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TNF, an inflammatory cytokine that is enriched in the tumor microenvironment, promotes tumor growth and subverts innate immune responses to cancer cells. We previously reported that tumors implanted in TNF receptor–deficient (Tnfr−/−) mice are spontaneously rejected; however, the molecular mechanisms underlying this rejection are unclear. Here we report that TNF signaling drives the peripheral accumulation of myeloid-derived suppressor cells (MDSCs). MDSCs expand extensively during inflammation and tumor progression in mice and humans and can enhance tumor growth by repressing T cell–mediated antitumor responses. Peripheral accumulation of MDSCs was drastically impaired in Tnfr−/− mice. Signaling of TNFR-2, but not TNFR-1, promoted MDSC survival through upregulation of cellular FLICE-inhibitory protein (c-FLIP) and inhibition of caspase-8 activity. Loss of TNFRs impaired the induction of MDSCs from bone marrow cells, but this could be reversed by treatment with caspase inhibitors. These results demonstrate that TNF-2 signaling promotes MDSC survival and accumulation and helps tumor cells evade the immune system.

Introduction

Inflammation has recently been recognized as one of the most important characteristics of tumors (1, 2). Among various inflammatory cells, myeloid-derived suppressor cells (MDSCs) have been demonstrated to be important negative immune regulators. In 1995, Schreiber’s group reported that elimination of granulocytes in nude mice by the Gr1-specific mAb RB6-8C5 inhibits tumor growth (3). Further studies identified that it is CD11b+Gr1+ myeloid cells (i.e., MDSCs) in mice that promote tumor progression (4, 5). MDSCs facilitate tumor growth mainly by suppressing antitumor responses of T cells and/or by promoting angiogenesis (6, 7). Reducing MDSC accumulation by direct depletion of these cells with RB6-8C5 retards tumor development and partly restores antitumor immunity in mice (3, 8). However, MDSCs are a heterogeneous population of cells consisting of myeloid progenitor cells and immature myeloid cells, making them almost impossible to be completely depleted. Therefore, knowing mechanisms governing MDSC accumulation may provide new strategies for targeting MDSCs in clinical trials against cancer.

TNF secreted by tumor or tumor stromal cells has been reported as an important regulator in the tumor microenvironment (1, 9). The role of TNF in tumor immunity is complex and remains controversial. Research using high doses of exogenous TNF or gene-modified tumor cell lines secreting TNF demonstrate the antitumor activity of TNF (10–13). TNF was also implicated in the rejection of transplantable 3-methylcholanthrene–induced (MCA-induced) sarcomas and immune surveillance against spontaneous pancreatic tumors (14, 15). However, TNF also has tumor-promoting effects in various tumor models. TNF knockdown in epithelial ovarian cancer cells led to diminished tumor growth and reduced vascular density (16). TNF knockout mice, as well as mice deficient in TNF receptor–1 (TNFR-1) and TNFR-2 (referred to herein as Tnfr1−/− and Tnfr2−/− mice, respectively), were more resistant to dimethylbenz-anthracene/tetrade-anoylphorbol-acetate-induced (DMBA/TPA–induced) skin carcinogenesis (17–19). In Tnfr1−/− mice, reduced liver metastasis of colon 26 cells after intrasplenic administration has been observed (20). TNF is also involved in the induction and maturation of macrophages and dendritic cells, indicative of a crucial role in the differentiation of myeloid cells, including MDSCs (21, 22).

By binding to TNFR-1 and TNFR-2, TNF activates distinct signaling pathways important for cell proliferation, cell survival, and immune responses (23–26). The default effect of TNF stimulation is to activate the NF-κB pathway and mediate inflammation. TNFR-1 mediates the cytotoxic effect through the death domain, and TNFR-2 is mainly involved in NF-κB activation. NF-κB regulates antiapoptotic products such as cellular FLICE-inhibitory protein (c-FLIP), blocks apoptosis initiated from the TRADD/FADD/RIP-1/caspase-8 complex, and maintains cell survival. However, whether the outcome of TNF regulation for cell apoptosis/survival depends on a balance between TNFR-1 and TNFR-2, on cell types, or on various inflammatory environments is currently not clear.

In the present study, we demonstrated that expression of TNFR-2 alone on MDSCs was necessary and sufficient for their survival, through upregulation of c-FLIP and downregulation of caspase-8 activities. Our findings may hold important significance for the prevention and treatment of inflammation-related cancers.

