Cancer-Expanded Myeloid-Derived Suppressor Cells Induce Anergy of NK Cells through Membrane-Bound TGF-β1[1]

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NK cells, the important effector of innate immunity, play critical roles in the antitumor immunity. Myeloid-derived suppressor cells (MDSC), a population of CD11b$^+$Gr-1$^+$ myeloid cells expanded dramatically during tumor progression, can inhibit T cells and dendritic cells, contributing to tumor immune escaper. However, regulation of NK cell innate function by MDSC in tumor-bearing host needs to be investigated. In this study, we found that the function of NK cells from liver and spleen was impaired significantly in all tumor-bearing models, indicating the impairment of hepatic NK cell function by tumor is a universal phenomenon. Then we prepared the orthotopic liver cancer-bearing mice as tumor model to investigate how hepatic NK cells are impaired. We show that down-regulation of NK cell function is inversely correlated with the marked increase of MDSC in liver and spleen. MDSC inhibit cytotoxicity, NKG2D expression, and IFN-γ production of NK cells both in vitro and in vivo. After incubation with MDSC, NK cells could not be activated to produce IFN-γ. Furthermore, membrane-bound TGF-β1 on MDSC is responsible for MDSC-mediated suppression of NK cells. The impaired function of hepatic NK cells in orthotopic liver cancer-bearing mice could be restored by depletion of MDSC, but not regulatory T cells. Therefore, cancer-expanded MDSC can induce anergy of NK cells via membrane-bound TGF-β1. MDSC, but not regulatory T cells, are main negative regulator of hepatic NK cell function in tumor-bearing host. Our study provides new mechanistic explanations for tumor immune escape. ***The Journal of Immunology,*** **2009, 182: 240–249.**

Natural killer cells play important roles in innate immunity, and are also involved in the activation of adaptive immunity by cross-talking with dendritic cells (DCs)[4] and promoting a Th1-mediated immunity (1). So, manipulation of NK cell activation has been regarded as an important approach to the immunotherapy of cancer and infectious diseases (2). The liver is a unique organ containing the highest percentage of NK cells, with 25–30% of intrahepatic lymphocytes in human and 15–20% in mice being NK cells (3). However, in addition to its anatomic characteristics, liver is also proposed as a tolerogenic organ prone to cancer metastasis and chronic infection such as chronic hepatitis B. Therefore, the mechanistic study for the regulation of NK cell function in liver will contribute to better understand the roles of NK cells in liver immunity and design of immunotherapy for the control of liver diseases such as liver cancer.

Myeloid-derived suppressor cells (MDSC), a population of CD11b$^+$Gr-1$^+$ myeloid cells at earlier stages of differentiation, represent ~20–30% of normal bone marrow cells and 1–4% of all nucleated cells in spleen (4, 5). MDSC expand dramatically during tumor progression, infection, and even immunization (4, 5). MDSC have been shown to inhibit T cell proliferation and activation, suppress maturation of DCs, which together contribute to the negative regulation of immune responses and the promotion of immune escape of tumors and pathogens (6, 7). In vivo depletion of MDSC with the monoclonal anti-Gr-1 Ab can improve T cell-mediated immune responses and suppress tumor growth in murine models (8). Dramatic reduction of MDSC is one of the mechanisms responsible for the potent antitumor effects of all-*trans*-retinoic acid used in vivo (9). Therefore, depletion of MDSC in tumor-bearing host has been proposed as a new approach for cancer immunotherapy. Although the regulation of adaptive immune response by MDSC is extensively studied, the roles of MDSC in the regulation of innate immunity, especially in the regulation of hepatic NK cell function, have not been completely elucidated.

Considering that the immunosuppressive MDSC expand dramatically during tumor progression and NK cells play important roles in the antitumor immunity, we investigated the regulation of NK cell function by MDSC in orthotopic tumor models including orthotopic liver cancer-bearing mice. We show that the cytotoxicity and IFN-γ production of NK cells in liver are decreased in all kinds of tumor-bearing mice we tested, suggesting a universal phenomenon of impairment of hepatic NK cell function in tumor-bearing host. We went further to investigate the underlying mechanisms for the down-regulation of hepatic NK cell function in tumor-bearing host, and demonstrated that MDSC could inhibit NK cell cytotoxicity, NKG2D expression and IFN-γ production through their membrane-bound TGF-β1. Also, after interaction with MDSC, NK cells are hyporesponsive to the activating stimuli, indicating MDSC induce NK cell anergy. So, we provide new mechanistic explanation for tumor immune escape by showing the negative regulation of hepatic NK cell-mediated innate immunity by MDSC.

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[4] Abbreviations used in this paper: DC, dendritic cell; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; MLN, mesenteric lymph node; MNC, mononuclear cell; VEGF, vascular endothelial growth factor; 7-AAD, 7-aminoactinomycin D; MFI, mean fluorescence intensity.

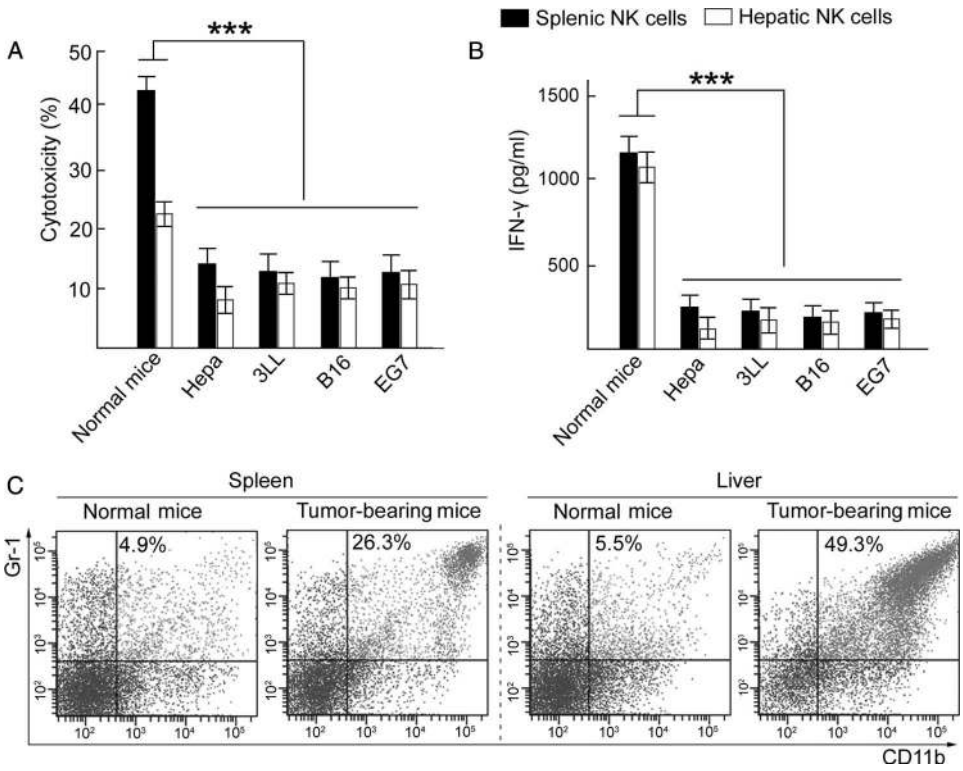

FIGURE 1. Down-regulation of NK cell function and increase of MDSC in spleen and liver of tumor-bearing mice. *A*, The decrease of splenic and hepatic NK cell cytotoxicity in tumor-bearing mice. The NK cells purified from spleen and liver of orthotopic liver cancer (Hepa), orthotopic lung cancer (3LL), s.c. melanoma (B16), or lymphoma (EG7) tumor-bearing mice were incubated with Yac-1 target cells at the indicated E:T ratio for 12 h, then NK cell cytotoxicity was examined by three-color flow cytometry assay (NK1.1-allophycocyanin, annexin V-FITC, and 7-AAD). Results are expressed as mean ± SEM of triplicate wells from one representative experiment of four experiments completed. ***, $p < 0.01$. *B*, The decrease of IFN-γ production by splenic and hepatic NK cells in tumor-bearing mice. A total of 1×10^6/ml splenic and hepatic NK cells purified from tumor-bearing mice or normal mice and stimulated with 25 ng/ml PMA plus 1 μg/ml ionomycin for 24 h. IFN-γ levels in the supernatants were determined using ELISA kit. Data are mean ± SEM of triplicate wells from one representative experiment of four as shown. ***, $p < 0.01$. *C*, The increased percentage of the splenic and hepatic MDSC in orthotopic liver cancer-bearing mice is shown. The MNC from spleen and liver of normal or liver cancer-bearing mice were stained with Gr-1-FITC and CD11b-PE. Data represent one of three independent experiments with similar results.

