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Tumor Cell-Released TLR4 Ligands Stimulate Gr-1⁺CD11b⁺ F4/80⁺ Cells to Induce Apoptosis of Activated T Cells

Yan-Yan Liu,*1 Ling-Cong Sun,*1 Jing-Jing Wei,* Dong Li,* Ye Yuan,* Bin Yan,* Zhi-Hui Liu,† Hui-Fen Zhu,† Yong Xu,† Bo Li,* Chuan-Wang Song,* Sheng-Jun Liao,* Zhang Lei,* Gui-Mei Zhang,* and Zuo-Hua Feng*

Gr-1⁺CD11b⁺F4/80⁺ cells play important roles in tumor development and have a negative effect on tumor immunotherapy. So far, the mechanisms underlying the regulation of their immunosuppressive phenotype by classical and alternative macrophage activation stimuli are not well elucidated. In this study, we found that molecules from necrotic tumor cells (NTC-Ms) stimulated Gr-1⁺CD11b⁺F4/80⁺ cells to induce apoptosis of activated T cells but not nonstimulated T cells. The apoptosis-inducing capacity was determined by higher expression levels of arginase I and IL-10 relative to those of NO synthase 2 and IL-12 in Gr-1⁺CD11b⁺F4/80⁺ cells, which were induced by NTC-Ms through TLR4 signaling. The apoptosis-inducing capacity of NTC-Ms–stimulated Gr-1⁺CD11b⁺F4/80⁺ cells could be enhanced by IL-10. IFN-γ may reduce the apoptosis-inducing capacity of Gr-1⁺CD11b⁺F4/80⁺ cells only if their response to IFN-γ was not attenuated. However, the potential of Gr-1⁺CD11b⁺F4/80⁺ cells to express IL-12 in response to IFN-γ could be attenuated by tumor, partially due to the existence of active STAT3 in Gr-1⁺CD11b⁺F4/80⁺ cells and NTC-Ms from tumor. In this situation, IFN-γ could not effectively reduce the apoptosis-inducing capacity of Gr-1⁺CD11b⁺F4/80⁺ cells. Tumor immunotherapy with 4-1BBL/soluble programmed death-1 may significantly reduce, but not abolish the apoptosis-inducing capacity of Gr-1⁺CD11b⁺F4/80⁺ cells in local microenvironment. Blockade of TLR4 signaling could further reduce the apoptosis-inducing capacity of Gr-1⁺CD11b⁺F4/80⁺ cells and enhance the suppressive effect of 4-1BBL/soluble form of programmed death-1 on tumor growth. These findings indicate the relationship of distinct signaling pathways with apoptosis-inducing capacity of Gr-1⁺CD11b⁺F4/80⁺ cells and emphasize the importance of blocking TLR4 signaling to prevent the induction of T cell apoptosis by Gr-1⁺CD11b⁺F4/80⁺ cells.


Abbreviations used in this paper: BM, bone marrow; BMDM, bone marrow–derived monocyte; DC, dendritic cell; IFR-3, IFN regulatory factor-3; MDSC, myeloid–derived suppressor cell; mTNF-α, membrane-associated TNF-α; N-BM, bone marrow of naive mice; N-BMDM, naive bone marrow–derived monocyte; NOS2, NO synthase 2; T-M, molecule from necrotic tumor cells; PC, peritoneal cavity; PD-1, programmed death-1; PM, peritoneal macrophage; Res, resveratrol; s, soluble form; sPD-1, soluble form of programmed death-1; T-BMDM, bone marrow–derived monocyte prepared from tumor-bearing mice; TAM, tumor–associated macrophage; T10-BM, bone marrow of tumor-bearing mice on day 10 after tumor inoculation; TRIF, Toll/IL-1R domain–containing adapter–inducing IFN-β.
Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells recruited into tumor microenvironment could be stimulated by both classical activation signal such as endogenous ligands for TLRs and the deactivator of macrophages such as IL-10. They may also be stimulated by IFN-γ, which could be increased in local microenvironment by tumor immunotherapy (22). So far, the effects of these stimuli on the capacity of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells to induce apoptosis of activated T cells are not well elucidated. In this study, we found that bone marrow-derived Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> monocytes could induce apoptosis of activated T cells because of their distinct response to TLR4 ligands released from damaged tumor cells. IFN-γ reduced the apoptosis-inducing capacity of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells if their response to IFN-γ was normal. Importantly, the apoptosis-inducing capacity of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells was not completely abolished in the process of tumor immunotherapy. Blockade of TLR4 signaling could further reduce the apoptosis-inducing capacity of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells and suppress tumor growth.

Materials and Methods

Animal and cell line

BALB/c mice, 6–8 wk old, were purchased from Center of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College (Wuhan, China). Mouse tumor cell line H22 (hepatocarcinoma) was purchased from China Center for Type Culture Collection (Wuhan, China) and cultured according to their guidelines.

