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Myeloid-Derived Suppressor Cells Regulate Growth of Multiple Myeloma by Inhibiting T Cells in Bone Marrow

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Myeloid-derived suppressor cells (MDSC) are one of the major factors limiting the immune response in cancer. However, their role in bone marrow (BM), the site of primary localization of multiple myeloma (MM), is poorly understood. In this study, we found a significant accumulation of CD11b+CD14+CD33+ immunosuppressive MDSC in BM of patients with newly diagnosed MM. To assess the possible role of MDSC in MM, we used immunocompetent mouse models. Immunosuppressive MDSC accumulated in BM of mice as early as 1 wk after tumor inoculation. S100A9 knockout (KO) mice, which are deficient in their ability to accumulate MDSC in tumor-bearing hosts, demonstrated reduced MDSC accumulation in BM after injection of MM cells compared with wild-type mice. Growth of the immunogenic MM cells was significantly reduced in S100A9 KO mice. This effect was associated with the accumulation of Ag-specific CD8+ T cells in BM and spleens of S100A9 KO mice, but not wild-type mice, and was abrogated by the administration of anti-CD8 Ab or adoptive transfer of MDSC. Thus, the accumulation of MDSC at early stages of MM plays a critical role in MM progression and suggests that MDSC can be considered a possible therapeutic target in this disease. The Journal of Immunology, 2013, 190: 000–000.

Multiple myeloma (MM) is a hematologic cancer characterized by the accumulation of malignant plasma cells within the bone marrow (BM). The BM microenvironment is known to be critically important for MM survival, growth, and chemosensitivity. Although the majority of studies have focused on the contribution of BM stroma and osteoclasts in MM pathogenesis (1–4), less attention has been paid to the role of other cells that constitute the BM microenvironment, including cells involved in the modulation of immune responses.

An impaired immune system plays an important role in tumor growth (5). In MM, abnormalities in T cells, including fewer peripheral blood (PB) CD4 and CD8 T cells, inversion of the CD4/CD8 ratio, abnormal Th1/Th2 CD4 ratio, downregulation of signal-transduction components, and normal T cell response, have been reported (6, 7). In addition, cellular immune defects, including abnormalities in macrophages, NK cells, and dendritic cells, have been described in MM. Despite the fact that MM localizes preferentially in BM, the majority of studies has focused on immunological alterations in PB of MM patients. At the same time, very little is known regarding the function of the immune system in MM BM, particularly the ability of the immune system to generate an anti-MM immune response in the BM tumor microenvironment (8, 9).

In recent years, the important role of myeloid-derived suppressor cells (MDSC) in the regulation of immune responses in cancer has been established. This heterogeneous group of myeloid cells consists of pathologically activated myeloid progenitors and immature myeloid cells (IMC), with a potent immunosuppressive activity (10). Under physiological conditions, IMC rapidly differentiate into mature myeloid cells. The accumulation of MDSC in cancer is the result of two sets of factors: one that promotes expansion of IMC and another that induces activation of these cells associated with a partial block in their differentiation. The first group of factors includes GM-CSF, M-CSF, VEGF, and others, which signal primarily via STAT3 and STAT5 transcription factors, whereas the second group consists of proinflammatory cytokines and signals via STAT1, IL-10, and NF-κB (11–13). MDSC inhibit the function of immune cells via a number of mechanisms involving NO, arginase, reactive oxygen species (ROS), Cox-2, and others (10, 12). In mice, MDSC are characterized by the coexpression of Gr1 and CD11b molecules (14). In recent years, two large groups of mouse MDSC were identified: CD11b+Ly6C+Ly6G+ polymorphonuclear MDSC (PMN-MDSC) and CD11b+Ly6C+Ly6G+ monocytic MDSC (M-MDSC). Although these cells share immunosuppressive activity, they are distinct with regard to their morphology and mechanisms of suppression (15, 16). Although PMN-MDSC use ROS to mediate T cell suppression, M-MDSC have increased levels of NO but undetectable levels of ROS (16). In humans, the phenotype of MDSC depends on the type of tumor. In most tumors, immunosuppressive MDSC are defined as CD11b+CD14+CD33+ or Lin−HLA-DR−CD33+ cells that can be subdivided into CD15+ PMN-MDSC and CD15−M-MDSC. In some tumors, M-MDSC also have been defined as CD14+HLA-DR+ (17, 18).
There is a wealth of information regarding the possible role of MDSC in the regulation of immune responses in solid tumors. However, little is known about the biology of MDSC and their possible immunosuppressive activity in hematologic malignancies, including MM. Although the presence of MDSC in PB of patients with MM was described (19), the possible role of MDSC in BM, the site most relevant for MM, has not been studied.