Materials and Methods

Mice, cell lines, and reagents

C57BL/6J mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China) and used at the age of 6–8 wk. Enhanced GFP-transgenic mice (ActbEGFP) obtained from The Jackson Laboratory, and Smad3-deficient mice established as previously described (10), were maintained under specific pathogen-free conditions. The murine liver carcinoma cell line (Hepa), Lewis lung carcinoma cell line (3LL), melanoma cell line (B16), lymphoma cell line (EG7), and leukemia cell line (Yac-1) were obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium (PAA Laboratories) supplemented with 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% (v/v) heat-inactivated (PAA Laboratories). Recombinant mouse TGF-β1, neutralizing anti-mouse vascular endothelial growth factor (VEGF) purified Ab, recombinant mouse IL-12, IL-18, anti-TGF-β1 Ab (1D11), and its isotype control mouse IgG1 mAb (clone 11711) were obtained from R&D Systems. The Abs for flow cytometry including allophycocyanin-labeled Abs against Gr-1 and NK1.1 (PK136), FITC-labeled Abs against CD3 (145-2C11) and biotin, PE-labeled Abs against CD11b (M1/70), Fas ligand (MFL3), and TRAIL (N2B2), biotin-labeled Ab against TGF-β1 (A75-3), and the respective isotype controls were obtained from BD Pharmingen. Purified anti-mouse NKG2D Ab (CX5), anti-IL-10 mAb (clone JES3-9D7), PE-labeled Abs against perforin (JAW246), and NKG2D (CX5) were from eBioscience. Neutralizing mouse anti-Gr-1 Ab (RB6-8C5), isotype control rat IgG2b mAb (clone A95-1), and purified Ab to CD16/CD32 (rat IgG2b; clone 2.4G2) were from BD Pharmingen. Brefeldin A, PMA, ionomycin and 1,3-PBIT (inhibitor of inducible NO synthase) were from Sigma-Aldrich.

Orthotopic tumor models

Orthotopic hepatic tumor model was established by subcapsular intrahepatic injection of Hepa cells (1×10^6/50 μl per mouse) into the left liver lobe of mice (11). Orthotopic lung cancer model was prepared by intrapulmonary inoculation with 3LL cells (1×10^6/50 μl per mouse) as previously described (12). The murine melanoma and lymphoma models were established by s.c. injection of B16 cells or EG7 cells (5×10^5/50 μl per mouse), respectively.

Isolation and purification of NK cells and MDSC

Single cell suspensions of splenocytes or liver mononuclear cells (MNC) from normal or tumor-bearing mice were prepared. For isolation of NK cells, the cells were first negatively selected by magnetic microbeads conjugated with anti-CD3 Ab, and then positively selected by magnetic microbeads conjugated with anti-DX5 Ab (Miltenyi Biotec) according to the manufacturer's instructions. Purity of NK cells was >90%. $CD11b^+Gr\text{-}1^+$ MDSC were prepared as previously described (13). Cells were incubated with biotinylated anti-Gr-1 and anti-CD11b microbeads. $CD11b^+Gr\text{-}1^+$ cells were positively selected. The purity of $CD11b^+Gr\text{-}1^+$ cells was confirmed by flow cytometry. For adoptive transfer, $CD11b^+Gr\text{-}1^+$ MDSC were sorted from splenocytes of tumor-bearing mice by MoFlo high-speed cell sorter (DakoCytomatix), the purity of which was confirmed to be >97%.

Isolation of liver MNC

Liver MNC were isolated and purified by the method of Richman et al. (14), with some modifications. MNC, resuspended in 40% Percoll, were gently overlayed onto 70% Percoll and centrifuged for 20 min at