Reagents and plasmids

Resveratrol (3,4',5-trihydroxy-trans-stilbene) and LPS were purchased from Sigma-Aldrich (St. Louis, MO). Murine M-CSF, IFN-γ, and IL-10 were purchased from PeproTech (Rocky Hill, NJ). Eukaryotic expression vector p4-1BBL carrying full-length cDNA of murine 4-1BBL (22), pSP-D carrying the cDNA encoding extracellular domain of murine programmed death-1 (soluble form of programmed death-1 [sPD-1]) (23), and pSPLR4 carrying the cDNA encoding extracellular domain of murine TLR4 (sTLR4) were constructed by insertion of cDNA into plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA). PE-anti–mouse F4/80 Ab, FITC-anti–mouse F4/80 Ab, PE-Cy7–anti-mouse CD11b Ab, allophycocyanin-anti–mouse Gr-1 Ab, allophycocyanin-anti–mouse CD8α Ab, PE-Cy7-anti mouse CD4 Ab, allophycocyanin-anti–mouse TNF-α Ab, Functional Grade Purified anti-mouse CD3e Ab, and anti-mouse CD28 Ab were purchased from eBioscience (San Diego, CA). Abs against mouse arginase I, NO synthase 2 (NOS2), IFN regulatory factor-3 (IRF-3), NF-κB p65, TLR4, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-STAT3, phospho-STAT3 (Tyr<sup>705</sup>), STAT1, and phospho-STAT1 (Tyr<sup>701</sup>) Abs were purchased from Cell Signaling Technology (Beverly, MA).

Generation of bone marrow-derived Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> monocytes

Bone marrow cells were harvested from femurs of mice and cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 1 mM HEPES, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Twenty-four hours later, the adherent cells were discarded, and the nonadherent cells were cultured in the presence of 10 ng/ml M-CSF. Forty-eight hours later, the adherent cells (bone marrow-derived monocytes [BMDMs]) were used for further experiments. BMDMs (CD11b<sup>+</sup>F4/80<sup>+</sup>) prepared in this way were mainly Gr-1<sup>+</sup>, identified by flow cytometry. The percentage of Gr-1<sup>+</sup> cells in different batches of BMDMs was <5%.

Assay of immunosuppressive function of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells

The immunosuppressive function of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells was evaluated by their inhibitory effect on proliferation of activated CD8<sup>+</sup> T cells. Splenocytes were seeded in triplicates at concentration of 2 × 10<sup>5</sup> cells/well into U-bottom 96-well plates and cultured in presence of anti-CD3 and anti-CD28 Abs (1 μg/ml each) for 72 h. The cells were cocultured with or without 1 × 10<sup>5</sup> Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells. BrdU was added during the last 16 h of a 72-h culture, and then BrdU<sup>+</sup> cells were determined by using FITC BrdU Flow Kit (BD Pharmingen, San Diego, CA), according to the manufacturer’s protocol. Allophycocyanin-anti–CD8 Ab was used to label CD8<sup>+</sup> T cells. The background BrdU<sup>+</sup> CD8<sup>+</sup> cells percentage (<1% in all tests) in splenocytes without stimulation was subtracted from the value of each sample.

Flow cytometric analysis

Cells were incubated with fluorescence-labeled Ab for flow cytometric analysis. Parameters were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

Isolation of F4/80<sup>+</sup> cells

Bone marrow cells were harvested from femurs of mice. F4/80<sup>+</sup> myeloid cells were isolated from bone marrow cells by using PE-anti–F4/80 Ab, magnetic microbeads, and MiniMACS columns (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s protocol. In other experiments, tumor tissues or the tissues at sites of tumor inoculation were digested with collagenase, hyaluronidase, and DNase. The single-cell suspensions were used for the isolation of F4/80<sup>+</sup> cells.

Preparation of molecules from necrotic tumor cells

H22 cells were washed with PBS and resuspended in PBS to a final concentration of 5 × 10<sup>7</sup>/ml. After four-round frozen-thaw cycles, the cell suspension was vortexed for 30s. Then, the cells were removed by...
centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant contained a mixture of molecules from necrotic tumor cells (NTC-Ms in this study). The concentration of NTC-Ms was defined by the concentration of protein, which was determined using Coomassie Bradford reagent (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's instructions.