The present study focused on defining the function of MDSC in the MM BM microenvironment. We demonstrated that immunosuppressive MDSC accumulate in BM of patients with MM, as well as in BM of MM-bearing immunocompetent mice.

To further understand the contribution of MDSC to regulation of the immune responses generated in BM of MM-bearing mice, we used an approach in which T cell responses to the model Ag were monitored in vitro. Using a mouse model of MM and knockout (KO) mice with defective MDSC accumulation, we showed that MDSC could be directly responsible for the tumor-specific immunosuppression observed in BM in this disease.

Materials and Methods

Isolation of human MDSC and functional assays

BM and PB samples were collected from newly diagnosed, nontreated patients with MM. The collection of samples was approved by the University of South Florida Institutional Review Board. BM and PB samples from healthy donors were purchased from Lonza (Allendale, NJ) and Florida Blood Services (St. Petersburg, FL), respectively. Mononuclear cells were isolated by Ficoll-Paque density-gradient centrifugation. CD11b+CD14+CD33+ populations of MDSC or IMC were isolated by flow sorting using a FACSAria instrument (BD). Human T cells were purified from PB mononuclear cells obtained from healthy donors, using T cell–enrichment columns (R&D Systems). Dendritic cells were generated in vitro from PB mononuclear cells obtained from a different donor. T cells (1 × 10^6) were stimulated with LPS-matured dendritic cells (1.5 × 10^6), with or without MDSC present in the coculture. The number of IFN-γ-producing T cells was evaluated in an ELISPOT assay on an automatic counter (Cellular Technology). Proliferation of T cells was measured by [3H]thymidine incorporation.

Mice and cell lines

C57BL/6 and FVB/N mice were purchased from the National Cancer Institute (Frederick, MD) and were crossed to obtain mice of a mixed FVB/N × C57BL/6 background. F1 progeny (6–8 wk old) were used. Mice were kept in pathogen-free conditions and handled in accordance with the requirements of the Guideline for Animal Experiments. S100A9KO mice on a C57BL/6 background were described previously (20). S100A9KO mice on a FVB/N background were obtained by the backcrossing of C57BL/6 S100A9KO mice to FVB/N mice for 10 generations. F1 progeny of S100A9KO FVB/N × C57BL/6 mice (6–8 wk old) were used for experiments.

MM cells BCM47BM (BCM), 38ATLN (ATLN), and DP42 were kindly provided by Dr. Brian Van Ness (University of Minnesota, Minneapolis, MN) and described previously (21). DP42-OVA cells were established by transfection of DP42 cells with a pAc-Neo-OVA1 vector expressing chimeric OVA protein, followed by selection with 1.4 mg/ml G418 to create stably transfected clones.

MM cells were cultured in RPMI 1640 medium (BioSource International), supplemented with 10% FBS, 5 mM glutamine, 50 μM 2-ME, 1% antibiotics (all from Invitrogen), and 0.5 ng/ml recombinant mouse IL-6 (R&D Systems). MM tumors were established by i.v. inoculation of syngeneic MM cells into mice tail veins (10^7 ATLN, 10^3 BCM, 3 × 10^3 DP42, and 10^3 DP42-OVA cells). In survival studies, mice were sacrificed when determined to be moribund according to Institutional Animal Care and Use Committee criteria.

For CD8+ T cell–depletion experiments, mice were treated i.p. with 200 μg CD8 mAb (clone 53-6.72; Bio X Cell, West Lebanon, NH) or control IgG2b (clone LTF-2; Bio X Cell) every 4 d, beginning 4 d after tumor cell injection. The mice received a total of six injections of CD8 mAb or control IgG.