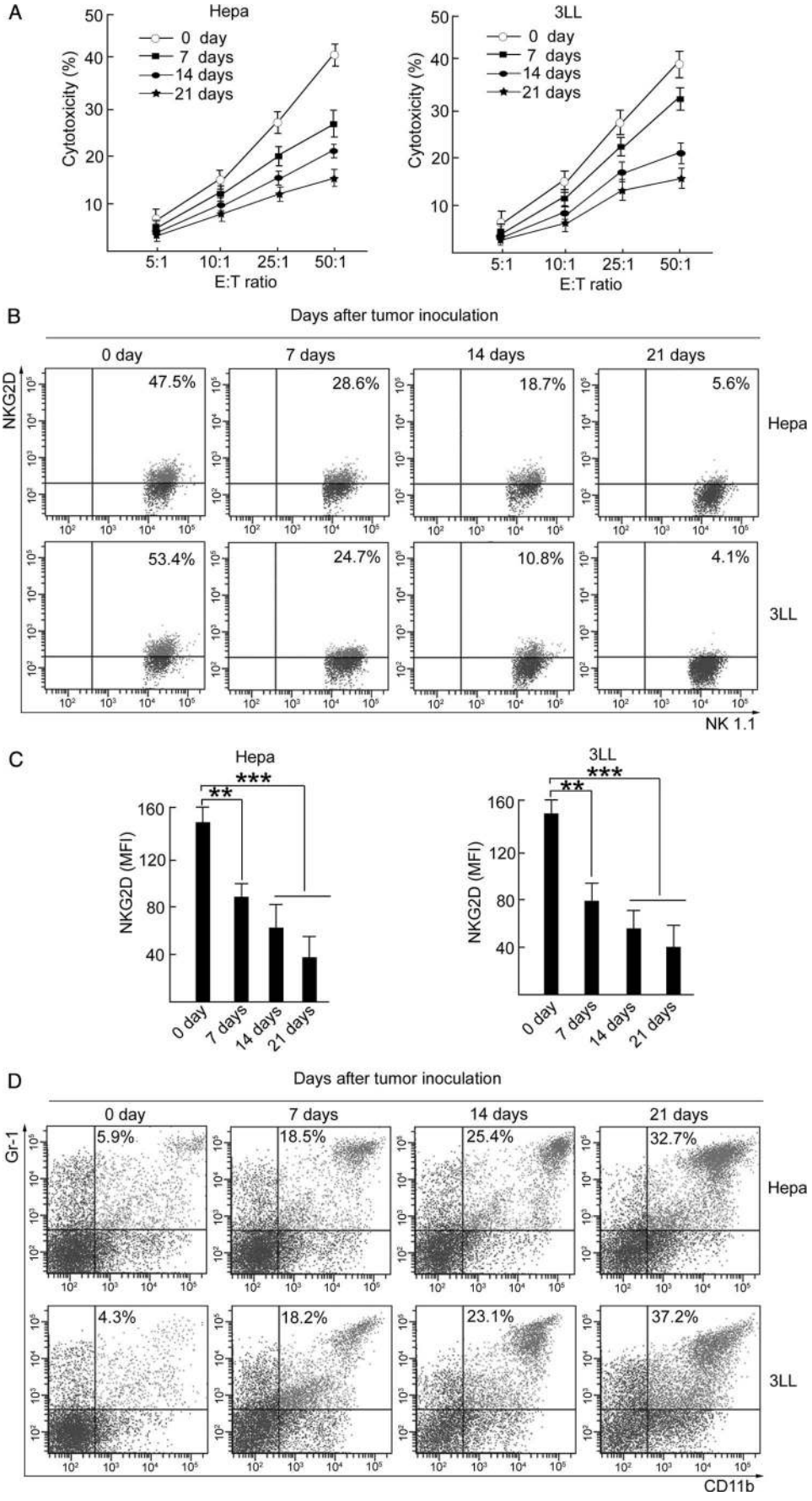

FIGURE 2. Dynamic observation of the decreased NK cell function and the increased MDSC in tumor-bearing mice. *A*, NK cell cytotoxicity was obviously decreased along tumor progression. The cytotoxicity of splenic NK cells from orthotopic liver (Hepa) or lung (3LL) cancer-bearing mice was examined by three-color flow cytometry assay. Results are expressed as mean ± SEM of triplicate wells from one representative experiment of four experiments completed. *B*, The percentage of NKG2D$^+$ NK cells on NK cells decreased obviously along tumor progression. *C*, MFI of NKG2D expression on NK cells decreased obviously along tumor progression. The splenocytes of orthotopic liver (Hepa) or lung (3LL) cancer-bearing mice at different stages were stained with CD3-FITC, NK1.1-allophycocyanin, and NKG2D-PE. CD3$^-$NK1.1$^+$ cells were gated in the FACS analysis. Dot plots represent one of four independent experiments. For MFI, data are mean ± SD of three experiments. *D*, The percentage of MDSC increased obviously along tumor progression. The splenocytes of orthotopic liver (Hepa) or lung (3LL) cancer-bearing mice at different stages were stained with Gr-1- and CD11b-specific mAbs. Dot plots represent one of four independent experiments. **, $p < 0.05$ and ***, $p < 0.01$.

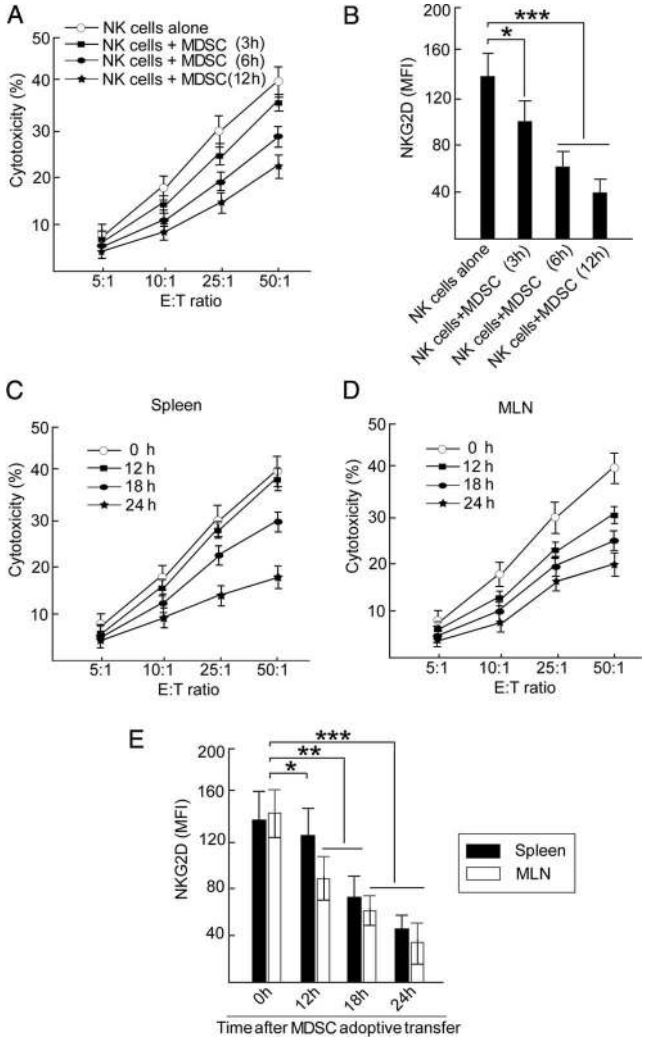

FIGURE 3. MDSC inhibit NK cell cytotoxicity and NKG2D expression in vitro and in vivo. Cytotoxicity (*A*) and NKG2D expression (*B*) of NK cells were determined after coculture with MDSC. NK cells purified from normal mice were cocultured with MDSC derived from spleens of orthotopic liver (Hepa) cancer-bearing mice at the ratio of 1:1 for 3, 6, or 12 h. Subsequently, NK cells were repurified and incubated with Yac-1 cells at the indicated E:T ratio for 12 h. Then the cells were collected and NK cell cytotoxicity was examined by three-color flow cytometry assay (NK1.1-allophycocyanin, annexin V-FITC, and 7-AAD) as described in Fig. 1*A*. Results are expressed as mean ± SEM of triplicate wells from one representative experiment of four experiments completed. For assay of NKG2D expression on NK cells (*B*), cells were collected after coculture as indicated, and analyzed by three-color flow cytometry using stain with CD3-FITC, NK1.1-allophycocyanin, and NKG2D-PE. $CD3^-NK1.1^+$ cells were gated in the FACS analysis as described in Fig. 2*C*. Data were presented as mean ± SD of three independent experiments. *, $p > 0.05$ and ***, $p < 0.01$. Cytotoxicity (*C* and *D*) and NKG2D expression (*E*) of NK cells from spleen and MLN cells were examined after adoptive transfer of MDSC (3×10^6/mouse) into normal C57BL/6 mice at time as indicated. MDSC were isolated from spleens of orthotopic liver (Hepa) cancer-bearing mice. NK cells purified from spleens or MLNs of transferred mice were incubated with Yac-1 target cells at the indicated E:T ratio. NK cell cytotoxicity was examined by three-color flow cytometry assay (NK1.1-allophycocyanin, annexin V-FITC, and 7-AAD) as described in Fig. 1*A*. Results are expressed as mean ± SEM of triplicate wells from one representative experiment of four experiments completed. Simultaneously, cells were analyzed for NKG2D expression as described in *B*. Data were presented as mean ± SD of three independent experiments. *, $p > 0.05$; **, $p < 0.05$; ***, $p < 0.01$.

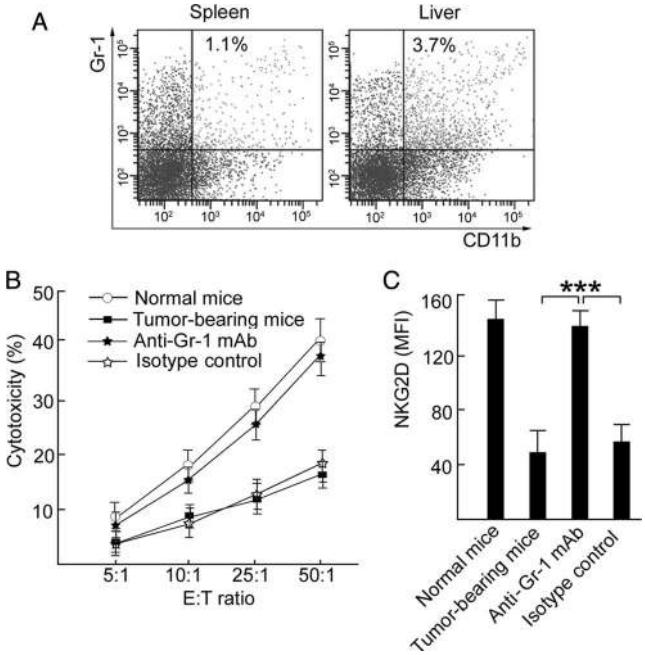

FIGURE 4. MDSC induce NK cell anergy both in vitro and in vivo. *A*, The FACS analysis of splenic and hepatic MDSC in tumor-bearing mice after $Gr\text{-}1^+$ cell depletion. To deplete $Gr\text{-}1^+$ cells, the orthotopic liver cancer-bearing mice were i.p. injected with 0.25 mg anti-Gr-1 Ab (BD Pharmingen). At 24 h later, splenic and hepatic MDSC were analyzed by two-color flow cytometry assay (Gr-1-allophycocyanin and CD11b-PE). The results represent one of three independent experiments with similar results. *B* and *C*, Depletion of MDSC restored NK cell cytotoxicity and NKG2D expression in orthotopic liver cancer-bearing mice. MDSC in the orthotopic liver cancer-bearing mice (21 days after tumor inoculation) were depleted with anti-Gr-1 mAb (RB6-8C5). At 24 h later, NK cell cytotoxicity (*B*) and NKG2D expression (*C*) were determined at the time as indicated. Results were representative of at least four independent experiments. For cytotoxicity assays, values were expressed as mean ± SEM of triplicate wells from one representative experiment of four experiments completed. For examination of NKG2D expression, results were presented as a change in MFI and presented as mean ± SD of four independent experiments. ***, $p < 0.01$.