Tumor growth and treatment experiments

In a coinoculation test, BALB/c mice were inoculated i.m. in the right hind limb with $1 \times 10^7$ H22 cells with or without $2 \times 10^5$ Gr-1$^+$CD11b$^+$F4/80$^+$ cells. In an intratumor injection test, mice were inoculated i.m. in the right hind limb with $1 \times 10^7$ H22 cells. On day 7 after inoculation, the mice with palpable tumors were randomly divided into two groups. A total of $5 \times 10^5$ Gr-1$^+$CD11b$^+$F4/80$^+$ cells in $50 \mu$L PBS were carefully injected into the palpable tumor. The mice of control group received intratumor injection of equal volume of PBS. The tumors were dissected and weighed on the indicated day after inoculation. In a parallel experiment, F4/80$^+$ cells were isolated from tumors for the analysis of gene expression and apoptosis-inducing capacity. For the analysis of tumor growth, the survival of mice was recorded after a 4-wk treatment. For the analysis of gene expression and apoptosis, F4/80$^+$ cells were isolated from tumors for the analysis of gene expression and apoptosis-inducing capacity, F4/80$^+$ cells were weighed on the indicated day after inoculation. In a parallel experiment, F4/80$^+$ cells were isolated from tumors for the analysis of gene expression.

In experiments of treatment with 4-1BBL and sPD-1, mice were inoculated i.m. in the right hind limb with $1 \times 10^7$ H22 cells. The mice of treatment group received i.m. injection of $100 \mu$g 4-1BBL or $50 \mu$g sPD-1 on days 2 and 3 after inoculation and then once every 2 d. The mice of control groups received i.m. injections of equal volume of saline or equal amount of pcDNA3.1. The tumors were dissected and weighed on day 15 after inoculation. In parallel experiments, F4/80$^+$ cells were isolated from tumors for the analysis of gene expression.

In experiments of treatment with psTLR4, mice were inoculated i.m. in the right hind limb with $1 \times 10^7$ H22 cells. The mice of treatment group received i.m. injection of $100 \mu$g psTLR4 on days 2 and 3 after inoculation and then once every 2 d. The mice of control groups received i.m. injections of equal volume of saline or equal amount of pcDNA3.1. The tumors were dissected and weighed on day 15 after inoculation. In parallel experiments, F4/80$^+$ cells were isolated from tumors for the analysis of gene expression.

Apoptosis assay

To analyze apoptosis of activated T cells, splenocytes were seeded in triplicates at concentration of $2 \times 10^7$/well in U-bottom 96-well plates, and cultured in presence of anti-CD3 and anti-CD28 Abs (1 $\mu$g/ml each) for 48 h. Gr-1$^+$CD11b$^+$F4/80$^+$ cells were then added to the wells ($1 \times 10^3$/well). The cells were cultured for another 24 h. To analyze apoptosis of nonstimulated T cells, $2 \times 10^5$ splenocytes were mixed with $1 \times 10^5$ Gr-1$^+$CD11b$^+$F4/80$^+$ cells, and cultured in U-bottom 96-well plates for 24 h. The apoptosis of T cells was evaluated by flow cytometry after staining with FITC-Annexin V (eBioscience), PE-Cy7–anti-CD4 Ab, and allophycocyanin-anti-CD8 Ab.

Analysis of gene expression by real-time RT-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen). Real-time RT-PCR assay was performed as described previously (24). The resulting data were analyzed with the comparative CT method for relative gene expression quantification against Actb gene. The primer sequences were as follows: arginase I, 5'-TGGGGAAGACAGCA GAGGA-3' (sense) and 5'-TCAGCTCCCTGCTTATGG 3' (antisense); NOS2, 5'-GGAGCG TTGTGTGATGTGTC-3' (sense) and 5'-TGGAGCTTGCTGCTGTA-3' (antisense); IL-10, 5'-GGGCGCATCTGCAACGCC-3' (sense) and 5'-GCTCTCTTGTATGGGTCG-3' (antisense) and 5'-GCTACATCCCTCTCCT-3' (sense) and 5'-GCTACATCCCTCTCCT-3' (sense) and 5'-CCACGCTCTCTTGCTCTAGT-3' (sense) and 5'-GCTACAGGCCTTGTCATCG3' (antisense) and 5'-TCAGGTTCCTGTAGTGGC-3' (antisense) and 5'-CGCTGTGGCAC-3' (sense) and 5'-AGGGAAATCGTTCCGAGT-3' (sense) and 5'-CGCTGTGGCAC-3' (antisense).

Western blot assay

Western blot assay was done as described previously (24). When nuclear extracts and cytoplasmic extracts were analyzed, nuclear extracts and cytoplasmic extracts were prepared by using Chemicon’s Nuclear Extraction Kit (Millipore, Bedford, MA), according to the manufacturer’s protocol.

Assay of arginase activity and NO production

BMDMs were cultured at the concentration of $5 \times 10^7$/ml in the absence or presence of NTC-Ms for 48 h. The cells and the culture supernatants were used for the assay of arginase activity and NO production, respectively. Arginase activity and NO production were measured as previously described by Kusmartsev et al. (20).

ELISA

Cell-free supernatants from untreated or treated cells were harvested after 48-h culture. IL-10 and IL-12p70 in the supernatants were quantified using ELISA kits (R&D Systems, Minneapolis, MN) as per manufacturer’s instructions.