Results

Transplanted tumors are often spontaneously rejected in Tnfr−/− mice. We previously reported that J558L tumors are often spontaneously rejected in mice deficient in both TNFRs (referred to herein as Tnfr−/− mice), for unknown reasons (27). To exclude possible artifacts due to the contamination of microorganisms, J558L cells were passed 3 times in nude mice and then injected into mice. As
were completely rejected in \( \text{Tnfr}^{-/-} \) mice, were also tested. As shown in Figure 1C, all 7 FD99 tumors and \( \text{Tnfr}^{-/-} \) fibrosarcoma FB61 cells were subcutaneously injected into and of splenic CD11b+Gr1+ myeloid cells in tumor-bearing mental Figure 2B). However, the percentages and absolute numbers of MDSCs causing tumor rejection, we established several \( \text{Tnfr}^{-/-} \) and \( \text{Tnfr}^{+/+} \) tumor cell lines by MCA-induced carcinogenesis in \( \text{Tnfr}^{-/-} \) and \( \text{Tnfr}^{+/+} \) mice. These cells did not express the corresponding TNFRs, and the downstream signaling pathways were defective, as confirmed for FB61 cells by flow cytometry and Western blot (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI64115DS1). Subsequently, the \( \text{Tnfr}^{+/+} \) fibrosarcoma FB61 cells were subcutaneously injected into \( \text{Tnfr}^{-/-} \) and \( \text{Tnfr}^{+/+} \) mice (Figure 1B). Whereas 6 of 7 FB61 tumors were spontaneously rejected within around 3 weeks in \( \text{Tnfr}^{-/-} \) mice, all 5 FB61 tumors in \( \text{Tnfr}^{-/-} \) mice grew progressively during the same time period. \( \text{Tnfr}^{-/-} \) FD99 tumor cells, originating from \( \text{Tnfr}^{-/-} \) mice, were also tested. As shown in Figure 1C, all 7 FD99 tumors were completely rejected in \( \text{Tnfr}^{-/-} \) mice, but none of the tumors did so in the \( \text{Tnfr}^{+/+} \) counterparts. These results demonstrated that the spontaneous tumor rejection in \( \text{Tnfr}^{-/-} \) mice was caused not by immunogenicity of TNFR proteins on transplanted tumor cells, but by TNFR expression on host cells.

**Tumor rejection is associated with impaired peripheral accumulation of MDSCs.** To address the mechanisms responsible for the aberrant tumor growth in \( \text{Tnfr}^{-/-} \) mice, we first analyzed immune cell populations in peripheral organs, such as tumors and spleens, at days 8–10, when tumors began to regress in \( \text{Tnfr}^{-/-} \) mice. No clear difference was found for CD11c+ dendritic cells, B220+ B cells, or CD8+ cytotoxic or CD4+ T cells (including CD4+Foxp3+ regulatory T cells and CD4+Th17 cells) between \( \text{Tnfr}^{+/+} \) and \( \text{Tnfr}^{-/-} \) mouse spleens (Supplemental Figure 2A). The density of blood vessels, as well as infiltrated CD4+ and CD8+ T cells in the tumor, were also similar between \( \text{Tnfr}^{-/-} \) and \( \text{Tnfr}^{+/+} \) mice (Supplemental Figure 2B). However, the percentages and absolute numbers of splenic CD11b+Gr1+ myeloid cells in tumor-bearing \( \text{Tnfr}^{-/-} \) mice were drastically reduced compared with those in their \( \text{Tnfr}^{+/+} \) counterparts (Figure 2, A and B). Decreased proportions of CD11b+Gr1+ cells in tumor-bearing \( \text{Tnfr}^{-/-} \) mice were also observed in the peripheral blood and within the tumor (Figure 2, C and D). CD11b+Gr1+ cells from both \( \text{Tnfr}^{-/-} \) and \( \text{Tnfr}^{+/+} \) tumor-bearing mice, but not from control mice, inhibited the proliferation of CD4+ T cells (Supplemental Figure 3A). Compared with the cells from control animals, they exhibited increased arginase and iNOS activities and produced higher levels of immune-suppressive cytokine IL-10 (Supplemental Figure 3, B–D). Therefore, the CD11b+Gr1+ cells from the tumor-bearing mice were also named as MDSCs (13, 28, 29).

