Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein

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Accumulation of myeloid-derived suppressor cells (MDSCs) associated with inhibition of dendritic cell (DC) differentiation is one of the major immunological abnormalities in cancer and leads to suppression of antitumor immune responses. The molecular mechanism of this phenomenon remains unclear. We report here that STAT3-inducible up-regulation of the myeloid-related protein S100A9 enhances MDSC production in cancer. Mice lacking this protein mounted potent antitumor immune responses and rejected implanted tumors. This effect was reversed by administration of wild-type MDSCs from tumor-bearing mice to S100A9-null mice. Overexpression of S100A9 in cultured embryonic stem cells or transgenic mice inhibited the differentiation of DCs and macrophages and induced accumulation of MDSCs. This study demonstrates that tumor-induced up-regulation of S100A9 protein is critically important for accumulation of MDSCs and reveals a novel molecular mechanism of immunological abnormalities in cancer.

Defective myeloid cell differentiation is one of the major factors underlying the immune nonresponsiveness of both solid tumors and hematological malignancies (1). Consequences of defective myeloid cell differentiation include decreased production of mature, functionally competent DCs and accumulation of myeloid-derived suppressor cells (MDSCs) (2–4). MDSCs are a mixed group of myeloid cells including immature granulocytes, macrophages, DCs, and myeloid progenitors. In mice, MDSCs with the phenotype of Gr-1+CD11b+ cells were detected in practically all tested tumor models. Significant accumulation of these cells was found in patients with various types of cancer (5–9). Numerous studies have demonstrated a critical role of MDSCs in the suppression of T cell responses and induction of T cell tolerance in cancer (10–13). MDSCs may account in large part for the limited effectiveness of cancer vaccines and other therapies such as anti–vascular endothelial growth factor (VEGF) treatment (8, 14, 15). Recent studies have also demonstrated an important function of these cells in infection, inflammation, and prevention of graft rejection (16–20).

Despite the wealth of information regarding the functional importance of MDSCs, the mechanism responsible for their accumulation remains unknown. Various tumor-derived factors (VEGF, IL-6, IL-10, M-CSF, and GM-CSF) can inhibit DC differentiation from hematopoietic progenitor cells (HPCs) in vitro and in vivo (1). In search of specific genes responsible for abnormal myeloid cell differentiation in cancer, we evaluated the effect of VEGF on HPC gene expression by differential display analysis and consistently found up-regulation of the mouse S100A9 gene. S100A9, also referred to as myeloid-related protein 14 (MRP14) or...
calgranulin B, is a member of the large family of S100 proteins. It is expressed together with its dimerization partner S100A8 (MRP8 or calgranulin A) in circulating neutrophils and monocytes, but not in resting tissue macrophages (21). S100A9 protein contains two helix-loop-helix motifs (EF-hand), which have high affinity for Ca$^{2+}$ (22). Upon elevation of intracellular calcium, S100A8 and S100A9 translocate from the cytosol to the cytoskeleton and the plasma membrane of myeloid cells (23) and are released as a heterodimer to recruit leukocytes to sites of inflammation or tumors (24). Until now, their role in myeloid cell differentiation remained unknown. We report here that up-regulation of S100A9 in myeloid precursors in cancer inhibits DC and macrophage differentiation and induces accumulation of MDSCs. This may represent a universal molecular mechanism of tumor-induced abnormalities in myeloid cells in cancer, directly linking inflammation and immune suppression.

RESULTS

S100A9 expression is associated with accumulation of MDSCs in cancer

S100A9 and S100A8 mRNAs and proteins were readily detectable in enriched bone marrow HPCs, but gradually decreased during culture with GM-CSF and IL-4 and were almost undetectable by day 7, when >70% of cells in culture were DCs (Fig. 1 A and Fig. S1, A and B, available at http://www.jem.org/cgi/content/full/jem.20080132/DC1). Thus, under physiological conditions, DC differentiation was associated with marked down-regulation of S100A8 and S100A9. To evaluate the effect of tumor-derived factors on S100A9 expression, HPCs were cultured for 7 d with GM-CSF and IL-4 in CT-26 tumor cell-conditioned medium (TCM) or 3T3 fibroblast-conditioned medium (FCM). TCM prevented the down-regulation of S100A9 mRNA expression during differentiation in vitro (Fig. 1 A). Previous studies have shown that in contrast to FCM, TCM inhibits the differentiation of DCs from HPCs and induces the accumulation of Gr-1$^+$CD11b$^+$ MDSCs (25–27). To verify the suppressive activity of these MDSCs, HPCs were cultured for 5 d with GM-CSF, IL-4, and TCM. Consistent with previous results, TCM substantially decreased the proportion of CD11c$^+$ DCs and increased the proportion of Gr-1$^+$CD11b$^+$ MDSCs (Fig. S2). Gr-1$^+$CD11b$^+$ cells were then sorted and added to splenocytes from C57BL/6 mice stimulated with anti-CD3/CD28 antibody or to OT-1 splenocytes (CD8$^+$ T cells from these mice have a TCR that recognizes the OVA-derived peptide SIINFEKL) stimulated with control or specific peptide. Gr-1$^+$CD11b$^+$ cells generated in TCM significantly suppressed T cell responses (Fig. 1 B).

We asked whether the observed up-regulation of S100A8 and S100A9 by TCM was associated with MDSC accumulation. Gr-1$^+$ cells isolated after a 5-d culture of HPCs with GM-CSF and IL-4 in TCM expressed substantially more S100A8 and S100A9 proteins than did Gr-1$^-$ cells (Fig. 1 C). No differences in the levels of S100A8 and S100A9 were found between Gr-1$^+$ cells isolated from populations cultured with FCM versus TCM.

The correlation between MDSC accumulation and S100A8/A9 levels was investigated in CT26 colon carcinoma-bearing mice. Consistent with previous observations (28, 29), the proportion of MDSCs in the spleens of these
mice increased gradually during tumor growth, and by 3 wk after inoculation was more than fivefold higher than in the spleens of control mice (unpublished data). Amounts of S100A8 and S100A9 increased in spleens of tumor-bearing mice in parallel with MDSC accumulation (Fig. 1 D). Similar to the in vitro experiments, S100A8/A9 proteins were detected in the Gr-1+, but not the Gr-1−, population, and no differences in protein levels were noted between Gr-1+ cells from naive and tumor-bearing mice (Fig. 1 E). Thus, the increased expression of S100A8 and S100A9 in spleens of tumor-bearing mice and the TCM-inducible up-regulation of S100A8 and S100A9 in vitro are associated exclusively with the accumulation of MDSCs.