Mouse cell isolation and functional assays

BM cells were obtained by flushing mice femurs and tibias with ice cold serum-free RPMI 1640 medium. Single-cell suspensions were prepared from spleens. RBC were lysed using ACK buffer. Gr1+CD11b+ mouse MDSC or IMC were isolated by FACS on a FACSAnia instrument (BD). The purity of the cell populations was 99%. Splenocytes from Pmel-1–transgenic mice were used as responder cells. The number of IFN-γ-producing cells, in response to stimulation with 0.1 μg/ml specific (EGSQRNQDWL) or control (SINVFEKL) peptides, was determined in an ELISPOT assay, performed as described earlier (16). The number of spots was counted in triplicate and calculated using an automatic ELISPOT counter (Cellular Technology). In parallel, T cell proliferation was evaluated using [3H]thymidine incorporation, as previously described (16).

Adaptive transfer of MDSC

Gr1+CD11b+ MDSC were isolated from BM of DP42-bearing mice by flow sorting 10 d after tumor cell inoculation. MDSC (5 × 10^6) were injected i.v. into the tail vein of DP42-OVA–bearing mice every 4 d, beginning on day 4 after DP42-OVA tumor cell injection. The mice received a total of four injections of MDSC.

Flow cytometry

Cells were labeled with specified Abs (all from BD Biosciences) for 30 min in the dark. Cells were washed twice with PBS, resuspended in PBS containing DAPI (Invitrogen) to exclude dead cells, and analyzed by flow cytometry. K+–SINFEKL pentamers (ProImmune) were used for detection of mouse Ag–specific T cells. At least 10,000 DAPI-negative events were acquired using a LSR II flow cytometer (BD). Data were analyzed using FlowJo software (TreeStar).

MTT assay

Cells were cultured in 96-well plates, with or without S100 proteins, for 48 h. MTT dye was added for the last 4 h of incubation. Insoluble formazan complexes were solubilized with DMSO, and absorbance was measured at 540 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA).

Histochemistry

Femur bones were collected from MM-bearing and tumor-free mice, fixed, decalcified, and embedded in paraffin. Slides were prepared, and standard H&E staining was performed.

Statistics

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software). Differences between groups were calculated using a two-tailed unpaired Student t test. A statistically significant difference was determined at p < 0.05. A log-rank test was used to evaluate the statistical significance in mouse survival experiments.

Results

MDSC in BM and PB of MM patients

The presence of different populations of MDSC was evaluated in BM and PB of MM patients: MDSC, CD11b+CD14+CD33+; PMN-MMDC, CD11b+CD14+CD33+CD15+; and M-MDSC, CD11b+CD14+CD33+CD15− or CD11b+CD14+HLA-DR+flow. Cells with the same phenotypes in control donors were called IMC. Because MM cells constitute a significant proportion of MM BM, the frequency of MDSC was evaluated as the proportion of these cells among the CD138− nonmyeloma cells of BM. Our data demonstrated a significant (p < 0.05) accumulation of MM BM in MM patients compared with healthy donors (Fig. 1A). MDSC represented 41.1 ± 5.3% (range, 13.3–75.9%) of BM cells in MM, whereas IMC with the same phenotype represented only 22.9 ± 2.8% (range, 7.7–33.3%). A significant accumulation of MDSC was also observed in PB of MM patients (Fig. 1A). PMN-MMDC represented the majority of MDSC in BM and was significantly increased in BM and PB of MM patients compared with healthy donors (Fig. 1B). In contrast, no differences in the proportions of CD11b+CD14−CD33−CD15− M-MMDC were found (Fig. 1C).

This was consistent with the lack of differences in the presence of CD11b+CD14−CD33−CD15− M-MMDC (Fig. 1C). Similarly, no differences were observed in the presence of CD11b+CD14+HLA-DR−flow monocytes (Fig. 1E). No correlation was found between the
proportion of MDSC and the extent of the disease evaluated by the proportion of MM cells in BM aspirates, core biopsies, and the concentration of H and L chain Ig in serum (data not shown).