To investigate local distributions of the CD11b+Gr1+ MDSCs in the absence of TNFR, immunofluorescence staining was performed in tumors and spleens. Although the number of CD11b+ and Gr1+ cells was reduced within \( \text{Tnfr}^{-/-} \) tumor sites, the distribution of cells did not differ in \( \text{Tnfr}^{-/-} \) and \( \text{Tnfr}^{+/+} \) mice (Figure 2E). In spleens, germinal center structures were disturbed in \( \text{Tnfr}^{-/-} \) mice, but again, the distribution patterns of CD11b+ and Gr1+ cells were similar to those of \( \text{Tnfr}^{+/+} \) controls. The activation status of \( \text{Tnfr}^{-/-} \) compared with \( \text{Tnfr}^{+/+} \) MDSCs showed no obvious changes in surface expression of the costimulatory molecules CD80, CD86, and B7-H1; the cytokine receptor IL-4R; the antigenic peptide–presenting molecules H2-Kd and I-Ad; and the apoptosis-related molecules CD95 and CD95L (Figure 2F). These results indicated that TNF signaling contributed to enhanced MDSC accumulation in spleen and tumor, but did not influence the cells’ immunosuppressive function and distribution in organs.

Adoptive transfer of \( \text{Tnfr}^{+/+} \) MDSCs restores tumor growth in \( \text{Tnfr}^{-/-} \) mice in a dose-dependent manner. To confirm that the lack of TNFRs on MDSCs was attributable to impaired \( \text{Tnfr}^{-/-} \) mouse tumor growth, we purified \( \text{Tnfr}^{-/-} \) and \( \text{Tnfr}^{+/+} \) spleen MDSCs and adoptively transferred them to \( \text{Tnfr}^{-/-} \) and \( \text{Tnfr}^{+/+} \) mice. Subsequently, \( \text{Tnfr}^{+/+} \) FB61 fibrosarcoma cells were used to challenge the MDSC recipient mice. In \( \text{Tnfr}^{-/-} \) recipients of \( \text{Tnfr}^{+/+} \) MDSCs (referred to

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**Figure 1**

Transplanted tumors are spontaneously rejected in \( \text{Tnfr}^{+/+} \) mice. \( \text{Tnfr}^{+/+} \) (4–5) and \( \text{Tnfr}^{-/-} \) (7) mice were subcutaneously injected with (A) \( 5 \times 10^5 \) J558L cells, (B) \( 1 \times 10^6 \) FB61 cells, or (C) \( 1 \times 10^6 \) FD99 cells. Tumor volumes after tumor cell inoculation are shown; each line represents the growth curve of a tumor in a single mouse. Similar results were obtained from 2 other independent experiments.
Peripheral accumulation of CD11b+Gr1+ cells is impaired in Tnfr–/– mice. (A) Tnfr+/+ and Tnfr–/– mice were subcutaneously injected with 1 × 10⁶ FB61 cells or PBS as control. 8–10 days after tumor cell inoculation, single splenocytes were stained for CD11b and Gr1 and assessed by flow cytometry. Left: Gated CD11b+Gr1+ cells. Right: Percent CD11b+Gr1+ cells in tumor-bearing Tnfr+/+ and Tnfr–/– mice and corresponding controls. Bars denote means. **P < 0.01. (B) Absolute number of CD11b+Gr1+ cells in spleens of tumor-bearing mice and corresponding controls. Data are mean ± SEM. *P < 0.05. (C and D) Percent CD11b+Gr1+ cells relative to total cells for (C) peripheral blood and (D) tumor tissues. n = 5 per group. Data are mean ± SEM. *P < 0.05. (E) CD11b+ and Gr1+ cells in tumor and spleen sections of Tnfr+/+ and Tnfr–/– mice were visualized by immunofluorescence staining. Nuclei were counterstained with DAPI. Images are representative of at least 3 mice per group. Original magnification, ×200 (tumor); ×100 (spleen). Scale bars: 300 μm. (F) Spleen cells as above were stained for flow cytometry analysis. Unstained Tnfr+/+ splenocytes were used as a control.
TNFR expression on MDSCs was necessary and sufficient for the MDSC-mediated tumor-promoting effect.

Enhanced apoptosis is responsible for reduced MDSC numbers in Tnfr−/− mice. It is well known that TNF elicits different cellular responses, such as cell proliferation or apoptosis (30). Because the reduced numbers of MDSCs in Tnfr−/− mice could be attributed to decreased proliferation or increased apoptosis, we used BrdU to determine the proliferation of MDSCs. BrdU incorporation in bone marrow MDSCs showed no differences between FB61 tumor–bearing Tnfr−/− and Tnfr+/+ mice (Figure 4A), which indicates that MDSC proliferation was not influenced. Similar results were obtained in spleen MDSCs (data not shown). However, in the same time period, when CD11b+Gr1+ cells were stained with annexin V antibody to detect apoptosis (Figure 4B), a drastic increase of annexin V+ cell numbers was found in Tnfr−/− mice compared with that in controls (24% ± 5% vs. 4% ± 3%; Figure 4C). These results suggest that the impaired accumulation of MDSCs in Tnfr−/− mice during tumor growth was not due to the decreased proliferation, but the increased apoptosis of these cells in the absence of TNFR.