750 × *g*. Finally purified MNC were collected from the interface for further analysis of hepatic MDSC, NK cells, and regulatory T cells (Tregs).

Flow cytometry

Cell phenotype was analyzed by flow cytometry with FACS LSRII (BD Biosciences) as previously described and data were analyzed with FACSDiva software (15). NKG2D expression was determined by mean fluorescence intensity (MFI) on $CD3^-NK1.1^+$ gated cells. Brefeldin A was added at 10 μg/ml for the last 6 h of culture. For intracellular staining, the cells were stained with the Abs against cell surface Ag, and then fixed and permeabilized (IC Fixation/Permeabilization buffer; eBioscience) for 20 min. The cells were labeled with cytokine-specific fluorescence-conjugated anti-IFN-γ mAbs or isotype-matched Ig controls.

Fluorescence confocal microscopy

To ascertain whether $CD11b^+Gr\text{-}1^+$ MDSC could express membrane-bound TGF-β1, we stained the freshly isolated MDSC with anti-Gr-1-allophycocyanin, DAPI (4′,6-diamidino-2-phenylindole), anti-TGF-β1 biotin, and anti-biotin FITC and then visualized with a Leica TCS SP2 confocal laser microscope (Leica Microsystem). All cell images were obtained using a 40X dry objective lens on the confocal microscopy with Leica Confocal Software.

Assay of NK cell cytotoxicity

NK cells from normal mice, tumor-bearing mice or Smad3-deficient mice were seeded at 5×10^5 cells/well in 96-well plates, incubated with MDSC

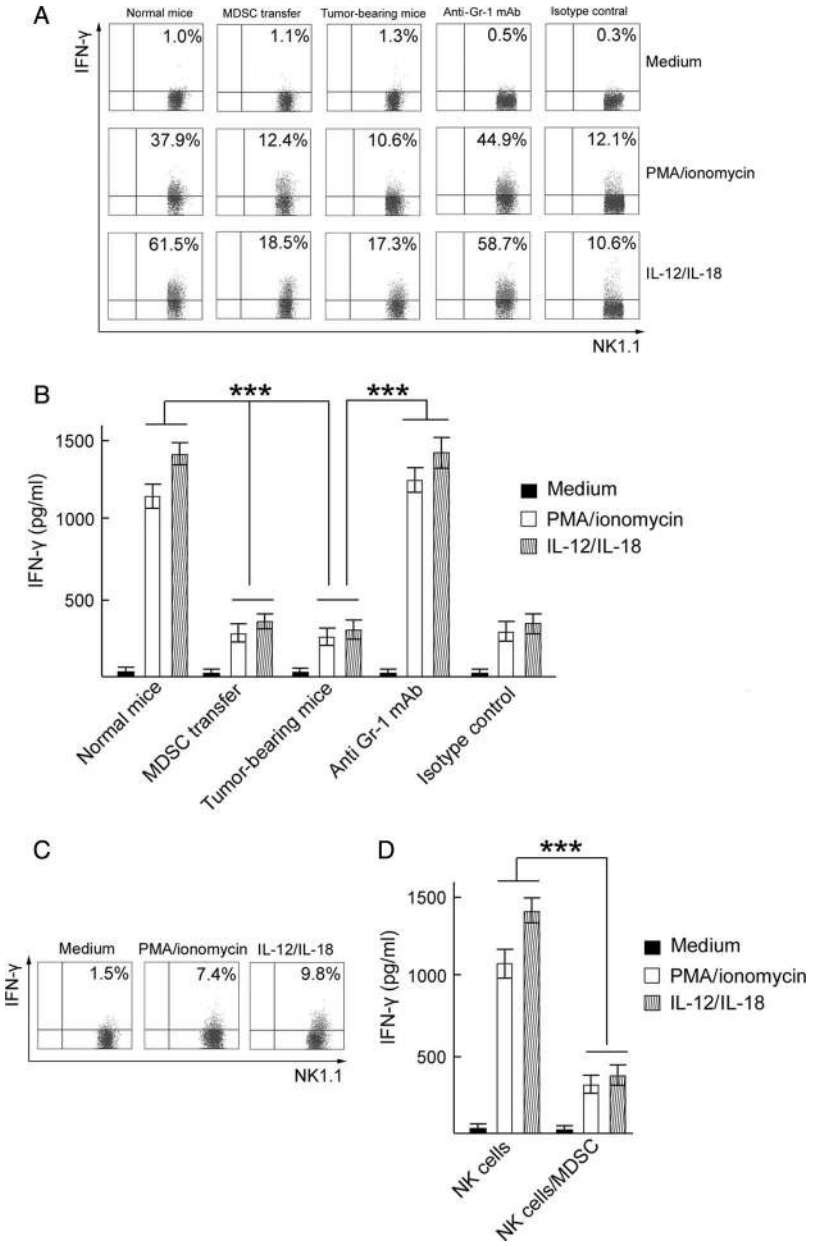

FIGURE 5. MDSC induce hyporesponsiveness of NK cells to produce IFN-γ toward activators. NK cells were purified from normal mice adoptively transferred with MDSC or not tranferred or from orthotopic liver cancer-bearing mice depleted with MDSC or not depleted. Then 1×10^6/ml NK cells were stimulated with 25 ng/ml PMA plus 1 μg/ml ionomycin or 10 ng/ml IL-12 plus 20 ng/ml IL-18 for 24 h. Cells and supernatants were harvested, and IFN-γ levels were determined with intracellular staining (*A*) and ELISA (*B*). For in vitro experiments, 1×10^6/ml NK cells purified from normal mice were cocultured with MDSC from tumor-bearing mice at the ratio of 1:1 for 6 h, then stimulated as described. At 18 h later, the level of IFN-γ was analyzed by FACS analysis (*C*) and ELISA (*D*). Mean ± SEM of triplicate wells from one representative experiment of four is shown. For intracellular staining, brefeldin A (BFA, 10 μg/ml) was added 6 h before the cells were harvested. *, $p > 0.05$ and ***, $p < 0.01$.

or cultured in the presence of 0.5 ng/ml recombinant mouse TGF-β1, 10 ng/ml anti-TGF-β1 Ab, 10 ng/ml isotype control Ab, or 15 μg/ml anti-NKG2D mAb, respectively, for the indicated times. NK cell cytotoxicity against Yac-1 cells, incubated for 12 h at 37°C at the indicated E:T ratio, was measured using three-color flow cytometry assay as described (16). At the indicated time points, cells were collected and incubated with anti-NK1.1 allophycocyanin and annexin V-FITC. After washing, cells were stained with 7-aminoactinomycin D (7-AAD) and analyzed by FACS LSRII. Live target cells were determined by gating on $NK1.1^-$annexin V^-7-AAD^- population. Percentage of cytotoxicity was calculated by the following equation: 100 × ($NK1.1^-$ cells − $NK1.1^-$annexin V^-7-AAD^- cells)/($NK1.1^-$ cells).