To verify whether the population of cells with the phenotype of MDSC that accumulates in BM of MM patients could be functionally defined as MDSC, we determined their immunosuppressive activity. MDSC were sorted from BM of MM patients, and their ability to inhibit the T cell response was tested in allogeneic MLR by IFN-γ ELISPOT assay and [3H]thymidine incorporation (24). IMC isolated from BM of healthy donors were used as control. A significant inhibition of T cell activity by MDSC was observed (Fig. 2A, 2C), whereas IMC lacked suppressive activity.

FIGURE 1. Accumulation of MDSC in patients with MM. (A–E) BM and PB samples were obtained from patients with MM (n = 15 and n = 11, respectively) or healthy donors (n = 6 and n = 10, respectively). Mononuclear cells were labeled with specified Abs and analyzed by flow cytometry. Proportion of CD11b+CD14+CD33+ cells (A), CD11b+CD14+CD33+CD15+ PMN-MDSC (B), CD11b+CD14+CD33+CD15+ M-MDSC (C), CD11b+CD14+HLA-DR−/lo M-MDSC (D), and CD11b+CD14+HLA-DR− monocytes (E) among CD138− nonmyeloma cells. *p < 0.05. N.S., not significant.

FIGURE 2. Suppressive activity of MDSC isolated from patients with MM. CD11b+CD14+CD33+ (A–D) or CD11b+CD14+HLA-DR−/lo (E, F) cells were isolated by sorting of BM from MM patients (A, C, E) or healthy donors (B, D, F). Sorted cells were added at a 1:1 ratio to purified T cells stimulated by allogeneic dendritic cells. (A and B) The secretion of IFN-γ was measured by ELISPOT after 48 h of incubation. (C–F) T cell proliferation was measured by [3H]thymidine incorporation. Each condition was set up in triplicate. *p < 0.05, ***p < 0.005, samples with versus without MDSC.
These data indicate that MDSC with immunosuppressive activity accumulated in BM of MM patients. Although the proportion of myeloid cells with the phenotype CD11b⁺CD14⁺ HLA-DR²/low did not increase in BM of MM patients, these cells were able to suppress T cell responses, whereas cells with a similar phenotype isolated from BM of healthy donors lacked this ability (Fig. 2E, 2F).

**Syngeneic murine model of MM and MDSC accumulation**

To better understand the role of MDSC in MM pathogenesis, we used syngeneic murine models of MM originally developed and characterized previously (21, 25). In these models, MM cells, injected i.v. into syngeneic mice, home to BM, and growth of the MM tumor closely resembles human disease (25). Because MM develops in BM of immunocompetent mice, these models allow investigation of MDSC in vivo in the BM milieu.

Three MM models were studied: BCM, DP42, and ATLN. Intravenous administration of these cells resulted in the development of MM in BM within 1–2 wk (Fig. 3A). These three tumor models demonstrated different kinetics of tumor growth. BCM was the fastest growing tumor, with a median survival of 18 d. The DP42 tumor grew more slowly, with a median survival of 21 d (range, 20–24 d). All mice injected with BCM or DP42 cells developed tumors. ATLN was the slowest growing tumor in mice, with a median survival of 25 d (range, 17–33 d). By day 40 after ATLN inoculation, only ~80% of the mice developed this tumor (Fig. 3B). Mice were euthanized at different time points after tumor cell inoculation, and the proportion of MM cells was evaluated in BM and spleens. Because MM cells are characterized phenotypically by surface expression of syndecan (CD138), we used this marker to distinguish MM (Fig. 3C). In the DP42 and ATLN models, MM cells were detected in BM within a week after tumor cell inoculation and had replaced the majority of hematopoietic cells in 2.5–3 wk (Fig. 3D). In spleens, MM was detected later, 3 wk after tumor inoculation (Fig. 3E). In the BCM model, MM cells accumulated in both BM and spleens within 7 d (Fig. 3D, 3E).