To further investigate why TNF signaling is crucial for protecting MDSCs from apoptosis, activities of apoptosis-related caspases were determined in splenic MDSCs. More than a 3-fold increase of caspase-8 activity was found in purified Tnfr−/− CD11b+Gr1+ cells (1.28 ± 0.18 vs. 0.32 ± 0.06; Figure 4D), but no increase of caspase-3 or caspase-9 activity was observed (data not shown). Western blot analysis confirmed this observation, since the levels of cleaved caspase-8 also increased in purified Tnfr−/− CD11b+Gr1+ cells was found in Tnfr−/− mice compared with that in controls (24% ± 5% vs. 4% ± 3%; Figure 4C). These results suggest that the impaired accumulation of MDSCs in Tnfr−/− mice during tumor growth was not due to the decreased proliferation, but the increased apoptosis of these cells in the absence of TNFR.
that lack of TNFR signaling led to enhanced caspase-8 activation and therefore accelerated MDSC apoptosis. Thus, TNFR signaling is necessary for maintaining antiapoptotic activities in MDSCs.

NF-κB-mediated c-FLIP expression is downregulated in Tnfr–/– MDSCs. c-FLIP is a natural inhibitor of caspase-8. To investigate whether c-FLIP is involved in influencing MDSC apoptosis, its expression was determined at the transcriptional level in purified splenic CD11b+Gr1+ cells. Corresponding to the enzymatic activities (Figure 4, D and E), significantly decreased c-FLIP — rather than Bcl-xL, another antiapoptotic protein that serves as a caspase-9 inhibitor — was found in Tnfr–/– MDSCs (Figure 5A). Similarly, Western blot confirmed decreased intracellular c-FLIP and increased cleaved caspase-8 in Tnfr–/– MDSCs, whereas Bcl-xL level did not markedly change (Figure 5B).

c-FLIP expression can be regulated by NF-κB pathway activation (26). To address whether the NF-κB pathway is involved here, recombinant mouse TNF was added to cultures of Tnfr+/+ or Tnfr–/– MDSCs, and phosphorylation of NF-κB p65, IκBα, and IKKα/β over time was studied. In Tnfr+/+ MDSCs, the protein level of p-IKKα/β steadily increased over 30 minutes, whereas p–NF-κB p65 and p-IκBα peaked at 15 minutes (Figure 5C). This was accompanied by an increase of c-FLIP and inhibited cleavage of caspase-8 within 48 hours. However, in MDSCs from Tnfr–/– mice, steady low levels of p–NF-κB p65 and absent p-IKKα/β and p-IκBα confirmed the lack of functional receptors of TNF. This was accompanied by reduced c-FLIP between 12 and 36 hours and a simultaneous increase in cleaved caspase-8. These data indicate that TNF induces c-FLIP upregulation through the NF-κB pathway in MDSCs.

TNFR-2 alone is crucial for the accumulation of MDSCs. TNF binds to 2 receptors, the ubiquitously expressed TNFR-1 and the hematopoietic cell–restricted TNFR-2. Flow cytometry analysis showed that both TNFR-1 and TNFR-2 were expressed on MDSCs (Sup-
NF-κB-mediated c-FLIP expression is downregulated in Tnfr<sup>−/−</sup>-MDSCs. (A) CD11b<sup>+</sup>Gr1<sup>+</sup> cells were freshly isolated from Tnfr<sup>+/+</sup> and Tnfr<sup>−/−</sup> tumor-bearing mice. Amounts of c-FLIP or Bcl-x<sub>L</sub> mRNA were determined by real-time RT-PCR and are shown relative to β-actin mRNA (mean ± SEM). *P < 0.05. (B) Total cell lysates were extracted from purified Tnfr<sup>+/+</sup> and Tnfr<sup>−/−</sup> MDSCs. Levels of c-FLIP, cleaved caspase-8, and Bcl-x<sub>L</sub> were determined by Western blot. β-actin served as internal control. (C) Purified MDSCs were stimulated with 20 ng/ml TNF for the indicated times. Levels of p-<sup>NF-κB p65</sup>, p-IκBα, and p-IκKα/β, as well as c-FLIP and cleaved caspase-8, were determined from total cell lysates by Western blot. β-actin served as internal control. Representative images are shown for 3 independent experiments.
MDSC accumulation is also influenced by many other factors, such as cyclooxygenase2, prostaglandins, SCF, M-CSF, GM-CSF, IL-1β, IL-6, S100A8/9, and VEGF (31–35). These factors are critical not only in the expansion of MDSCs, but also, more importantly, in their functional activation. Both processes are mainly regulated by the same set of transcription factors, i.e., STAT3, STAT1, STAT6, and NF-κB (36, 37). Blockade of factors such as S100A8/9 impairs MDSC accumulation (34). Besides, recent researches report that activation of the Fas/FasL pathway (38) or blockade of the IL-4Rα/STAT6 pathway (39) also promote MDSC apoptosis. It is not presently clear whether these factors function separately or synergistically or interact with TNF in MDSC accumulation. In-depth studies may further understanding of the general process of MDSC induction and accumulation in peripheral tissues. Moreover, since our experiments did not formally exclude that TNF signaling might also influence MDSC differentiation, other potential causes of peripheral MDSC accumulation remain to be studied.