S100A9 is essential for MDSC accumulation in cancer
To determine whether MDSC accumulation requires S100A9, we examined S100A9-deficient (S100A9KO) mice (32). No differences were found in the proportions of the populations of myeloid cells in spleens (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20080132/DC1), blood, and bone marrow (not depicted) between wild-type and S100A9KO mice. Likewise, no significant differences in the presence of the myeloid populations were observed after in vitro differentiation of HPCs (Fig. S3 B).

To evaluate differentiation of myeloid cells in the presence of tumor-derived factors, enriched HPCs isolated from S100A9KO mice and their wild-type littermates were cultured with GM-CSF for 5 d in TCM. Consistent with previous observations (for review see [1]), tumor-derived factors significantly reduced the differentiation of DCs and macrophages and substantially increased the production of Gr-1+CD11b+ MDSCs in wild-type populations. In contrast, TCM did not appreciably inhibit the differentiation of myeloid cells from S100A9KO mice (Fig. 2 A). Thus, loss of...
S100A9 prevents the defective differentiation of TCM-treated myeloid cells.

To evaluate the effect of S100A9 deficiency on tumor growth, control and S100A9KO mice were injected s.c. with $5 \times 10^5$ EL-4 lymphoma cells. By days 8–10 after inoculation, tumor growth was evident in all mice (Fig. 2 B). Tumors continued to grow aggressively in all 15 wild-type mice. In sharp contrast, tumors were rejected in 9 out of 12 S100A9KO mice ($P = 0.0002$). Mean tumor size in the remaining three S100A9KO mice was significantly smaller than in the wild-type mice (238.7 ± 51.8 vs. 521.5 ± 199.3, $P = 0.008$). Similar results were obtained in the C3 sarcoma tumor model (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20080132/DC1).

To investigate whether immunological mechanisms were involved in tumor rejection, S100A9KO mice were injected i.p. with 200 μg of anti-CD8 antibody 3 d and 1 d before tumor injection. This treatment reduced the level of CD8+ T cells in peripheral blood by more than fivefold (unpublished data). None of the S100A9KO mice treated with anti-CD8 antibody rejected their tumors. Tumor growth in these mice was similar to that in S100A9 wild-type mice (Fig. 2 B).

Splenocytes were obtained from S100A9KO and wild-type littersmates 12–13 d after injection of EL-4 cells and restimulated for 6 d with irradiated EL-4 tumor cells. T cells were collected and used as effectors in cytotoxicity assays against either EL-4 cells, unrelated B16 melanoma cells, or peritoneal macrophages obtained from naive C57BL/6 mice. T cells from S100A9KO mice showed substantially higher cytotoxicity against EL-4 cells than did T cells from wild-type mice. However, no differences were found in cytotoxicity against macrophages or B16 cells (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20080132/DC1). Tumors removed from wild-type mice 12 d after tumor injection had clearly visible infiltration of S100A9+ cells and Gr-1+ cells, whereas these cells were absent from tumors from S100A9KO mice (Fig. S6, A and B). Tumors from S100A9 KO mice had visible infiltration of CD8+ and CD4+ T cells, which was absent in tumors from S100A9 wild-type mice (Fig. S6, C and D). Tumor from S100A9 wild-type mice had viable cells and infiltrated adjacent adipose tissue. In contrast, tumors from S100A9KO mice had extensive necrosis and apoptosis at the periphery (Fig. S6 E). By day 15 after tumor cell injection, tumor-bearing wild-type mice expressed substantially more Gr-1+CD11b+ MDSCs than did tumor-free mice (11% in Fig. 2 C vs. 4% in Fig. 2 A). In contrast, the proportion of these cells in tumor-bearing S100A9KO mice was the same as in tumor-free S100A9KO mice and significantly ($P < 0.05$) lower than in tumor-bearing wild-type mice (Fig. 2 C). Thus, MDSCs do not accumulate in tumors in the absence of S100A9.

To address the need for S100A9 for MDSC generation in a tumor-independent model, we injected mice with CFA to mimic the conditions of infection and inflammation, which are known to stimulate production of MDSCs. The level of MDSCs was monitored in peripheral blood by flow cytometry. CFA induced a more than fivefold increase in the proportion of circulating MDSCs in wild-type mice, which peaked on days 6–9 after injection and returned to control level by day 13. In contrast, the number of circulating MDSCs did not increase in S100A9KO mice at any time point (Fig. 2 D). A similar pattern was observed in spleens on day 13 after CFA injection (Fig. 2 E). Thus, the absence of S100A9 protein prevents accumulation of MDSCs in response to CFA, as well as to tumor challenge.

To address the potential contribution of MDSC accumulation to tumor rejection, we performed adoptive transfers of MDSCs. S100A9KO mice were injected with EL-4 lymphoma cells, and then split into two groups of five mice each. One group was untreated and the other group was injected with MDSCs isolated from EL-4 tumor-bearing wild-type mice. 4 out of 5 untreated S100A9KO mice rejected their tumors by day 17, whereas all 5 S100A9KO mice injected with MDSCs developed rapidly growing tumors (Fig. 2 F). Collectively, these results suggest that the absence of MDSC accumulation in S100A9 KO mice is responsible for their ability to reject tumors.

**S100A9 overexpression impairs differentiation of DCs and induces accumulation of MDSCs**

We asked whether overexpression of S100A9 and A8 could directly regulate the differentiation of DCs and other myeloid cells. To address this question, we generated stable clones of R1 embryonic stem (ES) cells that overexpress S100A8, S100A9, or both (S100A8/9). R1 ES cells transfected with empty vector or another member of S100 family, S100A1, served as controls. Cells were subjected to in vitro differentiation of DCs, as previously described (33). The expression of S100A8 and S100A9 was verified by qRT-PCR, as well as by Western blotting (Figs. 3, A and B). Endogenous S100A8 and S100A9 genes were expressed in the control ES cells, but were shut down in embryoid bodies (EBs). The expression of these genes in transfected ES cells was continuously maintained during cell culture (Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20080132/DC1).