We evaluated the presence and function of the Gr-1⁺CD11b⁺ MDSC population in slower-growing MM models: ATLN and DP42. In the ATLN model, a significant increase in the proportion and absolute number of MDSC in BM was observed as early as 1 wk after tumor cell inoculation (Fig. 4A). From week two, the presence of MDSC in BM decreased gradually; 3 wk after tumor cell inoculation the proportion of MDSC in BM was significantly reduced because MM cells had expanded and replaced all of the hematopoietic cells in BM (Fig. 4A). As in BM, the proportion and absolute number of MDSC in spleens was significantly increased 1 wk after tumor cell inoculation. However, this cell population continued to grow during week two posttumor injection, reflecting the fact that MM cells accumulated in spleen at later time points and to a lesser extent than in BM. Only at the end of week three, when mice became moribund and MM cells expanded, had the presence of MDSC in spleens declined (Fig. 4B). The proportion and absolute number of MDSC in lymph nodes dramatically expanded by week three (data not shown). The kinetic
of populations of PMN-MDSC and M-MDSC in BM was similar to that of total MDSC, with a significant increase 1 wk postinoculation and a gradual decrease during tumor progression (Fig. 4C). Interestingly, M-MDSC were responsible for the increase in total MDSC in spleens 1 wk after tumor cell injection, whereas the number of PMN-MDSC dramatically increased during week two posttumor inoculation (Fig. 4D). The DP42 model showed a similar pattern of MDSC accumulation (Supplemental Fig. 1).

The hallmark of MDSC is the ability of this cell population to suppress immune responses. Although immunosuppressive properties of MDSC isolated from lymphoid organs or blood of mice with solid tumors have been demonstrated in many studies, the function of MDSC in BM has not been studied as much. To address this topic, we evaluated the ability of BM MDSC to inhibit Ag-specific T cell responses. Gr-1 +CD11b + MDSC were sorted from BM of ATLN-bearing mice 2 wk after the injection of tumor cells, and Gr-1 +CD11b + IMC were sorted from BM of tumor-free mice. MDSC and IMC were added to Pmel-1–transgenic splenocytes (responders) in the presence of control or specific peptides. Each condition was set up in triplicates. (E) MDSC or IMC were mixed with splenocytes in a 1:1 ratio. INF-γ production by T cells was measured in ELISPOT assay. Shown are the numbers of spots calculated by subtracting background values (cells stimulated with control peptides) from specific values (cells stimulated with specific peptide). Three mice per group were used. (F) Proliferation assay. Results are mean values obtained for three mice with each condition set up in triplicates. Proliferation was measured by [3H]thymidine incorporation. **p < 0.001, ***p = 0.000073, versus IMC.

FIGURE 4. MDSC in MM-bearing mice. MM tumors were established by i.v. tail vein injection of 10^5 ATLN cells into syngeneic mice. Mice were euthanized at indicated time points after tumor cell inoculation (n = 3–4 mice/time point). Tumor-free mice were used as a control (naive). Proportion (left panel) and absolute number (right panel) of Gr1 +CD11b + cells in BM (A) and spleen (B). Absolute number of CD11b +Ly6G^-Ly6C^- PMN-MDSC (left panel) and CD11b +Ly6G^hiLy6C^- M-MDSC (right panel) in BM (C) and spleen (D). (E and F) Gr1 +CD11b + cells isolated from BM of ATLN-bearing or naive mice were cultured with splenocytes from Pmel-1–transgenic mice in the presence of control or specific peptides. Each condition was set up in triplicates. (E) MDSC or IMC were mixed with splenocytes in a 1:1 ratio. INF-γ production by T cells was measured in ELISPOT assay. Shown are the numbers of spots calculated by subtracting background values (cells stimulated with control peptides) from specific values (cells stimulated with specific peptide). Three mice per group were used. (F) Proliferation assay. Results are mean values obtained for three mice with each condition set up in triplicates. Proliferation was measured by [3H]thymidine incorporation. **p < 0.001, ***p = 0.000073, versus IMC.

