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

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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-IBBL/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-IBBL/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. The Journal of Immunology, 2010, 185: 2773–2782.
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 Huabei 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 p41BBL carrying full-length cDNA of murine 4-1BBL (22), pSD-1 carrying the cDNA encoding extracellular domain of murine programmed death-1 (soluble form of programmed death-1 [sPD-1]) (23), and pSTLR4 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, allopurinocyanin-anti–mouse Gr-1 Ab, allopurinocyanin-anti–mouse CD8a Ab, PE-Cy7–anti mouse CD4 Ab, allopurinocyanin-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 (Tyr705), STAT-1, and phospho-STAT1 (Tyr701) 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 prepared in this way 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, allopurinocyanin-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 sites at 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

![Image](http://www.jimmunol.org/)
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 $1 \times 10^5$ 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 $1 \times 10^5$ 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 to the palpable tumor. The mice of control group received intratumor injection of equal volume of PBS. The tumors were dissected and weighed on day 15 after inoculation.

In the experiments of treatment with psTLR4, mice were inoculated i.m. in the right hind limb with $1 \times 10^5$ H22 cells. The mice of treatment group received the i.m. injection of 100 μg 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.

In experiments of treatment with 4-1BBL and sPD-1, mice were inoculated i.m. in the right hind limb with $1 \times 10^5$ H22 cells. The mice of treatment groups received i.m. injection of 200 μg plasmid DNA, once every 2 d starting from day 2 after inoculation. In each injection, 50 μg p4-1BBL and 50 μg sPD-1 were used in combination with 100 μg of either pcDNA3.1 or psTLR4. The mice of control groups received i.m. injection of pcDNA3.1 (200 μg), psTLR4 (100 μg each), and equal volume of saline, respectively. The tumors were dissected and weighed on the indicated day after inoculation. In a parallel experiment, the survival of mice was recorded after a 4-wk treatment. For the analysis of gene expression and apoptosis-inducing capacity, F4/80$^+$ cells were isolated from tumors or the tissues at sites of inoculation.

**Apoptosis assay**

To analyze apoptosis of activated T cells, splenocytes were seeded in triplicates at concentration of $2 \times 10^5$/well in U-bottom 96-well plates, and cultured in presence of anti-CD3 and anti-CD28 Abs (1 μg/ml each) for 48 h. 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. The expression of NOS2 was detected by Western blot assay 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'-TGGGAGACGACGAGGA-3' (sense) and 5'-TCAGTCCCTGGCTTATGG 3' (antisense); NOS2, 5'-GGTTTCCGGACTGGCTAA-3' (sense) and 5'-TGTCCTGGATCTGGGC-3' (antisense); IL-10, 5'-GGGAAACACTGCTAAGGC-3' (sense) and 5'-TGGCTTCTGGATCTGGGC-3' (antisense); IL-12a, 5'-GATCAACACAGCCTCCTCTG-3' (sense) and 5'-GATCAACACAGCCTCCTCTG-3' (antisense); and β-actin, 5'-AGGGAAATCGTGCGGAAGGA-3' (antisense) and 5'-GGTTTCCGGACTGGCTAA-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.

**Bioassay of arginase activity and NO production**

BMDMs were cultured at the concentration of $5 \times 10^5$/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 earlier 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 μg/ml) for 48 h and then cocultured with nonstimulated splenocytes or added to the splenocytes stimulated with anti-CD3 and anti-CD28 Abs (1 μg/ml each) for 48 h. Gr-1$^+$CD11b$^+$F4/80$^+$ cells were then added to the wells ($1 \times 10^5$/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'-TGGGAGACGACGAGGA-3' (sense) and 5'-TCAGTCCCTGGCTTATGG 3' (antisense); NOS2, 5'-GGTTTCCGGACTGGCTAA-3' (sense) and 5'-TGTCCTGGATCTGGGC-3' (antisense); and β-actin, 5'-AGGGAAATCGTGCGGAAGGA-3' (antisense) and 5'-GGTTTCCGGACTGGCTAA-3' (antisense). Arginase activity and NO production were measured as previously described by Kusmartsev et al. (20).
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 ± 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 Il12 and Tnf genes (Fig. 2B, 2F). NTC-Ms–stimulated BMDMs showed higher expression levels of arginase I and IL-10 relative to those of NOS2.
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 Ifi12 and Tnf 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 Ifi12, 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 intramuscular 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. 4A). 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. 4B). 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. 4C). 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. 4A). 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 Gr-1⁺CD11b⁺F4/80⁺ cells also expressed mTNF-α (Fig. 6B), which was not influenced by NTC-Ms. After stimulation 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 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 Il12 gene. The result showed that the transcription of 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 defectiveness 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 before i.m. inoculation of 1 mg/injection and then stimulated with IFN-γ to evaluate the inducing capacity of F4/80+ cells, which influences the function of F4/80+ cells through TLR4 signaling, as described in our previous study (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+) (26). Therefore, Gr-1+CD11b+F4/80+ cells recruited into tumor will contribute to tumor immunotherapy with 4-1BBL and sPD-1. The treatment with 4-1BBL/sPD-1 effectively suppressed tumor growth (Fig. 8A), increased the transcription of Nos2 and Il12 genes in F4/80+ cells, and promoted the differenti-ation of macrophages toward M1-like phenotype. However, 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 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.
Gr-1⁺CD11b⁺F4/80⁺ CELLS AND APOPTOSIS OF ACTIVATED T CELLS