On day 35 after the start of the cultures, cells generated from EBs were collected to evaluate their phenotype by flow cytometry. The total numbers of these cells were similar in all groups (unpublished data). More than 80% of cells generated from control- or S100A1-transfected ES cells had a phenotype of mature DCs (CD11c+IAb+ or CD11c+B7-2+; Fig. 3 C). In sharp contrast, ES cells transfected with S100A9 or S100A8/A9 displayed a dramatically reduced ability to generate DCs. Instead of DCs, these ES cells produced predominantly Gr-1+ cells. The effect of S100A8 on DC differentiation was similar, although much less pronounced. S100A8 did not increase the level of Gr-1+CD11b+ cells (Fig. 3 C). Reduced DC generation from S100A8/9-transfected ES cells was verified in a functional test using the allogeneic mixed leukocyte reaction, a hallmark of DC activity. Cells differentiated from S100A8/9 ES cells had a much lower ability to stimulate the proliferation of DCs.
shown high-level expression of targeted cDNA in hematopoietic cells from spleens, lymph nodes, and thymus (34), as well as in HPCs and stem cells in bone marrow (35). To better trace the expression of the transgene transcript, GFP was linked to the S100A9 cDNA behind an IRES sequence to permit independent translation (Fig. 4A). FVB/N mice derived from the zygotes injected with this H2K-S100A9-GFP transgenic construct were genotyped by slot blot hybridization to a GFP probe and phenotyped by FACS analysis for GFP expression in peripheral blood cells. Nine founder mice stably integrated the transgene. Expression of GFP protein in peripheral blood cells was significant in one founder mouse (Fig. 4B, left). Transgene-positive offspring from this founder line exhibited a consistent level of GFP expression during breeding for several generations to wild-type mice. The level of GFP expression was stable during the lifetime of these mice for at least 32 wk (the period of observation). The level of S100A9 protein in spleens obtained from allogeneic T cells than did cells differentiated from control ES cells (Fig. 3 D).

S100A9-, A8-, or 8/9-transfected progeny did not express CD34 or Sca-1 markers. However, more than half of these cells expressed c-kit (Fig. 4 E), suggesting that S100A8/9 might cause accumulation of myeloid progenitor cells. This hypothesis was tested in a colony formation assay. Cells differentiated from control ES cells did not form colonies, whereas a significant number of colonies were formed by cells differentiated from S100A9 or S100A8/9 ES cells (Figs. 3 F). Most of the colonies morphologically resembled granulocyte-macrophage CFU-GMs (Fig. S8, available at http://www.jem.org/cgi/content/full/jem.20080132/DC1).

To investigate the role of S100A9 in myeloid cell differentiation in vivo, we generated transgenic mice (S100A9Tg) that overexpress S100A9 in hematopoietic cells under control of the H2K-promoter/enhancer and Moloney MuLV enhancer/poly(A) site (34). This promoter previously demonstrated high-level expression of targeted cDNA in hematopoietic cells from spleens, lymph nodes, and thymus (34), as well as in HPCs and stem cells in bone marrow (35). To better trace the expression of the transgene transcript, GFP was linked to the H2K promoter/enhancer and Moloney MuLV enhancer/poly(A) site (34). This promoter previously demonstrated high-level expression of targeted cDNA in hematopoietic cells from spleens, lymph nodes, and thymus (34), as well as in HPCs and stem cells in bone marrow (35). To better trace the expression of the transgene transcript, GFP was linked to the H2K promoter/enhancer and Moloney MuLV enhancer/poly(A) site (34). This promoter previously demonstrated high-level expression of targeted cDNA in hematopoietic cells from spleens, lymph nodes, and thymus (34), as well as in HPCs and stem cells in bone marrow (35). To better trace the expression of the transgene transcript, GFP was linked to the H2K promoter/enhancer and Moloney MuLV enhancer/poly(A) site (34). This promoter previously demonstrated high-level expression of targeted cDNA in hematopoietic cells from spleens, lymph nodes, and thymus (34), as well as in HPCs and stem cells in bone marrow (35). To better trace the expression of the transgene transcript, GFP was linked to the H2K promoter/enhancer and Moloney MuLV enhancer/poly(A) site (34). This promoter previously demonstrated high-level expression of targeted cDNA in hematopoietic cells from spleens, lymph nodes, and thymus (34), as well as in HPCs and stem cells in bone marrow (35).
transgenic mice was substantially higher than in wild-type mice (Fig. 4 B, right).

To investigate the effect of S100A9 on the differentiation of myeloid cells, we isolated lineage-negative, c-kit-positive HPCs from bone marrow of control (wild-type) FVB/N mice and S100A9Tg mice. Lin− c-kit+GFP− and Lin− c-kit+GFP+ cells were sorted from S100A9Tg mice (Fig. 4 C). HPCs were cultured for 5 d with a cocktail of cytokines (Flt3, stem cell factor [SCF], IL-11, GM-CSF, and IL-3), followed by a 7-d incubation with GM-CSF; LPS was present in the culture medium for the last 24 h. As shown in Fig. 4 D, the cells generated from wild-type HPCs were CD11c+IAq+ DCs...
To evaluate the possible immunosuppressive activity of IMCs generated in S100A9Tg mice, Gr-1+CD11b+ cells were sorted from wild-type and transgenic tumor-free mice. Two experimental systems were used: peptide-specific CD8+ T cell response and allogenic mixed leukocyte reaction. In the first experimental system, peptide-specific CD8+ T cells were generated by immunizing naive FVB/N mice with DCs loaded with MHC class I (H2-Dq)-restricted rat HER-2/neu-derived peptide PSDLRDLSVF. Splenocytes were isolated and stimulated with control or specific peptide in the presence of IMCs. The cell response of both proteins (Fig. 6 F). Collectively, these data demonstrate that STAT3 up-regulates the expression of S100A9 and S100A9 both in vitro and in vivo.
independent assays were performed. First, splenocytes (10^6/ml) from control and S100A9Tg mice were cultured for 48 h, and extracellular levels of S100A8 and S100A9 proteins were measured by ELISA. The concentrations of the proteins were similar in both groups (150 ng/ml).