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MDSC promote MM growth in vivo

We used S100A9KO mice to address a possible role for MDSC in MM (26). Under physiological conditions, these mice demonstrated normal myeloid cell differentiation. However, when challenged with tumor cells, S100A9KO mice showed a reduced accumulation of MDSC compared with their wild-type (WT) counterparts (27). S100A9 was directly implicated in promoting MDSC accumulation (28).

Consistent with previously reported data, no differences in the presence of IMC in the spleens (Fig. 5A) and lymph nodes (data not shown) were found between WT and S100A9KO tumor-free mice. However, the presence of IMC was significantly reduced in BM of S100A9KO mice (Fig. 5A). To evaluate the effect of S100A9 deficiency on MM growth and mice survival from MM, BCM and DP42 tumors were established in WT or S100A9KO mice. No difference in BM tumor burden was observed between WT and S100A9KO mice during MM progression (Fig. 5B). Although S100A9KO BCM-bearing mice demonstrated a slight survival advantage compared with WT mice, the difference was not statistically significant (Fig. 5C). In DP42-bearing mice, no evidence of improved survival was observed (Fig. 5D). These data raised the question about what role, if any, MDSC plays in MM progression in BM. One of the major effects of MDSC is the inhibition of Ag-specific immune responses. Rapid growth of MM in BCM and

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DP42 models, 100% tumor take, and the lack of spontaneous rejection suggested that these tumor cells are likely to be poorly immunogenic. To investigate the effect of MDSC in the model of a more immunogenic tumor, we generated a DP42 cell line with a stable overexpression of OVA (DP42-OVA) (Supplemental Fig. 2A). DP42-OVA cells had similar kinetic of growth in vitro compared with parental DP42 cells (Supplemental Fig. 2B). However, S100A9KO mice, inoculated with DP42-OVA cells, demonstrated significantly improved survival ($p = 0.009$) (Fig. 6A) and delayed tumor growth in BM (Fig. 6B) compared with WT mice.

We compared the presence of Gr-1$^+$CD11b$^+$ MDSC in BM of WT and S100A9 mice 1 wk after MM inoculation. At this point, the number of tumor cells in BM (Fig. 6B) and spleen (Fig. 6C) of both groups of mice was the same. MDSC in BM of DP42-OVA–bearing WT mice was increased compared with tumor-free mice (Fig. 6D versus Fig. 5A). The presence of MDSC in BM of DP42-OVA–bearing S100A9KO mice was significantly lower ($p = 0.04$, Fig. 6D). MDSC isolated from BM of DP42-OVA–bearing WT mice 1 wk after tumor inoculation suppressed the Ag-specific T cell response, whereas MDSC from S100A9KO MM-bearing mice lacked this ability (Fig. 6E).

We asked whether S100A9 protein in the form of a homodimer or heterodimer with S100A8 protein could directly affect MM growth or the immunosuppressive activity of MDSC. Recombinant

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**FIGURE 5.** MM tumor growth in S100A9KO mice. (A) BM and spleen were obtained from tumor-free S100A9KO or WT mice. Proportions of Gr$^+$CD11b$^+$ IMC were determined by flow cytometry, and absolute numbers of IMC were calculated. (B) DP42-bearing S100A9KO or WT mice were euthanized at the indicated time points ($n = 3$–4 mice/time point), and tumor burden in BM was determined by measuring the proportion of CD138$^+$SSC$^{hi}$ MM cells. Survival of tumor-bearing S100A9KO and WT BCM ($n = 12$/group) (C) and DP42 ($n = 9$/group) (D) mice.

**FIGURE 6.** Growth of immunogenic MM tumors in S100A9KO mice. (A–D) DP42-OVA MM tumors were established in S100A9KO or WT mice. (A) Survival of S100A9KO ($n = 6$) and WT ($n = 8$) mice was evaluated. Log-rank analysis was used to determine the statistical significance between groups ($p = 0.009$). S100A9KO or WT DP42-OVA–bearing mice were euthanized at the indicated times ($n = 3$ mice/time point), and tumor burden in BM (B) and spleen (C) was determined by measuring the proportion of CD138$^+$SSC$^{hi}$ MM cells. (D) Absolute number of Gr$^+$CD11b$^+$ cells was determined in DP42-OVA S100A9KO and WT mice 1 wk after MM cell inoculation. Three mice/group were analyzed. (E) Sorted BM Gr$^+$CD11b$^+$ MDSC from DP42-OVA–bearing S100A9KO or WT mice 1 wk after tumor cell inoculation were mixed with Pmel-1 splenocytes at the indicated ratio in the presence of specific or control peptides. Proliferation was measured using $[^3H]$thymidine incorporation. Statistically significant difference between no MDSC and WT MDSC groups is shown. ***$p < 0.001$. 