Figure 6
TNFR-2 signaling maintains survival of CD11b⁺Gr1⁺ cells. (A) Tnfr⁺/⁺, Tnfr1⁻/⁻, Tnfr2⁻/⁻, and Tnfr⁻/⁻ mice were subcutaneously injected with 1 × 10⁶ FB61 cells. Tumor volumes were measured after tumor cell inoculation (mean ± SEM), n = 5–7 per group. *P < 0.05. (B) Spleen cells were prepared 8–10 days after tumor cell inoculation, stained for CD11b and Gr1, and analyzed by flow cytometry. Data (mean ± SEM) represent percent CD11b⁺Gr1⁺ cells of total spleen cells. *P < 0.05. (C) Accumulation of CD11b⁺ and Gr1⁺ cells in the spleen of tumor-bearing mice, determined by immunofluorescence staining (see Methods). Dotted outlines denote germinal centers. Images are representative of at least 3 mice. Original magnification, ×100 (CD11b); ×200 (Gr1). Scale bars: 300 µm. (D) Total cell lysates were prepared from isolated Tnfr1⁻/⁻ and Tnfr2⁻/⁻ CD11b⁺Gr1⁺ cells after stimulation with 20 ng/ml TNF for the indicated times. Levels of TRAF2, p–NF-κB p65, p-Iκκα, and p-Iκκα/β, c-FLIP, and cleaved caspase-8 were determined by Western blot. See Figure 5C for expression levels of respective molecules in Tnfr⁺/⁺ mice (controls). β-actin was used as an internal control. Representative images are shown for 3 independent experiments.
In tumor-bearing mice, MDSCs accumulate in central (bone marrow) and peripheral (spleen, blood, draining lymph nodes) organs as well as in tumor sites. Our results showed decreased proportions of MDSCs in spleen, peripheral blood, and tumor sites of Tnfr−/− mice (Figure 2, A–E). In bone marrow of tumor-bearing or CFA-injected mice, the proportion of MDSCs was comparable to that in corresponding Tnfr+/+ mice (Figure 2, A–E). In bone marrow of tumor-bearing mice, MDSCs accumulate not only by tumor growth, but also by many other kinds of chronic inflammation (28). In mice, TNF blockade inhibits tumor progression and/or metastasis in several transplanted and carcinogen-induced tumor models (12, 18). In humans, TNF blockade has shown some evidence of anticancer activity in phase I and phase II clinical trials (46). Moreover, in RA patients, the anti-TNF reagents etanercept and infliximab induce apoptosis in monocytes and macrophages in the synovial fluid and in the peripheral blood (49).

Targeting TNF is becoming a treatment option in both cancer (46) and immune-mediated chronic inflammatory diseases, such as rheumatoid arthritis and ankylosing spondylitis (47). In mice, TNF blockade inhibits tumor progression and/or metastasis in several transplanted and carcinogen-induced tumor models (12, 18). In humans, TNF blockade has shown some evidence of anticancer activity in phase I and phase II clinical trials (46). However, the anti-TNF therapy is still in the initial stages (43, 48).

In tumor-bearing mice, MDSCs accumulate in central (bone marrow) and peripheral (spleen, blood, draining lymph nodes) organs as well as in tumor sites. Our results showed decreased proportions of MDSCs in spleen, peripheral blood, and tumor sites of Tnfr−/− mice (Figure 2, A–E). In bone marrow of tumor-bearing or CFA-injected mice, the proportion of MDSCs was comparable to that in corresponding Tnfr−/− mice (data not shown). These findings underline the crucial role of TNFR signaling in peripheral MDSC accumulation, which mainly affects local but not systematic inflammatory responses, despite the diversity of MDSC populations distributing differently in every organ and the predominance of myeloid precursors in the bone marrow (45).