S100A9 regulates myeloid cell differentiation via reactive oxygen species (ROS)

It is possible that overexpression of S100A9 in myeloid cells results in increased secretion of the protein, which in turn inhibits HPC differentiation. To investigate this possibility, three
Second, Lin~c-kit~ myeloid progenitors derived from the bone marrow of naive FVB/N mice were placed in the bottom chambers of 24-well Transwell plates. Splenocytes (1.5 × 10^6 cells per ml) from wild-type or S100A9Tg mice were placed in the top chambers (which are separated from the bottom chambers by a semipermeable membrane) and were cultured with a cocktail of cytokines supporting myeloid differentiation. Splenocytes in the top chambers were replaced every 3 d. The progenitor cells differentiated into mature myeloid cells regardless of whether the splenocytes in the top chambers overexpressed S100A9 or not (unpublished data).

Third, Lin~c-kit~ progenitors were cultured in the presence of different concentrations of recombinant S100A8, S100A9, or S100A8/A9 heterodimer. None of these proteins at concentrations of 1 or 5 μg/ml significantly reduced the proportion of mature myeloid cells generated from myeloid progenitors. Collectively, the results suggest that overexpression of S100A9 inhibits myeloid differentiation primarily via intracellular mechanisms.

We and others have previously shown that one of the main characteristics of MDSCs from tumor-bearing mice is high production of ROS (28, 29, 37). Recent studies have implicated S100A9 protein in the regulation of ROS generation by NAPDH oxidase (23, 38–41). We hypothesized that tumor-induced up-regulation of S100A9 in progenitors might inhibit myeloid differentiation by increasing production of ROS. Consistent with this hypothesis, amounts of ROS in the spleen and bone marrow were four- to fivefold greater in Gr-1+GFP+ cells from S100A9Tg mice than in Gr-1+GFP/H11002 cells from wild-type and S100A9 mice (Fig. 7 A).

We determined whether tumor burden affected ROS levels in MDSCs in S100A9KO mice. EL-4 tumors were established in wild-type and S100A9KO mice, and ROS levels were evaluated in Gr-1+CD11b+ MDSCs in spleens at a time when all mice had similar tumor sizes (12 d after tumor inoculation). Consistent with previous reports, MDSCs from wild-type, tumor-bearing mice produced substantially more ROS than did Gr-1+CD11b+ IMCs from naive mice and (Fig. 7 B). ROS levels in IMCs from naive S100A9KO mice were only slightly lower than in IMCs from wild-type mice. However, ROS levels in MDSCs from tumor-bearing S100A9KO mice were substantially lower than in tumor-bearing wild-type mice and were equivalent to ROS levels in naive wild-type mice (Fig. 7 B).

We asked whether ROS affected the differentiation of MDSCs from tumor-bearing mice. EL-4 tumors were established in wild-type and gp91~−−~ mice, which lack a critical component of the NADPH complex and produce very little ROS. MDSCs (90–95% pure) were isolated from spleens 3 wk after tumor inoculation and cultured in the presence of GM-CSF and TCM for 7 d. MDSCs from tumor-bearing wild-type and gp91~−−~ mice expressed S100A9 (Fig. 7 C, inset). However, the lack of ROS in gp91~−−~ mice dramatically enhanced MDSC differentiation. Despite the presence of S100A9, <5% of gp91~−−~ cells retained an immature phenotype (Gr-1+CD11b+) after 7 d in culture, compared with...
This study demonstrates a novel role for the Ca²⁺ binding myeloid-related proteins S100A8 and S100A9 in myeloid cell differentiation. It suggests that up-regulation of these proteins in HPCs is primarily responsible for defective myeloid cell differentiation in cancer. S100A8 and S100A9 were previously implicated in trafficking of granulocytes and monocytes, especially during inflammation and metabolism of arachidonic acid (23). Here, we show that up-regulation of these proteins results in inhibition of DC differentiation and accumulation of MDSCs. The STAT3 transcription factor up-regulates the expression of S100A8 and S100A9, which is consistent with the previous observation that different tumor-derived factors mediate their immunosuppressive effects via hyperactivation of Jak2–STAT3 signaling in myeloid cells (27). Two of the cytokines (IL-10 and M-CSF) that were directly implicated in tumor-induced abnormalities of DCs (42, 43) were previously found to up-regulate S100A9 (44, 45). Consistent with these results, a direct connection between ROS and myeloid cell differentiation was observed.

Figure 7. S100A9 affects myeloid cell differentiation via up-regulation of ROS. (A) ROS levels in S100A9Tg mice. Splenocytes and bone marrow cells were collected from 3-wk-old wild-type and S100A9Tg FVB/N mice. Cells were stimulated with PMA, labeled with APC-conjugated anti-Gr-1 antibody, and loaded with the oxidation-sensitive dye hydroethidine. The level of ROS production was evaluated in GFP⁻ or GFP⁺ Gr-1⁺ cells. (left) Fluorescence histograms from one typical experiment. (right) Graphs representing mean fluorescence intensity (MFI) in all performed experiments. Red, Gr-1⁺ cells from wild-type mice; green, GFP⁻ Gr-1⁺ cells from S100A9 Tg mice; blue, GFP⁺ Gr-1⁺ cells from S100A9 Tg mice. Three experiments with the same results were performed. (B) EL-4 tumors were established in wild-type and S100A9KO mice as described in Fig. 3. Splenocytes were collected 12 d after tumor inoculation. Cells were stimulated with PMA and labeled with APC-conjugated anti-Gr-1 antibody and PE-conjugated anti-CD11b antibody. ROS were measured in Gr-1⁺CD11b⁺ cells using DCFDA. Two experiments with similar results were performed. (C) EL-4 tumors were established in wild-type C57BL/6 or gp91phox KO mice. 3 wk later, when tumors reached 1.5 cm in diameter, MDSCs were isolated from spleens using magnetic beads and cultured for 7 d in vitro, as described in Fig. 3 B. Proportions of different cell populations were evaluated. Cumulative results from three experiments are shown. (inset) The level of S100A9 protein in freshly isolated MDSCs. (D) Phenotypes of cells in F1 offspring from crosses between S100A9Tg and gp91phox KO mice or wild-type C57BL/6 mice. Lin⁻c-kit⁺ progenitor cells were sorted and cultured with cocktail of cytokines to generate myeloid cells. Proportions of different cell populations were evaluated by flow cytometry. Mean ± SD of cumulative results of two experiments is shown.