FIGURE 2. Immunosuppressive phenotype of BMDMs is induced by NTC-Ms. A, Apoptosis of activated T cells induced by NTC-Ms–stimulated BMDMs. N-BMDMs were stimulated with NTC-Ms (100 $\mu$g/ml) for 48 h and then cocultured with nonstimulated splenocytes or added to the splenocytes stimulated with anti-CD3 and anti-CD28 Abs (1 $\mu$g/ml each) for 48 h. B, Western blot analysis of the activation of Akt. C, Arginase activity and NO production were measured as described in Materials and Methods (D). The expression of mTNF-$\alpha$ was analyzed by flow cytometry after the cells were stained with FITC-anti-F4/80 Ab and allophycocyanin-anti–TNF-$\alpha$ Ab. The percentage of mTNF-$\alpha$-positive cells in F4/80$^+$ cells was calculated (E). The expressions of IL-10 and IL-12 were detected by ELISA (F).
instructions. Results were normalized between different samples and expressed as picograms per milliliter per $2 \times 10^6$ cells.

**Statistics**

Results were expressed as mean value $\pm$ SD and interpreted by one-way ANOVA. Differences were considered to be statistically significant when $p < 0.05$.

**Results**

Naive Gr-1+CD11b+F4/80+ cells promote tumor growth in established tumor microenvironment

To investigate the response of Gr-1+CD11b+F4/80+ cells to classical and alternative activation stimuli in tumor microenvironment, we first focused on the effect of tumor microenvironment on naive Gr-1+ CD11b+F4/80+ BMDMs, which could not inhibit T cell activation (Fig. 1A). In our tumor model, palpable tumor was formed 7 d after tumor inoculation. In either palpable tumor or the tissues at sites of inoculation before the formation of palpable tumor, F4/80+ cells were mainly Gr-1+ (Fig. 1B), similar to those in bone marrow and blood (Fig. 1C). We then prepared Gr-1+CD11b+F4/80+ BMDMs from bone marrow cells of naive mice to test their effect on tumor growth. The results showed that coinoculation of BMDMs with tumor cells suppressed tumor growth, whereas the injection of BMDMs into palpable tumor promoted the growth of tumor (Fig. 1D). Taken together, these data suggested that naive Gr-1+CD11b+F4/80+ cells were converted into tumor-promoting cells in tumor microenvironment even if they had antitumor function but not immunosuppressive function before they entered tumor microenvironment.

**NTC-Ms stimulate Gr-1+CD11b+F4/80+ BMDMs to induce apoptosis of activated T cells**

We next investigated whether naive Gr-1+CD11b+F4/80+ BMDMs could induce apoptosis of T cells after stimulation with NTC-Ms. The result showed that NTC-Ms–stimulated BMDMs induced apoptosis of splenic T cells activated in vitro but not nonstimulated T cells (Fig. 2A). Because coexpression of arginase I, NOS2, and membrane-associated TNF-α (mTNF-α) is required for the capacity of TAMs to induce apoptosis of activated T cells (20, 25), we then analyzed the expressions of these genes in naive Gr-1+ CD11b+F4/80+ BMDMs. NTC-Ms activated the transcription of Arg1 and Nos2 genes in BMDMs in a dose-dependent manner (Fig. 2B). Arginase I and NOS2 were induced by NTC-Ms (Fig. 2C, 2D), whereas mTNF-α was expressed in naive Gr-1+CD11b+F4/80+ BMDMs without stimulation with NTC-Ms (Fig. 2E). Meanwhile, we also detected the expressions of Il10 and Il12 genes (Fig. 2F, 2F). NTC-Ms–stimulated BMDMs showed higher expression levels of arginase I and IL-10 relative to those of NOS2.

FIGURE 3. Distinct TLR signaling is involved in the effect of NTC-Ms on BMDMs. A, Western blot analysis of p65 NF-κB subunit protein in nuclear extracts (Nu) and cytoplasmic extracts (Cy) of BMDMs and PMs, which were prepared from naive mice and stimulated with NTC-Ms (100 ng/ml) or LPS (100 ng/ml). B, Western blot analysis of IRF-3 protein in nuclear extracts and cytoplasmic extracts of BMDMs, which were prepared from naive mice and stimulated with NTC-Ms or LPS. C, Real-time RT-PCR analysis of Il12 and Tnf gene expressions in BMDMs and PMs, which were prepared from naive mice and stimulated with NTC-Ms or LPS for 24 h. D, Real-time RT-PCR analysis of gene expression in BMDMs, which were prepared from naive mice and stimulated with NTC-Ms in absence or presence of resveratrol (Res) for 24 h. E, Apoptosis of activated T cells induced by BMDMs untreated or treated with NTC-Ms in absence or presence of resveratrol (Res, 30 μM) for 48 h. F, sTLR4 expression after i.m. transfection. Naked plasmid DNA (micrograms per mouse) was injected into muscle of mice. The tissues at sites of injection were surgically excised 72 h later and homogenized. The proteins were analyzed by Western blot using anti-TLR4 Ab. G, Real-time RT-PCR analysis of gene expression in F4/80+ cells isolated from tumors on day 7 (palpable tumor) and day 15 (larger tumor) after the mice were inoculated with H22 cells and treated by i.m. injection of pcDNA3.1 or pSRL4 as described in Materials and Methods. H, Effect of sTLR4 on tumor growth. Tumors ($n=8$ in each group) were dissected and weighted on day 15 after inoculation in the experiment as described in G. Data are representative of three independent experiments (A, B, F) or are pooled from three independent experiments with a total of six independent samples (C–E, G). $p<0.05$; **$p<0.01$; ***$p<0.001$.
and IL-12 (Fig. 2C, 2F), further indicating that an immunosuppressive phenotype was induced by NTC-Ms.