Detection of IFN-γ

A total of 1×10^6/ml NK cells were cultured alone or with MDSC at a 1:1 ratio or reagents (0.5 ng/ml TGF-β1 or 10 ng/ml anti-TGF-β1 Ab or 10 ng/ml isotype control Ab) for 6 h. Then, NK cells were stimulated with 25 ng/ml PMA and 1 μg/ml ionomycin or 10 ng/ml IL-12 and 20 ng/ml IL-18 for 18 h. IFN-γ concentrations in the supernatants were determined by ELISA kit (R&D Systems).

Adoptive transfer or depletion of MDSC in vivo

To observe the in vivo effects of MDSC on NK cell functions, the purified MDSC were i.p. injected to normal C57BL/6 mice (3×10^6 per mouse). For depletion assay, 0.25 mg of anti-Gr-1 Ab (RB6-8C5) or isotype control rat IgG2b mAb (clone A95-1) was i.p. administered into tumor-bearing mice (21 days after tumor inoculation) (8). At 24 h later, the lymphocytes of spleen, liver, and mesenteric lymph nodes (MLN) were isolated and examined by FACS.

Depletion of $CD4^+CD25^+$ Tregs in vivo

Tregs in tumor-bearing mice (21 days after tumor inoculation) were depleted by i.p. injection of 500 μg of anti-CD25 mAb prepared from hybridoma PC61 (ATCC) (17). At 48 h later, lymphocytes from spleen and liver were isolated and analyzed to confirm the elimination of $CD25^+CD4^+$ T cells.

Statistical analysis

Data were analyzed for statistical significance using Student's *t* test. Statistical significance was determined for values of $p < 0.05$.

Results

Decrease of NK cell function in liver and spleen is inversely correlated to the increase of MDSC in tumor-bearing mice

As shown in Fig. 1, the cytotoxicity and IFN-γ production of NK cells from both liver and spleen were all suppressed significantly

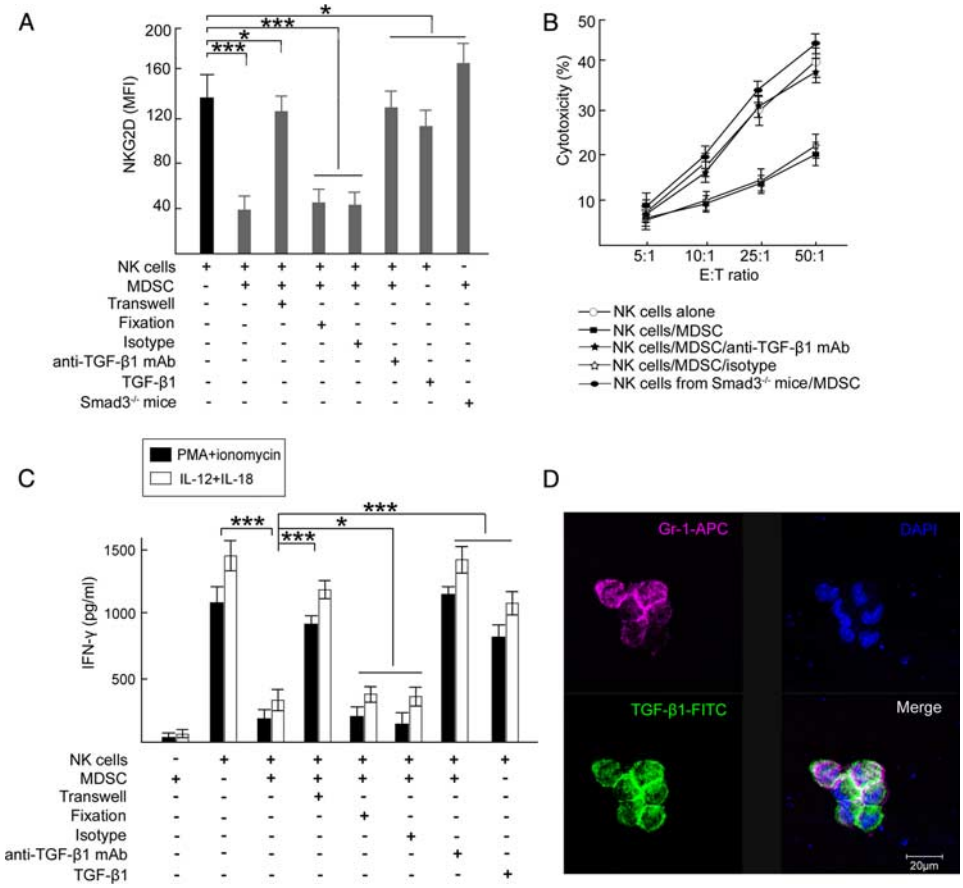

FIGURE 6. Membrane-bound TFG-β1 expressed on MDSC is responsible for suppression of NK cell function by MDSC. TFG-β1 and cell-to-cell contact were required for the MDSC-mediated inhibition of NKG2D expression (*A*) and NK cell cytotoxicity (*B*). NK cells were cultured with MDSC isolated from spleens of liver (Hepa) cancer-bearing mice at the ratio of 1:1 for 12 h under various conditions including Transwell (0.4 μm) separation and fixation of MDSC with 1% glutaraldehyde. Then NK cell cytotoxicity and NKG2D expression were detected as described. Otherwise, NK cells from normal mice or from Smad3-deficient mice were cocultured with MDSC at the ratio of 1:1 or in the presence of 10 μg/ml anti-TGF-β1 mAb, isotype Ab, or 0.5 ng/ml recombinant mouse TGF-β1 for 12 h, respectively, then NK cell cytotoxicity was analyzed by three-color flow cytometry and $CD3^-NK1.1^+$ were gated in the FACS analysis. Results are presented as MFI. Data are mean $\pm$ SD of three experiments. *, $p > 0.05$ and ***, $p < 0.01$. *C*, Membrane-bound TFG-β1 is the main factor for suppression of NK cell IFN-γ secretion. A total 1×10^6/ml NK cells from normal mice were cocultured with MDSC at the ratio of 1:1 or with 0.5 ng/ml TGF-β1 for 6 h, then stimulated with 25 ng/ml PMA plus 1 μg/ml ionomycin or 10 ng/ml IL-12 plus 20 ng/ml IL-18. At 18 h later, supernatants were harvested, and IFN-γ levels were determined using ELISA kit. Mean $\pm$ SEM of triplicate wells from one representative experiment of four is shown. *, $p > 0.05$ and ***, $p < 0.01$. *D*, Expression of membrane-bound TGF-β1 on MDSC. MDSC purified from spleen of liver cancer-bearing mice were stained with anti-Gr-1-allophycocyanin, anti-TGF-β1 biotin, anti-biotin FITC, and DAPI (nuclei), and then analyzed at a magnification of $\times$40 with Confocal Laser Microscope. The images are representative of three independent experiments.

in all tumor-bearing models including liver cancer, lung cancer, melanoma, and lymphoma-bearing mice. In contrast, the number of MDSC in both liver and spleen of tumor-bearing mice increased markedly. Then, we dynamically observed the changes of NK cell function and MDSC number by analyzing the NK cells and MDSC from tumor-bearing mice 7, 14, or 21 days after tumor implantation. We found that the cytotoxicity of NK cells was down-regulated dramatically in orthotopic liver cancer-bearing mice, with more pronounced reduction at the advanced stage of tumor burden (Fig. 2*A*). To further identify changes of the factors associated with NK cell cytotoxicity, we analyzed the expression of Fas ligand, perforin, TRAIL, and NKG2D by NK cells from different stages of tumor as indicated (Fig. 2, *B* and *C*). The number of $NKG2D^+$ NK cells and the MFI of NKG2D expression on NK cells decreased gradually along tumor progression, and the most pronounced decrease was present at the advanced stage of tumor burden. However, expression of Fas ligand, perforin, and TRAIL remained unchanged (data not shown). In contrary, the frequency of MDSC in splenocytes of tumor-bearing mice increased significantly to 20–40%, with more significant elevation at the advanced stage of tumor burden (Fig. 2*D*). The percentage of MDSC was as rare as 2–5% in spleen and MLN of normal mice bred in pathogen-free condition. In addition, we obtained similar results in orthotopic tumor model with 3LL lung cancer (Fig. 2). To confirm the critical role of NKD2D expression in NK cell cytotoxicity, we blocked NKG2D in the coculture system of NK cells and target cells Yac-1 by using neutralizing Ab against NKD2D (C7), and found that blockade of NKG2D did inhibit cytotoxicity of NK cells effectively (data not shown). These data demonstrated that there was definite inverse correlation of the decreased NK cell cytotoxicity, NKG2D expression with the increased frequency of MDSC in the tumor-bearing mice. Taken together with previous reports that MDSC can secrete many kinds of immunosuppressive factors and inhibit T cell activities, our data suggest that the impaired NK cell function in tumor-bearing mice might be due to the increase of MDSC.