Discussion

This study demonstrates a novel role for the Ca²⁺ binding myeloid-related proteins S100A8 and S100A9 in myeloid cell differentiation. It suggests that up-regulation of these proteins in HPCs is primarily responsible for defective myeloid cell differentiation in cancer. S100A8 and S100A9 were previously implicated in trafficking of granulocytes and monocytes, especially during inflammation and metabolism of arachidonic acid (23). Here, we show that up-regulation of these proteins results in inhibition of DC differentiation and accumulation of MDSCs. The STAT3 transcription factor up-regulates the expression of S100A8 and S100A9, which is consistent with the previous observation that different tumor-derived factors mediate their immunosuppressive effects via hyperactivation of Jak2–STAT3 signaling in myeloid cells (27). Two of the cytokines (IL-10 and M-CSF) that were directly implicated in tumor-induced abnormalities of DCs (42, 43) were previously found to up-regulate S100A9 (44, 45). Consistent with these results, a direct connection between ROS and myeloid cell differentiation was observed.

>30% of wild-type cells. Less than 10% of wild-type cells were F4/80⁺Gr-1⁻ macrophages or CD11c⁺ DCs. In sharp contrast, the majority of cells generated from gp91⁻/⁻ MDSCs were macrophages and DCs (Fig. 7C). These findings suggest that ROS mediate the inhibitory effects of S100A9 on myeloid cell differentiation.

To further explore this premise, F1 mice were generated by crossing S100A9Tg and gp91phox KO mice. As a control, we used the F1 cross of S100A9Tg mice with wild-type C57BL/6 mice. Mice that contained GFP-positive cells and exhibited sub-optimal ROS production in response to PMA stimulation were selected for experiments. Lin⁻c-kit⁺ progenitor cells were sorted and cultured with a cocktail of cytokines to generate myeloid cells. Progenitor cells from gp91phoxKO x S100A9Tg F1 mice produced significantly more CD11c⁺ DCs and significantly less Gr-1⁻CD11b⁺ cells than did progenitors from C57BL/6 x S100A9Tg mice (Fig. 7D). Collectively, these diverse data suggest that S100A9 may suppress myeloid cell differentiation via persistent up-regulation of ROS in progenitor cells.
STAT3 activity and S100A9 expression in breast cancer cells was recently reported (46).

Several lines of evidence presented here support the conclusion that up-regulation of S100A9 causes inhibition of DC and macrophage differentiation and accumulation of MDSCs.

First, S100A9 was crucial for the in vivo increase in the number of MDSCs in response to inoculation of mice with tumor cells or CFA. S100A9 was also critical for the inhibitory effects of tumor-derived factors on myeloid cell differentiation in vitro. Our data showing normal myeloid cell differentiation in naive S100A9KO mice are consistent with previous studies in which abnormalities in myeloid cells of S100A9KO mice were not detected (32, 47). Apparently, under physiological conditions, S100A9 is not essential for myeloid cell differentiation. However, in the pathological response to challenge with tumor-derived factors or infection, S100A9 is required for both hyperproduction of MDSCs and blockade of DC differentiation.

Second, overexpression of S100A9 in ES cells dramatically compromised their ability to differentiate into mature myeloid cells, particularly DCs and macrophages. The model of DC generation from ES cells has now been validated in several studies (33, 48, 49), and it was chosen for our studies because precursors of granulocytes and monocytes normally express high levels of S100A9 (23). Therefore, we hypothesized that if S100A9 is important for myeloid differentiation, it would most likely affect the differentiation of early progenitor cells. Our data, indeed, demonstrate that S100A9 overexpression blocks normal DC differentiation. This is consistent with the observation that mature DCs have very low or undetectable levels of this protein. Our results concur with those of Hashimoto et al., who performed serial analysis of gene expression in human DCs and found that, in contrast to monocytes, DCs did not express transcripts for S100A8 or S100A9 (50). Apparently, to progress toward macrophage and DC differentiation, myeloid cells must down-regulate S100A9 expression. If such down-regulation is prevented, DC and macrophage differentiation is halted. Instead, ES cells overexpressing S100A8/A9 produced Gr-1+ cells and myeloid progenitors able to form myeloid colonies, which is reminiscent of the phenomenon observed in tumor-bearing mice (1). Because S100A8 and S100A9 form heterodimers, our initial hypothesis was that overexpression of both proteins would have a stronger effect on DC differentiation than would S100A9 alone. However, although double-transfected ES cells formed myeloid colonies more readily than did S100A9 single transfectants, they affected myeloid cell differentiation equally. This may suggest that up-regulation of S100A9 homodimers is sufficient to block DC differentiation. In line with these findings was the observation that the effect of S100A8 overexpression on DC differentiation was less pronounced than that of S100A9. In addition, S100A8 failed to induce the accumulation of Gr-1+CD11b+ cells. Although different functions of S100A9 and S100A8 have been described in several experimental systems (51), more studies are needed to clarify the mechanism of this phenomenon.

Third, overexpression of S100A9 in myeloid cells of transgenic mice inhibited differentiation of macrophages and DCs and induced the accumulation of IMCs. Cells overexpressing S100A9 had a potent suppressive effect on T cell activity similar to that seen in tumor-associated MDSCs. Moreover, S100A9Tg mice were more susceptible to challenge with immunogenic tumors than wild-type mice. It is important to point out that this effect may not necessarily be caused entirely by accumulation of MDSCs or loss of DCs. Although our preliminary experiments indicate that T cells from S100A9Tg mice do not express S100A9 and exhibit normal function, we cannot yet exclude a possible contribution of lymphoid cells to immune suppression in this mouse model.

Use of the S100A9 expression vector containing the GFP reporter allowed us to isolate GFP-positive cells with S100A9 overexpression and to compare them to GFP-negative cells from the same mouse as an internal control to rule out contributions of systemic or microenvironmental effects. Our data strongly suggest that S100A9 exerts its effect on myeloid cell differentiation via intracellular mechanisms rather than through extracellular secretion of protein. GFP+ myeloid cells in S100A9Tg mice displayed a normal phenotype despite the fact that they were located in close proximity to GFP+ cells that were dramatically affected. In addition, splenocytes from control and Tg mice secreted similar levels of S100A9, as well as S100A8. Finally, soluble factors from S100A9Tg splenocytes, as well as recombinant proteins, did not affect the differentiation of wild-type myeloid cells.