FIGURE 8. Effect of tumor immunotherapy on F4/80⁺ cells in local microenvironment. A, Effect of sTLR4 on the therapeutic effect of 4-1BBL/sPD-1. Mice were inoculated with H22 cells and treated by i.m. injection of expression plasmids as described in Materials and Methods. Tumors (n = 8 in each group) were dissected and weighted on days 14 and 28 after tumor inoculation. B, Real-time RT-PCR analysis of gene expression in F4/80⁺ cells. In the experiments as described in A, F4/80⁺ cells were isolated from tumors or the tissues at sites of inoculation on days 14 and 28 after tumor inoculation. Gene expressions in F4/80⁺ cells were detected by real-time RT-PCR. C, Apoptosis of T cells induced by F4/80⁺ cells. In the experiments as described in A, the survival of mice was recorded (n = 16 in each group). Data are pooled from three independent experiments with a total of six independent samples (B, C). *p < 0.05; **p < 0.01.

may become fully functional along with the development of Gr-1⁺ CD11b⁺F4/80⁺ 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⁺CD11b⁺F4/80⁺ 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⁺CD11b⁺F4/80⁺ 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⁺CD11b⁺F4/80⁺ 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⁺CD11b⁺F4/80⁺ cells, because blockade of TLR4 ligand in tumor increased IL-12 expression in Gr-1⁺CD11b⁺F4/80⁺ 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⁺CD11b⁺F4/80⁺ 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⁺CD11b⁺F4/80⁺ cells without further stimulation. Although only part of Gr-1⁺CD11b⁺F4/80⁺ 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⁺CD11b⁺F4/80⁺ 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 II12 in Gr-1⁺CD11b⁺F4/80⁺ 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⁺ 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⁺CD11b⁺F4/80⁺ cells could be suppressed, which was in accordance with the increase of IL-12 expression in Gr-1⁺CD11b⁺F4/80⁺ cells. However, in the presence of tumor, IL-12 expression in Gr-1⁺CD11b⁺F4/80⁺ cells was attenuated. The attenuation was not due to defectiveness of STAT1 activation, because STAT1 was effectively activated by IFN-γ in Gr-1⁺CD11b⁺F4/80⁺ cells from bone marrow of both naive and tumor-bearing mice. The increase of active STAT3 in Gr-1⁺CD11b⁺F4/80⁺ 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⁺CD11b⁺F4/80⁺ 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⁺ myeloid cells to IFN-γ without influencing active STAT3, suggesting that tumor may attenuate IL-12 expression through complex mechanisms.

Phosphatidylserine exposed on apoptotic cells is used as a marker of apoptotic cells in Annexin V staining. Importantly, apoptotic
cells use phosphatidylserine as an “eat-me” signal (19). Multiple receptors have been identified to detect this eat-me signal, including stabilin-2, BAI, PSR, Tim-1, Tim-3, and Tim-4, which bind phosphatidylserine and mediate the clearance of apoptotic cells (41–44). Therefore, the induction of apoptosis is an important reason for depletion of the activated T cells in tumor microenvironment. For many years, different strategies for immunotherapy have been explored for tumor therapy, including the adoptive transfer of tumor-specific T cells that have been activated and expanded in vitro (45–48). The presence of immune suppressor cells has become a substantial obstacle to the success of tumor immunotherapy. Although TAMs and MDSCs could be temporarily depleted to improve the therapeutic effect of tumor immunotherapy, especially adoptive immunotherapy, Gr-1+CD11b+F4/80+ cells repleted by bone marrow will be recruited to tumor again. In the case of resection of primary tumor, monocytes derived from bone marrow will be recruited into residual tumor nodules. In all of these situations, TL4 receptors ligands released from damaged tumor cells will stimulate Gr-1+CD11b+F4/80+ cells to induce apoptosis of activated T cells. Moreover, IFN-γ may not effectively suppress the apoptosis-inducing capacity of Gr-1+CD11b+F4/80+ cells if their response to IFN-γ has already been attenuated by tumor. The response of Gr-1+CD11b+F4/80+ cells to IFN-γ may not be fully recovered after depletion of TAMs or MDSCs or resection of tumor. Therefore, both modulation of the response of Gr-1+CD11b+F4/80+ cells to IFN-γ and blockade of TL4 signaling in local microenvironment should be considered in designing new strategy for tumor immunotherapy. Moreover, given that TL4 signaling is also important for induction of antitumor immune response, because TL4 signaling can enhance the ability of dendritic cells (DCs) to process and present tumor Ags (49, 50), blockade of TL4 may influence antitumor immune response. 4-IBBL stimulation also enhances DC maturation and the ability of DCs to stimulate T cell proliferation in response to Ag (51, 52). The use of costimulatory factor such as 4-IBBL should be taken into consideration to compensate the effect of blocking TL4 signaling on DCs.

Disclosures

The authors have no financial conflicts of interest.

References