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S100A9, S100A8/A9, and S100A8 protein did not increase the survival and proliferation of MM cells (Supplemental Fig. 3). No S100A9-mediated conversion of IMC from naive tumor-free mice into immunosuppressive MDSC was observed (data not shown). S100 proteins also did not have a direct effect on T cell function (Supplemental Fig. 4). These results suggest that the loss of immunosuppressive activity in S100A9KO mice was likely the result of a decreased proportion of immunosuppressive MDSC among BM Gr1\(^-\)CD11b\(^+\) cells.

To investigate the involvement of the immune mechanisms in decreased tumor growth of MM in S100A9KO mice, we treated tumor-bearing mice with CD8 Ab. Administration of this Ab completely abrogated the improved survival of S100A9KO mice inoculated with DP42-OVA MM cells (Fig. 7A).

To investigate whether the decreased presence of MDSC in MM-bearing S100A9KO mice would result in an improved tumor-specific immune response, we measured the presence of OVA-specific CD8\(^+\) T cells in DP42-OVA-bearing mice using SIINFEKL-H2-K\(^b\) pentamers (Fig. 7B). After 1 wk, S100A9KO mice had more pentamer-positive CD8\(^+\) T cells in BM; however, the difference was not statistically significant. However, by week three, the number of pentamer-positive CD8\(^+\) T cells was significantly higher in BM of DP42-OVA-bearing S100A9KO mice than in WT mice (Fig. 7C). A similar kinetic was observed in spleens of these mice (Fig. 7D). However, the proportion of pentamer-positive CD8\(^+\) T cells in spleens was >10-fold lower than in BM at all time points (Fig. 7C, 7D), indicating that BM was the primary site of accumulation of Ag-specific CD8\(^+\) T cells in S100A9KO MM-bearing mice. We also evaluated the presence of IFN-\(\gamma\) (Th1-type) CD4\(^+\) T cells in MM-bearing mice. A slight increase in the proportion of these cells was observed in BM of S100A9KO mice 1 wk after tumor inoculation. A week later the differences became significant (Fig. 7E). There was no increase in the proportion of IFN-\(\gamma\) cells in spleen (Fig. 7F).

To confirm the contribution of MDSC in MM tumor growth, we adoptively transferred Gr1\(^-\)CD11b\(^+\) cells isolated from BM of WT DP42-bearing mice into recipient S100A9KO DP42-OVA-bearing mice. Transfer of WT MDSC into MM-bearing S100A9KO mice resulted in the significantly decreased survival of these mice (Fig. 7G).

**Discussion**

In this article, we report the direct role of MDSC in the regulation of BM antitumor immune responses and tumor progression in MM. MDSC are known to inhibit immune responses in solid tumors (29–31). Although the presence of MDSC in PB of MM