The molecular mechanism of S100A8/A9 effects on myeloid cell differentiation is currently not clear and requires further investigation. Several potential mechanisms could be explored.

These proteins may affect DC differentiation via their Ca2+ binding activity or by inhibiting casein kinase I and II activity, which may be associated with myeloid cell maturation and function. Up-regulation of S100A9 may sensitize myeloid cells to the effects of tumor-derived factors. For instance, we have shown that overexpression of S100A9 results in up-regulation of c-kit on the surface of myeloid cells. Tumor-derived SCF, the c-kit ligand, has been implicated in the accumulation of MDSCs in cancer (52). However, the fact that S100A8 overexpression induced up-regulation of c-kit, but did not affect production of Gr-1+CD11b+ cells argues against a c-kit–dependent mechanism. Recently, a novel role of S100A9 as an endogenous ligand for Toll-like receptor 4 (TLR4) was revealed (53). We have previously demonstrated that MDSC expansion requires signaling through the downstream target TLR MyD88 (16). It is possible that hyperactivation of TLR signaling may interfere with the normal process of myeloid cell differentiation, and S100A9 may be a major mediator of this effect.

Another possible mechanism by which S100A8/A9 might inhibit myeloid cell differentiation is linked to the ability of these proteins to facilitate ROS production by myeloid cells. S100A8/A9 is actively involved in the regulation of ROS generation by NAPDH oxidase (23). Studies demonstrated...
that S100A8 and S100A9 directly bind to p67phox and p47phox, critical components of the NADPH complex (38, 39). This binding potentiates NADPH oxidase activation in neutrophils. Furthermore, S100A9 inhibits the expression of a substantial number of genes in myeloid cells, including gp91phox (50), suggesting that differences in ROS production in the absence of S100A9 may reflect a general role of this protein during myeloid differentiation. ROS, in turn, are the major factors inhibiting differentiation of DCs in tumor-bearing mice (29). Our data ROS play an important role in S100A9-mediated effects on myeloid cell differentiation.

In recent years, it has become increasingly evident that inflammation plays a critical role in tumor progression (54). This study not only demonstrates a novel molecular mechanism responsible for the suppression of immune responses in cancer via accumulation of MDSCs but also provides a potential direct molecular link between immune suppression, MDSCs, and proinflammatory factors. Together with present and previous observations that lack of S100A9 or STAT3 function promotes tumor rejection via immunological mechanisms (31), these cumulative findings reveal a pathway that negatively regulates the immune response in cancer.

MATERIALS AND METHODS

Reagents. The following antibody-producing hybridomas were purchased from the American Type Culture Collection (ATCC) and used as isotypes: anti-CD4 (L3T4, TIB-207), anti-CD8 (Lyt-2.2, TIB-210), and anti-H-2M II (TIB-120). Anti-IAβ, IAd/IEd, Gr-1, CD4, CD6, CD11b, CD86 (B7-2), CD45, CD11c, CD34, Sca-1, c-KIT, isotype control antibodies, and mouse cell lineage depletion kit were obtained from Biocytex. Mouse and human lineage cell depletion kit from Miltenyi Biotec, anti–mouse S100A8, and anti-S100A9 from Santa Cruz Biotechnology. For immunohistochemical staining, anti-S100A9, anti–goat, anti–rat conjugated with biotin antibodies, and Vectastain ABC kit were purchased from Vector Laboratories. Myeloid Long-Term Culture Medium for Primitive Mouse Hematopoietic Cells (MyeloCult media) and hydrocortisone were purchased from StemCell Technologies. Low-Tox rabbit complement and Lymphotox M were obtained from Cedarlane Laboratories. Recombinant murine GM-CSF, IL-4, TNF-α, IL-3, IL-11, Flt3L, and SCF were obtained from R&D Systems. LPS was obtained from Sigma-Aldrich and Serotec. streptavidin microbeads and mouse lineage cell depletion kit from Miltenyi Biotec, anti–mouse S100A8, and anti-S100A9 from Santa Cruz Biotechnology. For immunohistochemical staining, anti-S100A9, anti–goat, anti–rat conjugated with biotin antibodies, and Vectastain ABC kit were purchased from Vector Laboratories. Myeloid Long-Term Culture Medium for Primitive Mouse Hematopoietic Cells (MyeloCult media) and hydrocortisone were purchased from StemCell Technologies. Low-Tox rabbit complement and Lympholyte M were obtained from Cedarlane Laboratories. Recombinant murine GM-CSF, IL-4, TNF-α, IL-3, IL-11, Flt3L, and SCF were obtained from R&D Systems. LPS was obtained from Sigma-Aldrich. Flow cytometry. For depletion of CD8+ T cells anti-CD8 Lyt-2.2 antibody (ATCC) were generated as ascitis (provided by E. Celis, H. Lee Moffitt Cancer Center, Tampa, FL). Recombinant S100A9 and A8 proteins were previously described (53). AVN mammary carcinoma cell line was provided by K. Knutson (Mayo Clinic, Rochester, MN).

Animals. 6–8-wk-old female BALB/c and C57BL/6 mice were purchased from Harlan. FVB/N mice were purchased from Charles River Laboratories. The gp91phoxKO and OT-1 mice were obtained from The Jackson Laboratory. S100A9-deficient mice were previously described (32) and housed in pathogen-free units of the Division of Comparative Medicine vivarium at H. Lee Moffitt Cancer Center, University of South Florida. These mice were backcrossed for six generations to C57BL/6 mice. Control groups included mice with wild-type genotype (S100A9+/−) from the same generation of backcross.

All animal experiments were approved by the Institutional Animal Care and Use Committee and performed in accordance with U.S. Public Health Service policy and National Research Council guidelines.