**Figure 7** Neutralization of endogenous TNF impairs transplanted tumor growth and peripheral accumulation of CD11b+Gr1+ cells. (A) Tnfr−/− mice were intraperitoneally injected with the TNF-neutralizing mAb V1q (n = 6) or isotype control mAb (n = 4) 2 days prior to subcutaneous injection of 1 × 10^6 FB61 cells. mAb injection was repeated 3 and 8 days after tumor cell inoculation. Each line represents the growth curve of a tumor in a single mouse; bars denote mean tumor size of each group at day 15. *P < 0.05. (B and C) Spleen or tumor cells were isolated after tumor cell inoculation and stained for flow cytometry. Shown are percent CD11b+Gr1+ cells in (B) total spleen cells and (C) total tumor cells at days 8–10 (mean ± SEM). n = 3–5 per group. *P < 0.05. (D) Bone marrow cells isolated from Tnfr−/− mice were induced for MDSC generation in vitro (see Methods). V1q, z-IETD, or DMSO (as control) was added to the culture after 6 hours. Cells were collected 5 days later and stained for CD11b and Gr1. Data (mean ± SEM) represent percent CD11b+Gr1+ cells within total living cells in the culture. *P < 0.05.
sible mechanism of anti-TNF reagent in treating such inflammatory diseases and also indicate a possible therapeutic benefit of anti-TNF reagent in clinical trials against cancers.

**Methods**

**Mice.** Tnf1−/−, Tnf2−/−, Tnfr−/−, and Tnfr+/− mice on BALB/c background were generated as described previously (13). The eGFP-transgenic mice, Tnf2−/− and their Tnfr−/− wild-type controls, on a C57BL/6 background were obtained from the Jackson Laboratory. All mice were bred in the specific pathogen-free barrier facility of Institute of Biophysics, Chinese Academy of Sciences. In all experiments described here, female mice aged 6–8 weeks were used.

**Cell lines.** J558L, a plasmacytoma cell line of BALB/c origin (50), expressed both TNFR-1 and TNFR-2 on the cell surface (data not shown). J558L cells were passed in nude mice for 3 generations before inoculation into Tnfr−/− and Tnfr+/− mice. To generate the Tnfr1−/− and Tnfr2−/− fibrosarcoma cell lines, MCA (Sigma-Aldrich) emulsified in sesame oil was intramuscularly injected into Tnfr1−/− or Tnfr2−/− mice (51). The MCA-induced tumors were excised at a size of 1 cm in diameter and cut into small fragments of about 3 × 3 × 3 mm. Tumor fragments were subsequently grafted twice onto nude mice, twice onto Tnfr1−/− or Tnfr2−/− mice, and twice onto Tnfr+/− BALB/c mice. Tumor nodules were then isolated, minced, and digested to obtain single-cell suspension using trypsin/EDTA solution. At least 5 passages in vitro, the Tnfr1−/− cell line FB61 (52) and the Tnfr2−/− cell line FD99 were randomly chosen for further experiments. MCA205 is a MCA-induced fibrosarcoma cell line of C57BL/6 origin (53). All tumor cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all Gibco).

**In vivo studies.** Exponentially growing tumor cells were harvested, washed, and injected subcutaneously into the abdominal region of mice. Tumor growth was monitored every 2–5 days, and tumor volume was calculated as (l × w × w) / 2. To neutralize endogenous TNF, each mouse was intraperitoneally injected with ascites containing 0.5 mg ml−1 anti-TNF antibody (BD Biosciences). Data were acquired with a FACS Calibur flow cytometer (BD Biosciences) and analyzed using Cell Quest-Pro (BD Biosciences) or FlowJo (FlowJo) software. To determine IL-10 or TNF production, 2 × 10⁶ purified CD11b+Gr1+ cells or 2 × 10⁵ J558L, FB61, or FD99 tumor cells were cultured in 96-well plates with 200 μl RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco), then stimulated with LPS (1 μg/ml; Sigma-Aldrich) and IFN-γ (50 ng/ml; PeproTech) as described previously (54). Supernatants were collected after 16 hours. The content of IL-10 or TNF was measured with the mouse inflammation cytometric bead array kit (CBA; BD Biosciences).