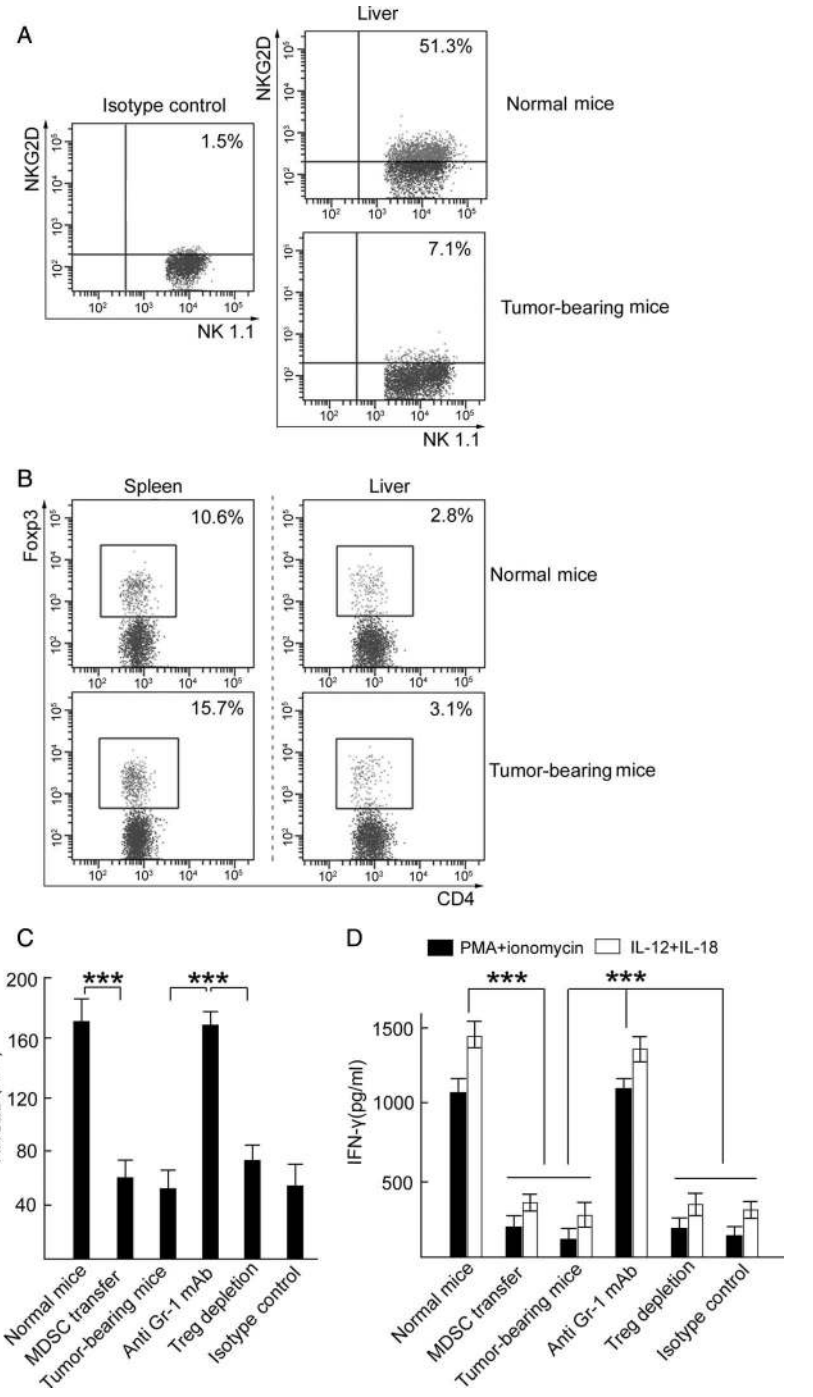

FIGURE 7. MDSC, but not Treg, are the main negative regulator of hepatic NK cells in liver cancer-bearing mice. *A*, The NKG2D expression of hepatic NK cells decreased in orthotopic liver cancer-bearing mice. The hepatic MNC from normal or orthotopic liver cancer-bearing mice were stained with CD3-FITC, NK1.1-allophycocyanin, and NKG2D-PE. For detection of NKG2D expression, $CD3^{-}NK1.1^{+}$ cells were gated in the FACS analysis. Data represented one of three independent experiments with similar results. *B*, Few Tregs were found in the liver of orthotopic liver cancer-bearing mice. The splenocytes and hepatic MNC from normal or orthotopic liver cancer-bearing mice were stained with CD4-FITC, Foxp3-PE, and CD25-allophycocyanin. $CD4^{+}$ T cells were gated in the FACS analysis and $Foxp3^{+}$ populations in the dot plots were all $CD25^{+}$. Data represented one of three independent experiments with similar results. *C*, NKG2D expression of hepatic NK cells was inhibited by MDSC, but not Tregs, in vivo. NKG2D expression on NK cells from liver MNC of the normal mice with or without MDSC transfer, orthotopic liver cancer-bearing mice with or without depletion of MDSC or Tregs was detected by three-color flow cytometry assay (CD3-FITC, NK1.1-allophycocyanin, and NKG2D-PE). $CD3^{-}NK1.1^{+}$ cells were gated in the FACS analysis and results were presented as changes in MFI. Data were mean ± SD of three independent experiments. ***, $p < 0.01$. *D*, Hepatic NK cell IFN-γ secretion was inhibited by MDSC, but not Tregs, in vivo. Hepatic NK cells purified from the normal mice with or without MDSC transfer, orthotopic liver cancer-bearing mice with or without depletion of MDSC, or Tregs 6 h later were stimulated with 25 ng/ml PMA plus 1 μg/ml ionomycin or 10 ng/ml recombinant murine IL-12 plus 20 ng/ml recombinant murine IL-18 for 18 h. Then supernatants were collected for ELISA of IFN-γ levels. Data represent mean ± SEM of triplicate wells. Results of one representative experiment of four were shown. ***, $p < 0.01$.

MDSC inhibit cytotoxicity and NKG2D expression of NK cells both in vitro and in vivo

To investigate whether the expanded MSC population was responsible for the impairment of NK cell function in liver cancer-bearing mice, we incubated the NK cells isolated from normal mice with MDSC purified from liver cancer-bearing mice in vitro. NK cell cytotoxicity was impaired even by incubation of NK cells with MDSC at a ratio of 1:1 for only 3 h, and more significant impairment was observed 12 h after NK or MDSC incubation (Fig. 3*A*). Accordingly, NKG2D expression on NK cells was down-regulated by incubation with MDSC in vitro (Fig. 3*B*). Thus, MDSC could significantly inhibit NK cell cytotoxicity and NKG2D expression in vitro.

To determine whether MDSC could impair NK cell cytotoxicity in vivo, MDSC (>97% purity) derived from liver cancer-bearing mice were prepared by sorting and then i.p. injection into normal mice. After adoptive transfer of MDSC, the splenocytes and MLN cells were collected for examination of NK cell cytotoxicity and NKG2D expression, respectively, at 12, 18, and 24 h. As shown in Fig. 3, the in vivo transfer of MDSC could down-regulate NK cell cytotoxicity (Fig. 3, *C* and *D*) and NKG2D expression (Fig. 3*E*) within 24 h. Interestingly, the significant down-regulation of NK cell cytotoxicity and NKG2D expression was observed at 12 h in MLN but at 18 h in spleen after i.p. transfer of MDSC, which may be attributed to the earlier presence of transferred MDSC in MLN than spleen

due to migration. Therefore, MDSC could inhibit NK cell cytotoxicity and NKG2D expression both in vitro and in vivo.