Preparation of cells. Spleens were collected from control or tumor-bearing mice. Single-cell suspensions of splenocytes were treated with ACK buffer to remove red blood cells. Bone marrow cells were harvested from the femur and tibia of mice and enriched for HPCs by depletion of lineage-specific cells, as previously described (55). In brief, bone marrow cells were incubated with a mixture of antibodies (TIB-207, TIB-210, TIB-120, anti-TER-119, anti-Gr-1, and anti-B220) for 30 min on ice, washed, and treated with complement for 1 h at 37°C. Dead cells were then removed by centrifugation over a Lympholyte M gradient. The resulting fraction contained <2% of lineage-positive cells as detected by flow cytometry. 500,000 enriched HPCs were placed into each well of 24-well plates in 2-ml of RPMI supplemented with 10% FBS, 20 ng/ml GM-CSF, and 10 ng/ml IL-4. This complete medium was replaced every 3 d, and cells were collected for further analysis at indicated time points. To assess the effects of tumor-derived factors on DC differentiation, HPCs were fed with complete medium supplemented with 25% medium conditioned for 48 h by subconfluent cultures of NIH 3T3 fibroblasts as a control, or the C3, CT26, or EL4 tumor cell lines. For DC activation, 5 ng/ml TNF-α was added to RPMI complete medium for 48 h or 1 μg/ml LPS for 24 h before cell analysis.

CTL assay. Splenocytes from EL4 tumor-bearing mice were cultured for 7 d with irradiated EL4 cells, in 10% FBS RPMI, supplemented with 20 ng/ml IL-15 and 20 ng/ml IL-21. On day 7, cytotoxicity was performed in a standard 6-h 3Cr-release CTL assay. The target cells were EL-4 incubated in duplicates at a serial effector/target ratio.

Real-time qPCR. Total RNA was extracted from cells with Trizol (Invitrogen). Traces of DNA were removed by treatment with DNase I. The cDNA was synthesized from 1 μg of total RNA using random hexamers and Superscript II reverse transcription (Invitrogen) according to the manufacturer’s protocol. PCR was performed with 2.5 μl cDNA, TaqMan Universal PCR Master Mix (Applied Biosystems), and target gene assay mix containing sequence-specific primers for S100A8 or S100A9 and 6-carboxyfluorescein (6-FAM) dye-labeled TaqMan minor groove binder (MGB) probe (Applied Biosystems). Amplification with 185 endogenous control assay mix was used for controls. Data quantitation was performed using the relative standard curve method. Expression levels of the genes were normalized by 18S rRNA.

RT-PCR and Southern blots. Total RNA was extracted from cells with Trizol. Traces of DNA were removed by treatment with DNase I. The cDNA was synthesized from 1 μg of total RNA using random hexamers and Superscript II reverse transcription according to the manufacturer’s protocol. Samples were subjected to initial denaturation at 94°C for 3 min and 24 cycles (for S100A8 and S100A9) or 28 cycles (for hprt) of PCR (94°C for 30 s, 54°C for 30 s, and 72°C for 45 s) with final extension for 7 min at 72°C. The number of cycles was selected after preliminary experiments to avoid saturation of the PCR products. PCR primer pairs used in this study include the following: S100A8 forward for HPC and DC cultures: 5′-GAAATGCGCTTCTACCTGG; reverse, 5′-CTTCGTACTCCTGTTGGCTTCT-3′, or S100A8 forward for ES transfectants, 5′-GGAAATCACCATGCTCCTACAA-3′; reverse, 5′-ATGCCACACCATTCTTTATACCC-3′. S100A9 forward for HPC and DC cultures, 5′-GGAGCCGCAGATACACCAACC-3′; reverse, 5′-GGTTGAGTACAGGCCAGACATCC-3′; or ES transfectants, 5′-ATGCATCACCCATTCTTTATACCC-3′; reverse, 5′-GCAACATCACATACACTCCTC-3′. hprt forward for HPC and DC cultures, 5′-GGAGCCGCAGATACACCAACC-3′; reverse, 5′-GGTTGAGTACAGGCCAGACATCC-3′; or ES transfectants, 5′-ATGCATCACCCATTCTTTATACCC-3′; reverse, 5′-GCCATACATCACATACACTCCTC-3′. hprt forward, 5′-GATTCAACATTCGCTCCTGAGGC-3′; reverse, 5′-GGTTGAGTACAGGCCAGACATCC-3′; or ES transfectants, 5′-ATGCATCACCCATTCTTTATACCC-3′; reverse, 5′-GCCATACATCACATACACTCCTC-3′. The PCR products were separated on 1% agarose gels. The sizes of PCR products were: 279 bp for S100A8 in HPC and DCs and 174 bp for ES transfectants; 378 bp for S100A9 in HPC and DC cultures and 206 bp for ES transfectants; and 164 bp for hprt. PCR products were transferred in an alkaline buffer (0.4 N NaOH, 1 M NaCl) onto Hybond N+ nylon transfer membranes (GE Healthcare), and probed with 32P-labeled oligonucleotide probes: S100A8, 5′-GGAGTTCCTTCTGGGATGGTATA-3′; S100A9, 5′-ACATCATGAGGACCTGGCACACA-3′; hprt, 5′-GTTGGTGGATATGTCCTTGGAC-3′. Western blot analyses were performed as previously described (27).
Chromatin immunoprecipitation assay (ChIP). 3D2 cells were cultured in 10% FBS RPMI 1640 supplemented with IL3. Preparation of chromatin-DNA and ChIP assay were performed with kit (Millipore) per the manufacturer’s instructions, using antibodies against STAT3 (Cell Signaling Technology), normal rabbit IgG (Santa Cruz Biotechnology), and protein A agarose/salmon sperm DNA (Millipore). Sonication was performed using a Branson Sonifier (model 450; VWR Scientific). After reversal of cross-linking, purified DNA was subjected to PCR with the following primers spanning the potential STAT3 binding site in the S100A9 promoter: forward 5’-AACGTCGTGTTGTTGTTGCTA-3’; reverse, 5’-TGGGTCTTCTTGCCCTGTTGCA-3’. Primers for S100A8 promoter: forward, 5’-ACACCTGTCAAAGCTGGAAACCACA-3’; reverse, 5’-TCAGCATCAGAAAGAGCCCCA-3’. Primers for β-actin: forward, 5’-TAGGTTAGACTTTTGACGACA-3’; reverse, 5’-AGGCTCTGTTGGTTCCAATACGTGT-3’.