**Induction of MDSCs in vitro.** Induction of CD11b+Gr1+ cells from bone marrow progenitors was done as described previously (55, 56). In brief, bone marrow cells were obtained from the femurs and tibias. 1 × 10⁶ bone marrow cells were cultured in 24-well plates in media conditioned by EL4 tumor cells, supplemented with 10% fetal calf serum (Gibco), 20 ng/ml GM-CSF (PeproTech), and 10 ng/ml IL-4 (PeproTech). Cells were collected 5 days later, and the proportion of CD11b+Gr1+ cells was analyzed by flow cytometry. Pan-caspase inhibitor z-VAD and caspase-8–specific inhibitor z-IETD (20 μM each; Bachem) were dissolved in DMSO. To block endogenous TNF, 5 μg/ml V1q mAb was added to the culture.

**BrdU incorporation.** Each mouse was intraperitoneally injected with 100 μl BrdU (10 mg/ml; Sigma-Aldrich) 3, 5, and 8 days after the inoculation of FB61 cells. 24 hours after each BrdU injection, the proportion of CD11b+Gr1+ cells with BrdU incorporation in total CD11b+Gr1+ cells in bone marrow was determined by flow cytometry analysis.

**Immunofluorescent staining.** Groups of mice were subcutaneously injected with 1 × 10⁶ FB61 cells; 8–10 days later, mice with a tumor about 200 mm³ in size were sacrificed. Tumors and spleens were fixed and prepared for cryostat sections as previously described (29). Tissue sections (7 μm for tumors; 5 μm for spleens) were incubated with rat anti-mouse mAbs specific to CD4, CD8, CD11b, CD31, or Gr1 (all from BD Biosciences) and anti-GFP rabbit serum (Life Technologies). For detection of primary antibody binding, sections were incubated with Alexa Fluor 555 goat anti-rabbit IgG (Life Technologies). Primary antibody bodies were omitted in negative controls. Nuclei were counterstained by DAPI (Sigma-Aldrich), and slides were mounted for fluorescence observation on an Olympus FX1000 confocal microscope. Tissue sections from at least 3 mice per group were evaluated.

**T cell proliferation assay.** The T cell proliferation assay was performed as previously described (28, 57), with slight modifications. To determine the immune-suppressive function of MDSCs, splenocytes of tumor-free Tnf+/− mice were labeled with 2.5 mM CFSE (Molecular Probes) for 10 minutes at 37°C and distributed in 96-well round-bottomed plates, with 3 × 10⁵ cells and 100 μl RPMI 1640 medium per well. These cells were cultured with or without 1 × 10⁵ CD11b+Gr1+ cells derived from Tnf+/−, Tnf−/−, or Tnfr2−/− FB61 tumor-bearing mice or tumor-free controls and stimulated with 2.5 mg/ml concanavalin A (Sigma-Aldrich) for 72 hours. For stimulating specific T cell proliferation, spleen cells from Tnf+/−→Tnf−/− or Tnfr1−/−→Tnfr−/− mice were CFSE labeled, and 3 × 10⁵ cells were plated in 100 μl medium per well as described above. FB61 cell lysate (made from 3 freeze-thaw cycles) was added at a 1:40 FB61/splenocyte ratio by cell counts. After 72 hours, cells were collected and stained. The dilution of CFSE in CD4+ and CD8+ T cells was determined by flow cytometry analysis.

**Real time RT-PCR.** Total RNA was extracted from 1 × 10⁶ isolated CD11b+Gr1+ cells with TRIzol (Invitrogen) and quantified on a ND-1000 spectrophotometer (NanoDrop Technologies). The cDNA was synthesized with 2 μg RNA, 9 nt random primer (TaKaRa), and M-MLV reverse transcrip
transcriptase (Promega). The amount of Bcl-xL or c-FLIP mRNA was determined using iQTM SYBR Green Supermix on MyiQTM system (Bio-Rad Laboratories). β-actin mRNA was used as internal control. Specific primers were as follows: Bcl-xL, 5′-ACAGGCACGCCAGCATGATT-3′ and 5′-ACCCGAGTCAAACCTCAT-3′; c-FLIP, 5′-TTGATTTGATTGGAAGC-3′ and 5′-AACCTGCTACTAAGCAGC-3′; β-actin, 5′-GAAAGTTGACGTT-GACATTCCCTA-3′ and 5′-CTAGGAGGGCACTAGTCTTGTA-3′.