![Figure 7](http://www.jimmunol.org/)
patients has been demonstrated (19, 32), the involvement of these cells in the pathogenesis of hematological malignancies, and specifically MM, was not clear. We found that one of the MDSC populations, CD11b<sup>+</sup>CD14<sup>+</sup>CD33<sup>+</sup> cells, significantly increased in BM of patients with MM. These cells demonstrated T-cell suppressive activity, whereas their control counterpart in healthy donors did not. Our data are consistent with a recent report showing the accumulation of MDSC in BM of patients with leukemia and lymphoma (23). In that study, most suppressive MDSC had a Lin<sup>−</sup>CD11b<sup>lo</sup>-<sup>−</sup> phenotype. Several populations of MDSC in humans were described (33). Although they share markers of myeloid cell lineage, they may differ in the extent to which some of the markers are expressed. It has now become evident that the specific phenotype of MDSC depends on the type of tumor. These populations most likely include cells with similar biology and function (22, 24). Our study revealed that, in MM patients, the major changes were observed in the population of PMN-MDSC. PMN-MDSC are the most abundant population of MDSC in PB of patients with many solid tumors (33). However, one study reported that M-MDSC accumulate more frequently in PB of patients with acute myelogenous leukemia (34). Brimnes et al. (35) suggested that CD14<sup>+</sup>HLA-DR<sup>−</sup>IMC-M-DSC was increased in PB of MM patients compared with healthy donors. However, the variation between patients samples was high, and the significance was not clear. Our data demonstrated no difference in the presence of CD14<sup>+</sup>HLA-DR<sup>−</sup>IMC in BM and PB of MM patients compared with healthy donors. However, the immunosuppressive activity of these cells was similar to that observed in the CD11b<sup>−</sup>CD14<sup>+</sup>CD33<sup>+</sup> population. More studies are needed to identify the true nature of MDSC in MM patients.

In this study, we attempted to understand the possible role of MDSC in the regulation of antitumor immune responses in MM. This question could not be addressed by using only patients samples; therefore, we used MM models that were established relatively recently in double-transgenic c-myc/Bcl-xL mice (16, 17). These mice spontaneously develop MM tumors, and several MM cell lines have been derived from those tumors. After i.v. injection in syngeneic mice, tumor cells home to BM and form MM. In these mice, the accumulation of immunosuppressive MDSC in BM was seen as early as 1 wk after tumor inoculation. During disease progression, MM cells expanded dramatically and replaced other hematopoietic BM cells, including MDSC, which may reflect the situation in patients with advanced disease. Such rapid accumulation of MDSC in BM is different from our observations in solid tumor mouse models, in which accumulation of MDSC in BM takes place much later during tumor progression (data not shown). These observations may reflect the fact that BM is a primary tumor site in MM and, therefore, the expansion of myeloid cells and their conversion into MDSC are directly affected by growing tumor.

In this study, we did not address the specific mechanism responsible for MDSC-mediated immunosuppression, because it has been studied extensively in many tumor models (10, 12, 32). The question that we addressed was whether a rapid, but transient, accumulation of MDSC during the initial phase of MM development was important for disease progression. We used S100A9KO mice, which have a defective ability to mount an MDSC response to tumors (27). These mice had a decreased presence of Gr-1<sup>+</sup>CD11b<sup>+</sup>IMC in BM and did not respond to injections of MM cells with an accumulation of MDSC in BM. However, this did not translate into improved survival. We hypothesized that it could be the result of an inability of the experimental MM cell line to generate a spontaneous immune response due to poor immunogenicity. To address this question, we overexpressed a model Ag (OVA) in a MM cell line. Growth of OVA-expressing MM tumors was significantly delayed in S100A9KO mice compared with WT mice. Adoptive transfer of MDSC isolated from BM of WT MM-bearing mice into S100A9KO MM-bearing mice resulted in significantly reduced survival of these mice, confirming the critical role played by MDSC in MM progression.

Our data indicated that, in the absence of MDSC, spontaneous expansion of Ag-specific T cells could be detected in BM as early as 1 wk after tumor inoculation, and it increased further during the following 2 wk. In contrast, the presence of these cells in spleen was barely detectable and was >10-fold lower than in BM. The delay in tumor progression was dependent on the presence of CD8<sup>+</sup> T cells, because administration of CD8 Ab completely abrogated the improved survival of MM-bearing S100A9KO mice. Thus, our data revealed that MDSC, present in BM tumors in MM, can block the activity of antitumor cytotoxic CD8<sup>+</sup> T cells in an Ag-specific manner, as well as decrease the presence of Th1 CD4<sup>+</sup> T cells. Taken together, these data provide insight into the mechanism of immune defects in MM. Our results support the notion that immunotherapeutic strategies aimed at improving Ag-specific T cell responses in MM need to be accompanied by depletion of MDSC.

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Disclosures
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References


