forward), 5′-GCAAAGC
CCTGGTTCTCAC-3′ (reverse); β-actin, 5′-TGGATCTCTTGGCATCCATGAAAC
-3′ (forward), 5′-TAAACGCAGCTCAGAATCCCG-3′ (reverse); 18s, 5′-TCC
GGAGAGGAGCCTGAGA-3′ (forward), 5′-GCACCA-GAGTGGCTCCCTCAA-3′ (reverse). Relative quantification of mRNA expression was calculated by the comparative threshold cycle (Ct) method.

Protein gel blot analysis
Proteins extracted from cells were prepared as described previously.19 Protein was resolved by 12% SDS-PAGE and transferred onto immobilon polyvinylidene fluoride (PVDF) membranes (Bio- tifully.19 Protein was resolved by 12% SDS-PAGE and transferred onto immobilon polyvinylidene fluoride (PVDF) membranes (Bio- tifully.19 Protein was resolved by 12% SDS-PAGE and transferred onto immobilon polyvinylidene fluoride (PVDF) membranes (Bio- tifully.19 Protein was resolved by 12% SDS-PAGE and transferred onto immobilon polyvinylidene fluoride (PVDF) membranes (Bio-

Transfection
G-MDSCs were planted into 24-well plates and then transfected with 100 nM NFIA siRNA or its negative control (Ribobio Co., Guangzhou, China) following the manufacturer’s protocol.

Detection of ROS levels and arginase activity
ROS produced by G-MDSCs was measured using the oxidation-sensitive dye 2, 7-dichlorofluorescin diacetate (Invitrogen, Carlsbad, CA). G-MDSCs were harvested and cultured with PMA (30 ng/mL) and oxidation-sensitive dye 2,7-dichlorofluorescin diacetate (2.5 nM) for 0.5 h. After that, cells were detected by flow cytometry.

Arginase activity was detected with the QuantiChrom Arginase Assay kit (BioAssay systems, Hayward, CA). The arginase activity was computed following the manufacturer’s instructions.

Flow cytometry
Cells were stimulated with PMA (Sigma-Aldrich, St. Louis, MO, 50 ng/mL), ionomycin (eBioscience, San Diego, CA, 1 μg/mL), monensin (eBioscience, 2 μg/mL) for 5 h. After that, cells were stained with anti-CD3 and anti-CD8 mAbs (eBioscience), fixed, permeabilized and stained with anti-IFNγ mAb (eBioscience) according to the Intracellular Staining Kit (Invitrogen, Carlsbad, CA) instructions. Cells obtained from tumor tissues were stained with anti-CD11b and anti-Ly6G mAbs (BioLegend).

G-MDSCs suppression assay in vitro
G-MDSCs isolated were transfected with NFIA siRNA or negative control. After that, different ratios of G-MDSCs were cocultured with CD4+ T cells sorted from wild-type C57BL/6 mice spleens using CD4 microbeads (MiltenyiBiotec, Bergisch Gladbach, Germany) in U-bottomed 96-well plates (Costar, Corning, NY) in the presence of 10 μg/mL anti-CD3 mAb and 5 μg/mL anti-CD28 mAb (Biolegend, SanDiego, CA) for 72 h and pulsed with 3H-thymidine (Pharmacia, Stockholm, Sweden, 1 μCi/well) for the last 16 h of culture.

In vivo experiments
G-MDSCs isolated from spleens of tumor-bearing C57BL/6 mice were transfected with NFIA siRNA. Then, 3 × 106 G-

Graphing and statistical analysis of data
Data are presented as Mean ± SD. Data from all experiments were entered into GraphPad Prism 5.0 (GraphPad, San Diego, CA) to generate bar graphs or graphs of tumor regression. The statistical significance of differences between groups was determined by the Student’s t-test. For analyzing survival of mice in different groups, long-rank test was used with the p values indicated. Correlations between variables were determined by Spearman’s correlation coefficient. Differences were considered significant at a p level less than 0.05.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental data for this article can be accessed on the publisher’s website.