Western blot. Cells were lysed by SDS lysis solution (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40, 0.5% [v/v] sodium deoxycholate, 1% [w/v] SDS, 1 mM EDTA) supplemented with 100 μM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 1 mM/ml sodium orthovanadate, and 50 μmol/l NaF. Aliquots of cell extracts were resolved on 10% SDS-PAGE gel, then transferred to nitrocellulose membrane (GE Healthcare) on a semidry transfer apparatus (Bio-Rad Laboratories). Primary antibodies used were as follows: Bcl-xL (H-5, 1:1,000; Santa Cruz), caspase-8 (D-8, 1:1,000; Santa Cruz), c-FLIP (catalog no. 3210, 1:1,000; Cell Signaling Technology), p–NF-κB (Ser536) (clone 33H1, 1:1,000; Cell Signaling Technology), TRAF2 (Cys192) (1:1,000; Cell Signaling Technology), p-IKKα/β (Ser176/180) (clone 16A6, 1:1,000; Cell Signaling Technology), p-IκBα (Ser32) (clone 14D4, 1:1,000; Cell Signaling Technology), and β-actin (1:8,000; Sigma–Aldrich). HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (Thermo) were used as secondary antibodies. After washing with PBST, the membrane was incubated with chemiluminescent substrate (Thermo) for 5 minutes. Specific bands were visualized by exposing the membrane to X-ray film (Kodak) in a dark room. To determine the extent of molecule involvement in the TNF signaling pathway, isolated CD11b+Gr1− cells were stimulated with murine recombinant TNF (20 ng/ml; PeproTech) for different time periods before collection. Primary antibody staining was determined using iQTM SYBR Green Supermix on MyiQTM system (Bio-Rad) with 100 μl of lysis solution (0.1% Triton X-100, 10 mM MnCl2, 25 mM Tris-HCl). Lysate was activated at 56°C for 10 minutes, supplemented with 200 μl of EDTA-CaPO4 substrate and incubated at 37°C for 1 hour. Absorbance at 400 nm was read in a microplate reader (Bio-Rad Laboratories).

Arginase activity. Arginase activity was determined by measuring the amount of urea generated from the hydrolysis of l-arginine, as described previously (29). Briefly, 1 × 106 isolated CD11b+Gr1− cells were solubilized with 100 μl lysis solution (0.1% Triton X-100, 10 mM MnCl2, 25 mM Tris-HCl). Lysate was activated at 56°C for 10 minutes, supplemented with 200 μl 0.5 M l-arginine (pH 9.7), and then incubated at 37°C for 4 hours. The reaction was stopped by 800 μl H2SO4/H3PO4/H2O (1:3:7 v/v/v). Subsequently, 40 μl α-isonitrosopropiophenone (dissolved in ethanol; Sigma–Aldrich) was added, followed by another heating at 100°C for 30 minutes. The concentration of urea was measured by 540 nm absorbance. 1 U enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per minute. Data are presented as mean ± SEM of triplicate wells.

iNOS activity. The activity of iNOS was measured by Nitric Oxide Synthase Assay Kit (Boeytime) according to the manufacturer’s instructions. Briefly, 2 × 104 isolated CD11b+Gr1− cells were cultured in 96-well plates, then stimulated with LPS (1 μg/ml; Sigma–Aldrich) and IFN-γ (50 ng/ml; PeproTech) with or without the iNOS-specific inhibitor 5-methylisothiourea sulfate (SMT; 1 mM; Sigma–Aldrich) for 8 hours at 37°C. Culture supernatant was removed, and 100 μl NOS assay buffer was added to each well, followed by 100 μl NOS assay reaction solution (50% NOS assay buffer, 39.8% MilliQ water, 5% l-arginine solution, 5% 0.1 mM NADPH, 0.2% fluorescent probes diaminofluorescin-diacetate), and incubated for 2 hours at 37°C. Relative fluorescence units (RFUs) were measured with a fluorescence plate reader (BioTek) at 485 nm excitation and 535 nm emission. iNOS activity was assessed as the difference between RFUs with stimulation alone and RFUs with stimulation plus SMT. Data were presented as mean ± SEM of triplicate wells.

Statistics. Differences between 2 groups were analyzed by 2-tailed Student’s t test. A P value less than 0.05 was considered significant.

Study approval. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee of Institute of Biophysics, Chinese Academy of Sciences.

Acknowledgments. The authors thank Miltenyi Biotec for providing mouse MDSC isolation kits. This work was supported by the Ministry of Science and Technology of China (2012CB917103 and 2012CB934003), the National Natural Science Foundation of China (81030049, 31071261, and 81001328), and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

Received for publication April 3, 2012, and accepted in revised form August 30, 2012.

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