Experiments with ES cells. S100A4, S100A8, and S100A9 were amplified from mRNA of HC cells isolated from BALB/c bone marrow using RT-PCR. The pairs of primers were as follows: for S100A1 forward, 5’-GCTCGAGGGGACCATGCCGTTGTTGG-3’ containing XhoI site; reverse, 5’-AGATTTCGTCCTCAACTTGTTGCCTCCA-3’; containing EcoRI site; S100A4 forward, 5’-CCCTAGGCACCCATGGGCTGTAAGTCTTGAC-3’, containing XhoI site at the 5’ end and modified Kozak sequence; reverse, 5’-CAGGAAGCTTGCGCTGCCAGAAG-3’, containing EcoRI site at 5’ end; S100A9 forward, 5’-AGATATTGACATCTGCCCCAGATCTGACCATGGCCACAGCAAGC-3’, containing EcoRV and BglII sites at the 5’ end and modified Kozak sequence; reverse, 5’-AGCTGCTGAGCTGTCCTCTTAATTTA-3’, containing XhoI site at 5’ end. For S100A1, the amplified 311-bp fragment was digested using XhoI and EcoRI, and subsequently inserted into pcDNA3.1(-) vector; for S100A4, the amplified 375-bp fragment was digested using EcoRV and XhoI, and inserted into pcDNA3.1(+). For S100A8, the latter gene was recloned using EcoRV and XhoI into pcDNA3-Hygro. The 311-bp fragment was cloned into pcDNA3.1(-). The vectors were verified by sequencing. To create cell lines expressing both S100A4 and S100A9, each vector was transduced into naïve ES cells and the cell lines were selected using puromycin. Some ES clones were selected using a FACSAria. To differentiate myeloid progenitor cells, 35,000 Lin−c-Kit+ GFP+ or Lin−c-Kit− GFP− cells were plated in each well of 24-well plates and cultured for 4 d in Myeloid Long-Term Culture Medium for Primitive Mouse Hematopoietic Cells, supplemented with 20 ng/ml of IL-3, IL-11, SCF, Flt3L and GM-CSF. 500,000 cells were cultured in RPMI supplemented with 10% FBS and 200 μg/ml of GM-CSF for another 7 d. 24 h before cell analysis, 1 μg/ml LPS was added to activate DCs.

Isolation of Gr-1+ cells. Gr-1+ cell isolation was performed using MiniMACS microbeads according to the manufacturer’s protocol (Miltenyi Biotec). The purity of Gr-1+ cells was consistently >95% in all samples. The depletion of Gr-1− cell was achieved by two rounds of isolation using magnetic beads. Negative fractions were collected and used as Gr-1− cells. They contained <5% Gr−1− cells. In some experiments, Gr-1+CD11b+ cells were sorted using FACSAria cell sorter (BD Biosciences).

IFN-γ ELISPOT assay. MDSCs were isolated from spleens of wild-type and S100A9Tg mice using cell sorting on FACSAria cell sorter. The purity of cell populations was >99%. As responder cells, we used splenocytes from FVB/N mice immunized twice with s.c. injection of 5 × 106 DCs loaded with MHC class I (H2-D9) restricted rat HER-2/neu-derived peptide PD-SLRDLSVF. MDSCs were mixed with splenocytes at 1:3 and 1:6 ratios. The number of IFN-γ-producing cells in response to stimulation with the specific (PD-SLRDLSVF) or control (RAHYNIVTF) peptides (10 μg/ml) was evaluated in a 42-h ELISPOT assay performed as previously described (57). The numbers of spots were counted in triplicates and calculated using an automatic ELISPOT counter (Cellular Technology, Ltd).

Allogeneic mixed leukocyte reaction. T cells were isolated from naive FVB/N mice using a T cell enrichment column (R&D Systems), and 106 cells were placed in each well of round-bottomed 96-well plates. DCs were generated from BALB/c mice using GM-CSF and IL-4. DCs were mixed with T cells at a 1:50 ratio. MDSCs isolated from wild-type and S100A9Tg cell were added to cultures at different MDSC/T cell ratios (from 1:2 to 1:16). Cell proliferation was evaluated in triplicates after a 96-h incubation using [3H]thymidine incorporation.

Measurement of ROS. ROS was measured by labeling cells either with the oxidation-sensitive dye DCFDA (28) for experiments with S100A9 KO mice and ES cells or hydroethidine (58) for experiments with S100A9Tg mice.

Hematoxylin-eosin and immunohistochemical staining of tumor tissues. EL4 tumor tissues were either fixed in paraformaldehyde or frozen with OCT. Hematoxylin-eosin staining was performed on paraffin embedding tissues. 5-μm-thick sections were stained with hematoxylin–eosin and examined under light microscopy. Immunohistochemical staining was
performed on frozen tissue sections using goat anti–mouse S100A9 antibody (R&D Systems), followed by staining with anti-goat IgG conjugated with biotin and ABC kit (Vector Laboratories) and counterstained with hematoxylin or stained with rat anti–mouse Gr-1 antibody (BD Biosciences), followed by staining with anti-rat IgG conjugated with biotin and ABC kit (Vector Laboratories) and counterstained with hematoxylin.

**Down-regulation of Stat3 by siRNA in 32D cells.** 32D cells (10⁴) were mixed with 100 nM Stat3 siRNA (Dharmacon) in 100 μl 32D nucleofector solution, and transfection was performed using Nucleofector 1 (Amaxa). As a control, nontargeting siRNA pool was used (siGenome). The gene expression was evaluated 36 h later using qRT-PCR, and protein levels were measured after 72 h by Western blotting.

**Statistics.** Statistical analysis was performed using parametric and nonparametric methods on JMP software (SAS Institute). In all cases, P values were calculated using two-sided t test.

**Online supplemental material.** Fig. S1 shows the expression of S100A9 and A8 in myeloid cells in tumor-bearing mice. Fig. S2 demonstrates that tumor cell-conditioned medium inhibits differentiation of DCs and induces accumulation of MDCs in vitro. Fig. S3 shows that differentiation of myeloid cells in S100A9-deficient naive mice is not impaired. Fig. S4 shows that C3 tumor is rejected by most of S100A9 KO mice. Fig. S5 provides the immunohistochemical evaluation of tumors from S100A9 KO mice. Fig. S7 demonstrates the expression of S100A9 and A8 in transfected ES cells. Fig. S8 shows the phenotype of myeloid cell colonies in cells transfected with S100A9/A8. Fig. S9 shows the colony formation by spleen and bone marrow cells from S100A9/Tg mice. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080132/DC1.

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