MDSC induce anergy of NK cells both in vitro and in vivo

Next, we depleted MDSC in orthotopic liver cancer-bearing mice (Fig. 4*A*) and then examined the change of NK cell cytotoxicity and NKG2D expression 24 h later. As expected, depletion of MDSC significantly restored cytotoxicity (Fig. 4*B*) and NKG2D expression (Fig. 4*C*) of NK cells in the liver cancer-bearing mice. Potent cytotoxicity and IFN-γ production are two important functional characteristics of the activated NK cells (18). We found that NK cells isolated from liver cancer-bearing mice produced much less IFN-γ than that from normal mice once stimulated by PMA and ionomycin in vitro (Fig. 1*B*). NK cells from the normal mice with adoptive transfer of MDSC 24 h before produced less IFN-γ in response to the activating stimuli, accordingly furthermore, depletion of MDSC in liver cancer-bearing mice could restore the capacity of NK cells to produce IFN-γ (Fig. 5, *A* and *B*). We also incubated NK cells from normal mice with MDSC from tumor-bearing mice in vitro, and then stimulated the NK cells with activating signals. We found that the production of IFN-γ by NK cells in response to the activating stimuli was down-regulated evidently (Fig. 5, *C* and *D*). So, MDSC expanded in liver cancer-bearing mice not only inhibit NK cell cytotoxicity and NKG2D expression, but also induce NK cells to be hyporesponsive and produce less IFN-γ toward the activating signals, suggesting that MDSC can induce anergy of NK cells.

Membrane-bound TGF-β1 on MDSC is critical for the suppression of NK cells

We further investigated the underlying mechanisms for the impairment of NK cell function by MDSC in liver cancer-bearing mice. To identify whether soluble molecules or cell-cell interaction involved in this process, we incubated NK cells and MDSC in the Transwell system (0.4 μM) or incubated NK cells with the fixed MDSC. The down-regulation of NK cell NKG2D expression and IFN-γ production by MDSC was lost in the Transwell system, whereas still existed when the fixed MDSC used (Fig. 6, *A* and *C*), suggesting that MDSC-mediated inhibition of NK cells was due to cell-to-cell interactions. We also confirmed that IL-10, VEGF and NO produced by MDSC are not involved in the suppression of NKG2D expression on NK cell because the Abs against these suppressive factors could not restore the suppression of NK cell activity by MDSC in the coculture system of NK cell and MDSC (see Supplemental Fig. 6).[5] As TGF-β1 is an important suppressive cytokine produced by MDSC and a critical factor for negative regulation of NKG2D expression (19, 20), we investigated whether MDSC could express membrane-bound TGF-β1, and if so, whether membrane-bound TGF-β1 was involved in the process. Neutralization of TGF-β1 in the coculture system of MDSC or NK cells could restore NKG2D expression (Fig. 6*A*), cytotoxicity (Fig. 6*B*), and IFN-γ production (Fig. 6*C*) of NK cells, whereas supplement of exogenous recombinant TGF-β1 in the culture system had no significant suppressive effect. As membrane-bound TGF-β1 was verified by confocal laser microscope to be expressed on MDSC (Fig. 6*D*), the data indicated that the membrane-bound TGF-β1 may be responsible for the impairment of NK cell function by MDSC. To further confirm this possibility, we isolated NK cells fron Smad3-deficient mice in transducing TGF-β1 signals, and found that MDSC derived from liver cancer-bearing mice could not down-regulate NKG2D expression (Fig. 6*A*) and cytotoxicity (Fig. 6*B*) of NK cells from Smad3-deficient mice. Our results convincingly demonstrate that MDSC inhibit NK cell function through their membrane-bound TGF-β1.

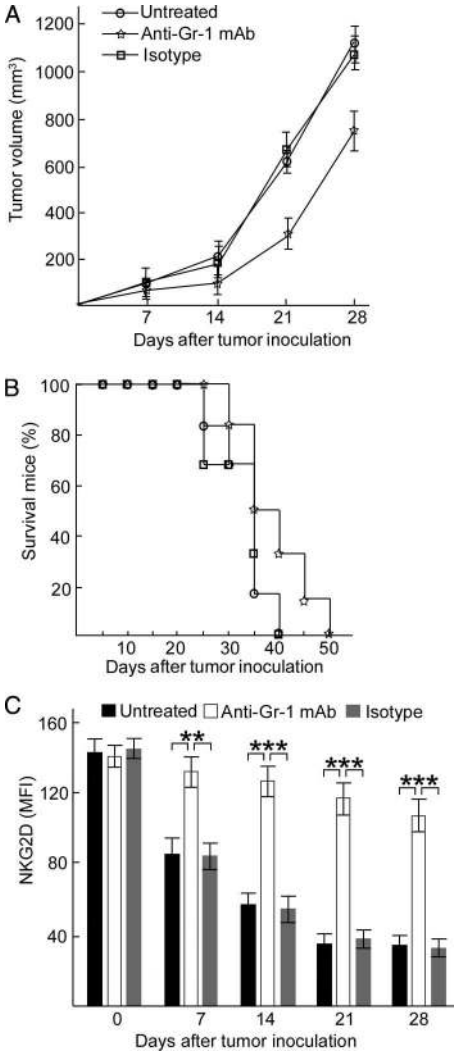

FIGURE 8. Depletion of MDSC inhibits tumor growth by restoring NK cell NKG2D expression. For depletion of Gr-1$^+$ cells in vivo, the (Hepa) tumor-bearing mice were i.p. administrated with 250, 125, 10, or 1 μg of anti-Gr-1 Ab (BD Pharmingen) on days 0, 6, 13, and 20 after tumor inoculation. The tumor size was measured every 7 days and the survival of the mice was observed every day. *A*, Tumor volumes were measured and expressed in cubic centimeters. Each data point represents the mean tumor volume $\pm$ SEM of $n = 6$ mice. *B*, The number of survival mice was indicated as a percentage of total mice ($n = 7$) injected. Data are representative of three independent experiments with similar results. *C*, NKG2D expression on NK cells from liver MNC of the described tumor-bearing mice with or without Gr-1+ cell depletion was detected by three-color flow cytometry assay (CD3-FITC, NK1.1-allophycocyanin, and NKG2D-PE). CD3-NK1.1$^+$ cells were gated in the FACS analysis and results were presented as a change in MFI. Data were mean $\pm$ SD of three independent experiments. **, $p < 0.05$ and ***, $p < 0.01$.

MDSC, but not Tregs, are the main negative regulator of hepatic NK cells in liver cancer-bearing mice

NK cells are enriched in liver and hepatic NK cells play important roles in the liver immunity. So, we wondered whether MDSC-mediated suppression of NK cells was a universal mechanism in tumor-induced impairment of NK activities in liver. We found that the expression of NKG2D by hepatic NK cells was significantly down-regulated in the orthotopic liver cancer-bearing mice (Fig.

[5] The online version of this article contains supplemental material.

7*A*), which was inversely correlated to the increase of MDSC (Fig. 1*C*). The frequency of liver MDSC was increased from 4–7% to 40–50%, and the expression level of NKG2D on NK cells was decreased from 30–50% to 4–9%. Adoptive transfer of MDSC to normal mice could reduce NKG2D expression and IFN-γ production of hepatic NK cells, accordingly, depletion of MDSC in the orthotopic liver cancer-bearing mice could restore NKG2D expression and IFN-γ production of hepatic NK cells (Fig. 7, *C* and *D*), of the findings were consistent with the observations that MDSC inhibited splenic NK cell function as shown in Fig. 3.