**Apoptosis-inducing capacity of BMDMs is induced by NTC-Ms through TLR4 signaling**

Many molecules released from necrotic tumor cells are endogenous ligands for TLR4 (26). To investigate whether the effect of NTC-Ms on BMDMs was mediated by TLR4 ligand, we detected the activation (nuclear translocation) of NF-κB and IRF-3 in BMDMs in response to NTC-Ms. LPS was used as control. The result showed that NF-κB was effectively activated in peritoneal macrophages (PMs) but not in BMDMs (Fig. 3A), whereas IRF-3 was activated in BMDMs (Fig. 3B), suggesting that Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF) pathway was effective, but MyD88-dependent pathway was defective in Gr-1⁺ CD11b⁺F4/80⁺ BMDMs. Consistently, NTC-Ms and LPS could not efficiently induce or promote transcription of Il12 and Il10 genes in BMDMs (Fig. 3C). We then stimulated BMDMs with NTC-Ms in the presence of resveratrol, an inhibitor of TRIF pathway, which inhibits TLR4 signaling but not TLR2 signaling (27). The transcription of Arg1, Nos2, Il10, and Il12 genes was inhibited by resveratrol (Fig. 3D). Consistently, resveratrol also suppressed the capacity of NTC-Ms–stimulated BMDMs to induce apoptosis of splenic T cells activated in vitro (Fig. 3E), indicating that the apoptosis-inducing capacity of Gr-1⁺ CD11b⁺F4/80⁺ BMDMs was induced by TLR4 ligands in NTC-Ms through TRIF signaling pathway.

To further confirm the effect of TLR4 ligands in vivo, we investigated the effect of blocking TLR4 ligand on F4/80⁺ cells by intramuscle transfection of sTLR4 expression vector (Fig. 3F). In palpable tumor, sTLR4 decreased the transcription of Arg1 gene and increased the transcription of the Il12 gene (Fig. 3G, left panel), further confirming that TLR4 signaling is required for the immunosuppressive phenotype of Gr-1⁺ CD11b⁺F4/80⁺ cells in tumor microenvironment. In line with this, sTLR4 significantly suppressed the growth of tumors (Fig. 3H). However, the effect of sTLR4 did not last in the larger tumor (Fig. 3G, right panel), probably because of a larger amount of TLR4 ligands in larger tumor.

**IL-10 enhances the apoptosis-inducing capacity of Gr-1⁺ CD11b⁺F4/80⁺ cells in presence of NTC-Ms**

We next investigated whether IL-10, a strong deactivator of macrophages (28), may influence the response of naive Gr-1⁺ CD11b⁺F4/80⁺ BMDMs to NTC-Ms. The results showed that IL-10 did not induce the transcription of Arg1, Nos2, Il10, and Il12 genes but suppressed the transcription of Nos2 and Il12 induced by NTC-Ms, resulting in further decrease of transcription levels of Nos2 and Il12 relative to those of Arg1 and Il10 (Fig. 4A). The expression of mTNF-α was not influenced by IL-10 (data not shown). Importantly, the apoptosis-inducing capacity of NTC-Ms–stimulated BMDMs was enhanced by IL-10 (Fig. 4B), suggesting that the increased apoptosis-inducing effect is in accordance with the decrease of the expression levels of Nos2 and Il12 relative to those of Arg1 and Il10 in Gr-1⁺ CD11b⁺F4/80⁺ cells. Furthermore, these data suggest that the apoptosis-inducing capacity of Gr-1⁺ CD11b⁺F4/80⁺ cells is mainly determined by TLR4 ligands in NTC-Ms and that this capacity might be further enhanced if IL-10 expression is increased in local microenvironment.

**Response of Gr-1⁺ CD11b⁺F4/80⁺ cells to IFN-γ influences the suppressive effect of IFN-γ on their apoptosis-inducing capacity**

We next investigated whether IFN-γ can reduce the capacity of Gr-1⁺ CD11b⁺F4/80⁺ cells to induce apoptosis of activated T cells. The result showed that IFN-γ alone induced the transcription of Nos2 and Il12 genes but not Arg1 and Il10 genes. IFN-γ increased the transcription levels of Nos2 and Il12 relative to those of Arg1 and Il10 in naive BMDMs (N-BMDMs) in the presence of NTC-Ms (Fig. 5A). IFN-γ also significantly increased the transcription of Nos2 and Il12 in BMDMs prepared from tumor-bearing mice (T-BMDMs), whereas the transcription was obviously attenuated (Fig. 5B). In this situation, IFN-γ did not effectively increase the transcription levels of Nos2 and Il12 relative to those of Arg1 and Il10 in T-BMDMs in the presence of NTC-Ms. Consistently, the apoptosis-inducing capacity of N-BMDMs, but not T-BMDMs, was effectively reduced by IFN-γ (Fig. 5C). Therefore, IFN-γ may suppress the apoptosis-inducing capacity of NTC-Ms–stimulated Gr-1⁺ CD11b⁺F4/80⁺ cells only if their response to IFN-γ is not attenuated.

**Nonadherent Gr-1⁺ CD11b⁺F4/80⁺ cells also induce apoptosis of T cells after stimulation with NTC-Ms**

When we prepared BMDMs, we found that there were still Gr-1⁺ CD11b⁺F4/80⁺ cells in nonadherent cells after 48-h culture of bone marrow cells in the presence of M-CSF. Furthermore, in both bone marrow and tumor, there were Gr-1⁺ CD11b⁺F4/80⁺ cells in nonadherent cells (Fig. 6A). Although nonadherent Gr-1⁺ CD11b⁺F4/80⁺ cells could develop into adherent cells (data not shown), so far it is unclear whether Gr-1⁺ CD11b⁺F4/80⁺ cells are able to induce apoptosis of activated T cells if they are not adherent cells. We therefore investigated whether NTC-Ms could stimulate nonadherent Gr-1⁺ CD11b⁺F4/80⁺ cells to induce apoptosis of activated T cells by isolating F4/80⁺ myeloid cells from bone marrow of mice and directly stimulating the cells with NTC-Ms. Nonadherent F4/80⁺ myeloid cells showed higher transcription levels of Arg1 and Il10 relative to those of Nos2 and Il12 (Fig. 6C) and were able to induce apoptosis of splenic T cells activated in vitro but not nonstimulated T cells (Fig. 6D). Moreover, the effects of IL-10 and IFN-γ on nonadherent Gr-1⁺ CD11b⁺F4/80⁺ cells were similar to those on adherent Gr-1⁺ CD11b⁺F4/80⁺ cells (data not shown). These data indicate that Gr-1⁺ CD11b⁺F4/80⁺ cells can induce apoptosis of activated T cells after stimulation with NTC-Ms.
apoptosis of activated T cells under the same conditions no matter whether they are adherent or nonadherent cells.

Potential of Gr-1+CD11b+F4/80+ cells to express IL-12 is attenuated by tumor before they leave bone marrow

To investigate whether the response of Gr-1+CD11b+F4/80+ cells to IFN-γ was altered by tumor before they leave bone marrow, we next investigated the response of F4/80+ myeloid cells to IFN-γ by detecting the transcription of \( \text{Il12} \) gene. The result showed that the transcription of \( \text{Il12} \) gene in both adherent and nonadherent F4/80+ myeloid cells in response to IFN-γ was attenuated along with the development of tumor (Fig. 7A), indicating that the potential of Gr-1+CD11b+F4/80+ cells to express IL-12 was attenuated by tumor before they left bone marrow. We then analyzed the activation of STAT1 and STAT3 in F4/80+ myeloid cells from naive and tumor-bearing mice. In unstimulated F4/80+ myeloid cells, active STAT1 was not detectable, whereas active STAT3 was detectable and increased in the presence of tumor (Fig. 7B). STAT1 in F4/80+ myeloid cells was effectively activated by IFN-γ (Fig. 7C), indicating that the attenuated response was not due to deficiencies of IFN-γ signaling. Importantly, active STAT3 in F4/80+ myeloid cells was further increased by not only IL-10 but also IFN-γ stimulation (Fig. 7C). We then investigated whether NTC-Ms might be involved in the alteration of the response of F4/80+ myeloid cells to IFN-γ. Tumor growth could be significantly promoted by continuous i.p. injection of NTC-Ms starting 10 d before tumor inoculation (Fig. 7D). The response of F4/80+ myeloid cells to IFN-γ was gradually attenuated after continuous i.p. injection of NTC-Ms (Fig. 7E), but active STAT3 in F4/80+ myeloid cells was not influenced (data not shown). Taken together, these data suggest that tumor may modulate the response of F4/80+ myeloid cells to IFN-γ through complex mechanisms.
F4/80+ cells were isolated from bone marrow of naive mice (N-F4/80) and tumor-bearing mice on day 10 (T10-F4/80) and day 20 (T20-F4/80) after tumor inoculation and directly used for analysis of STAT1 and STAT3 without stimulation. F4/80+ cells were isolated from bone marrow of naive mice (N-F4/80) and tumor-bearing mice (T-F4/80) on day 10 after tumor inoculation. F4/80+ cells were unstimulated (control) or stimulated with IFN-γ for 24 h. The expression of II12 gene in nonadherent and adherent cells was detected by real-time RT-PCR, respectively. Western blot analysis of phospho-STAT1, STAT1, phospho-STAT3, and STAT3 in F4/80+ myeloid cells. F4/80+ cells were isolated from bone marrow of naive mice (N-F4/80) and tumor-bearing mice on day 10 (T10-F4/80) and day 20 (T20-F4/80) after tumor inoculation and directly used for analysis of STAT1 and STAT3 without stimulation. Western blot analysis of phospho-STAT1, STAT1, phospho-STAT3, and STAT3 in F4/80+ myeloid cells. F4/80+ cells were isolated from bone marrow of naive mice (N-F4/80) and tumor-bearing mice (T-F4/80) on day 10 after tumor inoculation. F4/80+ cells were unstimulated (control) or stimulated with IFN-γ (50 ng/ml) or IL-10 (50 ng/ml) for 30 min and then used for analysis of STAT1 and STAT3. Effect of NTC-Ms on tumor growth. Mice received continuous i.p. injection of PBS or NTC-Ms, once a day starting 10 d before i.m. inoculation of 1 × 10^5 H22 cells. Tumors (n = 8 in each group) were dissected and weighted on day 15 after inoculation. E, Real-time RT-PCR analysis of II12 gene expression. F4/80+ cells were isolated from BM of mice on the indicated day after starting continuous i.p. injection of PBS or NTC-Ms (1 mg/injection) and then stimulated with IFN-γ for analysis of II12 gene expression as described in A. Data are representative of five independent experiments (B, C) or are pooled from three independent experiments with a total of six independent samples (A, E). *p < 0.01; **p < 0.001.