$CD4^+CD25^+$ Tregs, a critical player in the maintenance of peripheral tolerance, can suppress NK cell function in human and mice (21, 22). Tregs could be expanded markedly in tumor-bearing mice, thus Tregs may be another candidate in the impairment of NK cell function by tumor. However, we found that there were less $CD4^+CD25^+$ $Foxp3^+$ Tregs in liver than those in spleen (Fig. 7*B*). Furthermore, the frequency of hepatic Tregs remained almost unchanged in tumor-bearing mice, as compared with hepatic Tregs in normal mice. We depleted MDSC or Tregs from orthotopic liver cancer-bearing mice, and then analyzed NKG2D expression and IFN-γ production of hepatic NK cells. As shown in Fig. 7, *C* and *D*, depletion of MDSC restored NKG2D expression and IFN-γ secretion of hepatic NK cells significantly; however, depletion of Tregs could not up-regulate NKG2D expression and IFN-γ production of hepatic NK cells, suggesting that MDSC, but not Tregs, are the main negative regulator of hepatic NK cells in liver cancer.

Depletion of MDSC inhibits tumor growth by restoring NK cell activity

A shown in Fig. 8, in vivo depletion of $Gr\text{-}1^+$ cells in liver cancer-bearing mice by injecting anti-$Gr\text{-}1^+$Ab could inhibit tumor growth and prolong the survival of liver cancer-bearing mice significantly. Accordingly, the decreased NKG2D expression on NK cells was reversed, indicating that the suppressed NK cell activity was restored (Fig. 8*C*). The data indicate that in vivo depletion of MDSC may benefit tumor-bearing host by increasing NK cell activity.

Discussion

The liver is the most common organ of metastasis of gastrointestinal cancers (i.e., colorectal, gastric, and pancreatic) and other kinds of cancers. The liver is enriched in NK cells, and the liver-associated NK cells have been suggested to exert important roles in the liver immunity against cancer and infectious diseases. So, better understanding of the mechanisms for the regulation of hepatic NK cells is critical to the design of effective approaches for the immunotherapy of cancer and infectious diseases. However, the functions of NK cells are usually impaired in cancer patients (23), and tumors can escape NK cell-mediated cytotoxicity through their mechanisms of immune escape such as secretion of inhibitory cytokines and shedding of soluble ligands for activating receptors (24). Up to now, there are several kinds of regulatory cells that play important roles in the maintenance of immune tolerance and suppression of immune responses against cancer. For example, tumor growth promotes the expansion of Tregs and Tregs could suppress NK cell functions including cytotoxicity, NKG2D expression, and IFN-γ production in human and mice (21, 22). In addition to the direct negative regulation of NK cells by Tregs, is it possible for NK cells to be negatively regulated by other regulatory cells? MDSC can produce various immunosuppressive factors, including TGF-β1, IL-10, and VEGF, that can suppress the functions of T cells. MDSC can also inhibit T cell proliferation and activation through arginase-1 or NO synthase 2 (25, 26). Inspired by the findings that MDSC could be expanded rapidly and markedly and NK cell function was down-regulated significantly in tumor-bearing mice and cancer patients, the interaction of MDSC and NK cells in tumor-bearing mice was investigated more recently but the underlying mechanisms remained to be addressed (27). Interestingly, we found that, in addition to the impairment of splenic NK cells, hepatic NK cell functions including cytotoxicity and IFN-γ were impaired significantly in all tumor-bearing models we tested, including liver cancer, lung cancer, melanoma and lymphoma. So, the function of hepatic NK cells can be affected by the remote cancer outside liver. We found the decrease of NK cell function is inversely correlated to the increase of MDSC in tumor-bearing mice, thus suggesting one of mechanisms for the impairment of NK cell function is due to the cancer-expanded MDSC. Our results also can be helpful to explain why liver is an organ prone to cancer metastasis.

Tregs have been shown to be expanded systemically in tumor host and can inhibit NK cell functions. However, in our study, we observed that MDSC were accumulated rapidly and markedly in the liver in the orthotopic liver cancer-bearing mice while the number of Tregs in the liver remains almost unchanged in the same model, in which MDSC level was almost the same to that in the liver of normal mice. More importantly, depletion of MDSC can restore hepatic NK cell NKG2D expression and IFN-γ production in the orthotopic liver cancer-bearing mice; however, depletion of Treg has no such revering effect on the impaired function of hepatic NK cells. Interestingly, depletion of both MSC and Tregs can significantly restore splenic NK cell NKG2D expression and IFN-γ production in the orthotopic liver cancer-bearing mice (see Supplemental Fig. 1).[5] Although MDSC have been previously shown to be able to directly induce the generation of Tregs, taken together with the fact that MDSC appear and expand more rapidly and markedly than Tregs during tumor progression, we demonstrate that hepatic MDSC, but not the Tregs in liver, are the major negative regulator of hepatic NK cells, at least, in liver cancer.

The functions of NK cells are regulated by a balance of signals transmitted by inhibitory and activating receptors that can specifically interact with their ligands, including class I molecules and specific molecules expressed on tumor cells and virus-infected cells, respectively (28). Among the activation receptors for NK cells, NKG2D is the pivotal one that can be induced on abnormal cells such as stressed cells and tumor cells. Tumors may have several strategies to escape from NK cell-mediated lysis such as down-regulation of NKG2D expression and secretion of immunosuppressive cytokine TGF-β (24). TGF-β1 has been found to be increased markedly in tumor-bearing host, and induce dysfunction of T cells, DCs, and NK cells (29). Moreover, TGF-β1 produced by tumor cells or Tregs can exert its inhibitory effects in soluble or membrane-bound manner (19, 20). In our study, although we found that MDSC could secrete soluble TGF-β1, which was not involved in the suppression of NK cells by MDSC (data not shown), we for the first time show MDSC expanded in tumor-bearing mice can express membrane-bound TGF-β1. We also demonstrate that blockade of TGF-β1, deficiency of the TGF-β1 signaling in NK cells, and disassociation of NK cells and MDSC by Transwell all could reverse the suppression of NK cell cytotoxicity, NKG2D expression, and IFN-γ production by MDSC derived from tumor-bearing mice, convincingly confirming that MDSC can inhibit NK cell function through membrane-bound TGF-β1. We also analyzed the expression of other NK cell receptors including NKp46NKG2A, NKG2C, Ly49A, Ly49C, Ly49D, and Ly49F and found that their expression remained unchanged (see Supplemental Fig. 2).[5] In addition, we found that perforin expression on NK cells was not decreased as described by other

reports (see Supplemental Fig. 3).[5] Considering that NKG2D expression is crucial to NK cell cytotoxicity against target cells, MDSC-mediated down-regulation of NKG2D expression might be one of important reasons for the impairment of NK cell cytotoxicity by MDSC through membrane-bound TGF-β1. MDSC are heterogeneous with different subpopulations identified by different methods (30). Regulation of NK cells, and even T cell, B cell, and DC, by MDSC subpopulations needs to be investigated in the future.

The term anergy is used to describe tolerance phenomenon that the lymphocytes survive but appear to be functionally unresponsive (31). Anergy of T cell and B cell is an essential mechanism for immune tolerance (31, 32). However, anergy of NK cells is rarely mentioned. As the most important effector of innate immunity to kill target cells, NK cells can also can secret cytokines such as IFN-γ to control Th1 differentiation and regulate subsequent adaptive immune response. Therefore, understanding the mechanisms that control NK cell homeostasis and function will have important implications for maintenance of immune balance and self-tolerance. We showed that NK cells, once incubated with MDSC from tumor-bearing mice, could not be activated to secrete IFN-γ, thus proposing the concept of NK cell anergy to describe the impairment of NK cells in the tumor-bearing mice. In addition to the generally accepted Treg-mediated suppression of innate and adaptive immune response, our description will provide a mechanistic insight into tumor immune escape via negative regulation of NK cell innate function by MDSC.

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Disclosures

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