**Tumor immunotherapy reduces but not abolishes the apoptosis-inducing capacity of Gr-1⁺CD11b⁺F4/80⁺ cells**

Our previous study showed that immunotherapy with 4-1BBL and sPD-1 could increase IFN-γ expression and decrease IL-10 expression in local microenvironment and efficiently suppress the growth of tumor (22). Given that tumor cells damaged by immune cells in the process of tumor immunotherapy may also release TLR4 ligands to stimulate Gr-1⁺CD11b⁺F4/80⁺ cells, we next investigated the expression levels of Arg1 and II10 relative to those of Nos2 and II12 in Gr-1⁺CD11b⁺F4/80⁺ cells in the local microenvironment of tumor immunotherapy with 4-1BBL and sPD-1. The treatment with 4-1BBL/sPD-1 effectively suppressed tumor growth (Fig. 8A) and increased the transcription of Nos2 and II12 genes in F4/80⁺ cells in local microenvironment (Fig. 8B). However, Arg1 was also induced in F4/80⁺ cells, although the transcription level was much lower than that in F4/80⁺ cells in control groups. Consistently, treatment with 4-1BBL/sPD-1 effectively reduced, but did not abolish, the apoptosis-inducing capacity of F4/80⁺ cells (Fig. 8C). sTLR4 alone did not effectively influence gene expressions in F4/80⁺ cells in larger tumor but increased transcription levels of Nos2 and II12 relative to those of Arg1 and II10 in F4/80⁺ cells in the microenvironment of tumor immunotherapy (Fig. 8B) and further reduced the apoptosis-inducing capacity of F4/80⁺ cells, evaluated by coculturing F4/80⁺ cells with splenic T cells activated in vitro (Fig. 8C). Consistently, the suppressive effect of 4-1BBL/sPD-1 on tumor growth was enhanced if sTLR4 was expressed in the local microenvironment (Fig. 8A, 8D). The effect of sTLR4 might involve the blockade of TLR4 signaling in tumor cells, because TLR4 signaling in tumor cells also promotes tumor growth (29). Nevertheless, these results further suggest that the damage of tumor cells as a result of immunotherapy also influences the function of F4/80⁺ cells through TLR4 signaling, which may hinder the complete elimination of tumor cells even if the immunotherapy effectively suppresses the growth of tumor.

**Discussion**

Intracellular molecules of tumor cells are released due to the damage of tumor cells resulting from multiple reasons, especially the attack of immune cells. Many intracellular molecules are TLR4 ligands (26). Therefore, Gr-1⁺CD11b⁺F4/80⁺ cells recruited into tumor will be inevitably stimulated by endogenous TLR4 ligands. Although many factors in tumor microenvironment may influence the function of Gr-1⁺CD11b⁺F4/80⁺ cells, in this study, we found that Gr-1⁺CD11b⁺F4/80⁺ cells could induce apoptosis of activated T cells based on their distinct response to TLR4 ligands released from damaged tumor cells. The apoptosis-inducing capacity of Gr-1⁺CD11b⁺F4/80⁺ cells is basically determined by the attenuation of classical activation because of distinct signaling pathways and the modulation of them by tumor before they leave bone marrow.

TLR4 signaling usually induces gene expression through MyD88-dependent pathway and TRIF pathway and promotes the differentiation of macrophages toward M1-like phenotype. However, in this study, we found that TRIF pathway is functional, but MyD88-dependent pathway is defective in Gr-1⁺CD11b⁺F4/80⁺ cells. Consistently, TLR4 signaling could not efficiently promote the transcription of Tnf gene in Gr-1⁺CD11b⁺F4/80⁺ cells, because the defectiveness of MyD88-dependent pathway results in the defective expression of TNF-α (1). These results are consistent with a previous finding that LPS can efficiently induce TNF-α expression in more mature human monocytes (CD14⁺CD16⁺) but not in relatively immature monocytes (CD14⁺CD16⁻), suggesting that MyD88-dependent pathway may not be fully functional in relatively immature monocytes. Although MyD88-dependent pathway...
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may become fully functional along with the development of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells in tissue, tumor cell-released hyaluronan can induce the production of IL-1R–associated kinase-M (31), which inhibits MyD88-dependent pathway (32). Therefore, TLR4 signaling is mainly mediated by TRIF pathway in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells in tumor microenvironment.

TRIF signaling pathway plays a more important role in TLR4-mediated macrophage activation (33–35). However, TLR4 signaling through TRIF pathway only induces lower expressions of NOS2 and IL-12 (1, 20, 27, 34). Importantly, our data showed that TRIF pathway induced the expressions of not only NOS2 and IL-12 but also arginase I and IL-10 in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells, characterized by higher expression levels of arginase I and IL-10 relative to those of NOS2 and IL-12. On the basis of the stimulation with TLR4 ligands in NTC-Ms, IL-10 promoted further polarization of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells toward M2 phenotype by suppressing the expressions of NOS2 and IL-12. In contrast, our data showed that TLR4 ligands in tumor microenvironment reduced IL-12 expression in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells, because blockade of TLR4 ligand in tumor increased IL-12 expression in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells. This result is consistent with a recent report that TLR4 signaling attenuates IL-12 expression in macrophages by upregulating PD-1 expression (36). Therefore, tumor cell-released TLR4 ligands play a key role in the induction of immunosuppressive phenotype of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells in tumor microenvironment.

The coexpression of arginase I, NOS2, and mTNF-α is required for the capacity of TAMs to induce apoptosis of activated T cells (20, 25). In this study, we found that mTNF-α was expressed by Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells without further stimulation. Although only part of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells expressed mTNF-α, it was efficient for them to induce apoptosis of activated T cells. TLR4 signaling induced the expressions of both arginase I and NOS2 through TRIF pathway. Importantly, in this study, we found that coexpression of arginase I, NOS2, and mTNF-α in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells may not certainly result in an apoptosis-inducing effect. The apoptosis-inducing effect was actually in accordance with the relative higher expression of Arg1 and lower expression of IL12 in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells and could be suppressed by increasing IL-12 expression or decreasing arginase I expression. Importantly, our data showed that blocking TLR4 signaling could increase IL-12 expression and decrease arginase I expression in F4/80<sup>+</sup> cells in tumor microenvironment, suggesting an important strategy to prevent apoptosis of activated T cells in tumor microenvironment.

IFN-γ is the most potent cytokine to promote IL-12 expression in macrophages (37). In the presence of IFN-γ, the apoptosis-inducing capacity of NTC-Ms–stimulated naive Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells could be suppressed, which was in accordance with the increase of IL-12 expression in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells. However, in the presence of tumor, IL-12 expression in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells was attenuated. The attenuation was not due to defectiveness of STAT1 activation, because STAT1 was effectively activated by IFN-γ in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells from bone marrow of both naive and tumor-bearing mice. The increase of active STAT3 in Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells from tumor-bearing mice may partially explain their attenuated IL-12 expression. Active STAT3 is important for proliferation and survival of cells (38), which can explain the accumulation of Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells in tumor-bearing mice. STAT3 also opposes STAT1 functions and mediates the function of IL-10 to inhibit IL-12 expression in macrophages (28, 39, 40). Nevertheless, NTC-Ms could alter the response of F4/80<sup>+</sup> myeloid cells to IFN-γ without influencing active STAT3, suggesting that tumor may attenuate IL-12 expression through complex mechanisms.
cells use phosphatidylserine as an “eat-me” signal (19). Multiple receptors have been identified to detect this eat-me signal, including the receptor for phosphatidylserine (20, 21). The role of phosphatidylserine in the phagocytic clearance of apoptotic cells has already been attenuated by tumor. The receptor for phosphatidylserine in tumor cells is up-regulated by tumor as a mechanism to escape phagocytosis (22).

References


